INTEGRATED CULTURE OF FISH WITH MICROPROPAGATED PLANTS IN A RECIRCULATORY SYSTEM

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled **'Integrated culture of fish with micropropagated plants in a recirculatory system'** is a bonafide record of work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled **'Integrated culture of fish with micropropagated plants in a recirculatory system'** is a record of work done independently by Mrs. Navya R. under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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LIST OF ABBREVIATIONS

Ascorbic Acid	AA
2,4 – Dichloro Phenoxy Acetic Acid	2,4 – D
Benzyl Amino Purine/ Benzyl Adenine	6- BAP/BA
Days After Initiation	DAI
Filter System Ratio	FSR
Indole -3- Acetic Acid	IAA
Indole Butyric Acid	IBA
Kinetin	Kn
Light Emitting Diodes	LED
Murashige and Skoog Medium	MS
Naphthalene Acetic Acid	NAA
Parts per million	ppm
Plant Growth Regulators	PGR
Recirculating Aquaculture System	RAS
Thiodiazuron	TDZ
Total Ammonia Nitrogen	TAN
United States Environment Protection Agency	USEPA

Introduction

1. INTRODUCTION

Aquascaping with a multitude of aquarium plants is the concept of a modern day aquarium. A properly planted aquarium with gravel, rocks, bog wood and other materials can create an attractive natural environment in the confines of an aquarium. Fishes look better and comfortable in planted aquarium. Aquarium plants are traded around the world for their aesthetic role. Presently the global ornamental fish trade including aquarium plants, accessories and fish feed is worth US$ 15 billlion (Kurup *et al.*, 2012). In this, the aquarium plant trade is a multimillion–dollar industry with the leading producers being USA, Central Europe and Japan taking the lead role (Lehtonen and Arevalo, 2005). Variety of plants that are present in lakes, ponds, wetlands and sea are used and traded as aquarium plants. India has a rich biodiversity of aquatic plants and almost half of the world's flowering aquatic plants are reported from India (Singh and Singh, 2002). Inspite of the assetful resources, aquarium plant trade has not gained momentum in India for two reasons. The first and foremost one is that most of our aquatic flora are not properly documented. The available work pertains mainly to that of terrestrial and wetland flora descriptions and a detailed documentation of aquatic flora is few (Subramanyam, 1962; Joseph and Sivarajan, 1993 and Cook, 1996). The second one is that the art of growing plants successfully in aquariums is not familiar to the Indian aquarists.

Many of the attractive aquatic plant species that dominate in the trade are collected from the wild. This possibly has a devastating impact on rare species and affects the natural aquatic ecosystem (Yapabandara and Ranasinghe, 2002). It is necessary to discourage this wild collection to conserve precious aquatic biodiversity and develop mass propagation techniques to meet the export market. Wijesundara and Shantha Siri (2004) suggested that *in vitro* propagation is the best technology for mass production.

In vitro propagation is an efficient and cost effective method for propagating large number of clonal offsprings. According to Ailstock and Shafer (2006), the plants produced by *in vitro* propagation are genetically uniform, vigorous and free from associations with other organisms, an attribute particularly useful for the culture of underwater grasses where contaminating organisms can dominate other types of production systems. The continuous supply of sterile plantlets will overcome the contamination problem and reduce the time for sterilization process and also makes the quarantine procedures for export easy. Many tissue cultured water plant species show bushy growth with more adventitious shoots, qualities that many aquarists appreciate (Christensen, 1996).

Even though considerable progress has been achieved in developing *in vitro* micropropagation protocols in a variety of land plants, aquatic plants in general have lagged behind (Ozturk *et al.,* 2004). In India micropropagation of aquarium plants is a virgin area and hence the first part of the present work envisages to standardise the *in vitro* propagation techniques for five commercially important varieties of aquarium plants *viz. Bacopa caroliniana, Anubias minima, Aponogeton ulvaceus, Rotala rotundifolia* and *Nymphoides cristata.*

The benefit of any micropropagation system can be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro* which is the hardening (Hazarika, 2003). The second part of the work is to incorporate these micropropagated aquarium plants in a recirculatory system for hardening. Ammonia is one of the most important pollutants in the aquaria. Fish and bacteria excrete ammonia as a waste product of their metabolism. Aquatic plants have the capacity to detoxify ammonia by using it for synthesising proteins or by combining it with stored carbohydrates to

form ordinary amino acids (Ferguson and Bollard, 1969). Chakrabarti and Sharma (2008) reports that aquatic plants including seaweeds and fodder crops like *Marsilia*, *Azolla* etc. can be incorporated in recirculatory systems as a secondary crop in addition to fish. The micropropagated plants when incorporated into the recirculatory system will also help in purifying the water.

Recirculating Aquaculture Systems have been identified as one of the two main research areas in aquaculture (NOAA, 2001). Recirculating Aquacutlure Systems are better stated as the culture systems of the future to tide over the limitations of land and water usage. The main challenge of a recirculating system is the maintenance of optimum water quality parameters throughout the entire culture period without much cost implications. Unlike flow-through and pond production systems, the water reuse systems have minimum make-up flow and a constant volume of water is being recirculated throughout the operation. These production systems internally treat water contaminated by dissolved organics and ammonia by means of a biofilter rather than discharge them. Since filter medium is the heart of biofiltration, the type of filter medium used strongly influences the capital and operating costs of the biofilter.

There are a wide variety of commercially available biofilters and biofilter media in the market today. Rocks, shells, sand and plastic are the commonly used biofilter media. Proper selection and sizing of biofilters are critical to both the technical and economic success of a Recirculating Aquaculture System (RAS). These critical decisions are based on the filter performance measured from water quality parameters and nitrification rates. Nitrification is carried out in the biofilm which forms on the filter media. The nitrifying bacteria grows on the biofilm and converts ammonia to less toxic forms. Nitrification rates will be maximum in a filter media with highest surface area per unit volume. Van Rijn

(1996) reports that an ideal filter material (a) has a higher surface area per volume, (b) is low in cost, (c) is durable, (d) does not clog easily and (e) promotes a uniform spread of water to be treated. Plastic materials fulfill most of these criteria and are increasingly being used. In the present work, an environment friendly recirculatory system is designed with three indigenous filter media like coir, vetiver (Ramacham) and bamboo. Hence, the third part of the work studied the performance of three indigenous filter media by measuring the water quality parameters like ammonia, nitrite and nitrate in a plant fish integrated recirculatory system.

Considering the significance of aquarium plants in ornamental fish trade and the need for an environment friendly plant fish integrated recirculatory system, the present study entitled "Integrated culture of fish with micropropagated aquarium plants in a recirculatory system" was taken up with the following three objectives:

(1) *In vitro* propagation of five species of aquarium plants namely *Bacopa caroliniana, Anubias minima, Aponogeton ulvaceus, Rotala rotundifolia* and *Nymphoides cristata.*

(2) Hardening of micropropagated plants in a plant fish integrated recirculatory system.

(3) Studying the filter performance of three indigenous filter media like coir, vetiver and bamboo in the integrated recirculatory system based on the water quality parameters.

Review of Literature

2. REVIEW OF LITERATURE

In this chapter, reports pertaining to the richness of aquatic flora of India, emergence of aquarium plant trade, botanical description of selected varieties of aquarium plants, vegetative propagation techniques, *in vitro* propagation techniques and factors contributing to its success and recirculating aquaculture systems, nitrification processes in RAS, types of biofilters and water quality criterion in RAS are reviewed sequentially.

2.1 Aquatic plant diversity of India

The aquatic vegetation of India includes the aquatic plants that are spread along the rivers, lakes, ponds and wetlands. Aquatic plants grow either rooted in water or are free floating and their growth is favoured by water logging or submergence but usually perishing during prolonged dewatering. Approximately half of the aquatic flowering plants of the world are present in India. The aquatic families in Indian flora are Alismataceae, Aponogetonaceae, Azollaceae, Barclayaceae, Butomaceae, Cabombaceae, Callitrichaceae, Ceratophyllaceae, Hydrocharitaceae, Isoetaceae, Lemnaceae, Marsilieaceae, Najadaceae, Nelumbonaceae, Nymphaeaceae, Podostomaceae, Pontederiaceae, Potamogetonaceae, Ruppiaceae, Salviniaceae, Trapaceae, Typhaceae and Zanichelliaceae (Singh & Singh, 2002). According to Nagendran and Arekal (1981), a number of aquatic plants are endemic to India, of which Podostomaceae tops the list of about 24 endemic taxa.

2.2 Emergence of aquarium plant trade

A review of the paradigm changes in the role of plants in the 160 years of modern aquarium keeping reveals their position at the intersection of aesthetics, technology and commerce. Modern aquarium keeping can be said to have started with the "Aquarium mania" resulting from the opening of the first public

aquarium at the Regents Park Zoological Garden in London in 1853 (Hibberd, 1860). Initially the plants were given the status as water purifiers and their aesthetic qualities hold only second place (Weigel, 1973). However, it was in the 19th century that the keeping of exotic plants in the tropical glasshouses really took off (Sinisalo, 1997). The sword plants (genera *Echinodorus* and *Helanthium*, Alismataceae) are among the economically most important ornamental aquatic plants (Brunel, 2009), and have been in cultivation since the early 20th century (Wendt, 1952). Biotope aquariums to imitate nature evolved in 1950s and several aquarium design styles emerged later on like landscape style, dutch style which looked exactly like paintings of natural scenaries (Turner, 2005). The Japanese nature photographer Takashi Amanos' "Nature Aquarium" styles like "Karesansui style" and "Iwagumi style" is the most popular of the aquarium designs, where he recreated, emersed and not submersed scenes of nature (Adams, 2009). These gardens were developed from the representations of the old Taoist sansui paintings of stream, waterfall and mountains by reducing the scale and by the abstract use of substitutes: rocks, gravel and sand with only mosses used as vegetation (Douglas *et al.,* 1984). However, the popularisation of "Nature Aquarium" style is the major driving force that shaped the aquarium plant demand (Lehtonen and Falck, 2011).

Yapabandra and Ranasinghe (2002) has listed 13 most popular varieties of aquarium plants in modern day aquarium viz. *Anubias, Cryptocoryne, Echinodorus, Aponogeton, Hygrophyla, Bacopa, Hydrilla, Cabomba, Myriophyllum, Lagenandra, Vallisneria, Nympheae* and *Elodea*. The aquarium plant trade has grown into a multimillion–dollar industry with the leading producers as USA, Central Europe, Japan and SriLanka (Lehtonen and Arevalo, 2005).

2.3 Botanical description of aquarium plants selected for study

2.3.1 *Anubias minima* Chevalier

According to Crusio (1979) the systematic position of *Anubias minima* is as follows.

Kingdom	: Plantae
Phylum	: Tracheophyta
Class	: Magnoliopsida
Order	: Alismatales
Family	: Araceae
Genus	: *Anubias*
Species	: *Anubias minima*

Anubias is found in tropical West Africa and contains about twelve species growing along shady banks. The leaves are broadly lanceolate, ovate or elliptical, with an acuminate or rounded tip, tapering below into a petiole which is as long as the blade and sheathed below. The upper leaf surface is smooth and grass-green; the lower surface is lighter, a yellow-green, with distinct veins. The leaves are very strong and stiff. They are propagated by rhizome cuttings. A mature rhizome is separated from the parent plant and either divided along the dormant buds and left undisturbed, or the whole rhizome is put in water and divided only after the plantlets are formed. The plants rarely flower and proper identification is impossible without knowledge of the floral organs. *Anubias minima* is the smallest variety of *Anubias* where the leaf blades are narrowly elliptical to lance-shaped, 60 mm long and 20 to 30 mm wide. It is best suited for small aquariums or for planting in the foreground of medium-size tanks (Rataj and Horeman, 1977).

2.3.2 *Aponogeton ulvaceus* Baker

The systematic position of *Aponogeton ulvaceus* as given by Cronquist (1968) is as follows.

Kingdom : Plantae
Phylum : Magnoliophyta
Class : Liliopsida
Order : Najadales
Family : Aponogetonaceae
Genus : *Aponogeton*
Species : *Aponogeton ulvaceus*

Rataj and Horeman (1977) has given a detailed description of the species. The plant belongs to family Aponogetonaceae which has one genus and about 45 species (Ali, 2008). The members of this family are widely distributed in the tropical and subtropical regions of the Old World (Lye, 1989). *A. ulvaceus* originates from Madagascar. The leaf blades have a short or a long petiole and undulated margins, the base tapering gradually. Well-developed leaves are spirally coiled. Leaves arise from a cone-shaped lightly hairy tuber (James, 1997). The flowers are yellow, the inflorescence with two to five spikes. This species is propagated by seeds.

2.3.3 *Bacopa caroliniana* (Walter) B.L. Rob

A systematic classification of *Bacopa caroliniana* was given by Robinson (1908) as follows

Kingdom : Plantae
Phylum : Magnoliophyta
Class : Magnoliopsida

Order : Scrophulariales
Family : Scrophulariaceae
Genus : *Bacopa*
Species : *Bacopa caroliniana*

The genus *Bacopa* commonly called 'water hyssop' includes marsh, water or terrestrial plants growing in the tropical and subtropical zones. About 3000 species are included in this family and distributed throughout the world from the Arctic to the tropical zone. *Bacopa caroliniana* originated from Central America where it grows as a marsh or amphibious plant (Rataj and Horeman, 1977) . The emersed stem is about 2.5 to 5 mm thick, sparsely pilose, up to 1 m long, prostrate, and rooting at the nodes. The leaves are fleshy, opposite, ovate or elliptical, 20 to 30 mm long and 10 to 15 mm wide. The five petal blue flowers are solitary and arise from leaf axils (Kasselman, 2002).

2.3.4 *Rotala rotundifolia* (Roxb.) Koehne

Cook (1976) gave the systematic position of *Rotala rotundifolia* as follows:

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Myrtales
Family : Lythraceae
Genus : *Rotala*
Species : *R. rotundifolia*

The loosestrife family Lythraceae comprises herbs, bushes, or trees with opposite leaves (rarely whorled). The genus *Rotala*, has more than 46 species around the world, of which *Rotala indica* and *Rotala rotundifolia* are seen in

India (Vardhana, 2006). *Rotala rotundifolia* is an annual or perennial amphibious herb growing densely in marsh lands, paddyfields and stream sides of China, Bangladesh, Bhutan, India, Japan, Laos, Myanmar, Nepal, Thailand and Vietnam (Haining *et al.,* 2007). This is a popular aquarium plant on account of its decussate, elliptic leaves which get a beautiful red color when exposed to high light intensity. They are often arranged as a bunch plant in the aquarium. Bright rose petal flowers bloom on the emergent spike like inflorescences. This is a fast growing plant in the aquarium, but sensitive to unfavourable conditions, when it will react by producing smaller leaves (Jacobsen, 1979).

2.3.5 *Nymphoides cristata* (Roxb.) O. Ktze.

The taxonomic ranking of *Nymphoides cristata* as given by Kuntze (1891) is as follows:

Kingdom	: Plantae
Phylum	: Magnoliophyta
Class	: Magnoliopsida
Subclass	: Asteridae
Order	: Solanales
Family	: Menyanthaceae
Genus	: *Nymphoides*
Species	: *Nymphoides cristata*

The genus *Nymphoides* Seguier (Menyanthaceae), consisting of about 40 species, occurs mainly in wet place of temperate and tropical regions of both the Old World and the New World (Ho and Ornduff, 1995). Sivarajan and Joseph (1993) published a detailed account of *Nymphoides* spp. in India. *Nymphoides cristata* is commonly called 'Crested floating heart' because its petals are fused together along their margins, forming a crested appearance. These are annual plants with large floating stem, rooting at the nodes. Small clusters of tuberous

roots form just below the floating leaves, but they are slender and tapered. Each new plant develops roots and leaves and in their turn, its own offspring, each of which continues the process until something happens to break it all up. Leaves floating, lamina 2.5 to 7.5 (≈10) cm long, 2 to 8 mm broad, orbicular, cordate at base, with conspicuous dense brown glands on the under surface, margin entire or wavy, petiole 1 to 5 cm long, pedicel 0.5 to 5 cm long. Corolla lobes oblong, acute, approximately 4 x 1.5 to 2 mm, white, with yellow base and longitudnal folds along the margin and centre. Seeds (≈) 1 mm long and tuberculate. Li *et al.* (2002) reports that the species is distributed mainly in India, SriLanka, Malaysia and South China.

2.4 Vegetative propagation and generative reproduction

According to Rataj and Horemann (1977) aquatic plants have several modes of propagation. Some aquatic plants (*Cryptocoryne*) reproduce by rhizomes, others (*Vallisneria, Sagittaria*) by runners. In the genus *Echinodorus,* new plants arise on flower stems in the axil of the flowers. Numerous species of aquatic plants can be propagated by cuttings or by fragments of the plant body that can take root and continue growing. This is common in plants of the genera *Ludwigia, Rotala, Elodea, Ammania, Alternanthera,* and many others which sprout new branches from the axillary buds at each separate internode of the stem. In some plants this ability is so strongly developed that even torn leaves or their fragments may take root, as in *Hygrophila polysperma* and *Bacopa amplexicaulis.*

In plants with a cylindrical rhizome that is long and creeping, such as *Anubias, Acorus,* and some *Echinodorus* (*E. Osiris*), new plants will develop, form dormant buds on them and become separated in course of time. Another different method of propagation is seen in *Crinum* spp. which reproduces by means of small bulbs that develop on the main bulb from time to time (James, 1997). In perennial plants without the typical cylindrical rhizome, new plants

will develop around the perimeter of a globular woody rhizome as in the case of *Echinodorus berteroi*. The rhizomes of some perennial plants can also be divided into 2 to 6 parts, each of which is able to continue growing independently. Hiscock (2003) describes this mode of propagation as offset propagation, wherein the plantlets will be growing so close to the mother plants in clumps that they look as a single plant. Offsets can be carefully separated and replanted elsewhere and is common in the family Araceae, with *Aponogeton* species, and with all larger species of *Echinodorus*.

Generative propagation through seeds is used only for those species which cannot be reproduced vegetatively like *Aponogeton*, *Ottelia* and species such as *Echinodorus cordifolius* which reproduce faster and more abundantly by seed than vegetatively (Rataj and Horemann, 1977). One year of vegetative propagation might produce 1 to 30 new plants, while a pair of plants during the same time might produce over 1,000 viable seeds. To produce large number of new plants vegetatively would require large numbers of parent plants and correspondingly large tanks and floor space, while much less space is necessary for two or three flowering parents and their offspring. Although the plants produced from seeds may take twice as long to reach saleable size, the reduced need for space makes generative reproduction economically practical.

Vegetative propagation through stem cutting requires enough stocks of stem cutting and high labour inputs (Carneiro *et al.*, 1997). Seema *et al.* (2011) states that the major disadvantage with vegetative propagation is the lesser number of individuals that can be generated from a single plant. Hence, tissue culture is the only alternative method to prepare sufficient amount of plants within short time duration.

2.5 *In vitro* propagation

Many decorative plant species are collected from the wild, possibly causing devastating impact on rare species (Yapabandara and Ranasinghe, 2002). Wild collection for export without artificial propagation leads to an extinction of endemic aquatic plant species and also affects the natural aquatic ecosystem. However, *in vitro* micropropagation efforts have been very few in the case of aquarium plants and reported for *Anubias barteri, Aponogeton* sp., *Bacopa monnieri, Cryptocoryne wendtii, Myriophyllum aquaticum* and *Ludwigia repens* (Kukulezanga *et al.,* 1980; Huang *et al.,* 1994; Kane *et al.,* 1999; Ozturk *et al.,* 2004; Smitha *et al.,* 2007; Banerjee and Shrivastava, 2008). Aquarium plant *in vitro* propagation has minimised many of the problems associated with unreliable supply, over collection, variable plant quality, and frequent losses from poorly characterized water–transmitted diseases (Kukulezanka *et al.,* 1979, Petersen, 1996, Kauth *et al.,* 2006).

2.5.1 General aspects of plant tissue culture

Schleiden (1838) and Schwann (1839) postulated the cell theory , which revealed the totipotent nature of plant cells. This forms the basis for plant cell, tissue and organ culture. Haberlandt (1902) reported that isolated cells are capable of resuming uninterrupted growth. Skoog and Miller (1957) with their discovery of auxins and cytokinins made a landmark in the history of plant tissue culture. They put forth the concept of hormonal control of organ formation and showed the root and shoot differentiation was a function of auxin – cytokinin ratio and that it could be regulated by altering the relative concentrations of these growth regulators in the medium. Many pioneer investigations like White (1934), Gautheret (1939), Miller *et al.* (1956), Steward *et al.* (1958), Bergmann (1960) and Vasil and Hilderbrandt (1965) have contributed for successful development of plant tissue culture concepts. A completely defined nutrient medium for plant tissue culture was developed by Murashige and Skoog (1962).

Murashige (1974) advocated the possibility of three routes of *in vitro* propagule production, which included enhanced release of axillary buds, production of adventitious shoots through organogenesis and somatic embryogenesis. Vasil and Vasil (1980) reported that the tissue culture derived plantlets grow faster and mature earlier than seed propagated plants. The best commercial application of tissue culture is the production of true to type plants at a very rapid rate compared to the conventional methods (Levy, 1981).

Plant tissue culture is the science of growing plant cells, tissues or isolated organs on artificial media (George *et al.*, 2008). This technique is commonly used to mass produce relatively large quantities of desirable plants, helping to prevent extinction (Jang *et al.*, 2003), to preserve plants with specific genetic traits in an attempt to maintain the uniqueness of the species (Mol *et al.*, 1989), and as part of the engineering of certain plant crops for economic and commercial purposes (Zhao *et al.*, 2001). *In vitro* tissue culture techniques are also used to facilitate some *in vitro* experiments such as genetic transformation, protoplast fusion and investigation of the gene expression in saponin biosynthesis and for the production of interesting secondary metabolites. (Faizal *et al.*, 2011).

Many tissue cultured water plant species show a more bushy growth with more adventitious shoots, qualities that many aquarists appreciate (Christensen, 1996).

Tissue culture in turn is the only technique to produce plants of high and uniform quality in large quantity from any part of the plant in any season. This technique provides a rapid reliable system for production of large number of genetically uniform and even disease free plantlets (Sujana and Naidu, 2011).

2.5.2 Factors influencing success in micropropagation

Success of *in vitro* propagation depends on several factors directly and indirectly. These factors include the type of explant, surface sterilization, presence of systemic microbial contamination, presence or absence of other additives, nitrogen source and concentrations, physical conditions of the medium, pH, quality and intensity of light, temperature and relative humidity (Brown and Thorpe, 1986, Thorpe, 1995).

2.5.2.1 Explant

Skirvin (1980) pointed out that there is variation among the type of explant within each plant species and the most suitable explant for each plant species should be determined.

2.5.2.1.1 Type of explant

In aquatic plants the preferred tissues may include meristematic cells, the apical buds of photosynthetic stem and rhizomes, the meristems of over-wintering structures, or embryonic tissue that is protected by an intact seed coat (Madsen, 1985, Kyte and Kleyn, 1996, Cassells, 1997).

Kane *et al.* (1999) used basal shoot tip explants for micropropagation of *Cryptocoryne wendtii*, an aquarium plant native to southeast Asia and Indonesia. In *Nymphoides indica*, stolonic nodal explants with lateral buds and leaf petioles were used successfully to induce shoot organogenesis *in vitro* (Jenks *et al.*, 2000). Studies by Fracaro and Echeverrigaray (2001) mentions the excision of axillary buds for *in vitro* propagation of *Cunila galioides*, a medicinal plant. Despite a moderate multiplication rate, nodal segment remains a widely used explant for micropropagation of vines due to its operational feasibility and genotype stability (Torregrosa *et al.*, 2001). However, Tiwari *et al.* (2001) in

their works on *Bacopa monnieri* reveals that *in vitro* propagules can be successfully derived from nodal, internodal and leaf explants. Apical shoots and axillary buds of corms of *Alocasia* formed shoots on MS medium supplemented with 2-ip (Thao *et al.*, 2003). In *Pinellia tripartite*, a herbaceous medicinal plant propagated through tubers and bulbils, leaf and petiole explants were taken for the *in vitro* propagation and found that leaf explants were more appropriate than petiole for somatic embryogenesis (Kim *et al.*, 2005). *In vitro* propagation studies in *Drosera indica*, an insectivorous plant was successfully done with shoot tips (Jayaram and Prasad, 2007). Apart from this, buds from rhizome were used in shoot multiplication of lotus (*Nelumbo nucifera*) *in vitro* (Shou *et al.*, 2008).

2.5.2.1.2. Size of explant

The increasing size of explant shows an inverse relationship with growth rate (Caplin, 1963). But the results were contradictory in all the following citations. Norton and Norton (1986) studied the effects of explant length (2.5 to 2.0 mm), axillary bud number (0 to 6), presence or absence of apical dome and explant retrieval (from top, middle or base of plant canopy) in the case of *Prunus spirea*. The results showed that the number of shoots formed after four weeks increased with the explant length and decreased with the number of buds present.

Explant size plays significant role in shoot formation and growth (Salehi and Khosh – Khui, 1997). Uninodal segments of 0.5 to 1.0 cm were used for axillary bud breakage and callus regeneration in *Uraria picta* (Anand *et al.*, 1998) whereas nodal segments of 9.0 to 10 mm were used in the case of *Catharanthus roseus* (Pati *et al.*, 2011). *In vitro* propagation studies in *Lilium maculatum*, a wild lily in Japan were initiated from the inner scales of bulbs cut into middle sections of 1x1 cm^2 (Amaury *et al.*, 2007). Variations in bulblet induction rate was observed with variation in scale size (0.5 to 0.8 cm in length) and summarized that explant size may also affect the nutrient uptake by the scale

from the medium, leading to a variation in composition and quality of the scale cells. Findings of the work done by Shou *et al.* (2008) in Lotus (*Nelumbo nucifera*) reveals that buds with leaf primordia easily produced shoots than buds without leaf primordia.

Karimi *et al.* (2010) studied the effect of explant size on callus induction in *Cereus peruvianus* and found that explant size of 0.5 cm x 0.5 cm resulted in higher callus induction compared to explant size of 1 cm x 1 cm. Multinodal explants with 3 to 4 nodes were used effectively for *in vitro* multiplication of *Bacopa monnieri* in a semisolid medium supplemented with 0.2 mg l^{-1} BAP (Sharma *et al.*, 2010).

2.5.2.1.3. Position of explant

Ho and Lee (1985) reported that in *Lagerstroemia speciosa*, nodal segments were superior to shoot tips when cultured artificially. Yu and Meredith (1986) in their works demonstrated that survival and shoot production potential was greater in explants from axillary shoots than from terminal ones. Kane and Albert (1989) established that aerial-leaf explants of *Myriophyllum heterophyllum* exhibits high capacity for adventitious shoot formation when cultured *in vitro*.

Shoot regeneration frequency in *Ludwigia repens*, an aquarium plant was higher in apical meristems and first node and reduced in descending order to the third–fourth axillary buds (Ozturk *et al.*, 2004). Ibanez *et al.* (2005) opined that one of the major factors influencing the survival and *in vitro* proliferative response of grapevine explants is their position on the mother plant. Results show that cultures derived from nodal segments taken from the basal zones of the same shoots had serious contamination problems which considerably reduced their viability. At the same time, nodal segments belonging to the zones farthest

from the apex showed the highest percentage of explants with secondary shoots and axillary buds.

A different observation was made by Jenks *et al.* (2000) in their studies on effect of leaf tissues from different areas of leaf blade and petiole on shoot organogenesis in *Nymphoides indica*. Here they found that any part of the *Nymphoides* leaf can be used as a source of responsive explants for mutant induction without significant differences. Karimi *et al.* (2010) studied the effect of explant position on callus induction in *Cereus peruvianus* and found that apical explants gave the highest callus induction in all growth regulator combinations compared to lateral explants. The calluces formed from stem explants were friable. A comparison of shoot proliferation response by two types of explants viz. shoot tip and nodal segments in *Mentha peperita* showed that nodal explants produced more number of shoots per explant than shoot tip explants (Sujana and Naidu, 2011).

2.5.2.2 Surface sterilization of explant

Surface sterilization is done to remove all the microorganisms present on the explant with minimum damage to the plant parts. The first step in initiating a new cell culture is the availability of plant tissue free of all contaminating microorganisms (Hall, 2000). The presence of microbes results in increased culture mortality, variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Kane, 2003). Webster *et al.* (2003) suggested that prudent selection of explants from the healthy parent plants coupled with an effective surface sterilization method should be the goal in avoiding culture contamination.

Sodium hypochlorite (0.5 to 2.0 percent w/v), calcium hypochlorite (filtered 5.0 to 10.0 percent w/v) and mercuric chloride (0.05 to 0.1 percent w/v) are the commonly used surface sterilants (Chawla, 2002). As the surface

sterilants are toxic to the plant cells, it is necessary to wash the tissues twice or thrice in sterile distilled water to ensure dilution of the chemical (Hu and Wang, 1983).

Bonga (1982) advocated the use of alcohol alone or in combination with other chemicals for disinfection. Hall (2000) suggests the addition of a wetting agent like any house hold detergent or Tween to the surface sterilant to reduce the surface tension and improve the contact.

Dodds and Roberts (1985) reported that the use of antibiotics for sterilization should be avoided as they metabolise the plant tissues with unpredictable results. Leifert *et al.* (1994) states that arbitrary use of antibiotics might not yield any useful results as the majority of the bacteria infecting plant materials are gram-negative, which are less sensitive to the commonly used antibiotics.

Several authors have reported difficulties in establishment of aseptic aquatic plant cultures (Dore Swamy and Mohan Ram, 1969; Madsen, 1985; Godmaire and Nalewajko, 1986). Surface disinfection difficulties have been attributed to the presence of large bacterial and fungal epiphytic populations, mucilaginous coatings, endophytic organisms in highly lacunate tissues and lack of a cutinized epidermis (Madsen, 1985; Godmaire and Nalewajko, 1986). Meyberg (1988) reported that presence of numerous mucilage secreting trichomes in *Nymphoides* species account for the difficulties in surface sterilization.

Aseptic cultures of *Cryptocoryne wendtii* and *Nymphoides indica* were raised by surface sterilizing the explants with 50 percent ethanol for 1 minute and 1.05 percent sodium hypochlorite containing 1 drop Tween – 20 per 100 ml for 12 minutes, followed by three 5 minute rinses in sterile distilled deionized water (Kane *et al.*, 1999, Jenks *et al.*, 2000).

According to Sharma *et al.* (2010), *Bacopa monnieri* nodal segments require time specific surface sterilization treatment with 0.1 percent mercuric chloride for 4 to 5 minutes above which caused blackening of the tissues.

2.5.2.3 Culture medium

Selection of culture medium depends on the plant species and purpose of culturing. A wide variety of media have been reported by many researchers. The earliest and widely used basal media proposed were by White (1943) and Heller (1953). Since 1980, most researchers have been using Murashige and Skoog (MS), (Murashige and Skoog, 1962) medium. Other derivatives of MS medium include the B5 medium developed by Gamborg *et al.* (1968), SH (Schenk and Hilderbrandt, 1972) medium and the woody plant medium (WPM) developed by Lloyd and McCown (1980). The MS medium is characterized by high concentration of mineral salts. Skirvin (1980) suggested that reducing the strength of MS medium by half was more beneficial for culturing.

Response of an explant to different media depends on the plant species. Das (1992) reported that rhizome explants of *Agave sisalana* responded better in SH medium when compared to MS medium. Callusogenesis in *Rosa* species was different in MS and SH media. The calluses that developed in the MS medium were hard and dark green while those in the SH medium were soft, pale greenish and nodular (Datta *et al.*, 2002).

Full strength MS medium was found to be optimal for the tissue culture of aquatic plants (Kane and Gilman, 1991; Kane *et al.*, 1999; Jenks *et al.*, 2000; Ozturk *et al.*, 2004; Shou *et al.*, 2008; Sharma *et al.*, 2010).

2.5.2.3.1 Type of culture medium

The introduction of a liquid culture system for *in vitro* mass propagation helps in the substantial reduction of plantlet production costs (Sandal *et al.,* 2001) and is an important step towards automation (Aitken-Christie *et al.,* 1995). Further, a liquid medium offers other advantages in the form of uniform culturing conditions, renewal of the media without changing the container, sterilization by microfilteration, and ease of cleaning containers. However, several reports indicate that a liquid culture system promotes hyperhydricity (Deterz *et al.,* 1994; Aitken-Christie *et al.,* 1995) and is limited by a low oxygen content (Smith and Spomer, 1995).

A comparative study was conducted to evaluate the shoot proliferation intensity in liquid and solid medium in *Catharanthus roseus* (Pati *et al.,* 2011). The results showed that liquid medium performed better in terms of shoot length, shoot diameter, number of leaves and branches per shoot whereas shoots in agar gelled medium showed symptoms of chlorosis and leaf yellowing. Liquid culture also over performed solid culture in rooting response by producing roots faster (12 days) than in agar gelled medium (21 days). This work also threw light on several other findings that: elimination of agar can bring down the total cost up to 5.2 folds, reduction of quantity of medium required is less (20ml) in liquid culture at the place of 100 ml solid medium brings down cost by 5 folds, enhanced retention period in liquid medium (6 weeks) compared to 4 weeks in solid medium brings down cost by 1.5 fold. Apart from these, the extended subculture period in liquid culture also saves the cost in terms of labour.

Various compounds of cyanobacteria could be useful sources to enhance or substitute the influence of synthetic plant growth regulators on tissue cultures of different plants *in-vitro*. The approach of using cyanobacterial cultures would overcome many barriers of micropropagation where costly synthetic chemicals are involved (Banerjee and Sarkar, 2008). Cyanobacteria or Blue green algae are

prokaryotic photosynthetic microorganisms that produces a wide array of substances, including plant growth regulators (Metting and Pyne, 1996). Banerjee and Modi (2010) formulated an efficient and cost effective liquid culture medium comprising MS medium and cyanobacterial extract for *Bacopa monnieri.*

Several culture techniques have been employed over the years. These include liquid culture (Kao *et al.,* 1971), liquid over agar (Constabel, 1975), protoplasts embedded in agar (Nagata and Takebe, 1971), feeder layers (Raveh *et al.,* 1973; Cella and Galun, 1980), nurse cultures (Menezel *et al.,* 1978), hanging droplets (Potrykus *et al.,* 1976), and filter paper laid on agar (Partanen, 1981; Santos *et al.,* 1980).

2.5.2.3.2 Growth regulators

For a successful plant tissue culture, the selection and addition of growth regulators at the optimum level is one of the crucial factors (Skoog and Miller, 1957; Hilderbrandt *et al.*, 1963; Krikorian, 1982; Heyser *et al.*, 1983; Nabors *et al.*, 1983; Carman *et al.*, 1988 and Przetakiewicz *et al.*, 2003). Commonly used growth regulators in tissue culture include four groups such as auxins, cytokinins, gibberellins and retardants like abscissic acid. A significant difference in capability of shoot initiation found among explants is also influenced by the differences in degree of their sensitivity towards growth regulators (Sasaki, 1979; Nhut *et al.,* 2001).

2.5.2.3.2.1 Auxins

Auxins cause cell division, cell elongation and swelling of tissues, and the formation of adventitious roots but it often inhibits adventitious and axillary shoot formation (Chawla, 2002). Auxins most frequently incorporated in the medium to induce rooting are IAA, IBA and NAA (Hall, 2000). Monier and

Ochatt (1995) reported that the highest concentration of auxins resulted in maximal rooting in *Cotoneaster* genotypes.

Tiwari *et al.* (2001) reported that basal medium served as the rooting medium for *Bacopa monnieri in vitro* cultivars but addition of IBA significantly improved the rooting at 4.9 µM levels. Similarly in *Ludwigia repens*, rooting was better in MS growth regulator free medium compared to that of medium with TDZ and BA (Ozturk *et al.*, 2004).

2.5.2.3.2.2 Cytokinins

Cytokinins are derivatives of adenine and have an important role in shoot induction (Chawla, 2002). Benzyl adenine (BA), Kinetin (Kn), isopentyl adenine (2 – iP) and TDZ are the commonly used cytokinins (Hall, 2000). Cytokinins are generally considered as a critical factor for *in vitro* shoot production and there are many reports that BA exhibits beneficial effect (Rao and Purohit, 2006) over other cytokinins for shoot multiplication (Dantu and Bhojwani, 1987; De Bruyn and Ferreira, 1992; Lakshmanan *et al.*, 1997).

Differences in effectiveness of naturally occurring versus synthetic cytokinins to promote shoot organogenesis between aquatic plant species may reflect differences in cytokinins uptake and metabolism (Blakesley and Lenton, 1987). Jayaram and Prasad (2007) studied the effect of different growth hormones on shoot proliferation in *Drosera indica* and found that *in vitro* shooting was highest with Zeatin followed by Kinetin and then BA. They also found that high concentration of cytokinins inhibit growth and in extreme cases induce red pigmentation and necrosis and rooting was also poor.

Cytokinins overcome apical dominance, release lateral buds from dormancy, and promote shoot formation (George, 1993). Micropropagation experiments in *Cunila galioides* revealed that BA produce more shoots from

axillary buds than Kinetin and 2 –iP. However, an increase in hormone levels beyond optimum caused callusing, and hyperhydric and malformed shoots (Fracaro and Echeverrigaray, 2001).

Thidiazuron, a synthetic phenylurea derivative, is one of the most active cytokinin like compounds for woody plant tissue culture (Huetteman and Preece, 1993; Lu, 1993; Kim *et al.,* 1997; Thimmappaiah *et al.,* 2002; Ahmad and Anis, 2007). Unlike classic cytokinins, TDZ is competent of fulfilling both the cytokinin and auxin requirement of various regenerative responses of many different plant species (Jones *et al.,* 2007).

The TDZ stimulates shoot proliferation and inhibit their elongation. Inhibition of shoot elongation is a common problem with TDZ and it may be consistent with its superoptimal cytokinin activity, whereas the presence of a phenyl group in TDZ may be the possible cause of shoot bud fasciation (Huetteman and Preece, 1993; Steinitz *et al.,* 2003). The problem of shoot elongation was overcome by transferring shoot cultures on MS medium lacking TDZ.

Most frequently, however, root formation is inhibited by the cytokinins used to induce shoot multiplication (Pennazio, 1975). The inhibitory effect of TDZ has been reported in many plant species (Gray and Benton, 1991; Huetteman and Preece, 1993; Casanova *et al.,* 2004; Jaiswal and Sawhney, 2006; Ray and Bhattacharya, 2008).

2.5.2.3.2.3 Auxins and Cytokinins

The combination of cytokinins and auxins stimulate the *in vitro* multiplication and the growth of shoots of several plant species (George, 1993). Shoot organogenesis was completely inhibited in the presence of BA alone in *Myriophyllum hetrophyllum* and *Nymphoides indica* (Kane *et al.,* 1991; Jenks *et*

al., 2000). A combination of BA and NAA was effective in *in vitro* multiplication of Lotus (*Nelumbo nucifera*) where BA alone was effective for shoot formation but shoot length was disappointing which was compensated by the addition of NAA (Shou *et al.*, 2008).

Thirty three adventitious shoots of *Myriophyllum heterophyllum* were produced from a single leaf explant cultured for 28 days in the presence of Zeatin and IAA (Kane and Albert, 1989).

De Gyves and coworkers hypothesized that there is a synergism existing between TDZ and both endogenous and exogenous auxin (De Gyves *et al.*, 2001).

No shoots were induced on hormone-free medium or when cytokinins alone were added to the culture medium, implying that a combination of cytokinin and auxin was necessary for adventitious shoot formation in *Maesa* leaf explants. Similarly, a combination of TDZ and NAA had a synergistic effect on inducing multiple adventitious shoot formation in *Maesa* spp (Faizal *et al.*, 2011). The combination of TDZ and NAA has also been reported to induce shoot regeneration from apical meristerms and leaf explants of several plant species (Ozturk *et al.*, 2004; Espinosa *et al.*, 2006; Feng *et al.*, 2010; Zhou *et al.*, 2010).

2.5.2.4 Culture incubation

Giles and Friesen (1994) states that the physical form of the medium, whether liquid or semisolid, pH, other environmental factors like light, temperature, relative humidity and season of culture play an important role in the *in vitro* growth and differentiation.

2.5.2.4.1 Effect of temperature and photoperiod

Light requirement for differentiation involves a combination of several components namely intensity, quality and duration (Murashige, 1974). Brasileiro *et al.* (1999) reported that in onion and tomato, callus cultures were formed and maintained in darkness at 23 - 27°C. According to Murashige (1977) the optimum day light period required is 16 hours for a wide range of plants. During *in vitro* culture, the environmental temperature of the species at the original habitat should be taken into consideration (Yeoman, 1986).

Shou *et al.* (2008) reveals that temperature had significant effect on growth in *Nelumbo nucifera* and a temperature of 25°C was found to be optimum for growth in terms of number of leaves and shoots. A 16 hour photoperiod was sufficient for almost all the aquatic plants (Kane and Gilman, 1991; Jenks *et al.*, 2000; Ozturk *et al.*, 2004, Shou *et al.*, 2008).

2.5.2.4.2 Effect of light quality

Light is the major energy source for photosynthesis and regulates plant morphogenic (Mark *et al.*, 2000) and gene expression (Ma *et al.*, 2001). The traditional light source used in *in vitro* culture is fluorescent white light. Light emitting diodes (LEDs) as a new energy source have many advantages compared with the traditional light source, because LEDs have a long life, wavelength specificity and narrow bandwidth (Bula *et al.*, 1991; Brown *et al.*, 1995). In past studies, a mixture of red plus blue LEDs has been shown to improve the production of good quality *Doritaenopsis* plants, wheat plants, and *Withania somnifera* plantlets (Goins *et al.*, 1997; Lee *et al.*, 2007; Shin *et al.*, 2008). Red plus blue LEDs also promoted bulblet growth, producing bigger bulblets with higher fresh weights, dry weights, and dry matter accumulation (Lian *et al.*, 2002).White light enhanced growth and betacyanin accumulation of *S. salsa* calli (Zhao *et al.*, 2010). Red light induced the buds to elongate and to develop a morphology characteristic of etiolated shoots (Bonnett 1972) and increased shoot

height, internode length and rooting frequency. In contrast, blue light encouraged chlorophyll synthesis and stomatal development (Poudel *et al.*, 2008). *In vitro*, *Betula pendula* grew larger leaves and showed increased photosynthetic activity under blue light compared with red light (Sab *et al.*,1995). Another study showed that red and far-red LEDs promoted stem elongation and consequently fragile stems (Kim *et al.*, 2004). However, *in vitro* culture of *Azorina vidalii* under red plus far-red LEDs increased plant length and improved the production of good quality plants (Moreira and Debergh, 1997). Clearly, the reaction to different lighting conditions differs between species and their various growth stages (Lin *et al.*,2011).

2.5.2.5 Secondary product formation in plant tissue culture with special reference to phenolic compounds

The secondary product formation in plant tissue cuture has been reviewed by Collin (2001). In plants, the secondary products include alkaloids, terpenoids, phenolics, steroids and flavonoids, and these have a wide diversity in structure and size and are found in very large numbers throughout the plant kingdom. One of the functions of secondary compounds is that they form part of a biochemical defence mechanism against pathogens and predators (Bennet and Wallsgrove, 1994). A further view is that the secondary compounds may be a convenient sink, into which excess carbon and nitrogen can be derived away from an inactive part of primary metabolism. The secondary compounds are then degraded and the stored carbon and nitrogen recycled back into the primary metabolism, when there is a demand. Those factors, which determine the location and accumulation of secondary products in the intact plant, are important, since these same controls affect the production of secondary products in plant cell cultures. Plant secondary compounds normally accumulate *in vitro* at the later stages of the growth cycle when growth slows and excess carbohydrate and nitrogen becomes redirected into secondary pathways. Accumulation can be enhanced or reduced by changing the composition of the

nutrient medium, more specifically, the concentration and nature of the inorganic nitrogen and phosphate, carbon supply, growth regulators and the stage of growth of the culture at which these changes are made.

Many of these secondary products like diterpenoid alkaloids, anthocyanin, fragrant oils and pharmaceutical products are commercially extracted. But some compounds inhibit the growth of tissue in plant culture media like phenols. The browning and subsequent death of cultured explants is a major problem that is usually dependent on the phenolic compounds and the quantity of total phenols (Ozyigit, 2008). Phenolic compounds occur as secondary metabolites in all plant species (Antolovich *et al.*, 2000; Kefeli *et al.*, 2003). The phenols are synthesized by the plants and in many cases excreted and then oxidized (Ozyigit, 2008). In tissue culture studies, phenolic substances, especially oxidized phenols generally affect *in vitro* development negatively (Arnaldos *et al.*, 2001). Oxidized phenolic compounds may inhibit enzyme activity and result in the darkening of the culture medium and subsequent lethal browning of explants (Compton and Preece, 1986; Laukkanen *et al.*, 1999).The phenolic exudate problem was frequently encountered in genera *Strelitzia, Musa* and *Ensete* spp. (Zeweldu and Ludders, 1998; Birmeta and Welander, 2004; Diro and van Staden, 2004; Titov *et al.*, 2006; Martin *et al.*, 2007; Ko *et al.*, 2009).

Activated charcoal is commonly used in tissue culture media to improve cell growth and development (Pan and Van Staden, 1998; Thomas, 2008). The beneficial effects of AC may be attributed to its irreversible adsorption of inhibitory compounds in the culture medium and substantially reduce the toxic metabolites, phenolic exudation and exudate accumulation (Fridborg *et al.*, 1978; Thomas, 2008). This high adsorptive capacity is due to the structure of AC. It has a very fine network of pores with a large inner surface area on which many substances can be adsorbed (Pan and van Staden, 1998; Dąbrowski *et al.*, 2005; Thomas, 2008).

The antioxidant, ascorbic acid, has been used successfully in the past to inhibit the exudation of phenols (Strosse *et al.,* 2004) and to reduce oxidative browning in various plant species (Arditti and Ernst, 1993; George, 1996; Abdelwahd *et al.,* 2008). AA is able to scavenge oxygen radicals produced when the plant tissue is wounded, therefore protecting the cells from oxidative injury. The oxidative browning of explant tissue is reduced by AA detoxifying these free radicals (Titov *et al.,* 2006). Thus, AA is useful and effective in managing the problem of phenolics and improving plant growth *in vitro* (Abdelwahd *et al.*, 2008).

Phenolic concentration is often affected by several internal and external factors (Zapprometov, 1989). Some nutrients (Lux-Endrich *et al.*, 2000) and some stress factors like drought, water, radiation and pathogen infection from injured surfaces effect concentrations of the phenolics in plants (Zapprometov, 1989; Kefeli *et al.*, 2003). The various PGR concentrations may affect phenolic exudation as phenols are reactive compounds (Lux-Endrich *et al.,* 2000).

Studies by North *et al.* (2012) on phenolic exudates in micropropagation of *Strelitzia reginae* showed that activated charcoal was 53 percent more effective than ascorbic acid in controlling the phenolic compounds.

2.5.2.6 Hardening

Acclimatization is crucial to any micropropagation procedures since shoot and plantlets produced *in vitro* must be readapted to the environmental conditions outside the culture vessels. Plants produced by tissue culture techniques are generally more expensive than conventionally produced seedlings. The transfer of the plantlet from the culture vessels to the main field is time consuming, labour intensive and may vary with species or even with varieties. During the period of adaptation changes in both structures and physiology of shoots occur.

Leaves of *in vitro* cultured plantlets are characterized by the absence or reduced amount of epicuticular wax in comparison to the leaves of the green house or field grown plants (Grout, 1975; Sutter and Langhans, 1982). This affects the rate of water loss from the leaves. During acclimatization, as the humidity is gradually lowered, the density of wax on leaves increases (Wardle *et al.*, 1983).

Jenks *et al.* (2000) reports 100% acclimatization of *Nymphoides indica* in clay pots filled with peat moss and decomposed cow manure mixture in 3:1 ratio and immersed in culture tanks 10 cm below the water surface under full sun conditions. Rooted plantlets of *Cunila galioides* were acclimatized by transferring to plastic chambers containing a sterilized mixture of sand and soil (1:1) and covered with a plastic cap that was gradually opened within 2 weeks and transferred to greenhouse and then to outdoor conditions (Fracaro and Echeverrigaray, 2001).

Sharma *et al.*, (2010) carried out the hardening of *Bacopa monnieri* plantlets in polybags containing a mixture of sand, farmyard manure and soil in a ratio of 1:1:1 in a mist chamber irrigating with half strength MS medium for three weeks followed by transfer to an agro net open shade house for one month with tap water irrigation prior to transfer to field.

2.6 Recirculating Aquaculture Systems (RAS)

Recirculating aquaculture systems have been widely applied to the culture of aquatic animals in various regions of the world, since the systems reuse water with mechanical and biological treatment between each use. The benefits of RAS are the optimal use of space, lower water requirements compared to conventional aquaculture, capacity for high stocking densities and provision of a predictable and stable environment for the culture species (Itoi *et al.*, 2006). RAS offer more independence from the external environment (i.e.

increased levels of control), which can provide a basis for improved risk management (Rawlinson, 2002). In addition RAS provides growers with the geographical freedom to set up aquaculture systems in "non-traditional" farming areas (Shnel *et al.,* 2002).

However, Otte and Rosenthal (1979) states that the major disadvantage with RAS is that a high proportion of the total water volume is used for the biological filter unit, leaving only a minor volume for the intended fish cultivation making it an uneconomical enterprise.

The commercialization of RAS technology has only begun to show signs of maturity in recent times and the industry is widely accepted as being in its infancy in comparison to other aquaculture production techniques (Love and Langenkamp, 2003).

In recirculating fish growing systems the refreshment (make-up) water is used to reduce (purge) off-flavours (Timmons *et al.,* 2002), to add alkalinity, and to control temperature, atleast to some degree (Seginer *et al.,* 2008).

2.6.1 Microbial communities associated with RAS

A diversity of microbial populations are involved in nitrification, ammonification, nitrate reduction, denitrification, proteolysis and sulfate reduction on the filter material of the recirculating aquarium (Kawai *et al.,* 1964; Sugahara *et al.,* 1974). Thus, while it is known that the water quality of RAS is maintained by a diversity of microbial communities in the filter material, the difficulties associated with their cultivation have meant that the majority of these microbes have not yet been investigated in detail (Amann *et al.,* 1995). In a study of the microbial communities associated with well–conditioned filter material in recirculating aquaculture systems for carp *Cyprinus carpio* and gold fish *Carassius auratus*, it was found that a nitrite–oxidizing bacteria, *Nitrospira*,

comprised 8.0 – 9.8% of the bacterial communities in the filter material (Sugita *et al.*, 2005).

Schramm *et al.* (2000) studied the distribution of nitrifying bacteria *Nitrosomonas, Nitrosospira, Nitrobacter* and *Nitrospira* in a membrane-bound biofilm system with supply of oxygen and ammonium from opposite directions, in which oxic part of the biofilm, which was subjected to high ammonium and nitrite concentration was dominated by *Nitrosomonas europaea* like ammonia oxidizers and by members of the genus *Nitrobacter*, whereas *Nitrosospira* and *Nitrospira* were abundant at the oxic–anoxic interface of the biofilm. In the totally anoxic part of the biofilm, cell numbers of all nitrifiers were found relatively low.

In a study on changes in microbial community on the surface of filter material in RAS of the pufferfish by Itoi *et al.* (2006) found that large part of microflora was dominated by γproteobacteria, Flavobacteria and Sphingobacteria. Earlier Siegrist *et al.* (1998); Koch *et al.* (2000); Egli *et al.* (2003) have suggested that the lower layer of biofilm was composed by the filamentous bacteria including Cytophaga and Flavobacterium.

Studies by Nemergut and Schmidt (2002); Zehr and Ward (2002); Hipkin *et al.*(2004) and Joo *et al.* (2005) reveals that in some systems nitrite levels were stable even in the absence of nitrite–oxidizing bacteria. This is because some heterotrophic bacteria have the ability to oxidize nitrite, or maybe there is another pathway from ammonia to nitrate that does not require nitrite formation and oxidization. Several scientists report an anammox microbial process, where nitrite and ammonia combine, via microbial processes to form N_2 gas and water with no nitrate produced, and the organisms responsible for this novel metabolism have been identified as relatives of Planctomyces (Van Loosdrecht and Jetten, 1998; Strous *et al.*, 1999; Jetten *et al.*, 2001).

2.6.2 Biological nitrogen removal processes in aquaculture systems

The mineralization of organic matter associated with the decomposition of unconsumed food, dead aquaculture organisms and their faeces is one of the most important parameters in RAS. The nitrogen in the protein of these substances is decomposed to ammonia by proteases and deaminases produced by heterotrophic bacteria in the material of the filter. In addition, the fish also excrete ammonia directly. This toxic ammonia is converted into nitrate via nitrite by nitrifying bacteria that oxidise ammonia and nitrite mainly in the filter material (Kawai *et al.,* 1964; Sugahara *et al.,* 1974; Midlen and Redding, 1998). One of the main factors affecting bacterial growth is the amount of ammonia in the water. Generally values above 3 mg ammonium nitrogen per litre are recommended for maximal growth (Odegaard *et al.*, 1994). Experiments have demonstrated a reduction in *Nitrosomonas* activity with oxygen levels below 4 mg l^{-1} water, while corresponding value for *Nitrobacter* is 2 mg l^{-1} (Haug and McCarty, 1972).

Bacterial growth rate depends on the temperature. Bacterial activity occurs from 0 to 30°C and increases with temperature: the optimal range is around 30°C (Wortman and Wheaton, 1991). However, the bacteria may acclimate to lower temperatures over time, so high bacterial activity can also be achieved at lower temperatures (Zhu and Chen, 2002). Nitrification also depends on pH of water: the optimal values are between 8 and 9 (Henze and Harremoes, 1990). Nitrification will be reduced by increasing the carbon/nitrogen ratio (Zhu and Chen, 2001; Carrera *et al.*, 2004). According to Ling and Chen (2005), a 60 – 70% reduction in nitrification rate was observed when increasing the chemical oxygen demand/ nitrogen (COD/N) ratio from 0 - 3.0 for a substrate containing 10 mg TAN.

Nitrification is faster in freshwater than in seawater because the chloride ions inhibit bacterial growth (Nijhof and Bovendeur, 1990). Alleman and Preston

(1991) states that nitrification filters need to be shielded from light because it may reduce nitrification. Light is believed to oxidize cytochrome C in both species of bacteria. *Nitrobacter* is more sensitive to light because it contains less cytochrome C than *Nitrosomonas* (Olson, 1981). Horrigan *et al.* (1981) found similar results for light inhibitors and concluded complete darkness was superior to diurnal cycling of light regimes for nitrifying bacteria.

The total nitrification in the recirculatory system considers the ammonia removed by: (1) bacteria attached to the inside of pipes, fittings, and other unit processes, (2) suspended bacteria in the water column, and (3) bacteria attached to the filter media. Filter System Ratio values vary from 50 to 90% in laboratory systems (Malone and Beecher, 2000; Hargrove et al., 1995).The FSR is equal to the amount of TAN removed within the filter per day divided by the total amount of TAN removed in the entire system by nitrification (Colt *et al.,* 2006). Information on FSR is more important in small systems, because the ratio of filter: non-biofilter TAN removal may not scale linearly as the size of the system is increased. A low value of FSR for a laboratory system will increase the uncertainty of the estimation of the performance of larger systems.

Maintaining acceptable water quality constitutes the main bottleneck in RAS (Van Rijn, 1996; Menasveta *et al.,* 2001). The water quality parameters of greatest relevance in RAS are ammonia, nitrite and nitrate (Colt, 2006). Although nitrate is considered the least toxic of the different inorganic nitrogen forms, nitrate accumulation causes pH to decrease continuously (Sharma and Albert, 1977). In a fish culture system, dissolved oxygen can become critical in a matter of minutes, ammonia, carbon dioxide and pH levels over a few hours and alkalinity within a couple of days (Loyless and Malone, 1998). Various fish indigenous to soft water habitats and some commonly farmed invertebrate species are susceptible to elevated nitrate levels (Tal *et al.,* 2003).

2.6.3 Water quality parameters for biofilter operations

Important factors in the selection of water quality criteria for aquatic culture systems may depend strongly on the objectives of the project, the species, and life stage reared (Colt, 2006). On other hand, development of species or system specific water quality criteria is time consuming, expensive, and may be difficult to find in a peer-reviewed journal. Much of the published information is from relatively short-term experiments and does not cover the full production cycle. While there is documentation on the impacts of fixed and variable ammonia concentrations on fingerling channel catfish (1–10g) (Colt and Tchobanoglous, 1978; Hargreaves and Kucuk, 2001), there is little documentation for larger fish (>20–400 g). No freshwater or seawater water quality criteria are developed for either nitrate or nitrite (USEPA, 2002), two very important parameters in reuse systems. Important water quality parameters in water reuse systems are ammonia, nitrite, nitrate, faecal solids, oxygen, chlorine, salinity, pH, surface active compounds and colour compounds. Not all of the water quality parameters have major cost implications in reuse systems. The maintenance of ammonia, nitrite, faecal solids, carbon dioxide, dissolved oxygen and surface-active compounds may have the greatest costs implication in design and operation of reuse systems. For many parameters such as ammonia, nitrite, or dissolved oxygen, it is necessary to know not only the ''no effects'' levels but also the functional relationship between these parameters and parameters such as growth rate or product quality. The design level will depend both on the effects of these parameters on the culture animals and the costs of maintaining a given level. For other parameters such as heavy metals, chlorine residual, or biocides criteria based on a no-effects concentration may be appropriate. It may be desirable to keep influent ammonia and nitrite concentration in the undetectable range (<0.01 mg l^{-1}) as a general measure of water quality in broodstock or larval systems. An increase of ammonia to 0.15 mg l^{-1} indicates a problem with the biofilter performance that may adversely influence a number of other water quality parameters that are more difficult (or

impossible) to accurately monitor. The changes in water color or surface tension may be important indicator variables in reuse systems.

Compared to flow-through or ponds, reuse systems typically have significantly reduced make-up flows that can result in the build-up of some compounds. Also, the ability to independently control parameters such as DO or chloride ion concentration, may allow culture animals to tolerate higher levels of other water quality parameters (compared with other culture types).

2.6.3.1. Ammonia

Ammonia is produced in aquaculture systems by direct excretion from the fish and by breakdown of organic compounds by heterotrophic bacteria as the first inorganic compound produced via the nitrogen cycle (Wheaton *et al.*, 1991). Ammonia is toxic to fish in the range of 1 ppm (part per million).

Meade (1985) concluded that "a truly safe, maximum acceptable concentration of unionized, or total, ammonia for fish culture systems is not known." The apparent toxicity of ammonia is extremely variable and depends on more than the mean or maximum concentration of NH_3.

Detailed information on the toxicity of ammonia to fish has been reviewed by Tomasso (1994). The toxicity of ammonia is generally assumed to be due to the concentration of the un-ionized ammonia molecule (NH_3) because of its ability to move across cell membranes (Colt, 2006).While TAN, pH, and temperature determine the concentration of un-ionized ammonia, pH appears to have a significant impact on ammonia. At a pH of 9.0 and TAN = 5 mg l^{-1}, typical fish would be dead in hours, while with pH less than 6.0, ammonia would have negligible impacts.

Ammonia appears to have a direct effect on the growth of aquatic animals. Increasing the un-ionized ammonia concentration produces a linear reduction in growth of channel catfish (*Ictalurus punctatus*) (Colt and Tchobanoglous, 1978). Ammonia can have a serious effect on the incidence of disease, especially under less optimum conditions of temperature and dissolved oxygen. Low DO and variable NH_3 concentrations can increase the toxicity of ammonia while increasing salinity and sodium levels will reduce its toxicity.

Smith and Piper (1975) reported that six months of continuous exposure to 0.021 mg l^{-1} of non-ionized ammonia could promote pathological damages on gill tissues of rainbow trout. Smart *et al.* (1978) indicated that the exposition to sublethal levels produced a 3–fold increase in oxyen consumption. Burkhalter and Kaya (1977) found that 0.05 mg l^{-1} of non-ionized ammonia had a significant effect on the growth rate.

The commonly used un-ionized ammonia criterion in salmonid culture of 12.5 mg l^{-1} (Westers, 1981) is based on gill damage attributed to ammonia exposure (Smith and Piper, 1975). Recent work discounts the impact of ammonia on gill damage and suggests the un-ionized ammonia criteria could be at least 40 mg l^{-1} or higher (Meade, 1985) for state and federal hatcheries. For commercial production, the water quality criterion for un-ionized ammonia may be even higher than 40 mg l^{-1}.

2.6.3.2 Nitrite

Nitrite is the ionized form of the relatively strong acid, nitrous acid. At normal pH's, very little nitrous acid is present. Nitrous acid is freely diffusible across gill membranes while nitrite is not (Tomasso, 1994) but nitrite can be actively transported across gill membranes by the mechanism that normally transports chloride inward. The addition of chloride ions protect fish from the toxicity of nitrite by competitively excluding nitrite from uptake by the chloride

active transport mechanism in the gills. In pond culture of channel catfish, it is common to add NaCl to maintain a chloride: nitrite ratio of 4:1 on a molar basis or 10.1:1 on a weight basis (Schwedler and Tucker, 1983). High concentration of chloride in seawater may not be protective for all invertebrates (Tomasso, 1994). While the concentration of un-ionized nitrous acid increases at lower pH, Lewis and Morris (1986) concluded that there is no evidence of increased nitrite toxicity at lower pH. Because of the effects of buffer addition and acclimation issues in the experiments reviewed by Lewis and Morris (1986), the effects of pH on nitrous acid toxicity may have been confounded (Tomasso, 1994). If the pH of a reuse system is lowered, the toxicity of total nitrite nitrogen may be a more important problem even if chloride is added (Colt, 2006).

2.6. 3. 3 Nitrate

Reported maximum values of nitrate in recirculating systems are as high as 400– 500 mg NO_3-N/l (Otte and Rosenthal, 1979; Honda *et al.*, 1993). Nitrate reaches high concentrations in recirculating systems where nitrifying biofilters are used for ammonia removal. Maximum nitrate levels differ among recirculating systems and are dictated mainly by water exchange rates and the extent of nitrification and nitrate removal. Contrary to ammonia and nitrite, nitrate is relatively non-toxic to aquatic organisms. However, high nitrate concentrations can affect the growth of commercially cultured aquatic organisms such as: eel (Kamstra and van der Heul, 1998), octopus (Hyrayama, 1966), trout (Berka *et al.,* 1981) and shrimp (Muir *et al.,* 1991).

The toxicity of nitrate to freshwater fish is very low (96 h LC50s >1000 mg/L as N) and may be related to potential osmoregulation problems. The nitrate-N concentrations should be less than 500 mg/L for large marine fish (Pierce *et al.*, 1993), but marine tropical fish such as anemonefish (*Amphiprion ocellaris*) are more sensitive and a criterion of 20 mg/L was suggested for this

species (Frakes and Hoff, 1982). Walsh *et al.* (2002) recommended a criterion of 50 mg/L as N for the rearing of squid.

Increased efforts are now directed toward nitrate control in recirculating systems. Apart from the direct toxic effect on fish, nitrate removal is conducted for other reasons in recirculating systems: (1) environmental regulations associated with effluent discharge have permissible nitrate levels as low as 11.3 mg NO_3-N/l (European Council Directive, 1998); (2) prevention of high nitrite levels resulting from incomplete ''passive'' nitrate reduction; (3) stabilization of the buffering capacity; and (4) the concomitant elimination of organic carbon, orthophosphate and sulfide from the culture water during biological nitrate removal (Van Rijn *et al.*, 2006).

In some marine and freshwater organisms, lethal and sub–lethal effects of high concentrations of nitrates have been documented. An amount of 50 mg N/l is a generally accepted safe limit for nitrate nitrogen in fish culture, but this concentration varies widely for different species and development stages (Gutierrez–Wing and Malone, 2006). The reported LC50 for freshwater organism ranges from 5 to 2107 mg N/l of nitrate, with amphibians and invertebrates as the most sensitive groups. In marine species the ranges are 2.2 – 5050 mg N/l of nitrate with larvae and broodstock as the most sensitive stages (Environment Canada, 2003).

A nitrite concentration of 100mg N/l was clearly lethal to medaka fish (*Oryzias latipes*) when they were exposed to nitrite in both adult and growing phases (Colt and Armstrong, 1981) . Similarly, a nitrate concentration of 75 mg N/l reduced the fertilization rate, delayed hatching time, reduced the hatching rate of the eggs and decreased the growth rate of juveniles. Burgess (1995) reports that marine white spot disease is linked to nitrate concentrations above 30 mg N/l.

The problem of nitrate accumulation in RAS can be solved by integrating an anaerobic denitrification filter (Otte and Rosenthal, 1979). Anoxic conditions and the presence of high nitrate concentrations results in the development of a denitrifying heterobacteria population. They use the organic degradation products as sources of carbon and energy, and nitrite as an electron acceptor, dissimilate nitrate under anoxic conditions, via nitrite, nitric oxide and nitrous oxide to gaseous elemental nitrogen which is subsequently released into the atmosphere (Diab and Shilo, 1986; Hopkins *et al.,* 1994).

2.6.4 Types of biofilters

Many water re-use systems have already been described using biological filters for the degradation of accumulating organic compounds which originate from fish excretion and excessive feed (Scott and Gillespie, 1972; Siddall, 1974).

A biofilter is a fixed film reactor in which only the active biomass on filter medium is responsible for bio-oxidation of substrates, regardless of the total biomass present in biofilm (Lazarova *et al.,* 1994). Over time, biofilm grows thicker as a result of microbial populations built up on the medium and reaches a critical thickness of 15 – 25 μm (Liu and Capedville, 1996) and thereafter it sloughs off with overloading (Stewart, 1993). In a biofilter, growth and nitrification rate of biofilm are affected by different variables, e.g. ammonia concentration (Groeneweg *et al.,* 1994), organic loading (Hanaki *et al.,* 1990; Okabe *et al.,* 1996), dissolved oxygen (Cecen and Gonenc, 1992; Hao and Huang, 1996; Huang and Hao, 1996), pH value (Groeneweg *et al.,* 1994; Tseng *et al.,* 1996), temperature (Groeneweg *et al.,* 1994), and salinity (Tseng *et al.,* 1994).

RAS biofilter technologies can be divided in to two main categories: fixed film (attached growth) in which a media is provided for the microorganisms to attach and grow, and suspended growth in which the

microorganisms are maintained in suspension (Gutierrrez–Wing and Malone, 2006). Most of the biofiltration on recirculating systems has been focussed on aerobic, fixed film filters (Wortman and Wheaton, 1991; DeLosReyes and Lawson, 1996; Westerman *et al.,* 1996; Greiner and Timmons, 1998; Singh *et al.,* 1999; Malone and Beecher, 2000; Lekang and Kleppe, 2000; Sandu *et al.,* 2002) in which a substrate is provided for the growth of a biofilm that utilizes oxygen to convert ammonia and nitrites to nitrates and oxidise organic matter.

Nitrification is carried out in a variety of systems, which can be grouped into six general types: submerged filters, trickling filters, reciprocating filters, rotating biological contractors (RBC), rotating drums, and fluidized bed reactors (Wheaton *et al.,* 1991). RBCs are found to give the best performance with respect to specific ammonia removal efficiency (ammonia removal per surface area per time) and often gets operational failures (Van Rijn, 1996).

The effect of different surface materials (filter media) on nitrification has been examined mainly for trickling filters (Kruner and Rosenthal, 1983; Kikuchi *et al.,* 1994). According to them an ideal filter material should (a) have high surface area per volume (b) be low in cost (c) be durable (d) not clog easily and (e) promote a uniform spread of water to be treated. Plastic is the most widely used biofilter material (Kikuchi *et al.,* 1994).

Studies on trickle filter reveals that it requires a conditioning period of 3 –4 weeks to initiate the nitrification process (Anderson, 1974; Collins *et al.,* 1975; Carmignani and Benett, 1977). A steady-state in a biofilter is achieved when the TAN in the effluent stabilizes and a horizontal straight line can be drawn by eye through observed points on a TAN versus time graph (Colt *et al.,* 2006). Biofilters were considered fully conditioned when a steady-state culture was established, capable of keeping TAN and NO_2 - N levels below 0.7 mg/L in each system, under conditions of 8.64 g/d mass ammonia loading for 7 consecutive days (Sandu *et al.,* 2002). Compared to heterotrophic bacteria, the

autotrophic bacteria responsible for ammonia oxidation grows very slowly. It may take up to 60 d for a new filter to approach steady-state conditions, especially with some plastic media in seawater (Colt *et al.*, 2006). In general, the response of biofilters during the acclimation phase is not very important, except that this phase should be as short as possible.

Many studies have provided details of system design, operation and performance evaluations on fluidized bed reactors, floating bead filters, trickling filters and moving bead filters for their application in aquaculture systems (Kamstra *et al.*, 1998; Malone and Beecher, 2000; Yossi *et al.*, 2003; Summerfelt, 2006). However, information on process mechanism and kinetics relative to nitrification biofilters applied to aquaculture systems is still insufficient. In general, nitrification kinetics of fixed film reactors used in RAS was found to be affected mainly by water quality parameters (Chen *et al.*, 2006). The TAN concentrations, especially the minimum concentration that a biofilter can maintain and the relationship between nitrification rate and TAN concentrations are very important in the performance of a nitrifying biofilter. The substrate limitation rather than substrate inhibition is often the major concern for biofilter designs in RAS due to the low ammonia concentration in these systems (Wheaton *et al.*, 1994). Within the TAN concentration range that is common to RAS, the nitrification rate is proportional to the substrate concentration (Chen *et al.*, 2006). The flow rate into the bioreactor is another important criterion affecting the turbulence and thus has great impact on the mass transfer flux into biofilm as well as the nitrification rate. Stoodley *et al.* (1997) investigated the relationship between local mass transfer coefficients and fluid velocity in heterogeneous biofilms and found that the effects of biofilm heterogeneity on mass transport were strongly dependent upon the average flow velocity. Ling and Chen (2005) also reported higher nitrification rates in biofilters with high turbulence levels, suggesting that the nitrification rate may be significantly improved through increasing the turbulence.

Tseng and Wu (2004) found that ammonia removal rate of a submerged filter increases over time, reaches a maximum value, remains constant for a period of time and then decreases sharply. The duration of ammonia removal cycle of biofilter is affected by influent water temperature, and the concentrations of ammonia and suspended solids.

Itoi *et al.* (2006) used pebbles as the filter material to study the changes in microbial communities in recirculatory system for marine puffer fish, *Takifugu rubripes*.

Studies by Yao *et al.* (2007) on the effect of tourmailine on growth of nitrifying bacteria reveals that ceramsite medium supplemented with tourmaline performed better than ceramsite medium in terms of nitrification.

2.6.5 Aquarium plants as biofilters

Walstad (1999) has given a detailed account of role of plants in biofiltration in her book on 'Ecology of Planted Aquariums'. Her works reveal that plants can rapidly detoxify ammonia by combining with a hydrogen ion to form the non-toxic ammonium ion (NH_4^+) or they will be used to synthesise proteins or combined with stored carbohydrates to form ordinary amino acids. Thus plants that grow well can tolerate more ammonia, because they have more carbohydrates to combine with ammonia. When given a choice between ammonia and nitrite, plants readily take up ammonia because nitrite uptake and assimilation requires specific transporters and enzymes whereas ammonia uptake does not (Zsoldos *et al.*, 1993). Aquatic plants can absorb ammonia not only through their roots but also through the leaves as reported by Thursby and Harlin (1982) in the marine plant *Zostera marina*.

In aquatic environment, however ammonium predominates and most aquatic plant species have developed an ammonium – based nutrition (Lewis,

1986). Aquatic plants take up ammonia more quickly than nitrates. For example, the 'turnover time' for ammonium (at 0.4 ppm N) in *Pistia stratiotes* was found to be just 4 hours, while nitrate turnover required a full 20 hours (Nelson *et al.*, 1980). Exceptions are *Littorella uniflora, Lobelia dortmanna, Luronium natans* and *Echinodorus ranunculoides* which prefer root uptake of nitrate over the more common leaf uptake of ammonium (Schuurkes *et al.*, 1986). These plants are found to encourage nitrification by releasing particularly large amounts of oxygen into the root area (Roelofs *et al.*, 1984).

2.6.6 RAS in freshwater fish production

Broussard and Simco (1976); Rakocy and Allison (1981) and Buckling *et al.* (1993) have demonstrated the feasibility of raising freshwater species to market size in recirculating systems. Buckling *et al.* (1993) working in an ornamental fish production facility calculated that the average RAS savings in energy for heating and pumping was US$ 0.96 per pound of fish produced. Similarly the catfish and eel production facilities found in Netherlands also exist, in part because of the heating advantages presented by RAS systems (Bovendeur *et al.*, 1987; Kamstra *et al.*, 1998).

Water use issues are a major driving force for adoption of RAS technologies as demonstrated in the case of hybrid striped bass farming in deserts of California in the Western United States (Carlberg *et al.*, 2003) and in Israel (Barak and Van Rijn, 2000). Asano *et al.* (2003) describes a severe water threat to Hawaii's ornamental fish industry which is prompting an examination of RAS technologies. Likewise research works are going on to materialize RAS for coldwater fish production in the North East regions of the United States (Heinen *et al.*, 1996). Schuster and Stelz (1998) have designed a RAS for cold water trout farming with priority to water conservation.

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled "Integrated culture of fish with micropropagated plants in a recirculatory system" was carried out at the Department of Aquaculture, College of Fisheries, Panangad and the Centre for Plant Biotechnology and Molecular Biology, Kerala Agricultural University during the period 2007-2011. The study was undertaken through the conduct of the following two experiments.

Experiment 1:*In vitro* standardisation of five species of aquarium plants *viz. Bacopa caroliniana, Anubias minima, Aponogeton ulvaceus, Rotala rotundifolia* and *Nymphoides cristata.*

Experiment 2: Study of filter performance of recirculatory systems with three indigenous materials *viz.* coir, vetiver and bamboo strips as filter beds based on analysis of water quality parameters.

3.1. Micropropagation

3.1.1. Materials

3.1.1.1. Source of explants

Healthy stock of mother plants of the five varieties of aquarium plants were collected from the aquarium plant dealers in the Cochin city and maintained in the net house of Department of Aquaculture in cement and FRP tanks. The plant rearing tanks were prepared by washing pond soil thoroughly to remove all debris and filled the tank to a depth of 20 cm and surface layered with a mat of coir fibre as the bedding substratum. The tanks were filled to the full volume with tap water and plants were arranged at equidistant intervals inside the tank. The plants were regularly trimmed to prevent the crowding inside the tank and

water loss were compensated once in a week. The plates 1 to 5 show the five different aquarium plants used in the present study.

3.1.1.2. Culture medium.

3.1.1.2.1. Chemicals

The major and minor nutrients required for the preparation of media were of analytical grade and procured from M/s. Merck, Fischer Scientific, Qualigens and Nice Chemicals. The amino acids, vitamins, plant growth regulators and other media additives were obtained from M/s Merck, Sisco Research Laboratories, HiMedia and Loba Chemie.

3.1.1.2.2. Glasswares

Borosilicate glassware of Borosil/ Riviera brand were used for the study. The rhizomatous plants were grown in Jam bottles (350ml capacity) . Other glasswares include graduated measuring cylinder, petridishes, beakers and a range of pipettes. Before use, glasswares were thoroughly brushed with alkaline detergent teepol and then washed in running tap water. It was then treated with hot chromic acid (mixture of $K_2Cr_2O_7$ + H_2SO_4 + H_2O) followed by very thorough washing with tap water. Double distilled water (5 to 10ml) was poured into every culture vessel and tightly plugged. All the glasswares were autoclaved at a pressure of 15 lb\in^2 (121^{0}C) for 15 to 20 minutes (Dodds and Roberts, 1985).

3.1.2. Composition of media

During present investigation only MS medium (Murashige and Skoog 1962) was used. Composition of the media is given in Table.1.

Plate 1.

Plate 2.

Plate 3.

Plate 4.


Nymphoides cristata (Roxb.)O. Ktze
Kingdom : Plantae
Phylum : Magnoliophyta
Class : Magnoliopsida
Subclass : Asteridae
Order : Solanales
Family : Menyanthaceae
Genus : Nymphoides
Species : *Nymphoides cristata*

Plate 5.

3.1.3. Preparation of stock solutions

Standard procedures (Gamborg and Shyluk, 1981) were followed for the preparation of the medium. Major and minor nutrients were prepared as stock solutions of 50x and 100x concentrations first by dissolving the required quantity of chemicals in double distilled water and stored under refrigerated conditions in amber coloured bottles. Stock solutions for the major and minor nutrients were prepared afresh every three months. The vitamin stock solutions were prepared fresh every six to eight weeks and those of growth regulators were prepared fresh every four weeks.

3.1.4. Preparation of the culture medium

Specific quantities of the stock solution were pipetted out into a beaker. The required quantity of plant hormones was mixed. Sucrose and inositol were added fresh and well dissolved and volume made up to the required level using double distilled water. The pH of the solution was adjusted at 5.8 using 0.1 N NaOH or 0.1 N HCl. For obtaining a semisolid medium, agar was added at 0.75 percent level and the medium was boiled till a clear solution was obtained. About 15–20 ml of this molten medium was dispensed into the culture tubes or Jam bottles.

3.1.5. Sterilization of the culture medium

The tubes were plugged with adsorbent cotton and autoclaved at 121°C and 15 psi (1.06 kg/cm^2) for 20 minutes (Dodds and Roberts, 1985). The Jam bottles with the media were also double sealed with cellophane tape and autoclaved in the same way as described above. The medium was allowed to cool to room temperature and stored in a sterile room or inoculation chamber.

Table. 1. Composition of the MS medium used for the culture of aquarium plants (Murashige and Skoog, 1962)

Ingredients	Quantity (mg/l)
Inorganic constituents	
NH_4NO_3	1650
KNO_3	1900
KH_2PO_4	170
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
$FeSO_4.7H_2O$	27.8
Na_2EDTA	37.3
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.\ 5H_2O$	0.025
$CoCl_2.\ 6H_2O$	0.025
Organic constituents	
Myo-inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2
Sucrose	30,000

3.1.6. Preparation of explants

Nodal segments, rhizome buds, leaf petioles and lateral buds were taken as explants for the study. Nodal segments of approximately 6.0 to 7.0 cm length were carefully excised from the plants and defoliated and used as explants. For the leaf petiole explants, the mature and young leaves were collected and edges were trimmed off. Shoot pieces with lateral buds of approximately 3.0 to 4.0 cm length were excised as explants. For horizontal creeping rhizomes, 1.0 to 2.0 cm bits were separated carefully with rhizome buds and used as explants. For non-creeping type rhizome, one whole rhizome itself with the apical bud was taken after defoliation and removal of hairy roots. Pictures of explants used for the study are presented in plates 6 to 10.

The explants were immersed in 1.0 percent Teepol solution for five minutes with vigorous shaking and were thoroughly washed in running tap water to remove all traces of the foam. Fungicidal and insecticidal treatments were not given to the explants.

3.1.7. Transfer area and aseptic manipulations

All the aseptic manipulations such as surface sterilization of the explants, preparation and inoculation of the explants and subsequent subculturing were carried out in a clean laminar airflow chamber. The working table of the laminar airflow chamber was initially surface sterilized with absolute alcohol and then by switching on ultraviolet light for 30 minutes. The petridishes, forceps, knives and other inoculation aids were initially autoclaved and then flame sterilized before each inoculation. The hands were washed thoroughly with soap under running tap water. After drying, they were wiped with absolute alcohol before inoculation.

Plate 6: Rhizome buds of *Anubias minima*

Plate 7: Rhizome with apical bud in *Aponogeton ulvaceus*

Plate 8: Nodal explants of *Bacopa caroliniana*

Plate 9. Nodal explants of *Rotala rotundifolia*

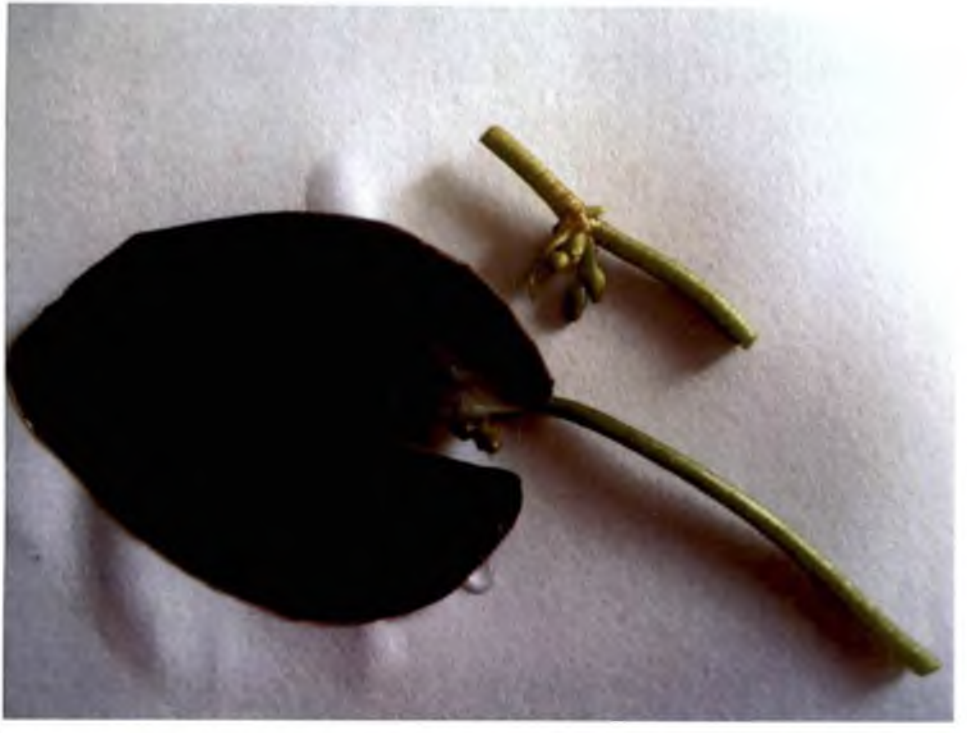

Plate 10: Leaf petiole and lateral buds of *Nymphoides cristata*

3.1.8. Culture establishment

3.1.8.1. Surface sterilization

The washed explants were put into the sterilant (depending on the method adopted either as a single step or multistep procedure) and kept immersed for the required period. They were continuously agitated manually to ensure thorough contact of the explants with the chemical. The different sterilization treatments tried for the explants are listed in Tables 2, 3, 4 and 5. The explants after surface sterilization were rinsed four times thoroughly with the sterilized distilled water to remove traces of the sterilant and drained over blotting paper and inoculated to the media. The incubated cultures were observed for contamination and percentage of survival.

3.1.8.2. Inoculation

The sterilized explants were inoculated under perfect aseptic conditions into the different basal media supplemented with varying quantities of growth regulators and cultured. For liquid culture using nodal explants, leaf petioles and lateral buds culture tubes (25x150mm) were employed. For establishment of rhizome cultures, semisolid media in wide mouthed jam bottles were used. Sub culturing was also carried out at regular intervals of 6 weeks under the same aseptic conditions in a laminar airflow.

3.1.8.3. Culture conditions

The cultures were incubated at 25 ± 2°C in an air-conditioned culture room with 12 hours photoperiod (1000 lux) supplied by cool white fluorescent light. Relative humidity in the culture room varied between 60 to 80 percent according to the climate prevailing.

Table 2. Different surface sterilization treatments carried out for the rhizome explants of *Anubias minima* before inoculation

Sterilant	Concentration (%)	Duration (minutes)
$HgCl_2$	0.1	3
	0.1	4
	0.1	5
	0.1	6
	0.1	7
$HgCl_2$	0.5	1
	0.5	2
	0.5	3
	0.5	4
Ethyl alcohol + $HgCl_2$ + Ethyl alcohol	70,0.5,30	Wipe, 1,2
	70,0.5,30	Wipe, 2,2
	70,0.5,30	Wipe, 3,2
	70,0.5,30	Wipe,4, 2

Table 3. Different surface sterilization treatments carried out for the rhizome bud explants of *Aponogeton ulvaceus*

Sterilant	Concentration (%)	Duration (minutes)
$HgCl_2$	0.1	3
	0.1	4
	0.1	5
	0.1	6
	0.1	7
$HgCl_2$	0.5	1
	0.5	2
	0.5	3
	0.5	4
Ethyl alcohol + $HgCl_2$ + Ethyl alcohol	70,0.1,30	Wipe, 3, 2
	70,0.1,30	Wipe, 4, 2
	70,0.1,30	Wipe, 5, 2
	70,0.1,30	Wipe, 6, 2
	70,0.1,30	Wipe, 7, 2
Ethyl alcohol + $HgCl_2$ + Ethyl alcohol	70,0.5,30	Wipe, 1,2
	70,0.5,30	Wipe, 2,2
	70,0.5,30	Wipe, 3,2
	70,0.5,30	Wipe,4, 2

Table 4. Different surface sterilization treatments carried out for the nodal explants of *Bacopa caroliniana* and leaf petiole and lateral bud explants of *Nymphoides cristata*

Sterilant	Concentration (%)	Duration (minutes)
$HgCl_2$	0.5	3
	0.5	2
	0.5	1
	0.1	3
	0.1	2
	0.1	1
	0.1	30 seconds

Table 5. Different surface sterilization treatments for nodal explants of *Rotala rotundifolia*

Sterilant	Concentration (%)	Duration (minutes)
Sodium hypochlorite solution	5.54%	40
	5.54%	35
	5.54%	30
	5.54%	25
	5.54%	20
	5.54%	15
	5.54%	10

3.1.9. Standardisation of medium

3.1.9.1. Effect of growth regulators on shoot multiplication

Studies were conducted to determine the effect of various growth regulators on multiple shoot bud induction from nodal segments. Different levels of hormone treatments tried for bud breakage and shoot induction for *Bacopa caroliniana* and *Rotala rotundifolia* from nodal segments are presented in Tables 6 and 7 respectively. Similarly the hormone concentrations tried for shoot organogenesis from leaf petiole explants and lateral buds are presented in Table 8. For rhizomatous plants the treatments to study the shoot induction from rhizomatous buds are presented in Tables 9 and 10. Surviving cultures were sub cultured at an interval of 6 weeks to the same medium in which they were inoculated, for a period of three months. Each treatment consist of 8 explants (only 6 explants in case of *Anubias minina* due to its slow growth and difficulty in availability). The response of the cultures in each subculture was recorded fortnightly. Survival rate in each subculture, days to initiate bud breakage, number of shoots and shoot length were recorded. The data were submitted to statistical analysis by ANOVA and the means compared by the Tuckey's test.

3.1.10. Hardening and acclimatization

Rooted plantlets were removed from the culture vessels after sufficient number of roots was formed. The plants that were taken out of the culture tubes were washed in running tap water to remove all the remnants of nutrients and agar. They were then transferred to FRP tanks with fine river sand overlaid with coir fibre in polyhouse.

3.2. Recirculating aquaculture system

3.2.1. Experimental set up

The experiments were conducted over a period of 70 days with four different filter materials *viz.* coir, split bamboo fibres and vetiver (locally called

Table 6. Standardisation of MS media with varying levels of cytokinins BA and Kinetin on *in vitro* propagation of *Bacopa caroliniana*

Treatment	Medium (mg l^{-1})
Control	½ MS + PGR* free media
T_1	½ MS + 0.1 BA + 0.1 Kinetin
T_2	½ MS + 0.1 BA + 0.2 Kinetin
T_3	½ MS + 0.5 BA + 0.1 Kinetin
T_4	½ MS + 0.5 BA + 0.2 Kinetin
T_5	½ MS + 1.0 BA + 0.1 Kinetin
T_6	½ MS + 1.0 BA + 0.2 Kinetin
T_7	½ MS + 1.5 BA + 0.1 Kinetin
T_8	½ MS + 1.5 BA + 0.2 Kinetin
T_9	½ MS + 2.0 BA + 0.1 Kinetin
T_{10}	½ MS + 2.0 BA + 0.2 Kinetin

*PGR – Plant Growth Regulator

Table 7. Standardisation of MS media with varying levels of auxin and cytokinin on *in vitro* propagation of *Rotala rotundifolia*

Treatment	Medium (mg l^{-1})
Control	½ MS + PGR free media
T_{11}	½ MS + 0.1 BA + 0.5 IAA
T_{12}	½ MS + 0.5 BA + 0.5 IAA
T_{13}	½ MS + 1.0 BA + 0.5 IAA
T_{14}	½ MS + 1.5 BA + 0.5 IAA
T_{15}	½ MS + 2.0 BA + 0.5 IAA
T_{16}	½ MS + 0.1 BA + 1.0 IAA
T_{17}	½ MS + 0.5 BA + 1.0 IAA
T_{18}	½ MS + 1.0 BA + 1.0 IAA
T_{19}	½ MS + 1.5 BA + 1.0 IAA
T_{20}	½ MS + 2.0 BA + 1.0 IAA

Table 8. Standardisation of MS media with different levels of auxin and auxin-cytokinin combination on *in vitro* propagation of *Nymphoides cristata*

Treatment	Medium (mg l^{-1})
Control	½ MS + PGR free media
T_{21}	½ MS + 0.1 BA
T_{22}	½ MS + 0.5 BA
T_{23}	½ MS + 1.0 BA
T_{24}	½ MS + 1.5 BA
T_{25}	½ MS + 2.0 BA
T_{26}	½ MS + 0.1 BA + 1 IAA
T_{27}	½ MS + 0.5 BA + 1 IAA
T_{28}	½ MS + 1.0 BA + 1 IAA
T_{29}	½ MS + 1.5 BA + 1 IAA
T_{30}	½ MS + 2.0 BA + 1 IAA

Table 9. Standardisation of MS media with auxin and cytokinin for *in vitro* propagation of *Anubias minima*

Treatment	Medium (mg l^{-1})
Control	MS + PGR free media
T_{31}	MS + 3BA + 0.1 Kn + 1 IAA
T_{32}	MS + 4 BA + 1 IAA
T_{33}	MS + 6 BA

Table 10. Standardisation of MS media with different levels of cytokinin for *in vitro* propagation of *Aponogeton ulvaceus*

Treatment	Medium (mg l^{-1})
Control	MS + PGR free media
T_{34}	MS + 15 BA
T_{35}	MS + 10 BA
T_{36}	MS + 8 BA
T_{37}	MS + 4 BA

'Ramacham') and a control without the filter media in four different recirculating systems, all with the same features. Each experiment was carried out in a rectangular glass aquarium (4x2x2 ft^3, water volume 340 L) tank with four chambers enabling gravity flow. The first chamber (CT_1)was designed for rearing fishes and the second chamber (CT_2) for holding the aquarium plants. The third chamber (CT_3) was with the biofilter material and the fourth was the collection sump (CT_4) where the filtered water finally accumulates. The water flowed from the CT_4 to CT_1 by means of a suction pipe fitted at the centre bottom of last chamber running parallel to the tank above it. The oxygen demand of the fishes were met by the influent water through the air stone fitted inside the suction pipe. The flow rate of each system was 1.2 L min^{-1}. Evaporative losses in the recirculatory systems were compensated once in a week. A working model of the above said recirculatory system is presented in the plate 11.

3.2.2. Experimental animals for the study

The fish selected for the study was gold fish (*Carassius auratus*). The fishes were collected from the aquarium fish shops in Cochin. About 60 nos. of fishes of uniform size were selected for the study (8.3 ± 0.85 cm TL, 10.61± 0.31 g). The fishes were acclimatized prior to introduction into the experimental tanks. A total of 10 uniform sized gold fish were exposed to each treatment. The fishes were fed once daily with commercial pellet diets with 25 percent protein at a rate of 3 percent of the body weight.

3.2.3. Establishment of biofilter

The biofilter materials selected for the study were coir (BF 1), bamboo strips (BF 2) and vetiver (BF 3). Plate 12 shows the biofilter materials used in the study. All the materials were presoaked in water for one month with water replacement every week to wash off the tannins. The materials were then drained and immersed in the third chamber of the glass tank. Water was filled in all the chambers to a particular height such that the water in the first chamber drained to the aquarium plant chamber through a seperation at the lower base of glass panel

Plate 11. Working model of the recirculating system

Bamboo

Coir

Vetiver

Plate 12. Biofilter materials used for the study

and from there it trickled down through the sides of the upper glass panel to the third biofilter chamber. The filtered water again drained to the collection sump through the lower base separation and from here the clean aerated water was airlifted to the first chamber and so on. A control (C) was set up without any filter media for a comparative performance study.

3.2.4. Conditioning of the tank

3M ammonium chloride (NH_4Cl) was added to each tank after the initial set up to attain a final concentration of 8 mg NH_4^+ per liter. Twenty-eight days after the initial dose of ammonium, dosing resumed with 3M NH_4Cl at the rate of 0.5 mg NH_4^+/litre/day. The conditioning helps in the establishment of microbial community.

3.2.5. Water sampling regime and analytical procedures

The recirculatory system modelled for second part of work was only a preliminary study focusing mainly on the incorporation of aquarium plants as a part of biofilter. The thrust was mainly to compare the three different filter media based on the water quality parameters attained in each system. Water was sampled from the systems daily to measure pH and temperature. pH was measured using a pH meter. Temperature was measured using a thermometer. Dissolved oxygen was analysed every two days using water test kit. To obtain the apparent nitrification performance of the biofilter, water sampled from the influent (CT_4) and effluent (CT_1) of each biofilter media was analysed initially every two days. Total ammonia nitrogen (TAN) was determined by the Nesslerization method, nitrite by the azo dye colorimetric method and nitrate by the Szechrome NAS reagent (diphenylamine sulfonic acid chromogene) method according to Gross and Boyd (1998).

Results

4. RESULTS

4.1. Micropropagation

4.1.1. Standardisation of surface sterilization of explants

All plant materials used for culture were treated with different sterilization agents to inactivate the microbes present on their surface. Each explant had its own optimum method for surface sterilization. Generally, the time of soaking or concentration of sterilant determines the survival of explant. Increasing the time of soaking or the concentration of the sterilant beyond the optimum adversely affects the survival of the explants.

The effects of the surface sterilization treatments on culture establishment in *Anubias minima* are presented in Table 11. The results showed that $HgCl_2$ treatment alone was not effective to arrest contamination in *Anubias minima*. The most effective surface sterilization treatment for *Anubias minima* was a combination treatment of 70 percent ethyl alcohol wipe followed by 0.5 percent $HgCl_2$ for 2 minutes. It was then followed by rinses in distilled water four times and then with 30 percent ethyl alcohol for 2 minutes and further washing with distilled water three times to produce 100 percent healthy cultures. The 0.5 percent $HgCl_2$ treatment for more than 2 minutes appeared to initiate scorching of the tissues. Hence, soaking the explant in 0.5 percent $HgCl_2$ was limited to 2 minutes.

The results of surface sterilization treatment for *Aponogeton ulvaceus* are presented in Table 12. In this case also, a combination treatment of 70 percent ethyl alcohol (wipe), 0.1 percent $HgCl_2$ (3 min) and 30 percent ethyl alcohol (2 min) gave 100 percent healthy cultures. $HgCl_2$ (0.1 percent, 0.5 percent) treatment alone was not as effective as combination treatments in terms of contamination and scorching. Increasing the soaking time in $HgCl_2$ (0.1 percent, 4 min) or increasing the $HgCl_2$ concentration (0.5 percent, 1 min) in combination

treatments resulted in browning and death of explants in 10 percent of the cultures even though there was no contamination.

The surface sterilization treatments for nodal segments of *Bacopa caroliniana* (Table 13) showed that $HgCl_2$ treatment alone was effective in controlling contamination rather than combination treatments as detailed above. Lowering the dose as well as concentration increased the percentage of healthy cultures and decreased the scorching. The most effective surface sterilization treatment was $HgCl_2$ (0.1 percent) for 30 seconds which produced 90 percent healthy cultures and 10 percent scorching.

A single step surface sterilization treatment with $HgCl_2$ alone was effective for leaf petiole and lateral bud explants of *Nymphoides cristata* (Table 14). In this case, eventhough higher concentration of $HgCl_2$ eliminated contamination, the tissues got scorched within one week. The most effective treatment was $HgCl_2$ (0.1 percent) for 2 minutes which gave 70 percent healthy cultures with only 10 percent scorching. The contamination was also less (20 percent) compared to other treatments.

Rotala rotundifolia having the most fragile stems of the selected explants were treated with sodium hypochlorite solution (5.54 percent active chlorine) which is the least toxic of the surface sterilants. The results of the experiment (Table 15) show that sodium hypochlorite rinsing for 35 – 40 minutes gave the highest percentage of healthy cultures (90 percent). Moreover, none of the treatments showed scorching.

Table 11. Effect of surface sterilization on survival of explants in *Anubias minima* (one week after inoculation)

Sterilant	Concentration (%)	Duration (minutes)	Contamination* (%)	Uncontaminated cultures	
				Scorched (%)	Healthy (%)
$HgCl_2$	0.1	3	100	Nil	Nil
	0.1	4	90	Nil	10
	0.1	5	90	Nil	10
	0.1	6	70	Nil	30
	0.1	7	70	Nil	30
$HgCl_2$	0.5	1	50	Nil	50
	0.5	2	50	Nil	50
	0.5	3	40	20	40
	0.5	4	20	30	50
Ethyl alcohol + $HgCl_2$ + Ethyl alcohol	70,0.5,30	Wipe, 1,2	20	Nil	80
	70,0.5,30	Wipe, 2,2	Nil	Nil	100
	70,0.5,30	Wipe, 3,2	Nil	20	80
	70,0.5,30	Wipe,4, 2	Nil	30	70

*Average of 10 observations

Table 12. Effect of surface sterilization on survival of explants in *Aponogeton ulvaceus* (one week after inoculation)

Sterilant	Concentration (%)	Duration (minutes)	Contamination* (%)	Uncontaminated cultures	
				Scorched (%)	Healthy (%)
$HgCl_2$	0.1	3	90	Nil	10
	0.1	4	90	Nil	10
	0.1	5	80	20	Nil
	0.1	6	60	20	20
	0.1	7	20	20	60
$HgCl_2$	0.5	1	20	30	50
	0.5	2	20	30	50
	0.5	3	10	50	40
	0.5	4	10	80	10
Ethyl alcohol + $HgCl_2$ + Ethyl alcohol	70,0.1,30	Wipe, 3, 2	Nil	Nil	100
	70,0.1,30	Wipe, 4, 2	Nil	10	90
	70,0.1,30	Wipe, 5, 2	Nil	40	60
	70,0.1,30	Wipe, 6, 2	Nil	90	10
	70,0.1,30	Wipe, 7, 2	Nil	90	10
Ethyl alcohol + $HgCl_2$ + Ethyl alcohol	70,0.5,30	Wipe, 1,2	Nil	10	90
	70,0.5,30	Wipe, 2,2	Nil	30	70
	70,0.5,30	Wipe, 3,2	Nil	60	40
	70,0.5,30	Wipe,4, 2	Nil	70	30

*Average of 10 observations

Table 13. Effect of surface sterilization on survival of explants in *Bacopa caroliniana* (one week after inoculation).

Sterilant	Concentration (%)	Duration (minutes)	Contamination* (%)	Uncontaminated cultures	
				Scorched (%)	Healthy (%)
$HgCl_2$	0.5	3	Nil	100	Nil
	0.5	2	Nil	100	Nil
	0.5	1	Nil	100	Nil
	0.1	3	Nil	80	20
	0.1	2	Nil	50	50
	0.1	1	Nil	30	70
	0.1	30 seconds	Nil	10	90

Table 14. Effect of surface sterilization on survival of explants in *Nymphoides cristata* (one week after inoculation).

Sterilant	Concentration (%)	Duration (minutes)	Contamination* (%)	Uncontaminated cultures	
				Scorched (%)	Healthy (%)
$HgCl_2$	0.5	3	Nil	100	Nil
	0.5	2	Nil	100	Nil
	0.5	1	Nil	70	30
	0.1	3	20	30	50
	0.1	2	20	10	70
	0.1	1	60	Nil	40
	0.1	30 seconds	70	Nil	30

Table 15. Effect of surface sterilization on survival of explants in *Rotala rotundifolia* (one week after inoculation).

Sterilant	Concentration (%)	Duration (minutes)	Contamination* (%)	Uncontaminated cultures (%)	
				Scorched	Healthy
Sodium hypochlorite solution	5.54%	40	10	Nil	90
	5.54%	35	10	Nil	90
	5.54%	30	20	Nil	80
	5.54%	25	40	Nil	60
	5.54%	20	70	Nil	30
	5.54%	15	70	Nil	30
	5.54%	10	80	Nil	20

4.1.2. Standardisation of growth regulators for shoot proliferation and rooting

4.1.2.1. Effect of BA and Kinetin on bud breakage and shoot proliferation in *Bacopa caroliniana*

Response of the nodal segments of *Bacopa caroliniana* to varying levels of BA and Kinetin are presented in the Table 16. From the results it can be inferred that cytokinins are necessary for bud breakage and shoot formation in *Bacopa caroliniana* as there was no response in the basal media. In almost all other cultures bud break occurred within 4 to 7 days (Plate 13). The results of the ANOVA are presented in Table 17. Statistical analysis showed highly significant difference in mean values for the 8 treatments. The most effective cytokinin concentration level in terms of highest percentage of bud break (72.2 %) and shoot formation (13.875) was 1.5mg l^{-1} BA and 0.1mg l^{-1} Kn (T_7). Both BA and Kn treatments resulted in multiple adventitious shoot buds formation in the nodal explants, but at higher levels of BA the bud break response started decreasing and the shoots showed a stunted growth. The average shoot length was also highest in T_7 (4.46 ± 0.065 cm). Control showed lowest value (0) among the 8 treatments. Tuckey's test revealed six homogenous groups of treatments (Table 16).Roots were also observed in all the media and callusing seen at higher concentrations of BA (Plate 13).

4.1.2.2. Effect of BA and IAA on shoot regeneration from the nodal segments of *Rotala rotundifolia*

Morphogenic response of the plants to varying levels of auxin-cytokinin combination trials is presented in Table 18 and Plate 14. The lower concentrations of BA (0.1, 0.5 mg l^{-1}) along with IAA (0.5 mg l^{-1}) was not much effective in inducing shoots as compared to the higher concentrations of BA (1.0, 1.5 and 2.0 mg l^{-1}) along with IAA (0.5, 1.0 mg l^{-1}). In all the media individual shoot development initiated within 7 to 14 days. Statistical analysis showed highly significant difference ($p<0.01$) in the mean values among the 8

Table. 16. Effect of BA and Kinetin in MS medium for regeneration of shoot from nodal explants of *Bacopa caroliniana* four weeks after inoculation

Treatments	Shoot formation (%)	No. of adventitious shoot bud induced per explant (*Mean ± SD)	Average length of shoot (cm) (Mean ± SD)
Control	0	0	0
T_1	22.2	1.375 ± 0.518^{a}	1.86 ± 0.071
T_2	16.6	2.625 ± 0.744^{b}	2.08 + 0.066
T_3	38.8	3.625 ± 0.744^{bc}	2.12 ± 0.053
T_4	44.4	4.875 ± 0.641^{cd}	2.24 ±0.065
T_5	55.5	5.125 ± 0.835^{d}	2.88 ± 0.079
T_6	50.0	8.5 ± 1.195^{e}	3.23 ± 0.075
T_7	72.2	13.875 ± 1.553^{f}	4.46 ± 0.065
T_8	66.7	7.00 ± 0.756^{e}	3.02 ± 0.064
T_9	55.5	4.875 ± 1.126^{cd}	2.33 ± 0.073
T_{10}	55.5	3.50 ± 1.069^{b}	2.40 ± 0.055

*Average of 8 observations

[1]Means followed by the same letters are not significantly different ($p<0.01$) using Tuckey's test

Table 17. ANOVA

	Sum of Squares	df	Mean Square	F	Sig
Between groups	53.570	10	5.357	150.379	0.000
Within groups	2.743	77	0.036		
Total	56.313	87			

*Data subjected to square root transformation

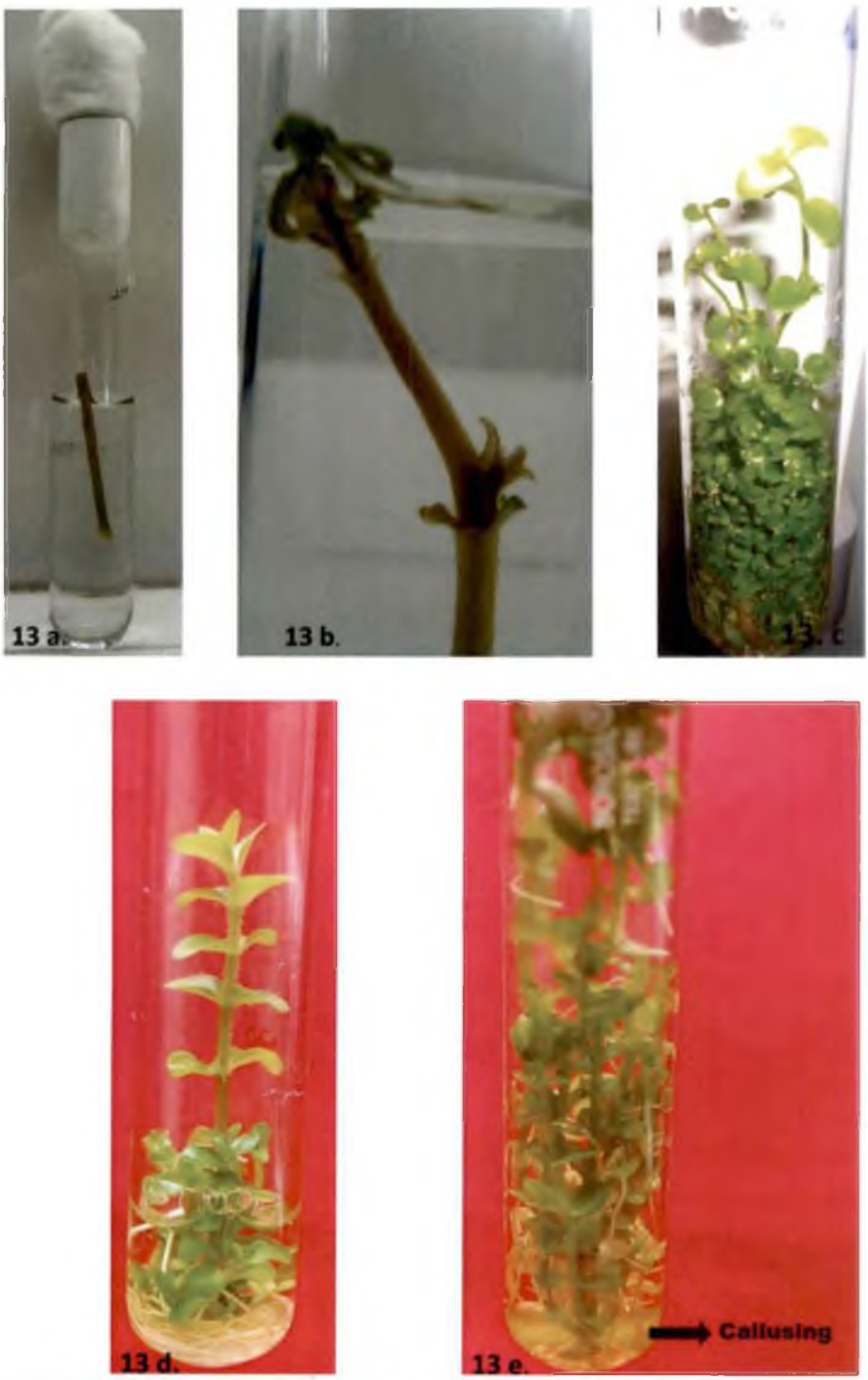

Plate 13. Micropropagation stages of *Bacopa caroliniana.* a) nodal explants in the medium b) lateral bud breakage c) shoot proliferation d) *in vitro* rooting e) callusing at 1.5 mg l^{-1} BA

Table 18. Effect of BA and IAA on MS medium for shoot regeneration from nodal segments of *Rotala rotundifolia* four weeks after inoculation

Treatments	Shoot formation (%)	No. of shoots induced per explant (Mean ± SD)	Average length of shoot (cm) (Mean ± SD)
C	11.1	10.25 ± 1.581^{a}	0.76 ± 0.002
T_{11}	27.7	16.0 ± 1.852^{b}	0.85 ± 0.915
T_{12}	22.2	21.5 ± 1.690^{c}	0.92 ± 0.006
T_{13}	44.4	28.25 ± 2.314^{cf}	1.31 ± 0.054
T_{14}	83.3	39.75 ± 1.909^{g}	1.58 ± 0.462
T_{15}	83.3	31.25 ± 2.052^{f}	0.64 ± 0.650
T_{16}	16.7	12.75 ± 1.832^{a}	0.71 ± 0.007
T_{17}	50.0	23.75 ± 2.605^{cd}	0.78 ± 0.221
T_{18}	77.7	32.25 ± 2.252^{f}	1.27 ± 0.035
T_{19}	88.8	48.75 ± 3.412^{h}	1.63 ± 0.710
T_{20}	77.7	25.5 ± 2.673^{dc}	0.85 ± 0.008

*Average of 8 cultures per treatment

[1]Means followed by the same letters are not significantly different ($p<0.01$) using Tuckey's test

Table 19. ANOVA

	Sum of Squares	df	Mean Square	F	Sig
Between groups	104.054	10	10.405	202.075	0.000
Within groups	3.965	77	0.051		
Total	108.019	87			

*Data subjected to square root transformation

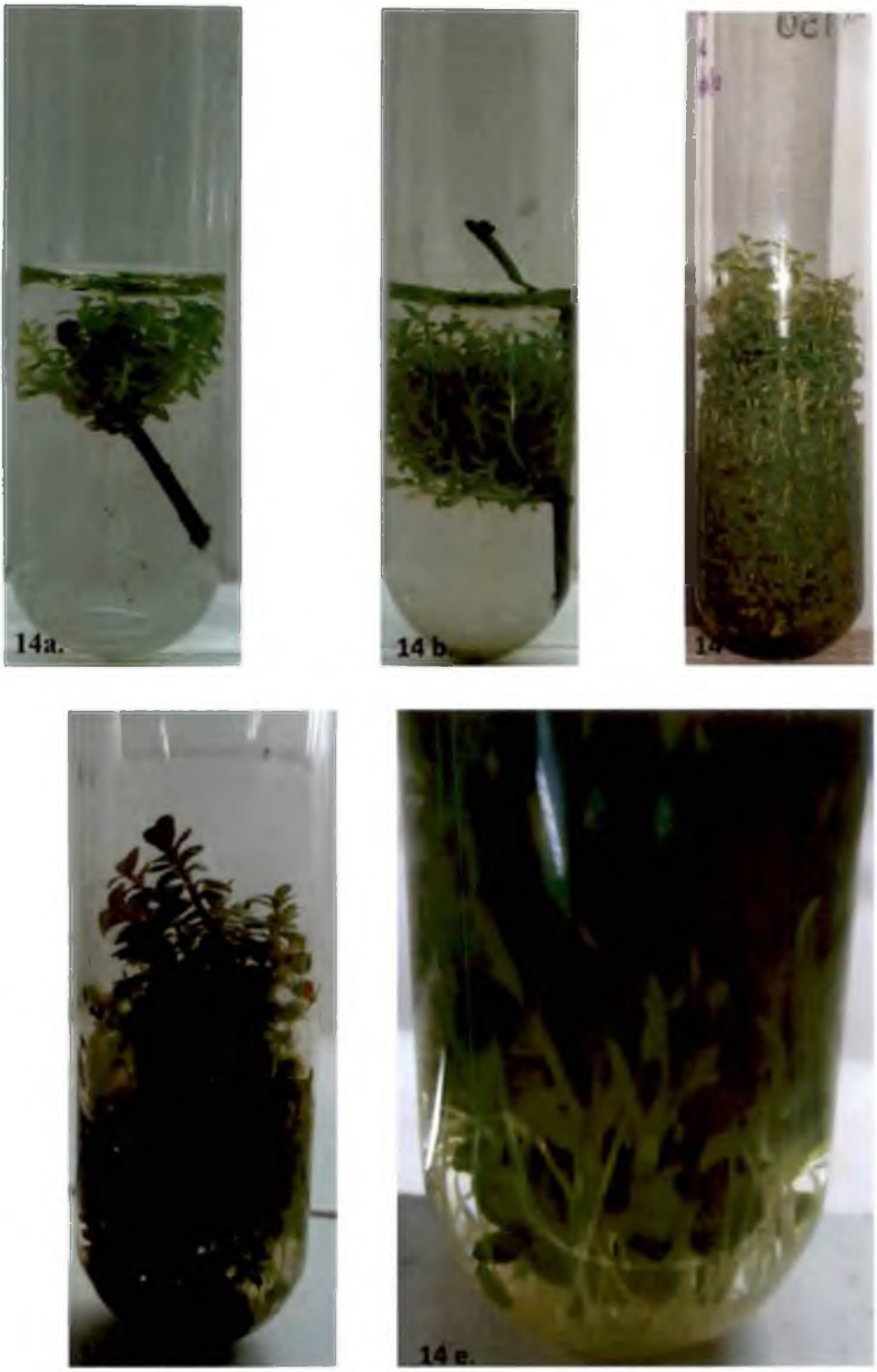

Plate 14. Micropropagation stages of *Rotala rotundifolia* a) shoot induction after 7 days b) Shoot induction after 14 days c) shoot multiplication d) *in vitro* plant at 1.5 mg l^{-1} BA e) *in vitro* rooting.

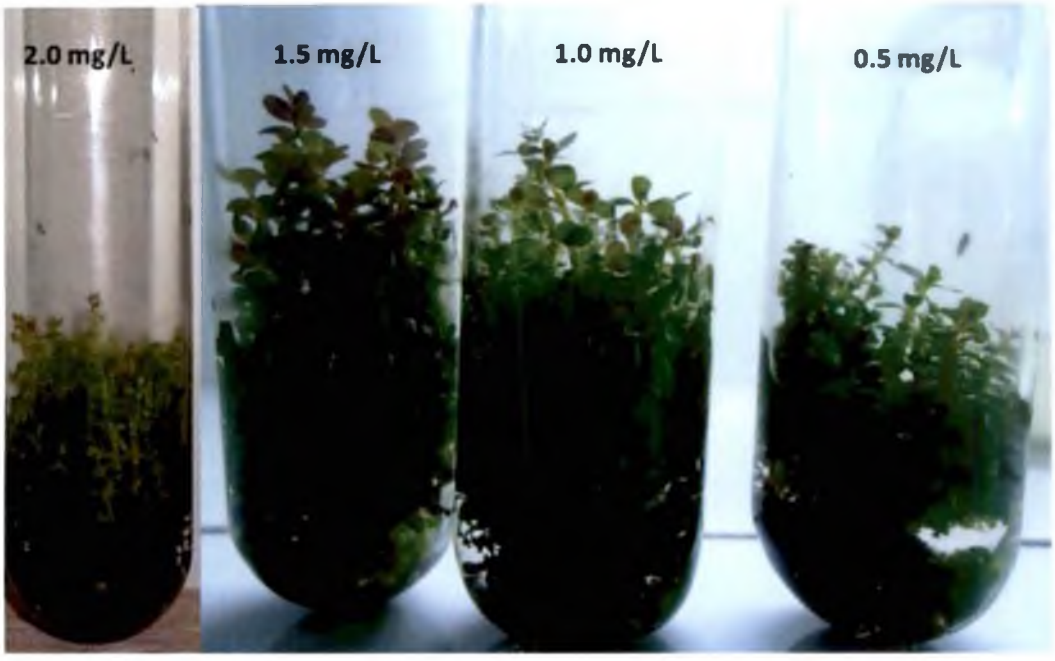

Plate 15. Effect of varying levels of BA on shoot number and length in *R. rotundifolia* four weeks after inoculation

treatments (Table 19). The highest percentage of shoot induction was observed in T_{19} (88.8 percent) with 1.5 mg l^{-1} BA and 1.0 mg l^{-1} IAA combination. The number of shoots produced increased as the BA concentration increased from 0 to 1.5 mg l^{-1} at both levels of IAA. However, explants on 2.0 mg l^{-1} BA produced stunted shoots indicating an upper limit on the BA concentration for the micropropagation of *R. rotundifolia* (Plate 15). The highest number of shoots per explant were obtained on 1.5 mg l^{-1} BA and 1.0 mg l^{-1} IAA, T_{19} (48.75), followed by T_{14}(39.75) and these are significantly different from the rest of the treatments. Control and T_{16} showed lowest values (10.25 and 12.75) among the 8 treatments.Tuckey's test revealed eight homogenous groups of treatments (Table 18).

4.1.2.3. Effect of BA and BA, IAA combinations on shoot organogenesis from the lateral buds and leaf petiole explants of *Nymphoides cristata*

The effect of the type of explant on shoot organogenesis in *Nymphoides cristata* is presented in Plate 16. Of the two types of explants studied for *in vitro* propagation lateral buds performed better in terms of shoot organogenesis as the shoots generated from leaf petioles were small and less in number. Hence lateral buds alone were chosen for *in vitro* propagation trials with varying hormone concentrations. Shoot organogenesis was promoted in the presence of both exogenous cytokinin and auxin. The results are presented in the Table 20. Statistical analysis showed highly significant difference ($p<0.01$) in mean values for the 8 treatments (Table 21). Adventitious shoot formation was highest in T_{28} with hormone concentration of 1.0 mg l^{-1} each of BA and IAA (20.13 nos.) followed by T_{23} with 1.0 mg l^{-1} of BA alone (16.50 nos.). The adventitious shoot formation percentages were 88.8 and 94.4 respectively. Even though the shoot number was highest in T_{28} (IBA and IAA combination) , remarkable increase in shoot length was observed in BA alone (13.2 ± 0.031 cm) T_{23} compared to 7.33 ± 0.962 cm in T_{28}. Rooting initiated in culture tubes within 14 days of initiation of culture(Plate 17). Tuckey's test revealed seven homogenous groups of treatments (Table 20).

Plate 16. a) shoot organogenesis from lateral buds of *Nymphoides cristata* b) shoot organogenesis from leaf petioles.

Plate 17. *In vitro* rooting in *Nymphoides cristata*

Table 20. Effect of BA and BA, IAA combination on MS medium for shoot organogenesis from lateral buds in *Nymphoides cristata* two weeks after inoculation

Treatments	Shoot formation (%)	No. of shoots induced per explant (Mean ± SD)	Average length of shoot (cm) (Mean ± SD)
Control	22.2	2.13 ± 1.356^{a}	2.21 ± 0.323
T_{21}	50.0	5.50 ± 1.773^{bc}	4.50 ± 0.630
T_{22}	61.1	10.75 ± 1.389^{dc}	7.86 ± 0.168
T_{23}	94.4	16.50 ± 2.268^{fg}	13.2 ± 0.031
T_{24}	83.3	10.50 ± 1.927^{dc}	13.0 ± 0.092
T_{25}	44.4	5.00 ± 1.772^{b}	6.51 ± 0.008
T_{26}	50.0	6.00 ± 2.204^{bc}	4.22 ± 0.334
T_{27}	72.2	13.25 ± 1.581^{ef}	6.88 ± 0.265
T_{28}	88.8	20.13 ± 2.588^{g}	7.33 ± 0.962
T_{29}	66.7	8.50 ± 2.00^{cd}	4.71 ± 0.023
T_{30}	33.3	4.375 ± 2.134^{b}	4.10 ± 0.001

*Average of 8 cultures per treatment

[1]Means followed by the same letters are not significantly different ($p<0.01$) using Tuckey's test

Table 21. ANOVA

	Sum of Squares	df	Mean Square	F	Sig
Between groups	64.923	10	6.492	51.575	0.000
Within groups	9.693	77	0.126		
Total	74.616	87			

*Data subjected to square root transformation

4.1.2.4. Effect of BA, kinetin and IAA on shoot regeneration in *Anubias minima*

The results of the experiment are presented in Table 22. The media combinations showed differences in shoot formation, number of shoot buds and shoot length. Of the two cytokinins tried, BA is more effective in initiating shoots when compared to Kn as indicated by the results of T_{31}, T_{32} and T_{33}. The combination of BA, Kn and IAA (3.0, 0.1, 1.0 mg l^{-1} respectively) failed to induce shoots in all the cultures experimented whereas in T_{32} with 4.0 mg l^{-1} BA and 1.0 mg l^{-1} IAA the shoot induction was 60 pecent with an average shoot number of 1.40 ± 0.51 Nos. The shoot induction percentage and the average number of shoots and shoot length increased with increasing levels of BA as the highest number of shoot buds per explant (4.167) and shoot length per explant (5.11 ± 0.71 cm) was observed in the higher levels of BA, T_{33} (6.0 mg l^{-1}). The shoot induction percentage was also highest in this treatment (T_{33}) i.e. 80 percent. Statistical analysis showed highly significant difference ($p<0.01$) in mean values for the 4 treatments (Table 23). Auxin (IAA) did not seem to have any effect on shoot induction since with a constant level of auxin and lower levels of BA and Kn (T_{31} and T_{32}), shoot induction was nil or poor. However, when BA alone was used at higher levels the shoot induction increased remarkably (T_{33}). This also served as the rooting media. The days to initiate the culture varied between 7 to 12 days except for basal media where the culture initiated around 21 days. The sequential steps in micropropagation are presented in Plates 18 and 19. Tuckey's test revealed three homogenous groups of treatments (Table 22).

4.1.2.5. Effect of BA on shoot organogenesis from apical rhizome buds of *Aponogeton ulvaceus*

The results of the *in vitro* trials for *Aponogeton ulvaceus* was not successful due to the exudation of phenolic extracts in all the treatments which eventually lead to the death of the tissues (Plate 20).

Table 22. Effect of BA and IAA on the *in vitro* propagation of *Anubias minima* from rhizome explants four weeks after inoculation

Treatments	Shoot formation (%)	No. of shoot buds induced per explant (Mean ± SD)	Average length of shoot (cm) (Mean ± SD)
Control	10	1.00 ± 0.894^{ab}	3.23 ± 0.15
T_{31}	No response	0^{a}	0
T_{32}	60	1.167 ± 0.983^{b}	2.80 ± 0.05
T_{33}	80	4.167 ± 0.753^{c}	5.11 ± 0.71

*Average of 6 explants per treatment

[1]Means followed by the same letters are not significantly different ($p<0.01$) using Tuckey's test

Table 23. ANOVA

	Sum of Squares	df	Mean Square	F	Sig
Between groups	6.610	3	2.203	23.874	0.000
Within groups	1.846	20	0.092		
Total	8.456	23			

*Data subjected to square root transformation

Shoot initiation 3 DAI

Multiple shoot induction 12 DAI

20 DAI

28 DAI

Plate 18. Micropropagation stages of *Anubias minima*

45 DAI

Plate 19. Adventitious shoot formation in *Anubias minima* 45 Days after initiation

Plate 20. Phenolic exudates in the *in vitro* propagation of *Aponogeton ulvaceus*

4.1.3. Hardening

Hardening was successful with the bedding substratum of pond soil and coir fibres in fibre tanks inside the net house. Results of the experiment showing percentage survival after two weeks of planting out are presented in the Table 24. Hardened plants in the fibre tanks are presented in Plate 21.

Table 24. Hardening success of the micropropagated plants in the bedding substratum of pond soil and coir fibres after two weeks of planting out.

Plant species	Bedding substratum	Survival (%) after 14 days*
Anubias minima	Pond soil with coir fibres	90
Aponogeton ulvaceus	-	-
Bacopa caroliniana	Pond soil with coir fibres	100
Nymphoides cristata	Pond soil with coir fibres	100
Rotala rotundifolia	Pond soil with coir fibres	70

***Average of 10 observations**

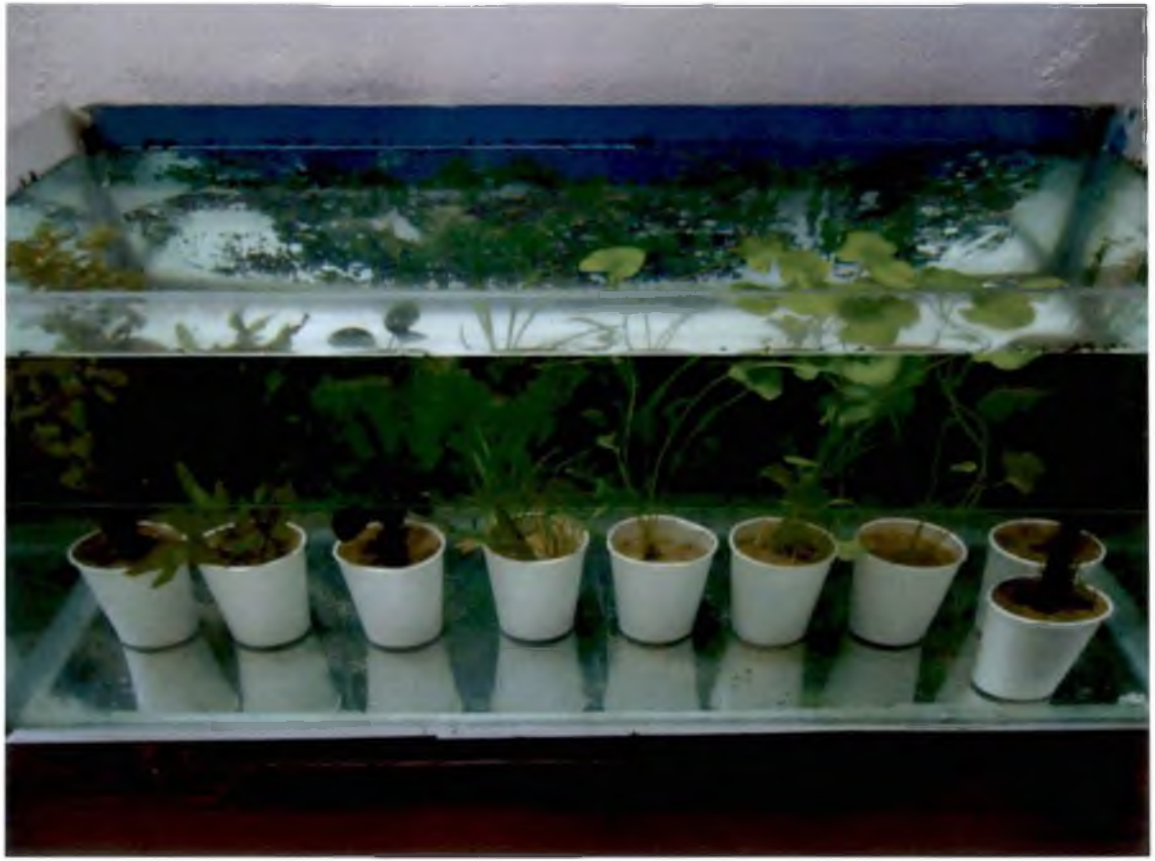

Plate 21. Hardening of micropropagated plants in tanks

4.2. Recirculating Aquaculture Systems

4.2.1. Water quality parameters

The water quality of the recirculating systems was stable throughout the experiment; there were no water quality problems for either the fish or the bacteria in the biofilter. The results of water quality analysis are presented in Table 25. pH levels of all systems were above 7.0 throughout the trial. The temperature did not vary much in the experimental units and was found to be in the range of 23.5 to 24.5 °C (Table 25). The concentrations of dissolved oxygen in the influent of the CT_1 ranged from 4.3 to 7.7 mg l^{-1} and the dissolved oxygen concentrations in each tank remained above 5.0 mg l^{-1}(Table 25). During the experiment, dissolved oxygen levels of the water in all of the biofilters were maintained above 2 mg l^{-1} level, which is considered necessary for satisfactory filter performance.

Overall, over the period of the first fortnight in setting up the tank, a marked cloudiness was observed in the control tank on days 2 to 5. In contrast, the treatments with biofilter materials remained clear during this time and indeed throughout the duration of the study.

All the nitrogenous compounds were measured as mentioned in the methodology described in Chapter 3. The data are presented in figures 1, 2 and 3 below. Overall, the data observed for the control tank are not unremarkable. The peak ammonia concentration was observed in all the systems on day 3 of operation. But there were variations in the time required to stabilize ammonia to the safe levels. Coir filter material was the first to stabilize ammonia concentrations to the safest level in 14 days followed by bamboo strips and vetiver filters. However, in the control system, the ammonia concentration stabilized around 45 days (Fig. 1).

Nitrites started appearing in the control tanks from day 7 onwards whereas they were detected in reasonable quantities from the coir filter systems

from day 2 to 3 and in bamboo strip and vetiver systems from day 3 to5. Nitrites peaked around days 9 to 14 in the case of coir filter system. The peak nitrite levels in coir and bamboo systems were observed during the days 16 to 21. However, in the control systems, the nitrite levels were progressing continuously towards the end of the experimental duration (Fig. 2).

Reasonable nitrate levels appeared in the control systems from day 14 -16 onwards and peaked around 42nd day and then started declining. In the coir system two peaks were observed during the experiment with the first peak around 7-9 days and then showed several upscaling and declining trends during the experimental period. Bamboo strips and vetiver filter systems also showed a peaking trend towards the initial phase of experiment (around 7 days) and declined and slowly started picking up around 36 to 42 days (Fig. 3).

Despite these variations, all the parameters remained within the safe limits for culture in recirculating systems. However, in control systems fish mortalities happened due to the uncontrolled chemical parameters.

Table 25. Average values of physicochemical parameters and nitrogenous compounds during the experiment.

Treatments	Sampling Days	pH	Temperature (°C)	DO (mg/l)
Coir (BF_1)	0	8.2(0.17)	24.5(1.0)	6.4(1.8)
	14	8.1(0.13)	24.5(1.2)	7.2(2.6)
	28	8.1(0.08)	23.5(1.0)	7.7(1.8)
	42	8.2(0.07)	24.0(1.0)	7.0(2.6)
	56	8.1(0.07)	24.0(1.0)	7.0(1.8)
Bamboo strips (BF_2)	0	8.0(0.17)	24.5(1.2)	6.3(1.8)
	14	7.9(0.13)	24.5(1.0)	6.1(1.8)
	28	8.0(0.08)	23.5(1.0)	5.8(1.8)
	42	8.1(0.07)	24.5(1.0)	5.9(1.7)
	56	8.1(0.07)	24.0(1.0)	5.8(1.7)
Vetiver (BF_3)	0	8.2(0.17)	24.0(1.0)	6.1(1.8)
	14	8.1(0.13)	23.5(1.2)	5.7(2.6)
	28	8.0(0.08)	24.0(1.0)	5.4(1.8)
	42	7.9(0.07)	23.5 (1.2)	5.3(1.7)
	56	7.8 (0.13)	23.5(1.1)	5.3(1.7)
Control	0	8.0(0.13)	24.5(1.2)	6.1(1.8)
	14	7.7(0.17)	24.0(1.0)	5.4(2.6)
	28	7.9(0.07)	24.5(1.1)	4.6(1.7)
	42	7.8(0.08)	24.0(1.0)	4.3(1.8)
	56	7.9(0.07)	24.0(1.0)	4.4(1.8)

*Figures in parenthesis indicate standard deviation

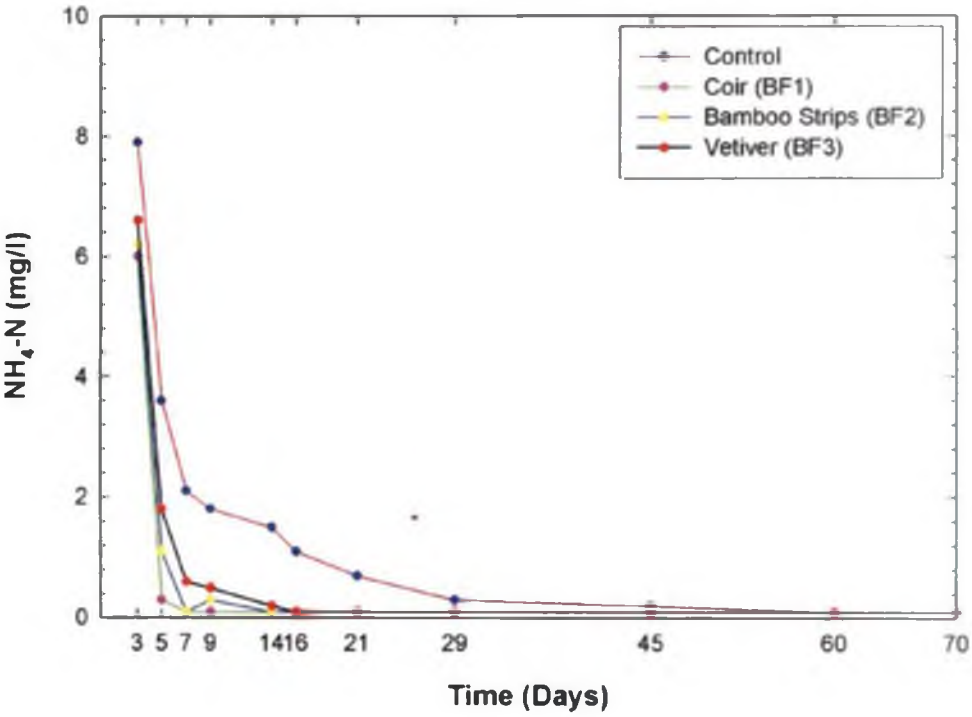

Figure 1. Variations in ammonia (NH_4-N) concentrations in the experimental treatment systems during 70 days of operation

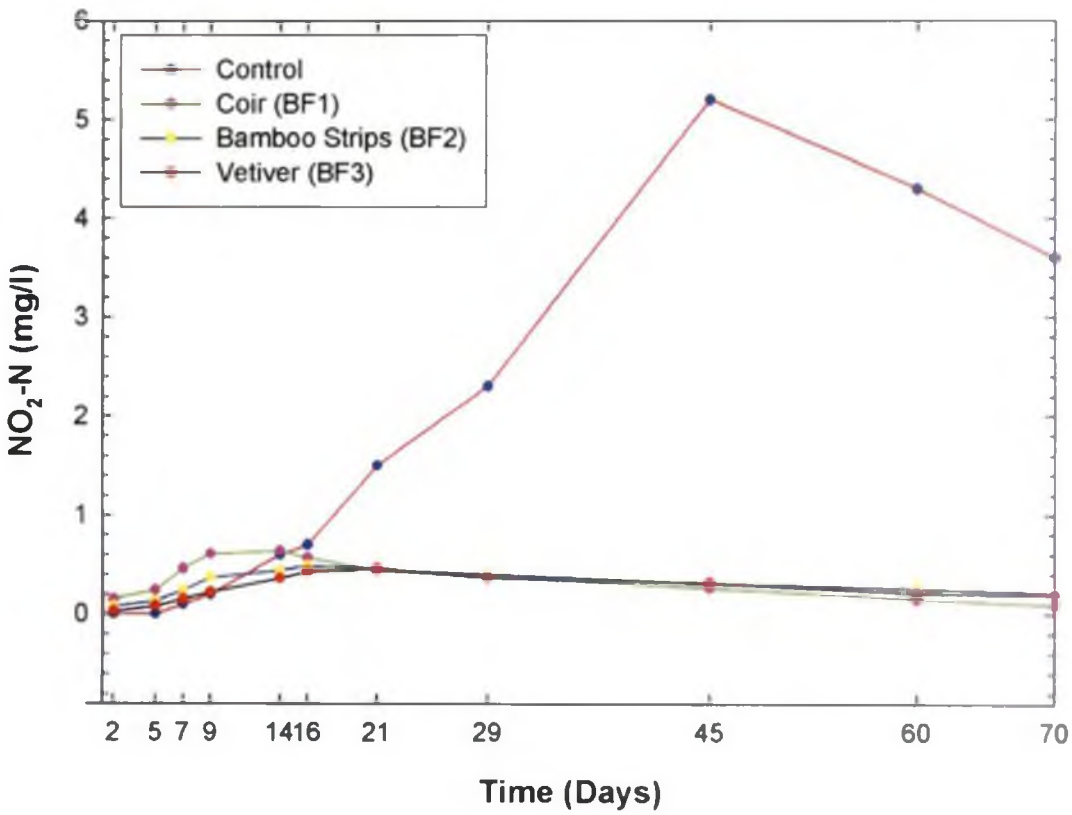

Figure 2. Variations in nitrite (NO_2-N) concentrations in experimental treatment systems during 70 days of operation.

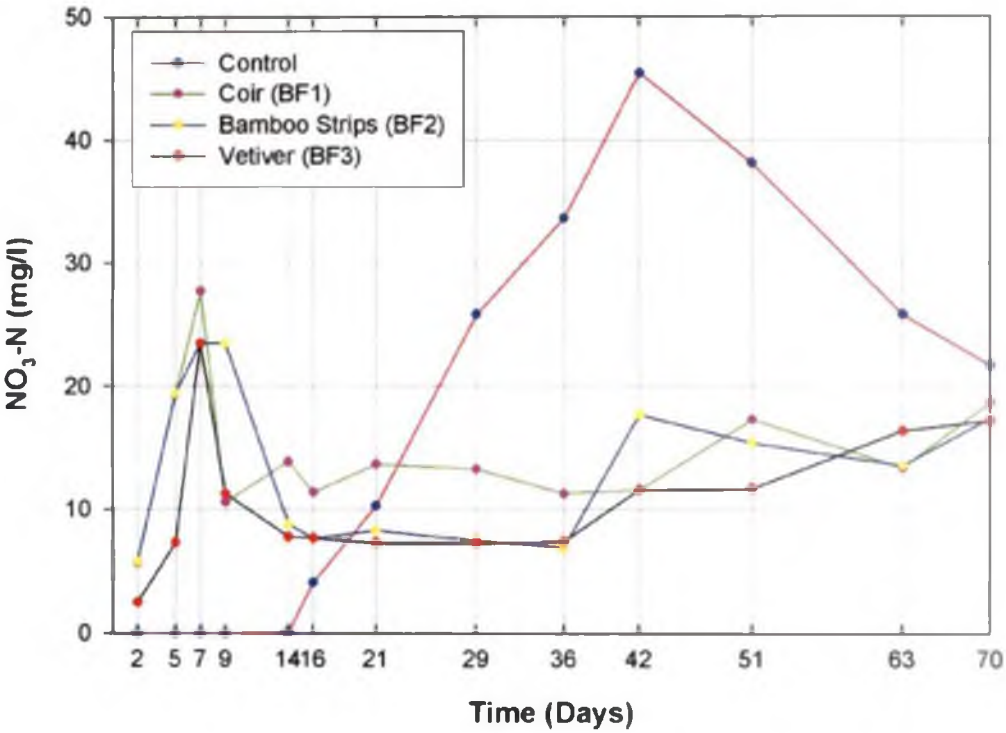

Figure 3. Variations in Nitrate (NO_3-N) concentrations in the experimental treatment units during 70 days operation

Discussion

5. DISCUSSION

Micropropagation of aquarium plants is a virgin area in India when compared to the top aquarium plant producers like USA, Central Europe and Japan. In the era of booming aquarium trade, aquarium plants deserve special attention not only for its aesthetic properties but also for its water rejuvenating properties. In view of these aspects, standardisation of *in vitro* propagation techniques for aquarium plants has great relevance in the production of true to type propagules at a cheaper rate within reasonable time and also as a conservation measure for our indigenous aquatic plant diversity.

Aquarium plants play the role of biofilters in recirculatory systems and helps in nutrient recycling. The water hardening of *in vitro* propagated aquarium plants can very well be carried out in recirculatory systems. But in high density fish culture systems, aquarium plant biofilter alone will not be sufficient and other substrata are needed to support the growth of nitrifying bacteria. The biofilter is the core of recirculatory system whose efficiency depends on the surface area, cost and life. Hence, attempts were made in the present work to incorporate three indigenous filter materials like coir, bamboo and vetiver as biofilters in the plant fish integrated recirculatory system.

5.1. Micropropagation of aquarium plants

5.1.1. Standardisation of surface sterilization of explants

Establishment of *in vitro* cultures often pose the problem of bacterial contamination in the primary cultures which can be considerably reduced by an effective surface sterilization (Anand *et al.,* 1998). The most effective surface sterilization treatment for *Anubias minima* was a combination treatment of 70 percent ethyl alcohol wipe followed by 0.5 percent $HgCl_2$ rinse for 2 minutes and rinsing in distilled water four times, then rinsing with 30 percent ethyl alcohol for 2 minutes and further washing with distilled water three times. This produced 100 percent healthy cultures. Bonga (1982) has advocated the use of alcohol

alone or in combination with other chemicals for disinfection. Anand *et al.* (1998) has established the *in vitro* cultures from the nodal explants of *Uraria picta*, a medicinal plant by surface sterilization with 70 percent ethanol alone for 3 minutes. Maridass *et al.* (2010) disinfected rhizome bud explants of *Dendrobium nanum* using 70 percent ethanol for 30 sec followed by 3 percent sodium hypochlorite with Tween 80 for 20 min.

George (1993) explained that the bactericidal action of hypochlorite solutions (bleach) is due to both hypochlorous acid (HOCl) and the OCl^- ion with the former being more active so that the disinfecting efficiency of chlorine is best in slightly acid hypochlorite solutions. However, the exposure of explants to the chemical beyond five minutes in low concentration (0.1 percent) and beyond two minutes in higher concentration (0.5 percent) was found to be deleterious. This is in confirmity with the findings of Krishnan and Seeni (1994) in *Woodfordia fruticosa*.

Similarly, the best surface sterilization treatment for *Aponogeton ulvaceus* is 70 percent ethyl alcohol (wipe), 0.1 percent $HgCl_2$ (3 min) and 30 percent ethyl alcohol (2 min). Basal shoot tip explants of *Cryptocoryne wendtii* were surface sterilized by successive immersion in 50 percent ethanol for 1 minute and 1.05 percent NaOCl containing 1 drop Tween–20 for 12 min and subsequent rinsing with sterile distilled water three times (Kane *et al.*, 1999).

After surface sterilization, rinsing the explants thoroughly with sterile distilled water ensured the removal of the traces of the chemical present on the explants. Hu and Wang (1983) opined that it is necessary to wash the tissues twice or thrice in sterile distilled water to ensure dilution of the chemical. Krishnan and Seeni (1994) also recommended rinsing the explants five to six times in sterile distilled water before inoculation.

The most effective surface sterilization treatment for *Bacopa caroliniana* nodal explants was $HgCl_2$ (0.1 percent) for 30 sec which produced 90 percent healthy cultures and 10 percent scorching. Similar surface sterilization treatments

were carried out for nodal explants of *Bacopa monnieri* but for a higher duration of 4 to 5 min (Sharma *et al.*, 2010). They also reported blackening of explants on longer exposure to the surface sterilant because they are phytotoxic chemicals.

In the case of explants of *Nymphoides cristata*, the best surface sterilization treatment was $HgCl_2$ (0.1 percent) wash for 2 minutes which gave 70 percent healthy cultures. Jenks *et al.* (2000) reported that a two-step surface sterilization using 50 percent ethanol and sodium hypochlorite was effective for explants of *Nymphoides indica*. However, there are morphological variations in relation to sturdiness among the two species which can be the cause for the difference in surface sterilization treatments. Meyberg (1988) reported that presence of numerous mucilage secreting trichomes in *Nymphoides* species account for the difficulties in surface sterilization.

Sodium hypochlorite solution (5.54 percent active chlorine) immersion for 35–40 min gave healthy cultures in *Rotala rotundifolia*. A similar surface sterilization treatment has been reported for apical meristems and axillary buds of aquarium plant, *Ludwigia repens* (Ozturk *et al.*, 2004). Baiyeri and Mbah (2006) has reported the surface sterilization of African breadfruit (*Treculia Africana* Decne) seedling with 3.5 percent sodium hypochlorite solution. Hypochlorite is known to be a very effective killer of bacteria; even micromolar concentrations are enough to reduce bacterial populations significantly (Nakagawara *et al.*, 1998). Moutia and Dookun (1999) reports that disinfection in either 2.7 percent solution of sodium hypochlorite (pH 6) or 1 percent mercuric chloride were equally good for sugarcane bud explants.

Surface sterilization techniques are unique for a species and it depends on the type of explant and sterilants involved. Increasing the duration of explants to the sterilant and scaling up the levels of sterilant has adverse effect on the survival of cultures as observed in the results discussed above.

5.1.2. Basal medium

In the present study, the culture establishment was best in MS medium. Best culture medium was identified based on their efficacy to initiate the maximum number of shoots, callus or bud breakage from the type of explants implanted. Full strength MS medium was found to be optimal for the tissue culture of aquatic plants (Kane and Gilman, 1991;Kane *et al.,* 1999; Jenks *et al.,* 2000; Ozturk *et al.,* 2004; Shou *et al.,* 2008; Sharma *et al.,* 2010). The strength of the MS basal medium also plays a role in the induction of shoots and roots in plant tissue culture. In the present study half strength MS medium (1/2 MS) was used for the *in vitro* propagation of *Bacopa caroliniana, Rotala rotundifolia* and *Nymphoides cristata* whereas full strength MS medium was used for the *in vitro* propagation of *Anubias minima* and *Aponogeton ulvaceus*. Studies by Jayaram and Prasad (2007) on the effect of strength of MS medium on shoot proliferation and rooting in *Drosera indica* showed that 1/4 MS was more effective when compared to 1/2, 1/3 and full MS. Since *Drosera* plants grow normally in nutrient poor habitats, 1/ 4 strength MS medium was sufficient for establishing the culture.

Use of full strength MS medium has been reported in *Ginkgo biloba* cultures (Carrier *et al.,* 1990), *Solanum mammosum* (Akram *et al.,* 1995), *Glycyrrhiza glabra* (Tailang *et al.,* 1997), *Alstroemeria* rhizome explants (Yousef *et al.,* 2007) etc.

5.1.2.1. Type of culture medium

Several types of culture medium like solid, liquid and semisolid culture mediums have been reported in plant tissue culture. In the present study, a liquid medium was used for *in vitro* propagation of *Bacopa caroliniana, Rotala rotundifolia* and *Nymphoides cristata*. The explants dried out when placed in the

agar medium since it is difficult to extract the nutrients from agar medium when compared to liquid medium.

Most of the reports of aquatic plant tissue culture refer to a culture in semisolid media using 0.7% agar (Kane and Gilman, 1991; Kane *et al.*, 1999; Ozturk *et al.*, 2004; Sharma *et al.*, 2010). Jenks *et al.* (2000) opines that a liquid basal media is good for the establishment of stock cultures of *Nymphoides indica*. The plants cultured in liquid medium produced leaves with elongated petioles. Similarly, in *Catharanthus roseus*, a medicinal plant liquid culture over performed solid media in *in vitro* propagation (Pati *et al.*, 2011). This work also demonstrated that elimination of agar can bring down the total cost upto 5.2 folds. Reduction of quantity of medium required in liquid culture in the place of 100 ml solid medium brings down cost by five folds. An enhanced retention period in liquid medium (6 weeks) compared to 4 weeks in solid medium brings down cost by 1.5 fold. Apart from these, the extended subculture period in liquid culture also saves the cost in terms of labour.

The introduction of a liquid culture system for *in vitro* mass propagation helps in the substantial reduction of plantlet production costs (Sandal *et al.*, 2001) and is an important step towards automation (Aitken-Christie *et al.*, 1995). Further, a liquid medium offers other advantages in the form of uniform culturing conditions, renewal of the media without changing the container, sterilization by microfiltration, and ease of cleaning containers.

Anubias minima and *Aponogeton ulvaceus* were raised on a semisolid media with full strength MS. There are several reports on the culture of rhizome explants in solid media in *Alstroemeria* (Yousef *et al.*, 2007) and *Dendrobium nanum* (Maridass *et al.*, 2010).

5.1.3. Type of explant

In the present study, the best explant for *in vitro* propagation in *Bacopa caroliniana* and *Rotala rotundifolia* was found to be nodal segments. Tiwari *et*

al. (2001) reports that *in vitro* propagules can be successfully derived from nodal, internodal and leaf explants in *Bacopa monnieri.*

Torregrosa *et al.* (2001) opines that nodal segments are the most widely used explant for micropropagation due to its operational feasibility and genotype stability. The response of nodal explants in inducing multiple shoots may be due to their inherent regenerative capacity and the balance between auxin present in the explant and the cytokinin present in the medium leading to the optimum cytokinin-auxin ratio.

Shoot tips of *Woodfordia fruticosa* were found to be the best in propagating multiple shoots in *in vitro* conditions by Krishna and Seeni (1994). The use of nodes in preference to shoot tips for proliferation of stem is also reported in medicinal plants like *Adhathoda beddomei* (Sudha and Seeni, 1994); *Aegle marmelos* (Ajithkumar and Seeni, 1998) and bitter almond (Kassim *et al.,* 2010). However, inferences in all these studies were based on relatively better morphogenic responses observed with nodal explant cultures compared to shoot tip cultures and not on exclusive regenerative capacity of the former.

Leaf petioles and lateral bud explants were selected for *in vitro* propagation of *Nymphoides cristata* in the present study. The lateral bud explants were found to be effective than petiole explants. Similarly, stolonic nodal explants and leaf petioles were taken as explants in the studies by Jenks *et al.* (2000) for *Nymphoides indica* where stolonic nodal explants propagated faster than petioles.

In *Anubias minima* and *Aponogeton ulvaceus*, rhizome buds were used for *in vitro* propagation. Similar works using rhizome explants were reported in the case of *Alstroemeria* (Yousef *et al.,* 2007) and *Dendrobium nanum* (Maridass *et al.,* 2010).

5.1.4. Standardisation of growth regulators

5.1.4.1. Effect of cytokinins on shoot proliferation

The best growth regulator combination for *in vitro* propagation of *Bacopa caroliniana* is 1.5 mg l^{-1} BA and 0.1 mg l^{-1} Kn. These results agree with the findings of Sharma *et al.* (2010) suggesting that cytokinins are essential for shoot proliferation in *Bacopa* species.

Tiwari *et al.*(2001) investigated the comparative performance of different cytokinins on multiple shoot induction in *Bacopa monnieri* and found that TDZ (6.8 μM) and BA (8.9 μM) were superior to all other treatments. There are also reports of BA exhibiting beneficial effects over other cytokinins (Dantu and Bhojwani, 1987; Lakshmanan *et al.*, 1997).

At higher levels of BA the shoot growth was stunted in the present study. Similar observation was made in *Drosera indica*, where high levels of cytokinin induced red pigmentation and necrosis in the *in vitro* propagules (Jayaram and Prasad, 2007). These findings are in agreement with those in Lotus (*Nelumbo nucifera*) where higher levels of BA concentration decreased the shoot length (Shou *et al.*, 2008).

Callusing was observed in the *Bacopa* culture tubes at higher levels of BA (2.0 mg l^{-1}). Fracaro and Echeverrigaray (2001) observed similar callusing when the hormone levels were increased beyond the optimum.

5.1.4.2. Effect of auxin-cytokinin combination on shoot induction

In *Rotala rotundifolia* a combination of BA (1.5 mg l^{-1}) and IAA (1.0 mg l^{-1}) was effective in inducing multiple shoots. Here also, the stunting of shoots was observed at higher levels of BA (> 2.0 mg l^{-1}). George (1993) has stated that

a combination of cytokinins and auxins stimulate the *in vitro* multiplication and the growth of shoots of several plant species. The results are in agreement with the findings of Kane *et al.* (1991) in *Myriophyllum heterophyllum* and Jenks *et al.*(2000) in *Nymphoides indica* where shoot organogenesis was completely inhibited with cytokinins alone. A combination of BA and NAA was effective in *in vitro* multiplication of Lotus (*Nelumbo nucifera*) where BA alone was effective for shoot formation but shoot length was disappointing which was compensated by the addition of NAA (Shou *et al.,* 2008). This, according to George (1993), is because cytokinins overcome apical dominance, release lateral buds from dormancy, and promote shoot formation rather than increasing the shoot length.

The growth regulator combination effective for *in vitro* propagation of *Nymphoides cristata* was 1.0 mg l^{-1} each of BA and IAA. BA alone at 1.0 mg l^{-1} also induced shoots from lateral buds in *N. cristata* but shoot numbers were less compared to BA-IAA combination. However shoot length was highest with BA at 1.0 mg l^{-1}. The maximum shoot number with BA-IAA combination can be attributed to the synergistic effect between auxin and cytokinins as explained by De Gyves and co-workers (2001) and Faizal *et al.* (2011). Jenks *et al.* (2000) reported that most efficient shoot organogenesis in *N. indica* that occurred on MS medium supplemented with 10µM BA and 20µM IAA. and 10µM BA alone completely inhibited the shoot organogenesis.

The growth regulators tried for *in vitro* propagation of *A. minima* in the present study were BA, Kn and IAA. The results showed that BA is more effective than kinetin in initiating shoots. The beneficial effects of BA over other cytokinins have been reported by several authors (Dantu and Bhojwani, 1987; De Bruyn and Ferreira, 1992; Tiwari *et al.,* 2001 and Rao and Purohit, 2006). However, a combination of BA, Kn and IAA failed to induce shoots in *A. minima* due to the lower levels of BA (3.0 mg l^{-1}) because shoot induction increased at higher levels of BA (>4.0 mg l^{-1}) and was maximum at 6.0 mg l^{-1}

of BA. Auxin (IAA) did not seem to have any effect on shoot induction since, with a constant level of auxin and lower levels of BA and Kn, shoot induction was nil or poor. Similar results were obtained for *Alstroemeria* rhizome explants with a higher concentration of BA and lower concentration of NAA (Yousef *et al.*, 2007). On the contrary, Pierik *et al.* (1997) reported that auxin has no effect on shoot growth in *Alstroemeria* hybrids.

5.1.4.3. Effect of phenolic extracts in shoot induction

In vitro propagation of *Aponogeton ulvaceus* was hindered by the exudation of phenolic extracts into the medium after 3 to 4 days of culturing. There are no reports on the *in vitro* propagation of *Aponogeton* species and data on the phenolic exudations during culture. Survival of explants were reduced by the phenolic excretions. However, phenolic exudates were reported in the case of woody plant species (Lenartowicz and Millikan, 1977; Lloyd and McCown, 1980). Hu and Wang (1983) advocates that providing dark culture conditions reduce the polyphenol interference in crop plants. But Collin (2001) reports that no significant difference in polyphenol exudation could be observed when the cultures were kept in dark. North *et al.* (2012) reports that adding activated charcoal and lowering the growth regulator concentrations in the culture medium reduced the release of phenolic compounds in *Strelitzia reginae*.

5.1.5. *In vitro* rooting

In all the five varieties of aquarium plants experimented, rooting was achieved in the same media. Tiwari *et al.* (2001) reported that basal medium served as the rooting medium for *Bacopa monnieri in vitro* cultivars but addition of IBA significantly improved the rooting at 4.9 µM levels. Similarly in *Ludwigia repens*, rooting was better in MS growth regulator free medium compared to that of medium with TDZ and BA (Ozturk *et al.*, 2004).

5.1.6. Hardening

Hardening of the micropropagated aquarium plants were carried out in FRP tanks with fine river sand overlaid with coir fibres in nethouse. Several authors have reported that aerial culture of *in vitro* propagated plants with mist irrigation is better than submersed culture in tanks. Similar findings by Kane *et al.* (1999) shows that aerial culture of *in vitro* derived plants in plug trays with MetroMix–500 soilless planting medium under shaded greenhouses with intermittent misting eliminates the chances for plant loss due to disease and algal problems frequently encountered in tank cultured plants. Jenks *et al.* (2000) achieved 100% acclimatization of the plantlets in clay pots filled with peat moss and decomposed cow manure mixture in tanks 10 cm below the water surface under full sun conditions. Rooted plantlets of *L. repens* were acclimated with 100% success in aquarium tanks (Ozturk *et al.*, 2004).

5.2. Recirculating Aquaculture systems

Toxic nitrogenous forms must be removed from aquaculture systems since high concentrations of nitrite and non-ionized ammonia can drastically reduce the growth rate, due to damage in gills and other internal organs. These compounds also predispose fish to diseases (Figueroa, 2007). Three potential removal methods for total ammonia nitrogen (TAN) in water reuse systems are generally applied: (1) air–stripping, (2) ion exchange and (3) biofiltration (Franco–Nava *et al.*, 2004). Biological nitrification is the commonest method used to eliminate toxic metabolites in high density semi-closed and closed aquaculture systems. However, oxidation of ammonia to relatively harmless nitrate forms by biological oxidation must be closely monitored in the system (Lucchetti and Gray, 1988). Biofilters used in aquaculture activities are designed to facilitate ammonia oxidation to nitrite and nitrate by nitrifying bacteria.

Physico-chemical parameters of water and their fluctuations play a decisive role in treatment efficiency. Some environmental factors could affect

ammonia and nitrite oxidizers such as substrate, dissolved oxygen concentration, organic matter, temperature, pH, alkalinity, salinity, turbulence level, products inhibition and light intensity (Chen *et al.,* 2006).

5.2.1. Changes in water quality

The results indicate that the three experimental systems behaved almost identically, as demonstrated in figures 1, 2, 3 and Table 25. Throughout the entire study, ammonia, nitrite and nitrate concentrations in the aquaculture tanks remained at low levels suitable for most aquatic life. The pH in the systems fluctuated between 7.7 and 8.2, a range that is considered by some authors as suitable for aquaculture growth (Boyd and Tucker, 1998). This pH range is also commonly cited as optimal for nitrifying biofilters (Hagopian and Riley, 1998). However, many studies have shown that the pH acceptable for aquatic animals varies, depending on type of animal and the salinity of the water, and that the pH is liable to drop rapidly unless a buffering agent is used (Akunna *et al.,* 1993; Skjolstrup *et al.,* 1998; Menasveta *et al.,* 2001; Shnel *et al.,* 2002; Vidal *et al.,* 2002; Singer *et al.,* 2008). This study applied a batchwise approach to solving the pH problem by periodically adding small amounts of lime. Addition of lime for controlling the pH is a common practice in aquaculture ponds or in RAS (Boyd and Tucker, 1998).

The temperature values in the systems were found to be in a range of 23.5 to 24.5 °C (Table 25). Temperature directly affects growth and nitrification rates of nitrifying bacteria. Basically, research on temperature and its effects on nitrification show that nitrification occurs and can be acclimated to conditions that are also favorable to aquatic species. Nitrification rates are slower at lower temperatures and increase linearly through the range of temperatures found in most aquacultural applications (Wortman, 1990).

The concentration of dissolved oxygen in the systems ranged between 4.3 to 7.7 mg l^{-1}. Oxidation of total ammonia nitrogen (TAN) to nitrate utilizes dissolved oxygen and can occur only if oxygen levels are such that development

of anaerobic conditions is prevented (Kruner and Rosenthal, 1987). To prevent dissolved oxygen from becoming a limiting factor, water entering a biofilter should have minimum oxygen levels of 2.0 mg l^{-1} (Espinosa and Stephenson, 1999).

Establishing and maintaining a robust population of nitrifying bacteria that is capable of removing the intended ammonia load is critical to success. Operators of recirculating aquacultural systems must acclimate the nitrifying bacteria population to unique conditions and develop a population that will be sufficient to remove levels of ammonia produced when fish are introduced into the system. The marked cloudiness in the experimental systems during the first few days is due to the start–up time lag of the nitrifying bacteria. Bower and Turner (1981 and 1984) concluded from their studies that seeding filters with filter media from established filters could significantly reduce new system start-up times.

Carmignani and Bennett (1977) reported that ammonia and nitrite levels above 15 to 20 mg l^{-1} can become toxic to nitrifying bacteria. In the experimental biofilters , ammonia levels were initially very high (more than 2 ppm) , probably due to the addition of ammonium chloride before the start of the experiment. However, approximately two weeks after the beginning of operation of nitrification filter, ammonia concentrations had fallen to very low levels (less than 0.1 mg l^{-1}), as shown in figure 1. However, there were variations in the ammonia stabilization period in the three types of filters. In the coir filter, ammonia stabilized in around 7 days, in bamboo filter around 14 days and in vetiver filter around 16 days. But, in the control system, ammonia stabilized around 45 days due to the lack of a substratum for colonization of nitrifying bacteria.

During the period of operation , the concentrations of nitrite–N (NO_2–N) also varied with time (Fig. 2), increasing to a maximum , decreasing gradually to a stable concentration and increasing again when the biofilter collapses. Nitrite

values peaked around 9 to 14 days in the case of coir filter and around 16 to 21 days in the case of bamboo strip and vetiver filter and then it gradually declined to levels below 0.5 ppm. However, in control the nitrite levels showed an increasing trend and peaked around 70 days and did not show a declining trend. Fish mortalities were reported from the first week onwards and continued till the end of the study period since the registered values were exceptionally high compared to those recommended for safe aquaculture. Jaffe (1964) reported that acceptable nitrite level in a recirculating system was around 0.55 mg l^{-1}. A nitrite concentration of 100mg N l^{-1} was clearly lethal to medaka fish (*Oryzias latipes*) when they were exposed to nitrite in both adult and growing phases (Colt and Armstrong, 1981) .

Nitrate reaches high concentrations in recirculating systems where nitrifying biofilters are used for ammonia removal. Maximum nitrate levels differ among recirculating systems and are dictated mainly by water exchange rates and the extent of nitrification and nitrate removal. Contrary to ammonia and nitrite, nitrate is relatively non-toxic to aquatic organisms. Nitrates started appearing in all the three experimental systems from day 2 onwards and peaked around 7 -9 days and were within the safe limits (less than 50 mg l^{-1}). However, in the control systems , nitrates started appearing from 14^{th} day onwards showing the delay in nitrification and peaked around 42^{nd} day. Highest nitrate peak was observed in coir filter showing the better conversion of ammonia followed by bamboo strips and vetiver. The nitrate graph showed a fluctuating trend due to the variations in pH which were corrected periodically by lime application. In general, nitrification is most efficient at pH levels ranging between 7.5 to 9.0. At the higher pH ranges (8.5 - 9.0), nitrification rates were faster when given sufficient ammonia. However, at the low ammonia concentrations usually found in aquacultural systems, operating at a pH of about 7.0 can be efficient. Because pH also effects the relative concentration of ionized and un-ionized ammonia in water and nitrifying bacteria use the ionized form, operating at a pH of about 7.0 usually increases the efficiency of the recirculating aquacultural system (Tseng

and Wu, 2004). Since, the toxicity of ammonia to fish increases with increasing pH, so operating in the lower range also reduces ammonia toxicity.

The efficiency of a biofilter depends on the hydraulic loading rate, surface area, fish biomass, water flow rate through the filter, media type and size (Lekang, 2007). In the present study, preliminary results showed that coir filter was more efficient in nitrification compared to bamboo strips and vetiver as represented in the Fig. 1 2 and 3. However, further studies are needed to ascertain the filter performance based on correlation studies between fish biomass, hydraulic loading, water flow rate and medium size.

5.3. Future line of research

In the present study, the multiplication rate or number of plantlets originating from the mother plant through micropropagation was lowest with *Anubias minima*. *Anubias minima* is one of the expensive plants in aquarium plant trade but a slow grower in vegetative propagation. Hence, further studies are needed to increase its multiplication rate and make way for mass production.

The *in vitro* propagation studies on *Aponogeton ulvaceus* were inhibited by the exudation of phenolic compounds into the medium. Several trials are needed like reducing the photoperiod, addition of antioxidants, activated charcoal, regulating the plant hormones, reducing the concentration of nutrients etc. to overcome the problems with phenolic compounds.

Works are to be undertaken to standardise the micropropagation techniques for high value aquarium plants in trade.

Recirculating systems integrating fish and micropropagated plants are to be standardized for many aspects like, volume of filter media needed for optimum nitrification at different fish loading rates, identifying the nitrifying bacteria in each filter media, estimating the total count of bacteria which develops in each system, start–up period required for the establishment of

biofilter, TAN removal rates and economics of the RAS with indigenous filter material.

6. SUMMARY

The present study was envisaged to standardise *in vitro* propagation techniques for five species of aquarium plants, *viz.*, *Bacopa caroliniana, Anubias minima, Aponogeton ulvaceus, Rotala rotundifolia* and *Nymphoides cristata* with successful hardening and integrating these plants with fish in a recirculatory system. The methodology, results and conclusions of the study are as follows:

In vitro propagation of above mentioned five species of aquarium plants were initiated by the collection of healthy mother plants. Mother plants were reared in cement tanks in net house with pond soil and coir fibres as the bedding substratum. The universally used plant culture medium Murashige and Skoog (MS) medium was used for the establishment of culture. Nodal segments, rhizome buds, leaf petioles and lateral buds were taken as explants for the study. The next step of surface sterilisation of explants were standardised by treatment with various sterilants like $HgCl_2$ (0.1, 0.5 percent), Ethyl alcohol (30, 70 percent), Hypochlorite solution (5.54 percent) for varying durations and the number of surviving cultures were recorded. Each surface sterilisation treatment had 10 replicates. Sterilized explants were inoculated in the media under a laminar airflow chamber.

The most effective surface sterilization treatment for *Anubias minima* was a combination treatment of 70 percent ethyl alcohol wipe followed by 0.5 percent $HgCl_2$ for 2 minutes which gave 100 percent healthy cultures. For *Aponogeton ulvaceus*, a combination treatment of 70 percent ethyl alcohol (wipe), 0.1 percent $HgCl_2$ (3 min) and 30 percent ethyl alcohol (2 min) gave 100 percent healthy cultures. The less sturdy stem plant, *Bacopa caroliniana* was effectively surface sterilised by $HgCl_2$ (0.1 percent) wash for 30 seconds which produced 90 percent healthy cultures. A single step surface sterilization treatment with $HgCl_2$ (0.1 percent for 2 minutes) alone was effective for leaf petiole and lateral bud explants of *Nymphoides cristata* which gave 70 percent healthy cultures with only 10 percent scorching. *Rotala rotundifolia* having the

most fragile stems were treated with a least toxic surface sterilising agent, sodium hypochlorite solution (5.54 percent active chlorine) for 35–40 minutes to get the highest percentage of healthy cultures (90 percent).

Standardisation of medium with optimum levels of growth regulators is the most important step in micropropagation. Studies were conducted to determine the effect of various growth regulators on multiple shoot bud induction from nodal segments. Auxins , IAA and Cytokinins BA and Kn were used for the study. Each treatment had 8 replicates (only 6 replicates in *Anubias minima* due to the difficulties in getting the healthy mother plant). The response of the cultures in each subculture was recorded fortnightly. Survival rate in each subculture, days to initiate bud breakage, number of shoots and shoot length were recorded. The cultures were incubated at 25 ± 2°C in an air-conditioned culture room with 12 hours photoperiod (1000 lux) supplied by cool white fluorescent light. The data for number of buds initiating from the explants were subjected to statistical analysis by ANOVA and the means compared by Tuckey's test.

For *Bacopa caroliniana* the most effective cytokinin concentration level in terms of highest percentage of bud break (72.2 %) and shoot formation (13.875) was 1.5mg l^{-1} BA and 0.1mg l^{-1} Kn. Liquid culture with ½ MS media were found to be efficient in establishing the cultures.

The highest percentage of shoot induction in *Rotala rotundifolia* was observed with 1.5 mg l^{-1} BA and 1.0 mg l^{-1} IAA combination (48.75 nos.). In *Nymphoides cristata,* among two types of explants studied for *in vitro* propagation lateral buds performed better in terms of shoot organogenesis as the shoots generated from leaf petioles were small and less in number. Adventitious shoot formation was highest with hormone concentration of 1.0 mg l^{-1} each of BA and IAA (20.13 nos.) followed by 1.0 mg l^{-1} BA with 16.5 nos. of shoots per explant. Tukeys test did not show any significant difference between the means of these treatments.

In *Anubias minima,* the highest number of shoot buds per explant (4.17 nos.) and shoot length per explant (5.11 ± 0.71 cm) was observed in the higher levels of BA (6.0 mg l^{-1}).

In vitro trials for *Aponogeton ulvaceus* was not successful due to the exudation of phenolic extracts in all the treatments which eventually lead to the death of the tissues. All the growth regulator optimization trials showed significant difference between the means of number of buds initiating from the explant.

The final step of micropropagation is hardening of *in vitro* grown plants in an external environment. *In vitro* cultivars were planted in a bedding substratum of pond soil overlaid with coir fibres in a glass tank. Out of the ten plants selected for hardening the results were promising. Hardening success was 90 percent, 100 percent, 70 percent, 100 percent in the case of *Anubias minima, Nymphoides cristata, Rotala rotundifolia* and *Bacopa caroliniana* respectively.

As a preliminary study, a recirculating system was designed with micropropagated aquarium plants and fish along with indigenous biofilter materials like coir, vetiver and bamboo splits. The study envisaged to compare the three different filter media as mentioned above. Experiment units were rectangular glass tanks of 4x2x2 ft^3 dimensions with four compartments. The first compartment for rearing fishes, second compartment for holding the aquarium plants, third compartment for arranging the filter materials and fourth compartment was the collection sump from where the water was airlifted to the first compartment and so on.The duration of the experiment was 70 days. A control with no filter media was set up for comparison.

Water quality parameters like ammonia, nitrite and nitrate were analysed regularly to plot the nitrification curves. Temperature, pH and dissolved oxygen were recorded once in 2 days. The graphs plotted indicate that ammonia, the most toxic of the nitrogenous compounds showed variations in stabilization period in the three different filter materials. In the coir filter, ammonia stabilized

in around 7 days, in bamboo filter around 14 days and in vetiver filter around 16 days. But, in the control system, ammonia stabilized around 45 days due to the lack of a substratum for colonization of nitrifying bacteria. The results showed that providing a substratum improves the nitrifying rates as compared to a substratum less medium (control).

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INTEGRATED CULTURE OF FISH WITH MICROPROPAGATED PLANTS IN A RECIRCULATORY SYSTEM

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ABSTRACT OF THE THESIS

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ABSTRACT

An experiment was designed to study an integrated recirculatory sytem with micropropagated aquarium plants, fish and indigenous filter materials like coir, vetiver and bamboo as the filter media. The plants selected for the study were *Bacopa caroliniana, Anubias minima, Aponogeton ulvaceus, Rotala rotundifolia* and *Nymphoides cristata.* The first part of the work was to standardise the micropropagation techniques for the above said plants. Murashige and Skoog medium (half and full strength) was used as the basal medium for the establishment of cultures. The explants varied from nodal segments, rhizome buds, leaf petioles and lateral buds.

Surface sterilization was carried out with a range of sterilants like mercuric chloride solution, ethyl alcohol, sodium hypochlorite solution etc. for varying durations and concentrations depending on the type of explant. Effect of growth regulators on explants were studied using auxin IAA and cytokinins, BA and Kn. A liquid culture media with 1.5mg l^{-1} BA and 0.1mg l^{-1} Kn concentrations was the best medium for *Bacopa caroliniana.* A combination of auxin cytokinin like 1.5 mg l^{-1} BA and 1.0 mg l^{-1} IAA in liquid culture was the best medium for *Rotala rotundifolia.* In *Nymphoides cristata,* hormone concentration of 1.0 mg l^{-1} each of BA and IAA in liquid culture gave the best results. In *Anubias minima*, a full MS solid medium with 6.0 mg l^{-1} of BA gave better results compared to lower levels of BA. *In vitro* trials for *Aponogeton ulvaceus* was not successful due to the exudation of phenolic extracts in all the treatments which eventually lead to the death of the tissues. Hardening success was 90 percent, 100 percent, 70 percent, 100 percent in the case of *Anubias minima, Nymphoides cristata, Rotala rotundifolia* and *Bacopa caroliniana* respectively.

The micropropagated plants were incorporated in the recirculatory system along with fish and three different biofilter materials like coir, vetiver and bamboo splits. The study focused mainly on the filtering efficiency of the

three filters based on the nitrification curves. The plants were included in the system to be a part of biofilter and their exclusive role in nitrification was not studied since this is a preliminary work.

A nitification graph was plotted with the observed values of ammonia, nitrite and nitrate in the three different biofilter systems and compared with a control. It was inferred that coir fibres were the best of filter materials tried in nitrification followed by bamboo and vetiver. The control system took more time (45 days) in stabilizing ammonia levels due to lack of a substratum for growing nitrifying bacteria.