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ROLE OF TISSUE CULTURE IN THE STUDY OF AQUATIC PLANTS

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ABSTRACT

There has been a revival of interest in the study of aquatic angiosperms. Escalating population pressure and dwindling resources have urged scientists to harness the untapped potential of aquatic plant wealth. Safe and effective control measures are needed to check the spread of water weeds. The problems and advantages of growing whole plants under axenic conditions have been summarised. The uses to which this technique can be put have been outlined. A brief account of the basic knowledge on growth, regeneration, heterophylly, flowering, pollination, reproduction, genetic variability and taxonomy gained from the tissue culture of aquatic plants has been presented.

INTRODUCTION

Aquatic angiosperms are fascinating objects for study on account of their perplexing forms and adaptive features. Compared to the large number of aquatic flowering plants known to science, only a few have been studied in detail. Inaccessibility of the materials, their fragile nature and cumbersome procedures involved in their collection and preservation are some of the reasons for their neglect.

The situation is further aggravated by the existence of a wide variability in morphology and the inherent difficulty in distinguishing between phenotypic and genotypic variation. Manuals of water plants feature only an inadequate representation of the range of variation in these plants and authentic identification is often difficult. The natural outcome has been an unsatisfactory state of nomenclature and an incomplete understanding of the relationships within several hydrophytic families. Examples are to be found among the habitually submerged tropical Podostemaceae which flower only when the water level recedes at the onset of the dry season and vegetative organs begin to die (Sculthorpe, 1967). Literature abounds in references to classical heterophyllous aquatic plants which bear leaves of varying form in different microhabitats. In such instances individuals of the same species collected in different seasons can be easily misidentified as belonging to two distinct species.

New need to study the biology of aquatic plants

(i) *Weed control and utilization of water plants*

The study of water plants in the past was principally motivated by the urge to satisfy natural curiosity. Whereas the knowledge gathered has thrown some light on the structural, functional and evolutionary aspects of water plants, special needs to investigate them have arisen to-day. Acute demographic pressure, coupled with the continually depleting plant resources on land have recently prompted intensification of studies for effective utilization of fresh water and marine plants. Several water plants have become insidious weeds of global importance on account of their propensity for adventive spread. Aquatic weeds ad-

versely affect many of man's activities by choking rivers and irrigation canals, by hindering navigation and impeding hydroelectric projects, by promoting evapotranspiration and causing drying up of lakes, by increasing water borne diseases and by interfering with rice and fish culture.

Aquatic weeds have always existed but their spread and effects have been magnified by the intensive use of natural water bodies, especially their modification into canals and dams and by polluting them with farm and city waste waters and introducing aggressive plant species into new locations (Anonymous, 1976).

The menace caused by aquatic weeds, notably water hyacinth, is reaching alarming proportions and the efforts being made to keep rivers and lakes free of this single plant have met with little success. It is difficult to wipe out the aquatic weeds completely by herbicides either because of the potential danger to the flora and fauna or because it is impossible to apply them on a large scale to cover every water body. Even a reasonable control is arduous and expensive. It is being increasingly realised that their utilization is the best method for their eradication (Anonymous, 1976). Lately water plants have attracted attention not only as potential sources of food, feed, fibre, paper pulp, green manure and biogas but also as important systems in waste water management and depollution studies (Boyd, 1968, 1970, 1972).

In developing any strategy for control, it is important to have a full knowledge of the biology of aquatic plants, their growth pattern, vegetative propagation, flowering, pollination, seed set, dispersal and germination, to establish the most vulnerable period in their life cycle for eradication.

The basic research needed to harness the potentialities of aquatic plants has been emphasized in a report of the panel set up by the Board on Science and Technology for International Development, under the National Academy of Sciences, Washington, D.C. U.S.A. (Anonymous, 1976). These recommendations are particularly relevant to the integrated rural development programmes in India, as the potential of aquatic plants has only been marginally utilized.

(ii) *Conservation*

Deforestation for extraction of timber, building of roads and bridges for agriculture and other purposes, hydroelectric and irrigation projects have led to the loss of special ecological niches which harbour unique aquatic plants. In his treatise on the biology of aquatic plants, Sculthorpe (1967) has stated "Aquatic habitats cannot be sharply distinguished from terrestrial habitats. In most climates there is a seasonal fluctuation of the water table. At no time there is an abrupt change from land to water but rather a gradual transition from dry through water logged to submerged soils". The reversion of vascular plants to aquatic life has involved colonization of all these transitional habitats as well as the water itself, and some of these marginal sites that are periodically flooded have come to possess their own distinctive plant associations (Noirfalise & Sougnez, 1961, cited in Sculthorpe, 1967). It is these colonizers as well as the inhabitants of similar special niches that face dangers of extinction today due to human encroachment. Such plants require proper identification, study and protection. It would not be out of place to mention the tropical Podostemaceae which are extremely polymorphic and exhibit astonishing reduction and diversity. In their vegetative state, they lack stem and roots and appear like creeping thalli. These plants are unique in their ability to colonize rocky substrates in torrential waters; their morphology at times being greatly influenced by the velocity of the water. To ensure that biologically interesting and specialized aquatic taxa are conserved, both *in situ* and *ex situ* approaches must be taken up.

Advantages of axenic whole plant culture

Tissue culture can serve as an effective tool to study and interpret a wide variety of plant phenomena. Growing whole plants rather than only their parts under precisely controlled conditions provides unlimited opportunities to understand the problems of growth, organization, integration and interaction (Mohan Ram, 1978). Aquatic plants are ideally suited for such studies because of the ease with which they grow in their natural surrounding and their prolific capacity for vegetative propagation. These plants can be used as systems for performing critical analytical work dealing with metabolism and in determining their sensitivity to chemical and biological control. Further, genetic variations which are normally eliminated in a highly competitive natural environment can be preserved under in vitro conditions (Mohan Ram, 1978).

Problems of raising pure cultures of aquatic plants for experimental meterial

Before proceeding to present the achievements and prospects of in vitro studies, it is necessary to point out the nature of difficulties experienced in using aquatic plants as experimental materials.

Raising and successful establishment of aseptic cultures of aquatic plants is a problematic task as these are invariably associated with countless epiphytic or endophytic microorganisms. Conventional methods of sterilization cannot be applied to obtain explants from naturally occurring aquatic plants on account of their fragile nature and sensitivity to chlorine. Elaborate procedures on these lines have, however, been evolved for culturing duckweeds (Hillman, 1961), although the percentage of plants surviving as starting material is low. In our laboratory, surface-sterilized fruits or seeds have proved to be excellent starting material for raising cultures of *Utricularia* (Mohan Ram & Doreswamy, 1966; Mohan Ram & Dutta, 1966), *Vallisneria* (Uma & Mohan Ram, 1972), *Ceratophyllum* (Mohan Ram & Kapoor, 1974) and *Limnophila* (Rao & Mohan Ram, 1981).

Establishment and growth of cultures

Once initial cultures become established, the aquatic plants can be grown on a defined mineral medium containing sucrose. Experiments have shown that without sucrose, growth is rather poor (Hillman, 1959; Doreswamy & Mohan Ram, 1969; Sehgal, 1976; Rao, 1981). This may be traced to the generally low light intensities used in culture rooms which do not support total autotrophy. Vitamins do not appear to be necessary, although in their presence growth is markedly enhanced (Doreswamy & Mohan Ram, 1969; Uma & Mohan Ram, 1972; Sehgal, 1976). Requirement of chelating agents such as EDTA (ethylene-diamine-tetraacetic acid) and EDDHA (ethylene-diamine-di-o-hydroxyphenylacetic acid) has been emphasised in the in vitro culture of several water plants.

Achievements of the technique of whole aquatic plant cultures

Axenic cultures of aquatic plants facilitate not only the understanding of patterns of development under controlled conditions, but also aspects such as rates of growth, regenerative abilities, mechanisms regulating phenomena such as germination, heterophylly, flowering, pollination biology, genetic variability and taxonomic delimitations. A few of the success stories are enumerated below:

(i) *Rates of growth*

Growth can be estimated in quantitative terms either by determination of fresh and dry weights (Doreswamy & Mohan Ram,

1969; Uma & Mohan Ram, 1972; Sehgal, 1976; Rao, 1981) or by calculating the multiplication rate (according to a formula by Clarke, 1925) in the case of duckweeds (Gupta & Maheshwari, 1970; Venkataraman *et al.*, 1970). Doubling time on a fresh weight basis may be as short as 7 days in *Utricularia inflexa* var. *stellaris* (Doreswamy, 1968) and *Ceratophyllum demersum* (Sehgal, 1976) during the log phase. Rate of multiplication of duckweeds is even faster; these form a new frond every 2—4 days (Gupta & Maheshwari, 1970; Venkataraman *et al.*, 1970; Hillman, 1976). Red light, cytokinins, chelating agents such as EDTA and EDDHA and casein hydrolysate are some factors that stimulate frond multiplication in duckweeds (Hillman, 1957, 1961; Gupta & Maheshwari, 1970; Venkataraman *et al.*, 1970). Abscisic acid (ABA) applied even at a concentration of 10^{-8}M has been reported to inhibit frond formation in *Lemna* (Van Overbeek & Mason, 1968) but the fact that growth is renewed on transfer to a medium without ABA shows that it is not an irreversible inhibition.

(ii) *Regeneration*

In contrast to terrestrial systems, hydrophytes exhibit an extraordinarily high propensity for regeneration and vegetative propagation. These are probably essential adaptations to turbulence and other mechanical disturbances which tend to break up the plant body into a myriad fragments (Sculthorpe, 1967). Fragmentation of the plant followed by regeneration from any part bearing a bud is a common feature. This has been substantiated by studies with *Utricularia* and *Ceratophyllum* in which explants of different sizes have been used (Doreswamy & Mohan Ram, 1969; Sehgal, 1976). Recently Rao and Mohan Ram (1981) reported the capacity of excised root tips of *Limnophila indica* to differentiate shoot buds even in a medium devoid of any growth regulators.

(iii) *Heterophylly*

Heterophylly—the presence of two or more distinct types of leaves differing in habit, shape or anatomical organisation on the same individual plant—is a characteristic feature of many aquatic angiosperms. The change from one foliar form to another may be abrupt at the water—air interphase or may be more gradual with intermediate types. The submerged leaves are thin, highly dissected, lack cuticle and stomata but have chlorophyllous epidermal cells. The aerial leaves are broad and thick, with entire margins, well-developed cuticle and mesophyll and numerous stomata. Heterophylly in some plants is a manifestation of heteroblastic development with juvenile leaves preceding adult ones, regardless of the influence of the environment. In others, the leaf rudiment is initially labile and can be modified in various ways, characteristic of the plant that bears it, the form depending on the environment in which the leaf develops (*Callitriche, Marsilea drummondii, Proserpinaca* etc.).

Heterophylly has been attributed to various internal and environmental causes (for details see Sculthorpe, 1967; Hutchinson, 1975 and references cited therein). Factors such as the depth of water, relative humidity, light intensity, photoperiod, temperature and variation in salt concentrations seem to be involved in determining leaf shape (Jones, 1955, 1956; McCallum, 1962; Davis, 1967; Cook, 1969). Allsopp was able to control heteroblastic development in the heterosporous fresh water fern *Marsilea,* by manipulating the sucrose concentration. He considered that heteroblastic development in vitro is regulated by nutritional factors such as sucrose and nitrogen on the shoot apex, a critical minimal nutritional status being necessary for the production of adult leaves (Allsopp, 1965, 1967).

Dissected water leaves can be initiated by submerging aerial stems (Bostrack & Millington, 1962) but it is more difficult to induce

aerial leaves under water. Floating leaf formation on submerged plants of *Potamogeton nodosus* by ABA treatment has been reported by Anderson (1978). Recently Mohan Ram and Rao (1982) have been able to induce aerial leaves and precocious flowering in vitro on submerged shoots of *Limnophila indica* — an amphibious heterophyllous aquatic plant by exogenously applied ABA. Under natural conditions aerial leaves and flowers are produced by *L. indica* only when the water recedes in the temporary pools. When the shoots emerge above water, they are subjected to desiccation. The effects caused by ABA in this plant may be interpreted as responses to induced stress (Mohan Ram & Rao, 1982).

Callitriche heterophylla develops water and aerial leaves, with distinctive morphological characteristics. The two contrasting leaf forms can be experimentally induced by a variety of treatments (Deschamp & Cooke, 1983). Application of 10^{-5}M gibberellic acid (GA_3) caused growth of water leaves on emergent shoots, whereas treatment with ABA (10^{-5}M) or mannitol (0.24 molal) led to the formation of aerial leaves on submerged apices. According to these workers cellular turgor pressure regulates leaf form. The various treatments that influence leaf form, control cell expansion by exerting their effects on turgor pressure or wall extensibility.

(iv) *Flowering*

The organ culture technique has been successfully employed to investigate floral evocation, to study the persistence of the induced state and also to determine the factors that support flower morphogenesis. Aquatic flowering plants, especially duckweed cultures have been used to probe into the mechanism of floral induction.

Kandeler (1955) was the first to report flowering in the cultures of *Lemna gibba*. Hillman (1957) induced flowering in *L. perpusilla* under short days by incorporating EDTA into the medium. It has now been established that EDTA and EDDHA are also essential for flowering in other members of the Lemnaceae — *Wolffia microscopica* (Maheshwari & Chauhan, 1963; Maheshwari & Seth, 1966a), *Lemna paucicostata* (Maheshwari & Gupta, 1967) and *L. gibba* (Pieterse *et al.*, 1970). However, *Wolffia papulifera* does not require EDTA for flowering (Maheshwari & Seth, 1966b).

Among the growth regulators, only cytokinins are able to bring about flowering in the duckweeds under non-inductive conditions — kinetin in *L. perpusilla* (Hillman, 1957) and zeatin in *W. microscopica* (Maheshwari & Venkataraman, 1966). Surprisingly, ABA induces flowering in *L. perpusilla* (Higham & Smith, 1969). Recently there have been reports on the induction of flowering in the fronds of *Lemna paucicostata* and *Spirodela polyrrhiza* SP 20, by inclusion of salicylic acid into the medium (Khurana & Maheshwari, 1978, 1980). Four weeks-old cultures of *Utricularia inflexa* var. *stellaris* when exposed to short-day regimes could be induced to flower in vitro (Mohan Ram & Doreswamy, 1966). However, organic nitrogen supplied as yeast extract inhibited flowering, while beef extract, tryptone, peptone, casamino acids and casein hydrolysate decreased the percentage of cultures flowering (Doreswamy & Mohan Ram, 1971). In *Utricularia inflexa* var. *stellaris*, cytokinins failed to bring about flowering under non-inductive conditions. Under short days, kinetin and benzyladenine enhanced flowering and zeatin depressed it. GA_3 also lowered the percentage of flowering cultures. A commercial sample of ethephon (2-chloroethyl phosphonic acid) induced flowering in all cultures kept under long days at 10^{-8}M. However, a highly purified sample caused induction but not flower development. Application of chlorflurenol, a morphactin caused an increase in the number of inflorescences and retardation of normal

flower development (Mohan Ram *et al.*, 1972).

(v) *Pollination and seed set*

The pollination biology of aquatic angiosperms is a fascinating subject of study. Plants whose flowers are not adapted for hydrophily raise their inflorescences above water allowing air or insects to bring about pollination. There are excellent accounts on the mechanism of pollination in *Vallisneria spiralis* (Wylie, 1917; Kausik, 1939) and *Hydrilla verticillata* (Ernst-Schwarzenbach, 1945).

The study of pollination under in vitro conditions has the advantage that observations can be restricted to a small volume of liquid under controlled conditions. In vitro studies on *Utricularia inflexa* var. *stellaris* by Mohan Ram and Doreswamy (1966) have shown that the flowers undergo self-pollination. The inflorescence is held erect by a circlet of floats. In an open flower the stamens are seen closely appressed to the funnel-shaped stigma. The pollen grains germinate in situ and the germinated grains are deposited en masse by the inward bending of the stamens and the dehiscence of the anthers. A good seed set has been recorded. The in vitro produced seeds can be cultured again and this way several generations of plants can be continuously raised. This material is a good system for scientific studies.

Pollination in vitro has also been investigated in detail in two species of *Ceratophyllum* by Sehgal (1976). The plants are monoecious. The male flowers bear a large number of spirally arranged stamens, each of which has floats at the apex. The presence of a continuous system of lacunae facilitates the accumulation of gases in the floats. At maturity the basal cells of the stamens give way and the stamens rise to the surface of the medium aided by the buoyancy of the floats. Pollen germination has been recorded even in intact stamens. Further stimulation in germination (nearly 100 per cent) occurs after the stamens abscise. After floating on the surface for a day, the anthers dehisce and the ungerminated and the germinated pollen become liberated. These gradually sink and bring about pollination on the way. Such a method of pollination is not confined to the plants cultured in vitro but also occurs in plants growing under natural conditions.

In many hydrophytes, after pollination is accomplished above water, there is a post-fertilization bending of the peduncle so that the development of fruits and seeds takes place under water. In *Vallisneria* pollination occurs at the water surface and after fertilization the peduncle of the female flower becomes spirally coiled and pulls the developing fruit under water (Kausik, 1939). Auxins such as indoleacetic acid and naphthaleneacetic acid have been reported to stimulate the coiling of the peduncle and the parthenocarpic development of the fruit (Funke, 1938, 1939). It would be interesting to analyse the hormonal regulation of pre-pollination elongation and post-fertilization coiling of the peduncle under in vitro conditions.

(vi) *Genetic variability*

Cultures of *Utricularia inflexa* var. *stellaris* raised from shoot tip or stem explants develop into fine thread-like stolons with profuse branches and shorter, sparingly dissected leaves (Mohan Ram *et al.*, 1972). This abnormal condition termed the "bushy clone" can be maintained in subculture. Such plants tend to sink and fail to flower under any photoperiodic treatment (the normal plants flower under short days). These types of plants have never been recorded in nature and it is likely that it is a spontaneous bud mutation.

"Bushy seedlings" had also been observed in the cultures of *Ceratophyllum demersum*

and *C. echinatum* along with normal plants by Sehgal (1976). These were characterised by absence of chlorophyll, slow growth rate, distorted fleshy cotyledons, condensed internodes, short leaves and small white shoot apices. These were produced both from excised embryos and embryos enclosed in fruits collected from plants growing in vivo and in vitro. Surprisingly, some of the bushy seedlings reverted to normal growth by the production of a normal axillary bud or by transformation of the shoot apex. Bushy plants have never been observed in nature. It is quite likely that such weaklings would not be able to survive due to severe competition and in vitro culture affords them protection.

(vii) *Taxonomic studies*

Water plants are taxonomically difficult because there is a lack of adequate herbarium material; a paucity of critical developmental details of various organs; a high degree of adaptability in form, structure and function in relation to the aquatic environment; an apparent simplicity resulting from reduction and a prevalence of convergence and parallelism (Sehgal & Mohan Ram, 1981). The in vitro culture technique offers opportunities to subject systems with taxonomic problems to extensive investigation under controlled conditions.

Sehgal and Mohan Ram (1981) have studied the comparative developmental morphology of two populations of *Ceratophyllum*. Their investigations under natural conditions and in aseptic culture have lead them to assign the two populations to two distinct species *C. demersum* L. and *C. echinatum* Gray. Curiously, the latter is a New World species not previously recorded from India. Their work on the effect of cytokinins on *Ceratophyllum* has brought to light some interesting features. The degree of dichotomy and the length of the segments of the leaf are directly related to the concentration of benzyladenine added to the medium (Mohan Ram & Sehgal, in press). The fact that delimitation of the species within the genus is based on the degree of dichotomy of the leaf (Fassett, 1953) merits a detailed analysis of the problem. *Ceratophyllum,* being a rootless aquatic plant, is an interesting material to investigate the site of synthesis of cytokinins.

FUTURE STUDIES

As can be judged from the above brief account, the in vitro culture technique can be fruitfully employed to probe into the many interesting facets of aquatic plants. It is an effective implement with which research in the future can be meaningfully directed towards solving problems that have a direct impact on man and his environment. Major thrust can be directed to areas devoted to test the utility of water plants in depollution and nutrient recovery from waste waters. Pursuits on their possible medical uses and as systems to test the efficacy of herbicides and insecticides can be fruitful. It will be also rewarding to obtain biological information about the lesser known species of aquatic plants. Cultivation and preservation of endangered plants such as *Aldrovanda,* coupled with the possibility of their reintroduction to the natural habitats from which they have disappeared make the in vitro studies of aquatic plants exciting as well as challenging.

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