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Intraspecies Diversity of Ricinus Communis L. in Bangladesh and Their in Vitro Conservation

Rahman, A. K. M. Abdur

University of Rajshahi

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INTRASPECIES DIVERSITY OF *RICINUS COMMUNIS* L. IN BANGLADESH AND THEIR *IN VITRO* CONSERVATION

THESIS SUBMITTED FOR THE DEGREE
OF
DOCTOR OF PHILOSOPHY
IN THE
INSTITUTE OF BIOLOGICAL SCIENCES
UNIVERSITY OF RAJSHAHI
BANGLADESH

BY
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BANGLADESH

June, 2013

DEDICATED
To
My Late Parents and Family

DECLARATION

I do hereby declare that the whole work submitted as a thesis entitled **"INTRASPECIES DIVERSITY OF *RICINUS COMMUNIS* L. IN BANGLADESH AND THEIR *IN VITRO* CONSERVATION"** in the Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205 for the degree of **Doctor of Philosophy** is the result of my own investigation and was carried out under the supervision of Dr. M A Bari Miah, Professor, Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh. No part of this work presented in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

.....

Rajshahi (A. K. M. Abdur Rahman)
June, 2013

CERTIFICATE

This is to certify that **A. K. M. Abdur Rahman** has carried out this research study under my supervision. I am pleased to forward his thesis entitled **"INTRASPECIES DIVERSITY OF *RICINUS COMMUNIS* L. IN BANGLADESH AND THEIR *IN VITRO* CONSERVATION"** which is the record of bona fide research carried out at the biotechnology laboratory and research farm in the Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh. He has fulfilled all the requirements of the regulations relating to the nature and prescribed period of research for submission of thesis for the award of Ph.D. degree.

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The author

INTRASPECIES DIVERSITY OF *RICINUS COMMUNIS* L. IN BANGLADESH AND THEIR *IN VITRO* CONSERVATION

ABSTRACT

The castor oil plant, *Ricinus communis*, is a species of flowering plant in the spurge family, Euphorbiaceae and it belongs to a monotypic genus, *Ricinus.* Castor seed is the source of castor oil, which has a wide variety of uses. The seeds contain between 40% and 60% oil that is rich in triglycerides, mainly ricinolein. The seed contains ricin, a toxin, which is also present in lower concentrations throughout the plant. The castor oil plant can vary greatly in its growth habit and appearance. In Bangladesh, castor plant growing all over the country without any special care. In the present aspect of climate change castor plant holds a great promise for producing environmentally friendly biofuel as a source of green energy .

In respect of research, castor provides a virgin field in Bangladesh as it is not a mandatory crop plant of any national research institute in the country. In the beginning of our research work we have made a survey on the existing germplasms of castor plant over the country in order to preserve the land races with their particular gene pools for breeding purposes. A number of varieties of castor plant found to grow here and there over the country with distinct morphological variation and peculiarities. In the present investigation, a thorough and extensive survey was made and different castor cultivars were collected from different corners of the country and established a germplasm bank in the Institute of Biological Sciences, Rajshahi University, Bangladesh. Under present study the diversity of castor was studied both on phenotypic and genotypic perspectives. Under the purview of morphological study a number of 28 characters of castor plant were analyzed during its growth period. An attempt was taken to assess the genotypic influences on the nature and extent of variation in different morphological characters of different castor germplasms collected from different corners of the country. The characters were categorized under this study depending on their morphological nature and they were (1) plant height (cm), (2) stem diameter (cm), (3) branch number, (4) branch length (cm), (5) node number, (6) internodal distance (cm), (7) leaf length (cm), (8) leaf breadth (cm), (9) leaf area (cm^2), (10) lobes number, (11) petiole length (cm), (12) petiole diameter (cm), (13) root length (cm), (14) lateral root number, (15) lateral root length (cm), (16) inflorescence number, (17) male flower's region (cm), (18) female flower's region (cm), (19) number of male flower, (20) number of female flower, (21) fruit

number, (22) thorn number, (23) flowering time (days). (24) seed number, (25) seed length (cm), (26) seed breadth (cm), (27) seed weight (gm), and (28) hundred seeds weight (gm). The results indicated the presence of substantial amount of genetic variability in the collected gerplasms and there exists ample scope for selection. The result also showed that phenotypic coefficient of variation (PCV) in general was higher than genotypic coefficient of variation (GCV) and very high estimate of broad sense heritability (more than 90%) were observed for all characters except number of seeds per fruit. Distinct variation was very obvious even in visual observation on stems, leaves, petioles, inflorescence and flowers.

The present study confirmed that the castor cultivars were significantly different in regards of vegetative growth and yield component parameters. The characters plant height, branch number, stem diameter, node number, internodal distance, length and breadth of leaves, leaf area and length of root were recognized as the most important characters in castor plant for evaluating their morphological characterization and they hold the merit of receiving greater importance and priority in selection under any breeding programme for the improvement of castor genotypes Following the study, six distinct cultivars were recognized as cultivar shabje, cultivar shadatae, cultivar roktima, cultivar lalchay, cultivar badami and cultivar dhusar based on local identity particulars.

An attempt was taken to confirm the molecular diversity and phylogenic relationship among six cultivars of castor by Random Amplified Polymorphic DNA (RAPD) markers. The RAPD provides a quick and efficient technique for DNA sequence-based polymorphisms at a very large number of loci. DNA fingerprinting of six cultivars of castor were performed using the seven RAPD markers in the present investigation. The seven primers used in our RAPD experiments were: OPA-8, OPA-9, OPA-10, OPB-17, OPC-17, OPD-3, OPE-6. RAPD experiment which showed promising result for the proper identification of the varieties using genetic marker. Among the seven primers used for DNA fingerprinting in six cultivars of castor 3 RAPD primers successfully generated distinct banding pattern. Three RAPD primers generated 50 bands and average 16.66 from the six cultivars of castor and the primers OPA-9, OPA-10 and OPB-17 produced 26, 17 and 7 bands respectively. Differential banding pattern was observed among four cultivars of castor. The result confirmed molecular diversity existed among the castor cultivars and considering the observed phenotypic and genotypic differences the collected germplasms of castor can be denoted as six distinct genotypes or cultivars.

Another potential avenue of present investigation was to establish protocols for *in vitro* propagation of six cultivars of castor. Cotyledonary node and shoot tip

segments were used as explants for proliferation of axillary shoots in MS, MMS_1 and MMS_2 medium with different concentrations (0.5-5.0 mg/L) of cytokinins viz. BAP, Kn and 2ip. Cotyledonary node explant showed better performance while MMS_1 was proved as the best medium and 2.0 mg/L BAP proved as the best hormonal supplement giving 100% direct shoot regeneration in this media formulation.

Callus induction was studied in six cultivars of castor from hypocotyl, internode and shoot tip explants in different concentrations of BAP (0.5-5.0 mg/L) with different concentrations (0.1-1.0 mg/L) of NAA, 2,4-D and IAA. Hypocotyl was proved as the best explant for callus induction producing 100% callus formation in suitable media formulation. On the other hand BAP 2.0 mg/L with 0.5 mg/L NAA proved the best formulation for callus induction. Nature of callus also varied in shape and colour, a very wide range of diversity existed in castor cultivars in producing whitish green, green compact, pinkish brown, pinkish nodular callus in their respective suitable media compositions. MMS_1 medium supplemented with 1.0 mg/L BAP + 0.2 mg/L NAA + 0.8 mg/L TDZ was recognized as the best media formulation for indirect organogenesis of cultivar shabje, cultivar roktima and cultivar lalchay among the six cultivars of castor. MMS_2 medium was identified as the best media formulation for root induction for castor cultivars. Particularly for root induction three auxins IBA, NAA and IAA were used in different concentrations (0.1 - 1.0 mg/L) and among them, 0.2 mg/L IBA was proved to be best for root formation. Rooting performance in castor cultivars were successfully enhanced by the application of additives to the artificial medium. Different concentrations of $AgNO_3$ and activated charcoal were used with 0.2 mg/L IBA but 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ showed best performance for root elongation of six cultivars of castor.

Investigation was carried out for cell suspension culture of six cultivars of castor. Free cells isolated from friable embryogenic calli drived from hypocotyl explant of six cultivars of castor were subjected to grow under different concentrations and combinations of auxins and cytokinin (NAA, 2,4-D and BAP) on cell suspension culture. All the cultivars of castor produced successfully cell suspension culture in liquid medium from their respective friable callus under continuous agitation in dark. The peak period of cell growth was observed within 4th - 6th days of suspension culture and the highest weight of the cells and cell aggregates of all cultivars were obtained on the 14th days of suspension culture in modified liquid MS medium supplemented with 2.0 mg/L BAP + 0.3 mg/L NAA accompanied with 1.0 mg/L biotin and 1.0 mg/L glutamine. The cultivar shabje showed the maximum cell weight which was 0.143±0.13 gm while the maximum cell weight of other cultivars viz. shadatae, roktima, lalchay, badami and dhusar were 0.133±0.12 gm,

0.138±0.13 gm, 0.135±0.13 gm, 0.126±0.12 gm and 0.129±0.12 gm respectively. For callus induction from the isolated single cells modified MS medium was used supplemented with 2.0 mg/L BAP with five different concentrations of NAA (0.1, 0.2, 0.5, 0.8 and 1.0 mg/L). Callus induction was observed in all BAP and NAA formulations. Growth regulator played very important role in callus induction from single cell culture in semisolid artificial medium and 2.0 mg/L BAP with 0.5 mg/L NAA showed the best performance in callus induction from isolated single cell culture.

Cell extracts of six cultivars of castor were taken after different periods of cell suspension culture and they were tested against five gram positive and six gram negative bacteria at the concentration of 25 µl/disc and they were compared with standard antibiotic ciprofloxacin 5 µg/disc. The cell extracts prepared for antibacterial test were taken from cell suspension cultures growing under different time regimes viz, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days and 16 days. All the castor cultivars showed antibacterial activity performing in different grades under different time periods. Over all the castor cultivars, cell extracts taken from 6 days to 16 days culture were found 100% effective against all the bacteria tested but the extracts taken from 4 days culture failed to show antibacterial activity against all the bacteria tested. But, the extract taken after 14 days from cell suspension culture showed the best antibacterial activities. Among the six cultivars of castor, comparatively cultivar roktima showed the highest level of lethality against all bacteria tested. The experimental results indicated that the castor cultivars also holds the diversity in the levels of their toxicity and mode of action in antibacterial activities.

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LIST OF ABBREVIATIONS

Abbreviation		Full Meaning
2n	:	Chromosome number
2,4-D	:	2,4-Dichlorophenoxy acetic acid
0.1N	:	0.1 Normal solution
%	:	Percent
µl	:	Micro liter
µM	:	Micro molar
ºC	:	Degree Celsius
AFLP	:	Amplified Fragment Length Polymorphism
APS	:	Ammonium persulphate
A260	:	Spectrophotometer Absorbance Reading at 260 nm
A280	:	Spectrophotometer Absorbance Reading at 280 nm
BAP	:	6-Benzyl amino purine
$CaCl_2.2H_2O$	:	Calcium chloride
$CoCl_3.6H_2O$	:	Cobalt chloride
CTAB	:	N-Cetyl-N,N,N-Trimethyl Ammonium Bromide
CuSO4.7H2O	:	Cupper sulphate
dw	:	Distilled water
dH2o	:	Distilled water
DNA	:	Deoxyribonucleic acid
dNTPs	:	Deoxy nucleotide triphosphates
DW	:	Distilled water
EDTA	:	Ethylene Diaminetera Acetic Acid
e.g.	:	Exempli gratia=for example
et al.	:	Et alia=and others
EtBr	:	Ethidium Bromide
Etc.	:	et. cet. Era=and the others
$FeSO_4.7H2O$	:	Ferrous sulphate

Fig.	:	Figure
Gm	:	Gram
GPR	:	Reagent grade
HCHO	:	Formaldehyde
H_3BO_3	:	Boric acid
$HgCl_2$	:	Mercuric chloride
HCl	:	Hydrochloric acid
i.e.	:	id est=that is
Kn	:	Kinetine (6-furfuryl amino purine)
Kg/cm^2	:	Killogram per centimeter square
KNO_3	:	Potassium nitrate
KH_2PO_4	:	Potassium bi-phosphate
KI	:	Potassium iodide
M	:	Molar
mg	:	Milligram
mg/l	:	Milligram per litre
$MgSO_4.7H_2O$	:	Magnesium sulphate
ml.	:	Milliliter
mM	:	Milli mole
$MnSO_4.7H_2O$	:	Manganese sulphate
MW	:	Molecular weight
MS	:	Murashige and Skoog
NH_4NO_3	:	Ammonium nitrate
N	:	Normal solution
NaCl	:	Sodium chloride
Na_2CO_3	:	Sodium carbonate
Na_2FDTA	:	Sodium salt of ferric ethylene diamine tetraacetate
$Na_2MoO_4.4H_2O$	:	Sodium molybdede
NaOH	:	Sodium hydroxide
NAA	:	α-Naphthyl Acetic Acid

Na_2-EDTA	:	Sodium di-ethylene tri-acetic acid
OD	:	Optical density
PCR	:	Polymerase chain Reaction
pH	:	Negative logarithm of hydrogen ion (H^+) concentration
PGRs	:	Plant growth regulators
PVP	:	Polyvinyl Pyrolidone
RAPD	:	Random Amplified Polymorphic DNA
RNA	:	Ribonucleic acid
rpm	:	Rotation per minute
$SddH_2O$	:	Sterile de-ionized distilled water
SDS	:	Sodium Dodecyl Sulphate
Sp.	:	Species
SSR	:	Simple Sequence Repeats
TEMED	:	N,N,N′,N′-tetraethyl-methylenediamine
UV	:	Ultra violet
viz.	:	Videlicet (=namely)
w/v	:	Weight per volume
wt.	:	Weight
$ZnSO_4$	:	Zinc sulphate

CHAPTER -I

1. GENERAL INTRODUCTION

1.1. Oil yielding plants

The plants producing vegetable oils are referred to as oil yielding plants supporting human life covering the areas of food, fuel and medicine. Plant synthesize oil essentially for the future survival of the seed. Usually oil accumulates in seeds, nuts and fruits and they are actually remains in the form of triglycerides which is an ester derived from glycerol and three fatty acids and it is an unique combination of hydroxyl group of the glycerol joining with the carboxyl group of the fatty acid. These vegetable oils have been of human culture for millennia. Oils derived from plants are mainly used as edible oil but some are non edible and used as fuel. A number of 287 plants are reported to produce oil but among them 45 plants produced oils as has been used as fuel (BIGR 2004).

We get edible oil mainly from twenty plants under six families. The families are, brassicaceae, rutaceae, asteraceae, papilionaceae, myristicaceae and pedaliaceae but the largest edible oil giving family is brassicaceae comprising of eight edible oil yielding plants of this family. The name of the plants are; *Brassica campestris* L. var. toria, *Brassica campestris* L. var. sarson, *Brassica comprestis* L. var. oleifera, *Brassica juncea* Hook. f. & Thoms. var. green, *Brassica napus* L. var. canola, *Brassica nigra* L. var. black, *Eruca sativ* L. var. arigula and *Sinapis alba*. L. var. white.

We get non edible oil from twenty five families, these are, Euphorbiaceae, Clusiaceae, Meliaceae, Apocynaceae, Burseraceae, Fabaceae, Lauraceae, Alangiaceae, Anacardiaceae, Asteraceae, Barringtoniaceae, Caesalpiniaceae, Caprifoliaceae, Clusiaceae, Dipterocarpaceae, Flacourtiaceae, Hernandiaceae, Icacinaceae, Malvaceae, Mimosaceae, Myristicaceae, Papaveraceae, Salvadoraceae, Sapotaceae, Sterculiaceae. Among the non edible oil yielding plants belongs to euphorbiaceae family and this is the largest family for non edible oil yielding plants. The largest non-edible oil giving family is Euphorbiaceae which includes six plants : *Jatrapha curcas* L., *Jatropha gossypifolia* L., *Joannesia princeps* Vell., *Putranjiva roxburghii* wall., *Drypetes roxburghii* (Wall) Hurusawa. and *Ricinus communis* Linn. The Euphorbiaceae family occurs mainly in the tropics, express mixed relationship with several other groups, and includes plants producing the most important economic products such as para rubber (*Hevea brasiliensis*), castor oil (*Ricinus communis* L.), tung oil (*Aleurites fordii*) and cassava meal (*Manihot esculenta*). Castor and

Jatrophas are commercially important non edible oilseed crops in the Euphorbiaceae with and croton forming very large genera (Hutchinson, 1964).

Oil yield for major non-edible and edible oil sources.

Type of oil	Oil yield (kg oil/ha)	Oil yield (wt%)
Non-edible oil		
Jatropha	1590	Seed: 35 – 40, kernel: 50 - 60
Rubber seed	80-120	40-50
Castor	1180	53
Pongamia pinnata	225 - 2250	30 - 40
Sea mango	N/A	N/A
Edible oil		
Soybean	375	20
Palm	5000	20
Rapeseed	1000	37 - 50

1.2. Castor

Ricinus communis is indigenous to north-eastern tropical Africa. It was already grown for its oil in Egypt some 6000 years ago and spread through the Mediterranean, the Middle East and India at an early date. It is now widely cultivated in most drier areas of the tropics and subtropics and in many temperate areas with a hot summer. It naturalizes easily and grows in many areas as a ruderal plant. *Ricinus communis* occurs across the African continent, from the Atlantic coast to the Red sea and from Tunisia to South Africa and in the Indian Ocean islands. The castor plant is a robust annual that they grow 6 to 15 feet (2-5 meters) in one season with full sunlight, heat and adequate moisture. In areas with mild, frost-free winters it may live for many years and become quite woody and tree-like.

The Castor Bean is the only member of the genus *Ricinus*, and it has no immediate relatives. As a member of the Spurge family of plants (Euphorbiaceae), it is distantly related to the poinsettia, true rubber tree, cassava, croton, tung oil tree, Chinese tallow tree and crown of thorns. While the Castor Bean is native only to Africa, people have introduced the species around the world. It has escaped from cultivation and can be found as a wild and sometimes invasive plant in Australia, many Pacific Islands, and in 27 states (including New Jersey). In tropical areas it grows as a shrub or a tree that can reach 40 feet in height along streams and rivers and on bottomlands with well-drained, nutrient rich soils. In temperate areas, Castor Beans are grown as annuals. In New Jersey they are grown as ornamentals and can be

directly sown into gardens in late spring, or started earlier indoors and transplanted outdoors when the weather warms. Castor Bean plants grow at an amazingly fast rate, if they are situated in full sun and provided with ample fertilizer and water. Ten-foot tall plants are not uncommon by late summer. They are intolerant of frost, and die as soon as the temperature drops below 32°F.

1.2.1. Classification

Kingdom – Plantae (Plants)
Subkingdom – Tracheobionta (Vascular plants)
Superdivision – Spermatophyta (Seed plants)
Division – Magnoliophyta – (Flowering plants)
Class – Magnoliopsida (Dicotyledons)
Subclass – rosidae
Order – Euphorbiales
Family - Euphorbiaceae (Spurge family)
Genus – *Ricinus* L. (ricinus)
Species – *Ricinus communis* L. (castor bean)

1.2.2. Castor oil

Castor oil is a vegetable oil obtained from the castor bean [technically *castor seed* as the castor plant, *Ricinus communis* (Euphorbiaceae), is not a member of the bean family]. Castor oil (CAS number 8001-79-4) is a colorless to very pale yellow liquid with mild or no odor or taste. Its boiling point is 313 °C (595 °F) and its density is 961 kg/m^3. It is a triglyceride in which approximately 90 percent of fatty acid chains are ricinoleic acid. Oleic and linoleic acid are the other significant components (Wikipedia 2012). The presence of hydroxyl group and double bonds in ricinoleic acid (D-12-hydroxyoctadec-cis-9-enoic acid) imparts unique chemical and physical properties for castor oil which makes castor oil a vital industrial raw material and stabilizes the oil against oxidation.

Average composition of castor oil:

Acid name	Average percentage range
Ricinoleic acid	85% to 95%
Oleic acid	6% to 2%
Linoleic acid	5% to 1%
Linolenic acid	1% to 0.5%
Stearic acid	1% to 0.5%
Palmitic acid	1% to 0.5%
Dihydroxystearic acid	0.5% to 0.3%
Others	0.5% to 0.2%

Ricinoleic acid, a monounsaturated, 18-carbon fatty acid, is unusual in that it has a hydroxyl functional group on the 12th carbon. This functional group causes ricinoleic acid (and castor oil) to be unusually polar, and also allows chemical derivatization that is not practical with most other seed oils. It is the hydroxyl group which makes castor oil and ricinoleic acid valuable as chemical feed stocks. Compared to other seed oils which lack the hydroxyl group, castor oil commands a higher price. As an example, in July 2007, Indian castor oil sold for about US$0.90 per kilogram (US$0.41 per pound) while U.S. soybean, sunflower and canola oilseeds sold for about US$0.30 per kilogram (US$0.14 per pound).

Industry uses yearly 600-800 million pounds of castor oil and its derivatives have applications in the manufacturing of soaps, lubricants, hydraulic and brake fluids, paints, dyes, coatings, inks, cold resistant plastics, waxes and polishes, nylon, pharmaceuticals and perfumes (Mutlu and Michael 2010).

1.2.3. Botanical description

Stems are succulent, herbaceous, very variable in all aspects. Stem and branches with conspicuous nodes and ring-like scars, shoots usually glaucous, variously green, light green, red or reddish brown often with a thin, waxy coating. Glands often present at nodes. The star-shaped leaves can reach 3 feet in long. Stipules 1-3 cm long, clasping the stem, deciduous; petiole 3.5-50 cm long, terete; blade palmately 5-12 lobed, up to 50-70 cm in diameter, membranous, lobes acuminate, median one up to 8-20 cm long, margins with glandular teeth. Tap root strong and with prominent lateral roots. The root system is well developed. Inflorescences are racemes 60-80 cm long. Flowers occur most of the year in dense terminal clusters (inflorescences). This species is clearly monoecious with separate male and female flowers on the same individuals. Female flowers are on the top half of the spike and have conspicuous red stigmas (the parts that receive the pollen). The female flowers are folded by reddish brown egg-shaped capsules, about an inch long, thickly covered with soft flexible spines. Petal absent in both sexes, sepals 3-5, greenish; stamens numerous, 5-10 mm long, ovary superior, 3-celled with a short style and 3 stigmas. Fruit a globose capsule 2.5 cm in diameter, on an elongated pedicel, usually spiny, green turning brown on ripening. The seed pod or capsule is composed of three sections or carpels which split apart at maturity. Each section (carpel) contains a single seed, and as the carpel dries and splits open, the seed is often ejected with considerable force. The shiny seeds of castor plants are somewhat large 0.5-1.5 cm long, ovoid shaped, tick-like and have very beautiful intricate

designs. At one end is small, spongy structure called the caruncle, which aids in the absorption of water when the seeds are planted. Like human faces, fingerprints or the spots on a leopard, no two seeds have exactly the same pattern. They are unquestionably among the most deadly seeds on earth, and it is their irresistible appearance that makes them so dangerous.

1.2.4. Cultivation

Castor is propagated by seed. Castor seed are large and slow to germinate; emergence of the seedlings may take 7 to 14 days. Seeds should be planted 6.3 to 7.6 cm deep, depending on texture and condition of the soil. If press wheels are used in contact with the seed, care should be taken that they do not crush the seed. Castor is planted in 0.96 to1.01 m row, with a seedling rate of 11.2 to 15.7 kg/ha and plant spacing of 20 to 25 cm within in the row. Special care must be taken to prevent crushing the fragile seed in the planter box. Adequate amounts of nitrogen, phosphorus and potassium must be available to produce high yields of castor seed. Levels of these nutrients should be determined by soil test. If the soil is deficient in nitrogen, 90 to 135 kg/ha of nitrogen usually are needed for maximum yields. A split application of nitrogen is often used, with the second half side dressed between the rows at last cultivation. If phosphorus is needed, application should be made before planting time. Potassium can be applied at planting time. A minimum of 37 to 56 kg/ha of P is needed for production of castor, and 15 to 19 kg/ha of K (Duke 1978). Where pre-plan furrow irrigation is applied, castor plants should not require irrigation until the first racemes appear on the plant. Under normal conditions, 12 to 14 days between irrigation should keep plants from stressing for moisture, but high temperatures and high winds during the peak growing and fruiting periods may cause the plants to need more frequent irrigation. Castor requires 20.6 to 24.7 cm/ha of water annually to produce high yields. The time of last irrigation is usually from 1 to 10 september. In intercropping, distance between rows of castor may be as much as 4-5 m, and it will receive the treatment of the main crop.

IMPUTE COSTS + VARIABLE COSTS + NET RETURN PER HECTARE IN CASTOR CULTIVATION: (As at August, 2003)

OUTPUT		UNITS	
YIELD KG		2000 KG/HECTARE	
Output at quoted price of € $ 0.42/KG		€ $ 830.56	
Variable costs per hectare	Unit	QTY	Cost per hectare
Seed	KG	4.5	€ $1.87
Labor	Man days	70	€ $ 23.26
Tractor hire	HRS	4	€ $ 21.26
Transportation	KG	2,000	€ $33.22
Total variable costs per hectare	Hect.	€	€ $ 79.61
Total impute costs per hectare	Hect.	€	€ $ 46.10
Castor seed oil production per hectare	KG	450 gm/KG	900 KG Castor oil
Liters of castor oil per hectare	Liter	1	990 Liters
Castor oil cost per KG including crushing	Liter	1 Liter	€ $ 0.13/ Liters
Castor oil to biodiesel production costs /KG	Liter	1 Liter	€ $ 0.15/ Liters
Total production costs	Liter	1 Liter	€ $ 0.28/ Liters
Biodiesel wholesale	Liter	1 Liter	€ $ 0.56/ Liters
Net return on biodiesel sales	Liter	1 Liter	€ $ 0.37/ Liters
Net return per hectare	Hect.	€	€ $ 366.30/Hect.

1.2.5. Castor production

India is the leading producer of castor oil in the world, followed by China and Brazil with 0.8 and 0.4 lakh tons respectively. The present annual world trade in castor oil is estimated at about 2.0-2.50 lakh tons. The major importers of castor oil in the world market are European Union, US and Japan. The world demand for castor oil is estimated to be growing at the rate of about 3 to 5 % per annum. Both Brazil and China have experienced a steady increase in their domestic castor oil consumption in the recent years and thus utilize almost their entire production. India consumes only a quarter of its castor oil production and exports the rest.

Castor (*R. communis* L.) is one of the most important non edible oilseed crops and is cultivated in 1.26 million hectare with an annual seed production of 1.14 m tons with an average seed yield of 902 kg/ha (FAO 2006) and 300-400 kg/ha when growing in soil without adequate moisture. Some improved open-pollinated varieties in Brazil and the United states yield 1,300 kg/ha, with exceptional yields up to 5,000 kg/ha. Average Indian yields are 560 kg/ha. Oil content of seeds varieties from 35 to

55 %, suggesting potential oil yields of 200- 275 kg/ha. During and since second World War, castor production increased in South America, Thailand and Haiti. In 1952, about 50,000 kg of castor oil was imported in the United States, mostly from India, Belgium, West Germany, Holland, Peru, Manchuria, China, Argentina, Mexico, Brazil and Paraguay. Wholesale prices for castor beans in 1969 from India was 15.2 cents /kg and from Europe 16.5 cents/kg. In 1971 prices from Brazil were $ 293/T. Prices vary: Italy 93.1 cents/kg producer price; Spain 66.6 cents/kg, export price; and the United States 85.3cents/kg, import price. In 1970 more than 469,000 MT of oil was produced in the world, representing about 1% of the oil market. (Duke 1983).

World top ten castor oil seed production, July 2008:

Country	Production (Tons)
India	830 000
China	210 000
Brazil	91 510
Ethiopia	15 000
Paraguay	12 000
Thailand	11 052
Vietnam	5 000
South Africa	4 900
Philippines	4 500
Angola	3 500
World	1209756

Source: Food and Agricultural Organization of United Nations: Economic and Social Department: The Statistical Division.

World castor oil production and consumption: 2004-2005 (in MT)

Castor seed		Castor oil	
Country	Production	Production	Consumption
India	8,50,000	3,36,000	82,000
China	2,50,000	1,05,700	1,67,000
Brazil	1,77,000	10,400	55,000
EU	-	4,600	1,30,000
USA	-	-	40,000
Japan	-	-	22,000
Others	50,000	30,600	42,000
Total	13,27,000	5,47,300	5,38,000

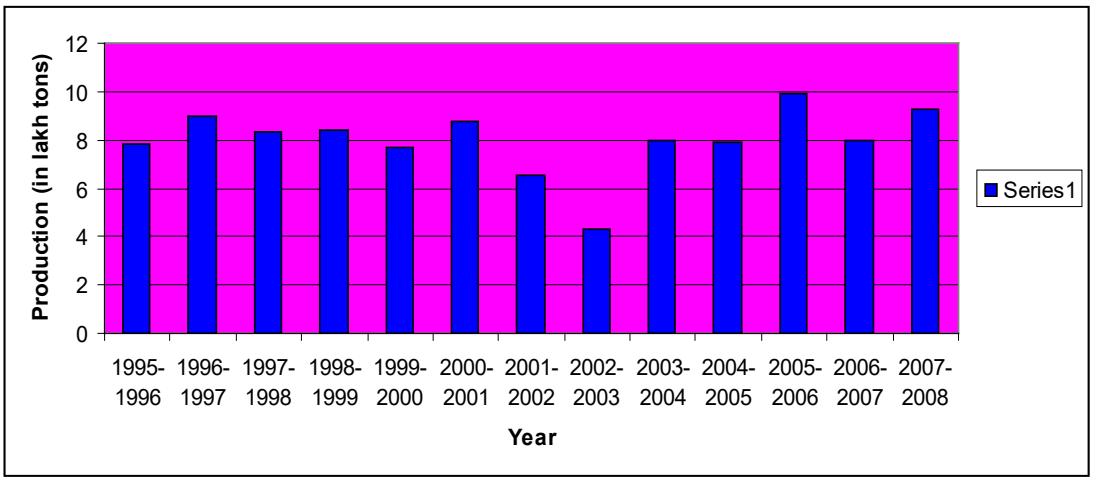
India is a leading country for castor production:

Castor (*R. communis L.*) is cultivated around the world because of the commercial importance of its oil. India is the world's largest producer of castor seed and meets most of the global demand for castor oil. India produces 8 to 8.5 lakh tones of castor seed annually, and accounting for more than 60% of the entire global production. Because of its unlimited industrial applications, castor oil enjoys tremendous demand world-wide. The current consumption of castor oil and its derivatives in the domestic market is estimated at about 300,000 tons. India is also the biggest exporter of castor oil and its derivatives at 87% share of the international trade in this commodity.

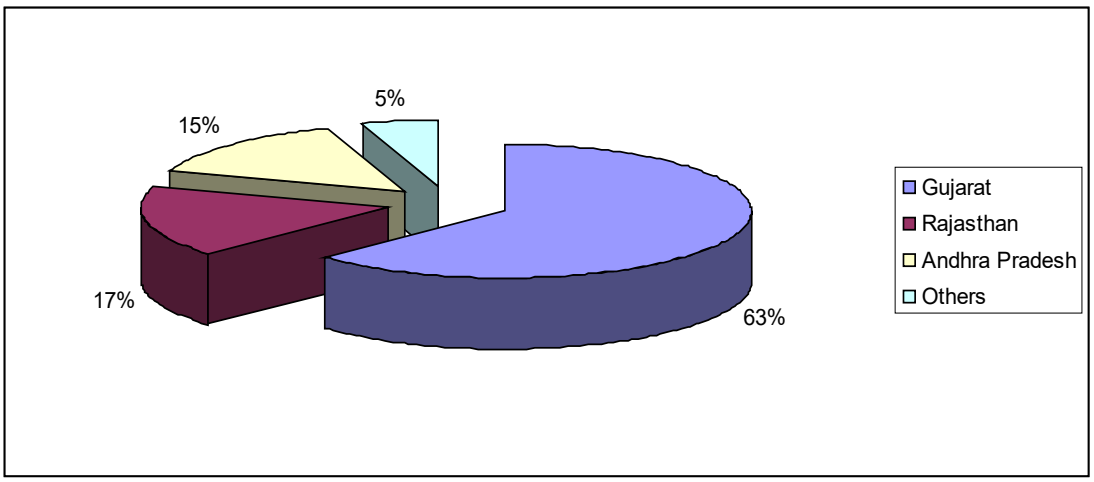
Indian Castor Seed Area, Production and Yield			
Year	**Area (000 ha)**	**Production (000 MT)**	**Yield (kg / ha)**
2001-02	716	652	911
2002-03	583	428	733
2003-04	732	801	1094
2004-05	820	790	963
2005-06	948	970	1023
2006-07	810	800	987

Source: Solvent Extraction Association (SEA) of India

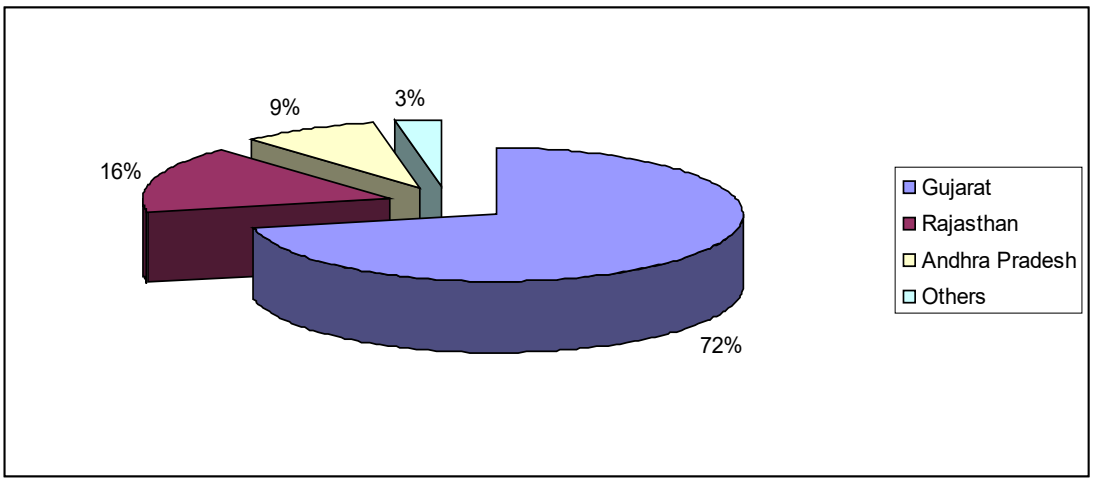
Castor seed production trends in India:



Statewise Castor Seed Production (in .000 tons) in India 2006-2007.



Statewise Castor Seed Production (in .000 tons) in India 2007-2008.



The Indian variety of castor has 48% oil content of which 42% can be extracted, while the cake retains the rest. India's castor oil production fluctuates between 2.5-3.5 lakh tons a year. In 2003-04, India's estimated castor oil production was 2.8 lakh tons. Gujarat accounts for 86% of India's castor seed production followed by Andhra Pradesh and Rajasthan. Castor is mainly grown in Mehsana, Banaskantha and Saurashtra/Kutch regions of Gujarat and Nalgonda and Mahboobnagar districts of Andhra Pradesh.

Castor is a Kharif crop. The sowing season of castor is from July to October and the harvesting season is from October to April. The annual domestic consumption of castor oil in India is only about 80 000-100 000 tons. Of this, the soap industry consumes about 25 000 tons, the paint and allied industries 35 000 tons and the

lubricant and derivatives industry 20,000 tons. India annually exports around 2.0-2.4 lakh tons of commercial castor oil.

1.3. Castor in uses

1.3.1. Use during the early time

In the Ebers-Papyrus, an ancient Egyptian medical treatise, castor oil is described for medicinal use. It was known to Herodotus, who calls it *Kiki*, and states that it furnishes oil much used by the Egyptians, in whose ancient tombs seeds of *Ricinus* were found. At the period when Herodotus wrote (the fourth century B.C.), it would appear to have been already introduced into Greece, where it is cultivated to the present day under the same ancient name. Cleopatra is reputed to have used it to brighten the whites of her eyes. Its use in India has been documented to 2000B.C.E. It was then regarded as a folk medicine and was used for burning in lamps for lighting and for unguents (Armstrong 1982).

Kiki is also mentioned by Strabo as a production of Egypt, the oil from which is used for burning in lamps and for unguents. Theophrastus and Dioscorides, in the first century, described the plant giving an account of the process for extracting the oil and saying that it is not fit for food, but is used externally in medicine, and stating that the seeds are extremely purgative. In the eighteenth century, its cultivation in Europe as a medicinal plant had, however, practically ceased, and the small supplies of the seeds and oil required for European medicine were obtained from Jamaica.

1.3.2. Modern uses

In the 20th century, chemical studies have discovered castor oil's multi-purpose potential. The presence of hydroxyl group and double bonds in ricinoleic acid (D-12-hydroxyoctadec-cis-9-enoic acid) imparts unique chemical and physical properties for castor oil which makes castor oil a vital industrial raw material and stabilizes the oil against oxidation. It is USFDA-approved for use in treating skin disorders. It is also a component of many modern drugs. Lipsticks, shampoos and other cosmetic products contain this oil. Castor oil helps in the manufacturing of soaps, plastics, synthetic resins, fibers, paints, varnishes, lubricants, dyes leather treatments, greases, hydraulic fluids, machining oils, rubbers, and sealants (Berman *et al*. 2011, Ogunniyi 2006, Scholz and Silva 2008). Certain chemicals such as sebacic acid and undecylenic acid were produced from castor oil. Undecylenic acid, which is one of the active compounds of castor oil, helps fight ringworm, a fungal infection. Castor oil contains ricinoleic acid, which delivers anti-inflammatory benefits to the skin. It provides treatment for minor cuts, burns, abrasions, sunburn and prevents skin

disorders such as acne and eczema. Aside from these, one of the benefits of castor oil is in skin conditioning. The only source of ricin is castor, which experimentally used to kill cancer cells and it improves the immune system of AIDS. In the search for more environmentally friendly fuels, the use of castor oil as “Biodiesel” has proven to have technical and ecological benefits, and stands as an opportunity for agricultural development in arid and impoverished areas throughout the tropics and sub tropics globally.

1.3.2.1. Industrial uses

Castor oil and its derivatives have applications in the manufacturing of soaps, lubricants, hydraulic and brake fluids, paints, dyes, coatings, inks, cold resistant plastics, waxes and polishes, nylon, pharmaceuticals pigments, leather treatments, refrigeration lubricants, rubbers, and perfumes. Since it has a relatively high dielectric constant (4.7), highly refined and dried castor oil is sometimes used a dielectric fluid within high performance high voltage (Reed 1976). Stems of castor are made into paper and wallboard (Reed 1976). In Russia the stem of the plant is used in the textile industry. In the food industry, castorial (food grade) is used in food additives, flavorings, candy (e.g., chocolate), as a mold inhibitor, and in packaging. Polyoxyethylated castor oil (e.g., Cremophor EL), is also used in the foodstuff industries (Wilson *et al.* 1998).

1.3.2.2. Medicinal uses

The United States Food and Drug Administration (FDA) has categorized castor oil as "generally recognized as safe and effective" (GRASE) for over the counter use as a laxative with its major site of action the small intestine. Castor oil is a stimulant and lubricating laxative. It is not a preferred treatment, because it can produce painful cramps, fecal incontinence and explosive diarrhea. Its action can go on for hours, sometimes unpredictably and powerfully causing an involuntary bowel movement at inconvenient locations and during sleep. It also has a lot of medicinal uses in ayurvedic and unani medicine. It is used as antidiabetic, Skin disease, Gastrointestinal problem, Gallbladder disease, Neurological problem, Antifungal, antibacterial, antiviral, Menstrual disorder, Constipation remedy, Ringworm treatment, Arthritis natural remedy, Stye treatment, Wrinkle treatment. Most importantly, current studies shows that it enhance the immune system and improves the AIDS. Recent research also showed that ricin is able to kill cancer cells. Several neurological problems have also been responsive to castor oil (Williams 1995). These

include; nerve inflammation such as sciatica, Parkinson's disease, Multiple sclerosis, Migraine headaches and Cerebral palsy.

1.4. Castor bean poisoning

The castor bean contains 40% oil, 1-5% ricin and 0.3-0.8% ricinin (Johnson *et al*. 2005). Ricin is one of the most potent and deadly plant toxins known. All parts of the plant contain the ricin but the seeds are particularly ricin which it and are extremely toxic and are most often associated with clinical toxicosis (Albretson *et al*. 2000). Ricin is also used for the treatment of cancer and AIDS, in bone marrow transplantation and in cell-based research (Johnson *et al*. 2005, Audi *et al*. 2005). The toxicity of castor beans due to the presence of ricin is well-known. The seeds containing 2.8-3% toxic substances (Duke 1978). According to the 2007 edition of the Ginness Book of World Records, this plant is the most poisonous in the world (Wikipedia 2012). As little as 0.5 mg can kill an adult. The toxicity varies among animal species, these have been given below (Wright and Robertus 1987).

Serial No.	Seeds	Name of animal
01	01	Kill a child
02	04	Kill a rabbit
03	05	Kill a sheep
04	06	Kill a horse
05	06	Kill an ox
06	07	Kill a pig
07	11	Kill a dog
08	80	Kill a duck

1.4.1. Ricin

Castor is poisonous because it contains ricin which is highly toxic, naturally occurring protein. Ricin is a toxic protein that is fatal to humans in extremely small doses The LD_{50} of ricin is around 22 micrograms per kilogram (1.78 mg for an average adult, around $^{1}/_{228}$ of a standard aspirin tablet/0.4 g gross) in humans if exposure is from injection or inhalation (Alexander *et al*. 2008). Oral exposure to ricin is far less toxic and a lethal dose can be up to 20–30 milligrams per kilogram.

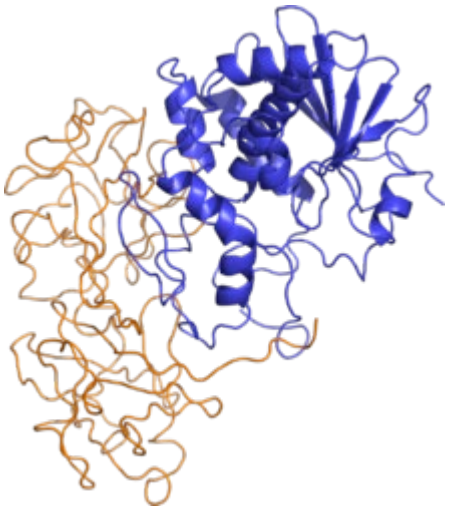
1.4.2. Classification of Ricin

Ricin is classified as a type 2 ribosome inactivating protein (RIP). Whereas Type 1 RIPs consist of a single enzymatic protein chain, Type 2 RIPs, also known as holotoxins, are heterodimericglycoproteins. Type 2 RIPs consist of an A chain that is functionally equivalent to a Type 1 RIP, covalently connected by a single disulfide bond to a B chain that is catalytically inactive, but serves to mediate entry of the A-B protein complex into the cytosol. Both Type 1 and Type 2 RIPs are functionally active against ribosomes *in vitro*, however only Type 2 RIPs display cytoxicity due to the lectin properties of the B chain. In order to display its ribosome inactivating function, the ricin disulfide bond must be reductively cleaved.

Structure: The tertiary structure of ricin was shown to be a globular, glycosylated heterodimer of approximately 60-65 kDA (Aplin and Eliseo 1997). Ricin toxin A chain and ricin toxin B chain are of similar molecular weight, approximately 32 kDA and 34 kDA respectively.

Ricin A chain (RTA) is an N-glycoside hydrolase composed of 267 amino acids (Olnes and Pihl 1973). It has three structural domains with approximately 50% of the polypeptide arranged into alpha-helices and beta-sheets (Weston *et al*. 1994). The three domains form a pronounced cleft that is the active site of RTA.

Ricin B chain (RTB) is a lectin composed of 262 amino acids that is able to bind terminal galactose residues on cell surfaces (Wales *et al*. 1991). RTB form a bilobal, barbell-like structure lacking alpha-helices or beta-sheets where individual lobes contain three subdomains. At least one of these three subdomains in each homologous lobe possesses a sugar-binding pocket that gives RTB its functional character.



Ricin structure. The **A** chain is shown in blue and the **B** chain in orange.

Many plants such as barley have the A chain but not the B chain. People do not get sick from eating large amounts of such products, as ricin A is of extremely low toxicity as long as the B chain is not present. (Wright and Robertus 1987).

1.4.3. How ricin works

Ricin works by getting inside the cells of a person's body. Ricin is a ribosome inactivating protein. It irrevocably damages the ribosomes that carry out protein synthesis in cells. Thus preventing the cells from making the proteins they need. Without the proteins, cells die. Eventually this is harmful to the whole body, and death may occur (Anonymous 2012).

1.4.4. Ricin in cancer research

Although it is a very potent poison, ricin has been shown to possess antitumor qualities and has been used in cancer research and chemotherapy during recent years. One of the most promising uses of ricin is in the production of immunotoxins, where the protein ricin is joined to monoclonal antibodies. The antibodies are produced in a test tube (*in vitro*), and have protein receptor sites that recognize the specific target cells of a tumor. The resulting ricin-antibody conjugate is called an immunotoxin. By arming these antibodies with ricin, the deadly toxin can be carried directly to the site of the tumor in a cancer patient. Thus, ricin can destroy the tumor cells, without damaging other cells in the patient (Armstrong 1982).

1.5. Biotechnology of castor development

1.5.1. Tissue culture

Earlier studies on tissue culture in Euphorbiaceae including castor were mostly with endosperm cultures (Thomas and Chaturvedi 2008). The interest in endosperm culture was mainly due to the large endospermic seeds that enabled easy culturability. The objectives were to obtain triploid plants and to understand the metabolism of glyoxylate pathway. Triploids will be useful for obtaining trisomic lines for genetic mapping. This can be accomplished through endosperm culture or through chromosome doubling (*in vitro* and *in vivo*) and crossing the resultant autotetraploids with diploids (Gmitter *et al.*1990, Sikdar and Jolly 1994, Chaturvedi *et al.* 2003). Attempts to grow endosperm tissue in cultures began in the 1930's and immature and mature endosperms of various angiosperm taxa have been successfully cultured (Johri *et al.* 1980). La Rue (1944) had reported organogenic differentiation in endosperm cultures of castor bean. However, extensive investigations by subsequent workers on maize and castor bean could not confirm La Rue's claim of achieving organogenesis.

In castor, callus initiation and plantlet regeneration from vegetative explants are restricted to young seedling tissues (Athma and Reddy 1983, Sarvesh *et al*. 1992, Ganesh Kumari *et al*. 2008). Athma and Reddy (1983), assessed the differences in callusing ability and organogenic potential of the various seedling explants and obtained shoots from the shoot tips and rhizogenesis from root explants, shoot and leaf tissues. Similarly, plant regeneration that has been reported to occur in the seedling explants was reported to involve pre-existing meristematic centers (Reddy *et al*. 1987b, Sangduen *et al*. 1987, Khumsub 1988, Sujatha and Reddy 1998, Sujatha and Sailaja 2005, Malathi *et al*. 2006). Multiple shoot induction reported by Sangduen *et al*. (1987) and Khumsub (1988) was from embryo explants. Further, Reddy and Bahadur (1989b) reported the genotypic response for shoot tip multiplication in castor. The shoot multiplication rates obtained from meristematic explants in most of these studies varied between 1 and 5.2 (Athma and Reddy 1983, Sangduen *et al*. 1987, Reddy and Bahadur 1989b, Molina and Schobert 1995).

Callus mediated regeneration is reported from hypocotyl sections (Reddy *et al*. 1987a), young stem segments (Genyu 1988), young leaves (Reddy and Bahadur 1989a) and epicotyls/cotyledons (Sarvesh *et al*. 1992). Sujatha and Reddy (2007) assessed the morphogenic competence of castor tissues on several basal media supplemented with a large number of growth regulators individually and in combination showing low caulogenic response of castor explants for direct as well as callus mediated shoot regeneration. Until very efficient and reproducible system of plant regeneration is available, genetic transformation experiments continued with meristem explants (Sujatha and Sailaja 2005, Malathi *et al*. 2006). Recently, protocols for highly efficient protocols of shoot regeneration and somatic organogenesis have been reported (Ahn *et al*. 2007, Ganesh Kumari *et al*. 2008). The frequency of callus-mediated shoot regeneration reported by Ganesh Kumari *et al*. (2008) is high (85%) but shoots appear 16 weeks after culture establishment. As genetic transformation involves several manipulations for gene introduction followed by selection for 2–3 subculture cycles, the efficiency of these regeneration systems for genetic transformation of castor need to be established.

1.5.2. Genetic transformation

Transgenic research in castor has been undertaken for development of insect resistant and ricin free genotypes (Auld *et al*. 2001, Malathi *et al*. 2006). The other interest in castor is for production of epoxy oil which offers all the advantages of a premium oil based paint without the liberation of volatile pollutants (Mc Keon and Chen 2001). The chemical structure of epoxy oil is very similar to that of castor oil

and only minor modifications are needed to cue the castor plant to make epoxy oil instead of castor oil. Castor plant also has the ability to produce industrially useful fatty acids (Ogunniyi 2006).

Recalcitrance *in vitro* has been a major problem for undertaking plant transformation experiments in castor. Mc Keon and Chen (2003) obtained genetically engineered plants by employing the method of Agrobacterium-mediated transformation through vacuum infiltration of wounded flower buds (US Patent No 6.620.986). The first successful attempt to develop a stable transformation system for castor using vegetative explants has been described by Sujatha and Sailaja (2005). In this protocol, co-cultivated explants were initially subjected to expansion and proliferation on Murashige and Skoog (MS) medium with 0.5 mg/L thidiazuron followed by 3 cycles of selection on medium with 0.5 mg/L BAP and increasing concentrations of hygromycin (20–40–60 mg/L).

1.6. Prospect of castor under present perspective

The oil extracted from the castor bean (*R. communis*) already has a growing international market, assured by more than 700 uses, ranging from medicines and cosmetics to substituting petroleum in the manufacturing of Biodiesel, plastics and lubricants. Each hectare of castor oil bean plants planted in arid and semi arid regions produces 350-900 kg of oil per hectare. *R. communis* L. oil is critical to many industrial applications because of its unique ability to withstand high and low temperatures

1.6.1. Biodiesel

Biodiesel is nothing but fatty acid methyl or ethyl esters made from edible and non-edible oils and animal fats. Biodiesel is about 10% oxygen by weight and contains no sulfur. The lifecycle production and use of biodiesel produces approximately 80% less carbon dioxide emissions, and almost 100% less sulfur dioxide. The main commodity sources for biodiesel in Bangladesh and India can be non-edible oils obtained from plant species such as Ratanjot (*Jatropha curcus*), Karanja (*Pongamia pinnata*), Castor (*R. communis*) oilseed etc. It contains no petroleum, but it can be blended at any level with petroleum diesel to create a biodiesel blend or can be used in its pure form. The biodiesel is found to burn more efficiently than diesel. The vegetable oil based fuel production is very attractive for developing countries like Bangladesh.

1.6.2. Genomics

The genome is an organism's complete set of DNA and genomics is the study of the genomes of organisms to understand the gene functions. Full genome analysis has been done in main crops to explore gene functions for human benefits. castor bean genomics is also relevant to biosecurity as the seeds contain high levels of ricin, a highly toxic, ribosome inactivating protein. Genomic science has greatly enhanced our understanding of the biological world. It is enabling researchers to "read" the genetic code of organisms from all branches of life by sequencing the four letters that make up DNA. Sequencing genomes has now become routine, giving rise to thousands of genomes in the public databases. A research team co-led by scientists from the J. Craig Venter Institute (JCVI) and the Institute for Genome Sciences (IGS), University of Maryland School of Medicine, today published the sequence and analysis of the castor bean (*R. communis*) genome in Nature Biotechnology. The results of this work show that the genome is 350 Mb and has an estimated 31,237 genes. Because of the potential use of castor bean as a biofuel and its production of the potent toxin ricin, the team focused efforts on genes related to oil and ricin production. They analyzed important metabolic pathways and regulatory genes involved in the production and storage of oils in the castor bean. The team discovered that the ricin gene family was larger than previously thought, and they revealed approximately 28 genes in the ricin producing family. The analyses could be important for comparative studies with other oilseed crops, and could also allow for genetic engineering of castor bean to produce oil without ricin.

1.6.3. GMOs: Ricin-free castor

Ricin, a lethal toxalbumin, inhibits protein synthesis by inactivating ribosomes and is deadly when inhaled, ingested or injected. The major objective of castor genetic transformation in USA is to develop ricin free castor plant varieties with the potential to create new economic opportunities for farmers and processors. The problem is tackled through a multiple approach. Through selection and traditional breeding methods, advanced generation lines (F6) with 70–75% reduction in ricin and *R. communis* agglutinin (RCA120) toxins have been developed (Auld *et al*. 2001, 2003). The second strategy is to develop transgenics using antisense genes for blocking ricin and allergen production that are highly expressed during seed development. The ricin gene is isolated and sequenced, and using mRNA expression of ricin, allergen and numerous lipid biosynthetic enzymes during seed development, promoters useful in expression of genes to suppress toxin and allergen production were identified. However, ricin and *R. communis* agglutinin production are controlled

by multiple genes and hence, transgenic strategies to completely eliminate the toxic components is necessary.

1.6.4. Drug development

The isolation and extraction of novel bioactive secondary metabolites from plants have a biomedical potential for future drug discovery and wide range of novel bioactive secondary metabolites exhibiting pharmacodynamic properties has been isolated from plant sources and many to be discovered. Plant cell cultures represent a potential source of valuable secondary metabolites which can be used as pharmaceuticals. As castor plant has been identified to hold the potential for innumerable medicinal properties its cell culture will lead to produce number of secondary metabolite through cell culture for new drug development .

1.7. Objectives

1) Collection of castor genotypes available in the country and establish a germplasm in the Institute of Biological Sciences, Rajshahi University, Bangladesh.
2) Characterization of genotypes through both genetical and morphological studies.
3) Study of genetic variability and intraspecific diversity.
4) Selection of elite genotypes holding the highest merit of producing non edible oil and lowest level of ricin content.
5) Identification and selection of suitable explant (s) for first response and better regenerative potentials.
6) Selection and standardization of media composition, growth regulator requirement and culture environment for consistently high production of plantlets.
7) Selection of auxin type and concentration and culture condition for efficient rooting of *in vitro* proliferated shoots.
8) Standardization of stable media for rapid callus induction and regeneration.
9) Establishment of cell culture as new and alternative methods for obtaining secondary metabolite from medicinal plant like castor.
10) Test of antibacterial activities by cell extract.

CHAPTER-II

2.1. INTRODUCTION

2.1.1. Brief description of castor

The spurge family (Euphorbiaceae) is one of the most diverse and numerous clades of the angiosperms, including several species of great economic importance as rubber tree (*Hevea brasiliensis*), cassava (*Manihot esculenta*), and some oil seed crops, as candlenut (*Aleurites moluccana*), physic nut (*Jatropha curcas*) and castor bean (*Ricinus communis*). Castor bean, the single member of the African genus *Ricinus* (subfamily Acalyphoideae), presents a wide variation regarding vegetative traits such as leaf and stem colors, number and size of leaf lobes and presence of wax covering the stem (Popova and Moshkin 1986, Savy-Filho 2005, Webster 1994). Depending on the environmental conditions, even the vegetative habit may vary, although it is more likely in a shrubby form (Webster 1994). However, the most conspicuous variability is related to reproductive characters, as color shape and size of seeds, number of flowers per raceme, peduncle length and fruit dehiscence as described by Popova and Moshkin (1986).

Castor oil, which has a long history of use for medicinal purposes (Gaginella *et al*. 1998), has been considered a promising raw material for the production of renewable energy in tropical countries. Besides, castor bean has been traditionally cultivated for the production of lubricants and paints (Berman *et al*. 2011, Ogunniyi 2006, Scholz and Silva 2008). Mainly in the semi-arid regions, a xerophytic-like as castor bean can be grown in areas with higher farming limitations, not intended for other crops (Ogunniyi 2006). Furthermore, the biodiesel derived from castor oil has several advantages over other vegetable oils due to the presence of 5% more oxygen, low levels of residual phosphorus and carbon, high cetan number, solubility in alcohol and absence of aromatic hydrocarbons (Ogunniyi 2006, Scholz and Silva 2008). The high viscosity of the castor oil is due to the high percentage of ricinoleic acid (a hydroxycarboxylic acid), which is a limiting factor for the use of pure castor bean diesel in the engines (Pinzi *et al*. 2009). However, the employment of this biodiesel blended with petrodiesel can be exploited in regions with severe winter. This is a highly recommended procedure because of its low freezing point and the lubricant power afforded by castor oil, as well as all other advantages associated to the utilization of renewable energy resources (Berman *et al*. 2011, Demirbas 2007, Ogunniyi 2006, Pinzi *et al*. 2009, Singh 2011).

2.1.2. Bangladesh perspective

In Bangladesh, castor plant growing all over the country without any special care. It is known as seed oil but recent time it is very important particularly for its holding merits for biofuel. In Bangladesh, castor oil is various used as industrial oil. It is reported that every year Bangladesh imports castor oil/castor seeds of 1000 crore taka from Srilanka, India, China and other countries (Wohab 2000). There is no utilization of castor as field crop in Bangladesh and has no recommended variety in our country. Improvement and designed of high yielding varieties of castor will be able reduce the foreign cost of our country.

A number of varieties of castor found to grow here and there over the country with distinct morphological varieties. In Bangladesh no research work has been done on the morphological study and characterization of these castor varieties grown in the country and no research endeavour is made to develop culture practices in its cultivation. The plant and its cultivation has been running out of the research peripheries of national research agencies as it is not a mandated crop of any agency. But it is a very important plant holding tremendous merit in regards of economic importance. Thus castor plant in Bangladesh provides a virgin field of research in variety evaluation, characterization and its improvement. In India there has been a national research centre named as Centre as Jatropha promotion and Biodiesel (CJP) with mandated two crops jatropha and castor and very recently they organized a world conference exclusively on castor held on 25-26 February at Jodhpur, India. In Bangladesh it is not a mandated crop of any national agencies no research endeavour was made in the area of improvement and exploitation of castor germplasm in the country.

2.1.3. Necessity of estimation of variation for improvement

Variation is the basis of improvement and germplasm represents the sum total of variability or hereditary materials or genes available in particular genus or species (Dandin 1989). Germplasm is the basic foundation of crop improvement and its importance was realized as far back as 1898 (Boraiah 1986). Which the advent of last decade, the major break through in the genetic improvement in crops has come through in the utilization of germplasm. The green revolution was basically due to the high utilization of “Norine-10” genes which resulted in doubling of the yield in wheat all over the world. Even in case of paddy, almost all the modern varieties of paddy has been developed through utilization of dwarf and high fertilizers responsive genotypes “Dee-geowoo” (Shivashankar 1989).

Germplasm is the raw material of crop improvement and considered as the living museum of the sum total of variability. Scientists all over the world are realizing its importance for future needs. Collection, conservation and evaluation of the existing gene pool are the prime task of plant breeding for all the crop improvement programme. Exploration of existing variability by systematic exploration and collection of primitive land races, wild relative, undomesticated forms and related weedy species are the need of the day. Preservation of this natural wealth is more important as their existence is under threat. Cultivation of species varieties and introduction of improved strains have resulted in the rapid elimination of primitive land races having wider adaptability. Extensive farming and over grazing have destroyed the natural undomesticated forms. To meet the demands of diverse nature, plant breeding programme requires wider spectrum of genetic base than ever before. Thinking over the future needs general awareness and concern have developed among the plant breeders all over the world to preserve the existing genetic wealth and prevent further loss of genotypes.

The conservation and utilization of genetic resources are the two vital components of varietals improvement programme. Without systematic evaluation on nature and magnitude of variation, existing strains, the available gene pool in the germplasm collection can not be utilized to the full extent. The most promising source of genes, controlling resistance to pest or tolerance to adverse conditions are likely to be found in minor varieties or special purpose types or primitive land races.

Under the present perspectives it is necessary to make a survey on the existing germplasms of castor over the country in order to preserve the land races with their particular gene pools for breeding purposes.

2.1.4. Morphological studies in castor

Plant morphology is useful in the visual identification of plants. Plant morphology or phytomorphology is the study of the physical form and external structure of plants whish is important to characterize the plant species particularly in classification. It "represents a study of the development, form, and structure of plants and by implication, an attempt to interpret these on the basis of similarity of plan and origin”. Plant morphology observes both the vegetative (somatic) structures of plants, as well as the reproductive structures. The vegetative structures of vascular plants includes the study of the shoot system, composed of stems and leaves, as well as the root system. The reproductive structures are more varied, and are usually specific to a particular group of plants. A living plant always has embryonic tissues. The way in which new structures mature as they are produced may be affected by the

point in the plants life when they begin to develop, as well as by the environment to which the structures are exposed. A morphologist studies this process, the causes, and its result.

It is generally recognized that intra-specific variation is multidimensional and that the unidimensional classification common in taxonomy is unlikely to deal satisfactory with the classification at the intraspecific level (Heywood 1963). It can also be then argued that the description of the variation at the intraspecific level is essential for proper taxonomic treatment of the variation at the species level. *R. communis* L. is only one species in the genus *Ricinus* morphological differences did not exit far apart what can widen the plant into different species. Morphological differences between the different land races always covers the range within intra species level. *R. communis* L. plants exhibit much variation regarding the size and ornamentation of the regma (fruit) and color and patterning of the seeds, fruits, leaves and pollens. On the basis of which nearly 20 varieties and some 20 formae have been recognized (Meikle 1985).

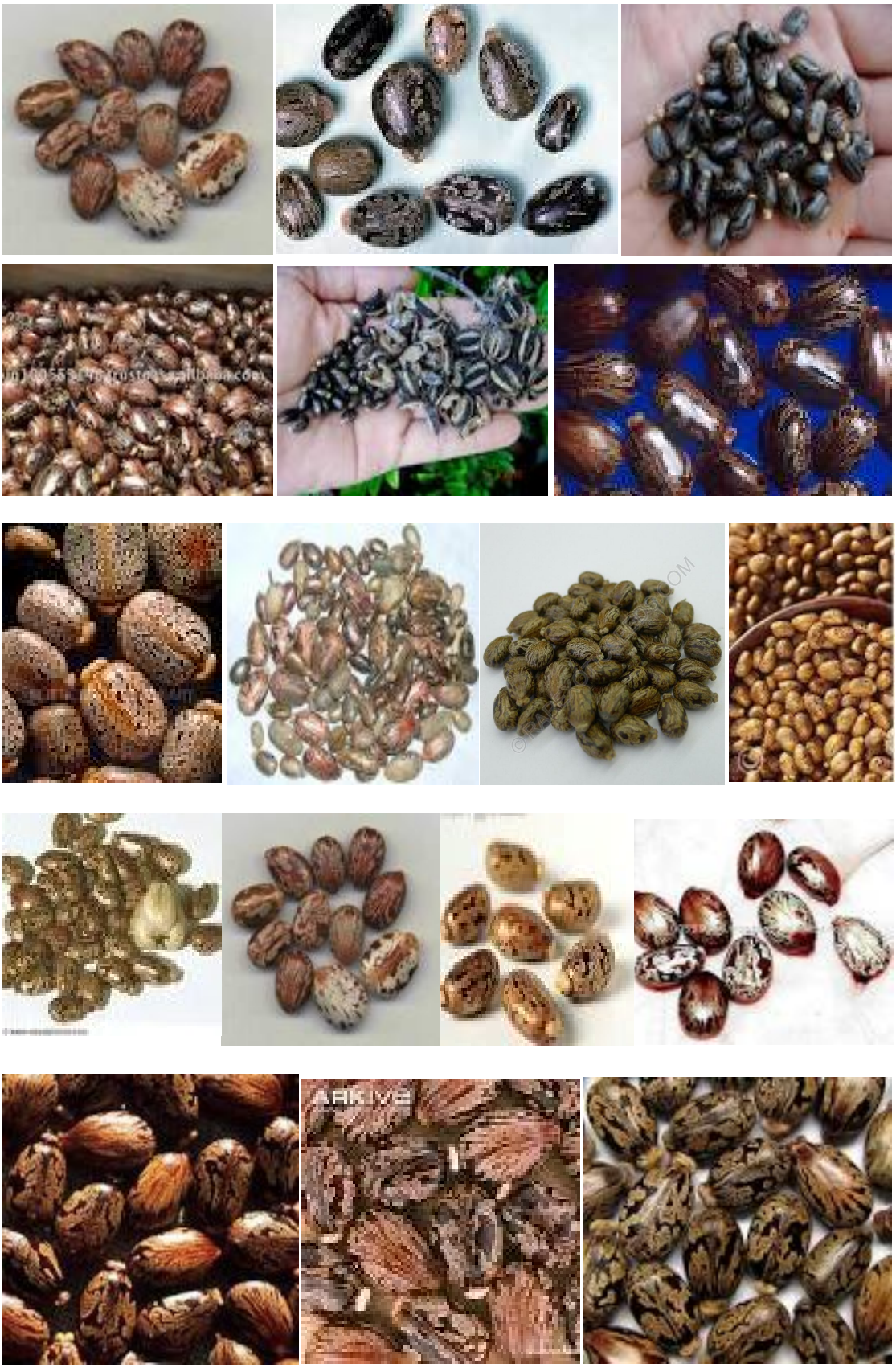
Ricinus L. is a monotypic genus probably native to north east tropical Africa, but now widely cultivated throughout the tropics, subtropics and warm temperate regions and often becomes naturalized presumably in the Sudanian and E. Africa regions (Meikle 1985). It is considerd a weed especially in the Mediterranean region, notably in Greece, Egypt, Italy and Morocco. It is subspontaneous in the hotter parts of Palestine, in the Dead sea area (Arnon River Delta) it occurs in dense groves; also common wadies, at road side, waste land, along the dried-up streams, abandoned gardens and in waste places (Zohary 1987). In Egypt, it is mainly localized in the irrigated lands of the nile valley and Delta with a few infestations in Egyptian desert (Wadi Allagi, Wadi Defiat, Egate, Elba, Sinai).

DIVERSITY IN MORPHOLOGICAL CHARACTERS IN CASTOR

DIVERSITY IN FLOWERING CHARACTERS IN CASTOR

DIVERSITY IN SEED POD FORMATION IN CASTOR

DIVERSITY IN SEED CHARACTERS IN CASTOR



A very wide range of morphological variations existent in the germplasms growing in the Bangladesh. Some grows annually but some live longer time more than one year. Some genotypes are dwarf in nature not growing much longer but produced number of stems presenting a bushy structure. Genotypes with longer shoots reaches up to 20 feet in height giving a number of lateral shoots around then particularly from upper part. Varieties or germplasms of castor growing here and there in the country are not properly cultivated and harvested but it grows spontaneously just dispersing the ripen seeds around then. No bodies are there found to care them but it grows well in Bangladesh giving two crops in a year. Castor plants mainly grows in homesteads, pond sides, road sides and other fellow lands but the plant can withstand extreme drought condition. It grows for three months to give flower and the color of the flower varieties greatly among the different genotypes. Flowers occur in dense terminal clusters (inflorescences), with female flowers just above the male flowers. The inflorescence size also varies according to the genotypes. This species is clearly monoecious with separate male and female flowers on the same individual. There are no petals and each female flower consists of a little spiny ovary (which develops into the fruit or seed capsule), and a bright colored feathery stigma lobes that receives pollen from male flowers. Each male flower consists of cluster of many stamens which shed pollen in a gust of wind.

2.1.5. Objectives

1) Collection of possible land races growing in the country and their characterization.
2) Study of morphological diversities of collected cultivars from different corners of the country.
3) To estimate the phenotypic and genotypic variability present in different characters.
4) The estimates of heritability for vegetative growth, reproductive growth, yield and yield components of castor genotypes in Bangladesh.
5) Selection of elite genotypes holding the highest merit of producing non-edible oil.

2.2. MATERIALS AND METHODS

2.2.1. Materials

The experiments was conducted in research field of the Institute of Biological Sciences in the University of Rajshahi, Bangladesh. We have collected all available genotypes from different corners of the country and established a germplasm in the research field during the year 2010. The seeds were sowing by Latin Square Design.

V_1	V_2	V_3	V_4	V_5	V_6
V_2	V_3	V_4	V_5	V_6	V_1
V_3	V_4	V_5	V_6	V_1	V_2
V_4	V_5	V_6	V_1	V_2	V_3
V_5	V_6	V_1	V_2	V_3	V_4
V_6	V_1	V_2	V_3	V_4	V_5

V_1= Cultivar shabje, V_2= Cultivar shadatae, V_3= Cultivar roktima, V_4= Cultivar lalchay, V_5= Cultivar badami, V_6= Cultivar dhusar.

2.2.2. Methods

Methodologies of the experiments under morphological studies are described below:

A. Stem characters:

1. Plant height (cm):

Plant height was measured from soil surface to the tip of plant. It was measured by centimeter. Height of the plant (cm) were estimated as follows:

$$\text{Plant height (cm): } = \frac{Sum\ of\ all\ plants\ height\ of\ a\ cultivar}{Number\ of\ plants\ in\ a\ cultivar}$$

2. Stem diameter (cm):

Average measurement was taken in middle position of the plant. Stem diameter (cm) were estimated as follows :

$$\text{Stem diameter (cm)} = \frac{Sum\ of\ diameter\ of\ all\ plants\ of\ a\ cultivar}{Number\ of\ plants\ in\ a\ cultivar}$$

3. Branch number:

More than thirty centimeter long branch was counted from bottom to the tip of plant of a cultivar. Number of main branch of a plant were estimated as follows:

$$\text{Branch number} = \frac{Sum\ of\ all\ main\ branch\ number}{Number\ of\ plants}$$

4. Branch length (cm):

Branch length (more than 30 cm long) measured from its base to the tip of branch. Branch length (cm) were estimated as follows:

$$\text{Branch length (cm)} = \frac{Sum\ of\ the\ length\ more\ than\ 30\ cm.long\ branches}{Number\ of\ more\ than\ 30\ cm.long\ branches}$$

5. Node number:

Node number was counted from bottom to the top of plant of a cultivar. Total number of node in a plant were estimated as follows:

$$\text{Node number} = \frac{Sum\ of\ the\ node\ number\ of\ all\ the\ plants\ of\ a\ cultivar}{Number\ of\ plants\ in\ a\ cultivar}$$

6. Internodal distance (cm):

$$\text{Internode length (cm)} = \frac{Sum\ of\ internode\ length\ of\ all\ the\ plants\ of\ a\ cultivar}{Number\ of\ in\ ternode\ of\ all\ plants\ in\ a\ cultivar}$$

B. Leaf Characters:

Leaf shape and size were very diverse in nature in different cultivars of Castor. For this study length of leaf, breadth of leaf, lobes number, petiole length and diameter and leaf area were measured.

7. Leaf length (cm):

Mature leaves were taken for this study and ten leaves were randomly collected from each plant under the same replication of each cultivar. Individual leaf length were measured from leaf base to leaf apex by scale. Then the total amount was divided by ten.

8. Leaf breadth (cm):

Mature leaves were taken for this study and ten leaves were randomly collected from each plant under the same replication of each cultivar. Individual leaf breadth were measured the cross length of one end to the other end of leaf lamina measured by scale. Then the total amount was divided by ten.

9. Lobes number:

Mature leaves were taken for this study and ten leaves were randomly collected from each plant under the same replication of each cultivar. Individual leaf lobes were counted. Then the total amount was divided by ten.

10. Petiole length (cm):

Ten petiole were randomly collected from each plant under the same replication of each cultivar and individual petiole length (the length between the base of the plant and point of insertion or junction of leaf) were measured by scale. Then the total amount was divided by ten.

11. Petiole diameter:

Ten petiole were randomly collected from each plant under the same replication of each cultivar and individual petiole diameter were measured by scale. Then the total amount was divided by ten.

12. Leaf area (cm^2):

Ten leaf were randomly collected from each plant under the same replication of each cultivar and individual leaf area were measured by leaf area meter. Then the total amount was divided by ten.

C. Root Characters:

Three plants were randomly taken of a cultivar in a replication at the first flowering time and uprooted carefully without damaging the root system. Roots were washed in pond water throughly to remove all the soil particles adhering to them. After clearing roots were separated and following data were taken.

13. Root length (cm) = $\frac{Sum\ of\ all\ tap\ root\ lendth\ in\ a\ cultivar}{Number\ of\ tap\ root\ in\ a\ cultivar}$

14. Lateral roots number = $\frac{Total\ number\ of\ lateral\ roots\ of\ the\ plants}{Number\ of\ plants}$

15. Lateral root length (cm) = $\frac{Total\ length\ of\ lateral\ roots\ of\ the\ plants}{Total\ number\ of\ lateral\ roots}$

16. Inflorescence number:

$$= \frac{Total\ number\ of\ inflorescence\ of\ all\ plants\ in\ a\ cultivar}{Total\ number\ of\ plants\ in\ a\ cultivar}$$

17. Number of male flower:

Ten inflorescences were randomly collected from each cultivar and were counted the number of male flowers. Then the total number was divided by ten separately.

18. Number of female flower:

Ten inflorescences were randomly collected from each cultivar and were counted the number of female flowers. Then the total number was divided by ten separately.

19. Male flower's region (cm):

Ten inflorescences were randomly collected from each cultivar and were measured by scale the region of male flower. Then the total amount was divided by ten.

20. Female flower's region (cm):

Ten inflorescences were randomly collected from each cultivar and were measured by scale the region of the region of female flower. Then the total amount was divided by ten.

21. Fruits number:

Ten inflorescences were randomly collected from each cultivar and were counted the fruit number. Then the total fruit number was divided by ten.

$$\text{Fruits number} = \frac{Total\ number\ of\ fruits}{Number\ of\ inflorescences}$$

22. Thorn number:

Open pollinated matured fruits were taken for this study and twenty fruits were randomly collected from each plant under the same replication of each cultivar. Individual fruit thorn were counted.

$$\text{Thorn number} = \frac{Total\ number\ of\ thorns}{Total\ number\ of\ fruits}$$

23. Flowering time (days):

Recorded the first flowering time (days) of all plants in a cultiver. The total number of time (days) was divided by the total number of plants.

$$\text{Flowering time (days)} = \frac{Total\ number\ of\ time\ (days)}{Total\ number\ of\ plants}$$

24. Seed number:

Twenty fruits were taken randomly from each cultivar and were crashed by the finger tip and seeds were separated and counted seeds. Then the total seeds number was divided by the total fruits number.

$$\text{Seed Number} = \frac{Total\ number\ of\ seeds}{Total\ number\ of\ fruits}$$

25. Seed Length (cm):

Twenty seeds were taken randomly from the each cultivar and measured the length in centimeter. Then the total amount was divided by the number of seeds.

$$\text{Length of seed} = \frac{Total\ length\ of\ seeds}{Number\ of\ seeds}$$

26. Seed Breadth (cm):

Twenty seeds were taken randomly from the each cultivar and measured the breadth in centimeter. Then the total amount was divided by the number of seeds.

$$\text{Seed breadth} = \frac{Total\ breadth\ of\ seeds}{Number\ of\ seeds}$$

27. Seed weight (gm):

Total seeds were collected separately from five plants. The plants were taken randomly in a cultivar and weight was taken by electronic balance. Then the total seeds weight (gm) was divided by the number of plants.

$$\text{Seed weight (gm)} = \frac{Total\ weight\ of\ seeds}{Total\ number\ of\ plants}$$

28. Hundred seeds weight (gm):

Hundred seeds were randomly taken separately from five plants of a cultivar and weight was taken separately by electronic balance. Then the total weight (gm) number was divided by the number of plants.

$$\text{Hundred seeds weight (gm)} = \frac{Total\ weight\ of\ seeds}{Total\ number\ of\ plants}$$

Techniques of analyzing data:

The collected data were analyzed following the biometrical techniques of analysis development by Mather (1949) based on mathematical model of Fisher *et al.* (1932). Mean, coefficient of variation and least significant difference were worked out by the method of analysis of variance. The techniques of analysis of data used are described under the following sub-heads:

i) Mean: It is the arithmetic mean or average and computed by dividing the sum of all observations in a sample by their number. It was computed by using the following formula:

$$\text{Mean} = \frac{\sum_{i=1}^{n} x_i}{n}$$

Where,

x = Individual reading recorded on each plant.

n = Number of observation.

i = 1, 2, 3.....n

Σ = Summation.

ii) Range: It is the difference between the lowest and highest values present in the observation included in the study. In this study lowest and highest values are shown to represent the range of variation.

iii) Analysis of variance: Variance analysis is a measure of dispersion of a population or sample. So, for testing the significant difference among the population or samples, the analysis of variance is necessary. It is expressed as the sum of square of deviations of all observations of a sample from its mean and divided by the degree

of freedom (n-1). It was computed by using formula following Sing and Chaudhary (1977) as follows:

$$\sigma^2 = \frac{\sum_{i-1}^{n} x_i^2 - \frac{\left(\sum_{i-1}^{n} x_i\right)^2}{n}}{n-1}$$

Where,

σ^2 = Variance.

x = Individual reading.

n = Total number.

Σ = Summation.

i = 1, 2, 3.....n

n-1 = Degree of freedom.

In analysis of variance, the fixed model was used and expected mean sum square (EMS) was determined as follows:

Analysis of variance (ANOVA)

Sources of variation	df	MS	EMS
Total	GR-1	MS_t	
Genotypes (G)	G-1	MS_g	$\sigma^2_e + r\,\sigma^2_g$
Replication (R)	R-1	MS_r	$\sigma^2_e + g\,\sigma^2_r$
Error	(G-1) (R-1)	MS_e	σ^2_e

Where,

G = Genotype.

R = Replication.

MS_t = Mean square of total.

MS_g = Mean square of genotypes.

MS_r = Mean square of replication.

MS_e = Mean square of error.

σ^2_g = Variance due to genotype.

σ^2_e = Variance due to environment.

iv) Component of variance: The components of variance were of three types viz. genotypic variance (σ^2_g), phenotypic variance (σ^2_p) and environmental variance (σ^2_e). These components were estimated following the methods as described by Sing and Chaudhary (1977).

Genotypic variance $(\sigma^2_g) = \frac{MSg - MSe}{r}$

Phenotypic variance $(\sigma^2_p) = \sigma^2_g + MS_e$

Environmental variance $(\sigma^2_e) = MS_e$

v) Coefficient of variability: Coefficient of variability at phenotypic, genotypic and environmental levels were computed following Johnson *et al*. (1955) and Burton and De Vane (1953) as follows:

(a) Phenotypic coefficient of variability $(CV_p) = \frac{\sqrt{\sigma^2_p}}{\bar{x}} \times 100$

(b) Genotypic coefficient of variability $(CV_g) = \frac{\sqrt{\sigma^2_g}}{\bar{x}} \times 100$

(c) Environmental coefficient of variability $(CV_e) = \frac{\sqrt{\sigma^2_e}}{\bar{x}} \times 100$

Where,

σ^2_p = Phenotypic variance.

σ^2_g = Genotypic variance.

σ^2_e = Environmental variance.

$\bar{x}$ = Grand mean.

vi) Heritability (h^2_b):

It is the ratio of genotypic variance to the phenotypic variance and it was worked by using following formula as suggested by Warner (1952).

$$h^2_b = \frac{\sigma^2_g}{\sigma^2_p} \times 100$$

Where,

h^2_b = Heritability in broad sense.

σ^2_g = Genotypic variance.

σ^2_p = Phenotypic variance.

2.3. RESULTS

2.3.1. Nature and extend of variations in colour in different cultivars of castor

In the present investigation an attempt was made to access the nature and extend of variations in some qualitative characters. The seeds were sowing by Latin Square Design. In this design every treatment is assigned to a row and a column only one. So, there is a double restriction instead of one. As a result, every row and every column looks like a complete replication or a block, because reduce experimental error in both directions. The results are presented in the Table 2.3.1. Data were taken at the first flowering time of all cultivars .

(1) Colour of stem: It was observed that the colour of stem of cultivar shabje was bright green, cultivar shadatae was whitish green, cultivar roktima was dark red, cultivar lalchay was reddish, cultivar badami was brown and cultivar dhusar was light brown in colour (Plate 2.3.3, A-F).

(2) Colour of node: It was observed that the colour of node of cultivar shabje was green, cultivar shadatae was whitish, cultivar roktima was dark red, cultivar lalchay was bright reddish, cultivar badami was pinkish brown and cultivar dhusar was reddish brown in colour (Plate 2.3.2, A-F) .

(3) Colour of leaf: The colour of upper surface and lower surface of leaf of cultivar shabje was noted green, the upper surface of cultivar shadatae was whitish green and lower surface was whitish. In cultivar roktima both the upper surface and lower surfaces were reddish green, in cultivar lalchay both the surfaces of leaf were bronze green, cultivar badami, the upper surface of leaf was light green. In cultivar dhusar, the upper surface was green but the lower surface was light green (Plate 2.3.5 and 2.3.6, A-F).

(4) Colour of petiole: In cultivar shabje it was observed that the colour of petiole was bright green, in cultivar shadatae was whitish, in cultivar roktima was reddish bronze, in cultivar lalchay was greenish bronze, in cultivar badami was pinkish white and cultivar dhusar was pinkish white in colour (Plate 2.3.9, A-F).

(5) Colour of vein junction: The colour of vein junction of leaf was also different in different cultivars and it was observed that in cultivar shabje was bright green, in cultivar shadatae was white, in cultivar roktima was reddish red, in cultivar lalchay was reddish, in cultivar badami was light reddish and cultivar dhusar was greenish brown in colour (Plate 2.3.5 and 2.3.6, A-F).

(6) Colour of male flower: It was observed that the colour of male flowers of all cultivars was whitish yellow (Plate 2.3.11, A-F).

(7) Colour of female flower: In cultivar shabje, the colour of stigma was yellowish and ovary was green, in cultivar shadatae, the stigma was yellowish and ovary was greenish white, in cultivar roktima the stigma was bright red and the ovary was reddish. In cultivar lalchay, the stigma was red and the ovary was light reddish. In cultivar badami, the stigma was magenta and the ovary was light green. In cultivar dhusar, the stigma was pinkish and ovary was light green (Plate 2.3.10, A-F).

(8) Colour of seed: The colour of seed in cultivar shabje was dark brown, in cultivar shadatae was dark brown. In cultivar roktima was light reddish brown, in cultivar lalchay it was brown, in cultivar badami the colour was brown and in cultivar dhusar it was brown (Plate 2.3.11, G-L).

(9) Shape of seed: It was observed that the shape of seed in all cultivars were egg shaped (Plate 2.3.11, G-L).

(10) Colour of seed pod: The colour of seed pod in cultivar shabje was green, in cultivar shadatae was greenish white, in cultivar roktima was light reddish green, in cultivar lalchay was reddish, in cultivar badami was green and in cultivar dhusar it was green in colour (Plate 2.3.12, A-F).

Table 2.3.1: Colour of different vegetative structures of different cultivars of castor plant.

2.3.2. Variations of some quantitative characters

Different genotypes of castor were collected from different places of the country and a germplasm bank was established in the Institute of Biological Sciences in Rajshahi University. We studied 28 morphological characters and characterized six distinct cultivars. Distinct variation is very obvious even in visual observation on stems, leaves, petioles, inflorescence and flower, six distinct cultivars were recognized as cultivar shabje, cultivar shadatae, cultivar roktima, cultivar lalchay, cultivar badami and cultivar dhusar based on local performance. Seeds of six cultivars were planted in May 2010 under the experimental plots and the plants attend maturity with in 3 months to 4 months.

Data were harvested on the parameter of the vegetative growth were at the time of first flowering. But other characters were recorded in maturity of the flower and harvesting of seed respectively.

In this piece of research work an attempt was taken to assess the genotypic influences on the nature and extent of variation in different morphological characters of different castor germplasms collected from different coners of the country. The characters under this study were arranged in three groups depending on their morphological nature.

(i) VEGETATIVE GROWTH: (1) Plant height (cm), (2) Stem diameter (cm), (3) Branch number, (4) Branch length (cm), (5) Node number, (6) Internodal distance(cm), (7) Leaf length (cm), (8) Leaf breadth (cm), (9) Leaf area (cm^2), (10) Lobes number, (11) Petiole length (cm), (12) Petiole diameter (cm), (13) Root length (cm), (14) Lateral root number, (15) Lateral root length (cm).

(ii) REPRODUCTIVE GROWTH: (1)inflorescence number, (2) Male flower's region (cm), (3) Female flower's region (cm), (4) Number of male flower, (5) Number of female flower, (6) Fruit number, (7) Thorn number, (8) Flowering time (days).

(iii) YIELD AND YIELD COMPONANTS: (1)Seed number, (2) Seed length (cm), (3) Seed breadth (cm), (4) Seed weight (gm), and (5) Hundred seeds weight (gm).

Mean performance of each cultivars are shown in the Table 2.3.2a, 2.3.2b and 2.3.2c with their CV (%) and LSD values. Analysis of variance were calculated for each character to test the significant difference among the cultivars studied and are shown in the Table 2.3.3a, 2.3.3b and 2.3.3c. Range with genetic parameters are

presented in the Table 2.3.4a, 2.3.4b and 2.3.4c. Results of each character is described under the following heads.

(i) VEGETATIVE GROWTH:

(1) Plant height (cm)

Plant height was measured for all the six cultivars at first flowering stage and their mean values are presented in the Table 2.3.2a. The Table 2.3.3a shows a significant difference existed in plant height in different cultivars of castor (Plate 2.3.1, A-F). Cultivar shabje showed the highest plant height (466.110. cm. a) and cultivar dhusar showed the lowest height (201.163. cm. d) among the cultivars included in the study. Range of variation and genetic parameter were calculated for plant height among the cultivars and presented in Table 2.3.4a. High range of variation (201.16 – 466.11) were present in the character among the cultivars. Phenotypic variance (12459.63) and coefficient of variation (36.226) were higher than the genotypic and environmental variance and coefficient of variation. Genotypic variance (10304.825) and coefficient of variability (32.945) were also higher than the environmental variance (2154.805) and coefficient of variability (15.065). High heritability (82.7) and low level of difference between phenotypic and genotypic variance indicates that the character is heritable in nature and the major portion of the phenotypic variance is contributed by the genotype.

(2) Stem diameter (cm)

At the first flowering stage stem diameter was measured for all the six cultivars and their mean values are presented in the Table 2.3.2a. The Table 2.3.3a shows a significant difference existed in stem diameter in different cultivars of castor. Cultivar shabje showed the highest value (25.770 cm. a) and lowest in cultivar dhusar (9.193 cm. e) among the cultivars included in the study. The Table 2.3.3a shows a significant difference existed in stem diameter in different cultivars of castor. Table 2.3.4a presents the range of variation was 9.19 – 25.77 cm. Phenotypic variance (48.908) and coefficient of variability (44.799) were higher than both the genotypic and environmental variances and coefficient of variabilities as expected. Environmental variance (0.081) and coefficient of variability (1.81) for this character was higher than its corresponding genotypic variance (48.907) and coefficient of variability (44.479). High heritability (99.9) for this character indicated that the character is heritable and very less influenced by the environment.

(3) Branch number.

The Table 2.3.2a shows the mean values of six cultivars of castor and the Table 2.3.3a shows that the characters are significantly different among the cultivars. Highest branch number (11.050 a) was found in cultivar shabje and lowest (5.440 d) in cultivar dhusar. Range of difference (5.44 – 11.05) is given in the Table 2.3.4a, pronounced the existence of high range of variability. Environmental variance (0.120) and coefficient of variability (4.358) was lower than both of its corresponding phenotypic and genotypic variances and coefficient of variabilities. Phenotypic variance (5.479) for this character was significantly higher than the genotypic variance (5.359) indicates that the major part of the phenotypic variation was due to genotypic in nature. Heritability (97.8) for this character was also significantly high.

(4) Branch length (cm)

Mean values of branch length are presented in the Tables 2.3.2a. Cultivar shabje showed the highest value (183.633 cm. a) and cultivar lalchay showed the lowest value (37.493 cm. e). The Table 2.3.3a shows that the cultivars under the study were significantly different in respect of branch length. Range of variation were 37.49 – 183.63 as shown in the Table 2.3.4a. Phenotypic variance (2924.302) and coefficient of variability (62.454) were higher than both the genotypic and environmental variance and coefficient of variation respectively. Heritability for this character as calculated from the component of variance was also high (99.6). Genetic variance (2911.906) was also found higher than environmental variance (12.396).

(5) Node number

Total node numbers were also significantly different among the cultivars as indicated in the Table 2.3.3a. Highest number of node was found in cultivar shabje (58.773 a) and lowest in cultivar dhusar (30.497 e) as presented in the Table 2.3.2a. Table 2.3.4a showed the high range of variation (30.49 – 58.77) in total number of node among the cultivars. Genotypic variance (162.611) and coefficient of variability (30.159) were more or less near to its phenotypic variance (162.753) and coefficient of variability (30.172) and higher than environmental variation (0.142) and environmental coefficient of variation (0.891) suggested that the major part of the phenotypic variation of this character was contributed by the genotypes. High heritability (99.9) for this character indicates that the character was very less influenced by the environment and heritable in nature.

(6) Internodal distance (cm)

Mean values of internodal distance are shown in the Table 2.3.2a. Cultivar shabje showed the highest internodal length (8.760 a) and cultivar dhusar showed the lowest length of internode (7.270 c) among the cultivars included in the study. Regarding this character a significant difference was found among the cultivars studied as shown in the Table 2.3.3a (Plate 2.3.4, A-F). The Table 2.3.4a shown the range of variation (6.11 – 8.76) were present in the character among the cultivars. Phenotypic variance (1.021) and coefficient of variation (13.408) were higher than the genotypic and environmental variance and coefficient of variation. Genotypic variance (0.972) and coefficient of variability (13.083) were also higher than the environmental variance (0.049) and coefficient of variability (2.937). High heritability (95.2) and low level of difference between phenotypic and genotypic variance indicates that the character is heritable in nature and the major portion of the phenotypic variance is contributed by the genotype.

(7) Leaf length (cm)

Leaf length was measured for all the six cultivars at first flowering stage and their mean values are presented in the Table 2.3.2a. The highest leaf length showed in cultivar shabje (81.293 cm. a) and lowest in cultivar dhusar (54.530 f).As shown in the Table 2.3.3a the leaf length (cm) was significantly different among the cultivars (Plate 2.3.7, A-F). Range of variation are presented in the Table 2.3.4a and it was 54.53 – 81.29. Phenotypic variance (106.329) and coefficient of variability (14.999) were higher than both the genotypic and environmental variances and coefficient of variabilities as expected. Environmental variance (0.126) and coefficient of variability (0.516) for this character was higher than its corresponding genotypic variance (106.203) and coefficient of variability (14.991). High heritability (99.9) for this character indicated that the character is heritable and very less influenced by the environment.

(8) Leaf breadth (cm)

The Tables 2.3.2a showed the leaf breadth of six cultivars of castor including in the study. Highest number of leaf breadth was found (51.723 a) in cultivar shabje and lowest (33.887 e) in cultivar dhusar. Table 2.3.3a showed that the character was significantly different among the cultivars (Plate 2.3.8, A-F). Range of difference (33.88 – 51.69) was given in the table 2.3.4a pronounced the existence of high range of variability. Environmental variance (0.260) and coefficient of variability (1.168) was lower than both of its corresponding phenotypic and genotypic variances and

coefficient of variabilities. Phenotypic variance (52.783) for this character was higher than the genotypic variance (52.523) indicates that the major part of the phenotypic variation was due to genotypic in nature. Heritability (99.5) for this character was also significantly high.

(9) Leaf area (cm^2)

Tables 2.3.2a shows the mean values of six cultivers of castor on contest of leaf area and the highest leaf area showed cultivar shabje (908.273 cm^2. ab) and lowest in cultivar dhusar (605.163 cm^2. c). Table 2.3.3a presented that the cultivars are significantly different among. Range of variation as shown in the Table 2.3.4a was 525.66 – 908.27. Phenotypic variance (37169.57) and coefficient of variability (24.201) were higher than both the genotypic and environmental variances and coefficient of variabilities as expected. Environmental variance (8834.420) and coefficient of variability (11.798) for this character was higher than its corresponding genotypic variance (28335.15) and coefficient of variability (21.129). Heritability (76.2) for this character indicated that the character is heritable and very less influenced by the environment.

(10) Lobes number

Mean values of lobes number of six cultivars of castor are shown in the Table 2.3.2a. Cultivar shabje showed the highest value (10.773 a) and cultivar badami showed the lowest value (8.663d). Table 2.3.3a revealed that the cultivars under the study were significantly different in respect of lobes number. Range of variation were 8.61 – 10.77 as shown in the Table 2.3.4a. Phenotypic variance (0.913) and coefficient of variability (9.869) were higher than both the genotypic and environmental variance and coefficient of variation respectively. Heritability for this character as calculated from the component of variance was also high (98.8). Genetic variance (0.902) was also found higher than environmental variance (0.011).

(11) Petiole length (cm)

Petiole length mean values are presented in the Table 2.3.2a. It was found the highest mean value showed cultivar shabje (50.560 a) and lowest in cultivar dhusar (34.107f). Table 2.3.3a also showed that the cultivars are significantly different among them (Plate 2.3.9, A-F). Table 2.3.4a showed the high range of variation (34.10 – 50.56) on the basis of petiole length. Genotypic variance (37.784) and coefficient of variability (14.543) were more or less near to its phenotypic variance (37.947) and coefficient of variability (14.574) and higher than

environmental variation (0.163) and environmental coefficient of variation (0.955) suggested that the major part of the phenotypic variation of this character was contributed by the genotypes. High heritability (99.6) for this character indicates that the character was very less influenced by the environment and heritable in nature.

(12) Petiole diameter (cm)

Regarding this character a significant difference was found among the cultivars studied which are presented in the Table 2.3.2a. Mean values are presented in the Table 2.3.3a where Cultivar shabje showed the highest diameter (4.083 cm. a) and cultivar dhusar showed the lowest diameter (3.013 cm. c) among the cultivars included in the study. Table 2.3.4a showed the range of variation (3.01 – 4.08) were present in the character among the cultivars. Phenotypic variance (0.225) and coefficient of variation (12.967) were higher than the genotypic and environmental variance and coefficient of variation. Genotypic variance (0.22) and coefficient of variability (12.822) were also higher than the environmental variance (0.005) and coefficient of variability (1.93). High heritability (97.8) and low level of difference between phenotypic and genotypic variance indicates that the character is heritable in nature and the major portion of the phenotypic variance is contributed by the genotype.

(13) Root length (cm)

Tables 2.3.2a presents that highest root length (65.887 a) was found in cultivar shabje and lowest (41.217 e) in cultivar dhusar (Plate 2.3.13, A-F). Table 2.3.3a showed that the character was significantly different among the cultivars. Range of difference (41.21 – 65.88) was given in the Table 2.3.4a pronounced the existence of range of variability. Environmental variance (1.547) and coefficient of variability (2.325) was lower than both of its corresponding phenotypic and genotypic variances and coefficient of variabilities. Phenotypic variance (97.811) for this character was higher than the genotypic variance (96.237) indicates that the major part of the phenotypic variation was due to genotypic in nature. Heritability (98.4) for this character was also significantly high.

(14) Lateral root number

Mean values are s presented in the Tables 2.3.2a. Cultivar shabje showed the highest value (34.273. a) and cultivar dhusar showed the lowest value (17.830. e). Table 2.3.3a revealed that the cultivars under the study were significantly different in respect of lateral root number. Range of variation were 17.83 – 34.27 as shown in the Table2.3.4a. Phenotypic variance (45.123) and coefficient of variability (27.636) were

higher than both the genotypic variance (44.767) and environmental variance (0.356) and genetic coefficient of variation (27.526) and environmental coefficient of variation (2.455) respectively. Heritability for this character as calculated from the component of variance was also high (99.2).

(15) Lateral root length

Highest lateral root length was found in cultivar shabje (62.743 a) and lowest in cultivar dhusar (35.323 d) as presented in the Table 2.3.2a. Lateral root length was also significantly different among the cultivars as indicated the Tables 2.3.3a. Table 2.3.4a showed the high range of variation (35.32 – 62.74) in lateral root length among the cultivars. Genotypic variance (129.828) and coefficient of variability (23.815) were more or less near to its phenotypic variance (133.075) and coefficient of variability (24.11) and higher than environmental variation (3.247) and environmental coefficient of variation (3.766) suggested that the major part of the phenotypic variation of this character was contributed by the genotypes. High heritability (97.6) for this character indicates that the character was very less influenced by the environment and heritable in nature.

(ii) REPRODUCTIVE GROWTH:

(1) Inflorescence number

Mean values are presented in the Table 2.3.2b. Cultivar shabje showed the highest inflorescence number (19.030 a) and cultivar dhusar showed the lowest inflorescence number (5.997 c) among the cultivars included in the study. Regarding this character a significant difference was found among the cultivars studied as presented in the Table 2.3.3b. As shown in the Table 2.3.4b range of variation (5.99 – 19.03) were present in the character among the cultivars. Phenotypic variance (32.428) and coefficient of variation (51.529) were higher than the genotypic and environmental variance and coefficient of variation. Genotypic variance (32.270) and coefficient of variability (51.803) were also higher than the environmental variance (0.158) and coefficient of variability (3.625). High heritability (99.5) and low level of difference between phenotypic and genotypic variance indicates that the character is heritable in nature and the major portion of the phenotypic variance is contributed by the genotype.

(2) Male flower's region (cm)

As shown in the Table 2.3.2b, male flower's region(cm) was the highest in cultivar shabje (12.363 cm. a) and lowest in cultivar dhusar (6.767 cm. e).2.3.3b and Table 2.3.4b showed that male flower's region (cm) was significantly different among

the cultivars included in the study. Range of variation as shown in the Table 2.3.4b was 6.76 – 12.36. Phenotypic variance (4.669) and coefficient of variability (26.822) were higher than both the genotypic and environmental variances and coefficient of variabilities as expected. Environmental variance (0.052) and coefficient of variability (2.831) for this character was higher than its corresponding genotypic variance (4.617) and coefficient of variability (26.672). High heritability (98.9) for this character indicated that the character is heritable and very less influenced by the environment.

(3) Female flower's region (cm)

Highest Female flower's region (36.337 a) was found in cultivar shabje and lowest (13.313 b) in cultivar dhusar as presented in The Table 2.3.2b. Table 2.3.3b showed that the character was significantly different among the cultivars. Range of difference (13.31 – 36.33) was given in the table 2.3.4b pronounced the existence of high range of variability. Environmental variance (1.203) and coefficient of variability (6.071) was lower than both of its corresponding phenotypic and genotypic variances and coefficient of variabilities. Phenotypic variance (82.035) for this character was higher than the genotypic variance (80.832) indicates that the major part of the phenotypic variation was due to genotypic in nature. Heritability (98.5) for this character was also significantly high.

(4) Number of male flower

Tables 2.3.2b shows the mean values of number of male flower. Cultivar shabje showed the highest value (156.703 a) and cultivar dhusar showed the lowest value (33.663). Table 2.3.3b revealed that the cultivars under the study were significantly different in respect of number of male flower. Range of variation were 32.38 – 156.7 as shown in the Table 2.3.4b. Phenotypic variance (3282.956) and coefficient of variability (84.960) were higher than both the genotypic and environmental variance and coefficient of variation respectively. Heritability for this character as calculated from the component of variance was also high (99.9). Genetic variance (8281.003) was also found higher than environmental variance (2.432).

(5) Number of female flower

Number of female flower was found highest in cultivar shabje (184.960 a) and lowest in cultivar dhusar (40.053 d) as presented in Tables 2.3.2b. Number of female flower was also significantly different among the cultivars as indicated the Table 2.3.3b Table 2.3.4b showed the high range of variation (40.05 – 184.96) in length

number of female flower of an inflorescence among the cultivars. Genotypic variance (3361.031) and coefficient of variability (86.689) were more or less near to its phenotypic variance (3361.031) and coefficient of variability (86.699) and higher than environmental variation (0.815) and environmental coefficient of variation (1.349) suggested that the major part of the phenotypic variation of this character was contributed by the genotypes. High heritability (99.9) for this character indicates that the character was very less influenced by the environment and heritable in nature.

(6) Fruits number

Table 2.3.2b presents the mean values of six cultivars of castor included in the study. Cultivar shabje showed the highest fruits number (170.620 a) and cultivar dhusar showed the lowest fruits number (32.387 d) among the cultivars. Regarding this character a significant difference was found among the cultivars studied. Results are presented in the Tables, 2.3.3b. Table 2.3.4b showed the high range of variation (32.38 – 170.62) were present in the character among the cultivars. Phenotypic variance (2988.509) and coefficient of variation (92.247) were higher than the genotypic and environmental variance and coefficient of variation. Genotypic variance (2987.151) and coefficient of variability (92.226) were also higher than the environmental variance (1.358) and coefficient of variability (1.966). High heritability (99.9) and low level of difference between phenotypic and genotypic variance indicates that the character is heritable in nature and the major portion of the phenotypic variance is contributed by the genotype.

(7) Thorn number

Mean values of thorn number shown in the Table 2.3.2b. Thorn number was the highest in cultivar shabje (219.973 a) and lowest in cultivar dhusar (111.887 c). Thorn number was significantly different among the cultivars as shown in the Table 2.3.3b. Table 2.3.4b presented the range of variation that was 111.88 – 219.97. Phenotypic variance (1660.475) and coefficient of variability (27.039) were higher than both the genotypic and environmental variances and coefficient of variabilities as expected. Environmental variance (225.049) and coefficient of variability (9.954) for this character was higher than its corresponding genotypic variance (1435.426) and coefficient of variability (25.140). High heritability (86.4) for this character indicated that the character is heritable and very less influenced by the environment.

(8) Flowering time (days)

The Tables 2.3.2b showed the mean values of flowering time. Flowering time was the highest (161.773 a) was found in cultivar shabje and lowest (49.330 d) in cultivar dhusar. Table 2.3.3b showed that the character was significantly different among the cultivars. Range of difference (44.10 – 161.77) was given in the table 2.3.4b pronounced the existence of high range of variability. Environmental variance (0.785) and coefficient of variability (0.889) was lower than both of its corresponding phenotypic and genotypic variances and coefficient of variabilities. Phenotypic variance (3434.454) for this character was significantly higher than the genotypic variance (3433.669) indicates that the major part of the phenotypic variation was due to genotypic in nature. Heritability (99.9) for this character was also significantly high.

(iii) YIELD AND YIELD COMPONANTS:

(1) Seed number

Results of mean values of seed number presented in the Table 2.3.2c and they are overall same. Table 2.3.3c revealed that the cultivars under the study were not significantly different in respect of seed number. All the cultivars showed the same results. There was no range of variation among them. Phenotypic variance, genotypic variance and environmental variance and their coefficient of variabilities showed same results. There was no heritability for this character.

(2) Seed length (cm)

Highest mean values of seed length was found in cultivar shabje (1.357 a) and lowest in cultivar dhusar (1.047 d) and it was presented in Table 2.3.2c. Seed length (cm) was also significantly different among the cultivars as indicated the Table, 2.3.3c. Table 2.3.4c showed the range of variation (1.04 – 1.35) in seed length (cm) among the cultivars. Genotypic variance (0.015) and coefficient of variability (10.522) were same to its phenotypic variance (0.015) and coefficient of variability (10.522). Environmental variation (0.000) and environmental coefficient of variation (0.000) was not found and suggested that this character was contributed by the genotypes. Highest heritability (100.00) for this character indicates that the character was not influenced by the environment and fully heritable in nature.

(3) Seed breadth (cm)

Cultivar shabje showed the highest mean value (0.900 a) and cultivar lalchay showed the lowest mean value (0.700 c) among the cultivars included in the study

regarding the character of seed breadth and it was presented in Table 2.3.2c. Table 2.3.3c shows the significant difference that was found among the cultivars studied. As shown in the Table 2.3.4c range of variation (0.7 – 0.9) were present in the character among the cultivars. Phenotypic variance (0.005) and coefficient of variation (9.231) were higher than the genotypic and environmental variance and coefficient of variation. Genotypic variance (0.005) and coefficient of variability (9.231) were also higher than the environmental variance. Environmental variation (0.000) and environmental coefficient of variation (0.000) was not found and suggested that this character was contributed by the genotypes. Highest heritability (100.00) for this character indicates that the character was not influenced by the environment and fully heritable in nature.

(4) Seed weight (gm)

Mean values are shown in the Table 2.3.2c regarding the character of seed weight. Seed weight per plant (gm) was the highest in cultivar shabje (2382.347 gm. a) and lowest in cultivar dhusar (64.147 e). Seed weight (gm) was significantly different among the cultivars. Range of variation as shown in the Table 2.3.4c was 64.116 – 2382.34. Phenotypic variance (811369.307) and coefficient of variability (158.468) were higher than both the genotypic and environmental variances and coefficient of variabilities as expected. Environmental variance (51.820) and coefficient of variability (1.266) for this character was higher than its corresponding genotypic variance (811317.487) and coefficient of variability (158.463). High heritability (99.9) for this character indicated that the character is heritable and very less influenced by the environment.

(5) Hundred seeds weight (gm)

The Table 2.3.2c shows the hundred seeds mean values it was the highest (22.430 a) showed cultivar roktima and lowest (12.453 e) in cultivar dhusar. Table 2.3.4c showed that the characters are significantly different among the cultivars. Range of difference (12.45 – 22.43) was given in the Table 2.3.4c pronounced the existence of high range of variability. Environmental variance (0.035) and coefficient of variability (1.091) was lower than both of its corresponding phenotypic and genotypic variances and coefficient of variabilities. Phenotypic variance (18.18) for this character was higher than the genotypic variance (18.145) indicates that the major part of the phenotypic variation was due to genotypic in nature. Heritability (99.8) for this character was also significantly high.

Table 2.3.2a: Mean values of vegetative growth parameters of six cultivars of castor.

To

Table 2.3.4c: Estimation of range and genetic parameter of yield and yield components of six cultivars of castor.

PLANT MORPHOLOGY

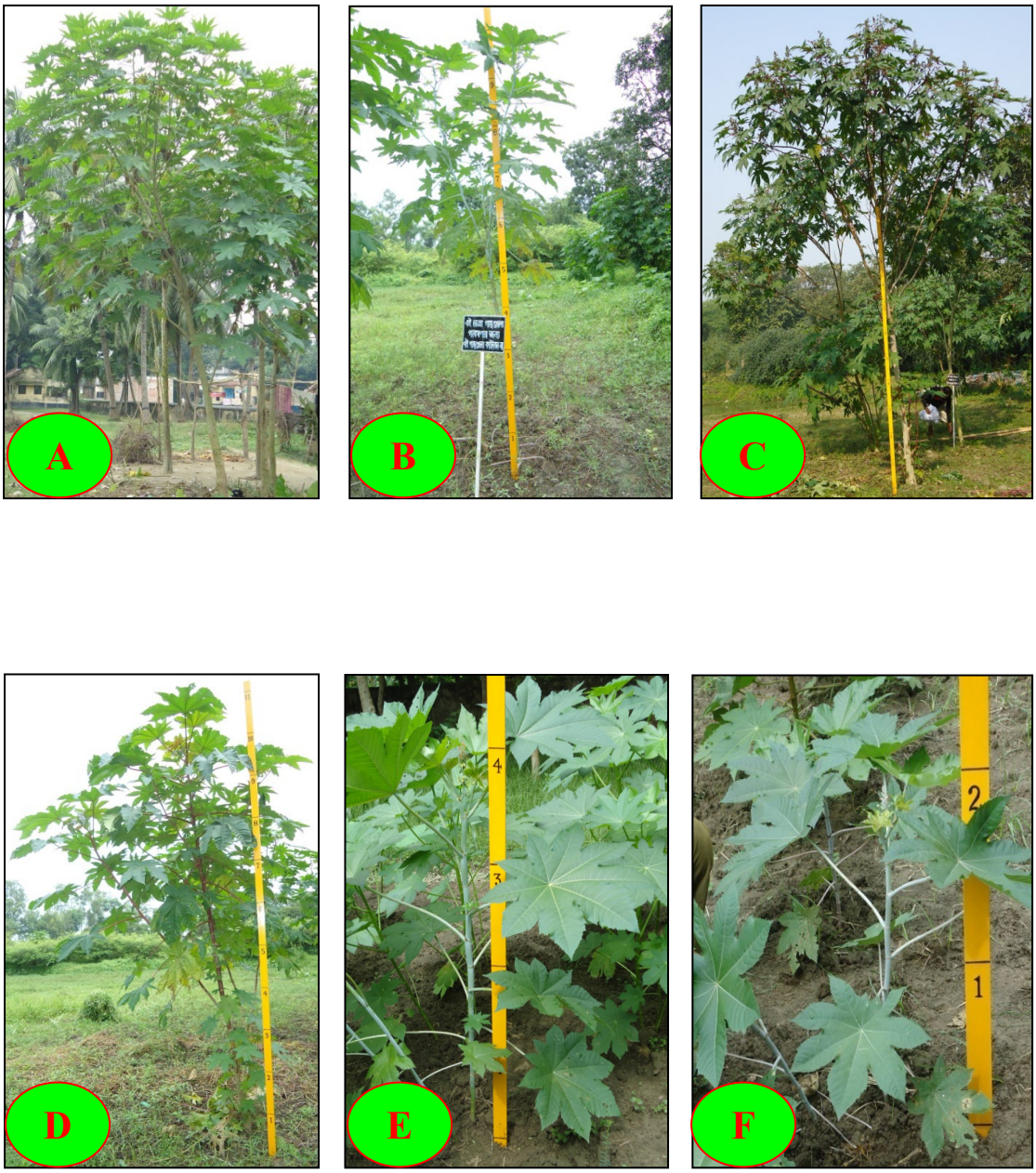


Plate- 2.3.1: Photographs showing the castor genotypes in Bangladesh. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

STEM COLOUR

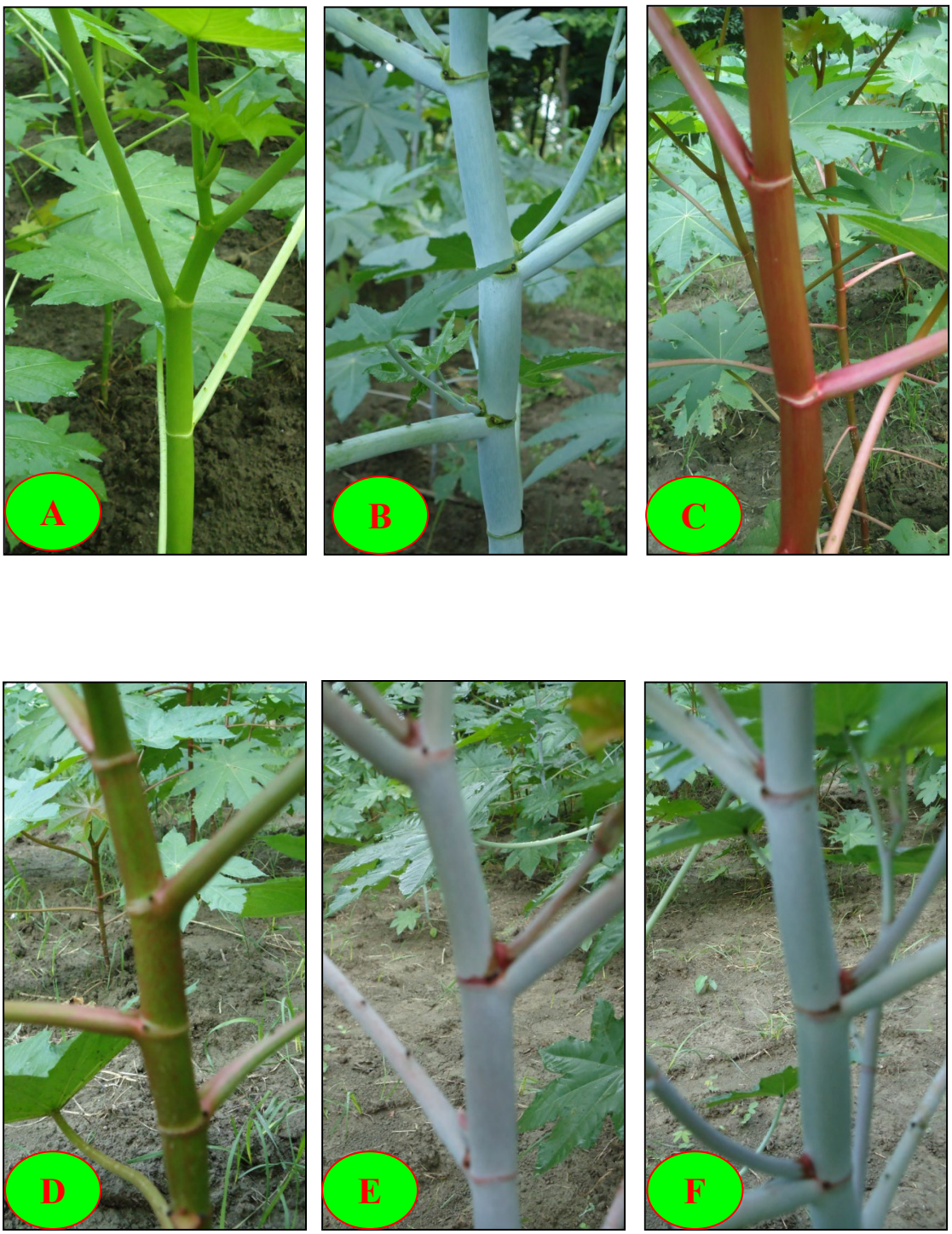


Plate- 2.3.2: Photographs showing the colour of stems and petioles of six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

STEM STRUCTURE

Plate- 2.3.3: Photographs showing the variation of stem structure in six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

INTERNODE LENGTH

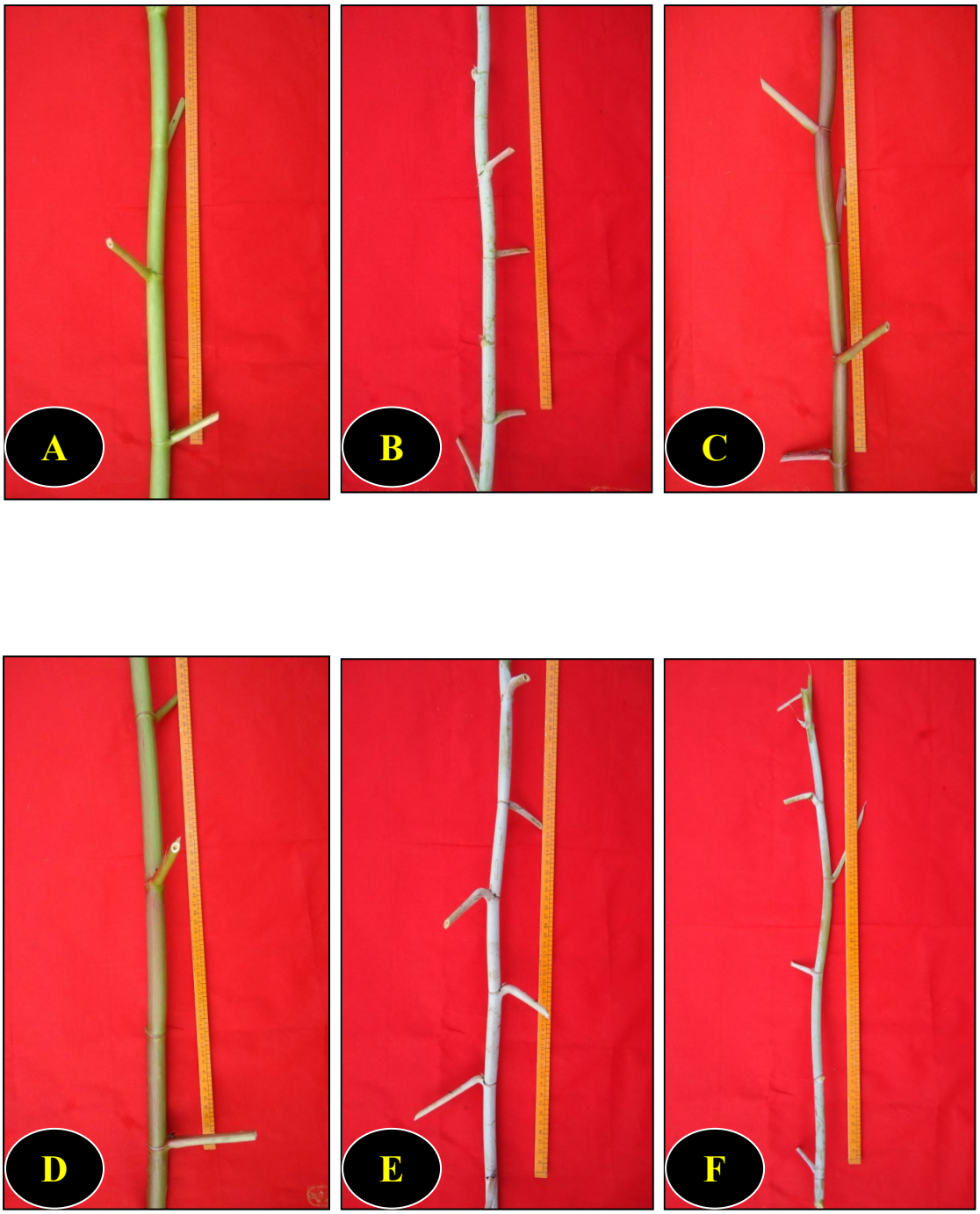


Plate- 2.3.4: Photographs showing the variation of internode length in six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

LEAF SURFACE (UPPER)

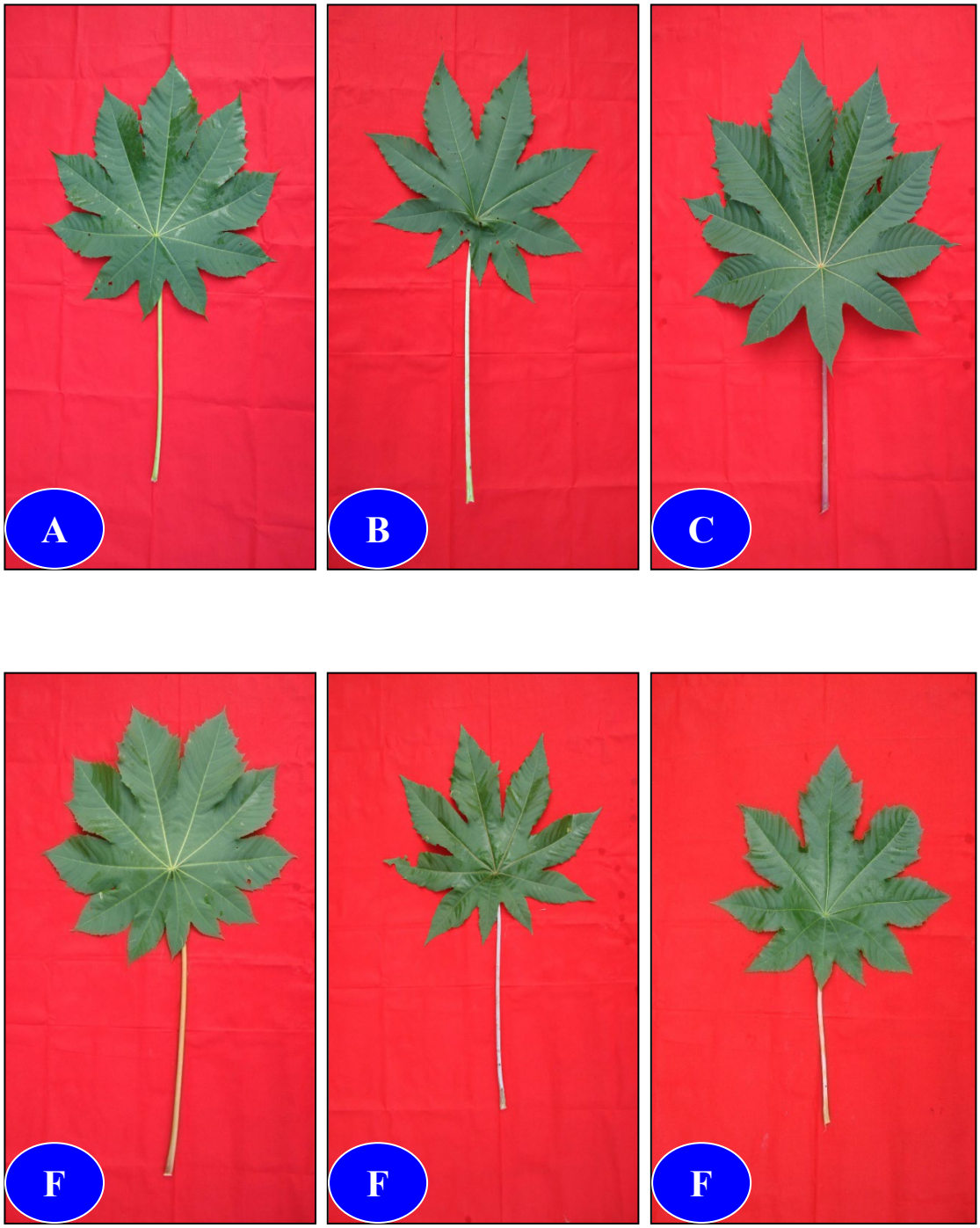


Plate- 2.3.5: Photographs showing the variation of upper surface of leaves in six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

LEAF SURFACE (LOWER)

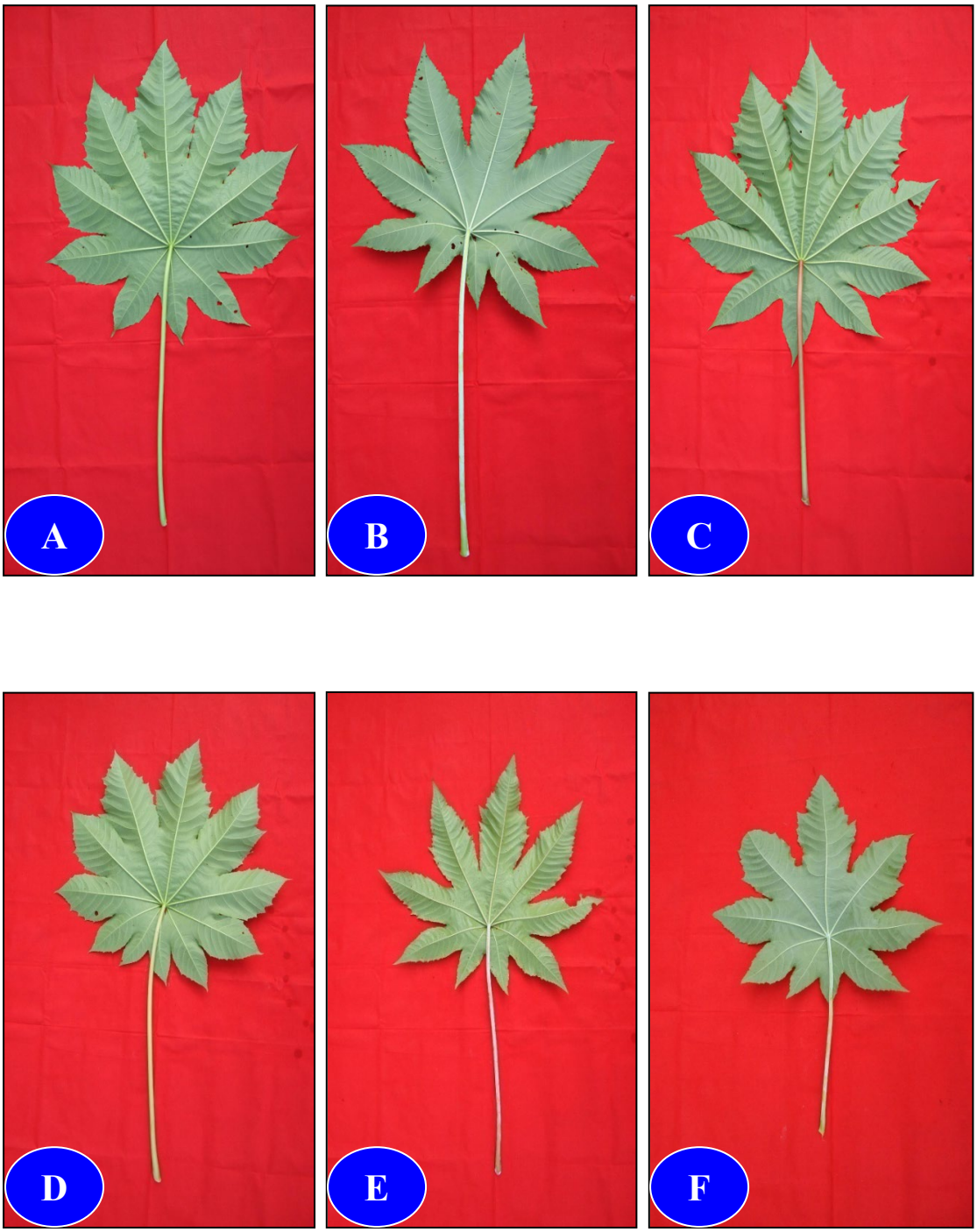


Plate- 2.3.6: Photographs showing the variation of lower surface of leaves in six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

LEAF LENGTH

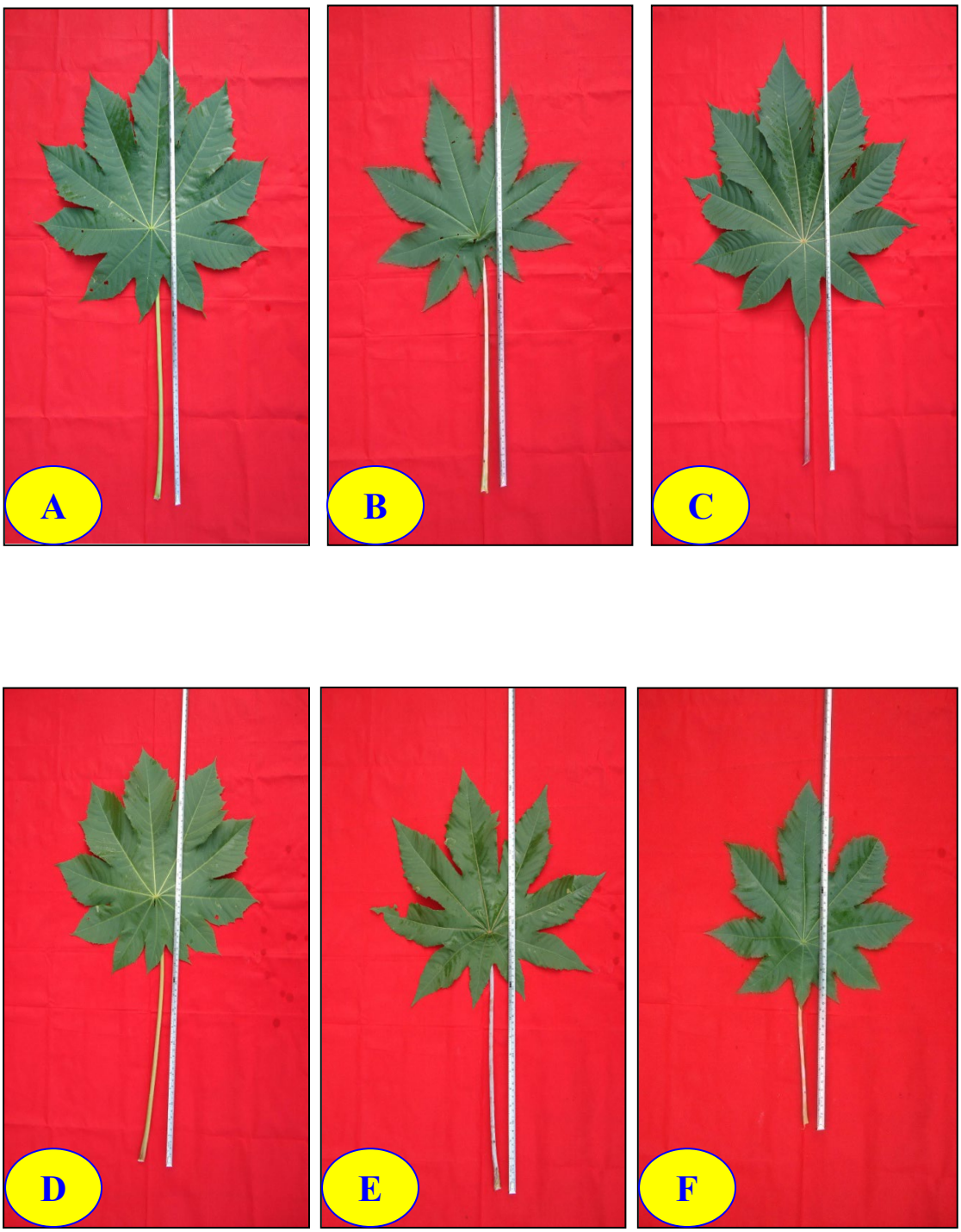


Plate- 2.3.7: Photographs showing the variation of leaf length in six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

LEAF BREADTH

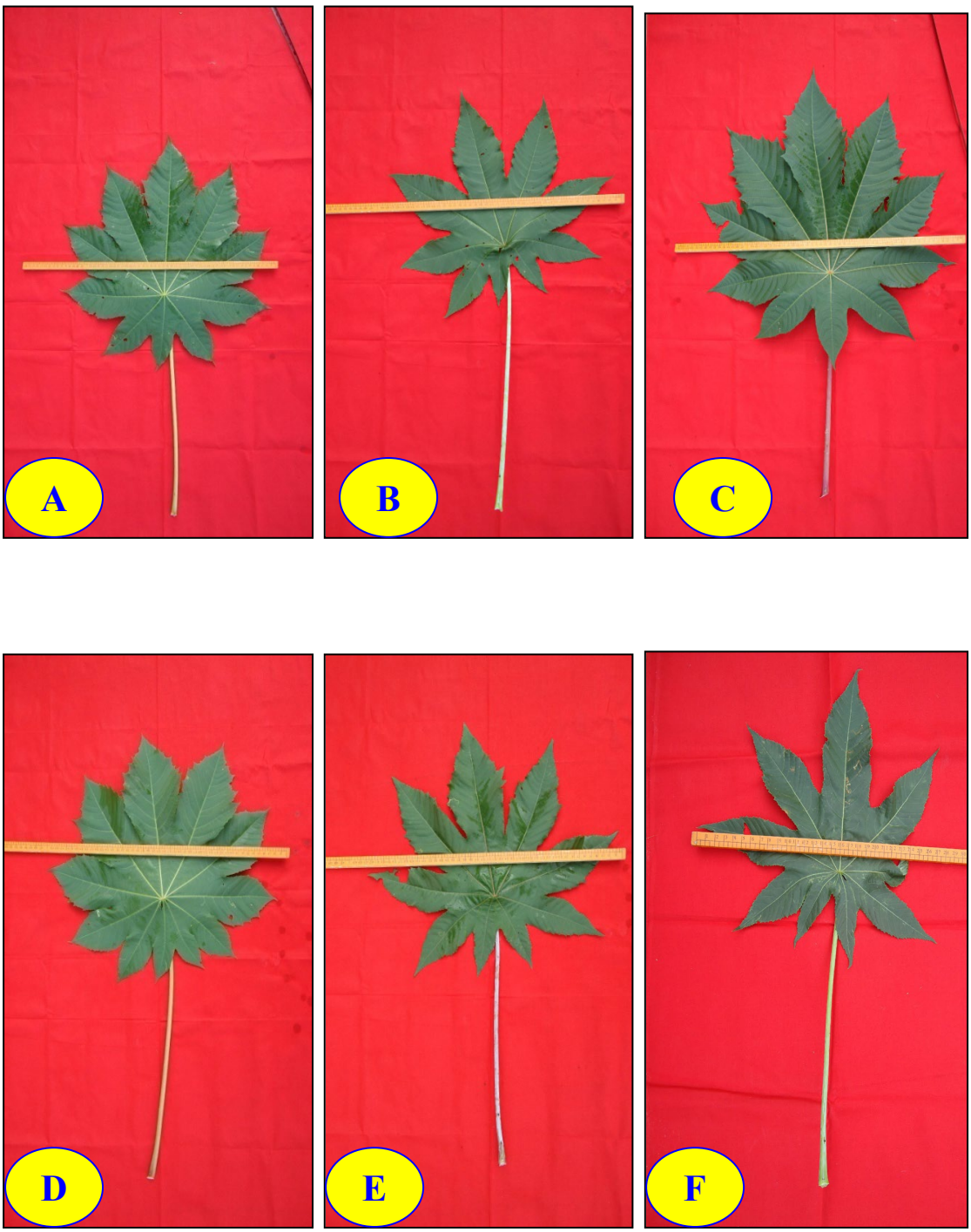


Plate- 2.3.8: Photographs showing the variation of leaf breadth in six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

PETIOLE LENGTH

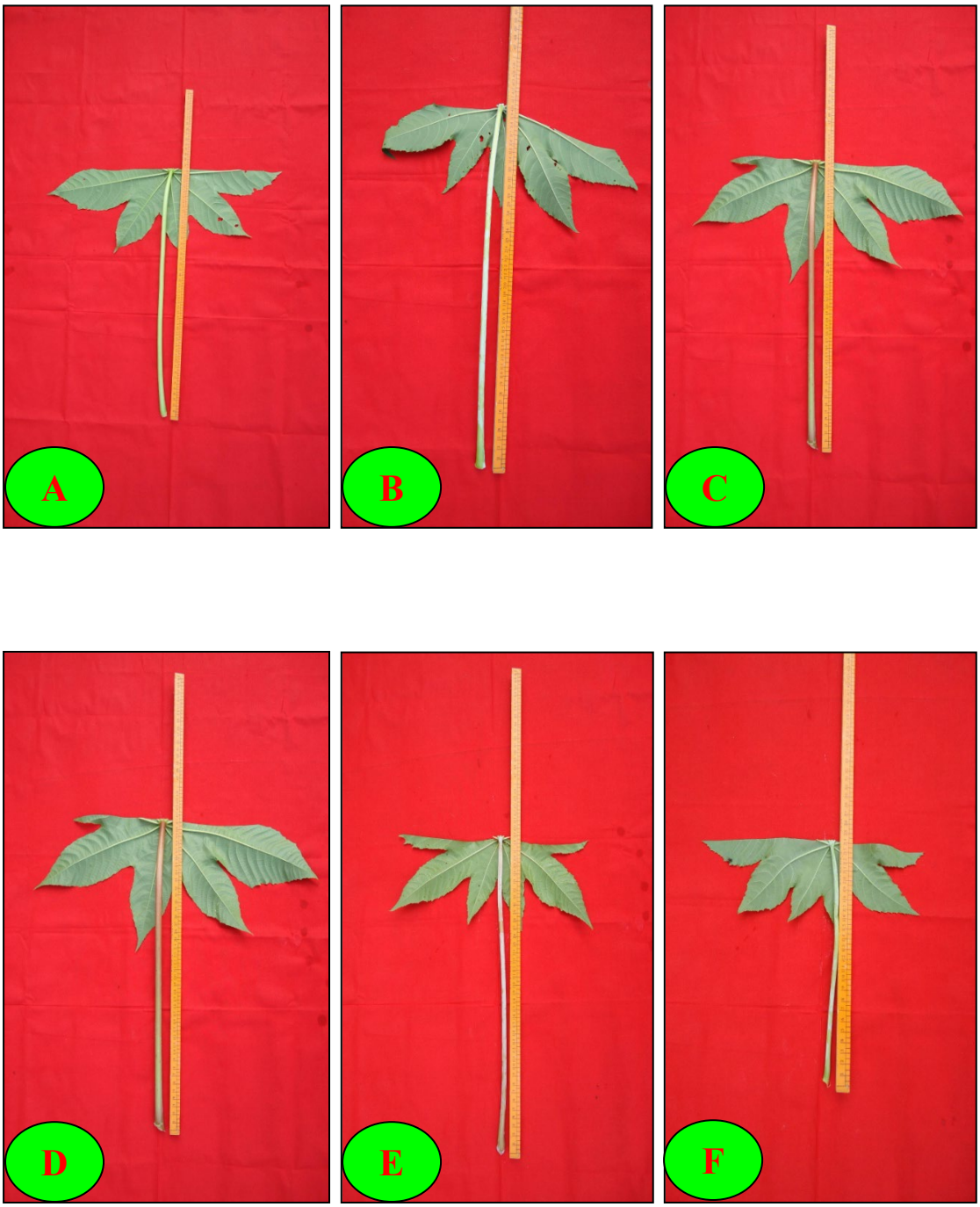


Plate- 2.3.9: Photographs showing the variation of petiole length in six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

FLOWERING INFLORESCENCE

Plate- 2.3.10: Photographs showing the variation of inflorescences in six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

MALE FLOWER AND SEED

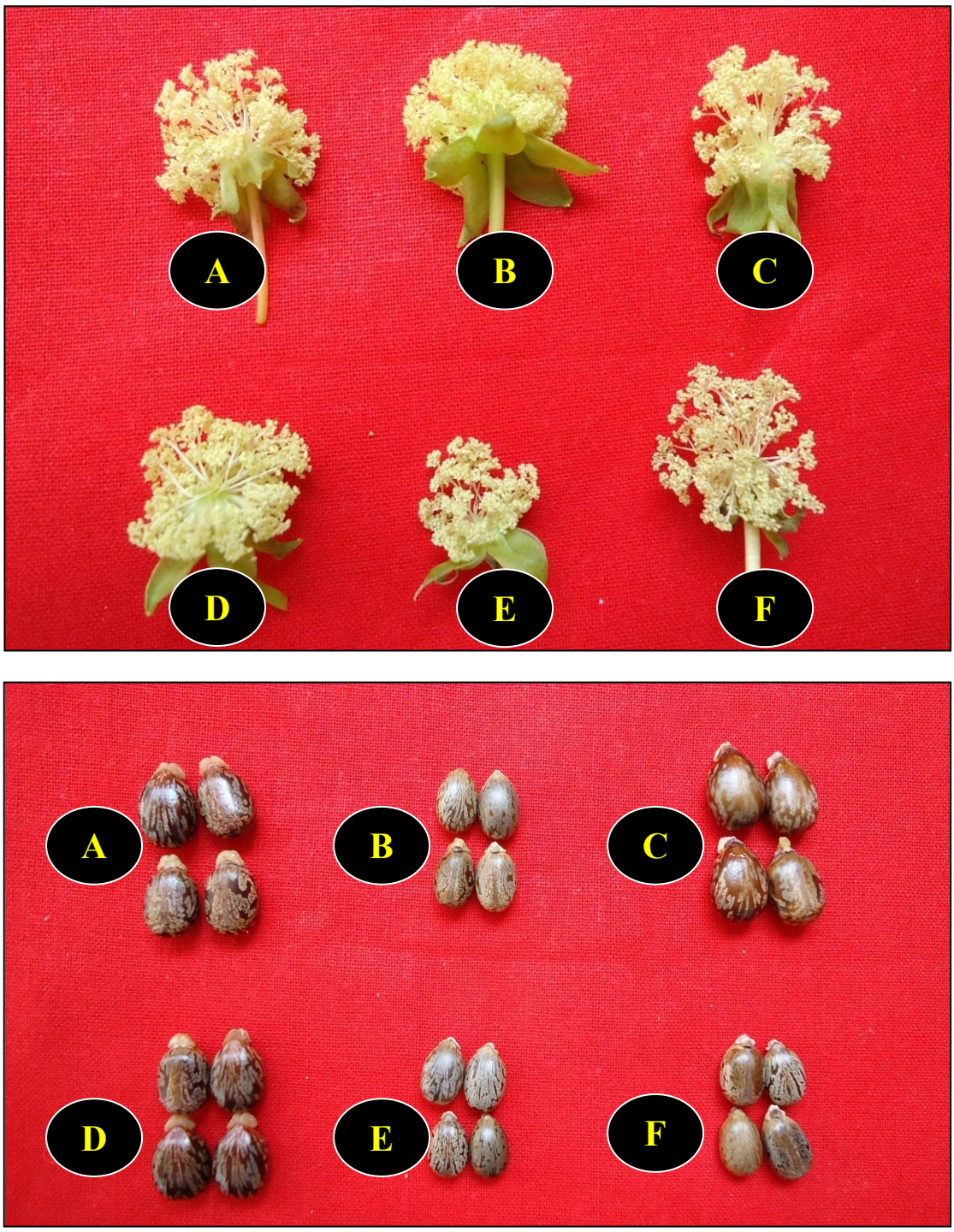


Plate- 2.3.11: Photographs showing the variation of male flowers (**A-F**) and seeds (**G-L**) in six cultivars of castor. **A** and **G:** Cultivar shabje. **B** and **H:** Cultivar shadatae. **C** and **I:** Cultivar roktima. **D** and **J:** Cultivar lalchay. **E** and **K:** Cultivar badami. **F** and **K:** Cultivar dhusar.

SEED POD

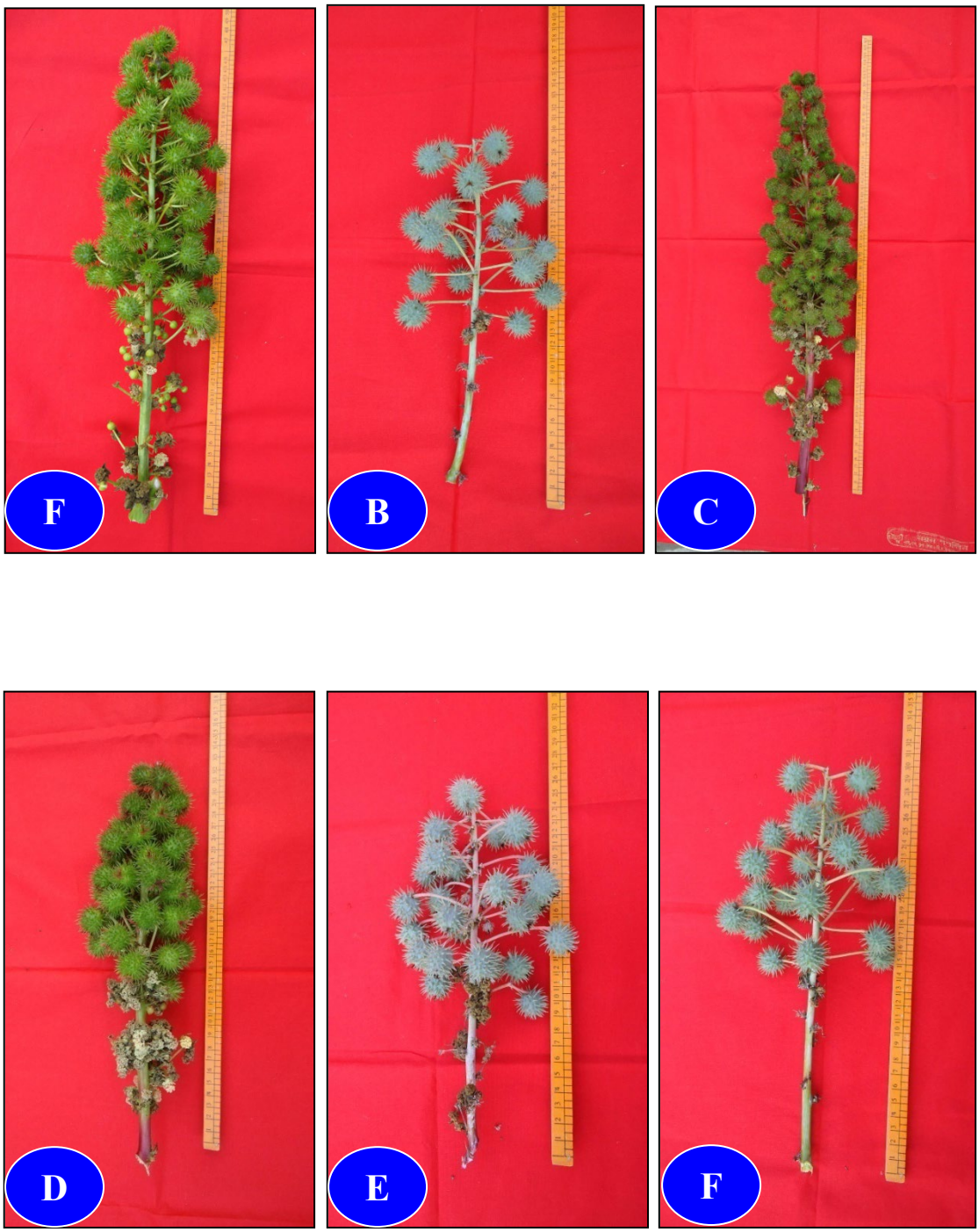


Plate- 2.3.12: Photographs showing the variation of seed pod in six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

ROOTING

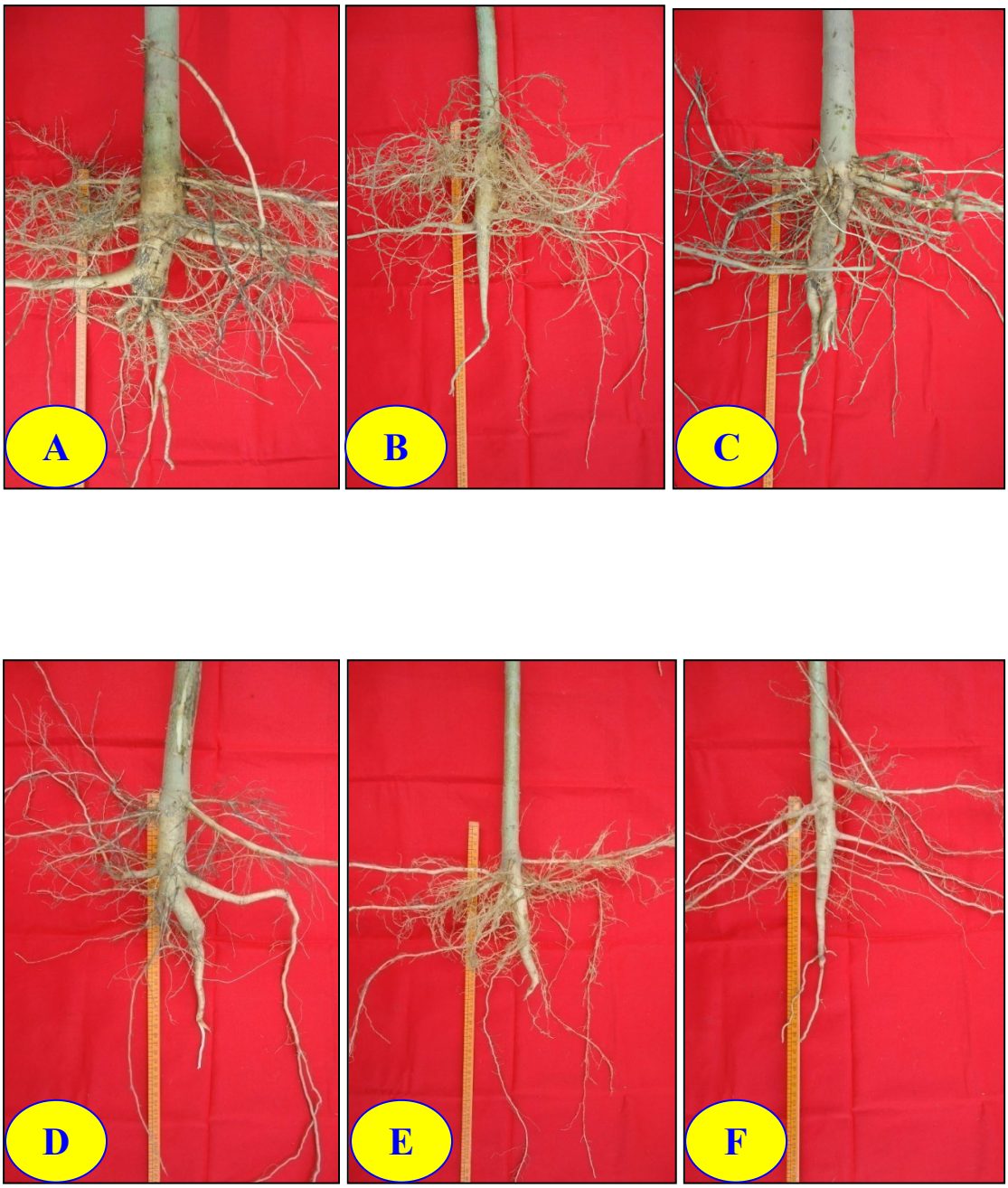


Plate- 2.3.13: Photographs showing the variation of roots in six cultivars of castor. **A:** Cultivar shabje. **B:** Cultivar shadatae. **C:** Cultivar roktima. **D:** Cultivar lalchay. **E:** Cultivar badami. **F:** Cultivar dhusar.

2.4. DISCUSSION

The genus *Ricinus* is monotypic and *R. communis* is the only species with the most polymorphic forms known (Weiss 2000). Several of these forms were designated as species (*R. communis*, R. *macrocarpus*, *R. microcarpus*) (Weiss 2000) but they are intercrossable and fertile and are not true species. All the varieties investigated cytologically are diploids (2n=20), and castor is presumed to be a secondary balanced polyploid with a basic number of x=5 (Singh 1976). Many of the morphological differences might be due to genic differences, cryptic inversions, duplications, etc., rather than to changes in the whole chromosome complement (Perry 1943). Cytological variations included presence or absence of a secondary constriction, presence or absence of a particular heterochromatic segment, size and length differences of particular heterochromatic segments (Paris *et al*. 1980). However variations in the degree of chromosome spreading and attenuation of heterochromatin could not be correlated with phenotypic characteristics (Paris 1981). Previously genetic improvement of castor was confined to the exploitation of naturally occurring genetic variability in the base population and limited selection for traits of interest to humans. Mass selection and pedigree methods have been employed for developing elite genotypes with desirable attributes (Moshkin 1986a, Weiss 2000, Hegde *et al*. 2003). India has made significant progress in development of hybrids in castor during the last three decades (Hegde *et al*. 2003). Based on the exotic pistillate line TSP-10R (Classen and Hoffman 1950), the first hybrid castor, GCH-3, was developed. Subsequently, the development of indigenous pistillate line, VP-1, based on TSP-10R, has given new impetus to hybrid castor development which resulted in the release of three hybrids, GAUCH-1, GCH-2 and GCH-4 during 1990s and ten more high yielding hybrids later on (Hegde *et al*. 2003).

Castor plant is a virgin plant in Bangladesh without having any research work but a comprehensive research works were performed in several countries particularly in India on castor diversities and variety characterization. Anjani *et al*. (1994) collected castor (*R. communis* L.) germplasms in north-eastern hill province of India and Anjani *et al*. (1999) collected castor (*R. communis* L.) germplasm in northwestern India. Particularly on morohological variation of castor germplasms were studied in Egypt (Shaheen 2002). AM Shaheen collected castor germplasms from different phytogeographic regions in Egypt and found morphological differences among the populations from different localities. In our study a number of twenty eight morphological characters were addressed as the castor parameter for characterization of existing germplasms in Bangladesh. During the growth period of castor twenty

eight morphological characters were chosen from three distinct morphological growth phases; vegetative growth, reproductive growth and yield and yield component and their comparative performance were stated under the following heads:

Diversity in vegetative growth

The agro-climatic condition of Bangladesh favors for the luxurious growth of castor particularly during the monsoon season when within three months it starts flowering. Depending on the morphological features we have selected fifteen parameters to evaluate the comparative performance in vegetative growth of different castor genotypes in Bangladesh.

Stem characters

Among the six genotypes cultivar shabje showed the highest height in vegetative growth over others and it attained 466.11cm at the flowering time which about 2½ times greater than that of cultivar dhusar. The cultivar roktima and cultivar lalchay were the next performer in height and these three cultivars, shabje, roktima and lalchay can be considered as the highest performers in vegetative growth in height. On the other hand, dhusar can be marked as the dwarf cultivar in Bangladesh (201.16cm) followed by cultivar shadatae and cultivar badami. Plant height was reported to vary within the same species in some other plants with shrubby nature, for example in case of rose the cultivar Ardabil attained the height 97.5 cm as compared to its dwarf relative Tehran cultivar 62.2 cm (Zeinali *et al*. 2009). In *Roza damascena* intraspecies plant height varried from 94.94-135.61 cm collected from different localities (Danyaei *et al*. 2012). Similarly, in other shrubby plant cotton, plant height varied from 88.73 – 127.8 cm among the different varieties under the same species (Khan 2011). Tahira *et al*. (2007) reported variation in intraspecies differences in plant height among the 8 cotton genotypes was 69.8-94.42 cm. Other stem characters including stem diameter, branch number, branch length, node number and internodal distance showed the similar performance in vegetative growth in all castor germplasms. Node number and internodal distance exhibit lower range of variation among the cultivars in respect of other stem characters in six cultivars of castor. Very wide range of variation was observed in plant height and other stem characters of the different genotypes in Bangladesh particularly stem diameter, branch number and branch length. Number of main branch was reported to vary among the different varieties under the same species of cotton which varied from 10.33-14.60 cm (Khan 2011) and in mulberry 11.11 cm-29.70 cm (Bari *et al*. 1989). Internodal distance variation 2.61-3.64 cm. was reported in mulberry plant among the different varieties

(Bari *et al*. 1989). In *Roza damascena* variation in intraspecies leaf area was reported from 7.26-10.94 cm^2 collected from different localities (Danyaei *et al*. 2012). Flowering time was reported to vary among the different varieties under the same species of In *Roza damascena* varied from 18.92-25.77 days (Danyaei *et al*. 2012).

All stem characters including plant height, stem diameter, branch length, branch diameter and internodal distance experienced high level of phenotypic and genotypic variation with high heritability proved that these characters are genetic in nature and hold the merit to give preference a varietal selection in the breeding programmee. Low level of difference between phenotypic and genotypic variations and high heritability in all these characters indicate that major of the phenotypic variation is contributed by genotype but in case of plant height high environmental coefficient of variation indicates that environmental conditions had a greater influence in plant height in regards of other stem characters. Similar to our result, Zeinali *et al*. (2009), observed the phenotypic coefficient of variation was higher than the corresponding genotypic coefficient of variation and high heritability in plant height and flowering period (days) in case of rose cultivars. Leaf characters including leaf length, leaf breadth, lobe number, petiole length also exhibit very wide range of variation among the different cultivars and all these differences in statistically significant. Greater size of leaf (leaf area) was observed in cultivar lalchay followed by cultivar roktima and cultivar shabje. Greater length and breadth of leaf were also observed in these three castor cultivars. The cultivar dhusar is always showed small leaf size and in regards of leaf area it stands about half of the size of cultivar lalchay. Petiole length in cultivar shabje is exceptionally greater than the other cultivars which projected its leaves for better exposure to the sunlight expecting greater role of photosynthesis in this plant. Low level of difference between phenotypic and genotypic variation and high level of heritability in all leaf characters indicates that the major portion of the phenotypic variance is contributed by the genotypes and these characters are heritable in nature. But there is one exception as observed in leaf area where environmental coefficient of variation stands very high including this character is relatively more venerable and is very prone to the environmental variation like existing agro climatic growth conditions.

Root characters

Low levels of variations were observed in root characters including root length, lateral root number and lateral root length but the difference were of significant level. High level of heritability and low level of difference between

phenotypic and genotypic variation proved genetic in nature and proves its importance in varietal selection.

Reproductive characters

Under our present study eight characters were considered as the reproductive parameters of the castor genotypes in Bangladesh viz. inflorescence number, male flower's region, female flower's region, male flower number, female flower number, fruit number, number of thorn and flowering time. Inflorescence number per plant is very important character as it determine the productive yield of the genotype. In castor very wide range of variation was found in florescence number. The cultivar shabje and roktima gave the highest inflorescence number which is about four times higher than the lowest performer. In respect of reproductive region female flower always occupied greater portion than that of male flower in an inflorescence. Number of female flower were also produced in great number than male flower in all castor genotypes. Significantly greater number of fruits were produced in cultivar shabje and the number did not vary widely among the other cultivars. Flowering time is another important character which was found to vary widely among the castor cultivars. The cultivar shadatae, cultivar badami and cultivar dhusar were noticed as the early flowering plants taking only 46, 47 and 49 days for flowering respectively. On the contrary, the cultivars shabje, lalchay and roktima were identified as the late blooming cultivars taking 161, 154 and 146 days for flowering respectively. In productive perspectives, the cultivar shabje holds the height merit of having greater number of inflorescence per plant being mounted by greater number of fruits per inflorescence. All the flowering characters including viz. inflorescence number, male flower's region, female flower's region, male flower number, female flower number, fruit number, number of thorn and flowering time were identified as very stable in nature as they exhibit very low level of difference between of phenotypic and genotypic variations accompanied by high level of heritability. exceptionally for the character of thorn per fruit. Production of thorn over the fruit surface was rather found to be influenced more by environmental changes as its coefficient of variation in environmental parameters exhibit greater values.

Yield and yield components

The seed yield in castor is very important as it is considered as the economic yield under castor cultivation. Under our present investigation five seed characters were selected for study to evaluate the yield performance of castor genotypes which were seed per fruit, seed length, seed breadth, total weight of seed per plant and

weight of hundred seeds. Number of seed per fruit is an exceptional character which did not vary and remains intact in number in all castor genotypes during its growth phases. Individual seed length and breadth were found significantly greater in cultivar shabje, both the characters did not show any wide range of variation among other castor genotypes. In regards of individual seed weight very wide range of variation was observed in different castor genotypes. Highest unit seed weight was observed in roktima followed by cultivar shabje which were about 100% greater than that of cultivar badami and cultivar dhusar. Cultivar shadatae and cultivar lalchay on the other hand produced seeds which were in intermediate in weight. The most important character is seed yield per plant which experienced the highest level of variation among the castor genotypes under our study. The cultivar shabje produced 2382 gm seed per plant whereas the cultivar dhusar produced only 64 gm which was about 37 times greater in yield. The second one is cultivar roktima producing 485 gm seed per plant followed by cultivar lalchay 206 gm. In this regard cultivar shadatae and cultivar badami were in intermediate in producing seed per plant 144 gm and 127 gm respectively. The results presented in genetic parameter indicated that the more genetically stable characters are seed length and seed breadth exhibiting 100% heritability and showing no difference between genotypic and phenotypic variations. Individual seed weight and seed yield per plant were also identified as genetically stable character specific value for specific cultivar and exhibit 99.9% heritability and very scantly level of difference among phenotypic and genotypic variations.

From the foregoing discussion it can be concluded that the castor cultivars cultivated in Bangladesh holds very high level of genetic diversity as has been ventilated by our experimental results. Among the castor cultivars shabje has been proved as the highest yielder in Bangladesh followed by roktima. The cultivar shabje also grows very first and producing greater biomass in addition to its greater seed yield.

The present study confirmed that the castor cultivars were very significantly different in regards of vegetative growth and yield component parameters. The characters plant height, branch number, stem diameter, node number, internodal distance, length and breadth of leaves, leaf area and length of root were recognized as the most important characters in castor plant for evaluating their morphological characterization and they hold the merit of receiving greater importance and priority in selection under any breeding programme for the improvement of castor genotypes.

Table 2.3.1: Colour of different vegetative structures of different cultivars of castor plant.

Cultivar	Colour of stem	Colour of node	Colour of leaf		Colour of petiole	Colour of vein-junction of leaf	Colour of male flower	Colour of female flower		Colour of seed	Shape of seed	Colour of seed pod
			Upper surface	Lower surface				Stigma	Ovary			
Shabje	Bright green	Green	Green	Green	Bright green	Bright green	Whitish yellow	Yellowish	Green	Dark brown	Egg shaped	Green
Shadatae	Whitish green	Whitish	Whitish green	Whitish	Whitish	White	Whitish yellow	Yellowish White	Greenish white	Dark brown	Egg shaped	Greenish white
Roktima	Dark red	Dark red	Reddish green	Reddish green	Reddish bronze	Red	Whitish yellow	Bright red	Reddish	Light reddish brown	Egg shaped	Reddish green
Lalchay	Reddish	Bright reddish	Bronze green	Bronze green	Greenish bronze	Reddish	Whitish yellow	Red	Light reddish	brown	Egg shaped	Reddish
Badami	Brown	Pinkish brown	Green	Light green	Pinkish white	Light reddish brown	Whitish yellow	Magenta	Light green	brown	Egg shaped	Green
Dhusar	Light brown	Reddish to purple	Green	Light green	Pinkish white	Greenish brown	Whitish yellow	Pinkish	Light green	brown	Egg shaped	Green

Table 2.3.2a: Mean values of vegetative growth parameters of six cultivars of castor.

Characters	Cultivars						CV (%)	LSD
	Shabje	Shadatae	Roktima	Lalchay	Badami	Dhusar		
(1) Plant height (cm)	466.110a	243.997cd	385.887ab	332.663bc	218.940cd	201.163d	15.07	120.1
(2) Stem Diameter (cm)	25.770a	11.137d	22.383b	16.097c	9.760e	9.193e	1.81	0.736
(3) Branch number	11.050a	6.883c	10.330a	8.107b	5.887d	5.440d	4.35	0.896
(4) Branch length(cm)	183.633a	70.323c	113.847b	37.493e	58.987d	55.240d	4.07	9.111
(5) Node number	58.773a	36.273c	57.927a	38.173b	32.050d	30.497e	0.89	0.975
(6) Internodal distance (cm)	8.760a	6.113d	8.330ab	7.947b	6.793c	7.270c	2.94	0.573
(7) Leaf length (cm)	81.293a	61.830e	77.813b	73.057c	63.943d	54.530f	0.52	0.918
(8) Leaf breadth (cm)	51.723a	42.420c	48.993b	48.353b	36.553d	33.887e	1.17	1.319
(9) Leaf area (cm^2)	908.273ab	658.997c	922.243a	1027.440a	657.773bc	605.163c	11.80	243.2
10) Lobe number	10.773a	8.613d	10.677a	9.973b	8.663d	9.393c	1.11	0.271
(11) Petiole length(cm)	50.560a	38.533d	48.450b	44.953c	36.997e	34.107f	0.95	1.045
(12) Petiole diameter(cm)	4.083a	3.743b	4.130a	3.823b	3.153c	3.013c	2.02	0.183
(13) Root length(cm)	65.887a	60.997b	59.883b	51.107c	44.663d	41.217e	2.32	3.247
(14) Lateral root number	34.273a	20.387d	30.530b	23.887c	18.933de	17.830e	2.45	1.544
(15) Lateral root length(cm)	62.743a	41.233c	61.053a	46.297b	40.413c	35.323d	3.77	4.663

Table 2.3.2b: Mean values of reproductive growth parameters of six cultivars of castor.

Characters	Cultivars						CV (%)	LSD
	Shabje	Shadatae	Roktima	Lalchay	Badami	Dhusar		
(1) Inflorescence number	19.030a	7.830d	17.053b	9.773c	6.110e	5.997e	3.63	1.029
(2) Male flower's region (cm)	12.363a	6.957c	7.817b	7.593b	6.840c	6.767c	2.84	0.590
(3) Female flower's region (cm)	36.337a	13.677b	15.670b	15.730b	13.673b	13.313b	6.07	2.838
(4) Number of male flower	156.703a	43.943b	38.170c	32.380d	39.883c	33.663d	2.43	3.616
(5) Number of female flower	184.960a	40.997d	50.097b	40.260d	44.887c	40.053d	1.35	2.336
(6) Fruits number	170.620a	35.940c	42.890b	36.680c	37.053c	32.387d	1.97	3.016
(7) Thorn number	219.973a	120.027c	150.940c	163.067b	138.327c	111.887c	9.95	38.82
(8) Flowering time (days)	161.773a	46.107e	146.220c	154.440b	47.217e	49.330d	0.88	2.293

Table 2.3.2c: Mean values of yield and yield components of six cultivars of castor.

Characters	Cultivars						CV (%)	LSD
	Shabje	Shadatae	Roktima	Lalchay	Badami	Dhusar		
(1) Seed number	3.000a	3.000a	3.000a	3.000a	3.000a	3.000a	0.00	0.026
(2) Seed length (cm)	1.357a	1.143c	1.250b	1.147c	1.040d	1.047d	0.88	0.025
(3) Seed breadth (cm)	0.900a	0.730c	0.780b	0.700d	0.747c	0.737c	1.50	0.025
(4) Seed weight (gm)	2382.347a	144.540d	485.637b	206.363c	127.467d	64.147e	1.27	18.63
(5) Hundred seeds weight(gm)	21.423b	16.113d	22.430a	18.477c	12.697e	12.453e	1.09	0.484

Table 2.3.3a: MS values of ANOVA for vegetative growth parameters of six cultivars of castor.

Characters	MS				VR	
	Total	**Genotype**	**Replication**	**Error**	**Genotype**	**Replication**
(1) Plant height (cm)	11244.05	33069.281**	2127.264	2154.805	15.3468	0.9872
(2) Stem diameter (cm)	43.261	146.802**	0.315	0.081	1819.9372	3.9090
(3) Branch number	4.867	16.196**	0.279	0.120	135.178	2.3294
(4) Branch length(cm)	2580.363	8748.113**	0.829	12.396	705.7317	0.0669
(5) Node number	143.695	487.976**	0.762	0.142	3445.3691	5.3816
(6) Internodal distance (cm)	0.909	2.966**	0.062	0.049	60.3539	1.2590
(7) Leaf length (cm)	93.897	318.735**	0.164	0.126	2534.4135	1.3011
(8) Leaf breadth (cm)	46.597	157.830**	0.203	0.260	606.7809	0.7797
(9) Leaf area (cm^2)	33844.500	93839.870**	8906.480	8834.420	10.6221	1.0082
(10) Lobe number	0.807	2.717**	0.014	0.011	236.9689	1.2535
(11) Petiole length(cm)	41.154	133.516**	0.076	0.163	821.3707	0.4704
(12) Petiole diameter(cm)	0.199	0.665**	0.002	0.005	121.9973	0.3128
(13) Root length(cm)	87.021	290.285**	6.098	1.574	184.4572	3.8751
(14) Lateral root number	40.093	134.656**	2.370	0.356	378.3578	6.6602
(15) Lateral root length(cm)	121.468	392.732**	34.416	3.247	120.9518	10.5993

**Indicates 1% level of significant.

Table 2.3.3b: MS values of ANOVA for reproductive growth parameters of six cultivars of castor.

Characters	**MS**				**VR**	
	Total	**Genotype**	**Replication**	**Error**	**Genotype**	**Replication**
(1) Inflorescence number	28.620	96.969**	0.059	0.158	612.0303	0.3737
(2) Male flower's region (cm)	4.124	13.904**	0.036	0.052	266.4419	0.6848
(3) Female flower's region (cm)	72.450	243.699**	0.570	1.203	202.6327	0.4737
(4) Number of male flower	2103.074	7144.962**	3.962	1.953	3657.7604	2.0284
(5) Number of female flower	2966.555	10083.909**	1.876	0.815	12368.0498	2.3006
(6) Fruits number	2637.020	8962.810**	0.859	1.358	6598.7926	0.6324
(7) Thorn number	1479.100	4531.328**	118.789	225.049	20.1349	0.5278
(8) Flowering time (days)	3030.445	10301.793**	0.379	0.785	13117.3334	0.4828

**Indicates 1% level of significant.

Table 2.3.3c: MS values of ANOVA for yield and yield components of six cultivars of castor.

Characters	MS				VR	
	Total	**Genotype**	**Replication**	**Error**	**Genotype**	**Replication**
(1) Seed number	0.000	0.000	0.000	0.000	0.000	0.000
(2) Seed length (cm)	0.013	0.045**	0.001	0.000	422.8325	6.6842
(3) Seed breadth (cm)	0.004	0.015**	0.000	0.000	113.6135	0.7983
(4) Seed weight (gm)	715917.595	2434004.280**	29.764	51.820	46970.8091	0.5744
(5) Hundred seeds weight(gm)	16.048	54.471**	0.056	0.035	1550.1648	1.5815

**Indicates 1% level of significant.

Table 2.3.4a: Estimation of range and genetic parameter of vegetative growth parameters of six cultivars of castor.

Characters	Range	Grand mean	Variation			Coefficient of variation			Heritability (bs)
			Phenotypic	Genotypic	Environ-mental	Phenotypic	Genotypic	Environ-mental	
(1) HP	201.16 - 466.11	308.127	12459.63	10304.825	2154.805	36.226	32.945	15.065	82.7
(2) DS	9.19 - 25.77	15.723	48.908	48.907	0.081	44.799	44.479	1.81	99.9
(3) BN	5.44 - 11.05	7.949	5.479	5.359	0.120	29.447	29.123	4.358	97.8
(4) BL	37.49 - 183.63	86.587	2924.302	2911.906	12.396	62.454	62.321	4.066	99.6
(5) NB	30.49 - 58.77	42.282	162.753	162.611	0.142	30.172	30.159	0.891	99.9
(6) ID	6.11 – 8.76	7.536	1.021	0.972	0.049	13.408	13.083	2.937	95.2
(7) LL	54.53 - 81.29	68.744	106.329	106.203	0.126	14.999	14.991	0.516	99.9
(8) LB	33.88 - 51.69	43.655	52.783	52.523	0.260	16.642	16.601	1.168	99.5
(9) LA	525.66 - 908.27	796.648	37169.57	28335.15	8834.420	24.201	21.129	11.798	76.2
(10) LB	8.61 - 10.77	9.682	0.913	0.902	0.011	9.869	9.809	1.083	98.8
(11) PL	34.10 - 50.56	42.267	37.947	37.784	0.163	14.574	14.543	0.955	99.6
(12) PD	3.01 - 4.08	3.685	0.225	0.22	0.005	12.967	12.822	1.93	97.8
(13) RL	41.21 - 65.88	53.959	97.811	96.237	1.574	18.329	18.181	2.325	98.4
(14) NRN	17.83 - 34.27	24.307	45.123	44.767	0.356	27.636	27.526	2.455	99.2
(15) RRL	35.32 - 62.74	47.844	133.075	129.828	3.247	24.11	23.815	3.766	97.6

(1) HP = Plant height (cm), (2) DS = Stem diameter (cm) , (3) BN = Branch number, (4) BL = Branch length(cm), (5) NB = Node number, (6) ID = Internodal distance (cm), (7) LL = Leaf length (cm), (8) LB= Leaf breadth (cm), (9) LB = Lobe number, (10) PL = Petiole length(cm), (11) PD = Petiole diameter(cm), (12) LA = Leaf area (cm^2), (13) RL = Root length(cm), (14) LRN = Lateral root number, (15) LRL = Lateral root length (cm).

Table 2.3.4b: Estimation of range and genetic parameter of reproductive growth parameters of six cultivars of castor.

Characters	Range	Grand mean	Variation			Coefficient of variation			Heritability (bs)
			Phenotypic	Genotypic	Environ-mental	Phenotypic	Genotypic	Environ-mental	
(1) IN	5.99 - 19.03	10.966	32.428	32.270	0.158	51.929	51.803	3.625	99.5
(2) MFR	6.76 - 12.36	8.056	4.669	4.617	0.052	26.822	26.672	2.831	98.9
(3) FFR	13.31 - 36.33	18.067	82.035	80.832	1.203	50.132	49.673	6.071	98.5
(4) NMF	32.38 - 156.7	57.457	2382.956	2381.003	1.953	84.960	84.925	2.432	99.9
(5) NFF	40.05 - 184.96	66.876	3361.846	3361.031	0.815	86.699	86.689	1.349	99.9
(6) FN	32.38 - 170.62	59.262	2988.509	2987.151	1.358	92.247	92.226	1.966	99.9
(7) TN	111.88 - 219.97	150.703	1660.475	1435.426	225.049	27.039	25.140	9.954	86.4
(8) FT	46.10 - 161.77	1.848	3434.454	3433.669	0.785	58.111	58.105	0.879	99.9

(1) IN = Inflorescence number, (2) MFR = Male flower's region (cm), (3) FFR = Female flower's region (cm), (4) NMF = Number of male flower, (5) NFF = Number of female flower, (6) FN = Fruit number, (7) TN = Thorn number, (8) FT = Flowering time (days).

Table 2.3.4c: Estimation of range and genetic parameter of yield and yield components of six cultivars of castor.

Characters	Range	Grand mean	Variation			Coefficient of variation			Heritability (bs)
			Phenotypic	Genotypic	Environ-mental	Phenotypic	Genotypic	Environ-mental	
(1) SN	3.0 - 3.0	3.000	0.000	0.000	0.000	0.000	0.000	0.000	0.00
(2) SL	1.04 - 1.35	1.164	0.015	0.015	0.000	10.522	10.522	0.000	100.0
(3) SB	0.7 - 0.9	0.766	0.005	0.005	0.000	9.231	9.231	0.000	100.0
(4) SW	64.116 - 2382.34	568.417	811369.307	811317.487	51.820	158.468	158.463	1.266	99.9
(5) HSW	12.45 - 22.43	17.145	18.18	18.145	0.035	24.695	24.671	1.091	99.8

(1) SN = Seed number, (2) SL = Seed length (cm), (3) SB = Seed breadth (cm), (4) SW = Seed weight (gm), (5) HSW = Hundred seeds Weight (gm).

CHAPTER-III

3.1. INTRODUCTION

3.1.1. Study on molecular diversity in plant

Molecular diversity refers to the variation of genes in molecular structure within species, that is, the heritable variation within and between populations of organisms. In the end, all variation resides in the sequence of the four base pairs that compose the DNA molecule and, as such, constitute the genetic code. The generation of new genetic variation occurs continuously in individuals through chromosomal and gene mutations, which, in organisms with sexual reproduction, are propagated by recombination. Plant genetic resources comprise the present genetic variation that is potentially useful for the future of humankind. These resources include traditional varieties, landraces, commercial cultivars, hybrids, and other plant materials developed through breeding; wild relatives of crop species; and others that could be used in the future for either agriculture or environmental benefits. Hence, plant genetic resources should be conserved, with the ultimate reason being to eventually use them as a source of potentially useful genetic variation.

To conserve and use genetic variation, it should first be assessed, that is, the extent and its distribution need to be determined. Variation can be evaluated on the phenotypic and genotypic levels. Assessment of phenotypic variation focuses on morphological traits those characteristics that define the shape and appearance of a set of individuals. Some of these traits can be considered as 'genetic' if their presence in related individuals is heritable and not dependent on the environment, meaning that they are associated with a particular DNA sequence.

Assessment of genotypic variation is at the level of the DNA molecule responsible for transmitting genetic information. The DNA molecule is composed of nucleotides, which are organized in a double-helix configuration in increasing levels of complexity up to the chromosomal units.

A genetic marker is a measurable character that can detect variation in either a protein or DNA sequence. A difference, whether phenotypic or genotypic, may act as a genetic marker if it identifies characteristics of an individual's genotype and/or phenotype, and if its inheritance can be followed through different generations.

A genetic trait may not have necessarily observable consequences on an individual's performance. Sometimes, however, this trait may be linked to, or correlated with, other traits that are more difficult to measure and do affect the individual's performance. In such cases, these unobservable genetic traits may be used as genetic markers for the linked traits because they indirectly indicate the presence of the characteristics of interest. The two measures can be correlated, using

an analysis of inheritance and studying the distribution of the characteristics in both parents and offspring.

Traditionally, diversity within and between populations was determined by assessing differences in morphology. These measures have the advantage of being readily available, do not require sophisticated equipment and are the most direct measure of phenotype, thus they are available for immediate use, an important attribute. However, morphological determinations need to be taken by an expert in the species, they are subject to changes due to environmental factors and may vary at different developmental stages and their number is limited.

To overcome the limitations of morphological traits, other markers have been developed at both the protein level (phenotype) and the DNA level (genotype). Protein markers are usually named 'biochemical markers' but, more and more, they are mistakenly considered as a common class under the so-called 'molecular markers'. Protein markers (seed storage proteins and isozymes) are generated through electrophoresis, taking advantage of the migrational properties of proteins and enzymes, and revealed by histochemical stains specific to the enzymes being assayed. Detecting polymorphisms detectable differences at a given marker occurring among individuals in protein markers is a technique that shares some of the advantages of using morphological ones. However, protein markers are also limited by being influenced by the environment and changes in different developmental stages. Even so, isozymes are a robust complement to the simple morphometric analysis of variation.

DNA polymorphisms can be detected in nuclear and organellar DNA, which is found in mitochondria and chloroplasts. Molecular markers concern the DNA molecule itself and, as such, are considered to be objective measures of variation. They are not subject to environmental influences; tests can be carried out at any time during plant development and best of all, they have the potential of existing in unlimited numbers, covering the entire genome.

Under the present investigation we worked with RAPD marker and it is one of the DNA based technology RAPD stands for random amplification of polymorphic DNA. It is a type of PCR reaction, but the segments of DNA that are amplified are random. RAPD markers are decamer (10 nucleotide length) DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way. It is used to analyze the genetic diversity of an individual by using random primers.

3.1.2. Molecular diversity in castor

Castor oil is the only vegetable oil soluble in alcohol, presenting high viscosity, and requiring less heating than others oils during the production of biodiesel (Jeong and Park 2009). Due to its unique chemical and physical properties, the oil from castor seed is used as raw material for numerous and varied industrial applications, such as: manufacture of polymers, coatings, lubricants for aircrafts, cosmetics, etc, and for the production of biodiesel. (Jeong and Park 2009). With more than 95% of the world's castor production concentrated in limited parts of India, China, and Brazil (Sailaja *et al*. 2008), and because of the ever increasing worldwide demand of castor for industrial use, there is a pressing need to increase the hectarage and productivity of castor. Castor is a cross pollinated crop and is usually cultivated as a hybrid in India, as hybrids give significantly greater yields than pure lines or varieties (Moll *et al*. 1962, Birchler *et al*. 2003, Reif *et al*. 2007). Higher magnitude of heterosis and genetically superior hybrids can be obtained by combining diverse parents in hybrid development. Conventional diversity analysis methods, in the field, are time consuming, laborious, resource intensive and drastically affected by environmental factors, therefore, a technique that is rapid and not affected by environment is needed for assessment of genetic diversity and selection of parental lines for use in hybrid development programmes. Genetic diversity assessment prior to developing hybrids can aid in better exploitation of heterosis. Assessment of genetic variation using molecular markers appears to be an attractive alternative to the conventional diversity analyses and can also aid in management and conservation of biodiversity. A basic analysis for the genetic diversity of the castor of Bangladesh is important for our current project to complement our phenotypic studies presented in the preceding chapter deciphering. Therefore, in the current chapter we have studied the genetic diversity of castor using RAPD marker method.

The development of new cultivars with traits of interest and adapted to specific microclimates is only possible when there is available knowledge about the extant genetic diversity of the species (Gepts 2004). Despite the recent publication of the castor bean genome (Chan *et al*. 2010), little is known about the actual genetic diversity of this species. Genetic diversity analyses of castor bean germplasm collections worldwide have showed low levels of variability and lack of geographically structured genetic populations, regardless of a marker system used (Allan *et al*. 2008, Foster *et al*. 2010, Qiu *et al*. 2010). Thus, the remarkable phenotypic variation observed in castor bean do not seem to reflect a high genetic diversity, similarly to the reported for physic nut, in which variations in epigenetic

mechanisms may have a more important role in the diversity of the species than genetic variability per se (Yi *et al*. 2010).

Several molecular markers are available for germplasm characterization and identification of cultivated plant varieties. The profile analysis of multi locus DNA markers, also called DNA fingerprinting, is a potential source of informative marker bands, which allows a reliable differentiation among cultivars (Tanya *et al*. 2011), wild populations (Andrade *et al*. 2009), species and even related genera (Simon *et al*. 2007). Additionally, molecular markers are very stable, in contrast to morphological characters, which may be influenced by environmental factors and having continuous variation and high plasticity (Weising *et al*. 2005).

Unlike other important oilseed crops, as oil palm (*Elaeis guineensis*), soybean (*Glycine max*), sunflower (*Helianthus annuus*), and some Euphorbiaceae species, as cassava and rubber tree, castor bean diversity is still poorly characterized by means of molecular marker systems (Billotte *et al*. 2010, Feng *et al*. 2009, Sayama *et al*. 2011, Sraphet *et al*. 2011, Talia *et al*. 2010). In fact, the species had been overlooked until the late 2000s, when analyses regarding genetic diversity of germplasm collections were first published (Allan *et al*. 2008). However, castor bean was the first member of the Euphorbiaceae family with the whole genome published (Chan *et al*. 2010), a fact that will be of great importance for characterizing the genetic base of the species.

3.1.3. Objectives

In my preceding chapters I have shown that the six cultivars of castor show significant variation among their morphological and phenotypic traits. However, till this point we were not sure whether the six cultivars are six variants of castor containing the same genotypic makeup or they have completely different genotypes. Therefore, to solve this intriguing question we setup experiments described in the current chapter. The aim of experiments presented in the current chapter is to confirm the genetic diversity and relationship among six cultivars of castor by Random Amplified Polymorphic DNA (RAPD) markers as it is important particularly for cultivar identification by DNA fingerprinting. Thus, the present work was undertaken to obtain the following major objectives.

1) To show that the six cultivars of the castor used in my current project are genotypically different.
2) To establish a protocol to identify six cultivars of castor by DNA fingerprinting using RAPD markers, and
3) To determine relationship among six cultivars of castor accessions.

3.2. MATERIALS AND METHODS

The present investigation was carried out at the biotechnology laboratory, in the Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh. The details of the materials used and methodologies followed for the current investigation have been described below.

3.2.1. Isolation of DNA

In the present investigation, CTAB method was used with minor modification for extraction of the total genomic DNA from six cultivars of Castor following the protocol reported by Winnepenninckx *et al*. 1993. The composition of the CTAB Buffer (100 ml) was as follows:

10 ml 1 M Tris HCl pH 8.0
28 ml 5 M NaCl
4 ml of 0.5 M EDTA
2 g of CTAB (cetyltrimethyl ammonium bromide)
The total volume was brought to 100 ml with ddH_2O.

Prior to starting extraction add polyvinyl-pyrrolidone (PVP) and β-mercaptoethanol was added according to the following table. Once these have been added the shelf life of the buffer is only 2-3 days.

CTAB Buffer	PVP	β-mercaptoethanol
0.5 ml	0.02 g	2.5 uL
5 ml	0.2 g	25 uL
20 ml	0.8 g	100 uL

3.2.1.1. Plant materials

We collected all available genotypes from different corners of the country and established a germplasm on the research field during the year 2010-2011 in the Institute of Biological Sciences (IBSc), University of Rajshahi, Bangladesh. Young leaves of six cultivars of castor were used as plant materials for DNA isolation. The cultivers of castor used in our current study is showed in the Table 3.2.1.

Table 3.2.1: List of Castor genotypes.

Serial No.	Cultivar
1	Cultivar Shabje (V_1)
2	Cultivar Shadatae (V_2)
3	Cultivar Roktima (V_3)
4	Cultivar Lalchay (V_4)
5	Cultivar Badami (V_5)
6	Cultivar Dhusar (V_6)

3.2.1.2. Preparation of stock solutions and working solutions used for DNA isolation

For preparing CTAB buffer and carrying out the DNA isolation procedures, the following stock solutions and working solutions were prepared.

3.2.1.2.1. 1M stock solution of tris HCl (pH 4.0)

For 100 ml solution, 12.114 gm of Trizma base, BioUltra (Sigma-Aldrich, USA; MW=121.14) was dissolved in 75 ml of sterile de-ionized distilled water (sdd H_2O). The pH of this solution was adjusted to 8.0 with 10 N HCl. About 5.6 mL of HCL (10 N) was added slowly by using dropper until the pH was adjusted to 8.0 and was kept at room temperature for several minutes. The volume of the solution was adjusted to a total of 100 ml with sterile deionized distilled water (sdd H_2O). Then it was sterilized by autoclaving and stored at 4ºC.

Function of Tris HCl: Tris HCl is used as buffer to maintain pH 8.0.

3.2.1.2.2. 0.5 M stock solution of EDTA (Ethylenediaminetetraacetic acid, pH 9.0)

For 100 ml solution, 18.612 gm of EDTA (Sigma-Aldrich, USA; MW=372.24) was added to 75 ml of sterile de-ionized distilled water and stirred vigorously with a magnetic stirrer. The EDTA salt was not dissolved in solution until the pH of the solution was adjusted to 8.0 using NaOH. Finally the volume of the solution was adjusted to 100 ml with sterile deionized distilled water (sdd H_2O).

3.2.1.2.3. 5 M sodium chloride (100 ml)

To prepare 100 ml 5M NaCl solution, 29.22 gm of sodium chloride (Fluka, Germany; MW=578.1 gm) was added gradually to 70 ml of sterile de-ionized distilled water and stirred vigorously with a magnetic stirrer overnight. Finally the volume of

the solution was adjusted to 100 ml with sterile deionized distilled water (sdd H_2O). Occasional heating was used to completely dissolve NaCl.

3.2.1.2.4. 7.5 M ammonium acetate (10 ml)

For 10 ml solution, 5.781 gm of Ammonium acetate (Sigma-Aldrich, USA; MW=578.1 gm) was added to 7 ml of sterile de-ionized distilled water and stirred vigorously with a magnetic stirrer overnight. Finally the volume of the solution was adjusted to 10 ml with sterile deionized distilled water (sdd H_2O).

3.2.1.2.5. 10% solution of CTAB (N-Cetyl-N, N, N-Trimethyl Ammonium Bromide)

In DNA extraction buffer 20gm/L CTAB salt (cetyltrimethyl ammonium bromide) was added directly and dissolved. 10% CTAB solution was also used in subsequent steps of washing of the extracted DNA. To prepare 100 ml of 10% CTAB solution solution, 10 gm of CTAB (MW=364.46) was added to 75 ml of sterile de-ionized distilled water (sdd H_2O). The total volume was adjusted to 100 ml with sterile de-ionized distilled water. Heat was provided to help dissolve. It was stored at room temperature. CTAB precipitates when the temperature is below 25ºC. Prior to use the solution heated to re-dissolve CTAB.

Function of CTAB: CTAB is used for solubilizing plant tissue and it along with more than 0.5M NaCl to remove polysaccharides.

3.2.1.2.6. Stock solution of TE (Tris EDTA) buffer (pH 8.0)

For storage of nucleic acid (DNA or RNA) TE-buffer, pH 8.0 is used universally. For 500 ml solution, 5 ml of 1M Tris HCl was added with 1 ml of 0.5M EDTA. The final volume was adjusted to 500 ml with sterile de-ionized distilled water. Then sterilized through disposable filter sterilizer of 0.5 µm pore size using syringe under laminar flow hood and stored at 4ºC. For the economic use of chemicals, different volume of TE buffer may be prepared as following **Table 3.2.2:**

Chemicals	Stock conc.	Final conc.	50 ml	100 ml	500 ml
Tris HCl (pH 8.0)	1M	10mM	0.5ml	1ml	5ml
EDTA (pH 8.0)	0.5M	1mM	0.1ml	0.2ml	1ml
SddH2O	-	-	Up to final volume		

Function of TE buffer: In TE buffer (Tris HCl with EDTA), Tris HCl is used as buffer to maintain pH at 8.0. It is essential to maintain the integrity of DNA because most lipolytic and lipoxygenase enzymes have pH optima between 5.0 and 6.0, whereas nuclear DNAses have the pH optima around 7.0. EDTA chelates Mg^{2+} or

removes Mg^{2+} which is required by the nuclease activity. Nuclease degrades DNA. Therefore, plant DNA is stored in TE (Tris HCl and EDTA).

3.2.1.2.7. TE saturated phenol

- The crystal phenol was melted in a water bath at 65ºC for 30 minutes.
- Then 50 ml of melted phenol was taken and same volume of TE buffer was added.
- With a magnetic stirrer, it was mixed for at least 10 minutes and then for 5 minutes it was kept in rest.
- At this stage, two distinct phases are visible, colorless upper phase and colorful lower phase.
- With the help of dropper, the upper phase was removed as much as possible. The same procedure was repeated until the pH raised up to 7.8 (it may be ranged 7.6-7.8) to the lower phase (TE buffer of volume 50 ml was added in every repeating stage after discarding the upper phase).
- This stage of operation was taken for several times. In our case, we did it for five times and it was taken about 3.5 hours to obtain the pH 7.7.
- After saturation, the phenol because the half of the initial volume.

Cautions: As phenol is very much corrosive and highly toxic, so adequate protective measures (Apron, Gloves and Musk) were taken.

3.2.1.2.8. Phenol: chloroform: isoamyl alcohol (25:24:1) solution

For 50 ml of this solution, 25 ml of TE saturated Phenol; 24 ml of Chloroform and 1 ml of Isoamyl Alcohol were added and mixed properly by vortexing. Mixing was done under fume hood for safety. The solution was then stored at 4ºC. It is recommended to vortex or shakes before use.

Function: Most proteins are removed by denaturation and precipitation from the extract using choloroform or phenol.

Table 2.2.3: Function of different components of extraction buffer of DNA isolation.

Components	Concentration	Function
Tris HCl (pH 8.0)	200Mm	Maintain pH
EDTA (pH 8.0)	50mM	Chelate Ca++, Mg++, inhibit DNase activity
NaCl	5M	Remove polysaccharides
CTAB	2%	Dissolve tissues
Ammonium Acetate		Precipitation
Beta mecaptoethanol		Reducing Agent
sddH_2O	Required	General solvent
Phenol: Chloroform: Isoamyl Alcohol		Phase seperation of DNA/RNA and proteins

3.2.1.3. Collection and handling of sample

Collection of sample is the most important part of the DNA isolation procedure. Due to the handing error during sampling, the quality of the isolated DNA varies considerably. In this experiment, collection of sample was carried out with special care. To prevent DNase or RNase contamination disposable latex examination gloves were used all along to handle the samples. Leaf segments were used as sample. The collection procedure of the samples is mentioned below.

3.2.1.3.1. Collection of young leaves

The young leaves of six cultivars of castor were collected from the research field. Then it was taken in the laboratory. The leaves were cut down in to small pieces (about 1.0 cm) with sterile scissors and 200-300 mg of leaf peaces were weight using a fine balance and used for DNA extraction.

3.2.1.4. Procedure used for genomic DNA isolation

3.2.1.4.1. List of equipments and materials needed for DNA Isolation

1. Autoclave machine
2. Balance
3. Beaker
4. Centrifuge machine
5. Conocal flasks
6. Eppendorf tubes and eppendorf tube rack
7. Falcon tubes
8. Ice machine
9. Incubator (Water bath 65ºC)
10. Micropipettes and Micropipette tips
11. Mortar and pestle
12. pH meter
13. Refrigerator
14. Water de-ionizer
15. Water distillation plant

3.2.1.4.2. Procedure followed for genomic DNA isolation

- The fresh leaves samples of six cultivars of castor were cut into about 1.0 cm strips. Placed 200 – 300 mg of sample in an autoclaved mortar and pestle.
- Liquid nitrogen was added in mortar for easy grinding of sample.
- 800 µl of Extraction buffer was added to the grinded sample and was transferred to a DNase/RNase/pyrogen free 2.0 ml axigen tubes.
- The tubes were incubated at 65ºC for 40 minutes in a water bath.
- Occasional mixed was performed by inversion 3-4 times during incubation.
- The samples were taken from the water bath and cooled down to room temperature.
- 1µl of RNase A (10 mg/ml stock solution) was added and incubated at 37° for 15 minutes to degrade RNA from the sample for obtaining pure DNA.
- An equal volume of Phenol: Chloroform: Isoamyl Alcohol at the ratio of 25:24:1 was added and mixed well by inversion, then centrifuged the tubes at 10,000 rpm for 10 minutes at room temperature.
- Aqueous phase (about 800 µl) was recovered and transferred carefully to a fresh ice-cold 2.0 ml eppendorf tubes.
- An equal volume of ice-cold Isopropanol, 50 µl of Ammonium acetate followed by 150 µl of 5M NaCl was added. DNA was precipitated immediately after addition of isopropanol as white cottony precipitate.
- To complete the precipitation the tube was incubated at -20ºC for at least 1 hour.
- Then the tubes were centrifuged at 10,000 rpm for 10 minutes at room temperature.
- The upper layer of the solution was discarded carefully by using adjustable micropipette.
- 250 µl of 10% CTAB was added to the pellet and the tubes were centrifuged at 10,000 rpm for 2 minutes at room temperature.
- Subsequently DNA was washed using 500 µl of ice-cold 70% ethanol twice.
- After final centrifugation step the solution was discarded carefully and the pellet was air dried for 30 minutes by putting the tubes upside down on a filter paper.
- Finally DNA was re-suspended in 50 µl of TE buffer and stored at -20ºC for subsequent experiments.

3.2.2. Qualification and quantification of isolated DNA

Sometimes isolated genomic DNA may contain a large amount of proteins, pigments and other substances which usually cause over estimation of DNA concentration on a UV spectophotometer. Thus the DNA sample was evaluated both qualitatively (visual estimation through agarose gel electrophoresis) and quantitatively (by spectrophotometer). Measurement of isolated DNA concentration can be done by comparing DNA with standard DNA markers on agarose gel electrophoresis or by estimating the absorbance of DNA by spectrophotometer at 260 nm and comparing it with the absorbance of protein at 280 nm. Both the methods were carried out in this investigation.

3.2.2.1. Measurement of DNA concentration and quality by agarose gel electrophoresis

Gel electrophoresis is a method that separates macromolecules on the basis of size, electric charge and other physical properties. The term electrophoresis describes the migration of charged particles under the influence of an electric field. The driving force for electrophoresis is the voltage applied to activated electrodes at either end of the gel. The properties of a molecule determine how rapidly an electric field can move it through a gelatinous medium. Electrophoresis is a standard method used to separate identity and purity of DNA fragments. The technique is simple, rapid to perform and capable of resolving fragments of DNA that can not be separated by other procedures. The following equipments and chemicals were used to conduct agarose gel electrophoresis.

1. A horizontal electrophoresis system and power supply
2. Gel casting trays
3. Sample combs, around which molten agarose is poured to form ssample wells in the gel
4. Electrophoresis buffer
5. loading the samples in the wells
6. A fluorescent dye for staining nucleic acids (Ethidium bromide)
7. UV-transelluminator

3.2.2.1.1. Preparation of stock solutions used for gel electrophoresis

For conducting the gel electrophoresis, the following stock solutions and other solutions were prepared.

3.2.2.1.1.1. 10 x TBE Buffer (pH 8.3)

For 100 ml of 10 x TBE buffer, 10.89 gm of Trizma base (MW=121.14) was dissolved into 75 ml of sterile de-ionized distilled water. Then 0.9 gm of EDTA.2H_2O (MW=372.2) was added to the solution. Finally, 5.5 gm of boric acid was added in it. They were mixed well. The pH of the solution was adjusted by mixing concentrated HCl (10N) at pH 8.3. The final volume of the solution was adjusted to 100 ml. For the economic use of chemicals, different volume of solution may be prepared as follows:

Chemicals	Final conc.	50 ml	100 ml	250 ml
Trisma base	0.9 M	5.445 gm	10.89 gm	27.225 gm
EDTA	-	0.465 gm	0.93 gm	2.325 gm
Boric acid	0.9 M	2.75 gm	5.5 gm	13.75 gm
SddH_2O	-	Up to the final volume		

3.2.2.1.1.2. 6X DNA loading dye

For 10 ml of 6X DNA loading dye, 25 mg of Bromophenol blue (i.e., the final concentration was 0.25%), 25 mg of Xylene cyanol FF (i.e., the final concentration was 0.25%) and 3 ml of 98% glycerol (i.e., the final concentration was 30%) were mixed throughly. Sterile de-ionized distilled water was then added 1o top up to 10 ml and mixed properly. The solution was then aliquot to 1.5 ml microcentrifuge tubes and provided heat by placing them on boiling water for 10 minutes supported by a float. These were cooled at room temperature and stored at 4ºC. For the economic use of chemicals, different volume of solution may be prepared as follows:

Chemicals	Final conc.	2.5 ml	5 ml	10 ml
Bromophenol blue	0.25%	6.25 mg	12.5 mg	25 mg
Xylene caynol	0.25%	6.25 mg	12.5 mg	25 mg
Glyceraol	30%	0.75 ml	1.5 ml	3 ml

3.2.2.1.1.3. Ethidium bromide solution

For 1ml solution, Ethidium Bromide 10mg was added to 1ml of sterile de-ionized distilled water. It was then mixed by hand shaking. The solution was then transferred to a dark bottle and stored at room temperature. As ethidium is a potent carcoinogen. Adequate precaution was taken while handling it. Latex examination gloves were using all along the experiments which involved ethidium bromide.

3.2.2.1.2. Preparation of 1.4% agarose gel

For agarose gel electrophoresis, 120 ml of 1.4% agarose gel was prepared (For tray size 15 cm x 15 cm, required 120 ml of agarose gel for thickness of 0.5 cm). For casting the gel, the following steps were followed:

- Initially, 1.68 gm molecular biology grade (QBiogene, USA) agarose powder was weighed out and placed into a 250 ml conical flask.
- Then 120 ml of electrophoresis buffer (1 × TBE buffer) was added in to the flask.
- The flask was then placed into a microwave oven. Using a low to medium setting, the timer was set for 1 minute. The oven was stopped and swirled the container gently to suspend the agarose which was not dissolved. The solution was heated again for 1 minute to dissolve small translucent agarose particles.
- When the agarose solution was cooled to about 50ºC (the flask was cool enough to hold comfortably with bare hand). 12 µl from 10 mg/ml solution of ethidium bromide was added, so that in the gel the concentration of ethidium bromide was maintained as 1 µg/ml (the final concentration of ethidium bromide in the melted agarose solution may be in the range of 0.5-1.0 µg/ml) and mixed well by gentle shaking.

3.2.2.1.3. Comb set-up

- The ends of the gal casting tray was sealed tightly with casting rubber in order to prevent from leakage.
- The gel tray was leveled on a leveling table or working bench using the leveling bubble.
- The comb was then placed in to the appropriate groove or slot of the tray.
- The molten agarose (about 50ºC) was poured onto the tray. Air bubbles were removed by pushing away to the side using a disposable tip.
- The gel was allowed to solidify at room temperature for 20-30 minutes.
- The comb was removed carefully from the solidified gel.
- The casting dams were removed from the edges of the gel tray. Care was taken when removing casting dams from the gel casting tray, so that the gel does not slide off the tray.

3.2.2.1.4. Preparation of DNA sample for electrophoresis

- At first on a piece of parafilm 2 µl 5x loading dye and 8 µl of 1X TBE buffer was dispensed using micropipette.

- 2 µl of DNA sample (after thawing from frozen stock) was added to the loading dye. Before loading of sample in to the agarose gel wells, it was mixed few times with the adjustable micropipette.

3.2.2.1.5. Loading the sample in the wells

The total volume of loading sample was 10 µl. Loading volume is dependent upon the type of comb used (i.e., well thickness and length) and thickness of the gel. The prepared samples were then loaded slowly to allow them to sink to the bottom of the wells. 2 µl of 1 kb DNA ladder (Fermentas, USA) was also loaded into the first well of the gel.

3.2.2.1.6. Electrophoresis

- The tray was placed by keeping the gel horizontal onto the base of the electrophoresis chamber, so that the sample wells were near the cathode (negative end generally marked as black). DNA sample would migrate towards the anode (positive end generally marked as red) during electrophoresis.
- Sufficient amount of 1x TBE buffer (about 600 ml) was added to cover the gel to a depth of about 5 mm/0.5 cm (1-5 mm may be used). The volume of the electrophoresis buffer should not be above the maximum buffer mark on electrophoresis system.
- Electrophoresis was carried out at 120 Volts for 1.5 hour. The separation process was monitored by the migration of the dye in the loading buffer. When the bromophenol blue dye had reached about three-fourths ($^3/_4$) of the gel length, the electrophoresis was stopped.

3.2.2.1.7. Documentation of the DNA sample

- After electrophoresis, the gel was taken out carefully from the electrophoresis chamber and placed on a UV transilluminator (Biometra TI 1, BDA, Germany) for checking bands.
- Photographed using a digital camera attached to the UV-transilluminator (Canon IXY Digital 220 IS, 12.1 Mega Pixels, Made in Japan).

3.2.2.2. Quantification and qualification of DNA by spectrophotometer

Different DNA extraction methods provide DNA of widely different concentrations and purity. Thus, it is necessary to optimize the amount of DNA used in the RAPD analysis to achieve reproducibility and also the strong signal. Below a certain concentration of genomic DNA, rapid amplification is no longer reproducible. Thus, it is essential to keep on above this critical concentration. UV-visible Spectophotometer (Shimadzu, Model UV-1200, Japan) was used in the Central Science Laboratory facility of Rajshahi University, Bangladesh for the measurement of DNA concentration and purity. Absorbance of nucleic (DNA/RNA) is taken at 260 nm. Another Absorbance of sample is taken at 280 nm to measure the presence of protein contamination in DNA (amino acid residue with aromatic ring absorbs 280 nm light). Good quality DNA should give the ratio (A260/A280) in range of 1.8-2.0. The A260/A280 higher than 2.0 generally indicates RNA contamination. For A260/A280 ratios lower than 1.8 normally indicates protein contamination during extraction process.

3.2.2.2.1. Set-up the spectrophotometer

To estimate genomic DNA concentration, absorbance reading of the DNA samples was recorded at 260nm using spectrophotometer. At first, the spectrophotometer's wavelength was set at 260 nm. Adequate time was given to warm the UV lamp prior to use the spectrophotometer.

- A square cuvette (the 'zero' or 'blank' cuvette) was filled with 2ml sterile deionized distilled water and placed on cuvette chamber and the absorbance reading was adjusted to zero for standardization.

3.2.2.2.2. Preparation of the DNA sample for spectrophotometer

- The test samples were prepared by taking 2 µl of each DNA sample in the cuvette containing 2 ml sterile distilled water. The samples were mixed well by using an adjustable micropipette.
- The reading was taken by viewing the monitor of spectrophotometer at 260 nm and at 280 nm respectively.
- After recording the absorbance readings, the cuvette was rinsed out this sterile distilled water, tamped out on a paper wipe and absorbance reading for each sample was recorded in the same way. The ratios of spectrophotometric absorption readings at 260 nm and 280 nm of different samples for quality determination of DNA.

3.2.2.2.3. Calculation of the concentration of DNA

By using the absorbance reading at 260 nm, the original sample concentrations were determined according to the following formula:

DNA concentration = A260 × Dilution factor × Conversion factor

$$= A260 \times \frac{\text{Volume of TE buffer } (\mu l)}{\text{Amount of the DNA sample } (\mu l)} \times 50$$

= (µg/ml)

= (ng/µl) [since 1 µg = 10^{-3}ng i.e., µg/ml = ng/µl]

A260: Spectrophotometric absorbance reading at 260 nm of DNA sample.

Dilution factor: the ratio of TE buffer (µl) to amount of DNA sample (µl).

Conversion factor 50: the 50 µg/ml of DNA contained in a solution which gives the Spectrophotometric absorbance reading at 260 nm equal to 1.

(**Note:** The results of the DNA concentration were obtained in µg/ml, this result containing a fraction. So, to avoid fraction and it was converted it into µg/µl (1µg = 10^{-3}ng). Therefore, the result was multiplied with 1000.)

3.2.2.2.4. Preparation of working solution (50 ng/µl) of DNA samples

Original stock solution concentration of each DNA sample was adjusted to a unique concentration (50 ng/µl) for RAPD PCR reaction using the following formula:

S_1 X V_1 = S_2 X V_2

V_2 = S_1 X V_1/S_2

= Final volume of DNA = [initial volume of DNA (V_1) + TE buffer]

Where,

S_1 = Initial concentration of DNA (ng/µl)

V_1 = Initial volume of DNA solution (µl)

V_2 = Final volume of DNA solution (µl)

S_2 = Final concentration of DNA (ng/µl)

3.2.3. Amplification of DNA by polymerase chain reaction (PCR) using RAPD markers

RAPD technique uses arbitrary primers that are usually ten nucleotides long to generate a set of DNA fragments based on the enzymatic amplification of different of DNA segments with a single arbitrary primer. Agarose gels (1.4%) were used to separate the amplification products that are visualized by ethidium bromide staining.

3.2.3.1. Principle of the amplification of RAPD markers

The RAPD technique is based on the polymerase chain reaction (PCR). A target DNA sequence is exponentially amplified with the help of arbitrary primers, a thermostable DNA polymerase, deoxy nucleotide triphosphates, $MgCl_2$ and reaction buffer. The reaction involves repeated cycles, each consisting of a denaturation, a primer annealing and an elongation step. In the first step the DNA is made single stranded by raising the temperature to 94ºC (denaturation). In the second step, temperature is lowered down to an optimal annealing temperature for optimal binding of primers to their target sequences on the template DNA (annealing step). In the third cycle, temperature is chosen as where the activity of the thermo-stable *Taq* DNA polymerase is optimal, i.e., usually 72ºC. The polymerase then extends the 3' ends of the DNA primer hybrids towards the other primer binding site. Repeating these three step cycles 40 to 50 times results in the exponential amplification of the target between the 5' ends of the two primer binding sites. Amplification products are separate by agarose gel electrophoresis and visualized by ethidium bromide staining.

3.2.3.2. Primer used

Seven primers of random sequences were used for RAPD experiments. Primer sequences were obtained from the operon technologies(USA) however the primers were purchased from 1st Base, Singapore). Primers were evaluated based on intensity of bands, consistency within individual, presence of smearing and potential for population discrimination. The details of the primers are given in the Table 3.2.4.

Table 3.2.4: List of 7 decamer RAPD primers with their sequences.

Serial No.	Primer Code	Sequence 5' → 3'	% G+C Content
01	OPA-8	GTGACGTAGG	60
02	OPA-9	GGGTAACGCC	70
03	OPA-10	GTGATCGCCG	70
04	OPB-17	AGGGAACGAG	60
05	OPC-17	TTCCCCCCAG	70
06	OPD-3	GTCGCCGTGA	70
07	OPE-6	AAGACCCCTC	60

3.2.3.3. Preparation of primers

Primers were delivered as 100 µM solution by 1st Base Singapore. 10 µM working solution were prepared from the stock of primers by diluting the primer 10 times using sterilized de-ionized water.

3.2.3.4. Preparation of dNTPs

The main stock of dNTPs was 100 mM (100 mM of dATP, dGTP dCTP, and dTTP), but the working solution of dNTPs for PCR reaction is 2.5 mM each. Therefore the equal amount of dNTPs were added in a 1.5 ml eppendrof tube, then to it was diluted 10 times with de-ionized water to make working stock solution for PCR reaction.

3.2.3.5. Preparation of master mix for RAPD based PCR reaction

Master mix for RAPD PCR reaction was prepared using sddH_2O, 10X polymerase Buffer with 15 mM $MgCl_2$, dNTPs (2.5 mM each of dATP, dCTP, dGTP and dTTP), template DNA and Fermentas Long PCR Enzyme mix (which is a mixture of Taq and pfu polymerase) (0.125U/10 µl reaction). The master mix was prepared according to following table 3.2.5. for 1 and 25 reactions of PCR cocktail of 10 µl.

Table 3.2.5: Preparation of master mix for RAPD based reaction

Sl. No.	Chemicals	Stock Concentration	Quantity for 1 reaction
1	Buffer with $MgCl_2$	10x	1.0 µl
2	dNTPs	2.5mM each	0.25 µl
3	Template DNA	50 ng/µl	1.0 µl
4	sddH_2O	-	7.475
5	Long PCR enzyme mix	5U/µl	0.025 µl
6	Primers	10µM	0.25
		Total	10.0 µl

3.2.3.6. Preparation of PCR reaction mixture/PCR cocktail

As we used DNA from six different varieties of castor and seven different primers master-mix was prepared for the reactions using dNTPs, polymerase, water and polymerase buffer. DNA template and primers were added to the individual tubes according to the quantity mentioned in the above table.

The reaction buffer (10 X polymerase buffer with $MgCl_2$, dNTPs, primer and DNA sample solutions) were thawed from frozen stocks, mixed by vortexing and kept on ice. First, master mix for PCR reaction was prepared using sddH_2O, Buffer (10X) with MgCl2, dNTPs (100 mM of ATP, dCTP, dGTP and dTTP) and DNA polymerase. Then 8.75 µl of master mixture was taken in 0.2 ml PCR tube. Then 0.25 µl of RAPD primer and 1 µl of template DNA was taken. Different combination of

the PCR reaction useing different primers and templets are shown in Table 3.2.6. This mixture was mixed up well by pipetting up and down and then briefly centrifused to collect everything at the bottom. The tubes were then sealed and placed in a thermo cycle and the cycling was started immediately. The DNA amplification was performed in oil-free Thermal Cycler (Bio Rad My Cycler, USA)

Table.3.2.6: PCR reactions of six cultivars of castor

3.2.3.7. PCR amplification

PCR reaction parameter was set as (Table. 3.2.7.) 95ºC for 3 minutes (initial denaturation/activation) followed by 7 cycles of 1 minute duration at 95ºC, 1 minute annealing at 35ºC and elongation or extension at 68ºC for 3.0 minutes. Followed by 40 cycles of 1 minute duration at 95ºC, 1 minute annealing 36ºC and elongation of extension at 68ºC for 3.0 minutes. After the last cycle, a final step of 10 minutes at 68ºC was added to allow the complete extension of all amplified fragments. Upon completion of the cycling programme, the reactions were held at 16ºC. To obtain accurate amplification we used Long PCR enzyme in our reaction mixture which has proof reading activity. It contains the mixture of *taq* and *pfu* polymerase and has an optimum extension temperature of 68ºC degree instead of 72ºC like normal *taq* polymerase. As the PCR reaction components are expensive we used 10µl reaction in stead of 50µl or 25µl of standard PCR reaction to cut down cost of our experimentation.

Table. 3.2.7: RAPD PCR reaction parameter for six cultivars of castor

95ºC Maximum 3 minutes

Steps	Temperature	Duration (Minute)	Cycle
Initial activation/ denaturation	95 ºC	3.00	1
Denaturation Anneling Extension	95 ºC 35 ºC 68 ºC	1.00 1.00 3.00	7 Cycles
Denaturation Anneling Extension	95 ºC 36 ºC 68 ºC	1.00 1.00 3.00	40 Cycles
Final Extension	68 ºC	10.00	1
Hold	16 ºC	α	

PCR products from each sample were visualized by running 1.4% agarose gel containing ethidium bromide. A molecular weight marker DNA (1 kb DNA ladder of Fermentas, USA) was also loadded on the gel. RAPD bands were observed under Ultra Violet light on a transilluminator and documented by taking photograph using a digital camera (Canon IXY Digital 220 IS, 12.1 Mega Pixel, Made in Japan) and Alpha View 3.2. (Cell Biosciences, Inc.) gel documented system.

3.3. RESULTS

DNA isolation, qualification and quantification are the prerequisite for fingerprinting and genetic diversity of varieties/accessions based on molecular markers. In the current study we used six cultivars of castor. An easy and efficient method for DNA isolation of castor was adopted using available chemicals and simple equipments. The isolation of DNA of six cultivars of castor accessions were successfully used for DNA fingerprinting using the seven primers. Results of the investigation have been presented and described in this chapter under the following headings and subheadings:

3.3.1. Isolation of DNA

A modified CTAB method reported by Winnepenninckx *et al*. 1993 was used to isolate total genomic DNA. Young leaves of six cultivars of castor were used as sample for DNA extraction.

3.3.2. Quality of DNA

Quality of DNA was determined by using agarose gel electrophoresis and spectrophotometric methods. To check the DNA quality, isolated genomic DNA was run on a 1.4% agarose gel for each sample of castor (Plate 3.3.1.). Agarose gel electrophoregram indicates presence of both degraded and good quality DNA. Our electrophorogram shows that the extraction was good and DNA with high quality without much of smearing. Smearing is the indication of shearing of DNA or degraded DNA. Enzyme such as RNase and Proteinase were used in our method of DNA extraction which eliminates the possibility of presence of RNA or protein with our DNA sample. High concentration of NaCl (5M) was used for removal of polysaccharides. DNA quality is affected by polymorphic compounds, which interact with DNA. The compounds such as PVP and CTAB adsorb polyphenols or prevent oxidations. They also inhibit the interaction of genomic DNA with oxidized Polyphenols, which affects DNA quality (Al-janabi *et al*. 1999).

Spectrophotometer is commonly used in laboratories for the measurement of DNA purity. The purity of the isolated DNA was also determined by spectrophotometric quantification. The DNA purity was measured by dividing the absorbance at 260 nm (A260) by the absorbance at 280 nm (A280). The ratio A260/A280 higher than 2.0 generally indicates RNA contamination. For A260/A280 ratios lower than 1.8 normally indicates protein contamination during extraction process. Good quality DNA showed give the A260/A280 in the range of 1.8-2.0. All

the DNA from six cultivars isolated in the current study has a 260/280 ratio within the range of 1.78 to 1.97 (Table 3.3.1).

3.3.3. Quantification of DNA

To find out the amount of isolation DNA, the visual estimation and spectrometric method were used. Visually the DNA was estimated by observing the DNA in the electrophoreses of agarose eg. In spectrophotometric method amount of DNA was determined by taking absorbance reading at 260 nm. The amount of DNA was observed 260/280 ratio near 1.8 for pure DNA. Elevated ratio usually indicates the presence of RNA, which can be confirmed by running a sample, 260/280 ratios below 1.8 often signal the presence of contamination protein, or phenol. This results agreed by Clark and Christopher (2000).

It was revealed form the spectrophotometric data (Table 3.3.1.) that the isolated DNA from the six cultivars of castor young leaves were of good quality to use in our subsequent RAPD experiments.

Table 3.3.1. Quality and quantity assessment of DNA isolated from young leaves of six cultivars of castor.

Cultivars	OD at 260 nm	OD at 280nm	OD 260/280	Total DNA (ng/µl)
V_1 (Shabje)	1.214	0.667	1.82	60.50
V_2 (Shadatae)	0.882	0.495	1.78	44
V_3 (Roktima)	1.027	0.552	1.86	51
V_4 (Lalchay)	1.135	0.576	1.97	56.50
V_5 (Badami)	0.381	0.209	1.82	19
V_6 (Dhusar)	0.414	0.231	1.79	20.50

*DNA was dissolved in 50 µl of TE buffer.

3.3.4. DNA fingerprinting using RAPD markers

The six cultivars of castor under study were identified using the 28 morphological characteristics. Result of the morphological variation was confirmed using the RAPD experiment. The RAPD technique has provided a quick and efficient for DNA sequence-based polymorphisms at a very large number of loci. The major advantage is that no prior DNA sequence information is required. DNA fingerprinting of six cultivars of castor accessions were performed using the seven RAPD markers developed by Operon Technologies Inc. However, the primers were ordered from 1st

base Singapore using the same sequence information. The seven primers used in our RAPD experiments are: OPA-8, OPA-9, OPA-10, OPB-17, OPC-17, OPD-3, OPE-6.

3.3.4.1. Number of bands

Our RAPD experiment shows promising result for the proper identification of the cultivars using genetic markers. Among the seven primers used for six cultivars of castor for DNA fingerprinting 3 primers produced bands but other 4 primers failed to show any DNA band in agarose gel electrophoresis. Number of amplified fragments scored against six cultivars of castor using 3 RAPD primers are presented in Table number 3.3.2. Three RAPD primers generated total 50 bands and average 16.66 from the six cultivars of castor. The primer OPA-9, OPA-10 and OPB-17 produced 26, 17 and 7 bands respectively and the number of total bands was 50. The primer OPA-9 produced the maximum number of bands (26) and OPB-17 produced the lowest number of bands (7) bands (Plate 3.3.2-3.3.4). Out of 50 bands, 23 bands were found polymorphic with average value for each primer was 7.67 (Table 3.3.3). Primer OPA-9 produced the 14 polymorphic bands with number of bands per variety was 4.33. Primer OPA-10 produced the 2 polymorphic bands with number of bands per variety was 2.83 and primer OPB-17 produced the 7 polymorphic bands with number of bands per variety was 1.67. The most polymorphic RAPD marker showed unique banding pattern (100%) by the primer OPB-17, followed by primer OPA-9 (53.85%) and OPA-10 (11.76%) respectively.

Table 3.3.2. Number of amplified fragments scored against six cultivars of castor using 3 RAPD primers.

Primer code	Cultivar Shabje (V_1)	Cultivar Shadatae (V_2)	Cultivar Roktima(V)	Cultivar Lalchay (V_4)	Cultivar Badami (V_5)	Cultivar Dhusar(V_6)
OPA-9	7	7	8	-	-	4
OPA-10	-	6	6	-	-	5
OPB-17	-	-	7	-	-	-

Table 3.3.3. RAPD primers with corresponding bands scored and their number of bands per variety, polymorphic bands and polymorphism in six cultivars of castor.

Primer code	Number of bands/variety	Polymorphic bands	Polymorphism
OPA-9	4.33	14	53.85%
OPA-10	2.83	2	11.76%
OPB-17	1.67	7	100.00%

Total = 23

Average =7.67

GEL ELECTROPHORESIS (ONLY DNA)

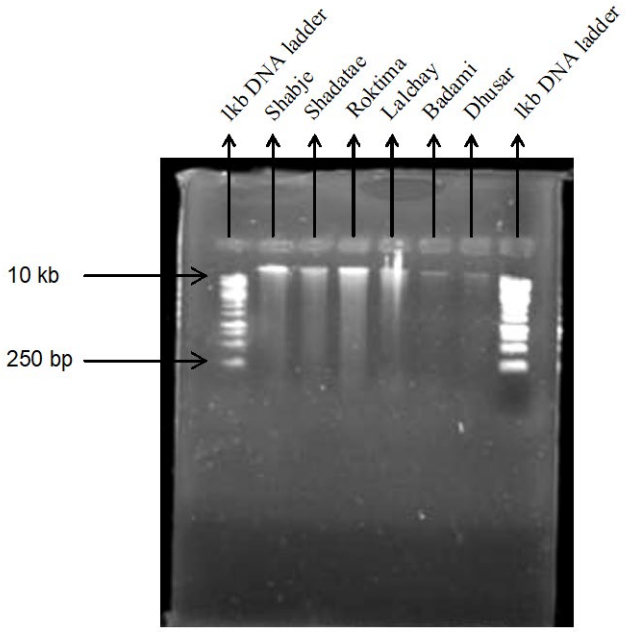


Plate- 3.3.1: Agarose gel photograph of isolated DNA stained with ethiduim bromide. 5 µl of DNA sample and 3 µl of DNA ladder were loaded on the gel using 5X loading dye. DNA ladder was loaded in 1st and 8th lane of the gel. DNA extracted from Shabje, Shadatae, Roktima, Lalchay, Badami and Dhusar cultivar was loaded in 2nd, 3rd, 4th, 5th, 6th and 7th lane respectively. All the extracted DNA was found above the highest band of the ladder (10 kb) which is indicative of a good quality DNA.

GEL ELECTROPHORESIS (DNA + OPA-9)

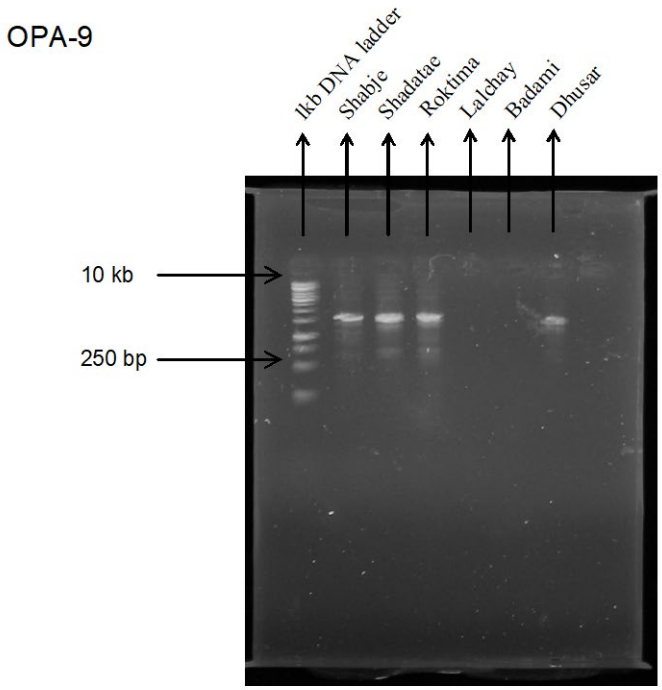


Plate- 3.3.2: Agarose Gel photograph of RAPD PCR product of six cultivars of castor using OPA-9 primer. DNA was stained using ethiduim bromide. 5 µl of PCR Product and 3 µl of DNA ladder were loaded on the gel using 5X loading dye. DNA ladder was loaded in 1st of the gel. PCR product derived from Shabje, Shadatae, Roktima, Lalchay, Badami and Dhusar cultivar was loaded in 2nd, 3rd, 4th, 5th, 6th and 7th lane respectively. Obtained bands are marked with solid arrow.

GEL ELECTROPHORESIS (DNA + OPA-10)

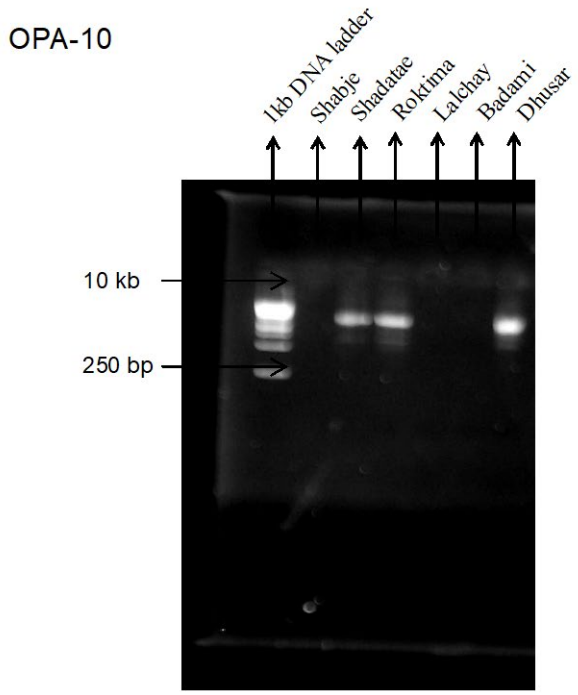


Plate- 3.3.3: Agarose Gel photograph of RAPD PCR product of six cultivars of castor using OPA-10 primer. DNA was stained using ethiduim bromide. 5 µl of PCR Product and 3 µl of DNA ladder were loaded on the gel using 5X loading dye. DNA ladder was loaded in 1st of the gel. PCR product derived from Shabje, Shadatae, Roktima, Lalchay, Badami and Dhusar cultivar was loaded in 2nd, 3rd, 4th, 5th, 6th and 7th lane respectively. Obtained bands are marked with solid arrow.

GEL ELECTROPHORESIS (DNA + OPB-17)

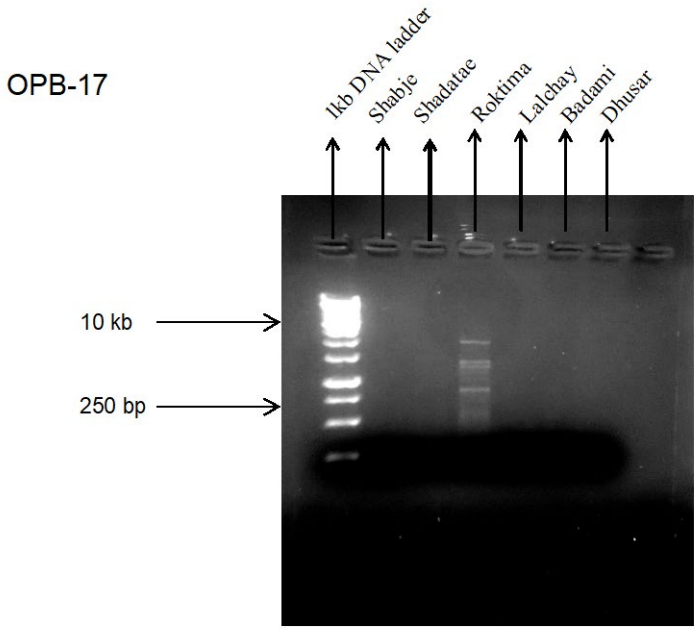


Plate- 3.3.4: Agarose Gel photograph of RAPD PCR product of six cultivars of castor using OPB-17 primer. DNA was stained using ethiduim bromide. 5 µl of PCR Product and 3 µl of DNA ladder were loaded on the gel using 5X loading dye. DNA ladder was loaded in 1st of the gel. PCR product derived from Shabje, Shadatae, Roktima, Lalchay, Badami and Dhusar cultivar was loaded in 2nd, 3rd, 4th, 5th, 6th and 7th lane respectively. Obtained bands are marked with solid arrow.

3.4. DISCUSSION

Germplasm is the raw material of crop improvement and considered as the living museum of the sum total of variability. In Bangladesh, castor grown all over the country without any special care and identity. Castor is not a mandated crop plant of any research organization, no scientific endeavor was made on its improvement including both in the field of its agronomy, taxonomy and genetics. A number of cultivars are found to grow in different places of Bangladesh but their proper identification and scientific characterization has not been made. Characterization of the cultivars and genetic identity are important for the cultivars of castor in Bangladesh as they hold tremendous potentiality for exploring its merit in different industrial uses. In Bangladesh, possibly it was the first attempt to pursue advance research works on castor towards its scientific characterization accompanied by the isolation of DNA for RAPD to check the genetic diversity among the six cultivars of castor. Despite the recent efforts to characterize castor bean germplasm collections, there are relatively few molecular markers available (Santelmo *et al*. 2012) curiously, the use of widely spread and low cost anonymous markers, as RAPD and ISSR to be used for genetic diversity analysis is still problematic and insufficient (Santelmo *et al*. 2012). In spite of the recent publication of the castor bean genome (Chan *et al*. 2010), little is known about the actual genetic diversity of this species. Unlike other important oilseed crops, as oil palm (*Elaeis guineensis*), soybean (*Glycine max*), sunflower (*Helianthus annuus*), and some Euphorbiaceae species, as cassava and rubber tree, castor bean diversity is still poorly characterized by means of molecular marker systems (Billotte *et al*. 2010, Feng *et al*. 2009, Sayama *et al*. 2011, Sraphet *et al*. 2011, Talia *et al*. 2010). In fact, the species had been overlooked until the late 2000, when analyses regarding genetic diversity of germplasm collections were first published (Allan *et al*. 2008). However, castor bean was the first member of the Euphorbiaceae family with the whole genome published (Chan *et al*. 2010), a fact that will be of great importance for characterizing the genetic base of the species.

The evaluation of varieties in distinct agro-climatic zones demonstrates significant levels of variation in response to the selection pressure prevailing in the zones (Singh *et al*. 1998). Both the RAPD and ISSR molecular markers have been used in population genetic studies (Parsons *et al*. 1997, Esselman *et al*. 1999, Li and Ge 2001). In the present investigation we have used only RAPD molecular markers. In our investigation we used seven primers against the six cultivars and among the seven primers three gave a total of 50 visible bands in gel electrophoresis with an

average of 16.66 bands from the six cultivars of castor which can be used for fingerprinting.

Cultivar shabje showed 7 bands in primer OPA-9, but did not show any band in primer OPA-10 and OPB-17. Cultivar shadatae showed 7 bands in primer OPA-9 and 6 bands in primer OPA-10, but did not produce any band in primer OPB-17. Cultivar roktima showed 8 bands in primer OPA-9, 6 bands in primer OPA-10 and 7 bands in primer OPB-17. Cultivar dhusar showed 4 bands in primer OPA-9, 5 bands in primer OPA-10 but did not show any band in primer OPB-17. Cultivar lalchay and cultivar badami did not show any band in any primer which were used in our experiment.

Primer OPA-9 produced 14 polymorphic bands out of total 26 bands and showed 53.85% polymorphism. Primer OPA-10 produced 2 polymorphic bands out of total 17 bands and showed 11.76% polymorphism. Primer OPB-17 produced 7 polymorphic bands out of total 7 bands and showed 100% polymorphism. Some researchers have considered RAPD markers to represent segments of DNA with non coding regions and to be selectively neutral (Bachmann 1997, Landergott *et al*. 2001), and some studies have shown that RAPD markers are distributed throughout the genome and may be associated with functionally important loci (Penner 1996). Dendogram has not been computed but observed result indicated that the cultivar lalchay and badami failed to produce any band against any one of the seven primers and confirming that these two cultivars are widely related to the other four castor cultivars. The cultivar shabje denoting its distant phylogenic position amplifying DNA bands only for one time against the primer OPA-9. The cultivars shadatae and cultivar dhusar are presumed to be more likely related as they amplified DNA bands against the two primers OPA-9 and OPA-10. While the cultivar roktima produced seven bands against the primer OPB-17 denoting its individual identity and very much different from other castor cultivars. In our investigation the cultivar roktima also has been appeared as a very distinct characters or very distinct genetic diversity in other sections of research including morphological parameters and cell culture and antibacterial activities. The castor cultivar roktima is virtually a unique cultivar showing very remarkable reddish in colour for its stem, petiole and flowering inflorescence. In a nutshell, in order to make more precise interference about the genetic diversity and phylogenic relationship among the castor cultivars further research is needed on detail analysis of RAPD and SSR markers.

However, from the foregoing discussion it can be concluded that highly differential banding pattern was observed among the cultivars of castor. The presence of wide level of genetic diversity existed among the different cultivars of castor in Bangladesh. Use of different primers exhibited different levels of DNA polymorphism in different castor cultivars proving a very wide level of intraspecies genetic diversity in Bangladeshi castor cultivars.

Table.3.2.6: PCR reactions of six cultivars of castor

V_1 (Cultivar shabje)		V_2 (Cultivar shadatae)		V_3 (Cultivar roktima)		V_4 (Cultivar lalchay)		V_5 (Cultivar badami)		V_6 (Cultivar dhusar)	
Reaction 1	OPA-8	Reaction 8	OPA-8	Reaction 15	OPA-8	Reaction 22	OPA-8	Reaction 29	OPA-8	Reaction 36	OPA-8
Reaction 2	OPA-9	Reaction 9	OPA-9	Reaction 16	OPA-9	Reaction 23	OPA-9	Reaction 30	OPA-9	Reaction 37	OPA-9
Reaction 3	OPA-10	Reaction 10	OPA-10	Reaction 17	OPA-10	Reaction 24	OPA-10	Reaction 31	OPA-10	Reaction 38	OPA-10
Reaction 4	OPB-17	Reaction 11	OPB-17	Reaction 18	OPB-17	Reaction 25	OPB-17	Reaction 32	OPB-17	Reaction 39	OPB-17
Reaction 5	OPC-17	Reaction 12	OPC-17	Reaction 19	OPC-17	Reaction 26	OPC-17	Reaction 33	OPC-17	Reaction 40	OPC-17
Reaction 6	OPD-3	Reaction 13	OPD-3	Reaction 20	OPD-3	Reaction 27	OPD-3	Reaction 34	OPD-3	Reaction 41	OPD-3
Reaction 7	OPE-6	Reaction 14	OPE-6	Reaction 21	OPE-6	Reaction 28	OPE-6	Reaction 35	OPE-6	Reaction 42	OPE-6

CHAPTER-IV

4.1: INTRODUCTION

4.1.1: Plant tissue culture

The primary advantage of micropropagation is the rapid production of high quality, disease-free and uniform planting material. The plants can be multiplied under a controlled environment, anywhere, irrespective of the season and weather, on a year-round basis. Production of high quality and healthy planting material of ornamentals, and forest and fruit trees, propagated from vegetative parts, has created new opportunities in global trading for producers, farmers, and nursery owners, and for rural employment. In present day plant tissue culture is a general term that encompasses and is concerned with the study of cells, tissues and organs maintained or grown *in vitro*. It includes the techniques of protoplast, cell, tissue and organ culture, followed by the success achieved in regenerating whole plant from the cultured plant materials. This field of plant biotechnology has advanced rapidly during the last fifty years. Currently it is used as an important tool in the both basic and applied researches as well as in commercial application. Tissue culture is now consider very promising for rapid and economic clonal multiplication of fruits and forest trees, production of virus-free stocks of clonal crops, creation of novel genetic variation through somaclonal and gametoclonal variation and transfer of novel and highly valuable genes (usually for herbicide and disease resistance) and that has opened up exciting possibilities in crop production, protection and improvement. It provides viable alternative methods of mass production of healthy plants with uniform characteristics. These techniques have the potentials not only to improve the existing cultivars but also for the synthesis of novel plants.

Plant tissue culture is now a well-established technology. Like many other technologies, it has gone through a series of stages of evolution; scientific curiosity /research tool/ novel application / mass exploitation/ maturation (rationalization). Today the application of plant tissue culture encompasses much more than clonal propagation and micro-propagation. The range of routine technologies has expanded to include somatic embryogenesis as well as the application of bioreactors to mass propagation. We can summarize the application of plant tissue culture as pointed below:

- Through tissue culture hundreds of plantlets can be grown from a small, even microscopic pieces of plant tissue over the year round.
- Rapid and large scale multiplication of genetically identical plants (clonal propagation) from a single "superior" stock plant by axillary bud growth, direct (adventitious) organogenesis, callus to organogenesis and somatic embryogenesis.

- Production of pathogen-free plants by meristem and callus culture. This also facilitates transfer of plant material through international borders.
- Germplasm storage and long term storage (cryopreservation) of stock plants through *in vitro* gene banks.
- Selection of mutants from spontaneous or induced mutations.
- Recovery of hybrids from incompatible species through either embryo or ovule culture.
- *In vitro* grafting.
- Production of haploid plants through anther or microspore culture. Haploid plants may be used to recover recessive mutations in breeding programmes. Subsequent regeneration of double haploid provides homozygous and thus pure breeding lines.
- Production of rooted micro cuttings in recalcitrant woody ornamental species.
- Protoplast culture and somatic fusion.
- DNA transformation system.
- Recovery of regeneration of transformed cells.
- Production of secondary metabolites.

Now-a-days, the application of plant tissue culture goes well beyond the bounds of agriculture and horticulture. It has found application in environmental remediation, mining, waste recycling, industrial processing etc. (Williams 2002).

4.1.2. Tissue culture of castor

Castor (*R. communis* L.) is a member of the family Euphorbiaceae or spurge family, mostly native of the tropical and subtropical regions (Weiss 2000). It is medicinally important oil seed crop containing approximately 60% oil (Kumari *et al*. 2008) and are the only commercial source of ricinolic acid that is used as industrial lubricants, paints, coatings, and plastics (Moshkin 1986b, Caupin 1997). In world trade, India is the largest castor oil producer, representing 60% of production, followed by China and Brazil (Weiss 2000, FAO 2006).

Most of the world's castor cultivars are vulnerable to insect attack. Reliable sources of resistance to the major insect pests are rather limited in the available germplasm of this monotypic genus (Sujatha and Sailaja 2005). Genetic engineering appears as one of the necessary tools for the improvement of cultivars of this species to reduce the levels of these hazardous proteins. Therefore, an efficient regeneration protocol for castor needs to be established, which is rapid, reproducible and applicable to a broad range of genotypes. However, castor is extremely recalcitrant to *in vitro* regeneration (Ahn *et al*. 2007). The previous reports on *in vitro* shoot multiplication

of castor (Athma and Reddy 1983, Reddy *et al*. 1987a, Sangduen *et al*. 1987, Reddy and Bahadur 1989a, Sarvesh *et al*. 1992) using vegetative tissue as explants was either inefficient or difficult to reproduce (Ahn *et al*. 2007). Then researchers started to focus on meristematic tissues to improve regeneration efficiency (Molina and Schbert 1995, Lakshmi and Bhadur 1997, Sujatha and Reddy 1998). Sujatha and Reddy (1998) reported the first reliable protocol using embryonic tips and shoot apex. However, all the shoots were derived from the preexisting meristem, and adventitious shoot formation was not observed. When applied in *Agrobacterium* mediated transformation, this protocol resulted in a low rate of putative transformant recovery (0.08%, one putative transformant in, 1,200 embryonic tips used (Sujatha and Sailaja 2005). The transformation efficiency of meristem based protocol was also developed by McKeon and Chen in 2003, but the efficiency was also very low. Therefore, it is necessary to develop an effective regeneration protocol by a range of different techniques which would widen the possibilites of developing transgenic lines and/or somaclonal variants. The present investigation was, therefore, undertaken to establish a high frequency plant regeneration system from the seedling explants of a local *Ricinus communis* L. cultivars using tissue culture technique.

4.1.3. Objectives

As the conventional and traditional methods for vegetative propagation of caster are either unreliable or unfeasible, a reliable *in vitro* method for the propagation of castor would have considerable benefits to the horticultural industry. Castor contains some toxic compounds like ricin (Hartley and Lord 2004) and hyperallergenic 2S albumins (Shewry *et al*. 2002) in its seeds, which restrict its commercial cultivation in USA (Ahn *et al*. 2007). Most of the world's castor cultivars are vulnerable to insect attack. Reliable sources of resistance to the major insect pests are rather limited in the available germplasm of this monotypic genus (Sujatha and Sailaja 2005). Genetic engineering appears as one of the necessary tools for the improvement of cultivars of this species to lower the toxicity of seed meal and to confer resistance to biotic stresses. The success of using such approach largely depends on an efficient *in vitro* regeneration system, which is rapid, reproducible and applicable to a broad range of genotypes. However, castor is extremely recalcitrant to *in vitro* regeneration (Ahn *et al*. 2007). The previous reports on *in vitro* shoot multiplication of castor (Athma and Reddy 1983, Reddy *et al*. 1987a, Sangduen *et al*. 1987, Reddy and Bahadur 1989a, Sarvesh *et al*. 1992) using vegetative tissues as explants was either inefficient or difficult to reproduce (reviewed by Ahn *et al*. 2007). While shoot induction from embryonic tips and shoot apex involved preexisting

meristem and inefficient in adventitious shoot formation (Molina and Schobert 1995, Sujatha and Reddy 1998). The transformation efficiency of meristem-based protocols was found very low (0.04%) (Sujatha and Sailaja 2005). Therefore, it is necessary to develop an effective regeneration protocol by a range of different techniques which would widen the possibilities of developing transgenic lines and/or somaclonal variants. Therefore, present investigation was undertaken to develop *in vitro* culture techniques for six cultivars of castor to evaluate the morphogenic potentialities of different explants for most efficient production of plantlets either through precocious axillary or adventitious bud proliferation with the following objectives:

1) Identification and selection of suitable explant(s) for fast response and better regenerative potentialities.
2) Selection and standardization of media composition, growth regulator requirement and culture environment for consistently high production of plantlets.
3) Selection of auxin type and concentration and culture condition for efficient rooting on *in vitro* proliferated shoots.
4) To develop the efficient protocol for extensive callus culture as the ultimate target for initiation for cell suspension culture to isolate cell extract.

4.2. MATERIALS AND METHODS

4.2.1. MATERIALS

4.2.1.1. Plant materials

In vitro grown seedlings of *Ricinus communis* L. six cultivers were used as source of explants to conduct different experiments in the present investigation.

The following parts were used as explants:

a) Shoot tip segment
b) Cotyledonary node segment
c) Nodal segment
d) Hypocotyl segment
e) Internode segment
f) Immature leaf segment

4.2.1.2. Nutrient medium

Unless otherwise mentioned, MS revised basal salt composition (Murashige and Skoog 1962) was used either as original formulation or as in modified forms to fulfill the medium used of the experiment, which are mentioned in appropriate places. Detailed chemical compositions of the media formulations used have been mentioned in the section-A.

4.2.1.3. Plant growth regulators

The following plant growth regulators were used in the present investigation :

Auxins :

Indole-3-acetic acid (IAA)
Indole-3-butyric acid (IBA)
α-napthalene acetic acid (NAA)
2,4-dichlorophenoxy acetic acid (2,4-D)

Cytokinins:

6-benzyl adenine (BAP)
6-furfuryl amino purine (Kinitin, Kn.)
2-Isopentenyl adenine (2ip)

Gibberellins :

Gibberellic acid (GA_3)
Thiodiazuron (TDZ)

4.2.1.4. Growth additives

Sucrose, agar powder and activated charcoal were also used in the nutrient medium as carbon source, gelling agent and polyphenol adsorbent respectively.

4.2.1.5. Sterility and surfactant

In the present investigation mercuric chloride ($HgCl_2$) and ethanol were used as sterilizing agents. Savlon (3% v/v cetrimide, an antiseptic plus detergent, ICI, U.K. marked in Bangladesh by ICI Bangladesh Ltd.) was used as detergent and surfactant.

4.2.2. METHODS

Experimental methods used for carrying out this investigation were accomplished through following steps:

4.2.2.1. Preparation of stock solution for culture media

The first step in the preparation of culture media was the preparation of the stock solution. Various constituents of the respective nutrient media were prepared into stock solutions for ready use during the preparation of media for different experiments. As different constituents were required in different concentrations, stock solutions of macro-nutrients, micro-nutrients, organic nutrients and growth regulators were prepared separately.

4.2.2.1.1. Stock solution of macro-nutrients (Solution A): This stock solution was made as 20 times of the final strength of the medium in 1000 ml of distilled water. At first 20 times the weight of each of the major salt required for one liter of the medium were weighed accurately, dissolved once at a time and sequentially in 750 ml of distilled water and then the final volume was made up to 1000 ml by further addition of distilled water. This stock solution was then poured in a clean glass container and was labeled by 20 × 1000 ml and stored in a refrigerator at 4-6ºC for several weeks.

4.2.2.1.2. Stock solution of micro-nutrients: Two separate stock solutions of micro-salts were prepared as follow:

(a) Stock solution of $FeSO_4$ and Na-EDTA (Solution B): This solution was prepared at 100x so that of required concentration. Requisite amount of $FeSO_4$ and Na-EDTA were taken and dissolved separately in clean glass beakers containing 225 ml of distilled water. Na-EDTA solution was then transferred in a 500 ml volumetric flask.

Subsequently the solution of $FeSO_4$ was poured to the volumetric flask slowly with constant stirring. The final volume of the solution was made up to 500 ml and placed in a incubator for 24 hours at 58ºC. pH of the solution was adjusted at 5.7 and after filtering it was stored at 4-6ºC in refrigerator.

(b) Stock solution of rest of the micro-nutrients (Solution C): This was made at 100x in 500 ml distilled water as described for solution A. All components were weighed separately and dissolved in 400 ml of distilled water. Finally, the volume of the solution was adjusted to 500 ml and after filtering stored at 4-6ºC in plastic bottle.

4.2.2.1.3. ***Stock solution of organic nutrients (Solution D):*** This stock solution was also prepared at 100x, dissolved in 100 ml distilled water as described for the stock solution A. The solution was stored at 4-6ºC in a plastic bottle.

4.2.2.1.4. ***Stock solution of growth regulators:*** Stock solution of different growth regulators was prepared separately. Details of the methods of preparation of stock solution are given in the Table 4.2.1.

Table 4.2.1. Different plant growth regulators and their solvents.

Growth regulators	Amount taken (mg)	Solvents (ml)	Final volume of the stock solution with distilled water (ml)	Strength of the stock solution (mg/ml)
IAA	10	0.1 N NaOH 1ml	100	0.1
IBA	10	0.1 N NaOH 1ml	100	0.1
NAA	10	0.1 N NaOH 1ml	100	0.1
2,4-D	10	70 % EtOH 0.5ml	100	0.1
BA	10	0.1 N NaOH 1ml	100	0.1
Kn	10	0.1 N NaOH 1ml	100	0.1
2Ip	10	0.1 N NaOH 1ml	100	0.1
GA_3	10	70 % EtOH 1ml	100	0.1
$AgNO_3$	10	Water	100	0.1

To prepare the stock solution of any of these growth regulators, 10 mg of powdered growth regulators was taken in a clean test tube and dissolved in required volume of appropriate solvent. The final volume of the solution was then made to 100 ml by adding distilled water. The solution was then poured in to a 100 ml glass reagent bottle and stored at 4-6ºC.

4.2.2.2. Surface sterilization

For surface sterilizing the plant materials to be cultured 0.1 % (w/v) $HgCl_2$ solution was used. This solution was prepared taking 250 mg of $HgCl_2$ in a 500 ml bottle and dissolved in 250 ml distilled water. Freshly prepared $HgCl_2$ was always used to ensure maximum effectiveness of the sterilization. Generally $HgCl_2$ solution was prepared 15-30 min before use.

Other substances like sucrose, agar, activated charcoal etc. were added directly to the media during preparation.

4.2.2.3. Preparation of nutrient media

The following steps were followed to make one liter of MS medium:

a) 50ml of stock solution A and 5 ml of each of the stock solutions B, C and D were taken in 1 liter volumetric flask containing 750 ml distilled water.
b) Different concentrations of hormonal supplements as required were added singly or in combination to the solution and throughly mixed. Since 1 ml of each of the hormonal stock solution contained 0.1 mg solute, therefore, addition of 10 ml stock solution of any of the hormone to one liter medium resulted 1 mg/l concentration. Hormonal concentration was made different by varying the volume of the stock solution as per requirement.
c) Other supplements such as activated charcoal, if required, were also added to the nutrient solution.
d) Unless otherwise mentioned, 8 gm/l agar (BDH) and 30 gm/l sucrose were added to the medium. With the special need of the experiment amount of any one of these supplements was varied keeping other conditions unchanged.
e) Final volume of the medium was then made 1 liter by adding distilled water.
f) Unless stated other wise the pH of the medium was adjusted to 5.7 ± 0.1using a digital pH meter by adding0.1N KOH or 0.1N HCl which ever necessary.
g) The medium was then transferred to 2 liter bottle and heated under low pressure in micro-oven for some time to melt the agar. The medium was shaked well to ensure uniform distribution of agar throughout the medium.

h) Requisite volume of the medium (while still hot) was then dispensed in to culture vessels (15-20 ml/25 mm test tube, 20-25 ml/150 ml conical flask and 45-50 ml/250 ml conical flask) of varying sizes.

i) The culture vessels were then plugged with non-absorbent cotton wrapped in cheese-cloth or heavy duty aluminum foil. Then the vessels were marked properly by glass marker to indicate the specificity of the medium.

j) Finally, the culture vessels containing nutrient medium were sterilized by autoclaving at 121ºC for 20 min. at 1.1 kg/cm² pressure. In case of flasks the medium were allowed to cool as vertically but the test tubes having media were allowed to cool as slants for shoot differentiation and as vertically for root induction and finally stored in the culture room (not more than a week) for ready use.

4.2.2.4. Culture techniques

The following techniques were employed in the present investigation for regenerating the complete plantlets and discussed under the following head.

4.2.2.4.1. Collection of seeds and raising of aseptic seedlings

Seeds from the ripe castor fruits of six cultivars were first collected and washed throughly under running tap water. Surface sterilization includes treatment of the seeds with 1 % savlon for 10 min., with constant shaking. Then the seeds were washed 3-4 times with distilled water and taken into laminar airflow cabinet and transferred to 250 ml sterilized conical flask. After rinsing in 80 % ethanol for 2 min. they were immersed in 0.1 % $HgCl_2$. While sterilization, the sterilant with seeds were constantly shaked for 5-7 min.. The remove every trace of the sterilant, the material was then washed with sterile distilled water at least 4-5 changes of water to make it completely free from $HgCl_2$. The sterilized seeds were then taken in a sterilized petridis and seed coats were carefully removed with the help of a sterilized surgical blade, forceps and needle. Aseptic seedlings were raised from the surface sterilized, decoated seeds on MS (Murashige and Skoog 1962) semi-solid medium supplemented with 3 % sucrose and with or without 1 mg/l BA. The aseptic seedlings thus grew attained a height of 70-80 mm after 4-5 weeks of culture. Different types of explants (8-10 mm segments) consisting of cotyledonary node, shoot tip, nodal segment, hypocotyl, internodal segment and immature leaf were prepared from the *in vitro* raised seedlings and were used as explants to conduct different experiments in the on-going investigation.

4.2.2.5. Culture environment

Unless mentioned specially, all cultures were maintained in a growth room under 16 h photoperiod with a light intensity of 50-70 $\mu Em^{-2}s^{-1}$ provided by warm-white florescent tubular lamp at 26±1ºC. For dark treatment, the cultures were raised in an incubator and maintained at desired temperature or the culture vessels were placed in a cardboard box in the same culture room. Humidity was not controlled for any of the experiment.

4.2.2.6. Setting and designing of experiments

Different experiments were conducted on various chemical and physical conditions (factors) of the medium for evaluating proper culture requirements for expressing the optimum morphological potentialities of different explants. Generally cultures were grown on to culture media supplemented with 3 % sucrose, 0.8 % agar (BDH) and pH at 5.7 ± 0.1. In order to determine the optimum condition of a specific factor, experiments were conducted with various notifications of one factor keeping the others constant. Most of the experiments were conducted following standard tissue culture method. All experiments had 10-15 replicated cultures per treatment and they were repeated two-three times. The cultures were grown for 1-4 consecutive passages of 4-5 week each and data of different parameters were recorded at the end of each passage.

4.2.2.7. Maintenance of culture

Different processes were followed for maintaining the proliferating cultures through subcultures and discussed under separate heads.

4.2.2.7.1. Axillary shoots proliferating cultures: Proliferating cultures of axillary shoots were established on suitable medium from shoot explants of *in vitro* raised seedlings. Cotyledonary node segments from these seedlings were found to be the best explants for axillary shoot formation on agar gelled MS medium with ½ strength major salts and 0.5-1.0 mg/l of BAP. On this medium axillary buds showed sprouting within one week of incubation. At this stage, the proliferating cultures were subcultured again in the same initial medium in order to increase budding frequency. After another 4 week incubation the proliferating culture were transferred to different media for bud elongation. The elongation shoots (usable shoots) were excised from the proliferated cultures and transferred individually to the rooting media. Some of the shoots after removing leaves, were cut into pieces having axillary buds and again cultured to freshly prepared medium for multiplication of axillary shoot. These

cultures again produced usable axillary shoots within 4 week of subculture. The process had been repeated for several times in order to establish continuous production of shoots.

4.2.2.7.2. Callus and adventitious shoot proliferating culture

Hypocotyl, internode and immature leaf explants of aseptically grown seedlings produced callus. These calli subcultured another medium for induction adventitious shoot formation and subsequently produced adventitious shoots from hypocotyl explants of three cultivars on suitable medium within 7-8 weeks.

4.2.2.8. Precaution to ensure aseptic condition

All inoculation and aseptic manipulations were carried out in a laminar air-flow cabinet. The air-flow was made on for half an hour before use and floor of the cabinet was cleaned with 90 % ethyl alcohol to reduce the contamination. The instruments like scalpels, forceps, needles, etc. were sterilized by an alcoholic dip and flaming method inside the inoculation chamber, while not in use these were kept immersed in alcohol. Other requirements like petridishes, bottles, conical flask, cotton, distilled water, etc. were sterilized by stem sterilization method. Before inoculation, hands were washed throughly by soap and then by spraying 70 % ethyl alcohol. Surgical operations were carried out taking all possible care to ensure contamination free condition.

4.2.2.9. Preparation and culture of microcuttings for rooting

In this stage the shoots with 3-4 cm in length grown on the multiplication media were separated aseptically from the shoot mass, basal leaves snapped off and 2-3 cm microcuttings were prepared. The individual micro cuttings were then transferred on to the freshly prepared rooting media supplemented with different concentrations of auxins with or without $AgNO_3$ and activated charcoal. For root induction the transferred cultures were maintained for three weeks of incubation under 16 h photoperiod at 26 ± 1ºC provided by warm white fluorescent tubes with a light intensity of about 2000 lux.

4.2.2.10. Computation and presentation of data

Different growth parameters were considered to record data on morphogenic responses of various explants while under different culture conditions. The parameters are-

i. *% of explants showing proliferation:* The number of explants that produced axillary shoots were expressed as % and data was recorded after 5-7 weeks of culture.
ii. *Number of shoot per culture:* Data on this character were recorded after 6-9 weeks of culture.
iii. *% of callus proliferating explants:* the number of explants that produced calli were expressed as % and data was recorded after 5-6 weeks of culture.
iv. *% of shoot (adventitious/ axillary) regenerating explants:* The explants those produced adventitious buds were recorded after 7-8 weeks of culture whereas % of axillary shoot regenerating explants were recorded after 5-7 weeks of culture.
v. *% of explants induced root development:* This parameter was used for rooting experiments only and data was recorded after 4 weeks of culture.
vi. *Number of root per rooted cutting:* It was recorded after 6 weeks of culture.
vii. *Shoot and root length:* Shoot height was measured in cm usually after 8 weeks of culture. Length of the root was measured in mm before the transplantation of plantation.

The data presented in the table in the form of percentage of explants / microcuttings showed proliferation / rooting for each experiment were computed as follow:

$$\text{\% of explants showing proliferation} = \frac{No.of\ explants\ formed\ shoots}{No.of\ explants\ cultured} \times 100$$

(excluding contaminated and dead cuttings)

$$\text{\% of cutting rooted} = \frac{No.of\ microcuttings\ formed\ root}{No.of\ microcuttings\ cultured} \times 100 \quad \text{d}$$

(excluding contaminated and dead cuttings)

4.2.2.10.1. Statistical analysis

All experiments were consisted of at least 15 replicate cultures and each of the experiment treated twice or thrice. Data were recorded at least from 10 randomly selected cultures and mean values were calculated separately for each replication.

4.2.2.10.2. Mean and standard error of mean

The mean of different batch of culture of different replications were worked out by taking arithmetic mean using the following formula:

$$\bar{X} = \frac{1}{n}\sum_{i=1}^{n} Xi$$

Where $\bar{X}$ = Arithmetic mean

N = number of observation

$\sum Xi$ = summation of variable

Standard error of mean (S.E.) where calculated as follows:

$$S.E. = \sqrt{S^2}/n \text{ or } S/\sqrt{n}$$

S^2 = Sample variance

S = Standard deviation

n = Number (viz. No. of culture & No. of microcuttings).

4.3. RESULTS

Results of the studies on *in vitro* plantlet regeneration of six cultivars of castor from different explants have been presented under the headings, viz. surface sterilization, axillary shoot proliferation, callus induction, adventitious shoot proliferation and rooting of shoots. Under these headings the results are elaborated based on the nature of the morphogenic responses showed by the explants so far used in this investigation.

4.3.1. SURFACE STERILIZATION

4.3.1.1. Effect of $HgCl_2$ for surface sterilization of seeds of six cultivars of castor.

Sterilization is the first condition to grow aseptic culture. Seeds were the primary explants and for their surface sterilization $HgCl_2$ was used. After sterilization, the seeds were used for germination. Duration of uncertain time caused unexpected results such as tissue killing and contamination. So the experiments were undertaken for standardization of duration of time for seed sterilization. Before treating with $HgCl_2$ solution the seeds were washed with 2 drops of tween 80. After washing the seeds were treated with 0.1 % of $HgCl_2$ in ten duration of times (5, 6, 7, 8, 9, 10, 11, 12, 15 and 20 minutes). Then the seeds were cultured on the semisolid MS (Murashige & Skoog, 1962) medium containing 1.0 mg/L of BAP. Data were taken after 7 days of culture and are presented in the Figure 4.3.1 and detail description of the experiment are given below.

i)Cultivar shabje: Out of ten duration of time 11 minute was found more effective, produce 100% contamination free culture. Some other duration like 9, 10, 12 and 15 minute of time also proved efficient for elimination of loose contamination. But 5 and 6 minute of time were totally failed to produce contamination free culture. Short duration of time failed to produce contamination free culture but long duration of time killed the tissues used.

ii) Cultivar shadatae: Among the ten duration of time 10 minute was found more effective, produced 95% contamination free culture. Five minute duration was totally failed to establish any contamination free culture and 20 minute to be high duration and it caused tissue killing and producing only 28% contamination free culture.

iii) Cultivar roktima: In this case, 100% contamination free cultures were obtained in 11 minute duration. Some other duration like 10, 12 and 15 minute also proved efficient for elimination of loose contamination but 5 and 6 minute duration

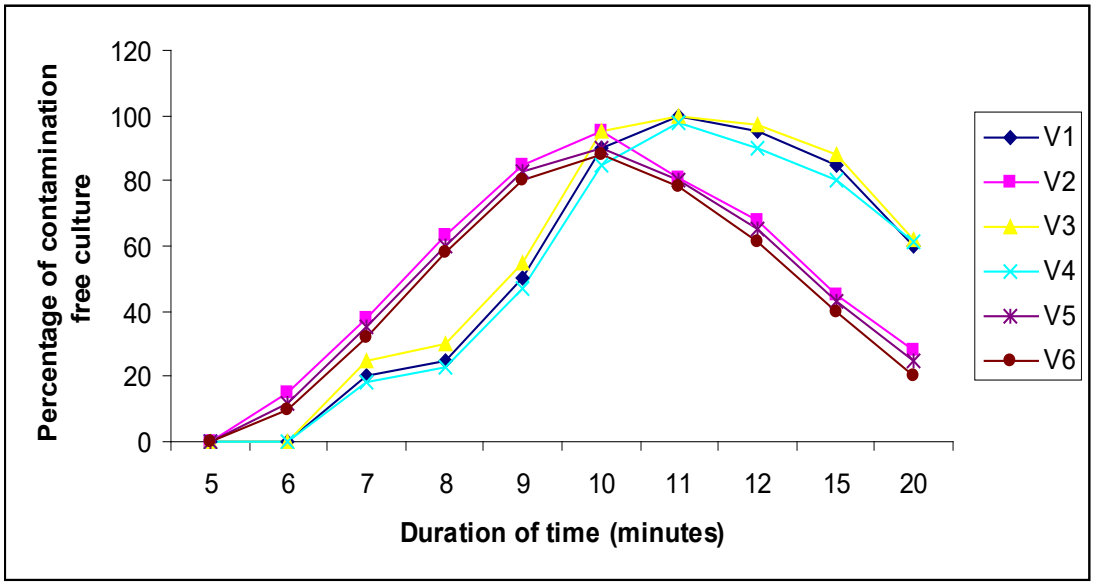
were totally failed to establish any contamination free culture and highest duration of 20 minute killing the tissue and producing 62% of contamination free culture.

iv) Cultivar lalchay: Highest contamination free culture was found in 11 minute duration of time $HgCl_2$ treated seed culture and it was 98%. 5 and 6 minute duration of time failed to produce any contamination free culture and 20 minute treated seeds producing 61% contamination free culture.

v) Cultivar badami: In this case highest contamination free culture was 90% obtained in 10 minute duration of time treated seed culture and lowest 12% contamination free culture in 6 minute duration of time treated seed culture. Five minute duration was totally failed to establish any contamination free culture and 20 minute caused high amount of tissue killing and most of the culture did not survive till germination of seed and showed 25% culture's seed germinated.

vi) Cultivar dhusar: Out of 10 duration of time 10 minute was found more effective, producing 88% contamination free culture. Five minute duration was totally failed to establish any contamination free culture and 20 minute to be high duration and caused tissue killing and producing 20% culture showed germination of seed.

Different cultivars of castor showed slide variation in response to surface sterilization under different time regime as because of their different seed size and different thickness of seed coat.



V1 = Cultivar shabje, V2 = Cultivar shadatae, V3 = Cultivar roktima, V4 = Cultivar lalchay, V5 = Cultivar badami, V6 = Cultivar dhusar.

Fig. 4.3.1: Effect of $HgCl_2$ (0.1%) treated in different duration of time on surface sterilization of seeds of six cultivars of castor.

4.3.2. AXILLARY SHOOT PROLIFERATION

The explants used in the present study were collected from aseptically raised *in vitro* seedlings (Plate 4.3.1-4.3.6, A). Cotyledonary node and shoot tip segment were used as explants for proliferating axillary shoots. Results on axillary shoot proliferation are presented in the following section.

4.3.2.1. Response of explants from *in vitro* grown seedlings.

Aseptic seedlings of six cultivars of castor were raised from the surface sterilized and decoated seeds on MS (Murashige & Skoog, 1962) semi-solid medium supplemented with 30 gm/L sucrose and with or without 1 mg/L BAP. The aseptic seedling thus grew, attained a height of 8-10 cm after 2-3 weeks of culture. Cotyledonary node and shoot tip were used as explants in the experiment for proliferation of axillary shoots. The results are discussed according to type of explants, chemical and physical factors of the culture under separate heads.

4.3.2.2. Axillary shoot proliferation efficiencies of six cultivars of castor.

In this experiment, two types of explants (shoot tip and cotyledonary node) of six cultivars of castor (10-15 mm shoot segments) were taken from the 4 week old seedlings raised *in vitro* on medium either with or without 1.0 mg/L BAP were used. The experiments were cultured on MS medium supplemented with BAP, Kn and 2ip at a single concentration of 1.0 mg/L for proliferating axillary shoots. Data on percentage of explant showing proliferation, number of total shoot per culture, number of usable shoot per culture and days to sprout the axillary buds were recorded from different treatments after six week of culture initiation. Initially, all the two types of explants responded in a similar way to the present treatments by producing shoots from the pre-existing axillary meristem and forming slow growing hard compact calli from the cut ends of the explants. But the frequency of explants showing proliferation and number of shoots per explants varied considerably depending upon the source (taken from with or without BAP containing medium) and type of the explants (shoot tip or cotyledonary node) cultured and type of the cytokinins (BAP, Kn or 2ip) tested. The first visual response of the explants was sprouting the axillary buds which was observed after one week of culture and that was followed by slight callus formation at the cut ends with the progression of the culture period. Seedlings grown on BAP containing medium were characterized with bushy thick shoots. In general, explants taken from the seedlings raised on BAP supplemented medium showed 5-12 days early sprouting of the axillary buds than those taken from the seedlings raised on BAP omitted medium. Among the two types

of the seedling explants used, proliferation efficiency of cotyledonary node segment, whether taken from seedling raised with or without BAP was at least 2 times greater than those of shoot tip in all six cultivars of castor (Table 4.3.1 & 4.3.2). Through this experiment it was apparent that, cotyledonary node segment was the most respective explant and the cytokinin BAP was comparatively more effective in proliferating axillary shoots in six cultivars of castor.

4.3.2.3. Effect of different strength of MS medium on axillary shoot proliferation.

In this experiment cotyledonary node was explant and cultured on MS medium with full strength of all major and minor salts (MS), only half strength of major salts (MMS_1) and half strength of both major and minor salts (MMS_2). All the media contained full strength of MS organic and vitamins, 8 gm/L agar, 30 gm/L sucrose and 1 mg/L BAP. The results are presented in Table 4.3.3. and discussed in following section.

i)Cultivar shabje: The percentage of explant showing proliferation was the highest 100% in MMS_1 medium and lowest 74.3% in MMS_2 medium. Number of shoot per culture was the highest 9.3 ± 2.25, number of usable shoot per culture was the highest 6.4 ± 0.97 and average length (mm) of the usable shoot was the highest 22.1 ± 3.51 obtained in MMS_1 medium. Axillary buds started growing in 5-7 days in MMS_1 medium and it was earlier than that on the other two salt concentrations.

ii) Cultivar shadatae: The percentage of explant showing proliferation was the highest 95.7% in MMS_1 medium and lowest 55.1% in MMS_2 medium. Number of shoot per culture was the highest 6.0±1.51, number of usable shoot per culture was the highest 3.9 ± 0.77 and average length (mm) of the usable shoot was the highest 20.5 ± 2.90 obtained in MMS_1 medium. Axillary buds started growing in 6-8 days in MMS_1 medium, and it was earlier than that on the other two salt concentrations.

iii) Cultivar roktima: The percentage of explant showing proliferation was the highest 100% in MMS_1 medium and lowest 71.4% in MMS_2 medium. Number of shoot per culture was the highest 8.2 ± 1.89, number of usable shoot per culture was the highest 5.8 ± 0.93 and average length (mm) of the usable shoot was the highest 21.0 ±3.19 obtained in MMS_1 medium. Axillary buds started growing in 5-7 days in MMS_1 medium and it was earlier than that on the other two medium concentration.

iv) Cultivar lalchay: The percentage of explant showing proliferation was the highest 100% in MMS_1 medium and lowest 70.1% in MMS_2 medium. Number of shoot per culture was the highest 7.4 ± 1.75, number of usable shoot per culture was the highest 5.1 ± 0.84 and average length (mm) of the usable shoot was the highest

20.5 ± 3.05 obtained in MMS_1 medium. Axillary buds started growing in 5-7 days in MMS_1 medium, and it was earlier than that on the other two medium concentrations.

v) Cultivar badami: The percentage of explant showing proliferation was the highest 94.5% in MMS_1 medium and lowest 51.8% in MMS_2 medium. Number of shoot per culture was the highest 6.5 ± 1.23 , number of usable shoot per culture was the highest 3.8 ± 0.70 and average length (mm) of the usable shoot was the highest 18.3 ± 2.80 obtained in MMS_1 medium. Axillary buds started growing in 6-8 days in MMS_1 medium, and it was earlier than that on the other two salt concentrations.

vi) Cultivar dhusar: The percentage of explant showing proliferation was the highest 94.2% in MMS_1 medium and lowest 50.9% in MMS_2 medium. Number of shoot per culture was the highest 6.4 ± 1.16, number of usable shoot per culture was the highest 3.5 ± 0.69 and average length (mm) of the usable shoot was the highest 18.1 ± 2.54 obtained in MMS_1 medium. Axillary buds started growing in 6-8 days in MMS_1 medium, and it was earlier than that on the other two salt concentrations.

From the over all observation it was noticed that MMS_1 medium was the best for axillary shoot proliferation than those on MMS_2 and MS media. Based on this finding, in the subsequent shoot proliferation experiments only MMS_1 medium was used as basal growth medium.

Table 4.3.1: Axillary shoot proliferation from two types of explants of *R. communis* L. from seedlings grown on medium with 1.0 mg/L of BAP. Data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture of the explants on MS medium containing 1.0 mg/L of BAP. Each treatment consisted of 15-20 explants.

Cultivar	Explant type	Seedlings grown on MS medium with BAP			
		% of explants showing propliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	* No. of usable shoot per culture ($\bar{x} \pm SE$)	Days to sprout the axillary buds
V_1	Cotyledonary node	100.0	9.5 ± 1.23	5.5 ± 0.92	5 - 7
	Shoot tip	81.7	4.6 ± 0.75	2.7 ± 0.65	7 - 10
V_2	Cotyledonary node	95.2	6.4 ± 0.91	3.0 ± 0.73	6 - 8
	Shoot tip	75.2	4.4 ± 0.60	2.1 ± 0.48	8 - 12
V_3	Cotyledonary node	100.0	8.2 ± 1.23	4.5 ± 0.81	5 - 7
	Shoot tip	80.5	4.3 ± 0.70	2.7 ± 0.61	7 - 10
V_4	Cotyledonary node	100.0	7.5 ± 1.00	4.2 ± 0.78	5 - 7
	Shoot tip	78.3	4.2 ± 0.68	2.6 ± 0.59	7 - 10
V_5	Cotyledonary node	94.1	6.4 ± 0.90	3.5 ± 0.51	6 - 8
	Shoot tip	71.8	3.5 ± 0.56	2.1 ± 0.43	8 - 12
V_6	Cotyledonary node	94.0	6.3 ± 0.84	3.4 ± 0.49	6 - 8
	Shoot tip	70.9	3.5 ± 0.39	2.1 ± 0.42	8 - 12

* Shoots length ≥ 10 mm were considered only.

V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar.

Table 4.3.2: Axillary shoot proliferation from two types of explants of *R. communis* L. seedlings grown on medium without BAP. Data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture of the explants on MS medium containing 1.0 mg/L of BAP. Each treatment consisted of 15-20 explants.

Cultivar	Explant type	Seedlings grown on MS medium without BAP			
		% of explants showing propliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	* No. of usable shoot per culture ($\bar{x} \pm SE$)	Days to sprout the axillary buds
V_1	Cotyledonary node	85.8	6.5 ± 0.85	3.3 ± 0.31	10 - 12
	Shoot tip	61.0	3.3 ± 0.47	2.1 ± 0.32	12 - 14
V_2	Cotyledonary node	71.7	3.7 ± 0.49	2.2 ± 0.25	12 - 14
	Shoot tip	53.1	2.8 ± 0.35	1.6 ± 0.18	14 - 16
V_3	Cotyledonary node	80.4	5.3 ± 0.87	3.3 ± 0.29	10 - 12
	Shoot tip	55.3	3.0 ± 0.38	2.0 ± 0.27	12 - 14
V_4	Cotyledonary node	78.3	4.5 ± 0.80	3.2 ± 0.27	10 - 12
	Shoot tip	54.7	2.9 ± 0.37	1.9 ± 0.25	12 - 14
V_5	Cotyledonary node	68.5	3.4 ± 0.48	2.1 ± 0.24	12 - 14
	Shoot tip	50.2	2.5 ± 0.33	1.7 ± 0.23	14 - 16
V_6	Cotyledonary node	65.2	3.1 ± 0.41	2.1 ± 0.21	12 - 14
	Shoot tip	50.0	2.4 ± 0.25	1.5 ± 0.19	14 - 16

* Shoots length ≥ 10 mm were considered only.

V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar.

Table 4.3.3: Effects of different strength of MS medium supplemented with 1.0 mg/L BAP on axillary shoot proliferation of different cultivars of castor. Data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture and at least 20 explants were used per treatment.

Cultivar	Different nutrient media	% of explant showing proliferation	No. of shoot per explant ($\bar{x} \pm SE$)	No. of usable shoot per explants ($\bar{x} \pm SE$)	Average length(mm) of the usable shoots ($\bar{x} \pm SE$)	Days to sprout of the axillary buds
V_1	MS	92.8	7.8 ± 1.24	3.4 ± 0.68	19.0 ± 3.10	8 - 10
	MMS_1	100.0	9.3 ± 2.25	6.4 ± 0.97	22.1 ± 3.51	5 - 7
	MMS_2	74.3	4.5 ± 0.87	3.0 ± 0.71	18.5 ± 2.72	10 - 12
V_2	MS	76.2	4.3 ± 0.97	2.9 ± 0.21	17.5 ± 2.09	8 - 11
	MMS_1	95.7	6.0 ± 1.51	3.9 ± 0.77	20.5 ± 2.90	6 - 8
	MMS_2	55.1	3.0 ± 0.62	2.3 ± 0.99	15.1 ± 2.46	10 - 13
V_3	MS	90.9	7.0 ± 1.12	3.3 ± 0.65	19.1 ± 3.23	8 - 10
	MMS_1	100.0	8.2 ± 1.89	5.8 ± 0.93	21.0 ± 3.19	5 - 7
	MMS_2	71.4	4.0 ± 0.73	2.9 ± 0.12	17.5 ± 2.53	10 - 12
V_4	MS	90.2	6.3 ± 1.00	3.2 ± 0.62	18.3 ± 2.81	8 - 10
	MMS_1	100.0	7.4 ± 1.75	5.1 ± 0.84	20.5 ± 3.05	5 - 7
	MMS_2	70.1	3.9 ± 0.73	2.8 ± 0.15	14.3 ± 2.51	10 - 12
V_5	MS	73.1	4.1 ± 0.91	2.8 ± 0.22	16.0 ± 2.19	8 - 11
	MMS_1	94.5	6.5 ± 1.23	3.8 ± 0.70	18.3 ± 2.80	6 - 8
	MMS_2	51.8	3.9 ± 0.71	2.3 ± 0.11	14.0 ± 2.33	10 - 13
V_6	MS	71.8	4.0 ± 0.88	2.7 ± 0.21	16.0 ± 2.07	8 - 11
	MMS_1	94.2	6.4 ± 1.16	3.5 ± 0.69	18.1 ± 2.54	6 - 8
	MMS_2	50.9	3.8 ± 0.70	2.2 ± 0.10	13.9 ± 2.11	10 - 13

MS = full strength.
MMS_1 = half major + full minor salt.
MMS_2 = half major + half minor salt.

* Shoots length ≥ 10 mm were considered only. V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar.

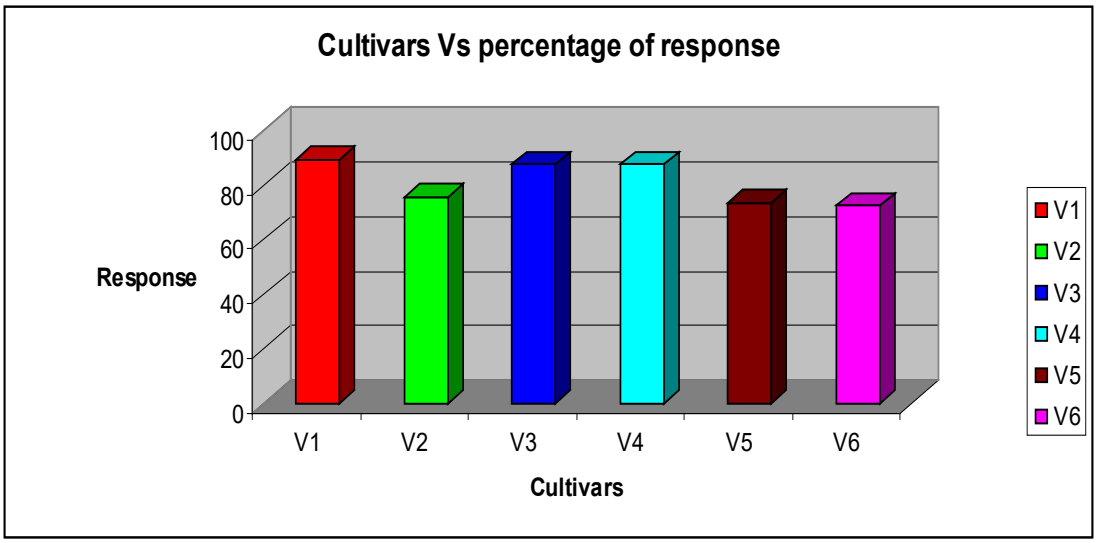


Fig.4.3.2: Percentage of response showing proliferation of six cultivars of castor in average of different concentrations of MS medium with BAP 1.0 mg/L from cotyledonary node explant. (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar).

4.3.2.4. Growth regulator selection for maximum proliferation.

In this experiment, cotyledonary node segment from the seedlings grown on 1.0 mg/L BAP were used for proliferating axillary shoots. These explants were cultured on MMS_1 medium contained 8 gm/L agar, 30 gm/L sucrose and supplemented with BAP, Kn and 2ip at cocentrations of 0.5, 1.0, 1.5, 2.0, 2.5 and 5.0 mg/L for selecting optimum cytokinin concentration for maximum shoot proliferation. Percentage of explants showing proliferation, number of total shoot per explant, number of usable shoot per explant, average length of shoot, days to sprout the axillary buds were considered as parameters for evaluating the experiment. Data on these parameters were recorded after six weeks of culture.

Regeneration of axillary shoot from cotyledonary node segment was influenced by the type of cytokinins as well as their concentrations used. The cytokinin BAP, at most of the concentrations, was comparatively more effective in producing axillary shoot. Whereas Kn and 2ip were considerably less effective in the present experiment. With the increase of cytokinin concentration from 0.5 - 2.0 mg/L, the percentage of explants showing proliferation and number of shoot per culture were increased gradually. Further increase in cytokinin concentration (2.5 mg/L) did not approve any of the parameter and the highest level (5.0 mg/L) of the cytokinins the explants failed to produce any axillary shoot.

i) Cultivar shabje: Among the different concentrations (0.5-5.0 mg/L) of BAP, Kn and 2ip indicated that fifteen concentrations successfully produced axillary shoots and three concentrations failed to produce any axillary shoot. The highest percentage of explant showing proliferation 100% were observed in 2.0 mg/L BAP and lowest 45.7% in 0.5mg/L 2ip. The highest number of total shoot per culture (9.4±1.23), highest number of usable shoot per culture (5.3±1.10) and highest average length of shoot (22.2±4.3 mm) produced in 2.0 mg/L of BAP (Plate 4.3.1, D). The lowest number of total shoot per culture (2.8±0.37), lowest number of usable shoot per culture (1.4±0.18) and lowest average length of shoot (14.2± 1.3 mm) produced in 0.5 mg/L 2ip (Plate 4.3.1, F). In different concentrations of Kn produced axillary shoot proliferation but it was lower than BAP 2.0 mg/L (Plate 4.3.1, E). Axillary buds started growing in 5-7 days in 2.0 mg/L BAP and it was the earliest time among the concentrations tested. From the over all observations, it was clear that BAP was found more effective and 2.0 mg/L BAP was the best concentration for axillary shoot proliferation of cultivar shabje and it is presented in Table 4.3.4.

ii) Cultivar shadatae: Different concentrations (0.5-5.0 mg/L) of BAP, Kn and 2ip were used in this experiment and observed that fifteen concentrations successfully produced axillary shoots and three concentrations failed to produce any axillary shoot. The highest percentage of explant showing proliferation 90% was showed in 2.0 mg/L BAP and lowest 44.2% in 0.5mg/L 2ip. The highest number of total shoot per culture was 7.0±0.92, highest number of usable shoot per culture was 3.8±0.82 and highest average length of shoot 22.4±3.9 mm produced in 2.0 mg/L of BAP (Plate 4.3.2, D). The lowest number of total shoot per culture 2.3±0.60, lowest number of usable shoot per culture 1.1±0.18 and lowest average length of shoot 13.2± 1.4 mm was noted in 0.5 mg/L 2ip (Plate 4.3.2, F). In different concentrations of Kn axillary shoot proliferation was lower than BAP 2.0 mg/L (Plate 4.3.2, E). Axillary buds started growing in 6-7 days in 2.0 mg/L BAP and it took lowest time among the concentrations tested. From the over all observations, it was clear that BAP was found to be more effective and 2.0 mg/L BAP was the best concentration for axillary shoot proliferation of cultivar shadatae and it is presented in Table 4.3.5.

iii) Cultivar roktima: A wide range of variation was observed in case of cultivar roktima. Different concentrations (0.5-5.0 mg/L) of BAP, Kn and 2ip were used in this experiment and indicated that fifteen concentrations successfully produced axillary shoots and three concentrations failed to produce any axillary shoot. The highest percentage of explant showing proliferation 100% were observed in 2.0 mg/L BAP and lowest 51.1% in 0.5mg/L 2ip. The highest number of total shoot per culture was 9.3±1.24, highest number of usable shoot per culture was 5.2±0.98 and

highest average length of shoot 22.0±4.2 mm produced in 2.0 mg/L of BAP (Plate 4.3.3, D). The lowest number of total shoot per culture 2.7±0.43, lowest number of usable shoot per culture 1.3±0.17 and lowest average length of shoot 14.1± 1.5 mm produced in 0.5 mg/L 2ip (Plate 4.3.3, F). In different concentrations of Kn produced axillary shoot proliferation but it was lower than BAP 2.0 mg/L (Plate 4.3.3, E). Axillary buds started growing in 5-7 days in 2.0 mg/L BAP and it was the earliest time among the concentrations tested. From the over all observations, it was clear that BAP was found more effective and 2.0 mg/L BAP was the best concentration for axillary shoot proliferation of cultivar roktima and it is presented in Table 4.3.6.

iv) Cultivar lalchay: Different concentrations (0.5-5.0 mg/L) of BAP, Kn and 2ip were used and observed that the highest percentage of explant showing proliferation 95.0% were noted in 2.0 mg/L BAP and lowest 50.5% in 0.5mg/L 2ip. The highest number of total shoot per culture 9.0±1.02, highest number of usable shoot per culture 5.0±0.81 and highest average length of shoot 22.3±3.9 mm produced in 2.0 mg/L of BAP (Plate 4.3.4, D). The lowest number of total shoot per culture was 2.5±0.61, lowest number of usable shoot per culture was 1.2±0.19 and lowest average length of shoot 14.0± 1.5 mm produced in 0.5 mg/L 2ip (Plate 4.3.4, F). In different concentrations of Kn produced axillary shoot proliferation but it was lower than BAP 2.0 mg/L (Plate 4.3.4, E). Axillary buds started growing in 5-7 days in 2.0 mg/L BAP and it was the earliest time among the other concentrations tested. From the over all observations, it was clear that BAP was found more effective and 2.0 mg/L BAP was the best concentration for axillary shoot proliferation of cultivar lalchay and it is presented in Table 4.3.7.

v) Cultivar badami: In this experiment the concentrations (0.5-5.0 mg/L) of Kn and 2ip was less effective than concentrations of BAP for axillary shoot induction. The highest percentage of explant showing proliferation 85.0% were observed in 2.0 mg/L BAP and lowest 43.1% in 0.5mg/L 2ip. The highest number of total shoot per culture was 7.1±0.93, highest number of usable shoot per culture was 3.9±0.62 and highest average length of shoot 21.3±3.8 mm produced in 2.0 mg/L of BAP (Plate 4.3.5, D). The lowest number of total shoot per culture was 2.1±0.63, lowest number of usable shoot per culture was 1.1±0.19 and lowest average length of shoot 13.1± 1.5 mm produced in 0.5 mg/L 2ip (Plate 4.3.5, F). In different concentrations of Kn produced axillary shoot proliferation but it was lower than BAP 2.0 mg/L (Plate 4.3.5, E). Axillary buds started growing in 6-7 days in 2.0 mg/L BAP and it was the earliest time among the other concentrations tested. From the over all observations, it was clear that BAP was found more effective and 2.0 mg/L BAP was the best

concentration for axillary shoot proliferation of cultivar badami and it is presented in Table 4.3.8.

vi) Cultivar dhusar: Among the different concentrations (0.5-5.0 mg/L) of BAP, Kn and 2ip indicated that fifteen concentrations successfully produced axillary shoots and three concentrations failed to produce any axillary shoot. The highest percentage of explant showing proliferation (89.0%) was observed in 2.0 mg/L BAP and lowest 43.0% in 0.5mg/L 2ip. The highest number of total shoot per culture was 6.9±0.83, highest number of usable shoot per culture was 3.8±0.51 and highest average length of shoot 20.0±3.6 mm produced in 2.0 mg/L of BAP (Plate 4.3.6, D). The lowest number of total shoot per culture was 2.0±0.62, lowest number of usable shoot per culture was 1.1±0.18 and lowest average length of shoot 13.1± 1.3 mm produced in 0.5 mg/L 2ip (Plate 4.3.6, F). In different concentrations of Kn produced axillary shoot but did not show good response and it was lower than BAP 2.0 mg/L (Plate 4.3.6, E). Axillary buds started growing in 6-7 days in 2.0 mg/L BAP and it was the earliest time among the other concentrations tested. From the over all observations, it was clear that BAP was found more effective and 2.0 mg/L BAP was the best concentration for axillary shoot proliferation of cultivar badami and it is presented in Table 4.3.9.

Table 4.3.4: Effect of different concentrations of cytokinins on axillary shoot proliferation of *R. communis* L. cultivar shabje (V_1) from cotyledonary node explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 cultures.

Concentrations of growth regulators (mg/L)		% of explants showing proliferation	No. of total shoot / culture ($\bar{x} \pm SE$)	* No. of usable shoot / culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	Days to sprout the axillary buds
BAP	0.5	64.0	4.7 ± 0.91	2.2 ± 0.68	17.0 ± 3.0	7 - 10
	1.0	76.0	6.5 ± 0.95	2.7 ± 0.91	19.1 ± 3.0	6 - 8
	1.5	86.3	7.4 ± 1.16	3.9 ± 0.95	20.0 ± 3.2	6 - 8
	2.0	100.0	9.4 ± 1.23	5.3 ± 1.10	22.2 ± 4.3	5 - 7
	2.5	79.4	8.5 ± 1.22	4.8 ± 1.00	21.1 ± 3.5	6 - 8
	5.0	-	-	-	-	-
Kn	0.5	53.3	3.0 ± 0.65	1.5 ± 0.27	16.1 ± 1.8	9 - 10
	1.0	65.1	3.8 ± 0.87	2.4 ± 0.63	17.5 ± 2.1	8 - 9
	1.5	75.0	5.1 ± 0.92	3.5 ± 0.71	18.1 ± 2.6	7 - 9
	2.0	85.9	7.5 ± 1.23	4.1 ± 0.91	20.3 ± 3.5	6 - 8
	2.5	68.6	6.3 ± 1.18	3.6 ± 0.88	19.1 ± 3.0	7 - 9
	5.0	-	-	-	-	-
2ip	0.5	45.7	2.8 ± 0.37	1.4 ± 0.18	14.2 ± 1.3	10 - 12
	1.0	58.2	3.2 ± 0.34	2.1 ± 0.23	15.4 ± 1.6	8 - 10
	1.5	64.6	4.0 ± 0.91	2.3 ± 0.27	16.2 ± 1.5	7 - 9
	2.0	70.9	5.8 ± 0.91	3.4 ± 0.68	18.0 ± 1.9	6 - 9
	2.5	62.5	5.7 ± 0.92	3.3 ± 0.69	17.6 ± 1.8	7 - 9
	5.0	-	-	-	-	-

* Shoots with length ≥ 10 mm were considered only.

Table 4.3.5: Effect of different concentrations of cytokinins on axillary shoot proliferation of *R. communis* L. cultivar shadate (V_2) from cotyledonary node explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 cultures.

Concentrations of growth regulators (mg/L)		% of explants showing proliferation	No. of total shoot / culture ($\bar{x} \pm SE$)	* No. of usable shoot / culture ($\bar{x} \pm SE$)	Average length of shoot (mm) ($\bar{x} \pm SE$)	Days to sprout the axillary buds
BAP	0.5	56.0	3.0 ± 0.69	2.0 ± 0.62	16.4 ± 3.1	8 - 10
	1.0	68.4	5.3 ± 0.75	2.1 ± 0.71	17.6 ± 2.9	7 - 8
	1.5	78.3	6.0 ± 0.80	3.0 ± 0.56	19.5 ± 3.0	7 - 8
	2.0	90.0	7.0 ± 0.92	3.8 ± 0.82	22.4 ± 3.9	6 - 7
	2.5	70.5	6.3 ± 0.81	3.4 ± 0.68	21.1 ± 3.8	7 - 8
	5.0	-	-	-	-	-
Kn	0.5	46.0	2.5 ± 0.60	1.2 ± 0.41	15.2 ± 1.7	10 - 12
	1.0	58.2	3.3 ± 0.64	2.0 ± 0.53	16.8 ± 2.0	8 - 9
	1.5	68.3	4.4 ± 0.81	2.9 ± 0.69	17.0 ± 2.8	8 - 9
	2.0	79.5	6.3 ± 1.17	3.5 ± 0.94	18.3 ± 3.5	7 - 8
	2.5	59.6	5.2 ± 1.05	3.1 ± 0.82	17.1 ± 3.6	8 - 9
	5.0	-	-	-	-	-
2ip	0.5	44.2	2.3 ± 0.60	1.1 ± 0.18	13.2 ± 1.4	12 - 14
	1.0	53.0	2.9 ± 0.58	1.6 ± 0.27	14.3 ± 1.7	8 - 10
	1.5	57.1	3.4 ± 0.75	2.0 ± 0.44	16.7 ± 1.8	8 - 10
	2.0	63.3	4.5 ± 0.81	2.7 ± 0.62	19.0 ± 1.7	7 - 10
	2.5	55.0	4.3 ± 0.82	2.5 ± 0.61	18.5 ± 1.6	8 - 10
	5.0	-	-	-	-	-

* Shoots with length ≥ 10 mm were considered only.

Table 4.3.6: Effect of different concentrations of cytokinins on axillary shoot proliferation of *R. communis* L. cultivar roktima (V_3) from cotyledonary node explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 cultures.

	Concentrations of growth regulators (mg/L)	% of explants showing proliferation	No. of total shoot / culture ($\bar{x} \pm SE$)	* No. of usable shoot / culture ($\bar{x} \pm SE$)	Average length(mm) of shoot ($\bar{x} \pm SE$)	Days to spfout the axillary buds
BAP	0.5	62.1	4.6 ± 0.91	2.2 ± 0.69	18.8 ± 3.0	7 - 10
	1.0	74.2	6.2 ± 0.96	2.5 ± 0.92	19.1 ± 3.2	6 - 8
	1.5	84.1	7.1 ± 1.00	3.8 ± 0.97	20.8 ± 3.3	6 - 8
	2.0	100.0	9.3 ± 1.24	5.2 ± 0.98	22.0 ± 4.2	5 - 7
	2.5	78.1	8.2 ± 1.15	4.6 ± 1.02	21.9 ± 3.4	6 - 8
	5.0	-	-	-	-	-
Kn	0.5	52.0	2.9 ± 0.68	1.4 ± 0.37	15.6 ± 1.7	9 - 10
	1.0	64.1	3.7 ± 0.90	2.2 ± 0.65	17.4 ± 2.1	8 - 9
	1.5	74.0	5.0 ± 0.93	3.3 ± 0.75	19.5 ± 2.5	7 - 9
	2.0	88.5	7.3 ± 1.14	4.0 ± 0.92	20.1 ± 3.7	6 - 8
	2.5	67.2	6.2 ± 1.09	3.5 ± 0.90	19.0 ± 3.2	7 - 9
	5.0	-	-	-	-	-
2ip	0.5	51.1	2.7 ± 0.43	1.3 ± 0.17	14.1 ± 1.5	10 - 12
	1.0	58.2	3.1 ± 0.42	1.9 ± 0.65	15.2 ± 1.7	8 - 10
	1.5	64.5	3.8 ± 0.91	2.1 ± 0.38	18.1 ± 1.6	7 - 9
	2.0	69.8	5.7 ± 0.92	3.0 ± 0.69	18.5 ± 1.8	6 - 9
	2.5	62.5	5.6 ± 0.93	2.9 ± 0.68	17.5 ± 1.7	7 - 9
	5.0	-	-	-	-	-

* Shoots with length ≥ 10 mm were considered only.

Table 4.3.7: Effect of different concentrations of cytokinins on axillary shoot proliferation of *R. communis* L. cultivar lalchay (V_4) from cotyledonary node explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 cultures.

	Concentrations of growth regulators (mg/L)	% of explants showing proliferation	No. of total shoot / culture ($\bar{x} \pm SE$)	* No. of usable shoot / culture ($\bar{x} \pm SE$)	Average length of shoot (mm) ($\bar{x} \pm SE$)	Days to sprout the axillary buds
BAP	0.5	62.0	3.2 ± 0.98	2.1 ± 0.71	18.5 ± 3.2	7 - 10
	1.0	73.7	6.1 ± 1.00	2.4 ± 0.93	19.8 ± 3.1	6 - 8
	1.5	83.0	7.0 ± 1.13	3.7 ± 0.95	20.9 ± 3.4	6 - 8
	2.0	95.0	9.0 ± 1.02	5.0 ± 0.81	22.3 ± 3.9	5 - 7
	2.5	79.1	7.9 ± 1.01	4.5 ± 0.86	21.0 ± 4.0	6 - 8
	5.0	-	-	-	-	-
Kn	0.5	52.3	2.8 ± 0.61	1.3 ± 0.45	15.4 ± 1.8	9 - 10
	1.0	63.5	3.5 ± 0.92	2.1 ± 0.69	17.0 ± 2.2	8 - 9
	1.5	73.6	4.9 ± 0.98	3.1 ± 0.77	19.3 ± 2.9	7 - 9
	2.0	85.0	7.0 ± 0.97	3.8 ± 0.95	20.0 ± 3.8	6 - 8
	2.5	65.1	5.9 ± 0.83	3.3 ± 0.88	19.5 ± 3.7	7 - 9
	5.0	-	-	-	-	-
2ip	0.5	50.5	2.5 ± 0.61	1.2 ± 0.19	14.0 ± 1.5	10 - 12
	1.0	57.1	3.0 ± 0.56	1.8 ± 0.59	15.1 ± 1.8	8 - 10
	1.5	62.3	3.6 ± 0.85	2.1 ± 0.43	17.8 ± 1.7	7 - 9
	2.0	74.9	5.5 ± 0.91	2.9 ± 0.65	18.5 ± 1.8	6 - 9
	2.5	61.0	5.3 ± 0.88	2.8 ± 0.66	17.3 ± 1.8	7 - 9
	5.0	-	-	-	-	-

* Shoots with length ≥ 10 mm were considered only.

Table 4.3.8: Effect of different concentrations of cytokinins on axillary shoot proliferation of *R. communis* L. cultivar badami (V_5) from cotyledonary node explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 cultures.

	Concentrations of growth regulators (mg/L)	% of explants showing proliferation	No. of total shoot / culture ($\bar{x} \pm SE$)	* No. of usable shoot / culture ($\bar{x} \pm SE$)	Average length (mm)of shoot ($\bar{x} \pm SE$)	Days to sprout the axillary buds
BAP	0.5	56.1	3.0 ± 0.65	2.0 ± 0.25	15.8 ± 3.0	8 - 10
	1.0	68.9	5.2 ± 0.71	2.2 ± 0.39	17.2 ± 2.7	7 - 8
	1.5	68.2	6.0 ± 0.78	3.1 ± 0.45	19.1 ± 0.29	7 - 8
	2.0	85.0	7.1 ± 0.93	3.9 ± 0.62	21.3 ± 3.8	6 - 7
	2.5	69.3	6.5 ± 0.80	3.5 ± 0.57	20.0 ± 3.7	7 - 8
	5.0	-	-	-	-	-
Kn	0.5	44.3	2.4 ± 0.61	1.1 ± 0.12	15.0 ± 1.8	10 - 12
	1.0	56.0	3.2 ± 0.65	1.9 ± 0.27	16.5 ± 2.0	8 - 9
	1.5	66.2	4.2 ± 0.82	2.8 ± 0.71	17.8 ± 2.7	8 - 9
	2.0	78.3	6.2 ± 0.95	3.4 ± 0.45	19.1 ± 3.4	7 - 8
	2.5	59.7	5.1 ± 0.72	3.0 ± 0.81	18.0 ± 3.5	8 - 9
	5.0	-	-	-	-	-
2ip	0.5	43.1	2.1 ± 0.63	1.1 ± 0.19	13.1± 1.5	12 - 14
	1.0	51.5	2.7 ± 0.55	1.5 ± 0.28	14.2 ± 1.9	8 - 10
	1.5	58.9	3.2 ± 0.76	1.9 ± 0.35	16.5 ± 1.6	8 - 10
	2.0	62.2	4.4 ± 0.85	25 ± 0.43	17.7 ± 1.7	7 - 10
	2.5	55.0	4.1 ± 0.81	2.3 ± 0.30	16.2 ± 1.8	8 - 10
	5.0	-	-	-	-	-

* Shoots with length ≥ 10 mm were considered only.

Table 4.3.9: Effect of different concentrations of cytokinins on axillary shoot proliferation of *R. communis* L. cultivar dhusar (V_6) from cotyledonary node explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 cultures.

Concentrations of growth regulators (mg/L)		% of explants showing proliferation	No. of total shoot / culture ($\bar{x} \pm SE$)	* No. of usable shoot / culture ($\bar{x} \pm SE$)	Average length of shoot (mm) ($\bar{x} \pm SE$)	Days to sprout the axillary buds
BAP	0.5	55.0	2.7 ± 0.66	1.8 ± 0.57	15.5 ± 2.8	8 - 10
	1.0	67.6	5.0 ± 0.72	2.1 ± 0.61	17.0 ± 2.6	7 - 8
	1.5	72.5	5.9 ± 0.79	3.0 ± 0.63	19.0 ± 2.8	7 - 8
	2.0	89.0	6.9 ± 0.83	3.8 ± 0.51	20.0 ± 3.6	6 - 7
	2.5	69.0	6.2 ± 0.85	3.3 ± 0.62	19.5 ± 3.5	7 - 8
	5.0	-	-	-	-	-
Kn	0.5	45.2	2.3 ± 0.19	1.5 ± 0.20	15.0 ± 1.1	10 - 12
	1.0	57.5	3.1 ± 0.41	1.8 ± 0.25	15.8 ± 1.8	8 - 9
	1.5	67.1	4.0 ± 0.70	2.7 ± 0.69	17.5 ± 2.4	8 - 9
	2.0	79.0	6.1 ± 0.94	3.3 ± 0.32	18.0 ± 2.9	7 - 8
	2.5	68.4	5.0 ± 0.87	2.9 ± 0.38	17.5 ± 2.8	8 - 9
	5.0	-	-	-	-	-
2ip	0.5	43.0	2.0 ± 0.62	1.1 ± 0.18	13.1 ± 1.3	12 - 14
	1.0	51.2	2.6 ± 0.59	1.4 ± 0.27	14.2 ± 1.8	8 - 10
	1.5	58.5	3.2 ± 0.75	1.8 ± 0.46	15.3 ± 1.4	8 - 10
	2.0	62.0	4.3 ± 0.84	2.4 ± 0.39	16.5 ± 1.2	7 - 10
	2.5	55.1	4.0 ± 0.85	2.2 ± 0.41	18.0 ± 1.9	8 - 10
	5.0	-	-	-	-	-

* Shoots with length ≥ 10 mm were considered only.

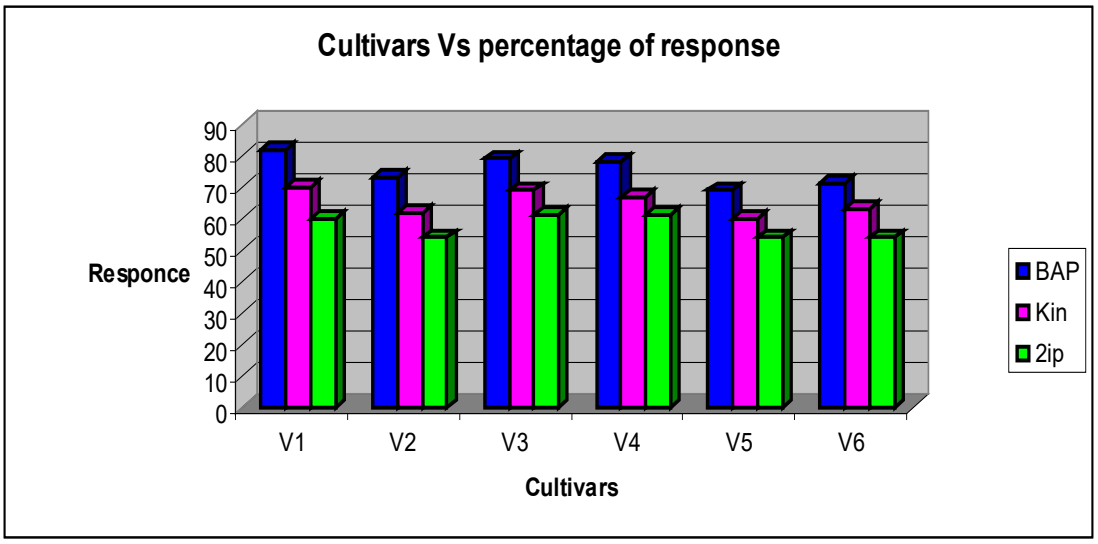


Fig.4.3.3: Average percentage of response showing proliferation in BAP, Kin and 2ip from cotyledonary node explant in MMS_1 medium of six cultivars of castor (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar).

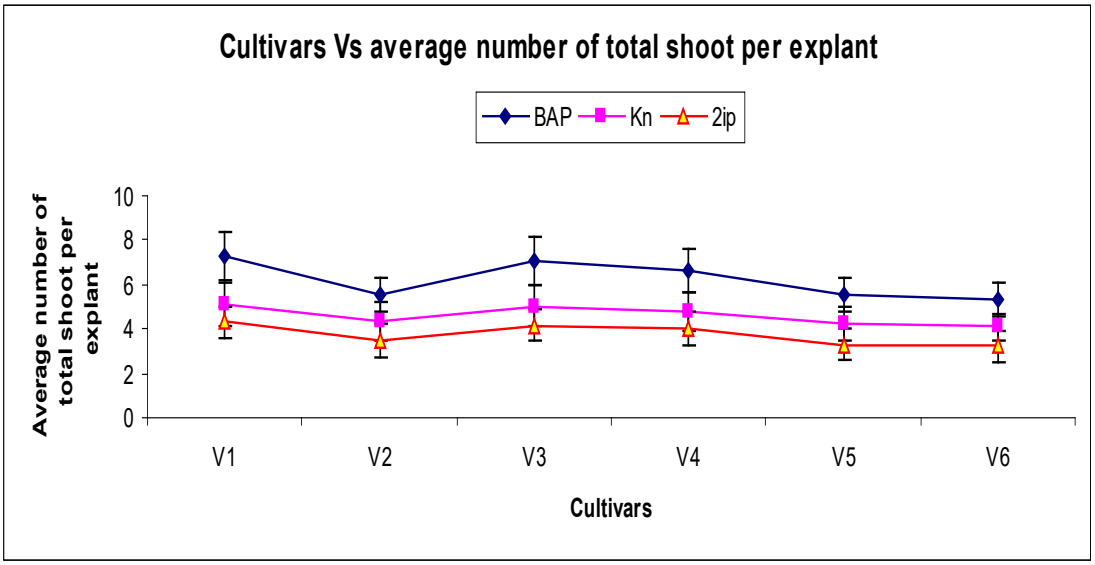


Fig.4.3.4: Average umber of total shoot per explant of six cultivars of castor in different concentrations of MMS_1 medium with BAP, Kn and 2ip from cotyledonary node explant. (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar).

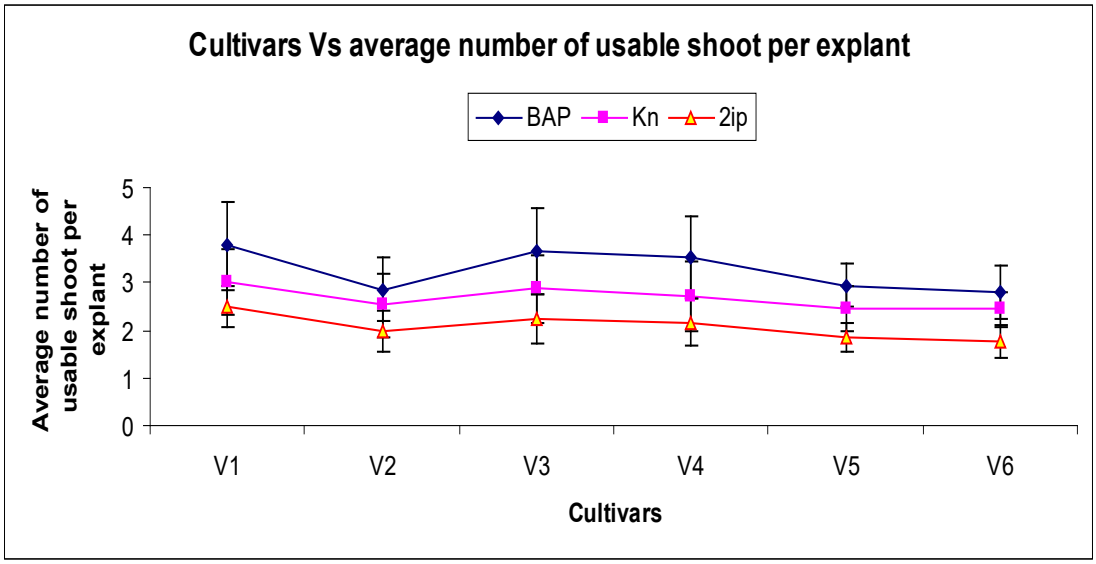


Fig.4.3.5: Average umber of usable shoot per explant of six cultivars of castor in different concentrations of MMS_1 medium with BAP, Kn and 2ip from cotyledonary node explant. (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar. Shoots with length ≥ 10 mm were considered only).

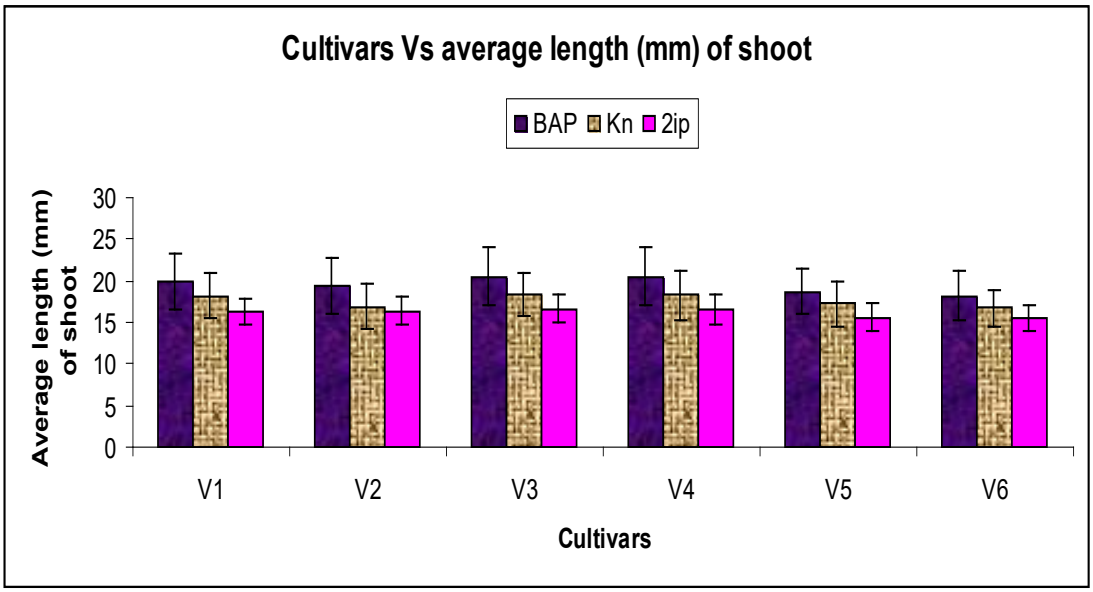


Fig.4.3.6: Average length of shoot per explant of six cultivars of castor in different concentrations of MMS_1 medium with BAP, Kn and 2ip from cotyledonary node explant. (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar.).

4.3.2.5: Effect of BAP in combination with NAA on axillary shoot proliferation.

The next phase of the investigation was to find out a suitable auxin-cytokinin concentration for optimizing axillary shoot production. In this experiment also cotyledonary node segments from seedlings grown on 1 mg/L BAP were used for proliferating axillary shoots. These explants were cultured on MMS_1 medium supplemented with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 5.0 mg/L) of BAP in all combinations with 4 different concentrations (0.05, 0.1, 0.2 and 0.5 mg/L) of NAA for selecting the best BAP-NAA combination in six cultivars of castor.

i) Cultivar shabje: Proliferation of axillary shoots from explants were influenced by the relative amounts and ratios of BAP and NAA. The results are presented in the Table 4.3.10. In most of the cases presence of NAA decreased the rate of normal shoot development and simultaneously increased the rate of callusing from the cut margins of the explants. Normal shoot formation was supported by the medium having comparatively a lower concentration (0.05-0.1 mg/L) of NAA and higher concentration (1.5-2.0 mg/L) of BAP. Among the different treatments, combination of 2.0 mg/L BAP and 0.05 mg/L NAA was found to be the best formulation where maximum frequency of axillary shoot proliferating culture (91.5%) and the highest number of shoot per culture were recorded (9.5±1.22), highest number of usable shoot per culture were recorded (5.3 ± 0.68) and average length of shoot was the highest (22.0 ± 3.8 mm) were recorded (Plate 4.3.1, C). Concentrations of 0.5 - 2.5 mg/L BAP + 0.05 - 0.1 mg/L NAA produced no callus formation. But 1.0 mg/L BAP + 0.2 mg/L NAA produced slight (+) callus formation and supplemented with 1.5 mg/L BAP + 0.5 mg/L NAA produced moderate (++) callus. In addition to different concentrations of NAA (0.1-0.5 mg/L) with 5.0 mg/L BAP failed to induce any axillary shoot but produced great amount of callus.

ii) Cultivar shadatae: Proliferation of axillary shoots from explants were influenced by the relative amounts and ratios of BAP and NAA. The results are presented in the Table 4.3.11. In most of the cases presence of NAA decreased the rate of normal shoot development and simultaneously increased the rate of callusing from the cut ends of the explants. Normal shoot formation was supported by having comparatively a lower concentration (0.05-0.1 mg/L) of NAA and higher concentration (1.5-2.0 mg/L) of BAP. Among the different treatments, combination of 2.0 mg/L BAP + 0.05 mg/l NAA was found to be the best formulation where maximum frequency of axillary shoot proliferating culture (79.5%) and the highest number of shoot per culture were recorded (8.4 ± 0.83), highest number of usable shoot per culture were recorded (4.8 ± 0.88) and average length of shoot was the

highest (20.6 ± 3.1 mm) (Plate 4.3.2, C). BAP 0.5 - 2.5 mg/L + NAA 0.05 - 0.1 mg/L produced no callus formation. But in 1.0 mg/L BAP + 0.2 mg/L NAA produced slight (+) callus formation and in 1.5 mg/L BAP + 0.5 mg/L NAA produced moderate (++) callus. Different concentrations of NAA (0.1-0.5 mg/L) with 5.0 mg/L BAP failed to induce any axillary shoot but produced great amount of callus.

iii) Cultivar roktima: Proliferation of axillary shoots from explants were influenced by the relative amounts and ratios of BAP and NAA. The results are presented in the Table 4.3.12. In most of the cases presence of NAA decreased the rate of normal shoot development and simultaneously increased the rate of callusing from the cut margins of the explants. Normal shoot formation was supported by having comparatively a lower concentration (0.05 - 0.1 mg/L) of NAA and higher concentration (1.5-2.0 mg/L) of BAP. Among the different concentration and combination tested, 2.0 mg/L BAP + 0.05 mg/L NAA was to be found the best formulation where maximum frequency of axillary shoot proliferating culture (90.1%) and the highest number of shoot per culture were recorded (9.3 ± 1.12), highest number of usable shoot per culture were recorded (5.2 ± 0.75) and the highest average length of shoot (21.8 ± 3.7 mm) was recorded (Plate 4.3.3, C). Concentrations of 0.5 - 2.5 mg/L BAP + 0.05 - 0.1 mg/L NAA produced no callus formation. But in 1.0 mg/L BAP + 0.2 mg/L NAA produced slight (+) callus formation and 1.5 mg/L BAP + 0.5 mg/L NAA produced moderate (++) callus formation. Different concentrations of NAA with 5.0 mg/L BAP failed to induce any axillary shoot but produced great amount of callus.

iv) Cultivar lalchay: The results are presented in the table 4.3.13. The table shows that in most of the cases presence of NAA decreased the rate of normal shoot development and simultaneously increased the rate of callusing from the cut margins of the explants. Normal shoot formation was supported by the medium having comparatively a lower concentration (0.05 - 0.1 mg/L) of NAA and higher concentration (1.5 - 2.0 mg/L) of BAP. Among the different concentrations and combinations of BAP and NAA were tested, 2.0 mg/L BAP + 0.05 mg/L NAA was found to be the best formulation where maximum frequency of axillary shoot proliferating culture (85.3%) and the highest number of shoot per culture were recorded (9.0±1.03), highest number of usable shoot per culture were recorded (5.1 ± 0.60) and the highest average length of shoot (21.7 ± 3.5 mm) was recorded (Plate 4.3.4, C). Concentrations of 0.5 - 2.5 mg/L BAP + 0.05 - 0.1 mg/L NAA produced no callus formation. But media supplemented with 1.0 mg/L BAP + 0.2 mg/L NAA produced slight (+) callusing and 1.5 mg/L BAP + 0.5 mg/L NAA produced moderate

(++) callus formation. Different concentrations of NAA with 5.0 mg/L BAP failed to induce any axillary shoot but produced great amount of callus.

v) Cultivar badami: Proliferation of axillary shoots from explants were influenced by the different concentrations and combinations of BAP and NAA. The results are presented in the Table 4.3.14. In most of the cases presence of NAA decreased the rate of normal shoot development and simultaneously increased the rate of callusing from the cut ends of the explants. Normal shoot formation was supported by the medium having comparatively a lower concentrations (0.05 - 0.1 mg/L) of NAA and higher concentrations (1.5 - 2.0 mg/L) of BAP. Among the different concentrations and combinations were tested, 2.0 mg/L BA + 0.05 mg/L NAA was found to be the best formulation where maximum frequency of axillary shoot proliferating culture (81.1%) and the highest number of shoot per culture were recorded (8.7 ± 0.98), highest number of usable shoot per culture were recorded (5.0 ± 0.91) and the highest average length of shoot (21.0 ± 3.2 mm) was recorded (Plate 4.3.5, C). Concentrations of 0.5 - 2.5 mg/L BAP + 0.05 - 0.1 mg/L NAA produced no callusing. But media supplemented with 1.0 mg/L BAP and 0.2 mg/L NAA produced slight (+) callus formation and 1.5 mg/L BAP + 0.5 mg/L NAA produced moderate (++) callus formation. Different concentrations of NAA with 5.0 mg/L BAP failed to induce any axillary shoot but produced great amount of callus.

vi) Cultivar dhusar: Proliferation of axillary shoots from explants were influenced by the relative amounts and ratios of BAP and NAA. The results are presented in the table 4.3.15. In most of the cases presence of NAA decreased the rate of normal shoot development and simultaneously increased the rate of callusing from the cut margins of the explants. Normal shoot formation was supported by the medium having comparatively a lower concentration (0.05 - 0.1 mg/L) of NAA and higher concentration (1.5 - 2.0 mg/L) of BAP. Among the different concentration and combination tested, 2.0 mg/L BAP + 0.05 mg/L NAA was found to be the best formulation where maximum frequency of axillary shoot proliferating culture (79.2%) and the highest number of shoot per culture were recorded (8.5 ± 8.85), highest number of usable shoot per culture were recorded (4.8 ± 0.87) and the highest average length of shoot (20.6 ± 3.0 mm) was recorded (Plate 4.3.6, C). Concentrations of 0.5 - 2.5 mg/L BAP + 0.05 - 0.1 mg/L NAA produced no callus formation. But in 1.0 mg/L BAP + 0.2 mg/L NAA produced slight (+) callus formation and 1.5 mg/L BAP + 0.5 mg/L NAA produced moderate (++) callusing. Different concentrations of NAA with 5.0 mg/L BAP failed to induce any axillary shoot but produced great amount of callus.

Table 4.3.10: Effects of different concentrations and combinations of BAP and NAA on axillary shoot proliferation of *R. communis* L. cultivar shabje (V_1) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length(mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	NAA					
0.5	0.05	53.2	4.7 ± 0.52	2.2 ± 0.32	16.7 ± 3.0	-
0.5	0.1	48.1	4.5 ± 0.56	2.1 ± 0.36	16.1 ± 2.8	-
0.5	0.2	42.0	4.3 ± 0.55	2.0 ± 0.31	15.9 ± 2.8	+
0.5	0.5	36.7	4.1 ± 0.59	1.9 ± 0.30	15.3 ± 2.7	++
1.0	0.05	65.2	6.6 ± 0.72	3.0 ± 0.48	18.6 ± 3.0	-
1.0	0.1	60.8	6.5 ± 0.77	2.9 ± 0.41	18.2 ± 2.9	-
1.0	0.2	54.0	6.3 ± 0.81	2.8 ± 0.43	17.8 ± 2.8	+
1.0	0.5	48.1	6.1 ± 0.78	2.7 ± 0.45	17.3 ± 2.7	++
1.5	0.05	75.0	7.5 ± 0.89	3.8 ± 0.53	19.8 ± 3.2	-
1.5	0.1	69.1	7.4 ± 0.88	3.7 ± 0.51	19.0 ± 3.0	-
1.5	0.2	63.6	7.2 ± 0.82	3.6 ± 0.48	18.5 ± 2.9	+
1.5	0.5	57.7	7.1 ± 0.78	3.5 ± 0.49	18.1 ± 2.7	++
2.0	0.05	91.5	9.5 ± 1.22	5.3 ± 0.68	22.0 ± 3.8	-
2.0	0.1	85.3	9.3 ± 1.17	5.2 ± 0.66	21.5 ± 3.1	-
2.0	0.2	79.2	9.2 ± 1.13	5.1 ± 0.73	20.8 ± 3.0	+
2.0	0.5	72.4	9.1 ± 1.11	5.0 ± 0.75	19.9 ± 2.8	++
2.5	0.05	73.3	8.6 ± 0.99	4.7 ± 0.51	20.9 ± 3.4	-
2.5	0.1	65.1	8.5 ± 0.95	4.6 ± 0.69	19.9 ± 3.1	-
2.5	0.2	57.0	8.4 ± 0.90	4.4 ± 0.63	19.3 ± 2.9	+
2.5	0.5	49.6	8.2 ± 0.92	4.2 ± 0.41	18.7 ± 2.7	++
5.0	0.05	-	-	-	-	+
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

Table 4.3.11: Effects of different concentrations and combinations of BAP and NAA on axillary shoot proliferation of *R. communis* L. cultiver shadatae (V_2) from nodal explant cultured on MMS_1 medium and data (X ± S.E) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture (X ± S.E)	No. of usable shoot per culture (X ± S.E)	Average length (mm) of shoot (X ± S.E)	* Callus formation
BAP	NAA					
0.5	0.05	46.0	3.7 ± 0.74	1.7 ± 0.46	16.0 ± 2.3	-
0.5	0.1	39.1	3.5 ± 0.70	1.6 ± 0.43	15.8 ± 2.1	-
0.5	0.2	33.3	3.3 ± 0.59	1.5 ± 0.37	15.6 ± 2.1	+
0.5	0.5	29.8	3.0 ± 0.32	1.3 ± 0.32	15.3 ± 2.0	++
1.0	0.05	58.1	5.6 ± 0.66	2.6 ± 0.81	18.1 ± 2.7	-
1.0	0.1	50.2	6.5 ± 0.50	2.5 ± 0.72	18.0 ± 2.5	-
1.0	0.2	45.6	5.3 ± 0.39	2.3 ± 0.61	17.5 ± 2.4	+
1.0	0.5	39.2	5.0 ± 0.35	2.1 ± 0.48	17.3 ± 2.3	++
1.5	0.05	68.5	6.6 ± 0.81	3.4 ± 0.80	19.2 ± 2.8	-
1.5	0.1	61.2	6.5 ± 0.72	3.3 ± 0.68	19.0 ± 2.5	-
1.5	0.2	51.0	6.3 ± 0.54	3.0 ± 0.59	18.8 ± 2.2	+
1.5	0.5	45.2	6.0 ± 0.39	2.8 ± 0.59	18.5 ± 2.1	++
2.0	0.05	79.5	8.4 ± 0.83	4.8 ± 0.88	20.6 ± 3.1	-
2.0	0.1	73.8	8.3 ± 0.77	4.7 ± 0.80	20.0 ± 2.7	-
2.0	0.2	67.9	8.0 ± 0.51	4.5 ± 0.65	19.8 ± 2.6	+
2.0	0.5	62.2	7.6 ± 0.45	4.1 ± 0.49	19.5 ± 2.5	++
2.5	0.05	63.1	7.0 ± 0.65	4.3 ± 0.87	20.0 ± 3.2	-
2.5	0.1	54.0	6.9 ± 0.61	4.2 ± 0.76	19.5 ± 2.8	-
2.5	0.2	47.1	6.7 ± 0.53	4.0 ± 0.59	19.0 ± 2.6	+
2.5	0.5	41.6	6.5 ± 0.43	3.6 ± 0.48	18.7 ± 2.5	++
5.0	0.05	-	-	-	-	+
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

Table 4.3.12: Effects of different concentrations and combinations of BAP and NAA on axillary shoot proliferation of *R. communis* L. cultivar roktima (V_3) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	NAA					
0.5	0.05	52.3	4.6 ± 0.61	2.1 ± 0.59	16.5 ± 2.9	-
0.5	0.1	47.2	4.3 ± 0.67	2.0 ± 0.51	16.0 ± 2.7	-
0.5	0.2	40.9	4.1 ± 0.65	1.9 ± 0.49	15.7 ± 2.5	+
0.5	0.5	35.1	3.9 ± 0.61	1.8 ± 0.39	14.9 ± 2.0	++
1.0	0.05	64.3	6.5 ± 0.80	2.9 ± 0.87	18.5 ± 3.0	-
1.0	0.1	58.7	6.3 ± 0.81	2.8 ± 0.83	17.9 ± 3.0	-
1.0	0.2	51.5	6.1 ± 0.73	2.7 ± 0.72	17.4 ± 2.9	+
1.0	0.5	43.0	5.9 ± 0.69	2.6 ± 0.65	16.9 ± 2.5	++
1.5	0.05	74.1	7.4 ± 0.88	3.7 ± 0.91	19.7 ± 3.1	-
1.5	0.1	68.2	7.2 ± 0.79	3.6 ± 0.89	18.8 ± 3.1	-
1.5	0.2	62.1	7.1 ± 0.98	3.5 ± 0.78	18.5 ± 3.0	+
1.5	0.5	55.0	6.9 ± 0.85	3.4 ± 0.73	17.8 ± 2.9	++
2.0	0.05	90.1	9.3 ± 1.12	5.2 ± 0.75	21.8 ± 3.7	-
2.0	0.1	84.2	9.1 ± 1.09	5.1 ± 0.68	20.9 ± 3.6	-
2.0	0.2	78.5	9.0 ± 1.05	5.0 ± 0.55	20.3 ± 3.4	+
2.0	0.5	72.1	8.8 ± 0.99	4.8 ± 0.48	19.8 ± 3.1	++
2.5	0.05	72.2	8.3 ± 0.88	4.6 ± 0.45	20.5 ± 3.4	-
2.5	0.1	64.3	8.1 ± 0.85	4.5 ± 0.41	20.0 ± 3.2	-
2.5	0.2	56.1	8.0 ± 0.78	4.4 ± 0.39	19.5 ± 3.0	+
2.5	0.5	47.9	7.9 ± 0.76	4.3 ± 0.37	19.0 ± 2.5	++
5.0	0.05	-	-	-	-	+
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

Table 4.3.13: Effects of different concentrations and combinations of BAP and NAA on axillary shoot proliferation of *R. communis* L. cultivar lalchay (V_4) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	NAA					
0.5	0.05	51.2	4.3 ± 0.57	2.0 ± 0.30	16.3 ± 2.5	-
0.5	0.1	46.1	4.1 ± 0.53	1.9 ± 0.32	16.0 ± 2.4	-
0.5	0.2	38.7	4.0 ± 0.64	1.7 ± 0.29	15.8 ± 2.3	+
0.5	0.5	32.0	3.8 ± 0.49	1.5 ± 0.22	15.5 ± 0.22	++
1.0	0.05	63.1	6.3 ± 0.71	2.8 ± 0.35	18.4 ± 2.9	-
1.0	0.1	56.3	6.2 ± 0.72	2.7 ± 0.43	18.0 ± 2.8	-
1.0	0.2	50.2	5.9 ± 0.59	2.5 ± 0.41	17.8 ± 2.5	+
1.0	0.5	41.1	5.5 ± 0.41	2.1 ± 0.43	17.3 ± 2.3	++
1.5	0.05	73.0	7.2 ± 0.85	3.6 ± 0.40	19.6 ± 3.0	-
1.5	0.1	67.1	7.1 ± 0.80	3.5 ± 0.41	19.5 ± 2.8	-
1.5	0.2	60.2	6.8 ± 0.79	3.3 ± 0.66	18.9 ± 2.7	+
1.5	0.5	52.1	6.5 ± 0.48	3.1 ± 0.58	18.5 ± 2.5	++
2.0	0.05	85.3	9.0 ± 1.03	5.1 ± 0.60	21.7 ± 3.5	-
2.0	0.1	79.4	8.8 ± 0.99	5.0 ± 0.68	20.8 ± 3.3	-
2.0	0.2	73.0	8.5 ± 0.88	4.8 ± 0.52	20.4 ± 3.0	+
2.0	0.5	67.3	8.3 ± 0.71	4.5 ± 0.44	19.9 ± 2.8	++
2.5	0.05	68.5	8.1 ± 0.89	4.5 ± 0.45	20.3 ± 3.2	-
2.5	0.1	59.2	8.0 ± 0.87	4.4 ± 0.42	20.0 ± 3.0	-
2.5	0.2	54.3	7.7 ± 0.61	4.2 ± 0.51	19.5 ± 2.7	+
2.5	0.5	45.4	7.3 ± 0.52	4.0 ± 0.48	19.1 ± 2.4	++
5.0	0.05	-	-	-	-	-
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

Table 4.3.14: Effects of different concentrations and combinations of BAP and NAA on axillary shoot proliferation of *R. communis* L. cultivar badami (V_5) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	NAA					
0.5	0.05	46.0	4.0 ± 0.55	1.8 ± 0.48	16.1 ± 2.3	-
0.5	0.1	41.3	3.9 ± 0.56	1.7 ± 0.45	16.0 ± 2.2	-
0.5	0.2	33.6	3.7 ± 0.49	1.5 ± 0.39	15.8 ± 2.1	+
0.5	0.5	27.5	3.5 ± 0.43	1.4 ± 0.35	15.6 ± 2.0	++
1.0	0.05	58.1	5.9 ± 0.70	2.7 ± 0.83	18.2 ± 2.7	-
1.0	0.1	50.2	5.8 ± 0.61	2.6 ± 0.78	17.5 ± 2.6	-
1.0	0.2	46.8	5.6 ± 0.58	2.4 ± 0.68	17.2 ± 2.3	+
1.0	0.5	40.0	5.3 ± 0.45	2.2 ± 0.59	17.0 ± 2.1	++
1.5	0.05	68.1	7.0 ± 0.89	3.5 ± 0.85	19.4 ± 2.8	-
1.5	0.1	61.2	6.8 ± 0.82	3.4 ± 0.77	19.1 ± 2.6	-
1.5	0.2	53.5	6.7 ± 0.76	3.2 ± 0.75	18.7 ± 2.5	+
1.5	0.5	46.0	6.4 ± 0.48	3.0 ± 0.61	18.3 ± 2.4	++
2.0	0.05	81.1	8.7 ± 0.98	5.0 ± 0.91	21.0 ± 3.2	-
2.0	0.1	74.3	8.5 ± 0.79	4.8 ± 0.81	20.7 ± 3.2	-
2.0	0.2	69.1	8.3 ± 0.61	4.5 ± 0.59	20.2 ± 2.8	+
2.0	0.5	62.4	7.8 ± 0.56	4.3 ± 0.48	19.7 ± 2.7	++
2.5	0.05	63.6	7.5 ± 0.88	4.4 ± 0.90	20.1 ± 3.1	-
2.5	0.1	54.1	7.3 ± 0.78	4.3 ± 0.71	19.5 ± 3.0	-
2.5	0.2	47.5	7.0 ± 0.65	4.1 ± 0.65	19.2 ± 2.8	+
2.5	0.5	31.3	6.9 ± 0.39	3.9 ± 0.51	19.0 ± 2.3	++
5.0	0.05	-	-	-	-	-
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

Table 4.3.15: Effects of different concentrations and combinations of BAP and NAA on axillary shoot proliferation of *R. communis* L. cultivar dhusar (V_6) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	NAA					
0.5	0.05	45.1	3.8 ± 0.75	1.7 ± 0.45	16.0 ± 2.3	-
0.5	0.1	40.4	3.5 ± 0.71	1.6 ± 0.42	15.8 ± 2.1	-
0.5	0.2	32.7	3.4 ± 0.62	1.5 ± 0.38	15.5 ± 2.0	+
0.5	0.5	28.4	3.2 ± 0.53	1.3 ± 0.35	15.0 ± 2.0	++
1.0	0.05	57.0	5.7 ± 0.68	2.6 ± 0.80	18.1 ± 2.6	-
1.0	0.1	49.4	5.5 ± 0.51	2.5 ± 0.78	18.0 ± 2.5	-
1.0	0.2	44.7	5.3 ± 0.42	2.3 ± 0.62	17.5 ± 2.4	+
1.0	0.5	26.1	5.1 ± 0.39	2.1 ± 0.49	17.3 ± 2.3	++
1.5	0.05	67.2	6.7 ± 0.82	3.4 ± 0.79	19.2 ± 2.7	-
1.5	0.1	60.3	6.6 ± 0.72	3.3 ± 0.71	19.0 ± 2.5	-
1.5	0.2	50.4	6.3 ± 0.55	3.0 ± 0.65	18.8 ± 2.3	+
1.5	0.5	44.1	6.0 ± 0.39	2.8 ± 0.59	18.5 ± 2.1	++
2.0	0.05	79.2	8.5 ± 0.85	4.8 ± 0.87	20.6 ± 3.0	-
2.0	0.1	73.1	8.3 ± 0.76	4.7 ± 0.81	20.0 ± 2.8	-
2.0	0.2	68.4	8.0 ± 0.48	4.5 ± 0.69	19.8 ± 2.7	+
2.0	0.5	61.5	7.8 ± 0.43	4.1 ± 0.48	19.5 ± 2.5	++
2.5	0.05	62.3	7.3 ± 0.72	4.3 ± 0.85	20.0 ± 3.0	-
2.5	0.1	53.0	7.0 ± 0.63	4.2 ± 0.78	19.5 ± 2.9	-
2.5	0.2	46.6	6.8 ± 0.56	4.0 ± 0.58	19.0 ± 2.7	+
2.5	0.5	40.5	6.5 ± 0.47	3.7 ± 0.49	18.8 ± 2.5	++
5.0	0.05	-	-	-	-	-
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

4.3.2.6: Effect of BAP in combination with IAA on axillary shoot proliferation.

Cotyledonary node segments from seedlings grown on 1 mg/L BAP were used for proliferating shoots. These explants were cultured on MMS_1 medium supplemented with different concentrations viz. 0.5, 1.0, 1.5, 2.0, 2.5 and 5.0 mg/L of BAP along with different concentrations of IAA viz. 0.05, 0.1, 0.2 and 0.5 mg/L for selecting the best BAP-IAA concentration in six cultivars of castor.

i) Cultivar shabje: Results on the influence of various concentrations and combinations of BAP and IAA on axillary shoot proliferation from explants are given in Table 4.3.16. The percent of explants showing proliferation was varied from 26.1-75.6%. The maximum number of 75.6% explants produced normal shoots when cultured in 2.0 mg/L BAP + 0.05 mg/L IAA. The total number of shoot per culture was the highest (98.3 ± 0.98), number of usable shoot per culture was the highest (4.0 ± 0.58), and average length of shoot was the highest (21.0 ± 3.7 mm) in 2.0 mg/L BAP + 0.05 mg/L IAA. From the over all observation, among all the concentrations and combinations tested it was clear that BAP 2.0 mg/L + IAA 0.05 mg/L was the best combination for axillary shoot proliferation (Plate 4.3.1, B). Combinations of 0.5 -1.5 mg/L BAP + 0.05 - 0.2 mg/L IAA and 2.0 - 2.5 mg/L BAP + 0.05 - 0.1 mg/L IAA produced no callus formation. But in 0.5 - 1.5 mg/L BAP + 0.5 mg/L IAA and 2.0 - 2.5 mg/L BA + 0.2 mg/L IAA produced poor (+) callusing. BAP 2.0 mg/L - 2.5 mg/L + IAA 0.5 mg/L produced moderate (++) callus. Concentrations of IAA (0.1-0.5 mg/L) with BAP 5.0 mg/L failed to induced any axillary shoot but produced great amount of callus.

ii) Cultivar shadatae: Results of various concentrations and combinations of BAP and IAA on axillary shoot proliferation from explants are given in Table 4.3.17. The percent of explants showing proliferation was varied from 17.1-67.4%. The maximum number (67.4%) of explants produced normal shoots when cultured in 2.0 mg/L BAP + 0.05 mg/l IAA. The total number of shoot per culture was highest (7.5 ± 0.68), number of usable shoot per culture was highest (3.5 ± 0.40), and average length of shoot was the highest (17.9 ± 3.2 mm) were showed in the same medium. From the over all observation, among the all concentrations and combinations tested it was clear that BAP 2.0 mg/L + IAA 0.05 mg/L was the best combination for axillary shoot proliferation (Plate 4.3.2, B). Different concentrations of BAP (0.5 - 2.0 mg/L) + 0.05 - 0.2 mg/L IAA and 2.5 mg/L BAP + 0.05 - 0.1 mg/L IAA failed to produce any callus. But 0.5 - 2.0 mg/L BAP + 0.5 mg/L IAA and 2.5 mg/L BAP + 0.2 mg/L IAA produced poor (+) callus. BAP 2.5 mg/L + 0.5 mg/L IAA produced moderate (++)

callus formation. Combination of IAA with BAP 5.0 mg/L failed to induce any axillary shoot but produced great amount of callus formation.

iii) Cultivar roktima: Results on the influence of various concentrations and combinations of BAP and IAA on axillary shoot proliferation from explants are given in Table 4.3.18. The maximum number (74.3%) of explants produced normal shoots in media having 2.0 mg/L BAP + 0.05 mg/L IAA with highest total number of shoot per culture (8.2 ± 0.90), highest number of usable shoot per culture (3.9 ± 0.590 and highest average length of shoot per culture (921.5 ± 3.5 mm). In this experiment the lowest (25.3%) response was noted in the media with 0.5 mg/L BAP + 0.5 mg/L IAA. From the over all observation, among the all concentrations and combinations tested it was clear that BAP 2.0 mg/L + IAA 0.05 mg/L was the best combination for axillary shoot proliferation (Plate 4.3.3, B). Combinations of 0.5 - 2.0 mg/L BAP + 0.05 - 0.2 mg/L IAA and 2.5 mg/L BAP + 0.05 - 0.1 mg/L IAA produced no callus. But in 0.5 - 2.0 mg/L BAP + 0.5 mg/L IAA and 2.5 mg/L BAP + 0.2 mg/L IAA produced poor (+) callus formation. But 2.5 mg/L BAP + 0.5 mg/L IAA produced moderate (++) callus. Combination of IAA with BAP 5.0 mg/L failed to induce any axillary shoot but produced great amount of callus.

iv) Cultivar lalchay: Different concentrations (0.5-5.0 mg/L) of BAP and IAA (0.05-5.5 mg/L) were used in this investigation and results are presented in Table 4.3.19. The percent of explants showing proliferation was varied from 24.2-72.1%. The maximum number of explants produced showing proliferation was the highest 72.1%, total number of shoot per culture was the highest (8.1 ± 0.87), number of usable shoot per culture was the highest (3.8 ± 0.51), and average length of shoot was the highest (18.2 ± 3.1 mm) showed in 2.0 mg/L BAP + 0.05 mg/L IAA. From the over all observation, among all the concentrations and combinations tested it was clear that BAP 2.0 mg/L + IAA 0.05 mg/L was the best combination for axillary shoot proliferation (Plate 4.3.4, B). BAP 0.5 - 2.0 mg/L + 0.05 - 0.2 mg/L IAA and 2.5 mg/L BAP + 0.05 - 0.1 mg/L IAA produced no callus. But 0.5 - 2.0 mg/L BAP + 0.5 mg/L IAA and 2.5 mg/L BAP + 0.2 mg/L IAA produced poor (+) callus. But 2.5 mg/L BAP + 0.5 mg/L IAA produced moderate (++) callus. Combination of IAA with BAP 5.0 mg/L failed to induce any axillary shoot but produced great amount of callus.

v) Cultivar badami: Results on the influence of different concentrations and combinations of BAP and IAA on axillary shoot proliferation from explants are given in Table 4.3.20. The maximum number (68.5%) and minimum number (20.0%) explants showing proliferation was observed in 2.0 mg/L BAP + 0.05 mg/L IAA and 0.5 mg/L BAP + 0.5 mg/L IAA respectively. The total number of shoot per culture

was the highest (7.8 ± 0.81), number of usable shoot per culture was the highest (3.7 ± 0.45), and average length of shoot was the highest (17.9 ± 3.1 mm) recorded in the same media. From the over all observation, among all the concentrations and combinations tested it was clear that BAP 2.0 mg/L + IAA 0.05 mg/L was the best combination for axillary shoot proliferation (Plate 4.3.5, B). Concentrations 0.5 - 2.0 mg/L BAP + 0.05 - 0.2 mg/L IAA and 2.5 mg/L BAP + 0.05-0.1 mg/L IAA produced no callus. But 0.5 - 2.0 mg/L BAP + 0.5 mg/L IAA and 2.5 mg/L BAP + 0.2 mg/L IAA produced poor (+) callus. But 2.5 mg/L BAP + 0.5 mg/L IAA produced moderate (++) callus. Combination of IAA with BAP 5.0 mg/L failed to induce any axillary shoot but produced great amount of callus.

vi) Cultivar dhusar: Results on the influence of various concentration and combination of BAP and IAA on axillary shoot proliferation from explants are given in Table 4.3.21. The maximum number (67.3%) of explants produced shoots in media having 2.0 mg/L BAP + 0.05 mg/L IAA. The total number of shoot per culture was the highest (97.7 ± 0.80), number of usable shoot per culture was highest (3.6 ± 0.41), and average length of shoot was highest (17.6 ± 3.1 mm) were recorded in 2.0 mg/L BAP + 0.05 mg/L IAA. Among all the concentrations and combinations tested it was clear that BAP 2.0 mg/L + IAA 0.05 mg/L was the best combination for axillary shoot proliferation (Plate 4.3.6, B). Concentrations of 0.5 - 2.0 mg/L BAP + 0.05 - 0.2 mg/L IAA and 2.5 mg/L BAP + 0.05 - 0.1 mg/L IAA did not produce any callus formation. But 0.5 - 2.0 mg/L BAP + 0.5 mg/L IAA and 2.5 mg/L BAP + 0.2 mg/L IAA produced poor (+) callus. Concentration of BAP 2.5 mg/L + 0.5 mg/L IAA produced moderate (++) callus. Combination of IAA with BAP 5.0 mg/L failed to induce any axillary shoot but produced great amount of callus.

Table 4.3.16: Effect of different concentrations and combinations of BAP and IAA on axillary shoot proliferation of *R. communis* L. cultivar shabje (V_1) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	IAA					
0.5	0.05	43.7	3.8 ± 0.51	2.0 ± 0.41	15.5 ± 2.9	-
0.5	0.1	37.0	3.6 ± 0.48	1.9 ± 0.32	14.8 ± 2.8	-
0.5	0.2	31.3	3.4 ± 0.36	1.8 ± 0.29	14.4 ± 2.6	-
0.5	0.5	26.1	3.3 ± 0.29	1.7 ± 0.25	14.0 ± 2.5	+
1.0	0.05	56.3	5.5 ± 0.59	2.5 ± 0.53	17.5 ± 3.0	-
1.0	0.1	51.5	5.4 ± 0.55	2.4 ± 0.50	17.0 ± 2.9	-
1.0	0.2	46.3	5.2 ± 0.51	2.3 ± 0.42	16.8 ± 2.7	-
1.0	0.5	40.0	5.1 ± 0.46	2.2 ± 0.31	16.4 ± 2.6	+
1.5	0.05	61.7	6.3 ± 0.91	3.1 ± 0.55	18.6 ± 3.1	-
1.5	0.1	53.0	6.2 ± 0.88	3.0 ± 0.51	18.3 ± 3.0	-
1.5	0.2	49.5	6.0 ± 0.73	2.9 ± 0.48	18.0 ± 2.9	-
1.5	0.5	43.6	5.8 ± 0.65	2.8 ± 0.41	17.6 ± 2.8	+
2.0	0.05	75.6	8.3 ± 0.98	4.0 ± 0.98	21.0 ± 3.7	-
2.0	0.1	69.2	8.2 ± 0.92	3.9 ± 0.56	20.6 ± 3.6	-
2.0	0.2	62.0	8.0 ± 0.89	3.8 ± 0.49	20.3 ± 3.4	+
2.0	0.5	56.1	7.8 ± 0.81	3.7 ± 0.35	20.0 ± 3.2	++
2.5	0.05	63.1	7.4 ± 0.85	3.3 ± 0.57	19.7 ± 3.2	-
2.5	0.1	56.3	7.2 ± 0.81	3.2 ± 0.55	18.9 ± 3.1	-
2.5	0.2	47.1	7.0 ± 0.79	3.1 ± 0.58	18.6 ± 3.0	+
2.5	0.5	39.6	6.8 ± 0.71	3.0 ± 0.56	18.3 ± 2.8	++
5.0	0.05	-	-	-	-	-
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

Table 4.3.17: Effect of different concentrations and combinations of BAP and IAA on axillary shoot proliferation of *R. communis* L. cultivar shadate (V_2) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	IAA					
0.5	0.05	35.1	3.3 ± 0.44	1.5 ± 0.27	14.5 ± 2.5	-
0.5	0.1	28.7	3.1 ± 0.42	1.4 ± 0.25	14.2 ± 2.4	-
0.5	0.2	23.1	3.0 ± 0.33	1.2 ± 0.23	14.0 ± 2.2	-
0.5	0.5	17.7	2.9 ± 0.29	1.1 ± 0.18	13.5 ± 2.0	+
1.0	0.05	47.2	5.0 ± 0.43	2.0 ± 0.35	16.7 ± 2.7	-
1.0	0.1	42.0	4.8 ± 0.40	1.9 ± 0.33	15.9 ± 2.6	-
1.0	0.2	38.1	4.5 ± 0.38	1.7 ± 0.29	15.5 ± 2.4	-
1.0	0.5	30.1	4.3 ± 0.35	1.6 ± 0.25	15.0 ± 2.1	+
1.5	0.05	52.8	5.5 ± 0.74	2.7 ± 0.37	17.7 ± 2.8	-
1.5	0.1	48.1	5.3 ± 0.68	2.6 ± 0.34	17.4 ± 2.6	-
1.5	0.2	41.7	5.1 ± 0.61	2.4 ± 0.31	17.2 ± 2.4	-
1.5	0.5	35.5	5.0 ± 0.59	2.3 ± 0.29	17.0 ± 2.1	+
2.0	0.05	67.4	7.5 ± 0.68	3.5 ± 0.40	17.9 ± 3.2	-
2.0	0.1	59.9	7.3 ± 0.61	3.4 ± 0.35	17.5 ± 3.0	-
2.0	0.2	53.0	7.0 ± 0.55	3.2 ± 0.31	17.1 ± 2.8	-
2.0	0.5	47.1	6.8 ± 0.48	3.0 ± 0.28	17.0 ± 2.6	+
2.5	0.05	53.0	6.8 ± 0.58	2.7 ± 0.50	19.4 ± 3.1	-
2.5	0.1	49.1	6.7 ± 0.49	2.6 ± 0.48	19.2 ± 3.0	-
2.5	0.2	39.1	6.4 ± 0.43	2.4 ± 0.42	19.0 ± 2.8	+
2.5	0.5	31.5	6.2 ± 0.39	2.2 ± 0.39	18.8 ± 2.6	++
5.0	0.05	-	-	-	-	-
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

Table 4.3.18: Effect of different concentrations and combinations of BAP and IAA on axillary shoot proliferation of *R. communis* L. cultivar roktima (V_3) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	IAA					
0.5	0.05	42.8	3.7 ± 0.52	1.9 ± 0.33	15.3 ± 2.9	-
0.5	0.1	36.2	3.6 ± 0.47	1.8 ± 0.34	14.8 ± 2.8	-
0.5	0.2	30.2	3.4 ± 0.39	1.7 ± 0.32	14.5 ± 2.6	-
0.5	0.5	25.3	3.2 ± 0.32	1.5 ± 0.29	14.1 ± 2.5	+
1.0	0.05	55.2	5.4 ± 0.58	2.4 ± 0.54	17.4 ± 3.1	-
1.0	0.1	49.4	5.3 ± 0.52	2.3 ± 0.49	17.0 ± 3.0	-
1.0	0.2	44.5	5.2 ± 0.46	2.2 ± 0.31	16.8 ± 2.9	-
1.0	0.5	38.6	5.0 ± 0.39	2.1 ± 0.29	16.5 ± 2.6	+
1.5	0.05	60.1	6.2 ± 0.87	3.0 ± 0.56	18.5 ± 3.2	-
1.5	0.1	54.1	6.0 ± 0.75	2.9 ± 0.49	18.1± 3.1	-
1.5	0.2	47.0	5.9 ± 0.68	2.8 ± 0.45	17.8 ± 3.0	-
1.5	0.5	30.7	5.7 ± 0.59	2.7 ± 0.43	17.5 ± 2.9	+
2.0	0.05	74.3	8.2 ± 0.90	3.9 ± 0.59	21.5 ± 3.5	-
2.0	0.1	68.0	8.1 ± 0.88	3.8 ± 0.55	20.0 ± 3.0	-
2.0	0.2	60.9	8.0 ± 0.79	3.7 ± 0.51	19.5 ± 2.9	-
2.0	0.5	55.5	7.8 ± 0.83	3.6 ± 0.42	19.1 ± 2.5	+
2.5	0.05	62.0	7.3 ± 0.83	3.2 ± 0.88	20.2 ± 3.6	-
2.5	0.1	55.1	7.1 ± 0.84	3.1 ± 0.53	20.0 ± 3.3	-
2.5	0.2	45.2	6.9 ± 0.81	3.0± 0.58	19.8 ± 3.1	+
2.5	0.5	38.3	6.7 ± 0.75	2.9 ± 0.49	19.4 ± 3.0	++
5.0	0.05	-	-	-	-	-
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

Table 4.3.19: Effect of different concentrations and combinations of BAP and IAA on axillary shoot proliferation of *R. communis* L. cultivar lalchay (V_4) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	IAA					
0.5	0.05	41.5	3.6 ± 0.50	1.8 ± 0.31	15.1 ± 2.8	-
0.5	0.1	35.1	3.4 ± 0.48	1.7 ± 0.29	14.9 ± 2.7	-
0.5	0.2	29.3	3.2 ± 0.42	1.6 ± 0.30	14.3 ± 2.6	-
0.5	0.5	24.2	3.0 ± 0.39	1.5 ± 0.31	14.0 ± 2.6	+
1.0	0.05	53.7	5.3 ± 0.57	2.3 ± 0.53	17.2 ± 3.0	-
1.0	0.1	48.9	5.1 ± 0.53	2.2 ± 0.51	16.8 ± 2.9	-
1.0	0.2	43.8	5.0 ± 0.49	2.1 ± 0.49	16.6 ± 2.8	-
1.0	0.5	37.5	4.8 ± 0.45	2.0 ± 0.47	16.3 ± 2.7	+
1.5	0.05	58.7	6.1 ± 0.86	2.9 ± 0.53	18.2 ± 3.1	-
1.5	0.1	53.8	6.0 ± 0.79	2.8 ± 0.48	18.0 ± 3.0	-
1.5	0.2	46.3	5.8 ± 0.71	2.7 ± 0.44	17.5 ± 3.0	-
1.5	0.5	41.9	5.6 ± 0.68	2.5 ± 0.41	17.2 ± 2.9	+
2.0	0.05	72.1	8.1 ± 0.87	3.8 ± 0.51	18.2 ± 3.1	-
2.0	0.1	66.3	8.0 ± 0.85	3.7 ± 0.52	18.0 ± 3.1	-
2.0	0.2	58.6	7.9 ± 0.81	3.5 ± 0.47	17.5 ± 3.0	-
2.0	0.5	52.4	7.7 ± 0.79	3.4 ± 0.43	17.3 ± 2.9	+
2.5	0.05	58.1	7.2 ± 0.81	3.1 ± 0.37	20.0 ± 3.3	-
2.5	0.1	53.6	6.8 ± 0.74	3.0 ± 0.38	19.5 ± 3.2	-
2.5	0.2	44.7	6.5 ± 0.75	2.8 ± 0.51	19.1 ± 3.1	+
2.5	0.5	37.3	6.3 ± 0.71	2.6 ± 0.48	19.0 ± 3.0	++
5.0	0.05	-	-	-	-	-
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

Table 4.3.20: Effect of different concentrations and combinations of BAP and IAA on axillary shoot proliferation of *R. communis* L. cultivar badami (V_5) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	IAA					
0.5	0.05	37.3	3.5 ± 0.48	1.6 ± 0.29	15.0 ± 2.7	-
0.5	0.1	31.0	3.2 ± 0.49	1.5 ± 0.27	14.8 ± 2.6	-
0.5	0.2	25.5	3.0 ± 0.41	1.4 ± 0.25	14.6 ± 2.5	-
0.5	0.5	20.0	2.9 ± 0.38	1.3 ± 0.23	14.3 ± 2.3	+
1.0	0.05	49.6	5.2 ± 0.54	2.1 ± 0.39	17.0 ± 2.9	-
1.0	0.1	44.9	5.0 ± 0.58	2.0 ± 0.37	16.8 ± 2.9	-
1.0	0.2	39.8	4.8 ± 0.45	1.9 ± 0.35	16.6 ± 2.8	-
1.0	0.5	33.3	4.6 ± 0.43	1.8 ± 0.32	16.3 ± 2.7	+
1.5	0.05	54.6	6.0 ± 0.86	2.8 ± 0.41	17.8 ± 3.0	-
1.5	0.1	49.7	5.8 ± 0.79	2.6 ± 0.39	17.5 ± 3.0	-
1.5	0.2	42.4	5.6 ± 0.73	2.5±0.35	17.3 ± 2.8	-
1.5	0.5	37.1	5.3 ± 0.70	2.3 ± 0.33	17.0 ± 2.7	+
2.0	0.05	68.5	7.8 ± 0.81	3.7 ± 0.45	17.9 ± 3.1	-
2.0	0.1	62.1	7.6 ± 0.79	3.6 ± 0.44	17.7 ± 3.0	-
2.0	0.2	54.4	7.4 ± 0.72	3.5 ± 0.42	17.5 ± 2.9	-
2.0	0.5	49.0	7.2 ± 0.68	3.3 ± 0.41	17.3 ± 2.9	+
2.5	0.05	54.1	7.0 ± 0.78	3.0 ± 0.68	19.7 ± 3.1	-
2.5	0.1	50.2	6.8 ± 0.75	2.9 ± 0.65	19.3 ± 3.0	-
2.5	0.2	40.5	6.6 ± 0.73	2.7 ± 0.63	19.0 ± 2.9	+
2.5	0.5	33.4	6.3 ± 0.72	2.5 ± 0.60	18.8 ± 2.8	++
5.0	0.05	-	-	-	-	-
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

Table 4.3.21: Effect of different concentrations and combinations of BAP and IAA on axillary shoot proliferation of *R. communis* L. cultivar dhusar (V_6) from nodal explant cultured on MMS_1 medium and data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture. Each treatment consisted of 10-15 explants.

Growth regulators mg/L		% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length (mm) of shoot ($\bar{x} \pm SE$)	* Callus formation
BAP	IAA					
0.5	0.05	36.0	3.4 ± 0.46	1.5 ± 0.28	14.5 ± 2.3	-
0.5	0.1	29.8	3.2 ± 0.45	1.4 ± 0.26	14.3 ± 2.5	-
0.5	0.2	24.4	3.0 ± 0.44	1.3 ± 0.23	14.2 ± 2.0	-
0.5	0.5	18.1	2.9 ± 0.40	1.2 ± 0.21	14.0 ± 1.9	+
1.0	0.05	48.3	5.1 ± 0.59	2.0 ± 0.32	16.7 ± 2.8	-
1.0	0.1	43.4	4.9 ± 0.58	1.9 ± 0.30	16.5 ± 2.7	-
1.0	0.2	38.5	4.7 ± 0.50	1.8 ± 0.28	16.3 ± 2.6	-
1.0	0.5	32.2	4.5 ± 0.46	1.7 ± 0.27	16.0 ± 2.5	+
1.5	0.05	53.3	5.8 ± 0.76	2.7 ± 0.38	17.6 ± 2.8	-
1.5	0.1	48.6	5.6 ± 0.74	2.6 ± 0.35	17.3 ± 2.7	-
1.5	0.2	41.2	5.3 ± 0.72	2.5 ± 0.32	17.1 ± 2.5	-
1.5	0.5	36.0	5.0 ± 0.70	2.4±0.30	17.0 ± 2.4	+
2.0	0.05	67.3	7.7 ± 0.80	3.6 ± 0.41	17.6 ± 3.1	-
2.0	0.1	61.0	7.5 ± 0.78	3.5 ± 0.40	17.4 ± 3.0	-
2.0	0.2	53.3	7.3 ± 0.75	3.4 ± 0.39	17.2 ± 2.9	-
2.0	0.5	47.9	7.1 ± 0.71	3.3 ± 0.38	17.0 ± 2.8	+
2.5	0.05	53.0	6.9 ± 0.65	2.9 ± 0.51	19.3 ± 3.0	-
2.5	0.1	49.1	6.7 ± 0.63	2.8 ± 0.49	19.1 ± 2.9	-
2.5	0.2	39.4	6.5 ± 0.60	2.7 ± 0.47	19.0 ± 2.9	+
2.5	0.5	32.6	6.3 ± 0.59	2.6 ± 0.45	18.8 ± 2.7	++
5.0	0.05	-	-	-	-	-
5.0	0.1	-	-	-	-	+
5.0	0.2	-	-	-	-	++
5.0	0.5	-	-	-	-	+++

* Rating scale of callus: - nil, + poor, ++ moderate, +++ high.

CULTIVAR SHABJE
(Direct regeneration)

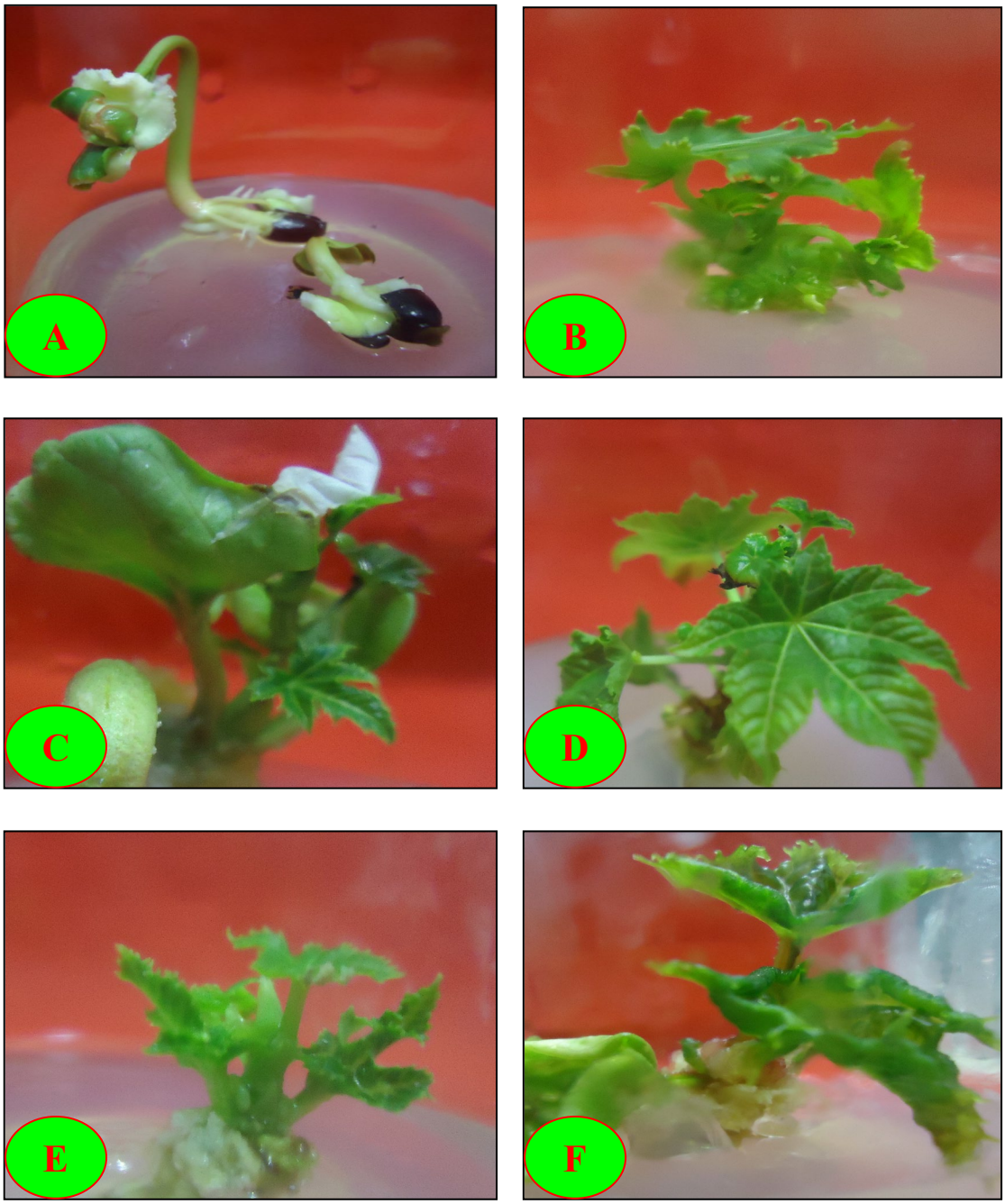


Plate- 4.3.1: Photographs showing the seed germination and axillary shoot proliferation of cultivar shabje. **A.** Seed germination in MS + BAP 1.0 mg/L**. B.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L + 0.05 mg/L IAA**. C**. Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L + 0.05 mg/L NAA. **D.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L. **E.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + Kn 2.0 mg/L. **F.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + 2ip 2.0 mg/L. Data were recorded after 4 weeks of culture for **A** and data were recorded after 6 weeks of culture for **B – F**.

CULTIVAR SHADATAE
(Direct regeneration)

Plate- 4.3.2: Photographs showing the seed germination and axillary shoot proliferation of cultivar shadatae. **A.** Seed germination in MS + BAP 1.0 mg/L**. B.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L + IAA 0.05 mg/L**. C**. Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L + NAA 0.05 mg/L. **D.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L. **E.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + Kn 2.0 mg/L. **F.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + 2ip 2.0 mg/L. Data were recorded after 4 weeks of culture for **A** and data were recorded after 6 weeks of culture for **B – F**.

CULTIVAR ROKTIMA
(Direct regeneration)

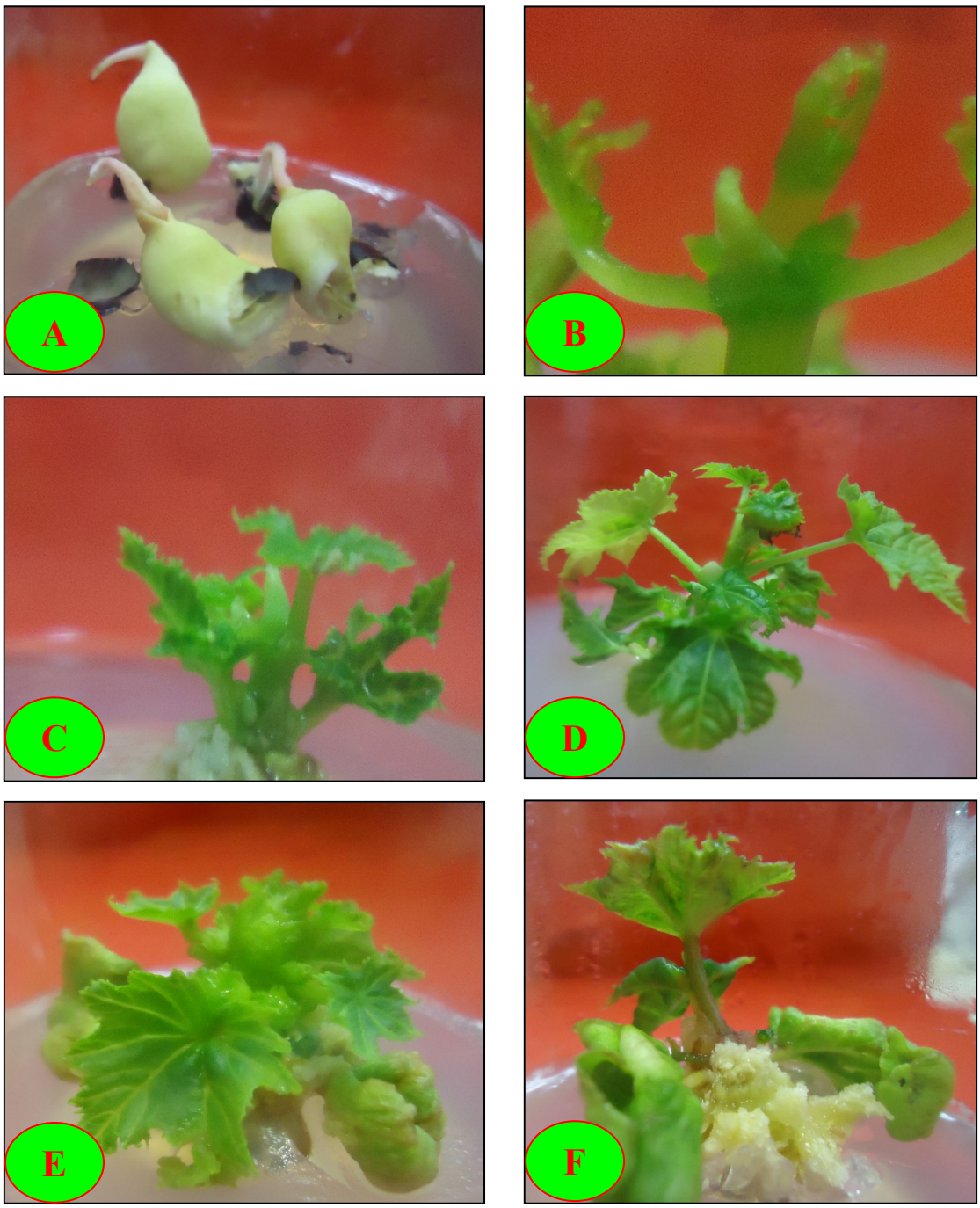


Plate- 4.3.3: Photographs showing the seed germination and axillary shoot proliferation of cultivar roktima. **A.** Seed germination in MS + BAP 1.0 mg/L**. B.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L IAA 0.05 mg/L**. C**. Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L + NAA 0.05 mg/L. **D.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L. **E.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + Kn 2.0 mg/L. **F.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + 2ip 2.0 mg/L. Data were recorded after 4 weeks of culture for **A** and data were recorded after 6 weeks of culture for **B – F**.

CULTIVAR LALCHAY
(Direct regeneration)

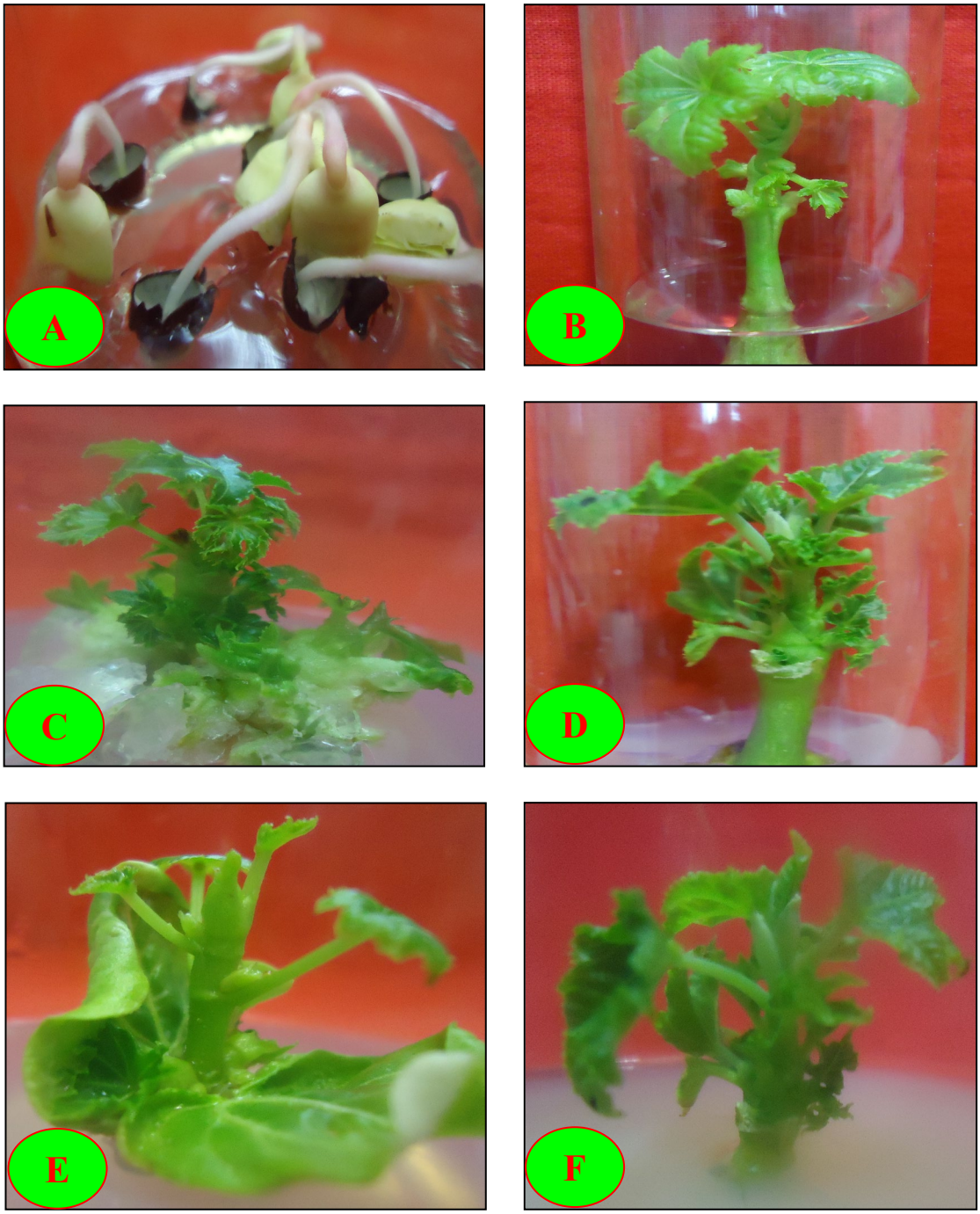


Plate- 4.3.4: Photographs showing the seed germination and axillary shoot proliferation of cultivar lalchay. **A.** Seed germination in MS + BAP 1.0 mg/L**. B.** Axillary shoot proliferation from cotyledonary node in MMS_1 + BAP 2.0 mg/L + IAA 0.05 mg/L**. C**. Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L NAA 0.05 mg/L. **D.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L. **E.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + Kn 2.0 mg/L. **F.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + 2ip 2.0 mg/L. Data were recorded after 4 weeks of culture for **A** and data were recorded after 6 weeks of culture for **B – F**.

CULTIVAR BADAMI
(Direct regeneration)

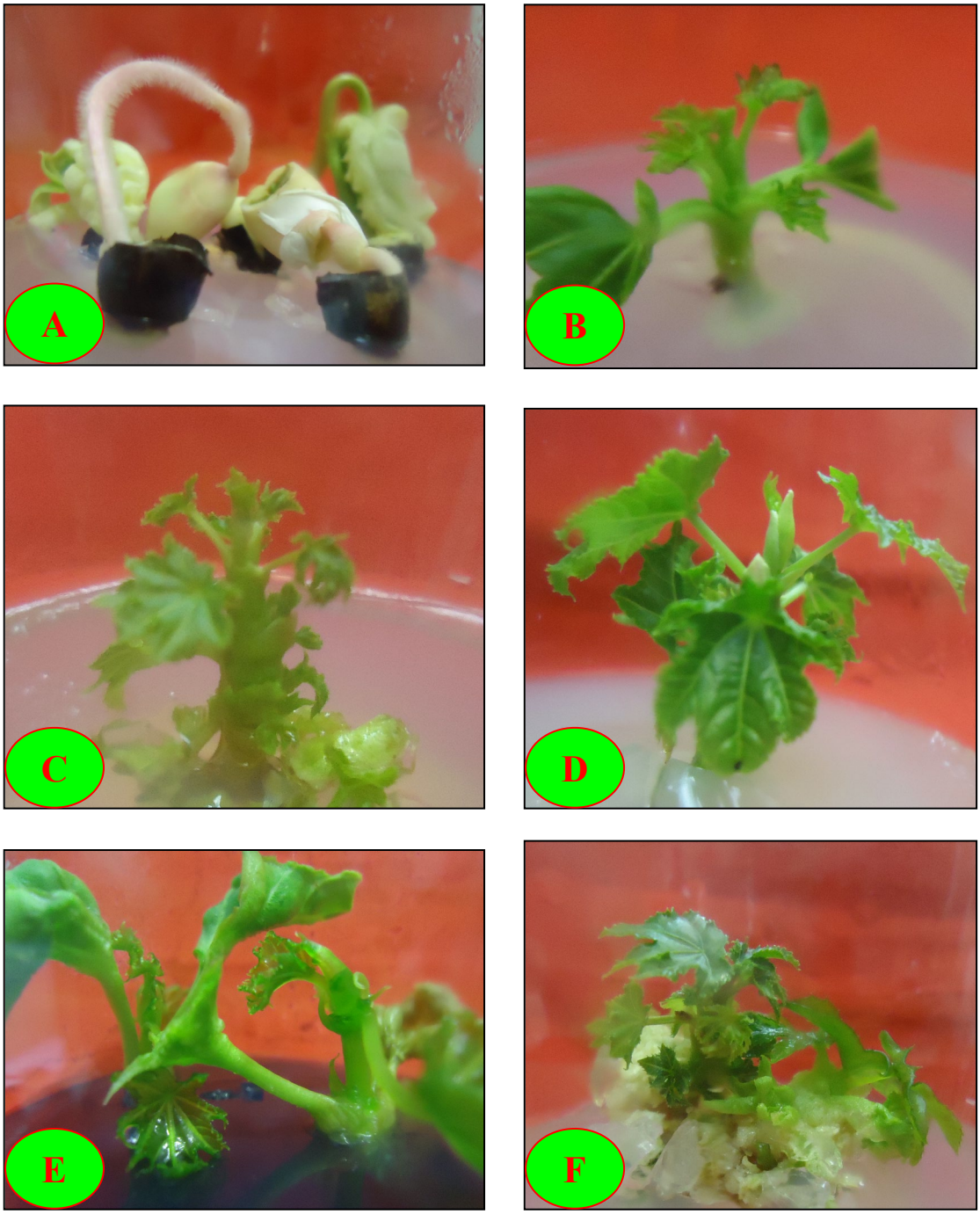


Plate- 4.3.5: Photographs showing the seed germination and axillary shoot proliferation of cultivar badami. **A.** Seed germination in MS + BAP 1.0 mg/L**. B.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L + IAA 0.05 mg/L**. C**. Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L + NAA 0.05. **D.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L. **E.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + Kn 2.0 mg/L. **F.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + 2ip 2.0 mg/L. Data were recorded after 4 weeks of culture for **A** and data were recorded after 6 weeks of culture for **B – F**.

CULTIVAR DHUSAR
(Direct regeneration)

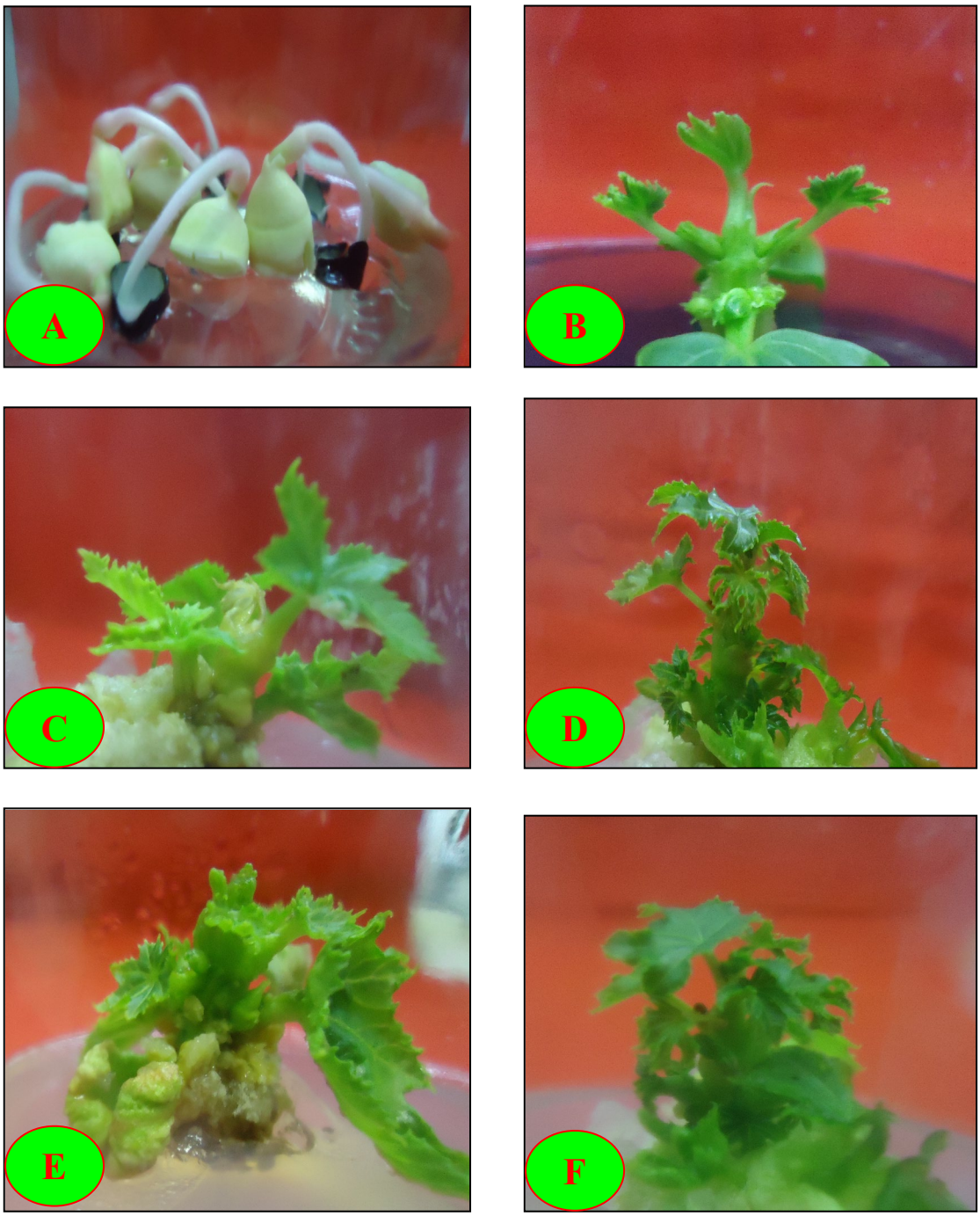


Plate- 4.3.6: Photographs showing the seed germination and axillary shoot proliferation of cultivar dhusar. **A.** Seed germination in MS + BAP 1.0 mg/L**. B.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L + IAA 0.05**. C**. Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L + NAA 0.05. **D.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + BAP 2.0 mg/L. **E.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + Kn 2.0 mg/L. **Fig. F.** Axillary shoot proliferation from cotyledonary node explant in MMS_1 + 2ip 2.0 mg/L. Data were recorded after 4 weeks of culture for **A** and data were recorded after 6 weeks of culture for **B – F**.

4.3.2.7: Effect of sucrose concentration on axillary shoot proliferation.

Sucrose is used as source of carbon and energy for optimum proliferation and growth of the *in vitro* grown cultures. An experiment was set up to study the optimum concentration of sucrose in MMS_1 medium for multiple shoot regeneration and development. The shoot segments were cultured on MMS_1 medium having 2.0 mg/L BAP, gelled with 8 gm/L agar at six different concentrations of sucrose viz, 10, 20, 30, 40, 50 and 60 gm/L and with a control treatment of without sucrose. The medium was adjusted to pH 5.7 ± 0.1 before autoclaving. After 6 weeks of culture the percentage of explants showing proliferation, number of total shoot per culture, number of usable shoot per culture, average length of shoot per culture and days to sprout the axillary buds were recorded of six cultivars of castor and described below in separate heads.

i) Cultivar shabje: The results of this investigation are presented in Table-4.3.22. Sucrose omitted medium failed to produce any axillary bud. Among the different sucrose concentrations tested 30 gm/L sucrose showed the highest percentage (100%) of explant showing proliferation and lowest (38.8%) in 60 gm/L. Number of total shoot per culture, number of usable shoot per culture and average length of shoot were also highest in 30 gm/L sucrose and they were 9.5 ± 1.17, 5.3 ± 0.99 and 23.5 ± 3.8 mm respectively and lowest in 60 gm/L sucrose and they were 4.1 ± 0.56, 2.8 ± 0.55, 17.1 ± 2.8 mm respectively. At the lowest concentration (10 gm/L) tested, shoots were leafy and fewer in number than those at 30 gm/L and 40 gm/L of sucrose. Days to sprout the axillary buds took the lowest in 30 gm/L and 40 gm/L sucrose and it was 5 - 7 days and highest in 10 gm/L sucrose and it was 10 - 15 days.

ii) Cultivar shadatae: The results of this investigation are presented in Table-4.3.23. No sprouting of any axillary bud was observed in sucrose omitted medium. Among the different sucrose concentrations tested highest (94.2%) and lowest (37.3%) percentage of explant showing proliferation were showed in 30 gm/L and 60 gm/L sucrose respectively. Number of total shoot per culture, number of usable shoot per culture and average length of shoot were also recorded in 30 gm/L sucrose and they were 7.0 ± 0.92, 3.8 ± 0.82 and 22.4 ± 3.9 mm respectively and lowest in 60 gm/L sucrose and they were 37.3%, 3.5 ± 0.76, 2.5 ± 0.41, 16.8 ± 2.7 mm respectively. At the lowest concentration (10 gm/L) tested, shoots were leafy and fewer in number than those at 30 gm/L and 40 gm/L of sucrose. Days to sprout the axillary buds took the lowest time in 30 gm and 40 gm sucrose and it was 6 - 8 days and highest 10 gm/L sucrose and it was 10 - 15 days.

iii) Cultivar roktima: The results of this investigation are presented in Table-4.3.24. No sprouting of any axillary bud was observed on the sucrose omitted

medium. Among the different sucrose concentrations tested 30 gm/L sucrose showed the highest percentage of explant showing proliferation and that was 100%. The optimum result showed in 30 gm/L sucrose for number of total shoot per culture, number of usable shoot per culture and average length of shoot were recorded 9.2 ± 1.18, 5.2 ± 0.71 and 23.3 ± 3.8 mm respectively. 60 gm/L sucrose showed the lowest percentage of explant showing proliferation, number of total shoot per culture, number of usable shoot per culture, average length of shoot per culture and they were 39.5%, 4.0 ± 0.74, 2.7 ± 0.36, 17.0 ± 2.7 mm respectively. At the lowest concentration (10 gm/L) tested, shoots were leafy and fewer in number than those at 30 gm/L and 40 gm/L of sucrose. Days to sprout the axillary buds took lowest time in having 30 gm and 40 gm sucrose and it was 5 - 7 days and highest on the medium having 10 gm/l sucrose and it was 10 - 15 days.

iv) Cultivar lalchay: The results of this investigation are presented in Table-4.3.25. No sprouting of any axillary bud was observed on the sucrose omitted medium. Among the different sucrose concentrations tested 30 gm/L sucrose showed the highest percentage of explant showing proliferation and that was 100%. 30 gm/L sucrose showed the optimum result for number of total shoot per culture, number of usable shoot per culture and average length of shoot were recorded 9.0 ± 1.02, 5.0 ± 0.81 and 23.0 ± 3.9 mm respectively. The lowest percentage of explant showed proliferation, number of total shoot per culture, number of usable shoot per culture and average length of shoot per culture were 37.8%, 3.9 ± 0.75, 2.7 ± 0.78, 17.0 ± 2.8 mm respectively in 60 gm/L sucrose. At the lowest concentration (10 gm/L) tested, shoots were leafy and fewer in number than those at 30 gm/L and 40 gm/L of sucrose. Days to sprout the axillary buds took the lowest (5 - 7 days) time in 30 gm and 40 gm sucrose and highest on 10 gm/L sucrose and it was 10-15 days.

v) Cultivar badami: The results of this investigation are presented in Table-4.3.26. No sprouting of any axillary bud was observed on the sucrose omitted medium. Among the different sucrose concentrations tested, 30 gm/L sucrose showed the highest percentage of explant showing proliferation and that was 95.0%. The optimum result for number of total shoot per culture, number of usable shoot per culture and average length of shoot were recorded 7.1 ± 0.93, 3.9 ± 0.65 and 21.5 ± 3.8 mm respectively30 gm/L sucrose. The lowest percentage of explant showed proliferation, number of total shoot per culture, number of usable shoot per culture, average length of shoot per culture and they were 37.1%, 3.5 ± 0.73, 2.5 ± 0.41, 16.5 ± 2.5 mm respectively in 60 gm/L sucrose. At the lowest concentration of sucrose (10 gm/L) tested, shoots were leafy and fewer in number than those at 30 gm/L and 40 gm/L of sucrose. Lowest time was taken to sprout by the axillary buds in 30 gm and

40 gm sucrose and it was 6 - 8 days but on the contrary, longest time was noted in 10 gm/L sucrose and it was 10 - 15 days.

vi) Cultivar dhusar: The results of this investigation are presented in Table-4.3.27. No sprouting of any axillary bud was observed on the sucrose omitted medium. Among the different sucrose concentrations tested 30 gm/L sucrose showed the highest percentage of explant showing proliferation and that was 95.5%. The optimum result showed for number of total shoot per culture in 30 gm/L sucrose but number of usable shoot per culture and average length of shoot were 6.9 ± 0.83, 3.8 ± 0.61 and 20.0 ± 3.6 mm respectively. The lowest percentage of explants were proliferated in 60 gm/L sucrose but number of total shoot per culture, number of usable shoot per culture, average length of shoot per culture and they were 37.2%, 3.5 ± 0.75, 2.5 ± 0.48, 16.4 ± 2.5 mm respectively. At the lowest concentration (10 gm/L) tested, shoots were leafy and fewer in number than those at 30 gm/L and 40 gm/L of sucrose. Days to sprout the axillary buds took the lowest (6 - 8 days) time in 30 gm/L and 40 gm/L sucrose and highest on 10 gm/L sucrose and it was 10-15 days.

Table 4.3.22: Effect of different concentrations of sucrose on proliferation and growth of axillary shoot taken from nodal explant of cultivar shabje (V_1). Data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture on MMS_1 medium supplemented with 2.0 mg/L of BAP and each treatment consisted of 10-15 explants.

Conc. of sucrose (gm/L)	% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length of shoot (mm) ($\bar{x} \pm SE$)	Days to sprout the axillary bud
Nil	-	-	-	-	-
10	65.8	5.3 ± 0.68	2.9 ± 0.62	19.1 ± 3.0	10 - 15
20	92.7	7.6 ± 0.81	4.0 ± 0.87	22.3 ± 3.4	5 - 10
30	100.0	9.5 ± 1.17	5.3 ± 0.99	23.5 ± 3.8	5 - 7
40	82.5	8.4 ± 0.99	5.2 ± 0.95	23.4 ± 3.7	5 - 7
50	55.9	6.7 ± 0.81	3.5 ± 0.82	21.0 ± 3.0	7 - 12
60	38.8	4.1 ± 0.56	2.8 ± 0.55	17.1 ± 2.8	10 - 12

Table 4.3.23: Effect of different concentrations of sucrose on proliferation and growth of axillary shoot taken from nodal explant of cultivar shadate (V_2). Data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture on MMS_1 medium supplemented with 2.0 mg/L of BAP and Each treatment consisted of 10-15 explants.

Conc. of sucrose (gm/L)	% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length of shoot (mm) ($\bar{x} \pm SE$)	Days to sprout the axillary bud
Nil	-	-	-	-	-
10	60.9	4.4 ± 0.59	2.5 ± 0.41	18.0 ± 2.5	10 - 15
20	88.3	6.4 ± 0.82	3.1 ± 0.57	20.1 ± 3.6	6 - 10
30	94.2	7.0 ± 0.92	3.8 ± 0.82	22.4 ± 3.9	6 - 8
40	78.1	6.3 ± 0.78	3.3 ± 0.55	21.2 ± 3.5	6 - 8
50	53.5	5.9 ± 0.72	3.0 ± 0.46	18.2 ± 3.1	7 - 12
60	37.3	3.5 ± 0.76	2.5 ± 0.41	16.8 ± 2.7	10 - 12

Table 4.3.24: Effect of different concentrations of sucrose on proliferation and growth of axillary shoot taken from nodal explant of cultivar roktima (V_3). Data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture on MMS_1 medium supplemented with 2.0 mg/L of BAP and Each treatment consisted of 10-15 explants.

Conc. of sucrose (gm/L)	% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length of shoot (mm) ($\bar{x} \pm SE$)	Days to sprout the axillary bud
Nil	-	-	-	-	-
10	64.5	5.2 ± 0.77	2.9 ± 0.35	19.0 ± 3.0	10 - 15
20	92.2	7.5 ± 0.85	4.0 ± 0.58	22.2 ± 3.4	5 - 10
30	100.0	9.2 ± 1.18	5.2 ± 0.71	23.3 ± 3.8	5 - 7
40	81.8	8.2 ± 0.98	5.0 ± 0.67	23.2 ± 3.7	5 - 7
50	56.0	6.6 ± 0.82	3.4 ± 0.45	21.1 ± 3.2	7 - 12
60	39.5	4.0 ± 0.74	2.7 ± 0.36	17.0 ± 2.7	10 - 12

Table 4.3.25: Effect of different concentrations of sucrose on proliferation and growth of axillary shoot taken from nodal explant of cultivar lalchay. (V_4). Data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture on MMS_1 medium supplemented with 2.0 mg/L of BAP and Each treatment consisted of 10-15 explants.

Conc. of sucrose (gm/L)	% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length of shoot (mm) ($\bar{x} \pm SE$)	Days to sprout the axillary bud
Nil	-	-	-	-	-
10	63.7	5.1 ± 0.79	2.8 ± 0.74	18.7 ± 2.9	10 - 15
20	90.2	7.4 ± 0.91	3.9 ± 0.78	22.1 ± 3.5	5 - 10
30	100.0	9.0 ± 1.02	5.0 ± 0.81	23.0 ± 3.9	5 - 7
40	80.5	8.1 ± 0.95	4.6 ± 0.77	22.1 ± 3.8	5 - 7
50	55.1	6.5 ± 0.83	3.3 ± 0.79	20.5 ± 3.2	7 - 12
60	37.8	3.9 ± 0.75	2.7 ± 0.78	17.0 ± 2.8	10 - 12

Table 4.3.26: Effect of different concentrations of sucrose on proliferation and growth of axillary shoot taken from nodal explant of cultivar badami (V_5). Data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture on MMS_1 medium supplemented with 2.0 mg/L of BAP and Each treatment consisted of 10-15 explants.

Conc. of sucrose (gm/L)	% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length of shoot (mm) ($\bar{x} \pm SE$)	Days to sprout the axillary bud
Nil	-	-	-	-	-
10	61.6	4.3 ± 0.56	2.5 ± 0.39	17.8 ± 2.7	10 - 15
20	89.1	6.9 ± 0.88	3.1 ± 0.57	19.2 ± 3.2	6 - 10
30	95.0	7.1 ± 0.93	3.9 ± 0.65	21.5 ± 3.8	6 - 8
40	78.3	7.0 ± 0.81	3.5 ± 0.61	20.1 ± 3.5	6 - 8
50	53.4	6.2 ± 0.76	3.0 ± 0.50	18.0 ± 3.1	7 - 12
60	37.1	3.5 ± 0.73	2.5 ± 0.41	16.5 ± 2.5	10 - 12

Table 4.3.27: Effect of different concentrations of sucrose on proliferation and growth of axillary shoot taken from nodal explant of cultivar dhusar. (V_6). Data ($\bar{x} \pm SE$) were recorded after 6 weeks of culture on MMS_1 medium supplemented with 2.0 mg/L of BAP and Each treatment consisted of 10-15 explants.

Conc. of sucrose (gm/L)	% of explants showing proliferation	No. of total shoot per culture ($\bar{x} \pm SE$)	No. of usable shoot per culture ($\bar{x} \pm SE$)	Average length of shoot (mm) ($\bar{x} \pm SE$)	Days to sprout the axillary bud
Nil	-	-	-	-	-
10	61.7	4.3 ± 0.57	2.5 ± 0.40	17.7 ± 2.6	10 - 15
20	88.5	6.3 ± 0.81	3.0 ± 0.56	19.1 ± 3.2	6 - 10
30	95.5	6.9 ± 0.83	3.8 ± 0.61	20.0 ± 3.6	6 - 8
40	78.2	6.2 ± 0.77	3.4 ± 0.59	19.5 ± 3.4	6 - 8
50	53.3	5.9 ± 0.71	3.0 ± 0.58	18.0 ± 3.2	7 - 12
60	37.2	3.5 ± 0.75	2.5 ± 0.48	16.4 ± 2.5	10 - 12

4.3.3: CALLUS INDUCTION

As a member of euphorbiaceae family *R. communis* exhibits a wide level of recalcitrance in tissue culture performance particularly in its *in vitro* regeneration but the plant found well suited for induction of callus in callus induction medium. In our experiment the plant produced abundant callus formation in a number of media formulations. We used three types of explants for induction of callus viz, hypocotyl, internode and immature leaf and MMS_1 was used as basal medium. All the explants produced callus induction but their levels of efficiency varied greatly for different cultivars. In these experiments only one cytokinin BAP in five concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) was used with all possible combinations of five different concentrations (viz, 0.1, 0.2, 0.5, 0.8 and 1.0 mg/L) of three auxins viz. NAA, 2,4-D and IAA.

Callus induced was observed in all the cultivars of castor from three types of explants but hypocotyl explant showed the best performance. When hypocotyl explants was cultured in different concentrations of auxins and cytokinin, explants of all six cultivars formed callus in all combinations. Percentage of explants showing callus formation was highest (100%) in cultivar shabje, cultivar roktima and cultivar lalchay followed by cultivar badami (92.5%), cultivar shadatae (92.2%) and cultivar dhusar (91.6%). When internode explant was used for callus induction from six cultivars of castor the rate of highest frequencies of callus induction was found in cultivar shabje (95.3%) followed by cultivar roktima (94.8%), cultivar lalchay (90.4%), cultivar badami (90.3%), cultivar dhusar (88.4%) and cultivar shadatae (88.3%). When immature leaf was used as explants for callus induction, it was observed that some concentrations and combinations of auxin and cytokinin failed to produce callus. Over all performance perspectives, the highest frequencies was found in cultivar shabje (63.3%) followed by cultivar roktima (62.1%), cultivar dhusar (62.3%), cultivar badami (61.1%), cultivar shadatae (60.4%) and cultivar lalchay (60.1%). Among all the concentrations and combinations were used for callus induction, 2.0 mg/L BAP with 0.5 mg/L and 0.8 mg/L NAA was found to give best result in terms of percentage of explants showing callus formation, degrees of callus growth and lowest time taken to induce callus.

The colour and nature of callus showed variations for different cultivars. Cultivar shabje showed greenish type of callus viz. green friable, green compact, green compact nodular and whitish green. Cultivar roktima showed pinkish brown, pinkish nodular, pinkish nodular compact, brown friable, yellow friable and yellow compact. Cultivar lalchay showed pinkish brown, pinkish nodular, pinkish friable, brown friable, yellow friable and white friable. Cultivar badami and cultivar dhusar

showed more or less brownish or whitish type of callus and cultivar shadatae showed greenish type of callus. So it is obvious that the cultivars of castors found in Bangladesh also have wide range of diversity in performing callus induction in callus induction medium.

A directory of coloured photography of callus formation from different explants of six cultivars of castor in Bangladesh under different hormonal combinations are presented in individual plates in the following section.

CULTIVAR SHABJE (Hypocotyl)

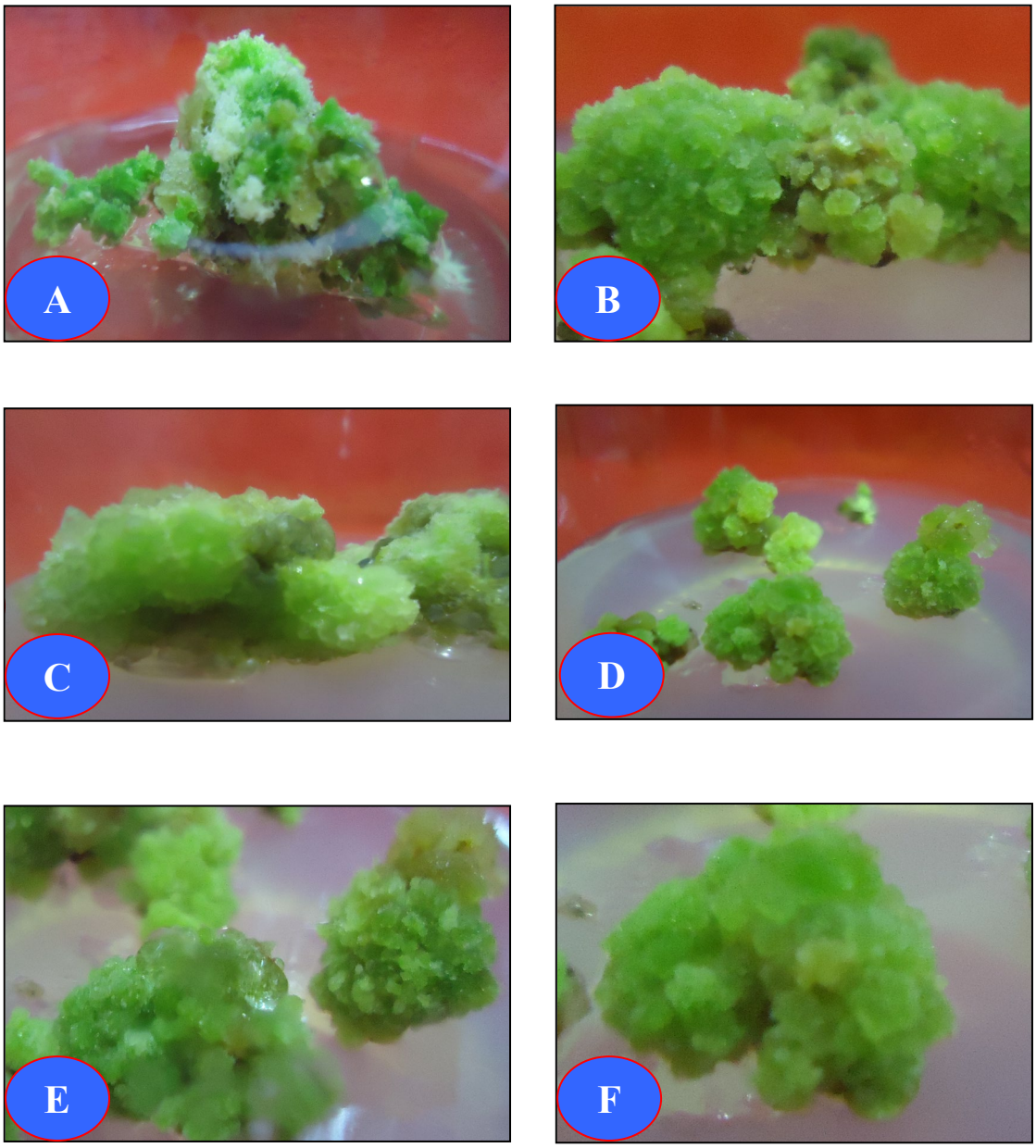


Plate- 4.3.7: Photographs showing the callus formation of cultivar shabje from hypocotyl explant in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Green friable callus in BAP 1.0 mg/L + NAA 0.2 mg/L. **B:** Green compact nodular callus in BAP 2.0 mg/L + 2,4-D 0.8 mg/L. **C:** Whitish green callus in BAP 1.5 mg/L + IAA 0.2 mg/L. **D:** Green compact callus in BAP 2.0 mg/L + NAA 0.5 mg/L. **E:** Green friable callus in BAP 1.0 mg/L + 2,4-D 1.0 mg/L. **F:** Green compact callus in BAP 2.0 mg/L + IAA 0.8 mg/L.

CULTIVAR SHABJE
(Internode and immature leaf)

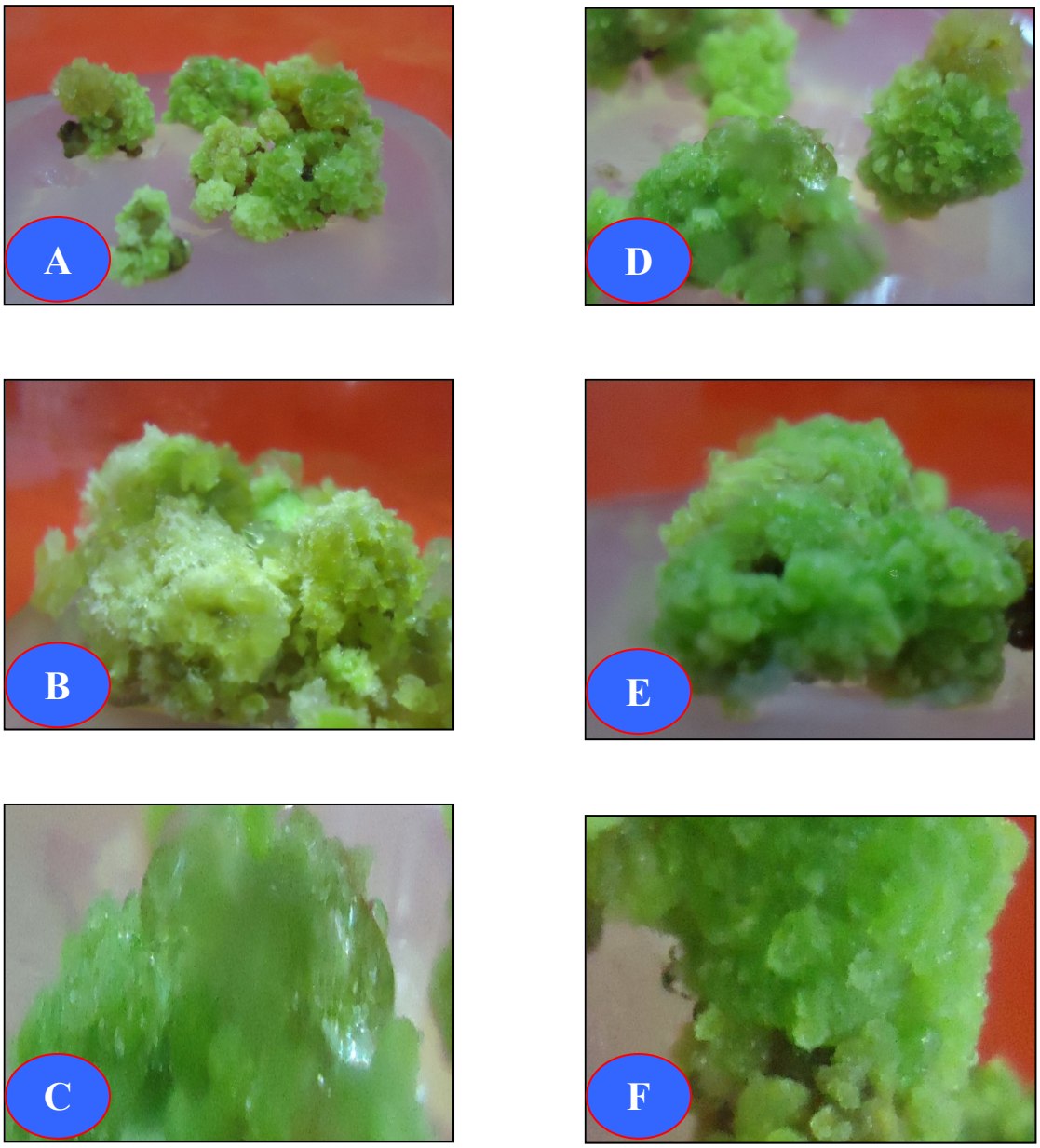


Plate- 4.3.8: Photographs showing the callus formation of cultivar shabje from internode explant (**A-C**) and immature leaf explant (**D-F**) in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Green friable callus in BAP 1.0 mg/L + NAA 0.2 mg/L. **B:** Green compact nodular callus in BAP 2.0 mg/L + 2,4-D 0.8 mg/L. **C:** Whitish green callus in BAP 1.5 mg/L + IAA 0.2 mg/L. **D:** Green compact callus in BAP 2.0 mg/L + NAA 0.5 mg/L. **E:** Green friable callus in BAP 1.0 mg/L + 2,4-D 1.0 mg/L. **F:** Green compact callus in BAP 2.0 mg/L + IAA 0.8 mg/L.

CULTIVAR SHADATAE (Hypocotyl)

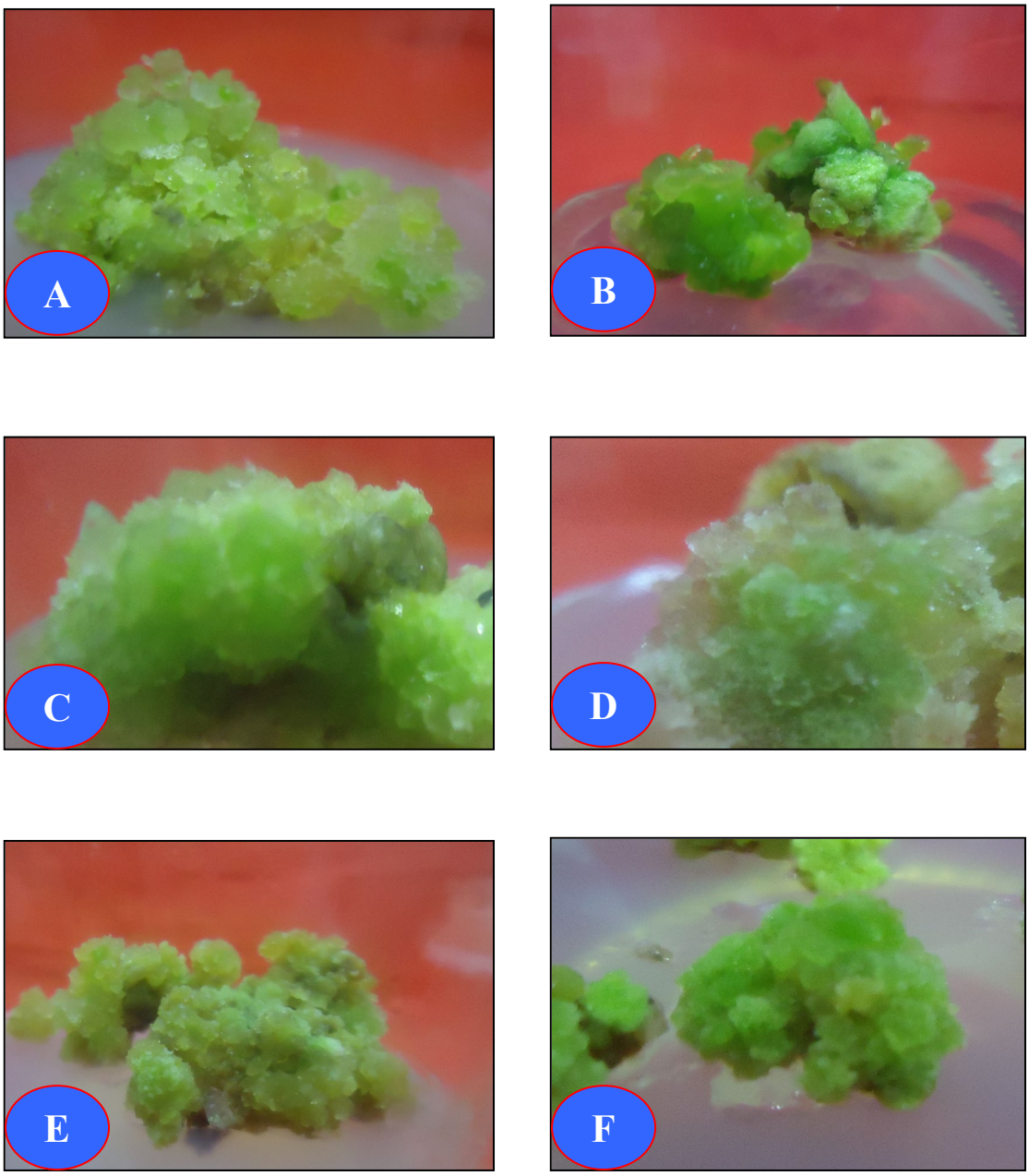


Plate- 4.3.9: Photographs showing the callus formation of cultivar shadatae from hypocotyl explant in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Whitish green callus from hypocotyl explant in BAP 2.0 mg/L + NAA 0.1 mg/L. **B:** Green compact callus from hypocotyl explant in BAP 2.0 mg/L + NAA 0.5 mg/L. **C:** Green friable callus from hypocotyl explant in BAP 2.0 mg/L + IAA 0.2 mg/L. **D:** Light green callus from internode explant in BAP 2.0 mg/L + NAA 0.2 mg/L. **E:** Green friable callus from internode explant in BAP 2.0 mg/L + 2,4-D 0.2 mg/L. **F:** Green compact callus from internode explant in BAP 2.0 mg/L + IAA 0.5 mg/L.

CULTIVAR SHADATAE
(Internode and immature leaf)

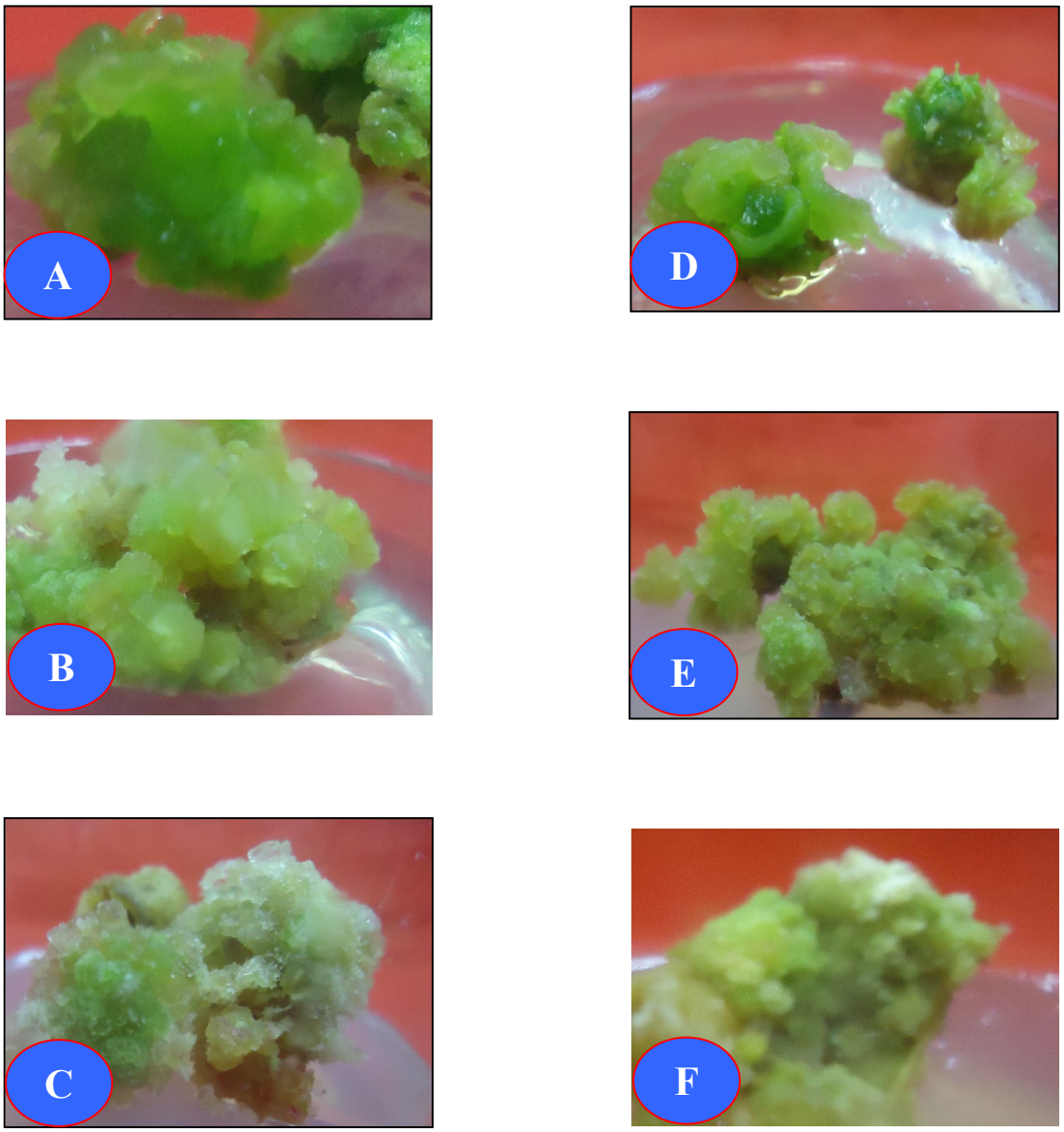


Plate- 4.3.10: Photographs showing the callus formation of cultivar shadatae from internode explant (**A-C**) and immature leaf explant (**D-F**) in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Green compact callus in BAP 2.0 mg/L + NAA 0.8 mg/L. **B:** Green friable callus in BAP 1.0 mg/L + 2,4-D 0.8 mg/L. **C:** Whitish green callus in BAP 1.5 mg/L + IAA 0.2 mg/L. **D:** Green compact callus in BAP 2.0 mg/L + NAA 0.5 mg/L. **E:** Green friable callus in BAP 1.0 mg/L + 2,4-D 1.0 mg/L. **F:** Whitish callus in BAP 2.0 mg/L + IAA 0.8 mg/L.

CULTIVAR ROKTIMA (Hypocotyl)

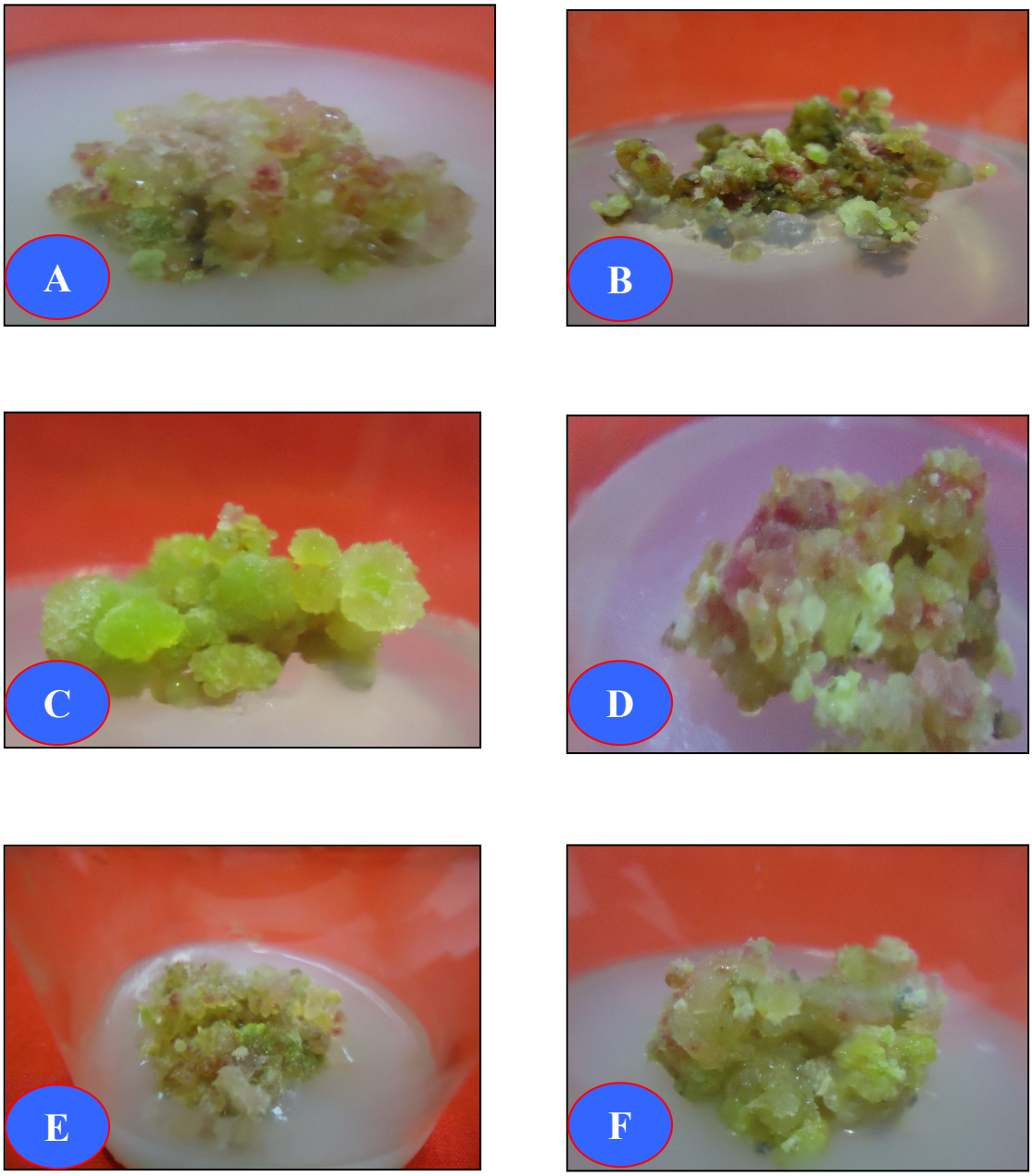


Plate- 4.3.11: Photographs showing the callus formation of cultivar roktima from hypocotyl explant in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Pinkish brown callus from hypocotyl explant in BAP 1.5 mg/L + NAA 0.8 mg/L. **B:** Pinkish nodular compact callus from hypocotyl explant in BAP 2.0 mg/L + 2,4-D 0.8 mg/L. **C:** Yellow friable callus from hypocotyl explant in BAP 1.0 mg/L + IAA 0.2 mg/L. **D:** Pinkish nodular callus from internode explant in BAP 1.5 mg/L + NAA 0.8 mg/L. **E:** Brown compact callus from internode explant in BAP 1.5 mg/L + 2,4-D 0.5 mg/L. **F:** Brown friable callus from internode explant in BAP 1.0 mg/L + IAA 1.0 mg/L.

CULTIVAR ROKTIMA
(Internode and immature leaf)

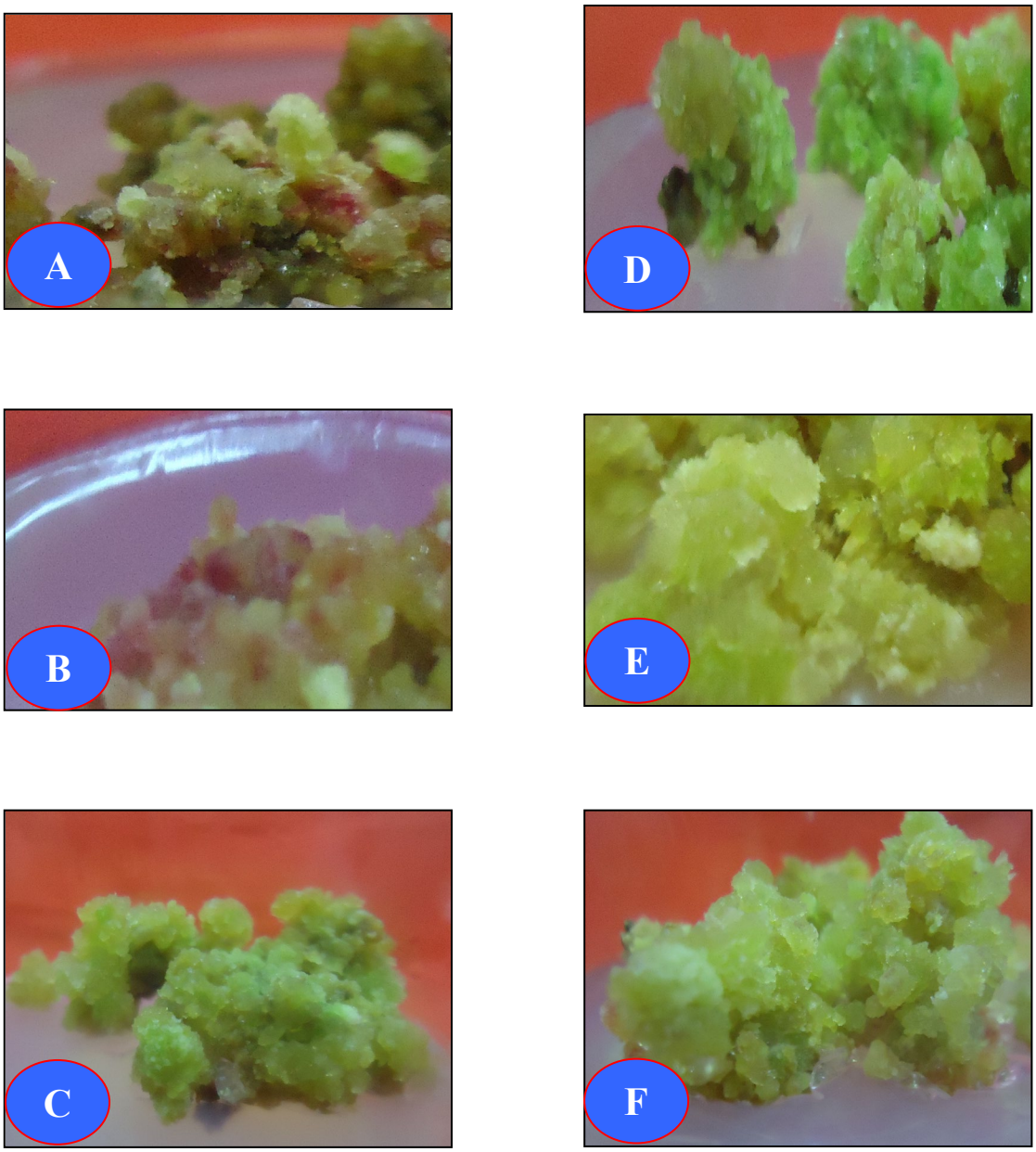


Plate- 4.3.12: Photographs showing the callus formation of cultivar roktima from internode explant (**A-C**) and immature leaf explant (**D-F**) in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Pinkish compact nodular callus in BAP 2.0 mg/L + NAA 0.2 mg/L. **B:** Pinkish compact nodular callus in BAP 2.0 mg/L + 2,4-D 0.8 mg/L. **C:** Green nodular callus in BAP 1.5 mg/L + IAA 0.2 mg/L. **D:** Green compact callus in BAP 2.0 mg/L + NAA 0.5 mg/L. **E:** Yellow friable callus in BAP 1.0 mg/L + 2,4-D 1.0 mg/L. **F:** Whitish compact callus in BAP 2.0 mg/L + IAA 0.8 mg/L.

CULTIVAR LALCHAY (Hypocotyl)

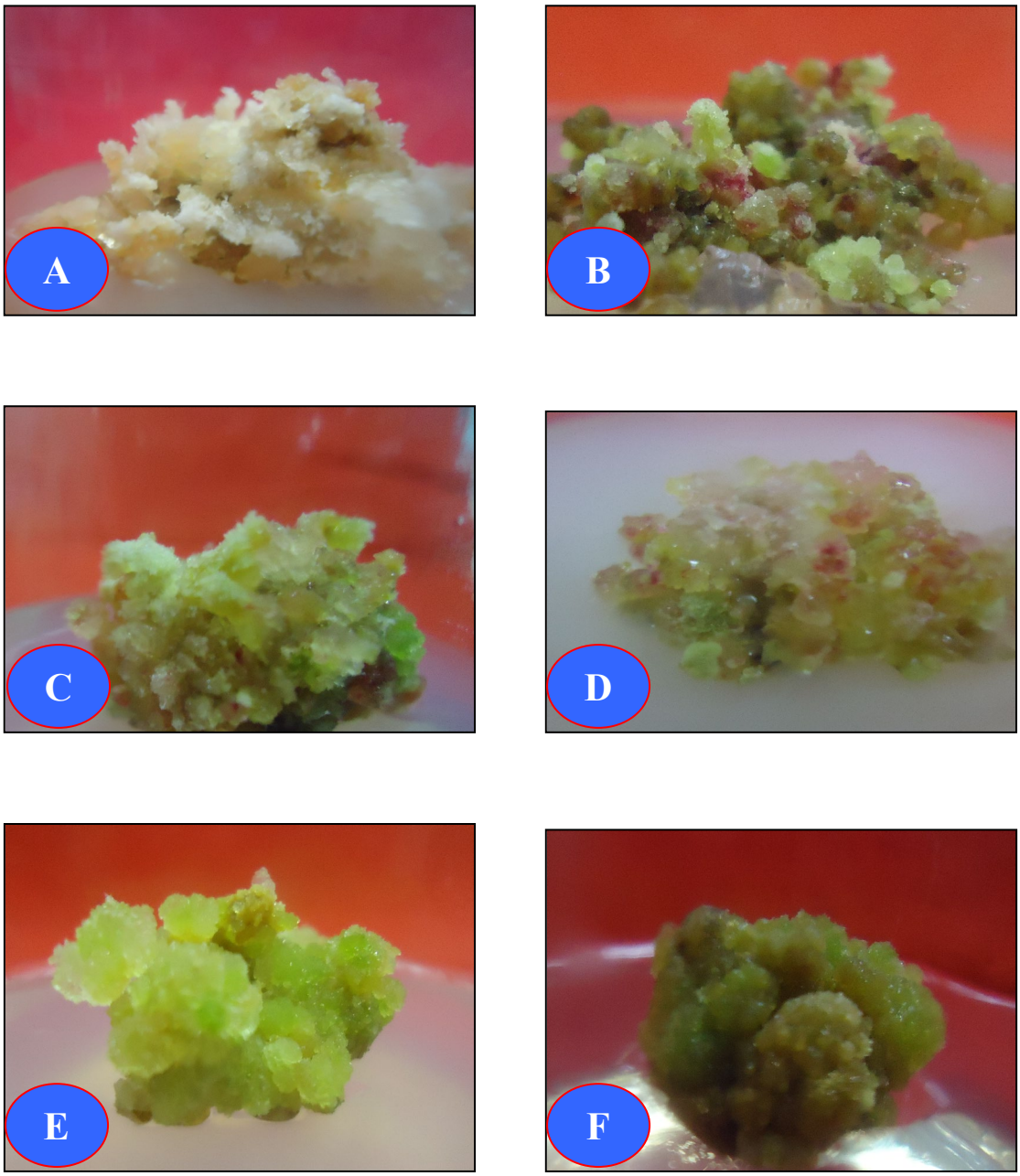


Plate- 4.3.13: Photographs showing the callus formation of cultivar lalchay from hypocotyl explant in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Whitish friable callus from hypocotyl explant in BAP 1.5 mg/L + NAA 0.1 mg/L. **B:** Pinkish nodular callus from hypocotyl explant in BAP 2.0 mg/L + NAA 0.8 mg/L. **C:** Brown compact callus from hypocotyl explant in BAP 1.5 mg/L + IAA 0.5 mg/L. **D:** Pinkish brown callus from internode explant in BAP 1.5 mg/L + NAA 0.5 mg/L. **E:** Yellow friable callus from internode explant in BAP 1.0 mg/L + 2,4-D 0.2 mg/L. **F:** Brown friable callus from internode explant in BAP 1.0 mg/L + IAA 1.0 mg/L.

CULTIVAR LALCHAY
(Internode and immature leaf)

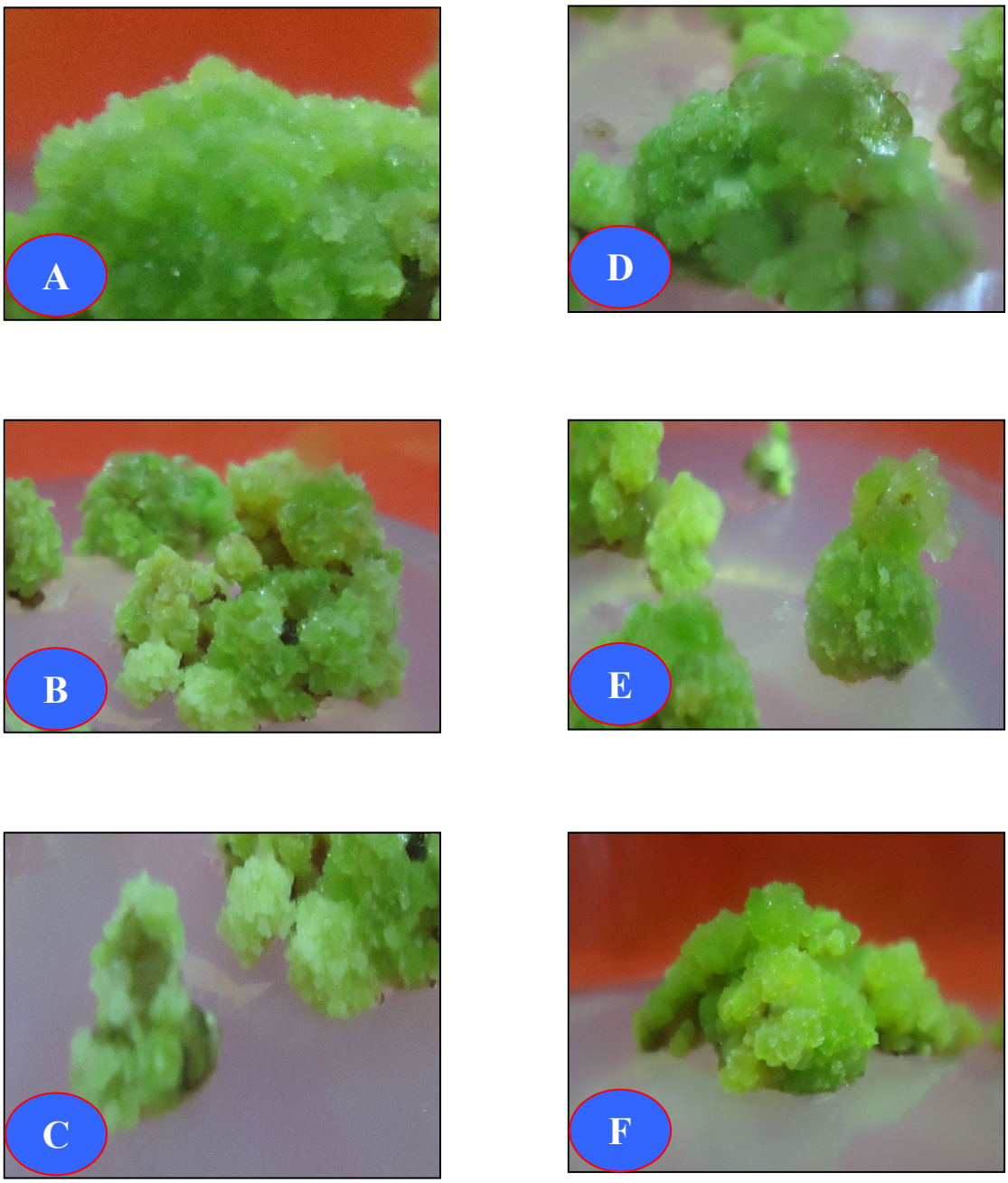


Plate- 4.3.14: Photographs showing the callus formation of cultivar lalchay from internode explant (**A-C**) and immature leaf explant (**D-F**) in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Green friable callus in BAP 1.0 mg/L + NAA 0.2 mg/L. **B:** Green compact nodular callus in BAP 2.0 mg/L + 2,4-D 0.8 mg/L. **C:** Whitish green callus in BAP 1.5 mg/L + IAA 0.2 mg/L. **D:** Green compact callus in BAP 2.0 mg/L + NAA 0.5 mg/L. **E:** Green friable callus in BAP 1.0 mg/L + 2,4-D 1.0 mg/L. **F:** Green compact callus in BAP 2.0 mg/L + IAA 0.8 mg/L.

CULTIVAR BADAMI (Hypocotyl)

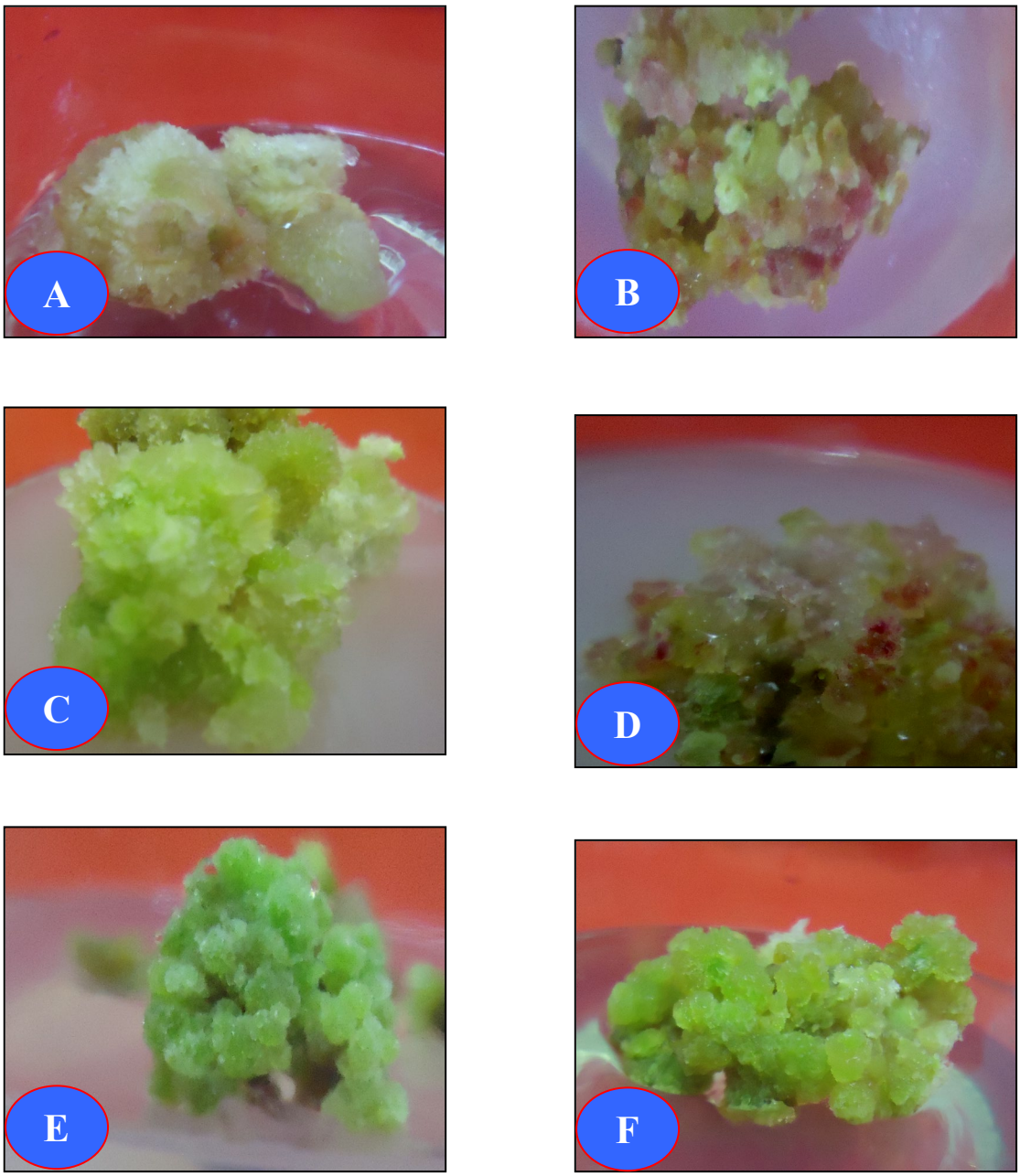


Plate- 4.3.15: Photographs showing the callus formation of cultivar badami from hypocotyl explant in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Whitish friable callus from hypocotyl explant in BAP 2.0 mg/L + NAA 0.1 mg/L. **B:** Pinkish brown callus from hypocotyl explant in BAP 2.0 mg/L + 2,4-D 0.8 mg/L. **C:** Whitish green callus from hypocotyl explant in BAP 1.0 mg/L + IAA 0.5 mg/L. **D:** Pinkish nodular callus from internode explant in BAP 2.0 mg/L + NAA 0.8 mg/L. **E:** Light green callus from internode explant in BAP 0.5 mg/L + 2,4-D 0.2 mg/L. **F:** Yellow friable callus from internode explant in BAP 3.0 mg/L + IAA 0.5 mg/L.

CULTIVAR BADAMI
(Internode and immature leaf)

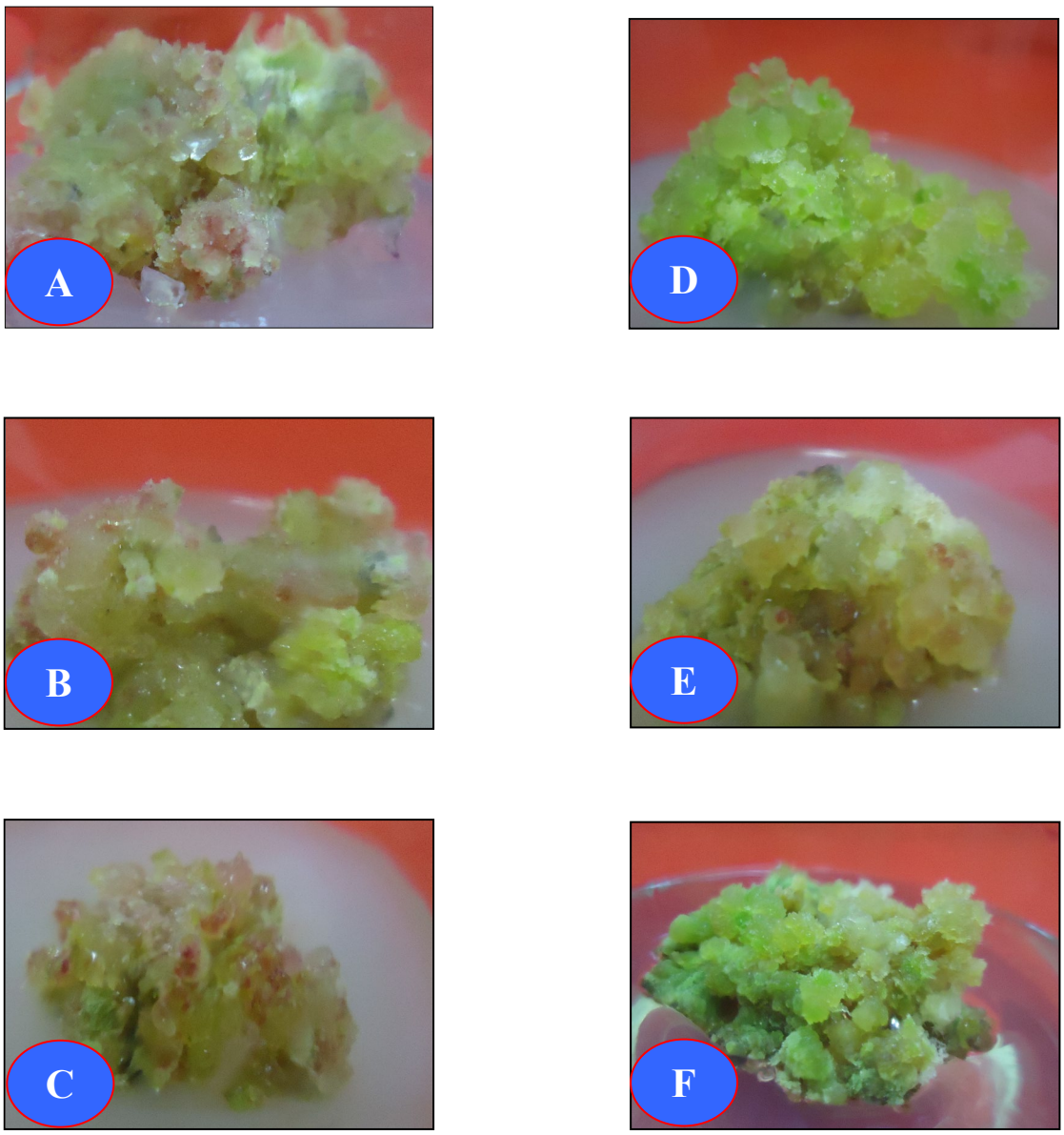


Plate- 4.3.16: Photographs showing the callus formation of cultivar badami from internode explant (**A-C**) and immature leaf explant (**D-E**) in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Pinkish white callus in BAP 1.0 mg/L + NAA 0.2 mg/L. **B:** Whitish brown callus in BAP 2.0 mg/L + 2,4-D 0.8 mg/L. **C:** Pinkish callus in BAP 1.5 mg/L + IAA 0.2 mg/L. **D:** Green friable callus in BAP 2.0 mg/L + NAA 0.5 mg/L. **E:** Whitish brown callus in BAP 1.0 mg/L + 2,4-D 1.0 mg/L. **F:** Green friable callus in BAP 2.0 mg/L + IAA 0.8 mg/L.

CULTIVAR DHUSAR (Hypocotyl)

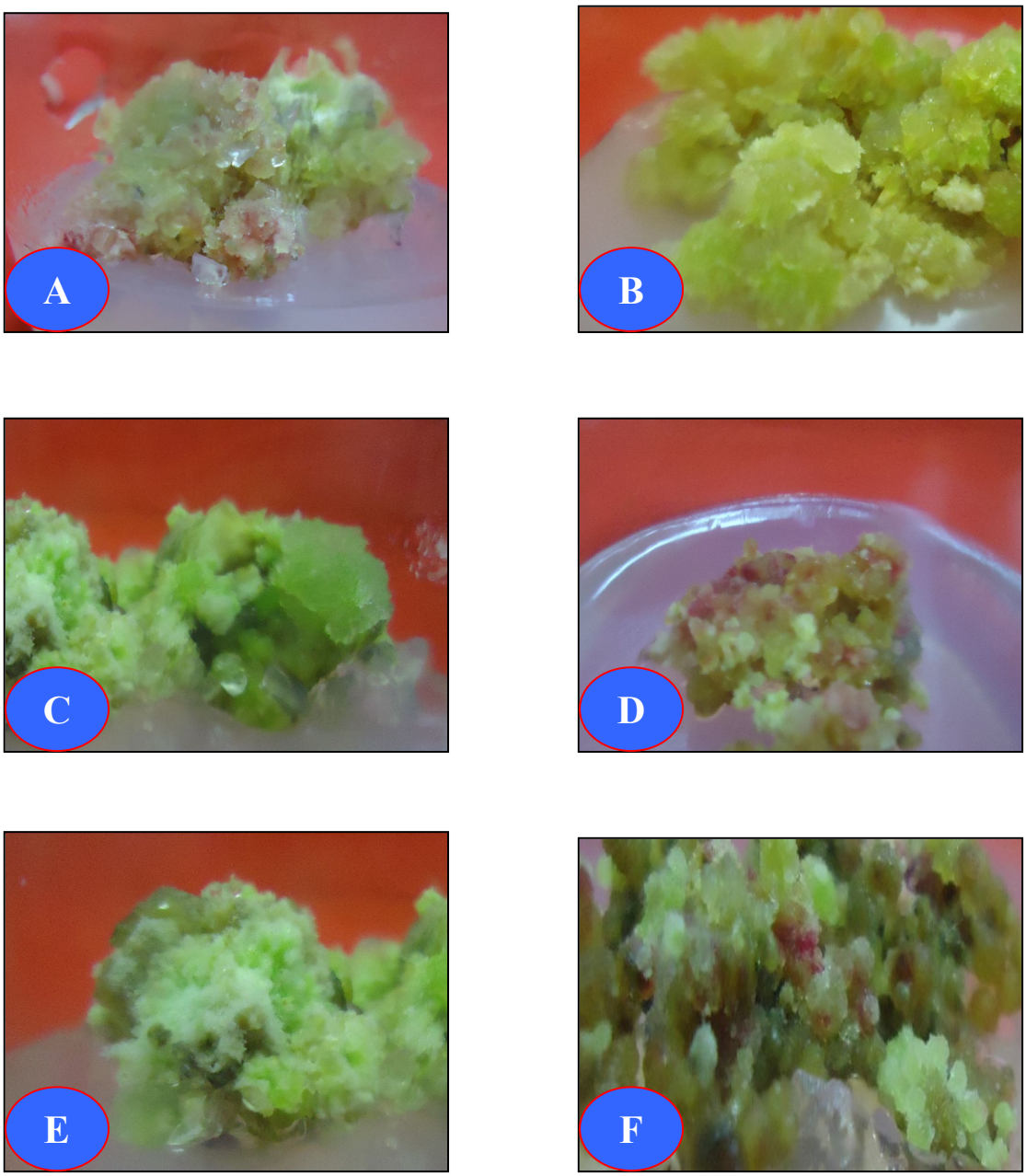


Plate- 4.3.17: Photographs showing the callus formation of cultivar dhusar from hypocotyl explant in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Whitish friable callus from hypocotyl explant in BAP 2.5 mg/L + NAA 0.1 mg/L. **B:** Whitish brown callus from hypocotyl explant in BAP 1.0 mg/L + 2,4-D 0.2 mg/L. **C:** Light green callus from hypocotyl explant in BAP 0.5 mg/L + IAA 0.1 mg/L. **D:** Pinkish nodular callus from internode explant in BAP 2.0 mg/L + NAA 0.8 mg/L. **E:** Whitish green callus from internode explant in BAP 0.5 mg/L + 2,4-D 0.8 mg/L. **F:** Pinkish friable callus from internode explant in BAP 1.0 mg/L + IAA 1.0 mg/L.

CULTIVAR DHUSAR
(Internode and immature leaf)

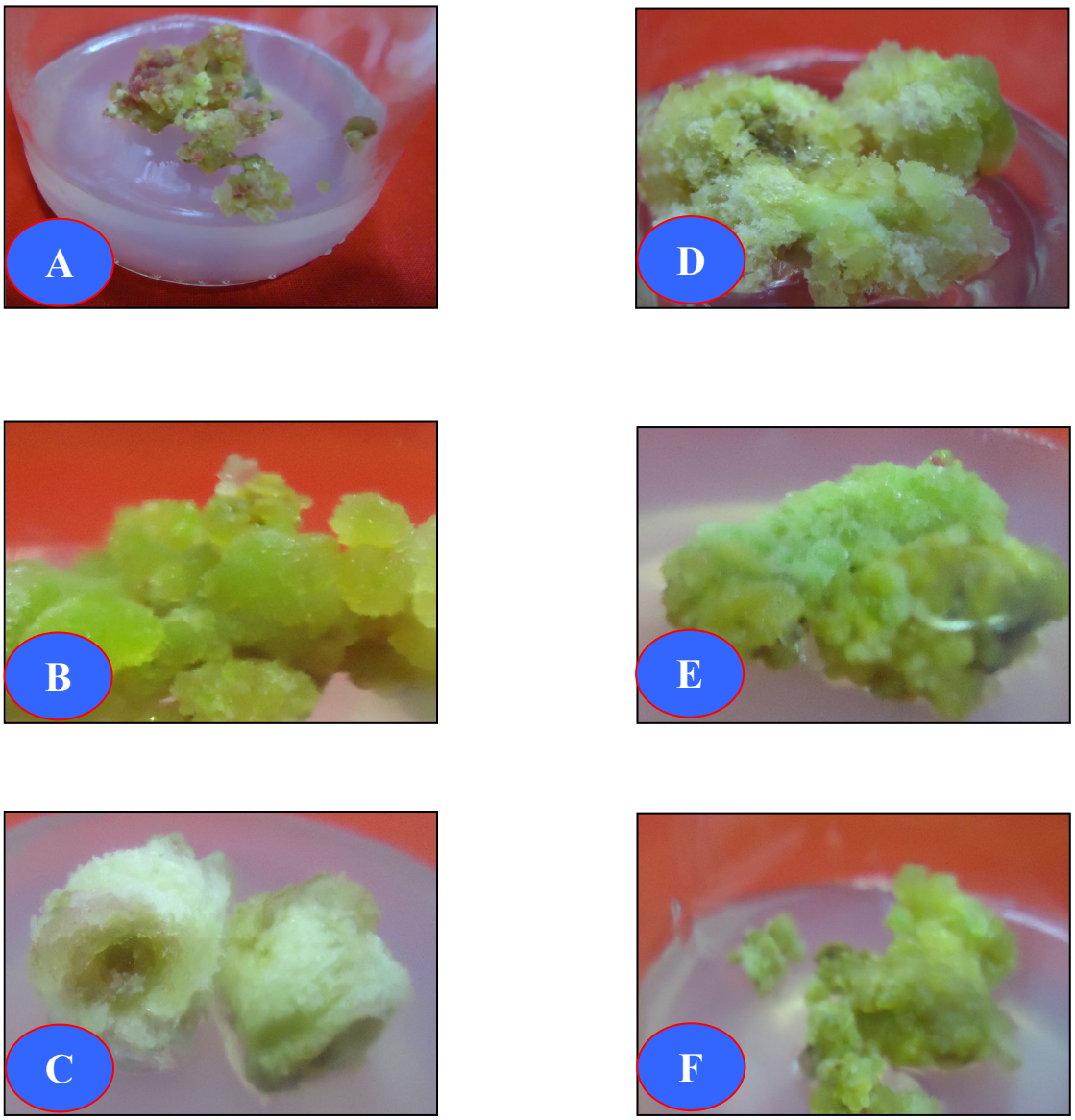


Plate- 4.3.18: Photographs showing the callus formation of cultivar dhusar from internode explant (**A-C**) and immature leaf explant (**D-E**) in MMS_1 medium. Data were recorded after 6 weeks of culture. **A:** Pinkish compact callus in BAP 1.0 mg/L + NAA 0.2 mg/L. **B:** Yellow friable callus in BAP 2.0 mg/L + 2,4-D 0.8 mg/L. **C:** Whitish callus in BAP 1.5 mg/L + IAA 0.2 mg/L. **D:** Brownish white callus in BAP 2.0 mg/L + NAA 0.5 mg/L. **E:** Green compact callus in BAP 1.0 mg/L + 2,4-D 1.0 mg/L. **F:** Greenish brown callus in BAP 2.0 mg/L + IAA 0.8 mg/L.

4.3.4: CALLUS REGENERATION.

4.3.4.1: Effect of BAP alone or in combination with NAA and GA_3 on callus regeneration from subcultured hypocotyl derived callus.

Unorganized green compact nodular calli that proliferated from the hypocotyl explants were subcultured on the MMS_1 medium supplemented with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) of BAP in all possible combinations with four different concentrations viz, 0.1, 0.2, 0.5, and 1.0 mg/L NAA and with four different concentrations viz, 0.1, 0.2, 0.5, and 1.0 mg/L GA_3 for callus regeneration. Data on percentage of explants showing shoot formation and number of shoot per explant were recorded after 8 weeks of culture. Results are presented on the basis of cultivar in separate heads.

i) Cultivar shabje: Results of this investigation are presented in the Table 4.3.28. Morphogenic responses were found to vary with growth regulators supplements. Irrespective of concentrations BAP failed to induce any regeneration of callus when used singly. Even in combined effect of high concentrations of BAP (2.5 – 3.0 mg/L), NAA (0.1 – 1.0 mg/L) and GA_3 (0.5 – 2.0 mg/L) also failed to induce any regeneration performance in callus. BAP 0.5 – 2.0 mg/L in combination with 0.1 – 1.0 mg/L NAA and 0.5 – 2.0 mg/L GA_3 concentrations showed the adventitious shoot proliferation and it was varied from 21.2% - 59.5%. The percentage of explants showing shoot formation was the highest 59.5% and number of shoot per culture was the highest 9.3 ± 1.91 in BAP 1.0 mg/L in combination with 0.2 mg/L NAA and 0.8 mg/L GA_3 (Plate 4.3.19. A).

ii) Cultivar roktima: Results of this investigation are presented in the Table 4.3.29. Irrespective of concentrations BAP failed to induce any regeneration of callus when used singly. Even in combined effect of high concentrations of BAP (2.5 – 3.0 mg/L), NAA (0.1 – 1.0 mg/L) and GA_3 (0.5 – 2.0 mg/L) also failed to induce any regeneration performance in callus. BAP 0.5 – 2.0 mg/L in combination with 0.1 – 1.0 mg/L NAA and 0.5 – 2.0 mg/L GA_3 concentrations showed the adventitious shoot proliferation and it was varied from 19.3% - 57.8%. The percentage of explants showing shoot formation was the highest 57.8% and number of shoot per culture was the highest 9.2 ± 1.82 at BAP 1.0 mg/L in combination with 0.2 mg/L NAA and 0.8 mg/L GA_3 (Plate 4.3.19. C).

iii) Cultivar lalchay: Results of this investigation are presented in the Table 4.3.30. Irrespective of concentrations BAP failed to induce any regeneration of callus when used singly. Even in combined effect of high concentrations of BAP (2.5 – 3.0 mg/L), NAA (0.1 – 1.0 mg/L) and GA_3 (0.5 – 2.0 mg/L) also failed to induce any regeneration performance in callus. BAP 0.5 – 2.0 mg/L in combination with 0.1 – 1.0

mg/L NAA and 0.5 – 2.0 mg/L GA_3 concentrations showed the adventitious shoot proliferation and it was varied from 17.0% - 52.1%. The percentage of explants showing shoot formation was the highest 52.1% and number of shoot per culture was the highest 8.8 ± 1.71 at BAP 1.0 mg/L in combination with 0.2 mg/L NAA and 0.8 mg/L GA_3 (Plate 4.3.19. E).

4.3.4.2: Effect of BAP alone or in combination with NAA and TDZ on callus regeneration from subcultured hypocotyl derived callus.

Unorganized green compact nodular calli that proliferated from the hypocotyl explants were subcultured on the MMS_1 medium supplemented with different concentrations (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) of BAP in all possible combinations with four different concentrations viz, 0.1, 0.2, 0.5, and 1.0 mg/L NAA and with four different concentrations viz, 0.1, 0.2, 0.5, and 1.0 mg/L TDZ for callus regeneration. Data on percentage of explants showing shoot formation and number of shoot per explant were recorded after 8 weeks of culture. Results are presented on the basis of cultivar in separate heads.

i) Cultivar shabje: Results of this investigation are presented in the Table 4.3.31. Morphogenic potentialities were found to vary with growth regulators supplements. Irrespective of concentrations BAP failed to induce any regeneration of callus when used singly. Even in combined effect of high concentrations of BAP (2.5 – 3.0 mg/L), NAA (0.1 – 1.0 mg/L) and TDZ (0.5 – 2.0 mg/L) also failed to induce any regeneration performance in callus. BAP 0.5 – 2.0 mg/L in combination with 0.1 – 1.0 mg/L NAA and 0.5 – 2.0 mg/L TDZ concentrations showed the adventitious shoot proliferation and it was varied from 23.9% - 63.7%. The percentage of explants showing shoot formation was the highest 63.7% and number of shoot per culture was the highest 9.5 ± 1.93 at BAP 1.0 mg/L in combination with 0.2 mg/L NAA and 0.8 mg/L TDZ (Plate 4.3.19. B).

ii) Cultivar roktima: Results of this investigation are presented in the Table 4.3.32. Irrespective of concentrations BAP failed to induce any regeneration of callus when used singly. Even in combined effect of high concentrations of BAP (2.5 – 3.0 mg/L), NAA (0.1 – 1.0 mg/L) and TDZ (0.5 – 2.0 mg/L) also failed to induce any regeneration performance in callus. BAP 0.5 – 2.0 mg/L in combination with 0.1 – 1.0 mg/L NAA and 0.5 – 2.0 mg/L TDZ concentrations showed the adventitious shoot proliferation and it was varied from 21.5% - 60.6%. The percentage of explants showing shoot formation was the highest 60.6% and number of shoot per culture was the highest 9.3 ± 1.90 at BAP 1.0 mg/L in combination with 0.2 mg/L NAA and 0.8 mg/L TDZ (Plate 4.3.19. D).

iii) Cultivar lalchay: Results of this investigation are presented in the Table 4.3.33. Morphogenic potentialities were found to vary with growth regulators supplements. Irrespective of concentrations BAP failed to induce any regeneration of callus when used singly. Even in combined effect of high concentrations of BAP (2.5 – 3.0 mg/L), NAA (0.1 – 1.0 mg/L) and TDZ (0.5 – 2.0 mg/L) also failed to induce any regeneration performance in callus. BAP 0.5 – 2.0 mg/L in combination with 0.1 – 1.0 mg/L NAA and 0.5 – 2.0 mg/L TDZ concentrations showed the adventitious shoot proliferation and it was varied from 19.6% - 58.2%. The percentage of explants showing shoot formation was the highest 58.2% and number of shoot per culture was the highest 9.1 ± 1.85 at BAP 1.0 mg/L in combination with 0.2 mg/L NAA and 0.8 mg/L TDZ (Plate 4.3.19. F).

Table 4.3.28: Effect of different concentrations and combinations of BAP, NAA and GA_3 on regeneration of adventitious shoot from callus of cultivar shabje (V_1) in MMS_1 medium. Each treatment consisted of 10-15 explants and data ($\bar{x} \pm SE$) were recorded after 8 weeks of culture.

Growth regulators mg/L			% of explants showing shoot formation	No. of shoot/culture ($\bar{x} \pm SE$)
BAP	NAA	GA_3		
0.5	-	-	-	-
0.5	0.1	0.5	38.2	5.0 ± 1.33
0.5	0.2	0.8	56.0	7.2 ± 1.81
0.5	0.5	1.0	45.3	5.6 ± 1.52
0.5	1.0	2.0	35.5	3.8 ± 0.90
1.0	-	-	-	-
1.0	0.1	0.5	41.7	5.2 ± 1.35
1.0	0.2	0.8	59.5	9.3 ± 1.91
1.0	0.5	1.0	48.2	6.4 ± 1.50
1.0	1.0	2.0	37.8	4.5 ± 1.07
1.5	-	-	-	-
1.5	0.1	0.5	32.1	4.5 ± 1.25
1.5	0.2	0.8	55.3	7.0 ± 1.51
1.5	0.5	1.0	40.4	5.1 ± 1.43
1.5	1.0	2.0	29.1	3.5 ± 0.71
2.0	-	-	-	-
2.0	0.1	0.5	25.6	3.4 ± 0.52
2.0	0.2	0.8	41.3	5.3 ± 1.23
2.0	0.5	1.0	30.5	4.2 ± 1.35
2.0	1.0	2.0	21.2	2.4 ± 0.40
2.5	-	-	-	-
2.5	0.1	0.5	-	-
2.5	0.2	0.8	-	-
2.5	0.5	1.0	-	-
2.5	1.0	2.0	-	-
3.0	-	-	-	-
3.0	0.1	0.5	-	-
3.0	0.2	0.8	-	-
3.0	0.5	1.0	-	-
3.0	1.0	2.0	-	-

Table 4.3.29: Effect of different concentrations and combinations of BAP, NAA and GA_3 on regeneration of adventitious shoot from the callus of cultivar roktima (V_3) in MMS_1 medium. Each treatment consisted of 10-15 explants and data ($\bar{x} \pm SE$) were recorded after 8 weeks of culture.

Growth regulators mg/L			% of explants showing shoot formation	No. of shoot/culture ($\bar{x} \pm SE$)
BAP	NAA	GA_3		
0.5	-	-	-	-
0.5	0.1	0.5	37.3	5.0 ± 1.31
0.5	0.2	0.8	55.1	7.1 ± 1.80
0.5	0.5	1.0	44.1	5.5 ± 1.42
0.5	1.0	2.0	33.5	3.7 ± 0.90
1.0	-	-	-	-
1.0	0.1	0.5	40.0	5.1 ± 1.33
1.0	0.2	0.8	57.8	9.2 ± 1.82
1.0	0.5	1.0	45.3	6.3 ± 1.54
1.0	1.0	2.0	36.1	4.2 ± 1.08
1.5	-	-	-	-
1.5	0.1	0.5	30.3	4.4 ± 1.21
1.5	0.2	0.8	53.4	6.8 ± 1.42
1.5	0.5	1.0	38.1	5.0 ± 1.35
1.5	1.0	2.0	27.2	3.4 ± 0.63
2.0	-	-	-	-
2.0	0.1	0.5	23.2	3.2 ± 0.31
2.0	0.2	0.8	38.9	5.1 ± 1.14
2.0	0.5	1.0	28.1	4.0 ± 1.23
2.0	1.0	2.0	19.3	2.2 ± 0.41
2.5	-	-	-	-
2.5	0.1	0.5	-	-
2.5	0.2	0.8	-	-
2.5	0.5	1.0	-	-
2.5	1.0	2.0	-	-
3.0	-	-	-	-
3.0	0.1	0.5	-	-
3.0	0.2	0.8	-	-
3.0	0.5	1.0	-	-
3.0	1.0	2.0	-	-

Table 4.3.30: Effect of different concentrations and combinations of BAP, NAA and GA_3 on regeneration of adventitious shoot from the callus of cultivar lalchay (V_4) in MMS_1 medium. Each treatment consisted of 10-15 explants and data ($\bar{x} \pm SE$) were recorded after 8 weeks of culture.

Growth regulators mg/L			% of explants showing shoot formation	No. of shoot/culture ($\bar{x} \pm SE$)
BAP	NAA	GA_3		
0.5	-	-	-	-
0.5	0.1	0.5	34.5	4.8 ± 1.11
0.5	0.2	0.8	52.0	6.6 ± 1.52
0.5	0.5	1.0	41.2	5.0 ± 1.31
0.5	1.0	2.0	30.6	3.3 ± 0.80
1.0	-	-	-	-
1.0	0.1	0.5	37.2	5.0 ± 1.35
1.0	0.2	0.8	52.1	8.8 ± 1.71
1.0	0.5	1.0	41.4	6.0 ± 1.42
1.0	1.0	2.0	32.5	3.9 ± 0.90
1.5	-	-	-	-
1.5	0.1	0.5	27.6	4.1 ± 1.22
1.5	0.2	0.8	47.8	6.2 ± 1.33
1.5	0.5	1.0	32.3	4.8 ± 1.12
1.5	1.0	2.0	21.5	3.1 ± 0.41
2.0	-	-	-	-
2.0	0.1	0.5	20.1	3.0 ± 0.30
2.0	0.2	0.8	35.2	4.8 ± 1.14
2.0	0.5	1.0	24.3	3.6 ± 1.03
2.0	1.0	2.0	17.0	2.1 ± 0.21
2.5	-	-	-	-
2.5	0.1	0.5	-	-
2.5	0.2	0.8	-	-
2.5	0.5	1.0	-	-
2.5	1.0	2.0	-	-
3.0	-	-	-	-
3.0	0.1	0.5	-	-
3.0	0.2	0.8	-	-
3.0	0.5	1.0	-	-
3.0	1.0	2.0	-	-

Table 4.3.31: Effect of different concentrations and combinations of BAP, NAA and TDZ on regeneration of adventitious shoot from callus of cultivar shabje (V_1) in MMS_1 medium. Each treatment consisted of 10-15 explants and data ($\bar{x} \pm SE$) were recorded after 8 weeks of culture.

Growth regulators mg/L			% of explants showing shoot formation	No. of shoot/culture ($\bar{x} \pm SE$)
BAP	NAA	TDZ		
0.5	-	-	-	-
0.5	0.1	0.5	41.3	5.1 ± 1.33
0.5	0.2	0.8	58.1	7.3 ± 1.80
0.5	0.5	1.0	47.5	5.7 ± 1.51
0.5	1.0	2.0	37.8	3.9 ± 0.95
1.0	-	-	-	-
1.0	0.1	0.5	44.2	5.3 ± 1.34
1.0	0.2	0.8	63.7	9.5 ± 1.93
1.0	0.5	1.0	51.0	6.5 ± 1.52
1.0	1.0	2.0	40.4	4.6 ± 1.08
1.5	-	-	-	-
1.5	0.1	0.5	35.0	4.6 ± 1.26
1.5	0.2	0.8	56.1	7.1 ± 1.52
1.5	0.5	1.0	42.5	5.1 ± 1.41
1.5	1.0	2.0	32.2	3.6 ± 0.73
2.0	-	-	-	-
2.0	0.1	0.5	27.5	3.4 ± 0.51
2.0	0.2	0.8	43.6	5.4 ± 1.25
2.0	0.5	1.0	34.1	4.3 ± 1.36
2.0	1.0	2.0	23.9	2.5 ± 0.48
2.5	-	-	-	-
2.5	0.1	0.5	-	-
2.5	0.2	0.8	-	-
2.5	0.5	1.0	-	-
2.5	1.0	2.0	-	-
3.0	-	-	-	-
3.0	0.1	0.5	-	-
3.0	0.2	0.8	-	-
3.0	0.5	1.0	-	-
3.0	1.0	2.0	-	-

Table 4.3.32: Effect of different concentrations and combinations of BAP, NAA and TDZ on regeneration of adventitious shoot from callus of cultivar roktima (V_3) in MMS_1 medium. Each treatment consisted of 10-15 explants and data ($\bar{x} \pm SE$) were recorded after 8 weeks of culture.

Growth regulators mg/L			% of explants showing shoot formation	No. of shoot/culture ($\bar{x} \pm SE$)
BAP	NAA	TDZ		
0.5	-	-	-	-
0.5	0.1	0.5	39.5	5.0 ± 1.32
0.5	0.2	0.8	57.2	7.1 ± 1.72
0.5	0.5	1.0	46.5	5.5 ± 1.50
0.5	1.0	2.0	35.7	3.7 ± 0.93
1.0	-	-	-	-
1.0	0.1	0.5	42.3	5.1 ± 1.31
1.0	0.2	0.8	60.6	9.3 ± 1.90
1.0	0.5	1.0	49.3	6.4 ± 1.54
1.0	1.0	2.0	38.2	4.5 ± 1.10
1.5	-	-	-	-
1.5	0.1	0.5	34.1	4.5 ± 1.25
1.5	0.2	0.8	54.2	7.0 ± 1.51
1.5	0.5	1.0	40.3	5.1 ± 1.42
1.5	1.0	2.0	30.5	3.5 ± 0.78
2.0	-	-	-	-
2.0	0.1	0.5	25.3	3.3 ± 0.49
2.0	0.2	0.8	41.4	5.2 ± 1.21
2.0	0.5	1.0	32.0	4.1 ± 1.35
2.0	1.0	2.0	21.5	2.4 ± 0.43
2.5	-	-	-	-
2.5	0.1	0.5	-	-
2.5	0.2	0.8	-	-
2.5	0.5	1.0	-	-
2.5	1.0	2.0	-	-
3.0	-	-	-	-
3.0	0.1	0.5	-	-
3.0	0.2	0.8	-	-
3.0	0.5	1.0	-	-
3.0	1.0	2.0	-	-

Table 4.3.33: Effect of different concentrations and combinations of BAP, NAA and TDZ on regeneration of adventitious shoot from callus of cultivar lalchay (V_4) in MMS_1 medium. Each treatment consisted of 10-15 explants and data ($\bar{x} \pm SE$) were recorded after 8 weeks of culture.

Growth regulators mg/L			% of explants showing shoot formation	No. of shoot/culture ($\bar{x} \pm SE$)
BAP	NAA	TDZ		
0.5	-	-	-	-
0.5	0.1	0.5	37.3	4.8 ± 1.31
0.5	0.2	0.8	55.1	7.0 ± 1.80
0.5	0.5	1.0	44.2	5.3 ± 1.49
0.5	1.0	2.0	33.5	3.2 ± 0.91
1.0	-	-	-	-
1.0	0.1	0.5	40.1	5.0 ± 1.29
1.0	0.2	0.8	58.2	9.1 ± 1.85
1.0	0.5	1.0	47.3	6.2 ± 1.51
1.0	1.0	2.0	35.9	4.2 ± 1.07
1.5	-	-	-	-
1.5	0.1	0.5	32.0	4.4 ± 1.26
1.5	0.2	0.8	52.1	6.8 ± 1.49
1.5	0.5	1.0	38.5	4.9 ± 1.39
1.5	1.0	2.0	28.3	3.3 ± 0.79
2.0	-	-	-	-
2.0	0.1	0.5	23.2	3.1 ± 0.42
2.0	0.2	0.8	39.3	5.1 ± 1.19
2.0	0.5	1.0	30.1	4.0 ± 1.33
2.0	1.0	2.0	19.6	2.3 ± 0.31
2.5	-	-	-	-
2.5	0.1	0.5	-	-
2.5	0.2	0.8	-	-
2.5	0.5	1.0	-	-
2.5	1.0	2.0	-	-
3.0	-	-	-	-
3.0	0.1	0.5	-	-
3.0	0.2	0.8	-	-
3.0	0.5	1.0	-	-
3.0	1.0	2.0	-	-

CALLUS REGENERATION

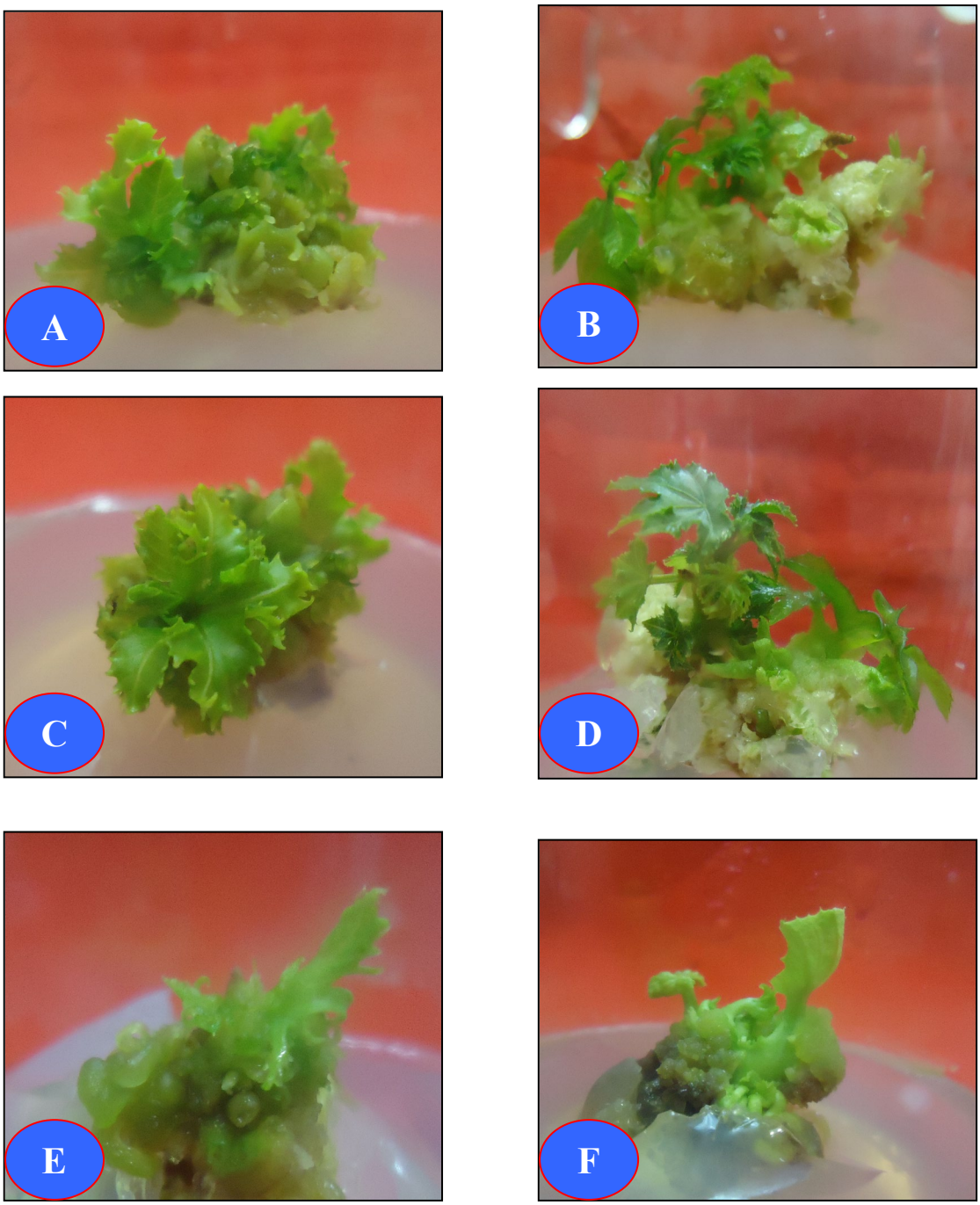


Plate- 4.3.19: Photographs showing the adventitious shoot formation from the callus of hypocotyl explants after 8 weeks of culture. **A:** Cultivar shabje in MMS_1 + BAP 1.0 mg/L + NAA 0.2 mg/L + GA_3 0.8 mg/L. **B:** Cultivar shabje in MMS_1 + BAP 1.0 mg/L + NAA 0.2 mg/L + TDZ 0.8 mg/L. **C:** Cultivar roktima in MMS_1 + BAP 1.0 mg/L + NAA 0.2 mg/L + GA_3 0.8 mg/L. **D:** Cultivar roktima in MMS_1 + BAP 1.0 mg/L + NAA 0.2 mg/L + TDZ 0.8 mg/L. **E:** Cultivar lalchay in MMS_1 + BAP 1.0 mg/L + NAA 0.2 mg/L + GA_3 0.8 mg/L. **F:** Cultivar lalchay in MMS_1 + BAP 1.0 mg/L + NAA 0.2 mg/L + TDZ 0.8 mg/L.

4.3.5: ROOTING OF MICROCUTTINGS

Axillary shoots that proliferated from different explants under different treatments attained to height 3-5 cm after 6-8 weeks of culture from the date of establishment of the explant under *in vitro* condition. Different experiments were conducted for rooting of the microcuttings prepared from these shoots. Results of these experiments are discussed below under different heads on the basis of types and objectives of the rooting experiments.

4.3.5.1: Selection of rooting medium and auxin type.

The experiment was designed with two concentrations (0.2, 0.4 mg/L) of IBA, IAA and NAA with singly in full strength MS and three modification of each of basal medium viz. MMS_1 (½ strength of major salts and full strength of minor salts and full organic), MMS_2 (½ strength of major salts and ½ strength of minor salts and full organic), and MMS_3 (¼ strength of major and minor salts and full organic.) for selecting suitable rooting medium and auxin type. All rooting media were supplemented with 30 gm/L sucrose, gelled with 3 gm/L phytagar and adjusted at pH 5.7 ± 0.1 before autoclaving. The experiments were carried out for four weeks of incubation under 16h photoperiod at 26 ± 1ºC provided by warm white fluorescent tubes with a light intensity of about 2000 lux. The data on rooting percentage, number of roots, root length (mm) and shoot length (mm) were recorded. Results are presented on the basis of cultivar in separate heads.

i) Cultivar shabje: The results of this investigation are presented in the Table 4.3.34. Among the three auxins and four types of MS and modified MS media were used, the lowest rooting was obtained in NAA whereas highest rooting was obtained in IBA at the concentrations tested. Rooting frequency increased gradually from 12.0% to 85.9%. The IAA also produced rooting but was not remarkable. Percentage of rooting was the highest 85.9%, number of roots was the highest 4.7 ± 0.52, root length was the highest 31.5 ± 3.2 mm, shoot length was the highest 27.4 ± 3.1 mm observed in MMS_2 medium in 0.2 mg/L IBA. From the overall observation it was clear that MMS_2 medium was the best among the all medium tested and IBA was the best auxin among the all auxin tested on adventitious root formation.

ii) Cultivar shadatae: The results of this investigation are presented in the Table 4.3.35. Among the three auxins and four types of MS and modified MS media were used, the lowest rooting was obtained in NAA whereas highest rooting was obtained in IBA at the concentrations tested. Rooting frequency increased gradually from 11.0% to 65.4%. The IAA were also produced rooting but was not remarkable. Percentage of rooting was the highest 75.7%, number of roots was the highest 4.0 ±

0.35, root length was the highest 26.5 ± 2.9 mm, shoot length was the highest 22.4 ± 2.7 mm observed in MMS_2 medium in 0.2 mg/L IBA. From the overall observation it was clear that MMS_2 medium was the best among the all medium tested and IBA was the best auxin among the all auxins tested on adventitious root formation.

iii) Cultivar roktima: The results of this investigation are presented in the Table 4.3.36. Among the three auxins and four types of MS and modified MS media were used, the lowest rooting was obtained in NAA whereas highest rooting was obtained in IBA. Rooting frequency increased gradually from 12.1% to 83.6%. The IAA was also produced rooting but was not remarkable. Percentage of rooting was the highest 83.6%, number of roots was the highest 4.5 ± 0.49, root length was the highest 30.5 ± 3.2 mm, shoot length was the highest 26.4 ± 3.2 mm observed in MMS_2 medium in 0.2 mg/L IBA. From the overall observation it was clear that MMS_2 medium was the best among the all medium tested and IBA was the best auxin among the all auxin tested on *in vitro* root formation.

iv) Cultivar lalchay: The results of this investigation are presented in the Table 4.3.37. Among the three auxins and four types of MS and modified MS media were used, the lowest rooting was obtained in NAA whereas highest rooting was obtained in IBA at the concentrations tested. Rooting frequency increased gradually from 12.0% to 82.7%. The IAA was also produced rooting but was not remarkable. Percentage of rooting was the highest 82.7%, number of roots was the highest 4.4 ± 0.48, root length was the highest 29.9 ± 3.2 mm, shoot length was the highest 25.5 ± 3.1 mm observed in MMS_2 medium in 0.2 mg/L IBA. From the overall observation it was clear that MMS_2 medium was the best among the all medium tested and IBA was the best auxin among the all auxin tested on adventitious root formation.

v) Cultivar badami: The results of this investigation are presented in the Table 4.3.38. Among the three auxins and four types of MS and modified MS media were used, the lowest rooting was obtained in NAA whereas highest rooting was obtained in IBA at the concentrations tested. Rooting frequency increased gradually from 11.1% to 79.8%. The IAA was also produced rooting but was not remarkable. Percentage of rooting was the highest 79.8%, root number was the highest 4.2 ± 0.38, root length was the highest 27.6 ± 3.0 mm, shoot length was the highest 23.7 ± 2.5 mm observed in MMS_2 medium in 0.2 mg/L IBA. From the overall observation it was clear that MMS_2 medium was the best among the all medium tested and IBA was the best auxin among the all auxin tested on adventitious root formation.

vi) Cultivar dhusar: The results of this investigation are presented in the Table 4.3.39. Among the three auxins and four types of MS and modified MS media were used, the lowest rooting was obtained in NAA whereas highest rooting was obtained

in IBA at the concentrations tested. Rooting frequency increased gradually from 11.0% to 77.5%. The IAA was also produced rooting but was not remarkable. Percentage rooting was the highest 77.5%, root number was the highest 4.1 ± 0.37, root length was the highest 26.9 ± 2.7 mm, shoot length was the highest 22.5 ± 2.5 mm observed in MMS_2 medium in 0.2 mg/L IBA. From the overall observation it was clear that MMS_2 medium was the best among the all medium tested and IBA was the best auxin among the all auxin tested on adventitious root formation.

Table 4.3.34: Effect of different modified MS media and auxin combination on adventitious root formation in cultivar shabje (V_1). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Strength of MS salts	Growth regulators (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Shoot length (mm) ($\bar{x} \pm SE$)
MS	0.2 IBA	35.2	1.9 ± 0.18	27.3 ± 3.0	20.8 ± 1.7
	0.4 IBA	29.3	1.5 ± 0.15	22.8 ± 2.5	16.5 ± 1.6
	0.2 IAA	15.5	1.2 ± 0.09	19.6 ± 1.8	14.6 ± 1.5
	0.4 IAA	14.7	1.1 ± 0.08	18.4 ± 1.5	13.3 ± 1.5
	0.2 NAA	17.9	1.5 ± 0.12	21.2 ± 2.1	12.3 ± 1.4
	0.4 NAA	14.2	1.4 ± 0.11	20.0 ± 2.0	11.2 ± 1.3
MMS_1	0.2 IBA	74.3	4.0 ± 0.58	29.4 ± 3.1	24.9 ± 2.9
	0.4 IBA	68.7	3.6 ± 0.51	25.5 ± 2.9	20.7 ± 2.5
	0.2 IAA	27.8	2.5 ± 0.27	21.7 ± 2.1	16.6 ± 1.6
	0.4 IAA	25.4	2.2 ± 0.25	20.3 ± 2.0	14.3 ± 1.5
	0.2 NAA	41.3	3.2 ± 0.21	23.5 ± 2.6	13.5 ± 1.4
	0.4 NAA	38.6	3.0 ± 0.27	22.2 ± 2.5	12.5 ± 1.2
MMS_2	0.2 IBA	85.9	4.7 ± 0.52	31.5 ± 3.2	27.4 ± 3.1
	0.4 IBA	78.5	3.8 ± 0.41	26.7 ± 3.0	22.3 ± 1.6
	0.2 IAA	34.7	2.5 ± 0.34	24.4 ± 2.8	18.4 ± 1.8
	0.4 IAA	26.2	2.2 ± 0.21	23.3 ± 2.5	16.5 ± 1.7
	0.2 NAA	52.7	3.5 ± 0.25	22.4 ± 2.4	14.4 ± 1.6
	0.4 NAA	44.3	3.2 ± 0.30	21.3 ± 2.2	13.2 ± 1.5
MMS_3	0.2 IBA	32.5	2.9 ± 0.21	22.3 ± 2.3	16.4 ± 1.5
	0.4 IBA	27.0	2.3 ± 0.17	20.8 ± 2.0	14.5 ± 1.4
	0.2 IAA	14.7	1.3 ± 0.12	18.3 ± 1.5	15.3 ± 1.4
	0.4 IAA	13.9	1.2 ± 0.11	17.2 ± 1.4	13.5 ± 1.4
	0.2 NAA	16.8	1.7 ± 0.16	14.2 ± 1.3	12.5 ± 1.3
	0.4 NAA	12.0	1.4 ± 0.15	12.1 ± 1.2	11.2 ± 1.1

MS = Full strength.

MMS_1 = ½ strength of major salts and full strength of minor salts and full organic.

MMS_2 =½ strength of major salts and ½ strength of minor salts and full organic.

MMS_3 =¼ strength of major and minor salts and full organic.

Table 4.3.35: Effect of different modified MS media and auxin combination on adventitious root formation in cultivar shadatae (V_2). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Strength of MS salts	Growth regulators (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Shoot length (mm) ($\bar{x} \pm SE$)
MS	0.2 IBA	30.0	1.3 ± 0.16	22.7 ± 2.5	16.5 ± 1.9
	0.4 IBA	24.1	1.2 ± 0.17	19.5 ± 2.0	15.6 ± 1.7
	0.2 IAA	12.2	1.2 ± 0.14	17.6 ± 1.8	12.4 ± 1.4
	0.4 IAA	11.4	1.1 ± 0.09	16.4 ± 1.5	11.7 ± 1.3
	0.2 NAA	14.0	1.3 ± 0.21	15.3 ± 1.4	12.5 ± 1.4
	0.4 NAA	12.8	1.2 ± 0.24	14.4 ± 1.3	10.4 ± 1.2
MMS_1	0.2 IBA	65.4	3.3 ± 0.38	24.6 ± 2.6	19.3 ± 2.3
	0.4 IBA	61.3	3.1 ± 0.36	20.7 ± 2.1	17.6 ± 1.9
	0.2 IAA	20.1	2.1 ± 0.31	18.5 ± 1.9	14.4 ± 1.5
	0.4 IAA	22.5	2.0 ± 0.29	17.4 ± 1.8	13.3 ± 1.4
	0.2 NAA	36.6	2.7 ± 0.27	16.6 ± 1.5	13.5 ± 1.4
	0.4 NAA	32.0	2.5 ± 0.23	15.5 ± 1.4	12.2 ± 1.2
MMS_2	0.2 IBA	75.7	4.0 ± 0.35	26.5 ± 2.9	22.4 ± 2.7
	0.4 IBA	66.3	3.1 ± 0.28	21.3 ± 2.3	19.4 ± 2.2
	0.2 IAA	30.0	2.6 ± 0.20	19.5 ± 1.9	16.3 ± 1.6
	0.4 IAA	22.8	2.1 ± 0.12	18.4 ± 1.8	15.3 ± 1.5
	0.2 NAA	45.1	3.0 ± 0.27	17.3 ± 1.6	14.2 ± 1.4
	0.4 NAA	38.2	2.8 ± 0.21	16.2 ± 1.4	13.0 ± 1.3
MMS_3	0.2 IBA	25.7	2.3 ± 0.16	18.3 ± 1.8	14.5 ± 1.7
	0.4 IBA	23.0	2.0 ± 0.18	16.4 ± 1.5	13.4 ± 1.5
	0.2 IAA	13.1	1.3 ± 0.17	14.9 ± 1.3	12.3 ± 1.3
	0.4 IAA	12.0	1.2 ± 0.13	12.5 ± 1.1	11.5 ± 1.2
	0.2 NAA	13.8	1.3 ± 0.13	11.4 ± 1.0	11.3 ± 1.2
	0.4 NAA	11.0	1.2 ± 0.09	10.6 ± 0.9	10.2 ± 1.1

MS = Full strength.

MMS_1 = ½ strength of major salts and full strength of minor salts and full organic.

MMS_2 =½ strength of major salts and ½ strength of minor salts and full organic.

MMS_3 =¼ strength of major and minor salts and full organic.

Table 4.3.36: Effect of different modified MS media and auxin combination on adventitious root formation in cultivar roktima (V_3). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Strength of MS salts	Growth regulators (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Shoot length (mm) ($\bar{x} \pm SE$)
MS	0.2 IBA	34.3	1.7 ± 0.21	26.6 ± 2.9	20.7 ± 1.8
	0.4 IBA	28.4	1.4 ± 0.16	22.3 ± 2.3	16.5 ± 1.7
	0.2 IAA	14.7	1.2 ± 0.10	21.4 ± 2.1	14.5 ± 1.5
	0.4 IAA	13.8	1.1 ± 0.09	20.2 ± 2.0	13.4 ± 1.4
	0.2 NAA	16.9	1.4 ± 0.16	19.4 ± 1.9	12.2 ± 1.3
	0.4 NAA	14.5	1.3 ± 0.15	18.1 ± 1.8	11.0 ± 1.2
MMS_1	0.2 IBA	73.1	3.8 ± 0.57	28.6 ± 3.1	23.5 ± 2.2
	0.4 IBA	67.8	3.5 ± 0.53	24.4 ± 2.6	19.6 ± 1.7
	0.2 IAA	24.7	2.4 ± 0.28	22.2 ± 2.3	16.3 ± 1.7
	0.4 IAA	26.5	2.1 ± 0.25	21.0 ± 2.2	14.3 ± 1.5
	0.2 NAA	41.0	3.1 ± 0.15	20.1 ± 2.0	13.2 ± 1.3
	0.4 NAA	37.9	2.9 ± 0.21	19.0 ± 1.8	12.1 ± 1.2
MMS_2	0.2 IBA	83.6	4.5 ± 0.49	30.5 ± 3.2	26.4 ± 3.2
	0.4 IBA	75.1	3.6 ± 0.42	25.6 ± 2.6	21.5 ± 1.9
	0.2 IAA	33.3	2.4 ± 0.35	23.7 ± 2.2	18.4 ± 1.6
	0.4 IAA	25.4	2.1 ± 0.23	22.1 ± 2.1	16.3 ± 1.5
	0.2 NAA	50.6	3.3 ± 0.28	21.1 ± 2.1	14.2 ± 1.4
	0.4 NAA	43.5	3.1 ± 0.27	20.0 ± 2.0	13.0 ± 1.3
MMS_3	0.2 IBA	31.3	2.8 ± 0.22	22.7 ± 2.4	16.7 ± 1.7
	0.4 IBA	26.1	2.7 ± 0.18	20.5 ± 2.1	15.6 ± 1.6
	0.2 IAA	14.3	1.3 ± 0.12	18.4 ± 1.9	14.3 ± 1.4
	0.4 IAA	13.7	1.2 ± 0.12	16.4 ± 1.6	12.4 ± 1.3
	0.2 NAA	16.5	1.7 ± 0.21	14.3 ± 1.5	12.1 ± 1.4
	0.4 NAA	12.1	1.4 ± 0.19	13.1 ± 1.4	11.0 ± 1.2

MS = Full strength.

MMS_1 = ½ strength of major salts and full strength of minor salts and full organic.

MMS_2 =½ strength of major salts and ½ strength of minor salts and full organic.

MMS_3 =¼ strength of major and minor salts and full organic.

Table 4.3.37: Effect of different modified MS media and auxin combination on adventitious root formation in cultivar lalchay (V_4). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Strength of MS salts	Growth regulators (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Shoot length (mm) ($\bar{x} \pm SE$)
MS	0.2 IBA	33.5	1.7 ± 0.18	25.6 ± 2.5	19.6 ± 2.0
	0.4 IBA	27.3	1.3 ± 0.20	21.3 ± 2.1	15.5 ± 1.8
	0.2 IAA	13.8	1.3 ± 0.12	20.4 ± 2.1	13.3 ± 1.5
	0.4 IAA	12.9	1.2 ± 0.11	19.5 ± 1.9	12.4 ± 1.3
	0.2 NAA	15.8	1.4 ± 0.10	18.4 ± 1.8	12.1 ± 1.4
	0.4 NAA	14.2	1.3 ± 0.19	17.0 ± 1.8	11.0 ± 1.2
MMS_1	0.2 IBA	71.3	3.7 ± 0.52	27.7 ± 3.0	22.7 ± 2.1
	0.4 IBA	66.1	3.5 ± 0.50	23.5 ± 2.4	18.5 ± 2.0
	0.2 IAA	25.6	2.3 ± 0.25	21.5 ± 2.1	16.6 ± 1.9
	0.4 IAA	23.4	2.1 ± 0.29	20.3 ± 2.0	14.3 ± 1.6
	0.2 NAA	40.3	3.0 ± 0.27	19.2 ± 1.9	13.1 ± 1.5
	0.4 NAA	36.5	2.8 ± 0.24	18.1 ± 1.8	12.2 ± 1.4
MMS_2	0.2 IBA	82.7	4.4 ± 0.48	29.9 ± 3.2	25.5 ± 3.1
	0.4 IBA	74.0	3.8 ± 0.43	24.5 ± 2.5	21.3 ± 2.1
	0.2 IAA	32.1	2.8 ± 0.34	22.7 ± 2.1	18.4 ± 1.9
	0.4 IAA	25.5	2.5 ± 0.23	21.4 ± 2.2	16.4 ± 1.6
	0.2 NAA	49.2	3.2 ± 0.29	20.5 ± 2.0	14.3 ± 1.5
	0.4 NAA	41.6	3.0 ± 0.26	19.2 ± 1.9	13.2 ± 1.5
MMS_3	0.2 IBA	30.2	2.7 ± 0.23	21.8 ± 2.1	16.5 ± 1.7
	0.4 IBA	25.4	2.3 ± 0.19	19.7 ± 1.9	15.4 ± 1.6
	0.2 IAA	14.2	1.4 ± 0.13	17.4 ± 1.6	14.5 ± 1.5
	0.4 IAA	13.5	1.3 ± 0.12	15.3 ± 1.5	13.3 ± 1.4
	0.2 NAA	15.6	1.6 ± 0.23	13.3 ± 1.2	12.0 ± 1.4
	0.4 NAA	12.0	1.4 ± 0.20	12.1 ± 1.1	11.2 ± 1.3

MS = Full strength.

MMS_1 = ½ strength of major salts and full strength of minor salts and full organic.

MMS_2 =½ strength of major salts and ½ strength of minor salts and full organic.

MMS_3 =¼ strength of major and minor salts and full organic.

Table 4.3.38 : Effect of different modified MS media and auxin combination on adventitious root formation in cultivar badami (V_5). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Strength of MS salts	Growth regulators (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Shoot length (mm) ($\bar{x} \pm SE$)
MS	0.2 IBA	31.6	1.5 ± 0.16	22.8 ± 2.3	17.6 ± 1.5
	0.4 IBA	26.0	1.3 ± 0.12	19.9 ± 2.0	15.6 ± 1.4
	0.2 IAA	12.2	1.2 ± 0.10	18.5 ± 1.9	11.7 ± 1.2
	0.4 IAA	11.8	1.1 ± 0.09	17.4 ± 1.8	10.2 ± 1.2
	0.2 NAA	14.5	1.3 ± 0.17	16.3 ± 1.5	12.3 ± 1.3
	0.4 NAA	13.3	1.2 ± 0.21	15.2 ± 1.4	11.0 ± 1.2
MMS_1	0.2 IBA	69.1	3.5 ± 0.49	24.9 ± 2.3	20.9 ± 2.0
	0.4 IBA	64.3	3.3 ± 0.41	21.4 ± 2.1	17.6 ± 1.5
	0.2 IAA	23.4	2.2 ± 0.27	19.3 ± 1.9	12.5 ± 1.4
	0.4 IAA	21.2	2.0 ± 0.25	18.2 ± 1.8	11.4 ± 1.3
	0.2 NAA	39.6	2.9 ± 0.31	17.2 ± 1.6	15.5 ± 1.5
	0.4 NAA	33.7	2.7 ± 0.26	16.1 ± 1.5	13.3 ± 1.4
MMS_2	0.2 IBA	79.8	4.2 ± 0.38	27.6 ± 3.0	23.7 ± 2.5
	0.4 IBA	71.5	3.6 ± 0.32	22.7 ± 2.2	20.5 ± 2.1
	0.2 IAA	31.0	2.3 ± 0.28	20.5 ± 2.0	13.6 ± 1.4
	0.4 IAA	24.2	2.1 ± 0.25	19.5 ± 1.9	12.4 ± 1.3
	0.2 NAA	47.3	3.2 ± 0.29	18.4 ± 1.8	17.5 ± 1.6
	0.4 NAA	40.9	3.0 ± 0.31	17.2 ± 1.8	15.3 ± 1.5
MMS_3	0.2 IBA	28.0	2.5 ± 0.21	19.6 ± 2.0	15.4 ± 1.6
	0.4 IBA	23.2	2.1 ± 0.18	17.5 ± 1.8	14.5 ± 1.5
	0.2 IAA	13.5	1.3 ± 0.14	15.4 ± 1.6	11.3 ± 1.3
	0.4 IAA	12.1	1.2 ± 0.13	13.4 ± 1.4	10.3 ± 1.2
	0.2 NAA	14.7	1.5 ± 0.21	11.3 ± 1.2	13.5 ± 1.4
	0.4 NAA	11.1	1.4 ± 0.19	10.0 ± 1.1	12.2 ± 1.3

MS = Full strength.

MMS_1 = ½ strength of major salts and full strength of minor salts and full organic.

MMS_2 =½ strength of major salts and ½ strength of minor salts and full organic.

MMS_3 =¼ strength of major and minor salts and full organic.

Table 4.3.39: Effect of different modified MS media and auxin combination on adventitious root formation in cultivar dhusar (V_6). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Strength of MS salts	Growth regulators (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Shoot length (mm) ($\bar{x} \pm SE$)
MS	0.2 IBA	30.1	1.4 ± 0.15	22.8 ± 2.4	16.7 ± 1.7
	0.4 IBA	25.2	1.2 ± 0.13	19.6 ± 1.9	15.5 ± 1.6
	0.2 IAA	12.0	1.1 ± 0.12	17.5 ± 1.6	11.6 ± 1.4
	0.4 IAA	11.5	1.2 ± 0.13	16.6 ± 1.5	10.4 ± 1.3
	0.2 NAA	14.1	1.3 ± 0.14	15.4 ± 1.3	11.6 ± 1.3
	0.4 NAA	12.9	1.2 ± 0.13	14.3 ± 1.2	10.3 ± 1.2
MMS_1	0.2 IBA	67.2	3.4 ± 0.42	24.9 ± 2.5	19.6 ± 2.0
	0.4 IBA	63.1	3.2 ± 0.39	20.5 ± 2.1	16.5 ± 1.7
	0.2 IAA	20.3	2.0 ± 0.28	18.5 ± 1.7	12.2 ± 1.4
	0.4 IAA	23.0	2.1 ± 0.23	17.3 ± 1.6	11.6 ± 1.3
	0.2 NAA	37.8	2.8 ± 0.30	16.4 ± 1.5	14.3 ± 1.5
	0.4 NAA	32.4	2.6 ± 0.24	15.3 ± 1.4	12.2 ± 1.3
MMS_2	0.2 IBA	77.5	4.1 ± 0.37	26.9 ± 2.7	22.5 ± 2.5
	0.4 IBA	70.2	3.2 ± 0.30	21.7 ± 2.2	19.4 ± 2.0
	0.2 IAA	30.1	2.1 ± 0.21	19.4 ± 2.0	13.4 ± 1.4
	0.4 IAA	23.0	2.7 ± 0.25	18.3 ± 1.9	12.3 ± 1.3
	0.2 NAA	45.6	3.0 ± 0.26	17.4 ± 1.7	16.4 ± 1.7
	0.4 NAA	39.9	2.8 ± 0.27	16.3 ± 1.5	14.2 ± 1.6
MMS_3	0.2 IBA	26.5	2.4 ± 0.22	18.5 ± 1.9	14.4 ± 1.6
	0.4 IBA	23.0	2.0 ± 0.19	16.6 ± 1.7	13.3 ± 1.5
	0.2 IAA	13.1	1.2 ± 0.14	14.5 ± 1.5	11.2 ± 1.3
	0.4 IAA	12.0	1.3 ± 0.15	12.4 ± 1.2	10.2 ± 1.2
	0.2 NAA	13.9	1.4 ± 0.22	11.3 ± 1.1	12.3 ± 1.4
	0.4 NAA	11.0	1.3 ± 0.20	10.1 ± 1.0	11.1 ± 1.2

MS = Full strength.

MMS_1 = ½ strength of major salts and full strength of minor salts and full organic.

MMS_2 =½ strength of major salts and ½ strength of minor salts and full organic.

MMS_3 =¼ strength of major and minor salts and full organic.

4.3.5.2: Effect of different concentrations of auxin on adventitious rooting.

Microcuttings (30 - 35 mm) prepared from the *in vitro* proliferated shoots were cultured in 25 X 150 mm tubes for adventitious rooting. In this experiment MMS_2 medium was used as standard medium but three auxins (IBA, NAA and IAA) were used in different concentrations (0.1 - 1.0 mg/L). Each treatment consisted of 15-20 cuttings. Percentage of rooting, root number, root length (mm), days to emergence of roots and intensity of callusing were considered as parameters for evaluating the experiment. Data on these parameters from different treatments were recorded after 4 weeks of culture. Results are presented on the basis of cultivar in separate heads.

i) Cultivar shabje: The results of this investigation are presented in the Table 4.3.40. The rooting emerged from microcuttings basal tissues and become visible with in 1 - 2 weeks of culture. The shoots which failed to produce roots within that period were unable to develop roots even after 6 weeks of culture. No rooting was found in the microcuttings cultured on auxin omitted medium. Among the three auxins tested, lowest rooting was obtained in IAA whereas highest rooting was obtained in IBA at the concentrations used. Rooting frequency increased gradually from 17.5% to 85.9% with the increase of auxin concentration from 0.1 – 0.2 mg/L. Further increase the auxin concentration, the rooting frequency did not increase and resulted basal callusing remarkably, particularly at concentrations 0.8 – 1.0 mg/L of NAA profuse callusing and malformation of roots were common (Plate 4.3.20. B). Contrary to that no callus formation and no malformed roots were found in 0.1 - 0.4 mg/L IAA and IBA. However slight callusing and considerable callusing but not malformed roots were seen in 0.6 – 1.0 mg/L of IAA and IBA. Number of roots was the highest 4.7 ± 0.52 and root length was the highest 31.9 ± 3.2 mm obtained in IBA 0.2 mg/L (Plate 4.3.20. A). Days to emergence of roots was found to be the minimum in 0.2 – 0.4 mg/L IBA and it was 7 - 10 days. From the overall observation it was noticed that 0.2 mg/L IBA was the best auxin concentration for adventitious root formation of cultivar shabje.

ii) Cultivar shadatae: The results of this investigation are presented in the Table 4.3.41. The rooting emerged from microcuttings basal tissues and become visible with in 1 - 2 weeks of culture. The shoots which failed to produce roots within that period were unable to develop roots even after 6 weeks of culture. No rooting was found in the microcuttings cultured on auxin omitted medium. Among the three auxins tested, lowest rooting was obtained in IAA whereas highest rooting was obtained in IBA at the concentrations used. Rooting frequency increased gradually from 19.5% to 75.7% with the increase of auxin concentration from 0.1 – 0.2 mg/L.

Further increase the auxin concentration, the rooting frequency did not increase and resulted basal callusing remarkably, particularly at concentrations 0.8 – 1.0 mg/L of NAA profuse callusing and malformation of roots were common (Plate 4.3.21 B). Contrary to that no callus formation and no malformed roots were found in 0.1 - 0.4 mg/L IAA and IBA. However slight callusing and considerable callusing but not malformed roots were seen in 0.6 – 1.0 mg/L of IAA and IBA. Number of roots was the highest 4.0 ± 0.35 and root length was the highest 26.4 ± 2.8 mm obtained in IBA 0.2 mg/L (Plate 4.3.21. A). Days to emergence of roots was found to be the minimum in 0.2 – 0.4 mg/L IBA and it was 7 - 10 days. From the overall observation it was noticed that 0.2 mg/L IBA was the best auxin concentration for adventitious root formation of cultivar shadatae.

iii) Cultivar roktima: The results of this investigation are presented in the Table 4.3.42. The rooting emerged from microcuttings basal tissues and become visible with in 1 - 2 weeks of culture. The shoots which failed to produce roots within that period were unable to develop roots even after 6 weeks of culture. No rooting was found in the microcuttings cultured on auxin omitted medium. Among the three auxins tested, lowest rooting was obtained in IAA whereas highest rooting was obtained in IBA at the concentrations used. Rooting frequency increased gradually from 23.4% to 83.6% with the increase of auxin concentration from 0.1 – 0.2 mg/L. Further increase the auxin concentration, the rooting frequency did not increase and resulted basal callusing remarkably, particularly at concentrations 0.8 – 1.0 mg/L of NAA profuse callusing and malformation of roots were common (Plate 4.3.22. B). Contrary to that no callus formation and no malformed roots were found in 0.1 - 0.4 mg/L IAA and IBA. However slight callusing and considerable callusing but not malformed roots were seen in 0.6 – 1.0 mg/L of IAA and IBA. Number of roots was the highest 4.5 ± 0.49 and root length was the highest 30.9 ± 3.1 mm obtained in IBA 0.2 mg/L (Plate 4.3.22. A). Days to emergence of roots was found to be the minimum in 0.2 – 0.4 mg/L IBA and it was 7 - 10 days. From the overall observation it was noticed that 0.2 mg/L IBA was the best auxin concentration for adventitious root formation of cultivar roktima.

iv) Cultivar lalchay: The results of this investigation are presented in the Table 4.3.43. The rooting emerged from microcuttings basal tissues and become visible with in 1 - 2 weeks of culture. The shoots which failed to produce roots within that period were unable to develop roots even after 6 weeks of culture. No rooting was found in the microcuttings cultured on auxin omitted medium. Among the three auxins tested, lowest rooting was obtained in IAA whereas highest rooting was obtained in IBA at the concentrations used. Rooting frequency increased gradually from 22.3% to 82.7%

with the increase of auxin concentration from 0.1 – 0.2 mg/l. Further increase the auxin concentration, the rooting frequency did not increase and resulted basal callusing remarkably, particularly at concentrations 0.8 – 1.0 mg/L of NAA profuse callusing and malformation of roots were common (Plate 4.3.23. B). Contrary to that no callus formation and no malformed roots were found in 0.1 - 0.4 mg/L IAA and IBA. However slight callusing and considerable callusing but not malformed roots were seen in 0.6 – 1.0 mg/L of IAA and IBA. Number of roots was the highest 4.4 ± 0.38 and root length was the highest 29.7 ± 2.8 mm obtained in IBA 0.2 mg/L (Plate 4.3.23. A). Days to emergence of roots was found to be the minimum in 0.2 – 0.4 mg/L IBA and it was 7 - 10 days. From the overall observation it was noticed that 0.2 mg/L IBA was the best auxin concentration for adventitious root formation of cultivar lalchay.

v) Cultivar badami: The results of this investigation are presented in the Table 4.3.44. The rooting emerged from microcuttings basal tissues and become visible with in 1 - 2 weeks of culture. The shoots which failed to produce roots within that period were unable to develop roots even after 6 weeks of culture. No rooting was found in the microcuttings cultured on auxin omitted medium. Among the three auxins tested, lowest rooting was obtained in IAA whereas highest rooting was obtained in IBA at the concentrations used. Rooting frequency increased gradually from 20.0% to 79.8% with the increase of auxin concentration from 0.1 – 0.2 mg/L. Further increase the auxin concentration, the rooting frequency did not increase and resulted basal callusing remarkably, particularly at concentrations 0.8 – 1.0 mg/L of NAA profuse callusing and malformation of roots were common (Plate 4.3.24. B). Contrary to that no callus formation and no malformed roots were found in 0.1 - 0.4 mg/L IAA and IBA. However slight callusing and considerable callusing but not malformed roots were seen in 0.6 – 1.0 mg/L of IAA and IBA. Number of roots was the highest 4.2 ± 0.35 and root length was the highest 27.7 ± 3.0 mm obtained in IBA 0.2 mg/L (Plate 4.3.24. A). Days to emergence of roots was found to be the minimum in 0.2 – 0.4 mg/L IBA and it was 7 - 10 days. From the overall observation it was noticed that 0.2 mg/L IBA was the best auxin concentration for adventitious root formation of cultivar badami.

vi) Cultivar dhusar: The results of this investigation are presented in the Table 4.3.45. The rooting emerged from microcuttings basal tissues and become visible with in 1 - 2 weeks of culture. The shoots which failed to produce roots within that period were unable to develop roots even after 6 weeks of culture. No rooting was found in the microcuttings cultured on auxin omitted medium. Among the three auxins tested, lowest rooting was obtained in IAA whereas highest rooting was obtained in IBA at

the concentrations used. Rooting frequency increased gradually from 19.5% to 77.5% with the increase of auxin concentration from 0.1 – 0.2 mg/L. Further increase the auxin concentration, the rooting frequency did not increase and resulted basal callusing remarkably, particularly at concentrations 0.8 – 1.0 mg/L of NAA profuse callusing and malformation of roots were common (Plate 4.3.25. B). Contrary to that no callus formation and no malformed roots were found in 0.1 - 0.4 mg/L IAA and IBA. However slight callusing and considerable callusing but not malformed roots were seen in 0.6 – 1.0 mg/L of IAA and IBA. Number of roots was the highest 4.1 ± 0.37 and root length was the highest 26.7 ± 2.7 mm obtained in IBA 0.2 mg/L (Plate 4.3.25. A). Days to emergence of roots was found to be the minimum in 0.2 – 0.4 mg/L IBA and it was 7 - 10 days. From the overall observation it was noticed that 0.2 mg/L IBA was the best auxin concentration for adventitious root formation of cultivar dhusar.

Table 4.3.40: Effect of different concentrations of auxins in MMS_2 medium on adventitious root formation in cultivar shabuje (V_1). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxins	Conc. of auxin (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus formation
Nil	-	-	-	-	-	-
IAA	0.1	17.5	1.4 ± 0.17	19.3 ± 1.7	12 - 15	-
	0.2	34.7	2.5 ± 0.34	22.8 ± 2.1	10 - 12	-
	0.4	26.2	2.3 ± 0.21	20.5 ± 2.0	10 - 12	-
	0.6	15.3	2.2 ± 0.16	17.6 ± 1.5	12 - 15	+
	0.8	9.4	2.1 ± 0.18	12.5 ± 1.2	15 - 20	++
	1.0	9.0	2.0 ± 0.15	12.1 ± 1.3	15 - 20	++
IBA	0.1	44.7	2.7 ± 0.39	23.4 ± 2.0	10 - 12	-
	0.2	85.9	4.7 ± 0.52	31.9 ± 3.2	7 - 10	-
	0.4	78.5	4.0 ± 0.41	26.7 ± 2.8	7 - 10	-
	0.6	72.1	3.5 ± 0.32	25.5 ± 2.2	10 - 12	+
	0.8	43.0	2.3 ± 0.19	17.3 ± 1.5	10 - 12	+
	1.0	21.2	1.5 ± 0.17	12.2 ± 0.9	12 - 15	++
NAA	0.1	28.4	2.3 ± 0.26	19.3 ± 1.3	12 - 15	+
	0.2	52.7	3.5 ± 0.25	24.5 ± 1.9	10 - 12	+
	0.4	44.3	3.2 ± 0.30	23.3 ± 1.9	12 - 15	++
	0.6	35.7	2.6 ± 0.35	20.4 ± 1.1	12 - 15	++
	0.8	21.0	2.0 ± 0.21	16.6 ± 1.0	15 - 20	+++
	1.0	10.6	1.3 ± 0.19	11.2 ± 0.9	15 - 20	+++

* Intensity of callusing:
- \- indicates no response.
- \+ slight callusing.
- ++ considerable callusing.
- +++ profuse callusing.

Table 4.3.41: Effect of different concentrations of auxins in MMS_2 medium on adventitious root formation in cultivar shadate (V_2). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxins	Conc. of auxin (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus formation
Nil	-	-	-	-	-	-
IAA	0.1	19.5	1.4 ± 0.15	15.5 ± 1.7	12 - 15	-
	0.2	30.0	2.1 ± 0.18	19.4 ± 1.8	10 - 12	-
	0.4	22.8	2.0 ± 0.20	18.6 ± 1.8	10 - 12	-
	0.6	11.9	1.9 ± 0.17	13.7 ± 1.5	12 - 15	+
	0.8	6.5	1.8 ± 0.16	11.9 ± 1.3	15 - 20	++
	1.0	6.4	1.8 ± 0.17	11.1 ± 1.4	15 - 20	++
IBA	0.1	43.2	2.2 ± 0.35	19.5 ± 1.8	10 - 12	-
	0.2	75.7	4.0 ± 0.35	26.4 ± 2.8	7 - 10	-
	0.4	66.3	3.1 ± 0.28	21.6 ± 2.1	7 - 10	-
	0.6	50.3	3.0 ± 0.31	20.3 ± 2.0	10 - 12	+
	0.8	37.2	2.1 ± 0.20	16.4 ± 1.7	10 - 12	+
	1.0	18.0	1.3 ± 0.18	10.2 ± 0.9	12 - 15	++
NAA	0.1	23.5	2.1 ± 0.26	16.6 ± 1.8	12 - 15	+
	0.2	45.1	3.0 ± 0.22	17.8 ± 1.8	10 - 12	+
	0.4	38.2	2.8 ± 0.21	16.5 ± 1.7	12 - 15	++
	0.6	26.0	2.1 ± 0.35	14.3 ± 1.5	12 - 15	++
	0.8	16.3	2.0 ± 0.28	12.1 ± 1.3	15 - 20	+++
	1.0	7.2	1.1 ± 0.15	10.0 ± 10	15 - 20	+++

* Intensity of callusing: - indicates no response.
+ slight callusing.
++ considerable callusing.
+++ profuse callusing.

Table 4.3.42: Effect of different concentrations of auxins in MMS_2 medium on adventitious root formation in cultivar roktima (V_3). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxins	Conc. of auxin (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus formation
Nil	-	-	-	-	-	-
IAA	0.1	23.4	1.7 ± 0.18	18.6 ± 1.9	12 - 15	-
	0.2	33.3	2.4 ± 0.35	22.5 ± 2.1	10 - 12	-
	0.4	25.4	2.2 ± 0.23	20.3 ± 1.9	10 - 12	-
	0.6	14.8	2.1 ± 0.15	16.4 ± 1.7	12 - 15	+
	0.8	9.0	2.0 ± 0.17	12.5 ± 1.3	15 - 20	++
	1.0	8.9	1.9 ± 0.16	12.2 ± 1.4	15 - 20	++
IBA	0.1	50.5	2.6 ± 0.38	22.8 ± 2.2	10 - 12	-
	0.2	83.6	4.5 ± 0.49	30.9 ± 3.1	7 - 10	-
	0.4	75.1	3.7 ± 0.42	25.6 ± 2.7	7 - 10	-
	0.6	60.0	3.4 ± 0.35	24.0 ± 2.3	10 - 12	+
	0.8	40.5	2.5 ± 0.20	16.5 ± 1.4	10 - 12	+
	1.0	20.3	1.5 ± 0.19	12.4 ± 0.9	12 - 15	++
NAA	0.1	26.7	2.3 ± 0.27	18.5 ± 1.9	12 - 15	+
	0.2	50.6	3.4 ± 0.35	22.6 ± 2.1	10 - 12	+
	0.4	43.5	3.1 ± 0.30	21.0 ± 2.0	12 - 15	++
	0.6	33.3	2.5 ± 0.28	18.3 ± 1.8	12 - 15	++
	0.8	19.1	2.2 ± 0.25	15.2 ± 1.6	15 - 20	+++
	1.0	8.5	1.3 ± 0.1.4	11.3 ± 1.2	15 - 20	+++

* Intensity of callusing: - indicates no response.
+ slight callusing.
++ considerable callusing.
+++ profuse callusing.

Table 4.3.43: Effect of different concentrations of auxins in MMS_2 medium on adventitious root formation in cultivar lalchay (V_4). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxins	Conc. of auxin (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus formation
Nil	-	-	-	-	-	-
IAA	0.1	22.3	1.6 ± 0.15	17.4 ± 1.8	12 - 15	-
	0.2	32.1	2.3 ± 0.24	21.3 ± 2.0	10 - 12	-
	0.4	25.5	2.1 ± 0.23	19.2 ± 1.9	10 - 12	-
	0.6	14.5	2.0 ± 0.20	15.3 ± 1.6	12 - 15	+
	0.8	8.8	1.9 ± 0.18	12.4 ± 1.3	15 - 20	++
	1.0	8.7	1.9 ± 0.19	12.0 ± 1.4	15 - 20	++
IBA	0.1	48.1	2.4 ± 0.22	21.6 ± 2.0	10 - 12	-
	0.2	82.7	4.4 ± 0.38	29.7 ± 2.8	7 - 10	-
	0.4	74.0	3.5 ± 0.33	24.3 ± 2.2	7 - 10	-
	0.6	58.2	3.2 ± 0.29	23.2 ± 2.1	10 - 12	+
	0.8	40.0	2.3 ± 0.21	16.0 ± 1.5	10 - 12	+
	1.0	20.1	1.4 ± 0.12	13.2 ± 1.1	15 - 20	++
NAA	0.1	25.1	2.2 ± 0.2.0	17.3 ± 1.8	12 - 15	+
	0.2	49.2	3.2 ± 0.31	19.5 ± 2.0	10 - 12	+
	0.4	41.6	3.0 ± 0.30	18.2 ± 1.9	12 - 15	++
	0.6	30.2	2.3 ± 0.24	16.4 ± 1.7	12 - 15	++
	0.8	19.0	2.1 ± 0.22	14.4 ± 1.5	15 - 20	+++
	1.0	8.5	1.2 ± 1.0	11.2 ± 1.0	15 - 20	+++

* Intensity of callusing: - indicates no response.
+ slight callusing.
++ considerable callusing.
+++ profuse callusing.

Table 4.3.44: Effect of different concentrations of auxins in MMS_2 medium on adventitious root formation in cultivar badami (V_5). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxins	Conc. of auxin (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus formation
Nil	-	-	-	-	-	-
IAA	0.1	20.0	1.5 ± 0.12	15.4 ± 1.6	12 - 15	-
	0.2	31.1	2.2 ± 0.21	19.6 ± 1.8	10 - 12	-
	0.4	24.2	2.0 ± 0.19	18.3 ± 1.7	10 - 12	-
	0.6	12.0	1.9 ± 0.15	14.3 ± 1.2	12 - 15	+
	0.8	6.7	1.8 ± 0.14	12.4 ± 1.0	15 - 20	++
	1.0	6.0	1.8 ± 0.15	12.0 ± 1.1	15 - 20	++
IBA	0.1	45.5	2.3 ± 0.22	19.3 ± 1.6	10 - 12	-
	0.2	79.8	4.2 ± 0.35	27.7 ± 3.0	7 - 10	-
	0.4	71.5	3.3 ± 0.31	22.3 ± 1.9	7 - 10	-
	0.6	54.2	3.1 ± 0.29	21.0 ± 2.0	10 - 12	+
	0.8	38.1	2.2 ± 0.21	16.4 ± 1.5	10 - 12	+
	1.0	18.6	1.3 ± 0.11	11.3 ± 1.0	15 - 20	++
NAA	0.1	24.1	2.1 ± 0.20	16.6 ± 1.7	12 - 15	+
	0.2	47.3	3.1 ± 0.29	18.4 ± 1.9	10 - 12	+
	0.4	40.9	2.9 ± 0.27	17.5 ± 1.8	12 - 15	++
	0.6	26.5	2.2 ± 0.21	15.2 ± 1.6	12 - 15	++
	0.8	16.1	2.0 ± 0.19	13.3 ± 1.2	15 - 20	+++
	1.0	7.9	1.1 ± 0.10	10.2 ± 0.9	15 - 20	+++

* Intensity of callusing: - indicates no response.
+ slight callusing.
++ considerable callusing.
+++ profuse callusing.

Table 4.3.45: Effect of different concentrations of auxins in MMS_2 medium on adventitious root formation in cultivar dhusar (V_6). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxins	Conc. of auxin (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus formation
Nil	-	-	-	-	-	-
IAA	0.1	19.5	1.4 ± 0.12	15.3 ± 1.6	12 - 15	-
	0.2	30.1	2.1 ± 0.21	19.5 ± 1.8	10 - 12	-
	0.4	23.0	2.0 ± 0.23	18.2 ± 1.7	10 - 12	-
	0.6	11.7	1.9 ± 0.17	13.2 ± 1.4	12 - 15	+
	0.8	6.3	1.8 ± 0.16	11.5 ± 1.2	15 - 20	++
	1.0	6.2	1.8 ± 0.17	11.3 ± 1.3	-	++
IBA	0.1	44.1	2.2 ± 0.23	19.5 ± 1.8	10 - 12	-
	0.2	77.5	4.1 ± 0.37	26.7 ± 2.7	7 - 10	-
	0.4	70.2	3.2 ± 0.28	21.6 ± 2.0	7 - 10	-
	0.6	53.0	3.0 ± 0.27	20.2 ± 1.9	10 - 12	+
	0.8	37.8	2.1 ± 0.20	16.6 ± 1.6	10 - 12	+
	1.0	18.0	1.3 ± 0.11	10.3 ± 0.9	12 - 15	++
NAA	0.1	24.0	2.1 ± 0.20	16.5 ± 1.7	12 - 14	+
	0.2	45.6	3.0 ± 0.27	17.7 ± 1.8	10 - 12	+
	0.4	39.4	2.8 ± 0.25	16.3 ± 1.8	12 - 15	++
	0.6	26.1	2.1 ± 0.19	14.4 ± 1.5	12 - 15	++
	0.8	16.0	2.0 ± 0.17	12.2 ± 1.3	15 - 20	+++
	1.0	7.4	1.1 ± 0.10	10.0 ± 1.1	15 - 20	+++

* Intensity of callusing: - indicates no response.
+ slight callusing.
+ + considerable callusing.
+ ++ profuse callusing.

4.3.5.3: Combined effect of $AgNO_3$, activated charcoal and IBA on adventitious rooting.

In this experiment $AgNO_3$ (0.1 – 1.0 mg/L) and activated charcoal (50 – 250 mg/L) were used as additive in MMS_2 medium supplemented with 0.2 mg/L IBA. Each treatment consisted of 15 – 20 cuttings. Percentage of rooting, root number, root length (mm), days to emergence of roots and intensity of callusing were considered as parameters for evaluating the experiments. Data on these parameters from different treatments were recorded after 4 weeks of culture. Results are presented on the basis of cultivar in separate heads.

i) Cultivar shabje: Results of this investigation are presented in the Table 4.3.46. When the explants cultured on IBA 0.2 mg/L with different concentrations of $AgNO_3$, the rooting frequency increased gradually from 85.0% to 92.7% with the increasing of $AgNO_3$ concentrations from 0.1 – 0.6 mg/L. Further increase the $AgNO_3$ concentration from 0.8 – 1.0 mg/L, the rooting frequency did not increase. Root number of was the highest 14.8 ± 0.95, Root length was the highest 42.2 ± 4.2 mm obtained in 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ (Plate 4.3.20. C). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA + 0.4 – 0.8 mg/L $AgNO_3$ and it was 5 – 8 days. No callus formation was found in IBA 0.2 mg/L + 0.1 – 0.6 mg/L $AgNO_3$, however slight callusing was found in IBA 0.2 mg/L + 0.8 – 1.0 mg/L $AgNO_3$. It is clear that 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ concentration was the best among the all concentrations tested.

When the explants cultured on IBA 0.2 mg/L with different concentrations (25 – 250 mg/L) of activated charcoal, percentage of cuttings rooted was the highest 85.2%, root number was the highest 7.9 ± 0.72 and root length was the highest 38.4 ± 3.9 mm obtained in IBA 0.2 mg/L + 150 mg/L activated charcoal (Plate 4.3.20. E). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA + 100 - 150 mg/L activated charcoal. No callus formation was found in all concentrations (Plate 4.3.20. D & F). So it is clear that 0.2 mg/L IBA + 150 mg/L activated charcoal was the best among the all concentrations tested.

ii) Cultivar shadatae: Results of this investigation are presented in the Table 4.3.47. When the explants cultured on IBA 0.2 mg/L with different concentrations of $AgNO_3$, the rooting frequency increased gradually from 69.0% to 78.1% with the increasing of $AgNO_3$ concentrations from 0.1 – 0.6 mg/L. Further increase the $AgNO_3$ concentration from 0.8 – 1.0 mg/L, the rooting frequency did not increase. Root number was the highest 11.6 ± 0.45, root length was the highest 33.8 ± 2.7 mm obtained in 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ (Plate 4.3.21. C). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA + 0.4 – 0.8 mg/L $AgNO_3$ and it

was 5 – 8 days. No callus formation was found in IBA 0.2 mg/L + 0.1 – 0.6 mg/L $AgNO_3$, however slight callusing was found in IBA 0.2 mg/L + 0.8 – 1.0 mg/L $AgNO_3$. It is clear that 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ concentration was the best among the all concentrations tested.

When the explants cultured on IBA 0.2 mg/L with different concentrations (25 – 250 mg/L) of activated charcoal, percentage of rooting was the highest 72.1%, root number was the highest 6.1 ± 0.43 and root length was the highest 30.2 ± 2.6 mm obtained in IBA 0.2 mg/L + 150 mg/L activated charcoal (Plate 4.3.21. E). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA with 100 - 150 mg/L activated charcoal. No callus formation was found in all concentrations (Plate 4.3.21. D & F). So it is clear that 0.2 mg/L IBA + 150 mg/L activated charcoal containing medium was the best among the all concentrations tested.

iii) Cultivar roktima: Results of this investigation are presented in the Table 4.3.48. When the explants cultured on IBA 0.2 mg/L with different concentrations of $AgNO_3$, the rooting frequency increased gradually from 83.5% to 90.3% with the increasing of $AgNO_3$ concentrations from 0.1 – 0.6 mg/L. Further increase the $AgNO_3$ concentration from 0.8 – 1.0 mg/L, the rooting frequency did not increase. Root number was the highest 14.4 ± 0.50, mean length (mm) of roots per shoot cutting was the highest 40.1 ± 3.5 obtained in 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ (Plate 4.3.22. C). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA + 0.4 – 0.8 mg/L $AgNO_3$ and it was 5 – 8 days. No callus formation was found in IBA 0.2 mg/L + 0.1 – 0.6 mg/L $AgNO_3$, however slight callusing was found in IBA 0.2 mg/L + 0.8 – 1.0 mg/L $AgNO_3$. It is clear that 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ concentration was the best among the all concentrations tested.

When the explants cultured on IBA 0.2 mg/L with different concentrations (25 – 250 mg/L) of activated charcoal, percentage of rooting was the highest 83.2%, root number was the highest 7.4 ± 0.51 and mean root length was the highest 36.3 ± 3.4 mm obtained in IBA 0.2 mg/L + 150 mg/L activated charcoal (Plate 4.3.22. E). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA with 100 - 150 mg/L activated charcoal. No callus formation was found in all concentrations (Plate 4.3.22. D & F). So it is clear that 0.2 mg/L IBA + 150 mg/L activated charcoal was the best among the all concentrations tested.

iv) Cultivar lalchay: Results of this investigation are presented in the Table 4.3.49. When the explants cultured on IBA 0.2 mg/L with different concentrations of $AgNO_3$, the rooting frequency increased gradually from 80.1% to 88.5% with the increasing of $AgNO_3$ 0.1 – 0.6 mg/L. Further increase the $AgNO_3$ concentration from 0.8 – 1.0 mg/L, the rooting frequency did not increase. Root number was the highest

12.1 ± 0.49, root length was the highest 38.1 ± 3.2 mm obtained in 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ (Plate 4.3.23. C). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA with 0.4 – 0.8 mg/L $AgNO_3$ and it was 5 – 8 days. No callus formation was found in IBA 0.2 mg/L with 0.1 – 0.6 mg/L $AgNO_3$, however slight callusing was found in IBA 0.2 mg/L + 0.8 – 1.0 mg/L $AgNO_3$. It is clear that 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ concentration was the best among the all concentrations tested.

When the explants cultured on IBA 0.2 mg/L with different concentrations (25 – 250 mg/L) of activated charcoal, percentage of rooting was the highest 80.0%, number of roots was the highest 7.1 ± 0.50 and root length cutting was the highest 34.1 ± 3.0 mm obtained in IBA 0.2 mg/L + 150 mg/L activated charcoal (Plate 4.3.23. E). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA with 100 - 150 mg/L activated charcoal. No callus formation was found in all concentrations (Plate 4.3.20, Fig. D & F). So it is clear that 0.2 mg/L IBA + 150 mg/L activated charcoal was the best among the all concentrations tested.

v) Cultivar badami: Results of this investigation are presented in the Table 4.3.50. When the explants cultured on IBA 0.2 mg/L with different concentrations of $AgNO_3$, the rooting frequency increased gradually from 70.9% to 81.5% with the increasing of $AgNO_3$ concentrations from 0.1 – 0.6 mg/L. Further increase the $AgNO_3$ concentration from 0.8 – 1.0 mg/L, the rooting frequency did not increase. Number of roots was the highest 11.8 ± 0.37, root length was the highest 35.5 ± 3.0 mm obtained in 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ (Plate 4.3.24. C). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA with 0.4 – 0.8 mg/L $AgNO_3$ and it was 5 – 8 days. No callus formation was found in IBA 0.2 mg/L with 0.1 – 0.6 mg/L $AgNO_3$, however slight callusing was found in IBA 0.2 mg/L with 0.8 – 1.0 mg/L $AgNO_3$. It is clear that 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ concentration was the best among the all concentrations tested.

When the explants cultured on IBA 0.2 mg/L with different concentrations (25 – 250 mg/L) of activated charcoal, percentage of rooting was the highest 75.5%, number of roots per shoot cutting was the highest 6.4 ± 0.45 and root length was the highest 32.2 ± 2.8 mm obtained in IBA 0.2 mg/L + 150 mg/L activated charcoal (Plate 4.3.21, Fig. E). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA with 100 - 150 mg/L activated charcoal. No callus formation was found in all concentrations (Plate 4.3.24. D & F). So it is clear that 0.2 mg/L IBA with 150 mg/L activated charcoal was the best among the all concentrations tested.

vi)Cultivar dhusar: Results of this investigation are presented in the Table 4.3.51. When the explants cultured on IBA 0.2 mg/L with different concentrations of

$AgNO_3$, the rooting frequency increased gradually from 70.1% to 80.0% with the increasing of $AgNO_3$ concentrations from 0.1 – 0.6 mg/L. Further increase the $AgNO_3$ concentration from 0.8 – 1.0 mg/L, the rooting frequency did not increase. Root number of was the highest 11.6 ± 0.42, root length (mm) was the highest 34.1 ± 2.8 obtained in 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ (Plate 4.3.25. C). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA with 0.4 – 0.8 mg/L $AgNO_3$ and it was 5 – 8 days. No callus formation was found in IBA 0.2 mg/L with 0.1 – 0.6 mg/L $AgNO_3$, however slight callusing was found in IBA 0.2 mg/L with 0.8 – 1.0 mg/L $AgNO_3$. It is clear that 0.2 mg/L IBA + 0.6 mg/L $AgNO_3$ concentration was the best among the all concentrations tested.

When the explants cultured on IBA 0.2 mg/L with different concentrations (25 – 250 mg/L) of activated charcoal, rooting percentage was the highest 74.1%, root number was the highest 6.2 ± 0.45 and root length was the highest 30.5 ± 2.7 mm obtained in IBA 0.2 mg/L + 150 mg/L activated charcoal (Plate 4.3.22, Fig. E). Days to emergence of roots was found to be the minimum in 0.2 mg/L IBA with 100 - 150 mg/L activated charcoal. No callus formation was found in all concentrations (Plate 4.3.25. D & F). So it is clear that 0.2 mg/L IBA + 150 mg/L activated charcoal was the best among the all concentrations tested.

Table 4.3.46: Effect of activated charcoal and $AgNO_3$ in MMS_2 medium with IBA on adventitious root formation in cultivar shabje (V_1). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxin and additives	Concen -trations (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus forma-tion
IBA + $AgNO_3$	0.2+0.1	85.0	7.9 ± 0.63	32.1 ± 3.0	7 - 10	-
	0.2+0.2	87.2	8.1 ± 0.75	35.2 ± 3.7	7 - 10	-
	0.2+0.4	88.6	10.4 ± 0.81	39.3 ± 3.9	5 - 8	-
	0.2+0.6	92.7	14.8 ± 0.95	42.2 ± 4.2	5 - 8	-
	0.2+0.8	90.3	11.5 ± 0.77	40.1 ± 4.1	5 - 8	+
	0.2+1.0	86.1	8.2 ± 0.69	35.0 ± 3.6	7 - 10	+
IBA + AC	0.2+25	75.3	6.1 ± 0.52	26.1 ± 2.1	7 -10	-
	0.2+50	79.2	6.5 ± 0.49	28.0 ± 2.3	7 - 10	-
	0.2+100	83.6	7.0 ± 0.53	31.7 ± 2.8	6 - 8	-
	0.2+150	85.2	7.9 ± 0.72	38.4 ± 3.9	6 - 8	-
	0.2+200	80.1	7.3 ± 0.61	35.2 ± 3.7	7 - 10	-
	0.2+250	79.4	7.0 ± 0.69	36.1 ± 3.6	7 - 10	-

* Intensity of callusing: - indicates no response.
+ slight callusing.

Table 4.3.47: Effect of activated charcoal and $AgNO_3$ in MMS_2 medium with IBA on adventitious root formation in cultivar shadate (V_2). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxin and additives	Concen-trations (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus forma-tion
IBA + $AgNO_3$	0.2+0.1	69.0	6.5±0.29	24.5±2.3	7-10	-
	0.2+0.2	72.1	6.8±0.32	27.1±2.5	7-10	-
	0.2+0.4	75.6	9.2±0.40	30.2±2.6	5-8	-
	0.2+0.6	78.1	11.6±0.45	33.8±2.7	5-8	-
	0.2+0.8	74.0	10.0±0.39	31.9±2.6	5-8	+
	0.2+1.0	67.4	6.8±0.34	27.0±2.5	7-10	+
IBA + AC	0.2+25	62.7	4.8±0.36	21.5±2.0	7-10	-
	0.2+50	65.0	5.2±0.38	22.4±2.1	7-10	-
	0.2+100	70.0	5.6±0.40	25.5±2.2	6-8	-
	0.2+150	72.1	6.1±0.43	30.2±2.6	6-8	-
	0.2+200	67.2	5.8±0.41	29.0±2.5	7-10	-
	0.2+250	62.7	5.1±0.38	27.1±2.4	7-10	-

* Intensity of callusing: - indicates no response.
+ slight callusing.

Table 4.3.48: Effect of activated charcoal and $AgNO_3$ in MMS_2 medium with IBA on adventitious root formation in cultivar roktima (V_3). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxin and additives	Concen-trations (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus forma-tion
IBA + $AgNO_3$	0.2+0.1	83.5	7.5±0.40	30.2±2.7	7-10	-
	0.2+0.2	85.7	7.7±0.45	33.1±2.8	7-10	-
	0.2+0.4	86.0	10.0±0.49	36.3±3.0	5-8	-
	0.2+0.6	90.3	14.4±0.50	40.1±3.5	5-8	-
	0.2+0.8	88.1	11.1±0.41	37.2±3.1	5-8	+
	0.2+1.0	82.2	7.8±0.39	32.0±2.9	7-10	+
IBA + AC	0.2+25	71.1	6.0±0.41	26.0±2.5	7-10	-
	0.2+50	74.5	6.3±0.43	28.1±2.6	7-10	-
	0.2+100	80.3	6.9±0.48	30.8±2.7	6-8	-
	0.2+150	83.2	7.4±0.51	36.3±3.4	6-8	-
	0.2+200	78.0	7.0±0.49	34.1±3.1	7-10	-
	0.2+250	74.9	6.5±0.37	32.1±3.0	7-10	-

* Intensity of callusing: - indicates no response.
+ slight callusing.

Table 4.3.49: Effect of activated charcoal and $AgNO_3$ in MMS_2 medium with IBA on adventitious root formation in cultivar lalchay (V_4). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxin and additives	Concen -trations (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus forma-tion
IBA + $AgNO_3$	0.2+0.1	80.1	7.2±0.37	28.3±2.6	7-10	-
	0.2+0.2	82.0	7.4±0.42	31.2±2.7	7-10	-
	0.2+0.4	84.2	9.7±0.47	34.0±3.0	5-8	-
	0.2+0.6	88.5	12.1±0.49	38.1±3.2	5-8	-
	0.2+0.8	83.9	10.8±0.40	35.0±3.0	5-8	+
	0.2+1.0	78.5	7.5±0.38	30.1±2.8	7-10	+
IBA + AC	0.2+25	67.8	5.7±0.56	24.1±2.5	7-10	-
	0.2+50	71.6	6.0±0.45	26.0±2.6	7-10	-
	0.2+100	77.2	6.6±0.47	28.5±2.7	6-8	-
	0.2+150	80.0	7.1±0.50	34.1±3.0	6-8	-
	0.2+200	75.1	6.7±0.48	32.0±2.9	7-10	-
	0.2+250	71.2	6.2±0.39	30.1±2.8	7-10	-

* Intensity of callusing: - indicates no response.
+ slight callusing.

Table 4.3.50: Effect of activated charcoal and $AgNO_3$ in MMS_2 medium with IBA on adventitious root formation in cultivar badami (V_5). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxin and additives	Concen-trations (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus forma-tion
IBA + $AgNO_3$	0.2+0.1	70.9	6.7±0.30	25.7±2.5	7-10	-
	0.2+0.2	75.3	7.0±0.32	28.5±2.6	7-10	-
	0.2+0.4	77.6	9.4±0.35	32.3±2.8	5-8	-
	0.2+0.6	81.5	11.8±0.37	35.5±3.0	5-8	-
	0.2+0.8	76.0	10.3±0.33	33.4±2.8	5-8	+
	0.2+1.0	71.7	7.1±0.32	29.2±2.5	7-10	+
IBA + AC	0.2+25	65.7	5.0±0.39	23.0±2.1	7-10	-
	0.2+50	68.5	5.5±0.41	24.1±2.2	7-10	-
	0.2+100	71.3	6.0±0.42	27.0±2.4	6-8	-
	0.2+150	75.5	6.4±0.45	32.2±2.8	6-8	-
	0.2+200	70.4	6.1±0.42	30.5±2.7	7-10	-
	0.2+250	66.3	5.5±0.43	29.1±2.5	7-10	-

* Intensity of callusing: - indicates no response.
+ slight callusing.

Table 4.3.51: Effect of activated charcoal and $AgNO_3$ in MMS_2 medium with IBA on adventitious root formation in cultivar dhusar (V_6). Data were recorded after 4 weeks of culture. Each treatment consisted of 15-20 microcuttings.

Type of auxin and additives	Concen-trations (mg/L)	% of rooting	Number of roots ($\bar{x} \pm SE$)	Root length (mm) ($\bar{x} \pm SE$)	Days to emergence of roots	* Callus forma-tion
IBA + $AgNO_3$	0.2+0.1	70.1	6.6±0.30	24.5±2.3	7-10	-
	0.2+0.2	74.2	6.9±0.33	27.2±2.5	7-10	-
	0.2+0.4	76.7	9.3±0.40	31.2±2.7	5-8	-
	0.2+0.6	80.0	11.6±0.42	34.1±2.8	5-8	-
	0.2+0.8	75.1	10.1±0.40	32.6±2.7	5-8	+
	0.2+1.0	69.5	6.9±0.34	28.0±2.6	7-10	+
IBA + AC	0.2+25	64.5	4.9±0.37	21.7±2.0	7-10	-
	0.2+50	67.3	5.3±0.40	22.5±2.1	7-10	-
	0.2+100	70.0	5.7±0.41	25.6±2.3	6-8	-
	0.2+150	74.1	6.2±0.45	30.5±2.7	6-8	-
	0.2+200	69.2	5.9±0.42	29.0±2.6	7-10	-
	0.2+250	65.3	5.2±0.40	27.1±2.4	7-10	-

* Intensity of callusing: - indicates no response.
+ slight callusing.

ROOTING IN CULTIVAR SHABJE

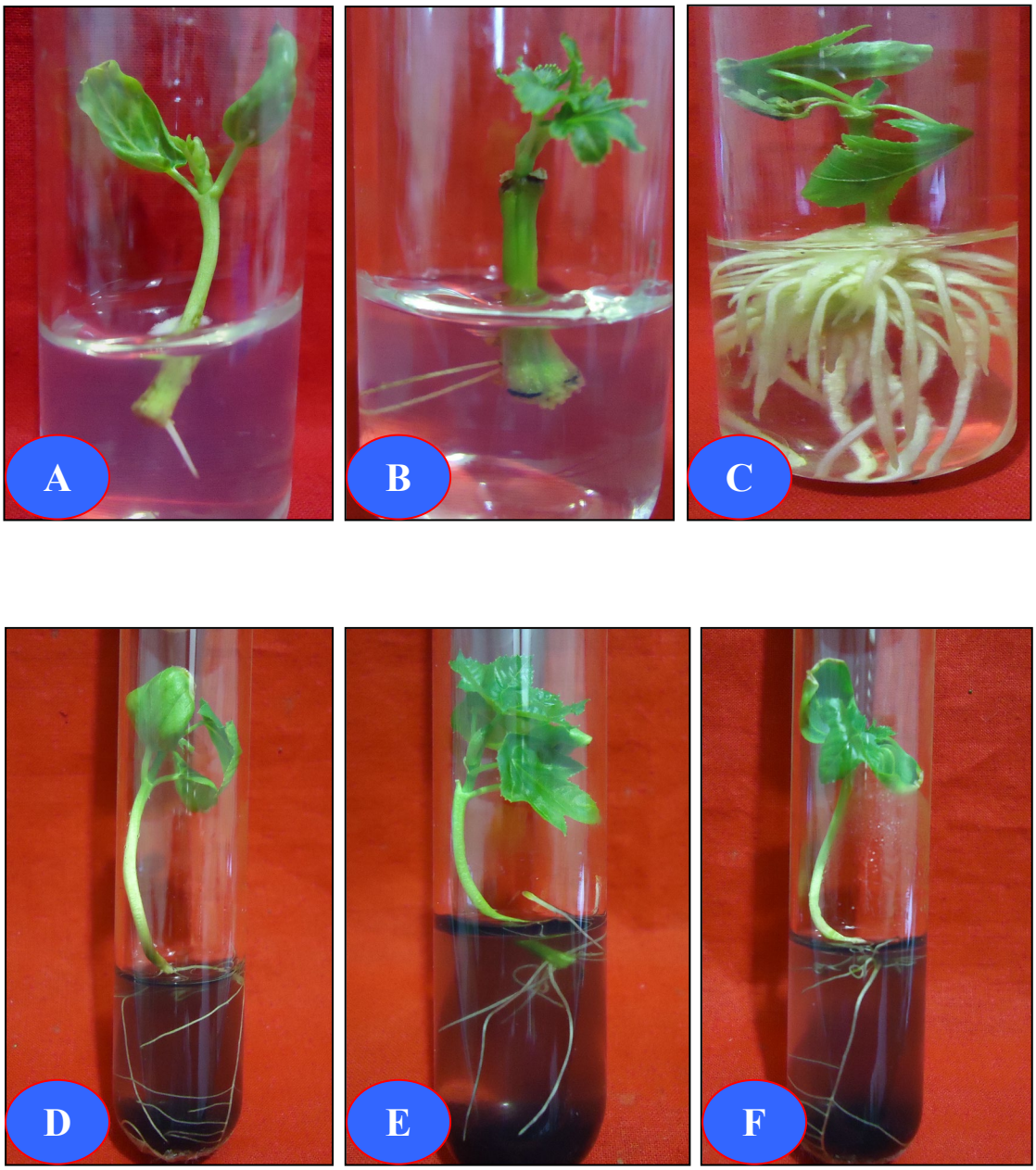


Plate- 4.3.20: Photographs showing the initiation and development of adventitious roots of cultivar shabje microcuttings. Data were recorded after 4 weeks of culture. **A:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L. **B:** Microcuttings cultured on MMS_2 + NAA 0.2 mg/L. **C:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + $AgNO_3$ 0.6 mg/L. **D:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 100 mg/L. **E:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 150 mg/L. **F:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 200 mg/L.

ROOTING IN CULTIVAR SHADATAE

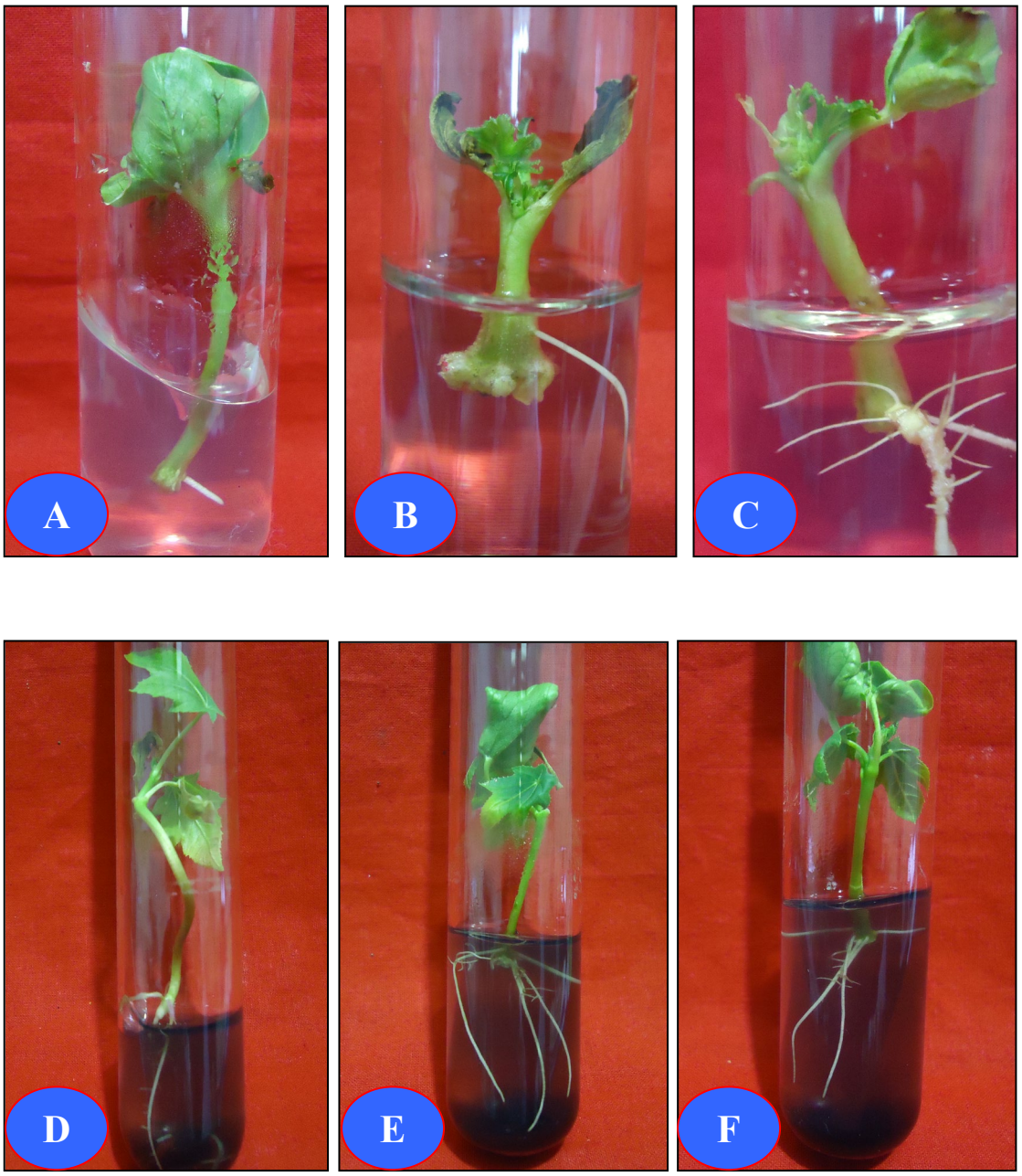


Plate- 4.3.21: Photographs showing the initiation and development of adventitious roots of cultivar shadatae microcuttings. Data were recorded after 4 weeks of culture. **A:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L. **B:** Microcuttings cultured on MMS_2 + NAA 0.2 mg/L. **C:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + $AgNO_3$ 0.6 mg/L. **D:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 100 mg/L. **E:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 150 mg/L.. **F:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 200 mg/L.

ROOTING IN CULTIVAR ROKTIMA

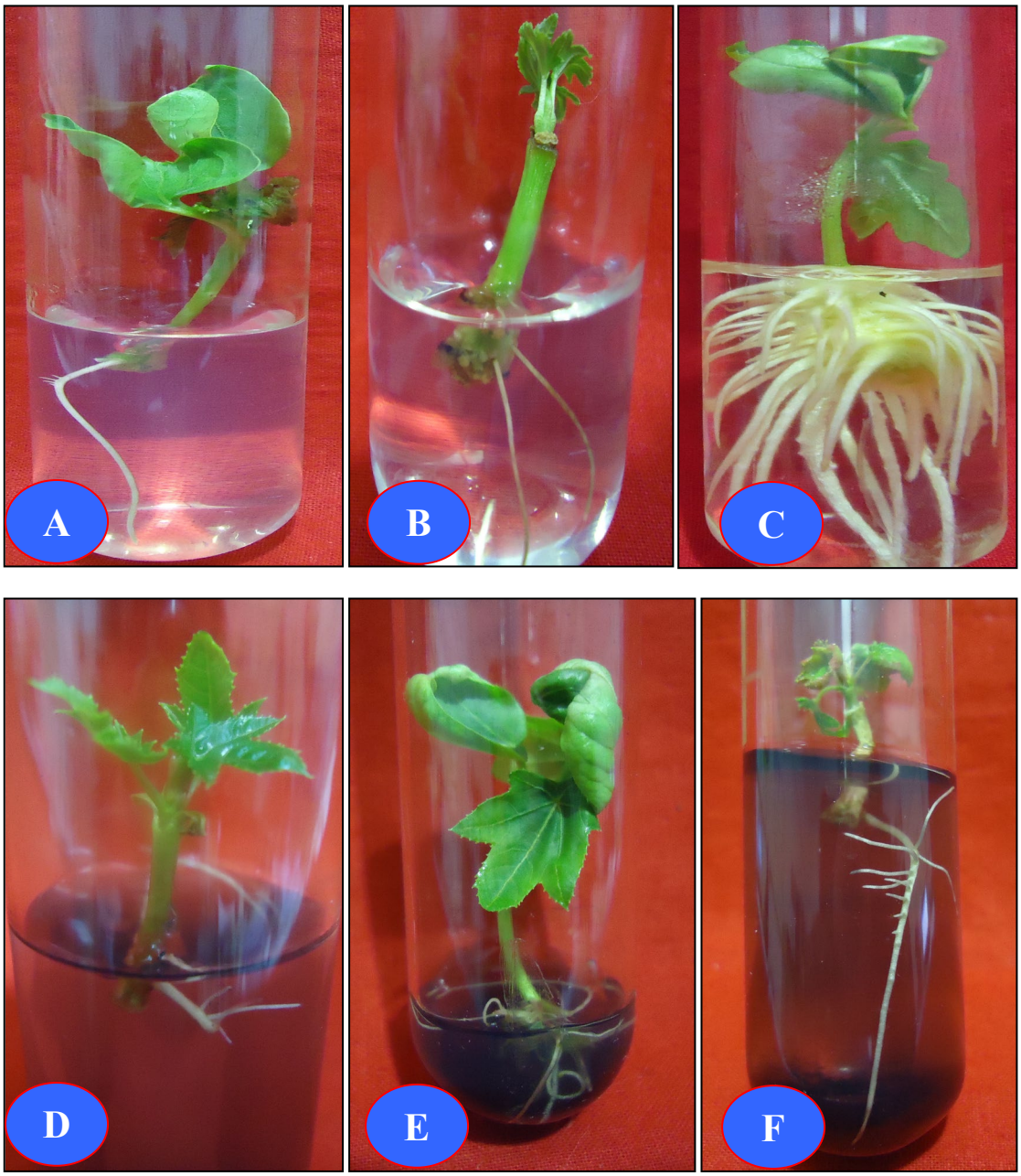


Plate- 4.3.22: Photographs showing the initiation and development of adventitious roots of cultivar roktima microcuttings. Data were recorded after 4 weeks of culture. **A:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L. **B:** Microcuttings cultured on MMS_2 + NAA 0.2 mg/L. **C:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + $AgNO_3$ 0.6 mg/L. **D:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 100 mg/L. **E:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 150 mg/L. **F:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 200 mg/L.

ROOTING IN CULTIVAR LALCHAY

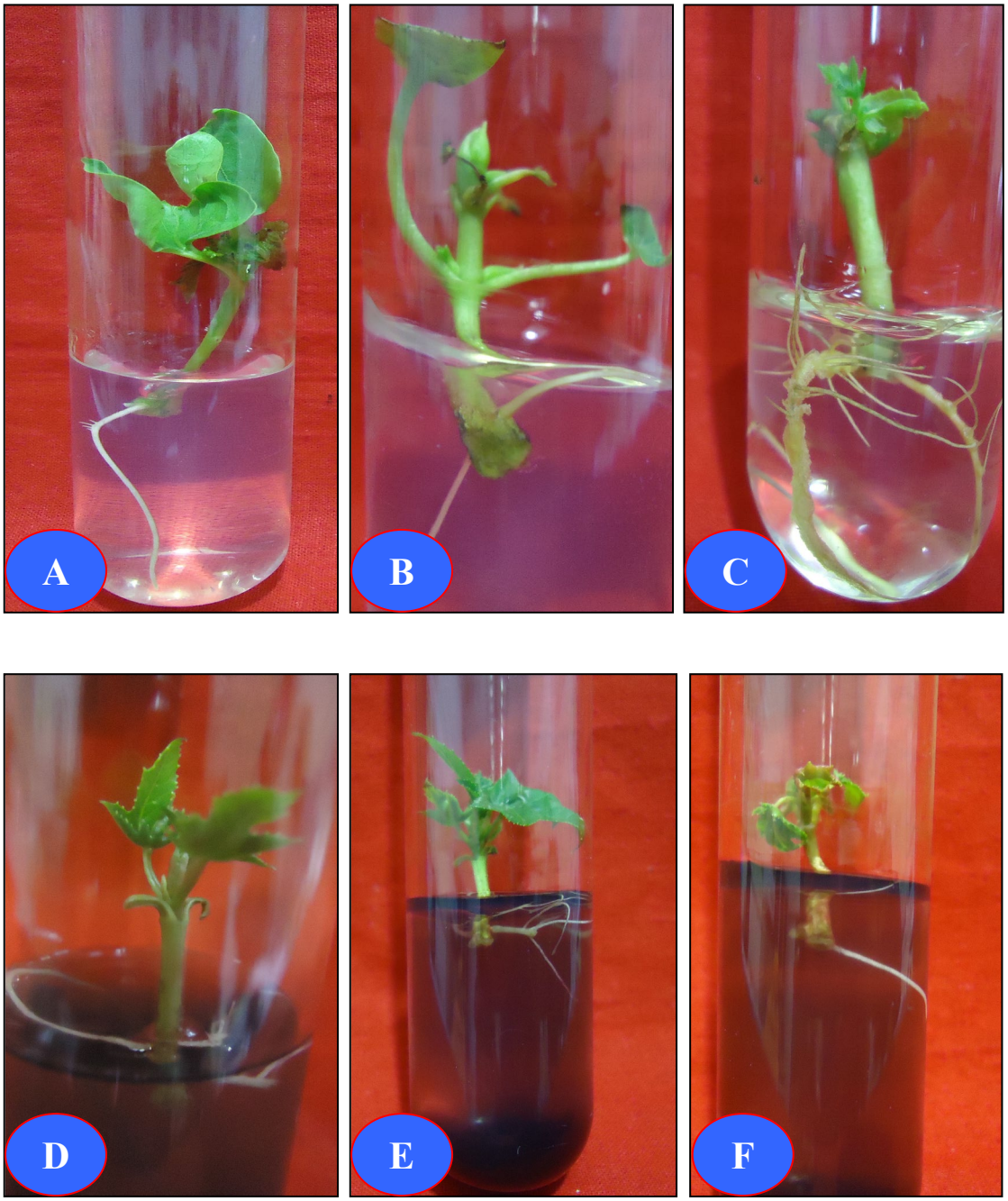


Plate- 4.3.23: Photographs showing the initiation and development of adventitious roots of cultivar lalchay microcuttings. Data were recorded after 4 weeks of culture. **A:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L. **B:** Microcuttings cultured on MMS_2 + NAA 0.2 mg/L. **C:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + $AgNO_3$ 0.6 mg/L. **D:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 100 mg/L. **E:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 150 mg/L. **F:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 200 mg/L.

ROOTING IN CULTIVAR BADAMI

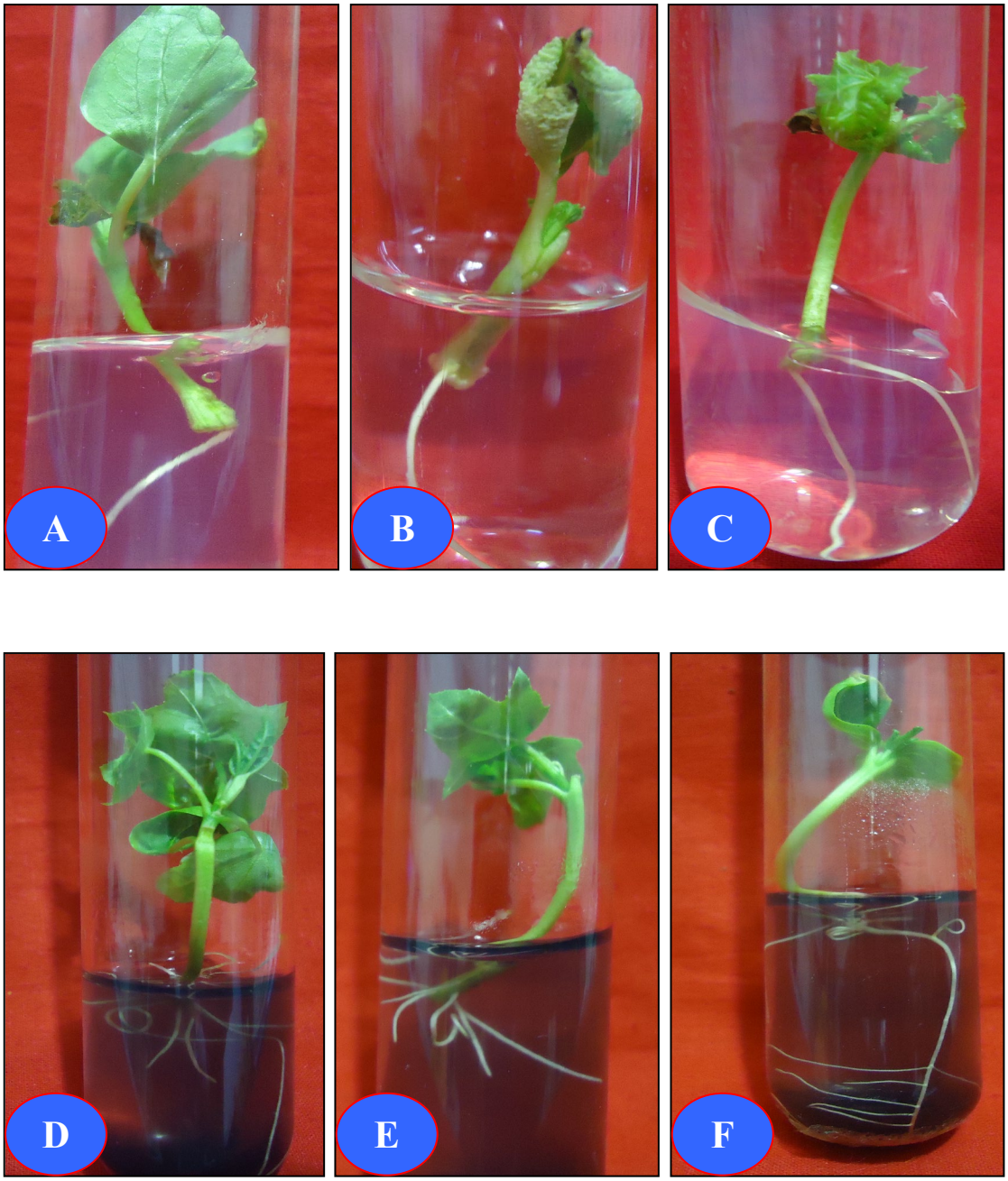


Plate- 4.3.24: Photographs showing the initiation and development of adventitious roots of cultivar badami microcuttings. Data were recorded after 4 weeks of culture. **A:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L. **B:** Microcuttings cultured on MMS_2 + NAA 0.2 mg/L. **C:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + $AgNO_3$ 0.6 mg/L. **D:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 100 mg/L. **E:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 150 mg/L. **F:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 200 mg/L.

ROOTING IN CULTIVAR DHUSAR

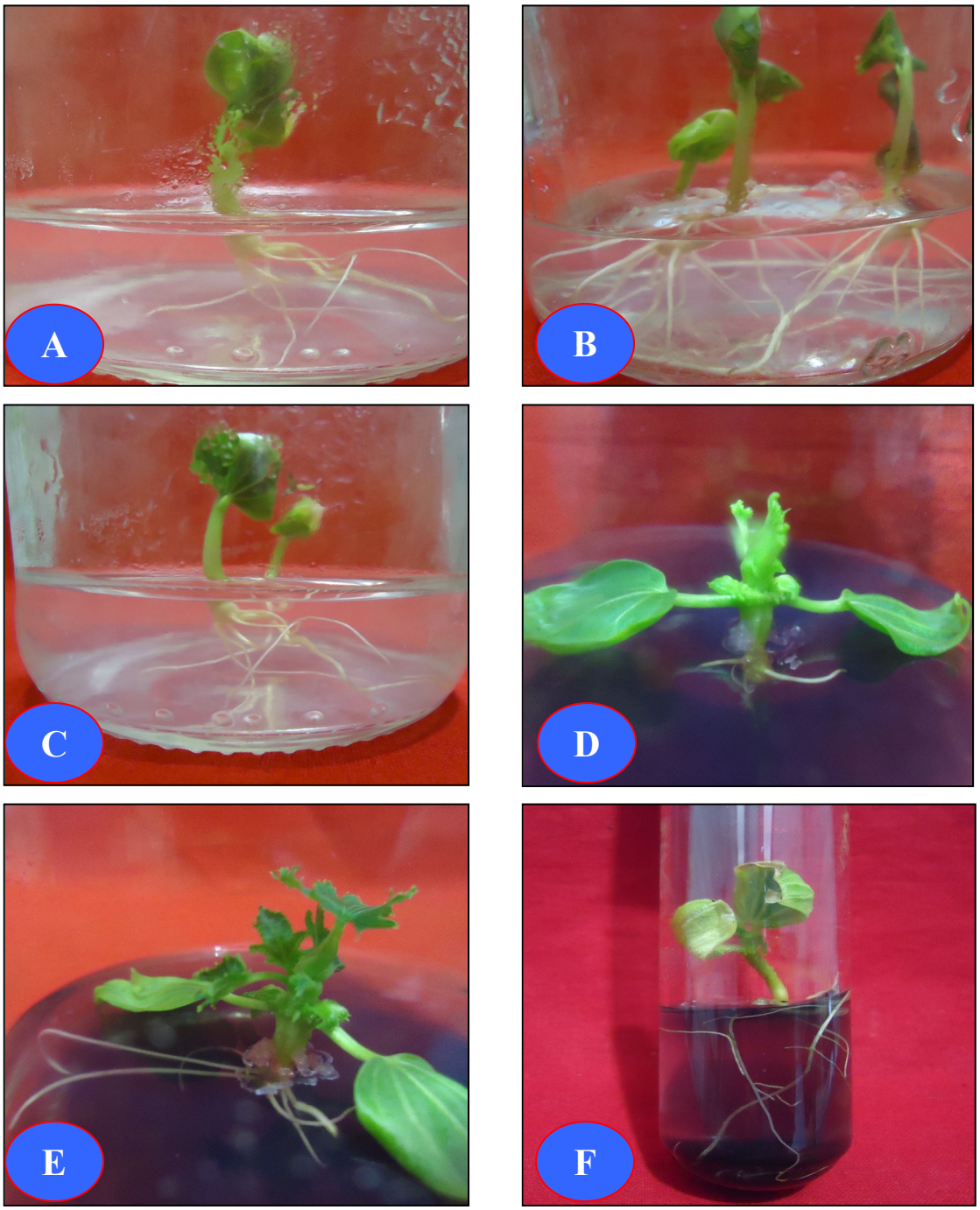


Plate- 4.3.25: Photographs showing the initiation and development of adventitious roots of cultivar dhusar microcuttings. Data were recorded after 4 weeks of culture. **A:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L. **B:** Microcuttings cultured on MMS_2 + NAA 0.2 mg/L. **C:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + $AgNO_3$ 0.6 mg/L. **D:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 100 mg/L. **E:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 150 mg/L. **F:** Microcuttings cultured on MMS_2 + IBA 0.2 mg/L + AC 200 mg/L.

4.4. DISCUSSION

From the embryo to the adult stage of a plant, definite structures and functions differentiate according to a developmental programme impregnated in their inherited genetic makeup. Within the plant body (*in vivo* system), this programme is realized by integrating the network of relation among apical and axillary shoot and root meristems, different organs, tissue and cells. Thus by defying the programme of development in an integrated plant body research at any level is rather difficult (Tran Thanh Van and Trinh 1990). Therefore investigation on *in vitro* regeneration from the dissected plant parts poses many problems that need special attention.

The efficient *de novo* regeneration of plants from cell and tissue cultures been recognized as a pre-requisite for the application of most modern genetic approaches to crop improvement. Only a few years ago, woody fruit crop species were considered to be difficult, or some times impossible, to regenerate *in vitro*. However, castor is extremely recalcitrant to *in vitro* regeneration (Ahn *et al*. 2007). The previous reports on *in vitro* shoot multiplication of castor (Athma and Reddy 1983, Reddy *et al*. 1987b, Sangduen *et al*. 1987, Reddy and Bahadur 1989b, Sarvesh *et al*. 1992) using vegetative tissue as explants was either inefficient or difficult to reproduce (Ahn *et al*. 2007). Then researchers started to focus on meristematic tissues to improve regeneration efficiency (Molina and Schbert 1995, Lakshmi and Bhadur 1997, Sujatha and Reddy 1998). Sujatha and Reddy (1998) reported the first reliable protocol using embryogenic tips and shoot apex. However, all the shoots were derived from the preexisting meristem, and adventitious shoot formation was not observed. When applied in *Agrobacterium* mediated transformation, this protocol resulted in a low rate of putative transformant recovery (0.08%, one putative transformant in, 1,200 embryonic tips used; Sujatha and Sailaja 2005). The transformation efficiency of meristem based protocol was also developed (McKeon and Chen 2003), but the efficiency was also very low. Therefore, it is necessary to develop an effective regeneration protocol by a range of different techniques which would widen the possibilites of developing transgenic lines and/or somaclonal variants. The present investigation was, therefore, undertaken to establish a high frequency plant regeneration system from the seedling explants of local six cultivars of castor using tissue culture technique.

In any preliminary study on the tissue culture of the recalcitrant species, it is customary to use juvenile tissues rather than those from mature plant (Bonga, 1987). It has been reported that juvenile tissue such as cotyledon and different parts of seedlings are good sources of starting material, specially important for callus

induction and successive plant regeneration through organogenesis or embryogenesis (Konar and Oberoi 1965, Arnold and Eriksson 1978, Ohyama and Oka 1980, Hammerschlag *et al*. 1985, Espinasse *et al*. 1989, Miller and Chandra 1990, Dong and Jia 1991). Moreover, mature tissues are also less responsive when compared with juvenile tissues (Sommer and Caldras 1981, Lazzeri *et al*. 1985). The present investigation was, therefore, desigened to conduct experiments using the explants mainly from the *in vitro* grown seedlings. The results of which are discussed in the following paragraphs.

Direct Regeneration

MS (Murashige and Skoog 1962) medium is a recognized as basal medium for tissue culture techniques and extensively used for *in vitro* propagation of various plant species including medicinal plants (Shudha *et al*. 1998, Rout *et al*. 2000, Selvakumar *et al*. 2001, Chandra *et al*. 2003, Sonia and Das 2002, Baksha *et al*. 2005, Binita *et al*. 2005, Sinha *et al*. 2005, Siddique *et al*. 2006, Senthikumar *et al*. 2007, Banu and Bari 2007). Three modified MS medium (MS, MMS_1 and MMS_2) were used for axillary shoot proliferation of six cultivars of castor in ortder to evaluate their comparative efficiencies in shoot proliferation in artificial medium. A number five parameters were selected to record their performance viz. percentage of explant showing proliferation, number of shoot per explant and average length of shoot and days to sprout the axillary buds. Half strength MS medium was proved as the best media formulation for in vitro propagation of castors cultivars as almost all the characters were significantly higher in the medium containing half strength of major salts and full strengths of minor salt and vitamin enriched MS medium (MMS_1). Alam *et al*. (2010) also used MS medium for cotyledonary node culture for the proliferation of auxiliary shoots in castor and obtained regeneration using full strength MS medium. But in our observation, we obtained more efficient regeneration in half strength MS medium than full strength MS medium and this our new finding indicating that MS medium containing half strength of major salts and full strengths of minor salt and vitamin induced better auxillary shoot proliferation in castor. Kamrun and Rira (2012) also obtained *in vitro* propagation from shoot tip explants of four months old castor plant using fortified full strength MS medium. All reported research workers working on *in vitro* regeneration on castor used full strength MS media and most of them encountered recalcitrance problem in shoot proliferation in castor. Half strength MS medium proved as an efficient alternative to avoid recalcitrance to some extent in shoot proliferation in castor. MS medium was formulated with comparatively higher concentrations of nutrient salts than other media commonly used in plant tissue

culture (Bhojawani and Razdan 1983). Although most plants exhibit varied degrees of responses to MS medium, the herbaceous species do better than the woody ones. Litz and Gray (1992) reported for supporting optimum morphogenesis in tissue culture of perennial fruit species variations of the standard MS medium are being increasingly utilized with the major salts diluted to half strength. However, requirement of a low salt medium for getting optimum proliferation response from the explants of woody fruit, forest and ornamental plants has also been suggested and reported by many others (Litz *et al*. 1986, Paranjothy *et al*. 1990, Thorpe *et al*. 1990).

Aseptic seedlings of six cultivars of castor were raised from the surface sterilized and decoated seeds on MS (Murashige & Skoog, 1962) semi-solid medium supplemented with 30 gm/L sucrose and 1.0 mg/L BAP. The aseptic seedling thus grew, attained a height of 8-10 cm after 2-3 weeks of culture. Cotyledonary node and shoot tip were used as explants in the experiment for proliferation of axillary shoots. The experiments were cultured on MS medium supplemented separately with BAP, Kn and 2ip in a single concentration of 1.0 mg/L for proliferating axillary shoots. Among the two types of explants used, proliferation efficiency of cotyledonary node explant was higher than the shoot tip explant. In case cotyledonary node explant, with in very short time it started proliferation, exhibiting greater percentage of response and produced greater number of shoot, number of usable shoot. Alam *et al*. 2010 also used cotyledonary node as an explant for castor and obtained better efficient regeneration. Kamrun and Rita (2012) also reported *in vitro* propagation of castor from shoot tip explants. Better response or calcitrance behavior are supposed to be caused by the indigenous hormonal difference in different plant parts. Variation in the indigenous hormonal levels of the buds present in different regions of the stem plays an important role in their sprouting (Lane 1978) but different *in vitro* responses of various explants taken from the of the same shoot is appeared to be more species specific (Deberg and Read 1990). Tarun *et al*. (2010) did efficient micropropagation of *Comnifera wightii* (Arn) a desart medicinal plant from cotyledonary node explant. Venkatachalan and Kavipriya (2012) reported that they established efficient method for *in vitro* plant regeneration from cotyledonary node explant of Peanut (*Arachis hypogaea* L.). A high efficiency shoot and root formation from cotyledonary nodes of cotton by Özyiğit and Gözükirmizi (2008). *In votro* plant regeneration from cotyledonary node of *Psoralea corylifolia* L. was established by Jeyakumar and Jayabalan (2002). In our experimental results proved that cotyledonary node in castor hold greater prosie in shoot proliferation than apical meristem.

In this experiment, cotyledonary node segment from the seedlings grown on 1.0 mg/L BAP were used for proliferating axillary shoots of six cultivars of castor viz.

cultivar shabje (V_1), cultivar shadatae (V_2), cultivar roktima (V_3), cultivar lalchay (V_4), cultivar badami (V_5) and cultivar dhusar (V_6). These explants were cultured on MMS_1 medium contained 8 gm/L agar, 30 gm/L sucrose and supplemented with BAP, Kn and 2ip at cocentrations of 0.5, 1.0, 1.5, 2.0, 2.5 and 5.0 mg/L for selecting optimum cytokinin concentration for maximum shoot proliferation. Percentage of explants showing proliferation showed 100% in 2.0 mg/L BAP of cultivar shabje and roktima. Number of total shoot per explant, number of usable shoot per explant, average length of shoot was also highest performance showed in cultivar shabje. From the overall observation it was clear that cultivar shabje showed the best performance among the six cultivars of castor.

Growth regulators are organic compounds naturally synthesized from higher plants which influence growth and development. There are two main classes of growth regulators that are specially important in tissue culture. These are the auxin and cytokinin. The other growth regulators viz. gibberellins, abscisic acid, ethylene etc are of minor importance. BAP, Kn and 2ip at a concentration range of 0.5-5.0 mg/L were tested for assessing the optimum concentrations of the cytokinins for early sprouting and maximum proliferation of axillary shoots. Explant taken from the six cultivars of castor seedlings raised on cytokinin-enriched medium showed early sprouting and development of axillary shoots than those taken from the seedlings grown on medium without cytokinin. The greater shoot proliferation ability of explants prepared from the seedlings raised on cytokinin supplemented medium could be attributed to the suppression of apical dominance and stimulation of growth of axillary buds by the cytokinin at an early stage of seedling growth. Similar effect was also noticed in case of monkey jack seedling explants (Rahman and Amin 1994). Although variation in the indigenous hormonal levels of the buds present in different regions of the stem plays an important role in their sprouting (Lane 1978) but different *in vitro* responses of various explants taken from the same shoot is appeared to be more species specific (Deberg and Read 1990). Growth regulators, especially cytokinins and auxins played significant role *in vitro* culture of higher plants. It can be said that *in vitro* culture is often impossible without growth regulators (Pierik 1987). BAP 2.0 mg/L was found to be more effective than either Kn or 2ip on the proliferation and development of six cultivars of castor shoots, an effect similar to that observed by Alam *et al*. (2010) in castor. Sujatha and Reddy (1998) were also used 2.0 mg/L BAP for best shoot induction and development. Danso *et al*. 2011, also reported that, of the three cytokinins used 2ip enhanced the highest shoot regeneration from meristem explants in castor. Superiority of BAP in inducing shoots has been described previously in castor (Sujatha and Reddy 1998). BAP has been reported to

be more beneficial than other cytokinins for micropropagation of various members of the Euphorbiaceae (Tideman and Hawker 1982, Ripley and Preece 1986, Nair *et al*. 1979). In the present study, higher concentrations of BAP reduced the shoot number as well as shoot length. Hu and Wang (1983) reported that higher concentrations of cytokinin reduced the number of micro-propagated shoots. Different concentrations of Kn and 2ip were also induced shoots, but the number of shoots was lower than that in BAP and was not encouraging. However, Kn has a profound effect on shoot length compared with BAP. Similar results were obtained earlier by Sujatha and Reddy (1998). Wareing and Phillips (1981) showed that synthetic cytokinin, such as BAP, was more active than naturally occurring cytokinin, such as 2ip, in shoot proliferation. Although both BAP and Kn are N_6 monosubstituted adenine derivatives but they differ in the nature of their substitution. The difference in efficiencies of BAP and Kn might be related to their difference in substitution position (Biondi and Thorpe 1982).

The plant species belong to the euphorbiaceae family particularly the castor has always been blamed to have recalcitrance property in tissue culture performance. The factors which influence on plant recalcitrance are ascribed to have three three main functions:

a) Whole plant physiology of the donor.

b) *In vitro* manipulations.

c) *In vitro* plant stress physiology (Benson 2000)

Among these three functions to are exclusively related to *in vitro* culture including *in vitro* manipulation and exposure to the physiological stress. In *in vitro* culture sugar, agar and pH status in medium impose physiological stress to the subjected to grow in *in vitro* culture. Under the present investigation experiments were conducted with sugar, agar and pH variation to understand there possible effect on recalcitrance *in vitro* regeneration of castor. Remarkable variation was observed in different concentration of sucrose and agar affected *in vitro* growth and proliferation of six cultivars of castor micro shoots. Complete inhibition of sprouting and development of shoot buds and eventual death of the culture on sucrose free medium confirmed the demand of a easily accessible energy source in the proliferation medium. The *in vitro* grown shoots despite being green, do not rely on photosynthesis and grow as heterotrophs (Bhojwani and Razdan 1983). In this investigation it has been proved without sucrose containing medium failed to produce any axillary bud. Among the different sucrose concentrations tested 30 gm/L sucrose showed the highest percentage (100%) of explant showing proliferation in cultivar shabje, roktima and lalchay followed by 95.5% in cultivar dhusar, 95.0% in cultivar badami and 94.2% in cultivar shadatae and lowest performance showed in 60 gm/L sucrose. Number of

total shoot per culture, number of usable shoot per culture and average length of shoot were also highest in 30 gm/L sucrose and lowest in 60 gm/L sucrose. At the lowest concentration (10 gm/L) tested, shoots were leafy and fewer in number than those at 30 gm/L and 40 gm/L of sucrose. Days to sprout the axillary buds took the lowest time in 30 gm/L and 40 gm/L sucrose highest in 10 gm/L sucrose. Alam *et al*. (2010), Kamrun and Rira (2012) were also used the same amount of sucrose in *in vitro* castor regeneration in their experiments. Yellowing of culture and inhibition of shoot growth on sucrose deficient have also been reported by Maene and Debergh (1985). The results of the present study and those of other (Kumar *et al*. 1984, Maene and Debergh 1985, Schnapp and Preece 1986) which reported the simulation of shoot growth up to certain level of sucrose (said option) and reduction of growth at concentrations beyond the optimum level (here it was 30 gm/L) are in affirmation with the apprehension that the sucrose not only acts as a carbon cum energy source in the medium but also acts as an osmoticum that differs according to the plant tissue under culture (Brown *et al*. 1979, Skirvin 1981). The lusterless, dry appearance of castor shoots proliferate at 50 gm/L of sucrose is in conformity with the appearance of monkey jack shoots grown with a high concentration of sucrose (Rahman and Amin 1995).

The early sprouting and rapid growth of shoot buds as were noticed on medium with lowest agar concentration (4 gm/L) could be attributed to the easy availablity of nutrient elements like Ca, K, Mg and Mn in the soft-gel medium (Deberg 1983). Contrary to this, reduced growth and less number of shoots on media gelled with 10 gm/L of agar could be due to restricted diffusion of macro-nutrients (Romberger and Tabor 1971) or reduced availability of organic matter and water (Stoltz 1971, Skirvin 1981, Debergh 1983). It is evident from results of present investigation and those of others (Debergh 1983) that concentration of agar in the media can affect the culture growth in many ways. Present study also indicate that lower concentration of agar (2-4 gm/L) resulted in the formation of vitrified shoot. It might be due to change in matric potential of media water (Debergh 1983, Arnold and Eriksson 1978, Gasper *et al*. 1987, Hossain *et al*. 1993). In the present investigation optimum agar concentration for the best shoot proliferation was found to be 8 gm/L for six cultivars of castor. Alam *et al*. (2010), Kamrun and Rira (2012) were also reported the same amount of sucrose in castor regeneration. Shoot proliferation was severely suppressed at higher concentration of agar (10 gm/L).

The pH of the culture medium is an important factor in the *in vitro* proliferation and growth of shoots. Therefore, optimization of media pH for healthy culture growth and multiplication rate of six cultivars of castor were recorded at pH

5.7. Alam *et al*. (2010) also adjusted pH at 5.7±0.01, but Kamrun and Rira (2012) adjusted pH at 5.8 for *in vitro* micropropagation of castor. Skirvin (1981) has stated that through most fruit tissue cultures are grown at pH 5.6 - 5.8 but some acid-loving fruit and ornamental plants like blueberry and rhododendron grown best at pH 4.5-4.8 (Anderson 1975, Zimmerman and Broome1981). Improvement of plant growth against recalcitrance can also be enhanced in some plants by ensuring more acidic media in their *in vitro* culture. Multiplication and elongation of chestnut shoots grown *in vitro* were also promoted by more acidic media (Chevre *et al*. 1983). This can be explained by the easy diffusion and availability of mineral in soft gel medium at 4.5 – 5.0. All these indicate species specific requirement of pH for satisfactory growth of cultures on nutrient medium. The present study revealed that both lower (4.7) and higher (6.7) pH level hindered axillary shoot proliferation and shoot elongation. The pH value 5.7 were found to be optimum at which maximum multiple shoots per explant were observed.

Indirect Regeneration

Three types of explants were used for induction of callus viz, hypocotyl, internode and immature leaf. Explants were taken from 3-4 week old aseptically grown seedlings. The explants (10-12 mm) were cultured on to MMS_1 medium supplemented with different concentrations and combinations of BAP with NAA, 2,4-D and IAA. Experiments were conducted to find out the optimum culture media for callus induction. Callus induction was achieved from three types of explants but hypocotyl explant proved as most efficient in callus induction and produced 100% callus showing height degrees of callus growth.

Callus induced was observed in all the cultivars of castor from three types of explants used for callus induction. In these experiments only one cytokinin BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) with all possible combinations of five different concentrations (viz, 0.1, 0.2, 0.5, 0.8 and 1.0 mg/L) of three auxins viz. NAA, 2,4-D and IAA were used. When hypocotyl explant was used in different concentrations of auxins and cytokinin for callus induction of six cultivars it was observed that callus induced from all combinations. Percentage of explants showing callus formation was highest (100%) in cultivar shabje, cultivar roktima and cultivar lalchay followed by cultivar badami (92.5%), cultivar shadatae (92.2%) and cultivar dhusar (91.6%). When internode explant was used for callus induction from six cultivars of castor the rate of highest frequencies of callus induction was found in cultivar shabje (95.3%) followed by cultivar roktima (94.8%), cultivar lalchay (90.4%), cultivar badami (90.3%), cultivar dhusar (88.4%) and cultivar shadatae (88.3%). When internode

explant was cultured on BAP with NAA and 2,4-D callus induction was observed from all cultivars but when cultured on different combinations and concentrations of BAP with IAA, callus did not induce from low levels of IAA (0.1 mg/L) formulations. Callus type/nature of callus showed variations on the basis of cultivars, such as cultivar shabje showed greenish type of callus viz. green friable, green compact, green compact nodular and whitish green, cultivar roktima showed pinkish brown, pinkish nodular, pinkish nodular compact, brown friable, yellow friable and yellow compact, cultivar lalchay showed pinkish brown, pinkish nodular, pinkish friable, brown friable, yellow friable and white friable, cultivar badami and cultivar dhusar showed more or less brownish or whitish type of callus and cultivar shadatae showed greenish type of callus. When immature leaf was used as explants for callus induction it was observed that some concentrations and combinations of auxin and cytokinin failed to produce callus formation mainly when added BAP with lower concentrations of NAA (0.1 mg/L), 2.4-D (0.1-0.2 mg/L) and IAA (0.1-0.5 mg/L). In these experiments the rate of highest frequencies was found in cultivar shabje (63.3%) followed by cultivar roktima (62.1%), cultivar dhusar (62.3%) cultivar badami (61.1%), cultivar shadatae (60.4%) and cultivar lalchay (60.1%). The type of callus was mainly greenish and whitish though cultivar roktima and lalchay showed some pinkish types of callus. In case of intensity of callusing cultivar shabje showed highest intensity followed by cultivar roktima and cultivar lalchay.

After investigation on the axillary shoot proliferation and growth in direct regeneration method, attention was given to induce adventitious shoots through callus culture derived from hypocotyl, internode and immature leaf explants. Many workers observed that MS medium supplemented with different concentrations and combinations of different auxins were more effective on callus induction than their respective individual action. In the present study, MMS_1 medium was used with different concentrations (0.5 - 3.0 mg/L) of BAP accompanied with different concentrations (0.1 - 1.0 mg/L) of NAA, 2,4-D and IAA. The combined action of BAP and NAA showed comparatively best callus formation among the other combinations and concentrations of BAP and auxins tested. Among the thirty BAP and NAA combinations tested, 2.0 mg/L BAP + 0.5 mg/L NAA was proved to be the best for induction and growth of callus from three types of explants of six cultivars of castor while lower as well as higher combinations were less effective in callus induction and callus growth for all cultivars. Similar results were reported by Kumari *et al.* (2008) in *R. communis* L. and they obtained callus induction from cotyledon explants using combination of BAP 2.0 mg/L + 0.8 mg/L NAA. Vila *et al*. (2003) was also reported the similar results in *Melia azedarach*, they obtained callus cultures

using combination of BA (4.4 μM) and NAA (0.46 μM) and successful regeneration of plantlets was obtained by using these callus cultures. Similar to our results in hormonal effects Hossain *et al*. (2007) obtained 100% callus induction rate in brinjal using MS medium containing NAA 2.0 mg/L + 0.05 mg/L BAP. Khafagi in 2007 induced calli from roots, hypocotyls and cotyledonary leaves of castor using BAP with NAA, 2,4-D and IAA. Callus growth and its further differentiation were found to be controlled by cytokinin and auxin ratios. High auxin concentrations resulted in the production of more friable, non-organic callus formation but a cytokinin to auxin ratio of 5:1 was found to be most suitable for differentiation of shoots. These observations support the classical-auxin balance hypothesis of Skoog and Miller (1957). Like our results, organogenic callus induction and plantlet multiplication was obtained in several plants species. In *Iris ensata*, *I. setosa* and *I. sanguinea* supplementation of BAP with NAA showed the best callus induction but NAA or BAP alone showed poor response (Boltenkov and Zarembo 2003). For organogenesis, callus induction from the different explants of *Malus domestica* was also obtained by the combined treatment of BAP and NAA (Caboni *et al*. 2000, Martin 2002). In *Dianthus caryophyllus* also organogenic callus induction was successfully induced by the supplementation of auxin and cytokinin synergistically (Kallak *et al*. 1997).

Regeneration

An attempt was taken for shoot regeneration from hypocotyl derived callus of six cultivars of castor. Kumari *et al.* (2008) reported that in castor, phenolic excretion and oxidation is a severy problem during callus induction and callus mediated regeneration and this problem was solved by the addition of some additives along with plant growth regulators (Ganeson and Jayabalam 2005). Unexpectedly, in this present investigation, excretion of phenolic compounds from explants to the medium was strictly avoid by regular subculturing of callus. For shoot induction from hypocotyl derived callus, MMS_1 medium was used either BAP alone or with different concentrations (0.5-3.0 mg/L) and different concentrations (0.1-1.0 mg/L) of NAA accompanied with GA_3 (0.5-2.0 mg/L) or TDZ (0.5-2.0 mg/L). In this investigation shoot induction was found in cultivar shabje, cultivar roktima and cultivar lalchay. No shoot induction was showed, when the callus cultured on the media having only BAP used or BAP 2.5-3.0 mg/L combined with 0.1-1.0 mg/L NAA fortified with 0.5-2.0 mg/L GA3 or 0.5-2.0 mg/L TDZ. The best result was also found in 1.0 mg/L BAP + 0.2 mg/L NAA + 0.8 mg/L TDZ containing medium. GA_3 containing media were also showed shoot induction, but the percentage were less than TDZ containing media. Similar to this result, TDZ-mediated shoot proliferation was obtained in castor by

Kumari *et al.* (2008). Many workers also reported the TDZ-mediated shoot proliferation was obtained in several plants (Fiola *et al*. 1990, Malik and Saxeha 1992). Usually TDZ was used for somatic embryogenesis in many plant species, either alone or in combination with other growth regulators (Murthy *et al*. 1998, Faisal and Anis 2006). Ahn *et al*. 2007, were also reported that TDZ was very effective in inducing adventitious shoots from hypocotyl tissue in castor.

Among the six cultivars of castor, callus regeneration was obtained from cultivar shabje, cultivar roktima and cultivar lalchay. Among them % of explants showing shoot formation was the highest in BAP 1.0 mg/L + NAA 0.2 mg/L + TDZ 0.8 mg/L of cultivar shabje. Under the statement cultivar shabje exhibited 63.7% callus regeneration followed by cultivar roktima 60.6% and cultivar lalchay 58.2%. Number of shoot per culture was observed highest (9.5 ± 1.93) in cultivar shabje followed by cultivar roktima (9.3±1.90) and cultivar lalchay (9.1±1.85). Other three cultivars shadatae, badami and dhusar produced excessive callus but failed to regenerate under the present investigation. Under the present investigation a complete and efficient protocol for regeneration was established in Bangaladashi castor cultivars. Development of protocol for efficient regeneration is very important and this is the prerequisite for accomplishing of any genetic transformation work. The results of our investigation proved that the Bangladeshi castor cultivars, shabje, roktima and lalchay were proved as the best candidates for performing genetic transformation. Suitable genes with commercial interest can be inserted in the genome of these three castor cultivars either throw direct method by using cotyledonary explant or by indirect method using callus culture.

Rooting

In tissue culture functions, the recalcitrance behavior of euphorbiaceae family is mainly exhibited by poor root formation. Root induction in artificial medium is particularly very hard in castor and for this region the workers are afraid of this trouble of root formation in artificial medium. In our experiment cultured microcuttings showed differential responses depending on strength of MS basal medium. The experiment was designed with two concentrations (0.2, 0.4 mg/L) of IBA, IAA and NAA with singly in full strength MS and three modification of each of basal medium viz. MMS_1 (½ strength of major salts and full strength of minor salts and full organic), MMS_2 (½ strength of major salts and ½ strength of minor salts and full organic), and MMS_3 (¼ strength of major and minor salts and full organic.) for selecting suitable rooting medium. All rooting media were supplemented with 30 gm/L sucrose, gelled with 3 gm/L phytagar and adjusted at pH 5.7 ± 0.1 before

autoclaving. The experiments were carried out by four weeks of incubation. Among the four strengths of MS basal medium, MMS_2 (half strengths of macro and micro nutrients and full strengths of vitamins) was most effective to support comparatively healthy roots.

Axillary shoots that proliferated from different explants under different treatments attained to height 3-5 cm after 6-8 weeks of culture from the date of establishment of the explant under *in vitro* condition. Different experiments were conducted for rooting of the microcuttings prepared from the six cultivars of castor. Among the six cultivars, the rooting frequency was observed highest (85.9%) in cultivar shabje followed by cultivar roktima (83.6%), cultivar lalchay (82.7%), cultivar badami (79.8%), cultivar dhusar (77.5%) and cultivar shadatae (75.7%). Number of root per shoot cutting, length of shoot was showed highest in cultivar shabje. For better root formation 0.2 IBA was used with different concentrations (0.1-1.0 mg/L) of $AgNO_3$ and 0.2 mg/L IBA + different concentrations (25-250 mg/L) activated charcoal were used for six cultivars of castor. Among them 0.2 mg/L IBA +0.6 mg/L $AgNO_3$ was proved to be best concentration for root formation for six cultivars of castor. Activated charcoal and silver nitrate were used was the additives on the culture media in order to induce rooting in the micro cutting but silver nitrate proved better responsive than activated charcoal in root induction of castor.

Basal medium strength, auxin sucrose, agar, pH, photoperiod and culture room temperature substantially affected the development of roots from the *in vitro* axillary and adventitious shoot that proliferated from different explant. Root induction in artificial media is very difficult in castor as the plant was denoted as recalcitrant by several authors (Ahn *et al*. 2007). Kumari *et al.* (2008) reported that root induction in castor-bean was difficult compared to other crop plants. Under the present investigation tremendous efforts has been made to produce root induction under different hormone treatments. For inducing adventitious rooting individual shoots were isolated, trimmed to 20-25 mm microcuttings and cultured on medium supplemented with 0.1-1.0 mg/L of either IAA, IBA, NAA or without any auxin. No rooting was found in the microcuttings cultured on auxin free medium confirmed the essential need of an exogenous auxin for rooting of six cultivars of castor. Similar results were also reported Sujatha and Reddy (1998), they observed hormone-free MS medium failed produce without any rooting. Among the concentrations tested (0.1-1.0 mg/L), rooting responses of microcuttings in 0.2 mg/L IBA containing medium was the best than in either IAA or NAA containing medium. Similar superior performance of IBA was observed during root induction in castor (Kumari *et al.* 2008). Sujatha and Reddy (1998) obtained highest rooting with least callusing using 1.0 mg/L IBA.

While Ahn *et al*. (2007) observed two different rooting pattern using IBA and NAA and percentage of rooting was inversely correlated to shoot development. Hutchinson (1981) and Litz and Jaiswal (1990) emphasized that the preferred auxin for adventitious rooting is IBA. But when $AgNO_3$ and activated charcoal were added with IBA, the root formation was comparatively better than IBA used singly. But the $AgNO_3$ containing medium showed the better result from activated charcoal containing medium. Similar effect was also reported in castor (Kumari *et al*. 2008) when they used $AgNO_3$. The results demonstrated that $AgNO_3$ can influence root emergence and growth and improve rooting efficiency (Bais *et al*. 2000). Similar to our results, it has been accepted that interaction of thiol compounds stimulate rooting *in vitro* (Biddington 1992). He demonostrated that the use of ethylene inhibitors such as $AgNO_3$ might promote root formation in shoot cultures of apple. From the foregoing discussion it can be concluded that the recalcitrant behaviour of castor in some extent is over-come by the use of $AgNO_3$ and activated charcoal in root induction.

It was observed in this study that activated charcoal improved the rooting percentage and root growth. However, presence of activated charcoal in the medium with auxin promoted early emergence and faster growth and inhibited malformation of roots irrespective of subculture period. Kumari *et al*. (2008) were also used activated charcoal for multiple shoot proliferation in castor. Similar result has also been reported by Snir (1983), Cheema and Sharma (1983) for apple root stocks. Anderson (1980) reported the beneficial effects of activated charcoal on rooting of raspberries and Strawberry.

From the foregoing discussion it can be concluded that MMS_1 is identified as the best medium for axillary shoot proliferation and cotyledonary node was identified as the best explant for axillary shoot induction for six cultivars of castor. On the other hand, BAP was proved as the best cytokinin for *in vitro* shoot induction in castor and among all the concentrations tested 2.0 mg/L BAP showed the best performance on axillary shoot proliferation. For callus induction hypocotyl explant proved to be the best explant and 2.0 mg/L BAP + 0.8 mg/L NAA was the best combination and concentration. Cultivar shabje and cultivar roktima showed good performance for callus induction. Shoot regeneration from callus was observed from cultivar shabje, cultivar roktima and cultivar lalchay and the regeneration medium was fortified 2.0 mg/L with 0.5 mg/L NAA and 0.8 mg/L YDZ. MMS_2 was recognized as the most suitable media for rooting in castor. BAP 0.2mg/L accompanied with 0.6 mg/L $AgNO_3$ was the best concentration and combination for adventitious shoot formation. Activated charcoal 150 mg/L also produced healthy rooting without basal callusing

but its efficiency was found lower than that of $AgNO_3$. Of course, basal callusing was obtained when high strength $AgNO_3$ was added to the medium. All over the world castor plant was recognized as one of the most recalcitrant plant, difficult in tissue regeneration. Out of tremendous efforts, we have developed relatively an efficient protocol for *in vitro* regeneration protocol for castor plant particularly in its rooting performance.

SUB CHAPTER 5.1

5.1.1. INTRODUCTION

5.1.1.1. Cell suspension culture

Single cell culture is a method of growing isolated single cell aseptically on a nutrient medium under controlled condition. On the other hand, cell suspension culture is a type of culture in which cells or small aggregates of cells multiply which suspended in agitated liquid medium. It is also referred to as cell culture or cell suspension culture. To achieve an ideal cell suspension, most commonly a friable callus is transferred to agitated liquid medium where it breaks up and readily dispersed. Ideally, suspension culture should consist of only single cells which are physiologically and biochemically uniform. Although this ideal culture has yet to be achieved, but it can be obtained if it is possible to synchronize the process of cell division, cell enlargement within the cell population. Movement of the cells in relation to nutrient medium facilitates gaseous exchange, removes any polarity of the cells due to gravity and eliminates the nutrient gradients within the medium and at the surface of the cells.

Haberlandt (1902) made pioneer attempt to isolate and culture single cell from the leaves of flowering plants. Hildebrandt (1960) attempted to culture single cells of tumerous hybrids tobacco plant (*Nicotiana tabacum X N. glutinosa*) using the microchamber technique. Later, it was shown that the callus tissue obtained from such highly differentiated cells could be induced to produce embryoids. Joshi and Noglle (1967) isolated cells from the mature leaves of groundnut by first tearing across the leaves to expose the mesophyll cells followed by scraping of the cells with a fine scalpel. Bergmann and Bergmann (1968) grew the single cells first using petridish plating technique. Takebe *et al*. (1968) first reported the large scale isolation of metabolically active mesophyll cells of tobacco by enzymatic method.

5.1.1.2. Priciples of single cell culture

The basic principles of single cell culture is the isolation of large number of intact living cells and culture them on a suitable nutrient medium for their requisite growth and development. Single cells can be isolated from a variety of tissue and organ of green plant as well as from callus and cell suspension. Single cells from the intact plant tissue (leaf, stem, root, cladode etc.) are isolated either mechanically or enzymatically. Mechanical isolation involves tearing or chopping the surface sterilized explants to expose the cells followed by scraping of the cells with a fine

scalpel to liberate the single cells hoping that it remains undamaged. But very few living cells are obtained for a lot of time and effort. Gentle grinding of surface sterilized explants in a sterilized mortar pestle followed by cleaning the cells by filtration and centrifugation is now widely used for the large scale mechanical isolation of viable cells. A considerable more efficient way of large scale isolation of free cells from the surface sterilized explant is to dissolve the intercellular cementing material, i.e. pectin, by pectinase or macerozyme treatment. The enzyme macerates the tissue from which large number of viable cells can be obtained. The special feature of enzymatic isolation of cell is that it has been possible to obtain pure preparation of viable cells with less effort and time.

The single cells are traditionally isolated from the established friable callus tissue and cell suspension culture. Mechanically single cells are carefully isolated from cell suspension or friable callus with a needle or fine glass capillary. Alternatively, the friable tissue is transferred to liquid medium and the medium is continuously agitated by a shaker. Agitation of liquid medium breaks and dispenses the single cells and cell clumps in the medium. As a result, it makes a cell suspension. The cell clumps and the filtrate is centrifuged to collect the single cells from the pellets. But under the present investigation the cells were isolated from the established friable callus tissue and cell suspension cultures with a needle or fine glass capillary. Alternatively, the friable tissue was transferred to liquid medium and the medium was continuously agitated by an orbital shaker. Agitation of liquid medium breaks and dispenses the single cells and cell clumps in the medium resulting cell suspension culture.

Interest in plant cell culture as a source of secondary products, stems from the fact that tissues could be cultured in season and out of season, free of pathogens and on chemically defined media with effective methods of altering bio-chemical pathwayes for the synthesis of specific compounds. Commercial applications and economic aspects of mass cell culture have been reviewed by Fowler (1983, 1986). In broad perspective, manipulations of cell culture to produce medicinals, stimulants, agrochemicals, fine chemicals and their utilization for biotransformation represent novel experimental designs that could be exploited to advantage for industrial purpose. Industrial application of plant cell culture for natural product synthesis is in its infancy and still under exploration and experimentation. Different situations need differing approaches and strategies, adaptable to suit particular backgrounds and there is no one system universally applicable to all situations.

5.1.1.3. Importance of cell suspension culture

The culture of single cells and small aggregates in moving liquid medium is an important experimental technique for a lot of studies that are not correctly possible to do from the callus culture. Such a system is capable of contributing much significant information about cell physiology, biochemistry, metabolic events at the level of individual cells and small cell aggregates. It is also important to build up an understanding of an organ formation or embryoid formation starting from single cell or small cell aggregates. The technique of plating out cell suspension on agar plates is of particular value were attempts are being made to obtain single cell clones. Suspension culture derived from medicinally important plants can be studied for the production of secondary metabolites such as alkaloids.

Suspension culture can be used for obtaining single cell clones by plating cell suspension on agar plates. Plants can be regenerated from such clones by the process of embryogenesis. Cell culture may be used for the whole or partial synthesis of secondary plants products, such as alkaloids, glycoside etc. Mutagenesis studies may be facilitated by the use of cell suspension culture to produce mutant cell clones from which mutant plants can be raised. Rapid studies in the field over the years have led to the possibility of growing plant cells as microorganisms in liquid media, for their utilization in the synthesis of a wide range of medicinals, insecticides, steroids, flavonoids and others, but commercial viability and economic feasibility are factors to be reckoned within such industrial ventures. Cell suspension culture are particularly suitable for physiological, biochemical and molecular studies of the process of somatic embryogenesis and its different stages-induction development, maturation and conservation. The other advantage of cell suspension culture is the use of single embryogenic cells and somatic embryos as a source for direct gene transfer via particle bombardment, transient gene expression and confocal microscopy observation.

5.1.1.3.1. Necessity of secondary metabolites

Since the early days of mankind, plants with secondary metabolites have been used by humans to treat infections, health disorders and illness (Wyk and Wink 2004). Only during the last 100 years have natural products been partly replaced by synthetic drugs, for which plant structures were a lead in many instances, for example salicylic acid and aspirin. Many higher plants are major sources of useful secondary metabolites which are used in pharmaceutical, agrochemical, flavor and aroma industries. The search for new plant-derived chemicals should thus be a priority in current and future efforts towards sustainable conservation and rational utilization of

biodiversity (Philipson 1990). Biotechnological approaches, specifically plant tissue culture plays a vital role in search for alternatives to production of desirable medicinal compounds from plants (Rao and Ravishankar 2002). On a global scale, medicinal plants are mainly used as crude drugs and extracts. Several of the more potent and active substances are employed as isolated compounds, including many alkaloids such as morphine (pain killer), codeine (antitussive), papaverine (phosphordiesterase inhibitor), ephedrine (stimulant), ajmaline (antirrhythmic), quinine (antimalarial), reserpine (antihypertensive), galanthamine (acetylecholine esterase inhibitor), scopolamine (travel sickness), berberine (psoriasis), caffeine (stimulant), capsaicin (rheumatic pains), colchicines (gout), yohimbine (aphrodisiac), pilocarpine (glaucoma), and various types of cardiac glycosides (heart insufficiency) (Wink *et al*. 2005).

The capacity for plant cell, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. The strong and growing demand in today's marketplace for natural, renewable products has refocused attention on *in vitro* plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression *in vitro*. However, it is not only commercial significance that drives the research initiatives. The deliberate stimulation of defined chemical products within carefully regulated *in vitro* cultures provides an excellent forum for in-depth investigation of biochemical and metabolic pathways, under highly controlled microenvironmental regimes. Plant-produced secondary compounds have been incorporated into a wide range of commercial and industrial applications, and fortuitously, in many cases, rigorously controlled plant *in vitro* cultures can generate the same valuable natural products. Plants and plant cell cultures have served as resources for flavors, aromas and fragrances, biobased fuels and plastics, enzymes, preservatives, cosmetics (cosmeceuticals), natural pigments, and bioactive compounds. There is a series of distinct advantages to producing a valuable secondary product in plant cell culture, rather than *in vivo* in the whole crop plant. These include:

a) Production can be more reliable, simpler and more predictable.
b) Isolation of the phytochemical can be rapid and efficient, as compared to extraction from complex whole plants.
c) Compounds produced *in vitro* can directly parallel compounds in the whole plant.

d) Interfering compounds that occur in the field-grown plant can be avoided in cell cultures.
e) Tissue and cell cultures can yield a source of defined standard phytochemicals in large volumes.
f) Tissue and cell cultures are a potential model to test elicitation.
g) Cell cultures can be radio labeled, such that the accumulated secondary products, when provided as feed to laboratory animals, can be traced metabolically.

Secondary products in plant cell culture can be generated on a continuous year-round basis; there are no seasonal constraints. Production is reliable, predictable, and independent of ambient weather. At least in some cases, the yield per gram fresh weight may exceed that which is found in nature. Disagreeable odours or flavors associated with the crop plant can be modified or eliminated *in vitro.* Plant cell culture eliminates potential political boundaries or geographic barriers to the production of a crop, such as the restriction of natural rubber production to the tropics or anthocyanin pigment production to climates with high light intensity. When a valuable product is found in a wild or scarce plant species, intensive cell culture is a practical alternative to wild collection of fruits or other plant materials. Extraction from the *in vitro* tissues is much simpler than extraction from organized, complex tissues of a plant. Plant tissue culture techniques offer the rare opportunity to tailor the chemical profile of a phytochemical product, by manipulation of the chemical or physical microenvironment, to produce a compound of potentially more value for human use. Intensive activity has centered on production of natural drugs or chemoprotective compounds from plant cell culture. Some of the most prominent pharmaceutical products in this latter category include ajmalicine (a drug for circulatory problems) from *C. roseus* and taxol (a phytochemical effective in treatment of ovarian cancer) from *Taxus* species.

Chemical synthesis of natural products is possible and commercially feasible, particularly for those with relatively simple chemical structures such as aspirin (derived from the natural product salicylic acid) and ephedrine (Wink *et al*. 2005). In many cases, however, the metabolite has a complex structure, which can include multiple rings and chiral centers, so that a synthetic production process becomes prohibitively costly. Many natural products used in cancer treatment, including compounds such as paclitaxel, vinblastine, and camptothecin, fall into this latter class, so an alternative method of supply is necessary like cell culture.

Depending on the nature of the plant, extraction directly from harvested plant tissue may be an option. Especially if a plant can be cultivated en masse, this can be

attractive on a commercial basis. The anticancer drugs vincristine and vinblastine, among other medicinally valuable metabolites such as ajmalicine and serpentine, are found in the Madagascar periwinkle *Catharanthus roseus* (Liu *et al*. 2007). Even though these important alkaloids, particularly vincristine and vinblastine, naturally occur at very low levels in *C. roseus* – less than 3 g per metric ton – the fast growing nature of the periwinkle makes field cultivation most practical at the present time (Wink *et al*. 2005). However, the relative inefficiency and high cost of whole plant extraction implies that an improved method of supply would be useful for these valuable anti-cancer agents. When natural supply is limited due to a combination of low yields and slow growth rates, an alternative method of supply is necessary like cell culture.

Many of the plant species that provide medicinal herbs have been scientifically evaluated for their possible medical applications. It has been mentioned that natural habitats for medicinal plants are disappearing fast and together with environmental and geopolitical instabilities; it is increasingly difficult to acquire plant-derived compounds. This has prompted industries, as well as scientists to consider the possibilities of investigation into cell cultures as an alternative supply for the production of plant pharmaceuticals.

Advances in biotechnology particularly methods for culturing plant cell cultures, should provide new means for the commercial processing of even rare plants and the chemicals they provide. These new technologies will extend and enhance the usefulness of plants as renewable resources of valuable chemicals. There has been considerable interest in plant cell cultures as a potential alternative to traditional agriculture for the industrial production of secondary metabolites (Dicosmo and Misawa 1995). Plant cell culture technologies were introduced at the end of 1960s as a possible tool for both studying and producing plant secondary metabolites. Different strategies using cell cultures systems have been extensively studied with the objective of improving the production of bioactive secondary metabolites. Cell culture systems could be used for the large scale culturing of plant cells from which secondary metabolites can be extracted. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products. The major advantages of cell cultures includes (i) synthesis of bioactive secondary metabolites is running in controlled environment, independently from climatic and soil conditions; (ii) negative biological influences that affect secondary metabolites production in the nature are eliminated (microorganisms and insects); (iii) it is possible to select cultivars with higher production of secondary metabolites; (iv) with automatization of cell growth control and metabolic processes regulation, cost price can decrease and production

increase. The objectives of many industries are to develop plant cell culture techniques to the stage where they yield secondary products more cheaply than extracting either the whole plant grown under natural conditions or synthesizing the product. Although the production of pharmaceuticals using plant cell cultures have been highlighted, other uses have also been suggested as new route for synthesis, for products from plants difficult to grow, or in short supply, as a source of novel chemicals and as biotransformation systems. It is expected that the use, production of market price and structure would bring some of the other compounds to a commercial scale more rapidly and *in vitro* culture products may see further commercialization. Recent research results indicate that plant cell suspension cells can be used for recombinant protein production under controlled conditions (Fischer *et al*. 1999).

5.1.1.3.2. Accumulation of secondary metabolites in plant cell cultures.

For plant cell culture techniques to become economically viable, it is important to develop methods that would allow for consistent generation of high yields of products from cultured cells (Berlin and Sasse 1985). Careful selection of productive cells and cultural conditions resulted in accumulation of several products in higher levels in cultured cells. In order to obtain yields in high concentrations for commercial exploitation, efforts have focused on the stimulation of biosynthetic activities of cultured cells using various methods (Ramachandra 2000, Dixon 1999, Ravishankar and Venkataraman 1993, Buitelaar and Tramper 1992). Culture productivity is critical to the practical application of plant cell culture technology to production of plant-specific bioactive metabolites. Until now, various strategies have been developed to improve the production of secondary metabolites using plant cell cultures. The tissue culture cells typically accumulate large amounts of secondary compounds only under specific conditions. That means maximization of the production and accumulation of secondary metabolites by plant tissue cultured cells requires (i) manipulating the parameters of the environment and medium, (ii) selecting high yielding cell clones, (iii) precursor feeding, and (iv) elicitation.

5.1.1.3.3. Selection of high-producing strains

Plant cell cultures represent a heterogeneous population in which physiological characteristics of individual plant cells are different. Synthesis of several products in high amounts using selection and screening of plant cell cultures have been described by Berlin and Sasse (1985). Cell cloning methods provide a promising way of selecting cell lines yielding increased levels of product. A strain of *Euphorbia milli* accumulated about 7-fold the level of anthocyanins produced by the

parent culture after 24 selections (Yamamoto *et al*. 1982). Selection can be easily achieved if the product of interest is a pigment (Fujita *et al*. 1984). Cell cloning using cell aggregates of *Coptis japonica* (Yamada and Sato 1981), and obtained strain, which grew faster and produced a higher amount of berberin and cultivated the strain in a 14 L bioreactor. Selected cell line increased growth about 6-fold in 3 weeks and the highest amount of alkaloid was produced 1.2 g/L of the medium and the strain was very stable, producing a high level of berberin even after 27 generations. Increased capsaicin and rosmarinic acid in PEP cell lines of *Capsicum annuum* were reported (Salgado and Ochoa 1990). Selective agents such as 5-methyltryptophan, glyphosate and biotin have also been studied to select high-yielding cell lines (Amrhein *et al*. 1985, Watanabe *et al*. 1982, Widholm 1974).

The use of *in vitro* plant cell culture for the production of chemicals and pharmaceuticals has made great strides building on advances in plant science. The increased use of genetic tools and an emerging picture of the structure and regulation of pathways for secondary metabolism will provide the basis for the production of commercially acceptable levels of product. The increased level of natural products for medicinal puroposes coupled with the low product yields and supply concerns of plant harvest has renewed interest in large-scale plant cell culture technology. Knowledge of biosynthetic pathways of desired phytochemicals in plants as well as in cultures is often still in its infancy, and consequently stratigies needed to develop an information based on a cellular and molecular level. These results show that *in vitro* plant cell cultures have potential for commercial production of secondary metabolites. The introduction of newer techniques of molecular biology, so as to produce transgenic cultures and to effect the expression and regulation of biosynthetic pathways, is also likely to be a significant step towards making cell cultures more generally applicable to the commercial production of secondary metabolites.

Castor is a medicinal plant as it contains ricinolic acid containing ricin. Ricin has been used experimentally in medicine to kill cancer cells. Castor oil enhance the immune system. Numerous AIDS patient have been able to increase their T-cell counts and clear up many of their problems by using castor oils (Anonymous 2012). In Bangladesh *R. communis* is widely grown all over the country holding great potential for producing castor oil as a source of green energy and also for commercial production of toxic protein ricin. We have developed the standard protocol for *in vitro* generation of *R. communis* and under the present investigation we have made efforts to develop the standard protocol for wide scale callus culture and cell culture of six cultivars of castor towards isolation of active protein ricin from these cultures.

5.1.1.4. Objectives

The present work was undertaken with the following objectives:

1. Standardization suitable culture media for the establishment of cell suspension culture of six cultivars of castor in Bangladesh.
2. Standardization of suitable hormone concentration for cell culture and their maintenance.
3. Measurement of cell growth in different duration of time.
4. Callus induction and culture from single cell clones.

5.1.2. MATERIALS AND METHODS

5.1.2.1. Materials

5.1.2.1.1. Experimental materials

Friable embryogenic calli containing rapidly dividing cells were used as experimental materials for cell suspension culture taken form six castor cultivars. These calli were harvested from *in vitro* grown callus of 35-42 days old and cultured in liquid medium.

5.1.2.1.2. Culture media

Modified (½ strength of major salts and full strength of minor salts and full organic.) MS (Murashige and Skoog, 1962) medium was used for the purpose of single cell culture.

5.1.2.1.3. Laboratory equipments

a) For media preparation:

Different types of glass vessels including conical flasks with plugs, beakers, measuring cylinders and pipettes each of various capacities were used for culturing single cell.

b) Chemicals:

All chemical compounds including micro- and macro-nutrients, growth regulators, sugar, agar, $HgCl_2$, alcohol etc., used in the present study, were the reagent grade product of Carollina (America) and Fluka (Germany). The vitamins, amino acids, and growth regulators were the product of Carollina Biological Supply Company of U.S.A.

c) Instruments for aseptic transfer and culture:

Autoclave, laminar airflow cabinet, forceps of various sizes, pipettes, petridishes, 500µ diameter pore sieve, rotary shaker, filter paper, media filter sterilizer, fire box, marker pen, spirit lamp, needle, scalpel blade, electronic balance were used in the present investigation.

d) For incubation:

Racks with light arrangement (16 h light and 8 h dark) and controlled temperature 25º ± 2ºC maintained with air cooler used in the dark room and light for culture room.

5.1.2.2. Methods

5.1.2.2.1. Preparation of culture media

a) Liquid media preparation for cell isolation:

Liquid media were prepared following MS medium preparation without adding agar and then autoclaved for 20 minutes under at 1.1 kg/cm² pressure and 121-˚c temperature.

b) Semisolid media preparation for callus induction from isolated cells:

Semisolid medium were prepared for callus induction from isolated single cells of cell suspension culture. It was prepared following the methods MS media when agar was used to solidify the medium and then autoclaved for 20 minutes under at 1.1 kg/cm² pressure and 121˚c temperature.

5.1.2.2.2. Cell isolation and culture.

a) Cell isolation from friable callus:

In this investigation, to isolate the cells, most commonly friable callus was transferred to agitate liquid medium where it was broken up and readily dispersed. In this purpose, at first friable callus was developed using the conventional method. Then this friable callus was transferred into the 250 ml conical flask containing 75 ml liquid (medium without agar) medium. Then the flasks were placed on the shaker and shake under 100 rpm. After 4 days, cells were isolated. At last the single cells were filtrated using sieve which have 500μ diameter pore. The used steps to get single cells were mentioned bellow-

1. An amount of 75 ml liquid medium (autoclaved) was taken in 250 ml conical flask
2. Pre-established friable callus were transferred aseptically to the conical flasks containing autoclaved liquid MS medium (75 ml).
3. Then the closures of the flasks were covered with brown papers and the flasks were placed within the clamps of an orbital shaker moving at 200 rpm.
4. After 4 days the contents of each flask were poured through a sterilized sieve (500 μ) and the filtrate was collected in a big sterilized container. The filtrate contained only free cells.

To ensure this scene, that is, the filtrate contained only single cells, some slides were prepared with the filtrate and photographs were taken for each of the slides.

b) Culture of isolated cells:

Free cells isolated from callus are grown as single cells under *in vitro* conditions using modified MS medium supplemented with different concentrations and combinations of hormones to observe the growth/division pattern of cells. The following steps are taken for this investigation.

1. The pre-filtrate containing single cells was allowed to settle for 20-25 minutes and finally poured off the supernatant.
2. Using the sterilized pipette, 5 ml residue cells re-suspended into the fresh liquid medium and dispensed the cells equally in several sterilized flasks of 250 ml which contain 70 ml liquid medium.
3. The flasks containing cells were placed on shaker with 200 rpm and allowed the free cells to grow.

5.1.2.2.3. Subculture of the cells for maintenance

When the cells reached the maximum in number in the liquid medium, the suspension was used to subculture. For subculture of cells the previous steps are repeated and taken 5 ml of residual cells as the inoculums for each flask and dispensed equally in the flasks and place them on shaker. All the works were done on the laminar air flow cabinet to avoid any contamination. The subcultured cells contained to proliferate and showed various types of response.

5.1.2.2.4. Callus induction from incubated cells.

For callus induction, the isolated cells six cultivars of castor harvested from the suspension were subcultured in the petridishes containing freshly prepared modified MS media supplemented with different concentrations and combinations of auxin and cytokinin. The head of petridish was removed inside the laminar air flow cabinet and the single cells were incubated with a needle in the petridishes containing media. Then the petridish was covered with its head followed by sealing with parafilm and sent to the dark chamber having 25±2ºC. A regular observation was performed to record the callusing response of the cells.

5.1.2.2.5. Data collection

a) Fresh weight of cells

The fresh weight of cells was taken carefully on the running laminar flow cabinet by electric balance using filter paper and media filter sterilizer. At first the weight of filter paper was taken and this was taken on the tip of media filter sterilizer.

Then the 5 ml growing cells was taken from liquid culture by a sterile pipette and was taken on the filter paper which was taken before. Then the media filter sterilizer was started. After some times, when the cells were dried and shown as powder then the weight was taken of the dry cells with filter paper. Then the weight of filter paper was debited from the weight of filter paper and dry cells weight. The weights were taken every after two days.

5.1.3: RESULTS

The present investigation was carried out for cell suspension culture of six cultivars of castor. The objective of this investigation was to establish a protocol for the cell suspension culture. Details of the results obtained from each of the experiments are described below-

5.1.3.1. Cell suspension culture.

In the present investigation, free cells isolated from friable embryogenic calli drived from hypocotyl of six cultivars of castor cv. Shabje (Plate 5.1.3.1, A), Shadatae (Plate 5.1.3.2, A), Roktima (Plate 5.1.3.3, A), Lalchay (Plate 5.1.3.4, A), Badami (Plate 5.1.3.5, A) and Dhusar (Plate 5.1.3.6, A) were used as explants and the effects of different concentrations and combinations of different auxins and cytokinin (NAA, 2,4-D and BAP) on cell suspension culture. The filtrated cells and cell aggregates were allowed to settle for 20-25 minutes and finally poured off the supernatant. Using the sterilized pipette, 5 ml residue cells were re-suspended into the fresh liquid medium and dispensed the cells equally in several sterilized flasks of 250 ml containing 70 ml liquid medium. The flasks containing cells were placed on the rotary shaker with 200 rpm and allowed the free cells to grow. Under agitated state the cells were grown in different sizes and growth stages. Every after 2 days of culture the liquid medium was filtrated through the sieve with pore having diameter of 500µ. This time, the filtrate contained only the free growing cell masses. The growing plant cells were confirmed by examining the drop of filtrate under microscopic examination (Plate 5.1.3.1, 5.1.3.2, 5.1.3.3, 5.1.3.4, 5.1.3.5 and 5.1.3.6, C). This cell suspension culture was maintained in the lab and served as the stock for starting any experiments with cell culture. Data (weight in gram) were recorded on cell weight per 10 ml cell suspension for 8 times with the interval of every after 2 days of culture. The experimental observation revealed that the cells grew gradually in the suspension culture of all hormonal concentrations and combinations. The effects so far obtained from each of the experiments of different concentrations and combinations of different hormones on cell suspension culture are mentions below:

5.1.3.1.1. Effect of different concentrations of NAA with 2.0 mg/L BAP on cell suspension culture

The experiment was conducted to find out the combined effect of different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mg/L) of NAA with 2.0 mg/L BAP, 1.0 mg/L biotin and 1.0 mg/L glutamine on cell suspension culture of six cultivars of castor cv. Shabje, Shadatae, Roktima, Lalchay, Badami and Dhusar. The cultures

were maintained under total dark regime at 25±2ºC temperature. The growth of the cell suspension was assessed by measuring the weight of cells and cell clusters. The obtained data are shown in Table 5.1.3.1.

The cells and the cell clusters of all cultivars responded in all the media formulations. The peak period of cell growth was observed within 4th - 6th days of suspension culture and the highest weight of the cells and cell aggregates of all cultivars were obtained on the 14th days of suspension culture in modified liquid MS medium supplemented with 2.0 mg/L BAP + 0.3 mg/L NAA accompanied with 1.0 mg/L biotin and 1.0 mg/L glutamine. The Shabje cultivar showed the maximum cell weight which was 0.143±0.13 gm while the maximum cell weight of other cultivars cv. Shadatae, Roktima, Lalchay, Badami and Dhusar were 0.133±0.12 gm, 0.138±0.13 gm, 0.135±0.13 gm, 0.126±0.12 gm and 0.129±0.12 gm in the above media formulation.

In this investigation, lower and higher concentrations of NAA with BAP were found less effective for cell growth. The minimum weight of cells of Shabje, Shadatae, Roktima, Lalchay, Badami and Dhusar were 0.079±0.08 gm, 0.043±0.06 gm, 0.068±0.07 gm, 0.074±0.07 gm, 0.056±0.07 gm and 0.045±0.06 gm respectively in MS medium having 2.0 mg/L BAP+ 0.1 mg/L NAA on the 2nd day of culture. The growth of cells was continued until 16 days of culture it was ceased down after 16th day.

5.1.3.1.2. Effect of different concentrations of 2,4-D with 2.0 mg/L BAP on cell suspension culture

This experiment was conducted using different concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mg/L) 2,4-D in combination with 2.0 mg/L BAP accompanied with 1.0 mg/L biotin and 1.0 mg/L glutamine to understand their combine effect on cell suspension culture in castor cultivars. Data were collected per 10 ml cell suspension for 8 times with the interval of every after 2 days of culture. The experimental observation revealed that the cells grew gradually with the increasing of time of all hormonal concentrations and combinations. The collected data were summarized in Table 5.1.3.2.

Among the five culture media formulations, 2.0 mg/L BAP in combination with 0.3 mg/L NAA, 1.0 mg/L biotin and 1.0 mg/L glutamine was found to be most effective on cell suspension culture for all the castor cultivars. Among the six cultivars, Shabje was found to be the most responsive genotype. The optimum period of cell growth was observed within 4th - 6th days of cell suspension culture in MS medium supplemented with 2.0 mg/L BAP + 0.3 mg/L 2,4-D. The Shabje cultivar

showed the maximum cell weight and it was 0.139±0.11 gm (Plate 5.1.3.1, B) while the maximum cell weight of other cultivars cv. Shadatae, Roktima, Lalchay, Badami and Dhusar were 0.130±0.10 gm (Plate 5.1.3.2, B), 0.138±0.12 gm (Plate 5.1.3.3, B), 0.135±0.12 gm (Plate 5.1.3.4, B), 0.126±0.11 gm (Plate 5.1.3.5, B) and 0.129±0.11 gm (Plate 5.1.3.6, B) respectively in the same hormonal concentration. The lowest weight of cells of Shabje, Shadatae, Roktima, Lalchay, Badami and Dhusar were 0.074±0.07 gm, 0.040±0.06 gm, 0.064±0.06 gm, 0.070±0.07 gm, 0.054±0.05 gm and 0.045±0.05 gm respectively in modified liquid MS medium having 2.0 mg/L BAP + 0.1 mg/L 2,4-D on the 2nd day of culture. The growth of cells was continued until 16 days of culture but it was ceased down after 16th day.

Table 5.1.3.1: Effect of different concentrations of NAA with 2.0 mg/L BAP, 1.0 mg/L biotin and 1.0 mg/L glutamine on cell growth of six cultivars of castor.

Concentrations of BAP+NAA (gm/l)	Days after incubation	Weight of cells of six cultivars of castor (gm)($\bar{x} \pm SE$)					
		Shabje (V_1)	Shadatae (V_2)	Roktima (V_3)	Lalchay (V_4)	Badami (V_5)	Dhusar (V_6)
2.0+0.1	2nd	0.079±0.08	0.043±0.06	0.068±0.07	0.074±0.07	0.056±0.07	0.045±0.06
	4th	0.092±0.10	0.053±0.07	0.082±0.09	0.084±0.08	0.068±0.08	0.060±0.07
	6th	0.112±0.12	0.103±0.09	0.107±0.10	0.102±0.11	0.088±0.09	0.100±0.10
	8th	0.120±0.12	0.110±0.10	0.118±0.11	0.112±0.11	0.100±0.10	0.112±0.10
	10th	0.125±0.11	0.116±0.10	0.122±0.12	0.118±0.12	0.108±0.12	0.116±0.11
	12th	0.128±0.12	0.120±0.11	0.125±0.13	0.122±0.12	0.114±0.11	0.118±0.12
	14th	0.130±0.13	0.128±0.12	0.128±0.13	0.125±0.14	0.116±0.12	0.120±0.12
	16th	0.130±0.14	0.128±0.13	0.128±0.14	0.125±0.14	0.116±0.13	0.120±0.13
2.0+0.2	2nd	0.084±0.08	0.046±0.06	0.071±0.07	0.078±0.07	0.061±0.06	0.048±0.05
	4th	0.098±0.10	0.058±0.07	0.086±0.08	0.088±0.08	0.072±0.07	0.063±0.06
	6th	0.118±0.12	0.108±0.09	0.111±0.09	0.107±0.09	0.092±0.08	0.104±0.08
	8th	0.125±0.11	0.116±0.10	0.123±0.19	0.116±0.10	0.104±0.09	0.115±0.09
	10th	0.131±0.11	0.122±0.10	0.128±0.11	0.123±0.10	0.112±0.11	0.120±0.11
	12th	0.135±0.12	0.126±0.11	0.131±0.11	0.128±0.11	0.119±0.10	0.123±0.11
	14th	0.138±0.12	0.130±0.11	0.134±0.12	0.130±0.12	0.122±0.11	0.125±0.11
	16th	0.138±0.13	0.130±0.12	0.134±0.13	0.130±0.13	0.122±0.12	0.125±0.12
2.0+0.3	2nd	0.087±0.09	0.049±0.07	0.074±0.08	0.082±0.08	0.064±0.07	0.051±0.06
	4th	0.102±0.11	0.061±0.08	0.090±0.09	0.092±0.09	0.077±0.08	0.066±0.07
	6th	0.122±0.13	0.112±0.10	0.115±0.10	0.112±0.10	0.097±0.09	0.109±0.09
	8th	0.130±0.12	0.121±0.11	0.128±0.11	0.120±0.11	0.109±0.10	0.120±0.10
	10th	0.136±0.12	0.127±0.11	0.132±0.12	0.127±0.11	0.117±0.12	0.125±0.12
	12th	0.140±0.13	0.131±0.12	0.135±0.12	0.133±0.12	0.123±0.11	0.128±0.12
	14th	0.143±0.13	0.133±0.12	0.138±0.13	0.135±0.13	0.126±0.12	0.129±0.12
	16th	0.143±0.14	0.133±0.13	0.138±0.14	0.135±0.14	0.127±0.13	0.129±0.13
2.0+0.4	2nd	0.085±0.10	0.047±0.08	0.072±0.09	0.080±0.09	0.062±0.08	0.049±0.07
	4th	0.100±0.12	0.059±0.09	0.088±0.10	0.090±0.10	0.074±0.09	0.064±0.08
	6th	0.119±0.14	0.110±0.11	0.113±0.11	0.109±0.11	0.094±0.10	0.106±0.10
	8th	0.127±0.13	0.118±0.12	0.125±0.12	0.118±0.12	0.106±0.11	0.117±0.11
	10th	0.133±0.13	0.124±0.12	0.129±0.13	0.125±0.12	0.114±0.13	0.122±0.13
	12th	0.137±0.14	0.128±0.13	0.132±0.13	0.130±0.13	0.120±0.12	0.125±0.13
	14th	0.140±0.14	0.132±0.13	0.135±0.14	0.132±0.14	0.123±0.13	0.126±0.13
	16th	0.140±0.14	0.132±0.14	0.135±0.15	0.132±0.15	0.123±0.14	0.126±0.14
2.0+0.5	2nd	0.082±0.09	0.045±0.07	0.070±0.08	0.076±0.08	0.060±0.07	0.047±0.06
	4th	0.096±0.11	0.056±0.08	0.084±0.09	0.086±0.09	0.070±0.08	0.062±0.07
	6th	0.116±0.13	0.106±0.10	0.109±0.10	0.105±0.10	0.090±0.09	0.102±0.09
	8th	0.123±0.12	0.114±0.11	0.120±0.11	0.114±0.11	0.102±0.10	0.113±0.10
	10th	0.128±0.12	0.120±0.11	0.125±0.12	0.121±0.11	0.110±0.12	0.118±0.12
	12th	0.132±0.13	0.121±0.12	0.128±0.12	0.125±0.12	0.116±0.11	0.120±0.12
	14th	0.135±0.13	0.130±0.12	0.131±0.13	0.127±0.13	0.119±0.12	0.122±0.12
	16th	0.135±0.14	0.130±0.13	0.131±0.14	0.127±0.14	0.119±0.13	0.122±0.13

Table 5.1.3.2: Effect of different concentrations of 2,4-D with 2.0 mg/L BAP, 1.0 mg/L biotin and 1.0 mg/L glutamine on cell growth of six cultivars of castor.

Concentrations of BAP+2,4-D (gm/l)	Days after incubation	Weight of cells of six cultivars of castor (gm)($\bar{x} \pm SE$)					
		Shabje (V_1)	Shadatae (V_2)	Roktima (V_3)	Lalchay (V_4)	Badami (V_5)	Dhusar (V_6)
2.0+0.1	2nd	0.074±0.07	0.040±0.06	0.064±0.06	0.070±0.07	0.054±0.05	0.045±0.05
	4th	0.087±0.09	0.049±0.06	0.078±0.07	0.081±0.06	0.062±0.06	0.059±0.06
	6th	0.108±0.08	0.098±0.07	0.102±0.08	0.100±0.07	0.085±0.07	0.097±0.07
	8th	0.115±0.10	0.105±0.08	0.113±0.07	0.110±0.08	0.096±0.07	0.110±0.07
	10th	0.120±0.09	0.111±0.09	0.118±0.09	0.115±0.09	0.105±0.08	0.114±0.08
	12th	0.123±0.09	0.115±0.10	0.120±0.09	0.119±0.10	0.112±0.08	0.116±0.09
	14th	0.125±0.10	0.123±0.09	0.123±0.08	0.121±0.10	0.114±0.09	0.117±0.09
	16th	0.125±0.11	0.123±0.10	0.123±0.09	0.121±0.11	0.114±0.10	0.117±0.10
2.0+0.2	2nd	0.078±0.07	0.046±0.07	0.068±0.08	0.074±0.07	0.059±0.07	0.046±0.05
	4th	0.094±0.08	0.054±0.08	0.083±0.09	0.085±0.08	0.070±0.08	0.060±0.06
	6th	0.114±0.09	0.100±0.10	0.108±0.10	0.104±0.09	0.090±0.09	0.100±0.07
	8th	0.121±0.08	0.114±0.10	0.120±0.10	0.112±0.09	0.100±0.10	0.112±0.08
	10th	0.127±0.09	0.118±0.10	0.125±0.10	0.120±0.10	0.108±0.12	0.118±0.08
	12th	0.131±0.10	0.123±0.11	0.127±0.09	0.124±0.10	0.116±0.11	0.120±0.09
	14th	0.134±0.10	0.126±0.11	0.130±0.10	0.125±0.11	0.118±0.12	0.122±0.10
	16th	0.134±0.11	0.126±0.12	0.130±0.10	0.125±0.11	0.118±0.13	0.122±0.10
2.0+0.3	2nd	0.085±0.09	0.049±0.07	0.074±0.08	0.082±0.08	0.064±0.06	0.051±0.06
	4th	0.100±0.11	0.059±0.08	0.090±0.09	0.092±0.08	0.077±0.07	0.066±0.07
	6th	0.119±0.13	0.110±0.10	0.115±0.10	0.112±0.10	0.097±0.08	0.109±0.08
	8th	0.127±0.12	0.118±0.09	0.128±0.10	0.120±0.11	0.109±0.09	0.120±0.10
	10th	0.133±0.12	0.125±0.11	0.132±0.11	0.127±0.11	0.117±0.11	0.125±0.10
	12th	0.137±0.13	0.128±0.11	0.135±0.11	0.133±0.12	0.123±0.10	0.128±0.11
	14th	0.140±0.13	0.130±0.10	0.138±0.12	0.135±0.12	0.126±0.11	0.129±0.11
	16th	0.140±0.14	0.130±0.11	0.138±0.13	0.135±0.13	0.127±0.12	0.129±0.12
2.0+0.4	2nd	0.084±0.08	0.044±0.07	0.070±0.08	0.077±0.08	0.060±0.07	0.042±0.06
	4th	0.100±0.10	0.057±0.08	0.084±0.09	0.086±0.09	0.072±0.08	0.062±0.07
	6th	0.119±0.11	0.105±0.10	0.110±0.10	0.105±0.10	0.090±0.09	0.104±0.09
	8th	0.127±0.11	0.115±0.11	0.120±0.11	0.115±0.11	0.103±0.10	0.115±0.10
	10th	0.133±0.10	0.120±0.11	0.124±0.12	0.121±0.11	0.111±0.12	0.120±0.12
	12th	0.136±0.11	0.124±0.12	0.127±0.12	0.125±0.12	0.117±0.11	0.122±0.12
	14th	0.139±0.11	0.128±0.12	0.131±0.13	0.128±0.13	0.120±0.12	0.123±0.12
	16th	0.139±0.12	0.128±0.13	0.131±0.14	0.128±0.14	0.120±0.13	0.123±0.13
2.0+0.5	2nd	0.080±0.09	0.044±0.07	0.068±0.08	0.074±0.09	0.058±0.07	0.047±0.06
	4th	0.093±0.11	0.054±0.08	0.082±0.09	0.083±0.09	0.068±0.08	0.060±0.07
	6th	0.113±0.10	0.103±0.10	0.105±0.10	0.102±0.11	0.087±0.09	0.100±0.09
	8th	0.120±0.11	0.111±0.10	0.116±0.12	0.112±0.11	0.100±0.09	0.112±0.10
	10th	0.125±0.11	0.117±0.11	0.123±0.12	0.118±0.12	0.108±0.10	0.115±0.11
	12th	0.130±0.13	0.119±0.11	0.126±0.12	0.122±0.12	0.114±0.11	0.117±0.12
	14th	0.132±0.13	0.127±0.12	0.129±0.13	0.125±0.14	0.115±0.11	0.120±0.13
	16th	0.132±0.13	0.127±0.13	0.129±0.13	0.125±0.14	0.115±0.12	0.120±0.13

5.1.3.2. Callus induction from isolated cells.

For callus induction, the isolated cells of six cultivars were cultured on modified MS media supplemented with 2.0 mg/L BAP with five different concentrations of NAA (0.1, 0.2, 0.5, 0.8 and 1.0 mg/L). Data were recorded on frequency of callus induction, percentage of explant responded and colour of callus. Data were recorded after 6 weeks of culture of six cultivars of castor and presented below in separate heads.

i) Cultivar shabje: Callus induction was observed in all BAP and NAA formulations (Table 5.1.3.3a). The response of the explants were found to vary with growth regulator concentrations used. Percentage of explants showing callus formation was the highest (6.7%) when the explants cultured on 2.0 mg/L BAP with 0.5 mg/L NAA (Plate 5.1.3.1, D). Callus induction from the explants were taken the lowest time in the same combination and concentration and it was 20 days. The colour of callus in this experiment were found whitish (W).

ii) Cultivar shadatae: Callus induction was observed in all BAP and NAA formulations (Table 5.1.3.3a). The response of the explants were found to vary with growth regulator concentrations used. Percentage of explants showing callus formation was the highest (6.0%) when the explants cultured on 2.0 mg/L BAP with 0.5 mg/L NAA (Plate 5.1.3.2, D). Callus induction from the explants were taken the lowest time in the same combination and concentration and it was 22 days. The colour of callus in this experiment were found whitish (W).

iii) Cultivar roktima: Callus induction was observed in all BAP and NAA formulations (Table 5.1.3.3a). The response of the explants were found to vary with growth regulator concentrations used. Percentage of explants showing callus formation was the highest (6.5%) when the explants cultured on 2.0 mg/L BAP with 0.5 mg/L NAA (Plate 5.1.3.3, D). Callus induction from the explants were taken the lowest time in the same combination and concentration and it was 20 days. The colour of callus in this experiment were found slight whitish (W).

iv) Cultivar lalchay: Callus induction was observed all in BAP and NAA formulations (Table 5.2.3.3b). The response of the explants were found to vary with growth regulator concentrations used. Percentage of explants showing callus formation was the highest (6.3%) when the explants cultured on 2.0 mg/L BAP with 0.5 mg/L NAA containing media (Plate 5.1.3.4, D). Callus induction from the explants were taken the lowest time in the same combination and concentration and it was 21 days. The colour of callus in this experiment were found whitish (W).

v) Cultivar badami: Callus induction was observed in all BAP and NAA formulations (Table 5.2.3.3b). The response of the explants were found to vary with

growth regulator concentrations used. Percentage of explants showing callus formation was the highest (6.1%) when the explants cultured on 2.0 mg/L BAP with 0.5 mg/L NAA (Plate 5.1.3.5, D). Callus induction from the explants were taken the lowest time in the same combination and concentration and it was 20 days. The colour of callus in this experiment were found whitish (W).

vi) Cultivar dhusar: Callus induction was observed in all BAP and NAA formulations (Table 5.2.3.3b). The response of the explants were found to vary with growth regulator concentrations used. Percentage of explants showing callus formation was the highest (6.0%) when the explants cultured on 2.0 mg/L BAP with 0.5 mg/L NAA (Plate 5.1.3.6, D). Callus induction from the explants were taken the lowest time in the same combination and concentration and it was 22 days. The colour of callus in this experiment were found whitish (W).

Table 5.1.3.3a: Effect of auxin (NAA) in combination with cytokinin (BAP) employed in MS medium on callus induction from isolated cells. Data were recorded after 6 weeks of culture.

CELL CULTURE
IN
CULTIVAR SHABJE

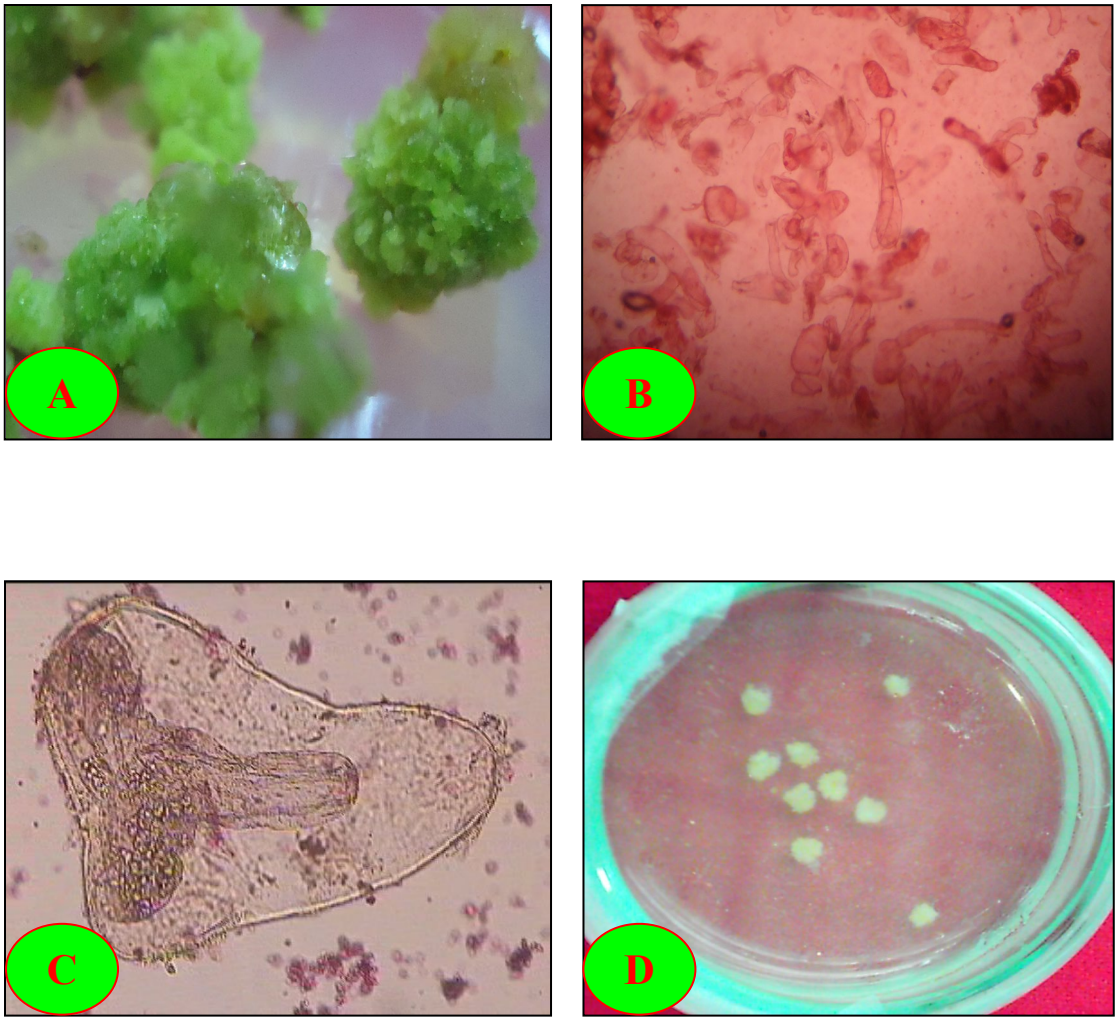


Plate- 5.1.3.1: A-D: Photographs showing the different stages of cell culture of cultivar shabje. **A:** Embryogenic calli. **B:** Isolated cells. **C:** Single cells. **D:** Callus induction from single cell.

CELL CULTURE
IN
CULTIVAR SHADATAE

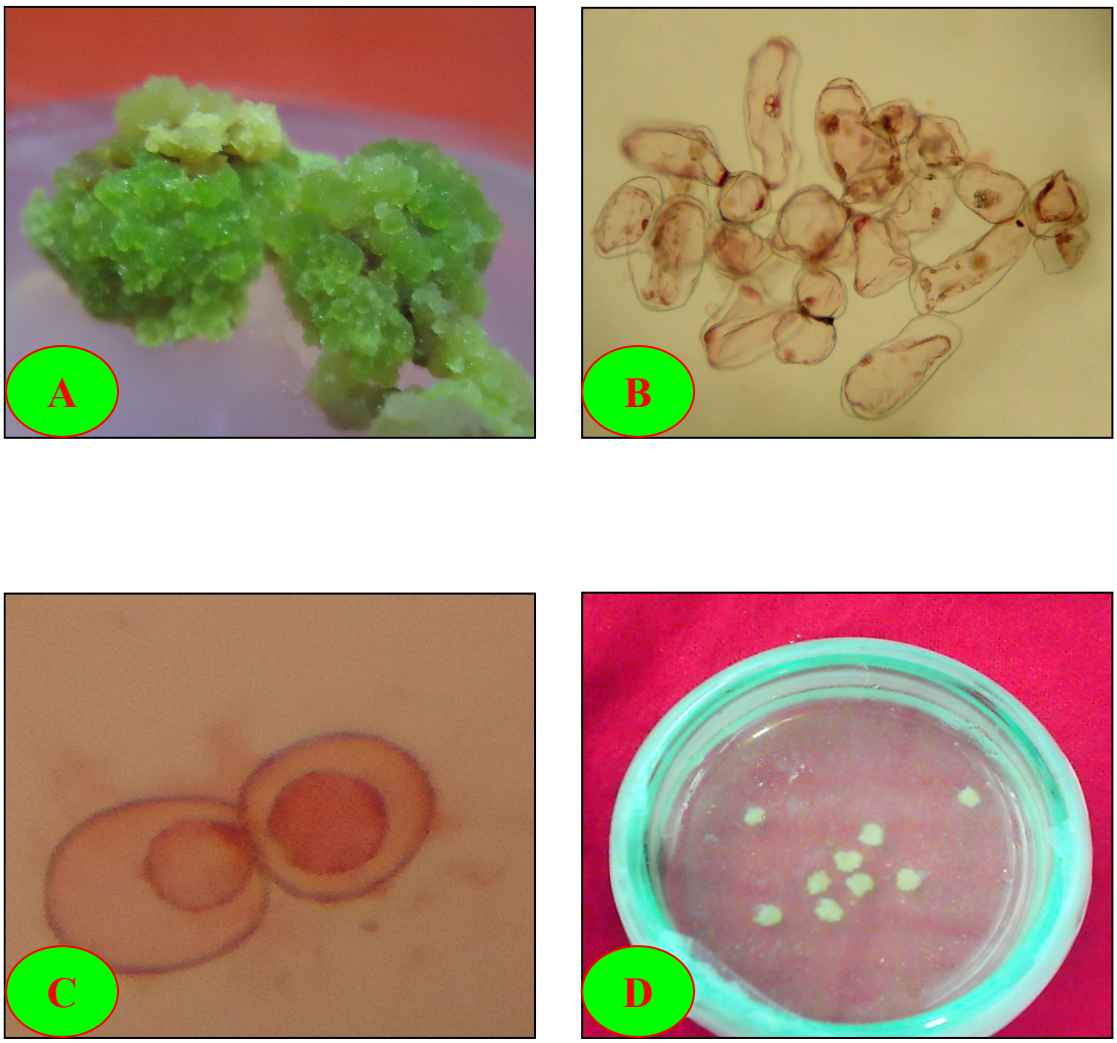


Plate- 5.1.3.2: A-D: Photographs showing the different stages of cell culture of cultivar shadatae. **A:** Embryogenic calli. **B:** Isolated cells. **C:** Single cells. **D:** Callus induction from single cell.

CELL CULTURE
IN
CULTIVAR ROKTIMA

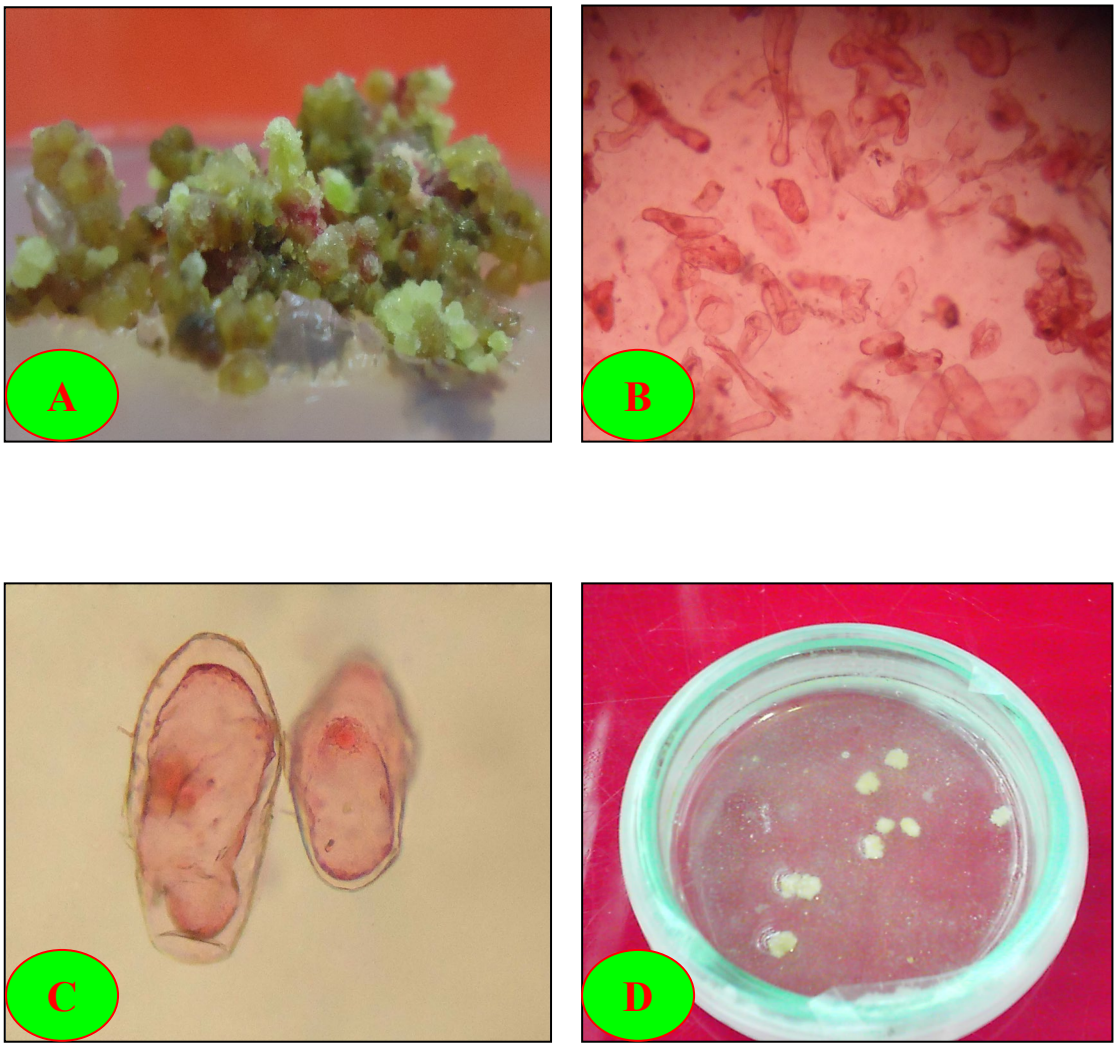


Plate- 5.1.3.3: A-D: Photographs showing the different stages of cell culture cultivar roktima. **A:** Embryogenic calli. **B:** Isolated cells. **C:** Single cells. **D:** Callus induction from single cell.

CELL CULTURE
IN
CULTIVAR LALCHAY

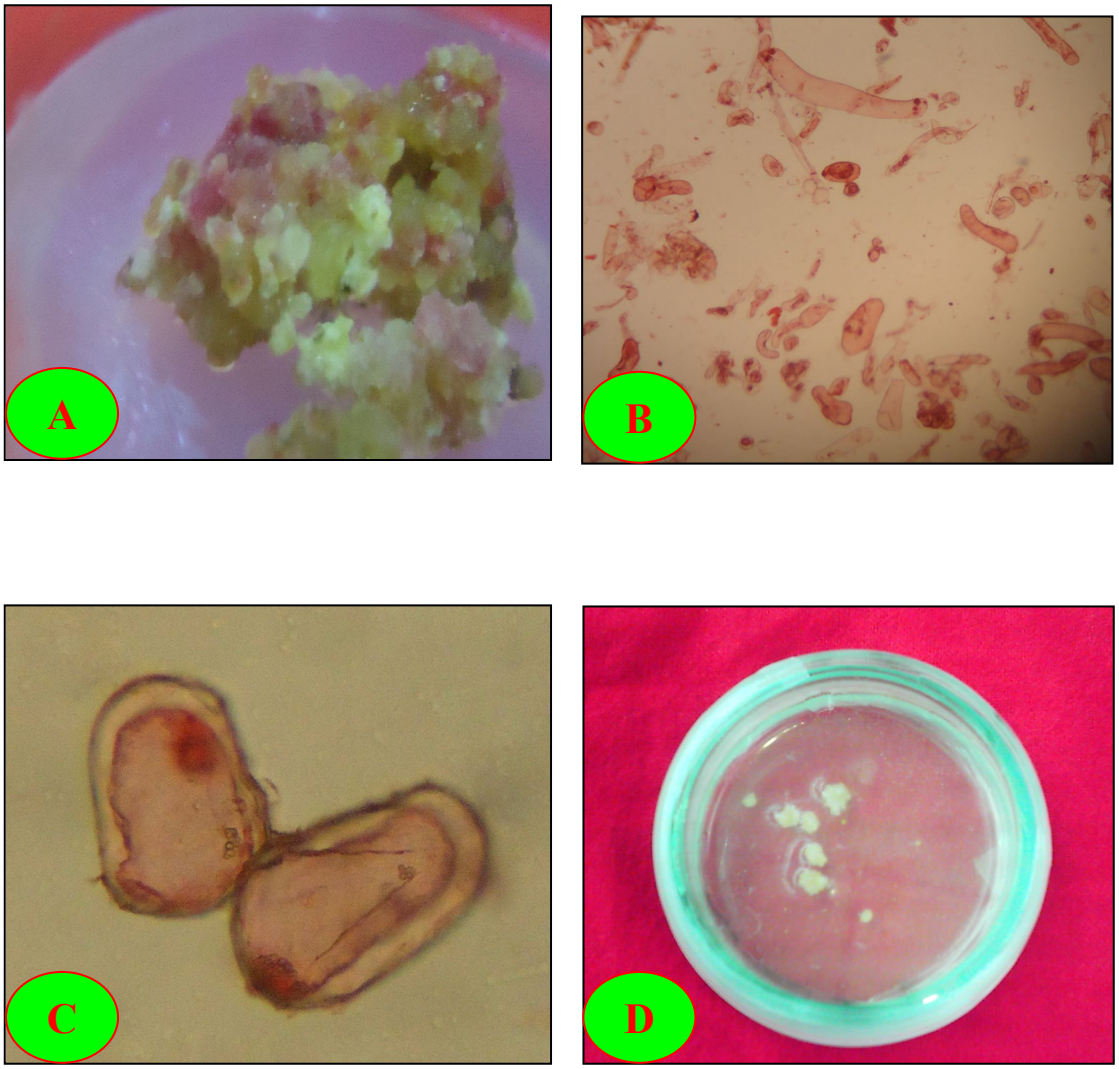


Plate- 5.1.3.4: A-D: Photographs showing the different stages of cell culture of cultivar lalchay. **A:** Embryogenic calli. **B:** Isolated cells. **C:** Single cells. **D:** Callus induction from single cell.

CELL CULTURE
IN
CULTIVAR BADAMI

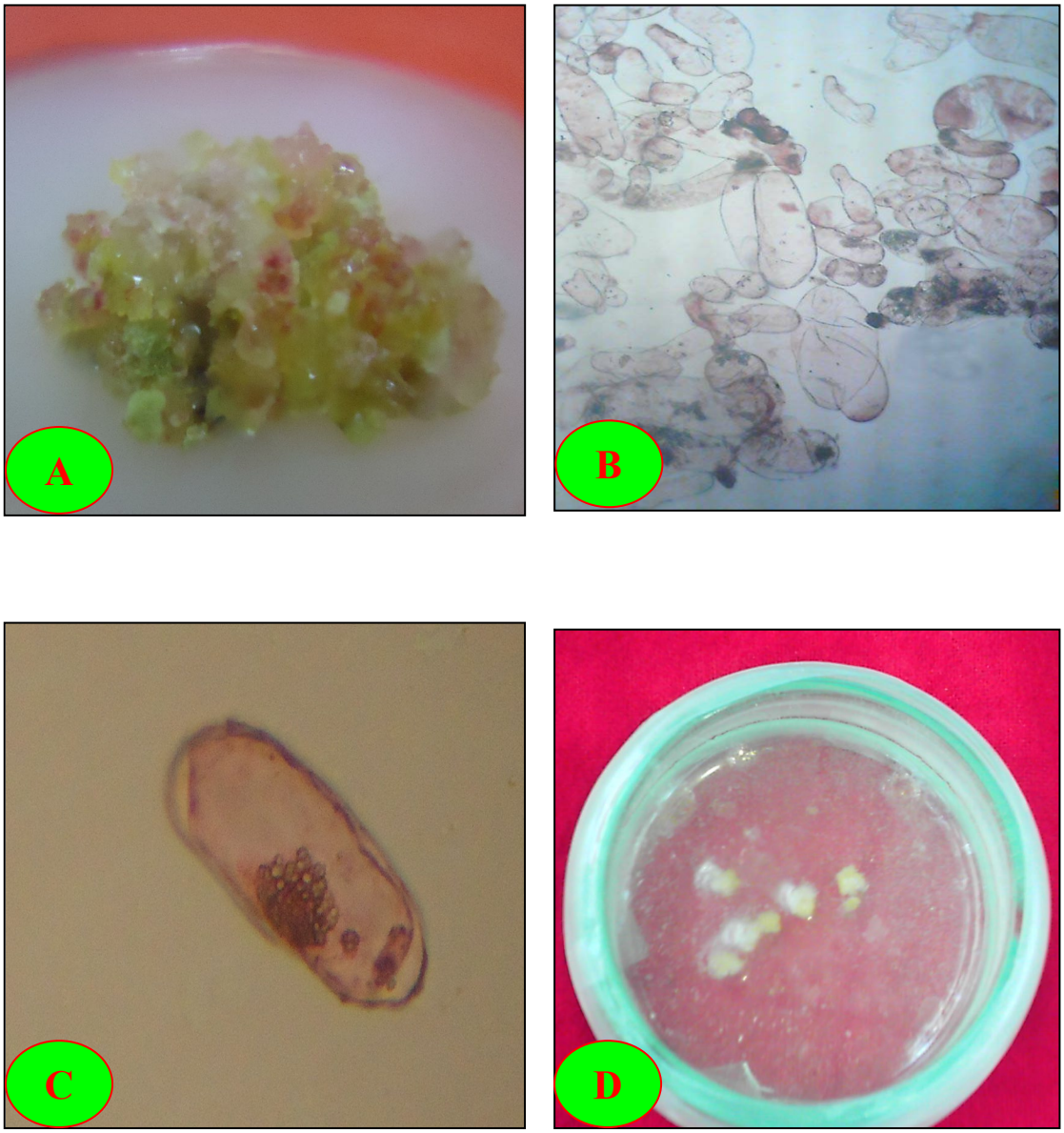


Plate- 5.1.3.5: A-D: Photographs showing the different stages of cell culture of cultivar badami. **A:** Embryogenic calli. **B:** Isolated cells. **C:** Single cells. **D:** Callus induction from single cell.

CELL CULTURE
IN
CULTIVAR DHUSAR

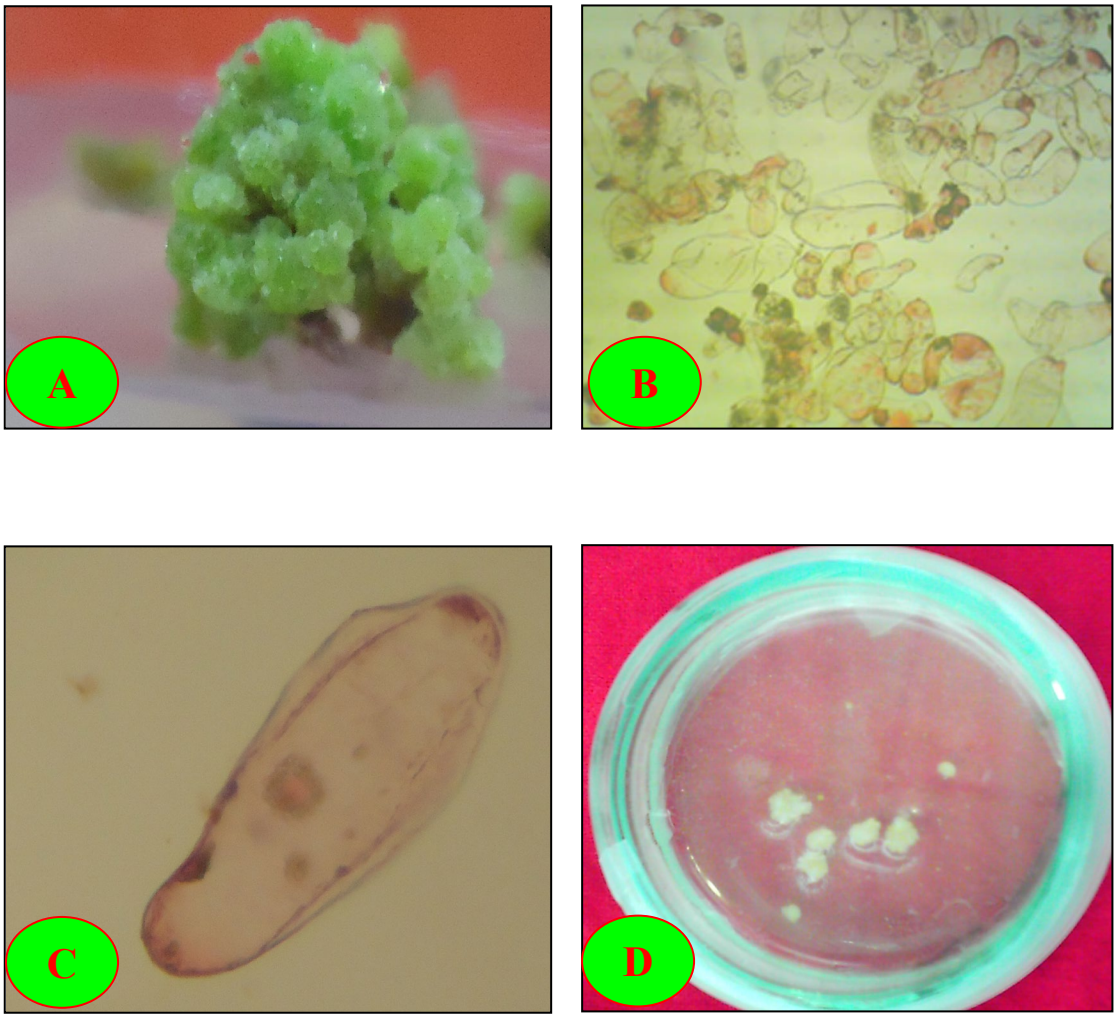


Plate- 5.1.3.6: A-D: Photographs showing the different stages of cell culture of cultivar dhusar. **A:** Embryogenic calli. **B:** Isolated cells. **C:** Single cells. **D:** Callus induction from single cell.

5.1.4. DISCUSSION

Growth conditions for plant culture must provide it with all essential nutrients, which are normally provided by the plant itself. Furthermore, these conditions must be optimized to promote the growth of the particular cell type. The medium in which the cells are grown, must contain a carbon source, vitamins, salts and other organic supplements. The organic nutrients come in different concentrations of macronutrients and micronutrients. Macronutrients include nitrogen, potassium, calcium and magnesium, which are always found in a concentration greater than 0.5 mM. The micronutrients include iron, copper, zinc and cobalt and can be adjusted for maximum growth of each culture type. In addition, medium must provide vitamins which are usually synthesized by plants. In addition to vitamins and nutrients, growth hormones can also influence the growth rate of plants growth in artificial environments. Many plants grown in a conventional manner produce their own growth hormones, however, in culture, artificial hormones are supplied to ensure optimal growth of the plants.

Besides having the appropriate nutrients in the media, the maintenance of sterile conditions is essential for the success of the cell culture allowing it to be free of microorganisms. This requires that all equipment used in creating a cell culture must be sterilized to ensure contamination free environment.

Many plant species can be regenerated *in vitro* through several approaches but all require a starting point. This can be anything from a single cell than can be reproduced, a tissue or organ part, or a cut out piece of differentiated tissue (or organ) known as an explant. Once the starting point has been determined, the culture used to grow this part must be considered. In case of six cultivars of castor, we used the starting point as friable calli for cell culture.

Cell suspension cultures are rapidly dividing homogenous suspensions of cells grown in liquid nutrient media from which samples can be taken. In a cell suspension, a mass of cells, called a callus, must first be collected. The callus can then be suspended in a liquid callus induction media containing all the required nutrients and elements to allow for optimal growth which acts to turn all cells into undifferentiated cells. The cell suspension is then placed on a shaker to allow the cell aggregates to disperse to form smaller clumps and single cells that are homogeneously distributed throughout the liquid media. The cells will continuously grow until one of the factors limiting causing cell growth to slow.

The suspensions contained both free cells and cell aggregates, in varying proportions between different cultures. Cell clusters up to 2 mm diameter not uncommon and formed by repeated divisions, held together by internal cross walls (plasmadesmata). Soon after the cell division phase, the cells enlarge and separated as

free cells from the aggregates. Plant cells in suspension possess a thin unspecialized cell wall (0.2 to 0.6 μ in diameter) and show a wide variety of shapes and sizes, ranging from nearly spherical to approximately cylindrical, and of linear dimensions in the range 20 to 40 μ diameter and 100 to 200 μ long.

Under the present investigation, embryogenic calli obtained from MS semisolid medium were used to initiate cell suspension. For the purpose of cell suspension culture the cells were cultured on to MS liquid medium supplemented with 2.0 mg/L BAP with different concentration of NAA and 2,4-D. After 2 days of culture under shaking resulted that, the production of some individual cells with small cell clusters in the liquid medium. Microscopic observation were made to characterize the individual cells isolated from the culture medium and in respect of their shape they may be regarded as spherical, elliptical and elongated shape. These cells were with dense cytoplasm and part of them possessed high embryogenic potential. The embryogenic single cells and cell clusters were capable to divide symmetrically and asymmetrically by transverse division which resulted in two cells stages. Iantcheva *et al*. 2005 observed similar asymmetrically cell division in *Medicago truncatula* in cell suspension culture.

In the present investigation a number of experiments were performed to study the effect of hormone on cell growth and the optimum cell division period in culture medium of six cultivars of castors. Among the different concentrations and combinations of different hormones, 2.0 mg/L BAP + 0.3 mg/L NAA was found to be most effective on cell culture of all cultivars. In this media formulation Shabje showed the highest performance (0.143±0.14 gm) while Badami showed the least performance (0.127±0.13 gm) on 16th days. Experimental results indicated that the peak period of cell growth runs from 7th days and after 16th days their growth seemed to be ceased down. The results revealed that 16th days period appeared as typical period for the culture of six cultivars and it is necessary to transfer the cell to the fresh medium after each 16th days of culture for its maintenance in the lab.

The peak period of cell growth was observed from 4th day to 6th day. Similar result was obtained in brinjal and cell growth showed the highest peak within 4-6 days of incubation (Hossain *et al*. 2007). Similar peak period of cell growth was obtained in *Abrus precatorius* where cells attained their highest peak within 6 – 8 days of growth (Bari *et al*. 2009). Similar results was also reported in sugar beets, using different concentrations and combinations of BAP and 2,4-D, the growth patterns of cell suspension cultures were examined during a range of culture periods (0, 3, 5, 7, 9, 11, 13 and 15 days). In all lines, the growth rates of cells were initially slow but as the culture proceeded, they increased significantly and accumulated great

amounts of biomass over a period of 15 days in Sugar Beet (Song *et al*. 2002). Plant cell growth and their growth measurement procedure was also very clearly described in PROTOCOL (Mustafa *et al.* 2010). In some countries endeavours are being made to design the cell culture biorector for commercial production of cell product in laboratory (Ozlem *et al* . 2010). But in banana cell suspension growth is absolutely different from castor, in banana, most of the cultivars showed the highest performance within 60-70 days of culture in liquid medium and the cell growth became stationary after 80 days of culture (Jamal and Bari 2011). The time required to establish the cell suspension culture varies greatly and depends on the tissue of the plant species and the medium composition. The use of fine suspension culture offers the opportunity to confirm single cell origin of somatic embryos and the asymmetry of the first cell division, which starts the process. The other advantage of cell suspension culture is the use of single embryogenic cells and somatic embryos as a source for direct gene transfer via particle bombardment, transit gene expression and confocal microscopy observation. Cell suspension cultures are particularly suitable for physiological, biochemical and molecular studies of the process of somatic embryogenesis and its different stages – induction development, maturation and conversion. The development of a genome and proteome database of model *Medicago truncatula* species together with the presence of protocol of cell suspension provided the opportunity to identify and characterized genes involved in the whole process of somatic embryogenesis (Iantcheva *et al*. 2006).

The isolated cells were also used for callus induction. For this purpose the cells were cultured in petridish containing MS medium supplemented with different concentrations and combinations of BAP and NAA. Micro calli were appeared after 2 weeks of culture in the petridish. Among the different media formulations, 2.0 mg/L BAP + 0.5 mg/L NAA was found to be the best for all six cultivars of castor. In this media formulation, cultivar Shabje the highest callusing rate (6.7%) and cultivar shadatae and cultivar dhusar showed the least (6.0%) callusing rate (Table 5.3.3).

From the foregoing discussion it may concluded that the auxins NAA, 2,4-D and IAA have great role in callus induction but 2.0 mg/l BAP + 0.5 mg/l NAA and 2.0 mg/l BAP + 0.8 mg/l NAA concentrations proved to be most suitable combinations for induction of embryonic calli of *R. communis*. Under this investigation NAA in combination with BAP proved to be suitable media composition for enhancing cell growth of *R. communis* in artificial medium. The present experiments clearly demonstrated that in *R. communis*, cell begins to grow only within 4 days of culture proving its potential for developing cell culture industry for production of important

toxic protein ricin as secondary metabolite. In regards of other medicinal plants *R. communis* proved suitable plant species for developing cell suspension culture in artificial medium and the present findings open up a new potential venture for obtaining secondary metabolites ricin as a alternative source from its cell culture.

SUB CHAPTER-5.2

5.2.1. INTRODUCTION

5.2.1.1. Overview of production of antimicrobial compounds by plants

The continuous use of antibiotics has resulted in multiresistant microbial strains all over the world and as expected, hospitals have become breeding grounds for human associated microorganisms (Mainous and Pomeroy 2001). Among fungi associated with human diseases, the *Candida* spp. were, for many years, the most frequently isolated from clinical specimens (Ainsworth 1986). Also the emerging of AIDS disease increased the risk of infections in the immunocompromised patients, caused by pathogenic yeast (known or emerging) which cause cutaneous or systemic infections (Anaissie and Bodey 1989, Cimolai *et al*. 1987). Another serious risk is caused by increasing development of fungal resistance to currently available antimycotic agents (Masiá Canuta and Gutiérrez Rodero, 2002). This phenomenon, firstly described in bacteria, is becoming a looming threat also for yeast, occasionally developing resistance also towards azoles and polyenes, which represent the two major classes of known antimycotic drugs (Sanglard and Odds 2002, Nguyen *et al*. 1996, Pfaller *et al*. 2001, Rex *et al*. 2000, Powderly *et al*. 1988). Also for bacterial strains, they have genetic ability to transmit and acquire resistance to drugs which are utilized as therapeutic agents (Cohen 1992). The knowledge acquired in the past two decades and the discovering of new groups of antimicrobial proteins make natural antibiotics the basic element of bacterial and fungal infections (De Lucca 2000, Hancock 2000, Welling *et al*. 2000, Selitrennikoff 2001). Among eukaryotes producing antimicrobial compounds, plants synthesize a vast array of secondary metabolites as defense mechanisms for protecting themselves against pathogen infections [bacteria, fungi and viruses (Osbourn 1996, Mendoza *et al*. 1997, Skadhange *et al*. 1997, Tereschuk *et al*.1997, Bois *et al*. 1999, Hou and Forman 2000, Rauha *et al*. 2000)]. The use of plant extracts and phytochemicals can be of great significance in therapeutic treatments. In the last years, a number of studies have been conducted in different countries to prove such efficiency (Almagboul *et al*. 1985, 1988, Artizzu *et al*. 1995, Ikram and Inamul 1984, Izzo *et al*. 1995, Kubo *et al*. 1993, Shapoval *et al*. 1994). Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in secondary metabolism of the plant. Hence, more studies pertaining to use of plants as therapeutic agents should be emphasized, especially those related to control of antibiotic resistant microbes.

5.2.1.2. Overview of production of new pharmaceutical compounds by plant tissue cultures

Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavor and fragrance ingredients, food additives, and pesticides (Balandrin and Klocke 1988). The search for new plant-derived chemicals should thus be a priority in current and future efforts toward sustainable conservation and rational utilization of biodiversity (Phillipson 1990). In the search for alternatives to production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Ramachandra and Ravishankar 2002). Cell suspension culture systems could be used for large scale culturing of plant cells from which secondary metabolites could be extracted.

The potential of plant cell cultures to produce useful compounds, especially for drug development, was perceived in the late 1960s. Thus Kaul and Staba (1967) and Heble *et al*. (1968) isolated visnagin and diosgenin respectively from cell cultures in larger quantities than from the whole plant. However, a large number of cultures failed to synthesize products characteristic of the parent plant. For instance, morphinan, tropane and quinoline alkaloids are synthesized only at extremely low levels in cell cultures (Berlin 1986). That is why, after a surge of interest, the trend of research declined.

In 1976, at an international congress held in Munich, Zenk and his coworkers demonstrated the outstanding metabolic capacities of plant cells and highlighted the spontaneous variability of plant cell biosynthetic capacity, which could explain the contradictory results obtained earlier. This natural variability is exploited to identify high-yielding cultures for use on an industrial scale (Tabata *et al*. 1976, Zenk *et al*. 1977, Zieg *et al*. 1983, Yamada 1984, Benjamin *et al*. 1986). Since the late 1970s, research and development in this area has seen a high increase in the number of patent applications field, especially by the scientific and corporate filed, and by the scientific and corporate sectors in the Federal Republic of Germany and Japan. In 1983, for the first time, a dye, shikonin, with anti-inflammatory and anti-bacterial properties, was produced by plant cell cultures on an industrial scale by Mitsui Petrochemical Industries Ltd (Fujita *et al*. 1982). However, although this was thought to be a major breakthrough, shikonin production is still the only plant product to be produced on a commercial scale by cell cultures.

Discoveries of cell cultures capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants have accelerated in the

last few years. New physiologically active compounds of medicinal interest have been found by bioassay. It has been demonstrated that the biosynthetic activity of cultured cells can be enhanced by regulating environmental factors, as well as by artificial selection or the induction of variant clones. Some of the medicinal compounds localized in morphologically specialized tissues or organs of native plants have been produced in culture system not only by inducing specific organized cultures, but also by undifferentiated cell cultures. The possible use of plant cell cultures for the specific biotransformation of natural compounds has been demonstrated (Cheetham 1995, Scragg 1997, Krings and Berger 1998, Ravishankar and Ramachandra 2000). Due to these advances, research in the area of tissue culture technology for production of plant chemicals has bloomed beyond expectations.

5.2.1.3. Secondary metabolites production from callus and cell culture

Plant-derived antibiotics often originate from plant defense mechanisms. Secondary metabolites mediate defense mechanisms by providing chemical barriers mostly against microbial predators. This chemical warfare between plants and their pathogen consistently provides new natural product (Wedge and Camper 2000). Opportunities of finding novel anti-infective drugs from plants may be increased by subjecting more plant extracts to broad array of bioassays and including extracts derived from plant cell, tissue and organ cultures too (Khafagi *et al*. 2003).

Assessment of the antibacterial (Khafagi, 1998), antifungal (Salvador *et al*. 2003, Shariff *et al*. 2006), antiviral and cytoxicity (Sokmen 2001) produced by *in vitro* cultures in contrast with their corresponding adult plants were documented in the literature. The past few decades have seen increasing scientific interest in the both growth of plant tissue culture and the commercial development of this technology as means of producing valuable phytochemicals (Havkin-Frenkel *et al*. 1997).Callus cultures from medicinal plants have been established under suitable conditions to enable production of antimicrobial substances *in vitro* (Chintalwar *et al*. 2003, Wolters and Eilert 1981). Recently, papers investigating the antimicrobial activity of extracts from calli of different medicinal plant species have been published (Sokmen *et al*. 1999, Furmanowa *et al*. 2002). Although very few plant cell processes are operating commercially, the most successful commercial pharmaceuticals produced from undifferentiated cell cultures are antibiotic compounds (Khafagi *et al*. 2003).

5.2.1.4. *In vitro* metabolites accumulation and antimicrobial test by castor

Biological activity of most medicinal plants lack information relegated to the variety used. Comparing metabolites accumulated in vitro in some plant varieties of the monotypic genus *Ricinus* L. may be valuable for future biotechnological application aiming at raising its *in vitro* secondary metabolite production. *R. communis* L. commonly known as castor bean belongs to family Euphorbiaceae. It is probably native to north East tropical Africa, but now widely cultivated throughout the tropics, subtropics and warm temperate regions (Meike 1985). *R. communis* L. plants exhibit much variation regarding the size and ornamentation of the fruit and color patterning of the seeds, fruits, leaves and pollens. Worldwide, 20 varieties of *R. communis* L. have been recognized (Meikle 1985).

Castor seeds contain a high percentage of oil that causes the purgative action, a toxic alkaloids, ricinine and a very toxic albumin called ricin (Dejey 1975, Zohary 1987). Castor oil is prescribed for infestation of intestinal worms. Infusion of the leaves was used as a remedy for rash, itch and eye inflammation. The decoction of roots is used for skin diseases, diarrhea and kidney and bladder troubles (Boulos 1983). Ethanol-water extracts of either roots or stems were reported to have *in vitro* antiamebic activity and cytotoxic effects against the 9KB carcinoma. *In vitro* antiviral activity and hypoglycemic activity were reported from leaf extracts (Ayensu 1978).

Ethno-pharmacological reputation of *R. communis* L. is remarkable. It is valued for its oil, abandoned for its toxicity and treasured for its biological activity. Despeyroux *et al*. (2000) found that a large extract of ricin heterogeneity was originated for various *R. communis* varieties.

In Bangladesh *R. communis* is widely grown all over the country holding great potential for producing castor oil as a source of green energy and also for commercial production of toxic protein ricin. We have developed the standard protocol for *in vitro* generation of *R. communis* and under the present investigation we have made efforts to develop the standard protocol for wide scale callus culture and cell culture of *R. communis* towards isolation of active protein ricin from these cultures. The aim of this study was to determine the *in vitro* antibacterial activity of crude extracts isolation from cell suspension cultures of six cultivars of castor against gram positive and gram negative bacteria using the disc diffusion and micro broth dilution methods.

5.2.1.5. Objectives

Castor is the world most toxic plant produces well reputed toxic element the ricin, in its body as a secondary metabolite. It is assumed that like living plant, the cell culture also produces this toxic element to the media. Thus our main objective was to make a through antibacterial screening of the cell culture extract to be obtained from cell culture of different castor cultivars in Bangladesh. Thus the objective of the present investigation was to a) develop efficient protocol for obtaining abundant cell extract from cell suspension culture of different castor cultivars; b) potent test of cell extract under different days of culture against gram positive and gram negative bacteria and to c) unveil the genetic diversity of different castor cultivars in regarde of toxicity in cell extract with projection a comparative analysis of their relative potentiality in future development.

5.2.2. MATERIALS AND METHODS

5.2.2.1. Assay for antibacterial screening

The essay can be performed *in vitro* by disc diffusion assay method (Bauer *et al*. 1966, Barry 1980), which includes

a) Plate diffusion test and
b) Streak test

The plate diffusion test utilizes different concentrations of a test compounds absorbed on sterile filter paper discs on the same plate whereas the streak test permits the determination of the antibacterial effect of a test compound on several microorganisms simultaneously and is suitable for the estimation of the spectrum of the activity. However, the plate diffusion test is commonly used.

5.2.2.2. Preparation of extracts

The liquid medium (filtrate) containing cell suspension culture were maintained in the laboratory. Every after two days, 5 ml liquid medium with growing cells centrifuged at 4000 rpm for 10 minutes. The cells were discarded and the clear supernatant was collected and used as cell extracts. Using 25 µl of the supernatant was applied on the test disc.

5.2.2.3. Test material used for the study

A. Isolated cell extract of cell culture were taken after 4 days, after 6 days, after 8 days, after 10 days, after 12 days, after 14 days and after 16 days. The extracts 25 µl/disc was applied on the discs.
B. Ciprofloxacin was used as (5 µgm/disc) standard disc.

5.2.2.4. Apparatus and reagents

i) Alcohol (95%)
ii) Filter paper discs (sterilized)
iii) Petridishes
iv) Inoculating loop
v) Sterile cotton
vi) Test tubes
vii) Sterile forceps
viii) Micropipette
ix) Nose mask and hand gloves
x) Spirit burner & match box

xi) Rectified sprit
xii) Nutrient agar (Difco)
xiii) Laminar air flow unit (BIO-CRAFT SCIENTIFIC INDUSTRIES, INDIA)
xiv) Refrigerator (ARTSTON, ITALY)
xv) Autoclave (ALP Co. Ltd. KT-30L, TOKYO)
xvi) Swab
xvii) Orbitary shaker
xvi) Centrifuge machine (Shanghai Surgical Instruments Factory, China)

5.2.2.5. Test organisms used for the study

Eleven pathogenic bacteria were selected for the test, five of which were Gram positive and the remaining were Gram negative . Pure culture of these organisms were collected from the Microbiology Laboratory, Institute of Biological Sciences, University of Rajshahi. The bacterial strains used for this investigation are listed in the Table 5.2.2.1.

Table 5.2.2.1: List of test pathogenic bacteria.

Name of test organism	
Gram positive	**Gram negative**
1. *Sarcina lutea*	1. *Shigella sonnei*
2. *Staphylococcus aureus*	2. *Klebsiella species*
3. *Bacillus megaterium*	3. *Proteus species*
4. *Bacillus subtillus*	4. *Escherichia coli*
5. *Bacillus halodurans*	5. *Pseudomonas aeruginosa*
	6. *Salmonella typhi*

5.2.2.6. Sterilization procedures

The antibacterial screening was carried out in a laminar air flow unit and all types of precaution were highly maintained to avoid any type of contamination during the test. UV light was switched on for half an hour before working in the laminar hood to avoid any accidental contamination. Petridishes and other glass wares were sterilized in the autoclaved at 121ºC temperature and a pressure of 15 lbs./sq. inch for 15 minutes. Micropipette tips, culture media, cotton, forceps, blank discs etc. were also sterilized (Carter 1987).

5.2.2.7. Culture media

For demonstrating the antibacterial activity and subculture of the test organisms nutrient agar media (Difco) was used.

5.2.2.8. Preparation of the nutrient agar medium

The instant nutrient agar (Difco) was weighed and dissolved in distilled water (28 gm/L) in a conical flask according to specification (2.3 % w/v). It was then heated in a water bath to dissolve the agar until a transparent solution was obtained.

5.2.2.9. Preparation of fresh culture of the pathogenic organisms

The nutrient broth medium was prepared and dispersed in a number of clean test for preparation of fresh culture of the bacteria. The nutrient broth powder was weighed and then reconstituted and dissolved 13 gm/L with distilled water in a conical flask. It was then heated in a water bath to dissolve the powder until a transparent solution was obtained. The medium was then dispersed in a number of clean test tubes (5 ml in each test tube). The test tubes were plugged with cotton and sterilized in an autoclave at 121ºC and temperature and a pressure of 15 lbs./sq. inch for 15 minutes. The test organisms were transferred to the test tubes from the supplied pure cultures with the help of an inoculating loop in an aseptic condition. The loop was burned after each transfer of microorganism to avoid contamination very carefully. The inoculated test tubes were then incubated at 37ºC for 24 hours to assure the growth of test organisms. The fresh cultures were used for the sensitivity test.

5.2.2.10. Culture media and inoculums

Solid media of nutrient agar was prepared by dissolving 28 gm/L water. About 25 ml of media was poured into a petridish. The inoculum was prepared by culturing a large number of organisms in a test tube containing 5 ml liquid media for bacterial strains and incubating over night at 37ºC. The agar plates for the assay were prepared by labeling them with the date, the name of the microorganism and the name (code) of the discs. The inoculi of bacteria were transferred into petridish containing solid nutrient media of agar using a sterile swab. The swab was used to spread the bacteria on the media in a confluent lawn. One swab was used for one species of bacteria.

5.2.2.11. Preparation of discs containing samples

For the preparation of discs containing samples the following procedure were utilized:

(a) Sample discs

Sterilized filter paper discs (5 mm in diameter) were taken by the forceps in the plates. The cell extracts taken from cell culture (25 µl/disc) was applied on the discs with the help of a micropipette in an aseptic condition. These discs were left for a few minutes in aseptic condition for complete removal of the solvent.

(b) Standard discs

These were used to compare the antibacterial activity of the test material. In the present study, ready-made ciprofloxacin containing 5 µg/disc of antibiotic ciprofloxacin were used as standard disc for comparison purpose.

5.2.2.12. Placement of discs and incubation

For the placement of the discs, the following procedure was used:

a) By means of a pair of sterile forceps, the dried sample impregnated discs and standard disc were placed gently on the solidified agar plates seeded with the test organisms to ensure contact with the medium.
b) The plates were then kept in a refrigerator at 4ºC for 24 hours in order to provide sufficient time to diffuse the antibiotics in to the medium.
c) Finally, the plates were incubated at 37ºC for 24 hours in an incubator.

Precaution:

The discs were placed in such a way that they were not closer than 15 mm to the edge of the plate and for enough apart to prevent overlaping the zones of inhibition.

5.2.2.13. Measurement of the zones of inhibition

After incubation, the antibacterial activities of the test samples were determined by measuring the diameter of inhibitory zones in term of mm with help of a transparent scale.

5.2.3. RESULTS

Cell extracts of six cultivars of castor were taken after different periods of cell suspension culture and they were tested against five gram positive and six gram negative bacteria at the concentration of 25 µl/disc and was compared with standard antibiotic ciprofloxacin 5 µg/disc. The results are presented in the Tables 5.2.3.1-5.2.3.7. It was found that most of the cultivars were effective against the test bacteria, when the extracts taken after 6 days to 16 days of cell suspension culture. But when the extract taken from 4 days of cell suspension culture were not so good effective against the bacteria.

5.2.3.1. Antibacterial activity of cell extract isolated after 4 days of cell suspension culture.

The results of this investigation were presented in the Table 5.2.3.1(Plate 5.2.3.1, A & B). In case of cultivar shabje the highest zone of inhibition was found 15 mm against the *Bacillus halodurans* and lowest zone of inhibition was found 8 mm against *Escherichia coli*, when the standard ciprofloxacin showed 30 mm and 26 mm zone of inhibition respectively. The extract of cultivar shadatae showed the highest zone of inhibition 7 mm against *Sarcina lutea and Bacillus megaterium,* when the standard ciprofloxacin showed 29 and 32 mm zone of inhibition respectively but did not show any activity against *Bacillus subtillus*, *Bacillus halodurans, Shigella sonnei*, *Klebsiella species*, *Proteus species, Escherichia coli, Pseudomonas aeruginosa and Salmonella typhi*. When the extract of cultivar roktima tested against all bacteria, the highest zone of inhibition was found against *Bacillus halodurans* and it was 17 mm, when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 9 mm against *Sarcina lutea,* when the standard ciprofloxacin showed 29 mm zone of inhibition. In case of cultivar lalchay the highest zone of inhibition was 16 mm against *Bacillus halodurans,* when the standard ciprofloxacin showed 30 mm zone of inhibition but against *Klebsiella species* did not show any activity. In case of cultivar Badami, the highest zone of inhibition was 10 mm against *Proteus species* when the standard ciprofloxacin showed 25 mm zone of inhibition. But it did not show any activity against *Bacillus subtillus* and *Klebsiella species.* In case of cultivar dhusar, the highest zone of inhibition was 9 mm against *Bacillus halodurans* when the standard ciprofloxacin showed 30 mm zone of inhibition. But it did not show any activity against *Bacillus subtillus, Shigella sonnei, Klebsiella species*, *Proteus species* and *Escherichia coli* .

5.2.3.2. Antibacterial activity of cell extract isolated after 6 days of cell suspension culture.

The results of this investigation were presented in the Table 5.2.3.2 (Plate 5.2.3.2, A & B). In case of cultivar shabje the highest zone of inhibition was found 17 mm against the *Bacillus halodurans* and lowest zone of inhibition was found 10 mm against *Escherichia coli*, when the standard ciprofloxacin showed 30 mm and 26 mm zone of inhibition respectively. The extract of cultivar shadatae showed the highest zone of inhibition 7 mm against *Sarcina lutea, Bacillus megaterium, Proteus species, and Pseudomonas aeruginosa* and the lowest zone of inhibition was found 6 mm against *Staphylococcus aureus, Bacillus subtillus, Shigella sonnei and Klebsiella species,* when the standard ciprofloxacin showed 34 mm, 26 mm, 22 mm and 25 mm respectively. The extract of cultivar roktima tested against all bacteria, the highest zone of inhibition was found against *Bacillus halodurans* and it was 19 mm when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 10 mm against *Sarcina lutea,* when the standard ciprofloxacin showed 29 mm zone of inhibition. In case of cultivar lalchay the highest zone of inhibition was 17 mm against *Bacillus halodurans* when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 7 mm against *Klebsiella species,* when the standard ciprofloxacin showed 25 mm zone of inhibition. In case of cultivar Badami, the highest zone of inhibition was 11 mm against *Bacillus halodurans,* when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 6 mm against *Bacillus subtillus and Klebsiella species,* when the standard ciprofloxacin showed 26 mm and 25 mm zone of inhibition. In case of cultivar dhusar, the highest zone of inhibition was 10 mm against *Bacillus halodurans* when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 6 mm against *Bacillus subtillus, Shigella sonnei and Klebsiella species,* when the standard ciprofloxacin showed 26mm, 22mm and 25 mm zone of inhibition respectively.

5.2.3.3. Antibacterial activity of cell extract isolated after 8 days of cell suspension culture.

The results of this investigation were presented in the Table 5.2.3.3 (Plate 5.2.3.3, A & B). In case of cultivar shabje the highest zone of inhibition was found 18 mm against the *Bacillus halodurans* and lowest zone of inhibition was found 11 mm against *Escherichia coli*, when the standard ciprofloxacin showed 30 mm and 26 mm zone of inhibition respectively. The extract of cultivar shadatae showed the highest zone of inhibition 8 mm against *Sarcina lutea, Bacillus megaterium, Proteus species,*

and Pseudomonas aeruginosa and the lowest zone of inhibition was found 6 mm against *Bacillus subtillus, Bacillus halodurans Shigella sonnei, Escherichia coli and Salmonella typhi,* when the standard ciprofloxacin showed 26 mm, 30 mm, 22 mm, 26 mm and 22 mm respectively. The extract of cultivar roktima tested against all bacteria, the highest zone of inhibition was found against *Bacillus halodurans* and it was 21 mm when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 11 mm against *Sarcina lutea,* when the standard ciprofloxacin showed 29 mm zone of inhibition. In case of cultivar lalchay the highest zone of inhibition was 18 mm against *Bacillus halodurans* when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 8 mm against *Klebsiella species,* when the standard ciprofloxacin showed 25 mm zone of inhibition. In case of cultivar Badami, the highest zone of inhibition was 14 mm against *Proteus species,* when the standard ciprofloxacin showed 25 mm zone of inhibition but the lowest zone of inhibition was found 6 mm against *Bacillus subtillus,* when the standard ciprofloxacin showed 26 mm zone of inhibition. In case of cultivar dhusar, the highest zone of inhibition was 11 mm against *Bacillus halodurans* when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 7 mm against *Bacillus subtillus, Shigella sonnei and Klebsiella species,* when the standard ciprofloxacin showed 26 mm, 22 mm and 25 mm zone of inhibition.

5.2.3.4. Antibacterial activity of cell extract isolated after 10 days of cell suspension culture.

The results of this investigation were presented in the Table 5.2.3.4 (Plate 5.2.3.4, A & B). In case of cultivar shabje the highest zone of inhibition was found 19 mm against the *Bacillus halodurans* and lowest zone of inhibition was found 12 mm against *Escherichia coli*, when the standard ciprofloxacin showed 30 mm and 26 mm zone of inhibition respectively. The extract of cultivar shadatae showed the highest zone of inhibition 9 mm against *Sarcina lutea,* and *Bacillus megaterium* and the lowest zone of inhibition was found 7 mm against *Bacillus subtillus, Bacillus halodurans, Shigella sonnei, Klebsiella species, Escherichia coli* and *Salmonella typhi,* when the standard ciprofloxacin showed 26 mm, 30 mm, 22 mm, 25 mm, 26 mm and 22mm respectively. The extract of cultivar roktima tested against all bacteria, the highest zone of inhibition was found against *Bacillus halodurans* and it was 22 mm when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 12 mm against *Sarcina lutea,* when the standard ciprofloxacin showed 29 mm zone of inhibition. In case of cultivar lalchay the highest

zone of inhibition was 19 mm against *Bacillus halodurans* when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 9 mm against *Klebsiella species,* when the standard ciprofloxacin showed 25 mm zone of inhibition. In case of cultivar Badami, the highest zone of inhibition was 15 mm against *Proteus species,* when the standard ciprofloxacin showed 25 mm zone of inhibition but the lowest zone of inhibition was found 7 mm against *Bacillus subtillus and Klebsiella species,* when the standard ciprofloxacin showed 26 mm and 25 mm zone of inhibition. In case of cultivar dhusar, the highest zone of inhibition was 12 mm against *Bacillus halodurans* when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 8 mm against *Bacillus subtillus, Shigella sonnei and Klebsiella species,* when the standard ciprofloxacin showed 26 mm, 22 mm and 25 mm zone of inhibition.

5.2.3.5. Antibacterial activity of cell extract isolated after 12 days of cell suspension culture.

The results of this investigation were presented in the Table 5.2.3.5 (Plate 5.2.3.5, A & B). In case of cultivar shabje the highest zone of inhibition was found 20 mm against the *Bacillus halodurans* and lowest zone of inhibition was found 13 mm against *Escherichia coli*, when the standard ciprofloxacin showed 30 mm and 26 mm zone of inhibition respectively. The extract of cultivar shadatae showed the highest zone of inhibition 10 mm against *Sarcina lutea,* and *Bacillus megaterium* and the lowest zone of inhibition was found 7 mm against *Bacillus subtillus and Bacillus halodurans,* when the standard ciprofloxacin showed 26 mm and 30 mm respectively. The extract of cultivar roktima tested against all bacteria, the highest zone of inhibition was found against *Bacillus halodurans* and it was 23 mm when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 13 mm against *Sarcina lutea* and *Klebsiella species,* when the standard ciprofloxacin showed 29 mm and 25 mm zone of inhibition respectively. In case of cultivar lalchay the highest zone of inhibition was 20 mm against *Bacillus halodurans* and *Escherichia coli* ,when the standard ciprofloxacin showed 30 mm and 26 mm zone of inhibition respectively but the lowest zone of inhibition was found 9 mm against *Klebsiella species,* when the standard ciprofloxacin showed 25 mm zone of inhibition. In case of cultivar Badami, the highest zone of inhibition was 16 mm against *Proteus species,* when the standard ciprofloxacin showed 26 mm zone of inhibition but the lowest zone of inhibition was found 8 mm against *Klebsiella species,* when the standard ciprofloxacin showed 25 mm zone of inhibition. In case of cultivar dhusar, the highest zone of inhibition was 12 mm against *Bacillus halodurans*

when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 8 mm against *Bacillus subtillus,* when the standard ciprofloxacin showed 26 mm zone of inhibition.

5.2.3.6. Antibacterial activity of cell extract isolated after 14 days of cell suspension culture.

The results of this investigation were presented in the Table 5.2.3.6 (Plate 5.2.3.6, A & B). In case of cultivar shabje the highest zone of inhibition was found 21 mm against the *Bacillus halodurans* and lowest zone of inhibition was found 13 mm against *Escherichia coli*, when the standard ciprofloxacin showed 30 mm and 26 mm zone of inhibition respectively. The extract of cultivar shadatae showed the highest zone of inhibition 10 mm against *Sarcina lutea,* and *Bacillus megaterium* and the lowest zone of inhibition was found 8 mm against *Bacillus subtillus and Bacillus halodurans, Shigella sonnei and Proteus species,* when the standard ciprofloxacin showed 26 mm, 30 mm, 22 mm and 25 mm respectively. The extract of cultivar roktima tested against all bacteria, the highest zone of inhibition was found against *Bacillus halodurans* and it was 24 mm, when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 14 mm against *Sarcina lutea* and *Klebsiella species* when the standard ciprofloxacin showed 29 mm and 25 mm zone of inhibition. In case of cultivar lalchay the highest zone of inhibition was 21 mm against *Escherichia coli,* when the standard ciprofloxacin showed 26 mm zone of inhibition but the lowest zone of inhibition was found 10 mm against *Bacillus subtillus, Klebsiella species,* when the standard ciprofloxacin showed 26 mm and 25 mm zone of inhibition respectively. In case of cultivar Badami, the highest zone of inhibition was 17 mm against *Proteus species,* when the standard ciprofloxacin showed 25 mm zone of inhibition but the lowest zone of inhibition was found 8 mm against *Bacillus subtillus,* when the standard ciprofloxacin showed 26 mm zone of inhibition. In case of cultivar dhusar, the highest zone of inhibition was 12 mm against *Bacillus megaterium, Bacillus halodurans* and *Pseudomonas aeruginosa*, when the standard ciprofloxacin showed 32 mm, 30 mm and 31 mm zone of inhibition respectively, but the lowest zone of inhibition was found 9 mm against, *Bacillus subtillus and Klebsiella species,* when the standard ciprofloxacin showed 26 mm and 25 mm zone of inhibition respectively.

5.2.3.7. Antibacterial activity of cell extract isolated after 16 days of cell suspension culture.

The results of this investigation were presented in the Table 5.2.3.7. In case of cultivar shabje the highest zone of inhibition was found 17 mm against the *Bacillus halodurans* and lowest zone of inhibition was found 11 mm against *Escherichia coli*, when the standard ciprofloxacin showed 30 mm and 26 mm zone of inhibition respectively. The extract of cultivar shadatae showed the highest zone of inhibition 9 mm *Bacillus megaterium,* when the standard ciprofloxacin showed 32 mm. But the lowest zone of inhibition was found 6 mm against *Shigella sonnei and Klebsiella species,* when the standard ciprofloxacin showed 22 mm and 25 mm zone of inhibition respectively. The extract of cultivar roktima tested against all bacteria, the highest zone of inhibition was found against *Bacillus halodurans* and it was 21 mm, when the standard ciprofloxacin showed 30 mm zone of inhibition but the lowest zone of inhibition was found 9 mm against *Sarcina lutea* when the standard ciprofloxacin showed 29 mm zone of inhibition. In case of cultivar lalchay the highest zone of inhibition was 17 mm against *Escherichia coli,* when the standard ciprofloxacin showed 26 mm zone of inhibition but the lowest zone of inhibition was found 8 mm against *Klebsiella species,* when the standard ciprofloxacin showed 25 mm zone of inhibition. In case of cultivar Badami, the highest zone of inhibition was 14 mm against *Proteus species,* when the standard ciprofloxacin showed 25 mm zone of inhibition but the lowest zone of inhibition was found 6 mm against *Bacillus subtillus,* when the standard ciprofloxacin showed 26 mm zone of inhibition. In case of cultivar dhusar, the highest zone of inhibition was 10 mm against *Bacillus megaterium, Bacillus halodurans* and *Pseudomonas aeruginosa*, when the standard ciprofloxacin showed 32 mm, 30 mm and 31 mm zone of inhibition respectively, but the lowest zone of inhibition was found 7 mm against*, Staphylococcus aureus, Bacillus subtillus, Klebsiella species and Proteus species,* when the standard ciprofloxacin showed 34 mm, 26 mm, 25 mm and 25 mm zone of inhibition respectively.

Table 5.2.3.1: Antibacterial activity of the cell extract isolated after 4 days of cell suspension culture of six cultivars of *R. communis* L. and standard ciprofloxacin.

Test organisms	V_1	V_2	V_3	V_4	V_5	V_6	Cipro.
	25 µl/disc						5µg/disc
	Diameter of zone of inhibition (mm)						
Gram positive bacteria							
Sarcina lutea	13	7	9	8	7	7	29
Staphylococcus aureus	11	6	13	12	8	6	34
Bacillus megaterium	13	7	11	13	6	7	32
Bacillus subtillus	10	-	12	6	-	-	26
Bacillus halodurans	15	-	17	16	9	9	30
Gram negative bacteria							
Shigella sonnei	11	-	12	10	6	-	22
Klebsiella species	11	-	10	-	-	-	25
Proteus species	10	-	13	10	10	-	25
Escherichia coli	8	-	13	13	9	-	26
Pseudomonas aeruginosa	13	-	14	8	8	7	31
Salmonella typhi	14	-	13	12	8	8	22

Table 5.2.3.2: Antibacterial activity of the cell extract isolated after 6 days of cell suspension culture of six cultivars of *R. communis* L. and standard ciprofloxacin.

Test organisms	V_1	V_2	V_3	V_4	V_5	V_6	Cipro.
	25 µl/disc						5µg/dic
	Diameter of zone of inhibition (mm)						
Gram positive bacteria							
Sarcina lutea	15	7	10	9	8	8	29
Staphylococcus aureus	13	6	16	15	10	7	34
Bacillus megaterium	15	7	13	15	8	9	32
Bacillus subtillus	12	7	14	8	6	6	26
Bacillus halodurans	17	-	19	17	11	10	30
Gram negative bacteria							
Shigella sonnei	13	6	14	11	8	6	22
Klebsiella species	13	6	11	7	6	6	25
Proteus species	12	7	16	13	13	7	25
Escherichia coli	10	-	16	17	11	7	26
Pseudomonas aeruginosa	15	7	16	10	10	8	31
Salmonella typhi	16	-	15	14	10	9	22

Table 5.2.3.3: Antibacterial activity of the cell extract isolated after 8 days of cell suspension culture of six cultivars of *R. communis* L. and standard ciprofloxacin.

Test organisms	V_1	V_2	V_3	V_4	V_5	V_6	Cipro.
	25 µl/disc						5µg/disc
	Diameter of zone of inhibition (mm)						
Gram positive bacteria							
Sarcina lutea	16	8	11	10	9	9	29
Staphylococcus aureus	14	7	17	16	10	8	34
Bacillus megaterium	16	8	14	16	8	9	32
Bacillus subtillus	13	8	15	9	6	7	26
Bacillus halodurans	18	10	21	18	12	11	30
Gram negative bacteria							
Shigella sonnei	14	6	15	11	9	7	22
Klebsiella species	14	7	12	8	7	7	25
Proteus species	13	8	17	14	14	8	25
Escherichia coli	11	8	17	18	12	8	26
Pseudomonas aeruginosa	16	8	17	11	11	8	31
Salmonella typhi	17	6	16	15	10	9	22

Table 5.2.3.4: Antibacterial activity of the cell extract isolated after 10 days of cell suspension culture of six cultivars of *R. communis* L. and standard ciprofloxacin.

Test organisms	V_1	V_2	V_3	V_4	V_5	V_6	Cipro.
	25 µl/disc						5µg/disc
	Diameter of zone of inhibition (mm)						
Gram positive bacteria							
Sarcina lutea	18	9	12	11	10	10	29
Staphylococcus aureus	15	8	18	17	11	9	34
Bacillus megaterium	17	9	15	18	9	10	32
Bacillus subtillus	13	7	17	10	7	8	26
Bacillus halodurans	19	10	22	19	13	12	30
Gram negative bacteria							
Shigella sonnei	15	7	16	12	10	8	22
Klebsiella species	15	7	13	9	7	8	25
Proteus species	14	8	18	14	15	9	25
Escherichia coli	12	9	18	19	13	9	26
Pseudomonas aeruginosa	17	8	18	12	11	10	31
Salmonella typhi	17	9	17	16	11	9	22

Table 5.2.3.5: Antibacterial activity of the cell extract isolated after 12 days of cell suspension culture of six cultivars of *R. communis* L. and standard ciprofloxacin.

Test organisms	V_1	V_2	V_3	V_4	V_5	V_6	Cipro.
	25 µl/disc						5µg/disc
	Diameter of zone of inhibition (mm)						
Gram positive bacteria							
Sarcina lutea	19	10	13	12	11	10	29
Staphylococcus aureus	15	9	19	17	11	10	34
Bacillus megaterium	18	10	16	19	9	11	32
Bacillus subtillus	14	10	18	10	8	8	26
Bacillus halodurans	20	11	23	20	14	12	30
Gram negative bacteria							
Shigella sonnei	15	8	16	12	11	9	22
Klebsiella species	16	9	13	9	8	9	25
Proteus species	15	9	19	14	16	10	25
Escherichia coli	13	9	19	20	14	10	26
Pseudomonas aeruginosa	18	9	19	12	11	11	31
Salmonella typhi	17	8	18	17	11	10	22

Table 5.2.3.6: Antibacterial activity of the cell extract isolated after 14 days of cell suspension culture of six cultivars of *R. communis* L. and standard ciprofloxacin.

Test organisms	V_1	V_2	V_3	V_4	V_5	V_6	Cipro.
	25 µl/disc						5µg/disc
	Diameter of zone of inhibition (mm)						
Gram positive bacteria							
Sarcina lutea	19	10	14	12	12	11	29
Staphylococcus aureus	15	9	19	18	11	10	34
Bacillus megaterium	18	10	17	20	9	12	32
Bacillus subtillus	14	12	18	10	8	9	26
Bacillus halodurans	21	11	24	20	14	12	30
Gram negative bacteria							
Shigella sonnei	15	7	17	12	11	11	22
Klebsiella species	16	7	14	10	9	9	25
Proteus species	15	9	20	15	17	10	25
Escherichia coli	13	9	20	21	14	11	26
Pseudomonas aeruginosa	18	9	20	12	11	12	31
Salmonella typhi	17	9	18	18	11	11	22

Table 5.2.3.7: Antibacterial activity of the cell extract isolated after 16 days of cell suspension culture of six cultivars of *R. communis* L. and standard ciprofloxacin.

Test organisms	V_1	V_2	V_3	V_4	V_5	V_6	Cipro.
	25 µl/disc						5µg/disc
	Diameter of zone of inhibition (mm)						
Gram positive bacteria							
Sarcina lutea	16	8	9	9	8	8	29
Staphylococcus aureus	13	8	13	13	17	7	34
Bacillus megaterium	15	9	15	16	7	10	32
Bacillus subtillus	12	7	15	8	6	7	26
Bacillus halodurans	17	7	21	16	12	10	30
Gram negative bacteria							
Shigella sonnei	12	6	14	10	8	8	22
Klebsiella species	13	6	11	8	8	7	25
Proteus species	13	7	17	12	14	7	25
Escherichia coli	11	7	16	17	12	9	26
Pseudomonas aeruginosa	15	8	16	10	9	10	31
Salmonella typhi	14	8	14	14	9	9	22

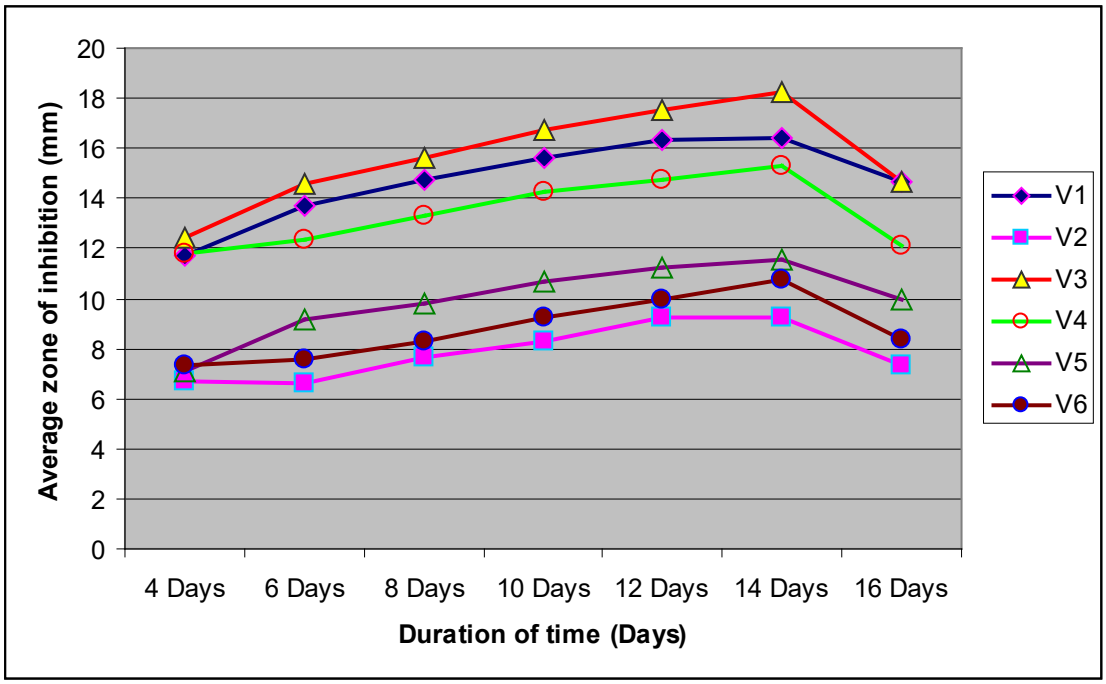


Fig. 5.2.3.1: Average zone of inhibition of six cultivars of castor from the cell extracts took from different duration of time (days).

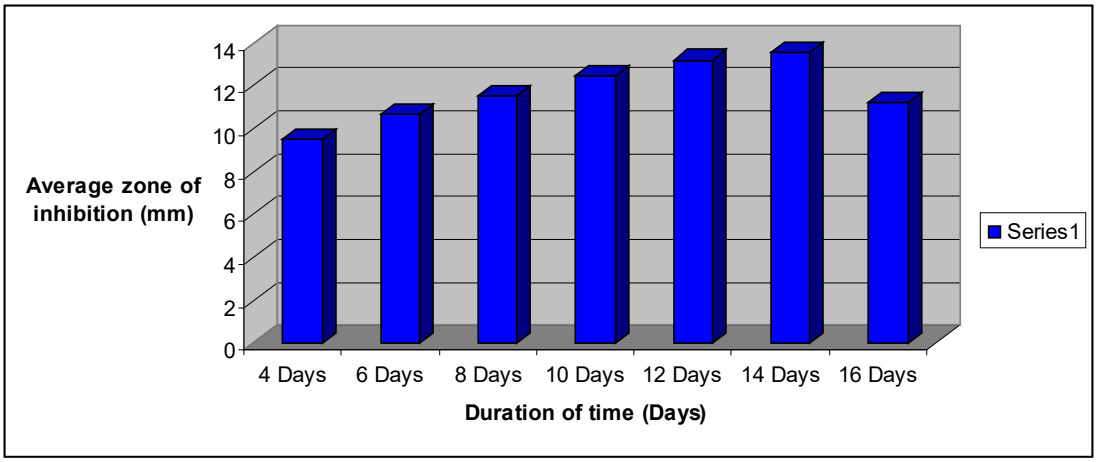


Fig. 5.2.3.2: Average zone of inhibition in different duration of time (days).

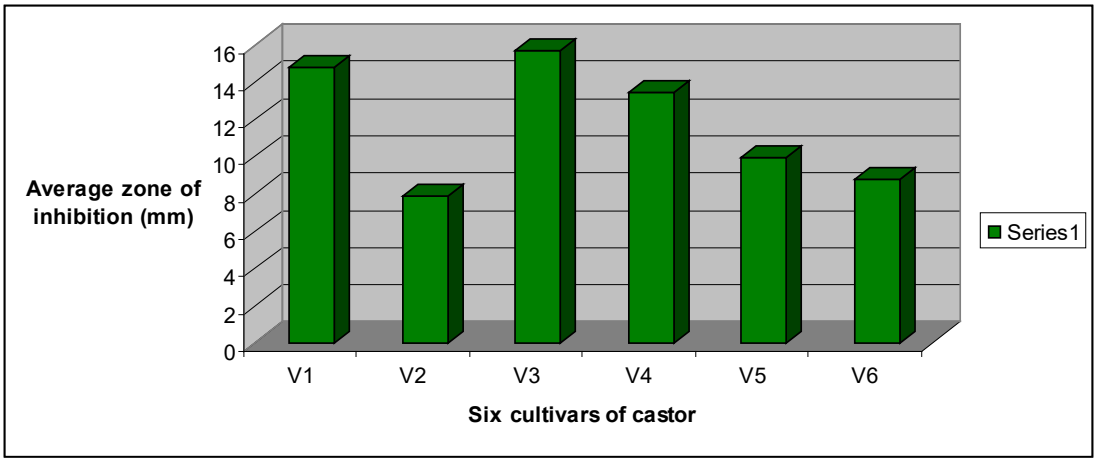
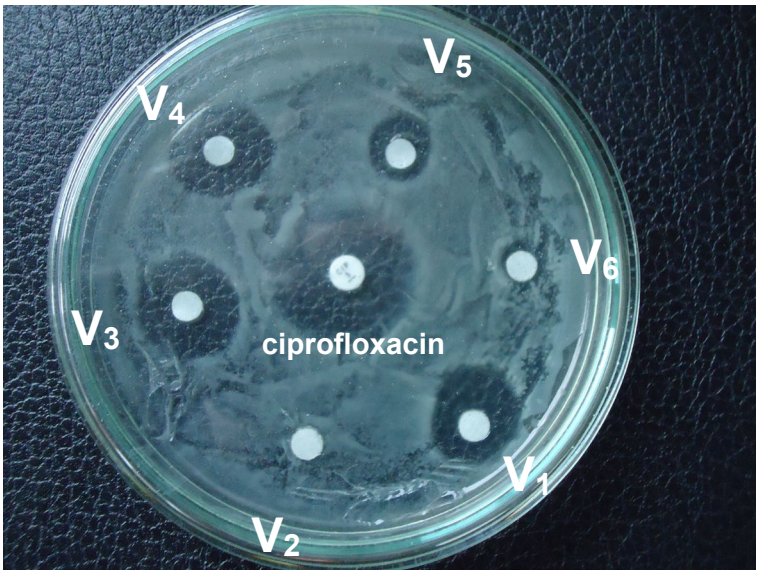


Fig. 5.2.3.3: Average zone of inhibition of different duration of time (days) of six cultivars of castor.

ANTIBACTERIAL ACTIVITY AFTER 4-DAYS CULTURE

A



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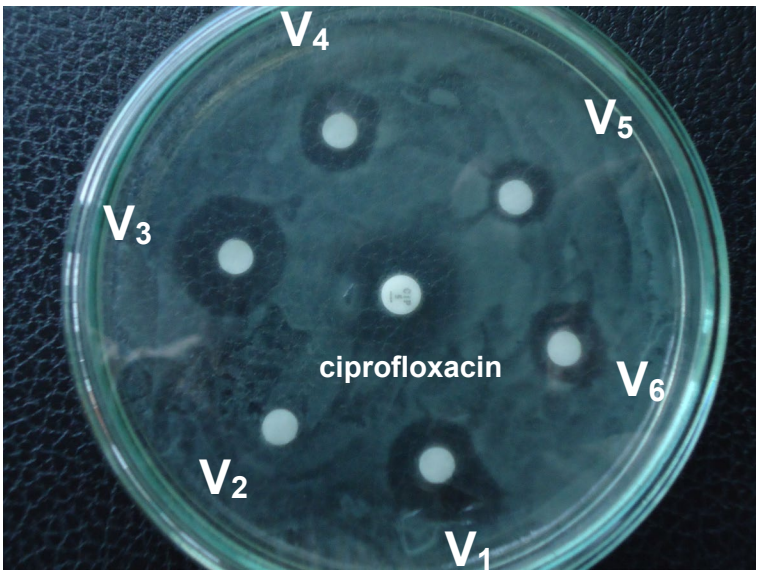
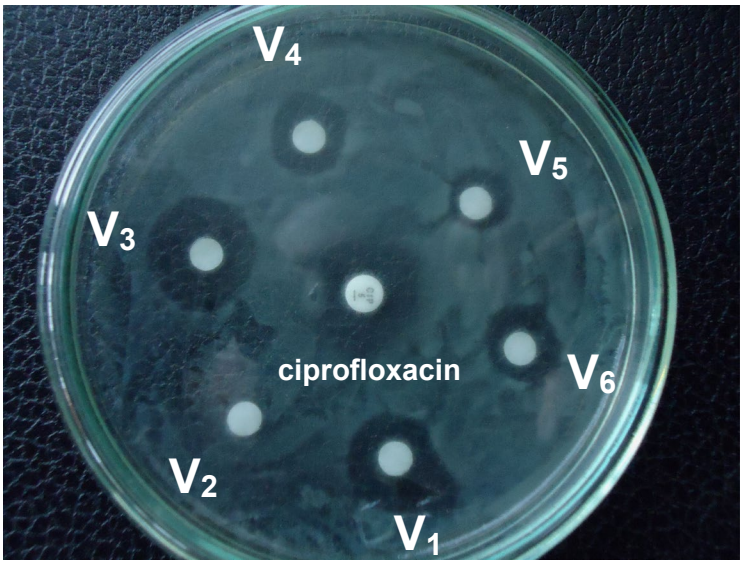


Plate- 5.2.3.1: A: Photographs showing the effect of cell extract (25 µl/disc) of six cultivars of castor taken after 4 days of cell suspension culture and standard ciprofloxacin (5 µg/disc) against *Bacillus holodurans*. **B:** Photographs showing the effect of cell extract (25µl/disc) of six cultivars of castor taken after 4 days of cell suspension culture and standard ciprofloxacin (5µg/disc) against *Pseudomonas aeruginosa*. (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar.)

ANTIBACTERIAL ACTIVITY AFTER 6-DAYS CULTURE

A



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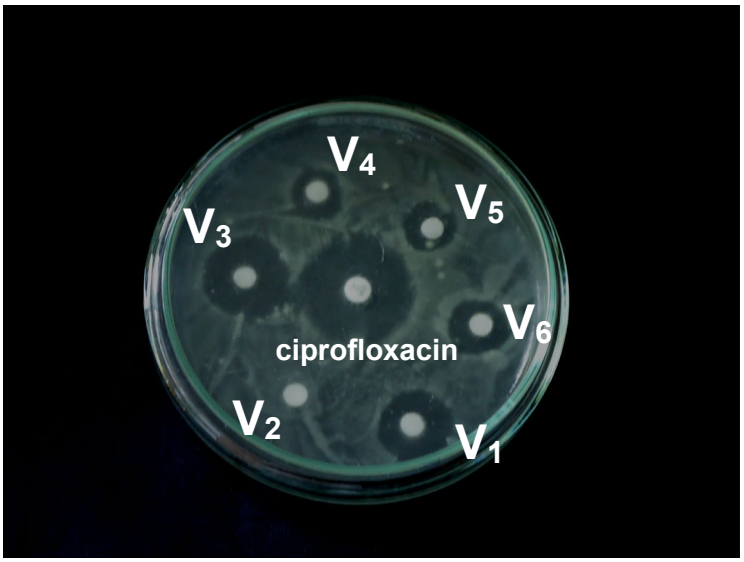
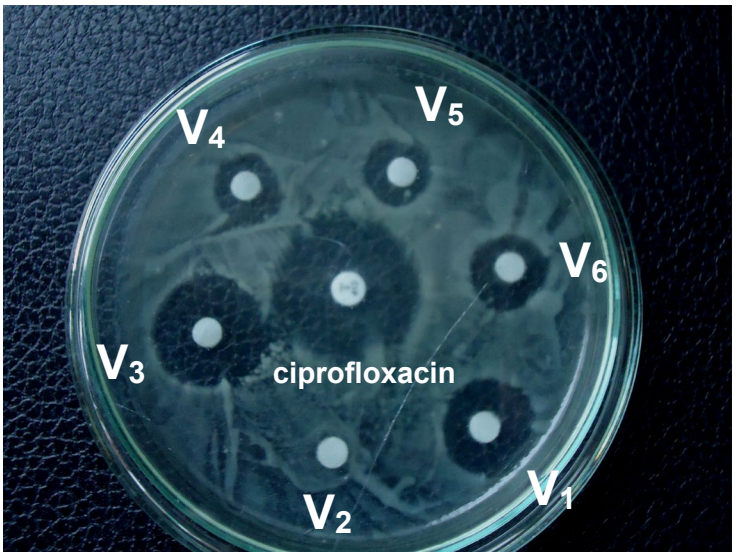


Plate- 5.2.3.2: A: Photographs showing the effect of cell extract (25µl/disc) of six cultivars of castor taken after 6 days of cell suspension culture and standard ciprofloxacin (5 µg/disc) against *Bacillus holodurans*. **B:** Photographs showing the effect of cell extract (25µl/disc) of six cultivars of castor taken after 6 days of cell suspension culture and standard ciprofloxacin (5µg/disc) against *Escherichia coli*. (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar.)

ANTIBACTERIAL ACTIVITY AFTER 8-DAYS CULTURE

A



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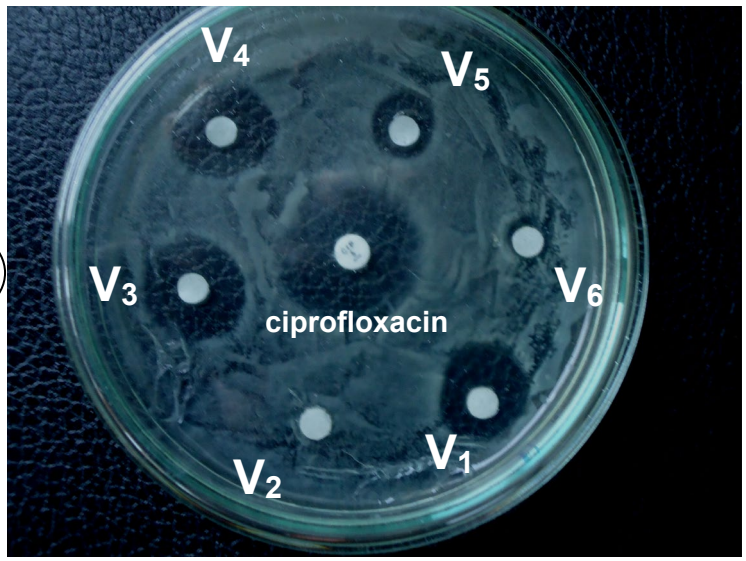
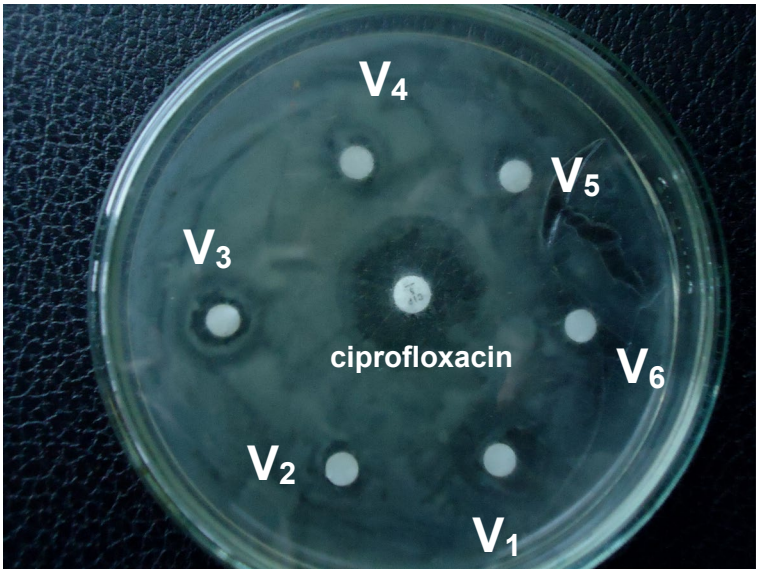


Plate- 5.2.3.3: A: Photographs showing the effect of cell extract (25 µl/disc) of six cultivars of castor taken after 8 days of cell suspension culture and standard ciprofloxacin (5 µg/disc) against *Shigella sonnei*. **B:** Photographs showing the effect of cell extract (25µl/disc) of sixcultivars of castor taken after 8 days of cell suspension culture and standard ciprofloxacin (5 µg/disc) against *Salmonella typhi*. (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar).

ANTIBACTERIAL ACTIVITY AFTER 10-DAYS CULTURE

A



B

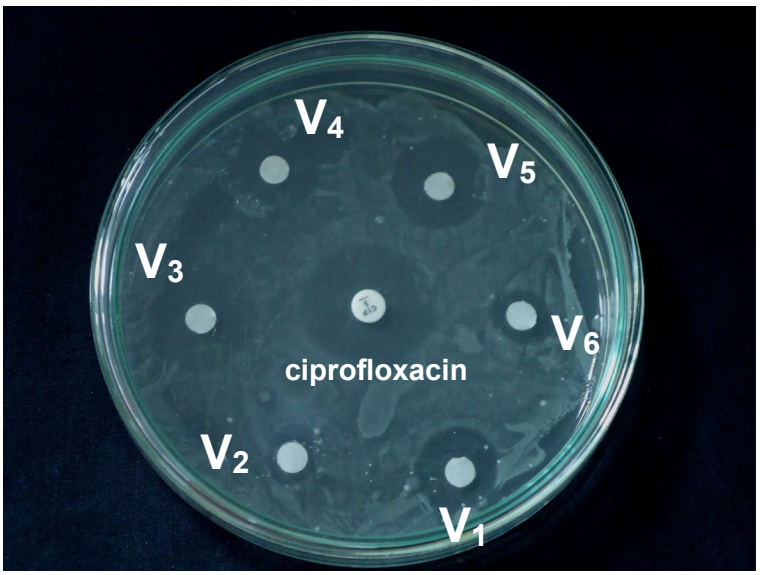
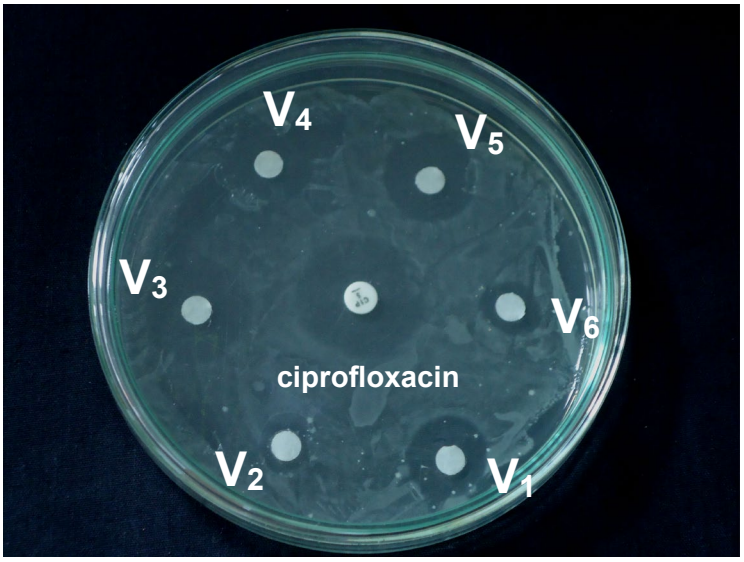


Plate- 5.2.3.4: A: Photographs showing the effect of cell extract (25 µl/disc) of six cultivars of castor taken after 10 days of cell suspension culture and standard ciprofloxacin (5 µg/disc) against *Klebsiella species*. **B:** Photographs showing the effect of cell extract (25 µl/disc) of six cultivars of castor taken after 10 days of cell suspension culture and standard ciprofloxacin (5 µg/disc) against *Bacillus subtillus*. (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar).

ANTIBACTERIAL ACTIVITY AFTER 12-DAYS CULTURE

A



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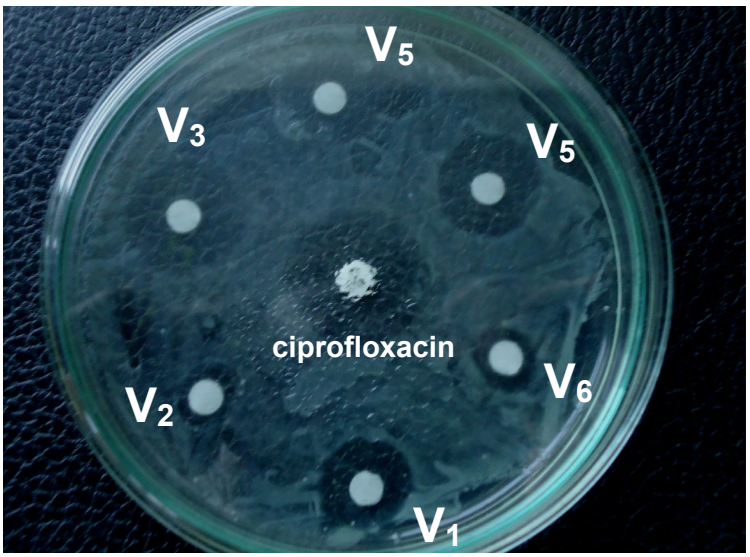
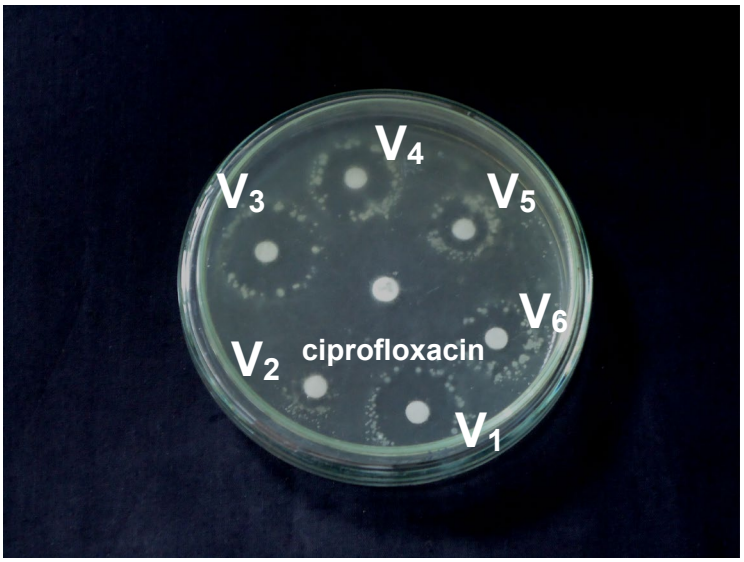


Plate- 5.2.3.5: A: Photographs showing the effect of cell extract (25 µl/disc) of six cultivars of castor taken after 12 days of cell suspension culture and standard ciprofloxacin (5 µg/disc) against *Shigella sonnei*. **Fig.** Photographs showing the effect of cell extract (25 µl/disc) of six cultivars of castor taken after 12 days of cell suspension culture and standard ciprofloxacin (5 µg/disc) against *Klebsiella species*. (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar).

ANTIBACTERIAL ACTIVITY AFTER 14-DAYS CULTURE

A



B

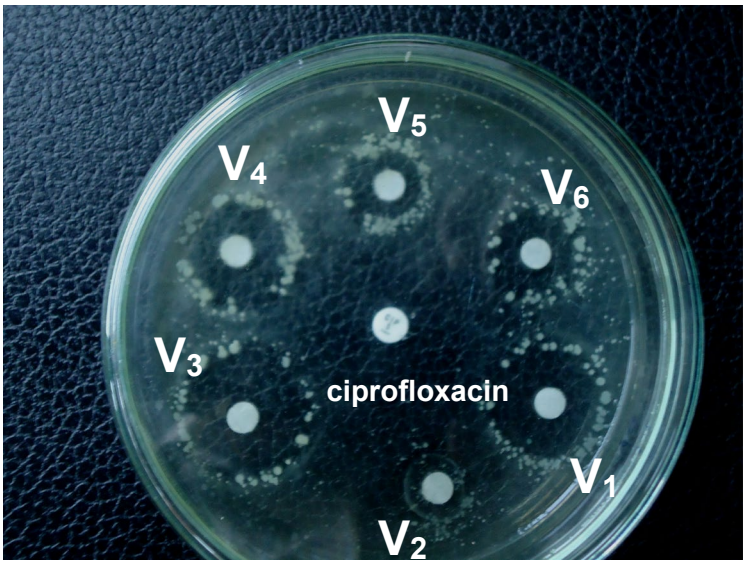


Plate- 5.2.3.6: A: Photographs showing the effect of cell extract (25 µl/disc) of six cultivars of castor taken after 14 days of cell suspension culture and standard ciprofloxacin (5 µg/disc) against *Salmonella typhi*. **B:** Photographs showing the effect of cell extract (25 µl/disc) of six cultivars of castor taken after 16 days of cell suspension culture and standard ciprofloxacin (5 µg/disc) against *Staphylococcus aureus*. (V_1 = Cultivar shabje, V_2 = Cultivar shadatae, V_3 = Cultivar roktima, V_4 = Cultivar lalchay, V_5 = Cultivar badami, V_6 = Cultivar dhusar).

5.2.4. DISCUSSION

Plant tissue culture approach has offered potent techniques, which may significantly contribute to the scheme of increasing levels of medicinally useful secondary metabolites (Ferreira and Duke 1997, Duke *et al*. 1999, Khafagi *et al*. 2003). For any drug development, antibacterial screening is obligatory and in the present investigation antibacterial screening against cell extract was performed with this objective.

The extracts from cell suspension cultures, which were collected from different periods viz. after 4 days, 6 days, 8 days, 10 days, 12 days, 14 days and 16 days of cell suspension culture. Every after two days, 5 ml liquid medium with growing cells were centrifuged at 4000 rpm for 10 minutes. The cells were discarded and the clear supernatant was collected which was used as cell extracts. Using 25 µl of the supernatant was applied on the test disc. Cell extracts of castor culture assumed to contain toxic material liberated by the growing cell to the culture medium and it is believed that the cell extracts harvested at different days would have different levels of lethal effect to the micro organisms. Our experimental results were also in support of this assumption as different grades of lethality shown under different periods of culture. The extracts collected after different periods showed different levels of antibacterial activities under the test. Especially the extract taken after 14 days from cell suspension culture showed the best antibacterial activities followed by 12 days, 10 days, 8 days, 16 days, 6 days and lowest in 4 days (Fig. 5.2.3.2). Minimum zone of inhibition was observed in antibacterial test with the cell extract of 4 days culture and over the varieties it was 9.51 mm while maximum zone of inhibition was noted in 14 days culture. It proved that cells growing for longer time in the suspension culture were able to produce more amount of toxic element to the culture making it more toxic to the bacterial growth. The experimental results indicated that the highest level of lethality was observed in the extract of 14 days culture which deteriorate thereafter synchronizing with cell growth as accomplished in the culture. It is very interesting to note that the highest level of cell growth occurred in 14 days culture similarly highest level of lethality (toxic effect) was also noted for 14 days culture. Thus, for castor plant, cell growth 14 days could be recommended as the standared period of cell culture for commercial use in isolation of toxic element (possibly ricin) from cell suspension culture.

Cell extract of six cultivars of castor viz. cultivar shabje, cultivar shadatae, cultivar roktima, cultivar lalchay, cultivar badami and cultivar dhusar were used to test the antibacterial activities against five gram positive and six gram negative

bacteria. Among the cultivars, comparatively cultivar roktima showed the highest lethality followed by cultivar shabje and these two cultivars showed the zone of inhibition against all bacteria tested. But the remaining cultivars failed to show perfect lethality against all the bacteria tested, particularly for the cell extract taken from 4 days and 6 days of cell suspension culture. It is important to note that all the castor cultivars were able to show the zone of inhibition against all bacteria after 8 days, 10 days, 12 days, 14 days and 16 days of extract from cell suspension culture (Fig. 5.2.3.3) but their lethality were lower than cultivar roktima and cultivar shabje. It was clear that cultivar roktima showed the highest lethality in antibacterial test followed by cultivar shabje. The result presented in the Fig. 5.2.3.1. clearly demonstrated that every cultivar of the castor exhibited a very specific range in the zone of inhibition when applied to the antibacterial test and their range of action are distinct from one another. The experimental results indicated that the castor cultivars also holds the diversity in the levels of their toxicity and mode of action in their antibacterial activities.

Khafagi (2007) work on induced callus in MS medium from roots, hypocotyls and cotyledonary leaves of castor minor and major and tested antibacterial activities of gram-positive and gram-negative bacteria by extracts of those callus. He observed ricin accumulation was significantly higher in callus tissues derived from those explants of *R. communis* L. variety minor than that accumulated in callus tissues derived from corresponding explants of variety major. Comparable results were reported by Wahab (2001), who found variations in the electrophoretic protein patterns in roots and leaves of *R. communis* L. populations of deserts and riverine, which are also corresponding to large-seed and small-seeded varieties. It was commonly established that slow growing callus cultures tend to accumulate higher levels of secondary metabolites compared with fast growing callus cultures (Ferreira and Duke 1997). Significant inhibitory activity was observed from callus culture extract of *Nigella* species. Since variations in chemical composition of callus tissues and intact plants have been described for number of plant species. According to the 2007 edition of the Guinness Book of world Records, the castor plant was mentioned as the most poisonous in the world. Fennell *et al*. (2004) reported that *R. communis* L. adult plant is one of the highly toxic plants that cause both DNA damage and chromosomal aberration.

Application of biotechnological systems to produce some compounds produced from such medicinally important *R. communis* L. plant should be more rational after throughly exploring various plant varieties for *in vitro* growth and major

metabolite production (Khafagi 2007). Despeyroux *et al*. (2000) highlights the existence of ricin heterogeneity that is originated for various *R. communis* varieties, which may necessitate the importance of exploring various *R. communis* L. varieties for active metabolites.

R. communis L. has broad medicinal uses that are documented in the ethnobotanical literature (Ayensu 1978, Boulos 1983, Rizk 1986). Recent ethnopharmacological research studies had regularly confirmed most of the therapeutic values reputed for such well-known medicinal plant. For instance, methanol extracts of *R. communis* L. root were reported to possess significant anti-inflammatory activity in acute and chronic inflammatory models in rats (Ilavarasan *et al*. 2006), stimulant effects of ricin on the central nervous system of mice, which cause memory-improving effect and seizure-eliciting properties was reported and may post ricin for the treatment of human amnesias (Ferraz *et al*. 1999), relation of the insecticidal properties of *R. communis* foliage extracts to the flavoniods quercetin and kaempferol (Upasani *et al*. 2003). Fennell *et al*. (2004) confirmed the anthelmintic activity of *R. communis* L. extracts. Immanuel *et al*. (2004) reported a potent antimicrobial action of n-butanol extract of *R. communis* L. against shrimp pathogen *Vibrio parahaemolyticus*. Sitton and west (1975) described the production of the anti-fungal diterpense casbene in cell-free extracts of *R. communis* L. seedlings. Antifungal activity of fatty acids against the symbiotic fungus *Leucoagaricus gongylophorus* and insecticidal activity of ricin against the leaf-cutting ant *Atta sexdens rubropilosa* were established (Bigi *et al*. 2004). Also, antifertility effects of 50% ethanol extract was revealed, which produced alternation in the motility, mode of movement and morphology of the sperms of male rats (Sandhyakumary *et al*. 2003). Bedouins usually utilize castor oil for the infection of intestinal worms (Zohary 1987, Rizk 1986). Fennel *et al*. (2004) reported that *R. communis* L. possess antihelmintic activity, without mentioning the variety used for the bioassay.

The present investigation demonstrated that the extracts of cell suspension culture of six cultivars of *R. communis* L. considered to be the potent source of antibacterial compounds and proved as an alternative source for production toxic compound ricin from castor. The experimental results confirm that there has been a wide range of genetic diversity in toxic behavior also existed in different castor genotypes of Bangladesh. Among the cultivars of castor in Bangladesh the cultivar roktima looking very dark reddish in colour proved to be most toxic and holds great potential for exploitation of commercial use in future toxic drug development.

Table 5.1.3.3a: Effect of auxin (NAA) in combination with cytokinin (BAP) employed in MS medium on callus induction from isolated cells. Data were recorded after 6 weeks of culture.

Growth regulators	Concentration of growth regulators mg/L	Cultivar shabje			Cultivar shadatae			Cultivar roktima		
		Days of callus initiation	% of explants responded	Colour of callus	Days of callus initiation	% of explants responded	Colour of callus	Days of callus initiation	% of explants responded	Colour of callus
BAP+NAA	2.0+0.1	32	4.8	W	34	4.5	W	32	4.7	W
	2.0+0.2	28	5.2	W	29	4.9	W	28	5.1	W
	2.0+0.5	20	6.7	W	22	6.0	W	20	6.5	W
	2.0+0.8	22	5.9	W	24	5.2	W	22	5.6	W
	2.0+1.0	24	4.1	W	26	3.8	W	24	4.0	W

W-Whitish.

Table 5.1.3.3b: Effect of auxin (NAA) in combination with cytokinin (BAP) employed in MS medium on callus induction from isolated cells. Data were recorded after 6 weeks of culture.

Growth regulators	Concentration of growth regulators mg/L	Cultivar lalchay			Cultivar badami			Cultivar dhusar		
		Days of callus initiation	% of explants responded	Colour of callus	Days of callus initiation	% of explants responded	Colour of callus	Days of callus initiation	% of explants responded	Colour of callus
BAP+NAA	2.0+0.1	33	4.7	W	32	4.5	W	33	4.5	W
	2.0+0.2	28	5.0	W	28	4.9	W	29	4.9	W
	2.0+0.5	21	6.3	W	20	6.1	W	22	6.0	W
	2.0+0.8	24	5.5	W	22	5.2	W	24	5.2	W
	2.0+1.0	25	4.0	W	24	3.9	W	26	3.9	W

W-Whitish

CHAPTER-VI

6. GENERAL DISCUSSION

Castor plant is a virgin plant in Bangladesh without having any research work but a comprehensive research works were performed in several countries particularly in India on castor diversities and variety characterization. Anjani *et al*. (1994) collected castor (*R. communis* L.) germplasms in north-eastern hill province of India and Anjani *et al*. (1999) collected castor (*R. communis* L.) germplasms in northwestern India. Particularly on morohological variation of castor germplasms were studied in Egypt (Shaheen 2002). AM Shaheen collected castor germplasms from different phytogeographic regions in Egypt and found morphological differences among the populations collected from different localities. In our study a number of twenty eight morphological characters were addressed as for evaluation of phenotypic diversity of castor and characterization of existing germplasms in Bangladesh. Diversity of different cultivars of castor has also been evaluated for tissue culture response in artificial media particularly to the application of different concentrations of growth hormones. Wide range of diversity was observed in their cell suspension culture and in the antibacterial activity test when cell extract was applied to different gram positive and gram negative bacteria.

6.1. Phenotypic and genotypic diversity

Under the present perspectives it is necessary to make a survey on the existing germplasms of castor over the country in order to preserve the land races with their particular gene pools for breeding purposes. A number of varieties of castor found to grow here and there over the country with distinct morphological variation. In the present investigation, a through and extensive survey was made throughout the country and castor germplasms were collected and established a germplasm in the Institute of Biological Sciences, Rajshahi University, Bangladesh.

During the growth period of castor twenty eight morphological characters were chosen from three distinct morphological growth phases; vegetative growth, reproductive growth and yield and yield component and their comparative performance were stated under the following heads:

The agro-climatic condition of Bangladesh favors for the luxurious growth of castor particularly during the monsoon season when within three months it starts flowering. Depending on the morphological features we have selected fifteen

parameters to evaluate the comparative performance in vegetative growth of different castor genotypes in Bangladesh.

Among the six genotypes cultivar shabje showed the highest height in vegetative growth over others and it attained 466.11cm at the flowering time which was about 2½ times greater than that of cultivar dhusar. The cultivar roktima and cultivar lalchay were the next performer in height and these three cultivars, shabje, roktima and lalchay can be considered as the highest performers in vegetative growth in height. On the other hand, dhusar can be marked as the dwarf cultivar in Bangladesh (201.16cm) followed by cultivar shadatae and cultivar badami. Other stem characters including stem diameter, branch number, branch length, node number and internodal distance showed the similar performance in vegetative growth in all castor germplasms. Node number and internodal distance exhibit lower range of variation among the cultivars in respect of other stem characters. Very wide range of variation was observed in plant height and other stem characters of the different genotypes in Bangladesh particularly stem diameter, branch number and branch length. All stem characters including plant height, stem diameter, branch length, branch diameter and internodal distance experienced high level of phenotypic and genotypic variation with high heritability proved that these characters are genetic in nature and hold the merit to give preference a varietal selection in the breeding programmee. Low level of difference between phenotypic and genotypic variations and high heritability in all these characters indicate that major of the phenotypic variation is contributed by genotype but in case of plant height high environmental coefficient of variation indicates that environmental conditions had a greater influence in plant height in regards of other stem characters.

Leaf characters including leaf length, leaf breadth, lobe number, petiole length also exhibit very wide range of variation among the different cultivars and all these differences were statistically significant. Greater size of leaf (leaf area) was observed in cultivar lalchay followed by cultivar roktima and cultivar shabje. Greater length and breadth of leaf were also observed in these three castor cultivars. The cultivar dhusar is always showed small leaf size and in regards of leaf area it stands about half of the size of cultivar lalchay. Petiole length in cultivar shabje is exceptionally greater than the other cultivars which projected its leaves for better exposure to the sunlight, expecting greater role of photosynthesis in this plant. Low level of difference between phenotypic and genotypic variation and high level of heritability in all leaf characters indicates that the major portion of the phenotypic variance is contributed by the genotypes and these characters are heritable in nature. But there is one exception as observed in leaf area where environmental coefficient of variation stands very high

indicating this character is relatively more venerable and is very prone to the environmental variation.

Low levels of variations were observed in root characters including root length, lateral root number and lateral root length but the differences were of significant level. High level of heritability and low level of difference between phenotypic and genotypic variation proved genetic in nature and deserves importance in varietal selection.

Under our present study eight characters were considered as the reproductive parameters of the castor genotypes in Bangladesh viz. inflorescence number, male flower's region, female flower's region, male flower number, female flower number, fruit number, number of thorn and flowering time. Inflorescence number per plant is very important character as it determine the productive yield of the genotype. In castor very wide range of variation was found in florescence number. The cultivar shabje and roktima gave the highest inflorescence number which is about four times higher than the lowest performer. In respect of reproductive region female flower always occupied greater portion than that of male flower in an inflorescence. Number of female flower were also produced in great number than male flower in all castor genotypes. Significantly greater number of fruits were produced in cultivar shabje and the number did not vary widely among the other cultivars. Flowering time is another important character which was found to vary widely among the castor cultivars. The cultivar shadatae, cultivar badami and cultivar dhusar were noticed as the early flowering plants taking only 46, 47 and 49 days for flowering respectively. On the contrary, the cultivars shabje, lalchay and roktima were identified as the late blooming cultivars taking 161, 154 and 146 days for flowering respectively. In productive perspectives, the cultivar shabje holds the height merit of having greater number of inflorescence per plant being mounted by greater number of fruits per inflorescence. All the flowering characters including inflorescence number, male flower's region, female flower's region, male flower number, female flower number, fruit number, number of thorn and flowering time were identified as very stable in nature as they exhibit very low level of difference between of phenotypic and genotypic variations accompanied by high level of heritability, exceptionally for the character of thorn per fruit. Production of thorn over the fruit surface was rather found to be influenced more by environmental changes as its coefficient of variation in environmental parameters exhibit greater values.

The seed yield in castor is very important as it is considered as the economic yield in castor cultivation. Under our present investigation five seed characters were selected for study to evaluate the yield performance of castor genotypes which were

seed per fruit, seed length, seed breadth, total weight of seed per plant and weight of hundred seeds. Number of seed per fruit is an exceptional character which did not vary and remains intact in number in all castor genotypes during its growth phases. Individual seed length and breadth were found significantly greater in cultivar shabje, both the characters did not show any wide range of variation among other castor genotypes. In regards of individual seed weight, very wide range of variation was observed in different castor genotypes. Highest unit seed weight was observed in roktima followed by cultivar shabje which were about 100% greater than that of cultivar badami and cultivar dhusar. Cultivar shadatae and cultivar lalchay on the other hand produced seeds which were in intermediate in weight. The most important character is seed yield per plant which experienced the highest level of variation among the castor genotypes under our study. The cultivar shabje produced 2382 gm seed per plant whereas the cultivar dhusar produced only 64 gm which was about 37 times greater in yield. The second one is cultivar roktima producing 485 gm seed per plant followed by cultivar lalchay 206 gm. In this regard cultivar shadatae and cultivar badami were in intermediate in producing seed per plant 144 gm and 127 gm respectively. The results presented in genetic parameter indicated that the more genetically stable characters are seed length and seed breadth exhibiting 100% heritability and showing no difference between genotypic and phenotypic variations. Individual seed weight and seed yield per plant were also identified as genetically stable character specific value for specific cultivar and exhibit 99.9% heritability and very scantly level of difference was observed among phenotypic and genotypic variations.

From the foregoing discussion it can be concluded that the castor cultivars cultivated in Bangladesh holds very high level of genetic diversity as has been ventilated by our experimental results. Among the castor cultivars shabje has been proved as the highest yielder in Bangladesh followed by roktima. The cultivar shabje also grows very first and producing greater biomass in addition to its greater seed yield.

6.2. Molecular diversity

Genetic identity is important for cultivar identification. In Bangladesh, possibly it was the first attempt to conduct isolation DNA and was used for RAPD to check the genetic diversity among the six cultivars of castor. Despite the recent efforts to characterize castor bean germplasm collections, there are relatively few molecular markers available (Santelmo *et al*. 2012) curiously, the use of widely spread and low cost anonymous markers, as RAPD and ISSR, in genetic diversity analysis is still

problematic and insufficient (Santelmo *et al*. 2012). The evaluation of varieties in distinct agro-climatic zones demonstrates significant levels of variation in response to the selection pressure in the zones (Singh *et al*. 1998). Both the RAPD and ISSR molecular markers have been used in population genetic studies (Parsons *et al*. 1997, Esselman *et al*. 1999, Li and Ge 2001). In the present investigation we have used only RAPD molecular markers. In our investigation we used seven primers against the six cultivars and among the seven primers three gave a total of 50 visible bands in gel electrophoresis with an average of 16.66 bands from the six cultivars of castor which can be used for fingerprinting.

Cultivar shabje showed 7 bands in primer OPA-9, but did not show any band in primer OPA-10 and OPB-17. Cultivar shadatae showed 7 bands in primer OPA-9 and 6 bands in primer OPA-10, but did not produce any band in primer OPB-17. Cultivar roktima showed 8 bands in primer OPA-9, 6 bands in primer OPA-10 and 7 bands in primer OPB-17. Cultivar dhusar showed 4 bands in primer OPA-9, 5 bands in primer OPA-10 but did not show any band in primer OPB-17. Cultivar lalchay and cultivar badami did not show any band in any primer which were used in our experiment.

Primer OPA-9 produced 14 polymorphic bands out of total 26 bands and showed 53.85% polymorphism. Primer OPA-10 produced 2 polymorphic bands out of total 17 bands and showed 11.76% polymorphism. Primer OPB-17 produced 7 polymorphic bands out of total 7 bands and showed 100% polymorphism. Some researchers have considered RAPD markers to represent segments of DNA with non coding regions and to be selectively neutral (Bachmann 1997, Landergott *et al*. 2001), and some studies have shown that RAPD markers are distributed throughout the genome and may be associated with functionally important loci (Penner 1996). Dendogram has not been computed but observed result indicated that the cultivar lalchay and badami failed to produce any band against any one of the seven primers used and confirming that these two cultivars are widely related to the other four castor cultivars. The cultivar shabje denoting its distant phylogenic position amplifying DNA bands only for one time against the primer OPA-9. The cultivars shadatae and cultivar dhusar are presumed to be more likely related as they amplified DNA bands against the two primers OPA-9 and OPA-10. While the cultivar roktima produced seven bands against the primer OPB-17 denoting its individual identity and very much different from other castor cultivars. In our investigation the cultivar roktima also has been appeared as a very distinct characters or very distinct genetic diversity in other sections of research including morphological parameters and cell culture and antibacterial activities. The castor cultivar roktima is virtually a unique cultivar

showing very remarkable reddish in colour for its stem, petiole and flowering inflorescence. In nutshell, in order to make more precise interference about the genetic diversity and phylogenic relationship among the castor cultivars further research is needed on detail analysis of RAPD and SSR markers.

However, from the foregoing discussion it can be concluded that highly differential banding pattern was observed among the cultivars of castor. The presence of wide level of genetic diversity existed among the different cultivars of castor in Bangladesh. Use of different primers exhibited different levels of DNA polymorphism in different castor cultivars proving a very wide level of intraspecies genetic diversity in Bangladeshi castor cultivars.

6.3. Diversity of castor in *in vitro* regeneration and conservation

Castor is extremely recalcitrant to *in vitro* regeneration (Ahn *et al*. 2007). The present investigation was, therefore, undertaken to establish a high frequency plant regeneration system from the seedling explants of local six cultivars of castor using tissue culture technique. Castor cultivars exhibited different levels of responses to tissue culture approaches in the area of direct and indirect regeneration showing their diversity in artificial medium. Under the present investigation the diversity of different cultivars of castor, as had been experienced in different tissue culture experiments, were evaluated.

MS (Murashige and Skoog 1962) medium is a recognized as basal medium for tissue culture techniques and extensively used for *in vitro* propagation of various plant species including medicinal plants (Shudha *et al*. 1998, Rout *et al*. 2000). Three modified MS medium (MS, MMS_1 and MMS_2) were used for axillary shoot proliferation of six cultivars of castor in order to evaluate their comparative efficiencies in shoot proliferation. Half strength MS medium was proved as the best media formulation for *in vitro* propagation of castors cultivars as almost all the characters were significantly higher in the medium containing half strength of major salts and full strengths of minor salt and vitamin enriched MS medium (MMS_1). In our observation, we obtained more efficient regeneration in half strength MS medium than full strength MS medium and this stands as one of our new findings indicating that MS medium containing half strength of major salts and full strengths of minor salt and vitamin, induced better axillary shoot proliferation in castor. Kamrun and Rira (2012) also obtained *in vitro* propagation from shoot tip explants of four months old castor plant using fortified full strength MS medium. All reported research workers working on *in vitro* regeneration on castor used full strength MS media and most of them encountered recalcitrance problem in shoot proliferation in castor. Half

strength MS medium proved as an efficient alternative to avoid recalcitrance to some extent in shoot proliferation in castor.

Cotyledonary node and shoot tip were used as explants in the experiment for proliferation of axillary shoots. The experiments were cultured on MS medium supplemented separately with BAP, Kn and 2ip in a single concentration of 1.0 mg/L for proliferating axillary shoots. Among the two types of explants used, proliferation efficiency of cotyledonary node explant was higher than the shoot tip explant. Alam *et al*. 2010 also used cotyledonary node as an explant for castor and obtained better efficient regeneration. Kamrun and Rita (2012) also reported *in vitro* propagation of castor from shoot tip explants. Better response or recalcitrance behavior are supposed to be caused by the indigenous hormonal difference in different plant parts. In our experimental results proved that cotyledonary node in castor showed better performance in shoot proliferation than apical meristem.

BAP, Kn and 2ip at a concentration range of 0.5-5.0 mg/L were tested for assessing the optimum concentrations of the cytokinins for early sprouting and maximum proliferation of axillary shoots. BAP 2.0 mg/L was found to be more effective than either Kn or 2ip on the proliferation and development of six cultivars of castor shoots, an effect similar to that observed by Alam *et al*. (2010) in castor. Sujatha and Reddy (1998) also used 2.0 mg/L BAP for best shoot induction and development of castor.

For axillary shoot proliferation, cultivar shabje showed the best performance followed by cultivar roktima and cultivar lalchay. In this case percentage of shoot induction, induced shoot number and shoot length were also showed the highest in cultivar shabje. Cultivar roktima and cultivar lalchay were in second position. Cultivar shadatae, badami and dhusar produced shoots but were not significant as cultivar shabje, roktima and lalchay. The experimental results proved that the cultivars of castor were distinct from one another performing unique level of efficiency in tissue culture performance including shoot proliferation and subsequent elongation.

Callus induced was observed in all the six cultivars of castor from three types of explants viz, hypocotyl, internode and immature leaf but their levels of efficiency were found different for different cultivers. In these experiments only one cytokinin BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/L) with all possible combinations of five different concentrations (viz, 0.1, 0.2, 0.5, 0.8 and 1.0 mg/L) of three auxins viz. NAA, 2,4-D and IAA were used.

Callus induced was observed in all the cultivars of castor from three types of explants used for callus induction. Among the three explants were used, hypocotyl

explant showed the best performance. When hypocotyl explant was used in different concentrations of auxins and cytokinin for callus induction of six cultivars it was observed that callus induced from all combinations. Percentage of explants showing callus formation was highest (100%) in cultivar shabje, cultivar roktima and cultivar lalchay followed by cultivar badami (92.5%), cultivar shadatae (92.2%) and cultivar dhusar (91.6%). When internode explant was used for callus induction from six cultivars of castor the rate of highest frequencies of callus induction was found in cultivar shabje (95.3%) followed by cultivar roktima (94.8%), cultivar lalchay (90.4%), cultivar badami (90.3%), cultivar dhusar (88.4%) and cultivar shadatae (88.3%). When immature leaf was used as explants for callus induction it was observed that some concentrations and combinations of auxin and cytokinin failed to produce callus formation. In these experiments the rate of highest frequencies was found in cultivar shabje (63.3%) followed by cultivar roktima (62.1%), cultivar dhusar (62.3%) cultivar badami (61.1%), cultivar shadatae (60.4%) and cultivar lalchay (60.1%).

The colour and nature of callus showed variations on the basis of cultivars, such as cultivar shabje showed greenish type of callus viz. green friable, green compact, green compact nodular and whitish green. Cultivar roktima showed pinkish brown, pinkish nodular, pinkish nodular compact, brown friable, yellow friable and yellow compact. Cultivar lalchay showed pinkish brown, pinkish nodular, pinkish friable, brown friable, yellow friable and white friable. Cultivar badami and cultivar dhusar showed more or less brownish or whitish type of callus and cultivar shadatae showed greenish type of callus. So it is clear that the cultivars of castor have widely variation in callus induction.

In the area of regeneration very high level of diversity was observed in different cultivars of castor, some cultivars failed to regenerate any shootlets from callus but others were found efficient to regenerate even more than fifty percent. In regeneration experiments MMS_1 medium was used either BAP alone or with different concentrations (0.5-3.0 mg/L) and different concentrations (0.1-1.0 mg/L) of NAA accompanied with GA_3 (0.5-2.0 mg/L) or TDZ (0.5-2.0 mg/L). The best result was also found in 1.0 mg/L BAP + 0.2 mg/L NAA + 0.8 mg/L TDZ. GA_3 containing media were also showed shoot induction, but the percentage were less than TDZ containing media.

Among the six cultivars of castor, callus regeneration was obtained from cultivar shabje, cultivar roktima and cultivar lalchay. Among them percentage of explants showing shoot formation was the highest in cultivar shabje. The cultivar shabje exhibited 63.7% callus regeneration followed by cultivar roktima 60.6% and

cultivar lalchay 58.2%. Number of shoot per culture was observed highest (9.5 ± 1.93) in cultivar shabje followed by cultivar roktima (9.3±1.90) and cultivar lalchay (9.1±1.85). Other three cultivars shadatae, badami and dhusar produced excessive callus but failed to regenerate shoot. So it can be demonstrated that every cultivar of the castor exhibited and unique range of variation in regeneration performance.

Root induction in artificial media is very difficult in castor as the plant was denoted as recalcitrant by several authors (Ahn *et al*. 2007). Kumari *et al.* (2008) reported that root induction in castor-bean was difficult compared to other crop plants. In tissue culture functions, the recalcitrance behavior of euphorbiaceae family is mainly exhibited by poor root formation. Root induction in artificial medium is particularly very hard in castor and for this region the workers are afraid of this trouble of root formation in artificial medium. Among the four strengths of MS basal medium, MMS_2 (half strengths of macro and micro nutrients and full strengths of vitamins) was found most effective in root induction.

No rooting was found in the microcuttings cultured on auxin free medium confirmed the essential need of an exogenous auxin for rooting of six cultivars of castor. Similar results were also reported Sujatha and Reddy (1998), they observed hormone-free MS medium failed produce without any rooting. Among the concentrations tested (0.1-1.0 mg/L), rooting responses of microcuttings in 0.2 mg/L IBA was the best than in either IAA or NAA. Sujatha and Reddy (1998) obtained highest rooting with least callusing using 1.0 mg/L IBA. While Ahn *et al*. (2007) observed two different rooting pattern using IBA and NAA and percentage of rooting was inversely correlated to shoot development. But when $AgNO_3$ and activated charcoal were added with IBA, the root formation was comparatively better than IBA used singly. But the $AgNO_3$ containing medium showed the better result from activated charcoal containing medium. Similar effect was also reported in castor (Kumari *et al*. 2008) when they used $AgNO_3$. The results demonstrated that $AgNO_3$ can influence root emergence and growth and improve rooting efficiency (Basis *et al*. 2000). From the foregoing discussion it can be concluded that the recalcitrant behavior of castor in some extent is over-come by the use of $AgNO_3$ and activated charcoal in root induction.

For root induction and elongation of the six cultivars of castor, rooting percentage, root number and root length showed the highest performance of cultivar shabje followed by roktima. Cultivar lalchay, shadatae, badami and dhusar were also produced root but was not remarkable as cultivar shabje and roktima. So it can be said that the six cultivars showed the diversity in root induction and elongation.

6.4. Diversity of castor in cell culture and its antibacterial activity

In the present investigation a number of experiments were performed to study the effect of hormone on cell growth and the optimum cell division period in culture medium of six cultivars of castors towards evaluating their diversity in cell suspension culture. Among the different concentrations and combinations of different hormones, 2.0 mg/L BAP + 0.3 mg/L NAA with 1.0 mg/L biotin and 1.0 mg/L glutamine was found to be most effective on cell culture of all cultivars. In this media formulation Shabje showed the highest performance (0.143±0.14 gm) while Badami showed the least performance (0.127±0.13 gm) on 16th days. Experimental results indicated that the peak period of cell growth runs from 7th days and after 16th days their growth seemed to be ceased down. The results revealed that 16th days period appeared as typical period for the culture of six cultivars and it is necessary to transfer the cell to the fresh medium after each 16th days of culture for its maintenance in the lab.

The isolated cells were also used for callus induction. For this purpose the cells were cultured in petridish containing MS medium supplemented with different concentrations and combinations of BAP and NAA. Micro calli were appeared after 2 weeks of culture in the petridish. Among the different media formulations, 2.0 mg/L BAP + 0.5 mg/L NAA was found to be the best for all six cultivars of castor. In this media formulation, cultivar Shabje the highest callusing rate (6.7%) followed by cultivar roktima (6.5%) and cultivar lalchay (6.3%). Cultivar badami (6.1%), cultivar shadatae and cultivar dhusar showed the least (6.0%) callusing rate. It is clear that the six cultivars of castor showed the diversity in callus induction from single cell culture.

Plant tissue culture approach has offered potent techniques, which may significantly contribute to the scheme of increasing levels of medicinally useful secondary metabolites (Ferreira and Duke 1997, Duke *et al*. 1999, Khafagi *et al*. 2003). For any drug development, antibacterial screening is obligatory and in the present investigation antibacterial screening against cell extract was performed with this objective.

The extracts from cell suspension cultures, which were collected from different periods viz. after 4 days, 6 days, 8 days, 10 days, 12 days, 14 days and 16 days of cell suspension culture. Every after two days, growing cells were centrifuged at 4000 rpm for 10 minutes. The cells were discarded and the clear supernatant was collected which was used as cell extracts. Using 25 µl of the supernatant was applied on the test disc. Cell extracts of castor culture assumed to contain toxic material liberated by the growing cell to the culture medium and it is believed that the cell

extracts harvested at different days would have different levels of lethal effect to the micro organisms. Our experimental results were also in support of this assumption as different grades of lethality shown under different periods of culture. The extracts collected after different periods showed different levels of antibacterial activities under the test. Especially the extract taken after 14 days from cell suspension culture showed the best antibacterial activities followed by 12 days, 10 days, 8 days, 16 days, 6 days and lowest in 4 days Minimum zone of inhibition was observed in antibacterial test with the cell extract of 4 days culture and over the varieties it was 9.51 mm while maximum zone of inhibition was noted in 14 days culture. It proved that cells growing for longer time in the suspension culture were able to produce more amount of toxic element to the culture making it more toxic to the bacterial growth. The experimental results indicated that the highest level of lethality was observed in the extract of 14 days culture which deteriorate thereafter synchronizing with cell growth as accomplished in the culture. It is very interesting to note that the highest level of cell growth occurred in 14 days culture similarly highest level of lethality (toxic effect) was also noted for 14 days culture. Thus, for castor plant, cell growth 14 days could be recommended as the standared period of cell culture for commercial use in isolation of toxic element (possibly ricin) from cell suspension culture.

Cell extract of six cultivars of castor viz. cultivar shabje, cultivar shadatae, cultivar roktima, cultivar lalchay, cultivar badami and cultivar dhusar were used to test the antibacterial activities against five gram positive and six gram negative bacteria. Among the cultivars, comparatively cultivar roktima showed the highest lethality followed by cultivar shabje and these two cultivars showed the zone of inhibition against all bacteria tested. But the remaining cultivars failed to show perfect lethality against all the bacteria tested, particularly for the cell extract taken from 4 days and 6 days of cell suspension culture. It is important to note that all the castor cultivars were able to show the zone of inhibition against all bacteria after 8 days, 10 days, 12 days, 14 days and 16 days of extract from cell suspension culture but their lethality were lower than cultivar roktima and cultivar shabje. It was clear that cultivar roktima showed the highest lethality in antibacterial test followed by cultivar shabje. The result demonstrated that every cultivar of the castor exhibited a very specific range in the zone of inhibition when applied to the antibacterial test and their range of action are distinct from one another. The experimental results indicated that the castor cultivars also holds the diversity in the levels of their toxicity and mode of action in their antibacterial activities.

From the foregoing discussion it can be concluded that the genotypes of *Ricinus communis* collected from different parts of the country belong to the same species but all of six cultivars are very much distinct from each other in respect of

morphological and molecular parameters. The cultivar roktima is remarkably distinct from others as exhibiting red body colour producing significantly distinct and lucrative flowering inflorescence. Molecular investigation by DNA marker (RAPD) also confirmed its distinctive nature producing DNA band unilaterally with primer OPB-17. The cultivar lalchay and badami were also very much distinct from others as well as their morphological features and molecular behaviors. Both of them failed to produce any band against the application of any primers used in this investigation. The cultivar shabje is really a unique cultivar with gigantic size both in its vegetative and reproductive growth also attained the highest position in producing greater seed yield following cultivar roktima. The cultivar shabje produced greater number of DNA bands under primer testing. But the other two cultivars shadatae and dhusar are more or less elite both in morphological and molecular parameters.

Intraspecies diversity as has been exhibited in tissue culture experiments indicated that the cultivar shabje is remarkable unique in *in vitro* regeneration followed by the cultivar lalchay and badami behave similarly in *in vitro* regeneration but their over all response in tissue culture and occupies the middle position between the responses as experienced by cultivar shabje and roktima in the upper and cultivar dhusar and shadatae at the lower in grading. The cultivar shadatae and dhusar exhibited lower performance in tissue culture response. But in the area of callus culture and callus regeneration the castor cultivars showed very diverse responses. Highest response in callus and callus regeneration was observed by cultivar shabje but the cultivars shadatae, badami and dhusar failed to any regeneration performance.

The present experiments clearly demonstrated that the six cultivars of castor cell begins to grow only within 4 days of culture proving its potential for developing cell culture industry for production of important toxic protein ricin as secondary metabolite. In regards of other medicinal plants castor proved suitable plant species for developing cell suspension culture in artificial medium and the present findings open up a new potential venture for obtaining secondary metabolites ricin as a alternative source from its cell culture. The isolation and extraction of novel bioactive secondary metabolites from plants having a biomedical potential for future drug discovery and wide range of novel bioactive secondary metabolites has been isolated from plant sources and many are to be discovered. Plant cell cultures present a potential source of valuable secondary metabolites which can be used as pharmaceuticals. The present investigation demonstrated that the extracts of cell suspension culture of six cultivars of castor considered to be potent source of antibacterial compounds and proved as an alternative source for production toxic compound ricin. As castor plant has been identified to hold the potential for innumerable medicinal properties its cell culture will lead to produce number of secondary metabolite through cell culture for new drug development.

CHAPTER-VII

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