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Traditional Knowledge and Commercial Cultivation of Medicinal Plants in Natore Oushodi Gram and in Vitro Conservation of Some Selected Species

University of Rajshahi

TRADITIONAL KNOWLEDGE AND COMMERCIAL CULTIVATION OF MEDICINAL PLANTS IN NATORE OUSHODI GRAM AND *IN VITRO* CONSERVATION OF SOME SELECTED SPECIES

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Dedicated

To My

Beloved Parents

DECLARATION

I do hereby declare that the whole work submitted as a thesis entitled **"TRADITIONAL KNOWLEDGE AND COMMERCIAL CULTIVATION OF MEDICINAL PLANTS IN NATORE OUSHODI GRAM AND *IN VITRO* CONSERVATION OF SOME SELECTED SPECIES"** in the Institute of Biological Sciences, University of Rajshahi, Bangladesh 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, 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.

_____

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CERTIFICATE

This is to certify that **Md. Shariful Islam** has carried out this research study under my supervision. I am pleased to forward his thesis entitled "**TRADITIONAL KNOWLEDGE AND COMMERCIAL CULTIVATION OF MEDICINAL PLANTS IN NATORE OUSHODI GRAM AND *IN VITRO* CONSERVATION OF SOME SELECTED SPECIES**" which is the record of bonafide research carried out at the Biotechnology Laboratory, Institute of Biological Sciences, University of Rajshahi, 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.

ABBREVIATIONS

Abbreviation	Full Meaning
%	Percent
2,4-D	2, 4-Dichlorophenoxy acetic acid
AC	Activated charcoal
BAP	6 – benzyl amino purine
cm	Centimeter (s)
CW	Coconut Water
DW	Distilled water
et al.	*Et alia* = and other people
Fig.	Figure
gm	Gram (s)
gm/l	Gram per litre
i.e.	*Id est* = that is
IAA	Indole – 3 – acetic acid
IBA	Indole – 3 – butyric acid
TK	Traditional knowledge
Kin	6 – Kinetine (6-furfuryl amino purine).
mg	Milligram (s)
mg/l	Milligram per litre
ml	Millilitre (s)
MS	Murashige and Skoog (1962) medium
NAA	α – naphthalene acetic acid
No.	Number
^{o}C	Degree Celsius
pH	Negative logarithm of hydrogen ion concentration
Sl.no.	Serial number
viz	*Videlicet*= Namely

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TRADITIONAL KNOWLEDGE AND COMMERCIAL CULTIVATION OF MEDICINAL PLANTS IN NATORE OUSHODI GRAM AND *IN VITRO* CONSERVATION OF SOME SELECTED SPECIES

ABSTRACT

Total area of Natore district is 1896.05 sq km, is bounded by Naogaon and Bogra districts on the north, Pabna and Kushtia districts on the south, Pabna and Sirajganj districts on the east, Rajshahi district on the west. The study area was Laxmipur-Kholabaria union of Natore sador upozilla under Natore district. Owing to the unique practices of commercial cultivation of medicinal plants made by the farmers, instead of agricultural crops, the area has been designated as the "Oushodi Gram" meaning the villages of medicine. Natore Oushodi Gram has become a resource centre for herbal medicine and imparting training to the new farmers motivated for the commercial cultivation of medicinal plants in the village area.

The livelihood of the local people of this area depends mostly on agriculture. They are poor community and the natural and cultivated medicinal plants became the earning source of their livelihood and also provide a major part of the medicines for treatment of different diseases. The traditional knowledge related to the use of medicinal plants is important to the context of exploitation of plant genetic resources and the local herbalist commonly known as kobiraj/herbal doctors have this kind of knowledge, which they inherited from their previous generation. The present study was undertaken to make a survey on traditional knowledge of medicinal plants in the area and cultivation of medicinal plants in homestead, agricultural lands and other fallow lands which collectively generated different avenues of income generation over the area.

Field survey was conducted during "October 2010 to September 2011" covering the seasons; summer, winter and rain. Under this study survey was made in eight villages; kholabaria, kanthalbaria, borobaria, toltolia para, khamar para, gazipur, ibrahimpur and laxmipur in Natore Oushodi Gram. Socioeconomic and ethnobotanical information were collected using a format of questionnaire interviews with 160 local respondents (medicinal plant farmer, kobiraj/herbal doctors, hawker, whole sale dealer, nursery owner). All interviewees were male except two were female. Results indicated that majority of the respondents were of middle age (43%), under the age

between 34-49 yrs. In terms of literacy, 17 percent of the respondents had no formal education, on the other hand 83 percent respondents had formal education in different levels. The respondents were selected from the local people in any way involved in the activity of medicinal plants and the results revealed that out of 160 respondents 72 percent were medicinal plant producer, 11 percent herbal doctor, 10 percent hawker, 3 percent nursery owner and 4 percent wholesaler.

One hundred and twenty medicinal plant species belonging to 51 families and 111 genera were identified and their ethnobotanical uses were studied. The predominating families were Fabaceae, Lamiaceae, Zingiberaceae, Euphorbiaceae, Acanthaceae, Compositae, Solanaceae, Liliaceae, Malvaceae, Apocynaceae, Combretaceae and Rutaceae. Among all the families Fabaceae contained the highest number of plant species with medicinal properties. Considering the habit of the medicinal plants, herbs were more prevalent than shrubs, trees, climbers and epiphytes. Seventy one diseases were addressed to be treated with these 120 medicinal plants. Fever, cough, asthma, sexual disease, diabetes, skin disease, rheumatism, weakness, stomachache were noted as the most common diseases being treated by these plant species. Leaves were recognized as the most frequently used part of the medicinal plants studied under this investigation and in most cases plants parts were used in the form of juice. In Natore Oushodi Gram cultivation of medicinal plants has appeared as the new venture of commercial cultivation for subsistence living. Now a day's many marginal and poor farmers were found to be fully dependent on the income of medicinal plants, which the traditional agriculture fails to compete.

Another potential investigation of the present study was to establish protocol for *in vitro* propagation of some important medicinal plant species. *In vitro* study was done in seven species; *Asparagus racemosus, Curcuma amada, Ipomoea mauritiana, Mentha arvensis, Paederia foetida, Rauwolfia serpentina* and *Smilax zeylanica*. Node, axillary bud, shoot tip, internode and leaf segments were used as explants under the tissue culture study. MS basal medium supplemented with different concentrations and combinations of auxins and cytokinins were used for the induction of direct and indirect organogenesis and rooting. Among the five types of explants, nodal segments were responded better but sometimes axillary bud responded also better than shoot tip, internode and leaf segments, and BAP was proved to be the most effective cytokinin than that of Kn in artificial culture of these medicinal plants.

Under *in vitro* study, MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA was determined as the best media formulation for the direct organogenesis in nodal explant of *Asparagus racemosus*. Callus induction was maximum in internode explants on MS + 0.5 mg/l BAP + 1.0 mg/l Kn + 1.0 mg/l 2,4-D. Formation of adventitious shoots from leaf derived callus was highest in MS + 1.0 mg/l BAP + 0.2 mg/l NAA. Rooting was best in half strength MS + 2.0 mg/l Kn + 0.5 mg/l NAA.

In *Curcuma amada*, shoot tip explants were proved best for the induction of direct organogenesis. Direct organogenesis was highest in 0.5 mg/l BAP + 0.5 mg/l NAA. Microrhizome induction was best in 1.0 mg/l BAP + 0.2 mg/l NAA of shoot tip explant. Rooting was best in half strength MS + 0.5 mg/l IBA.

In case of *Ipomoea mauritiana*, nodal segments were proved best for the induction of direct organogenesis. Direct organogenesis was highest in 1.0 mg/l BAP + 0.5 mg/l IAA. Callus induction was best in leaf explants on 1.0 mg/l 2,4-D + 2.0 mg/l BAP. Maximum (40%) shoot bud regeneration was obtained from internode derived callus in 1.0 mg/l BAP + 0.5 mg/l IAA. Rooting was best in half strength MS + 0.2 mg/l IBA.

In case of *Mentha arvensis*, nodal segments also proved best performer for the induction of direct organogenesis. Direct organogenesis was highest in 1.0 mg/l BAP + 0.2 mg/l NAA. Highest number of shoot multiplication and elongation was observed in MS + 0.5 mg/l GA_3. Callus induction was best in internode explants on 2.0 mg/l BAP +1.0 mg/l 2,4-D. Maximum (65%) shoot bud regeneration was obtained from leaf derived callus in 2.0 mg/l BAP + 1.0 mg/l Kn + 0.2 mg/l NAA. Rooting was best in half strength MS + 1.0 mg/l NAA.

In *Paederia foetida*, for direct organogenesis nodal segment perform best and the result was highest in 1.0 mg/l BAP + 0.5 mg/l IAA. Callus induction was best in internode explants on 2.5 mg/l 2,4-D + 0.5 mg/l IBA. Maximum (85%) shoot bud regeneration was obtained from internode derived callus in 1.0 mg/l BAP + 1.0 mg/l IAA. Rooting was best in half strength MS + 0.2 mg/l IBA.

Direct organogenesis in *Rauwolfia serpentina* was highest in nodal explants on 1.5 mg/l BAP + 0.2 mg/l NAA. Callus induction was best in internode explants on 2.5 mg/l 2, 4-D + 0.5 mg/l BAP. Rooting was best in half strength MS + 0.2 mg/l IBA + 0.2 mg/l NAA.

And in *Smilax zeylanica*, direct organogenesis was highest in nodal explants on 1.5 mg/l Kn + 0.5 mg/l L-glutamine. Rooting was best in half strength MS + 1.0 mg/l IBA.

Another potential avenue of this present investigation was to establish protocol for *in vitro* conservation of some important medicinal plant species by artificial seed production. Attempt was taken to produce artificial seed by encapsulating the shoot tip and nodal segments of plants. For germination of artificial seeds , encapsulated shoot tips (synthetic seed) and encapsulated nodal segments (synthetic seed) were cultured on MS basal media containing different concentrations and combinations of BAP, Kn and NAA used to induce shoot proliferation. In *Mentha arvensis* the highest 80% shoot formation was observed in MS medium containing 2.0 mg/l BAP + 0.2 mg/l NAA from nodal segments. For survivality test (viability) of encapsulated shoot tip and nodal explants of *M. arvensis* under different storage temperature, 4 ± 1ºC temperature proved best for storage of artificial seeds for about 60 days.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1. WORLD SCENARIO OF MEDICINAL PLANTS

Since time immemorial, people have gathered plant and animal resources for their needs. Examples include edible nuts, mushrooms, fruits, herbs, spices, gums, fodder, fibers, used for construction of shelter and housing, clothing or utensils; while plant or animal products for medicinal, cosmetic or cultural uses. Even today, hundreds of millions of people, mostly in developing countries, derive a significant part of their subsistence needs and income gathered from plant and animal products (Iqbal, 1993; Walter, 2001). Gathering of high value product such as mushrooms (morels, matsutake, truffles), medicinal plants (ginseng, black cohosh, goldenseal) also continuous in developed countries for cultural and economic reasons (Jones *et al.* 2002). Among these uses, medicinal plants play a central role, not only as traditional medicines used in many cultures, but also all trade commodities which meet the demand of often distant markets. Demand for a wide variety of wild species is increasing with growth in human needs, numbers of commercial trade. With the increased realization that some wild species are being over exploited, a number of agencies are recommending that wild species be brought into cultivation systems (BAH, 2002; Lambert *et al.*1997; WHO, IUCN and WWF, 1993). Cultivation can also have conservation impacts, however, these need to be better understood. Medicinal plant production through cultivation, for example, can reduce the extent to which wild populations are harvested, but it also may lead to environmental degradation and loss of genetic diversity as well as loss of incentives to conserve wild population (Anon, 2002).

On conservation perspectives, the relationship between *in situ* and *ex situ* conservation of species is an interesting topic with implication for local communities, public and privates land owners and managers, entire industries and of course wild species. Identifying the conservation benefits and costs of the different production systems for medicinal and aromatic plants should help guide policies as to whether species conservation should take place in nature or the nursery, or both (Bodeker *et al.* 1997). Besides global trends in the close relationship between cultivation and wild harvest of medicinal and aromatic plant species, than make recommendation on steps

that should be taken to achieve a balance between consumption, conservation and cultivation. According to the World Health Organization (WHO, 2000), over 80% of the world's population relies upon traditional plant based systems of medicines to provide them with primary health care (Bannerman *et al.* 1983). Fransworth & Soejarto (1991) and Shengji (2001) also echoed the same with their estimation that 70-80% of people worldwide rely chiefly on traditional, largely herbal, medicine to meet their primary healthcare needs. In the developed countries also, some 25% of prescriptions are filled with drugs whose active ingredients are extracted or derived from plants (Boerdeker, 1997). In South and South East Asia, including China, traditional systems of medicine use thousands of plant species to treat malaria, stomach ulcers, and various other disorders. Medicinal plants are used at the household level, especially by the women taking care of their families, at the village level by medicine men or tribal shamans and by the practitioners of classical traditional systems of medicine, such as the Ayurveda, Chinese medicine or Japanese Kampo system (Bannerman *et al*. 1983).

The global demand for herbal medicine is not only large but growing (Srivastava, 2000). The market for Ayurbedic medicines is estimated to be expanding at 20% annually in India (Subrat, 2002), while the quantity of medicinal plant obtained from just one province of China (Yunnan) has grown by 10 times in the last 10 years (Shengji, 2002). Factors contributing to the growth in demand for traditional medicine include the increasing human population and the frequently inadequate provision of western (allopathic) medicine in developing countries. There are many traditional systems of medicine. Following the practices in China , they may be classified into 3 broad categories: (1) Traditional medical system, with written tradition of documentation of knowledge, pharmacopoeias for doctors and institutions for training doctors: (2) Traditional medical knowledge (Folk medicine), which is orally transmitted and associated with households, communities or ethnic groups and (3) Shamanistic medicine, with a strong spiritual elements and which can only be applied by specialist practitioners (Shamans). Traditional medical systems are especially concentrated in Asia. Some of the more widely familiar are Chinese Traditional medicine, Tibetan medicine , Ayurveda, Siddha, Unani and western herbal medicine, the latter being rather ill defined. Herbal medicine is becoming ever more fashionable in richer countries, a market sectors which has grown at 10-20% annually in Europe and North America over recent years (Kate and Laird, 1999). In addition, there are many related botanical products sold as health foods, cosmetics, food supplements,

herbal teas and for various other purpose related to health and personal care. The extent to which herbal preparations are prescribed within conventional medicine varies greatly between countries, for instance being much higher in Germany than in the UK or USA. Plants have contributed hugely to western medicine, through providing in gradients for drugs or having played central roles in drugs discovery. Some drugs having botanical origins are still extracted directly from plants, others are made through transformation of chemical found within them while yet others are today synthesized from inorganic materials but have their historical origins in research into the active compounds found in plants (Mendelsohn and Balick, 1995).

1.2. BANGLADESH PERSPECTIVES

1.2.1. Medicinal plants in Bangladesh

In Bangladesh, a long tradition of indigenous herbal medicinal systems, based on the rich local plant diversity, is considered as very important component of the primary health care system. Many rural people of Bangladesh still depend on plant products and herbal remedies for treating their ailments. In our country about 546 plant species have been identified as medicinal plant because of their therapeutic properties (Ghani, 1998; Yusuf *et al.*1994). However, this list is not exhaustive since it is there, but have not been discovered or identified (Said, 1995; Ghani, 1998). In the mean time, a large number of industries (400 herbal factories) have been established in this country for producing Ayurvedic and Unani medicines. It has been estimated that Bangladesh has a market of about 100 core taka worth (125 million US $ approximately) herbal products annually (Alam *et al.* 1996). The pharmaceutical manufacturing industries of Bangladesh are solely dependent on imported raw materials for the production various drugs and medicines. The raw materials, particularly the active ingredients and also the major excipients, which these industries utilize for manufacturing purposes, can be obtained from indigenous sources. Some of these, which Bangladesh imports by spending a huge amount of foreign exchange, can be obtained or prepared from the indigenous medicinal plants of the country. It is estimated that if sincere efforts are made at least 30 percent, if not more, of the requirements of raw materials of active ingredients and major excipients can be met from the indigenous sources of Bangladesh. The importance of medicinal plants is increasing day by day. Bangladesh now feels the importance of medicinal plants but majority of the farmers are totally unaware about the profitability of medicinal plant cultivation. In Bangladesh,

medicinal plants are found to grow naturally in the forest, bushes and marginal land along the canal and other fallow lands. Many such medicinal plants especially the aromatic herbs are grown in the homestead/home garden or a crop field either in sole cropping or inter cropping systems and rarely as plantation crop (Padua *et al.* 1999). Home garden is increasing with a focus component of medicinal plants.

Environments of home gardens with different medicinal and aromatic plants could be good source of small scale or resources poor farmers. Over the country there are some cultivators who are trying to cultivate medicinal plants by their own initiatives. The cultivation is becoming both profitable and environmentally friendly. In Bangladesh sporadically developed some pocket areas of the cultivation of medicinal plants where farmers are growing medicinal plants in their homesteads or agricultural plots to sale their plants or plant products. In general, all the indigenous medicinal plants are extensively used in the preparation of Unani, Ayurvedic and Homeopathic medicines in Bangladesh. These plants also serve as important raw materials of many modern medicine preparations. Since there has been no systematic phytochemical survey of the medicinal plants of Bangladesh, it is quite possible that many other potential medicinal plants in this country still remain to be explored and evaluated. Its phytochemical and pharmacological investigations and research could play a vital role in bringing to the scientific world many useful remedies for alleviation of human sufferings.

In Bangladesh, where herbal medicines have been used for centuries, the most important markets are the rural consumers. Each year, companies producing herbal medicines, used to import huge amounts of raw plant ingredients from foreign countries. Increasing interest by multinational pharmaceutical companies and domestic manufacturers of herbal based medicines is contributing significant economic growth of the global medicinal plants sector.

The Government has encouraged the development of the industry since the Prime Minister in Bangladesh launched `plantation fortnight' in 2002 with a call to plant medicinal plants & fruit trees along with forest species. It is estimated that around 12,000 tones of dried medicinal plants are sold from the rural collection and production areas worth around 4.5 million US $ to the rural economy. The wholesale value is estimated to be US $ 6 million and the import of around 5,000 tons worth US$ 8 million. In summary the medicinal and aromatic plants sector in Bangladesh is estimated to be worth of US$ 14 million with local supply comprising of 70% by

volume and 40% by value (SEDF/IC, 2003). Dey (2006) using data obtained from Hamdard Laboratories Limited noted that the annual demand of medicinal plants is around 19250 tons in the country. Out of this medicine industry uses 10800 tons, herbal physicians use 6050 tons and cosmetic industries use 2400 tons. Therefore there conservation of such a valuable resource in the country is vitally important. Before marketing of medicinal plants raw or crude materials some activity of the local farmers, collectors, picker and beparies are noticeable. These activities are drying, cutting, grinding, grading and storage is mainly carried out by the farmers, collectors and local pikers. Interviews with 7 inter district beparis have been used to establish an outline of the supply chain for Bangladeshi medicinal plants from the more important areas (the Chittagong hill tracts and north central Bangladesh; Modhupur, Tangail, Mymensingh and Natore). These beparis claim that around 90% of all Bangladesh's medicinal plants are wild harvested and only 10% of medicinal plants are cultivated. Primary processing, i.e. drying, cutting, grinding, grading and storage, is mainly carried out by the farmers, collectors and local pikers, with the bepari concentrating on transport and distribution. The latter will sell to wholesalers and retailers and directly to a number of processors and possibly some herbal practitioners.

1.2.2. Healthcare system in Bangladesh

The healthcare systems practiced in Bangladesh include the traditional system, Ayurvedic, Unani, Homeopathic, Folk medicine systems and modern system.

1.2.2.1. Traditional system

Traditional system an art of healing based on traditional use of plants, animals, other natural substances, cultural habits, social practices, religious beliefs and in many cases, superstitions of the present and previous generations of people (Ghani, 1990). The basic concept of traditional medicine has been very comprehensively described by the World Health Organisation (WHO, 1976) in the following way: "Traditional medicine is the sum total of all knowledge and practice, whether explicable or not, used in the diagnosis, prevention and elimination of physical, mental or social imbalance, relying exclusively on practical experience and observations handed down from generation to generation, verbally or in writing." The forms of traditional medicine practiced today vary from highly organized and long established Ayurvedic and Unani systems to various folk medical practices, such as herbalism, spiritualism and religious medical practices. Because of their origin in the remote past and the fact

that most of them are practiced almost in the same way as in the past they are collectively called traditional medicine.

Bangladesh possesses a rich flora of medicinal plants. Out of the estimated 5000 species of different plants growing in this country more than a thousand are regarded as having medicinal properties. Use of these plants for therapeutic purposes has been in practice in this country since time immemorial. Continuous use of these plants as items of traditional medicine in the treatment and management of various health problems generation after generation has made traditional medicine an integral part of the culture of the people of this country. As a result, even at this age of highly advanced allopathic medicine, a large majority (75-80%) of the population of this country still prefer using traditional medicine in the treatment of most of their diseases even though modern medical facilities may be available in the neighborhood. Although the use of traditional medicine is so deeply rooted in the cultural heritage of Bangladesh the concept, practice, type and method of application of traditional medicine vary widely among the different ethnic groups. Traditional medical practice among the tribal people is guided by their culture and life style and is mainly based on the use of plant and animal parts and their various products as items of medicine. But the method of treatment and application of the medicament are greatly influenced by the religious beliefs of the different tribes and their concept of natural and supernatural causes of diseases. For that reason their medical practice also includes the use of a number of rituals like religious prayers, sacrifices, offerings in the name of the spirits and gods, incantations and sometimes tortures.

1.2.2.2. Ayurvedic system

Ayurvedic system is one of the oldest systems of medicine which has been practiced in this subcontinent for over 3,000 years. Ayurveda, meaning the science of life, is rooted to the social, cultural and philosophical principles that prevailed in India during the period 600 BC to 700 AD. Ayurveda considers the human being as a miniature universe. The properties found in the universe are believed to be present in the human body, which like the universe, consists of five gross elements: earth, water, fire, air, and the ethereal parts of the sky. These body constitutions are taken into consideration while treating a patient under this system. Curative treatment in Ayurvedic system consists of administration of medicine both internally and externally, minor surgical operations and psychosomatic treatment. The medicinal preparations employed in this system are mainly derived from plant materials and are

presented in the form of powders, semi-solid preparations, decoctions, elixirs and distillates. Many of them also contain inorganic chemical substances, minerals, and animal products. Alcoholic extracts and alcoholic solutions of the ingredients, tinctures and elixirs are also frequently used in Ayurvedic medicine. The materia medica of Ayurvedic medicine contains some 8,000 published recipes. Many more are held as secret information among certain families.

1.2.2.3. Unani system

Unani system originated in Greece and was named after the name of Unan province, which is regarded as the original place of development and practice of this system. Hakim Iskalibus of Greece was the first person to propagate the Unani system of medicine. However, this system flourished only when Arabian and Persian Muslim intellectuals like Al-Razi, Ibne-Sina, Al-Rashid, and others enriched it with newer scientific knowledge and discoveries in the 7th century. Because of the significant contributions of Arabian physicians to the development of this system, the Unani system is also known as the Greeko-Arab system. The famous medical book, 'Al-Kanun' (based on the Unani system) of Ibne-Sina (980-1037 AD) was the most prescribed book of medicine in Europe for several centuries. After the 13th century, although Muslim civilization declined, the Unani system of medicine was in full vigour and widely practiced as an effective system of treatment throughout the world.

According to the Unani system, the basic factors composing the human physique are four elements (fire, air, water, and earth), four types of temperament (hot and dry, hot and wet, cold and dry, and cold and wet), four humours (blood, phlegm, yellow bile and black bile) organs, vital spirit, powers and functions. Whole plants or their powders or pastes or products and their extracts, infusions, decoctions and distillates are major constituents of Unani medicine. Minerals, inorganic chemicals and animal products are also frequently used in preparing these medicines. However, tinctures or elixirs (which are alcoholic preparations) are not used in Unani medicine. Both Ayurvedic and Unani systems of traditional healthcare have taken firm roots in Bangladesh and are widely practiced all over the country. There are about 6,000 registered and 10,000 unregistered practitioners (Kaviraj/Herbal doctor of the Ayurvedic system and Hakims of the Unani system) of these two systems of medicine in Bangladesh. A total of 15 governments recognized and funded educational institutions are currently engaged in the teaching of traditional medicine in the country. Of them, 10 institutions are involved in teaching the Unani system and 5 in

Ayurvedic system. Each of these institutions has an attached out patient hospital which imparts internship training to graduates while giving medical services to out door patients. These institutions offer a four year diploma course and six month internship training. Annual intake of these institutions currently stands at about 400 students. Since the 1989-90 academic session a Government Unani and Ayurvedic Degree College, affiliated to the University of Dhaka, has been established in Dhaka. This college offers a five year degree course and one year internship training in an attached 100 bed Traditional Medical Hospital.

1.2.2.4. Homeopathic system

Homeopathic system of healthcare is not strictly an eastern medical system as it was developed in Europe by a German allopathic physician named Samuel Hahnemann (1755-1843) in the early 19th century from the allopathic system. In this system drugs are applied in very small and diluted doses. It is believed that the strength or curative power of a drug increases mathematically with the increasing degree of its dilution. There are about 1200 medicines in homeopathy, of which more than 500 are obtained from medicinal plants, a few from animals, and the rest from pure chemicals. Plant derived medicines in this system are used as mother tinctures. No excipient (preservative, colour, sweetener, flavour, etc) is used in preparing homeopathic medicine. This system of medicine is very popular in many Asian countries including Bangladesh.

1.2.2.5. Modern system

Modern system the highly advanced system of health management used in Bangladesh and the rest of the world. This system does not limit itself to only curative treatment of the patient but also endeavour's to extend its services to the prevention of diseases by immunization and improving the personal and environmental hygiene of the patient and the community. Well educated and professionally trained experts practice this system of medical treatment. Technologically advanced highly sophisticated equipment and methods are used in this system to attain precise diagnosis and treatment of diseases. Highly efficacious medicinal preparations prepared from purified synthetic or natural chemical substances are used in this system. It has developed sophisticated and precise method and technology of surgical operations and performs critical operations like open heart surgery, heart transplant, and transplantation of other vital organs of human body with high degree of precision

and safety. Organized and well equipped hospitals and clinics have been developed to effectively and properly offer healthcare services to people under this system. However, because of inadequacy of medical equipment and shortage of manpower and infra structural facilities, benefits of modern system of healthcare services cannot be extended to rural areas as adequately as needed. The cost involved in offering healthcare services under this system is also much higher than that of any other system of healthcare services available in Bangladesh (Ghani, 1990).

1.3. PRODUCTION OF TRADITIONAL MEDICINE

More than four hundred big and small manufacturers in Bangladesh are now engaged in manufacturing traditional medicine preparations in various dosage forms using local and imported raw materials. Some of the important raw materials of plant origin are derived from the rich tropical flora of Bangladesh. Many of them are imported from India and Pakistan. The Unani and Ayurvedic drugs manufactured in Bangladesh not only meet the local requirements but are also exported to the neighboring countries. Although many of these manufacturers are still using the traditional methods of producing these drugs, some of them, like Hamdard Laboratories (Waqf) Bangladesh, have substantially modernized their factories by installing modern equipment and machinery. They use modern methods and technology for the production and quality control of their traditional medicines. Some of these factories can be compared with any modern pharmaceutical factory of this and other countries. The presentation and quality of their products are as good as those of modern allopathic drugs. Many traditional medicine preparations in Bangladesh are now dispensed and sold from most of the modern allopathic drug stores, particularly those in the rural and peri urban areas, and some of them are even prescribed by the modern allopathic medicine practitioners. Modernization and utilization of modern technology and pharmaceutical knowledge in manufacturing and quality controlling of traditional medicines are now rapidly increasing in Bangladesh.

Traditional medicine systems, particularly Unani and Ayurvedic systems are now recognized and well accepted as good alternative systems of medicine in both rural and urban areas of Bangladesh. Considerable research is now going on in this country both privately and institutionally to improve the quality of these drugs. Establishment of a separate Research & Development laboratory by the Hamdard Laboratories of

Bangladesh, a manufacturer of Unani medicines, in order to undertake research programmes to improve the quality of its current products and to develop new drugs from indigenous natural sources, bears clear testimony to that.

The effort is not limited to that only. One of the objectives of the National Health Policy is to encourage systematic improvement in the practice of the indigenous systems of medicine and to utilize the additional manpower available in the health sector. The Government is also planning to incorporate traditional medicine in Primary Health Care (PHC) activities. In order to achieve the goal of providing basic health needs to maximum of the rural people in the shortest possible time with minimum expenditure, the Government is planning to bring traditional medicine into the mainstream of the organized public health services and health care delivery programmes of the country. In an attempt to integrate the traditional and modern allopathic medicine practices, the government has already started appointing qualified Hakims and Kavirajes/Herbal doctor in the rural hospitals and health complexes along with graduate allopathic medical doctors. With the encouragement and practical involvement of the World Health Organization, efforts are now in vogue in Bangladesh to utilize traditional medicine more and more in the health care programmes, particularly at the Primary Health Care level. And this is imparting a positive effect on the overall health management programmes of the country.

1.4. MEDICINAL PLANTS CONSERVATION

1.4.1. National and international perspective

The world conservation strategy (IUCN, UNEP & WWF, 1980) defines conservations as "the management for human use of the biodiversity so that it may yield the greatest sustainable benefit to present generation while maintaining its potential to meet the needs and aspirations of future generations". The above definition invokes two complementary components "conservation" and "sustainability.

Guidelines on the conservation of medicinal plants: WHO, IUCN and WWF, (1993) published detailed guidelines on the conservation of medicinal plants**.** The following extracts have been taken from that document. Conservation of medicinal plants require basic studies on traditional knowledge on the use of plants in health care and studies to identify the medicinal plants, outline their distributions and assess their abundance. These involve the following:

A. Basic

1. To study traditional knowledge on the use of plants in health care
2. To identify the medicinal plants, outline their distributions and assess their abundance

B. Utilization

3. Wherever possible, to cultivate the medicinal plants as the source of supply.
4. Cultivation practices should minimize the use of chemicals
5. Checklist of steps in bringing a species into cultivations
6. Botanic gardens and horticultural institutes should provide effective horticultural training and information
7. To ensure that any collection from the wild is sustainable

C. Conservation

8. To conserve population of medicinal plant species in natural habitats
9. To conserve populations of medicinal plant species

1.4.1.1. *In situ* conservation of medicinal plant

In situ conversation involves protection and establishment of plants and other biological resources in the location of their natural occurrence. In order to ensure that representative of wild populations of vulnerable medicinal plant species are maintained, core conservation areas or other protected habitats that will allow natural processes to continue undisturbed should be designated (Cunningham, 1997). Since, it is only in nature that plant diversity at genetic, species and ecosystem levels can be conserved on a long term basis, identification of ecosystems with diverse medicinal plant species is very essential. *In situ* conservation of medicinal plants can be accomplished through the active support and participation of people who dwell in or near and around the protected plant growing area. Involving the local mass in all phases of conservation programmes, such as planning, policy decision process, implementation etc. will be a significant component in achieving efficient management and utilization of medicinal plant resources. Natore Oushodi Gram presents an ideal example for *in situ* conservation of medicinal plants in Bangladesh organizing activities in a very public participatory approach. Indigenous medicinal plants are in plantation over the area with inherited traditional knowledge.

1.4.1.2. *Ex situ* conservation of medicinal plant

Conservation of medicinal plants can be accomplished by the *ex situ* i.e. outside natural habitat by cultivating and maintaining plants in botanic gardens parks, other

suitable sites, and through long term preservation of plant propagules in gene banks (seed bank, pollen bank, DNA libraries, etc.) and in plant tissue culture repositories and by cryopreservation). Botanical gardens can play a key role in *ex situ* conservation of plants, especially those facing imminent threat of extinction. Several gardens in the world are specialized in cultivation and study of medicinal plants, while some contain a special medicinal plant garden or harbor special collection of medicinal plants. Natore Oushodi Gram also may hold the merit of e*x situ* conservation, as a number of plants with medicinal properties collecting from different places are brought into the area for cultivation. Bangladesh Forest Research Institute has also established two preservation plots for conservation of 17 endangered tree species, 2 clone banks and arboretum for bamboo, cane and medicinal plants in different places of Chittagong district.

1.5. MEDICINAL PLANTS BIOTECHNOLOGY

Biotechnological tools are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in vitro* regeneration and genetic transformations. It can also be harnessed for production of secondary metabolites using plants as bioreactors.

In vitro propagation of plants holds tremendous potential for the production of high quality plant based medicines (Murch *et al*. 2000). This can be achieved through different methods including micropropagation. Micropropagation has many advantages over conventional methods of vegetative propagation, which suffer from several limitations (Nehra and Kartha, 1994). With micropropagation, the multiplication rate is greatly increased and it also permits the production of pathogen free material. Micropropagation of various plant species, including many medicinal plants, has been reported (Murashige, 1978; Skirvin, 1990; Withers and Anderson, 1986). Propagation from existing meristems yields plants that are genetically identical with the donor plants (Hu and Wang, 1983). Plant regeneration from shoot and stem meristems has yielded encouraging in the medicinal plants like *Catharanthus roseus, Cinchona ledgeriana and Digitalis spp, Rehmania glutinosa, Rauvalfia serpentina, Isoplexis canariensis* (Paek *et al.* 1995; Roy *et al.* 1994; Perez-Bermudez *et al*. 2002).

Numerous factors are reported to influence the success of *in vitro* propagation of different medicinal plants (Hu and Wang, 1983; Hussey, 1980; Bhagyalakshmi and Singh, 1988; Short and Roberts, 1991). The effects of auxins and cytokines on shoot multiplication of various medicinal plants have reported. Benjamin *et al.* (1987) has shown that 6-Benzylaaminopurine (BA), at high concentration (1-5 ppm), stimulates

the development of the auxillary meristems and shoot tips of *Atropa belladonna* . Lal and Ahuja (1996) observed a rapid proliferation rate in *Picrorhiza kurroa* using kinetin at 1.0-5.0 mg/l. Direct plantlet regeneration from male inflorescences of medicinal yam on MS medium supplemented with 13.94 µM kinetin has also be reported (Borthakur and Singh, 2002). The highest shoot multiplication of *Nothapodytes foetida* is achived on medium containing thidiazuron (TDZ) at a concentration of 2.2 µM (Rai, 2002). Similarly, it has been observed that cytokinin is required, in optimal quantity, for shoot proliferation in many genotypes but inclusion of low concentration of auxins along with cytikinin triggers the rate of shoot proliferation (Rout and Das, 1997; Das *et al.* 1999; Shasany *et al.* 1998; Tsay *et al.* 1989). Barna and Wakhlu (1988) has indicated that the production of multiple shoots is higher in *Plantago ovata* on a medium having 4-6 M kinetin along with 0.05 µM NAA. According to Faria and Illg (1995), the addition of 10 µM BA along with 5 µM indole-3- acetic acid (IAA) or 5 µM NAA induces a high rate of shoot proliferation of *Zingiber spectabile.*

Due to the toxic and adverse reactions of synthetic and chemical medicines being observed round the global herbal medicine has made a come back to improving the fulfillment of our present and future health needs. For further research into the biochemical compositions and potential medicinal values of this plant, an efficient *in vitro* regeneration system for the production of plants is required because field grown plants may be subject to seasonal and somatic variations, infestations of bacteria, fungi and insects as well as environmental pollutions that can affect the medicinal value of the harvested tissues (Geng *et al*. 2001; Tyler *et al*. 1981). In addition, *in vitro* propagation methods offer powerful tools for germplasm conservation and the mass multiplication of threatened plant species (Murch *et al*. 2000; Pakrashi and Shaha, 1978).

Cell suspension culture system, another area of tissue culture, could be used for large scale culturing of plants cell from which secondary metabolites could be extracted. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural product. 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 (Mulabagal and Tsay, 2004). Secondary metabolites were once believed to be waste products. They are not essential to the plant's survival, but the plant does suffer without them. Secondary metabolites considered as end products of primary metabolism and are in general not involved in metabolic activity,

viz. alkaloids, phenolics, essential oils, steroids etc. It has been demonstrated that the biosynthetic activity of cultured cells can be enhanced by regulating environmental factors, as well as artificial selection or 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 and Allan, 1997; Krings and Berger, 1998; Ravishanker and Rao, 2000; Facchini *et al*. 2004 and Grotewold, 2004). Due to these advantages, research in the area of tissue culture technology for production of plant chemicals has bloomed beyond expectations.

Under the present investigation effort has been made to collect the ethnobotanical information about 120 medicinal plants. Among these plants 17-20 species have been cultivated in agriculture plot and also identified some plants having propagation barrier. In spite of extended demand, the medicinal plants having propagation barrier suffer for mass propagation. It was necessary to develop the tissue culture protocols for all of these plants identified to have propagation barrier but considering volume of work and time limitation, seven medicinal plants (*Asparagus racemosus, Curcuma amada*, *Ipomoea mauritiana, Mentha arvensis*, *Paederia foetida, Rauwolfia serpentina and Smilax zeylanica*) were selected for tissue culture study under the present investigation. The present study was undertaken to examine the potential of different explants, to respond under *in vitro* conditions with the possibility of developing a protocol for their *in vitro* multiplication.

1.6. NATORE OUSHODI GRAM

Natore Oushodi Gram is situated in Natore sadar upazilla under Natore district at the northern part of the country and consists of several villages covering an area under laxmipur-kholabaria union. It consists of eight villages; kholabaria, kanthalbaria, borobaria, toltolia para, khamar para, gazipur, ibrahimpur and laxmipur. Over the area, the livelihood of the local people depends mostly on agriculture. The natural and cultivated medicinal plants provide a major part of the medicines for treatment of different diseases. Medicinal plants play a major role in people's healthcare especially for poor communities living in the area. The indigenous knowledge related to the use of medicinal plants is important to the context of exploitation of genetic resources and the local herbalist commonly known as kobiraj/local herbal doctor. These herbal practitioners have acquired practical knowledge of medicinal uses of plants, which

they inherited from their previous generation. Production of medicinal plants and their crude products in Natore Oushodi Gram obviously holds the promises of pharmaceutical industries and exporting the products to develop the opportunities for earnings and employments of the rural people. Natore Oushodi Gram has become a resources centre for herbal medicine and imparting training to the new farmers motivated for the commercial cultivation of medicinal plants in the village. Natore Oushodi Gram thus standing in the turning point that provides herbal raw materials to the pharmaceuticals companies and the suppliers of the same for the cosmetic industries and food companies in the country. Large number of local herbal doctors, farmers, nursery growers, traders and hawker have been engaged in different activities generated around the area by virtue of commercial cultivation of medicinal plants.

1.7. RATIONALE AND OBJECTIVES OF THE PRESENT STUDY

The importance of medicinal plants is increasing day by day because of its various uses. Due to natural causes and human interference medicinal plants there in declining rapidly. So it is an urgent need to survey and identify medicinal plants those are available in Natore Oushodi Gram with their traditional uses and profitability of cultivation and marketing medicinal plants. Many medicinal plants are facing rarity due to over exploitation and loss of biodiversity. Some of these now exist only in the domestic cultivation. Biotechnological methods particularly, *in vitro* propagation procedure can be applied for mass scale production and natural establishment of those medicinal plants, including artificial seed production for conservation. It can also help in meeting up the high demand of these plants. With all these above considerations the present research programme was designed to undertake a thorough survey of medicinal plants used by the local people of Natore Oushodi Gram and their cultivation in the area. For the purpose of micropropagation and conservation of medicinal plants some selected medicinal plants were undertaken for tissue culture and artificial seed production.

CHAPTER II

2. TRADITIONAL KNOWLEDGE AND COMMERCIAL CULTIVATION OF MEDICINAL PLANTS IN NATORE OUSHODI GRAM

2.1. INTRODUCTION

The global demand for medicinal plants is expressed from four identifiable sources: (i) pharmaceutical industries, (ii) traditional healthcare systems, (iii) individual traditional health practitioners, and (iv) women in family health care. The medicinal plants are not only using in medicines but also in cosmetics, detergents, dyes, insecticides, foods and paints etc. Approximately 25 % of drugs used in modern pharmacopoiea are derived from plants and many others are synthetic analogues built on prototype compounds isolated from plants.

Chemicals that make a plant valuable as medicinal plant are (1) Alkaloids (compounds has addictive or pain killing or poisonous effect and sometimes help in important cures), (2) Glycosides (use as heart stimulant or drastic purgative or better sexual health), (3) Tanins (used for gastro intestinal problems like diarrhoea, dysentery, ulcer and for wounds and skin diseases), (4) Volatile/essential oils (enhance appetite and facilitate digestion or use as antiseptic/insecticide and insect repellant properties), (5) Fixed oils (present in seeds and fruits could diminish gastric/acidity), (6) Gum resins and mucilage (possess analgesic property that suppress inflammation and protect affected tissues against further injury and cause mild purgative), and (7) Vitamins and minerals (Fruits and vegetables are the sources of vitamins and minerals and these are used popularly in herbals) (Ghani, 1998 and http://www.life.umd.edu/). A medicinal plant may have a number of such important characteristics and thus can facilitate in curing different disease rather than a single one. The variation in use of some medicinal plants in different ways might have arisen for this.

Traditional knowledge has been generated within different human communities based on their experience, observations and unknown trial based experiments for thousands of years in the course of their interaction with nature and plants of that area. In Bangladesh, a long tradition of indigenous herbal medicinal systems, based on the rich local plant diversity, is considered as very important component of the primary health care system. Many rural people of Bangladesh still depend on plant products and herbal remedies for treating their ailments. The present study was carried out to

document the activities of the medicinal plants growers, kobiraj/herbal doctors and other shakeholders, their livelihood improvements and in conservation of medicinal plants in local area of Natore Oushodi Gram. A number of villages are engaged in cultivation of medicinal plants under unique approach of commercial venture in the district of Natore is collectively denoted as Natore Oushodi Gram. Farmers and kobiraj/herbal doctors of these villages are traditionally cultivated medicinal plants both in their homesteads and agricultural lands.

In Bangladesh, medicinal plants are found to grow naturally in the forest, bushes and marginal land along the canal and in other fallow lands. Many such medicinal plants especially the aromatic herbs are grown in the home garden or as crop field either in sole cropping or inter cropping systems and rarely as plantation crop (Padua *et al*. 1999). Homestead garden has appeared as the potential venture for cultivation of medicinal plants in certain rural areas like Natore Oushodi Gram in Bangladesh. Environment of home gardens with different medicinal and aromatic plants could be good source of income of small scale or resource poor farmers (Agelet *et al*. 2000). Hundreds of rural farmers have been engaged in cultivation, propagation and processing of medicinal plants in the area. Natore Oushodi Gram has appeared as the burning example of commercial cultivation of medicinal plant in contrast to their agricultural crops over the area.

Because of huge rate of deforestation, natural disaster, liberal collection of medicinal plant, over exploitation many of the medicinal plant species have become rare and their wild population are declining rapidly and some might have been extinct. On this context, it is important to collect information on the indigenous knowledge and evaluate the present position of the existing medicinal plant resources of such area that would may help in planning programme for judicious exploitation and uses of these plant resources. Production of medicinal plants and their crude products in Natore Oushodi Gram obviously holds the promises of pharmaceutical and cosmetics industries and also exporting the products to develop the opportunities for additional earnings and employments of the rural people. Moreover, Natore Oushodi Gram presents a model for *in situ* and *ex situ* conservation of medicinal plants in the country.

Under the present investigation efforts have been made to make a survey on the farmers perfectly engaged in the cultivation of medicinal plants and for identification

and characterization of medicinal plants being cultivated over the area and documentation of their ethnobotanical uses.

The study aims to understand the possibility of medicinal plant cultivation as a sustainable livelihood option. The specific objectives are:

- To conduct a thorough survey of cultivated medicinal plants being used by the local people of Natore Oushodi Gram and to make scientific characterization of all medicinal plant species in order to address their correct taxonomic position.
- To make economic and social analysis of the cultivation of medicinal plants in Natore Oushodi Gram.
- To study on the ethnobotanical uses of medicinal plants and making documents on the traditional knowledge being persisted in the community over generations.
- To address the high valued medicinal plants cultivated in the area accompanied by propagation barrier which acts as limiting factor for its mass propagation.
- To explore the factors that will motivate the farmers in cultivating medicinal plants with its industrial promises.

2.2. MATERIALS AND METHODS

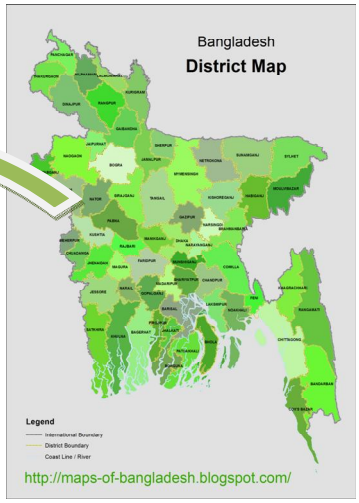
The study was conducted among the medicinal plant cultivators, user and kobiraj/herbal doctors belonging to the eight villages in the district of Natore situated at the northern part of the country. Owing to the unique practices of commercial cultivation of medicinal plant made by the farmers, instead of agricultural crops, the area has been designated as the Oushodi Gram meaning the "villages of medicine". Natore Oushodi Gram has become a resource centre for herbal medicine and imparting training to the new farmers motivated for the commercial cultivation of medicinal plants in the village. Natore Oushodi Gram thus standing in the turning point that provides herbal raw materials to the pharmaceuticals companies and the suppliers of the same for the cosmetic industries and food companies in the country. Large number of local herbal doctors, farmers, nursery growers, traders and hawker have been engaged in different activities generated around the area by virtue of commercial cultivation of medicinal plant. A questionnaire survey was made during 'October 2010 to September 2011' covering 3 season's summer, winter and rain. In order to record social status of different shakeholders involved in the campaign and to characterize the medicinal plants being cultivated over the area.

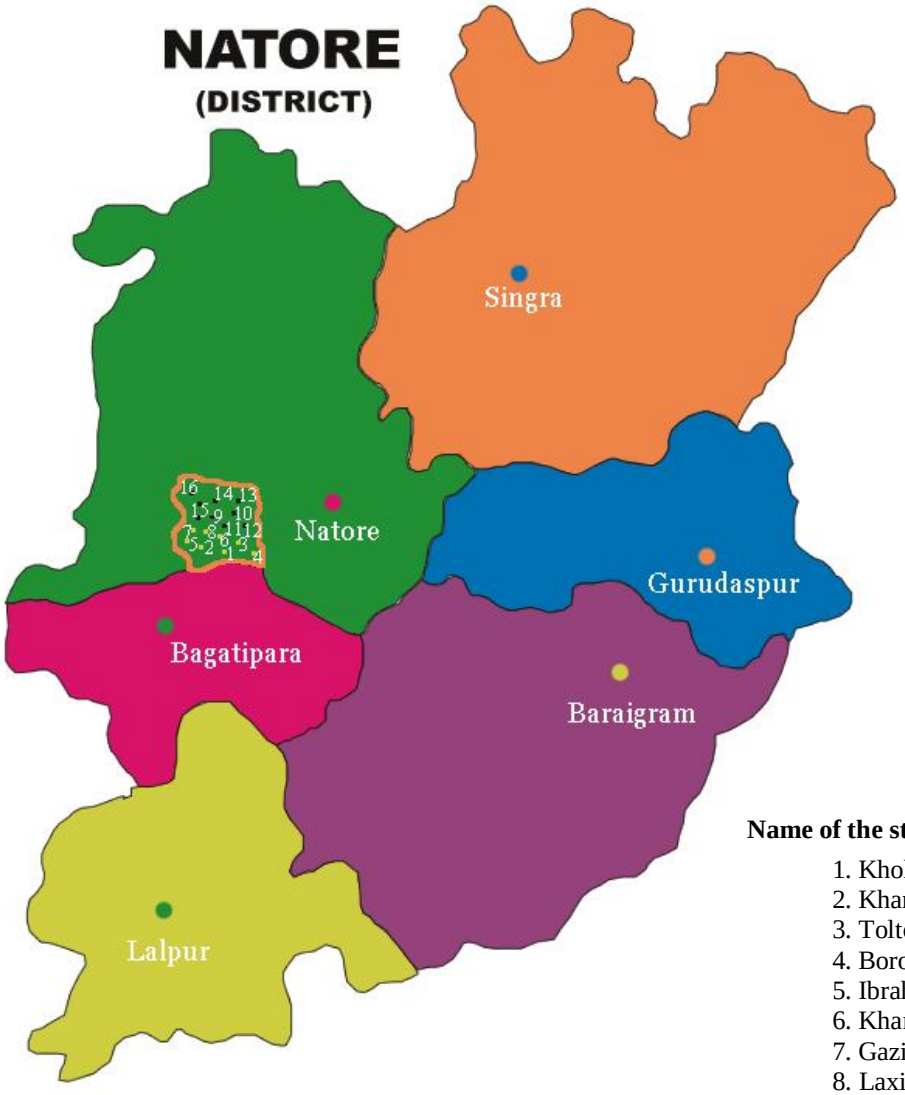
Socioeconomic and ethnobotanical information was collected using a format of questionnaire interviews with 160 local respondents (medicinal plant farmer, local herbal doctor, hawker, whole sale dealer, nursery owner). Local name of collected plants, processing and treatment procedure of diseases with a particular plant was confirmed by cross interviewing of local herbal doctors. Photographs of each species were taken and herbaria of mature plants were prepared for authentic identification of the specimens. The plants were arranged according to their scientific names, local name ,family, duration, propagation, plant form, parts used, mode uses and diseases or condition targeted. Voucher specimens and photographic documentation have been deposited at the Biotechnology Laboratory, Institute of Biological Sciences, Rajshahi University, Rajshahi, Bangladesh for their necessary identification.

2.2.1. Site Selection

In the present investigation Laxmipur Kholabaria union of Natore sadar upozilla under Natore district was selected. The survey has been carried out over the eight villages, these were; kholabaria, khantalbaria, borobaria, toltoliapara, ibrahimpur, khamarpara, gazipur, and laxmipur. The survey was carried out mainly based on information found available on commercial cultivation of medicinal plants over the area and traditional practices of medicinal plants through interview of selected respondents**.**

MAP OF NATORE DISTRICT INDICATING STUDY AREA





Name of the study village

1. Kholabaria
2. Khanthalbaria
3. Toltoliapara
4. Borobaria
5. Ibrahimpur
6. Khamarpara
7. Gazipur
8. Laximpur

Numeric number indicates the study area

2.2.2. Questionnaires formulation

Questionnaires for socioeconomic information of the respondents (medicinal plant cultivators, kobiraj/herbal doctors, hawker, wholesaler, nursery grower) were formulated and developed. Thorough surveys of cultivated and wild medicinal plants were made with their botanical characteristics and ethnobotanical uses. Intensified efforts have been made to develop a questionnaire during the initial period of investigation several formats were developed by studying the literature of similar research works undertaken by other workers. Direct field observation and through trial and error of the application of several formats, a final questionnaires format has been developed to hold every aspects of practical uses of medicinal plants and their occurrence in the locality. Reputed village doctors/ kaviraj were the main respondents for this questionnaires study but sometimes local medicinal plant cultivators, nursery owner, hawker with indigenous knowledge were also considered.

For collection of socioeconomic and ethnobotanical information from local herbal doctors/ other respondents with their indigenous knowledge, the following ethno botanical data sheet was used.

2.2.2.1. Socioeconomic information

Name:.....Father/Husband name.....
Village:.....Union.....Thana/Upozilla.....Dist.....
Education.....Religion.....Age.....Profession.....
Family members…….Male.....Female.....Total land……...
Medicinal plant land.....Number of patient (per week).....Income (yearly).....

2.2.2.2. Botanical and ethnobotanical information

Local name	Scientific name	Family	Monocot /dicot	Life span	Habit	Mode of propagation	Parts of plant used	How used	In which used

2.2.3. Collection of plant materials

The following tools were used during plant materials collection: a) Camera; b) Knife; c) Scissors; d) Polythene bags and e) Tags. Plants were collected systemically as

possible from the different villages of Natore Oushodi Gram. During the flowering time the plants were collected with flowers. In case of some of the plant parts were collected with the help of a long stick. During collection attempts were made to collect flowers, fruits and foliage from the same plant.

The study fields were visited for collection of specimens. The entire plants as far as possible with their flowers and fruits were collected and their photographs were taken focusing the particular organ of specific importance. In the field note books collection number, date of collection, locality, habit, habitat, flower colour and other characters which were not found after preparing herbarium or preservation of flower were recorded, if the flowers and fruits were not possible to collect with their vegetative body, they were collected separately.

2.2.4. Preservation and identification of plant materials

Preservation:

Special care was taken to preserve the plant specimens. The collected plants were preserved. Herbarium sheets were prepared following standard method. Sheets were always made in multiple sets. Insecticides were applied to protect the sheet from insect damage.

Identification:

After making the collection, the specimens were identified by the following methods.

i) Matching system
ii) With the help to taxonomic literature
iii) By experts

i) Matching system

The collected plants were identified by comparing the herbarium sheets preserved in Rajshahi University herbarium. The collected specimen was placed side by side with the herbariums which seem to be the same. After getting provisional identification the specimen was compared with the description provided by Hooker (1865-1885).

ii) Taxonomic literature used in identification

The collected plant specimen was identified with the help of books and journal (Huq, 1986; Hooker, 1865-1885; Khan and Huq 1975; Biswas *et al*. 1973) were consulted. For the current and up to date nomenclature (Huq, 1986; Bennet, 1987 and Pasha, 1988) were consulted. For medicinal information documents (books and journals) of Gilani *et al*. 2003; Ghani, 1998; Kirtikar and Basu, 1987 and 1995) were consulted.

iii) By experts

The collected plant specimens were identified with the help of botanical experts at the department of botany, Rajshahi University; department of botany, Dhaka University and National Herbarium Mirpur, Dhaka.

2.2.5. Data collection

Data for this study were conducted through personal interview of the respondents during "October 2010 to September 2011" using questionnaires prepared earlier. Secondary data were also collected from different sources according to needs. Data and information were collected from seminar library Institute of Biological Sciences, Rajshahi University, seminar library department of botany Rajshahi University, search from internet, and previous research and survey reports.

2.2.6. Data analysis

MS-Ward, MS-Excel, and SPSS were used to process all collected information by micro computer. Data were collected using the following parameters and the methods followed for data collection are given below:

Data were recorded on respondent frequency (%) and mean number of respondent (X) were calculated using formula below:

$$\text{Respondent frequency (\%)} = \frac{\text{No. of respondents}}{\text{Total no. of respondents}} \text{ X } 100$$

i) Average number of respondent

Average numbers of respondents were calculated using the following formula.

$$X = \frac{\sum Xi}{N}$$

Where, X = Average number of respondent.

$\sum$ = Summation.

Xi = Total number of respondent.

N = Number of observations.

2.3. RESULTS

Under the present investigation the information regarding medicinal plants in the Natore Oushodi Gram were carried out by questionnaires survey. We have developed the questionnaires for the survey of medicinal plants through trial and errors under several observations. There are many questionnaire survey used by different workers. Information collected by their survey was not totally applicable in my case and every one had some limitations on our perspectives. Most of the herbal doctors and medicinal plant cultivators were illiterate and in most cases inherited the expertise from their fore fathers and they were very much reluctant about sharing any knowledge. Herbal doctors were not agreed to show any medicinal plants even they were not agreed to talk any name of medicinal plants in some cases. Herbal doctors have strong belief on the effectiveness of medicinal plants. There had been superstition among the herbal practitioners and they believe if they share this knowledge, the medicinal plants will lose their activation. For collection of sufficient information many important medicinal plants or their seeds were supplied to the herbal doctors as the incentive to make them agreed for sharing knowledge.

Traditional knowledge and cultivation of medicinal plants is very important in Natore Oushodi Gram as inhabitants of the area find their living subsistence in this activity. The rural people engaged in this traditional knowledge and cultivation of medicinal plants were taken as the respondents for this survey study and economic condition of these respondents were very important. Under the presents investigation socioeconomic condition of these respondents were studied and their social and economic status were analyzed focusing on their number, age, profession, education, family size, land holding, earning and the results are described below in separate heads.

2.3.1. Socioeconomic study

Socioeconomic condition is related to the practice and cultivation of medicinal plants in Natore Oushodi Gram as the relatively rich farmers were not found to be engaged in this practice. Under the present study the respondents were selected from different profession and status of the rural society involved in medicinal plant cultivation and business of its products and process.

2.3.1.1. Selected respondents in the study area

The study was conducted among the medicinal plant cultivators belonging to the eight villages of Laxmipur-Kholabaria union of Natore sadar upozilla. A large number of local herbal doctors, farmers, nursery growers, traders and hawker have been engaged

in different activities generated around the area by virtue of commercial cultivation of medicinal plants. Number of respondents engaged in traditional practice and cultivation of medicinal plants, their family size including male and female members were studied and the results are given in Table 2.1.

Table 2.1 Respondents selected in different activities of medicinal plants cultivation in Natore Oushodi Gram.

Name of the village	Number of respondents	Total family member of respondents	Male	Female
Kholabaria	42	239	124	115
Kanthalbaria	30	168	83	83
Toltolia	31	158	78	80
Borobaria	16	86	48	38
Khamarpara	12	62	30	32
Ibrahimpur	05	26	13	13
Laxmipur	10	48	26	23
Gazipur	07	37	20	18
Others	07	43	23	20
Total	160	867	445	422

Total interviewees were 160 in number and their total family member were 867 (male 445 and female 442). The table shows that highest number (42 respondent) of respondent were in the village kholabaria (Table 2.1) followed by kanthalbaria, toltilia, borobaria, khamarpara, gazipur, ibrahimpur, and laxmipur.

2.3.1.2. Age of respondents

Age of the respondents was determined by the number of years counted from their date of birth to date of interview. The respondents were classified into three age groups such as young (18-33 yrs), middle (34-49 yrs) and old age (50 yrs and above). The result showed that about 33, 43 and 23 percent of the respondents were in young, middle and old age groups, respectively (Figure 2.1).

2.3.1.3. Profession of respondents

The respondent of the interviews were of different categories according to their profession, like medicinal plant cultivators, kobiraj/herbal doctor, hawker, nursery owner, whole seller. The figure 2.2 shows that highest numbers of respondent were medicinal plant cultivars 72 percent. Whereas, 11, 10, 4, 3 percent of respondent were in kobiraj/herbal doctor, hawker, whole seller and nursery owner respectively.

2.3.1.4. Education of respondents

Education helps an individual to acquire knowledge, to change the attitude, to exercise the modern practices and to promote their skill. Through education, one

becomes aware of new ideas, views and acquires the ability to analyze facts and phenomenon by scientific way. It was measured in terms of their schooling at different levels. The findings showed that 17 percent of the respondents had no formal education, while 68, 9 and 6 percent of the respondents had below S.S.C level, secondary school certificate (S.S.C) level, and above higher than secondary level education respectively (Figure 2.3). This result indicated that 83 percent of the respondents were literate.

2.3.1.5. Family size of respondents

Family size was measured by the total number of family members dependent fully or partially upon the respondent. The family size was classified into four categories based on their total family members. These categories were:

a. Single family (2 person /family)
b. Small family (3-4 person/family)
c. Medium family (5-7 person /family) and
d. Large family (8 person and above /family).

The figure 2.4 revealed that 44 percent of the respondents had small family, whereas 14 percent, 30 percent and 12 percent of the respondents had single, medium and large size family respectively.

2.3.1.6. Total land of respondents

The selected respondents were classified into four categories based on their total land size. These categories were:

a. Marginal farmer (less than 0.66 acre)
b. Small farmer (0.67-2 acre)
c. Medium farmer (2.01-3.33 acre)
d. Large farmer (3.34 acre-above)

It was evident from the figure 2.5 that 41 percent of the respondents belonged to marginal farmer category, whereas, 32, 15 and 12 percent respondents belonged to small, medium and large farmer categories respectively.

2.3.1.7. Medicinal plants plot size

The selected respondents were classified into four categories based on their medicinal plant plot size. These categories were:

a. Marginal farmer (less than 0.10 acre)
b. Small Farmer (0.11-0.33 acre)
c. Medium farmer (0.34-1.0 acre)
d. Large farmer (1.1 acre-above)

It was evident from the figure 2.6 that 52 percent of the respondents belonged to marginal medicinal plant plot categories, whereas, 33, 11 and 4 percent respondents belonged to small, medium and large medicinal plant plot categories, respectively.

2.3.1.8. Medicinal plants cultivation land

In the present investigation data was recorded on total land of the respondent and total medicinal cultivation land of the respondent. Total lands of the respondent were 243.70 acre and total medicinal plant cultivation lands were 34.42 acre. It was evident from the figure 2.7 that percentage of the medicinal land were 12% whereas, 88 % of non medicinal plant land of the respondent.

2.3.1.9. Yearly income of respondents

Large number of local herbal doctors, farmers, nursery growers, traders and hawker have been engaged in different activities generated around the area by virtue of commercial cultivation of medicinal plants in Natore Oushodi Gram. They sold crude materials of the medicinal plants to local kabiraj/herbal doctor, hawker or beparies. The selected respondents were classified into three categories based on their yearly income. These categories were:

a. Low income (less than 20,000 BDT)
b. Medium income (21,000-100,000 BDT)
c. High income (100,100 BDT- above)

It was evident from the figure 2.8 that 58.75 percent of the respondents belonged to medium income category, whereas, 30.62 and 10.62 percent respondents belonged to high and low income categories respectively.

The study was conducted among the medicinal plant cultivators and user in the district of Natore. Owing to the unique practices of commercial cultivation of medicinal plant made by the farmers, instead of agricultural crops, the area has been designated at as the Oushodi Gram meaning the “villages of medicine”. Natore Oushodi Gram has become a resource centre for herbal medicine and imparting training to the new farmers motivated for the commercial cultivation of medicinal plants in the village. When medicinal plants planted in one’s crop field boundary the land owner could get extra opportunity to additional income. People could easily get medicinal plants as and when they need. As a result, their treatment cost was reduced. For kabiraj/herbal doctor and villagers, medicinal plants appeared as the new source of their additional income.

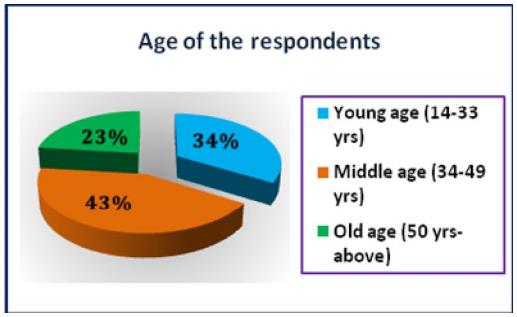


Fig. 2.1 Age of respondents

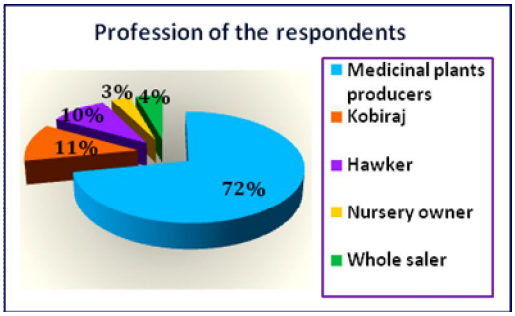


Fig. 2.2 Profession of respondents

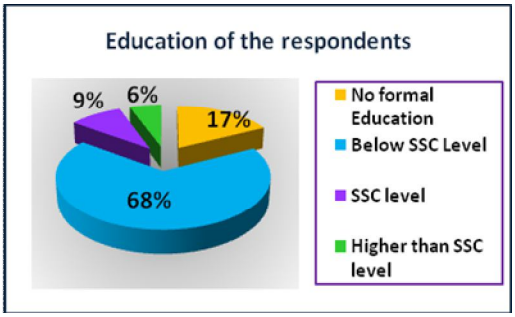


Fig. 2.3 Education of respondents

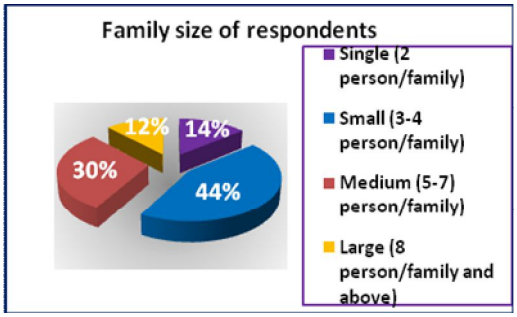


Fig. 2.4 Family size of respondents

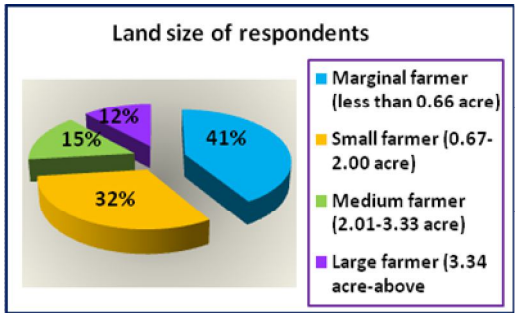


Fig. 2.5 Land size of respondents

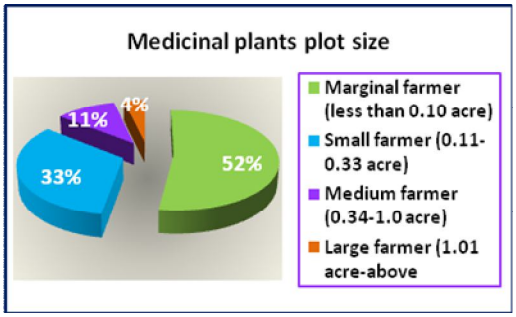


Fig. 2.6 Medicinal plants plot size

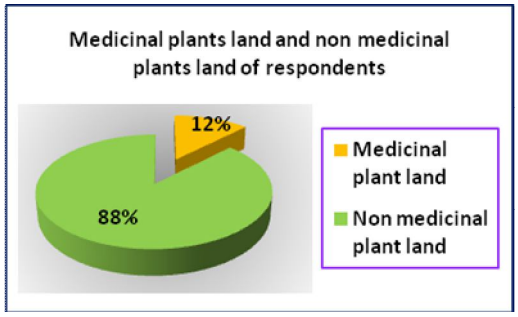


Fig. 2.7 Medicinal plants land of respondents

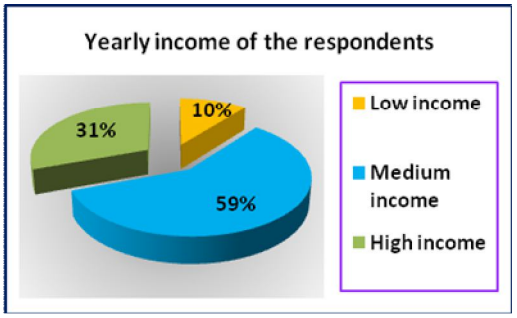


Fig. 2.8 Income of the respondents

2.3.2. Cultivation of medicinal plants

Cultivation of medicinal plants grows new challenge as the science of herbal medicine has been re-emerging all over the world including both developing and developed countries. Due to over exploitation of medicinal plants, its cultivation now imposing more importance on cultivation of medicinal plants in plots like some of agricultural crops. On this context Natore Oushodi Gram presents a typical examples for cultivation of medicinal plants both around homestead and also agricultural plots in Bangladesh. The farmers of Natore Oushodi Gram started to cultivate the important medicinal plants were recorded and their list is presented in the Table 2.2.

Table 2.2 List of commercially cultivated medicinal plants in Natore Oushodi Gram

Local Name	Scientific Name	Rank
Ghritakanchan	*Aloe vera*	1
Simul	*Bombax ceiba*	2
Sotomul	*Asparagus racemosus*	3
Basok	*Adhatoda vasica*	4
Arshagondha	*Withania somnifera*	5
Amada	*Curcuma amada*	6
Kalomegh	*Andrographis paniculata*	7
Bhui kumra	*Ipomoea mauritiana*	8
Pathorkuchi	*Kalanchoe pinnata*	9
Himsagor	*Kalanchoe spathulata*	10
Hostikorno-polash	*Leea macrophylla*	11
Sarpogondha	*Rauwolfia serpentina*	12
Ulot komble	*Ambroma augusta*	13
Key mul	*Costus spiciosus*	14
Turut chandal	*Desmodium motorium*	15
Holud	*Curcuma longa*	16
Misridana	*Kaempferia rotunda*	17
kalizira	*Nigella sativa*	18

A number of 18 medicinal plants found cultivated over the area but all of them were not cultivated by all the farmers. Some plant species were found commonly cultivated and some were cultivated in different numbers of the farmers.

Based on the scoring of farmers engaged in the cultivation of particular medicinal plant all the plant species were graded and according to the highest commercial value of cultivated medicinal plant was ghrita kanchan followed by simul, sotomul,basok, arshagondha, amada, kalomegh, bhui kumra, pathorkuchi, himsagor, hosti korna

polash, sarpogondha, ulot komble, key mul, turut chandal, hulud misridana, and kalozira etc.

2.3.2.1. Cultivation of medicinal plants in agricultural plots

For profitability against conventional agriculture many poor and marginal farmers of the local area were switchover against conventional agriculture and engaged in cultivation of medicinal plant in their agricultural plots. *Aloe vera, Bombax ceiba, Asparagus racemosus, Adhatoda vasica, Withania somnifera, Curcuma amada, Andrographis paniculata, Rauwolfia serpentina, Desmodium motorium, Kaempferia rotunda, Nizella sativa* plants were found to grow in agricultural plots in Natore Oushodi Gram. Among the cultivated medicinal plants in agricultural plots *A. vera* eventually covers the major cultivated lands and farmers looked into the multifarious economic benefit in its cultivation. Some farmers used to cultivate *A. vera* plants exclusively in agriculture plots more than one acre in one plot. The farmers made commercial linkage with the different industry to more necessary supply of *A. vera* leaf in regular harvest time. After *A. vera*, shimul, mint, sotomul, sorpogondha, arshogondha were made commercial supply to buyers (industry people) in large amounts.

2.3.2.2. Cultivation in homestead

In my observation of field survey, many local people of Natore Oushodi Gram specially women are engaged in cultivation of medicinal plants in a small area of their homestead. *Aloe vera, Asparagus racemosus, Adhatoda vasica*, *Azadirachta indica*, *Ocimum* spp, *Aegle marmelos, Leea macrophylla*, *Abroma augusta*, *Cassia alata*, *Vitex negundo*, *Hibiscus rosa sinensis*, *Vinca rosea* and many other medicinal plants were found to grow in homestead.

2.3.2.3. Cultivation in fallow lands

Local people of Natore Oushodi Gram were cultivated many medicinal plants in fallow land for their extra income. Specially poor people and women are engaged in these profession. *Terminalia arjuna, Terminalia bellirica, Terminalia chebula, Azadirachta indica, Cassia alata, Jatropha spp, Riccinus communis*, *Paederia foetida* and many other medicinal plants were found to grow in fallow lands.

The kabiraj/local herbalist, in Natore Oushodi Gram revealed that in the past they were self sufficient in terms of collecting medicinal plants from local sources, but now a days they were found to increasingly dependent on external supplies because of the exhaustion of local species due to indiscriminate collection. Inadequate knowledge accompanied by one time temporary gains rather motivated the plant

collectors to operate indiscriminate collection practices. These collectors normally inherit their knowledge of identification of plants, uses, collection and processing from their ancestors. Few experienced collectors in sometimes used to train local people in the identification of certain species, primary processing and drying of medicinal plants and develop them as their local agents or suppliers. The plant collectors employed by wholesalers or processors in Natore Oushodi Gram are usually termed as hawkers. They move to different plant growing areas and collect plant resources based on their commercial interest. They were found to develop their expertise in identifying plant species and properties but obviously not sincere enough to consider their sustainability.

Fig. 2.9 A: Local farmers engaged in cultivation of *Aloe vera* at Natore Oushodi Gram: **B:** A plot of *Aloe vera*; **C**: A plot of *Bombax ceiba*; **D:** A plot of *Withania somnifera*. **E:** A plot of *Nigella sativa*; **F:** A Nursery plot of mixed medicinal plants in Natore Oushodi Gram.

Fig. 2.10 A: A plot of *Curcuma amada*; **B:** A plot of *Kaempferia rotunda*; **C**: A plot of *Kalanchoe pinnata*; **D:** *Adhatoda vasica* cultivated in fallow land; **E:** *Mucuna pruriens* planted in homestead; **F:** *Adhatoda vasica* cultivated in land margins at Natore Oushodi Gram.

2.3.2.2. Processing, packaging and storage of the herbal materials

Processing activities involve cutting, drying, grading, storage and in some cases packaging with the bark of banana plant (in case of *Aloe vera*), is mainly carried out by the farmers, collectors and local pickers, the process is not standardized and subject to variation from farmer to farmer and bepari based on transport and distribution. The latter used to sell their materials to wholesalers and retailers and directly to a number of processors and possibly some herbal practitioners. Primary processing for the species observed in the study sites is currently performed by the cultivators and pickers. In many cases it was found that the harvested plant materials were spread over on a ragged mat in the yard of the farmer, where dusts and other polluting elements easily mixed with them. Other than this, several farmers were found to dry their plant materials on any available surface that included the roof of a nearby building or even on a bitumen road. The drying areas were usually not protected from contamination from birds, rodents or insects. As a matter of fact secondary processing activities for many species start at the production bases.

In the study at Oushudi Gram Natore, there was a grinding or flouring mill where the kabiraj, the beparies or pickers and in some cases the primary sellers get their dried materials grinded into a powdered form for sale or resale. Miserably, with the same milling machine, people of the locality get their spices and other stuffs ground, such as chilies, turmeric, rice and wheat. But the processor did not have any idea about Good Manufacturing Practices and they did not perform any test on the quality of raw materials, They took as an input supply from local bepari. They were quite ignorant about the active constituents of the raw materials and they were using and about the presence or possibilities of contaminant contents that their raw materials might have. Rather, dried herbs were stored on the floor in paper bags and mostly were found to be dirty and mouldy.

In order to make their herbal products marketable, another important primary processing activity is storage. As found at the study site, the storage activities were carried out in a substandard manner in many cases. In Laxmipur bazaar there are three medicinal plant materials wholesale stores. Ordinary packing with plastic and jute sacks was observed as well as open stacking and piling of materials in a noticeably dirty atmosphere. Such careless storing of materials (of dried form or raw) damages the properties of medicinal plants, i.e. the quality of their active ingredients.

Fig. 2.11 A: Drying of herbal material; **B:** Herbal paste; **C:** Herbal pill; **D:** Herbal dust; **E:** Leaf of *Aloe vera;* **F:** Dry stem of *Glycyrrhiza glabra*; **G:** Rhizome of *Curcuma amada*; **H:** Tuber of *Ipomoea mauritiana*; **I:** Tuber of *Kaemoferia rotunda;* **J:** Root of *Withania somnifera*; **K:** Root of *Bombax ceiba*; **L:** Root *of Asparagus recemosus*; **M:** Seeds of *Mucuna pruriens*; **N:** Dry fruits of *Emblica officinalis;* **O:** Dry fruits of *Terminalia chebula;* **P:** Dry fruits of *Terminalia bellirica;* **Q:** Dry stem of *Terminalia arjuna;* **R:** A farmer harvesting *Aloe vera* on his field and packaging with the bark of banana trees; **S:** A farmer harvesting *Kaempferia rotunda* in his field; **T-X:** Farmers, kobiraj/herbal doctor and whole seller involved in different activities; **Y:** Farmer showing medicinal plant.

2.3.3. Botanical and ethnobotanical studies

2.3.3.1. Medicinal plants cultivated/ growing in Natore Oushodi Gram

In the present investigation survey was made in 160 respondents over the area and under this survey 120 plants species were identified as medicinal plants either cultivated in agricultural plots or growing wildly around the homestead, roadsides, graveyards and other fallow lands in the area. Availability of medicinal plant species collected on respondent survey regarding local name, monocot/dicot, availability of medicinal plants species at respondents, cultivated /planted/ wild source and their position were analyzed and the results are presented in Table 2.3. The results revealed that all the plant species did not hold the same merit of importance in plantation by the respondents in Natore Oushodi Gram. Among the 120 medicinal plants the most frequently cultivated medicinal plant was *Aloe vera*, followed by *Bombax ceiba, Asparagus racemosus, Adhatoda vasica, Withania somnifera, Curcuma amada, Andrographis paniculata, Ipomoea mauritiana, Kalanchoe pinnata, Kalanchoe spathulata, Leea macrophylla, Rauwolfia serpentina, Abroma augusta, Desmodium motorium, Kaempferia rotunda, Nizella sativa, Costus spiciosus and different types of Ocimum spp*. Different medicinal plants had been identified predominantly growing in several micro sites over the area like homestead, road side and crop field, fallow land. The plants *Aloe vera, Asparagus racemosus, Adhatoda vasica, Azadirachta indica, Ocimum* spp, *Kalanchoe pinnata, Aegle marmelos, Leea macrophylla, Abroma augusta, Cassia alata, Vitex negundo, Hibiscus rosa sinensis and Vinca rosea* were predominantly found to grow in the homestead. The major medicinal plant species found in the road side were *Terminalia arjuna, Terminalia bellirica, Terminalia chebula, Azadirachta indica, Bauhinia racemosa, Cassia fistula, Glycosmis penthaphylla, Jatropha spp and Riccinus communis.* On the other hand, *Centella asiatica, Calotropis procera, Bacopa monnieri, Argemone mexicana, Paederia foetida and Chenopodium album* were mainly found in the crop fields and fallow land, mostly grown naturally.

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Table 2.3 List of medicinal plants cultivated/growing in Natore Oushodi Gram.

Sl. no	Name of the medicinal plant	Monocot/ Dicot	Number of growers (respondent)	Ranking	*Cultivated /Planted/Wild
1	*Aloe vera*	Monocot	142	1	C
2	*Bombax ceiba*	Dicot	51	2	C
3	*Asparagus racemosus*	Monocot	48	3	C
4	*Adhatoda vasica*	Dicot	46	4	C
5	*Withania somnifera*	Dicot	31	6	C
6	*Curcuma amada*	Monocot	31	6	C
7	*Andrographis paniculata*	Dicot	28	7	C
8	*Ipomoea mauritiana*	Dicot	28	7	C
9	*Kalanchoe pinnata*	Dicot	25	8	C
10	*Kalanchoe spatulata*	Dicot	22	9	C
11	*Leea macrophylla*	Dicot	22	9	C
12	*Rauwolfia serpentina*	Dicot	21	10	C
13	*Azadirachta indica*	Dicot	21	10	P
14	*Abroma augusta*	Dicot	21	10	C
15	*Costus spiciosus*	Monocot	18	11	C
16	*Ricinus communis*	Dicot	18	11	W
17	*Casia alata*	Dicot	18	11	W
18	*Mimosa pudica*	Dicot	18	11	P
19	*Leucas aspera*	Dicot	18	11	W
20	*Leonurus sibiricus*	Dicot	18	11	W
21	*Desmodium motorium*	Dicot	16	12	C
22	*Kaempferia rotunda*	monocot	16	12	C
23	*Curcuma longa*	Monocot	16	12	C
24	*Nigella sativa*	Dicot	15	13	C
25	*Aegle marmelos*	Dicot	15	13	P
26	*Mucuna pruriens*	Dicot	15	13	P
27	*Phlogacanthus thysiflorus*	Dicot	14	14	P
28	*Clitoria ternetea*	Dicot	14	14	P
29	*Boerhavia repens*	Dicot	14	14	W
30	*Vinca rosea*	Dicot	14	14	P
31	*Centella asiatica*	Monocot	14	14	W
32	*Emblica officinalis*	Dicot	12	15	P
33	*Barlaria lupulina*	Dicot	12	15	P
34	*Terminalia arjuna*	Dicot	12	15	P
35	*Aristolochia indica*	Dicot	10	16	P
36	*Paederia foetida*	Dicot	10	16	W
37	*Tarmarindus indica*	Dicot	10	16	P
38	*Ocimum basillicum*	Dicot	10	16	P
39	*Datura metel*	Dicot	10	16	W
40	*Achyranthes aspera*	Dicot	10	16	W

Table 2.3 List of medicinal plants cultivated/growing in Natore Oushodi Gram.

Sl. no	Name of the medicinal plant	Monocot / Dicot	Number of growers (respondent)	Ranking	*Cultivated / Planted/Wild
41	*Bacopa monnieri*	Dicot	10	16	W
42	*Hibiscus rosa sinensis*	Dicot	10	16	P
43	*Ecbolium viride*	Monocot	9	17	P
44	*Terminalia chebula*	Dicot	9	17	P
45	*Ocimum basilicum*	Dicot	9	17	P
46	*Wedelia calendulacea*	Dicot	9	17	W
47	*Smilax zeylanica*	Dicot	9	17	W
48	*Argemone mexicana*	Dicot	9	17	W
49	*Euphorbia pulcherrima*	Monocot	9	17	P
50	*Ocimum sanctum*	Dicot	9	17	P
51	*Cossinea cordifolia*	Dicot	9	17	W
52	*Curculigo orchioides*	Monocot	9	17	P
53	*Vitex negundo*	Dicot	8	18	P
54	*Cadariocalyx motorius*	Dicot	8	18	P
55	*Zanthoxylum rhetsa*	Dicot	8	18	P
56	*Nyctanthes arbortristis*	Dicot	8	18	P
57	*Pterocarpus santalinus*	Dicot	8	18	P
58	*Calotropis procera*	Dicot	8	18	W
59	*Achyranthes aspera*	Dicot	8	18	W
60	*Aegle marmelos*	Dicot	8	18	W
61	*Glycosmis pentaphylla*	Dicot	8	18	W
62	*Cissus quadrangualaris*	Monocot	8	18	P
63	*Ephedra garardiana*	Dicot	8	18	P
64	*Amorphophalus campanulatus*	Monocot	7	19	W
65	*Cajanus cajan*	Dicot	7	19	P
66	*Datura fastuosa*	Dicot	7	19	W
67	*Cuscuta reflexa*	Monocot	7	19	W
68	*Cassia occidentalis*	Dicot	7	19	W
69	*Cassia sophera*	Dicot	7	19	W
70	*Wedelia chinensis*	Dicot	7	19	W
71	*Duranta repens*	Dicot	6	20	P
72	*Malvaviscus arboreus*	Dicot	6	20	W
73	*Casia fistula*	Dicot	6	20	P
74	*Ocimum gratissmum*	Dicot	6	20	P
75	*Solanum nigram*	Monocot	6	20	W
76	*Mirabillis jalapa*	Dicot	6	20	P
77	*Alocasia indica*	Monocot	6	20	P
78	*Abrus precatorius*	Dicot	6	20	W
79	*Jatropha curcus*	Dicot	5	21	W
80	*Swertia chirata*	Dicot	5	21	P
81	*Saraca asoca*	Dicot	5	21	P
82	*Cannabis sativa*	Dicot	5	21	W

Table 2.3 List of medicinal plants cultivated/growing in Natore Oushodi Gram.

Sl. no	Name of the medicinal plant	*Monocot/ Dicot	Number of growers (respondent)	Ranking	*Cultivated /Planted/Wild
83	*Sesbania grandiflora*	Dicot	5	21	P
84	*Acalypha hispida*	Dicot	5	21	W
85	*Curcuma zeoderia*	Monocot	5	21	P
86	*Tragia involucrata*	Dicot	5	21	W
87	*Mikania cordata*	Dicot	5	21	W
88	*Mentha arvensis*	Dicot	4	22	P
89	*Sida cordifolia*	Dicot	4	22	W
90	*Aerva sanguinonenta*	Dicot	4	22	W
91	*Piper longum*	Dicot	4	22	P
92	*Eclipta alba*	Dicot	4	22	W
93	*Murdania nudiflora*	Dicot	4	22	W
94	*Madhuca indica*	Dicot	4	22	P
95	*Acalypha indica*	Dicot	4	22	W
96	*Cyperus rotundus*	Monocot	4	22	W
97	*Amaranthus spinosus*	Dicot	4	22	W
98	*Terninalia bellirica*	Dicot	4	22	P
99	*Curcuma roscoeana*	Monocot	4	22	P
100	*Glycyrrhiza glabra*	Dicot	4	22	P
101	*Oxalis corniculata*	Dicot	4	22	W
102	*Hemidesmus indicus*	Dicot	4	22	P
103	*Accacia farnesiana*	Dicot	3	23	P
104	*Tridax procumbens*	Dicot	3	23	P
105	*Indigofera tinctoria*	Monocot	3	23	P
106	*Bauhinia racemosa*	Dicot	3	23	P
107	*Blumea lacera*	Dicot	3	23	W
108	*Calotropis procera*	Dicot	3	23	W
109	*Calotropis gigantea*	Dicot	3	23	W
110	*Crocus sativus*	Dicot	3	23	P
111	*Aqillaria malacensis*	Dicot	3	23	P
112	*Acorus calamus*	Monocot	3	23	W
113	*Clerodendrum inerme*	Dicot	3	23	W
114	*Zingiber officinale*	Monocot	2	24	W
115	*Hyptis suaveolens*	Dicot	2	24	P
116	*Ipomoea fistulosa*	Dicot	2	24	W
117	*Jatropha gossypifolia*	Dicot	2	24	W
118	*Syzygium cumini*	Dicot	2	24	W
119	*Scorparia dulcis*	Dicot	2	24	W
120	*Urginea indica*	Monocot	2	24	P

* C: Cultivated; P: Planted; W: Wild

* Monocot: Monocotyledon; Dicot: Dicotyledon

2.3.3.2. Ethnobotanical information on 120 medicinal plant species in Natore Oushodi Gram

Traditional knowledge is as old as human civilization. It has been defined as the traditional knowledge of indigenous communities about surrounding plant diversity and as the study of how the people of a particular culture and region make use of indigenous plants. Ethnobotany has its roots in botany. Botany, in turn originated in part from an interest in finding plants to help fight illness. In fact medicine and botany have close ties. Many of today's drugs have been derived from plant resources.

The high costs of western pharmaceuticals put modern health care services out of reach of most of the world's population, which relies on traditional medicine and medicinal plants to meet their primary health care needs. Even where modern medical care is available and affordable, many people prefer more traditional practices. This is particularly true for first nations and immigrant populations, who have tended to retain ethnic medical practices. In the last decade, there has been considerable interest in resurrecting medicinal plants in western medicine, and integrating their use into modern medical systems.

The following figure prefers a list of medicinal plants, which have been observed in the study area. These plants were preferred by the local kobiraj / herbal doctor mainly for such reasons as (a) their adaptability to the edaphic and climatic conditions of the locality (b) their market potentiality and (c) the diverse use of many of them in different medicine preparation as the base ingredient.

In the present investigation ethnobotanical observation was made on 120 medicinal plant species during the course of survey in Laxmipur-Kholabaria union under sadar upozilla in Natore district. Among the plant species 17-20 plants were commercially cultivated in this area because of their medicinal values and market potentiality. Some plants were grown naturally or planted in the road side and crop field and fallow land. Ethnobotanical information collected on individual plant basis regarding scientific name, local name, family, parts of plant used and medicinal uses in the local people were analyzed and the results are presented in Figure 2.12.

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Abroma augusta*

Local name: Ulot komble

Family: Sterculiaceae

Parts used: Leaves, Stem, fruits

Medicinal uses in the local people: Menstrual disorder, weakness

Scientific name: *Accacia farnesiana*

Local name: Gui babul

Family: Fabaceae

Parts used: Leaves, root

Medicinal uses in the local people: Diarrhoea, sexual debility, eye disease

Scientific name: *Abrus precatorius*

Local name: Lal kuch

Family: Fabaceae

Parts used: Leaves, roots, seeds

Medicinal uses in the local people: Asthma, cough, piles

Scientific name: *Achyranthes aspera*

Local name: Apang

Family: Amaranthaceae

Parts used: Leaves, root

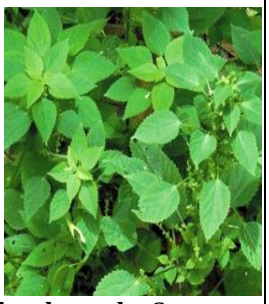
Medicinal uses in the local people: Diabetes, urinary infection

Scientific name: *Acalypha indica*

Local name: Muktajuri

Family: Euphorbiaceae

Parts used: Leaves



Medicinal uses in the local people: Sore, ring worm, toxic prevention

Scientific name: *Adhatoda vasica*

Local name: Basok

Family: Acanthaceae

Parts used: Leaves, whole plant

Medicinal uses in the local people: Bronchitis, whooping cough and asthma, removal of intestinal parasites

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Acorus calamus*

Local name: Borch

Family: Acoraceae

Parts used: Fruits, leaves

Medicinal uses in the local people: Diarrhoea, weakness, sore

Scientific name: *Aerva sanguinonenta*

Local name: Lal apang

Family: Amaranthaceae

Parts used: Leaves

Medicinal uses in the local people: To stop bleeding from cuts and wounds, fever

Scientific name: *Aegle marmelos*

Local name: Bel

Family: Rutaceae

Parts used: Leaves, Fruits

Medicinal uses in the local people: Digestives, energetic, tonic

Scientific name: *Alocasia indica*

Local name: Mankochu

Family: Araceae

Parts used: Whole plant

Medicinal uses in the local people: Weakness, blood purifier, alveolitis

Scientific name: *Amaranthus spinosus*

Local name:Kanta naty

Family: Amaranthaceae

Parts used: Whole plant

Medicinal uses in the local people: Snake bite, sexual debility, constipation

Scientific name: *Aloe vera*

Local name: Ghreta kanchan

Family: Liliaceae

Parts used: Leaves

Medicinal uses in the local people: Weakness, constipation, face brightness, health and vigor

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Amorphophalus campanulatus*

Local name: Ol

Family: Araceae

Parts used: Leaves
tuber

Medicinal uses in the local people: Weakness, Lumbago, Brain tonic, constipation

Scientific name: *Andrographis paniculata*

Local name: Kalo megh

Family: Acanthaceae

Parts used: Leaves

Medicinal uses in the local people: Snake bite, sexual debility, constipation

Scientific name: *Asparagus recemosus*

Local name: Sotomul

Family: Liliaceae

Parts used: Roots

Medicinal uses in the local people: Sexual debility, nervous disorders, general debility increases milk secretion during lactation

Scientific name: *Aqillaria malacensis*

Local name: Agor

Family: Thymmelacaceae

Parts used: Leaves

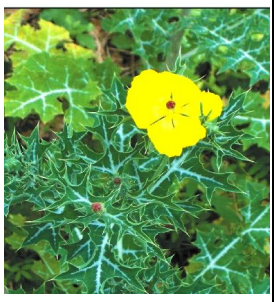
Medicinal uses in the local people: Tonic , constipation

Scientific name: *Argemone mexicana*

Local name: Seal kata

Family: Papaveraceae

Parts used: Seed, root



Medicinal uses in the local people: Skin diseases, itching, malarial fever

Scientific name: *Azadirachta indica*

Local name: Nim

Family: Meliaceae

Parts used: Leaves,
fruits

Medicinal uses in the local people: Abscess, itching, jaundice, sore healing

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Aristolochia indica*

Local name: Essarmul

Family: Aristolochiaceae

Parts used: Roots

Medicinal uses in the local people: Snake bite, leucorrhoea, sexual debility

Scientific name: *Bacopa monnieri*

Local name: Brahmilata

Family: Scrophulariaceae

Parts used: Whole plant

Medicinal uses in the local people: Brain tonic , dyspepsia

Scientific name: *Bombax ceiba*

Local name: Simul

Family: Bombacaceae

Parts used: Roots

Medicinal uses in the local people: Digestives, spermatorrhoea, constipation

Scientific name: *Bauhinia racemosa*

Local name: Kanchan

Family: Fabaceae

Parts used: Leaves

Medicinal uses in the local people: Sore itching, abscess, constipation

Scientific name: *Blumea lacera*

Local name: Kukur sunga

Family: Compositae

Parts used: Leaves, root

Medicinal uses in the local people: Cough stomach pain, itching

Scientific name: *Barlaria lupulina*

Local name: Kali chandal

Family: Acanthaceae

Parts used: Roots

Medicinal uses in the local people: Muscular pain, piles, abscess

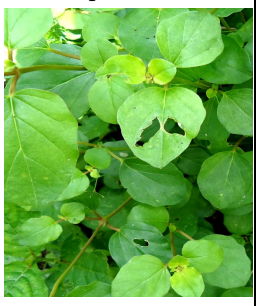
Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Boerhaavia repens*

Local name: Punorvoba

Family: Nyctaginaceae

Parts used: Whole plant



Medicinal uses in the local people: Cough , fever, weakness

Scientific name: *Calotropis procera*

Local name: Choto akondo

Family: Asclepiadaceae

Parts used: Leaves, root

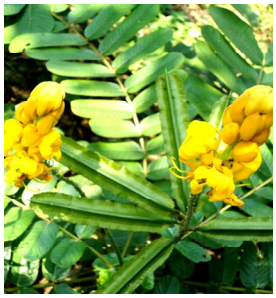
Medicinal uses in the local people: Ulcer, itching, eczema

Scientific name: *Cassia alata*

Local name: Dad mardon

Family: Fabaceae

Parts used: Leaves



Medicinal uses in the local people: Itching, tonic, eczema

Scientific name: *Cassia occidentalis*

Local name: Boro kolkasunda

Family: Fabaceae

Parts used: Leaves

Medicinal uses in the local people: Malarial fever, energetic, digestives

Scientific name: *Cassia sophera*

Local name: Choto kolkasundha

Family: Fabaceae

Parts used: Roots, leaves

Medicinal uses in the local people: Diarrhoea, weakness, sexual debility

Scientific name: *Cannabis sativa*

Local name: Bhang

Family: Cannabidaceae

Parts used: Leaves

Medicinal uses in the local people: Crazy, ring worm, pleurisy alveolitis

Scientific name: *Cadariocalyx motorius*

Local name: Bon chandal

Family: Fabaceae

Parts used: Roots

Medicinal uses in the local people: Rheumatism, tooth aches, eczema, elephantiasis

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Cassia fistula*

Local name: Sonalu

Family: Fabaceae

Parts used: Fruits, leaves, bark

Medicinal uses in the local people: Vomiting, nausea, abdominal pain

Scientific name: *Cajanus cajan*

Local name: Orohoro

Family: Fabaceae

Parts used: Roots, leaves, seeds

Medicinal uses in the local people: Jaundice, urinary disorder, bronchitis

Scientific name: *Clitoria tarnetea*

Local name: Aporajita

Family: Fabaceae

Parts used: Whole plant

Medicinal uses in the local people: Itching, pox, worm control

Scientific name: *Centella asiatica*

Local name: Thankuni

Family: Umbelliferae

Parts used: Leaves

Medicinal uses in the local people: Diarrhoea, dysentery, weakness

Scientific name: *Cissus quadrangularis*

Local name: Harzora

Family: Vitaceae

Parts used: Stem

Medicinal uses in the local people: Fractured bones, gleets bruise

Scientific name: *Clerodendrum inerme*

Local name: Bhat

Family: Verbanaceae

Parts used: Leaves, seeds

Medicinal uses in the local people: Ring worm, diabetes, obesity and hypertension

Scientific name: *Coccinea cordifolia*

Local name: Telakuch

Family: Cucurbitaceae

Parts used: Roots, leaves, seeds

Medicinal uses in the local people: Diabetes, itching, lactation

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Curculigo orchioides*

Local name: Talmuly

Family: Liliaceae

Parts used: Roots

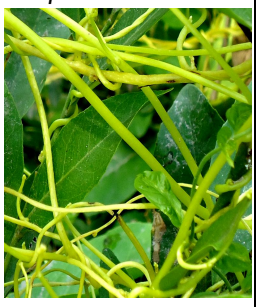
Medicinal uses in the local people: Piles, gonorrhea, jaundice, asthma

Scientific name: *Cuscuta reflexa*

Local name: Sornolata

Family: Cuscutaceae

Parts used: Stem



Medicinal uses in the local people: Rheumatism, sore, fever

Scientific name: *Curcuma roscoeana*

Local name: Shonkamul

Family:Zingiberaceae

Parts used: Rhizome

Medicinal uses in the local people: Sexual debility, gonorrhea, constipation

Scientific name: *Curcuma longa*

Local name: Halud

Family: Zingiberaceae

Parts used: Rhizome

Medicinal uses in the local people: Digestive, blood purifier, Lowering blood cholesterol, dyspepsia

Scientific name: *Curcuma amada*

Local name: Amada

Family: Zingiberaceae

Parts used: Rhizome

Medicinal uses in the local people: Digestive, blood purifier, gonorrhea, dyspepsia

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

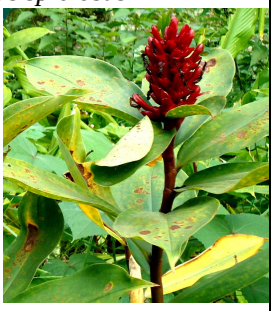
Scientific name: *Costus spiciosus* **Local name**: Key mul **Family**: Zingiberaceae **Parts used**: Roots  **Medicinal uses in the local people**: Leprosy, constipation, skin diseases, worm infection	**Scientific name**: *Curcuma zeoderia* **Local name**: Ekangi **Family**: Zingiberaceae **Parts used**: Rhizome  **Medicinal uses in the local people**: Weakness, cough, diabetes
Scientific name:*Cyperus rotundus* **Local name**: Muthua ghas **Family**: Cyperaceae **Parts used**: Whole plant  **Medicinal uses in the local people**: Dysentery, diabetes, dyspepsia	**Scientific name**: *Desmodium motorium* **Local name**: Turut chandal **Family**: Fabaceae **Parts used**: Leaves  **Medicinal uses in the local people**: Eye strain, ocular diabetic neuropathy, glaucoma
Scientific name: *Datura metel* **Local name**: Datura **Family**: Solanaceae **Parts used**: Leaves, fruits  **Medicinal uses in the local people**: Asthma, chronic bronchitis, chronic pain	**Scientific name**: *Duranta repens* **Local name**: Mehede **Family**: Verbenaceae **Parts used**: Leaves  **Medicinal uses in the local people**: Edema, hair fall, graying of hair, skin diseases
Scientific name: *Datura fastuosa* **Local name**: Kalo Datura **Family**: Solanaceae **Parts used**: Leaves, fruits  **Medicinal uses in the local people**: Asthma, chronic bronchitis, chronic pain	**Scientific name**: *Ecbolium viride* **Local name**: Nilkontho **Family**: Acanthaceae **Parts used**: Leaves, fruits  **Medicinal uses in the local people**: Dysuria, jaundice, rheumatism

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Eclipta alba*

Local name: Kess raj

Family: Compositae

Parts used: Whole plant

Medicinal uses in the local people: Promote hair growth, stimulate liver function

Scientific name: *Ephedra garardiana*

Local name: Dodraj

Family: Ephedraceae

Parts used: Leaves, roots

Medicinal uses in the local people: Asthma, urinary disorders

Scientific name: *Emblica officinalis*

Local name: Amloki

Family: Phyllanthaceae

Parts used: Fruits, leaves

Medicinal uses in the local people: Sore, vtamine-C, tonic

Scientific name: *Euphorbia hirta*

Local name: Dudhea

Family: Euphorbiaceae

Parts used: Roots

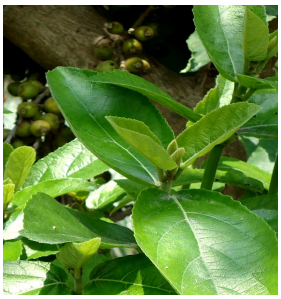
Medicinal uses in the local people: Fever, blood dysentery, itching

Scientific name: *Ficcus racemosa*

Local name: Joga dumur

Family: Moraceae

Parts used: Leaves, fruits



Medicinal uses in the local people: Diabetes, heart disease, energetic

Scientific name: *Glycyrrhiza glabra*

Local name: joshti modhu

Family: Fabaceae

Parts used: Whole plant

Medicinal uses in the local people: Fever, cough, weakness

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Glycosmis pentaphylla*

Local name: Datmajon

Family: Rutaceae

Parts used: Whole plant

Medicinal uses in the local people: Piles, toothache, anemia

Scientific name: *Heliotropium indicum*

Local name: Hatisur

Family: Boraginaceae

Parts used: Whole plant

Medicinal uses in the local people: Ulcer, sores, wounds, skin affections,

Scientific name: *Hemidesmus indicus*

Local name: Anontomul

Family: Asclepiadaceae

Parts used: Roots

Medicinal uses in the local people: Fever, lactation, menstrual regulation

Scientific name: *Hibiscus rosa sinensis*

Local name: Joba

Family: Malvaceae

Parts used: Flowers, leaves

Medicinal uses in the local people: Menstrual regulation, leucorrhoea

Scientific name: *Hyptis suaveolens*

Local name: Tocma

Family: Lamiaceae

Parts used: Leaves, fruits

Medicinal uses in the local people: Itching, weakness, sexual sensation, urinary complications

Scientific name: *Indigofera tinctoria*

Local name: Neel

Family: Fabaceae

Parts used: Leaves

Medicinal uses in the local people: Carbuncle, chest pain, dye colour blue

Scientific name: *Ipomoea fistulosa*

Local name: Dhol kolmi

Family: Convolvulaceae

Parts used: Whole plant

Medicinal uses in the local people: Weakness, diabetes, constipation

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Ipomoea mauritiana*

Local name: Bhui kumra

Family: Convolvulacae

Parts used: Tuber


Medicinal uses in the local people: Stimulate the digestive system, bile movement, boost sexual desire

Scientific name: *Jatropha curcas*

Local name: Jamal kota

Family: Euphorbiaceae

Parts used: Leaves, fruits

Medicinal uses in the local people: Parasitic skin diseases, gonorrhea, leprosy

Scientific name: *Jatropha gossypifolia*

Local name: Lal jamal kota

Family: Euphorbiaceae

Parts used: Leaves, fruits

Medicinal uses in the local people: Sore, bio diesel, ringworm

Scientific name: Misridana

Local name: *Kaempferia rotunda*

Family: Zingiberaceae

Parts used: Tuber


Medicinal uses in the local people: Anti helminthes, reducing swellings, gastric pain

Scientific name: *Kalanchoe spathulata*

Local name: Himsagor

Family: Crassulaceae

Parts used: Leaves

Medicinal uses in the local people: Ulcers toothaches, earaches, eye infections, wounds

Scientific name: *Kalanchoe pinnata*

Local name: Pathor kuchi

Family: Crassulaceae

Parts used: Leaves

Medicinal uses in the local people: Ulcers, diarrhea, vomiting, blood vomiting

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

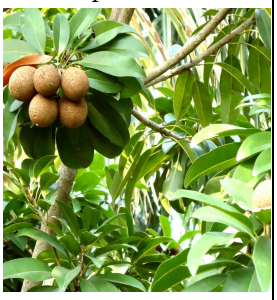
Scientific name: *Leea macrophylla* **Local name**: Hasti karno polash **Family**: Vitaceae **Parts used**: Leaves, roots  **Medicinal uses in the local people**: Piles, jaundice, asthma, gonorrhea	**Scientific name**: *Leonurus sibiricus* **Local name**: Roctodron **Family**: Lamiaceae **Parts used**: Leaves  **Medicinal uses in the local people**: Fever, cough, weakness
Scientific name: *Leucas aspera* **Local name**: Shatodron **Family**: Lamiaceae **Parts used**: Leaves  **Medicinal uses in the local people**: Ulcers, hemorrhoids, boils, diarrhea, blood vomiting	**Scientific name**: *Madhuca indica* **Local name**: Mohua **Family**: Sapotaceae **Parts used**: Leaves  **Medicinal uses in the local people**: Impotency, ophthalmic
Scientific name: *Malvaviscus arboreus* **Local name**: Morich joba **Family**: Malvaceae **Parts used**: Leaves  **Medicinal uses in the local people**: Digestive, menstrual regulation	**Scientific name**: *Manilkara zapota* **Local name**: Sofeda **Family**: Sapotaceae **Parts used**: Fruits, seeds  **Medicinal uses in the local people**: Diarrhea, dysentery, pulmonary complaints
Scientific name: *Mentha arvensis* **Local name**: Pudina **Family**: Lamiaceae **Parts used**: Whole plant  **Medicinal uses in the local people**: Rhinitis, sore throat, good blood cleanser, liver tonic	**Scientific name**: *Mikania cordata* **Local name**: Assam lata **Family**: Compositae **Parts used**: Leaves  **Medicinal uses in the local people**: Stomach disease, digestion, blood cleanser

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Mimosa pudica*

Local name: Sada lazzaboti

Family: Mimosaceae

Parts used: Roots

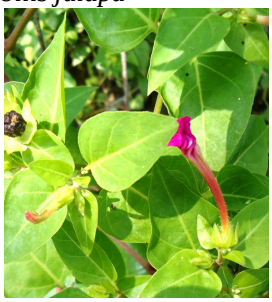
Medicinal uses in the local people: Spermatorrhoea, piles, abscess

Scientific name: *Mirabilis jalapa*

Local name: Sandha rani

Family: Nyctaginaceae

Parts used: Leaves



Medicinal uses in the local people: Fever, syphilis, general debility

Scientific name: *Mucuna pruriens*
Local name: Alkusi

Family: Fabaceae

Parts used: Seeds, root

Medicinal uses in the local people: Male fertility enhancer, nervous system disorders such as parkinson's disease

Scientific name: *Nigella sativa*

Local name: Kalozira

Family: Ranunculaceae

Parts used: Seeds

Medicinal uses in the local people: Blood pressure, diabetes, itching, puerperal

Scientific name: *Murdania nudiflora*

Local name: Kurulla

Family: Commelinaceae

Parts used: Leaves

Medicinal uses in the local people: Scabies, stomach problems, leprosy

Scientific name: *Nyctanthes arbortritis*

Local name: Sheuli

Family: Oleaceae

Parts used: Roots, leaves

Medicinal uses in the local people: Fungal infection, dry cough, bronchitis

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

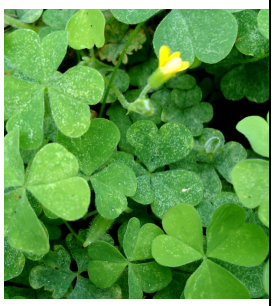
Scientific name: *Ocimum americanum* **Local name**: Tulsi (white) **Family**: Lamiaceae **Parts used**: Leaves  **Medicinal uses in the local people**: Cough, fever, expectorant, stimulant	**Scientific name**: *Ocimum basilicum* **Local name**: Babui tulsi **Family**: Lamiaceae **Parts used**: Leaves  **Medicinal uses in the local people**: Fever, cough, bronchitis
Scientific name: *Ocimum gratissimum* **Local name**: Ram tulsi **Family**: Lamiaceae **Parts used**: Whole plant  **Medicinal uses in the local people**: Sore, itching, abscess, constipation	**Scientific name**: *Ocimum sanctum* **Local name**: Tulsi (black) **Family**: Lamiaceae **Parts used**: Roots, leaves  **Medicinal uses in the local people**: Tonic cough, asthma, bronchitis
Scientific name: *Oxalis corniculata* **Local name**: Amrul **Family**: Oxalidaceae **Parts used**: Whole plant  **Medicinal uses in the local people**: Dyspepsia, dysentery, diarrhea, dysmenorrhoea	**Scientific name**: *Paederia foetida* **Local name**: Gandha vadulia **Family**: Rubiaceae **Parts used**: Whole plant  **Medicinal uses in the local people**: Rheumatism, abdominal pain, snakebite
Scientific name: *Passiflora foetida* **Local name**: Jhumka lata **Family**: Passifloraceae **Parts used**: Whole plant  **Medicinal uses in the local people**: Snakebite, leucorrhoea, sexual debility	**Scientific name**: *Phlogacanthus thysiflorus* **Local name**:Kalo basok **Family**: Acanthaceae **Parts used**: Leaves, root  **Medicinal uses in the local people**: Fever, cough, asthma, bronchitis

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Piper longum*

Local name: Pipul

Family: Piperaceae

Parts used: Dried spikes, roots

Medicinal uses in the local people: Arthritis, dyspepsia, asthma, bronchitis, fever

Scientific name: *Pterocarpus santalinus*

Local name: Rocto chandon

Family: Fabaceae

Parts used: Roots, leaves

Medicinal uses in the local people: Abscess, ulcer, sore

Scientific name: *Rauwolfia serpentina*

Local name: Sarpo gandha

Family: Apocynaceae

Parts used: Roots, leaves

Medicinal uses in the local people: Hypertension, remove anxity, painful affections of bowel

Scientific name: *Ricinus communis*

Local name: Venna

Family: Euphorbiaceae

Parts used: Seeds, leaves, roots

Medicinal uses in the local people: Bronchitis, leprosy, skin diseases, colic

Scientific name: *Santalum album*

Local name: Sweta chandon

Family: Santalaceae

Parts used: Heart wood, roots

Medicinal uses in the local people: Gonorrhea, excessive sweating and fevers, menstrual problems

Scientific name: *Saraca asoca*

Local name: Asok

Family:Caesalpiniaceae

Parts used: Leaves

Medicinal uses in the local people: Menorrhogia, leucorrhoea, bleeding though anus

Scientific name: *Smilax zeylanica*

Local name: Kumarilata

Family: Smilacaceae

Parts used: Roots, young stem with leaves

Medicinal uses in the local people: Syphilis, gonorrhea, leucorrhoea, impotency and general weakness

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Scoparia dulcis*

Local name: Bondhonia

Family: Scrophulariaceae

Parts used: Whole plant

Medicinal uses in the local people: Asthma, anemia, diabetes

Scientific name: *Sesbania grandiflora*

Local name: Bokful

Family: Fabaceae

Parts used: Roots

Medicinal uses in the local people: Fever, richest source of vit-A

Scientific name: *Sida cordifolia*

Local name:Berela

Family: Malvaceae

Parts used: Stem, leaves

Medicinal uses in the local people: Tonic, treatment of respiratory system related troubles

Scientific name: *Solanum nigrum*

Local name: Tit begun

Family: Solanaceae

Parts used: Whole plant

Medicinal uses in the local people: Cough, asthma, arthritis, anti-cancerous drugs

Scientific name: *Swertia chirata*

Local name: Chirata

Family: Gentianaceae

Parts used: Leaves, stem

Medicinal uses in the local people: Bitter tonic, stomachic, appetizer, laxative

Scientific name: *Syzygium cumini*

Local name: Kalo jam

Family: Myrtaceae

Parts used: seed, bark, leaves,

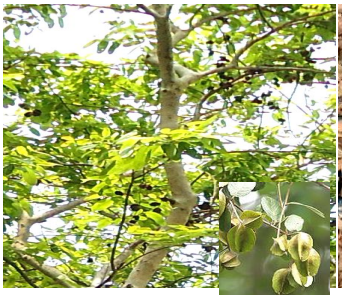
Medicinal uses in the local people: Diabetes, sore throat, bronchitis, asthma and dysentery

Scientific name: *Terminalia arjuna*

Local name: Arjun

Family: Combretaceae

Parts used: Barks



Medicinal uses in the local people: Heart disease, blood circulation, blood pressure

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Terminalia bellirica*

Local name: Bohera

Family: Combretaceae

Parts used: Fruits


Medicinal uses in the local people: Antifungal, reduces levels of lipids, asthma, piles and worms

Scientific name: *Terminalia chebula*

Local name: Haritoki

Family: Combretaceae

Parts used: Fruits


Medicinal uses in the local people: Hair tonic, dental caries, bleeding gums

Scientific name: *Tarmarindus indica*

Local name: Tetul

Family: Fabaceae

Parts used: Fruits, leaves, seed

Medicinal uses in the local people: Indigestion, dyspepsia, constipation, urinary retention, reduce cholesterol

Scientific name: *Tragia involucrata*

Local name: Besatu

Family: Euphorbiaceae

Parts used: Leaves

Medicinal uses in the local people: Toxic prevention, worm control, sore

Scientific name: *Trigia involucreta*

Local name: Tridhara

Family: Compositae

Parts used: Leaves

Medicinal uses in the local people: Sore, toxic prevention, ring worm

Scientific name: *Urgenia indica*

Local name: Jongli piaz

Family: Liliaceae

Parts used: Leaves, bulb

Medicinal uses in the local people: Jaundice, digestive, flatulence

Figure: 2.12 Ethnobotanical information (Traditional knowledge) of 120 medicinal plant species in Natore Oushodi Gram.

Scientific name: *Vitex negundo*

Local name: Nishinda

Family: Verbenaceae

Parts used: Leaves, roots

Medicinal uses in the local people: High blood pressure, diabetes, urinary disorders

Scientific name: *Vinca rosea*

Local name: Nayantara

Family: Apocynaceae

Parts used: Roots

Medicinal uses in the local people: High blood pressure, diabetes, anxiety

Scientific name: *Withania somnifera*

Local name: Arshogandha

Family: Solanaceae

Parts used: Roots, while plant

Medicinal uses in the local people: Stress, anxiety and depression, sexual health

Scientific name: *Wedelia chinensis*

Local name: Moha vungaraj

Family: Compositae

Parts used: Leaves

Medicinal uses in the local people: Hair growth , skin disease

Scientific name: *Wedelia calendulacea*

Local name: Vungaraj

Family: Asteraraceae

Parts used: Leaves, root

Medicinal uses in the local people: Headache, protect hair fall, worm infestation

Scientific name: *Zanthoxylum rhetsa*

Local name: Tezbol

Family: Rutaceae

Parts used: Leaves

Medicinal uses in the local people: Asthma, bronchitis, cardiac ailments

Scientific name: *Zingiber officinale*

Local name: Ada

Family: Zingiberaceae

Parts used: Rhizome

Medicinal uses in the local people: Abdominal pain, jaundice, flatulence

2.3.3.3. Identification of medicinal plant species with their families

The identified 120 plants were studied on their salient features in order to locate their taxonomic position. Results of this study revealed that 120 plant species belongs to 51 families and 111 genera. Among these Fabaceae, Lamiaceae, Zingiberaceae, Euphorbiaceae, Acanthaceae, Compositae and Solanaceae were identified as the major families predominantly composing of these medicinal plants in Natore Oushodi Gram. Highest number of 19 medicinal plants found to belong to the family of Fabaceae but the family Lamiaceae contained 8 medicinal plants, Zigiberaceae 7, Euphorbiaceae 6, Acanthaceae and compositae included 5 medicinal plants, Liliaceae and Solanaceae each contained 4 number of medicinal plants, Amaranthaceae, Asclepiadaceae, Combretaceae, Malvaceae, Rutaceae, and Verbenaceae each had 3 medicinal plants. The remaining families only contained a few number of medicinal plant species.

Table 2.4 Families and their belonging medicinal plant species studied in Natore Oushodi Gram.

Sl. no.	Name of Family	No. of plants	Sl. no.	Name of Family	No. of plants	Sl. no.	Name of Family	No. of plants
1.	Fabiaceae	19	18.	Nyctagnaceae	2	35.	Smilacaceae	1
2.	Lamiaceae	8	19.	Crassulaceae	2	36.	Sterculiaceae	1
3.	Zingiberaceae	7	20.	Convolvulaceae	2	37.	Moraceae	1
4.	Euphorbiaceae	6	21.	Sapotaceae	2	38.	Umbelliferae	1
5.	Acanthaceae	5	22.	Vitaceae	1	39.	Papaveraceae	1
6.	Compositae	5	23.	Myrtaceae	1	40.	Meliaceae	1
7.	Liliaceae	4	24.	Ranuneculaceae	1	41.	Acoraceae	1
8.	Solanaceae	4	25.	Rubiaceae	1	42.	Thymmelacaceae	1
9.	Amaranthaceae	3	26.	Cyperaceae	1	43.	Cucurbitaceae	1
10.	Rutaceae	3	27.	Vitaceae	1	44.	Cuscutaceae	1
11.	Malvaceae	3	28.	Aristolochiaceae	1	45.	Ephedraceae	1
12.	Asclepiadaceae	3	29.	Bombacaceae	1	46.	Oleaceae	1
13.	Verbenaceae	3	30.	Boraginaceae	1	47.	Oxalidaceae	1
14.	Combretaceae	3	31.	Cannabinaceae	1	48.	Passifloraceae	1
15.	Araceae	2	32.	Commelinaceae	1	49.	Piperaceae	1
16.	Apocynaceae	2	33.	Santalaceae	1	50.	Gentiaceae	1
17.	Scrophulariaceae	2	34.	Phyllanthaceae	1	51.	Asteraceae	1

2.3.3.4. Life span of medicinal plants

On life span aspect, out of 120 medicinal plant species, 44 plants were found annual and 76 plants were identified as perennial species (Figure 2.13) in Natore Oushodi Gram.

2.3.3.5. Monocot and Dicot species

On cotyledon aspect, out of 120 medicinal plant species, 99 plants were dicot and 21 plants were identified as monocot plants (Figure 2.14).

2.3.3.6. Habit of medicinal plants

In the present investigation 120 medicinal plants were recorded based on habit and the results are shown in Figure 2.15. The figure shows that 38 percent of plants were herb, 32 percent shrub, 17 percent tree, 11 percent climber and only 2 percent were of epiphytic habit. Considering the habit of the medicinal plants in these areas, herbs were more prevalent than shrub, trees, climber and epiphytes.

2.3.3.7. Parts used in different medicinal plants

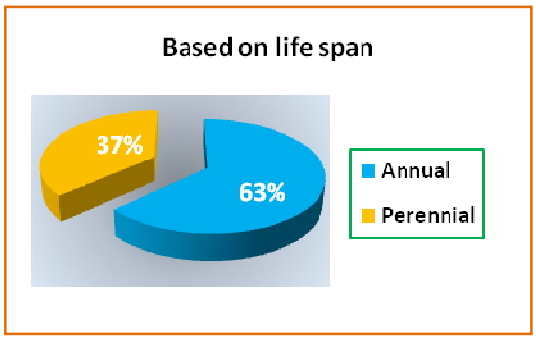
Leaf is the most frequently used part followed by roots, whole plant part, seed, fruits, stem bark, rhizome. Among the 120 plants species, the local herbal doctors used leaves of 36% plants, roots of 18% plants, fruits of 6% plants, stem of 4% plants, bark of 3% plants, whole plants of 13% plants, seeds of 10% plants, rhizome of 4% plants, tuber of 2% plants, bulb of 2% plants and flower of 2% plants were used for medicinal purpose (Figure 2.16).

2.3.3.8. Medicinal plants application.

The method of application varies according to the nature of ailment. The plants were used in the form of juice, paste, powder, decoction and even soaked seed and raw plants were also found to be used. Generally juice and powder used to be given, paste was used externally and decoction was taken either orally or externally. In the present investigation 62% plants were used of juice for feeding, whereas 7% powder forms, 3% paste, 20% surface used, 5% direct used and 3% used as boiled seeds forms (Figure. 2.17).

2.3.3.9. Propagation of medicinal plants

On the propagation aspect, out of 120 medicinal plants species, 77% plants were mainly propagated by seeds, whereas 12% by stem cutting, 7% rhizomes and 1% mainly propagated by 1% sucker, 1% tuberous roots, 1% leaves and 1% bulbs (Figure. 2.18).



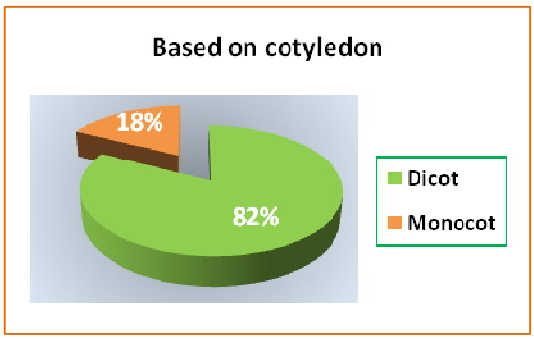
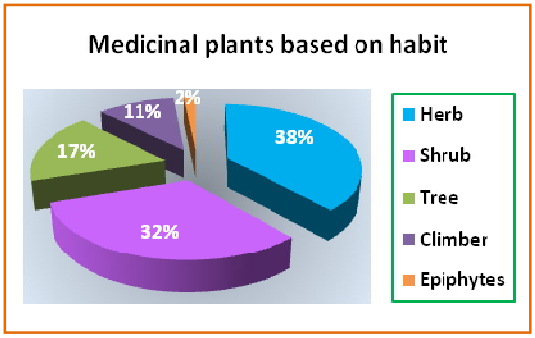


Fig. 2.13 Medicinal plants based on life span **Fig. 2.14 Medicinal plants based on cotyledon**



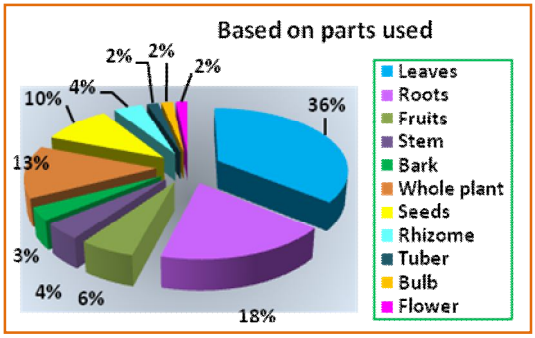
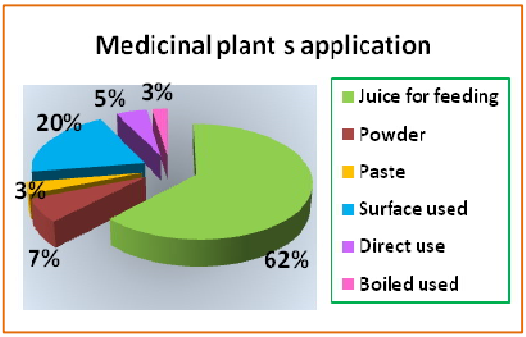


Fig. 2.15 Medicinal plants based on habit **Fig. 2.16 Medicinal plants based on parts used**



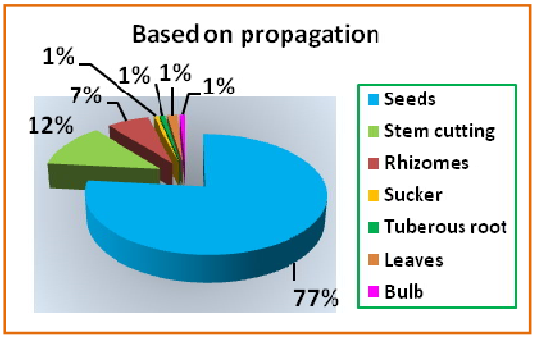


Fig. 2.17 Medicinal plants application **Fig. 2.18 Medicinal plants based on propagation**

2.4. DISCUSSION

Local knowledge generated by people living within a particular community is unique and it can contribute to a sustainable development strategy that's accounts for the potential of the local environment and the experience and wisdom of the indigenous population. The local people of Natore Oushodi Gram possess such kind of knowledge particularly from their healthcare viewpoint. Which is transmitted from generation to generation by Guru mode of instruction and this system is still prevailing in the area. In most cases, the knowledge is kept strictly within the family circle. The apprentices lived with and learnt at the feet of masters who maintained a conventional oral tradition. The inhabitants of the areas were mainly dependent on herbal medicine for treating their diseases. Proper study of various medicinal plants in the area and their stock assessment were thoroughly carried out. When due to indiscriminate deforestation, over exploitation, habitat destruction, injudicious collection and natural habitats of medicinal plants in the country are being gradually declined. Moreover modern pharmaceuticals have replaced many natural remedies. In these consequences, traditional knowledge of healing system is disappearing rapidly in the country. If this situation continues, it may be completely lost. It is our moral responsibility to restore such indigenous knowledge system of traditional medicine that inherited from generation to generation and persisted in the community of local people. Besides these, most of the modern medicines have many bad side effects, so now a day's interest towards the herbal medicine is increasing again. On this perspective we have made an investigation on Natore Oushodi Gram to analysis of traditional knowledge of medicinal plants accompanied by their commercial cultivation and the status of biodiversity conservation of medicinal plants growing over the area.

In Natore study area farmers started their cultivation totally on their own initiative inspired by a local herbal doctor named Afaz Uddin popularly called Afaz pagla and until now there is hardly any effort from the government sector to cater the needs of the farmer. It is possible to improve livelihood of rural marginal farmers by creating possibilities for a commercial cultivation of medicinal plants. The present study experienced the potential of medicinal plant as the means of earning of livelihood through studying the farmers in the laxmipur kholabaria union who improved their living standard considerably. Among 160 respondents, 52% respondents were marginal farmers and they changed to medicinal plant cultivation considering it would give a quick return compared to general agriculture. Now a days, many marginal and

poor farmers are fully dependent on the income of medicinal plants, which the traditional agriculture fails to compete. From our study, it was revealed that many new farmers were switching over from agriculture to medicinal plants cultivation and its better income motivated them to engage their agricultural lands in this practice. The farmers of these villages were found to cultivate medicinal plants both in their homesteads and agricultural fields. Besides this, mixed cropping technique can help reducing disease infestation and maximum utilization of scarce land resources as observed in the study.

Medicinal plants were also reported in India for commercial cultivation in homestead and in agricultural lands. In India, more than 400 plants species used for production of medicine by the Indian herbal industry, fewer than 20 species are currently under cultivation in different parts of the country (Uniyal *et al.* 2000).

Lange and Schippmann (1997) stated that of the 1543 species traded in Germany, only 50-100 species (3-6 percent) are exclusively sourced from cultivation. In China, about 5000 medicinal plants have been identified and about 1000 are more commonly used, but only 100-250 species are cultivated (Xiao, 1991). In Hungery, a country with a long tradition of medicinal and aromatic plant cultivation, only 40 species are cultivated for commercial production (Bernath, 1999; Palevitch, 1991). In Europe as a whole, only 130-140 medicinal and aromatic species are cultivated (Pank, 1998; Verlet and Lecercq, 1999). A global survey on the extent of medicinal and aromatic plant cultivation in terms of species, volume and values would be highly desirable. On the other hand, however, we recognized that many more medicinal and aromatic plant species are cultivated on a small scale in home gardens, either as home remedies or herbalist or cultivation by local people can take place as enrichment planting.

The cultivation of medicinal plants originates popular activities in the community and the people of all ages have been engaged in these activities. Literate, illiterate, adult, old and women also involved in medicinal plant cultivation. All interviewees were male except 2 were female, results indicated that majority of the respondents were of middle age (43%) under the age between 34-49 yrs. In terms of literacy 17 percent of the respondent had no formal education; on the other hand 83 percent respondents had formal education in different levels. The respondents were taken from the local people in any way involved in the activity of medicinal plants and the results revealed that out of 160 respondents 72 percent were medicinal plants producer, 11 percent herbal doctor, 10 percent hawker, 3 percent nursery owner and 4 percent wholesaler. In the present investigation out of 160 respondents, total land area under the possession of

all the respondents were 243.70 acre and total medicinal plant land were 34.42 acre. It was evident that the land under medicinal plants cultivation was 12.38% out of their total land holdings.

Following taxonomical characteristics 120 medicinal plants, being used by the people of the area, were identified and found to belong 51 families and among these families predominating families were Fabaceae, Lamiaceae, Zingiberaceae, Euphorbiaceae, Acanthaceae, Compositae, Solanaceae, Liliaceae, Malvaceae, Apocynaceae, Combretaceae, Rutaceae. Considering the habit of the medicinal plants, herbs were more prevalent than shrubs, trees, climbers and epiphytes. The use of medicinal plants for treatment varies from place to place depending upon the local demand. Moreover, different use of particular plant species was also noted among the local community. In case of *Boerhaavia diffusa*, whole plant was used in the treatment of pulmonary tuberculosis, plant powder was used abdominal tumor, dysentery and renal disease flowers and seeds were used as contraceptives. Roots were used in the treatment of jaundice, anemia, gonorrhea, blood purifier and as stimulant. On the other hand, more than one plant species was found to use for the treatment of same diseases for example 16 plant species were used for fever, 18 plants for cough and asthma, 25 plants for weakness, 12 plants for skin disease, 10 plants for sex disease, 8 plants for menstrual disorder , 8 plants for abdominal pain and ulcer, 6 plants for heart disease and blood pressure, 6 plants species were used for diabetics, 5 plants for worm control and 4 plants for liver complaints and jaundice.

Seventy one different diseases could be treated with 120 medicinal plants. Among these diseases fever, cough and asthma and general weaknesses were more common diseases. Other more prevalent diseases were skin disease, constipation, weakness, stomach pain, piles, worm control, sexual debility, jaundice, bronchitis, rheumatism, toothaches, eczema, venereal disease, dysmenorrhoea, osteoporosis, liver disease, diabetes, hypertension, dysentery, sore, pyorrhea, headache etc. Leaf is the most frequently used part followed by roots, whole plant part, seed, fruits, stem bark and rhizome. The local herbal doctors used leaves of 46 plants, roots of 24 plants, whole plant part of 15 plants, seeds of 12 plants, stem bark of 4 plants, fruits of 8 plants, rhizome of 5 plants, tuber of 3 plants, bulb of 1 plant and flower of 2 plants. The method of application varies according to the nature of ailment. The plants were used in the form of juice, paste, powder, decoction, and even soaked seed and raw plants were also used. Generally juice and powder were given, paste was used externally and decoction was given to take either orally or externally. Grita kanchan is the most cultivated medicinal plant in the study area. The popular mixed drink with harvested

juice of ghritakumari keeps the stomach cool and also helps in mitigating constipation.

Moreover, combinations of different plant parts were used for better treatment of many diseases. Such as one *Curcuma roscoeana* with betel leaf and that act immediately to increase sexual power; tuber powder of *Ipomoea mauritiana* with *Nigella sativa* were used among the lactating mother to increase breast milk; *Kaempferia rotunda* with *Terminalia arjuna* bark were used to control high blood pressure and heart problem; *Kalanchoe spathulata* with *Ecbolium viride* popular for treating teeth pain; *Andrographis paniculata* with *Keampferia rotunda* were used to treat jaundice, *Paederia foetida* with *Allium sativum* were used to treat rheumatic pain. Some organic and inorganic substances such as egg, honey, spices, latex, resin, meat of fox and black hen, shell and flesh of snail were found to be added in the preparation of herbal medicine for the treatment of various diseases prevailing over the area.

Bangladesh government formed a cell for medicinal plant in the "Ministry of Environment and Forest". The cell is working in different dimension for developing the medicinal plant sector, like (1) a research center for medicinal plant, (2) promoting nim plantation, (3) medicinal plant seedling production through tissue culture and (4) leasing of land for medicinal plant cultivation. From the above discussion, it is clear that the medicinal plant cultivation is still in a rudimentary stage. There are very few farmers those who are cultivating medicinal plant by their own initiatives.

Identification of some promising opportunities specially generated in the Natore Oushodi Gram:

1. Cultivation of medicinal plants gives better income over agricultural crops

In Natore Oushodi Gram a large number of local herbal doctors, farmers, nursery growers, traders and hawker have been engaged in different activities generated around the area by virtue of commercial cultivation of medicinal plants considering it would give a quick return compared to general agriculture. Now a day's many new farmers were switching over from agriculture to medicinal plants cultivation and its better income motivated them to engage their agricultural lands in this practice.

2. Ideal example for both *in situ* and *ex situ* conservation of medicinal plants

Due to indiscriminate deforestation, over exploitation, habitat destruction, injudicious collection and natural habitats of medicinal plants in the country are being gradually declined. That necessity to develop proper conservation programs and strategy to conserve the medicinal plant in the country. The local people of these villages cultivated

/planted many medicinal plants in their homestead and agriculture plot. Among these plants, some plants were grown in naturally in this area and some were imported from home and abroad. So, we can say it's an ideal example for *in situ* and *ex situ* conservation of medicinal plants in this area.

3. Prospect of exploring industries

At present medicinal plants from Natore Oushodi Gram are being used in different sectors i.e Ayurvedic, Unani, Homeopathy, Allopathic, cosmetics, beverage etc and their demands increasing day by day. There should be exposure in different mass media to promote awareness of the true economic value of these medicinal plant products and by products to support the national health system, especially primary health care system and promoting national economy. Different extension campaign using folk media should be effectively organized for this purpose.

4. Training centre for new growers of other regions

Natore Oushodi Gram has become a resource centre for herbal medicine and also can be a training centre for the new farmers motivated for the commercial cultivation of medicinal plants in the village. Training should be provided to both local Kabiraj and local people for proper cultivation, collection and processing methods of medicinal plants, and also for quality maintenance during preparation of medicine.

5. Means of employment generation particularly for women

In most of the cases, men take part in the cultivation process of medicinal plants. In all cases except two it was found that the women are the owner of the land but the women are putting labour to the cultivation. The women may be the owner's wife or mother or in few cases daughter. Others those help in medicinal plant cultivation were owner's son, brother and nephew. In very few cases the cultivators hired labour for cultivation. The involvement of women is also noticeable in the medicinal plant cultivation. So, cultivation in the homestead land could be good source of income for the rural women not only that many men and women were engaged in harvesting, drying, processing and carrying. So we can say, medicinal plant cultivation create employment generation particularly for women in this area.

6. Driving force for social awareness

Among the various reasons cited for cultivating medicinal plants, the majority mentioned profitability. Many of them said that, the maximum return by cultivating small portion of land make 'cultivation of medicinal plants lucrative'. The effects of neighborhood cultivating medicinal plants and getting easy money out of this are also playing a role for spreading medicinal plant cultivation.

2.5. SUMMARY

Medicinal plants play a significant role in providing primary healthcare services to the people and a large number of medicinal plants are now widely used in Bangladesh including Natore Oushodi Gram at Natore district for production of both traditional and modern drugs. The present study experienced the potentials of medicinal plant as for earning livelihood which improved their living standard considerably. A questionnaire documentation data sheet was developed during the initial period of investigation to hold every aspect of practical uses of medicinal plants and their occurrence in the locality.

Among 160 respondents, 52% respondents were marginal farmers and they changed to medicinal plant cultivation considering it gave a quick return compared to general agriculture. Now a day's many marginal and poor farmers were fully dependent on the income of medicinal plants, which the traditional agriculture fails to provide.The farmers of these villages traditionally cultivated medicinal plants both in their homesteads and agricultural fields. From our study, it was revealed that many farmers found to increase their buying capacity and even enabled them to buy new land with the money earning from the cultivation of medicinal plants. .

Majority of the respondents are of middle aged (43%), and their age between 34-49 yrs. 17 percent of the respondent have no formal education, on the other hand 83 percent respondents having formal education in different levels. The respondents were different categories according to their profession, out of 160 interviewees 72 percent of the respondent were medicinal plant producers, 11 percent herbal doctors, 10 percent hawker, 3 percent nursery owner and 4 percent wholesaler. Out of 160 respondents, total lands of the respondent were 243.70 acre and total medicinal plant lands were 34.42 acre. It was evident that the land for medicinal plant cultivation was 12.38% out of their total land holdings.

In the present investigation, extensive survey was made on 160 respondents over the study area and 120 plant species belonging to 51 families were identified with medicinal use by questionnaire survey. Among these Fabaceae, Lamiaceae, Zingiberaceae, Euphorbiaceae, Acanthaceae, Solanaceae, and Compositae were identified as the major families predominantly consisting of these medicinal plants in Natore Oushodi Gram. Highest number of medicinal plants (19)found to belong to the family Fabaceae; Lamiaceae consists of 8 medicinal plant species ; Zigiberaceae consists of 7 species ; Euphorbiaceae consists of 6 species; Acanthaceae and compositae included 5 medicinal plants, Liliaceae and Solanaceae, each included 4

number of medicinal plants, Amaranthaceae, Asclepiadaceae, Rutaceae, Verbenaceae and Combretaceae each included 3 medicinal plants. And each of other families bearing only a few of the remaining plant species. Among 120 identified medicinal plants 44 numbers of plants were annual and 76 numbers of plants were perennial. In regards of propagation, 89 numbers of plants were propagated by seeds, 20 numbers of plants were propagated by vegetative means and 11 numbers of plants exhibited both seeds and vegetative propagation. The habitats of medicinal plants were mainly village thicket, road side, jungle, graveyard and fallow lands. They were also found in homestead, edge of pond, waste place and sometimes in cultivation of agricultural plot. In consideration of plant habit 46 plants were herb, 38 plants were shrub, 21 plants were tree, 13 plants were climber and 2 plants were epiphytes. On cotyledon aspect, out of 120 medicinal plant species 99 plants were dicot and 21 plants are identified as monocot.

Under the present ethnobotanical survey a number 71 diseases were addressed by the village doctors being treated with herbal medicine. Among these diseases cough, fever, ulcer and acidity, jaundice, diabetics, skin diseases, gonorrhoea, asthma, leucorrhoea, sexual debility, weakness, rheumatism, ring worms, toxic prevention, stomachache were predominant diseases for herbal remedies. Field survey indicated that 25 plants were used for cough, 18 plants were used for weakness, 16 plants were used for fever, 12 plants were used for asthma and constipation, 12 plants were used for sexual debility, 10 plants were used for diabetics and jaundices, 9 plants were used for tonic and 7 plants were used for skin diseases, rheumatism and ulcer or stomachache. In most cases decoction of root, leaves or bark, sometimes total parts (for small plants) were used as herbal remedy.

The study is of great importance to preserve the knowledge of medicinal plants used by the local people of Natore Oushodi Gram and exploit the knowledge in treatment of various diseases. Moreover, Natore Oushodi Gram presents an unique pieces of model for both *in situ* and *ex situ* conservation of medicinal plants in the country. A large number of local herbal doctors, farmers, nursery growers, traders and hawker have been engaged in different activities generated around the area by virtue of commercial cultivation of medicinal plants considering it highly remunerative over the other conventional agricultural crops.

CHAPTER III

3. *IN VITRO* CONSERVATION OF SOME SELECTED MEDICINAL PLANT SPECIES BY TISSUE CULTURE

3.1. INTRODUCTION

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions (Thorpe T, 2007) often to produce the clones of plants. The resultant clones are true-to-type of the selected genotype. The controlled conditions provide the culture an environment conducive for their growth and multiplication. These conditions include proper supply of nutrients, pH medium, adequate temperature and proper gaseous and liquid environment.

Plant tissue culture technology is being widely used for large scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have, in recent years, become of major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites. Small pieces of tissue (named explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand of plants in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis (Akin-Idowu *et al*. 2009). Endangered, threatened and rare species have successfully been grown and conserved by micropropagation because of high coefficient of multiplication and small demands on number of initial plants and space.

In addition, plant tissue culture is considered to be the most efficient technology for crop improvement by the production of somaclonal and gametoclonal variants. The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well adapted high yielding genotypes with better disease resistance and stress tolerance capacities (Brown and Thorpe, 1995). In addition, certain type of callus cultures give rise to clones that have inheritable characteristics different from those of parent plants due to the possibility of occurrence of somaclonal variability (George, 1993), which leads to the development of commercially important improved varieties. Commercial production of plants through micropropagation techniques has several advantages over the traditional methods of propagation through seed, cutting, grafting and air layering. It is rapid propagation process that can lead to the production of virus free plants (Gercia-

Gonzales *et al.* 2010). Recent advances in the techniques and applications of plant cell culture and plant molecular biology have created unprecedented opportunities for the genetic manipulation of plants. The potential impact of these novel and powerful biotechnology on the genetic improvement of medicinal plants has generated considerable interest, enthusiasm and optimism in the scientific community and is in part responsible for the rapid expansion of biotechnology industry over the world.

3.1.1. Medicinal Plants and Tissue culture

Plant tissue culture refers to growing and multiplication of cells, tissues and organs of plants on defined solid or liquid media under aseptic and controlled environment. Plant tissue culture on the other hand has given a new dimension in multiplication, conservation and isolation of secondary metabolites in these research perspectives in medicinal plants. A good number of research works have been reported in tissue culture, cell culture and organ culture of medicinal plant. Plant regeneration through somatic embryogenesis from stem, petiole and leaf explants of Indian chicory (*Cichorium intybus* L) has been achieved (Abdin and Ilah, 2007; Rehman *et al*. 2003). The commercial technology is primarily based on micropropagation, in which rapid proliferation is achieved from tiny stem cuttings, shoot tip and meristem tip culture, nodal or axillary buds, and to a limited extent from somatic embryos, cell clumps in suspension cultures and bioreactors. Plant was regenerated from excised nodes from juvenile shoot apices of *Paederia foetida* (Alam *et al*. 2010). An efficient method was developed for high frequency shoot regeneration and plant establishment from nodal explants of *Rauwolfia serpentina* (Alatar *et al*. 2012), The plant has been successfully regenerated from callus and shoot explants (shoot tips and nodes) of *Asparagus recemosus* (Pant and Joshi, 2009). Direct *in vitro* regeneration of explants without passing through callus phase, in order to keep integrity of the genome, offers a mean for conservation of vegetatively propagated plant species through tissue culture. Different *in vitro* culture protocols have been used for: *Z. officinale* (Bhagyalakshmi and Singh, 1988; Rout *et al*. 2000); *C. longa* (Salvi *et al*. 2002) and *C. amada* (Prakash *et al*. 2004; Barthakur and Bordoloi, 1992). Direct multiple shoot induction was achieved in excised nodal explants of *Plumbago indica* (Biswas, 2006), Direct multiple shoot induction in excised nodal and shoot tip explants of *Aristolochia indica* (Siddique *et al.* 2006). Mint is valued for its multipurpose uses in the field of pharmaceuticals, cosmetics as well as for flavoring foods, beverages and tobacco (Ohloff, 1994). Clonal propagation of *Mentha arvensis* L. Through nodal explant was

reported by Chishti *et al*. (2006). Complete plants have already been regenerated from callus culture, excised anthers, isolated protoplast of many medicinal plants. Many of the regenerated plants showed somaclonal variation and selections were made for high alkaloid content cell lines. In many cases, accumulation of active compounds was found to be in embryo tissues and embryo culture systems provide "good harvest" of the compounds. Plant suspension culture is the most profitable system for secondary metabolite synthesis but the technique yields lower quantities of medicinally important alkaloids than the whole plant grown in the field.

Huge quantities of plant materials and extracts are imported for the manufacture of Ayurvedic, Unani and Homeopathic medicines in Bangladesh. Making health care and medical facilities available to the people is now a major concern of a large number of countries (Chopra *et al.* 1958; Suffness and Douros, 1982). Due to the toxic and adverse reactions of synthetic and chemical medicines being observed round the global herbal medicine has made a comeback to improving the fulfillment of our present and future health needs. For further research into the biochemical compositions and potential medicinal values of this plants, an efficient *in vitro* regeneration system for the production of plants is required because field grown plants may be subject to seasonal and somatic variations, infestations of bacteria, fungi and insects as well as environmental pollutions that can affect the medicinal value of the harvested tissues (Geng *et al.* 2001; Tyler *et al.* 1981). In recent years screening of plants for biological activities has resulted in the development of therapeutics used in the treatment of various diseases like cancer, AIDS and others. In order to conserve the natural flora and meet the increasing demand for plant based drugs certain alternative methods have become more important. The biotechnological approach such as tissue cultures initiated from medicinal plants is a vital method for the production of therapeutic compounds. *In vitro* propagation technology makes it possible to produce a large number of disease free and uniform plants of medicinally important species. Such plants can be used for the extraction of medicinally important compounds or for pharmacological studies. In addition, *in vitro* propagation methods offer powerful tools for germplasm conservation and the mass multiplication of threatened plant species (Murch et *al*. 2000a; Pakrashi and Shaha, 1978).

In Bangladesh Natore Oushodi Gram has appear as the burning show piece in commercial cultivation and *in situ* conservation of medicinal plants in the country holding a great promise in exploring the pharmacological values of medicinal plants

in the country. Under the present investigation, effort has been made to collect the ethnobotanical information for 120 medicinal plants traditionally cultivated over the area both in homestead, fallow lands and cultivated land. Under the biological study of these plants a number of bottle necks and limitations have been identified which are presenting hindrance in their massive propagation over the area. Among these plants some species have been identified as experiencing propagation barrier, facing profound hindrance in mass propagation. It was necessary to develop the tissue culture protocols for all of these identified species having propagation barrier but considering volume of work and time limitation, seven medicinal plants (*Asparagus racemosus, Curcuma amada, Ipomoea mauritiana, Paederia foetida, Mentha arvensis, Rauwolfia serpentina and Smilax zeylanica*) were selected for tissue culture study under the present investigation.

3.1.2. A brief description of seven important medicinal plants selected for *in vitro* propagation

3.1.2.1. *Asparagus racemosus* Wild

Asparagus racemosus Wild locally known as "sotomuli" belongs to the family Asparagaceae (formerly known as Liliaceae). It is an under shrub climber with extensively branched woody stems, growing upto 2 m in height. The succulent tuberous roots are 30-100 cm long and 1-2 cm thick in bunch attached at the stem base. The leaves are reduced to small scales or needle like spines called cladodes. The flowers are small, white, fragrant and in simple or branched racemes. Its fruits are globular or obscurely 3-lobed, pulpy berries, that are purplish black when ripe. This plant can be found growing naturally in the tropical and sub tropical forest. In Bangladesh it grows wild in forests and is planted in gardens. It is widely used for multiple purposes and its medicinal importance has been recognized by Ayurveda for centuries. *Asparagus racemosus* is considered to be of medicinal importance because of the presence of steroidal saponins and sapogenins in various parts of the plant (Hayes *et al.* 2006; Oketch and Rabah, 1998). All parts such as leaves, flowers, fruits, tubers, roots and bark of this species contains diosgenin, glycosides, sterols and their glycosidese and are very important for the treatment of diarrhoea, dysentery, diabetes, jaundice and other urinary disorders (Ghani, 1998).

Traditionally the roots are used mainly to promote milk secretion and as a demulcent, diuretic, tonic, alterative, antiseptic, antidiarrheal and antispasmodic. It is also used to treat debility, especially in woman and infertility, impotence, menopause, stomach ulcers, hyperacidity, dehydration, delay ageing process and form health food ingredients in several Ayurvedic formulations (Hayes *et al*. 2006).

Asparagus racemosus is usually propagated by planting the separated tuberous roots along with shoot apex. Since roots are the organs used for medicinal purpose there has been a practice of using seeds for plant propagation; but there are few technical problems involved in seed derived multiplication and seed germination is very low. Seed propagated plants are slow grower and existence of heterozygosity in seed germinated plants. These conditions collectively create a hindrance in its massive propagation and it ultimately experiencing a propagation trouble and these needs an alternative method of propagation like micropropagation.

Asparagus racemosus with Fruits Roots

Fig 3.1 *Asparagus racemosus*

3.1.2.2. *Curcuma amada*

Common bengali name is amada and in english it is called mango ginger. It is a perennial, rhizomatous aromatic herb belonging to the family Zingiberaceae. The genus originated in the Indo Malayan region, and is widely distributed in the tropic of Asia to Africa and Australia (Sasikumer, 2005). The plant grows to a height of 1 m. The leaves are long, oblong, lanceolate, radical, sheathed, petiolate and in tufts. Each plant bears 5 to 6 pairs of leaves. Mango zinger rhizomes are fleshy, buff coloured, 5-10 cm long, and 2-5 cm in diameter and demarcated into nodes and internodes. At the rhizomes nodes scaly leaves are arranged circularly giving the appearance of growth rings with scars on the surface. The rhizomes are branched, and the branching is sympodial. The rhizomes emulate a raw mango flavour and taste pungent. The mango zinger rhizome was found to be a rich source of fibers and starch (Lakshminarayana *et al*. 1963). Rhizome, being a storehouse of bioactive compounds, has extensive use. Mango ginger is used medicinally as a coolant, aromatic and astringent and to promote digestion. A rhizome paste has traditionally been used for healing of wounds, cuts and itching (Srivastava *et al.* 2006). The rhizome has combinative properties, as well as being useful as a stomachic (Hussain *et al.* 1992). Very few reports are available on the aerial parts of the plant. However , a whole plant paste with crushed long peppers (*Piper longum*) is reported to be effective for the treatment of piles and a decoction of the rhizome with common salt is an effective treatment for colds and coughs and is used to improve blood quality (Kapoor, 1990).

Mango ginger is propagated vegetatively using rhizome pieces. However, the planting material has a very low multiplication rate. In addition, lack of seed set in this species

hinders breeding efforts by conventional techniques. Moreover, many plants are susceptible to soft rot disease (Balachandran *et al*. 1990). Hence, huge amounts of planting material are required every year for plantation purposes (Barthakur and Bordoloi, 1992). The inconveniences encountered in its normal propagation process creating problems for its commercial propagation which ultimately has limited its cultivation over the area. The plant holds the merit for extensive cultivation but due to prevailing bottle necks, the cultivators could not find available propagules during its propagation seasons. Hence, tissue culture can present an alternative source for providing plantlets to the farmers for its extensive plantation.

Plant Rhizome TS of rhizome

Fig. 3.2 *Curcuma amada*

3.1.2.3. *Ipomoea mauritiana* Jacq

Common bengali name is “bhuikumra” and in english it is called the giant potato. It belongs to the family Convolvulaceae. It has a large glabrous twinner with tuberous root. Leaves are 10-15 cm long, deeply palmately divided into 7-9 segments, each segments ovate, lanceolate or oblong, acuminate. Flowers are few to many in axillary or terminal cymes. Corolla are funnel shaped, 5-6 cm long, light purple in colour. Capsule is ovoid, 8-13 mm in long. In Bangladesh it grows in forests of Chittagong, Chittagong Hill Tracts, and Sylhet. Now many people planted in their homestead and garden in Natore district Bangladesh. *Ipomoea mauritiana* ensured the presence of Alkaloids, Tannins, Steroids, Gums, Glycosides, Carbohydrate and Saponins (Monjur *et al*. 2013). The plant has extensive medicinal uses. It is used in skin diseases, in the treatment of anorexia, fever, inflammation and burning sensation (Mishra and Datta, 1962). Tuber contains β-sitosterol and glycoside- paniculatin (Asolkar *et al*. 1992). Tubers are used for the treatment of sexual disabilities in Khagrachari (Yusuf *et al*.

2009). Ether soluble fraction of the plant is hypotensive and muscle relaxant (Asolkar *et al*. 1992). It is also used to promote breast milk production (Martin *et al*. 1969).
The climbing plant produces fruits and seeds but during growing season seed are mostly failed to germinate. So it is very hard to propagate the plant by seeds. On the other hand, in practical approaches the plant is generally propagated by tuber which usually develop the emerging shoot. Also in vegetative propagation the plant faces acute difficulty in providing available propagules like germinating tubers as they usually remains in a very few numbers. *In vitro* propagation by tissue culture is invariably needed for the plants to be widely propagated among the interested farmers.

Plant with flower Tuber

Fig 3.3 *Ipomoea mauritiana*

3.1.2.4. *Mentha arvensis* L

Common bengali name is pudina and in english it is called mint or corn mint. It belongs to the family Lamiaceae. It is a herbaceous perennial plant growing to 10–60 cm (rarely to 100 cm) tall. The leaves are in opposite pairs, simple, 2–6.5 cm long and 1–2 cm broad, hairy and with a coarsely serrated margin. The flowers are funnel shaped with 4 spreading lobes, white to light purple or pink, 4-7 mm long, numerous in compact, separate whorls, borne in the axils of the middle and upper leaves. Calyx hairy, 2.5-3 mm long, with short, triangular, pointed lobes. Flowering times: July-September. The Fruits contain 4 small, egg shaped nutlets (Blamey and Wilson, 1989). Plants have a minty aroma. It is native to the temperate regions of Europe and Western and Central Asia, east to the Himalaya and eastern Siberia and North America. The leaves of wild mint are edible, raw or cooked. Having a quite strong

minty flavor with a slight bitterness, they are used as a flavoring in salads, soups or cooked foods. A herb tea can be made from the fresh or dried leaves. The leaves contain about 0.2% essential oil. Wild mint is often used as a domestic herbal remedy, The whole plant is anesthetic, antispasmodic, antiseptic, aromatic and has agents that counteract inflammation, that relieve and remove gas from the digestive system, induce sweating, promote or assist the flow of menstrual fluid, promote secretion of milk, relieve fever and thirst, give strength and tone to the stomach and is a stimulant. The plant is propagated mainly by shoot cuttings, sucker and scanty seed. Seed setting is very poor, but they are mostly sterile. The sucker cannot be stored for more than a few days because they deteriorate rapidly owing to heat or dehydration. In dry places and during summer season, the propagation of this plant is very difficult. Thus the plant facing in its extensive multiplication and cultivation problem and experiencing an acute propagation barrier demanding an alternative method like its micropropagation.

Whole plant Flower

Fig. 3.4 *Mentha arvensis*

3.1.2.5. *Paederia foetida* L

The common bengli name is “Gandhavadali” and in english it is called Chinese flower. It belongs to the family Rubiaceae. *P. foetida* is a climbing twining shrub emitting a bad smell. The plant is indigenous to the Indian subcontinent and it grows wild in different forests and village groves of Bangladesh. It is an important gregarious medicinal plant. The leaves are rich in carotene and vitamin C; also contain a high amount of keto alcohol, keto compound and alkaloid. The major classes of chemical constituents present in this plant are iridoid glycosides, sitosterol, stigmasterol, alkaloids, carbohydrates, proteins, amino acids and volatile oils (Blatter

et al. 1981; Nandkoni, 2002; Steinmetz, 1961; Khare, 2007). This species is reported to have ethnomedicinal uses both in Bangladesh (Hannan *et al.* 2008; Reza *et al.* 2008) and India (Hynniewta and Kumar, 2008). It is traditionally used for stomach ailments by Garo (Mia *et al.* 2009) and Santal tribes (Hanif *et al.* 2008) in Bangladesh. The various plant parts are utilized in traditional medicine of Bangladesh in different ways. Leaf juice is astringent and given to children for treatment of diarrhea; poultice of leaves are used to relieve distention due to flatulence, in herpes infections and during retention of urine; decoction of leaves are used to dissolve vesical calculi and acts as diuretic; fruit is specific against toothache (Ghani, 1998). It is also reported to be used in gout, diarrhea, dysentery, piles, inflammation of the liver and as an emetic (Blatter *et al.* 1981; Nandkoni, 2002). Different pharmacological reports exist for this species which includes anti inflammatory effect (Srivastava *et al.* 1973; De *et al.* 1994), relief in gastrointestinal disorder by helminthic infections (Roychoudhury *et al.* 1970); and antidiarrheal effects (Afroz *et al.* 2006). Fresh leaves reportedly have antioxidant properties (Osman *et al.* 2009).

P. foetida can be propagated from seeds and cutting, seeds mainly dispersed by bird and mostly are damaged. Fruits persist through winter and are shiny brown and are typically 0.7 cm wide. Insides are two seeds that are black. It is mainly propagated by cutting and the cuttings are easily perishable and cannot be stored for long time. Seeds are dispersed by the bird through eating ripe fruits, the plant also experienced propagation barrier and tissue culture would be an alternatives means for its propagation.

Whole plant Stem with leaf

Fig. 3.5 *Paederia foetida*

3.1.2.6. *Rauwolfia serpentina* Benth

Common name in bengali “sarpagondha” and in english it is called Indian snakeroot, It belongs to the family Apocynaceae. Sarpagandha is an erect, evergreen shrub and merely 15 to 45 cm high. Its leaves are large, leaves in whorls of 3, thin, lanceolate, acute, bright green above and pale beneath, flowers in irregular corymbose cymes, white, often tinged with violet and flowering time March to May. Its fruit are tiny, oval, fleshy which turn a shiny purple black when ripe. Chemical constituents the major alkaloid present in root, stem and leaves of the plant is reserpine varies from 1.7 to 3.0 %. The root barks has more than 90% of the total alkaloids. The minor alkaloids present in the plant are Ajmalicine, ajmaline, isoajmaline, ajmalinine, rauwolfinine, reserpiline, reserpinine, sarpagine, serpentine and yohimbine (Sidduqui and Siddiqui, 1931). *Rauwolfia* is native to India also found in Sri Lanka, Burma, Bangladesh and Thailand. The roots of the plant that are mainly used for medicinal purposes. *Rauwolfia* constituent ajmaline not only lowers blood pressure, but also has a potent anti arrhythmic effect which used to treat high blood pressure (hypertension). *Rauwolfia* work by controlling nerve impulses along with certain nerve pathways. As a result, they act on the heart and blood vessels to lower blood pressure. Also root is a valuable remedy for dysentery and painful affections of bowel.

The propagation of *R. serpentina* through seeds is difficult due to less viability and very low germination percentage. Propagation by direct sowing of seeds in the field has not been found successful. Sun dried and stored seeds generally gave a low rate of germination and seeds store more than 7 - 8 months practically did not germinate. The germination percentage of seed is very poor and variable (25% - 50%) and is often as low as 10 percent that is due to the presence of cinnamic acid derivatives (Mitra, 1976). Thus the plant facing in its extensive multiplication and cultivation problem and experiencing an acute propagation barrier demanding an alternative method like its micropropagation.

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Plant Flower with immature fruit

Fig 3. 6 *Rauwollfia serpentina*

3.1.2.7. *Smilax zeylanica* Linn

Smilax zeylanica L. is a large woody climber grows wild in Chittagong and sporadically in other areas throughout the country (Ghani, 2003). They also grow over trees and other plants up to 10 m high, their hooked thorns allowing them to hang into and scramble over branches. The plant belongs to the family Smilacaceae. In English it is called Indian smilax or Sarsaparilla but in bengali it is commonly known as 'Kumarilata'. The leaves are heart shaped and vary from 4–30 cm in long. Plants flower in May and June with white/green clustered flowers. The fruit stays intact through winter, It grows best in moist woodlands with a soil pH between 5 and 6. The seeds have the greatest chance of germinating after being exposed to a freeze. The plant contains 1–3% steroidal saponins, phytosterols, starch, resin, sarsapic acid and minerals. Leaves and root contain diosgenin (Kar and Sen, 1984). The common people of Bangladesh, especially the tribals use the roots and rhizomes of *S. zeylanica* as tonic. Sarsaparilla root has been used for centuries by the indigenous peoples of Central and South America for sexual impotence, rheumatism, skin ailments and as a general tonic for physical weakness. European physicians considered sarsaparilla root a tonic, blood purifier, diuretic and sweat promoter.

The conventional propagation method of this plant is growing stem cutting, but the stem cutting very rarely produce any root, tissue culture may be an alternatives sources of its propagation.

Whole plant Green fruits Ripe fruits

Fig 3.7 *Smilax zeylanica*

3.1.3. Aims and objectives

Medicinal plants play a vital role in the health care of local people of Natore Oushodi Gram. The indigenous people in this area possess the knowledge of traditional healing system using medicinal plants since time immemorial and till now it is one of the main way to treat different ailments in the local people. Due to human interference the wild populations of medicinal plants are declining rapidly. Many of them have become rare and some are facing extinction**.** In these consequences, the local herbal doctors and farmers of these villages are traditionally cultivated medicinal plants both in their homesteads and agricultural fields for commercial purpose. The results of the survey revealed that these medicinal plants were found important for the local kobiraj/herbal doctors. It is therefore considered important to develop mechanism for mass scale propagation of such plant species to meet up the local demand as well as their conservation through their reestablishment. Adoption of *in vitro* technique may be a practically feasible answer to this programme. With this background the seven important medicinal plants of Natore Oushodi Gram were selected for developing tissue culture protocol for their micropropagation.

Therefore, the present investigation was undertaken with a view to develop *in vitro* mass propagation techniques for seven important medicinal plant species of Natore Oushodi Gram namly *A.racemosus, C. amada, I. mauritiana, M. arvensis, P. foetida, R. serpentina* and *S. zeylanica* with the following objectives:

i) Identification and selection of suitable explants from the plants experienced with propagation barrier for their rapid and large scale propagation.
ii) Selection and standardization of appropriate media composition, growth regulator requirement and culture environment for regular high production of plantlets.
iii) Acclimatization and transplantation of *in vitro* grown plants into the soil for raising under natural condition.

3.2. MATERIALS AND METHODS

3.2.1. Materials

To conduct the present investigation following materials and equipments were used.

3.2.1.1. Plant materials

Different types of plant propagule (seedling, seeds, rhizome and rootstock) were collected from natural habitats during survey and planted in the experimental field of the Institute of Biological Sciences, Rajshahi University. The seven different medicinal plants (*Asparagus racemosus, Curcuma amada, Ipomoea mauritiana, Paederia foetida, Mentha arvensis, Rauwolfia serpentina and Smilax zeylanica*) were used as experimental materials in this investigation. Young shoots were used as explant source. Immature leaf, shoot tip, nodal and intermodal explants of two month old twigs were used as explants for direct and indirect organogenesis.

3.2.1.2. Nutrition Media

All the cultures were grown on MS (Murashige and Skoog, 1962) media for the purpose of multiple shoot induction, callus induction, shoot regeneration from callus and root initiation.

3.2.1.3. Plant growth regulators

The basal media were supplemented with various concentrations of different plant growth regulators. The following plant growth regulators were employed for the present investigation.

Auxins:

2,4-dichlorophenoxy acetic acid (2,4-D)

α-naphthalene acetic acid (NAA)

Indole-3-butyric acid (IBA)

Indole-3-acetic acid (IAA)

Cytokinins:

6-benzylaminopurine (BAP)

Kinetin (6-furfuryl aminopurine) (Kn)

Additives:

L-glutamine, GA_3, Charcol

3.2.1.4. Laboratory equipments

For media preparation: Different types of glass vessels including culture bottles, test tube, conical flask with plugs, beakers, petridishes of various capacities measuring cylinders, pipette pump, parafilm, aluminium foils, marker pen, hot plate, magnetic stirrer, analytical loading single pan balance with precision of ± 0.001g, refrigerator, electric hot air oven range up to 250 ± 20C, digital pH meter, autoclave preferably horizontal, continuous of single and double distilled water.

Chemicals: Inorganic and organic salts, vitamins, amino acids, growth regulators/hormones, sucrose and agar or gelrite etc.

Instrument for aseptic transfer: Autoclave**,** laminar airflow cabinets, scissors and scalpel handles with blades, forceps of various size and spirit lamp, 95% and 70% alcohol.

For incubation: Racks with light arrangements (16h light and 8h dark) and controlled temperature 20º ± 2C maintained with air cooler.

3.2.1.5. Sterilizing and cleaning agents

In the present investigation mercuric chloride ($HgCl_2$) and ethanol (95% and 70%) were used as sterilizing agents. Tween- 80 and Savlon were also used as cleaning agents.

3.2.2. Methods

3.2.2.1. Preparation of culture media

In order to prepare different media stock solutions of salts, organic compounds and growth regulators were first prepared taking different combinations of chemicals.

3.2.2.2. Preparation of stock solution

Different constituents of the culture media formulations were prepared into stock solutions as macronutrient, micronutrient, organic components and growth regulators separately for ready use during the preparation of culture media.

a. Stock solution of macronutrients (Soln. A)

The stock solution of macro salts was prepared at 20X that of the required concentration. Required amount of all the macro salt components prescribes for a particular medium formulation was weighed accurately with electronic balance and dissolved separately in substantial volume of double autoclave distilled water

(DDW).The solutions were sequentially poured into a 1 litre volumetric flask. Final volume of the solution was made into 1 litre by adding sufficient amount of DDW. Special care was taken during dissolving calcium chloride ($CaCl_2$).The solution after filtering through Whatman No. 1 filter paper, was poured into clean plastic bottole and stored into refrigerator at 4ºC.

b. Stock solution of micronutrients

Two separate stock solutions of macro salts were prepared as follows:

(i) Stock solution of FeSO4 and Na-EDTA (Soln. B)

This solution was prepared at 20X to that of required concentration. Requisite amount of FeSO4 and Na-EDTA were taken and dissolved separately into clean glass beakers containing 225 ml of DDW. Na-EDTA solution was then transferred in a 500 ml volumetric flask. Subsequently the solution of FeSO4 was pored to the volumetric flask slowly with constant stirring. The final volume of the solution was made up to 500 ml. The pH of the solution was adjusted to 5.7 and after filtering it was stored at 4ºC in refrigerator.

(ii) Stock solution of rest of the micronutrients (Soln. C)

Stock solution of micronutrients was made at 20 and 200X in 500ml DDW. All components were weighed (except $CaCl_2$) separately and dissolved in 400ml of DDW. $CaCl_2$ was dissolved separately and added to the solution. Finally , the volume of the solution was adjusted up to 500 and after filtering was stored at 4ºC in a plastic bottle.

c. Stock solution of organic components (Soln. D)

Organic components were made into stock solution separately. Ten times of each of the require ingredients were taken in a measuring cylinder and dissolved in 100 ml of distilled water. Then the stock solution was labeled and stored in refrigerator at 4ºC for several weeks.

d. Stock solution of plant growth regulators

Stock solution of different phytohormones was prepared separately. For preparation of plant growth regulators (PGR) stock solution 10 mg of PGR were taken in clean 100 ml reagent bottle and then dissolved in required volume of appropriate solvent. The final volume of the solution was then made to 10 ml adding DDW. The solution was then stored in a refrigerator at 4ºC for two or three weeks. The stock solutions of all the growth regulators were made in the same way.

3.2.2.3. Sterilent solution

$HgCl_2$ solution at various concentrations generally 0.1% was used for surface sterilization of plant materials. To prepare 0.1% solution, 0.1 g of $HgCl_2$ was taken in a 0.5 litre bottle and dissolved in 100 ml DW. Freshly prepared $HgCl_2$ was always used. Generally $HgCl_2$ solution was prepared 1 hour before use.

3.2.2.4. Preparation of culture medium

To prepare 1 litre of any of the above mentioned culture medium the following steps were involved:

i) 30 g of sucrose was dissolved in 500 ml of distilled water and filtered in a 1000 ml volumetric flask.

ii) 100 ml stock solution of macronutrients, 10 ml from each of the micronutrients stock solutions and 10 ml from each vitamin stock solutions were added to the sucrose solution and mixed them well.

iii) Different concentration of hormonal supplements were added either singly or in different concentrations to the solution were mixed thoroughly. Since each hormonal stock solution contained 10 mg of the chemical in 10 ml of stock solution, the addition of 1 ml of any hormonal stock solution to prepare 1 litre of medium resulted in 1.0 mg/ml concentration of the particular hormone

iv) The whole mixture was then made up to 1 liter with further addition of distilled water.

v) The pH of all the medium was adjusted to 5.7 ± 0.1 using a pH meter with the help of 0.1N NaOH or 0.1N HCl whichever necessary.

vi) The required quantities of agar were added to the medium and the whole mixture was then gently heated in a microwave oven till complete dissolution of agar.

vii) 10 to 20 ml of hot medium was taken into culture vessels namely test tubes, culture bottle, conical flask. The culture vessels were plugged with non absorbent cotton or with plastics caps, which were inserted tightly at the mouth of the culture vessels. and marked with different codes with the help of a glass marker pen to indicate specific hormonal supplements.

viii) The culture vessels with medium were then autoclaved at 120°C for 20 minutes at 1.1 kg/cm2 pressure. After sterilization the medium was allowed to cool and stored in the culture room (not more than a week) for ready use.

3.2.2.5. Preparation of surface sterilizing solution

In this experiment, mercuric chloride ($HgCl_2$) solution [0.1% (w/v)] was used as surface sterilizing agent. To prepare 200 ml of 0.1% of mercuric chloride, 0.2 mg of mercuric chloride were taken in a 500 ml conical flask and dissolved in 200 ml sterile distilled water and mixed well. Generally the solution was made 15 to 20 minutes before use.

3.2.2.6. Culture techniques

The following techniques were employed in the present investigation for induction and maintenance of callus induction, shoot induction, root induction as well as regeneration of complete plantlets:

a) Surface sterilization of plant materials
b) Preparation of explants and inoculation
c) Incubation
d) Subculture

a) Surface sterilization of plant materials

Juvenile twigs were collected and thoroughly washed under running tap water. The materials were then separated into short pieces (shoot tips, nodal leaves and internodal segments); surface sterilized with 1% savlon few drops 80% tween for 5-10 minutes with constant shacking. The materials were then washed 3-4 times with distilled water for complete removal of detergent and taken under running laminar airflow cabinet and transferred 500 ml sterilized conical flask. After rinsing with 70% ethanol for less than 60 seconds, they were immersed in 0.1% $HgCl_2$ for different duration of time. To remove every trace of the sterilant, the plant materials were washed 3-4 times with sterile distilled water.

b) Preparation of explants and inoculation:

Explants were laid on the sterile petridish using sterile forceps. During this action hands were made sterile as far as possible with absolute alcohol to avoid contamination.

i) Shoot tip: Shoot tips of approximately 1 to 2 cm in length were cut from the sterilized shoot of mature plants and then inoculated into the culture bottle/test tubes having different concentrations and combinations of hormones.

ii) Nodal segments: Following same way explants with 1-2 nodes were cut from the sterilized shoots and were inoculated into culture bottle/test tubes.

iii) Internodal segments: In the same way mentioned above internodal segments approximately 1 cm in length were cut from the shoots and were inoculated into culture bottle/test tubes.

iv) Leaves: In the same way mentioned above tender leaves were cut and placed into the culture bottle/test tubes.

c) Incubation

The culture tubes containing inoculation were incubated to light in the growth chamber. The growth chamber maintained at 16 hrs photoperiod with a light intensity of 2000-3000 lux (50-70µ $E.m^{-2}S^{-1}$) provided by 40W cool-white florescent tubes. The temperature of the incubation chamber was 22 ± 2°C but humidity was not controlled for any of the experiments.

d) Subculture techniques

The following *in vitro* culture techniques were employed during the course of culturing of different explants.

i) **Subculture maintenance**: Proliferated multiple shoots were rescued very carefully in the aseptic conditions and divided into clusters of 2-3 shoots using a sterile sharp scalpel. Then they were transferred to same or different media for further response.

ii) **Root induction**: Proliferated shoots of 2-4 cm in length were rescued aseptically from the culture vessels and cultured on freshly prepared medium containing different combinations of hormonal supplements for root induction.

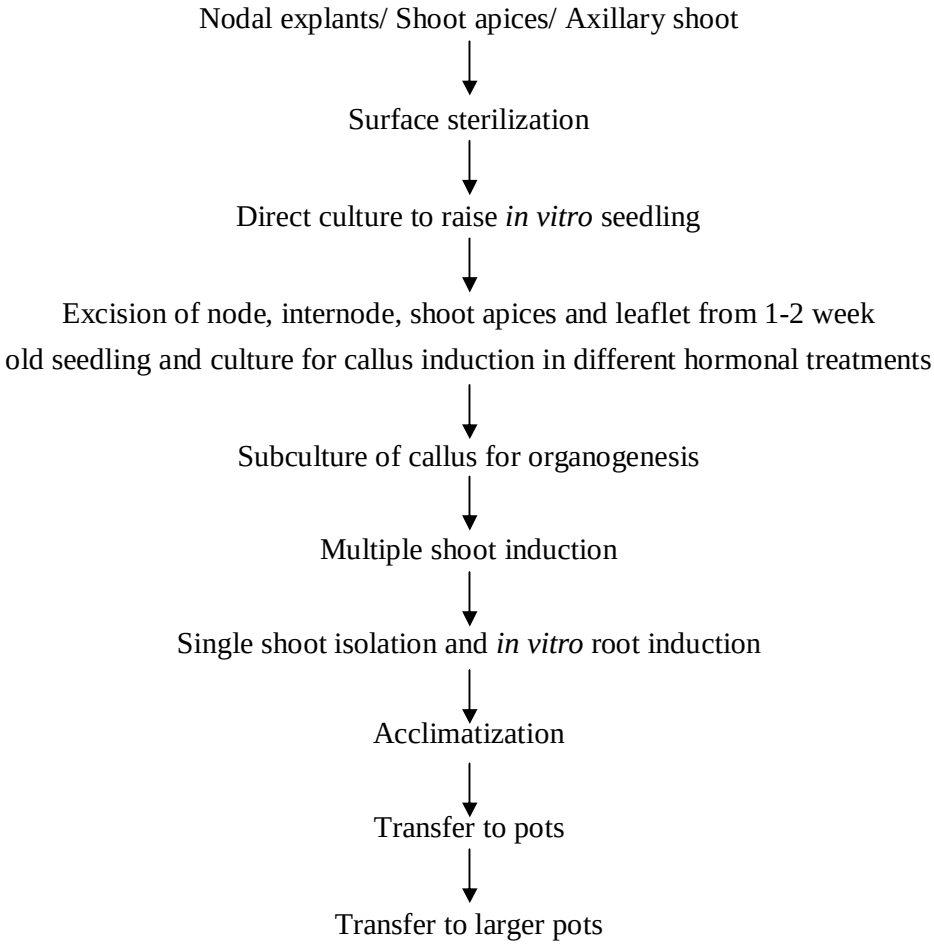
iii) **Callus induction**: The *in vitro* grown shoots of 3-5 cm in length were rescued aseptically from the culture vessels and internodes as well leaves were cultured on freshly prepared medium containing different concentrations and combinations of hormonal supplements for callus induction.

3.2.2.7. Transplantation

After sufficient growth of shoot and root systems, the plantlets were considered ready to transfer in soil. The plantlets grown inside the test tubes/flasks were brought out of the control environment of growth chamber and were kept in the room temperature from 5-7 days to bring them in contact of normal temperature. The plantlets were then

rescued very carefully from the culture vessels. Agar attached to the root system was gently washed out under running tap water. Then the plantlets were transplanted to small polythene bags containing garden soil and compost in the ratios of 2:1. The soil substance was treated with 0.1% Agrosan (fungicide) solution. Immediately after transplantation, the plantlets along with the polythene bags were covered with a large moist polythene bag to prevent desiccation. To obtain higher humidity around the plantlets, all the bags were checked up and the interior of the polythene bags were sprayed with water at every 24 hours. The polythene bags were gradually perforated to expose the plantlets to the outer environment and subsequently removed after 10 days. By this time new leaves emerged out and the regenerants became established in the soil being complete plantlets. They were then transferred to garden soil.

3.2.2.8. Flow chart for direct and indirect plant regeneration:



3.2.2.9. Computation and presentation of data

3.2.2.9.1. Data collection

Data were collected using the following parameters and the methods followed for data collection are given below:

i) Average number of shoots and length of the longest shoot: Number of multiple shoots per explant was counted after 4-6 weeks of inoculation. Average number of shoots per explant were calculated using the following formula.

$$X = \frac{\sum Xi}{N}$$

Where, X = Average number of shoots

$\sum$ = Summation

Xi = Total number of shoots

N = Number of observations

ii) Shoot height was recorded separately with meter scale after 4-6 weeks of inoculation:

Average height of the longest shoot were calculated by the following formula,

$$\bar{X} = \frac{\sum Xi}{N}$$

Where, $\bar{X}$ = Average length of the longest shoots

$\sum X$ = Total length of the longest shoots

N = Number of observations

iii) Average number of roots per shoot and length of the longest shoot: Number of main roots were counted after 4-6 weeks of inoculation. Average number of roots per explant was calculated using the following formula.

$$X = \frac{\sum Xi}{N}$$

Where, X = Average number of main roots

X = Total number of main roots

N = Number of observations

Average length of the longest root was calculated by the formula used in the shoot length calculation.

3.2.2.9.2. Data recorded

Data were recorded on callus induction and plant regeneration frequency (%), development of no. of shoots per calli and length of the longest regenerated shoot (cm). Callus induction and plant regeneration frequency (%) were calculated using formula below:

$$\textbf{Callus induction frequency (\%)} = \frac{\text{No. of explants responsed to callus initiation}}{\text{No. of explants cultured}} \times 100$$

$$\textbf{Plant regeneration frequency (\%)} = \frac{\text{No. of callus induction shoot}}{\text{No. of callus culture}} \times 100$$

Rate of callus forming explants: Cultured explants which showed callus formation were recorded after 35-42 days of inoculation. The degree of callus formation was recorded as follows :

- \- = No callus
- \+ = Trace callus development (1-30%)
- ++ = Moderate callus development (31-50%)
- +++ = Massive callus formation (51-100%)

3.2.2.9.3. Standard Error (SE):

Standard Error was calculated according to following formula:

$$\text{Standard Deviation (SD)} = \sqrt{\frac{\sum x^2 - (\sum x)^2/n}{n-1}}$$

$$\text{Standard Error (SE)} = \frac{SD}{\sqrt{n}}$$

3.2.2.9.4. Statistical analysis

Experiments were consisted of 5-15 explants and each of the experiment repeated thrice and mean values were calculated separately for each replication**.**

3.3. RESULTS

3.3.1. *In vitro* regeneration and conservation of *Asparagus racemosus*

Experiments on direct and indirect organogenesis were carried out using different types of explants viz. shoot apex, node, internode, and leaf from two months old field grown plants. Surface sterilization was done separately according to the explant types. In case of nodal and internodal segments effective surface sterilization was performed with 0.1% $HgCl_2$ for 5-6 min. On the other hand, shoot apex and leaf explant took 3-4 min for contamination free cultures in the same concentrations of $HgCl_2$. Sterilized explants were then cut into appropriate size (1.0-2.0 cm) and cultured on plant growth regulators (PGR) supplemented media for induction of direct and indirect organogenesis. Both auxins (NAA, IBA and 2,4-D) and cytokinins (BAP and Kn) were used in the media either single or in combinations. For root induction, micro shoots obtained from direct and indirect organogenesis were transferred to natural conditions through successive phages of acclimatization. The results of *in vitro* culture of direct and indirect organogenesis are described below:

3.3.1.1. Direct shoot regeneration in *A. racemosus*

Two types of explants *viz.* shoot apex and nodal explants were cultured for direct shoot regeneration. Explants were cultured on MS medium with BAP and Kn used alone and in combinations with each other or with NAA. Data on number of days taken for shoot initiation, percentage of explant induced shoot development, average number of shoot per culture and average length of shoot per culture were collected after 6 weeks of culture. Shoot apex and nodal explants responded on almost all of the supplemented cultured medium. The results are described according to types of explants under separate heads:

3.3.1.1.1. Direct multiple shoot induction in nodal explants of *A. racemosus*

Nodal explants of two months old *A. rarcemosus* were aseptically cultured on different concentrations (0.2-3.0 mg/l) of BAP and Kn either alone or in combinations with different concentrations (0.2-2 mg/l) of NAA. Nodal explants found to be most suitable for multiple shoot induction on almost all of the supplemented culture media, but morphogenic responses of the cultured explants varied depending upon the culture media formulation. Effects of different concentrations and combinations of auxins and cytokinins on multiple shoot induction from nodal explants are described below:

Experiment. 1 Effect of BAP and Kn singly and in combination on multiple shoot induction

In this present investigation five concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and Kn used alone and in combination of three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP with four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of Kn were treated in MS medium for the purpose of multiple shoot induction from nodal explants of *A. racemosus*. Data were taken after 6 weeks of inoculation and percentage of shoot formation, number of shoot/culture and length of shoot/culture were measured. The results are presented in Table 3.1. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Shoot proliferation ranged from 20.00-67.00%. Highest percentage (67.00%) of multiple shoot formation was observed in MS medium containing 2.0 mg/l BAP + 0.5 mg/l Kn followed by 62.00% in MS medium containing 1.0 mg/l BAP + 0.5 mg/l Kn. The lowest percentage (20.00) of multiple shoot formation was observed in media having 0.5 mg/l Kn. Highest mean number of shoots was 8.00 ± 0.05 in media having 1.0 mg/l BAP + 0.5 mg/l Kn followed by 7.20 ± 0.11 in MS medium containing 1.0 mg/l BAP + 1.0 mg/l Kn and 2.0 mg/l BAP + 0.5 mg/l Kn. Lowest mean number of shoot was 2.50 ± 0.15 in media containing 0.5 mg/l Kn. Average length of shoots gradually increased after induction of shoot. Highest average length was recorded 6.60 ± 0.17 cm in 1.0 mg/l BAP + 0.5 mg/l Kn followed by 6.30 ± 0.17 cm in 0.5 mg/l BAP + 0.5 mg/l Kn and 1.0 mg/l BAP + 1.0 mg/l Kn. The lowest average length was 4.00 ± 0.11 cm in 3.0 mg/l BAP and 0.5 mg/l Kn. Experimental results revealed that, 1.0 mg/l of BAP and 2.0 mg/l Kn alone and combination of 1.0 mg/l BAP + 0.5 mg/l Kn, 2.0 mg/l BAP + 0.5 mg/l Kn were found most effective concentrations for multiple shoot induction in *A. racemosus*. Experiment were set for the induction of multiple shoot but sometimes callus was found to grow at the basal part of the growing shoots in many media formulations.

Experiment. 2 Effect of different concentrations and combinations of BAP and Kn with NAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentrations of BAP (1.0, 2.0 and 3.0 mg/l) and Kn (1.0, 2.0 and 3.0 mg/l) combined with different concentrations of NAA (0.2, 0.5, 1.0 and 2.0 mg/l). Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.2. The efficiency of BAP + NAA was better than BAP alone and Kn + NAA combination on direct shoot

regeneration. All the used media compositions formed multiple shoots and the results were superior to the results obtained from shoot apex explants. Addition of lower concentration of NAA along with higher concentration of BAP was found more suitable than that of other concentrations. Among the combinations of BAP + NAA the highest percentage (82%) of shoot proliferation was noted in MS medium containing 2.0 mg/l BAP + 0.5 mg/l NAA and followed by (73%) MS medium containing 2.0 mg/l BAP + 1.0 mg/l NAA. The lowest percentage (44%) of shoot proliferation was noted in 3.0 mg/l BAP + 2.0 mg/l NAA. Highest mean number of shoot per culture was 18.00±0.28 in media having 3.0 mg/l BAP + 1.0 mg/l NAA followed by 12.00 ± 0.34 in media having 2.0 mg/l BAP + 0.5 mg/l NAA. The lowest mean number of shoot per culture was 6.50 ± 0.28 in media having 1.0 mg/l BAP + 2.0 mg/l NAA. Highest average length of shoot per culture was 8.00 ± 0.23 cm in the media having 2.0 mg/l BAP + 0.5 mg/l NAA and the lowest length of shoot per culture was 4.50 ± 0.28 cm in the media containing 1.0 mg/l BAP + 2.0 mg/l NAA (Plate 3.1).

On the other hand the efficiency of Kn + NAA was better than Kn alone but inferior than that of BAP + NAA. Among the combinations of Kn + NAA the highest percentage (73%) of shoot proliferation was noted in the media having 2.0 mg/l Kn + 0.5 mg/l NAA. The lowest percentage (40%) of shoot proliferation was noted in 3.0 mg/l Kn + 2.0 mg/l NAA. highest mean number of shoot per culture was 12.00 ± 0.34 in media having 2.0 mg/l Kn + 0.5 mg/l NAA followed by 11.60 ± 0.10 in media having 1.0 mg/l Kn + 2.0 mg/l NAA and 2.0 mg/l Kn + 1.0 mg/l NAA. The lowest mean number of shoot per culture was 6.00 ± 0.17 in media having 3.0 mg/l Kn + 0.2 mg/l NAA. Highest average length of shoot per culture was 7.93 ± 0.29 cm in the media having 2.0 mg/l Kn + 0.5 mg/l NAA and the lowest length of shoot per culture was 4.00 ± 0.20 cm in the media containing 3.0 mg/l Kn + 2.0 mg/l NAA (Plate 3.1**)**. Experimental results revealed that, combination of 2.0 mg/l BAP+ 0.5 mg/l NAA, 2.0 mg/l BAP+ 1.0 mg/l NAA, 3.0 mg/l BAP + 1.0 mg/l NAA and 2.0 mg/l Kn + 0.5 mg/l NAA were found most effective combinations for multiple shoot induction in *A. racemosus*. Experiments were set for the induction of multiple shoot but sometimes callus was found to grow at the basal part of the growing shoots in many media formulations.

Table 3.1 Effect of different concentrations of BAP and Kn singly and in combinations on multiple shoot induction from nodal explants of *A. racemosus*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Days taken for shoot initiation	% of shoot formation	*Average number of shoots / culture (mean ± SE)	*Average length (cm) of shoots / culture (mean ± SE)	Base callusing
BAP					
0.2	-	-	-	-	
0.5	12-15	30	3.00 ± 0.17	4.30 ± 0.11	-
1.0	12-15	63	4.00 ± 0.20	6.00 ± 0.11	+
2.0	12-15	54	6.00 ± 0.20	5.20 ± 0.11	+
3.0	12-15	42	4.00 ± 0.05	4.00 ± 0.11	+
Kn					
0.2	-	-	-	-	
0.5	12-15	20	2.50 ± 0.15	4.00 ± 0.17	-
1.0	12-15	40	4.00 ± 0.20	6.20 ± 0.20	+
2.0	12-15	52	5.30 ± 0.05	5.50 ± 0.11	+
3.0	12-15	36	3.00 ± 0.17	4.50 ± 0.17	-
BAP + Kn					
0.5 + 0.2	12-15	33	4.00 ± 0.05	5.00 ± 0.17	-
0.5 + 0.5	10-12	43	4.00 ± 0.17	6.30 ± 0.17	+
0.5 + 1.0	10-12	52	6.50 ± 0.20	6.10 ± 0.05	+
0.5 + 2.0	10-12	40	4.20 ± 0.11	5.50 ± 0.17	+
1.0 + 0.2	12-15	27	5.00 ± 0.20	5.27 ± 0.14	-
1.0 + 0.5	10-12	62	8.00 ± 0.05	6.60 ± 0.17	+
1.0 + 1.0	10-12	55	7.20 ± 0.11	6.30 ± 0.17	+
1.0 + 2.0	10-12	47	5.30 ± 0.17	5.27 ± 0.14	+
2.0 + 0.2	10-12	30	4.50 ± 0.05	5.40 ± 0.23	+
2.0 + 0.5	10-12	67	7.20 ± 0.20	5.20 ± 0.11	+
2.0 + 1.0	10-12	56	6.00 ± 0.28	6.00 ± 0.11	+
2.0 + 2.0	10-12	50	4.00 ± 0.11	6.20 ± 0.11	-

–: Absent of base callus **+ :** Present of base callus

*Values are the mean of three replicates with 10 explants.

Table 3.2 Effect of different concentrations and combinations BAP and Kn with NAA on multiple shoot induction from nodal explants of A. *racemosus.*

Growth regulators (mg/l)	Days taken for shoot initiation	% of shoot formation	*Average number of shoots / culture (mean ± SE)	*Average length (cm) of shoots / culture (mean ± SE)	Base callusing
BAP + NAA					
1.0 + 0.2	10-12	52	7.93 ± 0.29	5.00 ± 0.17	-
1.0 + 0.5	10-12	70	10.00 ± 0.15	6.00 ± 0.17	+
1.0 + 1.0	10-12	66	10.10 ± 0.05	6.00 ± 0.11	+
1.0 + 2.0	10-12	54	6.50 ± 0.28	4.50 ± 0.28	+
2.0 + 0.2	10-12	60	7.00 ± 0.15	6.00 ± 0.11	-
2.0 + 0.5	8-10	82	12.00 ± 0.34	8.00 ± 0.23	+
2.0 + 1.0	8-10	73	10.00 ± 0.17	7.00 ± 0.11	+
2.0 + 2.0	10-12	52	8.00 ± 0.28	5.00 ± 0.15	+
3.0 + 0.2	10-12	56	10.00 ± 0.11	5.00 ± 0.17	-
3.0 + 0.5	8-10	67	11.60 ± 0.10	6.00 ± 0.23	+
3.0 + 1.0	10-12	56	18.00 ± 0.28	7.00 ± 0.15	+
3.0 + 2.0	10-12	44	12.00 ± 0.11	6.00 ± 0.11	-
Kn + NAA					
1.0 + 0.2	10-12	45	9.00 ± 0.11	6.00 ± 0.11	-
1.0 + 0.5	10-12	62	10.00 ± 0.11	6.00 ± 0.11	+
1.0 + 1.0	10-12	71	11.00 ± 0.11	7.00 ± 0.17	+
1.0 + 2.0	10-12	63	11.60 ± 0.10	5.00 ± 0.15	+
2.0 + 0.2	10-12	56	10.00 ± 0.11	6.00 ± 0.34	-
2.0 + 0.5	8-10	73	12.00 ± 0.23	7.93 ± 0.29	-
2.0 + 1.0	8-10	66	11.60 ± 0.10	7.00 ± 0.28	+
2.0 + 2.0	10-12	60	10.00 ± 0.26	6.00 ± 0.17	+
3.0 + 0.2	10-12	52	6.00 ± 0.17	6.00 ± 0.28	+
3.0 + 0.5	10-12	63	8.20 ± 0.10	6.00 ± 0.26	+
3.0 + 1.0	10-12	57	10.00 ± 0.23	7.00 ± 0.11	+
3.0 + 2.0	10-12	40	8.00 ± 0.30	4.00 ± 0.20	-

–: Absent of base callus + : Present of base callus

* Values are the mean of three replicates with 10 explants.

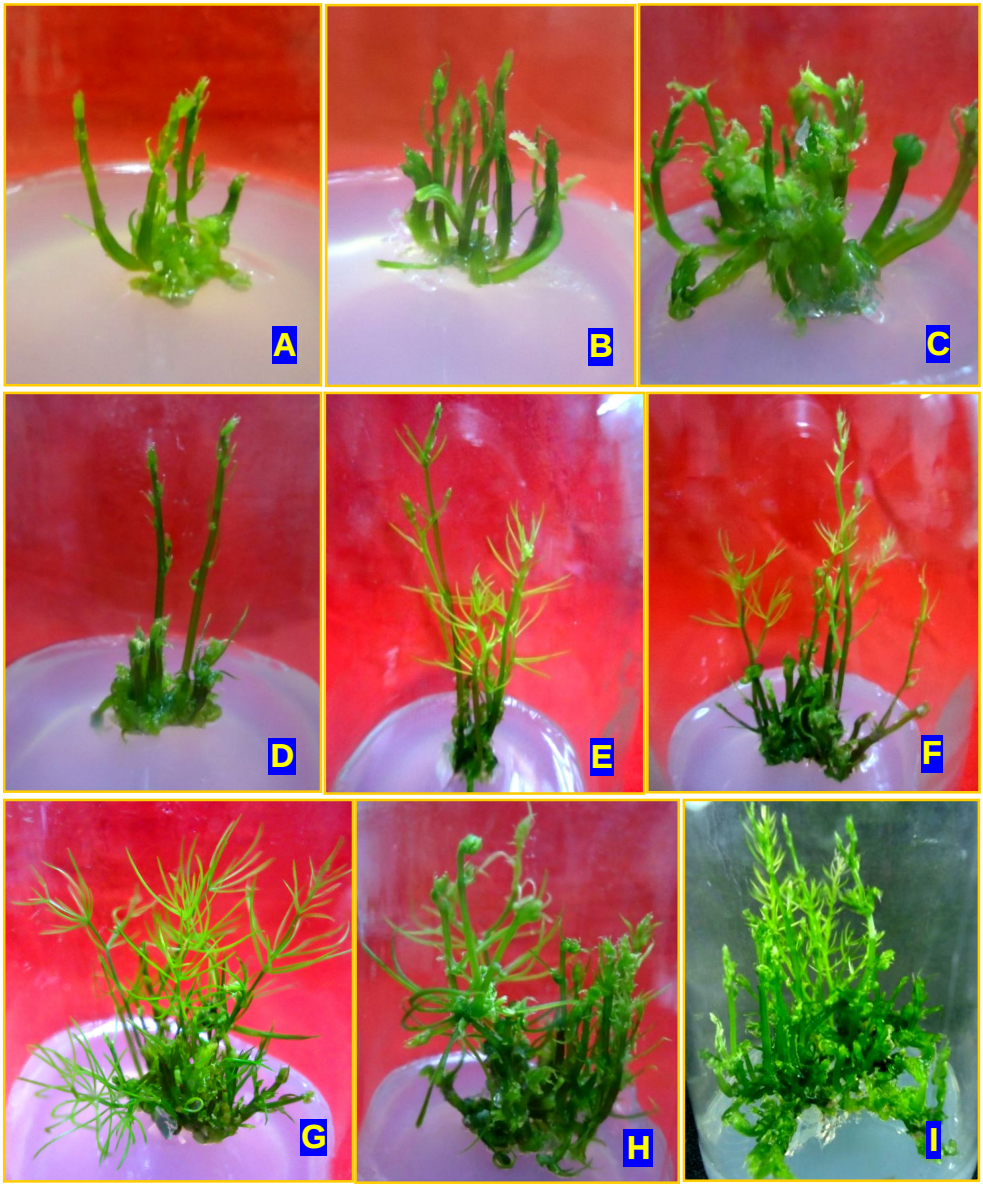


Plate 3.1 Development of multiple shoot buds from nodal explants of *A. racemosus* through the process of direct organogenesis with their subsequent development.

A-B: Initiation of multiple shoot bud formation in nodal explants after 3 weeks of inoculation in media having 2.0 mg/l BAP + 0.5 mg/l NAA; **C-F**: Proliferation of multiple shoot buds in the same medium after two subcultures at 14 days interval; **G-I**: Further proliferation and elongation of shoot buds after 8 weeks of culture in the same medium.

3.3.1.1.2. Direct multiple shoot induction in shoot apex explants of *A. racemosus*

Shoot apex explants were aseptically cultured on different concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and Kn either alone and in combination with each other or in combination with different concentrations (0.2, 0.5, 1.0 and 2 mg/l) of NAA. Shoot apex explants found to be suitable for multiple shoot induction on almost all of the supplemented culture media but morphogenic responses of the cultured explants varied depending upon the culture media formulation. Effects of different concentrations and combinations of auxins and cytokinins on multiple shoot induction from shoot apex explants are described below:

Experiment. 1 Effect of BAP and Kn singly and in combination on multiple shoot induction

In this present investigation five concentrations of BAP (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l), five concentrations of Kn (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) alone and in combination of three concentration of BAP (0.5, 1.0 and 2.0 mg/l) with four concentration of Kn (0.2, 0.5, 1.0 and 2.0 mg/l) were treated in MS medium for the purpose of multiple shoot induction from shoot apex explants of *A. racemosus*. Data were taken after 6 weeks of inoculation and percentage of shoot formation, number of shoot/culture, length of shoot/culture and base callusing were measured. The results are presented in Table 3.3**.** Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Shoot proliferation was not noticed in all media formulations. Shoot proliferation ranged from 24.00-62.00%. Highest percentage (62.00%) of multiple shoot formation was observed in MS medium containing 1.0 mg/l BAP + 1.0 mg/l Kn and 2.0 mg/l BAP + 1.0 mg/l Kn followed by (57.00%) in MS medium containing 1.0 mg/l BAP and 0.5 mg/l BAP + 1.0 mg/l Kn. The lowest percentage (24.00%) of multiple shoot formation was observed in media having 0.5 mg/l Kn. Highest mean number of shoots was 10.00 ± 0.25 in media having 1.0 mg/l BAP + 0.5 mg/l Kn and followed by 8.00 ± 0.32 in media having 1.0 mg/l BAP +1.0 mg/l Kn. The lowest mean number of shoot was 3.00 ± 0.15 in media containing 3.0 mg/l BAP. Average length of shoots gradually increased after induction of shoot. Highest average length was recorded 7.00 cm in 1.0 mg/l BAP + 0.2 mg/l Kn and followed by 6.50 ± 0.05 cm in 1.0 mg/l BAP + 0.5 mg/l Kn and 2.0 mg/l BAP + 0.5 mg/l Kn. The lowest average length was 4.00 ± 0.28 cm in 3.0 mg/l Kn. Experimental results revealed that, 1.0 mg/l of BAP and 2.0 mg/l Kn alone and combination of 1.0 mg/l BAP + 1.0 mg/l Kn and 2.0 mg/l BAP + 1.0 mg/l Kn were found most effective concentrations for multiple shoot induction.

Experiment were set for the induction of multiple shoot but sometimes callus was found to grow at the basal part of the growing shoots in many media formulations.

Experiment. 2 Effect of different concentrations and combinations of BAP and Kn with NAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentrations of BAP (0.5, 1.0 and 2.0 mg/l) and Kn (0.5, 1.0 and 2.0 mg/l) combined with different concentrations of NAA (0.2, 0.5, 1.0 and 2.0 mg/l). Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.4. The efficiency of BAP + NAA was better than BAP alone and Kn + NAA combination on direct shoot regeneration. All the used media compositions formed multiple shoots and the results were inferior to the results obtained from nodal explants. Addition of lower concentration of NAA along with higher concentration of BAP was found more suitable than that of other concentrations. Among the combinations of BAP + NAA the highest percentage (78%) of shoot proliferation was noted in the media having 2.0 mg/l BAP + 0.5 mg/l NAA and followed by (75%) in1.0 mg/l BAP + 0.5 mg/l NAA. The lowest percentage (52%) of shoot proliferation was noted in media having 0.5 mg/l BAP + 0.2 mg/l NAA. Highest mean number of shoot per culture was 16.00 ± 0.17 in media having 1.0 mg/l BAP + 1.0 mg/l NAA followed by 14.20 ± 0.11 in media having 1.0 mg/l BAP + 0.5 mg/l NAA .The lowest mean number of shoot per culture was 6.10 ± 0.11 in media having 2.0 mg/l BAP + 0.2 mg/l NAA. Highest average length of shoot per culture was 8.00 ± 0.11 cm in the media having 2.0 mg/l BAP + 0.5 mg/l NAA and the lowest length of shoot per culture was 4.00 ± 0.20 cm in the media containing 0.5 mg/l BAP + 2.0 mg/l NAA.

On the other hand the efficiency of Kn + NAA was better than Kn alone but inferior than the combined effect of that BAP + NAA. Among the combinations of Kn + NAA the highest percentage (72%) of shoot proliferation was noted in the media having 1.0 mg/l Kn + 0.5 mg/l NAA and 2.0 mg/l Kn + 0.5 mg/l NAA and the lowest percentage (48%) of shoot proliferation was noted in 0.5 mg/l Kn + 0.2 mg/l NAA. Highest mean number of shoot per culture was 12.00 ± 0.17 in media having 1.0 mg/l Kn + 2.0 mg/l NAA followed by 10.63 ± 0.20 in media having 0.5 mg/l Kn + 1.0 mg/l NAA. The lowest mean number of shoot per culture was 6.00 ± 0.36 in media having 0.5 mg/l Kn + 0.2 mg/l NAA and 2.0 mg/l Kn + 0.5 mg/l NAA. Highest average length of shoot per culture was 7.20 ± 0.11 cm in the media having 1.0 mg/l Kn + 0.5 mg/l NAA and the lowest length of shoot per culture was 5.00 ± 0.28 cm in the media containing 0.5 mg/l Kn + 2.0 mg/l NAA.

Table 3.3 Effect of different concentrations of BAP and Kn singly and in combinations on multiple shoot induction from shoot apex explants of *A. racemosus.* Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Days taken for shoot initiation	% of shoot formation	*Average number of shoots / culture (mean ± SE)	*Average length (cm) of shoots / culture (mean ± SE)	Base callusing
BAP					
0.2	-	-	-	-	-
0.5	12-15	36	5.50 ± 0.05	5.50 ± 0.17	-
1.0	12-15	57	6.30 ± 0.17	6.30 ± 0.17	+
2.0	12-15	45	6.00 ± 0.23	6.00 ± 0.11	+
3.0	12-15	32	3.00 ± 0.15	5.00 ± 0.17	+
Kn					
10.2	-	-	-	-	-
0.5	12-15	24	5.00 ± 0.28	5.30 ± 0.17	-
1.0	12-15	45	6.30 ± 0.17	5.60 ± 0.11	-
2.0	12-15	52	4.30 ± 0.17	6.10 ± 0.10	+
3.0	12-15	36	5.00 ± 0.17	4.00 ± 0.28	+
BAP + Kn					
0.5 + 0.2	12-15	36	4.00 ± 0.25	5.00 ± 0.23	-
0.5 + 0.5	10-12	52	4.00 ± 0.17	6.30 ± 0.17	-
0.5 + 1.0	10-12	57	6.30 ± 0.15	5.30 ± 0.17	+
0.5 + 2.0	10-12	40	4.00 ± 0.15	6.00 ± 0.17	+
1.0 + 0.2	12-15	40	4.00 ± 0.57	7.00 ± 0.11	+
1.0 + 0.5	10-12	47	10.00 ± 0.25	6.50 ± 0.05	+
1.0 + 1.0	10-12	62	8.00 ± 0.32	6.00 ± 0.25	+
1.0 + 2.0	10-12	52	4.30 ± 0.17	5.60 ± 0.17	+
2.0 + 0.2	10-12	45	5.00 ± 0.23	5.30 ± 0.17	+
2.0 + 0.5	10-12	55	6.00 ± 0.11	6.50 ± 0.05	+
2.0 + 1.0	10-12	62	6.50 ± 0.20	6.30 ± 0.11	+
2.0 + 2.0	10-12	52	4.30 ± 0.15	6.00 ± 0.17	+

–: Absent of base callus + : Present of base callus

* Values are the mean of three replicates with 10 explants.

Table 3.4 Effect of different concentrations and combinations of NAA with BA, and Kn on multiple shoot induction from shoot apex explants of *A. racemosus.*

Growth regulators (mg/l)	Days taken for shoot initiation	% of shoot formation	*Average number of shoots /culture (mean ± SE)	*Average length (cm) of shoots / culture (mean ± SE)	Base callusing
BAP + NAA					
0.5 + 0.2	10-12	52	8.00 ± 0.32	5.30 ± 0.17	-
0.5 + 0.5	10-12	64	10.00 ± 0.23	6.60 ± 0.11	-
0.5 + 1.0	10-12	72	12.00 ± 0.23	6.00 ± 0.45	+
0.5 + 2.0	10-12	61	10.00 ± 0.28	4.00 ± 0.20	+
1.0 + 0.2	10-12	56	10.00 ± 0.23	6.30 ± 0.15	+
1.0 + 0.5	10-12	75	14.20 ± 0.11	7.00 ± 0.17	+
1.0 + 1.0	10-12	66	16.00 ± 0.17	6.50 ± 0.11	+
1.0 + 2.0	10-12	60	12.00 ± 0.34	5.00 ± 0.20	+
2.0 + 0.2	10-12	62	6.10 ± 0.11	5.30 ± 0.17	+
2.0 + 0.5	10-12	78	10.00 ± 0.34	8.00 ± 0.11	+
2.0 + 1.0	12-15	72	14.00 ± 0.28	6.60 ± 0.11	+
2.0 + 2.0	12-15	55	10.00 ± 0.30	6.30 ± 0.17	+
Kn + NAA					
0.5 + 0.2	12-15	48	6.00 ± 0.36	6.20 ± 0.11	-
0.5 + 0.5	10-12	57	8.00 ± 0.32	6.60 ± 0.11	-
0.5 + 1.0	10-12	65	10.63 ± 0.20	7.00 ± 0.17	-
0.5 + 2.0	10-12	52	8.50 ± 0.23	5.00 ± 0.28	+
1.0 + 0.2	10-12	51	8.00 ± 0.28	6.00 ± 0.25	+
1.0 + 0.5	10-12	72	8.00 ± 0.34	7.20 ± 0.11	+
1.0 + 1.0	10-12	65	10.00 ± 0.35	7.30 ± 0.17	+
1.0 + 2.0	10-12	60	12.00 ± 0.17	6.60 ± 0.17	+
2.0 + 0.2	10-12	54	8.00 ± 0.20	6.17 ± 0.17	+
2.0 + 0.5	10-12	72	6.00 ± 0.50	6.60 ± 0.17	+
2.0 + 1.0	10-12	67	10.00 ± 0.23	7.30 ± 0.15	+
2.0 + 2.0	10-12	50	8.00 ± 0.17	6.00 ± 0.17	+

–: Absent of base callus + : Present of base callus

* Values are the mean of three replicates with 10 explants.

3.3.1.2. Induction of callus on different explants in *A. racemosus*

Experiment 1. Effect of different concentrations and combinations of phytohormones on callus induction and characteristics of callus derived from internode explants

In this present investigation internode explants excised from *in vitro* grown seedlings of *A. racemosus* were used to investigate the effect of different hormonal concentrations and combinations. Explants were cultured on to MS media supplemented with different concentrations (0.2, 0.5,1.0 and 2.0 mg/l) of 2,4-D and NAA alone and in combinations of BAP + 2,4-D, Kn + 2,4-D, BAP + NAA + 2,4-D and BAP + Kn + 2,4-D in different concentrations. Data were recorded after 6 weeks of culture. Cultures were maintained under 16 h light and 8 h dark regime. Results obtained on morphogenic response of the cultured explants are shown in Table 3.5. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was noticed in all media formulations. But there was a wide variation in morphological nature and percentage of callus formation among them. Callus initiation occurred within 10-15 days depending upon the concentration and combination of hormones. BAP + NAA + 2,4-D and BAP + Kn + 2,4-D induced callus within 10-12 days, BAP + 2,4-D and Kn + 2,4-D took also same days but NAA and 2,4-D alone took 12-15 days to initiate callus formation. Percentage of callus formation ranged from 10.00-95.00 %. Highest percentage 95.00% of callus formation occurred in MS medium containing 0.5 mg/l BAP + 1.0 mg/l Kn + 1.0 mg/l 2,4-D (Plate 3.2 E) followed by 85.00% in MS medium containing 0.5 mg/l BAP +1.0 mg/l NAA + 1 mg/l 2,4-D and 0.5 mg/l BAP + 1.0 mg/l Kn + 0.5 mg/l 2,4-D. The lowest percentage (10.00%) of callus formation was observed in media having 2.0 mg/l 2,4-D alone. In most cases colour of calli was whitish, green, light green , dark green and creamy and texture of calli were compact and friable.

Experiment. 2 Effect of different concentrations and combinations of phytohormones on callus induction and characteristics of callus derived from leaf explants

In this present investigation leaf explants excised from *in vitro* grown seedlings of *A. racemosus* were used to investigate the effect of different hormonal concentrations and combinations. Explants were cultured on to MS media supplemented with different concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of 2,4-D and NAA used alone and in combinations of BAP + 2,4-D, Kn + 2,4-D, BAP + NAA + 2,4-D and

BAP +Kn + 2,4-D in different concentrations. Data were recorded after 6 weeks of culture. Cultures were maintained under 16 h light and 8 h dark regime. Results obtained on morphogenic response of the cultured explants are shown in Table 3.5. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was noticed in all media formulations. But there was a wide variation in morphological nature and percentage of callus formation among them. Callus initiation occurred within 10-15 days but the duration varied depending upon the concentration and combination of hormones. BAP + NAA + 2,4-D and BAP + Kn + 2,4-D induced callus within 10-12 days, BAP + 2,4-D and Kn + 2,4-D took also same days but NAA and 2,4-D took 12-15 days to initiate callus formation. Percentage of callus formation ranged from 15.00-85.00%. Highest percentage (85.00%) of callus formation occurred in MS medium containing 0.5 mg/l BAP + 1.0 mg/l NAA+ 1.0 mg/l 2,4-D and that was followed by (80.00%) in MS medium containing 0.5 mg/l BAP + 0.5 mg/l NAA + 1 mg/l 2,4-D and 0.5 mg/l BAP +1.0 mg/l Kn+1.0 mg/l 2,4-D. The lowest percentage (15.00%) of callus formation was observed in media having 0.5 mg/l 2,4-D . In most cases colour of calli was whitish, green, light green , dark green and creamy and texture of calli were compact and friable.

Table 3.5 Effect of different concentrations and combinations of phytohormones on callus induction and characteristics of callus derived from leaf and internode explants of *A. racemosus*. Data were collected after 6 weeks of inoculation.

Hormonal combination (mg/l)	Leaf explant			Internode explant		
	Days of callus initiation	% of explant induced callus	*Callus colour and texture	Days of callus initiation	% of explant induced callus	*Callus colour and texture
2,4-D						
0.2	12-15	10	wf	12-15	15	wf
0.5	12-15	15	wf	12-15	20	wf
1.0	12-15	25	wf	12-15	25	wf
2.0	12-15	35	cf	12-15	10	cf
NAA						
0.2	12-15	15	cf	12-15	20	cc
0.5	12-15	20	cf	12-15	30	cc
1.0	12-15	35	cf	12-15	35	wc
2.0	12-15	25	cf	12-15	25	cc
BAP + 2,4-D						
0.5 + 0.5	10-12	25	lgf	10-12	25	lgf
0.5 + 1.0	10-12	45	lgf	10-12	60	lgf
0.5 + 2.0	10-12	40	lgf	10-12	40	lgf
1.0 + 0.5	10-12	40	lgf	10-12	40	lgf
1.0 + 1.0	10-12	50	gf	10-12	50	lgf
1.0 + 2.0	10-12	45	gf	10-12	45	lgf
Kn + 2,4-D						
0.5 + 0.5	10-12	20	lgf	10-12	30	lgf
0.5 + 1.0	10-12	40	lgf	10-12	50	lgf
0.5 + 2.0	10-12	35	lgf	10-12	40	lgf
1.0 + 0.5	10-12	30	lgf	10-12	35	gf
1.0 + 1.0	10-12	45	gf	10-12	45	dgf
1.0 + 2.0	10-12	35	gf	10-12	40	dgf
BAP + NAA + 2,4-D						
0.5 + 0.2 + 0.5	10-12	50	lgf	10-12	50	lgf
0.5 + 0.5 + 0.5	10-12	65	lgc	10-12	60	lgf
0.5 + 1.0 + 0.5	10-12	70	dgc	10-12	70	gf
0.5 + 2.0 + 0.5	10-12	60	dgc	10-12	65	gf
0.5 + 0.2 + 1.0	10-12	60	dgf	10-12	60	lgf
0.5 + 0.5 + 1.0	10-12	80	dgf	10-12	70	lgc
0.5 + 1.0 + 1.0	10-12	85	gf	10-12	85	lgc
0.5 + 2.0 + 1.0	10-12	65	lgf	10-12	70	lgc
BAP + Kn + 2,4-D						
0.5 + 0.2 + 0.5	10-12	34	lgf	10-12	50	gf
0.5 + 0.5 + 0.5	10-12	55	gf	10-12	65	gf
0.5 + 1.0 + 0.5	10-12	70	dgc	10-12	85	gf
0.5 + 2.0 + 0.5	10-12	60	dgc	10-12	70	gc
0.5 + 0.2 + 1.0	10-12	50	dgc	10-12	65	dgc
0.5 + 0.5 + 1.0	10-12	65	dgc	10-12	75	dgc
0.5 + 1.0 + 1.0	10-12	80	dgc	10-12	95	dgf
0.5 + 2.0 + 1.0	10-12	70	gc	10-12	70	gf

***wf**: white friable, **wc**: white compact, **cf**: creamy friable, **cc**: creamy compact, **lgf:** light green friable, **gf:** green friable, **dgf:** dark green friable, **gc:** green compact, **dgc**: dark green compact.

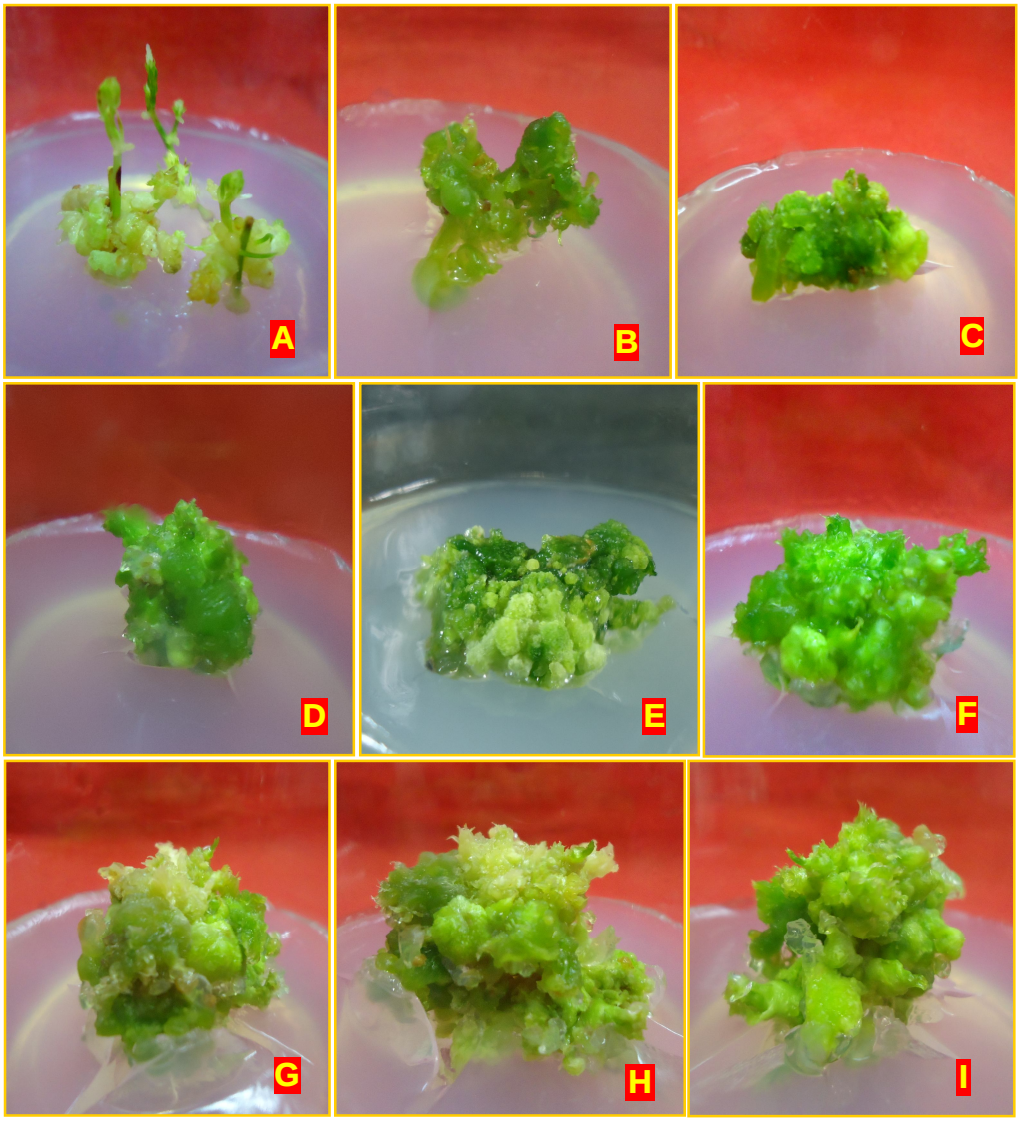


Plate 3.2 Callus induction and multiple shoot bud initiation from intermodal explants of *A. racemosus*.

A: Light green colour and friable callus induction from nodal explant in MS + 0.5 mg/l NAA + 2.0 mg/l 2,4-D from internodal explants after 4 weeks; **B-D**: Dark green colour and compact callus induction from internodal explant in MS + 0.5 mg/l BAP + 1.0 mg/l Kn + 1.0 mg/l 2,4-D after 6 weeks of culture; **E-F**: Greenish colour and compact callus induction from internodal explant in MS + 0.5 mg/l BAP + 1.0 mg/l NAA + 1.0 mg/l 2, 4-D after 6 weeks of culture; **G-I**: Further development and shoot bud initiation in the same medium after 8 weeks of culture.

3.3.1.3. Plant regeneration from different types of callus in *A. racemosus*

Regeneration of callus is more important than induction of callus and a number of experiments were undertaken with the aim of establish media combination for organogenesis of calli derived from two types of explants. A broad spectrum of cytokinin (BAP and Kin) and auxin (NAA) supplemented media were used to scrutinize the organogenic response. Among the different types of calli light green friable, light green compact and green compact found to be best regenerative calli on different plant growth regulator supplemented media. Leaf derived callus showed better performance than internode derived callus. Findings of the experiments are described below:

3.3.1.3.1. Plant regeneration from leaf derived callus

Different experiments were conducted to investigate plant regeneration ability through callus culture from leaf explants. Data on percentage of organogenic calli induced, average number of shoot per calli and average length of shoot per calli were collected after 8 weeks of culture.

Experiment. 1 Effect of different concentrations of BAP and Kn alone and in combinations with NAA on adventitious shoot regeneration from leaf derived callus of *A. racemosus*

In this experiment, leaf derived calli of *A. racemosus* were used to investigate the effect of different hormonal concentrations and combinations. For shoot differentiation, light green compact calli were subcultured on MS medium supplemented with five concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and Kn used alone and in combinations of two concentrations (1.0 and 2.0 mg/l) of BAP or Kn with four concentrations (0.1, 0.2, 0.5 and 1.0 mg/l) of NAA to scrutinize the organogenic response. Data were recorded after 6 weeks of culture and presented in Table 3.6. Among all the concentrations it was observed that 3.0 mg/l BAP, 0.2 mg/l Kn and 3.0 g/l Kn alone and in combination of 2.0 mg/l BAP + 1.0 mg/l NAA and 2.0 mg/l Kn + 1.0 mg/l NAA altogether failed to differentiate any shoots. The percentage of calli produced shoots was ranged from 10.00-85.00 %. Highest percentage 85.00 % of adventitious shoots regeneration occurred in MS medium containing 1.0 mg/l BAP + 0.2 mg/l NAA followed by 70.00% in MS medium containing 2.0 mg/l BAP + 0.2 mg/l NAA and 1.0 mg/l Kn + 0.2 mg/l NAA respectively. The lowest percentage (10.00%) of adventitious shoots regeneration was observed in media having 0.2 mg/l BAP. The highest number of adventitious shoot regeneration per callus was 12.00 ± 0.15 in media having 1.0 mg/l BAP + 0.2 mg/l NAA followed by 10.00 ± 0.41 in 1.0

mg/l BAP + 0.5 mg/l NAA. The lowest number of adventitious shoots regeneration per callus was 2.60 ± 0.11 in media having 0.2 mg/l BAP. Highest length of shoot 6.00 ± 0.11 cm was recorded in 1.0 mg/l BAP + 0.2 mg/l NAA and the lowest length of shoots 2.50 ± 0.05 cm was recorded in media only having 0.2 mg/l BAP.

3.3.1.3.2. Plant regeneration from internode derived callus

Different experiments were conducted to investigate plant regeneration ability through callus culture from internode explants. Data on percentage of organogenic calli induced, average number of shoot per calli and average length of shoot per calli were collected after 6 weeks of culture.

Experiment. 1 Effect of different concentrations of BAP and Kn singly and combinations with NAA on adventitious shoot regeneration from internode derived callus of *A. racemosus*

Under this experiment internode derived calli of *A. racemosus* were used to investigate the effect of different hormonal concentrations and combinations. For shoot differentiation, light green and dark green compact calli were subcultured on MS medium supplemented with five concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and Kn used alone and in combinations of two concentrations (1.0 and 2.0 mg/l) of BAP or Kn with four concentrations (0.1, 0.2, 0.5 and 1.0 mg/l) of NAA to scrutinize the organogenic response. Data were recorded after 6 weeks of culture and presented in Table 3.6. Among all the concentrations it was observed that 0.2 mg/l BAP, 0.2 mg/l Kn and 3.0 mg/l Kn alone and in combination of 2.0 mg/l BAP + 1.0 mg/l NAA and 2.0 mg/l Kn + 1.0 mg/l NAA altogether failed to differentiate any shoots. The percentage of calli produced shoot was ranged from 10.00-80.00%. Highest percentage (80.00%) of adventitious shoots regeneration occurred in MS medium containing 1.0 mg/l BAP + 0.2 mg/l NAA (plate 3.3) followed by (70.00%) in MS medium containing 1.0 mg/l Kn + 0.2 mg/l NAA. The lowest percentage (10.00%) of adventitious shoots regeneration was observed in media having 3.0 mg/l BAP. The highest number of adventitious shoots regeneration per callus was 10.00 ± 0.41 in media having 1.0 mg/l BAP + 0.5 mg/l NAA followed by 9.30 ± 0.17 in the media having1.0 mg/l BAP + 0.2 mg/l NAA and 1.0 mg/l Kn +0.2 mg/l NAA. The lowest number of adventitious shoots regeneration per callus was 3.50 ± 0.17 in media having 3.0 mg/l BAP. Highest length of shoot 6.00 ± 0.11 cm was recorded in 1.0 mg/l BAP + 0.5 mg/l NAA and the lowest length of shoots 3.00 ± 0.28 cm was recorded in 3.0 mg/l BAP and 2.0 mg/l Kn containing media.

Table 3.6 Effect of BAP and Kn alone and combinations with NAA on adventitious shoot regeneration from leaf and internode derived callus in *A. racemosus*. Data were collected after 6 weeks of inoculation.

Hormonal combination (mg/l)	Leaf derived callus			Internode derived callus		
	% of regenerable calli	*Average number of shoot/calli (mean ± SE)	*Average length (cm) of shoot /calli (mean ± SE)	% of regenerable calli	*Average number of shoot/calli (mean ± SE)	*Average length (cm) of shoot /calli (mean ± SE)
BAP						
0.2	10	2.60 ± 0.11	2.50 ± 0.05	-	-	-
0.5	50	4.50 ± 0.28	3.30 ± 0.17	25	6.00 ± 0.23	3.60 ± 0.15
1.0	60	6.00 ± 0.11	3.50 ± 0.17	40	8.00 ± 0.23	4.50 ± 0.28
2.0	30	5.30 ± 0.17	3.00 ± 0.20	20	4.60 ± 0.11	3.30 ± 0.15
3.0	-	-	-	10	3.50 ± 0.17	3.00 ± 0.28
Kn						
0.2	-	-	-	-	-	-
0.5	50	5.50 ± 0.28	4.00 ± 0.28	30	5.00 ± 0.28	4.00 ± 0.28
1.0	40	4.30 ± 0.17	2.50 ± 0.05	45	6.30 ± 0.11	4.60 ± 0.11
2.0	15	4.00 ± 0.28	3.00 ± 0.28	20	4.00 ± 0.28	3.00 ± 0.28
3.0	-	-	-	-	-	-
BAP + NAA						
1.0 + 0.1	60	6.00 ± 0.28	4.00 ± 0.30	55	6.50 ± 0.11	4.30 ± 0.20
1.0 + 0.2	85	12.00 ± 0.15	6.00 ± 0.11	80	9.30 ± 0.17	5.50 ± 0.25
1.0 + 0.5	65	10.00 ± 0.41	5.30 ± 0.17	60	10.00 ± 0.41	6.00 ± 0.11
1.0 + 1.0	45	5.50 ± 0.28	3.50 ± 0.25	35	4.00 ± 0.23	3.50 ± 0.11
2.0 + 0.1	40	8.30 ± 0.17	4.60 ± 0.17	40	8.00 ± 0.28	4.00 ± 0.23
2.0 + 0.2	70	7.30 ± 0.15	3.60 ± 0.17	60	7.30 ± 0.17	4.50 ± 0.20
2.0 + 0.5	40	5.00 ± 0.25	3.60 ± 0.11	30	5.60 ± 0.11	3.60 ± 0.11
2.0 + 1.0	-	-	-	-	-	-
Kn + NAA						
1.0 + 0.1	50	5.40 ± 0.20	3.50 ± 0.25	45	5.30 ± 0.15	3.50 ± 0.25
1.0 + 0.2	70	8.00 ± 0.23	5.30 ± 0.17	70	9.30 ± 0.17	5.30 ± 0.17
1.0 + 0.5	55	7.30 ± 0.17	4.30 ± 0.05	50	8.00 ± 0.11	4.00 ± 0.25
1.0 + 1.0	40	5.00 ± 0.25	4.00 ± 0.28	30	4.60 ± 0.11	3.30 ± 0.15
2.0 + 0.1	40	4.30 ± 0.05	3.30 ± 0.20	40	8.00 ± 0.28	4.00 ± 023
2.0 + 0.2	60	7.60 ± 0.17	5.00 ± 0.17	60	8.50 ± 0.05	4.50 ± 0.20
2.0 + 0.5	50	6.00 ± 0.26	3.00 ± 0.17	35	6.60 ± 0.11	3.60 ± 0.11
2.0 + 1.0	-	-	-	-	-	-

– : Failed to differentiate any shoots.

* Values are the mean of three replicates with 10 explants.

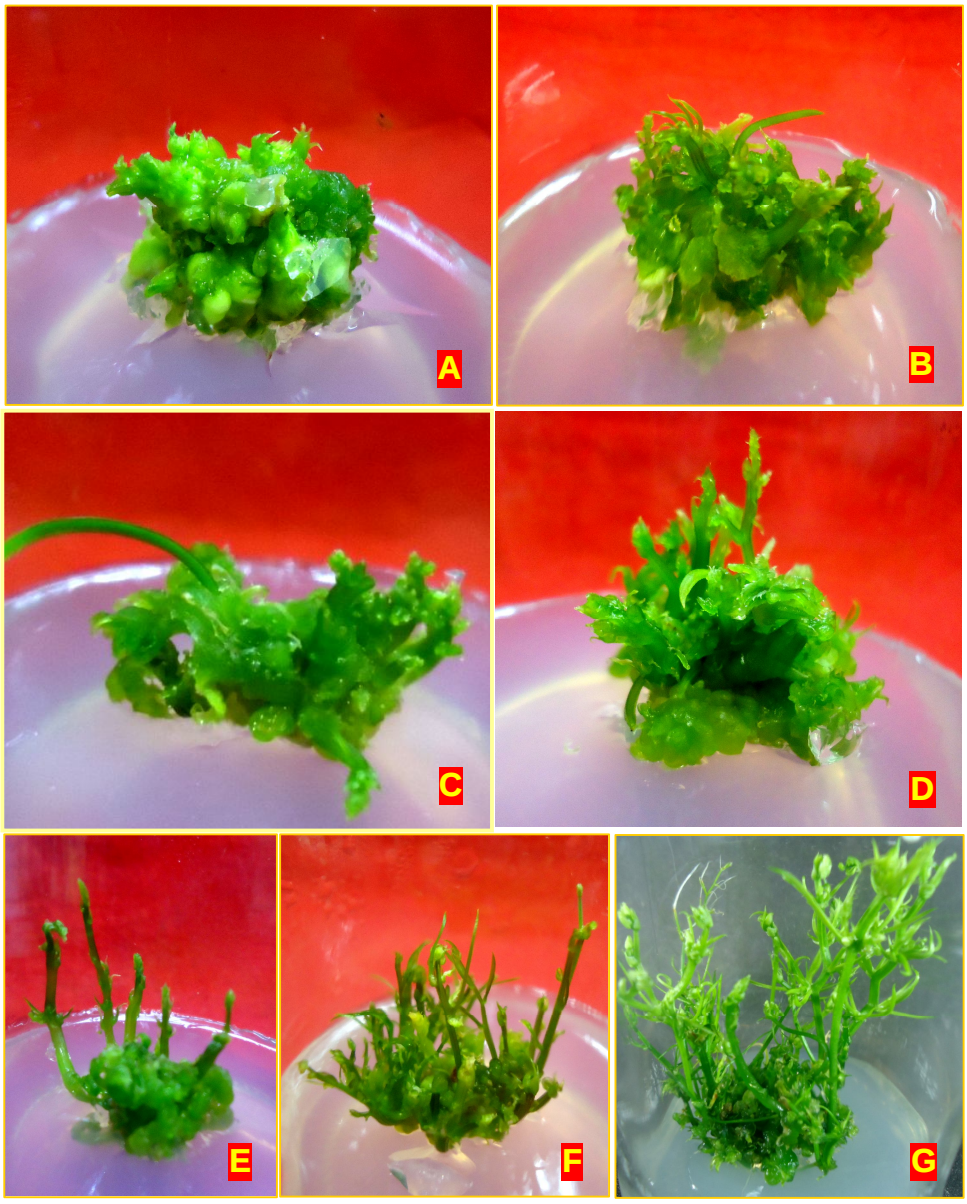


Plate 3.3 Callus regeneration and development of multiple shoot bud from nodal explants of *A. racemosus* through the process of indirect organogenesis and their subsequent development.

A: Differentiation of callus tissue into multiple shoot buds in MS + 1.0 mg/l BAP + 0.2 mg/l NAA after 3 weeks of subculture; **B-D**: Proliferation of multiple shoot buds in the same medium after 4-6 weeks of culture and **E-G**: Further proliferation and elongation of shoot buds in the same medium after 6-8 weeks of subculture.

3.3.1.4. Rooting of *in vitro* grown shoots in *A. racemosus*

Shoot cuttings 5-7 cm long *in vitro* grown shoots were separated and transferred to rooting media. Half strength MS medium fortified with different concentrations (0.1, 0.2, 0.5, 1.0 and 2.0 mg/l) of NAA alone and combinations of NAA (0.2 and 0.5 mg/l) with four concentrations (0.5, 1.0, 2.0 and 3.0 mg/l) of BAP or Kn were used for rooting experiment. Data on days to root initiation, percentage (%) of root formation, average number of root per culture and average length of root per culture were recorded after 8 weeks of culture. The results are described experiment wise:

Experiment 1. Effect of ½ strength MS medium with different concentrations of NAA alone and in combination with BAP or Kn on root induction from *in vitro* grown shoot

For adventitious root formation, the shoots obtained from *in vitro* grown shoot explants were excised and cultured on MS medium with different concentrations of NAA alone and combination with BAP and Kn. Results obtained for root induction, percentage of root formation, morphology, average number and length of roots are shown in Table 3.7. Percentage range of cultures produced roots varied from 8.00-30.00%. Highest 30.00% of root regeneration was recorded in 0.5 mg/l NAA + 2.0 mg/l Kn followed by 24.00 % in media having 0.5 mg/l NAA + 1.0 mg/l BAP. The lowest 8.00 % of root regeneration was recorded in media having 1.0 mg/l NAA. The highest average number of roots per shoot was recorded 38.00 ± 0.52 in media having 0.5 mg/l NAA + 2.0 mg/l Kn (Plate 3.4.E) followed by 20.00 ± 0.23 in combinations of 0.2 mg/l NAA + 2.0 mg/l Kn. The lowest average number of root per shoots was recorded 5.00 ± 0.23 in media having 1.0 mg/l NAA. Highest length of roots 8.00 ± 0.17 cm was recorded in 0.5 mg/l NAA + 2.0 mg/l Kn and the lowest length of roots 5.60 ± 0.15 cm was recorded in 1.0 mg/l NAA and 0.5 mg/l NAA + 3.0 mg/l Kn . In most cases morphology of roots was healthy, thick and long.

Table 3.7 Effect of ½ strength MS medium with different concentrations of NAA alone and in combination with BAP or Kn on root induction in *A. racemosus.*

Growth regulators (mg/l)	Days taken for root initiation	% of explant induced root development	*Average number of root per culture (mean ± SE)	*Average length (cm) of root per culture (mean ± SE)
NAA				
0.1	15-20	-	-	-
0.2	15-20	16	6.00 ± 0.11	6.30 ± 0.17
0.5	15-20	20	10.00 ± 0.30	6.60 ± 0.11
1.0	15-20	8	5.00 ± 0.23	5.60 ± 0.15
2.0	15-20	-	-	-
NAA + BAP				
0.2 + 0.5	12-15	10	6.00 ± 0.17	7.00 ± 0.17
0.2 + 1.0	12-15	15	8.00 ± 0.23	7.30 ± 0.17
0.2 + 2.0	-	-	-	-
0.2 + 3.0	-	-	-	-
0.5 + 0.5	12-15	12	10.00 ± 0.23	7.60 ± 0.17
0.5 + 1.0	12-15	24	16.00 ± 0.23	6.50 ± 0.11
0.5 + 2.0	12-15	10	6.00 ± 0.17	6.60 ± 0.11
0.5 + 3.0	-	-	-	-
NAA + Kn				
0.2 + 0.5	12-15	-	-	-
0.2 + 1.0	12-15	10	8.00 ± 0.23	6.60 ± 0.11
0.2 + 2.0	12-15	12	20.00 ± 0.23	6.00 ± 0.23
0.2 + 3.0	-	-	-	-
0.5 + 0.5	12-15	12	8.00 ± 0.23	6.30 ± 0.17
0.5 + 1.0	12-15	16	12.00 ± 0.34	7.00 ± 0.17
0.5 + 2.0	12-15	30	38.00 ± 0.52	8.00 ± 0.17
0.5 + 3.0	12-15	15	12.00 ± 0.34	5.60 ± 0.15

–: Failed to any initiation of root.

*Values are the mean of three replicates with 10 explants.

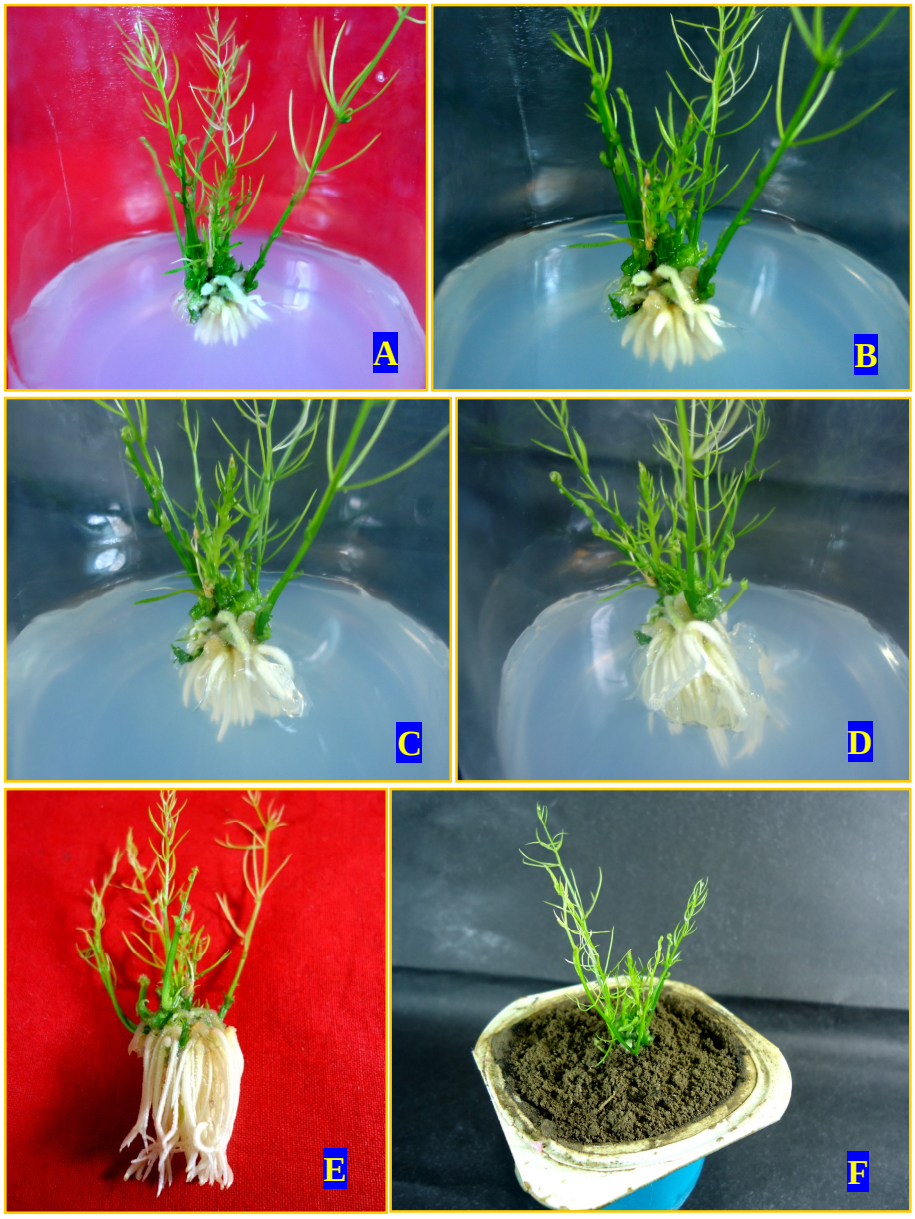


Plate 3.4 Induction of adventitious roots and establishment in soil of *A. racemosus.*

A-B: Induction of adventitious roots on shoots regenerated from nodal explants in half strength MS medium containing 0.5 mg/l NAA + 2.0 mg/l Kn after 6 weeks of culture; C-**D:** Further proliferation and elongation of adventitious root in the same medium after 8-10 weeks of culture; **E**: *A. racemosus* with adventitious roots outside of culture bottle; **F**: The *in vitro* grown seedlings acclimatized to grow in pots in outside environment after 4 weeks.

3.3.2. *In vitro* regeneration and conservation of *Curcuma amada*

Experiments on direct and indirect organogenesis were carried out using two types of explants *viz*. shoot tip and axillary bud in *C. amada*, shoot tip and axillary bud explants were cultured for direct shoot regeneration. Explants were cultured on the MS (Murashig and Skoog, 1962) agar gelled medium supplemented with different concentrations of auxins and cytokinins which were used singly or in combinations to investigate the initiation of shoot and its subsequent regeneration. For root induction, micro shoots obtained from direct and indirect organogenesis were transferred to rooting media. Finally well developed plants with both shoots and roots were transferred to natural conditions through successive phages of acclimatization. Details of the results so far obtained from each of the experiments are described under following heads:

3.3.2.1. Direct regeneration

Two types of explants *viz*. shoot tip and axillary bud explants were cultured for direct shoot regeneration. Explants were cultured on MS medium with BAP and Kn used alone and in combinations with each other or with NAA and IAA. Data on number of days taken for shoot initiation, percentage of explant induced shoot development, average number of shoot per culture and average length of shoot per culture were collected after 4-6 weeks of culture. Shoot tip and axillary bud explants responded on almost all of the supplemented cultured medium. The results are described according to types of explants under separate heads:

3.3.2.1.1. Shoot tip explants

Shoot tip explants were aseptically cultured on five concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and Kn either alone and in combinations with each other. Another experiment set in combinations of three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP or Kn with four concentrations (0.2, 0.5, 1.0 and 2 mg/l) of NAA or IAA. Shoot tip explants found to be suitable for multiple shoot induction on almost all of the supplemented culture media but morphogenic responses of the cultured explants varied depending upon the culture media formulation. Effects of different concentrations and combinations of auxins and cytokinins on multiple shoot induction from shoot tip explants are described below:

Experiment 1. Effect of different concentrations of BAP and Kn alone and combinations on multiple shoot induction from shoot tip explants of *C. amada*

Five concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and Kn alone and combinations of three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP with four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of Kn were treated in MS medium for the purpose of multiple shoot induction from shoot tip explants of *C. amada*. Data were taken after 6 weeks of inoculation and percentage of shoot formation, number of shoot/culture and length of shoot/culture were measured and the results are presented in Table 3.8. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Shoot proliferation ranged from 20.00-80.00%. Highest percentage (80.00%) of multiple shoot formation was observed in MS medium containing 1.0 mg/l BAP + 0.5 mg/l Kn followed by 75.00% in MS medium containing 0.5 mg/l BAP + 0.5 mg/l Kn and 1.0 mg/l BAP. The lowest percentage (20.00) of multiple shoot formation was observed in media having 3.0 mg/l Kn. Highest mean number of shoots was 6.00 ± 0.17 in media having 1.0 mg/l BAP + 0.5 mg/l Kn and lowest mean number of shoot was 1.0 ± 0.17 in media containing 0.2 mg/l Kn. Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 28 days of culture. Highest average length was recorded 7.50 ± 0.28 cm in 1.0 mg/l BAP + 1.0 mg/l Kn and the lowest average length was 4.50 ± 0.28 cm in 3.0 mg/l BAP and 3.0 mg/l Kn. Experimental results revealed that, 1.0 mg/l of BAP and 1.0 mg/l Kn alone and combination of 0.5 mg/l BAP + 0.5 mg/l Kn, 1.0 mg/l BAP+ 0.5 mg/l Kn, 1.0 mg/l BAP + 1.0 mg/l Kn were found most effective concentrations and combinations for multiple shoot induction in *C. amada*.

Experiment 2. Effect of different concentrations and combinations of BAP with NAA and IAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentrations of BAP (0.5, 1.0 and 2.0 mg/l) combined with different concentrations of NAA (0.2, 0.5, 1.0 and 2.0 mg/l) and IAA (0.2, 0.5, 1.0 and 2.0 mg/l). Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.9. The efficiency of BAP + NAA was better than BAP + IAA on direct shoot regeneration. All the used media compositions formed multiple shoots and the results were inferior to the results obtained from axillary bud explants. Among the combinations of BAP + NAA and BAP + IAA the highest percentage (86%) of shoot

proliferation was noted in the media having 0.5 mg/l BAP + 0.5 mg/l NAA followed by (80%) in 1.0 mg/l BAP + 0.5 mg/l NAA and 0.5 mg/l BAP + 1.0 mg/l IAA. Highest average number of shoot per culture 6.00 ± 0.17 was noted in the media having 0.5 mg/l BAP + 0.5 mg/l NAA followed by 5.00 ± 0.17 in the media having 0.5 mg/l BAP + 1.0 mg/l NAA and 2.0 mg/l BAP + 0.5 mg/l NAA. The lowest average number of shoot 1.00 ± 0.11 was noted in the media having 0.5 mg/l BAP+ 0.2 mg/l IAA. Highest average length of shoot per culture was 8.00 ± 0.28 cm in the media concentration of 1.0 mg/l BAP + 0.5 mg/l NAA and the lowest length per culture was 5.00 ± 0.11 cm in media having 2.0 mg/l BAP + 2.0 mg/l IAA. Experimental results revealed that, 0.5 mg/l BAP + 0.5 mg/l NAA, 1.0 mg/l BAP + 1.0 mg/l NAA, 1.0 mg/l BAP + 0.5 mg/l IAA and 0.5 mg/l BAP + 1.0mg/l IAA were more effective combinations for multiple shoot induction in *C. amada*.

Experiment 3. Effect of different concentrations and combinations of Kn with NAA and IAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentrations of Kn (0.5, 1.0 and 2.0 mg/l) combined with different concentrations of NAA (0.2, 0.5, 1.0 and 2.0 mg/l) and IAA (0.2, 0.5, 1.0 and 2.0 mg/l). Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.10. The efficiency of shoot induction in Kn + NAA was better than Kn + IAA. All the used media compositions formed multiple shoots. Among the combinations of Kn + NAA and Kn + IAA the highest percentage (80%) of shoot proliferation was noted in the media having 1.0 mg/l Kn + 0.5 mg/l NAA followed by 75% in 2.0 mg/l Kn + 0.5 mg/l NAA and 1.0 mg/l Kn+ 0.5 mg/l IAA. The lowest percentage (30%) of shoot proliferation was noted in the media having 2.0 mg/l Kn + 2.0 mg/l IAA. Highest average number of shoot per culture was 5.50 ± 0.11 in the media having 1.0 mg/l Kn + 0.5 mg/l NAA. The lowest number of shoot per culture was 1.50 ± 0.15 in the media having 0.5 mg/l Kn + 2.0 mg/l IAA and 2.0 mg/l Kn + 2.0 mg/l IAA. Average highest length of shoot 6.50 ± 0.28 cm per culture was found in the combinations of 1.0 mg/l Kn + 0.5 mg/l NAA and 1.0 mg/l Kn +1.0 mg/l IAA. The lowest average length of shoot was 4.50 ± 0.17 cm per culture in 2.0 mg/l Kn + 2.0 mg/l NAA. Experimental results revealed that, 1.0 mg/l Kn + 0.5 mg/l NAA, 2.0 mg/l Kn + 0.5 mg/l NAA, 1.0 mg/l Kn + 0.5 mg/l IAA and 0.5 mg/l Kn + 1.0 mg/l NAA were found most effective combinations for multiple shoot induction in *C. amada*.

Table 3.8 Effect of different concentrations of BAP and Kn singly and in combinations with each other on multiple shoot induction from shoot tip explants of *C. amada*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP				
0.2	7-8	50	2.00 ± 0.11	5.00 ± 0.17
0.5	7-8	60	3.00 ± 0.11	5.50 ± 0.28
1.0	6-7	75	5.00 ± 0.11	6.00 ± 0.17
2.0	6-7	65	4.00 ± 0.17	5.00 ± 0.28
3.0	7-8	40	2.00 ± 0.23	4.50 ± 0.28
Kn				
0.2	7-8	30	1.00 ± 0.17	5.00 ± 0.11
0.5	7-8	40	2.00 ± 0.17	6.00 ± 0.11
1.0	7-8	60	4.00 ± 0.30	7.00 ± 0.28
2.0	7-8	50	2.00 ± 0.17	6.00 ± 0.11
3.0	7-8	20	2.00 ± 0.23	4.50 ±0 .28
BAP + Kn				
0.5 + 0.2	7-8	50	2.00 ± 0.05	6.00 ± 0.28
0.5 + 0.5	7-8	75	3.00 ± 0.11	7.00 ± 0.17
0.5 + 1.0	7-8	60	4.00 ± 0.23	7.00 ± 0.17
0.5 + 2.0	7-8	45	2.00 ± 0.23	6.50 ± 0.28
1.0 + 0.2	6-7	60	3.00 ± 0.17	5.00 ± 0.28
1.0 + 0.5	6-7	80	6.00 ± 0.17	6.50 ± 0.28
1.0 + 1.0	6-7	70	4.00 ± 0.17	7.50 ± 0.28
1.0 + 2.0	6-7	50	2.00 ± 0.27	6.00 ± 0.28
2.0 + 0.2	7-8	50	2.00 ± 0.11	5.00 ± 0.17
2.0 + 0.5	7-8	60	4.00 ± 0.17	6.00 ± 0.28
2.0 + 1.0	7-8	55	3.00 ± 0.17	7.30 ± 0.28
2.0 + 2.0	7-8	30	3.00 ± 0.17	5.50 ± 0.28

* Values are the mean of three replicates with 10 explants.

Table 3.9 Effect of different concentrations and combinations of BAP with NAA and IAA on shoot multiplication from shoot tip explants of *C. amada.* Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP + NAA				
0.5 + 0.2	7-8	60	2.00 ± 0.30	5.60 ± 0.17
0.5 + 0.5	7-8	86	6.00 ± 0.17	6.50 ± 0.11
0.5 + 1.0	7-8	75	5.00 ± 0.17	7.00 ± 0.17
0.5 + 2.0	7-8	72	3.00 ± 0.11	6.00 ± 0.28
1.0 + 0.2	7-8	60	3.00 ± 0.11	6.00 ± 0.23
1.0 + 0.5	6-7	80	4.00 ± 0.28	8.00 ± 0.28
1.0 + 1.0	6-7	66	4.00 ± 0.28	7.00 ± 0.28
1.0 + 2.0	6-7	56	3.00 ± 0.11	6.70 ± 0.11
2.0 + 0.2	7-8	66	2.00 ± 0.11	6.60 ± 0.26
2.0 + 0.5	7-8	75	5.00 ± 0.17	6.50 ± 0.28
2.0 + 1.0	7-8	65	4.00 ± 0.17	6.20 ± 0.05
2.0 + 2.0	7-8	54	3.00 ± 0.28	6.00 ± 0.17
BAP + IAA				
0.5 + 0.2	8-10	50	1.00 ± 0.11	5.30 ± 0.17
0.5 + 0.5	8-10	72	2.00 ± 0.11	6.00 ± 0.28
0.5 + 1.0	8-10	80	3.00 ± 0.17	6.50 ± 0.28
0.5 + 2.0	8-10	62	3.00 ± 0.17	6.00 ± 0.28
1.0 + 0.2	7-8	60	2.00 ± 0.17	5.60 ± 0.11
1.0 + 0.5	7-8	75	4.00 ± 0.17	5.50 ± 0.28
1.0 + 1.0	7-8	72	4.00 ± 0.17	6.00 ± 0.17
1.0 + 2.0	7-8	64	3.00 ± 0.17	5.30 ± 0.17
2.0 + 0.2	8-10	50	3.00 ± 0.17	5.30 ± 0.17
2.0 + 0.5	8-10	72	4.00 ± 0.17	6.50 ± 0.17
2.0 + 1.0	8-10	65	4.00 ± 0.17	5.30 ± 0.17
2.0 + 2.0	8-10	60	3.00 ± 0.17	5.00 ± 0.11

* Values are the mean of three replicates with 10 explants.

Table 3.10 Effect of different concentration and combinations of Kn with NAA and IAA on multiple shoot induction from shoot tip explants of *C. amada*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
Kn + NAA				
0.5 + 0.2	7-8	50	3.00 ± 0.23	5.50 ± 0.28
0.5 + 0.5	6-7	65	4.00 ± 0.23	6.30 ± 0.17
0.5 + 1.0	6-7	60	5.00 ± 0.17	6.00 ± 0.28
0.5 + 2.0	7-8	40	3.00 ± 0.11	5.00 ± 0.17
1.0 + 0.2	7-8	60	4.00 ± 0.11	5.50 ± 0.17
1.0 + 0.5	6-7	80	5.50 ± 0.11	6.50 ± 0.28
1.0 + 1.0	6-7	70	3.00 ± 0.17	6.00 ± 0.17
1.0 + 2.0	7-8	50	2.00 ± 0.17	5.00 ± 0.17
2.0 + 0.2	7-8	60	2.00 ± 0.11	5.00 ± 0.17
2.0 + 0.5	7-8	75	3.00 ± 0.11	5.50 ± 0.28
2.0 + 1.0	7-8	65	2.00 ± 0.17	5.50 ± 0.11
2.0 + 2.0	7-8	40	1.60 ± 0.15	4.50 ± 0.17
Kn + IAA				
0.5 + 0.2	7-8	50	2.00 ± 0.30	5.50 ± 0.28
0.5 + 0.5	7-8	60	2.00 ± 0.11	6.30 ± 0.17
0.5 + 1.0	7-8	70	2.00 ± 0.11	6.00 ± 0.28
0.5 + 2.0	7-8	45	1.50 ± 0.15	5.00 ± 0.11
1.0 + 0.2	7-8	65	3.00 ± 0.11	5.00 ± 0.28
1.0 + 0.5	7-8	75	3.00 ± 0.11	6.00 ± 0.28
1.0 + 1.0	7-8	60	2.00 ± 0.17	6.50 ± 0.11
1.0 + 2.0	7-8	50	2.00 ± 0.17	5.50 ± 0.11
2.0 + 0.2	7-8	40	2.00 ± 0.11	5.20 ± 0.11
2.0 + 0.5	7-8	60	4.00 ± 0.28	5.50 ± 0.28
2.0 + 1.0	7-8	50	2.00 ± 0.11	6.00 ± 0.17
2.0 + 2.0	7-8	30	1.50 ± 0.15	5.00 ± 0.11

* Values are the mean of three replicates with 10 explants.

3.3.2.1.2. Axillary bud explants

Axillary bud explants were aseptically cultured on different concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and Kn either alone and combinations with each other. Another experiment sets in combinations of three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP or Kn with four concentrations (0.2, 0.5, 1.0 and 2 mg/l) of NAA or IAA. Axillary bud explants also found to be suitable for multiple shoot induction on almost all of the supplemented culture media but morphogenic responses of the cultured explants varied depending upon the culture media formulation. Effects of different concentrations and combinations of auxins and cytokinins on multiple shoot induction from shoot tip explants are described below:

Experiment 1. Effect of different concentrations of BAP and Kn alone and combinations on multiple shoot induction from axillary bud explants of *C. amada*

Under this investigation five concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and Kn used alone and combination of three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP with four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of Kn were treated in MS medium for the purpose of multiple shoot induction from axillary bud explants of *C. amada*. Data were taken after 6 weeks of inoculation and percentage of shoot formation, number of shoot/culture and length of shoot/culture were measured. The results are presented in Table 3.11. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Shoot proliferation ranged from 30.00-80.00%. Highest percentage (80.00%) of multiple shoot formation was observed in MS medium containing 1.0 mg/l BAP + 1.0 mg/l Kn followed by 75.00% in MS medium containing 0.5 mg/l BAP + 1.0 mg/l Kn and 1.0 mg/l BAP + 0.5 mg/l Kn. The lowest percentage (30.00%) of multiple shoot formation was observed in media having 3.0 mg/l Kn. Highest mean number of shoots was 5.00 ± 0.28 in media having 1.0 mg/l BAP + 0.5 mg/l Kn and lowest mean number of shoot was 1.00 ± 0.17 in media containing 3.0 mg/l BAP and 3.0 mg/l Kn. Average length of shoots gradually increased after induction of shoot. Highest average length was recorded 7.00 ± 0.28 cm in 1.0 mg/l BAP+ 1.0 mg/l Kn and the lowest average length was 4.00 ± 0.23 cm in media containing 3.0 mg/l Kn. Experimental results revealed that, 1.0 mg/l of BAP and 1.0 mg/l Kn alone and combination of 1.0 mg/l BAP + 1.0 mg/l Kn, 1.0 mg/l BAP + 0.5 mg/l Kn and 0.5 mg/l BAP + 1.0 mg/l Kn were found most effective concentrations and combinations for multiple shoot induction in *C. amada*.

Experiment 2. Effect of different concentrations and combinations of BAP with NAA and IAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentrations of BAP (0.5, 1.0 and 2.0 mg/l) combined with different concentrations of NAA (0.2, 0.5, 1.0 and 2.0 mg/l) and IAA (0.2, 0.5, 1.0 and 2.0 mg/l). Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.12. The efficiency of BAP + NAA was observed better than that of BAP + IAA on direct shoot regeneration. All the used media compositions formed multiple shoots and the results were superior to the results obtained from shoot tip explants. Addition of lower concentration of NAA and IAA along with higher concentration of BAP was found more suitable than other concentrations. Among the combinations of BAP + NAA and BAP + IAA, the highest percentage (90%) of shoot proliferation was noted in the media having 0.5 mg/l BAP + 0.5 mg/l NAA followed by (85%) 1.0 mg/l BAP + 0.5 mg/l NAA. The lowest percentage (40%) of shoot proliferation was noted in media having 2.0 mg/l BAP + 2.0 mg/l IAA. The average highest number of shoot per culture was 7.00 ± 0.28 in the combinations of 0.5 mg/l BAP + 0.5 mg/l NAA and the lowest average number of shoot per culture were 1.30 ± 0.05 in the combination of 0.5 mg/l BAP + 0.2 mg/l IAA. The highest length of shoot per culture was 7.50 ± 0.28 cm in the media containing 0.5 mg/l BAP + 0.5 mg/l NAA and the lowest length of shoot per culture was 4.50 ± 0.11 cm in the media containing 0.5 mg/l BAP + 0.2 mg/l IAA. Experimental results revealed that, 0.5 mg/l BAP + 0.5 mg/l NAA, 1.0 mg/l BAP + 0.5 mg/l NAA, 0.5 mg/l BAP + 1.0 mg/l NAA and 0.5 mg/l BAP + 1.0 mg/l IAA were found most effective combinations for multiple shoot induction in *C. amada*.

Experiment 3. Effect of different concentrations and combinations of Kn with NAA and IAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentrations of Kn (0.5, 1.0 and 2.0 mg/l) combined with different concentrations of NAA (0.2, 0.5, 1.0 and 2.0 mg/l) and IAA (0.2, 0.5, 1.0 and 2.0 mg/l). Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.13. The combined effect of Kn + NAA was better than that of Kn + IAA on direct shoot regeneration. All the used media compositions formed multiple shoots and the results were superior to the results obtained from shoot tip explants. Addition of lower concentration of NAA and IAA along with higher concentration of Kn was found more suitable than that of other concentrations. Among the combinations, the combined

effect of Kn + NAA and Kn + IAA gave the highest percentage (80%) of shoot proliferation and in the media having 1.0 mg/l Kn + 0.5 mg/l NAA produced the highest performance followed by 75% in 0.5 mg/l Kn + 0.5 mg/l NAA. The lowest percentage (40%) of shoot proliferation was noted in the media having 2.0 mg/l Kn + 2.0 mg/l NAA. The average highest number of shoot per culture was 6.50 ± 0.28 in the media having 1.0 mg/l Kn + 0.5 mg/l NAA (Plate 3.5) and the lowest number of shoot per culture was 1.00 ± 0.11 in the media having 0.5 mg/l Kn + 0.2 mg/l IAA. Average highest length of shoot 7.00 ± 0.28 cm per culture was found in the combination of 1.0 mg/l Kn + 1.0 mg/l NAA and lowest average length 4.50 ± 0.11 cm per culture was found in the media having 2.0 mg/l Kn + 0.2 mg/l NAA and 0.5 mg/l Kn + 0.2 mg/l IAA respectively. Experimental results revealed that, 1.0 mg/l Kn + 0.5 mg/l NAA, 0.5 mg/l Kn + 0.5 mg/l NAA and 1.0 mg/l Kn + 1.0 mg/l NAA were found most effective combinations for multiple shoot induction in *C. amada*.

Table 3.11 Effect of different concentrations of BAP and Kn singly and in combinations with each other on multiple shoot induction from axillary bud explants of C. *amada*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP				
0.2	5-7	40	2.00 ± 0.11	5.30 ± 0.17
0.5	5-7	60	2.00 ± 0.15	6.00 ± 0.28
1.0	5-7	72	3.00 ± 0.11	6.50 ± 0.28
2.0	5-7	65	2.00 ± 0.17	5.50 ± 0.28
3.0	5-7	50	1.00 ± 0.11	5.00 ± 0.28
Kn				
0.2	7-8	40	2.00 ± 0.11	5.00 ± 0.11
0.5	7-8	50	2.00 ± 0.17	5.50 ± 0.17
1.0	7-8	66	3.00 ± 0.11	6.00 ± 0.28
2.0	7-8	60	2.00 ± 0.17	5.50 ± 0.17
3.0	7-8	30	1.00 ± 0.17	4.00 ± 0.23
BAP + Kn				
0.5 + 0.2	5-7	50	2.00 ± 0.17	6.00 ± 0.28
0.5 + 0.5	5-7	66	3.00 ± 0.17	6.50 ± 0.11
0.5 + 1.0	5-7	75	3.00 ± 0.17	6.50 ± 0.17
0.5 + 2.0	5-7	60	2.00 ± 0.17	5.00 ± 0.11
1.0 + 0.2	5-7	60	2.00 ± 0.17	6.00 ± 0.17
1.0 + 0.5	5-7	75	5.00 ± 0.28	6.50 ± 0.28
1.0 + 1.0	5-7	80	4.00 ± 0.11	7.00 ± 0.28
1.0 + 2.0	5-7	62	2.00 ± 0.17	5.67 ± 0.16
2.0 + 0.2	5-7	50	2.00 ± 0.11	5.00 ± 0.17
2.0 + 0.5	5-7	66	4.00 ± 0.11	6.00 ± 0.28
2.0 + 1.0	5-7	70	2.00 ± 0.17	6.50 ± 0.17
2.0 + 2.0	5-7	52	2.00 ± 0.11	5.50 ± 0.28

* Values are the mean of three replicates with 10 explants.

Table 3.12 Effect of different concentrations and combinations of BAP with NAA and IAA on shoot multiplication from axillary bud explants of *C. amada.* Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP + NAA				
0.5 + 0.2	5-7	60	3.00 ± 0.23	5.00 ± 0.28
0.5 + 0.5	5-7	90	7.00 ± 0.28	7.50 ± 0.28
0.5 + 1.0	5-7	72	4.00 ± 0.17	6.00 ± 0.17
0.5 + 2.0	5-7	52	4.00 ± 0.23	6.00 ± 0.28
1.0 + 0.2	5-7	60	4.00 ± 0.11	5.00 ± 0.17
1.0 + 0.5	5-7	85	6.50 ± 0.28	6.00 ± 0.28
1.0 + 1.0	5-7	66	6.00 ± 0.28	7.00 ± 0.28
1.0 + 2.0	5-7	55	3.00 ± 0.23	6.50 ± 0.11
2.0 + 0.2	5-7	65	3.00 ± 0.17	5.00 ± 0.17
2.0 + 0.5	5-7	75	4.00 ± 0.11	6.00 ± 0.28
2.0 + 1.0	5-7	52	3.00 ± 0.17	6.20 ± 0.11
2.0 + 2.0	5-7	42	3.00 ± 0.23	5.50 ± 0.28
BAP + IAA				
0.5 + 0.2	7-8	60	1.30 ± 0.05	4.50 ± 0.11
0.5 + 0.5	7-8	72	2.00 ± 0.11	5.50 ± 0.11
0.5 + 1.0	7-8	80	4.00 ± 0.17	6.00 ± 0.11
0.5 + 2.0	7-8	66	3.00 ± 0.11	6.30 ± 0.17
1.0 + 0.2	7-8	50	2.00 ± 0.17	5.00 ± 0.17
1.0 + 0.5	7-8	66	4.00 ± 0.28	6.00 ± 0.28
1.0 + 1.0	7-8	70	5.00 ± 0.17	6.00 ± 0.17
1.0 + 2.0	7-8	55	3.00 ± 0.11	5.50 ± 0.11
2.0 + 0.2	7-8	60	2.00 ± 0.11	6.00 ± 0.28
2.0 + 0.5	7-8	72	3.00 ± 0.23	6.50 ± 0.28
2.0 + 1.0	7-8	55	3.00 ± 0.17	7.00 ± 0.17
2.0 + 2.0	7-8	40	2.00 ± 0.11	6.30 ± 0.17

* Values are the mean of three replicates with 10 explants.

Table 3.13 Effect of different concentration and combinations of Kn with NAA and IAA on shoot proliferation from axillary bud explants of *C. amada*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
Kn + NAA				
0.5 + 0.2	6-7	52	2.00 ± 0.17	5.20 ± 0.11
0.5 + 0.5	6-7	75	3.00 ± 0.17	6.00 ± 0.17
0.5 + 1.0	6-7	70	4.00 ± 0.23	6.50 ± 0.28
0.5 + 2.0	6-7	55	4.00 ± 0.23	6.30 ± 0.17
1.0 + 0.2	6-7	55	2.00 ± 0.17	5.50 ± 0.28
1.0 + 0.5	6-7	80	6.50 ± 0.28	6.50 ± 0.28
1.0 + 1.0	6-7	72	4.00 ± 0.11	7.00 ± 0.28
1.0 + 2.0	6-7	66	4.00 ± 0.11	6.30 ± 0.17
2.0 + 0.2	6-7	50	2.00 ± 0.11	4.50 ± 0.11
2.0 + 0.5	6-7	67	3.00 ± 0.11	5.50 ± 0.28
2.0 + 1.0	6-7	60	4.00 ± 0.17	5.00 ± 0.11
2.0 + 2.0	6-7	40	2.00 ± 0.11	5.00 ± 0.28
Kn + IAA				
0.5 + 0.2	7-8	45	1.00 ± 0.11	4.50 ± 0.11
0.5 + 0.5	7-8	60	2.00 ± 0.11	5.50 ± 0.11
0.5 + 1.0	7-8	70	4.30 ± 0.17	5.00 ± 0.17
0.5 + 2.0	7-8	56	3.00 ± 0.11	5.30 ± 0.17
1.0 + 0.2	7-8	60	2.00 ± 0.17	5.50 ± 0.11
1.0 + 0.5	7-8	72	5.00 ± 0.11	6.50 ± 0.28
1.0 + 1.0	7-8	66	6.00 ± 0.28	5.60 ± 0.17
1.0 + 2.0	7-8	60	3.00 ± 0.11	5.00 ± 0.17
2.0 + 0.2	7-8	50	1.60 ± 0.15	5.00 ± 0.28
2.0 + 0.5	7-8	70	3.00 ± 0.23	6.00 ± 0.28
2.0 + 1.0	7-8	62	4.00 ± 0.11	6.50 ± 0.28
2.0 + 2.0	7-8	54	3.00 ± 0.17	6.30 ± 0.17

* Values are the mean of three replicates with 10 explants.

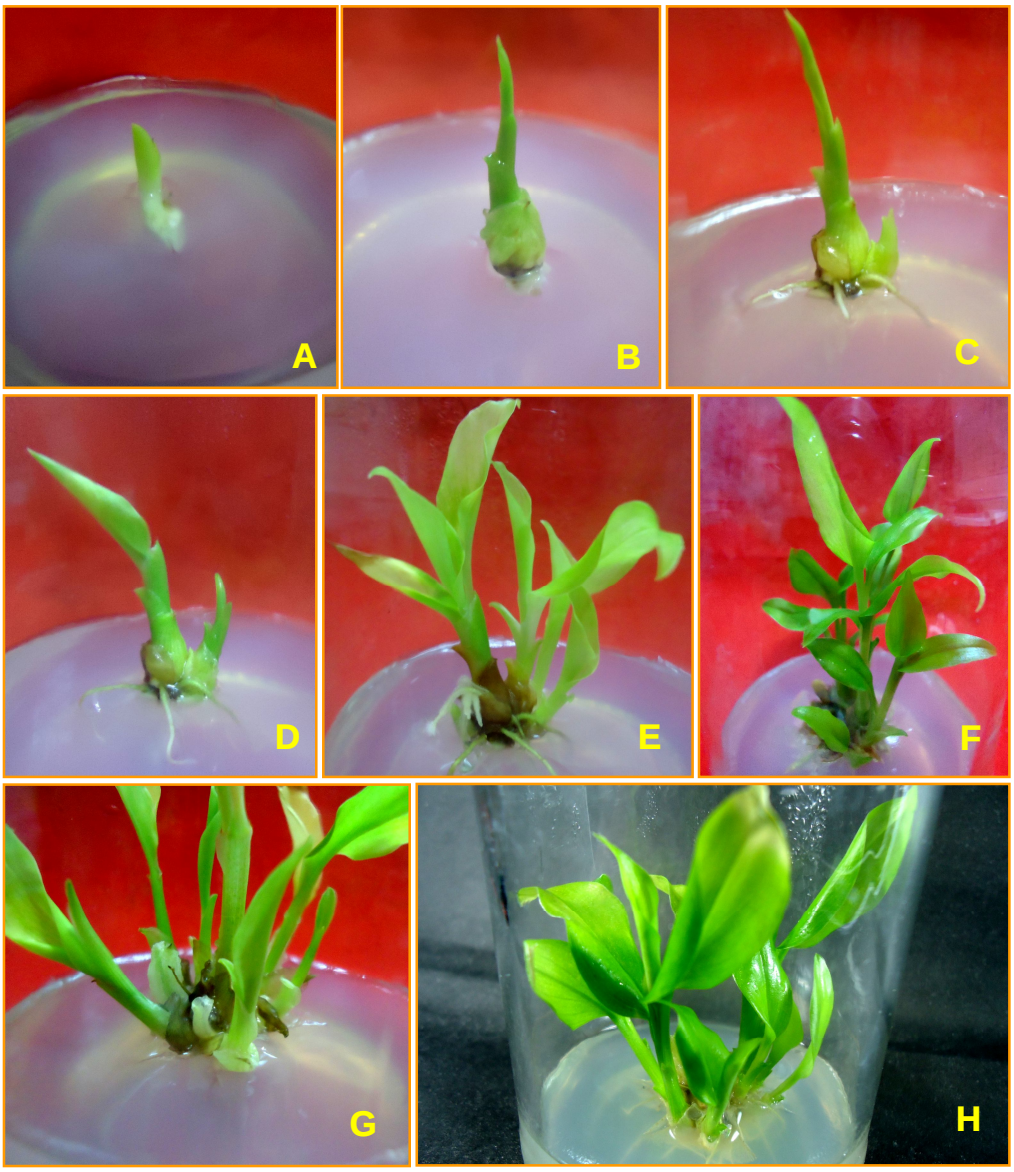


Plate 3.5 Development of multiple shoot from axiliary bud and nodal explants of *C. amada*.

A: Explant inoculation in culture bottle; **B-D**: Single and double shoot initiation from axillary bud explant in 0.5 mg/l BAP + 0.5 mg/l NAA after 2-4 weeks of culture; **E-F**: Multiple shoot proliferation from axillary bud explant in the same medium after 4-6 weeks of culture; **G-H**: Further multiple shoot proliferation and elongation from axillary bud explant in the same medium after 6-8 weeks of culture.

3.3.2.2. Microrhizomes production from shoot tip and axillary bud explants of *C. amada*

Two types of explants viz. shoot tip and axillary bud explants were cultured for microrhizomes production. Explants were cultured on MS medium with BAP alone and combinations with NAA. Data were taken after 10 weeks of inoculation. Days taken for initiation of micro rhizomes, number of shoots producing microrhizomes , number of microrhizomes per shoot were measured.

Experiment 1. Effect of different concentrations of BAP alone and in combinations with NAA on microrhizome production from shoot tip and axillary bud explants of *C. amada*

Four concentrations of BAP (0.5, 1.0, 2.0 and 3.0mg/l) alone and in combination of three concentrations of BAP (0.5, 1.0 and 2.0 mg/l) with three concentrations of NAA (0.1, 0.2 and 0.5 mg/l) were treated in MS medium for the purpose of microrhizomes production from shoot tip and axillary bud explants of *C. amada*. Data were taken after 10 weeks of inoculation and days taken for initiation of micro rhizomes, number of shoots producing microrhizomes , number of microrhizomes per shoot were measured. The results are presented in Table 3.14. The results obtained in the present investigation using different concentrations of growth regulators showed significant influence on the number of days taken to show initiation of microrhizomes of shoot tip explants, the lowest number of days (36-40) was required for induction of microrhizomes in medium containing 1.0 mg/l BAP with 0.2 mg/l NAA (Plate 3.6), followed by (40-45 days) in 1.0 mg/l BAP alone and in combinations of 1.0 mg/l BAP with 0.5 mg/l NAA. These three treatments were significantly superior to other treatments. The highest number of days (60-65) was recorded in plain MS. The data in Table 3.14 indicates that the highest number of shoots producing microrhizomes 6.00 ± 0.17 was observed in medium supplemented with 2.0 mg/l BAP + 0.2 mg/l NAA followed by (5.00 ± 0.11) in 1.0 mg/l BAP + 0.2 mg/l NAA. Among the treatments, plain MS medium significantly recorded lower 1.00 ± 0.11 number of shoots producing microrhizomes. The number of microrhizomes per shoot was significantly influenced by different concentrations of growth regulator. The highest number of microrhizomes per shoot was recorded 6.00 ± 0.11 (Plate 3.6) in the media having 1 mg/l BAP + 0.2 mg/l NAA, which were followed by 5.00 ± 0.28 in 1.0 mg/l BAP and 1.0 mg/l BAP + 0.1 mg/l NAA. Whereas, the lowest number of microrhizomes per shoot 2.00 ± 0.11 was recorded in plain MS medium which significantly differed with other treatments.

But of axillary bud explants the lowest number of days (38-40) was required for induction of microrhizomes in medium containing 1 mg/l BAP + 0.2 mg/l NAA, followed by (40-45 days) in 1 mg/l BAP + 0.1 mg/l NAA .These two treatments were significantly superior to other treatments. The highest number of days (60-65) was recorded in plain MS medium. The data in Table 3.14, indicates that the highest number of shoots producing microrhizomes was 5.00 ± 0.28 observed in medium supplemented with BAP 1.0 mg/l and NAA 0.2 mg/l. Among the treatments, plain MS medium significantly recorded lower number of shoots 1.00 ± 0.11 producing microrhizomes. The number of microrhizomes per shoot was significantly influenced by different concentrations of growth regulator. The highest number of microrhizomes per shoot 5.80 ± 0.11 was recorded in 0.5 mg/l BAP + 0.2 mg/l NAA, which was followed by 5.00 ± 0.28 in 1.0 mg/l BAP alone and in combination of 1.0 mg/l BAP + 0.2 mg/l NAA. Whereas, the lowest number of microrhizomes per shoot 2.00 ± 0.11 was recorded in plain MS medium which significantly differed from other treatments.

Table 3.14 Effect of growth regulators on microrhizome production on shoot tip and axillary bud explant of *C. amada*. Data were recorded up to 10 weeks of culture.

Plant growth regulators (mg/l)	Shoot tip explant			Axillary bud explant		
	Days taken for initiation of micro rhizomes	*No. of shoot producing microrhizomes (mean ± SE)	*No. of micro rhizomes per shoot (mean ± SE)	Days taken for initiation of micro rhizomes	*No. of shoot producing microrhizomes (mean ± SE)	*No. of micro rhizomes per shoot (mean ± SE)
Plain MS	60-65	1.00 ± 0.11	2.00 ± 0.11	60-65	1.00 ± 0.11	2.00 ± 0.11
BAP						
0.5	45-50	2.00 ± 0.11	4.00 ± 0.17	45-50	4.00 ± 0.11	3.00 ± 0.17
1.0	40-45	4.00 ± 0.20	5.00 ± 0.28	42-45	4.60 ± 0.11	5.00 ± 0.28
2.0	45-50	4.00 ± 0.17	4.00 ± 0.17	48-50	2.00 ± 0.17	2.00 ± 0.17
3.0	-	-	-	-	-	-
BAP + NAA						
0.5 + 0.1	45-50	3.00 ± 0.17	3.50 ± 0.11	45-50	4.00 ± 0.11	4.00 ± 0.17
0.5 + 0.2	45-50	3.50 ± 0.11	4.00 ± 0.17	42-45	4.50 ± 0.05	5.80 ± 0.11
0.5 + 0.5	45-50	4.00 ± 0.17	4.00 ± 0.23	42-45	3.00 ± 0.17	2.30 ± 0.17
1.0 + 0.1	42-45	4.00 ± 0.17	5.00 ± 0.28	40-45	4.00 ± 0.17	4.00 ± 0.28
1.0 + 0.2	36-40	5.00 ± 0.11	6.00 ± 0.11	38-40	5.00 ± 0.28	5.00 ± 0.17
1.0 + 0.5	40-45	4.00 ± 0.23	4.00 ± 0.23	42-46	3.00 ± 0.17	3.30 ± 0.15
2.0 + 0.1	45-50	3.00 ± 0.11	4.00 ± 0.17	48-50	4.00 ± 0.17	3.00 ± 0.17
2.0 + 0.2	45-50	6.00 ± 0.17	3.00 ± 0.23	45-50	4.00 ± 0.34	4.00 ± 0.23
2.0 + 0.5	50-55	4.00 ± 0.28	3.00 ± 0.17	50-55	3.00 ± 0.28	3.00 ± 0.17

* Values are the mean of three replicates with 5 explants.

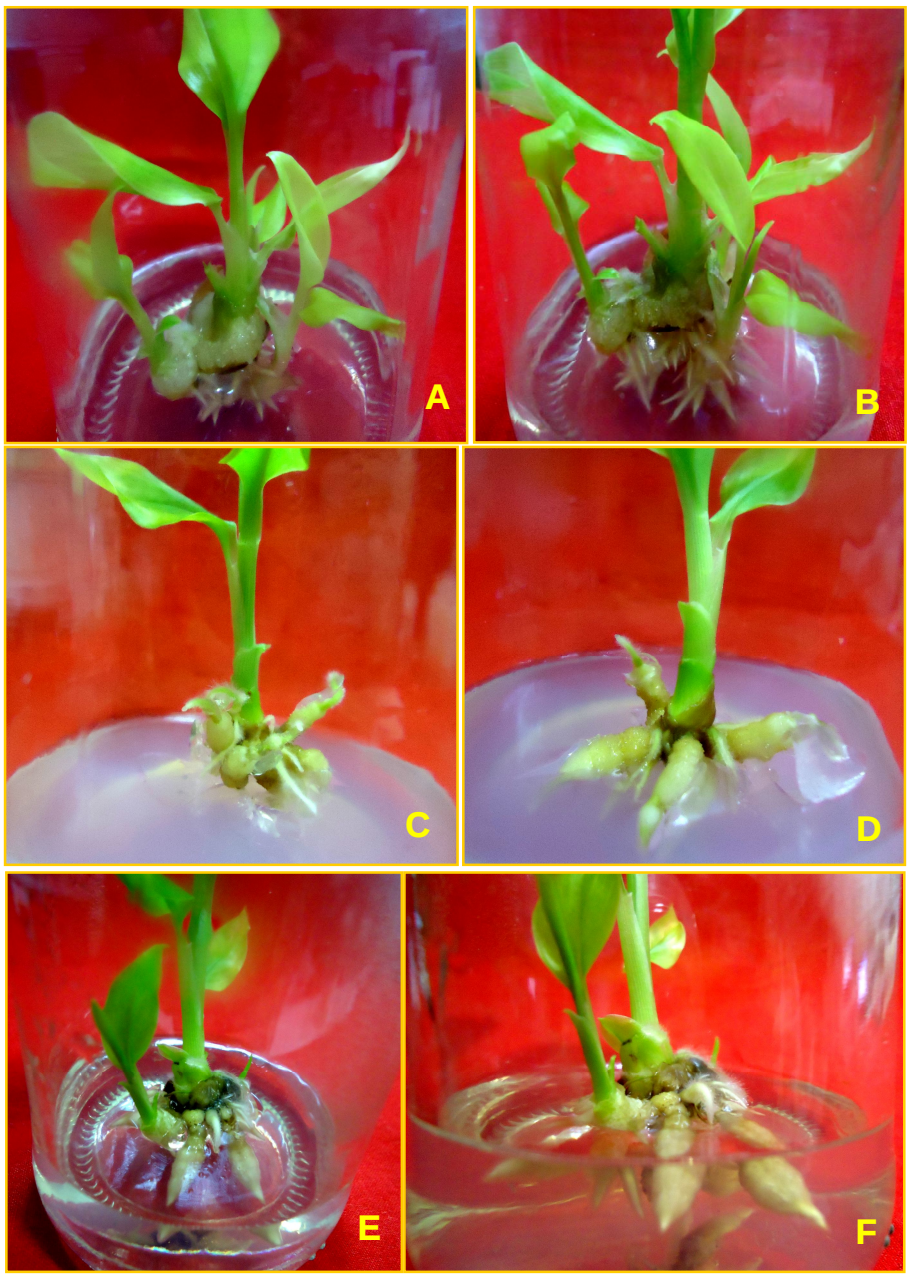


Plate 3. 6 Development of microrhizome from shoot tip explants of *C. amada*.

A-B: Microrhizome initiation from shoot tip explant in 1.0 mg/l BAP + 0.2 mg/l NAA after 5-6 weeks of culture; **C-D**: Proliferation of microrhizome in the same medium after 7-8 weeks of sub culture; **E-F**: Further proliferation and elongation of shoot buds in the same medium after 10 weeks of subculture.

3.3.2.3. Rooting of *in vitro* grown shoots in *C. amada*

Shoot cuttings 5–7 cm long were separated from *in vitro* grown shoots and transferred to rooting media. MS plain (without hormones) and half strength MS medium fortified with different concentrations of IAA, NAA and IBA (0.2, 0.5, 1.0, 1.5 and 2.0 mg/l) were used for rooting experiment. Rooting was started within 7 to 10 days of culture. Data on days to root initiation, frequency of root formation (%), average number of root per culture and average length of root per culture were recorded after 6 weeks of culture

Experiment 1. Effect of different concentrations of IAA, NAA, IBA and half MS=0 on root induction from *in vitro* grown shoots of *C. amada*

For adventitious root formation, the shoots obtained from *in vitro* grown shoot explant were excised and cultured on half strength MS medium with different concentrations (0.2, 0.5, 1.0, 1.5 and 2.0 mg/l) of IAA NAA , IBA and MS=0 (without hormones). Results obtained for root induction, frequency of root formation, morphology, average number and length of roots were observed and the results are shown in Table 3.15. Percentage range of cultures produced roots varied from 30.00-90.00%. Highest 90.00% of root formation was recorded in 0.5 mg/l IBA followed by 75.00 % in media with 0.5mg/l NAA. The lowest 30.00 % of root regeneration was recorded in media having 2.0 mg/l IAA. The highest average number of roots per shoot was recorded 22.00 ± 0.57 in media having 1.5 mg/l IBA (Plate 3.7 B-C). The lowest average number of root per shoots was recorded 4.00 ± 0.17 in media having 2.0 mg/l IAA and 0.2 mg/l NAA. Highest length of roots was 6.30 ± 0.17 cm recorded in 1.0 mg/l IAA and the lowest length of roots was 3.50 ± 0.11 cm recorded in 2.0 mg/l IBA. In most cases morphology of roots was thin but someone looked swollen and long.

Table 3.15 Effect of different concentrations of IAA, NAA and IBA in half strength MS medium for root induction from *in vitro* grown shoot of *C. amada*. Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	Number of days taken for shoot initiation	Percentage (%) of root formation	*Average number of roots / explants (mean ± SE)	*Average length (cm) of root / explant (mean ± SE)
MS=0	10-12	25	4.00 ± 0.17	3.50 ± 0.11
IAA				
0.2	8-10	40	6.00 ± 0.28	5.00 ± 0.25
0.5	8-10	60	8.00 ± 0.23	5.60 ± 0.11
1.0	8-10	65	9.00 ± 0.23	6.30 ± 0.17
1.5	8-10	45	12.00 ± 0.11	6.00 ± 0.23
2.0	8-10	30	4.00 ± 0.11	5.30 ± 0.17
NAA				
0.2	7-10	50	4.00 ± 0.17	4.60 ± 0.17
0.5	7-10	75	6.00 ± 0.17	5.00 ± 0.28
1.0	7-10	60	10.00 ± 0.23	5.50 ± 0.28
1.5	7-10	45	8.00 ± 0.23	5.70 ± 0.05
2.0	7-10	35	5.00 ± 0.17	5.30 ± 0.17
IBA				
0.2	7-10	60	8.00 ± 0.23	3.60 ± 0.17
0.5	7-10	90	12.00 ± 0.23	4.30 ± 0.17
1.0	7-10	55	16.00 ± 0.17	4.60 ± 0.11
1.5	7-10	40	22.00 ± 0.57	4.00 ± 0.11
2.0	7-10	40	15.00 ± 0.11	3.50 ± 0.11

* Values are the mean of three replicates with 10 explants.

Plate 3.7 Induction of adventitious roots and establishment in soil of *C. amada*.

A-B: Induction of adventitious roots on shoots regenerated from axiliary bud explants in half strength MS medium containing 0.5 mg/l IBA after 4-6 weeks of culture; **C**: Picture of *C. amada* with roots outside of culture bottle; **D**: The *in vitro* grown seedlings acclimatized to grow in pots in outside environment after 4 weeks.

3.3.3. *In vitro* regeneration and conservation of *Ipomoea mauritiana*

Experiments on direct and indirect organogenesis were carried out using different types of explants viz. shoot tip, node, internode, and leaf from two months old field grown plants. Surface sterilization was done separately according to the explant types. In case of nodal and internodal segments effective surface sterilization was performed with 0.1% $HgCl_2$ 5-6 min. On the other hand, shoot tip and leaf explant took 3-4 min for 100% contamination free cultures in the same concentrations of $HgCl_2$. Sterilized explants were then cut into appropriate size (1.0-2.0 cm) and cultured on plant growth regulators (PGR) supplemented media for induction of direct and indirect organogenesis. Both auxins (NAA, IAA, IBA and 2,4-D) and cytokinins (BAP and Kn) PGR supplements were used in the media either single or in combinations. For root induction, micro shoots obtained from direct and indirect organogenesis were transferred to natural conditions through successive phages of acclimatization. The results of *in vitro* culture of direct and indirect organogenesis are described below:

3.3.3.1. Direct shoot regeneration in *I. mauritiana*

Two types of explants *viz*. shoot tip and nodal explants were cultured for direct shoot regeneration. explants were cultured on MS medium with BAP, Kn and IAA used alone, and in combinations of BAP or Kn (0.5, 1.0 and 2.0 mg/l) with NAA or IAA (0.2, 0.5, 1.0 and 2.0 mg/l). Data on number of days taken for shoot initiation, percentage of explant induced shoot development, average number of shoot per culture and average length of shoot per culture were collected after 6 weeks of culture. Shoot tip and nodal explants responded on almost all of the supplemented cultured medium. The results are described according to types of explants under separate heads:

3.3.3.1.1. Direct multiple shoot induction from nodal explants of *I. mauritiana*

Nodal explants of two months old *I. mauritiana* were aseptically cultured on MS medium with different concentrations (0.2–4.0 mg/l) of cytokinins viz. BAP and Kn either alone or in combinations (0.5, 1.0 and 2.0 mg/l) with different concentrations (0.2–2.0 mg/l) of NAA and IAA. Among the four types of explants nodal segments found to be most suitable for multiple shoot induction. Though nodal segments responded on almost all of the supplemented culture media but morphogenic responses of the cultured explants varied depending upon the culture media formulation. Effects of different concentrations and combinations of auxins and cytokinins on multiple shoot induction from nodal explants are described below:

Experiment 1. Effect of BAP, Kn and IAA alone on multiple shoot induction from nodal explants

Six concentrations (0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) of BAP, Kn and IAA alone were treated in MS medium for the purpose of multiple shoot induction from nodal segments of *I. mauritiana*. Data were taken after 6 weeks of inoculation and days taken for shoot initiation, percentage of shoot induction, number of shoot/culture and length of shoot/culture were measured. The results are presented in Table 3.16. The table shows that BAP was more effective than Kn and IAA for multiple shoot induction from nodal explants. Though nodal explants responded in all the concentrations (0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) of BAP, Kn and IAA but the percent of explant showing proliferation was found varied from 30-80%. The maximum 80% cultures induced multiple shoots in media having 1.0 mg/l BAP followed by 70% in 0.5 mg/l BAP and 1.0 mg/l Kn. The lowest 30% response was noted in the media with 4.0 mg/l IAA. The highest average number of shoot per culture was 4.00 ± 0.28 noted in the media having 1.0 mg/l IAA. The lowest average number of shoot per culture was 1.40 ± 0.13 noted in the media having 4.0 mg/l Kn. The highest average length of shoot per culture was 5.80 ± 0.29 cm in the media having 1.0 mg/l BAP followed by 5.50 ± 0.28 cm in media having 1.0 mg/l Kn. And the lowest average length of shoot per culture 3.80 ± 0.11 cm in the media having 4.0 mg/l Kn. Experimental results revealed that, 0.5, 1.0 mg/l of BAP and 1.0 mg/l Kn and IAA was proved as the effective concentrations for multiple shoot induction.

Experiment 2. Effect of different concentrations and combinations of BAP with NAA and IAA on multiple shoot induction from nodal explants

Explants were cultured on MS medium supplemented with three concentration (0.5, 1.0 and 2.0 mg/l) of BAP combined with four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of NAA and IAA. Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.17. The efficiency of BAP + IAA was found better than that of BAP alone and BAP + NAA on direct shoot regeneration. All the used media compositions formed multiple shoots and the results were superior to the results obtained from shoot tip explants. Addition of lower concentration of NAA and IAA along with higher concentration of BAP was found more suitable than that of other concentrations. Among the combinations of BAP + NAA and BAP + IAA, the highest 95% of shoot proliferation was noted in the media having 1.0 mg/l BAP + 0.5 mg/l IAA followed by 90% in 1.0 mg/l BAP + 1.0 mg/l IAA and 1.0 mg/l BAP + 0.5 mg/l NAA respectively. The lowest percentage 60% of shoot proliferation was noted in media having 0.5 mg/l BAP + 0.2 mg/l IAA. The

highest number of average shoot per culture was 5.80 ± 0.27 in the media having 1.0 mg/l BAP + 0.5 mg/l IAA (Plate 3.8 F-G) and the lowest number of average shoot was 2.20 ± 0.08 per culture noted in the media having 0.5 mg/l BAP +0.2 mg/l NAA. The highest average length of shoot was 7.50 ± 0.15 cm per culture found in the combination of 1.0 mg/l BAP + 0.5 mg/l IAA. Average lowest length of shoot 4.30 ± 0.17 cm per culture were found in the combination of 2.0 mg/l BAP +2.0 mg/l IAA. Experimental results revealed that, 1.0 mg/l BAP + 0.5 mg/l IAA, 1.0 mg/l BAP + 1.0 mg/l IAA, 1.0 mg/l BAP + 0.5 mg/l NAA and 0.5 mg/l BAP + 1.0 mg/l NAA were found more effective combinations for multiple shoot induction in *I. mauritiana*.

Experiment 3. Effect of different concentrations and combinations of Kn with NAA and IAA on multiple shoot induction from nodal explants

Explants were cultured on MS medium supplemented with three concentrations (0.5, 1.0 and 2.0 mg/l) of Kn combined with four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of NAA or IAA. Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.18. All the used media compositions formed multiple shoots and the results were superior to the results obtained from shoot tip explants. Addition of lower concentration of NAA and IAA along with higher concentration of Kn was found more suitable than that of other concentrations. Among the combinations of Kn + NAA and Kn + IAA the highest percentage (85%) of shoot proliferation was noted in the media having 1.0 mg/l Kn + 1.0 mg/l IAA followed by (80%) in 1.0 mg/l Kn +0.5 mg/l NAA and 1.0 mg/l Kn + 0.5 mg/l IAA respectively. The lowest percentage (52%) of shoot proliferation was noted in the media having 2.0 mg/l Kn + 2.0 mg/l IAA .The average highest number of shoot per culture was 5.00 ± 0.05 in the media having 1.0 mg/l Kn + 1.0 mg/l NAA followed by 4.00 ± 0.11 in 1.0 mg/l Kn + 0.5 mg/l NAA and 1.0 mg/l Kn + 1.0 mg/l IAA respectively. The lowest number of shoot per culture was 1.30 ± 0.05 in the media having 2.0 mg/l Kn +2.0 mg/l NAA. Average highest length of shoot was 6.90 ± 0.08 cm per culture found in the combination of 1.0 mg/l Kn + 1.0 mg/l NAA followed by 6.50 ± 0.28 cm per culture was found in the media having 1.0 mg/l Kn + 0.5 mg/l NAA and 2.0 mg/l Kn + 0.5 mg/l NAA. The lowest length of shoot per culture was 4.10 ± 0.12 found in 2.0 mg/l Kn + 2.0 mg/l IAA. Experimental results revealed that, 1.0 mg/l Kn + 0.5 mg/l NAA, 1.0 mg/l Kn + 1.0 mg/l NAA, 1.0 mg/l Kn + 0.5 mg/l IAA and 1.0 mg/l Kn + 1.0 mg/l IAA were found more effective combinations for multiple shoot induction.

Table 3.16 Effect of different levels of BA, Kn and IAA used alone on multiple shoot induction from nodal explants of *I. mauritiana*. Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	Number of days taken for shoot initiation	Percentage (%) of shoot induction	Base callusing	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP					
0.2	7-8	40	-	2.40 ± 0.17	4.5 ± 0.12
0.5	7-8	70	+	3.40 ± 0.14	5.2 ± 0.26
1.0	7-8	80	+	3.60 ± 0.12	5.8 ± 0.29
2.0	7-8	66	+	2.50 ± 0.20	5.4 ± 0.32
3.0	7-8	60	+	1.80 ± 0.12	5.2 ± 0.17
4.0	7-8	52	+	1.50 ± 0.08	4.6 ± 0.29
Kn					
0.2	7-10	40	-	1.80 ± 0.11	4.4 ± 0.26
0.5	7-10	62	-	2.80 ± 0.17	5.1 ± 0.37
1.0	7-8	70	+	3.60 ± 0.20	5.5 ± 0.28
2.0	7-8	66	+	2.80 ± 0.20	5.0 ± 0.26
3.0	7-8	52	+	2.40 ± 0.12	4.3 ± 0.17
4.0	7-10	40	+	1.40 ± 0.13	3.8 ± 0.11
IAA					
0.2	7-10	40	-	1.90 ± 0.14	4.5 ± 0.23
0.5	7-10	50	-	2.70 ± 0.14	4.8 ± 0.20
1.0	7-8	70	+	4.00 ± 0.28	5.4 ± 0.14
2.0	7-8	60	+	3.70 ± 0.14	5.1 ± 0.12
3.0	7-8	55	+	2.60 ± 0.20	4.7 ± 0.20
4.0	7-10	30	+	1.50 ± 0.14	4.2 ± 0.14

– : Absent of base callus **+ :** Present of base callus

*Values are the mean of three replicates with 10 explants.

Table 3.17 Effect of different concentrations and combinations of BAP with NAA and IAA on shoot multiplication from nodal explant of *I. mauritiana*. Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	Number of days taken for shoot initiation	(%) of shoot induction	Base callusing	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP + NAA					
0.5 + 0.2	7-8	70	-	2.20 ± 0.08	5.80 ± 0.14
0.5 + 0.5	7-8	75	+	2.90 ± 0.20	5.80 ± 0.17
0.5 + 1.0	7-8	85	+	3.50 ± 0.26	6.10 ± 0.03
0.5 + 2.0	7-8	70	+	2.70 ± 0.11	5.50 ± 0.20
1.0 + 0.2	7-8	70	+	3.00 ± 0.26	5.00 ± 0.08
1.0 + 0.5	7-8	90	+	4.40 ± 0.18	5.80 ± 0.15
1.0 + 1.0	7-8	80	+	4.00 ± 0.15	5.70 ± 0.20
1.0 + 2.0	7-8	75	+	2.60 ± 0.05	5.40 ± 0.03
2.0 + 0.2	7-8	65	+	3.10 ± 0.08	5.70 ± 0.12
2.0 + 0.5	7-8	80	+	4.10 ± 0.12	6.20 ± 0.08
2.0 + 1.0	7-8	70	+	3.40 ± 0.26	5.90 ± 0.20
2.0 + 2.0	7-8	65	+	2.50 ± 0.12	5.40 ± 0.12
BAP + IAA					
0.5 + 0.2	6-8	60	-	2.70 ± 0.12	5.70 ± 0.12
0.5 + 0.5	6-8	75	+	3.50 ± 0.20	5.90 ± 0.13
0.5 + 1.0	6-8	80	+	3.40 ± 0.17	6.40 ± 0.08
0.5 + 2.0	6-8	70	+	4.00 ± 0.17	5.10 ± 0.13
1.0 + 0.2	6-8	70	+	2.70 ± 0.11	6.00 ± 0.18
1.0 + 0.5	6-8	95	+	5.80 ± 0.27	7.50 ± 0.15
1.0 + 1.0	6-8	90	+	4.00 ± 0.17	6.90 ± 0.12
1.0 + 2.0	6-8	80	+	3.50 ± 0.40	6.70 ± 0.14
2.0 + 0.2	6-8	70	+	2.40 ± 0.14	6.00 ± 0.08
2.0 + 0.5	6-8	80	+	3.70 ± 0.17	6.10 ± 0.12
2.0 + 1.0	6-8	75	+	3.10 ± 0.18	5.50 ± 0.20
2.0 + 2.0	6-8	70	+	2.60 ± 0.06	4.30 ± 0.17

– : Absent of base callus, **+ :** Present of base callus

*Values are the mean of three replicates with 10 explants.

Table 3.18 Effect of different concentrations and combinations of Kn with NAA and IAA on shoot multiplication from nodal explant of *I. mauritiana.* Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	Number of days taken for shoot initiation	(%) of shoot induction	Base callusing	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
Kn + NAA					
0.5 + 0.2	7-8	60	-	2.00 ± 0.11	5.90 ± 0.08
0.5 + 0.5	7-8	66	+	3.00 ± 0.15	6.10 ± 0.14
0.5 + 1.0	7-8	74	+	3.00 ± 0.11	6.20 ± 0.11
0.5 + 2.0	7-8	55	+	2.00 ± 0.20	6.00 ± 0.11
1.0 + 0.2	7-8	72	+	2.00 ± 0.11	5.90 ± 0.08
1.0 + 0.5	7-8	80	+	4.00 ± 0.11	6.50 ± 0.28
1.0 + 1.0	7-8	76	+	5.00 ± 0.05	6.90 ± 0.08
1.0 + 2.0	7-8	62	+	2.00 ± 0.05	6.10 ± 0.08
2.0 + 0.2	7-8	60	+	2.00 ± 0.14	5.70 ± 0.12
2.0 + 0.5	7-8	72	+	3.00 ± 0.11	6.50 ± 0.28
2.0 + 1.0	7-8	75	+	2.00 ± 0.17	6.00 ± 0.23
2.0 + 2.0	7-8	55	+	1.30 ± 0.05	5.30 ± 0.11
Kn + IAA					
0.5 + 0.2	7-8	65	-	1.50 ± 0.05	4.50 ± 0.20
0.5 + 0.5	7-8	75	+	2.00 ± 0.11	5.00 ± 0.17
0.5 + 1.0	7-8	72	+	3.00 ± 0.11	5.40 ± 0.15
0.5 + 2.0	7-8	55	+	2.00 ± 0.15	5.00 ± 0.12
1.0 + 0.2	7-8	65	+	3.00 ± 0.11	5.00 ± 0.15
1.0 + 0.5	7-8	80	+	3.00 ± 0.15	5.20 ± 0.26
1.0 + 1.0	7-8	85	+	4.00 ± 0.05	5.80 ± 0.08
1.0 + 2.0	7-8	62	+	2.00 ± 0.05	5.00 ± 0.26
2.0 + 0.2	7-8	72	+	2.00 ± 0.11	4.80 ± 0.05
2.0 + 0.5	7-8	75	+	2.00 ± 0.11	5.00 ± 0.14
2.0 + 1.0	7-8	66	+	3.00 ± 0.15	5.00 ± 0.28
2.0 + 2.0	7-8	52	+	2.00 ± 0.30	4.10 ± 0.12

– : Absent of base callus, + : Present of base callus.

* Values are the mean of three replicates with 10 explants.

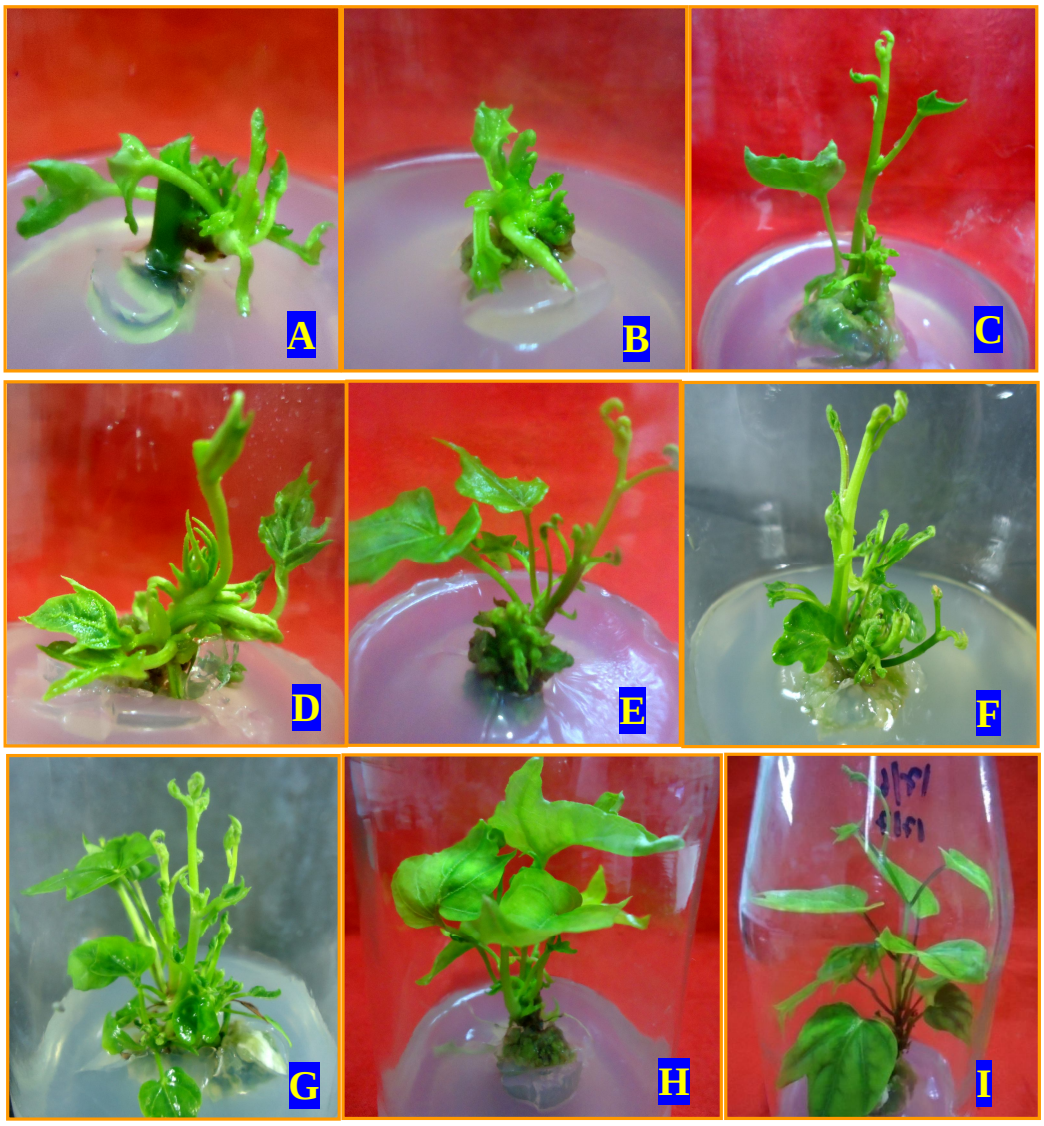


Plate 3.8 Development of multiple shoot from nodal explants of *I. mauritiana* in direct organogenesis and their subsequent development.

A: Initiation of multiple shoot formation in nodal segment after 2 weeks of inoculation in media having 1.0 mg/l BAP + 0.5 mg/l IAA; **B-G**: Proliferation of multiple shoot in the same medium after two subcultures at 14 days interval: **H-I**: Further proliferation and elongation of shoot after 8 weeks of culture in the same medium.

3.3.3.1.2. Direct multiple shoot induction from shoot tip explants of *I. mauritiana*

Shoot tip explants of two months old twigs were aseptically cultured on MS medium with different concentrations (0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) of BAP and Kn either alone or in combinations of BAP and Kn (0.5 mg/l, 1.0 mg/l and 2.0 mg/l) with different concentrations (0.2, 0.5, 1.0 and 2 mg/l) of NAA or IAA. Shoot tip explants found to be inferior for multiple shoot induction than nodal explant. Though shoot tip explant responded on almost all of the supplemented culture media but morphogenic responses of the cultured explants varied depending upon the culture media formulation. Effects of different concentrations and combinations of auxins and cytokinins on multiple shoot induction from shoot tip explants are described below:

Experiment 1. Effect of BAP, Kn and IAA alone on multiple shoot induction from shoot tip explant

Six concentrations (0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) of BAP , Kn and IAA alone were treated in MS medium for the purpose of multiple shoot induction from shoot tip explants of *I. mauritiana*. Data were taken after 6 weeks of inoculation and days taken for shoot initiation, percentage of shoot induction, number of shoot/culture and length of shoot/culture were measured. The results are presented in Table 3.19. The table shows that BAP was more effectives than Kn and IAA for multiple shoot induction from shoot tip explants. Though shoot tip explants respondent in all the concentrations (0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) of BAP, Kn and IAA but the percentage of explant showing proliferation was found varied from 20-65%. The maximum 65% cultures induced multiple shoots in media having 2.0 mg/l BAP followed by 60% in 1.0 mg/l BAP and 1.0 mg/l IAA. The lowest 20% response was noted in the media with 0.2 mg/l Kn and 4.0 mg/l IAA. The highest average number of shoot per culture 3.60 ± 0.05 was noted in the media having 1.0 mg/l BAP followed by 3.00 ± 0.05 in 2.0 mg/l BAP and 1.0 mg/l IAA. The lowest average number of shoot per culture 1.00 ± 0.05 were noted in the media having 0.2 mg/l Kn. The highest average length of shoot per culture was 5.80 ± 0.23 cm in the media having 2.0 mg/l IAA. And the lowest average length of shoot per culture was 4.00 ± 0.17 in the media having 0.2 mg/l BAP and 0.2 mg/l Kn. Experimental results revealed that, 1.0 mg/l and 2.0 mg/l of BAP, 2.0 mg/l Kn and 1.0 mg/l IAA were proved as the effective concentrations for multiple shoot induction in *I. mauritiana*. Experiment were set for the induction of multiple shoot but sometimes callus was found to grow at the basal part of the growing shoots in many media formulations.

Experiment 2. Effect of different concentrations and combinations of BAP with NAA and IAA on multiple shoot induction.

Explants were cultured on MS medium supplemented with three concentrations of BAP (0.5, 1.0 and 2.0 mg/l) combined with four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of NAA and IAA. Data were recorded after 4 weeks of culture and results on different parameters are presented in the Table 3.20. The efficiency of BAP + IAA was found better than BAP alone but more or less same of BAP + NAA on direct shoot regeneration. All the used media compositions formed multiple shoots and the results were inferior to the results obtained from nodal explants. Addition of lower concentration of NAA and IAA along with higher concentration of BAP was found more suitable than that of other concentrations. Among the combinations of BAP + NAA and BAP + IAA the highest percentage (80%) of shoot proliferation was noted in the media having 1.0 mg/l BAP + 1.0 mg/l IAA followed by (75%) in 1.0 mg/l BAP + 0.5 mg/l NAA. The lowest percentage (30%) of shoot proliferation was noted in media having 0.5 mg/l BAP + 0.2 mg/l NAA and 0.5 mg/l BAP + 0.2 mg/l IAA. The highest average number of shoot per culture was 4.00 ± 0.17 in the media having 1.0 mg/l BAP + 1.0 mg/l IAA and the lowest average number of shoot was 1.00 ± 0.57 per culture noted in the media having 0.5 mg/l BAP + 0.2 mg/l NAA and 0.5 mg/l BAP + 0.2 mg/l IAA. The highest average length of shoot 6.00 ± 0.25 cm per culture was found in the combination of 1.0 mg/l BAP + 1.0 mg/l IAA. Average lowest length of shoot 4.00 ± 0.11 cm per culture was found in the combination of 0.5 mg/l BAP + 2.0 mg/l NAA. Experimental results revealed that, 1.0 mg/l BAP + 0.5 mg/l NAA, 1.0 mg/l BAP + 1.0 mg/l NAA, 1.0 mg/l BAP + 1.0 mg/l IAA and 2.0 mg/l BAP + 0.5 mg/l IAA were more effective combinations for multiple shoot induction. Experiments were set for the induction of multiple shoot but sometimes callus was found to grow at the basal part of the growing shoots in many media formulations.

Experiment 3. Effect of different concentrations and combinations of Kn with NAA and IAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentrations (0.5, 1.0 and 2.0 mg/l) of Kn combined with four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of NAA or IAA. Data were recorded after 4 weeks of culture and results on different parameters are presented in the Table 3.21. All the used media compositions formed multiple shoots and the results were found inferior to the results obtained from nodal

explants. Addition of lower concentration of NAA and IAA along with higher concentration of Kn was found more suitable than that of other concentrations. Among the combinations of Kn + NAA and Kn + IAA, the highest percentage (75%) of shoot proliferation was noted in the media having 1.0 mg/l Kn + 1.0 mg/l NAA followed by 72% in 2.0 mg/l Kn +0.5 mg/l NAA and 1.0 mg/l Kn + 1.0 mg/l IAA. The lowest percentage (40%) of shoot proliferation was noted in the media having 2.0 mg/l Kn + 2.0 mg/l NAA. The average highest number of shoot per culture was 3.60 ± 0.17 in the media having 1.0 mg/l Kn + 0.5 mg/l IAA and the lowest number of shoot per culture was 1.00 ± 0.05 in the media having 0.5 mg/l Kn + 0.2 mg/l NAA and 0.5 mg/l Kn + 0.2 mg/l IAA. Average highest length of shoot was 6.00 ± 0.23 cm per culture found in the combination of 1.0 mg/l Kn + 1.0 mg/l NAA and the lowest length of shoot was 4.00 ± 0.05 cm per culture in 0.5 mg/l Kn + 0.2 mg/l IAA. Experimental results revealed that, 1.0 mg/l Kn + 1.0 mg/l NAA, 1.0 mg/l Kn + 1.0 mg/l IAA, 1.0 mg/l Kn + 0.5 mg/l NAA and 1.0 mg/l Kn + 0.5 mg/l IAA were more effective combinations for multiple shoot induction from shoot tip explants in *I. mauritiana*. Experiments were set for the induction of multiple shoot but sometimes callus was found to grow at the basal part of the growing shoots in many media formulations.

Table 3.19 Effect of different levels of BA, Kn and IAA used alone on multiple shoot induction from shoot tip explants of *I. mauritiana*. Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	Number of days taken for shoot initiation	Percentage (%) of shoot induction	Base callusing	Average number of shoot per culture (mean ± SE)	Average length (cm) of shoot per culture (mean ± SE)
BAP					
0.2	10-15	25	-	2.00 ± 0.11	4.00 ± 0.11
0.5	10-12	45	+	2.60 ± 0.10	4.50 ± 0.15
1.0	10-12	60	+	3.60 ± 0.05	5.50 ± 0.11
2.0	10-12	65	+	3.00 ± 0.05	4.80 ± 0.05
3.0	10-12	50	+	2.00 ± 0.17	4.50 ± 0.05
4.0	10-15	35	+	1.30 ± 0.05	4.63 ± 0.29
Kn					
0.2	10-15	20	-	1.00 ± 0.05	4.00 ± 0.17
0.5	10-15	30	+	2.30 ± 0.05	4.60 ± 0.11
1.0	10-12	45	+	2.00 ± 0.15	5.20 ± 0.11
2.0	10-12	55	+	2.00 ± 0.10	5.50 ± 0.20
3.0	10-12	40	+	3.00 ± 0.17	5.00 ± 0.11
4.0	10-15	30	+	2.00 ± 0.05	4.50 ± 0.05
IAA					
0.2	10-15	30	-	2.00 ± 0.05	4.50 ± 0.28
0.5	10-12	40	+	2.00 ± 0.20	5.00 ± 0.11
1.0	10-12	60	+	3.00 ± 0.20	5.47 ± 0.14
2.0	10-12	40	+	2.60 ± 0.05	5.80 ± 0.23
3.0	10-15	40	+	2.00 ± 0.11	5.00 ± 0.11
4.0	10-15	20	+	2.00 ± 0.11	4.20 ± 0.11

–: Absent of base callus, + : Present of base callus

* Values are the mean of three replicates with 10 explants.

Table 3.20 Effect of different concentrations and combinations of BAP with NAA and IAA on shoot multiplication from shoot tip explants of *I. mauritiana*. Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot induction	Base callusing	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP + NAA					
0.5 + 0.2	7-10	30	-	1.00 ± 0.57	4.00 ± 0.11
0.5 + 0.5	7-10	40	+	2.00 ± 0.15	4.50 ± 0.23
0.5 + 1.0	7-10	60	+	3.00 ± 0.30	4.20 ± 0.11
0.5 + 2.0	7-10	50	+	2.30 ± 0.11	3.80 ± 0.05
1.0 + 0.2	7-10	60	+	2.00 ± 0.05	5.00 ± 0.23
1.0 + 0.5	7-10	80	+	3.60 ± 0.11	5.00 ± 0.11
1.0 + 1.0	7-10	70	+	3.00 ± 0.28	5.20 ± 0.11
1.0 + 2.0	7-10	60	+	2.00 ± 0.11	4.80 ± 0.17
2.0 + 0.2	7-10	50	+	2.60 ± 0.11	4.30 ± 0.11
2.0 + 0.5	7-10	60	+	3.00 ± 0.15	4.80 ± 0.15
2.0 + 1.0	7-10	70	+	2.60 ± 0.17	5.07 ± 0.23
2.0 + 2.0	7-10	50	+	1.30 ± 0.05	4.50 ± 0.20
BAP + IAA					
0.5 + 0.2	10-12	30		1.00 ± 0.11	5.00 ± 0.20
0.5 + 0.5	10-12	60	-	2.30 ± 0.11	5.50 ± 0.05
0.5 + 1.0	10-12	65	+	3.00 ± 0.30	4.50 ± 0.05
0.5 + 2.0	10-12	50	+	2.50 ± 0.11	4.20 ± 0.11
1.0 + 0.2	10-12	60	+	3.00 ± 0.15	5.00 ± 0.17
1.0 + 0.5	10-12	70	+	3.30 ± 0.15	5.00 ± 0.11
1.0 + 1.0	10-12	75	+	4.00 ± 0.17	6.00 ± 0.25
1.0 + 2.0	10-12	50	+	2.00 ± 0.11	5.00 ± 0.05
2.0 + 0.2	10-12	40	+	2.60 ± 0.11	4.80 ± 0.11
2.0 + 0.5	10-12	60	+	3.00 ± 0.12	5.50 ± 0.15
2.0 + 1.0	10-12	70	+	2.30 ± 0.17	5.20 ± 0.11
2.0 + 2.0	10-12	45	+	1.60 ± 0.11	5.00 ± 0.23

– : Absent of base callus, + : Present of base callus

* Values are the mean of three replicates with 10 explants.

Table 3.21 Effect of different concentrations and combinations of Kn with NAA and IAA on shoot multiplication from shoot tip explants of *I. mauritiana.* Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	No. of days taken for shoot initiation	(%) of shoot induction	Base callusing	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
Kn + NAA					
0.5 + 0.2	8-10	45	-	1.00 ± 0.05	4.90 ± 0.11
0.5 + 0.5	8-10	50	+	1.30 ± 0.05	5.00 ± 0.05
0.5 + 1.0	8-10	66	+	2.00 ± 0.20	5.50 ± 0.11
0.5 + 2.0	8-10	50	+	1.60 ± 0.05	5.00 ± 0.23
1.0 + 0.2	8-10	52	+	2.60 ± 0.15	5.00 ± 0.11
1.0 + 0.5	8-10	66	+	3.00 ± 0.28	5.57 ± 0.08
1.0 + 1.0	8-10	75	+	3.30 ± 0.11	6.00 ± 0.23
1.0 + 2.0	8-10	55	+	2.00 ± 0.11	5.20 ± 0.11
2.0 + 0.2	8-10	62	+	3.00 ± 0.17	4.63 ± 0.14
2.0 + 0.5	8-10	72	+	2.60 ± 0.17	5.00 ± 0.11
2.0 + 1.0	8-10	62	+	2.30 ± 0.11	5.07 ± 0.23
2.0 + 2.0	8-10	40	+	1.60 ± 0.11	4.80 ± 0.17
Kn + IAA					
0.5 + 0.2	10-12	45		1.00 ± 0.05	4.00 ± 0.05
0.5 + 0.5	10-12	66	-	1.60 ± 0.11	5.20 ± 0.11
0.5 + 1.0	10-12	70	+	1.60 ± 0.17	5.60 ± 0.11
0.5 + 2.0	10-12	60	+	1.40 ± 0.10	4.80 ± 0.17
1.0 + 0.2	10-12	55	+	2.60 ± 0.05	5.00 ± 0.11
1.0 + 0.5	10-12	66	+	3.60 ± 0.17	5.20 ± 0.11
1.0 + 1.0	10-12	72	+	3.30 ± 0.15	5.60 ± 0.17
1.0 + 2.0	10-12	60	+	2.00 ± 0.11	5.00 ± 0.20
2.0 + 0.2	10-12	52	+	2.00 ± 0.11	4.50 ± 0.05
2.0 + 0.5	10-12	60	+	3.00 ± 0.17	4.80 ± 0.15
2.0 + 1.0	10-12	66	+	2.60 ± 0.17	5.00 ± 0.17
2.0 + 2.0	10-12	45	+	1.30 ± 0.05	4.50 ± 0.17

– : Absent of base callus, + : Present of base callus

* Values are the mean of three replicates with 10 explants.

3.3.3.2. Induction of callus on different explants in *I. mauritiana*

For the induction of callus internode and leaf explants were cultured on MS medium supplemented with different concentrations of 2,4–D singly and in combination with BAP and Kn. Data on days of callus initiation, percentage of explant induced callus, color and texture of callus were recorded after 8 weeks of culture. Internode explant showed better performance than leaf explant. The results are described according to types of explants under separate heads.

3.3.3.2.1. Callus induction from internode explants

Experiment 1. Effect of different concentrations and combinations of 2,4-D, BAP and Kn on callus induction from internode explants

In order to induce of callus, internode explants were cultured on MS medium supplemented with different concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of 2,4–D used alone and in combinations of three concentrations (0.5, 1.0 and 2.0 mg/l) of 2,4-D with four concentrations (0.5, 1.0, 2.0 and 3.0 mg/l) of BAP or Kn. Cultures were maintained under 16 h light and 8 h dark regime and data on different parameters are summarized in Table 3.22. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was not noticed in all media formulations. But there was a wide variation in morphological nature and percentage of callus formation among them. Callus initiation occurred within 8-14 days depending upon the concentration and combination of hormones. 2,4-D with BAP induced callus within 8-10 days, 2,4-D with Kn took same days and 2,4-D alone took 10-14 days to initiate callus. Percentage of callus formation ranged from 30.00-75.00 %. Among the tested combinations MS medium having 1.0 mg/l 2,4-D + 2.0 mg/l BAP found highest percentage (75%) of callus formation (Plate 3.9 B-F) followed by 70% in 2.0 mg/l 2,4-D+ 2.0 mg/l BAP and 1.0 mg/l 2,4-D +1.0 mg/l Kn. The lowest percentage (30%) of callus formation was obtained in media having 0.5 mg/l 2,4-D but 0.2 mg/l of 2,4-D did not produce any callus. The calli were green, white, brown, light green in color and nodular, compact and friable in texture.

3.3.3.2.2. Callus induction from leaf explant

Experiment 1. Effect of different concentrations and combinations of 2, 4-D, BAP and Kn on callus induction from leaf explant

In order to induce of callus, leaf explants were cultured on MS medium supplemented with different concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of 2,4–D used alone and in combination of three concentration of 2,4-D (0.5, 1.0 and 2.0 mg/l) with four concentrations (0.5, 1.0, 2.0 and 3.0 mg/l) of BAP or Kn. Cultures were maintained under 16 h light and 8 h dark regime and data on different parameters are summarized in Table 3.22. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was not noticed in all media formulations. But there was a wide variation in morphological nature and percentage of callus formation among them. Callus initiation occurred within 8-12 days depending upon the concentration and combination of hormones. 2,4-D with BAP induced callus within 8-10 days, 2,4-D with Kn took same days and 2,4-D alone took 10-12 days to initiate callus. Frequency of callus formation ranged from 40.00-90.00 %. Among the tested combinations MS medium having 1.0 mg/l 2,4–D + 2.0 mg/l BAP found highest percentage (90%) of callus formation (Plate 3.9 G-H) followed by 75% of 0.5 mg/l 2,4–D + 2.0 mg/l BAP and 0.5 mg/l 2,4–D + 2.0 mg/l Kn. The lowest percentage (30%) of callus formation was obtained in media having 0.5 mg/l 2,4–D but 0.2 mg/l of 2,4-D did not produce any callus. The calli were green, brown, light green in color and nodular, hard, compact and friable in texture.

Table 3.22 Effect of different concentrations and combinations of 2,4-D with BA and Kn on callus induction from leaf and internode explants of *I. mauritiana*. Data were taken after 6 weeks of culture.

Hormonal combination (mg/l)	Leaf explant			Internode explant		
	Days of callus initiation	% of explant induced callus development	Callus colour and texture	Days of callus initiation	% of explant induced callus development	Callus colour and texture
2,4-D						
0.2	-	-	-	-	-	-
0.5	10-12	40	bf	10-14	30	bf
1.0	10-12	50	bf	10-14	50	bf
2.0	10-12	65	bf	10-14	60	bn
3.0	10-12	55	bf	12-14	45	bn
2,4-D + BA						
0.5 + 0.5	8-10	50	wgf	8-10	40	lgf
0.5 + 1.0	8-10	60	wgf	8-10	50	lgf
0.5 + 2.0	8-10	75	lgc	8-10	65	lgf
0.5 + 3.0	8-10	65	lgc	8-10	55	wgf
1.0 + 0.5	8-10	60	bn	8-10	50	wgf
1.0 + 1.0	8-10	70	bn	8-10	60	wgf
1.0 + 2.0	8-10	90	wf	8-10	75	wgf
1.0 + 3.0	8-10	65	wf	8-10	55	wgf
2.0 + 0.5	8-10	50	lgc	8-10	50	wgf
2.0 + 1.0	8-10	60	lgc	8-10	60	wgf
2.0 + 2.0	8-10	80	lgc	8-10	70	wgf
2.0 + 3.0	8-10	65	lgc	8-10	50	wgf
2,4-D + Kn						
0.5 + 0.5	8-10	50	bn	8-10	40	bn
0.5 + 1.0	8-10	65	bn	8-10	50	bn
0.5 + 2.0	8-10	75	bn	8-10	65	bn
0.5 + 3.0	8-10	60	lgc	8-10	50	bn
1.0 + 0.5	8-10	50	wgn	8-10	60	bn
1.0 + 1.0	8-10	65	wgn	8-10	70	bn
1.0 + 2.0	8-10	80	wgn	8-10	65	wgn
1.0 + 3.0	8-10	70	wgn	8-10	50	wgn
2.0 + 0.5	8-10	50	bf	8-10	50	bn
2.0 + 1.0	8-10	60	bf	8-10	60	bn
2.0 + 2.0	8-10	70	bf	8-10	65	wgf
2.0 + 3.0	8-10	65	lgc	8-10	50	wgf

* Values are the mean of three replicates with 10 explants.

–: No response

bf: Brown friable, **bn:** Brown nodular, **lgc:** Light green compact, **lgf:** Light green friable, **wgf:** White green friable, **wgn:** White green nodular, **gc:** Green compact, **gf:** Green friable

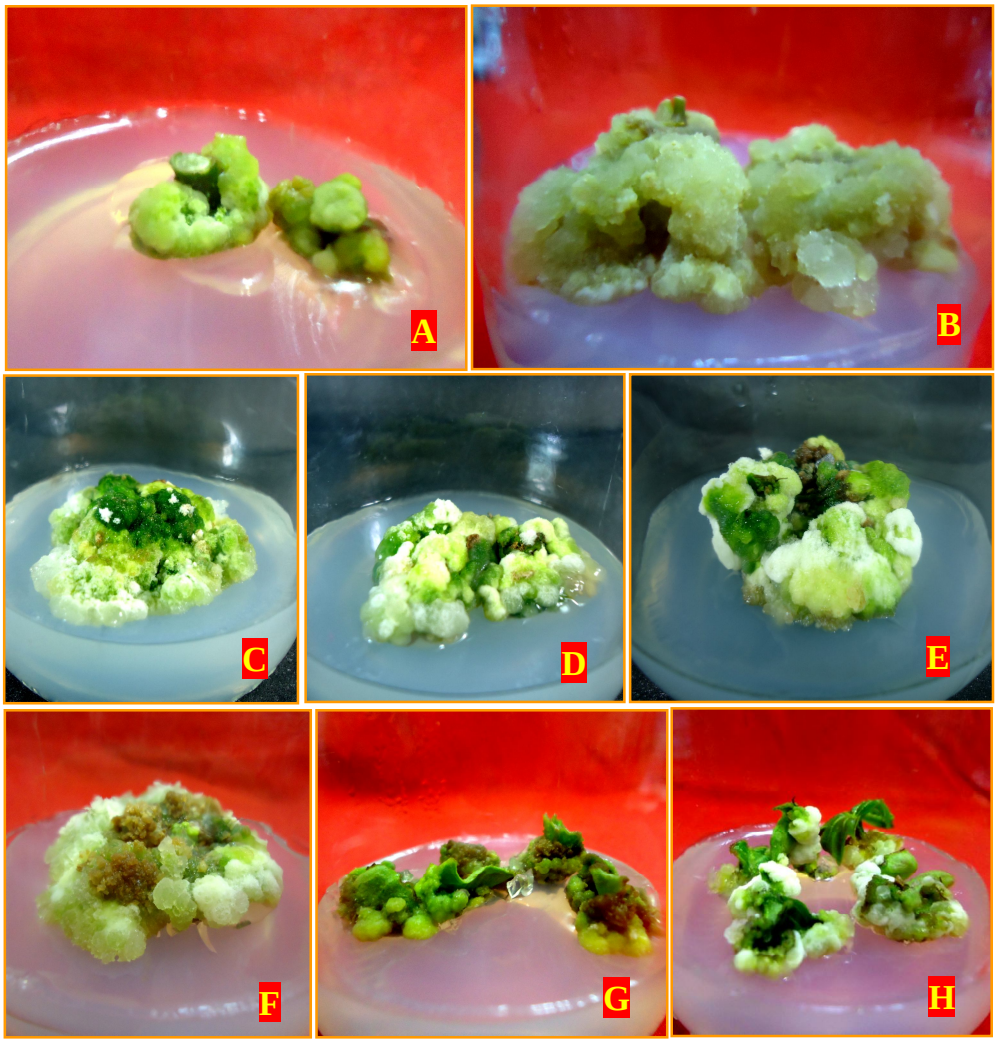


Plate 3.9 Callus induction from *in vitro* grown nodal and leaf explants of *I. mauritiana*.

A: Light green colour and compact callus induction from internodal segment in MS + 2.0 mg/l BAP + 1.0 mg/l 2,4-D after 4 weeks of culture; **B:** Brown colour and friable callus induction from internodal explants after 8 weeks of culture; **C-D:** Greenish colour compact callus induction from internodal segment in MS + 2.0 mg/l Kn + 1.0 mg/l 2,4-D after 6 weeks of culture; **E-F:** Further development of callus in the same medium after 8 weeks of culture; **G-H:** Light green colour and soft callus induction from leaf segment in MS + 2.0 mg/l BAP + 1.0 mg/l 2, 4-D after 4 weeks of culture.

3.3.3.3. Adventitious shoot regeneration from different types of calli in *I. mauritiana*

Calli produced in different plant growth regulators supplemented on MS medium did not differentiate into shoot buds in the same medium after subsequent subcultures. For the development of adventitious shoot buds from various types of calli, different media formulations were needed. A wide range of auxin and cytokinin supplemented media were used to standardize the organogenic response of calli. Among the different types of calli only brown soft and light green nodular callus found to be regenerative on different plant growth regulators supplemented media. The results are discussed according to types of explants under separate heads.

3.3.3.3.1. Plant regeneration from leaf derived callus

Different experiments were conducted to investigate plant regeneration ability through callus culture from leaf explants. Data on percentage of organogenic calli induced, average number of shoot per callus and average length of shoot per callus were collected after 8 weeks of culture. Findings of the experiments are described below:

Experiment 1. Effect of different concentrations and combinations of BAP, Kn, NAA and IAA on organogenesis of leaf derived callus

In this experiment leaf derived calli of *I. mauritiana* were used to investigate the effect of different hormonal concentrations and combinations on organogenesis. For shoot differentiation, light green nodular calli were sudcultured on to MS medium supplemented with different concentrations of BAP and Kn alone and in combination with different concentrations of BAP + NAA, Kn + NAA, BAP + IAA and Kn + IAA. Morphogenic potentialities of cultured calli varied with hormonal treatments (Table 3.23). Highest 30.00% of shoot regeneration was recorded in 1.0 mg/l BAP with 0.2 mg/l NAA and 1.0 mg/l BAP + 0.5 mg/l IAA followed by 25% in media having 1.0 mg/l Kn with 0.5 mg/l NAA and 1.0 mg/l BAP + 0.2 mg/l IAA. The lowest 10.00% of shoot regeneration was recorded in the media of 1.0 mg/l Kn. The highest number of shoots per callus was recorded 5.00 ± 0.17 in media having 1.0 mg/l BAP with 0.2 mg/l NAA followed by 4.50 ± 0.28 in 1.0 mg/l BAP + 0.2 mg/l IAA. The lowest number of shoots per callus was recorded 1.00 ± 0.05 in media having 0.5 mg/l Kn with 0.5 mg/l NAA. Highest length of shoot 6.00 ± 0.11 cm was recorded in 1.0 mg/l BAP + 0.2 mg/l IAA and followed by 5.60 ± 0.05 cm in BAP 1.0 mg/l + IAA 0.5 mg/l. and the lowest length of shoots 4.00 ± 0.17 cm was recorded in 1.0 mg/l Kn.

3.3.3.3.2. Plant regeneration from internode derived callus

Different experiments were conducted to investigate plant regeneration ability through callus culture from internode explants. Data on percentage of organogenic calli induced root and shoot, average number of shoot per callus and average length of shoot per callus were collected after 8 weeks of culture. Findings of the experiments are described below:

Experiment 1. Effect of different concentrations and combinations of BAP, Kn, NAA and IAA on organogenesis of internode derived callus

In this experiment internode derived calli of *I. mauritiana* were used to investigate the effect of different hormonal concentrations and combinations on organogenesis. For shoot differentiation brown nodular and light green nodular calli were sudcultured on to MS medium supplemented with different concentrations of BAP and Kn alone and in combination with different concentrations of BAP + NAA, Kn + NAA, BAP + IAA and Kn + IAA. Morphogenic potentialities of cultured calli varied with hormonal treatments (Table 3.23). Highest 40.00% of shoot regeneration was recorded in 1.0 mg/l BAP + 0.5 mg/l IAA (Plate 3.10) followed by 30% in media having 1.0 mg/l BAP + 0.2 mg/l NAA and 1.0 mg/l Kn + 0.2 mg/l NAA. The lowest 10.00% of shoot regeneration was recorded in media having 2.0 mg/l BAP and 1.0 mg/l Kn. The highest number of shoots per callus was recorded 5.30 ± 0.17 in media having 1.0 mg/l BAP + 0.2mg/l IAA. The lowest number of shoots per callus was recorded 1.00 ± 0.05 in media having 0.5 mg/l Kn + 0.5 mg/l NAA. Highest length of shoot 5.60 ± 0.05 cm was recorded in 1.0 mg/l BAP +0.5 mg/l IAA and the lowest length of shoots 4.00 ± 0.11 cm was recorded in 1.0 mg/l BAP and 1.0 mg/l Kn.

Table 3.23 Effect of BAP and Kn used singly and in combinations with NAA or IAA on adventitious shoot regeneration from leaf and internode derived callus of *I. mauritiana*. Data were recorded after 6 weeks of culture.

Hormonal combination (mg/l)	Leaf derived callus			Internode derived callus		
	% of regenerable calli	*Average number of shoot/calli (mean ± SE)	*Average length (cm) of shoot /calli (mean ± SE)	% of regenerable calli	*Average number of shoot/calli (mean ± SE)	*Average length (cm) of shoot /calli (mean ± SE)
BAP						
0.5	-	-	-	-	-	-
1.0	15	2.00 ± 0.11	4.60 ± 0.17	20	3.00 ± 0.17	4.00 ± 0.11
2.0	-	-	-	10	2.00 ± 0.17	4.30 ± 0.11
Kn						
0.5	-	-	-	-	-	-
1.0	10	2.00 ± 0.15	4.00 ± 0.17	10	2.00 ± 0.15	4.00 ± 0.17
2.0	-	-	-	-	-	-
BAP + NAA						
0.5 + 0.2	-	-	-	-	-	-
0.5 + 0.5	20	3.00 ± 0.15	4.60 ± 0.11	25	3.00 ± 0.15	4.60 ± 0.11
0.5 + 1.0	15	4.00 ± 0.17	5.00 ± 0.17	15	4.00 ± 0.17	5.00 ± 0.17
1.0 + 0.2	27	5.00 ± 0.17	5.60 ± 0.17	30	4.00 ± 0.17	5.00 ± 0.11
1.0 + 0.5	22	4.00 ± 0.17	4.60 ± 0.17	25	4.00 ± 0.17	4.60 ± 0.17
1.0 + 1.0	15	2.00 ± 0.11	4.40 ± 0.26	15	2.00 ± 0.11	4.40 ± 0.26
Kn + NAA						
0.5 + 0.2	-	-	-	-		
0.5 + 0.5	15	1.00 ± 0.05	4.60 ± 0.23	15	1.00 ± 0.05	4.60 ± 0.23
0.5 + 1.0	-	-	-	-	-	-
1.0 + 0.2	20	3.00 ± 0.11	5.60 ± 0.23	30	2.00 ± 0.17	5.30 ± 0.17
1.0 + 0.5	25	2.00 ± 0.17	5.00 ± 0.23	20	2.00 ± 0.17	5.00 ± 0.23
1.0 + 1.0	15	2.00 ± 0.11	4.30 ± 0.17	15	2.00 ± 0.11	4.30 ± 0.17
BAP + IAA						
0.5 + 0.2	-	-	-	-	-	-
0.5 + 0.5	-	-	-	-	-	-
0.5 + 1.0	-	-	-	-	-	-
1.0 + 0.2	25	4.50 ± 0.28	6.00 ± 0.11	25	5.00 ± 0.11	5.30 ± 0.17
1.0 + 0.5	30	4.00 ± 0.34	5.60 ± 0.05	40	5.30 ± 0.17	5.60 ± 0.05
1.0 + 1.0	15	2.00 ± 0.11	5.00 ± 0.11	20	2.00 ± 0.11	5.00 ± 0.11
Kn + IAA						
0.5 + 0.2	-	-	-	-	-	-
0.5 + 0.5	-	-	-	-	-	-
0.5 + 1.0	-	-	-	-	-	-
1.0 + 0.2	16	3.00 ± 0.26	4.30 ± 0.11	20	2.00 ± 0.17	4.60 ± 0.17
1.0 + 0.5	20	3.00 ± 0.11	5.00 ± 0.17	25	3.00 ± 0.11	5.00 ± 0.17
1.0 + 1.0	12	2.00 ± 0.11	5.00 ± 0.11	15	2.00 ± 0.11	5.00 ± 0.11

*Values are the mean of three replicates with 10 explants.

–: No response

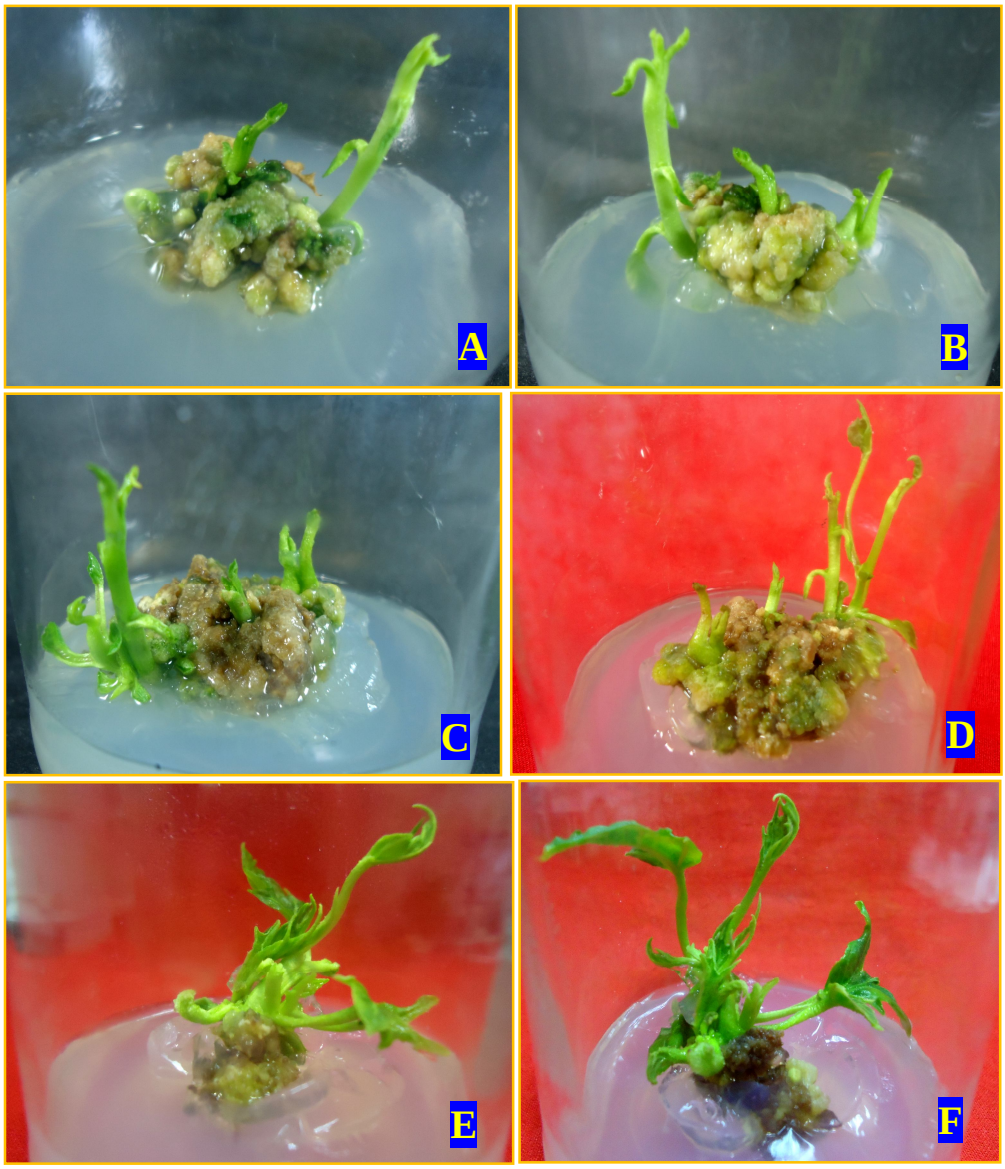


Plate 3.10 Plant regeneration from internode derived callus of *I. mauritiana* through the process of indirect organogenesis and their further growth.

A: Differentiation of callus tissue into multiple shoot bud in MS + 1.0mg/l BAP + 0.2 mg/l NAA after 6 weeks of subculture; **B-D**: Proliferation of multiple shoot buds in the same medium after 8 weeks of subculture and **E-F**: Further proliferation and elongation of shoot buds in the same medium after 10 weeks of subculture.

3.3.3.4. Rooting of *in vitro* grown shoots in *I. mauritiana*

Shoot cuttings 5–7 cm long taken from *in vitro* grown shoots were separated and transferred to rooting media. Half strength MS medium fortified with different concentrations (0.1, 0.2, 0.5, 1.0 and 2.0 mg/l) of IBA alone and in combinations of BAP and Kn (0.5, 1.0 and 2.0 mg/l) with different concentrations (0.1, 0.2, 0.5 mg/l) of IBA were used for rooting experiments. Rooting started within 8 to 15 days of culture. Data on days to root initiation, frequency of root formation (%), average number of root per culture and average length of root per culture were recorded after 6 weeks of culture. Findings of the experiments are described below:

Experiment 1. Effect of different concentrations and combinations of IBA, BAP and Kn on root induction from *in vitro* grown shoot explants of *I. mauritiana*

For adventitious root formation, the shoots obtained from *in vitro* grown shoot explants were excised and cultured on MS medium with different concentrations of IBA used singly and in combination with BAP and Kn. Results obtained for root induction, frequency of root formation, morphology, average number and length of roots are shown in Table 3.24. Percentage range of cultures produced roots varied from 20.00-90.00%. Highest 90.00% of root regeneration was recorded in 0.2 mg/l IBA ((Plate 3.11) followed by 85.00 % in media 1.0 mg/l BAP + 0.2 mg/l IBA. The lowest 20.00 % of root regeneration was recorded in media having 1.0 mg/l IBA. The highest average number of roots per shoot was recorded 56.00 ± 0.23 in media having 0.2 mg/l IBA. The lowest average number of root per shoots was recorded 8.00 ± 0.51 in media having 0.5 mg/l Kn + 0.1 mg/l IBA and 0.5 mg/l Kn + 0.5 mg/l IBA. Highest length of roots 10.00 ± 0.30 cm was recorded in 1.0 mg/l BAP+ 0.2 mg/l IBA followed by 9.00 ± 0.26 cm in media having 1.0 mg/l BAP + 0.1 mg/l IBA and 2.0 mg/l BAP + 0.2 mg/l IBA. The lowest length of roots 5.00 ± 0.32 cm was recorded in 0.5 mg/l Kn + 0.1 mg/l IBA. In most cases morphology of roots was thin, thick and long.

Table 3.24 Effect of different concentrations of IBA used alone and in combinations with BAP or Kn in half strength MS medium for root induction from *in vitro* grown shoots of *I. mauritiana*. Data were recorded after 6 weeks of culture.

Plants growth regulators (mg/l)	Days to root initiation	Percentage (%) of root formation	*Average number of roots/explants (mean ± SE)	*Average length (cm) of root/explant (mean ± SE)
IBA				
0.1	10-15	35	16.00 ± 0.51	6.00 ± 0.11
0.2	10-15	90	56.00 ± 0.23	8.00 ± 0.28
0.5	10-15	70	32.00 ± 0.35	7.00 ± 0.25
1.0	10-15	20	12.00 ± 0.41	6.00 ± 0.23
2.0	-	-	-	-
IBA + BAP				
0.1 + 0.5	8-12	35	10.00 ± 0.23	7.00 ± 0.23
0.1 + 1.0	8-12	70	36.00 ± 0.17	9.00 ± 0.26
0.1 + 2.0	8-12	80	16.33 ± 0.48	8.00 ± 0.28
0.2 + 0.5	8-12	40	22.00 ± 0.28	8.00 ± 0.11
0.2 + 1.0	8-12	85	48.00 ± 0.15	10.00 ± 0.30
0.2 + 2.0	8-12	60	26.33 ± 0.17	9.00 ± 0.34
0.5 + 0.5	8-12	20	18.00 ± 0.23	6.00 ± 0.17
0.5 + 1.0	8-12	60	26.33 ± 0.71	7.00 ± 0.23
0.5 + 2.0	8-12	40	22.00 ± 0.43	8.00 ± 0.28
IBA + Kn				
0.1 + 0.5	10-15	30	8.00 ± 0.51	5.00 ± 0.32
0.1 + 1.0	10-15	70	28.00 ± 0.25	7.00 ± 0.17
0.1 + 2.0	10-15	40	16.00 ± 0.11	6.00 ± 0.23
0.2 + 0.5	10-15	30	10.00 ± 0.34	6.00 ± 0.20
0.2 + 1.0	10-15	75	30.00 ± 0.15	8.00 ± 0.28
0.2 + 2.0	10-15	40	22.00 ± 0.11	7.00 ± 0.11
0.5 + 0.5	10-15	20	8.00 ± 0.20	5.00 ± 0.32
0.5 + 1.0	10-15	60	22.00 ± 0.26	7.00 ± 0.23
0.5 + 2.0	10-15	40	14.00 ± 0.15	6.00 ± 0.28

–: No response

* Values are the mean of three replicates with 5 explants.

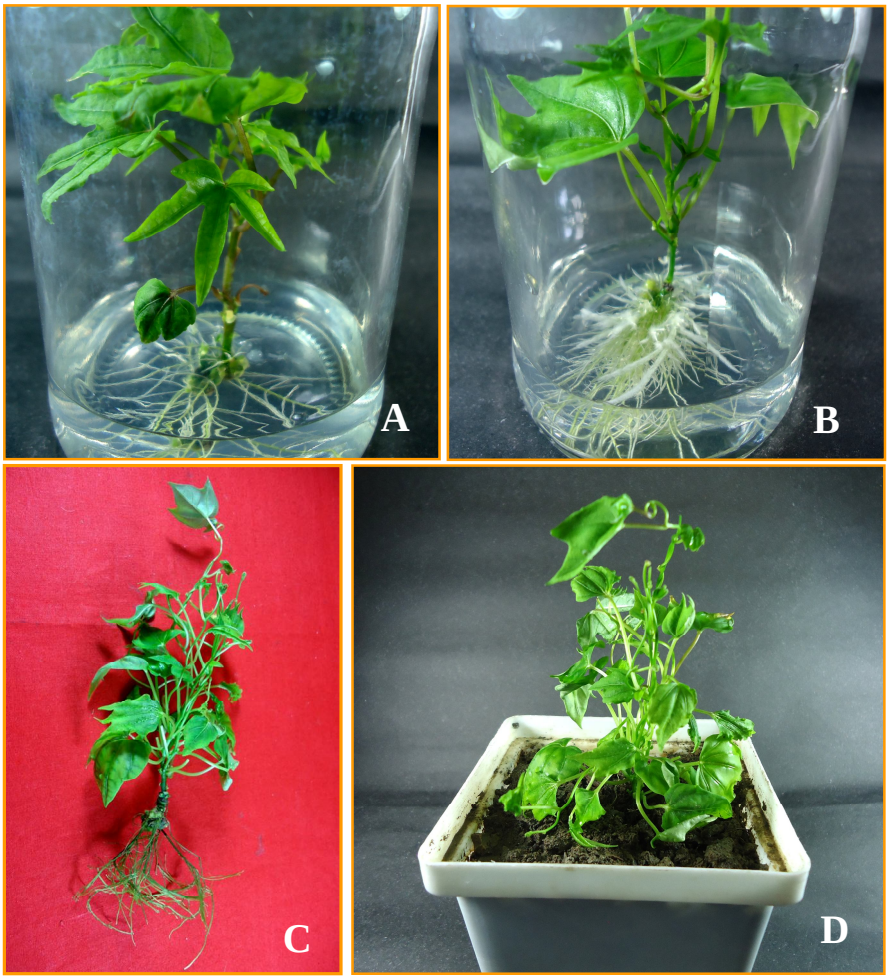


Plate 3.11 Induction of adventitious roots and establishment in soil in *I. mauritiana.*

A-B: Induction of adventitious roots on shoots regenerated from nodal explants in half strength MS medium containing 1.0 mg/l BAP + 0.2 mg/l IBA after 6 weeks of culture; **C:** *I. mauritiana* with roots outside of culture bottle; **D:** The *in vitro* grown seedlings acclimatized to grow in pots in outside environment after 6 weeks.

3.3.4. *In vitro* regeneration and conservation of *Mentha arvensis*

Experiments on direct and indirect organogenesis were carried out using different types of explants *viz*. shoot tip, nodal explants, internodal explants and leaves. Shoot tip and nodal explants were cultured for direct shoot regeneration. Explants were cultured on MS agar gelled medium supplemented with different concentrations of auxins and cytokinins used singly or in combinations to investigate the initiation of shoot and its subsequent regeneration. For root induction, micro shoots obtained from direct and indirect organogenesis were transferred to rooting media. Finally well developed plants with both shoots and roots were transferred to natural conditions through successive phages of acclimatization. Details of the results so far obtained from each of the experiments are described under following heads:

3.3.4.1. Direct regeneration

Two types of explants *viz*. shoot tip and nodal explants were cultured for direct shoot regeneration. Explants were cultured on MS medium with BAP, Kn, NAA used alone and in combinations with each other. Data on number of days taken for shoot initiation, percentage of explant induced shoot development, average number of shoot per culture and average length of shoot per culture were collected after 6 weeks of culture. Among the two types of explants nodal explants responded on almost all of the supplemented cultured medium. The results are described according to types of explants under separate heads.

3.3.4.1.1. Shoot tip explants

Experiment 1. Effect of different concentrations of BAP and Kn used alone and combination on multiple shoot induction from shoot tip explants of *M. arvensis*

In this present investigation five concentrations of BAP (0.2-3.0 mg/l), five concentrations of Kn (0.2-3.0 mg/l) used alone and in combination with three concentrations of BAP (0.5-2.0 mg/l) and four concentration of Kn (0.2-2.0 mg/l) were treated in MS medium for the purpose of multiple shoot induction from shoot tip segments of *M. arvensis*. Data were taken after 4 weeks of inoculation and percentage of shoot formation, number of shoot/culture and length of shoot/culture were measured. The results are presented in Table 3.25. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Shoot proliferation was not noticed in all media formulations. Shoot proliferation ranged from 20.00-82.00%. Highest percentage (82.00%) of multiple shoot formation was observed in MS medium containing 1.0 mg/l BAP + 0.5 mg/l Kn followed by

(75.00%) in MS medium containing 2.0 mg/l BAP + 0.5 mg/l Kn, 1.0 mg/l BAP + 1.0 mg/l Kn and 1.0 mg/l BAP. The lowest percentage (20.00%) of multiple shoot formation was observed in media having 3.0 mg/l Kn. Highest mean number of shoots was 12.00 ± 0.17 in media having 1.0 mg/l BAP + 0.5 mg/l Kn and lowest mean number of shoot was 2.00 ± 0.17 in media containing 0.2 mg/l Kn. Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 28 days of culture. Highest average length was recorded 8.00 ± 0.34 cm in 1.0 mg/l BAP + 0.5 mg/l Kn and followed by 7.60 ± 0.17 cm in 1.0 mg/l BAP + 1.0 mg/l Kn. On the other hand lowest average length was 3.47 ± 0.14 cm in 3.0 mg/l Kn. Experimental results revealed that, 1.0 mg/l of BAP and 2.0 mg/l Kn alone and in combinations of 1.0 mg/l BAP + 0.5 mg/l Kn and 2.0 mg/l BAP + 0.5 mg/l Kn were found more effective concentrations and combinations for multiple shoot induction in *M. arvensis*.

Experiment 2. Effect of different concentrations and combinations of BAP and Kn with NAA on multiple shoot induction from shoot tip explants of *M. arvensis*

Explants were cultured on MS medium supplemented with three concentrations of BAP (1.0, 2.0 and 3.0 mg/l) and Kn (1.0, 2.0 and 3.0 mg/l) combined with four concentrations of NAA (0.1, 0.2, 0.5 and 1.0 mg/l). Data were recorded after 4 weeks of culture and results on different parameters are presented in the Table 3.26. The efficiency of BAP + NAA was better than BAP alone and Kn + NAA combination on direct shoot regeneration. All the used media compositions formed multiple shoots and the results were inferior to the results obtained from nodal explants. Addition of lower concentration of NAA along with higher concentration of BAP was found more suitable than that of other concentrations. Among the combinations of BAP + NAA and Kn + NAA, the highest percentage (85%) of shoot proliferation was noted in the media having 1.0 mg/l BAP + 0.2 mg/l NAA followed by 75% in 1.0 mg/l BAP + 0.5 mg/l NAA and1.0 mg/l Kn + 0.2 mg/l NAA. And the lowest percentage (40%) of shoot proliferation was noted in media having 2.0 mg/l Kn + 1.0 mg/l NAA. Highest mean number of shoot per culture was 16.00 ± 0.17 in the media having1.0 mg/l BAP + 0.2 mg/l NAA followed by 14.00 ± 0.28 in 2.0 mg/l BAP + 0.5 mg/l NAA and 1.0 mg/l Kn + 0.2 mg /l NAA. The lowest mean number of shoot per culture was 6.00 ± 0.36 noted in the media having 2.0 mg/l BAP +0.1 mg/l NAA. Highest average length of shoot per culture was 8.50 ± 0.23 cm in the media having 1.0 mg/l Kn + 0.2 mg/l NAA. Average

lowest length of shoot 4.57 ± 0.23 cm per culture was found in the combination of 0.5 mg/l BAP +1.0 mg/l NAA.

Experiment 3. Effect of different concentrations of GA_3 alone and in combinations with BAP and Kn on *in vitro* shoot multiplication and elongation from shoot tip explants of *M. arvensis*

Effect of different levels of GA_3 (0.1, 0.2, 0.5, 1.0 and 2.0 mg/l) alone and combination of three concentrations GA_3 (0.2, 0.5 and 1.0 mg/l) with three concentrations of BAP (0.5, 1.0 and 2.0 mg/l) or Kn (0.5, 1.0 and 2.0 mg/l) were treated in MS medium for the purpose of multiple shoot induction and elongation from shoot tip segments of *M. arvensis*. Data were taken after 4 weeks of inoculation and frequency of shoot elongation, number of shoot per culture and length of shoot per culture were measured. The results are presented in Table 3.27. Morphogenic responses of shoot elongation were found to vary with hormonal formulations present in the culture media. Shoot elongation was not noticed in all media formulations. Shoot elongation ranged from 40.00–95.00%. Highest percentage (95.00%) of multiple shoot elongation was observed in MS medium containing 0.5 mg/l GA_3 and followed by (85.00%) in MS medium containing 1.0 mg/l BAP + 0.5 mg/l GA_3 and 1.0 mg/l Kn + 0.5 mg/l GA_3. Lowest percentage (40.00%) of multiple shoot elongation was observed in media having 0.1 mg/l GA_3. Highest mean number of shoots elongation was 26.90 ± 0.55 in media having 1.0 mg/l BAP + 0.5 mg/l GA_3 followed by 24.93 ± 0.38 in 1.0 mg/l BAP + 1.0 mg/l GA_3. Lowest mean number of shoot elongation was 6.30 ± 0.17 in media containing 0.1 mg/l GA_3. Elongation of average shoot length gradually increased after induction of shoot. Length of shoots elongation was recorded at 28 days of culture. Highest average length of shoot elongation was recorded 12.10 ± 0.05 cm in 0.5 mg/l BAP + 0.5 mg/l GA_3 followed by 11.60 ± 0.17 cm in 0.5 mg/l GA_3. Average lowest length of shoot elongation was 7.53 ± 0.12 cm in 0.5 mg/l Kn + 0.2 mg/l GA_3. Experimental results revealed that, 0.5 mg/l of GA_3 alone and combination of 1.0 mg/l BAP +0.5 mg/l GA_3 , 1.0 mg/l Kn+ 0.5 mg/l GA_3 were found more effective concentrations and combinations for multiple shoot elongation in *M. arvensis*.

Table 3.25 Effect of different concentrations of BAP and Kn alone and combinations on multiple shoot induction from shoot tip explants of *M. arvensis*.

Plant growth regulators (mg/l)	Number of days taken for shoot initiation	Percentage(%) of shoot regeneration	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP				
0.2	7-10	30	3.60 ± 0.20	4.47 ± 0.18
0.5	6-8	64	4.87 ± 0.20	5.30 ± 0.11
1.0	7-10	75	5.97 ± 0.20	5.20 ± 0.11
2.0	7-10	70	5.53 ± 0.20	6.20 ± 0.11
3.0	7-10	45	3.17 ± 0.08	3.60 ± 0.20
Kn				
0.2	7-10	25	2.00 ± 0.17	4.70 ± 0.20
0.5	7-10	50	2.40 ± 0.17	6.50 ± 0.28
1.0	6-8	60	5.50 ± 0.17	7.00 ± 0.17
2.0	6-8	70	5.00 ± 0.11	5.97 ± 0.13
3.0	7-10	20	2.80 ± 0.20	3.47 ± 0.14
BAP + Kn				
0.5 + 0.2	5-7	40	4.30 ± 0.17	4.53 ± 0.12
0.5 + 0.5	5-7	54	6.17 ± 0.12	5.50 ± 0.17
0.5 + 1.0	5-7	70	8.00 ± 0.32	6.60 ± 0.11
0.5 + 2.0	5-7	70	10.00 ± 0.34	6.10 ± 0.10
1.0 + 0.2	5-7	50	8.50 ± 0.23	6.50 ± 0.05
1.0 + 0.5	5-7	82	12.00 ± 0.17	8.00 ± 0.34
1.0 + 1.0	5-7	75	10.00 ± 0.35	7.60 ± 0.17
1.0 + 2.0	5-7	70	10.63 ± 0.20	6.20 ± 0.20
2.0 + 0.2	5-7	55	8.00 ± 0.32	5.17 ± 0.12
2.0 + 0.5	5-7	75	10.00 ± 0.30	7.30 ± 0.17
2.0 + 1.0	7-10	60	8.00 ± 0.11	5.50 ± 0.05
2.0 + 2.0	7-10	42	6.20 ± 0.11	6.10 ± 0.10

* Values are the mean of three replicates with 10 explants.

Table 3.26 Effect of different concentration and combinations of BAP and Kn with NAA on multiple shoot induction from shoot tip explants of *M. arvensis.*

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	Average number of shoots / culture (mean ± SE)	Average length (cm) of shoots / culture (mean ± SE)
BAP + NAA				
0.5 + 0.1	7-10	60	8.00 ± 0.32	5.00 ± 0.23
0.5 + 0.2	5-7	70	10.00 ± 0.35	6.60 ± 0.17
0.5 + 0.5	5-7	70	10.63 ± 0.20	6.10 ± 0.11
0.5 + 1.0	5-7	50	8.50 ± 0.23	4.57 ± 0.23
1.0 + 0.1	7-10	65	8.30 ± 0.17	6.60 ± 0.11
1.0 + 0.2	5-7	80	16.00 ± 0.17	8.30 ± 0.17
1.0 + 0.5	5-7	75	12.00 ± 0.34	7.20 ± 0.20
1.0 + 1.0	5-7	60	10.30 ± 0.30	5.40 ± 0.32
2.0 + 0.1	7-10	60	6.00 ± 0.17	5.27 ± 0.14
2.0 + 0.2	7-10	75	12.00 ± 0.34	6.30 ± 0.20
2.0 + 0.5	7-10	70	14.00 ± 0.28	7.30 ± 0.15
2.0 + 1.0	7-10	45	11.60 ± 0.10	6.50 ± 0.05
Kn + NAA				
0.5 + 0.1	5-7	50	10.30 ± 0.30	6.70 ± 0.17
0.5 + 0.2	5-7	65	10.00 ± 0.17	6.10 ± 0.10
0.5 + 0.5	5-7	70	12.00 ± 0.11	7.60 ± 0.17
0.5 + 1.0	5-7	60	12.00 ± 0.34	5.17 ± 0.12
1.0 + 0.1	7-10	60	10.63 ± 0.20	6.10 ± 0.10
1.0 + 0.2	5-7	75	14.00 ± 0.28	8.50 ± 0.23
1.0 + 0.5	5-7	65	12.00 ± 0.34	7.30 ± 0.17
1.0 + 1.0	5-7	60	10.00 ± 0.17	6.20 ± 0.11
2.0 + 0.1	7-10	50	6.00 ± 0.36	6.50 ± 0.20
2.0 + 0.2	7-10	60	8.00 ± 0.28	6.60 ± 0.17
2.0 + 0.5	7-10	45	10.63 ± 0.20	7.30 ± 0.17
2.0 + 1.0	7-10	40	8.00 ± 0.32	6.20 ± 0.11

* Values are the mean of three replicates with 10 explants.

Table 3.27 Effect of different concentrations of GA_3 alone and combination with BAP or Kn on *in vitro* shoot multiplication and elongation from shoot tip explants of *M. arvensis.*

Plant growth regulators (mg/l)	Percentage (%) of shoot elongation	*Average number of shoot elongation per culture (mean ± SE)	*Average length (cm) of shoot elongation / culture (mean ± SE)
GA_3			
0.1	40	6.30 ± 0.17	8.20 ± 0.10
0.2	70	8.20 ± 0.10	10.67 ± 0.21
0.5	95	10.63 ± 0.20	11.60 ± 0.10
1.0	75	12.00 ± 0.11	9.30 ± 0.17
2.0	60	10.10 ± 0.05	9.00 ± 0.11
BAP + GA_3			
0.5 + 0.2	50	8.00 ± 0.23	9.50 ± 0.26
0.5 + 0.5	70	18.30 ± 0.05	12.10 ± 0.05
0.5 + 1.0	75	16.00 ± 0.23	10.67 ± 0.21
1.0 + 0.2	70	22.00 ± 0.11	9.50 ± 0.26
1.0 + 0.5	85	26.90 ± 0.55	10.30 ± 0.15
1.0 + 1.0	70	24.93 ± 0.38	10.30 ± 0.15
2.0 + 0.2	60	12.40 ± 0.05	9.57 ± 0.21
2.0 + 0.5	70	16.30 ± 0.25	10.50 ± 0.20
2.0 + 1.0	50	16.00 ± 0.23	10.20 ± 0.11
Kn + GA_3			
0.5 + 0.2	55	10.30 ± 0.15	7.53 ± 0.12
0.5 + 0.5	70	18.30 ± 0.05	9.57 ± 0.21
0.5 + 1.0	60	18.47 ± 0.17	9.20 ± 0.11
1.0 + 0.2	70	15.77 ± 0.67	9.10 ± 0.05
1.0 + 0.5	85	22.50 ± 0.15	10.67 ± 0.21
1.0 + 1.0	60	18.00 ± 0.28	9.40 ± 0.11
2.0 + 0.2	45	8.00 ± 0.23	8.27 ± 0.24
2.0 + 0.5	60	12.57 ± 0.14	9.20 ± 0.11
2.0 + 1.0	50	10.67 ± 0.21	8.50 ± 0.05

* Values are the mean of three replicates with 10 explants.

3.3.4.1.2. Nodal explants

Experiment 1. Effect of different concentrations of BAP and Kn used alone and combinations on multiple shoot induction from nodal explants of *M. arvensis*

In this present investigation five concentrations of BAP (0.2-3.0 mg/l), five concentrations of Kn (0.2-3.0 mg/l) alone and combination of three concentrations (0.5-2.0 mg/l) of BAP with four concentrations (0.2-2.0 mg/l) of Kn were treated in MS medium for the purpose of multiple shoot induction from nodal segments of *M. arvensis*. Data were taken after 4 weeks of inoculation and percentage of shoot formation, number of shoot/culture and length of shoot/culture were measured. The results are presented in Table 3.28. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Shoot proliferation was not noticed in all media formulations. Shoot proliferation ranged from 25.00-90.00%. Highest percentage (90.00%) of multiple shoot formation was observed in MS medium containing 1.0 mg/l BAP + 0.5 mg/l Kn followed by 80.00% in MS medium containing 1.0 mg/l BAP alone and 2.0 mg/l BAP + 0.5 mg/l Kn. Lowest percentage (25.00%) of multiple shoot formation was observed in media having 3.0 mg/l Kn. Highest mean number of shoots was recorded 12.30 ± 0.10 in media having 1.0 mg/l BAP + 0.5 mg/l Kn followed by 10.63 ± 0.20 in 1.0 mg/l BAP + 1.0 mg/l Kn And lowest mean number of shoot per culture was 2.30 ± 0.17 in media containing 3.0 mg/l Kn. Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 28 days of culture. Highest average length was recorded 8.30 ± 0.15 cm in 1.0 mg/l BAP + 1.0 mg/l Kn and the lowest average length was 4.20 ± 0.11cm in 3.0 mg/l BAP. Experimental results revealed that, 1.0 mg/l of BAP and 2.0 mg/l Kn alone and combination of 1.0 mg/l BAP+ 0.5 mg/l Kn, 2.0 mg/l BAP+ 0.5 mg/l Kn were found more effective concentrations for multiple shoot induction in *M. arvensis* from nodal segment.

Experiment 2. Effect of different concentrations and combinations of BAP and Kn with NAA on multiple shoot induction from nodal explants of *M. arvensis*

Explants were cultured on MS medium supplemented with three concentrations of BAP (1.0, 2.0 and 3.0 mg/l) and Kn (1.0, 2.0 and 3.0 mg/l) combined with four concentrations of NAA (0.1, 0.2, 0.5 and 1.0 mg/l). Data were recorded after 4 weeks of culture and results on different parameters are presented in the Table 3.29. The

efficiency of BAP + NAA was better than BAP alone and Kn + NAA combination on direct shoot regeneration. All the used media compositions formed multiple shoots and the results were superior to the results obtained from shoot tip explants. Addition of lower concentration of NAA along with higher concentration of BAP was found more suitable than that of other concentrations. Among the combinations of BAP + NAA and Kn +NAA, the highest percentage (95%) of shoot proliferation was noted in the media having 1.0 mg/l BAP + 0.2 mg/l NAA. The lowest percentage (30%) of shoot proliferation was recorded in 2.0 mg/l Kn + 1.0 mg/l NAA. Highest average number of shoot per culture was recorded in 16.00 ± 0.23 in media having 1.0 mg/l BAP + 0.5 mg/l NAA and 1.0 mg/l Kn + 0.2 mg/l NAA. The lowest average number of shoot per culture was recorded 6.30 ± 0.17 in 2.0 mg/l Kn + 0.1 mg/l NAA and 0.5 mg/l BAP + 0.1 mg/l NAA (Plate 3.12). Highest average length of shoot per culture was recorded in 8.00 ± 0.17 cm in 1.0 mg/l Kn + 0.2 mg/l NAA. The lowest average length of shoot per culture was recorded 4.27 ± 0.14 cm in 0.5 mg/l BAP + 1.0 mg/l NAA. Experimental results revealed that, 1.0 mg/l BAP + 0.2 mg/l NAA, 1.0 mg/l BAP + 0.5 mg/l NAA, 1.0 mg/l Kn + 0.2 mg/l NAA and 1.0 mg/l Kn + 0.5 mg/l NAA were found most effectives combinations for multiple shoot induction.

Experiment 3. Effect of different concentrations of GA_3 alone and combination with BAP and Kn on *in vitro* shoot multiplication and elongation from nodal explants

Effect of different levels of GA_3 (0.1, 0.2, 0.5, 1.0 and 2.0 mg/l) alone and combination of three concentrations (0.2, 0.5 and 1.0 mg/l) of GA_3 with three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP or Kn were treated in MS medium for the purpose of multiple shoot induction and elongation from nodal segments of *M. arvensis*. Data were taken after 4 weeks of inoculation and frequency of shoot elongation, number of shoot elongation/culture and length of shoot elongation/culture were measured. The results are presented in Table 3.30. Morphogenic responses of shoot elongation were found to vary with hormonal formulations present in the culture media. Shoot elongation was not noticed in all media formulations. Shoot elongation ranged from 46.00-100.00%. Highest percentage (100.00%) of multiple shoot elongation was observed in MS medium containing 0.5 mg/l GA_3 followed by (90.00%) in MS medium containing 1.0 mg/l BAP + 0.5 mg/l GA_3 and 1.0 mg/l GA_3. The lowest percentage (46.00%) of multiple shoot elongation was observed in media having 0.1 mg/l GA_3. Highest mean number of shoots elongation was 32.07 ± 0.46 in media having 1.0 mg/l BAP + 0.5 mg/l GA_3 followed by 22.37 ± 0.12 in the media having 1.0 mg/l BAP + 1.0 mg/l GA_3.

Lowest mean number of shoot elongation was 8.00 ± 0.32 in media containing 2.0 Kn + 0.2 mg/l GA_3. Elongation of average shoot length gradually increased after induction of shoot. Length of shoot elongation was recorded at 28 days of culture. Highest average length of shoot elongation was recorded 12.30 ± 0.10 cm. in 1.0 mg/l BAP + 1.0 mg/l GA_3 followed by 11.60 ± 0.10 cm in 0.5 mg/l GA_3 and the lowest average length of shoot elongation was 7.53 ± 0.12 cm in 2.0 mg/l Kn + 1.0 mg/l GA_3. Experimental results revealed that, 0.5 mg/l of GA_3 alone and combination of 1.0 mg/l BAP +0.5 mg/l GA_3 and 1.0 mg/l Kn+ 0.5 mg/l GA_3 were found more effective concentrations and combinations for multiple shoot elongation in *M. arvensis.*

Table 3.28 Effect of different concentrations of BAP and Kn used alone and combinations on multiple shoot induction from nodal explants of *M. arvensis*.

Plant growth regulators (mg/l)	Number of days taken for shoot initiation	Percentage (%) of shoot regeneration	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP				
0.2	7-10	40	4.53 ± 0.12	4.30 ± 0.17
0.5	7-10	60	6.30 ± 0.20	5.50 ± 0.17
1.0	5-7	80	8.50 ± 0.23	6.60 ± 0.11
2.0	5-7	75	6.50 ± 0.28	6.00 ± 0.20
3.0	7-10	66	4.70 ± 0.20	4.20 ± 0.11
Kn				
0.2	-	-	-	-
0.5	7-10	50	6.30 ± 0.20	6.30 ± 0.20
1.0	6-8	66	6.60 ± 0.17	6.70 ± 0.17
2.0	6-8	70	2.80 ± 0.20	7.30 ± 0.17
3.0	7-10	25	2.30 ± 0.17	5.00 ± 0.23
BAP + Kn				
0.5 + 0.2	5-7	40	4.50 ± 0.28	4.70 ± 0.20
0.5 + 0.5	5-7	60	8.50 ± 0.23	5.50 ± 0.20
0.5 + 1.0	5-7	70	10.30 ± 0.15	6.43 ± 0.08
0.5 + 2.0	5-7	70	8.00 ± 0.32	5.30 ± 0.11
1.0 + 0.2	5-7	50	8.30 ± 0.17	5.03 ± 0.17
1.0 + 0.5	5-7	90	12.30 ± 0.10	7.20 ± 0.20
1.0 + 1.0	5-7	75	10.63 ± 0.20	8.30 ± 0.15
1.0 + 2.0	5-7	60	10.20 ± 0.11	5.77 ± 0.12
2.0 + 0.2	5-7	55	6.50 ± 0.28	5.23 ± 0.17
2.0 + 0.5	5-7	80	10.30 ± 0.15	7.20 ± 0.20
2.0 + 1.0	7-10	60	8.30 ± 0.15	6.50 ± 0.28
2.0 + 2.0	7-10	40	6.50 ± 0.28	5.17 ± 0.12

* Values are the mean of three replicates with 10 explants.

Table 3.29 Effect of different concentration and combinations of BAP and Kn with NAA on multiple shoot induction from nodal explants of *M. arvensis.*

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of total shoots / culture (mean ± SE)	*Average length of shoots / culture (mean ± SE)
BAP + NAA				
0.5 + 0.1	7-10	65	6.30 ± 0.20	5.07 ± 0.08
0.5 + 0.2	5-7	80	10.00 ± 0.11	6.50 ± 0.28
0.5 + 0.5	5-7	70	10.20 ± 0.11	6.17 ± 0.12
0.5 + 1.0	5-7	50	8.30 ± 0.20	4.27 ± 0.14
1.0 + 0.1	7-10	60	8.10 ± 0.05	6.23 ± 0.08
1.0 + 0.2	5-7	95	10.30 ± 0.15	6.97 ± 0.12
1.0 + 0.5	5-7	75	16.00 ± 0.23	7.53 ± 0.12
1.0 + 1.0	5-7	60	10.50 ± 0.20	5.00 ± 0.20
2.0 + 0.1	7-10	70	10.20 ± 0.11	5.13 ± 0.37
2.0 + 0.2	7-10	80	12.40 ± 0.11	6.50 ± 0.28
2.0 + 0.5	7-10	70	12.10 ± 0.05	7.30 ± 0.10
2.0 + 1.0	7-10	45	8.00 ± 0.28	6.20 ± 0.11
Kn + NAA				
0.5 + 0.1	5-7	50	10.00 ± 0.23	6.20 ± 0.11
0.5 + 0.2	5-7	65	10.30 ± 0.15	6.50 ± 0.28
0.5 + 0.5	5-7	70	12.57 ± 0.14	7.30 ± 0.10
0.5 + 1.0	5-7	60	12.10 ± 0.05	5.53 ± 0.28
1.0 + 0.1	7-10	60	10.30 ± 0.15	5.07 ± 0.26
1.0 + 0.2	5-7	80	16.00 ± 0.23	6.50 ± 0.28
1.0 + 0.5	5-7	65	12.30 ± 0.10	7.30 ± 0.10
1.0 + 1.0	5-7	60	10.50 ± 0.20	6.30 ± 0.17
2.0 + 0.1	7-10	50	6.30 ± 0.17	6.20 ± 0.11
2.0 + 0.2	7-10	65	8.10 ± 0.05	6.50 ± 0.28
2.0 + 0.5	7-10	45	10.20 ± 0.11	7.30 ± 0.10
2.0 + 1.0	7-10	30	8.10 ± 0.05	6.20 ± 0.11

* Values are the mean of three replicates with 10 explants.

Table 3.30 Effect of different concentrations of GA3 alone and in combination of BAP and Kn on *in vitro* shoot elongation from nodal explants of *M. arvensis.*

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of total shoots / culture (mean ± SE)	*Average length of shoots / culture (mean ± SE)
GA3				
0.1	5-7	46	8.30 ± 0.15	8.10 ± 0.05
0.2	5-7	74	12.00 ± 0.23	9.40 ± 0.11
0.5	5-7	100	16.00 ± 0.23	11.60 ± 0.10
1.0	5-7	90	14.20 ± 0.11	9.20 ± 0.11
2.0	5-7	62	10.00 ± 0.15	8.10 ± 0.05
BAP + GA3				
0.5 + 0.2	5-7	70	10.20 ± 0.11	9.10 ± 0.05
0.5 + 0.5	5-7	80	16.30 ± 0.25	10.67 ± 0.21
0.5 + 1.0	5-7	75	16.00 ± 0.23	9.40 ± 0.11
1.0 + 0.2	5-7	75	15.00 ± 0.28	8.50 ± 0.05
1.0 + 0.5	5-7	90	32.07 ± 0.46	12.30 ± 0.10
1.0 + 1.0	5-7	70	22.37 ± 0.12	10.67 ± 0. 21
2.0 + 0.2	5-7	65	16.00 ± 0.23	9.57 ± 0.21
2.0 + 0.5	5-7	75	24.20 ± 0.11	10.20 ± 0.11
2.0 + 1.0	7-10	70	14.20 ± 0.11	10.67 ± 0.21
Kn + GA3				
0.5 + 0.2	5-7	50	10.30 ± 0.15	9.10 ± 0.05
0.5 + 0.5	5-7	70	16.30 ± 0.25	9.50 ± 0.26
0.5 + 1.0	5-7	75	18.27 ± 0.24	10.20 ± 0.11
1.0 + 0.2	5-7	60	16.30 ± 0.25	8.30 ± 0.15
1.0 + 0.5	5-7	80	20.20 ± 0.23	9.57 ± 0.21
1.0 + 1.0	5-7	70	18.27 ± 0.24	9.10 ± 0.05
2.0 + 0.2	5-7	50	8.00 ± 0.32	8.50 ± 0.05
2.0 + 0.5	5-7	65	12.57 ± 0.14	9.40 ± 0.11
2.0 + 1.0	7-10	50	12.10 ± 0.05	7.53 ± 0.12

* Values are the mean of three replicates with 10 explants.

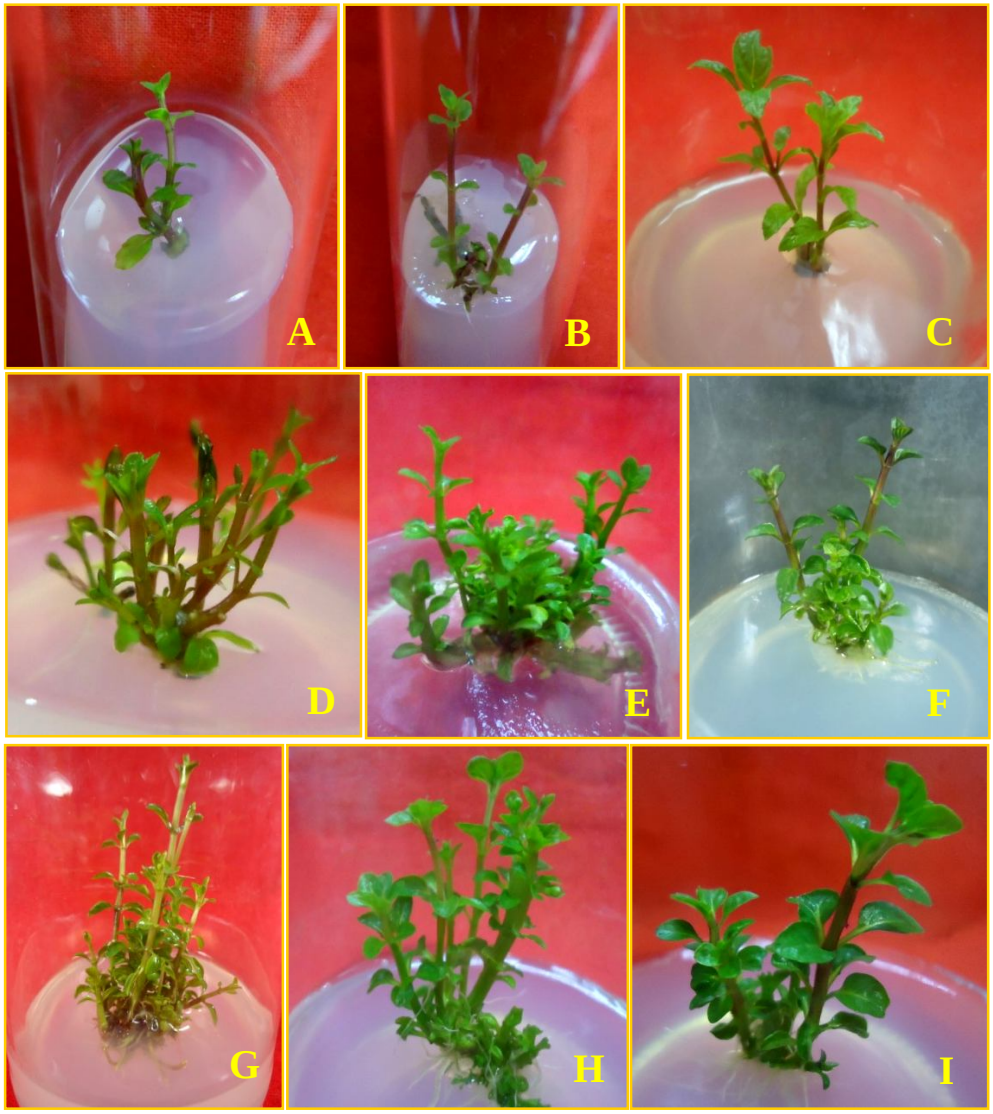


Plate 3.12 Development of multiple shoot from nodal explants of *M. arvensis* in direct organogenesis and their subsequent development.

A-C: Multiple shoot formation in nodal explants after 2-3 weeks of inoculation in media having 1.0 mg/l BAP; **D-G:** Proliferation of multiple shoot in medium having 1.0 mg/l BAP + 0.2 mg/l NAA after 4-6 weeks of culture; **H-I:** Further proliferation of shoot in the same medium after 2 sub culture at 14 days intervals.

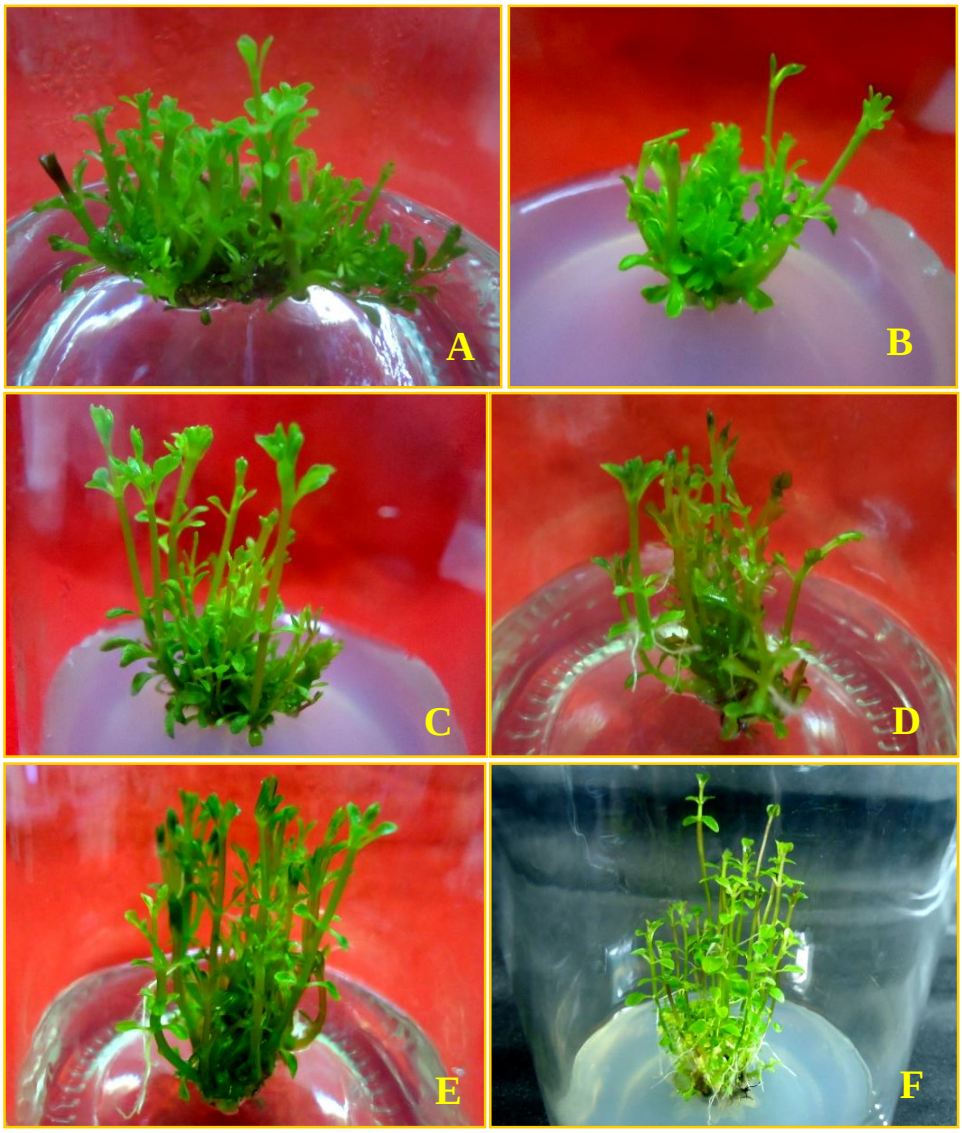


Plate 3.13 Development and elongation of multiple shoot from nodal explants of *M. arvensis* in direct organogenesis and their subsequent development.

A-B: Multiple shoot elongation from *in vitro* grown shoot explants after 2-3 weeks of inoculation in media having 0.5 mg/l GA_3; C-**E:** Further proliferation of shoot in the same medium after 2 sub culture at 14 days intervals; F**:** Further development of shoot and root initiation in the same medium after 6 weeks of culture.

3.3.4.2. Induction of callus on different explants in *M. arvensis*

Five concentrations (0.2, 0.5, 1.0, 2.0, 3.0 mg/l) of 2, 4-D used alone and combinations of four concentrations of 2,4-D (0.2, 0.5, 1.0 and 2.0 mg/l) with three concentrations (1.0, 2.0 and 3.0 mg/l) of BAP or Kn were used to investigate the initiation of callus and its subsequent regeneration. Leaf and internode were used as explants for callus induction. In preliminary experiments it was observed that when the leaf and internode explant were placed horizontally on the media surface the initiation of callus took place from both the cut ends. But when they were placed vertically, callus initiation took place only from the cut end dipped in the agar medium. Horizontally oriented explants produced callus throughout their entire surface more rapidly than those, which were placed vertically. Therefore internodes explants were always placed horizontally on the agar surface to induce callus in all these experiments. The effect of different combinations and concentrations of phytohormones, days of callus initiation, percentage of explants induced callus development, callus colour and texture from different explants are discussed experiment wise:

3.3.4.2.1. Callus induction from leaf explant

Experiment 1. Effect of different concentrations and combinations of 2,4-D, BAP and Kn on callus induction from leaf explant

In order to induce of callus, leaf segments were cultured on MS medium supplemented with five concentrations (0.2, 0.5, 1.0, 2.0, 3.0 mg/l) of 2,4–D singly or in combination with three concentrations (1.0, 2.0 and 3.0 mg/l) of BAP or Kn. Cultures were maintained under 16 h light and 8 h dark regime and data on different parameters are summarized in Table 3.31. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was not noticed in all media formulations. But there was a wide variation in morphological nature and percentage of callus formation among them. Callus initiation occurred within 10-15 days depending upon the concentration and combination of hormones. 2,4-D with BAP induced callus within 10-12 days, 2,4-D with Kn took 12-15 days and 2,4-D alone took 12-15 days to initiate callus. Percentage of explants induced callus formation ranged from 15.00-70.00%. Among the tested combinations MS medium having 2.0 mg/l BAP + 1.0 mg/l 2,4-D found highest percentage (70%) of callus formation (plate 3.14 D) followed by 65% of 3.0 mg/l BAP + 1.0 mg/l 2,4-D and 2.0 mg/l BAP + 1.0 mg/l 2,4-D. The lowest percentage (15%) of callus formation was obtained in media

having 0.5 mg/l 2,4–D but 0.2 mg/l of 2,4-D did not produce any callus. The calli were green, light green, brown and creamy in color and compact, nodular and friable in texture.

3.3.4.2.2. Callus induction from internode explant

Experiment 1. Effect of different concentrations and combinations of 2,4-D, BAP and Kn on callus induction from internode explant

In order to induce of callus from internode, segments were cultured on MS medium supplemented with five concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of 2, 4–D singly or in combination with three concentrations (1.0, 2.0 and 3.0 mg/l) of BAP or Kn. Cultures were maintained under 16 h light and 8 h dark regime and data on different parameters are summarized in Table 3.31. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was not noticed in all media formulations. But there was a wide variation in morphological nature and percentage of callus formation among them. Callus initiation occurred within 10-15 days depending upon the concentration and combination of hormones. 2,4-D with BAP induced callus within 10-12 days, 2,4-D with Kn took 12-15 days and 2,4-D alone took 12-15 days to initiate callus. Percentage of explants induced callus formation ranged from 25.00-80.00 %. Among the tested combinations MS medium having 2.0 mg/l BAP + 1.0 mg/l 2, 4-D found highest percentage (80%) of callus formation (plate 3.14 A-C) followed by 70 % of 1.0 mg/l BAP + 1.0 mg/l 2, 4-D and 2.0 mg/l BAP + 0.5 mg/l 2,4-D. The lowest percentage of callus formation (25%) was obtained in media having 3.0 mg/l Kn + 2.0 mg/l of 2,4-D. The calli were green, light green, brown and creamy in color and hard, compact, nodular and friable in texture.

Table 3.31 Effect of different concentrations of plants growth regulators in MS medium on induction of callus from leaf and internode explants of *M. arvensis*.

Hormonal combination (mg/l)	Leaf explant			Internode explant		
	Days of callus initiation	% of explant induced callus	Callus colour and texture	Days of callus initiation	% of explant induced callus	Callus colour and texture
2,4-D						
0.2	-	-	-	-	-	-
0.5	12-15	15	lgn	12-15	30	lgn
1.0	12-15	25	lgn	12-15	40	gf
2.0	12-15	20	lgn	12-15	30	gf
BAP + 2,4-D						
1.0 + 0.2	-	-	-	-	-	-
1.0 + 0.5	10-12	35	gf	10-12	60	bf
1.0 + 1.0	10-12	50	gf	10-12	70	bf
1.0 + 2.0	10-12	45	gf	10-12	50	bf
2.0 + 0.2	-	-	-	10-12	50	lgn
2.0 + 0.5	10-12	50	lgc	10-12	70	cc
2.0 + 1.0	10-12	70	lgc	10-12	80	cc
2.0 + 2.0	10-12	45	lgc	10-12	45	cc
3.0 + 0.2	-	-	-	10-12	40	lgn
3.0 + 0.5	10-12	50	cf	10-12	70	lgn
3.0 + 1.0	10-12	65	cf	10-12	60	lgn
3.0 + 2.0	10-12	55	cf	10-12	50	lgn
Kn + 2,4-D						
1.0 + 0.2	-	-	-	12-15	45	lgn
1.0 + 0.5	12-15	45	cf	12-15	50	lgn
1.0 + 1.0	12-15	60	lgn	12-45	60	gf
1.0 + 2.0	12-15	40	lgn	12-15	40	gf
2.0 + 0.2	-	-	-	12-15	35	lgn
2.0 + 0.5	12-15	50	lgn	12-15	60	lgn
2.0 + 1.0	12-15	65	lgn	12-15	70	lgn
2.0 + 2.0	12-15	55	lgn	12-15	55	lgn
3.0 + 0.2	-	-	-	12-15	45	bf
2.0 + 0.5	12-15	60	cf	12-15	60	bf
3.0 + 1.0	12-15	55	lgn	12-15	55	lgn
3.0 + 2.0	12-15	25	lgn	12-15	25	lgn

lgn: light green nodular; **gf:** green friable; **lgc:** light green compact; **cf:** creamy friable; **bn:** brown nodular; **cc:** creamy compact.

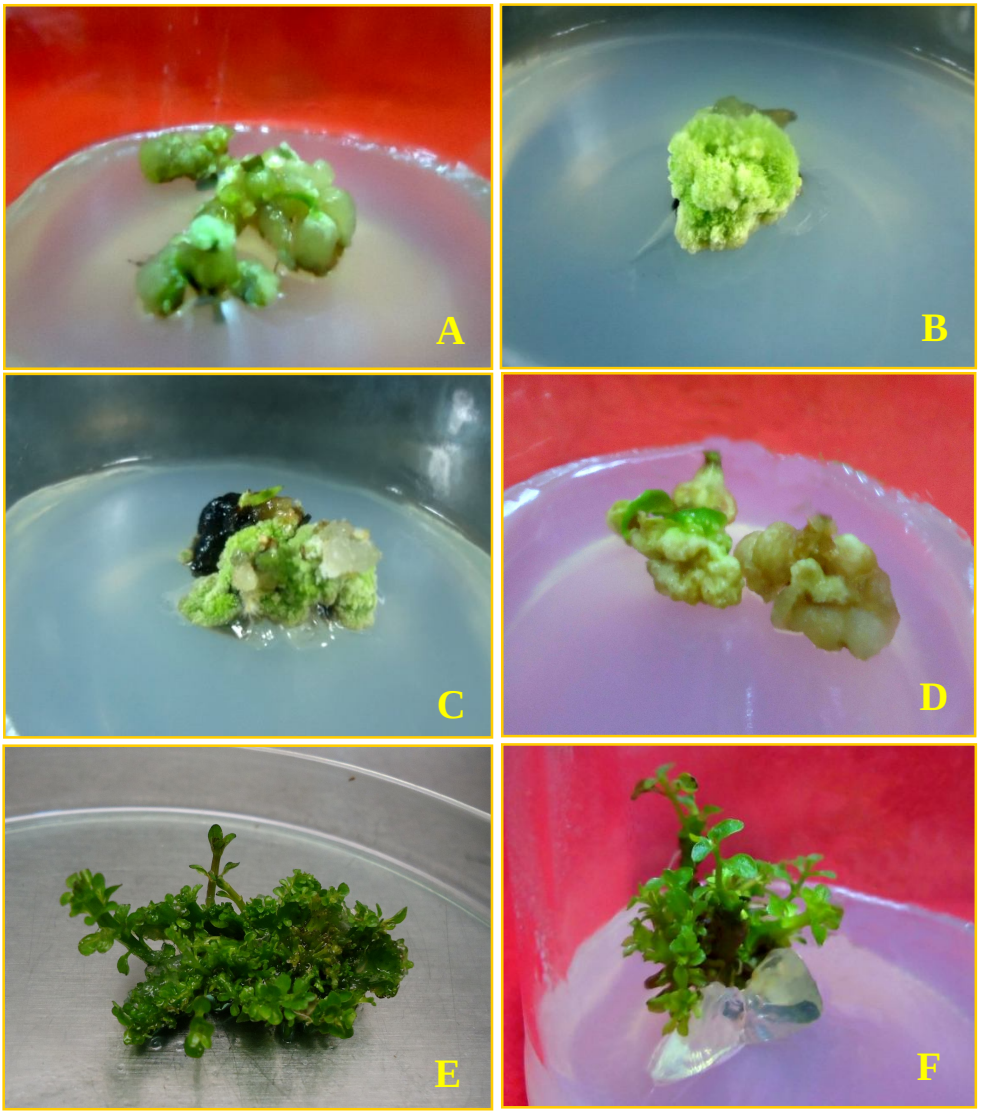


Plate 3.14 Callus induction from leaf and internode segment and plant regeneration from internode derived callus of *M. arvensis.*

A-C: Callus induction from internode segment in 2.0 mg/l BAP + 1.0 mg/l 2,4-D; **D:** Callus induction from leaf explant in 2.0 mg/l BAP + 1.0 mg/l 2,4-D; **E:** Initiation of callus regeneration in 1.0 mg/l BAP + 0.2 mg/l NAA after 4 weeks of culture from leaf derived callus and **F:** Callus regeneration and proliferation of multiple shoot buds in the same medium after 8 weeks of subculture.

3.3.4.3. Adventitious shoot regeneration from different types of calli in *M. arvensis*

Calli produced in different plant growth regulators supplemented on MS medium did not differentiate into shoot buds in the same medium after subsequent subcultures. So to induce adventitious shoot buds from various types of calli, different media combinations were needed to initiate regenerations. A wide range of auxin and cytokinin supplemented media were used to standardize the organogenic response of calli. Among the different types of calli only light green nodular callus found to be regenerative on different plant growth regulators supplemented media. The results are discussed according to types of explants under separate heads:

3.3.4.3.1. Plant regeneration from leaf derived callus

Different experiments were conducted to investigate plant regeneration ability through callus culture from leaf explants. Data on percentage of organogenic calli induced root and shoot, average number of shoot per callus and average length of shoot per callus were collected after 8 weeks of culture. Findings of the experiments are described below.

Experiment 1. Effect of different concentrations and combinations of BAP, Kn and NAA on organogenesis of leaf derived callus

In this experiment leaf derived calli of *M. arvensis* were used to investigate the effect of different hormonal concentrations and combinations. For shoot differentiation light green nodular calli were sudcultured on to MS medium supplemented with different concentrations of BAP and Kn alone and in combination with each other or in different concentrations of NAA. Morphogenic potentialities of cultured calli varied with hormonal treatments (Table 3.32). Highest 65.00% of shoot regeneration was recorded in 2.0 mg/l BAP + 1.0 mg/l Kn + 0.2 mg/l NAA (Plate 3.14 E) followed by 60.00% in media having 1.0 mg/l BAP + 0.5 mg/l NAA and 2.0 mg/l BAP + 1.0 mg/l Kn + 0.1 mg/l NAA. The lowest 25.00% of shoot regeneration was recorded in media having 3.0 mg/l BAP. The highest number of shoots per callus was recorded 12.00 ± 0.23 in media having 1.0 mg/l BAP+ 1.0 mg/l Kn +0.2 mg/l NAA. The lowest number of shoots per callus was recorded 4.00 ± 0.20 in media having 0.2 mg/l BAP. Highest length of shoot 4.30 ± 0.11 cm was recorded in 1.0 mg/l BAP + 1.0 mg/l Kn + 0.5 mg/l NAA and the lowest length of shoots 2.23 ± 0.08 cm was recorded in 3.0 mg/l BAP.

3.3.4.3.2. Plant regeneration from internode derived callus

Different experiments were conducted to investigate plant regeneration ability through callus culture from internode explants. Data on percentage of organogenic calli induced regeneration, average number of shoot per callus and average length of shoot per callus were collected after 8 weeks of culture. Findings of the experiments are described below:

Experiment 1. Effect of different concentrations and combinations of BAP, Kn and NAA on organogenesis of internode derived callus

In this experiment internode derived calli of *M. arvensis* were used to investigate the effect of different hormonal concentrations and combinations. For shoot differentiation brown nodular and light green nodular calli were sudcultured on to MS medium supplemented with different concentrations of BAP and Kn alone and in combination with each other or in different concentrations of NAA. Morphogenic potentialities of cultured calli varied with hormonal treatments (Table 3.33). Highest 54.00% of shoot regeneration was recorded in 2.0 mg/l BAP + 1.0 mg/l Kn + 0.2 mg/l NAA followed by 52% in 1.0 mg/l BAP + 0.2 mg/l NAA and 1.0 mg/l BAP + 1.0 mg/l Kn + 0.2 mg/l NAA (Plate 3.14 B). The lowest 20.00% of shoot regeneration was recorded in media having 0.2 mg/l BAP. The highest average number of shoots per callus was recorded 12.50 ± 0.15 in media having 1.0 mg/l BAP + 0.2 mg/l NAA followed by 10.00 ± 0.23 in 1.0 mg/l BAP + 1.0 mg/l Kn + 0.2 mg/l NAA. The lowest average number of shoots per callus was recorded 4.70 ± 0.20 in media having 3.0 mg/l BAP. Highest length of shoot 4.60 ± 0.11 cm was recorded in 1.0 mg/l BAP + 1.0 mg/l Kn + 0.5 mg/l NAA and the lowest length of shoots 2.00 ± 0.17 cm was recorded in 0.2 mg/l BAP.

Table 3.32 Indirect shoot organogenesis from callus derived from leaf explants of *M. arvensis.*

Plant growth regulators (mg/l)	Days taken for shoot regeneration	Regeneration frequency (%)	*Average number of shoots/callus (mean ± SE)	*Average length (cm) of shoots/callus (mean ± SE)
BAP				
0.2	20-25	32	4.00 ± 0.20	2.47 ± 0.12
0.5	20-25	45	5.23 ± 0.17	2.50 ± 0.15
1.0	20-25	52	6.60 ± 0.11	3.60 ± 0.20
2.0	20-25	56	5.97 ± 0.20	2.50 ± 0.20
3.0	20-25	25	4.50 ± 0.28	2.23 ± 0.08
BAP + Kn				
0.5 + 0.5	20-25	45	5.20 ± 0.26	2.63 ± 0.20
0.5 + 1.0	20-25	52	6.30 ± 0.20	3.27 ± 0.14
0.5 + 2.0	20-25	42	7.20 ± 0.20	2.73 ± 0.14
1.0 + 0.5	20-25	50	8.30 ± 0.20	3.60 ± 0.20
1.0 + 1.0	20-25	55	7.60 ± 0.17	2.80 ± 0.20
1.0 + 2.0	20-25	36	5.30 ± 0.17	2.50 ± 0.20
BAP + NAA				
0.5 + 0.2	15-20	40	5.83 ± 0.27	2.30 ± 0.17
0.5 + 0.5	15-20	47	6.50 ± 0.28	3.50 ± 0.11
0.5 + 1.0	15-20	45	7.20 ± 0.20	2.80 ± 0.20
1.0 + 0.2	15-20	48	10.00 ± 0.11	4.20 ± 0.11
1.0 + 0.5	15-20	60	7.50 ± 0.11	3.30 ± 0.17
1.0 + 1.0	15-20	52	5.50 ± 0.20	3.17 ± 0.08
BAP+ Kn+ NAA				
1.0 + 1.0 + 0.1	15-20	48	6.10 ± 0.05	2.40 ± 0.17
1.0 + 1.0 + 0.2	15-20	58	12.00 ± 0.23	4.30 ± 0.11
1.0 + 1.0 + 0.5	15-20	50	10.00 ± 0.17	3.30 ± 0. 17
2.0 + 1.0 + 0.1	15-20	60	8.00 ± 0.28	3.80 ± 0.11
2.0 + 1.0 + 0.2	15-20	65	9.30 ± 0.17	3.60 ± 0.20
2.0 + 1.0 + 0.5	15-20	55	7.60 ± 0.17	3.27 ± 0.14

*Values are the mean of three replicates with 10 explants.

Table 3.33 Indirect shoot organogenesis from callus derived from internode explants of *M. arvensis.*

Plant growth regulators (mg/l)	Days taken for shoot regeneration	Regeneration frequency (%)	*Average number of shoots/callus (mean ± SE)	*Average length (cm) of shoots/callus (mean ± SE)
BAP				
0.2	20-25	20	5.13 ± 0.37	2.00 ± 0.17
0.5	20-25	36	5.53 ± 0.28	2.60 ± 0.26
1.0	20-25	42	6.60 ± 0.11	3.27 ± 0.14
2.0	20-25	45	6.10 ± 0.10	2.80 ± 0.20
3.0	20-25	25	4.70 ± 0.20	2.17 ± 0.12
BAP + Kn				
0.5 + 0.5	20-25	35	5.53 ± 0.28	2.50 ± 0.20
0.5 + 1.0	20-25	47	6.60 ± 0.11	3.30 ± 0.17
0.5 + 2.0	20-25	42	7.60 ± 0.17	2.90 ± 0.20
1.0 + 0.5	20-25	45	8.50 ± 0.23	3.50 ± 0.40
1.0 + 1.0	20-25	50	6.30 ± 0.17	2.60 ± 0.05
1.0 + 2.0	20-25	36	5.17 ± 0.12	2.23 ± 0.08
BAP + NAA				
0.5 + 0.2	15-20	35	6.50 ± 0.28	2.50 ± 0.15
0.5 + 0.5	15-20	45	6.80 ± 0.77	3.60 ± 0.11
0.5 + 1.0	15-20	48	7.50 ± 0.11	3.17 ± 0.08
1.0 + 0.2	15-20	52	12.50 ± 0.15	4.00 ± 0.17
1.0 + 0.5	15-20	45	8.30 ± 0.17	3.40 ± 0.17
1.0 + 1.0	15-20	35	5.20 ± 0.11	2.90 ± 0.20
BAP+ Kn+ NAA				
1.0 + 1.0 + 0.1	15-20	45	6.23 ± 0.08	2.47 ± 0.14
1.0 + 1.0 + 0.2	15-20	52	9.20 ± 0.11	3.50 ± 0.11
1.0 + 1.0 + 0.5	15-20	42	8.50 ± 0.05	3.30 ± 0.17
2.0 + 1.0 + 0.1	15-20	47	7.30 ± 0.10	3.80 ± 0.11
2.0 + 1.0 + 0.2	15-20	54	10.00 ± 0.23	4.60 ± 0.11
2.0 + 1.0 + 0.5	15-20	38	6.97 ± 0.12	3.17 ± 0.08

*Values are the mean of three replicates with 10 explants.

3.3.4.4. Rooting of *in vitro* grown shoots in *M. arvensis*

Shoot cuttings 5–7 cm long *in vitro* grown shoots were separated and transferred to rooting media. Half strength MS medium fortified with different concentrations (0.1, 0.2, 0.5, 1.0 and 2.0 mg/l) of IAA, NAA and IBA were used for rooting experiment. Roots started within 10 to 15 days of culture. Among the tested combinations half strength MS medium having 1.0 mg/l NAA found highest percentage (95%) of root formation and the lowest percentage (30%) of root formation was obtained in media having 0.1 mg/l IAA. Data on days to emergence of root, percentage of root formation (%), average number of root per culture and average length of root per culture were conducted and were recorded after 6 weeks of culture. Findings of the experiments are described below.

Experiment 1. Effect of different concentrations of IAA, NAA and IBA on root induction from shoots derived from nodal explants of *M. arvensis*

For adventitious root formation, the shoots obtained from *in vitro* grown nodal segments were cultured on half strength MS medium with different concentrations of IAA, NAA and IBA. Results obtained for root induction, percentage of root formation, morphology, average number and length of roots are shown in Table 3.34. Percentage range of cultures produced roots varied from 30-95.00%. Highest 95.00% of root regeneration was recorded in 1.0 mg/l NAA followed by 75.00 % in media 1.0 mg/l IAA and 0.2 mg/l IBA. The lowest 30.00 % of root regeneration was recorded in media having 0.1 mg/l IAA and 0.1 mg/l IBA. The highest average number of roots per shoot was recorded 22.50 ± 0.15 in media having 1.0 mg/l NAA (Plate 3.15 C) followed by 20.20 ± 0.23 in 0.2 mg/l IBA. The lowest average number of root per shoots was recorded 6.43 ± 0.08 in media having 0.1 mg/l IAA. Highest average length of roots 7.30 ± 0.10 cm was recorded in 0.5 mg/l NAA and the lowest length of roots 3.50 ± 0.11 cm was recorded in 2.0 mg/l IBA. In most cases morphology of roots was thin, thick and long.

Table 3.34 Effect of different concentrations of IAA, NAA and IBA in half strength MS medium for root induction from *in vitro* grown shoot explants of *M. arvensis*. Data were taken after 6 weeks of culture.

Plant growth regulators (mg/l)	Days to emergence of roots	Percentage (%)of root formation	*Average number of roots/explants (mean ± SE)	*Average length of root/explant (cm) (mean ± SE)
IAA				
0.1	12-15	30	6.43 ± 0.08	4.50 ± 0.17
0.2	12-15	50	8.50 ± 0.23	5.07 ± 0.08
0.5	12-15	66	10.63 ± 0.20	6.50 ± 0.28
1.0	12-15	75	12.00 ± 0.34	7.20 ± 0.20
2.0	12-15	45	8.50 ± 0.23	4.50 ± 0.28
NAA				
0.1	10-15	42	8.00 ± 0.30	4.50 ± 0.05
0.2	10-12	66	10.00 ± 0.11	5.50 ± 0.17
0.5	10-12	72	14.00 ± 0.28	6.50 ± 0.28
1.0	10-12	95	22.50 ± 0.15	7.30 ± 0.10
2.0	12-15	52	15.77 ± 0.67	4.20 ± 0.11
IBA				
0.1	10-12	30	9.00 ± 0.11	4.00 ± 0.17
0.2	10-12	75	20.20 ± 0.23	5.20 ± 0.11
0.5	10-12	70	14.20 ± 0.11	7.00 ± 0.17
1.0	10-12	62	10.00 ± 0.17	5.97 ± 0.20
2.0	10-12	46	12.00 ± 0.28	3.50 ± 0.11

*Values are the mean of three replicates with 5 explants.

Plate 3.15 Induction of adventitious roots and plants establishment in soil in *M. arvensis*

A: Induction of adventitious roots from *in vitro* grown shoot explants in half strength MS medium containing 1.0 mg/l NAA after 4 weeks of culture; **B:** Further development of root in the some medium after 5 weeks of culture; **C:** *M. arvensis* with roots outside of culture bottle; **D:** The *in vitro* grown seedlings acclimatized to grow in pots in outside environment after 8 weeks.

3.3.5. *In vitro* regeneration and conservation of *Paederia foetida*

Experiments on direct and indirect organogenesis were carried out using different types of explants viz. shoot tip, node, internode and leaves. Shoot tip and nodal explants were cultured for direct shoot regeneration. Explants were cultured on the MS (Murashig and Skoog, 1962) agar gelled medium supplemented with different concentrations of auxins and cytokinins used singly or in combinations to investigate the initiation of shoot and its subsequent development. For root induction micro shoots obtained from direct and indirect organogenesis were transferred to rooting media. Finally well developed plants with both shoots and roots were transferred to natural conditions through successive phages of acclimatization. Details of the results emerging out from each of the experiments are described under following heads:

3.3.5.1. Direct regeneration

Two types of explants *viz*. shoot tip and nodal explants were cultured for direct shoot regeneration. Explants were cultured on MS medium with BAP and Kn used alone and in combinations with each other or with NAA and IAA. Data on number of days taken for shoot initiation, percentage of explant induced shoot development, average number of shoot per culture and average length of shoot per culture were collected after 4-6 weeks of culture. Among the two types of explants nodal explants responded on almost all of the supplemented cultured media. The results are described according to types of explants under separate heads:

3.3.5.1.1. Shoot tip explants

Experiment 1. Effect of different concentrations of BAP and Kn used alone and in combinations on multiple shoot induction from shoot tip explants of *P. foetida*

In this present investigation five concentrations of BAP (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l), five concentrations of Kn (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) alone and in combination of three concentrations of BAP (0.5, 1.0 and 2.0 mg/l) with four concentrations of Kn (0.2, 0.5, 1.0 and 2.0 mg/l) were treated in MS medium for the purpose of multiple shoot induction from shoot tip explants of *P. foetida*. Data were taken after 6 weeks of inoculation and percentage of shoot formation, average number of shoot/culture and average length of shoot/culture were measured. The results are presented in Table 3.35. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. The percentage of shoot proliferation varied from 25.00-75.00%. Highest percentage (75.00%) of multiple

shoot formation was observed in MS medium containing 1.0 mg/l BAP + 0.5 mg/l Kn followed by (72.00%) in MS medium containing 0.5 mg/l BAP + 0.5 mg/l Kn and 1.0 mg/l BAP + 1.0 mg/l Kn. The lowest percentage (25.00%) of multiple shoot formation was observed in media having 0.2 mg/l Kn. Highest mean number of shoots was 5.00 ± 0.28 in media having 1.0 mg/l BAP + 0.5 mg/l Kn followed by 4.30 ± 0.11 in the combination of 1.0 mg/l BAP and 1.0 mg/l BAP + 1.0 mg/l Kn. The lowest mean number of shoot was 1.30 ± 0.05 in media containing 0.2 mg/l Kn. Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 6 weeks of culture. Highest average length was recorded 6.50 ± 0.28 cm in 0.5 mg/l BAP followed by 6.00 ± 0.11 cm in 0.5 mg/l BAP + 0.5 mg/l Kn and 1.0 mg/l BAP + 0.5 mg/l Kn. On the other hand lowest average length was 3.00 ± 0.20 cm in 0.2 mg/l Kn. Experimental results revealed that, 1.0 mg/l of BAP and 1.0 mg/l Kn alone and combination of 0.5 mg/l BAP + 0.5 mg/l Kn, 1.0 mg/l BAP+ 0.5 mg/l Kn, 1.0 mg/l BAP + 1.0 mg/l Kn were found most effective concentrations for multiple shoot induction in *P. foetida*.

Experiment 2. Effect of different concentrations and combinations of BAP with NAA and IAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentration of BAP (0.5, 1.0 and 2.0 mg/l) combined with four concentrations of NAA (0.1, 0.2, 0.5 and 1.0 mg/l) and IAA (0.1, 0.2, 0.5 and 1.0 mg/l). Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.36. The efficiency of BAP + NAA was better than BAP alone but inferior than BAP + IAA on direct shoot regeneration. All the used media compositions formed multiple shoots (except 0.5 mg/l BAP + 0.1 mg/l NAA and 2.0 mg/l BAP + 0.1 mg/l NAA) and the results were inferior to the results obtained from nodal explants. Addition of lower concentration of NAA and IAA along with higher concentration of BAP was found more suitable than that of other concentrations. Among the combinations of BAP + NAA and BAP + IAA the highest percentage (85%) of shoot proliferation was noted in the media having 0.5 mg/l BAP + 0.5 mg/l IAA followed by (80%) in 1.0 mg/l BAP + 0.5 mg/l NAA and 1.0 mg/l BAP + 0.5 mg/l IAA. The lowest percentage (45%) of shoot proliferation was noted in media having 2.0 mg/l BAP + 1.0 mg/l NAA. The highest average number of shoot per culture was 6.00 ± 0.11 in the combination of 1.0 mg/l BAP + 0.5 mg/l IAA and 1.0 mg/l BAP + 0.5 mg/l NAA followed by 5.00 ± 0.17 in the combinations of 0.5 mg/l BAP + 0.5 mg/l IAA. The

lowest number of average shoot 1.00 ± 0.11 per culture was noted in the media having 0.5 mg/l BAP + 0.1 mg/l NAA and 2.0 mg/l BAP + 0.1 mg/l NAA. Average highest length of shoot 7.50 ± 0.28 cm per culture was found in the combination of 1.0 mg/l BAP + 0.5 mg/l IAA and the lowest length of shoot was 3.30 ± 0.17cm per culture in the media having 0.5 mg/l BAP + 0.1 mg/l NAA. Experimental results revealed that, 0.5 mg/l BAP + 0.5 mg/l IAA, 1.0 mg/l BAP + 0.5 mg/l NAA, 1.0 mg/l BAP + 0.2 mg/l IAA and 1.0 mg/l BAP + 0 2mg/l NAA were found most effective combinations for multiple shoot induction in *P. foetida*.

Experiment 3. Effect of different concentrations and combinations of Kn with NAA and IAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentration of Kn (0.5, 1.0 and 2.0 mg/l) combined with four concentrations of NAA (0.1, 0.2, 0.5 and 1.0 mg/l) and IAA (0.1, 0.2, 0.5 and 1.0 mg/l). Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.37. The efficiency of Kn + NAA was better than Kn alone but inferior than Kn + IAA on direct shoot regeneration. All the used media compositions formed multiple shoots (except 0.5 mg/l Kn + 0.1 mg/l NAA and 2.0 mg/l Kn + 1.0 mg/l IAA) and the results were inferior to the results obtained from nodal explants. Addition of lower concentration of NAA and IAA along with higher concentration of Kn was found more suitable for multiple shoot induction than that of other concentrations. Among the combinations of Kn + NAA and Kn + IAA the highest percentage (80%) of shoot proliferation was noted in the media having 1.0 mg/l Kn + 0.5 mg/l IAA followed by 75% in 0.5 mg/l Kn + 0.5 mg/l IAA. The lowest percentage (40%) of shoot proliferation was noted in the media having 2.0 mg/l Kn + 1.0 mg/l IAA. The highest average number of shoot per culture was 4.00 ± 0.25 in the media having 0.5 mg/l Kn + 0.5 mg/l IAA and 1.0 mg/l Kn + 0.5 mg/l IAA. The lowest number of shoot per culture was 1.00 ± 0.05 in the media having 0.5 mg/l Kn + 0.1 mg/l NAA and 2.0 mg/l Kn + 1.0 mg/l IAA. Average highest length of shoot 6.00 ± 0.25 cm per culture was found in the combination of 1.0 mg/l Kn + 0.5 mg/l IAA followed by 5.60 ± 0.17 cm per culture was found in the media having 1.0 mg/l Kn + 0.5 mg/l NAA and 1.0 mg/l Kn + 0.2 mg/l IAA respectively. Experimental results revealed that, 0.5 mg/l Kn + 0.5 mg/l IAA, 1.0 mg/l Kn + 0.5 mg/l IAA, 1.0 mg/l Kn + 0.5 mg/l NAA and 0.5 mg/l Kn + 0.5 mg/l NAA were found most effective combinations for multiple shoot induction in *P. foetida*.

Table 3.35 Effect of different levels of growth regulators (BA and Kn alone and combination) on shoot proliferation from shoot tip explants of *P. foetida*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of total shoots/culture (mean ± SE)	*Average length (cm) of shoots/culture (mean ± SE)
BAP				
0.2	7-8	40	1.50 ± 0.15	4.00 ± 0.26
0.5	7-8	62	2.00 ± 0.17	6.50 ± 0.28
1.0	7-8	70	4.30 ± 0.11	5.50 ± 0.11
2.0	8-10	50	2.60 ± 0.23	5.00 ± 0.28
3.0	8-10	30	2.60 ± 0.23	3.50 ± 0.17
Kn				
0.2	8-10	25	1.30 ± 0.05	3.00 ± 0.20
0.5	8-10	55	2.00 ± 0.17	3.50 ± 0.17
1.0	7-8	65	4.00 ± 0.30	4.00 ± 0.30
2.0	7-8	50	2.30 ± 0.17	5.00 ± 0.17
3.0	8-10	35	2.00 ± 0.23	3.50 ± 0.11
BAP + Kn				
0.5 + 0.2	7-8	55	1.60 ± 0.17	4.50 ± 0.11
0.5 + 0.5	7-8	72	2.00 ± 0.17	6.00 ± 0.11
0.5 + 1.0	7-8	66	2.60 ± 0.11	5.50 ± 0.11
0.5 + 2.0	7-8	50	1.60 ± 0.05	3.80 ± 0.11
1.0 + 0.2	7-8	60	2.00 ± 0.23	4.50 ± 0.28
1.0 + 0.5	7-8	75	5.00 ± 0.28	6.00 ± 0.57
1.0 + 1.0	7-8	72	4.30 ± 0.11	5.00 ± 0.11
1.0 + 2.0	8-10	55	2.00 ± 0.17	4.00 ± 0.28
2.0 + 0.2	8-10	52	1.60 ± 0.15	3.50 ± 0.11
2.0 + 0.5	8-10	66	2.60 ± 0.11	5.00 ± 0.20
2.0 + 1.0	8-10	60	2.00 ± 0.23	5.50 ± 0.17
2.0 + 2.0	8-10	40	1.30 ± 0.05	4.00 ± 0.26

*Values are the mean of three replicates with 10 explants.

Table 3.36 Effect of different concentrations and combinations of BAP with NAA and IAA on shoot multiplication from shoot tip explants of *P. foetida.* Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of total shoots/culture (mean ± SE)	*Average length (cm) of shoots/culture (mean ± SE)
BAP + NAA				
0.5 + 0.1	6-7	56	1.00 ± 0.05	3.30 ± 0.17
0.5 + 0.2	6-7	60	2.00 ± 0.15	4.00 ± 0.26
0.5 + 0.5	6-7	72	4.00 ± 0.23	5.30 ± 0.17
0.5 + 1.0	6-7	52	2.60 ± 0.11	4.00 ± 0.28
1.0 + 0.1	6-7	62	2.00 ± 0.05	3.60 ± 0.17
1.0 + 0.2	6-7	70	4.00 ± 0.28	4.6 ± 0.23
1.0 + 0.5	6-7	80	6.00 ± 0.17	5.60 ± 0.17
1.0 + 1.0	6-7	65	3.00 ± 0.11	4.00 ± 0.17
2.0 + 0.1	7-8	40	1.00 ± 0.11	4.00 ± 0.17
2.0 + 0.2	7-8	62	2.30 ± 0.17	4.60 ± 0.17
2.0 + 0.5	7-8	55	4.00 ± 0.23	7.00 ± 0.28
2.0 + 1.0	7-8	45	2.00 ± 0.11	4.30 ± 0.17
BAP + IAA				
0.5 + 0.1	6-7	55	2.00 ± 0.30	4.50 ± 0.17
0.5 + 0.2	6-7	72	4.00 ± 0.26	6.00 ± 0.26
0.5 + 0.5	6-7	85	5.00 ± 0.17	6.60 ± 0.17
0.5 + 1.0	6-7	65	2.30 ± 0.05	7.00 ± 0.17
1.0 + 0.1	6-7	62	2.00 ± 0.05	5.50 ± 0.17
1.0 + 0.2	6-7	70	4.00 ± 0.28	6.00 ± 0.15
1.0 + 0.5	6-7	80	6.00 ± 0.11	7.50 ± 0.28
1.0 + 1.0	6-7	66	2.60 ± 0.11	6.00 ± 0.26
2.0 + 0.1	6-7	50	2.00 ± 0.11	5.00 ± 0.28
2.0 + 0.2	6-7	70	4.00 ± 0.28	6.00 ± 0.23
2.0 + 0.5	7-8	66	2.60 ± 0.17	6.50 ± 0.11
2.0 + 1.0	7-8	55	2.30 ± 0.17	5.60 ± 0.11

*Values are the mean of three replicates with 10 explants.

Table 3.37 Effect of different concentrations and combinations of Kn with NAA and IAA on shoot proliferation from shoot tip explants of *P. foetida.* Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of total shoots/culture (mean ± SE)	*Average length (cm) of shoots/culture (mean ± SE)
Kn + NAA				
0.5 + 0.1	7-8	52	1.00 ± 0.05	3.60 ± 0.11
0.5 + 0.2	7-8	60	2.00 ± 0.15	4.60 ± 0.11
0.5 + 0.5	7-8	70	2.60 ± 0.11	5.00 ± 0.17
0.5 + 1.0	7-8	55	2.30 ± 0.17	4.30 ± 0.15
1.0 + 0.1	7-8	55	2.00 ± 0.05	4.00 ± 0.11
1.0 + 0.2	6-7	66	2.60 ± 0.11	5.00 ± 0.11
1.0 + 0.5	6-7	70	3.00 ± 0.25	5.60 ± 0.17
1.0 + 1.0	6-7	52	2.30 ± 0.15	4.60 ± 0.17
2.0 + 0.1	7-8	50	1.30 ± 0.15	4.30 ± 0.17
2.0 + 0.2	7-8	66	3.00 ± 0.11	4.60 ± 0.17
2.0 + 0.5	8-10	60	2.00 ± 0.11	5.30 ± 0.17
2.0 + 1.0	8-10	45	1.60 ± 0.11	4.00 ± 0.17
Kn + IAA				
0.5 + 0.1	7-8	50	1.30 ± 0.05	3.60 ± 0.17
0.5 + 0.2	7-8	65	2.00 ± 0.11	4.60 ± 0.11
0.5 + 0.5	7-8	75	4.00 ± 0.25	5.00 ± 0.17
0.5 + 1.0	7-8	62	2.00 ± 0.15	4.00 ± 0.28
1.0 + 0.1	8-10	60	1.60 ± 0.11	4.60 ± 0.11
1.0 + 0.2	7-8	66	2.60 ± 0.11	5.60 ± 0.17
1.0 + 0.5	7-8	80	4.00 ± 0.26	6.00 ± 0.25
1.0 + 1.0	7-8	55	1.93 ± 0.27	4.30 ± 0.11
2.0 + 0.1	8-10	50	1.60 ± 0.15	3.60 ± 0.17
2.0 + 0.2	8-10	62	2.00 ± 0.17	5.00 ± 0.28
2.0 + 0.5	7-8	70	1.30 ± 0.05	5.60 ± 0.17
2.0 + 1.0	8-10	40	1.00 ± 0.05	4.00 ± 0.17

*Values are the mean of three replicates with 10 explants.

3.3.5.1.2. Nodal explants

Experiment 1. Effect of different concentrations of BAP and Kn alone and combinations on multiple shoot induction from nodal explants of *P. foetida.*

In this present investigation five concentrations of BAP (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l), five concentrations of Kn (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) alone and combination of three concentrations of BAP (0.5, 1.0 and 2.0 mg/l) with four concentrations of Kn (0.2, 0.5, 1.0 and 2.0 mg/l) were treated in MS medium for the purpose of multiple shoot induction from nodal explants of *P. foetida.* Data were taken after 6 weeks of inoculation and percentage of shoot formation, number of shoot/culture and length of shoot/culture were measured. The results are presented in Table 3.38. Shoot proliferation ranged from 30.00-85.00%. Highest percentage (85.00%) of multiple shoot formation was observed in MS medium containing 0.5 mg/l BAP + 1.0 mg/l Kn followed by 80.00% in MS medium containing 1.0 mg/l BAP + 0.5 mg/l Kn. The lowest percentage (30.00%) of multiple shoot formation was observed in media having 2.0 mg/l BAP + 2.0 mg/l Kn. Highest mean number of shoots was 5.00 ± 0.28 in media having 1.0 mg/l BAP + 0.5 mg/l Kn followed by 4.60 ± 0.11 in 0.5 mg/l BAP + 1.0 mg/l Kn. The lowest mean number of shoot was 2.00 ± 0.23 in media containing 3.0 mg/l BAP, 3.0 mg/l Kn and 2.0 mg/l BAP + 2.0 mg/l Kn. Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 28 days of culture. Highest average length was recorded 6.00 ± 0.20 cm in 1.0 mg/l BAP+ 1.0 mg/l Kn and the lowest average length was 3.00 ± 0.20 cm in media containing 3.0 mg/l Kn. Experimental results revealed that, 1.0 mg/l of BAP and 1.0 mg/l Kn alone and combination of 0.5 mg/l BAP + 1.0 mg/l Kn, 1.0 mg/l BAP+ 0.5 mg/l Kn, 0.5 mg/l BAP + 0.5 mg/l Kn were found most effective concentrations for multiple shoot induction in *P. foetida*.

Experiment 2. Effect of different concentrations and combinations of BAP with NAA and IAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentrations of BAP (0.5, 1.0 and 2.0 mg/l) combined with four concentrations of NAA (0.1, 0.2, 0.5 and 1.0 mg/l) and IAA (0.1, 0.2, 0.5 and 1.0 mg/l). Data were recorded after 4 weeks of culture and results on different parameters are presented in the Table 3.39. The efficiency of BAP + NAA was better than BAP alone but inferior than BAP + IAA on direct shoot regeneration. All the used media compositions formed multiple shoots and the results were superior to the results obtained from shoot tip explants. Among the combinations of BAP + NAA and BAP + IAA the highest percentage (95%) of shoot proliferation was noted in the media having 1.0 mg/l BAP + 0.5 mg/l IAA (Plate 3.16) followed by

85% in 0.5 mg/l BAP + 0.5 mg/l NAA and 0.5 mg/l BAP + 0.5 mg/l IAA. The lowest percentage (50%) of shoot proliferation was noted in media having 2.0 mg/l BAP + 0.1 mg/l NAA. Average highest number of shoot per culture was 6.00 ± 0.17 in the combinations of 1.0 mg/l BAP + 0.5 mg/l NAA, 0.5 mg/l BAP + 0.5 mg/l IAA and 1.0 mg/l BAP + 0.5 mg/l IAA. Lowest number of shoot per culture was 2.00 ± 0.17 in the media of 0.5 mg/l BAP + 0.1 mg/l NAA and 2.0 mg/l BAP + 1.0 mg/l IAA. The highest length of shoot per culture was 7.00 ± 0.11 in the media containing 1.0 mg/l BAP + 0.5 mg/l IAA and the lowest length of shoot per culture was 4.00 ± 0.25 cm in the media containing 0.5 mg/l BAP + 1.0 mg/l NAA. Experimental results revealed that, 1.0 mg/l BAP + 0.5 mg/l IAA, 0.5 mg/l BAP + 0.5 mg/l NAA, 0.5 mg/l BAP + 0.5 mg/l IAA and 1.0 mg/l BAP + 0. 5 mg/l NAA were found more effective combinations for multiple shoot induction in *P. foetida*.

Experiment 3. Effect of different concentrations and combinations of Kn with NAA and IAA on multiple shoot induction

Explants were cultured on MS medium supplemented with three concentrations of Kn (0.5, 1.0 and 2.0 mg/l) combined with different concentrations of NAA (0.1, 0.2, 0.5 and 1.0 mg/l) and IAA (0.1, 0.2, 0.5 and 1.0 mg/l). Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.40. All the used media compositions formed multiple shoots and the results were superior to the results obtained from shoot tip explants. Addition of lower concentration of NAA and IAA along with higher concentration of Kn was found more suitable than that of other concentrations. Among the combinations of Kn + NAA and Kn + IAA the highest percentage (85%) of shoot proliferation was noted in the media having 0.5 mg/l Kn + 0.5 mg/l IAA followed by 80% in 1.0 mg/l Kn + 0.2 mg/l NAA and 1.0 mg/l Kn + 0.5 mg/l IAA. The lowest percentage (50%) of shoot proliferation was noted in the media having 2.0 mg/l Kn + 1.0 mg/l IAA. The average highest number of shoot per culture was 6.00 ± 0.17 in the media having 0.5 mg/l Kn + 0.5 mg/l IAA and the lowest number of shoot per culture was 2.00 ± 0.11 in the media having 2.0 mg/l Kn +0.1 mg/l IAA (Plate 3.16). Average highest length of shoot was 6.50 ± 0.11 cm per culture was found in the combination of 1.0 mg/l Kn + 0.5 mg/l IAA. The lowest average length of shoot per culture was 3.60 ± 0.11cm in the media having 0.5 mg/l Kn + 0.1 mg/l NAA and 0.5 mg/l Kn + 0.1 mg/l IAA. Experimental results revealed that, 0.5 mg/l Kn + 0.5 mg/l IAA, 1.0 mg/l Kn + 0.5 mg/l IAA, 1.0 mg/l Kn + 0.5 mg/l NAA and 0.5 mg/l Kn + 0.5 mg/l NAA were found more effective combinations for multiple shoot induction.

Table 3.38 Effect of different levels of growth regulators (BA and Kn singly and combination) on shoot proliferation from nodal explants of *P. foetida*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of total shoots/culture (mean ± SE)	*Average length (cm) of shoots/culture (mean ± SE)
BAP				
0.2	7-10	55	2.60 ± 0.17	4.00 ± 0.26
0.5	6-8	66	3.00 ± 0.11	4.50 ± 0.15
1.0	6-8	75	4.00 ± 0.30	5.50 ± 0.11
2.0	6-8	62	2.93 ± 0.06	5.00 ± 0.28
3.0	7-10	40	2.00 ± 0.23	4.00 ± 0.11
Kn				
0.2	7-10	52	2.00 ± 0.17	3.60 ± 0.11
0.5	6-8	65	2.60 ± 0.17	4.00 ± 0.11
1.0	6-8	72	3.00 ± 0.25	5.00 ± 0.28
2.0	6-8	62	2.30 ± 0.17	4.30 ± 0.17
3.0	6-8	55	2.00 ± 0.23	3.00 ± 0.20
BAP + Kn				
0.5 + 0.2	6-7	65	2.30 ± 0.17	4.50 ± 0.11
0.5 + 0.5	6-7	75	4.00 ± 0.11	5.00 ± 0.28
0.5 + 1.0	6-7	85	4.60 ± 0.11	5.60 ± 0.05
0.5 + 2.0	6-7	62	2.60 ± 0.11	5.00 ± 0.28
1.0 + 0.2	6-8	65	3.00 ± 0.23	5.00 ± 0.28
1.0 + 0.5	6-7	80	5.00 ± 0.28	5.40 ± 0.23
1.0 + 1.0	6-7	72	4.00 ± 0.25	6.00 ± 0.20
1.0 + 2.0	6-7	50	2.60 ± 0.11	5.00 ± 0.28
2.0 + 0.2	6-8	50	2.60 ± 0.11	4.60 ± 0.17
2.0 + 0.5	6-8	72	3.00 ± 0.17	5.60 ± 0.05
2.0 + 1.0	6-8	62	4.00 ± 0.20	5.00 ± 0.25
2.0 + 2.0	6-8	30	2.00 ± 0.11	4.60 ± 0.11

*Values are the mean of three replicates with 10 explants.

Table 3.39 Effect of different concentrations and combinations of BA + NAA and BA + IAA on shoot proliferation from nodal explants of *P. foetida*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of total shoots/culture (mean ± SE)	*Average length (cm) of shoots/culture (mean ± SE)
BAP + NAA				
0.5 + 0.1	7-8	60	2.00 ± 0.17	4.00 ± 0.25
0.5 + 0.2	7-8	72	2.30 ± 0.11	4.50 ± 0.11
0.5 + 0.5	7-8	85	4.00 ± 0.30	5.00 ± 0.17
0.5 + 1.0	7-8	66	4.50 ± 0.17	5.60 ± 0.17
1.0 + 0.1	7-8	65	2.60 ± 0.11	4.60 ± 0.11
1.0 + 0.2	7-8	70	4.00 ± 0.28	5.00 ± 0.25
1.0 + 0.5	7-8	80	6.00 ± 0.28	6.50 ± 0.11
1.0 + 1.0	7-8	62	4.60 ± 0.11	6.00 ± 0.28
2.0 + 0.1	8-10	50	2.50 ± 0.20	4.60 ± 0.11
2.0 + 0.2	8-10	60	3.00 ± 0.17	5.50 ± 0.17
2.0 + 0.5	8-10	72	4.00 ± 0.20	6.00 ± 0.11
2.0 + 1.0	8-10	56	2.60 ± 0.17	4.30 ± 0.17
BAP + IAA				
0.5 + 0.1	7-8	60	2.60 ± 0.17	4.30 ± 0.05
0.5 + 0.2	6-8	72	3.00 ± 0.11	5.00 ± 0.17
0.5 + 0.5	6-8	85	6.00 ± 0.17	6.00 ± 0.23
0.5 + 1.0	6-8	65	4.00 ± 0.17	6.50 ± 0.17
1.0 + 0.1	6-8	65	2.60 ± 0.11	5.00 ± 0.23
1.0 + 0.2	6-8	80	4.00 ± 0.28	5.60 ± 0.17
1.0 + 0.5	6-8	95	6.00 ± 0.23	7.00 ± 0.11
1.0 + 1.0	6-8	72	3.00 ± 0.17	6.00 ± 0.28
2.0 + 0.1	7-8	55	2.60 ± 0.17	4.60 ± 0.11
2.0 + 0.2	7-8	65	3.00 ± 0.17	5.30 ± 0.17
2.0 + 0.5	7-8	72	4.00 ± 0.20	5.60 ± 0.11
2.0 + 1.0	7-8	52	2.00 ± 0.11	5.00 ± 0.11

*Values are the mean of three replicates with 10 explants.

Table 3.40 Effect of different concentrations and combinations of Kn with NAA and IAA on shoot proliferation from nodal explants of *P. foetida*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of total shoots/culture (mean ± SE)	*Average length (cm) of shoots/culture (mean ± SE)
Kn + NAA				
0.5 + 0.1	7-8	52	2.60 ± 0.17	3.60 ± 0.11
0.5 + 0.2	7-8	60	2.30 ± 0.11	4.50 ± 0.11
0.5 + 0.5	7-8	75	4.00 ± 0.30	5.00 ± 0.17
0.5 + 1.0	7-8	55	2.30 ± 0.17	5.00 ± 0.25
1.0 + 0.1	7-8	62	2.30 ± 0.17	4.00 ± 0.17
1.0 + 0.2	7-8	80	4.00 ± 0.28	4.60 ± 0.11
1.0 + 0.5	7-8	72	4.00 ± 0.15	6.00 ± 0.15
1.0 + 1.0	7-8	66	2.60 ± 0.17	4.60 ± 0.17
2.0 + 0.1	8-10	55	2.60 ± 0.11	4.00 ± 0.23
2.0 + 0.2	8-10	65	3.00 ± 0.17	5.00 ± 0.17
2.0 + 0.5	8-10	72	4.00 ± 0.20	5.60 ± 0.11
2.0 + 1.0	8-10	52	2.30 ± 0.17	4.60 ± 0.17
Kn + IAA				
0.5 + 0.1	7-8	60	2.60 ± 0.17	3.60 ± 0.11
0.5 + 0.2	7-8	72	4.00 ± 0.11	4.50 ± 0.11
0.5 + 0.5	7-8	85	6.00 ± 0.17	5.50 ± 0.17
0.5 + 1.0	7-8	55	5.00 ± 0.23	4.60 ± 0.17
1.0 + 0.1	7-8	62	2.60 ± 0.11	5.00 ± 0.23
1.0 + 0.2	7-8	75	3.00 ± 0.26	5.60 ± 0.17
1.0 + 0.5	7-8	80	4.00 ± 0.23	6.50 ± 0.11
1.0 + 1.0	7-8	56	2.30 ± 0.17	5.30 ± 0.17
2.0 + 0.1	8-10	60	2.00 ± 0.11	4.00 ± 0.23
2.0 + 0.2	8-10	72	2.60 ± 0.11	5.00 ± 0.17
2.0 + 0.5	8-10	66	4.00 ± 0.20	5.60 ± 0.11
2.0 + 1.0	8-10	50	2.60 ± 0.11	5.60 ± 0.17

*Values are the mean of three replicates with 10 explants.

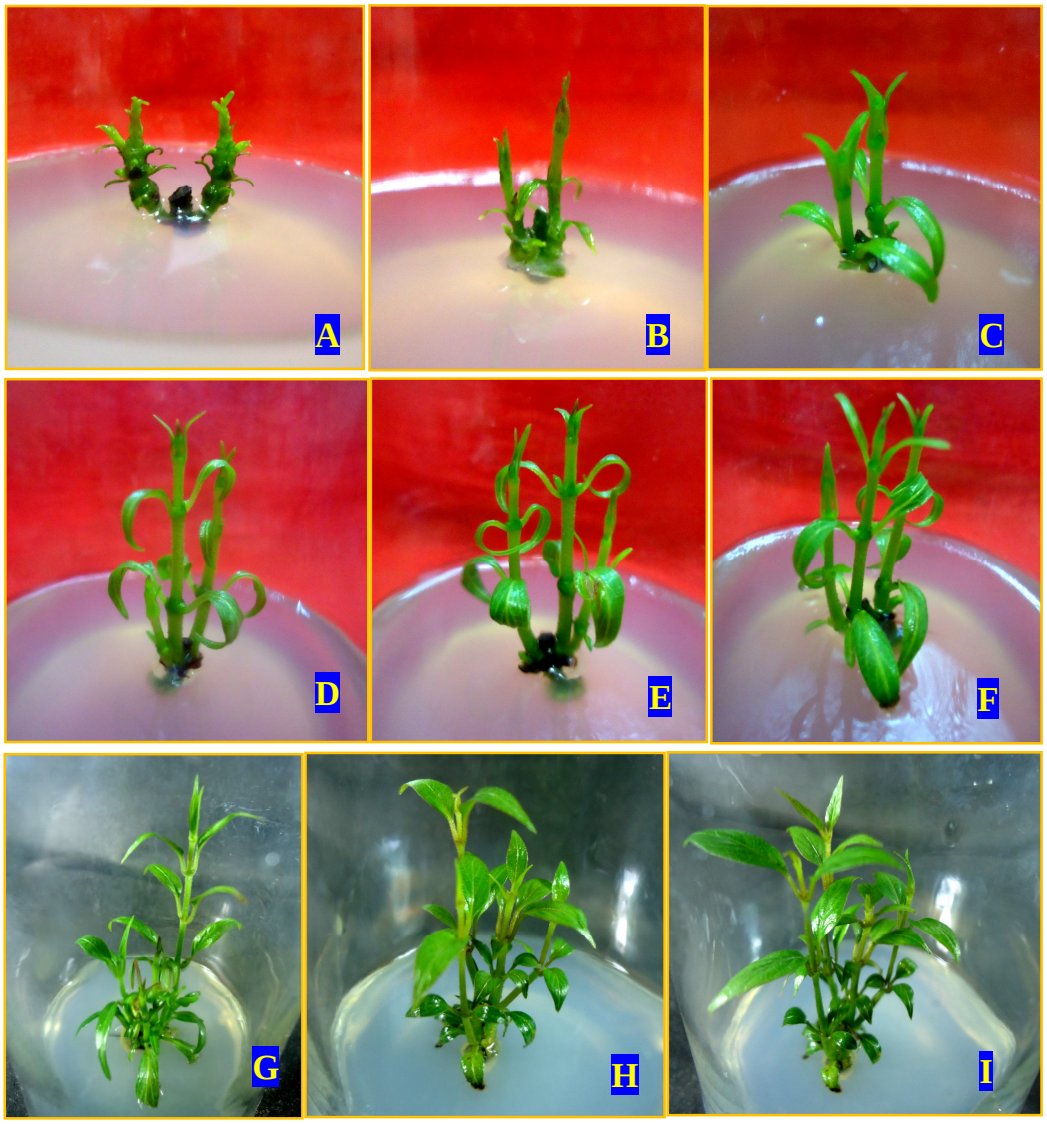


Plate 3.16 Development of multiple shoot from nodal segments of *P. foetida* in direct organogenesis and their subsequent development.

A-B: Initiation of multiple shoot formation in nodal explant after 2 weeks of inoculation in media having 1.0 mg/l BAP + 0.5 mg/l NAA; **C-F:** Proliferation of multiple shoot in the same medium after two subcultures at 14 days interval; **G-I:** Further proliferation and elongation of shoot after 7-8 weeks of culture in the same medium.

3.3.5.2. Effect of phytohormones on callus induction from different explants of *P. foetida*

Five concentrations (0.5, 1.0, 2.0, 2.5 and 3.0 mg/l) of 2, 4-D used singly and two concentrations (1.0 and 2.0 mg/l) of 2,4-D in combinations with three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP or Kn. In another experiments sets in three concentrations (1.0 and 2.0 and 2.5 mg/l) of 2,4-D in combinations with three concentrations of IBA (0.5, 1.0 and 2.0 mg/l) were used to investigate the initiation of callus and its subsequent development. Leaf and internode were used as explants for callus induction. In preliminary experiments it was observed that when the leaf and internode explant were placed horizontally on the media surface the initiation of callus took place from both the cut ends. But when they were placed vertically callus initiation took place only from the cut end dipped in the agar medium. Horizontally oriented explants produced callus throughout their entire surface more rapidly than those, which were placed vertically. Therefore internodes explants were always placed horizontally on the agar surface to induce callus in all these experiments. The effect of different combinations and concentrations of phytohormones, days of callus initiation, percentage of explants induced callus development, callus colour and texture from different explants are discussed experiment wise:

3.3.5.2.1. Callus induction from leaf explant of *P. foetida*

Experiment 1. Effect of different concentrations and combinations of 2,4-D, BAP, Kn and IBA on callus induction from leaf explant. Data were taken after 6 weeks of culture

In order to induce of callus, leaf explants were cultured on MS medium supplemented with different concentrations (0.5, 1.0, 2.0, 2.5 and 3.0 mg/l) of 2, 4-D singly and two concentrations (1.0 and 2.0 mg/l) of 2,4-D in combination with three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP or Kn. In another set of experiments three concentrations (1.0 and 2.0 and 2.5 mg/l) of 2,4-D in combination with three concentrations of IBA (0.5, 1.0 and 2.0 mg/l). Cultures were maintained under 16 h light and 8 h dark regime and data on different parameters are summarized in Table 3.41. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was not noticed in all media formulations. But there was a wide variation in morphological nature and

percentage of callus formation among them. In case of leaf explants, callus initiation occurred within 10-15 days depending upon the concentrations and combinations of hormones. Percentage explant induce callus formation ranged from 15.00-80.00 %. Among the tested combinations MS medium having 2.0 mg/l 2,4-D + 1.0 mg/l IBA found highest percentage (80%) of callus formation (Plate 16B-C) followed by 75% in 1.0 mg/l 2,4-D + 1.0 mg/l IBA and 2.5 mg/l 2,4-D + 1.0 mg/l IBA. The lowest percentage (15%) of callus formation was obtained in media having 2.5 mg/l 2, 4-D. The calli were light green, brown and creamy in color and nodular and soft in texture.

3.3.5.2.2. Callus induction from internode explant

Experiment 2. Effect of different concentrations and combinations of 2,4-D, BAP and Kn on callus induction from internode explant.

For callus, internode segments were cultured on MS medium supplemented with four concentrations (0.5, 1.0, 2.0 and 3.0 mg/l) of 2,4–D used singly and in combinations of three concentrations (1.0, 2.0 and 3.0 mg/l) of BAP or Kn. In another experiments sets, three concentrations (1.0, 2.0 and 2.5 mg/l) of 2,4-D in combinations of three concentrations (0.5, 1.0 and 2.0 mg/l) of IBA. Cultures were maintained under 16 h light and 8 h dark regime and data on different parameters are summarized in Table 3.41. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was not noticed in all media formulations. But there was a wide variation in morphological nature and percentage of callus formation among them. Callus initiation occurred within 10-15 days of culture depending upon the concentrations and combinations of hormones. Percentage of explant induced callus formation ranged from 20.00-90.00 %. Among the tested combinations MS medium having 2.5 mg/l 2,4-D + 0.5 mg/l IBA found highest percentage (90%) of callus formation (Plate 16 A) followed by 80% in 2.0 mg/l 2,4-D + 1.0 mg/l IBA and 2.5 mg/l 2,4-D + 1.0 mg/l IBA. The lowest percentage (20%) of callus formation was obtained in media having 2.5 mg/l 2,4-D. The calli were light green , brown and creamy in color and nodular soft and friable in texture.

Table 3.41 Effect of different concentrations and combinations of 2,4-D alone and with BA, Kn or IBA on callus induction from leaf and internode explants of *P. foetida*. Data were recorded after 6 weeks of culture.

Hormonal combination (mg/l)	Leaf explant			Internode explant		
	Days of callus initiation	% of explant induced callus (mean ± SE)	Callus colour and texture	Days of callus initiation	% of explant induced callus (mean ± SE)	Callus colour and texture
2,4-D						
0.5	-	-	-	-	-	-
1.0	12-15	20	bn	12-15	25	bn
2.0	12-15	30	"	12-15	30	"
2.5	12-15	15	"	12-15	20	"
3.0	-	-	-	-	-	-
2,4-D + BA						
1.0 + 0.5	12-15	40	cs	12-15	50	lgn
1.0 + 1.0	10-15	60	"	12-15	60	"
1.0 + 2.0	10-15	55	"	10-12	45	"
2.0 + 0.5	12-15	60	"	10-15	60	"
2.0 + 1.0	12-15	70	lgn	12-15	75	cs
2.0 + 2.0	12-15	65	"	12-15	65	"
2,4-D + Kn						
1.0 + 0.5	12-15	30	bs	12-15	40	bf
1.0 + 1.0	12-15	55	"	12-15	60	"
1.0 + 2.0	12-15	50	"	10-12	50	"
2.0 + 0.5	10-15	60	lgs	12-15	60	lgs
2.0 + 1.0	10-15	70	"	12-15	70	"
2.0 + 2.0	10-15	50	"	10-12	50	"
2, 4-D + IBA						
1.0 + 0.5	10-12	50	lgs	10-12	50	bfs
1.0 + 1.0	10-12	75	"	10-12	75	"
1.0 + 2.0	10-12	60	"	10-12	60	"
2.0 + 0.5	10-12	60	"	10-12	60	"
2.0 + 1.0	10-12	80	"	10-12	80	"
2.0 + 2.0	10-12	70	bs	10-12	70	bs
2.5 + 0.5	10-12	70	"	10-12	90	"
2.5 + 1.0	10-12	75	"	10-12	80	"
2.5 + 2.0	10-12	65	"	10-12	70	"

* Values are the mean of three replicates with 10 explants.

bn: brown nodular, **cs:** creamy soft**, lgn:** light green nodular, **bs:** brown soft, **lgs:** light green soft, **bfs:** brown friable soft.

3.3.5.3. Adventitious shoot regeneration from different types of calli in *P. foetida*

Calli produced in different plant growth regulators supplemented on MS medium did not differentiate into shoot buds in the same medium after subsequent subcultures. So to induce adventitious shoot buds from various types of calli for their formulations with different media combinations were needed. A wide range of auxin and cytokinin supplemented media were used to standardize the organogenic response of calli. Among the different types of calli only light green nodular callus found to be regenerative on different plant growth regulators supplemented media. The results are discussed according to types of explants under separate heads.

3.3.5.3.1. Plant regeneration from leaf derived callus

Different experiments were conducted to investigate plant regeneration ability through callus culture from leaf explants. Data on percentage of organogenic calli induced, average number of shoot per callus and average length of shoot per callus were collected after 8 weeks of culture. Findings of the experiments are described below:

Experiment 1. Effect of different concentrations and combinations of BAP, Kn, NAA and IAA on organogenesis of leaf derived callus

In this experiment leaf derived calli of *P. foetida* were used to investigate the effect of different hormonal concentrations and combinations. For shoot differentiation light green nodular calli were subcultured on to MS medium supplemented with three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP and Kn alone and two concentrations of BAP or Kn (1.0 and 2.0 mg/l) in combinations with three concentrations (0.2, 0.5 and 1.0 mg/l) of NAA or IAA. Morphogenic potentialities of cultured calli varied with hormonal treatments (Table 3.42). Highest 70.00% of shoot regeneration was recorded in 1.0 mg/l BAP with 1.0 mg/l IAA followed by 60% regeneration in media having 1.0 mg/l BAP with 1.0 mg/l NAA and 1.0 mg/l Kn with 1.0 mg/l IAA. The lowest 10.00% of shoot regeneration was recorded in media having 1.0 mg/l Kn. The highest number of shoots per callus was recorded 5.00 ± 0.17 in media having 1.0 mg/l BAP with 1.0 mg/l IAA. The lowest number of shoots per callus was recorded 1.00 ± 0.05 in media having 2.0 mg/l Kn with 1.0 mg/l NAA. Highest length of shoot 6.60 ± 0.11 cm was recorded in 1.0 mg/l BAP+ 1.0 mg/l IAA and the lowest length of shoots 3.60 ± 0.11 cm was recorded in 1.0 mg/l Kn.

3.3.5.3.2. Plant regeneration from internode derived callus

Different experiments were conducted to investigate plant regeneration ability through callus culture from internode explants. Data on percentage of organogenic calli induced root and shoot, average number of shoot per callus and average length of shoot per callus were collected after 8 weeks of culture. Findings of the experiments are described below.

Experiment 1. Effect of different concentrations and combinations of BAP, Kn, NAA and IAA on organogenesis of internode derived callus

In this experiment internode derived calli of *P. foetida* were used to investigate the effect of different hormonal concentrations and combinations on regeneration performance. For shoot differentiation light green nodular calli were sub cultured on to MS medium supplemented with different concentrations of BAP and Kn (0.5, 1.0 and 2.0 mg/l) alone and two concentrations of BAP or Kn (1.0 and 2.0 mg/l) in combinations with different concentrations of NAA or IAA (0.2, 0.5 and 1.0 mg/l). Morphogenic potentialities of cultured calli varied with hormonal treatments (Table 3.42). Highest 85.00% of shoot regeneration was recorded in 1.0 mg/l BAP with 1.0 mg/l IAA (Plate 3.17) followed by 70% regeneration in media having 1.0 mg/l BAP with 0.5 mg/l IAA and 1.0 mg/l Kn with 1.0 mg/l IAA. The lowest 15.00% shoot regeneration was recorded in media having 1.0 mg/l Kn. The highest number of shoots per callus was recorded 6.00 ± 0.11 in media having 1.0 mg/l BAP with 1.0 mg/l IAA. The lowest number of shoots per callus was recorded 1.00 ± 0.11 in media having 2.0 mg/l BAP with 1.0 mg/l IAA and 1.0 mg/l Kn with 0.2 mg/l IAA. Highest length of shoot 6.50 ± 0.28 cm was recorded in 1.0 mg/l BAP with 1.0 mg/l IAA and the lowest length of shoots 3.50 ± 0.11 cm was recorded in 1.0 mg/l Kn.

Table 3.42 Effect of BAP and Kn singly and combination with NAA and IAA on adventitious shoot regeneration from leaf and internode derived callus of *P. foetida*. Data were recorded after 8 weeks of culture.

Hormonal combination (mg/l)	Leaf derived callus			Internode derived callus		
	% of regenerative calli	*Average number of shoot / calli (mean ± SE)	*Average length (cm) of shoot /calli (mean ± SE)	% of regenerative calli	*Average number of shoot /calli (mean ± SE)	*Average length (cm) of shoot /calli (mean ± SE)
BAP						
0.5	-	-	-	-	-	-
1.0	25	2.00 ± 0.17	4.00 ± 0.17	30	2.00 ± 0.17	4.00 ± 0.17
2.0	15	2.00 ± 0.11	4.00 ± 0.23	20	2.00 ± 0.11	4.50 ± 0.11
Kn						
0.5	-	-	-	-	-	-
1.0	10	2.00 ± 0.17	3.60 ± 0.11	15	2.00 ± 0.17	3.50 ± 0.11
2.0	-	-	-	-	-	-
BAP + NAA						
1.0 + 0.2	30	2.00 ± 0.17	5.30 ± 0.17	40	2.00 ± 0.17	5.00 ± 0.17
1.0 + 0.5	40	3.00 ± 0.17	6.00 ± 0.28	50	4.00 ± 0.17	5.60 ± 0.17
1.0 + 1.0	60	4.00 ± 0.30	4.60 ± 0.11	65	4.00 ± 0.30	4.00 ± 0.25
2.0 + 0.2	40	2.00 ± 0.26	5.00 ± 0.17	40	2.00 ± 0.26	5.00 ± 0.17
2.0 + 0.5	50	3.00 ± 0.11	5.60 ± 0.11	45	3.00 ± 0.11	5.30 ± 0.17
2.0 + 1.0	30	3.00 ± 0.11	4.60 ± 0.17	35	2.00 ± 0.23	4.60 ± 0.17
Kn + NAA						
1.0 + 0.2	-	-	-	40	2.00 ± 0.11	5.00 ± 0.11
1.0 + 0.5	30	2.00 ± 0.20	5.60 ± 0.17	50	4.00 ± 0.23	6.00 ± 0.17
1.0 + 1.0	50	3.00 ± 0.23	4.60 ± 0.17	60	4.00 ± 0.17	5.50 ± 0.28
2.0 + 0.2	-	-	-	25	2.00 ± 0.25	5.00 ± 0.17
2.0 + 0.5	40	3.00 ± 0.23	5.60 ± 0.11	30	2.00 ± 0.11	5.30 ± 0.17
2.0 + 1.0	30	1.00 ± 0.05	4.00 ± 0.17	-	-	-
BAP + IAA						
1.0 + 0.2	30	2.00 ± 0.17	5.60 ± 0.11	50	3.00 ± 0.11	5.30 ± 0.17
1.0 + 0.5	50	4.00 ± 0.17	6.00 ± 0.28	70	4.00 ± 0.17	6.00 ± 0.28
1.0 + 1.0	70	5.00 ± 0.17	6.60 ± 0.11	85	6.00 ± 0.11	6.50 ± 0.28
2.0 + 0.2	35	3.00 ± 0.17	5.00 ± 0.17	60	2.00 ± 0.26	5.60 ± 0.17
2.0 + 0.5	50	4.00 ± 0.11	5.30 ± 0.17	40	3.00 ± 0.11	5.30 ± 0.17
2.0 + 1.0	40	2.00 ± 0.17	4.60 ± 0.17	30	1.00 ± 0.15	4.30 ± 0.17
Kn + IAA						
1.0 + 0.2	35	3.00 ± 0.23	5.30 ± 0.17	40	1.00 ± 0.11	5.60 ± 0.17
1.0 + 0.5	50	4.00 ± 0.17	5.60 ± 0.11	50	2.00 ± 0.11	6.00 ± 0.11
1.0 + 1.0	60	4.00 ± 0.17	4.60 ± 0.11	70	3.00 ± 0.11	5.50 ± 0.28
2.0 + 0.2	30	2.00 ± 0.17	5.00 ± 0.17	25	3.00 ± 0.17	5.90 ± 0.75
2.0 + 0.5	40	3.00 ± 0.20	5.60 ± 0.11	30	2.00 ± 0.11	5.30 ± 0.17
2.0 + 1.0	-	-	-	-	-	-

*Values are the mean of three replicates with 10 explants.

Plate 3.17 Callus induction from leaf and internode explant with plant regeneration from leaf derived callus of *P. foetida*.

A: Callus induction from internode explant in 2.0 mg/l 2, 4-D + 0.5 mg/l IBA; **B-C:** Callus induction from leaf segment in 2.0 mg/l 2, 4-D + 1.0 mg/l IBA; **D:** Base callus induction of shoot tip explants in 1.0 mg/l 2,4-D + 1.0 mg/l BAP; **E:** Initiation of callus regeneration in 1.0 mg/l BAP + 1.0 mg/l IAA after 6 weeks of culture from leaf derived callus; **F:** Proliferation of multiple shoot buds in the same medium after 8 weeks of sub culture and **G-I:** Further proliferation and elongation of shoot buds in the same medium after 10 weeks of subculture.

3.3.5.4. Rooting of *in vitro* grown shoots in *P. foetida*

Shoot cuttings 5–7 cm long from *in vitro* grown shoots were separated and transferred to rooting media. Half strength MS medium fortified with different concentrations (0.1, 0.2, 0.3, 0.5 and 1.0 mg/l) of NAA, IBA and IAA were used for rooting experiment. Rooting started within 10 to 15 days of culture. Among the tested concentrations half strength MS medium having 0.2 mg/l, 0.3 mg/, IBA and 0.2 mg/l NAA found highest percentage (60-80%) of root formation. The lowest percentage of root formation (20%) obtained in media having 0.3 mg/l IAA and 0.1 mg/l NAA. Data on days to root initiation, percentage (%) of root formation, average number of root per culture and average length of root per culture were conducted and were recorded after 6 weeks of culture. Findings of the experiments are described below:

Experiment 1. Effect of different concentrations of IBA, NAA and IAA on root induction from *in vitro* grown shoots of *P. foetida*

For adventitious root formation, the shoots obtained from *in vitro* grown shoots were excised and cultured on half strength MS medium with different concentrations of IBA, NAA and IAA. Results obtained for root induction, percentage (%) of root formation, morphology, average number and length of roots are shown in Table 3.43. Percentage range of cultures produced roots varied from 20.00-80.00%. Highest 80.00% of root regeneration was recorded in 0.2 mg/l IBA followed by 70.00 % regeneration in media 0.3mg/l IBA. The lowest 20.00 % of root regeneration was recorded in media having 0.1 mg/l NAA and 0.3 mg/l IAA. The highest average number of roots per shoot was recorded 12.00 ± 0.28 in media having 0.2 mg/l IBA (Plate 3.18B) followed by 8.00 ± 0.23 in 0.3 mg/l IBA and 0.2 mg/l NAA. The lowest average number of root per shoots was recorded 3.00 ± 0.17 in media having 0.3 mg/l IAA. Highest length of roots 6.00 ± 0.28 cm was recorded in 0.3 mg/l NAA and the lowest length of roots 4.00 ± 0.26 cm was recorded in 0.1 mg/l IBA and 0.3 mg/l IAA. In most cases morphology of roots was thin, thick and long.

Table 3.43 Effect of different concentrations and combinations of auxins on adventitious root formation from the *in vitro* grown nodal explant on half strength MS medium in *P. foetida*.

Different concentration of auxin (mg/l)	Number of days to root initiation	Percentage (%)of root formation	*Average number of root/ culture (mean ± SE)	*Average length(cm) of root/ culture (mean ± SE)
IBA				
0.1	12-15	40	4.00 ± 0.26	4.00 ± 0.26
0.2	12-15	80	12.00 ± 0.28	5.60 ± 0.11
0.3	12-15	70	8.00 ± 0.23	5.00 ± 0.17
0.5	12-15	50	6.00 ± 0.28	5.00 ± 0.28
1.0	-	-	-	-
NAA				
0.1	12-15	20	4.00 ± 0.26	4.60 ± 0.11
0.2	12-15	50	8.00 ± 0.23	5.00 ± 0.30
0.3	12-15	60	6.00 ± 0.28	6.00 ± 0.28
0.5	12-15	30	4.00 ± 0.23	4.50 ± 0.17
1.0	-	-	-	-
IAA				
0.1	-	-	-	-
0.2	-	-	-	-
0.3	12-15	20	3.00 ± 0.17	4.00 ± 0.17
0.5	12-15	30	6.00 ± 0.17	4.50 ± 0.28
1.0	-	-	-	-

*Values are the mean of three replicates with 10 explants.

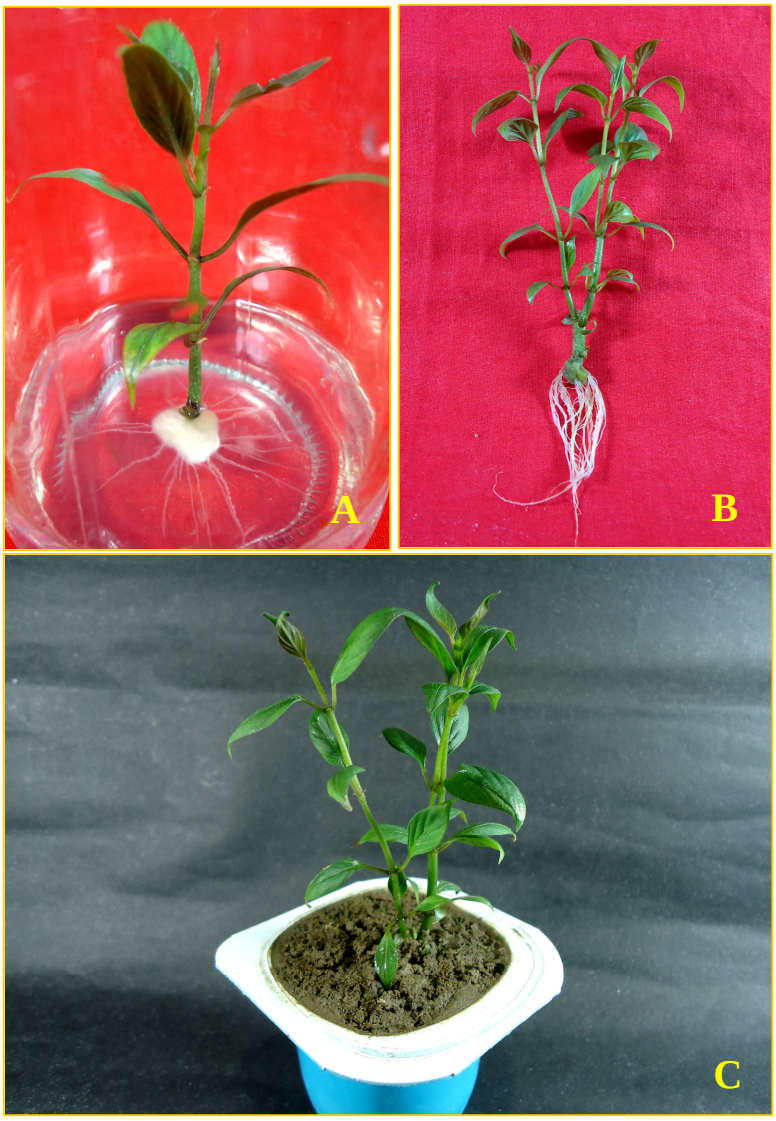


Plate 3.18 Induction of adventitious roots and establishment in soil in *P. foetida.*

A: Induction of adventitious roots on shoots regenerated from nodal explants in half strength MS medium containing 0.2 mg/l IBA after 6 weeks of culture; **B:** *P. foetida* with roots outside of culture bottle; **C:** The *in vitro* grown seedlings acclimatized to grow in pots in outside environment after 20 days.

3.3.6. *In vitro* regeneration and conservation of *Rauwolfia serpentina*

Experiments on direct and indirect organogenesis were carried out using different types of explants viz. axillary bud, nodal explants, internodal explants and leaves. Axillary bud and nodal explants were cultured for direct shoot regeneration. Explants were cultured on the MS (Murashig and Skoog, 1962) agar gelled medium supplemented with different concentrations of auxins and cytokinins used singly or in combinations to investigate the initiation of shoot and its subsequent regeneration. For root induction, micro shoots obtained from direct organogenesis were transferred to rooting media. Finally well developed plants with both shoots and roots were transferred to natural conditions through successive phages of acclimatization. Details of the results so far obtained from each of the experiments are described under following heads:

3.3.6.1. Direct regeneration

Axillary bud and nodal explants were used for direct shoot regeneration in *R. serpentina*. Explants were cultured on MS medium with BAP, Kn used alone and in combinations with NAA or IAA. Data on number of days taken for shoot initiation, percentage (%) of explant induced shoot development, average number of shoot per culture and average length of shoot per culture were collected after 6 weeks of culture. Among the two types of explants nodal explants responded better in the supplemented cultured medium. The results are described according to types of explants under separate heads:

3.3.6.1.1. Axillary bud explants

Experiment 1. Effect of different concentrations of BAP alone and combinations with NAA or IAA on multiple shoot induction from axillary bud explants. Data were taken after 6 weeks of culture

Five concentrations (0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) of BAP alone and combination of two concentration (1.5, and 2.0 mg/l) of BAP with four concentrations (0.1, 0.2, 0.5 and 1.0 mg/l) of NAA or IAA were treated in MS medium for the purpose of multiple shoot induction from axillary bud explants of *R. serpentina*. Data were taken after 6 weeks of inoculation and days taken for shoot initiation, percentage of shoot induction, number of shoot/culture and length of shoot/culture were measured. The results are presented in Table 3.44. The efficiency of BAP + NAA was better than BAP alone and BAP + IAA on direct shoot regeneration from axillary bud explants.

All the used media compositions formed shoot proliferation and the results were inferior to the results obtained from nodal explants. Addition of lower concentration of NAA with higher concentration of BAP was found more suitable in direct regeneration. Among the combinations of BAP + NAA and BAP + IAA, the highest percentage (80%) of shoot proliferation was noted in the media having 1.5 mg/l BAP + 0.2 mg/l NAA followed by 70% regeneration in 1.5 mg/l BAP + 0.5 mg/l NAA and 2.0 mg/l BAP + 0.2 mg/l NAA. The lowest percentage (15%) of shoot proliferation was observed in 0.5 mg/l BAP alone. The highest average number 10.00 ± 0.11 of shoot per culture was observed in the media having 1.5 mg/l BAP + 0.2 mg/l NAA followed by 8.00 ± 0.32 of 1.5 mg/l BAP + 0.5 mg/l NAA. The lowest number of shoot per culture 1.60 ± 0.05 was noted in the media having 0.5 mg/l BAP. Highest average length 4.60 ± 0.17 cm of shoot per culture were noted in the media having 2.0 mg/l BAP + 0.2 mg/l IAA and the lowest average length 2.30 ± 0.17 was observed in 3.0 mg/l BAP. Experimental results revealed that, 1.5 mg/l BAP + 0.2 mg/l NAA, 1.5 mg/l BAP + 0.5 mg/l NAA, 2.0 mg/l BAP + 0.2 mg/l NAA and 2.0 mg/l BAP + 0.2 mg/l IAA were more effective combinations for multiple shoot induction. Experiment was set for the induction of multiple shoot but sometimes callus was found to grow at the basal part of the growing shoots in many media formulations.

Experiment 2. Effect of different concentrations of Kn used alone and in combinations with NAA or IAA on multiple shoot induction from axillary bud explants. Data were taken after 6 weeks of culture

Explants were cultured on MS medium supplemented with five concentrations (0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) of Kn alone and combination of two concentrations (1.5, and 2.0 mg/l) of Kn with four concentrations (0.1, 0.2, 0.5, 1.0 mg/l) of NAA or IAA were treated in MS medium for the purpose of multiple shoot induction from axillary bud explants of *R. serpentina*. Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.45. The efficiency of Kn + NAA was better than Kn alone and Kn + IAA on direct shoot regeneration. All the used media compositions formed multiple shoots. Addition of lower concentration of NAA with higher concentration of Kn was found more suitable than that of other concentrations. Among the combinations of Kn + NAA and Kn + IAA, the highest percentage (70%) of shoot proliferation was noted in the media having 1.5 mg/l Kn + 0.2 mg/l NAA followed by 65% regeneration in 2.0 mg/l Kn + 0.2mg/l NAA. The lowest percentage (10%) of shoot proliferation was noted in 0.5 mg/l Kn. The highest

average number 6.00 ± 0.11 of shoot per culture was observed in the media having 2.0 mg/l Kn + 0.2 mg/l NAA followed by 5.50 ± 0.11 in 1.5 mg/l Kn + 0.2 mg/l NAA. The lowest number of shoot per culture 1.30 ± 0.05 were noted in the media having 0.5 mg/l Kn. Highest average length 4.60 ± 0.28 cm of shoot per culture were noted in the media having 2.0 mg/l Kn + 0.2 mg/l NAA and the lowest average length 2.00 ± 0.17 cm was observed in 0.5 mg/l Kn. Experimental results revealed that, 1.5 mg/l Kn + 0.2 mg/l NAA, 1.5 mg/l Kn + 0.5 mg/l NAA, 2.0 mg/l Kn + 0.2 mg/l NAA and 2.0 mg/l Kn + 0.2 mg/l IAA were more effective combinations for multiple shoot induction. Experiment was set for the induction of multiple shoot but sometimes callus was found to grow at the basal part the growing shoots in many media formulations.

Table 3.44 Effect of different concentrations of BAP used singly and combinations with NAA and IAA on multiple shoot induction from axillary bud explants of *R. serpentina.* Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	Degree of callusing	*Average number of shoots / culture (mean ± SE)	*Average length (cm) of shoots /culture (mean ± SE)
BAP					
0.5	8-10	15	-	1.60 ± 0.17	2.60 ± 0.23
1.0	7-8	40	-	2.00 ± 0.30	3.00 ± 0.20
1.5	7-8	55	+	3.30 ± 0.17	4.00 ± 0.30
2.0	7-8	60	+	3.60 ± 0.17	3.60 ± 0.11
3.0	8-10	45	+	1.80 ± 0.11	2.30 ± 0.17
BAP + NAA					
1.5 + 0.1	7-8	65	-	6.00 ± 0.28	2.60 ± 0.23
1.5 + 0.2	7-8	80	-	10.00 ± 0.11	3.50 ± 0.11
1.5 + 0.5	7-8	70	-	8.00 ± 0.32	4.00 ± 0.25
1.5 + 1.0	7-8	55	-	4.00 ± 0.57	3.30 ± 0.17
2.0 + 0.1	7-8	50	-	4.00 ± 0.30	3.00 ± 0.20
2.0 + 0.2	7-8	70	-	8.00 ± 0.34	4.00 ± 0.30
2.0 + 0.5	7-8	56	-	5.30 ± 0.17	3.60 ± 0.11
2.0 + 1.0	7-8	50	-	4.30 ± 0.11	3.30 ± 0.17
BAP + IAA					
1.5 + 0.1	7-8	45	-	3.50 ± 0.11	3.60 ± 0.11
1.5 + 0.2	7-8	60	-	4.00 ± 0.30	4.00 ± 0.25
1.5 + 0.5	7-8	48	+	4.30 ± 0.11	4.50 ± 0.28
1.5 + 1.0	7-8	36	+	3.00 ± 0.20	3.67 ± 0.12
2.0 + 0.1	7-8	40	-	4.00 ± 0.30	3.50 ± 0.11
2.0 + 0.2	7-8	65	-	5.30 ± 0.17	4.60 ± 0.11
2.0 + 0.5	7-8	56	+	4.60 ± 0.11	3.60 ± 0.11
2.0 + 1.0	7-8	45	+	3.60 ± 0.11	3.30 ± 0.17

+ : Base callus present, **–:** Base callus absent.

* Values are the mean of three replicates with 10 explants.

Table 3.45 Effect of different concentrations of Kn used singly and combinations with NAA and IAA on multiple shoot induction from axillary bud explants of *R. serpentina.* Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	Degree of callusing	*Average number of shoots / culture (mean ± SE)	*Average length (cm) of shoots /culture (mean ± SE)
Kn					
0.5	8-10	10	-	1.30 ± 0.05	2.00 ± 0.17
1.0	8-10	30	-	1.60 ± 0.17	2.60 ± 0.23
1.5	8-10	45	+	2.00 ± 0.23	3.30 ± 0.17
2.0	8-10	50	+	2.60 ± 0.11	3.00 ± 0.20
3.0	8-10	37	+	2.30 ± 0.17	2.60 ± 0.23
Kn + NAA					
1.5 + 0.1	7-8	50	-	4.00 ± 0.30	3.50 ± 0.11
1.5 + 0.2	7-8	70	-	5.50 ± 0.11	4.30 ± 0.11
1.5 + 0.5	7-8	62	-	5.00 ± 0.28	3.60 ± 0.11
1.5 + 1.0	7-8	45	-	2.00 ± 0.23	3.00 ± 0.20
2.0 + 0.1	7-8	40	-	4.00 ± 0.30	3.00 ± 0.15
2.0 + 0.2	7-8	65	-	6.00 ± 0.11	4.60 ± 0.28
2.0 + 0.5	7-8	55	-	5.00 ± 0.23	4.30 ± 0.17
2.0 + 1.0	7-8	45	-	3.30 ± 0.17	3.30 ± 0.17
Kn + IAA					
1.5 + 0.1	7-8	40	-	3.50 ± 0.11	4.00 ± 0.15
1.5 + 0.2	7-8	62	-	4.30 ± 0.11	4.50 ± 0.28
1.5 + 0.5	7-8	52	+	5.00 ± 0.23	4.30 ± 0.11
1.5 + 1.0	7-8	40	+	3.30 ± 0.17	3.50 ± 0.11
2.0 + 0.1	7-8	36	-	4.00 ± 0.30	3.60 ± 0.11
2.0 + 0.2	7-8	60	-	5.00 ± 0.28	4.60 ± 0.11
2.0 + 0.5	7-8	45	+	5.30 ± 0.17	4.30 ± 0.15
2.0 + 1.0	7-8	30	+	4.30 ± 0.15	3.60 ± 0.11

+ : Base callus present, **–:** Base callus absent.

*Values are the mean of three replicates with 10 explants.

3.3.6.1.2. Nodal Explants

Experiment 1. Effect of different concentrations of BAP used alone and in combinations with NAA or IAA on multiple shoot induction from nodal explants. Data were taken after 6 weeks of culture

Five concentrations (0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) of BAP alone and combination of two concentrations (1.5, and 2.0 mg/l) of BAP with four concentrations (0.1, 0.2, 0.5, 1.0 mg/l) of NAA or IAA were treated in MS medium for the purpose of multiple shoot induction from nodal explants of *R. serpentina*. Data were taken after 6 weeks of inoculation and days taken for shoot initiation, percentage of shoot induction, number of shoot/culture and length of shoot/culture were measured. The results are presented in Table 3.46. The efficiency of BAP + NAA was better than BAP alone and BAP + IAA on direct shoot regeneration from nodal explants. All the used media compositions formed shoot proliferation and the results were superior to the results obtained from axillary bud explants. Addition of lower concentration of NAA with higher concentration of BAP was found more suitable than that of other concentrations. Among the combinations of BAP + NAA, the highest percentage (90%) of shoot proliferation was noted in the media having 1.5 mg/l BAP + 0.2 mg/l NAA followed by 80% regeneration in 2.0 mg/l BAP + 0.2 mg/l NAA The lowest percentage (20%) of shoot proliferation was noted in media having 0.5 mg/l BAP. The highest average number 12.00 ± 0.34 of shoot per culture was observed in the media having 1.5 mg/l BAP + 0.2 mg/l NAA (Plate 3.19A-B) followed by 8.00 ± 0.34 in 2.0 mg/l BAP + 0.2 mg/l NAA. The lowest average number of shoot per culture 2.00 ± 0.17 was noted in the media having 0.5 mg/l BAP. Highest average length 4.70 ± 0.20 cm of shoot per culture was noted in the media having 2.0 mg/l BAP + 0.2 mg/l IAA and the lowest average length 2.60 ± 0.23 cm per culture was observed in 2.0 mg/l BAP + 1.0 mg/l NAA. Experimental results revealed that, 1.5 mg/l BAP + 0.2 mg/l NAA, 2.0 mg/l BAP + 0.2 mg/l NAA, 2.0 mg/l BAP + 0.2 mg/l IAA and 1.5 mg/l BAP were found more effective combinations and concentrations for multiple shoot induction. Experiment was set for the induction of multiple shoot but sometimes callus was found to grow at the basal part the growing shoots in many media formulations.

Experiment 2. Effect of different concentrations of Kn used alone and in combinations with NAA or IAA on multiple shoot induction from nodal explants. Data were taken after 6 weeks of culture

Explants were cultured on MS medium supplemented with five concentrations (0.5, 1.0, 1.5, 2.0 and 3.0 mg/l) of Kn used alone and combination of two concentrations (1.5, and 2.0 mg/l) of Kn with four concentrations (0.1, 0.2, 0.5, 1.0 mg/l) of NAA or IAA were treated in MS medium for the purpose of multiple shoot induction from nodal explants of *R. serpentina*. Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.47. The efficiency of Kn + NAA was better than Kn alone and Kn + IAA on direct shoot regeneration. All the used media compositions formed multiple shoots and the results were superior to the results obtained from axillary bud explants. Addition of lower concentration of NAA with higher concentration of Kn was found more suitable than that of other concentrations. Among the combinations of Kn + NAA, the highest percentage (75%) of shoot proliferation was noted in the media having 2.0 mg/l Kn + 0.2 mg/l NAA followed by 70% regeneration in 1.5 mg/l Kn + 0.2 mg/l NAA. The lowest percentage (15%) of shoot proliferation was noted in 0.5 mg/l Kn. The highest average number 6.50 ± 0.28 of shoot per culture was observed in the media having 1.5 mg/l Kn + 0.2 mg/l NAA followed by 6.00 ± 0.36 in 1.5 mg/l Kn +0.5 mg/l NAA and 2.0 mg/l Kn + 0.2 mg/l NAA. The lowest average number of shoot per culture 1.30 ± 0.05 was noted in the media having 0.5 mg/l Kn and 3.0 mg/l Kn. Highest average length 5.00 ± 0.28 of shoot per culture was noted in the media having 1.5 mg/l Kn + 0.5 mg/l IAA and the lowest average length 2.60 cm was observed in 0.5 mg/l Kn. Experimental results revealed that, 1.5 mg/l Kn + 0.2 mg/l NAA, 1.5 mg/l Kn + 0.5 mg/l NAA and 2.0 mg/l Kn + 0.2 mg/l NAA were more effective combinations for multiple shoot induction. Experiment was set for the induction of multiple shoot but sometimes callus was found to grow at the basal part the growing shoots in many media formulations.

Table 3.46 Effect of different concentrations of BAP used singly and in combinations with NAA or IAA on multiple shoot induction from nodal explants of *R. serpentina*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	Degree of callusing	*Average number of shoots /culture (mean ± SE)	*Average length (cm) of shoots /culture (mean ± SE)
BAP					
0.5	7-8	20	-	2.00 ± 0.17	3.00 ± 0.20
1.0	7-8	46	-	2.60 ± 0.23	3.60 ± 0.11
1.5	7-8	70	+	3.00 ± 0.25	4.00 ± 0.25
2.0	7-8	64	+	2.30 ± 0.17	3.50 ± 0.11
3.0	7-8	40	+	2.30 ± 0.17	3.30 ± 0.17
BAP + NAA					
1.5 + 0.1	7-8	64	-	6.00 ± 0.11	3.30 ± 0.17
1.5 + 0.2	7-8	90	-	12.00 ± 0.34	4.70 ± 0.20
1.5 + 0.5	7-8	72	-	6.60 ± 0.17	4.00 ± 0.30
1.5 + 1.0	7-8	55	-	4.00 ± 0.25	3.30 ± 0.17
2.0 + 0.1	7-8	50	-	3.00 ± 0.15	3.00 ± 0.11
2.0 + 0.2	7-8	80	-	8.00 ± 0.34	4.30 ± 0.17
2.0 + 0.5	7-8	62	-	6.00 ± 0.50	3.30 ± 0.17
2.0 + 1.0	7-8	50	-	4.00 ± 0.15	2.60 ± 0.23
BAP + IAA					
1.5 + 0.1	7-8	54	-	4.00 ± 0.30	3.50 ± 0.11
1.5 + 0.2	7-8	64	-	6.00 ± 0.25	4.30 ± 0.17
1.5 + 0.5	7-8	60	+	5.00 ± 0.28	4.50 ± 0.28
1.5 + 1.0	7-8	45	+	5.30 ± 0.17	4.00 ± 0.30
2.0 + 0.1	7-8	40	-	3.00 ± 0.15	3.70 ± 0.17
2.0 + 0.2	7-8	70	-	6.00 ± 0.50	3.60 ± 0.11
2.0 + 0.5	7-8	62	+	4.00 ± 0.25	4.00 ± 0.30
2.0 + 1.0	7-8	40	+	4.30 ± 0.05	3.50 ± 0.11

+: Base callus present, **–:** Base callus absent.

*Values are the mean of three replicates with 10 explants.

Table 3.47 Effect of different concentrations of Kn used singly and in combinations with NAA on multiple shoot induction from nodal explants of *R. serpentina*. Data were taken after 4 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	Degree of callusing	*Average number of shoots / culture (mean ± SE)	*Average length (cm) of shoots / culture (mean ± SE)
Kn					
0.5	7-8	15	-	1.30 ± 0.05	2.60 ± 0.23
1.0	7-8	40	-	1.60 ± 0.15	3.00 ± 0.20
1.5	7-8	46	-	2.00 ± 0.23	3.30 ± 0.17
2.0	7-8	52	+	2.30 ± 0.17	3.60 ± 0.11
3.0	7-8	40	+	1.30 ± 0.05	3.00 ± 0.11
Kn + NAA					
1.5 + 0.1	7-8	54	-	4.00 ± 0.11	3.30 ± 0.15
1.5 + 0.2	7-8	70	-	6.50 ± 0.28	4.00 ± 0.05
1.5 + 0.5	7-8	62	-	6.00 ± 0.36	4.30 ± 0.11
1.5 + 1.0	7-8	50	-	3.00 ± 0.11	3.60 ± 0.11
2.0 + 0.1	7-8	54	-	3.50 ± 0.20	3.50 ± 0.11
2.0 + 0.2	7-8	75	-	6.00 ± 0.50	4.00 ± 0.30
2.0 + 0.5	7-8	64	-	4.60 ± 0.17	3.30 ± 0.17
2.0 + 1.0	7-8	50	-	3.00 ± 0.11	3.00 ± 0.20
Kn + IAA					
1.5 + 0.1	7-8	52	-	4.00 ± 0.30	3.50 ± 0.11
1.5 + 0.2	7-8	60	-	4.50 ± 0.28	4.30 ± 0.17
1.5 + 0.5	7-8	56	+	4.80 ± 0.05	5.00 ± 0.28
1.5 + 1.0	7-8	46	+	3.50 ± 0.11	4.00 ± 0.15
2.0 + 0.1	7-8	55	-	3.00 ± 0.15	3.60 ± 0.11
2.0 + 0.2	7-8	66	-	8.00 ± 0.34	4.00 ± 0.30
2.0 + 0.5	7-8	60	+	5.00 ± 0.28	4.30 ± 0.11
2.0 + 1.0	7-8	45	+	3.50 ± 0.11	3.30 ± 0.17

+: Base callus present, **–:** Base callus absent.

*Values are the mean of three replicates with 10 explants.

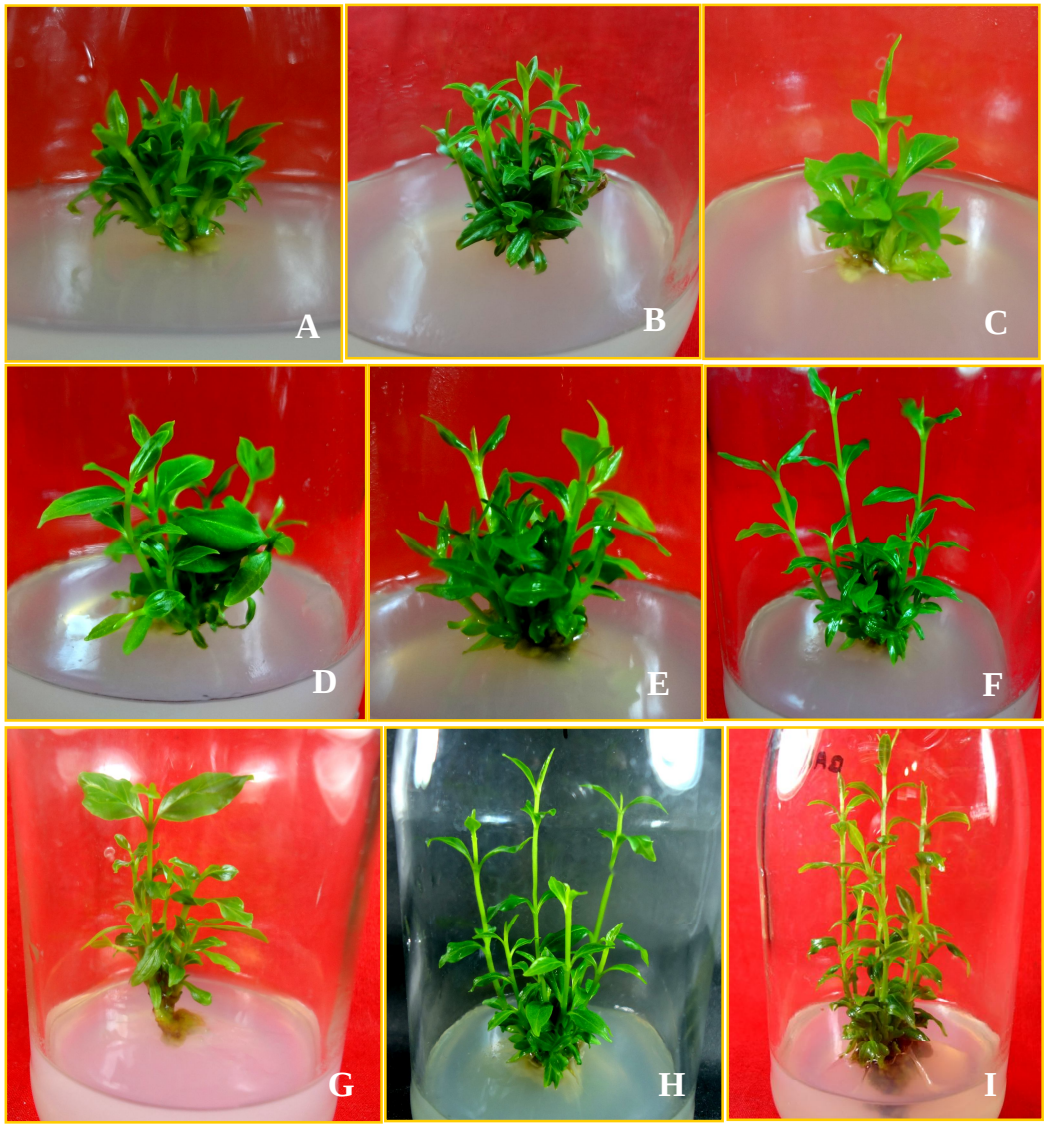


Plate 3.19 Development of multiple shoot from nodal explants of *R. serpentina* in direct organogenesis and their subsequent development.

A-B: Multiple shoot formation in nodal explants after 3 weeks of inoculation in media having 1.5 mg/l BAP + 0.2 mg/l NAA; **C-F:** Proliferation of multiple shoot in the same medium after one subcultures at 14 days interval; **G-I:** Further proliferation and elongation of shoot after 6-8 weeks of culture in the same medium.

3.3.6.2. Effect of phytohormones on callus induction from different explants of *R. serpentina*

Five concentrations (0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) of 2, 4-D, NAA, IBA and IAA used singly and three concentrations (1.0, 2.0 and 3.0 mg/l) of 2,4-D in combination with four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of BAP or Kn were used to investigate the initiation of callus and its subsequent regeneration. Leaf and internodes were used as explants for callus induction. In preliminary experiments it was observed that when the leaf and internode explants were placed horizontally on the media surface the initiation of callus took place from both the cut ends. But when they were placed vertically callus initiation took place only from the cut end dipped in the agar medium. Horizontally oriented explants produced callus throughout their entire surface more rapidly than those, which were placed vertically. Therefore internodes explants were always placed horizontally on the agar surface to induce callus in all these experiments. The effect of different combinations and concentrations of phytohormones, days of callus initiation, percentage of explants induced callus development, callus colour and texture from different explants are discussed experiment wise.

3.3.6.2.1. Callus induction from internode and leaf explants in *R. serpentina*

Experiment 1. Effect of different concentrations of 2, 4-D, NAA, IBA and IAA on callus induction from internode and leaf explant

In order to induce of callus from internode and leaf explants were cultured on MS medium supplemented with different concentrations (0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) of 2, 4–D, NAA, IBA and IAA used singly. Cultures were maintained under 16 h light and 8 h dark regime and data on different parameters are summarized in Table 3.48**.** Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was not noticed in all media formulations. But there was a wide variation in morphological nature and percentage of callus formation among them. Callus initiation occurred within 7-12 days depending upon the concentration and combination of hormones. In case of internode explants, percentage of induced callus formation ranged from 15-85.00 %. Among the tested combinations, MS medium having 2.5 mg/l 2, 4-D found highest percentage (75%) of callus formation followed by 62% regeneration in 2.0 mg/l 2,4-D. The lowest percentage of callus formation (15%) was obtained in media having 1.0

mg/l IAA. The calli were whitish, light green in color and compact and friable in texture.

But in case of leaf explants, percentage of induced callus formation ranged from 10-70 %. Among the tested combinations, MS medium having 2.5 mg/l 2,4-D found highest percentage (66%) of callus formation followed by 56% of 2.0 mg/l 2,4-D. The lowest percentage of callus formation (10%) was obtained in media having 1.0 mg/l IAA. Here, the calli were whitish, green and light green in color and compact and friable in texture.

Experiment 2. Effect of different concentrations and combinations of 2,4-D, BAP and Kn on callus induction from internode and leaf explants

In order to induce of callus, internode explants were cultured on MS medium supplemented with three concentrations (1.0, 2.0 and 3.0 mg/l) of 2, 4-D and four concentrations (0.2 , 05, 1.0, and 2.0 mg/l) of BAP and Kn. Cultures were maintained under 16 h light and 8 h dark regime and data on different parameters are summarized in Table 3.49. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was noticed in all media formulations. But there was a wide variation in morphological nature and percentage of callus formation among them. Callus initiation occurred within 8-12 days depending upon the concentrations and combinations of hormones. Incase of internode explants, percentage of induced callus formation ranged from 50-90.00 %. Among the tested combinations, MS medium having 2.0 mg/l 2,4-D + 0.5 mg/l BAP gave highest percentage (90%) of callus formation followed by 82% in 2.0 mg/l 2,4-D + 1.0 mg/l BAP and 2.0 mg/l 2,4-D + 0.5 mg/l Kn. The lowest percentage of callus formation (50%) was obtained in media having 3.0 mg/l 2,4–D + 0.2 mg/l Kn. The calli were greenish and pinkish in color and they were nodular soft and friable in texture.

But in case of leaf explant, percentage of explant induced callus formation ranged from 30-70.00%. Among the tested combinations, MS medium having 2.0 mg/l 2,4-D + 1.0 mg/l BAP produced highest percentage (70%) of callus formation and the lowest percentage (45%) in media having 1.0 mg/l 2, 4 – D + 0.2 mg/l BAP and 1.0 mg/l 2, 4 – D + 0.2 mg/l Kn . The calli were greenish in color and compact in texture.

Table 3.48 Effect of different concentrations of auxins on callus induction from internode and leaf explants of *R. serpentina*.

Hormonal combination (mg/l)	Internode explants			Leaf explants		
	Days of callus initiation	% of explant induced callus	Callus colour and texture	Days of callus initiation	% of explant induced callus	Callus colour and texture
2,4-D						
0.5	8-10	10	wc	8-10	8	gf
1.0	7-8	42	wc	8-10	32	gf
2.0	7-8	62	wc	8-10	56	gf
2.5	7-8	75	wc	8-10	66	gf
3.0	7-8	54	wc	8-10	52	gf
NAA						
0.5	-	-	-	-	-	-
1.0	8-10	12	wf	8-10	20	gf
2.0	8-10	20	wf	8-10	40	gf
2.5	8-10	25	wf	8-10	45	gf
3.0	8-10	18	wf	8-10	25	gf
IBA						
0.5	-	-	-	-	-	-
1.0	8-10	20	wf	10-12	20	gf
2.0	8-10	40	wf	10-12	30	gf
2.5	8-10	36	wf	10-12	35	gf
3.0	8-10	35	wf	10-12	32	gf
IAA						
0.5	-	-	-	-	-	-
1.0	8-10	15	wf	10-12	10	gf
2.0	8-10	35	wf	10-12	20	gf
2.5	8-10	45	wf	10-12	30	gf
3.0	8-10	40	wf	10-12	25	gf

–: No response.

wc: whitish compact, **wf:** whitish friable, **gf:** greenish friable.

Table 3.49 Effect of different combinations of 2,4-D with BAP and Kn on callus induction from internode explants of *R. serpentina*.

Hormonal combination (mg/l)	Internode explants			Leaf explants		
	Days of callus initiation	% of explant induced callus	Callus colour and texture	Days of callus initiation	% of explant induced callus	Callus colour and texture
2,4-D + BAP						
1.0 + 0.2	7-8	65	gf	7-8	45	gc
1.0 + 0.5	7-8	75	gf	7-8	52	gc
1.0 + 1.0	7-8	80	gf	7-8	60	gc
1.0 + 2.0	7-8	75	gf	7-8	50	gc
2.0 + 0.2	7-8	80	gf	7-8	54	gc
2.0 + 0.5	7-8	90	gf	7-8	62	gc
2.0 + 1.0	7-8	82	gf	7-8	70	gc
2.0 + 2.0	7-8	80	gf	7-8	45	gc
3.0 + 0.2	7-8	60	gf	7-8	52	gc
3.0 + 0.5	7-8	80	gf	7-8	66	gc
3.0 + 1.0	7-8	75	gf	7-8	55	gc
3.0 + 2.0	7-8	60	gf	7-8	42	gc
2, 4-D + Kn						
1.0 + 0.2	7-8	60	pn	7-8	45	gc
1.0 + 0.5	7-8	70	pn	7-8	60	gc
1.0 + 1.0	7-8	75	pn	7-8	64	gc
1.0 + 2.0	7-8	65	pn	7-8	52	gc
2.0 + 0.2	7-8	70	pn	7-8	55	gc
2.0 + 0.5	7-8	82	pn	7-8	65	gc
2.0 + 1.0	7-8	76	pn	7-8	52	gc
2.0 + 2.0	7-8	66	pn	7-8	35	gc
3.0 + 0.2	8-10	50	pn	7-8	40	gc
3.0 + 0.5	8-10	65	pn	7-8	60	gc
3.0 + 1.0	8-10	70	pn	7-8	52	gc
3.0 + 2.0	8-10	55	pn	7-8	30	gc

gf: greenish friable, **pn:** pinkish nodular, **gc:** green compact.

Plate 3.20 Callus induction and multiple shoot bud initiation from nodal explants of *R. serpentina*.

A: Light green colour and compact callus induction from internode explant in MS + 2.0 mg/l 2,4-D + 0.5 mg/l BAP after 4 weeks of culture; **B-C:** Greenish colour compact callus induction from leaf explant in MS + 2.0 mg/l 2,4-D + 1.0 mg/l BAP after 6 weeks of culture; **D-E:** Greenish colour and friable callus induction from internodal explant in MS + 2.0 mg/l 2,4-D + 1.0 mg/l BAP after 8 weeks of culture: **F:** Whitish colour and friable callus induction from internode explant in MS + 1.0 mg/l IBA after 6 weeks of culture; **G:** Pinkish colour and nodular callus induction from internode explant in MS + 2.0 mg/l 2,4-D + 0.5 mg/l Kn; **H-I:** Light green colour and soft base callus induction from nodal explants in MS + 3.0 mg/l BAP after 4 weeks of culture.

3.3.6.3. Rooting of *in vitro* grown shoots in *R. serpentina*

Shoot cuttings 5–7 cm long *in vitro* grown shoots were separated and transferred to rooting media. Half strength MS medium fortified with four concentrations (0.1, 0.2, 0.3 and 0.5 mg/l) of IAA, NAA and IBA used alone and in combination of IBA (0.2 mg/l) with three concentrations (0.2, 0.3 and 0.5 mg/l) of NAA or IAA were used for rooting experiment. Rooting started within 10 to 15 days of culture. Among the tested concentrations half strength MS medium having 0.2 mg/l, 0.3 mg/l IBA and combination of NAA+ IBA found highest percentage (60-80%) of root formation. Data on days to root initiation, frequency of root formation (%), average number of root per culture and average length of root per culture were recorded after 6 weeks of culture.

Experiment. 1 Effect of different concentrations of IAA, NAA, IBA used alone and in combination of NAA + IBA, IAA + IBA on root induction from *in vitro* grown shoots of *R. serpentina*

For adventitious root formation, the shoots obtained from *in vitro* grown explant were excised and cultured on half strength MS medium with different concentrations (0.1, 0.2, 0.3 and 0.5 mg/l) of IAA, NAA, IBA used alone and in combination of IBA (0.2 mg/l) with three concentrations (0.2, 0.3 and 0.5 mg/l) of NAA or IAA were used for rooting experiment. Results obtained for root induction, percentage of root formation, average number and length of roots are shown in Table 3.50. Percentage range of cultures produced roots varied from 15.00-80.00%. Highest 80.00% of root regeneration was recorded in 0.2 mg/l NAA + 0.2 mg/l IBA (Plate 3.21) and the lowest 15.00 % of root regeneration was recorded in media having 0.5 mg/l IAA. The highest average number of roots per shoot was recorded 8.00 ± 0.32 in media having 0.2 mg/l IBA + 0.2 mg/l NAA. The lowest average number of root per shoots was recorded 2.20 ± 0.20 in media having 0.5 mg/l NAA. Highest average length of roots 4.00 ± 0.17 cm was recorded in 0.2 mg/l IBA + 0.2 mg/l NAA and the lowest length of roots 1.60 ± 0.15 cm was recorded in 0.5 mg/l IAA. In most cases morphology of roots was healthy, thick and long.

Table 3.50 Root inductions from *in vitro* regenerated shoots of *R. serpentina* in half strength MS medium supplemented with different concentrations of IAA, NAA and IBA exclusively or in combination.

Growth regulators (mg/l)	Number of days taken for root initiation	% of root induction	*Average number of roots / explant (mean ± SE)	*Average length (cm) of roots / explant (mean ± SE)
IAA				
0.1	-	-	-	-
0.2	10-15	35	4.00 ± 0.30	2.30 ± 0.17
0.3	10-15	26	3.00 ± 0.15	2.30 ± 0.17
0.5	10-15	15	3.00 ± 0.15	1.60 ± 0.15
NAA				
0.1	-	-	-	-
0.2	10-12	30	3.00 ± 0.20	2.60 ± 0.23
0.3	10-12	25	3.00 ± 0.15	2.90 ± 0.20
0.5	10-12	22	2.50 ± 0.20	2.00 ± 0.17
IBA				
0.1	10-15	30	3.00 ± 0.11	2.00 ± 0.17
0.2	10-15	62	6.00 ± 0.28	2.60 ± 0.05
0.3	10-15	55	5.00 ± 0.05	3.00 ± 0.11
0.5	10-15	30	4.00 ± 0.05	2.30 ± 0.05
NAA + IBA				
0.2 + 0.2	10-12	80	8.00 ± 0.32	4.00 ± 0.15
0.3 + 0.2	10-12	75	6.00 ± 0.36	3.60 ± 0.11
0.5 + 0.2	10-12	60	4.00 ± 0.30	3.00 ± 0.20
IAA + IBA				
0.2 + 0.2	10-12	70	6.00 ± 0.11	2.60 ± 0.23
0.3 + 0.2	10-12	50	4.00 ± 0.30	2.00 ± 0.23
0.5 + 0.2	10-12	35	3.30 ± 0.17	1.80 ± 0.11

*Values are the mean of three replicates with 10 explants.

Plate 3.21 Induction of adventitious roots and establishment in soil in *R. serpentina*.

A: Initiation of adventitious roots on shoots regenerated from nodal explants in half strength MS medium containing 0.2 mg/l IBA+ 0.2 mg/l NAA after three weeks of culture; **B:** Induction of root on shoot regenerated from nodal explants in the same medium after 4 weeks of culture; **C:** Further elongation of adventitious roots on shoots regenerated from nodal explants in the same medium after 6 weeks of culture; **D:** The *in vitro* grown seedlings acclimatized to grow in pots in outside environment after 4 weeks.

3.3.7. *In vitro* regeneration and conservation of *Smilax zeylanica*

In case of *S. zeylanica* shoot tips and nodal explants were used as the explants for direct regeneration. Explants were cultured on the MS agar gelled medium supplemented with different concentrations of auxins and cytokinins used singly or in combinations to investigate the initiation of shoot and its subsequent regeneration. For root induction, micro shoots obtained from direct organogenesis were transferred to rooting media. Finally, well developed plants with both shoots and roots were transferred to natural conditions through successive phages of acclimatization. Results of the experiments are described under following heads:

3.3.7.1. Direct regeneration

Two types of explants *viz*. shoot tip and nodal explants were cultured for direct shoot regeneration. Explants were cultured on MS medium with BAP and Kn used alone and or combinations with each other or with NAA and IAA. Data on number of days taken for shoot initiation , percentage of explant induced shoot development, average number of shoot per culture and average length of shoot per culture were collected after 6 weeks of culture. Among the two types of explants nodal explants responded better on almost all of the supplemented cultured medium. The results are discussed according to types of explants under separate heads.

3.3.7.1.1. Nodal explants

Experiment 1. Effect of different concentrations of BAP and Kn used alone and their combinations on multiple shoot induction from nodal explants of *S. zeylanica*

Five concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and Kn used alone and combination of three concentrations BAP (0.5, 1.0 and 2.0 mg/l) with four concentrations of Kn (0.2, 0.5, 1.0 and 2.0 mg/l) were treated in MS medium for the purpose of multiple shoot induction from nodal explants of *S. zeylanica*. Data were taken after 6 weeks of inoculation and percentage of shoot formation, average number of shoot/culture and average length of shoot/culture were measured. The results are presented in Table 3.51. Shoot proliferation ranged from 30.00-75.00%. Highest percentage (75.00%) of multiple shoot formation was observed in MS medium containing 1.0 mg/l BAP + 0.5 mg/l Kn followed by 72.00% regeneration in MS medium containing 0.5 mg/l BAP + 1.0 mg/l Kn. The lowest percentage (30.00%) of

multiple shoot formation was observed in media having 3.0 mg/l BAP. Highest mean number of shoots was 3.50 ± 0.11 in the media having 1.0 mg/l BAP + 0.5 mg/l Kn and lowest mean number of shoot was 1.00 ± 0.57 in media containing 0.2 mg/l mg/l Kn. Average length of shoots gradually increased after induction of shoot. Highest average length was recorded 6.60 ± 0.17 cm in media having 2.0 mg/l BAP + 1.0 mg/l Kn followed by 6.30 ± 0.17 cm in 1 mg/l BAP + 1 mg/l Kn. And the lowest average length was 3.60 ± 0.11 cm in 0.2 mg/l Kn. Experimental results revealed that, 1.0 mg/l of BAP and 2.0 mg/l Kn alone and combination of 2.0 mg/l BAP + 1.0 mg/l Kn, 1.0 mg/l BAP+ 0.5 mg/l Kn, 1.0 mg/l BAP + 1.0 mg/l Kn were marked as the most effective concentrations for multiple shoot induction.

Experiment 2. Effect of different concentrations and combinations of phytohormones on multiple shoot induction from node explants on *S. zeylanica*

Explants were cultured on MS medium supplemented with two concentrations (1.0 and 2.0 mg/l) of BAP and Kn combined with three concentrations (0.1, 0.2 and 0.5 mg/l) of NAA or IAA. Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.52. All the used media compositions formed multiple shoots and the results were superiors to the results obtained from shoot tip explants. Addition of lower concentration of NAA and IAA along with higher concentration of BAP and Kn was found more suitable than that of other concentrations. Among all the combinations, the highest percentage (80%) of shoot proliferation was noted in the media having 2.0 mg/l Kn + 0.2 mg/l NAA followed by 75% regeneration in 2.0 mg/l BAP + 0.2 mg/l NAA and 1.0 mg/l Kn + 0.2 mg/l NAA. The lowest percentage (45%) of shoot proliferation was noted in media having 2.0 mg/l Kn + 0.5 mg/l IAA. The highest average number of shoot per culture was 4.00 ± 0.25 noted in the combination of 1.0 mg/l Kn + 0.2 mg/l NAA (Plate 3.22) followed by 3.60 ± 0.17 in 2.0 mg/l BAP + 0.2 mg/l NAA. The lowest number of average shoot 1.30 ± 0.05 per culture was noted in the media having 2.0 mg/l BAP +0.1 mg/l IAA and 2.0 mg/l Kn + 0.1 mg/l IAA. Highest average length was recorded 6.00 ± 0.50 cm in media having 1.0 mg/l BAP + 0.2 mg/l IAA. Average lowest length of shoot 4.00 ± 0.28 cm per culture was found in the combination of 2.0 mg/l Kn + 0.5 mg/l NAA. Experimental results revealed that, 2.0 mg/l BAP + 0.2 mg/l NAA, 1.0 mg/l BAP +

0.2 mg/l IAA, 1.0 mg/l Kn + 0.5 mg/l IAA and 1.0 mg/l Kn + 0 2mg/l NAA were more effective combinations for multiple shoot induction.

Experiment 3. Effect of different levels of L-glutamine with BAP or Kn on multiple shoot induction from *in vitro* grown nodal explants of *S. zeylanica* on half strength MS medium

Different levels of L-glutamine (0.1, 0.2, 0.5, and 1.0 mg/l) in combination with three concentrations (0.5, 1.0 and 1.5 mg/l) of BAP or Kn were treated in half strength MS medium for the purpose of multiple shoot induction from *in vitro* grown nodal explants of *S. zeylanica*. Data were taken after 6 weeks of inoculation and percentage of multiple shoot induction, average number of shoot /culture and average length of shoot /culture were measured. The results are presented in Table 3.53. Morphogenic responses of shoot induction were found to vary with hormonal formulations present in the culture media. Multiple shoot proliferation ranged from 45.00-90.00%. Highest percentage (90.00%) of multiple shoot proliferation was observed in half strength MS medium containing 1.5 mg/l Kn + 0.5 mg/l L-glutamine followed by 75.00% in half strength MS medium containing 1.5 mg/l BAP + 0.5 mg/l L-glutamine and 1.5 mg/l Kn + 0.2 mg/l L-glutamine. The lowest percentage (45.00) of multiple shoot proliferation was observed in media having 0.5 mg/l BAP + 0.1 mg/l L-glutamine. Highest mean number of shoots proliferation was 6.00 ± 0.25 in media having 1.5 mg/l Kn + 0.5 mg/l L-glutamine and followed by 5.00 ± 0.28 in 1.5 mg/l BAP + 0.5 mg/l L-glutamine and 1.5 mg/l Kn + 0.2 mg/l L-glutamine. The lowest mean number of shoot proliferation was 2.00 ± 0.17 in media containing 0.5 mg/l BAP + 0.1 mg/l L-glutamine. Average shoot length gradually increased after induction of shoot. Highest average length of shoot proliferation was recorded 5.50 ± 0.11 cm in 1.5 mg/l Kn + 0.2 mg/l L-glutamine and lowest average length of shoot proliferation was 3.00 ± 0.20 cm in 0.5 mg/l Kn + 0.1 mg/l L-glutamine. Experimental results revealed that, 1.5 mg/l Kn + 0.5 mg/l L-glutamine, 1.5 mg/l BAP + 0.5 mg/l L-glutamine and 1.5 mg/l Kn + 0.2 mg/l L-glutamine were more effective concentrations and for multiple shoot proliferation.

Table 3.51 Effect of different concentrations of BAP and Kn alone and in combinations with each other on multiple shoot induction from nodal explants of *S. zeylanica*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoots / culture (mean ± SE)	*Average length (cm) of shoots / culture (mean ± SE)
BAP				
0.2	7-8	45	1.00 ± 0.17	4.00 ± 0.11
0.5	7-8	56	1.50 ± 0. 14	4.50 ± 0.11
1.0	7-8	70	2.00 ± 0.15	5.50 ± 0.28
2.0	7-8	64	2.30 ± 0.15	5.00 ± 0.28
3.0	7-8	30	1.60 ± 0.11	4.50 ± 0.11
Kn				
0.2	7-8	40	1.00 ± 0.57	3.60 ± 0.11
0.5	7-8	60	1.50 ± 0.14	4.60 ± 0.17
1.0	7-8	66	1.87 ± 0.12	5.00 ± 0.17
2.0	7-8	70	2.50 ± 0.20	4.30 ± 0.15
3.0	7-8	35	1.00 ± 0.17	4.00 ± 0.11
BAP + Kn				
0.5 + 0.2	7-8	55	1.60 ± 0.11	5.00 ± 0.17
0.5 + 0.5	7-8	64	2.00 ± 0.11	5.30 ± 0.17
0.5 + 1.0	7-8	72	2.73 ± 0.14	6.00 ± 0.28
0.5 + 2.0	7-8	62	2.30 ± 0.15	5.60 ± 0.17
1.0 + 0.2	6-7	70	2.60 ± 0.11	5.00 ± 0.11
1.0 + 0.5	6-7	75	3.50 ± 0.11	6.00 ± 0.28
1.0 + 1.0	6-7	66	3.00 ± 0.17	6.30 ± 0.17
1.0 + 2.0	6-7	52	2.00 ± 0.17	5.30 ± 0.17
2.0 + 0.2	7-8	50	2.00 ± 0.11	5.00 ± 0.28
2.0 + 0.5	7-8	55	2.60 ± 0.11	6.00 ± 0.25
2.0 + 1.0	7-8	66	2.00 ± 0.17	6.60 ± 0.17
2.0 + 2.0	7-8	34	1.60 ± 0.11	5.67 ± 0.16

*Values are the mean of three replicates with 10 explants.

Table 3.52 Effect of different levels of growth regulators on shoot proliferation from nodal explants of *S. zeylanica.* Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoots / culture (mean ± SE)	*Average length (cm) of shoots / culture (mean ± SE)
BAP + NAA				
1.0 + 0.1	7-8	62	3.00 ± 0.17	4.00 ± 0.30
1.0 + 0.2	7-8	70	3.30 ± 0.17	5.60 ± 0.17
1.0 + 0.5	7-8	50	3.50 ± 0.11	4.60 ± 0.17
2.0 + 0.1	7-8	72	2.00 ± 0.15	5.00 ± 0.28
2.0 + 0.2	7-8	75	3.60 ± 0.17	5.60 ± 0.11
2.0 + 0.5	7-8	55	2.30 ± 0.17	4.30 ± 0.17
BAP + IAA				
1.0 + 0.1	8-10	65	2.50 ± 0.20	5.00 ± 0.28
1.0 + 0.2	8-10	70	3.00 ± 0.20	6.00 ± 0.50
1.0 + 0.5	8-10	62	2.60 ± 0.11	5.30 ± 0.17
2.0 + 0.1	8-10	52	1.30 ± 0.05	5.30 ± 0.17
2.0 + 0.2	8-10	66	2.50 ± 0.20	5.60 ± 0.11
2.0 + 0.5	8-10	55	2.00 ± 0.23	4.60 ± 0.17
Kn + NAA				
1.0 + 0.1	8-10	66	2.00 ± 0.23	4.60 ± 0.17
1.0 + 0.2	8-10	75	4.00 ± 0.25	5.00 ± 0.11
1.0 + 0.5	8-10	60	2.50 ± 0.20	4.30 ± 0.17
2.0 + 0.1	8-10	60	2.00 ± 0.57	4.30 ± 0.17
2.0 + 0.2	8-10	80	2.60 ± 0.11	5.00 ± 0.17
2.0 + 0.5	8-10	55	1.50 ± 0.14	4.00 ± 0.28
Kn + IAA				
1.0 + 0.1	8-10	60	2.00 ± 0.30	4.60 ± 0.17
1.0 + 0.2	8-10	70	3.00 ± 0.11	5.00 ± 0.11
1.0 + 0.5	8-10	50	2.50 ± 0.20	5.00 ± 0.28
2.0 + 0.1	8-10	50	1.30 ± 0.05	5.30 ± 0.17
2.0 + 0.2	8-10	66	2.00 ± 0.11	5.00 ± 0.28
2.0 + 0.5	8-10	45	1.50 ± 0.14	4.30 ± 0.15

*Values are the mean of three replicates with 10 explants.

Table 3.53 Effect of different levels of L-glutamine with BAP and Kn on multiple shoot proliferation from *in vitro* grown nodal explants of *S. zeylanica* on half strength MS medium. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoots/culture (mean ± SE)	*Average length (cm) of shoots/culture (mean ± SE)
BAP + L-glu				
0.5 + 0.1	7-10	45	2.00 ± 0.17	4.00 ± 0.30
0.5 + 0.2	7-10	60	2.50 ± 0.20	4.30 ± 0.11
0.5 + 0.5	7-10	66	3.00 ± 0.20	4.50 ± 0.28
0.5 + 1.0	7-10	50	2.30 ± 0.17	4.50 ± 0.28
1.0 + 0.1	7-10	60	2.00 ± 0.23	3.60 ± 0.11
1.0 + 0.2	7-10	72	4.00 ± 0.30	4.50 ± 0.28
1.0 + 0.5	7-10	66	4.50 ± 0.28	4.00 ± 0.30
1.0 + 1.0	7-10	55	3.00 ± 0.20	4.30 ± 0.11
1.5 + 0.1	7-10	50	2.50 ± 0.20	4.00 ± 0.30
1.5 + 0.2	7-10	65	4.60 ± 0.11	4.50 ± 0.28
1.5 + 0.5	7-10	75	5.00 ± 0.28	5.00 ± 0.11
1.5 + 1.0	7-10	55	3.50 ± 0.11	4.30 ± 0.11
Kn + L-glu				
0.5 + 0.1	7-10	52	2.30 ± 0.17	3.00 ± 0.20
0.5 + 0.2	7-10	70	2.50 ± 0.20	4.30 ± 0.11
0.5 + 0.5	7-10	66	3.00 ± 0.20	4.50 ± 0.28
0.5 + 1.0	7-10	55	2.60 ± 0.23	5.00 ± 0.28
1.0 + 0.1	7-10	50	3.00 ± 0.20	4.00 ± 0.30
1.0 + 0.2	7-10	70	3.60 ± 0.11	4.50 ± 0.28
1.0 + 0.5	7-10	62	4.60 ± 0.11	5.30 ± 0.17
1.0 + 1.0	7-10	55	2.50 ± 0.20	5.00 ± 0.28
1.5 + 0.1	7-10	60	3.00 ± 0.25	4.50 ± 0.28
1.5 + 0.2	7-10	75	5.00 ± 0.28	5.00 ± 0.11
1.5 + 0.5	7-10	90	6.00 ± 0.25	5.50 ± 0.11
1.5 + 1.0	7-10	55	3.50 ± 0.11	5.30 ± 0.17

*Values are the mean of three replicates with 10 explants.

3.3.7.1.2. Shoot tip explant

Experiment 1. Effect of different concentrations of BAP and Kn alone and in combinations on multiple shoot induction from shoot tip explants of *S. zeylanica*

Five concentrations (0.2, 0.5, 1.0, 2.0 and 3.0 mg/l) of BAP and Kn used alone and in combination of three concentrations BAP (0.5, 1.0 and 2.0 mg/l) with four concentrations of Kn (0.2, 0.5, 1.0 and 2.0 mg/l) were treated in MS medium for the purpose of multiple shoot induction from shoot tip explants of *S. zeylanica*. Data were taken after 6 weeks of inoculation and percentage of shoot formation, mean number of shoot/culture and mean length of shoot/culture were measured. The results are presented in Table 3.54. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Shoot proliferation ranged from 20.00-65.00%. Highest percentage (65.00%) of multiple shoot formation was observed in MS medium containing 1.0 mg/l BAP + 0.5 mg/l Kn, and the lowest percentage (20.00) of multiple shoot formation was observed in media having 0.2 mg/l BAP. Highest mean number of shoots was 3.00 ± 0.17 in media having 2.0 mg/l BAP + 1.0 mg/l Kn and lowest mean number of shoot were 1.00 ± 0.57 in media containing 0.2 mg/l BAP and 0.2 mg/l Kn. Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 6 weeks of culture. Highest average length was recorded 6.30 ± 0.17 cm in 2.0 mg/l BAP+ 1.0 mg/l Kn and the lowest average length was 3.60±0.17 cm in media containing 0.2 mg/l Kn. Experimental results revealed that, 1.0 mg/l of BAP and 2.0 mg/l Kn alone and combination of 1.0 mg/l BAP + 0.5 mg/l Kn and 2.0 mg/l BAP+ 1.0 mg/l Kn were found more effective concentrations for multiple shoot induction.

Experiment 2. Effect of different concentrations and combinations of phytohormones on multiple shoot induction from shoot tip explants on *S. zeylanica*

Explants were cultured on MS medium supplemented with two concentrations (1.0 and 2.0 mg/l) of BAP and Kn combined with three concentrations (0.1, 0.2 and 0.5 mg/l) of NAA and IAA. Data were recorded after 6 weeks of culture and results on different parameters are presented in the Table 3.55. The efficiency of BAP + NAA was recorded better than BAP alone but inferior than Kn + NAA on direct shoot regeneration. All the used media compositions formed multiple shoots. Addition of

lower concentration of NAA and IAA along with higher concentration of BAP and Kn was found more suitable than that of other concentrations. Among all of the combinations, the highest percentage (66%) of shoot proliferation was noted in the media having 2.0 mg/l Kn + 0.2 mg/l NAA followed by 62% in 2.0 mg/l BAP + 0.2 mg/l NAA and 1.0 mg/l Kn + 0.2 mg/l NAA. The lowest percentage (40%) of shoot proliferation was noted in media having 2.0 mg/l Kn + 0.5 mg/l IAA. The average highest number of shoot per culture was 4.00 ± 0.28 in the combinations of 1.0 mg/l Kn + 0.2 mg/l NAA and lowest number of shoot per culture 1.00 ± 0.05 in the combination of 1.0 mg/l BAP + 0.1 mg/l IAA and 1.0 mg/l Kn + 0.1 mg/l IAA. The highest length of shoot per culture was recorded 6.00 ± 0.57 in the media containing 1.0 mg/l BAP + 0.2 mg/l IAA and the lowest length of shoot per culture was 4.00 ± 0.11 cm in the media containing 2.0 mg/l Kn + 0.5 mg/l NAA. Experimental results revealed that, 1.0 mg/l Kn + 0.2 mg/l NAA, 2.0 mg/l BAP + 0.2 mg/l NAA, 1.0 mg/l BAP + 0.2 mg/l IAA and 2.0 mg/l Kn + 0. 2 mg/l NAA were the most effective combinations for multiple shoot induction from shoot tip explants.

Experiment 3. Effect of different levels of L-glutamine with BAP or Kn on multiple shoot induction from *in vitro* grown shoot tip explants of *S. zeylanica* on half strength MS medium

Different levels of L-glutamine (0.1, 0.2, 0.5, and 1.0 mg/l) in combination with three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP or Kn were treated in half strength MS medium for the purpose of multiple shoot induction from *in vitro* grown shoot tip explants of *S. zeylanica*. Data were taken after 6 weeks of inoculation and percentage of multiple shoot induction, average number of shoot /culture and average length of shoot /culture were measured. The results are presented in Table 3.56. Morphogenic responses of shoot induction were found to vary with hormonal formulations present in the culture media. Multiple shoot proliferation ranged from 40.00-70.00%. Highest percentage (70.00%) of multiple shoot proliferation was observed in half strength MS medium containing 1.5 mg/l Kn + 0.5 mg/l L-glutamine followed by 66.00% in half strength MS medium containing 1.5 mg/l BAP + 0.5 mg/l L-glutamine and 1.0 mg/l Kn + 0.2 mg/l L-glutamine. The lowest percentage (40.00%) of multiple shoot proliferation was observed in media having 0.5 mg/l BAP + 0.1 mg/l L-glutamine. Highest mean number of shoots proliferation was 4.00 ± 0.30 in media having 1.5 mg/l Kn + 0.5 mg/l L-glutamine and lowest mean number of shoot proliferation was 2.00 ± 0.17 in media

containing 0.5 mg/l BAP + 0.1 mg/l L-glutamine. Average shoot length gradually increased after induction of shoot. Highest average length of shoot proliferation was recorded 5.30 ± 0.17 cm in 1.5 mg/l Kn + 0.5 mg/l L-glutamine and lowest average length of shoot proliferation was 3.00 ± 0.20 cm in 0.5 mg/l Kn + 0.1 mg/l L-glutamine. Experimental results revealed that, 1.5 mg/l Kn + 0.5 mg/l L-glutamine, 1.5 mg/l BAP + 0.5 mg/l L-glutamine and 1.5 mg/l Kn + 0.2 mg/l L-glutamine were found most effective concentrations and combinations for multiple shoot proliferation.

Table 3.54 Effect of different concentrations of BAP and Kn alone and combinations with each other on multiple shoot induction from shoot tip explants of *S. zeylanica*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoots/culture (mean ± SE)	*Average length (cm) of shoots/culture (mean ± SE)
BAP				
0.2	8-10	20	1.00 ± 0.57	4.00 ± 0.26
0.5	8-10	35	1.30 ± 0.05	4.60 ± 0.17
1.0	8-10	50	1.50 ± 0.14	5.30 ± 0.17
2.0	8-10	42	1.30 ± 0.05	5.00 ± 0.28
3.0	8-10	25	1.30 ± 0.05	4.50 ± 0.17
Kn				
0.2	8-10	25	1.00 ± 0.11	3.60 ± 0.17
0.5	8-10	33	1.30 ± 0.05	4.60 ± 0.17
1.0	8-10	50	1.80 ± 0.11	5.00 ± 0.28
2.0	8-10	60	1.50 ± 0.14	5.30 ± 0.17
3.0	8-10	40	1.30 ± 0.05	4.50 ± 0.11
BAP + Kn				
0.5 + 0.2	7-8	40	1.30 ± 0.05	5.00 ± 0.17
0.5 + 0.5	7-8	60	1.60 ± 0.15	5.60 ± 0.17
0.5 + 1.0	7-8	52	2.30 ± 0.11	4.60 ± 0.17
0.5 + 2.0	7-8	45	1.50 ± 0.14	4.30 ± 0.17
1.0 + 0.2	7-8	50	1.50 ± 0.14	5.00 ± 0.11
1.0 + 0.5	7-8	65	2.00 ± 0.30	5.60 ± 0.17
1.0 + 1.0	7-8	50	2.50 ± 0.20	6.00 ± 0.28
1.0 + 2.0	7-8	42	2.30 ± 0.23	5.30 ± 0.17
2.0 + 0.2	8-10	40	2.00 ± 0.11	5.00 ± 0.17
2.0 + 0.5	8-10	56	2.50 ± 0.20	6.00 ± 0.17
2.0 + 1.0	8-10	50	3.00 ± 0.17	6.30 ± 0.17
2.0 + 2.0	8-10	33	2.30 ± 0.11	5.00 ± 0.28

*Values are the mean of three replicates with 10 explants.

Table 3.55 Effect of different levels of growth regulators on shoot proliferation from shoot tip explants of *S. zeylanica*. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoots/culture (mean ± SE)	*Average length (cm) of shoots/culture (mean ± SE)
BAP + NAA				
1.0 + 0.1	7-8	52	2.00 ± 0.30	4.00 ± 0.17
1.0 + 0.2	7-8	60	3.50 ± 0.11	5.60 ± 0.11
1.0 + 0.5	7-8	50	3.60 ± 0.11	4.60 ± 0.11
2.0 + 0.1	7-8	55	2.00 ± 0.05	5.00 ± 0.17
2.0 + 0.2	7-8	62	3.00 ± 0.11	5.60 ± 0.17
2.0 + 0.5	7-8	55	2.50 ± 0.20	4.30 ± 0.17
BAP + IAA				
1.0 + 0.1	8-10	45	1.00 ± 0.05	5.00 ± 0.11
1.0 + 0.2	8-10	52	3.00 ± 0.20	6.00 ± 0.57
1.0 + 0.5	8-10	55	2.50 ± 0.20	5.30 ± 0.17
2.0 + 0.1	8-10	50	1.50 ± 0.15	5.30 ± 0.17
2.0 + 0.2	8-10	60	2.00 ± 0.15	5.60 ± 0.11
2.0 + 0.5	8-10	45	2.00 ± 0.11	4.60 ± 0.17
Kn + NAA				
1.0 + 0.1	8-10	45	2.00 ± 0.11	4.60 ± 0.17
1.0 + 0.2	8-10	62	4.00 ± 0.28	5.00 ± 0.11
1.0 + 0.5	8-10	55	2.50 ± 0.20	4.00 ± 0.30
2.0 + 0.1	8-10	60	2.00 ± 0.17	4.30 ± 0.17
2.0 + 0.2	8-10	66	2.30 ± 0.17	5.00 ± 0.11
2.0 + 0.5	8-10	52	1.60 ± 0.15	4.00 ± 0.11
Kn + IAA				
1.0 + 0.1	8-10	50	1.00 ± 0.57	4.60 ± 0.11
1.0 + 0.2	8-10	60	3.00 ± 0.20	5.00 ± 0.17
1.0 + 0.5	8-10	55	2.00 ± 0.23	5.00 ± 0.17
2.0 + 0.1	8-10	54	1.50 ± 0.15	5.30 ± 0.17
2.0 + 0.2	8-10	60	2.30 ± 0.17	5.00 ± 0.28
2.0 + 0.5	8-10	40	1.60 ± 0.17	4.30 ± 0.11

*Values are the mean of three replicates with 10 explants.

Table 3.56 Effect of different levels of L-glutamine with BAP and Kn on multiple shoot proliferation from *in vitro* grown shoot tip explants of *S. zeylanica* on half strength MS medium. Data were taken after 6 weeks of culture.

Growth regulators (mg/l)	Number of days taken for shoot initiation	% of shoot formation	*Average number of shoots/culture (mean ± SE)	*Average length (cm) of shoots/culture (mean ± SE)
BAP + L-glu				
0.5 + 0.1	7-8	40	2.00 ± 0.17	3.50 ± 0.11
0.5 + 0.2	7-8	60	2.30 ± 0.17	4.00 ± 0.30
0.5 + 0.5	7-8	55	3.00 ± 0.25	4.30 ± 0.11
0.5 + 1.0	7-8	52	2.60 ± 0.23	3.80 ± 0.11
1.0 + 0.1	7-8	45	2.00 ± 0.23	3.30 ± 0.17
1.0 + 0.2	7-8	62	2.60 ± 0.23	4.30 ± 0.11
1.0 + 0.5	7-8	55	3.00 ± 0.20	4.00 ± 0.30
1.0 + 1.0	7-8	52	2.50 ± 0.20	3.50 ± 0.11
1.5 + 0.1	7-8	45	2.30 ± 0.17	4.00 ± 0.30
1.5 + 0.2	7-8	60	3.00 ± 0.20	4.30 ± 0.11
1.5 + 0.5	7-8	66	3.60 ± 0.11	4.87 ± 0.20
1.5 + 1.0	7-8	50	2.50 ± 0.20	3.50 ± 0.11
Kn + L-glu				
0.5 + 0.1	7-8	52	2.30 ± 0.17	3.00 ± 0.20
0.5 + 0.2	7-8	55	2.60 ± 0.23	4.00 ± 0.30
0.5 + 0.5	7-8	62	3.30 ± 0.17	4.30 ± 0.11
0.5 + 1.0	7-8	45	2.50 ± 0.20	4.50 ± 0.28
1.0 + 0.1	7-8	50	2.30 ± 0.05	4.00 ± 0.26
1.0 + 0.2	7-8	66	3.00 ± 0.25	4.50 ± 0.28
1.0 + 0.5	7-8	62	3.60 ± 0.11	5.00 ± 0.11
1.0 + 1.0	7-8	55	2.50 ± 0.20	4.30 ± 0.11
1.5 + 0.1	7-8	60	3.00 ± 0.20	4.00 ± 0.30
1.5 + 0.2	7-8	62	3.50 ± 0.11	5.00 ± 0.28
1.5 + 0.5	7-8	70	4.00 ± .0.30	5.30 ± 0.17
1.5 + 1.0	7-8	55	3.30 ± 0.17	5.13 ± 0.37

*Values are the mean of three replicates with 10 explants.

Plate 3.22 Development of single and multiple shoot formation from nodal and shoot tip explants of *S. zeylanica* in direct organogenesis and their subsequent development.

A: Single shoot initiation from nodal explant in 0.5 mg/l BAP + 1.0mg/l IAA after 2 weeks of culture; **B-D:** Shoot formation from nodal explant in 2.0 mg/l BAP + 0.2 mg/l IAA after 3-6 weeks of culture; **E-H:** Multiple shoot induction and their further development from nodal explant in 2.0 mg/l Kn + 0.5 mg/l L-glutamone after 4-8 weeks of culture; **I-J:** Multiple shoot initiation from shoot tip explant in 1.0 mg/l BAP + 1.0 mg/l Kn + 0.5 mg/l L-glutamine after 6 weeks of culture; **K-L:** Multiple shoot induction and their subsequent development from shoot tip explant in the media having 0.5 mg/l BAP + 2.0mg/l Kin + 0.5 mg/l L-glutamine after 6 weeks of culture.

3.3.7.2. Rooting of *in vitro* grown shoots in *S. zeylanica*

Shoot cuttings 5–7 cm long *in vitro* grown shoots were separated and transferred to rooting media. MS plain (without hormones) and half strength MS medium fortified with different concentrations of NAA, IAA and IBA (0.1, 0.2, 0.5, 1.0 and 2.0 mg/l) were used for rooting experiment. Rooting started produced within 7 to 10 days of culture. Among the tested concentrations half strength MS medium having 0.5 mg/l, 1.0 mg/l IBA, and1.0 mg/l IAA found highest percentage (50-70%) of root formation. The lowest percentage of root formation (10%) was obtained in media having 0.2 mg/l NAA. Data on days to root initiation, percentage (%) of root formation, average number of root per culture and average length of root per culture were recorded after 6 weeks of culture.

Experiment 1. Effect of different concentrations of NAA, IAA, IBA and MS= 0 on root induction from *in vitro* grown shoots of *S. zeylanica*

For adventitious root formation, the shoots obtained from *in vitro* grown shoots were excised and cultured on half strength MS medium with different concentrations of NAA, IAA, IBA and half strength MS=0 (without hormones). Results obtained for root induction, percentage (%) of root formation, average number and length of roots are shown in Table 3.57. Percentage range of cultures produced roots varied from 10.00-70.00%. Highest 70.00% of root regeneration was recorded in 1.0 mg/l IBA (Plate 3.23) followed by 66.00 % in media 0.5mg/l IBA. The lowest 10.00 % of root regeneration was recorded in media having 0.2 mg/l NAA. The highest average number of roots per shoot was recorded 7.00±0.11 in media having 1.0 mg/l IBA followed by 4.60 ± 0.11 in 1.0 mg/l NAA and 0.5 mg/l IBA. The lowest average number of root per shoots was recorded 2.00 ± 0.15 in media having 0.2 mg/l NAA and 0.2 mg/l IAA. Highest average length of roots was 4.70 ± 0.20 cm in 1.0 mg/l IBA and the lowest length of roots was 3.30 ± 0.17 cm in 0.2 mg/l NAA and 0.2 mg/l IAA. In most cases morphology of roots was healthy, thin and long.

Table 3.57 Effect of different concentrations and combinations of auxins on adventitious root formation from the *in vitro* grown shoot on half strength MS medium. Data were recorded after 6 weeks of culture.

Plants growth regulators (mg/l)	Number of days taken for root initiation	Percentage (%) of root formation	Average number of root/micro cutting (mean ± SE)	Average length (cm) of root/micro cutting (mean ± SE)
Half MS=0	-	-	-	-
NAA				
0.1	-	-	-	-
0.2	12-15	10	2.00 ± 0.17	3.30 ± 0.17
0.5	12-15	20	4.00 ± 0.26	3.50 ± 0.11
1.0	12-15	15	4.60 ± 0.11	4.00 ± 0.28
2.0	-	-	-	-
IAA				
0.1	-	-	-	-
0.2	12-15	20	2.00 ± 0.15	3.30 ± 0.17
0.5	12-15	30	2.60 ± 0.23	4.30 ± 0.11
1.0	12-15	50	3.50 ± 0.11	4.50 ± 0.28
2.0	12-15	25	3.00 ± 0.20	3.60 ± 0.11
IBA				
0.1	10-12	42	3.00 ± 0.20	3.50 ± 0.11
0.2	10-12	55	3.60 ± 0.17	3.60 ± 0.23
0.5	10-12	66	4.60 ± 0.11	4.00 ± 0.28
1.0	10-12	70	7.00 ± 0.11	4.70 ± 0.20
2.0	10-12	45	3.50 ± 0.11	4.27 ± 0.14

*Values are the mean of three replicates with 5 explants.

Plate 3.23 Development of adventitious root from *in vitro* grown shoot and establishment in soil of *S. zeylanica*.

A-B: Root formation from shoot tip explant in half strength MS medium with 1.0 mg/l IBA after 5-8 weeks of culture; **C-D:** Root formation from nodal explant in half strength MS medium with 0.5 mg/l IBA after 6 weeks of culture; **E:** *S. zeylanica* with roots outside of culture bottle; **F:** The *in vitro* grown seedlings acclimatized to grow in pots in outside environment.

3.4. DISCUSSION

Numerous drugs or their precursors used in the current pharmacopoeias originate from plant sources. Natural products or natural product derived drugs comprise nearly 28% of all the new chemical entities launched into the market in the last 20 years (Goyal *et al*. 2008). Medicinal plant based drugs have the added advantage of being simple, effective, and offering a broad spectrum of activity with well documented prophylactic or curative actions. Medicinal plant products have also proved useful in minimizing the adverse side effects of various chemotherapeutic agents (Gómez-Galera *et al*. 2007; Leonard *et al*. 2009). Approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from plant substances. Many of the modern medicines are produced indirectly from medicinal plants, for example aspirin and digitalis. The World Health Organization (WHO) has estimated that the present demand for medicinal plants is approximately US $14 billion per year. The demand for medicinal plant based raw materials is growing at the rate of 15 to 25% annually, and according to an estimate of WHO, the demand for medicinal plants is likely to increase more than US $5 trillion in 2050.

Medicinal plants are the most important source of life saving drugs for the majority of the world's population. The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants. *In vitro* regeneration holds tremendous potential for the production of high quality plant based medicine. Plant tissue culture studies were carried out for the preservation of medicinal plant resources and efficient production of pharmaceutically important secondary metabolites. Plant tissue culture technology is being widely used for large scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have in recent years, become of major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites. Small pieces of tissue (named explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis. Endangered, threatened and rare species have successfully been grown and conserved by micropropagation because of high coefficient of multiplication and small demands on number of initial plants and space.

In plant cell culture, plant tissues and organs are grown *in vitro* on artificial media, under aseptic and controlled environment. The technique depends mainly on the

concept of totipotentiality of plant cells which refers to the ability of a single cell to express the full genome by cell division. Along with the totipotent potential of plant cell, the capacity of cells to alter their metabolism, growth and development is also equally important and crucial to regenerate the entire plant. Plant tissue culture medium contains all the nutrients required for the normal growth and development of plants. It is mainly composed of macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in case of solid medium Murashige and Skoog medium (MS medium) is most extensively used for the vegetative propagation of many plant species *in vitro*. The pH of the media is also important that affects both the growth of plants and activity of plant growth regulators. It is adjusted to the value between 5.4 - 5.8. Both the solid and liquid medium can be used for culturing. The composition of the medium, particularly the plant hormones and the nitrogen source has profound effects on the response of the initial explant. Plant growth regulators (PGR's) play an essential role in determining the development pathway of plant cells and tissues in culture medium. The auxins, cytokinins and gibberellins are most commonly used plant growth regulators. The type and the concentration of hormones used depend mainly on the species of the plant, the tissue or organ cultured and the objective of the experiment. Auxins and cytokinins are most widely used plant growth regulators in plant tissue culture and their amount determined the type of culture established or regenerated. The high concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. A balance of both auxin and cytokinin leads to the development of mass of undifferentiated cells known as callus.

In some medicinal plant species seed propagation has not been successful. Moreover, most of the plant raised through seeds are highly heterozygous and show great variations in growth, habit and yield and may have to be discarded because of poor quality of products for their commercial release. Likewise, majority of the plants are not amenable to vegetative propagation through cutting and grafting, thus limiting multiplication of desired cultivars. However, *in vivo* vegetative propagation techniques are time consuming and expensive and the propagules carry the diseases and pest from the mother plant to the seedlings.

under *in vitro* conditions rather than have indifferent populations. Tissue culture has now become a well established technique for culturing and studying the physiological behavior of isolated plant organs, tissues, cells, protoplasts and even cell organelles

under precisely controlled physical and chemical conditions. Most of the medicinal plants either do not produce seeds or seeds are too small and do not germinate in soils. Thus mass multiplication of disease free planting material is a general problem. In this regard the micropropagation holds significant promise for true to type, rapid and mass multiplication under disease free conditions. Besides, the callus derived plants exhibit huge genetic variation that could be exploited for developing superior clones/varieties particularly in vegetatively propagated plant species.

In this present investigation effort has been made to collect the ethnobotanical information about 120 medicinal plants growing in Natore Oushodi Gram. Among these plants, 17-20 plant species have been cultivated in agriculture plots in Natore Oushodi Gram on commercial purpose. Biological study was done addressing the bottle necks as exhibited by these plants for commercial and massive culture and also identified some plants that have propagation barrier. It was necessary to develop the tissue culture protocols for all of these identified medicinal plants experiencing propagation barrier, but considering volume of work and time limitation, seven medicinal plants (*Asparagus racemosus, Curcuma amada, Ipomoea mauritiana, Mentha arvensis, Paederia foetida, Rauwolfia serpentina* and *Smilax zeylanica*) was selected for tissue culture study under the present investigation because of their medicinal value and market potentiality. The present study was undertaken to examine the potential of different explants, to respond under *in vitro* conditions with the possibility of developing a protocol for the *in vitro* multiplication. As for conservation strategy artificial seed production has been successfully developed in case of *Mentha arvensis*. At the advent of plant biotechnology, since 1980, efforts have been made to develop tissue culture protocol for the commercially important plants, including medicinal plants for their large scale production, but in this investigation, emphasis was given particularly on plant species that has been commercially cultivated in Natore Oushodi Gram. Due to some propagation barriers their multiplication occur in nature at very slow rate (Millar *et al*. 1991; Lee 1994; Sudha *et al.* 1998; Chetia and Handique, 2000; Rout *et al.* 2000; Selvakumer *et al*. 2001; Chandramu *et al.* 2003; Hassan and Roy, 2004; Baksha *et al*. 2005; Binita *et al*. 2005; Sinha *et al.* 2005). Tissue culture not only give the opportunity of cloning plants at a higher rate but also gives many other opportunities such as conservation (Engelmann, 1991) including a somaclonal variation (Camerson *et al*. 1989; Drew and Smith, 1990), facilitated desirable gene transfer (David *et al*. 1984) and

production of important secondary metabolites (Nigra *et al*. 1987; Jha *et al*. 1988; Pilli and Oliveira, 2000) by callus tissue or cell culture.

The nature of explants and age of the source plant is an important factor related to success of *in vitro* culture. Generally explants from juvenile plants give better response than those of aged plant (Sommer and Cladera*s*, 1981). Direct and indirect organogenesis have been successfully established in many medicinal plant species using shoot tips (Huang *et al*. 2000; Soniya and Das, 2002; Baksha *et al*. 2005; Binita *et al*. 2005; Banu and Bari, 2007; Karuppusamy and Pullaiah, 2007), node (Handique and Bora, 1999; Chetia and Handique, 2000; Banu *et al*. 2001; Barua and Handique, 2002; Das and Handique, 2002; Chandramu *et al*. 2003; Faisal *et al*. 2005; Sinha *et al*. 2005; Karuppusamy and Pullaiah, 2007), internode (Azad and Amin, 1998; Chen *et al*. 2001; Martin, 2002) and leaf (Das and Rout, 2002; Banu and Bari, 2007; Senthilkumar *et al*. 2007) as explants. However, for all the seven plant species five kinds of explants namely shoot tip, node, axillary bud, internode and leaf explants of about 2-4 months old plants were used for culture. The explants were taken from 1-2 months old juvenile tissue so that phenolics and other inhibitory compounds secreted by the tissue could not inhibit the growth of tissue in culture.

Surface sterilization of explants is an important step for *in vitro* propagation of any plants because the nutrient culture media, which are generally used in tissue culture techniques, are most suitable for the growth of microbes and pathogens. For this purpose, a well known surface sterilizing agent ($HgCl_2$) was employed at 0.1% for sterilization of explants but treatment duration was different according to explants and plant species. In case of *Asparagus racemosus* shoot apex, node and leaf explants took 5 min; internode segments took 6-7 min for getting cent percent contamination free culture. On the other hand, effective surface sterilization was done at 5-6 min for shoot tip and axillary bud explants of *Curcuma amada*. In *Mentha arvensis* shoot tip, node and internode segments took 4-5 min and 4-5 min for leaf segments. In *Ipomoea mauritiana and Paederia foetida* shoot tip and node segments took 5 min and leaf took 4 min for contamination free culture. In case of *Rauwolfia serpentina* axillary bud and shoot tip explant took 6 min and in leaf 4-5 min for contamination free culture. Best case of *Smilax zeylanica* shoot tip and node when surface sterilization was done with an aqueous solution of 0.1 % $Hgcl_2$ for 5-6 min. In our study all the seven selected plant species gave response to the PGR supplemented media and the

kind of response was dependent on species, explant and hormone supplement of the media.

MS (Murashige and Skoog, 1962) medium is a recognized basal medium for tissue culture techniques and extensively used for *in vitro* propagation of various plant species including medicinal plants (Sudha *et al*. 1998; Rout *et al*. 2000; Selvakumar *et al*. 2001; Chandramu *et al.* 2003; Hassan and Roy, 2004; Soniya 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; Biswas, 2006). Throughout the investigation MS medium was used as the basal medium for direct and indirect organogenesis of seven important medicinal plants *Asparagus racemosus, Curcuma amada, Ipomoea mauritiana, Mentha arvensis, Paederia foetida, Rauwolfia serpentina* and *Smilax zeylanica*. Growth regulators, especially cytokinins and auxins played significant role in *in vitro* culture of higher plants and it can be said that *in vitro* culture is often impossible without growth regulators (Pierik, 1987). Auxins are generally responsible for elongation and swelling of tissues, cell division and formation of adventitious roots, the inhibition of adventitious and axillary shoot formation and often embryogenesis in suspension culture. With low auxin concentrations adventitious root formation predominates, whereas with high auxin concentrations root formation fails to occur and callus formation takes place (Pierik, 1987). Cytokinins usually promote cell division, especially if added together with an auxin. In higher concentration they can induce adventitious shoot formation, but root formation is generally inhibited. They promote axillary shoot formation by decreasing apical dominance (Pierik, 1987).During the present investigation BAP and Kin were applied as cytokinin and NAA, IAA, IBA and 2,4-D used as auxin. L-glutamine, GA_3 and charcoal were used as additives.

3.4.1. *In vitro* culture of *Asparagus racemosus*

A. racemosus is an important medicinal plants, which is localy known as "Shatamuli" and the plant growing as one of the important plants in Natore Oushodi Gram. It also grows wild in forests and is planted in homestead and home garden in Bangladesh. *A. racemosus* plants are widely used in Ayurvedic medicinal preparations as refrigerant, demulcent, diuretic, aphrodisiac, antispasmodic, antidiarrhoeaic, expectorant, galactogougue tonic and for renal and reproductive system disorders (Kirtika and Basu 1937). *A. racemosus* is usually propagated by planting the separated tuberous

roots along with shoot apex. Since roots are the organs used for medicinal purpose there has been a practice of using seeds for plant propagation, but there are few technical problems involved in seed derived multiplication. Such as the germination % of seeds is very low and the seed grown plants are slow in growth. So it was decided to develop a protocol for clonal propagation of elite high root yielding plants of *A. racemosus* by in *vitro* technique using shoot apex and nodal explants.

In case of *A. racemosus* available reports regarding *in vitro* mass propagation is limited. Pant and Joshi (2009) develop a protocol on *in vitro* multiplication of wild Nepalese *A. racemosus* through shoots and shoots induced callus culture. They obtained high frequency multiple shoots in MS media having different concentrations of BAP alone (0.5 mg/l was best) and combination with NAA (2.0 mg/l BAP + 0.1 mg/l NAA was best). It was remarkable to note that higher concentration of BAP alone and higher concentration with NAA caused to reduce bud differentiation. Another report from Bopana and Saxena (2008) developing a protocol *in vitro* propagation of a high value medicinal plants. *A. racemosus* wild. They obtained high frequency multiple shoot in MS media having BAP and Kn alone and in their combination. Another report for regeneration of multiple shoot obtained by Sharan *et al.* (2011) in *A. racemosus* from shoot apex and nodal explants. They found high frequency multiple shoot in MS media in BAP and Kn alone (in different concentrations) and in combination with NAA. Afroz *et al.* (2010) reported multiple shoot formation in *A. racemosus* from axillary buds. They found multiple shoot induction in different concentrations and combinations of BAP alone and combination of BAP + NAA and BAP + IBA.

In the present study BAP and Kn were used alone in different concentrations or in combination with BAP + Kn, BAP + NAA and Kn + NAA. Among the different types of explants (shoot apex, node, internode and leaf), nodal explants was proved to be the best and most efficient explant for tissue culture of *A. racemosus* as it responded better than shoot apex for direct organogenesis in all media compositions. When BAP and Kn applied singly proliferation response was found to be poor with only 2-6 shoots. On the other hand, multiple shoot induction enhanced remarkably when low concentration of auxins was used in combination with higher concentration of BAP and Kn. The synergistic effect of BAP in combination with an auxin has been demonstrated in many medicinal plants (Casado *et al*. 2002; Fraternale *et al*. 2002; Salvi *et al.* 2002; Martin 2003). Tripepi (1997) reported that combination of BAP

with NAA was found most effective in shoot organogenesis. Results of the present study proved that combination of BAP + NAA was very efficient for multiple shoot induction from shoot apex and node explant of *A. racemosus*. The highest percentage (82%) of multiple shoot induction was noted from nodal explant in the media having 2.0 mg/l BAP + 0.5 mg/l NAA. The highest mean number of shoot per culture 18.00 ± 0.28 was noted in 3.0 mg/l BAP + 1.0 mg/l NAA and the highest length of shoot per culture 8.00 ± 0.23 was noted in the media having 2.0 mg/l BAP + 0.5 mg/l NAA. On the other hand, maximum (78%) shoot proliferation was noted from shoot apex explant in the media having 2.0 mg/l BAP + 0.5 mg/l NAA. The highest average length of shoot was noted in the same medium and the highest average number of shoot per culture 16.00 ± 0.17 noted in media of 1.0 mg/l BAP + 1.0 mg/l NAA. The present finding are in agreement with those observed in other medicinal plant species such as *Rehum emodi* (Lal and Ahuja, 1989) , *Gentiana kuroo* (Sharma *et al*. 1993), *Vitex negundo* (Chandramu *et al.* 2003), *Aloe barbadensis* (Liao *et al.* 2004), *Plumbago indica* L (Biswas *et al*. 2009) and *Bupleurum distichophyllum* (Karuppusamy and Pullaiah, 2007).

Indirect organogenesis of *A. racemosus* was also carried out using leaf and internode segments and cultured them on MS medium supplemented with different concentrations of 2,4-D and NAA alone or in combinations of BAP + 2,4-D, Kn + 2,4-D, BAP + Kn + 2,4-D and BAP + NAA + 2,4-D. Results showed that leaf and internode explants produced white friable and compact, creamy friable, light green friable and compact and green compact color callus. Among all tested concentrations and combinations, 2,4-D and NAA alone produced poor percentage of callus induction. In another experiment, when 2,4-D with BAP or Kn used together the satisfactory callus formation was observed in both explants and in another experiment, when BAP + Kn + 2,4-D, BAP + NAA + 2,4-D used together better callus formation was observed in both explants. Here, internode explants performed better than leaf explants. The highest 60% light green friable callus induction was obtained from internode explants in MS medium having 0.5 mg/l BAP + 1.0 mg/l 2,4-D within 6 weeks of culture. But when BAP + Kn + 2,4-D were used together optimum callus formation was achieved in both explants. Here, internode explants also performed better than leaf explants. The highest 95% dark green friable callus induction was obtained from internode explants in MS medium having 0.5 mg/l BAP + 1.0 mg/l Kn + 1.0 mg/l 2,4-D within 6 weeks of culture. On the other hand, highest

85% green friable callus induction was obtained from leaf explants in 0.5 mg/l BA + 1.0 mg/l Kn + 1.0 mg/l 2,4-D. Pant and Joshi (2009) found creamy, nodular and friable callus in MS medium with 0.1 mg/l NAA from internode explants of *A. racemosus* which slightly differs from our present result.

For callus regeneration, the leaf and internode derived callus were cultured on MS medium supplemented with different concentrations of BAP and Kin singly or in combination with NAA. Results showed that BAP + NAA combination was better than that of other combinations. Among all tested media combinations the leaf derived callus gave better performance than internode derived callus to regeneration of shoot. However, the best regeneration of shoots was obtained from 1.0 mg/l BAP + 0.2 mg/l NAA. In this concentrations 85% callus produced adventitious shoots with a highest average 12.00 ± 0.15 numbers of shoots per culture and average 6.00 ± 0.11 cm length of shoot per culture. Similar results have been reported by Sarker *et al.* (1996) in *Rauvolfia serpentina,* Azad and Amin (1998) in *Adhatoda vasica*, Munshi *et al.* (2001) in *Mimosa pudica*, Rao *et al.* (2004) in *Physalis pubescens*, Sultana *et al.* (2004) in *Citrullus lanatus* , Biswas (2006) in *Plumbago indica* L by using BAP and NAA.

For induction of adventitious rooting from shoot cuttings cultured on half strength of MS medium with NAA alone and in combination with BAP or Kn. Half strength MS with Kn + NAA was found to be the most effective hormonal supplemented medium than that of other growth regulators. The best response (30%) observed in half strength MS medium with 2.0 mg/l Kn + 0.5 mg/l NAA. The highest average number of roots 38.00 ± 0.52 per culture and highest average length of roots 8.00 ± 0.17 cm per culture also found in the same medium from nodal explants after 6 week of transfer in rooting medium. On the other hand, 94% shoots produced an average of 7.64 ± 0.43 roots per culture and 3.50 ± 0.22 cm length of root per culture from shoot tip explants in the same concentration. Half strength of MS supplemented with NAA alone was effective for rooting reported in other medicinal plant species (Siddique *et al.* 2001; Munshi *et al.* 2001; Islam *et al.* 2001; Amin *et al.* 2003; Chandramu *et al.* 2003; Joshi and Dhar 2003; Shyamol and Sinha 2003; Das *et al.* 2010; Sadia *et al.* 2009; Alatar *et al.* 2012**).** On the other hand, full strength of MS medium with different concentrations of NAA alone and combination of BAP or Kn failed to induce any roots from *in vitro* grown shoots.

3.4.2. *In vitro* culture of *curcuma amada*

C. amada is another important herbaceous medicinal plant cultivated in Natore Oushodi Gram and to the best of our knowledge very limited report was available regarding *in vitro* mass propagation of this plant. Prakash *et al*. (2004) develop a protocol on efficient regeneration of *C. amada* Roxb. They obtained high frequency multiple shoots in MS media having different concentrations of BAP with NAA (MS+BA 4.4 µM + NAA 1.0 µM /MS + BAP 8.88 µM + NAA 2.7 µM). Another work reported from Roychaudhari (2004) in developing a protocol on in *vitro* regeneration and estimation of *curcuma* in content in four species of *Curcuma* plant. They also obtained high frequency multiple shoots in MS media having different combinations of Kn and NAA (MS + NAA 4.0 mg/l + Kn .05 mg/l was best). Prakash *et al*. (2004) developed a protocol on efficient regeneration of *C. amada* Roxb. producing plantlets from rhizome and leaf sheath explants. They found high frequency multiple shoots in MS media having different combinations of BAP and NAA was proved better than BAP alone. Pramila *et al*. (2011) developed a protocol on microrhizome production in turmeric (*Curcuma longa* L). Best response was obtained in medium containing 1.0 mg/l BAP with 0.2 mg/l NAA (31.33 days) followed by 1.0 mg/l BAP alone (43.33 days) for early induction of microrhizomes. More number of microrhizomes and bigger microrhizomes were produced in MS medium having lower concentration of BAP.

In the present study single medium based efficient and large scale micropropagation protocol was established using two types of explants *viz*. shoot tip and axillary bud through direct organogenesis and microrhizome production. Here, direct shoot multiplication was achieved from shoot tip and axillary bud explants. For direct organogenesis BAP and Kin were used singly or in combination with NAA and IAA. Among the two types of explants axillary bud segment proved most efficient explant for tissue culture of *C. amada*. Similar results have been reported by Karuppusamy and Pullaiah (2007) in *Bupleurum distichophyllum*, Rahman *et al*. (2004) in *Ficus benghalensis*, Islam *et al*. (2001) in *Catharanthus roseus*. The axillary bud explants underwent direct organogenesis when cultured on MS medium using various concentrations of BAP (0.2–3.0 mg/l) and Kin (0.2–3.0 mg/l) separately or in combination of high concentration of BAP or Kn with low concentrations (0.2–2.0 mg/l) of NAA or IAA. BAP is considered to be one of the most useful cytokinin for achieving the micropropagation and showed highest effect with respect to the

multiplication of axillary buds (Joshi and Dhar, 2003; Martin, 2002). Present investigation revealed that between the two cytokinins tested, BAP was more effective than Kn in terms of shoot induction in *C. amada*. Of the various levels of BAP supplemented to MS, 1.0 mg/l BAP developed 72% multiple shoots with a mean of 3.00 ± 0.11 shoot per culture and average 6.50 ± 0.28 cm length of shoot per culture. Addition of lower concentrations of auxins (NAA, IAA) along with BAP and Kin increased multiple shoots induction and elongation of shoots. The best response was achieved using 0.5 mg/l BAP + 0.5 mg/l NAA from axillary bud segments. In this combination 90% explants showed proliferation and highest average number of shoot and length (cm) of shoot per culture was 7.00 ± 0.28 and 7.50 ± 0.28 respectively. Similar experiments with the use of BAP and NAA were also carried out for induction of multiple shoot were reported in other medicinal plants such as *Azadirachta indica* (Sarker *et al*. 1997, Abubacker and Alagumanian, 1999), *Adhatoda vasica* (Azad *et al*. 1999), *Rheum emodi* (Lal and Ahuja, 2000), *Centella asiatica* (Hossain *et al*. 2000), *Aristolochia indica* and *Hemidesmus indicus* (Siddique *et al*. 2006), *Boerhaavia diffusa* (Biswas, 2006), *Bupleurum distichophyllum* (Karuppusamy and Pullaiah, 2007).

Induction of rhizomes under *in vitro* condition is known as microrhizome production. Shoots regenerated *in vitro* can be transferred to microrhizome induction media. Two types of explants viz. shoot tip and axillary bud explants were cultured for microrhizomes production. Explants were cultured on MS medium with BAP alone and combinations with NAA. Four concentrations (0.5, 1.0, 2.0 and 3.0mg/l) of BAP alone and three concentrations (0.5, 1.0 and 2.0 mg/l) of BAP with three concentrations (0.1, 0.2 and 0.5 mg/l) of NAA were treated in MS medium for the purpose of microrhizomes production from shoot tip and axillary bud explants of *C. amada.* Incase of shoot tip explant the lowest number of days (36-40) was required for induction of microrhizomes in medium containing 1.0 mg/l BAP with 0.2 mg/l NAA. The highest number of microrhizomes per shoot 6.00 ± 0.11 was recorded in the same medium. Highest number of shoots producing microrhizomes 6.00 ± 0.17 was observed in medium supplemented with 2.0 mg/l BAP + 0.2 mg/l NAA. Similar experiments with the use of BAP and NAA were also carried out for induction of microrhizome were reported in Pramila *et al*. (2011), microrhizome production in turmeric (*Curcuma longa* L), Chirangini *et al*. (2005), *in vitro* propagation and microrhizome induction in *Kaempferia galangal* L and *K. rotunda* L.

Rooting experiments were conducted on half strength of MS medium with 3% sucrose supplemented with different concentrations of auxins (IAA, NAA and IBA). Among the three types of auxins used, IBA was found best for root induction. These findings are in agreements with those observed in other medicinal plants such as *Plumbago zeylanica* (Selvakumar *et al*. 2001), *Lilium nepalense* (Wawrosch *et al*. 2001), *Holostemma ada–kodien* (Martin, 2002), *Vitex nigundu* (Chandromu *et al*. 2003), *Phellodendrom amurense* (Azad *et al*. 2005), *Plumbago indica* and *Boerhaavia diffusa* (Biawas *et al*. 2006). After two weeks of inoculation, profuse root formation was noticed from basal cut portion of the shoot in half strength MS medium having 0.5 mg/l IBA with a fairly good length (4.30 ± 0.17) and number (12.0 ± 0.23 cm). The rooting medium consisted of half strength MS supplemented with IBA was also reported in other plant species such as *Azadirachta indica* (Sarker *et al*. 1997), *Centella asiatica* (Hossain *et al*. 2000), *Carica papaya* L. (Islam *et al*. 2001), *Catharanthus roseus* L. (Islam *et al*. 2001), *Mimosa pudica* (Munshi *et al*. 2001), *Saussurea obvallata* (Joshi and Dhar, 2003).

3.4.3. *In vitro* culture of *Ipomoea mauritiana*

I. mauritiana is an important medicinal plant used for diabetes, urinary infection, sexual debilities and muscle relaxant. To the best of our knowledge, no report on *in vitro* mass propagation of *I. mauritiana* was available. Here single medium based micropropagation protocol was established using four types of explants *viz.* shoot tip, node, leaf and internode segments through direct and indirect organogenesis. For direct shoot regeneration shoot tip and node segments were cultured on MS medium without growth regulators. All of the explants failed to show shoot proliferation in MS_0 medium. The experiment proved that the growth regulator played an important role on shoot proliferation. On the other hand, BAP, Kin and IAA were used singly or in combination with NAA when shoot tip and node segment produced shoot proliferation. When BAP and Kin applied singly proliferation response was found to be moderate type with 1–4 shoots per explants. But shoot induction enhanced 2–6 per explants when low concentrations of NAA or IAA were used in combinations with BAP and Kin and the best combination was found in BAP + IAA combination. The significant effect of BAP in combination with IAA or NAA has been demonstrated is many medicinal plants such as W*ithania somnifera* (Sen and Sharma, 1991), *Dictyospermum ovalifolium* Wight. (Thoyajaksha and Rai, 2001), *Holostemna adakodien* (Martin, 2003), *Bupleurum fruticosum* (Fraternale *et al*. 2002), *Limonium*

wrightii (Huang *et al.* 2000), *Acmella calva* (Senthilkumar *et al*. 2007), *Rauwolfia serpentina* (Mallick *et al*. 2012), *Smilax zeylanica* (Thirugnanasampandan *et al*. 2009), *Paderia foetida* (Alam *et al*. 2010). Among the two type of explants the highest frequency (95%) of shoot induction was noted from node segment in media having 1.0 mg/l BAP + 0.5 mg/l IAA. The mean number of shoot per culture and mean length of shoot per culture were also highest in the same combination and those were 5.80 ± 0.27 and 7.50 ± 0.15 cm respectively. On the other hand, the highest frequency (80%) of shoot induction was noted from shoot tip segment in media having 1.0 mg/l BAP + 1.0 mg/l NAA and average 4.00 ± 0.17 number of shoot per culture and 6.00 ± 0.25 cm mean length of shoot per culture were found in the same medium. The present findings are in agreement with those observed in other plant species such as *Albizzia lebbeck* (Reza *et al*. 1995), *Adhatoda* vasica (Banu *et al*. 1997), *Rauwolfia serpentina* (Roy 1994; Rosa *et al*. 1996), *Catharanthus roseus* (Islam *et al*. 2001), *Physalis pubescens* L. (Rao *et al.* 2004). In the present experiment it was observed that, base callusing was observed in many concentrations The calli were cream in color, compact and nodular in texture. The growth of callus was rapid and within few days the explant with proliferated shoots were covered with callus. The results are in agreement with Banu and Bari (2007), Banu *et al.* (2001) in *Ocimum sanctum.* They reported that increase of BAP level and BAP, NAA combination in the medium explants produced base callus with shooting.

Experiments on callus induction of *I. mauritiana* was also carried out using leaf and internode segments and cultured on MS medium supplemented with different concentrations of 2,4-D alone or in combination with 2,4-D + BAP and 2,4-D + Kn. Among all the tested growth regulators 2,4-D + BAP was more effective for callus induction from leaf segments than internode segments. Leaf explants started callus formation within 8 –12 days of culture and 90% cultures produced callus in medium containing 1.0 mg/l 2,4-D + 2.0 mg/l BAP. These calli were whitish or light green in colour and friable in texture. On the other hand, 75% callus induced from internode explants within 8–14 days of culture with production of whitish green and friable callus. The results agrees with the findings of Rao *et al*. (2004) in *Physalis pubescens*, Farooq *et al.* (2001) in wheat, Siddique *et al.* (2001) in *Dendrobium aphyllum*, Rani and Groven (1999) in *Withania somnifera*, Torres *et al*. (1988) in *Physalis peruviana*, Bapat and Rao (1977) in *Physalis minima*, Pant and Joshi (2009) in *Asparagus*

racemosus, Zhang *et al.* (2011) in *Curcuma kwangsiensis,* Sujana and Naidu (2011) in *mentha piperita* L.

Successful shoot differentiation from callus was achieved when BAP and Kin were used singly or in combination of BAP with NAA or IAA and Kin with NAA or IAA. Among the two types of explant, internode derived callus was proved most effective for regeneration of *I. mauritiana*. However, the highest 40% culture induced shoots from internode derived callus in MS medium supplemented with 1.0 mg/l BAP + 0.5 mg/l IAA with an highest average 5.30 ± 0.17 shoot per culture and 5.60 ± 0.05 cm length of shoot per culture were noted in the same medium. It was noted that when internode derived callus was cultured on 0.5 mg/l BAP and 0.5 mg/l Kin singly the calli failed to produce any shoots. Similarly 0.5 mg/l BAP with 0.2-1.0 mg/l IAA and 0.5 mg/l Kn with 0.2-1.0 mg/l IAA also failed to produce any shoot in both leaf and internode derived callus. On the other hand, highest 30% culture induced shoots from leaf derived callus in MS medium supplemented with 1.0 mg/l BAP + 0.5 mg/l IAA and highest average 4.00 ± 0.34 shoot per culture and highest 6.00 ± 0.11 cm length of shoot per culture were noted in the same medium. Effects of BAP + NAA and BAP + IAA combination on callus regeneration were suitable in many species such as *Physalis pubescens* (Rao *et al*. 2004), *Mimosa pudica* (Munshi *et al*. 2001), *Rauwolfia serpentina* (Sarker *et al*. 1996) *Abrus precatorius* L (Rahman *et al*.2008).

Rooting experiments were conducted on half strength of MS medium with 3% sucrose supplemented with different concentrations of IBA alone and combination with BAP or Kn. Among these concentrations and combinations, IBA was the best for root induction. These findings were in agreements with those observed in other medicinal plants such as *Plumbago zeylanica* (Selvakumar *et al*. 2001), *Lilium nepalense* (Wawrosch *et al*. 2001), *Holostemma ada–kodien* (Martin, 2002), *Vitex nigundu* (Chandromu *et al*. 2003), *Phellodendrom amurense* (Azad *et al*. 2005), *Aristolochia indica and Hemidesmus indicus* (Siddique, 2005), *Asparagus racemosus* (Afroz *et al*. 2010). After two weeks of inoculation, profuse root formation (90%) was noticed from basal cut portion of the shoot in half strength MS medium having 0.2 mg/l IBA with a fairly good length (8.00 ± 0.28 cm) and number (56.00 ± 0.23). The rooting medium consisted of half strength MS supplemented with IBA was also reported in other plant species such as *Azadirachta indica* (Sarker *et al*. 1997), *Carica papaya* L. (Islam *et al*. 2001), *Catharanthus roseus* L. (Islam *et al*. 2001), *Mimosa pudica*

(Munshi *et al*. 2001), *Saussurea obvallata* (Joshi and Dhar, 2003), *Withania somnifera* (Siddique, 2005), *Paederia foetida* (Alam *et al*. 2010).

3.4.4. *In vitro* culture of *Mentha arvensis*

Mint is valued for its multipurpose uses in the field of pharmaceuticals, cosmetics as well as for flavoring foods and tobacco (Ohloff, 1994). The plant is aromatic, stimulant and carminative. The infusion of leaves affords a remedy for rheumatism and indigestion (Anonymous, 1962). The plant is propagated mainly by shoot cuttings, seed setting is very poor. Besides this, in dry places and during summer season, the propagation of this plant is very difficult. In case of *M. arvensis* available reports regarding *in vitro* mass propagation is limited. Maity *et al*. (2011) developed a protocol in rapid and large scale micropropagation of true to type clone of *M. arvensis* Linn. In this experiment they found that the best shoot proliferation (8.81 shoot per explant) was achieved on BAP (1.0 mg/l). *In vitro* shoots could be easily rooted on MS medium containing 1 mg/l IBA. Plantlets were successfully acclimatized in green house and field conditions. Another report was available from Chishti *et al*. (2006) develop a protocol in clonal propagation of *M. arvensis* L. through nodal explant. They used BAP and Kn alone in different concentrations and combinations with either NAA or IAA. They also found BAP + IAA was best combination of multiple shoot induction from nodal explant. Mehta *et al*. (2012) reported a efficient protocol for clonal micropropagation of *Mentha piperita* L. They found the induction of multiple shoots from nodal segments was the highest in MS medium supplemented with 0.5 mg/l BAP + 3.0 mg/l Kn. For rooting different concentration of IBA were used and highest rooting was recorded on MS medium with 2.0 mg/l IBA.

We report here a rapid and efficient method for *in vitro* propagation of menthol producing plants through shoot tip and nodal explant both direct and indirect proliferation. Here single medium based micropropagation protocol was established using four types of explants *viz*. shoot tip, node, leaf and internode segments through direct and indirect organogenesis. For direct shoot regeneration shoot tip and node segments were cultured on MS medium with BAP, Kin alone or in combination with BAP + Kn, BAP + NAA and Kn + NAA. Shoot tip and node segment produced shoot proliferation. When BAP and Kin applied singly proliferation response was found to be moderate type with 2–8 shoots per explant. But shoot induction enhanced 6–16 when low concentrations of NAA or IAA were used in combination with BAP and

Kin and the best combination was found in BAP + NAA combination. The significant effect of BAP in combination with IAA or NAA has been demonstrated is many medicinal plants such as W*ithania somnifera* (Sen and Sharma, 1991), *Dictyospermum ovalifolium* Wight. (Thoyajaksha and Rai, 2001), *Holostemna ada–kodien* (Martin, 2003), *Bupleurum fruticosum* (Fraternale *et al*. 2002), *Limonium wrightii* (Huang *et al*. 2000), *Acmella calva* (Senthilkumar *et al*. 2007), *Vanasushava pedaata* (Karuppusamy *et al*. 2006), *Rauwolfia serpentina* (Mallick *et al*. 2012), *Smilax zeylanica* (Thirugnanasampandan *et al*. 2009), *Paderia foetida* (Alam *et al.* 2010). Among the two types of explants the highest percentage (95%) of shoot induction was noted from node segment in media having 1.0 mg/l BAP + 0.2 mg/l NAA. The highest mean number of shoot per culture (16.00 ± 0.23) and highest mean length of shoot per culture (7.53 ± 0.12 cm) were recorded in the combination of 1.0 mg/l BAP + 0.5 mg/l NAA. On the other hand, the highest percentage (80%) of shoot induction was noted from shoot tip segment in media having 1.0 mg/l BAP + 0.2 mg/l NAA and highest average 16.00 ± 0.17 number of shoot per culture and 8.30 ± 0.17 cm mean length of shoot per culture were found in the same medium. The present findings are in agreement with those observed in other plant species such as *Albizzia lebbeck* (Reza *et al*. 1995), *Adhatoda* vasica (Banu *et al*. 1997), *Rauwolfia serpentina* (Roy *et al.* 1994; Roja *et al*. 1996), *Catharanthus roseus* (Islam *et al*. 2001), *Physalis pubescens* L. (Rao *et al.* 2004). In the present experiment, it was observed that base callusing was observed in many concentrations. The calli were cream in color and compact and nodular in texture. The growth of callus was rapid and within few days the explant with proliferated shoots were covered with callus and they prevents to elongate the shoots. The results are agreement with Banu and Bari (2007), Banu *et al.* (2001) in *Ocimum sanctum*. They reported that increase of BAP level and BAP, NAA combination in the medium explants produced base callus with shooting. Finally, GA3 was used alone and combinations with BAP or Kn for elongation and shoot multiplication. Here, the highest (100%) shoot induction and elongation was noted from nodal explants in media having 0.5 mg/l GA_3 but highest average number of shoot 32.07 ± 0.46 cm per culture and 12.30 ± 0.14 cm mean length of shoot per culture were found in the media having 1.0 mg/l BAP + 0.5 mg/l GA_3. Similar results have been reported by Ghanti *et al*. (2004) in *Mentha piperita* L, Chinnamadasamy *et al.* (2010) in *Plumbago zeylanica*.

Experiments on callus induction of *M. arvensis* was also carried out using leaf and internode segments and cultured on MS medium supplemented with different concentrations of 2,4-D alone or in combination with 2,4-D + BAP and 2,4-D + Kn. Callus was not induced in 0.2 mg/l 2,4-D alone or in combination with BAP and Kn. However, among all the tested growth regulators 2, 4-D + BAP were more effective for callus induction from internode segments than leaf segments. Leaf explants started callus formation within 10–15 days of culture and 70% cultures produced callus in medium containing 2.0 mg/l BAP +1.0 mg/l 2,4-D. These calli were light green in colour compact in texture. On the other hand, 80% callus induced from internode explants within 10–14 days of culture with production of cream and compact callus in the same medium. The results agrees with the findings of Rao *et al*. (2004) in *Physalis pubescens*, Farooq *et al*. (2001) in wheat, Siddique *et al*. (2001) in *Dendrobium aphyllum*, Rani and Groven (1999) in *Withania somnifera*, Torres *et al*. (1988) in *Physalis peruviana*, Bapat and Rao (1977) in *Physalis minima,* Ilahi *et al*. (2007) in *Rauwolfia serpentina,* Zhang et al. (2011) in *Curcuma kwangsiensis,* Biwas, (2006) in *Stemona tuberosa* Lour.

Successful shoot differentiation from callus was achieved when BAP and Kin were used singly or in combination of BAP with NAA and Kin with NAA. Leaf and internode were used for callus induction. Among the two types of explant derived callus, leaf derived callus was proved most effective for regeneration of *M. arvensis*. However, the highest 65% culture induced shoots from leaf derived callus in MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l Kn + 0.5 mg/l NAA and highest average 12.00 ± 0.23 shoot per culture and highest 4.30 ± 0.11 cm length of shoot per culture were found in the media having 1.0 mg/l BAP + 1.0 mg/l Kn + 0.2 mg/l NAA. On the other hand, highest 54% culture induced shoots from internode derived callus in MS medium supplemented with 2.0 mg/l BAP + 1.0 mg/l Kn + 0.2 mg/l NAA with an average 10.00 ± 0.23 shoot per culture and 4.60 ± 0.11 cm length of shoot per culture. Effects of BAP + NAA and BAP + Kn + NAA combination on callus regeneration were suitable in many species such as *Physalis pubescens* (Rao *et al*. 2004), *Mimosa pudica* (Munshi *et al*. 2001), *Rauwolfia serpentina* (Sarker *et al*. 1996), *Plumbago indica* L and *Boerhaavia diffusa* L (Biswas, 2006) .

Rooting experiments were conducted on half strength of MS medium with 3% sucrose supplemented with different concentrations of auxins (NAA, IAA and IBA). Among the three types of auxin used, NAA was the best for root induction. These findings are

in agreements with those observed in other medicinal plants such as *Plumbago zeylanica* (Selvakumar *et al*. 2001), *Lilium nepalense* (Wawrosch *et al*. 2001), *Holostemma ada–kodien* (Martin, 2002), *Vitex nigundu* (Chandromu *et al*. 2003), *Phellodendrom amurense* (Azad *et al*. 2005), *Rauwolfia serpentina* (Alatar *et al.* 2012)**,** *Curcuma* sp. (DAS, *et al.* 2010). After 4 weeks of inoculation, profuse root formation was noticed from basal cut portion of the shoot in half strength MS medium having 1.0 mg/l NAA with highest (95%) root induction and a fairly good number 22.50 ± 0.15 and length 7.30 ± 0.10 cm. The rooting medium consisted of half strength MS supplemented with NAA was also reported in other plant species such as *Azadirachta indica* (Sarker *et al.* 1997), *Carica papaya* L. (Islam *et al*. 2001), *Catharanthus roseus* L. (Islam *et al*. 2001), *Mimosa pudica* (Munshi *et al*. 2001), *Saussurea obvallata* (Joshi and Dhar, 2003).

3.4.5. *In vitro* culture of *paederia foetida*

P. foetida is one the potential herb in Bangladesh used for various medicinal practices. This species is reported to have ethnomedicinal uses both in Bangladesh (Hannan *et al.* 2008; Reza *et al.* 2008) and India (Hynniewta and Kumar, 2008). It is traditionally used for stomach ailments by Garo (Mia *et al.* 2009) and Santal tribes (Hanif *et al.* 2008) in Bangladesh. The plant having bitter taste with foul smell. *In vitro* propagation has a number of advantages over the sexual one in a large scale propagation programme (Abbott, 1978). In sexual methods superior genotypes are sometimes lost through gene recombination. By means of micropropagation, superior gene combinations can be preserved practically unaltered. Limited reports regarding *in vitro* mass propagation of this plant was available. A report from Alam *et al.* (2010) for developing a protocol *in vitro* regeneration of *P. foetida*: a widely used medicinal vine in Bangladesh. They used MS media having different concentrations of BAP alone and in combination of BAP with Kn and IAA. They found best combination for multiple shoots induction media was 1.5 mg/l BAP + 0.5 mg/l IAA. Another report from Amin *et al.* (2003) for *in vitro* clonal propagation of *P. foetida* L. A medicinal plant of Bangladesh. They used MS media having different concentrations of BAP and Kn alone and in combination of BAP with NAA and IAA but they found 1.0 mg/l BAP alone was best for multiple shoot induction.

The present study was undertaken to establish a protocol for *in vitro* clonal propagation of *P. foetida*. In this experiment, we used BAP and Kn alone in different

concentrations or in combinations with BAP +Kn, BAP + NAA, BAP + IAA, Kn +NAA and Kn + IAA used in MS media. Among the different types of explants (shoot tip, node, internode and leaf), nodal explants was proved to be the best and most efficient explant for tissue culture of *P. foetida* as it responded better than shoot tip for direct organogenesis in all media compositions. When BAP and Kn applied singly proliferation response was found to be moderate with only 2-4 shoots but multiple shoot induction enhanced remarkably when low concentration of auxins (NAA, IAA) were used in combination with higher concentration of BAP and Kn. The synergistic effect of BAP in combination with an auxin (NAA, IAA) has been demonstrated in many other medicinal plants (Casado *et al*. 2002; Fraternale *et al*. 2002; Salvi *et al*. 2002; Martin. 2003), *Smilax zeylanica* (Thirugnanasampandan *et al.* 2009), *Plumbago indica* L and *Boerhaavia diffusa* (Biswas *et al*. 2011), *Aristolochia indica* and *Hemidesmus indicus (*Siddique *et al.* 2006) reported that combination of BAP with IAA is most often used for shoot organogenesis. Results of the present study proved that combination of BAP + IAA was very efficient for multiple shoot induction from shoot tip and node explant of *P. foetida*. The highest percentage (95%) of multiple shoot induction was noted from nodal explant in the media having 1.0 mg/l BAP + 0.5 mg/l IAA. The highest mean number of shoot per culture 6.00 ± 0.23 and the highest length of shoot per culture 7.00 ± 0.11 were noted in the same media. On the other hand, maximum (80%) shoot proliferation was noted from shoot tip explant in the media having 1.0 mg/l BAP + 0.5 mg/l IAA. The highest average length of shoot 7.50 ± 0.28 and the highest average number of shoot per culture 6.00 ± 0.11 noted in the same medium. The present finding corroborated with those observed in other medicinal plant species such as *Rehum emodi* (Lal and Ahuja, 1989) , *Gentiana kuroo* (Sharma *et al.* 1993), *Vitex negundo* (Chandramu *et al.* 2003), *Aloe barbadensis* (Liao *et al*. 2004) and *Plumbago indica* L (Biswas *et al.* 2011) *Bupleurum distichophyllum* (Karuppusamy and Pullaiah, 2007).

Indirect organogenesis of *P. foetida* was also carried out using leaf and internode explants and cultured on MS medium supplemented with different concentrations of 2, 4-D alone or in combination with BAP, Kn and IBA. Results showed that leaf and internode explant produced poor response in 2,4-D (1.0, 2.0 and 2.5 mg/l) but in 0.5 mg/l and 3.0 mg/l and above concentrations failed to produce any callus. But when BAP + 2,4-D and Kn + 2,4-D used in different concentrations moderate type of callus formation was achieved in both explants. But in other experiments when 2,4-D and

IBA were used together the optimum callus formation was achieved in both explants. Here, internode explants performed better than leaf explants. The highest 90% brown and soft callus induction was obtained from internode explants in MS medium having 2.5 mg/l 2,4-D + 0.5 mg/l IBA within 5-6 weeks of culture. On the other hand, highest 80% light green and soft callus induction was obtained from leaf explants in 2.0 mg/l 2,4-D + 1.0 mg/l IBA. Thirupathi *et al*. (2011) found light green, nodular and friable callus in MS medium with 2.5 mg/l 2,4-D + 1.0 mg/l IBA from leaf explants of *P. foetida* which slightly different from the present result.

Regeneration of shoots from different types of calli was carried out using BAP and Kn alone and in combination with either NAA or IAA. Among all the concentrations and combinations, results showed that BAP + IAA produced highly regenerable media both internode and leaf explants. 0.5 mg/l BAP and 0.5 mg/l Kn, 2.0 mg/l Kn failed to produce any morphogenic response. The best regeneration of shoot was obtained from 1.0 mg/l BAP + 1.0 mg/l IAA. In this combination 85% callus produced adventitious shoots from internode derived callus explants with a highest average number 6.00 ± 0.11 shoot per culture and average 6.50 ± 0.28 cm length of shoot per culture in 1.0 mg/l BAP + 1.0 mg/l IAA. On the other hand, highest 70% callus produced adventitious shoots from leaf derived callus and highest average 5.00 ± 0.17 shoot per culture and average 6.60 ± 0.11 cm length of shoot per culture was found in the same medium. Similar results have been reported by Sarker *et al*. (1996) in *Rauwolfia serpentina*.

Rooting experiments were conducted on half strength MS basal medium with 3% sucrose supplemented and different concentrations of auxins (IBA, NAA and IAA) used alone. Among the different hormonal concentrations a half strength MS medium supplemented highest 80% root regeneration was recorded in 0.2 mg/l IBA. The highest average 12.00 ± 0.28 roots per culture and highest 5.60 ± 0.11 cm mean length of root per culture found in the same medium. It has been reported that IBA alone is the suitable auxin for adventitious root induction and it was also found superior to IAA or NAA for its more stable nature (Litz and Jaiswal, 1990). The efficacy of half strength of MS medium with IBA at lower concentrations in *in vitro* rootings has been reported in many plant species such as *Ficus benghalensis* (Rahman *et al*. 2004), *Mimosa pudica* (Munshi *et al*. 2001), *Catharanthus roseus* L (Islam *et al*. 2001), *Dendrobium aphyllum* (Siddique *et al*. 2001), *Carica papaya* L. (Islam *et al*. 2001), *Smilax zeylanica* (Thirugnanasampandan *et al*. 2009), *Saussurea obvallata*

(Joshi and Dhar, 2003), *Withania somnifera* (Siddique, 2005), *Paederia foetida* (Alam *et al*. 2010).

3.4.6. *In vitro* culture of *Rauwolfia serpentina*

R. serpentina Benth localy known as "Sarpgandha" is an endangered medicinal plant found in Bangladesh, Bhutan, China, Indonesia, India, Malaysia, Myanmar, Nepal, Pakistan, Sri Lanka and Viet Nam (Dey and De, 2010). This herbal plant is used as medicine for high blood pressure, insomnia, anxiety and other disorders of the central epilepsy. Reserpine is a potent indole alkaloid first isolated from this plant which is being widely used as an antihypertensive (Anonymous, 2003). Due to poor seed germination rate, large scale and indiscriminate collection of wild plants for pharmaceutical purposes and insufficient attempts to either allow its replenishment or its cultivation, Therefore, it is necessary to devise an efficient method for the development of a potentially large scale multiplication protocol for commercial production of this endangered species. Under these circumstances, propagation through tissue culture offers a viable alternative for this species rehabilitation, and conservation or utilization of genetic resources. Further, *in vitro* plant regeneration through auxiliary bud culture is an economic way for obtaining large number of consistently uniform and true-to-plants type within a short time span.

There are several earlier attempts, to develop *in vitro* propagation protocol of *R. serpentina* via callus, shoot tips, and nodal segments (Stockgit *et al*. 1981; Yamamoto and Yamada, 1986; Benjamin *et al*. 1993; Roja and Heble, 1996; Ilahi *et al*. 2007; Bhatt *et al*. 2008). The existing protocol produced only a few numbers of shoot. The objectives of the study reported here were to optimize the culture conditions applied for the initiation and proliferation of shoots from nodal explants of *R. serpentina* through enhanced auxiliary branching, and induced rooting in microshoots and establish the plantlets in outdoor conditions.

In the present study BAP and Kn were used alone in different concentrations or in combinations with BAP + NAA, BAP + IAA, Kn + NAA and Kn + IAA used in MS medium. Among the different types of explants (axillary bud, node, internode and leaf), nodal explants was proved to be the best and most efficient explant for tissue culture of *R. serpentina* as it responded better than axillary bud for direct organogenesis in all media compositions. When BAP and Kn applied singly proliferation response was found to be poor with only 1-2 shoots, but multiple shoot

induction enhanced remarkably when low concentration of auxins (NAA or IAA) were used in combination with higher concentration of BAP and Kn. The synergistic effect of BAP in combination with an auxin (NAA or IAA) has been demonstrated in many medicinal plants (Casado *et al*. 2002; Fraternale *et al*. 2002; Salvi *et al*. 2002; Martin, 2003; Tripepi, 1997)**,** *Smilax zeylanica***, (**Thirugnanasampandan *et al*. 2009), *Plumbago indica* L and *Boerhaavia diffusa*, (Biswas, 2006), *Aristolochia indica* and *Hemidesmus indicus, (*Siddique et al. 2006) and they reported that combination of BAP with NAA is most often used for shoot organogenesis. Results of the present study proved that combination of BAP + NAA was very efficient for multiple shoot induction from axillary bud and nodal explant of *R. serpentina*. The highest percentage (90%) multiple shoot induction was noted from nodal explant in the media having 1.5 mg/l BAP + 0.2 mg/l NAA. The highest mean number of shoot per culture 12.00 ± 0.34 and the highest length of shoot per culture 4.70 ± 0.20 were noted in the same medium. On the other hand, maximum 80% shoot proliferation was noted from axillary bud explant in the media having 1.5 mg/l BAP + 0.2 mg/l NAA. The highest average number of shoot per culture 10.00 ± 0.11 and the highest average length of shoot 4.60 ± 0.11cm were noted. The present findings corrborated with those observed in other medicinal plant species such as *Rehum emodi* (Lal and Ahuja, 1989) , *Gentiana kuroo* (Sharma *et al*. 1993), *Vitex negundo* (Chandramu *et al*. 2003), *Paederia foetida* (Amin *et al*. 2003**),** *Aloe barbadensis* (Liao *et al*. 2004), *Bupleurum distichophyllum* (Karuppusamy and Pullaiah, 2007) *and Asparagus racemosus* (Pant and Joshi, 2009).

Experiments on callus induction of *R. serpentina* was also carried out using leaf and internode explants and cultured on MS medium supplemented with different concentrations of 2,4-D, NAA, IBA and IAA alone or in combination with 2,4-D + BAP and 2,4-D + Kn. Callus induction was poor in different concentrations of NAA, IBA and IAA when used singly in MS medium. However, among all the tested growth regulators 2,4-D alone and in combination of 2,4-D + BAP and 2,4-D + Kn were more effective for callus induction from internode segments over leaf segments. Leaf explants started callus formation within 8–12 days of culture and 70% cultures produced callus in medium containing 2.0 mg/l 2,4-D + 1.0 mg/l BAP. These calli were green and compact. On the other hand, 90% callus induced from internode explants within 7–10 days of culture with production of green and friable callus. Similar findings were also reported by Rao *et al.* (2004) in *Physalis pubescens*,

Farooq *et al.* (2001) in wheat, Siddique *et al.* (2001) in *Dendrobium aphyllum*, Rani and Groven (1999) in *Withania somnifera*, Torres *et al*. (1988) in *Physalis peruviana*, Bapat and Rao (1977) in *Physalis minima*, Biswas (2006) in *Stemona tuberosa* L.

Rooting experiments were conducted on half strength MS basal medium with 3% sucrose supplemented and different concentrations of auxins (IAA, IBA and NAA) alone or in combination with IBA + IAA and IBA + NAA were used. Among the different hormonal concentrations a half strength MS medium supplemented with 0.2 mg/l IBA + 0.2 mg/l NAA gave the best response, here highest 80% root induced within 5–6 weeks of culture with an average 8.00 ± 0.32 roots per culture and 4.00 ± 0.15 cm mean length of root per culture found in the same medium. It has been reported that IBA alone or IBA + NAA is the suitable auxin for adventitious root induction and it was also found superior to IAA or NAA for its more stable nature (Litz and Jaiswal, 1990). The efficacy of half strength of MS medium with IBA at lower concentrations in *in vitro* rootings has been reported in many plant species such as *Ficus benghalensis* (Rahman *et al*. 2004), *Mimosa pudica* (Munshi *et al*. 2001), *Catharanthus roseus* L (Islam *et al*. 2001), *Dendrobium aphyllum* (Siddique *et al*. 2001), *Carica papaya* L. (Islam *et al*. 2001), *Smilax zeylanica* (Thirugnanasampandan *et al*. 2009), *Paederia foetida* (Alam *et al*. 2010).

3.4.7. *In vitro* culture of *Smilax zeylanica*

S. zeylanica Vent. localy called “Kumarilata”,The roots are used to treat syphilis, gonorrhea, swellings, abscesses and boils (Nadkarni, 1976). Species of *Smilax* are gaining importance as a source of steroidal saponins isolated from the roots (Sautour *et al.* 2005). The plant number has declined presumably due to several biotic and abiotic factors. The major threat factor for seed which is severely damaged by insects (Mutharaian, 2003). Very limited reports regarding *in vitro* mass propagation of this plant are available. Thirugnanasampandan *et al*. (2009), developed a protocol for *in vitro* propagation and free radical studies of *S. zeylanica* Vent. They found single shoot from nodal explants of half strength MS media with BAP (0.5 mg/l) + IAA (1.0 mg/l) and then *in vitro* shoots subcultured in the media of Kn (2.0 mg/l) with L- glutamine (0.5 mg/l) and they found two shoots. Finally, rooting of the microshoots was achieved in half strength MS medium fortified with IBA (1 mg/l) within three weeks.

The present investigation aims to establish a protocol of *in vitro* propagation in different explants (shoot tip and nodal) used in half strength media with different hormonal concentrations and combinations. In the present study, BAP and Kn were used alone in different concentrations or in combinations with BAP + NAA, BAP + IAA, Kn + NAA, Kn + IAA, BAP + L-glutamine and Kn + L-glutamine used in half strength MS medium. Between the two types of explants (shoot tip and node), nodal explants was proved to be the best and most efficient explant for tissue culture of *S. zeylanica* as it responded better than shoot tip for direct organogenesis in all media compositions. When BAP and Kn applied singly or in combination with each other proliferation response was found to be poor with only 1-2 shoots, but when BAP + NAA, BAP + IAA, Kn +NAA, Kn + IAA was applied the proliferation response found moderate with 2-4 shoots. But multiple shoot induction enhanced remarkably when high concentration of cytokinins (BAP, Kn) were used in low concentration of L-glutamine with 3-6 shoot. Thirugnanasampandan *et al*. (2009), found 2 shoots in half strength MS medium with 2.0 mg/l Kn + 0.5 mg/l L- glutamine from nodal explants of *S. zeylanica* which slightly differs from the present result.

Results of the present study proved that combination of Kn + L-glutamine was very efficient for multiple shoot induction from nodal explant of *S. zeylanica*. The highest percentage (90%) of multiple shoot induction was noted from nodal explant in the media having 1.5 mg/l Kn + 0.5 mg/l L-glutamine. The highest mean number of shoot per culture 6.00 ± 0.25 and the highest length of shoot per culture 5.50 ± 0.11 was noted in the same medium. On the other hand maximum (70%) shoot proliferation was noted from shoot tip explant and the highest average number of shoot per culture 4.00 ± 0.30 and the highest average length of shoot 5.30 ±0.17 was noted in the same medium. The present findings are in agreement with those observed in other medicinal plant species such as *Vitex negundo* (Chandramu *et al*. 2003), *Paederia foetida* (Amin, 2003), *Aloe barbadensis* (Liao *et al*. 2004), *Bupleurum distichophyllum* (Karuppusamy and Pullaiah, 2007) *and Asparagus racemosus* (Pant and Joshi, 2009).

Rooting experiments were conducted on half strength MS basal medium with 3% sucrose supplemented and different concentrations of auxins (NAA, IAA and IBA) alone. Among the different hormonal concentrations in half strength MS medium supplemented with 1.0 mg/l IBA gave the best response, here highest 70% root induced within 5–6 weeks of culture with an average 7.00 ± 0.11 roots per culture and

4.70 ± 0.20 cm mean length of root per culture found in the same medium. The efficacy of half strength of MS medium with IBA at lower concentrations in *in vitro* rootings has been reported in many plant species such as *Ficus benghalensis* (Rahman *et al.* 2004), *Mimosa pudica* (Munshi *et al*. 2001), *Catharanthus roseus* L (Islam *et al*. 2001), *Dendrobium aphyllum* (Siddique *et al.* 2001), *Carica papaya* L (Islam *et al.* 2001), *Smilax zeylanica* (Thirugnanasampandan *et al*. 2009), *Paederia foetida* (Alam *et al*. 2010).

3.5. SUMMARY

The main aim of the *in vitro* culture experiments was to develop efficient and repeatable protocol for mass propagation of seven important medicinal plants namely, *Asparagus racemosus, Curcuma amada, Ipomoea mauritiana, Mentha arvensis, Paederia foetida, Rauwolfia serpentina* and *Smilax zeylanica* cultivated or planted in Natore Oushodi Gram. It was considered important that if such protocol could be developed that would be of great use in supporting the future demand as well as conservation of such uncared medicinal plant species. The experiments were conducted with the use of different kinds of explants viz. nodal , shoot tip, axillary bud, intermodal and leaf segments culture in MS medium fortified with different kinds of auxins (NAA, IAA, IBA and 2,4-D) and cytokinins (BAP and Kn) at various concentrations either at individual or combination levels.

Both direct and indirect organogenesis took place in all seven plant species and the response was dependent on the nature of explants and hormonal supplements as applied in the medium. In case of *Asparagus racemosus* best results was obtained in direct organogenesis *via* shoot bud formation in nodal segments and 2.0 mg/l BAP + 0.5 mg/l NAA combination of growth regulators induced maximum buds per explants. Incase of indirect organogenesis, with the development of shoot buds from green nodular callus that developed best in leaf segments on 1.0 mg/l BAP + 0.2 mg/l NAA supplemented media. In many cases cultured explants produced white and friable callus that usually failed to differentiate.

Among the two kinds of explants in *Curcuma amada*, axillary bud explants was proved best for generating shoot buds through direct organogenesis. The plants growth regulators supplements play a great role in the process of organogenesis. Combination of 0.5 mg/l BAP + 0.5 mg/l NAA in MS medium gave the best direct regeneration frequency. Similarly, better microrhizome production took place at higher frequency in shoot tip segment compared to that in axillary buds segments. Among the different plant growth regulators, combinations of 0.5 mg/l BAP + 0.5 mg/l NAA was proved most efficient in direct organogenesis.

Ipomoea mauritiana also exhibited the similar trend of response as compared to *Asparagus racemosus.* Nodal segments gave the best response compared to the shoot tip explants. Here also organogenesis took place either directly or indirectly depending on the plant growth regulators supplements in the media and culture conditions. Direct organogenesis took place best in the media supplemented with 1.0

mg/l BAP + 0.5 mg/l IAA. Incase of indirect organogenesis best response was obtained in the media supplemented with 1.0 mg/l BAP + 0.5 mg/l IAA.

In *Mentha arvensis* nodal segments gave the best response compared to the shoot tip explants in direct organogenesis and 1.0 mg/l BAP + 0.2 mg/l NAA combination of growth regulators induced maximum buds per explants. Incase of indirect organogenesis, better shoot buds initiated from leaf derived green nodular callus in the media supplemented with 2.0 mg/l BAP + 1.0 mg/l Kn + 0.2 mg/l NAA.

Nodal segments in *Paederia foetida* also gave the best response compared to the shoot tip explants in direct organogenesis and 1.0 mg/l BAP + 0.5 mg/l IAA combination of growth regulators induced maximum buds per explants. But in indirect organogenesis 1.0 mg/l BAP + 1.0 mg/l IAA supplemented media proved best in developing shoot buds from light green nodular callus derived from nodal segments.

In *Rauwolfia serpentina,* nodal segments also gave the best response compared to the axillary bud explants in direct organogenesis and 1.5 mg/l BAP + 0.2 mg/l NAA combination of growth regulators induced maximum buds per explants.

Finally, in case of *Smilax zeylanica,* nodal segments gave the best response compared to the shoot tip explants in direct organogenesis and 1.5 mg/l Kn + 0.5 mg/l L-glu combination of growth regulators induced maximum buds per explants.

Considering overall performance, among the seven species nodal segments was found best as a source of explants in six species for *in vitro* mass propagation. Application of plant growth regulators played an important role in *in vitro* regeneration in all medicinal plants and combination of auxins and cytokinins were proved better than their individual use. When the shoot buds developed either through direct or indirect organogenesis attained a height of 3-5 cm. They were individually grown on half strength MS medium supplemented with auxins but in some cases auxins was used in combinations with BAP where rooting was found to grow at the base of bud. Half MS + 2.0 mg/l Kn + 0.5 mg/l NAA was best for producing root system in *Asparagus racemosus*, half MS + 0.5 mg/l IBA was best for producing root system in *Curcuma amada,* half MS + 1.0 mg/l BAP + 0.2 mg/l IBA was best for producing root system in *Ipomoea mauritiana*. On the other hand, half MS + 1.0 mg/l NAA was best for producing root system in *Mentha arvensis,* half MS + 0.2 mg/l IBA was best for producing root system in *Paederia foetida*, half MS + 0.2 mg/l IBA + 0.2 mg/l NAA was best for producing root system in *Rauwolfia serpentina* and half MS + 1.0 mg/l IBA was best for producing root system in *Smilax zeylanica*. Through successive

phages of acclimatization the *in vitro* grown plantlets in seven plant species were transferred finally to earthen pots in outside environment.

The protocols developed here can reliably be used in mass scale production of plant of these seven medicinal plants. It also offers the opportunities of selecting desirable somaclones if originate in the course of indirect organogenesis. The findings also indicate that it is possible to develop similar protocols for *in vitro* propagation of other medicinal plants of Natore Oushodi Gram and that can be used in mass propagation and conservation programmes.

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CHAPTER IV

4. MICRO CONSERVATION THROUGH ARTIFICIAL SEED PRODUCTION

4.1. INTRODUCTION

Synthetic seeds are defined as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions and that retain this potential also after storage. Synthetic seeds can be stored for a long time in appropriate condition and synthetic seed production and used technology is rapidly growing branch of seed biotechnology. Toshio Murashige in 1970 first coined the term synthetic seed but the idea of artificial seeds was proposed by Murashige in 1977 (Bajaj, 1995; Cyr, 2000), but first reports on their development were published a few years later. The artificial seeds (also called somatic seeds, synthetic seeds, clonal seeds, synseeds, somseeds) may also be defined as an alternative to botanic seeds analogue consisting of somatic embryos surrounded by artificial coats. This definition, also popular in these days, is based on the similarity of somatic embryos with zygotic embryos in morphology, physiology and biochemistry (Redenbaugh *et al*. 1986 and 1988). Synthetic seeds can be produced either as coated or non coated, desiccated somatic embryos or as embryos encapsulated in hydrated gel (usually calcium alginate) (Redenbaugh *et al*. 1993). A few years earlier Kamada (1985) defined artificial seed as 'a capsule prepared by coating a cultured matter, a tissue piece or an organ which can grow into a plant body and nutrients with an artificial film'. This artificial seed concept comprised of 'an external film for strengthening the seed' which possibly implies the seed coat and 'an internal film for encapsulating nutrients required for growth of the cultured matter and plant hormones for controlling germination', a layer that probably simulates the endosperm tissue (Khor and Loh, 2005). The currently used broader definition of synthetic seed is 'an artificially encapsulated somatic embryo, shoot or any other meristematic tissue which can develop into a plant under *in vitro* or *in vivo* conditions' (Bapat and Mhatre, 2005). The following two types of artificial seeds are known: desiccated and hydrated. The first type is produced from plant material either naked or encapsulated in polyoxyethylene glycol followed by its desiccation. In this context, the desiccated artificial seeds can be produced only in plant with desiccation tolerant propagules.

The hydrated artificial seeds are produced by encapsulating plant material in hydrogel coats. The second type of synseeds is produced in those species in which the propagules are recalcitrant and/or sensitive to desiccation (Jaiswal *et al*. 2000). This method has been employed as a suitable alternative for the use of somatic embryos (Srivastava *etal.* 2009) and became an important asset in micropropagation e.g. plants of medicinal value.

Successful utilization of synthetic seeds as propagules of choice requires an efficient and reproducible production system and a high percentage of post planting conversion into vigorous plants. Artificial coats and gel capsules containing nutrients, pesticides and beneficial organisms have long been thought as substitutes for seed coat and endosperm (Bajaj, 1995). However, this technology is still in the developmental stage, and currently cannot compete with the other methods of commercial plant propagation (Cyr, 2000).

Earlier, synthetic seeds were referred only to the somatic embryos that were of economic use in crop production and plant delivery to the field or greenhouse (Gray and Purohit, 1991; Janick *et al*. 1993). In the recent past, however, other micropropagules like shoot buds, shoot tips, organogenic or embryogenic calli etc. have also been employed in the production of synthetic seeds. Thus, the concept of synthetic seeds has been set free from its bonds to somatic embryogenesis, and links the term not only to its use (storage and sowing) and product (plantlet) but also to other techniques of micropropagation like organogenesis and enhanced axillary bud proliferation system (Hossain *et al*. 2000).

Artificial seed production is a potential technique for plant multiplication and preservation, especially as it has been considered to be promising for propagation of no seed producing plants, transgenic plants and other plants that need to keep superior traits by means of asexual propagation (Saiprasad, 2001). Plant artificial seed in a narrow sense, means the beads formed by encapsulating somatic embryo with coating materials. Its effect varied with different species, coating materials, maintained solutions and its concentration and condition (Nhut *et al*. 2005). Kamada (1985) presented a general concept of plant artificial seed, in which all kinds of plant explants with germination ability can be used for artificial seed production. Now a days, it is widely used in many plants (Slade *et al*. 1989; Fukai *et al*. 1994; Stephen and Jayabalan, 2000; Ipekci and Gozukirmizi, 2003; Halmagyi *et al*. 2004; Nhut *et al.*

2005). Artificial seed production is an outstanding technique used to propagate and preserve plants and has been applied on many plants (Wang and Qi, 2010).

Artificial seed would provide an easy and novel propagation system for the elite as well as difficult to root species (Bapat *et al*. 1987). Also, encapsulation of propagules that were produced *in vitro* could reduce the cost of micropropagation of plantlets for commercialization and final delivery (Chu, 1995) this technology may be of value in breeding programs and allows the propagation of many elite genotype derived plants in a short time (Nieves *et al*. 1998). In some of the horticultural crops seeds propagation is not successful due to: a. heterozygosity of seeds particularly in cross pollinated crops, b. minute seed size eg; orchids, c. presence of reduced endosperm, d. some seeds require mycorrhizal fungi association for germination eg: orchids, e. no seeds are formed. These crop species can be propagated by vegetative means like micro propagation and clonal propagation.

Several aspects of the technique are still under developed and hinder. Its commercial application (Brischia *et al*. 2002). This technology also has been employed for germplasm storage and exchange purposes as reported by Danso and Lloyd (2003). The first indications of artificial seed propagation were reported in annual crops such as alfalfa (*Medicago sativa* L.) and sugar cane (*Saccharum* spp.). Currently, systems of artificial seed production have progressed substantially in this area, the most advanced being in seeding under *ex vitro* or field conditions, obtaining high percentages of conversion to plants (Fujii *et al*. 1987; Nieves *et al*. 2003). Several researchers suggest that to control growth and facilitate the germination of somatic embryogenesis, the synthetic endosperm can simulate an endosperm of sexual origin, containing one or several compounds such as: nutrients, growth regulators, anti pathogens, herbicides, biocontrollers and bio fertilizers, among others, with the aim of ensuring the conversion of the plant and its development in the field (Castillo *et al*. 1998; Kumar *et al*. 2004; Malabadi and Staden, 2005).

Development of efficient *in vitro* techniques to ensure its safe conservation is therefore of paramount importance. Due to its ease of handling and quick of conversion to plantlets, *in vitro* regenerated bulblet seems to be the sutiable explant for germplasm preservation of garlic. *In vitro* bulblets formation of garlic largely dependent on growth regulators and sucrose in culture media (Matsubara and Chen, 1989 ; Nagakubo *et al*. 1993), as well as other conditions such as cultivar, photoperiod and temperature. The further spreading and application of artificial seed

is based on many factors, including the efficiency of the existing explants regeneration system, relative cost of a specific application for a given plant species, etc. For example, the synthetic seed of seedless watermelon would be less costly than the conventional seed (Saiprasad, 2001). Because garlic is sterile, it is vegetatively propagated by cloves and air bulbils (Shawky, 2006). Encapsulation of *in vitro* grown bulblets of garlic is a suitable system for mid term storage of garlic tissue cultures since encapsulation saves space, time and resources and it demonstrates advantages over conventional method and this method considered a very good tool to exchange the garlic germplasm between countries and international plant gene banks. Huda and Bari (2007) found that artificial seed in eggplant can offer a new avenue for supporting the program of genetic engineering providing an exciting asexual propagation bridge for readily multiplication of transformed plants. Gona and Omid (2008) demonstrated that somatic embryos could successfully be converted to fully formed plants; this work should facilitate genetic transformation and artificial seed production in strawberry. It was successful in developing and optimizing a procedure of the production and storage of sweet corn synthetic seeds by encapsulating somatic embryos in calcium alginate hollow beads and retrieving plantlets, as an alternative sweet corn propagation practice (Thobunluepop *et al*. 2009). It was successful to production and storage of synthetic seeds in *Coelogyne breviscapa* (Mohanraj *et al*. 2009). Genetically uniform production of synthetic seeds of cucumber by standardizing culture conditions to induce somatic embryogenesis synchronously and at high frequency in cell suspension culture.

Since the production of genetically stable and true-to-type plant is desired for production of synthetic seeds, AFLP analysis is linked to ensure the genetic integrity of mother plants (Tabassum *et al*. 2010). Olive plantlets regenerated from synthetic seeds, and well developed root system was successfully acclimatized under *ex vitro* conditions. The protocol can be used for germplasm exchange of woody trees and preparation of synthetic seed (Muhammad *et al*. 2010). Ma *et al*. (2011) showed that *P. heterophylla* artificial seed production and the formation of *in vitro* plants derived from these synthetic seeds were feasible, and the benefit that could be conferred by their use would be very great.

4.1.1. Characteristics of synthetic seeds

1. High volume and large scale propagation method
2. Maintains genetic uniformity of plants
3. Direct delivery of propagules to the field, thus eliminating transplants
4. Lower cost per plantlet
5. Rapid multiplication of plants.

4.1.2. Application of synthetic seeds

By combining the benefits of a vegetative propagation system with the capability of long term storage and with the clonal multiplication, synthetic seeds have many diverse applications in the field.

1) Multiplication of non seed producing plants, ornamental hybrids or polyploids plants.
2) Propagation of male or female sterile plants for hybrid seed production
3) Germplasm conservation of recalcitrant species
4) Multiplication of transgenic plants.

4.1.3. Objectives

Cultivation of medicinal plants in most case have been suffering from low seed viability, lack abundant propagules, scanty seed size, easily damage by pathogen and accompanied by other limitations making hindrance in massive seed multiplication. On this context, enhancement of seed viability, storage capacity and propagule diversity undoubtly would ensure better cultivation practice in medicinal plants. The objectives of the present investigation was to standardize artificial seed production technologies for medicinal plants particularly experiencing propagation barrier.

In this respect *Mentha arvensis* was taken as a model for standardizing protocol of artificial seed production by encapsulating vegetative organ with sodium alginate.

4.2. MATERIALS AND METHODS

4.2.1. Materials

4.2.1.1. Plant materials

At the beginning somatic embryos were used as plant material for artificial seed production. During the last few years, considerable efforts have been made for encapsulation of non embryogenic *in vitro* derived plant material. This strategy was developed for plants in which the process of somatic embryogenesis has not been documented. The various types of unipolar vegetative propagules: microtubers, microbulbs, corms, rhizomes, microcuttings: shoots and nodal segments with apical or axillary buds; differentiating aggregates: organogenic callus and primordia or polar vegetative propagules: protocorms and protocorm like bodies, can be used as explants for the preparation of synthetic seeds (Standardi and Piccioni, 1998). There is a wide range of reports on the use of unipolar synthetic seeds from the last two decades. Among several non-embryogenic materials shoot tip explants are most responsive because of its mitotic activity in the meristem (Ballester *et al.* 1997).

Shoot tip and nodal segments of *Mentha arvensis* were used as explants in this investigation for artificial seed production. Shoot tips and nodal segments 3-5 mm long were aseptically excised from *in vitro* cultured plants regenerated by the method described by Maruyama, 1996.

Procedure for production of artificial seeds:

Establishment of shoot regeneration

↓

Segment the *in vitro* plantlets

↓

Encapsulation of explants

↓

Evaluation of explants and plant conservation

↓

Mass production of synthetic seeds

↓

Planting in field / green house

4.2.2. Methods

Sodium alginate beads were produced by encapsulation according to the method of Kinoshita and Satio (1990). The method involved in this investigation can be described under following heads:

A. Media preparation

Culture media were prepared following MS (Murashige and Skoog, 1962) medium preparation technique and agar was added at the rate of 8 gm/l and then autoclaved for 20 minutes under 121˚C temperature.

B. Preparation of solution for encapsulation of artificial seed

Following steps were taken for the preparation of solution for explants encapsulation:

i) Media preparation

200 ml ½ strength MS medium was prepared and 0.8 gm. sucrose was first added to 150 ml of ½ MS and then different concentrations of hormones ie 0.5, 1.0, 1.5, 2.0 and 2.5 mg./1 BAP, Kn and NAA were also added to it. After mixing the solution it was filled up to 200 ml.

ii) Alginate solution

20 ml of the above mentioned solution was taken and added required growth regulator. Then 0.8 gm (800 mg) of sodium alginate was also added and taken in a small beaker (50 ml beaker). With a small piece of glass rod efforts were made to mix the alginate in solution. Alginate was partially dissolved and it was then kept aside. During autoclaving alginate was completely dissolved.

iii) $CaCl_2$ solution

50 ml of above mentioned solution (½ MS + Sug + hormones) was taken in a small beaker. An amount of 0.7 gm (700 mg) $CaCl_2$ was added to it and dissolved. Out of 200 ml. ½ strength solution 70 ml (50 + 20) was used during the preparations of alginate and $CaCl_2$ and another 130 ml was remained reserved which after autoclaving it was used during washing the encapsulated explants.

iv) Autoclaving:

a. Culture flack containing different solutions.

b. Alginate solution.

c. $CaCl_2$ solution.

d. Several petridishes

Remaining 130 ml of ½ MS + Sugar + hormone solution

C. Encapsulation of explants

Several gels like agar, sodium alginate, polyco 2133 (Bordon Co.), carboxy methyl cellulose, carrageenan, gelrite (Kelko. Co.), guargum, sodium pectate, tragacanth gum, etc. were tested for synthetic seed production, out of which sodium alginate encapsulation was found to be more suitable and practicable for synthetic seed production. Alginate hydrogel is frequently selected as a matrix for synthetic seed because of its moderate viscosity and low spinnability of solution, low toxicity for somatic embryos and quick gellation, low cost and biocompatibility characteristics. Only *in vitro* grown plants were used for this experiment. Explants were taken in an autoclaved petridishes and nodal segments, shoot tips were cut carefully removing the internodal zones. The nodal segments with active buds and shoot tips were placed to the beaker containing alginate solution. The buds were dipped in alginate solution. The dipping explants were taken by a forcep and placed to the beaker of $CaCl_2$. During picking up the explants, the forceps also took some addition alginate solution together with explants. The rolling explants with the liquid of alginate were dropped in to the $CaCl_2$ solution. The explants were kept inside the alginate solution for about 30 minutes. After 30 minutes each explant become a hardball encoated by alginate. They were then washed well with remaining solution of MS + Sucrose + hormones (Different concentrations and combinations of BAP, Kn and NAA).

D. Storage of artificial seeds and plants recovery

Conservation of germplasm using artificial seeds can be visaged either as short term and mild term storage or as long term cryopreservation (Arora *et al*. 2010). The artificial seeds may be carried out in different levels of time intervals. Encapsulated plant material is, in general, short term stored for 6–12 months in closed sterile container at a temperature of 4°C. During long term conservation in liquid nitrogen (–196°C) in different durations, cell division, metabolic and biochemical processes are arrested. For this reason the artificial seed matrix should be supplemented with nutrients playing an important role in storage (e.g. sucrose, salicylic acid, mannitol, paclobutrazol). Sucrose increases the tolerance to dehydratation and maintains the tissue viability (Katouzi *et al*. 2011), salicylic acid plays a role during the plant response to abiotic stress such as low temperature (Janda *et al*. 2007), mannitol and paclobutrazo increase the tolerance of drought and cold stress (Lisek and Orlikowska,

2004). *In vitro* conservation involves the maintenance of plant material in a pathogen free environment. Long term storage allows maintaining genes collections but storage in liquid nitrogen cannot be applied to all genotypes. However, encapsulated in calcium alginate shoot tips and nodal segments of all genotypes can be stored at low temperature.

To summarize, the retention of biosynthetic potential of the encapsulated *in vitro* derived plant material, implicates the use of this method for the storage of high secondary metabolites producing cells and tissue cultures for pharmaceutical purposes. At the end of each storage period, synthetic seeds were immediately transferred to fresh germination medium and placed for the recovery of plantlets. After storage period the artificial seeds were grown under *in vitro* conditions on nutrient media supplemented or not with phytohormones for shoot development and root induction, followed by their hardening and growth in soil under *ex vitro* conditions. A number of research workers reported the techniques used for encapsulation and storage of synthetic seeds and as an alternative way for soil cultivation artificial seeds were placed in soil pits in greenhouse, and then in ground (Srivasta *et al*. 2009; Bach *et al*. 2004; Singh *et al*. 2009; Lata *et al*. 2009; Lisek and Orlikowska, 2004; Maruyama *et al*. 1997; Katouzi *et al*. 2011; Ma *et al*. 2011; Germana *et al*. 1998; Singh *et al*. 2006).

E. Inoculation of encapsulated explants

The encapsulated explants or synthetic seeds were washed with 130 ml half strength MS liquid medium (as mentioned above). After washing the synthetic seeds were placed on right culture medium for subsequent growth.

F. Culture incubation

Inoculated glass vessels were incubated in growth chamber providing a special culture environment. In growth chamber photoperiod was maintained generally 16 hours light 8 hours dark. The culture vessels were checked daily to note the germination.

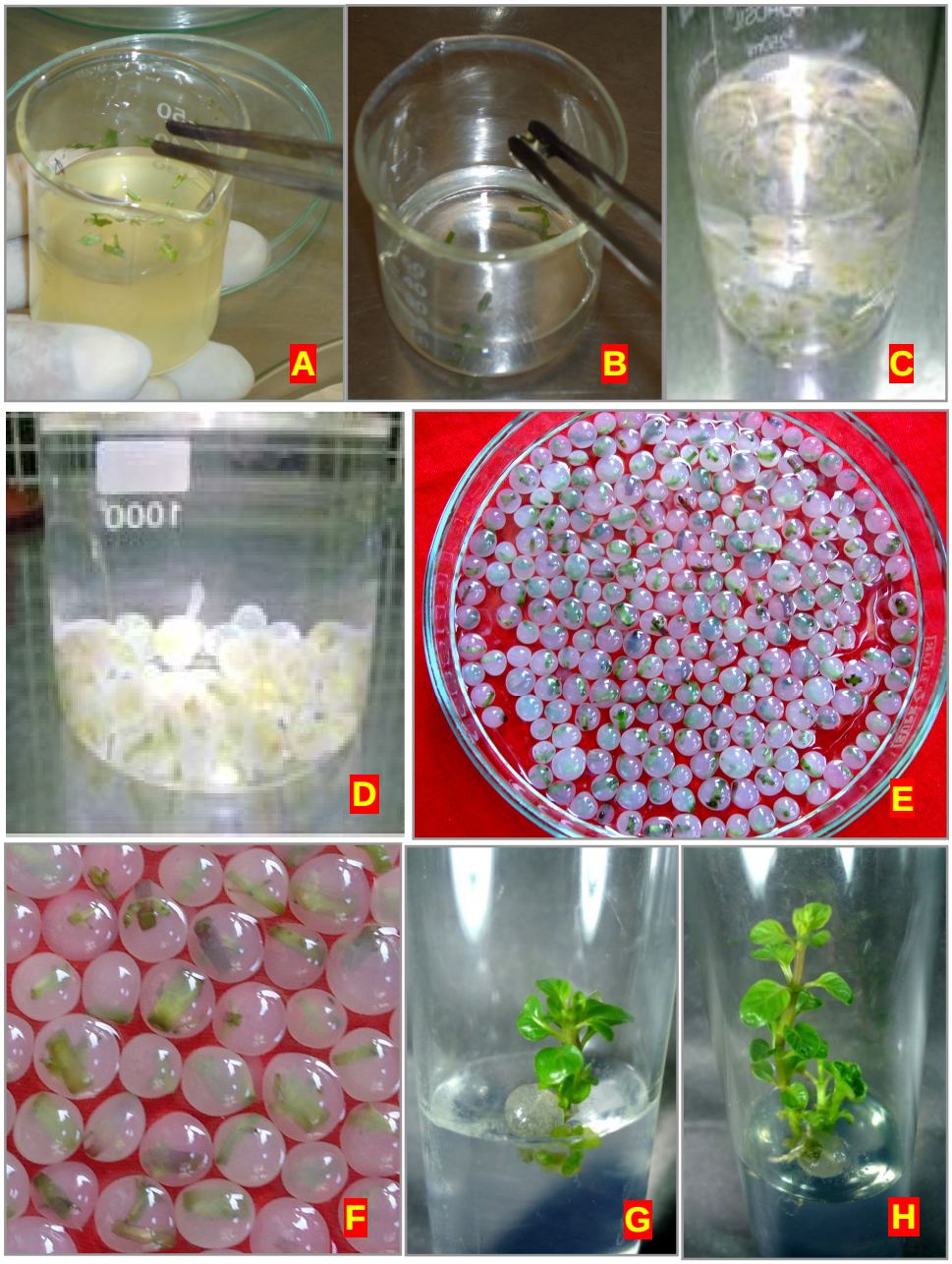

Plate 4.1 Procedure of artificial seed production.

B: Nodal explants mixed with sodium alginate; B-**C:** Encapsulated seed dropped in calcium chloride solution; **D:** Artificial seeds rinsed in distill water; **E-F:** Artificial seeds; **G:** Spouting of artificial seed; **H:** Further development of artificial seed.

G. Data collection

Percentage of germination: Percentage of germination of synthetic seed was calculated using the following formula:

$$\% \text{ of germination} = \frac{\text{No. of germinated seed}}{\text{No. of seed inoculated}} \times 100$$

No. of shoot: Number of shoots per explant was counted after 4 weeks of culture. Average number of shoots per explants was calculated using following formula:

$$\text{Average number of shoot} = \frac{\text{Total number of shoots}}{\text{No. of germinated seeds}}$$

Shoot length: Length of shoot was measured in cm. For each plant average length of shoot was calculated using following formula:

$$\text{Average length of shoot} = \frac{\text{Total length of shoots}}{\text{No. of shoots}}$$

Standard error: Standard error was calculated using following formula:

$$\text{Standard error} = \frac{SD}{\sqrt{\text{N}}}$$

$$\textbf{Standard Deviation (SD)} = \sqrt{\frac{\sum \text{X}^2 - \left(\sum \text{X}\right)^2 / n}{n-1}}$$

4.3. RESULTS

Mentha arvensis plant was selected for the study of artificial seed production. *In vitro* grown explants were used as the source for providing necessary explants. Different concentrations of auxins and cytokinins were used alone or in combinations to investigate the induction of shoot regeneration. These hormonal concentrations were tested in MS basal media (Murashige and Skoog, 1962). Nodes and shoot tips were used as explants for artificial seed production. Details of the results so far obtained from each of the experiments are described under following heads:

4.3.1. Alginate encapsulated explants culture for shoot regenerations

The application of encapsulated shoot tips and nodal segments may contribute to the protection of rare and threatened medicinal plants. Although the artificial seed technique has been reported for more than two decades, for medicinal plants this method has not been developed sufficiently. The main limitations in conventional propagation of some species with medicinal value are: reduced endosperm, low germination rate and seedless varieties (Saiprasad, 2001). Many taxons also are desiccation sensitive or have recalcitrant seeds, for this reason they can be stored only for limited time. The above mentioned reasons indicate the need for the production of artificial seeds as a technique which combines the advantages of clonal multiplication with those of seed propagation and storage. Despite this research, practical implementation of this technique encounters a number of difficulties associated with plant material maturation, development and regeneration. The parameters of encapsulation (mostly alginate coat matrix), suitable storage temperature and duration should be optimized for selected medicinal species to achieve a high percentage of regeneration and conservation.

4.3.2. Encapsulated artificial seed regenerations from different explants of *M. arvensis*

Different concentrations of auxins and cytokinins were used alone or in combinations to investigate the initiation of shoot and its subsequent regeneration. Encapsulated shoot tips and encapsulated nodal explants were cultured for shoot regeneration. Encapsulated artificial seeds were cultured on to the MS agar gelled media supplemented with different concentrations of two cytokinins (BAP and Kn) and one auxins (NAA). It was observed that IBA, 2,4-D alone or in combinations with cytokinin failed to initiate any shoot. Therefore, IBA and 2,4-D were not used in these

experiments. Data on days of germination, percentage of germination, average number of shoot per culture and average length of shoot per culture were collected after 6 weeks of culture. The results are presented according to types of explants used under separate heads:

4.3.3. Encapsulated shoot tip explants

Experiment 1. Effect of different concentrations and combinations of plant growth regulators on artificial seed proliferation from shoot tip explants of *M. arvensis*

In this present investigation four concentrations (0.5, 1.0, 2.0 and 3.0 mg/l) of BAP used alone and combinations of two concentrations (1.0 and 2.0 mg/l) of BAP with four concentration of Kn (0.2, 0.5, 1.0 and 2.0 mg/l) or with four concentrations of NAA (0.1, 0.2, 0.5 and 1.0 mg/l) were treated in MS medium for the purpose of multiple shoot induction from encapsulated shoot tip explants of *M. arvensis*. Data were taken after 6 weeks of inoculation and days of germination, percentage of germination, average number of shoot/culture and average length of shoot/culture were measured. The results are presented in Table 4.1. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Shoot germination started within 6-12 days. Shoot germination ranged from 20.00-65.00%. From artificial seed highest percentage (65.00%) of multiple shoot formation was observed in MS medium containing 2.0 mg/l BAP + 0.5 mg/l Kn and 1.0 mg/l BAP + 0.2 mg/l NAA. The lowest percentage (20.00%) of multiple shoot formation was observed in media having 0.5 mg/l BAP. Highest mean number of shoots was 6.20 ± 0.23 in media having 1.0 mg/l BAP + 0.5 mg/l NAA followed by 4.60 ± 0.11 in 1.0 mg/l BAP + 1.0 mg/l Kn. The lowest mean number of shoot was 1.33 ± 0.06 in media containing 0.5 mg/l BAP. Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 42 days of culture. Highest average length was recorded 6.43 ± 0.08 cm in 1.0 mg/l BAP + 0.2mg/l NAA and the lowest average length was 3.73 ± 0.17 cm in 0.5 mg/l BAP. Experimental results revealed that, 1.0 mg/l of BAP alone and combination of 1.0 mg/l BAP + 0.5 mg/l Kn, 1.0 mg/l BAP + 0.2 mg/l NAA and 1.0 mg/l BAP + 0.5 mg/l NAA were found most effective concentrations for multiple shoot induction.

4.3.4. Encapsulated nodal explants

Experiment 1. Effect of different concentrations and combinations of plant growth regulators on artificial seed proliferation in encapsulated nodal explants of *M. arvensis*

Four concentrations (0.5, 1.0, 2.0 and 3.0 mg/l) of BAP used alone and combinations of two concentrations (1.0 and 2.0 mg/l) of BAP with four concentrations (0.2, 0.5, 1.0 and 2.0 mg/l) of Kn or with four concentrations of NAA (0.1, 0.2, 0.5 and 1.0 mg/l) were treated in MS medium for the purpose of multiple shoot induction from encapsulated shoot tip explants of *M. arvensis*. Data were taken after 6 weeks of inoculation and days of germination, percentage of germination, average number of shoot/culture and average length of shoot/culture were measured. The results are presented in Table 4.2. Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Shoot germination started within 6-12 days. Shoot germination ranged from 25.00-80.00%. From artificial seeds highest percentage (80.00%) of multiple shoot formation was observed in MS medium containing 2.0 mg/l BAP + 0.2 mg/l NAA (plate 4.1) followed by 75% in MS medium containing 1.0 mg/l BAP + 0.5 mg/l NAA. The lowest percentage (25.00%) of multiple shoot formation was observed in media having 3.0 mg/l BAP. Highest mean number of shoots was 9.87 ± 0.58 in media having 2.0 mg/l BAP + 0.2 mg/l NAA followed by 7.87 ± 0.33 in 1.0 mg/l BAP + 0.2 mg/l NAA and lowest mean number of shoot was 2.13 ± 0.17 in media containing 1.0 mg/l BAP + 2.0 mg/l Kn. Average length of shoots gradually increased after induction of shoot. Length of shoots was recorded at 42 days of culture. Highest average length was recorded 6.27 ± 0.29 cm in 1.0 mg/l BAP + 0.5 mg/l NAA and the lowest average length was 4.13 ± 0.17 cm in 0.5 mg/l BAP. Experimental results revealed that, 1.0 mg/l of BAP alone and combination of 2.0 mg/l BAP + 0.2 mg/l NAA, 1.0 mg/l BAP + 0.5 mg/l NAA and 1.0 mg/l BAP + 0.2 mg/l NAA were found most effective concentrations for multiple shoot induction in *M. arvensis*.

Table 4.1 Effect of different concentrations and combinations of plant growth regulators on artificial seed proliferation in shoot tip explants of *M. arvensis*. Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	Days of germination	Percentage (%) of germination	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP				
0.5	8-12	20	1.33 ± 0.06	3.73 ± 0.17
1.0	8-12	50	2.20 ± 0.20	4.27 ± 0.17
2.0	8-12	40	4.20 ± 0.30	4.93 ± 0.29
3.0	8-12	30	2.60 ± 0.23	4.47 ± 0.24
BAP + Kn				
1.0 + 0.2	7-10	45	2.60 ± 0.23	5.40 ± 0.11
1.0 + 0.5	7-10	60	2.93 ± 0.06	5.80 ± 0.23
1.0 + 1.0	7-10	52	4.60 ± 0.11	5.53 ± 0.24
1.0 + 2.0	7-10	46	3.93 ± 0.13	5.20 ± 0.11
2.0 + 0.2	7-10	44	2.80 ± 0.11	5.13 ± 0.06
2.0 + 0.5	7-10	65	4.33 ± 0.17	6.13 ± 0.29
2.0 + 1.0	7-10	54	3.60 ± 0.23	5.33 ± 0.06
2.0 + 2.0	7-10	40	2.87 ± 0.17	4.73 ± 0.17
BAP + NAA				
1.0 + 0.1	6-8	50	2.60 ± 0.11	5.33 ± 0.06
1.0 + 0.2	6-8	65	4.27 ± 0.29	6.43 ± 0.08
1.0 + 0.5	6-8	55	6.20 ± 0.23	6.20 ± 0.11
1.0 + 1.0	6-8	45	4.53 ± 0.17	5.67 ± 0.17
2.0 + 0.1	6-8	52	2.87 ± 0.17	4.00 ± 0.11
2.0 + 0.2	6-8	60	4.20 ± 0.11	5.33 ± 0.13
2.0 + 0.5	6-8	55	4.53 ± 0.06	4.53 ± 0.17
2.0 + 1.0	6-8	50	2.67 ± 0.17	4.40 ± 0.11

* Values are the mean of three replicates with 5 explants.

Table 4.2 Effect of different concentrations and combinations of plant growth regulators on artificial seed proliferation in nodal explants of *M. arvensis*. Data were recorded after 6 weeks of culture.

Plant growth regulators (mg/l)	Days of germination	Percentage (%) of germination	*Average number of shoot per culture (mean ± SE)	*Average length (cm) of shoot per culture (mean ± SE)
BAP				
0.5	7-10	42	2.33 ± 0.06	4.13 ± 0.17
1.0	7-10	64	5.67 ± 0.29	4.40 ± 0.11
2.0	7-10	56	4.20 ± 0.11	5.07 ± 0.17
3.0	7-10	25	2.33 ± 0.06	4.60 ± 0.11
BAP + Kn				
1.0 + 0.2	7-10	50	4.00 ± 0.11	5.40 ± 0.11
1.0 + 0.5	7-10	65	5.87 ± 0.17	5.67 ± 0.06
1.0 + 1.0	7-10	45	3.40 ± 0.11	5.73 ± 0.06
1.0 + 2.0	7-10	30	2.13 ± 0.17	4.80 ± 0.11
2.0 + 0.2	7-10	55	4.47 ± 0.17	4.93 ± 0.17
2.0 + 0.5	7-10	72	6.47 ± 0.13	6.07 ± 0.17
2.0 + 1.0	7-10	52	4.40 ± 0.11	5.53 ± 0.24
2.0 + 2.0	7-10	36	3.60 ± 0.11	4.60 ± 0.11
BAP + NAA				
1.0 + 0.1	5-8	50	6.00 ± 0.11	5.40 ± 0.11
1.0 + 0.2	5-8	72	7.87 ± 0.33	5.93 ± 0.06
1.0 + 0.5	5-8	75	7.60 ± 0.30	6.27 ± 0.29
1.0 + 1.0	5-8	62	4.47 ± 0.06	5.20 ± 0.30
2.0 + 0.1	5-8	54	4.33 ± 0.29	4.27 ± 0.17
2.0 + 0.2	5-8	80	9.87 ± 0.58	5.27 ± 0.24
2.0 + 0.5	5-8	66	5.47 ± 0.17	4.87 ± 0.17
2.0 + 1.0	5-8	55	3.53 ± 0.17	4.33 ± 0.17

* Values are the mean of three replicates with 5 explants.

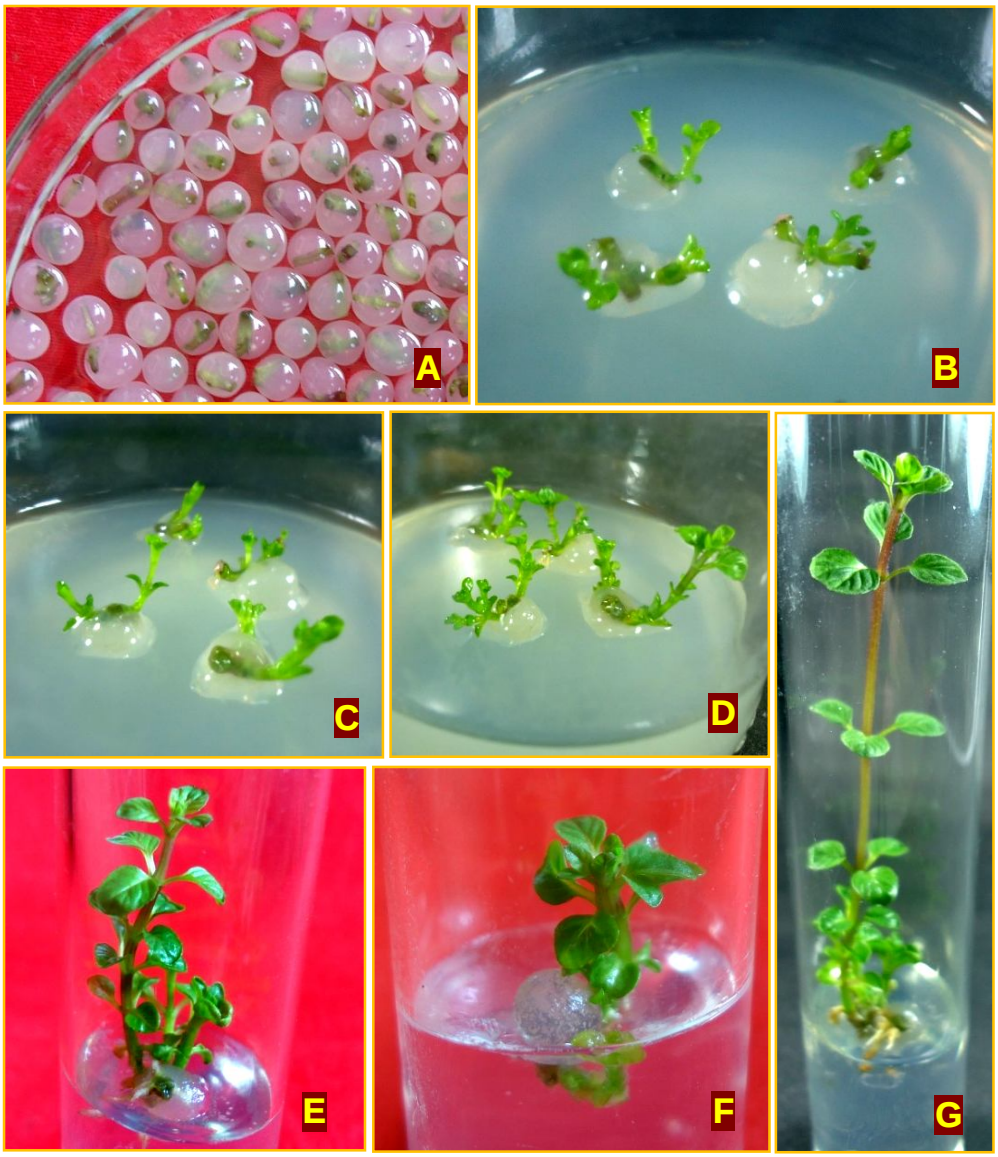

Plate 4.2 Artificial seeds and different stages of germination.

A: Artificial seeds encapsulated by sodium alginate; **B:** Germinated artificial seeds (nodal explants) in 2.0 mg/l BAP + 0.2 mg/l NAA; **C-E:** Artificial seed derived plant of nodal explants in same medium after 2-4 weeks of culture; F**:** Artificial seed derived plant of shoot tip explants in 2.0 mg/l BAP + 0.2 mg/l NAA after 4 weeks of culture; **G:** Root induction from germinated artificial seeds.

4.3.5. Survival test of artificial seeds in *M. arvensis* under different storage temperatures.

In the present investigation, 45 artificial seeds were kept in storage under growth chamber at 20 ± 2°C, 50 were kept in refrigerator at 4 ± 1°C and 50 were kept in refrigerator at 0°C for survival test. Both shoot tip and nodal encapsulated artificial seeds were used for this survival test. At the end of each storage period, artificial seeds were immediately transferred to fresh germination medium and placed for the recovery of plantlets. After storage period the artificial seeds were regrown under *in vitro* conditions on nutrient media for shoot development and root induction.

The survival percentage of encapsulated shoot tips and nodal explants decreased significantly with increased storage periods and temperature. However, the reduction in viability was recorded more at 20 ± 2° C in contrast to storage at 4 ± 1° C and O° C. Under the storage of 50 artificial seeds, the mean number of encapsulated shoot tip and nodal explants that survived after 15 days at 20 ± 2°C storage temperature was 25-45 percent, whereas the survival percentage of encapsulated explants at the storage of 4 ± 1° C was 70-73 percent. After 30 days of storage at 20 ± 2° C, 0-20 percent was found while it was 62-64 percent at 4 ± 1° C. After 45 days of storage at 20 ± 2°C, no survived encapsulated explants was found, while it was 54-55% at 4 ± 1° C. But after 60 days of storage at 20 ± 2°C, no survived encapsulated explants was found, while it was 44-46% at 4 ± 1°C. On the other hand, storage at 0º C temperature no survived encapsulated explants was found. After 60 days and above no survived encapsulated explants were found at 20 ± 2° C. On the other hand, survivability found extended around 44-46 % under storage temperature 4 ± 1°C (Table 4.3).

Table 4.3 Effect of storage on the viability of artificial seed (For each treatment 15 explants were used).

Sl. No.	Storage period (days)	Survival percentage (shoot tip explants)			Survival percentage (nodal explants)		
		Storage temperature			Storage temperature		
		Storage at 20 ± 2° C	Storage at 4± 1° C	Storage at 0° C	Storage at 20 ± 2°C	Storage at 4± 1° C	Storage at 0° C
1	07	52	80	-	60	80	-
2	15	25	73	-	45	70	-
3	30	-	62	-	20	64	-
4	45	-	54	-	-	55	-
5	60	-	46	-	-	44	-
6	90	-	-	-	-	-	-

4.4. DISCUSSION

Plant propagation using artificial or synthetic seeds developed from somatic tissue opens up new vistas in agriculture. Artificial seeds make a promising technique for propagation of transgenic plants, non seed producing plants, polyploids with elite traits and plant lines with problems in seed propagation. Encapsulation can be considered an important application of micropropagation, to improve the success of *in vitro* culture to synthetic seed technology. Studies on somatic embryogenesis and the related encapsulation of somatic embryos have recently been reported by Redenbaugh *et al.* (1993). Less attention has been given to the possibility of encapsulating non-embryogenic *in vitro* derived vegetative propagules. Nonetheless, some authors have tried to encapsulate shoot tips or axillary buds of different species, with promising results (Ganapathi *et al*. 1992; Bapat *et al*. 1993). This kind of capsule could be useful in exchanges of sterile material between laboratories, due to the small size and relative ease in handling the structures, or in germplasm conservation, with proper preservation techniques (Fabre and Dereuddre, 1990), or even in plant propagation and nurseries, if the development of the plant could be properly directed towards proliferation, rooting, elongation etc. (Bapat, 1993; Mathur *et al*. 1989). Synthetic seeds will be useful for propagation of valuable hand polinated hybrids, elite germplasm and genetically engineered plants, particularly those with sterile unstable genotypes.

In some medicinal plant species seed propagation has not been successful. This is mainly due to heterozygosity of seed, minute seed size, presence of reduced endosperm and the requirement of seed with mycorrhizal fungi association for germination (eg. Orchids) and also in some seedless varieties of crop plants. Some of these species can be propagated by vegetative means. However, *in vivo* vegetative propagation techniques are time consuming and expensive and the propagules carry the diseases and pest from the mother plant to the seedlings. Development of artificial seed producing technology is currently considered as an effective and efficient alternate method of propagation in several commercially important agronomic and horticultural crops. It has been suggested as a powerful tool for mass propagation of elite plant species with high commercial value.

A number of encapsulating agents have been tried out of which agar, agarose, alginate, carragenan, gelrite and polyacrylamide are important (Kitto *and* Janick.

1985). However, it has been suggested that most suitable encapsulating agent is sodium alginate (Bapat *et, al*. 1987) due to its solubility at room temperature and its ability to form completely permeable gel with calcium chloride ($CaCl_2 + 2H_2O$). Our findings have revealed that this method provides an efficient mechanism for encapsulating the shoot tip and nodal segment in *Mentha arvensis*. Alginate is one of the most commonly used polymers for immobilization of plant cells and production of manufactured seeds because it is available in large quantities, is inert, non-toxic, cheap and easily handled (Endress, 1994). However, studies on *in vitro* germplasm conservation using alginate encapsulation techniques have been reported for only a few species. Embryogenic tissue of *Samtalum album* (Bapat and Rao, 1988) and axillary buds of *Morus indica* (Bapat *et al.* 1987) have been encapsulated in alginate beads.

In the present investigation *in vitro* shoot tip and nodal segments of *Mentha arvensis* were encapsulated in sodium alginate prepared using MS basal medium. Different concentrations and combinations of BAP, Kin and NAA were used in alginate bead. Four different concentrations (0.5,1.0, 2.0 and 3.0 mg/l) of BAP used alone and in combinations of two different concentrations (1.0 and 2.0 mg/l) of BAP with four concentrations of Kn (0.2, 0.5, 1.0 and 2.0 mg/l) or four concentration of NAA (0.1, 0.2, 0.5 and 1.0 mg/l) were used in artificial seed beads. These encapsulated synthetic seeds were cultured on MS medium containing same growth regulators. Among the different concentrations of phytohormone, highest 80% of shoot formation was observed in MS medium containing 2.0 mg/l BAP + 0.2 mg/l NAA from nodal segments of *M. arvensis*. Highest average number of shoot 9.87 ± 0.58 formation was observed in the same medium and average highest length of shoot 6.27 ± 0.29 cm was found in the media having 1.0 mg/l BAP + 0.5 mg/l NAA. Same phytohormone was also best in seed bead of Mulberry (Machii, 1992).

The influence of storage at 20 ± 2°C, 4ºC and 0ºC temperature on germination rate was also examined. This investigation indicates that synthetic seed could be stored 20 ±2º C for 15 days and the germination rate was 25% and after 15 days at 20±2º C no response of the artificial seeds. And at 4ºC for 60 days the germination rate was 45% after 60 days synthetic seed did not give any response being stored at 4 ºC. Ipekci and Gozukirmizi (2003) also observed that the encapsulated embryos of *Paulownia elongate* was survived when the synthetic seeds were stored at 4ºC for 60 days and the

germination rate was 32.40%. This type of result was also supported the result of Alfalfa seeds (Redenbaugh *et al*., 1987), *Asparagus cooperi* (Ghosh and Sen, 1994), *Eucalyptus citrisdora* (Muralidharan and Mascarenhas, 1995), *Camellia* (Janeiro *et al*. 1995), Mulberry (Machii, 1992 and Bapat *et al*. 1987). But no result found at 0°C temperature.

4.5. SUMMARY

For conservation of important medicinal plants attempt was taken to produce artificial seed by encapsulating the shoot tips and nodal segments of a important medicinal plants *Mentha arvensis.* For germination of artificial seeds, encapsulated shoot tips (synthetic seed) and encapsulated nodal segment (synthetic seed) were cultured in MS basal media containing different concentrations and combinations of BAP, Kn and NAA to induce shoot proliferation. In *M. arvensis* the highest 80% of shoot formation was observed in MS medium containing 2.0 mg/l BAP + 0.2 mg/l NAA from nodal segments which was followed by 75% in MS medium containing 1.0 mg/l + 0.5 mg/l NAA. The lowest percentage (25%) of shoot formation was observed in media having 0.5 mg/l BAP. On the other hand highest percentage (65%) of shoot proliferation was observed from shoot tips in MS medium containing 2.0 mg/l BAP + 0.5 mg/l Kn and 1.0 mg/l BAP + 0.2 mg/l NAA followed by (60%) in MS medium containing 1.0 mg/l BAP + 0.5 mg/l Kn and 2.0 mg/l BAP + 0.2 mg/l NAA. The lowest percentage of (20%) of shoot formation was observed in media having 0.5 mg/l BAP. The experiment results indicated that cytokinins play an important role in germination of artificial seeds, being encapsulated by sodium alginate.

Another experiment was laid out on the survival (viability) test of encapsulated shoot tip and nodal explants of *M. arvensis* under different storage temperature regime. Under this experiment three storage temperatures were maintained for storing the artificial seed *viz.* 20 ± 2° C, 4 ± 1° C and 0º C for different periods. Highest survival percentage was noted after 60 days of storage at 4±1° C, when 44-46% of survivality was observed in encapsulated explants. But after 60 days of storage at 20 ± 2° and 0º C no survival encapsulated explants was found.

CHAPTER V

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Asparagus racemosus
Asparagus racemosus


Curcuma amada
Curcuma amada

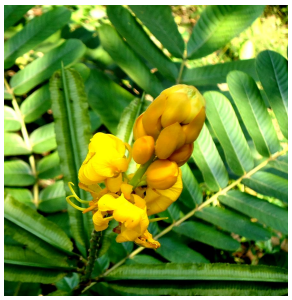

Ipomoea mauritiana
Ipomoea mauritiana


Mentha arvensis
Mentha arvensis


Paederia foetida
Paederia foetida


Rauwolfia serpentina
Rauwolfia serpentina


Smilax zeylanica
Smilax zeylanica



CHAPTER I

GENERAL INTRODUCTION

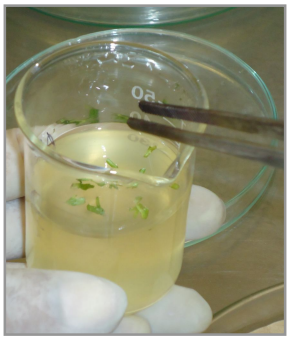
GENERAL INTRODUCTION

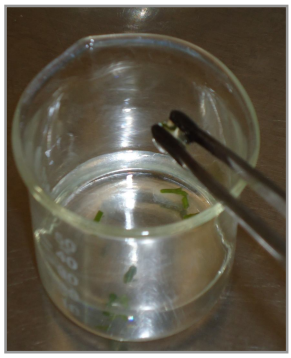
CHAPTER II

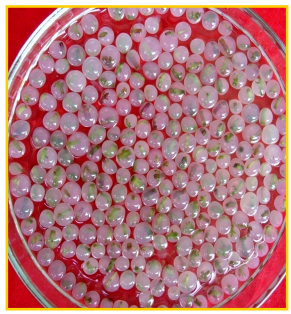
TRADITIONAL KNOWLEDGE

CHAPTER III

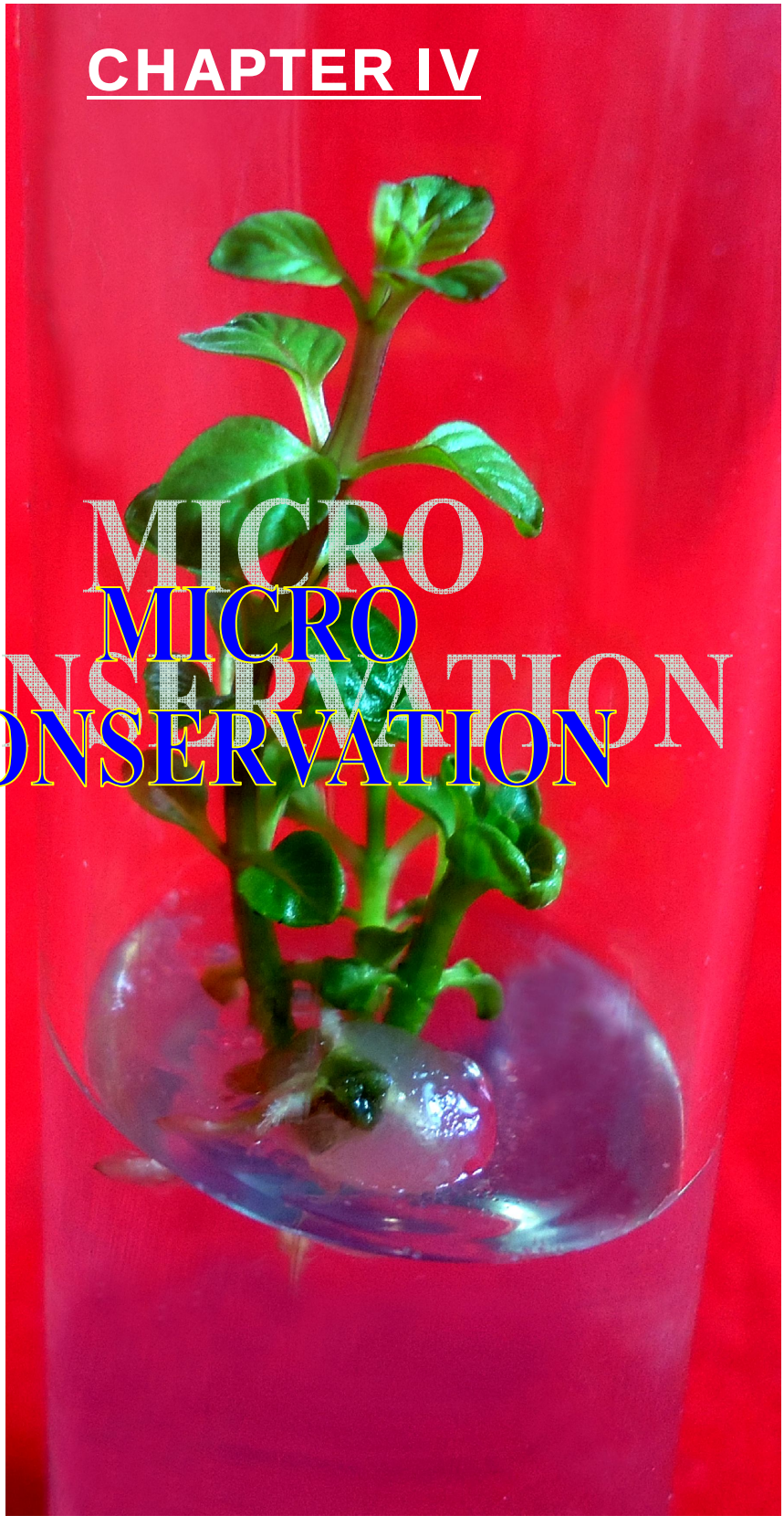
MICRO
PROPAGATION





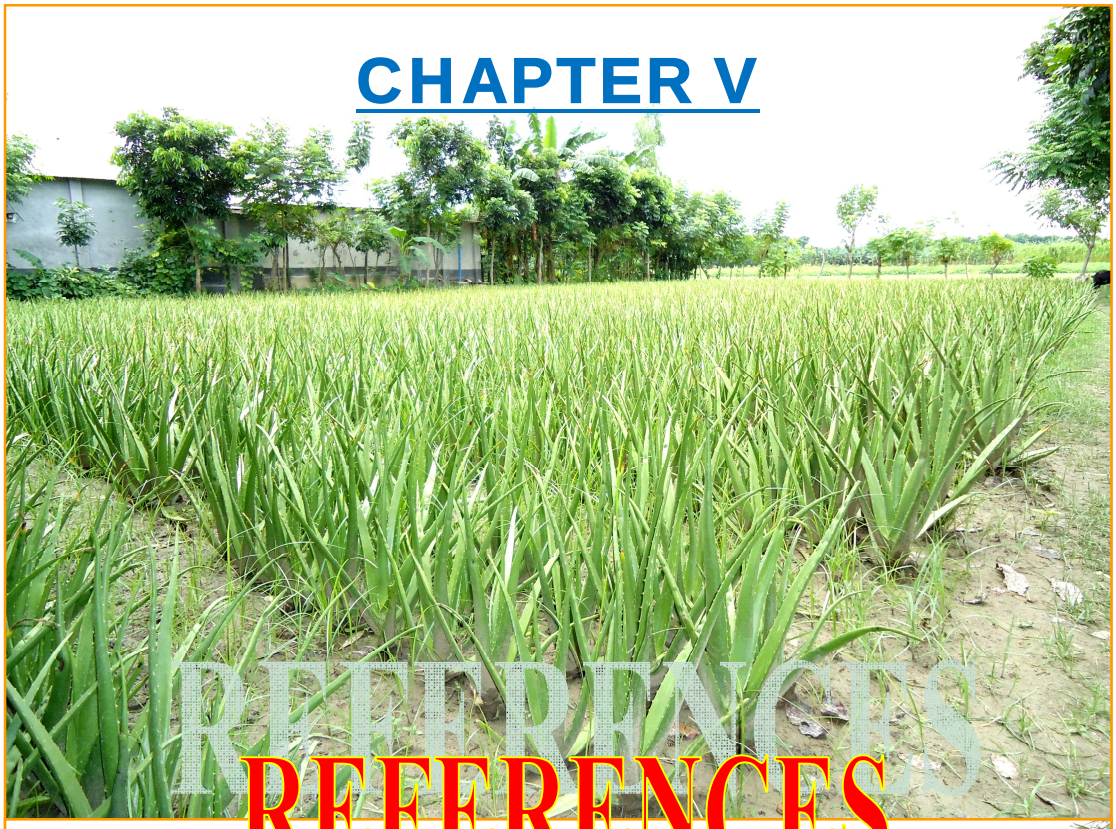


CHAPTER IV



MICRO CONSERVATION

CHAPTER V



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