Biotechnology of Spices, Medicinal & Aromatic Plants

Proceedings of the National Seminar on Biotechnology of Spices and Aromatic Plants.

24 - 25 April 1996, Calicut, India.

Indian Society for Spices

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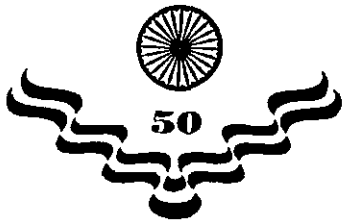

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Foreword

India is the land of spices and the centre of origin of the King of Spices - Black Pepper and the Queen of Spices- Cardamom. India is a major producer of ginger, turmeric, seed spices and tree spices. Vanilla, the Princess of Spices, is also gaining importance in our country. Though most of the spices produced are consumed domestically, a good percentage of the produce is also exported. India's export earnings from spices alone during 1996-97 was Rs. 1180 crores.

Though India maintains a prominent place in global spices trade, higher productivity and low cost of production enable other producing countries to outbid India in terms of the prices in the international market, particularly in black pepper, cardamom and ginger. In order to maintain our supremacy in spices production and also to recapture the past glory of India in global spices trade, integrated programmes involving high productivity and quality oriented research are the needs of the day. Low productivity of spices in the country is attributed to many factors such as Phytophthora foot rot disease of black pepper, rhizome rot of ginger, 'Katte' virus disease of cardamom and narrow genetic variability of tree spices such as nutmeg, clove and vanilla. Modern biotechnological tools including biocontrol approaches in conjuction with the conventional techniques of crop improvement, crop production and protection will go a long way in solving many of the problems of the spices sector. At this juncture, a consolidation of our achievements involving biotechnological approaches in the area of spices will be most appropriate and timely.

This publication, ***Biotechnology of Spices, Medicinal & Aromatic Plants*** *contains the proceedings of the National Seminar on Biotechnology of Spices and Aromatic Plants organised by the Indian Society for Spices at Calicut during April 24-25, 1996, fills the gap in this field. I am sure that this publication will be very useful to all those involved in spices research and farming.*

I congratulate the Indian Society for Spices for conducting such a relevant seminar and bringing out the proceedings in time.

Manju Sharma

2 October 1997

(Manju Sharma)

PREFACE

The Indian Society for Spices is devoted for the advancement of research and development of spices and related crops. The Society has successfully organised three national seminars so far with focal themes on Crop Improvement, Crop Protection and Post Harvest Technology of Spices, Medicinal and Aromatic Plants.

Realising the importance and rapid progress made in crop improvement, crop protection through biotechnological approaches in several crops, the Indian Society for Spices organised a National Seminar on Biotechnology of Spices and Aromatic Plants during 24-25 April, 1996 at Calicut, Kerala in collaboration with Indian Institute of Spices Research, Calicut.

This publication "Biotechnology of Spices, Medicinal & Aromatic Plants" is the proceedings of this National Seminar. A total of 43 papers were presented covering spices, medicinal and aromatic crops under four technical sessions. A few papers presented in the seminar could not be included in this proceedings as we could not receive the full text of these papers.

The editors are grateful to Dr. (Mrs.) Manju Sharma, Secretary, Department of Biotechnology, Ministry of Science & Technology , Government of India for giving the 'Foreword' for this publication. The editors are highly grateful to Dr. K.V. Peter, President, Indian Society for Spices and Director, Indian Institute of Spices Research, Calicut for his support and encouragement. The Indian Society for Spices gratefully acknowledge the financial assistance given by Spices Board, Cochin in publishing this proceedings. The editors express their appreciation to M/s Lucos Offset Prints, Calicut for bringing out the proceedings in attractive form.

-Editors

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Tissue culture and *in vitro* conservation of spices - an overview

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Abstract

Crop improvement in majority of spice crops is a difficult and time consuming programme due to long pre bearing age and other crop specific problems. Biotechnology offers an alternative to conventional crop improvement in spices. This paper reviews the present status of biotechnology of spices with emphasis on the work done in India on micropropagation and rapid clonal multiplication of high yielding 'elite' genotypes to generate good quality planting material; exploiting somaclonal variation and genetic engineering techniques for crop improvement; *in vitro* selection for resistance to biotic and abiotic stresses; *in vitro* conservation and safe exchange of germplasm and production of flavour and volatile constituents in tissue culture.

Key words: biotechnology, *in vitro* conservation, spices, tissue culture.

Introduction

The productivity of many of spice crops is considerably low due to various factors such as inadequate availability of high yielding varieties, absence of genotypes resistant to pests and diseases and absence of variability in many of the introduced crops. Biotechnology with its apparently unlimited potential offers new and exciting opportunities to address the above crop specific problems. Some of the important applications of biotechnology in spices are; micropropagation and rapid clonal multiplication of high yielding 'elite' genotypes to generate good quality planting material; exploiting somaclonal variation and genetic engineering techniques for crop improvement; *in vitro* selection for resistance to biotic and abiotic stresses; *in vitro* conservation and safe exchange of germplasm and production of flavour and volatile constituents in culture.

This paper reviews the present status of biotechnology of spices with emphasis on the work done in India.

Cardamom

'Katte' disease caused by virus is one of the major production constraints in cardamom. Utilisation of virus-free planting material is considered the most important input in disease management

programme and to check disease spread.

Clonal multiplication

Cardamom is multiplied vegetatively as well as through seed. Being a cross pollinated crop, clones are ideal for generating true to type planting materials from high yielding clumps. However, due to inadequate availability of clonal planting material, farmers still prefer seedlings. *In vitro* propagation method for clonal propagation of cardamom hase been standardized. (Nadgauda, Mascarenhas & Madhusoodanan 1983; Vatsya *et al.* 1987; Reghunath & Gopalakrishnan 1991).

High rate of multiplication coupled with additional advantage of obtaining uniform and disease free planting material makes micropropagation a preferred method to conventional method. Field evaluation of tissue cultured plants was carried out at Indian Institute of Spices Research (IISR). Preliminary results showed that the micropropagated plants performed on par with suckers of the original mother plant. However, Nadgauda, Mascarenhas & Madhusoodanan (1983) reported that the tissue cultured plants were superior to that of seedling progenies.

Regeneration of plantlets from callus

Rao *et al.* (1982) reported successful regeneration of plantlets from callus of seedling explants of cardamom. Protocols for organogenesis and plant regeneration from rhizome and vegetative bud-derived callus cultures were also standardised (with about 20-50 plantlets per culture) at IISR which are being used for large scale production of somaclones and selection of useful genotypes. High amount of variability was noticed among the somaclones for the morphological characters in the culture vessels itself. Somaclones are being evaluated in the field at IISR for their genetic variability. Efficient plant regeneration systems are essential for future genetic manipulation to evolve resistant genotypes for combating biotic and abiotic stress.

Inflorescence culture

Immature inflorescences form an excellent source for clonal multiplication of cardamom through tissue culture especially when other sources are prone to high rate of contamination. Kumar *et al.* (1985) reported the successful conversion of immature floral buds to vegetative buds and subsequently to plantlets.

Black Pepper

The conservation of the precious genetic resources of black pepper is highly essential. *Phytophthora* foot rot and slow decline diseases are major diseases affecting pepper plantations. None of the existing genotypes is resistant to these diseases.

Clonal multiplication

There are many reports of *in vitro* multiplication of black pepper from mature shoot tip explants (Nazeem *et al.* 1993; Philip *et al.* 1992; Nirmal Babu *et al.* 1993 a,b) and from seedlings (Mathews & Rao 1984; Nirmal Babu *et al.* 1993b). The multiplication rate is around 6 shoots per culture in about 90 days. Earlier reports on micropropagation of black pepper reported phenolic exudates from the cut surface (Chua 1981; Fitchet 1988 a,b) and bacterial contamination (Raj Mohan 1985; Fitchet 1988

a,b) severely hampered the establishment phase. Methods for reducing phenolic interference and systemic contamination in the *in vitro* cultures have been reported (Fitchet 1988 a; Nazeem *et al.* 1993).

Callus cultures and regeneration of plantlets

Efficient plant regeneration system is an important step in genetic manipulation attempts. Protocols for plant regeneration were standardised and plants were regenerated from leaf tissues directly without intervening callus phase (NRCS 1991) and leaf callus (Nirmal Babu *et al.* 1993 a,b; Nazeem *et al* 1993). Shylaja *et al.* (1996) reported *in vitro* screening of black pepper for tolerance to *Phytophthora capsici* using concentrated culture filters of the fungus.

Micropropagation of related species of *Piper*

Piper betle is an economically important species cultivated extensively in India. *Piper longum* L. (Indian long pepper) and *Piper chaba* Hunt. (Java long pepper) are another group of medicinally important spices. *Piper colubrinum* is a South American species found to be resistant to *Phytophthora capsici* and *Radopholus similis*. *Piper barberi* Gamble is an endangered species from South India (Nirmal Babu *et al.* 1992 b).

Protocols for rapid clonal multiplication of *P.longum* from shoot tip explants are available (Sarasan *et al.* 1993; Nirmal Babu *et al.* 1994 d) Micropropagation of *P.chaba* (Nirmal Babu *et al.* 1994 d), *P.betle* (Nirmal Babu *et al.* 1992 e), *P.colubrinum* (Nirmal Babu *et al.* 1996a) and *P.barberi* (Nirmal Babu *et al.* 1996b) have been standardised. Plants were regenerated from leaf and stem explants of *P.longum, P.chaba* and *P.colubrinum* through both direct and indirect organogenesis (Rema, John & Mini 1995). In *P.betle*, different explants from shoot, leaf and root developed multiple shoots and regenerated into plantlets either directly or through intervening callus phase (Nirmal Babu *et al.* 1992e; Rema, John & Mini 1995). Conversion of root meristem into shoot meristem and its subsequent development to plantlets were reported in *P.longum* and *P.colubrinum* (Nirmal Babu *et al.* 1993b). Micropropagated plantlets of these species are being evaluated for their field performance at IISR.

Ginger

Rhizome rot caused by *Pythium aphanidermatum,* bacterial wilt caused by *Pseudomonas solanacearum* and leaf spot caused by *Phyllosticta zingiberi* are major diseases of ginger. Shoot borer (*Conogethes punctiferalis)* and rhizome scale (*Aspidiella hartii)* are major pests and plant parasitic nematodes like *Meloidogyne incognita* and *Pratylenchus* sp. are affecting ginger production. These diseases and pests are mostly spread through infected seed rhizomes. Crop improvement programmes in ginger are hampered by lack of seed set leading to limited variability. Somaclonal variation could be an important source of variability that could be exploited to evolve high-yielding, high-quality lines and to develop lines resistant to disease and pests. Tissue culture techniques could also be used for *in vitro* pollination, embryo rescue and possible production of 'seed'

in ginger.

Clonal multiplication

Clonal multiplication of ginger from vegetative buds has been reported by many workers (Hosoki & Sagawa 1977; Nadgauda *et al.* 1980; Pillai & Kumar 1982; Balachandran, Bhat & Chandel 1990; Choi 1991; Ilahi & Jabeen 1992). Tissue culture technique would help in production of infection free planting material.

At IISR, field evaluation of tissue cultured plants indicated that they cannot be used directly for commercial planting as two crop seasons are required for the micropropagated plants to develop rhizomes of normal size that can be used as seed rhizomes.

Regeneration of plantlets from callus

Regeneration of plantlets through callus phase has been reported (Nirmal Babu, Samsudeen & Ratnambal 1992; Kacker *et al.* 1993) from leaf explants. The techniques for plant regeneration from leaf, ovary and vegetative buds were standardized. This system could be used for inducing somaclonal variability in ginger. This is very important in crops where conventional breeding is hampered by lack of seed set. Somaclones could also be used for screening against biotic and abiotic stress.

In vitro *selection*

Kulkarni, Khuspe & Mascarenhas (1984) reported isolation of *Pythium*-tolerant ginger by using culture filtrate. *In vitro* selection for resistant types to *Pythium* and *Pseudomonas* is in progress at IISR using culture filtrates of the pathogen or pathotoxin.

Inflorescence culture and in vitro *development of fruit*

In nature, ginger fails to set fruit. However, by supplying required nutrients to immature inflorescence in culture, it was possible to make the ovary develop *in vitro* into 'fruit' and subsequently plants could be recovered from the fruits. It is also possible to convert the immature floral buds into vegetative buds and their subsequent development into complete plants (Nirmal Babu, Samsudeen & Ravindran 1992).

In vitro *rhizome development*

In vitro rhizome formation was reported in 10 per cent of the cultures when ginger plantlets were maintained in the culture medium for more than 100 days (NRCS 1991; Bhat, Chandel & Kacher 1994). *In vitro* formed rhizome may form a source of disease-free planting material. Probably they can also be coated with alginate to facilitate longer storage and transportation.

Turmeric

Rhizome rot caused by *Pythium graminicolum* is a major production constraint. Curcumin is the important colouring material obtained from turmeric and development of varieties with high recovery of curcumin is the need of the hour.

Micropropagation

Successful micropropagation of turmeric has been reported (Nadgauda *et al.* 1978; Shetty, Hariharan & Iyer 1982; Yasuda *et al.* 1988). Micropropagation of turmeric was standardized at IISR using young vegetative buds as explants (Nirmal Babu *et al.* 1993 a).

Plant regeneration from callus

Organogenesis and plantlet formation

were achieved from the callus cultures of turmeric (Shetty, Hariharan & Iyer 1982; Nadgauda, Khuspe & Mascarenhas 1982). Variants with high curcumin content were isolated from tissue cultured plantlets (Nadgauda, Khuspe & Mascarenhas 1982).

Vanilla

In India vanilla is an introduced crop and practically there is no variability in the cultivated form of this spice. Vanilla, though produces numerous minute seeds, these do not germinate under natural condition. Hence, the crop is commercially propagated by means of stem cuttings. Further, root rot, caused by *Fusarium batatatis* var. *vanillae,* is a devastating disease which wipes out a plantation in a short period. Tissue culture techniques could be used for germination of seeds, rapid multiplication and for getting disease-free planting material.

Micropropagation

Micropropagation of vanilla using apical meristem of aerial roots was standardized for large scale multiplication of disease free and genetically stable plants (Philip & Nainar 1986; Minoo *et al.* 1996).

Vanilla is known to have many meiotic and post-meiotic chromosomal abnormalities (Ravindran 1979). As a result of these aberrations, it is quite possible to get various cytotypes in the seed progenies. Culturing of seeds can thus give many genetically variant types.

In vitro germination of vanilla seeds has been reported (Nirmal Babu *et al.* 1993 a). A population of vanilla progenies are being established at IISR so that the variations among them could be used for crop improvement. Variations among the progenies from seed and ovule cultures were reported based on morphological and isozyme characterization (Minoo *et al.* 1996). Callus induction and shoot regeneration were also reported (Davidonis & Knorr 1991; IISR unpublished).

Saffron

Saffron is a triploid and sterile genotype propagated vegetatively by means of corms.

Micropropagation

Reports are available on the micropropagation and plant regeneration in saffron. However, most of the reports in this crop are on the *in vitro* proliferation of stigma and *in vitro* synthesis of colour components and metabolites. Successful micropropagation and regeneration were reported from corms (Ding *et al.* 1981; Illahi, Jabeen & Firdous 1987; Isa & Ogasawara 1988).

Tree spices

Clove, cinnamon, allspice and nutmeg are important tree spices grown in India. Though all these crops could be propagated vegetatively, non-availability of enough quality planting material is the limiting factor. Long pre bearing period makes crop improvement programmes time consuming. In all these perennial tree crops, identification and clonal multiplication of high-yielding genotypes become an immediate priority. Standardization of micropropagation methods will help in rapid multiplication of 'elite' planting materials in these crops.

Micropropagation

Micropropagation of clove (Jaga-

dishchandra & Rai 1986; Mathew & Hariharan 1990) and cinnamon (Jagadishchandra & Rai 1986) from seedling explants have been reported. Successful micropropagation from shoot tip of mature cinnamon (Mini *et al.* 1996) has been reported. Micropropagation of *in vitro* raised *Murraya koenigii* was reported by Hazarika, Nagaraju & Parthasarathy (1995).

In vitro *proliferation of mace and synthesis of flavour components in culture*

Nutmeg and mace are the two important spices obtained from nutmeg tree. Nutmeg is the kernel while mace is dried aril that surrounds the seed. *In vitro* proliferation of mace tissue has been reported by Nirmal Babu *et al.* (1992 a). A ten-fold increase in the fresh weight of the tissue within two weeks was observed. Proliferated tissue not only retained the colour but also the flavour of original mace. Gas chromatographic analysis of the mace oil extracted from the cultured tissue was similar to that of original in qualitative profile. This technique, if refined further, has tremendous potential for industrial production of mace tissue and *in vitro* production of myristicin and myristic acid.

Seed and herbal spices

Work on seed and herbal spices has been reviewed recently by Rao *et al.* (1994). Micropropagation protocols for 15 seed and herbal spices like coriander, celery, thyme, lavender, anise, savory, ocimum, oregano, basil, sage, fennel, parsley, dill, peppermint and spearmint were standardized at IISR. Plant regeneration was successfully induced from callus cultures of coriander, fennel, lavender, anise and sage (Sajina *et al.* 1996 a).

Production of secondary metabolites

Biotechnology can be utilized to exploit the potential of spices for production of useful plant metabolites. The use of tissue culture for the biosynthesis of secondary metabolites particularly in plants of pharmaceutical significance holds an interesting alternative to control production of plant constituents. This technique is all the more relevant in recent years due to the ruthless exploitation of plants in field leading to reduced availability.

Production of flavour components and secondary metabolites using bio-reactors and culture flasks is ideal for spice crops. Callus and cell cultures were established in nutmeg, clove, camphor, ginger, lavender, mint, thyme, celery etc. Cell immobilization techniques have been standardized in sage, anise and lavender (Sajina *et al.* 1996 b). Accumulation of essential oils by *Agrobacterium tumefaciens* transformed shoot cultures of *Pimpinella anisum* was reported (Salem & Charlwood 1995). Total essential oil accumulation by transformed shoot cultures was lower than the untransformed cultures but the relative amount of principle component in the essential oils of the transformed shoots was similar to those present in the parent plant. *In vitro* production of capsanthin, an alkaloid obtained from the green fruits of *Capsicum annuum* and *C.frutesence,* has been standardized at CFTRI, Mysore (Ravishankar, Johnson & Venkataraman 1993).

Reports on the *in vitro* synthesis of

crocin, picrocrocin and safranal from saffron stigma (Himeno & Sano 1987) and colour components from cells derived from pistils (Hori, Enamoto & Nakaya 1988) are available for further scaling up.

Synseed technology

Synthetic seeds form an ideal system for conservation and exchange of germplasm. 'Synseeds' in black pepper, cardamom, ginger, turmeric, vanilla, lavender, fennel, camphor, cinnamon and seed spices were produced by encapsulating the embryoids and shoot buds in sodium alginate (Sajina *et al.* 1996 b).

In vitro conservation of spices germplasm

India is known as the home of spices. However, ecological disturbances in recent years have eroded much of these valuable materials from its natural habitats. Conservation of these valuable germplasm, which is our national heritage, is important. Preservation of the germplasm in *in vitro* gene bank is a safe alternative to protect these from the vagaries of nature.

The National Repository for Spices Germplasm at IISR maintains the world's largest collection of black pepper germplasm. It also maintains large collections of cardamom, ginger, turmeric, nutmeg, clove, cinnamon and allspice germplasm. They are at present conserved in clonal field repositories. Establishment of *in vitro* gene bank will be a safe alternative in protecting the genetic resources from epidemic diseases. It also helps in maintaining species native to different agroclimatic zones.

Conservation of cardamom and ginger germplasm in *in vitro* gene banks by slow growth was reported (Dekkers, Rao & Ghosh 1991; Geetha, Manjula & Sajina 1995). The *in vitro* gene bank of spices germplasm functions both at IISR and National Bureau of Plant Genetic Resources. A total of 350 accessions of spices germplasm are currently kept in *in vitro* repository of IISR.

Protoplast isolation and culture

Successful isolation of protoplast is a prerequisite for many genetic transformation experiments for developing transgenics. Successful isolation of protoplasts was achieved in black pepper, ginger, cardamom and vanilla (IISR, unpublished). Organogenesis from isolated protoplasts has been achieved in a few spice crops like chillies (Fari & Czako 1981; Agarwal 1988), fennel (Miura & Tabata 1986), fenugreek (Sen & Gupta 1979; Multani 1981), peppermint (Sato *et al.* 1993) and saffron (Isa, Ogasawara & Kaneko 1990).

Genetic transformation

Preliminary studies have been carried out on *Agrobacterium* transfer system in *P.nigrum* (Sasikumar & Veluthambi 1996). Transformation system can be used for the transfer of disease resistance to *Phytophthora* foot rot from *P.colubrinum* to *P.nigrum*.

Isolation of DNA and studies on biochemical/molecular markers

The recent advances in the mapping of the genome of important crop species through RFLP analysis and the use of PCR technology will not only be useful in breeding programmes but also crucial in the identification and cloning of important genes and the understanding

of their inter relationship at molecular level.

Isozyme studies using PAGE of esterases and SOD indicated the presence of high variability among seed generated progeny of vanilla.

DNA was isolated from 3 species of *Piper* and two lines of ginger. RAPD profiles of black pepper and long pepper were recorded using 15 types of random primers. Most of them gave positive differences between these two species.

Conclusion

Protocols for micropropagation are available for most of the spices. However, intensive research is needed for application of genetic manipulation and transgenic techniques for production of resistant types to biotic and abiotic stresses. Anther and pollen culture for production of homozygous lines will increase the speed and efficiency of conventional plant breeding. Use of molecular markers for genetic characterisation of germplasm as well as plants needs to be given importance especially in post GATT scenario. Though programmes have been initiated in many laboratories for the production of secondary metabolites *in vitro*, these techniques are to be refined and scaled up for possible industrial use. Because of their commercial possibilities, intensification of biotechnological research in spices is important in the coming years.

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In vitro propagation of black pepper(*Piper nigrum* L.)

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Abstract

An *in vitro* propagation method for black pepper was developed using shoot tips and axillary buds from field grown mature vines. Shoot proliferation was induced on White's medium with 1.5 mgl $^{-1}$ benzyladenine (BA) and 0.01 - 0.05 mgl $^{-1}$ napthalene acetic acid (NAA). Each explant produced 6 to 8% shoots within 12 to 14 weeks in 4 successive transfers. Rooting was 100 % in White's medium with or without activated charcoal. Rooted shoots established in the soil with 85 to 90 per cent success. Field survival was over 95 %.

Key words: black pepper, micropropagation.

Introduction

Black pepper (*Piper nigrum*) is an important spice crop cultivated mainly in Kerala, Karnataka and Tamil Nadu. One of the major production constraints of black pepper is foot rot disease caused by *Phytophthora*. Apart from the conventional multiplication technique, *in vitro* propagation also has great relevance in the rapid multiplication of any elite line.

This paper describes a method for micropropagation of black pepper and the clonal variation in responce to the propagation system.

Materials and methods

Shoot tips along with two axillary buds of black pepper vines from the field were collected. Without removing sheath, the shoot tips were surface sterilized sequentially with 70% ethanol for 1 min, 0.05% mercuric chloride ($HgCl_2$) for 20 min followed by rinsing with sterile distilled water. After removing the terminal bud, cross sections (approximately 5 mm length) containing one axillary bud, were cut asceptically and placed individually in test tubes containing 10 ml of culture medium. The cultures were incubated in the dark at 20°C. After 24 h of incubation, explants were further sterilized with 0.05% mercuric chloride for 15 min and washed with sterile distilled water. Explants were then inoculated on to fresh medium after trimming the cut ends.

The culture media used were based on the formulations of Murashige & Skoog (1962) and White (1943). The pH of the media was adjusted to 5.6 $\pm$ 0.02 before autoclaving, and solidified with 0.8% (w/v) agar. For preliminary experiments on

explant establishment, growth and shoot multiplication, the media were normally supplemented with BA at 0.5, 1.0, 1.5, 2.0 and 2.5 mgl $^{-1}$. The cytokinin was used alone or in combination with either NAA or IBA at 0.01 and 0.05 mgl $^{-1}$. The cultures were incubated at 25 ± 2^{0} C under 16 h photoperiod.

Freshly sprouted shoots were transferred to fresh medium after 2 to 3 weeks initially and thereafter, every 3 to 4 weeks. For rooting, shoots were excised at 2 to 3 cm height and placed on the medium supplemented with or without auxins and activated charcoal.

Rooted plantlets were washed free of agar with running tap water and 5 to 6 plants were placed in pots containing potting mixture of sand and peatmoss (1:1). The plants were placed under shade provided by coirmat and irrigated weekly for the first 30 days. In pot culture the plants were covered with polythene sheet to maintain high humidity. The plants were transferred to field 12 to 16 weeks later. All the experiments were repeated atleast five times with 25 replicates per treatment.

Results and discussion

Preliminary observations showed that White's medium was superior to MS medium as the latter induced more phenolics at the explant establishment stage and produced excessive callus at the base of the explants at the shoot multiplication stage. Hence White's medium was used throughout the study as a basal medium. Results obtained after 90 days of culture on the basal medium with the auxin and cytokinin are summarised in Table 1. BA promoted shoot elongation, leaf expansion and shoot proliferation. Shoot proliferation, however, varied between the varieties studied with Panniyur-1 rating first.

The lateral buds, particularly nodal segments, showed better explant growth but in general their proliferation rate was lower than shoot tips. Initial culture of lateral buds usually developed only 1 or 2 shoots, atleast for two subscultures, resulting in lower shoot multiplication.

Suppressed growth and abnormal leaf formation was noticed in both varieties when inoculated onto media with BA concentration higher than 4.0 mgl $^{-1}$. Similarly NAA concentration higher than 0.5 mgl $^{-1}$ promoted callus formation. When IBA was used instead of NAA, even the lower most concentration (0.05 mg l $^{-1}$) produced enormous callus and the percentage of good quality shoots was very poor. At times these cultures also produced roots in the multiplication medium as observed in the earlier work (Philip *et al.* 1992). Variation in proliferation was observed between these two varieties grown on media containing different com-

Table 1. Effect of BA & NAA on shoot multiplication in two cultivars of black pepper (after 90 days).

BA (mgl $^{-1}$)	NAA 0.01 mgl^{-1}	NAA 0.05mg l^{-1}
PANNIYUR		
0.5	1.07 ±0.03*	0.98 ± 0.04
1.0	2.13 ± 0.04	4.07 ± 0.16
1.5	5.32 ± 0.18	8.10 ± 0.07
2.0	7.12 ± 0.21	6.98 ± 0.15
2.5	4.92 ± 0.17	4.38 ± 0.13
KARIMUNDA		
0.5	1.51 ± 0.06	1.23 ± 0.04
1.0	4.10 ± 0.12	3.90 ± 0.08
1.5	6.30 ± 0.03	4.80 ± 0.12
2.0	5.07 ± 0.09	4.90 ± 0.02
2.5	4.67 ± 0.11	4.32 ± 0.04

* SD of three replications

binations of BA and NAA. The auxin requirement also varied for both cultivars.

For initiation of roots, single shoots were isolated from the shoot clump and transferred to both White's and MS media of half and full strength containing 0.05 mgl^{-1} and 1 mgl^{-1} NAA and without any growth regulators. The number of roots and average root length decreased with increasing auxin concentration. Considering these factors and percentage of rooted shoots, full-strength White's medium without growth regulators was selected as the best rooting medium (Table 2). This finding is contrary to the earlier reports (Nazeem *et al.* 1990; Philip *et al.* 1992) which showed that MS with 1 mgl^{-1} NAA was optimal for rooting of shoots. When 1 mgl^{-1} NAA was used, the number of roots increased, but the root elongation was suppressed by the high concentration of NAA and also callus formation was observed at the basal cut end within 4 - 5 days. Though number of adventitious roots induced were less compared to the earlier report, they elongated within 14 days in this rooting medium which helped the *in vitro* plantlets for transferring to nursery within 2 weeks thereby reducing the period under *in vitro* condition.

The rooted cultures were transplanted to pots and the percentage of establisment was 85 - 90. About 2400 plants propagated by this procedure have been transferred to the field and the plants established well. This protocol is now being used for propagating some of the superior hybrid pepper selections namely HP-34, HP-105 and HP-813.

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Table 2. Effect of media strength and additions on rooting of shoots *in vitro*.

Medium	Rooting % after		No. of	Elongation
	7 days	14 days	roots	of shoots (cm)
Half strengthMS without growth regulators	69	92	2-3	3.0-3.4
Half strength MS+ Charcoal 0.15% without growth regulators	54	88	2-3	3.0-3.4
Half strength MS+ NAA 0.5 mgl^{-1}	72	100	2-3	3.0-3.2
Full strength White's without growth regulators	100	100	3-5	3.0-4.0

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In vitro responses of *Piper* species on activated charcoal supplemented media

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Abstract

Methods were developed to effectively minimise/control the browning of the culture medium and explants in different species of *Piper* namely *P.longum*, *P.attenuatum*, *P. betle* and to some extent in *P. nigrum*, both cultivated (Panniyur-1) and wild accessions. Explants from stem (nodal and internodal segments), petiole and leaf were planted on MS basal medium supplemented with different concentrations and combinations of growth regulators and activated charcoal (AC) (50-250 mgl $^{-1}$). All the treatments except control (without AC) showed a positive effect in controlling the rapid and inhibitory browning of medium and explants. Out of the 5 treatments, AC (200 mgl $^{-1}$) proved to be the best with least browning. Among the species studied, *P. longum* showed less browning whereas *P. nigrum* collected from wild showed maximum browning. The morphogenetic response of *P. longum* was maximum in terms of number of plantlets regenerated.

Key words : activated charcoal, *Piper*.

Introduction

One of the reasons for low success rate *in vitro* culturing of *Piper* species (especially *P. nigrum* L.) is the rapid browning of the medium as well as explants (Jacquiot 1964; Nitsch & Strain 1969). This is mainly due to the presence of phenolics, which were reported to undergo oxidation to produce toxic polyphenols/ quinones, which on accumulation have an inhibitory effect on the growth of explants, resulting in ultimate death of the tissues (Scalbert , Montiers & Favre 1990).

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The genus *Piper* contains high concentration of phenols and due to this, explants when cultured *in vitro* turn brown quickly resulting in a considerable loss of cultures. The aim of the present study was to alleviate the damage due to browning by using activated charcoal (AC) in various concentrations and thereby increasing the morphogenetic capability of the various explants of different species of *Piper*.

Materials and methods

Nodal, internodal, petiole and leaf explants from cultivated black pepper (Panniyur-1), wild black pepper, *P. betle*, long pepper (*P.longum*) and *P.attenuatum* were utilised in this study. The explants were taken from actively growing plants and washed in running tap water for 30 min followed by rinsing with Tween-20 for 5-10 min and then in DDH_2O. The explants were then surface sterilized with 0.1% mercuric chloride ($HgC1_2$) for 3-5 min and washed 3-4 times with sterile DDH_2O to remove all traces of $HgC1_2$

The explants on MS basal medium (Murashige & Skoog 1962) with different concentrations and combinations of growth regulators and activated charcoal (50-250 mgl^{-1}) (Sarabhai Merck Chemicals, Baroda). The pH of the medium was adjusted to 5.8 and was gelled with agar.

Subculturing of the explants calli was done once in 20 days. All experiments were carried out thrice, with 20 replications. The extent of phenolic leaching into medium was assessed on the basis of colour change in medium from creamish brown to almost black and also on the basis of whether the browning was confined to the surrounding area of the explant or has spread to the entire medium. AC was not added in the control.

Results and discussion

Leaching of polyphenols into the medium was effectively controlled to a certain extent in all the explants tried (except in wild *P. nigrum*), by incorporating AC into the culture medium. In case of wild *P. nigrum*, the first sign of browning in the explants was visible right after dissection, in other cases the explants turned brown soon after their transfer to the culture media. The same was true in the case of control in all the species studied, but with varying intensities i.e. highest browning in *P. nigrum* and least in *P. longum*.

In the present investigation, addition of AC (100-250 mgl^{-1})) greatly reduced the incidence of browning of the explants of *P. longum*. In *P.betle* and *P. attenuatum* AC was also found to be helpful in reducing the browning to a certain extent besides in *P. nigrum* cultivar (Panniyur-1). However, it was not much helpful in the case of *P.nigrum* (wild). The intensity of browning was reduced during subsequent subculturing. Maximum browning was noticed in *P.nigrum* (wild) and least in *P. longum* (Table 1). The *in vitro* response of *P.longum*, *P.betle* and *P.attenuatum* was also studied in the basal medium without AC and in few instances (less than 20%) some growth was observed.

In order to control browning of medium and explants *in vitro*, many workers have tried incorporating non-specific adsorbents like activated charcoal (AC), antioxidants like and poly vinyl pyrollidone (PVP) ascorbic acid (AA) into the culture medium but only met with limited success (Fridborg *et al.* 1978; Weatherhead, Burdon & Henshaw 1979; Bharadwaj & Ramawat 1993). AC can absorb inhibitory compounds secreted from phenolic inhibitors (George & Sherrington 1984). Recently, Druart &

Table 1. Effect of activated charcoal on plant-regeneration and browning of medium/explants.

Species	Explant	Degree of browning at the end of 1st week of inoculation — Conc. of AC (mgl $^{-1}$)					Culture after 7 weeks	
		0	50	100	200	250	Browning (%) at 200mgl $^{-1}$ of AC	Plantlet regeneration(%)
P.nigrum (wild)	Stem	++++	++++	++++	+++	+++	95.0	-
	Petiole	++++	++++	++++	+++	++	90.0	-
	Leaf	++++	++++	+++	++	++	89.5	-
P.nigrum Cultivar (Panniyur-1)	Stem	++++	++++	++	+	+	88.8	-
	Petiole	++++	++++	++	+	+	85.0	-
	Leaf	++++	+++	++	+	+	85.0	-
P.attenuatum	Stem	+++	+++	++	-	-	35.0	**58.3**
	Petiole	+++	++	+	-	-	30.0	-
	Leaf	+++	+	+	-	-	27.2	-
P.betle	Stem	+++	++	+	-	-	33.3	**66.6**
	Petiole	++	+	-	-	-	31.2	-
	Leaf	++	+	+	-	-	25.0	-
P.longum	Stem	++	++	+	-	-	15.0	83.3
	Petiole	++	+	+	-	-	12.5	56.2
	Leaf	++	+	-	-	-	15.4	73.3

++++ dark brown (almost black), +++ brown, ++ light brown, + light grey (below the explant)
- no colouration (no exudation)

Wulf (1993) reported that addition of AC to the medium reduces browning as well as the catalysis of sucrose hydrolysis during autoclaving.

Though AC is known to absorb toxic substances and impurities present in the media, it is also found to absorb growth regulators (auxins), thus limiting the utilization of auxins in the culture and therefore, the potency of these growth regulators (Constantin, Henke &Mansur 1977; Ebert & Taylor 1990).

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Micropropagation of ginger *(Zingiber officinale* Rosc.)- interaction of growth regulators and culture conditions

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Abstract

Zingiber officinale Rosc. cvs. Suruchi and Suprabha were micropropagated on Murashige & Skoog (1962) medium supplemented with 4.0 - 6.0 mgl $^{-1}$ BA , 1.0 -1.5 mgl $^{-1}$ IAA, 100 mgl $^{-1}$ adenine sulphate and 3% (w/v) sucrose. The effect of media variables and light conditions on micropropagation was investigated. Shoot bud multiplication decreased as BA concentration increased from 6 mgl $^{-1}$ to 8 mgl $^{-1}$; while IAA had intermediary effect; NAA, IBA and 2, 4-D were ineffective to induce multiple shoots. Though high rate of multiplication of shoots was noted under both 14 h and 24 h illumination, shoot elongation was poor under the 14 h photoperiod. The rate of shoot multiplication was positively correlated with the concentration of hormones in the nutrient media. The isolated shoots were rooted on half strength basal MS mineral salts supplemented with 0.5-1.0 mgl $^{-1}$ IBA (Indole-3-butyric acid) or IAA and 2% (w/v) sucrose. Liquid media (agitated or static) were less effective compared to the solid (agar-gelled) media for root development. The *in vitro* derived plantlets were hardened in the green house and successfully establilshed in soil.

Introduction

Ginger (*Zingiber officinale* Rosc.) is an important spice and medicinal crop. Ginger is vegetatively propagated through rhizome and two of the serious diseases of ginger viz., bacterial wilt and soft rot are spread through infected rhizome.

In vitro culture techniques especially meristem culture provide an alternative way of plant propagation which help to propagate disease free planting material. Clonal multiplication of ginger through multiple shoot induction has been reported (Hosoki & Sagawa 1977; Bhagyalaksmi & Singh 1988; Hu & Wang 1983; Wang 1989; Balachandran, Bhat & Chandel 1990). No systematic work however, has been done on the factors such as light requirements affecting shoot mul-

tiplication of ginger. The present paper deals with the influence of various growth regulators and culture conditions on high frequency shoot multiplication of ginger.

Materials and methods

Plant material

Rhizomes of ginger cvs. Suruchi and Suprabha were incubated in a bed of sterile moist sandy soil to induce sprouting. The palewhite sprouting buds were collected and washed with 2% (v/v) detergent solution 'Teepol' for 15 min and subsequently surface sterilised by using 0.2% (w/v) aqueous solution of mercuric chloride for 25 min followed by several rinses in sterile distilled water. The shoot buds were asceptically dissected under a dissecting microscope and meristems with the apical dome or with one or two leaf primordia ranging in size from 0.5-1.0 mm were removed and used as explants.

Culture medium

Isolated shoot meristems were placed on Murashige and Skoog (MS) basal salts supplemented with various concentrations of cytokinins i.e. 6-benzyladenine (BA: 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 mgl $^{-1}$, kinetin (Kn: 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0, mgl $^{-1}$), adenine sulphate (Ads: 50, 100 and 150mgl $^{-1}$and auxins like indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 1-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) in various combinations and pH of the media was adjusted to 5.8.

Culture conditions

During bud sprout, all cultures were incubated under a 14h photoperiod from cool, white flourecent lamps at 25±2^{0} C. For shoot multiplication, the culture flasks were incubated under 14h photoperiod or continuous illumination with a constant light intensity of 55 $EM^{-2}s^{-1}$

Scoring of data

All the cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. There were 20 cultures per treatment and each experiment was repeated atleast three times. Subculturing was carried out at an interval of 4 weeks. The effects of different treatments were quantified on the basis of percent of cultures showing response and the number of shoots/explant, percentage of rooting and percentage of survival. The data pertaining culture response to multiple shoots were analysed using Duncan's Multiple Range Test.

Introduction of rooting and transplantation

The *in vitro* grown shoots (about 0.5-1.0 cm) were excised from the parent culture and inoculated into liquid or semi-solid basal half-strength MS medium supplemented with IAA or IBA (0,0.25, 1.0 and 1.5mgl $^{-1}$) and 2% (w/v) sucrose for induction of root. The incubation conditions were the same as mentioned earlier for bud break.

The rooted plantlets were removed from the culture vessels, washed gently under running tap water and planted in earthen pots containing a mixture of sand, soil (3:1). The plantlets were kept in the green house for acclimatization and were subsequently transferred to the open field conditions.

Results and discussion

Effect of growth regulator on bud break

Depending on the type, concentration of growth regulators used, the number of days required to bud break varied from 6-10 in two cultivars. However, in most of the treatments, the percentage of explants showing bud break was more than 80-90 and 75-80 in cvs. Suruchi and Suprabha, respectively. In media devoid of growth regulators, the time required for bud sprout was always more than 20 days. Among all the growth regulators tested, the media containing BA (2.0-4.0 mgl $^{-1}$) induced bud sprout in about 6-7 days of culture. Kinetin was ineffective in stimulating bud sprout as compared to BA.

Effect of growth regulators on shoot multiplication

The rate of multiplication varied with the cultivars. The medium containing BA (4.0-6.0mgl $^{-1}$) 1.0-1.5mgl $^{-1}$ IAA and 100 mgl $^{-1}$ adenine sulphate showed higher rate of shoot multiplication in both the cultivars (Table 1). The percentage of cultures with multiple shoots varied from 52.8 to 86.8 and 58.6 to 72.4 in cvs. Suruchi and Suprabha, respectively, after 4 weeks of culture. Similar effects were reported earlier in other cultivars (Nadgauda *et al.* 1980). The number of shoots/explant was also different depending the cultivar and growth regulators. Shoot bud multiplication decreased as the concentration of BA increased from 6.0-8.0mgl $^{-1}$ while IAA had intermediary effect. NAA, IBA and 2,4-D were ineffective in inducing multiple shoots (data not shown). Similar results have been reported on the other varieties of ginger (Hosoki & Sagawa 1977; Nadgauda *et al.* 1980). The present study indicates that the inclusion of IAA at a concentration higher than 2.5mgl $^{-1}$ suppressed the rate of shoot multiplication and induced

Table 1. Effect of various treatments on induction of multiple shoots (A) and number of shoots per culture (B) in ginger (4 weeks of culture under 24 h photoperiod).

MS basal salt + growth regulators mgl $^{-1}$			Cultivar			
			Suruchi		Suprabha	
BA	IAA	Ads	A	B	A	B
0	0	0	0	0	0	0
2.0	1.0	50	10.2 ± 0.2 a	6.2 ± 0.3 a	14.6 ± 0.8 a	7.2 ± 0.2 a
4.0	1.0	100	23.4 ± 0.6 b	12.3 ± 0.6 b	26.4 ± 0.6 b	16.8 ± 0.3 c
6.0	1.0	100	58.8 ± 0.8 f	16.4 ± 0.3 c	62.6 ± 0.4 f	20.3 ± 0.6 c,d
4.0	1.5	50	52.6 ± 0.4 e	18.8 ± 0.4 c	66.2 ± 0.2 g	24.6 ± 0.4 e
6.0	1.5	100	82.4 ± 0.5 h	28.3 ± 0.6 f	88.4 ± 0.4 i	32.8 ± 0.6 f
4.0	2.0	100	38.6 ± 0.4 c	19.4 ± 0.6 d	41.2 ± 0.8 d	16.3 ± 0.8 c
6.0	1.5	150	86.6 ± 0.4 i	31.2 ± 0.8 g	72.4 ± 0.5 h	26.4 ± 0.4 e
4.0	1.5	100	64.4 ± 0.5 g	24.4 ± 0.6 e	58.6 ± 0.6 e	18.9 ± 0.6 c
6.0	2.0	100	42.6 ± 0.3 d	20.2 ± 0.4 d	36.5 ± 0.7 c	12.2 ± 0.4 b

Means having the same letter in a column are not significantly different as per Duncan's Multiple Range Test ($P \leq 0.05$).

stunted growth. The frequency of shoot multiplication was increased by 6 fold at every 4 week interval. The number of multiple shoots per explant increased upto 6th sub culture and thereafter the rate of multiplication became almost static (Table 1).

Effect of photoperiod on shoot multiplication

The multiplication frequency and elongation were higher under continuous light (24 h) than 14 h photoperiod. The rate of shoot multiplication was, however, inhibited under both photoperiods when the medium was supplemented with high dose of IAA (<2. 5mg l^{-1}). The ability of the cultures to produce multiple shoots declined significantly when cultured on MS medium with either BA + NAA or TBA under 14 h or continuous illumination. The effects of light on *in vitro* morphogenesis have been reported earlier (Murashige 1974; Seibert & Kadkade 1980). The variation of shoot multiplication was observed in both the cultivars tested under 14 h and 24 h photoperiod (Fig.1). The result showed that the rate of shoot multiplication was positively correlated with the concentration of hormones in the nutrient media and illumination conditions.

Induction of rooting and fields establishment

The growing shoots (1-2 cm) were excised from parent culture and implanted into medium containing half strength MS basal salts supplemented with different concentrations of IAA and IBA in both solid and liquid medium. The results showed that the liquid medium was less effective as compared to the solid medium for root development. Percentage of rooting was higher in half strength MS basal salts supplemented with 0.5 to 1.0 mg l^{-1} IBA as compared with IAA (Table 2). Root initials formed within 7-8 days which developed into a good root system in 12-14 days. Root initiation was delayed at higher concentration of IAA or IBA or without growth regulators. Root initiation could also be achieved at a higher concentration of IAA or IBA but with loss of chlorophyll in the growing shoots. Subsequently, the shoot became yellowish in colour. The percentage of rooting was maximum (90-95%) in both the cultivars

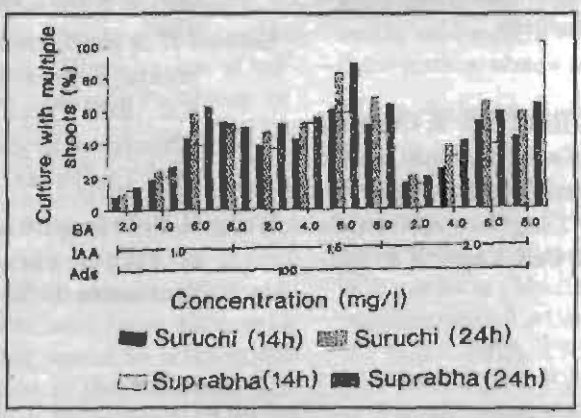

Fig. 1. Effect of growth regulators and photoperiod on shoot multiplication of ginger

Table 2. Effect of various media (half strength basal MS + 2% sucrose + different concentrations of auxins) on rooting of shoots in ginger (after two weeks of culture).

Auxin (mgl^{-1})		Percentage of shoots rooted	
IAA	IBA	Suruchi	Suprabha
0	0	0	0
0.25	0	62.4 ± 0.8	68.4 ± 0.4
0.5	0	80.2 ± 0.6	82.6 ± 0.8
1.0	0	86.2 ± 0.4	86.1 ± 0.2
1.5	0	54.8 ± 0.6	58.2 ± 0.3
0	0.25	67.6 ± 0.4	78.2 ± 0.6
0	0.5	83.2 ± 0.8	87.4 ± 0.3
0	1.0	92.4 ± 0.4	94.4 ± 0.2
0	1.5	68.4 ± 0.5	60.6 ± 0.8

with half MS supplemented with $1.0mgl^{-1}$ IBA (Table 2). The rooted plantlets were transferred to pots containing sand: soil (3:1) and kept in the green house for acclimatization. After 1 month of transfer 95-98% of the plants survived in the field conditions.

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Micropropagation of turmeric (*Curcuma longa* L.) by *in vitro* microrhizomes

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Abstract

A method was developed to induce microrhizomes at the base of the turmeric plantlets. MS medium supplemented with 0.3 mg l^{-1} BAP, 0.1 mgl^{-1} NAA, 0.5 mgl^{-1} ancymidol and 10% sucrose was found to be the best for induction of microrhizomes. Microrhizomes harvested at a maturity period of 8 weeks in the induction medium was the best stage for germination under field conditions, without an intervening phase of hardening.

Key words: *Curcuma longa*, microrhizomes.

Introduction

Turmeric of commerce is the processed and dried rhizomes of plant *Curcuma longa* L., belonging to family Zingiberaceae. Turmeric is conventionally propagated by whole or split mother or finger rhizomes. Propagation of turmeric by *in vitro* methods has been reported (Nadgauda *et al.* 1978; Balachandran, Bhat & Chandel 1990). One of the disadvantages of the micropropagated turmeric plants is the poor rhizome formation observed in field conditions in the first year than conventional plants obtained from rhizomes. Another disadvantage of *in vitro* methods of propagation is the laborious and difficult hardening stage required by the tissue cultured plantlets. The cost of hardening tissue culture plantlets has been estimated to be 40 to 80 % of total production cost. This paper describes a modified *in vitro* method of propagation of turmeric by microrhizomes induced at the base of the plantlets and planted directly under field conditions, without an intervening phase of hardening.

Materials and methods

One high curcumin veriety of turmeric collected from Wynad, was selected for this study. Newly sprouted, unopened buds from rhizomes were inoculated in MS medium (Murashige & Skoog 1962) supplemented with 3.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA, using standard inoculation procedures. By repeated subcultures at 8 week intervals, required quantity of stock cultures for the experiments were developed.

For the induction of microrhizomes, single cultures from the stock cultures were cultured in different media combinations containing MS medium supplemented with BAP (0.1, 0.3 and 0.5 mgl^{-1}), IAA (0.5 and 1.0 mgl^{-1}), NAA (0.1 and 0.5 mg l^{-1}) and with / without 0.5 mgl^{-1} ancymidol in various combinations with different concentrations of sucrose (30, 50, 100, 150 and 200 g l^{-1}). The experimental cultures were incubated in light at 2000 lux and 16 h photoperiod. Twenty five percent of the experimental cultures from all media combinations were harvested after 8 weeks of incubation in induction medium and isolated the microrhizomes developed at the base of the plantlets by removing 2 aerial shoots by cutting and peeling the leaf sheaths. Twenty five percent each of the remaining cultures were harvested after 12, 16 and 20 weeks respectively of incubation in induction medium and were analyzed for microrhizome parameters like length and weight in all media combinations.

The harvested microrhizomes were then washed thoroughly in water and treated with 0.2% Indofil M45 for 15 min. Fifty percent of the treated microrhizomes from all media combinations, harvested at different maturity periods like 8, 12 16 and 20 weeks, were planted seperately on soaked cotton kept in petri plates under laboratory conditions and the remaining 50 percent of the treated microrhizomes were planted under field conditions by broadcasting on levelled beds. Percentage germination of microrhizomes in different experiments under laboratory and field conditions were taken. All experiments and trials were repeated for three times.

Results and discussion

Sufficient quantitiy of stock cultures for the experiments were developed from the inoculated cultures by about 12-16 weeks.

In the different media combinations tested for the induction of *in vitro* microrhizomes, MS medium supplemented with 0.3 mgl^{-1} BAP + 0.1 mgl^{-1} NAA + 0.5 mgl^{-1} ancymidol with 10% sucrose was the best by producing the biggest size and highest in weight of the microrhizomes (Table 1). Media combinations without ancymidol or with surcose concentrations lesser or higher than 10% were found to be inhibitory in microhizome formation. Development of microrhizomes in media combinations containing ancymidol and 10% sucrose showed variations. Microrhizomes harvested after maturity periods of 12, 16 and 20 weeks in induction medium did not show any further increase in size or weight than harvested after 8 weeks.

The microrhizomes planted under laboratory and field conditions started germination 2-3 days after planting. The highest percentage of germination of 93% under laboratory conditions and 89% under field conditions were observed in samples of microrhizomes harvested after 8 weeks of maturity in induction medium. Percentage germination of microrhizomes gradually increased when the maturity of microrhizomes in induction medium was increased to 12, 16 and 20 weeks both under laboratory and field conditions.

The present study shows the role of ancymidol and high sucrose concentration in culture medium in inducing microrhizomes in turmeric. The role of high sucrose concentration in culture medium in inducing microtubers in potato (*Solanum tuberosum*) has been reported earlier (Wang & Hu 1982; Hussey & Stacey 1984). The effect of ancymidol,

Table 1. Effect of growth regulators on induction of *in vitro* microrhizomes in turmeric. *

Growth regulator (mgl^{-1})	Sucrose (gl^{-1})	Microrhizome **	
		Length (mm)	Weight (mg)
BAP 0.1+1AA 0.5+ ancymidol 0.5	100	6	943
BAP 0.1+1AA 1.0+ ancymidol 0.5	100	5	825
BAP 0.1+NAA 0.5+ ancymidol 0.5	100	7	1075
BAP 0.1+NAA 0.5+ ancymidol 0.5	100	6	959
BAP 0.3+1AA 0.5+ ancymildol 0.5	100	5	890
BAP 0.3+1AA 1.0+ ancymidol 0.5	100	6	969
BAP. 0.3+ NAA 0.1+ anciymidol 0.5	100	8	1250
BAP 0.3+NAA 0.5+ ancymidol 0.5	100	7	1095
BAP 0.5+1AA 0.5+ ancymidol 0.5	100	6	977
BAP 0.5+1AA 1.0+ ancymidol 0.5	100	5	850
BAP 0.5+NAA 0.1+ ancymidol 0.5	100	7	1020
BAP 0.5+NAA 0.5+ ancymidol 0.5	100	5	866

* Data on media combinations without ancymidol and with sucrose concentrations lesser or higher than 10% are not given.

** Fresh size and weight of average 25 microrhizomes, harvested after 8 weeks of incubation in induction medium.

a growth retardant, in inducing microrhizomes in turmeric is evident from the results, but its role is not known. It may act by suppressing vegetative growth, thereby inducing rhizome formation.

The present method described for the micropropagation of turmeric by *in vitro* microrhizomes is a definite and simple one. The microrhizomes can be planted directly under field conditions, thereby eliminating the difficult and laborious stage of hardening, which the normal tissue culture plantlets require. Turmeric plants raised through *in vitro* microrhizomes may also solve some of the problems like poor rhizome formation observed in normal tissue culture plants planted under field conditions.

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In vitro shoot initiation from explants of field grown trees of nutmeg (*Myristica fragrans* Houtt.)

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Abstract

Standardisation of protocol for *in vitro* multiplication of elite nutmeg trees is important because of the dioecious nature of the plant. Explants collected from field grown trees with a systemic fungicide were pretreated and cultured on different basal media. Among the different basic media tried, SH medium was found to be the best. Response was obtained only from single node segments of orthotropic shoots from field-grown trees. Nodal segments gave better results than terminal bud. Bud burst and leaf expansion from pre-existing axillary meristem could be achieved in 3 to 4 weeks in SH medium supplemented with BA and AC. Consistent results could be obtained from trees of different locations indicating lack of genotypic influence.

Key words: micropropagation, *Myristica fragrans*, nutmeg.

Introduction

Nutmeg (*Myristica fragrans* Houtt.) is an important tree in the spices industry valued for the two spices obtained from its fruits- 'nutmeg', the kernel of the seed and 'mace',the aril that surrounds the seed. Both nutmeg and mace are valued as spices and in medicine. Nutmeg is a dioecious tree usually propagated through seeds. Seed propagation always poses problems because of the occurrence of about 50 per cent of male trees in the progeny which is much more than the required number of pollinators in the population. The sex cannot be determined until the trees begin to flower. Vegetative propagation has considerable use for the rapid multiplication of elite female trees. The conventional methods used are grafting, layering etc. and these are also not easily and quickly achieved. Therefore, efficient micropropagation technique would be useful for mass multiplication of selected female trees within a short period of time. So far not much work was done in the *in vitro* culture of nutmeg explants from field grown trees. Yapabendera, Litz & Desanayake (1986) reported *in vitro* shoot tip culture of nutmeg from three year old plants in Anderson medium supplemented with BA 2 and NAA 0.2 ppm.

The present paper reports micro propagation of nutmeg from explants of field grown trees.

Materials and methods

Green orthotropic shoots having a basal diameter of 7-8 mm arising from plagiotropic branches of field grown elite trees were used for preparing the explants.

In the freshly harvested shoots, the leaves were trimmed retaining part of the lamina and these shoots were swabbed with 70% ethanol. They were cut into single node segments. These explants were treated with the systemic fungicide,Bavistin. The fungicide treatment was given in an orbital shaker at a speed of about 1000 rpm. The duration as well as concentration of Bavistin were standardised during the study.

The fungicide was drained and the explants were surface sterilized with 0.1% $HgCl_2$ for 15 min and thoroughly washed 3 times with sterile distilled water. The explants were allowed to dry for 15-20 min on sterile blotting paper and were inoculated in the media contained in culture tubes of 150 x 25 mm. Both axillary and terminal segments were cultured.

Different basal media initially tried were MS (Murashige & Skoog 1962) half strength MS, Woody plant medium (Lloyd & Mc Cown 1980) and SH (Schenk & Hildebrandt 1972). Based on the preliminary results, SH medium was used for later studies. The effects of different levels of BA (1,2,3 and 4 mgl $^{-1}$) as well as AC (0,0.25,0.5 and 1%) were also studied. The pH of medium was adjusted to 5.8 before autoclaving and was solidified with 0.7% agar. The cultures were grown at 26±2°C under a 14 h photoperiod.

Results and discussion

Fungicidal pretreatment of explants

Pretreatment of explants using a systemic fungicide was found to be essential for overcoming fungal contamination. Since the explants were collected from field grown trees, shaking the explants in an orbital shaker was more effective for better penetration. Among the two concentrations tried, 0.1% was found to be better, higher concentration being toxic, especially at longer durations (Table 1). The optimum combination for maximum culture establishment and minimum toxicity was found to be the treatment with 0.1% Bavistin for 10 min.

Table 1. Effect of Bavistin pretreatment of nutmeg explants on contamination.*

Bavistin concentration(%)	Duration of treatment (min)	Explants (%)		
		Contaminated	Dried	Established
0	-	94.7	3.0	2.3
0.1	5	88.2	7.8	4.0
	10	7.5	26.0	66.5
	15	4.2	73.8	22.0
0.2	5	30.7	54.8	14.5
	10	3.5	64.3	32.2
	15	1.7	93.5	5.0

* Explants - Single node segments of field grown trees cultured in SH + BA 2 mgl $^{-1}$ NAA 1 mgl $^{-1}$ + AC0.5% and observed after six weeks. 60 cultures per treatment

Table 2. Effect of different basal media on bud burst from nodal segments of nutmeg.

Medium	Time taken for bud burst (days)	Percentage explants with bud burst	Percentage explants with leaf expansion
MS	25-30	22.3	5.7
1/2 MS	20-25	50.1	10.6
SH	15-20	60.4	20.8
WPM	25-30	20.7	6.0

Other ingredients : BA 2 mgl^{-1} + NAA 1 mgl^{-1}+ Sucrose 3% + AC 0.5%

Basal media

Among the four different basal media tried, SH medium was found to be better (Table 2). The percentage explants with bud burst as well as leaf expansion was more in this medium. Precocity of bud burst and superiority of burst were also observed in this. The superiority of SH medium may be due to the higher content of K^+ ions or the higher level of inositol in this medium. It may be noted that in nutmeg, a higher dose of K is recommended for its cultivation. Compared to MS medium, the concentration of other mineral salts is less in SH medium. Several tree spices prefer low salt concentrations for their survival and multiplication in culture (Sharma & Thorpe 1990). Yapabendera (1986) used 'Anderson' medium for the establishment and proliferation of nutmeg shoot tip from 3 year old plant.

Position of the explant

The position of the nodal segment on the parent shoot exercised a notable effect on bud burst (Table 3). The shoot tip and the terminal nodal segments usually exhibited lower potential for bud burst and leaf expansion. Often the terminal bud showed some elongation but unfurling of leaves was never observed. Axillary buds, on the other hand, showed a much better response to culture. Variation in response was exhibited by axillary buds of varying ages. This was clear from the positive correlation observed between diameter of stem and the vigour of the shoot. In explants with thick stem, the emerging shoot was healthy with expanded leaves. Hence, explants from base of the shoot were found to be more effective, while those from the explants from immature shoots dried off in the media. Thus there was a gradual basipetal gradient in response of nodal explants in terms of both bud burst and leaf expansion. This indicates physiological differences among different nodes of the shoot. Pereira *et al.* (1995) also reported physiological differences of the explants on micropropagation of *Maytenus ilicifolia*. They obtained more shoots from axillary buds located at distant positions from the apical bud.

The size as well as the nature of the nodal explant also influence the culture response. In the explant, retention of the petiole along with a part of the lamina as well as maximum length of the lower internode was essential for bud break and

Table 3. Effect of position of the nodal segment on the parent shoot on the rate of bud burst and leaf expansion.

Position of the node on the shoot	Bud break (%)	Leaf expansion (%)	Dried (%)
Terminal	10.4	0	89.6
Near terminal	36.4	1.5	62.1
Distant	73.0	8.6	18.4

Medium : SH + BA 2 mgl $^{-1}$ + NAA 1.0 mg l $^{-1}$ + AC 0.5%, Growth period - 6 weeks, 50 cultures per treatment.

Table 4. Effect of BA concentration on bud burst and leaf expansion in nodal explants of nutmeg.

BA concentration (mgl $^{-1}$)	Explants (%)	
	with bud burst	with leaf expansion
1.0	30.0	1.7
2.0	53.3	16.7
3.0	36.7	6.7
4.0	36.7	5.0

Medium : SH + 1 mgl ^{1} NAA + 3% sucrose + AC 0.5%, 60 cultures per treatment.

leaf expansion. Bhojwani, Mullins & Cohen (1987) also reported that retention of the parent tissues plays a significant role in the survival and growth of the bud. He found that in *Feijoa sellowiana*, the presence of the parent tissue along with the basal node gave rise to maximum shoots whereas lateral shoots without any parent tissue dropped their leaves and dried.

Effect of BA

Addition of BA to SH medium with 3% sucrose, 1mgl $^{-1}$ NAA and 0.5 % AC favoured bud burst and shoot growth. Among the various BA concentrations tested viz., 1.0, 2.0, 3.0 and 4.0 mgl $^{-1}$ the concentration 2.0 mgl $^{-1}$ was found to be superior both in terms of bud burst and leaf expansion (Table 4).

Generally a cytokinin or a combination of auxin and cytokinin is required for *in vitro* shoot proliferation (Sharma & Thorpe 1990). In nutmeg also, a combination of auxin and cytokinin helped to induce bud break and subsequent growth of the shoot.

Effect of activated charcoal

Addition of AC 0.25 to 1 % to the medium helped in better culture establishment (Table 5). Cultures without any AC in the medium dried without any bud burst. AC was found to have a profound influence on nutmeg cultures in retention

Table 5. Effect of activated charcoal on shoot initiation from nodal segments of nutmeg.

Activated charcoal (percentage)	Explants (%)		
	with bud break	with leaf expansion	dried
0	2.0	0	98.0
0.25	32.1	3.6	64.2
0.50	38.6	2.2	59.2
1.0	4.8	0	95.2

Medium : SH + BA 2 mgl $^{-1}$ + NAA 1.0 mgl $^{-1}$ + Sucrose 3%, Growth period - 6 weeks, 50 cultures per treatment.

of their green colour, better culture establishment as well as better bud burst. Fridborg & Eriksson (1975) reported generally better growth responses of plant tissues associated with addition of AC to the medium. The beneficial effects of AC are attributed to the removal of inhibitory substances from the media produced either on autoclaving the media or by tissue culture itself (Fridborg *et al.* 1978).

Sharma & Thorpe (1990) also reported that the inclusion of AC in the medium (0.1-1%) has been found to be beneficial during shoot multiplication, development and root formation in cultures of several species. Nutmeg is known to contain different alkaloids and chemicals in its tissues and it is only reasonable to assume that some of these substances by itself or their oxidation products may be acting as inhibitory substances in the culture medium. Removal of these substances probably is effectively done by AC in the medium. In the present study, the increase in the concentration of AC above 0.5 % was found to be ineffective. The excess charcoal may be adsorbing the growth regulators making them unavailble to the explant. Elbert, Taylor & Blake (1993) also reported that AC may reduce the availability of necessary growth regulators in the TC media.

The results of the preliminary trials on the micropropagation of nutmeg showed that agitation of the field explants in a systemic fungicide solution helped to reduce contamination. Maximum response could be obtained with thick single nodal segments from the base of the shoot. SH medium supplemented with BA 2 mg, NAA 1 mgl^{-1}, sucrose 3 and AC 0.50 % gave maximum cultures with bud burst and leaf expansion. Presence of AC in the medium was found to be essential for better survival and establishment.

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Micropropagation of *Cinnamomum verum* (Bercht & Presl.)

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Abstract

Protocols were standardised for micropropagation of cinnamon from mature explants and plant regeneration from immature explants and immature seeds by somatic embryogenesis. Multiple shoots upto six were induced from the shoot tips and nodal segments of mature trees on Woody Plants Medium (WPM) fortified with 3 mgl $^{-1}$ BA and 1mgl^{-1} Kinetin. Rooting was achieved in WPM supplemented with activated charcoal 2 g ml $^{-1}$ (alone or with 0.5 mgl $^{-1}$ IBA and o.5 mgl $^{-1}$ NAA). Rooted plantlets were established in a mixture of garden soil, soilrite and sand in equal proportion with 80% success after maintaining them in humid chamber for 30-40 days.

Key words: cinnamon, micropropagation, somatic embryogenesis.

Introduction

Cinnamon, *Cinnamomum verum* Bercht and Presl., true cinnamon of commerce, is an important tree spice used as a spice and also in medicines. Conventional vegetative propagation methods like rooted cuttings and layers are not sufficient for the production of large scale clonal progenies. Micropropagation is ideal for clonal multiplication of selected elite lines of cinnamon to augment the conventional vegetative propagation methods. Micro propagation of elite trees also helps to circumvent seedlings variability.

The earlier report of cinnamon micropropagation mentioned the use of seeds as explants (Ravishankar Rai & Jagadish Chandra 1987). Successful micropropagation of *Cinnamomum cassia* or chinese cassia was also reported (Inomoto & Kitani 1989). This paper reports the micropropagation of *Cinnamomum verum* from mature explants and regeneration of plantlets by embryogenesis when young seeds are used as explants for the first time.

Materials and methods

Explants and culture media

Shoot tips and nodal segments containing activated axillary buds were collected from 7-8 year old trees. The explants

were cleaned with a detergent solution, then immersed in a 3% fungicide solution (Dithane M-45 or Bavistin) for half an hour. Then they were washed and surface sterilised with 0.1% solution of $HgCl_2$ for 8-10 min depending upon the maturity of explants. Explants were washed aseptically 4-5 times in sterile water and inoculated on the medium.

The basal medium tried was Woody Plants Medium. After initial establishment of cultures in Woody Plants Medium supplemented with 0.5mgl $^{-1}$ kinetin, they were transferred to multiple shoot induction medium. For multiple shoot induction cytokinins like BAP, Kinetin (Kn) and Zeatin (Zn) were tried separately or in combination in a concentration of 0.5mgl $^{-1}$ to 3 mgl $^{-1}$ (Table 1). For rooting IBA, NAA and activated charcoal were tried separately or in combination. IBA and NAA were used in a concentration of 0.5 mgl $^{-1}$ to 2 mgl $^{-1}$ (Table 2). The pH of the media was adjusted to 5.8 before autoclaving. The medium was gelled with 0.7% (w/v) agar.

For the production of somatic embryos 4 to 5 month old seeds were collected , surface sterilised with 0.1% $HgCl_2$ for 8 min, the seed coats were removed aseptically and the embryos with cotyledons were inoculated. The somatic embryos and exbryogenic calli produced from the cotyledonary axis were subcultured on WPM supplemented with 3 mgl $^{-1}$ BA and 1mgl $^{-1}$ Kinetin for maturing and development into plantlets. The cultures were incubated at 25°C ± 2°C under 14h photoperiod at 2500 lux.

Hardening and planting

In vitro plantlets were transplanted to soilrite in cups, covered with polybags and kept in humid chamber for 30-40 days. Then they were transferred to polybags containing a mixture of garden soil, soilrite and sand in equal proportion.

Results and discussion

MS medium or WPM fortified with 0.5 mgl $^{-1}$ Kinetin and 2% sucrose was ideal for the initial establishment of the cultures. Within 15-20 days of establishment the cultures were transferred to WPM supplemented with BAP, Kinetin and Zeatin in various concentrations (Table 1). WPM supplemented with 3 mgl $^{-1}$ BAP and 1 mgl $^{-1}$ Kinetin was the best in which multiple shoots more than 6 could be induced within 30-40 days of subculture (Fig 1.1).

The regenerated shoots were transferred to the Woody Plants Medium supplemented with NAA, IBA and activated charcoal in various concentrations (Table

Table 1. Effect of cytokinins on multiple shoot induction in cinnamon (30-40 days).

WPM + additives (mgl $^{-1}$)	Average No.* of shoots
WPM alone	1.33
0.5 BAP	1.66
1 BAP	2.33
2 BAP	3.00
3 BAP	5.00
3 BAP + 1 Kn	6.83
1 Kn	2.17
2 Kn	2.66
1 Zn	2.33
2 Zn	2.83
1 BAP + 1 Kn	2.66
1 BAP + 2 Kn	3.33
2 BAP + 1 Kn	4.00
2 BAP + 2 Kn	4.66
1 Kn + 1Zn	2.83
1 Kn + 2 Zn	3.33
2 Kn + 1 Zn	3.50
2 Kn + 2 Zn	3.66

* *Means of 3 replicates*

Table 2. Effect of auxins and charcoal on rooting in cinnamon (30-40 days).

WPM + additives	Rooting (%)	Average no.* of shoots	Remarks
WPM alone	17	0.16	No callus formation
2 CH	100	3.83	Best medium for rooting. 3to 4 roots, 4-5 cms long were produced. No callus formation
1 IBA	50	0.50	No callus formation
1 IBA +2 CH	100	1.66	No callus formation
1 NAA	100	1.50	Callus formation at the cut end of the shoot
1 NAA + 2 CH	100	1.83	No callus formation
2 IBA	100	3.33	3-4 thick and stout roots, 1-2 cms long with callus formation at the cut end of the shoots
2 IBA + 2 CH	83	1.33	No callus formation
2 NAA	100	6.16	5-8 thick and stout roots 0.5 to 1 cm long with vigorous callus formation at the cut end and basal portion of the shoots.
2 NAA + 2 CH	100	2.00	No callus formation
0.5 IBA + 0.5 NAA	100	2.16	Callus formation at the base
0.5 IBA +0.5 NAA +2 CH	100	3.00	Good medium for rooting No callus formation.
1 IBA + 1 NAA	100	1.66	1-2, small, about 0.5cm long roots were produced with callus.
1 IBA + 1 NAA + 2 CH	56	0.83	No callus information

(Growth regulators : mgl^{-1} Charcoal : gml^{-1})

2) for rooting. Roots were induced in all the media used and there was callus production in all the media except where activated charcoal is used. Among the different media tried WPM supplemented with $2mgl^{-1}$ activated charcoal without growth regulators was the best. Similar results were obtained in Woody Plants Medium supplemented with $0.5mgl^{-1}$ IBA, $0.5mgl^{-1}$ NAA and $2 mgl^{-1}$ activated charcoal.

With the increase of auxin concentration the callus formation at the cut end of the shoots also increases and the roots become thicker and shorter. Addition of charcoal induces normal and healthy root development and prevents callus formation along with the roots. Auxin supplemented Woody Plants Medium without charcoal or with low concentration of charcoal induces callus formation at the cut end of the shoots.

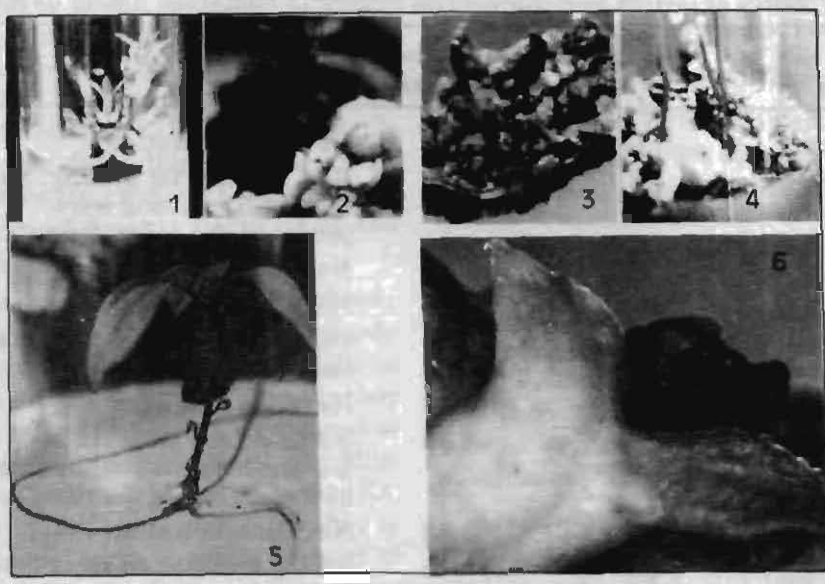

Fig. 1. Micropropagation of cinnamon. 1.1. Production of multiple shoots from mature explants, 1.2. Embryogenic callus with globular somatic embryos, 1.3. Shoot development from embryos, 1.4. Plant regeneration from somatic embryos, 1.5. *In vitro* rooting of shoot & 1.6. Close up view of an embryo.

When young seeds are inoculated on Woody Plants Medium supplemented with 0.5mgl $^{-1}$ Kinetin, about 20-30 percent cultures showed production of somatic embryos as well as embryogenic calli from the cotyledonary axis within 30-40 days of inoculation (Fig.1. 2 & 1.3). Initially the embryos were white and globular which later turn to cup shaped structures. After 2-3 subcultures on Woody Plants Medium supplemented with 3mgl $^{-1}$ BA and 1mgl $^{-1}$ kinetin, indications of shoot development were seen in the same medium (Fig.1.4 & 1.6). The cup shaped structures developed into torpedo structures, then cotyledon stages and developed into plantlets. The number of somatic embryos ranges from 75 to 100 in each culture tube, of these about 5% have matured and developed into plantlets (Fig.1.5). In WPM basal medium without growth regulators, the embryos multiplied very well but there was no differentiation into plantlets.

Acknowledgements

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Tissue culture studies on tree spices

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Abstract

Four tree spices viz. clove (*Syzygium aromaticum*) nutmeg *(Myristica fragrans)*, tamarind *(Tamarindus indica)* and curry leaf *(Murraya koenigii)* were subjected to tissue culture studies. *In vitro* shoot multiplication, elongation and rooting were achieved for clove, tamarind and curry leaf whereas nutmeg exhibited only single shoot proliferation. The media used were based on MS and WPM with growth regulator supplements. Callus cultures were also established in these crops.

Key Words : clove, curry leaf, nutmeg, tamarind, tissue culture.

Introduction

Concerted efforts on tissue culture propagation of spices especially tree spices have been few when compared to those on ornamentals, fruits, vegetables and other agricultural crops, though conventional vegetative propagation of tree spices has not been able to meet the demand of planting material. Literature survey on tissue culture of spice crops (Rao *et al* 1994) revealed that reports on tree spices are limited to those on regeneration of plantlets in cinnamon (Rai & Chandra 1987), clove (Mathew & Hariharan 1990), nutmeg (Mariska, Gatidan & Sukmadjaja 1989) and tamarind (Mascarenhas *et al.* 1987). We report here the results of our studies on the *in vitro* regeneration potential of 4 tree spices viz. clove, nutmeg, tamarind and curry leaf.

Materials and methods

For shoot multiplication, tender, actively growing shoots from elite trees of clove, nutmeg, tamarind and curry leaf as well as seeds of clove and tamarind were used as explant sources. Nodal segments from the shoots were collected and washed under running tap water for 1 h and surface sterilised using standard procedures. Subsequently, dissected nodal segments of clove were suspended in sterile distilled water for 30 min to allow leaching out of harmful phenolic oxidation products. The embryos of clove were isolated from fresh ripened fruits and those that were olive green were selected, washed, surface sterilised and planted radical tip downwards on different media. Likewise, seeds of tamarind were surface sterilised and implanted horizontally on the medium. The sterilant used in all cases was mercuric chloride (0.10%).

Medium based on Murashige & Skoog (1962) and WPM (Llyod & Mc Cown 1980) containing a variety of plant growth regulator combinations was designed for different explants. The plant growth regulators used were BAP, KN, 2 ip, IAA, NAA and IBA.

For callus cultures, leaf segments from the seedlings of clove, internodal and leaf pieces of nutmeg and internodes, cotyledons, hypocotyles and roots of tamarind were utilised. Plant growth regulators like 2, 4 -D, NAA, PCPA, KN and BAP were used in different combinations.

The cultures were incubated at 16 h photoperiod (2000 lux) at 25 ± 1° C.

Results and discussion

Clove

Nodal segments from mature clove trees displayed excessive leaching of oxidised phenolic products into the culture medium, proving lethal to the explants. Inclusion of PVP, ascorbic acid, citric acid and activated charcoal in the medium did not prove effective in adsorbing the phenolic products. Though frequent transfer of explants to fresh medium was moderately effective, high contamination (95%) in the explants could not be evaded. The response of surviving explants was also poor with regard to multiple shoot regeneration. However, cultured embryos exhibited relatively higher regenerative potential, enhanced percentage of multiple shoot formation. When planted on MS medium with low levels of BA (<1mgl $^{-1}$), the embryos germinated into single plantlets. At higher levels of BAP (>2 to 3 mgl^{-1}) combined with IAA and NAA (0.2 mgl $^{-1}$ each), 3-5 shoots initiated from each embryo, from the cotyledonary nodal region within 40 days in culture. The shoots elongated on the same medium to 4-5 cm and developed 2 to 3 nodes (Fig. 1a) ruling out the otherwise essential intervention of a shoot elongation medium. Nodal segments from seedlings also responded well enough producing 8-10 buds at a range of 0.5 cm to 2 cm shoot length (Table 1). The shoots could be rooted at half strength MS media supplemented with NAA and IBA at 1 mgl $^{-1}$ (Fig. 1b)

Segments from tender leaves were cultured on MS medium employing several auxins (2,4-D, IAA, IBA, PCPA) and cytokinins (KN, BA) at varying ratios

Table 1. Shoot multiplication response from different explants of clove.

Medium (MS) + Growth regulator (mgl $^{-1}$)	Response (No. of multiple shoots)		
	Embryos	Nodes from seedlings	Nodes from mature trees
BA 0.5	1	1-2	1
KN 0.5 to 1	1	1	-
BA 2 to 3+ IAA/NAA 0.2	3-5	8-10	1-2
KN 2 to 3 + IAA/NAA 0.2	1-2	1	-
2ip 2 + IAA/NAA 0.2	1-2	1-2	-

Table 2. Effect of different auxins on callus morphology of clove leaf cultures.

Medium (MS) + Growth regulator (mgl $^{-1}$)	Callus texture
2,4 - D 3 to 5 + KN 0.5	Friable, spongy, dry
2,4 - D 2 + KN 0.5	Friable but nodular
2,4 - D 0.3 to 0.5 *	Embryogenic callus, occasional rhizogenesis
BA 1 + KN 0.5	Callus green, compact
BA 1 + 2,4-D 0.5	do
BA 1 + KN 1 + PCPA 0.5	Callus green, shiny, compact

*Embryogenic callus obtained on subculture.

(Table 2). 2,4-D at higher concentration (>3-5 mgl $^{-1}$) induced dry spongy, creamy white friable callus, whereas at lower levels (<2.0 mgl $^{-1}$) gave rise to creamy, friable but nodular callus (Fig 1c). The nodular callus upon transfer to medium having still lower levels of 2,4-D (<1mgl^{-1}) developed embryogenic nodules. Rhizogenesis was also observed occasionally. Though BAP promoted green compact callus from explants, failed to evoke any regenerative response.

Nutmeg

Actively growing orthotrophic shoot segments of elite nutmeg trees, cultured on various media based on WPM (Llyod & Mc Cown 1980) displayed a very poor response. Axillary buds appeared highly dormant. Medium containing a mixture of BAP, KN, 2 ip, NAA and IBA stimulated single shoot development within 2-3 months in culture (Fig. 1d). Shoot proliferation could not be accelerated irrespective of modifications in concentrations of the above growth regulators. Earlier reports on nutmeg micro propagation by Mariska, Gatidan & Sukmadijaja (1989) revealed similar culture response. Culture of seedling explants is not desirable in nutmeg due to segregation of sex in progenies from seeds. An attempt was made for regeneration through callus cultures using tender leaves and other vegetative plant parts from mature trees.

Tender leaf segments exhibited capability of callus production on medium supplemented with 2,4-D and BAP as growth regulators. Callus though slow growing, appeared green and compact with several nodular regions. Shoot like out growths (2-3 mm long) were observed to develop from one of the calli on medium with high levels of BAP (>1mgl $^{-1}$). However, the cultures failed to evoke any further response.

Tamarind

Seeds inoculated on MS basal media devoid of growth regulators germinated within 10 - 15 days. Shoot tips and nodal segments excised from 4 -5 cm tall *in vitro* seedlings were cultured on MS medium containing BAP, KN, NAA, IAA, and IBA (Table 3). On MS medium containing 0.5 mgl $^{-1}$ BAP, 0.5 mgl $^{-1}$ KN and 0.1 mgl $^{-1}$ IAA, 2-3 multiple shoots developed from nodes and shoot tips (Fig 1e). However, the same medium when used for direct regeneration from seeds, generated callus. At higher concentrations of BAP

Table 3 . Effect of growth regulators on multiple shoot regeneration in tamarind.

Medium (MS) + Growth regulator (mgl $^{-1}$)	Response (No. of multiple shoots / callus) Seed	Nodes from seedlings
No growth regulator	Single shoot	-
BA 0.5 + KN 0.5 + IAA 0.1	1-2 shoots but callus at base	2-3 shoots
BA 1 + Kn 1 + IAA 0.1	2-3 shoots with callus	2-3 shoots with callus
BA 2 + IAA/ NAA 0.2	Stunted shoots with callus	3-5 shoots with callus

(>2mgl $^{-1}$) with IAA/ NAA (0.2 mgl $^{-1}$) 3 to 5 multiple shoots developed but at the base of the shoots callus formation was noticed. The multiple shoots attained a length of 2-3 cm within 45 days. Individulised shoots were planted on medium containing IAA, NAA and IBA each at 0.2 mgl^{-1} for root initiation. Rhizogenesis occurred within 5 weeks in about 50% cultures exhibiting small thin roots.

Tamarind, though mostly seed propagated, conventional methods like grafting and rooting of cuttings have also been tried with varying degrees of success. Micropropagation technique can be followed for tamarind for its large scale production. This method is comparatively quicker and may be used to circumvent problems associated with conventional vegetative propagation methods.

Callus initiation was observed in almost all explants cultured viz. cotyledon, internodes, hypocotyl and root segments excised from *in vitro* seedlings. Callus was cream white to brown, soft but compact. 2,4-D and Kinetin were the growth regulators used in MS medium for callus induction.

Curry leaf

Tender shoot sections from curry leaf trees when cultured on MS medium containing BAP (1-2 mgl $^{-1}$) and NAA / IAA (0.1 to 0.5 mgl $^{-1}$) developed 4 to 6 multiple shoots over a period of 3 weeks. The cultures were rapidly proliferating with multiple buds developing asynchronously from the nodal regions as well as adventitiously around the nodal regions (Fig 1f & 1g). The shoot buds at a range of 0.5 cm to 2 cm in length elongated on a medium with relatively low BAP (0.5 mgl $^{-1}$). Rooting of cultured shoots could be facilitated on medium supplemented with 1 mgl $^{-1}$ IBA and activated charcoal at 500 mgl $^{-1}$. Rooting efficiency was about 60%. Curry leaf is commercially propagated through seeds. Inadequate availability of planting materials of vegetative origin could be circumvented by using *in vitro* methods as an alternative propagation method, the latter being faster and the progenies true to type.

The present study thus indicates the possibility of *in vitro* direct organogenesis (through multiple shoots) from axillary buds for clove, tamarind and curry leaf, whereas, the same is difficult in the case of nutmeg. In nutmeg, there are several factors such as seasonal, explant juvenility, unresponding tissues etc. that might affect development of a successful *in vitro* regeneration system. The callus path way seems to be feasible in nutmeg and can be utilised, provided undesirable varients are checked.

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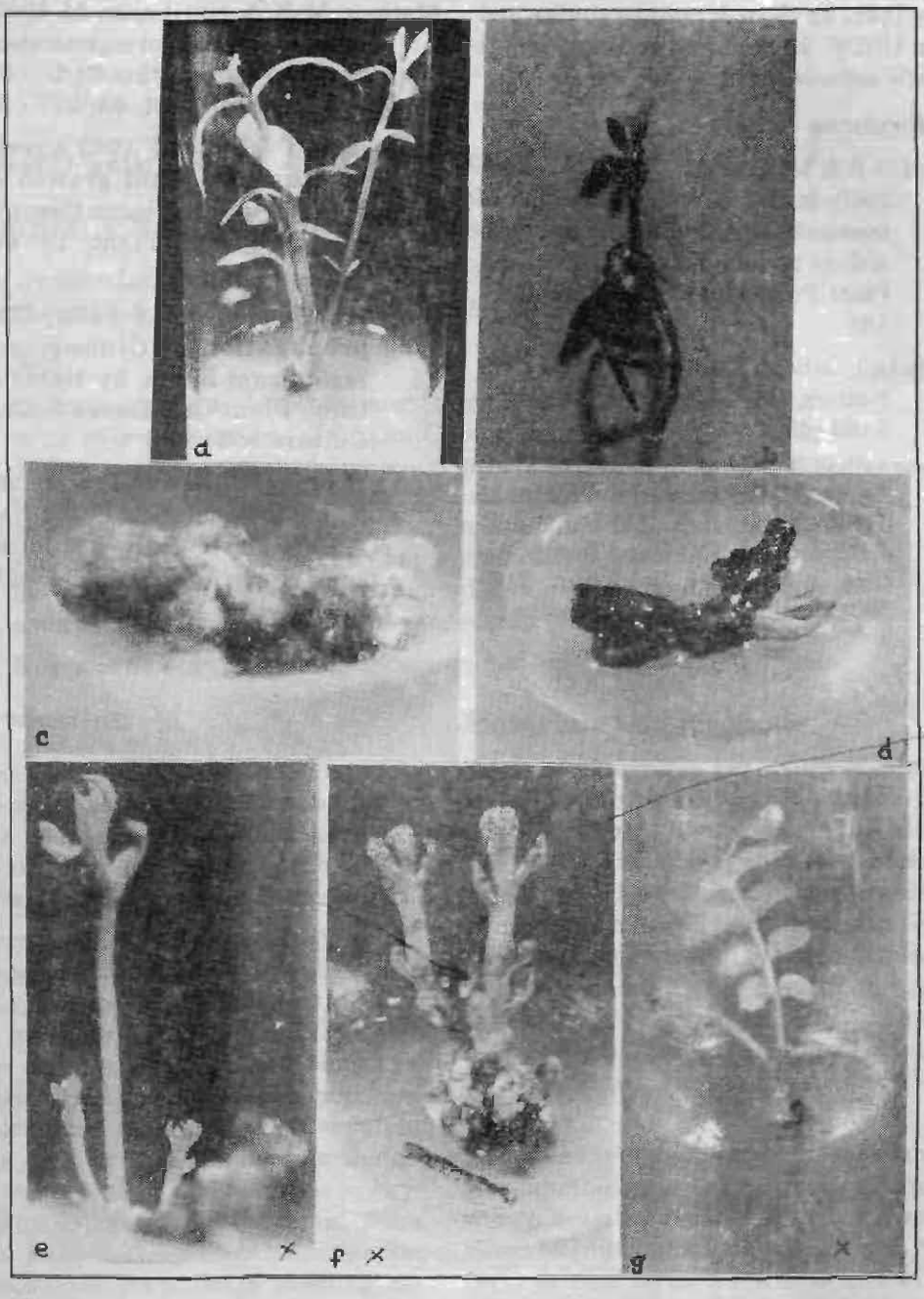

Fig. 1. *In vitro* responses in tree spices. **a.** Elongated *in vitro* shoots of clove ready for rooting, **b.***In vitro* plantlet of clove with developed roots, **c.** Embryogenetic nodules in clove callus, and **d.** Single shoot through axillary bud in nutmeg, **e.** Multiple shoots in tamarind, **f & g.** Multiple shoots in curry leaf .

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In vitro studies in cumin (*Cuminum cyminum* L.)

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Abstract

Callus induction and establishment were studied using different explants of 12 diverse genotypes of cumin (*Cuminum cyminum* L.). Among the different explants studied, hypocotyl gave maximum callus induction on semisolid MS medium supplemented with IAA and Kn except in genotypes, GC-1, MC-43, JC-19 and Hairy cumin wherein maximum response was obtained in cotyledon explant without any subculturing. On transfer to MS medium containing reduced levels of IAA and same Kn, the embryoids developed into heart shaped and torpedo stages.

Key words : callus, cumin, *Cuminum cyminum,* somatic embryoids.

Introduction

Cumin (*Cuminum cyminum* L.) is an important seed spice cultivated mainly in north Gujarat and some parts of Rajasthan. *Alternaria* blight and *Fusarium* wilt are the major constraints.

Conventional breeding methods to develop resistant varieties are handicapped by the absence of resistance sources. *In vitro* induction of variability offers some scope under such situation. However, inspite of its high value as spice crop, very few attempts are made to grow cumin under *in vitro* condition barring the report on the production of somatic embryoids from hypocotyl callus of cumin (Dave & Batra 1995). The present investigation describes the response of seedling explants of diverse genotypes of cumin to different phytohormones for callus induction and induction of embryoids in callus.

Materials and methods

Seeds of 12 diverse genotypes of cumin viz. GC-1, MC-43, JC-15, JC- 19, JC-65, JC-106, JC-126, JC-136, JC-147, JC-165, EC-109635 and Hairy cumin were soaked in water for 1 h followed by surface sterilization with 0.1% $HgCl_2$ for 2 min. The sterilized seeds were thoroughly rinsed in sterile double distilled water and germinated on Whatman No. 1 filter paper aseptically in liquid Murashige & Skoog (MS) basal medium incubated at 25 ± 2°C.

Explants (hypocotyl, cotyledon) of 10 mm size obtained from 15 day old seedlings were inoculated aseptically on 0.8 % agar based MS medium supplemented with 2% (w/v) sucrose as carbon source and with different phytohormones (IAA & kinetin at 4 levels with all possible combinations). The embryonic response of the callus cul-

tures was studied in all the genotypes after 8 weeks of inoculations on optimal semisolid MS medium (MS + IAA 1mgl $^{-1}$ + Kn 1mgl $^{-1}$). The pH of the medium was adjusted to 5.8 and incubated at 25 $\pm$ 2^{0} C with a 16 h photoperiod (3000 lux).

Results and discussion

Different explants reacted differently to callus induction treatments. Among the explants, hypocotyl was found to respond most favourbly in producing callus, followed by cotyledon. In general, callus growth in terms of fresh weight and dry weight after 4 weeks of inoculation was found significantly higher in hypocotyl explant cultures as compared to cotyledon.

In the present study, IAA and Kn each at 1.0 mgl $^{-1}$ have been found to be optimal for profuse callusing in cumin. Higher or lower levels suppressed callus growth. Requirment of specific auxin (IAA) and cytokinin (Kn) levels (1.0 mgl $^{-1}$ each) for maximum response for embryogenic callus (i.e. friable dark green callus) by cotyledon, but not so by hypocotyl, further emphasizes the inherent variations for embryogenic response in different explants tried as also observed by previous workers (Mroginski & Kartha 1981; Dave & Batra 1995).

The embryognic responses after 8 weeks of inoculation of callus cultures using hypocotyl and cotyledon explants from 12 diverse cumin genotypes on optimal semi solid growth medium (MS + 1.0 mgl $^{-1}$ IAA + 1.0 mgl $^{-1}$ Kn+2 % sucrose + 0.8 % agar) are presented in Table 1. Among the genotypes GC-1, MC-43, JC-19 and Hairy cumin showed better callus growth as compared to other genotypes, irrespective of the explants. However, the callus turned embryogenic only when cotyledon was used as explant in these 4 genotypes. The present study reiterates that auxin is essential for the induction of embryogenesis.

Cotyledon explants are found a better source of embryogenic callus in the present case which is contradictory to earlier experiments (Jasrai *et al.* 1992; Dave & Batra 1995) suggesting hypocotyl is ideal source for embryogenic callus.

The embryogenic callus obtained from cotyledon explants of 4 genotypes viz. GC-1, MC-43, JC-19 and Hairy cumin when subjected to reduced level of IAA (0.1 mgl $^{-1}$), keeping the level of Kn constant (i.e. 1.0 mgl $^{-1}$) in MS medium, showed globular to heart and torpedo shape embryoids development in very large numbers throughout the callus mass within 3 weeks.

Gradual withdrawal of auxin has been reported as essential for the induction of somatic embryoids in many systems (Ammirato 1983).

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Table 1. Response of cumin genotypes for different callus parameters from hypocotyl and coyledon explants.

Genotype	Explant	Days for callus initation	Callus initation (%)	Callus type	Callus colour	Fresh wt (mg/ explant)	Dry wt (mg/ explant)	Dry matter (%)	Remark
GC-1	H	8	100	Friable	Yellowish green	4.838	0.445	9.20	
	C	10	100	Friable	Greenish	2.137	0.201	9.41	Embryogenic callus
MC-43	H	8	100	Friable	Yellowish green	4.036	0.371	9.19	
	C	10	100	Friable	Greenish	1.179	0.109	9.24	Embryogenic callus
JC-15	H	8	80	Semifriable	Yellowish	2.430	0.213	8.76	
	C	13	55	Compact	Brownish	0.263	0.022	8.36	
JC-19	H	8	85	Semifriable	Yellowish green	2.038	0.174	8.53	
	C	10	90	Semifriable	Yellowish green	0.981	0.083	8.46	Embryogenic callus
JC-65	H	11	60	Compact	Pale yellow	0.625	0.053	8.48	
	C	12	50	Compact	Yellowish green	0.390	0.032	8.20	
JC-106	H	10	80	Semicompact	Yellowish	1.281	0.109	8.50	
	C	11	60	Semicompact	Yellowish green	0.881	0.074	8.39	
JS-126	H	9	80	Semifriable	Yellowish	1.880	0.159	8.45	
	C	11	80	Semifriable	Yellowish green	0.976	0.083	8.50	
JC-136	H	9	85	Semicompact	Yellowish	2.005	0.173	8.62	
	C	13	50	Compact	Yellowish brown	0.161	0.013	8.07	

Table 1. contd.

Genotype	Explant	Days for callus initiation	Callus initiation (%)	Callus type	Callus colour	Fresh wt (mg/explant)	Dry wt (mg/explant)	Dry matter (%)	Remarks
JC-147	H	10	80	Semi compact	Yellowish	1.470	0.126	8.57	
	C	12	55	Compact	Yellowish bown		0.273	0.023	8.42
JC-165	H	9	75	Semi friable	Yellowish	1.651	0.129	8.41	
	C	11	60	Compact	Yellowish green	0.651	0.055	8.44	
EC-1.9635	H	10	65	Semi friable	Yellowish	0.897	0.072	8.52	
	C	10	85	Semi friable	Yellowish green	0.880	0.075	8.52	
Hairy cumin	H	9	100	Semi friable	Yellowish	1.017	0.086	8.45	
	C	10	90	Semi friable	Yellowish green	1.001	0.086	8.59	**Embryogenic callus**
S. Em.(Explant x Genotype)						0.017	0.009		
C.D. at 5% (Explant x Genotype)						0.047	0.027		
C.V.%						2.900	18.800		

Observation after 8 weeks. Data represent aggregate of 20 tubes
H= hypocotyl, C= cotyledon

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In vitro responses of fennel (*Foeniculum vulgare* Mill.)

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Abstract

The potential of leaf and stem explants of fennel was investigated *in vitro*. Callus induction in stem explants on MS medium fortified with wide range of auxins + cytokinins was higher in comparison to leaf explants. Auxins or cytokinins when individually included in the MS media were not conducive for callus induction. Regeneration was observed when the callus was maintained on MS media fortified with 1 mgl $^{-1}$ NAA and 0.5 mgl $^{-1}$ kinetin.

Key words: fennel, *Foeniculum vulgare*, tissue culture.

Introduction

The technique of plant cell and tissue culture are increasingly applied to economically important plants with the objective to develop superior clones. However, seed spices have not received any attention in this direction. The crop improvement programme in seed spices is hampered due to low variability. Attempts have been made to differentiate the callus from explant of fennel with the objective to develop protocol for regeneration to be used in crop improvement programme.

Materials and methods

Young shoot and leaf tissues from fennel (variety RF 101) were collected from field grown plants. They were surface sterilised with 0.1% $HgCl_2$ solution for 2-3 min and washed 3-4 times with sterile distilled water. The surface sterilised shoot and tender leaf were inoculated in the culture media under aseptic conditions. MS medium (Murashige & Skoog 1962) with 3% sucrose, 2,4 D and NAA at 0.4 mgl $^{-1}$ and cytokinins-6 furfurul aminopurine (kinetin) at 0.5 mgl $^{-1}$ individually or in combinations was used (Table1). The pH of the medium was adjusted to 5.8. The medium was solidified with 0.8% agar . The cultures were incubated in environmental chamber at 26± 2°C with 16 h photoperiod (2000 lux).

Results and discussion

Callus was obtained from stem and leaf of fennel. Callus induction was observed within 10-12 days after inoculation. The

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Table 1. Influence of NAA and 2,4 D in combination with kinetin ($0.5 mgl^{-1}$) on callus induction in fennel.

Concentration (mgl^{-1})	Callus from leaf explants	Callus from stem explants
NAA		
0	-	-
0.5	-	-
1.0	+	-
2.0	++	-
3.0	+	+
4.0	-	-
2,4-D		
0	-	-
0.5	-	-
1.0	-	+++

-no response, + low response, ++ medium response, +++ high response.

volume of the callus gradually increased up to 21 days after which no appreciable growth was observed. Besides, browning of callus was also observed after 21 days.

The response of stem and leaf explant for callus initiation was different. No callusing was observed either with the auxin or cytokinin individually at any concentration. Callusing was however observed in the combined treatment of cytokinin and auxin. Callus was induced with 2,4 D from stem explants. However, NAA induced the callusing from leaf explant. 2,4 D did not induce callus from leaf explants (Table 1). To induce regeneration, sub culturing was done using different concentrations of cytokinins and auxins. Transfer of calli derived from stem or leaf to MS+2,4 D or NAA with higher level of BA or kinetin ($4mgl^{-1}$) did not induce any differentiation of shoot or root.

For maintenance and multiplication, the callus was transfered to the same media on which the callus was induced. It was observed that the new leaf emerged from the MS media fortified with $1mgl^{-1}$ NAA+0.5 mgl^{-1} kinetin. There was no regeneration from 2,4 D induced callus. The higher or lower concentration of the hormones did not induce regeneration. This indicates that NAA in proper combination with kinetin induce regeneration.

In conclusion, it may be said that MS medium supplemented with 1 mgl^{-1} 2, 4-D and 0.5 mgl^{-1} kinetin is the best combination for callus induction from fennel shoot explants and 2 mgl^{-1} NAA and 0.5 mgl^{-1} kinetin is the best combination for callus induction from fennel leaf explants.

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Rapid *in vitro* multiplication of *Eryngium foetidum* L., an aromatic spice, through shoot multiplication and organogenesis

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Abstract

Rapid *in vitro* multiplication methods were established in an aromatic spice, *Eryngium foetidum* L. through regeneration from callus, direct from leaf explants and through shoot-tip multiplication. Shoot tips cultured on Murashige and Skoog (MS) medium containing 4-8 mgl^{-1} benzyl adenine (BA) alone or 2-8 mgl^{-1} BA along with 2-4 mgl^{-1} oc naphthalene acetic acid (NAA) or 2-5 mgl^{-1} gibberrelic acid (GA_3) showed different rates of axillary branching. Leaf discs cultured on MS medium with BA alone or BA along with NAA developed shoot buds directly from them. Maximum multiplication was recorded in an 8 mgl^{-1} BA supplemented medium. Leaf derived callus showed shoot organogensis when treated with 2 mgl^{-1} NAA and 6 mgl^{-1} BA fortified MS medium. Polyvinyl pyrrolidone or phloroglucinol incorporated into the medium as additives reduced tissue blackening and favoured healthy shoot proliferation. Rooting was achieved in MS medium containing 3-4 mgl^{-1} indole-3-acetic acid and the plantlets transferred to soil after hardening in liquid basal MS, showed 81% survival, under green house conditions.

Key words : callus, *Eryngium foetidum*, organogenesis, shoot multiplication.

Introduction

Eryngium foetidum L. (false coriander), a minor spice belonging to the family Apiaceae, is used as a substitute for coriander for flavouring rice and curries (Anonymous 1952) in addition to its use in indigenous system of medicine. There is no tissue culture work reported in this spice and therefore, this paper describes

protocols for its rapid multiplication.

Materials and methods

Shoot tips and leaf segments from mature green house grown *Eryngium foetidum* were used as explants. The explants were soaked in 10% (v/v) labolene (a commerical neutral detergent, Qualigens, India) solution for 5 min and washed in running tap water for 1h before sterilization with 0.1% mercuric chloride solution for 3-5 min MS (Murashige & Skoog 1962) medium supplemented with 3% sucrose and solidified with 0.8% agar was used in all experiments. The pH of the medium was adjusted to 5.8 before autoclaving. The cultures were incubated at 25 ± 2°C under 12/12h photoperiod (3000 lux).

MS medium supplemented with 4-8 mgl $^{-1}$ BA alone or along with 0.5-8.0 mgl $^{-1}$ NAA/2.0-6.0 mgl $^{-1}$ GA_3 was used for shoot multiplication. Leaf segments (1cm^2) were cultured on MS medium fortified with 2.0-10 mgl $^{-1}$ BA alone or along with 2-8 mgl $^{-1}$ NAA for direct adventitious shoot formation. For induction of callus, leaf segments were planted on MS medium containing 2-4 mgl $^{-1}$ BA and 0.1-2.0 mgl $^{-1}$ NAA supplemented with either 200 mgl $^{-1}$ PVP or 2.0 mgl $^{-1}$ PG. The medium used for callus regeneration contained 2-8 mgl $^{-1}$ BA and 2-4 mgl $^{-1}$ NAA. Rooting medium comprised of basal MS, supplemented with different concentrations of NAA, IAA, IBA, BA & Kin. The rooted plants were hardened in1/2 strengh MS basal liquid medium and subsequently transferred to vermiculite and later to pots containing garden soil and sand under green house conditions.

Results and discussion

Shoot tips cultured on MS medium supplemented with 2-8 mgl $^{-1}$ BA along with 0.5-4.0 mgl $^{-1}$ NAA/4.0-8.0 mgl $^{-1}$ GA_3 developed mutliple shoots with different rates of shoot proliferation (Table 1). Six mgl $^{-1}$ BA alone in the medium induced about 8 shoots, while 10 shoots developed from an explant inoculated in combination of 6 mgl $^{-1}$ BA and 2 mgl $^{-1}$ NAA. Four mgl $^{-1}$ GA_3 with 8 mgl $^{-1}$ BA was found to be the best combination for maximum shoot multiplication (12 shoots/explant). The stimulating impact of cytokinins on axillary bud proliferation is well established (Purohit, Dave & Kukda 1994; Monier & Ochatt 1995). Cytokinins are known to promote cell divisons and act as vital to bud production (George & Sherrington 1984) of which BA was reported to be the most effective one (Hu & Wang 1983). Incorporation of NAA along with BA increased the number of shoots produced per explant, similar to the results obtained in *Foeniculum vulgare* (Paupardin, Garcia-Rodriquz & Bricout 1980). Supplementing the medium with GA_3 was also found to be very effective in stimulating shoot elongation and multiplication than the most favouable auxin-cytokinin combination in *Eryngium* similar to the findings in *Atriplex* shoot cultures (Wochok & Sluis 1990).

Leaf segments cultured on MS medium supplemented with different concentrations of BA/ Kin individually or BA in combination with NAA showed *de novo* shoot organogenesis within 15 days of culture from more than 90% of the explants. The shoot buds arose along the veins and no shoot buds sprouted from

Table 1. Micropropatation of *Eryngium foetidum* through shoot multiplication.

Growth regulator (mgl $^{-1}$)			No. of shoots* per explant	Rate of** proliferation	No. of plants survived	
BA	NAA	GA3			After*** rooting	After**** transfer to pots
2	-	-	3±0.09	2.87	9±0.79	7±0.14
4	-	-	7±0.13	6.84	48±0.13	44±0.34
6	-	-	8±0.32	7.39	59±0.01	52±0.00
8	-	-	4±0.43	4.00	16±0.14	16±0.12
4	2	-	7±0.17	3.12	12±0.17	10±0.17
6	2	-	10±0.71	9.33	93±0.14	82±0.52
8	2	-	9±0.13	8.14	73±0.74	61±0.74
4	4	-	4±0.29	3.91	16±0.12	14±0.04
6	4	-	2±0.07	2.00	4±0.00	3±0.00
8	4	-	2±0.17	1.71	3±0.03	1±0.11
4	-	2	1±0.73	1.00	1±0.00	1±0.00
6	-	2	1±0.14	1.00	1±0.07	0±0.00
8	-	2	2±0.13	1.71	4±0.10	2±0.10
4	-	4	9±0.74	8.41	76±054	70±0.54
6	-	4	0±0.51	8.22	74±0.12	71±0.34
8	-	4	12±0.02	11.71	141±0.31	129±0.21
4	-	6	3±0.31	2.91	9±0.19	6±0.19
6	-	6	1±0.19	1.00	1±0.12	1±0.03
8	-	6	1±0.00	1.00	0±0.00	0±0.00

* Values represent mean ± SE of 7 replicates after 27 days of culture
** Values represent mean proliferation rate of 7 replicates after 54 days of culture
*** Values represent mean ± SE of 7 replicates after 54 days of culture
**** Values represent mean ± SE of 7 replicates after 81 days of culture and subsequent transfer to greenhouse

the cut ends. Maximum number of shoots (10 shoots/ explant) developed from leaf segments cultured in 6 mgl^{-1} BA alone. Leaf segments inoculated on MS medium containing 2-4 mgl $^{-1}$ BA and 0.1-2.0 mgl $^{-1}$ NAA also developed callus from the excised regions, which became brown and necrotic within 25 days. However, addition of 200 mgl^{-1} PVP or 2.0 mgl^{-1} PG reduced the browning and influenced the formation of friable callus. The necrotic callus also developed pockets of new friable callus upon transfer to medium supplemented with the additives. The revival of growth and prevention of blackening by the addition of PVP may be because the phenolics are adsorbed by PVP thus preventing their oxidation (George & Sherrington 1984). Apart from PVP, 2 mgl^{-1} PG was also found to be effective in preventing blackening of the callus in *Eryngium*. PG has been successfully used to reduce blackening, revive growth and induce morphogenesis in some spices (Sarasan & Nair 1991). Maximum number of shoots (25) developed from callus initiated in PVP containing medium upon transfer to medium containing 4.0 mgl^{-1}

NAA along with 6.0 mgl^{-1} BA and 2.0 mgl^{-1} PG.

Though roots developed along with shoot initiation in most of the experiments conducted, they did not support the growth of the plants in the field and resulted in high rate of mortality. Hence to overcome this, the shoots were freshly planted onto rooting medium comprising of various hormones, after removing all the roots developed primarily (Table 2). MS medium with 3.0 mgl^{-1} IAA induced maximum profuse thick roots within 20 days which is recommended as the best rooting medium in this species. Rooted plants were hardened in liquid basal MS medium and transferred to cups containing vermiculite. The plantlets were nourished with 1/10 strength Hoagland's solution for 10 days (Eptstein 1972). Subsequently they

Table 2. Response of excised shoots of *Eryngium foetidum* to rooting treatments of semi solid medium (MS medium + 0.8% agar + 3% sources).

Plant growth regulator	Concentration (mgl^{-1})	Shoots rooted * (%)	Nature of roots formed	Number of days taken*
NAA	0.1-1.0	35	Long and slender	15
	2.0-3.0	70	Very thick and small	35
	above 4.0	-	No rooting	125
IAA	0.1-1.0	87	Long and slender	18
	1.0-20	85	Long and slender	25
	3.0-4.0	63	Thick and profuse	20
	above 4.0	-	No rooting	50
IBA	0.1-1.0	44	Short and slender	16
	2.0-3.0	67	Short and small	30
	above 4.0	-	No rooting	55
IB	0.1-1.0	37	Long and slender	25
	2.0-4.0	42	Long and slender	35
	4.0-6.0	51	Short and slender	60
	above 6.0	-	No rooting	70
Kin	0.1-2.0	60	Long and slender	20
	2.0-4.0	65	Long and slender	35
	above 4.0	-	No rooting	45
Control	Basal MS+ 0.8% agar + 3% sucrose	20	Long and slender	17

* Values represent mean ± SE of 7 replicates

were transferred to pots containing soil/ sand mixture and grown under green house conditions. Almost 81% of the plants transferred this way survived in the field and exhibited luxuriant growth.

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Propagation of *Pogoestemon patchouli* Hook through tissue culture

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Abstract

Plant regeneration from callus culture of *Pogoestemon patchouli* was achieved through indirect organogenesis. The component callus was initiated from the leaf bit, stem bit and petiole bit tissue using Murashige and Skoog medium supplemented with 1.0 mgl $^{-1}$ indole-3-acetic acid and 5.0 mgl^{-1} kinetin. The optimum growth regulator concentrations for shoot induction and shoot elongation were found to be 5.0 mgl $^{-1}$ kinetin, and 1.0 mgl^{-1} indole-3- acetic acid with 7.0mgl^{-1} kinetin, respectively. Elongated individual shoots were rooted on Murashige and Skoog medium containing 5.0 mgl^{-1} naphthalene acetic acid. The isolated plantlets, hardened in vermiculite were transferred to pots containing garden soil and sand.

Key words: callus, indirect organogenesis, *Pogoestemon patchouli*, tissue culture.

Introduction

Patchouli (*Pogoestemon patchouli* Hook), an aromatic herbaceous perennial plant, is highly valued for its essential oil-patchouli oil, which is a perfume by itself. Cultivation is not profitable owing to a lot of constraints, the foremost being the non-availability of bulk planting material of desired types.

Conventionaly, patchouli is cultivated through stem cuttings, and only a limited number of planting material can be obtained per plant. Coupled with this is the problem of the crop being susceptible to mosaic virus (Subba Rao 1986), root knot nematode and insect pests (Ramani & Bhamanavar 1990). Therefore, to meet the demand of the growers, a rapid mass propagation of elite material through tissue culture has become imperative. The present paper describes the induction of callus from leaf bit, stem and petiole segments, and the subsequent regeneration

and planting out of the plantlets.

Materials and methods

The strain of Malaysian type of *Pogoestemon patchouli* was established in pots from stem cuttings obtained from Indian Institute of Horticultural Research, Bangalore. Various explants like leaf bits, nodal and petiole segments were collected from 2-3 months old plants grown in the green house. These explants were thoroughly washed in running tap water for 15 min and soaked in 1% Teepol for 10-15 min. Then, these explants were surface sterilized with 0.1% mercuric chloride solution for 3, 5 and 6 min for leaf, stem and petiole segments, respectively, followed by washing 5-7 times in sterile double distilled water. Before inoculation in to the media, the surface sterilized explants were trimmed to a size of 0.5 cm.

The culture medium consisted of Murashige and Skoog (1962) medium, containing 1.5% coconut water, 100 mgl $^{-1}$ myo-inosital, 3% sucrose and 8 % agar. This was supplemented with auxins (IAA, NAA and 2, 4-D) and cytokinins (Kinetin and BA) at various factorial combinations (1.0, 2.5 and 5.0 mgl $^{-1}$). pH of the media was adjusted to 5.8. Fifteen ml of medium was transferred evenly to clean culture tubes of 25 x 150 mm size and autoclaved . The cultures were incubated at a light intensity of 1000 - 3000 lux with 16 h photoperiod, at a temperature 25 $\pm$ 2°C, under 75% relative humidity. After 5-6 weeks, the induced callus was sub-cultured for further calli proliferation. After 30-35 days, the proliferated greenish, matured callus was transfered to shooting media and then to rooting media at various concentrations of cytokinins (Kinetin and BAP) and auxins (IAA, NAA and IBA), respectively. Well rooted plantlets were transferred from test tubes to small pots filled with sterile vermiculite. At this stage, plants were initially covered with plastic bags (upto 15 days).

Results and discussion

Leaf , stem and petiole explants were able to produce callus in Murashige and Skoog medium with auxins alone (IAA and NAA) or in combination with cytokinins (Kinetin and BA) at different concentrations (1.0, 2.5, 5.0 mgl^{-1}). Among the various types of explants and various growth regulator combinations tried, leaf bit has shown maximum callusability (70%), followed by stem bit (42%) and petiole (30%) at the growth regulator combination of 1.0 and 5.5 mgl $^{-1}$ of IAA and kinetin, respectively. After 25 days, higher callus proliferation rate was observed in the leaf bit callus at 2.5 mgl $^{-1}$ IAA with 5 mgl $^{-1}$ kinetin (Table 1). The leaf bit callus was found to be friable and compact, with reddish green pigmentation of variable intensity. The stem bit and petiole callus showed lesser compactness.

In general, cytokinins alone or in combination with auxins are known to produce the most friable callus with greater morphogenic responses. In patchouli also, friable callus was produced at higher concentrations of kinetin (7.0 mgl $^{-1}$) in combination with IAA (1.0 mgl^{-1}). In earlier reports by Grnat & Fuller (1968), the friable callus of *Vicia faba* exhibited larger

Table 1. *In vitro* response of leaf explants of *Pogoestemon patchouli*.

Hormone (mgl^{-1})	Callus* induction (%)	Average number of shoots per callus **	Average number of roots per shootlet**
IAA 1.0	27	0	21 ± 1.39
IAA 2.5	35	0	3 ± 1.23
IAA 5.0	43	0	7 ± 1.08
Kin 1.0	25	10 + 0.32	0
Kin 2.5	30	13 + 0.40	0
Kin 5.0	28	19 + 0.59	0
Kin 7.0	20	30 + 0.39	0
IAA 1.0 ± Kin 1.0	35	7 + 0.92	1± 0.97
IAA 1.0 ± Kin 2.5	55	11 + 1.02	0
IAA 1.0 ± Kin 5.0	70	18 + 1.11	0
IAA 2.5 ± Kin 1.0	36	0	3 ± 1.63
IAA 2.5 ± Kin 2.5	55	2 + 1.12	2 ± 1.96
IAA 2.5 ± Kin 5.0	65	5 + 0.98	2 ± 1.77
IAA 5.0 ± Kin 1.0	30	0	6 ± 1.33
IAA 5.0 ± Kin 2.5	35	0	2 ± 1.40
IAA 5.0 ± Kin 5.0	43	21+ 1.09	1 ±1. 88
NAA 1.0	5	0	5 ± 1.92
NAA 2.5	12	0	9 ± 1.49
NAA 5.0	15	0	12 ± 1.56
Hormone free	20	1	0

* After 45 days of culture
** After 30 days of culture

number of meristematic nodules than the non-friable ones. These nodular structures may be induced to emerge as roots or shoots according to the auxin/cytokinin balance of the media (Yeoman & Street 1977).

Shoot bud differentiation occured in all the callus obtained from different explants, after 30-40 days of inoculation on MS medium supplemented with kinetin alone. The callus derived from leaf bit explants produced the highest frequency of shoot bud formation at 7.0 mgl^{-1} kinetin. Differences in regeneration potential were also observed in the callus derived from the stem and petiole explants.

In general, higher cytokinin concentration has induced more shootlets from the callus, while cytokinins in combination with auxins or auxin alone has reduced the shoot bud development. In *Pogoestemon patchouli*, the shoot bud formation and differentation from the callus were drastically reduced at lower concentrations of kinetin and in combination with IAA. Similar results were also obtained in *Clitoria ternata* (Pradipkumar *et al*. 1993), *Vigna aconitifolia* (Eapen, Gill & Rao 1982) and *Vicia faba* (Selva, Scouffs & Briguet 1989).

Individual shootlets of 2 cm length were rooted on MS medium containing different concentrations of NAA and IAA (1-5 mgl^{-1}). Among the two auxins tried, NAA was found to show a better response than IAA. Profuse roots developed from the individual shoots at 5.0 mgl^{-1} of NAA, but concentrations above or below 5.0 mgl^{-1} suppressed the root induction. These findings are in agreement with that of Debergh & Maene (1978) and Somari, John & Thengane (1989).

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Growth and regeneration of vetiver (*Vetiveria zizanioides* (L.) Nash) callus tissue under varied nutritional status

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Abstract

Callus could be induced from pretreated immature inflorescence of *Vetiveria zizanioides* on culture in media with 2,4-D and kinetin. Of the three media tried, MS, Y3 and SH, maximum callusing within the shortest time was obtained in MS medium. Among the different carbon sources tried, sucrose at 3 % was found optimum. No promotory effect on callus initiation was observed by the addition of various organic sources of nitrogen such as glutamine, casein hydrolysate and yeast extract. Addition of adenine sulphate to the basic regeneration medium containing benzyl amino purine increased the number of shoots formed. Rooting of shoots was satisfactory at 2 % sucrose level and at half the concentration of MS salts. Regeneration of shoots was better in shaken liquid media while higher percentage of rooting was obtained in solid media.

Key words: callus, nutrient source, *Vetiveria zizanioides*.

Introduction

Vetiveria zizanioides (L.) Nash is an aromatic grass yielding an essential oil highly valued and used in the manufacture of perfumes, soaps and chewing tobacco products. Development of superior strains of vetiver through the induction of somaclonal variation is an attractive option. This paper describes the nutritional requirements for callus initiation, maintenance and plantlet regeneration from callus cultures of vetiver.

Materials and methods

Callus was induced from immature inflorescence segments of vetiver as per the procedure described earlier (Keshavachandran & Khader 1996). MS, SH (Schenk & Hildebrandt (1972) and Y3 (Eeuwens 1976) media were tried with varying concentrations and combinations of auxins and cytokinins for induction of callus. In order to standardise the optimum quantities of various medium supplements used for induction of callus,

different carbon sources, different concentrations of the best carbon source and different nitrogen sources were tried. The effect of different concentrations of 2,4-D and Kin on maintenance and proliferation of callus was studied by sub-culturing approximately 20 mg of the calli formed into MS media containing different concentrations of 2,4 - D and Kin.

The calli formed were sub-cultured in three different media with varying concentrations of BAP, Kin, 2 ip and 2,4-D with NAA. In order to determine the effect of addition of adenine sulphate to the basic regeneration medium, 5 levels of adenine sulphate (0, 20, 40, 80 and 160 mgl $^{-1}$) were added.

The efficacy of solid media as against liquid media was tested by varying concentrations of agar and using stationary liquid cultures with the explant supported on filter paper supports and shaken liquid cultures kept on a rotary shaker.

The effect of the different auxins viz., IBA, IAA and NAA in induction of rooting was studied with solid and liquid media. Varying concentrations of sucrose were used to determine the optimum concentration for rooting. The effect of reducing or increasing the strength of the media on rooting was observed by using the basic MS medium at 0.24, 0.5, 1.0, 1.5 and 2.0 strengths.

Results and discussion

Maximum callus induction was obtained from immature inflorescence segments in the presence of 2, 4 - D supplemented with low concentration of Kin. Pre-treatment of the immature inflorescence seg-

Table 1. Effect of different carbon sources on callus induction and growth in vetiver.

Carbon source (3%)	Cultures* initiating callus (%)	Days to callus induction	Fresh weight (g)	Dry weight (g)
Glucose	70 (56.79)	8	0.364	0.029
Fructose	55 (47.87)	9	0.303	0.028
Dextrose	50 (40)	9	0.272	0.027
Galactose	20 (26.56)	11	0.161	0.015
Mannitol	0 (5.24)	0	0	0
Sucrose	87 (68.87)	8	0.405	0.032
CD ($P \leq 0.05$)	6.46	2.05	3.07	2.91
SE ±	2.10	0.67	9.97	9.43

(Medium MS + 2,4-D 2.5 mgl $^{-1}$ + Kin 1 mgl $^{-1}$, Growth period 30 days)

* Values in parentheses indicate angular transformed data

Table 2. Effect of sucrose concentration on induction and growth of roots in vetiver.

Sucrose concentration (%)	Rooting* (%)	Days to root induction	No.of roots	Dry weight (g)	Fresh weight (g)
1	70 (56.79)	31	9	2.550	0.213
2	100 (84.73)	27	10	2.510	0.228
3	100 (84.73)	27	10	2.450	0.254
4	90 (71.56)	34	10	2.325	0.259
5	70 (56.79)	34	7	2.260	0.281
6.	40 (39.23)	37	7	2.200	0.314
CD ($P \leq 0.05$)	11.03	2.52	1.66	0.34	0.033
SE±	3.58	0.82	0.53	0.11	0.01

* Values in parentheses indicate angular transformed data

(Medium : MS + IBA 2.5 mgl $^{-1}$; Growth period 50 days)

ments in MS liquid medium with 2,4-D (0.5 to 1.0 mgl $^{-1}$) + BAP (0.5 to 1.0 mgl $^{-1}$) + Kin (0.5 to 1.0 mgl $^{-1}$) was found to initiate callus in 80-100 % of the cultures when cultured on media with 2,4-D 2.5 mgl $^{-1}$ + Kin 1.0 mgl $^{-1}$.

Of the three media tried, maximum callusing within the shortest time occured in the MS medium. The MS medium as such or modifications of MS is the most widely used medium for culture initiation, maintenance and plant regeneration is aromatic grasses (Mathur *et al.* 1988; Sreenath & Jagadishchandra 1989; Baruah & Bordoloi 1991).

Among the different carbon sources tried such as glucose, fructose, dextrose, galactose, mannitol and sucrose, sucrose showed significant difference in induction of callus and on the days taken for callus initiation (Table1). Among the different concentrations of sucrose tried, maximum callusing of 80 % was obtained with 3 % within the shortest time of seven days for callus initiation (Table 2). Eapen & Rao (1984) observed in the case of triticale, sucrose, fructose and glucose produced good callus growth while galactose was found unsuitable. Sheridan (1975) also reported that sucrose was distinctly superior to other carbon sources and that galactose was found to be inhibitory for callus growth.

There was no significant effect on callus initiation by the addition of various sources of nitrogen such as glutamine, casein hydrolysate and yeast extract to the basic callus induction medium. The percentage of callusing was maximum in medium without the addition of the nitrogen sources and minimum when yeast extract was added to the medium. Eapen & Rao (1984) reported that yeast extract was inhibitory for the growth of rye and triticale callus.

The callus formed showed maximum proliferation in MS medium with 2,4-D 5mgl^{-1} and Kin 1mgl $^{-1}$. The callus continued to proliferate and could be maintained for 15 cultures with a culture passage interval of 30 days without loss of proliferation potential.

The maximum regeneration percentage was obtained in the MS medium followed

by the Y3 and the SH medium. The regeneration of shoots was faster and the number of shoots formed per culture was greater in MS medium. Skoog & Miller (1957) postulated that the cytokinin:auxin ratio was important in deciding the type of morphogenesis obtained *in vitro*. In the present study, transfer of the callus formed to the medium supplemented with high cytokinin and lower auxin levels induced regeneration of shoots. Maximum regeneration (100%) was obtained in MS with BAP 5 mgl $^{-1}$ + NAA 0.5 mgl $^{-1}$ in 29 days with a mean number of 21 shoots per culture. Mathur *et al.* (1988) obtained optimal shoot bud induction in Java citronella when calli were put on MS medium supplemented with 1.0 mgl $^{-1}$ IAA and 0.5 mgl $^{-1}$ Kin. Addition of adenine sulphate (80 mgl $^{-1}$) to the basic regeneration medium increased the number of shoots formed and reduced the days to shoot initiation and considerably increased the number of shoots formed per culture. A mean number of 32 shoots was obtained in 11 days in shaken liquid cultures. Uhring (1982) obtained 30 % more shoots from *Scutellaria* shoot tips in shaken liquid cultures. In the present study, the number of shoots formed increased by 60 % and the number of days taken for shoot initiation was reduced by 62 % in the shaken liquid cultures compared with cultures on 0.8 % agar.

Higher percentage of rooting was obtained in solid media than in liquid stationary media. Among the auxins tried, maximum percentage of rooting and the fastest initiation of roots was obtained with IBA at 5 mgl $^{-1}$ concentration. The number of days taken for rooting showed a progressive decline with increase in auxin concentration.

The presence of sugar in the medium has been found essential for *in vitro* rooting of many species. George & Sherrington (1984) stated that the optimum level of

Table 3. Effect of strength of medium on induction and growth of roots in vetiver.

Strength of the medium	Rooting* (%)	Days to root induction	No. of roots	Fresh weight (g)	Dry weight (g)
0.25	70 (56.79)	31	5	1.542	0.141
0.50	100 (84.73)	27	12	2.475	0.225
1.00	100 (84.73)	27	12	2.620	0.229
1.50	100 (84.73)	29	10	2.220	0.185
2.00	60 (50.77)	35	4	1.210	0.105
CD (P=0. 05)	6.73	2.06	1.65	0.16	0.28
SE ±	2.23	0.68	0.55	0.05	0.09

* Values in parentheses are angular transformed data

(Medium: MS+ IBA 25 mgl^{-1}, Growth period 50 days)

sucrose for root formation will vary according to the medium used and will differ from one species to another. With vetiver, maximum rooting within the shortest time and the maximum number of roots occured at 2 and 3 % sucrose levels. Increasing the sucrose concentration reduced the number of roots and extended the number of days needed for root initiation.

Increasing the strength of the medium 1.5 times or reducing to half concentration did not affect the percentage of rooting of vetiver, while reducing the concentration further or doubling the concentration adversely affected the rooting percentage (Table 3). The time taken for root initiation was extended by reducing the media concentration beyond half concentration or increase it from normal concentration. Likewise the number of roots formed was reduced by increasing the media concentration beyond normal or decreasing beyond half concentration.

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Production of synthetic seeds in a few spice crops

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Abstract

'Synthetic seeds' have been proposed as a safe means for germplasm conservation and exchange. Synthetic seeds were successfully produced and stored upto four to ten months in some important spices like black pepper, ginger, cardamom, turmeric, cinnamon, camphor, vanilla and in some herbal spices like anise, lavender and sage by encapsulating shoot buds, somatic embryos and regenerating calli in sodium alginate gel matrix.

Key words: encapsulation, somatic embryos, spices, synseed.

Introduction

Synthetic seeds are developed by coating a tissue or an organ, which can grow into a complete plant, alongwith nutrients in an artificial film or covering. Production of artificial seeds or 'synthetic seeds', consisting of somatic embryos or shoot buds enclosed in a biodegradable protective coating, is a low cost, high volume propagation system. Artificial seed production using different gelling agents has been discussed by Redenbaugh (1986). This paper reports successful production of 'synseeds' of ten spices viz., black pepper, ginger, cardamom, turmeric, cinnamon, camphor, vanilla, anise, lavender and sage and their storage.

Materials and methods

In vitro regenerated shoot buds, somatic embryos and regenerating calli were used in the present study as propagules for encapsulation.

The propagules were separated aseptically and placed in a gel matrix containing MS salts and sodium alginate at different concentrations (3%, 4%, 5% 6% and 7%). The explants along with the medium were dropped into a solution of calcium chloride (1.036 g/150 ml) using a pipette or a glass tube. After 40 min the beads were recovered by decanting the $CaCl_2$ solution and washed in 3-4 changes of sterile water.

The synthetic seeds were stored in 250 ml flasks sealed with aluminium foil and different media like sterile water, MS basal medium with or without growth regulators were used to soak these seeds to avoid drying. The flasks

were incubated at three different temperature regimes (5°C, 15°C, 22±2°C) to study the effect of temperature on storage. The synseeds were transferred to germination medium at different stages of storage. The culture medium used for *in vitro* multiplication of each species was used for germination. Observations were recorded on germination of encapsulated beads to assess their viability.

Results and discussion

Shoot buds, somatic embryos and regenerating calli of spice crops could be encapsulated in 4% and 5% sodium alginate gel and could be successfully stored at 22+2°C for four to ten months with 60-80 per cent survival (Table 1) (Fig. 1 & 2). Sodium alginate at 4% level was sufficient to produce good quality rigid beads in vanilla and turmeric, whereas in black pepper, cardamom, ginger, cinnamon, camphor, anise, lavender and sage an alginate concentration of 5% was necessary. Higher concentrations of alginate (6% and 7%) were not suitable as they produced very hard matrix which hindered the emergence of shoot buds and thereby affecting the germination. Lower concentration (3%) of alginate produced beads which are very fragile and difficult to handle. Shoot buds of 0.4-0.5 cm size are suitable for encapsulation. Smaller sized buds failed to survive the storage and lost their viability within 1 month. Most of the earlier workers have used alginate concentration of 3-4% for synthetic seed production (Fermandes, Bapat & Rao 1994; George & Eapen 1995; Hasan & Takagi 1995; Padmaja, Reddy & Reddy 1995). The alginate matrix provides nutrients and protects the beads from decay and desication when transferred to soil (Bapat & Rao 1990).

In all the crops studied, laboratory temperature of 22°C was ideal for storage. At low temperatures (5°C, 15°C) the beads could be stored only up to 15-30 days in the different species studied. Within 1 month all the beads lost their viability.

In *Piper nigrum* shoot buds of 0.5 cm were optimum for production of synthetic seeds and could be stored upto 5 and 9 months in sterile water with 80% and 65% viability, respectively. The shoot buds emerged out of the matrix and regenerated into plantlets on transfer to multiplication medium (WPM with 3 mgl^{-1} BAP and 1 mgl^{-1}Kin). Synthetic seeds of zingiberaceous crops such as cardamom, ginger and turmeric could be stored in MS supplemented with 1.0 mgl^{-1} BAP and 0.5 mgl^{-1}IBA upto 9, 6 and 7 months respectively. The encapsulated beads, on transfer to MS medium supplemented with 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA, regenerated and produced normal plantlets. Successful cases of disease free encapsulated shoot buds, has been reported in ginger (Sharma, Sing & Chauhan 1994). The encapsulated shoot buds (0.4-0.5 cm) and somatic embryos of cinnamon could be stored for 4 months in WPM containing 3 mgl^{-1} BAP and 1 mgl^{-1} Kin whereas camphor could be stored upto 9 months in sterile water. After storage the encapsulated shoot buds germinated and multiplied on transfer to the same medium. The somatic embryos regenerated in MS with 0.5 mgl^{-1}Kin. Encapsulated shoot buds of vanilla could be stored for 10 months in sterile water. The beads germinated and multiplied normally in MS

Table 1. Production and storage of synthetic seeds in spices

Crop	Explant	Alginate* conc.(%)	Storage medium (months)**	Period (%)	Viability
Black pepper	Shoot buds	5	Sterile water	9	65
Ginger	Somatic embryos	5	MS+BAP(1)+IBA(0.5)	9	75
Cardamom	Callus	5	MS+BAP(1)+IBA(0.5)	6	70
Turmeric	Adv.buds	5	MS+BAP(1)+IBA(0.5)	7	60
Vanilla	Shoot buds	4	Sterile water	10	70
Camphor	Shoot buds	5	Sterile water	9	80
Cinnamon	Shoot buds	5	WPM+BAP(3)+K(1)	4	70
	Somatic embryos	5	WPM+BAP(3)+K(1)	4	60
Sage	Callus	5	MS+BAP(1)+IBA(0.5)	6	65
Lavender	Callus	5	MS+BAP(1)+IBA(0.5)	6	60
Anise	Callus	5	MS+BAP(1)+IBA(0.5)	6	60

*MS medium was used; **Stored at 22+2°C

medium supplemented with 1.0 mgl^{-1} BAP and 0.5 mgl^{1} IBA after storage. In lavender, sage and anise synseeds could be stored upto 7 months in MS medium supplemented with 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} IBA and the callus proliferated on transfer to MS medium with 2.0 mgl^{-1} 2, 4-D. The proliferated callus regenerated successfully in the same medium used for storage. The encapsulation of callus can be used as a system for immobilisation of cells for the production of secondary metabolites from cell cultures. In this study, synthetic seeds germinated after storage, when transferred to the multiplication medium with over 70% viability, under sterile conditions. Sharma, Singh & Chauhan (1994) reported the possibility of germinating such beads under non-sterile environment by adding broad spectrum fungicides in the alginate beads. Improvement in the germination percentage of encapsulated beads of mulberry by addition of carbendazim in the matrix has been reported by Bapat & Rao (1990). Synthetic seeds were developed by encapsulating somatic embryos in carrot (Kitto & Janick 1985), *Santalum album* (Bapat & Rao 1988; Fernandes, Bapat & Rao 1994), *Eleusine coracana* (George & Eapen 1995), groundnut (Padmaja, Reddy & Reddy 1995), celery (Kim & Janick 1990) axillary or apical shoot buds in *Morus indica* (Bapat, Mhatre & Rao 1987; Bapat & Rao 1990), *Dioscorea* species (Hasan & Takagi 1995). Plant regeneration from protoplasts encapsulated in alginate beads was reported in *Pogostemon cablin* (Kageyama, Honda & Sugimura 1995). Synthetic seeds can be an ideal system for low cost plant movement, propagation, conservation and exchange of germplasm. Since it is important to produce disease free plantlets, encapsulation of disease free buds utilising tissue culture techniques serves as an easy and safe delivery system (Bapat, Mhatre & Rao 1987). Hasan & Takagi (1995) reported an alternative method for *in vitro* exchange

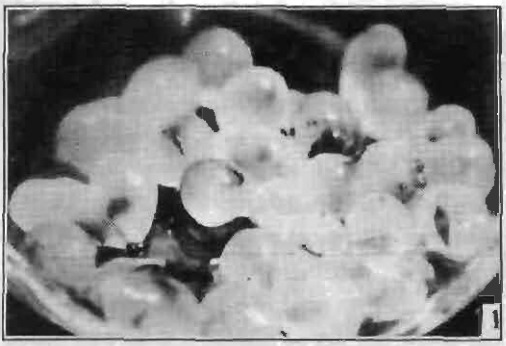

Fig. 1. Synthetic seeds in camphor

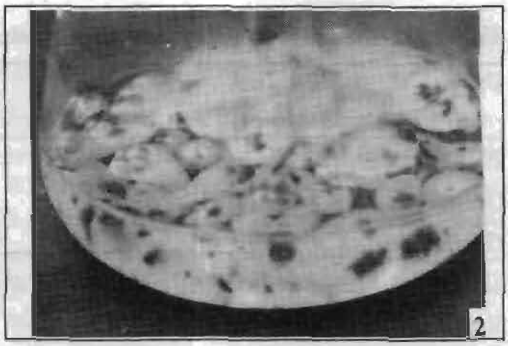

Fig. 2. Synthetic seeds in sage

of yam germplasm using encapsulated nodal segments. Gray & Compton (1993) reported the application of synthetic seed technology to germplasm conservation in grapes. This method of alginate encapsulation was used to develop an encapsulation - dehydration/ vitrification technique for cryopreservation of germplasm (Dereuddre, Blandin & Hassen 1991a, 1991b; Niino & Sakai 1992; Scotez *et al.* 1992; Paulet, Engelmann & Glaszmann 1993; Hatanaka *et al.* 1994; Fukai, Togasni & Gor 1994).

The present report on production and storage of synthetic seeds using different explants has great relevance as this system can be used as a disease free plant propagation system and means for conservation and exchange of germplasm of spices. The encapsulation of callus can be used as a system for immobilisation of cells for the production of secondary metabolites from cell cultures.

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Micropropagation of *Ruta graveolens* L. through organogenesis and somatic embryogenesis

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Abstract

Reproducible in *vitro* multiplication protocols were established in *Ruta graveolens* through shoot tip/nodal segment culture, direct/indirect organogenesis and somatic embryogenesis. Shoot tips cultured on Murashige and Skoog's (MS) medium containing 0.5 mgl^{-1} IAA, 4mgl^{-1} BA developed the maximum number of shoots. Multiple shoots were also induced from nodal and internodal segments and cultured in medium supplemented with 0.01-0.03 mgl^{-1} IAA and 3mgl^{-1} Kin/ 4mgl^{-1} BA alone. The nodal segment showed both axillary bud proliferaton as well as *de novo* organogenesis from the upper cut ends. Leaf segments incubated on MS medium containing 12 mgl^{-1} IAA, 2-6 mgl^{-1} 2,4-D and 0.3 mgl^{-1} Kin along with a supplement of 4 mgl^{-1} Adenine sulphate developed organogenetic callus. Shoots sprouted from this callus upon transfer to medium containing 0.8 mgl^{-1} IAA and 8 mgl^{-1} Kin. Petiole derived callus became embryogenic in the same medium and the plantlets were recovered when transferred to MS medium containing 2 mgl^{-1} BA alone. The isolated shoots were rooted in 1/2 MS basal medium and after hardening, the plantlets were grown in soil. Electrophoretic patterns of isozymes of peroxidase and esterase showed considerable variation in shoot and root forming callus cultures.

Keywords : isozymes, micropropagation, organogenesis, *Ruta graveolens*.

Introduction

'Rue' (*Ruta graveolens* Linn.), a valuable aromatic herb of the family Rutaceae, is extensively used in the indigenous systems of medicine. Conventionally, the plant is propagated through seeds and from dividing clustures in dry, stoney and sunny soils. The *in vitro* studies conducted in this plant were primarily focused on the exploitation of the secondary metabolites (Petit-Paley *et al.* 1989). Owing to the high medicinal value and the lack of efficient multiplication meth-

ods the present work was undertaken for establishing rapid multiplication through efficient *in vitro* routes of organogenesis and embryogenesis.

Materials and methods

Mature explants (40days old) of *Ruta graveolens* were washed in tap water, soaked and washed again in 0.2% (v/v) labolene (Qualigens, India) and finally surface disinfected with 0.1% (w/v) mercuric chloride for 90 sec. The sterilized explants were washed 4-5 times in sterile double distilled water under asceptic conditions and cultured on MS (Murashige & Skoog 1962) medium with hormones and additives in different concentrations (Tables 1 and 2). The medium pH was adjusted to 5.8, gelled with 0.8% (w/v) agar and autoclaved for 15 min at 121°C and 15lbs/inc^2. The cultures were incubated at 25±2°C under a 12 h photoperiod at a light intensity of 3000 lux.

Shoots, measuring 4-4.5cm, were excised from proliferated shoot cultures and rooted in 1/2 MS basal medium. They were hardened in 1/2 MS basal liquid medium on filter paper bridges for 10 days and subsequently for 10 days in vermiculite. The plantlets were nourished with 1/10 strength modified Hoagland's solution (Epstein 1972) while in vermiculite and subsequently transferred to graden soil and sand (1:3) under greenhouse conditions.

Isozyme analysis

For isozyme analysis, leaves from source plant, callus at different stages of organogenesis (viz. undifferentiated callus, callus showing initiations of shoot buds and with developed shoots/roots/both shoots and roots) and leaves from regenerated plants were harvested and extracted according to Arnison & Boll (1974). Polyacrylamide gel electrophoresis was performed as per the methodology of Laemmli (1970). Proteins were resolved on a 7.5% (w/v) acrylamide gel, pH 8.3 at 4°C in dark with a current of 10-15 mA. Peroxidase (PRX. EC.1.11.17) was stained using benzidine and H_2O_2 (Arinson & Boll 1974) and esterase (EST. EC. 3.11.1) with α -naphthyl acetate and fast blue RR salt (Allichio *et al.* 1987). The zymograms were prepared and analysed as per Moore & Litz (1984).

Results and discussion

Multiple shoots are initiated from young shoot tips (1-1.5 cm) cultured on MS medium containing IAA and BA (Table 1). Cultured shoots initially responded through enlargement of main apex and subsequently through axillary bud breaking. An average of 3-4 shoots/explant was initiated within 21 days in 4 mgl^{-1} BA and 0.5 mgl^{-1} IAA fortified medium. BA above this concentration along with 0.5 mgl^{-1} IAA was inhibitory to shoot proliferation (Table 1). Eventhough BA, generally is beneficial to shoot multiplication (Nair, Kartha & Gamborg 1979), at higher concentrations it normally suppresses the growth and multiplication rate of axillary buds (Sarasan, Alex & Nair 1991). After 3 passages (63 days), the multiplication process was terminated by rooting the individual shoot and transferred after hardening to the field. The survival rate of plants generated in high concentrations of BA was less than that recovered in the lower concentration of the hormone (Table 1). Earlier reports by Patience Alderson (1987) in liliac and Christopher & Rajam (1994) in *Capsicum annuum* have indicated that the high concentration of BA/Kin in the multiplica-

Table 1. Shoot-tip nodal segment culture and direct organogeneisis in *Ruta graveolens.*

Explant	PGR (mgl $^{-1}$)			No. of shoots/ explant	No. of plantlets survived		Survival (%)
	IAA	KIN	BA		Hardening	Field	
Shoot tip	-	-	1	13.1± 0.62*	12.12 ± 0.72	11.1± 0.29	91.58
	0.5	-	2	33.7 ± 0.21	33.14 ± 0.81	32.9 ± 0.41	99.2
	0.5	-	4	59.8 ± 0.48	56.70 ± 0.72	55.1 ± 0.76	97.1
	0.5	-	6	16.5 ± 0.31	15.52 ± 0.68	14.4 ± 0.38	92.7
Nodal segment	0	3	-	Initation of buds			
	0.01	3	-	26.2± 0.64**	25.8 ± 0.43	25.4 ± 0.48	98.4
	0.02	3	-	18.1 ± 0.76	17.8 ± 0.59	17.2 ± 0.32	96.6
	0.04	3	-	6.7 ± 0.29	6.2 ± 0.38	5.8 ± 0.74	93.5
Internodal	-	-	2	24.6 ± 0.84#	23.8 ± 0.72	23.4 ± 0.41	98.3
segment	-	-	4	38.9 ± 0.32	38.4 ± 0.48	38.2 ± 0.7	99.4
	-	-	6	16.1 ± 0.21	15.8 ± 0.32	15.2 ± 0.21	96.2
Leaf	-	-	1	3.1 + 0.29 ##	2.9 ± 0.49	2.7 ± 0.48	96.4
	-	-	2	11.1 ± 0.48	10.8 ± 0.51	10.6 ± 0.31	98.4
	-	-	3	4.7 ± 0.38	4.2 ± 0.48	4.1 ± 0.28	97.1

* Data scored for 3 subcultures (63 days) with values representing a mean of 7 replicates

** Data scored for the initial 30 days in culture with value representing a mean of 7 replicates

\# Data scored for the initial 40 days in culture with value representing a mean of 7 replicates

\#\# Data scored for the initial 60 days in culture with value reprisenting a mean of 7 replicates

tion medium was responsible for the inhibitition of rooting and subsequent survival in the field. The present study also supports this interpretation, since the roots developed in such media were unhealthy and with poor branching. Nodal segments planted in a medium containing 0.01 mgl^{-1} IAA and 3 mgl^{-1} Kin showed simultaneous axillary proliferation from the nodes and *de novo* shoot development from the upper cut ends, 3-4 and 18-72 shoots, respectively within 30 days (Table1). This suggests that the medium used is conducive for both types of regeneration and injury as the primary cause for triggering the proliferation of large number of shoots *de novo*. It is also of interest to note that the shoots developed through forced axillary branching grew faster than those developed *de novo*. In the former, the incipient shoots which were already differentiated, developed and elongated while in the latter case the competence for nutrients among the large number of developing shoots might have resulted in their fragile nature.

Shoots were also induced directly from leaf and internodal segments. Leaf segments cultured on MS medium containing 2 mgl^{-1} BA developed 4-6 shoot buds initially from the midrib portion and later from the entire leaf lamina. Internodal segments planted vertically in the medium containing 4 mgl^{-1} BA developed 35-40 shoots, clustured at the cut end. Survival rate of such field transferred plantlets was to the tune of 96-98% (Table 1).

Pale yellow friable callus was apparent on the cut surfaces of the leaf and petiole explants within 10-14 days, inoculated in MS containing combinations of 2 mgl^{-1} IAA, 6 mgl^{-1} 2,4-D, 0.3 mgl^{-1} Kin and 4 mgl^{-1} Ads. The leaf derived callus became compact and regenerated shoots in 0.8 mgl^{-1} IAA, 8 mgl^{-1} Kin containing medium within 40 days (Table 2).

The friable, petiole derived callus made into suspension in MS agitated liquid medium containing 0.8 mgl^{-1} IAA and 8 mgl^{-1} Kin formed embryogenic structures (viz. globular, heart shaped, and torpedo stages) within 90 days of incubation (Table 2). Embroys were also developed from callus transferred to MS solid medium containing similar hormonal milieu, albiet to a lesser number. The embryos germinated upon transfer to MS containing 2 mgl^{-1} BA or to $^1/_2$ MS basal medium with profuse root proliferation (Table 2).

Rooting, hardening and transplanting

Microcuttings obtained from various routes of organogenesis developed 6-8 roots/shoot in $^1/_2$ MS basal medium. The use of low salt medium for obtaining rooting response has been reported by several workers. (Maene &Debergh 1985; James & Thurbon 1981). The plantlets initially hardened on filter paper bridges (10 days) and later in vermiculite (10 days) showed varying rates of survival in the soil (Table 1,2).

Isozyme analysis

Both the enzymes, peroxidase and esterase showed intense activity during calougenic and rhizogenic events. Isoperoxidases resolved using benzidine HCl showed (Fig .1) the emergence of a new band (Rf 0.4; lane 4) at the onset of shoot induction which was absent in non-organogenetic callus. This band was retained during further stages of development including the regenerated plant. The rhizogenesis was characterised by the expression of 4 new bands (Rf 0.42;

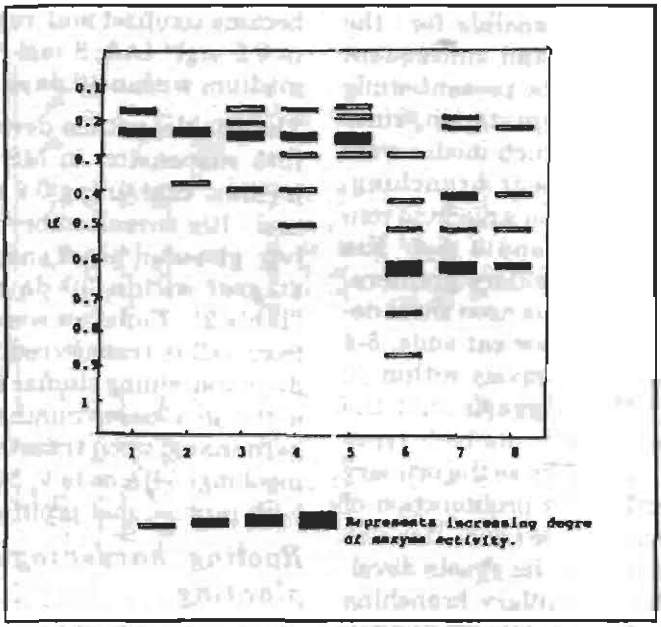

Fig. 1. Isozyme profile of peroxidases

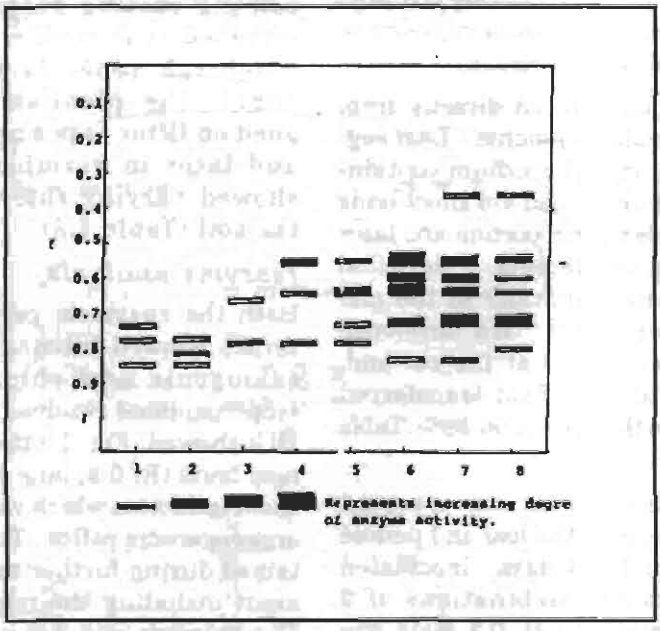

Fig. 2. Isozyme profile of esterases

Table 2. Callus regeneration and somatic embryogenesis

Callus source		PGR mgl $^{-1}$		Response		Survival		
	Medium	IAA	KIN	Mean no of shoots, buds/embryo	Mean no of shoots record/ germinated	Hardening	Field	Survival%
Leaf	MS	0.5	5	Shoot bud initiation				
		0.5	8	4.8*	3.8	3.2 ± 0.41	3.1 ± 0.38	96.87
		0.8	8	7.2	6.9	6.4 ± 0.48	6.1 ± 0.38	95.31
		0.8	9	3.1	2.5	2.2 ± 0.21	2.1 ± 0.81	95.45
Petiole	MS solid	0.8	8	Globular and heart shaped embryos (7-9)**	3.4#	3.1 ± 0.61	2.9 ± 0.29	93.5
	MS liquid	0.8	8	18-12	10.2	9.1 ± 0.86	8.1 ± 0.11	89
	1/2 MS	-	-	CP ##	-	-	-	-
	MS	-	-	CP	-	-	-	-

* Mean no. of shoots scored after 35 days in regeneration medium
** Mean no. of embryos formed after 90 days in culture medium
\# Mean on of germinated shoots in MS+ 2 mgl^{-1} IBA
Callus proliferation

0.50; 0.75 and 0.86; lane 6) besides an increasingly intense band at Rf 0.64. The appearance of new isoperoxidases, during caulogenesis was earlier observed by Arnison & Boll (1974) in bush bean, indicating a possible role of this enzyme in root and shoot organogenetic events.

Esterase bands with specificity for naphthyl acetate substrate showed (Fig.2) a relative increase in the activity of isoforms at Rf 0.76 through different stages of development from the callus (lane 5-8). In addition, a fresh minor band at Rf 0.36 was apparent in lane 7 and 8 (callus with shoot and root/regenerated plant). The increase in the enzyme activity during shoot/root morphogenesis and its absence in undifferentiated callus clearly substantiate the earlier findings of increased activity of the enzyme during vascular differentiation (Gahan 1981) and subsequent shoot/root morphogenesis (Everett, Wach & Ashwarth 1985).

Thus, through the present work, we have suggested protocols for increased regeneration and multiplication from various explants of *Ruta graveolens* using nodal segment culture, direct and indirect organogenesis and somatic embryogenesis. The protocols developed for nodal and internodal segment culture can be made use for the rapid *in vitro* clonal multiplication in large scale of this valuable aromatic plant and for the future improvement programmes through the other routes of regeneration.

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Micropropagation of some important herbal spices

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Abstract

Protocols were standardised for micropropagation of 13 herbal spices viz. thyme, spearmint, peppermint, sage, marjoram, oregano, anise, sacred basil, lavender, parsley, fennel, dill and celery using shoot tip and nodal explants derived from *in vitro* germinated seedlings. Regeneration of plantlets from callus was achieved in anise, dill, fennel, lavender and sage. Slow growth was induced and shoot cultures of these herbs could be maintained for 4-12 months without subculture in minimal growth medium.

Key words: herbal spices, micropropagation, plant regeneration.

Introduction

Herbal spices are fragrant herbaceous plants of which the whole plants, twigs, leaves, flowers, fruits, seeds etc., fresh or dried are used as flavouring agents. Once an elite genotype is identified it can be multiplied rapidly through tissue culture. Tissue culture studies were reported in anise (Ernst & Osterhelt 1984; Ernst 1989), celery (Gosal & Greval 1991; Kim & Janick 1991; Sakamoto *et al.* 1991; Toth & Lacy 1992), fennel (Du Manior, Desmarest & Saussay 1985; Hunault, Desmarest & Du Manior 1989; Song, Oh & Cho, 1991a, 1991b; Theiler-Hedtrich & Kagi, 1991; Hunault & Du Manior, 1992), thyme (Furmanowa & Olszowska 1992), mint (Rech & Pires 1986; Van Ech & Kitto 1990, 1992; Cellarova 1992; Kukreja, 1996), lavender (Panizza & Toghoni 1992). This paper reports tissue culture methods in thirteen herbal spices viz., anise (*Pimpinella anisum* L.), celery (*Apium graveolens* L.), dill (*Anethum graveolens* L.), fennel (*Foeniculum vulgare* Mill.), parsley (*Petroselinium crispum* Mill.), thyme (*Thymus vulgaris* L.), spearmint (*Mentha spicata* L.), peppermint (*Mentha piperita* L.), sage (*Salvia officinalis* L.), marjoram (*Marjorana hortensis* L.), oregano (*Origanum vulgare* L.), sacred basil(*Ocimum sanctum* L.) and lavender (*Lavendula angustifolia* Mill.) for rapid multiplication, plant regeneration from callus and minimal growth storage.

Materials and methods

Explants

Seeds of herbal spices used in this study were obtained from Lockhart Seeds Inc. 3 North Welsonway, Stockton, California. Seeds were treated with 0.01% fungicide (copper oxychloride) solution for 15 min and washed thoroughly several times with filtered water. They were then surface sterilised with 0.1% mercuric chloride for 3-4 min under aseptic conditions and washed with 5-6 changes of sterile distilled water. Seeds were then placed on culture medium for germination and the seedlings were allowed to grow for 30-60 days. Shoot tips and nodal segments were excised from these seedlings and used for micropropagation, callus regeneration and *in vitro* conservation.

Culture media

Murashige & Skoog basal medium supplemented with 0.5 mg l^{-1}kinetin and 2% sucrose, gelled with 0.7% agar was used for the germination of seeds. The MS basal medium supplemented with Kin, BAP, NAA, and IBA (0.5 mg l^{-1} and 1.0 mg l^{-1}) alone and in combination, sucrose (3%), gelled with agar (0.7%, 0.8% and 0.9%) was used to study the *in vitro* responses like multiplication, callus induction and plant regeneration.

MS basal medium at full and half strength, with sucrose (30 gl^{-1}, 20 gl^{-1}, 10 gl^{-1}) alone and in combination with mannitol (10 gl^{-1}, 20gl^{-1}), supplemented with 0.9% agar was used to induce minimal growth in cultures and to increase the subculture interval.

Culture conditions

The pH of the medium was adjusted to 5.8 and autoclaved at 121°C temperature and 1.5 kg/cm^2 pressure for 20 min. All the cultures were incubated at 25°C and 12 h photoperiod of 2500 lux. Observations were recorded on multiple shoot production, callus induction and regeneration.

Results and discussion

In vitro seed germination

The rate of germination and time taken for germination varied in different species. Seeds germinated within 10-20 days in the medium. Crops like anise, celery, dill, fennel, marjoram, oregano, peppermint, spearmint, sacred basil, sage and thyme showed 70-90% germination whereas in parsley and lavender the germination was only 20-30%.

Micropropagation and callus regeneration

Shoot tips and nodal segments excised from 30-60 days old seedlings gave rise to multiple shoots, rooting and callus regeneration in MS medium supplemented with different concentrations of auxins and cytokinins (Table 1). Multiple shoots could be induced in all the crops studied whereas successful plant regeneration from callus was observed in anise, dill, fennel, lavender and sage (Fig.1.1 to 1.8). In celery, marjoram, oregano, parsley, peppermint, spearmint, sacred basil and thyme, plant regeneration could not be induced. Multiplication rate was very high in plants belonging to Lamiaceae and produced 10-20 fold increase in the biomass in 45 days of culture.

In the case of fennel, MS supplemented with BAP (1.0 mgl^{-1}) and IBA (0.5 mgl^{-1}) was suitable for both multiple shoot formation and callus regeneration. Mul-

tiple shoots ranging from 4-7 were produced in 6 weeks of culture in 70 per cent of the cultures. Callusing was observed and plant regeneration was achieved in 60 per cent of the culture. The callus on transfer to fresh medium regenerated to shoots. Rooting was achieved in MS medium devoid of growth regulators. Micropropagation, callus regeneration and somatic embryogenesis were reported in fennel (DuManior, Desmarest & Saussay 1985; Miura, Fukui & Talata 1987; Hunault, Desmarest & Du Manior 1989; Hunault & DuManior 1992; Lourdes & Alfermann 1994; Hunault & Maatar 1995). Hunault, Desmarest & D Manior (1989) reported the difficulty in regenerating plants from callus but in the present study regeneration of shoots could be achieved in 60 per cent of cultures.

Among all the media combinations tried MS supplemented with IBA (0.5 mgl^{-1}) was suitable for the production of 10-15 multiple shoots in about 85 per cent of the cultures in celery and rooting was also achieved in the same medium. Micropropagation of celery was reported by Toth & Lacy (1992). In the present study no callus formation was observed in any of the combinations tried. Multiple shoots were produced in all the combinations but the highest rate was observed in MS medium with 0.5 mgl^{-1} IBA.

In dill 60 per cent of the cultures responded in MS with 0.5 mgl^{-1} kinetin. Multiple shoots (1:7) as well as callus initiation were noticed in this medium. Profuse callusing was obtained in MS supplemented with 2, 4-D (2 mgl^{-1}). The proliferated callus on transfer to MS basal medium regenerated to plantlets (50%). In anise multiple shoots and plant regeneration from callus was induced in MS with (1.0 mgl^{-1}) BAP and 0.5 mgl^{-1} IBA in 80% and 75% of the cultures respectively. Somatic embryogenesis and cell culture studies were reported in anise (Ernst & Osterhelt 1984; Ernst 1989).

In lavender, oregano, parsley, peppermint, spearmint, sacred basil and sage, MS medium with IBA (0.5 mgl^{-1}) and BAP (1.0 mgl^{-1}) was suitable for the production of multiple shoots (Fig. 1.1-1.6). In sage and lavender callus regeneration was obtained in MS with BAP (1.0 mgl^{-1}) and IBA (0.5 mgl^{-1}). Marjoram and thyme produced multiple shoots initially in MS with 0.5 mgl^{-1} kinetin and later on multiple shoots and rooting was obtained in growth regulator free medium. Micropropagation and regeneration of thyme was reported by Furmanowa & Olszowska (1992). They used Nitsch & Nitsch as the basal medium with different growth factors for thyme multiplication by shoot cultures, nodal segments and cotyledon segment culture. Clonal propagation of *Mentha piperata* was reported (Venkitaraman & Ravisankar 1986; Repcakova *et al.* 1986). Cellarova (1992) reported peppermint can be clonlly propagated in Linsmaier & Skoog medium with 2 mgl^{-1} BAP solidified with 0.4% agar. In the present report peppermint seeds germinated in MS with 0.5 mgl^{-1} kinetin and the *in vitro* derived shoot tips and nodal segments multiplied at the rate of 1:30 in 45 days of culture in MS supplemented with 0.5 mgl^{-1} IBA and 1.0 mgl^{-1} BAP. In the present study lavender could be micropropagated from shoot tips and nodal segments from *in vitro* derived

Table 1 *In vitro* response of herbal spices.

Plant species	Growth regulator (mgl^{-1})*	Response	Response (%)*
Anise	BA(1)+IBA (0.5)	MS, CR	80
Celery	IBA(0.5)	MS	90
Dill	Kin (0.5)	MS	60
	2,4-D (2)	CI	
	MS basal medium	CR	
Fennel	BA(1)+IBA (0.5)	MS, CR	70
Parsley	BA(1)+IBA(0.5)	MS	70
Lavender	BA(1)+IBA(0.5)	MS,CR	75
Sacred basil	BA(1)+IBA(0.5)	MS	70
Oregano	BA(1)+IBA(0.5)	MS	80
Marjoram	MS+Kin(0.5)	MS	75
Peppermint	BA(1)+IBA(0.5)	MS	85
Spearmint	BA(1)+IBA(0.5)	MS	80
Sage	BA(1)+IBA(0.5)	MS,CR	75
Thyme	Kin(0.5)	MS	80

*MS basal medium was used. **Mean of 10 replications.

MS: Multiple shoots, CR: Callus regeneration, CI: Callus induction.

seedlings in MS supplemented with BAP (1 mgl^{-1}) and IBA (0.5 mgl^{1}) and callus regeneration (80%) was also obtained in the same medium. In this medium on an average of 5-7 shoots were obtained in 40-45 days of culture. In lavender, callus induction and regeneration of callus were also obtained in the same medium in 60 days. Micropropagation by axillary budding and plant regeneration *via* callus was reported in lavender (Panizza & Toghoni 1988, 1992; Onisei, Toth & Amariei 1994). In sage, plant regeneration from callus was obtained in 80% of the cultures.

To our knowledge no detailed study has been made on tissue culture of spearmint, sage, oregano, marjoram and ocimum. In oregano, prevention of vitrification associated with *in vitro* shoot cultures by *Pseudomonas* spp. was reported (Shetty *et al.* 1995). In the present study also species belonging to the family Apiaceae showed vitrification and they require frequent subculture to maintain healthy cultures. Increase in agar concentration from 0.7 to 0.9% helped in reducing vitrification to some extent.

Multiplication rate was high in thyme, peppermint, oregano, celery and spearmint. Upon transferring to multiplication medium, they produced shoots and plantlets which appeared like a clump filling the culture vessel within 30-40 days. Growth rate is very high and healthy growth ceases within 30-40 days of subculture. This may be due to depletion of nutrients in culture medium. Response was faster in mints compared to other crops. Rooting could

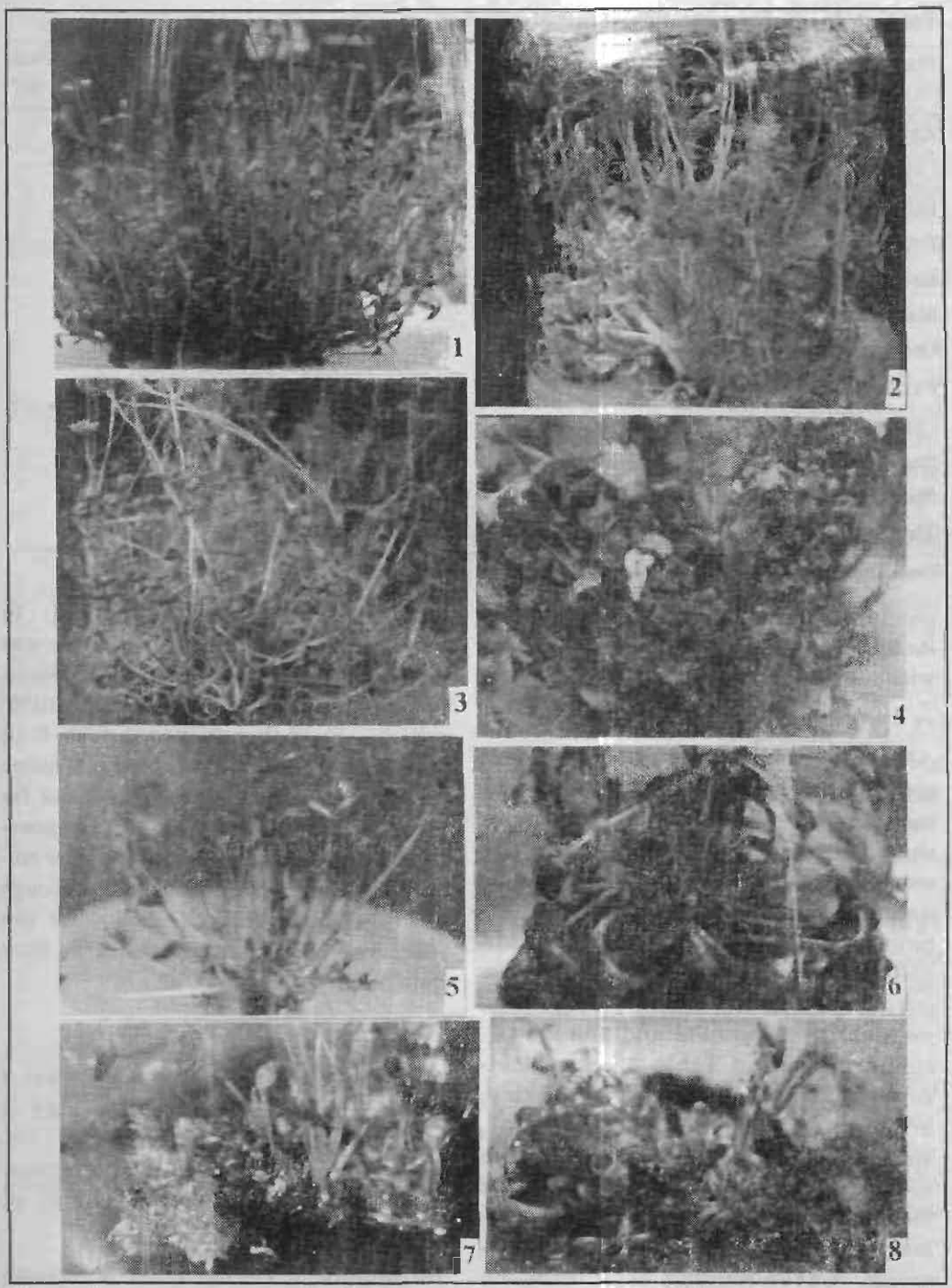

Fig. 1. *In vitro* responses of herbal spices. Micropropagation of thyme (1.1), fennel (1.2), mint (1.3), parsley (1.4), celery (1.5) and sage (1.6). Plant regeneration from callus of anise (1.7) and lavender (1.8).

Table 2 *In vitro* conservation of herbal spices

Plant species	Medium	Storage period (months)	Survival (%)*
Anise	$^1/_2$ MS+ 20 gl^{-1}S	6	60
Celery	$^1/_2$ MS+20gl^{-1}S	5	70
Dill	$^1/_2$ MS+ 20 gl^{-1}S	4	60
Fennel	$^1/_2$ MS+ 20 gl^{-1}S	4	65
Lavender	$^1/_2$ MS+ 20 gl^{-1}S + 10 gl^{-1}M	8	70
Marjoram	$^1/_2$ MS+ 20 gl^{-1}S	8	75
Oregano	$^1/_2$ MS+ 20 gl^{-1}S	8	70
Parsley	$^1/_2$ MS+ 20 gl^{-1}S	4	65
Peppermint	$^1/_2$ MS+ 20 gl^{-1}S	12	85
Sage	$^1/_2$ MS+ 20 gl^{-1}S+ 10 gl^{-1}M	6	65
Spearmint	$^1/_2$ MS+ 20 gl^{-1}S	6	65
Thyme	$^1/_2$ MS+ 20 gl^{-1}S	12	80

*Mean of 10 replications; S:Sucrose; M: Mannitol

easily be achieved in the same basal medium containing charcoal (1 gl^{-1}) or without any growth regulators.

In vitro conservation

All the species studied are very fast growing under *in vitro* conditions and maintenance of healthy cultures more than thirty days was difficult under normal conditions. Among the combinations of media tried, reduction of sucrose and/or reduction of nutrients helped in reducing the growth rate of *in vitro* cultures. The media combination, percentage of survival and storage period are given in Table 2. Shoot cultures of dill, fennel, celery, parsley, oregano, marjoram, peppermint, spearmint, anise, thyme and ocimum could be stored upto a period of 4-12 months without subculture in ½ MS medium supplemented with 20 gl^{-1} sucrose. Lavender and sage could be stored upto six months in ½ MS medium with 20 gl^{-1} sucrose and 10 gl^{-1} mannitol. Agar was used at 9 gl^{-1}, so that vitrification could be reduced. *In vitro* conservation of herbal spices was not reported so far except in *Mentha* species (Gunning & Lagerstedt 1986) which could be stored up to 6-13 months. The techniques standardised in the present study can be used for large scale multiplication of elite genotypes, production of biomass in the culture vessel, creating variability through callus regeneration and also for the maintenance of germplasm in *in vitro* genebank

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Induction of *Phytophthora* foot rot tolerance in black pepper through *in vitro* culture system

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Abstract

Three black pepper varieties namely Panniyur 1, Panniyur 4 and Karimunda were utilised to induce variability for tolerance to *Phytophthora* foot rot. *In vitro* culture system was developed for indirect organogenesis and the regenerants were screened against the disease using the culture discs of the live organism through leaf and stem inoculation. The toxic metabolite isolated from the fungal culture was used to induce *in vitro* stress during callus induction and regeneration. The toxic metabolite was found to act at cellular level and all the levels of the toxic metabolite tried were found to inhibit the regeneration potential of the callus. The regenerants were found to be highly sensitive to the infection thereby indicating the inefficiency of such *in vitro* stress for the induction of tolerance/resistance against *Phytophthora capsici*. However, the callus regenerant developed in the absence of *in vitro* stress from toxic metabolite exhibited good tolerance indicating the potential for exploitation of somaclonal variation in black pepper improvement.

Key words: black pepper, disease resistance, *Phytophthora capsici*, somaclonal variation.

Introduction

Conventional breeding programmes so far have not succeeded in inducing resistance against *Phytophthora* foot rot disease of black pepper. The great potential of plant cell and tissue culture technology has been exploited in various crops to increase the efficiency of crop improvement programmes especially through somaclonal variation. Hammerschlag *et al.* (1994) have suggested *in vitro* culture and screening for somaclonal variation for obtaining disease resistance in peach. Mendel *et al.* (1995) have reported great degree of somaclonal variation for sheath blight resistance in rice.

The present paper reports the attempts made to induce variability through *in vitro* culture system in black pepper.

Materials and methods

Callus induction and regeneration

In vitro cultures of black pepper varieties viz., Panniyur 1 (P_1), Panniyur 4 (P_4) and Karimunda (Ka) were established using leaf segments as explants. The protocol developed by Nazeem *et al.* (1993) was followed for callus induction and regeneration. The leaf segments were surface sterilised with 0.1 per cent mercuric chloride for 5 min and cultured in modified MS basal medium supplemented with 3% sucrose, 0.7 % agar, 1.0 ppm IAA, 1.0 ppm BA and 0.5 ppm NAA for callus induction. The calli induced were subcultured in the same medium for further proliferation and later transferred to the medium supplemented with 1.0 ppm BA, 1.0 ppm IAA and 15 ppm silver nitrate, for shoot regeneration. The regenerated shoots were rooted and planted out following the procedure standardised earlier (Joseph *et al.* 1994).

Isolation of toxic metabolite

Pure cultures of *Phytophthora capsici* was maintained in oatmeal dextrose agar and later cultured in modified Rebeiro's medium (Rebeiro *el al.* 1975) for enhanced production of toxic metabolite. After seven days incubation, the culture filtrate was separated and concentrated to 1/10th volume to get concentrated culture filtrate (CCF). The toxic metabolite was fractionated by passing the CCF through ion exchanger columns of Dowex 1 and Dowex 50, modifying the procedure Keen *et al.* (1975). The fractions collected at near neutral pH was tested for phytotoxicity on intact black pepper plants and detached leaves. This fraction was used as absolute toxic metabolite and was incorporated into the medium after desired dilution.

In vitro *stress for induction of variability*

The calli induced and proliferated were repeatedly subcultured upto 15 subculture (3 week cycle) and the regenerated shoots were rooted and planted out. Biotic stress was induced by incorporating the toxic metabolite at varying concentrations in the culture induction and regeneration media. The regenerants were later rooted and planted out. Atleast 30 cultures were observed under each treatment in all the experiments.

Screening for disease tolerance

The regenerants were screened for disease tolerance using culture discs of *Phytophthora capsici*. Pin pricks were made on the detached shoots, on the leaf and the stem before placing the culture disc. The shoots were incubated at 26±2°C with high humidity (>80%). The shoots were observed for six days and the symptom developed was recorded at two days interval. Spread of dark brown, necrotic spot/patches on the leaf/stem was measured and scored as very low, low, medium and high.

Bioassay for electrolyte leakage

The leakage of electrolytes when infiltrated with the toxic metabolite was assayed using a precision conductivity meter of ORION make, model 120, following the procedure described by Vidyasekharan *et al.* (1986). The

youngest fully opened leaves of the tolerant and susceptible plants were inoculated with the live cultures (culture discs) of *Phytophthora capsici*. Three days after inoculation, 1.0 cm^2 area of leaf lamina from near the inoculated region was cut into fine pieces and random sample of 100 mg was enclosed in cheese cloth and placed in scintillation vials. Three ml toxic metabolite (10%) was infiltrated into the leaf sections in vacuum for 30 min. The samples were then rinsed with water and placed in another vial with 10 ml glass distilled water. The vials were slowly shaken and the conductance of ambient solution was measured at 30 min interval. The sample infiltrated with distilled water was used as control.

The electrolyte leakage from callus cultured in the presence and absence of biotic stress was also determined by taking random samples of 100 mg. The electrolyte leakage was expressed as μmhos.

Results and discussion

Callus induction and regeneration in the presence of toxic metabolite

The callus induction and regeneration potential were found inhibited in the presence of toxic metabolite in the culture medium. The response was almost same in all the three varieties tried (Table 1). Among the three varieties tried, Panniyur 4 recorded maximum callus induction and regeneration in the medium identified. The toxic metabolite incorporated in the culture medium, to induce the *in vitro* stress, reduced callus induction to the extent of 29 % and callus regeneration to the extent of 90%. The negative response was the highest in variety Panniyur 1. The calli cultured in toxin incorporated medium turned black with high phenol exudation.

Table 1. Influence of toxic metabolite from *P.capsici* on callus induction and regeneration in black pepper.

Culture medium	Response of varieties (%)		
	P_1	P_4	Ka
I. Callus induction			
Callus induction medium (M_1)	76	94	89
M_1 + 50 ppm toxic metabolite	68(11)	87(7)	73(18)
M_1 + 100 ppm toxic metabolite	54(29)	82(13)	65(27)
II Callus regeneration			
Callus regeneration medium (M_R)	48	78	55
M_R + 50 ppm toxic metabolite	20(58)	46(41)	32(42)
M_R + 100 ppm toxic metabolite	5(90)	16(79)	9(84)

P_1 = Panniyur 1, P_4 = Panniyur - 4, Ka = Karimunda
(Values in parentheses indicate % reduction over control)

Culture filtrates and toxic metabolites of causal organisms are often used for *in vitro* screening. Lee (1973) has reported the possibility of using toxic metabolite of *Phytophthora* sp. for screening black pepper against 'foot root' disease. The *toxic metabolite from P.capsici* incorporated in the culture

medium in the present study could induce the stress as indicated by the reduction in callus induction and regeneration.

Screening of calli clones

The callus regenerants were screened using culture discs of *P.capsici*. All the plantlets regenerated in the presence of toxic metabolite were found highly susceptible to infection with live organism (Table 2). However, the callus cultures that had passed more than 12 subculture cycles and had regenerated in the absence of stress induced by the toxic metabolite showed great variation for the expression of disease symptom. Two plants out of 200 screened escaped the infection. These plants did not develop the disease symptoms even after repeated inoculation with the fungus. Generation of variability for disease resistance through tissue culture has been found successful in various plant species. Sebastiani *et al.* (1994) could screen out *Verticillium* resistant lines from among the calli clones of potato. Arnold *et al.* (1995) identified crook rot resistant watercress from somaclones.

Table 2. Response of tissue culture derived plants when inoculated with culture discs of *P.capsici*.

Type of plant	Percentage infected				
	Nil	Very Low	Low	Medium	High
Callus regenerants	1.0	9.5	22.0	25.5	42.0
Calli clones regenerated in the presence of toxic metabolite	-	-	-	12.0	88.0
Bud culture derived plants	-	-	-	62.0	38.0

Several reasons have been attributed to the variations that occur in tissue culture cycle. Variability is most likely to be the result of both pre-existing genetic instability and genetic alteration induced during the process of cell culture and subsequent plant regeneration (Evans 1988). Some such changes would have taken place in the prolonged culture period of callus regenerants of black pepper in the present study and that could account for the variation observed for foot rot tolerance.

General resistance mechanisms in plants against *Phytophthora* species include structural features of the host, preformed chemical inhibitors, induced structural barriers and presence of phytoalexins (Keen Yoshikawa 1983). The *in vitro* stress induced in the present study by incorporating toxic metabolite in the culture medium was found to reduce the *in vitro* response in black pepper. Such stress might not be sufficient to improve the resistance mechanism in black pepper. Moreover, the toxic metabolite that might act upon the cell wall components reduced the regeneration potential and vigour of the regenerants which was expressed as increased sensitivity to infection with the organism. Thus the stress induced by the toxic metabolite did not improve the resistance mechanism of black pepper regenerants in the present study.

Bioassay for electrolyte leakage

The leakage of electrolytes from intact and infected cells/tissues is presented in Table 3. The leakage measured as electrical conductance induced with the toxic metabolite in intact calli (control) i.e., callus cultured in the absence of toxic metabolite was only 68 µmhos within one hour whereas it was 122 to 138 for the calli cultured in the medium having toxic metabolite. This clearly indicates the *in vitro* stress induced in the presence of the toxic metabolite. The stress induced did not vary much among the varieties Panniyur 1, Panniyur 4 and Karimunda.

Table 3. Electrolyte leakage expressed by tolerant and susceptible types.

Treatment	Electrolyte leakage (µmhos) (1 h after infiltration)
Callus stage	
Intact callus	68
Callus in M_R + 100 ppm toxic metabolite	
P_1	138
P_4	122
Ka	124
Whole plant level	
Callus regenerant with high infection	131
Callus regenerant with no infection	86
P.colubrinum	84

The intensity of electrolyte leakage from cells of infected plants was found to vary according to the subsceptibility to the disease. The callus regenerant which showed high infection range when inoculated with the culture disc recorded the highest value for electrolyte leakage (131 µmhos). The calli clone that escaped the symptoms and which is expected to be tolerant, recorded a leakage value of 86 µmhos. This was comparable with the leakage recorded for *Piper colubrinum* and exotic species identified to be resistant to *P.capsici*.

The data clearly indicated the high tolerance level of the 'escapes' identified among the calli clones of black pepper.

The electrolyte leakage bioassay is an efficient technique to determine the intensity of damage occurred to the cell wall and thereby indicate the tolerance level of cells/tissue assayed. Vidyasekharan *et al.* (1986) have suggested this technique for determining the host specificity of toxin produced by *Helminthosporium oryzae* in rice. Shylaja *et al.* (1996) have reported variations in electrolyte leakage level in different varieties of black pepper when infiltrated with concentrated culture filtrate of *P.capsici*. They have reported a positive relation between disease susceptibility and electrolyte leakage. The low electrolyte leakage and failure to develop disease symptoms when infected with *P.capsici* indicate the high tolerance of the somaclone identified in the present study.

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Micropropagation and genetic fidelity studies in *Piper longum* L.

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Abstract

Multiple shoots were induced from mature plants of *Piper longum* in MS medium supplemented with cytokinins. Maximum shoot proliferation without callus formation was observed in MS medium supplemented with 0.5 mgl^{-1} BA and 0.5mgl^{-1} Kn and 100% rooting was achieved in half strength MS medium. Comparison of RAPD fingerprints of twenty micropropagated plants and the mother plant was carried out by amplification of genomic DNA using 10-mer universal primers. Thirtyseven out of 65 amplified products were polymorphic and only one primer produced all monomorphic fragments.

Key words: micropropagation, *Piper longum*, RAPD.

Introduction

Among the cultivated species of *Piper*, *Piper longum* L. (long pepper) is the third most economically important species which is commonly used in seasoning food and as medicine. Long pepper is conventionally propagated through cuttings. However, tissue culture techniques could successfully be employed in mass multiplication of disease-free clones and elite genotypes for cultivation as well as *in situ/ex situ* conservation. Though *in vitro* multiplication in *P.longum* has been reported by Bhat, Kackar & Chandel (1992); Bhat, Chandel & Malik (1995) the present paper reports the micropropagation of *P.longum* using mature explants and genetic fidelity analysis of the micropropagated plants using RAPD (Williams *et al.* 1990).

Materials and methods

Plant material and culture conditions

Single nodal segments (3-3.5.cm) from pot raised plants were excised, washed with detergent (Tween-20) under running water. The explants were given a pre-treatment with a mixture of systemic fungicide, Bavistin (1% w/v) and Fytolan(0.5% w/v) in detergent (Tween-20) for 5 min before surface sterilizing with 0.1% $HgCl_2$ for 15min. The shoots were trimmed (1-1.5cm) and inoculated on MS medium (Murashige & Skoog,

1962) with different concentrations of growth regulators. For initiation, MS medium supplemented with 0.1-2.0 mgl^{-1} IBA was used. Subculturing was carried out regularly at three week interval on MS medium with different combinations of BA (0.1-2.0mgl^{-1}) and kinetin (0.1-2.0mgl^{-1}) seperately or in combination to evaluate multiple shoot induction. The elongated shoots were cut into individual segments (3-4cm) and rooted on half strength MS medium.

All media were adjusted to pH 5.8, gelled with 0.8% agar(w/v). Cultures were maintained at 25±2°C with 16h photoperiod at 3,000 lux. For each treatment, twelve replicates were maintained and each experiment was performed thrice.

Hardening and establishment in mist chamber

In vitro raised plants were transferred to sterile potting mixture of soil: sand(2:1) under controlled conditions (28±2°C, 80±5% RH and 14 hr photoperiod) in the growth chamber (Nippon LP-1PH). After about four weeks, the hardened plants were transferred to soil and kept in mist chamber for four weeks before transferring them to field.

Fingerprinting by RAPD

RAPD analysis was carried out by PCR amplification of genomic DNA using ten 10-mer universal primers (Operon Tech USA). Genomic DNA was extracted from leaf tissue using the standard CTAB method (Saghai Maroof *et al.* 1984). For RAPD analysis, the reaction mixture of 25 l containing 10 ng of genomic DNA, 2.5µl of 10X assay buffer (100mM Tris-HCI, 500mM KCI and 0.01% gelatin), 2mM $MgCl_2$, 100µM each of dATP, dGTP, dCTP, dTTP, 15 ng of primer and 1.0U of Taq DNA polymerase was prepared. Each reaction mixture was overlaid with equal volume of mineral oil and DNA amplification was performed in Perkin Elmer DNA Thermal Cycler 480. The amplification condition included a total of 30 cycles with 1 min (3 min for the first cycle) at 94°C, 1 min at 40°C and 2 min at 72°C followed by incubation at 72°C for 15 min. The amplification products were analysed by electrophoresis (on 1.2% agarose gel) in 1X TAE buffer at 50V for 3 hours.

Results and discussion

Micropropagation

Bud break was readily observed in the media supplemented with 0.5mgl^{-1} BA. Various combinations of BA or kinetin were tested for morphogenetic potential of the uninodal segments. Maximum shoot proliferation per nodal segment was recorded in MS medium with 0.5 mgl^{1} IBA and 0.5 mgl^{-1} kinetin (Table 1). Trimming of growing apices and subsequent subculturing on fresh set of media overcame apical dormancy. No callus formation was observed. Combinations of BA and kinetin showed better response when compared to treatments of BA or kinetin alone. The proliferating shoots were sub-cultured on elongation medium. Root induction and development were achieved with 100% success in half-strength MS medium within two weeks. Rooted plants were hardened and established in soil with 85% survival rate.

Genetic stability of micropropagated plants

Ten random primers were used for assessing the RAPD fingerprint profiles of 20 randomly selected micropr-

Table 1. Effect of different combinations of cytokinins (BA and Kn) on shoot proliferation @

BA(mgl^{-1})	Kinetin (mgl^{-1})	No. of shoot ± SD*
0.1	-	2± 0.94
0.5	-	4.4±1.75
0.75	-	2.66±0.86
1.00		2.46±0.84
-	0.1	3.5±1.26
-	0.5	7.2±1.91
-	0.75	6.5±1.35
0.1	0.5	12.36±2.5
0.5	0.5	29.24±5.08
1.0	0.5	7.36±2.41
2.0	0.5	5.56±2.4

@ on MS basal medium * Standard Deviation.

opagated plants and the mother plant (Table 2). A total of 65 fragments were amplified with an overall size range from 257 (OPD 13) to 4381 bp (OPA 15). The number of amplification products per primer ranged from four (OPD 12) to eight (OPD 01, OPA 08, OPD 11). Except OPD 13 in which all the fragments were monomorphic, the other primers produced 2 (OPD 12) or more polymorphic fragments (upto 8 by OPA 08).

Table 2: Primers used, total number and polymorphic bands, and size range of the amplification products.

Primer (Operon Tech)	Sequences	No.of amplified fragments		Size range (bp)
		Total	Polymorphic	
OPA05	AGGGGTCTTG	5	4	467-1639
OPA08	GTGACGTAGG	8	4	416-1757
OPA15	TTCCGAACCC	7	5	559-4381
OPA18	AGGTGACCGT	6	3	287-3206
OPA20	GTTGCGATCC	6	5	369-1394
OPD01	ACCGCGAAGG	8	8	290-2706
OPD11	AGCGCCATTG	8	3	464-1743
OPD12	CACCGTATCC	4	2	515-1629
OPD13	GGGGTGACGA	7	0	257-3308
OPD14	CTTCCCCAAG	6	3	481-2623

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Variability in the tissue cultured cardamom plants

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Abstract

The Indian Cardamom Research Institute took up a large scale field evaluation of tissue cultured cardamom clones vis-a-vis open pollinated seedling progeny during 1988-89. In Karnataka, performance of 8 high yielding micropropagated clones and open pollinated progenies were evaluated at 56 locations planted in an area of 37.5 hectares. Variability was observed in the clonal population as change in the type of panicle, capsule shape and size and sterility. The overall variability in tissue cultured cardamom plants was 4.5% as against 3.0% in open pollinated seedling progeny for the given set of characters. The variability in individual clones vis-a-vis their seedling progeny, the possible cause of these variabilities are discussed.

Key words: cardamom, micropropagation, tissue culture, variability.

Introduction

Small cardamom (*Elettaria cardamomum* Maton) is an indigenous spice crop cultivated in an area of over 83,000 ha spread over Kerala, Karnataka and Tamil Nadu states. The productivity of cardamom in India is low (113 Kg/ha) compared to other cardamom producing countries like Guatemala (250 Kg/ha), on mainly due to lack of sufficient high yielding planting materials, use of seedlings having high degree of variability and unscientific cultivation practices.

Tissue culture is expected to produce true to mother type planting material. Micropropagation (tissue culture) techniques were standardised for cardamom as early in 80's in different institutes (Rao *et al.* 1982; Nadaguada *et al.* 1983; Kumar *et al.* 1985). However, no scientific information is available on the adaptability and suitability of tissue culture plantlets in field conditions. Hence, Spices Board, Govt. of India, Ministry of Commerce, in collaboration with the Department of Biotechnology (DBT), New Delhi, formulated a project with an objective to evaluate the performance of high yielding tissue cultured clones vis-a-vis the open pollinated seedlings of cardamom under different agroclimatic conditions, in the planters' fields. The present paper re-

ports the variability in tissue cultured plants as compared to that of open pollinated (OP) seedlings.

Materials and methods

Tissue cultured cardamom plants (TC) of 8 high yielding clones selected by ICRI, Regional Station, Sakleshpur, were micropropagated and supplied by two private firms for the project. These were compared with the open pollinated seedlings (OP) of the same mother plants. A total planted area of 37.5 ha spread over a 56 planters' fields in Karnataka was selected for the study. Planting was done during 1990 & 1991 and the plants started yielding in 1992 & 1993, respectively.

Survey was conducted covering 30 plantations having trial plots (35,500 TC plants and 5,600 OP seedlings) for variability. Only variabilities which were found adversely affecting the yield were considered in the present study.

The clones tested were all prostrate panicle types and any change in the type of panicle i.e. semi-erect to erect were classified as semi-erect (S.E.) panicles. Plants having no capsules at all were classified as completely sterile plants and plants with less than 5 capsules/panicle were classified as partially sterile plants. Plants having average capsule diameter of 10 mm or less were taken as microcapsules.

The variability in the tissue cultured plants were compared with that of open pollinated seedlings of each clone using paired "t" test (Panse & Sukhatme 1985). Observations from 100 plants each were taken for the analysis.

Results and discussion

In the present study no macromorphological variations were observed with respect to growth characters. However, four types of variability affecting the yield were recorded. They were change in the nature of panicle i.e. from prostrate to semi erect/erect type, partially or completely sterile plants and plants having micrcapsules. In all the clones studied, one or the other type of variability was noticed. In the seedling progeny also these variabilities were noticed.

In general, tissue cultured plants had more variability than seedling progeny. Variability regarding nature of panicle was observed in all clones. It was significantly higher in clones 2,3,5,6 and 8 than the seedling progeny. Complete sterility was noticed in clones 5,6,7& 8 and it was absent in clones 1, 2, 3, & 4. Sterile TC plants were significantly higher in clone 5 compared to seedlings. A more number of partially sterile plants was observed in clones 3, 5 & 8. Partial sterility was, however, absent in clones 1 & 2. Microcapsules were also not observed in clone 2. But clones 1, 3, 4, 7 & 8 had significantly more microcapsules. In general, among the tissue cultured (TC) clones, clone 2 had least variability followed by clone 1, 3 and 4. All the four types of variabilities were observed in clone 5, 6, 7 and 8.

In the present study, the overall percentage of variability was 4.53 in tissue cultured plants compared to 3.04 in the open pollinated seedlings (Table 1). Micro capsules accounted for the major share of variation in TC plants (8.4%), next in order being panicle variation (1.3%) followed by partial sterility (0.8%).

One of the main advantages of

Table1 . Variability in tissue cultured cardamom plants

Clone	No.of yielding plants	Variability						Yield Kg/ha
		S.E. panicles	Complete sterility	Part. sterility	Micro capsules	Total	%	
TC1	2972	5	1	15	52	73	2.45	682
OP1	435	-	-	5	8	13	2.98	399
TC2	982	24	-	-	-	24	2.44	241
OP2	182	-	-	-	-	-	-	141
TC3	497	11	1	5	8	25	5.03	324
OP3	125	-	-	-	-	-		231
TC4	1219	5		2	29	36	2.95	572
OP4	363	1	-	-	1	2	0.5	353
TC5	3254	74	12	38	22	146	4.48	284
OP5	490	1	-	5	9	15	3.06	163
TC6	2296	35	8	28	34	105	4.57	234
OP6	581	1	2	17	24	44	7.57	185
TC7	7719	41	17	59	374	491	6.36	354
OP7	1431	13	1	17	49	80	5.59	259
TC8	11578	212	14	115	138	479	4.13	711
OP8	2076	7	2	3	7	19	0.91	487
Grand Total TC	30517	407 (1.3%)	53 (0.17%)	262 (0.8%)	657 (2.1%)	1379	4.53	427
OP	5683	23 (0.4%)	3 (0.05%)	47 (0.8%)	98 (1.7%)	173	3.04	277

smicropropagation technique is mass multiplication of plantlets which are genetically similar and phenotypically uniform. However, occurrence of variability among tissue cultured plants have been reported in oil palm (Corely *et al* 1986), black berry (Harry *et al.* 1983), coleus (Marcatrigiano *et al.* 1990), strawberry (Moore *et al.* 1991) and plantains (Vuylsteke *et al.* 1988; Yair Israeli *et al.* 1991). Various reasons have been assigned to the occurrence of variability in *in vitro* propagated plants. The probable causes are adventitious bud formation during micropropagation via axillary buds, genetic instability of adventitious meristem and tissue culture induced disorganisation of meristems.

According to Hammer Schlag (1992) somaclonal variation can be either eliminated or minimised if special efforts are made to distinguish between axillary and adventitious shoot that are produced during *in vitro* propagation and rogue out the adventitious shoots.

Inspite of the occurrence of variability in tissue cultured plants, 34% higher yield was obtained in the TC plants

over the open pollinated seedlings. The present study in cardamom indicates that the optimum number of transfer cycles should be standardised to bring the variability among tissue cultured plants to a minimum level. The higher amount of variability observed in the clones where more plantlets were supplied indicate it would have taken larger cycles to produce more number of plantlets.

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Preliminary yield trial of tissue cultured cardamom selections

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Abstract

Tissue cultured cardamom selections tested for their yield performance for three years revealed TC 5, TC 6 and TC 7 are promising compared to other five lines. These three selections also differed among themselves for yield and yield attributes. Clonal crop of the two ruling varieties viz., Mudigere 1 and Mudigere 2 yielded sigificantly low compared to TC 5 but was on par with other seven tissue cultured selections.

Key words: *Elettaria cardamomum*, tissue culture, yield evaluation.

Introduction

Cardamom (*Elettaria cardamomum* Maton) is an important spice of export value. Lack of availability of sufficient planting material of high yielding varieties is one of the production constraints. Of late tissue culture technique is being used to multiply elite lines and varieties of cardamom. But studies on the yield performance of the tissue cultured plantlets are limited. Hence the present study was undertaken to evaluate the yield performance of eight tissue cultured cardamom selections along with two clonal lines.

Materials and methods

Hardened plants of eight tissue cultured selections viz., TC 5, TC 8, TC 1, TC 7, TC 4, TC 6, TC 3 and TC 2, and suckers of the two local check varieties viz., Mudigere 1 and Mudigere 2 were planted in an upper slope of the Regional Research Station, Mudigere, Karnataka, during November 1991 in a randomized block design, replicated thrice in single row plots of 14 plants each with a spacing of 1.8 x 0.9m. All the ten entries in the study were malabar type with spreading panicle.

Protective irrigation was given once in 10-15 days during summer months. High mortality of plants in some of the tissue cultured selections and unavoidable damage to the suckers of check varieties resulted in variation in the number of plants available for observation and data collection in each replication.

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Data were collected from ten middle plants leaving two plants on either end in most of the entries and in a few entries the number of plants available for observation varied from 3 to 5. In view of this t- test (Little & Hills 1975) was employed for testing differences between the entries.

Results and discussion

The data collected on yield attributes and yield (1993-96) and capsule characters (1995-96) are presented in Tables 1, 2 & 3. Maximum height of psuedostem was observed in TC2 followed by TC7. Mudigere-1 & Mudigere - 2 were significantly shorter as compared to the tissue cultured lines (TC2, TC7, TC5 & TC6). The tissue culture lines TC5, TC6, TC4, TC8, TC 1 and TC3 did not differ significantly among themselves. Bearing and total suckers per clump were significantly higher in TC1 followed by TC3 and both the entries were on par. The entries did not differ as regards to new suckers produced. TC5 had maximum panicles per clump next in order being TC6 and TC7 which were on par with it. TC7 was on par with Mudigere 1 and Mudigere 2. TC4, TC8, TC1, TC3 and TC2 were on par with each other and with Mudigere 1 and Mudigere 2. Panicle length in TC5 was significantly longer compared to others. TC7 was next in rank and was significantly superior than all other entries which were on par with each other. Nodes per panicle were significantly lower in Mudigere 1 compared to TC 5, TC 7 and TC 1 and all other entries were on par with each other. Flowers per node were significantly more in TC 5 compared to TC 2 and TC 1 and others were on par with it.

Yield during first year was maximum in TC 6 and it was on par with TC 5, TC 7, TC 4 and Mudigere 2. However, during the second and third years

Table 1. Yield attributes of tissue cultured promising cardamom selections in preliminary yield trial, 1993-96.

Entries	Psuedostem height (cm)	Suckers/Clump			Panicles/ clump	Panicle length	Nodes/ panicle	Flowers/ panicle
		Bearing	New	Total				
TC 1	228 cd	6.0 a	9.2	15.2 a	7.9 c	44 c	16.2 a	6.0 b
TC 2	260 a	4.9 ab	8.3	13.2 ab	7.5 c	43 c	15.9 ab	6.5 b
TC 3	227 cd	4.8 b	8.0	12.8 b	7.3 c	40 c	14.6 ab	7.2 ab
TC 4	231 cd	5.2 ab	8.7	13.9 ab	8.3 c	44 c	15.1 ab	7.2 ab
TC 5	240 bc	5.4 ab	8.0	13.3 ab	13.0 a	64 a	16.8 a	8.8 a
TC 6	239 bc	5.9 ab	9.4	15.2 a	11.6 a	44 c	14.6 ab	7.3 ab
TC 7	254 ab	5.7 ab	7.9	13.6 ab	11.4 ab	54 b	16.2 a	8.0 ab
TC 8	227 cd	5.1 ab	9.0	15.1 a	8.1 c	41 c	14.6 ab	7.3 ab
M 1	214 d	5.3 ab	9.0	14.1 ab	8.7 bc	36 c	13.3 b	7.4 ab
M 2	216 d	5.3 ab	9.7	15.1 a	8.8 bc	42 c	14.3 ab	7.3 ab
Mean	234	5.4	8.8	14.1	9.2	45	15	7.3
t-test	*	*	NS	*	*	*	*	*

Table 2. Performance of tissue cultured promising cardamom selections in preliminary yield trial, (1993-96).

Entries	Yield (Kg/ha dry capsules)			
	1993-94	1994-95	1995-96	Average
TC 1	41 bcd	54 c	83 ab	56 cde
TC 2	40 bcd	55 bc	71 ab	55 de
TC 3	16 d	49 c	49 b	38 e
TC 4	54 abcd	115 abc	69 ab	79 bcd
TC 5	77 ab	175 a	184 a	145 a
TC 6	93 a	93 bc	110 ab	99 b
TC 7	62 abc	134 ab	91 ab	96 bc
TC 8	20 cd	89 bc	142 ab	84 bcd
M 1	37 bcd	87 bc	46 b	57 bcde
M 2	54 abcd	98 abc	118 ab	90 bcd
Mean	50	94	95	79
t-test	*	*	*	*

Table 3. Dry capsule characteristics of tissue cultured cardamom selections in preliminary yield trial, (1995-96).

Entry	100 capsule weight (g)	Volume (cc)	Length (cm)	Breadth (cm)	l/b ratio
TC 1	30.88 ab	73.32 abc	1.68 ab	0.747 a	2.25
TC 2	26.28 ef	56.00 de	1.52 cd	0.717 ab	2.12
TC 3	27.56 cde	65.32 cd	1.67 bc	0.700 ab	2.39
TC 4	29.60 bcd	62.68 cde	1.64 bc	0.740 ab	2.22
TC 5	32.64 a	81.32 a	1.73 ab	0.750 a	2.31
TC 6	30.22 abc	78.68 ab	1.63 bc	0.703 ab	2.32
TC 7	27.16 de	68.68 bcd	1.83 a	0.750 a	2.44
TC 8	27.16 de	62.68 cde	1.60 bcd	0.767 a	2.09
M 1	23.68 f	52.00 e	1.45 d	0.650 b	2.23
M 2	32.52 a	72.00 abc	1.68 ab	0.783 a	2.15
Mean	28.76	67.08	1.65	0.73	
t-test	*	*	*	*	

maximum yield was obtained from TC 5. Average yield data for the three years showed TC 5 to be significantly superior than all the other entries. TC 6 was next in rank and was significantly superior compared to TC 3, TC 2 and TC 1 and was on par with others. TC 3 was significantly lower yielding

than others except Mudigere 1, TC 1 and TC 2. Thus TC 5 was relatively superior in yield compared to others and TC 6, TC 7, Mudigere 2, TC 8 and TC 4 were next in rank and the others were low yielders.

TC 5 showed maximum capsule weight and volume and it was significantly superior to others except Mudigere 2, TC 1 and TC 6 which were on par with it. Mudigere 1 showed least capsule weight and was on par with TC 2. TC 7 showed maximum capsule length and was significantly superior compared to others except TC 5, TC 1 and Mudigere 2 which were on par with it. Mudigere 1 showed least capsule length and TC 8 and TC 2 were on par with it. Mudigere 2 showed maximum capsule breadth and it was on par with others except Mudigere 1. Mudigere 1 also showed least capsule breadth and TC 4, TC 2, TC 6 and TC 3 were on par with it. Thus TC 5, TC 6 and TC 7 were relatively superior compared to others in capsule characteristics.

Besides higher yield, TC 5 recorded higher values for number of panicles per clump, panicle length, flowers per node, weight and volume of dry capsules. TC 6 and TC 7 were next in rank with higher panicles per clump. TC 6 also recorded higher values for capsule weight and volume and TC 7 recorded higher values for capsule and panicle length. Thus among the eight entries evaluated these three entries merit further large scale testing.

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In vitro seed set and seed development in ginger, *Zingiber officinale* Rosc.

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Abstract

Natural seed set is absent in ginger. Seed set and seed development was obtained through *in vitro* pollination and fertilization. The mean seed set per culture after placental pollination was 61.56 per cent. The mean number of well developed seeds per culture at 80 days after pollination (DAP) was 6.87. The *in vitro* produced seeds grew rapidly at the initial 20 DAP and later growth was comparatively little. Maturity period of seeds of ginger can be 2 to 3 months under *in vitro* condition as observed from the change in colour of these seeds.

Keywords: ginger, *in vitro* pollination, maturity, seed set.

Introduction

Ginger (*Zingiber officinale* Rosc.) fail to set seed under natural conditions and this pose a problem in crop improvement of ginger through breeding. *In vitro* pollination and fertilization are useful under such conditions where pre fertilization barriers come in the way of seed set (Bhojwani & Razdan 1983).

Materials and methods

Ginger cultivars Maran and Rio-de Janeiro were used as female and male parents, respectively. The flower buds on the day of anthesis were collected 3 h prior to opening i.e. by 12 noon and surface sterilized. They were later pollinated by *in vitro* placental pollination technique (Bhojwani & Razdan 1983). The pollen grains suspended in ME_3 medium (Valsala 1994; Leduce *et al.* 1990) were used for pollination. The pollinated ovules with placental tissues were inoculated in four suitable media combinations (Table 1). The cultures were kept in diffused light at 26±2°C and observed for seed set 20 days after pollination (DAP). Eighty days after pollination, number of well developed seeds per culture was also examined. The seed development in terms of length and breadth of the ovule were measured at intervals of 20 days commencing from 20 DAP to 80 DAP. Morphological changes associated with seed development were also recorded and presented. Structure of the seed was examined at 80 DAP.

Results and discussion

Seed set after in vitro *pollination*

The mean seed set per culture after placental pollination was 61.56 per cent. The flowers of the cultures studied, recorded a mean ovule number of 24.43. Hence, theoretically each flower has the capacity of producing 24 seeds. The maximum seed set recorded was 66.84 per cent and it was in the medium of ½ MS + sucrose 8% + BAP 5 mgl^{-1} + NAA 1 mgl^{-1} + coconut water 15% v/v. The minimum value of 54.03 was recorded in ½ MS + sucrose 3% + BAP 5 mgl^{-1} + NAA 1 mgl^{-1} + coconut water 15% v/v.

The mean number of well developed ovules (good size) per culture at 80 DAP was 6.87. The maximum value of 8.24 was recorded in the medium of ½ MS + 6% sucrose + BAP 5 mgl^{-1} + NAA 1 mgl^{-1} + CH 500 mgl^{-1}. The minimum value of 5.10 was recorded in ½ MS + sucrose 3% + NAA 1 mgl^{-1} + BAP 5 mgl^{-1} + 15% coconut water v/v.

The comparative efficiency of four favourable media combinations with respect to setting per cent was tested. The media having high sucrose, i.e. 6% to 8% registered a high setting per cent of 61.39 to 66.84 compared to medium having 3.0 % sucrose (54.03). The same trend was observed with respect to number of well developed ovules. So it can be concluded that higher levels of sucrose, to the extent of 6% to 8% per cent, is favourable for the development of *in vitro* fertilized ginger ovule.

Seed development after in vitro *pollination*

The ovules on the day of anthesis measured a length of 0.54 mm and breadth of 0.32 mm. There was substantial increase in the size of the seeds within 20 DAP as they recorded a mean length of 1.82 mm and breadth of 0.92 mm by that time. Forty days after pollination, the seeds recorded a mean length of 2.05 mm and breadth of 1.16 mm. They registered a length of 2.30 mm and breadth of 1.55 mm at 60 DAP and 2.30 mm and 1.60 mm at 80 DAP. There was four fold increase in length and six fold increase in breadth. It can be seen that the increase in size of the seed within 20 DAP is very rapid. Then they showed a slow phase and at 60 DAP the growth was comparatively nil. There was not much difference in size of the seeds developed in different media, eventhough the media differed significantly with respect to sucrose and media supplements.

The colour of the ovules at the initial stage was creamy white and in the course of development about 14.95 % of cultures development purple red colour as if they were ripe (Table 1).

The mean number of days taken by ovules to become purple red colour was 30.65. The ovules later turned black in the course of development and approximately 56 days after pollination few ovules per culture showed blackening . The time required for blackening of

Figure 1: Black arillate seed of ginger

Table 1 : Physical change of the ginger seeds during *in vitro* development. *

Medium	Colour of ovule at initial state	Percentage of cultures showing ripening**	Mean No. of days for ripening	Mean No. of days for blackening
½ MS + sucrose 3% + BAP 5 mgl^{-1} + NAA 1 mgl^{-1} + CW 15% v/v + Agar 0.8%	Creamy white	13.25	33.52	53.33
½ MS + sucrose 6% + BAP 5 mgl^{-1} + NAA 1 mgl^{-1} + CW 15% v/v	"	15.09	27.20	57.55
½ MS + sucrose 6% + KIN 2 mgl^{-1} + NAA 0.5 mgl^{-1} + CH 500 mgl^{-1}	"	16.20	30.66	56.00
½ MS + sucrose 8% + BAP 5 mgl^{-1} + NAA 1 mgl^{-1}	"	15.25	31.22	58.50
Mean		14.95	30.65	56.27

*Average of 18 observations

** Ripening - colour purple red

Explant - Ovules after placental pollination

whole ovules of a culture varied from 60 to 90 DAP. Thus the maturity period of ginger seeds can be 2 to 3 months.

Seed structure

The small black arillate seeds had two seed coats (Fig. 1). The outer one being thick and the inner being thin. The seed coat encloses a cavity which is typical of monocots. In the cavity, endosperm with embedded embryo is seen.

It was interesting to note that under *in vitro*, the seeds grew initially at a rapid pace and later developed red colour as if they were ripe. The developed seeds had all the structures of a true seed. The maturity period of ginger seeds can be two to three months as most of them turned black within that period.

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Anther callus from diploid and tetraploid ginger (*Zingiber officinale* Rosc.)

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Abstract

Zingiber officinale is the source of important spice ginger. It does not set seed and hence conventional breeding is not possible. Development of haploids and dihaploids are important in increasing the breeding efficiency. thereby obtaining variation in ginger. A five factor experiment viz., ploidy level (diploid and tetraploid), growth regulator in media, physical condition of media (solid and liquid), cold treatment 1, 7, & 9 days) and incubation condition (dark and light), was conducted for induction of androgemic callus. Anthers from diploid and tetraploid plants gave callus in media with 2.0 mgl^{-1} 2, 4-D. Solid media were better than liquid media. Seven days cold treatment and incubation in light were the other conditions required for callus formation.

Key Words: anther culture, ginger, *Zingiber officinale*

Introduction

Induction of androgenesis by the culture of excised anther for haploid production is routinely used in many plant species. (Reinert & Bajaj 1977). Haploids are important in induction of mutation, gametoclonal variation and for the production of homozygous plants. *Zingiber officinale* is an important spice crop with limited options for breeding owing to the absence of seed set. Anther culture also offers a new method for creating variation (gametoclonal variation) and for production of homozygous. In addition anther culture in tetraploid ginger can be used to get diploids. The resultant variants in the population with desirable traits can be directly exploited to develop new varieties.

Materials and methods

Anthers from diploid cv.Maran and induced tetraploid of cv. Maran were used for this work. Inflorecences were collected during August/September, washed in running water and kept in refrigerator (2^0c) for cold treatment (1,7 & 9 days). After cold treatment indi-

vidual flowers were separated from infloresences and surface sterilized with 0.1% $HgCl_2$ for 10 minutes. They were then washed three times in sterile water to remove $HgCl_2$. Flowers were dissected and anthers were separated from flowers. Each stamen was cut into four pieces and inoculated.

MS (Murashige & Skoog 1962) basal nutrients, vitamins, 2% sucrose, 0.7% agar and 2, 4-D (0.5 mg l^{-1} or 2.0 mgl^{-1}) were used to prepare medium. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C at 15 lb pressure. Each medium was tired in solid as well as liquid state.

Cultures were incubated in dark or light (approximately 2000 lux for 16h photoperiod) at 25±2°C temperature. Cultures in liquid medium were kept on a rotary shaker. Observations were recorded for 8 weeks.

Results and discussion

Out of the three different cold treatment durations tried 7 days treatment has helped in inducing callus from anthers. Effect of cold treatment on callus formation is not fully understood. It was suggested that cold treatment kills weak and non-viable anthers and only vigorous anthers survived for further growth; microspores were arrested during the first mitosis and this increasing the possibility of subsequent development; cold treatment retards ageing of anther wall allowing a higher proportion of microspores to change their development pattern from gametophytic to sporophytic, are some of the reasons (Wenzel & Foroughi-Wehr 1984). Callus could be produced from both diploid and tetraploid clones of ginger. Callus obtained from diploid anthers was more fraible and colourless. Callus from anthers of tetraploid plants was hard and white in colour. Callus developed within 4 weeks in tetraploid anthers while it took 6 weeks for diploid anthers.

Growth regulator concentration in the media is critical for callus development. 2, 4-D at 2.0 mgl^{-1} was required to induce callusing from both tetraploid and diploid anthers. Though growth regulators are not essential for anther cultures in monocotyledons, their addition increased the response considerably. In ginger anther culture, no callus developed when growth regulator were removed from the medium.

Ginger anthers developed callus in solid medium. In liquid medium only a few cultures developed callus after more than 8 weeks of culture. Occassional development of roots were observed in liquid cultures. Liquid medium is generally considered better for anther culture (Keller 1984). This study has shown that solid medium is better for ginger anther culture. Earlier workers on ginger anther culture also used solid medium (Ramachandran & Chandrashekharan Nair 1992).

Cultures were incubated in light and dark conditions. Callus developed from anther cultures kept in light where as no callus developed from cultures kept in dark. This is in contradiction to the earlier report, (Ramachandran & Chandrashekharan Nair 1992).

Thus callus could be obtained from anthers of diploid and tetraploid clones of ginger cv.Maran by culturing seven day cold pretreated anthers on agar solidified MS medium with 2, 4-D at 2.0 mgl^{-1} and incubating them in light. Though

anther callus was reported in tetraploid clones of ginger (Ramachandran & Chandrashekharan Nair 1992), this is the first report of anther callus from diploid clones of ginger.

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Ovule culture of vanilla and its potential in crop improvement

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Abstract

Vanilla is a cross pollinated crop and hence good amount of variability can be expected among the seedling progenies. In the present study vanilla seeds were cultured on Murashige & Skoog medium supplemented with cytokinins and auxins for germination, development and subsequent multiplication of plantlets in the same medium. Individual shoots produced multiple shoots and conversion of root meristem into shoots was also induced in MS medium supplemented with BAP (1 mgl^{-1}) and IBA (0.5 mgl^{-1}). Plantlets could be transferred to soil with 80% success. Studies on various morphological parameters like leaf size, internodal length and growth rate and isozyme analysis of leaf tissue by Native PAGE, indicated genetic variability among the progenies.

Key Words: crop improvement, ovule culture, *Vanilla planifolia*, variability

Introduction

Cultivated vanilla, *Vanilla planifolia* (Salisb.) Ames (*V.fragrans* Andrews.), belongs to the family Orchidaceae. Commercial cultivation of vanilla is limited to Kerala, Karnataka and Tamil Nadu. Much of the planting material was originated from limited clonal source and practically no variability is available for crop improvement programmes.

Vanilla is suspected to be highly heterozygous with extensive genetic variations because of its cross pollinated nature. Germination of orchid seeds, which are ex-albuminous, under natural conditions is rare, unless associated with a fungus. *In vitro* seed germination (ovule culture) of vanilla has been reported by Knudson (1950) and Withner (1955)

The objective of the present study is to estimate and exploit the genetic variations among seedling progenies developed through *in vitro* culture of seeds/ ovule and search for isozyme markers which could be useful for indexing genetic variability. An attempt was also made to standardise a micropropa-

gation protocol for vanilla.

Materials and methods

In vitro *seed germination*

Vanilla flowers were hand pollinated and capsules (4-9 months old) were harvested. Surface sterilisation of these capsules was carried out under aseptic conditions by dipping in 95% ethanol and flaming. The capsules were then split opened and the seeds were transferred to sterilised medium.

MS (Murashige & Skoog 1962) medium fortified with 2% sucrose was tried as basal medium for germination of vanilla seed *in vitro*. Medium was used both as liquid phase and gelled with 0.65% agar-agar. MS medium supplemented with auxins and cytokinins were tried to study protocorm proliferation, multiple shoot induction and rooting.

Micropropagation

Shoot tips and nodal segments of vanilla plants were collected and washed, cleaned with weak detergent solution and surface sterilised with 0.1% mercuric chloride for 5-7 min in the laminar flow chamber. Then they were washed thoroughly with sterilised distilled water and a fresh basal cut was made before inoculating onto a culture medium.

MS medium fortified with 2% sucrose and gelled with 0.65% agar was used as basal medium. Growth regulators; cytokinins (BAP and Kin) and auxins (IBA and NAA) were supplemented to the basal medium singly or in combination at two levels (0.5 and 1.0 mgl^{-1}).

Culture conditions

The pH of the culture medium was adjusted to 5.8 in all cases prior to autoclaving at 15 lbs pressure and 120°C temperature for 20 min. Initial seed cultures were made in liquid media and kept in rotary shakers at 80 rpm. The germinating seeds were transferred to solid medium. All cultures were incubated at 25±2°C with a photoperiod of 14 h.

Hardening and Planting

Micropropagated and the plantlets generated from seeds were transferred to polybags with sand, garden soil and vermiculite in equal proportions. They were kept in humid chamber for 30 days for hardening and establishment.

Assessment of variability

To assess genetic variability, observations were recorded on

i) morphological characters *viz.*, leaf size, growth rate, internodal distance etc. of one year old plantlets.

ii) Isozyme profiles of 10 genotypes using Native PAGE were carried out in Phast system. The gels were stained for peroxidase and superoxide dismutase isozymes. Leaf samples were homogenised in Tris extraction buffer (Bhat & Chandel 1992). Electrophoresis was carried out at 40°C at 80 volts for 15 min and then at 250 V, till the run was completed, after which the gel was immediately immersed in staining solution. Staining procedure described by Shaw & Prasad (1970) was followed.

Paired Affinity Indices (PAI) were calculated by the formula

$$PAI = \frac{Number\ of\ similar\ bands}{Total\ Number\ of\ bands}$$

The PAIs expressed as percentage indicated the similarity (%) between any

two seedling progenies.

Results and discussion

Micropropagation

Of the 10 combinations tested only BAP when used alone or in combination with IBA induced multiple shoots (Table 1) . The best medium was MS supplemented with IBA (0.5 mgl^{-1}) and BAP (1.0 mgl^{-1}). In this medium multiple shoots ranging from 5-10 were induced in 90 days of culture (Fig. 1.1). Nodal segments gave better response with a mean of 7 shoots per culture compared to the shoot tips (4 shoots per culture). In the present study, Kin either alone or in combination with BAP, NAA or IBA has no effect on multiple shoot induction. Similar cytokinin specificity was reported earlier in *Castanea* (Vieitez & Vieitez 1980). NAA either alone or in combination with IBA induced roots and the combination of IBA (1.0 mgl^{-1}) and NAA (0.5 mgl^{-1}) being the best. Clonal propagation of *Vanilla* was reported earlier (Philip & Nainar 1986; George, Ravishankar & Venkataraman 1995).

Table 1 : *In vitro* responses in vanilla

Growth regulator	Response
Kinetin (0.5 mgl^{-1})	Single shoot growth
BAP (0.5 mgl^{-1})	Multiple shoot induction
NAA (0.5 mgl^{-1})	Root induction
IBA (0.5 mgl^{-1})	Root induction
Kin (0.5 mgl^{-1}) + BAP (0.5 mgl^{-1})	Single shoot
Kin (1..0 mgl^{-1}) + NAA (0.5 mgl^{-1})	Single shoot
Kin (1.0 mgl^{-1}) + IBA (0.5 mgl^{-1})	Single shoot
BAP (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1})	Multiple shoot
BAP (1.0 mgl^{-1}) + NAA (0.5 mgl^{-1})	Single shoot
NAA (0.5 mgl^{-1}) + IBA (1.0 mgl^{-1})	Good root system

In vitro *seed germination*

Seeds/ovules collected from six month old pods were cultured on MS medium supplemented with IBA (0.5 mgl^{-1}) and BAP (1.0 mgl^{-1}). Seeds were initially cultured in liquid medium and rotated at 80rpm on gyratory shakers for better aeration and germination. Necessity of aeration for protocorm formation and proliferation in shoot cultures of different orchids has been reported by Kunisaki, Kim & Sogawa (1972). The seeds germinated in about 45 days and were immediately transferred to solid medium of same composition for better growth.

The seeds developed into protocorms in 60 days (Fig. 1.2) and into well developed plantlets in 90-100 days (Fig. 1.3 & 1.4). The plantlets developed roots and multiple shoots in the medium of same composition. The individual plantlets were transferred to either growth regulator free medium or medium supplemented with IBA (0.5 mgl^{-1}) and NAA (0.5 mgl^{-1}) for bet-

ter rooting before they were planted out for hardening. Seed culture of vanilla was first reported by Knudson (1950).

Hardening and planting

Rooted shoots were carefully removed from culture vessels, washed thoroughly of nutrient medium, treated with 0.2% Indofil and transferred to polybags containing potting mixture (sand, garden soil and vermiculite in equal proportions). These plantlets were kept in humid chamber for 30 days for hardening. Over 80% of the plantlets established in polybags. The established plantlets were transferred to pots for further observation.

Variations among seedling progenies

In vitro responses of germinated protocorms indicated variability among the progenies. Four percent of germinated progenies were albinos and did not grow further. Twentyfive percent of the germinated progenies developed callus and regenerated into plantlets while the rest of them did not show callus development but produced multiple shoots. Conversion of root meristem into shoots was observed in some seedlings (Fig. 1.5). This phenomenon was reported earlier by Philip & Nainar (1988).

Observations on various morphological characters like leaf size, internodal length, plant growth were recorded in 10 randomly selected progenies (Table 2). Leaf size ranged from 4.5-12.5 cms in length and 1.5-3.2 cms in breadth. Progeny V_8 exhibited the maximum growth followed by V_1, whereas V_{12} and V_6 had the lowest growth. V_4 and V_{10} exhibited maximum leaf size (length and breadth) while V_{12} had the smallest leaf size (Fig. 1.6).

Isozyme profiles of superoxide

Table 2. Morphological variations in one year old seedling progenies.*

Progeny	Plant-height(cm)	Internode length (cm)	Leaf size (cm	
			Length	Breadth
V_1	140.0	7.5	9.5	3.0
V_2	**120.0**	6.5	10.5	**3.0**
V_3	**65.0**	6.0	**9.5**	**3.0**
V_4	**55.0**	5.5	12.5	**3.2**
V_6	**5.0**	2.5	**5.5**	**1.7**
V_7	105.0	5.5	**7.5**	**1.9**
V_8	170.0	6.5	**8.5**	**2.5**
V_{10}	**90.0**	5.0	12.0	**3.0**
V_{11}	**9.0**	2.0	**4.7**	**1.8**
V_{12}	**4.5**	1.0	**4.5**	**1.5**

*Mean of ten observations

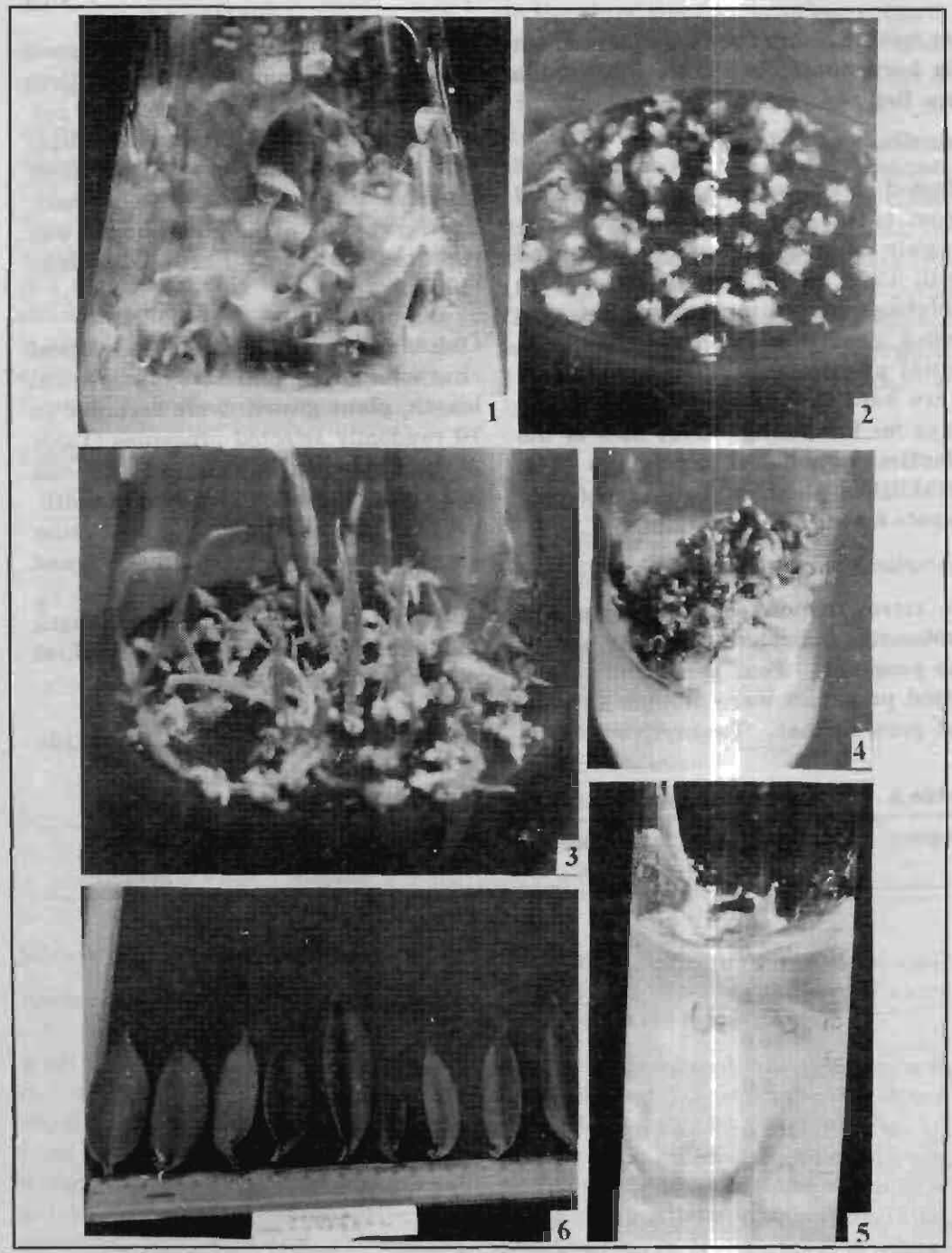

Fig. 1. *In vitro* responses in vanilla.

1.1 Induction of multiple shoots from shoot explants, 1.2 & 1.3 Germination of seeds and development of plantlets, 1.4 Induction of multiple shoots from seedlings, 1.5 Conversion of root meristem into shoot and 1.6 Variablity in leaf size among seedling progenies

dismutase (SOD) and peroxide (PRX) were studied in 10 seedling progenies of vanilla. Profiles clearly indicated differences among progenies as expressed by the presence and absence of specific bands. Variations in isozyme profiles were used to estimate genetic variability in many crop plants (Fedak & Rajhathy 1972; Yndgaard & Iloskuldsson 1985).

The Paired Affinity Indices (PAI) calculated to estimate the percentage similarity between any two progenies are indicated in Table 3. From the PAI it was evident that the maximum similarity (47.37%) existed between V_1 and V_8 V_4 and V_2 were 45.45% similar, followed by V_{10} and V_{24} (44.44%). The progenies that differed most from each other were V_3 and V_{10} (with only 25% similarity).

Table 3 Paired Affinity Indices (% similarity) among seedling progenies of vanilla

	V_1	V_2	V_3	V_4	V_7	V_8	V_{10}	V_{11}	V_{14}	V_{24}
V_1	-	28.57	34.70	36.37	40.91	47.37	36.84	39.13	33.33	42.11
V_2		-	28.57	45.45	31.82	26.32	31.58	36.09	37.50	36.84
V_3			-	30.43	43.48	35.00	25.00	37.50	28.00	35.00
V_4				-	37.50	33.00	38.09	32.00	42.31	42.86
V_7					-	38.09	38.09	36.00	34.62	42.86
V_8						-	33.33	36.36	34.78	38.89
V_{10}							-	31.82	34.78	44.44
V_{11}								-	40.74	36.36
V_{14}									-	34.78
V_{24}										-

The possible origin of vanilla as a hybrid has been suggested by Nair and Ravindran (1994) and Ravindran (1979). They observed somatic association and abnormal mitotic behaviour as well as asynchrony in pollen grain mitosis. The variations observed among the progenies in the present study may be due to segregation of characters in seedling progenies coupled with cytological abnormalities during gamete formation. Thus seedling progenies offer rich source of variability for fu ther selection and crop improvement in vanilla.

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In vitro selection for salt tolerance in *Trigonella foenum graceum* using callus and shoot tip cultures

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Abstract

Selection of salt-tolerant *Trigonella foenum graceum,* cell lines or plants have been carried out by screening callus and shoot tip explants *in vitro* on high concentrations of NaCl. Shoot tip based selection was unsatisfactory, as only 2 out of 1153 showed salt-tolerance. Whereas, salt-tolerant selections made *via* callus culture were found to be more stable. However, they failed to regenerate into plantlets.

Key words: *in vitro* screening, salt tolerance *Trigonella foenum-graceum*

Introduction

Fenugreek, *Trigonella foenum graceum,* is an important seed spice, belonging to the family Fabaceae. It is an annual crop cultivated widely due to its medicinal value.

The paper reports the work done on inducing salt tolerance in fenugreek through *in vitro* system which may help in developing salt tolerant varieties.

Materials and methods

Hypocotyls from 6 day old, aseptically germinated seedlings of *Trigonella foenum graceum* were cultured on MS (Murashige & Skoog 1962) agar medium supplemented with 2.0 mgl^{-1} IAA & 0.2 mgl^{-1} Kn for the production of callus (MS-1). Similarly, excised shoot tips were cultured on MS agar medium supplemented with 3 mgl^{-1} Kn for multiple shoot formation (MS-2).

Inhibitory concentrations of NaCl were determined by introducing small callus pieces or shoot tips into the MS agar medium (MS-1 for callus induction and MS-2 for shoot tips) supplemented with 0.025, 0.05, 0.75, 1.00, 1.25 & 1.50% (w/v) NaCl. The lowest NaCl concentration which completely inhibited callus growth and shoot regeneration was taken as the 'inhibitory' concentration. Subsequently, selection for salt-tolerance was carried out in MS-1 & MS-2 medium containing NaCl concentrations slightly higher than that determined to be inhibitory (Table-1) and cultures were scored for growing callus

and shoots after 2 months of incubation. Tolerance stability was tested by growing callus or multiplying shoots on NaCl-free medium for 2-3 months and then transferring them back to a stressful 1.0% NaCl containing medium. Later experiment was conducted in triplicate and the variant callus/shoots which grew in the NaCl medium, were selected as stable salt tolerant variants. Increase in fresh weight of calli' per culture after 4 weeks was determined according to the following equation (Meredith 1978).

$$\text{Increase in fresh wt.} = \frac{\text{final wt.} - \text{initial wt.}}{\text{initial wt}} \times 100$$

Salt tolerance of the shoot tip cultures was assessed on the basis of visual observations on shoot multiplication and their growth and discolouration on the selection medium containing NaCl.

Results and discussion

Callus initiation as well as multiple shoot formation in shoot tip explants and cell growth in cultured callus pieces decreased with increasing NaCl concentrations in the medium and were completely inhibited at 0.50% NaCl (for shoot tip explant) and 1.25% NaCl (for callus growth) (Table 1).

Table 1. NaCl - tolerant clones/plants from callus and shoot tip explant cultures of *Trigonella foenum graceum*

Explant	Inhibitory concentration of NaCl (%)	Concentration of NaCl (%) in the selection medium	No.of explant or callus pieces cultured	Salt tolerant lines/plants	
				Isolated (no)	Stable (no)
Callus pieces (25 mg each)	1.25	1.50	428	9	3
Shoot tips	0.50	0.75	1153	2	-

Shoot morphogenesis in shoot tip explant was very sensitive to NaCl stress. On NaCl-free medium, more than 85% of explants regenerated whereas only 7% of the explants could regenerate in 0.25% NaCl. At higher levels, shoot organogenesis was completely inhibited. However, some of the explants showed a little callus formation at 0.5% NaCl (the inhibitory level). After about 2 months of culturing in selection medium, 9 and 2 salt tolerant lines plant , respectively were isolated from callus and shoot tip culture. However, out of the 9 clones selected from callus culture, only 3 survived in still higher concentration of NaCl (1%). Further, this selection is likely to be inefficient and uncertain as all the cells in the callus pieces were not uniformly exposed to the selective agent and this can result in stress avoidance due to cross-feeding between the cells in close contact with each other (Meredith 1984). The two shoots isolated by screening shoot tip explants could not withstand the stability test at 1% NaCl.

The three survived salt-tolerant calli grew slowly in 1% NaCl for the first 10-days after which the growth rate is increased. In a 30-days experiment at 1.25% level, the 3 selected tolerant

lines had over twice the fresh weight of the non-selected line. At 1.0% & 1.5% the non-selected calli ceased to proliferate whereas the selected calli continued to increase in mass. Infact, the tolerant callus at 1.5% NaCl-treatment, showed fresh weight increase comparable to that of the control at 0.5% NaCl. However, the callus based selections failed to regenerated shoots on MS media. Similar results were obtained by Mc Hugen (1987) and Vajrabhaya *et al.* (1989).

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Screening for drought tolerance in coriander through tissue culture

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Abstract

Drought tolerant cell lines were selected by screening callus pieces of hypocotyl, cotyledon and root segments *in vitro* on high concentration of polyethylene glycol (PEG). Root segments based selection was unsuccessful. Hypocotyl and cotyledon derived tolerant calli showed some response. Three out of 753 screened hypocotyl calli, were stable whereas 9 out of 821 PEG tolerant selected cotyledonary calli showed stability. The tolerant calli failed to regenerate shoots.

Key words: *Coriandrum sativum*, drought tolerance, *in vitro* screening.

Introduction

Cariander sativum L., is an important spice crop. A decline in productivity due to drought necessitates the development of drought tolerant varieties. *In vitro* systems have been used in inducing drought tolerance in many crop plants (Iraki *et al.* 1989; Sumaryati *et al.* 1992). The present paper describes isolation and characterization of drought tolerant lines of coriander using PEG as the osmotic compound in the *in vitro* system.

Materials and methods

Seeds of coriander were surface sterilised for 30 sec. in 70% ethanol solution followed by 0.1% $HgCl_2$ solution for 4-5 min. After repeated washings with sterile distilled water, the seeds were cultured on the hormone free MS medium (Murashige & Skoog 1962) for germination.

Explant culture and selection of PEG tolerant callus

From 15-days old seedings, explants like hypocotyl, cotyledon and root segments were excised and cultured in test tubes (25 mm x 150 mm) containing 20ml MS medium or MS medium supplemented with 0, 0.25, 0.50, 0.75, 1.0, 1.25 & 1.50% (w/v) of PEG. All the cultures were incubated for 16h photoperiod at 2.5±2°C. The number of explants showing callus formation was recorded after 3 weeks of incubation. The lowest concentration of PEG which completely inhibited callus initiation and proliferation was taken as the 'in-

Table 1 PEG tolerant clones from callus cultures of coriander.

Explant	Inhibitory concentration of PEG (%)	Concentration of PEG (%) in the selection medium	No.of explant or callus pieces cultured	salt tolerant cell lines	
				Isolated (no.)	Stable (no.)
Hypocotyl callus	1.00	1.25	753	11	3
Cotyledon callus	1.25	1.50	821	18	9

were also grown in medium containing PEG (1.5%). Cotyledon calli responded in a similar manner as of hypocotyl calli.

Out of a total 753 hypocotyl explants cultured on PEG added medium (1.25%) 11 callus clones were selected as PEG tolerant calli and among them 3 showed stability. On the other hand out of 821 cotyledon explants cultured, 18 were found tolerant to PEG treatment (1.5%) and from these tolerant calli only 9 were found to be stable.

The stability was further tested by estimating the difference in fresh weight of the clones. The selected tolerant calli showed a continuous increase in their mass, whereas the non-selected clones ceased to grow.

This study revealed that the cotyledon callus was the best for *in vitro* selection experiments on coriander which possesses a comparatively high potential to proliferate under drought conditions. Similar results were obtained by Bressan *et al.* (1981), Gulati & Jaiwal (1990), Santos-Diaz & Neftali (1994).

However, Meredith (1984) opposed callus selection which is likely to be inefficient and uncertain. He explained that all the cells in the callus pieces were not uniformly exposed to the selective agent and this can result in stress avoidance due to cross-feeding between the cells in close contact with each other.

All the selected calli, failed to regenerate shoots when transferred to the shoot induction medium. The inability of selected calli to regenerate shoots may therefore be the result of increased somatic age (Murata & Orton 1987). Moreover, the role of osmotic potential and accumulation of ions of the PEG tolerant calli are yet to be understood.

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In vitro selection for resistance to *Alternaria* blight in cumin (*Cuminum cyminum* L.)

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Abstract

Alternaria blight of cumin (*Cuminum cyminum* L.) is a devastating disease. Since resistance source to this disease is not available in the existing germplasm, attempts were made to isolate cell lines resistant to *Alternaria* blight using *in vitro* system. Various dilutions (2 to 80%) of culture filtrate subjected to different durations of exposure (6-96 h) at callus stage were studied in twelve genotypes of cumin. The exposure of callus to culture filtrate resulted in gradual browning and subsequent death of the cells. The genotype GC-1 could survive better at 5% culture filtrate exposed for a period of 24 h.

Key words: *Alternaria* blight, cumin, *in vitro* selection.

Introduction

Cumin is an important cash crop of North Gujarat and Rajasthan. Cumin blight (*Alternaria burnsii*) is the major constraint affecting the cultivation of this spice. There is no source of resistance to this disease in the existing germplasm. Induction of variability through *in vitro* system becomes useful under such situation. The present paper reports the result of *in vitro* induction of variability for resistance to *Alternaria* blight in cumin.

Material and methods

Plant material

The callus cultures were established on semi-solid MS (Murashige & Skoog 1962) medium supplemented with IAA (1.0 mgl^{-1}), Kn (1.0 mgl^{-1}), 2 % sucrose and 0.8% agar using cotyledon explants of 12 diverse genotypes of cumin aseptically grown on MS basal media.

Preparation of culture filtrate

The fungal isolate of *Alternaria burnsii* was grown on PDA medium at room temperature. Pure cultures were maintained by subsequent transfer on PDA slants. To confirm the pathogenicity of the isolate, 45-50 days old healthy cumin plants of twelve different genotypes grown in three replications were artificially inoculated with the fungal suspension under field conditions.

Culture filtrate of fungus was prepared

from stationary liquid medium of modified Richard's solution (Hendrix & Nielson 1958). After 30 days of incubation the culture was filtered through Whatman No.1 filter paper to remove the mycelium and pH of the filtrate was adjusted to 5.8 before autoclaving. The culture filtrate was stored at 0.5°C. The toxicity of the culture filtrate was tested on aseptically grown healthy seedlings.

Effect of culture filtrate on callus growth

The test of culture filtrate toxicity involved dilution series in which the culture filtrate was diluted to 2, 5, 10, 20, 50 and 80% with callus growth media (MS + 1.0 mgl^{-1} IAA + 1.0 mgl^{-1} Kn) along with the control (callus growth media without culture filtrate) in two replications.

Lots of 25 pieces of seven week old callus of each cumin line (approximately 615 mg fresh weight each) were placed in the Erlenmeyer flask containing callus growth medium and culture filtrate (15ml) and incubated under diffuse light at 25° ± 2°C for various exposure periods (duration) of 6, 12, 18, 24, 48, 72 and 96 h on a gyrotary shaker at a speed of 90 rpm. As *Alternaria* culture filtrate looses its activity when placed under direct light all tests using culture filtrate were carried out in diffuse light. The rate of callus browning after exposure to the culture filtrate was visually evaluated as the percentage of exposed callus that had turned brown. Subsequently the callus from all treatments and control were transferred to callus growth media and fresh weight and dry weight of callus were determined after 20 days of incubation.

Sensitivity of calli to culture filtrate

The sensitivity of calli to culture filtrate was assayed using a single genotype (GC-1). The callus of GC-1 was exposed to culture filtrate (2-80% concentration) at different durations (5, 12, 18, 24, 48, 72 & 96 h).

Results and discussion

None of the genotypes tested was found resistant or tolerant to *Alternaria* blight under field conditions.

The callus of GC-1 survived better and showed inhibited growth at 5% culture filtrate when exposed for a period of 24 h, thus 5% culture filtrate was selected as the test concentration for screening calli of other genotypes.

The calli of the twelve genotypes subjected to different exposures (6-96 h) of 5% culture filtrate showed differential behaviour in gradual browning, growth inhibition and subsequent death of cells. Almost all genotypes survived well up to the exposure of 12 h to culture filtrate except the genotypes JC-106, JC-147 & JC-15 in which browning effect was much pronounced. At the exposure of 18 h, calli of GC-1 and MC-43 became necrotic but showed small clumps of surviving cells (i.e. 22% and 8% callus survival, respectively). The genotype GC-1 showed poor survival whereas a complete browning of callus was observed in remaining genotypes at the exposure of 24 h indicating the complete inhibition of callus growth (Table 1).

The surviving calli of different genotypes at various exposures to 5% culture filtrate were subsequently placed on non toxic (optimal callus growth) media and percentage increase in fresh

Table 1 *In vitro* response of cumin calli to *Alternaria burnsii* culture filtrate (5%)

Genotype	Exposure time (h)															
	0		6		12		18		24		48		72		96	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
GC-1	100	0	56	44	38	62	22	78*	14	86*	0	100	0	100	0	100
MC-43	100	0	52	48	32	68	8	92	0	100	0	100	0	100	0	100
JC-15	100	0	48	52	18	82	0	100	0	100	0	100	0	100	0	100
JC-19	100	0	50	50	22	78	0	100	0	100	0	100	0	100	0	100
JC-65	100	0	42	58	16	84	0	100	0	100	0	100	0	100	0	100
JC-106	100	0	40	60	8	92	0	100	0	100	0	100	0	100	0	100
JC-126	100	0	40	60	30	70	2	98	0	100	0	100	0	100	0	100
JC-136	100	0	50	50	22	78	0	100	0	100	0	100	0	100	0	100
JC-147	100	0	28	72	18	82	0	100	0	100	0	100	0	100	0	100
JC-165	100	0	38	62	28	72	2	98	0	100	0	100	0	100	0	100
EC-109635	100	0	46	54	24	76	6	94	0	100	0	100	0	100	0	100
Hairy cumin	100	0	50	50	26	74	4	96	0	100	0	100	0	100	0	100

*Significant at 5% level

A = Percentage of survival; B= Percentage of browning

weight was measured after 3 weeks. A high amount of variation in fresh weight increase was observed among the genotypes. There was less increase in fresh weight on toxic medium than on non toxic medium (i.e. control). Each genotype tested showed decrease in callus growth with increase in the exposure period. The genotypes showing cent percent browning (i.e.no survival) could not regain growth.

Similar results were reported in wheat and barley (Chawla & Wenzel 1989) and alfalfa (Arcioni *et al.* 1987).

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Evaluation of *Pseudomonas fluorescens* isolates for control of *Meloidogyne incognita* in black pepper (*Piper nigrum* L.)

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Abstract

Thirty isolates of fluorescent psedomonads, isolated from the rhizoplane of black pepper, were tested for their interaction with root knot nematodes (*Meloidogyne incognita*), in two trials under green house conditions. Black pepper OP seedlings were treated with the bacterial suspension of *Pseudomonas fluorescens* alone or *P. fluorescens* followed by second stage juveniles of *M.incognita*. Plants treated with *M. incognita* alone and those treated with neither bacteria nor nematodes served as controls. Growth of the seedlings and nematode infestation were assessed two months and three months after inoculation in the first and second trial, respectively. None of the isolates could give absolute protection against *M. incognita* infestation. However, isolates No. 10, 14, 17 and 31 reduced the nematode damage significantly and isolates No. 20, 27, 33 promoted the growth of the seedlings though they had no effect on nematodes.

Key words: black pepper, fluorescent pseudomonads, *Meloidogyne incognita*, *Piper nigrum*, *Pseudomonas fluorescens*, root knot nematode.

Introduction

Biological control of plant parasitic nematodes is receiving more attention due to the growing public concern over chemical pesticides. Several organisms are reported as antagonists of nematodes. Rhizobacteria, bacteria that colonise the rhizosphere or that are rhizosphere - competent are being used for suppression of soil borne diseases. Their versatile metabolism, fast growth, active movement and ability to colonise the root surface readily make them good candidates for biological control. Antagonistic rhizobacteria, especially plant health promoting rhizobacteria (PHPR), have been tried against cyst and root knot nematodes

(Becker *et al.* 1988; Oostendrop & Sikora 1986; Racke & Sikora 1986). In this study several local strains of *Pseudomonas fluorescens,* a known PHPR, are tested for their efficacy in suppressing root knot nematodes, an important nematode pest of black pepper.

Materials and methods

Test organisms

Thirty isolates of *Pseudomonas fluorescens* Migula, collected and maintained in the Plant Pathology laboratory from different black pepper gardens in India, were used for this study. The bacterial cultures were grown in King's B - Agar medium for two days and suspended in sterile water (about 10^7cfu/ml). Root knot nematode, *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949, population was originally collected from black pepper and was maintained in the greehouse on black pepper seedlings. Egg masses of *M.incognita*, hand picked from black pepper roots, were kept in sterile distilled water and second stage juveniles were collected.

Screening of isolates

Isolates of *P.fluorescens* were screened for their activity in the greenhouse using black pepper (Panniyur-1) OP seedlings as the test plants. Six each one month old seedlings, raised in sterile soil, were drenched with 2ml of the bacterial suspension of each isolate. Out of this, three plants were inoculated with an aqueous suspension of *M. incognita* (500 juveniles/plant). Plants inoculated with root knot nematodes alone and those treated with neither nematodes nor bacteria were the controls. In the first trial only seven isolates (No. 4,7,10,12, 26,29 and 34) were used, while in the second trial 23 strains were tested. The experiment were concluded after two and three months in the first and second trial, respectively. Observations on height of the plant, total biomass (fresh weight), root weight (fresh weight, only for first trial) and root galling on a 0-5 scale (0-no galling and 5 - maximum galling) were recorded.

Statistical analysis

The data were analysed for a split plot design with nematode-treated and untreated being the main plot treatments and different isolates of *P. fluorescens* and the untreated control constituting the subplot treatments. Analysis of variance was carried out and the means were separated using Duncan's Multiple Range Test.

Results and discussion

The effects of rhizobacteria, *P. fluorescens* on plant growth and root knot infections varied considerably. No isolate used in the first trial showed any statistically significant difference in growth improvement or reduction in root galling (Table 1). The beneficial effects of rhizobacteria might not have been expressed as the experiment was concluded after two months. However, isolate No. 4 and 10 increased the total biomass of plants and reduced the gall index, even in the presence of root knot nematodes. Similarly, no isolate in the second trial also gave statistically significant results (Table 2). But many strains caused a detectable decrease in gall index and increase in total biomass. Isolate No. 14,17,31,35 and 49 increased the total biomass by 26.7 - 55.6 % and reduced the root galling by

Table 1. Effect of seven isolates of fluorescent pseudomonads on growth of black pepper seedlings and root galling due to root knot nematodes

Isolate No	Height (cm)			Total biomass (g)			Root wt. (g)			Gall index
	Alone	With RKN	Difference	Alone	With RKN	Difference	Alone	With RKN	Difference	
4	27.83	27.67	-0.16	11.66	13.03	1.37	3.57	4.17	0.60	3.67
7	32.67	46.33	13.66	19.11	15.72	-3.39	5.07	4.05	-1.02	3.00
10	29.00	37.83	8.83	10.93	16.16	5.23	3.49	4.16	0.67	3.00
12	29.75	24.67	-5.08	12.78	11.18	-1.60	2.93	2.73	-0.20	2.00
26	71.00	40.67	-30.33	16.51	13.47	-3.04	5.32	4.34	-0.98	3.00
29	45.67	24.67	-21.00	14.33	8.08	-6.25	5.29	2.32	-2.97	3.33
34	53.67	22.50	-31.17	24.25	9.04	-15.21	6.05	3.10	-2.95	4.00
Control	41.33	30.16	-11.17	18.35	11.75	-6.60	4.93	3.10	-1.83	4.33
$LSD_{0.05}$	N.S.	N.S.	-	N.S.	N.S.	-	N.S.	N.S.	-	N.S.

Data are means of three replications. RKN - root knot nematodes, N.S. - not significant

30.0 - 39.9%, even when the plants are infected with *M. incognita*. These observations suggest that many strains used in this study possess nematode antagonistic activity. Several reasons can be attributed for the non significant results obtained.

Generally siderophore and antibiotic production by fluorescent pseudomonads is often proposed as the mechanism of action in reduction of soil borne plant diseases or in plant growth stimulation (Kloepper & Schroth 1981). Nematode activity is greatly influenced by production of antibiotics than siderophore porduction. Phenazine antibiotics, the main factor contributing to the biological control activity of these bacteria, were identified in root washings of seedlings inoculated with fluorescent pseudomonads and then grown in natural soil (Thomashow *et al.* 1990). The antibiotics alter hatch, attraction or host recognition by nematodes (Sikora 1992). The extent and thoroughness of colonization determines whether sufficient bacterial metabolities will be produced to inhibit, interrupt or stimulate certain processes in the nematodes' life cycle or specific root exudates necessary to complete the nematode life cycle will be metabolised. In the present study, bacteria were incorporated only after one month and this might have affected their colonisation in black pepper roots. Wilting or too frequent watering can also interfere with nematode infection or the activity of bacteria. Similarly, inoculum density as well as soil moisture conditions are also reported to influence the antagonistic activity (Oostendrop & Sikora 1989).

Similar results were observed by Becker *et al.*(1988) in tomato and cucumber. Oostendrop and Sikora (1989, 1990) found eight bacterial isolates (three of them were *P.fluorescens*) from the rhizosphere of sugarbeet which reduced penetration of *Heterodera schachtii*, with some isolates reducing nematode numbers in roots by as much

Table 2. Growth and gall index of black pepper seedlings inoculated with 24 different isolates of *Pseudomonas fluorescence*

Isolate	Height(cm)			Total biomass (g)			
No.	*Alone*	*With RKN*	*Difference*	*Alone*	*WithRKN*	Difference	Gall Index
2	65.00	76.67	11.67	34.33	48.33	14.00	4.67
6	77.00	59.33	-17.67	41.67	35.00	-6.67	3.00
9	76.33	52.67	-23.66	32.33	26.67	-5.66	3.67
11	41.67	77.67	36.00	21.67	36.67	15.00	3.67
13	84.00	77.00	7.00	40.00	33.33	-6.67	2.33
14	71.33	73.00	1.67	21.33	43.33	22.00	1.67
16	84.00	32.67	-51.33*	42.33	16.67	25.66	2.00
17	30.00	42.00	12.00	15.00	20.67	5.67	2.00
19	50.33	48.67	-1.66	26.67	24.33	-2.66	4.33
20	92.33	94.33	2.00	50.00	43.33	-6.67	2.33
21	53.67	41.67	-12.00	25.00	18.33	-6.67	2.33
23	53.33	43.33	10.00	25.00	27.33	2.33	1.33
27	107.00	92.67	-14.33	58.33	53.33	-5.00	2.33
28	100.70	72.33	-28.37	41.67	46.67	5.00	1.33
30	95.33	107.00	11.67	48.33	54.33	6.00	4.33
31	35.67	83.00	47.33*	22.33	38.00	15.67	2.00
33	86.00	76.67	-9.33	53.33	40.00	-13.33	3.33
35	72.33	94.00	21.67	40.33	46.67	6.34	2.00
36	78.67	85.33	6.66	35.00	45.00	10.00	4.00
37	80.00	54.00	-26.00	35.33	37.33	2.00	4.33
43	68.67	72.00	3.33	41.67	45.00	3.33	3.33
49	91.00	109.70	18.70	40.00	46.33	6.33	2.33
51	107.70	60.67	-47.03*	47.00	35.33	-11.67	2.00
Control	116.00	61.33	-54.67*	51.00	30.00	-21.00	3.33
$LSD_{0.05}$	36.6	36.6	-	N.S	N.S	-	2.1

Data are means of three replications. RKN - root knot nematodes, N.S. - not significant, * - significant difference between the means in a row

as 75% and producing significant increases in the yield under field conditions. Nematodes like *Meloidogyne* which are with multiple generations and cause damage throughout the season are more difficult targets for *P. fluorescens* (Sikora 1992). Being root pathogens, there may be locally adapted strains for each nematode, possibly on each crop, and possibly for each soil. Therefore, use of mixture of compatible strains may be ideal.

The problems encountered in the present study can be overcome by improved inoculation techniques, strain mixing and incorporating them right form the initial planting.

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Repellent and insecticidal properties of *Piper retrofractum* against insect pests of crops and stored grain

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Abstract

The fruit oil of *Piper retrofractum* obtained by steam distillation was evaluated for repllent action towards stored grain insect, *Tribolium castaneum*. Four types of extracts of the fruit were also evaluated for insecticidal property against *Spilosoma obliqua* and *Spodoptera litura*. The oil exhibited high repellency in a laboratory bioassay test. Among the extracts tested, acetone extract was found to be highly toxic to both the insect pests but are highly photo-unstable.

Key words: insecticidal property, *Piper retrofractum*, repellent, *Spilosoma obliqua*, *Spodoptera litura*, *Tribolium castaneum*.

Introduction

Botanicals are now well recognized in the control of insect pests. Plants species belonging to family Piperaceae are important due to the presence of lignan and amide group compounds (Bernard *et al.* 1995).

A wide range of phytochemicals from several species of *Piper* have been reported to show biological, pesticidal and medicinal properties. *P. retrofractum* (syn, *Piper chaba* or *Piper officinarium*) sold in Indian market in the name of large peepal is an important ingredient of traditional system of medicine. However this species has not been fully evaluated for insecticidal properties.

Extracts from fruits of *P. retrofractum* were evaluated for insecticidal activity against two crop pests *Spilosoma obliqua* and *Spodoptera litura* and repellent action against stored grain insect, *Tribolium castaneum*. Photodegradability of crude acetone extract was also studied.

Materials and methods

Insect culture

Larvae of *S.obliqua* and *S.litura* were obtained from mass culture maintained on the leaves of its natural host, castor (*Ricinus communis*) in a B.O.D. incuba-

tor maintained at 27±2°C and 16h/18h light/dark photoperiod (Tripathi, Singh & Jain 1990).

Adult red flour beetles, *T. castaneum* were collected from the local provision market and raised in glass jars on artificial diet of whole-meal consisting of wheat flour mixed with brewer's yeast in 18: 1 ratio. The rearing and experimental conditions were 12 h light and dark periods at 27±2°C and 70±5% relative humidity.

Extraction

Fruits of *P.retrofractum* were ground and extracted in a soxhlet apparatus in petroleum ether for 6 h. The marc was then extracted successively with acetone, ethanol and methanol, each for 6h. Solvents from each extract were evaporated in a rotatory vaccum evaporator under reduced pressure and yeilded the petroleum ether, acetone, ethanol and methanol extracts, respectively. The oil from fruits was obtained by steam distillation.

Bioassays

Topical application*: (contact toxicity)*. For contact toxicity assay, 12 days old larvae of *S.obliqua* and *S.litura* from the laboratory bred cultures maintained on castor leaves were used. The crude extracts of *P.retrofractum* fruits were diluted using suitable solvents to get 2.00, 1.50, 1.00, 0.75, 0.50 and 0.25% concentrations. The test solutions were applied on insects as topical spray using thin layer chromatograph sprayer. Four replications with 5 insects/replication were used for each concentration. Mortality of the insects were recorded after 24 h and the data subjected to probit analysis to get lethal dose (LD_{50}) (Abbott 1987) and compared by Duncans Multiple Range Test.

Repellent action: Repellent activity was tested by modified method of filter paper technique (Mc Donald, Guy & Speiss 1970). Filter paper (Whatman No.1) was cut into circles of 12 cm diameter and each circle was cut into two equal halves. One half of each circle was dipped in the oil of *P. retrofractum* fruit made in acetone at different concentrations viz., 2.00, 1.00 and 0.50 %, acetone (2.0%) and ethanol (2.0%) extract for 1 min and then air dried for 15 min under fan. Each treated half circle was then attached to half circle treated with acetone by cellulose tape to have a full circle and placed in a petridish. Ten adult beetles (7 days old) were released in the middle of each filter paper circle and covered. For each concentration, 4 replications were maintained. Number of beetles settled on each half of the filter-paper was counted at hourly interval till 5 h. The average of counts was converted to express the percentage repulsion (PR) as follows.

PR = (Control-5) X 20

Positive values (+) express repellence and negative values (-) attraction. Data were analysed using analysis of variance after correction of the percentage repulsion using Abbott (1987) formula and transformation into percentage values. The averages were then assigned to different classes using the following scale.

Class repulsion	Percentage
O	0.01 - 0.1
I	0.1 - 20
II	20.1 - 40
III	40.1 - 60
IV	60.1 - 80
V	80.1 - 100

Photodegradability of crude acetone extract

Crude acetone extract was diluted in acetone to make 2% solution. Two sets of vials having 10 ml solution each were taken. One set of vials having crude acetone extract and acetone alone were exposed to sunlight separately for 2 h, 4h, 6h and 8 h. The second set of vials were kept in dark for corresponding periods. Soon after exposure to sunlight for fixed period as stated earlier, samples of both sets were analysed on High Performance Liquid Chromatography (HPLC) to observe any degradation of peaks as compared to unexposed set kept under dark. Simultaneously biological evaluation in terms of insecticidal activity was done using the test insects as per procedure already described with 4 replications and 4 insects per replication to correlate the HPLC data with biological activity.

Results and discussion

Repellent action

The extracts of *P.retrofractum* showed very low repellent action to red flour beetle, *T. castaneum*. (Table 1). Among the four extracts only acetone and ethanol extracts showed some repellent activity for one hour. Statistical analysis of the data revealed that other extracts showed attractancy towards the beetle. However the fruit oil of *P. retrofractum* gave 90.00, 76.20 and 51.80 % repellency at 2.00, 1.00 and 0.50% concentration, respectively. The weak repellent effect of the extract might be related to the fact that extracts have no vapour effects thereby not interacting with olfactory senses of the beetle.

Table 1. Repellency of *P.retrofractum* fruit extracts and oils*

Extract/oil	Concentration	Repellency rate (%) at different intervals (h)					Repellency
	%	1	2	3	4	5	class
Acetone	2.0	23^{C}	29^{C}	39^{BC}	29^{CD}	13^{BC}	II
Ethanol	2.0	39^{BC}	30^{C}	33^{C}	13^{D}	7^{D}	II
Oil	2.0	90^{A}	90^{A}	90^{A}	90^{A}	90^{A}	V
	1.0	90^{A}	90^{A}	67^{AB}	67^{B}	67^{A}	IV
	0.5	54^{B}	58^{B}	70^{AB}	42^{C}	35^{B}	III
F value	18.76	17.27	5.48	22.87	16.04		

* Original data transformed into arcsin √ percentage values during ANOVA test.
Figures followed by same letter are not significantly different as per Duncans Multiple Range Test.

Contact toxicity

Acetone extract was highly toxic to both the crop pests *S. obliqua* and *S. litura* followed by ethanol extract (Table 2). Other extracts were not effective. Comparison of LD_{50} values based on probit analysis showed that acetone extract exhibited 0.8284, 0.8401 LD_{50} values and ethanol extract with 1.1505, 1.0788 LD_{50} values against *S. obliqua* and *S.litura*, respectively. Lower doses of acetone extract were not very effective.

The probit statistics, estimates of LD_{50}

and their 95% fiducial limits are presented in Table 2 The probit regression lines for the effects of extracts on the stored grain insect showed a clear linear relationship between percentage mortality and extract concentrations (Fig. 1). The slopes of the probit lines were steeper as concentration increased which may be due to more amount of active principle in terms of mg/kg body weight due to higher concentration. Comparison of regression line also demonstrates the higher efficacy of acetone extract may be attributed to the presence of amides and lignans in *Piper* (Chandhoke *et al.* 1979).

Table 2. Probit analysis for contact toxicity at 24 h after topical application of fruit extract of *P. retrofractum* to *S. obliqua* and *S.litura* *

Extract	Insect (N)	LD_{50} (ppm)	95% fiducial limit	Regression equation
Spodoptera litura				
Acetone	**120**	0.8401	0.758-0.912	Y = 2.333 + 3.173 X
Ethanol	**120**	1.0788	0.969-1.188	Y = 2.372 + 2.436 X
Spilosoma obliqua				
Acetone	**120**	0.8284	0.738-0.918	Y = 2.645 + 2.843 X
Ethanol	120	1.1505	1.027-1.273	Y = 2.236 + 2.402

* Values are based on six concentrations and four replicates of 5 insects each.

Photodegradability of acetone extract

HPLC analysis of the crude acetone extracts showed that on exposure to sunlight the extracts started degradation within 14 of exposure to sunlight and substantial degradation had occurred by 4 h. The degradation pattern is well correlated with insecticidal potential of the extract (Table 3). After one hour of exposure to sunlight, the insecticidal activity of the extract was at par with unexposed sample in case of *S.obliqua* but less than unexposed sample in case of *S.litura*. However, for more than 2 h, the extracts had little insecticidal potential.

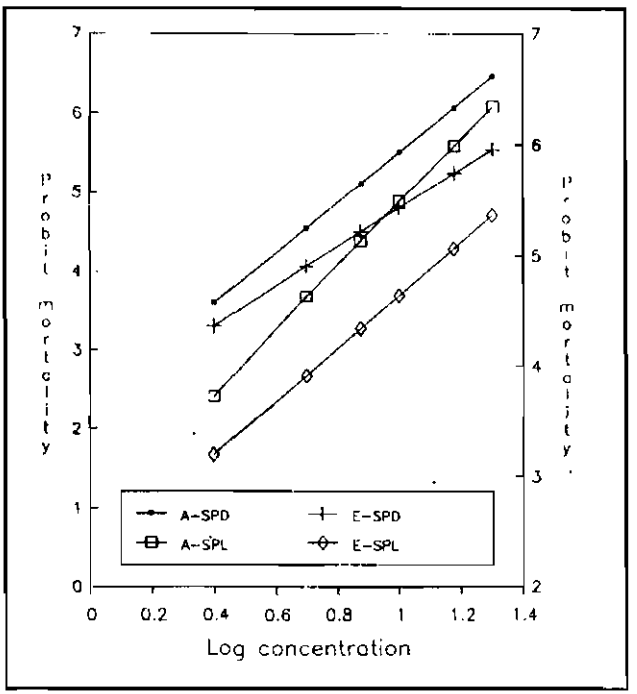
Table 3. Effect of sunlight on the insecticidal potential of acetone extract (2%) of *P. retrofractum* fruit

Exposure time (h)	Mortality	
	S.obliqua	*S. litura*
1	71.3^{A}	63.8^{B}
2	30.0^{B}	30.0^{C}
4	26.3^{B}	22.5^{C}
6	7.5^{B}	7.5^{C}
8	7.5^{B}	7.5^{C}
Unexposed	82.5^{A}	90.0^{A}
F-value	10.8	14.3

[a] Original data were transformed into arcsine √percentage values during ANOVA test

Figure followed by same letter within a column are non-significantly different at 0.05 per cent level in Duncans Multiple Range Test

The present laboratory studies reveal the highest repellency by *P.retrofractum* fruit oil against *Tribolium castaneum*, indicating its potential use in management of stored grain insects. The acetone extract exhibited highest insecticidal efficacy against *S. obliqua* and *S. litura* under laboratory conditions but its high photodegradation rate limits its use under field condition.



SPD : Spodoptera litura;
SPL : Spilosina obliqua;
A : Acetone extract;
E : Ethanol extract

Fig. 1. Effect of extracts of *P. retrofractum* against *S. obliqua* and *S. litura*

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Mode of action of *Trichoderma* and *Gliocladium* on *Pythium aphanidermatum*

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Abstract

The potential of 12 isolates belonging to 8 species of *Trichoderma* and two isolates of *Gliocladium virens* were tested for their biocontrol efficacy and mode of action on *Pythium aphanidermatum*, the causal organism of rhizome rot of ginger. Considerable variations were noticed among these isolates and *Trichoderma hamatum* (ISO.2) only showed typical inhibition of the pathogenic fungus in the dual culture tests. *T.harzianum* (ISO.2), *T.hamatum* (ISO.2) and *T.polysporum* are efficient in hyphal interactions. Abnormal granules, cytoplasmic vacuolation and coagulation are some of the changes in the hyphal structures noticed. Volatile and non volatile metabolites of the isolates tested suppressed the growth of the pathogenic fungus.

Key Words: biological control, *Gliocladium, Trichoderma.*

Introduction

Ginger is an important spice and vegetable crop grown extensively in India. Rhizome rot caused by *Pythium aphanidermatum* Edson. Fitz., is one of the major production constraints in ginger. Disease management in ginger is mainly confined to seed treatment and soil drenching with fungicides. Concerted efforts have not been made on the biological control of this disease.

The potential of *Trichoderma* and *Gliocladium* as biological control agents to check soil-borne root infecting fungal pathogens has been well established (Baker & Cook 1974; Chet 1987; Papavizas 1985). The suppressive effects of *Trichoderma* and *Gliocladium* on *Pythium* spp., *Fusarium* spp. and *Rhizoctonia* spp. have been reviewed (Mukhopadhyay 1987). Competition, mycoparasitism, production of antibiotics (volatile and non-volatile) are the reported mechanisms by which these fungi suppress soil-borne plant pathogens (Chet 1987). Investigations were carried out on the mode of action of 8 species of *Trichoderma* and *G.virens* collected from different ginger growing tracts in Kerala on *P.aphanidermatum* and are presented in this paper.

Materials and methods

Fourteen isolates viz., two isolates each of *T.viride*, *T.harzianum*, *G.virens*; 3 isolates of *T.hamatum* and one each of *T.aureoviride*, *T.polysporum*, *T.pseudokoningii*, *T.longi bracheatum* and *T.koningii* were selected for the present study based on the preliminary screening for their antagonism.

Hyphal interaction

The studies were carried out on potato dextrose agar (PDA) medium and culture discs from the edges of the 72 h old cultures of both *P.aphanidermatum* (ISO.P.11) and antagonist fungus were used. Hyphal interaction was studied as per the methods of Dennis & Webster (1971b). Culture discs of *P.aphanidermatum* and test fungi were paired in petridishes keeping them at 5 cm apart and inocubated at 26-28°C. Colony diameter of both fungi were measured after 48 and 72 h of incubation and the inhibition percentages of *P.aphanidermatum* were calculated. Pre-inoculation of antagonist 24 h prior to the pathogen and also simultaneous pairing of *Trichoderma* and *Pythium* were studied.

Volatile antibiotics

To test the effect of volatile antibiotics produced by the antagonistic fungi on the pathogen, the procedure of Dennis & Webster (1971a) was followed. *P.aphanidermatum* and the test isolates of *Trichoderma* and *G.virens* were raised separately in petriplates and were incubated for 48 h at 22-24°C. Then the lid of each petriplate was replaced by a bottom plate of a petridish containing *P.aphanidermatum* and were sealed with adhesive tape. Plain PDA bottom covers with bottom plates containing *P.aphanidermatum* served as control. After 24 h and 48 h of incubation, the colony diameter of the pathogen was measured and the percentage of inhibition was calculated.

Non-volatile antibiotics

Sterile cellophane discs (9cm dia.) were placed aseptically in petridishes containing PDA and were left overnight to allow excess moisture to evaporate. Culture discs (5mm) of *Trichoderma* and *Gliocladium* were placed at the centre of the petriplates containing cellophane discs and incubated at 22-24°C. After 48 h of incubation, the cellophane discs were removed and replaced with 5mm discs of *P.aphanidermatum* and incubated at room temperature (26-28°C). Cellophane discs removed from uninoculated plates and inoculated with culture discs of *P.aphanidermatum* served as control. Four replications were maintained for each treatment.

Results and discussion

Hyphal interaction

Macroscopic and microscopic changes were observed in the hyphae of *P.aphanidermatum* due to the interaction with antagonistic fungi (Table 1). *T.hamatum* (ISO.2) exhibited typical inhibition zone. *T. viride* (ISO.2), *T.harzianum* (ISO. 1&2), *T.hamatum* (all three isolates), *T.polysporum*, and *G.virens* (ISO. 1& 2) overcovered the entire *Pythium* colony after 7 days. At 20 days *Pythium* colony was not visually discernible in all these cases. This indicated clearly the competitive ability of *Trichoderma*. Aflattening of *Pythium* colony was observed with *T.harzianum* (ISO.2), *T.hamatum* (all isolates) and *G.virens* (ISO. 1&2) (Table1).

Table 1. Macroscopic and microscopic changes in *P.aphanidermatum* due to interaction with *Trichoderma* & *Gliocladium*.

Organism	Isolate	Macroscopic			Microscopic				
		IZ	OA	AFL	GS	HC	L	O	
T.viride	ISO-1		-	-	-	-	-	-	-
	ISO 2	-	+	-	+	+	-	-	
T. harzianum	ISO 1	-	+	-	-	-	-	-	
	ISO 2	-	+	+	+	+	-	-	
T. hamatum	ISO 1	-	+	+	+	-	-	Abnormal granules & vacoulation	
	ISO 2	+	+	+	+	+	-		
	ISO 3	-	+	+	+	+	-		
T. aureoviride		-	-	-	-	-	-	Vacoulation	
T. psuedokoningii		-	-	-	-	-			
T. Koningii		-	-	-	-	-	-	Cytoplasmic coagulation	
T.polysporum		-	+	-	-	-	+		
T. longibracheatum		-	-	-	-	-	+		
G. virens	ISO 1	-	+	-	+	-	-	Cytoplasmic coagulation	
	ISO 2	-	+	-	+	-	-	-do-	

IZ - Inhibition zone, OA - Overgrowth of antagonist, AFL - Aflattening of test organism, GS - General stunting of pathogen mycelia, HC - Hyphal coiling, LI - Lysis, O - Other abnormal microscopic changes.

T.viride (ISO.2), *T.harzianum* (ISO.2) and *T.hamatum* (ISO.2 & 3) and *T.longibracheatum* showed hyphal coiling around *Pythium*. Hyphal lysis was found in the case of *T.polysporum* and *T.hamatum*.

Abnormal structures like granule formation and vacuolation were found in the hyphae of *P.aphanidermatum* when paired with *T.hamatum* and vacuolation alone was observed when paired with *T.aureoviride*. *G.virens* and *T.koningii* induced coagulation of cytoplasm in *P.aphanidermatum* (Table 1).

In the first method viz., pre-inoculation with antagonist followed by post inoculation of *P.aphanidermatum*, the percentage inhibition ranged from 47.43 to 65.38 after 72 h of incubation (Table 2). The maximum inhibition (65.38%) was by *T.harzianum* (ISO.2) followed by *T.polysporum*.

In the second method viz., simultaneously paired cultures, the inhibition of *P.aphanidermatum* ranged from 21.11 to 61.95%. In general, the inhibition by the antagonist was less in this method compared with pre-inoculation.

Table 2. Growth inhibition in *P.aphanidermatum* by *Trichoderma / Gliocladium* spp. (percentage)

Organism	Isolate	*Pre inoculation		Simultaneous	
		48hrs	72hrs	48hrs	72hrs
T. viride	ISO 1	26.20	55.42	29.45	21.11
	ISO 2	46.15	59.18	37.68	46.68
T. harzianum	ISO 1	29.26	60.26	29.56	30.46
	ISO 2	37.24	65.38	26.36	61.95
T. hamatum	ISO 1	30.72	62.86	31.37	42.78
	ISO 2	45.76	63.49	38.28	43.89
	ISO 3	32.45	57.49	27.16	37.31
T. aureoviride	---	29.99	50.41	32.87	40.56
T. psueudokoningii	---	30.98	56.71	41.28	33.43
T. Koningii	---	23.42	47.43	34.77	44.63
T. polysporum	---	46.29	63.97	36.17	46.76
T. longibracheatum	---	30.46	49.61	33.97	44.44
G. virens	ISO 1	23.80	52.95	38.58	42.78
	ISO 2	46.15	59.18	29.86	46.67
CD		4.72	2.47	3.69	3.71

* Transplanting of *Trichoderma* discs 24 h earlier to dual culture test.

T.harzianum (ISO.2) gave the maximum (61.95%) inhibition followed by *T.polysporum* (46.76%) (Table 2). Thus in the dual-culture technique, and the inhibition of the pathogenic fungus by the antagonists was always high with the pre-inoculation compared to simultaneous inoculation. Gowda & Nambiar (1991) and Usman & Nambiar (1992) reported similar results with *Trichoderma* spp. against *Thielaviopsis paradoxa*, the stem bleeding pathogen of coconut.

Volatile and non-volatile antibiotics

Inhibition of *P.aphanidermatum* varied with *Trichoderma* spp. *T.viride* (ISO.2) gave maximum inhibition after 48 h of incubation (39.35%). The inhibition percentage of *T.hamatum* (ISO.2) was 37.72. Two isolates of *T.hamatum* (ISO.1 & 3) showed 17.97% and 17.9% inhibition respectively. *T.pseudokoningii* exhibited 16.21% inhibition of *Pythium*. *T.harzianum* (ISO.1) did not show any inhibition initially but showed 13.33% inhibition with *T.aureoviride*, *T.polysporum* and *T.longibracheatum*. Both the isolates of *G.virens* showed a meagre suppression of the pathogen (Table 3). *Trichoderma* isolates were reported to produce volatile compounds inhibitory to the growth of other fungi (Dennis & Webster 1971 a; Webber & Hedger 1985).

The results indicated that within *Trichoderma* spp. volatile antibiotic production varied considerably and non-volatile producers were also present. Production of 6

Table 3 Effect of volatile compounds of *Trichoderma* and *Gliocladium* on the growth of *P. aphanidermatum*.

Organism		Inhibition (%)	
	Isolate	24 hours	48 hours
T. viride	ISO 1	19.28	39.98
	ISO 2	26.19	39.35
T.harzianum	ISO 1	+1.30	13.33
	ISO 2	07.84	04.97
T. hamatum	ISO 1	04.08	17.97
	ISO 2	35.19	37.72
	ISO 3	13.52	17.90
T. aureoviride		+1.11	+0.92
T.psuedokoningii		03.09	16.21
T.koningii		07.52	04.39
T.polysporum		+3.34	01.73
T.longibracheatum		01.12	00.00
G.virens	ISO 1	04.17	03.32
	ISO 2	03.02	03.02
CD		13.93	16.57

pentyl α pyrone was reported from *T.viride* and the production varied among *T.viride* isolates studied (Zeppa *et al.* 1990). Further studies are needed on the nature of volatile and non-volatile metabolites in different *Trichoderma* and *Gliocladium* isolates used in the present study.

The results also showed that the inhibition increased with time. In the case of *T.hamatum* and *T.viride* (ISO.2) the colony growth of *P.aphanidermatum* was totally inhibited at 48 h of incubation and there was no change even after 10 days. Further studies are warranted on the age and stage of the *Trichoderma* on volatile production and also their mode of action on different phases of the pathogen, *P.aphanidermatum*. *Pythium* colony did not show the normal fluffiness, instead the colony was adpressed and became flat. However, the reisolation of the mycelium of *Pythium* specific P_{10} VP medium (Tsao 1970) gave positive isolation thereby indicating fungistatic nature of the volatiles.

T. viride (ISO.2), *T. harzianum* (ISO 1 &.2), *T.hamatum* (ISO.2), *T.koningii*, *T.polysporum* and *T.longibracheatum* showed complete inhibition of the pathogen after 24 h of inoculation of the pathogen. However, the inhibition effect in *T.viride* (ISO.2) was reduced after 48 h. *G.virens* reduced mycelial growth of the pathogen by 94.63% and 76.3% after 24 h and 48 h. respectively. *T.aureoviride* did not inhibit growth of the pathogen thereby indicating the absence of non volatile antibiotics (Table 4).

Dennis & Webster (1971a) reported the production of non-volatile compounds

Table 4. Effect of non-volatile compounds of *Trichoderma* and *Gliocladium* on the growth of *P.aphanidermatum*

Organism	Isolate	Inhibition (%)	
		24 hours	48 hours
T. viride	ISO 1	38.02	34.69
	ISO 2	100.00	100.00
T.harzianum	ISO 1	100.00	85.19
	ISO 2	100.00	85.10
T.hamatum	ISO 1	94.61	70.37
	ISO 2	100.00	95.35
	ISO 3	96.39	75.64
T. aureoviride		01.19	00.00
T. pseudokoningii		61.01	62.39
T. koninglii		100.00	79.81
T. polysporum		100.00	84.08
T. longibracheatum		100.00	93.14
Gliocladium virens	ISO 1	94.63	76.30
	ISO 2	78.53	43.33
C.D		16.03	26.56

by *Trichoderma* spp. The ability to produce such antibiotics varied between different species and also different isolates of the same species. Jackson *et al.* (1991) studied some antifungal metabolites of *Trichoderma* spp. and *G.virens* antagonistic to *Sclerotium cepivorum* and *Botrytis cinerea.*

In the present study *T.viride* (ISO.2) exhibited 100% inhibition even at 48 h. The *Trichoderma* spp. viz. *T.harzianum* (ISO.1 & 2), *T.hamatum* (ISO.2), *T.koningii, T.polysporum, T.longibracheatum* which gave 100% inhibition after 24 h showed a decreased inhibition after 48 h. This might be due to the diffusion of antibiotics in to the media. Bettucci, Lup & Silvan (1988) studied the control of growth of wood rotting fungi by non-volatile and metabolites into agar medium. Except, *T.aureoviride*, all the other species of *Trichoderma* and *Gliocladium* studied were active antibiotic producers (Table 4).

All the isolates studied showed considerable variations on the production of volatile and non-volatiles based on the inhibitory effects on *P.aphanidermatum*. In general, the inhibitory effect of non-volatiles was more compared to volatiles. *T.aureoviride* did not produce volatile or non-volatile antibiotics. Slight stimulation of growth of host mycelium with *T.harzianum* (ISO.2), *T.aureoviride* and *T.polysporum* noticed after 24 h did not persist after 48 h of incubation where suppression of growth of *P.aphanidermatum* was noticed. Even though non-volatile and volatile compounds are considered to play major role in suppression of *P.aphanidermatum*, it is essential to check for their inhibitory effect on the host plant also since bio-control agents without any inhibitory effect on host are of practical importance.

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Management of rhizome-rot of ginger by fungal antagonists

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Abstract

Suppression of *Phythium aphanidermatum* and rhizome rot of ginger by fungi viz. *Aspergillus niger, A. terreus, Penicillium* species and *Absidia cylindrospora* are reported. The former three fungi inhibited *P.aphanidermatum* upto 100% by producing fungitoxic non-volatile metabolites. *A.cylindrospora* expressed mild inhibition (7.03%). *A.cylindrospora* and *P.aphanidermatum* also exhibited mutual overgrowth in dual culture. *A.niger* showed good protection against rhizome rot. Severity of rhizome rot infection was low when infested soil was treated with *A. terreus, Penicillium* species and *A.cylindrospora*. Highest yield was recorded with *A.niger*.

Keywords: disease management, ginger, *Pythium aphanidermatum*, rhizome rot, *Zingiber officinale*.

Introduction

Ginger (*Zingiber officinale* Rosc.) is one of the most valuable spices of commerce. Rhizome rot casued by *Pythium aphanidermatum* is the main constraint in production of ginger. Existing disease management practices which consist mainly of seed rhizome treatment and soil drenching with fungicides have provided only partial control of the disease. Many *Trichoderma* species have been isolated and evaluated under field conditions and an efficient strain of *T.harzianum* has been selected. The present study deals with other biocontrol agents viz. *Aspergillus niger, A. terreus, Penicillium* sp. and *Absidia cylindrospora* and their efficacy in suppression of rhizome rot.

Materials and methods

Isolation of resident biocontrol agents was made from rhizosphere soil of ginger by dilution plate method. For isolation of fungi, 10 dilution of soil was plated on rosebengal-agar medium and incubated at room temperature (20°C) for 96 h. Different fungal colonies were subcultured and maintained on Potat Dextrose Agar (PDA) slants. Identification of fungi was made by analysing macro and micro morphological charac-

teristics using taxonomic keys.

Evaluation of antagonistic potential *in vitro* was based on the tests used by Dennis & Webster (1971a, b, c) and Royse & Ries (1978). The tests include identification of antagonists that produce non-volatitle metabolites, volatile metabolites or that are potential mycoparasites. The following tests were conducted.

Dual culture tests

Dual culture tests were used to observe the type of colony and hyphal interaction by visual as well as microscopic examination of the interaction zone. A culture disc of the antagonist (5mm) was placed on one side of solidified agar medium and allowed to grow for 48h. Then the culture disc of the pathogen was placed on the opposite side of the plate, 4-5cm away from the antagonist and incubated. Colony and hyphal interactions were observed after 24 h and followed upto 5 days.

Test for non-volatile metabolities

This test was performed by placing a 5mm agar disc of the antagonist centrally on cellophane covered agar. After 3 days, the cellophane was removed along with the antagonist. A 5mm agar disc of the pathogen was then placed centrally on the agar. Growth of the pathogen was recorded after 48 h. When complete inhibition of pathogen was observed, the pathogen inoculum was transferred to fresh medium and examined for regrowth to assess fungitoxic/fungistatic nature of the metabolites.

Test for volatile metabolites

The antagonists were grown on PDA for 48 h at 22 - 24 ^{0}C. The lid of each plate was then replaced by another base containing pathogen colonised agar disc on PDA. The two petridish bases were then sealed using adhesive tape. Percentage inhibition of growth was assessed by comparing the growth of pathogen in the presence of the antagonist with that of control.

Effect of antagonists on rhizome rot of ginger

Effect of antagonistic fungi on rhizome rot of ginger was studied in pot culture tests. Ten kilograms of potting mixture (soil: farmyard manure: sand in 3:1:1 ratio) was filled in 12" pots and watered. Soil in each pot was infested with 50ml of mycelial suspension of *P.aphanidermatum*. Mycelial suspension was prepared using mycelial mat grown in 200ml potato broth in Roux bottles for 7 days. Mycelial mat was washed in sterile distilled water and blended at low speed in strile distilled water for 2 min to get the suspension. The suspension was diluted to 200ml and 50ml was used for inoculation per pot. Infested soil in each pot was treated with 50g sorghum grain preparation of respective antagonist one weak after infestion. Four bits of seed rhizomes (20g each) were planted in each pot. Germination and disease incidence were recorded periodically and yield of rhizome (fresh wt.) was taken after six months.

Results and discussion

Among the four antagonistic fungi tested *in vitro* for colony and hyphal interactions, *A. niger* and *Penicillium* species developed distinct inhibition zone in dual culture. *A. terreus* and *P.aphanidermatum* colonies stopped growth at the point of contact.

A.cylindrospora and *P.aphanidermatum* expressed mutual overgrowth beyond the point of contact but there was no distinct hyphal interaction. In the test for non-volatile antifungal metabolites production *A.nigher, A.terreus* and *Penicillium* species inhibited *P.aphanidermatum* completely (100%). *A.cylindrospora* expressed only weak inhibition of the pathogen (7.03%). None of the fungi produced volatile antifungal metabolites (Table 1).

Table 1. Inhibition of *Pythium aphanidermatum* by fungal antagonists *in vitro*

Antagonist	Inhibition of growth after 48 hrs. (%)	
	NVM	VM
Aspergillus nigher	100.0*	0.0
A. terreus	100.0*	0.0
Penicillium sp.	100.0*	0.0
Absidia cylindrospora	7.02	0.0

* Fungitoxic, NVM = Non-volatile metabolite, VM = Volatile metatbolite

Germination of rhizome was improved in all treatments except *A.cylindrospora*. *A.nigher* showed good protection against rhizome rot. Rhizome rot infection was low when infested soils were treated with sorghum grain preparation of *A.terreus, Penicillium* species or *A.cylindrospora*. In untreated infested control disease incidence was highest (64.83%). Highest yield was recorded in *A.nigher* treatment (331g/pot). Yield in *A.terreus, Penicillium* species and *A.cylindrospora* treatments were 233g, 192.7g and 140.3g respectively as against 43.5g in control (Table 2).

Table 2. Effect of antagonists on rhizome rot of ginger

Antagonist	Germination (%)	Disease incidence (%)	Disease severity (%)	Yield (g)
Aspergillus nigher	100.0A	0.0 C	0.0 C	331.0 A
A. terreus	97.5 A	20.0 BC	12.0 BC	233.0 B
Penicillium sp.	100.0A	25.0 BC	19.25 BC	192. 7 C
Absidia cylindrospora	80.0B	30.0BC	30.0 B	140.3 C
Control	75.0B	72.5 A	64.83A	43.5 D

In each column values followed by different letters are significant according to Duncans multiple range test.

Antagonistic potential of *Penicillium* species against *Pythium* spp. is known (Windels 1981; Windels & Kommedahl 1982a, b). *A.cylindrospora* antagonistic to *P.ultimum* has been isolated and tested in Himachal Pradesh, India for the management of rhizome rot disease. *A. nigher* and *A.terreus* are less investigated organisms with respect to their antagonism on *Pythium* species. Efficacy of *A.nigher, Penicillium* species and *A.terreus* in suppression of rhi-

zome rot is related to their biological activity against *Pythium aphanidermatum in vitro*. *A.cylindrospora* did not produce any antifungal metabolite strongly inhibitory to the pathogen *in vitro*. Correspondingly its efficacy in suppression of rhizome rot is also less.

This preliminary study indicates the involvement of organisms other than *Trichoderma* spp. in suppression of rhizome rot infection and stresses the need for concerted effort in exploitation of such organisms for effective management of the disease.

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Compatibility of potassium phosphonate (Akomin-40) with different species of *Trichoderma* and *Gliocladium virens*

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Abstract

Biocontrol agents viz., *Trichoderma* spp and *Gliocladium virens* showed varying degrees of inhibition on growth of *Phytophthora capsici*, the foot rot pathogen of black pepper. Potassium phosphonate (Akomin), an antifungal compound has been found to check root rot in black pepper. In integrated disease management programme, compatibility of biocontrol agents with the fungicide is crucial. In the present study sensitivity of 8 species of *Trichoderma* and one species of *Gliocladium* to potassium phosphonate was tested. No significant effect was noticed even at concentration as high as 1200 ppm.of potassium posphonate, while in *Trichoderma aureoviride* and in *Trichoderma pseudokoningii*, increased sporulation was noticed with potassium phosphonate compared to control. Of the isolates tested *Gliocladium virens*, *Trichoderma harzianum* and *Trichoderma hamatum* were found effective in the inhibition of the pathogen. These isolates showed different degrees of protection against root rot. The present results clearly established the compatibility of biocontrol agents with potassium phosphonate, which forms a component in the integrated disease management programme.

Key words: *Gliocladium virens*, *Phytophthora capsici*, potassium phosphonate, *Trichoderma*

Introduction

Phytophthora foot rot is the major production constraint in black pepper and an integrated approach involving cultural, chemical and biological control methods coupled with host resistance is the strategy adopted for disease management (Sarma, Anandaraj & Rajan 1994). The efficacy of systemic fungicides viz., Metalaxyl, Fosetyl-Al has been reported (Ramachandran & Sarma 1991). The efficacy of potassium phosphonate (Akomin), an antifungal compound showing systemic effect in

checking *Phytophthora* infection in black pepper and also of biocontrol agents viz., *Trichoderma* spp. and *Gliocladium virens* has been reported (IISR 1995). The compatibility of agrochemicals with biocontrol agents viz., *Trichoderma* spp, *Gliocladium virens* and *Pseudomonas fluorescens* used in checking *Phytophthora* infection in black pepper has been highlighted (Sarma *et al.* 1996). The present study was undertaken to test the sensitivity of *Trichoderma* spp. and *Gliocladium virens* to potassium phosphonate which is now being used extensively in the management of *Phytophthora* caused plant diseases.

Materials and methods

Eight *Trichoderma* species viz., *T.aureo viride*, *T.hamatum*, *T.harzianum*, *T.koningii*, *T.longibracheatum*, *T.polysporum*, *T.pseudokoningii*, *T.viride* and *Gliocladium virens* from culture collection of Indian Institute of Spices Research were used in the present study. Stock cultures of these antagonistic fungi were maintained on PDA slants, stored in BOD incubator at 20°C for further studies.

Potassium phosphonate formulation supplied by M/S Rallis Agrochemicals, Bangalore, India was used for the studies. Potassium phosphonate formulation was passed through a sterile G5 filter for sterilisation and stored in refrigerator for further studies. For all the studies, 3 concentrations of the chemical viz; 400, 800 and 1200 ppm were used. For each treatment four replications were maintained and experiments were conducted at room temperature.

Growth of antagonistic fungi

To study the compatibility of potassium phosphonate with different biocontrol agents, 3 concentrations of the chemical (400,800 and 1200 ppm) were incorporated in sterile PDA medium at the time of pouring into the petri plates. Culture discs (0.5cm) of the test isolates were taken from actively growing cultures (48 h old) and plated. Culture raised on PDA without potassium phosphonate served as control for each isolate. Growth of each isolate was recorded at 24 h interval upto 72 h.

Sporulation of antagonistic fungi

To study the effect of potassium phosphonate on sporulation, cultures of different biocontrol agents were raised on PDA incorporating the different concentrations of potassium phosphonate. After 96 h, 10 discs (0.5cm.) per plate were randomly taken in 80ml of sterile double distilled water in 250ml beakers and agitated thoroughly with the help of magnetic stirrer and the contents were made upto 90ml with sterile double distilled water. Spores in each treatment were counted with the help of hoemocytometer.

Data were analysed by using statistical software MSTATC. Factorial analysis with the concentrations (3) as one factor and species (9) as second factor with four replications was done.

Results and discussion

Growth of different biocontrol agents differed considerably during the test period. *T.aureoviride*, *T.viride* and *Gliocladium virens* attained maximum radial growth within 24 h. But after 48 h, *T.pseudokoningii*, *T.viride*, *T.aureoviride*, *T.harzianum* and *G.virens* attained maximum radial growth (45.00mm) and the least growth was recorded in *T.hamatum* (32.67 mm). After

72 h, all species of *Trichoderma* and *G.virens* attained maximum growth in all control plates.

In plates treated with 400ppm of potassium phosphonate, all the species of *Trichoderma* and *G.virens* showed significant difference in the growth. After 24 h *T.harzianum* attained maximum growth (30.25mm) compared to all other isolates tested and significantly least growth was recorded in *T.polysporum* (12.75mm). The growth of *T.hamatum* and *T.aureoviride* was at par (Table 1).

Table 1. Effect of potassium phosphonate on growth of different species of *Trichoderma* and *Gliocladium virens*

Conc.	Radial growth in mm (after 24 h)								
	T.pse	*T.koni*	*T. Poly*	*T.long*	*T.ham*	*T.harz*	*T.viri*	*T.aure*	*G.vir*
400ppm	19.75	17.75	12.75	19.00	16.00	30.25	23.00	16.00	20.25
800ppm	21.50	17.00	13.75	16.50	14.00	18.25	23.25	18.25	17.25
1200pp	21.00	18.00	13.50	14.50	11.00	17.50	19.75	20.75	17.00
Control	22.75	16.25	17.50	21.75	15.75	22.00	24.00	24.75	24.00
	CD ($p<0.05$)=1.244								
	(after 48 h)								
400ppm	45.00	35.50	31.45	33.88	30.42	40.63	43.63	40.63	42.08
800ppm	43.75	32.08	31.36	30.92	27.55	35.88	38.92	40.75	30.33
1200pp	45.00	33.08	28.83	26.67	22.50	32.15	35.83	34.92	30.83
Control	45.00	34.15	37.58	39.90	32.67	45.00	45.00	45.00	45.00
	CD ($p<0.05$)=1.714								
	(after 72 h)								
400ppm	45.00	35.50	31.45	33.88	30.42	40.63	43.63	40.63	42.08
400ppm	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00
800ppm	45.00	45.00	45.00	45.00	42.00	45.00	45.00	45.00	45.00
1200pp	45.00	45.00	45.00	45.00	24.00	45.00	45.00	45.00	45.00
Control	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00
	CD ($p<0.05$)= 0.7563								

After 48 h *T.pseudokoningii*, *T.harzianum*, *T.viride*, *T.aureoviride* and *G.virens* attained maximum radial growth (45.00mm) and considerably least growth was recorded in *T.hamatum* both in treated and control plates (Table 1). In plates treated with 800 ppm of potassium phosphonate, *T.viride* attained maximum radial growth (23.25mm). After 48 h *T. pscudokoningii* attained maximum growth (43.74mm) followed by *T.aureoviride* (40.75mm) and least was in *T.hamatum* (27.55mm).

Cultures treated with 1200ppm of po-

tassium phosphonate showed significant differences in growth. *T.pseudokoningii* attained maximum growth (21.10mm), within 24 h compared to all other isolates, followed by *T.aureoviride* (20.75mm) and least was in *T.hamatum* (11.0 mm) and *T.polysporum* (13.50mm).

After 48h, *T.pseudokoningii* attained maximum growth (45.00 mm) followed by *T.viride* and least growth was observed in *T.hamatum* (22.50mm).

After 72 h, all the species of *Trichoderma* and *G.virens* attained maximum radial growth (45.00 mm) in all the treated and untreated plates except in *T.hamatum* (Table 1). *T.hamatum* attained 24.00 mm (46.44% reduction) in 1200 ppm treated plates. Not much difference was observed in growth of biocontrol agents at different concentrations of the chemical.

From the sporulation study, highest spore count was observed in *T.harziaum* in treated and nontreated plates, followed by *Gliocladium virens* and the least spore count was noticed in *T.longibracheatum* (Table 2). Even after 96 h. *T.viride* did not sporulate even in control plates. Out of eight species of *Trichoderma* and *Gliocladium virens* tested, *T.pseudokoningii* recorded highest spore load in 400 ppm treated plates (342503 spores per ml).

Table 2. Effect of Potassium phosphonate (Akomin) on sporulation of different species of *Trichoderma* and *Gliocladium virens*

Conc.	Spore count per ml. after 96 h								
	T.pse	*T.koni*	*T. Poly*	*T.long*	*T.ham*	*T.harz*	*T.viri*	*T.aure*	*G.vir*
Con	10130	12500	62480	2522	3123	250616	0.0	17880	87480
400.ppm	342503	1872	00.00	3540	00.00	13750	0.0	8750	10620
800.ppm	38120	1252	00.00	3748	00.00	75.03	0.0	15070	6251
1200 ppm	265033	1248	00.00	2510	00.00	10040	0.0	13750	4372

CD)P<0.05)=30.32

Spore count/ml.after 168 h

	T.polysporum	T.hamatum	T.viride
Control	63030	31250	00.00
400ppm	18750(7025%)*	8248(73.60%)*	00.00
800 ppm	13750(78.15%)*	1248(96.00%)*	00.00
1200ppm	1248 (98.01%)*	1248(96.00%)*	00.00

*Reduction in spore count CD (p<0.05) = 12.30

In *T.polysporum*, *T.hamatum* the sporulation was not observed in treated plates and in non treated plates of *T.viride*. *T.viride* did not sporulate even after 196 h. In *T.pseudokoningii*, increment of spore load was observed in an 1200 ppm treated plates.

Potassium phosphonate has no negative effect on the host and plant products (Johri & Chourasia 1995) and it was also reported that it enhances the

overall growth of the plants (Merwe & Dev Van Der 1993) and yield in avocado (Pegg *et al.* 1987). Potassium phosphonate is found very effective against many phytopathogenic fungi. The mode of its action on pathogens was described (Smillie, Grant & Guest 1989; Guest & Bompeix 1990).

Potassium phosphonate was found compatible with *Enterobacter aerogens* (Utkhede & Smith 1993) and also reported that it has no negative effect on other soil microbes in pepper corn and avocado field (Wongwathanarat & Sivasithamparam 1991). Recent studies showed that, this fungicide is compatible with *Trichoderma harzianum* (Sharma & Ashok Mishra 1995).

Potassium phosphonate (Akomin) is widely used to control the foot rot disease of black pepper. Field trials, clearly showed that the biocontrol agents like *Gliocladium virens*, *T.harizianum* and *T.hamatum* are found very effective in disease suppression. From this compatibility study it is clear that this chemical can be used along with the biocontrol agents to control the disease in field.

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Biotechnological approaches for production of saffron and capsaicin - a perspective

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Abstract

Central Food Technological Research Institute, Mysore had done pioneering work in the *in vitro* production of saffron, the highly priced spice which contains crocin (colour), picrocrocin (taste) and saffranol (flavour) principles. Protocols for development of saffron like structures have been achieved using floral explants in defined media. Scaling up of this protocol is still to be perfected. Though the technology has not reached commercial application level, it may possibly be achieved in the years to come. Capsaicin from *Capsicum annuum* is important pharmaceutically and in food industry. Protocol for *in vitro* production of capsaicin using placental tissues was standardised. Capsaicin was released into the medium at the rate 1600 mg/g of fresh cells over a 10 day period. Nitrogen stress resulted in two fold increase in capsaicin production. Biotransformation using ferulic acid and valine has also been achieved. The system of immobilized cells has been successfully tried in 2 l column reactors. Commercialization of this technology is very promising. The future for plant cell culture to produce selected spice and aromatic principles appears feasible now as the initial problems have now been overcome.

Key words: biotransformation, capsaicin, immobilization, *in vitro* production, saffron.

Introduction

India has been a traditional leader in production and export of spices. With the present biotechnological approaches new outlook is needed in the production of spices and aromatic plants. One such method is *in vitro* culturing of plant cells in bioreactors with assured quality and yield.

Plant cells cultured *in vitro* produce wide range of primary and secondary metabolites of economic value (Heble & Chada 1986). The advantages being production under controlled conditions

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free from agroclimatic constraints, besides uniform and rapid production. Production of phytochemicals from plant cell cultures has been presently used for pharmaceutical products. (Ravishankar & Venkataraman 1990). It is now realized that this technology can be used for production of food additives and spice principles, biopesticides, sweeteners etc. However, production of such compounds are to be attempted only if their value is higher than US $ 500/Kg.

Successful cultivation of plant cells and organs in liquid medium has opened up new vistas towards using them as industrial microbes. It is now possible to grow plant cells in 50 to 50,000 l capacity bioreactors under precise control of industrial processes. Shikonins from *Lithospermum erythrorhizon* (Fujita *et al.* 1982), ginsenosides from *Panax ginseng* and vinblastine from *Catharanthus roseus* have now been commercialized. The cell culture methods for production of pharmaceutically important compounds viz., shikonin, sanguinarine, berberine and β - methyl digoxin were successful. The application of this technology for food additives is now catching up and the important candidates being anthocyanin, capsaicin and vanillin.

Central Food Technology Research Institute (CFTRI) has pioneered the *in vitro* production of saffron and capsaicin.

Saffron

The saffron of commerce is the dried stigma of the plant saffron (*Crocus sativus*). Saffron has a very high value with the current international market price of over US $ 2000/Kg. Saffron finds a variety of uses in Europe and in East as perfume, drug, dye, culinary adjunct, therapeutic, flavouring and colouring of foods.

In vitro propagation

Any attempt to modernize the cultivation of saffron will require efficient mass production of pathogen free corms or saffron plants for which tissue culture method is ideal. Homes, Legros & Laziu (1987) and Ilahi, jabeen & Firdaus (1986) tried to establish propagation protocol for saffron with limited success. Isa & Ogasamara (1988) reported the formation of organized nodules on 2,4-D and zeatin supplemented medium which regenerated into shoots upon changing growth regulators to NAA and BA medium. Low temperature such as 15-20°C was the best suited for plantlet regeneration and the plants grew further under aseptic conditions only.

Propagation through direct corm/shoot regeneration from the explant

Callus was induced in saffron using 2, 4-D and zeatin which developed into small spherical organ like structures which could be regenerated into shoots marked the first step towards *in vitro* corm production followed by other reports (Homes, Lergros & Laziu 1987; Gui *et al.* 1987).

Direct shoot regeneration

Successful shoot regeneration directly from the ovary explants was also achieved. Such shoots completed their growth cycle by producing corms at the base. The corms thus produced could be multiplied *in vitro* as abnormal corm like structures.

Production of stigma and stigma constituents

Stigma of saffron comprises a very small portion of the plant produced only once a year explains the high specificity and cost in the production. For these reasons, production in artificial environment has always been a highly challenging task for tissue culturists. Enthused by the report of Hicks & McHughen (1974) on the formation of stigma like structures in tobacco cultures, Sano & Himeno (1987) of Ajinomoto Co., Japan explored for the first time the possibility of saffron stigma proliferation *in vitro* aiming for the industrial production of exotic spice-saffron. They cultured young stigma and ovaries of saffron on LS medium (Table 1) which directly produced stigma like structures (SLS) containing yellow pigments, crocins, comparable to those in natural stigmas. This report (Agricell Report, Vol.9(6): 1987) induced several other groups of scientists all over the world to work on saffron.

Workers at Hiroshima Pharmaceutical Institute also produced similar results on the same LS medium and growth regulators (BA and NAA). The results differed with those of Sano and Himeno that the stigma like structures with all the stigma compounds originated from the opaque hard callus mass which was induced from young stigma explants. Researchers at Ajinomoto (Himeno, Matsushima & Sano 1988) extended their studies confirming the SLS by scanning electron microscopy. Several patterns of stigma and style-like primordium formation were observed in sections of several kinds of cultured floral explants. The stigmatic surfaces of SLS were morphologically and biologically functional as pollen receptors confirming the ontogeny of stigmas in field grown flowers. In addition, they matured developing a deep orange colour.

Table - 1. Explants, media and incubation conditions for proliferation of sigma-like - structures (SLS)

Explant	Medium	Response	Reference
Young stigma and overies	LS+Kn (1&5)+NAA (1&10) or LS + IBA 20)+ BA (1&5) at 20°C in dark	Direct/indirect proliferation of stigma like structures with stigma colour components	Sano & Himeno 1987
Immature half ovaries, anthers stigma, & petals	White NAA(4)+ Leatin (4) at 25°C in dark	Stigma-like structures with intense orange colour & saffron aroma on all the explants with or without callus	Fakhrai & Evans 1990
Stigma	MS+NAA (10) +BA (1) at 20°C in dark	Intensely orange coloured stigma like structure	Sharma *et al.* 1990

Detailed reports on screening of different floral explants, their growth stage,

Table - 2. Different types of callus cultures of saffron grown on specific medium with varied ability to synthesize saffron stigma metabolites

Culture type	Medium	Metabolite			
		crocin	crocetin	picrocrocein	safranal
Yellow callus	Ms+NAA (2) +Kn (4) or MS+2, 4-D(1) +Kn (0.5)	-	-	-	-
Red friable callus	MS+IBA (2) + Kn (4) or MS+activated charcoal (1%)	+	+	-	-
Red filamentous structures (RFS)	MS+Kn (0.5%)	+	+	+	+
Red globular callus	MS half strength	+	+	+	+

+ Presence - Absence.

influence of growth regulators and incubation conditions for best stigma formation are available (Table 3). Fakhrai & Evans (1990) reported stigma development from half ovaries on White's medium with zeatin and NAA whereas Sharma *et al.* (1990, 1991) observed highly pigmented SLS on MS with BA and NAA from both maturing stigmas and anthers.

Stigma metabolites in cultured cells and tissues

Very fascinating responses towards SLS formation were reported by several workers, However, these SLS failed to proliferate continuously. Hence a different approach was accomplished by Venkataraman *et al.* (1989) for the first time. They attempted the induction of stigma metabolites directly from cultured cells and tissues with partial success.

Callus cultures raised on MS medium supplemented with 2, 4-D and Kinetin when transferred to the same medium with different set of growth regulators (IBA and Kinetin) produced red pigmentation. When separated on TLC, the spots were of same Rf obtained for authentic saffron and analysed by HPLC. They also reported the formation of characteristic flavour of saffron, safranal, when an ethanol extract of callus was evaporated and hydrolysed with 0.1 N NaOH at 37°C. Visvanath, Ravishankar & Venkataraman (1990) later developed different types of culture tissues with an array of organisation and colour for which a specific medium appeared responsible. In addition different levels of stigma

metabolites were synthesized by each callus type which are summarised in Table 2. Stigma metabolites in red callus and red filamentous structures (RFS) were two fold more (0.13%) than in the former (0.05) on dry weight basis. However, the safranal content (1.2%) in the red globular callus was almost comparable to that of dry floral stigma (1.4%). Very interesting observation made by these workers (Visvanath Ravishankar & Venkataraman 1990) was the ability of red globular tissue to synthesize high level of picrocrocin (18.7%) which was nearly six times higher than that present in dry floral stigmas.

Economic aspects of in vitro saffron production

Though the possibility of *in vitro* production of saffron stigma and its chemical constituents appear fascinating, the various market considerations dictate the potential for production *via* plant tissue culture. The position of biotechnology based products versus existing products, including the lower priced synthetic product and the highly priced whole plant derived natural product, is of basic consideration. This has been well projected in a recent review (Sahai 1994) emphasizing the research need for natural food colours and flavours through tissue culture despite the product being several fold more expensive than its synthetic analogue. Saffron colours (crocin) and flavour (picrocrocin) in pure form (active solids) cost US $ 7,500/Kg with an estimated world market of 15-20 US $ million per year (Sahai 1994). The saffron crop is subject to fluctuations in yield, quality, agro-climatic conditions, political and sociological considerations in producing countries in addition to its labour intensive processes. Thus saffron stigma constituents form highly priced low volume product and tissue culture method appears commercially feasible.

Basic information on synthesis of various stigma metabolites are meagre. Studies on the formation of organised stigma and enzymes involved in such defined growth are not available. Similarly investigations on the molecular biological aspects which complement the bio-process production of saffron *in vitro* are urgently needed.

Capsaicin

Capsaicin is highly important food additive in India and other Asian countries and also as a pharmaceutical compound. It finds its use in formulated foods and therapeutically as counter irritant, fungistatic and bacteriostatic agent (Sooch, Thakur & Kaur 1977). Capsaicin is a constituent of *Capsicum fruitescens*. Commercial production of capsaicin by separation from capsicum oleoresin and subsequent purification would involve several steps and the cost of the production reaches Rs. 25000/Kg. It is one of the compounds which can be produced by immobilized cell cultures, which leaches out to the medium facilitating easy separation and purification. In addition immobilized cell cultures of capsicum offer a continuous method of producing capsaicin under controlled conditions *in vitro*.

In vitro *procedures*

Plant material and culture conditions

C. annuum var. Selection 1 of Indian Institute of Horticultural Research was used to develop callus cultures from seedling callus on Murashige & Skoog's me-

dium supplemented with phytohormones. The unorganised callus masses could be sequentially subcultured on semisolid agar medium with an interval of 3 weeks (Ravishankar *et al.* 1988). The callus tissues were transferred to 40 ml liquid MS medium with phytohormones and were maintained by subculturing at 2 week interval to fresh medium (Sudhakar Johnson, Ravisankar & Venkataraman 1990). The cells were periodically tested for their ability to biosynthesize capsaicin by using a sensitive HPLC method developed by (Sudhakar Johnson, Ravishankar & Venkataraman 1992).

Immobilization of cells and placenta in calcium alginate matrix

The cells were mixed in sodium alginate solution (2.5% w/v) and extruded into calcium chloride solution to obtain 3mm beads which were washed with distilled water and cultured in liquid medium. In another method developed, placenta from the *Capsicum frutescens* fruits were isolated and made into 2mm segments which were encapsulated in sodium alginate matrix as mentioned above.

The relative performance of immobilized cells and immobilized placenta were analysed. It was found that the immobilized placenta exhibited higher productivity of capsaicin due to the fact that the placenta has the biochemical machinery expressed for capsaicin synthesizing activity (Sudhakar Johnson, Ravishankar & Venkataraman 1990). The immobilization of cells in alginate matrix enhances the production of cells by over hundred fold as reported by British workers (Lindsey & Yeoman 1984). This along with other features like release of the capsaicin from cells to the medium, easy separation of biomass from the medium and continuous utilization of biomass makes scaling up of the system much easier.

Enhancement of capsaicin production by media manipulations

A carefull analysis of secondary metabolite profile during the culture of plant cells indicates that most of the secondary compounds are produced during post exponential or stationary phase of growth (Lindsey 1985). The biochemical factor underlying this phenomenon is the channelling of precursors from growth related processes to secondary metabolism (Ravishankar *et al.* 1988). The above situation could be induced by subjecting the cells to nutrient stress. We found that nitrate and phosphate stress enhances the capsaicin in comparison with free cell culture systems. The capsaicin production was further enhanced by feeding ferulic acid as precursor.

Influence of elicitors

Secondary metabolite production in plant cell cultures can be elicited using a range of elicitors (DiCosmo & Misawa 1985). Elicitation is envisaged to overcome the problem of low productivity of plant cells for industrial applications (Knorr *et al.* 1990). Treatment of immobilized cells and placental tissues with various elicitors such as fungal extracts *(Aspergillus niger and Rhizopus oligosporus)* and bacterial polysaccharides, curdlan and xanthan were done. It was found that curdlan was most effective in eliciting capsaicin synthesis and it increased by almost two fold compared to control. Immobilized cells responded more effectively than placental tissue to curdlan treatment. Curdlan and xanthan in combination enhanced capsaicin production

by nearly 8 fold. Mycelial extracts of both the fungal species resulted in an increase of capsaicin by about 25% in immobilized placental tissues. Similarly, chitosan enhanced capsaicin production by over 5 fold (Table 3).

Table 3 Influence of chitosan on capsaicin production in cultured cells

Day	Treatment [b]	Capsaicin content[a]			
		Cells[c] (μg/g)	Medium[d] (μg/40ml)	Total[e] (μg/culture)	Capsaicin medium (%)
5	---	95(+1.44)	80(+1.23)	175(+2.67)	45.7
6	Control	72(+0.96)	128(+1.74)	200(+2.70)	64.0
	Treated (150 μg chito san/ml medium	124(+1.02)	432(+3.62)	556(4.64)	77.6
7	Control	62(+1.75)	216 (+1.73)	278(+3.48)	77.6
	Treated (150 μg chitos an/ ml medium	144(+1.41)	220(+1.84)	364(+3.25)	60.4

a. Data are mean of five replicates in two repetitive experiments.

b. One gram cells (30 days old) transferred to 40 ml MS medium with or without (control) the addition of chitosan.

c. Capsaicin in μg per gram fresh weight of cells.

d. Capsaicin in μg per 40 ml medium.

e. Total capsaicin in 1 g cells and 40 ml medium.

Data in parenthesis indicate ± S.E.

Separation techniques

A HPLC procedure was developed to separate vanillyamine, L- phenylalanine, caffeic acid, coumaric acid, ferulic acid, cinnamic acid, capsaicin and dihydrocapsaicin. Using the HPLC method, concentrations of intermediates were determined in placenta, pericarp, cell cultures, immobilized cells and immobilized placenta (Sudhakar Johnson, Ravishankar & Venkataraman 1991). The profile of intermediates showed higher capacity of biotransformation in immobilized placenta over immobilized cells. Vanillylamine was not a limiting intermediate for capsaicin production. Three-week-old placenta separated from fruit had a higher quality of capsaicin than in pericarp.

Biotransformations

Immobilized plant cell cultures of *Capsicum frutescens* were treated with the ferulic acid and vanillylamine in order to study their biotransformation ability. It was found that ferulic acid and vanillylamine were biotransformed to capsaicin and vanillin. Ferulic acid treated cultures produced more vanillin than capsaicin. This trend was expected since ferulic acid and vanillylamine are the nearest precursors to vanillin and capsaicin, respectively. Maximum vanillin (315μg/culture was observed in ferulic acid (2.5

mM) treated cultures on the 15th day and maximum capsaicin (190µg/culture) was in vanillylamine (11.0 mm) treated cultures on the 6th day. It was shown that immobilized cell cultures of *C. frutescens* can perform oxidative deamination, a reversible reaction which was evident by conversion of vanillylamine to vanillin. This study also provides an example for an alternative route to formation of vanillin by cell cultures.

Bio-reactor

Capsaicin production in 2 liter airlift column reactor has been successfully used at CFTRI. Immobilized placental cells were used and run for 2 weeks with standard medium with elicitor and precursor as discussed earlier and 1148 mg/100g fresh placenta /15 days or 7.6% of capsaicin on dry weight basis could be produced. However, several improvements to enhance the productivity, stability and viability of cell in cultures and elimination of microbial contamination have to be made. Since major part of capsaicin comes in to the medium, recovery of the same by cycling of medium from the culture medium also needs to be optimised.

Conclusion

Though the feasibility of production of spice principles of saffron and capsicum by plant cell cultures been clearly established, scaling up the methodology had to be reproducibly established, before it can reach commercial levels. Plant cells *in vitro* are more complex to grow in bioreactors in comparison to microbial cells. The problems are the tendency of plant cells to aggregate in suspension cultures besides being fragile and oxygen sensitivity of plant cells. The concept that the cells have to reach certain stage of differentiation before producing the metabolites needs to be reexamined. Coming years will see significant developments in the scaling up of cell cultures and India should not loose the initiative in this regard.

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Mass propagation of *Artemisia annua* plantlets in bioreactor and production of terpenoids

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Abstract

Bioreactor systems have been developed for the mass propagation and production of terpenoids in plantlet cultures of *Artemisia annua*. Callus tissues interspersed with shoot buds were obtained from seedling portions on Murashige and Skoog's medium containing 6-benzylaminopurine (BA) (4.44 μM) and α - napthalene acetic acid (NAA) (5.37 μM). Shoot buds elongated when transferred to MS medium supplemented with BA (0.44 μM) and indole-3-acetic acid (IAA) (5.71 μM). Plantlets were produced on MS medium containing NAA (5.37 μM) and Kinetin (0.46 μM) Chemical analysis of multiple shoot cultures revealed trace amount of terpenoids whereas fully grown plantlets with roots produced camphor (0.81 mg % FW), 1,8-cineole (0.07 mg % FW) and β-caryophyllene (0.025 mg % FW). Mass propagation of multiple shoots and plantlets was achieved in shake flasks and bioreactor under controlled defined parameters. However, multiple shoots did not produce any terpenoids in shake flask and bioreactor. The plantlet cultures propagated from bioreactor synthesised 1.08 mg % FW camphor closely followed by 0.14 mg % FW 1,8-cineole and 0.65 mg % FW β-caryophyllene. Around 69 well developed plantlets were harvested from bioreactor and the final biomass of the plantlets was 113 g FW. Addition of gibberellic acid and ethephon to the culture medium in the bioreactor stimulated the synthesis of terpenoids. The cultures produced 3.41 mg % FW camphor after 15 days of cultivation in the bioreactor with ethephone addition.

Key words: *Artemisia annua*, bioreactor, camphor; β-Caryophyllene, 1,8-Cineole, plantlet cultures.

Introduction

Artemisia annua L. is known for the drug Qinghaosu (Artemisinin), a potent antimalarial, effective against both

chloroquinine resistant and chloroquinine sensitive strains of *Plasmodium falciparum* and useful in the treatment of cerebral malaria (Klayman 1985; Luo & Shen 1987). In addition to artemisinin, *A.annua* contains terpenoids up to 3% in the leaves, which includes sesquiterpenes and monoterpenes, which are used in fragrance, perfumery and cosmetic products (Lawrence 1982). The terpenoids of *Artemisia* have antipyretic, anti-inflammatory, anti-conceptive and anti-bacterial activities and is used in the treatment of certain skin moulds (Li *et al.* 1993). In the present paper we report the production of terpenoids by plantlet cultures of *A.annua* in bioreactor.

Materials and methods

Seed sterilisation and culture conditions

A.annua seeds were surface sterilized first with dettol (3 min) and 70% alcohol (2 min) followed by 0.1% $HgCl_2$ solution (2 min). The seeds were subsequently washed 6 times with sterile water and aseptically placed on moist filter paper in sterile petri dishes for germination. The 2-4 mm segments of 7 day old seedlings were cultured on Murashige and Skoog medium (1962) containing 30 gl^{-1} sucrose and supplemented with different combinations of growth regulators such as 6-benzylaminopurine (BA), Kinetin(KIN), α Naphaleneacetic acid (NAA), Indol-3-acetic acid (IAA). The pH of the medium was adjusted to 5.8 and sterilized at 120°C for 20 min. The cultures were incubated under white fluorescent light 50 mol $m^{-2}s^{-1}$ with 16 h photoperiod at 24±1°C.

Initiation of callus and plantlet cultures

Callus cultures interspersed with shoot buds were initiated from the germinated seedlings on MS medium supplemented with BA (4.44μM) and NAA (5.37 μM). The cultures were maintained on a fresh medium of the same composition by subculturing every 15 days. For development of plantlets the callus interspersed with shoot buds (base culture) was transferred to the MS medium supplemented with BA (0.44 μM), IAA (5.71 μM), NAA (5.37 μM) and KIN (0.46 μM) in various combinations.

Submerged culture

Shoot cultures were established in 250 ml Erlenmeyer flask containing 70 ml MS medium supplemented with BA (0.44 μM) and IAA (5.71 μM). The plantlet cultures were initiated in MS medium with NAA (5.37 μM) and KIN (0.46 μM) in 250 ml flasks and kept on rotary shaker at 90 rpm. All cultures were subcultured once in every 15 days.

Growth measurement

For fresh weight (FW) determination, the shoots were gently pressed on filter papers to remove excess water and weighed. The shoots were dried subsequently in an oven at 60°C for 16 h and the dry weight (DW) was recorded. Sucrose concentration was determined according to method employed by Fulzele & Heble (1994).

Bioreactor

One litre bioreactor having 450 ml working volume was used for the mass propagation of shoots and plantlet cultures. Sterile air was supplied through air sparger (size 60 mm). The orifice of the circular air sparger was spaced at a distance of 0.5 cm and the sparger was located at 0.85 cm height from the bottom of the bioreactor (Fiδ.1). Aeration

was achieved at the rate of 0.09 vvm. Air inlet and exhaust were connected with PALL disposable filters (PALL, Pall Europe Ltd., England). The bioreactor was inoculated with 15 day old multiple shoot cultures and were allowed to grow for 30 days.

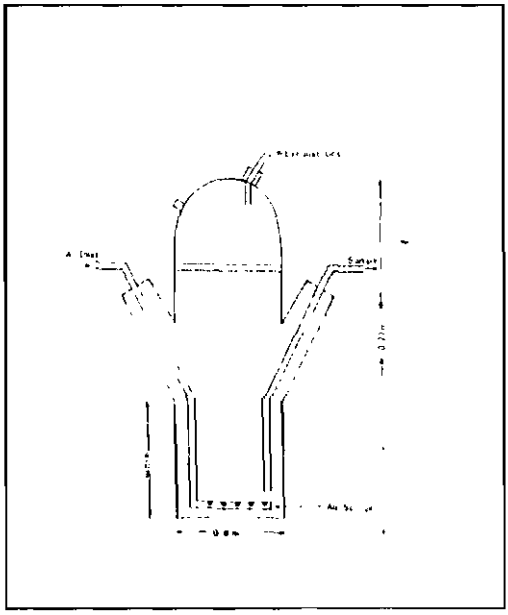

Fig.1 Schematic diagram of the 1 l capacity bioreactor for the growth and production of terpenoids by *Artemisia* plantlet cultures.

Control unit and air sterilization process

Outlet of compressor was fitted with metal pipe for carrying air from compressor to the bioreactor system. Air was sterilized through different processes before passing into the bioreactor. A moister absorber (Fulflo filter) was fitted between compressor and air regulator. The Fulflo filter was used to reduce to moisture contained in the air. The pressure of air absorber was maintained through non-return valve. The outlet of the Fulflo moisture absorber was connected to the air flow regulator (AR100P SMC, Germany) by silicon rubber tube. Air pressure was regulated between 0.5 to 1.0 bar. A control unit (B.Braun, Germany) was used to regulate the valve of the air regulator and digital display of pH, dissolved oxygen (PO^2) and temperature. Air flow meter (KROHNE, Germany, range 0.800 Nl/hr) was connected with air pressure regulator by silicon rubber tube and the valve position was maintained by control unit. Air flow rate was maintained between 0.06 to 0.08 vvm. For bioreactor system air sterilization process starts after air flow meter. Sterile air inlet and exhaust were connected with PALL disposable membrane filter (PALL, Pall Europe Ltd., England) to maintained rigid sterility of air (Fig.2)

Extraction and gas chromatography

Cultured tissues were ground in a mortar with sand and soxhlet with petro-

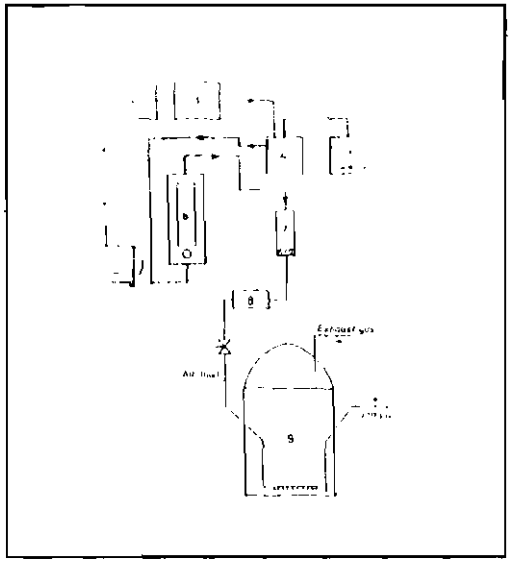

Fig.2 Flow diagram of air sterilization system cobined with control unit and 1 l bio reactor. (1) Compressor, (2) Moisture absorber, (3) Air pressure guage, (4) Air flow controller, (5) Control unit, 6. Air flow meter, (7). Filter with glass wool, (8) Disposable filter, and (9) Bioreactor.

leum ether (30-60°C). The petroleum ether was evaporated to dryness and aliquots were used for the gas chromatography (Fulzele, Sipahimalani & Heble 1991).

Results and discussion

Terpenoid synthesis in plantlet cultures grown on agar media

Seedling segments cultured on MS medium contained sucrose 30 g l^{-1} and supplemented with BA (4.44 µM) and NAA (5.37 µM) produced friable callus mass interspersed with shoot buds. In order to obtain multiple shoots callus interspersed with shoot buds were cultured on MS basal medium with BA (0.44 µM) and IAA (5.71 µM) (Fig. 3). Chemical analysis of multiple shoots revealed the presence of trace amounts of terpenoids. When the shoots, were isolated and grown on MS medium with NAA (5.37 µM) and KIN (0.46 µM) root formation occurred and complete plantlets were developed. Analysis of 21 day-old plantler cultures yielded 0.81 mg % FW of camphor and 0.042 mg % FW of 1,8 cineole.

Multiple shoot cultures and terpenoid synthesis in shake-flasks

Two-week-old shoot cultures from static culture media were transferred to the 250 ml Erlenmeyer flask containing 75 ml MS medium supplemented with BA (0.44 µM) and IAA (5.71 µM). The initial inoculum concentration was 3.5 g/ flask and kept on shaker at 90 rpm. After two weeks, dark green profuse multiple shoots became visible (Fig.3). The shoot cultures showed uniform growth and over a period of 18 days five fold growth was achieved (Fig.4). At the end of 18 days sucrose was completely consumed. Chemical analysis of the harvested multiple shoot extracts showed trace amounts of terpenoids.

Plantlet cultures and terpenoid synthesis in shake-flasks.

Two-week-old shoot cultures grown on BA (0.44 µM) and IAA (5.77 µM) were

Fig.3. Multiple shoots of *A. annua* growing in shake flask

aseptically transferred to 250 ml Erlenmeyer flask containing 75 ml MS medium supplemented with NAA (5.37 µM) and KIN (0.46 µM). The inoculum concentration was maintained at 45 gl^{1}. Root formation occurred in the same medium in 12 days. The plantlets grew actively and around 6-fold growth was achieved in 28 days. At the end of cultivation period sucrose in the medium was completely consumed and the pH was around 5.71 (Fig.5). The cultures grown with 15% inoculum yielded a biomass of 14.8 g l^{-1} DW. Chemical analysis of extracts showed significant production of camphor (0.81 mg % FW) closely followed by 1,8 cineole (0.07 mg % FW) and B-caryophyllene (0.025 mg % FW). To establish plants in soil, well developed plantlets were harvested

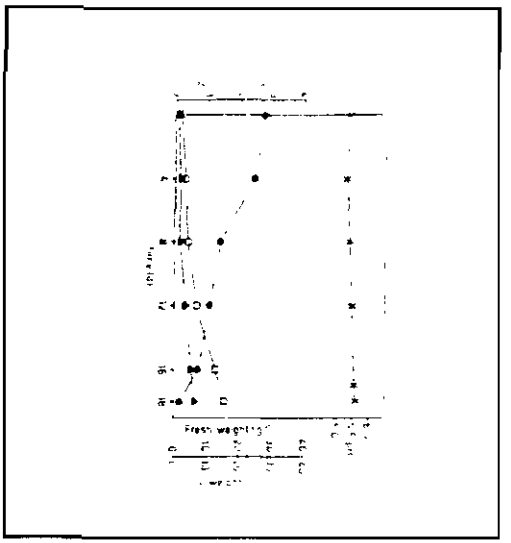

Fig.4. Kinetics of growth and sucrose concentration in shake-flask cultures of A. annua multiple shoots ot 90 rpm. [], Fresh wt; , Dry weight, X, pH and , sucrose concentration.

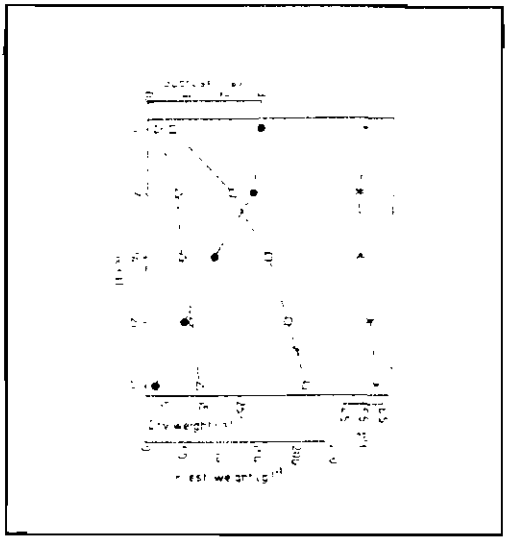

Fig. 5 Kinetics of growth and sucrose concentration in shake-flask cultures of A. annua planlet cultures shoots at 90 rpm. □, Fresh weight;Δ, Dry weight; X, pH and ●, sucrose concentration. 7. Mass propagation and production of terpenoids from A. annua planlet cultures in bioreactor.

from shake flask and transferred to the paper cups containing sterilized soil and maintained in green house under controlled conditions.

Mass propagation of plantlets and terpenoids synthesis in bioreactor

A. annua shoots were cultivated in one litre bioreactor under submerged condition containing 250 ml MS medium supplemented with NAA (5.37 μM) and KIN (0.46 μM). Fifteen days old shoot cultures (16 g of FW) were used as inoculum giving an initial biomass to liquid ratio of 0.064 g ml^{-1} FW. Root formation occurred in the same medium in two weeks. The incubation was stopped 30 days after the inoculation. At the end of 30 days sucrose was completely consumed by the tissue. The final biomass of plantlets was 74.8 g FW, i.e. 4-5-fold increase in biomass. The cultures produced significantly high amounts of camphor (1.08 mg % FW) followed by 1,8 cineole (0.14 mg % FW) and β-caryophyllene (0.65 mg % FW)

Effect of gibberellic acid on growth and terpenoid synthesis

To investigate the effect of gibberellic acid 15 days old shoot cultures were inoculated in the bioreactor containing MS basal medium supplemented with NAA (5.37 μM) and KIN (0.46 μM) and gibberellic acid (5.77 μM). The inoculum giving initial biomass to liquid ratio was 0.068 g ml^{-1} FW (Table 1). The shoots were dark green and root formation occurred in 9 days. Bioreactor operation was terminated on day 15 and the harvested fresh biomass of 113 g FW. Analysis of the plantlets showed the camphor concentration in the cultures which is a major constituent increased from 1.08 to 2.42 mg % FW.

Effect of ethephon on growth and terpenoid synthesis

In this experiment ethephon (10 mg l^{-1})

was incorporated into the MS medium containing NAA (5.37 μM) and KIN (0.46 μM). The initial biomass of shoots to liquid volume ratio was maintained at 0.061 g ml^{-1} FW. The shoots developed roots in 8 days. The plantlets grew uniformly for 15 days. The incubation was terminated on day 18 since the cultures started browning at this stage. Final fresh biomass of the cultures was 80 g FW. Maximum production of terpenoids was achieved in this experiment. A major constituent of terpenoids produced was camphor 3.42 mg % FW followed by 0.41 mg % FW 1,8-cineole, and 0.65 mg % FW β-caryophyllene in 18 days cultivation in bioreactor. The production of terpenoids was significantly higher as compared with the cultures grown in normal medium and with gibberellic acid.

Synthesis of terpenoids in *in vitro* cultures has been studied extensively by several research groups. Structural identification and quantification of the terpenoids of *A.annua* has been reported (Duke, Palul & Lee 1988; Woerdenbag *et al.* 1993; Ahmad & Misra 1994). Undifferentiated callus cultures of *A.annua* could not synthesize the lower terpenoids (Brown 1994). Lappin, Stride & Thampion (1987) reported that no terpenoids could be detected from callus cultures of *Lavindula angustifolia*. Our studies have also shown the similar results.

Jain *et al.* (1991) reported that 40 day-old *in vitro* shoots of *Rosmarinus officinalis* produced appreciable quantity of terpenoids involving major constituents, camphor and 1,8-cineole. We could not detect any lower terpenoids in *in vitro* shoots of *A.annua*. However, plantlet cultures of *A.annua* from static and submerged conditions produced significant levels of terpenoids. The static plantlet cultures yielded 0.81 mg % FW camphor and 0.042 mg % FW of 1,8-cineole in 21 day old culture.

Recent developments in the high-tech area of design and development of bioreactor systems have resulted in the successful cultivation of shoots in the large bioreactor of 500 l capacity (Akita *et al.* 1994). A number of possible scale-up techniques for shoot and plantlet cultures have been described (Takayama & Misawa 1981; Hagimori, Matsumo & Mikami 1984; Levin *et al.* 1988). Park, Hu & Staba (1989) used a bioreactor for the growth of *A.annua* plantlet cultures.

Our results have demonstrated that *A.annua* can be propagated in bioreactor under submerged conditions with controlled aeration. Using a bioreactor we have observed that plantlet cultures increase the biomass upto 5 fold (296 g l^{-1}) in 30 days as compared with the biomass increase in the shake-flask (284 gl^{-1}). Park, Hu & Staba (1989) reported that in *A.annua* cultures roots formation occurred shortly after glucose depletion. Our results also demonstrated that roots were induced after 3 weeks in the same reactor vessel when sucrose was completely consumed by the tissues. The plantlets produced in the reactor and shake-flasks have been analysed and shown the synthesis of lower terpenoids. However, the production of camphor was higher (1.08 mg % FW) in the bioreactor than that of the shake-flask culture (0.81 mg % FW). Therefore, the cultivation of shoots and plantlets under controlled aeration did not affect the growth but increased the yield of

lower terpenoids.

Gibberellic acid is known to influence the formation of terpenoids and other secondary metabolites (Evans 1989). In cell culture of *Taxus brevifolia* and *Taxus cuspidata*, Saito, Dhashi & Hibi (1992) obtained 10 fold increase in taxol production in gibberellic acid (GA) supplemented medium than that of the dried bark of the plant. In contrast, Fett-Neto *et al.* (1993) reported that the use of GA enhanced the callus growth without affecting taxol yield. Our studies showed that the treatment of GA with plantlet cultures stimulated a 6 fold growth as compared with the shake-flask cultures. The synthesis of terpenoids were also enhanced by addition of GA acid into the bioreactor.

Kim, Pederson & Chin (1991) reported that in *Thalictrum rugosum* addition of ethephon into the 2l air-lift bioreactor the berberin content was increased 2 fold. Cho *et al.* (1988) observed that supplement of ethephon improved the production of berberin (30%) and caffein (85%). In contrast, Buitelaar, Cesario & Tramper (1992) have shown that by the addition of ethephon to the root cultures of *Tagetes patula*, the production of thiophenes dropped dramatically. Our studies have shown that the incorporation of ethephon in the culture media significantly increased the terpenoid synthesis compared with the culture without ethephon. It was also observed that the growth rate of tissue was reduced when the cells were treated with ethephon. The present studies have demonstrated that mass propagation of shoots and plantlet cultures under controlled conditions in the bioreactors is a viable technique for growth and product biosynthesis.

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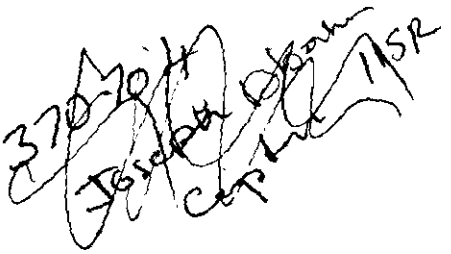

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