SOMATIC EMBRYOGENESIS OF TEA (*Camellia sinensis* (L.) O.KUNTZE) AND THEIR EVALUATION UNDER IN VIVO CONDITIONS

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BY

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DECLARATION

I do hereby declare that the thesis entitled **SOMATIC EMBRYOGENESIS OF TEA (*Camellia sinensis* (L.) O.KUNTZE) AND THEIR EVALUATION UNDER IN VIVO CONDITIONS** submitted to the Bharathiar University, Coimbatore, for the award of the Degree of Doctor of Philosophy in **BOTANY,** is a record of original and independent research work done by me during 1994 to 1998 under the supervision and guidance of **Dr. L. MANIVEL, MSc.(Ag) Ph.D.,** Head, Department of Plant Physiology, UPASI Tea Research Institute, Valparai-642 127. Coimbatore District, and it has not previously formed the basis for the award of any Degree, Diploma, Associateship, Fellowship or other similar title to any candidate of any university.

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CONTENTS

I - *INTRODUCTION*

INTRODUCTION

Tea is considered as world's most popular and cheapest beverage. Tea was first mentioned in a Chinese dictionary published in 350 B.C. Tea drinking became so popular in China at the time of the Tang dynasty that a book entitled "Cha Ching" (Tea classic) was published by Lu Yu (618-907 AD). He discussed the history of tea, methods of preparation as well as the rituals of drinking tea. For several centuries China was the only supplier of this commodity and in the eighth century Buddhist priests brought it to Japan. The Arabs became aware of tea in the 9th century when an Arab merchant, Solimon, visited China and wrote about this wonderful beverage. During the 12th century, Japan began to grow tea plants from seeds obtained from China. Europe came to know the delights of the new drink only in the 16th century. The Dutch lost no time in commencing trade with the Chinese and by 1600 AD the former started growing tea plants in Java (Indonesia). The English also succeeded in opening trade with the Chinese and in 1793 a British delegation was sent to that country. They collected seeds from the tea growing areas

and some of them were planted in the Botanical Gardens at Calcutta. However, these plants did not thrive well to become the first tea producing plants in India. Meanwhile, individual adventures and dedicated officials continued their search for the elusive tea plant in the foot hills of the Himalayas, especially in the north eastern part of India. It was around 1823, the Bruce brothers discovered the tea plants growing wild in Sadiya, in Assam. It appears that the leaves of these `Assam plants' were used by the people in that area as a vegetable and also for making a drink. Tea planting started on a large scale in north eastern India in the first half of the 19th century and during 1823 this plant was introduced into southern India.

The tea plant is indigenous to a vast fan shaped area marked at the north west by Assam, at the north east by the China coast and at the south by southern Cambodia and Vietnam (Hardy, 1979). A recent study showed that the narrow region of Wenshan and Honghe, located at 22° 40'-24° 10' N and 103° 10'-105° 20' E is the centre of origin of the tea plant (Yu, 1986). All the commercially cultivated tea plants belong to the heterogeneous mixture of hybrids of *Camellia sinensis* (L.) O.Kuntze, the 'China jat' or type, *C. assamica* (Masters) Wight, the ' Assam jat' and *C. assamica lasiocalyx* (Planchon ex Watt) Wight, the' Cambod jat', all belonging to the family Camelliaceae and having 2n = 30

chromosomes. It is believed that many wild species of tea have also contributed to the present day hybrid population of cultivated tea plants. *C. sinensis* has relatively small, erect, dark green leaves whereas in *C. assamica* the leaves are supple, light green, flat or semi-erect. The leaves are broadly elliptic, erect and denticulate in *C. assamica lasiocalyx*. Under natural conditions, the tea plant grows to a height of 9 m or more. The tea flowers are either solitary or occur in clusters of two to four; they are 2.5 - 3.2 cm across, pedicellate, white and often mildly scented. The fruit is a capsule with 2-3 locules. The seeds are brown, nearly spherical and measure 1.2 -1.8 cm in diameter.

Tea grows in the tropical to temperate regions with an annual precipitation of about 1150 -8000 mm, air temperature that fluctuates between - 8^{0}C and 35^{0}C and day length of 9.4 -15.0 hours (Ranganathan and Natesan, 1985). The optimum soil pH for growing tea is 4.5 - 5.5 though certain tolerant varieties can be grown at higher pH levels of 6.0 - 6.5. Tea plants are raised either from seeds or by vegetative propagation, the latter being the most widely accepted practice today. In the fields, plants undergo several cultural operations like 'training' the young plant by 'centering', 'pruning' at regular intervals with varying intensities and 'tipping' the primary shoots emerging from pruned frames at appropriate height. The most important cultural operation in tea

is 'plucking' (harvesting) the succulent shoots. In southern India, tea shoots consisting of a terminal bud (active or dormant) and two/ three leaves are plucked at appropriate intervals (seven to ten days) throughout the year. The harvested shoots are processed in the factories to make black tea. Management of pests, diseases and weeds is another important operation in tea cultivation. At present, tea is commercially grown in more than 30 countries, mostly in Asia and Africa. China, with a history of nearly 3000 years of tea cultivation, has more than one million hectares under this crop (Chen and Chen, 1989). But India, with nearly 400,000 ha of tea, is the major producer, consumer and exporter of this commodity in the world. In India, tea is cultivated in north eastern region of Assam, West Bengal, certain pockets of Himachal Pradesh, Uttar Pradesh in Northern India covering an area of 33,946 ha and in the Western Ghats of the three states, Karnataka, Kerala and Tamil Nadu of southern India covering an area of 85,000 ha.

While about two - third of tea produced is consumed in India, only about one - third is exported. The government has stressed that India needs to attain 1000 M.Kg mark on the production front by 2000 A.D. to meet the country's demand and to maintain the present level of export. The current level of output is 750 M.Kg. Out of this, approximately 170 - 180 M.Kg. is contributed by south Indian tea industry (Sarronwala,1996). Since the horizontal

expansion is very much limited, the increase in demand could mainly be met by vertical growth in productivity. In order to achieve this, research work is further geared up in utilising the advantages of biotechnology in tea culture.

Classification

Wight (1962) gave a concise description of the China varieties of tea while proposing their specific ranks.

Camellia sinensis (L.) O.Kuntze or the China tea plant is a big shrub, 1-3 m tall with many virgate stems arising from the base of the plant near the ground, leaf hard, thick and leathery; surface matt, marginal veins indistinct and appear sunken in lamina; blade elliptic with obtuse or broadly obtuse apex; base cuneate, margin bluntly serrulate to sinuate - serrulate with more or less incurved teeth, glabrous above and villose below when young, becoming sparsely villose as the leaf ages, ultimately becoming glabrous. Petiole short, 3 - 7 mm long, stout, usually giving the leaf an erect pose.

Flowers are borne singly or in pairs in the cataphyllary axils. Pedicels 6-10 mm long, clavate, glabrous with 2-3 sub - opposite scars little below the middle, marking the position of cauducous bractioles 2-5 mm long. Sepals 5-6, imbricate, persistent, leathery, ovate or orbicular, 3-6 mm long, glabrous, green. Petals 7-8, shallowly cup - shaped, 1.5 - 2 cm long, broad - oval to sub

- orbicular, generally white sometimes with pale pink pigmentation. Stamen numerous, arranged in two whorls, inner ones shorter and fewer in number, outer longer and numerous numbers, 8-13 mm long, united at the base for a few mm with the corolla lobes. Ovary white, densely hairy, 3 locular, ovules 3-5 in each loculus, placentation axile. Style generally 3, sometimes upto 5, free for the greater part of their length. occasionally free upto the base of the ovary. Stigma apical. Capsule 1,2 or 3, coccate, containing 1 to 3 nearly spherical seeds, 10 - 15 mm in diameter (Wight 1962).

Time of flowering

Geographical location determines the time of flowering of the tea plants. The tea trees in South India begin flowering towards the end of December and continue to flower up to the end of March, with the peak flowering in February, though, odd flowers do appear on the trees, particularly, on 'China type' (C.sinensis (L). O. Kuntze) throughout the year (Sharma *et al.*, 1981). Flower bud initiation depends on phasic growth of the tea plant which takes 120 -150 days from initial appearance of flower buds to their anthesis. Maximum fertilisation occurs during the peak flowering period. Although the peak flowering time does not usually extend beyond December, there is considerable

variation between different seed trees of each race of tea in flowering time and occurrence of the peak period.

Pollination

Pollen grains of the three races of tea are morphologically similar. The grains of diploid plants are triangular, tricarporate and binucleate. Wellensiek (1938) and Visser *et al.*, (1958) observed that tea pollen germinated well in distilled water. Sugar solution of 10 per cent strength was found to be the best; at 25 percent the growth of the pollen tube was retarded (Bezbaruah, 1971). At 12- 25°C ambient temperature, all freshly collected pollen grains started germinating within two hours and germination was complete within eight hours. The germination rate was slow at lower temperature.

Fertilisation and Fruitset

Bezbaruah (1975) reported that no fixed time was observed in North East India for the opening of tea flowers, however, the flowers in all the clones were observed to open only in the morning, commencing from 6.00 h; the flowers continued to open up to 11.30 h there was a sharp decline in the number of flowers that opened afterwards.

The stigma becomes receptive of pollen about 24 hours before anthesis and remains so until the corolla shows signs of

withering. According to Salisbury and Ross (1977), the opening of flowers is influenced to some extent by temperature and atmospheric vapour pressure, but the primary factor controlling this phenomenon is the signal relayed by dawn or dusk. As per this explanation, dusk signal appears to be in operation for tea flowers to open in the morning in South India. The time taken for full expansion of the flowers varied not only between clones but also between the flowers in the same clone and the tree (Sharma, 1981). Some flowers drop off the trees, two - three days after detachment of the corolla lobes while in others the persistent calyx lobes close in over the ovary, leaving the style exposed. The style and the stigma gradually wither off.

Syngamy and triple fusion are reported to occur simultaneously after fertilisation. The endosperm remains nuclear in the beginning which changes to cellular afterwards (Wu, 1960; Balasethi, 1965). Development of the ovary during the first three - four months after fertilisation is very slow. Under conditions of South India, the external signs of development of the fruit become evident by about March - April when the tree starts growth of the new season. After the initial phase, the rate of development of the fruit becomes rapid and by the end of May it reaches almost one half of the final size. The fruit attains its full size by August. The fruit usually contains one round seed, sometimes two round or half seeds and occasionally, three or more seeds (Barua, 1989).

In the mature seed, two large cotyledons are enclosed by a hard, brown testa formed by the outer integument. The pericarp encloses one to three and rarely more seeds and is made of thick parenchymatous tissue when young, becoming sclerotic on maturity (Bezbaruah, 1971). The ripening of the fruit continues till the next flowering season in North eastern India. Dehiscence starts in October and continues till late November. Thus, from the time of flowering till dehiscence of the mature fruits, it takes about a year and this happens in all tea growing regions of the world. However, the timing of the various phases of development of the embryo varies from place to place due to difference in the time of flowering (Barua, 1989).

Abscission of fruits

In tea, as in most other fruit crops, fruits continue to drop off the trees from the time of blossoming till dehiscence of the mature fruits. Abscission of tea fruits occurs in perceptible waves at different stages of development. This happens everywhere but more prominently in seasonal climate. Examination of the premature dropped fruits during the second and third phases showed normal development of the pericarp and testa, often with mucilaginous endosperm inside. Bezbaruah (1971) was of the view that the abscised fruits were parthenocarpic and that

abscission was caused by lack of pollination leading to failure of syngamy or triple fusion. (Barua, 1989).

Biotechnology is defined as "any technique that uses living organisms or substances from these organisms to make or modify a product, to improve plants or to develop microorganisms for specific uses" (Rao *et al.*, 1996). Traditional biotechnology covers well established and widely used technologies based on commercial use of living organisms. These include biotechnologies currently used in brewing, food fermentation, etc. Modern biotechnology encompasses the uses of more recently developed technologies:

1. Cell and tissue culture
2. Recombinant DNA technology and
3. Monoclonal antibodies.

Plant cell and tissue culture techniques coupled with recombinant DNA technologies have played a vital role in the genetic improvement of crops by providing materials such as haploids, genetic variants, wide-cross hybrids, disease-free stocks and large numbers of plants as well by transfer of genetic material from alien germplasm through protoplast fusion and genetic engineering for the creation of new plant types (Rao *et al.*, 1996).

The idea that isolated plant cells had the ability to divide and grow into complete plants, was conceived early in this century

by Haberlandt (1902) and later detailed experiments by White, Gautheret and Nobecourt in 1930s laid the foundation that led to the defined conditions for cultivation of plant cells and regeneration of plants from them.

Biotechnology is emerging as the innovative tool in life sciences with several benefits in agriculture, medicine and industry. For the developing countries, biotechnology is surely an alternative, if appropriate technologies are employed for specific and need-based problems. Globally, some economically important crops have attracted much attention in terms of research and product development, both by government and private sector (Rao *et al.*, 1996).

However, there is a need to boost the improvement of germplasm of under-researched crop species. Biotechnology is sure to play a prominent role in the development of sustainable agriculture and in conjunction with conventional breeding techniques, biotechnological tools can be supplementive in offering rapid solutions for many agricultural problems. For the industry, viable production and processing systems can be developed which are cost effective and have global relevance (Rao *et al., 1996).*

Drought is caused by the combination of soil moisture stress and high ambient temperature aggravated by dry wind. Ameliorative steps commence from the planting stage, extended

upto productivity and continued. The steps encompass short term soft options to long term cost intensive permanent ones. Tea plantations lose almost 30 per cent of the crop due to the impact of drought. It is a national waste to lose this much of crop. If ameliorative steps were taken in the existing plantations, the health of the bushes and the productivity could be improved which will additionally benefit the planters and exchequer.

Plants absorb water from the soil through the feeder roots along with the nutrients which are translocated to the growing shoots and leaves, mediated by the transpiration pull. The pressure gradient or water deficit in the leaves and shoots caused by transpiration, sustains the absorption and flow of water. Transpiration thus assists the plants in cooling, absorption of water and nutrients. Intensity of stomata and their opening in leaves regulate the transpiration and gas exchange. Transpiration sets in motion series of metabolic functions that affect the growth.

As a first step to cope up with the deficit of soil moisture, partial closure of stomata takes place to build the required turgidity. Number of growing points and the growth rate are reduced for conserving energy. Photosynthates are diverted to produce proline and related amines to build up the osmoticum to avoid desiccation and wilting. If the water stress is intense and prolonged, the leaves switch over from the synthesis of gibberellic acid (a growth promoter) to abscisic acid (an inhibitor) to retard

the growth, forcing the buds to become dormant (banji). Further stress results in wilting of tender shoots and abscission of mature leaves (Manivel and Abdul Kareem,1996).

Due to the restricted gas exchange, the photosynthetic rate is subdued to the level of subsistence while the rate of respiration is accelerated due to triggered metabolism to sustain the increased demand of energy. Starch in storage organs - root, stem, bark etc. are reduced to simple sugars to cope up with demand due to surge in respiration. A crucial stage is reached when the root reserve is utilised to meet the increased demand for metabolites. Thus, the reserve in roots which is the sign of health of bush is depleted, weakening the bushes, predisposing to pest and diseases. Prolonged drought is a critical phase leading to dieback and death of the bushes causing vacancies. A balanced shoot : root ratio enables the plant to minimise the impact of the moisture stress (Manivel and Abdulkareem, 1996).

Almost all facets of plant growth are affected by drought. The important physiological and biochemical processes affected are clearly understood in recent years. From the agronomic point of view, crop establishment, development of the crop canopy, establishment of the sink size, pollination, fertilization and survival of the sink and even the partitioning of carbohydrates are affected by stress.

Plants adapt through several mechanisms to sustain growth under stress for better recovery and growth for alleviation. The different adaptive mechanisms either favour survival under stress or help in sustaining productivity under stress. It is more important for a drought resistant crop to have characteristics associated with maximum productivity under drought situation rather than possessing mechanisms which ensure mere survival. Some attempts have been made in recent times to classify some of the adaptive mechanisms based on their effect on productivity.

Major emphasis still is to identify drought resistant genotypes suitable for a given situation. A number of resistant mechanisms have been identified and correlation of these traits with drought resistance suggests the importance of these traits in crop improvement for drought situations. However, many of the mechanisms are still not amenable for large scale screening and hence, empirical screening technique by field evaluation under water deficit conditions is still widely adopted. Though empirical assessment is still an useful technique, it will be difficult to pinpoint the adaptive mechanisms associated with resistance. Hence, it is important to identify physiological and biochemical mechanisms and their genetic control for better understanding. Reliable techniques have been developed to measure a few parameters directly or indirectly which have relevance to stress tolerance and productivity.

During the last two decades, considerable amount of information has accumulated to establish the embryogenic potential of somatic plant cells. Virtually every plant organ has been shown to form embryoids. According to Kohlenbach (1977), somatic embryogenesis has been reported in 80 species belonging to 33 different families. Reviewing the book, "Recent Advances in the Embryology of Angiosperms", edited by Maheshwari (1950); Kohlenbach, remarked "The phenomenon of embryogenesis is not confined to the reproductive cycle. It can be inferred from the culture of single cells of higher plants in which irreversible differentiation has not proceeded too far, if placed in appropriate medium, develop embryo-like way and produce a complete plant. The whole complex sexual apparatus is not, therefore, an essential prerequisite for the removal of the effects of ageing and the re-establishing of embryonic properties. The events occurring in the ovule after fertilization thus provide only a special case of embryogeny".

Kohlenbach (1977) has proposed the following classification of embryos.

1. Zygotic embryos - those formed by fertilized egg, or the zygote.
2. Non-zygotic embryos - those formed by cells other than the zygote.

(i) Somatic embryos - those formed by the sporophytic cells (except zygote),

(a) Adventive embryos - somatic embryos arising directly from other embryos or organs, e.g., stem embryos in carrot (*Daucus carota L.*).

(ii) Parthenogenetic embryos - those formed by the unfertilized egg.

(iii) Androgenetic embryos - those formed by the male gametophyte (microspores, pollen grains).

It is a process by which somatic cells develop into embryos which ultimately give rise to whole plants. This phenomenon has been reported in more than 150 species (Thorpe, 1994). Somatic embryogenesis can be induced from a variety of plant tissues such as germinating seedlings, shoot meristems, young inflorescences, nucellus, leaf, anther, immature embryos, floral parts, root etc. Somatic embryos proceed through similar developmental stages like that of seed embryos, acquire root and shoot meristem connected by a common vascular system and develop into a plant (Rao *et al.*, 1996).

Among the growth regulators, auxin play a crucial role. The process of somatic embryogenesis normally takes place in two stages: first, the induction of cells with embryogenic competence referred to as proembryonic cell massed or clumps, proembryos etc., in the presence of high auxin and second, the development

of embryogenic masses or proembryos into embryos in the absence or lower concentration of auxin (Thorpe, 1994).

In the past few years, plant regeneration via somatic embryogenesis has been reported for an increasing number of dicotyledonous plant species. The development of some horticulturally desirable new hybrids particularly from many crosses in *C. sinensis* is often hampered by poor fertility and low viability of hybrid embryos. Micropropagation by somatic embryogenesis from immature zygotic embryos before abortion provides an embryo rescue technique that circumvents the stated shortcoming and also provides a method of plant multiplication (Wachira and Ogada, 1995).

Drought is a recurring phenomenon in tea plantations causing extensive damage to the plants. Irrigation though suggested as a remedial measure, it could not be adopted due to limitations. Plant growth regulators and antitranspirants are advocated as ameliorants against drought to an extent. Evolving drought tolerant tea cultivars through breeding is the ultimate solution. The following techniques could be used for the purposes of evolving drought tolerant cultivars :

a. Back crossing
b. Clonal selection
c. Gene transformation

d. Mutation breeding and

e. Plant improvement through somaclonal variation.

Plant improvement through somaclonal variation can be adopted to evolve a drought tolerant cultivar or to improve upon existing tea clones. Though stress tolerance is a multigenic factor, changes in a single allele can still increase tolerance. Variations in tolerance occur within a plant species and they can also be induced through mutations. Desirable mutations occur infrequently in field conditions while the increased mutation rate from tissue culture is extremely useful in selecting tolerant ones.

Induction of somatic embryoids and development of protocol for regeneration of plants for possible selection of drought tolerant lines have been chosen as the primary objective of the this thesis using a moderate drought tolerant UPASI clone UPASI- 10,a china cultivar. Plant regeneration through somatic embryogenesis has several advantages over the other routes of *in vitro* plant production and appears most promising for future large scale rapid plant propagation. The totipotency of cells finds best expression in the formation of somatic embryos from single cells and their growth and development to form a complete plant. Somatic embryogenesis has great implications in tissue culture technology. As somatic embryos have preformed root and shoot meristems, regeneration of plantlets becomes easier. If the single cell orgin of somatic embryos is universal, then potentially all

single cells in cell suspensions could be induced to form embryoids in a prodigious way (Mascarenhas,1993).

Many researchers in the area of hardwood biotechnology regard somatic embryogenesis as the system of choice for mass propagation of superior and genetically engineered forest tree genotypes (Gupta *et al.*, 1991). For both coniferous and hardwood species, somatic embryogenesis has a number of advantages over other micropropagation techniques. The advantages include very high multiplication rates, and the potentials for scale - up in liquid culture (i.e., bioreactors) and for direct delivery to the greenhouse or field as artificial seeds (Merkle *et al.*, 1990). Such features make it likely that clonal propagules produced via somatic embryogenesis will have significantly lower per unit costs than those produced using other micropropgation systems (Merkle, 1995).

Bud regeneration through undifferentiated cell callus has often given rise to abnormal organogenesis resulting from cellular mitotic anomalies, largely due to aneuploidization and polyploidization (Kasperbauer and Collins, 1972). The observed changes in chromosome numbers of cultured cells of *Daucus carota L.* (Bayliss, 1977) and *Crepis capillaris L.* (Sacristan, 1971), have for example furnished additional documentation of the occurrence of mitotic irregularities. Such *in vitro* anomalies have widely been used in isolation and selection of mutant plants.

Cold tolerant and salt tolerance cells have also been selected using the callus culture techniques. Despite the wide applicability of callus culture techniques in plant improvement and its wide success in the other woody plants, not much has been done in tea.

Major limitations to hardwood tree somatic embryogenesis

Two major limitations to the application of somatic embryogenesis for propagation and genetic manipulation of hardwood are noteworthy. The first of these is low multiplication rates - that is, the least number of field - plantable clonal plantlets produced per embryogenic culture. Of the 81 species for which somatic embryogenesis had been reported, only 34 had produced plantlets capable of growing in soil. Thus, not only the somatic embryo derived plantlet production to be either low or unquantified, but in over half of the cases plantlets are not regenerated at all. (Merkle. 1995).

The second major limitation to application of somatic embryogenesis to hardwood species is the inability to initiate embryogenic cultures from mature trees. Most of the embryogenic systems reported for hardwood trees to date have relied on tissues from seeds or seedlings for explanting material. Thus the material being propagated is of unproven genetic value and juvenile in nature. Most reports of somatic embryogenesis in

woody perennials describe direct embryogenesis followed by repetitive embryogenesis or "embryo cloning", in which the zygotic embryo is induced to replicate itself indefinitely. This type of embryogenesis has been described as coming from "pre-embryogenic determined cells" (Sharp *et al.*, 1980), and represents a relatively straightforward process whereby the explanted cells, which are already close to the embryonic state, are simply induced to maintain a program of repetitive embryo production (Merkle. 1995).

Conventional methods of breeding are long - term and laborious, and seed is therefore very expensive. As a result, such seed is not used to provide seedlings, but rather is grown to maturity in seed production nurseries. Plants from these seeds will vary in performance and quality. Somatic embryogenesis offers a method for propagating large numbers of uniform plants in less time and possibly at less cost than from tree breeding programs. Moreover, the method can also be applied in a breeding program to propagate trees which have been selected for superior performance and with desirable traits. Somatic embryogenesis removes the uncertainty of breeding. (Stephen *et al.*, 1995).

Considering the recurring drought in tea plantation and the crop loss incurred, evolution of a drought tolerant clone is important. Generation of embryoids and their evaluation for drought tolerance was an attempt in this direction. The techniques adopted and the results obtained are discussed.

1.1. REVIEW OF LITERATURE

1.1.1. WOODY PERENNIALS

Early observations of *in vitro* somatic embryogenesis were made in *Daucus carota* (Reinert, 1958, 1959; Steward *et al.*, 1958). Since then this species has been widely used to investigate various aspects of *in vitro* somatic embryogenesis (Steward *et al.*, 1970; Jones, 1974; McWilliam *et al.*, 1974; Smith and Street, 1974). Other woody plants in which this phenomenon has been studied are *Ranunculus sceleratus* (Konar and Nataraja, 1969; Konar *et al.*, 1972;Thomas *et al.*, 1972), *Macleaya cordata* (Kohlenbach, 1977), *Citrus sp.* (Rangaswamy, 1963; Sabharwal, 1963; Rangan *et al.*, 1968; Kochba and Spieger-Roy, 1977; Tisserat and Murashige, 1977).

In *Ranunculus sceleratus* various floral parts as well as somatic tissues proliferate to form callus on a medium containing coconut milk (10%) with or without IAA. Within three weeks, numerous embryoids appear on the callus. The embryoids originate from the peripheral as well as deep-seated cells of the callus (Konar and Nataraja, 1969). In the embryological texts (Maheshwari, 1950; Bhojwani and Bhatnagar, 1978), *Citrus* is commonly cited as an example of natural polyembryony . In several species of this genus the nucellar tissue forms 1- 40 adventive embryos

per seed (Furusato *et al.*, 1957) of which many attain maturity and form plantlets following seed germination.

The embryogenic potential of *Citrus* nucellar cells is also maintained in cultures. The embryos may arise directly from the nucellar cells (Rangan *et al.*, 1968) or from the callus derived from them. In the latter case embryogenesis occurs in repeated subcultures (Kochba and Spieger-Roy, 1977). Regeneration of plants through the embryogenic pathway is of special significance in mutagenic studies. Unlike shoots, somatic embryos arise from single cells and, therefore, the new strains of plants obtained through adventive embryogenesis in tissue cultures would be solid mutants (Bhojwani and Razdan, 1983).

Citrus trees propagated from nucellar embryos are free of virus as are the plants raised from zygotic embryos. The progeny from nucellar embryos is also a clone. Nucellar embryogeny is the only practical approach to raising virus-free clones of polyembryonate *Citrus* varieties in nature because shoot tip grafting developed by Navarro *et al.*, (1975) is very tedious. A few commercially important clones of Citrus are either monoembryonate or seedless, there is no *in vivo* method for raising virus-free clones. However, it can be achieved by culturing their nucelli and inducing somatic embryogenesis artificially (Button, 1977).

Somaclonal variation is a variation among regenerated plants that occurs as a result of tissue culture of any type. It may arise from preexisting or induced variation. Somaclonal variation will be heritable through a sexual cycle. It is not always possible or feasible to demonstrate heritability because of complex sexual incompatibilites, seedlessness, polyploidy, or long generation times. Hence, it may be difficult or impossible to verify the heritable nature of somaclonal variation (Skirvin *et al.*, 1994).

Such variation is a serious problem confronting researchers or propagators who require uniformity. However, the natural variability associated with tissue culture represents a pool upon which selection pressure can be imposed to isolate unique forms of clone. The amount of variation that can be expected will vary with the clone, age of the culture, use of mutagenic agents, and selection pressure applied to cell suspensions or callus cultures of clones for stress conditions such as salt concentration, herbicides, microorganisms or their by-products, and specific metabolites (Skirvin, 1978). The exploitation of such natural and induced variation seems especially applicable to old woody plant cultivars, such as 'Bartlett' pear (*Pyrus communis L.*) and 'Delicious' apple (*Malus domestica Borkh.*) (Skirvin, 1977).

Somaclonal variation appears to result from both preexisting genetic variation within the explants and variation induced during

the tissue culture phase (Evans *et al.*, 1984). There appears to be two types of somaclonal variation:

Heritable and epigenetic

Heritable variation is stable through the sexual cycle or repeated asexual propagation; epigenetic variation may be unstable even when asexually propagated. Somaclonal variation may involve either single or multiple genes and can be due to alterations in DNA bases, genes, chromosomes, or entire sets of chromosomes (Orton, 1984).

Explants from mature parents adapt to the *in vitro* environment by becoming more juvenile in a step-wise manner. Tissue cultures may be at any stage of development, from mature to fully juvenile. Plants regenerated from such tissues will vary, depending on the developmental stage that the tissue had attained when the stimulus to regenerate was received. Shoot regeneration from dedifferentiated cultures can yield and immature form of the parental clone that may or may not be stable over time. For instance, *Eucalyptus* cultures could yield regenerants with sessile leaves, a juvenile trait. However, with time, such juvenile traits cease to be expressed and the parental type reappears (Skirvin *et al.*, 1994).

The causes of somaclonal variation are not well understood and have not been elucidated. However, among the heritable

types of variation, single base-pair changes, chromosome deletions, translocations, and changes in ploidy have been encountered (D'Amato, 1991; Evans and Sharp,1986). Variation is not limited to nuclear genes, Gengenbach and Umbeck (1982) have used restriction enzyme analysis of isolated mitochondrial DNA to demonstrate variation in mitochondrially controlled male sterility. The broad spectrum of variation available through both nuclear and cytoplasmic characters suggests that all classes of variation could be recovered and used for crop improvement.

Tissue culture-induced variation

Callus is so often associated with somaclonal variation that some callus-derived variants have been called "calliclones" to denote their callus orgin (Skirvin and Janick, 1976). However, variation is not limited to callus regenerants. Evans (1988) reports considerable variation for morphological traits (flower shape, leaf shape, plant height), pollen viability, and chromosome number among adventitious *Nicotiana alata* Link & Otto, plants regenerated directly from leaf explants without a callus intermediate. Variation also occurs among plants regenerated via embryogenesis.

Growth regulators

Growth regulators such as 2,4-D and 6 - benzylaminopurine (BAP) have been implicated in the induction of variability

(Evans, 1988; Griesbach *et al.*,1988; Shoemaker *et al.*,1991), but their direct relationship to this phenomenon is still debated. Although the effect of specific growth regulators on variation remains unclear, most tissue culturists agree that variation rates are increased as the overall concentrations of growth regulators rise. High growth regulator concentrations also can alter the frequency of ploidy changes vs. point mutations. The excising process itself is probably enough to stimulate wound responses that growth regulators enhance (Mc Clintock, 1984).

Cultivar

The amount of variation encountered *in vitro* is not the same for all cultivars of a species (Kurtz and Lineberger, 1983). Some cultivars show excessive variation and others are relatively stable.

Cultivar age

Although older asexually propagated clones would be expected to show more preexisting variation than newer clones, there seems to be little correlation between clonal age and the amount of variation observed in a clone. Shepard *et al.*, (1980) were among the first to report somaclonal variation.

Explant source

When exploring a new species or cultivar for somaclonal variation, it is a good idea to use several different explants for comparison. Not all explants should be assumed to exhibit variation equally. In general, variation is less likely to be observed from preformed shoots (axillary buds, shoot tips, and meristems) than from explants that have no preformed shoot meristems, such as leaves, roots (Shepard *et al.*, 1980).

Length of time

The length of time that a culture has been maintained *in vitro* is among the most important factors involved in inducing somaclonal variation. To maintain clonal stability, samples are drawn regularly *ex vitro* in a field or greenhouse to make sure that the characteristics of the particular cultivar are maintained. In addition, limitation on the number of subcultures that can be made from an explant is also restricted. For instance, after one year all cultures established from a particular explant will be harvested. The clone will then be replaced with another explant taken from the original mother plant. To ensure continuity, new cultures are established regularly and older ones eliminated on schedule (Reed, 1990; Reed and Lagerstedt, 1987; Sherman, 1987).

Selection pressure

In vitro selection pressure has been used to select cell lines with resistance to disease, herbicides, particular compounds, drought, etc. Bringham and Mc Coy (1986) reported the selection of alfalfa cell lines with resistance or tolerance to ethionine, salt, and a fungal culture filtrate. However, among plants regenerated from these cultures, only the disease-resistant character was expressed at the whole-plant level. Widholm (1988) has reviewed the use of tissue culture to select cell lines of value to researchers and industry. He states that direct selection for single gene traits is more likely to be successful than for those with multigenic control.

For selection pressure schemes to be of long-term value for plant improvement, the trait selected at the cellular level must be expressed at the whole-plant level. However, it is not safe to assume that all genes that are expressed at the cellular level will be expressed in the same manner at the whole-plant level. For instance, Scowcroft and Larkin, (1988) reported that a cell line of tobacco that produced exceptional quantities of nicotine, but regenerated plants produced only normal levels. However, high nicotine production levels were re-established in callus cultures of the regenerated plants. This difference indicated that the high-nicotine gene was present, but that it was only expressed at the cellular level. In contrast, for pharmaceutical products

produced by cells, it may be less expensive to harvest the product directly from the cells and avoid the greater expense of maintaining whole plants.

Hammerschlag (1990) and Hammerschlag *et al.*, (1991) have isolated peach (*Prunus persica* (*L.*) Batsch.) clones with putative resistance to *Xanthomonas campestris* pv. pruni. Some of these clones have continued to exhibit disease resistance for 2 years, while in others, the trait has proved transient. Seeds are now being collected from these plants to study the heritability of the trait among somaclones (Hammerschlag *et al.*, 1991).

Widholm (1988), for instance, has suggested that tissue culture conditions are so unnatural for plant cells that only the most rapidly growing cells survive due to repeated subculturing. Furthermore, he suggested that cell and tissue cultures do not grow equally well in all laboratories because they are unintentionally selected under the specific media and environmental conditions of a particular laboratory.

Cultural conditions

Genomic irregularities in plant cell cultures occur most often when cells are removed from the stabilizing and controlling influences exerted by an intact organism and placed into an unnatural environment within a culture vessel. When a plant is

excised and wounded in preparation for transfer to tissue culture, the control systems that regulate a whole plant are interrupted. For instance, in a whole plant, callus is a wound response that rarely results in sufficient organization to produce shoots or roots. However, in tissue culture, callus is a common phase through which excised plant parts commonly pass before organizing into a meristem that can yield roots, shoots, or both (Skirvin *et al.*, 1994).

Although rates of somaclonal variation seem high, random phenomenon that can occur at any location in the genome. These random changes are pooled to arrive at the total somaclonal variation rates. The true rate of somaclonal variation is difficult to ascertain because there are so many individual genes to examine.

More realistic level of somaclonal variation expected *in vitro* is probably about 1% to 3% of their regenerants to mutate at a particular locus, but it does mean that 1% to 3% of the regenerants will vary from their parent in some physical or biochemical manner. Although this rate of variation sounds less useful than the 25% to 100% levels that all of this variation occurs within a clone. Selection pressure can result in isolating unique forms of the parental clone. Conventional breeding procedures involving backcrossing can also yield improved forms of a clone, but the process can be time consuming, unsuccessful, and only work for

plants that are self-compatible. Many important fruit crops, such as apple and pear, are either self-incompatible or exhibit severe inbreeding depression (Skirvin *et al.*, 1994).

Protocol has been developed for the *in vitro* propagation of Mexican weeping Bamboo through somatic embryogenesis from zygotic embryo explants (Woods *et al.*, 1992). Mature seeds and excised embryos were cultured in the light or in the dark on both MS and Gamborg's B5 basal media with various supplements. Optimal somatic embryogenesis and plant regeneration were obtained by culture in the dark on MS basal medium supplemented with 3 mg/l 2,4-D and 0.5 mg/l BAP and 2.0% sucrose. More than 95% of the germinating somatic embryos developed shoots and roots, and were transferred to soil with 85% success (Woods *et al.*, 1992).

In apple, nucellar tissue was isolated from developing seeds of Golden Delicious, separated into micropylar and chalazal halves, and cultured on growth regulator free MS medium in the dark (Eichholtz *et al.*, 1979). Several of the micropylar halves of the nucellus produced a few adventive embryoids. When the embryoids were recultured in the dark, additional embryo like structures formed on the cotyledons (Zimmerman, 1984). Somatic tissue of embryo origin represents a good source for adventitious regeneration to study somaclonal variation, mutation breeding, gene transfer etc. (Kolova and Stoyanov, 1993).

Seed sections (cotyledon and hypocotyl segments) of *in vitro* stratified immature zygotic embryos of apple *Malus pumila* Mill. were used for embryoid induction. The effects of type and concentration of mineral, organic and growth regulator components of culture media and its pH value on regenerative capacity of apple embryonic tissues were studied. Upto 48% cotyledon sections produced adventitious somatic embryoids supplemented with 2 mg BAP, 0.5 mg NAA and 0.1 mg gibberellic acid/l. Somatic embryoids developed into normal plants only after being stored for 5 to 7 weeks at 4°C (Kolova and Stoyanov, 1993).

Callus cultures of *Encephalartos cycadifolius,* a small cycad endemic to south Africa, were established from zygotic embryo explants on a modified B5 medium containing 1 mg 2,4-D and 1 mg kinetin/l (Jager and Van, 1996). Dicotyledonary embryoids formed at the distal end of the suspensor. The embryoids turned green in light. When transferred to a medium containing 1 mg abscisic acid/l, the somatic embryoids matured. The suspensors desiccated and the embryoids rooted when transferred to a medium without growth regulators (Jager and Van, 1996).

Immature zygotic embryos of *Ginkgo biloba L.* were taken, at various developmental stages from ovules. The highest frequency of embryogenic tissues (90 - 95%) and the highest number of somatic embryoids per explant (9.6) were obtained with BAP as the sole exogenous growth factor (Laurain *et al.*, 1996).

Somatic embryogenesis has been reported in three Eucalyptus species so far. Initial success is reported on the somatic embryoid induction of fourth species, *Eucalyptus dunnii L.* Somatic embryoids were induced from 3- day old seedlings in the presence of NAA (5.5 or 16.5 µM) alone or in combination with 2,4-D (4.5 µM). Either 10% coconut milk or 1g/l hydrolysed casein enriched auxin free medium induced the development of somatic embryoids (Termignoni *et al.*, 1996).

Maturation of slash pine *Pinus elliottii* Engelm. somatic embryoid was achieved by testing the concentrations of abscisic acid (ABA) (0,15 and 30 µM) and carbohydrate source 30 µM ABA and 6% maltose were deemed the best combination. Embryoid germination was accomplished in a continuously lighted environment and embryoids receiving a cold pretreatment (Liao and Amerson,1995). With a survival rate of 33% after acclimation in a mist system, more than 25 plants from somatic embryoids have been established in a green house.

All established plants obtained from somatic embryogenesis appear normal in morphology (Liao and Amerson, 1995). Direct regeneration of somatic embryoids was obtained from immature zygotic embryos of *Dalbergia latifolia. Roxb.* Immature embryos dissected from green pods 90 days after flowering gave the highest frequency of somatic embryo formation (Rao and Sita, 1996). Embryoids were separated and transferred to the

maturation medium containing MS salts and 0.5 to 1.0 mg BAP/l, where embryoids developed into plantlets. Somatic embryoids failed to convert into complete plants without BAP treatment. This method of direct regeneration of somatic embryoids without a callus phase has direct application for genetic manipulation studies (Rao and Sita, 1996).

Plant regeneration via somatic embryogenesis was achieved from callus derived from immature cotyledons of *Acacia catechu L.* on woody plant medium (WPM) supplemented with 1.39 µM NAA. The addition of 0.9-3.5 µM L-Proline to the medium influenced development of somatic embryoids (Rout *et al.*, 1995). The light green somatic embryoids germinated on half-strength MS medium supplemented with 2% sucrose. Somatic embryoids germinated into plantlets and were transferred to the field (Rout *et al.*, 1995).

Somaclonal variation can be used for plant improvement. To date, somaclonal variation has been a random process that will become more useful as researchers learn to control and direct it to achieve specific goals. The development of regeneration protocol for recalcitrant species will facilitate access to natural and induced variations (Skirvin *et al.*, 1994).

Synthetic seeds by encapsulation of somatic embryoids

Recent developments in the field of plant propagation via., organogenesis and somatic embryogenesis is diversified

considerably and rapid propagation for large scale commercial multiplication of elite clones has been accomplished in many ornamentals, crops and trees. During the past few years, noteworthy progress has been made in preparation of synthetic seed technology, which are designated as somatic embryoid engineered to be of use in commercial plant propagation (Redenbaugh *et al.*, 1986).

In general, somatic embryoids produced in large quantities have been individually isolated and encapsulated in a mixture of alginate matrix to prepare synthetic seeds. However, in plant species which do not regenerate somatic embryoids in culture, other vegetative parts like nodal cutting, axillary buds and meristem have been encapsulated to prepare synthetic seeds (Bapat, 1993; Ganapathi *et al.*, 1992). Such encapsulated somatic embryoids / vegetative parts would be an efficient delivery system as compared to large parcels of cutting from one place to another. Moreover, the acclimatization step of tissue cultured plants could be avoided if direct sowing of encapsulated somatic embryoids/ vegetative parts is achieved in soil under sterile conditions. This investigation is aimed at to evaluate the effect of encapsulation of embryoids of tea and to optimise the conditions for germination.

Encapsulation of embryoids with alginate, as reported by Redenbaugh *et al.*, (1986), might serve as a potential method for delivery of fragile conifer somatic embryoids through mechanical

handling to automated planting. With woody tree species, the only example of plant recovery after somatic embryoid encapsulation is for *Santalum album L.* (Bapat and Rao, 1992). Plant recovery after somatic embryoid encapsulation for conifer species has thus far not been reported (Gupta and Durzan, 1986).

Selection of the proper nutrient composition in the alginate capsule might be used to obtain a reliable germination in conifer species. In addition, the hydrated state of alginate capsules requires a storage method to retain embryoid viability and capsule integrity. Cold storage was a method investigated by several authors (Bapat and Rao, 1992), but with variable success.

1.1.2. PLANTATION CROPS

The role of ethylene in the formation of somatic embryoids in *Coffea canephora L.* from leaf discs cultured on MS medium supplemented with 5 µM isopentenyladenine was investigated by Hatanaka *et al.*, (1995). The results indicated that ethylene plays an important role in regulating somatic embryogenesis in leaf cultures of *Coffea canephora L.*

Direct somatic embryogenesis from young leaf explants was obtained in robusta coffee (*Coffea canephora L.*) clone B6 which is tolerant of the nematode *Pratylenchus coffeae L.* (Sumayono and Tahardi, 1993). Embryoid maturation was done in liquid medium,

and mature embryoids were transferred onto agar medium for shoot growth and root development. In *Coffea arabica L.* also somatic embryoids develop only when a callus grown on 2,4-D containing medium is transferred to 2,4-D free medium (Sondahl and Sharp, 1977).

Embryoids were produced from leaf callus of *coffea eugenioides L.* (Marques, 1993). Direct embryo production was achieved from leaves averaged over 3 - 15 embryos per responsive leaf (Pedroso and Pais, 1995 b). A simplified culture system is developed for somatic embryogenesis from leaf explants of Coffea species using a defined medium containing cytokinin and the preparation of competent embryogenic callus proliferating on 2,4-D were reported. Explants from *C. arabica L.* and *C. canephora L.* reacted in different ways to the culture system. In *C. canephora L.*, somatic embryoids formed from the cut edges of cultured young leaf explants in contact with cytokinin medium. In *C. arabica L.*, embryogenic callus was only induced on medium containing cytokinin after a long period of time (Yasuda *et al.*, 1995).

In witloof chicory, *Cichorium intybus L.* explants were taken from the distal third part of the leaf vein and cultured on MS medium containing 100 mg caseinhydrolysate/l, 1.3 µM 2,4-D and 1.3 µM kinetin. A pale yellowish nodular callus was formed after 4 weeks.

Embryo like structures appeared upon transfer to liquid MS medium containing 1.8 µM BAP. Embryo germination was accomplished in half-strength MS medium with 1g activated charcoal/l (Mohamed Yasseen and Splittstoesser,1995).

In *Theobroma cacao L.*, while immature embryos (2.5-10 mm) give rise to somatic embryoids from their cotyledons, the older embryos, ovules, pericarp or leaf pieces form only unorganized calli (Pence *et al.*, 1979). An *in vitro* regeneration protocol based on somatic embryogenesis was developed for cocoa clone DR1. Embryogenic calluses were initiated on petals from immature flower buds cultured on modified MS medium supplemented with 1.2 mg 2,4-D and 0.25 mg kinetin/l (Tahardi and Mardiana, 1995). Embryo maturation and germination were achieved after the somatic embryoids had been transferred to half-strength MS medium and cultured under 12-h days (Tahardi and Mardiana, 1995).

Embryoids were induced from slices of immature seeds of *Hevea brasiliensis* (Veisseire *et al.*, 1994). *Hevea brasiliensis* calluses were grown on culture media supplemented with different concentrations of 2,4-D, BAP or kinetin to induce somatic embryoginesis slightly (SE medium), moderately (MEC medium) or strongly (HEC medium). During first phase of culture, nitrate uptake was lowest in SEC medium and highest in HEC medium

(Montoro *et al.*, 1995). Free aminoacids accumulated temporarily at the end of second culture phase. Free glutamic acid and glutamine concentrations decreased in the order of calli > proembryoids > developed somatic embryoids. It is concluded that the type and amount of plant growth regulator added to the culture medium may play an important role in the ammonium and nitrate uptake of the developing callus (Montoro *et al.*, 1995).

Somaclonal variation have been observed in bananas obtained from tissue culture. The most common off-type in the Cavendish banana sub group is dwarfism, accounting for 75% of total variants (Sandoval *et al.*, 1995). Giant variants have also been observed, but with reduced frequency. GA3 and GA20 were quantified in leaf extracts from true-to-type and somaclonal variants (dwarf and giant) of banana.

For normal and giant plants, the endogenous GA3 concentrations were respectively 3.6 and 4.6 times higher. GA3 concentration in dwarf plants was 811 ng/g DW and in giant plants it was 2919 - 3730 ng/g. The GA20 concentration in giant plants was 68 ng/g DW. This concentration was, respectively, 4.6 and 7.3 times higher than those of normal and dwarf plants. It is suggested that somaclonal variation affecting banana plant height are associated with modifications in GA metabolism (Sandoval *et al.*, 1995).

1.1.3. REVIEW OF WORK IN TEA - CURRENT STATUS

The potential of tissue and organ culture for rapid mass multiplication and improvement of tea (*Camellia sinensis* (L.) O.Kuntze) has been fully recognized, and attempts are underway in a number of laboratories in India and abroad to achieve these objectives (Palni *et al.*, 1991).

Tea improvement, as in other highly heterozygous, long - lived and cross pollinated crops, through conventional breeding methods, is a difficult and time consuming task. Tissue culture of cotyledon segments can be a useful method of producing a large number of plants, from incompatible crosses where premature fruit drop occurs. Further more, somatic embryogenesis has the potential for rapid, mass production of bipolar structures which give rise to rooted plants following germination. Although a number of reports on cotyledon culture of tea are available (Wu *et al.*, 1981; Abraham and Raman, 1986; Arulpragasm *et al.*, 1988; Nakamura, 1988; Jha and Sen, 1991; Palni *et al.*, 1991; Raj Kumar and Ayyappan, 1992) information on morphogenetic events leading to organogenesis is fragmentary (Kato, 1986; Bano *et al.*, 1991). Cotyledon explants of *Camellia sinensis* produced somatic embryos without intermediate callus (Wachira and Ogada, 1995).

Although extensive studies in induction of somaclonal variants in other important crop plants have been done, no serious

attempts have been made for tea. Rapid *in vitro* multiplication of tea is in progress in India , Sri Lanka and other tea growing nations. Somatic embryogenesis of tea, through callus culture, cotyledons have been reported from Sri Lanka (Arulpragasam *et al.*, 1988) and India (Abraham and Raman, 1986; Palni *et al.*, 1991; Raj Kumar and Ayyappan, 1992) respectively. Somatic embryogenesis of tea have been reported from UPASI using mature / immature cotyledons (Abraham and Raman, 1986; Raj Kumar and Ayyappan,1992). Direct shoot bud formation was noticed in cotyledon callus at varying concentration of cytokinin and kinetin. Rapid proliferation and rooting of individual shoots was also obtained. However, establishment of the plantlets could not be achieved. No reports are available on performance of plantlets derived from somatic embryoids and their morphological, cytological, biochemical and physiological behaviour.

Caulogenesis and subsequent organogenesis has been relatively extensively studied in woody plants (Sondahl *et al.*, 1979 a. ; Oka and Ohyama, 1981). Moreover leaves may be the explants of choice for genetic engineering projects, as leaf regeneration methods have already been used for the genetic transformation of several plant species (Horsch *et al.*, 1987; Klee *et al.*, 1987).

Somatic embryoids and shoot bud formation were observed and plantlets had been raised from both the methods using leaf

fragments of *Camellia japonica L.* and *Camellia reticulata L.* cultivar 'Mouchang'(San-Jose and Vieitez, 1993). Direct shoot bud was produced from cotyledon callus of *Camellia sinensis* (Sood *et al.*, 1991). Friable callus obtained from stem explant is being tried for shoot bud differentiation. Only rooting was obtained from the cut end and near vascular strands developed (Palni *et al.*, 1991). Roots of *in vitro* raised plants gave 3 types of responses from cut or damaged portions. 1. callusing 2. shoot bud formation and 3. embryogenesis. (Palni *et al.*, 1991).

Inability to control the regeneration of plants from cultured cells has been a major problem in tea culture techniques. The loss of morphogenetic capacity that occurs on propagation of cell cultures has often been the single most intractable and frustrating obstacle to the application of *in vitro* tea callus culture method. (Wachira, 1990).

A few successful attempts have however been described by scientists largely from Taiwan and Sri Lanka. Wu (1976) used the basal MS medium supplemented with 2 -4 ppm IAA, 2 - 4 ppm Kinetin and 2 - 8 ppm 2,4 -D to induce callus. To induce differentiation, he increased the concentration of Kinetin to 10 ppm and reduced that of IAA to 1 ppm. Similar success was later reported by Kato (1985) when he managed to induce plantlets from stem callus using the basal MS medium.

Sarathchandra *et al.*, (1988) used nodal explants and juvenile leaves in amended Vacin and Went medium. Embryoid -like structures were formed on the nodal explants but plantlets have not been regenerated. Li *et al.*, (1987) used modified medium to induce callus and regeneration of plantlets from unripe embryos of tea.

The successful differentiation of cotyledon callus was first achieved by Wu *et al.*, (1981) and Kato (1986,1987) both of them developed new clones and embryoids respectively using this technique. Arulpragasm *et al.*, (1988) devised differently modified MS media supplemented with different growth regulators for the callus initiation and maintenance, embryoid regeneration, and plant regeneration respectively. In all the cases reported, genotype variations in response to the media used were observed. Cotyledon explants of *Camellia sinensis* produced somatic embryos without intermediate callus (Wachira and Ogada, 1995).

Further work therefore still needs to be done to perfect plant regeneration from embryogenic totipotent cells in view of the fact that no media devised to date is universal. New cell lines with spontaneously acquired favourable traits can then be selected and regenerated into plantlets. Cell lines of tea resistant to cold, water stress et cetera can be isolated and regenerated in to plants (Wachira, 1990).

Invariably, callus culture techniques coupled with mutagenesis would be important in creation of new variant tea plant materials. Mutagens which induce chromosome instability can be used in undifferentiated callus cells. According to Skirvin (1978), the most frequently reported variation induced by such mutagens has been polyploidy and aneuploidy. The former has been attributed to selective growth of normally non - dividing polyploid cells pre - existing in the original explant (D' Amata 1977) whereas the latter is caused by selective disintegration and loss of some somatic chromosomes. Both non - ionizing and ionizing radiations as well as chemical mutagens can be used in such experiments.

1.2. SCOPE OF THE PRESENT STUDY

The scope of the present investigation with the objective of evolving drought tolerant plants is highlighted as follow:

1. Standardization of physiological maturity of the seed stage for rapid embryoid production
2. Induction of somatic embryoids
3. Maturation of embryoids
4. Germination of embryoids
5. Rapid multiplication of embryoids
6. Encapsulation of embryoids
7. Histological studies of origin of embryoids
8. Hardening of somaclonal plants
9. Progeny characteristics and
10. Evaluation of progeny in the nursery.

II - *MATERIALS AND METHODS*

MATERIALS AND METHODS

In this chapter, the materials used for the investigation and various methods adopted to evaluate *in vitro* somatic embryogenesis and *in vivo* screening are described hereunder.

2.1 EXPLANT

Both developing and mature seeds were collected from established grown up trees of Chinery type (*Camellia sinensis* (L.) O. Kuntze) from open pollinated UPASI - 10 clone (Figs.1 and 2) growing in the UPASI Tea Research Institute at Valparai. Seeds were cracked open and cotyledon pieces were taken out. Many forms of explants namely, cotyledon with embryo axis, cotyledon without embryo axis, cotyledon half sliced, and cotyledon segments were used. Also, the position of explant both abaxial and adaxial were tried. Seeds were inoculated at different developmental stages (Figs.3 and 4)

Seeds were surface sterilized with 0.05% mercuric chloride for 10 minutes and rinsed in sterilized demineral water for 3 times. The excised cotyledons were cultured on full and half strength (Murashige and Skoog, 1962) MS medium for one week to screen out

Fig.1 UPASI-10 (chinery type) mother tree for seed collection.


WEST-10

Fig.2 Seed of UPASI-10 clone. Ready for explant collection.

Fig.3 Development stages of seeds


UPASI-10
STAGES OF SEED DEVT.
4-5 MONTHS OLD
6-7 MONTHS OLD
7- MONTHS OLD
8-9 MONTHS OLD

contaminated cultures; from the remaining clean ones, 4-7 mm long segments of cotyledon with or without embryo axis were then transferred to variously modified MS media.

2.2 CULTURE CONDITIONS

Cultures were incubated under continuous light from fluorescent tubes ($\cong$ 200 - 400 µ mol m^{-2} s^{-1}) at a constant temperature of 25 $\pm$ 2° C and 80 $\pm$ 5% relative humidity, unless otherwise mentioned. Each culture bottle (250 ml capacity) with 40 ml medium (0.8% agar, and 3.0% sucrose) was sterilised in an autoclave for 20 minutes under 1 kg/ cm^2 pressure at 120°C. For maturation, embryoids were transferred to 150 x 25 mm test tubes with 50 ml of capacity.

2.3 NUTRIENT MEDIUM

The nutrient medium consisted of MS (Murashige and Skoog 1962) salts and organics with 3.0 % sucrose and 50 ppm casein hydrolysate. The pH was adjusted to 5.6 - 5.8 with 1N NaOH or 1N HCl prior to the addition of 0.8% agar and autoclavied. Both full strength and half strength MS medium were used for embryoid induction, maturation and germination, quarter strength MS liquid medium was used for the root induction.

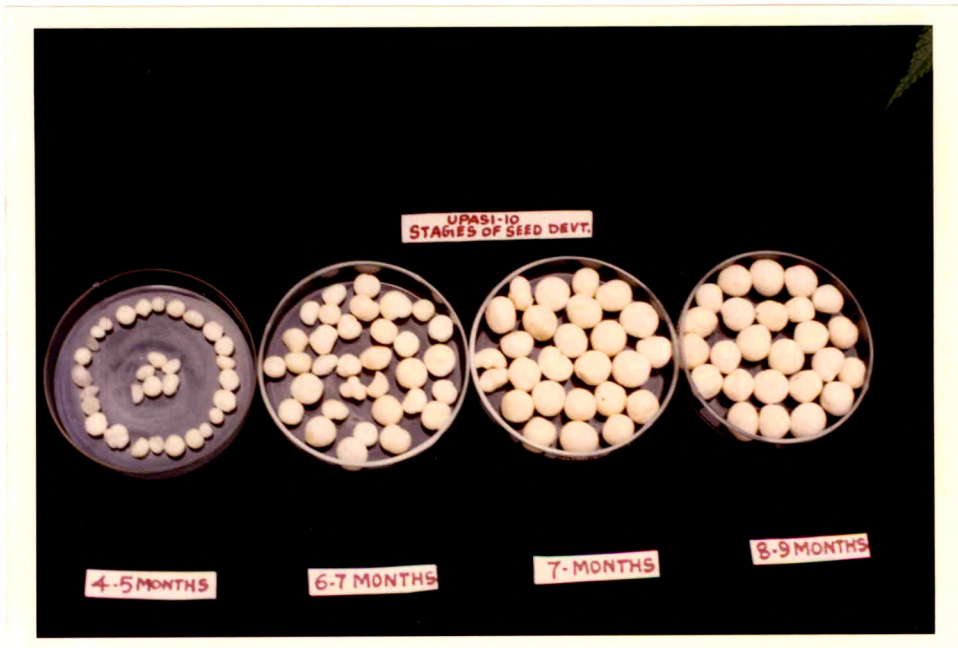
2.4 PLANT GROWTH REGULATORS

In the somatic embryogenesis studies, the cotyledon explants from immature seeds were placed (Fig. 5) into several primary media combinations namely, IAA (2.85, 8.56, 14.27 and 17.13 µM) IBA (2.46, 4.90, 9.80 and 14.70 µM), 2,4 - D (4.52, 9.05, 13.57, and 22.62 µM) gibberellic acid (2.89,8.60, and 11.55 µM) and combinations of IBA (2.46, 4.90, 14.70 µM) and BAP (4.40, 13.30, 17.80, 22.19, 35.50, 44.38 µM), thidiazuron (TDZ) (1.50, 3.75, 15.0) and NAA (0.54, 2.69, 5.37, 8.06, 10.74, 13.43, 16.11 µM), finally TDZ and IAA , BAP and NAA were tried. The explant remained on the primary medium for 12 weeks. After rapid embryoid induction, they were transferred to secondary medium for maturation. Effect of ABA (0.76, 1.89, 3.78, 5.67 µM) and PEG - 6000 (1.5, 3.0, 3.5, 4.0%) on maturation of somatic embryoids in 4 weeks of culture were studied. Matured embryoids were transferred to tertiary medium for germination. Effect of BAP (4.40, 8.90, 13.30, 17.80, 22.19 µM) and gibberellic acid (0.6, 0.14, 0.58, 1.44, 2.89 µM) on conversion of somatic embryoids into plantlets were studied. Strength of the MS germination medium (Half and full strength) played an important role in stimulating the growth of juvenile plants.

Apart from direct somatic embryogenesis, indirect somatic embryogenesis and shoot bud induction were also attempted. Cotyledon callus, leaf callus and internodal callus were used for the

Fig.4 Development stages - shelled seeds (cotyledon)

Fig.5 Cotyledon in the initial inoculation stage.


UPASI-10
STAGES OF SEED DEVT.
4-5 MONTHS
6-7 MONTHS
7-MONTHS
8-9 MONTHS

above studies. All explants were transferred every 2 weeks to fresh medium.

2.5 IN VITRO STUDIES

Several factors were investigated in the preliminary development of a protocol for embryoids induction, maturation, and germination. Experiments were conducted to determine how the embryogenesis induction and developments were affected by :

1. Physiological maturity of the explant
2. Hormonal combinations (Auxin, Cytokinin and Gibberellin concentrations)
3. The apical, middle or basal portions of the cotyledon as explants
4. Factors affecting maturation of embryoids
5. Factors affecting germination of embryoids
6. Maximum number of embryoid induction per cotyledon pieces
7. Maximum number of multiple shoots from a single growing embryoid and
8. Hardening of somaclonal plants.

2.5.1 Encapsulation of embryoids

Uniform sized mature embryoids (Ca. 5 and 8 mm diameter) were selected from the cultures maintained in the tissue culture laboratory. Embryoids were dipped in a sterile pre-cooled mixture of 2% sodium alginate prepared in MS medium (Murashige and Skoog,

1962) and various combinations of growth regulators and were picked up with a wide mouthed pipette and dropped into sterile cool 0.6 mM (1.016 g/l) calcium chloride solution. The calcium alginate beads thus formed were incubated onto a rotary shaker under $\cong$ 120 - 150 μ M m^{-2} s^{-1} light for 20 min. After the incubation period, the beads containing embryoids were recovered from the calcium chloride solution, washed with sterile distilled water, blotted and were germinated under different conditions to check their ability to regenerate plantlets.

Each embryoid was encapsulated and inoculated in the MS germination medium as well as in the soil and sand, vermiculite, perlite mixtures. Embryoid derived rooted plants were transferred into fibre glass mist house (9 m x 4.5 m). The minimum and maximum temperatures maintained at 21° C (night) and 30 $\pm$ 5° C (day), respectively and relative humidity of 98% to 100%. Day temperature and relative humidity were maintained through misting the chamber at hourly interval for one minute.

2.6 IN VIVO STUDIES

2.6.1 Morphological parameters

Plantlets transferred to soil and sand mixture were hardened under greenhouse conditions, where minimum and maximum temperatures were maintained at 20°C and 25 $\pm$ 2°C, respectively

and relative humidity at 95%. Day temperature and RH were maintained through misting chamber at hourly interval for one minute. All plants were given soil nutrients at fortnightly interval and foliar spray of Zinc sulphate (1%) and Magnesium sulphate (500 ppm) at weekly interval as per the nursery practices. Growth performance of the plants under greenhouse conditions was monitored periodically.

2.6.2 Leaf characteristics

Morphological leaf characteristics were studied both in control as well as in somaclonal plants by observing their leaf shape, tip, base, pose, petiole length, serration, venation and leaf area. Leaf area was measured by portable leaf area meter (LI-COR ; LI- 3000A USA). Leaves from five somaclonal plants with control were taken for studying the leaf characteristics.

2.6.3 Morphological growth rate

Morphological growth pattern was observed over a period of nine months. Number of leaves, internodal length and total height were recorded as the growth parameters. Five somaclonal plants and two vegetatively propagated plants were taken for studying the growth rate.

2.7 PROGENY CHARACTERISTICS

2.7.1 Histology

Plant tissue culture methods often are applied to fundamental studies of plant morphology and development. Such studies require familiarity with histological techniques for light microscopy. Individual somatic embryos at different developmental stages, origin of embryoids from the cotyledon, were examined histologically to confirm the origin of embryoids from cotyledonary tissues. The procedure of fixing, dehydrating and paraffin embedding of tissues was adopted as per the procedure described by Feder and O' Brien, 1968.

2.7.2 Proline estimation

Proline content was estimated following the procedure described by Bates *et al.*, 1973. The materials required and methodology as described are given below:

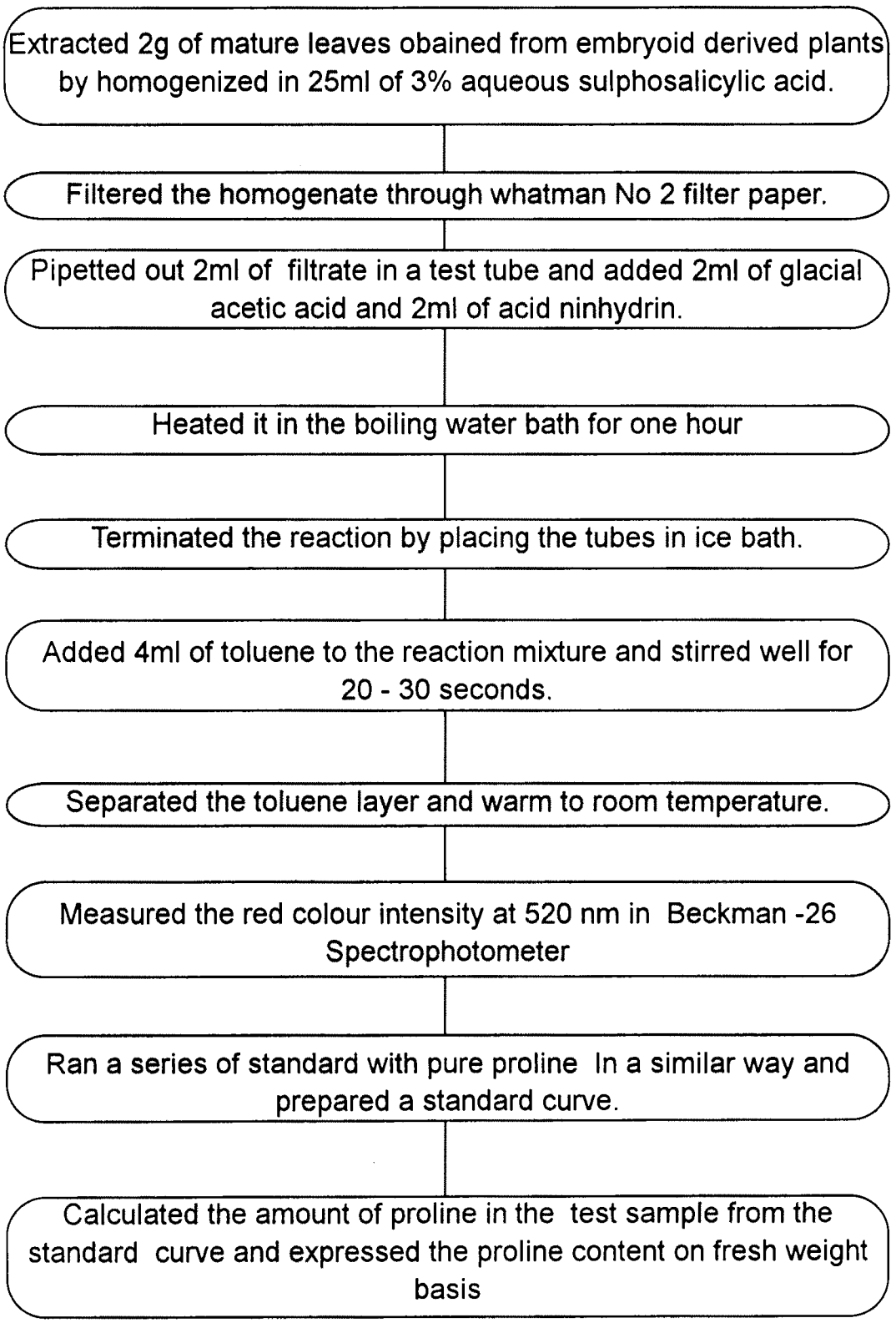
2.7.2.1 Materials

1. Preparation of acid ninhydrin:

   Warmed 1.25 g of ninhydrin in 30 ml of glacial acetic acid and 20 ml of 6M phosphoric acid with agitation until dissolved.

2. 3% Aqueous sulphosalicylic acid
3. Glacial acetic acid
4. Toluene
5. Proline

2.7.2.2 Methods



Seven somaclonal plants were compared with conventionally propagated plant as control for the above studies.

Calculation:

$$\left.\begin{array}{l}\mu \text{ moles proline} \\ \text{per g tissue}\end{array}\right\} = \frac{\mu g \text{ proline/ ml x ml toluene}}{115.5} \times \frac{5}{\text{g sample}}$$

Where, 115.5 is the molecular weight of proline.

2.7.3 Net photosynthetic rate (Pn)

The differential level of carbon dioxide concentration between reference and sample cells are measured to monitor the net photosynthesis which is an important parameter associated with productivity.

Intact, photosynthetically active, recently mature leaves were selected to measure the carbon dioxide exchange rates with portable Infra Red Gas Analyser (IRGA, Model LCA-3, Analytical Development Company, UK). Carbon dioxide uptake and shoot water potential of mature clonal teas were monitored in seven somaclonal plants with conventionally propagated plant as control. The experiment was repeated for three times.

2.7.4 Stomatal counting to study transpiration loss

Small leaves were collected from three somaclonal plants and a control to study the transpiration loss. Epidermis had been peeled

off from the lower surface of the leaves and observed under photomicroscope (Zeiss Germany; Photomicroscope III). Number of stomata were counted in mm^2 area of young leaves. Four replications were taken and analysed statistically.

2.7.5 Response of somaclonal plants to artificial stress

2.7.5.1 Imparting stress in the growth chamber

Somaclonal plants at five to six leaves stage were selected and subjected to different ambient temperature regimes(20,25 and 30^{0}C) with and without watering in a growth chamber (Heraeus Votsch; HPS 1500 Germany) for a period of six months. Five somaclonal plants and three control plants were taken for stress studies. The experiment was repeated for three times. Growth rate (time taken to unfold the bud) were monitored from time to time.

2.7.5.2 Inducing artificial stress by an osmoticum poly ethylene glycol (PEG-6000)

The objective was to study the response of somaclonal plants to short term moisture stress. The survived plants under higher percentage of PEG will be chosen and observed for their tolerance to drought.

Plantlets with 5 cm high were selected and dipped in poly ethylene glycol for five and ten minutes time at the concentration of

10,20 and 30 percent. After one week, the response of plants to PEG treatment was recorded. Four somaclonal plants were taken for PEG stress studies and the experiment was repeated two times.

2.8 STATISTICAL ANALYSIS

Data on all the growth hormone combinations were subjected to analysis of variance (Steel and Torrie, 1960) to find out the treatment effect of Auxin, Cytokinin, Gibberellic acid and interaction among those plant growth regulators. The critical difference (CD) was computed and the means were compared using Duncan's (1955) Multiple range Test (DMRT).

III - *RESULTS*

RESULTS

3.1 EXPLANT

The most critical aspect of induction of direct somatic embryogenesis is, selection of appropriate explant material. Seven months old immature cotyledon explants were used for direct embryogenesis studies. Stem segment, leaf bits, and root bits were also tried for indirect embryogenesis. Of the different stages of cotyledon inoculated, it was observed that six to seven months old cotyledon with embryo axis gave higher rate of embryoid production (Table 1). Cotyledon inoculated on the adaxial side (Lower surface) only responded for induction. The abaxial tissue (Upper surface) did not show any sign of embryoid induction.

After two weeks of culture, explants were changed from pure white to pale cream yellow and after two months they became swollen (Fig.6). Starch grains were being metabolized preferentially in the adaxial tissues. Large nuclei appeared and mitotic activity was initiated in cells with few starch grains near the adaxial surface. Division continued within the adaxial regions of the cotyledon.

Table 1. Influence of the physiological maturity of the cotyledon for embryoid induction.

Number of cotyledons inoculated	*Age of the cotyledon				
	4-5	5-6	6-7	7-8	8-9
	cultures forming somatic embryos (%)				
200	0	0	20	0	0
250	0	0	30	0	0
300	0	0	25	0	0
350	0	0	40	0	0
400	0	0	35	0	0

* Only 6-7 months old cotyledon produced embryoids

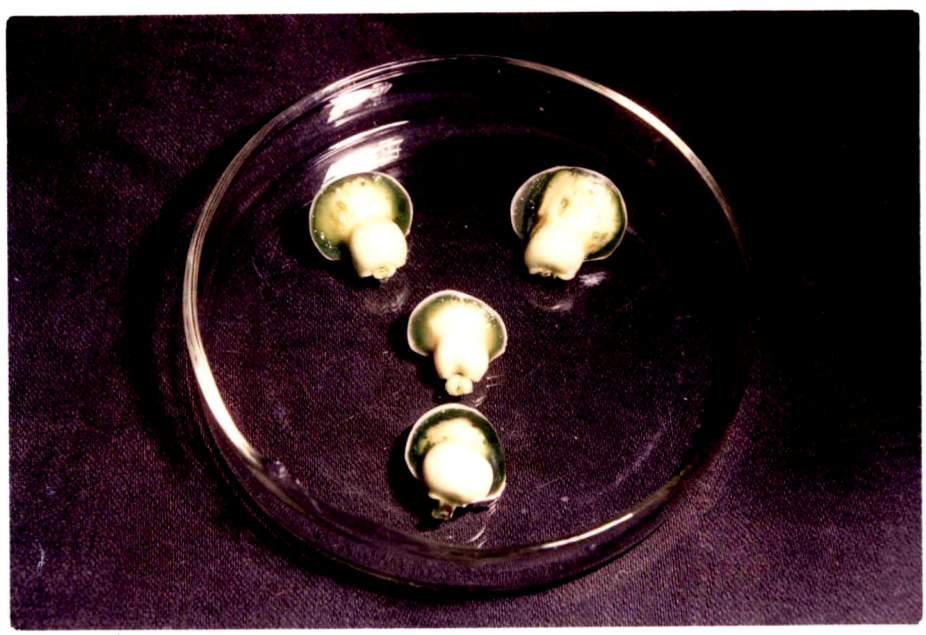
The development of raised areas of tissue on the adaxial surface was associated with this cell division activity. Such raised areas often developed at the edges of the explant (Fig.7). When cell division activity reached the adaxial surface, further divisions occurred within groups of adjacent surface cells. These divisions gave rise to tissues composed of smaller cells with densely staining cytoplasm and large nuclei which, on continued development, formed protuberances and ridges of embryogenic tissues. As embryogenic tissues developed, disorganized cell proliferation in abaxial tissues resulted in friable callus formation. After three months, primary embryoid started emerging from the swollen portion (Fig.8).

After 6 months of inoculation, different stages of embryoids namely globular stage, heart shaped, torpedo stages were observed. The embryoid induction rate was 1:70 i.e.. from one cotyledon 70 numbers of embryoids were isolated after nine subcultures at 20 days interval. Of the other explants tried for indirect embryogenesis, more callusing was noticed in the leaf bits compared to nodal segments. Primary, secondary and tertiary root bits produced only rhizogenic callus (Fig.9). In these stages, they were subjected to different media supplemented with 2,4 - D, IBA, NAA and BAP in combination and observed for response.

It was found that in all the media tested for indirect somatic embryogenesis, there were no embryoid induction. Instead, profused

Fig.6 Cotyledon after two weeks of inoculation in MS medium.

Fig.7 Raised areas developed on the lower surface after two months of inoculation.



root formation and more callus growth were observed. Histological studies revealed the vascular bundle formation in both root and callus grown explants (Fig.9).

3.2 CULTURE CONDITIONS

The pH adjusted to 5.6 - 5.8 was optimum for the embryogenesis. The concentration of agar at 0.8% was sufficient for supporting the cotyledon. Incubation under continuous light from fluorescent tube (=200 - 400 μ mol m^{-2} s^{-1}) at a constant temperature of 25 $\pm$ 2°C and 80 $\pm$ 5% relative humidity were optimum for the maintenance of culture.

3.3 NUTRIENT MEDIUM

Both full strength and half strength MS were tried for embryoid induction. Profuse embryoids were produced only in the full strength MS medium, whereas the half strength medium did not show any sign of embryoid initiation though the required hormone combinations were used.

3.4 PLANT GROWTH REGULATORS

3.4.1 Effect of auxin, cytokinin and gibberellic acid on embryoid induction:

Among the 200 growth regulator combinations tested in culture media, only two of them allowed the differentiation of somatic

Fig.8 Primary embryoids from the swollen protion after three months of inoculation.

Fig.9 Primary, secondary and tertiary root bits produced rhizogenic callus.

embryoids. The auxins (IAA, IBA, 2,4 - D and NAA), cytokinin (BAP) and gibberellic acid were tried individually and in combinations.

It was found that individual hormones did not produce embryoids but more callus growth was observed. IBA - 14.7 µM, (Table 2), BAP 44.38 µM (Table 3) 2,4-D 22.62 µM (Table 4) and 17.80 µM of BAP resulted in more calli. Gibberellic acid in combination with IBA, BAP, NAA and 2,4 - D did not respond, whereas gibberellic acid alone 8.66 µM gave prominent swelling on the adaxial side of the cotyledon (Table 5) . This was the initial sign of embryoid induction. However on subculture in various embryoid induction media, further development was totally absent (Fig.10).

3.4.2 Effect of thidiazuron (TDZ) and auxin on embryoid induction:

Cotyledons were cultured on MS medium supplemented with a substituted phenyl urea, thidiazuron (TDZ) and NAA. TDZ (1.50,3.75, and 15.0 µM) and NAA (0.54, 2.96, 5.37, 8.06, 10.74, 13.49 and 16.11 µM) were used. After eight weeks of culture it was observed that there were yellowish swelling on the cotyledon surface.

Hundred cotyledons were inoculated in each treatment. The maximum number of cotyledon, with 90% swelling was observed TDZ 3.75 µM and NAA 10.74 µM combination after six weeks of

Table 2. Effect of IBA concentration on induction of embryogenic mass.

PGR	Conc. μM	Total No. of Explants Inoculated	No. of explants Responded
IBA	2.46	100	0
	4.90	150	0
	9.80	50	0
	14.70	200	0

IBA at different concentrations did not respond for embryogenesis

Table 3. Effect of BAP concentration on induction of embryogenic mass.

PGR	Conc. μM	Total No. of Explants Inoculated	No. of explants Responded
	4.40	50	0
	13.30	175	0
BAP	17.80	75	0
	22.19	150	0
	35.50	200	0
	44.38	250	0

BAP alone produced no embryoids

Table 4. Effect of various concentrations of 2,4-D on induction of embryogenic mass.

PGR	Conc. μM	Total No.of explant inoculated	No. of explant responded
2,4-D	4.52	300	0
	9.05	150	0
	13.57	200	0
	22.62	100	0

Lower and higher concentrations of 2,4 -D failed to express embryoids

Table 5. Effect of various concentrations of GA3 on induction of embryogenic mass.

PGR	Conc. μM	z Mean No. of cotyledon responded
	2.89	3.8 *a
GA3	8.66	3.8 a
	11.55	3.8 a

z - Mean number of cotyledon responded for localized swellings

Mean of 4 replicates

S.E = 1.76

C.D = 4.4

* statistically not significant

cultures. The lowest response (5%) was noticed in TDZ 15.0 µM and NAA 5.37 µM. There was no embryoid induction from the swellings in all the treatments. These cotyledon cultures were transferred to embryoid induction medium (NAA 10.74 µM and BAP 13.30 µM where, all the inoculum failed to express the embryoid formation. TDZ (3.75, 7.50 and 22.50 µM) in combinations with other auxin namely with IAA (0.57, 2.85, 5.71, 8.56, 11.42,14.27 and 17.13 µM) were tried. Here also there was no embryoid induction from the localized swellings (Table 6 & 7).

3.5 IN VITRO STUDIES

Effect of benzyl aminopurine (BAP) and naphthyl acetic acid (NAA) on embryoid induction

Stage I Medium

Auxin or Cytokinin alone did not produce any embryoids. But combinations of Auxin (NAA) and Cytokinin (BAP) were required for embryoid induction. Various concentrations of BAP (0.89, 8.90, 13.30, 17.80 and 22.20 µM) and NAA (5.37, 10.74, 16.11 and 21.48 µM) were tried (Table 8) (Fig. 11) . Combinations of BAP 13.30 µM and NAA 10.74 µM resulted in higher rate of embryoid induction per cotyledon. A single cotyledon segment produced 70 embryoids with different stages of development in a period of six months after nine subcultures at 20 days interval in BAP 13.30 µM and NAA 10.74

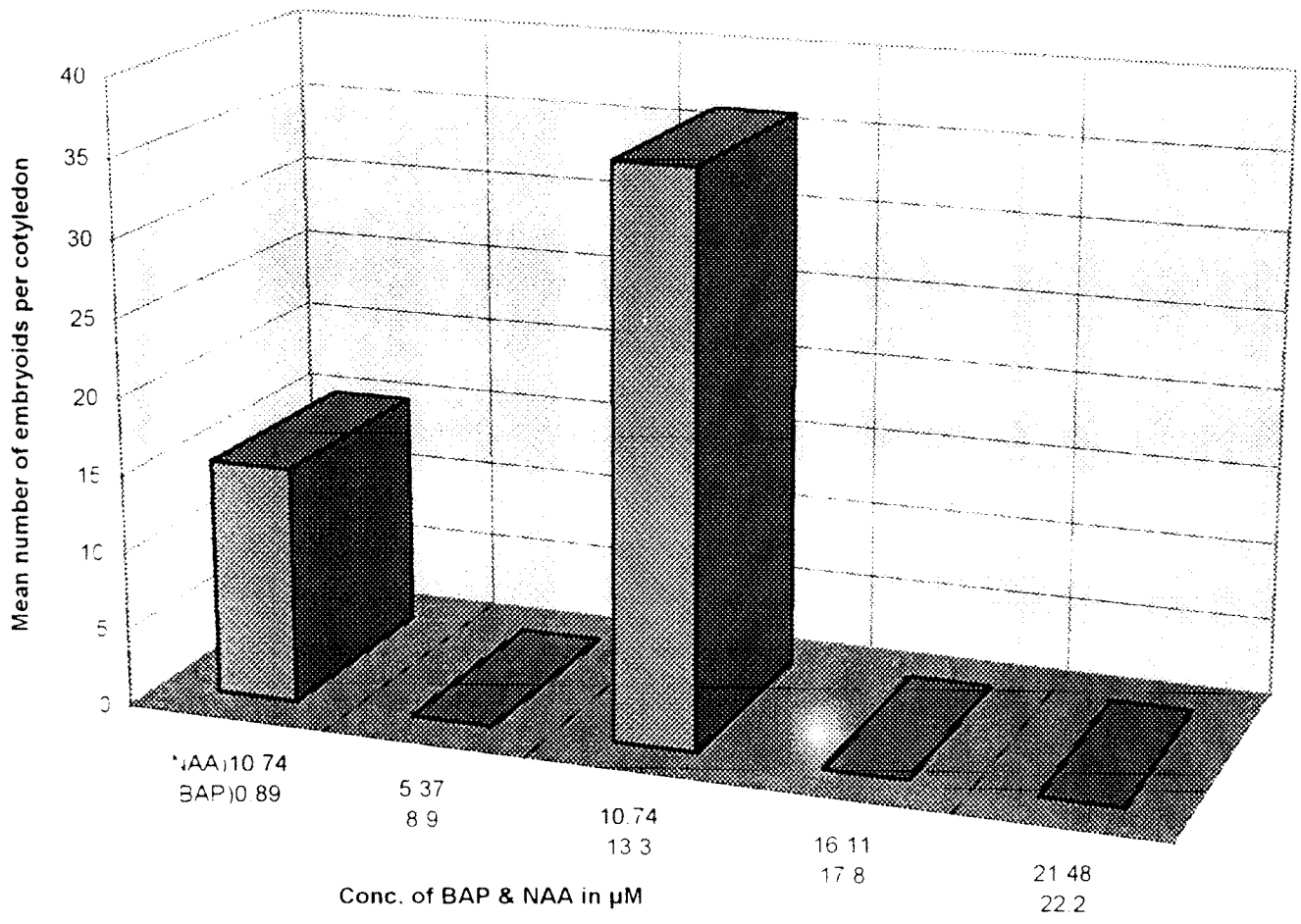


Fig.11. Effect of NAA & BAP on embryoid induction

Table 6. **Effect of thidiazuron (TDZ) and NAA on embryoid induction.**

Plant growth regulators concentration		z Weeks after inoculation					
TDZ(μM)	NAA	3	4	5	6	7	8
		*cultures forming embryogenic swellings					
	0.54	0	10	0	10	0	0
1.50	2.69	0	15	25	25	0	0
	5.37	0	0	30	70	0	0
	8.06	0	25	60	65	0	0
3.75	10.74	0	20	40	90	0	0
	13.43	0	16	0	40	0	0
	2.69	0	22	0	0	0	0
15.0	5.37	0	0	0	0	0	0
	16.11	0	8	0	0	0	0

z - No somatic embryos formed after swellings

* Embryogenic swellings from the cotyledon after 8 weeks of inoculation

Table 7. Effect of thidiazuron (TDZ) and IAA on embryoid induction.

Plant growth regulators concentration		z Weeks after inoculation					
TDZ(μM)	IAA	3	4	5	6	7	8
		*cultures forming embryogenic swellings					
	0.57	0	0	0	0	0	0
3.75	2.85	0	0	0	0	10	10
	5.71	0	15	20	10	0	0
	8.56	0	10	30	30	0	60
7.50	11.42	0	20	0	10	0	50
	14.27	0	0	40	50	0	40
	2.85	0	0	10	15	0	20
22.50	5.71	0	15	50	70	15	70
	17.13	0	10	0	0	0	25

z - No somatic embryos formed after swellings

* Embryogenic swellings from the cotyledon after 8 weeks of inoculation

Table 8. Effect of NAA and BAP on embryoid induction.

Plant Growth Regulators μM		z Mean number of embryoid per cotyledon
BAP	NAA	
0.89	10.74	*15.2 b
8.90	5.37	0.0 a
13.30	10.74	*36.2 c
17.80	16.11	0.0 a
22.20	21.48	0.0 a

z - Mean of 5 replicates

S.E = 1.3

C.D = 2.8

* significant at 1% level

µM (Fig. 12). BAP 0.89 µM and NAA 10.74 µM produced 30 embryoids per cotyledon in the same period. Initially all somatic embryoids were yellowish in colour. After one month, the embryoids turned into green in colour. These embryoids germinated after maturation in poly ethylene glycol - 6000 (PEG - 6000) and abscisic acid (ABA). Other combinations of BAP (8.90,17.80 and 22.20 µM) and NAA (5.37,16.11 and 11.48 µM) proved ineffective in embryoid induction. The frequency of somatic embryogenesis and the number of somatic embryoids were not modified by the increase in NAA concentrations. Secondary embryoids emerged out in the same induction medium after harvesting the primary embryoids (Fig. 13).

3.5.1 Encapsulation of embryoids (synthetic seed production)

Various concentrations of sodium alginate (2 to 5%) and calcium chloride (0.7%) were tried to standardize the optimum dose for encapsulation of embryoids (Table 9). Among different concentrations tried, combinations of sodium alginate 2% and calcium chloride 0.7% had given more number of beads and increased the strength of the gel bead (Fig. 14). In the present investigation, 2% sodium alginate prepared in MS medium supplemented with various growth regulators and additives were used for encapsulation. The calcium alginate beads containing the tea embryoids had a diameter ranging between 5 and 8 mm (Fig. 15). The encapsulated embryoids

Fig.10 Prominent swellings on the adaxial side of the cotyledon.

Fig.12 Single cotyledon produced seventy embryoids

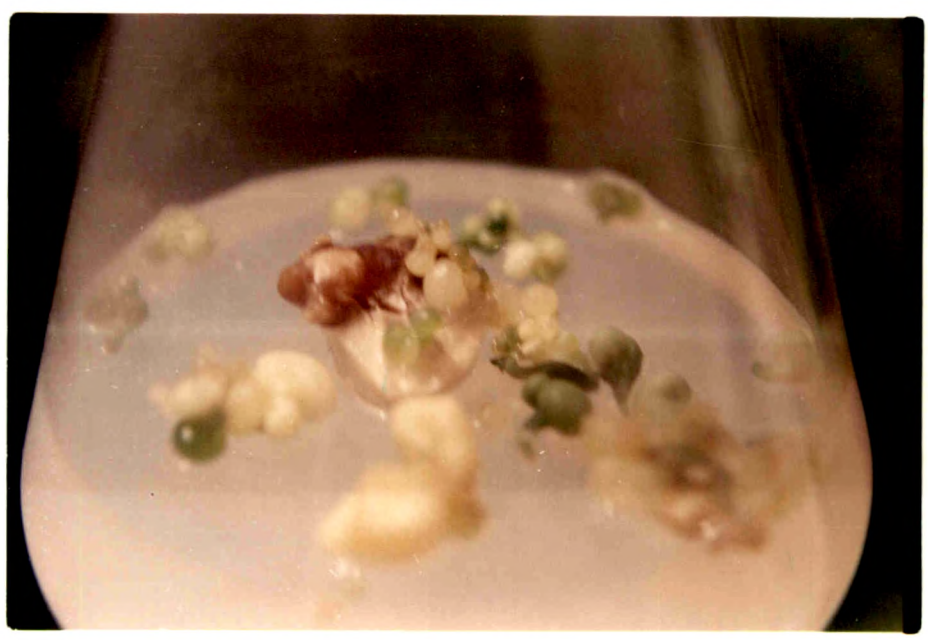
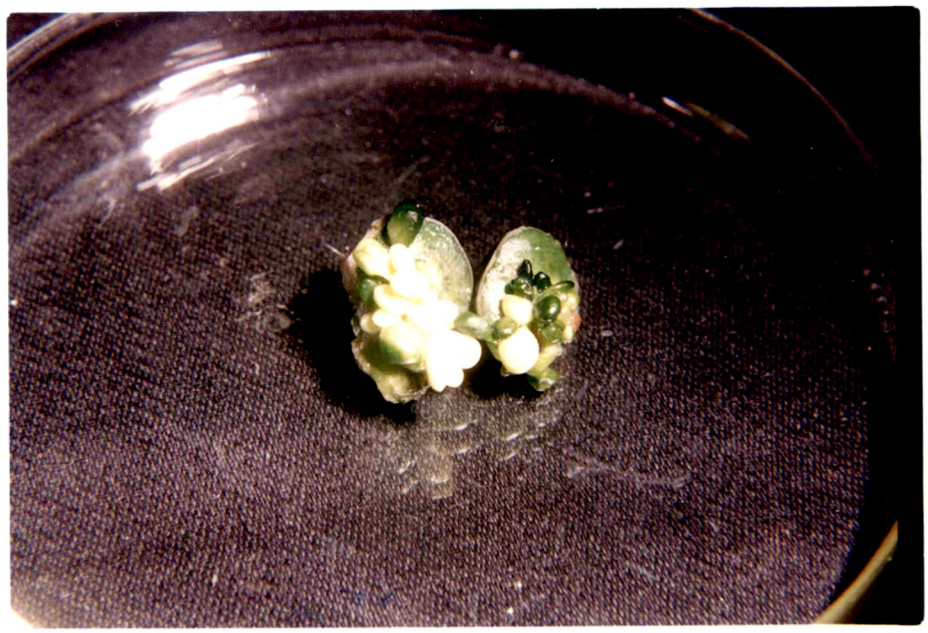


Fig.13 Initiation of secondary embryoids.

Fig.14 Encapsulation of embryoids in sodium alginate (synthetic seeds)



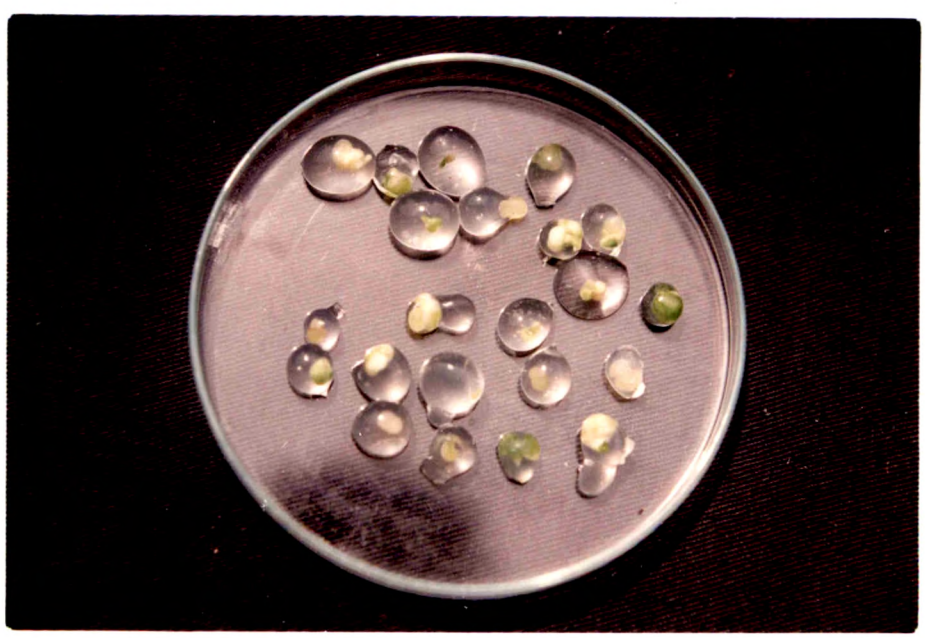


Table 9. Strength of sodium alginate and calcium chloride on the consistency of encapsulated gel beads.

Sodium Alginate %	Calcium Chloride %	Percent of synthetic seed conversion
1	0.5	50
2	0.7	90
3	1.0	35
4	2.0	25
5	3.0	30

were germinated in full strength MS medium as well soil-sand mixture to check the ability to regenerate plantlets (Fig. 16). These synthetic seeds were sown in the nursery soil. This gel bead gradually swelled and became brittle, finally cracked spontaneously and germinated into plantlets in the MS medium within one month (Fig. 17). Those beads which were sown in the soil pots did not germinate. Those embryoids which were sown on soil-sand mixture lost their vegetative potential, gradually turned black and remained dormant. The encapsulated embryoids inoculated on full strength MS medium germinated well and regenerated into plantlets (Fig. 18).

Storage of synthetic seeds (cryopreservation)

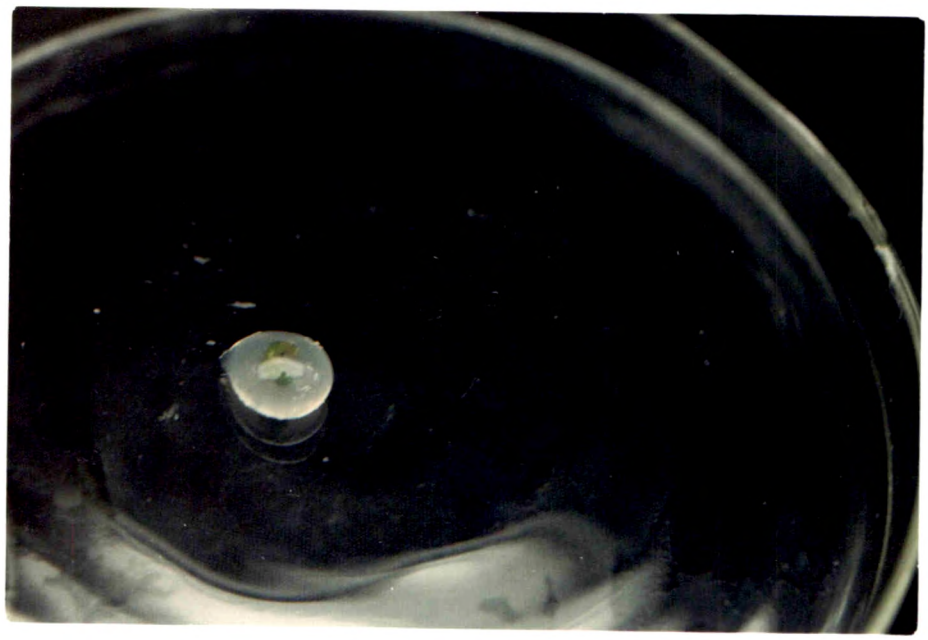
Encapsulated synthetic seeds were kept in - 5°C to - 10°C in deep freeze for one month and then germinated (Table 10). There was an impact on the germination efficiency after preservation in the low temperature. This has proved that these encapsulated embryoid could be cryopreserved for a long period and germinated readily. Storage in low temperature is preferred.

3.5.2 Effect of auxin, cytokinin and thidiazuron on indirect embryogenesis

Four / Five Various concentrations of auxins (IAA - 2.85, 8.56, 14.27 and 17.13 µM, IBA 2.46, 4.90, 9.80 and 14.70 µM NAA 2.69, 8.06, 16.11, 11.48 µM, and 2,4 - D 2.26, 6.78, 11.31 and 13.57 µM),

Fig.15 An encapsulated bead ranging between 5-8 mm diameter.

Fig.16 Synthetic seeds inoculated in the full MS medium.



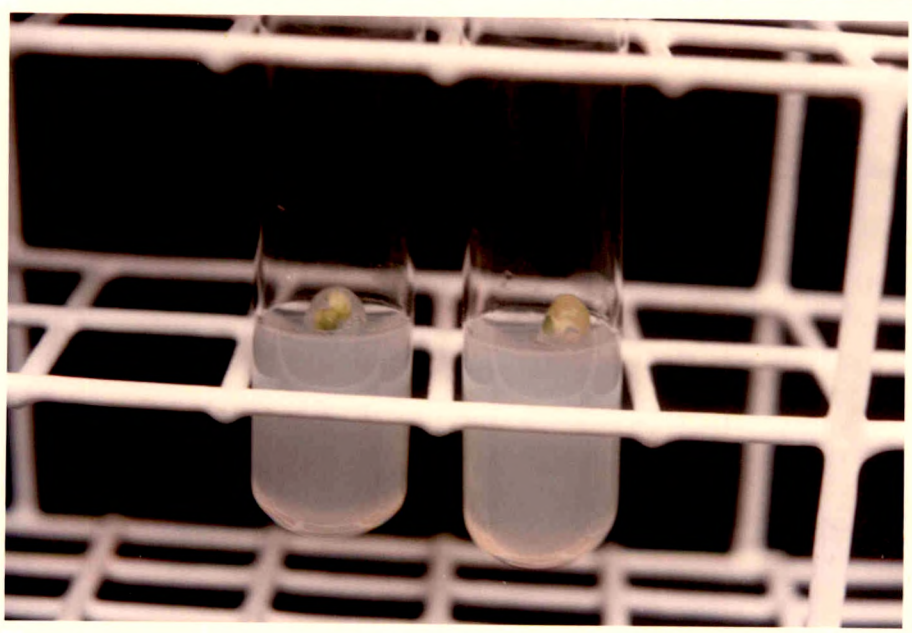


Fig.17 Gel bead gradually swells and becomes brittle

Fig.18 Gel bead split spontaneously and germinated

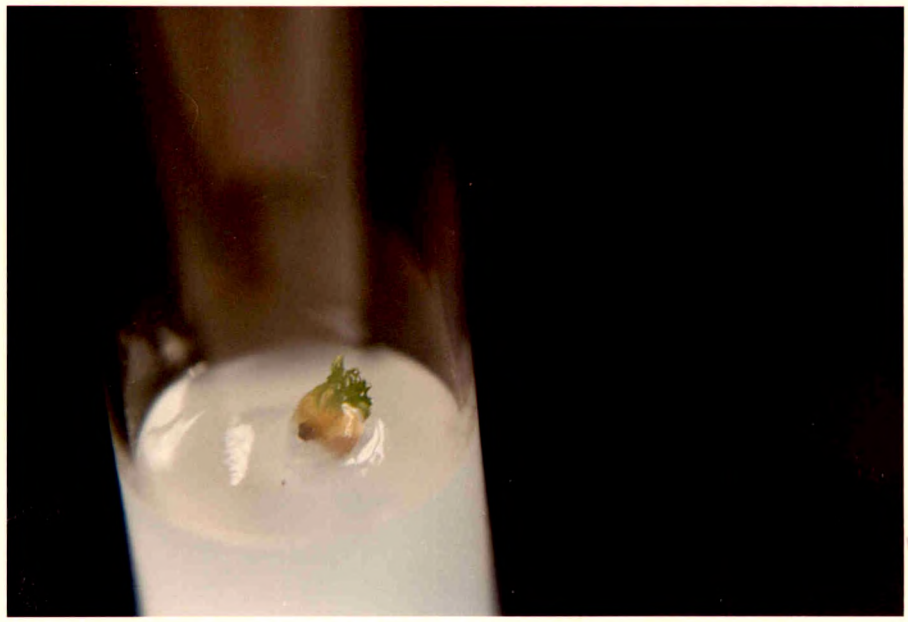


Table 10. Effect of low temperature (storage) on germination of encapsulated embryoids in full strength MS medium.

Temperature C^0	Number of beads stored	Germination after one month z (Mean)
- 5	20	7.0 a
- 7	25	9.7 b
- 8	15	13.3 c
- 10	30	16.0 d

S.E = 0.82

C.D = 2.1

significant at 1% level

z - Mean of 3 replicate

cytokinin BAP 4.40, 11.09, 13.30, 14.40, 15.52, 17.80, 22.19 µM and thidiazuron TDZ (3.75, 7.50, 11.25, 15.0, 22.50, 30.0 µM) were tested for induction of embryoids (Tables 11,12 &13). The above PGRs tested in combination with one another at different concentrations. Monthly observations were made. These treatments failed to induce embryoids.

The response differed depending upon the type of explants used. Leaf bits produced more callus in thidiazuron supplemented medium. The calli were friable, greenish but failed to respond on transfer to embryogenic medium (Table 14 & 15). Root bits and inter nodes produced more roots in IBA supplemented medium. Conversion of root tip in to shoot bud had resulted in more root formation. BAP and kinetin supplemented media had expressed more cell division and thereby more callus growth in immature cotyledon and inter nodes. Further development such as organogenesis or embryogenesis from these initial products were absent.

3.5.3 Abscisic acid (ABA) and polyethylene glycol (PEG-6000) on maturation of somatic embryos

Abscisic acid (ABA) and polyethylene glycol (PEG - 6000) were found useful on maturation of somatic embryos. During embryoid developmental period embryoid moisture content decreased from about 95% to 40 - 55% during the late cotyledonary stage. Dehydration seemed essential to promote normal germination and

Table 11. Effect of BAP and IBA on embryogenic and non embryogenic callus initiation from cotyledon surface.

	responding explants (mean %)									
	BAP (μM)									
IBA (μM)	0		4.40		13.30		17.80		22.19	
	E'	C	E'	C	E'	C	E'	C	E'	C
0.0	0		0	0	0	0	0	0	0	0
2.46	0		0	25	0	60	0	20	0	0
4.90	0		0	40	0	70	0	30	0	10
9.80	0		0	0	0	100	0	10	0	60
14.70	0		0	0	0	68	0	50	0	90

E' = Embryogenic callus

C = Non - embryogenic callus

Table 12. Effect of BAP and IAA on embryogenic and non embryogenic callus initiation from cotyledon surface.

IAA (μM)	Average percentage of responding explants									
	BAP (μM)									
	0		14.40		13.30		17.80		22.19	
	E'	C	E'	C	E'	C	E'	C	E'	C
0.0	0		0	0	0	0	0	0	0	0
2.85	0		0	0	0	20	0	0	0	20
8.56	0		0	50	0	40	0	15	0	50
14.27	0		0	75	0	65	0	20	0	75
17.13	0		0	100	0	70	0	70	0	90

E' = Embryogenic callus

C = Non - embryogenic callus

Table 13. Effect of BAP and 2,4-D on embryogenic and non embryogenic callus initiation from cotyledon surface.

2,4 - D (μM)	Average percentage of responding explants									
	BAP (μM)									
	0		14.40		11.09		15.52		22.19	
	E'	C	E'	C	E'	C	E'	C	E'	C
0.0	0		0	0	0	0	0	0	0	0
2.26	0		0	30	0	40	0	20	0	20
6.78	0		0	50	0	70	0	60	0	40
11.31	0		0	55	0	65	0	25	0	20
13.57	0		0	100	0	90	0	75	0	80

E' = Embryogenic callus

C = Non - embryogenic callus

Table 14. Effect of explant type on shoot bud formation.

Source of explant from which callus derived	per cent calli forming	* Number of shoot bud formed per callus
Immature cotyledon	50	0
Mature cotyledon	30	0
Immature leaf bits	20	0
Inter node segments	20	0

* Eighteen week old callus from different explants inoculated on embryoid induction medium with BAP 0.89 μM and NAA 10.74 μM

Table 15. Effect of explant type on shoot bud formation.

Source of explant from which callus derived	per cent calli forming	* Number of shoot bud formed per callus
Immature cotyledon	70	0
Mature cotyledon	40	0
Immature leaf bits	50	0
Inter node segments	20	0

* Eighteen week old callus from different explants inoculated on embryoid induction medium with BAP 13.30 μM and NAA 10.74 μM

growth following maturation with PEG and ABA.

ABA (0.76,1.89,3.78,5.67 µM) and PEG - 6000 (1.5, 3.0, 3.5, 4.0%) were tried (Table 16) (Fig. 19). Among different concentrations tested, ABA at 1.89 µM and PEG - 6000 3% had resulted in higher proportions of fully developed mature embryoids (Fig. 20) with prominent root pole (Fig. 21) followed by different developmental stages such as globular, heart,torpedo and cotyledonary (Fig. 22). One month period was taken for maturation.

3.5.4 Effect of BAP, gibberellic acid and medium strength on conversion of somatic embryos into plantlets

Germination and conversion of somatic embryoids into plantlet is another important aspect (Fig. 23). Among different concentrations of BAP (4.40,8.90,13.30,17.80,22.20 µM) and gibberellic acid (0.6, 0.14, 0.58, 1.44, 2.89 µM) tried, BAP at 22.20 µM and gibberellic acid 2.89 µM in the media had produced more shoot apex in one month (Table 17) and stimulated the germination (Figs. 24 and 25). Out of 30 embryoids cultured, 20 embryoids produced active shoot tips. BAP 8.90 µM and gibberellic acid 0.14 µM had resulted in next higher conversion rate (Table 17).

Media strength played a role in stimulation of germination. BAP 22.20 and gibberellic acid 2.89 µM with MS full strength showed more germination percentage compared with the half strength MS medium (Table 18). Out of 30 embryoids inoculated in the full strength

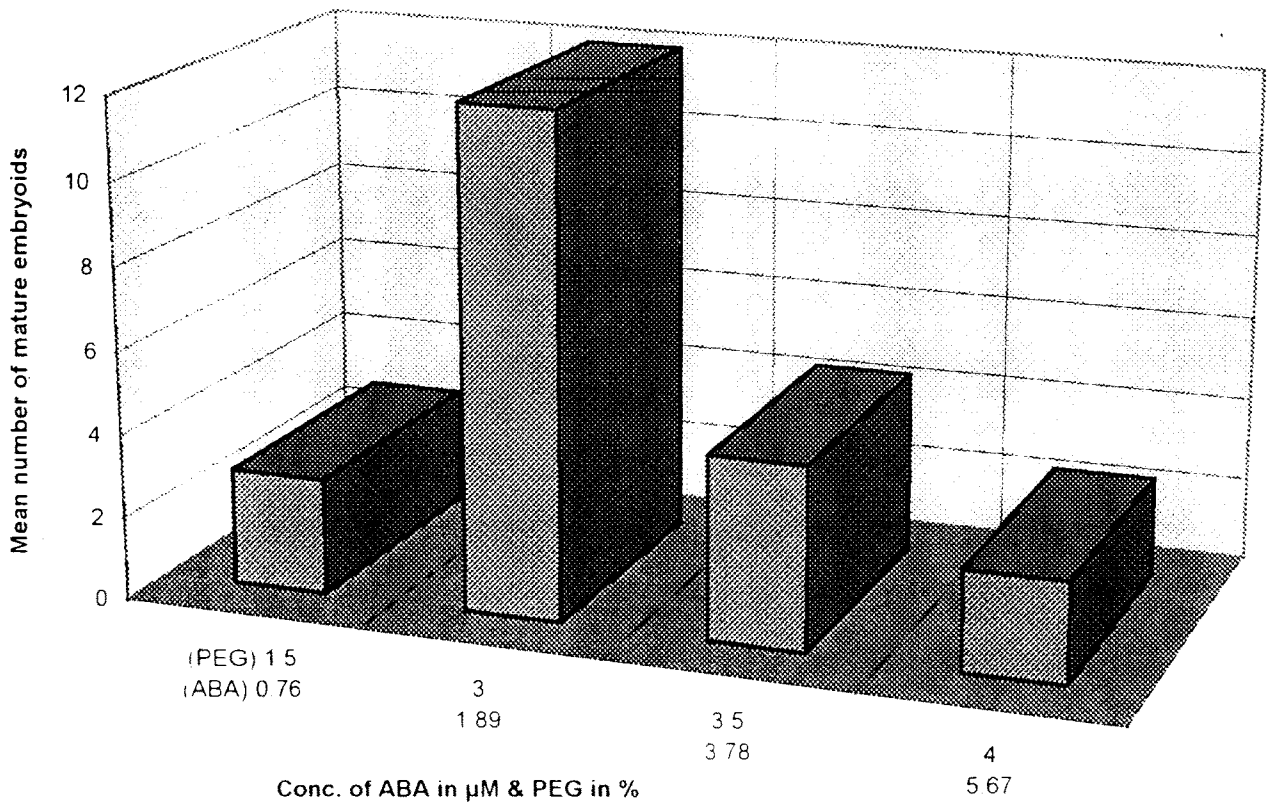


Fig. 19. Effect of ABA and PEG on maturation of somatic embryoids

Fig.20 Fully developed mature embryoids

Fig.21 Prominent rootpole developed after maturation

Fig.22 Globular, heart shaped, torpedo and cotyledonary stages of embryoid development.

Fig.23 Matured embryoids ready for germination.

Fig.24 Embryoids germinated and meristematic tissues sprouted in the MS medium.

Fig.25 Stages of germination of embryoids.

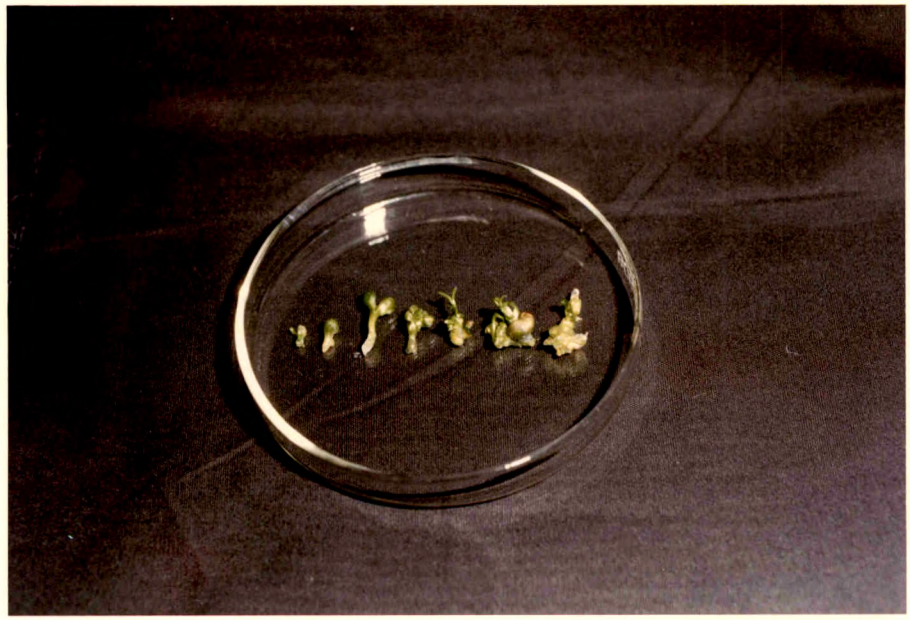


Table 16. Effect of ABA and PEG - 6000 on maturation of somatic embryoids after 4 weeks of culture.

Additives		z
ABA μM	PEG - 6000 (%)	Mean number of mature embryoids
0.76	1.5	2.8 ab
1.89	3.0	12.0 * c
3.78	3.5	4.4 b
5.67	4.0	2.4 a

S.E = 0.78

C.D = 1.70

* significant at 1% level

z - Mean of 5 replicates

Table 17. Effect of BAP and GA3 combination on conversion of somatic embryoids into plantlets.

Level of PGR μM		z Plantlet conversion (Mean)
BAP	GA3	
4.40	0.6	14.0 ab
8.90	0.14	21.0 c
13.30	0.58	11.0 a
17.80	1.44	18.0 bc
22.20	2.89	32.0 * d

S.E = 2.54

C.D = 5.4

* significant at 1% level.

z - Mean of 5 replicates

Table 18. Influence of nutrients on conversion of somatic embryoids into plantlets.

Level of		Conversion of somatic embryoids into plantlets		z Mean
BAP μM	GA3 μM	MS full strength	MS half strength	
4.40	0.6	12.2 a	13.2 a	12.7
8.90	0.14	15.2 ab	13.6 ab	14.4
13.30	0.58	20.2 abc	19.2 abc	19.7
17.80	1.44	23.8 c	21.6 bc	22.7
22.20	2.89	42.0 d	23.8 c	32.9
	Mean	22.7	18.3	-

S.E = 3.99

C.D = 8.06

significant at 1% level.

z - Mean of 5 replicates

medium, 22 embryoids germinated and produced well developed prominent shoot tip (Fig. 26). Half strength medium effected delayed germination and slow shoot growth. Germination resulted in normal looking plantlets with elongated hypocotyls and well developed roots in one month period.

3.5.5 Benzylaminopurine (BAP) and gibberellic acid (GA_3) on multiple shoot formation and multiplied shoot elongation from single embryoid

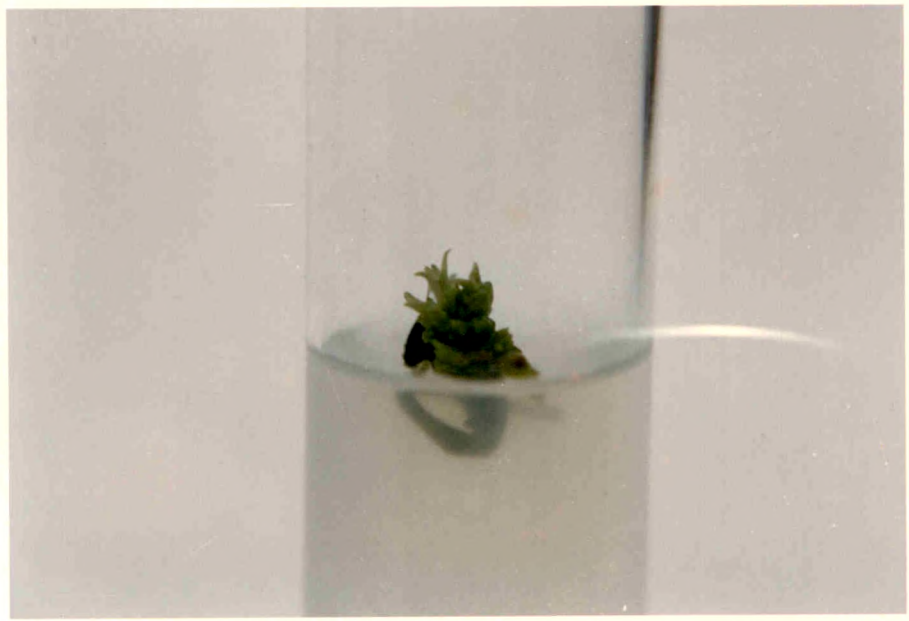
Normally single embryoid will give rise to a single plant. But in this study a few embryoids produced more multiple shoots (Fig. 27) within a single embryoid. In the beginning, multiple shoots produced root pole and after shoot proliferation it was suppressed (Fig. 28) It was observed that in the same BAP and gibberellic acid concentrations and medium strength, some of the embryoids expressed high rate of multiple shoot formation from a single embryoid. After six subcultures at 10 days interval (two months), a clump of multiple shoots containing small tiny shoots with actively growing points were emerged. Around 100 small tiny shoots were isolated and inoculated in the test tubes accounting for multiplication rate of 1 : 100 (Figs. 29 and 30). The present multiplication rate works out to 1:1000 (Fig. 31). This multiplication rate was achieved after three subcultures at 10 days interval. After three months, on

Fig.26 Germinated embryoids producing shoots.

Fig.27 Proliferation of multiple shoots from single embryoid.

Fig.28 Multiplied shoots producing root initial

Fig.29 1:10 multiplication rate achieved after first subculture.



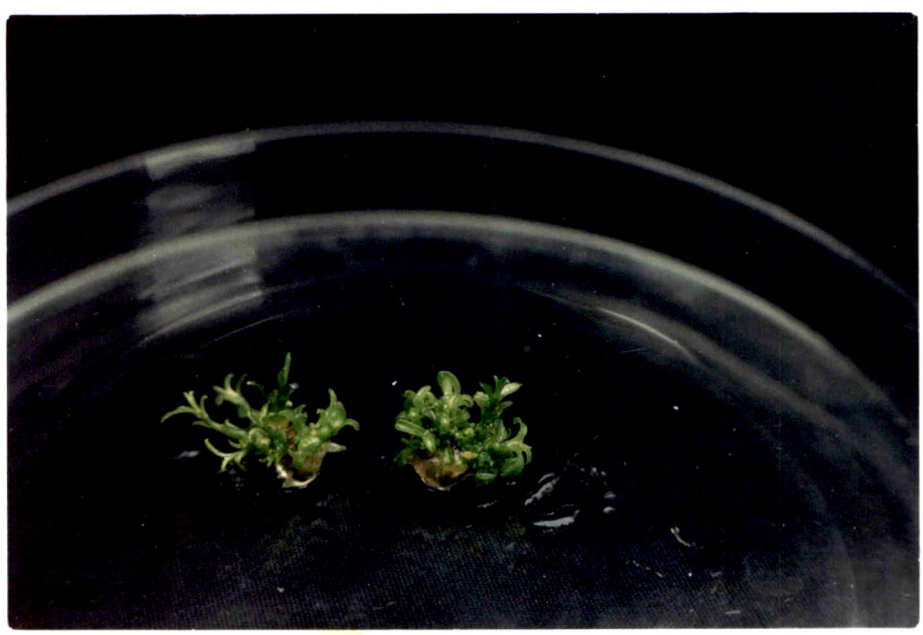


Fig.30 1:100 multiplication rate achieved after second month of subculture.

Fig.31 Further proliferation of vigorous multiple shoots followed by third month of subculture and accounts for 1:1000 multiplication rate.

subculture to elongation medium these shoots were further multiplied and elongated.

This finding has got great scope to produce more shoots from a limited number of embryoid and thereby production of more somaclones for screening purposes could be obtained. Also, this system can be considered as another model for rapid micropropagation to produce more plants within a short period (Fig. 32).

These micro shoots were inoculated in the elongation medium. Gibberellic acid level at 4.33 µM had effected in quicker elongation of multiple shoot within one month (Fig. 33) These elongated shoots were inoculated for rooting in liquid quarter strength MS liquid medium supplemented with IBA 19.60 µM (Fig. 34).

3.5.6 Rooting

During maturation stage, 70% of the embryoids produced well developed roots before shoot meristem develops. After root formation, the pseudo cotyledons were opened and shoot meristem had emerged out in the germination medium. Embryoids attained the growth of complete plantlets after one month. These plantlets possessed prominent root and shoot. More number of root initials were observed in quarter strength MS liquid medium with IBA 19.60 µM when compared to 14.70 µM IBA in one month period (Fig. 35) This is the ideal stage to transfer them to small pots for

Fig.32 Multiple shoots separated from the clump and subcultured for further multiplication

Fig.33 Multiplied shoots elongating in the MS medium

Fig.34 Separated multiple shoots rooted in the quarter strengtl MS liquid medium

Fig.35 More number of root initials in IBA 19.60 µM compare to 14.70 µM.

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hardening. All the rooted plants had radially distributed fibrous root system (Fig. 36) Multiple shoots were elongated after one month and individual shoots were separated and inoculated for rooting in quarter strength MS liquid medium supplemented with IBA 19.60 µM (Fig. 37), whereas plantlets emerged from individual embryoid were hardened directly since they had well developed root initials (Fig. 38).

3.6 IN VIVO STUDIES

3.6.1 Hardening of embryoid derived plants

Plants rooted in either way were transferred to small pots containing soil : sand mixtures at 1 : 1 ratio (v / v); bottom of the poly pot filled with soil and sand mixture and topdressed with red soil. The pH of the soil was adjusted to 4.8 as tea grows well at this pH. Rooted plants were transferred into fibre glass mist chamber (9 m x 4.5 m) (Fig. 39).

Hardening in the mist house:

Plants rooted directly from the embryoids were tested in different hardening substrates such as soil and sand, vermiculite, perlite and peat moss. It was found that soil and sand recording 60 per cent survival rate was adjudged as the best (Fig. 40). All the plants were given soil nutrients at fortnightly interval and foliar spray of zinc

Fig.36 Initiated roots produced radially distributed fibrous root system.

Fig.37 Multiple shoot derived plantlet. Ready for hardening.

Fig.38 Plantlets produced from single embryoid without the mediation of multiple shoot. Ready for hardening.

Fig.39 A view of fibre glass mist chamber used for hardening (9 cm x 4.5 m)

Fig.40 Hardening in the small pots containing soil and sand 1:1 ratio.

sulphate at weekly interval to boost the growth as per the nursery practices (Fig. 41). In the case of embryoid multiple shoots, they rooted well in quarter strength MS liquid medium supplemented with IBA 19.60 µM. These plants survived well in soil and sand media recording 80 per cent survival. Plants were transferred to bigger pots for further growth after 60 days (Fig. 42). Hardened plants were transferred to the field after six months for further field evaluation.

3.6.2 Morphological parameters

3.6.2.1 Leaf characteristics

when compared to conventionally propagated control, somaclonal plants expressed many morphological variations. All the plants had elliptic leaf shape but plant number three had narrowly elliptic and number four had broadly elliptic. Leaf tip was acuminate except in plant number four where it was acute. Leaf base was obtuse but truncate in the case of number four.

Leaf pose was erect and pubescent in all the plants. Leaf area was recorded more in number four (67.29 sq.cm.) and in number five (61.16 sq.cm) over the control and other embryoid derived plants. Petiole length was normal. Leaves were serrate but bluntly serrated in number two; deeply serrated (sharp) in number four and deeply serrated (blunt) in number five. Leaf venation appeared normal in all the plants and number three had prominent (matted) venation

Fig.41 Plants given soil nutrients at fortnightly interval and foliar spray at weekly interval.

Fig.42 Grownup plants transferred to bigger pots for further growth.

(Table 19). The above results confirmed 70 percent phenotypic variation. The conventionally propagated plants were looking normal (Fig. 46) but, in some of the somaclonal plants, the leaf was totally shrinked, reduced in area and appeared cream yellow colour where the chlorophyll was absent (Fig. 47). This confirms the malformation of leaves from embryogenesis.

3.6.2.2 Morphological growth pattern

The total height of the plants was showing normal growth during first four months and their height increased two fold in ninth month over the conventionally propagated control. Internodal length was normal during first four months and increased in ninth month. number of leaves also have shown the increased pattern of growth (Table 20). The observations confirmed the increased vigour of embryoids derived plants compared to the control (Fig. 48).

3.7 EVALUATION OF PROGENY IN THE NURSERY

3.7.1 Histological examination

Histological observations of the embryo - producing region of the cotyledon explants revealed that the zone of preferential cell division arose as patches of parenchymatous cells. Early stages of somatic embryogenesis were observed within the cotyledonary tissue as distinct and organized zones (Fig. 43). Globular or torpedo -

Fig.46 Normal leaves of conventionally propagated control plant.

Fig.47 Variation in the leaf structure and pigmentation of somaclonal plant.

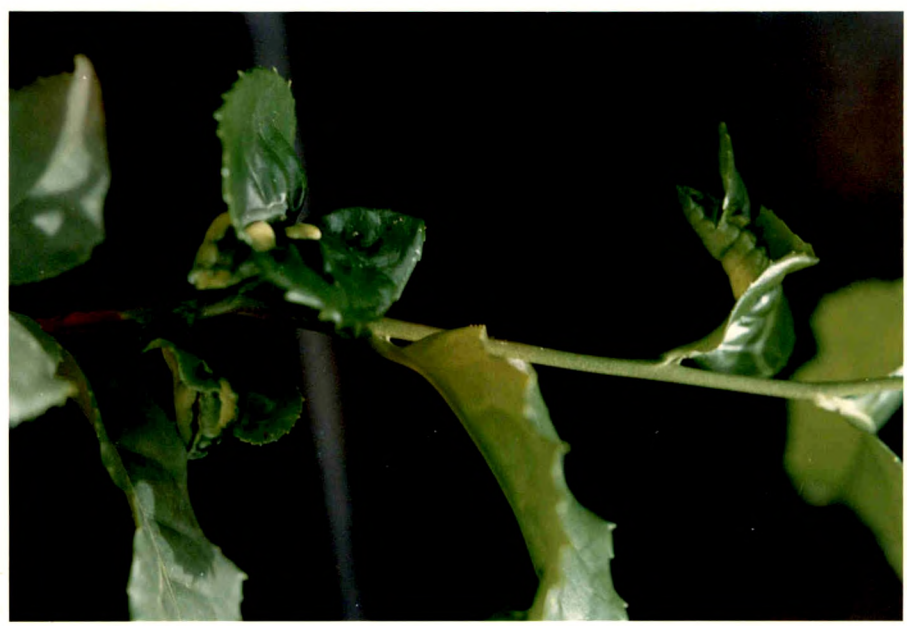


Fig.48 Improved vigour of somaclonal plants over conventionally propagated control.


C
SC
SC
SC

Table 19. Leaf characteristics of somaclonal plants.

Plant No.	Leaf shape	Leaf tip	Leaf base	Leaf pose	Pube-sent	Leaf area (Sq.cm)	Petiole length (mm)	Serra-tion	Vena-tion
C	E	AC	OB	Erect	Present	26.52	4	SE	N
SC-1	E	AC	OB	do	do	52.19	5	SE	N
SC-2	E	AC	OB	do	do	54.61	6	BSE	N
SC-3	NE	AC	OB	do	do	40.0	3	SE	P
SC-4	O	A	TR	do	do	67.29	6	DSEs	N
SC-5	BE	AC	OB	do	do	61.16	5	DSEb	N

C- Conventionally propagated plant

SC- Somaclonal plants

E- Elliptic

NE- Narrowly elliptic

BE- Broadly elliptic

AC- Acuminate

OB- Obtuse

O- Ovate

SE- Serrate

BSE- Bluntly serrated

DSEs- Deeply serrated (sharp)

DSEb- Deeply serrated (blunt)

N - Normal

P - Prominent (matted above)

TR- Truncate

A- Acute

Table 20. Morphological growth pattern of somaclonal plants.

Plant No.	Total height (cm)			Internodal length (cm)			No.of leaves		
	months			months			months		
	1	4	9	1	4	9	1	4	9
Control	3.5	9.5	13.5	0	1	2	4	6	8
SC-1	5.0	10	28.5	0	1	2.5	3	5	8
SC-2	5.5	10.3	16.5	0	1	1.7	3	5	8
SC-3	4.0	9.3	20.5	0	0.5	3.5	2	6	14
SC-4	1.5	11	24.5	0	0.4	2.5	3	7	11
SC-5	2	12	27.4	0	0.7	3.0	3	6	19

SC - Somaclonal plants

Control - Conventionally propagated plant

shaped somatic embryos developed towards the outer surface of the cotyledonary explant. Root and shoot meristems were connected to each other by vascular strands.

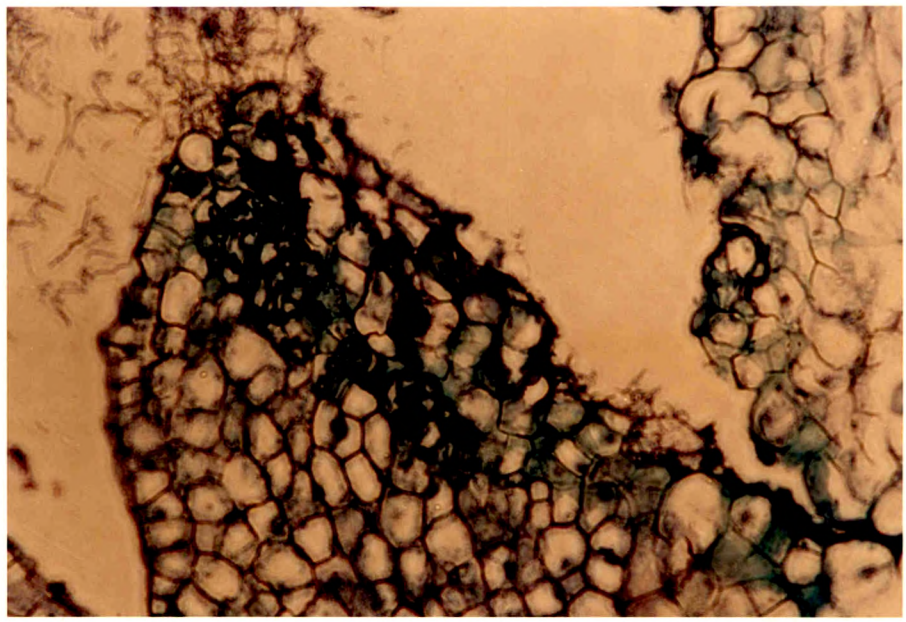
Freshly isolated cotyledon pieces were composed of large uniform cells containing numerous starch grains. There were no apparent differences between the lower surface (adaxial) and upper surface (abaxial) tissues. After 2 weeks of culture, explants had become swollen and had changed from pure white to green in colour. Starch grains were being metabolized preferentially in the adaxial tissues.

Large nuclei appeared and mitotic activity was initiated in cells with few starch grains near the adaxial surface. Divisions continued within the central and adaxial regions of the cotyledon (Fig. 44). The development of the raised areas (localized swellings) of tissue on the adaxial surface was associated with this cell division activity. Such raised areas often developed around wounded tissues and at the edge of the explant.

When cell division activity reached the adaxial surface, further divisions occurred within groups of adjacent surface cells. These divisions gave rise to tissues composed of smaller cells with densely staining cytoplasm and large nuclei which, on continued development, formed embryonic lobes and ridges of embryogenic tissue was noticed in L.S. of an embryoid clump (Fig. 45). As embryogenic

Fig.43 Embryogenesis observed within cotyledonary tissue as distinct and organized zones. - L.S. of an embryoid.

Fig.44 Mitotic activity continued within the central and adaxial regions of the cotyledon - L.S.of an embryoid.



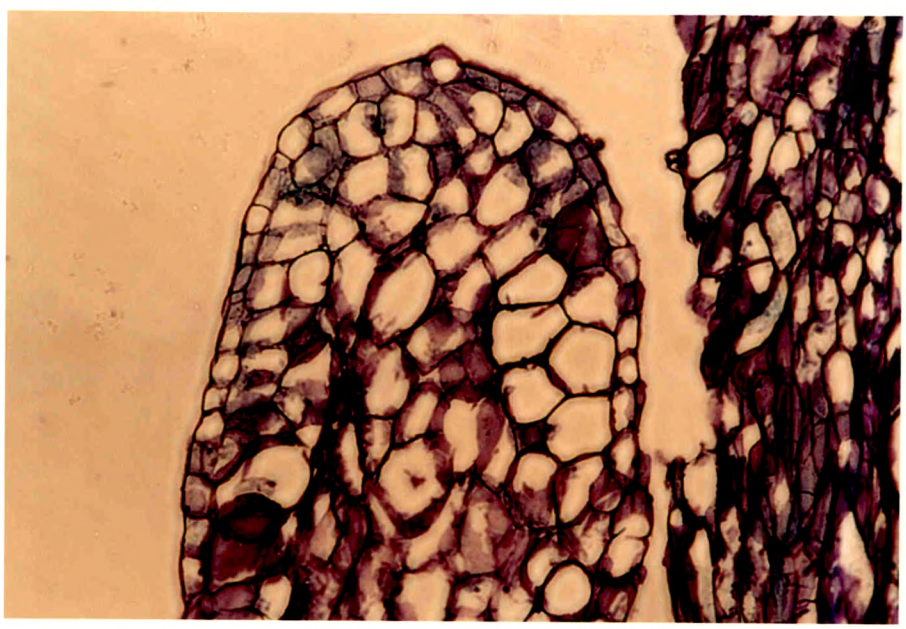
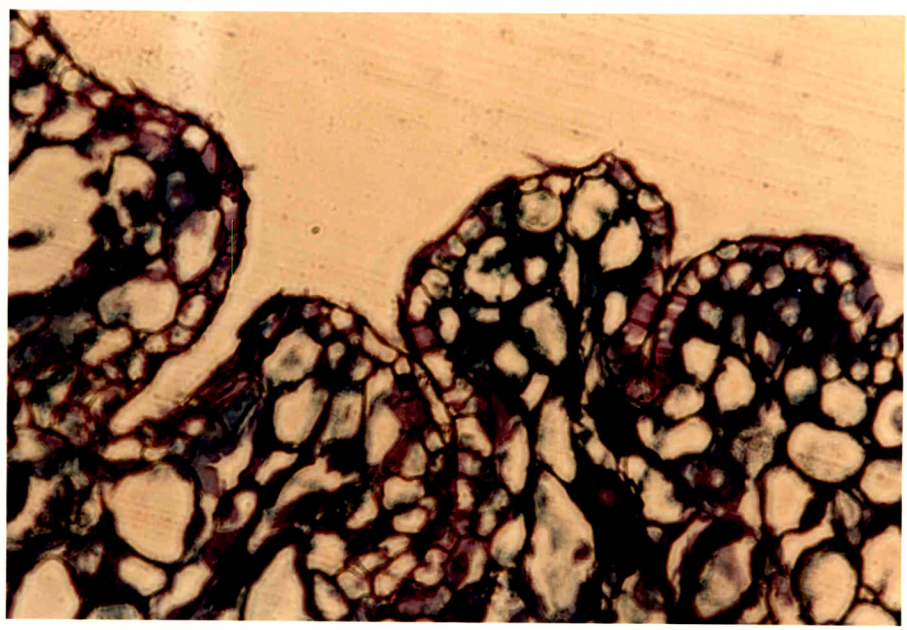


Fig.45 Embryonic lobes and ridges formed on continued development followed by further cell division - L.S.of an embryoid clump.



tissues developed, disorganized cell proliferation in abaxial tissues resulted in friable callus formation .

3.7.2 Proline status

Proline is a basic amino acid found in high percentage in basic proteins. Free proline is said to play a role in plants under stress conditions. Though the molecular mechanism has not been established for the increased level of proline, one of the hypotheses refers to break down of proteins into amino acids and conversion to proline for storage.

Proline content was estimated for seven somaclonal plants. The results indicated that the proline content was normal in all the plants and there was no significant increase in the proline content over the control (Table 21).

3.7.3 Net photosynthetic rate (Pn)

The Pn rate was high in plant number four and five (4.8 and 4.3 respectively) with concomitant increase in water use efficiency (1.18 and 0.81 respectively) (Fig. 49). Other plants were showing normal rate as the control (Table 22).

3.7.4 Stomatal counting

The intensity of stomata was more in control plant than in embryoid derived plants (Fig. 50). Number of stomata in mm^2 area of leaf in somaclonal plants were 152 while in control it was 184.

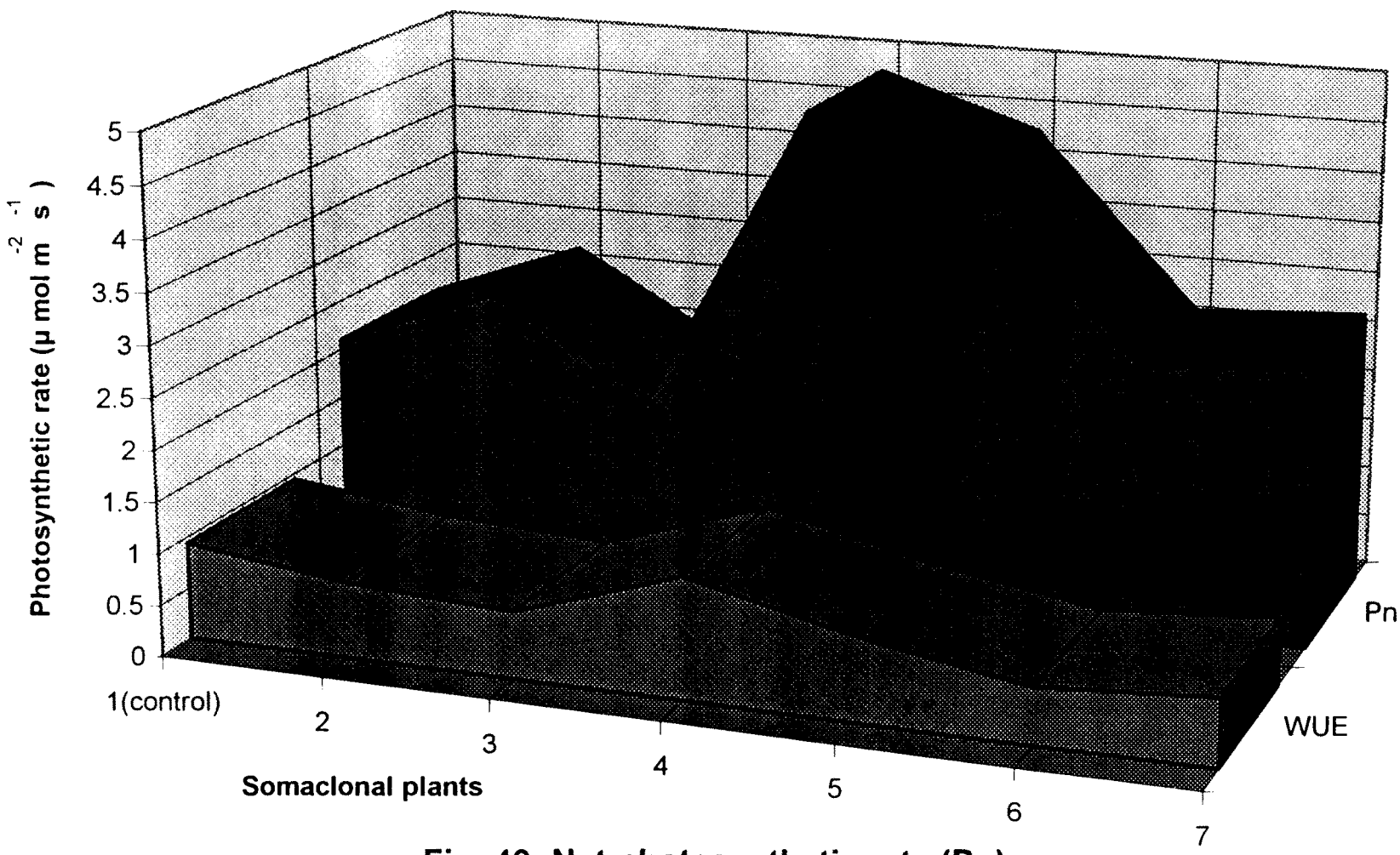


Fig. 49 Net photosynthetic rate (Pn)

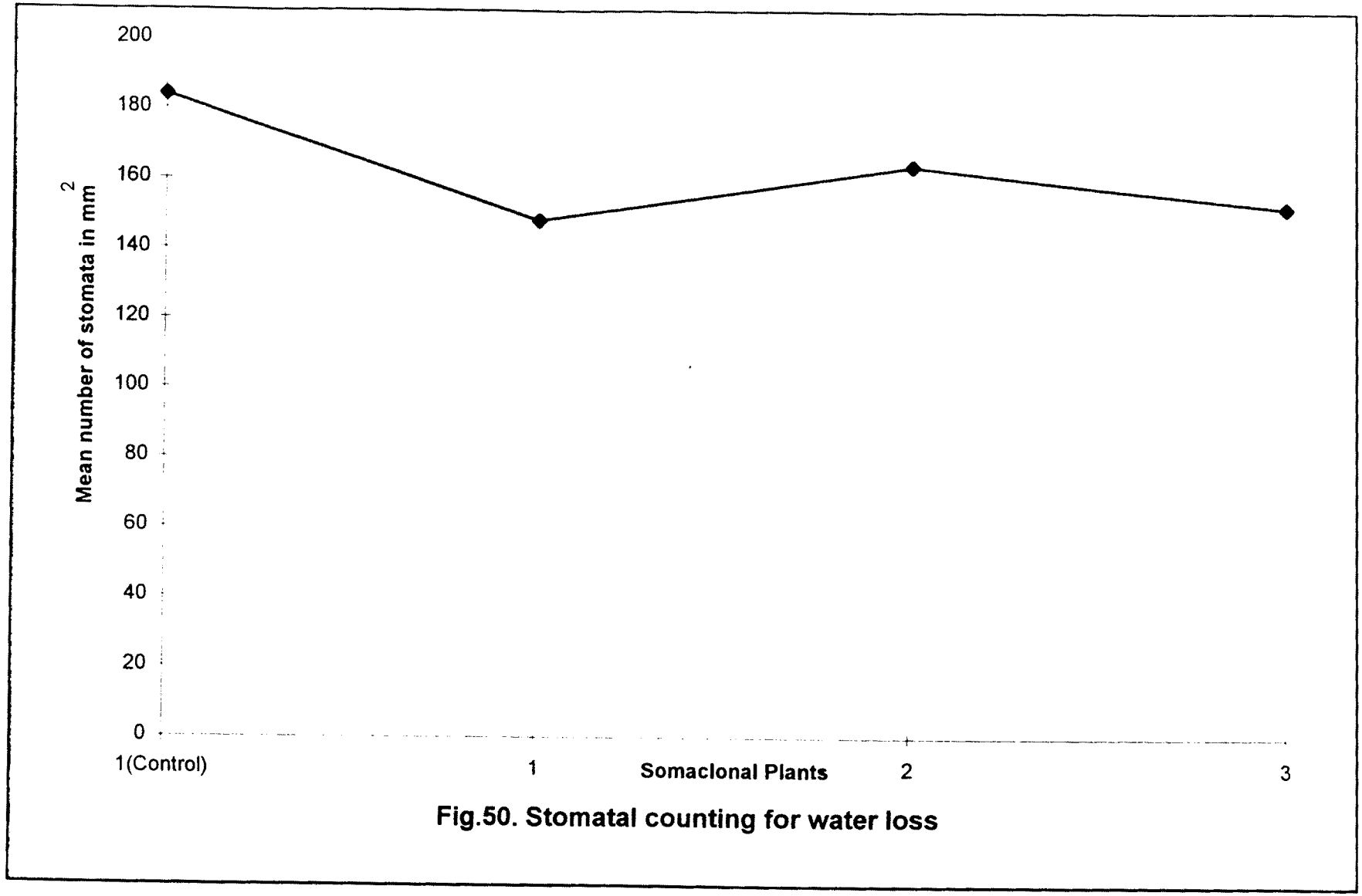


Fig.50. Stomatal counting for water loss

Table 21. Proline status of embryoid regenerated plantlets.

Plant No	proline content	
	ppm	μ mol/g
control	43.4	0.3757
SC-1	41.2	0.3567
SC-2	54.5	0.4718
SC-3	43.0	0.3722
SC-4	47.0	0.4069
SC-5	57.0	0.4935
SC-6	65.0	0.5627
SC-7	62.0	0.5367

SC - Somaclonal plants

Control - Conventionally propagated plant

Table 22. Net photosynthetic rate (Pn).

Somaclonal plants	WUE *(mean)	Pn *(mean)
1 (control)	0.94	2.1
2	0.74	2.7
3	0.65	1.8
4	1.18	4.8
5	0.81	4.3
6	0.54	2.6
7	0.65	2.7

Control - Conventionally propagated plant

* Mean of three observations

SEt	:	0.34	0.82
C.D. at P 0.05%	:	0.75	1.78

Embryoid derived plants exhibited less number of stomata per unit area than control (Table 23).

3.7.5 Response of somaclonal plants to moisture stress

3.7.5.1 Response to temperature and moisture stress

Plants with watering (four days interval) at 20° C has taken six to ten days to unfold the bud following to seven to eleven days at 25° C and ten to thirteen days at 30° C respectively (Fig. 51).

Plants without watering at 20° C has taken seven to eleven days to unfold following to ten to twelve days at 25° C and twelve to fifteen days at 30° C (Fig. 52). Control plants grown in the mist chamber at 25° C with intermittent misting recorded three to five days to unfold the bud. The rate of growth of plant was affected by both high temperature and drought. There was no morphological variation between control and somaclonal plants except the growth rate. It was found that stresses affect adversely the growth rate of plants.

3.7.5.2 Response to osmoticum & water stress

Exposure of somaclonal and control plants to PEG at 10,20 and 30 percent for 10 min. was enforced. After 24 hours it has been observed that 10 percent PEG exposed for 10 min. had no symptoms of wilting whereas PEG with 20 and 30 percent had wilting of leaves. After a weeks time, plantlets exposed for 10 min. in 10 percent PEG

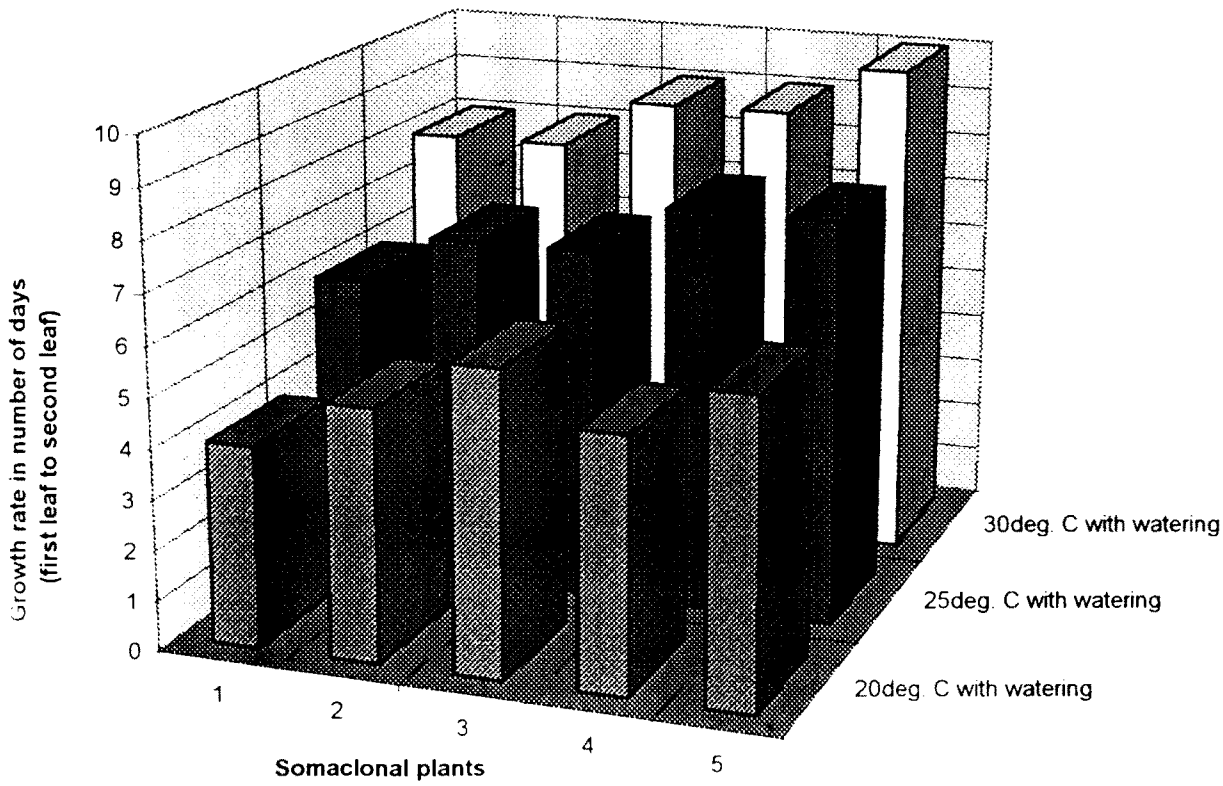


Fig. 51. Studies on the growth rate of embryoid plants under artificial stress conditions with watering

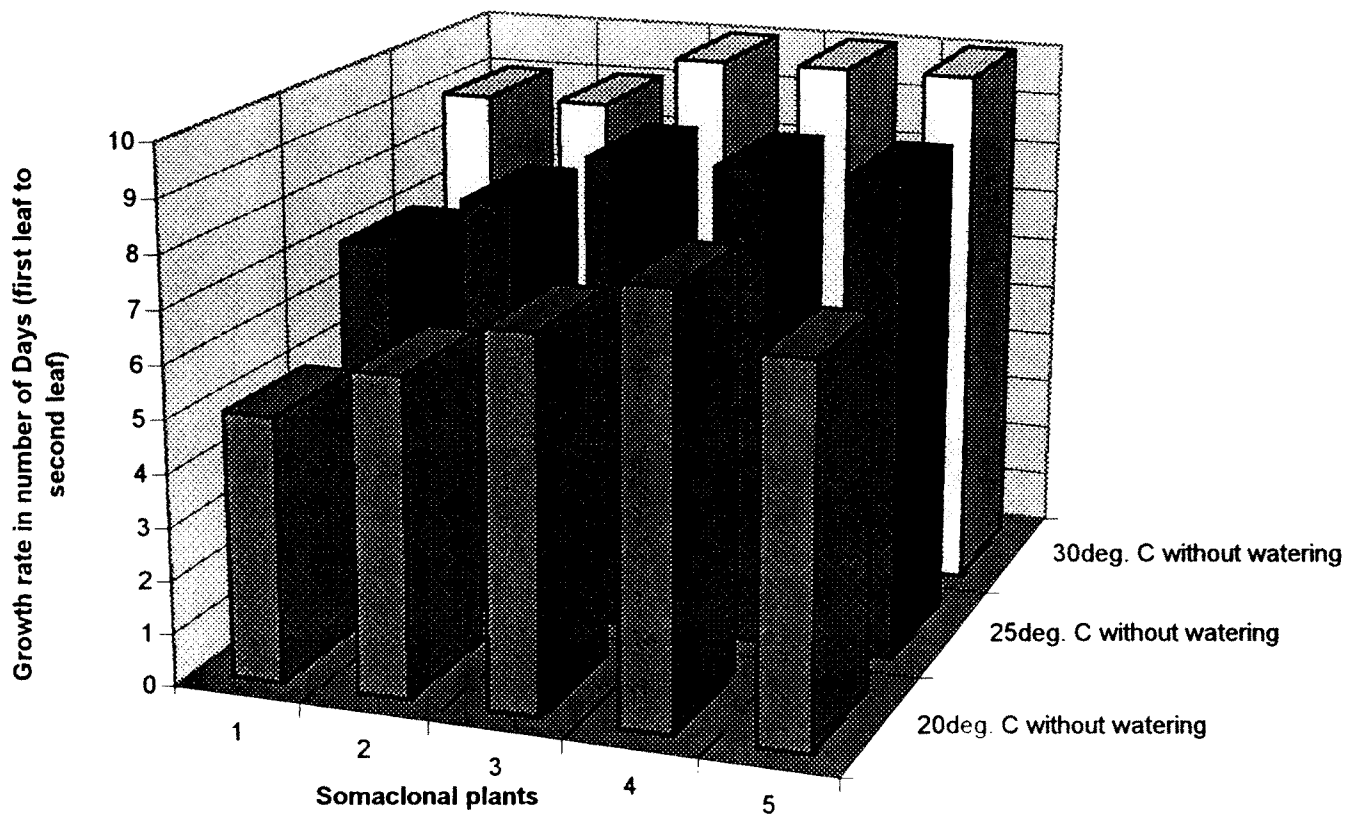


Fig. 52. Studies on the growth rate of embryoid plants under artificial stress conditions without watering

Table 23. Stomatal counting to study the transpiration loss in somaclonal plants.

Plant No.	* Mean Number of stomata per mm^2
Control	184 a
SC-1	148 ab
SC-2	164 ab
SC-3	152 b
Total	648

SE : 14.48

C.D at 5% : 32.8

Control - Conventionally propagated plant

* Mean of four replication

* No. of stomata in mm^2 of young leaves

SC-1 is significant

SC-2 & SC-3 are nonsignificant

remained same without wilting and 20 and 30 percent concentrations resulted in complete wilting of plantlets. The survived plants under higher percentage of PEG should be further tested for their tolerance to stress.

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