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Implementation of Advanced Cultivation Technique- of Water Chestnut in Bangladesh

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IMPLEMENTATION OF ADVANCED CULTIVATION TECHNIQUE OF WATER CHESTNUT IN BANGLADESH

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BY

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DEDICATED
TO
MY BELOVED PARENTS
AND
SUPERVISOR

DECLARATION

I do hereby declare that, the whole research work submitted as a Ph.D thesis entitled **"Implementation of Advanced Cultivation Technique of Water Chestnut in Bangladesh"** in the department of Agronomy and Agricultural Extension, University of Rajshahi, Rajshahi- 6205, Bangladesh for the degree of **DOCTOR OF PHILOSOPHY** in Agronomy and Agricultural Extension, University of Rajshahi, Rajshahi- 6205, Bangladesh is the result of my own investigation and was carried out under the supervision of **Dr. M. Aminul Hoque**, Professor, department of Agronomy and Agricultural Extension, University of Rajshahi, Rajshahi- 6205, Bangladesh. The thesis has not already been submitted in the substance for any degree or qualification of this or any other university or other institutions of learning.

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CERTIFICATE

I do hereby certify that **Md. Anowar Razvy** has been working on his thesis entitled **"Implementation of Advanced Cultivation Technique of Water chestnut in Bangladesh"** under my supervision, which is the record of bonafide research carried out at the department of Agronomy and Agricultural Extension and Plant Breeding & Gene Engineering Laboratory, Dept. of Botany, University of Rajshahi, Rajshahi- 6205, Bangladesh. The results of the investigation, which are embodied are original and have not been submitted before in substance for any degrees of this University. He has fulfilled all the requirements of regulations relating to the nature and prescribed period of research for submission of thesis for the partial fulfillment of Doctor of Philosophy (Ph.D) degree in the department of Agronomy and Agricultural Extension, University of Rajshahi, Rajshahi- 6205, Bangladesh.

Research Supervisor

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May Allah give me courage to study further research work.

The Author

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CONTAINS

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ABSTRACT

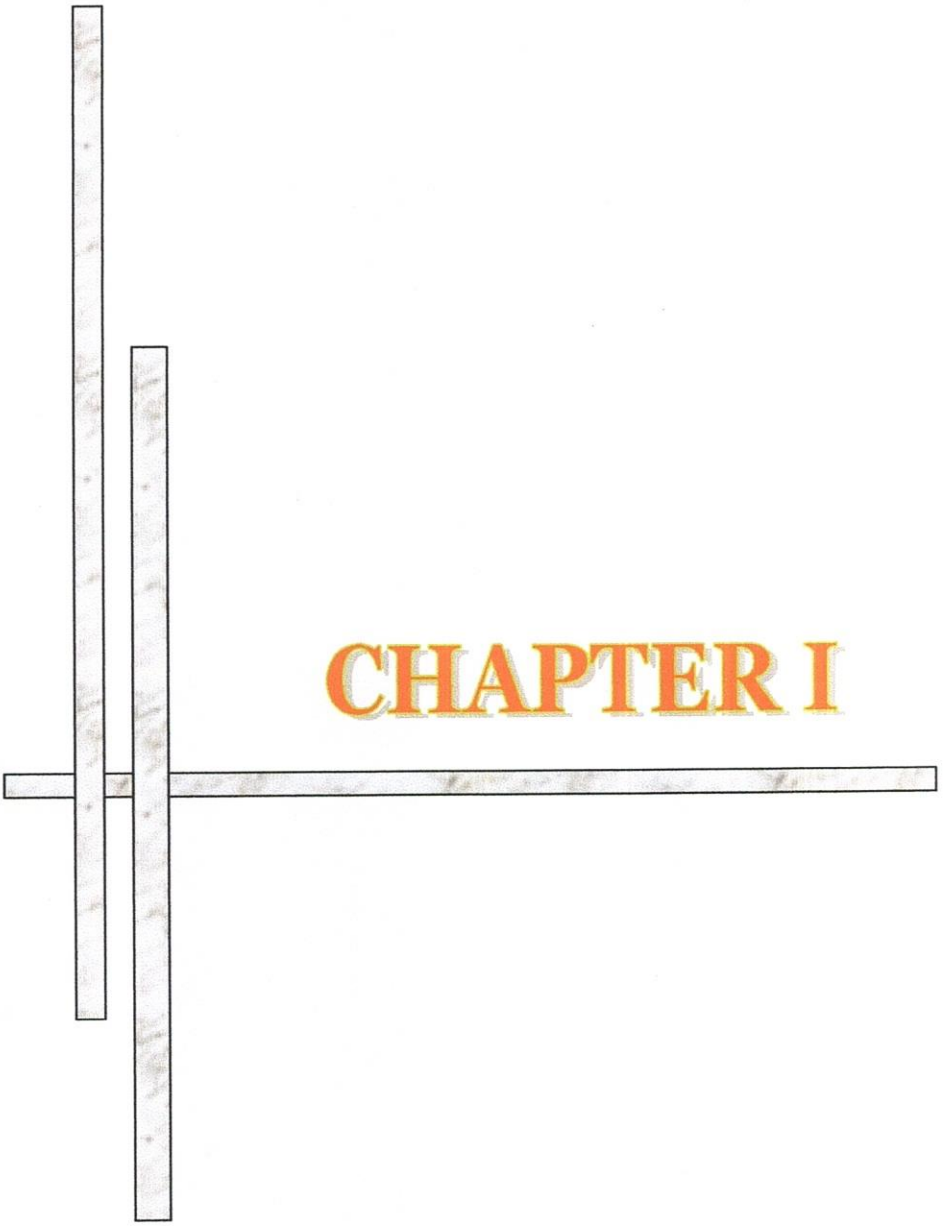
The water chestnut is an annual aquatic minor fruits and available grown in every where in watery bog place in Bangladesh. Water chestnut has been considered as a minor crop because of its low yield performance and lack of optimum physiological data and advanced cultivation techniques. There is little information on procedures to improve its cultivation and performance and little effort has been directed to the development of cultivation methods and productions. However, growers now demand information on any improved varieties and their production technology. In order to increase the biological performance and production of water chestnut in paddy fields and mass cultivation in field condition, the first requirement is its mass propagation, seedling quality improvement, possibly involving chemical/hormonal treatment optimization, breeding and micropropagation through axenic culture to multiply existing germplasms of water chestnut. To improve the varietals performance and modern cultivation techniques fruits chemical analysis and this relevant study, hormonal treatment and micropropagation were carried out in different three varieties of water chestnut. Among three varieties red water chestnut produce large sized fruits with rich food nutrients but green water chest optimized as effective variety in different parameters as much number fruit production with average amount of food nutrients, produced much number of lateral branch from coteyledonary shoot, much flower initiation and more sensitive to *in vitro* micropropagation, overall it forms excellent vegetative canopy and over fruits production in controlled cultivation field. Green water chestnut rich in protein, lipid, sugar, starch, phenol, fiber, minerals, amino acids and vitamins than the red water chestnut but wild water chestnut shows more antibacterial and antifungal activities than both green and red varieties. Green and red water chestnuts are suitable for commercial cultivation while wild water chestnut required to varietals improvement for further commercial cultivation. Besides in plantlets multiplication and micropropagation green water chestnut showed better performance to callus induction at 1+1mh/l BA+GA_3, best shoot initiation (at BA), shoot elongation, root formation (best at NAA+GA_3), To multiply much branches, enhance shoot initiation and elongation, in physically hormonal treatment in *in vitro* condition, best shoot proliferated hormone found at 1-5ml/l BA, NAA shoot length proliferation performance found at 1mg/l. These findings could have a considerable socio-economic impact to optimization physical, chemical and micropropagation has become an important aspect for commercial nursery development and commercial cultivation of water chestnut, and this relevant information needs to be disseminated to more effectively multiply, commercial cultivation on a much larger scale in Bangladesh.

ABBREVIATIONS

The following abbreviations have been used throughout the text:

Sign	Abbreviation
%	Percentage
°C	Degree Celsius.
0.1N	0.1 Normal solution
½ MS	Half strength of Murashige and Skoog (1962) medium
BA	6-benzyl adenine.
BAP	6-benzyl amino purine
cm	Centimeter (s)
e.g.	Example gratia = For example
EDTA	Ethylene diamine tetra acetate
et al.	et alile = Other people
etc.	Etcetera = And others
Fig.	Figure (s)
g	Gram (s)
g/l	Gram per liter
GA_3	Gibberellic acid grade 3
h	Hour
HCI	Hydrochloric acid
$HgCl_2$	Murcuric chloride
IBA	Indol-3-butyric acid
kg	Kilogram (s)
KIN	6-furfuryl amino purine (Kinetin)
KOH	Potassium hydroxide
L.	Linneaus (Carolus Linneaus)
lb	Pound (s)
mg	Milligram
mg/l	Milligram per liter.
min	Minute
ml	Milliliter
MS	Murashige and Skoog (1962) medium
MS0	Growth regulator free MS medium
Na_2-EDTA	Sodium salt of ferric enthylene diamine tetra acetate
NAA	α-napthalene acetic acid
NaOH	Sodium hydroxide
No.	Number(s)
NS	Nodal segment
PGR	Plant growth regulator
pH	Negative logarithm of hydrogen ion (H^+) concentration
RT	Runner tip
sp./spp.	Species

var.	Variety (s)
viz.	Namely
rpm	Revolutions per minute
vit	Vitamin(s)
nm	
N	Normal (Normality)
H_2So_4	Sulfuric acid
HNO_3	Nitric acid
cu	Cupper
ca	Calcium
Fe	Iron
µ	Micron
ß	Beta



CHAPTER I

GENERAL INTRODUCTION

A General Account

Water chestnut is an annual aquatic angiospermic plant in which the flower appears above the level of water but starchy spined fruits development occurs inside water and creates four-horned, barbed, nut-like structure. The water chestnut plant usually grown shallow fresh water basin in the tropical subtropical and temperate zones of the world and abundant in India, Indonesia, South-east Asia, Southern part of China, in the eutrophic waters in Japan, Italy and tropical America (Hoque *et. al.*, 2000). In many countries in the torrid and temperate zones, people eat the fruit as raw, boiled or roasted and the stem or leaves as vegetables. The starchy fruit contains 67.5% starch (Porter field, 1928), 5% protein (Subrahmenyan *et. al.*, 1954) and significant amount of calcium, phosphorous, iron, thiamin and ascorbic acid (Khan and Chugtai, 1955; Bargale *et. al.,* 1987).

Trapa natans Linn var. *bispinosa* Makino is native of China. The name of the nut is 'Ling Ko', meaning 'spiritual horn' Ling Ko is found all over China. In Bangladesh many previously water chestnut grown in shallow areas of freshwater lakes, ponds and bog places. The fruit is a minor but very popular for its crispy consumptions. Now the few quantities of water chestnut began to cultivate in Bangladesh. It is also a very useful plant because shallow fresh water, bog and watery paddy field can be used for fruits production. Some times it occur nuisance because mature *Trapa* nuts drift to shore where their sharp spines may hurt human or animal feet (Haber, 1999). It produce large canopy thus reduces oxygen levels, which may increase the potential for fish kills or oxygen shortage in water.

History

Water chestnut in North America was first observed near Concord, Massachusetts in 1859 (Worobel, 1996). The exact path or reason for the

introduction is a mystery. Harvard botanist Asa Gray cultured the organism in his botanical garden in 1877. Its escape to local waters occurred by 1879 (Worobel, 1996) and populations were documented in New York by the late 1800s. Further spread occurred through water-ways and into Vermont and Massachusetts.

The first population of water chestnut in Maryland was documented in 1923 in a two acres patch on the Potomac river outside of Washington D.C. Within a few years, the plant had spread over 40 river miles on the Potomac. The 10,000 acres coverage of water chestnut reaching past Quantico, Virginia prompted removal efforts by the Army corps of engineers in 1939 Water chestnut was found in the Bird river, Baltimore county in 1955 and subsequently in the Sassafras river, Kent County in 1964.

The most problematic populations currently occur in the Potomac and Hudson rivers and in Connecticut River valley, Lake Champlain region. In 1998, water chestnut was found in the South River in Quebec, which is connected to the Lake Champlain outlet via the Richelieu River. Its spread has continued because of the suitability of habitat; in 2001, for example, water chestnut was discovered in the Pike River, which flows into Misssissquoi Bay.

The dispersal of water chestnut by human hands to the United States (see USGS map) and other parts of the world can be attributed to its status as an ornamental plant having medicinal and nutritional value. In many parts of Asia, the fruit is a staple food source and used for livestock feed. The fruit has been used medicinally to treat elephantitus, pestilent fevers, rheumatism and skin complaints (Worobel, 1996).

Water chestnut has been declared a noxious weed in Arizona, Massachusetts, New Hampshire, North Carolina and South Carolina, and sale is prohibited through most Southern states (USDA).

In Europe, water chestnut populations have been dwindling in Belgium, Holland and Sweden; it has been listed as a strictly protected species by the Bern Convention in March 1998 (Council of Europe, convention on the conservation of European wildlife and natural habitats); in 1981 Germany issued stamps featuring four aquatic plants including water chestnut.

Background of *Trap* in Bangladesh

Water chestnut (*Trapa bispinsoa* Roxb. or *T. natans* Linn. var. *bispinosa* Makino) is a starch producing annual hydrophytic plant, belonging to the family Trapaceae and grown shallow fresh water basins. Fruits of water chestnut contain high quality starch that also contain significant amount of protein, calcium, phosphorous, iron, thiamine and ascorbic acid. Fruit is also consumed as raw, boiled or roasted and stem with leaves as vegetable. The fruits with two spined known as 'Paniphol' (*T. bispinosa* Roxb) and the four spined fruits known as 'Singhara' (*T. quadrispinosa* Roxb.) in Bangladesh. It is our native but minor fruits. Generally its other common name are Bull nut, Singhara nut, Jesuit nut, Water nut, Horn chestnut, Water caltrops, Buffalo head fruit etc. In our country available it naturally grows in fresh water marshy pond or flat bog place.

Description of the plant/Morphology

Morphologically water chestnut plant is divided into root, stem, leaf, flower, fruit etc. The primary root is entirely lacking. Lateral roots from the hypocotyls curve down and anchor the seedling. Lateral adventitious roots developed at the nodes of the main stem and its branches. They are green and are able to perform both assimilating and absorbing functions.

Floating leaves

The leaves develop in a radial pattern on the surface of the water, with swollen, air-filled petioles that keep the upper part of the plant afloat. Upper leaves are diamond-shaped with toothed edges and are shiny on the upper side and dull

with fine hairs underneath. They are alternately arranged on inflated, spongy stalks and occur in clusters up to 50cm across. Opposite submersed leaves are long and narrow. Green feather-like structures often replace the linear underwater leaves.

Stem

The stems of water chestnut are stoloniferous and branched with long internodes, the latter being longer towards the base of the plant. The leaves are simple, petiolate, stipulate, crowded at the tips of the branches, rhomboidal in shape with an entire basal margin and a serrate apical margin. The petioles are longer on the leaves towards the base of the plant and gradually decrease on the uppermost leaves. The latter are villous beneath with spongy dilations near the apex. Their upper surfaces are glabrous and dark green and red in color; the lower surfaces are woolly and light green or reddish-purple. Leaves at the seedling stage are strap-shaped with a serrated margin, the normal, adult leaves developing later.

Roots

Root develops on shoots. Lower roots are unbranched and thread-like, while upper roots are sparsely branched and fibrous. Some botanists consider the feather shaped underwater structures to be roots instead of leaves. Lateral roots grow down from the hypocotyl to anchor the young seedlings. The adventitious roots that develop at the nodes of the main stem and its branches contain photosynthetic pigments, particularly chlorophyll, and perform both photosynthesis and the absorption of nutrients.

Inflorescence

The inflorescence of water chestnut is solitary and axillary, the pedicels being generally shorter than the petioles. The flowers are perigynous, with a disc above the stamens, bisexual and actinomorphic. The calyx is gamosepalous, four-lobed and pubescent; two lobes persist and become spines of the fruit. The corolla is polypetalous, about twice as long as the calyx-tube, crenulated on the margin, pure white or slightly purple in color. The androecium consists of four, two-celled, dorsifixed anthers that dehisce longitudinally. The gynaecium is half-inferior, pendulous and two-celled, with a solitary ovule in each cell. After pollination, the flowers submerge beneath the water surface to facilitate fruit formation. The floral biology of water chestnut has been reviewed by Kadono and Schneider, 1986; Arima *et. al.*, 1999.

Fruit

The fruit of water chestnut is a one-seeded, triangular-shaped drupe, the fleshy layer of which soon disappears, leaving the hard endocarp. The latter bears upwardly projecting thorns or is thorn less. The seeds are ex-endospermic and ripen under water, and become detached when ripe. Morphologically the fruit is a dry nut of with rich nutritional (Pandit and Quadmi, 1986) and medicinal (Korsuge *et. al.*, 1985) value.

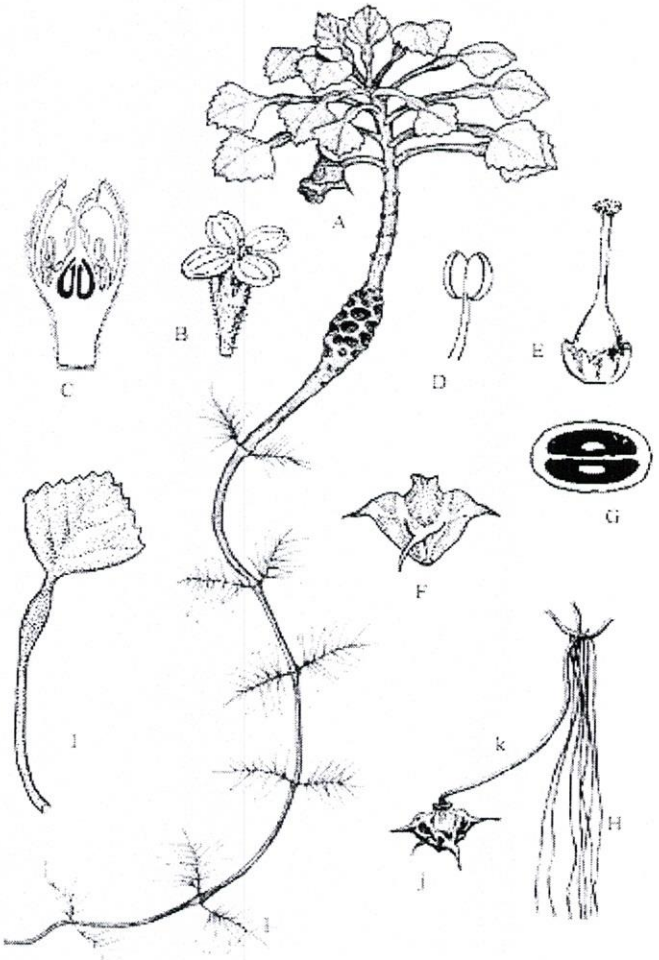


Figure. Diagram of water chestnut plants and its different parts
A. Main plant of water chestnut, B. Single flower, C. LS of flower, D. Anther, E. Ovary, F. Mature fruit, G. LS of ovary, H. Cotyledonary root, I. Single leaf, j. Germinated fruit, k. Cotyledonary inter-node, l. Fibrous roots (secondary roots)

Habitat

Water chestnut requires full sun, sluggish, nutrient-rich, fresh waters and soft substrate (Winne, 1950; Kiviat, 1993). Water chestnut grows in waters 0.3 to 3.6m deep but is most abundant in sheltered bodies of water about 2m deep with soft, muddy bottoms (Muenscher, 1937; Countryman, 1978; Bogucki *et al.*, 1980). Water chestnut rarely grows where the substrate is low in organic matter; in addition, swiftly flowing water and sharp fluctuations in water level can be detrimental to its survival (Winne, 1950; Vuorela and Aalto, 1982). Seeds germinated most quickly in a substrate with a pH of 7.9 to 8.3 and more slowly at a pH of 4.2 to 5.2; slowest germination occurred in sediments with a

pH of 5.3 to 7.8. After 2 weeks, however, seedlings grown in both alkaline and acidic substrates died, but those grown in moderately acidic to neutral substrates developed well (Apinis, 1937; Winne, 1950).

Water chestnut is restricted to fresh waters because its seeds fail to germinate when NaCl concentrations exceed 0.1% (Vuorela and Aalto, 1982) maximum salinity of about 7ppt where water chestnut is limited to an area apparently receiving fresh groundwater. The plant is also intolerant of $Ca(HCO_3)_2$ and $Mg(HCO_3)_2$ (Vuorela and Aalto, 1982).

Systematic position

This annual floating-leaved aquatic herb plants taxonomically it is placed in the family Onagraceae along with Lythraceae and Samydaceae by the Bentham and Hooders (1879). Engler and prantl's (1924) classification system, it is placed in a new family Hydrocaryaceae which includes only one genus *Trapa.* Hutchinson (1926) it is placed in the family Onagraceae along with Oliniaceae and Halorrhagaceal but most of taxonomic interest has arisen due to the unique dicot nature of this naonotypic genus (Maheshwari, 1950; Ram, 1956; Nakano, 1964). Now it belongs to the natural order Trapanaceae, family- Trapacea, genus *Trapa* (unigenic) with 30 species derived from the Onagraceae.

Now its taxonomic position is -

Kingdom	Plantae - Plants
Subkingdom	Tracheobionta - vascular plants
Superdivision	Spermatophyta - seed plants
Division	Magnoliophyta - flowering plants
Class	Magnoliopsida - dicotyledons
Subclass	Rosidae
Order	Myrtales
Family	Trapaceae - water chestnut family
Genus	*Trapa* L. - water chestnut
Species	*Trapa* sp.

Chromosome number

The chromosome number of *Trapa* generally diploid 2n = 48, 56 (Kumar and Subramaniam, 1987) but the four species *T. conocarpa*, *T. media, T. muzzarensis* and *T. natans* available have shown 2n = 48. (Trela-sawicka, 1965). *T. incisa* and *T. natans* var. *Japonica* were diploid (2n = 48) or tetraploid (4x = 2n = 48) and *T. Japonica, T. natans* var. *pumila* were tetroploid (4x = 2n = 96) or octaploid (8x = 2n = 96) reported that Oginuma *et al.*, (1996) in Japan. *Trapa natans* an intra specific polyploidy (or aneuploidy) occurs 2n = 36 (Palmygren, 1943), 2n = 40 and 2n = 48 (Trela-sawicka, 1965).

Distribution

Water chestnut is native to the tropical and warm temperature regions of Asia, Europe and Africa. It has also become naturalized in Australia and northeastern North America. These plants were apparently introduced into Switzerland around 3000 B.C and were first introduced into North America in 1874. Water chestnuts were cultured in 1877 in the botanical garden of Harvard University. By the late 1970s water chestnut was common in almost all states of northern India (Little, 1979; Kaul *et. al.*, 1976) noted that, water chestnut inhabited water bodies of the valley of Kashmir in the Himalayas where lakes, ponds, ditches, and streams. Water chestnut is now very rare in Europe where it is considered endangered or extirpated in many countries (Zvelebil, 1987; Cronk and Fennessy, 2001). However it is well distributed world wide in fresh water basin.

Alien range

Country wide *Trapa* sp. distributed China, Japan, Korea, Poland, Sweden, Norway, Portugal, Switzerland, Hungary, Holland, Belgium, Spain, France, Germany, Russia, United estates, Italy, Nepal, Myanmar, Pakistan, India, Bangladesh

Native range

It grows every where in Bangladesh from the ancient time in still water shallow basin, bill, bog pond, road side canal and marshy land and best grows at the district Sylhed, Moulovibajar, Habigonj, Sunamganj, Brahmanbaria, Kishorganj, Netrokona, Maymansing, Foridpur, Rajshahi, Naogaon, Natore, Bogra, Pabna, Sirajgong etc.

Reproduction

Water chestnut can multiply by sexual and commonly vegetative in process by axillary shoot formation. A fruit of annual water chestnut reproduces by over wintering seeds, single seeded woody fruits germination in the early spring. A single seed may give rise to 10-15 plant rosettes. Each rosette can produce up to 15-20 seeds. Ungerminated seeds may remain viable up to 12 years. However, most seeds probably germinate in the first two years. It can germination under a wide range of water depths and grows best at 0.5m. The maximum water depth should never be more than 1m though the plant can grows to a depth of around 3m. The plant requires full sunlight and the water level should be full by March - August. The pond water must have a high organic content and should be free of high concentration of salts. Neutral to some what alkaline pH are being best for proper growth of the plant.

Dispersal/Spread

The fruit of water chestnut may be dispersed when individual plants are uprooted and float down-stream. These plants can also be dispersed by fragmentation. Due to the large size and weight of the sinking seeds, it is unlikely that water-fowl or water currents can transport seeds to any great extent.

Life cycle biology

The insect-pollinated bisexual flowers stalks curve downward after fertilization and fruits develop underwater. Ripen Fruits fall to the floor of the water body,

over winter the seeds germinate in the late spring. Each seed can give rise to 10-15 rosettes and each rosette can produce as many as 20 seeds. Over winter it become germination and going towards maturation with long stem, flower and fruits.

Ecology of the plants

The plant prefer eutrophic fresh of low and flat water body, lake, canal or pond which are unsuitable for fish cultures are being utilized for farming of this fruit crops. It is best grows in shallow perennial water bodies, which hold abundant water throughout the year and light (sandy), medium (loamy), heavy (clay) soils with full sunny weather (Flora Europapea, 1964). It can grows water up surface to 60cm depth and can not grows in the shade.

Economic importance of water chestnut

A vast amount of water chestnut grows all over Bangladesh from the ancient times, but few people cultivate it as an agricultural crop fruit. Now, it is going to be popular for its rich nutritional food value (Pandit and Quadri, 1986) and over food demands. The nutritive value of the fruit is not less than that of wheat (Kusum and Chandra, 1980).

The starchy fruit contains up to 50% starch (Uphof, 1959), another report 16% starch, 3% protein (Rosengarten, 1984) and 15% protein, 7.5% fat (Komarov, 1968). with significant amount of calcium (Ca), phosphorous (P), iron (Fe), potassium (K), zinc (Zn), thiamine (vitamin- B_1) and ascorbic acid (vit- C) (Khan and Chugtai, 1955; Bargale *et. al.*, 1987). People eat the fruit as a raw or cooked (Hedrick, 1972; Simmons, 1972; Triska, 1975; Harrison *et. al.*, 1975; Muhlberg, 1982; Howes, 1948; Komarov, 1968; Polunin, 1969 of the native stocks.

It is sold both fresh and boiled in street markets in Bangladesh, China, and India. The immature pulp of the fruit, called milky water chestnut, is eaten raw or cooked, while the mature pulp is used to prepare dishes after boiling and

drying. Fresh and boiled water chestnuts are used not only as vegetables, but also in tea and in preparing curries. Some times its crispy nut used as stir-fries, salads combining with vegetable. The kernels are dried and sold as nuts, and are also ground into flour for bread. The nutritive value of the fruits is similar to that of wheat (Kusum and Chandra, 1980). Water chestnut is also used for preparing tea in Japan, in the commercial production of wine and for special food during festivals. A paste prepared by diluting the dough of water chestnut is an excellent diet for some patients. Its medicinal value is great (Kosuge *et. al.*, 1985). The fruits are also used in bilious attacks and diarrhea, and are sometimes applied externally as poultices (Kusum and Chandra, 1980). In Myanmar and India, the fruits of *T. natans* are made into rosaries that are sold in Italy (Ahmad and Singh, 1998).

Bangladesh is generally low lying, it is regularly affected by floods and, every year, vegetables and other crops are damaged, creating a serious impact on national food security. Water chestnut, being a floating aquatic plant, is little affected by over flooding and is potentially an important food crop in low-lying areas, especially as an emergency crop during floods. It is also a potential source of high quality starch for industrial use (Hoque *et. al.,* 2000).

Sometimes raw nut contains few amount of toxins that are destroyed by cooking the seed (Huxley, 1992; Lark, 1991) and some lakes treated as a weed because its vigorous propagation resulting forms eutrophication of the fresh water areas (Smith, 1955; Brezny *et. al.*, 1973; Methe *et. al.*, 1993; Groth *et. a.l*, 1996) as a result it reduces oxygen (O_2) levels of the water which may increase the potential for fish kills and water contamination.

Biological and ecological impacts

Due to its dense growth, the species impedes navigation and its low food value for wildlife potentially can have a substantial impact on the use of an area by waterfowl and other native species. Decomposition of the abundant detritus

produced in the fall of each year as the plants senesce, could contribute to lower oxygen levels in shallow waters and thus impact other aquatic organisms (http://www.paflora.org). Such low oxygen conditions could potentially cause fish kills (www.nps.gov). With four hard, half-inch spines that are sharp enough to penetrate shoe leather and large enough to keep people off beaches. Additionally, water chestnut threatens native bay grasses by forming a complete canopy with up to three layers of leaves, blocking all sunlight from reaching the sediment surface and preventing the growth of other, desirable aquatic plant species. Water chestnut prevents nearly all water use where it occurs, creates breeding grounds for mosquitoes and provides only marginal habitat for native fish and invertebrates. There are many readily available anecdotes highlighting problems from areas with water chestnut (e.g. boats or motorboats and sailing craft could not use and affected water areas formerly available during the summer months. Sanitary problems arose because of the fact that the thick beds collected and held quantities of organic waste, thus creating water pollution hazards, and swarms of mosquitoes bred prolifically among the plants.

Improvements of water chestnut

Until now, water chestnut has been considered a minor crop because of its low yield performance and lack of optimum physiological data and advanced cultivation techniques. There is little information on procedures to improve its cultivation and performance (Arima *et. al.*, 1999; Lalit *et. al.*, 2007). Interestingly, little effort has been directed at seedling establishment, the latter being fundamental to longer-term performance, since the latter depends on optimal growing conditions at the seedling stage. However, growers now demand information on any improved varieties and their production technology. In order to increase the biological performance and production of water chestnut in paddy fields and mass cultivation, the first requirement is its mass propagation, seedling quality improvement, possibly involving chemical /hormonal treatment, breeding and micropropagation through axenic culture to multiply existing germplasms. The most significant advantages offered by axenic procedures of clonal propagation compared to conventional methods is that many plants can be produced from a single individual as source material. Such procedures can underpin conventional plant multiplication approaches. Indeed, optimization physical, chemical and micropropagation has become an important aspect of commercial nursery development and commercial cultivation of many plants, especially of ornamentals with possible extension to crops such as water chestnut (Zimmerman *et. al.*, 1986). Micropropagation techniques have been explored to a limited extent with water chestnut, and this relevant information needs to be disseminated more effectively in order to effectively multiply the plant lets/plants on a much larger scale. Additionally, some somaticqa cell technologies could be exploited to develop salt tolerant cultivars in order to increase the potential cultivation of water chestnut in both fresh and marginal (saline) wet land areas. This could have a considerable socio-economic impact.

Water chestnut has considerable potential as a crop, although considerable effort will be required to establish plant collections with well documented and characterized germplasm. Currently, all species of *Trapa* are classified according to their floral, fruit and leaf morphologies. Comprehensive phyolgenetic relationships amongst *Trapa* species has yet to be established. The exploitation of molecular fingerprinting technologies should facilitate the establishment of such relationships and the identification of germplasms for incorporation into genetic improvement programmes. More needs to be know about crossing in water chestnut as a basis for organized breeding programmes. Alongside these requirements is the need to develop the production of water chestnut on a more agriculturally organised scale. In this respect, advanced breeding and micropropagation has considerable potential in the mass production of propagules, which is now feasible, at least on a small scale, through the ability to culture and propagate explants under defined conditions. It is clear that climate change will have a serious impact on land utilization in the future through the effects of increased flooding, salinization of aquifers, increased population density and food requirements. Rising water levels, for example, may inundate one third of the existing farm land in Bangladesh. Water chestnut, because of its morphology and growth habit, could become an important alternative crop to cereals and the opportunity to cultivate it on marginal lands should be taken seriously by the scientific community.

Objectives of improvement of water chestnut

Though water chestnut (*Trapa* sp.) is one of the most important fruit crop in Bangladesh but the yield of this crop is not at all satisfactory as compared to grown in other countries of the world as like India, China, Japan and others fruits in Bangladesh.

It can multiply in nature but that is very low. If we can optimize environmental, physiological and genetical parameter and increase the yield performance, then we can keep more production which would be helpful to national food security

in Bangladesh. Yield in the major consideration for improvement of water chestnut for the potential value of the future. So, the adjectives of the present study are described in below:

i) To get an advanced cultivation techniques and over production of water chestnut.

ii) To obtained highest production of water chestnut from local water chestnut stocks and seeds.

iii) Overcome the seasonal effects on seed germination and increase proper physical growth and fruit/seed production of water chestnut plant.

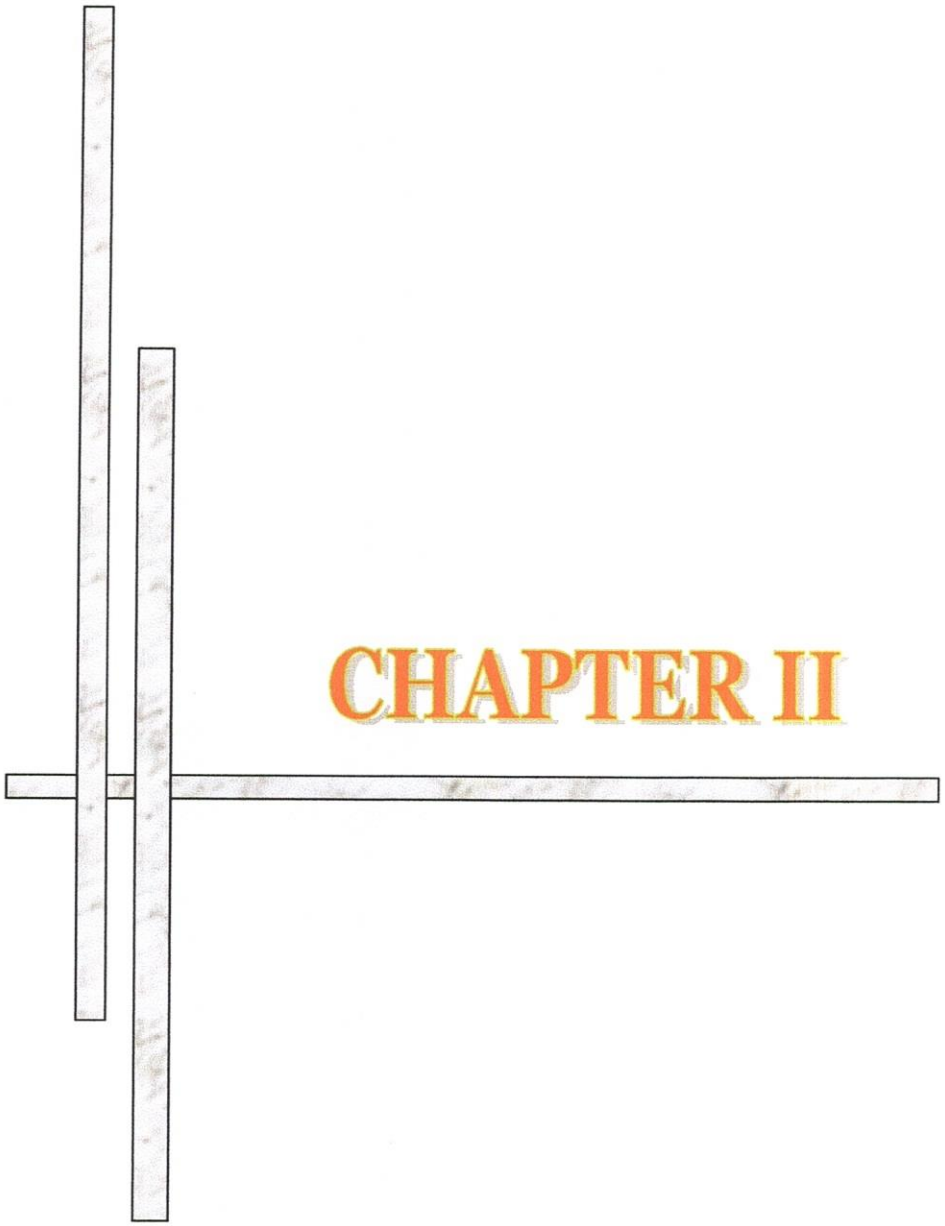
iv) Increase resistance capacity from the past, diseases and salted/alcalic/basic water of water chestnut.

v) To establish a advanced Bangladeshi variety of water chestnut. Over all, the experiments will standardize some eco-physiolocal data which helps to build up a model of varietals improvement, increase production and sustain cultivation technique of water chestnut in Bangladeshi climate.

IMPROVEMENT OF CULTIVATION AND PRODUCTION

Upto present water chestnut known as a minor crop because of its poor seedlings growth qualities, low fruit production, lack of sustain cultivation methods, harvesting problems etc. During the flowering period, the Water chestnut plant bearing many flowers but the fruit setting percentage is very low due to eco-physiological or pollination problem, it is not clear. Yield performance of any plants depends on its good seedling growing conditions. Seedling growth is highly sensitive to environmental conditions, especially light intensity (Arima *et. al.,* 1996). In the aquatic plants, water depth as well as light intensity is an important factor for their seedling growth. There is little information on the improvement of its cultivation techniques and yield Performance (Arima *et. al.,* 1990, 1992a, 1992b, 1993, 1994). However, any attempts were not been taken on its seedling growing conditions.

Until the present, studies on the water chestnut have been carried out mainly in the field of botany, and thus, its varietal characteristics, eco-physiology and cultivation methods have been largely ignored. In the present series of experiments, we have aimed at establishing a high yielding culture method by elucidating the physiology, ecology, breeding and biotechnology of the water chestnut, and at the same time, are striving to obtain basic data for improving seedlings growth qualities and productivity as well as minor crop to valuable crop. From these points of view, the present investigations were done as varital improvements and implementation of advanced cultivation methods of water chestnut in Bangladeshi climatic condition in agricultural trends.



CHAPTER II

Experiment

Food Nutrient Analysis of three varieties water chestnut (*Trapa* spp) Fruits Available in Bangladesh

INTRODUCTION

Good health is achieved by eating the right kinds of food such as fruits and vegetables. Well balanced human foods such as fruits and vegetables should contain adequate amounts of nutrients. A large number of populations in Bangladesh have been suffering from malnutrition. Protein energy malnutrition in infants and children is one of the most common nutritional problems of the rural people in Bangladesh (Ngosom and Abondo, 1989) today. There are many kinds of fruits available in Bangladesh, which are rich in nutrients. For the ignorance of people, they do not know the nutritive value of different kinds of fruits.

In Bangladesh, water chestnut is being cultivated as a minor crop by marginal and landless farmers in roadside water bodies during rainy season (April to September). Bangladesh has one third low land of the total area that regularly affected with flood. Every year paddy, vegetable and other crops are damaged by flood that creates salted soil of near ocean and shortage food of national foodstuff. Some times water chestnut plant absorb low amount of arsenic molecules and it can grows in low concentration of saline water. However, a water chestnut is the floating aquatic crop, is not affected with flood and low concentration of saline water, even water level increases (Hoque *et. al.,* 2000). Therefore, from this point of view, water chestnut would be a very useful food crop for cultivating in low land or saline water especially during flood as an emergency crop and water chestnut can be considered as an alternative source of high quality starch that has multipurpose uses for food and industrial purposes (Rashid, 2005). On the other hand, with global warming, water level is rising which will inundate one third of the farm land of Bangladesh. In the forth-coming changed environmental condition, water chestnut would be an important alternative crop as environmental benefit that must be taken into consideration by the scientific community.

In most road side water bodies and bog place in Bangladesh, there abound numerous plants whose leaves and fruits are potential sources of rural foods, water chestnut fruit one of them. The starchy water chestnut fruits are extensively used in many traditional medicines plants (Guo, *et. al.,* 2001) which are good sources of carbohydrates, proteins, fats, vitamins (vit- B_1, B_2, B_3, C & A), crude fiber, flavonoid, thiamin, niacin, pyridoxine, kobalamin, glucose, phenolic, betacyanins, polyphenol (Wang, *et. al.,* 1969), carotene, phosphorus, phytoalbumin, minerals and amino acids (Bellec, *et. al.,* 2006), which are reflected by the inability to imbibe sufficient water to fully rehydrate and aromatic substances used to enhance the flavour of foods.

There are many varieties of water chestnut (*Trapa* sp.) in our country but their nutritive values are not known in these varieties and a few studies have reported on the potential of water chestnut to be used as a starch and to determine its potential applications to others food products, and alternative carbohydrate sources (Murty *et. al.,* 1962). The concentration of these nutrients also varies with different varieties. Therefore, in the present investigation, three varieties (var. green, var. red and var. wild) of water chestnut (*Trapa* sp.) have been selected to analyze their nutrient composition.

Nutritional assay

Two major problems facing the world are global climate change and food security. Globally, over 800 million people are undernourished and up to 2 billion people lack food security intermittently (FAO, 2008). Most of the countries of the world have been facing malnutrition problems. This malnutrition problem is due to the deficiency of protein in human food and animal feed is well recognized. The need of the good quality of proteins has been increasing due to rapid growth of population. Protein is a food substance essentially required by bodies and cannot be substituted by other compounds in the whole body tissues for its nitrogen contents; hence it must be provided in food. The population in developing countries generally obtains the protein from

cereals and fruits. Protein is required for normal growth, production and health, thus an ideal protein must possess amino acid patterns in line with the human and animal requirements (Unander, 1989).

Fresh Fruits and vegetables are a major source of macronutrients such as carbohydrate, fiber and micronutrients, minerals, vitamins, antioxidants and phytochemicals that are potentially protective against various diseases. They also play an important role in weight management. Vegetables contain a great quantity of non-nutritional antioxidants, such as flavoniods, flavone and total phenolic contents (Guo, *et. al.*, 2001; Wang, *et. al.*, 1996.). Flavonoids act as natural antioxidants (Pratt and Hudson, 1990; Nuutila *et. al.*, 2002).

Recently, researchers have become convinced that nutrients found in fruits and vegetables do more than just prevent deficiency diseases for instance beriberi or rickets. The most publicized finding reveals that certain vitamins or vitamin precursors in produce, notably vitamin C; betacarotene as well as polyphenols are powerful antioxidants (Consumer Reports on Health, 1998). Antioxidants help prevent molecular damage caused by oxidation in that the protection offered may help fend off many diseases such as cancer, cardiovascular diseases and muscular degeneration (Islam *et. al.*, 2002).

The dietary requirement for a micronutrient is defined as an intake level which meets specified criteria for adequacy, thereby minimizing risk of nutrient deficit or excess.

Functional assays are presently the most relevant indices of sub-clinical conditions related to vitamin and mineral intakes. Ideally, these biomarkers should be sensitive to changes in nutritional state while at the same time be specific to the nutrient responsible for the sub-clinical deficiency. Often, the most sensitive indicators are not the most specific; for example, plasma ferritin, a sensitive indicator of iron status, may change not only in response to iron supply, but also as a result of acute infection or chronic inflammatory processes. Similarly anaemia, the defining marker of dietary iron deficiency,

may also result from, among other things, deficiencies infolate, vitamin B_{12} or copper (Saltveit, 2000; Pratt and Hudson, 1990; Nuut *et. al.*, 2002). Unfortunately, the information base to scientifically support the definition of nutritional needs across age ranges, sex and physiologic states is limited for many nutrients. Where relevant and possible, requirement estimates presented here include an allowance for variations in micronutrient bioavailability and utilization. The use of nutrient balance to define requirements has been avoided whenever possible, since it is now generally recognized that balance can be reached over a wide range of nutrient intakes.

Definition of terms

The following definitions relate to the micronutrient intake from food and water required to promote optimal health, that is prevent vitamin and mineral deficiency and avoid the consequences of excess. Upper limits of nutrient intake are defined for specific vitamins and minerals where there is a potential problem with excess either from food or from food in combination with nutrient supplements. The methods used to estimate nutritional requirements have changed over time. Four currently used approaches are briefly outlined below:

Plant analysis, normally, is a laboratory analysis of collected plant parts. Using established critical or standard values, or sufficiency range, a comparison is made between the laboratory analysis results with one or more of these known values or ranges in order to access the plant's nutritional status (Jones *et. al.*, 1991; Kelling, *et. al.*, 2000). The use of plant analysis as a diagnostic tool has a history dating back to studies of plant ash content in the early 1800's. Quantitative methods for interpreting these relationships in a manner that could be used for assessing plant nutrient status arose from the work of Macy (1936). Since then, much effort has been directed towards plant analysis as diagnostic tool. Plant analysis is carried out as a series of steps that include sampling and sample preparation followed by laboratory analysis and interpretation of analytical data.

MATERIALS AND METHODS

Plant materials

Freshly harvested three varieties (var. green, var. red and var. wild) of water chestnut (*Trapa* sp.) fruits were collected from the cultivated field of botanical garden at Rajshahi University, Rajshahi, Bangladesh which originally obtained from the road side canal at Niamatpur, Manda and Sapahar Upazila of Naogaon District, Bangladesh for the conducted experiment. The chemical parameters were studied by the following methods. The chemicals and reagents used in this study were of analytical grade.

Identification of the plant

The plant of water chestnut originally collected from Naogaon District, Bangladesh during February to April 2009 furthermore, that was the main source of experimental materials and the plant taxonomically identified by departmental herbarium, department of Botany, University of Rajshahi, Bangladesh.

Physical measurement

The whole acorn consists of cupules, cotyledon and starch. An average of three replications of measurement was recorded for each variety of water chestnut.

METHODS

Determination of pH

Juice Extraction from water chestnut

About 70-90g of water chestnuts (*Trapa* sp.) was the fruits-starch were crushed thoroughly in a mortar with a pestle and then filtered through two layers of muslin cloth. The filtrate was then centrifuged for 10 min at 3000rpm and the clear supernatant was collected.

Preparation of standard buffer solution

Buffer tablet (BDH Chemicals Ltd. Poole, England) of pH 4.0 or 7.0 was dissolved in distilled water and made up to the mark of 100ml with distilled water.

Experimental procedure

The electrode assembly of the pH meter was dipped into the standard buffer solution of pH 7.0 taken in a clear and dry beaker. The temperature correction knob was set to 28°C and the fine adjustment was made by asymmetry potentially knob to pH 7.0. After wash the electrode assembly was then dipped into a solution of standard pH 4.0 and adjusted to the required pH by fine asymmetry potentially knob. The electrode assembly was raised, washed twice with distilled water, rinsed off with juice of the cultivars and then dipped into the juice of the water chestnut (*Trapa* sp.). The pH of the juice was noted.

Determination of total Titratable Acidity (TTA)

The total titratable acidity was determined by Folin method (Oser, 1965).

Reagents:

a) Standard NaOH solution (0.1N).

b) 1% Phenolphthalein solution.

Experimental Procedure

Water chestnuts (*Trapa* sp.) juice extract (as described in section 2.2.1.1) was taken in a conical flask. Two to three (2-3) drops of phenolphthalein indicator was added and shaked vigorously. It was then titrated immediately with 0.1N NaOH solutions from a burette till a permanent pink color was appeared. The volume of NaOH solution required for titration was noted.

Determination of moisture content

Moisture content was determined by the conventional procedure (ICOMR, 1971).

Materials

a) Porcelain crucible

b) Electrical balance

c) Oven

d) Desiccators

Experimental procedure

About 5-6g of water chestnuts (*Trapa* sp.) was weighed in a porcelain crucible (which was previously cleaned and heated to about 100°C, cooled and weighed). The crucible with the sample was heated in an electrical oven for about six (6) hours at 100°C. It was then cooled in a desiccator and weighed again.

Calculation:

Percentage of moisture content [g per 100g of water chestnuts (*Trapa* sp.)]

$$= \frac{\text{Amount of moisture}}{\text{Weight of the water chestnuts}} \times 100$$

Determination of DRY matter

Dry matter content was calculated from the data obtained for percentage of moisture content.

Determination of Ash

Ash content was determined following the method of A.O.A.C. (1980).

Materials

a) Porcelain crucible

b) Muffle furnace

c) Electrical balance

d) Desiccator

Experimental Procedure

About 25-30g of water chestnuts (*Trapa* sp.) starch was placed in a weighed porcelain crucible (which was previously cleaned and heated to about 100°C, cooled and weighed). The crucible was placed in a muffle furnace for about 6 hours at about 600°C. It was then cooled in a desiccator and weighed. To ensure completion of ashing, the crucible was again heated in the muffle furnace for half an hour, cooled and weighed again. This was repeated till two consecutive weights were the same and the ash was almost white in color.

Calculation

Percent of ash content [g per 100g of water chestnuts (*Trapa* sp.) starch]

$$= \frac{\text{Amount of ash}}{\text{Weight of the water chestnuts}} \times 100$$

Determination of water-soluble protein

Water-soluble protein concentration was determined following the method of Lowry *et. al.*, (1951) using BSA as standard. The extraction was carried out with distilled water.

Reagents

a) 2% Na_2CO_3 solution in 0.1N NaOH.

b) 0.5% copper sulphate in 1% sodium potassium tartrate,

c) Folin-Ciocalteau's reagent (FCR),

d) Standard protein solution: 10mg/ml in distilled water.

Experimental procedure

Reagents 'a' and 'b' were mixed in the ratio of 50:10 and reagent 'c' was diluted with twice the volume of water.

For the construction of standard curve, 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8ml of the standard protein solution were taken into different test tubes and made up to 1ml with distilled water.

The extract (1ml) was also taken in duplicate in different test tubs and 5ml of a:b mixture was added to each of the test tube. After 10 min 0.5ml of FCR was added to each test tube, mixed well and kept for 30 minutes. Then spectrophotometric reading at 650nm was recorded. A graph was constructed by plotting concentration against absorbance (OD) and the concentration of protein was calculated from the graph (Fig 2.1).

Calculation

$$\text{Amount of protein [mg/100g of water chestnuts (}\textit{Trapa}\text{ sp.)]} = \frac{\text{Amount of the protein obtained}}{\text{Weight of the water chestnuts}} \times 100$$

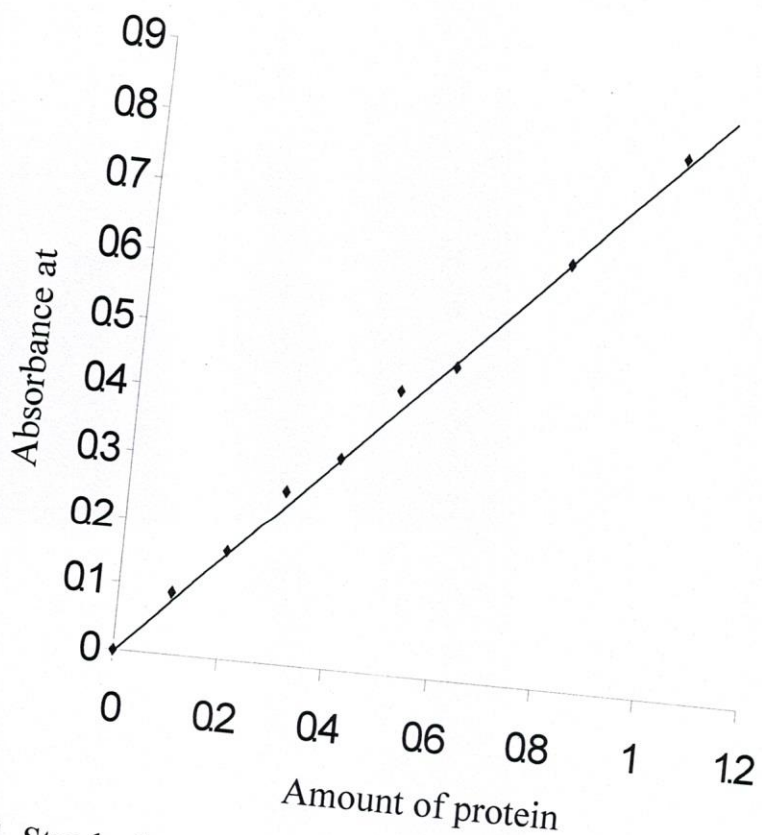


Fig. 2.1. Standard curve for the determination of protein concentration.

Determination of Lipid

Lipid content of the water chestnuts (*Trapa* sp.) was determined by the method of Bligh and Dyer (1959).

Reagent

A mixture of chloroform and ethanol (2:1 V/V)

Experimental procedure

About 1g of water chestnuts (*Trapa* sp.) fruit was first pasted in a mortar with about 10ml of distilled water. The pasted flesh was transferred to a separating funnel and 30ml of chloroform-ethanol mixture was added. After mixing well, it was kept overnight at room temperature in the dark. At the end of this period 20ml of chloroform and 20ml of water were further added and mixed. Generally three layers were seen. A clear lower layer of chloroform containing the entire lipid, a colored aqueous layer of ethanol with all water-soluble materials and a thick pasty interphase were seen.

The chloroform layer was carefully collected in a pre-weighed beaker (50ml) and then placed on a steam bath for evaporation. After evaporation of the chloroform, the weight of the beaker was determined again. The difference in weight gives the amount of the lipid.

Calculation

Percentage of lipid content (g per 100g of fruits of water chestnut)

$$= \frac{\text{Amount of of lipid obtained}}{\text{Weight of the water chestnuts}} \times 100$$

Determination of Total Sugar

Total sugar content of water chestnuts (*Trapa* sp.) fruits was determined colorimetrically by the anthrone method as described in Laboratory Manual in Biochemistry (Jayaraman, 1981).

Reagents

a) Anthrone reagent: The anthrone reagent was prepared by dissolving 2g of anthrone in 1 liter of concentrated H_2SO_4.

b) Standard glucose solution: A standard solution of glucose was prepared by dissolving 10mg of glucose in 100ml of distilled water.

Extraction of sugar

Extraction of sugar from water chestnuts (*Trapa* sp.) kernel was done as described by Loomis and Shull (1937).

Five to six gram (5-6g) of water chestnuts (*Trapa* sp.) opened fruit were plunged into boiling ethyl alcohol and allowed to boil for 5-10 minutes (5-10ml of alcohol was used for every gram of fruits). The extract was cooled and crushed thoroughly in a mortar with a pestle. Then the extract was filtered through two layers of muslin cloth and re-extracted the pasted tissue for three min. in hot 80 percent (80%) alcohol, using 2-3ml of alcohol for every gram (g) of sample. This second extraction ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extracts were filtered through Whatman No.41 filter paper.

The volume of the extract was evaporate to about 1/4th of the volume over a steam bath and cooled. This reduced volume of the extract was then transferred to a 100ml volumetric flask and made up to the mark with distilled water. Then 1ml of the diluted solution was taken into another 100ml volumetric flask and made up to the mark with distilled water (working standard).

Experimental procedure

Aliquot of 1ml of the fruit extract from each level was pipette into test tubes and 4ml of the enthrone reagent was added to each of this solution and mixed well. Glass marbles were placed on the top of each tube to prevent loss of water by evaporation. The test tubes were heated for 10 minutes in a boiling water bath and then cooled. A reagent blank was prepared by taking 1ml of water and 4ml of enthrone reagent in a tube and treated similarly. The absorbance of the blue green solution was measured at 680nm in a colorimeter.

A standard curve of glucose was prepared by taking 0.0, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0ml of standard glucose solution in different test tubes containing 0.0, 10, 20, 60, 80, and 100μg of glucose respectively and made the volume up to 1.0 ml with distilled water. Then 4ml of enthrone reagent was added to each test tube and mixed well. All these solutions were treated similarly as described above. The absorbance was measured at 680nm using the blank containing 1ml of water and 4ml of enthrone reagent.

The amount of total sugar was calculated from the standard curve of glucose (Fig. 2.2). Finally, the percentage of total sugar present in the water chestnuts (*Trapa* sp.) was determined using the formula given below.

Calculation

Percentage of total sugar [g per100g of water chestnuts (*Trapa* sp.)]

$$= \frac{\text{Amount of of sugar obtained}}{\text{Weight of the water chestnuts}} \times 100$$

Determination of reducing sugar

Reducing sugar content of the water chestnuts (*Trapa* sp.) fruits was determined by dinitrosalicylic acid method (Miller, 1972).

Reagents

a) Dinitrosalicylic acid (DNS) reagent: Simultaneously 1g of DNS, 200mg of crystalline phenol and 50mg of sodium sulphite were placed in a beaker and mixed with 100ml of 1% NaOH solution by stirring. If it is need to store then sodium sulphite must be added just before use.

b) 40% solution of Rochelle salt: 40g Na-k tartarate with water of 100ml. Sugar extract from water chestnuts (*Trapa* sp.) was done according to the method described in section (2.2.8.2.).

Experimental procedure

Aliquot of 3ml of the extract was pipetted into test tubes and 3ml of DNS reagent was added to each of this solution and mixed well. The test tubes were heated for 5minutes in a boiling water bath. After developing the color, 1ml of 40% Rochelle salt was added when the contents of the tubes were still warm. The test tubes were then cooled under a running tap water. A reagent blank was prepared by taking 3ml of water and 3ml of DNS reagent in a tube and treated similarly. The absorbance of the solution was measured at 575nm in a colorimeter.

The amount of reducing sugar was calculated from the standard curve of glucose (Fig. 2.3).

Calculation

The percentage of reducing sugar [g /100g of water chestnuts (*Trapa* sp.) fruits]

$$= \frac{\text{Amount of of reducing sugar obtained}}{\text{Weight of the water chestnuts}} \times 100$$

Determination of non-reducing Sugar or Sucrose

Non-reducing sugar or Sucrose content was calculated from the following formula (Ranganna, 1979).

Percentage (%) of Sucrose or non-reducing sugar = (Percentage (%) of total sugar – Percentage (%) of reducing sugar) × 0.95

Determination of Starch

The starch content of the water chestnuts (*Trapa* sp.) fruits was determined by the Anthrone method, as described in Laboratory Manual in Biochemistry (Jayaraman, 1981).

Reagents

a) Anthrone reagent (0.2% in conc. H_2SO_4).

b) Standard glucose solution (10mg / 100ml).

c) 1M HCl

Experimental procedure

About 5g of water chestnuts (*Trapa* sp.) kernel were cut into small pieces and homogenized well with 20ml of water. It was then filtered through double layer of muslin cloth. To the filtrate, twice the volume of ethanol was added to precipitate the polysaccharide, mainly starch. After keeping overnight in cold, the precipitate was collected by centrifugation at 3,000rpm for 15minutes. The precipitate was then dried over a steam bath. Then 40ml of 1M HCl was added to the dried precipitate and heated to about 70°C. It was then transferred to a volumetric flask and diluted to 100ml with 1M HCl. Then 2ml of diluted solution was taken in another 100ml volumetric flask and diluted to 100ml with 1M HCl.

Aliquot of 1ml of the extract of each cultivar was pipetted into test tubes and 4ml of another one reagent was added to the solution of each tube and mixed well. Glass marbles were placed on top of each tube to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for 10minutes, then removed and cooled. A reagent blank was prepared by taking 1ml water and 4ml of anthrone reagent in a test tube and treated similarly. The absorbance of the blue-green solution was measured at 680nm in a colorimeter.
The amount of starch present in water chestnuts (*Trapa* sp.) was calculated from standard curve of glucose (Fig. 2.2).

Calculation

The percent of starch content [g/100 g of water chestnut (*Trapa* sp.) fruits]

$$= \frac{\text{Amount of starch obtained}}{\text{Weight of the water chestnuts}} \times 100$$

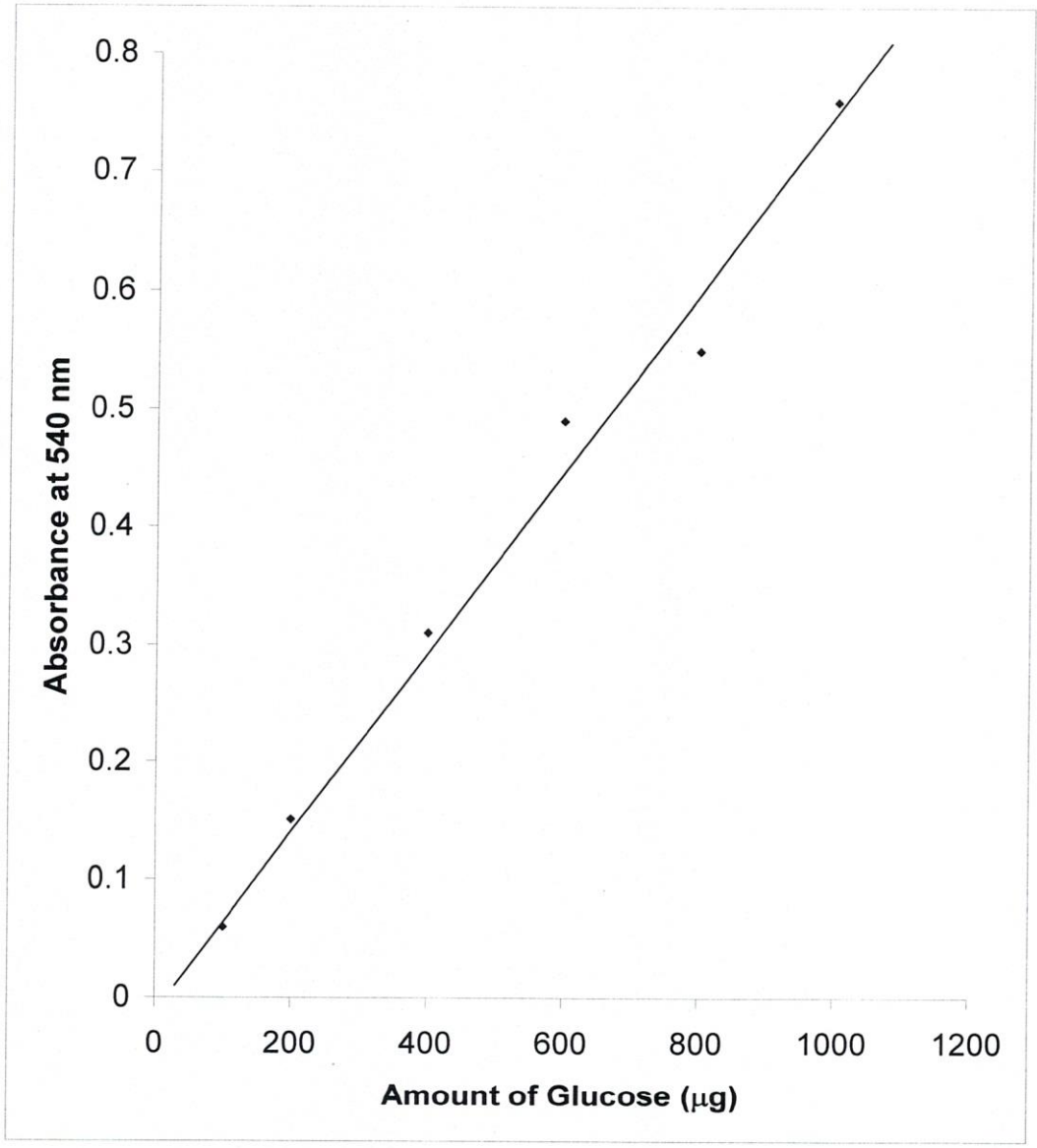


Fig. 2.2. Standard curve of glucose for estimation of total sugar and starch

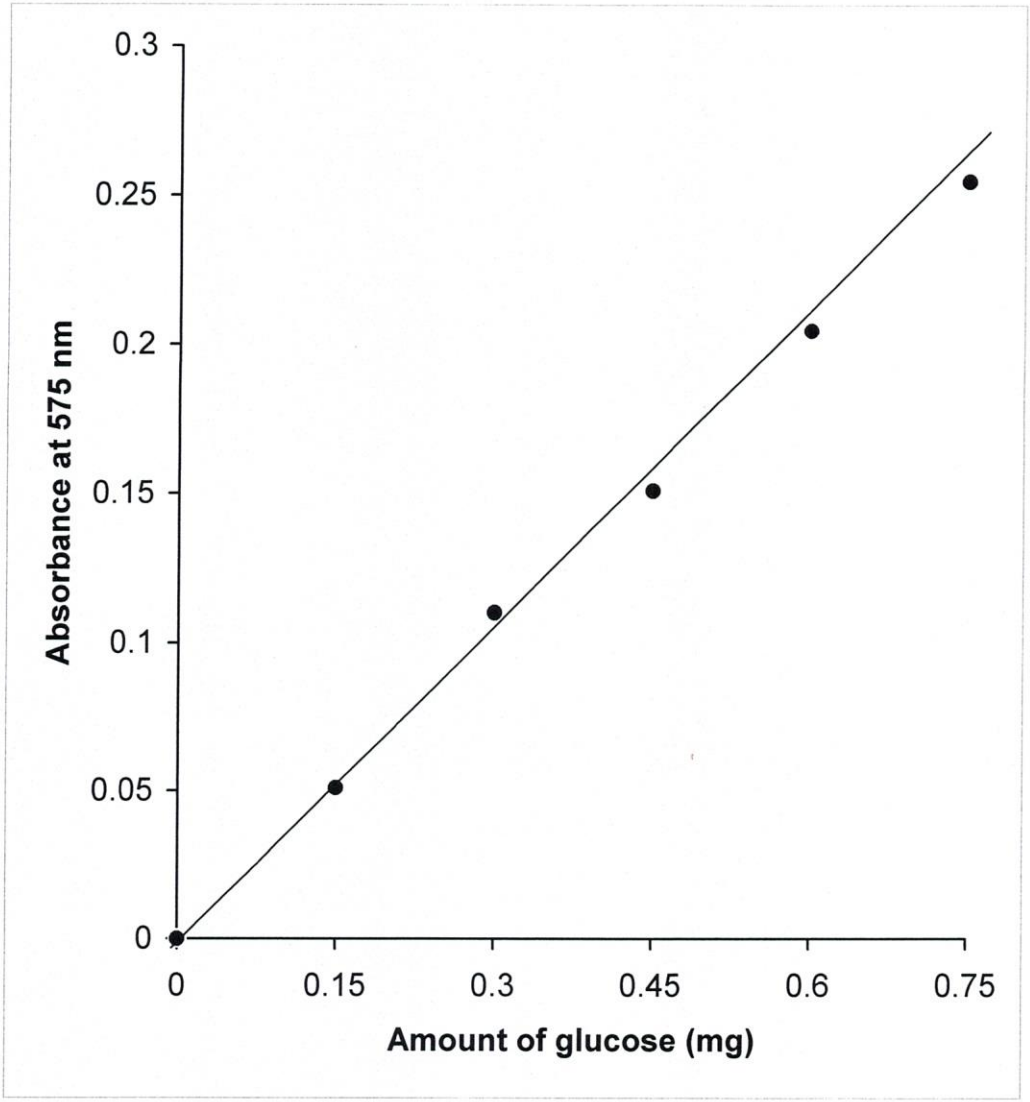


Fig. 2.3. Standard curve of glucose for estimation of reducing sugar.

Determination of vitamin 'C'

Vitamin 'C' content of water chestnuts (*Trapa* sp.) fruits was determined by the titrimetric method (Bessey and King, 1933).

Reagents

a) Dye solution: 200mg of 2,6-dichlorophenol indophenol and 210mg of sodium bicarbonate were dissolved in distilled water and made up to 100ml. The solution was then filtered.

b) 3% metaphosphoric acid reagent: 3g of metaphosphoric acid was dissolved in 80ml of acetic acid and made up to 100ml with distilled water.

c) Standard vitamin 'C' solution (0.1mg/ml): 10mg of pure vitamin 'C' was dissolved in 3% metaphosphoric acid and made up to 100ml with 3% metaphosphoric acid.

Experimental procedure

Ten (10ml) ml of standard vitamin 'C' solution was taken in a conical flask and titrated it with the dye solution.

Five to six gram (5-6g) of water chestnuts (*Trapa* sp.) fruit kernel were cut into small pieces and homogenized well with 3% metaphosphoric acid (approximately 20ml) and filtered it through double layer of muslin cloth. The filtrate was centrifuged at 3,000rpm for 10minutes. and the clear supernatant was titrated with 2, 6-dichlorophenol indophenol solution. The amount of vitamin 'C' in the extract was determined by comparing with the titration result of standard vitamin 'C' solution.

Calculation

Percentage of vitamin 'C' content [mg/100 g of water chestnut (*Trapa* sp.) fruit]

$$= \frac{\text{Amount of vitamin - C obtained}}{\text{Weight of the water chestnuts}} \times 100$$

Determination of β-Carotene

β-Carotene content of water chestnuts (*Trapa* sp.) kernel was determined according to the procedure reported in the Methods of Vitamin Assay (Anon, 1960) and Methods of Biochemical analysis (Click, 1957)

Reagents

a) Ammonium sulphate

b) Acetone

c) Petorleum ether (40-60^{0}C)

d) n-Hexane

e) Activated alumina (BDH chemicals Ltd.)

f) *Standard solution of β-carotene: A Standard solution of β-carotene (BDH* chemicals Ltd.) was prepared by dissolving 50mg of β-carotene in 100ml of petroleum ether.

g) 5.6% KOH solution: 5g of KOH dissolved in 100ml distill water

Column preparation

A column was prepared by using alumina as a packing material. 10% acetone in petroleum ether was used as an eluent buffer.

Experimental procedure

Five gram (5g) of fresh water chestnuts (*Trapa* sp.) fruit and about four gram (4g) of ammonium sulphate were taken in a mortar and rubbed to an even paste with pestle. The extraction was carried out with acetone and small amount of hexane. Extraction was continued until the acetone extract became colorless. Potassium hydroxide solution (10ml, 5.6%) was added to the extract and it was kept in a dark place for half an hour. The mixture was then transferred to a separating funnel; then, 20ml of petroleum ether few milliliter of hexane and 10ml of water were added to the process was repeated until the petroleum ether layer became colorless. The petroleum ether, was concentrated by gentle heating, the concentrated extract (1-2ml) was applied on to the top of the alumina column and eluted with 10% acetone in petroleum ether. The absorbance of the eluent was taken at 440nm in a spectrophotometer.

Construction of standard curve of β-Carotene

A standard curve (Fig-8) was prepared by taking 0.0, 0.1, 0.2, 0.4, 1.6, 0.8, and 1.0ml standard solution of β-carotene and the volume was made up to 5ml with petroleum ether and mixed well. The absorbance of the solutions was taken at 440nm in a spectrophotometer and a standard curve of β-carotene was prepared by plotting the data.

The amount of β-carotene content in each variety of water chestnut (*Trapa* sp.) fruit was calculated by using the standard curve of β-carotene.

Calculation

Amount of β-carotene [μg / 100 g of water chestnuts (*Trapa* sp.) fruits]

$$= \frac{\text{Amount of } \beta\text{ - carotene obtained}}{\text{Weight of the water chestnuts}} \times 100$$

Determination of total phenol

Total phenol content of water chestnuts (*Trapa* sp.) fruits was determined colorimetrically by Folin-Ciocalteau's method (Bray and Thorpe, 1954).

Reagents

a) Folin-Ciocalteau's reagent (FCR)

b) Sodium carbonate, 20%

c) Catechol (0.1mg / ml).

Extraction of phenol

Extraction of phenol from water chestnuts (*Trapa* sp.) kernel was done according to the methods described by Loomis and Shull, (1937).

Five to six gram (5-6g) of water chestnuts (*Trapa* sp.) fruit were cut into small pieces and immediately plunged into boiling ethyl alcohol and allowed to boil for 5-10minutes (5 to 10ml of alcohol was used per gram of fruits). The extract was cooled and crushed thoroughly in a mortar with a pestle. Then the extract filtered

through two layers of muslin cloth and re-extracted the tissue for three min. in hot 80% alcohol, using 2-3ml of alcohol for each gram of tissue. This second extraction ensured complete removal of alcohol soluble substances. The extract was cooled and passed through muslin cloth. Both the extracts were filtered through Whatman No.41 filter paper.

This alcohol extract was used for the estimation of total phenol.

Experimental procedure

Aliquot of 1ml of the extract was pipetted into test tubes and 1ml of Folin-Ciocalteau's reagent followed by 2ml of Na_2CO_3 solution were added to each tube and mixed well. The tubes were placed in a boiling water bath for exactly 2minutes and then removed and cooled. The blue solution was transferred to a 25ml volumetric flask and made up to the mark with distilled water. Then the solution was filtered. A reagent blank was prepared by taking 1ml of water and 1ml of Folin-Ciocalteau's reagent in a tube and treated similarly. The absorbance of the blue solution was measured at 650 nm in a colorimeter. The amount of total phenols was calculated from the standard curve of catechol (Fig. 2.4).

Calculation

Percentage of total phenols [mg / 100g of water chestnuts (*Trapa* sp.)]

$$= \frac{\text{Amount of phenols obtained}}{\text{Weight of the water chestnuts}} \times 100$$

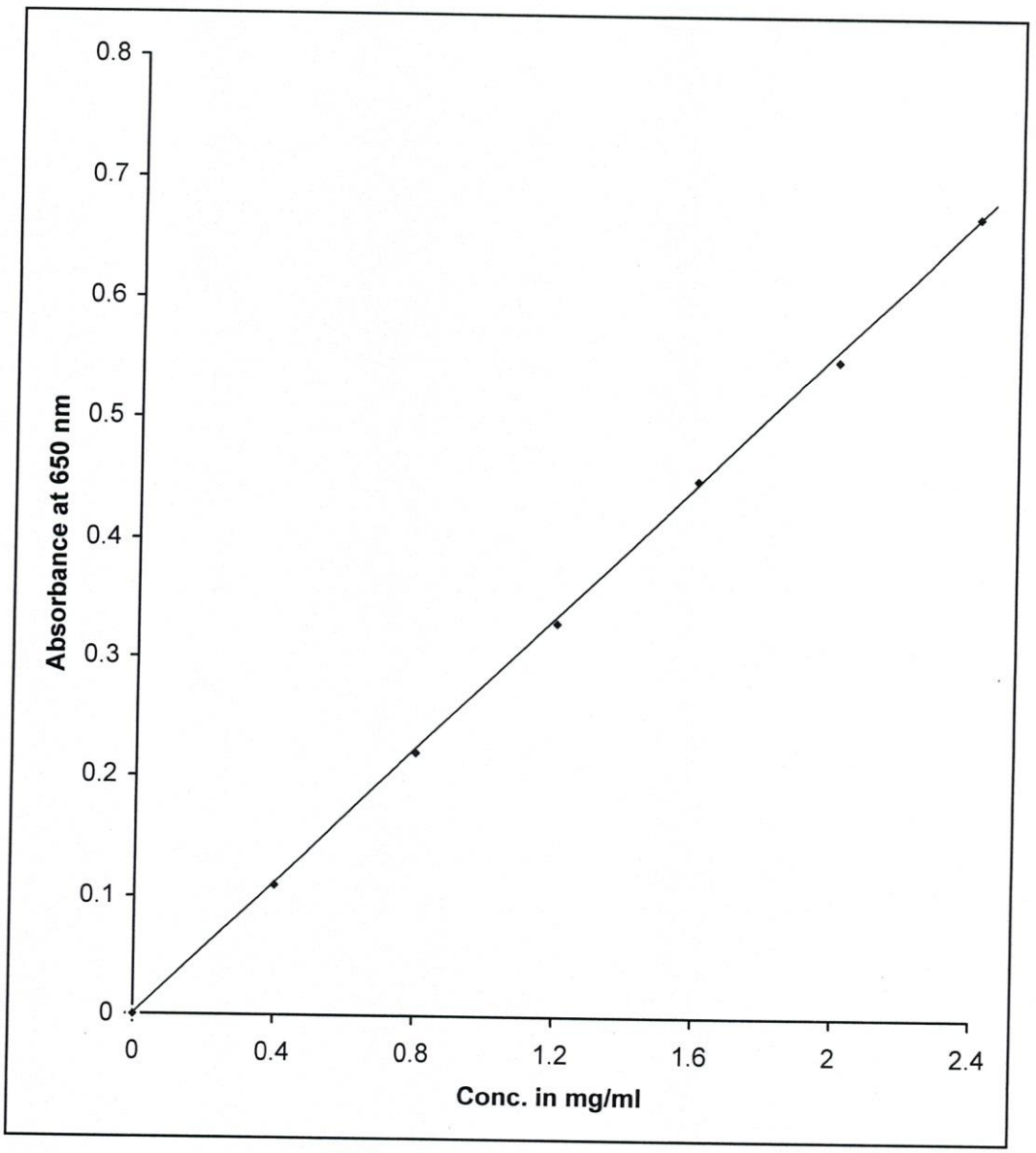


Fig. 2.4. Standard curve of catechol for estimation of total phenols.

Determination of Crude Fiber

Crude fiber of water chestnut fruits was determined by the following method (AOAC, 1980)

Reagents

a) H_2SO_4 (0.26N)

b) NaOH (1.25%)

c) Ethanol

d) Ether

Experimental Procedure

Three gram (3g) of water chestnuts (*Trapa* sp.) fruit were taken into a 500ml beaker and 200ml boiling 0.26N H_2SO_4 was added to it. The mixture was then boiled for 30minutes, keeping the volume constant by the addition of water at frequent intervals (a glass rod inserted in the beaker to help smooth boiling). At the end of this period, the mixture was filtered through a muslin cloth and the residue was washed with hot water till free from acid.

The extract was then transferred into the same beaker and 200ml of boiling 11.25% NaOH was added. After boiling for 30minutes (keeping the volume constant as before) the mixture was filtered through muslin cloth. The extract was washed with hot water until free from alkali, followed by washing with some ethanol and ether. It was then transferred to a crucible dried overnight at 80°C-100°C and weighed.

The crucible was then heated in a muffle furnace at 600°C for three hours, cooled and weighed again. The difference in the weight represented the weight of crude fiber. The percentage of crude fiber (on dry basis) was calculated from the formula given below.

Calculation

Crude fiber content [g/100g of water chestnuts (*Trapa* sp.) fruits]

$$= \frac{\text{Amount of Crude fibre obtained}}{\text{Weight of the water chestnuts}} \times 100$$

DETERMINATION OF MINERALS

Minerals compositions were determined previously described by Black, C.A. in 1965.

Preparation of Plant Samples for Analysis

Drying

A clean container (dish or beaker) was placed in an oven at 105^0C overnight and allowed the container to cool in desiccators and weigh it. The samples were

put in to the container and weight was taken. Then the container was placed in the oven at 105^0C for 24 hours and allowed the container to cool in a desiccator and weight was taken again. Drying cooling and weighting were repeated until the weight becomes constant. The dried sample was stored in an airtight container. The moisture content in the sample was calculated.

Grinding

If necessary, the dried plant material was cut into small pieces with a knife or scissors. The sample was grinded in a plant grinder fitted with a suitable screen. If the grinding takes a long time, the sample will absorb moisture and it necessary to dry the sample again in the oven at 105^0C overnight.

Estimation of Minerals

Organic matter was digested and Calcium (Ca), Potassium (K), Sodium (Na), Iron (Fe), Cupper (Cu) and Phosphorus (P) were released by digestion with nitric acid. Ca, Fe and Cu were determined by atomic absorption spectrophotometry.

Reagents

a) Iron accelerator

b) Copper accelerator

c) Concentrated sulphuric acid

d) Catalyst mixture

e) 33% sodium hydroxide

f) 0.0500M sodium hydroxide

g) 0.0500M Hydrochloric acid

h) Methyl red- metyl blue indicatore solution

i) 68% Nitric acid

j) 1:20 diluted HNO_3

k) 1:100 diluted HNO_3

l) 5M HNO_3

m) $CaCl_2$ solution

n) Acetate buffer solution

o) Azomethine-H reagent

p) Perchloric acid

q) Hydrochloric acid 1:1

r) 0.5M Barium chloride solution

s) Silver nitrate solution

t) Used two stock solutions and one slandered solution of each mineral at different concentration.

Digestion

Total 0.5g dried plant material (water chestnut fruits powder) was weighed into each of 38 nitrogen digestion tubes. The two remaining tubes were blanks. 5.0 ml 68% nitric acid was added to each of all 40 tubes. The content in each tube was mixed and left overnight. The tubes in the digester were placed and covered the tubes with the exhaust manifold. The temperature was set to 125^0C. The digester was turn on and continued the digestion for 4 hours after boiling has started. Precaution was taken so that no tubes became dry.

After cooling, the digestion mixture was transferred with distilled water to a 100ml volumetric flask. The flask was made up to volume with water and mixed and filtered on a dry filter into a dry bottle, which was closed with a screw cap. The filtrate was kept in the closed bottle. Ca, K, Na, Fe, Cu and P contents in the filtrates were determined.

Per Procedure of Minerals Estimation

Using a pipette, transfer 20ml of filtrate was transferred to a 100ml volumetric flask. The flask was made up to volume with distilled water and was mixed well.

Estimation of 'Ca'

The 20ml diluted filtrate was transferred into a 50ml volumetric flask and the flask was made up to volume with distilled water and mixed. The content of 'Ca' was measured by atomic absorption spectrometer (AAS). If the reading is higher than the reading of the highest standard solution, then it is need to larger dilution, e.g. 10ml filtrate into a 50ml volumetric flask. In this case 1:100 diluted HNO_3 must be added to the volumetric flask to make the total volume of 1:100 diluted HNO_3 and filtrate equal to 20ml.

Estimation of 'K' and 'Na'

10ml diluted filtrate was transferred into a 50ml volumetric flask using a pipette. The flask was made up to volume with water and mixed. The content of 'K' and 'Na' were measured by flame photometer. A larger dilution is needed if the reading is higher than the reading of the highest standard solution, e.g. 5ml filtrate transferred into a 50ml volumetric flask. In this case 0:100 diluted HNO_3 must be added to the volumetric flask to make the total volume of 1:100 diluted HNO_3 and filtrate equal to 10ml.

Estimation of 'P'

The 5ml diluted filtrate was transferred to a 50ml volumetric flask. Water (approximately 30ml), added to it and mixed well followed by addition of 10ml ammonium molybdate-ascorbic acid solution. The flask was made up to volume with water and mixed. After 15minutes, the absorbance on a spectrophotometer at 890nm was measured. If the absorbance is higher than that of the highest standard solution, the procedure was repeated using a smaller amount of filtrate. In this case 1:100 diluted HNO_3 must be added to the volumetric flask to make the total volume of 1:100 HNO_3 and filtrate equal to 5ml.

If the content of 'P' is very high, it is necessary to dilute the filtrate further before transferring into the 50ml flask. The dilution is made with water using pipette and volumetric flask. After transfer of 5ml diluted filtrate to the 50ml

volumetric flask. 5ml 1:100 diluted HNO_3 and water to approx. 30ml are added. Then 10ml ammonium molybdate-ascorbic acid is added, the 50ml volumetric flask is made to volume with water and the absorbance is measured at 890nm after 15 minutes.

Estimation of 'Fe' and 'Cu'

The content of these elements were measured by atomic absorption spectrometer (AAS) directly in the undiluted filtrate.

Calculations:

Ca, K, Na, P

$$\text{mg per kg plant material} = \frac{a \times 25000}{b \times c}$$

Where

a = mg/l Ca, K, Na or P measured on atomic absorption spectrometer, flame photometer or spectrophotometer, flame photometer or spectrophotometer,

b = ml diluted filtrate transferred into the 50ml volumetric flask for determination of Ca, K, Na. or P,

c = Amount of plant material (g) weighed into the digestion tube.

If an additional dilution is made before the transfer to the 50ml volumetric flask, the result is multiplied by the dilution factor.

Cu, Fe,

$$\text{mg per kg plant material} = \frac{d \times 100}{c}$$

Where

d = mg/l Cu, Fe measured on atomic absorption spectrometer or spectrophotometer,

c = gram plant material weighed into the digestion tube.

IDENTIFICATION OF FREE AMINO ACIDS

Free amino acids content were determined in the present examination of three varieties (var. green, var. red and var. wild) of water chestnut (*Trapa*) as follows by Jayaraman (1985).

Reagents and Materials

a) Chromatographic paper (whatman no. 1, 20″×20″).

b) Chromatographic tank with lid.

c) 20 standard amino acids (Sigma Chemicals Ltd.) solution.

d) Developing solvents (4).

i) n-Butanol: Glacial acetic acid: Water (3:1:1).

ii) Phenol: Water (4:1).

e) Spray reagent: Ninhydrin solution (200mg of ninhydrin in 100ml acetone). Free amino acid present in the seed cake were identified by two dimensional paper chromatography.

Experimental Procedure

One gram (1gm) of water chestnuts fruit was taken in a morter and pested with distilled water. The pested mixture was filtered through filter paper (whatman no.40). The filtrate was centrifuged at 8000rpm for 10minutes and the clean solution was used for the experiment. The free amino acids were separated from the filtrate by two-dimensional paper chromatography following the conventional procedure (J. Jayraman, 1985) and identified by ninhydrin spray. The following solvents were used during chromatographic separation. First dimension: n-Butanol: acetic acid: water (3:1:1) second dimension: phenol: water (4: 1).

RESULTS AND DISCUSSION

The pH and TTA Content of water chestnuts

The pH of different varieties of water chestnuts (*Trapa* sp.) is given in table 1

Table 1: pH value of three varieties of water chestnut (*Trapa*).

Parameters	Values		
	var. wild	var. green	var. red
pH	5.37±0.03	5.88±0.04	5.11±0.03

The pH of water chestnuts (*Trapa* sp.) was found in the acidic range for different varieties.

TTA of the water chestnuts

TTA content of water chestnuts (*Trapa* sp.) at different varieties is given in table 2.

Table 2: Total Titratable Acidity (TTA) of three varieties of water chestnut (*Trapa*).

Parameters	Values		
	var. wild	var. green	var. red
TTA (ml of 0.1N NaOH required/100g)	7.2	7.1	7.3

From the results of Total Titratable Acidity (TTA) it may be concluded that the acidity of the water chestnuts (*Trapa* sp.) decreased in green variety. This decrease in TTA is consistent with the increase in red and wild variety.

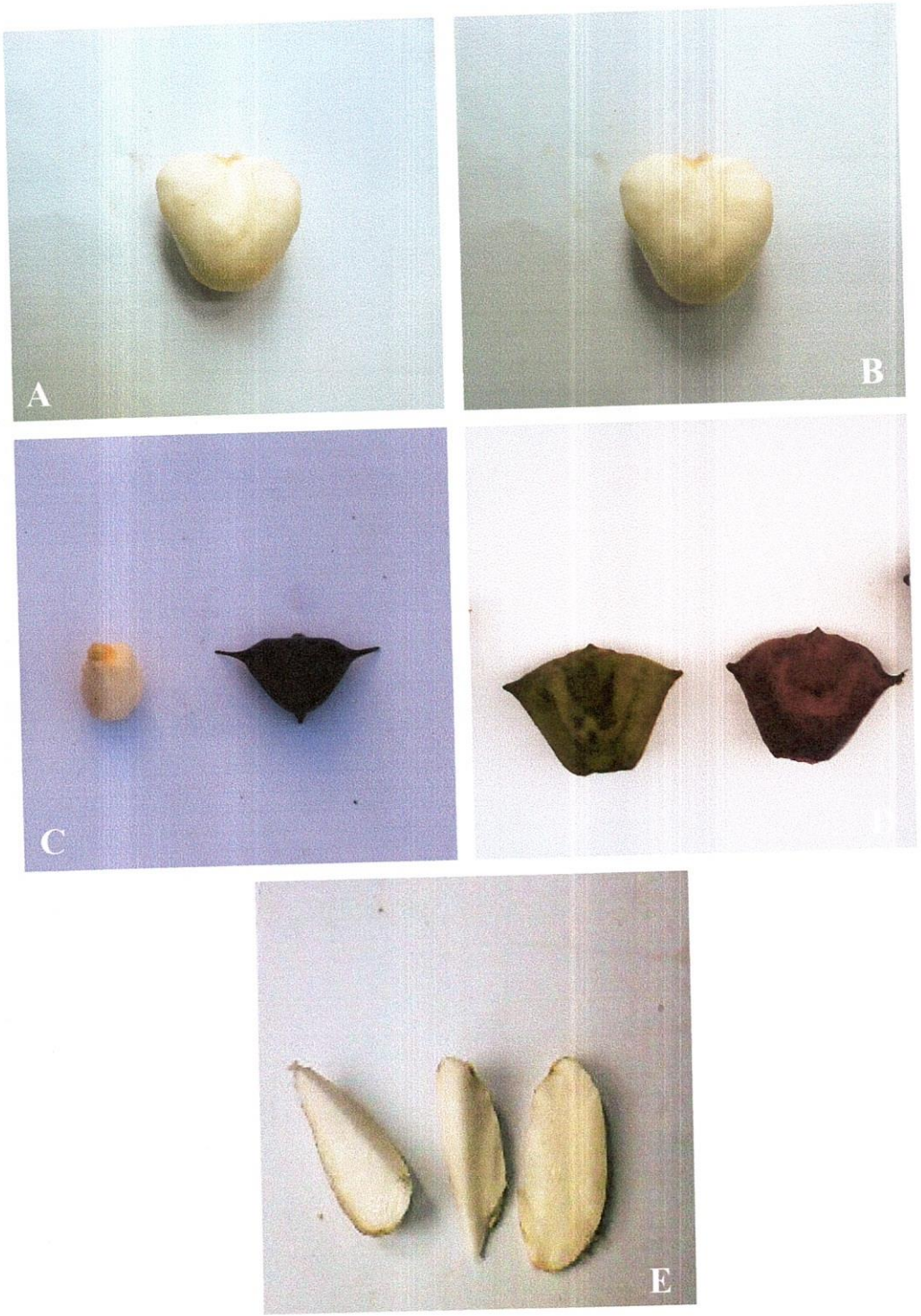


Figure: Nutritional analysis of different water chestnut fruits (green, red & wild)
A. Kernel of green water chestnut fruit, B. Kernel of red water chestnut fruit, C. Mature fruit and kernel of wild water chestnut fruit, D. Mature fruits of green and red water chestnut, E. Slice of water chestnut starch.

Moisture Content

Moisture plays an important role in the growth activities of trees. Water is indispensable for the absorption and transport of food, to carry out photosynthesis, to metabolize materials and to regulate moisture in plants, as in all other living systems. It contributes as much as to the essential properties of life as do the other constituents like Carbohydrate, Protein. Moisture is also essential for most of the physiological reactions in the plant tissues and in its absence life does not exist. Therefore, in the present study, moisture contents of red, green and wild varieties were determined. As shown in table 03, the moisture content were found 60.09±1.11 in wild variety, 62.5±1.16 in green variety and 62.70±1.19 in the red varieties respectively of water chestnuts.

Dry Matter Content

Data on changes in dry matter content of water chestnuts (*Trapa* sp.) at different varieties derived from percentage of moisture content and are shown in table 03.

The results indicated that the dry matter content of water chestnuts (*Trapa* sp.) increased in red variety (17.30±1.22) as compared to wild (16.20±0.97) and green variety (11.50±0.15). The increased in dry matter content shows good correlation with the decrease in moisture content

Ash Content

The ash content of water chestnuts (*Trapa* sp.) is shown in the table 03. Most of the inorganic constituents or minerals are present in ash.

The ash content was observed at 1.02±0.40, 1.04±0.02 and 1.09±0.03 in wild, green and red varieties of *Trapa*, respectively. The results suggesting that the ash content is almost similar in both of the varieties of water chestnuts examined.

Water Soluble Protein Content

Protein plays an important role in all the biological processes. The protein constituents of fruits and vegetables, although occurring in low concentration,

are of primary importance not only as component of nuclear and cytoplasmic structures, but also including, as they must the full complement of enzymes involved in metabolism during growth, development, maturation of fruit and vegetables (Hansen, 1970).

Water soluble protein content was determined by Lowry method as described in the materials and methods. Water chestnuts (*Trapa*) contained 0.237±03 0.275±0.03 and 0.251±0.04 gram (per 100g) of water soluble protein in wild, green and red varieties respectively which present in table 03.

Total Lipid Content

Lipid is more useful in animal body. Fats serve as efficient source of energy and insoluble material. Dietary fat helps in the absorption of fat soluble vitamins, lipoproteins are important cellular constituents. Lipids are essential components of cell membrane, source of metabolic energy for cell maintenance, reproduction and embryogenesis in insects.

In water chestnuts fruits were found to contain a very low amount of lipid (table 03), i.e., 0.87±0.03, 0.84±0.02 and 0.83±0.02 in wild, green and red varieties respectively.

Total Sugar Content

The total soluble sugar content of water chestnuts (*Trapa*) fruit was analyzed and the result is summarized in table 03. The amount of total soluble sugar was estimated to be 0.81±0.02 (wild), 0.92±0.0 (green) and 0.90±0.05 (red) percent.

Reducing Sugar Content

In the table 03 Shows reducing sugar content of water chestnuts (*Trapa*). It was found that water chestnuts (*Trapa*) fruit contained low amount of reducing sugar and the content of reducing sugar was estimated to be 0.28±0.03, 0.33±0.02 and 0.30±0.03 percent in wild, green and red varieties respectively. In case of water chestnuts (*Trapa*) the reducing sugar content is affected by several factors including variety, growing conditions, maturity and the storage environment.

Non-reducing Sugar or Sucrose Content

In the table 03 shows the Non-reducing sugar or sucrose content of water chestnut (*Trapa*) fruit at different varieties.

Sucrose content of water chestnuts (*Trapa*) was found to be almost similar in different varieties (for wild 0.63±0.02, green 0.59±0.01and red 0.60±0.03 percent) tested.

Starch Content

Starch is the most important source of carbohydrate in human diet. As presented in table 03, in all varieties was found to contain a significant amount of starch, i.e, 8.30±0.03, 8.70±0.03 and 8.20±0.02 in wild, green and red varieties respectively.

Vitamin 'C' Content

Vitamin 'C' takes part in the formation of tissue collagen. Recent research has established the role of ascorbic acid in the conversion of folic acid to a physiologically active form tetrahydrofolic acid. Vitamin 'C' also involves in oxidation reduction reaction in cells.

The amounts of vitamin 'C' present in water chestnuts (*Trapa* sp.) at different varieties are given in table 03. It may be concluded from the results that the vitamin 'C' content of water chestnuts (*Trapa*) fruit was higher (1.10±0.02mg/100g) in green variety compared to wild variety (0.73±0.03mg/100g) while red variety shows (0.90±0.02 mg/100g). These results suggest that water chestnuts (*Trapa* sp.) are a good source of vitamin 'C'.

β-Carotene Content

β-Carotenes are precursors of vitamin A. Animal cannot synthesize it but can convert it to vitamin A through enzymatic reaction. In plants, it is very necessary for growth and development of soft tissues through its effect upon protein synthesis. Vitamin A also plays a role in the maintenance of normal epithelial structure.

β-carotene content of two varieties of water chestnuts (*Trapa* sp.) is given in the table 03. Examined all varieties of *Trapa* β-carotene content was higher in red variety (92.0±3.73µg) and lower in green variety (60.0±2.06µg) and wild variety shows average range (88.0±2.39µg) β-carotene between the both varieties.

Total Phenol Content

Phenolic compounds enjoy a distribution in the plant kingdom, and they are particularly prominent in fruits and vegetables where they are important in determining color and flavor (Buren, 1970).

The amount of phenol present in water chestnuts (*Trapa*) at different the varieties is given in table 03

Water chestnuts (*Trapa* sp.) fruits contained low amount of phenol. In green variety contents low range of phenol, the amount of phenol was 0.50±0.01 (mg per 100g), wild variety contents higher amount phenol, the amount of phenol was 0.99±0.02 (mg per 100g) and red variety contents average scale of phenol, the amount of phenol was 0.60±0.02 (mg per 100g). It may be concluded from the results that the phenol content of water chestnuts (*Trapa*) was slightly higher in wild variety.

Crude fiber Content

The crude fiber content is commonly used as a measure of the nutritive value of poultry and livestock feeds and also in the analysis of various foods and food products to detect adulteration and quantity. The amounts of crude fiber in three varieties of the water chestnuts fruits were determined to be 2.28±0.04, 2.13±0.03 and 2.75 ±0.05 for wild, green and red varieties respectively witch shown in table 03.

Table 03: Nutrient Content of three varieties of water chestnuts (*Trapa*) fruits.

Parameters	Amounts		
	wild	green	red
Moisture (%)	60.09±1.11	62.5±1.16	62.70±1.19
Dry Matter (%)	16.20±0.97	11.50±0.15	17.30±1.22
Ash (%)	1.03±0.40	1.04±0.02	1.09±0.03
Water Soluble Protein (mg/100g)	0.237±03	0.275±0.03	0.251±0.04
Total Lipid (g%)	0.87±0.03	0.84±0.02	0.83±0.02
Total Sugar (g%)	0.81±0.02	0.92±0.03	0.90±0.05
Reducing Sugar (g%)	0.28±0.03	0.33±0.02	0.30±0.03
Non-reducing Sugar or Sucrose (g%)	0.63±0.02	0.59±0.01	0.60±0.03
Starch (g%)	8.30±0.03	8.70±0.03	8.20±0.02
Vitamin-C (mg)	0.73±0.03	1.10±0.02	0.90±0.02
β- Carotene (µg)	88.0±2.39	60.0±2.06	92.0±3.73
Total Phenol (mg)	0.99±0.02	0.50±0.01	0.60±0.02
Crude Fiber (%)	2.28±0.04	2.13±0.03	2.75 ±0.05

Minerals Content of water chestnuts

Minerals are inorganic elements exist in the body and in food as organic and inorganic combination. In foods mineral elements are present as salt. They combined with organic compound, e.g. iron in hemoglobin. Minerals are required for the teeth and bone formation. Minute amount of mineral elements are constituents of various regulatory compounds such as vitamins, enzymes and hormones .For example, some enzymes require calcium for their activities as lipases and succinate dehydrogenase. Iron requiring enzymes are ferredoxin, catalase, indophenol oxidase, aldehyde oxidase etc. The mineral elements present in the intra and extra cellular fluid maintained water and acid-base balance. They regulate transmission of impulses and contraction of muscles. The deficiencies of minerals cause many disease in human being.

The amount of potassium, sodium, calcium, phosphorus, sulpher, Iron, cupper, manganese and zinc present in water chestnuts (*Trapa*) are shown in table 04.The results indicated that the amount of potassium was slightly lower in Green variety. Potassium content of water chestnuts (*Trapa.*) was 6.13%, 5.22% and 5.32 % was found for wild, green and red varieties respectively.The sodium content of water chestnuts was found markedly higher in wild variety (0.72%) as compared to red variety (0.59%) and average (0.64%) sodium content shows in green variety. A similar amount of calcium was observed in the three varieties of water chestnuts i,e. 0.12%, 0.25% and 0.26% in wild, green and red varieties respectively. Calcium is an important nutrient element for human body. It plays an important role in formation of bone and teeth. It plays an important role as second messenger for some hormone action. Calcium acts through calmodulin component of phosphorylase kinase and activates phosphorylase. Ca^{++} activates some enzymes e.g. lipase, ATP-ase, succinate dehydrogenase etc.

Phosphorus content of water chestnut fruit was found 5.98% for wild variety, 6.77% for green variety and 6.77% for red variety. Green and red variety shows similar quantity as 6.77% and wild variety store low amount of Phosphorus than the both cultivars. Like calcium Phosphorus is also essential for bone and teeth formation, as well as acid base regulation. Phosphorus is also used in the form of phosphate (as high energy compounds e.g. ATP, UTP, CTP, creatinine phosphate, GTP etc.), in the synthesis of phospholipids, constituents of cell membranes and nerve tissues etc.

Sulpher content of water chestnuts (*Trapa*) was 0.31%, 0.38% and 0.38% in wild, green and red varieties respectively. Sulpher content high and same (**0.38**%) of the two cultivers than wild variety (0.31%) of water chestnut.

Iron content was 237 ppm in wild and 200ppm in both cultivars (green and red) of water chestnut fruits. Iron content was same in green and red varieties. The amount

of iron was 200ppm in both varieties and wild variety content rich amount (237ppm) iron than the others. The primary function of iron is to form hemoglobin, and for the formation and maturation of red blood cells. It carries oxygen in the blood in the form of oxyhemoglobin myoglobin is an iron containing chromoprotein. There is some iron requiring enzymes, such as Xanthine oxidase, Cytochrome-C reductase, Aconitase, Acyl Co-A dehydrogenase, Succinate dehydrogenase etc.

Copper content of water chestnut (*Trapa*) fruit was 432ppm, 430ppm and 450ppm in wild, green and red varieties respectively indicating that red variety contained a higher amount of copper (Cu).

Manganese content was 133ppm in wild, 90ppm in green and 110ppm in red variety, wild variety rich (133ppm) in with manganese and green variety content was very low (90ppm) than red variety while store average amount of manganese of water chest nut.

Zinc content of water chestnut fruit was 499 ppm, 600ppm and 650ppm in wild, green and red variety and Zink content was significantly higher in red variety (650ppm) as compared to wild variety (499ppm) and green variety (600ppm).

Table 04: Mineral Contents of three varieties of water chestnut fruit.

Parameters	Amounts		
	wild	green	red
Potassium (%)	6.13	5.22	5.32
Sodium (%)	0.72	0.64	0.59
Calcium (%)	0.12	0.25	0.26
Phosphorus (%)	5.98	6.77	6.77
Sulpher (%)	0.31	0.38	0.38
Iron (ppm)	237	200	200
Cupper (ppm)	432	430	450
Manganese (ppm)	133	90	110
Zinc (ppm)	499	600	650

Free Amino Acids Content

The free amino acids present in the different varieties of water chestnuts were identified by two dimensional paper chromatography and were tabulated in table 05. It was found that eleven (11) amino acids detected in wild varieties but eight (08) amino acids were detected in red and nine (09) amino acid found in green variety. The amino acid Alanine, Glutamic acid, Leucine, Lysine, Tyrosine were found in three varieties, Asparagine, Glutamine, Proline and Tryptophan present in two varieties. Arginine, Cystine, Histidine and Serine present in a one variety but in examined amino acids of Valine, Hydroxyproline, Isoleucine, Methionine, Phenylalanine and Threonine were not found in all studied variety of water chestnut of Bangladesh.

Table 05. Free amino acid content of the three varieties of water chestnuts fruits.

Amino acid	Varieties		
	wild	**green**	**red**
Alanine	+	+	+
Arginine	-	+	-
Aspartic acid	+	-	-
Asparagine	+	-	+
Cystine	-	+	-
Valine	-	-	-
Glutamine	+	-	+
Glutamic acid	+	+	+
Histidine	+	-	-
Hydroxyproline	-	-	-
Isoleucine	-	-	-
Leucine	+	+	+
Lysine	+	+	+
Methionine	-	-	-
Phenylalanine	-	-	-
Proline	+	+	-
Serine	+	-	-
Threonine	-	-	-
Tyrosine	+	+	+
Tryptophan	-	+	+

SUMMARY

From the experiment fruit extracts of water chestnut found different level chemical compounds such as moisture contents of red, green and wild varieties of moisture content were found 60.09±1.11 in wild variety, 62.5±1.16 in green variety and 62.70±1.19 in the red varieties of water chestnuts. Dry matter content of water chestnuts (*Trapa* sp.) increased in red variety (17.30±1.22) as compared to wild (16.20±0.97) and green variety (11.50±0.15). The ash content was observed at 1.02±0.40, 1.04±0.02 and 1.09±0.03 in wild, green and red varieties of *Trapa*. The results suggesting that the ash content is almost similar in both of the varieties of water chestnuts examined.

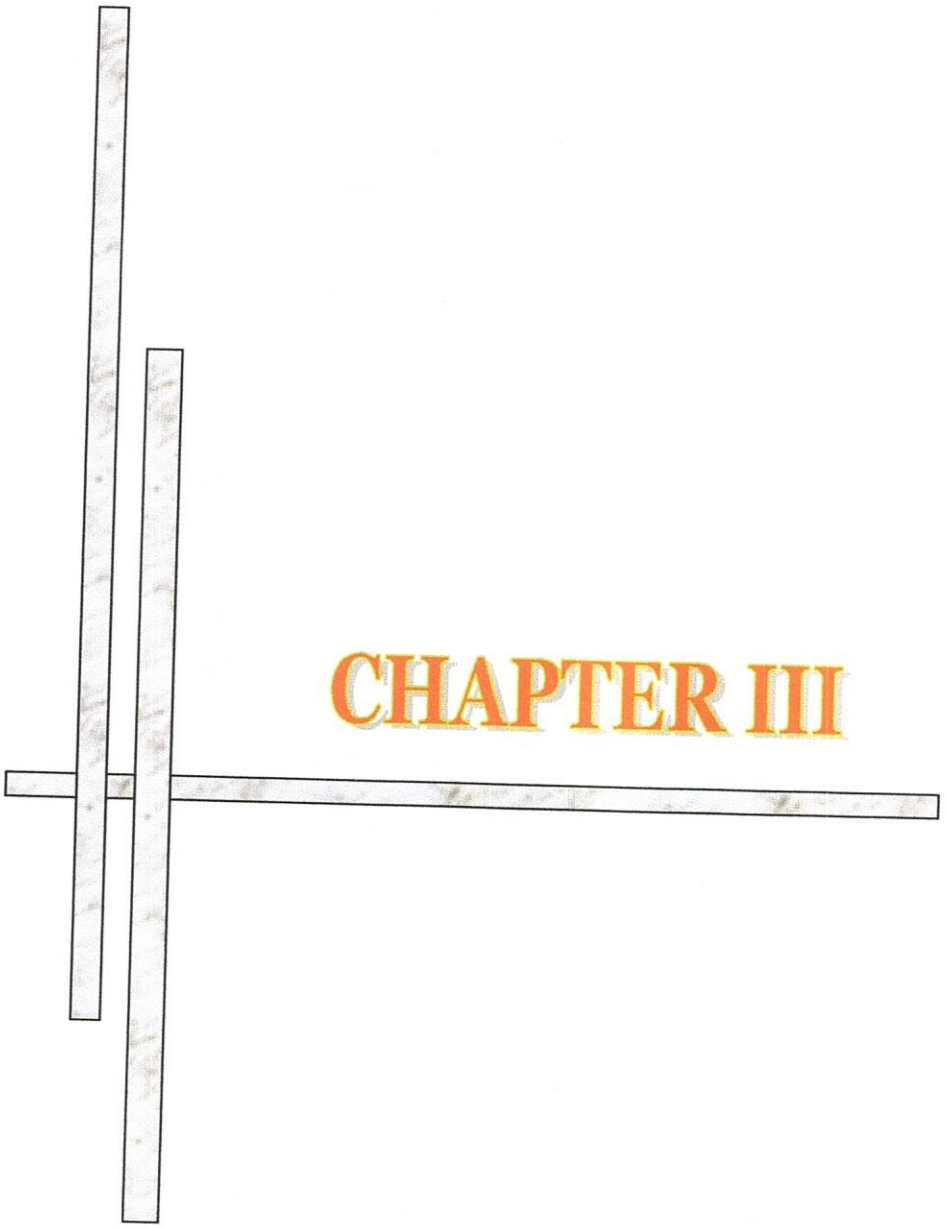
The protein constituents of fruits and vegetables, although occurring in low concentration, are of primary importance not only as component of nuclear and cytoplasmic structures, but also including, as they must the full complement of enzymes involved in metabolism during growth, development, maturation of fruit and vegetables (Hansen, 1970). Water soluble protein content was determined by Lowry method. Water chestnuts (*Trapa*) contained 0.237±03 0.275±0.03 and 0.251±0.04 gram (per 100g) of water soluble protein in wild, green and red varieties, and found a very low amount of lipid, i.e., 0.87±0.03, 0.84±0.02 and 0.83±0.02 in wild, green and red varieties. The amount of total soluble sugar was estimated to be 0.81±0.02 (wild), 0.92±0.0 (green) and 0.90±0.05 (red) percent. Reducing sugar content of water chestnuts were found that to be 0.28±0.03, 0.33±0.02 and 0.30±0.03 percent in wild, green and red varieties respectively. Sucrose content of water chestnuts (*Trapa*) was found to be almost similar in different varieties (for wild 0.63±0.02, green 0.59±0.01and red 0.60±0.03 percent) tested. Starch is the most important source of carbohydrate in human diet and the amount of starch found 8.30±0.03, 8.70±0.03 and 8.20±0.02 in wild, green and red varieties respectively.

Vitamin 'C' takes part in the formation of tissue collagen. Recent research has established the role of ascorbic acid in the conversion of folic acid to a physiologically activities. Vitamin 'C' also involves in oxidation reduction reaction in cells. The amounts of vitamin 'C' content of water chestnuts (*Trapa*) fruit was higher (1.10±0.02mg/100g) in green variety compared to wild variety (0.73±0.03mg/100g) while red variety shows (0.90±0.02 mg/100g). β-carotene content was higher in red variety (92.0±3.73µg) and lower in green variety (60.0±2.06µg) and wild variety shows average range (88.0±2.39µg) β-carotene between the both varieties. Phenolic compounds enjoy a distribution in the plant kingdom, and they are particularly prominent in fruits and vegetables where they are important in determining color and flavor (Buren, 1970). Water chestnuts (*Trapa* sp.) fruits contained low amount of phenol. In green variety contents low range of phenol, wild variety contents higher amount phenol, the amount of phenol was 0.99±0.02 (mg per 100g) and red variety contents average scale of phenol. The amounts of crude fiber in three varieties of the water chestnuts fruits were determined to be 2.28±0.04, 2.13±0.03 and 2.75 ±0.05 for wild, green and red varieties.

In foods mineral elements are present as salt. They combined with organic compound, e.g. iron in hemoglobin. Minerals are required for the teeth and bone formation, some enzymes require calcium for their activities as lipases and succinate dehydrogenase. The amount of potassium, sodium, calcium, phosphorus, sulpher, Iron, cupper, manganese and zinc present in water chestnuts, indicated that the amount of potassium was slightly lower in Green variety. Potassium content of water chestnuts (*Trapa.*) was 6.13%, 5.22% and 5.32 % was found for wild, green and red varieties. The sodium content of water chestnuts was found markedly higher in wild variety (0.72%) as compared to red variety (0.59%) and average (0.64%) sodium content shows in green variety. A similar amount of calcium was observed in the three varieties of water chestnuts i,e. 0.12%, 0.25% and 0.26% in wild, green and red varieties

respectively. Phosphorus, sulpher, iron, copper, manganese, zinc was found in different range in green and red varieties, wild variety store low amount of phosphorus than the both cultivars. Like calcium phosphorus is also essential for bone and teeth formation, as well as acid base regulation.

The free amino acids present in the different varieties of water chestnuts were identified by two dimensional paper chromatography. It was found that eleven amino acids detected in wild varieties but eight amino acids were detected in red and nine amino acid found in green. From the experiment green water chest nut found that the vigor than red and wild variety with rich amount of protein, surge, minerals and free amino acids, In red variety found average food nutrient but less than the green, wild varieties contains rich amount of phenols and minerals, but low amount contained amino acids. Red water chestnut found large sized fruit with high amount of sucrose but others compound was not similar to green. Over all green water chestnut was the nutritionally rich and red is similar to green, So the both variety will be suggest a valuable fruits to the common people in Bangladesh.



CHAPTER III

Experiment

Anti bacterial activities test of water chestnut (*Trapa* spp)

INTRODUCTION

Natural products play an important role in drug development programs of the pharmaceutical industry (Baker *et. al.,* 1995; Cordell, 1995). The modern medicine has brought with it an array of drugs, none of which is non-toxic and quite safer for human consumption. Over 50% of all advanced clinical drugs re made of natural product (Stuffness and Douros, 1982). There are hundreds of medicinal plants which have a long history of curative properties against various diseases. Besides microorganisms have developed resistance to many antibiotics and this has created vast clinical inconvenience in the treatment of infectious diseases (Davis, 1994). The increase in resistance to microorganisms due to the indiscriminate use of antimicrobial drugs forced scientists to search for new antimicrobial substances from various sources including medicinal plants (karaman *et. al.,* 2003). Another driving factor for the renewed interest in past 20 years has been the rapid rate of plant species extinction. Infectious diseases account for high proportion of health problems in the developing countries (sashi *et. al.,* 2003). The demand on plant-based therapeutics is increasing in both developing and developed countries due to their recognition as natural products, non-narcotic, readily biodegradable, has no adverse side-effects and availability at affordable prices.

Higher plants have the capacity to produce a large number of organic phytochemicals with complex structural diversity that is known as secondary metabolites. Some of these secondary metabolites are produced for plant's self defense (Evans *et. al.,* 1986). Over the last 20 years, a large number of secondary metabolites from different plant species have been evaluated for their antimicrobial activity.

However, screening of plants for their activity is very essential and needs urgent attention in order to know the value of the higher plant. The screening of the plants for their biological activity is done on the basis of their chemotaxonomic investigation or ethno-botanical knowledge for a particular disease. Identification of a particular compound against a specific disease is a challenging issue.

The nutritive value of *Trapa spp.* (family-Trapaceae) fruit is not less than wheat (Kusum and Chandra, 1980). The plant has a folkloric reputation as a cure for various diseases. The acrid juice is used for diarrhoea and dysentery (Vhotracharcho, 1987) from the ancient and fruit are used in aphrodisiac, astringent to the bowels, leprosy, inflammations, urinary discharges, fractures, sore throat, bronchitis, leucorrhoea, bad teeth and malaria (Kirtikar and Basu, 1994). It is also a drug of good reputation in 'Yunani' and 'Ayurvedic' medicine in Indian subcontinent; still the plant is being used by the rural people of the northern part of Bangladesh in the treatment of diarrhea and dysentery. The present study was undertaken to characterize some secondary compounds from the three types [wild water chestnut (*T. quadrispinosa)* and two varieties of cultivars e.g. red water chestnut (var. red- *T. bispinosa)* and green water chestnut (var. green- *T. bispinosa*)] of water chestnut and investigate their antibacterial effect.

MATERIALS AND METHODS

MATERIALS

Plant materials

Mature and fresh fruits of *T. bispinosa* Raxb. (var. red and var. green variety) and *T. quadrispinosa* Roxb. called water chestnut were used the present antibacterial response test witch was originated from the experimental cultivated field of botanical garden at Rajshahi University, Rajshahi, Bangladesh during September 2010.

Microbial strain

Eight pathogenic bacteria including five strains of gram-positive (*Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus megaterium*, *Sarcina lutea* and *Bacillus cereus*) and three strains of gram-negative (*Escherichia coli, Salmonella typhi* and *Shigella sonnei*) were used for the bioassay study. The pure strain was identified and obtained from Gene Engineering and Biotechnology Laboratory, Department of Botany, University of Rajshahi, Rajshahi, Bangladesh.

Apparatus and reagents

(a) Standard disc (Kanamycine-50μg/disc)

(b) Ethyl acetate

(c) Ethanol (95%)

(d) Filter paper discs (sterilized)

(e) Petridishes

(f) Inoculating loop

(g) Sterile cotton

(h) Test tubes

(i) Sterile forceps

(j) Micropipette

(k) Spirit burner

(l) Nutrient agar (Difco)

(m) Laminar airflow unit (Bio-Craft & Scientific Industries, India)

(n) Incubator (OSK-9639A, Japan)

(o) Refrigerator (Aritstion, Italy)

(p) Autoclave (ALP Co. Ltd. KT-30L, Tokyo)

METHODS

Sterilization procedure

Antimicrobial screening was carried out in a laminar airflow unit and all types of precautions were highly maintained to avoid any contamination during the test. UV light was switched on one hour earlier of the start of the experiment to avoid contamination. Petridishes and other glassware were sterilized by an autoclave machine at a temperature of 121°C and pressure of 15lb/sq inch for 30 minutes. Blank discs were also sterilized and kept in laminar hood under UV light for 30 minutes.

Microbial culture media

Bacterial cultures were pre-grown on nutrient broth at 37.5°C for 24h. Cultures were spread on nutrient agar media (Difco). For preparing of 100ml nutrient agar media, 0.5g peptone, 1g yeast extract, 0.5g sodium chloride and 2g agar were dissolved in distilled water. This composition of the nutrient was maintained constantly throughout the work.

Preparation of fresh culture

The nutrient agar medium was prepared and dispersed in a number of clean test tubes to prepare slants (5ml in each test tube). The test tubes were plugged with cotton and sterilized for 30minutes. After sterilization, the test tubes were kept in an inclined position (45°C) for solidification. The test organisms were transferred to the agar slants from the supplied pure cultures with the help of an inoculating loop in an aseptic condition. The inoculated slants were then incubated at 37.5°C for 24 hours to assure the growth of test organisms. These fresh cultures were used for the sensitivity test.

Preparation of the test plates

Nutrient agar media were transferred to the sterile petridishes in sterile area. The media were poured into petridishes in such a way to keep a uniform depth of approximately 4mm. The petridishes were rotated several times, initially

clockwise and then anticlockwise. The 200µl of test organism cultured in nutrient broth media was spread on the surface of solid nutrient agar media and kept for applying samples and standard discs.

The test plates were prepared according to the following procedure:

(a) A number of petridishes and nutrient agar media were sterilized in an autoclave for 30 minutes and were transferred into laminar air flow unit.

(b) Nutrient agar media was then transferred to the sterile petridishes in aseptic area. The media were poured into petridishes in such a way as to give a uniform depth of approximately 4mm. The petridishes were rotated several times, first clockwise and then anticlockwise.

(c) 200µl of cultured test organism in nutrient broth media, was spread on the surface of solid nutrient agar media and kept preserved for applying of sample and standard discs.

Preparation of test sample

For preparing the test sample, three different amounts (5mg, 10mg and 20mg) of aquas extract (AE), methanol extracts (ME) and petroleum extract (PE) of each water chestnuts sample (wild, green and red) were dissolved in 0.5ml water in separate glass vial. Thus, for each extract the concentrations were 100µg/µl, 150µg/µl and 300µg/µl, respectively for each extract.

Preparation of discs

Three types of discs were prepared for antibacterial screening. That is

a) Sample discs: Sterilized (BBL, U.S.A) filter paper discs (5mm in diameter) were prepared with the help of punch machine and were taken in a blank petridis. Sample solution of desired concentration (10µl/disc) was applied on the discs with the help of a micropipette in an aseptic condition.

b) Standard discs: These were used to compare the antibacterial activity of test material. In our investigation, *Kanamycin* (50µg/disc) was used as standard disc.

C) Solvent control discs: These were prepared using same filter paper (5mm diameter) and same volume of residual solvent without sample following the same process and condition. These were used, as negative control to ensure that the residual solvent and the filter paper themselves was not active.

Placement of the discs and incubation

The following procedure was adopted for the placement of the discs:

(a) The dried crude extract discs and standard discs were placed gently on the solidified agar plates seeded with the test organisms with the help of a pair of sterile forceps to ensure contact with the medium.

(b) Then the plates were kept in a refrigerator at 4°C for 24 hours in order to provide sufficient time to diffuse the antibiotics into the medium.

(c) Finally, the plates were incubated at 37.5°C for 24 hours in an incubator.

Precaution

The discs were placed 20mm apart from each other and 15mm far from the edge of the plate to prevent overlapping the zones of inhibition.

Measurement of the zones of inhibition

After incubation, the antibacterial activities of the test samples were determined by measuring the diameter of inhibitory zones in terms millimeter (mm) with a transparent scale. The antimicrobial activity of the different extract (AE, ME and PE) having different concentration (100µg/disc, 150µg/disc and 300µg/disc), was tested against pathogenic eight bacteria. *Kanamycin* disc (50µg/disc) was used for comparing the bioassay test.

RESULTS AND DISCUSSIONS

RESULTS

The aqueous extract was found to be effective against all the pathogenic bacteria by disc diffusion assay. It exhibited reasonable antibacterial activity against all the tested bacteria.

Inhibition zone most effected found by the activity of isolated petroleum extract (PE) for red variety of water chestnut against gram positive *Bacillus megaterium, Bacillus subtilis, Bacillus megaterium* bacteria that's were 23, 21 and 19mm at 300µg/disc dosage respectively which was more than positive control of 50µg *Kanamycin.* Less sensitivities found in extracted of menthol extract (ME) and Aqua's extract (AE). These sensitivities were so less than the control standard (19mm) that's are present in table 1,2,3.

Green water chestnut showed similar activities in petroleum extract but it also active (18mm) in gram negative bacteria (*Escherichia colli* (- ve)) at 300µg/disc doze. *Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus* were inhibited 19mm, 16mm and 16mm in 300µg/disc with the petroleum extract (PE), which were less effective to inhibit of red water chestnut, described in table 2. The extract of wild water chestnut showed most effectiveness to *Bacillus megaterium* by 300µg/disc menthol extract (ME) compared to 50µg *Kanamycin* and the inhibition zone was 32mm to the same bacteria were *Kanamycin* inhibit 33mm to *Staphylococcus aureus* bacteria, and petroleum extract (PE) showed average inhibition effects and aqua's extract (AE) showed poor performance of inhibition to the experimental bacteria but that was less inhibited performance than the inhibition of *Kanamycin* shown in table 3. *Escherichia colli* showed full resistance to the all extract and all concentrations. The fruit extract of green water chestnut was more effective than red water chestnut and wild variety is most effective against all the pathogenic bacteria tested by the disc diffusion assay.

Table 1. *In vitro* antibacterial activity in different extracts of red water chestnut (*T. bispinosa* Roxb.) compared to *Kanamycin.*

Bacterial Strain (Pathogen)	AE			ME			PE			PC	NC		
	Inhibition zone (µg/disc)			Inhibition zone (µg/disc)			Inhibition zone (µg/disc)			Iz	AE	NE	PE
	100	150	300	100	150	300	100	150	300	50			
Staphylococcus aureus (+ ve)	R	R	3	R	5	9	1	10	18	11	-	-	-
Bacillus subtilis (+ ve)	R	R	6	R	4	8	1	11	21	10	-	-	-
Bacillus megaterium (+ ve)	R	R	7	R	8	13	1	12	23	19	-	-	-
Sarcina lutea (+ ve)	R	R	3	R	3	10	1	11	19	11	-	-	-
Escherichia colli (- ve)	R	R	R	R	6	12	R	10	17	12	-	-	-
Bacillus Cereus (+ ve)	R	R	4	R	7	10	R	9	15	13	-	-	-
Shigella sonnei (- ve)	R	R	2	R	6	11	1	10	17	11	-	-	-
Salmonella typhi (-ve)	R	R	3	R	5	9	1	9	16	9	-	-	-

*** AQ=Aqua's extract, ME= Methanol extract, PE= Petroleum ether extract, PC= Positive control, NC= Negative Control (*Kanamycin*), Iz= Inhibition zone and R= Resistance

Table 2. *In vitro* antibacterial activity in different extracts of green water chestnut (*T. bispinosa* Roxb.) compared to *Kanamycin.*

Bacterial Strain (Pathogen)	AE			ME			PE			PC	NC		
	Inhibition zone (µg/disc)			Inhibition zone (µg/disc)			Inhibition zone (µg/disc)			Iz	AE	NE	PE
	100	150	300	100	150	300	100	150	300	50			
Staphylococcus aureus (+ ve)	R	R	3	R	4	8	1	10	16	13	-	-	-
Bacillus subtilis (+ ve)	R	R	4	R	5	9	1	11	17	15	-	-	-
Bacillus megaterium (+ ve)	R	R	5	R	8	12	1	12	19	16	-	-	-
Sarcina lutea (+ ve)	R	R	2	R	3	9	1	10	15	10	-	-	-
Escherichia colli (- ve)	R	R	R	R	3	6	R	10	18	9	-	-	-
Bacillus Cereus (+ ve)	R	R	3	R	2	5	R	9	10	10	-	-	-
Shigella sonnei (- ve)	R	R	1	R	3	7	1	8	11	12	-	-	-
Salmonella typhi (-ve)	R	R	R	R	R	8	R	5	8	9	-	-	-

*** AQ= Aqua's extract, ME= Methanol extract, PE= Petroleum ether extract, PC= Positive control, NC= Negative control (*Kanamycin*), Iz= Inhibition zone and R= Resistance

Table 3. *In vitro* antibacterial activity in different extracts of wild water chestnut (*T. quadrispinosa* Roxb.) compared to *Kanamycin*.

Bacterial Strain (Pathogen)	**AE**			**ME**			**PE**			**PC**	**NC**		
	Inhibition zone (µg/disc)			**Inhibition zone** (µg/disc)			**Inhibition zone** (µg/disc)			**Iz**	**AE**	**NE**	**PE**
	100	150	300	100	150	300	100	150	300	50			
Staphylococcus aureus (+ ve)	R	R	9	18	23	20	14	14	19	33	-	-	-
Bacillus subtilis (+ ve)	R	13	21	11	11	18	21	22	29	31	-	-	-
Bacillus megaterium (+ ve)	8	12	13	27	29	34	21	22	26	32	-	-	-
Sarcina lutea (+ ve)	8	12	12	15	18	21	17	24	26	28	-	-	-
Escherichia colli (- ve)	R	R	R	R	R	R	R	R	R	25	-	-	-
Bacillus Cereus (+ ve)	8	10	10	18	23	26	10	18	23	27	-	-	-
Shigella sonnei (- ve)	R	R	7	19	27	31	20	22	22	28	-	-	-
Salmonella typhi (-ve)	R	R	2	5	6	27	14	19	27	28	-	-	-

*** AQ= Aqua's extract, ME= Methanol extract, PE= Petroleum ether extract, PC= Positive control, NC= Negative control (*Kanamycin*), Iz= Inhibition zone and R= Resistance

DISCUSSIONS

The antimicrobial activity of the aqua's extract (AE), methanol extract (ME), petroleum ether extract (PE) were tested against eight bacteria at the concentration of 100µg/ disc, 150µg/ disc and 300µg/ disc. Standard antibiotic disc, *kanamycin* (50µg/disc) was used for the comparison of the bioassay. The petroleum ether extract of water chestnut (red variety) showed notable antibacterial efficiency (15-23mm) against most of the tested organisms. Highest antibacterial activity was observed against *Bacillus megaterium* (23mm) bacteria when applied 300µg/disc dosages (Fig. 1.1,1.2 & 1.3). Poor efficiency was found against the *Bacillus Cereus* (23mm) which were lower than *kanamycin* (19mm). It was revealed that fruit extract of water chestnut (green variety) showed the most effectiveness (8-16mm) against all pathogenic bacteria. Highest inhibitory activity was found against *Bacillus megaterium* (19mm) bacterium having concentration of 300µg/disc, whereas lowest activity was observed against *Shigella sonnei* (1mm). *Salmonella typhi, Escherichia coli* and *Bacillus Cereus* were resistant to the green variety and *Escherichia coli Bacillus Cereus* were resistant to the red variety. The produced zone of inhibition for isolated methanol extract (ME) of wild water chestnut against gram positive bacteria showed that 300µg/disc dose was most effective because there was no resistant bacteria found and maximum 32mm inhibition zone produced. Whereas, except *Escherichia colli* bacterial strains in all other treatments showed resistant against to experimental extract. Furthermore, in case of petroleum ether extract (PE) of wild water chestnut showed average activities (range14-27mm) against the tested organisms where maximum inhibition zones were produced in 300µg/disc dose by all bacterial strains. The highest 34mm zone of inhibition was produced by *Bacillus megaterium* in gram positive and maximum 32mm zone of inhibition was formed by *Staphylococcus aureus* bacteria [table 3, Fig. 1(i)]. Also among three extracts studied, the highest diameter of inhibitory zone was found in methanol extract

(34mm) and the lowest was recorded in both aquas and petroleum ether extracts (7mm). The petroleum ether extract of green variety of water chestnut fruit was found to be the most potential antibacterial activities that showed inhibitory activity against both gram positive and gram negative bacteria besides red varieties showed inhibition only gram positive bacteria with the 300µg/disc. The results of different studies provide evidence that some medicinal plants might indeed be potential sources of new antibacterial agents (Kone *et al.,* 2004).

This consequence was comparable to the previous findings (Razvy *et. al.,* 2011) where methanol extract of wild variety of water chestnut showed maximum inhibitory zone against gram positive bacteria and remarkable antibacterial efficiency (18-34mm) were recorded against most of the tested organisms. Also extracts of some other plants have been used for the antimicrobial activity such as antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* where they have found significant potency of aqueous and methanol extracts (Karaman *et. al.,* 2003). No resistant bacteria strain was found in petroleum ether extract experiment and in case of all strains, maximum inhibition zone was not recorded in red and green water chestnut. Therefore antibacterial activities of three different extracts were preeminent in 300µg/disc dose. It was also perceptible that in low concentration of aqua's extract, all gram negative bacteria were not found as resistant. In positive control, significant inhibitory zone was formed such as maximum 33mm and minimum 25mm zone of inhibition was produced, in contrary, not a single inhibitory zone was formed in negative control. Previous study (Parekh and Chanda, 2007) also documented antibacterial activity of *T. natans* fruit extracts against different gram positive and gram negative bacteria. The increase of antibiotic resistance of microorganisms to conventional drugs has necessitated the exploration for new, efficient and cost effective way for the control of infectious diseases.

The result of different studies provides that some medicinal plants might be potential sources of new antibacterial agents (Kone *et. al.,* 2004). From this study, we can conclude that, this medicinal plant has a wide range of antibacterial activity and supports the traditional use of these plants as medicine. This study demonstrated that herbal medicine could be as effective as modern medicine to combat pathogenic microorganisms. This work has highlighted the antimicrobial effects of fruit of *T. quadrispinosa* and *T. bispinosa* on some of the medically important pathogens (fig. 2.1,2.2, & 2.3). This is in fact a promising result, with the comparable standard antibiotics and suggests the potency of these extracts. Hence, *T. quadrispinosa* fruit could be used as a guide in our continuing search for being used in the field of biopharmaceutical industry or in ethno-medicine.

SUMMARY

Antibacterial activities of the water chestnut fruit extract of three varieties (green, red and wild varieties) of water chestnut by the disc diffusion method from aqua's extract, methanol extract and petroleum ether were studied. The extract of wild variety of water chestnut showed high antibacterial potential (34mm) against *Bacillus megaterium* with the concentration of 300µg. On the other hand, green variety showed highest antibacterial activities (19mm) against *Bacillus megaterium* with the concentration of 300µg/disc and red variety showed highest antibacterial activities (23mm) against *Bacillus megaterium* with the concentration of 300µg in petroleum ether extract based on 50µg *kanamycin* used as standard. In this disc diffusion assay, the petroleum ether extract of red and green variety were found to have a significant antibacterial efficiency, and wild varieties in methanol extract was found most and effective sensitivities to inhibition (34mm). These findings pinpoint the efficiency of these extracts to inhibit microbial growth. It may lead to the development of a new phyto-medicine such as inhibition compound of *Bacillus megaterium* bacrerie.

PHOTOS

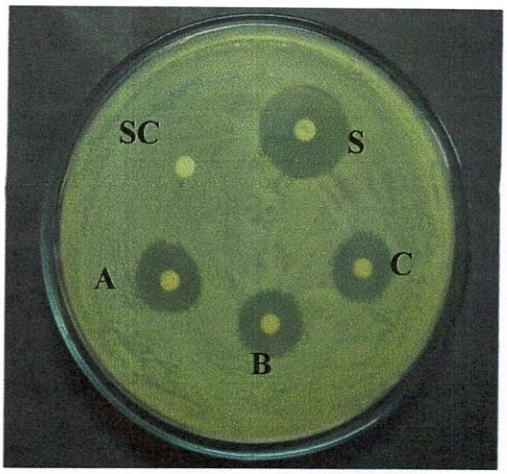


Fig. 1.1. Effect of methanol extract (red) on *Bacillus megaterium*

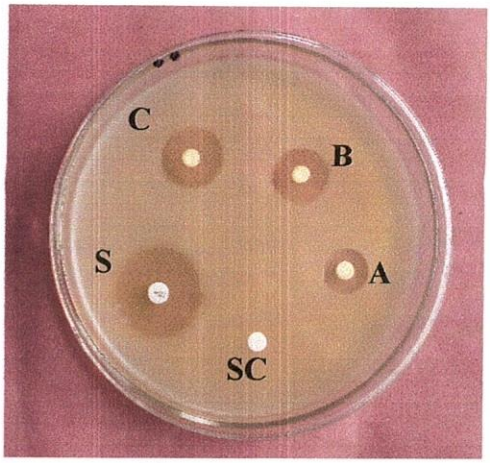


Fig. 1.2. Effect of methanol extract (green) on *Bacillus megaterium*

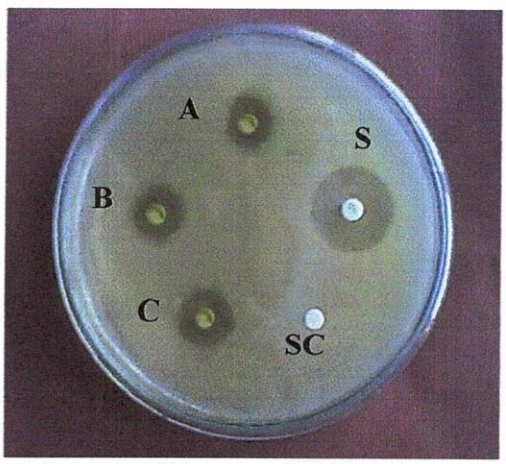


Fig. 1.3. Effect of methanol extract (wild) on *Bacillus megaterium*

SC - Solvent control
A - 200 µg/disc
B – 400 µg/disc
C - 600 µg/disc
S- Kanamycin (30µg/disc)

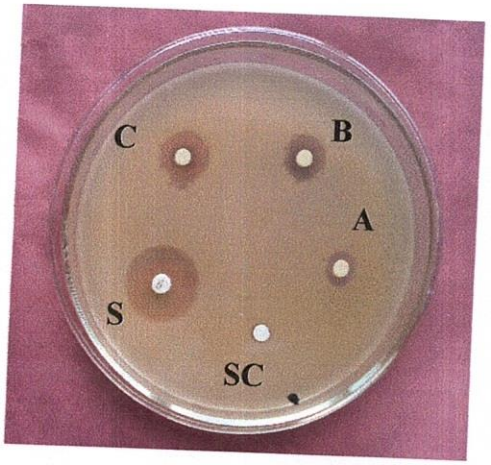


Fig. 2.1. Effect of ethyl acetate extract (Red) on *Bacillus subtilis*

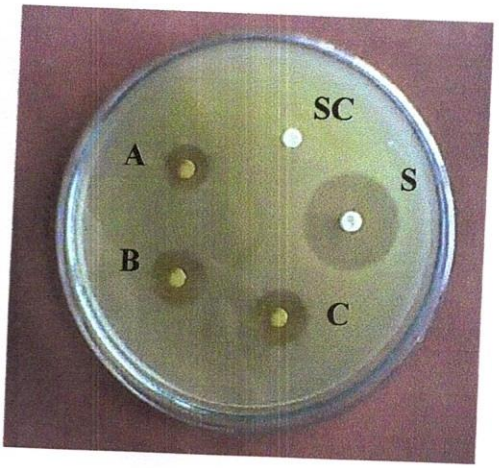


Fig. 2.2. Effect of ethyl acetate extract (green) on *Bacillus subtilis*

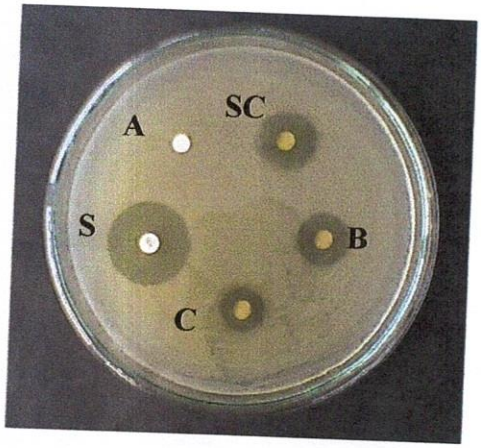
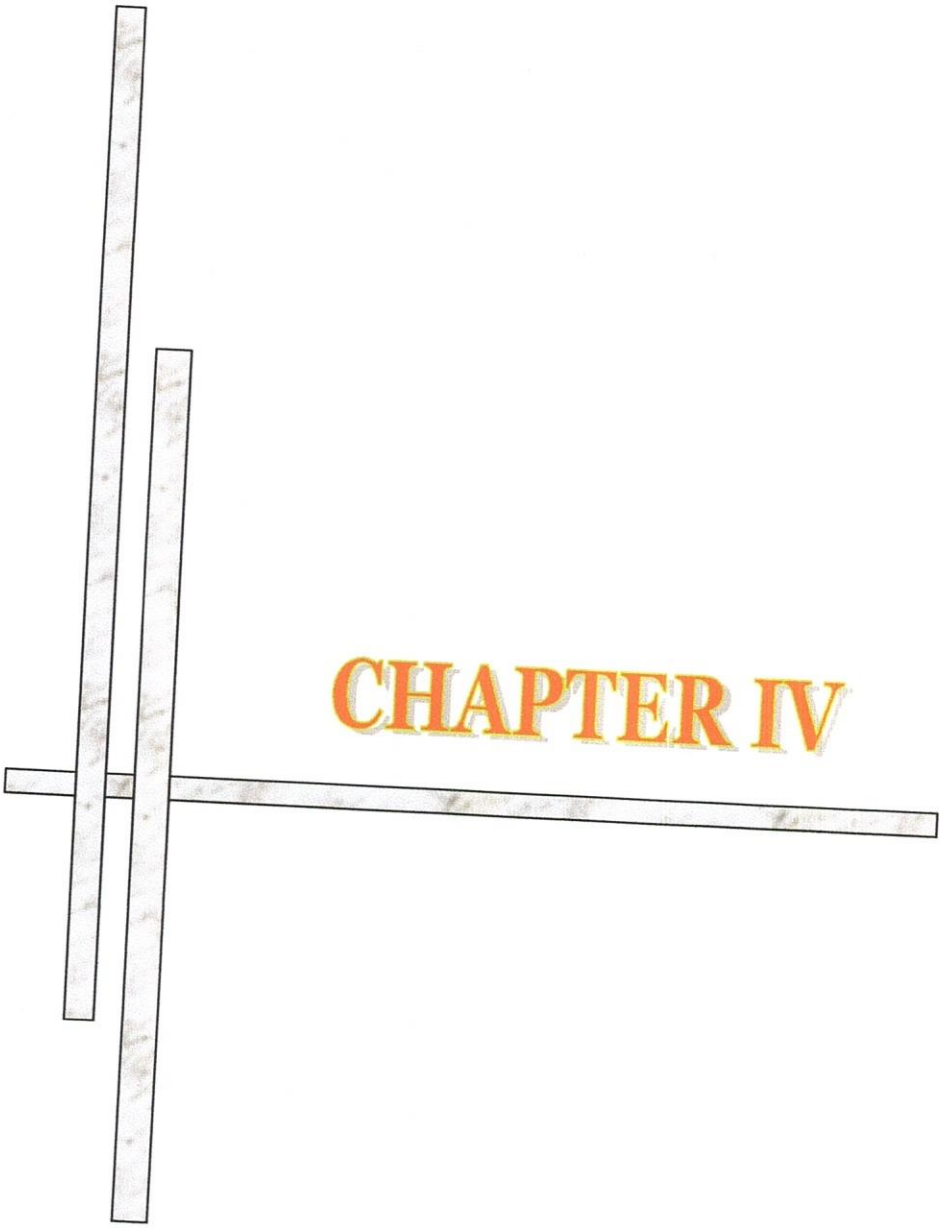


Fig. 2.3. Effect of ethyl acetate extract (wild) on *Bacillus subtilis*

SC - Solvent control
A - 200 µg/disc
B – 400 µg/disc
C - 600 µg/disc
S- Kanamycin (30µg/disc)

Figure: Best performance or antibacterial activities of water chestnut fruit extruct



CHAPTER IV

Experiment
Determination of antifungal activity of fruit extracts of different water chestnut varieties in Bangladesh

INTRODUCTION

Medicinal plants have been used for centuries before the advent of orthodox medicine. Leaves, flowers, stems, roots, seeds, fruit and bark of plant can all be constituents of herbal medicines (Broekaert *et. al.,* 1997). Secondary metabolites (photochemical) of plant are extensively found at different levels in various medicinal plants and used in herbal medicine to treat diverse ailments such as cough, malaria, wounds, toothache and rheumatism diseases (Exarchou, *et. al.,* 2002) and protection of crops (Chandra and Sarbhoy, 1997). In recent years, the growing demand for herbal product has led to a quantum jump in volume of plant materials traded within and across the countries. While species used in traditional medicines continue to be the most reliable sources for the discovery of useful compounds, the screening of plants (Ben *et. al.*, 1992; Dubery *et. al.,* 1999; Kruger and Manion, 1994; Mohamed and Sehgal, 1997; Pernas *et. al.*, 2000) has provided yet another source for compounds with useful activities against different microbes. Therefore there is a constant need to establish and develop antimicrobial drugs from natural origin that are much safe, reliable and less expensive.

Fungi are significant destroyers of crop, food stuffs and grains (Hanawa *et. al.,* 1992). A significant portion of the agricultural produce in the country and the world over become unfit for human consumption due to mycotoxins contamination of grains, especially those produced by species of *Aspergillus* (Devi *et. al.*, 2001; Janardhana *et. al.*, 1999). The main toxic effects are carcinogenicity, genotoxicity, terratogenicity, nephrotoxicity, hepatotoxicity, reproductive disorders and immunosuppression (Desjardins *et. al.*, 2000; Lacey, 19888). A sizeable portion of the world population living below poverty line in the developing and underdeveloped countries are suffering from health problems associated with consuming mycotoxin contaminated grains and cereals (Majumder, 1997).

Even though effective and efficient control of different fungi of can be achieved by the use of synthetic chemical fungicides, the same can not be applied. Thus, there is a need to search for alternative compound to protect the damage of crop, store grains/cereals and various diseases or infections without toxicity problems that are ecofriendly and not capital intensive. Plant extracts of many higher plants have been reported to exhibit antibacterial, antifungal and insecticidal properties under laboratory trails (Bouamama *et. al.,* 2006; Ergene *et. al.*, 2006; Kiran and Raveesha, 2006; Mohana and Raveesha, 2006; Okigbo and Ogbonnaya, 2006; Satish *et. al.,* 1999; Shariff *et. al.,* 2006). Plant metabolites and plant-based pesticides appear to be one of the better alternatives as they are known to have minimal environmental impact and danger to consumers in contrast to the synthetic pesticides (Verma and Dubey, 1999).

The present study investigated antimicrobial properties in different varieties of water chestnut, hydrophytes fruit species found on rainy season in Bangladesh Antifungal activities of different extracts of water chestnut were tested against six fungi by using disc diffusion technique, because it is essentially a quantitative or semi-quantitative test indicating the sensitivity or resistance of fungus to the test material.

MATERIALS AND METHODS

Materials

Plant materials

Plant materials used as mature fresh fruit of three varieties of *Trapa* spp for extraction. One of them wild variety (*Trapa quadrispinosa* Roxb.) and two cultivars (Green and Red) of *Trapa bispinosa* Roxb were collected from the experimental field of Botanical Garden at Rajshahi University, Bangladesh, which was originally collected from Naogaon, Bangladesh.

Fungal strain

Total six pathogenic fungal strain, *Penicilium* sp, *Aspergillus flavus*, *Aspergillus niger*, Fusarium sp, *Aspergillus fumigatius* and *Candida albicans* were used for the present sensitivity test.

1. *Penicilium sp.*
2. *Aspergillus flavus*
3. *Aspergillus niger*
4. *Fusarium species*
5. *Aspergillus fumigatius*
6. *Candida albicans*

Test materials

Three solvents (aqua's, ethanol and petroleum ether) were used for the antifungal activity study. The concentrations of test sample in different extract were 100µg/l 200µg/l and 250µg/l respectively. The standard antifungal reagent was Dithane- M_{45} fungicides at the fixed concentration of 100µg/l used as positive control.

Experimental apparatus and materials

(a) Filter paper discs

(b) Petridish

(c) PDA and Seaboard media

(d) Sterile cotton

(e) Sterile forceps

(f) Micropipette (10-100µl)

(g) Distilled water

(h) Test tube

(i) Vial

(j) Bunsen burner

(k) Autoclave (KT-30L)

(l) Refrigerator

(m) Punch machine

(n) p^H meter

(o) Alcohol (95%)

(p) Incubator (9639A, OSK)

Methods

Preparation of culture media

Potato Dextrose Agar (PDA) media was used to perform the antifungal activity test and for subculture of the test organism. The composition of the culture media was 200gm peeled and sliced potato, 40gm dextrose, 20gm agar and 1000m distill water.

Preparation of culture media

Potato Dextrose Agar (PDA) media was used to perform the antifungal activity test and for subculture of the test organism. The composition of the media was as follows.

Potato Dextrose Agar (PDA) media

Materials	Amounts
Peeled and sliced potato	200gm
Dextrose	40gm
Agar	20gm
Distill water	1000ml

Preparation of the test plates

About 10ml in quantity of distilled water was poured in several clean test tubes and plugged with cotton and 6 ml of the medium was poured carefully in the medium sized Petri-dishes in each. The Petri-dishes were rotated several times, first clockwise and then anticlockwise to assure homogenous thickness of the medium and allowed to cool and solidify at about 30°C. The test tubes containing distilled water were inoculated with fresh culture of the test fungi and were shaken gently to form a uniform suspension of the organism because of their high prevalence sporulation process. Then a piece of cotton was immerged in the test tubes with the help of individual glass rod and then gentle rubbed the medium and the cotton was discarded and finally the plates were stored in a refrigerator (4°C) for overnight.

Preparation of the test plates

The test plates were prepared according to the following procedure:

(a) About 10 ml in quantity of distilled water was poured in several clean test tubes and plugged with cotton.

(b) The test tubes, a number of petridishes, glass rods, a piece of cotton and the medium were sterilized by autoclave and then transferred to the laminar air flow cabinet.

(c) About 6 ml of the medium was poured carefully in the medium sized petridishes in each. The Petri dishes were rotated several times, first clockwise and then anticlockwise to assure homogenous thickness of the medium and allowed to cool and solidify at about 30°C.

(d) The test tubes containing distilled water were inoculated with fresh culture of the test fungi and were shaken gently to form a uniform suspension of the organism because of their high prevalence sporulation process.

(e) A piece of cotton was immerged in the test tubes with the help of individual glass rod and then gentle rubbed the medium and the cotton was discarded.

(f) Finally, the plates were stored in a refrigerator (4°C) for overnight.

Preparation of discs

Two types of antifungal discs were prepared for antifungal screening one of them Sample discs and another type discs was Standard discs by antifungal reagent Dithane- M_{45} fungicides. Sample discs were sterilized metrical (BBL, Cocksrvile, USA) filter paper discs (5mm diameter) were taken in a blank Petridis. Sample solution of desired amount was applied on the discs with the help of micropipette in and aseptic condition. The discs were left for a few minutes in an aseptic condition for complete removal of solvent and standard discs was prepared with the concentration of (Dithane- M_{45} fungicides) 100µg/disc.

Preparation of discs

Two types of antifungal discs were prepared for antifungal screening. They were:

(a) Sample discs

(b) Standard discs (Nastatin)

Sample discs

Sterilized metrical (BBL, Cocksrvile, USA) filter paper discs (4mm diameter) were taken in a blank petridish. Sample solution of desired amount (10µl/ disc) was applied on the discs with the help of micropippette in and aseptic condition. The discs were left for a few minutes in an aseptic condition for complete removal of solvent.

Standard discs

It was also prepared by the same process of sample discs preparation. Thus the concentration of standard nystatin was 100µg/disc.

Placement of the discs and incubation

The dried crude extract discs and standard disc were placed gently on the solidified agar plates seeded with the test pathogenic fungus ensure contact

with the medium with optimum precaution by the sterile forceps. The plates were then kept in a refrigerator at 4°C for 24 hours in order to provide sufficient time to diffuse the antibiotics into the medium then the plates were incubated at 37.5°C for 24 hours in an incubator. After incubation, the antibacterial activities of the test samples were determined by measuring the diameter of inhibitory zones in term of mm with a transparent scale

Placement of the discs and incubation

For the placement of the discs, the following procedure was adopted:

(a) By means of a pair of sterile forceps, the dried crude extract discs and standard disc were placed gently on the solidified agar plates seeded with the test organisms to ensure contact with the medium.

(b) The plates were then kept in a refrigerator at 4°C for 24 hours in order to provide sufficient time to diffuse the antibiotics into the medium.

(c) Finally, the plates were incubated at 37.5°C for 24 hours in an incubator.

Precaution

The discs were placed in such a way that they were not closer than 15 mm to the edge of the plate and apart from each other to prevent overlapping the zones of inhibition.

Measurement of the zones of inhibition

After incubation, the antibacterial activities of the test samples were determined by measuring the diameter of inhibitory zones in term of mm with a transparent scale

RESULTS AND DISCUSSION

Different concentration of aqua's, ethanol and petroleum extract of water chestnut fruit extract has been tested against fungal growth. In was observed that all three varies of water chestnut exhibited some sort of antifungal activity resulting inhibition zone ranging from 1.1±0.03mm to 9.9±0.71mm in diameter. Highest inhibition zone (9.9±0.71mm) was found against *Aspergillus flavus* while treated with 250 µg/disc of ethanol extract of wild variety (Figure 1). It was revealed that both ethanol and petroleum extracts seemed to be more efficient to inhibit the growth of all *Aspergillus* species compare to aqua's extract (table 1). It was reported that ethanolic extract of *Anogeissus leiocarpus* and *Terminalia avicennioides* proved to be more efficient than the methanolic, chloroform, or aqueous extracts against all the test fungi (18). In comparison, wild variety showed highest antifungal activity against all the fungi used in this study, which reveals the presence of toxic substances in wild water chestnut compare to red and green variety. It was interesting to be noted that petroleum extract found to be more efficient than other two against *Penicilium* sp., *Fusarium sp. and Candida albicans* (table 2).

Table 1. Evaluation of in vitro antifungal activity against different *Aspergillus* species.

PM	Ext.	Disc/Zone of Inhibition (mm)								
	µg/disc	*Aspergillus fumigatius*			*Aspergillus flavus*			*Aspergillus niger*		
		100	200	250	100	200	250	100	200	250
V_1	AQ	1.7±0.07	2.6±0.06	2.2±0.04	1.75±0.07	2.0±0.10	2.1±0.21	2.2±0.21	2.9±0.21	2.9±0.39
	ET	5.0±0.12	4.0±0.21	4.0±0.09	4.0±0.11	5.0±0.16	5.0±0.44	2.0±0.13	1.7±0.09	3.0±0.36
	PE	4.1±0.23	3.2±0.14	3.2±0.20	3.1±0.18	3.1±0.18	4.0±0.11	4.0±0.28	4.1±0.33	3.1±0.44
V_2	AQ	2.3±0.06	2.1±0.11	2.1±0.12	3.4±0.22	3.6±0.45	3.3±0.81	3.1±0.41	2.9±0.28	2.7±0.52
	ET	5.1±0.10	5.1±0.21	5.2±0.25	6.8±0.05	5.9±0.28	6.2±0.29	4±.3±0.33	5.8±0.07	5.8±0.12
	PE	5.1±0.12	5.3±0.26	5.8±0.66	5.9±0.17	4.7±0.21	6.1±0.41	5.1±0.36	5.1±0.25	5.2±0.38
V_3	AQ	2.4±0.20	2.5±0.09	2.7±0.10	2.3±0.14	2.8±0.35	3.6±0.61	2.3±0.14	2.5±0.33	3.5±0.41
	ET	7.3±0.33	7.5±0.07	8.1±0.19	8.6±0.80	9.1±0.45	9.9±0.71	8.2±0.32	7.6±0.29	7.5±0.59
	PE	7.1±0.10	6.9±0.22	7.8±0.21	9.1±0.38	9.2±0.18	9.2±0.60	7.7±0.21	7.9±0.51	7.9±0.11
NC	AQ	+	+	+	+	+	+	+	+	+
	ET	+	+	+	+	+	+	+	+	+
	PE	+	+	+	+	+	+	+	+	+

* PM = Plant Materials, AQ = Aqua's extract, ET = Ethanol Extract, PE = Petroleum Extract, PC = Positive control (Disc containing Antifungal reagent), NC = Negative control (Disc containing only solvent), (+) = Growth, (-) = no sensitivity, V_1 = Green, variety, V_2 = Red variety, V_3 = Wild variety, Inhibition zone excluding disc (5mm) space

Table 2. Evaluation of in vitro antifungal activity against other fungi.

PM	Ext.	Disc/Zone of Inhibition (mm)								
	µg/disc	*Penicilium* sp			*Fusarium* sp.			*Candida albicans*		
		100	200	250	100	200	250	100	200	250
V_1	AQ	1.1±0.03	1.7±0.01	1.9±0.01	1.3±0.12	2.2±.02	2.0±0.22	2.4±0.16	2.9±0.09	2.6±0.04
	ET	3.0±0.22	2.0±0.22	3.0±0.22	1.5±0.34	2.0±0.32	2.0±0.21	3.0±0.30	3.0±0.06	3.0±0.01
	PE	4.0±0.01	3.0±0.34	3.1±0.30	3.1±0.54	4.0±0.41	3.0±0.07	3.0±0.45	4.0±0.09	4.0±0.42
V_2	AQ	1.3±0.11	1.3±0.11	1.2±0.28	1.7±0.70	2.2±0.11	2.9±0.11	2.3±0.31	2.3±0.34	2.1±0.55
	ET	4.3±0.19	4.3±0.51	5.2±0.35	4.1±0.24	4.7±0.44	3.8±0.23	4.5±0.43	3.3±0.41	3.1±0.21
	PE	5.1±0.08	3.9±0.22	4.8±0.22	4.4±0.54	3.8±0.11	4.1±0.29	4.3±0.22	4.1±0.44	3.8±0.07
V_3	AQ	2.3±0.28	2.3±0.44	2.8±0.38	3.1±0.23	3.5±0.10	3.6±0.11	2.5±0.21	2.9±0.21	2.9±0.22
	ET	6.1±0.21	5.9±0.24	5.9±0.11	5.3±0.66	5.2±0.25	5.5±0.05	4.2±0.07	4.9±0.07	4.8±0.36
	PE	7.1±0.58	6.8±0.34	6.5±0.25	5.2±.08	5.1±0.44	4.9±0.08	6.1±0.35	6.3±0.02	6.4±0.11
NC	AQ	+	+	+	+	+	+	+	+	+
	ET	+	+	+	+	+	+	+	+	+
	PE	+	+	+	+	+	+	+	+	+

* PM = Plant Materials, AQ = Aqua's extract, ET = Ethanol Extract, PE = Petroleum Extract, PC = Positive control (Disc containing Antifungal reagent), NC = Negative control (Disc containing only solvent), (+) = Growth, (-) = no sensitivity, V_1 = Green, variety, V_2 = Red variety, V_3 = Wild variety, Inhibition zone excluding disc (5mm) space.

Lowest mean growth diameter (4.2±0.07mm) was recorded against *Candida albicans* while highest growth diameter (7.1±0.58mm) was obtained against *Penicilium* sp (table 2). Hypothetically increase in the antifungal activity of any extract supposed to be found by increase concentration, which is not observed in this study. Application of plant extract to inhibit fungal or bacterial growth is a common practice. In this study fruits of water chestnut especially the wild variety found to be efficient against some fungal genotypes and it also indicates

the potentially to work on other fungi or molds (table 1 and table 2). Use of different parts of plant are also been noticed in literature. Leaves extract of *Pistacia lentiscus* and *Pistacia atlantica* have been proved to be very effective against eight bacteria, five moulds and yeast by disc diffusion method (Benhammou *et. al.,* 2008). Plant extracts also act as an inhibiting agent against some bacteria. It was reported that *Caryophyllus aromaticus* and *Syzygyum joabolanum* extracts found to be promising to inhibit the growth of *Pseudomonas aeruginosa* (Nascimento *et. al.,* 2000).

CONCLUSIONS

It could be concluded that fruit extract of water chestnut have great potential as antimicrobial compounds against microorganisms. This may open a new window in the treatment of infectious diseases caused by resistant microbes.

SUMMARY

The antifungal activity of three varieties (red, green and wild) of water chestnut fruit extracts were studied against a number of fungal species. A strong antifungal activity of ethanol and petroleum extract was found against the treated fungi resulting remarkable inhibition zone compare to Dithane- M_{45} fungicide and control. It has also been evident that wild variety of water chestnut was comparatively more efficient in respect to antifungal activity compared to the red and green variety.

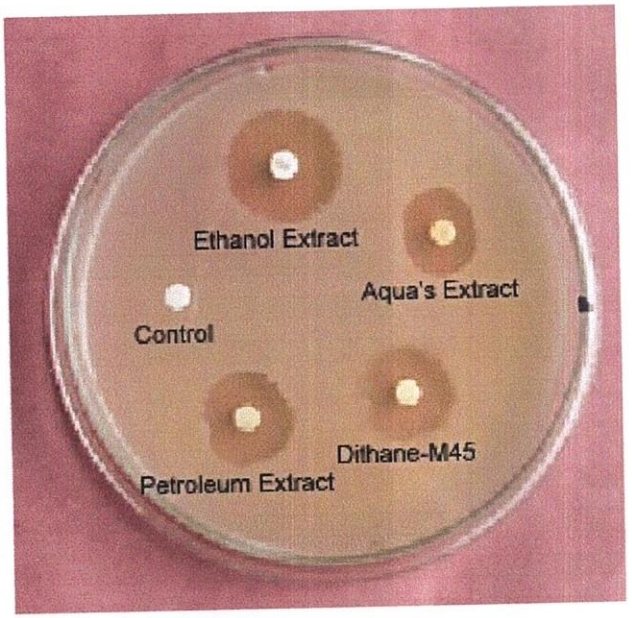


Figure I. Antifungal activity of water chestnut

Experiment

Effects of plant growth regulators on the formation of axillary shoots and adventitious roots in water chestnut (*Trapa* spp.)

INTRODUCTION

In addition to an increasing world population, there are numerous reasons for serious concern about sufficient future global production of food from crop plants. First, the availability of arable land is decreasing because of non-sustainable farming, soil erosion and degradation, availability of water for agriculture will declines, global climate changes will not only seriously affect crop growth but will also threaten the conservation of cultivated land. Droughts, storms, floods, heat waves and rises in sea level are predicted to occur more frequently, salinity and other soil toxicities are likely to be much more problematic in some areas in Bangladesh. To overcome these problems, new agricultural technologies will be needed to ensure global food security. In particular, crop improvements that confer tolerance to environmental stresses and soil toxicity, as well as high yield and biomass, will be required. To achieve this goal we have to improve knowledge for crop improvement, including varietals improvement, cultivation technique improvement, breeding strategies and genetic modification for over hunger, poverty reduction and undoubtedly headed off a global food crisis.

Up to now water chestnut (*Trapa*) considered as a minor crop because of its low yield, lack of suitable cultivation techniques, harvesting problems and strong marketing channel etc. There is little information on the improvement of its cultivation techniques and yield performance (Arima *et. al.*, 1990, 1992a, 1992b, 1993, 1994). However, any attempts were not been taken on its seedling growing conditions. Yield performance of any plants depends on its good seedling growing conditions. Seedling growth is highly sensitive to environmental conditions, especially light intensity (Arnim *et. al.*, 1996).

In the aquatic plants, water depth as well as light intensity is an important factor for their seedling growth.

Until the present, studies on the water chestnut have been carried out mainly in the field of botany, and thus, its varietal characteristics, eco-physiology and cultivation methods have been largely ignored. In the present series of experiments, we have aimed at establishing a high yielding culture method by elucidating the physiology, ecology, breeding and biotechnology of the water chestnut, and at the same time, are striving to obtain basic data for improving productivity as well as minor crop to valuable crop in Bangladesh. To increase the water chestnut cultivation area, demand and development a flood/ salt tolerant variety is necessary to study.

From these points of view, the present investigation on analysis of ecophysiology of water chestnut was undertaken with the view to optimization of seed germination rate and branches multiplication from few seeds.

With the correlation of this experiment, some important experiments will be carried out in future with three varieties of water chestnut collected from different place of Bangladesh, and their vegetative growth characteristics, hormonal effects on their growth as well as yield, selection a salt and cold tolerance variety to increase the cultivation area and shown in flow diagram.

Objectives of improvement of water chestnut

Though water chestnut (*Trapa* spp) is one of the most important fruit crop in Bangladesh but the yield of this crop is not at all satisfactory as compared to cultivated others agricultural crops in Bangladesh.

It can multiply in nature but that is very low. If we can increase the multiply the number of shoots, root with the hormonal treatment in the *in vitro* or *in vivo* condition, then we can keeps more plantlets or branch from the few seed and

get more fruits production of water chestnut. Yield in the major consideration for improvement of water chestnut for the potential value of the future in Bangladesh and Bangladeshi food stuffs. So, the adjectives of the present study are described in below:

i) To get more plant lets or branches from the few seeds or stolon of water chestnut this helps to get over fruit production with low cost.

ii) To obtained highest production of water chestnut from few quantities of seeds from the small cultivated area in Bangladesh.

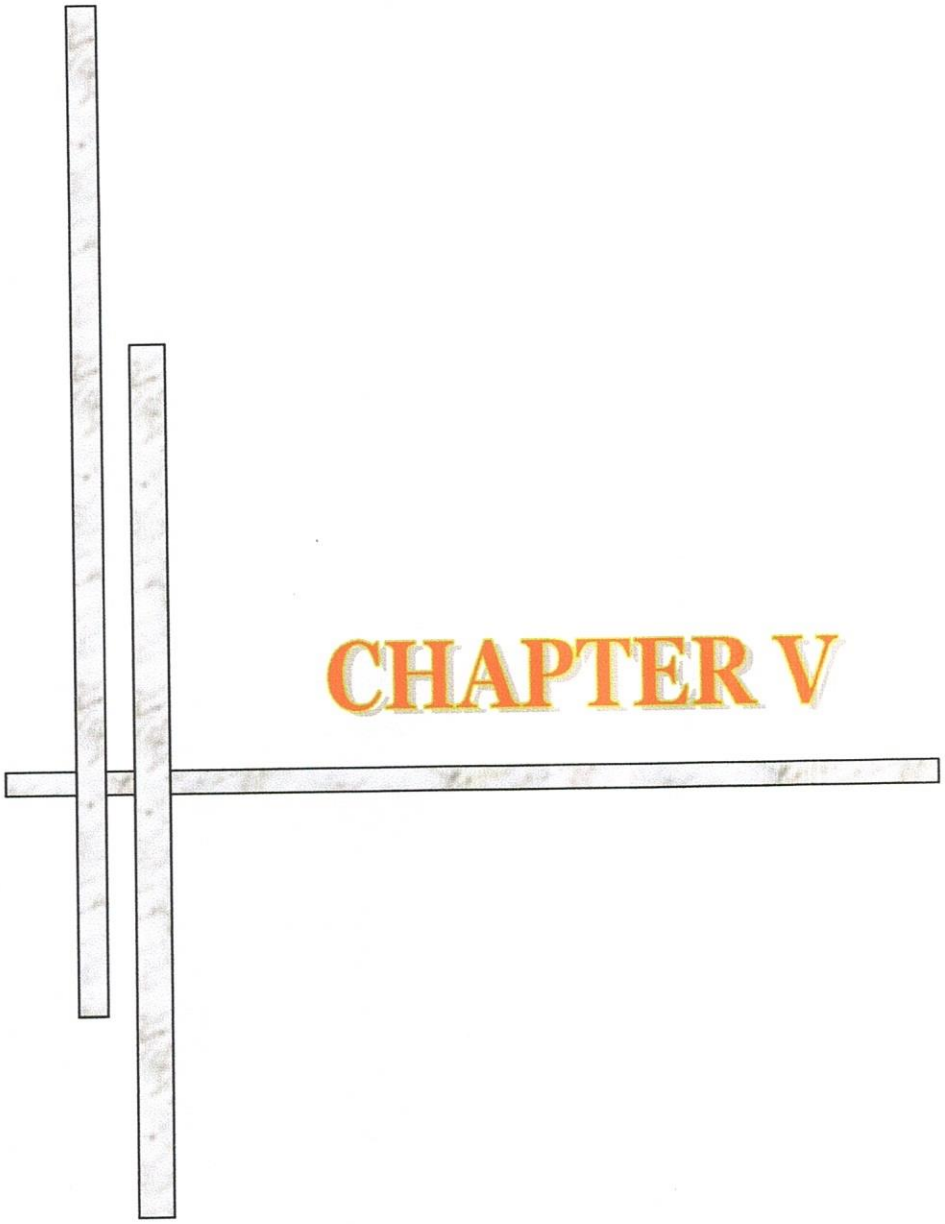
iii) Overcome the seasonal effects on seed germination and increase proper physical growth and fruit/seed production of water chestnut plant.

iv) Enhance to multiplication of shoot and rood number which produce more quantities of water chestnut fruits.

v) To obtain a large number of plant lets or branch within the short time through the year.

vi) Increase resistance capacity from the past, diseases and salted/alcalic/basic water of water chestnut.

Over all, the experiments will standardize some ecophysiolocal data which helps to build up a model of varietals improvement, increase production and sustain cultivation technique of water chestnut in Bangladeshi climate with advanced Bangladeshi variety.



CHAPTER V

MATERIALS AND METHOD

MATERIALS

Plant materials

Mature fresh fruits/seeds of *T. bispinosa* Rox. (var. green, var. red), mature fruits/seeds of *T. quadrispinosa* Rox. (var. wild) and cutting of those rossate with 3/4 node which were used as experimental materials in the present investigation collected during April/May 2011 from the early cultivated watery field of botanical garden at University of Rajshahi, Rajshahi, Bangladesh.

Chemical materials

Plant growth regulators (PGRs)

Plant growth regulators (artificial plant hormones) play significant role of physical and biological on plants. The following PGRs were used as the stimulant agents on water chestnut plants. Auxins and cytokinin were added to the stimulating media, GA_3 was used for germination stimulating media.

Auxins

Auxin promote cell enlargement and root initiation by Kyte, 1987; the following two type of auxin were used to fulfill the present experiments.

α-Napthalene acetic acid (NAA)

Indole acetic acid (IAA)

Cytokinins

Cytokinins promote cell division and shoot initiation by Kyte, 1987; the following two types of cytokinins were used to fulfill the present investigation.

6-Benzyl adenine (BA)

Indole- 3- butyric acid (IBA)

Gibberellins

Gibberellins influence cell enlargement and elongation by Kyte, 1987; the following Gibberellic acid was used to fulfill the present experiment.

Gibberellic acid (GA_3)

Fertilizers

Triple super phosphate (TSP)- [$3CaH_4(PO_4)_2\,4H_2O$]

Murate of potash (MOP)- [KCl]

Urea- [$CO(NH_2)_2$]

Glassware and instruments

Different Glassware and instruments were used for plant growth regulators preparation and measuring, fertilizer and water measuring. The following were used in the present experiment.

Others Laboratory Equipments

10 piece chari (large round mud pot)

10 piece plastic pots (small)

Plastic container (water carrier)

Beaker

Measuring slender

Scale

Filter paper

Marker pen

Level tag

METHODS

Preparation for stock solution of PGR

To preparation for stock solution of those growth regulators 10mg of solid powder were placed into a clean test tube and then dissolved in specific individual solvent. The volume of the solution was made up to 100ml by adding distilled water. Then it was stored in a refrigerator for ready use in time.

Table 1. Stock solution preparation of plant growth regulators (PGRs).

	Plant growth regulators	Amount taken as powder (mg)	Appropriate solvent to dissolve the PGR powder.	Final volume of the stock solution distilled water (ml)	Strength of the final solution	Storage temperature
Auxin	IBA	10	0.1N, KOH	100	0.1	-0^0C
	NAA	10	0.1N, KOH	100	0.1	$0\text{-}5^0$C
	IAA	10	0.1N, KOH	100	0.1	-0^0C
	GA_3	10	0.1N, H_2SO_4	100	0.1	-0^0C
Cytokini	BA	10	0.1N, KOH	100	0.1	$0\text{-}5^0$C
	IBA	10	0.1N, KOH	100	0.1	-0^0C

Preparation of treatment medium with different PGRs

For the shooting the medium was perpetrated to 15 liters fresh tap water with appropriate volume and strength follows that.

Table 2. Preparation of shooting medium with different PGRs

Require concentration of medium		Concentration of PGR	Fresh tap water (liter)	PGR have taken into the medium
NAA/IAA/ IBA/BA	1.0	0.1%	15	150ml
	2.0	0.1%	15	300ml
	5.0	0.1%	15	750ml
	10.0	0.1%	15	1500ml
	20.0	0.1%	15	3000ml

For the rooting the treatment medium was prepared to 30 liter fresh water with appropriate volume and strength follows that.

Table 3. Preparation of rooting medium with different PGRs

Require concentration of medium		Concentration of PGR	Fresh tap water (liter)	PGR have taken into the medium
IBA/NAA/IAA	0.1	0.1%	30	30ml
	0.2	0.1%	30	60ml
	0.5	0.1%	30	150ml
	1.0	0.1%	30	300ml
	2.0	0.1%	30	600ml
	5.0	0.1%	30	1500ml
	10.0	0.1%	30	3000ml

Germination test of water chestnut nut seeds

Cold treatment

At first fresh water chestnut seeds were taken in refrigerator with the 0-5^{0}C temperature for 120 hours continuously for breaking seed dormancy; after the period seeds were kept out from refrigerator and placed in germinating medium with GA_3 to promote germination.

Stimulation for germination

After cold treatment of water chestnut seeds were placed in two types medium as water and water with GA_3 (0.5ml/l) for 3-7 days besides different two types experiments was set in water and water with GA_3 without cold treatments for 3-7 days. The germination performances were record at 3, 5 and 7 days intervals respectively after further analysis.

Induction of axillary shoot of water chestnut

Water chestnut fruit/seeds which have started germination were treated (soaked) in three types plant growth regulator solutions (NAA, IAA and BA) at five concentrations (1.0, 2.0, 5.0, 10.0 and 20.0mg/l) in the room temperature (23.2 ± 2^{0}C) with 12hours (50μmol^{-2}5^{-1}) daily light for 7 days. Then the seeds

were steeped into a plastic pot (25×30×25cm), separately with 15 liters fresh tap water in Plant Breeding and Gene Engineering Laboratory of Rajshahi University (at 23.2±2^{0}C under at 12hr photo period and light intensity 1200μmol^{-2}5^{-1}). Two milliliter (2ml) of HYPON$_e$X^R (N:P:K= 1:2:1= 5:10:5g/15l) chemical fertilizer solution was applied in each pot. The experiment was randomized with four replications and per treatment 20 seeds have been taken. After 14 and 28 days of germination, main shoot (cotyledonary) number, main shoot (cotyledonary) length, cotyledonary branch number and cotyledonary branch length of the seedlings were observed and recorded on the data sheet for further analysis which shown in below.

Induction of adventitious root of water chestnut

Uniformly grown 20cm long shoot cutting (rossate) with 3/4 node of water chestnut (*Trapa*) plant were cut from the nursery bed of botanical garden at Rajshahi University. For each treatment 8 shoots were treated with three types plant growth regulators (NAA, IAA and IBA) solution at seven concentrations (0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0mg/l) for 5hours in the Plant Breeding and Gene Engineering Laboratory at the room temperature (23±2^{0}C). After the treatment the shoots were steeped into the water tank (32×35×32) contain 30 liters fresh tap water. Two milliliter (2ml) HYPON$_2$X^R and 4gm chemical fertilizer solution (mixture with 100gm urea, 100mg T.S.P and 100gm MOP = 1:1:1) was applied in the water and stored under the same laboratory and same condition which describe above.

After 14 and 21 days, formatted adventitious root number and length from the nodes of cotyledonary stem were observed and recorded on the data sheet which shown in below.

Data collection

After 20 and 30 days of treatment, shoot and root number of different stems (cotyledonary stem and lateral stem) was counted separately. Shoot and root length were carefully examined with the help of small scale.

Analysis of data

Mean ($\overline{X}$)**:** Data on the individual plant were added together then divided by the total number of observation that was obtained as follows:

Mean: $\overline{X} = \frac{\sum Xi}{N}$

Here, $\overline{X}$ = Mean

Xi = The individual reading recorded on each plant

X = The individual reading recorded from each plant

N = Number of observation

Σ = Summation.

Range: Highest value – Lowest value (shoot and root number/length)

Standard Deviation (SD) = (S)

Standard deviation is the average deviation at the individual observation from the mean. It was calculated as the square root of the variance of follows

SD: $S = \sqrt{S}^2$

S^2 = Variance and

$S^2 = \frac{(X - X)^2}{N}$

Here, $\overline{X}$ = The individual reading record from each plant.

N = Number of observation

Another,

Standard deviation (SD):

If instead of taking one sample, several samples are taken it is found that the standard deviations of the different samples also vary. The sample variation was measured by Standard deviation (SD of mean) which was determined as follows:

$$\text{SD: } S = \sqrt{\frac{\sum X^2 - (\sum X)^2 / N}{N - 1}}$$

Where,

SD = Standard Deviation of mean

X = Total number of shoots or roots

Σ = Summation

N = Number of observation

Yield Performance of water chestnut

After 60 days of cultivation (counted from plantation) the total yield performance was observed as total branch number (including branch length), total mass weight of plants, total flower number, total fruit number, total fruit number and yield per area by used different statistical terms to count yield or mass performances of water chestnut.

RESULT AND DISCUSSION

Germination performance in different medium with cold treatment

For seed germination, different treatment were used such as only fresh water with cold treatment, water without cold treatment, water and GA_3 solution with cold treatment and water plus GA_3 solution without cold treatment. In all treatment 30 seeds were used. Data were collected as percentage of germination and tabulated in table 4.

In the fresh water without cold treatment, 5 seeds (30%) were germinated to green, red, and 20% wild water chestnut after 7 days incubation. When 63% green, red, and 83% wild water chestnut seed germinated with 120 hours cold treatment after 7 days cultured period. Besides, in water + GA_3 solution green, red 40% and wild 33% found germination rate without cold treatment, and green 93%, red 80% and wild 97% showed germination performance with cold treatment after 7 days intervals of culture period.

Several times, it found cold treatment increase germination rate within short time. Water and GA_3 solution was more effective than only water and water with GA_3 without cold treatment was less effective than the water and GA_3 with cold treatment. GA_3 and cold treatment successfully stimulate germination rate than normal condition, besides it decries time elongation. So, in this experiment it is clearly found that, water and GA_3 solution will needed for better germination rate within short time (result shown in table 4). And cold treatment also needed to breakdown seed dormancy of water chestnut. It prevents more seeds loss and more time being for water chestnut cultivation in Bangladesh.

Table 4. *In vitro* germination performance in different medium with different cold treatment period 30 seeds (nuts) were used for the germination sensitivity test of water chestnut.

Treatment	**Germination**					
	var. green		**var. red**		**var. wild**	
With cold treatment	Water	GA_3 + water	Water	GA_3 + water	Water	GA_3 + water
3 days	3	5	2	4	6	7
5 days	7	10	8	9	8	9
7 days	9	13	9	14	11	13
% of germination	63%	93%	63%	80%	83%	97%
Without cold treatment						
3 days	1	2	2	2	1	2
5 days	3	4	2	4	2	4
7 days	5	6	5	6	3	4
% of germination	30%	40%	30%	40%	20%	33%

*Cold treatment carried with 05-06^{0}C

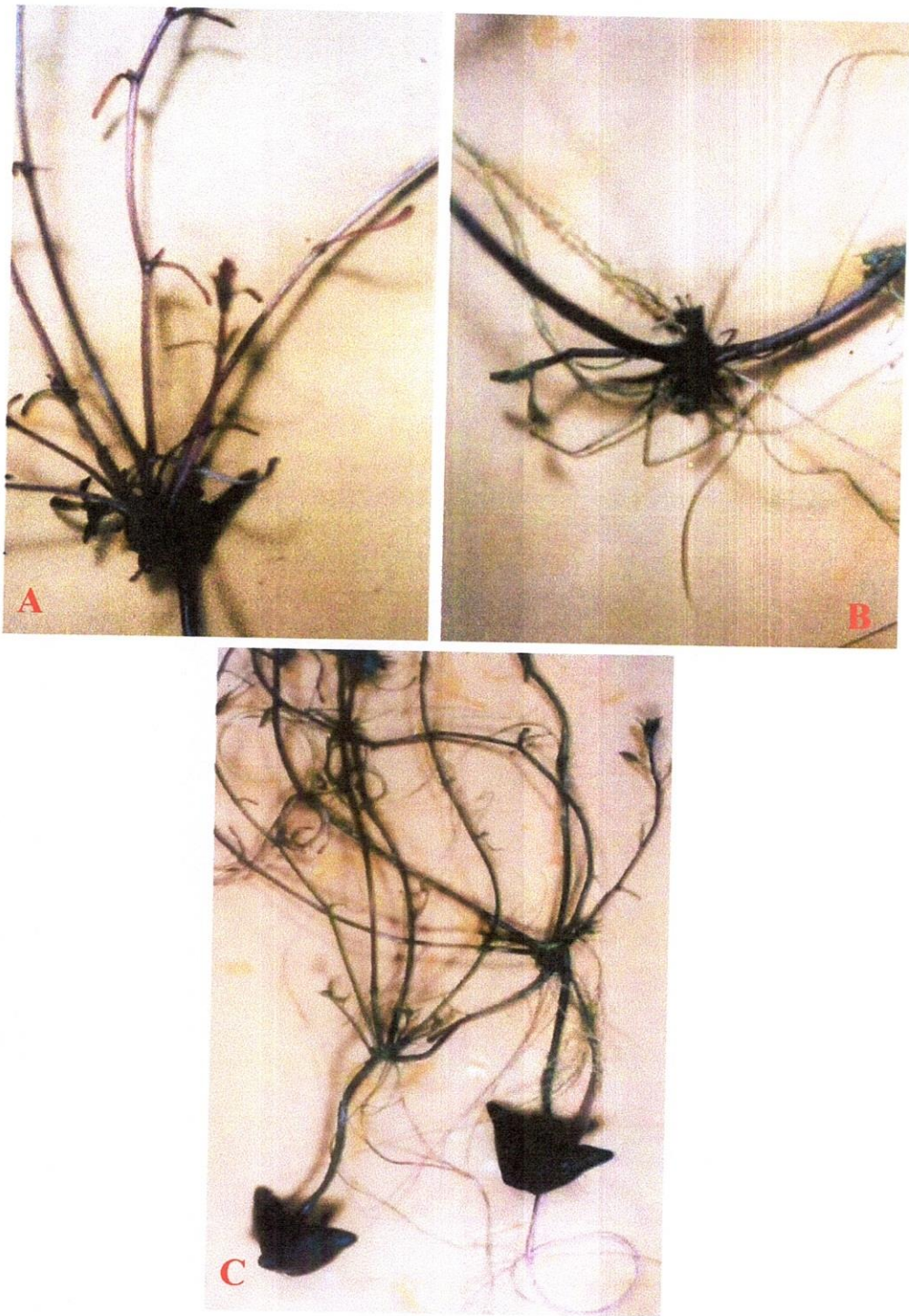


Figure 1. Growth regulators performance on multiple shoots and roots induction of water chestnut

A. Shoot multiplication, found the top result, B. Root initiation from coteyledonary node of water chestnut, C. Multiple shoot and root initiation of water chest nut after hormonal treatments

Axillary shoot formation

Seedlings of water chestnut treated for 7 days showed better performances of shoot induction and axillary shoot production than the control. Here we describe that (table 5, 6, 7, 8 and figure 2abc, 3abc, 4abc, 5abc) the result obtained for the former. Among the various (NAA, IAA and BA) growth regulators BA caused more than twofold shoot proliferation form the cotyledonary node of water chestnut seedlings 28 days after germination. Green water chestnut showed effective response to shoot multiplications than the red and wild varieties. Thus shoot length and axillary shoot number more proliferated. So, BA is known to be the most effective synthetic cytokinine of increase axillary shoot production in different plant (picture) and among the different concentration of 5.0mg/l BA induced the best responded for shoot proliferation and axillary shoot production from the cotyledonary node of water chestnut plantlets. Under *in vitro* conditions similar effect was reported by Ganogpadhgay *et. al.* (1998) in sesame and they found that maximum number of shoots were optimized at the concentration of 2.0-8.0mg/l.

NAA promoted only shoot length but few responses to the axillary shoot production (figure 2abc, 3abc, 4abc, 5abc). IAA similarly responded to NAA as less performed to multiple shoot formations. Shoot length up to increase based on the high concentration of PGR, besides axillary shoot number caused few and small. IAA is more effective to stimulating shoot length and axillary shoot production than NAA growth regulator.

We know that auxin inhibits axillary bud growth where as cytolinine (BA) promotes it. Auxin and cytokinine in the release of buds. Generally, axillary shoots energy from lower node to upper node of main shoot gradually but untreated. Shoot do not produce axillary shoots up to 40days after germination. The length of axillary shoots up to the 6^{th} node position in shown in the length of 1^{st} and 2^{nd} emerged shoot fro the cotyledonary node of treated plant was always shorter than that of control. Though growth regulator treatment, shoot

number of seedlings increases significantly with BA, but the growth of each shoot decreased uniformly because of the loss of apical dominance. Therefore an alternate technology to promote growth of an individual, shoot will be necessary to sue BA treatment for better shoot production.

Table 5. Average shoot number induction from coteylednary node after 14 days sowing of 7 days of hormonal treatment on different nodal position from the base.

PGR	**Shoot number**						
	Con. (mg/l)	**var. green**		**var. red**		**var. wild**	
		Average	**SD**	**Average**	**SD**	**Average**	**SD**
NAA	1	1.39	0.04	1.29	0.33	1.21	0.04
	2	1.54	0.21	1.42	0.41	1.25	0.21
	5	1.62	0.13	1.66	0.13	1.33	0.13
	10	1.71	0.28	1.89	0.42	1.48	0.40
	20	2.10	0.07	2.87	0.87	1.63	0.34
IAA	1	1.98	0.20	1.29	0.30	1.44	0.37
	2	2.55	0.06	1.53	0.24	1.57	0.06
	5	2.60	0.47	1.65	0.47	1.65	0.33
	10	1.90	0.17	2.36	0.38	1.72	0.31
	20	2.42	0.22	2.88	0.35	2.10	0.09
BA	1	2.51	0.15	2.38	0.46	1.50	0.22
	2	3.63	0.21	3.57	0.53	2.78	0.07
	5	5.03	0.77	5.25	0.62	4.02	0.82
	10	3.46	0.12	3.46	0.28	3.46	0.77
	20	3.39	1.05	3.32	0.05	3.32	0.39
Control		3.06	0.42	2.49	0.33	2.32	0.11

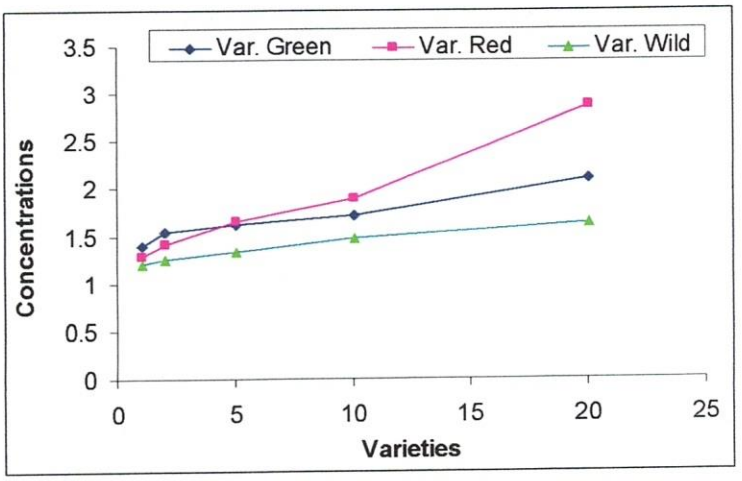


Fig. 2a. For NAA

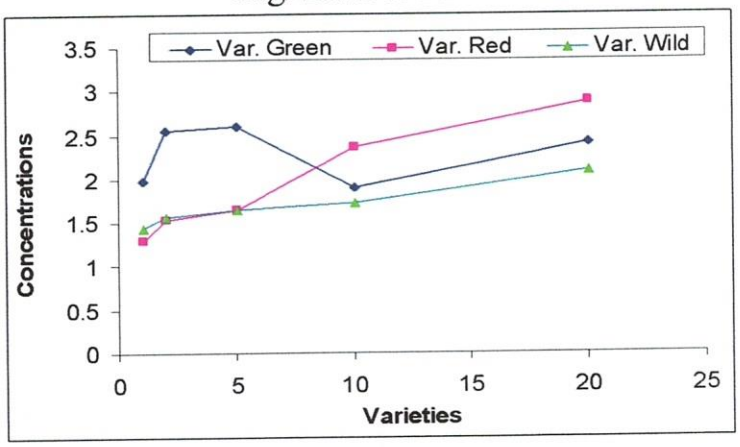


Fig. 2b. For IAA

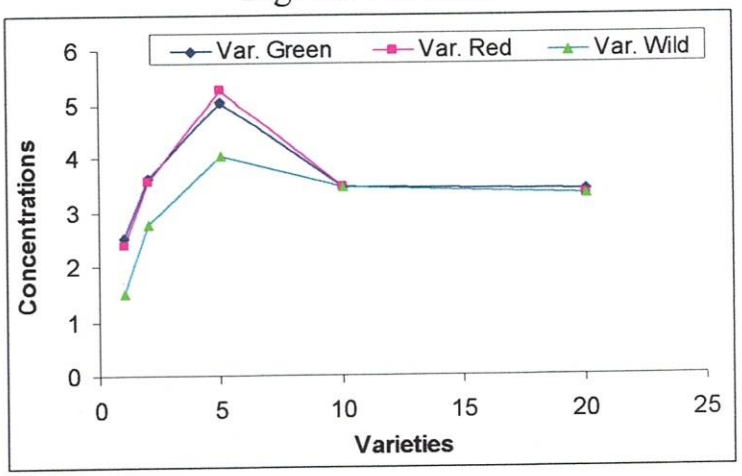


Fig. 2c. For BA

Figure 2 (Fig. 2a- 2c.). Average shoot number induction from coteylednary node after 14 days sowing of 7 days of hormonal treatment on different nodal position from the base.

In order to shoot induction performance after 14 days sowing of 7 days hormonal treatments in BA 5ml/l showed better performance of shoot number formation, green variety showed best performance than red variety (5.02mg/l) and wild variety showed less response compared to green and red variety, but wild variety formed small number of shots. When in NAA (1.71 for green, 2.87 for red & 1.63 for wild) and IAA (2.60mg/l for green, 2.88mg/l for red & 2.10mg/l for wild) found low response for shoot induction than BA, NAA and IAA shows better performance in high concentration (5mg/l - 20mg/l) but those responses was much varied with BA in different variety. Control plant shows only more than single (1.36) shoot. Green variety showed much performance than the red and wild varieties, red showed moderate response and wild showed less response, presented in table 5 and fig. 2abc.

Table 6. Average shoots number induction from coteylednary node after 28 days sowing of 7 days of hormonal treatment on different nodal position from the base.

PGR	Con. (mg/l)	Shoot number					
		var. green		var. red		var. wild	
		Average	SD	Average	SD	Average	SD
NAA	1	1.32	0.62	1.21	1.06	1.12	1.01
	2	1.46	0.22	1. 33	0.31	1.27	0.41
	5	1.56	1.15	1.46	1.08	1.46	2.06
	10	2.78	2.05	1. 67	2.33	1.67	1.08
	20	2.86	1.02	2. 34	1.09	1.84	1.02
IAA	1	1.19	2.25	1.30	0.25	1.19	0.25
	2	1.02	0.29	1.33	2.29	1.34	2.04
	5	2.11	1.42	1.66	0.03	1.55	0.29
	10	2.21	0.47	2.47	2.25	1.63	3.01
	20	2.35	1.59	2.51	0.59	1.78	2.59
BA	1	2.21	2.16	2.31	2.91	2.16	2.91
	2	2.17	1.30	2.58	0.27	2.61	1.28
	5	6.31	0.02	5.92	1.31	5.07	3.06
	10	2.25	2.73	2.12	2.73	2.87	1.21
	20	2.02	1.46	1.79	0.46	1.54	0.11
Control		4.08	0.21	2.91	0.22	2.85	0.14

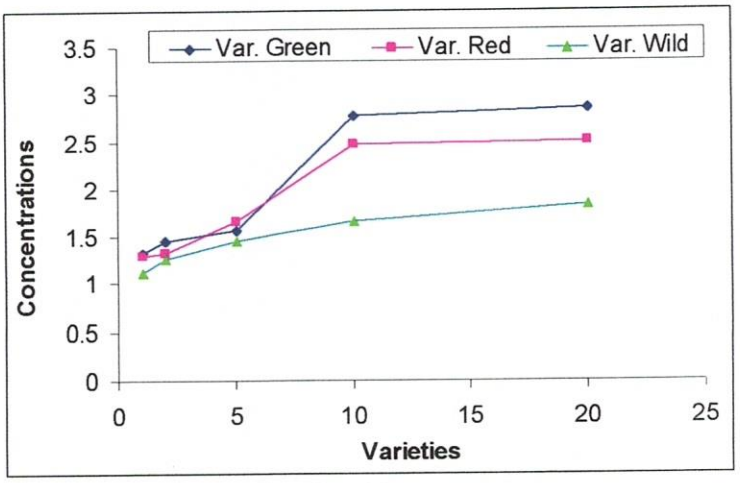


Fig. 3a. For NAA

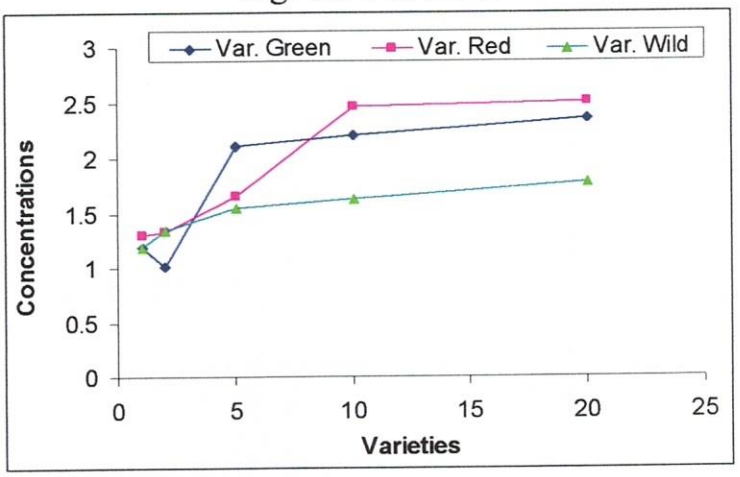


Fig. 3b. For IAA

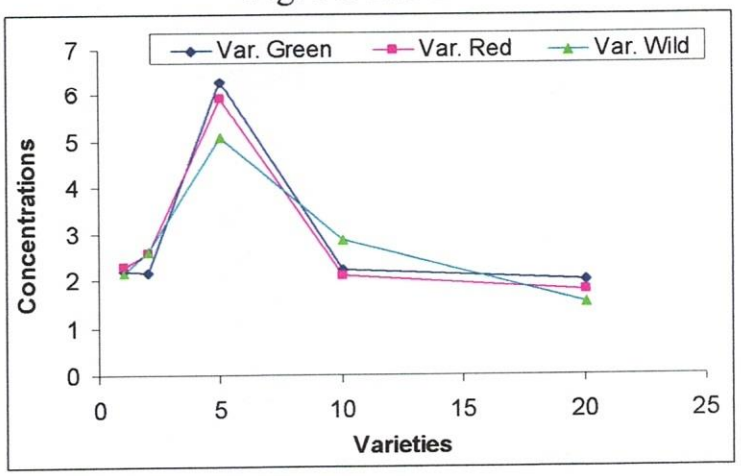


Fig. 3c. For BA

Figure 3 (Fig. 3a- 3c.)**.** Average shoots number induction from coteylednary node after 28 days sowing of 7 days of hormonal treatment on different nodal position from the base.

In the table 6 and fig. 3abc, 28 days sowing after 7 days PGR treatment in different concentration (1mg/l, 2mg/l, 5mg/l, 10mg/l, 20mg/l), BA found the most effective response of shoot induction (6.31) and 0.5mg/l concentration formatted most response among the different concentrations. NAA (2.86 for green, 2.34 for red, 1.84 for wild at 20mg/l) and IAA (2.35 for green, 2.51 for red, 1.78 for wild at 20mg/l) showed moderate performance in high concentration but the response was not significant compared to BA response.

Table 7. Average shoot length proliferation of induced axillary shoots after 14 days sowing of 7 days of hormonal treatment.

PGR	**Shoot length** (cm)					
	Con. (mg/l)	**var. green**		**var. red**		**var. wild**	
		Average	**SD**	**Average**	**SD**	**Average**	**SD**
NAA	1	9.35	0.61	8.43	1.21	7.91	1.01
	2	9. 48	0.73	9. 79	0.22	8.59	0.44
	5	10.62	1.05	10.02	0.81	8.79	1.06
	10	10.86	1.12	10.23	1.52	9. 07	0.33
	20	11.23	0.17	11.48	1.28	9.25	1.04
IAA	1	10.32	0.01	9.90	1.34	8.54	0.12
	2	11.25	0.06	10.08	0.62	9.03	0.43
	5	11.58	1.44	10.36	0.91	9.61	1.02
	10	12.22	0.02	11.39	1.08	9.77	0.61
	20	12.36	1.20	11.51	0.70	9.94	1.09
BA	1	11.80	0.22	10.80	0.45	9.80	0.36
	2	12.60	1.53	11.60	0.66	9.60	1.06
	5	15.05	2.19	14.76	1.93	12.72	2.89
	10	14.70	0.42	13.89	0.43	12.05	1.02
	20	13.42	1.33	12.30	1.27	11.30	0.86
Control		6.32	0.61	6.26	0.32	5.44	0.65

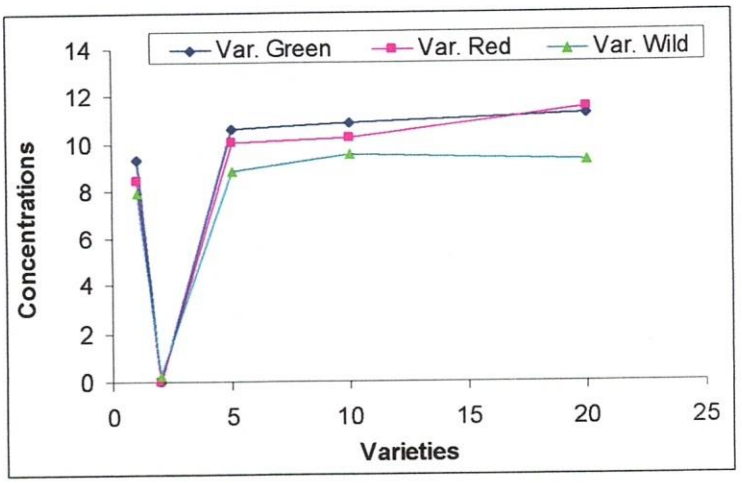


Fig. 4a. For NAA

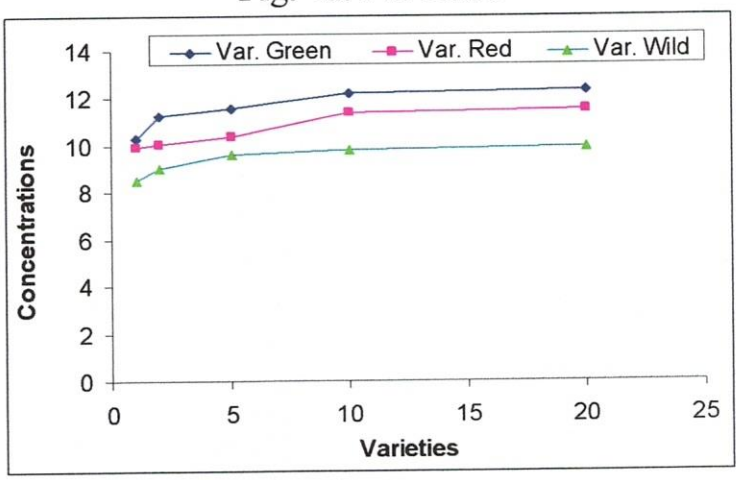


Fig. 4b. For IAA

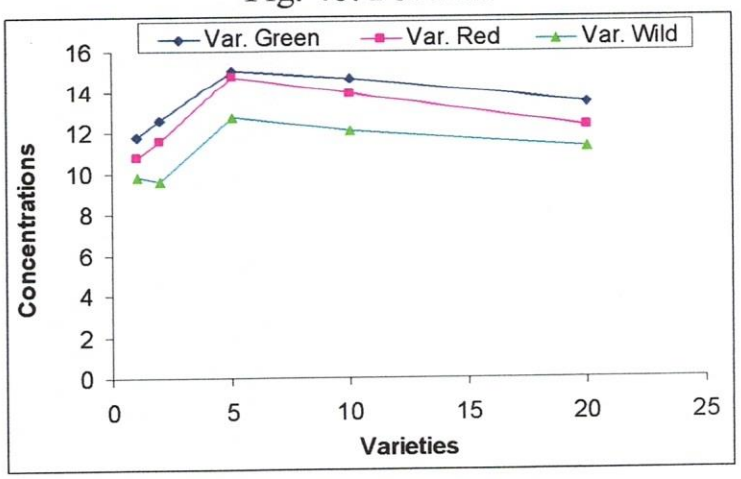


Fig. 4c. For BA

Figure 4 (Fig. 4a- 4c.). Average shoot length proliferation of induced axillary shoots after 14 days sowing of 7 days of hormonal treatment.

In table 7 and fig. 4abc presented here that, hormonal treatment of water chestnut seedling showed the better performance than the control. BA in 5mg/l found most proliferated response average 15.05 in green variety, red and wild variety respond same to green, varied little intervals of shoot length. NAA and IAA showed effective response than the control but less than BA, NAA showed moderate response as 11.23 for green, 11.48 for red & 9.25 for wild and IAA response was optimized than NAA response as 12.36 for green, 11.51 for red & 9.94 for wild in high concentration. IAA caused better response than NAA for shoot elongation and proliferation.

Table 8. Average shoot length proliferation of induced axillary shoots after 28 days sowing of 7 days of hormonal treatment.

PGR	**Shoot length** (cm)						
	Con. (mg/l)	**var. green**		**var. red**		**var. wild**	
		Average	**SD**	**Average**	**SD**	**Average**	**SD**
NAA	1	16.02	1.05	15.61	1.80	14.21	1.05
	2	16.31	1.09	15.41	1.76	14.33	1.21
	5	16.73	2.18	16.95	2.92	14.37	2.04
	10	16. 45	3.15	16.15	3.13	14.48	3.03
	20	16.53	2.02	15.58	2.33	14.73	2.01
IAA	1	16.42	3.03	16.54	1.21	14.26	2.03
	2	16.66	1.27	16.67	2.90	14.44	2.34
	5	17.08	2.79	18.44	3.22	14.68	3.07
	10	17.30	2.03	18.64	2.05	14.81	2.01
	20	17.27	1.34	18.50	1.85	14.98	1.03
BA	1	16.30	2.27	17.38	2.06	14.32	2.09
	2	17.52	2.15	18.05	3.15	14.64	3.01
	5	20.21	3.04	19.13	2.22	14.92	3.24
	10	18.87	3.21	19.25	3.55	16.09	3.05
	20	17.78	2.17	18.72	2.07	14.88	2.02
Control		8.84	0.54	8.55	1.01	7.9	0.22

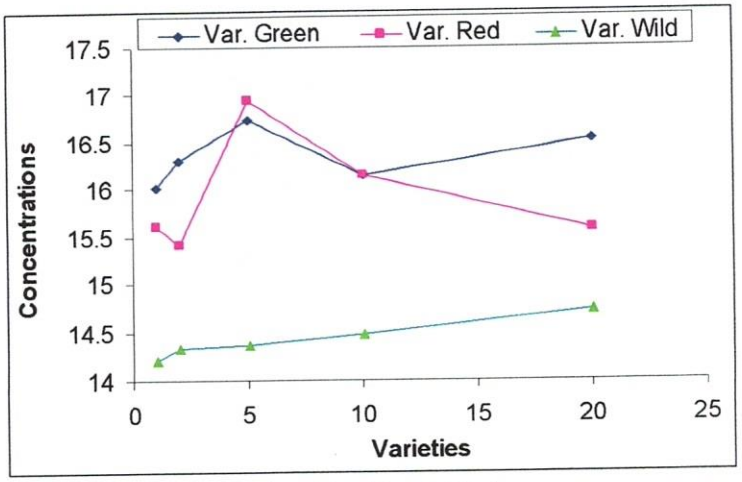


Fig. 5a. For NAA

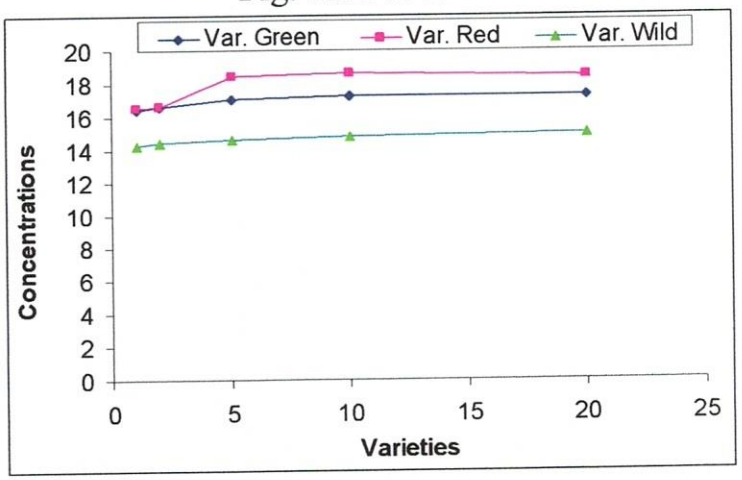


Fig. 5b. For IAA

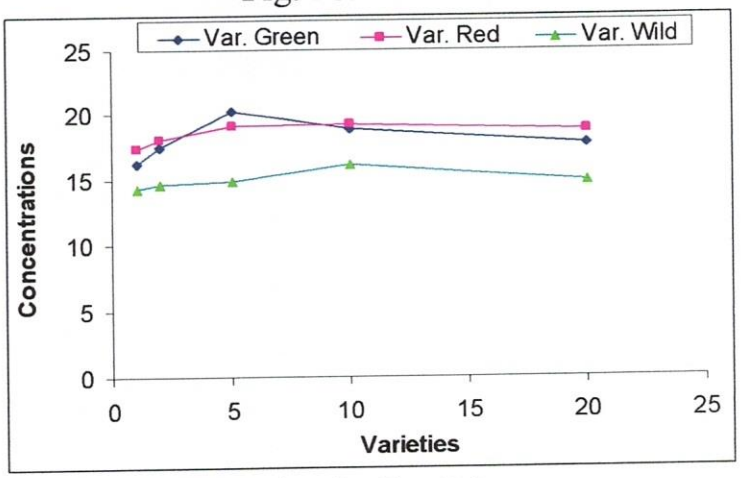


Fig. 5c. For BA

Figure 5 (Fig. 5a- 5c.)**.** Average shoot length proliferation of induced axillary shoots after 28 days sowing of 7 days of hormonal treatment.

After 7 days hormonal treatment and 28 days of sowing period observed that BA increased best shoot length (20.21) in 5mg/l concentration of green water chestnut, 19.25 found in 10mg/l for red and 16.09 found for wild water chestnut at the same concentration, With BA green variety response at low concentration (5mg/l) and red & wild variety respond at high concentration (10mg/l). Same response found in 10mg/l for green (17.30) and red (18.64), was the top shoot length record with the IAA, while red variety more effective respond and wild water chestnut respond in high concentration in IAA. NAA showed top result at 5mg/l for green (16.73) and red (16.95) and wild variety showed better result (14.73) at the 10mg/l. Over all BA showed best result (at 5mg/l) for shoot length elongation, IAA showed moderate and NAA showed average result respectively which is shown in table 8 and fig. 5abc.

Adventitious root formation

In order to the various concentration of 1mg/l NAA caused the more root number, it is most effective to stimulating the root number and root length. IAA cause the longest root at higher concentration (10.0mg/l) but less than IBA caused at 1mg/l concentration increase maximum root length. Thus the NAA proliferated maximum root number and average root length (Table 9, 10, 11, 12 and fig 6abc, 7abc, 8abc, 9abc).

IBA, NAA and IAA are the generally used auxins for root induction. (Ellyard, 1981; Nemeth, 1986; Rajani & Urs, 1988). The number of root per seedlings after 21 days of steeping with 5th treatment of IBA and IAA showed the same growth rate, but root length significantly by NAA grater than the performance of 14 days with 5hr treatment.

After 21 days of steeping 0.2mg/l IBA, 0.1mg/l NAA and 10.0mg/l IAA induced greatest root length of and respectively up to the 5 node from the base. Different concentration of PGR are important factors for effective to induction maximum number and length of roots formed and most effective for adventitious root induction, followed by NAA which can be helpful in early establishment of plants during transplanting of water chestnut.

Table 9. Average root number of axillary shoots after 14 days of sowing 5 days of hormonal treatment.

PGR	Con. (mg/l)	Shoot number					
		var. green		var. red		var. wild	
		Average	SD	Average	SD	Average	SD
NAA	0.1	3.55	2.02	3.64	2.06	3.33	1.41
	0.2	3.61	2.30	3.79	3.03	3.60	2.03
	0.5	3.70	0.33	4.55	1.54	4.56	1.24
	1	4.22	1.32	4.61	1.11	4.47	1.09
	2	5.51	0.83	5.51	0.74	6.55	0.36
	5	4.92	2.18	4.55	2.56	5.83	1.08
	10	4.45	2.20	4.39	2.23	5.71	2.09
IAA	0.1	2.70	2.15	2.45	2.08	3.49	2.25
	0.2	3.89	1.32	3.67	0.07	3.55	0.18
	0.5	3.87	0.21	3.28	2.23	4.17	2.16
	1	4.14	1.43	4.09	1.05	5.38	0.12
	2	3.78	0.01	3.56	2.33	4.27	2.29
	5	3.51	1.07	3.44	0.26	4.20	1.21
	10	3.56	0.26	3.19	1.03	4.16	2.02
IBA	0.1	3.65	2.20	2.56	2.34	3.44	2.02
	0.2	3.63	1.55	3.29	2.56	3.94	1.11
	0.5	3.77	0.01	3.37	2.01	3.88	0.01
	1	4.20	2.41	4.06	2.04	4.40	2.28
	2	4.42	1.29	4.26	2.29	4.58	1.04
	5	4.01	0.05	3.63	2.09	4.45	2.42
	10	3.94	2.21	3.44	2.35	3.23	0.05
Control		2.39	0.32	2.66	0.51	2.15	0.58

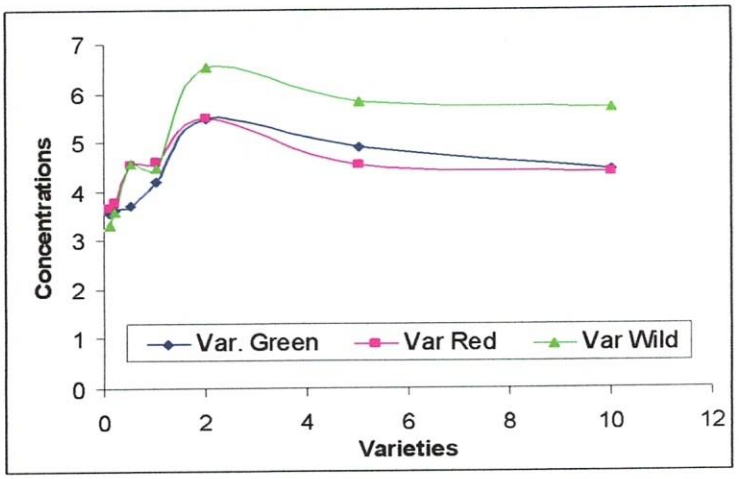


Fig. 6a. For NAA

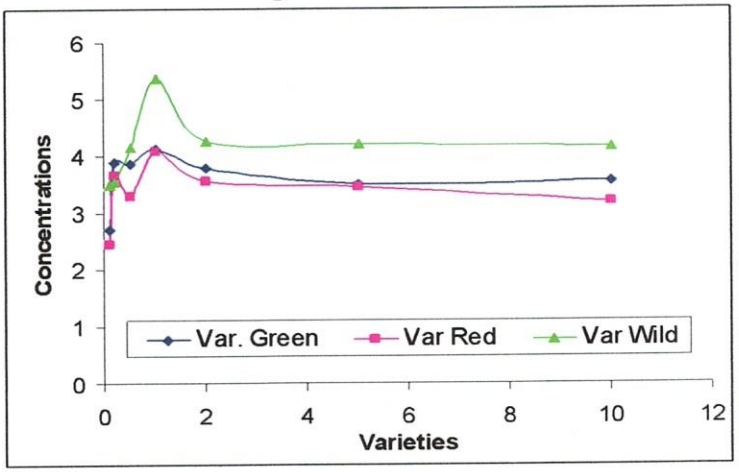


Fig. 6b. For IAA

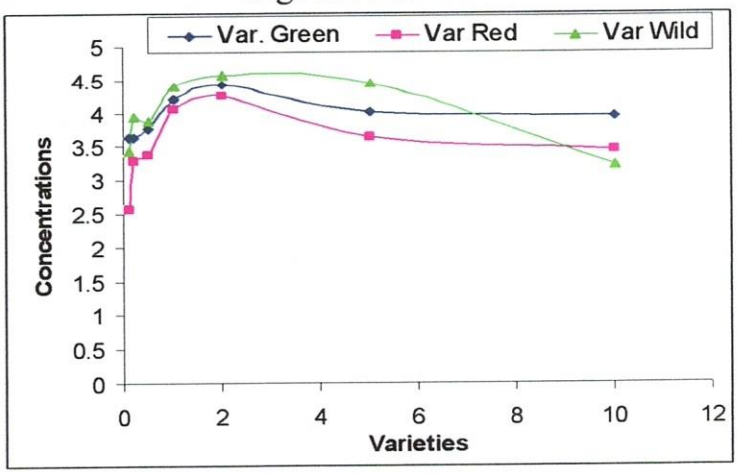


Fig. 6c. For IBA

Figure 6 (Fig. 6a- 6c.)**.** Average root number of axillary shoots after 14 days of sowing 5 days of hormonal treatment.

For root initiation in seven concentrations and three type's (NAA, IAA and IBA) hormone were treated on different varieties of water chestnut (table 9 and fig. 6abc). Wild water chestnut showed best performance in different hormone at 1- 2mg/l hormonal concentration and NAA showed best root initiation (6.55) at 2mg/l, while second performance found 5.38 of root initiation at 1mg/l IAA and 4.58 at 2mg/l IBA. NAA showed the best performance than IBA and IAA. NAA caused best rooting 5.51 for green and red water chestnut at the 2mg/l, when IAA caused best rooting (4.14 for green and 4.09 for red), and IAA showed less performance (4.01 for green 3.94 for red varieties) compare to others different at 1mg/l respectively.

Table 10. Average root number of axillary shoots after 21 days of sowing 5 days of hormonal treatment.

PGR	Con. (mg/l)	Shoot number					
		var. green		var. red		var. wild	
		Average	SD	Average	SD	Average	SD
NAA	0.1	5.03	2.83	4.22	0.07	4.11	2.64
	0.2	5.00	1.97	5.21	0.18	3.34	1.07
	0.5	5.92	4.01	5.62	1.16	3.50	3.02
	1	8.25	0.09	8.18	0.32	3.65	0.11
	2	7.50	0.08	7.72	0.17	5.32	2.67
	5	5.17	0.52	4.13	0.16	4.61	0.14
	10	5.53	0.39	4.25	0.08	4.56	0.05
IAA	0.1	4.58	0.32	3.12	2.23	4.26	0.26
	0.2	4.05	2.14	3.44	0.09	4.30	1.43
	0.5	4.57	0.04	3.51	1.21	4.45	0.20
	1	4.26	0.08	3.64	0.34	4.51	1.03
	2	5.78	1.75	5.61	1.51	4.64	0.55
	5	5.00	0.74	5.01	0.52	4.98	0.67
	10	3.44	0.90	3.55	1.25	4.77	0.23
IBA	0.1	4.11	0.29	3.31	0.67	4.23	0.08
	0.2	4.78	1.53	3.19	1.08	4.47	1.34
	0.5	3.81	1.04	3.36	0.09	4.36	0.12
	1	4.66	0.79	4.51	2.14	4.11	1.23
	2	4.89	0.09	4.85	0.67	5.02	1.02
	5	5.93	0.18	5.71	0.34	5.61	1.12
	10	5.42	1.82	5.30	1.54	5.26	0.07
Control		4.21	0.65	4.34	0.23	4.02	0.34

According three types of plant growth regulators NAA caused the best performance of adventitious root induction, Green and red water chestnut best performed (8.25 & 8.18) at 1mg/l NAA, but wild water chestnut showed best performance (5.32) at 2mg/l NAA. Relatively best perforce of root initiation found in IBA (5.93 for green, 5.71 for red & 5.61 for wild water chestnut) at the same concentration (5mg/l) of different varieties water chestnut but less than NAA. IAA induced moderate rooting, that were 5.78 in green, 5.61 in red and 4.98 in wild. In IAA green and red varieties showed best performance (5.78 and 5.61) at the same concentration (2mg/l) but wild water chestnut best performed found (4.98) at 5mg/l, shown in table 10 and fig. 7abc.

Table 11. Average root length of formatted axillary shoots after 14 days of sowing 5 days of hormonal treatment.

PGR	Con. (mg/l)	Root length (cm)					
		var. green		var. red		var. wild	
		Average	SD	Average	SD	Average	SD
NAA	0.1	1.59	0.09	1.84	0.81	1.04	0.65
	0.2	1.78	0.11	2.06	0.11	1.18	0.03
	0.5	2.30	1.08	2.31	0.84	2.21	0.57
	1	2.63	0.06	2.36	1.07	2.29	1.04
	2	2.58	0.05	2.28	0.81	2.27	0.45
	5	1.41	1.01	2.07	1.06	1.65	1.08
	10	1.34	0.30	1.66	0.67	1.53	0.06
IAA	0.1	2.44	1.06	1.21	1.47	1.03	0.09
	0.2	2.51	0.76	2.43	2.04	2.12	0.05
	0.5	2.65	1.14	2.67	1.14	2.73	1.14
	1	2.83	2.07	2.75	0.03	2.74	0.43
	2	2.33	0.43	2.96	0.80	2.79	1.01
	5	2.28	1.02	2.53	1.72	2.68	1.02
	10	2.10	0.04	2.29	0.74	2.32	0.74
IBA	0.1	3.22	1.36	3.10	1.71	2.28	0.05
	0.2	3.38	0.05	3.23	0.02	3.33	1.09
	0.5	3.43	0.98	3.54	0.01	3.41	0.68
	1	3.44	2.46	3.65	1.13	3.36	1.03
	2	3.40	1.06	3.52	1.37	3.53	1.07
	5	3.22	0.24	3.34	0.60	3.35	1.21
	10	2.17	0.60	3.21	0.05	2.19	0.75
Control		2.67	0.03	3.29	0.17	2.07	0.12

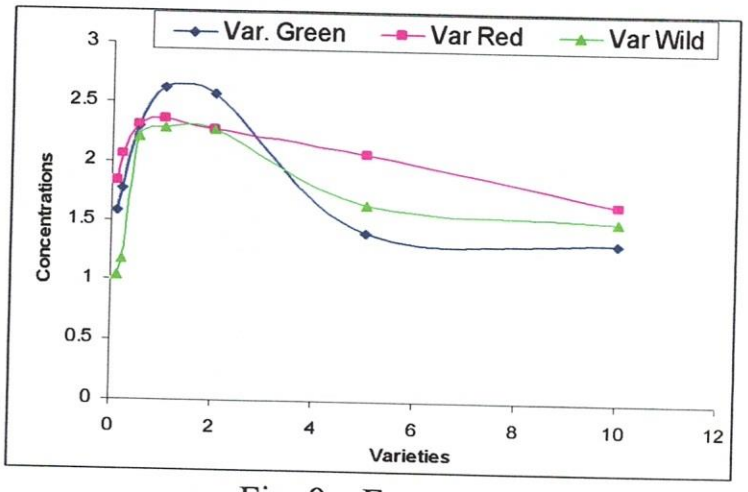


Fig. 8a. For NAA

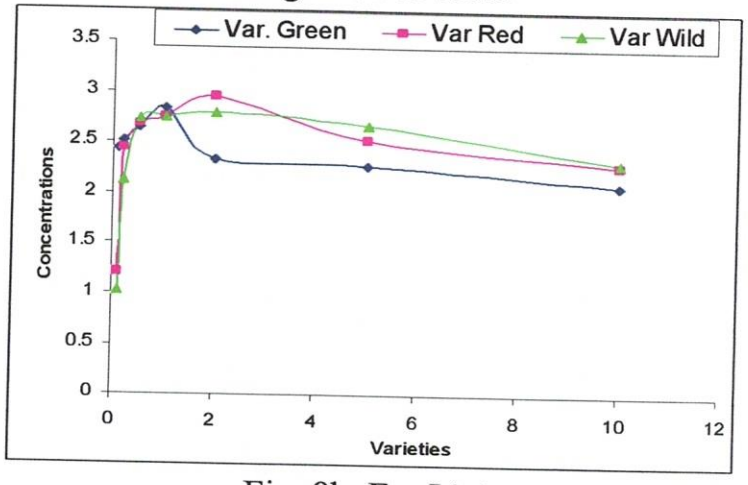


Fig. 8b. For IAA

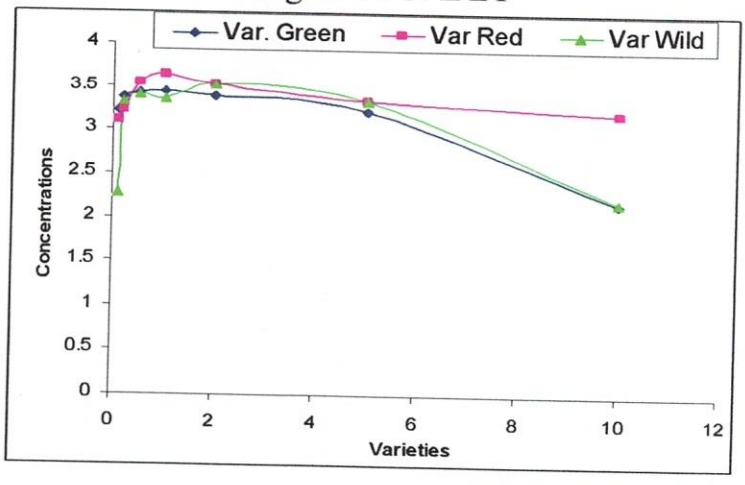


Fig. 8c. For IBA

Figure 8 (Fig. 8a- 8c.). Average root length of formatted axillary shoots after 14 days of sowing 5 days of hormonal treatment.

The length of adventitious root was successfully increased than the control in different varieties in different concentrations. IBA showed the best performance to root length elongation for green was 3.44, for red was 3.56 and for wild was 3.36 at the same concentration, that was 1mg/l. In IAA found top result 2.83 at 1mg/l of green water chestnut, 2.96 at 2mg/l while wild variety showed better performance 2.79 at 2mg/l. Average performance found in NAA (2.63 at 1mg/l in green, 2.26 and 2.29 at 2mg/l in red and wild water chestnut). In IAA showed different stimulating performance among the seven concentrations, red and wild verities showed similar nature to top elongation respond were found at the higher concentration (2mg/l) than IBA and NAA but in IBA and IAA, three varieties found top result in similar concentration (1mg/l) with varied performance, presented in table 11 and fig. 8abc.

Table 12. Average root length of formatted axillary shoots after 21 days of sowing 5 days of hormonal treatment.

PGR	Con. (mg/l)	Root length (cm)					
		var. green		var. red		var. wild	
		Average	SD	Average	SD	Average	SD
NAA	0.1	3.11	2.29	4.65	2.32	3.53	1.65
	0.2	3.05	1.53	5.56	1.04	5.05	0.11
	0.5	4.56	2.04	4.65	2.01	3.52	2.29
	1	4.05	2.21	4.32	0.08	4.05	1.02
	2	4.26	2.09	4.05	1.75	4.26	3.04
	5	5.01	2.18	4.99	0.74	4.01	0.33
	10	4.10	1.82	3.67	2.32	4.46	2.03
IAA	0.1	4.81	2.29	4.87	2.76	3.60	1.34
	0.2	5.11	1.53	5.28	1.53	4.43	0.21
	0.5	5.78	1.03	5.53	1.04	4.68	1.34
	1	6.72	2.04	6.56	0.79	4.87	2.07
	2	5.89	0.09	5.64	1.90	4.49	1.27
	5	5.61	1.18	5.23	2.05	4.37	0.03
	10	6.32	0.07	4.43	1.04	3.05	1.29
IBA	0.1	6.07	1.32	5.53	3.43	5.67	2.03
	0.2	6.61	0.09	6.04	1.21	5.73	0.53
	0.5	7.92	0.65	6.71	0.75	5.92	3.01
	1	8.07	2.41	7.79	2.02	6.90	0.04
	2	7.70	1.57	6.73	0.96	6.56	2.06
	5	6.46	0.60	5.99	1.53	5.40	0.02
	10	5.81	0.75	5.12	1.29	5.23	1.06
Control		5.16	0.66	5.10	0.34	4.88	0.54

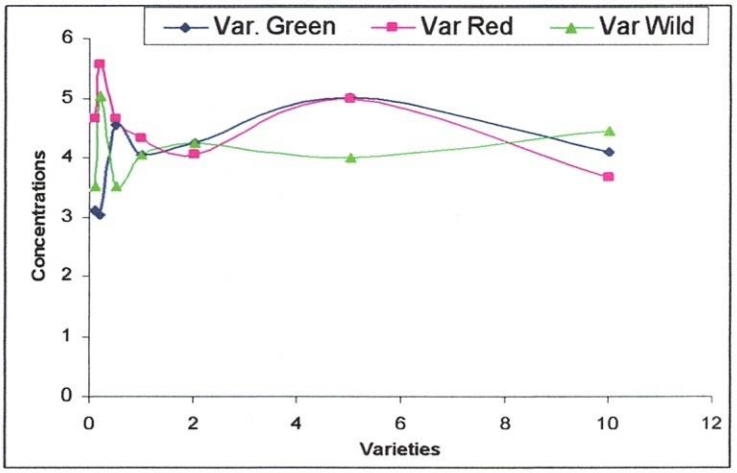


Fig. 9a. For NAA

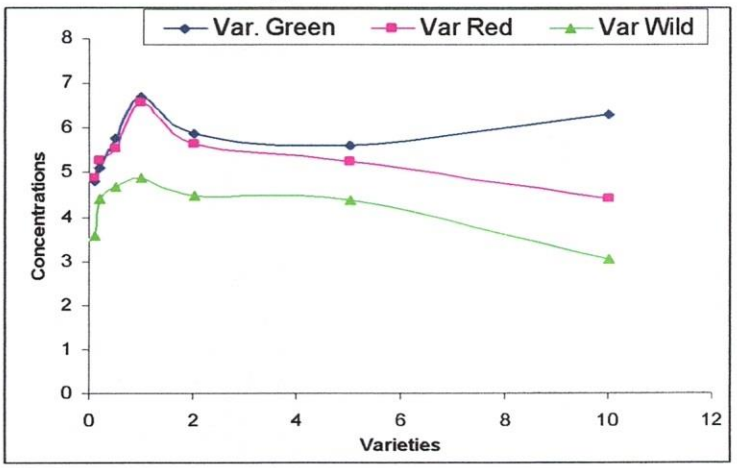


Fig. 9b. For IAA

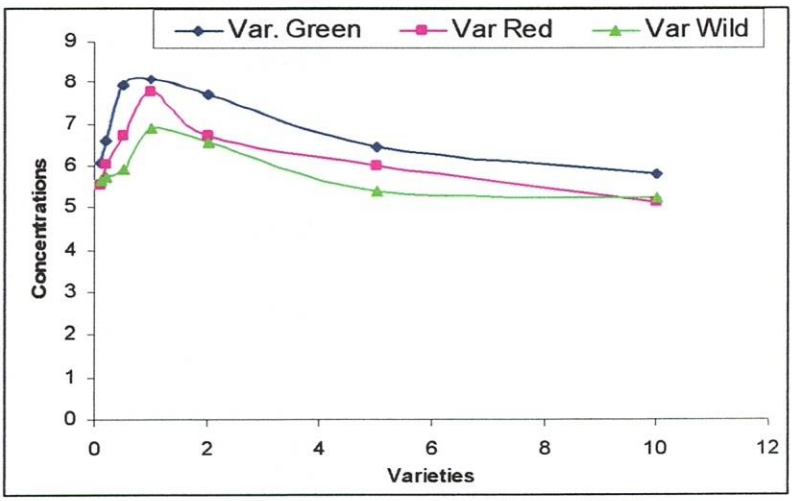


Fig. 9c. For IBA

Figure 9 (Fig. 9a- 9c.)**..** Average root length of formatted axillary shoots after 21 days of sowing 5 days of hormonal treatment.

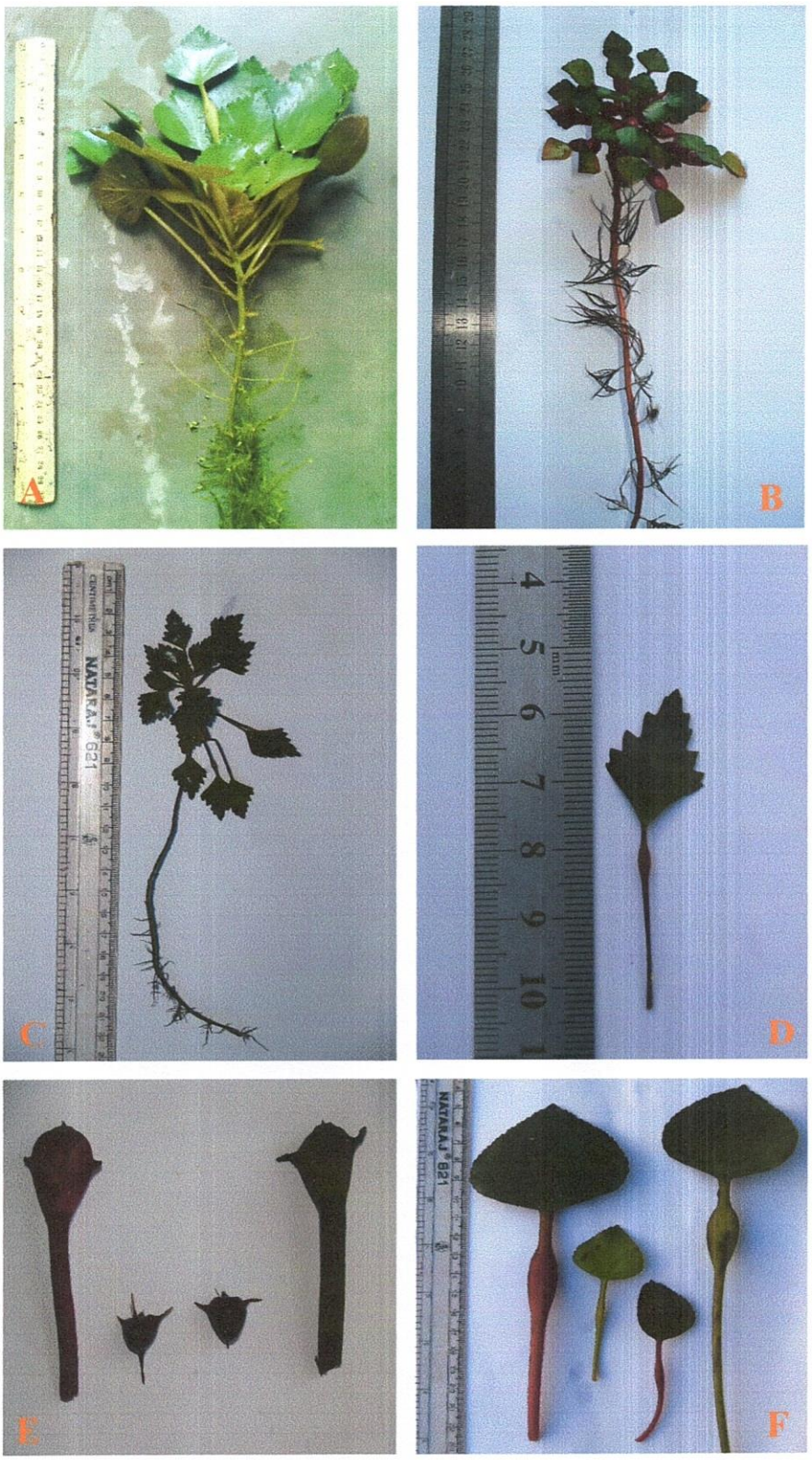


Figure 3: Represent the comparative study of three different varieties of water chestnut

A. Main plant of green water chestnut, B. Main plant of red water chestnut, C. Main plant of wild water chestnut, D. A single leaf of wild water chestnut, E. Fruit sample of red, wild and green water chestnut, F. Leaves of red and green water chestnut

IBA was the most effective PGR of root length elongation in different varieties. Green variety caused best performance (8.07) at the 1mg/l when red and wild shows 7.79 and 6.90 at the 1mg/l. IAA performed moderate effect (6.72 for green, 6.56 for red and 4.87 for wild water chestnut) at 1mg/l, uniformly NAA caused poor and anomalous performance (5.01 at 5mg/l in green, 5.56 at 0.2mg/l in red and 5.05 at 0.2mg/l in wild varieties) than IBA and IAA. Wild variety shows relatively poor performance than others variety in IBA, IAA & NAA, and differ result found after 21 days than the 14 days sowing with the 5 days hormonal treatment for root length elongation of different varieties water chestnut present in table 12 and fig. 9abc.

Yield performance of water chestnut in cultivation plot

The red water chestnut produce large size of fruits than the green varieties, and wild water chestnut produced small sized fruits. Treated plant produce large sized and number of fruits than the control of each varieties. Red variety being 25g, green 23g and wild 17g of average weighted. Multiple shoot much formatted in the green variety (5), while 4 multiple shoot found in red and 3 in wild variety. And also much branched character was found in green than green, evaluate here by the 13 no table and fig 3.

Table 13. Comparative yield performance with the different parameters of mature condition of water chestnut plant in cultivation field.

	var. green		**var. red**		**var. wild**	
Parameters	Cont.	Treated	Cont.	Treated	Cont.	Treated
Fresh weight (g/plant)						
Fruits	21	23	24	25	14	17
Dry weight (g/plant)						
Fruits	15	18	14	19	9	11
Soot Number	1	5	1	4	1	3
Branch number	13	20	9	19	25	30
Flower number	30	45	28	39	20	25
Fruit number	15	21	11	18	17	22

* Data of yield performance after 60 days of plantation

In this field evaluation green water chestnut was more vigor in the shoot multiplication, much branching, flower formation and over fruit settings while red water chestnut produced large fruit but total production is more effective in green. Wild water chestnut showed much branch and fruits setting ratio more than red and green water chestnut but it is vary natural and reduce total production, so wild is not suitable for mass cultivation, red and green water chestnut should be selective varieties for mass cultivation and especially green variety will be desire cultivated variety for the farmer of Bangladesh data were present in table 13.

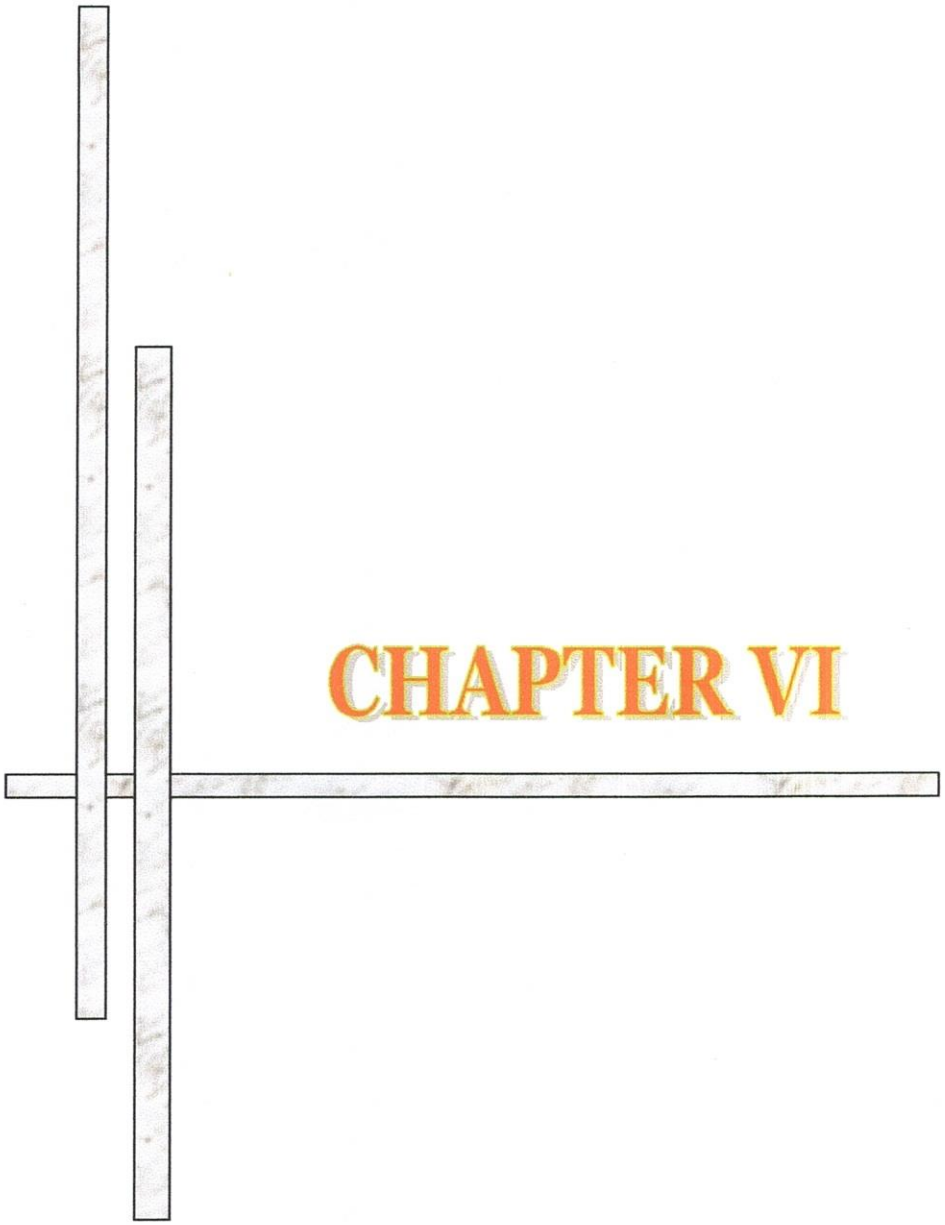
SUMMARY

In order to determining the maximum shoot and root length induct efficiency from the germinating fruit as well as cutting of stolon (stem) seedlings of *Trapa* (Bangladeshi variety) by the various concentration in different plant growth regulators with different duration of soaking treatment.

BA concentration 5.0mg/l induced maximum shoot proliferation from cotyledonary node of germinated fruit as seedling with 28 days 7 days treatment in green water chestnut it found most sensitive for shoot production than red and wild water chestnut.

IBA caused the more root number; it is most effective stimulation the root number and root length. NAA caused the longest root higher concentration (1.0mg/l) but less than IAA caused at 1.0mg/l concentration increase maximum root length, but less than NAA. Thus IBA proliferated maximum root number and IAA caused top root length.

So the present study, we can agree that plant growth regulators treatment require to improve shoot and root production in length of germination of (seeds) and stolon (stem) cutting in green water chestnut for success propagation and successful commercial cultivation in Bangladesh.



CHAPTER VI

Experiment

Successful *in-vitro* clonal propagation of three cultivars of water chestnut (*Trapa* spp.) through embryonic explants found in Bangladesh

INRTODUCTION

Water chestnut (*Trapa* spp) plants morphologically is divided into root, stem, leaf, flower, fruit etc. which produces two or four sharp spined fruits in fresh water environment. The plant grown in shallow fresh water basins in the tropical, sub tropical and temperate part of the world (A. Hoque *et. al.*). In many countries in the torrid and temperate zones people eat the fruit as raw, boiled or roasted and stem or leaves as vegetables (Mazumdar, 1985). It cultivate as a secondary crop in many countries as Japan, China, India, Bangladesh ctc. The fruit contains a large food value like Starch 67.5% (Porter field, 1928), Protein 5% (Subrahmenyan *et. al.* 1954), Calcium phosphorous, iron-Significant amount, Thiamin and ascorbic acid (Khan and chugtai, 1955; Bargale *et. al.,* 1987)

Up to now water chestnut considered as a minor crop because of its low yield, lack of suitable cultivation techniques, harvesting problems etc. During the flowering period, the water chestnut plant bearing many flowers but the fruit setting percentage is very low due to eco-physiological or pollination problem, it is not clear. There is little information on the improvement of its cultivation techniques and yield performance (Arima *et. al.* 1990, 1992a, 1992b, 1993, 1994). However, any attempts were not been taken on its seedling growing and mass propagation. Yield performance of any plants depends on its good seedling and growing conditions. Seedling growth is highly sensitive to environmental conditions specially, light intensity (Arima *et. al.* 1996). In the aquatic plants, water depth as well as light intensity is an important factor for their seedling growth.

To improve the optimum production technology of water chestnut in the Bangladeshi environment, the first requirement is mass number of uniform propagates in short period of time. Micropropagation utilities technique of aseptic tissues culture for the multiplication is important for the water chestnut cultivars. Therefore the present investigation on *in-vitro* culture effects of Bangladeshi water chestnut varieties was under taken with the following aims.

MATERIALS AND METHODS

MATERIALS

Plant materials

Mature fresh fruits of three varieties (cv. red, cv. green and var. wild) of water chestnut (*Trapa* sp) were harvested in 10 August, 2010 from the water chestnut experimental field in botanical garden at Rajshahi University campus which were originally collected from road side aquatic basin in Niamatpur, Manda and Shapaher Upozila at Naogaon District, Bangladesh. (two cultivated and one wild variety). Collected seeds were stored in water at 5°C and used for the conducted study.

Chemicals:

All chemical compounds including macro and micro-nutrients, sugar, agar, ethyl alcohol etc. used in the present study.

Sterilizing Agents:

In present investigation 90% ethyl alcohol and 0.1% mercuric chloride were used as surface sterilizing agents.

Basal nutrient media:

In this investigation MS (Murashige and Skoog, 1962) medium was used for shoot differentiation, subculture for shoot multiplication, root induction and for callus induction and maintenance. All the media were solidified with agar.

Growth regulators

The following growth regulators were used in the present investigation. These growth regulators were dissolved separately in appropriate solvent as shown below:

Auxin: Indole-3-acetic acid (IAA) INdole-3-butyric acid (IBA) α-naphthalene acetic acid (NAA) 2,4-dichlorophenoxy acetic acid (2,4-D)

Cytokinins: 6-Benzylaminopurine (BAP) 6-furfurylamino purine (kinetin, Kin) Gibbrellic acid (GA_3) 6-(4-hydroxy 3-methylbut-trans-2enyl) amino purine (Zeatin), 6-benzyladenine

Other materials

Culture vessels such as test tubes (150×25mm), conical flasks (250ml, 500ml, 1000ml) Measuring cylinder, separating funnel, pipette, forceps, cotton, firebox, marker pen, spirit lamp, needle, sharp blade, electronic balance, pH meter, autoclave, laminar air-flow machine etc. were also used in the present experiment.

Methods

Experimental method used for carrying out this experiment was accomplished through following steps.

Media used for callus culture and plant regeneration.

In the present set of experiments culture media with various growth regulators were used for various purpose that is presented bellow:

MS (Murashige and Skoog 1962) medium supplement with different concentrations and combinations of 2, 4-D, Kin, BAP, NAA, IAA and Zeatin with various amount of sucrose solidified with 0.6% agar.

Preparation of stock solution

The stock solution of different ingredients was prepared as the first step of media preparation practice. Different constituents of different culture media formulations were prepared in to stock solution as macronutrients, micro-nutrients, organic components and growth regulators separately for ready use during the preparation of the culture media.

Stock solution-A (macro-nutrients)

Stock Solution of macro-salt was prepared at 10X that of the required concentration. Required amount of all the macro-salt components prescribed for a particular medium formulation was weighed accurately with electronic balance and dissolved separately in substantial volume of double distilled water (DDW). The solution was sequentially poured in to a 1 litter volumetric flask. Final volume of the solution was made up to 1 liter by adding sufficient amount of DDW. Special care was taken during dissolving calcium chloride (C_aCI_2). The solution was filtered through whatman's No. 1 filter paper ot remove all solids contaminants like dust, cotton etc. and was poured into a colored glass reagent bottle and was lableled by 10×1000ml and stored in refrigerator at 4-6°C temperature for several weeks.

Stock solution-B (micro-nutrients)

Two separate stock solutions of micro-salts were prepared as follows:

Stock solution of micro nutrient was made up to 100X of the final strength of the medium in 100ml of distilled water as described for the final strength of the stock solution of the macro-nutrients. All the salts (except $FeSo_4$ and Na_2EDTA) were weighted accurately and dissolved one at a time, mixed them well, filtered and stored in a refrigerator at 4-6°C for several weeks.

2. This stock solution was also made up to hundred times of the final strength of the medium in 100ml of distilled water. In this case two constituents $FeSo_4$ and Na_2EDTA were dissolved separately in 100ml of distilled water and was heated for at 58°C until the salts dissolved completely. Finally the solution was filtered and stored in refrigerator at 4-6°C for several weeks.

Stock solution-C (organic components)

This stock solution was also prepared at 100X dissolved in 100ml DDW as described for the stock solution A. Then the solution was filtered poured in to a suitable glass container, labeled and stored at 4°C in a refrigerator for several weeks.

Stock solution of growth regulators

Stock solution of different growth regulators was prepared separately. Details of the methods of preparation of stock solution are given in the following Table which were used in the present investigation (Following the sigma plant cell cultural catalogue 1992)

Plant growth regulator and their appropriate solvent

IAA	10	1N NaOH 1m	10	1
IBA	10	1N NaOH 1m	10	1
NAA	10	1N NaOH 1m	10	1
2,4-D	10	70% ethano l 0.5ml	10	1
BAP	10	1N NaOH 1m	10	1
KIN	10	1N NaOH 1m	10	1
GA_3	10	1N NaOH 1m	10	1
Zeatin	10	1N NaOH 1m	10	1
BA	10	70% ethanol 1ml	10	1

To prepare stock solution, 10gm of any of the growth regulators were taken in a clean test tube and dissolved in required volume of appropriate solvent. Final volume of the solution was made up to 10ml by adding distilled water. The solution was then poured into a 100ml glass reagent bottle, labeled 1mg/ml and stored in refrigerator at 4°C.

Surficant preparation

$HgCl_2$ solution 0.1% was used for surface sterilization of plant materials. To prepare 0.1% solution, 0.1gm of $HgCl_2$ was taken in a bottle and dissolve 100ml distilled water. Generally $HgCl_2$ solution was prepared 1 hour before use.

Media Preparation

The steps as followed to make on one liter of any of the above media (except special modification) were based on the instruction chart given in the book of Bhojwani and Razdan (1983). The fall strength of one liter MS mekium was prepared as follows:

Assembling of the Medium Components

At first 100ml of stock solution of macro-nutrients, 10ml of each of the stock solution of micro nutrients and 10ml of stock solution of organic components and 30mg of sucrose were added to 750ml distilled water and mixed then well.

Addition of growth regulators

Different concentration of hormonal supplements were added either singly or in different combinations to the solution of step-1 and were mixed them well. Since each hormonal stock solution contained 1mg/ml, the addition of 1ml of any hormonal stock solution to prepare 1 liter of medium resulted in 1mg/ml concentration of that specific hormone. Similarly 0.5, 1.0, and 2.0mg/ml of any hormonal supplement required addition to 0.5, 1, 2ml amounts of specific hormonal stock solution for 1 liter of medium. The whole mixture was them made up to 1 liter by getting further addition of distilled water.

P^H adjustment

The pH of the medium was adusted to 5.8 with a digital p^H meter (TOA, Japan) with the help of 1 (N) NaOH or 1 (N) HCI, which ever necessary.

Sucrose and agar adding

After the adjustment of pH, sucrose 30gm/I and 0.61gm (at 0.61%) of sigma brand bactoagar was added to the medium and to dissolve the agar quickly, the whole mixture was heated in a microwave oven (National Japan).

Medium dispensing to culture vessels

Fixed volume of hot medium was dispensed in to culture vessels i.e. test tubes or conical flask. The culture vessels were plugged with absorbent cotton and marked with the help of a glass marker to indicate special hormonal supplement.

Sterilization

The culture vessels were then autoclaved at 15-26 1b/incs pressure at 121°C temperatures for 20 minutes. (Autoclave Hirayama, Japan). In case of test tubes the medium was allowed to cool as stints after sterilization. As GA_3 is degraded at higher temperature and by autoclaving, this hormone was filter sterilized and added to cool autoclaved medium using micro filter of pore size 0.2μm.

Culture technique

The following methods were employed in the present investigation for primary establishment of meristem culture, subculture and maintenance of culture.

Collection of plant materials

Mature fruits were collected. The excess unnecessary parts were removed from the collected materials and the remaining part were cut into convenient size (1-1.5cm). Then the materials were washed thoroughly under running tap water for several times to reduce the dust and surface contaminants.

Surface sterilization

Surface sterilization was carried out in front of running laminar-air flow cabinet. The materials were transferred to 250ml sterilized conical flask. Surface sterilization was carried out by dipping the materials in 0.1% $HgCI_2$

solution for different duration of time to ensure contamination free culture. Then the sterilized explants were washed 6-7 times with sterile distilled water immediately to remove all traces of $HgCI_2$.

Inoculation techniques

All inoculations and aseptic manipulations were carried out in a laminar air flow cabinet. The cabinet was switched on for half an hour before use and cleaned with 70% ethyl alcohol to reduce the chances of contamination. All instruments like scalpels, needle, forceps, tiles, petridishes etc. were covered with aluminum foil and sterilized by an alcoholic dip and flaming method inside the inoculation chamber. To ensure complete aseptic condition, both hands were also wiped by 70% ethanol. Before every use the above instruments were rinsed with absolute alcohol and flamed over a spirit lamp for several times. Other requirements like distilled water, blotting paper were sterilized by stem sterilization procedures and a binocular dissection microscope was required. The stage of the microscope was wiped with 70% ethyl alcohol after isolating each meristem from sterile shoot tips.

After sterilization explant materials were laid on the sterile tiles using sterile forceps. Then the isolated embryos were carefully inoculated on half strength MS medium supplemented with different concentration and combinations of hormones. The neck of the tube was flamed with spirit lamp and then plugged. After incubation, the culture tubes were labeled by glass market and were ready for incubation.

Culture incubation

The inoculated culture tubes were incubated in a growth chamber providing a special culture environment. The tubes were placed on the providing a special culture environment. The tubes were placed on the shelves of culture rack in the growth chamber. It may be mentioned specially that, all cultures were grown in the growth chamber illuminated by 40 watts white fluorescent tubes

fitted at a distance of 30-40 cm from the culture shelves. The cultures were maintained at 25°C ± 2°C with light intensity varied from 2000-3000 lux. The photoperiod was maintained generally 16 hours light and 8 hours dark. The culture tubes were checked daily to note the morphogenic response of culture explants in different experiments conducted in the present investigation.

Subculture of callus

After callus induction from the explants, the calli were transferred into the fresh medium for further proliferation and maintenance.

Plant regeneration

Plants were regenerated by transferring selected calli in us semisolid medium supplement with BAP, NAA, IAA, KIN, in various concentrations and combinations. Within 4-6 weeks of sub-culturing each callus developed in to a mass of 15-20 shoots which was sub divided again and cultured repeatedly. This culture was incubated at 25± 1°C under which light.

Root induction from regenerated shoot

After having sufficient micro shoots proliferation experiments were conducted for root induction from shoot proliferated in vitro for rooting. The shoot with 2-3 cm in length grown on the regeneration media aseptically snapped of and shoots were prepared fro rooting. The individual shoots were then transferred on to the freshly prepared rooting media supplement with different concentration of growth regulation or without any growth regulators.

Transplantation

When the regenerated plantlet and balblets produced sufficient roots and attained a height 10-15 cm were considered ready to transfer in soil. The plantlets grow inside the tubes were brought out from the growth chamber. After deplugging of the culture tubes were kept in room temperature for 5-7 days to bring them in contact to normal temperature. The plantlets were then

rescued very carefully from the tubes. Agar attached to the root was gently and carefully washed out under running tap water. Then the plantlets transferred to small pots containing fresh water or fresh water with nutrient solvent. The pots with plantlets kept in shady place and covered with perforated polythene sheet to prevent sudden desiccation. The soil in pots was sprayed with water everyday to maintain high humidity. After herding the pot was exposed to outer environment. After 25-3 days plantlet were finally transplanted to field, where they eventually developed into mature plants.

Data collection and analysis

For shoot induction

A. Percentage of explants induced shoot:

Percentage of expalnts showing proliferation

$$= \frac{\text{Number of cutue induced shoots}}{\text{Total number of explants cultured}} \times 100$$

B. *Mean Number of shoots per explant:*

Number of shoots per explant was counted after required days of culture. Mean number of adventitious shoots per explant was calculated using following formula.

Were, $\overline{X} = \frac{\sum xi}{N}$

Xi = Total no. of shoot, $\overline{X}$ = Average no. of shoot.

Σ = Summation.

N = No. of observation.

C. *Length of the longest shoo:t*

Length of the longest shoot was measured in cm scale for each explant. Average length of the longest shoot was calculated by using the formula like previous calculation.

For Root Induction

A. *percentage of shoots induced to form root:*

Percentage of shoots induced to develop roots were calculated by using the following formula.

$$\% \text{ of shoots induced root} = \frac{\text{Number of culture explant fromed shoot}}{\text{Total number of shoot cultured}} \times 100$$

B. *Mean number of roots per shoot:*

Number of roots per shoot was calculated after required days of culture. Mean number of roots per shoot was calculated using the same formula mentioned for number of shoots per explant.

RESULTS

In-vitro technique for plantlets regeneration via callus culture was done in different varieties of *Trapa* sp. of Bangladesh. The results obtained from different experiments of the present investigation are described and tabulated under the following heads.

1. Surface sterilization of the explants for collecting aseptic manipulation.
2. Callus induction and plant regeneration from embryonic explants.
3. Rooting of callus derived shoots.
4. Acclimatization and establishment of *in-vitro* regenerated plants in *ex-vitro* condition on the soil.

Surface sterilization of the explants for collecting aseptic manipulation.

Surface sterilization of field grown explants is essential for establishing the aseptic culture. Standardization for surface sterilization was carried out by a trial and error experiments. The pre washed explants (embryo) were treated with 0.1% $HgCl_2$ at different duration of time viz. 1, 2, 3, 4, 5, 6, 7 and 8 minutes. The effects of different treatment duration on surface sterilization of embryos are summarized in (table 1). When the embryos were treated for 1 and 2 minutes, the cultures were contamination with microorganism about 90% of explants were found contamination free and survive result were found in cv.

green was 90%, cv. red was 85% and var. wild was 80% when explants were treated for 7min. However when the explants were treated for long duration (8-12min) no contamination was observed, but more rates or complete tissue killing was noted. So, for surface sterilization of embryonic treatment with 0.1 % $HgCl_2$ for 7min. was found effective for the establishment of aseptic embryonic culture of water chestnut.

The response of medium

The response of embryonic explants on liquid media conditions were better and earlier than solid and semisolid media, shoot initiation and formation was the great than shoot proliferation and shoot proliferation rate was increased in liquid medium, followed by solid and semi-solid medium (Table-2) as with solid medium decrease shoot length related to sami-solid and solid media after 10 days of culture none of the shoots elongated more than 6-7cm turned black or red and gradually died from the tip. This tendency was observed in both cases of treated and control explants in all combinations of growth regulators for the different varieties. Under the different media conditions the number of shoots per explants and their length in the presence of same growth regulators combinations were highest and best (15.1 ± 0.7c) in to the liquid media conditions (Table 2) and performance was better than the cv. red (12.4 ± 0.5c) and var. wild (8.9 ± 0.3c), among these varieties var. wild showed poor performance than cv. green, cv. red respond average between cv. green and var. wild thus it can be concluded for water chestnut varieties that shoot proliferation in liquid medium could be better due to the easy diffusion of nutrients. The highest shoot length was found 14.7 ± 03c in cv. green than 10.2 ± 0.2c in cv. red and 6.1± 0.2c in var. wild. The wild vatiety showed better performance in liquid medium, which was less than cv. red (10.2 ± 0.2c) for shoot elongation.

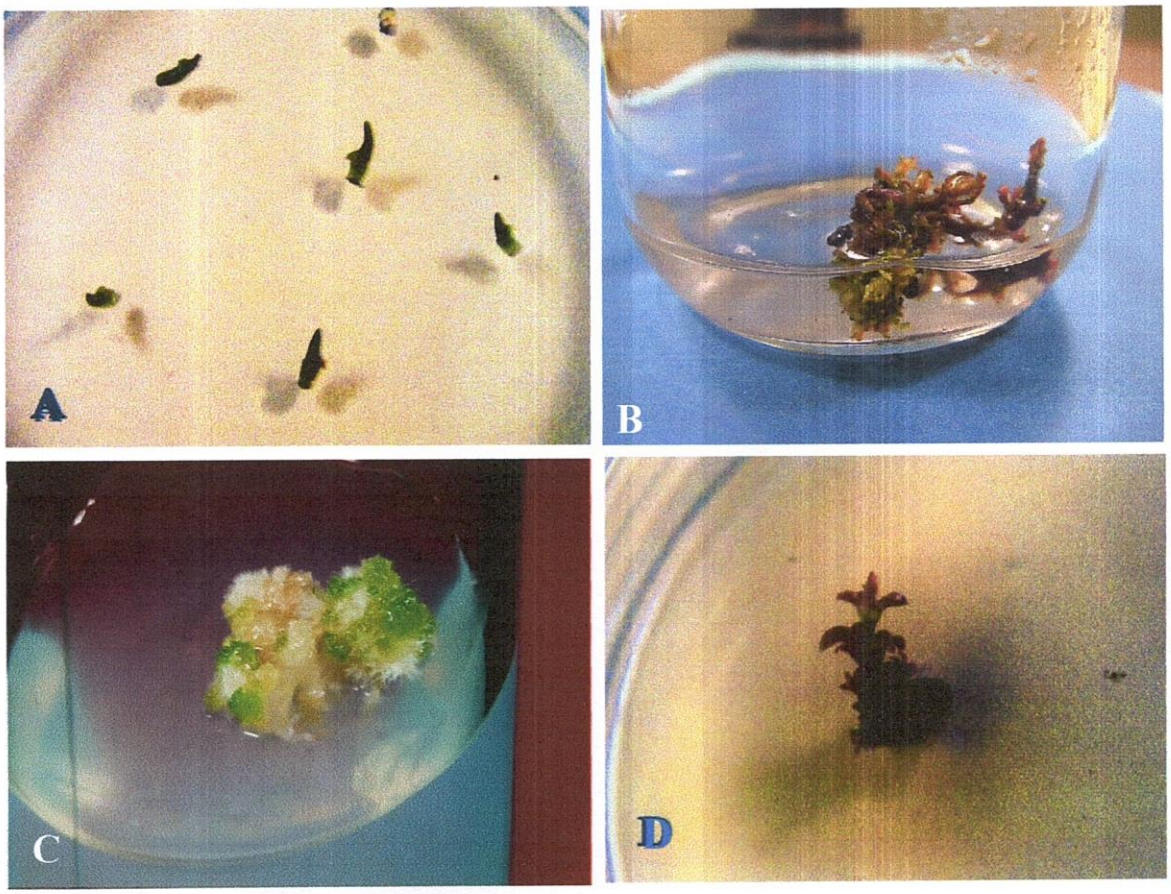


Figure: Micropropagation from embryonic explants of water chestnut
A. Mature embryo cultured in MS medium, B. Shoot multiplications of water chestnut, C. Callus induction from embryonic explants of water chestnut fruits, D. Survived embryonic explants with reduced phenolic compounds in culture media

Figure: Shows the *ex vivo* performance of water chestnut
A. Cultivation of green water chestnut, B. Cultivation of red water chestnut, C. Mature red water chestnut plants in *ex vivo* conditions, D. Cultivation field of water chestnut plant, E. Sowing of water chestnut plant in watery pot.

Overcoming phenolic accumulation during callus induction

Callus production in water chestnuts is different due to the copious amount of phenolic substances leached into the medium from the explants. Explants and the medium turned brown, eventually leading to poor callus growth or death. Blackening of explants and medium was reported to be due to the oxidation of phenolic substance and other growth inhibiting substances that are excreted into the culture medium. Accumulation of phenolic substances was reduced and callus formulation was promoted by the addition of PG. PG is an effective antioxidant that prevent phenolic oxidation (*George and Sherrington*, 1984) in water chestnut, it failed to evoke morphogenesis in the calluses on all media examined. The best medium for cllus induction was half-strength MS medium supplemented with 2.0μm, 2,4-D. 108.0μm PG. PG seemed to rejuvenate the old brown callus of the 15-30°C temperature levels examined explants cultured at 30°C showed the best callus induction on a half-strength MS medium supplemented with 2.0μm 2,4-D + 108.0μm CH and 108.0μm PG and 108.0μm CH, organogenesis occurred in the presence of BA. BA is the most effective synthetic cytokinin for stimulating organogenesis in various plant species (A. Hoque e*t. al*, 2000). Organogenic callus was produced at various temperatures. Optimal organogenesis occurred on the calluses. Cultured at 25°C for 4 week and sub-cultured on the half-strength MS medium supplemented with 0.27μm BA at 28°C under a 14hr photoperiod for 3 weeks

Callus induction and plant regeneration from embryonic explants

This investigation was carried out for callus induction from embryonic explants and maintenance for further growth of different varieties of water chestnut. Explant were cultured on to agar solidified MS medium supplemented with different concentrations and combinations of 2, 4-D with BA and BA with NAA in order to find out the most suitable culture media formulation to induce the explants to develop maximum callus. The response to callus induction

varied greatly with different concentration and combinations of growth regulator formulations; usually callus proliferation was started from the cut surface of the explants and finally covered the whole explants. Suitable callus formatted (80%) in 2.0 mg/l 2,4-D with 1.0mg/l BA for cv. green, when cv. red callus formatted 80% in 1.0mg/l 2,4-D + 0.5mg/l, secondly 60% callus induced at 2.0mg/l 2,4-D with 1.0mg/l BA and 0.5mg/l NAA + 0.5mg/l BA besides, var. wild formatted 70% callus at 1.5mg/l 2,4-D with 1.0mg/l BA. Compare the results for callus induction of water chestnut optimized that 2,4-D + BA combination were better than NAA + BA in different varieties and wild varieties less responded than red and green varieties which are present in (Table-3, Fig-C) respectively. Among the different combinations of 2,4-D + BA and NAA + BA, the highest degree of callus formation as well as the number was found in same conditions as 2.0mg/l 2,4-D + 1.0mg/l BA for cv. green, 1.0mg/l 2,4-D + 0.5mg/l BA for cv. red and 1.5mg/l 2,4-D + 1.0mg/l BA. In these case the explants showed highest tendency of callus formation to BA with 2,4-D.

Effect of NAA with BA on callus induction

In *Trapa* sp. Among all the treatments of 2, 4-D with BA and NAA with BA, the maximum explants induced to from callus from embryo in medium having 0.5 mg/l NAA + 0.5mg/l BA. The second highest degrees of callusing are noted in 0.5mg/l NAA + 1.0mg/l BA. The callus developed in this medium was light green in color and degree of callusing was moderate effect of 2,4-D with BA on callus induction present at (Table-3, Fig-C).

In *Trapa* sp. among all the treatments of 2, 4-D with BA the highest (80%) explants was induced for callus formation in medium having 2.0mg/l of 2, 4-D + 1.0mg/l BA. The second highest (70%) explants induced callus formation in the medium having 1.0mg/l 2, 4-D + 0.5mg/l BA. These calli were light green in color but the degree of callus formation was moderate.

Shoot induction from callus

High frequency of plant regeneration efficiency through organogenesis from somatic cell is a prerequisite for using para sexual method of plant breeding for crop improvement. The investigation conducted to see the effect of subculture of callus derived from embryonic explants of *Trapa* sp. on morphological differentiation. Calli were sub-cultured for shoot initiation in media supplemented with in differentiation. Calli were sub-cultured for shoot initiation in media supplemented with different concentrations and combinations of cytokinins. Results obtained from these experiments are described under the following heads.

Effect of BA+ NAA+GA_3 on shoot induction

From the various cytokinins with the combinations of NAA, percentage of shoot formation increased in presence of BA and GA_3 combinations on liquid media conditions (Table-4, Fig-E). Among the different combinations of BA and NAA the highest degree of shoot formation as well as number was found (8.2 ± 0.1b) in liquid media recorded into the combination with 1.1µm BA + 0.5µm NAA and shoot length was found as 13.2 ± 0.2d at the same concentration and combination that other combination respectively and highest shoot number (7.8± 0.6b) and shoot length was found 15.1 ± 0.2c containing 1.1µm BA + 0.5µm NAA + 0.5µm GA_3 for the green variety of water chestnut. In red water chestnut, the best shoot number initiation was found 7.6 ± 0.4b at 1.1µm BA + 0.5µm NAA + 0.5µm GA_3 and shoot length was moderate performed as 12.3 ± 0.7c when shoot number was 4.9 ± 0.5a and best result of shoot length (13.3 ± 0.1bd) was found at the combination of 2.2µm BA + 1.1µm NAA + 0.5µm. The finalized result were optimized that highest shoot number initiate in low doze and top shoot length was found in high doze of plant growth regulators. Whereas comparatively result found in wild water chestnut in the same plant regulators at the combination of 1.1µm BA + 0.5µm NAA + 0.5µm GA_3 as best shoot number showed that 6.9 ±0.9b and best shoot

length showed that 10.1 ± 0.1c but treated explants always showed the highest tendency of shoot proliferation as well as number in all growth regulator combinations. (A. Hoque *et. al.*, 2000).

Root induction

For establishment a plantlet, root induction is essential. The callus derived axillary shoots were transferred in the liquid rooting medium at IBA & GA_3 either singly or in different concentration and combination containing medium for successful root induction. The summaries of the results are presented in (Table-5, Fig-F). The highest roots per plants (12.14 ± 0.65) formatted in IBA containing medium with 1.1mg/l relatively average root length was top (11.78 ± 0.71) whereas GA3 showed poor performance and IBA + GA3 conjugally showed moderate performance as Roots/shoots was 10.86 ± 0.53 and Average root length was 9.52 ± 0.47 with the 8-12 days of emergence and shoot formation frequency was 100% in the green water chestnut. So in IBA found the best growth regulator for root formation and elongation. At the same concentration (1.1mg/l IBA) wild water chestnut performed less than red and green varieties. Red water chestnut induced root/plants 11.33 ± 0.43 and wild showed 9.82 ± 0.81, when average root length for red 10.77 ± 0.71 and wild 8.61 ± 0.22 found respectively. *Trapa* sp. showed that earliest root emergence was started after 5 days of inculcation in liquid MS medium. So for this experiment it is concluded that liquid MS medium was most effective medium formulation for rooting of callus regenerated shoots. After growth of roots, plantlets were removed from the growth chamber and rooted micro cutting have been successfully transplanted to the paddy field and were survived 95%.
In conclusion, the protocol described here provides an efficient and rapid shoot regeneration system, which is suitable for micropagation of water chestnut and could be a potential as well as sustainable method for raising a water chestnut nursery in the paddy field.

Table 1. Effect of different time duration 0.1% $HgCl_2$ treatments for surface sterilized embryonic explants of water chestnut.

Variety	Treatment period of 0.1% $HgCl_2$ in min	Total of culture	% of explants contained		Degree of tissue killing	% of explants survived
			After 5 days	After 10 days		
cv. **green**	3	15-20	45	20	-	30
	4	15-20	35	15	-	35
	5	15-20	10	10	-	70
	6	15-20	10	NC	-	75
	7	15-20	NC	NC	-	**90**
	8	15-20	NC	NC	+	70
	9	15-20	NC	NC	+	60
	10	15-20	NC	NC	++	50
	11	15-20	NC	NC	+++	40
cv. **red**	3	15-20	35	20	-	30
	4	15-20	35	15	-	35
	5	15-20	10	10	-	60
	6	15-20	10	NC	-	70
	7	15-20	NC	NC	-	**85**
	8	15-20	NC	NC	+	60
	9	15-20	NC	NC	+	60
	10	15-20	NC	NC	++	50
	11	15-20	NC	NC	+++	40
var. **wild**	3	10-15	30	20	-	30
	4	10-15	35	20	-	35
	5	10-15	10	15	-	40
	6	10-15	10	NC	-	60
	7	10-15	NC	NC	-	**80**
	8	10-15	NC	NC	-	70
	9	10-15	NC	NC	-	60
	10	10-15	NC	NC	+	40
	11	10-15	NC	NC	++	50

NC = No contamination
'-' = No tissue killing
'+' = Partial tissue killing
'++' = Moderate tissue killing
'+++' = Complete tissue killing.

Table 2. Effect of medium solidification on shoot proliferation and elongation of embryonic explants of water chestnut after 4 weeks culture.

Variety	Media condition	No. of shoots/cm	Length of shoots cm/cx
cv. green	Solid	7.3 ± 0.3a	5.7 ± 0.1a
	Semi solid	9.8 ± 0.46	8.2 ± 0.16
	Liquid	13.2 ± 0.7c	12.7 ± 0.3c
cv. red	Solid	5.5 ± 0.2a	5.2 ± 0.3a
	Semi solid	7.3 ± 0.25	6.8 ± 0.13
	Liquid	12.4 ± 0.7c	10.2 ± 0.2c
var. wild	Solid	4.1 ± 0.3a	2.3 ± 0.2a
	Semi solid	6.6 ± 0.11	4.2 ± 0.16
	Liquid	8.9 ± 0.3c	6.1 ± 0.2c

Table 3. Effect of different concentration of 2, 4-D with BA and NAA with BA in MS medium on callus induction from embryonic explants of *Trapa* sp.

Variety	Growth regulator (mg/l)	% of explant induced callus	Callus color	Degree of callus formation
	2,4-D + BA			
	0.5 + 0.5	50	LG	+
	1.0 + 0.5	70	LG	+
	2.0 + 1.0	**80**	**LG**	++
cv. green	1.5 + 1.0	50	LG	+
	1.0 + 1.5	40	LG	+
	NAA + BA			
	0.5 + 1.0	50	LG	+
	0.5 + 0.5	60	LG	+
	1.0 + 1.0	40	LG	+
	2,4-D + BA			
	0.5 + 0.5	60	LG	+
	1.0 + 0.5	**80**	**LG**	++
	2.0 + 1.0	60	LG	+
cv. red	1.5 + 1.0	50	BG	+
	1.0 + 1.5	40	BG	+
	NAA + BA			
	0.5 + 1.0	50	LG	+
	0.5 + 0.5	60	LG	++
	1.0 + 1.0	40	LG	+
	2,4-D + BA			
	0.5 + 0.5	50	LG	+
	1.0 + 0.5	60	LG	+
	2.0 + 1.0	50	LG	+
	1.5 + 1.0	**70**	**LG**	++
var. wild	1.0 + 1.5	50	LG	+
	NAA + BA			
	0.5 + 1.0	50	LG	+
	0.5 + 0.5	60	LG	+
	1.0 + 1.0	40	LG	+

LG = Light Green
BG = Blackish Green
++ = Moderate Callus
+ = Slight Callus
* Data were recorded after 6 weeks of culture.

Table 4. Main effect of growth regulators concentrations and combination on shoot multiplication and elongation from embryonic explants of water chestnut after 4 weeks culture.

Variety	Regulators			Number of shoots/ explants	Shoot length (cm)/ explants
	BA	**NAA**	**GA_3**		
	1.1	0	0	5 ± 0.3a	9.5 ± 0.2ac
	2.2	0	0	6.3 ± 0.2ac	8.1 ± 0.1ad
	1.1	0.5	0	8.2 ± 0.1b	13.2 ± 0.2d
	2.2	0.5	0	6.1 ± 0.5ad	11.6 ± 0.3c
cv. green	1.1	1.1	0	7 ± 0.8bc	8 ± 0.4d
	2.2	1.1	0	7.3 ± 0.4bc	9.2 ± 0.3d
	1.1	**0.5**	**0.5**	**7.8 ± 0.6b**	**15.1 ± 0.2c**
	2.2	0.5	0.5	6.3 ± 0.3cd	14.8 ± 0.3c
	1.1	1.1	0.5	6.3 ± 0.3bc	14.7 ± 0.6c
	2.2	1.1	0.5	5.2 ± 03ad	14.4 ± 0.4bc
	1.1	0	0	4.8 ± 0.6ad	5.5 ± 0.1ab
	2.2	0	0	7.1 ± 0.1ac	6.1 ± 0.3ad
	1.1	0.5	0	7.9 ± 0.2b	9.4 ± 0.5d
	2.2	0.5	0	6.6 ± 0.3ad	8.5 ± 0.4d
	1.1	1.1	0	7 ± 0.6ba	8.2 ± 0.2c
cv. red	2.2	1.1	0	7.3 ± 0.2bc	8.9 ± 0.1d
	1.1	**0.5**	**0.5**	**7.6 ± 0.4b**	**12.3 ± 0.7c**
	2.2	0.5	0.5	6.1 ± 0.6cd	13.9 ± 0.5b
	1.1	1.1	0.5	6.2 ± 0.2bc	13.7 ± 0.4c
	2.2	**1.1**	**0.5**	**4.9 ± 0.5a**	**13.3 ± 0.1bd**
	1.1	0	0	3.7± 0.1b	5.5 ± 0.3c
	2.2	0	0	4.3 ± 0.3ab	7.1 ± 0.2ad
	1.1	0.5	0	**5.2 ± 0.2ba**	**9.2 ± 0.4d**
	2.2	0.5	0	5.7 ± 0.3ad	9.1 ± 0.5cd
var. wild	1.1	1.1	0	6.1 ± 0.6ba	7.1 ± 0.3d
	2.2	1.1	0	6.3 ± 0.5bc	8.2 ± 0.5b
	1.1	**0.5**	**0.5**	**6.9 ± 0.9b**	**10.1 ± 0.1c**
	2.2	0.5	0.5	6.2 ± 0.2cd	9.7 ± 0.4c
	1.1	1.1	0.5	6.1 ± 0.1bd	9.6 ± 0.7a
	2.2	1.1	0.5	4.9 ± 02ab	9.1 ± 0.2bc

* Comparison of mean values obtained in treatments were made using Duncan's multiple range test. Values with different letters within column are significantly different at $p < 0.05$

Table 5. Effect of different concentrations and combinations of GA_3 and IBA for root induction from callus derived shoots. Data were recorded on days of emergence, average length of roots (cm) root/shoots and root formation frequency at 3 weeks after inoculation.

Treatment	**Rooting parameters**	**Cultivar/ Variety of *Trapa.* sp**		
		cv. green	**cv. red**	**var. wild**
IBA + GA_3 (2.0mg/1+.5mg/1)	Days of emergence	8-12	8-12	8-12
	Average length of root (cm)	9.52±0.47	8.44±0.68	6.21±0.82
	Roots/shoots	10.86±0.53	9.92±0.49	10.86±0.36
	Root formation frequency	90	80	80
GA_3 (0.5mg/1)	Days of emergence	12-15	12-15	12-15
	Average length of root (cm)	4.82±0.90	4.1±0.60	4.69 ± 0.78
	Roots/shoots	9.02±0.12	5.10±0.19	3.12±0.43
	Root formation frequency	80	70	70
IBA (1.1mg/1)	Days of emergence	8-10	8-10	8-10
	Average length of root (cm)	11.78±0.71	10.77±0.53	8.61±0.22
	Roots/shoots	12.14±0.65	11.33±0.43	9.82±0.81
	Root formation frequency	100	100	100

DISCUSSION

Embryonic explants from fresh fruits were cultured no to the different concentrations and combinations of auxin cytokinin and gibberelic acid with half-strength MS salts solidification with 0.6% agar from the obtained results, the combination of 2.7μm 2, 4-D with 1 μm BA was shown the best performance for embryonic germination. Explants after 40 days of harvesting were cultured on the media composition as mentioned above and get the germination rate 65% after 10 days of inoculation lower percentage of embryonic germination as well as shoot proliferation in the control explants indicated the presence of deep dormancy state. Because fresh seeds of water chestnut could not geminate due to dormancy.

Solidification effect of media is clear that the germination of embryonic explants on solid and semi-solid media condition were better and earlier than liquid medium but shoot proliferation was increased in liquid medium followed by semi-solid medium as with solid medium decreased in the number of shoots and after 10 days of culture none of the shoot elongated more than 6-7 cm. Turned red and gradually died from the tip, this tendency was observed in control explants in all combinations of growth regulators. Thus it can be concluded for water chestnut that, shoot proliferation in liquid medium could be better due to easy diffusion of nutrients.

During callus induction copious amount of phenolic substance leached into the medium from the explants. Accumulation of phenolic substance was reduced and callus formation was promoted by the addition of PG. The best medium for callus induction was half-strength MS medium supplemented with 2.7μm 2, 4-D, 108.0μm CH and 10.8μm PG. PG seemed to rejuvenate the old brown callus.

The effect of various cytokinins with the combinations of 0.5μm NAA percentage of shoot formation increased in presence of BA and NAA combinations. Among the different combinations of BA and NA, the highest degree of shoot formation as well as number was found in media having 1.1μm BA and 0.5μm NAA.

Evaluation of different hormonal concentrations and combinations the greatest number of shoots was recorded in liquid media containing 1.1μm BA with 0.5μm NAA and highest shoot length was also recorded onto the aforesaid combination with 0.5μm GA_3 than other combinations respectively. At lower or higher concentrations of BA or NAA. The number of multiple shoot and their length per explant was always lower to that obtained from earlier cited combinations. It was found necessary to transfer the in vitro axillary shoots to medium supplemented with 0.5μm GA_3 also with BA and NAA to ensure their normal elongation. GA_3 is hnown to have stimulatory effect on stem elongation in different plants (phinney, 1984; Manjula *et al.,* 1997).

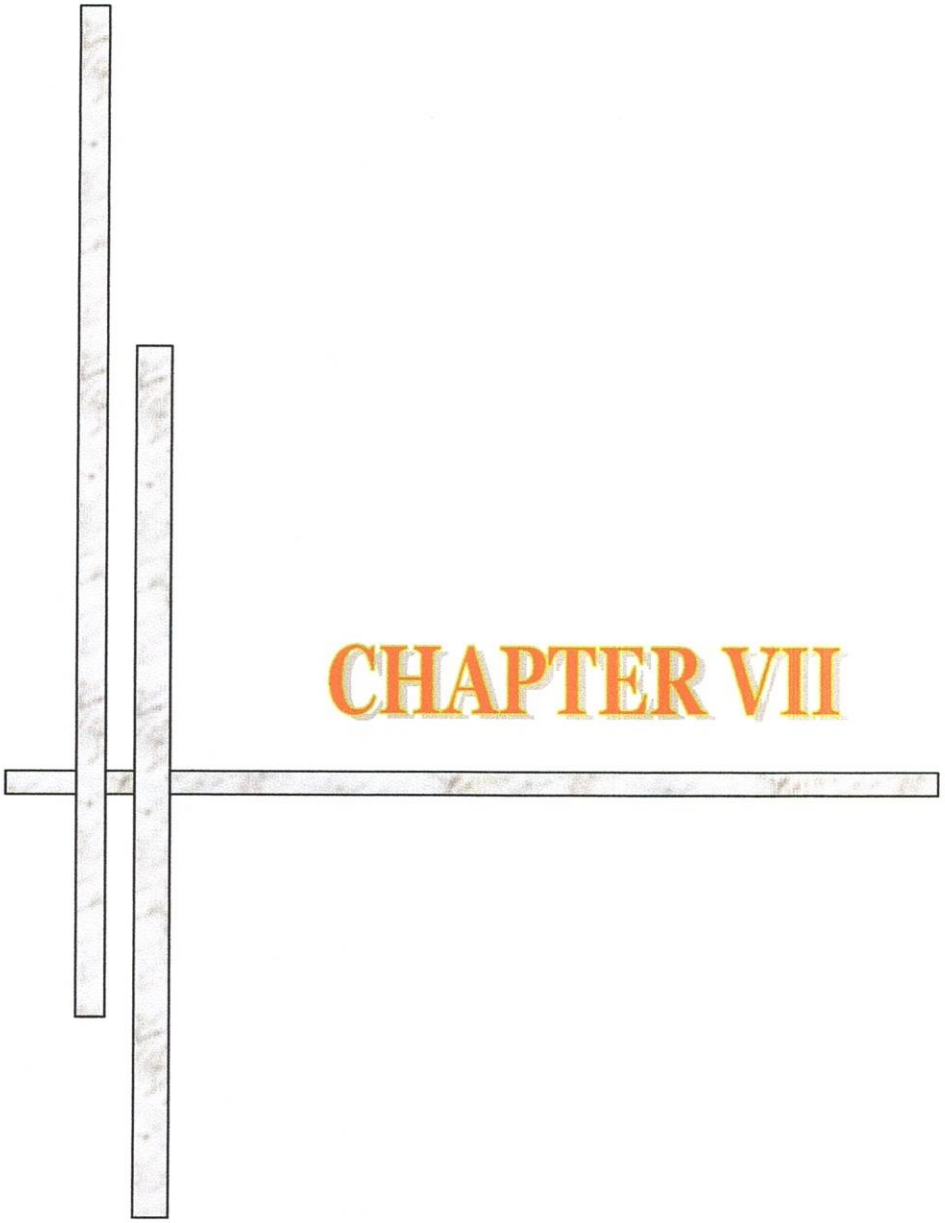
For root proliferation, mainly axillary shoots were excised from the shoots of the primary culture and placed in the liquid rooting medium. In most of the cases, two roots emerged from the node of nearest cut end of the stem within 8-10 days in presence of 1.1 μm IBA. After growth of root, plantlets were brought out from the growth chamber and rooted micro cuttings have been successfully transplanted to the paddy field.

SUMMARY

In vitro plant regeneration via callus culture have been done in water chestnut (*Trapa* sp.). In Bangladesh. For callus induction embryo were used as explants. Among different media formulation 2.7 μm 2, 4-D with 1μm BA (108.0μm CH and 10.8μm PG for antioxidant of phenol) and half-strength MS medium showed best performance for callus induction.

Selected calli of explant were transferred liquid MS medium containing various concentration and combination of plant growth regulators. Among used media formulation 1.1μm BA+0.5μm NAA was found most effective for plant regeneration from the calli. But the most effective formulation for shoot elongation was adding 0.5μm GA_3 with the medium.

The regenerated shoots were well developed rooted when them culture in liquid $\frac{1}{2}$ MS medium with the best concentration and combination of 1.1μm IBA, and the regenerated plantlets were successfully established in the water chestnut grown paddy field, and the survival rate of plantlets were satisfactory.



CHAPTER VII

GENERAL DISCUSSION

In future changing environmental conditions associated with global warming, water chestnut many become an important alternative crop that should be considered seriously by the scientific community. However, there is at present no recognised high yielding water chestnut in countries such as Bangladesh. The introduction of high yielding cultivars, coupled with enhanced cultivation, would increase significantly the earning capacity of many landless farmers.

The reports of food nutrient analysis of water chestnut in Bangladesh is poor. But some work have been done in India and Japan, such as Chemical analysis of fruits of Japani water chestnut. Chemical analysis and review of food compontnt (Bargale *et. al.,* 1987, Kiviat 1993, Hoque *et. al*., 2005a), micropropagation (Hoque *et. al*., 2001; Worobel, 1996; Pandit and Quadmi, 1986) hormonal treatment Korsuge *et. al*., 1985; Apinis 1937; Winne 1950 Zimmerman *et al.,* 1986 etc.

The protein constituents of fruits and vegetables, although occurring in low concentration, are of primary importance not only as component of nuclear and cytoplasmic structures, but also including, as they must the full complement of enzymes involved in metabolism during growth, development, maturation of fruit and vegetables (Hansen, 1970). Lipid is more useful in animal body. Fats serve as efficient source of energy and insoluble material. Dietary fat helps in the absorption of fat soluble vitamins, lipoproteins are important cellular constituents. In water chestnuts fruits were found to contain a very low amount of lipid in wild, green and red varieties respectively. It was found that water chestnuts (*Trapa*) fruit contained low amount of reducing sugar and the content of reducing sugar, In case of water chestnuts (*Trapa*) the reducing sugar content is affected by several factors including variety, growing conditions, maturity and the storage environment. Sucrose content of water chestnuts (*Trapa*) was found to be almost similar in different varieties (for wild 0.63±0.02, green 0.59±0.01and red 0.60±0.03 percent) tested. Starch is the most

important source of carbohydrate in human diet. in all varieties was found to contain a significant amount of starch, i.e, 8.30±0.03, 8.70±0.03 and 8.20±0.02 in wild, green and red varieties respectively.

Animal cannot synthesize it but can convert it to vitamin A through enzymatic reaction. In plants, it is very necessary for growth and development of soft tissues through its effect upon protein synthesis. Vitamin A also plays a role in the maintenance of normal epithelial structure. Phenolic compounds enjoy a distribution in the plant kingdom, and they are particularly prominent in fruits and vegetables where they are important in determining color and flavor (Buren, 1970). Water chestnuts (*Trapa* sp.) fruits contained low amount of phenol. In green variety contents low range of phenol, the amount of phenol was 0.50±0.01 (mg per 100g), wild variety contents higher amount phenol, the amount of phenol was 0.99±0.02 (mg per 100g) and red variety contents average scale of phenol. It may be concluded from the results that the phenol content of water chestnuts (*Trapa*) was slightly higher in wild variety. The amounts of crude fiber in three varieties of the water chestnuts fruits were determined to be 2.28±0.04, 2.13±0.03 and 2.75 ±0.05 for wild, green and red varieties.

Minerals are inorganic elements exist in the body and in food as organic and inorganic combination. In foods mineral elements are present as salt. They combined with organic compound, e.g. iron in hemoglobin. Some enzymes require calcium for their activities as lipases and succinate dehydrogenase. They regulate transmission of impulses and contraction of muscles. The deficiencies of minerals cause many disease in human being. The amount of potassium, sodium, calcium, phosphorus, sulpher, Iron, cupper, manganese and zinc present in water chestnuts (*Trapa*) are shown different water chestnut varieties. The results indicated that the amount of potassium was slightly lower in green variety. Potassium content of water chestnuts (*Trapa.*) was 6.13%, 5.22% and 5.32 % was found for wild, green and red varieties (Kaul *et. al.*,

1976). The sodium content of water chestnuts was found markedly higher in wild variety (0.72%) as compared to red variety (0.59%) and average (0.64%) sodium content shows in green variety. A similar amount of calcium was observed in the three varieties of water chestnuts i,e. 0.12%, 0.25% and 0.26% in wild, green and red varieties respectively. Calcium is an important nutrient element for human body. It plays an important role in formation of bone and teeth. Phosphorus content of water chestnut fruit was found 5.98% for wild variety, 6.77% for green variety and 6.77% for red variety. Green and red variety shows similar quantity as 6.77% and wild variety store low amount of phosphorus than the both cultivars. Sulpher content high and same (**0.38**%) of the two cultivars than wild variety (0.31%) of water chestnut. Iron content was 237 ppm in wild and 200ppm in both cultivars (green and red) of water chestnut fruits.

It was found that eleven (11) amino acids detected in wild varieties but eight 8 amino acids were detected in red and nine 9 amino acid found in green variety. There is little information on procedures to improve its cultivation and performance (Arima *et. al.*, 1999, Lalit *et. al.*, 2007) and little effort has been directed at seedling establishment, the latter being fundamental to longer-term performance, since the latter depends on optimal growing conditions at the seedling stage. The most significant advantages offered by axenic procedures of clonal propagation compared to conventional methods is that many plants can be produced from a single individual as source material and micropropagation has become an important aspect of commercial nursery propagation of many plants, especially of ornamentals with possible extension to crops such as water chestnut (Zimmerman *et. al.*, 1986).

The antimicrobial activity of the aqua's extract, methanol extract, petroleum ether extract were tested against eight bacteria at the concentration of 100µg/ disc, 150µg/ disc and 300µg/ disc. Standard antibiotic disc, *kanamycin*

(50μg/disc) was used for the comparison of the bioassay. The petroleum ether extract of water chestnut (red variety) showed notable antibacterial efficiency (15-23mm) against most of the tested organisms. Highest antibacterial activity was observed against *Bacillus megaterium* (23mm) bacteria when applied 300μg/disc dosages (Little, 1979). Poor efficiency was found against the *Bacillus Cereus* (23mm) which were lower than *kanamycin* (19mm). It was revealed that fruit extract of water chestnut (green variety) showed the most effectiveness (8-16mm) against all pathogenic bacteria. Highest inhibitory activity was found against *Bacillus megaterium* (19mm) bacterium having concentration of 300μg/disc. The produced zone of inhibition for isolated methanol extract of wild water chestnut against gram positive bacteria showed that 300μg/disc dose was most effective because there was no resistant bacteria found and maximum 32mm inhibition zone produced. Also among three extracts studied, the highest diameter of inhibitory zone was found in methanol extract (34mm) and the lowest was recorded in both aquas and petroleum ether extracts (7mm). The results of different studies provide evidence that some medicinal plants might indeed be potential sources of new antibacterial agents (Kone *et. al.,* 2004).

This consequence was comparable to the previous findings (Razvy *et. al.,* 2011) where methanol extract of wild variety of water chestnut showed maximum inhibitory zone against gram positive bacteria and remarkable antibacterial efficiency were recorded against most of the tested organisms. Also extracts of some other plants have been used for the antimicrobial activity such as antimicrobial activity of aqueous and methanol extracts of *Juniperus oxycedrus* where they have found significant potency of aqueous and methanol extracts (Karaman *et. al.,* 2003). Previous study (Parekh and Chanda, 2007) also documented antibacterial activity of *T. natans* fruit extracts against different gram positive and gram negative bacteria. The increase of antibiotic resistance of microorganisms to conventional drugs has necessitated the

exploration for new, efficient and cost effective way for the control of infectious diseases.

The result of different studies provides that some medicinal plants might be potential sources of new antibacterial agents (Kone *et. al.,* 2004). From this study, we can conclude that, this medicinal plant has a wide range of antibacterial activity and supports the traditional use of these plants as medicine. This work has highlighted the antimicrobial effects of fruit of *T. quadrispinosa* and *T. bispinosa* on some of the medically important pathogens. This is in fact a promising result, with the comparable standard antibiotics and suggests the potency of these extracts. Hence, *T. quadrispinosa* fruit could be used as a guide in our continuing search for being used in the field of biopharmaceutical industry or in ethno-medicine.

Different concentration of aqua's, ethanol and petroleum extract of water chestnut fruit extract has been tested against fungal growth. In was observed that all three varies of water chestnut exhibited some sort of antifungal activity resulting inhibition zone ranging from 1.1±0.03mm to 9.9±0.71mm in diameter. Highest inhibition zone (9.9±0.71mm) was found against *Aspergillus flavus* while treated with 250 µg/disc of ethanol extract of wild variety. It was revealed that both ethanol and petroleum extracts seemed to be more efficient to inhibit the growth of all *Aspergillus* species compare to aqua's extract. It was reported that ethanolic extract of *Anogeissus leiocarpus* and *Terminalia avicennioides* proved to be more efficient than the methanolic, chloroform, or aqueous extracts against all the test fungi (18). In comparison, wild variety showed highest antifungal activity against all the fungi used in this study, which reveals the presence of toxic substances in wild water chestnut compare to red and green variety.

In order to determining the maximum shoot and root length induct efficiency from the germinating fruit as well as cutting of seedlings of *Trapa* by the

various concentration in different plant growth regulators with different duration of soaking treatment. BA concentration 5.0mg/l induced maximum shoot proliferation from cotyledonary node of germinated fruit as seedling with 28 days 7 days treatment in green water chestnut it found most sensitive for shoot production than red and wild water chestnut.

IBA caused the more root number; it is most effective stimulation the root number and root length. NAA caused the longest root higher concentration (1.0mg/l) but less than IAA caused at 1.0mg/l concentration increase maximum root length, but less than NAA. Thus IBA proliferated maximum root number and IAA caused top root length. present study, we can agree that plant growth regulators treatment require to improve shoot and root production in length of germination of seeds stem, multiplication of stem or cutting in water chestnut for success propagation and successful commercial cultivation in Bangladesh, this research work were to perform a study on traditional utilization of wild fruits in Bangladesh and to generate data on the proximate composition and other characteristics of three minor and wild fruits as a basis for the selection of fruits suitable for processing. The long-term goal was to promote and increase the utilization and consumption of local fruits.

Cozza *et. al.* (1994) reported that seeds of *T. natans* exhibited a low percentage of germination (about 6%) accompanied by a delay in germination. In contrast, when seed was exposed to a low temperature, the germination increased to 60%, chilling enhancing maturation of the embryo. In *T. natans* var. *japonica*, Kurihara and Ikusima (1991) reported that a period of low temperature was effective in breaking dormancy. In order to exploit this observation and to investigate this phenomenon in detail, Hoque *et al.* (2001) developed a robust protocol to chill seeds, followed by excision of embryos as explants as a basis for subsequent micropropagation. The percentage germination increased to 72% for embryos excised from seeds that were stored, confirming the benefit

of post-harvest storage. Storage for 40 days at 5°C increased germination to 100% with proficient shoot proliferation.

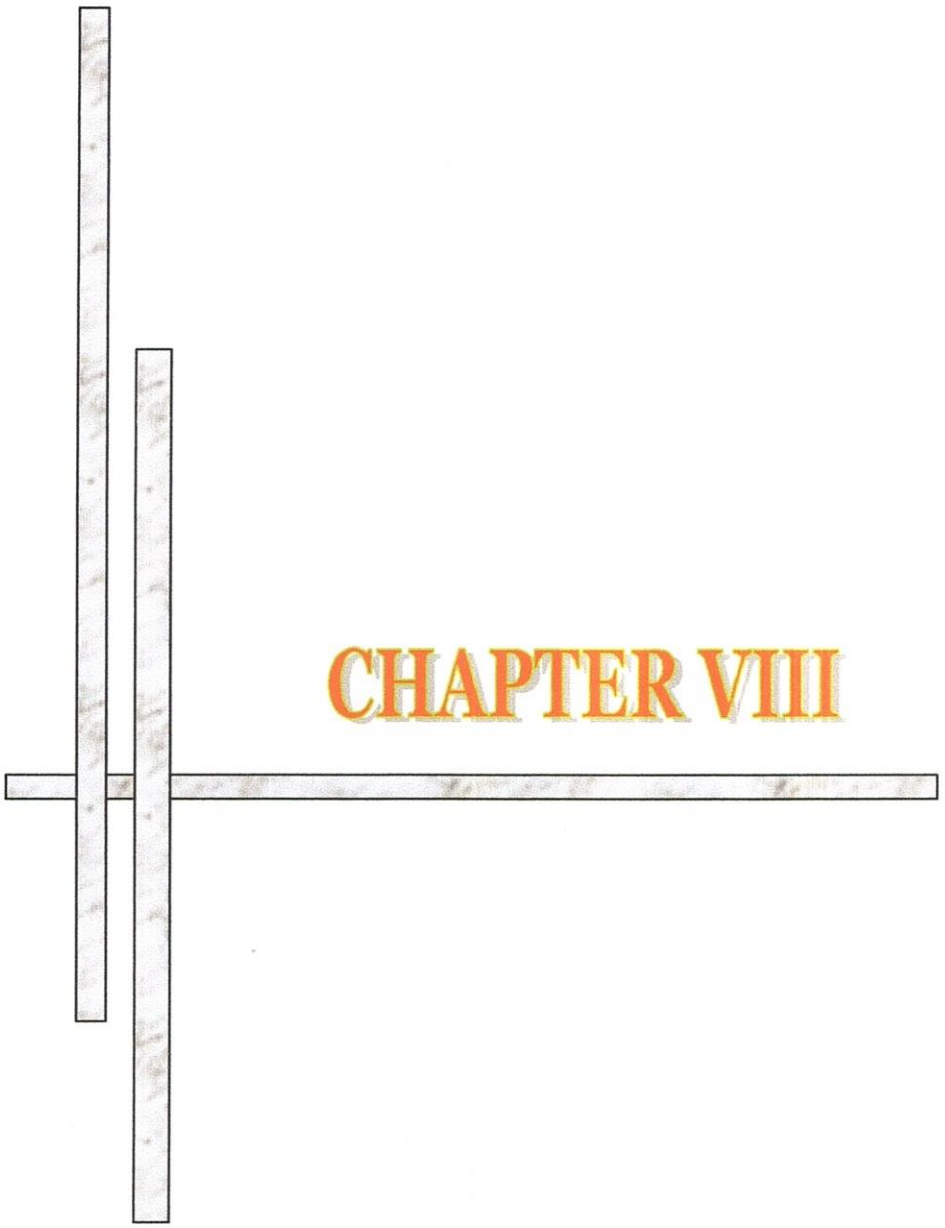
The number of shoots per explant and their length were optimum in liquid medium, possibly reflecting the hydrophytic nature of the plant resulting in maximum uptake of nutrients (Arrebola *et. al.,* 1997). Optimum shoot regeneration was in medium with 1.1 µM BA and 0.5 µM NAA, chilling treatment promoting shoot with BA in the range 1.1 - 4.4 µM, resulting in 100% shoot proliferation.

Shoot elongation was stimulated in the same medium to which had been added 0.5µM GA_3. Shoot multiplication from these explants occurred not only from the axillary buds of the explants, but each node of the *in vitro*-derived shoots also produced one or two axillary shoots. Secondary axillary shoots also proliferated earlier from chilling treated explants. For root induction, *in vitro*-derived shoots were transferred to liquid medium containing 1.1µM IBA. In most cases, two roots emerged from the node nearest to the cut end of the stem within 7-10 days. After roots had elongated (7.2 ±1.4 cm), rooted plants, each 22.4 ± 2.3 cm in height, were transferred to cultivated field.

RAPD analysis was also a suitable technique with which to investigate the phylogenetic relationships between 18 cultivars of water chestnut (Hoque *et. al.,* 2001). Indeed, this could be the first step towards more efficient identification of water chestnut. The protocol used mature leaves and CTAB to isolate DNA that was undegraded, dissolved readily in Tris EDTA buffer and that could be amplified.

In respect of all the above mentioned aspects, we hope that an enhanced cultivation techniques related with the selection of salt tolerant water chestnut variety and transgenic plant would be possible through the advanced studies promises to provide a means for the mitigation in developing countries which would be expand the water chestnut cultivation area through the utilization of

saline water near the sea as well as fresh lowland and would be helpful for future growth of population and industrial purposes. At present, there is an increasing interest both in industry and scientific research in useful plants which could be recognized as a nutritious food or dietary supplement as well as an important source of biologically active compounds of medicinal n Mozambique, a large number of wild food plants are widely distributed throughout the country (Hoque *et. al.,* 2005a). It is clear that climate change will have a serious impact on land utilization in the future through the effects of increased flooding, salinization of aquifers, increased population density and food requirements. Rising water levels, for example, may inundate one third of the existing farm land in Bangladesh. Water chestnut, because of its morphology and growth habit, could become an important alternative crop to cereals and the opportunity to cultivate it on marginal lands should be taken seriously by the scientific community.



CHAPTER VIII

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