In Vitro CLONING OF CHINESE GUAVA
(*Psidium friedrichsthalianum* (Berg.) Nierdz.)

Thesis

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By

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Dedicated to

My

Dedicated to

Beloved and

Venerable Mother

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CERTIFICATE

This is to certify that the thesis entitled "***In vitro* cloning of chinese guava (*Psidium friedrichsthalianum* (Berg.) Nierdz.)**" submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy with major in Horticulture of the College of Post Graduate Studies, Govind Ballabh Pant University of Agriculture & Technology, Pantnagar, is record of *bona fide* research carried out by Mr. Rajesh Kumar, I.D. No. 25814, under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

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CERTIFICATE

We, the undersigned, members of the Advisory Committee of **Mr. Rajesh Kumar, I.D. No. 25814**, a candidate for the degree of Doctor of Philosophy with major in Horticulture, agree that the thesis entitled **"*In vitro* cloning of chinese guava (*Psidium friedrichsthalianum* (Berg.) Nierdz.)"**, may be submitted in partial fulfilment of the requirement for the degree.

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CONTENTS

	CHAPTER	PAGE NO.
1.	Introduction	1
2.	Review of Literature	5
3.	Materials and Methods	31
4.	Experimental Results	42
5.	Discussion	65
6.	Summary and Conclusion	72
	Bibliography	75
	Vita	

INTRODUCTION

INTRODUCTION

Guava (*Psidium guajava* L.), the apple of tropics, is an important tropical fruit crop. It is native of tropical America and now cultivated in all tropical and sub-tropical areas of the world (Martin, 1984). Guava is a rich source of vitamin C, pectin, calcium, phosphorus and iron. Miller and Bazore (1945) stated that guava in Hawaii is a fair source of vitamin A, but a poor source of thiamine and riboflavin. Guava fruit is relished when mature or ripe. Excellent salad and pudding are prepared from the shell of ripe fruit. Guava jelly is well-known to all and the common sour wild guava make the best jelly. It can also be canned in sugar syrup or made into fruit butter. In some countries, the leaves are used for curing diarrhoea.

Guava occupies fourth position among the fruit crops in India (Samuel, 1999) whereas in Uttar Pradesh its production is maximum followed by Bihar, Madhya Pradesh, Haryana, Gujarat and Andhra Pradesh. The excellent quality of guava fruits are produced in Uttar Pradesh.

Guava is quite hardy, prolific bearer and highly remunerative even without much care but its cultivation is in serious threat because of wilt disease which causes about 20 to 100 per cent mortality under different agroclimatic and soil conditions. The disease in known to occur for the past few decades but its actual cause is not yet known (Rawal, 1993). This disease is known to occur in

Uttar Pradesh, West Bengal, Bihar, Rajasthan, Madhya Pradesh, Punjab, Haryana and Andhra Pradesh. It is a serious malady causing heavy losses, but information is scanty on its etiology (Madan *et al.,* 1985). The disease was first noticed in 1935 in Allahabad (Prasad *et al.,* 1952). This is a soil borne fungal disease. Different genera of fungus are associated with guava wilt disease e.g., *Fusarium* spp., *Colletotrichum* spp., *Rhizoctonia* sp., *Macrophomina* spp. These fungi are mainly affecting root system and ultimately xylem part of the plant.

The guava wilt may be controlled either by using wilt resistant *Psidium* species as a rootstock for cultivated varieties or improving soil environment. Edward (1960a) reported that chinese guava (*Psidium friedrichsthalianum* (Berg.) Nierdz.) is resistant against guava wilt, but due to its lesser economic value, it is not cultivated by the farmers for fruit production. The chinese guava is characterized by small leaves and short internodal length. The chinese guava is traditionally propagated through inarching, stooling, air layering or stem cuttings but these methods are time consuming, slow, season dependent, cumbersome and require large quantity of propagating material. To overcome these problems, tissue culture technique may be used to produce a large number of high quality, true to type rootstock plants in shorter duration (Hartmann *et al.*, 1993).

The tissue culture technique was first used by Haberlandt (1902). Shoot tip and axillary bud culture as tool of rapid clonal multiplication were initiated as technique was first achieved by Ball (1946). After successful cloning of orchid (Morel, 1965), the technique was also employed in herbaceous strawberry (Boxus, 1974). Druat and Gruselle (1986) suggested the use of shoot tip or meristem as explant to avoid the chances of variability especially in case of fruit trees. The production of apple and peach rootstocks was perhaps the first major application of tissue culture technique in woody fruit species. Although, some work has been done in the field of tissue culture in subtropical and tropical woody fruit trees but not as it was needed, because of several problems like wide occurrence of bacterial and fungal contamination, presence of phenolic compounds in plants and their oxidation resulting in death of explants.

The micropropagation of guava have been tried in seedlings (Amin and Jaiswal, 1988; Loh and Rao, 1989; Papadatou *et al.,* 1990) and cultivar Sardar (Parkash and Tiwari, 1996) with some success but so far no work has been reported for multiplication of chinese guava (*Psidium friedrichsthalianum* (Berg.) Nierdz.) which is considered resistant to guava wilt and can be used as rootstock for various cultivars. Therefore, there is an urgent need to develop the protocol for the multiplication of chinese guava. Taking above points into consideration, the present studies were undertaken with the following objectives:

i) To standardize the time and season of explant collection.

ii) To standardize the effect of various surface sterilants and their concentration on the establishment of explants.

iii) To study the effect of different antioxidants in controlling the browing of explants.

iv) To study the effect of various plant bio-regulators on shoot proliferation and rooting of microshoots.

v) To workout the suitable potting mixture for survival and growth of rooted microshoots.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Classical botany has long recognized the complex nature of plant, where inter tissue relationships and interactions among the cell constitute profound influence on the fate of the organism. Schwann's (1839) cell theory which proposed that each living cell of an organism, if provided proper environment would be capable of independent development gave birth to the concept of 'totipotency'. The theory of 'totipotency' provides that it should be possible to reproduce an organism from any one of its nucleated cells since all the information needed to specify an organism is contained in its DNA (Steward and Krikorian, 1978).

The major steps in experimental plant tissue culture were made by Robbins (1922) and Kotte (1922) using intact meristem excised from the roots of grass seedling. Thereafter, White (1963) was able to grow tomato roots continuously *in vitro* by supplying them with yeast extract. The discovery of cytokinins and hormonal control of shoot and root generation from tobacco callus by Skoog and Tsui, (1948) at the University of Wisconsin established the basis for manipulating organ initiation and provided the principles on which micropropagation depended (Hartmann *et al.,* 1993). This foundation work encouraged others to exploit the field of micropropagation and first successful experiment under sterile conditions was carried out at the turn of this century

(Murashige, 1974). Muir *et al.,* (1954) transferred fragments of callus tissue to agitated liquid medium and succeeded in obtaining suspension containing single cell and small cell clumps.

The basic advantages of *in vitro* propagation lie mainly in the rapidity with which plant multiplication can be achieved and the number of plants that can be produced in short period of time. Murashige (1974) has stated that a million fold increase per year in the rate of clonal multiplication over conventional methods is not unrealistic. Many pathogens are also eliminated during clonal micro-propagation, which carried out under aseptic conditions facilitates the international transport and exchange of germplasm without the inherent risks of spreading pest and pathogen. The technique is especially useful in those cases where a newly developed cultivar must be multiplied for many years before it is available in sufficient quantities to be released for commercial production (Zimmerman, 1985).

Stages of micropropagation

Various stages of micropropagation have been described by different workers. Murashige (1974) and Hammerschlag (1982) reported three stages: stage I (establishment of an aseptic tissue culture of a plant), stage II (multiplication i.e., rapid numerical increase of organs or other structure) and stage III (rooting of shoot cutting, hardening of plant parts and initiating the change from the heterotropic to autotropic state). Murashige and Skoog (1962) added another stage IV which involves the transfer of the plantlets to an environment out of the culture i.e., acclimatization of the plants in greenhouse. Debergh and Maene (1981), Debergh (1987) and Torres (1988) added one more stage in the process i.e., stage 'O' which involves the preparation of the stock or mother plant from which the primary explant is to be derived.

Selection of explant

In tissue culture apparently healthy plants are selected for explant as starting material (Debergh and Maene, 1981). However, most of the symptomless plants may be infected with pathogens (Kanus, 1976; Holdgale and Aymestey, 1977). Torres (1988) suggested that a careful attention should be made to make sure that stock plant is disease free, preferably grown in either growth chamber or greenhouse on a regularly maintained pesticide and fertilizer programme. Keeping stock plants in controlled dry condition decreases the percentage of infection (Jones and Hopgood, 1979 and Rosati *et al.,* 1980). In the greenhouse, strict insect and disease control is easier than in the field because of this explants of greenhouse are often less contaminated than those of plants in the field (Ahuja, 1993). Ivanivcka and Pretova (1986) and Debergh (1987) observed the beneficial effects of homogeneous and hygienic status of the explants. Such material needs less sterilization which helps to minimize hypersensitivity while starting the cultures. Micropropagation has now been applied successfully to many fruit trees including rootstock and scion cultivars of guava, *Malus, Prunus* and *Pyrus* (Bajaj, 1985). Hartmann and Kester (1986), McCown (1986) and Pierik (1987) observed some fundamental problems of woody species related to juvenility, maturation and growth pattern. In general, larger the explant, greater the chance of success with culture establishment. Plant species with continuous growth pattern throughout the year or season are usually easier to culture than species characterized by episodic and flushing growth (McCown, 1986). Pierik (1987) reported that buds taken from woody plants still in resting state (late autumn or early winter) were more difficult to culture *in vitro* than buds from plants which were no longer dormant (spring).

Part of explant used

Healthy, young and soft (actively growing) shoots are generally more amenable for culture than older woody tissue (Pierik, 1987, Hartmann and Kester, 1986). The contamination was reported to be minimal when small sized explant was used (Murashige, 1974). New vegetative growth from the base of main stem during the period of vegetative growth in guava (Amin and Jaiswal, 1987) has been reported to be reliable source of explant. The majority of workers have used actively growing shoot tips as a explants in various fruits like apple (Jones, 1965; Walkey, 1972; Jones and Hatfield, 1976; Jones *et al.* 1979; James and Thurbon, 1979 and 1981; Lundergan and Janick, 1980; Snir and Erez, 1980; Ma *et al.,* 1990; Baraldi *et al.* 1991; Schuch and Peters, 1993; Turovskaya, 1994; Jun *et al.*, 1996), peach (Hammerschlog, 1980), pear (Lane, 1979; Banno *et al.*, 1988 and 1989; Kim *et al.*, 1991), quince (Morini and Scuitti, 1991), pecan (Wood, 1982), citrus (Barless and Skene, 1982; Singh *et al.*, 1994; Otoni and Teixeira, 1991; Rawat, 1996), jackfruit (Rahman and Blake, 1988; Singh and Tiwari, 1998), guava (Loh and Rao, 1989, Papadatou *et al.*, 1990, Leon –de-Sierralta *et al.*, 1997), black plum (Yadav *et al.*, 1990), litchi (Yu, 1991), banana (Jyothi *et al.*, 1993; Swamy *et al.*, 1991), pomegranate (Mahishni *et al.*, 1991) raspberry (Karakullukchu *et al.*, 1993). However, using juvenile materials as a source of explant may be dubious value for clonal propagation (Debergh, 1987). Shoot tips and nodal explants for mature tree of plum (Hammerschlag, 1982), apple (Marks and Simpson, 1990), peach (Zuccherelli *et al.*, 1978), pear (Rosati *et al.*, 1980), banana (Bower and Fraser, 1982; Cronauer and Krikorian, 1984 a), citrus (Broertjes and Harten, 1978; Barless and Skene, 1982), grapes (Chee and Pool, 1982; Li and Eaton, 1984), guava (Jaiswal and Amin, 1987; Amin and Jaiswal, 1988; Fitchet, 1989) and jackfruit (Roy *et al.*, 1990) have responded readily in cultures. Hutchinson (1985) found that nodal explants produced twice as many shoots as shoot tip of cherry and pear. Pierik (1987) and Hartmann and Kester (1986) reported that healthy, young and soft explants (actively growing shoots) are generally more amenable for culture than older woody tissue. Sidarovich *et al.* (1993) reported that physiological state of explant (juvenile, young and mature) and type of explants have direct influence on regeneration ability of plant. In pear, Dwivedi and Bist (1999) used 0.5 – 1.5 cm long shoot tips as explant where as 2.0 cm long shoots were used as explant by Singha (1980). Banno *et al.* (1988 and 1989) preferred less than 0.5 mm long shoot tips for culturing. Jones (1965) took 2-3 cm long apple shoot tips and dissected out to 1.0 cm long shoot tips after sterilization just prior to inoculation. Majority of workers (Snir and Erez, 1980; Zimmerman and Broome 1985 and Sriskandarajah and Mullins, 1981) used 1.0 cm long shoot tips in apple as explant.

Sterilization of explant

The explant should be free from micro-organism before placing on the nutrient media otherwise this would result in the death of the explant. Surface sterilization of juvenile explant is generally not difficult. However, if older plant parts are used, contamination of explant is some times a serious problem unless the tree produces juvenile sprouts. Explant from branch of 5 years old *Eucalyptus grandis* were all contaminated after surface sterilization while more than half of those taken from its juvenile sprouts were clean. Explant taken from current growth are some times easier to surface sterilize than similar explants from older section of the branch (Ahuja, 1993).

The contamination may be due to the presence of bacteria, fungi and yeast either on surface or internally in the plant tissues. The external contamination may be removed by surface sterilization without killing the plant tissue (Nudgauda, 1993). Plant tissue is sterilized by treating with disinfecting solution (Torres, 1988). The contamination of sterilizing agent and duration of the treatment varied with the plant material (Haendre, 1993). For this purpose, many surface sterilants such as calcium hypochloride, chlorine water, bleaching powder, mercuric chloride, hydrogen peroxide, ethylene oxide and ethanol are used. A general range of concentration and exposure time for these chemicals as suggested by Anonymous (1994) is given below:

Disinfectants	Chemical formula	Concentration (%)	Exposure time (min)
Calcium hypochloride	Ca OCl	9-10	5-30
Sodium hypochloride	Na OCl	0.5-5.0	5.-30
Hydrogen peroxide	H_2O_2	3-12	5.0-15.0
Ethyl alcohol	CH_3CH_2OH	70-95	0.1-5.0
Mercuric chloride	$Hg\ Cl_2$	0.1-1.0	2-10
Silver nitrate	$Ag\ No_3$	1.0	5.30
Benzyl konium chloride	$C_2H_{25}N(CH_3)_2{-}C_7H_7\,Cl$	0.01-0.1	5.20

Washing the plant material with clean water followed by distilled water at the beginning of sterilization process drastically reduced the infection (Hughes, 1981). The infectious contamination have been reported to be removed by placing the plant material in 70 per cent ethanol for few seconds, followed by 5.25 min. dip in 1 per cent NaOCl containing a few drops of Tween-20 as wetting agent to reduce the surface tension and washing 3-4 times with sterilized distilled water in *Monstera* (Fonnesbech and Fonnesbech, 1980), pear (Banno *et al.*, 1988; 1989; Dwivedi and Bist 1999) guava (Loh and Rao, 1989; Papadatou *et al.*, 1990; Khattak *et al.*, 1990; Parkash and Tiwari, 1996), jackfruit (Amin and

Jaiswal, 1988, Rajmohan and Mohanakumaran, 1988; Roy *et al.*, 1990; 1991; Singh and Tiwari, 1998), plum (Hammerschlag, 1982) and apple (Skirvin *et al.*, 1986).

Debergh and Maene (1981) sterilized the explants in $HgCl_2$ followed by NaOCl and washed the explants 3-4 times with sterilized distilled water. Ethanol (70%) and solution of $HgCl_2$ were toxic to coffee explants. On the other hand solution of Ca and Na hypochloride were effective and non-toxic (Sondahl *et al.*, 1986). Lane (1979) surface sterilized Bartlett pear by using 2.5 per cent sodium hypochloride. Singh and Tiwari (1998) used 1.5 per cent sodium hypochloride to sterilize jackfruit explants. Actively growing peach explants were successfully sterilized with 0.5 per cent and 0.01 per cent Tween-20 for 15 minutes followed by 100-200 ppm solution of penicillin and streptomycin for 15 minutes by Hammerschlag (1980).

Internal contamination also posed serious problems in woody plants and this problem has been overcome by adding 10 ppm benomyl to the culture medium or by treating the tissues with these before disinfection with commercial bleach as normal disinfection techniques failed to remove the internal contamination (Torres, 1988). It has been found that some-times bacterial contaminations are observed on the cultures at the time of establishment and later during proliferation mainly in woody plants (Zimmerman, 1985). James and Thurbon (1979) observed bacterial contamination even after several weeks on apple cultures. In most of the cases, the problem seems due to bacteria growing internally in the tissue (Debergh and Maene, 1984; Zimmerman, 1985). Culture indexing at the time of explant establishment is advisable, but some contaminants appear only after a number of subcultures. Maurice *et al.*, (1985) used Alcide and it has been mentioned to be an effective bactericidal product for *Phoenix dactylifera*. A technique using four antibiotics simultaneously has been reported in a number of different plant genera (Young *et al.*, 1984). Phillips *et al.* (1981) reported that Rifampicin at 50 μg/l alone was fully effective against contaminating bacteria without affecting the rate of cell division of *Helianthus tuberosus*. Studies done by Umiel and Golden (1976) already indicated that potential of antibiotics, particularly the aminoglycocides, to be phytotoxic, perhaps due to inhibition of protein synthesis. Phillips *et al.* (1981) observed that certain antibiotics were non toxic and effective. However, Debergh and Maene (1984) and Bastiaens *et al.* (1983) reported that the use of antibiotics never appear to be a solution for the problem. They can have a bacteriostatic effect, but they never were bactericidal.

Control of phenolics

Establishment of *in vitro* cultures of woody plants is greatly hampered by the browning of the explants and culture medium is generally considered to result from the oxidation of phenolic compounds released from the cut ends of the explants by polyphenol-oxidases (Mayer and Harel, 1979), peroxidase (Loomis and Battlaile, 1966) or air (Robinson 1983). The oxidized products, quinones are known to be highly reactive and inhibit enzyme activity leading to the death of explants (Hu and Wang, 1983).

Various techniques have been suggested either to neutralize or avoid the build up of toxic substance in the medium. These include (i) choice of juvenile explants or new growth flushes during the active growth period (Bon *et al.*, 1988), (ii) culture in dark (Pieper and Zimmer, 1976; Cassels and Minas, 1983, Leon-de-Sierralta *et al.*, 1997), (iii) transfer of explant to fresh medium at short intervals (Broome and Zimmerman, 1978; Lloyd and McCown , 1980 and Singh and Tiwari, 1998) (iv) culture in liquid medium (Ishii *et al.*, 1979) (v) inclusion of antioxidants in culture medium or soaking explants in water or solution containing antioxidants prior to inoculation (Gupta *et al.*, 1980; Ziv and Halwy, 1983; Dalsaso and Guevara, 1989; Siddiqui and Farooq, 1996), (vi) use of adsorbing agents such as activated charcoal, polyvinylpolypyrrolidone (PVPP) (Gupta *et al.*, 1980, Siddiqui and Farooq 1996; Loh and Rao, 1989; Amin and Jaiswal, 1988, Parkash and Tiwari, 1996; Singh and Tiwari, 1998), (vii) use of low salt medium and proper growth regulators (Lloyd and McCown, 1980; Hilderbrandt and Harney, 1988) and (viii) sealing the cut ends with paraffin wax.

Browning of cultures was overcome by the combined effect of reducing agents and/or antioxidants and frequent transfer of explants to fresh medium (Song *et al.*, 1988; Amin and Jaiswal, 1987; Cronaur and Krikorian, 1984 b;

Rathore *et al.*, 1992; Block and Lankes, 1995). The synthesis and oxidation of phenolic compounds are light induced (Creasey, 1968; Davies, 1972, Leon-de-Sierralta *et al.*, 1997) and these may remain active in explants grown under normal conditions of field irradiance. Phenolic oxidation causing loss to explant was minimized or eliminated when cultures of apple and grape were initiated *in vitro* either from dark-grown field stock plants or those exposed to low irradiance (Marks and Simpson, 1990); Yu and Meredith, 1986). Cherve *et al.*, (1983) found that the explants cut from tree at the beginning of the spring vegetative cycle had a lower content of inhibitory tanins. Browning was also prevented by placing the rinsed explant of guava in petridishes to dry in an air flow for 30-45 minutes so that non of the surface was moist when the explants were incubated (Fitchet, 1989).

Shoot proliferation

Media

Regeneration of explant from guava have been successfully achieved mainly in modified Murashige and Skoogs medium (Amin and Jaiswal, 1987; Jaiswal and Amin, 1987; Fitchet, 1989; Loh and Rao, 1989, Papadatou *et al.*, 1990; Siddiqui and Farooq, 1996; Mohamad *et al.*, 1995 and Parkash and Tiwari, 1996), jamun (Yadav *et al.*, 1990; Roy *et al.*, 1996; Roy *et al.*, 1996), ber (Yan *et al.*, 1990), litchi (Yu, 1991; Kantharajah *et al.*, 1997), strawberry (Petrovic and Jacimovic, 1990), Kiwi(Wiyaporn *et al.* 1990), pomegranate (Mahishni *et al.*

1991), jackfruit (Rahman and Blake, 1988; Roy *et al.* 1990; Singh and Tiwari, 1998), banana (Bower and Fraser, 1982; Cronaur and Krikorian, 1984a; Bayona, 1991; Silayoi and Kaewsompong, 1992; Swamy *et al.*, 1991), *Citrus* (Barless and Skene, 1982; Pontikis and Sapontzaki, 1984; Pasqual and Ando, 1989, Rahaman *et al.*, 1992; Liu *et al.*, 1991; Singh *et al.*, 1994).

There is no single medium to suit every crop. Therefore, in past two-three decades a large number of reports have appeared on modification of about two dozens basic media compositions (Street and Shillito, 1997; Pierik, 1987; Torres 1988). The MS (Murashige and Skoog, 1962) or LS (Linsmaier and Skoog 1965) have been found satisfactory especially in procedure where plant regeneration is the object. The Driver Kuniyuki Walnut medium (DKW) (Driver and Kuniyuki, 1984) and the WPM medium (Lloyd and McCown, 1980) are used widely for culture of woody species. It is often possible to use same medium for culture initiation and shoot multiplication.

Plant bio-regulators

For obtaining maximum proliferation and growth of shoots per culture different plant bioregulator concentrations have been reported by different workers. It was observed by Amin and Jaiswal (1987) and Jaiswal and Amin (1987) that concentration of 4.5 μM BA alone produced 3-6 shoots in 12 weeks *in vitro* of guava explant. Loh and Rao (1989) obtained 3.2 shoots/segment after 8 weeks in MS medium with 0.5 mg l^{-1} BA in guava. Maximum proliferation in guava was achieved with 2 mg l^{-1} BA (Papadatou *et al.,* 1990). Parkash and Tiwari (1996) reported that better shoot proliferation in guava with BA (1 mg l^{-1}) + IBA (0.2 mg l^{-1}). Yadav *et al.* (1990) obtained multiple shoots from nodal and shoot tip segments of 10-15 days old seedlings of *Syzygium cuminii* on modified MS medium supplemented with BA (0.23-8.9 μM) individually or in combination with NAA, IAA or IBA. Excised shoots were placed were again transferred for further multiplication in same medium and gave multiple shoots. Rathore *et al.* (1992) developed protocols for cloning of *Zizyphus nummnlaria* and *Z. mauritiana* from nodal explants. Five to seven shoots proliferated on MS medium containing BAP 5.0 mg l^{-1} + IAA 0.05mg l^{-1} while on MS + 7.5 mg l^{-1} BAP + 0.1 mg l^{-1} IAA only four to five shoots were produced. Goyal and Arya (1985) described methods for multiplication of *Z. mauritiana* (cv. Gola) but numbers of shoots induced were less.

Roy *et al.*, (1990) cultured nodal explant of *Artocarpus heterophyllus* on MS medium with 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} Kinetin and developed 5-7 new shoots. Rahman and Blake (1988) observed shoot proliferation in jackfruit in a cytokinin medium and rhizogenesis in an auxin supplemented medium. Singh and Tiwari (1998) used axillary buds of jackfruit on MS medium supplemented with 18 mg l^{-1} BA in combination with 0.2 mg l^{-1} IBA. Highest number (4-5) of usable shoots were developed after 4 subcultures on medium supplemented with 2 mg l^{-1}, BA and 0.2 mg l^{-1}, IBA. Higher concentration of BA in shoot proliferation medium produced increase number of adventitious shoots.

Yadav *et al.* (1990) found better shoot multiplication of mulberry with benzylaminopurine (BAP) than kinetin. Cronaur and Krikorian (1984a) established culture of desert banana and plantain clones on a modified MS medium supplemented with 5.0 mg l^{-1} BA. Jyothi *et al.* (1993) cultured banana shoot tip, eye bud and floral explants and obtained 9-12 per explants on medium supplemented with 100 ppm BA regardless of NAA concentration. Silayoi and Kaewsompong (1992) cultured fourteen cultivars with differing genome (4 AA, 5AAA, 2 AAB, 2ABB and 1 ABBB) on MS medium supplemented with 15% coconut milk and 5 mg benzyladenine/litre. Number of plantlets produced per explant did not differ among genomes but cultivars with AA, AAA and AAB genomes generally produced plantlets over shorter period than those with ABB and ABBB genomes.

Rahaman *et al.* (1992) cultured shoot apices of papaya cv. Rajshahi Red on MS medium supplemented with different concentrations and combinations of BAP and NAA. After 6 weeks of culture, successfully established explants were transferred to MS medium supplemented with BA, kinetin or isopentenyladenosine (0.5 or 1 mg l^{-1}) and NAA (0.1 mg l^{-1}), with or without GA_3 (0.5 mg l^{-1}) for shoot multiplication. The survival rate of shoot apices increased with increasing concentration of BA and NAA. Greatest percentage of multiple shoot formation was obtained with 10 mg BA + 1 mg NAA/litre. The addition of GA_3 to multiplication medium resulted in better shoot growth.

Kumar *et al.* (1992) observed the effect of different auxins viz., IBA, NAA and 2,4-D added to the basal medium on callus differentiation in *Carica papaya, Carica monoica* and medium also containing kinetin, 0.5 mg l^{-1}. Shoot induction was obtained from the callus when medium containing various combinations of NAA + kinein or 1BA + kinetin. Chawdhury (1991) cultured apical and lateral shoots of papaya on MS medium containing all combinations of NAA at 0.186 or 0.372 mg l^{-1} and BA at 0.113, 0.225, 0.452 or 0.904 mg l^{-1}. The best combination for shoot development was 0.186 mg NAA + 0.225 mg BA/litre but shoots did not elongate sufficiently for nodal explants to be available for mass propagation unless 1 mg GA_3 per litre was included in culture medium.

Werner and Boe (1980) and Snir and Erez (1980) used MS medium for shoot proliferation. The BAP has been the most commonly used cytokinin to induce shoot proliferation in apple (Hutchinson, 1985; Lundergan and Janick, 1980). Snir and Erez (1980) used 1 mg l^{-1} BAP and 1 mg l^{-1} IAA for shoot proliferation in many apple rootstocks. The PBRs concentration ranging 1-2 mg l^{-1} BAP and 0.1-0.5 mg l^{-1} IBA was found most favourable for shoot multiplication in apple (James and Thurbon, 1981). Kader *et al.* (1992) found optimal concentration of different PBRs for shoot proliferation for vegetative axillary buds of apple varieties, Granny Smith, Spur Golden Delicious and Mutsu as 1 mg. 0.5 mg and 0.7 mg BA/l, respectively. Lee *et al.* (1990) reported that the best shoot multiplication of apple explant was obtained with 0.5 mg NAA +

2.0 mg BA/l while Ma *et al.* (1990) found 1.0-1.5 mg BA + 0.5 mg IBA/litre optimum concentration of PBRs for shoot proliferation of apple. Schuch and Peters (1993) obtained lighter number of shoots/explant of apple rootstock cv Marubakaido and scion cultivar Megumi on MS medium with 2.0 mg BA/litre with or without GA_3. Turovskaya (1994) obtained better shoot proliferation of rootstock in MS medium with 2.0 – 3.0 mg BA/litre for stock 62-396 and 1-3 mg BA/litre for stock 2-46-77 and 54-118.

Banno *et al.* (1989) recommended 1.0 mg l^{-1} BA and 0.1-0.5 mg l^{-1} IBA for the optimum shoot growth and proliferation of pear cultivars. Shibli *et al.* (1997) reported that successful shoot proliferation of pear (*Pyrus syriaca* – a potential rootstock) can be obtained on MS medium supplemented with 1.5 mg BA/litre. Chiariotti and Antonelli (1988) reported that BA of 5.0 mg l^{-1} induced vitrification in peach and could be needed only for one sub-culture. To obtain a higher proliferation rate in following subculture, lower BA concentration (1.4 and 2.8 mg l^{-1}) had to be used. The optimum concentration for culturing stem explant was 1.0-1.5 mg l^{-1} BA and 0.5 mg l^{-1} IBA. Higher concentrations caused vitrified shoots (Ma *et al.*, 1990).

Islam *et al.* (1993) developed a protocol for organogenesis from leaf explants of *in vitro* grown seedlings of *Aegle marmelos* and adventitious buds were initiated on MS medium containing various concentrations of BA and IAA. The maximum frequency of shoot organogenesis (61.2%) and the highest number of shoots per explant (38.4%) were obtained when 1.5 mg BA and 0.5 mg IAA per litre were used. They also reported that when mature seeds were cultured on MS medium supplemented with 0-5 mg BA/litre, 1-2 mg BA + 0.1 mg IAA per litre increased shoot proliferation. Hossain *et al.* (1994) cultured cotyledon of *Aegle marmelos* seedling on MS medium supplemented with 20 mg BA + 0.2 mg IAA per litre found optimal for shoot regeneration efficiency.

The first attempt to regenerate mango plant from callus culture was made by Rao *et al.* (1982). They achieved the regeneration of only roots from callus of mature cotyledons. Litz *et al.* (1982; 1984) reported somatic embryogenesis in five polyembryonic cultivars and two other cultivars. However, most of the somatic embryos did not mature and plantlets ceased to grow to a transplantable size (Litz, 1986).

Grinblat (1972) observed that concentration of 10 mg l^{-1} BAP, 0.1 mg l^{-1} NAA and 500 mg l^{-1} malt extract gave maximum initiation of buds from citrus stem explant *in vitro*. Similarly, Kitto and Young (1981) reported that basal medium supplemented with BAP 5 mg l^{-1} was best for shoot multiplication of Carrizo citrange. Barless and Skene (1982) observed maximum shoot production from both juvenile and mature nodes and induced adventitious shoot formation from juvenile internodes by use of 10 μM BA/litre. Pontikis and Sapontzaki (1984) obtained rapid propagation by culturing shoot tips of Troyer citrange on MS medium supplemented with 0.5 mg BA + 0.1 mg IBA + 1 mg GA_3 per litre.

Petrovic and Jacimovic (1990) cultured apical meristem of strawberry on MS medium supplemented with 1.0 mg BA + 0.1 mg/IBA and 0.1 mg GA_3 per litre for leaf rosette development and 1.0 mg BA and 1.0 mg IBA for shoot multiplication. Wongkaew *et al.* (1991) observed maximum shoot proliferation from leaf primordia, shoot apices and seedlings of date palm when MS medium was supplemented with 1.0 mg NAA + 10 mg kinetin per litre. Jin *et al.* (1992) achieved rapid proliferation of the axillary buds of raspberry (*Rubus hirsutus, Rubus lambertiamus*) on MS medium containing GA_3 and kinetin. GA_3 was essential for proliferation for *Rubus* species. In black berry, BA (0.5-3.0 mg l^{-1}) induced better shoot proliferation than kinetin (0.5-3.0 mg l^{-1}), and similar results were obtained with black raspberry (Upadyshev and Vysotskii, 1991). Karakullukchu *et al.* (1993) cultured shoot tip of raspberry cv. Schonemann on MS media containing IBA and BA, 0.01 or 0.5 mg l^{-1} and 0.5 mg l^{-1}, respectively gave highest rate of establishment. Shoot proliferation was greatest on media containing 0.5 mg IBA + 0.5 mg BA/litre.

Yu (1991) reported that when shoot segment of litchi was cultured on half strength MS medium with addition of 1.0 mg BA, 0.2 mg IAA and 0.6 mg GA_3/litre, the highest regeneration rate in the segments was born. Sharma *et al.* (1992) propagated cherry rootstock-Colt *in vitro* and got multiple shoots on MS medium supplemented with 0.5 mg l^{-1} each of BA and kinetin together. Ma *et al.* (1992) obtained on an average 4.7 proliferated shoots of *Ribes nigrum* when MS medium was supplemented with 1-2 mg BA and 0.1 mg IAA/litre. Good shoot regeneration of avocado was obtained with 2.0 mg BA/litre + 0.5 mg IBA (Dalsaso and Guevara, 1989).

Root induction

Media

In vitro derived shoots are induced to produce roots during the stage III which is generally one generation length and lasts 2-4 weeks during which the plantlets are rooted and conditioned to certain degree (Upreti, 1993). There is no single medium as well as same constituent of medium to suit every stage of *in vitro* cloning e.g., medium that was used for establishment and suit proliferation may or may not be suitable for root induction. In most of the cases, half strength MS medium was used for rooting of microcutting in many fruit corps like guava (Amin and Jaiswal, 1993). Papadatou *et al.* (1990) used Rugini olive medium, pH was adjusted to 5.2 for induction of roots in axillary shoots of guava. Some times activated charcoal was also used for enhanced rooting percentage in guava (Jaiswal and Amin, 1987; Amin and Jaiswal, 1987; Mohamed *et al.*, 1995; Parkash and Tiwari 1996), jamun (Yadav *et al.*, 1990), papaya (Pandey and Rajeevan, 1987) and pear (Dwivedi and Bist, 1999). Concentration of sugar and agar also varies in root inducing media from crop to crop as 1.5 per cent sucrose + 0.6 per cent agar was found better in guava (Papudatou *et al.*, 1990), and apple (Liu *et al.*, 1991).

Plant bioregulators

Among various auxins, NAA was found to be highly effective for rooting (Ancora *et al.*, 1991; Kitto and Young, 1981 and Vinterhalter and Vinterhalter, 1992) but some workers reported IBA as most effective for rooting (Capuana and Giannini, 1997). In an experiment with axillary shoots of *Castenea sativa*, IBA produced longer, thinner and more fibrous roots than IAA (Vieitez and Vieitez, 1983). Since developing shoots are rich source of auxin, and addition of auxin is not required in many species for *in vitro* rooting such as Narcissus (Seabrook *et al.*, 1976). The suitability of auxin for rooting has also been reported by various workers in different crops like apple (Zimmerman, 1988), pear (Lane, 1979; Dwivedi and Bist, 1999), guava (Amin and Jaiswal, 1988; Papadatou *et al.*, 1990; Parkash and Tiwari, 1996) and jackfruit (Singh and Tiwari, 1998). Auxins most frequently incorporated into medium to induce rooting are IAA, NAA, 2,4-D and IBA. The response of shoots to these auxins and the formation of roots in their presence is species dependant (Torres, 1988). Root initiation was not influenced by salt concentration but root growth was enhanced when salt content of the rooting media was reduced (Harris and Stevenson, 1979). Sriskandarajah *et al.* (1982) induced adventitious rooting *in vitro* in difficult to root cultivars of apple by the use of liquid medium. James and Thurbon (1979) observed that microcuttings of M-9 apple rootstock rooted well when shoots cultured in IBA containing medium upto four days and then transferred to hormone free media. This procedure prevented callus formation and led to a three fold increase in root number per culture compared to continuous contact with hormone. Al-Maarri *et al.* (1986) obtained best rooting with 0.1 mg l^{-1} NAA in quince. The optimum concentration for rooting *in vitro* for apple was 0.3 mg l^{-1} or 0.3 mg IBA + 0.1 mg NAA or 0.15 mg NAA/l (Liu *et al.*, 1991).

Amin and Jaiswal (1990) found that optimum concentration of IBA and NAA in half MS medium forced rooting from proliferation stage of guava. Amin and Jaiswal (1987) obtained adventitious rooting after subculturing excised shoots of guava on half MS medium supplemented with 1 μM each of the IBA and NAA. Shoots produced from guava explants were rooted in media containing 9.8 μM IBA (Mohamed *et al.*, 1995). Parkash and Tiwari (1996) obtained best rooting when half strength MS media was supplemented with 0.2 + 0.2 mg l^{-1} NAA + IBA and incubation of micro-cuttings for one week in dark resulted in early and better rooting. *In vitro* grown microshoots of jackfruit rooted in half MS medium containing 1.0 mg l^{-1} IBA with 58.7% rooting frequency (Singh and Tiwari, 1998). Amin and Jaiswal (1993) achieved 60-80% rooted microshoots of jackfruit on ½ MS medium containing 10 μM IBA or NAA. Rooting of excised shoots of jackfruit was achieved on half strength MS medium supplemented with NAA and IBA each at 1.0 mg l^{-1} (Roy *et al.*, 1996). Islam *et al.*, (1993) obtained better rooted plants when medium was supplemented with 0.1 mg IBA/litre in case *Aegle marmelos*. Islam *et al.* (1996)

reported that adventitious shoots of bael (*Aegle marmelos*) rooted well when MS medium was supplemented with IBA. *In vitro* raised shoots of jamun (*Syzygium cumini* L.) were rooted with half strength MS medium containing 0.5 mg l^{-1} each of IBA, NAA and IAA (Roy *et al.*, 1996). Studies of 4 cultivars of peach for rooting *in vitro* conducted by Kornova (1995) who found that rooting was successful with auxin: IBA and IAA at 1 mg and 2 mg l^{-1} respectively for Fantasia, IBA at 2 mg l^{-1} for Redhaven and Andros; IBA at 1 mg l^{-1} for Aheloy. Elongated shoots of kiwi fruit were rooted when medium was supplemented with 0.5 mg IBA + 0.5 NAA and 3 g activated charcoal (Wiyaporn *et al.*, 1990). Morini *et al.* (1990) obtained highest rooting in myrobalan plum in MS medium containing 0.5 mg IBA/litre while Aier and Sharma (1990) obtained rooting in plum by 1.0 mg IBA/litre. Wongkaew *et al.* (1991) achieved better root development in proliferated shoots of date palm with MS medium containing 0.1 mg NAA/l.

Hussain *et al.* (1998) developed protocol for mango cv. Amrapalli. IBA was the most potent among the 3 auxins (IBA, IAA and NAA) tested for promoting efficient rooting and growth. The best rooting (89.71%) was observed on auxin free agar medium after a 24 hours pulse treatment with 5 mg IBA/litre in liquid root induction medium in dark.

Petrovic and Jacimovic (1990) propagated *in vitro* to the strawberry cv. Senga Sengana and best rooting was achieved by IBA at 0.5 mg l^{-1} concentration in the medium. Lopez *et al.* (1994) conducted *in vitro* culture experiment with strawberry and reported that addition of 500 mg activated charcoal/litre enhanced rooting. In contrast, high BA concentration during multiplication decreased the rooting capacity of shoots. Kumar *et al.* (1992) obtained rooting from papaya callus when it was transferred to medium containing various combinations of NAA + Kinetin or IBA + Kinetin. Root was induced from all shoots obtained from papaya callus in half strength modified MS medium containing 20 mg IBA per litre (Mondal *et al.*, 1994). Microcuttings dipped in 12.3 mm IBA and then cultured in a medium of distilled water plus 1 x 10^{-2} kg agar/m^3 produced normal roots without any callus farming at the basal end (Teo and Chan, 1994). Rahaman *et al.* (1992) obtained better root induction in papaya microshoots when they were transferred to half strength MS medium containing 1 mg IBA/litre.

Chaturvedi and Mitra (1974) were first to produce roots from callus regulated excised shoots of *C. grandis* (L.) osbeck and *Citrus sinensis* (L) Osbeck in a treatment containing 0.5 mg l^{-1} NAA. Root primordia were visible after 10-30 days of incubation. Kitto and Young (1981) performed rooting studies on Carrizo citrange and reported that enhanced rooting (80% rooting; 10.2 roots per shoots) with NAA as compared to IBA or IAA supplemented to Murashige and Tucher media. According to Barless and Skene (1982), *in vitro* grown citrus shoots exhibited a requirement for high level of exogenous auxin (upto 10 μM% NAA) to achieve rooting. Edriss and Burger (1984) reported a system providing a rapid method of propagation of Troyer citrange where MS media supplemented with 2 mg l^{-1} NAA was used to root the *in vitro* regenerated shoots. Duran Vila *et al.* (1989) stated that optimal concentration of NAA to induce root formation on stem segments was 10 mg l^{-1} for sweet orange and lime while it was 3 mg l^{-1} for citron Research carried out by Lukman *et al.* (1990) on *in vitro* derived shoots of Troyer citronge indicate that NAA did not promote rooting.

Hardening of rooted microcutting

Success in micropropagation requires that shoot or plantlets that have been growing heterotrophically under condition of very high humidity, to become autotrophic and to grow under condition of moderate to low humidity. Change in both structure and physiology of shoots occur during this period of adaptation (Zimmerman, 1988). Factors that should be considered during transplanting of shootlets are infection and desiccation and several factors including lack of sufficient epicuticular wax and abnormal stomata on the leaf result in excessive dehydration and poor control of gas exchange. So, there should be gradual change in humidity and light intensity environment (Hartmann *et al.* 1997). Murashige (1974) reported that high light intensity before transplanting is beneficial but survival was not increased. Subjecting plantlets above a certain level of light intensity, light above 600 lux, was found to be detrimental for establishing papaya plantlets in soil (Pandey and Rajeevan,1987). During acclimatization, humidity is greatly reduced over period of 2-3 weeks during which the plants undergo morphological and physiological adaptations enabling them to develop typical terrestrial plant water control (Brainerd and Fuchigami, 1981).

Laszloffy *et al.* (1992) studied the *in vitro* propagation of July red apple. *In vitro* plantlets were acclimatized to greenhouse conditions by transferring the plantlets to sterile perlite soaked with ¼ MS salts and 0.5 per cent sucrose for four weeks prior to transferring to greenhouse. Singha (1980) hardened the micro-propagated pear plants by covering with beaker under room conditions prior to transferring them to the field. Lane (1978); Snir and Erez (1980) and Werner and Boe (1980) also reported the successful transferring of apple plantlets to soil but provided no details of survival rates. Cheng (1978) got success in the establishment of plantlets under mist by creating a finest thin water film over the plantlets.

Pandey and Rajeevan (1987) reported eighty per cent survival of plantlets when they were transplanted 30 days after root initiation to 1:1:1 mixture of sand, soil and cowdung in papaya.

Singh *et al.* (1994) transferred shoots of *Citrus* species to MS inorganics and 1% agar after 5 weeks, and after 8 weeks *in vitro* grown plantlets were thoroughly washed with tap water to remove agar from the shoots which were then soaked in 0.2% bavistin, a fungicides for 5-10 minutes. The plantlets were potted in a sterilized mixture of sand and soil (1:1). They were initially irrigated with half MS solution for one week and subsequently with water. The plants were acclimatized at 27 $\pm$ 2°C with a 16 hrs photoperiod for 3-4 weeks.

Polythene cover was used to ensure high humidity around plants. About 60% shoots/plantlets were established in soil. Dwivedi and Bist (1999) reported that the rooted plantlets survived for 15 days in plastic pot with a mixture of sand, soil and FYM in a ratio of 1:1:1 in open room condition at 24 $\pm$ 2°C temperature at 16/8 hr photoperiod. Rooted shootlets of jackfruit were subjected to hardening treatments and they readily established (80%) in sterilized mixture of sand, soil and farm yard manure (Singh and Tiwari, 1998).

Amin and Jaiswal (1990) reported that for transferring of guava rooted plantlets to the soil, plantlets were taken out of culture tubes, washed thoroughly to remove any remaining medium, and planted in small plastic pots filled with garden soil and compost (1:1). During the first 7-10 days, the potted plantlets were covered with glass beakers to provide high humidity. Parkash and Tiwari (1996) transferred rooted plantlets for hardening treatments, 30 days after rooting and 86% of survival was recorded in the pot containing a mixture of sand, soil and FYM (1:1:1).

MATERIALS AND METHODS

MATERIALS AND METHODS

The present investigation was carried out in Tissue culture laboratory of the Department of Horticulture, College of Agriculture, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, District Udham Singh Nagar (Uttaranchal) during the year 1998-2000. The explants were collected from the trees of chinese guava (*Psidium friedrichsthalianum* (Berg.) Nierdz.) grown at the Garden Section of this university. The materials and methods followed during the course of experimentation are presented in this chapter under the following head.

Medium

Modified M.S. medium (Murashige and Skoog, 1962) was used during the study. The composition of the medium is given below:

Compounds	**Quantity ($mg\ l^{-1}$)**	**Volume (ml) drawn for one litre**	
		MS	½ MS
A. Major inorganic nutrients (20 x)			
KNO_3	38,000		
NH_4NO_3	38,000	50	25
KH_2PO_4	3,400		
$Mg\ SO_4.\ 7H_2O$	7,400		
$CaCl_2.\ 2H_2O$	8,800		
B. Minor inorganic Nutrients (200 X)			
$ZnSO_4$	2,120		

KI	166		
H_3BO_3	1,240		
$MnSO_4.H_2O$	3,380		
$Na_2MoO_4.2H_2O$	50		
Cu $SO_4.5H_2O$	5	5	2.5
$CoCl_2.6H_2O$	5		
C. Iron EDTA (200 X)			
Na_2 EDTA	7,460	5	2.5
$FeSO_4.7H_2O$	5,560		
D. Vitamins (200 X)			
Pyridoxine HCl	100		
Nicotinic Acid	100	5	2.5
Thiamine HCl	100		
Glycine	400		
Myoinositol	20,000		
E. Other constituents			
Sucrose	30,000		
Agar	7,000		
PH	5.7 ± 0.1		

pH was adjusted with 0.1N NaOH and 0.1N HCl

Cleaning of glassware

Glassware were treated with chromic acid solution for six hours followed by thoroughly washing with running tap water and then washed with liquid detergent 'Teepol' and rinsed with tap water. These were finally rinsed with double distilled water. The washed glassware were oven dried for about three hours at 120°C and stored in dust free cupboards.

Preparation of stock solution

Media

Modified MS medium was used for the experimentation. It contained macro and micro nutrients with vitamins, sucrose and agar in different specified proportions. For the preparation of stock solutions, appropriate amount of each chemical was transferred to volumetric flask after dissolving them separately in a beaker with a few millilitres of distilled water and then the required volume was made-up by adding distilled water. For the preparation of Fe EDTA, stock solution Na_2 EDTA and $FeSO_4.7H_2O$ were dissolved separately in 100 ml of distilled water. Then Na_2 EDTA solution was boiled and added to $FeSO_4.7H_2O$ and volume was made up to mark.

Plant bioregulators

The basal medium was supplemented with varying concentrations of auxins (IBA, NAA) and cytokinin (BAP). Stock solutions of plant bioregulators was in a few ml of 0.1N NaOH (for IBA and NAA) or 0.1N HCl (for BAP) in 100 ml volumetric flask and then volume was made up by adding sufficient amount of distilled water as given in following table (Table 2).

Table 2. Preparation of stock solutions for plant bioregulator

Plant bioregulators	Quantity taken (mg)	Initially dissolved in	Final volume (ml)	Strength (mg/ml)
Naphthalene acetic acid (NAA)	100	1 ml 0.1N NaOH or KOH	100	1
Indole-3-butyric acid (IBA)	100	-do-	100	1
6 Benzyl adenine (BA)	100	1 ml 0.1N HCl	100	1
Gibberellic acid (GA_3)	100	1 ml 0.1N NaOH or KOH	100	1

Sterilization

Media

The required quantities of the prepared hot medium was poured in 2.5 x 15 cm culture tubes or 100, 150 or 250 ml conical flask. After plugging tightly with cotton plug, prepared by packing non-absorbent cotton in muslin cloth, the medium was sterilized in an autoclave operated under steam pressure of 1.02 kg/cm^2 pressure and timing was adjusted as per the volume of medium as given in Table 3.

Table 3. Minimum autoclaving time for plant tissue culture media (Anon., 1994)

Volume of medium per vessel (ml)	Minimum autoclaving time (minutes)
25	20
50	25
100	28
250	31
500	35
1000	40

Instrument box

The instrument box containing 2 pairs of petriplates, 4 beakers (250 ml), 2 forceps, scalpel with surgical blade, filter paper, cotton wrapped in aluminium foil were steam sterilized at 1.02 kg/cm^2 pressure for the period of 20 minutes.

Laminar air flow cabinet

All the aseptic manipulations like surface sterilization, preparation and inoculation of explants and subsequent subculturing were carried out under the hood of clean air flow cabinet.

Plate 1: A field grown chinese guava tree

The working platform of laminar air flow cabinet was surface sterilized by wiping the open surfaces with 96 per cent ethanol. Thereafter, all the sterilized materials to be used (except the living plant tissues) were kept inside the chamber and exposed to U.V. light for 30 minutes. The laminar airflow device was switched on 10 minutes prior to inoculation or subculturing.

Culture room

The culture room used for incubating the cultures was maintained at temperature of 25 $\pm$ 2°C which was regulated by room air conditioner or thermostatically controlled heating system as per requirement. Fluorescent light tubes were fixed for supplying 3000-3200 lux light intensity for 16 hours and 8 hours as a dark period.

Stages of micropropagation

Stock plant selection and maintenance

The stock plant of chinese guava (*Psidium friedrichsthalianum* (Berg.) Nierdz.) was selected from the garden section. The plant received uniform cultural treatments during the course of investigation. The plant was treated with insecticides and pesticides as and when required and the explants (shoot tips) were collected from this plant.

Culture establishment

Explant collection

Shoot tips (2.0-2.5 cm long) were collected from new growth. These were collected in a 250 ml conical flask containing 100 ml distilled water + 2 ml Tween-20 + 1 mg Bavistin + 1 mg streptomycin and brought to the laboratory. Shoot tips were washed gently with liquid detergent and kept under running tap water for 50-60 minutes.

Sterilization of explant

The washed explants were brought to sterilized working Laminar flow cabinet for surface sterilization treatments (Table 4). The explants were treated with 70% ethyl alcohol for 30 seconds and then washed with sterilized distilled water. One minute dip in 1 per cent autoclave KCl was given to shoots to remove excess Hg^{++} ions. The observations on the per cent aseptic culture and percentage bacterial and fungal contaminations, dead and survival were recorded at 21 and 28 days intervals.

Table 4. Details showing sterilization treatments for explants

Serial Number	Exposure time			
	Ethanol (70%) (sec)	$HgCl_2$ (0.1%) (min)	NaOCl (1%) (min)	KCl (1%) (min)
1.	Control	-	-	-
2.	30	-	-	-
3.	30	4	-	1
4.	30	-	4	-
5.	30	4	4	1
6.	30	4	6	1
7.	30	5	6	1
8.	30	5	8	1
9.	30	6	8	1
10.	30	6	10	1
11.	30	8	10	1

Medium for shoot proliferation

The modified MS medium supplemented with varying concentrations of BA and IBA were tried in combination for proliferation as given below:

i) BAP : 0.0, 1.0 , 1.5, 2.0, 2.5, 3.0 mg l^{-1}

ii) IBA : 0.0, 0.1, 0.2, 0.3 mg l^{-1}

Explant inoculation

After surface sterilization, explants were placed on the modified MS medium containing different concentrations of BA and IBA. Cultures were kept under fluorescent light intensity of 3000-3200 lux with 16 and 8 hours light and dark periods, respectively at a temperature of 25 $\pm$ 2°C and 80% relative humidity. The observations on fungal and bacterial contaminations, browning and sprouting of explants were recorded.

Control of phenolics

The cultures were initially exposed to various illumination levels viz., no light, 1000 lux and 3200 lux for 24 hours, 48 hours and 72 hours. After this initial treatment, all the cultures were given an illumination of 3200 lux with 16/8 hours light-dark photoperiod.

Table 5. Details of initial exposure of varying levels of light intensity to control phenols

Light condition	Time in (hours)
Complete dark	24
Complete dark	48
Complete dark	72
Light 1000 lux	24
Light 1000 lux	48
Light 1000 lux	72
Light 3200 lux	24
Light 3200 lux	48
Light 3200 lux	72

In case of media additives, excised shoot tips were cultured on the establishment medium fortified with antioxidants or phenol binding agents at various concentrations. These include PVP, PVPP, citric acid, ascorbic acid and activated charcoal. The details are given below in Table 6.

Table 6. Details of various phenol binding and antioxidant chemicals for controlling the browning of explant.

Antioxidants/phenol binding compounds	Concentration (per litre)
Control	-
Polyvinyl pyrrolidone (PVP)	1 g
	2 g
	3 g
Polyvinyl polypyrrolidone (PVPP)	1 g
	2 g
	3 g
Citric Acid	100 mg
	500 mg
	1000 mg
Ascorbic Acid	100 mg
	250 mg
	500 mg
Activated Charcoal	250 mg
	500 mg
	1000 mg

Proliferation

After a period of 2-3 weeks, the growing culture was transferred to proliferation medium containing BA and IBA for rapid proliferation and were subcultured at 3 weeks interval.

Root induction

Proliferated shootlets of at least 2 cm length were placed in the half strength MS media for rooting containing different levels of IBA and NAA in combination in addition to 3 per cent sucrose, 0.6% agar and activated charcoal 0.1%. These were kept in dark for a period of 7 days.

Acclimatization

Sand soil and decomposed farmyard manure were mixed and autoclaved for 2 hours at 1.02 kg/cm^2. This autoclaved mixture of sand, soil and farmyard manure (1:1:1) was filled in clean pots. The plantlets were taken out of culture and washed gently with water to remove medium and to avoid microbial infection. One plant was transplanted in each pot. After thorough watering with hand sprayer, the pots were covered with transparent beakers to maintain high humidity. After 5 days, the beakers were removed and humidity was maintained at 75%. The humidity was further reduced gradually to harden the plants. After 15 days, the potted plants were transferred to green house and later on to nursery.

Statistical analysis

The data were analysed according to the procedure of analysis of variance for completely randomized design (Snedecor and Cochran, 1988). There were 10 cultures in each treatment and each treatment was replicated thrice. The significant of variance among the treatments were observed by applying 'F' test and CD at 5 per cent level of significance was calculated to compare the treatment means for all the characters. The per cent data were transformed angularily before the statistical analysis. The results are presented with the help of tables, graphs and photographs.

RESULTS

RESULTS

The results obtained during the course of investigation in different sets of experiments are being presented in this section under different heads.

Effect of exposure time of surface sterilants.

Aseptic culture

Data on the effect of surface sterilants and their exposure time on the extent of aseptic culture are presented in Table 7 and Fig 1. The data indicate that for obtaining aseptic culture, surface sterilization was necessary. None of the cultures were found aseptic when sterilants were not applied. Thirty second exposure to 70% ethanol alone was also not sufficient. Sequential exposure to more than one sterilant was required for better results. With constant exposure of explant to 70% ethanol for 30 second and increase in the exposure time of other sterilants viz., 0.1% $HgCl_2$ and 1% NaOCl resulted in higher percentage of aseptic culture. Maximum percentage (93.33) of aseptic culture was obtained when the explants were exposed to $HgCl_2$ for 8 minutes and NaOCl for 10 minutes. Sterilization procedure was also accompanied with rinsing in 70 per cent ethanol and 1 per cent KCl for all the treatments except control.

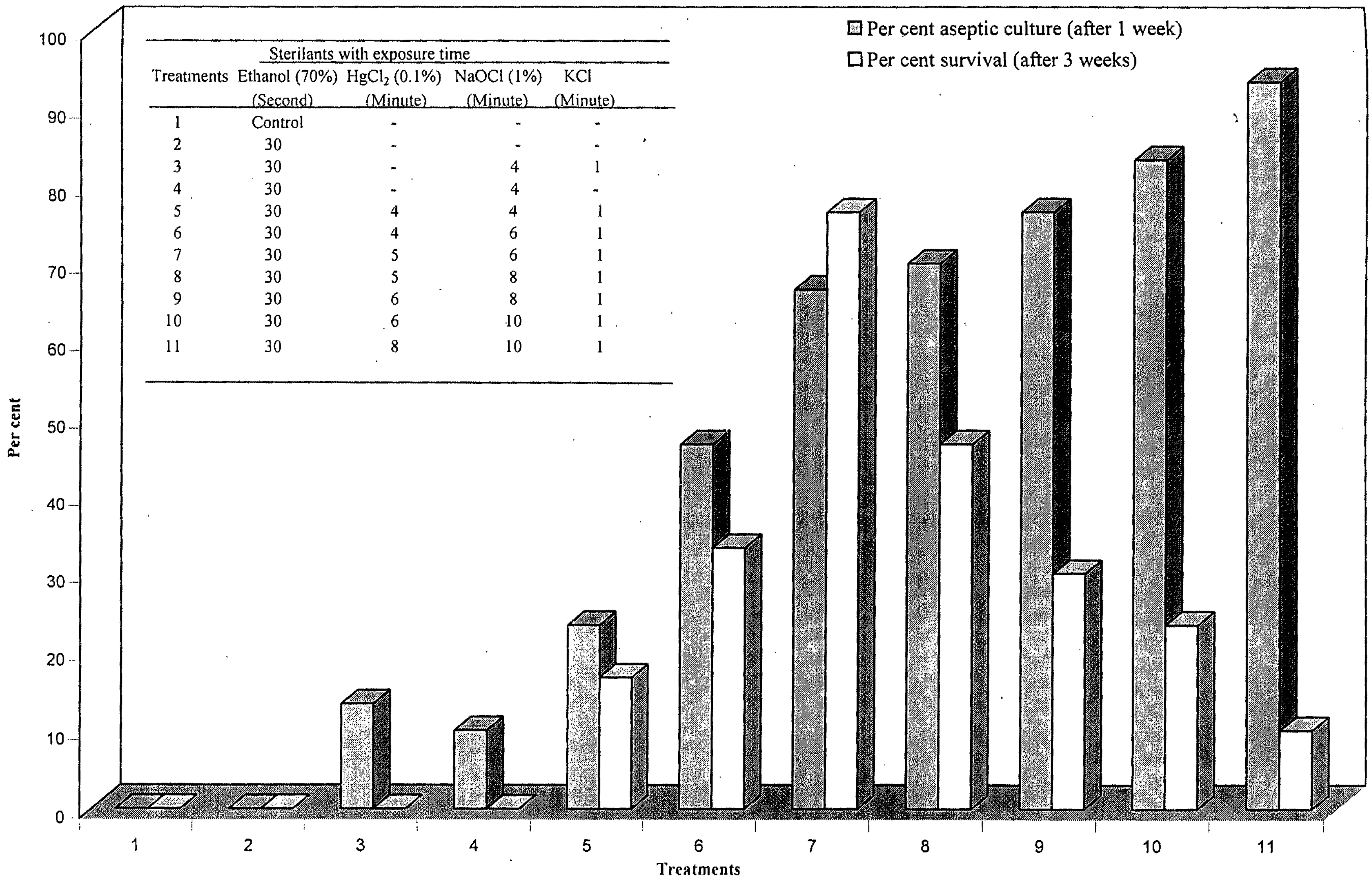
Survival of explant

Data presented in Table 7 and Fig. 1 reveal that increased time of exposure to surface sterilants increased the per cent survival of explants upto a

Table 7: Effect of surface sterilants on aseptic culture and survival of explants

Treatments	Sterilants with exposure time				Per cent aseptic culture (after one week)		Per cent survival of explant (after 3 weeks)	
	Ethanol (70%) (Second)	$HgCl_2$ (0.1%) (Minute)	NaOCl (1%) (Minute)	KCl (Minutes)				
1	Control	-	-	-	0.00	(0.00)	0.00	(0.00)
2	30	-	-	-	0.00	(0.00)	0.00	(0.00)
3	30	-	4	1	13.33	(21.14)	0.00	(0.00)
4	30	-	4	-	10.00	(18.43)	0.00	(0.00)
5	30	4	4	1	23.33	(28.78)	16.67	(23.85)
6	30	4	6	1	46.67	(43.07)	33.33	(35.21)
7	30	5	6	1	66.66	(54.78)	76.66	(61.22)
8	30	5	8	1	70.00	(56.78)	46.67	(43.08)
9	30	6	8	1	76.66	(61.22)	30.00	(33.21)
10	30	6	10	1	83.33	(66.14)	23.33	(28.78)
11	30	8	10	1	93.33	(77.71)	10.00	(18.43)
S.Em. ±						(2.52)		(1.50)
C.D. at 5%						(7.39)		(4.41)

Figures in parentheses indicate transformed value.



Treatments	Sterilants with exposure time			
	Ethanol (70%) (Second)	$HgCl_2$ (0.1%) (Minute)	NaOCl (1%) (Minute)	KCl (Minute)
1	Control	-	-	-
2	30	-	-	-
3	30	-	4	1
4	30	-	4	-
5	30	4	4	1
6	30	4	6	1
7	30	5	6	1
8	30	5	8	1
9	30	6	8	1
10	30	6	10	1
11	30	8	10	1

Fig. 1: Effect of different sterilants on aseptic culture and survival of expalnts

Plate 2: Establishment of explant

Plate 3: Sprouting of explant after 3 weeks

certain limit, after that there was decrease in the survival rate. The maximum survival (76.66%) was obtained with sequential exposure to 70 per cent ethanol for 30 second followed by 5 minutes in 0.1 per cent $HgCl_2$, 1 minute in 1 per cent KCl and 6 minutes in 1 per cent NaOCl and it was minimum (10.00%) with 30 second ethanol (70%) followed by 8 minutes $HgCl_2$ (0.1%), 1 minute KCl (1%) and 10 minutes NaOCl (1%).

Effect of explant size

Fungal contamination

Data in Table 8 and Fig.2 show that size of explant of chinese guava played a significant role in fungal contamination. Per cent contamination of fungus increased with increase in the length of shoot tips. The minimum (10%) fungal contamination was observed when explant was 0.5 cm in size while maximum (33.33%) contamination was observed when explant size was 2.0 cm. The explant size of 1.0 cm and 1.5 cm showed 13.33% and 26.67% fungal contamination, respectively.

Bacterial contamination

Data in Table 8 and Fig. 2 indicate the effect of size of explant on bacterial contamination in cultures. The maximum bacterial contamination (26.67%) was observed in explant of the largest size (2.0 cm) whereas, it was minimum (10%) with explant size of 0.5 cm and 1.0 cm. Similar result was

Table 8: Effect of explant size on fungal and bacterial contamination and sprouting of explant

Explant size (cm)	Fungal contamination (%)		Bacterial contamination (%)		Sprouting (%)	
0.5	10.00	(18.43)	10.00	(18.43)	43.33	(41.45)
1.0	13.33	(21.14)	10.00	(18.43)	56.67	(48.84)
1.5	26.67	(30.99)	16.66	(23.85)	83.33	(66.14)
2.0	33.33	(35.21)	26.67	(30.99)	86.67	)68.85)
S.Em. ±		(2.01)		(1.75)		(2.34)
C.D. at 5%		(6.57)		(5.70)		(7.65)

Figures in parentheses indicate transformed value.

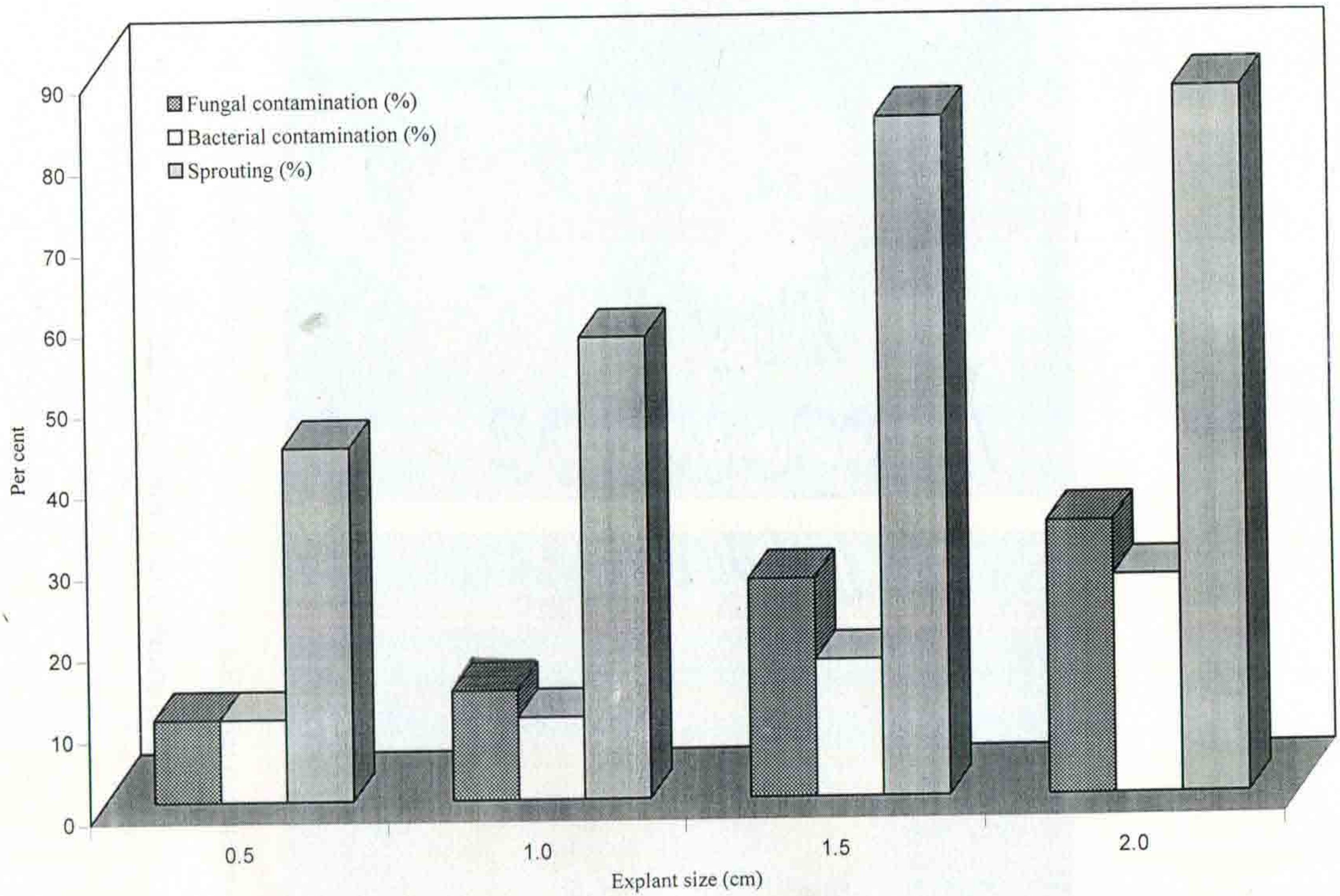


Fig. 2: Effect of explant size on fungal and bacterial contamination and sprouting of explant

a) Fungal contamination

b) Bacterial contamination

Plate 4: Contamination in culture (a & b)

obtained in 0.5 cm and 1.0 cm sized explant in case of bacterial contamination. Intermediate intensity of contamination was recorded in 1.5 cm explant size.

Sprouting of explant

Data in Table 8 and Fig. 2 show that the sprouting was maximum (86.67%) in explant of largest size (2.0 cm) as compared to the smallest (0.5 cm) size of explant. There was no significant difference between the sprouting of explant of 1.5 cm and 2.0 cm size.

Effect of season of explant (shoot tip) collection

Fungal contamination

The season of explant collection showed a significant effect on fungal contamination (Table 9 and Fig. 3). The fungal contamination was minimum (13.33%) in winter followed by spring (20%), summer (23.33%) and autumn (26.67%) while it was maximum (46.67%) when collection of explant was made in rainy season.

Bacterial contamination

Data presented in Table 9 and Fig. 3 show significant effect of season on bacterial contamination. The explant collected during winter, spring, summer and autumn seasons showed significantly lower (<16.67%) bacterial contamination than explants collected during rainy season (43.33%).

Table 9: Effect of season of explant collection on asepsis of culture and sprouting of explant

Season	Per cent fungal contamination (after one week)		Per cent bacterial contamination (after three week)		Per cent of Sprouted explants (after three week)	
Winter	13.33	(21.14)	10.00	(18.43)	16.66	(23.85)
Spring	20.00	(26.56)	16.67	(23.85)	83.33	(66.14)
Summer	23.33	(28.78)	13.33	(21.15)	63.33	(52.77)
Rainy	46.67	(43.08)	43.33	(41.15)	53.33	(46.92)
Autumn	26.67	(30.99)	10.00	(18.43)	33.33	(35.21)
S.Em. $\pm$		(2.04)		(1.92)		(2.29)
C.D. at 5%		(6.43)		(6.04)		(8.27)

Figures in parentheses indicate transformed value.

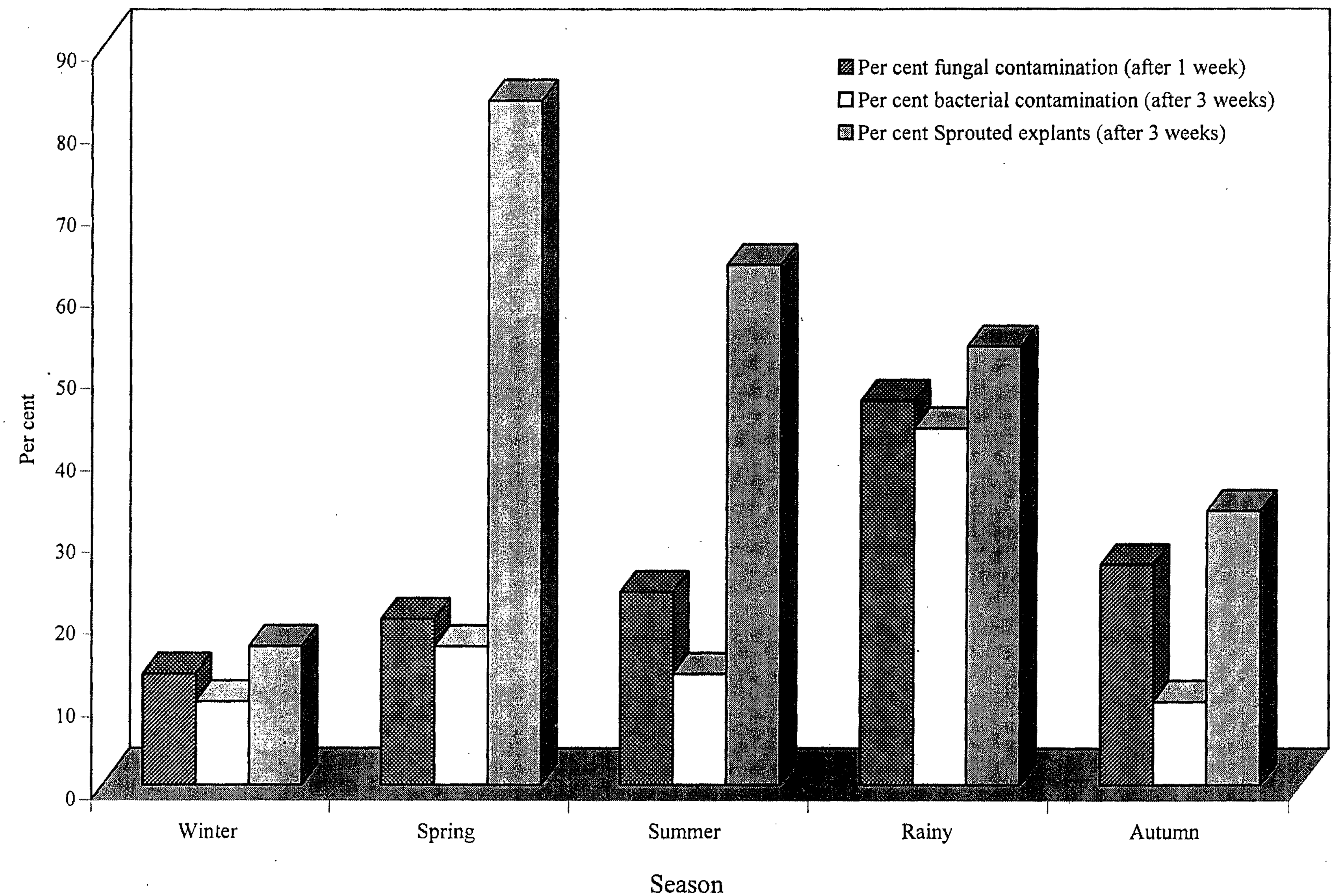


Fig 3: Effect of season of explant collection on asepsis of culture and sprouting of explant

Sprouting of Shoot tips

Data in Table 9 and Fig. 3 show that sprouting of explant was significantly affected by their collection season. The minimum sprouting (16.66%) was observed when the explants were collected during winter season. The explants collected during spring season showed significantly higher (83.33%) sprouting followed by summer season (63.33%).

Control of phenolics:

Incubation light intensity

The data presented in Table 10 and Fig. 4 showed that initial exposure to complete dark for 72 hours (L_0 T_3) resulted in the lowest (33.33%) explant browning while maximum browning (80%) was recorded when full intensity light/illumination (3200 lux) was given for 72 hours continuously.

Data on survival of explant given in Table 10 and Fig. 4 indicate that maximum survival (80%) was observed when complete dark was maintained for 24 hours followed by L_1 T_3 (1000 lux for 72 hours) while minimum survival (43.33%) was recorded when complete dark was maintained for 72 hours.

Effect of antioxidant and phenol binding agents on the control of phenolic and survival of explant

Data presented in Table 11 and Fig. 5 reveal that various antoxidents and phenol binding agents significantly reduced the browning and improved the survival of explant as compared to control. In this regard PVP 2 g/l,

Table 10: Effect of initial exposure to varying levels of light intensity on the establishment of shoot tip

Treatments	Per cent browning of explant	Per cent survival of explants
$L_0 T_1$	56.66 (48.84)	80.00 (63.43)
T_2	50.00 (44.99)	60.00 (50.76)
T_3	33.33 (35.21)	43.33 (41.15)
$L_1 T_1$	46.66 (43.07)	60.00 (50.76)
T_2	43.33 (41.15)	63.33 (52.77)
T_3	36.66 (37.22)	70.00 (56.78)
$L_2 T_1$	53.33 (46.92)	56.66 (48.84)
T_2	66.66 (54.92)	63.33 (52.77)
T_3	80.00 (63.43)	56.66 (49.22)
S.Em. $\pm$	(1.72)	(2.76)
C.D. at 5%	(5.13)	(8.20)

L_0	=	No light	T_1	=	24 hours
L_1	=	1000 lux light	T_2	=	48 hours
L_2	=	3200 lux light	T_3	=	72 hours

Figures in parentheses indicate transformed values.

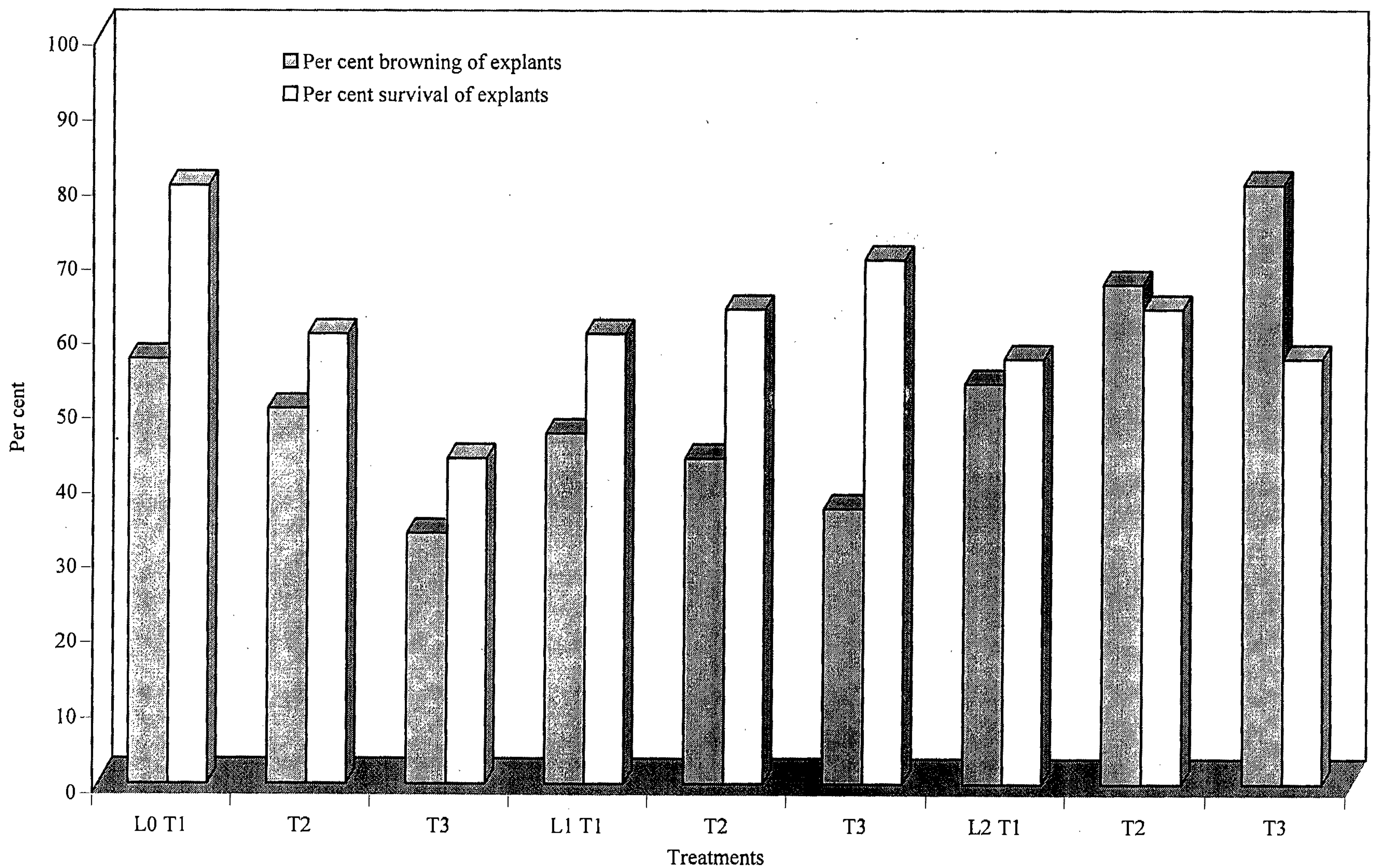


Fig. 4: Effect of initial exposure to varying levels of light intensity on the establishment of explants

Table 11: Effect of phenol binding and antioxidants additives on the degree of browning and survival of explants

Treatments (antioxidant /binding agents)		Per cent browning of explant		Per cent survival	
Control		100.00	(90.00)	23.33	(28.78)
PVP	1 g/l	93.33	(77.70)	40.00	(39.23)
	2 g/l	46.66	(43.07)	56.66	(48.84)
	3 g/l	53.33	(46.92)	36.66	(37.22)
PVPP	1 g/l	83.33	(66.14)	33.33	(35.21)
	2 g/l	63.33	(52.77)	66.66	(54.78)
	3 g/l	76.66	(61.21)	50.00	(44.99)
Citric Acid	100 mg/l	96.66	(83.85)	63.33	(52.77)
	500 mg/l	73.33	(59.00)	66.66	(54.78)
	1000 mg/l	36.66	(37.21)	76.66	(61.21)
Ascorbic Acid	100 mg/l	100.00	(90.00)	33.33	(35.21)
	250 mg/l	86.66	(68.85)	36.66	(37.22)
	500 mg/l	73.33	(59.00)	43.33	(41.15)
Activated	250 mg/l	66.66	(54.78)	66.66	(54.78)
Charcoal	500 mg/l	56.66	(48.84)	70.00	(56.78)
	1000 mg/l	33.33	(35.21)	83.33	(66.14)
S.Em. ±			(2.87)		(2.05)
C.D. at 5%			(8.27)		(5.93)

Figures in parentheses indicate transformed values.

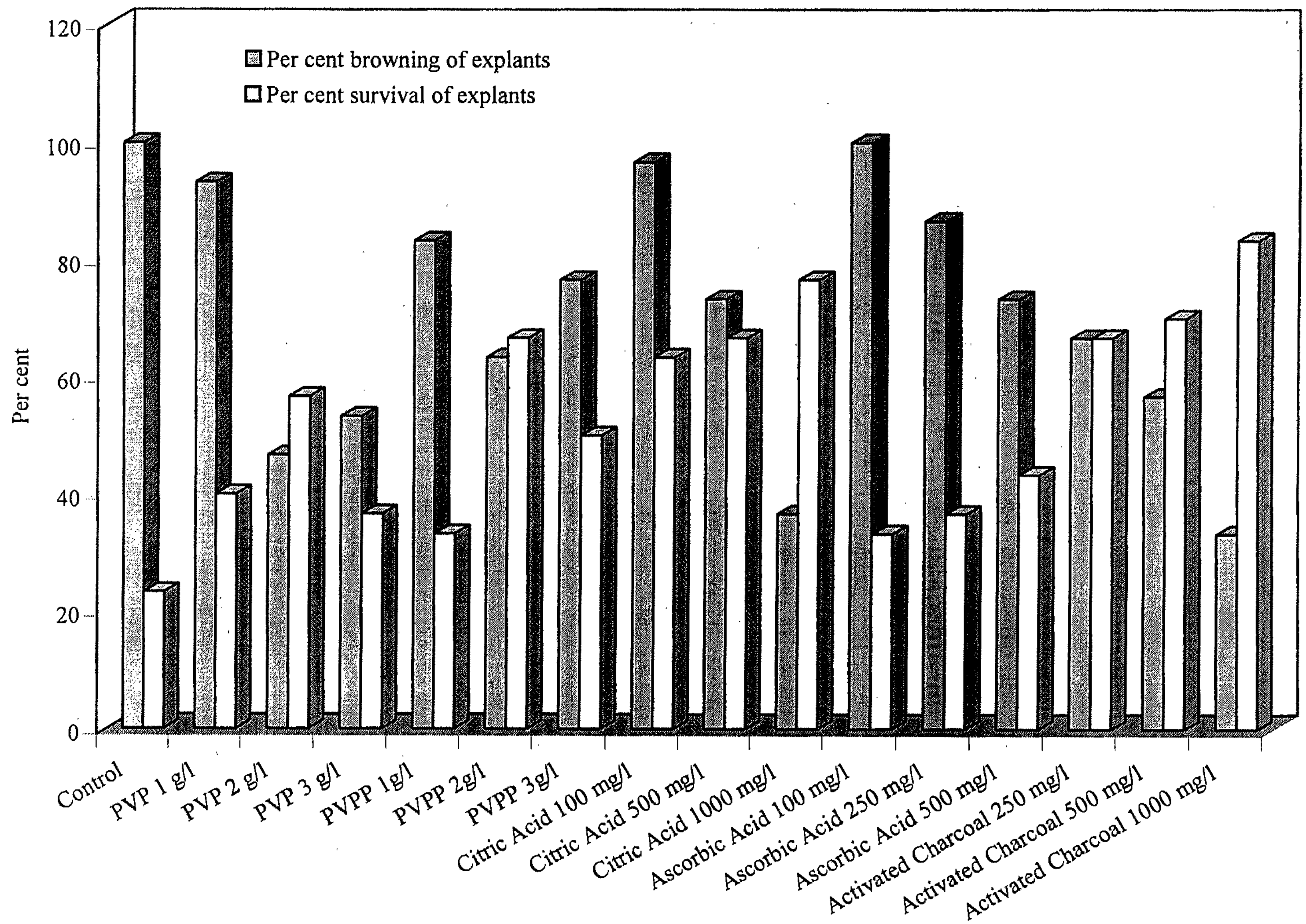


Fig. 5: Effect of phenol binding and antioxidant additives on the degree of browning and survival of explants

Plate 5: Browning of medium

PVPP 2g/l, citric acid 1000 mg/l, ascorbic acid 500 mg/l and activated charcoal, 1000 mg/l. Proved their superiority. However, when overall assessment of all the treatments was made, citirc acid 1000 mg/l proved highly effective in reducing the browning of explant and ultimately it gave highest percentage (76.66%) of survival.

Effect of varying levels of cytokinins and auxins on sprouting, shoot proliferation and growth:

Explant sprouting

Data on explant sprouting presented in Table 12 and Fig. 6 show that various combinations of BA + IBA significantly influenced the sprouting of explants. The maximum (93.33%) sprouting of explant was obtained with BA + IBA (3.0 + 0.2 mg/l) followed by BA + IBA (3.0 + 0.0 mg/l) and BA + IBA (4.0 + 0.2 mg/l). Where cytokinin and auxin (IBA) were not incorporated in the media upto 2 weeks of incubation, the sprouting of explant was prevented.

Number of shoots per explants at establishment stage

The number of shoots per explant (Table 12, Fig. 6) as affected by plant bio-regulators indicate maximum number of shoots per explant at establishment stage with cytokinin (BA) + auxin (IBA) (3.0 + 0.2 g/l) followed by BA + IBA (1.0 + 0.4 mg/l), however, the difference among these treatments was found to be non significant.

Table 12: Effect of PBRs (BA and IBA) on the sprouting of explants at the establishment stage

Treatments (BA + IBA) (mg/l)	Sprouting explants (%)		Mean number of shoots per culture
0.0 + 0.0	0.00	(0.00)	0.00
1.0 + 0.0	16.66	(23.85)	1.00
2.0 + 0.0	43.33	(41.15)	1.66
3.0 + 0.0	73.33	(59.00)	1.33
4.0 + 0.0	66.66	(54.78)	0.33
1.0 + 0.2	20.00	(26.56)	1.33
2.0 + 0.2	40.00	(39.23)	1.66
3.0 + 0.2	96.33	(77.70)	2.00
4.0 + 0.2	63.33	(52.77)	1.00
1.0 + 0.4	10.00	(18.43)	1.66
2.0 + 0.4	16.66	(23.85)	1.33
3.0 + 0.4	63.33	(52.77)	1.66
4.0 + 0.4	50.00	(44.99)	1.33
1.0 + 0.6	13.33	(21.14)	0.66
2.0 + 0.6	40.00	(39.23)	1.66
3.0 + 0.6	56.66	(48.84)	1.33
4.0 + 0.6	60.00	(50.76)	1.00
S.Em. ±		(2.22)	0.29
C.D. at 5%		(6.39)	0.84

Figures in parentheses indicate transformed values.

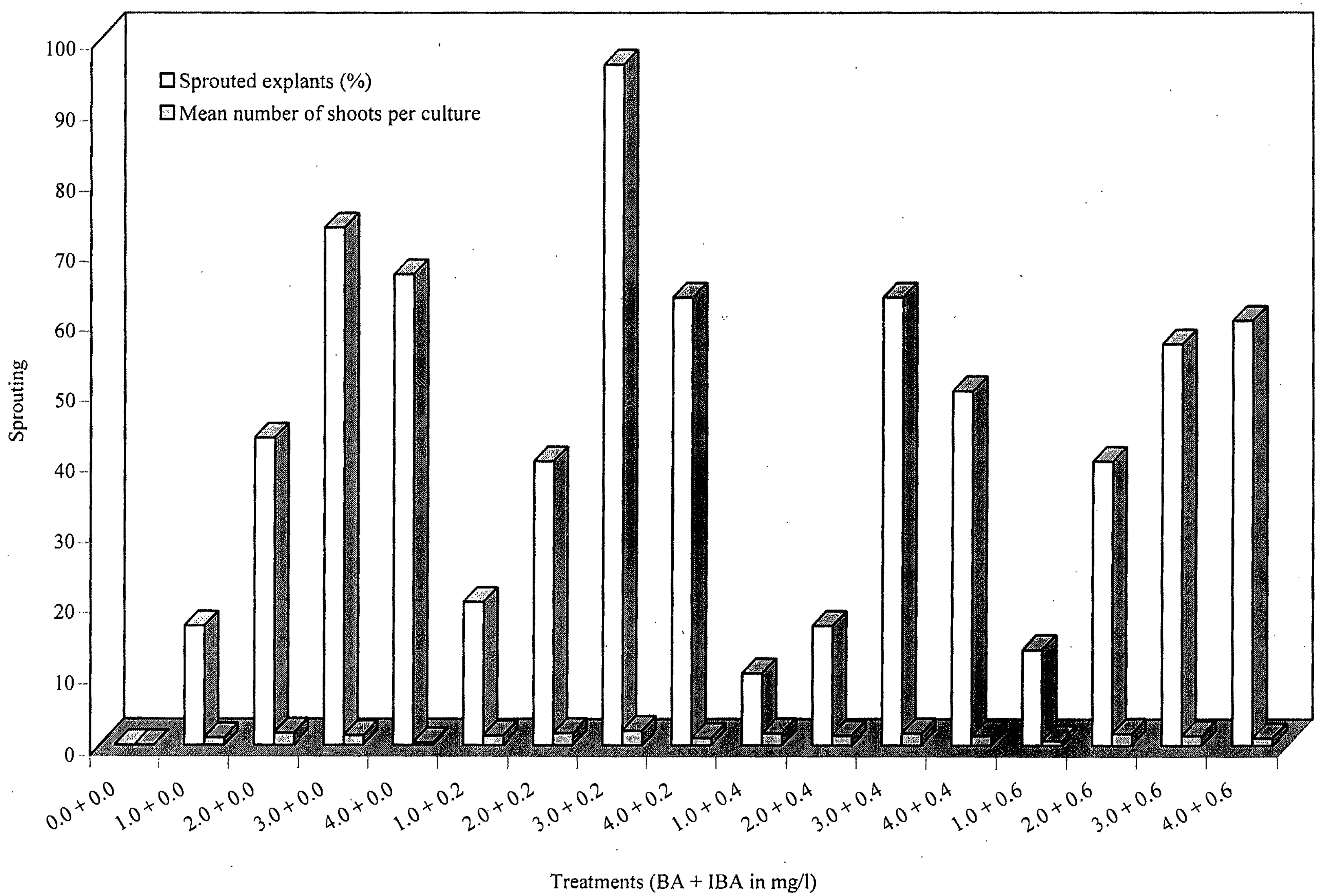


Fig. 6: Effect of PBRs (BA and IBA) on the sprouting of explants at the establishment stage

Effect of plant bio-regulators on shoot proliferation and growth:

Shoot proliferation

The data presented in Table 13 and Fig. 7 show maximum (13.33) shoot proliferation (Plate 6 and 7) with 2.0 mg/l BA and 0.2 mg/l IBA followed by 1.5 mg/l BA and 0.3 mg/l IBA combination (8.33). The number of shoots per culture increased significantly with the increase in BA concentration upto certain level (<2.0 mg/l).

Shoot growth

The shoot growth recorded in terms of length was significantly influenced by different levels of BA and IBA. The combination of 2.0 mg/l BA and 0.2 mg/l IBA proved the best in this regard where the average shoot length was 3.17 cm followed by 1.0 mg/l BA and 0.2 mg/l IBA. Increase in concentration of cytokinin (BA) level reduced shoot growth in general (Table 13 and Fig. 7).

Rooting of microshoots

The early root initiation and better rooting were recorded in microshoots when incubated in dark for one week than microshoots at normal light intensity and photoperiod (Plate 8 a & b).

Table 13: Effect plant bio-regulators (BA and IBA) on shoot proliferation of cultured shoot tips and growth after 4 weeks

Treatments (BA + IBA) (mg/l)	Average no. of proliferated shoots	Average length of proliferated shoots
0 + 0	1.33	0.46
1.0 + 0	2.66	0.56
1.5 + 0	4.33	0.46
2.0 + 0	3.66	0.43
3.0 + 0	2.33	0.43
1.0 + 0.1	2.33	1.00
1.5 + 0.1	4.66	1.33
2.0 + 0.1	3.66	1.42
3.0 + 0.1	2.66	0.96
1.0 + 0.2	3.33	1.42
1.5 + 0.2	5.66	2.66
2.0 + 0.2	13.33	3.17
3.0 + 0.2	7.33	2.16
1.0 + 0.3	3.66	1.83
1.5 + 0.3	8.33	2.07
2.0 + 0.3	5.66	1.58
3.0 + 0.3	4.33	1.33
S.Em.	0.38	1.10
C.D. at 5%	1.11	0.29

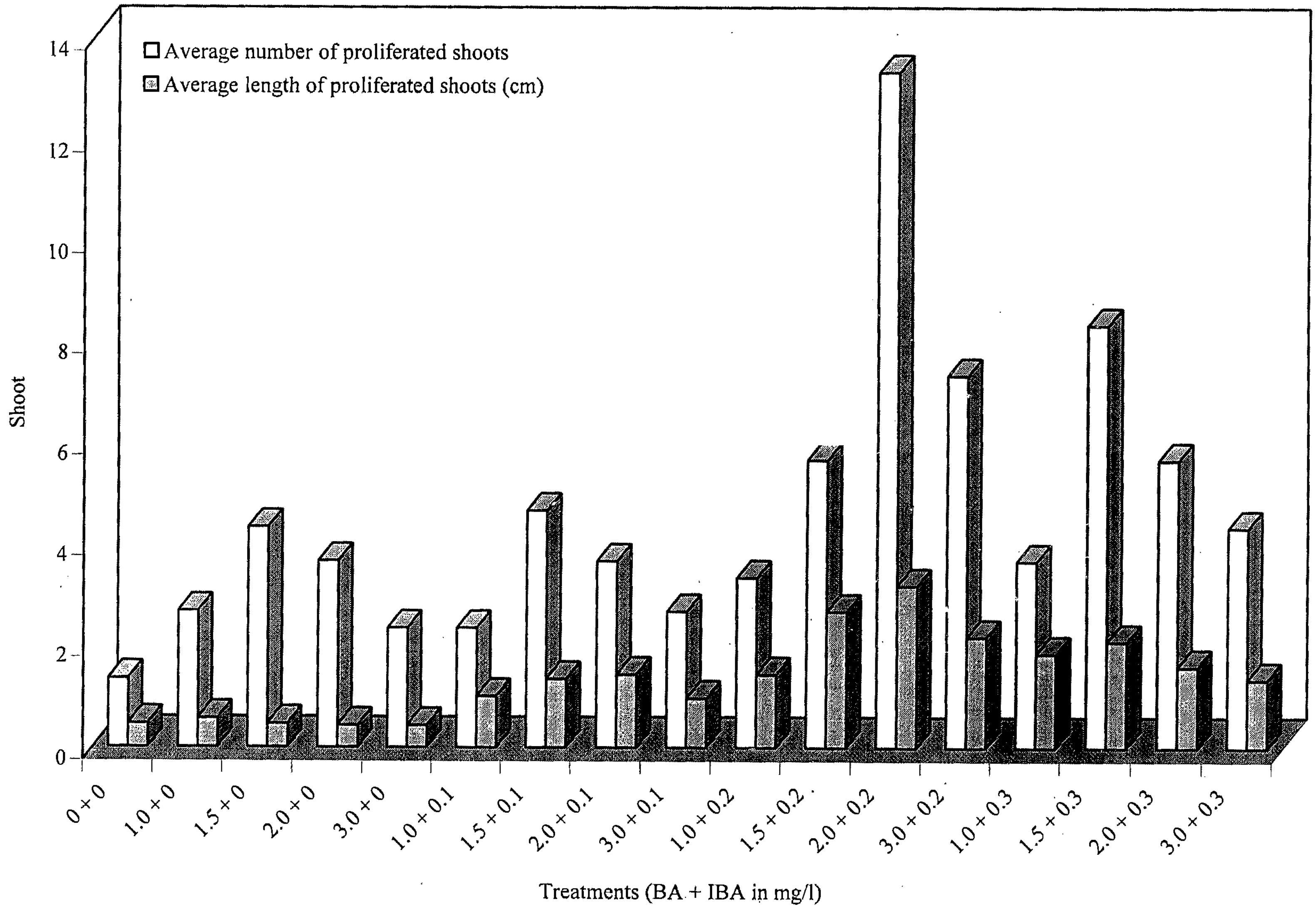


Fig. 7: Effect plant bioregulators (BA and IBA) on shoot proliferation and growth

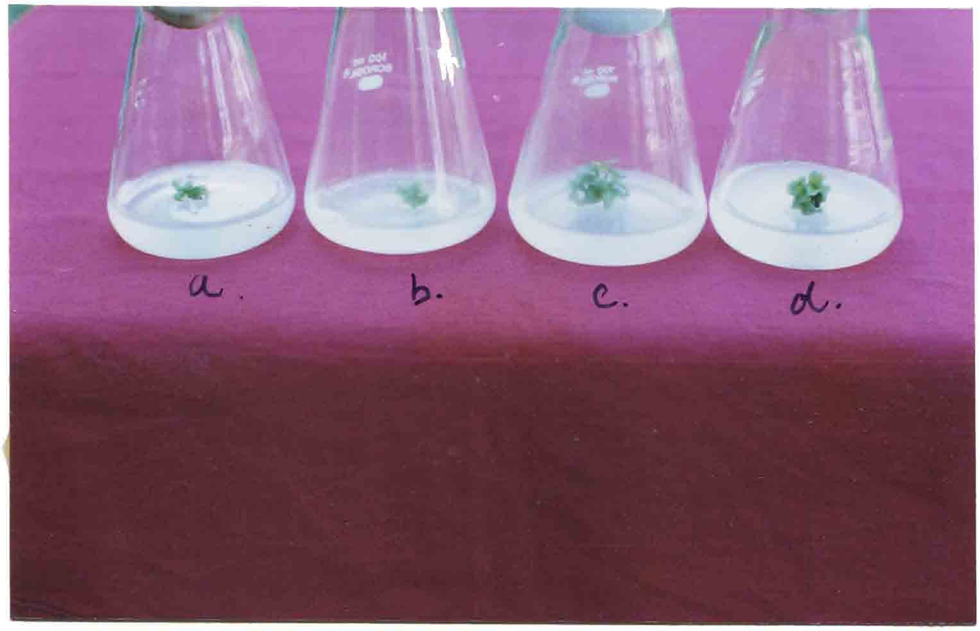


Plate 6: Effect of BA and IBA combinations on shoot proliferation
a) 1.0 mg/l BA+0.2 mg/l IBA
b) 1.5 mg/l BA+0.2 mg/l IBA
c) 2.0 mg/l BA+0.2 mg/l IBA
d) 3.0 mg/l BA+0.2 mg/l IBA

Plate 7: Multiplication of axillary shoots
(2.0 mg/l BA+0.2 mg/l IBA)

Effect of auxins on rooting:

Number of rooted cuttings

The number of rooted cuttings was found maximum with IBA + NAA (0.2 + 0.2 mg/l) closely followed by IBA + NAA (0.4 + 0.4 mg/l), however, no rooting was observed where auxins were not incorporated in the media. Further increase of IBA and NAA concentrations decreased rooting.

Average number of roots/microshoots

The Table 14 and Fig. 8 indicate maximum average number of roots per microshoots with IBA + NAA (0.2 + 0.2 mg/l). Further, increase in concentrations of these auxins significantly decreased the number of roots per cutting.

Average length of root microshoot

The average length of roots per shoot (Table 14, Fig. 8) was found maximum with IBA + NAA (0.2 + 0.2 mg/l) followed by IBA + NAA (0.4 + 0.4 mg/l). However, further increase in concentration caused decrease in length of roots.

Survival of rooted plantlets

After hardening, rooted plantlets were transplanted in the mixture of sand, soil and F.Y.M. (1:1:1) and 80 per cent survival was recorded (Plate 9).

Table 14: Effect of auxins on rooting behaviour of microshoots

Treatment (IBA + NAA) (mg/l)	Rooting (%)		Number of roots	Length of roots (cm)
0.0 + 0.0	0.00	(0.00)	0.00	0.00
0.1 + 0.1	53.33	(46.92)	1.66	1.25
0.2 + 0.2	86.66	(68.85)	4.33	4.83
0.4 + 0.4	83.33	(66.14)	2.66	1.83
1.0 + 1.0	53.33	(46.92)	2.33	1.25
1.5 + 1.5	36.66	(37.22)	2.00	0.89
S.Em.		(2.08)	0.27	0.12
C.D. at 5%		(6.42)	0.84	0.39

Figures in parentheses indicate transformed values.

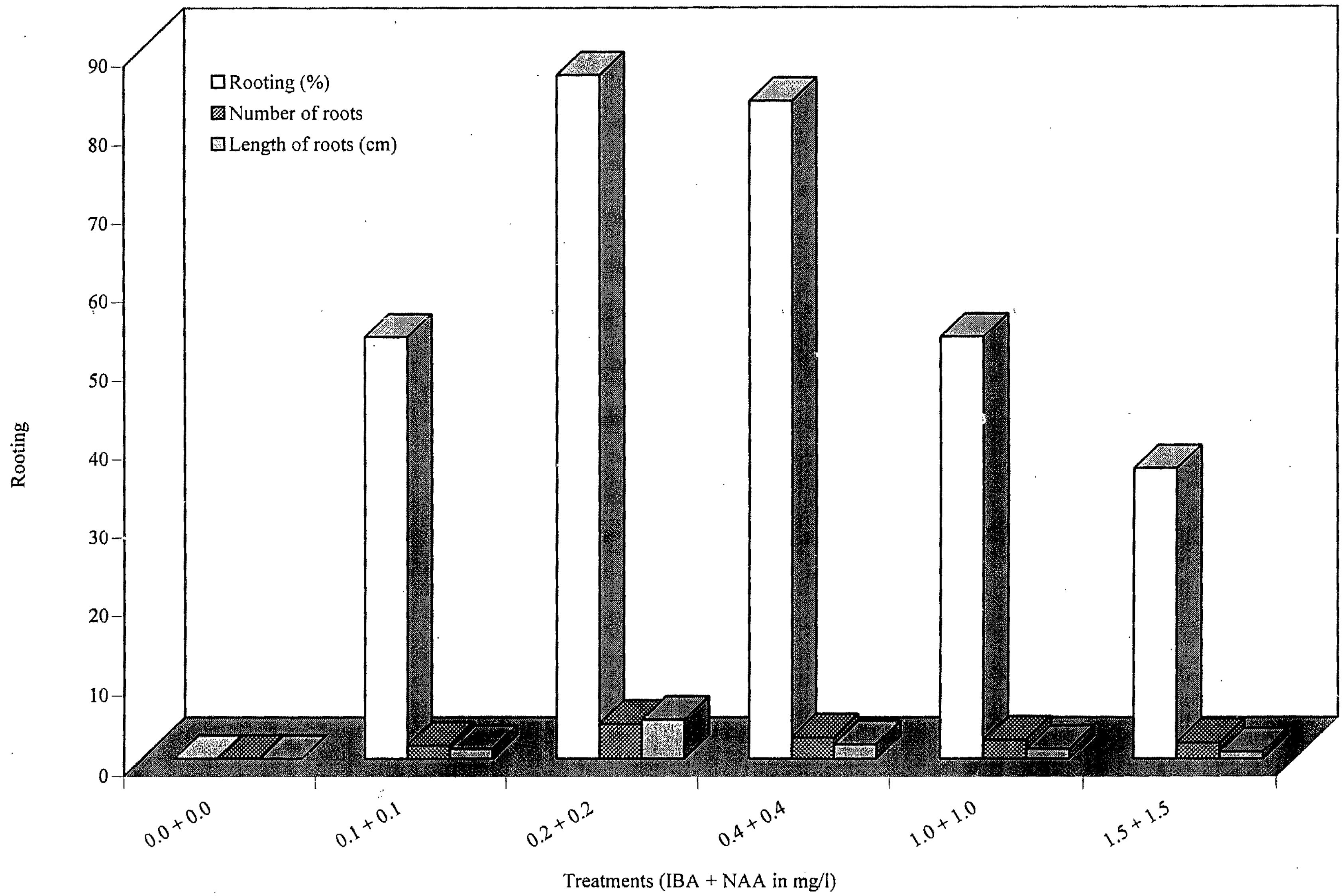
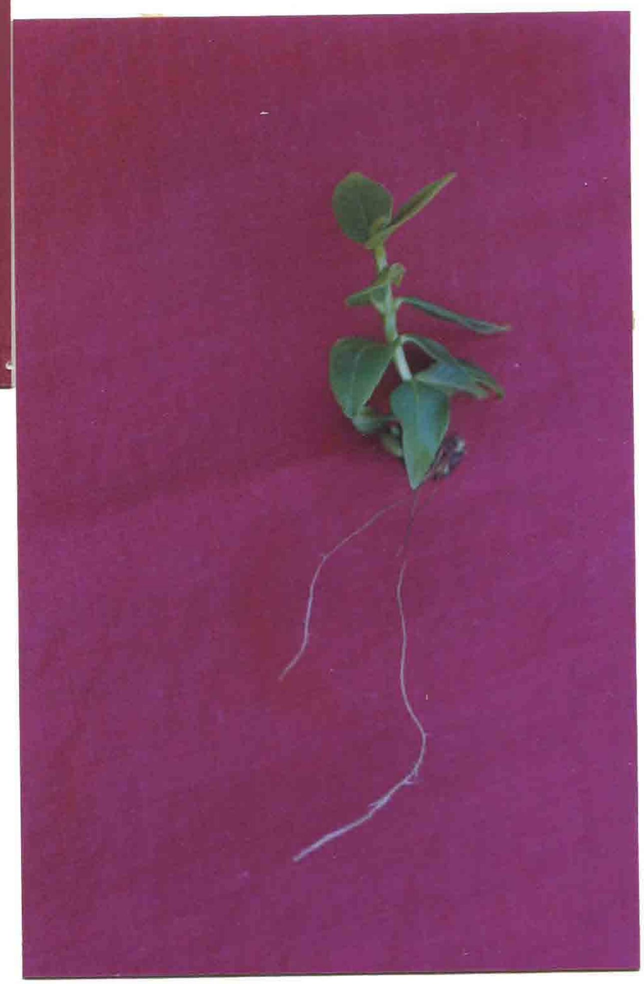


Fig. 8: Effect of auxins on rooting of shootlets

a) In culture tube



b) Rooted plant removed from tube

Plate 8: Rooting of shootlet

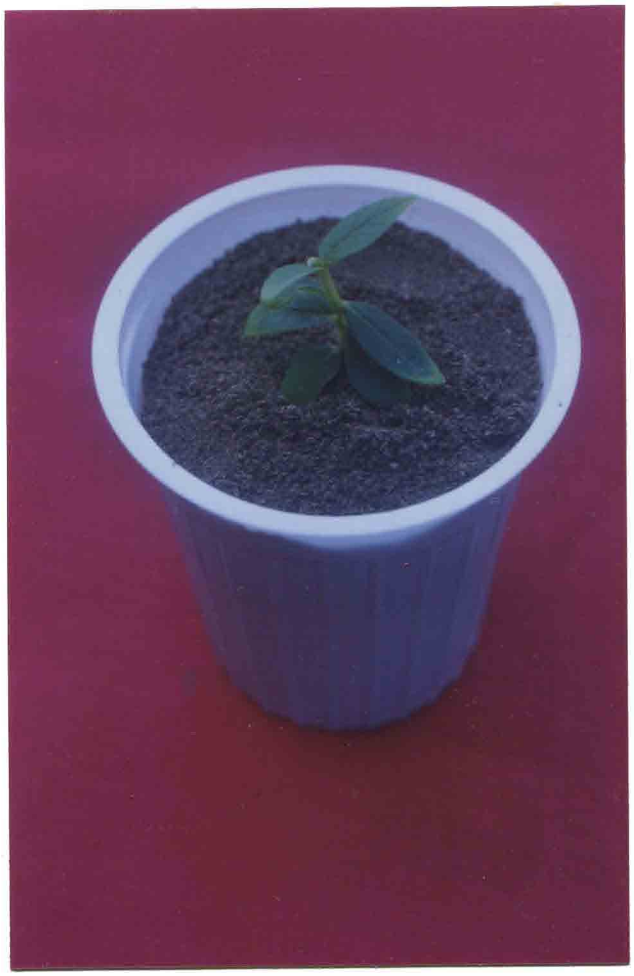


Plate 9: Rooted plant in pot

DISCUSSION

DISCUSSION

In the present study an attempt has been made to develop a protocol for *in vitro* propagation of chinese guava (*Psidium friedrichsthalianum* (Brig.) Nierdz). Different experiments viz., effect of various surface sterilants on aseptic culture, size of explant on fungal and bacterial contamination, season of explant collection, culture establishment, shoot proliferation, multiplication and rooting were conducted. The rooted plantlets were subjected to acclimatization for establishment. In this chapter, the findings of the present investigation have been discussed in the light of the results obtained by other workers.

In present study, apical portion i.e., shoot tip of recently emerged shoots of chinese guava were collected from branches of selected mother plant (Plate 1). Prior to inoculation, the explants were treated with ethanol (70%), $HgCl_2$ (0.1%) and NaOCl (1%) at varying time intervals to standardize the optimum exposure time of shoot tip (Table 7, Plate 2). The best surface sterilization of explants was achieved by sequential application of 70 per cent ethanol for 30 seconds, 0.1 per cent $HgCl_2$ for 5 minutes and 1 per cent NaOCl for 6 minutes which resulted in 66.66 per cent aseptic culture with 76.66 per cent survival of explants (Table 7, Plate 3). Further increase in exposure time to sterilants led to the killing of explants. One of the probable reasons for the death of explants, when they are exposed to surface sterilants for longer period of time may be contamination of mercury (Hg^{2+}) present in mercuric chloride ($HgCl_2$), causing phytotoxicity for the survival of the explant. These results are in conformity with those of Parkash and Tiwari (1996), who successfully used $HgCl_2$ and NaOCl for surface sterilization of guava explant. Amin and Jaiswal (1987); Khattak *et al.* (1990), Loh and Rao (1989); Papadatou *et al.* (1990), also confirmed the above results in guava. Beneficial effects of $HgCl_2$ followed NaOCl have been observed in other woody plants (Debergh and Maene, 1981; Marks and Simpson, 1990).

Different sized explant (0.5 – 2 cm) were used after disinfecting them with different surface sterilants prior to inoculation. It was found that smallest explant showed the minimum fungal and bacterial contamination (Table 8) Hammerschlag (1980) and Hartmann *et al.* (1997) also found similar results. Smaller apical shoot tips appear to have lesser chance of microbial contamination than the large sized explants (Table 8). Yet smaller explants such as apical meristem have been used for obtaining disease free plants (Hartmann *et al.*, 1997). Bacterial contamination came either from internally infected explant or by using inadequate disinfection procedure. Bacterial contamination is serious problem in tropical and subtropical woody plants (James and Thurbon, 1979).

Sprouting of explant was significantly influenced by the size of explant. Maximum sprouting (Plate 3) was observed in large (2.0 cm) explant compared to that of the smallest shoot tip (0.5 cm). The use of larger explant for better sprouting, as reported by Pieirik (1987); Parkash and Tiwari (1996) and Hartmann *et al.* (1997) match with present findings where largest shoot tips

(2.0 cm) resulted in maximum sprouting as compared to the smallest shoot tips (0.5 cm). In general, it is more difficult to induce growth in very small explants because the isolated plant parts have its own supply of food reserve and hormones (Pieirik, 1987) and thus larger explant size was easier to induce growth (Parkash and Tiwari, 1996).

The season of explant collection is one of the most important factors and it should be taken into consideration at the time of explant collection. The explant collected during winter season (Table 9) followed by spring and autumn showed minimum bacterial and fungal contamination (Plate 4 a & b). The explants collected during rainy season, showed maximum microbial contamination. These results are in agreement with the findings of Parkash and Tiwari (1996) in guava, Singh and Tiwari (1998) in jackfruit and Dwivedi and Bist (1999) in pear. High microbial contamination during rainy season may be due to inadequacy of applied levels of sterilants to eliminate large populations of microbs present on the explants. High temperature coupled with high relative humidity during rainy season has been reported to be favourable for the growth of microorganisms.

The shoot tips collected and cultured during spring season showed higher sprouting which was significantly greater than other seasons (Table 9, Plate 3). The presence of higher growth promoting substances and low growth inhibitors in the actively growing shoot tips during spring season (Moore, 1986) may be the plausible explanation for this success. These results are in agreement with the earlier findings of Lane (1978), Banno *et al.* (1988) and Dwivedi and Bist (1999) who also got higher success in sprouting of pear shoot tips when cultured during spring season. The shoot tips collected during winter showed minimum sprouting which may be due to the presence of resting buds.

Browning and death of explants is generally observed as a result from exposure to surface sterilants and exudation of phenols in the nutrient medium (Mayer and Harel, 1979). Hence the browning in *in vitro* culture of chinese guava may be due to exudations and oxidation of phenolic compounds released from cut ends of the explants (Plate 5) by polyphenoloxidases (Mayer and Harel, 1979), peroxidase (Loomis and Battlaile, 1966) or air (Robinson, 1983). The oxidized products, quinones are known to be highly reactive and inhibit enzyme activity leading to the death of explants (Hu and Wang, 1993). However, in the present investigation, the problem of browning was successfully solved by the exposure of explants to different levels of light intensity (i.e. 00 lux, 1000 lux and 3200 lux) for varying period (24, 48, 72 hours). The effect of light intensity was highly significant due to the treatment of initial exposure to complete darkness for 72 hours which gave minimum browning of the media. This can be explained by well known favourable effect of illumination on the synthesis and accumulation of phenolic compounds in cell and tissue culture (Dalal *et al.*; 1992) while survival of explant in dark for longer period increase probably due to reduced synthesis of phenols.

The excised shoot tips were inoculated in medium fortified with antioxidants and phenol binding agents at various concentrations (Table 12). Addition of citric acid and activated charcoal to the MS medium were significantly effective in reducing the browning and improving the survival of explant, whereas, PVPP and ascorbic acid were not much efficient to control the phenols. PVP was found to reduce phenols but survival percentage was not appreciable. The beneficial effect of activated charcoal was, however, associated with reduced vigour of surviving explants. The effectiveness of activated charcoal in improving explant browning may be due to binding of phenols while reduced growth may be attributed to its absorption of organic molecules and auxins. This observation is in agreement with those of Gupta *et al.* (1980); Ziv and Halwy (1983), Siddiqui and Farooq (1996); Loh and Rao, (1989); Amin and Jaiswal (1988); Parkash and Tiwari (1996) and Singh and Tiwari (1998) who used adsorbing agent like activated charcoal and PVPP for controlling phenols. The best control of phenols was achieved by frequent subculturing/transfer of explants to fresh media containing antioxidants. The similar findings have been reported by Song *et al.* (1988); Amin and Jaiswal (1987); Cronaur and Krikorian, (1984b) Rathore *et al.* (1992) and Block and Lankes (1995).

One of the most important step in tissue culture is to adopt the plant tissue to heterotrophic mode of nutrition. This has been termed as establishment stage (Murashige 1974). In the present investigation, the maximum explant sprouting was observed with BA + IBA (3.0 + 0.2 mg/l) (Table 12). Further increase in cytokinin (BA) concentration reduced the sprouting of explants and number of shoot per explant, however the lowest number of sprouted explant were observed in cytokinin and auxin free medium (control).

Murashige and Skoog medium supplemented with 2.0 mg per litre BA and 0.2 mg/l IBA gave significantly higher shoot proliferation and growth of proliferated shoots (Table 13, Plate 7). The concentration of BA upto 2 mg/l in the medium significantly increased the number of shoots, however, the shoot length was decreased with increased level of cytokinin (BA). These results are in conformity of Parkash and Tiwari (1996); Amin and Jaiswal (1987) in guava and Singh and Tiwari (1998) in jackfruit. Further increase in plant bio-regulators was not effective in rooting of shoots possibly due to excessive callusing at the base of explants (Sadhu, 1989; Joshi and Thengene, 1996). However, the plant species specific for specific cytokinin to auxin ratio are reported to have role in the shoot proliferation and growth of explants (Salisbury and Ross, 1986).

When combinations of IBA + NAA were tried (Table 14, Plate 8 a & b), the maximum rooting (86.66%), average number of roots/shoot and mean length of root were observed with IBA + NAA (0.2 + 0.2) mg/l), however, no rooting was observed in control (auxins free medium). Further increase in concentration of these auxins decreased the rooting percentage, number and length of roots/shoot and induced more number of deformed roots and callusing in microshoots. Higher concentration of auxins reduced rooting percentage and induced basal callusing in pear (Banno *et al.*, 1989) and black plum (Yadav *et al.,* 1990) also. Combination of IBA and NAA was also found to be effective by Williams *et al.* (1985) in apple, Parkash and Tiwari (1996) and Amin and Jaiswal (1988) in guava, Roy *et al.* (1990) and Singh and Tiwari (1998) in jackfruit. This is probable due to the synergism between these two auxins. Activated charcoal @ 1 g/l applied in rooting medium induced early rooting. Similar results were obtained by Cronauer and Krikorian (1984a) and Song *et al.* (1988). Johnson (1983) pointed out that activated charcoal absorbs the toxic and growth inhibiting substances secreted by micro-cuttings. In this investigation microshoots incubated in dark for one week gave early and better rooting. Similar beneficial effects of etiolation has also been recorded in apple (Hammerschlag, 1982), pear (Banno *et al,* 1989) and guava (Parkash and Tiwari, 1996). They pointed out that in light, it was probably due to either inhibition of synthesis of rooting cofactors or photo inactivation of root promoting factors.

The rooted micro plantlets were subjected to hardening treatments in pots containing sand, soil and FYM in (1:1:1) ratio which gave 80% success in terms of survival of plantlets (Plate 9). Similar observations were also made by Pandey and Rajeevan (1987) who reported maximum survival of rooted microshoots of papaya in potting mixture containing FYM sand and soil (1:1:1). Parkash and Tiwari (1996) transferred rooted guava plantlets for hardening treatment 30 days after rooting in mixture of sand, soil and FYM (1:1:1) and obtained maximum survival of rooted plantlets.

SUMMARY AND CONCLUSION

SUMMARY AND CONCLUSION

Studies on *in vitro* culture of chinese guava (*Psidium friedrichsthalianum* (Berg.) Nierdz.) were carried out in the Department of Horticulture, Gobind Bhallabh Pant University of Agriculture and Technology, Pantnagar, District, Udham Singh Nagar (Uttaranchal) from 1998-2000. The experiments were conducted to standardize the optimum size of explants, suitable season for explant collection, suitable sterilants and their exposure time, optimum level of plant bioregulators in medium for shoot proliferation. Studies were also made to standardize the optimum level of plant bioregulators on rooting of microshoots and acclimatization of new shootlets. The salient findings of the investigation are summarized below:

1) Surface sterilants 0.1 per cent $HgCl_2$ (5 minutes) + 1 per cent NaOCl (6 minutes) were found to be better sterilants for shoot tips resulting in 66.66 per cent aseptic culture and 76.66 per cent survival of explants.

2) Minimum bacterial and fungal contaminations were recorded with the smallest sized (0.5 cm) explant, however, sprouting percentage was the lowest. Explant of 1.5 cm size was found to be most suitable for *in vitro* culture.

3) The shoot tips cultured during winter, spring, summer and autumn registered significantly lower (<27%) fungal contamination than rainy season (46.67%).

4) A significantly lower (<16.67%) bacterial contamination was observed when explants were collected during autumn, winter, spring and summer seasons, whereas shoot tips collected in rainy season showed maximum bacterial contamination (43.33%).

5) The explant collected during spring season sprouted profusely (83.33%) whereas it was minimum in winter season (16.66%).

6) The best control of phenolics was achieved when medium was supplemented with 1 g/l citric acid followed by rapid transfer of media. Explants may be kept in complete dark for 24 hours also.

7) The higher concentration of cytokinin i.e., BA (3 mg/l) and low concentration of auxin i.e. IBA (0.2 mg/l) were found more effective for sprouting and number of shoot per explant at establishment stage. Further increase in cytokinin reduced the explant sprouting.

8) Relatively lower concentration of cytokinins (BA) (2.0 mg/l) and auxin (IBA) (0.2 mg/l) resulted 13.33 shoots per culture with mean length of 2.66 cm per microshoots after 4 weeks of culture.

9) Best rooting (66.66%) was recorded with IBA + NAA (0.2 + 0.2 mg/l). Media fortified with activated charcoal improved the root quality. Microshoots incubated in dark for one week gave early rooting.

10) Rooted plantlets showed 80 per cent survival in the pots containing a mixture, of sand soil and FYM (1:1:1) when planted after 30 days of hardening treatment.

Conclusion .

On the basis of present investigation it is conduced that *in vitro* culture technique can be successfully applied for the multiplication of chinese guava on a large scale within a short period. For getting best results shoot tip of 1.5 cm size may be collected in early spring season for culture. It is also evident that, before *in vitro* culture, the explant should be surface sterilized in 0.1% $HgCl_2$ for 5 minutes followed by 1% NaOCl for 6 minutes. MS medium fortified with BA 2.0 mg/l and IBA 0.2 mg/l proved better for shoot proliferation. Rooting may be induced in shootlets with IBA + NAA (0.2 + 0.2 mg/l) in ½ MS medium and rooted plantlets can be successfully planted in pots containing a mixture of sand, soil and FYM (1:1:1).

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* Original not seen.

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ABSTRACT

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Thesis Title: *In vitro* cloning of chinese guava (*Psidium friedrichsthalianum* (Berg.) Nierdz.)

Present investigation was carried out at Tissue Culture Laboratory, Department of Horticulture, G.B. Pant University of Agriculture & Technology, Pantnagar (Uttaranchal), during 1998-2000 for *in vitro* cloning of chinese guava (*Psidium friedrichsthalianum* (Berg.). Nierdz.) for mass multiplication. The shoot tips of 0.5 to 2.0 cm size were collected during different seasons viz., winter, spring, summer, rainy and autumn. These shoot tips were treated with different sterilants viz., ethyl alcohol, mercuric chloride and sodium hypochloride for various time periods to control contamination. Different phenol binding agents like PVP, PVPP and activated charcoal and antioxidants like citric acid and ascorbic acid were used in various concentrations to control browning of media. In other experiment, various illumination levels viz., dark, 1000 and 3200 lux for various time periods as 24, 48 and 72 hours were given. The established plants were inoculated in proliferation medium containing different levels of BA (1.0, 1.5, 2.0 and 3.0 mg/l) and IBA (0.1, 0.2 and 0.3 mg/l) for shoot proliferation. For rooting micro-shoots were inoculated in half strength MS medium containing different levels of auxins (IBA + NAA). The rooted plantlets were hardened and planted in pot mixture containing sand, soil and FYM (1:1:1).

Results showed that shoot tips of 1.5 cm size collected in early spring season sprouted well with least contamination. Ethyl alcohol (70%) for 30 seconds, mercuric chloride (0.1%) for 5 minutes and NaOCl (1.0%) for 6 minutes in sequential order were found better for the control of contamination with maximum survival of explants. Out of various antioxidants, phenol binding agents, activated charcoal (1g/l) and citric acid (1g/l) significantly reduced browning and improved the survival of explants. Complete dark for 24 hours showed minimum browning of explants. Maximum proliferation of shoots were achieved in medium containing relatively lower level of BA (2.0 mg/l) and IBA (0.2 mg/l). Results on rooting of micro-shoots showed that IBA and NAA (0.2 + 0.2 mg/l) gave maximum rooting followed by IBA and NAA (0.4 + 0.4 mg/l). The rooted plantlets recorded 80 per cent survival in potting mixture containing sand, soil and FYM (1:1:1) after hardening.

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