REFINEMENT OF *IN VITRO* PROPAGATION TECHNIQUE IN PINEAPPLE VAR. MAURITIUS AND MASS MULTIPLICATION OF ELITE CLONES

BY

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THESIS

submitted in partial fulfilment of the requirement
for the degree of

Master of Science in Horticulture

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1996

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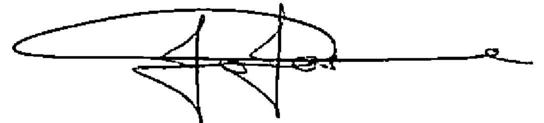

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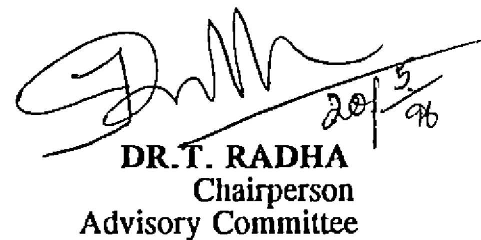

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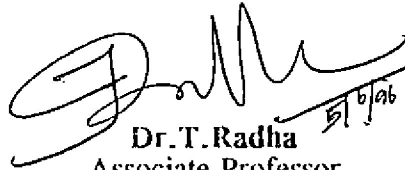

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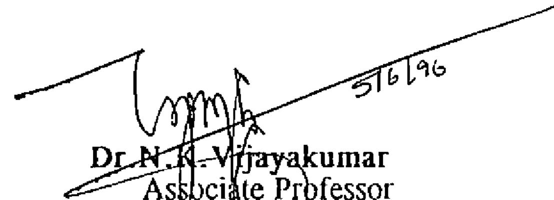

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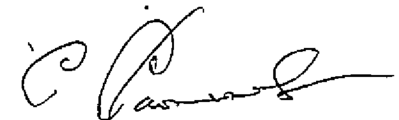

EXTERNAL EXAMINER

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JO JOSE, C.

Dedicated to my parents

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ABBREVIATION

MS	-	Murashige and Skoog
BAP	-	N^6-Benzyladenine
KIN	-	Kinetin
NAA	-	α-Napthalene acetic acid
IBA	-	Indole-3-butyric acid
IAA	-	Indole-3-acetic acid

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Introduction

INTRODUCTION

Pineapple (*Ananas comosus* L. Merr.) a member of the family Bromeliaceae, is one of the choicest fruits of the tropical and subtropical countries. It is an amazingly versatile and delicious fruit used both for table purposes and processing.

The leading pineapple growing states in India are Assam, Kerala, West Bengal, Meghalaya and Karnataka. An area of 5033 ha (FIB, 1995) is under pineapple cultivation in Kerala, the main growing areas being in Ernakulam and Kottayam districts.

Though, two varieties, namely, Mauritius and Kew are grown in the state, Mauritius occupies more than 80 percentage of the area in the above districts (KHDP Final Report, 1990). It is the choice variety for fresh consumption and for extraction of juice.

Eventhough, Mauritius is considered to be the leading pineapple variety of Kerala, practically no work has been carried out for the improvement of this variety. Wide variations exist in this variety,with regard to fruit weight and other characters. This variability can be commercially exploited through clonal selection of plants for higher yield and other desirable fruit characters.

Clonal selection is much easier and requires less time to secure the start of a new variety, than to develop one by hybridization and selection. Selection of elite clones from a population and their evaluation for confirming their superiority can be used as an effective tool of crop improvement in Mauritius variety.

Once the superior types are located, they must be subjected to replicated trials, so as to reliably estimate the value of that clone. For this sufficient number of homogeneous plants need to be produced within a short period. The propagation through traditional methods, namely, suckers, crowns, slips, stem bits and butts are inefficient and about 6 years would be required to obtain sufficient planting material for a replicated plot test (Collins, 1960). Also the difference in growth pattern and asynchronous flowering among the plants propagated through different propagules make the evaluation tests rather difficult and tedious. In view of the above facts, there must be an upsurge of interest in the field of rapid clonal propagation of Mauritius variety of pineapple applying micro propagation technique in crop improvement programmes, wherein rapid multiplication of selected clones are possible.

Aghion and Beauchesne (1960) were the first to report successful micro propagation in pineapple. There after many workers like Mathews *et al.* (1976), Wakasa *et al.* (1978), Fitchet (1985), Prabha (1993) and Kiss *et al.* (1995) worked in the field of pineapple tissue culture and protocols were developed for different varieties of pineapple. However, a suitable *in vitro* propagation technique for rapid clonal propagation of Mauritius variety of pineapple is still lacking. In this context, the present studies were undertaken with the following major objectives.

i. Selection of elite clones of Mauritius variety of pineapple.

ii. Refinement of the *in vitro* propagation technique in pineapple to suite for Mauritius variety, and

iii. Mass multiplication of selected elite clones, using the refined micro-propagation technique for further evaluation and confirmatory trials.

Review of Literature

2. REVIEW OF LITERATURE

The present studies on the refinement of *in vitro* propagation technique in pineapple var. Mauritius and mass multiplication of elite clones were carried out at Kerala Horticulture Development Programme, Kerala Agricultural University, Vellanikkara during 1993-95. Literature on clonal selection and *in vitro* multiplication in pineapple is reviewed in this chapter.

2.1 Clonal selection of elite plants

Clonal selection and multiplication offers a viable tool for crop improvement in pineapple. Clone is a group of plants produced from a single plant through asexual reproduction. Thus asexually propagated crops will have a large number of clones.

Though pineapple is propagated asexually, variations in the plant and fruit characters in a single variety are very often observed. These variations are mostly due to spontaneous bud mutations (Singh *et al.*, 1976). Reports on existence of variations in both plant and fruit characters in pineapple are available. Collins (1960) reported several types of mutations in Cayenne group of pineapple. Spiny leaves, multiple crown, enlarged fruitlets, increased fruit length etc. are certain types of mutations reported by the author. Marr (1965) described several mutated forms in pineapple and stressed the need for selection of plants for yield and quality. Chimera and gene mutations for some leaf characters in cultivar Kew was reported by Singh *et al.* (1976). According to him, 'Kew' appears to be in a heterozygous form for smooth spiny tip character, where in a mutation leading to homozygous recessive spineness.

Elite types of pineapple are selected based on both plant and fruit characters. Vegetative vigour of a plant can be an index of its yield potential. Vegetative vigour is usually assessed based on biometric characters, such as, plant height, leaf number, 'D' leaf area and general appearance of plant. Brown (1953) indicated the list of characters that should be looked for, while selecting superior plants in pineapple. It included, general vigour of plant and large sized fruit with good qualities. He also suggested certain methods for obtaining a rapid increase of planting material from selections, such as, stem slices from sucker, crown, slips and stimulation of basal suckering.

Collins (1960) listed some of the desirable characters to be considered for plant selection in pineapple. They were hardy and vigorous plants with good shoot and root system, larger fruit size, good shaped fruits, good flavour and aroma, non-fibrous juicy flesh, high sugar content and resistance to diseases like heart rot and root rot.

Reports on correlation studies between vegetative and fruit characters in pineapple are also available. Su (1958) observed positive correlation of leaf length and width with the fruit weight in pineapple. Chen and Chi (1963) reported that fruit weight decreased with the number of leaves removed for fibre extraction. A positive correlation of 'D' leaf area with fruit weight was reported by Wu and Su (1965).

Chadha *et al.* (1977) conducted an extensive study on plant characters affecting both fruit weight and quality. Fruit weight was found to increase significantly with an increase in the number of suckers and the leaf number, one year after planting. An increase of a single sucker contributed 0.252 kg towards fruit weight.

Similarly an additional leaf at one year after planting contributed 2.8 g to the fruit weight, when the fruit weight was 1.3987 kg and above. Total soluble solids content of the juice was positively correlated with leaf number and the potential leaf area index.

The major variable contributing to a larger part of variation in fruit yield was the number of leaves per plant, as per Prabhakaran and Balakrishnan (1978). An increase in one leaf at the time of flowering, would be followed, on an average by an expected increase of 13 g in fruit weight.

Similar reports on effect of leaf population on yield have been reported in other crops like sweet potato (Shanmughavelu and Ramaswamy, 1973).and grapes (Winkler, 1930).

Many workers have successfully employed clonal selection for crop improvement in pineapple. Wee (1974) reported that, Masmerah is a selection variety of pineapple from a cultivar population of Malaysia. The fruit size of this improved cultivar was about 50 per cent better than that of the contemporary Singapore Spanish.

At Kerala Agricultural University an attempt was made to assess the natural variability and to select the promising types in Kew. Out of the 19 clones collected and tested, seven were found to be superior than the local clone, with respect to yield (KAU, 1987).

2.2 *In vitro* propagation

Following the successful rapid multiplication of orchids through shoot meristem culture by Morel (1965) and Wimber (1965) there has been in recent

years, an increasing interest in the application of tissue culture technique as an alternate means of asexual propagation of horticultural plants. Since then, several crops are micro propagated and protocals are now available (Bhojwani and Razdan, 1983). Application of tissue culture for rapid clonal propagation has been reported as highly successful for many economically important tropical fruit crops (Litz *et al.*, 1985). Literature on *in vitro* studies in pineapple pertaining to the present study are briefly reviewed hereunder.

Murashige (1974) reported three possible routes for any *in vitro* propagule multiplication, namely,

i) enhanced release of axillary buds
ii) production of adventitious buds through organogenesis, and
iii) somatic embryogenesis

Only the first two routes have so far been reported in the case of pineapple till date.

Aghion and Beauchesne (1960) were the first to report successful *in vitro* culturing of pineapple. Since then, various aspects of successful *in vitro* propagation of pineapple have been reported by several workers (Lakshmisita *et al.*, 1974; Mathews *et al.*, 1976; Wakasa *et al.*, 1978; Mathews and Rangan, 1979; Rao *et al.*, 1981; Zepeda and Sagawa, 1981; Cabral *et al.*, 1984; Fitchet, 1985; Evans and Moore, 1987; Dewald *et al.*, 1988; Sharrock, 1992; Prabha, 1993; Sudhadevi *et al.*, 1994 and Kiss *et al.*, 1995).

2.2.1 Explants

For any *in vitro* propagation technique to be successful, selection of proper explant is very important. The different plant materials used as explant for *in vitro* propagation in pineapple include shoot tips and lateral buds from suckers, crowns, slips and also syncarp slices. The culture of excised buds of pineapple was first described by Aghion and Beauchesne (1960). They used axillay buds from crown as explants. Mapes (1973) selected shoot tips of the pineapple cv. Smooth Cayenne as explant. Lakshmisita *et al.* (1974) reported successful technique for *in vitro* meristem culture of pineapple using explants from 1.0-1.5 months old slips. Teo (1974) observed callus induction after 30 days of inoculation and this differentiated into plantlets after another 30 days. Mathews *et al.* (1976) used terminal or axillary buds isolated from crown for large scale multiplication of pineapple plantlets.

Crown axillary leaf bud was used as explant by Pannetier and Lanaud (1976). Good response was obtained by using various plant parts such as syncarp, axillary buds of suckers or slips, young crowns and young slips for *in vitro* culturing of pineapple. Wakasa *et al.* (1978) and Mathews and Rangan (1979) obtained multiple plantlets from dormant axillary buds excised from crown. They could successfully regenerate plantlets from leaf explants of *in vitro* obtained plants.

Drew (1980) reported micro propagation of pineapple using lateral buds from crown and suckers. Induction of embryonic callus from Kew x Queen hybrid seed was observed by Rao *et al.* (1981). Yang (1981) reported the use of stem tip for *in vitro* culturing of pineapple.

According to Zepeda and Sagawa (1981) pineapple could be propagated by *in vitro* culture of axillary buds from crowns of mature fruit. Axillary buds from off shoots of cv. Smooth Cayenne were used as explants by Cabral *et al.* (1984).

In a study conducted by Fitchet (1985) using pineapple cultivars Queen and Cayenne, the culture of basal buds from crown leaves proved successful. Evans and Moore (1987) suggested the use of axillary buds for clonal multiplication of Smooth Cayenne and Cambray pineapples.

Clonal propagation of pineapple plants by meristem culture was reported by Fitchet (1987). Liu *et al.* (1987) reported the *in vitro* propagation of spineless Red Spanish pineapple using lateral buds (0.4-0.8 mm) and meristem slips from crowns. Dewald (1988) and Fitchet (1990a) suggested lateral buds from crown, slips and stems as explant.

Fitchet (1990b) induced callus from crown apical region of Queen pineapple. Hirimburegma and Wijesinghe (1992) and Sharrock (1992) achieved culture initiation from shoot apices. Fitchet and Purnell (1993) reported a technique involving *in vitro* culture of lateral buds obtained from crowns as well as easily visible buds, and sections of the crown apical dome region for maximum utilization of crown for pineapple micro propagation.

Prabha (1993) and Sudhadevi *et al.* (1994) adopted shoot tip culture for *in vitro* multiplication of pineapple. Kiss *et al.* (1995) demonstrated a novel method for rapid propagation of pineapple using decapitated *in vitro* plantlets as explants.

2.2.2 Surface sterilization of explants

Explants collected from external environment are invariably harbouring numerous microorganisms which when inoculated into the nutrient medium cause contamination and compete adversely with plant material growing *in vitro*. Hence explants must be made contaminant free using suitable sterilants before inoculation.

Wakasa *et al.* (1978) used sodium hypochlorite solution at different concentrations for various explants. The explant material was initially washed well with tap water. After removing the leaves of suckers and slips, the small dormant axillary buds at the leaf bases were excised along with a part of shoot. These buds were then sterilized using five per cent sodium hypochlorite solution for 10 to 20 minutes followed by rinsing the material several times with sterile water. Small crowns and small slips used for culturing were also decontaminated in the same way. From the young syncarps of about 3.5 to 4 cm length, sepals, petals stamens and pistils were removed and then the material was immersed in 20 per cent sodium hypochlorite solution for 10-20 minutes. After removing the outside layer thinly, the syncarp was sliced 1 to 2 mm thick horizontally at first, then cut into small pieces. These pieces were thoroughly rinsed with sterile water before culturing.

Mathews and Rangan (1979) suggested the use of 0.1 per cent mercuric chloride for three minutes and then rinsing in sterile distilled water for surface sterilizing dormant lateral buds from crown.

Zepeda and Sagawa (1981) disinfected small crowns from mature fruit, after removing all leaves in 0.5 per cent sodium hypochlorite (10% clorox) with three drops of wetting agent (Tween 20) for 60 minutes. Axillary buds were then excised and sterilised using one per cent clorox for about 20 minutes.

Cabral *et al.* (1984) reported surface sterilization of axillary buds from off shoots with sodium hypochlorite solution (0.5 to 2%).

Dewald *et al.* (1988) suggested a detailed procedure for surface sterilizing axillary buds from crowns and stems of various pineapple cultivars. Initially the crowns and stems were rinsed in water, after defoliation. Then the materials were surface sterilized by agitation in 20 per cent clorox (1% sodium hypochlorite) with two to three drops of a surfactant (Tween 20) for 20 minutes followed by three rinses of 10 minutes each in sterile water. Under aseptic conditions, terminal and axillary buds were excised and disinfected using two per cent clorox solution for 10 minutes followed by three rinses of 10 minutes each in sterile water.

Fitchet (1990a) used 0.2 per cent sodium hypochlorite solution for sterilising lateral buds from the crown.

Zee and Munekata (1992) excised axillary bud pieces of size 1 cm^3 from mature green house grown plants and decontaminated with 15 per cent clorox solution (5.25% sodium hypochlorite) containing Tween-20 (two drops/100 ml) for 15 minutes followed by 10 per cent clorox + Tween-20 for another 10 minutes. Explant size was then reduced to a size 5 mm^3 in five per cent clorox and Tween-20 and soaked for one hour in one per cent clorox solution. Buds were then rinsed in sterile water for 10 minutes.

Prabha (1993) reported that for minimum contamination and maximum explant survival, treatment with Emisan 0.1 per cent for 30 minutes followed by 0.1 per cent mercuric chloride for 10 minutes was the best.

2.2.3 Culture medium

Successful *in vitro* culture as a means of plant propagation depends largely on the choice of nutrient medium, including its chemical composition and physical form (Murashige, 1974). For proper growth and morphogenesis of plant tissue, the medium should provide both major plant nutrients like salts of nitrogen, phosphorus, potassium, magnesium and sulphur and micronutrients such as salts of iron, manganese, zinc, boron, copper, molybdenum, cobalt and also carbohydrates, usually sucrose, less weight organic compounds like vitamins, aminoacids and plant growth regulators. Medium containing the Murashige and Skoog (MS) mineral salt mixture (Murashige and Skoog, 1962 is found to be suitable for *in vitro* culture of pineapple.

Aghion and Beauchesne (1960) stimulated growth of plantlets from axillary buds on Knop's medium. According to Mapes (1973) MS basal medium was ideal for the production of meristematic protocorm like bodies and plantlets. Lakshmisita *et al.* (1974), used Knudson's basal medium with Nitsch's micro elements for *in vitro* propagation of pineapple.

Several other workers such as Pannetier and Lanaud, 1976; Wakasa *et al.*, 1978; Mathews and Rangan, 1979; Cabral *et al.*, 1984; Ramirez, 1984; Evans and Moore, 1987; Liu *et al.*, 1987; Rosa-Marquez *et al.*, 1987; Dewald *et al.*, 1988; Marchal and Alvard, 1988; Zee and Munekata, 1992; Bordoloi and Sharma, 1993; Prabha, 1993 and Kiss *et al.*, 1995 have reported MS medium as most ideal for *in vitro* culturing of pineapple.

Yang (1981) developed pineapple cultures initially in half strength MS medium. Fitchet (1990a) reported the use of Murashige and Tucker's (MT) medium for pineapple *in vitro* propagation.

2.2.4 Growth regulators

Plant growth regulators are essential for manipulation of growth and development of explants *in vitro*. Their concentration and ratio in the medium often determine the pattern of development in culture (Krikorian, 1982).

Auxins and cytokinins are the two major classes of growth regulators which are of special importance in plant tissue culture. The most commonly used auxins include NAA, IBA, IAA and 2,4-D. Among cytokinins, BAP, kinetin and 2ip are important (Pierik, 1987). The interaction and balance of these two principal group of growth regulators decide the type of morphogenesis in culture. Shoot induction is promoted when cytokinin level is higher than that of auxin, while rooting is enhanced by high auxin concentration. Intermediate concentration of these will favour unorganised callus growth.

According to Murashige (1974) cytokinin could break apical dominance and enhance growth of lateral buds from leaf axils. Among the various cytokinins, BAP was found most effective and widely used for shoot tip, meristem and bud culture followed by kinetin and 2ip. High cytokinin content was deleterious to the initiation and elongation of roots of plants (Lo *et al.*, 1980).

Exogeneous auxins were not always needed for culture establishment and axillary shoot proliferation, but their presence could improve culture growth (Hu and

Wang, 1983). According to Lundergan and Janick (1980) the possible role of auxin in culture proliferation medium is to nullify the suppressive effect of high cytokinin concentration on axillary shoot elongation, thereby restoring normal shoot growth. Hasegawa (1980) reported that very high concentration of auxin may induce callus.

2.2.5 The routes

The two major routes adopted for *in vitro* multiplication of pineapple are

1. enhanced release of axillary buds and
2. indirect somatic organogenesis.

Various growth regulators are used in different combinations or alone to achieve growth manipulation.

2.2.5.1 Axillary bud release

Lakshmisita *et al.* (1974) inoculated shoot tips in Knudson's basal media with Nitsch's micro elements supplemented with NAA 1 ppm. Clusters of thick roots and leaves developed, which was subcultured at fourth leaf stage into a medium of same constitution.

Pannetier and Lanaud (1976) have reported two methods of micro propagation of pineapple, both based on initial culture of crown axillary leaf bud on MS medium containing BA and NAA. In the first method the plantlet obtained is sectioned horizontally or longitudinally and the fragments replanted on a similar medium. In the second method in which a single bud can theoretically produce about a million plants in two years, the bud is grown for 10 years to a size of about 3 mm. It is then detached from the mother fragment and transferred to a medium containing

BA and IBA, where intense ramification occurs, with the formation of numerous buds.

Mathews and Rangan (1979) achieved enhanced release of axillary buds from dormant lateral buds. Shoot buds appeared from lateral bud explant after 8-10 weeks on MS medium containing 1.8 mg NAA, 2.0 mg IBA and 2.1 mg KIN per litre. These regenerated buds when transferred to fresh medium, developed numerous multiple buds and this cycle was repeatable. The outline of an improved procedure for rapid multiplication of pineapple was published by Drew (1980). Four stages were envisaged for the recovery of multiple shoots from axillary buds excised from the base of leaves. The first stage was concerned with the establishment of sterile cultures. The second stage involved placing the sterile tissue on a medium which support shoot growth and produce a strong single shoot. The third stage of culture aimed at the production of multiple buds either by changing the media or by stimulation of lateral bud growth through vertical dissection of the shoot. Transfer of single shoots on to a medium which would initiate roots brought up the final stage of multiplication.

Zepeda and Sagawa (1981) reported repeated proliferation of axillary buds from crown axillary buds. The explants were cultured in MS medium with 25 per cent coconut water. Explants were subcultured every two weeks into fresh medium consisting 4 ml half strength MS plus 25 per cent coconut water. Solitary shoots with five to eight leaves were obtained within two months after initial culture.

These were subcultured into half strength MS supplemented with BA 1 mg/litre. At least three axillary shoots were produced within 30 days. Further multiplication was obtained by separation of axillary shoots and subculturing four times in same medium.

Evans and Moore (1987) and Fitchet (1987) have also reported stimulation of precocious axillary branches by meristem culture in MS medium containing BA. Bordoloi and Sharma (1993) observed multiple shoot initiation on MS medium supplemented with IBA (10 mg l^{-1}), KIN (5 mg l^{-1}) and casein hydrolysate (200 mg l^{-1}).

2.2.5.2 Indirect somatic organogenesies

Mapes (1973) described a procedure for inducing callus (globular bodies) from pineapple axillary buds on modified MS medium containing adenosine (30 mg l^{-1}) or adenine (20 mg $^{-1}$) following subculture of buds in a medium with adenine and 20 per cent coconut water.

Mathews *et al.* (1976) reported that supernumerary shoots arose from terminal buds of crown after callus formation. Wakasa *et al.* (1978) observed differentiation of plants from protocorm like globular bodies. The dormant axillary buds from suckers, slips and crowns were initially grown on MS medium containing 2 mg l^{-1} of NAA and 1 mg l^{-1} of BA.

After 45 days all growing buds were transferred to MS medium supplemented with 2 mg l^{-1} of NAA and BA. Within one or two months all buds produced lumpish tissues, looking like an aggregation of small globes called globular bodies. Slices of syncarp also produced callus on the medium with 10 mg l^{-1} each of NAA and BA. The globular bodies with a number of leaves and buds on its surface were transferred to regeneration medium, containing 2 mg l^{-1} BA, where both leaves and small buds became complete shoots. Further growth of small shoots and root induction were achieved by transferring them to MS medium without hormones.

Mathews and Rangan (1979) reported proliferation of callus from cut ends of leaves of *in vito* obtained plants when explants were cultured on MS medium supplemented with NAA (1.8 mg l^{-1}) + IBA (2.0 mg l^{-1}) + KIN (2.1 mg l^{-1}) or BA (2.2 mg l^{-1}).

Mathews and Rangan (1981) developed callus cultures from the basal region of *in vitro* obtained shoot buds of pineapple on MS medium with NAA (10 mg l^{-1}), coconut water (15%) and casein hydrolysate (400 mg l^{-1}). Hundred percent regeneration of callus was obtained on MS medium containing five per cent coconut water. Differentiation of meristemoids from hybrid embryo callus on MS medium supplemented with 2.0 mg NAA, 2.0 mg IBA and 2.5 mg BA per litre (Rao *et al.*, 1981).

Rosa-Marquez *et al.* (1987) achieved callus induction from tissue explants using MS medium with benzyl adenine. Regeneration of plants were obtained by transferring the callus to MS medium containing NAA (4 mg l^{-1}).

Fitchet (1990b) induced callus from the crown apical region on Murashige and Tucker's medium supplemented with casein hydrolysate (400 mg l^{-1}), coconut water five per cent and NAA (40 mg l^{-1}). He also reported that regeneration of plants was by indirect adventitious organogenesis and not by somatic embryogenesis.

Bordoloi and Sharma (1993) reported callus production from axillary buds cultured on MS medium containing IAA, IBA and KIN (5 mg l^{-1} each).

According to Prabha (1993) maximum intensity of globular bodies with leaves and shoots were formed within 5.96 days on subculturing to MS medium supplemented with BA 5.0 mg l^{-1} and NAA 1.0 mg l^{-1}. Plant regeneration was high on MS basal medium with cent per cent of cultures developing vigorous dark green shoots. Sudhadevi *et al.* (1994) reported maximum rate of formation of shoot bud primordia in medium containing BA 4.0 mg l^{-1} and NAA 1.0 mg l^{-1}. Shoot formation was best in MS basal media.

2.2.6 Medium supplements

Medium supplements are certain organic additives which influence the establishment and growth of *in vitro* cultures. In many cases these organic supplements have constituents of an undefined nature. However, the success achieved with these compounds in tissue culture cannot be ignored. These include protein (casein hydrolysate), adenine, adenine sulphate, yeast and malt extract, coconut water, tomato juice, banana homogenate, activated charcoal etc.

Mapes (1973) reported production of protocorm like bodies and plantlets from the shoot tips in MS basal medium amended with adenosine (30 mg l^{-1}) or adenine (20 mg l^{-1}).

According to Mathews and Rangan (1981) *in vitro* obtained shoot buds induced callus when cultured on MS medium with NAA (10 mg l^{-1}), coconut 15 per cent and casein hydrolysate (400 mg l^{-1}). Role of coconut water for *in vitro* culturing of pineapple has been reported by Zepeda and Sagawa (1981). Fitchet (1990a) reported the use of casein hydrolysate (400 mg l^{-1}) and 15 per cent coconut water along with NAA (40 mg l^{-1}) for callus induction from crown apical region.

2.2.7 Physical condition of media

The rate of morphogenesis under *in vitro* conditions is seen to be affected by consistency of medium. The most commonly used gelling agent for solid media preparation is agar. The availability of medium ingredients to the plant material cultured on solid medium, will in any case be less compared to tissue in shaken liquid culture, as only less than half of the explant or callus surface is in contact with the medium. Shaken cultures induced rapid proliferation of single shoot apices of orchid (Wimber, 1965). *Nicotiana rustica* (Walkey and Woolfitt, 1968) and bromeliads (Mapes, 1973).

Mathews and Rangan (1979) reported that shaking the cultures during growth promoted the formation of multiple shoots. A three fold increase in the number of multiple shoots formed per culture was observed as compared to stationery cultures, suggesting possible commercial use of shake culture in obtaining large number of clonal plants rapidly. Zepeda and Sagawa (1981) opined that liquid culture medium on a TC-3 rollerdrum at 1/5 rpm at $26\pm2^{\circ}$C under continuous light of about 2100 lux appeared to be most suitable for obtaining rapid proliferation.

Fitchet (1985) and Dewald *et al.* (1988) also reported that shake cultures gave better results.

2.2.8 Culture environment

Culture environment greatly influences the process of growth and differentiation of *in vitro* cultures. Hence cultures are incubated under conditions of well

controlled temperature, humidity, illumination and air circulation. Murashige (1974) found that the optimum light intensity for shoot formation in many herbaceous species was to be around 1000 lux. The optimum day light period was considered to be 16 hours for a wide range of plants. Hu and Wang (1983) observed that for most meristem, shoot tip and bud cultures, constant incubation temperature used ranged between 24 and 26°C. The optimum humidity requirement range between 20 and 98 per cent.

Laskshmisita *et al.* (1974) cultured *in vitro* plantlets under an increased light intensity of 200 FC. Wakasa *et al.* (1978) incubated the cultures at 26-28°C under 12 hour illumination or at 30°C under natural light. Mathews and Rangan (1979) maintained the cultures at 25±2°C, in a relative humidity of 50-60 per cent and in continuous diffused light (950-1000 lux).

Zepeda and Sagawa (1981) grew cultures on a TC-3 Rollerdrum at 1/5 rpm at 26±2°C under continuous light of about 2.1 klx. Rosa Marquez *et al.* (1987) have reported callus regeneration of Red Spanish pineapple into green plantlets 2-3 weeks after incubation in different light intensities at 26°C.

2.2.9 Root induction

In vitro produced plants must have a strong and functional root system for proper establishment and growth *in vivo*. The roots produced may be taproot or adventitious root depending on the species. According to Hu and Wang (1983) there are three stages for rhizogenesis, viz., induction, initiation and elongation. The roots can be induced under *in vitro* conditions and the rooted plantlets are transferred to suitable planting medium, the process generally referred as *in vitro* rhizogenesis.

Rooting of plantlets can also be achieved under *in vivo* conditions where in the propagules are subjected to suitable rooting treatments before planting.

2.2.9.1 *In vitro* rhizogenesis

Root induction hormones, commonly auxins are used for *in vitro* rooting. Auxins most frequently incorporated into media are NAA, IBA and IAA, of which IBA and NAA have been most effective for root induction (Ancora *et al.*, 1981). However, high concentrations of auxin inhibited root elongation (Thimman, 1977).

Lakshmisita *et al.* (1974) reported that clusters of thickroots were obtained in *in vitro* pineapple plantlets on Knudson's basal media with Nitsch's micro elements with NAA 1 mg l^{-1}.

Wakasa *et al.* (1978) achieved rooting of *in vitro* produced shoots by transferring them to the medium without any growth regulators. Rooting has also been accomplished on MS solid medium in the presence of 0.18 mg l^{-1} NAA and 0.4 mg l^{-1} IBA (Mathews and Rangan, 1979).

Mathews and Rangan (1981) reported that pineapple plantlets were rooted on modified Whites medium supplemented with 0.05 mg l^{-1} NAA and 0.4 mg l^{-1} IBA. Yang (1981) induced rooting in MS medium containing 1 mg l^{-1} NAA.

Cabral *et al.* (1984) observed rooting of *in vitro* plantlets in MS medium incorporated with 0.18 mg l^{-1} NAA and 0.4 mg l^{-1} BA. Contrary to these reports Dewald *et al.* (1988) stated that transferring to a medium with or without growth regulators to induce root formation was not necessary for plantlet survival in pineapple. Plantlets without roots produced in a multiplication medium with plant

growth regulators had a high survival rate, it being nearly 100 per cent if plantlets larger than 13 cm were used. According to Fitchet (1990b) plantlets were rooted on MT medium with 1 mg l^{-1} NAA and 500 mg l^{-1} malt extract. Bordoloi and Sharma (1993) found that half strength MS medium containing 2 mg l^{-1} IBA was the best for *in vitro* rooting of pineapple.

According to Prabha (1993) MS basal medium is sufficient for root induction, even though rooting was accomplished in MS medium supplemented with IBA, 2 mg l^{-1}. Sudhadevi *et al.* (1994) suggested the use of oxins such as IBA and NAA at the concentrations of 1.0 and 2.0 mg l^{-1} for *in vitro* rooting in pineapple. Kiss *et al.* (1995) also reported *in vitro* rooting of pineapple plantlets on a growth regulator free MS medium.

2.2.9.2 *Ex vitro* rhizogenesis

Debergh and Maene (1981) emphasized that, the *in vitro* rooting of propagated shoot is expensive accounting for 35-75 per cent of the total cost of micro propagated plants. Also in many species *in vitro* roots lack root hairs and chances of root mortality is high. An alternate system suggested for rooting of plantlets is *ex vitro* rooting, where in shoots are rooted outside aseptic conditions on a suitable rooting substrate. This facilitates elimination of a separate rooting stage as it combines rooting with acclimatization (George and Sherrington, 1984).

In *ex vitro* rooting, the micro propagated shoots are treated as normal cuttings. They are subjected to auxin treatment by dipping the cut basal end in auxin before planting into a suitable rooting substrate and then keeping them under high humidity or intermittent water mists.

Such technique was found successful in black berry and blue berry (Zimmerman and Broome, 1981) and in apple (Simmonds, 1983).

2.2.10 Hardening and planting out

Micro propagation on a large scale can be successful only when plants after transfer from culture conditions to soil have high survival rate and better growth and the cost involved in the process is low. Micropropagated plants differ from field grown plants in many ways. They have poor photosynthetic efficiency, decreased epicuticular wase and malfunctioning of stomata. These characters render them vulnerable to transplanting shock and hence transfer of plantlets from aseptic tissue culture conditions to external environment must be done carefully in a step-wise procedure (George and Sherrington, 1984).

Light, temperature and relative humidity are the three major factors to be controlled during acclimatization. The most essential requirement for successful transplantation is to maintain plants under very high relative humidity (90-100%) for the first 10-15 days, by keeping them covered with glass or clear, transparent plastic with holes for aeration or by subjecting them to intermittent water misting (Bhojwani, 1980).

Prior to hardening, the plantlets should have attained sufficient size with well proportioned shoots and roots that are capable of supporting each other (Sommer and Caldas, 1981).

Fitchet (1985) used plantlets of 100 mm length for hardening studies in green house condition. In a report by Dewald *et al.* (1988) pineapple plantlets were

transferred to individual pots or to flats containing commercial soil mixture and enclosed in plastic bags creating a green house effect. These plants were incubated in a growth chamber at 28°C under flourescent lamps and gradually hardened off by removing the plastic covers. Folliot and Marchal (1991) found that *in vitro* produced plantlets of one to two gram reached a fresh weight of 106 g/plant after 4.5 months of acclimatization in green house. The rapid growth rate attained during acclimatization was due to controlled conditions in the green house.

Rhizosphere environment also influence plant growth during hardening. Folliot and Marchal (1990) carried out hardening trials for *in vitro* produced pineapple plantlets using eight different rooting substrates. Leaf development, fresh weight, dry weight gain and root development were studied and the mineral content of the substrate and the plants were analysed. The peat based substrate gave the best results. Compost + perlite performed almost similarly as well and was more reliable.

Prabha (1993) observed that potting media such as cocopeat soilrite and biofibe were found better for inducing vigorous growth in *in vitro* produced plantlets.

Nutrition of *in vitro* produced plants during hardening has been found to influence their growth and development. Cent per cent establishment and better vigour of the *in vitro* produced vettiver plantlets was noticed with the application of NPK fertilizer solution (10:5:10 g l^{-1}) at weekly intervals. Treatment with half strength MS nutrients also gave similar results (Keshavachandran, 1991).

In pineapple, during initial stages healthy plants were produced by the application of NPK fertiliser solution (10:5:20 @ 5 g l^{-1}) or 1/4 strength MS nutrient solution at weekly intervals. While in later stages treatment with NPK fertilizer solution (10:5:20, @ 5 g l^{-1}) once in two weeks or Hogland's solution once in a week gave better results (Prabha, 1993).

2.2.11 Economics of tissue culture plants

Commercial micropropagation is a labour intensive procedure. The cost of producing *in vitro* plants is mainly depending on the suitability of the species for micropropagation, multiplication rate achieved, success rate of planting out and the demand for the species.

The multiplication rates achieved in the case of pineapple through tissue culture has been satisfactory enough to warrant its commercial adoption. Zepeda and Sagawa (1981) estimated that by the application of micropropagation technique 5000 plants can be produced in 12 months from a single crown, while Fitchet (1985) projects 2000 plants from a single crown containing about 40 buds. Dewald *et al.* (1988) could obtain 210 to 380 plants for Perlora, 300-350 plants for PR-1-67 and 40 to 85 plants for Smooth Cayenne by the culture of a single bud for a period of an year.

Materials and Methods

3. MATERIALS AND METHODS

The present study on the 'refinement of *in vitro* propagation technique in pineapple var. Mauritius and mass multiplication of elite clones' was carried out as two parts, namely,

1. Selection of elite clones of Mauritius variety of pineapple
2. Refinement of *in vitro* propagation technique and mass multiplication of selected elite clones.

3.1 Selection of elite clones

The selection of elite plants was done based on a survey, collection and detailed studies of located plants. The details regarding the methodology and analytical techniques adopted are presented in this chapter.

A detailed survey was conducted during the period of January to April, 1994, in the major pineapple growing areas, namely, Vazhakulam and Muvattupuzha areas of Ernakulam district. Based on different biometric characters, namely, plant height, leaf number, D leaf area and general vigour, 20 plants each receiving similar cultural practices, were tagged in 5 different locations, namely, Kaloor, Kalloorkadu, Vazhakulam, Thodupuzha and Koothattukulam, thus tagging 100 plants in total. The following vegetative characters of the plants were considered for initial selection.

3.1.1 Plant characters

(a) Plant height

Height of the individual plants was recorded at the time of flowering from ground level to the tip of the longest leaf and was expressed in cm.

(b) Leaf number

The total number of leaves per plant was recorded.

(c) 'D' leaf area

'D' leaf (5th leaf from top) was taken out and measurements, namely, length and breadth were recorded 'D' leaf area of each plant was calculated using the formula, length x breadth x 0.725 (Balakrishnan *et al.*, 1978).

(d) Number of suckers

The number of suckers produced per plant was recorded at the time of harvesting of fruits.

3.1.2 Fruit characters

From the 100 plants marked initially based on plant characters, fruits were harvested, when they reached the physiological maturity (appearance of yellow colour at the bottom 1/3rd of fruits). The average weight of Mauritius fruit in this area is 1.25 to 1.50 kg and hence for further selection, fruits weighing 2.0 kg or more alone were considered. Thus the number of selected accessions were reduced to 25. The fruits of these 25 accessions were subjected to detailed quantitative and qualitative analysis.

3.1.2.1 Quantitative characters of fruit

(a) Fruit weight

Immediately after harvest, fruits with crown intact were weighed and recorded in kg. Being the most important factor, fruit weight was considered as the first selection criterion. Plants producing fruits weighing less than 2.0 kg were eliminated from the further selection procedure.

(b) Fruit length and breadth

The length of the fruit and the breadth at the top 3/4, middle and bottom 1/4 were recorded and expressed in cm.

(c) Taper ratio

Taper ratio was worked out from the formula

$$\text{Taper ratio} = \frac{\text{Breadth at top 3/4}}{\text{Breadth at bottom 1/4}}$$

(d) L/B ratio

L/B ratio was arrived at using the equation,

$$\text{L/B ratio} = \frac{\text{Fruit length}}{\text{Mean fruit breadth}}$$

(e) Peel weight

Peeling of fruits was done carefully and peel weight was noted down and recorded in g.

(f) Pulp weight

After removing the peel and central core, the weight of the pulp in kg was recorded for each fruit.

(g) Peel/pulp ratio

Peel/pulp ratio was calculated by dividing the weight of the peel by the weight of the pulp for each fruit.

(h) Pulp percentage

Pulp percentage was worked out from the above observation as

$$\frac{\text{Pulp weight}}{\text{Weight of fruit without crown}} \times 100$$

(i) Juice content

A known weight of fruit pulp was squeezed in a muslin cloth to extract the juice and juice content was calculated and expressed in percentage.

$$\text{Juice content} = \frac{\text{Weight of juice}}{\text{Weight of pulp taken}} \times 100$$

3.1.2.2 Qualitative characters of fruit

For the qualitative analysis, samples were taken from each fruit from three portions, namely, top, middle and bottom, pooled and macerated in a waring blender. Triplicate samples from this were used for analysis.

(a) Total soluble solids

Total soluble solids were measured using a pocket refractometer and were expressed as percentage.

(b) Acidity

Ten gram of macerated sample was mixed with distilled water and made upto a known volume. An aliquot of the filtered solution was titrated against 0.1N sodium hydroxide using phenolphthalein as indicator. The acidity was expressed as percentage of citric acid (AOAC, 1960).

(c) Reducing sugar

The reducing sugar of the samples was determined as per the procedure described by AOAC (1960). To a known quantity of macerated pulp, distilled water was added. The solution was then filtered and an aliquot of this was titrated against a mixture of Fehling's solution A and B using methylene blue as indicator. The content of reducing sugar was expressed as percentage.

(d) Total sugars

Total sugars were determined as per the method described by AOAC (1960). Five ml of concentrated hydrochloric acid was added to a known volume of clarified solution and was kept overnight. Using 1N sodium hydroxide, the solution was neutralised and titrated against Fehling's solution A and B. Total sugar content was worked out from the titre value and expressed as percentage.

(e) Non-reducing sugar

Non-reducing sugar in percentage was obtained by substracting the amount of reducing sugar from the total sugar.

3.1.3 Identification of elite clones

The vegetative and fruit characters of all the 25 selected accessions were tabulated and compared. For selecting the elite plants, a scoring procedure was followed. Firstly the average value of all the characters compared were calculated. Then the plants were given a score of one each for favourable character and zero for unfavourable character. Based on the total score, five elite clones with maximum score were selected. The suckers, crowns and slips of these five superior plants were planted at Vellanikkara, to be mass multiplied through refined *in vitro* propagation technique for further evaluation.

3.2 Refinement of *in vitro* propagation technique and mass multiplication of elite clones

The work on refinement of *in vitro* propagation and mass multiplication of elite clones was carried out at the Tissue Culture Laboratory of Kerala Horticulture Development Programme, Kerala Agricultural University, Vellanikkara, during the year 1993-'95.

The protocol for *in vitro* propagation of pineapple variety, Kew has already been developed (Mathews, 1976; Prabha, 1993). In the present study a protocol for rapid *in vitro* propagation of Mauritius was developed by refining the procedure and media developed earlier.

3.2.1 Materials

3.2.1.1 Explant

The shoot tips from suckers, crowns, slips and lateral buds from suckers and plant stem collected from farmers field formed the explants for the present study.

3.2.1.2 Culture medium

The basal medium used for the study was the one developed by Murashige and Skoog in 1962 (MS medium). The major and minor elements, organic supplements as well as growth regulators were modified to develop a suitable medium for the variety Mauritius. The standard chemical composition of MS medium is given in Appendix-I.

3.2.1.3 Glassware and chemicals

Borosil brand borosilicate glassware available at the tissue culture laboratory, Kerala Horticulture Development Programme, Vellanikkara were utilised for the study. The items of glassware were initially soaked in potassium dichromate solution in sulphuric acid for 10-12 hours followed by thorough washing with jets of tap water followed by cleaning in soap solution (Labolen) for 10 hours and consequently washed thoroughly with tap water. The glassware were then rinsed with double distilled water before sterilizing in an autoclave at 15 lbs for 20 minutes. The sterilized glassware were utilised for various studies.

The analytical grade chemicals procured from British Drug House (B.D.H.), Sisco Research Laboratories (S.R.L.), Merck and Sigma Chemicals, U.S.A. were utilized for the study.

3.2.2 Methods

3.2.2.1 Preparation of stock solution

The standard procedure putforth by Gamborg and Shyluk (1981) was strictly followed for preparation of M.S. media. Separate stock solutions were prepared for various constituents.

a) Inorganic constituents

Separate stock solutions such as stock solution A (Ammonium nitrate + Potassium nitrate + Potassium dihydrogen phosphate + Magnesium sulphate), stock solution B (calcium chloride), stock solution C (Iron source) and stock solution D (Minor nutrients) were prepared by dissolving specific quantity (Appendix-II) of each component separately in double distilled water and then making up the required volume with double distilled water.

b) Organic constituents

Stock solutions of various vitamins (stock E) was prepared by separately dissolving each of the vitamins in a small quantity of distilled water and then making up to the standard volume with double distilled water (Appendix-II). Stock solutions of vitamins were prepared afresh once in two months and stored in refrigerator at 5-10°C.

c) Growth regulators

Stock solutions of growth regulators were prepared by dissolving specific quantities in specific solvents recommended for each and then making up the standard volume with suitable diluent (Appendix-III). Fresh stock of growth regulators were prepared once in a month and stored in refrigerated conditions until use.

3.2.2.2 Preparation of media

Specific quantities of various stock solutions were pipetted out into a 1000 ml beaker. Five hundred ml of double distilled water was added to the beaker. Required quantities of sucrose and inositol were weighed using an electronic balance and dissolved fully. The volume was then made up to 995 ml with double distilled water. The pH of the medium was measured using a pH meter (Elico) and adjustment was effected by adding 0.1N NaOH/0.1N HCl to bring the pH to 5.6 to 5.8 range. Finally the volume was made upto 1000 ml using double distilled water.

Agar was then added to the medium and dissolved it by keeping in a water bath maintained at a temperature of 90-95°C until the medium became clear. The hot medium was then distributed to autoclaved test tubes at the rate of 15 ml each or to screw capped jam bottles/conical flasks at the rate of 50 ml each. The test tubes and conical flasks were tightly plugged with sterile nonabsorbent cotton plugs and bottles were screw capped thoroughly. The containers with the medium were then autoclaved for 20 minutes at a temperature of 121°C and 15 Psi (Dodds and Roberts, 1982). After autoclaving, the medium was allowed to cool to room temperature and then stored under aseptic conditions in the culture room until use.

3.2.2.3 Preparation of explants

3.2.2.3.1 Collection of explants

Shoot tip explants were collected from suckers, crowns and slips were prepared by carefully removing all the leaves surrounding the shoot apex. All the sides and bottom stem portion were trimmed and finally with a sharp knife, the shoot tip measuring 0.8 cm^3 with the meristem at the centre was excised. The explants prepared in such manner was collected in double distilled water contained in a 250 ml conical flask and brought to the laboratory without delay for further work.

For the collection of lateral buds situated concealed in the leaf axils of stem, the leaf bases were first carefully removed to expose the lateral buds. The lateral buds were then excised with a portion of stem tissue at the base.

All the explant materials were washed well with sterile water and were given surface sterilization treatments.

3.2.2.3.2 Surface sterilization of explants

The explants were subjected to surface sterilization treatments to make them free of contaminations and microbes.

Initially the explants were treated with 0.1 per cent emisan followed by thorough washing with sterile water for three times. The explants were then taken inside the laminar air flow chamber and final sterilization using 0.1 per cent mercuric chloride was given. The duration of treatment with these two sterilants were varied to standardise the optimum treatment time for maximum culture success.

After this, explants were rinsed three times with sterile distilled water and dried by carefully keeping them on sterile filter paper placed on sterile petri dishes.

A sterilized cube of tissue with shoot tip was placed on a sterile petri plate and using sterile forceps and scalpel the superficial tissue exposed to the sterilant during disinfection was cut away from all the sides so that the explant size was reduced to 0.5 cm^3. Lateral buds along with the stem tissue were also trimmed in the same manner without damaging the bud portion.

Observations on percentage of contamination and explant survival were recorded 15 days after inoculation.

3.2.2.4 Inoculation of explants.

All inoculation operations were done inside a laminar air flow chamber under perfect aseptic conditions.

To inoculate the explants on the culture medium, the cotton wool plug of the culture vessel was removed and the vessel neck was first flamed over a gas burner kept inside the chamber. While showing the mouth of the container to the flame, the sterile explants were quickly transferred in to the medium, using sterile forceps. The neck of culture vessel was again flame sterilized and the cotton wool plug was replaced immediately.

3.2.2.5 Incubation of cultures

After inoculation of explants, the cultures were properly labelled and transferred to the culture room with controlled temperature and light regimes. The temperature was maintained at $26\pm2^{\circ}C$ and artificial lighting was provided by cool-

white fluorescent tubes. The light intensity was maintained at 1500 lux and a photoperiod of 16 hours per day was provided.

3.2.2.6 Standardisation of media for culture establishment

In order to find out the best culture establishment medium for pineapple variety Mauritius, separate experiments were conducted by varying the hormone levels in the media. The treatments consisted of seven levels of BAP (1.0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}).

Observations on the number of days taken for greening and number of days for initiation of growth were recorded at three day interval upto 25 days after inoculation.

The medium which was found most suitable for establishment of shoot tip explants from suckers were tried for crowns and slips.

3.2.2.7 Standardisation of media for the enhanced release of axillary buds

The established shoot tip cultures from suckers were divided into four similar segments and they were used for studying the effect of different media on the enhanced release of axillary buds. The experiment was carried out using MS medium supplemented with cytokinins, namely, BAP and Kinetin (1,0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in combination with NAA (0.5 and 1.0 mg l^{-1}).

The number of axillary buds produced was recorded 25 days after first subculturing. The experiment was repeated for second and third subculture, each time one axillary bud from the axillary bud aggregate was carefully separated and inoculated. Each subculture cycle consisted of 25 days.

3.2.2.8 Effect of medium supplements on enhanced release of axillary buds

Effect of different medium supplements on the enhanced release of axillary buds was studied in a separate experiment. The details of different supplements tried in MS medium + BAP 4.0 mg l^{-1} are furnished in the Table 1.

Number of axillary buds produced were recorded 25 days after culturing. This experiment was repeated for two more subculturing, each time transferring a single axillary bud to fresh medium. Each subculture cycle consisted of 25 days.

3.2.2.9 Standardisation of media for the initiation of adventitious buds (indirect somatic organogenesis)

An experiment was carried out to study the effect of different media on initiation of adventitious buds. Axillary buds obtained from shoot tip cultures of suckers as well as lateral buds excised from stem were repeatedly subcultured in MS medium supplemented with BAP and Kinetin at different concentrations (1.0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in combination with NAA at two levels (0.5 and 1.0 mg l^{-1}) until the initiation of adventitious buds. Each subculture cycle consisted of 25 days. The number of days for initiation of adventitious buds were calculated from the day of first subculturing.

The medium which induced fastest response in the case of shoot tip of suckers was tried for shoot tip of crowns and slips.

3.2.2.10 Standardisation of media for proliferation of adventitious buds

Uniform mass of multiplying culture of adventitious buds were transferred to MS solid medium supplemented with cytokinins, viz., BAP and kinetin at

Table 1. Types and levels of medium supplements used for enhanced release of axillary buds

Treatment No.	Medium supplements	Concentrations
T_1	Caesin hydrolysate	50 mg l^{-1}
T_2	Caesin hydrolysate	100 mg l^{-1}
T_3	Coconut water	5%
T_4	Coconut water	10%
T_5	Tomato juice	10%
T_6	Control (BAP)	4 mg l^{-1}

different levels (1.0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in combination with NAA at two levels (0.5 and 1.0 mg l^{-1}) to standardise the suitable media for maximum proliferation of adventitious buds.

Observations were taken on the intensity of adventitious bud aggregate produced, 25 days after culturing by visual scoring. A '+' mark was given for every one time increase of initial culture used for inoculation.

3.2.2.11 Effect of physical condition of the media on proliferation of adventitious buds

Uniform mass of multiplying culture of adventitious buds were transferred to MS solid medium and liquid medium supplemented with four levels of BAP (2.0, 3.0, 4.0 and 5.0 mg l^{-1}) in combination with two levels of NAA (0.5 and 1.0 mg l^{-1}), to study the effect of physical condition of media on proliferation of adventitious buds.

Liquid medium was kept in an orbital shaker with automatic temperature regulator system. The number of rotations per minute was adjusted to 60 and the temperature was maintained at 26°C. A light intensity of 1500 lux for a photoperiod of 16 hours per day was provided.

Observations were recorded on increase in mass of adventitious buds produced, 25 days after culturing by visually scoring as for the previous experiment. The results were compared with that of response in corresponding solid medium.

3.2.2.12 Standardisation of media for regeneration of shoots from adventitious buds

Under the different hormonal levels, aggregates of adventitious buds

were produced. These buds were to be kept in suitable regeneration media to get plantlets. A study to standardise the best regeneration media was conducted.

Effect of different levels of cytokinins, viz., BAP and kinetin alone or in combination with NAA on multiple shoot production was studied. All the treatment combinations tried for proliferation of adventitious buds were repeated for this experiment also.

The observations on days for shoot initiation, number of shoots produced, average length of shoots and nature of shoots were recorded at three days interval upto 40 days after culturing.

3.2.2.13 Effect of physical condition of the media on regeneration of shoots from adventitious buds

Uniform mass of adventitious buds were transferred to MS solid and liquid media supplemented with BAP 1.0 mg l^{-1} to study the effect of physical condition of media on regeneration of shoots.

Liquid medium was kept in an orbital shaker with automatic temperature regulator system. The number of rotations per minute was adjusted to 60 and the temperature was maintained at 26°C. A light intensity of 1500 lux for a photoperiod of 16 hours per day was provided.

Observations on days for shoot initiation were recorded at three days interval and number of shoots produced, average length of shoots and nature of shoots were recorded 40 days after culturing. The results were compared with that of response in corresponding solid medium.

3.2.2.14 Induction of rooting

3.2.2.14.1 *In vitro* rooting

Studies on *in vitro* rooting was carried out on regenerated shoot with three or four leaves in solid MS medium and in liquid medium without agar using filter paper supports. The MS medium was supplemented with various auxins viz., IAA, IBA and NAA each at different concentrations of 1.0, 2.0 and 3.0 mg l^{-1}.

Observations on number of days for root initiation were recorded at three days interval and observations on average number of roots, average length of roots and nature of roots were recorded 40 days after culturing.

3.2.2.14.2 *Ex vitro* rooting

Ex vitro rooting trials were carried out using uniform 5 cm long regenerated shoots. The rooting treatments tried for *ex vitro* rooting are given below.

1. IBA 100 mg l^{-1}
2. IBA 200 mg l^{-1}
3. NAA 100 mg l^{-1}
4. NAA 200 mg l^{-1}
5. Control (distilled water)

The cut ends of the shoots were dipped in the above auxins for a period of one hour and shoots were planted in poly bags filled with sterilized sand. Observations on the percentage of plants showing rooting, days for rooting and mean number and length of roots were recorded at three days interval upto 40 days after planting.

3.2.2.15 Hardening and *ex vitro* establishment

The rooted plantlets from aseptic culture conditions were transferred to external environment for further hardening studies.

The plug of the culture vessel was removed and using a forceps the rooted plantlets having 4-5 leaves and 3-6 cm height with well developed roots were taken out carefully. The agar adhering to the roots were completely removed by thorough washing with distilled water. Then the plantlets were treated with a weak fungicide solution (Bavistin 0.1%) for five minutes. Those plantlets were used for various hardening studies.

3.2.2.15.1 Effect of potting media on growth and development of plants under *ex vitro* condition

In order to study the effect of potting media on the growth of plantlets, they were grown in different potting media in poly bags. The different potting media namely, coarse sand, soilrite, palmpeat, vermiculite + soil (1:1 ratio) and potting mixture (1:1:1 sand:red earth:cowdung) were tried.

The plants were kept under shade house for one week. Later they were transferred to the green house and watered once in two days.

Observations were made on the plant height, average leaf number, length and width of largest leaf, at the time of planting and 60 days after planting out.

3.2.2.15.2 Effect of containers on growth and development of plants under *ex vitro* condition

The following containers were used for the experiment

1. Transparent poly bags (14 x 10 cm) with sufficient holes.
2. Black plastic pots (8 x 6 cm) with sufficient holes.
3. Mud pots (8 x 6 cm) with sufficient holes.
4. Por-trays (4 x 3 cm).

The potting medium used for the study was coarse sand. The standard transferring procedure adopted as in the previous experiment was followed here also.

The relative growth rate of plants as evidenced by increase in plant height, average number of leaves, length and width of the largest leaf were recorded at the time of planting and 60 days thereafter.

3.2.2.15.3 Effect of nutrient starter solution on growth and development of plants under *ex vitro* condition

This experiment was conducted using uniform pineapple *in vitro* plants (30 days old having 7-8 leaves) grown in poly bags in sand medium. The various nutrient solutions used are listed below.

1. Urea 1 per cent solution
2. Urea 0.5 per cent solution
3. MS nutrient solution at full strength with pH adjusted to 5.7
4. MS nutrient solution at ½ strength with pH adjusted to 5.7
5. MS nutrient solution at 1/4 strength with pH adjusted to 5.7
6. Fertilizer solution (NPK - 10:5:20 @ 5 g/litre)
7. Control (Tap water)

The nutrient starter solutions were applied at an interval of 15 days at the rate of 5 ml for each plant and watering was done in alternate days with tap water.

Observations on plant height, average number of leaves, length and width of the largest leaf were recorded initially and 60 days thereafter.

3.2.2.15.4 Effect of shoot size on growth and development of plants under *ex vitro* conditions

In vitro produced shoots were categorized into the following five groups based on biometric characters, to standardise the optimum shoot size for maximum growth and development (Table 2).

The shoots were planted in poly bags in coarse sand medium and watered in alternate days.

Observations on height of plant, average number of leaves, length and width of largest leaf and weight of plant were recorded initially and 60 days thereafter and percentage increase was calculated.

3.2.2.16 Statistical analysis

The data obtained from various experiments were subjected to statistical analysis in completely randomised design, wherever necessary, following Panse and Sukhatme (1985).

Table 2. Categorization of shoots based on biometric characters to standardise the optimum shoot size for maximum growth and development

Group number	Height of shoot (cm)	Average number of leaves	Length of largest leaf (cm)	Width of largest leaf (mm)	Weight of shoot (mg)
I	10.1-12	11-13	6.1-8	8.1-10	801-1000
II	8.1-10	8-10	5.1-6	6.1-8	601-800
III	6.1-8	6-7	4.1-5	5.1-6	401-600
IV	4.1-6	4-5	3.1-4	4.1-5	201-400
V	2-4	2-3	2-3	3-4	100-200

Results

4. RESULTS

Results of the studies on refinement of *in vitro* propagation technique in pineapple var. Mauritius and mass multiplication of elite clones are presented in this chapter.

4.1 Selection of elite clones

A detailed survey was conducted at the major pineapple growing areas, namely, Muvattupuzha and Vazhakulam of Ernakulam district, for selecting elite plants of Mauritius variety of pineapple. One hundred plants were initially tagged based on general vigour and biometric observations were recorded. At the time of harvest, further selection was made based on the fruit weight. Plants which produced fruits of two kilograms and above alone were selected, the number being only 25 out of the 100. Fruits from these 25 accessions were subjected to quantitative and qualitative analysis. Observations on the different biometric and fruit characters of these accessions are presented in Tables 3, 4 and 5.

The best five accessions out of these 25 were found out by utilising the scoring technique described in materials and methods. Seventeen different plant and fruit characters were taken for the scoring. The details of scores obtained for each accession are given in Table 6. Data on the various characters and the score obtained by the five selected accessions are given in Table 7 and Fig.1. The highest score was obtained by the accession TK3 (16) followed by KT5 with a score of 15. The accession numbers KV17 and KT2 recorded a score of 14 each and accession TK18 scored 13 points.

Table 3. Vegetative characters of selected 25 accessions

Sl. No.	Accession No.	Height of plant (cm)	No. of leaves	Area of 'D' leaf (cm^2)	No. of suckers	No. of slips
1	TK2	89.0	49.0	259.19	3	0
2	TK3	90.0	56.0	315.38	2	0
3	TK6	88.0	53.0	313.20	3	1
4	TK9	83.0	49.0	269.70	2	0
5	TK13	84.0	53.0	255.20	1	1
6	TK15	82.0	45.0	235.48	3	0
7	TK 16	84.0	50.0	273.75	2	0
8	TK17	87.0	46.0	300.15	2	0
9	TK18	87.0	48.0	285.59	2	0
10	TK20	88.0	46.0	300.73	2	0
11	KV1	88.0	54.0	290.87	2	0
12	KV2	90.0	50.0	317.55	2	0
13	KV11	88.0	46.0	267.16	1	0
14	KV15	85.0	57.0	262.31	1	0
15	KV16	86.0	54.0	317.55	2	0
16	KV17	87.0	50.00	278.04	3	1
17	KT2	85.0	52.0	307.98	2	0
18	KT5	89.0	54.0	277.53	2	0
19	KT10	86.0	55.0	304.50	2	0
20	KT16	90.0	55.0	290.87	2	0
21	J1	87.0	47.0	290.87	2	1
22	J2	88.5	48.0	278.04	2	0
23	J4	88.5	52.0	295.80	2	1
24	A1	87.5	49.0	282.32	1	0
25	A12	87.5	55.0	291.45	2	0
	Mean	87.00	50.92	286.45	2.16	

Table 4. Quantitative characters of fruits of selected 25 accessions

Sl. No.	Accession No.	Weight of fruit + crown (kg)	Weight of crown (g)	Weight of fruit alone (kg)	Weight of peel (g)	Weight of fruit after peeling (kg)	Weight of core (g)	Weight of pulp (kg)	Peel pulp ratio	Percent-age of pulp	Juice content (%)	Taper ratio	L/B ratio
1	TK2	2.045	145.0	1.900	500	1.400	250	1.150	0.43	60.53	78.80	0.79	1.86
2	TK3	2.380	150.0	2.230	510	1.720	215	1.505	0.34	67.45	78.90	0.84	1.80
3	TK6	2.300	125.0	2.175	520	1.656	235	1.420	0.37	65.29	78.00	0.76	1.94
4	TK9	2.022	120.0	1.902	530	1.372	212	1.160	0.46	60.99	76.50	0.77	2.00
5	TK13	2.122	165.0	1.957	520	1.437	244	1.193	0.44	60.96	78.30	0.70	1.86
6	TK15	2.000	180.0	1.820	500	1.320	220	1.100	0.45	60.44	77.60	0.80	2.00
7	TK16	2.000	120.0	1.880	450	1.430	210	1.220	0.37	64.89	79.00	0.84	1.86
8	TK17	2.260	140.0	2.120	530	1.590	250	1.340	0.40	63.21	76.60	0.78	1.86
9	TK18	2.300	125.0	2.175	500	1.675	200	1.475	0.34	67.82	78.60	0.83	1.83
10	TK20	2.300	125.0	2.175	520	1.655	260	1.395	0.37	64.14	78.00	0.78	1.93
11	KV1	2.290	200.0	2.090	520	1.570	265	1.305	0.40	62.44	76.00	0.79	2.00
12	KV2	2.320	150.0	2.170	510	1.660	230	1.430	0.36	65.90	77.20	0.84	1.91
13	KV11	2.100	195.0	1.905	500	1.405	240	1.165	0.43	61.15	78.90	0.81	1.89
14	KV15	2.250	170.0	2.080	535	1.545	275	1.270	0.42	61.06	77.00	0.77	1.83
15	KV16	2.170	210.0	1.960	550	1.410	235	1.175	0.47	59.95	78.50	0.82	1.97
16	KV17	2.315	155.0	2.160	520	1.640	230	1.410	0.37	65.28	78.10	0.84	1.85
17	KT2	2.320	160.0	2.170	500	1.670	250	1.420	0.35	65.44	79.20	0.82	1.85
18	KT5	2.325	150.0	2.175	500	1.675	220	1.455	0.34	66.90	79.50	0.80	1.83
19	KT10	2.150	130.0	2.020	520	1.500	250	1.250	0.42	61.88	77.00	0.78	2.05
20	KT16	2.300	200.0	2.100	480	1.620	260	1.360	0.35	64.76	78.10	0.75	1.94
21	J1	2.200	200.0	2.000	525	1.475	200	1.275	0.41	63.75	79.20	0.78	2.06
22	J2	2.310	140.0	2.170	500	1.670	240	1.430	0.35	65.90	78.70	0.83	2.04
23	J4	2.300	180.0	2.120	500	1.620	210	1.410	0.35	66.51	79.60	0.72	2.00
24	A1	2.200	200.0	2.000	550	1.450	250	1.200	0.46	60.00	79.30	0.79	1.88
25	A12	2.310	160.0	2.150	470	1.680	250	1.430	0.33	60.51	79.10	0.79	1.90
	Mean	2.224	159.8	2.064	510.4	1.554	236.04	1.318	0.39	63.73	78.69	0.79	1.91

Table 5. Qualitative characters of fruits of selected 25 accessions

Sl. No.	Accession No.	T.S.S.	Acidity (%)	Total sugar (%)	Reducing sugar (%)	Non-reducing sugar (%)
1	TK2	15.33	0.43	13.27	3.25	10.02
2	TK3	16.00	0.38	13.79	3.97	9.82
3	TK6	16.33	0.45	13.31	3.21	10.10
4	TK9	14.66	0.48	13.28	3.15	10.13
5	TK13	14.33	0.45	13.72	3.48	10.24
6	TK15	15.00	0.38	13.73	3.91	9.82
7	TK16	15.33	0.45	13.33	3.25	10.08
8	TK17	15.00	0.49	13.40	3.50	9.90
9	TK18	16.00	0.40	13.75	3.95	9.80
10	TK20	14.00	0.51	13.12	3.24	9.88
11	KV1	14.00	0.48	12.90	3.10	9.80
12	KV2	14.00	0.49	13.42	3.45	9.97
13	KV11	14.00	0.48	13.62	3.59	10.03
14	KV15	15.00	0.42	13.45	3.54	9.91
15	KV16	15.33	0.42	12.93	3.28	9.65
16	KV17	15.66	0.40	13.90	4.05	9.85
17	KT2	16.33	0.39	13.75	3.83	9.92
18	KT5	16.00	0.39	13.81	3.95	9.86
19	KT10	16.00	0.42	13.89	3.69	10.20
20	KT16	14.33	0.40	13.67	3.96	9.71
21	J1	14.33	0.42	13.69	4.01	9.68
22	J2	13.33	0.39	12.95	3.27	9.68
23	J4	16.00	0.45	13.70	3.54	10.16
24	A1	16.00	0.40	13.76	3.81	9.95
25	A12	14.33	0.45	13.83	3.83	10.00
	Mean	15:19	0.43	13.52	3.59	9.93

Table 6. Scoring of selected 25 accessions

Character	Average value	Criteria*	TK2	TK3	TK6	TK9	TK13	TK15	TK16	TK17	TK18	TK20	KV1	KV2	KV11	KV15	KV16	KV17	KT2	KT5	KT10	KT16	J1	J2	J4	A1	A12
			Accession number																								
1. Height (cm)	87.00	<	1	1	1	0	0	0	0	0	1	1	1	1	1	0	0	1	0	1	0	1	0	1	1	1	1
2. Leaf number	50.92	<	0	1	1	0	1	1	0	1	0	0	1	0	0	1	1	0	1	1	1	1	0	0	1	0	1
3. Area of 'D' leaf (cm²)	286.45	<	0	1	1	0	0	0	0	1	0	1	1	1	0	0	1	0	1	0	1	1	1	0	1	0	1
4. No.of suckers	2.16	<	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
5. Weight of fruit with crown (kg)	2.224	<	0	1	1	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	0	1	0	1	1	0	1
6. Weight of crown (g)	159.80	>	1	1	1	1	0	0	1	0	1	1	0	1	0	0	0	1	0	1	1	0	0	1	0	0	0
7. Weight of fruit without crown (kg)	2.064	<	0	1	1	0	0	0	0	1	1	1	1	1	0	1	0	1	1	1	0	1	0	1	1	0	1
8. Weight of fruit after peeling	1.554	<	0	1	1	0	0	0	0	1	1	1	1	1	0	0	0	1	1	1	0	1	0	1	1	0	1
9. Weight of pulp	1.318	<	0	1	1	0	0	0	0	1	1	1	0	1	0	0	0	1	1	1	0	1	0	1	1	0	1
10. Peel pulp ratio	0.39	>	0	1	1	0	0	0	1	0	1	1	0	1	0	0	0	1	1	1	0	1	0	1	1	0	1
11. Pulp percentage	63.73	<	0	1	1	0	0	0	1	0	1	1	0	1	0	0	0	1	1	1	0	1	1	1	1	0	0
12. Juice content (%)	78.69	<	1	1	1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	1	1	1
13. Taper ratio	0.79	<	0	1	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1	1	0	0	0	1	0	0	0
14. L/B ratio	1.91	>	1	1	0	0	1	0	1	1	1	0	0	0	1	1	0	1	1	1	0	0	0	0	0	1	1
15. T.S.S. °brix	15.19	<	1	1	1	0	0	0	1	0	1	0	0	0	0	0	1	1	1	1	1	0	0	0	1	1	0
16. Acidity %	0.43	>	0	1	0	0	0	1	0	0	1	0	0	0	0	1	1	1	1	1	1	1	1	1	0	1	0
17. Total sugar %	13.52	<	0	1	0	0	1	1	0	0	1	0	0	0	1	0	0	1	1	1	1	1	1	0	1	1	1
Total score			6	16	13	1	3	5	7	7	13	9	6	10	4	5	5	14	14	15	6	11	5	10	12	6	11

* < = more than the average value
> = less than the average value

Among the selected five accessions, plant height ranged from 85 cm (KT2) to 90 cm (TK3) and total leaf number varied from 48.0 (TK18) to 56.0 (TK3). With respect to 'D' leaf area, accession TK3 recorded the highest value of 315.38 cm^2.

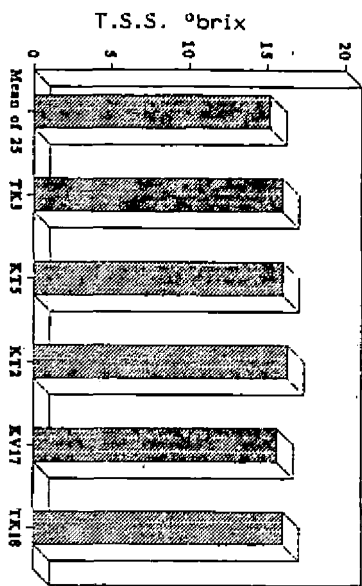
Fruit weight with crown varied among different accessions. This was the highest (2.380 kg) in TK3, followed by KT5 (2.325 kg), KT2 (2.320 kg), KV17 (2.315 kg) and KT18 (2.300 kg). The pulp weight ranged from 1.410 kg (KV17) to 1.505 kg (TK3). The lowest value of peel-pulp ratio (0.34) was recorded in accessions, TK3, TK18, KT5 and with regard to pulp percentage, the highest value was observed in accession TK18 (67.82%) followed by TK3 (67.45%).

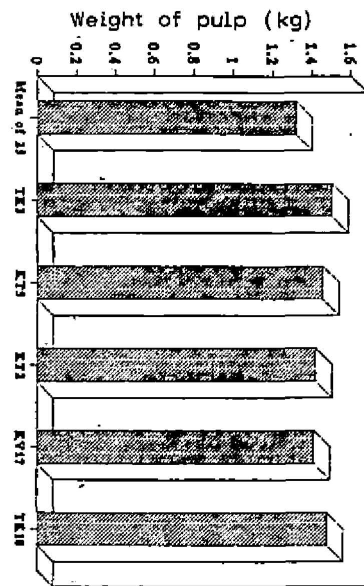
Juice content is an important character, particularly when the fruits are used for juice extraction. Juice content of the selected five accessions ranged from 78.10 per cent to 79.50 per cent, the highest being in accession KT5, followed by 79.20 per cent in KT2.

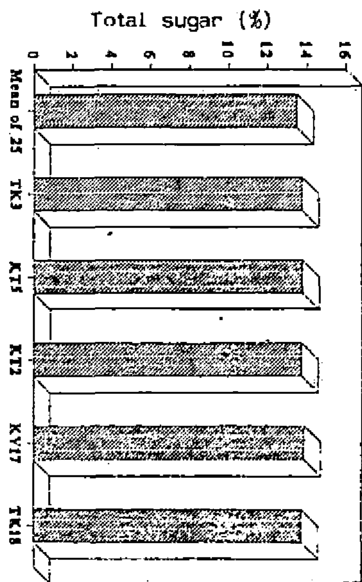
Taper ratio and L/B ratio are the two characters determing the fruit shape, which in turn decides the suitability for efficient and economic processing. With regard to taper ratio, the most superior accessions are TK3 and KV17 wherein the taper ratio was found to be 0.84. While considering the L/B ratio, accession TK3 with L/B ratio of 1.80 was found most superior followed by TK18 and KT5 with a L/B ratio of 1.83 each.

Total soluble solids and total sugar content are the major indicators of sweetness. TSS was the highest in accession KT 2 (16.33 °brix). The total soluble solids observed in other accessions were 16.00 °brix (TK3, TK18 and KT5) and

Sl. No.	Character	value accessions	TK3				
1	Plant height (cm)	87.00	90.00	89.00	85.00	87.00	87.00
2	Number of leaves	50.92	56.00	54.00	52.00	50.00	48.00
3	Leaf area (cm²)	286.45	315.38	277.53	307.98	278.04	285.59
4	Number of suckers	2.16	2.00	2.00	3.00	3.00	2.00
5	Weight of fruit (kg) with crown	2.224	2.380	2.325	2.320	2.315	2.300
6	Weight of crown (g)	159.80	150.00	150.00	160.00	155.00	125.00
7	Weight of fruit (kg) without crown	2.064	2.230	2.175	2.170	2.160	2.175
8	Weight of fruit after peeling (kg)	1.554	1.720	1.675	1.670	1.640	1.675
9	Weight of pulp (kg)	1.318	1.505	1.455	1.420	1.410	1.475
10	Peel-pulp ratio	0.39	0.34	0.34	0.35	0.37	0.34
11	Pulp percentage	63.73	67.45	66.90	65.44	65.28	67.82
12	Juice content %	78.69	78.90	79.50	79.20	78.10	78.60
13	Taper ratio	0.79	0.84	0.80	0.82	0.84	0.83
14	L/B ratio	1.91	1.80	1.83	1.85	1.85	1.83
15	T.S.S. °brix	15.19	16.00	16.00	16.33	15.66	16.00
16	Acidity %	0.43	0.38	0.39	0.39	0.40	0.40
17	Total sugar %	13.52	13.79	13.81	13.75	13.90	13.75
18	Reducing sugar %	3.59	3.97	3.95	3.83	4.05	3.95
19	Non-reducing sugar %	9.93	9.82	9.86	9.92	9.85	9.80
	Score		16	15	14	14	13


T.S.S. °brix
Mean of 25
TK3
KT5
KT2
KV17
TK18


Weight of pulp (kg)
Mean of 25
TK3
KT5
KT2
KV17
TK18


Total sugar (%)
Mean of 25
TK3
KT5
KT2
KV17
TK18

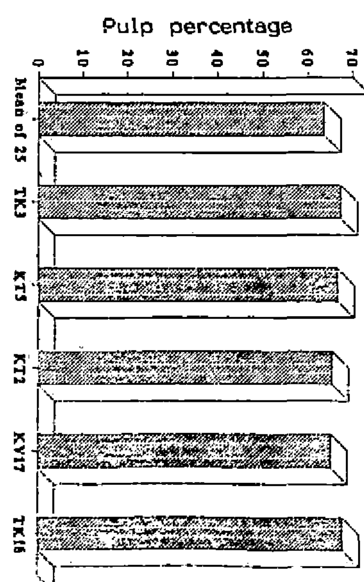
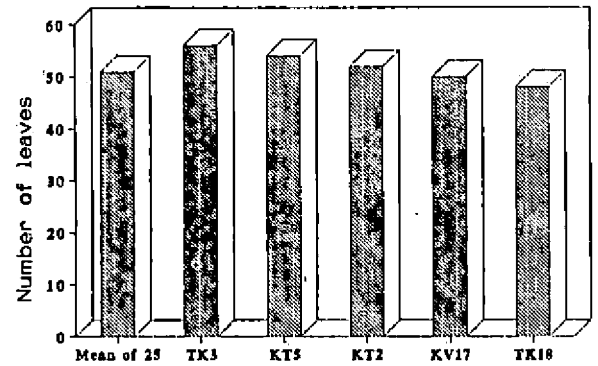
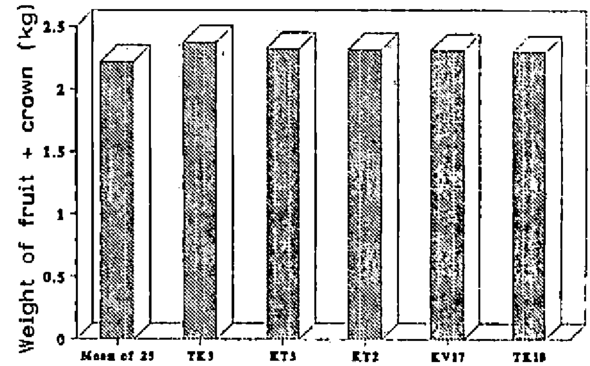
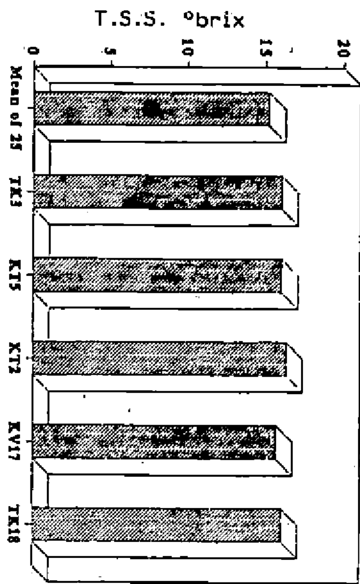
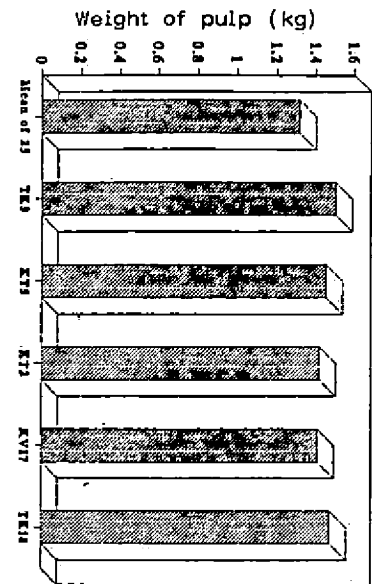

Pulp percentage
Mean of 25
TK3
KT5
KT2
KV17
TK18

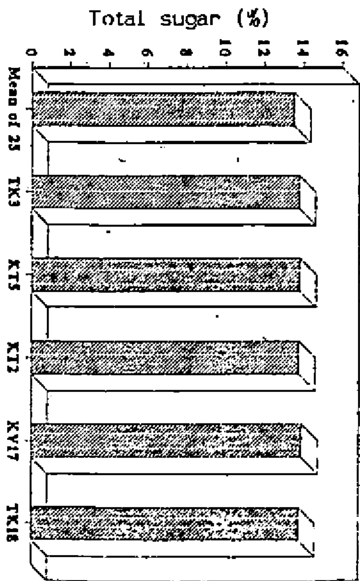
Fig.1. Plant and fruit characters of five selected elite plants of pineapple






T.S.S. °brix
Mean of 25
TK3
KT5
KT2
KV17
TK18


Weight of pulp (kg)
Mean of 25
TK9
KT5
KT2
KV17
TK18


Total sugar (%)
Mean of 25
TK3
KT5
KT2
KV17
TK18

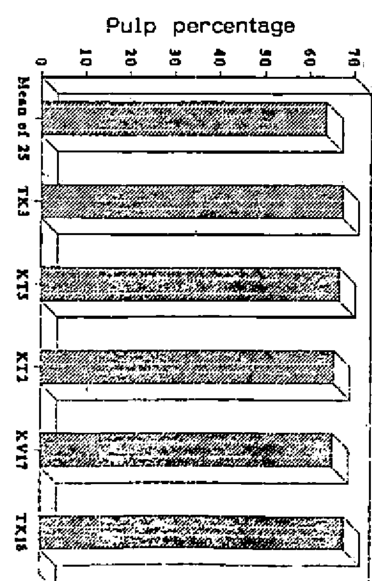
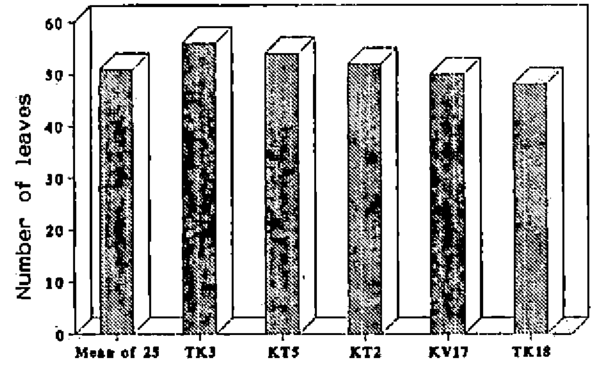
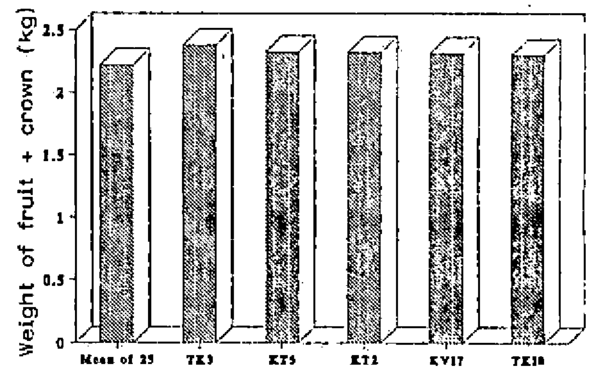

Pulp percentage
Mean of 25
TK3
KT5
KT2
KV17
TK18

Fig.1. Plant and fruit characters of five selected elite plants of pineapple





15.66 °brix in KV17. Total sugar was observed to be maximum in KV17 (13.90%). Accessions KT5 and TK3 recorded a total sugar of 13.81 per cent and 13.79 per cent respectively, while in accessions TK18 and KT2 it was 13.75 per cent.

4.2 Refinement of *in vitro* propagation technique

4.2.1 Standardisation of surface sterilization of explants

The results of the experiments on surface sterilization of explants are presented in Tables 8 and 9.

Shoot tip explants when surface sterilized with Emisan (0.10%) for 35 minutes followed by mercuric chloride (0.10%) for 10 minutes (T_{11}) was found to give maximum percentage of survival (91.66%). Treatments T_9 (Emisan 0.1% for 25 minutes followed by mercuric chloride 0.1% for 10 minutes) and T_{10} (Emisan 0.1% for 30 minutes followed by mercuric chloride 0.1% 10 minutes) have produced 75 per cent contamination free cultures.

Increased.exposure of explants to surface sterilants was found to reduce explant survival. Eventhough the rate of contamination was less (8.33%) in T_{12} (Emisan 0.1% for 40 minutes followed by mercuric chloride 0.1% for 10 minutes) and T_{13} (Emisan 0.1% for 30 minutes followed by mercuric chloride 0.1% for 15 minutes), which was on par with T_{11} (Emisan 0.1% for 35 minutes followed by mercuric chloride 0.1% for 10 minutes), out of the resultant explants, mortality rate was 25 per cent and 33.33 per cent in T_{12} and T_{13} respectively.

Among the various treatments tried for surface sterilization of lateral buds, treatment T_5 (Emisan 0.1% for 10 minutes followed by mercuric chloride 0.1% for 3 minutes) was found to give 83.33 per cent contamination free cultures.

Table 8. Standardisation of surface sterilization of shoot tip explants of pineapple

Treatment No.	Surface sterilization treatment	Percentage of contamination	Percentage of explant mortality	Percentage of explant survival
T_1	Emisan 0.1% 10 mts + Mercuric chloride 0.1% 5 mts	100.00	0.00	0.00
T_2	Emisan 0.1% 15 mts + Mercuric chloride 0.1% 5 mts	100.00	0.00	0.00
T_3	Emisan 0.1% 20 mts + Mercuric chloride 0.1% 5 mts	91.66	0.00	8.33
T_4	Emisan 0.1% 25 mts + Mercuric chloride 0.1% 5 mts	83.33	0.00	16.66
T_5	Emisan 0.1% 30 mts + Mercuric chloride 0.1% 5 mts	75.00	0.00	25.00
T_6	Emisan 0.1% 10 mts + Mercuric chloride 0.1% 10 mts	66.66	0.00	33.33
T_7	Emisan 0.1% 15 mts + Mercuric chloride 0.1% 10 mts	58.33	0.00	41.66
T_8	Emisan 0.1% 20 mts + Mercuric chloride 0.1% 10 mts	41.66	0.00	58.33
T_9	Emisan 0.1% 25 mts + Mercuric chloride 0.1% 10 mts	25.00	0.00	75.00
T_{10}	Emisan 0.1% 30 mts + Mercuric chloride 0.1% 10 mts	25.00	0.00	75.00
T_{11}	Emisan 0.1% 35 mts + Mercuric chloride 0.1% 10 mts	8.33	0.00	91.66
T_{12}	Emisan 0.1% 40 mts + Mercuric chloride 0.1% 10 mts	8.33	25.00	66.66
T_{13}	Emisan 0.1% 30 mts + Mercuric chloride 0.1% 15 mts	8.33	33.33	58.33

Observation recorded 15 days after inoculation

Table 9. Standardisation of surface sterilization of lateral bud explants

Treatment No.	Surface sterilization treatment	Percentage of contamination	Percentage of explant mortality	Percentage of explant survival
T_1	Emisan 0.1% 5 mts + Mercuric chloride 0.1% 1 mts	100.00	0.00	0.00
T_2	Emisan 0.1% 10 mts + Mercuric chloride 0.1% 1 mts	91.66	0.00	8.33
T_3	Emisan 0.1% 15 mts + Mercuric chloride 0.1% 1 mts	83.33	0.00	16.66
T_4	Emisan 0.1% 5 mts + Mercuric chloride 0.1% 3 mts	50.00	0.00	50.00
T_5	Emisan 0.1% 10 mts + Mercuric chloride 0.1% 3 mts	16.66	0.00	83.33
T_6	Emisan 0.1% 15 mts + Mercuric chloride 0.1% 3 mts	16.66	16.66	66.66
T_7	Emisan 0.1% 5 mts + Mercuric chloride 0.1% 5 mts	16.66	33.33	50.00

Observations recorded 15 days after inoculation

Increasing the exposure time of lateral buds to surface sterilants was found to reduce the survival rate of explants. Treatment T_6 (0.1% Emisan for 15 minutes followed by 0.1% mercuric chloride for 3 minutes) and treatment T_7 (0.1% Emisan for 5 minutes followed by 0.1% mercuric chloride for 5 minutes) have shown mortality percentage of 16.66 and 33.33 and survival percentage of 66.66 and 50.00 respectively, where as in both cases rate of contamination was same (16.66%) as in the case of T_2.

4.2.2 Culture establishment

Culture establishment trials were carried out using shoot tip explants from suckers, crowns, slips and lateral buds from stem in MS medium supplemented with different concentrations of BAP.

4.2.2.1 Standardisation of media for culture establishment from shoot tip explants of suckers

The data on number of days taken for culture establishment as evidenced by greening of excised explants from suckers are presented in Table 10.

MS medium supplemented with BAP induced fastest greening of explants compared to basal MS medium without any growth regulator (Plate 1). The mean number of days taken for greening was shortest (10.12 days) in T_3 (MS + BAP 3 mg l^{-1}) followed by 10.25 days in T_4 (MS + BAP 4.0 mg l^{-1}).

Higher concentrations of BAP (above 5.0 mg l^{-1}) was found to increase the days for greening of explants. Treatments T_5 (BAP 5.0 mg l^{-1}), T_6 (BAP 7.5 mg l^{-1} and T_7 (BAP 10.0 mg l^{-1}) took 11.23, 14.12 and 14.82 days

Table 10. Effect of media on culture establishment of shoot tip explants from suckers

Treatment No.	Growth regulator concentration	Days taken for explant greening
T_1	BAP 1.0 mg l^{-1}	12.84
T_2	BAP 2.0 mg l^{-1}	11.66
T_3	BAP 3.0 mg l^{-1}	10.12
T_4	BAP 4.0 mg l^{-1}	10.25
T_5	BAP 5.0 mg l^{-1}	11.23
T_6	BAP 7.5 mg l^{-1}	14.12
T_7	BAP 10.0 mg l^{-1}	14.82
T_8	Control (basal MS)	16.43
F		**
CD		1.19
SEm±		0.21

** Significant at 0.01 level
Observations recorded at three days interval upto 25 days

respectively for greening of explants. In basal MS medium, explants took 16.43 days for greening.

4.2.2.2 Effect of source of shoot tip explants on culture establishment

MS medium supplemented with BAP 3.0 mg l^{-1}, which was found most superior in culture establishment as evidenced by greening of shoot tips from suckers was used for this study.

The mean number of days for establishment of shoot tip explants from suckers, crowns and slips were 10.12, 10.66 and 10.48 days respectively which was statistically on par with each other, Table 11.

4.2.2.3 Lateral bud explant

The data on number of days taken for culture establishment from lateral bud explants as evidenced by greening of cultures are presented in Table 12.

When the lateral buds were used as explants, the minimum number of 13.05 days for greening was observed in treatment T_4 (BAP 4.0 mg l^{-1}) and treatment T_3 with BAP 3.0 mg l^{-1} (13.25 days), which were significantly superior to all other treatments. Basal MS without any growth regulator took 19.88 days for greening (Plate 2).

Higher concentrations of BAP (above 5.0 mg l^{-1}) delayed culture establishment. In treatments T_6 (BAP 7.5 mg l^{-1}) and T_7 (BAP 10.0 mg l^{-1}) greening of cultures was noticed in 15.13 and 16.08 days respectively.

Table 11. Effect of source of shoot tip explants on culture establishment

Treatment No.	Explant source	Days taken for explant greening
T_1	Sucker	10.12
T_2	Crown	10.66
T_3	Slips	10.48
F		NS
SEm±		0.22

NS - Non significant
Observations recorded at three days interval up to 25 days of inoculation

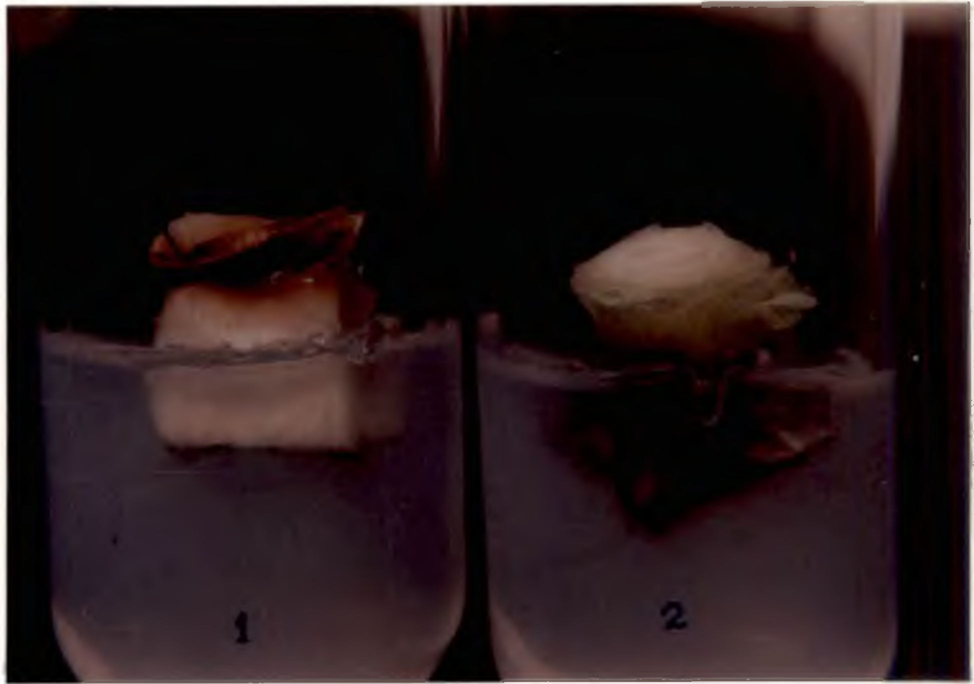
Table 12. Effect of media on culture establishment from lateral bud explants

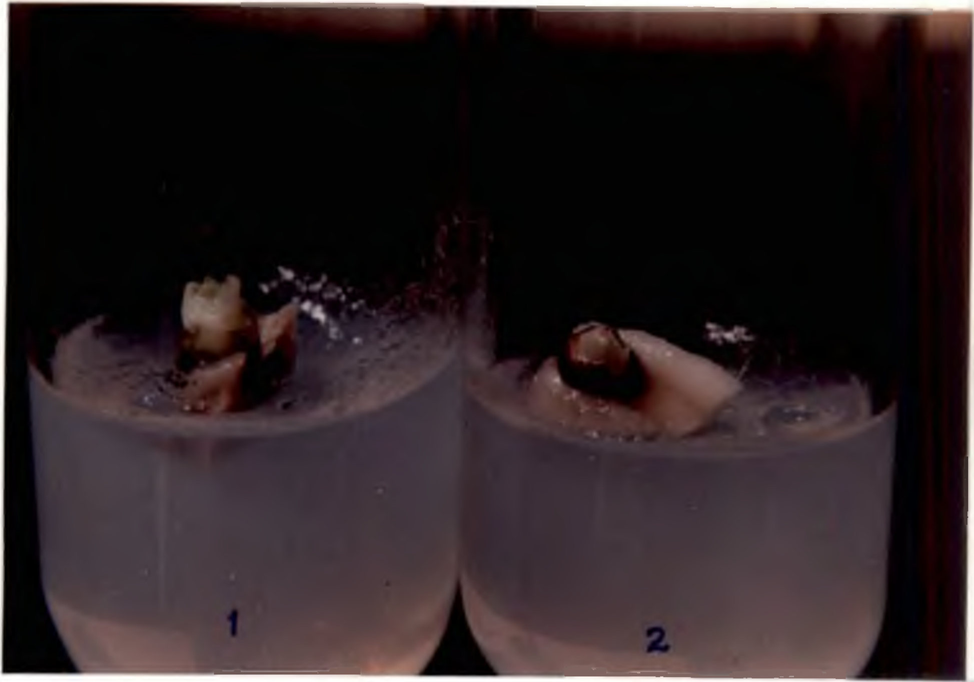
Treatment No.	Growth regulator concentration	Days taken for explant greening
T_1	BAP 1.0 mg l^{-1}	14.13
T_2	BAP 2.0 mg l^{-1}	13.77
T_3	BAP 3.0 mg l^{-1}	13.25
T_4	BAP 4.0 mg l^{-1}	13.05
T_5	BAP 5.0 mg l^{-1}	13.95
T_6	BAP 7.5 mg l^{-1}	15.13
T_7	BAP 10.0 mg l^{-1}	16.08
T_8	Control (basal MS)	19.88
F		**
CD		1.00
SEm±		0.18

** Significant at 0.01 level
Observations recorded at three days interval up to 25 days of inoculation

Plate 1. Culture establishment of shoot tip explants from suckers in basal MS (1) and MS + BAP 3.0 mg l^{-1} medium (2) (11 days after culturing)

Plate 2. Culture establishment of lateral bud explants in MS + BAP 4.0 mg l^{-1} (1) and basal MS medium (2) (14 days after culturing)


1
2


1
2

4.2.3 Standardisation of media for growth initiation

4.2.3.1 Shoot tip explants from suckers

Days for initiation of growth of shoot tip explants from suckers ranged from 12.22 days to 19.25 days among different treatments (Table 13, Plate 3).

Growth initiation was achieved by 12.22 days in treatment T_4 (BAP 4.0 mg l^{-1}), which was on par with treatment T_3 (BAP 3.0 mg l^{-1}), where in growth initiation was observed in 12.29 days.

Concentrations of BAP above 4.0 mg l^{-1} delayed initiation of growth as exhibited in treatments T_5, T_6 and T_7, where in the cultures took 12.98, 15.07 and 16.47 days respectively for initiation of growth. The cultures in basal MS medium took 19.25 days for this response.

4.2.3.2 Effect of source of shoot-tip explants on growth initiation

The treatment, BAP 4.0 mg l^{-1} which was found most suited for initiation of growth for shoot tip explants from suckers was used to compare the response of various shoot tip explants. The number of days for growth initiation from shoot tip explants from suckers, crowns and slips were 12.22, 12.22 and 12.57 days respectively which was statistically on par with each other, Table 14.

4.2.3.3 Lateral bud explants

Number of days taken for initiation of growth from lateral bud explant is presented in Table 15.

Table 13. Effect of media on growth initiation from sucker shoot tips

Treatment No.	Growth regulator concentration	Days taken for initiation of growth
T_1	BAP 1.0 mg l^{-1}	13.89
T_2	BAP 2.0 mg l^{-1}	13.65
T_3	BAP 3.0 mg l^{-1}	12.29
T_4	BAP 4.0 mg l^{-1}	12.22
T_5	BAP 5.0 mg l^{-1}	12.98
T_6	BAP 7.5 mg l^{-1}	15.07
T_7	BAP 10.0 mg l^{-1}	16.47
T_8	Control (basal MS)	19.25
F		**
CD		1.16
SEm±		0.21

** Significant at 0.01 level
Observations recorded at three days interval up to 25 days of inoculation

Table 14. Effect of source of shoot tip explants on growth initiation

Treatment No.	Source of explants	Days taken for initiation of growth
T_1	Sucker	12.22
T_2	Crown	12.22
T_3	Slips	12.57
F		NS
SEm±		0.52

NS - Non significant
Observations recorded at three days interval up to 25 days of inoculation

Table 15. Effect of media on growth initiation from lateral bud explants

Treatment No.	Growth regulator concentration	Days taken for initiation of growth
T_1	BAP 1.0 mg l^{-1}	15.79
T_2	BAP 2.0 mg l^{-1}	15.33
T_3	BAP 3.0 mg l^{-1}	13.96
T_4	BAP 4.0 mg l^{-1}	13.53
T_5	BAP 5.0 mg l^{-1}	14.14
T_6	BAP 7.5 mg l^{-1}	16.75
T_7	BAP 1.0 mg l^{-1}	17.52
T_8	Control (basal)	21.05
F		**
CD		1.13
SEm±		0.20

** Significant at 0.01 level
Observations recorded at three days interval up to 25 days of inoculation

Among different treatments tried, the number of days taken for initiation of growth was shortest (13.53 days) in treatment T_4 (BAP 4.0 mg l^{-1}), which was statistically superior to all other treatments (Plate 4).

Higher concentrations of BAP (above 5.0 mg l^{-1}) in the medium delayed the response in cultures initiated with lateral bud explants.

4.2.4 Culture multiplication

4.2.4.1 Standardisation of media for enhanced release of axillary buds

4.2.4.1.1 Effect of different levels of BAP on enhanced release of axillary buds

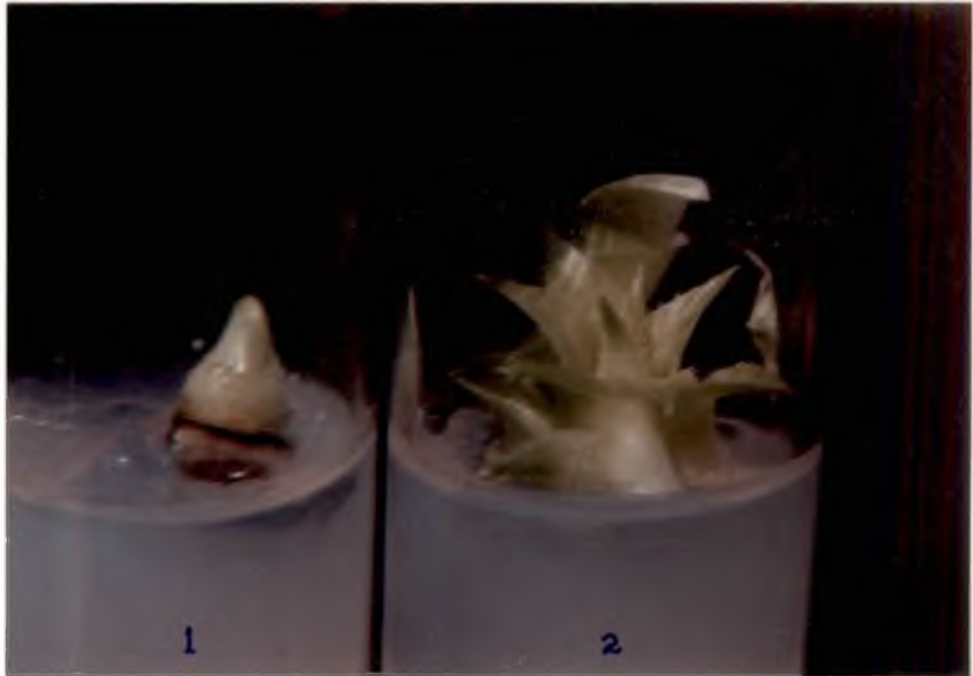
Result of the experiment on effect of BAP at the following different levels (1.0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in combination with NAA at two levels (0.5 and 1.0 mg l^{-1}) on axillary bud production during different subculture stages are presented in Table 16.

a) First subculture

Among the different treatments tried, the treatment T_4 (BAP 4.0 mg l^{-1}) resulted in the highest number of axillary buds of 3.32 per culture during first subculture, which was statistically superior to all other treatments. In the case of treatment T_3 (BAP 3.0 mg l^{-1}), the mean number of axillary buds produced per culture was 2.80. At higher levels of BAP above 4.0 mg l^{-1} axillary bud production was reduced as exhibited by treatment T_5 (BAP 5.0 mg l^{-1}), T_6 (BAP 7.5 mg l^{-1}) and T_7 (BAP 10.0 mg l^{-1}); where in the mean number of axillary buds produced was only, 1.95, 1.40 and 1.30 respectively.

Plate 3. Growth initiation of sucker shoot tips in MS + BAP 4.0 mg l^{-1} (13 days after culturing)

Plate 4. Growth initiation of lateral bud explants in basal MS (1) and MS + BAP 4.0 mg l^{-1} medium (2) (14 days after culturing)



1
2

Table 16. Effect of different levels of BAP on enhanced release of axillary buds

Treatment No.	Growth regulator concentration	Mean number of axillary bud		
		I subculture	II subculture	III subculture
T_1	BAP 1.0 mg l^{-1}	1.56	1.97	1.57
T_2	BAP 2.0 mg l^{-1}	1.93	2.29	2.40
T_3	BAP 3.0 mg l^{-1}	2.80	3.80	3.03
T_4	BAP 4.0 mg l^{-1}	3.32	4.11	3.40
T_5	BAP 5.0 mg l^{-1}	1.95	2.11	1.90
T_6	BAP 7.5 mg l^{-1}	1.40	1.50	1.40
T_7	BAP 10.0 mg l^{-1}	1.30	1.18	0.88
T_8	BAP 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}	1.10	1.80	1.08
T_9	BAP 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}	1.03	1.20	1.02
T_{10}	BAP 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}	1.10	1.87	1.66
T_{11}	BAP 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}	0.98	1.30	1.04
T_{12}	BAP 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}	1.38	2.00	0.32
T_{13}	BAP 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}	1.25	1.12	0.18
T_{14}	BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}	1.52	1.77	0.28
T_{15}	BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}	1.46	1.67	0.18
T_{16}	BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}	1.89	1.02	0.00
T_{17}	BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}	1.11	0.93	0.00
T_{18}	BAP 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}	1.09	0.95	0.00
T_{19}	BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}	1.01	0.89	0.00
T_{20}	Control (basal MS)	0.56	0.75	0.67
F		**	**	**
CD		0.10	0.12	0.12
SEm±		0.02	0.02	0.02

** Significant at 0.01 level
Observations recorded 25 days after each subculturing

The production of axillary buds was adversely affected by the inclusion of NAA to the media as evidenced in the combination treatments T_{14} (BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{15} (BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}), where in the number of axillary buds produced was 1.52 and 1.46 respectively as against 3.32 buds produced in media with BAP 4.0 mg l^{-1} alone (T_4).

Axillary bud production was minimum in basal MS medium without any growth regulator (T_{20}), where in the mean number of buds per culture was only 0.56.

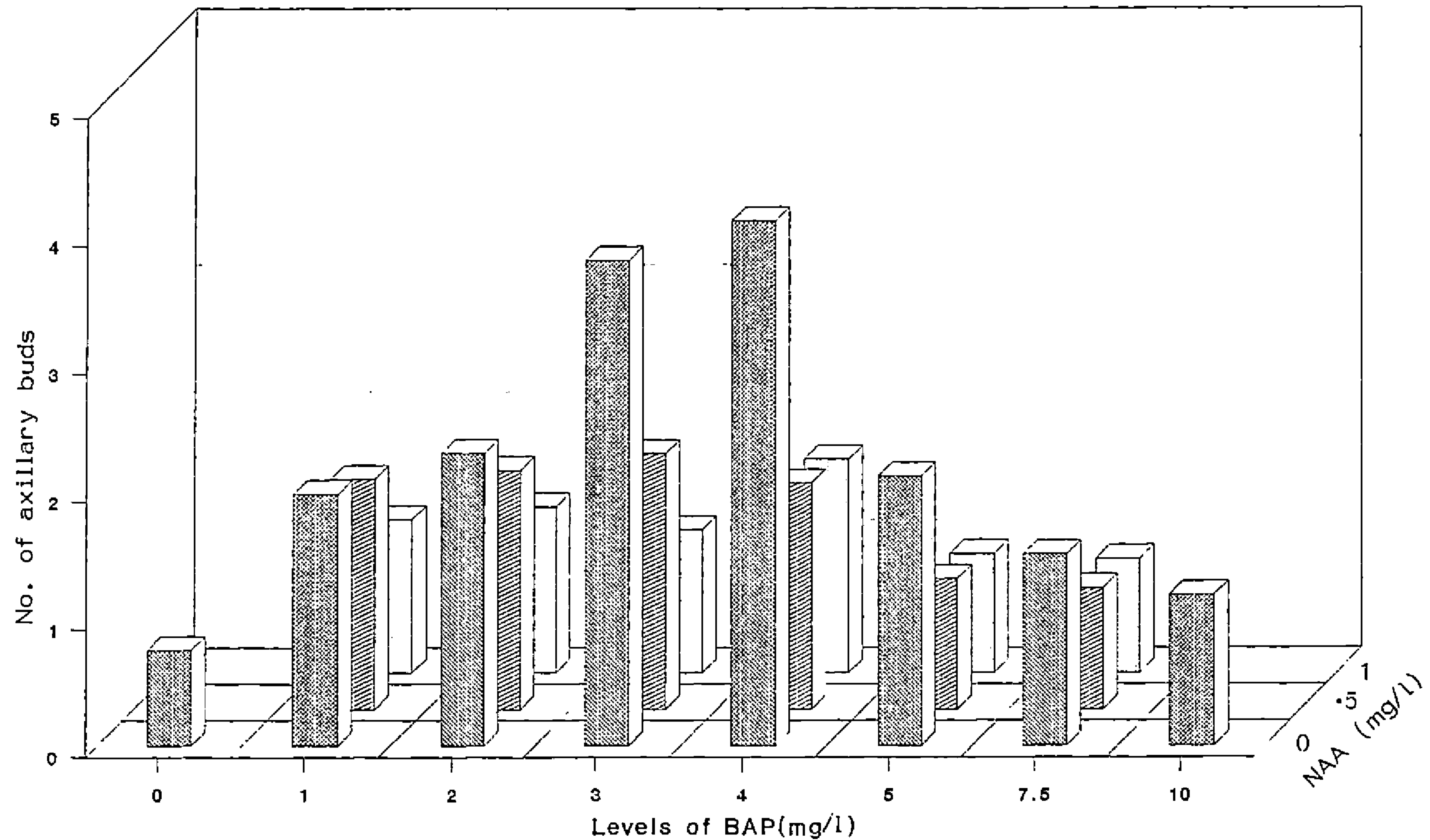
b) Second subculture

The axillary bud production during second subculture recorded a similar trend as that of first subculture, but for the higher number of buds produced (Fig.2). A maximum of 4.11 axillary buds per culture was recorded in treatment T_4 (BAP 4.0 mg l^{-1}). This was significantly superior to all other treatments (Plate 5). The treatment T_3 with BAP 3.0 mg l^{-1} resulted in the production of 3.80 buds per culture.

In combination treatments of BAP and NAA, axillary bud production was reduced as evidenced by treatments T_{14} (BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}), T_{15} (BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}); where in the mean number of buds per culture was 1.77 and 1.67 respectively as against 4.11 buds produced in treatment T_4 with BAP 4.0 mg l^{-1} alone.

The number of axillary buds was only 0.75 per culture in basal MS medium.

Fig.2 Effect of different levels of BAP on enhanced release of axillary buds (during second subculture stage)



c) Third subculture

As in the case of first and second subculture stages, the significant superior treatment with regard to axillary bud production was treatment T_4 (BAP 4.0 mg l^{-1}); where in the mean number of axillary buds produced was 3.40 followed by 3.03 buds produced in T_3 (BAP 3.0 mg l^{-1}).

Comparing to second subculture, in third subculture treatment with these two levels of BAP (4.0 mg l^{-1} and 3.0 mg l^{-1}) resulted in lesser production of axillary buds.

In treatments T_{16} (BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}), T_{17} (BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}; T_{18} (BAP 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{19} (BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}) axillary bud production was lacking but these treatments resulted in initiation and growth of adventitious buds (globular bodies).

4.2.4.1.2 Effect of different levels of kinetin on enhanced release of axillary buds

Results of the experiment conducted to study the effect of kinetin at different levels (1.0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in combination with NAA at two levels (0.5 and 1.0 mg l^{-1}) on axillary bud production during different subculture stages are presented in Table 17.

a) First subculture

Among different treatments tried, treatment T_5 (kinetin 5.0 mg l^{-1}) produced 2.40 axillary buds per culture, which was statistically superior to all other treatments. This was followed by treatment T_6 (kinetin 7.5 mg l^{-1}) with 2.21 buds per culture.

Table 17. Effect of different levels of kinetin on enhanced release of axillary buds

Treatment No.	Growth regulator concentration	Mean number of axillary buds		
		I subculture	II subculture	III subculture
T_1	Kinetin 1.0 mg l^{-1}	0.71	1.06	0.74
T_2	Kinetin 2.0 mg l^{-1}	1.08	2.04	1.86
T_3	Kinetin 3.0 mg l^{-1}	1.86	2.28	2.15
T_4	Kinetin 4.0 mg l^{-1}	2.03	2.99	2.17
T_5	Kinetin 5.0 mg l^{-1}	2.40	3.39	2.45
T_6	Kinetin 7.5 mg l^{-1}	2.21	2.83	2.03
T_7	Kinetin 10.0 mg l^{-1}	0.68	1.06	0.68
T_8	Kinetin 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}	0.65	0.87	0.62
T_9	Kinetin 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}	0.67	0.85	0.66
T_{10}	Kinetin 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}	0.78	0.95	0.68
T_{11}	Kinetin 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}	0.76	0.89	0.66
T_{12}	Kinetin 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}	0.94	1.65	0.99
T_{13}	Kinetin 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}	0.94	1.57	0.97
T_{14}	Kinetin 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}	1.03	1.69	0.95
T_{15}	Kinetin 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}	1.02	1.50	0.82
T_{16}	Kinetin 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}	1.14	1.82	0.61
T_{17}	Kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}	1.10	1.21	0.50
T_{18}	Kinetin 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}	0.99	0.97	0.00
T_{19}	Kinetin 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}	0.96	0.95	0.00
T_{20}	Control (basal MS)	0.57	0.74	0.67
F		**	**	**
CD		0.13	0.12	0.11
SEm±		0.02	0.02	0.02

** Significant at 0.01 level
Observations recorded 25 days after each subculturing

In combination with NAA, kinetin produced lesser number of axillary buds, as observed in treatment T_{16} (kinetin 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{17} (kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}); where in the number of axillary buds produced were only 1.14 and 1.10 respectively, while in the treatment T_5 with kinetin 5.0 mg l^{-1} alone the mean number of buds produced was 2.40.

In basal MS medium, the number of axillary buds produced was 0.57 per culture, which was the lowest among all other treatments.

b) Second subculture

The maximum number of 3.39 axillary buds was produced in treatment T_5 (kinetin 5.0 mg l^{-1}), which was statistically superior to all other treatments (Fig.3, Plate 6).

In combination with NAA, axillary bud production was less. The treatments T_{16} (kinetin 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{17} (kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}), the number of axillary buds produced per culture was 1.82 and 1.21 respectively, while in the same concentration of kinetin alone (5.0 mg l^{-1}), the number of axillary buds produced was 3.39.

In basal medium without any growth regulator, the number of axillary buds produced was only 0.74 per culture.

c) Third subculture

During third subculture stage also, treatment T_5 (kinetin 5.0 mg l^{-1}) was statistically superior to all other treatments. The treatment produced 2.45 axillary

Fig.3 Effect of different levels of kinetin on enhanced release of axillary buds (during second subculture stage)

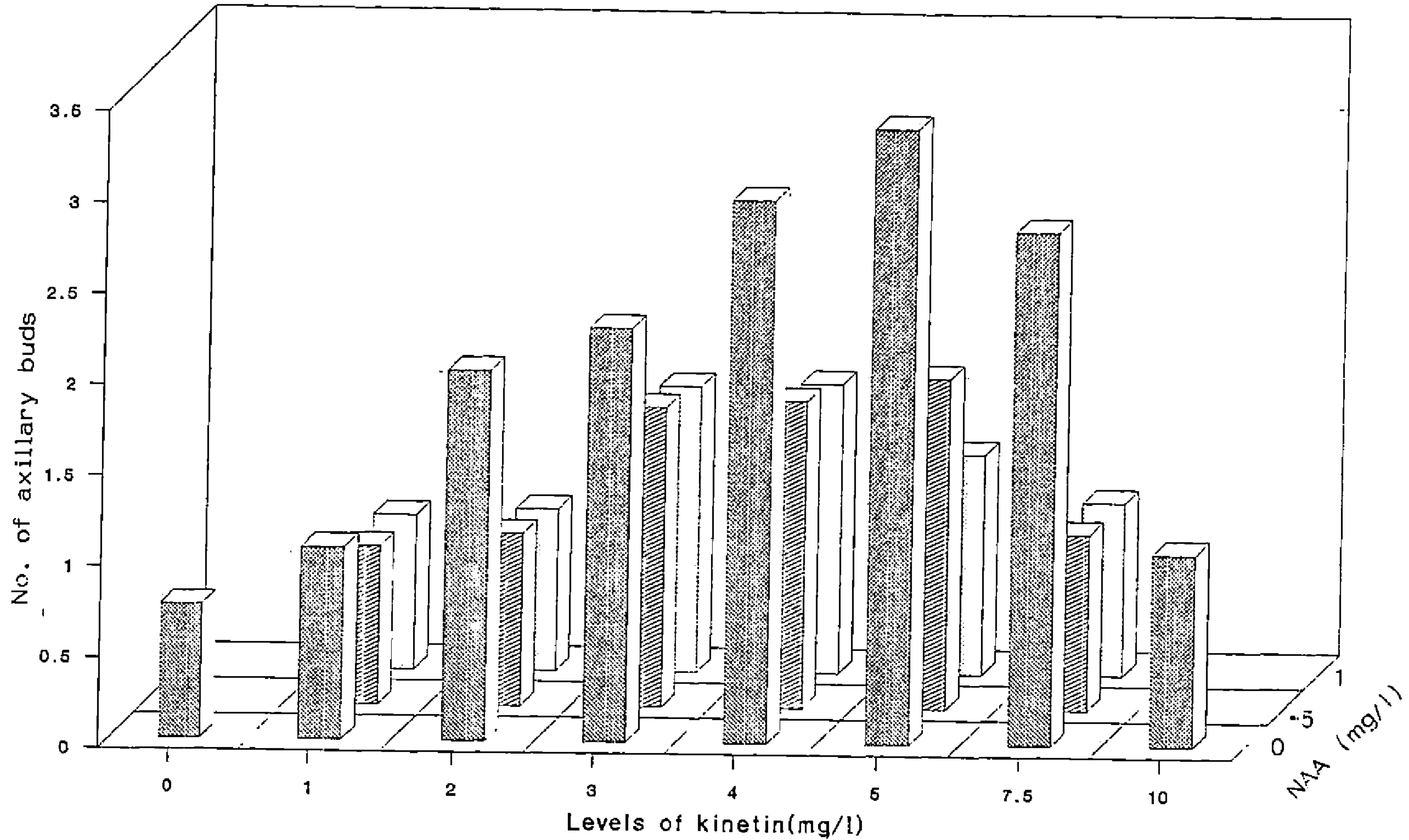
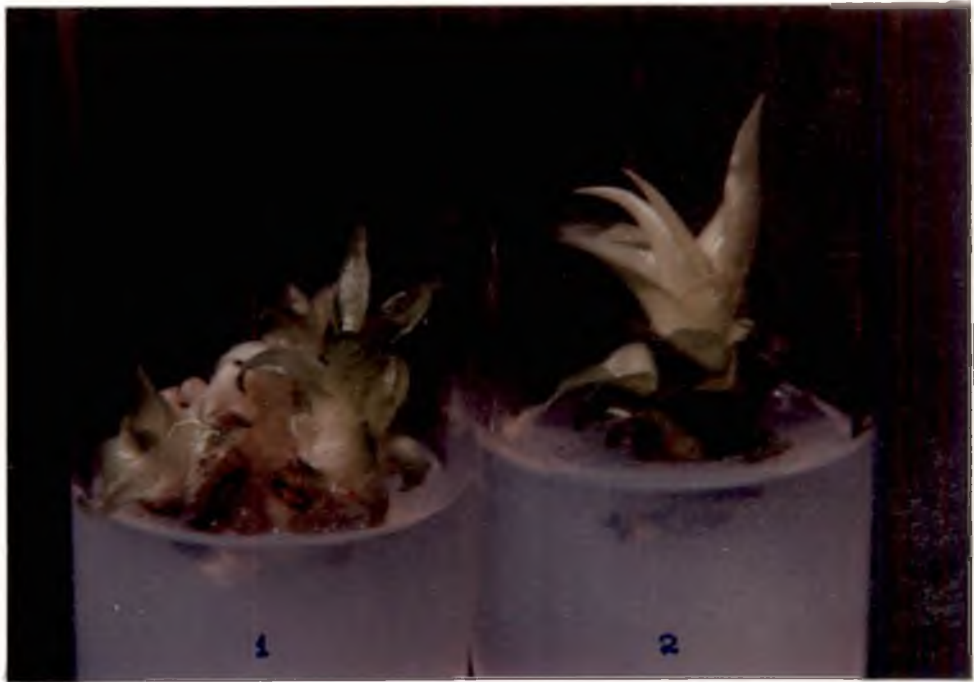


Plate 5. Enhanced release of axillary buds in MS + BAP 4.0 mg l^{-1} (1) and basal MS medium (2) (25 days after second subculturing)

Plate 6. Enhanced release of axillary buds in MS + kinetin 5.0 mg l^{-1} (1) and basal MS medium (2) (25 days after second subculturing)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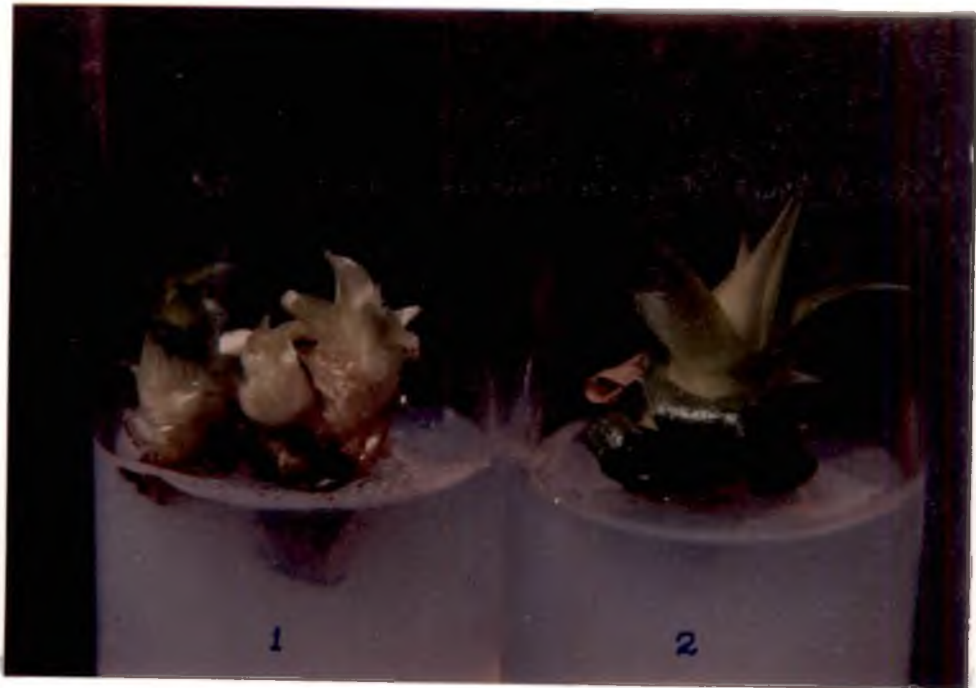

1
2

buds per culture. This was followed by 2.17 buds in treatment T_4 with kinetin 4.0 mg l^{-1}, which was statistically on par with treatment T_3 (kinetin 3.0 mg l^{-1}) producing 2.15 buds.

In treatments T_{18} (kinetin 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{19} (kinetin 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}) axillary bud production was lacking, rather adventitious buds were initiated.

4.2.4.1.3 Effect of medium supplements on enhanced release of axillary buds

The effects of different medium supplements, namely, caesin hydrolysate 50.0 mg l^{-1} and 100.0 mg l^{-1}, coconut water five per cent and 10 per cent, Tomato juice 10 per cent on axillary bud production during different subculture stages were studied in a separate experiment. The multiplication medium containing MS + BAP 4.0 mg l^{-1} along with the medium supplements was used for the study. Results of the experiment are presented in Table 18.

a) First subculture

A maximum number of 3.66 axillary buds per culture was produced in treatment T_2 with caesin hydrolysate 100.0 mg l^{-1}. This was significantly superior to all other treatments. In treatments T_1 (caesin hydrolysate 50.0 mg l^{-1}), T_5 (tomato juice 10%) and T_3 (coconut water 5%), the number of axillary buds produced was 3.49, 3.46 and 3.40 respectively which was statistically on par to each other and with Control (BAP 4.0 mg l^{-1}) producing 3.37 axillary buds per culture. The lowest number of axillary buds of 3.09 was produced in treatment with coconut water 10 per cent.

Table 18. Effect of medium supplements on enhanced release of axillary buds

Treatment No.	Medium supplements	Mean number of axillary buds		
		I subculture	II subculture	III subculture
T_1	Caesin hydrolysate 50 mg l^{-1}	3.49	4.10	3.44
T_2	Casein hydrolysate 100 mg l^{-1}	3.66	4.48	4.02
T_3	Coconut water 5%	3.40	4.12	3.47
T_4	Coconut water 10%	3.09	3.78	3.12
T_5	Tomato juice 10%	3.46	4.27	3.46
T_6	Control (BAP 4.0 mg l^{-1})	3.37	4.08	3.43
F		**	**	**
CD		0.41	0.59	0.60
SEm±		0.07	0.10	0.10

** Significant at 0.01 level
Observations recorded 25 days after each subculturing

b) Second subculture

During the second subculture stage, the maximum number of axillary buds of 4.48 was produced in treatment T_2 with caesin hydrolysate 100.0 mg l^{-1}, which was significantly superior to all other treatments (Plate 7). The mean number of axillary buds produced was 4.27, 4.12 and 4.10 respectively in treatments with tomato juice 10 per cent (T_5); Coconut water 5 per cent (T_3) and Caesin hydrolysate 50.0 mg l^{-1} (T_1), which were statistically similar to each other and were on par with 4.08 buds of the MS + BAP 4.0 mg l^{-1} (T_6). The number of axillary buds produced was lowest in treatment T_4 (Coconut water 10%) where a mean number of 3.78 buds was formed per culture.

In all the treatments, during second subculture stage, axillary bud production was higher comparing to first subculture stage.

c) Third subculture

Among the different treatments, T_2 (Caesin hydrolysate 100.0 mg l^{-1}) producing a mean of 4.02 axillary buds per culture was statistically superior to all other treatments. Treatments T_3 (Coconut water 5%); T_5 (Tomato juice 10%) and T_1 (Caesin hydrolysate 50.0 mg l^{-1}) and the control T_6 (BAP 4.0 mg l^{-1}) were statistically on par with respect to this parameter producing 3.47, 3.46 and 3.44 and 3.43 buds per culture. The minimum response was observed in treatment T_4 (Coconut water 10%) where a mean number of 3.12 buds was formed.

All the treatments in third subculture, showed lesser production of axillary buds comparing to second subculture stage.

Plate 7. Effect of caesin hydrolysate 100.0 mg l^{-1} on enhanced release of axillary buds (25 days after second subculturing)

4.2.4.2 Standardisation of media for initiation of adventitious buds

4.2.4.2.1 Effect of different levels of BAP on adventitious bud initiation

Trials were conducted with BAP at different levels (1.0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in combination with two levels of NAA (0.5 and 1.0 mg l^{-1}) on adventitious bud initiation using shoot tip explants from suckers. The number of days taken for adventitious bud initiation was calculated from the first subculture stage and the results are presented in Table 19.

The minimum number of days for initiation of adventitious buds was taken by the medium supplemented with BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} (T_{17}), where bud initiation took place in 24.29 days (Plate 8). In treatment T_{16} (BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}) adventitious bud initiation was achieved in 25.21 days. These two treatments were statistically on par to each other and were superior to all other treatments. While treatments T_{15} (BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}) and T_{14} (BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}) took 32.64 and 36.07 days respectively for the response.

When BAP was used alone in the medium, this response was delayed as comparing to its combination treatments with NAA. In treatment T_5 (BAP 5.0 mg l^{-1}) adventitious buds were initiated in 76.41 days as against 24.29 and 25.21 days respectively in treatments T_{17} (BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}) and T_{16} (BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}).

When BAP was used alone in the medium, higher concentrations of BAP (above 5.0 mg l^{-1}), induced faster response, comparing to its lower levels (below

Table 19. Effect of BAP on adventitious bud formation in sucker shoot tip explants

Treatment No.	Growth regulator concentration	Mean number of days for Adventitious bud initiation
T_1	BAP 1.0 mg l^{-1}	147.72
T_2	BAP 2.0 mg l^{-1}	141.75
T_3	BAP 3.0 mg l^{-1}	125.75
T_4	BAP 4.0 mg l^{-1}	101.57
T_5	BAP 5.0 mg l^{-1}	76.41
T_6	BAP 7.5 mg l^{-1}	75.63
T_7	BAP 10.0 mg l^{-1}	73.11
T_8	BAP 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}	98.02
T_9	BAP 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}	67.54
T_{10}	BAP 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}	73.51
T_{11}	BAP 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}	62.22
T_{12}	BAP 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}	54.46
T_{13}	BAP 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}	48.12
T_{14}	BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}	36.07
T_{15}	BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}	32.64
T_{16}	BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}	25.21
T_{17}	BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}	24.29
T_{18}	BAP 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}	44.28
T_{19}	BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}	41.47
T_{20}	Control (basal MS)	159.11
F		**
CD		6.68
SEm±		1.26

** Significant at 0.01 level

Observations recorded at three days interval up to 25 days of inoculation

4.0 mg l^{-1}), as evidenced by treatment T_5 (BAP 5.0 mg l^{-1}), where adventitious bud initiation took place in 76.41 days, while in treatment with BAP 1.0 mg l^{-1} (T_1) buds were formed in 147.72 days. This trend was followed in combination treatments containing BAP and NAA also. In treatment T_{17} (BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}) adventitious buds were formed in 24.29 days, while in treatment T_9 (BAP 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}) adventitious bud initiation took place only in 67.54 days.

In basal MS medium without any growth regulator, 159.11 days were required to form the adventitious buds.

4.2.4.2.2 Effect of different levels of kinetin on initiation of adventitious buds

Results of the experiment on effect of kinetin at different levels (1.0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in combination with NAA at two levels (0.5 and 1.0 mg l^{-1}) on adventitious bud initiation using shoot tip explants from suckers are presented in Table 20.

The number of days taken for adventitious bud initiation varied significantly from 57.25 days to 159.63 days in different treatments. Minimum days for the response was observed in treatment T_{17} with kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} (57.25 days) (Plate 9). This treatment was on par with treatments T_{19} (kinetin 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}) and T_{18} (kinetin 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}), where adventitious bud initiation took place in 57.31 and 59.45 days respectively.

When kinetin alone was used in the medium, adventitious bud initiation was delayed, comparing to its combination treatments with NAA. In treatment T_5

Table 20. Effect of kinetin on initiation of adventitious buds in shoot tip explants from suckers

Treatment No.	Growth regulator concentration	Mean number of days for adventitious bud initiation
T_1	Kinetin 1.0 mg l^{-1}	159.49
T_2	Kinetin 2.0 mg l^{-1}	154.37
T_2	Kinetin 3.0 mg l^{-1}	135.92
T_4	Kinetin 4.0 mg l^{-1}	121.39
T_5	Kinetin 5.0 mg l^{-1}	112.65
T_6	Kinetin 7.5 mg l^{-1}	110.31
T_7	Kinetin 10.0 mg l^{-1}	107.19
T_8	Kinetin 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}	110.22
T_9	Kinetin 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}	100.52
T_{10}	Kinetin 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}	104.51
T_{11}	Kinetin 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}	90.73
T_{12}	Kinetin 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}	91.12
T_{13}	Kinetin 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}	79.94
T_{14}	Kinetin 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}	73.61
T_{15}	Kinetin 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}	65.79
T_{16}	Kinetin 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}	62.72
T_{17}	Kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}	57.25
T_{18}	Kinetin 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}	59.45
T_{19}	Kinetin 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}	57.31
T_{20}	Control (basal MS)	159.63
F		**
CD		5.04
SEm±		0.95

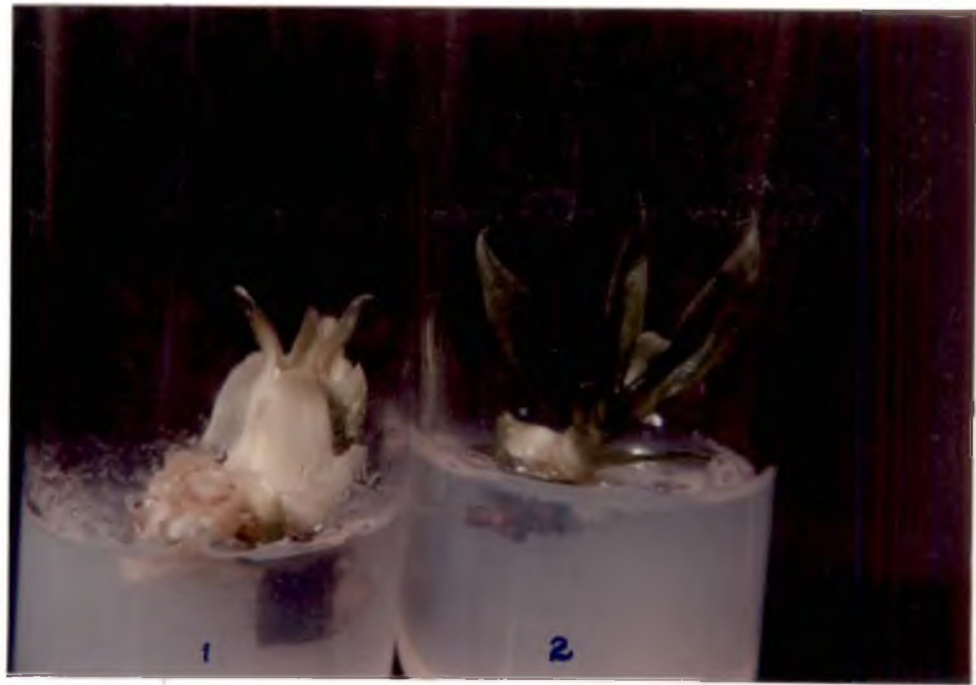
** Significant at 0.01 level

Observations recorded at three days interval up to 25 days of inoculation

Plate 8. Adventitious bud initiation in shoot tip explants from suckers in MS + BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} (1) and basal MS medium (2) (25 days after first subculturing)

Plate 9. Adventitious bud initiation in shoot tip explants from suckers in MS + kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} (1) and basal MS medium (2) (8 days after third subculturing)

1
2


1
2

(kinetin 5.0 mg l^{-1}) adventitious buds were initiated in 112.65 days, while treatment T_{17} (kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}) took only 57.25 days for the response.

Adventitious buds production was delayed in treatments containing lower levels of kinetin comparing to its higher levels, as evidenced by treatment T_1 (kinetin 1.0 mg l^{-1}), where in buds were initiated only in 159.49 days, while in treatment T_7 (kinetin 10.0 mg l^{-1}), which took only 107.19 days for adventitious bud formation. Similar trend was observed in combination of treatments of kinetin and NAA also. Treatment T_9 (kinetin 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}), took 100.52 days for adventitious bud initiation, while in treatment T_{17} (kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}), the response was noticed in 57.25 days.

Initiation of adventitious buds was noticed in basal MS medium only at 159.63 days.

4.2.4.2.3 Effect of source of shoot tip explants on initiation of adventitious buds

The number of days taken for adventitious bud formation among different cultures initiated with shoot tip explants excised from suckers, crowns and slips are presented in Table 21.

Among the different shoot tip explants used, there existed no significant difference with regard to formation of adventitious buds. The number of days taken for initiation of adventitious buds from shoot tip explants of suckers, crowns and slips were 24.29, 26.06 and 26.28 days respectively, in the MS medium supplemented with BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}.

Table 21. Effect of source of shoot tip explants on initiation of adventitious buds

Treatment No.	Source of shoot tip explants	Mean number of days for adventitious bud initiation
T_1	Sucker	24.29
T_2	Crown	26.06
T_3	Slips	26.28
F		NS
SEm±		0.96

NS - Non significant
Observations recorded at three days interval up to 25 days of inoculation

4.2.4.2.4 Effect of different levels of BAP on initiation of adventitious buds in lateral bud explants

In order to study the effect of different levels of BAP on initiation of adventitious buds from lateral bud explants, a separate experiment with 7 levels of BAP (1.0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in combination with NAA at two levels (0.5 and 1.0 mg l^{-1}) was conducted. The data on observations recorded are furnished in Table 22.

Among different treatments tried, treatment T_{19} (BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}) was more effective in inducing adventitious buds, where in the bud initiation took place in 33.40 days (Plate 10). The treatment was statistically superior to all other treatments. Treatment with BAP 7.5 mg l^{-1} + NAA 0.5 mg l^{-1} (T_{18}) produced this response in 38.01 days.

Different treatments with BAP alone was found to take more time for initiation of adventitious buds, comparing to its combination treatments with NAA. Treatment T_6 (BAP 7.5 mg l^{-1}) took 99.48 days for initiating adventitious buds, while in treatments T_{18} (BAP 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{19} (BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}) adventitious buds were formed in 38.01 and 33.40 days respectively.

When BAP was used alone, its higher concentrations was found to induce adventitious bud formation more faster, comparing to lower concentrations of BAP. In treatment T_7 (BAP 10.0 mg l^{-1}) adventitious buds were produced in 94.77 days, while in treatments T_1 (BAP 1.0 mg l^{-1}) and T_2 (BAP 2.0 mg l^{-1}) this response was observed in 166.42 and 155.39 days respectively. Similar trends were

Table 22. Effect of BAP on adventitious bud initiation in lateral bud explants

Treatment No.	Growth regulator concentrations	Mean number of days for adventitious bud initiation
T_1	BAP 1.0 mg l^{-1}	166.42
T_2	BAP 2.0 mg l^{-1}	155.39
T_3	BAP 3.0 mg l^{-1}	147.16
T_4	BAP 4.0 mg l^{-1}	120.44
T_5	BAP 5.0 mg l^{-1}	110.90
T_6	BAP 7.5 mg l^{-1}	99.48
T_7	BAP 10.0 mg l^{-1}	94.77
T_8	BAP 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}	115.45
T_9	BAP 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}	95.90
T_{10}	BAP 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}	103.22
T_{11}	BAP 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}	85.19
T_{12}	BAP 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}	70.07
T_{13}	BAP 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}	59.29
T_{14}	BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}	53.54
T_{15}	BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}	46.00
T_{16}	BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}	43.79
T_{17}	BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}	41.77
T_{18}	BAP 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}	38.01
T_{19}	BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}	33.40
T_{20}	Control (basal MS)	173.57
F		**
CD		4.43
SEm±		0.83

** Significant at 0.01 level

Observations recorded at three days interval up to 25 days of inoculation

Plate 10. Adventitious bud initiation in lateral bud explants in MS + BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1} medium (9 days after second subculturing)

observed in combination treatments of BAP and NAA as evidenced by treatments T_{18} (BAP 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{19} (BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}), where in adventitious buds were formed in 38.01 and 33.40 days respectively, while treatments T_8 (BAP 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_9 (BAP 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}) took 115.45 and 95.90 days respectively for this response.

Maximum days for production of adventitious buds was taken by the basal MS medium without any growth regulator (173.57 days).

4.2.4.3 Standardisation of media for proliferation of adventitious buds

4.2.4.3.1 Effect of different levels of BAP on proliferation of adventitious buds

Results of the experiment on effect of different levels of BAP on proliferation of adventitious buds are presented in Table 23 and Fig.4.

Among different treatments, maximum multiplication of adventitious buds was observed in treatments T_{14} (BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{15} (BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}) (Plate 11).

When BAP alone was used in the medium, the proliferation rate was lower, when compared to combination treatments of both BAP and NAA as evidenced by treatment T_4 (BAP 4.0 mg l^{-1}) and treatments T_{14} (BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{15} (BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}).

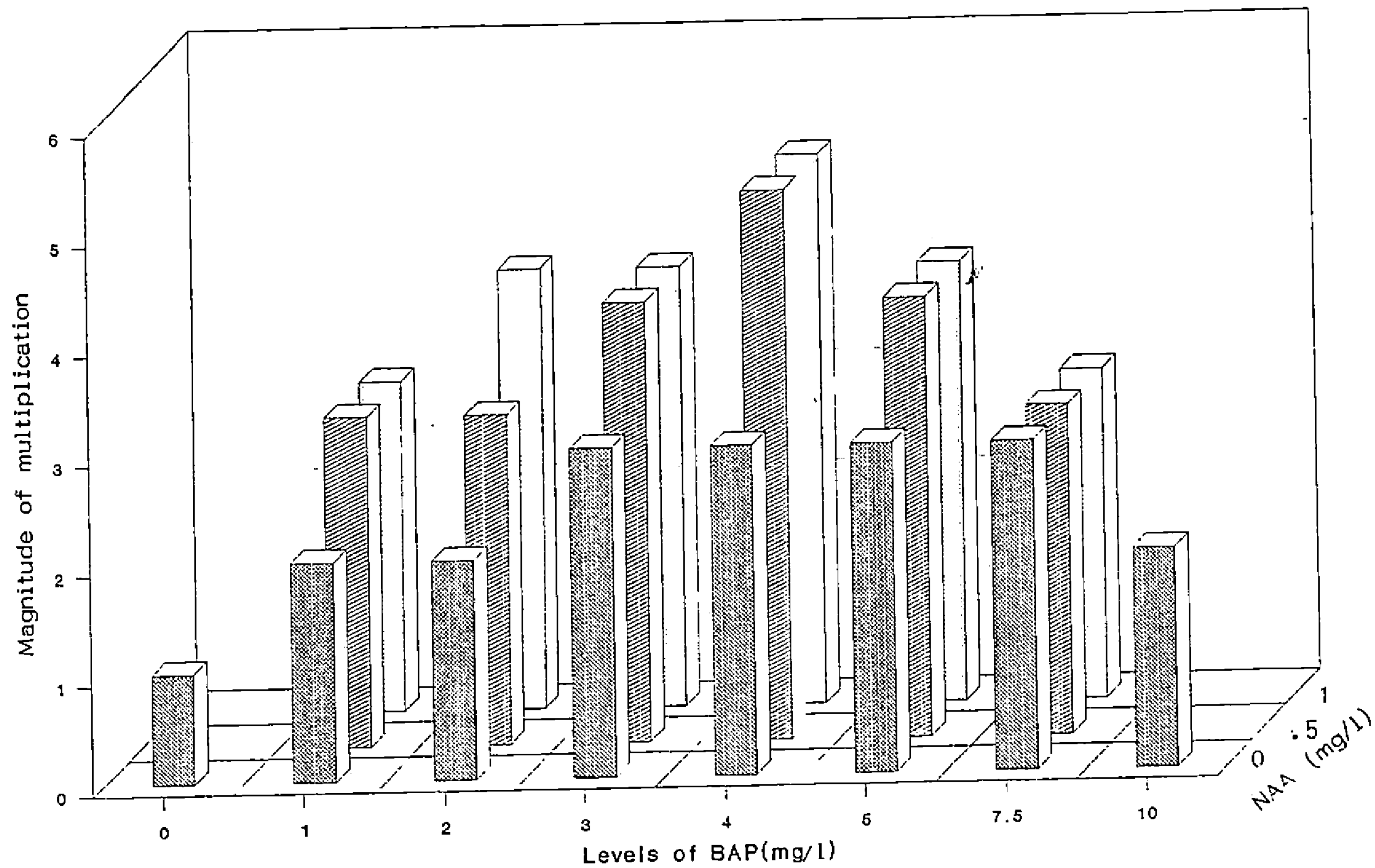
BAP in the media at lower concentrations (below 3.0 mg l^{-1}) and at higher concentrations of above 7.5 mg l^{-1} were found to reduce the proliferation rate. Likewise, when used in combination with NAA also, both lower levels of BAP

Table 23. Effect of BAP on proliferation of adventitious buds

Treatment No.	Growth regulator concentrations	Intensity of adventitious bud proliferation
T_1	BAP 1.0 mg l^{-1}	++
T_2	BAP 2.0 mg l^{-1}	++
T_3	BAP 3.0 mg l^{-1}	+++
T_4	BAP 4.0 mg l^{-1}	+++
T_5	BAP 5.0 mg l^{-1}	+++
T_6	BAP 7.5 mg l^{-1}	+++
T_7	BAP 10.0 mg l^{-1}	++
T_8	BAP 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}	+++
T_9	BAP 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}	+++
T_{10}	BAP 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}	+++
T_{11}	BAP 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}	++++
T_{12}	BAP 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}	++++
T_{13}	BAP 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}	++++
T_{14}	BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}	+++++
T_{15}	BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}	+++++
T_{16}	BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}	++++
T_{17}	BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}	++++
T_{18}	BAP 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}	+++
T_{19}	BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}	+++
T_{20}	Control (basal MS)	+

Observations recorded 25 days after inoculation

Fig.4 Effect of BAP on proliferation of adventitious buds



(below 3.0 mg l^{-1}) and higher levels (above 4.0 mg l^{-1}), showed lesser multiplication of adventitious buds.

Lowest rate of proliferation of adventitious buds was observed in basal MS medium (T_{20}).

4.2.4.3.2 Effect of different levels of kinetin of proliferation of adventitious buds

Effect of kinetin at different levels in the media on the proliferation of adventitious buds were recorded and presented in Table 24 and Fig.5.

With regard to rate of multiplication of adventitious buds, among different treatments, maximum rate of proliferation was noticed in treatment T_{17} with kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} (Plate 12). Other treatments with kinetin alone or in combination with NAA showed lesser rate of proliferation of adventitious buds.

Lowest rate of multiplication was observed in treatment T_{20} (basal MS medium without any growth regulator).

4.2.4.3.3 Effect of physical condition of the media on proliferation of adventitious buds

Observations on the effect of physical condition of the media on proliferation of adventitious buds are presented in Table 25 and Fig.6.

Among different treatments with solid media maximum rate of multiplication of adventitious buds was observed in treatments T_5 (BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_6 (BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}). In liquid medium under shake culture conditions, the highest rate of proliferation of adventitious buds

Table 24. Effect of kinetin on proliferation of adventitious buds

Treatment No.	Growth regulator concentration	Intensity of adventitious bud proliferation
T_1	Kinetin 1.0 mg l^{-1}	++
T_2	Kinetin 2.0 mg l^{-1}	++
T_3	Kinetin 3.0 mg l^{-1}	++
T_4	Kinetin 4.0 mg l^{-1}	++
T_5	Kinetin 5.0 mg l^{-1}	+++
T_6	Kinetin 7.5 mg l^{-1}	++
T_7	Kinetin 10.0 mg l^{-1}	++
T_8	Kinetin 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}	+++
T_9	Kinetin 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}	+++
T_{10}	Kinetin 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}	+++
T_{11}	Kinetin 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}	+++
T_{12}	Kinetin 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}	+++
T_{13}	Kinetin 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}	+++
T_{14}	Kinetin 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}	+++
T_{15}	Kinetin 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}	+++
T_{16}	Kinetin 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}	+++
T_{17}	Kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}	++++
T_{18}	Kinetin 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}	++
T_{19}	Kinetin 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}	++
T_{20}	Control (basal MS)	+

Observations recorded 25 days after inoculation

Fig.5 Effect of kinetin on proliferation of adventitious buds

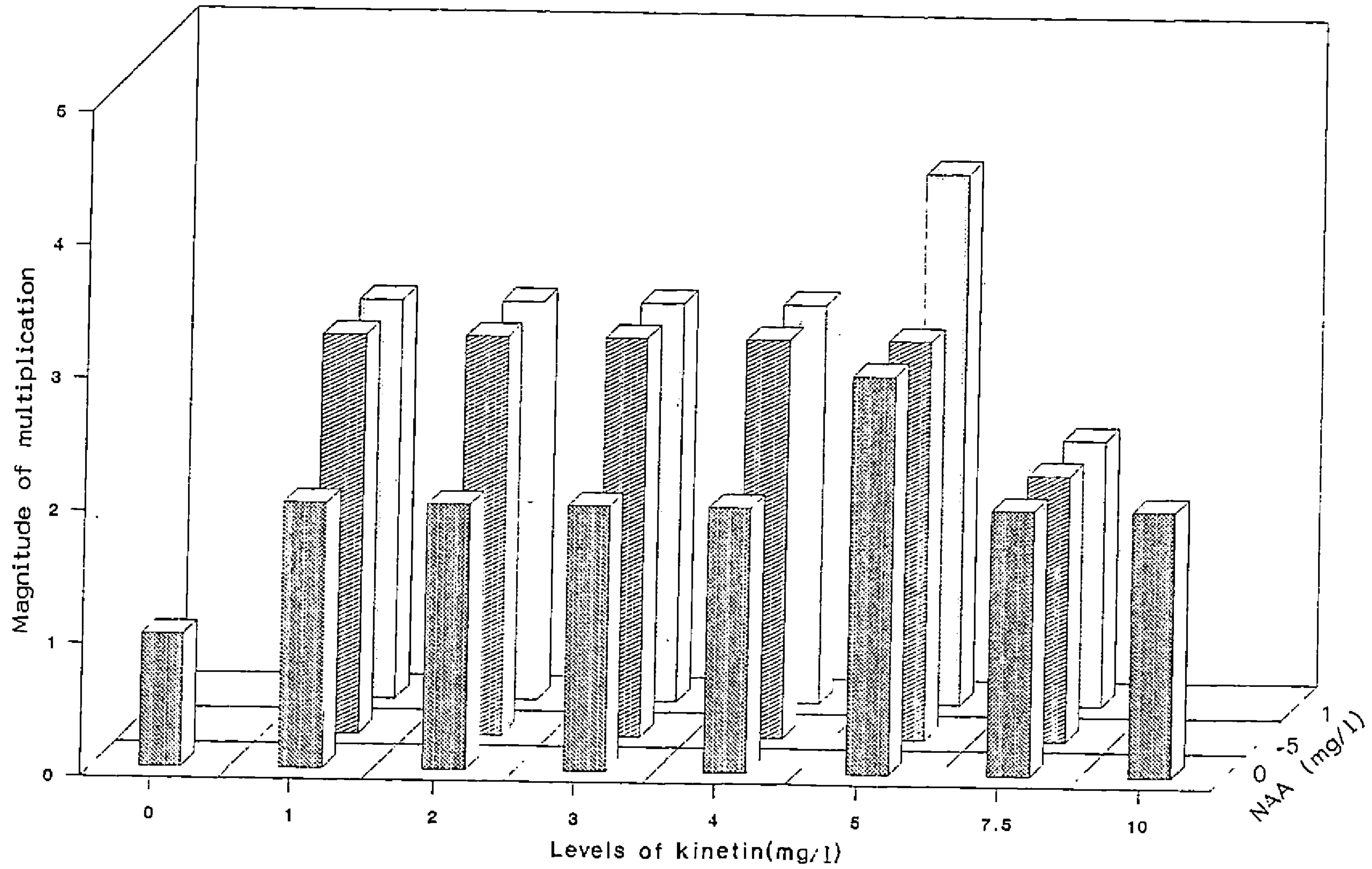


Plate 11. Culture showing adventitious bud proliferation in MS + BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1} medium (25 days after culturing)

Plate 12. Culture showing adventitious bud proliferation in MS + kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} medium (25 days after culturing)

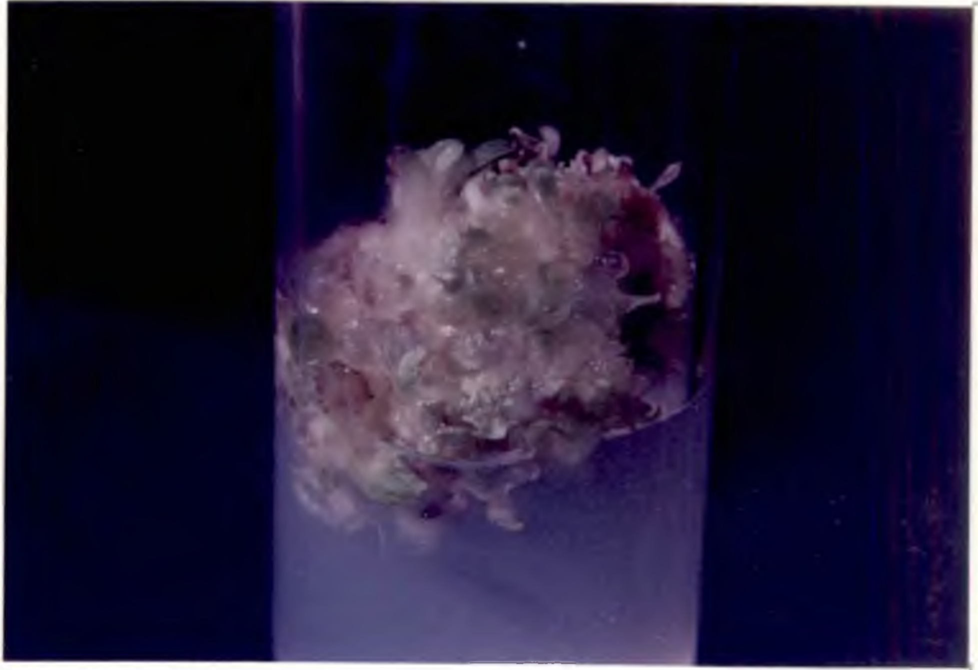
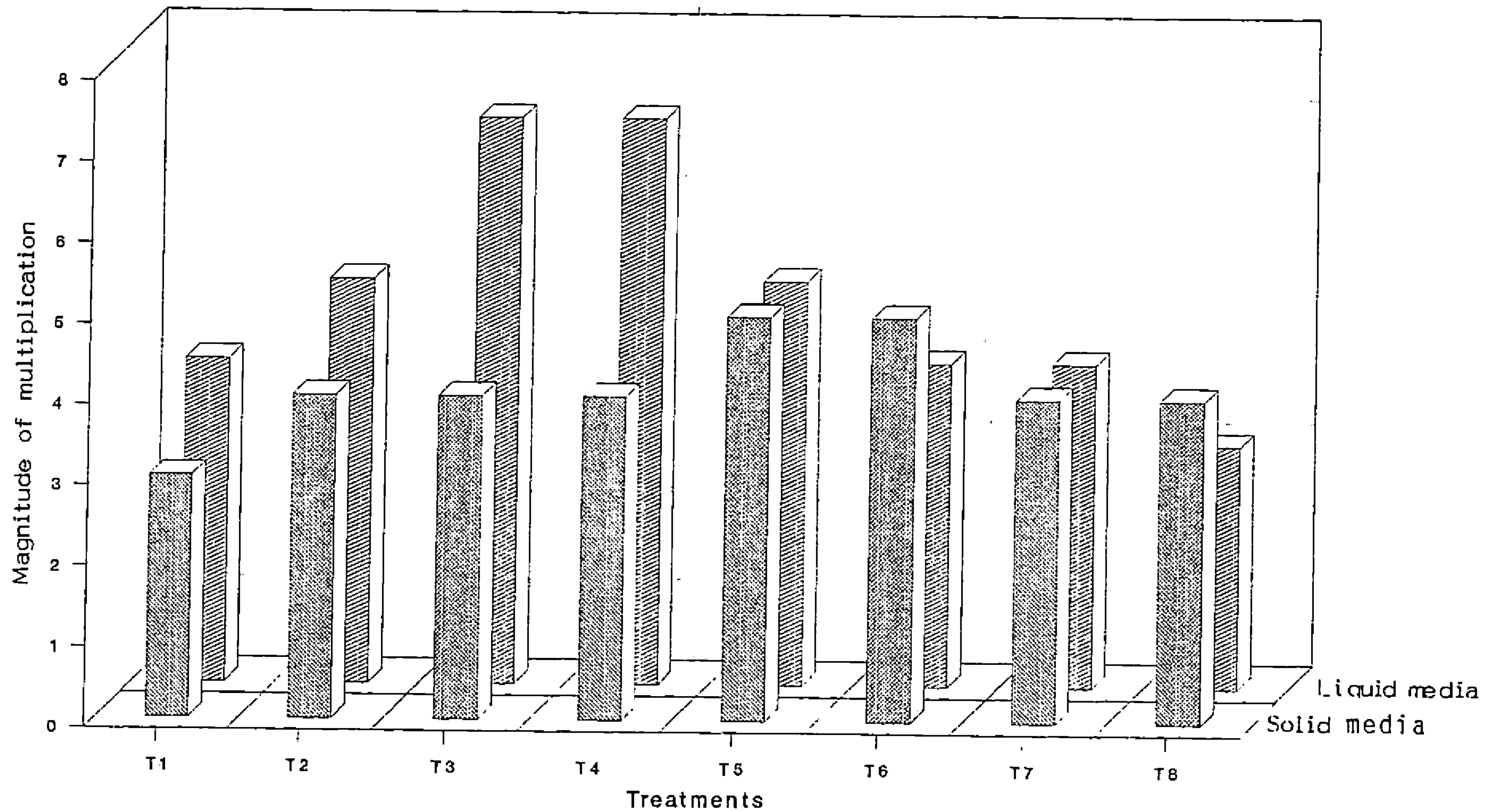


Table 25. Effect of physical condition of media on proliferation of adventitious buds

Treatment No.	Growth regulator concentrations	Intensity of adventitious bud proliferation	
		Solid MS media	Shake culture (Liquid media)
T_1	BAP 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}	+++	++++
T_2	BAP 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}	++++	+++++
T_3	BAP 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}	++++	+++++++
T_4	BAP 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}	++++	+++++++
T_5	BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}	+++++	+++++
T_6	BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}	+++++	++++
T_7	BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}	++++	++++
T_8	BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}	++++	+++

Observations recorded 25 days after inoculation

Fig.6 Effect of physical condition of media on proliferation of adventitious buds



was observed in treatments T_3 (BAP 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_4 (BAP 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}).

Proliferation rate achieved in the best treatments in liquid medium namely, T_3 (BAP 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_4 (BAP 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}) was higher than that observed in best treatments in solid medium, namely T_5 with BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1} and T_6 with BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1} (Plate 13).

4.2.4.4 Standardisation of media for regeneration of shoots from adventitious buds

4.2.4.4.1 Effect of different levels of BAP on shoot growth

Cluster of adventitious buds was transformed to MS medium supplemented with BAP at different levels (1.0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in combination with NAA at two levels (0.5 and 1.0 mg l^{-1}) to study the effect of BAP at different levels on regeneration of multiple shoots. Number of days taken for shoot initiation, number of shoots per culture, mean length of shoots were recorded and are presented in Table 26.

a) Days for shoot initiation

Among different treatments, shoot initiation was fastest in basal MS medium without any growth regulator (T_{20}), where in shoot production was observed in 10.15 days (Plate 14). This treatment was statistically superior to all other treatments. This was followed by treatment T_1 (11.91 days).

Plate 13. Cultures showing adventitious bud proliferation in liquid shake culture (1) and in solid medium (2) (25 days after culturing)

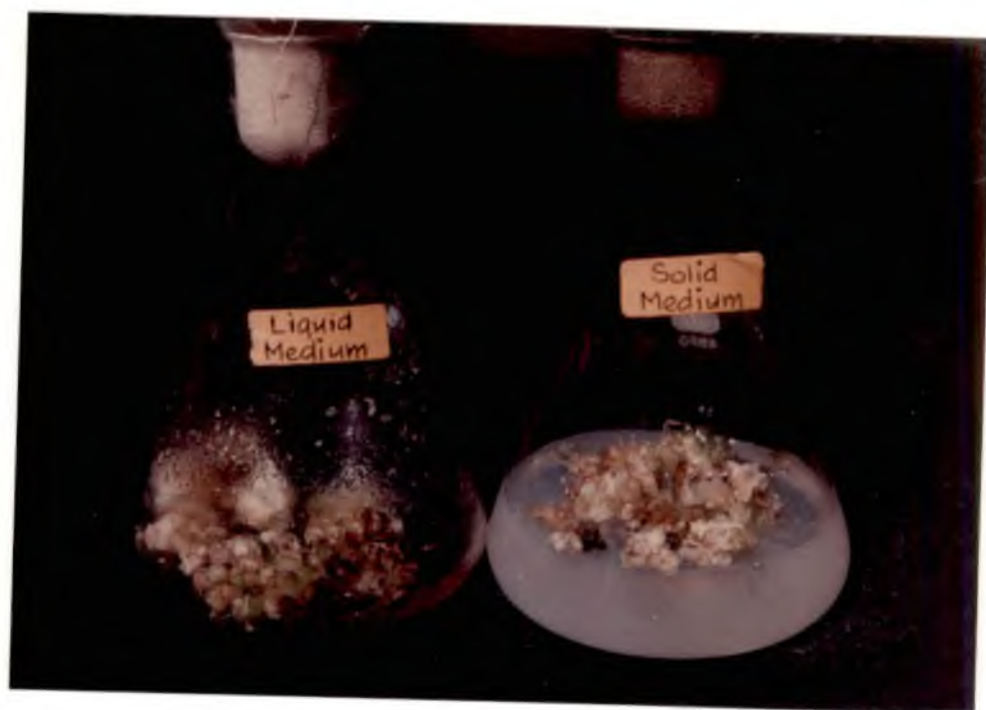

Liquid
Medium
Solid
Medium

Table 26. Effect of different levels of BAP on shoot growth

Treatment No.	Growth regulator concentration	Mean number of days for shoot initiation	Mean number of shoots	Mean length of shoots (cm)	Nature of shoots
T_1	BAP 1.0 mg l^{-1}	11.91	27.01	1.35	Shoot, medium vigorous shoots
T_2	BAP 2.0 mg l^{-1}	13.06	26.16	1.06	,,
T_3	BAP 3.0 mg l^{-1}	13.12	24.99	1.03	,,
T_4	BAP 4.0 mg l^{-1}	13.99	17.60	0.46	Very short condensed shoots
T_5	BAP 5.0 mg l^{-1}	14.38	16.08	0.33	,,
T_6	BAP 7.5 mg l^{-1}	14.65	Numerous shoot initials	Length cannot be measured	Leafy proliferation
T_7	BAP 10.0 mg l^{-1}	> 40	0.00	0.00	Blackening of adventitious bud from 15 days onwards
T_8	BAP 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}	13.43	22.02	1.00	Short shoots
T_9	BAP 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}	14.41	20.29	0.54	Short condensed shoots
T_{10}	BAP 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}	18.55	20.98	0.62	,,
T_{11}	BAP 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}	19.01	19.81	0.39	Very short condensed shoots
T_{12}	BAP 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}	19.70	18.54	0.33	,,
T_{13}	BAP 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}	19.86	18.05	0.32	,,
T_{14}	BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}	19.99	17.87	0.32	,,
T_{15}	BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}	20.36	17.01	0.32	,,
T_{16}	BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}	20.63	Numerous shoot initials	Length cannot be measured	Leafy proliferation
T_{17}	BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}	20.88	,,	,,	,,
T_{18}	BAP 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}	> 40	0.00	0.00	Blackening of adventitious bud from 15 days onwards
T_{19}	BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}	> 40	0.00	0.00	,,
T_{20}	Control (basal MS)	10.15	18.77	2.46	Long, highly vigorous shoot with dark green leaves
	F	**	**	**	
	CD	0.26	0.98	0.12	
	SEm±	0.05	0.19	0.02	

** Significant at 0.01 level

Observations recorded at three days interval upto 40 days after inoculation

Higher concentrations of BAP delayed shoot initiation. In treatment T_6 with BAP 7.5 mg l^{-1}, shoot development started in 14.65 days, while in treatment T_7 (BAP 10.0 mg l^{-1}), shoot initiation was lacking during the culture period of 40 days.

When BAP was used in combination with NAA, cultures took more time for the response. In treatments T_8 with BAP 1.0 mg l^{-1} + NAA 0.5 mg l^{-1} and T_9 with BAP 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}, the shoot initiation took place in 13.43 days and 14.41 days respectively, while in T_1 with same concentration of BAP alone (1.0 mg l^{-1}), the response was observed in 11.91 days.

b) Number of shoots per culture

Among different treatments, more number of adventitious shoots (27.01) were observed in treatment T_1 with BAP 1.0 mg l^{-1}, which was statistically superior to other treatments (Fig. 7 and Plate 15). In basal MS medium the mean number of shoots produced was 18.77. Shoot production was arrested in treatments with very high concentrations of BAP. Shoot production was lacking in treatment with BAP 10.0 mg l^{-1} (T_7), BAP 7.5 mg l^{-1} + NAA 0.5 mg l^{-1} (T_{18}) and BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1} (T_{19}) during the culture period of 40 days.

Numerous shoot initials as leafy proliferations were observed in treatments T_6 (BAP 7.5 mg l^{-1}), T_{16} (BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{17} (BAP 0.5 mg l^{-1} + NAA 1.0 mg l^{-1}) and resulted in condensed shoots which were difficult to be distinguished as stem, leaf etc.

c) Length of shoot

The longer shoots were produced in basal medium (T_{20}), where the

Fig.7 Effect of different levels of BAP on shoot growth

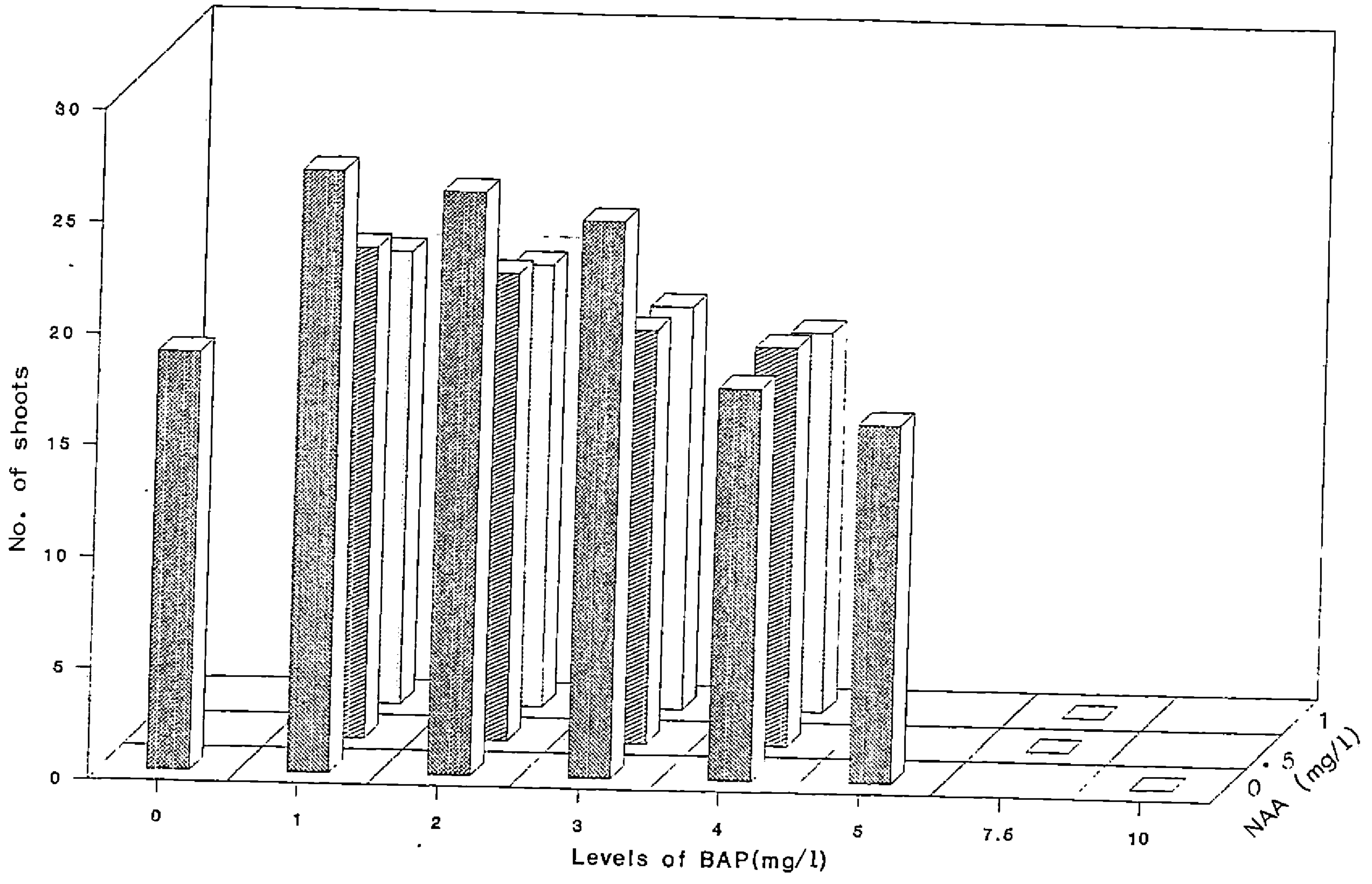
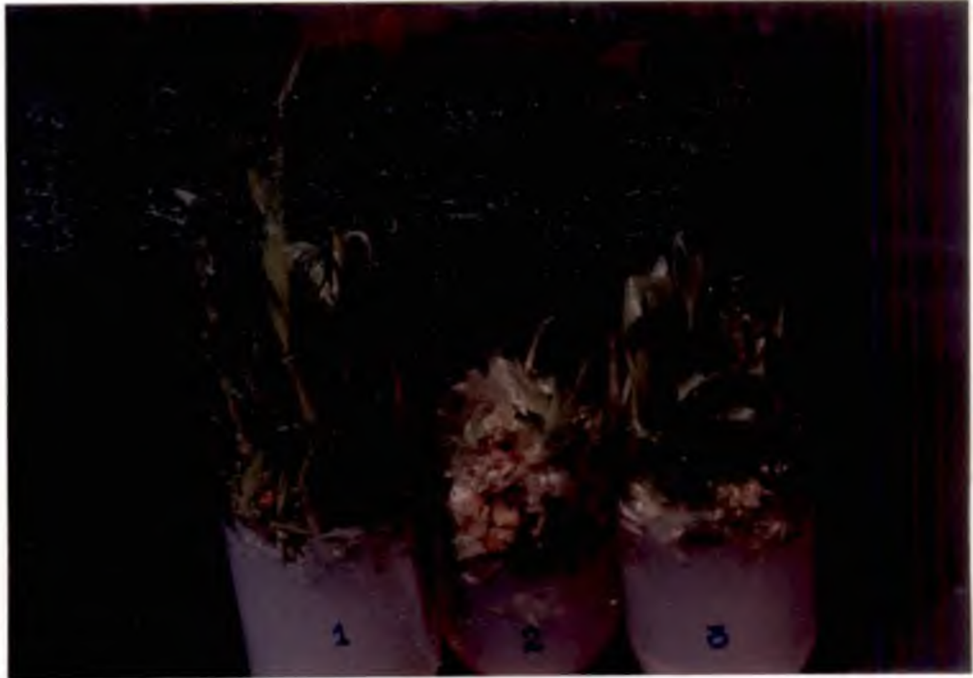


Plate 14. Shoot initiation in MS + BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1} (1) and basal MS medium (2) (11 days after inoculation)

Plate 15. A comparison of shoot growth in basal MS medium (1), MS + BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} (2) and MS + BAP 1.0 mg l^{-1} medium (3) (40 days after culturing)

1
2


1
2
3

mean shoot length was 2.46 cm. This effect was significantly superior to all other treatments. This was followed by T_1 with BAP 1.0 mg l^{-1} (1.35 cm).

Higher concentrations of BAP, both alone or in combination with NAA was found to reduce the shoot length. Treatments T_5 with BAP 5.0 mg l^{-1} and T_{15} with BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1} produced shoots with a mean length of 0.33 cm and 0.32 cm respectively, while in treatments T_6 with BAP 7.5 mg l^{-1}, T_{16} (BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{17} (BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}), numerous shoot initials appeared as small leafy proliferations.

d) Nature of shoots

Both the appearance and nature of shoots varied between different treatments. Long, highly vigorous shoots with dark green leaves were produced in basal MS medium (T_{20}) while treatments T_1 (BAP 1.0 mg l^{-1}), T_2 (BAP 2.0 mg l^{-1}) and T_3 (BAP 3.0 mg l^{-1}) pro[illegible]uced comparatively short shoots with medium vigour.

Higher concent[illegible]ions of BAP alone (4.0 mg l^{-1} and 5.0 mg l^{-1}) and levels of BAP more than 1 mg l^{-1} upto 4.0 mg l^{-1} in combination with NAA produced very short conden[illegible] shoots as evidenced in treatments T_{10}, T_{11}, T_{12}, T_{13}, T_{14} and T_{15}. Numer[illegible] shoot initials with leaf proliferations were produced in T_6, T_{16} and T_{17}.

4.2.4.4.2 Effect of differe[illegible] of kinetin on shoot growth

In order to [illegible]e effect of kinetin on shoot growth, cluster of adventitious buds (globula[illegible] was transferred to medium supplemented with kinetin at different levels ([illegible].0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in

mean shoot length was 2.46 cm. This effect was significantly superior to all other treatments. This was followed by T_1 with BAP 1.0 mg l^{-1} (1.35 cm).

Higher concentrations of BAP, both alone or in combination with NAA was found to reduce the shoot length. Treatments T_5 with BAP 5.0 mg l^{-1} and T_{15} with BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1} produced shoots with a mean length of 0.33 cm and 0.32 cm respectively, while in treatments T_6 with BAP 7.5 mg l^{-1}, T_{16} (BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{17} (BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}), numerous shoot initials appeared as small leafy proliferations.

d) Nature of shoots

Both the appearance and nature of shoots varied between different treatments. Long, highly vigorous shoots with dark green leaves were produced in basal MS medium (T_{20}) while treatments T_1 (BAP 1.0 mg l^{-1}), T_2 (BAP 2.0 mg l^{-1}) and T_3 (BAP 3.0 mg l^{-1}) produced comparatively short shoots with medium vigour.

Higher concentrations of BAP alone (4.0 mg l^{-1} and 5.0 mg l^{-1}) and levels of BAP more than 1.0 mg l^{-1} upto 4.0 mg l^{-1} in combination with NAA produced very short condensed shoots as evidenced in treatments T_{10}, T_{11}, T_{12}, T_{13}, T_{14} and T_{15}. Numerous shoot initials with leaf proliferations were produced in T_6, T_{16} and T_{17}.

4.2.4.4.2 Effect of different levels of kinetin on shoot growth

In order to study the effect of kinetin on shoot growth, cluster of adventitious buds (globular body) was transferred to medium supplemented with kinetin at different levels (1.0, 2.0, 3.0, 4.0, 5.0, 7.5 and 10.0 mg l^{-1}) alone or in

combination with NAA at two levels (0.5 mg l^{-1} and 1.0 mg l^{-1}). Data pertaining to the study are presented in Table 27.

a) Days for shoot initiation

Among different treatments, the fastest response with regard to shoot initiation was observed in basal MS medium (T_{20}), where in shoot production started in 10.16 days, which was statistically superior to other treatments (Plate 16). Following this the fastest response was achieved in T_1 with kinetin 1.0 mg l^{-1} (12.51 days).

Higher concentrations of kinetin delayed shoot initiation. In treatment T_6 with kinetin 7.5 mg l^{-1}, shoot development was initiated in 19.08 days, while in treatment T_7 with kinetin 10.0 mg l^{-1} no shooting was observed during the culture period of 40 days.

Addition of NAA into the medium in combination with kinetin was found to delay shoots initiation. In treatments T_8 with kinetin 1.0 mg l^{-1} + NAA 0.5 mg l^{-1} and T_9 with kinetin 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}, shoot initiation was observed in 15.42 days and 15.91 days respectively; while in T_1 with same concentration of kinetin alone (1.0 mg l^{-1}) the response was observed in 12.51 days.

b) Number of shoots per culture

Among different treatments, more number of adventitious shoots were recorded in treatment with basal MS medium without any hormone (T_{20}) (Fig. 8 and Plate 17). Average number of shoots formed per culture in the treatment was 18.50. This was statistically superior to other treatments. The treatments T_2 (kinetin

Table 27. Effect of different levels of kinetin on shoot growth

Treatment No.	Growth regulator concentration	Mean number of days for shoot initiation	Mean number of shoots	Mean length of shoots (cm)	Nature of shoots
T_1	Kinetin 1.0 mg l^{-1}	12.51	11.68	1.95	Long, medium vigorous shoots
T_2	Kinetin 2.0 mg l^{-1}	13.21	12.09	1.54	,,
T_3	Kinetin 3.0 mg l^{-1}	15.31	11.10	1.45	,,
T_4	Kinetin 4.0 mg l^{-1}	16.23	10.95	1.35	,,
T_5	Kinetin 5.0 mg l^{-1}	18.39	9.34	0.75	Short shoots with pale leaves
T_6	Kinetin 7.5 mg l^{-1}	19.08	8.49	0.52	,,
T_7	Kinetin 10.0 mg l^{-1}	> 40.0	0.00	0.00	Blackening of adventitious buds from 15 days onwards
T_8	Kinetin 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}	15.42	8.69	0.97	Short pale leaved shoots
T_9	Kinetin 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}	15.91	7.96	0.90	,,
T_{10}	Kinetin 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}	16.64	7.64	0.76	,,
T_{11}	Kinetin 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}	16.96	7.46	0.63	,,
T_{12}	Kinetin 3.0 mg l^{-1} + NAA 0.5 mg l^{-1}	17.13	7.33	0.56	,,
T_{13}	Kinetin 3.0 mg l^{-1} + NAA 1.0 mg l^{-1}	17.68	7.29	0.45	,,
T_{14}	Kinetin 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}	18.68	6.85	0.45	,,
T_{15}	Kinetin 4.0 mg l^{-1} + NAA 1.0 mg l^{-1}	19.04	6.70	0.41	,,
T_{16}	Kinetin 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}	19.94	Numerous shoot initials	Length cannot be measured	Shoot initials with leafy proliferation
T_{17}	Kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}	20.11		,,	,,
T_{18}	Kinetin 7.5 mg l^{-1} + NAA 0.5 mg l^{-1}	>40.00	0.00	0.00	No shoots
T_{19}	Kinetin 7.5 mg l^{-1} + NAA 1.0 mg l^{-1}	>40.00	0.00	0.00	No shoots
T_{20}	Control (basal MS)	10.16	18.50	2.46	Long highly vigorous shoots with dark green leaves
	F	**	**	**	
	CD	0.48	0.25	0.12	
	SEm±	0.09	0.05	0.02	

** Significant at 0.01 level
Observations recorded at 40 days after inoculation

Fig.8 Effect of different levels of kinetin on shoot growth

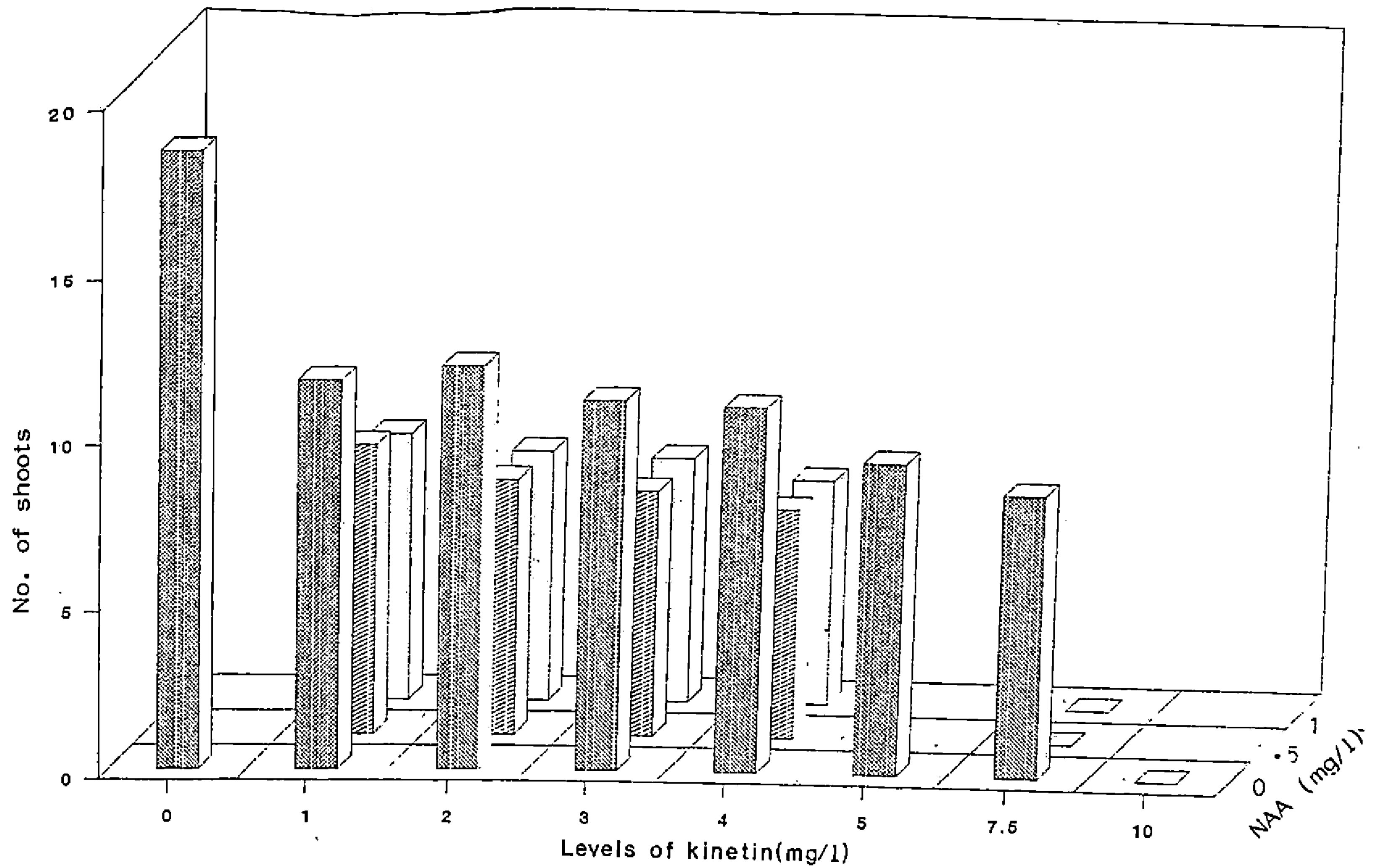


Fig.8 Effect of different levels of kinetin on shoot growth

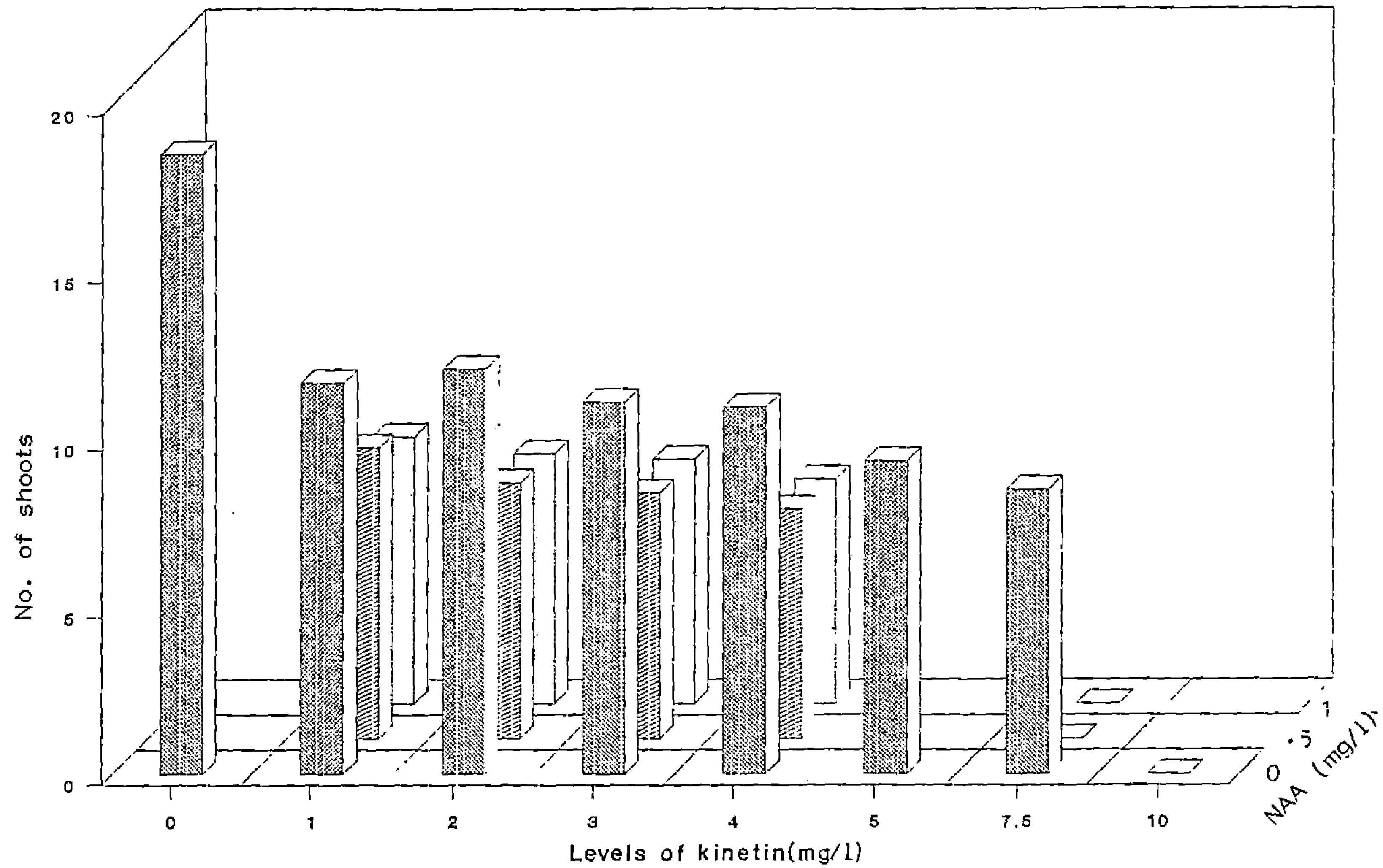
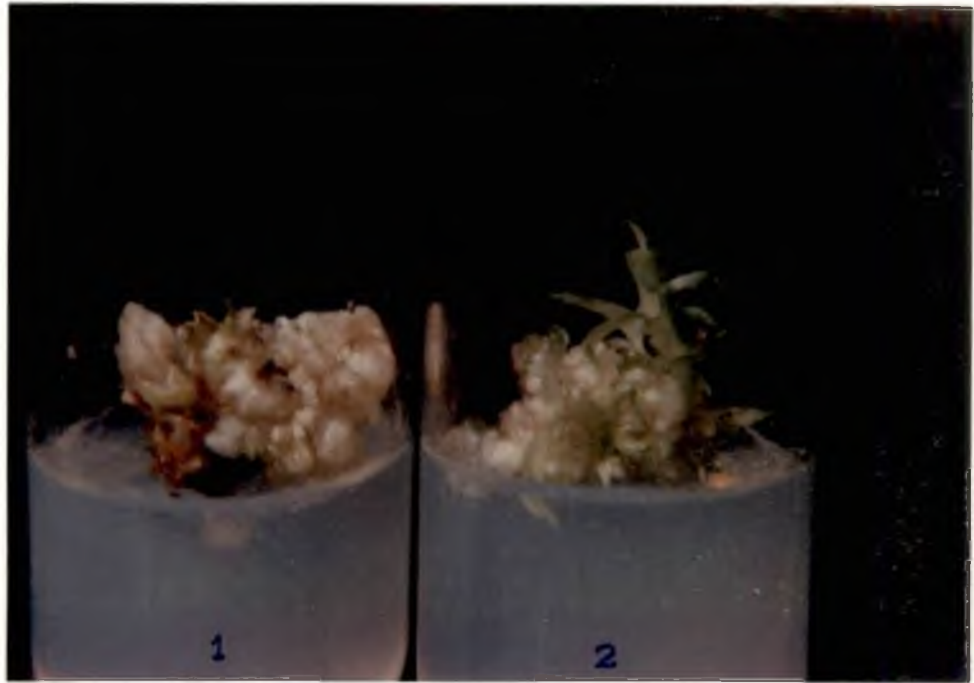
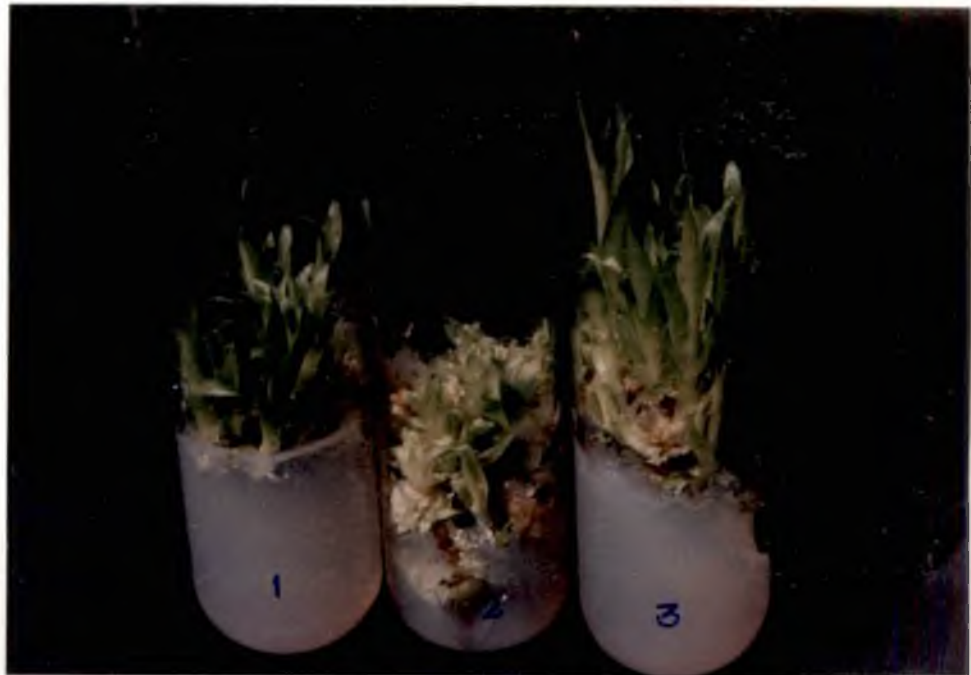


Plate 16. Shoot initiation in MS + kinetin 7.5 mg l^{-1} + NAA 1.0 mg l^{-1} (1) and basal MS medium (2) (11 days after culturing)

Plate 17. A comparison of shoot growth in MS + kinetin 2.0 mg l^{-1} (1), MS + kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} (2) and basal MS medium (3) (40 days after culturing)


1
2


1
2
3

2.0 mg l^{-1}) produced 12.09 shoots and T_1 (kinetin 1.0 mg l^{-1}) produced 11.68 shoots.

Higher concentrations of kinetin with 7.5 mg l^{-1} (T_6) reduced the number of shoots formed (8.49).

Higher concentrations of kinetin in combination with NAA produced numerous shoot initials as leafy proliferations as in the case of T_{16} and T_{17}.

c) Length of shoot

The shoots were the longest in basal medium (T_{20}), where the value being 2.46 cm. This was significantly superior to all other treatments. This was followed by treatments T_1 (kinetin 1.0 mg l^{-1}) with a mean shoot length of 1.95 cm and T_2 (kinetin 2.0 mg l^{-1}) with 1.54 cm.

Higher concentrations of kinetin T_6 (7.5 mg l^{-1}) reduced the average shoot length (0.52 cm) and in higher concentrations of kinetin along with NAA produced numerous shoot initials with leafy proliferations as in the case of treatments (T_{16}) with kinetin 5.0 mg l^{-1} + NAA 0.5 mg l^{-1} and (T_{17}) with kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}.

d) Nature of shoots

The appearance and nature of shoots varied among different treatments. Long, highly vigorous shoots with dark green leaves were formed in basal MS medium (T_{20}) (Plate 18). Treatments T_1 with kinetin 1.0 mg l^{-1}, T_2 with kinetin 2.0 mg l^{-1}, T_3 with kinetin 3.0 mg l^{-1} and T_4 with kinetin 4.0 mg l^{-1} produced long, medium vigorous shoots.

Plate 18. Cultures with plantlets ready for planting out (50 days after culturing in basal MS medium)

Higher concentrations of kinetin (5.0 mg l^{-1} and 7.5 mg l^{-1}) alone and all levels of kinetin from 1.0 mg l^{-1} to 4.0 mg l^{-1} in combination with NAA (0.5 mg l^{-1} and 1.0 mg l^{-1}) produced short condensed shoots with pale green leaves.

In treatments, T_{16} (kinetin 5.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and T_{17} (kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}) numerous shoot initials with leafy proliferation were formed.

4.2.4.4.3 Effect of physical conditions of the media on shoot growth from adventitious buds

Results of the experiment on effect of physical conditions of the media on shoot growth are presented in Table 28.

a) Days for shoot initiation

Shoot initiation was observed to be faster in liquid medium, where shoot initials appeared in 10.53 days. This treatment was statistically superior to treatment T_1 (solid medium), where in the response was noticed in 11.91 days.

b) Number of shoots

The mean number of shoots produced per culture was more in liquid medium, producing 36.98 shoots. In solid medium it was 27.02 (Plate 19).

c) Length of shoot

The mean length of shoots varied significantly among the treatments. The shoots were longer in solid medium, the value being 1.34 cm, as against 1.02 cm in liquid medium.

Table 28. Effect of physical conditions of the media on shoot growth from adventitious buds

Treatment No.	Type of culture	Mean number of days for shoot initiation	Mean number of shoots	Mean length of shoot (cm)
T_1	Stationary culture (Solid media)	11.91	27.02	1.34
T_2	Shake culture (Liquid media)	10.53	36.98	1.02
F		**	**	*
CD		0.92	3.57	0.15
SEm±		0.12	0.48	0.03

** Significant at 0.01 level
* Significant at 0.05 level
Observations recorded at three days interval up to 40 days of inoclation

Plate 19. Effect of physical condition of media on shoot growth (40 days after culturing)

Solid Medium
Liquid Medium

4.2.5 Induction of rooting

Uniform *in vitro* grown shoots with three or four leaves were used for root induction studies.

4.2.5.1 *In vitro* rooting

4.2.5.1.1 Standardisation of media for *in vitro* rooting

a) Solid media

Results of the experiment on standardisation of media for *in vitro* rooting using different auxins, namely, IAA, IBA and NAA each, at three levels (1.0, 2.0 and 3.0 mg l^{-1}) in solid media are presented in Table 29.

Number of days for root initiation

The number of days taken for *in vitro* rooting ranged from 5.99 days to 11.98 days in different treatments. Root initiation was observed in 5.99 days in basal MS medium without any growth regulator (T_{10}), which was significantly superior to other treatments.

Among the different auxins tried, NAA was found to cause early root induction as evidenced by treatments T_8 (NAA 2.0 mg l^{-1}) and T_7 (NAA 1.0 mg l^{-1}), where in rooting was noticed in 9.53 and 9.54 days, respectively. Treatment T_{10}, with NAA 3.0 mg l^{-1} took 9.79 days for root induction.

Next to NAA, the more effective auxin for inducing faster rooting was IBA. Different treatments with IBA, namely T_4 (IBA 1.0 mg l^{-1}), T_5 (IBA

Table 29. Effect of different auxins on *in vitro* rooting in solid medium

Treatment No.	Levels of auxin	Mean number of days for rooting	Mean number of roots	Mean length of roots (cm)	Nature of roots
T_1	IAA 1.0 mg l^{-1}	10.79	4.44	1.02	Normal roots
T_2	IAA 2.0 mg l^{-1}	11.03	4.80	1.00	Normal roots
T_3	IAA 3.0 mg l^{-1}	11.98	5.64	0.96	Short roots
T_4	IBA 1.0 mg l^{-1}	10.85	8.83	1.04	Normal roots
T_5	IBA 2.0 mg l^{-1}	10.85	10.66	1.00	Normal roots
T_6	IBA 3.0 mg l^{-1}	10.94	13.23	0.82	Short roots
T_7	NAA 1.0 mg l^{-1}	9.54	18.57	0.65	Very short roots
T_8	NAA 2.0 mg l^{-1}	9.53	19.93	0.47	Very short roots, callus like growth
T_9	NAA 3.0 mg l^{-1}	9.79	20.16	0.22	Very short with callus like growth
T_{10}	Control (basal MS)	5.99	6.49	1.61	Normal roots with secondaries and root hairs
F		**	**	**	
CD		0.25	0.24	0.08	
SEm±		0.05	0.04	0.02	

** Significant at 0.01 level
Observations recorded at three days interval up to 40 days of inoculation

2.0 mg l^{-1}) and T_6 (IBA 3.0 mg l^{-1}) took 10.85, 10.85 and 10.94 days, respectively for rooting.

Among different concentrations of IAA, the treatment T_1 (IAA 1.0 mg l^{-1}) induced faster rooting (10.79 days), which was on par with treatments T_4 (IBA 1.0 mg l^{-1}) and T_5 (IBA 2.0 mg l^{-1}), where in root initiation took place in 10.85 days.

All the auxins tried, delayed rooting at their higher levels (above 2.0 mg l^{-1}) comparing to lower levels. Treatments T_9 (NAA 3.0 mg l^{-1}), T_6 (IBA 3.0 mg l^{-1}) and T_3 (IAA 3.0 mg l^{-1}) took 9.79, 10.94 and 11.98 days respectively for inducing *in vitro* rooting in solid medium.

Number of roots

The mean number of roots produced in different treatments ranged significantly from 4.44 to 20.16 (Plate 20).

A maximum number of 20.16 roots was observed in treatment T_9 (NAA 3.0 mg l^{-1}) followed by 19.93 in T_8 (NAA 2.0 mg l^{-1}) and 18.57 roots in T_7 (NAA 1.0 mg l^{-1}).

Among the different treatments with IAA, the highest number of roots (5.64) was produced in treatment T_3 (IAA 3.0 mg l^{-1}). Among different levels of IBA in the medium, more number of roots was produced in treatment T_6 with IBA 3.0 mg l^{-1} (13.23).

In basal MS medium, the mean number of roots produced per culture was 6.49, which was superior to IAA at all levels.

Length of roots

Comparing the different treatments, the mean root length was the highest in basal MS medium, with a mean root length of 1.61 cm. This was statistically superior to other treatments.

Among the different treatments with auxins, treatments T_5 (IBA 2.0 mg l^{-1}), T_2 (IAA 2.0 mg l^{-1}), T_1 (IAA 1.0 mg l^{-1}) and T_4 (IBA 1.0 mg l^{-1}), producing a mean root length of 1.00, 1:00, 1.02 and 1.04 cm respectively were on par with each other and were superior to all other treatments with auxins.

Treatments with NAA produced smaller roots comparing to other auxins as evidenced by treatments T_7 (NAA 1.0 mg l^{-1}), T_8 (NAA 2.0 mg l^{-1}) and T_9 (NAA 3.0 mg l^{-1}), where in the mean root length was 0.65, 0.47 and 0.22 respectively.

Nature of roots

Normal slender roots with secondaries and root hairs were produced in basal MS media (T_{10}).

Treatments with IAA and IBA at lower concentrations of 1.0 mg l^{-1} (T_1 and T_4) and 2.0 mg l^{-1} (T_2 and T_5) produced normal roots with branching nature, while both these auxins at 3.0 mg l^{-1} (T_3 and T_6) produced short roots.

In treatments with NAA, at low concentrations of 1.0 mg l^{-1} (T_7) roots were very short, thick and had hairy appearance. At higher levels of NAA

2.0 mg l^{-1} (T_8) and 3.0 mg l^{-1} (T_9) roots were very short and produced callus like growth at the shoot base.

b) Liquid media

Results of the experiment on standardisation of media for *in vitro* rooting using different auxins, namely, IAA, IBA and NAA each at three levels (1.0, 2.0 and 3.0 mg l^{-1}) in liquid media are presented in Table 30.

Number of days for root initiation

The minimum number of days for root initiation (5.25 days) was observed in basal MS media (T_{10}) which was statistically superior to all other treatments.

Among the trials conducted with different auxins, faster rooting (8.34 days) was observed in treatment with NAA at 1 mg l^{-1} (T_7) which was on par with (T_1) with IAA at 1 mg l^{-1} (8.43 days). Among different levels of IBA, faster response was noticed in T_4 with 1 mg l^{-1} IBA (8.92 days) which was on par with IBA 2 mg l^{-1} (8.93 days).

All the treatments with auxins delayed rooting at their higher concentrations. NAA, IBA and IAA at 3 mg l^{-1} (T_9, T_6 and T_3) initiated rooting in 8.68 days, 9.04 days and 9.04 days respectively.

Number of roots

The mean number of roots produced in different treatments in liquid medium ranged from 3.8 to 13.78 (Plate 21). Maximum number of roots (13.78)

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Table 30. Effect of different auxins on *in vitro* rooting in liquid medium

Treatment No.	Levels of auxins	Mean number of days for rooting	Mean number of roots	Mean length of roots (cm)	Nature of roots
T_1	IAA 1.0 mg l^{-1}	8.43	3.80	2.16	Normal roots
T_2	IAA 2.0 mg l^{-1}	8.66	3.92	2.01	Normal roots
T_3	IAA 3.0 mg l^{-1}	9.04	3.97	1.87	Normal roots slightly thicker
T_4	IBA 1.0 mg l^{-1}	8.92	4.17	2.17	Normal roots
T_5	IBA 2.0 mg l^{-1}	8.93	5.00	2.15	Normal roots
T_6	IBA 3.0 mg l^{-1}	9.04	5.03	2.12	Slightly thicker roots
T_7	NAA 1.0 mg l^{-1}	8.34	12.51	0.73	Short thick roots
T_8	NAA 2.0 mg l^{-1}	8.54	13.24	0.58	Short thick roots
T_9	NAA 3.0 mg l^{-1}	8.68	13.78	0.35	Very short, thick, coiled roots
T_{10}	Control (basal MS)	5.25	4.53	2.18	Normal slender roots with secondaries and root hairs
F		**	**	**	
DE		0.16	0.24	0.11	
SEm±		0.03	0.04	0.02	

**Significant at 0.01 level

Observation recorded at three days interval up to 40 days after inoculation

Plate 20. Root production in MS solid medium + NAA 2.0 mg l^{-1} (1), MS + IBA 2 mg l^{-1} (2) and basal MS medium (3) (40 days after culturing)

Plate 21. Root production in MS liquid medium + NAA 2.0 mg l^{-1} (1), MS + IBA 2.0 mg l^{-1} (2) and basal MS medium (3) (40 days after culturing)

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1
2
3

was formed in treatment with NAA 3 mg l^{-1} (T_9) followed by T_8 with NAA 2 mg l^{-1} (13.24).

Among different levels of IAA tried, maximum number of roots (3.97) were observed in treatment with IAA 3 mg l^{-1} (T_3) and in the case of treatments with IBA, higher number of 5.03 roots were produced in treatment containing IBA 3 mg l^{-1} (T_6) which was on par with IBA 2 mg l^{-1} (T_5) producing a mean of 5.0 roots per culture. In basal MS liquid media (T_{10}) the mean number of roots produced was 4.53.

Length of roots

The average length of roots produced in different treatments in liquid media ranged significantly from 0.35 cm to 2.18 cm. The maximum length of roots (2.18 cm) was observed in basal MS medium which was on par with that of medium supplemented with 1 mg l^{-1} IBA (2.17 cm), 1 mg l^{-1} IAA (2.16 cm), 2 mg l^{-1} IBA (2.15 cm) and 3 mg l^{-1} IBA (2.12 cm).

Among the various auxins, treatments with NAA produced smaller roots. Minimum root length was observed in treatments T_9 with NAA 3 mg l^{-1} (0.35 cm) and T_8 with NAA 2 mg l^{-1} (0.58 cm).

Nature of roots

Normal slender roots with secondaries and root hairs were produced in basal MS media (T_{10}). Among the auxins tried, IAA and IBA were found to produce roots of normal nature. But these two auxins at higher concentrations of 3 mg l^{-1} (T_3 and T_6) produced roots of slightly thick nature. In the treatments with NAA, at all levels, the roots were very short, thick and sometimes coiled.

4.2.5.2 *Ex vitro* rooting

An *ex vitro* rooting trial was carried outby dipping regenerated shoots of uniform size with 5 cm length, for one hour in different auxins, namely, IBA and NAA both at two concentrations (100 mg l^{-1} and 200 mg l^{-1}). The data pertaining to the study are presented in Table 31.

4.2.5.2.1 Percentage of rooting

Among the different treatments, rooting percentage was the highest in treatment T_2 (IBA 200.0 mg l^{-1} for a period of one hour), where in the rooting was 87.5 per cent. This was followed by 75.0 per cent in T_5 (control with distilled water).

Rooting percentage was less in treatment with NAA as evidenced by treatments T_3 (NAA 100.0 mg l^{-1} for a period of one hour) and T_4 (NAA 200.0 mg l^{-1} for a period of one hour), where in the rooting was 43.75 per cent and 50.0 per cent, respectively.

4.2.5.2.2 Days for root initiation

Number of days for rooting among different treatments ranged from 12.19 days to 16.14 days. The fastest response was noticed in treatment T_4 (NAA 200.0 mg l^{-1}) producing roots in 12.19 days, which was statistically on par with T_3 (NAA 100.0 mg l^{-1}), where in root initiation took place in 12.33 days.

Treatments T_1 (IBA 100.0 mg l^{-1}) and T_2 (IBA 200.0 mg l^{-1}) took 15.16 and 14.06 days, respectively, for rooting and in treatment T_5 (control) roots were produced in 16.14 days, which took highest number of days for rooting.

Table 31. Effect of auxins on *ex vitro* rooting

Treatment No.	Levels of auxins	Percentage of rooting	Mean number of days for rooting	Mean number of roots	Mean length of roots (cm)	Nature of roots
T_1	IBA 100 ppm	62.50	15.16	5.67	2.05	Normal roots
T_2	IBA 200 ppm	87.50	14.06	5.83	2.23	Normal roots
T_3	NAA 100 ppm	43.75	12.33	3.30	0.57	Short and thick roots
T_4	NAA 200 ppm	50.00	12.19	4.33	0.82	Short and thick roots
T_5	Control	75.00	16.14	4.38	2.23	Normal roots
F			**	**	**	
CD			1.38	1.84	1.02	
SEm±			0.22	0.29	0.16	

** Significant at 0.01 level
Observations recorded at three days interval up to 40 days after planting out

4.2.5.2.3 Number of roots

The mean number of roots produced was the highest in treatment T_2 (IBA 200.0 mg l^{-1}), producing 5.83 roots per shoot. This treatment was statistically on par with T_1 (IBA 100.0 mg l^{-1}), where in the mean number of roots produced was 5.67.

Treatments T_5 (control), T_4 (NAA 200.0 mg l^{-1}) and T_3 (NAA 100.0 mg l^{-1}) produced 4.38, 4.33 and 3.30 roots, respectively and these three treatments were statistically similar to each other.

4.2.5.2.4 Length of roots

The mean root length was the highest in treatments T_2 (IBA 200 mg l^{-1}) and T_5 (control) producing roots with mean length of 2.23 cm. These treatments were statistically on par with treatment T_2 (IBA 200.0 mg l^{-1}), where in the mean root length was 2.05 cm.

Treatments with NAA produced small roots as evidenced by treatments T_3 (NAA 100.00 mg l^{-1}) and T_4 (NAA 200.0 mg l^{-1}) with a mean root length of 0.57 and 0.82 cm respectively.

4.2.5.2.5 Nature of roots

In all the treatments, roots were normal with secondaries and root hairs. But in treatments with NAA, the roots were comparatively smaller and were more thick with less number of secondaries.

4.2.6 Hardening and *ex vitro* establishment

4.2.6.1 Effect of potting media on growth and development of plants under *ex vitro* condition

Results of the experiment on the effect of potting media on growth and development of plants under *ex vitro* condition are presented in Table 32 (Fig. 9 and Plate 22).

4.2.6.1.1 Percentage of establishment

Establishment of pineapple plants under *ex vitro* condition was cent per cent for plants grown in sand (T_1), soilrite (T_2), palmpeat (T_3) and vermiculite + soil (T_4), while in the case of potting mixture (T_5) only 91.67 percentage of establishment was recorded.

4.2.6.1.2 Plant height

Among the different treatments tried, plant height was maximum in plants grown in (T_2) soilrite (5.59 cm), which was significantly superior to other treatments, followed by (T_5) potting mixture (5.30 cm) and (T_1) sand (5.26 cm) which were on par with each other. Minimum height for plants was observed in (T_4) vermiculite + soil (3.68 cm) while for plants grown in palmpeat (T_3) the average height was 4.36 cm.

4.2.6.1.3 Number of leaves

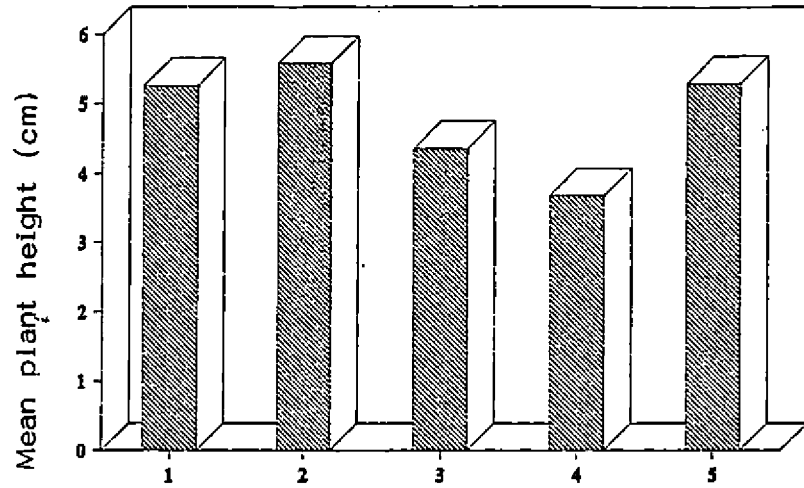
The average number of leaves produced per plant ranged from 3.10 to 4.56 among various treatments. The highest number of leaves (4.56) was observed in plants grown in soilrite (T_2), which was statistically superior to all other treat-

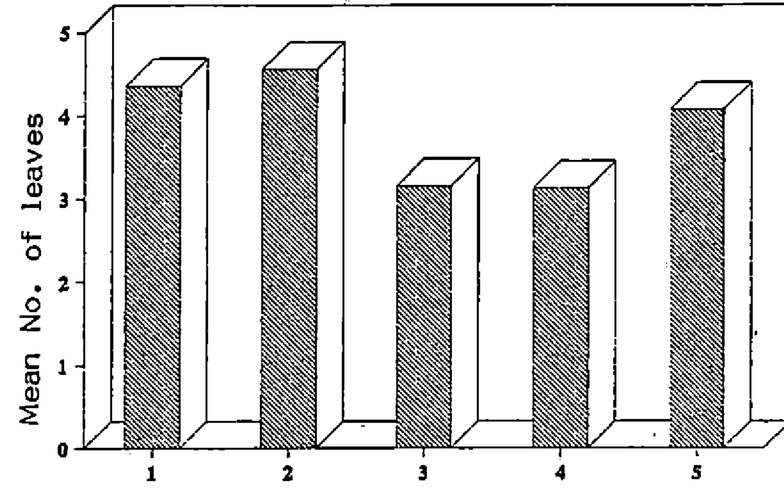
Table 32. Effect of potting media on growth and development of plants under *ex vitro* condition

Treatment No.	Potting media	Percentage of establishment	Mean plant height (cm)	Mean number of leaves	Mean length of largest leaf (cm)	Mean width of largest leaf (cm)
T_1	Sand	100.0	5.26	4.36	4.77	0.52
T_2	Soilrite	100.0	5.59	4.56	4.60	0.49
T_3	Palmpeat	100.0	4.36	3.10	4.24	0.43
T_4	Vermiculite + soil (1:1)	100.0	3.68	3.13	3.78	0.40
T_5	Potting mixture	91.67	5.30	4.07	4.25	0.40
F			**	**	**	**
CD			0.46	0.75	0.37	0.09
SEm±			0.08	0.13	0.06	0.02

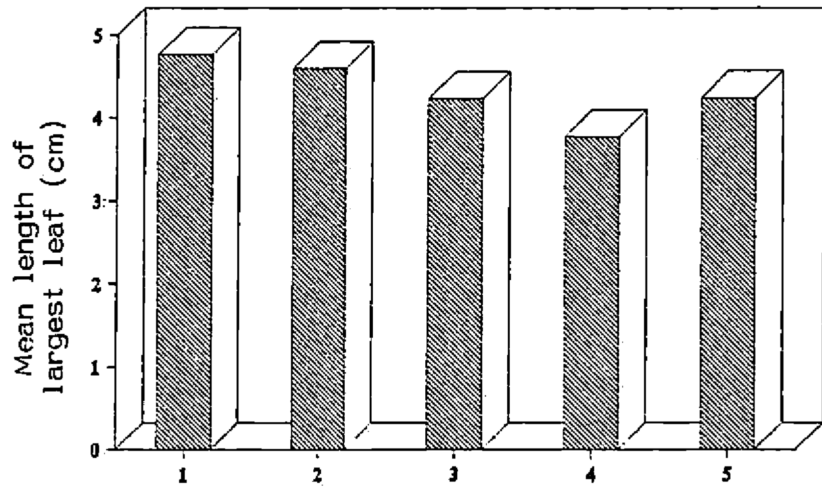
** Significant at 0.01 level
Observations recorded at 60 days after planting out

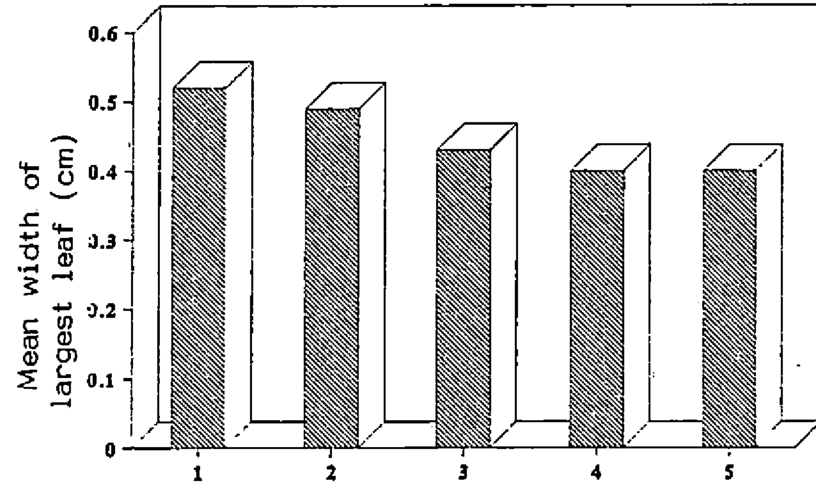
Fig.9. Effect of potting media on growth and development of plants under *ex vitro* condition





1. Sand; 2. Soilrite, 3. Palmpeat
4. Vermiculite + soil (1:1); 5. Potting mixture





ments. This was followed by sand (T_1) and potting mixture (T_5), where in the average number of leaves produced was 4.36 and 4.07 respectively. The lowest number of leaves were produced by plants grown in (T_3) palmpeat (3.10).

4.2.6.1.4 Length of largest leaf

Among the different potting media used, maximum length of largest leaf was observed in plants grown in sand (4.77 cm) which was statistically on par with soilrite (T_2), in which the value was 4.60 cm. For plants grown in potting mixture (T_5) and palmpeat (T_3) the average length of largest leaf was 4.25 cm and 4.24 cm, respectively. Length of largest leaf observed to be minimum for plants grown in the mixture of vermiculite and soil (3.78 cm).

4.2.6.1.5 Width of largest leaf

Width of largest leaf was observed to be maximum for plants grown in (T_1) sand (0.52 cm), followed by soilrite (0.49 cm). These two treatments were statistically superior to all others. For plants grown in palmpeat (T_3), potting mixture (T_5) and vermiculite + soil (T_4), the values were 0.43 cm, 0.40 cm and 0.40 cm, respectively which were statistically on par with each other.

4.2.6.2 Effect of containers on growth and development of plants under *ex vitro* condition

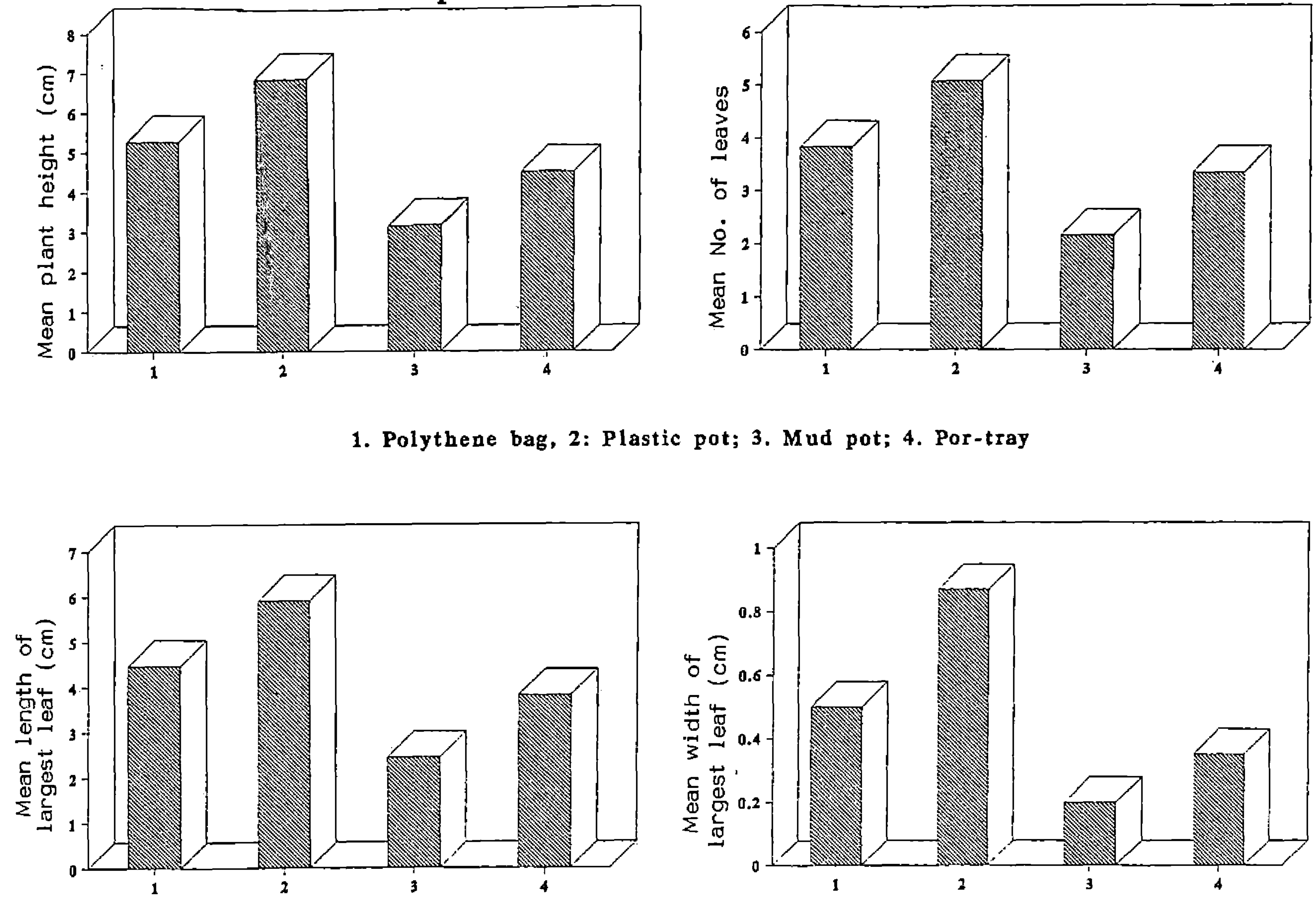
The data relating to the effect of containers on the growth and development of plants during acclimatisation are given in Table 33 (Fig.10 and Plate 23).

Table 33. Effect of containers on growth and development of plants under *ex vitro* condition

Treatment No.	Containers	Mean plant height (cm)	Mean number of leaves	Mean length of longest leaf (cm)	Mean width of longest leaf (cm)
T_1	Polythene bag	5.29	3.84	4.48	0.50
T_2	Plastic pot	6.88	5.08	5.90	0.87
T_3	Mud pot	3.18	2.16	2.44	0.20
T_4	Por-tray	4.52	3.35	3.81	0.35
F		**	**	**	**
CD		0.26	0.29	0.19	0.07
SEm±		0.04	0.05	0.03	0.01

** Significant at 0.01 level
Observations recorded at 60 days after planting out

Fig.10. Effect of containers on growth and development of plants under *ex vitro* condition



1. Polythene bag, 2: Plastic pot; 3. Mud pot; 4. Por-tray

Plate 22. *Ex vitro* establishment and growth of plantlets as influenced by different potting media (60 days after planting out)

Plate 23. Effect of containers on growth and development of plantlets under *ex vitro* condition (60 days after planting out)

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4.2.6.2.1 Plant height

The mean height of plants among different treatments varied significantly from 3.18 cm to 6.88 cm. The maximum height was recorded in T_2 with plastic pot (6.88 cm), which was statistically superior to other treatments. The plant height recorded was 5.29 cm, 3.18 cm and 4.52 cm, respectively in treatments T_1 (polythene bag), T_4 (por-tray) and T_3 (mud pot).

4.2.6.2.2 Number of leaves

The mean number of leaves was highest in T_2 (plastic pot) producing 5.08 leaves. This treatment was significantly superior to other treatments. The lowest number of leaves (2.16) was found in treatment with mud pot.

4.2.6.2.3 Length of largest leaf

The mean length of largest leaf among different treatments followed the same pattern as for plant height and number of leaves. The mean length of largest leaf was maximum in treatment T_2 (plastic pot) with a mean length of 5.90 cm. The mean length of largest leaf was lowest in mud pot (2.44 cm).

4.2.6.2.4 Width of largest leaf

The mean leaf width was maximum (0.87 cm) in treatment (T_2) with plastic pot which was significantly superior to other treatments. In treatments T_1 (polythene bag), T_4 (por-tray) and T_3 (mud pot), the mean width was 0.50, 0.35 and 0.20 cm, respectively.

4.2.6.3 Effect of nutrient starter solution on growth and development of plants under *ex vitro* condition

Results of the experiment on effect of different nutrient starter solutions on growth and development of pineapple plants under *ex vitro* condition are presented in Table 34 (Fig.11 and Plate 24).

4.2.6.3.1 Plant height

Among the different treatments tried, plant height varied from 1.78 cm to 5.39 cm. Treatment (T_3) with full MS salts recorded a maximum mean height of 5.39 cm. This was significantly superior to other treatments. With ½ MS salts (T_4), plants were of 4.39 cm height, which was on par with T_6 (N:P:K mixture), where in the mean plant height was 3.90 cm. In treatments T_5 (1/4 MS salts), T_1 (urea 1% solution) and T_2 (urea 0.5% solution) the mean plant height were 2.46, 2.16 and 2.94 cm, respectively.

4.2.6.3.2 Number of leaves

The mean number of leaves produced in different treatments, ranged from 2.11 to 5.67. Treatment T_3 (full MS salt solution) recording the maximum value. This was followed by T_6 (N:P:K mixture solution), where in the mean number of leaves was 4.44.

4.2.6.3.3 Length of largest leaf

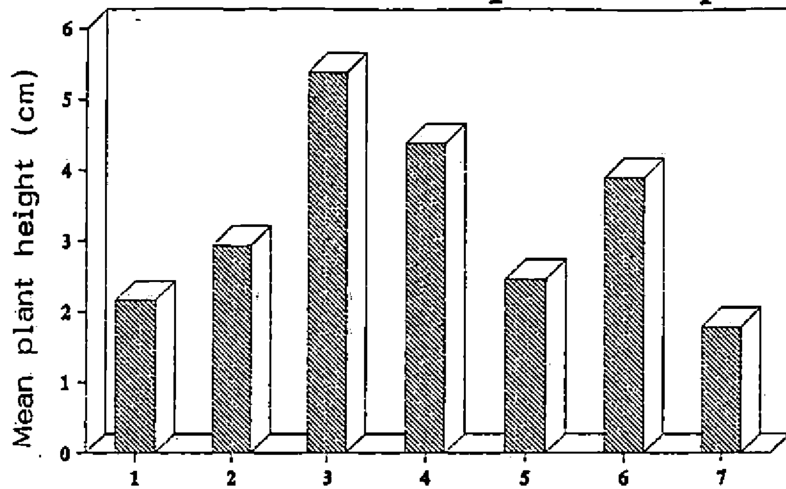
The mean length of largest leaf recorded a highest value of 4.03 cm in treatment (T_3) with full MS salt solution. This treatment was on par with T_4 (½ MS

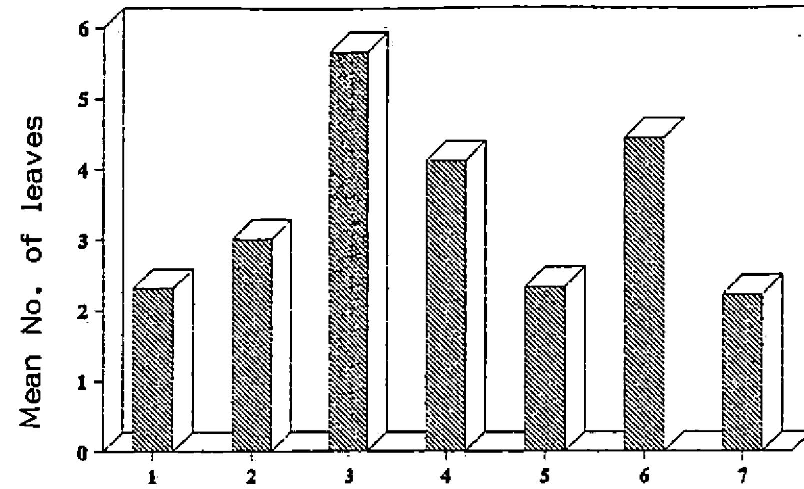
Table 34. Effect of nutrient starter solutions on growth and development of plants under *ex vitro* condition

Treatment No.	Nutrient starter solution	Mean plant height (cm)	Mean number of leaves	Mean length of largest leaf (cm)	Mean width of largest leaf (cm)
T_1	Urea 1% solution	2.16	2.31	1.68	0.22
T_2	Urea 0.5% solution	2.94	3.00	1.93	0.23
T_3	Full MS salt solution	5.39	5.67	4.03	0.75
T_4	½ MS salt solution	4.39	4.11	3.51	0.59
T_5	1/4 MS salt solution	2.46	2.33	1.96	0.29
T_6	NPK (10:5:20) mixture	3.90	4.44	3.13	0.57
T_7	Control	1.78	2.22	1.67	0.10
F		**	**	**	**
CD		1.58	2.29	1.76	0.30
SEm±		0.27	0.38	0.30	0.05

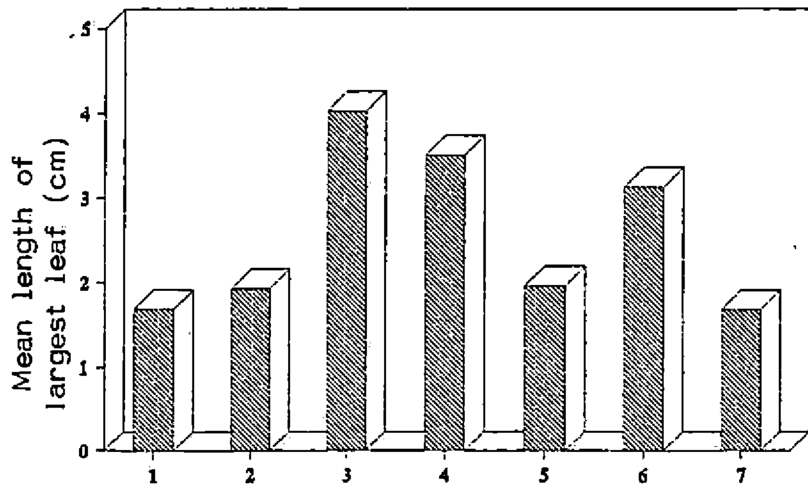
** Significant at 0.01 level
Observations recorded at 60 days after treatment

Fig.11. Effect of nutrient starter solutions on growth and development of plants under *ex vitro* condition





1. Urea 1% solution; 2. Urea 0.5% solution
3. Full MS salt solution; 4. 1/2 MS salt soltuion
5. 1/4 MS salt solution; 6. NPK (10:5:20) mixture
7. Control



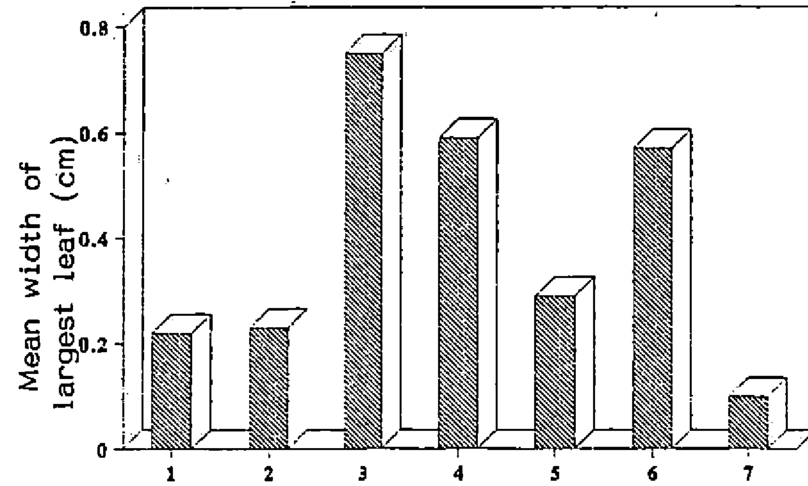


Plate 24. Establishment and growth of plantlets as influenced by the application of different nutrient starter solutions (90 days after planting out)

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salt solution) and T_6 (N:P:K mixture solution), where in the values were 3.51 and 3.13 cm, respectively.

A minimum value of 1.67 cm was recorded in treatment T_7 (control).

4.2.6.3.4 Width of largest leaf

Mean width of largest leaf was maximum in treatment T_3 (0.75 cm). This treatment was statistically superior to other treatments. Treatment (T_4) with ½ MS salt solution recorded a mean width of 0.59 cm for largest leaf, which was on par with T_6 (0.57 cm).

4.2.6.4 Effect of shoot size on growth and development of plants under *ex vitro* condition

In vitro produced shoots were categorised into different groups, based on the following biometric characters, namely, length of shoot, leaf number, length of largest leaf, width of largest leaf and shoot weight, for finding out the optimum shoot size for establishment and growth. Observations were recorded as percentage increase of individual characters and are presented in Table 35 (Fig.12).

4.2.6.4.1 Percentage of establishment

Establishment of *in vitro* produced shoots was cent per cent in all the groups except group 5 where the percentage of establishment was found to be 83.33 per cent.

4.2.6.4.2 Plant height

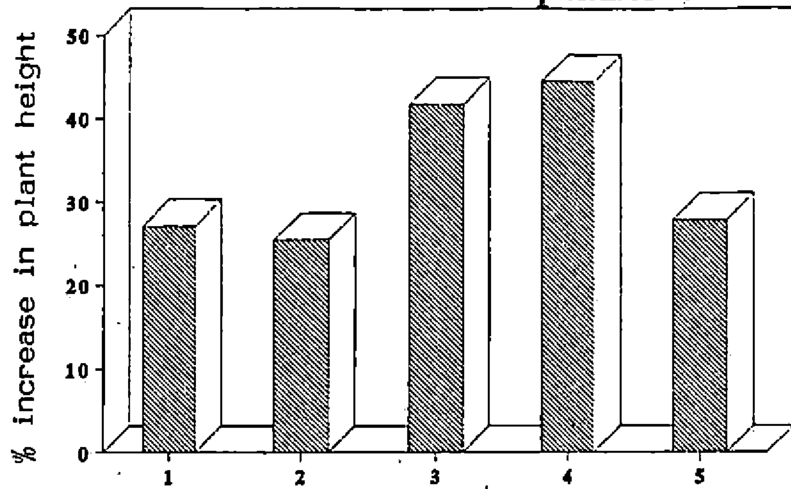
Among the different groups, the rate of increase in plant height was

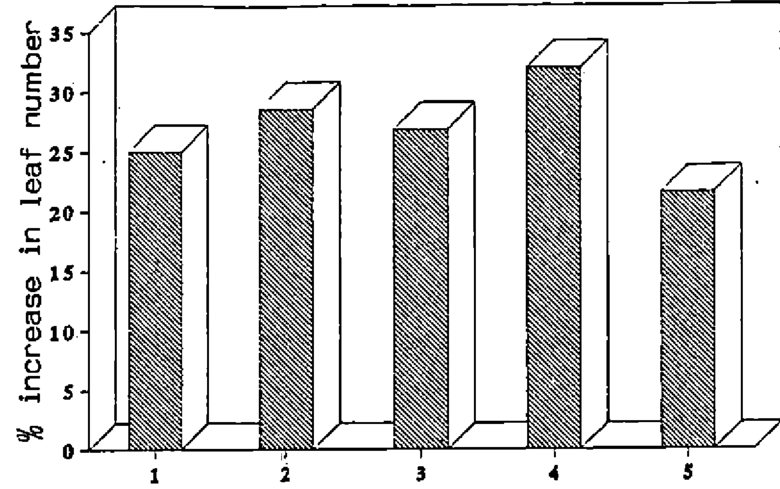
Table 35. Effect of shoot size on growth and development of plants under *ex vitro* condition

Treatment No.	Group	Percentage of establishment	Percentage increase in plant height	Percentage increase in leaf number	Percentage increase in length of largest leaf	Percentage increase in width of largest leaf	Percentage increase in plant weight
T_1	Group I	100	27.12	25.00	43.93	65.08	167.23
T_2	Group II	100	25.51	28.60	52.36	79.90	250.22
T_3	Group III	100	41.80	26.89	55.50	87.92	276.00
T_4	Group IV	100	44.52	32.02	53.97	58.60	231.48
T_5	Group V	83.33	27.91	21.56	44.02	84.86	184.12
F			**	*	*	**	**
CD			12.80	8.47	12.96	32.65	60.06
SEm±			2.17	1.99	3.04	5.54	14.11

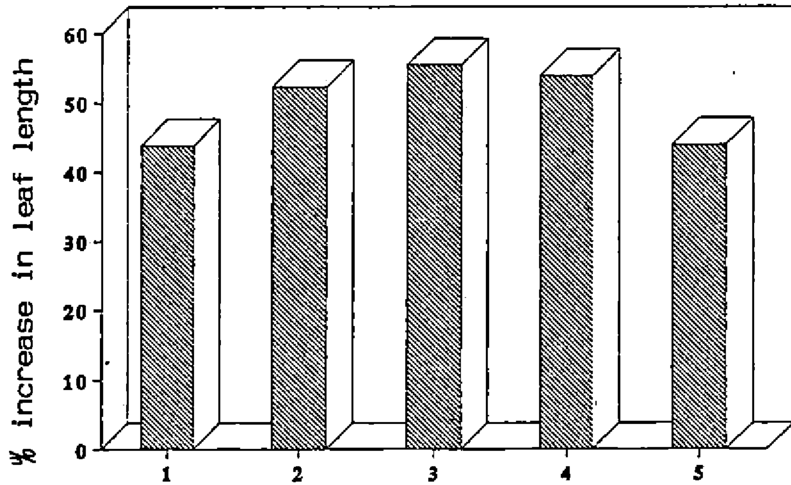
* Significant at 0.05 level
** Significant at 0.01 level
Observations recorded at 60 days after planting out

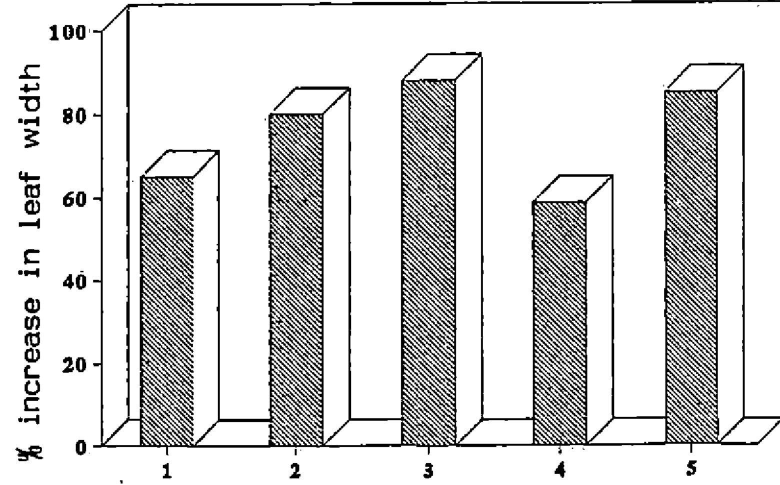
Fig.12. Effect of shoot size on growth and development of plants under *ex vitro* condition





1. Group I; 2. Group II; 3. Group III; 4. Group IV
5. Group V





maximum in group 4 (44.52%) which was statistically on par with group 3 (41.80%). Groups 5, 1 and 2 were on par with each other in this respect.

4.2.6.4.3 Leaf number

Group number 4 recorded the maximum value (32.02%) with regard to percentage increase in leaf number which was statistically similar to group 2 (28.60%) and group 3 (26.89%).

4.2.6.4.4 Length of largest leaf

A maximum increase in length of largest leaf was noticed in group 3 (55.50%) which was on par with group 4 (53.97%). Minimum value was recorded in group 1 (43.93%).

4.2.6.4.5 Width of largest leaf

As in the case of length of largest leaf, percentage increase in width of largest leaf was maximum in group 3 (87.92%) which was statistically on par with group 5 (84.86%). Minimal increase in width of largest leaf was observed in group 4 and group 1, 58.59 per cent and 65.08 per cent respectively.

4.2.6.4.6 Plant weight

Among the different groups, percentage increase in plant weight was highest in group 3 (276.00%) which was statistically on par with group 2 (250.22%).

4.3 Mass multiplication of elite clones

The refined *in vitro* protocol developed through the present study (Fig.13) was used for mass multiplication of selected five elite clones. Two hundred and fifty plants, each belonging to these clones are maintained in the nursery for further evaluation (Plate 25).

Fig.13. Schematic representation of refined *in vitro* protocol for pineapple var. Mauritius

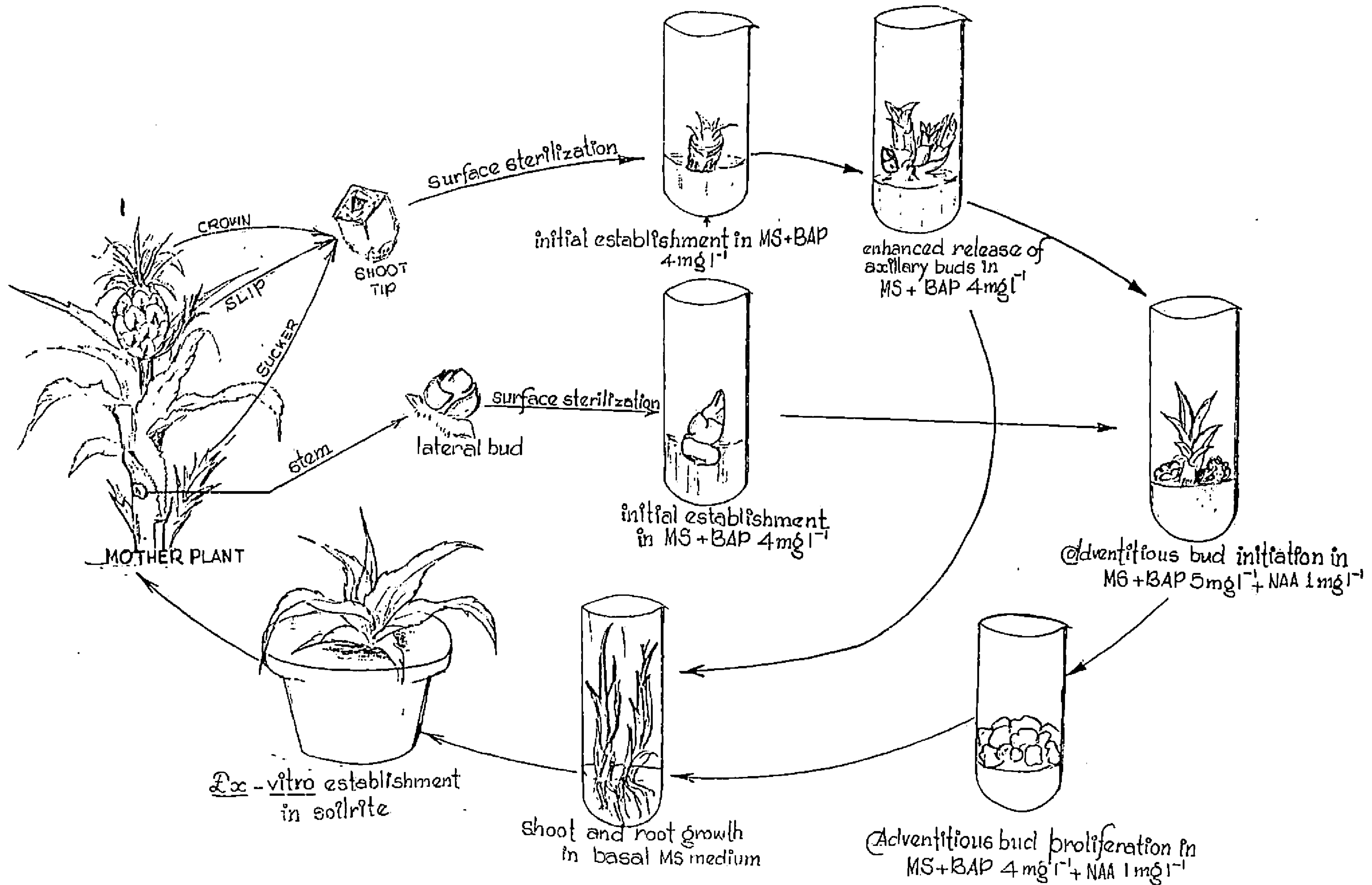


Plate 25. General view of the plantlets of the selected five accessions in the green house during weaning period (90 days after planting out)

KV 17
KT 2
KT 5
TK 3

Discussion

5. DISCUSSION

The present investigations on the refinement of *in vitro* propagation technique in pineapple var. Mauritius and mass multiplication of elite clones were carried out at Kerala Horticulture Development Programme, Kerala Agricultural University, Vellanikkara during 1993-95. The results of the study are discussed in this chapter.

The pineapple (*Ananas comosus* (L) Merr) belonging to the family Bromeliaceae is considered as one of the most important commercial fruits of the tropics and subtropics. Its characteristic pleasant flavour qualifies it as a choice fruit both for fresh consumption and processing. Mainly two varieties, namely, Mauritius and Kew are grown in Kerala. However, Mauritius occupies major part of area under pineapple cultivation in the State. It is the choice variety for table purpose and for extraction of juice.

Pineapple is an asexually propagated crop, in which suckers, crowns, slips, stem bits and stumps are the major propagules. Though propagated asexually, variations in the plant and fruit characters in a single variety are very often observed. These variations may be due to spontaneous bud mutations (Singh *et al.*, 1976). Occurrence of several types of mutations in pineapple were reported (Collins, 1960; Marr, 1965 and Singh *et al.*, 1976). In Mauritius variety also variations with regard to plant and fruit characters were noticed. In the present study, exploitation of such variations through clonal selection and mass multiplication of the selected clones were attempted.

5.1 Selection of elite clones of pineapple var. Mauritius

Vegetative vigour of a plant is a reasonable index of its yield potential (Chadha *et al.*, 1977). Initial selection of plants was done based on plant characters. Based on general appearance and vegetative vigour, one hundred plants were selected from among the bulk population in major areas growing under identical agrodynamic conditions.

Plant characters such as height, leaf number, 'D' leaf area and number of suckers have profound influence on fruit yield. In the present study, initial selection of plants was carried out based on these characters. Influence of vegetative characters on yield in pineapple have already been reported by Brown (1953) and Collins (1960), who indicated the importance of general vigour and vegetative characters in clonal selection. Further reports on the correlation studies between vegetative characters and yield characters are also available (Su, 1958; Wu and Su, 1965; Chadha *et al.*, 1977 and Prabhakaran and Balakrishnan, 1978).

In the present study, fruit weight was taken as an important parameter and plants producing fruits of 2.00 kg or more alone were considered in the further selection procedure. The base population for clonal selection was thus brought down to 25. Quantitative characters of the fruits, namely, fruit weight with crown and without crown, crown weight, weight of fruit after peeling, weight of pulp, peel pulp ratio, pulp percentage, juice content, taper ratio and L/B ratio were separately recorded and utilized for final selection of five accessions for further multiplication. Similar procedure of plant selection has already been reported in pineapple by Brown (1953) and Collins (1960).

Higher fruit weight with crown is a desirable character, from the point of view of the seller. But a consumer prefers a condition, where in the contribution of crown to the total fruit weight is less. Being the choice variety for fresh consumption, characters like high pulp weight, lower peel pulp ratio and high pulp percentage were given due importance in the selection. Higher juice content also was opted, considering the suitability of Mauritius for juice making. When used in processing industry shape of the fruit is an important character. Mauritius is a variety noted for its peculiar conical shape, with the fruit girth progressively decreasing from the base towards the top. This character renders the variety not suitable for canning as wastage will be very high while mechanical methods for peeling are resorted to. So during selection of elite types, a taper ratio nearer to one was preferred. Also an L/B ratio nearer to 1.5 was considered as superior character. The ideal taper and L/B ratio for most profitable canning are reported to be as 1 and 1.5, respectively (Gopimony *et al.*, 1978).

Different qualitative characters of fruit, namely, T.S.S., acidity, total sugar, reducing sugar and non reducing sugar were analysed. Sweetness is an important character to be given higher importance, particularly when the fruit is used for table purposes. Sweetness was reported to be directly correlated with T.S.S. and total sugar (Gopimony *et al.*, 1978). So fruits with higher T.S.S. and total sugar were selected during clonal selection.

Both plant characters and fruit characters of the selected 25 accessions were tabulated and compared. After thorough comparison and scoring, five elite accessions with maximum number of desirable characters were finally selected.

5.2 Refinement of *in vitro* propagation technique

Although there are reports available on rapid *in vitro* propagation of different varieties of pineapple, namely Smooth Cayenne strain Mitsubishi (Wakasa, 1989). Kew (Prabha, 1993) and Cayenne de oriental and Espanola Raja (Kiss *et al.*, 1995), an optimal *in vitro* technique for rapid colonal propagation of Mauritius, needed to be developed.

The present studies were carried out to refine the *in vitro* propagation technique in pineapple, to suite the variety Mauritius and to rapidly multiply the selected elite plants for further trial and evaluation.

Since the explants collected from external environment harbour numerous micro organisms, they need to be surface sterilized before inoculation. In the present studies, after detailed surface sterilisation experiments, it was found necessary to standardise the surface sterilization procedure with optimum concentration of different chemicals for optimum duration for each explant. For shoot tip explants it was found that, an initial surface sterilization with 0.1 per cent emisan for 35 minutes followed by 0.1 per cent mercuric chloride for 10 minutes give a maximum contamination free cultures of 91.66 per cent without any mortality of the explants (Table 8). For lateral bud explants, treatment with 0.1 per cent emisan for 10 minutes followed by 0.1 per cent mercuric chloride for three minutes resulted in least rate of contamination (16.66%) and maximum percentage (83.33) of explant survival (Table 9). Increased exposure of explants to surface sterilants increased their mortality rate. The duration of exposure of lateral bud explants, to the surface sterilants, for getting least percentage of contamination and maximum explant

survival was less when compared to shoot tip explants. This may be due to the fact that, lateral buds are directly exposed to the sterilization chemicals, unlike in shoot tip explants, which are kept concealed by the surrounding leaf primodea.

For surface sterilizing crown explants, Prabha (1993) used 0.1 per cent emisan for 30 minutes followed by 0.1 per cent mercuric chloride for 10 minutes, so as to get a maximum percentage of explant survival (45.8%). In the present study a new technique was developed to reduce contamination and increase survival percentage of establishment. Biggest sized explants after surface sterilisation were cut to size of 0.5 cm^3 within the laminar air flow chamber, under aseptic condition, before inoculation on to the establishment media. This resulted in 91.66 per cent explant survival, as against 45.8 per cent reported by Prabha (1993). Mathews and Rangan (1979) suggested the use of 0.1 per cent mercuric chloride for three minutes for surface sterilising lateral buds from crown. In the present study, it was revealed that considering the least percentage of contamination and maximum rate of explant survival, treatment with 0.1 per cent emisan for 10 minutes, followed by 0.1 per cent mercuric chloride for three minutes was the effective surface sterilisation treatment for lateral bud explants.

Many workers have reported MS medium as most suitable for *in vitro* multiplication of pineapple (Mapes, 1973; Wakasa *et al.* 1978; Mathews and Rangan, 1979; Evans and Moore, 1987; Dewald *et al.*, 1988; Bordoloi and Sharma, 1993 and Prabha, 1993). Using MS medium as basal medium, growth regulator manipulations were done with BAP at different levels, to standardise the optimum medium for culture establishment (as indicated by greening of explants), for different explants. For shoot tip explants from suckers, MS medium supplemented

with BAP 3.0 mg l^{-1} and 4.0 mg l^{-1} were found to be the best for culture establishment as evidenced by the less number of days taken for greening of explants, wherein greening was observed in 10.12 and 10.25 days, respectively (Table 10). There existed no significant difference among different shoot tip explants from different sources, namely, suckers, crowns and slips with regard to time taken for culture establishment (Table 11). Lateral bud explants took more time for greening, (13.05 and 13.25 days), respectively in MS medium containing BAP 4.0 mg l^{-1} and 3.0 mg l^{-1} (Table 12). Higher concentration of BAP (above 5.0 mg l^{-1}) delayed explant greening. A similar response has been reported by Prabha (1993) in Kew. The pattern of response was similar with regard to the initiation of growth in different explants (Tables 13 and 14). But comparing to shoot tip explants, in lateral buds initiation of growth was late (Table 15). The reason for delayed greening and growth initiation of lateral buds may be attributed to the dormant nature of lateral buds. Because of apical dominance, the lateral buds were in a state of dormancy, and they took more time for getting into active growing stage, unlike in shoot tip explants, which are already in their active stage of growth, responded quickly in the establishment medium.

Prabha (1993) reported that MS medium supplemented with BAP 5.0 mg l^{-1} alone or in combination with NAA 1.0 mg l^{-1} was most suitable for culture establishment and initiation of growth in shoot tip explants from crown. In the present study, it was observed that, for Mauritius variety of pineapple, MS medium containing BAP 3.0 or 4.0 mg l^{-1} alone was sufficient for faster culture establishment and initiation of growth. This difference may be due to the physiological variations in the varieties Mauritius and Kew.

Favourable effects of cytokinins on enhanced release of axillary buds have been reported by Murashige (1974). Among the different levels of BAP, during the different subculture stages, (first, second and third) axillary bud production was higher in MS medium containing BAP 4.0 mg l^{-1}, while in the case of kinetin a concentration of 5.0 mg l^{-1} was found producing maximum number of axillary buds (Tables 16 and 17). On comparing these two cytokinins, BAP was found to be more effective for enhanced release of axillary buds. This is in confirmation with the findings of Bhaskar (1990) in banana. Higher levels of cytokinin were found to have deleterious effect on axillary bud production. Similar reports have been made by Hussey (1976) in gladiolus. Inclusion of NAA into the medium, particularly along with higher levels of cytokinin, adversely affected the axillary bud production. In the combination treatments containing both auxins and cytokinins, axillary buds and adventitious buds were produced simultaneously by the second subculture stage. This may be due to the auxin-cytokinin synergism reported by George and Sherington (1984). NAA suppressed axillary bud release and enhanced the formation of adventitious buds (callus formation in other species). Similar results have been reported by Kefford and Goldacre (1960) in tobacco. While auxin alone leads to cell enlargement, both auxin and cytokinin are essential for the initiation of cell division and callus formation in tobacco pith. When different subculture stages were compared, high rate of axillary bud production could be observed in second subculture stage, with lower levels of cytokinin alone or in combination with NAA, followed by third subculture stage. Axillary bud production was the lowest in first subculture stage. This may be due to the fact that, the plant material needed a minimum exposure period in culture medium with cytokinin for the response, namely, release of axillary buds. With the higher levels of cytokinin alone or in

combination with NAA, axillary bud production was progressively decreased through different subculture stages, leading to complete arrest of axillary bud release in third subculture period and multiplication of adventitious bud alone could be seen in combination treatments of cytokinins and auxins.

The possibility of using different medium supplements in the micro propagation of pineapple have been reported by many workers (Mapes, 1973; Mathews and Rangan, 1981; Zepeda and Sagawa, 1981 and Fitchet, 1990a). In the present investigation, it was found that addition of caesin hydrolysate 100.0 mg l^{-1} into the medium containing BAP 4.0 mg l^{-1}, increased the rate of production of axillary buds during different subculture stages (Table 18). Caesin hydrolysate, being an amino acid supplement, significantly enhanced the release of axillary buds at a concentration of 100.0 mg l^{-1}. Coconut water at high concentrations of 10 per cent reduced the multiplication rate of axillary buds. This may be attributed to the presence of cytokinin like substances in coconut water (Straus and Rodney, 1960) and hence when used in combination with BAP 4.0 mg l^{-1}, the net concentration of cytokinin in the medium might have been increased beyond favourable limits and in turn resulted in the lesser production of axillary buds.

Culture multiplication through adventitious bud production (indirect somatic organogenesis) was also attempted. Many workers have reported rapid multiplication of pineapple through adventitious buds (also called as globular bodies). Wakasa *et al.* (1978) induced formation of adventitious buds by culturing axillary buds in MS medium supplemented with NAA abd BA 2.0 mg l^{-1} each. Mathews and Rangan (1981) reported proliferation of globular bodies from shoot buds of pineapple, in MS medium containing NAA 10.0 mg l^{-1}, coconut water 15

per cent and caesin hydrolysate (400.0 mg l^{-1}). Bordoloi and Sharma (1993) induced callus from axillary buds cultured on MS medium containing IAA, IBA and KIN (5.0 mg l^{-1} each). Prabha (1993) reported that, faster induction and highest proliferation of adventitious buds were achieved in MS medium containing BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}. In the present study, it was found that, among the different treatments involving BAP, the medium supplemented with BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} induced faster initiation of adventitious buds in shoot tip explants and in the case of kinetin, treatment with kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} was more effective (Tables 19 and 20). This is in confirmation with the findings of Prabha (1993). When the two cytokinins were compared for adventitious bud initiation, BAP was found more promising than kinetin. When cytokinins were used alone in the medium, their higher concentrations induced faster response than the lower levels. This could be due to the high levels of cytokinin, which reduced the apical dominance in both primary axillary buds and in laterals leading to a non-distichous arrangement of growing axillaries and finally resulting in the formation of adventitious buds. In combination treatments involving both cytokinins and auxins, adventitious bud formation was faster as against their corresponding levels of cytokinin alone. The reason for this may be attributed to the favourable effect of auxins on callus formation through auxin-cytokinin synergism (George and Sherington, 1984). There existed no significant difference among different shoot tip explants from various sources, namely, suckers, crowns and slips, with regard to adventitious bud formation (Table 21). But lateral buds differed both in favourable concentration of growth regulator and in number of days taken for adventitious bud production (Table 22). In the case of lateral buds, MS medium containing BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1} was more effective in inducing adventitious buds.

Regarding the rate of proliferation of adventitious buds, it was maximum, in MS medium supplemented with BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1} and BAP 4.0 mg l^{-1} + NAA 1.0 mg l^{-1} (Table 23). This is in line with the findings of Sudhadevi *et al.* (1994). Among different levels of kinetin, maximum multiplication of adventitious buds was observed with kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} (Table 24). Results indicated that out of the two cytokinins, tried BAP was more effective for adventitious bud proliferation. As for initiation of adventitious buds, its multiplication also was increased by the inclusion of NAA in the medium, along with cytokinin.

In the attempt to standardise the physical condition of the media for maximum proliferation of adventitious buds, it was found that, the proliferation rate was higher in liquid medium under shake culture condition as compared to solid medium (Table 25). Similar findings, have been reported by Zepeda and Sagawa (1981); Fitchet (1985) and Dewald *et al.* (1988). Moreover the requirement of BAP for maximum rate of multiplication of adventitious buds was also lower in liquid medium. This could be attributed to more contact and availability of medium ingredients including hormones to the tissues in liquid medium than in solid medium.

After standardising the media for initiation and proliferation of adventitious buds, regeneration of plantlets was attempted. In the present study, it was found that, with regard to faster regeneration of shoots and vigour of the shoots, MS medium without any growth regulator was most ideal (Table 26). This is in confirmation with the findings of Wakasa *et al.* (1978) and Prabha (1993). Lower levels of BAP enhanced shoot regeneration. The mean number of shoots was highest

in medium containing BAP 1.0 mg l^{-1}, but the mean length of shoot was less when compared to that in growth regulator free MS medium. This suggested the possibility of starting the shoot regeneration in medium containing BAP 1.0 mg l^{-1} and further growth of shoots is obtained in growth regulator free medium so that more number of vigorous shoots could be produced. Wakasa *et al.* (1978) reported regeneration of shoots in the MS medium supplemented with BA 2.0 mg l^{-1} and further growth was achieved in MS medium without any hormones. Prabha (1993) reported that growth regulator free MS medium was most suitable for regeneration of shoot. Comparing the two cytokinins, BAP was found more effective than kinetin for shoot regeneration (Table 27). Addition of kinetin into the medium delayed shoot initiation and reduced the number of shoots drastically. This is in contradictory to the findings of Prabha (1993), who reported that KIN at 10.0 and 5.0 mg l^{-1} was more effective in inducing formation of shoots in large numbers in Kew. This may be due to the difference between Mauritius and Kew with regard to cytokinin preference for shoot regeneration. Superiority of BAP over kinetin for shoot regeneration, from cormel tip derived callus has been reported in gladiolus by Hussain (1995). The cytokinins at their higher levels adversely affected shoot regeneration. This may be because of favourable effect of high levels of cytokinin on adventitious bud formation and suppressive effect on shoot regeneration. Addition of NAA in the medium delayed shoot regeneration as well as reduced the shoot number and length. With high levels of cytokinins, NAA produced small condensed shoots and with still higher levels only small leafy proliferations were obtained and sometimes shoot regeneration itself was lacking. This may be due to the fact that, auxins and high levels of cytokinins favoured porliferation of adventitious buds, rather than their differentiation to shoot. These results are in

confirmity with the earlier findings of Wakasa *et al.* (1978) and Bordoloi and Sharma (1993).

Physical condition of the media also had significant effect on shoot regeneration from adventitious buds with respect to both shoot initiation and mean number of shoots, liquid medium under shake culture conditions was found superior than solid medium (Table 28). Similar results have been reported earlier by Mathews and Rangan (1979), wherein a three fold increase in the number of shoots was achieved using shake cultures. Shake cultures induced rapid proliferation of single shoot apices of orchid (Wimber, 1965), *Nicotiana rustica* (Walkey and Woolfitt, 1968) and bromeliads (Mapes, 1973). The success of a micropropagation programme is largely dependant on the multiplication rate and hence shake culture technique can be used as a efficient method for producing large number of clonal plants rapidly. In liquid medium, the mean length of shoots was comparatively less than in solid medium. Since the mean number of shoots was higher in liquid medium, the growth rate of individual shoot might have reduced resulting in the production of comparatively shorter shoots.

Next stage in micropropagation of pineapple is *in vitro* rooting. In the present studies, different factors affecting rooting, namely, levels and types of different auxins and physical conditions of the media were standardised. Faster rooting was observed in MS medium without any growth regulator (Table 29). This is in confirmation with the findings of Wakasa *et al.* (1978), Prabha (1993) and Kiss *et al.* (1995). Among the different auxins tried, NAA 2.0 and 1.0 mg l^{-1} were found to be the best with respect to root initiation. This is in line with the findings of Lakshmisita *et al.* (1974) who reported the use of NAA 1.0 mg l^{-1} for root

induction in pineapple. Yang (1981) also suggested that NAA 1.0 mg l^{-1} was best for faster root induction in pineapple. The mean number of roots was also higher in medium supplemented with NAA. However, NAA drastically reduced root length. Root length was maximum in growth regulator free MS medium. It was observed that, out of different auxins tried, both IBA and IAA at their lower levels of 1.0 and 2.0 mg l^{-1} was superior with regard to mean root length. Bordoloi and Sharma (1993) reported that ½ strength MS medium containing IBA 2.0 mg l^{-1} was effective for *in vitro* rooting of pineapple. Favourable effect of NAA and IBA on *in vitro* rooting in pineapple have been reported by Mathews and Rangan (1979 and 1981). Normal slender roots with secondaries and root hairs were produced in hormone free MS medium. Both IBA and IAA produced comparatively shorter roots, while NAA formed very short thick roots without any secondaries. These results are in line with the findings in cardamom as reported by Lane (1979).

Physical condition of the media also had greatly influenced *in vitro* rooting. Faster rooting, with higher root length was achieved in liquid medium (Table 30). This can be attributed to the higher availability of medium constituents in liquid medium. Also in liquid medium, roots could grow more freely without any resistance, unlike in solid medium, wherein the root growth was obstructed by the solid consistency of medium. Investigations on *in vitro* rooting in pineapple revealed that, with regard to root induction and mean length of roots, growth regulator free MS medium was superior. Although the mean number of roots was less when compared to NAA treatments, both the nature and appearance of roots were good in basal MS medium without hormones.

Better rooting was achieved in basal MS medium, which was as good for shoot regeneration also, by keeping the adventitious buds for a period of 40 days in the hormone free medium itself, simultaneous rooting could be achieved. Thus by combining both shoot and root regeneration in a single step a separate rooting phase could be avoided.

Debergh and Maene (1981) indicated that, *in vitro* rooting was the most labour-intensive and hence expensive part of micro propagation, accounting for 35-75 per cent of the total cost of micro propagation depending upon the species. Moreover, in many species *in vitro* roots lack root hairs and are easily damaged on transfer and there by chances of plant mortality will be high. Also removal of agar, adhering to the roots, without damaging the roots is not easy and any traces of agar left in the roots will cause their decay and ultimately high plant mortality. An alternate system, suggested for rooting of plantlets is *ex vitro* rooting, wherein both rooting and initial acclimatization are combined. This eliminates a separate rooting stage and is more economical, besides producing good quality roots. *Ex vitro* rooting is achieved by dipping the basal ends of *in vitro* produced shoots in suitable auxins.

An experiment was conducted to combine both rooting process and initial hardening in pineapple. Rooting percentage was highest in treatment with dipping the shoots in IBA 200.0 mg l^{-1} (for a period of one hour) to the tune of 87.5 per cent (Table 31). Seventy five per cent of the shoots produced roots even without any growth regulator treatment. Results indicated that faster rooting was achieved in treatment with NAA 200.0 and 100.0 mg l^{-1} (dipping the shoots for one hour period). Considering the percentage of rooting, mean number of roots and mean root length, IBA 200.0 mg l^{-1} was found better than other treatments for *ex vitro* rooting

in pineapple. The IBA 100.0 mg l^{-1} for one hour was also effective. Prabha (1993) reported that mean number of roots was highest when treated with IBA 200.0 mg l^{-1} as quick dip.

Micro propagation on a large scale can be successful only when plants after transfer from *in vitro* conditions to soil have high survival rate, better growth and development, during hardening and in subsequent stages. Transfer of *in vitro* produced plants to *ex vitro* conditions and their subsequent acclimatization is a critical step in micro propagation.

In the present investigation also, experiments were conducted to standardise the most suited potting media, container, nutrient starter solution and plant size for better growth and development of *in vitro* plants under *ex vitro* condition.

One of the important factors, which greatly influences the growth and development of *in vitro* plants under *ex vitro* condition, is the type of potting media. Potting media vary in their water holding capacity, mineral content and ability to provide sufficient aeration and drainage in the rhizosphere. Results indicated that, potting media such as sand, soilrite, palmpeat, and vermiculite + soil (1:1 ratio) were equally good in plant survival (Table 32). The survival was only 91.67 per cent in ordinary potting mixture, but all other treatments resulted in cent per cent establishment. The growth of plants under different potting media was found to differ significantly. The plants grown in soilrite was statistically superior in plant height, mean number of leaves and mean length and breadth of largest leaf and in general vigour than in other media tried. With regard to mean length and mean width of largest leaf, both sand and soilrite were found equally good. Better

performance of soilrite may be attributed to its ability to maintain optimum moisture and to provide sufficient aeration in the rhizosphere. The nutrient content of soilrite was also higher than other potting media. Eventhough, sand was found, not as good as soilrite, for hardening of plantlets, particularly with respect to plant height and mean number of leaves, since it performs equally well with soilrite with regard to mean length and width of largest leaf, it can be considered as an efficient potting media for *in vitro* produced pineapple plantlets. Moreover, sand being cheaper than soilrite, on economic ground also sand is preferred.

Folliot and Marchal (1990) reported that peat based substrate is the most ideal potting media for pineapple. Prabha (1993) observed that, potting media such as cocopeat, soilrite, biofibe and vermiculite were better for inducing vigorous growth in pineapple plantlets. Keshavachandran (1991) found that a mixture of soilrite and potting mixture gave the highest establishment percentage and vigour of vettiver plants.

Type of containers used during acclimatization also affects the growth and development of plantlets. From the present study, it was observed that, considering the plant height, mean number of leaves, mean length and width of largest leaf, plastic pots could be chosen as the most ideal container for transplanting *in vitro* produced pineapple plantlets (Table 33). This agrees with the findings of Prabha (1993). Polythene bag was found to be the second best container for inducing better vigour for the plants. Being cheaper and more economic when compared to plastic pot, polythene bag also could be considered as suitable for hardening of pineapple plants. Also transplanting to the main field was much easier in the case of plants grown in polythene bags. Plants grown in mudpots showed less vigour and

growth. This is in confirmation with the findings in Jack plantlets (Ramesh *et al.*, 1993).

Addition of inorganic nutrients, into the potting media during hardening influences the growth and development of *in vitro* produced plants. Results of the present study indicated that, with regard to the plant height, mean number of leaves, mean length and width of largest leaf, treatment with full MS salt solution was superior to all other nutrient solutions (Table 34). Better results obtained with full MS salt solution may be due to its higher nutrient status. Effects of ½ MS salt solution and NPK fertiliser mixture solution were on par to each other and stood next to full MS salt solution. Urea at both the concentrations of one per cent and 0.5 per cent was found less effective in inducing better growth of plants. Prabha (1993) reported that in pineapple, in the early stages of hardening, application of NPK solution (10:5:20 @ 5 g/litre) once in a week or 1/4 strength MS salt solution produced vigorous plants. At later stages of plant growth, application of Hogland's solution or the NPK fertiliser mixture solution twice a week was more effective. Reghunath (1989) suggested the use of 5-10 ml nutrient solution, containing MS inorganic salts at half strength, at weekly intervals, for enhancing the growth and vigour of cardamom plantlets.

Size of the plantlets when transferred to *ex vitro* condition is an important factor influencing the survival and growth of plants. Prior to hardening, the plants must have sufficient size, with well proportioned shoots and roots, that are capable of supporting each other. Plants also should have sufficient photosynthetic area for becoming quickly self supporting.

Results of the present study showed that, plants belonging to group 3 (with a plant height of 6.1-8.0 cm, 6-7 leaves, 4.1-5.0 cm and 5.1-6.0 mm length and width respectively, for largest leaf and with a plant weight of (401-600 mg) registered highest growth rate as indicated by percentage of establishment, percentage increase in plant height, length and width of largest leaf and plant weight (Table 35). Even in bigger sized plants rate of growth was lesser when compared to plants of group 3. Dewald *et al.* (1988) reported that shoots larger than 3.0 cm length had 100 per cent survival, when transferred to soil. This indicated that, a plant size as that of group 3 was most optimum for transferring *in vitro* produced pineapple plants to *in vivo* conditions. This suggested earlier transfer of plantlets and saving of both time as well as expense on media.

The refined *in vitro* protocol for mass multiplication of Mauritius developed through the present study was utilized for the multiplication of selected five elite clones of Mauritius. Two hundred and fifty plantlets, each belonging to these clones are maintained in the nursery for further evaluation.

Summary

SUMMARY

Investigations on 'refinement of *in vitro* propagation technique in pineapple var. Mauritius and mass multiplication of elite clones' were carried out at the Kerala Horticulture Development Programme, Kerala Agricultural University, Vellanikkara. The main objective was to refine the *in vitro* propagation technique in pineapple, to suite for Mauritius variety and mass multiplication of elite clones, using the refined *in vitro* technique. The results and salient findings are summarised in this chapter.

1. Based on the detailed survey conducted at the major Mauritius growing areas, namely, Vazhakulam, Muvattupuzha regions of Ernakulam district, five elite plants with higher yield and desirable fruit characters were selected. Suckers, crowns and slips collected from the selected types were planted at Vellanikkara for their mass multiplication through tissue culture.

2. Treatment with 0.1 per cent emisan for 35 minutes followed with 0.1 per cent mercuric chloride for 10 minutes was ideal for surface sterilization of shoot tip explants, wherein the percentage survival of explant was 91.66 per cent. For lateral bud explants, treatment with 0.1 per cent emisan for 10 minutes followed with 0.1 per cent mercuric chloride for 3 minutes was found to be the ideal surface sterilization treatment, which gave a survival rate of 83.33 per cent.

3. MS medium supplemented with BAP 3.0 and 4.0 mg l^{-1} was found to be best for culture establishment in shoot tip explants from suckers, wherein the greening of explants was noticed in 10.12 and 10.25 days, respectively. Source of shoot tip explants had no significant effect on culture establishment.

4. For lateral bud explants, fastest culture establishment was noticed in MS medium supplemented with BAP 4.0 and 3.0 mg l^{-1} (13.05 and 13.25 days, respectively). Lateral bud explants took more time for establishment when compared to shoot tip explants. Higher concentrations of BAP (above 5.0 mg l^{-1}) delayed the establishment of cultures.

5. For initiation of growth from sucker shoot tips, MS medium containing. BAP 4.0 and 3.0 mg l^{-1} was found most ideal, wherein culture growth was initiated in 12.22 and 12.29 days, respectively. Source of shoot tip explant had no significant effect on culture growth initiation.

6. In lateral buds, fastest initiation of growth (13.53 days) was observed in MS medium supplemented with BAP 4.0 mg l^{-1}. When compared to shoot tip explants, culture growth initiation was delayed in lateral bud explants. Higher concentrations of BAP (above 5.0 mg l^{-1}) in the medium delayed the initiation of growth.

7. Among different treatments with BAP, highest number of axillary buds was produced in MS medium containing BAP 4.0 mg l^{-1} (3.32, 4.11 and 3.40 axillary buds, respectively in first, second and third subculture stages).

8. Out of different treatments with kinetin, MS medium supplemented with kinetin 5.0 mg l^{-1} was found most ideal for enhanced release of axillary buds, wherein the mean number of axillary buds produced was 2.40, 3.39 and 2.45, respectively in first, second and third subculture stages.

9. BAP was found more effective for enhanced release of axillary buds when compared to kinetin. Out of the different subculture stages, enhanced release of axillary buds was higher in second subculture stage.

10. Higher levels of cytokinin had deleterious effect on axillary bud release. Inclusion of NAA into the medium, drastically reduced the production of axillary buds.

11. Among the different medium supplements tried, inclusion of caesin hydrolysate 100.0 mg l^{-1}, in the medium containing BAP 4.0 mg l^{-1} was found most effective for enhanced release of axillary buds. The treatment produced a mean number of 3.66, 4.48 and 4.02 axillary buds, respectively during first, second and third subculture stages.

12. Adventitious bud initiation from suckers shoot tips was fastest in MS medium supplemented with BAP 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} followed by BAP 5.0 mg l^{-1} + NAA 0.5 mg l^{-1} (24.29 and 25.21 days, respectively).

13. Of the different levels of kinetin, MS medium supplemented with kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1} was ideal for adventitious bud production (57.25 days).

14. When comparing the two cytokinin sources (BAP and kinetin), BAP was found more effective for adventitious bud formation. Combination treatments containing both cytokinin and auxin were found best for initiating adventitous buds, than cytokinins alone. Source of shoot tip explants, namely, suckers, crowns and slips had no significant effect on initiation of adventitious buds.

15. In the case of lateral bud explants, adventitious bud initiation was delayed when compared to shoot tip explants. Also higher levels of BAP, namely BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1} was required for inducing fastest response (33.40 days).

16. Rate of proliferation of adventitious buds was maximum in MS medium supplemented with BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1} and BAP 4.0 mg l^{-1} + NAA 0.5 mg l^{-1}.

17. Among different levels of kinetin, maximum multiplication of adventitious buds was observed in MS medium containing kinetin 5.0 mg l^{-1} + NAA 1.0 mg l^{-1}.

18. Between the two sources of cytokinins (BAP and kinetin) tried BAP was found ideal for proliferation of adventitious buds.

19. Proliferation rate of adventitious buds was higher in liquid medium under shake culture conditions, than in solid medium. In liquid medium under shake culture conditions, the concentration of BAP required for maximum proliferation of adventitious bud was lower.

20. With regard to faster shoot regeneration, mean length and vigour of the shoots, MS medium without any growth regulator was most superior. In this medium shoot initiation started in 10.15 days and the mean shoot length was 2.46 cm. The mean number of shoots was the highest (27.01) in MS medium supplemented with BAP 1.0 mg l^{-1}.

21. Between the cytokinin sources, BAP was found more effective than kinetin for shoot regeneration. Addition of kinetin into the medium delayed shoot initiation and reduced the number of shoots drastically.

22. Higher levels of cytokinins adversely affected shoot regeneration. Inclusion of NAA in the medium was found to have deleterious effect on regeneration of shoots.

23. Liquid medium under shake culture condition was superior to solid medium with respect to both shoot initiation and mean number of shoots produced. In liquid medium shoots were initiated in 10.53 days and the mean number of shoots was 36.98. However, mean length of shoots was higher in solid medium (1.34 cm).

24. Fastest rooting was observed in growth regulator free MS medium (5.99 days in solid medium and 5.25 days in liquid medium).

25. Among the auxins tried, in solid medium faster rooting was observed in MS medium containing NAA 2.0 mg l^{-1} wherein roots were initiated in 9.53 days and in liquid medium fastest rooting (8.34 days) was seen in treatment with NAA 1.0 mg l^{-1}.

26. Mean number of roots was the highest in treatment with NAA 3.0 mg l^{-1} (20.16 and 13.78 in solid and liquid medium, respectively). However, the roots were very short and hair like. Mean root length was the highest in growth regulator free MS medium (1.61 cm, 2.18 cm in solid and liquid medium, respectively). Among the different auxins tried, both IBA and IAA at their lowest levels 1.0 and 2.0 mg l^{-1} was found superior with regard to mean root length.

27. Normal slender roots with secondaries and root hairs were produced in MS medium without any growth regultor.

28. Stationary liquid medium was found superior to solid medium with regard to root initiation and mean root length. However, mean root number was comparatively lesser in liquid medium.

29. By keeping the adventitious buds for a period of 40 days in the hormone free MS medium, both shoot and root regeneration could be achieved simultaneously.

30. For *ex vitro* rooting of pineapple, dipping the shoots in IBA 200.0 mg l^{-1} for a period of one hour, before planting was the most ideal. The treatment resulted in the highest rate of rooting (87.5%), maximum number of roots (5.83 roots per shoot) and higher mean root length (2.23 cm).

31. Different potting media such as soilrite, sand, palmpeat and vermiculite + soil (1:1 ratio) were good for *ex vitro* survival of *in vitro* produced pineapple plantlets. However, with regard to plant height, mean number of leaves, mean length and breadth of largest leaf and general vigour of plantlets, soilrite was most ideal for *ex vitro* establishment and growth of plants. With regard to mean length and width of largest leaf; sand performed equally well with soilrite. Moreover, sand being cheaper than soilrite, can be considered as an efficient potting media for *ex vitro* planting of pineapple plantlets.

32. Among the different containers used, plastic pot was found most ideal for *ex vitro* growth and development of pineapple plantlets. Polythene bag was found to be the second best container for better growth and development of plants.

Being cheaper and more economic than plastic pot, polythene bag also could be chosen as an ideal container.

33. Among the different nutrient starter solutions tried, with regard to the plant height, mean number of leaves, mean length and width of largest leaf, full MS salt solution was the best.

34. Plants belonging to the size group 3 (with a plant height of 6.1-8.0 cm, 6-7 leaves, 4.1-5.0 cm and 5.1-6.0 mm mean length and width, respectively for the largest leaf and having a weight of 401-600 mg registered highest growth rate as indicated by the rate of establishment (100.0%), percentage increase in plant height (41.80), leaf number (26.89), length (55.50) and width (87.92) of largest leaf and plant weight (276.00).

35. Making use of the refined micro propagation technique, clonal multiplication of the selected elite types of Mauritius variety of pineapple was done. Two hundred and fifty plants, from each selected five elite types were produced. These plants will be subjected to further evaluation and yield trials.

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* Originals not seen

Appendices

APPENDIX-I

Chemical composition of MS medium

Chemical	Quantity (mg l)
INORGANIC	
Major elements	
Stock A	
NH_4NO_3	1650
KNO_3	1900
KH_2PO_4	170
$MgSO_4$	370
Stock B	
$CaCl_2.2H_2O$	440
Iron source	
Stock C	
$Na_2.EDTA$	37.3
$FeSO_4.7H_2O$	27.8
Minor elements	
Stock D	
$MnSo_4.4H_2O$	22.30
$ZnSO_4.7H_2O$	10.60
H_3BO_3	6.20
KI	0.83
$Na_2MOO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
Organic	
Stock E	
Glycine	2
Nicotinic acid	0.5
Pyridoxine.HCl	0.1
Thiamine.HCl	0.1
Myo-inositol	100.0
Sucrose (in g)	30
Agar (in g)	8
pH	5.7

APPENDIX-II

Composition and preparation of stock solution of MS medium

Ingredient	Quantity (mg/l)	Concentration of stock	Quantity chemical to prepare this stock (per ltr.) (g/l)	Volume of stock per ltr. of medium
Major elements				
Stock A				
NH_4NO_3	1650.0		165.0	
KNO_3	1900.00	50 x	190.0	10 ml
KH_2PO_4	170.00		17.0	
$MgSO_4.7H_2O$	370.0		37.0	
Stock B				
$CaCl_2.2H_2O$	440.0	50 x	44.0	10 ml
Iron source				
Stock C				
Na_2.EDTA	37.30	100 x	7.4	5 ml
$FeSO_4.7H_2O$	27.80		5.6	
Minor elements				
Stock D				
$MnSO_4.4H_2O$	22.30		4.46	
$ZnSO_4.7H_2O$	10.60		2.12	
H_3Bo_3	6.20		1.24	
KI	0.83	100 x	166 mg l^{-1}	5 ml
$Na_2NO\ O_4.2H_2O$	0.25		50 mg l^{-1}	
$CoCl_2.6H_2O$	0.025		5 mg l^{-1}	
$CuSO_4.5H_2O$	0.025		5 mg l^{-1}	
Organic (Vitamins)				
Stock E				
Glycine	2 mg l^{-1}	1000 ppm	100 mg/100 ml	2 ml
Pyridoxine.HCl	0.5 mg l^{-1}	1000 ppm	100 mg/100 ml	0.5 ml
Nicotinic acid.HCl	0.5 mg l^{-1}	1000 ppm	100 mg/100 ml	0.5 ml
Thiamine.HCl	0.1 mg l^{-1}	1000 ppm	100 mg/100 ml	0.1 ml

APPENDIX-III

Preparation and storage of plant growth regulators

Sl. No.	Growth regulator	Common abbreviation	Solvent	Diluent	Regulators quantity to prepare 1000 ppm in 100 ml (mg)	Preparation	Storage temperature
1	Cytokinin						
a.	N^6-Benzyladenine	BAP	1N NaOH	Distilled water	100	Dissolved in 5 ml of suitable solvent and made upto 100 ml using double distilled water	0-5°
b.	Kinetin	KIN	1N NaOH	Distilled water	100		-0°
2	Auxins						
a.	α-Napthalene acetic acid	NAA	1N NaOH	Distilled water	100	Dissolved in 5 ml of suitable solvent and made upto 100 ml using suitable diluent	RT
b.	Indole-3-butyric acid	IBA	1N NaOH	,,	100		0-5°
c.	Indole-3-Acetic acid	IAA	1N NaOH	,,	100		-0°

REFINEMENT OF *IN VITRO* PROPAGATION TECHNIQUE IN PINEAPPLE VAR. MAURITIUS AND MASS MULTIPLICATION OF ELITE CLONES

BY

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ABSTRACT OF A THESIS

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ABSTRACT

The studies on refinement of *in vitro* propagation technique in pineapple var. Mauritius and mass multiplication of elite clones were conducted at Kerala Horticulture Development Programme, Kerala Agricultural University, Vellanikkara during 1993-'95.

Based on the survey conducted at the major pineapple growing areas, namely, Vazhakulam-Muvattupuzha regions of Ernakulam district, five elite clones of Mauritius variety with higher yield and other desirable fruit characters were selected propagules from the selected clones were planted at Vellanikkara for taking explants for their mass multiplication through refined *in vitro* propagation techniques.

For shoot tip explants, treatment with emisan 0.1 per cent for 35 minutes followed with mercuric chloride 0.1 per cent for 10 minutes and for lateral bud explants treatment with emisan 0.1 per cent for 10 minutes followed by 0.1 per cent mercuric chloride for three minutes was found to be the ideal surface sterilization treatment.

Culture establishment and growth initiation of shoot tip explants from different sources (suckers, crowns and slips) and lateral bud explants were better in MS medium supplemented with BAP 3 and 4 mg l^{-1}. Explants from shoot tips were found to be better than those from lateral buds for achieving faster culture establishment and growth initiation.

Enhanced release of axillary buds was the maximum in MS medium containing BAP 4.0 mg l^{-1}. Among the different subculture stages, multiple axillary bud production was higher in second suculture stage. Addition of caesin hydrolysate 100.0 mg l^{-1} in to the medium with BAP 4.0 mg l^{-1} favoured the production of axillary buds.

Adventitious bud initiation from sucker shoot tips was fastest in MS medium supplemented with BAP 5.0 mg l^{-1} + NAA 1.0 or 0.5 mg l^{-1}. For adventitious bud production from lateral buds, treatment with BAP 7.5 mg l^{-1} + NAA 1.0 mg l^{-1} was the best.

Proliferation rate of adventitious buds was maximum in MS medium supplemented with BAP 4.0 mg l^{-1} + NAA 0.5 or 1.0 mg l^{-1}. Rate of multiplication of adventitious buds was higher in liquid medium under shake culture condition, than in solid medium.

Faster shoot regeneration and increased vigour of the shoots were resulted in growth regulator free MS medium. However, highest number of shoots were produced in MS medium supplemented with BAP 1.0 mg l^{-1}. Liquid medium under shake culture condition was found superior to solid media with respect to initiation of shoots and mean number of shoots, however, the latter resulted in longer shoots.

In vitro rooting was fastest in MS medium, without any growth regulator, which produced longer and normal roots with secondaries and root hairs. Though addition of NAA (3 mg l^{-1}) resulted in increased number of roots, they were very short and hair like. Stationary liquid medium was found superior to solid