IN VITRO TECHNOLOGY FOR GENETIC CONSERVATION OF SOME GENERA OF ZINGIBERACEAE

Thesis submitted to the **University of Calicut**

for the award of degree of **Doctor of Philosophy** in Botany

By

Geetha S Pillai

UNIVERSITY OF CALICUT

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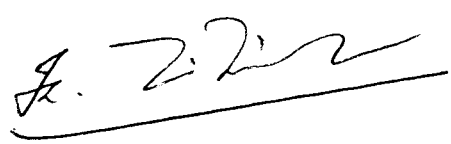
2002

CERTIFICATE

This is to certify that the thesis entitled '*In vitro technology for genetic conservation of some genera of Zingiberaceae.*' submitted by Mrs. Geetha S Pillai, for the award of the degree of Doctor of Philosophy in Botany, University of Calicut, contains the results of bonafide research work done by her during 1995-2002 at University of Calicut and at Indian Institute of Spices Research, Calicut, under my supervision and guidance. No part of this thesis has been submitted to any other university for the award of any other degree or diploma. All sources of help received by her during the course of this investigation have been duly acknowledged.



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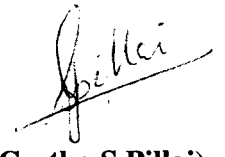
DECLARATION

I hereby declare that this thesis entitled '*In vitro technology for genetic conservation of some genera of Zingiberaceae*' submitted by me for the award of the degree of Doctor of Philosophy in Botany, University of Calicut, contains the results of bonafide research work done by me at University of Calicut, Calicut, and at the Department of Biotechnology, Indian Institute of Spices Research, Calicut under the supervision and guidance of Dr. K. Unnikrishnan. This thesis or part of it has not been submitted to any other university for the award of any other degree or diploma. All sources of help received by me during the course of this study have been duly acknowledged.



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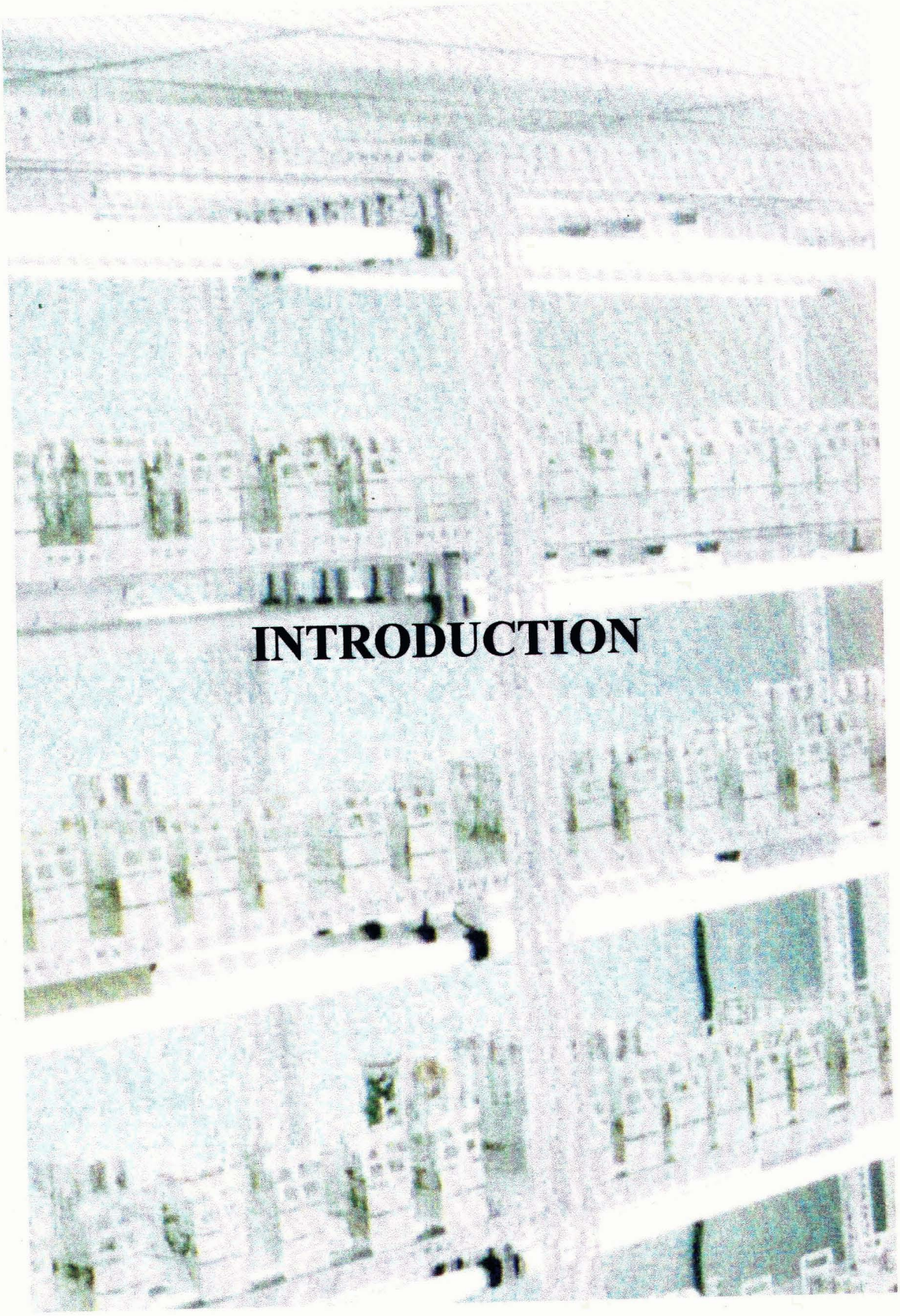
Geetha S Pillai

CONTENTS



INTRODUCTION

Biological resources are not only essential components of our life support system but also provide raw materials for meeting human needs. India is one of the 12 identified centres of origin of cultivated plants and has 2 of the 18 identified mega diversity 'hotspots'. Thus India is represented by the rich germplasm of plants of agri-horticultural, medicinal and industrial values.

The rapid degradation of major ecosystems and their biological components has become a major international concern. This biological diversity is threatened due to fragmentation of habitats, deforestation, over exploitation of genetic resources, rapid changes in hydrological regimes and land use patterns, soil degradation, air and water pollution, adverse impact of developmental projects and ever increasing population.

Conservation and sustainable use of biological resources is of great importance to meet the demand of both present and future generations. Developing and establishing adequate conservation measures and mechanisms for sustainable utilization of biological resources pose a multidimensional challenge involving scientific, socio-economic, administrative, legal and political issues (Chauhan, 1996; 1998). So is the case with plant germplasm conservation, which forms the foundation on which sustainable and increased food production is based.

Zingiberaceae

Zingiberaceae is one of the angiosperm families with many economically important species. It consists of about 50 genera and 1400 species distributed mainly in tropics and subtropics with the centre of diversity in Indo-Malayan region, but extending through tropical Africa to Central and South America. It is a monocotyledonous family consisting of perennial rhizomatous herbs characterized by

the possession of a tuberous, often creeping rhizome, which are used in food and medicine. The members of the family have considerable economic potential, with economically important genera such as cardamom (*Elettaria cardamomum* Maton), ginger (*Zingiber officinale* Rosc.), turmeric (*Curcuma longa* L.), kasturi turmeric (*C. aromatica* Salisb.), mango ginger (*C. amada* Roxb.), large cardamom (*Amomum subulatum* Roxb.), *Aframomum* spp. and *Kaempferia* spp. The other important genera are *Costus*, *Alpinia, Hedychium,* etc. Many of these species are used in ayurvedic and other native systems of medicine from time immemorial, and some are important spices. Among the spices cardamom, ginger and turmeric are the most important.

Cardamom, popularly known as "Queen of Spices" is the dried fruits of *Elettaria cardamomum.* The plant is endogenous to the evergreen rain forests of Western Ghats of South India. Cardamom is used directly as a flavouring material and is also processed to extract essential oil and oleoresin. The major use of cardamom is for culinary purpose for flavouring food. It is also used in medicine as an aromatic stimulant, carminative and flavouring agent.

Cardamom and their wild relatives/allied genera occur in natural preserves. The representative collections of cardamom germplasm are conserved in field gene banks maintained in different agro-climatic conditions (Madhusoodanan *et al.*, 1994).

Serious diseases of viral and bacterial origin, such as katte, Nilgiri necrosis and Azhukal disease threaten crop cultivation as well as field germplasm repositories (Venugopal, 2001). Cardamom is also infested by various insects and nematode pests, among which thrips (*Sciothrips cardamomi* Ramk.) shoot and capsule borer (*Conogethes punctiferalis* Guen.), root grub (*Basilepta fulvicorne* Jacoby) and root knot nematode (*Meloidogyne incognita* Kofoed *et* White) is important (Premkumar

and Madhusoodanan, 1995, Ramana and Eapen, 1992). Hence development of alternate strategies for conservation is important and *in vitro* methods may be ideal.

Ginger is the underground rhizome of *Zingiber officinale*, a herbaceous perennial, cultivated as an annual. Ginger has been prized since ancient times, for its flavour and medicinal properties. The rhizome has a volatile oil containing aromatic compounds such as camphene, phellandrene, zingiberene and zingerone. The pungency of ginger is attributed to zingerone, shogaol and gingerol. The primary flavouring constituents of the oil include cineole, borneol, geraniol, linalool and farmascene (Farrell, 1985). Ginger is one of the world's most popular medicinal spices, used in folk medicine and for culinary purpose (Dobelis, 1990). In early Indian systems of medicine, ginger was recommended for liver complaints, flatulence, anemia, rheumatism, piles and jaundice (Westland, 1987). Ginger is also used as an aphrodisiac. Ginger is widely used for culinary purposes as fresh ginger, dried ginger, preserved ginger, ground ginger, pickled ginger, crystalline ginger and gingerbread. It has numerous applications in sweet and savory cooking, as an essential ingredient in curry powder, spice blends, biscuits, cakes, puddings, soups, candies, sauces and soft drinks. The essential oil from ginger is also used in soaps and perfumery.

Ginger is propagated only vegetatively and is widely cultivated in tropical and subtropical regions of Asia, West Africa, West Indies and Australia. Its origin is presumed to be in the South-East Asia, probably India or China (Bailey, 1949; Purseglove, 1975). Wild forms of *Z. officinale* have not been found in nature though the maximum variability of ginger cultivars exists in North East India (Nayar and Ravindran, 1995). The germplasm collections include both exotic and indigenous types. All these germplasm are conserved in *ex situ* field gene banks (Mohanthy and Panda, 1994).

Various diseases especially of fungal and bacterial origin affect Ginger. Major diseases such as soft rot caused by *Pythium aphanidermatum* (Eds) Fitz, yellows caused by *Fusarium oxysporum* Schlecht, bacterial wilt caused by *Pseudomonas solanacearum* Smith (Yabuuchi) and leaf spot caused by *Phyllosticta zingiberi* Ramakr. result in considerable crop losses (Dake, 1995). Shoot borer (*C. punctiferalis*) and root knot nematode (*M. incognita*) are the major insect and nematode pests (Koya *et al.*, 1991; Mohanty *et al.*, 1995; Ramana and Eapen, 1995). These diseases and pests seriously threaten conservation of genetic resources in field gene banks.

Turmeric is obtained from the rhizomes of *Curcuma longa* L. (Syn. *C. domestica* Val.) and is an important spice in India, South East Asia and Indonesia. Turmeric is indispensable in the preparation of curry powders. In addition to its use as a spice, it has medicinal properties and also used as a dye. Turmeric is used to some extent as a stomachic, tonic and blood purifier in the Indian system of medicine. It is also prescribed as an antiperiodic alternative. Turmeric powder is a main ingredient in many of the cosmetics.

The centre of origin of this crop is believed to be South East Asia and some species are naturalized in northeastern regions of India and Java. India is the largest producer and exporter of the crop. Genetic resources of turmeric include popular cultivars/types, high yielding selections and promising pre-release cultures, local cultivars/types and semi wild and related species.

Leaf spot caused by *Taphrina maculans* Butl. and rhizome rot by *Pythium graminicolum* Subram. are serious diseases of turmeric in India. The shoot borer, *C. punctiferalis,* is the most serious pest of turmeric (Koya *et al.,* 1991; Sarma *et al.* 1994).

Kaempferia is the genus of rhizomatous herbs distributed in the tropics and subtropics of Asia and Africa. About 10 species occur in India, but those of economic value are mainly *K. galanga* and *K. rotunda. K. galanga* is a herb found throughout the plains of India and is cultivated for its aromatic rhizomes and also for its ornamental value. The herb is used as a flavouring agent for rice preparations. The rhizomes are considered stimulatory, expectorant, carminative and diuretic. The rhizomes and roots are used for flavouring food and also in medicine in South East Asia where they are widely cultivated. *K. rotunda* is cultivated in gardens for the beauty and fragrance of its flowers. The rhizomes and young leaves are used as a flavouring agent. The juice of rhizomes is used as eye drops for removing cataract of the eye and also as a cure for night blindness. The tubers are widely used as a local application for tumours, swellings and wounds. They are also considered stomachic and given in gastric complaints (Bhattacharjee, 2000).

Genetic resources and their conservation

Plant genetic resources are of immense value to mankind as they provide food, fodder, fuel, shelter and industrial products and above all they provide a reservoir of genetic variation to plant breeders for crop improvement. However, in the wake of spread of high yielding varieties, there is a gradual erosion of this genetic variability resulting in large-scale depletion of variability (Frankel, 1975). In addition, deforestation and disturbances in the ecological niches of these plant species also cause depletion of wild relatives and species at an alarming rate. Hence, it is necessary to adopt appropriate conservation strategies (whether seeds, pollen, root, tubers, bulbs, other vegetative material or cell, meristem and other tissue culture systems) to conserve the genetic resources of plants.

In vegetatively propagated crops, the clonal material carries variable gene combinations, and hence is conserved in field clonal repositories. But conservation in field gene banks tends to be expensive due to high labour costs, exposure to environmental hazards and requirement of large amount of space. The more serious problem faced by field gene banks is the vulnerability of such clones to pests and pathogens leading to sudden loss of valuable germplasm. Hence alternate strategies are required for conservation. This led to the consideration of *in vitro* techniques for germplasm conservation (Withers and Alderson, 1986). With *in vitro* techniques, it is now possible to provide a germplasm storage procedure, which uniquely combines the possibilities of disease elimination and rapid clonal propagation (Henshaw and Grout, 1977).

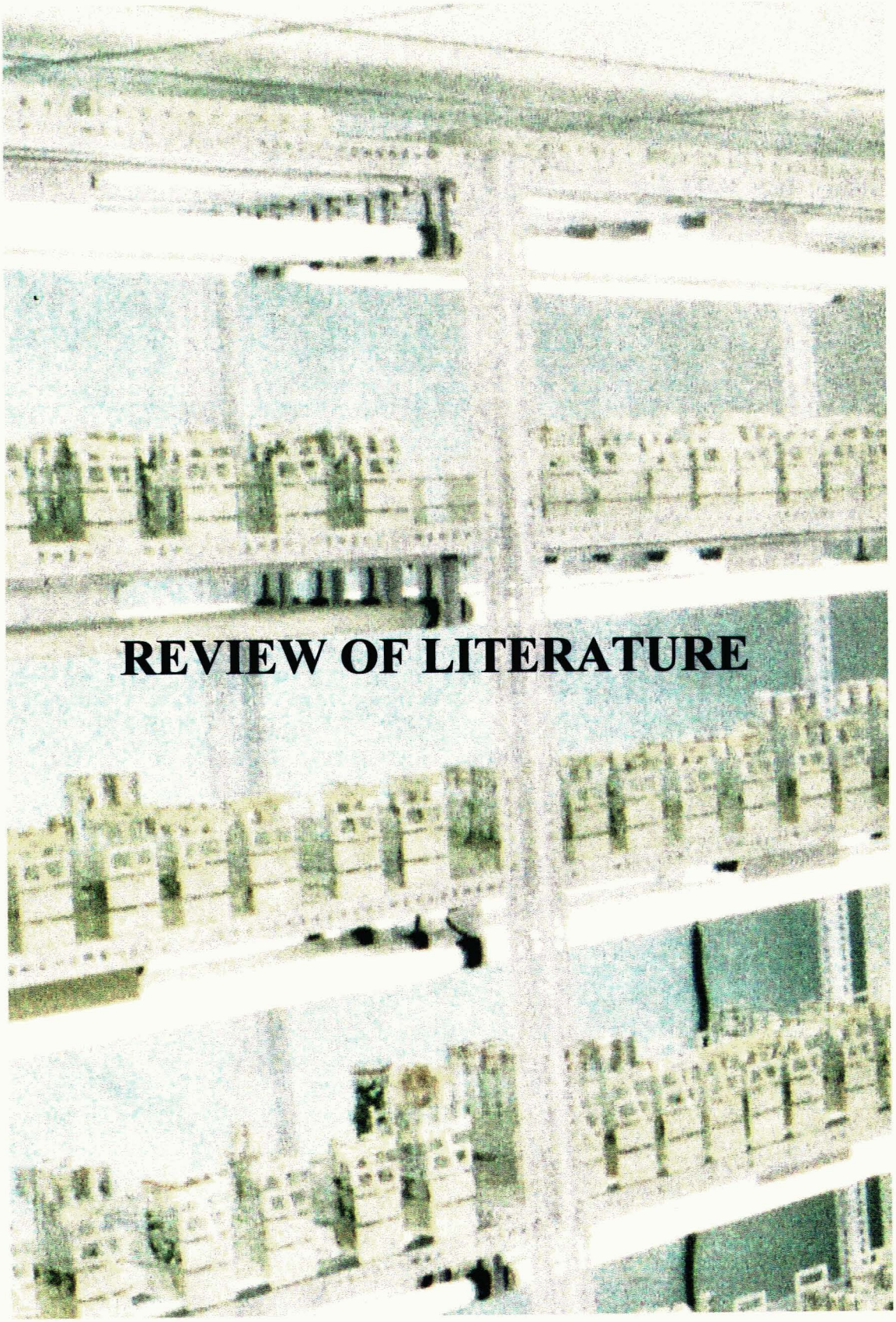
Two basic approaches are followed to maintain germplasm collections *in vitro*, namely minimal growth and cryopreservation (Withers, 1980). The advantage of slow growth approach for short to medium term storage is that the cultures can be readily brought back to normal culture conditions to produce plants based on the requirement. Cryopreservation at the temperature of liquid nitrogen (LN_2) (-196^0C) offers the possibility for long-term storage of germplasm. *In vitro* conservation by slow growth is achieved in a wide variety of crops such as *Vitis* spp., *Solanum* spp., *Colocasia* spp., potato, cassava, banana, strawberry, apple, sugarcane, etc. Successful cryopreservation was also achieved in a variety of crop species like *Dianthus caryophyllus*, tomato, potato, cassava, etc.

Zingiberaceous species such as cardamom, ginger, turmeric, *K. galanga*, *K. rotunda*, etc., are propagated vegetatively. The germplasm of all these economically important crops are conserved in clonal field repositories. Most of these crops are threatened by serious soil-born diseases, which can wipe out the germplasm

completely. There is also high risk of mixing up of germplasm due to planting in the same area year after year through the left over rhizome pieces especially in ginger, turmeric and *Kaempferia* spp., which are harvested and replanted annually. Hence, it is important to formulate *in vitro* conservation strategies as a complement to field gene banks for conservation of these species.

The present study aims at developing *in vitro* conservation strategies for short to medium term as well as long-term conservation through slow growth and cryopreservation, respectively, in important zingiberaceous crops such as cardamom, ginger, turmeric, *K. galanga* and *K. rotunda*. As micropropagation forms the backbone of any *in vitro* conservation strategy, the present study also includes standardization of protocols for micropropagation of these crop species. Use of synthetic seeds and microrhizomes (in ginger and turmeric) as a tool for germplasm conservation and exchange was also explored. In addition, this study also attempts to include morphological, biochemical and molecular tools for characterization of *in vitro* conserved plants to estimate their genetic fidelity.

Thus, development of efficient *in vitro* conservation strategies helps in collection and conservation of samples of crops that are normally vegetatively propagated or those that produce recalcitrant seeds. This method offers a safe alternative or additive to field gene bank and can also be used for efficient pest and pathogen free germplasm exchange. This will form an important component in the overall strategy of genetic resources management and utilization in these crop species.



REVIEW OF LITERATURE

GENETIC DIVERSITY OF ZINGIBERACEAE

References have been made to cardamom, ginger and turmeric in ayurveda, the world-renowned medicinal system of India, which dates back to 1500 BC. However the earliest scientifically described and published record of Zingiberaceous taxa in India is in *Hortus Indicus Malabaricus* by Hendric Andrian van Rheede (1678–1693). In his *Flora Indica*, Roxburgh (1820–1824) described 65 species of Zingiberaceae under the class Monandria Monogynia distributed over 8 genera. Wallich (1830–1832) described some Zingibers in his *Plantae Asiaticae Rariores*. Wight (1838–1853) explored the South Indian flora and included 7 genera and 14 species of Zingiberaceae in his *Icones Plantarum Indiae Orientalis*. Graham (1839) enumerated 28 species of Zingiberaceae in 7 genera in his *Catalogue of Plants Growing in Bombay* and Dalzell and Gibson (1861) listed 25 species in 7 genera in *Bombay Flora*. Later, Cooke (1907) enumerated 9 genera and 27 species from the same region in his *Flora of Presidency of Bombay*. Hooker's (1890–1892) *Flora of British India* described 19 genera and 224 species of Zingiberaceae. Rama Rao (1914) in his *Flowering Plants of Travancore* gave a systematic account of Zingiberaceae of a greater portion of Kerala. The *Flora of the Presidency of Madras* by Gamble (1916–1935) is an authentic record on this family. Fischer (1928) described 34 species under 9 genera, namely, *Alpinia, Amomum, Costus, Curcuma, Elettaria, Globba, Hedychium, Kaempferia* and *Zingiber* in the *Flora of the Presidency of Madras*. Sabu (1991) conducted a detailed study on the taxonomy and phylogeny of South Indian Zingiberaceae. A few commercially important plant species in

Zingiberaceae are cardamom (*Elettaria cardamomum* Maton), ginger (*Zingiber officinale* Rosc.), turmeric (*Curcuma longa* L.), kasturi turmeric (*C. aromatica* Salisb.), mango ginger (*C. amada* Roxb.), large cardamom (*Amomum subulatum* Roxb.), *Aframomum* spp., *Kaempferia* spp., etc. Many are used in ayurvedic and other native systems of medicine from time immemorial, and some are important spices.

CARDAMOM (*ELETTARIA CARDAMOMUM* MATON)

The cardamom, the spice of commerce, are the dried fruits of a perennial herb *Elettaria cardamomom* Maton. The plant is endogenous to South India and Sri Lanka. The plant has been described in great detail in a monograph by Ravindran and Madhusoodanan (2001). Rosengarten (1969) states that cardamom was an article of Greek trade during the fourth century BC. Ridley (1912) and Burkill (1966) doubted whether the Greeks and Romans had the true cardamom (Purseglove *et al.*, 1981). It is said that cardamom grew in the gardens of King Babylon in 720 B. C. (Morris and Mackley, 1997). Holttum (1950) describes one species from Malaysia and says that the genus occurs in Ceylon and South India, Malaysia, Sumatra and Borneo. According to Willis (1966) *Elettaria* has seven species in Indo Malaysia. Some controversy exists regarding the systematics, local races, types and grades of *E. cadamomum*. Two botanical varieties, namely var. *major* Thwaites and var *cardamomum* (syn. var *minor* Watt; var. *miniscula* Burkill), have been recognized based on the size of the fruit (Purseglove *et al.*, 1981). Based on the nature of panicles, three varieties of cardamom namely, Malabar, Mysore and Vazhukka, are recognized (Sastri, 1952).

The ancient Indian ayurvedic texts, *Charaka Samhita* and *Susrutha Samhitha*, (1400–600 BC) mention cardamom on many occasions; though the identity of '*ela*' in those texts is not sure (Ravindran, 2001). Earlier in the 4 BC Indian ayurvedic medicine used the spice to remove fat and as a cure for urinary and skin complaints. The ancient Egyptians chewed cardamom to whiten their teeth and simultaneously sweeten their breath. Cardamom was used in perfumes by ancient Greeks and Romans and also recommended by the Apicius, a famous Roman empire to counteract over indulgence (Morris and Mackley, 1997).

Cardamom is a tall herbaceous perennial with branched subterranean rhizomes from which arise several erect leafy shoots and erect or decumbent panicles. There is a stout horizontal rhizome with numerous fibrous roots in the surface layer. Leafy shoots are composed of leaf sheaths and are borne in thick clumps. The shoots are 10–20 in number and 2.0–5.5 m tall. The leaves are distichous with lanceolate acuminate lamina, 25–90 cm wide, dark green and glabrous above, and paler beneath. The panicles emerge from the rootstock at the base of the leafy shoots and are 60–120 cm long. They are slender, erect and recumbent or decumbent. The flowers are hermaphrodite, zygomorphic and about 4 cm long and 1.7 cm across. The bracteole, as is the calyx, is tubular which is green; shortly three toothed and is persistent. The corolla tube is about the same length as of the calyx with three narrow strap shaped, spreading, pale green lobes about 1 cm long. The labellum is composed of three modified stamens, about 1.8 cm long with undulating edge. There are two rudimentary staminodes and one functional stamen. The stamen has a short broad filament, with a longer anther, and a connective with a short crest at the apex. The ovary is inferior, consisting of three united carpels with numerous ovules in axile

placentation and a slender style with a small capitate stigma. The fruit is a trilocular capsule, fusiform globose, pale green to yellow in colour, varying in size according to the variety. The fruit contains 15–20 seeds and are dark brown in colour, angled, aromatic, about 3 mm long and with a thin mucillagenous aril. They contain some white perisperm and a small embryo. The seed contains some white perisperm and a small embryo (Purseglove, *et al.* 1981).

Cardamom is grown at altitudes between 760 m and 1400 m in areas with an annual rainfall of 1500 to 7000 mm and a temperature range of 10–35^{0}C. Cardamom is very susceptible to wind and require good drainage and cannot tolerate water logging (Mayne, 1942; Sastri, 1952). The crop thrives best under moderate natural shade (Purseglove *et al.* 1981).

Cultivation of cardamom is mostly concentrated in the evergreen forests of Western Ghats in South India. Besides India, cardamom is cultivated in Guatemala, Tanzania, Sri Lanka, El Salvador, Vietnam, Thailand, Cambodia and Papua and New Guinea. Today its production is concentrated mainly in India and Guatemala. Cardamom cultivation in India is concentrated mainly in those regions, which form the natural habitat of the species (Purseglove *et al*, 1981), except for a small area in Uttar Kannada and adjoining southern districts in Karnataka and Wynad District in Kerala where it is grown as a subsidiary crop in arecanut gardens. It is also not uncommon as a mixed crop with coffee in suitable pockets. The important areas of cultivation are Uttar Kannada, Shimoga, Hassan and Chickmangalur districts and hills of Kodagu (Coorg) in Karnataka; northern and southern foothills of Nilgiris, Madurai, Salem and Tirunelveli, Annamalai and parts of Coimbatore districts of Tamil Nadu, Nelliampathy, Wynad and Idukki (Prasath *et al.* 2001b).

In India, cardamom is cultivated in an area of about 81,000 ha, in the states Kerala, Karnataka and Tamil Nadu with a production of about 10400 tonnes during 1999–2000. India earned a foreign exchange of Rs. 5654.7 lakhs, by exporting 1,100 tonnes of cardamom during 2000–2001 (Source: Director General of Commercial Intelligence and Statistics-DGCI & S). Currently, Guatemala produces 13,000–14,000 tonnes of cardamom annually. The world production of cardamom is about 24,953 tonnes. India has a large domestic market for cardamom, consuming about 7000 tonnes per year (George and John, 1998; Ravindran, 2001).

Cardamom is propagated mostly through seeds and also by vegetative means. For large-scale production it is usual to sow the selected seeds in specially prepared nursery beds. The seedlings are transplanted into secondary nursery beds, and finally to the field. For vegetative propagation, rhizomes from large clumps of growing plants are taken out, separated into small clumps, each consisting of at least one old and one young shoot, and planted in prepared pits (Purseglove *et al.*, 1981). The first crop, which is usually obtained in the third year after planting, is small. Higher yields are obtained in subsequent years up to the 10th or 15th year (Sastri, 1952).

Gregory (1936) described the basic chromosome number of *Elettaria* as x = 12 and the somatic chromosome number of *E. cardamomum* as 2n = 48. Darlington and Wylie (1955) also gave the same chromosome number, quoting Gregory (1936). Reports of Ramachandran (1969) and Sudharshan (1987, 1989) also confirmed the findings of the earlier workers. However, Chandrasekhar and Kumar (1986) observed variation in number as well as in the morphology of chromosomes of var. Mysore and var. Malabar and concluded that aneuploidy as well as structural alterations in

chromosomes had contributed to the varietal differentiation. Meiosis is quite normal and pollen fertility is high (Sudharshan, 1989).

Cardamom is affected by a number of diseases caused by viruses, fungi, bacteria and nemotodes. Diseases of cardamom were reviewed by Chattopadhyay (1967), Agnihothrudu (1987), Naidu and Thomas (1994), Venugopal (1995, 2001) and Thomas and Bhai (2001). Cultivation of cardamom in India and Guatemala is threatened due to the severe incidence of *katte* disease caused by Car MV that is an aphid transmitted poty virus. Resistant sources to this disease were identified by collecting 134 disease escapes from hotspots of virus infection in South India and screening them in greenhouse, sick plot and hotspots. Testing of promising collections in four hotspots and also against natural infection confirmed the resistant nature of the collections (Venugopal, 1999). A high yielding and resistant to rhizome rot line (RR-1) has been developed from Indian Institute of Spices Research, Cardamom Research Centre, Appangala (Karnataka). Three promising katte tolerant lines are in advanced stage of evaluation and release (Venugopal unpublished). Occurrence of cardamom necrosis virus (Nilgiri necrosis virus) and cardamom vein clearing disease (*kokke kandu*) in some endemic zones are also matters of concern to the cardamom industry (Venugopal, 2001). The major fungal diseases are capsule rot (*Azhukal* disease) caused by *Phytophthora* spp., rhizome rot caused by *Pythium vexans*, *Rhizoctonia solanii* and *Fusarium oxisporum* and leaf blight caused by *Colletotrichum gloeosporioides* (Thomas and Bhai, 2001). Nematode infestation in cardamom is a major problem often amounting to heavy crop losses. Root knot nematodes are widely observed in almost all cardamom plantations and nurseries (Ramana and Eapen, 1992).

Natural variability is fairly high in cardamom since it is cross-pollinated. Genetic variability in cardamom for various quantitative characters such as plant height, tiller number, number of panicles, yield etc were reported (Gopal *et al.* 1995; Prasanth, 2001a). The primary requirement of any crop improvement programme is the availability of a diverse genetic stock in that crop variety. Hence, collection conservation, evaluation and exploitation of germplasm deserves utmost importance in breeding strategies. Explorations for germplasm collection are made by different research organizations in India and a number of accessions (1350) are presently available with different centres (Madhusoodanan *et al.*, 1999).

Cardamom is amenable to both sexual and vegetative propagations. Selection, hybridization, mutation and polyploidy breeding are used as means of crop improvement. Hybridization within improved cultivars provides ample scope to combine the desirable characters of yield, *katte* resistance and drought tolerance. Research efforts being made at various research institutes have led to the development of high yielding heterotic recombinants. So far 10 high yielding varieties have been developed and released for cultivation; they are Mudigere-1, PV-1, CCS-1, ICRI-1, ICRI-2, ICRI-3, RR-1, IISR Avinash (RR1), IISR Vijetha-1 and TDK-4. Njallani Green Gold is a high yielding clonal selection developed by a farmer in Idukki District (Babu *et al.*, 2001; Madhusoodanan *et al.* 2001,). Among the released varieties, IISR Vijetha-1 and IISR Avinash are resistant to *katte* virus and rhizome rot, respectively.

Conservation of cardamom in *in situ* conditions is taken care by the natural population occurs in protected forest areas, where a sizable population of cardamom plants in their natural habitat exists. *Ex situ* conservation in field gene banks is being

undertaken by different organizations such as Cardamom Research Centre (Indian Institute of Spices Research), Appangala (Karnataka); Indian Cardamom Research Institute, Myladumpara, Idukki District, Kerala; Cardamom Research Centre (Kerala Agricultural University), Pampadumpara, Idukki (Kerala) and Regional Research Station, Mudigere (Karnataka) (Madhusoonan *et al.*, 2001) (Table 1).

Table 1. Germplasm holdings of cardamom in India

Institutions	Indigenous/ cultivated	Wild and related spp	Total
Indian Institute of Spices Research, Cardamom Research Centre, Appangala, Karnataka	314	13	327
Indian Cardamom Research Institute (Spices Board), Myladumpara, Idukki, Kerala	600	12	612
All India Coordinated Research Project on Spices (AICRPS) centre, Cardamom Research Station, Pampadumpara, Idukki, Kerala	78	15	93
AICRPS Centre, Regional Research Station (University of Agricultural Sciences, Bangalore), Mudigere, Karnataka	245	7	252

Ravindran *et al.* 2001

Ex situ conservation in cardamom is being carried out in field gene banks, and they are used for preliminary evaluation, maintenance as well as for characterization. Many variations in morphological, agronomical and chemical characters and in yield have been recorded in these collections (Zachariah and Luckose, 1992; Zachariah *et al.*, 1998). *Ex situ* conservation is always at risk due to a variety of reasons, mainly biotic and abiotic stress factors.

GINGER (*ZINGIBER OFFICINALE* ROSC.)

Ginger (*Zingiber officinale* Rosc.), was first described by Rheede (1692) in *Hortus Indicus Malabaricus* (Ravindran *et al,* 1994; Nayar and Ravindran, 1995). The name is believed to have originated from the Sanskrit word *Singabera* meaning

'shaped like a horn', and this has further evolved to the Greek word *Zingiberi* and subsequently the Latin word *Zingiber*. Ginger has a long reported history as a spice. Its origin lies in either India or China, where it was mentioned in 500 BC in the writings of the philosopher Confucius. Arab traders from the Orient introduced ginger to Greece and Rome, and it is quite likely that the invading Romans carried it to Britain. The ginger rhizome was easily transported, allowing the Arabs to introduce it to East Africa and the Portuguese to take it to West Africa in the 13th century. The Spanish expanded the trade by taking ginger to Mexico and West Indies, especially Jamaica, which still claims to produce the best quality of ginger. By the 14th century ginger was the most common spice after black pepper (Morris and Mackley, 1997).

Fischer (1928) reported seven species from South India including *Z. officinale*. Sabu (1991) described eight species from Western Ghats and adjacent areas. The *Zingiber* species that occur in India are given in Table 2.

Table 2. *Zingiber* species occur in India

Species	Species	Species
Z. officinale Rosc.	*Z. cernuum* Dalz.	*Z. marginatum* Roxb.
Z. zerumbet (L.) Sm.	*Z. capitatum* Roxb.	*Z. intermedium* Baker
Z. purpureum Rosc.	*Z. cylindricum* Moon	*Z. chrysanthum* Rosc.
Z. roseum (Roxb.) Rosc.	*Z. ligulatum* Roxb.	*Z. rubens* Roxb.
Z. wightianum Thw.	*Z. spectabilis* Griff.	*Z. squarrosum* Roxb.
Z. macrostachyum Dalz.	*Z. clarkii* King *ex* Benth.	*Z. elatum* R. Br.

Ravindran *et al.*, 2001

Ginger is a slender, 30–100 cm tall perennial herb, usually cultivated as an annual. The underground rhizome (stem) is robust, borne horizontally near the surface bearing leafy shoots (pseudostems), which are formed of sheathing leaf bases. The pseudostems are annual, bearing 8–12 distichous leaves. Ginger flowers rarely, but in India it flowers profusely (Holttum, 1950). The stipitate inflorescence arises directly

from the rhizome and is cone-like, formed of elliptic appressed bracts. Flowers are produced in the axils of the bracts and are fragile and short-lived. The calyx is thin and spathaceous. The corolla tube is 2.0–2.5 cm long with three yellow coloured lobes. The stamen has a short filament and the anther is about 9 mm long and cream coloured. The connective is prolonged into a bean shaped appendage. The stigma protrudes just below the apex of the appendage and has a circular apical aperture surrounded by stiff hairs. The inferior ovary is trilocular with several ovules in each locule. The fruit, which is rarely produced, is a thin walled, three-valved capsule with small black arillate seeds (Purseglove *et al.*, 1981).

In India ginger is cultivated in about 77,610 ha and produced 263170 tonnes in the year 1999–2000. India earned a foreign exchange of Rs. 2295.40 lakhs by exporting about 6580 tonnes of ginger during 2000–01 (Source: Director General of Commercial Intelligence and Statistics-DGCI & S). India is the largest producer and exporter of dry ginger contributing to 50% of world's production. China, Taiwan, Nigeria, Jamaica, Australia and Japan are the other major ginger producing countries.

Ginger is propagated vegetatively by rhizome pieces, weighing 20–40 gm each, with one or two good undamaged buds. The yield depends on rhizome size; use of larger seed rhizomes gives significantly higher yields than planting smaller pieces (Aiyadurai, 1966). Seed rhizome weighing 20–25 g is considered optimum for economic yield. In Kerala, about 1500–1800 kg of seed rhizomes per hectare is recommended (NRCS, 1989). Due to the incidence of major soil and seed borne diseases and pests, seed rhizomes are collected from disease and pest free plantations. Prophylactic measures like treating the seed rhizomes with fungicides and insecticides are taken before planting. At the time of planting the rhizomes are broken into pieces

with one to two buds and are planted in loosely tilled soil in raised beds or in ridges at a spacing of 20–25 cm along as well as between the rows. They start germinating in 10 to 15 days and are ready for harvest in 7 to 10 months depending upon the area of cultivation. However, the optimum time for harvesting ginger in India is at 245–260 days after planting (Aiyadurai, 1966).

Cytological studies showed the somatic chromosome number of ginger as 2n=22 (Sugiura, 1936; Raghavan and Venkatasubban, 1943; Chakravorti, 1948; Sato, 1960; Ramachandran, 1969; Ratnambal, 1979; Omanakumari and Mathew, 1985). Ginger is considered a diploid and the basic chromosome number x=11. B-chromosome (2n=22 + 2B) has also been observed in ginger (Beltram and Kam, 1984). Studies on meiosis indicated the formation of multivalents and these coupled with self-incompatibility were cited as main reasons for lack of seed set (East, 1940; Ramachandran, 1969; Pillai *et al.*, 1978; Ratnambal, 1979; Nair *et al.*, 1980). Pollen fertility is also as low as 35% (Pillai *et al.*, 1978). Pollen fertility increased to 85% in tetraploid (Ramachandran and Nair, 1992 a, b) but did not result in seed set.

Several cultivars are recognized in India, which differ in rhizome size and yield and fibre and moisture contents. The cultivars are generally are named after the area in which they are mostly cultivated. Some of the popular cultivars are Maran, Nadia, Jorhat, Burdwan, Wynad Local, Karakkal, Himachal, Kurrupumpadi, Narsapattam, China, Rio-de-Janeiro and Jamaica. The last three are exotic cultivars from China, Brazil and Jamaica, respectively. Maran, an Assam cultivar, is popular because of its high yield and high percentage recovery of dry ginger.

Variability in various morphological characters and yield attributes like, yield per plant, plant height, number of tillers per plant, rhizome size, leaf size, etc. have

been reported in ginger (Mohanty and Sharma, 1979; Sreekumar *et al.*, 1980; Mohanty *et al.*, 1981; Sasikumar *et a.l*, 1992; Mohanty, 1984; Ratnambal *et al.*, 1980; Rattan *et al.*, 1988; Mohanty *et al.*, 1990; Mohanty and Panda, 1994; Ravindran *et al.*, 1994). Variability was also reported for various quality parameters like dry recovery, oleoresin, essential oils and crude fibre (Mohanty *et al.*, 1990; Sreekumar *et al.*, 1980; Nybe *et al.*, 1980; Muralidharan and Sakunthala, 1975; Zachariah *et al.*, 1993; Ravindran *et al*, 1994). No accession was found to be tolerant to rhizome rot disease (Sarma *et al.*, 1979). Crop improvement in ginger was confined to selection of lines with high yield and quality from the germplasm. Five high yielding, high quality varieties have been released for cultivation so far. All of them are clonal selections from the germplasm. High yielding selections namely, Suprabha, Suruchi, Suravi and Varada, were also released (Edison *et al.*, 1991; Sasikumar *et al.*, 1996a). Polyploidy breeding resulted in induction of tetraploids but promising lines could not be identified from them (Ratnambal, 1979; Ramachandran, 1982; Ratnambal and Nair, 1982; Ramachandran and Nair, 1992a, 1992b). There are also reports on mutation breeding using ionizing radiations and chemical mutagens (Gonzalez *et al.*, 1969; Raju *et al.*, 1980; Giridharan, 1984; Datta and Biswas, 1985; Jayachandran, 1989).

Conservation of ginger germplasm is being done in two ways namely, as nucleus gene bank (in concrete tubs in nursery, for safeguarding the material from soil borne pathogens, maintained under 50% shade (to avoid the *Phyllosticta* leaf spot incidence) and avoid mixing up of varieties) and in *in vitro* gene bank by slow growth. The National Conservatory for Ginger Germplasm at IISR, Calicut has 530 accessions, all being maintained in cement tubs. The other important centres of ginger conservation are High Altitude Research Station, Pottangi (Orissa) and

Department of Vegetable Crops, Dr. Y.S. Parmar University of Horticulture and Forestry, Solan (Himachal Pradesh). Small collections are maintained in a few other centres also (Table 3). Though variability for yield attribute and quality parameters are reported in ginger (Ravindran *et al.*, 1994: Sasikumar *et al.*, 1992), none of the cultivars possess resistance to serious diseases such as rhizome rot and bacterial wilt.

Table 3. Germplasm holdings of ginger in India

Institutions	No. of accessions
Indian Institute of Spices Research (IISR), Calicut, Kerala	530
National Bureau of Plant Genetic Resources (NBPGR), Regional Station, Trichur, Kerala	173
AICRPS Centre, High Altitude Research Station (Orissa University of Agriculture and Technology, Pottangi, Orissa	163
AICRPS Centre, Y.S. Parmar University Horticulture and Forestry, Solan, Himachal Pradesh	196
AICRPS Centre, Rajendra Agricultural University, Dholi Campus, Bihar	19
AICRPS Centre, Narendra Dev University of Agriculture and Technology, Kumarganj, Faizabad, Uttar Pradesh	12
AICRPS Centre, Uttar Bangla Agricultural University, Pundibari, West Bengal	18

Ravindran *et al.* (2001)

TURMERIC (*CURCUMA LONGA* L.)

Turmeric is the rhizomes of *Curcuma longa* L. (syn. *C. domestica* Val.) and is an important spice in India, South East Asia and Indonesia and is indispensable in the preparation of curry powders. The name turmeric is believed to have originated from the Latin word *terra merita,* meaning merit of the earth. The turmeric he found in Southern China fascinated Marco Polo, "There is also a vegetable which has all the properties of true saffron, as well the smell and the colour, and yet it is not really saffron". Turmeric is much revered by Hindus and associated with fertility. In

Malaysia a paste of turmeric is spread on the mother's abdomen and on the umbilical cord after childbirth, not only to warn off evil spirits, but also for its medicinal value, as turmeric is known to be antiseptic (Morris and Mackley, 1997; Khanna, 1999). In addition to its use as a spice, it has medicinal properties and also used as a dye (Filho *et al.* 2000). Turmeric powder is a main ingredient in many of the cosmetics.

The genus *Curcuma* is mainly distributed in the Indo-Malayan region and about a 100 species are known. Baker (1890) described 27 species in *Flora of British India.* He subdivided the genus into three sections – *Exantha, Mesantha* and *Hitcheniopsis.* The section *Exantha* consists of 14 species including turmeric and other economically important species such as *C. angustifolia* Roxb. (Indian arrow root), *C. aromatica* Salisb. and *C. zedoaria* Rosc. From South India, 16 species of *Curcuma* were reported (Sabu, 1991), out of which 9 are endemic to India. The distribution of *Curcuma* species in India is given in Table 4.

India is the largest producer and exporter of the crop. Turmeric is cultivated in about 1,61,300 ha with a production of over 653600 tonnes. India earned a foreign exchange of Rs.9106 lakhs by exporting about 34,500 tonnes of ginger during 2000–01 (Source: Director General of Commercial Intelligence and Statistics-DGCI & S).

Turmeric was domesticated in southern or South East Asia. Turmeric requires a hot and moist climate (Purseglove *et al.* 1981) and can be cultivated in most areas of the tropics and subtropics.

Turmeric is a tall perennial herb, usually cultivated as an annual. The pseudostem is tall, robust with oblong / elliptic leaves narrowed at the base. In long duration varieties the plant height is 67–83 cm and consists of 8–10 leaves. In short duration varieties, the height is 67–69 cm and the leaf number ranges from 7–8. Spike

length ranges from 10–18 cm with a diameter of 5 cm. Flowers are pale yellow in colour equating the bract (Bailey, 1961). Flowering is rare and the species is a sterile triploid (3n = 63) and do not set viable seeds (Nair *et al.*, 1980). The rhizomes are bigger in size with a stout mother rhizome with branching primary and secondary fingers exhibiting yellow to bright orange yellow core. The rhizomes are rich in curcumin and moderate in volatile oil (Rao and Rao, 1994).

Table 4 – Distribution of *Curcuma* species in India

Species without fingers or rhizome	Region of occurence	Species with fingers or rhizome	Region of occurence
C. nilamburensis	Ke	*C. montana.*	AP
C. karnatakensis	Ka	*C. brog*	NE
C. cannanorensis	Ke	*C. soloensis*	NE
C. lutea	Ke/Ka	*C. latifolia*	NE
C. albiflora	Ka	*C. malabarica*	Ka/Ke
C. oligantha	Ke	*C. zedoaria*	Ind
C. decipiens	Ke	*C. comosa*	NE
C. vellanikkaveriensis	Ke	*C. caesia*	NE
C. ecalcarata	Ke	*C. aeruginosa* Roxb.	NE
C. aurantiana	Ke	*C. raktakanta*	Ke
C. nilgherrensis	SI	*C. haritha* Sabu	Ke
C. pseudomontana	SI & M	*C. sylvatica*	NE/Ke
C. kudagensis	Ka	*C. aromatica*	Ka/Ke
C. thalakkaveriensis	Ka	*C. amada*	Ind
C. coriaceae	Ke	*C. amarassima*	NE
C. longa L.	Ind	*C. vamana*	Ke

Ke: Kerala, Ka: Karnataka, NE: North East India Mh: Maharashtra, SI: South India, AP: Andhra Pradesh, Ind: India; Velayudhan *et a.l.,* 1999

Sugiura (1936) was the first to report chromosome number in *C. longa* (Nair *et. al.*, 1980). A chromosome number of 2n = 32 was reported by Sato (1960) and he concluded that the species seems to be an allotetraploid with a basic number of x = 8,

based on karyomorphology. According to Ramachandran (1961, 1969) the chromosome number of turmeric is 2n=63. He studied the cytology of seven cultivars of *C. longa* and also the meiosis of the species (2n = 63) and reported that the sterility of the species was probably due to its autotriploid nature. Nambiar (1979) reported that the somatic chromosome number of *C. longa* is 2n = 63. The various reports on the studies of in 8 Indian species by different authors have clearly identified 6 distinct cytological entries having chromosome counts 2n=32, 42, 62, 63, 64, and 86 (Velayudhan. *et al.* 1994).

Genetic resources of turmeric include popular cultivars/types, high yielding selections, local cultivars/types and semi wild and related species. Turmeric types are known by trade names, based on the appearance, rhizome thickness, colour intensity, aroma and hardness of the core and duration (Rao and Rao, 1994). Turmeric cultivars/types of Andhra Pradesh are classified into three groups namely, short duration 'Kasturi' types, medium duration 'Kesari' types and long duration types belonging to *C. longa*. Some of the popular cultivars are, Duggirala, Mydukur, Armoor, Sugandham, Erode, Salem, Alleppey and Mannuthy local (Muthuswamy and Ahamad Shah, 1982; Srirama Rao, 1982; Rao and Rao, 1994).

Efforts have been made to identify and select turmeric types with high yield potential, curcumin content and curing percentage. Some of the important released varieties are Krishna, Suvarna, Roma, Suguna, Sudarsana, Prabha, Prathibha and BSR-2 (Pujari *et al.*, 1986a; Edison *et al.*, 1991; Sasikumar *et al.*, 1996b; Chezhiyan and Shanmugasundaram, 2000).

Variability with respect to yield components and quality in turmeric, were reported by several workers (Mohanthy, 1979; Philip *et al.*, 1982; Philip, 1983; Philip

and Nair 1986; Pujari *et al.*, 1986b; Babu *et al.* 1993; Hazra *et al.* 2000). Muralidharan *et al.* (1980) observed that number of tillers, plant height and number of fingers had significant correlation with yield. The diameter of the mother rhizome is reported to be the main determinant of the yield in turmeric. Turmeric is always propagated vegetatively by finger or rhizomes with one or two buds. Leaf spot caused by *Taphrina maculans* Butl. is commonly present in India, wherever the crop is grown. Rhizome rot caused *by Pythium graminicolum* Subram. is another serious disease reported in India. The shoot borer, *Conogethes punctiferalis* Guen. is the most important pest of turmeric (Sarma, *et al.*, 1994).

Turmeric is grown in most of the states in India, and there is rich cultivar diversity Cultivtion of turmeric is concentrated in Andhra Pradesh, Tamil Nadu, Maharashtra, Madhya Pradesh, Uttar Pradesh and Bihar. The total turmeric production in India is around 550,000 tonnes from an area of about 1,40,000 ha. Some cultivars such as Alleppey grown in Central Kerala are regarded as high yielding and of high quality. The cultivars are mainly known after the place where the particular type is grown traditionally. Velayudhan *et al.* (1990; 1991; 1994; 1999) described 6 new species, identified 21 different morphotypes, 6 taxonomic groups and several promising lines in *Curcuma.*

Collection and conservation of genetic resources of turmeric is mainly being carried out by Indian Institute of Spices Research (IISR), Calicut and National Bureau of Plant Genetic resources (NBPGR) Regional Station, Thrissur. The National Conservatory for turmeric at IISR has currently more than 800 accessions, all maintained in large cement tubs to maintain purity (Table 5).

Table 5. Germplasm holdings of turmeric and their related species in India

Institutions	No. of accessions
Indian Institute of Spices Research, Calicut, Kerala	800
National Bureau of Plant Genetic resources (NBPGR) Regional Station, Trichur, Kerala	962
AICRPS Centre, High Altitude Research Station, Pottangi, Orissa.	187
AICRPS Centre, Regional Agricultural Research Station, Jagtial, Andhra Pradesh	188
AICRPS Centre, Dr. Y. S. Parmar University of Horticulture and Forestry, Solan, Himachal Pradesh	172
AICRPS Centre, Rajendra Agricultural University, Dholi, Bihar	51
AICRPS Centre, Horticultural Research Station, Bhavanisagar (Tamil Nadu Agricultural University), Coimbatore, Tamil Nadu	124
AICRPS Centre, Indira Gandhi Agricultural University, Raigarh Station, Chattisgarh	34
AICRPS Centre, Narendra Dev University of Agriculture and Technology, Kumarganj, Uttar Pradesh	48
AICRPS Centre, Uttar Bangla Agricultural University, Pundibari, West Bengal	65
AICRPS Centre, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu	234

Ravindran *et al.*, 2001

KAEMPFERIA SPECIES (*K. GALANGA* AND *K. ROTUNDA*)

Kaempferia is the genus of rhizomatous herbs distributed in the tropics and subtropics of Asia and Africa. This genus comprises of about 70 species among which 11 are native to India. These tuberous and fleshy rooted plants are named after Engelbert Kaempfer (1651–1716), a German physician who wrote a book on Japanese plants (Bhattacharjee, 2000). (Kaempfer's most important archievement was the first description of Japanese plants by a European scientist; for example, he first named and described the *ginkgo* tree, maidenhair tree, *Ginkgo biloba*).

Table 6. List of *Kaempferia* species (Baker, 1890)

Species	Species	Species	Species
K. anguistifolia	*K. involucrata*	*K. parvoflora*	*K. speciosa*
K. andasoni	*K. linearis*	*K. parvula*	*K. sikkimensis*
K. candida	*K. marginata*	*K. prainiana*	*K. siphonantha*
K. concinna	*K. macrochlamys*	*K. roscoeana*	*K. scaposa*
K. elegans	*K. ovalifolia*	*K. rotunda*	
K. galanga	*K. pandurata*	*K. secunda*	

In Hooker's *The Flora of British India*, Baker (1890) described 22 species in *Kaempferia* (Table 6). Among the species that occur in India, *K. galanga* and *K. rotunda* are of economic value. These plants are used for flavouring food and also in medicine in South East Asia where they are both widely cultivated.

K. galanga

K. galanga is a stem-less perennial herb, presumably native of tropical Asia and hardly found growing wild. These plants attain a height of 30 cm; leaves sessile, ovate, flowers are white to purple, and appearing with leaves and fragrant. The plant is cultivated in India, Malaya and Central and West Java. It grows well in sandy soils. This species is propagated by fragments of its rhizomes. It requires fertile soils and is planted at distances of 40–60 cm^2 (IBPGR, 1981; Bhattacharjee, 2000). In Kerala, cultivation of *K. galanga* is restricted to some localized tracts and the productivity of the crop is low ranging from 2 to 5 tones of fresh rhizomes per hectare. There is acute shortage of planting material and the absence of seed set limits the scope for generative breeding (Kurian *et al.*, 1993).

K. galanga is cultivated for its aromatic rhizomes and also as an ornament. *K. galanga* is used extensively as a spice throughout tropical Asia and has a long history of medicinal use. The rhizome is chewed and ingested. The herb is used as flavouring for rice. The rhizomes are considered stimulatory, expectorant, carminative and diuretic. They are used in the preparation of gargles and administered with honey in cough and pectoral affections. In Philippines, a decoction of the rhizomes is used for dyspepsia, headache and malaria. The juice of the plant is an ingredient in the preparation of some tonic preparations. The rhizomes and roots are used for flavouring food and also in medicine in South East Asia (CSIR, 1959; 1992). Lesser galanga rhizome contains about 2.5 to 4% essential oil, whose main components are ethyl cinnamate (25%), ethyl-*p*-methoxycinnamate (30%) and *p*-methoxycinnamic acid and a monoterpene ketone compound, 3-carene-5-one (Kiuchi *et al.,* 1987). The rhizome is also reported to display cytotoxic properties. Volatile oil (2.4–3.9%) is obtained through steam distillation of dried rhizomes. Compounds of volatile oil are pentadecane, ethyl p-methoxycinnamate, careen, camphene, borneol and p-methoxystyrene. It is used in flavouring curries, in perfumery and also for medicinal purposes (Bhattacharjee, 2000). Slices of the dried rhizome may be cooked with vegetable or meat dishes, but mostly the spice is used fresh and grated or crushed. It is essential for Javanese cooking (*Rijstafel*) and especially much used on the Indonesian island of Bali.

Cytological studies showed that the somatic chromosome number of *K. galanga* is 2n=54 (Ramachandran, 1969). Moore (1973), in his '*Index to Plant Chromosome Numbers*', also indicated the same chromosome number quoting Ramachandran (1969).

K. rotunda

K. rotunda is a attractive leaved species of the ginger family, native to tropical Asia and is cultivated in gardens for the beauty and fragrance of its flowers. Its rhizomes are short but robust and branched. The roots are yellowish white, cylindrical towards the apex becoming swollen into spindle or egg shaped. The plants attain a height of 25–65 cm bearing 3–5 petiolate leaves which are oblong lanceolate, their upper surface is green with brown flame-like markings, but wooly and dark violet in the lower surface. The inflorescences are short-stalked, sprouting from the rhizomes before the leaves appear. These inflorescences consists of 4–16 flowers, but only 1–2 flowers open at a time, their sepals are white and ornamented with some purple stripes (IBPGR, 1981).

K. rotunda has been cultivated in almost all tropical countries of the world. In East Java this plant grows wild in the teak forests and brushwoods below 750 m altitudes. This species is propagated by rhizome cuttings and is grown in shady and rather moist places (IBPGR, 1981).

The rhizomes and tubers have a bitter, pungent, camphoraceous taste. The rhizomes and young leaves are used for flavouring. The juice of rhizomes is used as eye drop for removing cateract of the eye and also as a cure for night blindness. The tubers are widely used as a local application for tumours, swellings and wounds. They are also considered stomachic and given in gastric complaints (CSIR, 1959; 1992). The rhizomes are one of the ingredients of Javanese medicines, while the tubers are used as a tranquilizer. They are usually mixed with ginger, pepper and brown sugar. The rhizomes are also used in cosmetics, while the leaves are used as body lotion. The

rhizomes and tubers yield an essential oil on distillation, which contains the camphor smelling sineol (IBPGR, 1981).

Cytological studies showed that the somatic chromosome number of *K. rotunda* is 2n=44 (Ramachandran, 1969). According to Mahanty (1970) the somatic chromosome number of *K. rotunda* is 2n=33. Moore (1973), in his '*Index to Plant Chromosome Numbers*', quoted both the above-mentioned authors and he also indicated the basic chromosome number x=16+1 quoting Bhattacharya (1968).

CONSERVATION OF PLANT GENETIC RESOURCES

Biological diversity encompasses the total variability of life forms existing on earth. Plant genetic resources, a subset of biodiversity, contain the genetic material, which is the source of the vast variety of plant life on the planet. Plant genetic resources hold the key to food security and sustainable agricultural development (Iwananga, 1994). Plant genetic resources include land races primitive cultivars, advanced/improved varieties and wild relatives of crop plants. Activities that relate to conservation and use of plant genetic resources include: exploration and collecting, characterization and evaluation, conservation, assessment of variation and identification of useful genes, and exchange and genetic enhancement (Rao and Riley, 1994). Exploration and collection of plant genetic resources have been carried out to study phylogenetic relationships among plant species and their speciation, origin and dispersal of cultivated plants, ethno botanical studies on the relationship between plants and man and breeding of new cultivars and systematic introduction of exotic cultivars (Sakamoto, 1994). Vavilov and several other explorers recognized South Asia to be the cradle of life and agriculture as seen from the rich biodiversity and also from archeological evidences. This region is known for immense genetic variability in

rice, small millets, leguminous crops, vegetables, tropical fruits, fibre crops, sugarcane, grasses, oilseeds, spices, condiments, and also medicinal and aromatic plants. In addition, wild progenitors and related taxa of domesticated species contain valuable genes for adaptation to stress environments (Rana, 1994). Plant genetic resources in agri-horticultural crops and their wild relatives are of immense value to mankind as they provide food, fodder, shelter and industrial products. Plant breeders require reservoir of genetic variation for crop improvement. Due to the spread of high yielding varieties and selection pressure the genetic variability is gradually getting eroded resulting in large-scale depletion of variability. This situation thus demands priority action to conserve such germplasm (Frankel, 1975).

Conservation includes the management and preservation of known genetic resources. The plant genetic resources must be conserved in such a manner that there should be minimum genetic changes and losses. There are two main approaches for conservation of plant genetic resources namely, *in situ* and *ex situ*. *In situ* conservation approach has been defined, as the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings or, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties (Article 2, Convention on Biological Diversity). *Ex situ* conservation maintains germplasm outside its original habitats, in facilities that have been specifically created such as the seed, field and *in vitro* genebanks or botanical gardens (Frankel and Soule, 1981; Rao and Riley, 1994). *In vitro* genebanks is meant for the conservation of crop species with recalcitrant seeds and those, which are vegetatively propagated. This system

provides a cost effective alternative to field genebanks, nurseries or green houses (Tayler, 1997).

Appropriate storage methods need to be formulated for different crop species. For crops that produce seeds capable of storage under conditions of low temperature and low humidity their genetic conservation can be managed very adequately by seed genebanks (Ellis *et al.*, 1985). There are certain problem crops, included under two categories, namely, recalcitrant seed producers and clonal crops. Recalcitrant seeds die when exposed to desiccating conditions or low temperatures (Chin and Pritchard, 1988; Roberts and King, 1982). There are a number of crops, which are normally vegetatively propagated such as potato, sweet potato, yams, cassava, several fruit tree species, ginger, turmeric, vanilla, sugarcane, etc. In some of these crops, seed production is impossible due to sterility; in others it is undesirable to produce seeds for conservation as this would break up highly heterozygous clonal genotypes. For both types of problem crops, conservation needs are met, traditionally by the use of the field genebank ('orchard', 'clonal repository' etc.). For reasons of high labour costs, vulnerability to environmental hazards and requirement of large amount of space, the field genebank has many disadvantages (Withers, 1980; Withers and Williams, 1985; 1986; Withers and Engles, 1990; Chandel and Pandey, 1991; Withers, 1991). Its advantages are few and relate to the opportunities that are offered for evaluation of the germplasm.

Realizing the importance of developing suitable conservation strategies for recalcitrant seeds and clonal crops, efforts have been initiated to formulate alternate methods of conservation. Under these circumstances, biotechnology is a useful tool and is ideally suited for conservation of plant genetic resources. If genotypes can be

inoculated into *in vitro* culture for purposes of clonal propagation and germplasm distribution, it can be used for storage by prolonging the *in vitro* phase. The realization of the potential of *in vitro* conservation came in the early 1970s (Meryman, 1966; Nag and Street, 1973; Henshaw, 1975).

BIOTECHNOLOGY AND CONSERVATION

The past few years have witnessed a dramatic increase in our ability to manipulate and study plant tissues and cells in *in vitro* cultures. The different techniques that comprise plant cell and tissue culture have permitted investigations at molecular, cellular and organism levels. These have not only permitted better understanding in many aspects like genetics, biochemistry, physiology and cell biology but also resulted in developing novel organisms that otherwise could not have been derived from sexual reproduction. The growing realization of the potential of plant tissue and cell culture for plant propagation and breeding has resulted in commercial plant propagation of many ornamentals and orchids using shoot tip cultures, development of new varieties and new breeding lines *via* somaclonal variation, anther culture and protoplast fusion. Various techniques of gene transformations in plants are also based nowadays on these *in vitro* procedures, which have greatly benefited plant biotechnology as they aim to propagate useful cultivars and produce new ones rapidly (Cocking, 1994; Bhojwani and Razdan, 1996). Over the same time scale, *in vitro* conservation has moved from speculation to development and implementation (Withers, 1991).

IN VITRO CONSERVATION - FOUNDATION TECHNOLOGIES

The foundation technologies that make up an *in vitro* conservation system are collecting, disease eradication and indexing, culture initiation, multiplication, storage

and distribution (IBPGR, 1986; Withers, 1989; Withers and Williams, 1985; 1986). As in conventional conservation, there are points where monitoring should take place to check viability and genetic stability as well as the important task of characterization and documentation.

In vitro conservation has active and base components, which gives it an advantage over the field genebank, which, as indicated earlier, is an active conservation mode. *In vitro* active conservation is achieved by maintenance of cultures in the growing state, usually with growth slowed down by limiting physical, environmental or culture medium factors. *In vitro* base conservation is achieved by cryopreservation - storage in liquid nitrogen at or near a temperature of -196^0C (Withers, 1980; 1985a; 1985b; 1985c; 1986; 1987a; 1989). The linkage between the different foundation technologies of *in vitro* conservation and their relationship to the field genebank is shown in Fig.1 (Withers, 1991).

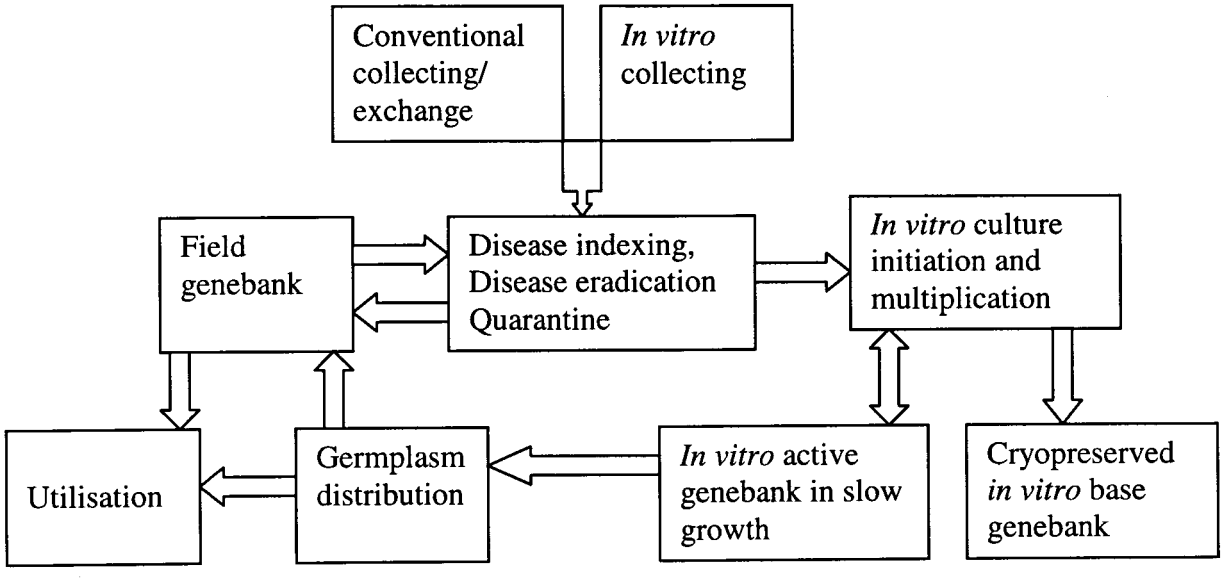


Fig. 1 The components of an *in vitro* conservation system, showing the flow of germplasm and the relationship between *in vitro* conservation, the field genebank and the user

Culture initiation, multiplication and plant regeneration form the cycle of events that is the backbone of an *in vitro* conservation scheme. The way in which the cycle is carried out has implications for the success of the conservation effort. There are two main areas, which is taken into consideration, when adopting any strategy for conservation. The first is in relation to genetic stability. Certain culture systems are less stable than others, being more prone to somaclonal variation (Scowcroft, 1984). The choice of culture system for *in vitro* conservation largely relate to the second area of concern in the *in vitro* culture cycle, ie, amenability to storage (Withers, 1991).

Micropropagation

Micropropagation is the true-to-type propagation of a selected genotype using *in vitro* culture techniques (Debergh and Read, 1991). Following the successful rapid multiplication of orchids by shoot meristem culture (Morel, 1960; 1965) there has been increasing interest, in recent years, in the application of tissue culture techniques as an alternative means of asexual propagation of plant species (Hu and Wang, 1983). Presently there are about 1000 plant species that can be micropropagated (Murashige, 1989; George, 1996). They include, *Anthurium* spp., *Gerbera* sp., *Gladiolus* sp., *Chrysanthemum* sp., *Dianthus* sp., *Phlox* sp, *Asparagus* sp., *Cicer* sp., *Arachis* sp., *Beta* sp., *Brassica* sp., *Tectona* sp., *Dalbergia* sp., *Santalum* sp., *Lolium* sp., *Vigna* sp., *Zea* sp., *Prunus* sp., *Pyrus* sp., *Malus* sp., *Coffea* sp., *Rosa* sp., *Grevillea* sp., *Eucalyptus* sp., *Thuja* sp., *Populas* sp., oil palm etc. Now micropropagation of plants is a multibillion-dollar industry being practiced in hundreds of small and large nurseries and biotech laboratories throughout the world (Bajaj, 1991c). Presently it is the only component of plant biotechnology, which has been commercially exploited on such a large scale. It is employed especially for propagation of ornamental and

foliage plants and recently in plantation crops, medicinal and aromatic plants (Bajaj, 1988; 1989b; 1991a) and trees (Bajaj, 1986; 1989a; 1991b).

The possibility of elimination of virus is another important aspect of shoot apex (meristem) culture. This began with Morel's demonstration of virus elimination from *Dahlia* stocks through shoot apex culture (Morel, 1960). Virus elimination was achieved after successful meristem culture in many crop species like garlic, pineapple, *Brassica* sp., *Caladium* sp., *Colocassia* sp., *Chrysanthemum* sp., dahlia, carnation, *Fragaria* sp., *Freesia* sp., *Gladiolus* sp., apple, cassava, banana, geranium, *Phaseolus* sp., sugarcane, potato etc (Quak, 1977; Love *et al.*, 1987).

Murashige (1974) has developed the concept of three developmental stages in micropropagation. They are explant establishment, multiplication of propagules and rooting and hardening for planting in soil. It has been further expanded and is now accepted that there are five stages critical for successful micropropagation. They are preparative stage to minimize contamination, initiation of culture, multiplication, elongation and root development and transfer to greenhouse conditions (Debergh and Read, 1991).

There are many factors contributing to the development of a successful micropropagation protocol for commercial multiplication. Various parameters like growing mother plants under more hygienic conditions, changing the physiological state of the stock plant, choice and nature of explant, phenolic exudates and presence of endogenous bacteria will influence the establishment of axenic cultures. The explant type, culture environment, number of subcultures, propagation systems and multiplication rate are the considerations at multiplication stage. Rooting can either be *in vitro* or *ex vitro*. *Ex vitro* rooting is cheaper and more effective in plant species that

are easy to root (Debergh and Maene, 1981; Debergh and Read, 1991). The survival, multiplication and field establishment of cultures depends upon a variety of factors such as origin of culture, physiological stage of explant, endogenous hormone level and culture environment like nutrient media, photoperiod, CO_2 concentration, temperature, etc. Each species is unique in these requirements.

Culture initiation

For micropropagation, the explant chosen is mostly the apical or axillary bud. This is to maintain the genetic fidelity of micropropagated plants and to minimize the chances of obtaining off type plants. Plants regenerated from shoot apex cultures may occasionally include variants (Denton *et al.*, 1977). However most reports content that genetic stability is preserved in shoot tip cultures (D'Amato, 1975; Murashige, 1974; Morel, 1975). According to D'Amato (1977) this is because the shoot apex provides the structure in which cell division and DNA replication is strictly controlled and the cells with genetic defects are eliminated because of competition. The developmental stages of an explant as well as its size can determine the success of a procedure (George and Sherrington, 1984; Franchet *et al.*, 1987).

The size of the explant determines the survival of cultures. In general, the larger the explant, there is more the chances of survival. However, where elimination of viruses is one of the objectives, meristem of smallest size should be used (Kartha and Gamborg, 1975; Hu and Wang, 1983).

Explants from the tip of the shoot that are in younger stage of development are optimum for shoot regeneration. Since only one terminal bud is available many workers also used axiallary buds. Actively growing shoot tips are recommended for

meristem, shoot tip and bud cultures because of their strong growth potential and low virus contamination.

Culture media and culture conditions

Though there are many formulations of nutrient media available, MS medium (Murashige and Skoog, 1962), sometimes with minor modifications, is the one, which is used most frequently with greatest success. The growth additives and salt concentration requirements of the medium vary with species to species especially from one stage of culture development to another.

Although there are endogenous levels of growth regulators and small quantities of cytokinins may be synthesized by shoots growing *in vitro* (Koda and Okazawa, 1980), the nutrient medium is required to be supplemented with cytokinins and auxins. Kinetin (kin), Benzyl aminopurine (BAP) and N^6-(2-isopentenyl)-adenine (2iP) are the most common cytokinins used for shoot multiplication.

Most workers in meristem and shoot tip cultures select a constant incubation temperature between 20–28^{0}C. In order to maximize the growth in tissue cultures 12–24 h photoperiod is used with a light intensity ranging from 1000–10,000 lux.

Shoot multiplication and* in vitro *rooting

For multiplication of the propagules, 'axillary shoot proliferation' is ideal. Since high multiplication rates may include variations, optimum multiplication rates have to be determined for each crop species.

Indole-3 acetic acid (IAA), Indole 3-butyric acid (IBA), α-Naphthalene acetic acid (NAA) and 2, 4-Dichlorophenoxy acetic acid (2,4-D), are the common auxins used in plant tissue cultures for root initiation and elongation. Among these, NAA is the most preferred (Hu and Wang, 1983). Juvenility of explant (de Fossard *et al.*,

1974) and subculture process (Gupta *et al.*, 1981) was most important parameters affecting root induction especially in hard to root species.

In general, increased light intensity results in better culture growth, which helps in successful transfer to pots (Bhojwani, 1980; Ziv, 1979). Care should be taken so that the high intensity of light does not directly inhibit root growth.

Acclimatization and planting out

High water loss was recorded from leaves of transplanted tissue cultured plants immediately after transplantation. This is due to reduced quantity of epicuticular wax (Grout and Aston, 1977; Sutter and Langhams, 1979), high volumes of mesophyll intercellular space (Brainerd *et al.*, 1981) and slowness of stomatal response to water stress (Brainerd and Fuchigami, 1981). A period (2–4 weeks) of humidity acclimatization, help newly transferred plants to undergo morphological and physiological adaptations required for terrestrial plant water control (Grout and Aston, 1977; Brainerd and Fuchigami, 1981).

***In vitro* storage**

The normal approach to tissue culture is to find a medium and set of growth conditions, which favour the most rapid rate of growth and promote the desired pattern of development. For storage the approach must be changed. The principle of slow growth storage is that the safety of *in vitro* culture be ensured without disadvantages of frequent subculturing. Thus the risk of contamination at each transfer interval, inputs in terms of labour and consumables are reduced. Several methods, such as temperature reduction, medium modification, use of osmoticums, etc., have been found to reduce the rate of growth of tissue cultures, so that it can be

kept unattended for moderate length of time (Withers, 1980; 1987a; 1991; Withers and Williams, 1986).

Tremendous workload is involved in maintaining any tissue culture in continuous growth as stocks for use in experimental work and it requires transfer to fresh medium every week, fortnight or month. It is necessary to find alternative storage methods to reduce the workload, time and resources. By changing the growth kinetics of the cultures, the subculture intervals can be extended to quarterly, half yearly or annual and even too much longer times, up to centuries. There are two basic approaches for *in vitro* storage, namely *slow growth* for short-to-medium term storage and *cryopreservation*, for long term storage in the active and base collections, respectively (Withers, 1980; 1985a; b).

Slow growth or minimal growth storage

Slow growth or minimal growth can be induced by different factors, such as reduction in temperature, raising the osmolarity of the medium, addition of growth retardants to the medium, reduction of respiration of the explant by an over layer of oil, starvation of the cultures, induction of dormancy, induction of special organs as microtubers and dehydration of the cultures (Reed, 1995; Staritsky, 1997). Thus by altering the growth kinetics, the subculture intervals can be increased significantly. The most commonly used method is reduction of temperature. Plant species vary in the length of time (6–24 months) during which their cultures can be stored.

Isolated protoplasts, cells from suspension or callus cultures, meristem tips or other suitable explants, propagules of different types at various developmental stages, organized plants, single shoots, somatic embryos, rooted plantlets and even seeds are the materials suitable for *in vitro* storage. Among these, plantlets are most ideal. Shoot

cultures have been the most commonly used plant material, mainly due to their presumed higher genetic stability with respect to other culture systems (Hammerschlag, 1992; Withers, 1992). Rooted plantlets of *Mentha* sp., *Rubus* sp., blackberry and *Vaccinium* sp. could be stored for 6–17 months under normal culture conditions in culture vessels sealed with caps or films which allow gaseous exchange but prevent escape of moisture (Gunning and Lagerstedt, 1986). Rooted plantlets of *Pestacia* sp. could be stored for 1 year (Barghchii, 1986) and those of *Coffea arabica* for more than 2 years (Kartha *et al.*, 1981) under normal conditions.

The interval between transfers can be greatly increased by keeping the cultures in weak light or in the dark and at a temperature less than the temperature optimal for active growth. The optimum temperature and light requirement for storage, is genotype dependent. Shoots and plantlets of many temperate crops were stored successfully at temperatures close to freezing. Storing materials in cultures at nonfreezing reduced or low temperatures (1–9^{0}C) is generally practiced for plants like *Fragaria virginiana*, *F. vesca*, *Lolium multiflorum*, *L. corniculatus*, *Malus domestica*, *Medicago sativa*, *Rubus* sp., *Trifolium* sp. and *Vitis vinifera* (Razdan, 1993). Virus-free strawberry plants were maintained for 6 years at 4^{0}C by simply adding a few drops of the liquid medium to the cultures at periodic intervals (Mullin and Schlegel, 1976). Yearly transfers on a hormone-free medium likewise ensured maintenance of straberry germplasm for more than 17 years *in vitro* (Jungnickel, 1988). Morel (1975) reported maintenance of about 800 cultivars of grape plants under similar conditions for over 15 years. A temperature range of 0^{0}C to 5^{0}C was found suitable for conserving shoot cultures of many temperate woody species (Lundergan and Janick, 1979; Chun and Hall, 1986; Aitken-Christie and Singh, 1987). Somatic embryos can

be stored at a temperature just above freezing (4^0C to 10^0C) (Durzan, 1988; Lutz *et al*, 1985). Encapsulated embryos of *Santalum album* were stored for 45 days at 4^0C. Dark storage is satisfactory for some species. Shoot cultures of *Kalmia* sp., *Populus* sp. and *Malus* sp. can be kept in the dark at -3^0C and 4^0C respectively, for 1 to 2 years (Chun and Hall, 1986; McCulloch, 1988; Negri *et al.* 2000). Shoot cultures of *Prunus* sp. could be stored up to 6 to 28 months with 100–60% survival at -3^0C to 5^0C under dark and/light conditions (Marino *et al.*, 1985; Eckard, 1989; Regnard *et al.*, 1990; Dorian *et al.*, 1994; Janero *et al.*, 1995; Marino, 1997). The cold storage of herbaceous species has been reviewed by Bhojwani and Razdan (1983) and that of woody plants by Aitken-Christie and Singh (1987).

Plantlets and shoot cultures of some temperate plants, many tropical and sub-tropical species loose their viability if stored at low temperature. For many of these species minimal growth is best achieved at 14^0C to 20^0C (Henshaw *et al.*, 1980). Cassava shoot cultures deteriorated if stored at temperature lower than 20^0C (Roca *et al.*, 1982). The international germplasm collection of *Musa* sp. is conserved as shoot cultures stored at 15^0C and an illuminance of 1000 to 2000 lux. *Musa* cultures die within 6 weeks if kept at 5^0C and within 3 months at 10^0C, but at 15^0C they can be kept for 13–17 months according to their genotype (Banerjee and De Langhe, 1985; Banerjee, 1989).

Placing cultures in an environment with a low partial pressure of oxygen appears to have potential for limiting *in vitro* growth. Caplin (1959) used mineral oil ovelay to conserve callus cultures. He attributed the decrease in growth rates to oxygen being prevented from reaching the plant tissue. Substances like autoclaved silicon (Moriguchi *et al*, 1988), liquid paraffin oil (Mathur, 1991) were also used for

the same effect. Remarkable successes have been reported on the application of an overlay of liquid paraffin in combination with mannitol in the medium (Dekkers *et al.*, 1991).

Manipulation of chemical constituents in the culture medium using abscisic acid (ABA) or high levels of sucrose also influences conditions in favour of slow growth of cultures (Withers and Williams, 1986). Nitzsche (1978, 1980, 1983) reported that carrot callus can survive storage up to 2 years if it is pretreated with 10 mgl^{-1} of abscisic acid and dried on sterile filter paper in the air stream of laminar flow cabinet before storage. Dormancy could be induced in encapsulated somatic embryos by the use of abscisic acid followed by drying. *Medicago sativa* somatic embryos, matured in the presence of abscisic acid and dried to a moisture content of 8–15%, have remained fully viable at room temperature for 12 months (Redenbaugh *et al.*, 1991). Dried embryos may survive for longer periods if they are encapsulated in a polymer coating (Kitto and Janick, 1985a,b). Conservation of *Coleus Forkohlii* through the use of *in vitro* cultures and encapsulation technique was reported by Bhattacharyya *et al.*, (2001). Shoot cultures of *Eucalyptus grandis* could be maintained for up to 10 months by the addition of 10 mgl^{-1} ABA to the growth medium or by halving the nutrient supply and removal of exogenous plant growth regulators (Watt *et al.*, 2000). Thyagi *et al.* (2001) reported *in vitro* conservation of shoot cultures of *Vanilla planifolia* up to 290 days in a medium containing polyamines like putresine and spermidine. At Indian Institute of Spices Research, Calicut, shoot cultures of *Vanilla* spp. (*V. planifolia* and *V. aphylla*) could be stored up to 2 years under minimal growth conditions by the addition of mannitol into the culture medium (Minoo, Unpublished). *In vitro* storage of *V. walkeriae* under slow

growth conditions, in mannitol containing medium was reported by Agrawal *et al.* (1992). Conservation of *Coleus forkohli*, a medicinal plant through the use of *in vitro* cultures and encapsulation technique was reported by Bhattacharyya *et al.* (2001).

Decreasing the carbohydrate/nutrient supply could also result in reduced growth rate (Gunning and Lagerstedt, 1986). Similar results were reported in coffee (Kartha *et al.*, 1981; Roca *et al.*, 1982). Reduction of macronutrient availability gave better results in *Vitis* shoot cultures (Moriguchi and Yamaki, 1989).

Changing the osmotic potential by increasing the sucrose levels will have an inhibitory effect on plant cell growth and thus can be used to maintain cultures in a dormant condition for 1 or 2 years (Schenk and Hildebrandt, 1972). Addition of mannitol to the culture medium can also prevent rapid cell death (Codron *et al.*, 1979). It was suggested that mannitol preserves membrane integrity and prevents solute leakage. Addition of mannitol and increase in sucrose concentration were found to restrict growth and enhance the viability of cells in *Cinchona* sp. (Hunter *et al.*, 1986) and potato (Henshaw *et al.*, 1980) respectively.

Growth regulators are normally added to the culture medium to promote and regulate plant growth *in vitro*. Withdrawal of these chemicals can assist in *in vitro* storage of certain plants. Gunning and Langerstedt (1986) reported this in strawberry cultivars. Certain growth regulators like abscisic acid are able to induce dormancy in plant meristems and somatic embryos of caraway (Ammirato, 1974) and potato (Roca *et al.*, 1982).

Starvation does not seem to be a proper method to slow down the growth of cultures either. The manifestation of starvation of cultures is certainly a deduction in growth but in general at the cost of weakening of cultures, which results in a very low

regrowth rate. However, starvation may induce dormancy or the formation of survival organs such as microtubers (Staritsky, 1997). The induction of microtubers as a method of medium term preservation and for exchange of germplasm is presently applied in potato and yam (Ng *et al*, 1992).

Longevity of *in vitro* cultures can be extended for varying periods of time by using different types of closures for the culture vessel, which can prevent evaporation from the medium as well as from the plant surfaces. Shoot cultures of *Ravolfia serpentina* were maintained for 9 months at 15^0C or 25^0C in a standard shoot culture medium by replacing cotton plugs with polypropelene caps as enclosures for culture tubes (Sharma and Chandel, 1992). Sharma (2001) reported storage of *in vitro* cultures of *Gentiana kurroo*, a medicinal plant, up to 7 months at 25^0C by replacing the cotton plugs with polypropelene caps as enclosure for culture tubes.

Minimal growth storage is clearly useful for preservation of clones, which are required as stocks for continued propagation *in vivo* or *in vitro*, or as parents in plant breeding programmes. The technique is not ideal for long-term storage of genotypes, because the periods during which cultures can be left unattended are relatively short. Nevertheless, in the absence of a reliable method, minimal growth storage is being used in conjunction with shoot or node culture, to maintain genotypes in germplasm banks (George, 1993).

Cryopreservation

Cryopreservation is defined as the viable freezing of biological material and their subsequent storage at ultra low temperatures, preferably at that of liquid nitrogen. Preservation of both plant and animal germplasm is an integral component of sustainable agriculture system. From a crop improvement perspective, preservation

of all valuable germplasm is being accorded a high priority (IBPGR, 1985). Although several decades later, the development of cryopreservation strategy for plant cells and organs has followed the advances made with mammalian systems (Kartha and Engelmann, 1994). One of the earliest report on the survival of plant tissues exposed to ultra-low temperature was made by Sakai (1956) when he demonstrated that very hardy mulberry twigs, upon induction of dehydration mediated by extra-cellular freezing are capable of survival following immersion in liquid nitrogen provided the frozen samples are subsequently re-warmed slowly at an air temperature of 0 ^{0}C. Sun (1958) achieved partial success when desiccated seedlings of *Pisum sativum* were immersed in liquid nitrogen. Quatrano (1968) reported the first record on the use of cryoprotectant for the freezing of plant cells. The first successful attempt to cryopreserve cultured cells of species is that with cell cultures of *Daucus carota* (Latta, 1971) and this has become a model species to study cryobiology of cultured cells. Many reports were available on the cryopreservation of carrot cell cultures (Nag and Street, 1973; 1975a; 1975b; Dougall and Wetherell, 1974; Bajaj, 1976; Withers and Street, 1977; Dougall and Whitten, 1980; Weber *et al.*, 1983).

An array of plant material could be considered for cryopreservation as dictated by the actual needs *vis-a-vis* preservation. These include meristems, cell, callus and protoplast cultures, somatic and zygotic embryos, anthers, pollen or microspores and whole seeds (Withers, 1985a, b, c; Kartha, 1985).

Plant cells contain high amounts of cellular water and freezing of plant cells implies conversion of some or all of their liquid water to ice, whereas thawing is reversal of this transition. Since most of the experimental systems (meristems, shoot tips, cultured cells etc.) contain high amount of cellular water and hence extremely

sensitive to freezing injury, protection from freezing and thawing has to be imposed artificially. This involves various strategies such as the use of cryoprotectants or other manipulations (Kartha and Engelmann, 1994).

However, only by freezing at ultra low temperatures such as -196^{0}C, the temperature of liquid nitrogen, growth in plant tissue and cell cultures can be entirely suspended. Since very few cultures have a natural resistance to freezing, special manipulations of explant and culture conditions are required to prepare a specimen with maximum freeze tolerance. Application of certain cryoprotectants like dimethyl sulphoxide (DMSO), glycerol in combination with other compounds like mannitol, sorbitol, glucose, sucrose, proline or polyethylene glycol (PEG) will bring about changes in cell permeability, freezing point and response to the stresses of freezing and thawing essential for survival.

Cryopreservation involves essentially three steps namely, specimen treatment and freezing, storage at ultra-low temperature and thawing and recovery. Before commencing proper cryopreservation procedures different culture systems need to be given specific manipulations and or culture conditions in order to present a specimen with maximal freeze tolerance. An ideal cryoprotectant should protect cells from all the factors, which would affect the viability of the frozen biological sample during all these stages. Since viability loss is not expected to occur at ultra-low temperatures, protection of the cells during freezing and thawing is of paramount importance. A number of compounds such as glycerol, dimethyl sulphoxide (DMSO), ethylene glycol, polyethylene glycol (PEG), sugars and sugar alcohols either alone and in combination protect living cells against damage during freezing and thawing. Such compounds can lower the temperature at which freezing first occurs and can alter the

crystal habit of ice when it separates (Withers, 1980; Kartha and Engelmann, 1994). Although the exact mechanism of action of cryoprotective compounds is still poorly understood, the colligative properties of the cryoprotectants can minimize the deleterious action of excessive electrolyte concentration resulting from removal of water and conversion of water to ice (Nash, 1966).

Cryoprotectants generally used for freezing biological specimens fall into two categories namely, permeating and non-permeating. The most commonly used permeating additives are DMSO and glycerol; the former permeates rapidly and is more toxic when compared to the latter. Generally a concentration of 5–10% for DMSO and 10–20% for glycerol is adequate for most material. In instances where application of a single cryoprotectant does not result in survival, a mixture of cryoprotectants has been beneficial (Finkle and Ulrich, 1979; Chen *et al.*, 1984, Finkle *et al.*, 1985; Withers, 1985a, b, c). Application of cryoprotectant to the cells and the removal of cryoprotectants from the thawed samples should always be a gradual process to alleviate the problems associated with plasmolysis and deplasmolysis, respectively (Kartha and Engelmann, 1994). Cryoprotectants are usually prepared in culture medium and the material to be cryopreserved is incubated prior to their freezing (Withers, 1985a, b, c; Kartha and Engelmann, 1994).

There are different freezing methods such as slow freezing, rapid freezing, droplet freezing, vitrification, etc. A number of factors such as cooling rates, pretreatment and cryoprotection, type and physiological state of the experimental material and the terminal freezing temperature, influence the success of slow freezing method. The most commonly used method of freezing meristems and cell cultures is by regulated slow cooling at a rate of 0.5 to 1.0 ^{0}C /min down to either –30^{0}C, –35^{0}C

or –40 ^{0}C with the help of a programmable freezer, followed by storage in liquid nitrogen. Meristems, cell cultures and somatic embryos of a number of species have been cryopreserved using slow freezing methods (Kartha, 1985; Withers, 1985a, b, c).

New cryopreservation techniques offer practical advantages in comparison to classical ones (Sakai, 1995) Rapid freezing is the simplest form of cryopreservation since the procedure does not require sophisticated and expensive equipment. Rapid freezing has successfully cryopreserved meristems and somatic embryos of a few plant species. It is suggested that the viability of cells may be maintained by preventing the growth of intracellular ice crystals formed during rapid cooling by rapidly passing the tissue through the temperature zone in which lethal ice formation occurs (Sakai, 1995; Engelmann, 1997).

The technique of droplet freezing was originally developed for cryopreservation of cassava meristems (Kartha *et al.*, 1982). After dissection, apices are precultured with DMSO for a few hours and frozen rapidly in droplets of cryoprotective medium placed on aluminium foils. This procedure has been applied successfully to more than 150 varieties of potato with an average recovery rate of 40% (Schäfer-Menuhr, 1996).

The development of vitrification technique for the cryostorage of cultured plant cells and organs is of recent origin although Sakai (1958) succeeded in obtaining the survival of hardy mulberry cortical tissue in liquid nitrogen by vitrification. In the vitrification method, cells or meristems must be sufficiently dehydrated with a highly concentrated vitrification solution at 25 ^{0}C or 0 ^{0}C without causing injury prior to immersion in liquid nitrogen. Vitrification method of cryopreservation was reported

in 5 apple species or cultivars, 8 pear cultivars (Niino *et al.*, 1992a) and 13 mulberry species or cultivars (Niino *et al.*, 1992b; 1992c).

For freezing of differentiated tissues and organs such as apices zygotic and somatic embryos, new techniques have been developed (Deruddre, 1992; Engelmann, 1997; 2000). They are based on the removal of most or all freezable water by physical or osmotic dehydration of explants followed by ultra-rapid freezing which results in vitrification of intracellular solutes, i.e. the formation of an amorphous glassy structure without occurrence of ice crystals which are detrimental to cellular structural integrity. Their main advantages in comparison to classical procedures are their simplicity, since they do not require the use of a programmable freezer, and their applicability to a wide range of genotypes. They include encapsulation-dehydration, vitrification, encapsulation-vitrification, desiccation, pregrowth, pregrowth-desiccation and droplet-freezwth, pregrowth-desiccation and droplet freezing (Engelmann, 1997; 2000).

The encapsulation-dehydration procedure is based on the technology developed for the production of artificial seeds. Explants are encapsulated in alginate beads, pregrown in liquid medium enriched with sucrose for several days, partially desiccated down to a water content around 20% (fresh weight basis), then frozen rapidly. Survival rates are high and growth recovery of cryopreserved samples is generally rapid and direct, without callus formation. This technique has been developed for apices of various species from temperate origin such as apple, pear, grape, eucalyptus, and of tropical origin such as sugarcane and cassava (Dereuddre, 1992; Engelmann, 1997; Engelmann and Takagi, 2000).

Vitrification consists of placing explants in the presence of a highly concentrated cryoprotective solution, then freezing them rapidly. This technique has been experimented with cell suspensions, apices and somatic embryos of around 20 plant species (Sakai, 1993; 1997, 2000).

Encapsulation-vitrification is a combination of the above techniques, where explants are encapsulated in alginate beads and treated with vitrification solutions before freezing. It has been applied to apices of lily and wasabi (Matsumoto *et al.*, 1995; Sakai and Matsumoto, 1996).

Desiccation is the simplest procedure since it consists of dehydrating explants, then freezing them rapidly by direct immersion in liquid nitrogen. This technique has been applied to embryos of a large number of recalcitrant and intermediate seed species (Engelmann, 1992; Engelmann *et al.*, 1995, Dumet *et al.*, 1997). Optimal survival rates are generally obtained when samples are frozen with a water content comprised between 10 and 20% (fresh weight basis).

Pregrowth involves preculturing the plant material on a medium containing cryoprotectants, then freezing explants rapidly. An efficient pregrowth procedure has been developed recently for meristematic clumps of *Musa* sp. (Panis, 1995a; b; Panis and Thinh, 2001).

In a pregrowth-desiccation procedure, explants are pregrown in the presence of cryoprotectants (generally sugars such as sucrose or glucose), dehydrated under the laminar airflow cabinet or with silica gel, then frozen rapidly. This method has currently been applied to coconut zygotic embryos and oilpalm somatic embryos only (Assy-Bah and Engelmann, 1992b; Dumet *et al.*, 1993). Survival rates obtained after freezing are high and growth recovery is rapid and direct.

Cryopreservation methods have been developed for more than 80 different plant species in various forms like cell suspensions, calluses, apices, somatic and zygotic embryos (Kartha and Engelmann, 1994; Engelmann, 1997). However, their routine utilisation is still restricted almost exclusively to the conservation of cell lines in research laboratories. The method is routinely used in oilpalm for which more than 80 clones of somatic embryos are stored for the long term in liquid nitrogen (Dumet, 1994) and potato (Schäfer-Menuhr, 1996).

Technique of *in vitro* conservation has been made dramatic progress in the last 10 years and methods have been standardized for quite a lot of plant species (Table 7). Slow growth techniques are immediately applicable in most cases, whereas cryopreservation techniques often require further improvement before they can be applied routinely.

Table 7: Summarized information on the present status of development of *in vitro* conservation for different plant species

Crop/Application	Technique	Reference
Cassava		
Disease eradication	Meristem culture	IPGRI / CIAT, 1994
Propagation	Shoot culture	IPGRI / CIAT, 1994
Slow growth storage	Plantlets at 23 ^{0}C	IPGRI/CIAT, 1994; Mabanza *et al.*, 2001
Cryopreservation	Zygotic embryos / seeds, shoottips; Somatic embryos	Marin *et al.*, 1990; Escobar *et al.*, 1993; 1995; 1997; 2000; Engelmann *et al.*, 1994; Escobar and Roca, 1997; Sudarmonowati and Henshaw, 1990; Stewart, *et al.* 2001
Distribution	*In vitro* plantlets	Roca *et al.*, 1984
Potato		
Disease eradication	Meristem culture	Cassels and Long, 1982
Propagation	Shoot cultures,	Goodwin, 1966
Slow growth storage	Microtubers	Hussey and Stacey, 1981
	Shoot culture at 10 ^{0}C,	Mix 1984, Thieme and Pett, 1982
Cryopreservation	Shoot apices; meristems	Schafer-Menuhr, 1995; Grospietsch *et al.*, 1999; Hirai and Sakai, 2000
Distribution	*In vitro* plantlets	Roca *et al.*, 1979
***Musa* spp.**		
Collection	Shoot meristem	FAO / IPGRI, 1997

Crop/Application	Technique	Reference
Disease eradication	Meristem culture	Gupta, 1986
Propagation	Shoot culture, somatic embryogenesis	Vuylsteke and Swennen, 1990, Novak *et al.*, 1989; Panis and Swennen, 1993
Slow growth storage	Shoot culture at 10^{0}C	Van den houwe *et al.*, 1995
Cryopreservation	Zygotic embryos, meristematic clumps	Abdelnour-Esquivel *et al.*, 1992a; Panis, 1995a; b; Panis, *et al.*, 2000; Panis and Thinh, 2001
Distribution	*In vitro* plantlets	Frison and Putter, 1989
Coconut		
Collecting	Zygotic embryos	Assy-Bah *et al.*, 1987
Propagation	Zygotic embryo culture	Assy-Bah *et al.*, 1987
Somatic embryogenesis	From leaf/inflorescence callus	Verdelliand Buffard-Morel, 1995
Cryopreservation	Zygotic embryo	Assy-Bah and Engelmann 1992a; 1992b; 1993
Distribution	Zygotic embryo	Frison *et al.*, 1993
***Prunus* spp**		
Slow growth storage	Shoots at 0-4 ^{0}C	Wilkins *et al.*, 1988
Cryopreservation	Shoot apices, zygotic embryos	Brizon *et al.*, 1995
***Allium* spp.**		
Disease eradication	Meristem culture and thermotherapy	Conci and Nome, 1991
Propagation	Shoot culture from bulb cuttings	Hussey, 1978 Novak and Havel, 1981, Keller, 1991
Slow growth storage	Shoots from inflorescence, Microbulbets	El-Gizawy and Ford-Lloyd, 1987
	Shoot culture; Microbulbets	Keller, 1991
Cryopreservation	Apical meristem	Niwata, 1995
***Citrus* spp.**		
Collection	Budwoods	FAO/IPGRI, 1997
Disease eradication	Shoot tip grafting; *In vitro* quarantine	Navarro, 1992; Navarro *et al.*, 1984; Frison and Taher, 1991
Propagation	Shoot culture	Duran-Vila *et al.*, 1989
	Somatic embryogenesis	Ollitrault *et al.*, 1992
Slow growth storage	*In vitro* plantlets	Marin and Duran-Vila, 1991
Cryopreservation	Seeds; Somatic embryos	Duran Vila, 1995; Marin and Duran Vila, 1992
Distribution	Nucellar callus and cell suspensions	Engelmann *et al.*, 1994
***Coffea* spp.**		
Collection	Cuttings	FAO / IPGRI, 1997
Propagation	Shoot cultures	de Pena, 1984
Slow growth storage	*In vitro* plantlets	Bertrand-Desbrunais and Charrier, 1989

Crop/Application	Technique	Reference
Cryopreservation	Apices	Mari *et al.*, 1995
	Zygotic embryos	Abdelnour-Esquivel *et al.*, 1992b
	Somatic embryos	Bertrand-Desbrunais *et al.*, 1988
	Seeds	Normah and Vengadasalam, 1992; Dussert *et al.*, 2000.
Colocasia esculenta		
Disease eradication and indexing	Meristem culture	Ng, 1983a
	Tested for DMV (ELISA), taro bobone rhabdovirus (ISEM), TBV	Jackson, 1990
Propagation	Shoot culture, Corm tissue	Ng 1983b, Islam *et al.*, 1994
Slow growth storage	Shoot culture at 28/24^{0}C	Bessembinder *et al.*, 1993
Cryopreservation	Somatic embryos, meristematic clumps; shoot tips	Islam *et al.*, 1994; Takagi *et al.*, 1994; 1997
***Dioscorea* spp.**		
Disease eradication and indexing	Meristem tip culture, ELISA, RT PCR	Mantell *et al.*, 1980; Ng, 1983a; Saleil *et al.*, 1990
Propagation	Shoot culture	Malaurie *et al.*, 1998; Ng, 1992; Malaurie *et al.*, 1995
Slow growth storage	Reduced temperature	Ng and Ng, 1994
	Modified medium	Malaurie *et al.*, 1993
	Mineral overlay, reduced temperature	Takagi *et al.*, 1994
		Mandal and Chandel, 1993
	Nodal segments at 25^{0}C	Mandal and Chandel, 1993;
Cryopreservation	Encapsulation/Dehydration of shoot tips, *In vitro* plantlets	Mandal, 2000; Mandal and Chandel, 1995; Ng, 1991
Distribution	Microtubers; Minitubers; Alginate coated nodal segments	Malaurie *et al.*, 1993; Hassan and Takagi, 1995
Ipomoea batatas		
Disease eradication and indexing	Meristem culture	Green and Lo, 1989; Love *et al.*, 1987
Propagation	Axillary bud culture	Jarret and Florkowski, 1990
	Somatic embryos	Chee and Cantliffe, 1988
Slow growth storage	Chemical and environmental inhibition, ABA inhibition	Jarret and Gawel, 1991a
		Jarret and Gawel, 1991b
Cryopreservation	Shoot tip vitrification	Towill and Jarret, 1992
	Somatic embryo desiccation	Shimonishi *et al.*, 1994
Distribution	Axillary bud, *in vitro* plantlets	Jarret, 1989
***Pyrus* spp.**		
Disease eradication and indexing	Thermotherapy and meristem culture	Postman, 1994

Crop/Application	Technique	Reference
Propagation	Shoot culture	Berardi *et al.*, 1993
Slow growth storage	1^0C or 4^0C for 1–4yr; 4^0C, 16h photoperiod; 1^0C dark	Reed and Chang, 1997; Wanis *et al.*, 1986; Moriguchi *et al.*, 1990
Cryopreservation	Slow freezing; Encapsulation	Reed, 1990a; b; Reed *et al.* 1998a; b
	Vitrification, Encapsulation and vitrification	Scottez *et al.*, 1992; Niino *et al.*, 1992a; Niino and Sakai, 1992
Distribution	*In vitro* plantlets	Hummer, 1994
***Saccharum* spp.**		
Propagation	Shoot culture	Feldman *et al.*, 1991
	Somatic embryogenesis	Guiderdoni *et al.*, 1995
Slow growth storage	*In vitro* plantlets at 18^0C	Paulet and Glaszmann, 1994
Cryopreservation	Encapsulation/Dehydration of apices	Gonzalez-Arnao *et al.*, 1993; 1999; Paulet *et al.*, 1993
Distribution	*In vitro* plantlets	Paulet and Glaszmann, 1994
***Vitis* spp.**		
Disease eradication and indexing	Meristem culture	Alleweldt *et al.*, 1990
Slow growth storage	Shoot culture at 9.5^0C	Barlass and Skene, 1983
Cryopreservation	Shoot tip encapsulation	Plessis *et al.*, 1991; Zhao, *et al.*, 2001; Wang *et al.*, 2000
	Slow freezing cell suspension	Dussert *et al.*, 1991
Distribution	Disease free plantlets	Cao, 1990

Ever so many recent reports are available on slow growth storage and cryopreservation of crops such as cassava (Mabanza *et al.*, 2001); rice (Watanabe *et al.*, 1999; Wang *et al.* 2001), kiwifruit (Bachiri *et al.*, 2001; Wu, *et al.*, 2001), *Rubus* (Chang and Reed, 1999), *Zoysia* sp. and *Lolium* sp. (Chang *et al.* 2000); orchid seed and its fungal symbiont (Wood *et al.*, 2000), *Eucalyptus* hybrid (Blakesley and Kiernan, 2001), *Auricula* (Hornung, *et al.*, 2001) etc.

Pollen cryopreservation

The pollen longevity of different species varies between minutes and years depending primarily on the taxonomic status of the plant and on abiotic environmental conditions. For a number of agronomically important taxa, including the short-lived graminaceous pollen, special storage conditions are needed to preserve the viability

and fertilizing ability of pollen for a long period. There are a large number of crop species, including vegetables, fibre and fruit crops, forages and cereals, for which pollen storage strategies are desirable (Barnabas and Kovacs, 1997). According to them pollen storage has been used to satisfy the following practical needs:

1. To hybridize plants that flower at different times and locations or show nonsynchronous flowering
2. To provide a constant supply of short lived (recalcitrant) pollen
3. To facilitate supplementary pollination for improving yields
4. To eliminate the need to grow male lines continuously in breeding programmes
5. To obviate the variability incidental to the daily collection of pollen samples
6. To study pollen allergens and the mechanism of self incompatibility
7. To provide materials for international germplasm exchange
8. To ensure the availability of pollen throughout the year without using nurseries or artificial climate for plant growth

Earlier reports on storage of pollen has been reviewed by Knowlton (1922), Johri and Vasil (1961), Linskens (1964), Stanley and Linskens (1974), Shivanna and Johri (1985), Shivanna and Sawhney (1997) and Towill and Walters (2000).

Various methods are available for short term as well as long-term storage of pollen. Cryogenic procedures seem to show promise for long-term preservation of pollen viability (Barnabas and Kovacs, 1997). Knowlton (1922) was the first to observe that pollen could survive extremely low temperatures. Since then numerous studies have been conducted on cryopreservation of pollen (Table 8).

Table 8: Successful cryostorage of pollen from various crop species

Taxa	Storage temp. (^{0}C)	Duration of storage	Quality of stored pollen	References
Beta vulgaris	-196	1year	fertile	Hecker *et al.*, 1986
Brassica oleracea	-196	16 months	fertile	Crips and Grout, 1984
Capsicum annum	-196	42 months	fertile	Alexander *et al.*, 1991
Carya illinoensis	-196	1year	fertile	Yates and Sparks, 1990
Carica papaya	-196	485 days	viable	Ganeshan, 1986
Glycine max	-192	21 days	fertile	Collins *et al.*, 1973
Gossipium hirsutum	-192	10 days	viable	Collins *et al.*, 1973
Helianthus annus	-196	4 years	fertile	Frank *et al.*, 1982
Humulus lupulus	-196	2 years	fertile	Haunold and Stanwood, 1985
Juglans nigra	-196	2 years	viable	Farmer and Barnett, 1974
Lycopersicon esculentum	-190	1062 days	fertile	Visser, 1955
Narcissus cv.	-196	351 days	fertile	Bowes, 1990
Persea sp.	-196	1 year	fertile	Sedgley, 1981
Pistacia sp.	-196	1 year	fertile	Vithanage and Alexander, 1985
Prunus persica	-196	1year	fertile	Jiang and Gao, 1989
Rosa spp.	-196	8 weeks	fertile	Marchant *et al.*, 1993
Solanum tuberosum	-196	24 months	fertile	Towill, 1984
Trifolium pratense	-196	24 weeks	fertile	Collins *et al.*, 1973
Vicia faba	-196	1 month	viable	Telaye *et al.*, 1990
Vitis vinifera	-196	5 years	fertile	Ganeshan and Alexander, 1988

Shivanna and Sawhney, 1997

Recently Rajasekharan and Ganeshan (2001) reported cryostorage of pollen in various vegetable and ornamental species such as onion, tomato, *Capsicum* spp., *Solanum* species, rose, gladiolus etc. Indian Institute of Horticultural Research, Bangalorehas established a pollen cryobank, which maintains more than 600 tropical pollen accessions, collected over different seasons and years, kept under constant cryogenic condition (Rajasekharan and Ganeshan, 2001).

Synthetic seeds

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 (Redenbaugh *et al.*, 1986). Two types of artificial seeds, namely *hydrated* and *desiccated*, have been developed. Hydrated artificial seeds consists of somatic embryos or propagules individually encapsulated in a hydrogel such as, calcium alginate. Desiccated artificial seeds are produced by coating somatic embryos, roots and callus in polyoxyethylene glycol. The coated mixture is allowed to dry for several hours on a teflon surface in sterile hood, before culturing for germination. At present, these methods are not completely satisfactory. Hydrated capsules are more difficult to store because of the requirement of embryo respiration; moreover these capsules dry out quickly unless they are kept in a humid environment or coated with a hydrophobic membrane (Redenbaugh *et al.*, 1987a; b; Gray, *et al.* 1995). In desiccated artificial seeds the desiccation process itself may damage the propagules.

The use of somatic embryos as artificial or synthetic seeds was long felt as a promising alternative to conventional propagule production in many crops. The main advantages of somatic embryos are that their production could be completely automated, and large quantities of embryos could be produced in closed system without much manual labour. However, somatic embryogenesis is not common in tissue cultures of many of the crops. In crop species where somatic embryogenesis is not common, attempts were made to encapsulate *in vitro*-derived vegetative propagules in an appropriate gel and to use the encapsulated segments as artificial or synthetic seeds. This technique offers tremendous opportunities for production of

large quantities of disease-free propagules from *in vitro* plantlets in protected condition within a limited time and space (Uozumi and Kobayashi, 1995; Piccioni and Standardi, 1995; Standardi and Piccioni, 1998).

Synthetic seeds were developed by encapsulating somatic embryos in carrot (Kitto and Janick, 1985a, b), groundnut (Padmaja *et al.*, 1995), *Eleusine coracana* (George and Eapen, 1995), *Santalum album* (Fernandes *et al.*, 1994), etc., axillary or apical shoot buds in *Morus indica* and sandalwood (Bapat *et al.*, 1987; Bapat and Rao, 1990; Bapat, 1993), alfalfa (McKersie and Bowley, 1993), *Valeriana wallichii* and *Dioscorea* sp. (Hasan and Takagi, 1995), *Coleus forskhlii* (Bhattacharyya *et al.*, 2001), flower buds in citrus (Mitra and Chaturvedi, 1972) and apple root stocks (Sakamoto *et al.*, 1995; Piccioni, 1997; Capuano *et al.*, 1998; Sicurani *et al.*,2001). The encapsulation of shoot buds and production of synthetic seeds were reported in orchids like *Phaius tankervillae* (Malemnganba *et al.*, 1996) and *Spathoglottis spicata* (Nayak *et al.*, 1998), *Geodorum densiflorum* (Datta *et al.*, 1999). Plant regeneration from protoplasts encapsulated in alginate beads was reported in *Pogostemon cablin* (Kageyama *et al.*, 1995).

There is potential for the storage of artificial seeds, or the distribution of germplasm in the form of encapsulated embryos or apices (Hassan and Takagi, 1995). The advantages of synthetic seeds over sexually produced seeds are, i) they are rapidly produced at any time of the year, ii) They can be produced in large numbers, and iii) they are genetically the same as the original plant. Thus, the major interest is in the commercial production of seeds by a controlled process, with the possibility of production whenever demand occurs. There is considerable potential for the use of synthetic seeds for germplasm exchange, and perhaps for storage of plant germplasm.

The latter will require the development of synthetic seeds, which can withstand both desiccation and low temperature regimes (Seneratna and McKersie, 1989; Ashmore, 1997). Synthetic seeds can be an ideal system for low cost plant movement, propagation, conservation and exchange of germplasm.

***In vitro* storage organ formation**

Many plant species are normally propagated and stored in the form of vegetative storage organs like bulbils, cormlets, protocorms, microtubers, etc. Such storage structures can be induced to form in tissue cultures of several plant species and they often provide a convenient means of micropropagation and genotype storage. Cormlets were induced in *Gladiolus* sp. either directly from explants or from callus cultures (Ziv *et al.*, 1970; Ziv and Halevy, 1972). Microtubers are miniature tubers developed under tuber inducing conditions *in vitro*. These small dormant tubers are particularly convenient for handling, storage and distribution. Unlike micropropagated plantlets, they do not require time-consuming hardening period in greenhouses, and may be adapted easily to large scale mechanized planting in the field. Many protocols have been developed to induce microtubers in potato. Explants can be nodal cuttings, excised stolons, microshoot cuttings or whole microplants. Wang and Hu (1982) first reported *in vitro* mass tuberization in potato and successful integration of this technology into virus-free seed potato production programme in Taiwan. Subsequently, this technique was adopted at International Potato Centre (CIP), Lima, Peru (Estrada *et al.*, 1986) and many other organizations in developing complementary methods of clonal propagation for commercial potato production. Miniature tubers or microtubers were induced in potato (Wang and Hu, 1980; Hussey and Stacey, 1981) and yams (Ng, 1988) on medium containing high cytokinin levels.

These miniature storage organs have great advantage as they can be readily removed from culture flasks in a dormant condition and can be stored *ex vitro*. On transfer to soil, they behave as normal tubers and produce plants from axillary shoots and thus can be a source of disease free planting material (George, 1993).

Much of the published work on potato microtuber induction *in vitro* is focussed on the use of growth regulators. In particular, emphasis has been given on the role of cytokinins, especially N^6-benzyladenine (BA) to stimulate the process (Wang and Hu, 1982; Estrada *et al.*, 1986). Various other substances including abscisic acid (Hussey and Stacey, 1984), chlorocholine chloride (Tovar *et al.*, 1985; Vecchio *et al.*, 2000), ancymidol (Ochotorena *et al.*, 1999) and jasmonic acid (Castro *et al.*, 2000) have also been investigated. Potato microtubers can also be induced on medium free of growth regulating substances (Garner and Blake, 1989).

In addition to growth regulating factors, other important nutritional factors that affect potato microtuberization are i) type and concentrations of carbon source, and ii) inorganic nitrogen and potassium contents of the induction medium. Sucrose is the most effective carbon source for potato microtuber induction. An increase in sucrose concentration from 1 to 8 % induces early tuberization (Wang and Hu 1982; Hussey and Stacey, 1984), but at sucrose levels above 8% the tuberization is inhibited (Garner and Blake, 1989). It has been reported that as high as 60% sucrose is utilized during *in vitro* tuberization, and there is no quantitative relation between sucrose absorption and reducing sugar appearance in the medium (Khuri and Moorby, 1995). Increased sucrose utilization results in an increase in microtuber weight and yield, but does not have any effect on microtuber number.

In general, high nitrogen is inhibitory to microtuberization in potato. In the absence of growth regulating substances, a reduction in total nitrogen supply or an increase in the ratio of ammonium to nitrate reduces the size and number of microtubers (Garner and Blake, 1989). However, cytokinin-induced microtuberization is not sensitive to the inhibitory effect of high nitrogen level in the medium (Wang and Hu, 1982).

Temperature and photoperiod are two important physical factors that affect potato microtuber induction *in vitro*. The optimum temperature for *in vitro* tuberization is 20°C with a constant temperature being more effective than alternating day-night temperature (Wang and Hu, 1982; Veramendi *et al.*, 2000). Temperatures below 12° C and above 28° C have been found to be inhibitory to potato microtuber production (Wang and Hu, 1982). It has also been shown that in the absence of growth regulating substance, especially cytokinin, the lower temperatures become the essential tuber-inducing agents. It has been suggested that the critical factor determining the photoperiod requirement during potato microtuberization is whether cytokinin is used for induction or not. In general, optimum microtuberization occurs under continuous darkness during cytokinin-induced tuberization, but a longer photoperiod with higher light intensity is required when cytokinin is not used. The density of explants in the culture vessels during microtuberization period also influences microtuber size and weight (Sarkar *et al.*, 1997).

The effect of low doses of gamma irradiation on the production of microtubers in different cultivars was also investigated. Irradiation of explants with 2.5 Gy of gamma radiation led to a significant increase of about 38% over the control in the number of microtubers without any genetic change (Al-safadi *et al.*, 2000).

The harvested microtubers are dormant, and, therefore, required to be stored at 5–6° C for 3–4 months before planting on nursery beds or in the field. Microtubers have been reported to exhibit no dormant period (Hussey and Stacey, 1981, 1984) and have dormant periods of 1 to 7 months (Estrada *et al.*, 1986).

There are several reports on the investigations conducted on *in vitro* micro-tuber formation and their application in medium-term to long-term conservation of potato germplasm (Sylvestre, 1983; Tovar *et al.*, 1985; Bohac and Miller, 1988; Kwiatkowshi *et al.*, 1988; Mitten *et al.*, 1988; Ashmore, 1997; Steinitz, 1997; Malaurie *et al.*, 1998).

GENETIC STABILITY OF CONSERVED GERMPLASM

In vitro culture methods are essential to conserve germplasm resources for species where it is impractical, difficult or impossible to conserve seed. An extensive literature has developed on *in vitro* methods for conserving plant germplasm resources (Kartha, 1982; Withers and Williams, 1982; Wilkins *et al.*, 1982; Engelmann, 1997; Razdan and Cocking, 1997). The principle of genetic conservation that materials retrieved should represent the materials accessed has caused concern about the use of *in vitro* systems because of the occurrence of somaclonal variation; hence the emphasis, on adequate monitoring in the *in vitro* genebanks (Withers, 1986). It is particularly important with *in vitro* collections to monitor the genetic stability of collections, since somaclonal variation can arise in plant tissue culture. The need to maintain genetic integrity in conserved germplasm is implicit, whilst accepting that absolute stability is not the norm in nature and may not even be desirable in evolutionary terms. This variation has possible benefits as an adjunct to

plant improvement but is a serious disadvantage for *in vitro* germplasm conservation and exchange (Scowcroft, 1984; Israeli *et al.*, 1994).

TISSUE CULTURE INSTABILITY AND SOMACLONAL VARIATION

Variation occurs at all tissue culture levels. The phenomenon of genetic variation during culture has been recognized for many years and has been observed in many plant species. Variations in callus and suspension cultures is referred to as tissue culture instability and variation observed among plants regenerated from tissue cultures is described as somaclonal variation (Larkin and Scowcroft, 1981; Scowcroft, 1984). Tissue culture instability and somaclonal variation is ubiquitous. Genetic instability and somaclonal variation has been recorded in callus, liquid suspensions, cultured protoplasts, anther culture, adventitious shoots, somatic embryos, in plants regenerated from tissue cultures and among seed and vegetative propagules of regenerants. *In vitro* plant culture systems may be ranked for instability (low to high) as meristem multiplication, adventitious shoots, somatic embryogenesis, organogenesis from callus and cultured protoplasts (Scowcroft, 1984). As a consequence of instability there is variation in ploidy levels and chromosome structure, morphological and biochemical traits and changes at the nucleotide level have been observed for both nuclear and cytoplasmic genomes.

The genetic and phenotypic changes include changes in chromosome number and structure, biochemical changes such as loss of secondary product production, changes at the DNA level, or changes in characteristics such as disease resistance or plant height which are detected when whole plants are regenerated from culture (Lee and Phillips, 1988; D'Amato, 1989; Brown *et al.* 1991; Karp, 1994). Some of these

changes are the result of heritable genetic mutations, whereas others may be epigenetic changes which will not be transmitted to the next generation.

The underlying causes of somaclonal variation remain undetermined and unpredictable. In addition to the external factors such as genotype or type of explant, factors such as culture environment, the level and types of plant growth substances supplied to the medium and the degree of cellular organization seem to affect the level and type of variation (Vuylsteke and Swennen, 1990; Karp, 1994). Extensive experiments have not yet been conducted to define the relative impact of various components and aspects of tissue culture on somaclonal variation. However several features of tissue culture are already known to have an influence on the level of somaclonal variation in plants. The factors influencing somaclonal variation are described as sexual versus asexual species, preexisting versus culture induced variation, genotype of the donor plant, explant type and culture mode and culture conditions such as media composition, time of culture etc. Somaclonal variation is more likely to be enhanced, a) in asexually propagated species relative to seed propagated species, b) if callus formation is a significant phase in the regeneration cycle, c) with increased duration of culture, d) in certain genotypes, and e) from specific explants (Scowcroft, 1984; Gould, 1986).

The degree of genetic instability can range from minimal to considerable depending upon the culture systems used. Somaclonal variation is more common in disorganized culture such as callus or suspension culture and occurs less frequently where organized plant structures are maintained in culture (Scowcroft, 1984; Karp, 1994). Any *in vitro* technique utilized in the conservation of plant germplasm should be ideally based on the use of existing meristem, and should attempt to restrict the

development of adventitious meristems in culture, and particularly avoid disorganized callus growth. This is sometimes difficult to realize, and a balance is vital between the need for the multiplication of the material and maintenance of its genetic integrity. In *Musa* spp., even where single plantlets are directly regenerated from an existing meristem, somaclonal variation has been observed (Vuylsteke and Swennen, 1990).

Crop species also vary considerably in the type and extent of somaclonal variation observed. In *Musa* spp. somaclonal variation occurs quite frequently (from 2.4% to as high as 69.0%), but the variants are commonly mutations that also arise as natural mutations during field propagation of this species (Vuylsteke, 1989). Micropropagation of *Vitis vinifera* also has been shown to result in the production of plants with both morphological modifications and reduced bud fertility in the first generation (Grenan, 1994).

IN VITRO CONSERVATION – IMPACT OF CULTURE INSTABILITY AND SOMACLONAL VARIATION

In vitro germplasm storage currently uses slow growth techniques rather than cryopreservation. Minimal growth conditions put germplasm integrity at serious risk because of directional genetic change in response to selection, and somaclonal variation is possibly enhanced, particularly where regeneration is adventitious. Cryopreservation of meristem and shoot tips provides greatest security against genetic instability during *in vitro* germplasm conservation (Scowcroft, 1984). Although the risk of genetic instability may be minimized through the use of highly organized culture systems, careful monitoring of cultures will still be required. This may prove difficult while plantlets remain in culture vessels as many mutations may only be recognized in later stages of development of the plant, or morphological features of

the plant may be reversibly modified in the tissue culture environment. Some regular assessment must be made of plants in field conditions as well as during culture. Ideally, simple monitoring systems need to be developed for each crop species (Ashmore, 1997).

Eventual plant regeneration will be the aim in the majority of cases of tissue storage. Thus it is important that morphogenic potential be retained. *In vitro* material presents a special challenge in that the morphological features, which help, define the plant in question are likely to be either absent or modified in culture. Therefore alternative methods of characterization and evaluation are particularly available. These include nuclear cytology, isozyme analysis and secondary product synthesis. Molecular biological techniques are likely to offer further possibilities (Withers, 1980). The simplest way of retaining the morphogenic potential is to ensure that the culture remains in an organized form throughout. Thus meristem tips can be cultured to produce clonal plantlets from nodal/axillary meristems (Morel, 1975; Murashige, 1977; Mantell *et al.*, 1978; Walkey, 1978). The possibility of local mutations or abnormal mitoses producing variant genotypes must be entertained along with the more serious consideration of adventitious bud formation on deformed or callused explants. Once these possibilities are considered, meristem culture is as vulnerable to genetic change as is a callus or suspension culture, with resultant genetic variability in the progeny (Murashige, 1977).

Some of the earliest reports of genetic instability in culture were made on the basis of chromosome counts (D'Amato, 1975) but it is known that this approach will reveal only crude genetic change. Correlation between the incidence of chromasomal instability and disorganized growth are cited widely. Cytological check of

appropriately selected accessions from the *in vitro* gene bank will be essential without over estimation of the resolution of the information, particularly in the case of no apparent change (Withers, 1989).

Bayliss (1980), following D'Amato (1978), comprehensively reviewed the reported instances of chromosomal variation in tissue cultures. The classes of variant cells included polyploid and aneuploid changes, structural changes in chromosome morphology, and mitotic aberrations including multiplolar spindles, lagging chromosomes, fragments and asymmetric chromatid separation. Tissue cultured plants with extensive chromosome rearrangements were reported in *Lolium* sp. (Ahloowalia, 1983) and *Medicago sativa* (Reisch and Bingham, 1981).

Clonal variation in culture morphology and growth rate has often been described (Blakely and Steward, 1964; Sievert and Hildebrandt, 1965; Davey *et al.*, 1971; Snijman *et al.*, 1977). Denton *et al* (1977) reported phenotypic variation in plants of *Solanum tuberosum* regenerated directly from shoot tip culture. Genetic changes alone are not thought to be completely responsible for the loss of morphogenic potential, although ploidy shifts, aneuploidy and the possibility of non-morphogenic cell lines having a selective advantage in culture have been proposed (Murashige and Nacano, 1967; Smith and Street, 1974). Careful adjustments of the culture conditions and medium constituents may be required to reveal morphogenic potential during prolonged culture (Asuwa, 1972; Drew, 1979; Koblitz and Schumann, 1976). Although some genetic changes in culture will lead to the loss of morphogenic potential, this will not always be the case. Regenetant plants may embody genetic changes produced in the preceding culture period. Although some morphological differences between parent and progeny plants may be attributable

e.g., to virus eradication (Kartha and Gamborg, 1979; Skirvin, 1978), chimeral segregation (Skirvin, 1978; Skirvin and Janick, 1976) or exposure to certain hormones during the culture period (Ibrahim, 1969), there are many cases where a novel genetic changes are involved.

IN VITRO CONSERVATION – ASSESSMENT OF GENETIC STABILITY

The *in vitro* genebank will hold very large numbers of cultures maintained under conditions where their phenotype is either modified as an essential component of the storage procedure or inaccessible to inspection during storage as in cryopreservation. Visual inspection of cultures in slow growth will check for viability loss and microbial contamination, but meaningful monitoring of stability by eye will be difficult as vigour is intentionally being depressed. Most species once committed to culture cease to resemble the parent plant, especially at the level of discrimination of the species. Furthermore, the *in vitro* phenotype can change both randomly and progressively with time with respect to behavior and morphology. In any storage methods it is essential that reliable descriptors and descriptor state be determined for the *in vitro* condition. Additionally plants should be transferred to *in vivo* growth at appropriate intervals to verify assumptions made *in vitro* (Withers, 1989).

BASIC TOOLS FOR ASSESSMENT OF GENETIC STABILITY - A BRIEF REVIEW

Germplasm in the form of *in vitro* cultures cannot be characterized by using the normal procedures of morphological measurement of those characteristics that are highly heritable. Genetic stability of cultured material can be analyzed through cytological, biochemical and molecular markers, in addition to morphological evaluation.

Morphological characterization

The success of any *in vitro* culture technique depends either on the ability to clone the genotypes for production of uniform planting material, or the ability to bring about variations, which can be exploited in crop improvement programmes. The genetic uniformity of plants multiplied by tissue culture depends on a number of factors, the two most important being the method of multiplication and the genotype. Accumulated information now shows that plants propagated by precocious shoots show no more spontaneous mutation than those propagated by conventional means. The evidence for this comes mainly from the successful large-scale operation in commercial tissue culture laboratories throughout the world. In contrast, plants regenerated from callus or cell suspension cultures may include a varying proportion showing structural or physiological abnormalities depending upon the species, origin and the age of culture (Yeoman, 1986), various other factors like growth regulators (Singh and Harvey, 1975; D'Amato, 1978; Zakhlenyuk and Kunakh, 1987), composition of the culture medium (Bayliss, 1977; Feng and Quyang, 1988), culture conditions (Cerutti, 1985; Jackson and Dale, 1988) and culture method (Wilson *et al*, 1976) influence somaclonal variation. The reasons for variations in micropropagated plants can be due to the variation that existed in the source plant (pre-existing variation), epigenetic or physiological effects and genetic changes (Swartz, 1991; George, 1996). Extensive studies conducted during the last decade have shown that the cell and callus cultures especially on periodical subculture undergo various morphological and genetic changes i.e., polyploidy, aneuploidy, chromosome breakage, deletions, translocations, gene amplifications, inversions, and mutations (Nagl, 1972, D'Amato, 1985). In addition, there are changes at the molecular and

biochemical level including changes in the DNA, rearrangement of genes, somatic crossing over, altered nucleotide methylation, perturbation of DNA replication by altered nucleotide pools, slicing or activation of genes by mutations in associated non coding regions and transposons (Scowcroft, 1984) and enzymes (Cullis, 1983; Day and Ellis, 1984; Ball and Seilleur, 1986; Brettel *et al*, 1986).

Most of the somaclonal variations observed among tissue cultured plants are either genetic or epigenetic in nature. According to Phillips *et al* (1990), most of the mutational events occurring in tissue culture are directly or indirectly related to alterations in the state of DNA methylation. When DNA is highly methylated, gene activity is suppressed. A decrease in methylation correlates with increased gene activity. An increase or decrease in DNA methylation, might account for quantitative mutations such as those controlled by single recessive genes, for increased transposable element activity for simultaneous changes in quantitative characters and for the mutations caused by chromosome breakage. It is also suggested that increased transposable element activity could be responsible for the genetic changes in callus cell culture, which occur during prolonged incubation. DNA methylation was also found to affect DNA replication (George, 1993).

Biochemical characterization using *isozyme analysis*

Electrophoretic examination using isozyme analysis is a relatively simple and potentially widely applicable technique. Isozyme analysis may also be used as tool for assessing the stability of cultures. IBPGR has used this technique for cocao, taro and *Musa* sp. (Withers, 1985d) and a report is published by Simpson and Withers (1986).

Markert and Mullor (1959) proposed the term 'isozyme' (synonymous with 'isoenzyme') for multiple molecular forms of an enzyme sharing a catalytic activity,

derived from a tissue of a single organism. Proteins are attractive for direct genetic study because they are primary products of structural genes. Changes in coding base sequence will under any circumstances, result in corresponding changes in the primary structure proteins. Even simple amino acid substitutions deletions or additions can have marked effects on the migration of proteins under an electric field during electrophoresis. Isozymes are different forms of an enzyme exhibiting the same catalytic activity but differing in charge and electrophoretic mobility. In isozyme analysis, crude plant extracts are subjected to electrophoresis using starch or polyacrylamide gels. Following electrophoresis the enzymes of interest are detected by treating the gels with specific activity stains. Variations in banding patterns obtained between individual samples can be used to sort out genetically the varieties tested. The advantages of these techniques are, it is relatively simple and less expensive. The disadvantages are, availability of limited number of enzyme loci, and developmental and season dependent expression of activity.

Isozyme analysis of population variation can be carried out at intervals for a period of time to monitor genetic changes. This may be applied in studies aimed at detecting loss of genetic integrity during maintenance of samples in genebanks (Simpson and Withers, 1986). A new role of isozymes as monitors of variation has arisen with the development of tissue culture methods of germplasm storage (Withers, 1980; Withers and Williams, 1982; 1985). Some attention has been given to the isozyme complement of *in vitro* cultures and examples are listed in Table 9. Isozyme technology affords a unique opportunity of estimating the degree of genetic stability in cultures stored by methods of slow growth or cryopreservation (Withers and Williams, 1985). Isozyme markers could be used to assess some aspects of

biochemical stability in stored cultures; however, little attention has been given to the application of isozyme analysis (Withers, 1985d; Finkle *et al*, 1983). Marin *et al* (1993) analyzed the genetic stability of *Citrus sinensis* plantlets recovered after cryopreservation using peroxidase and esterase enzymes and no variability was observed except for minor differences in intensity of the bands. Similar observations have been reported in sugarcane also (Paulet *et al.* 1993).

Table 9. List of species in which isozymes have used in the *in vitro* studies

Family	Genus and Species	Reference
Caricaceae	*Carica* spp.	Moore and Litz (1984)
Caryophyllacae	*Dianthus caryophyllus*	McCown *et al* (1970)
Graminae	*Hordeum* spp.	Orton (1980a; 1980b)
	Saccharum spp.	Heinz and Mee (1971)
	Triticum aestivum	Larkin *et al* (1984)
	Zea mays	Khavkin and Sukhorzhevskaia (1979)
	Zea mays	Rhodes and Green (1981)
	Zea mays	Zeleneva and Khavkin (1980)
Leguminosae	*Phaseolus vulgaris*	Arnison and Boll (1974; 1975)
Linaceae	*Linum usitatissimum*	Liau *et al* (1974)
Palmae	*Phoenix dactylifera*	Finkle *et al* (1983)
Rosaceae	*Malus* spp	Vinterhalter (1979)
Solanaceae	*Nicotiana tabacum*	Jong *et al* (1968)
	Nicotiana tabacum	Thorpe and Gasper (1978)
Umbelliferae	*Apium graveolens*	Orton (1983)
	Daudus carota	Chourey and Wildholm (1980)
	Daudus carota	Lee and Dougall (1973)

Simpson and Withers (1986)

Analysis by electrophoresis of families of polymorphic enzymes provides a means of describing phenotypes at the level of primary gene product (Simpson and Withers, 1986). It provides information on genetic identity that is generally less prone to perturbation by environmental conditions than by either morphology or agronomic

evaluation. However several enzymes should be examined in any one study and isozyme analysis should be used in parallel with other methods of evaluation (Withers, 1989). Several authors illustrate the usefulness of this technique in plant breeding and other applied areas, as well as in basic studies of genetics and developmental biology (Markert, 1975; Rattazzi *et al.*, 1983; Tanksley and Orton, 1983).

Shamina *et al.*, (1997) studied the variations in total free amino acids, proteins total proteins and isozymes in 25 accessions of ginger germplasm and the variability for the isozyme loci in the population was generally low. Fifteen accessions of *Curcuma longa* were studied for variations based on isozyme polymorphism and a high degree of variability was reported (Shamina *et al.*, 1998).

Molecular characterization

One of the earliest biochemical markers to be used was isozyme patterns and they have been the method of choice in several cases. However isozyme analysis has its inherent disadvantages like limited number of enzyme loci and development and season dependant enzyme expression. With the advent of molecular biology techniques, DNA based markers replaced enzyme markers in germplasm identification and characterization and in gene tagging. Owing to its plasticity, ubiquity and stability, DNA is the ideal molecule for such analysis (Caetano-Anolles *et al.*, 1991). Polymorphism in the nucleotide sequence is sufficient for it to function as a molecular marker in mapping.

There is currently a great deal of interest in the use of molecular markers as an alternative or additional technique for monitoring the genetic stability of the conserved materials. PCR based technologies may be the most useful for this purpose

because of the simplicity of use and its relatively low cost so that it can be routinely used in genebanks. The simplest of these PCR techniques, RAPDs, may not, however, be very reproducible and either RFLP or AFLP markers may be more appropriate as reliable markers for further studies on somaclonal variations (Karp and Edwards, 1997).

Isolation and purification of genomic DNA

Isolation and purification of high molecular weight DNA is a fundamental requirement for the identification and isolation of genes for plant genetic engineering as well as for the study of gene organization and gene expression at the molecular level. Besides their use in gene cloning and gene construction, nucleic acid isolation and analysis techniques are integral to many procedures used to determine the physical structure and expression of transferred genes in both transformed tissues and whole regenerated plants (Draper and Scott, 1988).

There are a number of procedures for the preparation of genomic DNA. They all start with some form of cell lysis, followed by deproteinisation and recovery of DNA. The main differences between various approaches lie in the extent of deproteinisation and in molecular weight of the DNA produced. A critical factor in the isolation of plant DNA is the efficient disruption of the plant cell wall. Most of the protocols used for isolation of DNA consist of two parts, a technique to lyse gently the cells and solubilise the DNA, followed by one of several basic enzymatic or chemical methods to remove contaminating proteins, RNA and other macromolecules (Ausubel *et al*, 1995). Unfortunately, many techniques for breaking open cells also shear DNA and thus any method must be a compromise between DNA length and yield. During DNA isolation from plant extracts, large amounts of polysaccharides,

tannins and pigments were often found as contaminants and are difficult to separate from DNA. These contaminants interfere with quantification of nucleic acids by spectrophotometric methods and also can inhibit the activity of most of the restriction enzymes and other DNA modifying enzymes and cause problems during genome cloning.

Plant cells are usually disrupted in aqueous solutions, containing chelating agents to inhibit nuclease action and detergents for solubilising membranous materials. The commonly used detergents are cetyl triethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) (Murray and Thompson, 1980; Rogers and Benedicht, 1985). The proteins from the extract are denatured and precipitated using phenol or chloroform-octanol (Kirby, 1957; Penman, 1966; Palmiter, 1974). Ethanol precipitation is useful for concentrating the remaining DNA solution and for removing residual phenol and chloroform from the deproteinised aqueous solutions. It is also useful for isolating DNA that is relatively free of solute molecules and ethanol precipitation and washing of the pellet will effectively desalt DNA.

DNA can be isolated from various types of tissues, which includes whole seedlings leaves, cotyledons, seeds/grains, endosperm, embryos, tissue culture callus and pollen. Milligram amounts of tissue can be used when sample size is limited (Dellaporta, *et al.*, 1983).

DNA can be isolated from lyophilised tissue, fresh tissue, protoplasts, nuclei, mitochondria and choloroplasts, depending upon the need and purity requirements of the isolated DNA. Each follows a specific protocol (Draper and Scott, 1988). Wherever pure DNA preparations are required, the DNA in the dissolved nucleic acid

preparation can be purified on a cesium chloride (CsCl) density gradient by ultra centrifugation.

The aim of any genomic DNA preparation technique is to isolate high molecular weight DNA of sufficient purity. Two factors affects the size of the DNA isolated namely, shear force and nuclease activity. As noted in the protocol lysates should be treated gently to minimize shear forces. Plant cells are rich in nucleases. To reduce nuclease activity, the tissue should be frozen quickly and thawed only in the presence of an extraction buffer that contains detergent and high concentration of EDTA (Ausubel. *et al.* 1995).

Plant DNA isolated using the basic protocol should be in the range of 50 kb in length which is quite acceptable for most of the applications. In some cases it may be necessary to modify the steps in order to reduce contamination by polysaccharides, phenolics and other molecules that interfere with DNA isolation. Polysaccharides pose the most common problem affecting plant DNA purity. These carbohydrates can be removed by chloroform extractions of lysates in the presence of 1% CTAB and 0.7 molar NaCl as described by Murray and Thompson (1980).

The CTAB purification ensures 100 to 500 µg of DNA per gram of fresh plant tissue. DNA > 50 kb can be obtained if care is taken not to shear it (by using wide bore pipettes and gentle mixing) and if nucleases are avoided (by keeping tissue frozen or lyophilized and thawing or dehydrating, only in the presence of CTAB extraction buffer). The CTAB method should take between 2 to 6 h depending on the species and quantity of starting material desired, purity and yield (Ausubel, *et al.*, 1995).

Measuring the DNA content of a sample is usually done using a scanning spectrophotometer between 200 and 300 nm. The DNA shows a clear absorbance peak at 260 nm (1.0 OD_{260} = 50 µg/ml). Pure DNA solutions are transparent and have an OD_{260} : OD_{280} ratio of 1.8 (Draper and Scott, 1988).

DNA was isolated from many plant species using one of the protocols available; however, the most commonly used protocols are CTAB method or SDS method (Ausubel *et al.*, 1995). The CTAB method of DNA isolation is widely used in plants because of its versatility. The total genomic DNA has been isolated from many genera of monocotyledons and dicotyledons adopting these techniques (Murray and Thompson 1980; Rogers and Benedicht, 1985).

DNA based molecular markers

Genetic information that make up genes in higher plants is stored in the DNA molecules. There is such an enormous amount of DNA in higher plant cells, that no two organisms are likely to be identical in the DNA base sequence. Natural variation in DNA sequence can be detected in several ways. Advances in molecular biology during the last two decades have proved a new class of genetic markers, which can detect polymorphism at DNA level. Molecular markers are now used for plant classification and breeding. Polygenic characters, which are very difficult to analyze using traditional methods, can be easily tagged using molecular markers. Molecular markers can be used for, characterization of germplasm, varietal identification, clonal fidelity testing, assessment of genetic diversity, validation of genetic relationship, phylogenetic and evolutionary studies, and marker assisted selection (Waycott and Fort, 1994; Fang *et al.*, 1998; Gilbert *et al.*, 1999; Bhat, 1997; Wolfe and Liston, 1998; Smith and Beavis, 1998).

The first five uses have widespread application in management of genetic resources and in crop improvement, while marker assisted selection has virtually revolutionized breeding programmes. Molecular markers could be used to trace linkage with traits of importance, especially multigenic or quantitative traits that are difficult to deal with when relying on phenotypic assay alone. This approach permits the breeder to make an early decision about his selections while examining fewer plants. Molecular markers have been used to develop linkage maps for many important crop species (Helentjaris *et al.* 1985; Bernatzsky and Tanksley 1986; Mc Couch *et al* 1988;) with concentrated efforts on cereals.

Restriction Fragment Length Polymorphism (RFLP) is based on hybridization of a probe to a fragment of genomic DNA following digestion with restriction enzymes (Vos *et al.*, 1995). A probe is a specific DNA sequence designed to hybridize and thus detect a target sequence or sequences in the unknown sample. Usually the probes are either cDNA or genomic clones. Differences in the sequences at or around the sequence with which the probe hybridizes may result in differences (polymorphisms) in the length of the fragments detected by the probe. Genomic DNA from the sample being tested is digested with a restriction endonuclease. These enzymes cleave the DNA at specific sites with sequences (four or more base pairs) recognized by the enzyme. The resulting DNA fragments are separated by an agarose gel and transferred by blotting on to a nylon membrane to allow the hybridization with a probe. The DNA is cross-linked to the membrane and hybridizes to the pre-labeled probe under conditions of appropriate stringency. From the nylon membrane the autoradiogram is developed (Vos *et al.*, 1995).

The remaining DNA markers are based on Polymerase Chain Reaction (PCR). Amplified fragment length polymorphism (AFLP) is a method for PCR amplification of restriction digests of genomic DNA following the ligation of oligonucleotides. Sequence Characterized Amplified Region (SCAR) is a term used to describe PCR methods developed by sequencing markers amplified in arbitrary primer experiments. The sequence of amplified product is used to design longer primers that offer great specificity. Simple sequence repeats (SSR) Di or Tri nucleotide repeats are common in plant genomes. These are also known as microsattellites. Micro satellites have been established as useful genetic markers in many plant species.

Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) is the molecular technique which utilizes the short oligonucleotide primers of arbitrary (random) sequence for the synthesis of DNA fragment mediated by *Taq* polymerase and this technique has been adopted widely. The profile obtained may be influenced by any variation in the method used to prepare the DNA template and the exact reaction compositions and conditions used in the PCR. This means the variation in certain factors like primer or template can result in the amplification of different products. Thus obtaining reliable results depends upon standardizing these conditions or identifying combinations of conditions that give consistent results. Standard primer, nucleotide and magnesium concentrations, exact reproduction of temperature cycling conditions and DNA polymerase type and activity are essential. However the key requirement for reliable and reproducible RAPD results is a consistent approach to sample preparations and DNA isolation i.e., both quality and quantity of DNA isolated.

For most plants primers that are 9 to 10 nucleotides are predicted to generate an average of 2 to 10 amplification products. The primers are generally random sequence biased to contain at least 50% Gs and Cs and to lack internal repeats. The products are easily separated by standard electrophoretic techniques and visualized by ultra violet illumination of ethidium bromide stained gels polymorphism results in change in either sequence of primer binding sites (eg. point mutations) which prevent stable association with primer or from changes, which alter the size or prevent successful amplification of target DNA. As a rule, size variants are only rarely deleted and individual amplification products represent one allele per locus. In inheritance studies amplification products are transmitted as dominant markers (Waugh and Powell, 1992).

This technique has wide application in the field of molecular biology. The DNA fragment polymorphism obtained were used for varietal identification and percentage determination gene tagging and in the construction of genetic maps in generating phylogenetic trees especially at intra specific level (Welsh *et al.*, 1992) and in studies on population genetics as done by Van and Buchman (1992) in *Microseries elegance* of Asteraceae. The important applications as relevant to this study are reviewed here under.

Varietal identification and percentage determination

Williams *et al.* (1990) showed that single primers detected polymorphism between amplification products. Welsh and Mc Clelland (1990) used DNA polymorphisms amplified using oligo nucleotide primers for DNA fingerprints in bacteria and rice. Caetano–Anolles *et al.*, (1991) used DNA amplification fingerprints to detect amplification length polymorphism in soybean cultivars.

RAPDs were shown to be applicable to interspecific comparison in the genus *Lycopersicon* (Klein-lankhort *et al.*, 1991).

Fukuoka *et al.* (1992) used RAPD for identification of rice accessions. They found that the number of amplification products increased with increase in GC content of the primer and demonstrated that RAPDs were useful polymorphisms in rice and superior to RFLPs for their technological simplicity. Vierling and Nguyen (1992) brought about high incidences of polymorphism between two diploid wheat species and He *et al.* (1992) detected DNA sequence polymorphism among wheat varieties.

Genetic fingerprints of *Theobroma* clones using RAPD markers have reported by Wilde *et al.* (1992). Lashermes *et al.* (1993) used RAPD fragments as genetic markers in *Coffea*. RAPD markers appeared to be of high value for characterization analysis and utilization of *Coffea* genetic resources.

RAPD markers indicated a low level of genetic variability within grain sorghum (Tao *et al.*, 1993) and cassava germplasm (Beeching *et al.*, 1993). RAPD markers gave the greatest discriminations among the cultivars of sweet potato (Connolly *et al.*, 1994). dos Santos *et al.* (1994) indicated that RAPDs provide a level of resolution equivalent to RFLPs for determination of genetic relationships among the genotypes.

RAPD markers are also utilized for studying the genetic diversity of coffee for genetic improvement (Anthony *et al.*, 1997) discrimination of Basidiomycetous species and strains (Yasuhiro *et al.*, 1999) identification of maize inbred lines (Zhang *et al.*, 1998), molecular polymorphism between root-knot nematode resistant and susceptible rice cultivars (Bose *et al.*, 1998)

GENETIC STABILITY ANALYSIS OF TISSUE CULTURED AND *IN VITRO* CONSERVED MATERIAL–SOME CASE STUDIES

Very little work has been undertaken or published on the comparative effects of slow growth and cryogenic storage on the stability of plant material. The most comprehensive study has been performed as a joint project between CIAT and IPGRI, which aimed at monitoring the genetic stability of cassava shoot cultures stored *in vitro* (IPGRI/CIAT, 1994; Harding, 1996; Engelmann, 1997). Limite reports are available on the use of morphological, biochemical and molecular characterization of tissue cultured or *in vitro* conserved material tom assess the genetic fidelity.

Genetic stability analysis of *in vitro* derived potato plants has been reported by Potter and Jones (1991). Morphological examination of cassava plants regrown in the field did not reveal any modification after 10 years of storage under slow growth (Angel *et al.*, 1996).

Plantlets of *Citrus sinensis* recovered after cryopreservation was evaluated under *in vivo* conditions and no phenotypic abnormalities were observed during a growth period of 2 years (Marin *et al.*, 1993). Plants regenerated from cryopreserved apices of strawberry and cassava was phenotypically normal (Kartha *et al.*, 1980; Bajaj, 1983). No differences were noted in the vegetative and floral development of several hundreds of oil palms regenerated from control and cryopreserved somatic embryos (Engelmann, 1991). In contrast, Fukai *et al.* (1994b) showed that a high percentage of plants regenerated from apices of a percinally chimeric chrysanthemum cultivar frozen using a classical protocol had an altered flower colour. The same group compared the effect of freezing carnation apices using a classical protocol and the encapsulation/dehydration technique (Fukai *et al.*, 1994a).

Encapsulation/dehydration ensured 100% recovery after freezing, and regrowth was rapid and direct, in contrast to slow and controlled freezing, where recovery rate was only 50%. This gives an emphasis on the importance of selecting the most appropriate freezing technique for any given material, not only as regards recovery rates but also as regards recovery pattern and genetic stability.

It has been shown that cell suspensions of numerous species maintain their biosynthetic and morphogenic potential after cryopreservation (Withers, 1985c; Kartha, 1987; Seitz, 1987). Lavender cell suspensions exposed to successive freeze-thaw cycles, exhibited an increase in the number of colonies recovered from cryopreserved cells with increase in number of freeze-thaw cycles. But the biosynthetic capacities of the recovered cells remain unchanged, suggesting a change in the population structure rather than genetic change (Watanabe *et al.*, 1985). Bercetche *et al.* (1990) noted that cryopreserved *Picea abies* embryogenic calluses recovered faster than non-frozen controls and resulted in the production of a more homogenous population with higher embryogenic potential.

Mannonen *et al.* (1990) showed that the production of secondary metabolites by cell cultures of *Panax ginseng* and *Catheranthus roseus* decreased drastically after 6 months of culture either in standard conditions with weekly subculture or in slow growth under mineral oil, whereas the productivity of cell suspensions cryopreserved and stored in liquid nitrogen during the same period was identical to that of the original culture. The ploidy level of plants regenerated from oil seed rape somatic embryos and sensitive dihaploids of potato was not modified by cryopreservation (Uragami *et al.* 1993; Ward *et al.* 1993).

Isozyme analysis of cassava plants conserved for 10 years under slow growthdid not show any vriability (Angel *et al.*, 1996). Isozymes analysis to determine genetic changes in cryopreserved sugarcane plants indicated that some changes occurred in the cryopreserved and field propagated plantlets (Glaszmann *et al.*, 1996).

Many reports are available on the genetic fidelity analysis of tissue culture derived plants using DNA markers such as RFLP, RAPD and AFLP. RFLP has been used for molecular characterization of tissue culture raised plants such as *Saccharum* sp. (Chaudhary and Vasil, 1993), *Frestuca* pratensis (Valles *et al.*, 1993); wheat (Chaudhary *et al.*, 1994) and *Angelica acutiloba* (Watanabe *et al.*, 1998). There are many reports on molecular characterization of tissue culture derived plants by the RAPD technique, in crops such as *Picea mariana* (Isabel *et al.*, 1993), *Triticum* sp. (Brown *et al.*, 1993), *Populus deltoids* (Rani *et al.*, 1995), cavendish bananas (Damasco *et al.* 1996); rose (Matsumoto and Fukui, 1996), sugar beet (Munthali *et al.*, 1996), peach (Hashmi *et al.* 1997; ginger (Rout *et al.*, 1998), rice (Yang *et al.*, 1999), *Fragaria X ananasa* (Kumar *et al.*, 1999); *Allium* sp. (Al Zahim *et al.*, 1999), *Lilium* sp. (Varshney *et al.*, 2001) and turmeric (Salvi *et al.*, 2001).

Molecular markers were used for the genetic stability analysis of *in vitro* derived potato plants (Potter and Jones, 1991). RFLP patterns of plants regenerated from sugarcane embryogenic cell suspensions were identical to those unfrozen controls (Chaudhary and Vasil, 1993). Several molecular types were uncovered in plants recovered from both control and cryopreserved sugarcane apices, thus indicating that the variation was not due to freezing but was preexisting among the *in vitro* mother plants (Glaszmann *et al.*, 1996). DNA analysis showed that a gene

integrated in the genome of a navel orange cell suspension was maintained after freezing and 1 year storage in liquid nitrogen (Kobayashi *et al.*, 1994). Modifications in the RFLP pattern were observed in potato plants stored for 6 months under slow growth on a medium supplemented with mannitol, whereas no such modifications were noted in plants regenerated from cryopreserved apices (Harding, 1991).

DNA analysis (RFLPs with homologous probes, RAPDs with 20 primers and DNA finger printing with the M13 probe) as well as examination of the morphology of cassava plants regrown in the field did not reveal any modification after 10 years of storage under slow growth (Angel *et al.*, 1996). Harding (1991) studied the molecular stability of ribosomal RNA genes in both slow growth cultures and cryopreserved shoot tips of potato (*Solanum tuberosum* L.). RFLPs were detected in 2 out of 16 of the plants recovered from slow growth, but no changes were observed in plantlets recovered from cryopreservation, or in control plants. Further investigation of potato plants grown on the mannitol supplemented slow growth medium indicated that hypermethylation of rDNA had occurred (Harding, 1994). Such changes in the methylation status may be heritable. Cryopreserved shoot-tips of *S. tuberosum* cultivar Brodick using the encapsulation/dehydration technique was analysed for stability of the nuclear-chloroplast genomic complex and identical chloroplast-DNA fragments were detected in all regenerated plantlets. This suggests that the observed nuclear DNA fragments and the chloroplast genome are stable in potato plants regenerated from cryopreserved shoot-tips. This study extends the range of molecular biological techniques, which can be applied to examine post-cryogenic storage stability in cryopreserved plant germplasm (Harding and Benson, 2000). The

application of microsatellite in the genetic stability analysis of cryopreserved germplasm of *Solanum tuberosum* was reported by Harding and Benson (2001).

RFLP was used to determine genetic changes in sugarcane plants regenerated after cryopreservation, and compared with field-propagated plants (Glaszmann *et al.*, 1996). The results indicated that some changes occurred in the field propagated and cryopreserved plantlets, although in the case of the cryopreserved material the changes were not the result of freezing, but were either present in the original explants or arose during the *in vitro* propagation of material prior to cryopreservation. These results suggest that freezing *per se* did not induce genetic instability, and that any variation *in vitro* may either be related to the original explant material, or may also occur as frequently in the field conserved material.

Conservation of DNA - DNA banking

Concurrent with the advancements in gene cloning and transfer has been the development of technology for the removal and analysis of DNA. DNAs are routinely extracted and immobilized on to nitrocellulose membrane where the DNA can be probed with numerous cloned genes. In addition, the rapid development of polymerase chain reaction (PCR) could routinely amplify specific oligonucleotides or genes from the entire mixture of genomic DNA. These advances, coupled with the prospect of loss of significant plant genetic resources throughout the world, have led to the establishment of DNA bank-net, an international network of DNA repositories for the storage of genomic DNA (Adams, 1997).

DNA is the basis of all genetic information contained in a plant and through techniques of gene sequencing, this information can be read and stored and the genes themselves can also be stored as DNA and used for germplasm conservation. This

option has to be considered where no other alternatives are available. This method of using *in vitro* biotechnology and particularly cryostorage of DNA and DNA rich materials is thoroughly discussed and currently being used in 40 institutions representing 25 nations that have expressed interest in DNA bank (Adams, 1988, 1990; Adams and Adams, 1991; Giannasi, 1991; Mattich *et al.*, 1992; Adams *et al.* 1994).

The conserved DNA will have numerous uses, such as, molecular phylogenetics and systematics of extant and extinct taxa, production of previously characterized secondary compounds in transgenic cell cultures, production of transgenic plants using genes from gene families, *in vitro* expression and study of enzyme structure and function and genomic probes for research laboratories (Adams, 1997). Specific structure, function, operation and general requirements for DNA bank net is described by Adams and Adams (1991) and Adams (1997).

Structure and operation of DNA bank

The recent advances in technology for the extraction and immobilization of DNA, coupled with the prospect of the loss of significant plant genetic resources throughout the world, has led to the establishment of DNA Bank. DNA Bank is involved in preserving DNA as well as *in vitro* cryopreservation of plant cells. DNA Bank should have both working nodes (DNA dispensing nodes) and reserve (base) nodes. The working nodes will be engaged in collection of plant material (by taxonomists), DNA extraction (by molecular biologists or trained staff), long-term preservation of DNA rich materials and/or extracted DNA in liquid nitrogen, DNA analysis/gene replication (by molecular biologists or trained staff) and distribution of DNA (genes, gene segments, oligonucleotides, etc.). Reserve (base) nodes are meant

for long term DNA preservation in liquid nitrogen and monitoring of potential DNA degradation, act as genetic reserve buffer for working nodes, replenishment of DNA if a working node experiences the catastrophic loss of storage parameters and DNA (Adams, 1997).

Each DNA sample should be split initially in to at least two or three portions. One portion should be available for the working node and the other one or two to the reserve node. Collection and preservation of DNA rich material in liquid nitrogen is a preferred method and in this case DNA should be extracted from this material as and when required (Adams, 1997).

CELL AND TISSUE CULTURE WORK IN ZINGIBERACEOUS GENERA

The genera included in the study are economically important crops in many parts of the world and hence reports are available from different laboratories across the world on biotechnological work on these crops. These reports, though mostly concentrated on micropropagation, include various other aspects like plant regeneration from callus cultures, *in vitro* conservation, synthetic seeds, production of flavour and flavour components using cell culture and exploiting somaclonal variation for disease resistance.

CARDAMOM

In vitro methods for clonal propagation of cardamom from vegetative buds have been standardized (Nadgauda *et al.*, 1983; Priyadarsan and Zachariah, 1986; Vatsy *et al.*, 1987; Reghunath and Gopalakrishnan, 1991). Kumar *et al.* (1985) reported the successful conversion of immature floral buds to vegetative buds and subsequently to plantlets. Many commercial laboratories are using micropropagation techniques for large-scale production of cardamom planting material. Field evaluation

of tissue cultured plants of cardamom showed that the micropropagated plants performed on par with suckers (Luckose *et al.*, 1993). Sudharshan *et al.* (1997) and Chandrappa *et al.* (1997) have also reported the performance of tissue-cultured plants. Rao *et al.* (1982) reported plant regeneration from callus cultures of cardamom. Reghunath and Priyadarsan (1992) reported occurrence of somaclonal variation in cardamom derived from axenic cultures of juvenile shoot primordia during their large-scale production.

Rao *et al.* (1982) reported the successful regeneration of plantlets from callus of seedling explants of cardamom. Priyadarshan and Zachariah (1986) reported plantlet formation via adventitious shoots from callus cultures. Protocols for organogenesis and plant regeneration from rhizome and vegetative bud-derived callus cultures were also standardized at IISR (Babu *et al.*, 1997). This excellent regeneration system (with about 20–50 plantlets per culture) is being used at present for large-scale production of somaclones and selection of useful genotypes from them. High variability could be noticed among the somaclones for the morphological characters in the culture vessels itself. The somaclones are being evaluated in the field at IISR for realistic estimation of the genetic variability and few katte tolerant lines could be isolated and they are under advanced stages of screening (Peter *et al.*, 2001). Cryopreservation of cardamom seeds in liquid nitrogen (LN_2) was reported by Chaudhary and Chandel (1995).

GINGER

Micropropagation of ginger through tissue culture was reported by various authors (Hosoki and Sagawa, 1977; Nadgauda *et al.*, 1980; Pillai and Kumar, 1982; Sato *et al.*, 1987; Ilahi and Jabeen, 1987; 1992; Bhagyalaksmi and Singh, 1988; Inden *et al.*, 1988; Noguchi and Yamakawa, 1988; Sakamura and Suga, 1989, Wang, 1989;

Saradha and Padmanabhan, 1989; Balachandran *et al.*, 1990; Choi, 1991a; 1991b; Choi and Kim, 1991; Huang, 1995; Samsudeen, 1996; Babu, 1997; Palai, *et al.*, 1997; Sharma and Singh, 1997; Arimura, *et al.* 2000). Various explants like young sprouts, dormant buds, rhizome bits, meristem, pseudostem, etc. were used as explants. The nutrient medium used was mostly MS (Murashige and Skoog, 1962). Though other media like Gamborg's B5 (Gamborg *et al*, 1968), White's (White, 1963), Smith's (Kassanis 1967) and Schenk and Hildebrandt's (Schenk and Hildebrandt, 1972) were also used by some workers. The most commonly used growth regulators were cytokinins (BAP and kinetin) and auxins (2.4-D, IAA and IBA and in one case Decamba). Sharma and Singh (1997) reported high frequency *in vitro* multiplication of disease-free clones by culturing small and active buds of ginger on MS medium supplemented with 2 mgl^{-1} kinetin and plantlet regeneration was obtained in MS with 2 mgl^{-1} kinetin and 2 mgl^{-1} NAA.

There are a few reports on successful regeneration of ginger plantlets *via* intervening callus phase. Callus could be successfully induced from young sprouts, leaves and ovary tissue of ginger with the addition of auxins like 2,4-D., NAA, Decamba and plantlets were regenerated from them using either BAP or BAP with very low concentration of 2,4-D. Sometimes, removal of growth regulators from the culture medium resulted in plant regeneration and their further multiplication. The plant regeneration was either by organogenesis or by embryogenesis or both (Nadgauda *et al.*, 1980; Kulkarni *et al.*, 1987; Ilahi and Jabeen, 1987; Malamug *et al.*, 1991; Babu *et al.*, 1992a; 1996a; 1996b; Kackar *et al.*, 1993). Yeoman (1987) reported immobilization of ginger cells in polyurethane blocks.

Reports are also available on the anther culture of ginger. Callus formation and development of roots and rhizome like structures from excised ginger anthers cultured on MS medium containing 2,4-D and coconut milk were reported (Ramachandran and Nair, 1992b). Plant regeneration from anther derived callus cultures was also reported (Babu, 1997; Samsudeen *et al.*1997; 2000; Kim *et al.* 2000). Babu *et al.* (1992b) reported the successful conversion of immature floral buds to vegetative buds and subsequently to plantlets.

There are only two reports on evaluation of somaclones for useful agronomic characters. Kulkarni *et al.* (1987) reported variation in somaclones for their reaction to *Pythium* infection. They subjected ginger cell suspensions to *Pythium* culture filtrate by adding it to culture medium and subsequently regenerated plantlets from surviving cells. Three lines tolerant to *Pythium* sp. were isolated from the regenerating somaclones. Bhagyalakshmi and Singh (1994) reported that the micropropagated plants were on par with conventionally propagated ones except that they need longer (additional 2 months) crop duration for the same effect.

Another study showed that the somaclones have significant exploitable variations with regard to various morphological and biochemical characters like, number of tillers per plant, number of leaves per plant, plant height, rhizome yield per plant, contents of oleoresin, dry recovery and fibre contents (Samsudeen, 1996).

Since ginger rhizomes are affected by nematode infestations use of infested seed rhizomes can result in transmitting the nematodes to new areas and causing considerable crop losses. De Langhe *et al.* (1987) reported that when sprouting buds

were used as explants, the resultant plants were free of nematodes and suggested that this system was ideal for production of nematode-free planting material of ginger.

Ginger does not set seed naturally. Sharma *et al.* (1994) reported that the shoot buds of ginger could be encapsulated in calcium alginate beads to make synthetic seeds. These seeds germinated *in vitro* and thus can be used for planting material production and exchange. *In vitro pollination,* fertlization, fruit development and regeneration of plantlets from *in vitro* raised seeds was also reported (Babu *et al.*, 1996a; Nazeem *et al.*, 1996; Valsala *et al*, 1997; Babu, 1997).

Some attempts have been made to use tissue culture for conservation of ginger germplasm. The subculture interval could be extended up to 7 months to 1 year by using mannitol as osmoticum, polypropylene caps to minimize evaporation loss and application of mineral oil overlay (Balachandran *et al.*, 1990; Dekkers *et al.*, 1991; Babu, 1997).

In vitro rhizome formation and their germination were also reported by various workers (Sakamura *et al*, 1986; Sakamura and Suga, 1989; Bhat *et al*, 1994; Sharma and Singh, 1995; Babu, 1997). The *in vitro* rhizomes are good propagules and hence could be used for production and exchange of disease free planting material. Quality analysis of *in vitro* developed rhizomes indicated that they contain the same constituents as the original rhizome but with quantitative difference. The composition of basal medium seems to effect the composition of oil (Sakamura *et al*, 1986; Sakamura and Suga, 1989; Charlwood *et al*, 1988). Rout *et al.* (1998) reported genetic stability of micropropagated plants of ginger using RAPD markers.

TURMERIC

Successful micropropagation of turmeric has been reported (Nadgauda *et al*, 1978; Shetty *et al* 1982; Yasuda *et al*, 1988; Babu *et al*, 1997; Mukhri and Yamaguchi, 1986; sit and Tiwari, 1997). Plant regeneration from cultured immature inflorescence of turmeric was also reported (Salvi *et al.*, 2000). This technique could be used for production of disease-free planting material of elite plants. Organogenesis and plant regeneration was achieved from the callus cultures of turmeric (Shetty *et al*, 1982; Salvi *et al.*, 2001). Variants with high curcumin content were also isolated from tissue-cultured plantlets (Nadgauda *et al*, 1982). Salvi *et al.* (2001) reported genetic variability among the plants regenerated from the callus cultures of turmeric using RAPD markers.

In vitro microrhizomes could be induced in turmeric also. *In vitro* microrhizomes were produced in in MS medium with different concentrations of sucrose and / BAP and ancymidol (Reghu Rajan, 1997; Nayak, 2000). These microrhizomes are disease free propagules and hence could be used for production and exchange of disease free planting material.

KAEMPFERIA SPECIES

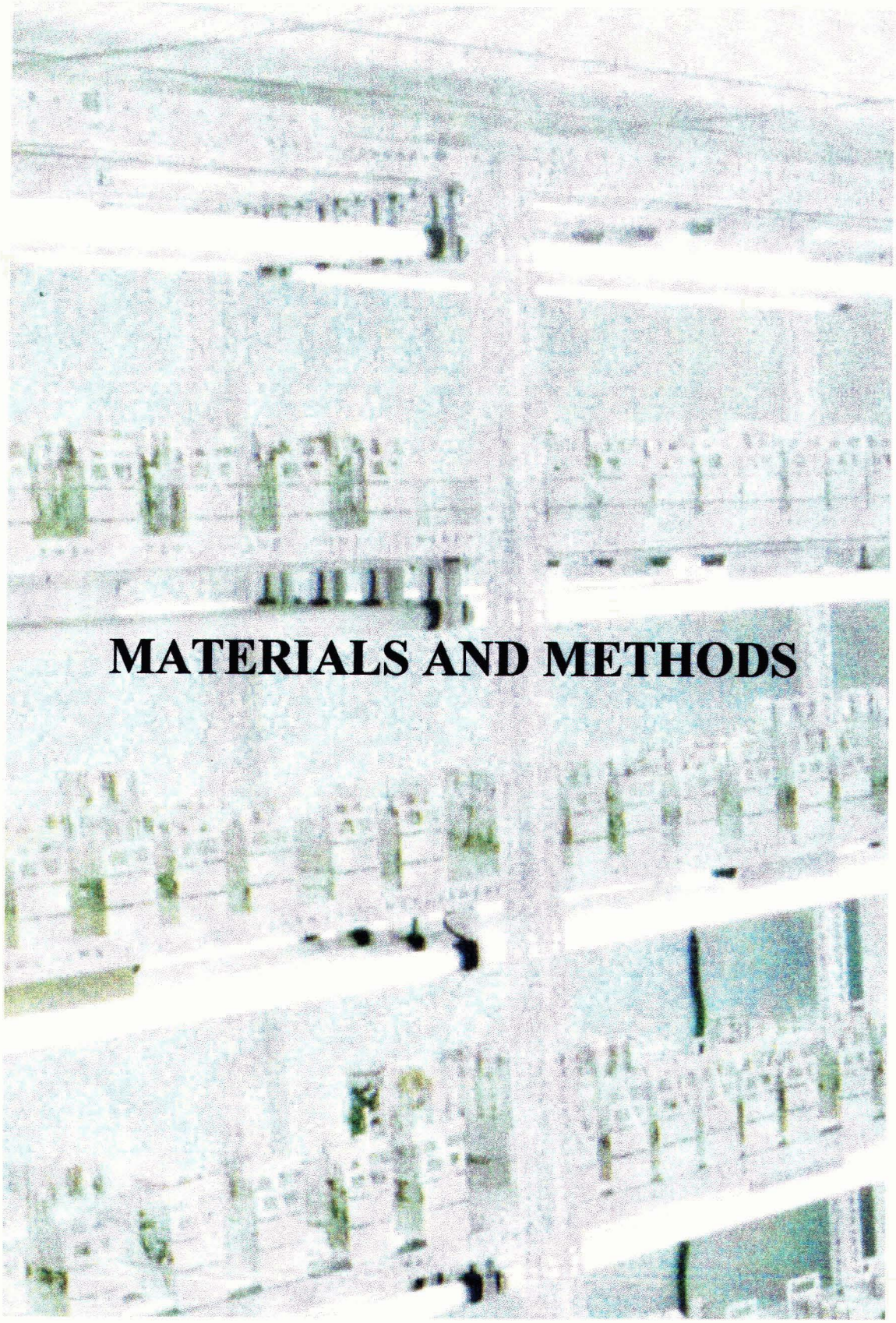
Economically and medicinally important zingiberaceous species like *K. galanga* and *K. rotunda* could be micropropagated (Vincent *et al*, 1992; Geetha *et al*, 1997). Successful plant regeneration and variations among regenerated plants were reported in *K. galanga* (Kumar and Seeni, 1995).

RELEVANCE OF THE PRESENT STUDY

Zingiberaceous species such as cardamom, ginger, turmeric, *K. galanga*, *K. rotunda* etc., are commercially important crops in India. These crops are propagated

vegetatively, and the germplasm of all these species are conserved in clonal field repositories. These crops are threatened by serious soil borne and seed borne diseases of bacterial, fungal and viral origin and outbreak of any of these diseases can wipe out the germplasm completely. In the case of cardamom, the crop is seriously affected by viral diseases and meristem isolation and culture is the only viable method for production of disease free planting material and storage. There is a high risk of mixing up of germplasm due to planting in the same area year after year through the leftover rhizome pieces especially in ginger and tumeric. Hence it is important to formulate *in vitro* conservation strategies as a complement to the field genebank. The *in vitro* technology can also be used for disease-free exchange of germplasm from one location to another. This technology can also be used for *in vitro* collection of crop genetic resources. In addition *in vitro* genebanks can conserve the plant material from any part of the world, from temperate, arid, tropical and alpine regions of the world, while the field genebank can conserve only crop species that are grown in a given agro-climatic region where it is located. In addition, the molecular techniques standardized to estimate the genetic fidelity can be used to characterize the valuable germplasm.

The present study is taken up keeping all the above in view.



MATERIALS AND METHODS

MATERIALS

Plant species studied

In the present study, plants belonging to the family Zingiberaceae, namely cardamom (*Elettaria cardamomum* Maton. Clone-37), ginger (*Zingiber officinale* Rosc. *var.* Maran), turmeric *(Curcuma longa, var.* Sudarsana), *Kaempferia galanga* L. and *K. rotunda* L. were used.

Rhizome materials were collected from the Experimental Farm of Indian Institute of Spices Research, Peruvannamuzhi (Kozhikode District, Kerala), and were planted in earthen pots (12 inches diameter). The plants were multiplied and maintained and used as mother plants for source of explants.

Explants

Spouting vegetative buds from freshly harvested rhizome were used as explant in all the plant species studied.

Culture medium

MS (Murashige and Skoog, 1962) basal medium, the most extensively used medium for plant tissue culture, was used in the present study (Table 10). MS medium in full strength was used in all experiments, except for minimal growth storage experiments where both full strength as well as half strength medium was used. The chemicals used for micro and macronutrients were obtained from Hi-Media, Bombay, while the vitamins and growth regulators were from Sigma, USA.

Carbon source

Sucrose (Qualigens, Bombay) was used as the carbon source at the rate of 30 gml^{-1} in all the multiplication experiments, except for the *in vitro* conservation experiments, where it was used in combination with mannitol (Hi-Media).

Table 10. Composition of Murashige and Skoog* basal medium

Components		Concentration (mgl^{-1})
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650.00
Potassium nitrate	KNO_3	1900.00
Calcium chloride	$CaCl_2.2H_2O$	440.00
Potassium orthophosphate	KH_2PO_4	170.00
Magnesium sulphate	$MgSO_4.7H_2O$	370.00
Micronutrients		
Sodium EDTA	Na_2EDTA	37.30
Ferrous sulphate	$FeSO_4.7H_2O$	27.80
Boric acid	H_3BO_3	6.20
Manganese sulphate	$MnSO_4.4H_2O$	22.30
Potassium iodide	KI	0.83
Zinc sulphate	$ZnSO_4.7H_2O$	8.60
Sodium molybdate	$Na_2MoO_4.2H_2O$	0.25
Copper sulphate	$CuSO_4.5H_2O$	0.025
Cobalt chloride	$CoCl_2.6H_2O$	0.025
Vitamins		
Myo-inositol	$C_6H_{12}0_6$	100.00
Thiamine HCl	$C_{12}H_{17}CIN_4OS.HCl$	0.10
Nicotinic acid	$C_6H_5NO_2$	0.50
Pyridoxine HCl	$C_8H_{11}NO_3.HCl$	0.50
Amino acid		
Glycine	$C_2H_5NO_2$	2.00

• Murashige and Skoog, 1962

Growth regulators

Auxins: Two major auxins, namely ∝-naphthalene acetic acid (NAA) and Indole 3-butiric acid (IBA) were used in this study at concentrations of 0–1 mgl^{-1}.

Cytokinins: Two cytokinins namely, 6-Benzylaminopurine (BAP) and 6-fufurylamino purine (kinetin – Kin) were used at a concentration of 0–1 mgl^{-1}.

Gelling agents

For solidifying the culture medium, 'Qualigens' bacteriological grade agar agar was used at the concentration of 7 gl^{-1}.

Culture vessels

Borosil culture tubes (150 mm x 25 mm) were used for culture initiation. For plantlet growth and multiplication, Borosil culture tubes as well as borosilicate glass bottles and Erlenmeyer flasks (500 ml) were used. For *in vitro* conservation experiments borosil culture tubes with various enclosures (screw cap, aluminium foil, polypropylene cap and cotton plugs) were used.

Culture vessel closures

The culture tubes were closed with cotton plugs made of non-absorbent cotton covered with cheesecloth for culture initiation, multiplication and micropropagation studies. Erlenmeyer flasks were closed with cotton plugs or aluminum foil. For the *in vitro* conservation studies cotton plugs, screw caps, aluminium foil and polypropylene caps were used to close the culture tubes.

Distilled water

Double glass distilled water or Millipore 'Milli Q' water was used for preparation of stocks, growth regulators and enzyme stocks, media, buffers, etc.

Instruments

Inoculation under aseptic conditions was done in Klenzaids horizontal laminar airflow chamber. 'Nat steel' horizontal autoclave was used for sterilizing the culture media, stock solutions and other instruments like blades, forceps, needles, etc. For filter sterilization of thermolabile chemicals and growth regulators, Millipore filter

sterilization system with 0.22μ pore size filter was used. Certomat-R (B-Braun Biotech International) was used for the liquid cultures. Nikon SMZ-U Stereomicroscope, Leica Inverted Fluorescent microscope and Olympus microscope were used for isolation of meristem, pollen and anatomical studies respectively. NK Systems, MT-3 automatic plant microtome was used for taking anatomical sections. NK Systems, Biotron LPH-200 growth chamber with temperature, light and humidity control was used for the incubation of cultures under 5^0C, 10^0C and 15^0C. Horizontal electrophoresis unit from Bangalore Genie was used for the isozyme studies and Gene Cycler (Thermal cycler) from Bio-Rad was used for Polymerase Chain Reaction (PCR) studies. Gel-Doc 1000 from BIO-Rad was used for documentation of images,

METHODS

Experiments were conducted to standardize micropropagation protocols, *in vitro* conservation strategy by slow growth and cryopreservation, synthetic seed technology, *in vitro* production of microrhizomes and DNA storage in the Zingiberaceous genera. Studies were carried out to assess the genetic fidelity of the conserved material through morphological, biochemical and molecular characterization.

Micropropagation

Micropropagation (culture initiation, multiplication, plant regeneration, *in vitro* rooting and establishment of plants in soil) form the cycle of events that form the backbone of *in vitro* conservation scheme and hence an attempt was made to standardize micropropagation protocols in the zingiberaceous genera mentioned.

Collection of explant

Freshly harvested rhizomes were cleaned of debris, washed and treated with 0.3% Dithane-M45 for 30 min. The rhizomes were dried in shade for 1h and planted

in plastic trays containing sterilized sand. This was done to minimize the high rate of fungal contamination of cultures usually seen when sub-soil tissues were used as explants. The developing sprouts were collected and used as explants.

Surface sterilization and preparation of explants

Sprouting buds along with a part of rhizome was cut, removed and washed in running tap water to remove sand, dead plant parts, etc. The material was treated with 0.3% solution of copper oxychloride and dilute Tween 20 (1 drop per 100 ml of solution) for 30-60 min based on the hardiness of the material and washed thoroughly in 3–4 changes of sterile double distilled water. The explants were taken to the Laminar Air Flow Chamber and treated with 0.1% mercuric chloride ($HgCl_2$) and Tween 20 (1 drop per 100 ml of the solution) for 8 -10 min. The explants were rinsed in 4 to 5 changes of sterile double distilled water to remove all the traces of surface sterilant. One more layer of scale leaf was removed and they were trimmed to about 1–2 cm size and inoculated on to the culture medium.

Culture medium

MS (Murashige and Skoog, 1962) medium was used as nutrient medium. Separate stocks were prepared for macronutrients, micronutrients, vitamins, amino acid and growth regulators. Stocks of calcium chloride, ferrous sulphate, Na_2EDTA and glycine were prepared separately. Separate stocks were prepared for each of the growth regulators used (Table 11).

Sucrose (20 gl^{-1}) and agar-agar (7 gl^{-1}) were added directly to the medium. pH was adjusted to 5.8 before adding agar. The agar was melted to ensure uniform distribution in the medium. The medium was poured in to the culture vessels, closed with appropriate closures and then autoclaved at 121^0C at 16psi for 20 min.

Table 11. Stock solutions for MS medium

Stock	Composition	Stock strength	Quantity per 1litre medium	
			Full strength	Half strength
A	**Macronutrients**	x 20	50 ml	25 ml
	NH_4NO_3			
	KNO_3			
	$CaCl_2.2H_2O$*			
	KH_2PO_4			
	$MgSO_4.7H_2O$			
B	**Micronutrients**	x 100	10 ml	5 ml
	H_3BO_3			
	$MnSO_4.4H_2O$			
	KI			
	$ZnSO_4.7H_2O$			
	$Na_2MoO_4.2H_2O$			
	$CuSO_4.5H_2O$*			
	$CoCl_2.6H_2O$*			
C	**Micronutrients**	x 100	10 ml	5 ml
	Na_2EDTA*			
	$FeSO_4.7H_2O$*			
D	**Vitamins**	x 100	10 ml	5 ml
	Thiamine HCl			
	Nicotinic acid			
	Pyridoxine HCl			
E	**Amino acid**	x 100	10 ml	5 ml
	Glycine			
F	Myo-inositol	x 100	10 ml	5 ml
	Growth regulators			
	NAA	50 mg/100 ml		
	IBA	50 mg/100 ml		
	BAP	50 mg/100 ml		
	Kin	50 mg/100 ml		

- Dissolved separately before mixing in the final stock

Growth regulators

For direct plant regeneration from vegetative buds, NAA, IBA, BAP and Kin (0 to 1 mgl^{-1}) were tried in various combinations.

Sterilization of culture medium

The medium was sterilized by autoclaving at 121^{0}C for 20 minutes at 1.08 kg/cm^2 (16 psi) pressure.

Incubation conditions

The cultures were incubated at 22±2^{0}C and were given a photoperiod of 12 h with a light intensity of 2500–3000 lux, provided by 'Philips' cool white fluorescent tubes for culture initiation, multiplication and rooting trials. For *in vitro* conservation by slow growth, the cultures were incubated at 5^{0}C, 10^{0}C, 15^{0}C and 22±2^{0}C, with a light intensity of 2500–3000 lux.

Culture initiation

The excised shoot buds were inoculated on to culture initiation medium (MS + 0.5 mgl^{-1} Kin) and observed for responses and contamination.

In vitro *multiplication*

Contamination free cultures, which showed bud break and emergence of shoot, were subsequently transferred to different combinations of multiplication medium. The cultures were subcultured to fresh medium every 5–6 weeks. During subculture the plantlets were separated, roots cut and removed and a top cut was given at a height of 1.5 to 2.0 cm.

Observations

Observations were recorded on number of cultures developing roots and shoots, rate of multiplication and the number of roots per shoot at weekly intervals. The most promising treatments for multiple shoot induction and root development were repeated to confirm the repeatability.

Hardening and planting out

Healthy *in vitro* plants with good roots were taken out from the culture vessel and agar was gently washed off from the roots. The cluster of plantlets were separated into individual plants and then dipped in 0.3% Dithane-M45 for 5 min and transplanted into thermocol cups, containing a mixture of garden soil, sand and vermiculite in equal proportions, without damaging the fragile roots. The transplanted plantlets were kept in humid chamber for 3–4 weeks for hardening and establishment. The plantlets were then taken out and allowed to acclimatize in the nursery for 1 month. The plantlets were then transplanted into polybags and kept in the nursery for another 6 to 9 months. In the third year they were transferred to earthen pots (12 inches diameter) or to field for evaluation.

***In vitro* conservation**

The normal approach of tissue culture is to find a medium and set of conditions that favour the most rapid rate of growth with a subculture interval of 20–30 days. For *in vitro* germplasm storage, the approach must be changed. There are two basic approaches to *in vitro* germplasm storage, namely, minimal growth and cryopreservation.

Minimal growth storage

Minimal growth storage for short to medium term conservation can be achieved in several ways. Those include, maintenance of shoot cultures at a reduced nutritional status, particularly reduced carbon, by induction of osmotic stress with mannitol or sucrose, reduced temperature and/or light, incorporation of growth retardants and by minimizing the loss of moisture through evaporation. The principle behind slow growth is the reduction of growth rate and thereby extending the subculture interval up to a minimum period of 1 year. A detailed experiment was

conducted in cardamom for the induction of minimal growth and selected treatments were tried in other genera to find out the individual requirements for inducing minimal growth and the details are given in the following sections.

Experiment to induce minimal growth in cardamom cultures

In experiments to induce minimal growth in cardamom cultures, the following parameters were tried for inducing slow growth and thereby extending the subculture interval up to a minimum period of 1 year.

1) Low temperature : 5, 10, 15, 22 ± 2^0C

2) Culture vessel closures : Cotton plug, screw cap

3) Basal medium (MS) : Full and half strength

4) Carbon source (sucrose) : 30, 20, 15, 10 gl^{-1}

5) Osmoticum (mannitol) : 0, 10, 15, 20 gl^{-1}

1) Low temperature: Four different temperature regimes (5^0C, 10^0C, 15^0C and 22±2^0C) were tried. For incubation under 5^0C, 10^0C and 15^0C the cultures were kept in NK Systems Biotron LPH-200 growth chamber with temperature, light and humidity control. Temperature of 22±2^0C was adjusted in the plant growth room.

2) Culture vessel closures: The culture tubes were closed with cotton plug as well as screw caps with the intention of studying the effect of moisture loss from the plant and from the medium.

3) Basal medium: MS medium was used as the basal medium in all the experiments. Both full strength and half strength of the medium were used. Details of the media preparation are given in Table 10 and 11.

4) Carbon source (Sucrose): Sucrose at the rate of 20 gl^{-1} was used in all the normal multiplication experiments. In this experiment sucrose was used alone

at 30 gm/l and 20 gl^{-1} and in combination at 20 gl^{-1}, 15 gl^{-1} and 10gl^{-1} by adding 10 gl^{-1}, 15gl^{-1} and 10 gl^{-1} mannitol, respectively.

5) Osmoticum (mannitol): Mannitol was used only in combination with sucrose. The sucrose-mannitol ratio was 0: 30, 0: 20, 10:10, 15:15 and 20:10 with full as well as half strength of the basal medium.

No growth regulators were used in conservation experiments, as use of the growth regulators such as 2, 4-D are reported to induce somaclonal variation.

All these factors were tried singly and in combination constituting 80 treatments (Table.12). Twenty-five ml of the medium was poured per culture tube. Uniform sized plantlets of about 2 cm size with new emerging roots were inoculated in all the treatments. Each treatment was replicated with 20 culture tubes. The cultures were incubated with 12 h photoperiod of 2500–3000 lux.

Table 12. Treatments for inducing minimal growth in cardamom cultures

Basal medium concentration (MS)	Sucrose (S) + Mannitol (M) (gl^{-1})	Temperature (^0C)	Closure type
Full strength	30S + 0M	5	CP
Full strength	30S + 0M	5	SC
Full strength	30S + 0M	10	CP
Full strength	30S + 0M	10	SC
Full strength	30S + 0 M	15	CP
Full strength	30S + 0M	15	SC
Full strength	30S + 0M	22	CP
Full strength	30S + 0 M	22	SC
Full strength	20S + 0M	5	CP
Full strength	20S + 0M	5	SC
Full strength	20S + 0M	10	CP
Full strength	20S + 0M	10	SC
Full strength	20S + 0M	15	CP
Full strength	20S + 0M	15	SC
Full strength	20S + 0M	22	CP
Full strength	20S + 0M	22	SC
Full strength	20S + 10M	5	CP

Basal medium concentration (MS)	Sucrose (S) + Mannitol (M) (gl^{-1})	Temperature (^0C)	Closure type
Full strength	20S + 10M	5	SC
Full strength	20S + 10M	10	CP
Full strength	20S + 10M	10	SC
Full strength	20S + 10M	15	CP
Full strength	20S + 10M	15	SC
Full strength	20S + 10M	22	CP
Full strength	20S + 10M	22	SC
Full strength	15S + 15M	5	CP
Full strength	15S + 15M	5	SC
Full strength	15S + 15M	10	CP
Full strength	15S + 15M	10	SC
Full strength	15S + 15M	15	CP
Full strength	15S + 15M	15	SC
Full strength	15S + 15M	22	CP
Full strength	15S + 15M	22	SC
Full strength	10S + 10M	5	CP
Full strength	10S + 10M	5	SC
Full strength	10S + 10M	10	CP
Full strength	10S + 10M	10	SC
Full strength	10S + 10M	15	CP
Full strength	10S + 10M	15	SC
Full strength	10S + 10M	22	CP
Full strength	10S + 10M	22	SC
Half strength	30S + 0M	5	CP
Half strength	30S + 0M	5	SC
Half strength	30S + 0M	10	CP
Half strength	30S + 0M	10	SC
Half strength	30S + 0M	15	CP
Half strength	30S + 0M	15	SC
Half strength	30S + 0M	22	CP
Half strength	30S + 0M	22	SC
Half strength	20S + 0M	5	CP
Half strength	20S + 0M	5	SC
Half strength	20S + 0M	10	CP
Half strength	20S + 0M	10	SC
Half strength	20S + 0M	15	CP
Half strength	20S + 0M	15	SC
Half strength	20S + 0M	22	CP

Basal medium concentration (MS)	Sucrose (S) + Mannitol (M) (gl^{-1})	Temperature (^0C)	Closure type
Half strength	20S + 0M	22	SC
Half strength	20S + 10M	5	CP
Half strength	20S + 10M	5	SC
Half strength	20S + 10M	10	CP
Half strength	20S + 10M	10	SC
Half strength	20S + 10M	15	CP
Half strength	20S + 10M	15	SC
Half strength	20S + 10M	22	CP
Half strength	20S + 10M	22	SC
Half strength	15M + 15S	5	CP
Half strength	15M + 15S	5	SC
Half strength	15M + 15S	10	CP
Half strength	15M + 15S	10	SC
Half strength	15M + 15S	15	CP
Half strength	15M + 15S	15	SC
Half strength	15M + 15S	22	CP
Half strength	15M + 15S	22	SC
Half strength	10S + 10M	5	CP
Half strength	10S + 10M	5	SC
Half strength	10S + 10M	10	CP
Half strength	10S + 10M	10	SC
Half strength	10S + 10M	15	CP
Half strength	10S + 10M	15	SC
Half strength	10S + 10M	22	CP
Half strength	10S + 10M	22	SC

S= Sucrose; M=Mannitol; CP=Cotton plugs; SC= Screw caps

Experiment to induce minimal growth in ginger and turmeric cultures

The following parameters were tried to induce minimal growth in cultures so as to increase the subculture intervals up to a minimum period of 1 year.

i) Low temperature : 15, 22 ± 2^0C

ii) Culture vessel closures : Cotton plug, screw cap

iii) Basal medium (MS) : Full and half strength

iv) Carbon source (sucrose) : 30, 20, 15, 10 gl^{-1}

v) Osmoticum (mannitol) : 0, 10, 15, 20 gl^{-1}

Low temperatures of 5^0C and 10^0C were not tried in this experiment. All other parameters were same as those in experiments conducted for cardamom. All these factors singly and in combination constituted 40 treatments. The details of the experiment are given in Table 13.

Table 13. Treatments for inducing minimal growth in ginger and turmeric cultures*

Basal medium concentration (MS)	Sucrose (S) + Mannitol (M) (gm/l)	Temperature (^{0}C)	Closure types
Full strength	30S + 0M	15	CP
Full strength	30S + 0M	15	SC
Full strength	30S + 0M	22	CP
Full strength	30S + 0M	22	SC
Full strength	20S + 0M	15	CP
Full strength	20S + 0M	15	SC
Full strength	20S + 0M	22	CP
Full strength	20S + 0M	22	SC
Full strength	20S + 10M	15	CP
Full strength	20S + 10M	15	SC
Full strength	20S + 10M	22	CP
Full strength	20S + 10M	22	SC
Full strength	15S + 15M	15	CP
Full strength	15S + 15M	15	SC
Full strength	15S + 15M	22	CP
Full strength	15S + 15M	22	SC
Full strength	10S + 10M	15	CP
Full strength	10S + 10M	15	SC
Full strength	10S + 10M	22	CP
Full strength	10S + 10M	22	SC
Half strength	30S + 0M	15	CP
Half strength	30S + 0M	15	SC
Half strength	30S + 0M	22	CP
Half strength	30S + 0M	22	SC
Half strength	20S + 0M	15	CP
Half strength	20S + 0M	15	SC
Half strength	20S + 0M	22	CP
Half strength	20S + 0M	22	SC

Basal medium concentration (MS)	Sucrose (S) + Mannitol (M) (gm/l)	Temperature (^{0}C)	Closure types
Half strength	20S + 10M	15	CP
Half strength	20S + 10M	15	SC
Half strength	20S + 10M	22	CP
Half strength	20S + 10M	22	SC
Half strength	15M + 15S	15	CP
Half strength	15M + 15S	15	SC
Half strength	15M + 15S	22	CP
Half strength	15M + 15S	22	SC
Half strength	10S + 10M	15	CP
Half strength	10S + 10M	15	SC
Half strength	10S + 10M	22	CP
Half strength	10S + 10M	22	SC

S= Sucrose; M= Mannitol; CP= Cotton plugs; SC= Screw caps

Experiments to induce minimal growth in K. galanga *and* K. rotunda *cultures*

Some selected treatments, which were found favourable for the storage of cardamom, ginger and turmeric cultures were tried to induce minimal growth in cultures of *K. galanga* and *K. rotunda* so as to increase the subculture intervals up to a minimum period of 1 year.

i) Low temperature : 22 ± 2^0C

ii) Culture vessel closures : Cotton plug, Screw cap

iii) Basal medium (MS) : Full and half strength

iv) Carbon source (sucrose) : 30, 20, 15, 10 gl^{-1}

v) Osmoticum (mannitol) : 0, 10, 15, 20 gl^{-1}

A single temperature regime of 22 ± 2^0C was tried in the experiment. All these factors singly and in combination constituted 10 treatments and the details of the experiment are given in Table 14.

Table 14. Treatments for inducing minimal growth in *Kaempferia* spp. cultures

Treatment number	Basal Medium concentration (MS)	Sucrose (S) + Mannitol (M) (gml^{-1})	Closure types
1	Full strength	30S + 0 M	SC
2	Full strength	30S + 0 M	CP
3	Full strength	20S + 0M	SC
4	Full strength	20S + 0M	CP
5	Full strength	20S + 10M	SC
6	Full strength	20S + 10M	CP
7	Full strength	15S + 15M	SC
8	Full strength	15S + 15M	CP
9	Full strength	10S + 10M	SC
10	Full strength	10S + 10M	CP
11	Half strength	30S + 0 M	SC
12	Half strength	30S + 0 M	CP
13	Half strength	20S + 0M	SC
14	Half strength	20S + 0M	CP
15	Half strength	20S + 10M	SC
16	Half strength	20S + 10M	CP
17	Half strength	15S + 15M	SC
18	Half strength	15S + 15M	CP
19	Half strength	10S + 10M	SC
20	Half strength	10S + 10M	CP

M=Mannitol; S=Sucrose; CP = Cotton plug; SC = Screw cap

Observations

In *in vitro* conservation experiments observations were taken on growth rate (number of multiple shoots and increase in height over initial height), general vigour of the plant, symptoms of deficiency, percentage of survival at monthly intervals, to draw conclusions.

Recovery and growth

The cultures under slow growth were transferred to medium for multiplication and rooting. Observations were taken on the time taken to resume normal growth, multiplication rate and the general morphological appearance of culture to assess the viability.

Cryopreservation

Cryopreservation experiments were conducted in ginger and cardamom and the materials and methods used are described below. Two methods namely cryoprotective dehydration (Niino *et. al.*, 1992a) and encapsulation/dehydration (Fabre and Dereuddre, 1990) were adopted for cryopreservation studies in ginger.

Cryopreservation of ginger shoot buds by cryoprotective dehydration

Shoot buds of about 2–5mm were dissected out from *in vitro* grown cultures of ginger and used for cryopreservation experiments. Shoot buds were excised and precultured on MS liquid medium supplemented with 0.5 and 0.75M sucrose for 3 days on a gyratory shaker at 70 rpm. The dehydrated buds were transferred to an open petridish in the laminar flow chamber. The shoot buds were desiccated for 0, 1, 2, 3 and 4 h. The dehydrated shoot buds were transferred to cryovials, plunged directly into liquid nitrogen and stored for 24 h. On thawing the vials were taken out and immersed in 38–40^{0}C water bath for about 5min. After thawing the shoot buds were aseptically cultured on to recovery medium.

The shoot buds (control as well as cryopreserved) were cultured on liquid recovery medium (MS basal medium supplemented with 3% sucrose, 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA) and kept on a on a gyratory shaker at 70 rpm in dark for two weeks. The post freeze recovery was assessed initially by microscopic observation up to 2 weeks of culture. After two weeks the cultures were transferred to solid medium and incubated under1500-2000 Lux light intensity for further growth responses. The number of shoot buds that show greening and expansion of the leaf primordial were recorded. Weekly assessment was performed and the stages of recovery was recorded as leaf expansion, shoot and plantlet regeneration and callus formation

Cryopreservation of ginger shoot buds by Encapsulation/Dehydration

Shoot buds were transferred to a 3% (v/v) alginate solution, swirled gently so as to submerge the shoots. The alginate solution along with the shoot buds was withdrawn using a Pasteur pipette. The droplets of the alginate were dispensed into calcium chloride solution. The beads were sorted out, which contained the shoot tips, transferred to a dish, and left for 30 min to polymerize (The detailed method for encapsulation is given under *Synthetic seeds*). The beads were transferred to a liquid dehydration medium containing 0.75M sucrose and cultured for 3 days on a shaker with gentle agitation. The beads were taken out from the sucrose solution and transferred to sterilized aluminium foil kept on a laminar airflow bench and desiccated for 0, 1, 2, 3 and 4 h and plunged directly into liquid nitrogen and stored for 24 h. On thawing, the vials were taken out and immersed in 38–40^{0}C water bath for about 5 min. After thawing the shoot buds were aseptically cultured on to recovery medium.

The beads/shoot buds (control as well as cryopreserved) were cultured on recovery medium (MS basal medium supplemented with 3% sucrose, 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA). The post freeze recovery was assessed initially by microscopic observation up to 2 weeks of culture. The number of shoot buds that show greening and expansion of the leaf primordial were recorded. After 2 weeks, the beads were broken and the shoot buds were cultured on the same combination of solid medium for further growth responses. Weekly assessment was performed and the stages of recovery was recorded as leaf expansion, shoot and plantlet regeneration and callus formation.

Cryopreservation of cardamom pollen

Pollen was collected from mature flowers prior to opening. Pollen from 10 flowers were pooled and used for the experiments Pollen germination studies were

carried out in Brewbaker and Kwack's medium with sucrose at 10, 15 and 20% concentrations to optimize the germination conditions. The pollen grains were then desiccated for 10–30min in the air current of laminar flow chamber. The desiccated pollen was transferred to cryovials containing 5 and 10% of Dimethyl sulfoxide (DMSO), kept for 30min and then plunged directly into liquid nitrogen. The post freeze viability was assessed by staining in acetocarmine, fluorescein diacetate and also by *in vitro* germination.

Synthetic seeds

Synthetic seeds were prepared by encapsulating shoot buds of cardamom, ginger and turmeric in sodium alginate beads.

Preparation of sodium alginate matrix

Sodium alginate (3% w/v) was made with MS basal liquid medium. The alginate was dissolved by placing the liquid medium on a magnetic stirrer and heated to boiling while agitating vigorously and small amount of alginate was added step by step. The medium was then autoclaved.

Encapsulating propagules

The propagules *i.e.*, *in vitro* developed shoot buds were transferred to a 3% (v/v) alginate solution, swirled gently so as to submerge the shoots. The alginate solution along with the shoot buds was withdrawn using a Pasteur pipette with cut end, for easy passage of propagules. The droplets of the alginate were dispensed into calcium chloride solution (100 µM). The propagules were allowed to remain in the solution for 30–40 min on gyratory shaker for polymerization and proper bead formation. The beads were recovered by decanting the $CaCl_2$ solution and later washed in one or two changes of sterile water. The beads were sorted out, which contained the shoot tips and transferred to a dish. The synthetic seeds or 'synseeds'

thus produced were stored in 50 ml flasks in sterile water or MS medium for storage and germination. For storage the synthetic seeds were incubated at three different temperatures regimes namely, 5^0C, 10^0C and 22 ± 2^0C. Observations were made on the regeneration of plantlets from the beads after storage.

Microrhizome formation in ginger and turmeric

Microrhizome formation was noticed under slow growth storage conditions in ginger and turmeric and hence an experiment was conducted with the aim of enhancing the microrhizome formation. MS basal medium without growth regulators were used in the expreiment. Sucrose alone at four levels (3%, 9%, 10% and 12%) and sucrose and mannitol at various levels (1% each, 1.5% each, 2% + 1%, 3%+3%, 3%+6%, 5%+5%, 6%+6%) were used. Observations were made on time taken for induction, number of microrhizomes per culture, fresh weight, and size, percentage of establishment in soil, yield and rhizome morphology. Transverse sections of microrhizomes were taken and stained in 1% saffranin and observed under microscope and compared with normal rhizome anatomy.

Genetic stability analysis after storage

Morphological characterization

Recovery and growth: *In vitro* cultures of all the species in which minimal growth storage was successful, were transferred to the multiplication medium and afterwards to rooting medium specified for each species after periodic intervals to assess the tissue viability.

Planting out and establishment in the soil: Healthy *in vitro* plants with good roots were taken out and washed carefully for removing the traces of medium sticking to the roots. They were then dipped in 0.3% Dithane-M45 for 5–10 min and transplanted in polybags containing a mixture of garden soil, sand and vermiculite in equal

proportions. The transplanted plantlets were kept in humid chamber for 3–4 weeks for hardening and establishment. The plantlets were then taken out and kept in the nursery during the first year. Later they were transferred to earthen pots (12 inches diameter) for morphological evaluation.

Biochemical characterization using Isozyme profiles

Extraction: Leaf tissue was collected from *in vitro* cultures of the conserved material and also from control (the source plant used to initiate *in vitro* cultures). Leaf tissue (3.0–3.5g) was extracted in 5 ml of tris-HCl buffer (0.05M, pH–7.4) containing 0.1% ascorbic acid, 0.1% cysteine HCl and 17% sucrose. The above homogenate was filtered through two layers of muslin cloth and centrifuged at 40C at 7000 rpm for 30 min. Two drops of bromophenol blue was added to the supernatant and kept frozen until use. The extract was used as such for the separation on native gels.

The following reagents and buffer solutions were prepared and used in gel mixture (Hames, 1994).

1) Acrylamide-bis acrylamide: Acrylamide (30 g) and methylene-bis acrylamide (0.8 g) were dissolved and made up to 100 ml with double distilled water. The solution was filtered through a Whatman No.1 filter paper and stored at 4^0C in amber coloured bottles.

2) Resolving gel buffer stock: Tris-HCl (3M) was prepared in double distilled water and the pH adjusted to 8.8.

3) Stacking gel buffer stock: Tris-HCl (0.5M) was prepared in double distilled water and the pH adjusted to 6.8.

4) Riboflavin (0.004%): 4 mg riboflavin dissolved in 100 ml double distilled water.

5) Ammonium per sulfate (1.5%): Solution was prepared fresh and used.

Separating gel (7.5%) and stacking gel (2.5%) were cast as shown in Table 15.

Table 15. Stock solutions for separating and stacking gel mixtures

Stock solutions	Separating gel mixture (ml)	Stacking gel mixture (ml)
Acrylamide-bis acrylamide	7.50	1.25
Separating gel buffer stock	3.75	-
Stacking gel buffer stock	-	2.50
Ammonium per sulfate (1.5%)	1.50	-
Riboflavin (0.004%)	-	1.25
Double distilled water	17.25	5.00
Temed	0.005	0.008

Electrophoretic run: Mini dual model of Bangalore Genei, vertical slab gel electrophoresis system was used. Gels (8.0 x 7.0 x 0.1 cm) with seven wells were cast and 25µm of sample was loaded. Three wells were loaded with the control sample and the other four wells with the samples of the conserved material. Native reservoir buffer stock (0.025M Tris-glycine with pH 8.3) was taken in the upper as well as lower buffer tank. Electrophoretic run was carried out at cool temperature (in the refrigerator) under constant current of 70V until proper stacking was achieved, and then at 120V until the tracking dye reached the end of the slab gel.

After the completion of the run, the gel was taken and stained for isozyme patterns. The staining procedures used for the four isozymes are given below.

1) Superoxide dismutase (SOD) (Ravindranath and Fridovich, 1975): The gel was covered with stain solution (7.5 mg Na_2 EDTA, 4 mg riboflavin and 10 mg nitroblue tetrazolium salt dissolved in 100 ml of 0.05M Tris-HCl with pH 8.2) and incubated at 37^0C for 20min in dark. Then it was removed from dark and placed under light to resolve the bands of SOD activity as clear areas in the gel.

2) Esterase (Harris and Hopkinson, 1976): The stain solution was prepared by dissolving Na_2 EDTA (30 mg), Fast blue RR salt (60 mg) and α-naphthyl acetate (40 mg in 2 ml acetone) in 100 ml of 0.1M potassium phosphate buffer of pH 7.2. The gel was covered with filtered stain solution and incubated at 37^0C for 1 h for the appearance of esterase activity, as dark areas on a clear background.

3) Peroxidase (Shimoni and Renneui, 1988): The gels were soaked in 15 mM sodium phosphate buffer (pH 6.0), containing 1.0 mM hydrogen peroxide and 0.1 mM O-methoxy phenol (guaiacol). The gels were rinsed with de-ionised water and fixed in 7% acetic acid.

4) Polyphenol oxidase (PPO) (Holstein *et al.*, 1967): The gel was incubated for 1–2h in a solution containing 80 mg L-DOPA in 50 ml double distilled water and then fixed in 7% acetic acid.

The gels were then photographed, scanned in Gel-Doc 1000 and the bands were marked as such and zymographs were prepared.

Molecular characterization (Random Amplified Polymorphic DNA - RAPD)

Plant material

RAPD analysis was employed in the present study to evaluate genetic fidelity of the *in vitro* conserved lines of cardamom, ginger, *K. galanga* and *K. rotunda* plantlets, conserved under minimal growth storage conditions for 3 years. In all these species six replicates each were used for the study and compared with the control (conventionally propagated mother plant). Young and fresh leaves, from healthy *in vitro* cultures of cardamom, ginger, *K. galanga* and *K. rotunda* and also from the parent plants were collected.

DNA extraction procedure

CTAB method (Ausubel *et al.,* 1995) was used for isolation of genomic DNA from the leaf tissue. The procedure is as follows:

1) Grind 4 g of cleaned, frozen leaf tissue in liquid nitrogen to a fine powder using pre-chilled mortar and pestle.

2) Transfer the powder to 50 ml oak ridge containing 16 ml preheated CTAB extraction buffer. Use spatula to dispense the material completely.

3) Incubate the sample at 60°C for 30-60 min with occasional mixing by gentle swirling.

4) Add equal volume of chloroform : isoamyl alcohol (24:1) and mix by gentle spin at 10,000 rpm, 10 min at 4°C.

5) Transfer the aqueous phase to fresh tubes with cut tips.

6) Add 2/3 volume of ice cold Isopropanol and mix by gentle inversions.

7) Incubate at 4°C for 30 m.

8) Centrifuge at 10,000 rpm, for 15 min, at 4°C.

9) Discard supernatant, add 70% ethanol (7 ml) and wash the precipitate by gentle swirling for 3–4 min.

10) Spin at 10,000rpm, 15 min at 4°C. Pour off supernatant, invert the tubes for 15 min to drain off excess alcohol and leave the pellet to air dry, overnight.

11) To the dried pellet, 500 µl of TE added to re-dissolve the DNA and transferred the solution to 1 ml sterile microfuge tubes.

DNA purification procedure

(1) Add 2 µl of DNase free RNase (10 mg /ml) and incubate at 37 °C for 1 h.

(2) Extract twice with equal volume of chloroform: isoamyl alcohol, spin at 10,000 rpm and transfer aqueous phase to fresh tubes.

(3) Add 1/10 volume of 3M sodium acetate (pH 5.2) and 2.5 times absolute alcohol and mix by quick gentle inversion to precipitate DNA.

(4) Centrifuge at 10,000 rpm for 5 min.

(5) Decant supernatant carefully, wash pellet with 70% cold ethanol.

(6) Air dry pellet and dissolve in 500 µl of TE.

Preparation of buffers and stocks

The buffers and stocks required for the study were prepared as per the methods given by Sambrook *et al.* (1989) (Tables 16 & 17).

Table 16: Stock solutions prepared for DNA extraction*

Solutions	Method of preparation
1M Tris (pH 8)	Dissolve 60.55 g Tris base (Sigma) in 300 ml distilled water. Adjust pH to 8 by adding concentrated HCl. Adjust volume to 500ml.
0.5 M EDTA pH 8.0	Dissolve 93.05g of EDTA-disodium salt (sigma) in 300 ml of water. Adjust pH to 8 by adding NaOH pellets. Adjust volume to 500 ml.
5 M NaCl	Weigh 146.1g NaCl (Merck) add 200ml of water and mix well. When the salts get completely dissolved, adjust the final volume to 500 ml.
3 M Sodium acetate (pH 5.2)	Dissolve 61.523 g of anhydrous sodium acetate (Qualigens) in 200 ml of water and mix well. When dissolved completely adjust the pH of the solution to 5.2 with glacial acetic acid (99–100%).
Ethidium bromide – 10 mg/ml	Add 1g Ethidium bromide to 100 ml of distilled water. Keep on magnetic stirrer to ensure that the dye has dissolved completely. (Dispense to amber coloured reagent bottle and store at 4°C).
70% ethanol	Take 360 ml. of ethanol; mix with 140 ml of distilled water. (Dispense to reagent bottle and store at 4°C).
Chloroform: isoamyl alcohol (24:1)	Measure 450 ml of chloroform and 20 ml of isoamyl alcohol. Mix well and store in reagent bottle in room temperature.
1 M $MgCl_2$	Weigh 20.33 g of $MgCl_2$, dissolve in double distilled water, make up to 100ml.

* All the stock solutions were dispensed into reagent bottles and autoclaved

Table 17. Buffers prepared for extraction of DNA

	Buffer	Method of preparation
1	CTAB Extraction Buffer (1 litre) 100 mM Tris HCl (pH 8.0) 20 mM EDTA (pH 8.0) 1.4 M NaCl, 2% CTAB (w/v) 0.2% B-mercapto ethanol (v/v)	Measure 100 ml Tris (1 M), 280 ml of NaCl, 40ml of EDTA (0.5 M). Mix with about 400ml of hot distilled water, add 20 g of CTAB to this. Adjust final volume to 1 litre. Dispense to reagent bottles and autoclave. Just before use, add 0.2% β-mercaptoethanol.
2	TE (0.1mM) buffer 100ml 100 mM Tris HCl (pH 8.0) 0.1 mM EDTA (pH 8.0)	Take 1 ml of Tris HCl (1M), 20ml of EDTA (0.5 M). Mix with 99ml of sterile distilled water taken in a reagent bottle mix thoroughly, autoclave.
3	TAE buffer 10x :1 litre	Weigh 48.4g of Tris base; add 20ml of EDTA (0.5M); 11.42 ml of Glacial acetic acid and around 150ml distilled water. Dissolve the salt and adjust volume to 1litre. Autoclave.
4	Gel loading buffer (6x) 100ml 0.25% Bromophenol blue (BPB) (Sigma), 30% Glycerol (Merck)	Dissolve 0.25 g of BPB in 99 ml of 30% Glycerol. Keep on magnetic stirrer for several hours to get the dye completely dissolved. Dispense to reagent bottles and keep in 4 °C.

Quality and quantity analysis of DNA

Qualitative and quantitative analysis of DNA was performed by electrophoresis on 1% agarose gel (Sigma, St. Louis, USA). Electrophoresis was carried out at 90–100 volts for 3 h to allow proper resolution–λ weight marker (mgl^{-1}) was used as marker to quantify the genomic DNA. The image is visualized under UV Transilluminator in Biorad Gel Doc 1000 system and stored for further reference.

Polymerase Chain Reaction (PCR)

Optimization of PCR reaction components

1.Template DNA concentration

One of the most appealing features of PCR is that the quantity and quality of the DNA sample to be subjected to amplification need not be high. When DNA of

known concentration is available, amounts of 50–100ng are typically used for amplifications of single copy loci (Innis *et al.*, 1990). Genomic DNA diluted to a concentration of 25 to 75 ng/µl were tested in separate reactions.

2. Buffer

The standard buffer contain 50 mM KCl, 10 mMTrisHCl (pH 8.4), 1.5 mM $MgCl_2$, and 100 µl/ml of gelatin will be adequate for the majority of genomic PCR (Innis *et al.*, 1990). 10 x buffer from Boehringer Mannheim was used, from which 2µl was directly added for each reaction.

3. Deoxy Nucleotide Triphosphates (dNTPs)

The four dNTPs-dATP, dTTP, dCTP and dGTP are usually used in concentrations between 20-200µM each result in the optimal balance among yield, specificity and fidelity. All the four dNTPs were used at equivalent concentrations to minimize incorporation errors (Innis *et al* 1990). A ready made10 mM dNTPs of Boehringer Mannheim were used. Three dNTP concentrations (125,150 and 175 µM) were tested for optimal PCR.

4. Enzyme

The optimal concentration of *Taq* DNA polymerase is about 2 U/100 µl. As a rule, concentrations in excess of 4 U tend to result in the accumulation of non-specific amplification products, whereas amounts less than 1 U usually reduce the yield of the desired product (Innis *et al.*, 1990). *Taq* polymerase, from Bangalore Genei having a concentration of 3 U/µl, were used in the study. When optimizing PCR, two enzyme concentrations 2.5U and 5U were tested for 25 µl reaction.

5. Primers

Primer concentrations between 0.1–0.5 µM are generally optimal. Higher primer concentrations may promote mispriming and accumulation of non-specific

product and may increase the probability of generating a template independent artifact termed primer-dimer. Non specific products and primer dimer artifacts are themselves substrates for PCR and compete with the desired product for enzyme, dNTPs and primers resulting in a low yield of the desired product (Innis *et al.*, 1990). Two different primer concentrations namely, 5 and 10 picomoles /µl were tested, in the present study.

Twenty arbitrary 10 mer primers (Operon Technologies Inc. Alameda, California) were screened (Table 18) using two genotypes of cardamom (CL 37 and APG 50) and ginger (Varada and Maran) for amplification and polymorphism, and primers which gave best polymorphism were used to estimate genetic stability, following the protocol of Williams *et al.* (1990) with minor modifications. Each of the primer contains at least 60–70 % GC content and no self-complementary ends.

The primers used and their base sequences with the GC% is given in the Table 18. Each of the 10-mer samples was re suspended in 1 ml TE (pH 7) so that the final concentration was 5 picomoles/µl.

6. Magnesium ion (Mg^{2+}) concentration

The Mg^{2+} concentration may affect all the following steps, primer annealing, strand dissociation temperature of both template and PCR product, product specificity, formation of primer dimer artifacts, enzyme activity and fidelity. Taq DNA polymerase requires free Mg^{2+} top of that bound by template DNA, primers and dNTPs. PCR should contain 0.5–2.5 mM Mg^{2+} over the total dNTP concentration. The presence of EDTA or other chelators in the primer stocks or template DNA may disturb the apparent Mg^{2+} optimum. Therefore, though the buffer contains Magnesium, an additional 10 mM solution was added separately. For 10mM solution, 10 µl of 1M stock was diluted with 990µl of sterile water, aliquoted and kept in 4°C.

Table 18. Operon primers used in RAPD analysis and their base sequences

Sl. No	Primer number	Base sequence	% of GC
1	OPA 01	5' CAGGCCCTTC 3'	70
2	OPA 03	5' AGTCAGCCAC 3'	60
3	OPA 05	5' AGGGGTCTTC 3'	60
4	OPA 06	5' GGTCCCTGAC 3'	70
5	OPA 08	5' GTGACGTAGG 3'	60
6	OPC 10	5' GTGATCGCAG 3'	60
7	OPA 12	5' TCGGCGATAG 3'	60
8	OPB 02	5' TGATCCGTGG 3'	60
9	OPC 02	5' GTGAGGCGTC 3'	70
10	OPC 04	5' CCGCATCTAC 3'	60
11	OPC 05	5' GATGACCGCC 3'	70
12	OPC 06	5' GAACGGACTC 3'	60
13	OPC 07	5' GTCCCGACGA 3'	70
14	OPC 19	5' GTTGCCAGCC 3'	70
15	OPD 01	5' ACCGCGAAGG 3'	70
16	OPD 02	5' GGACCCAACC 3'	70
17.	OPE 01	5' CCCAAGGTCC 3'	70
18	OPE 02	5' GGTGCGGGAA 3'	70
19	OPF 01	5' ACGGATCCTG 3'	60
20	OPF 02	5' GAGGATCCCT 3'	60

The reaction mixture for PCR was prepared as follows:

	1x
1. Sterile distilled water	17.15 µl.
2. 10x PCR buffer	2.5 µl.
3. dNTPs (10 mM)	0.3 µl.
4. Primer (5picomoles/µl)	2.0 µl.
5. $MgCl_2$, (10 mM)	1.25 µl
6. Taq polymerase (3 U/µl)	0.8 µl
7. Template DNA (25 ng/µl)	1.0 µl
Total reaction volume	25µl

Optimisation of PCR conditions

1. Denaturation time and temperature

The most likely cause for failure of a PCR is incomplete denaturation of the target-template and/or the PCR product. Incomplete denaturation reduce the product yield, while denaturation steps that are too high and/or too long lead to unnecessary loss of enzyme activity The typical denaturation conditions are 95 °C for 30 sec / 97 °C for 15sec. However, higher temperature may be appropriate especially for G+C rich targets. Only 94 °C for 2 min in the first cycle and rest of the cycles at 94 °C for 1 min was tested for denaturation.

2. Primer annealing

The temperature and length of time required for primer annealing depend upon the base composition, length and concentration of amplification primers. Primer annealing was done at 37 °C for 1 min.

3. Primer extension

Extension time depends upon the length and concentration of the target sequence and upon temperature. Primer extensions are traditionally performed at 72 °C because this temperature is near optimal for extending primers on an M-13 based model template (Innis *et al.*, 1990). However, longer extension times may be helpful in early cycles if the substrate concentration is very low and at late cycles when product concentration exceeds enzyme concentration. Primer extension was done at 72 °C for 2 min for all the cycles except the last one and for the final cycle the extension time was increased to15 min.

4. Cycle number

The optimum number of cycles will depend mainly upon the starting concentration of target DNA when other parameters are optimized .Too many cycles

can increase the amount and complexity of non specific background products while too few cycles give low product yield (*Innis et al.*, 1990). Thirty-five cycles were used in the present study.

The PCR reaction mix was prepared using all the seven components and in each tube (sterile, fresh, 0.2 ml microfuge tube) the final reaction volume was made up to 20 µl and given a spin in the centrifuge and the vials were loaded in the wells in the PCR machine. (Genecycler TM, BIORAD) and the instrument was made to run according to the loaded program. The total time taken by the PCR machine to complete the reaction was around 5 hours.

The PCR reaction profiles tested were given below:

Reaction 1 Cycles: 3

Ist cycle:	94°C for 2 min	
	37 °C for 1 min	
	72 °C for 2 min	Cycle repeats: 1
IInd cycle:	94 °C for 1 min	
	37 °C for 1 min	
	72 °C for 2 min	Cycle repeats: 38
IIIrd cycle :	94 °C for 1 min	
	37 °C for 1 min	
	72 °C for 15 min	Cycle repeats: 1

Reaction I1 Cycles: 3

Ist cycle:	94°C for 2 min	
	37 °C for 1 min	
	72 °C for 2 min	Cycle repeats: 1
IInd cycle:	94 °C for 1 min	
	37 °C for 1 min	
	72 °C for 2 min	Cycle repeats: 35
IIIrd cycle :	94 °C for 1 min	
	37 °C for 1 min	
	72 °C for 15 min	Cycle repeats: 1

Reaction I11 Cycles: 3

Ist cycle:	95°C for 2 min	
	40 °C for 1 min	
	72 °C for 1 min	Cycle repeats: 1
IInd cycle:	95 °C for 1 min	
	40 °C for 1 min	
	72 °C for 2 min	Cycle repeats: 33
IIIrd cycle :	95 °C for 1 min	
	40 °C for 1 min	
	72 °C for 15 min	Cycle repeats: 1

After the reaction is over the gel-loading dye was added to the amplification product and then separated by electrophoresis in 1% agarose gel, stained with ethydium bromide. The gels were then scanned in Gel-doc 1000 and the images were documented for further data analysis.

Scoring of bands and data analysis

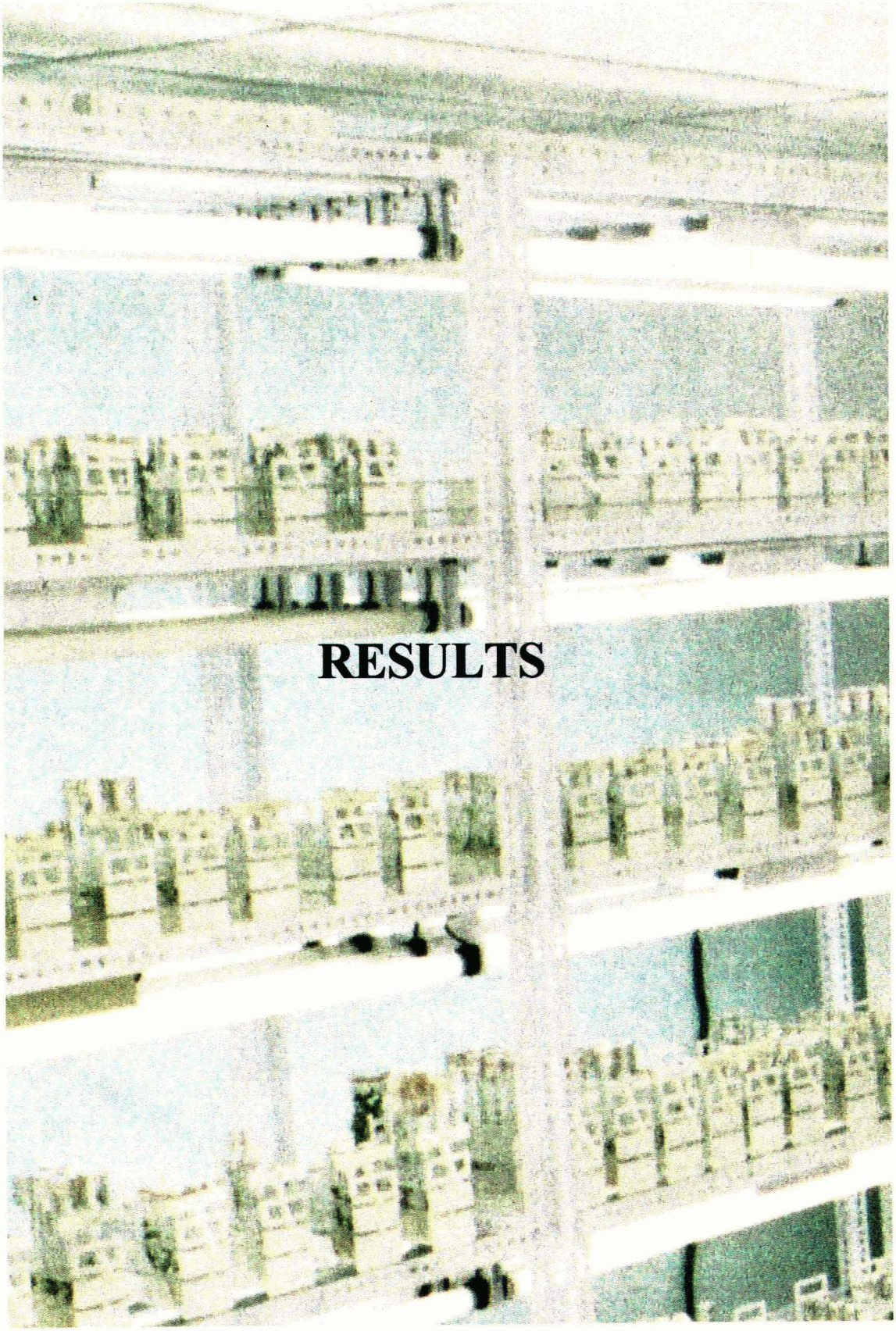
Each amplified product was scored across all the samples. Presence of amplified product was represented as '1' and its absence marked as '0'. Intensity of the bands was also considered while scoring. The bands appeared as artifacts of PCR were eliminated.

DNA Bank - Storage of DNA

Genomic DNA was isolated from 50 lines each of cardamom, ginger, and turmeric and 20 lines each of *K. galanga* and *K. rotunda*. DNA was dissolved in 500 µl TE buffer and stored at –20°C. The quality of the stored DNA samples was analyzed by agarose gel electrophoresis. The quantity of DNA was measured against the standard λ DNA marker.

Statistical analysis

In the micropropagation experiments and *in vitro* conservation experiments, the analysis of variance was worked out. The values were CD (critical difference) and CV (co-efficient of variance), mean and standard error were given to corresponding treatments wherever applicable. The F-values were worked out with corresponding degrees of freedom.



RESULTS

The present study was aimed to standardize an efficient strategy for *in vitro* conservation of genetic resources in zingiberaceous genera, namely, cardamom, ginger, turmeric and two species of *Kaempferia* (*K. galanga* and *K. rotunda*) for medium term conservation.

In vitro conservation is considered as an important additive for safe conservation of genetic resources, especially those of horticultural crops, which are vegetatively propagated. Micropropagation forms the backbone of *in vitro* conservation strategy and hence, the present study includes standardization of protocols for micropropagation (culture initiation, multiplication, rooting, hardening and planting out) of these crop species. Various approaches like slow growth, cryopreservation and DNA storage was attempted for *in vitro* conservation of genetic resources. The utility of other novel methods such as synthetic seeds and microrhizomes in the overall strategy of germplasm conservation was also tried. Finally genetic fidelity of conserved material was assessed by morphological, biochemical and molecular markers wherever applicable.

MICROPROPAGATION AND *IN VITRO* CONSERVATION

CARDAMOM

Vegetative bud explants from rhizomes were used to study *in vitro* responses on MS basal medium supplemented with cytokinins (BAP and Kin) and auxins (NAA and IBA), with the objective to standardize micropropagation protocols for direct clonal multiplication in cardamom (Fig. 2a).

In vitro culture initiation

Cultures were initiated on MS medium supplemented with 0.5 mgl^{-1} Kin using vegetative buds as explants. Freshly sprouting vegetative buds (1–2 cm long) from the

base of the clump gave vigorous and fast growing cultures and hence was used for culture initiation. Fungal and bacterial contamination appeared within 5–10 days in culture and the contaminated cultures were discarded. Only 50% of the explants could be established while the rest were lost due to contamination. The explants took 25–30 days for exhibiting the first signs of growth and bud break (Fig. 2b). The shoot tips turned green in 3–4 weeks and within 5–6 weeks axillary shoot buds also emerged out (Fig. 2c). Explants less than 2 cm size gave slow response and more than 50% mortality though the contamination was low (<30%). The initial response of cardamom cultures was comparatively slow.

Meristem isolation and culture

Apical and axillary meristems of about 0.2– 0.5 mm sizes could be isolated from *in vitro* established cultures and established with 30–40% survival, when cultured in MS medium containing 0.5 gl^{-1} Kin. The death of the tissue was mainly due to dissection damage and small size of the explant. Contamination rate was 10–20%. The growth of the meristem was observed as swelling of the meristem tip; followed by development of new leaf primordia, shoot initiation and root initiation. This sequence of growth took place in 1–6 months. The meristem develops into a dome shaped structure within a week (Fig. 2d) and leaf primordial initiation starts from apical dome by third week. The leaves completely cover the shoot apex within 5 weeks (Fig. 2e). The meristem develops into whole plants within 4–6 months.

***In vitro* multipliation**

Contamination free cultures, which exhibited initial growth, were transferred to MS medium with different combinations of growth regulators (BAP, Kin, NAA

and IBA) to find out suitable media combination for micropropagation of cardamom. The culture responses in various combinations of the media are given in Table 19.

Multiple shoots as well as roots were produced in all the growth regulator concentrations tried, though there were differences in degree of response in terms of multiple shoot production as well as rooting.

MS medium supplemented with 1 mgl^{-1} IBA gave lowest culture response (30%) for production of multiple shoots or roots, while that supplemented with 1 mgl^{-1} NAA alone or 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA gave the highest response (90%). The culture response of other treatments ranged from 50–80%, but the difference in culture response between the treatments was not significant

MS basal medium with cytokinins (Kin and BAP) alone showed significant difference in response in terms of both multiple shoot production and rooting. All the media combinations produced an average of 1.7 multiple shoots and 1.5 roots. Both these cytokinins when use in combination, the one with 1 mgl^{-1} each gave significant difference in multiple shoot production. In terms of rooting all the combinations except one with 0.5 mgl^{-1} Kin and 1 mgl^{-1} BAP gave significant difference.

When auxins (NAA and IBA) were tried alone, IBA either at 0.5 mgl^{-1} and or at 1 mgl^{-1} was significantly different in terms of multiple shoots, but rooting did not show any significant difference. Among the various combination of both these auxins two treatments, i.e., MS with 0.5 mgl^{-1} each of IBA and NAA and MS with 0.5 mgl^{-1} NAA and 1 mgl^{-1} IBA, gave significant difference with respect to multiple shoot production but there was no significant difference in rooting.

Table 19. Effect of growth regulators on multiple shoot and root induction from vegetative bud explant in cardamom on MS basal medium

Growth regulators				Response frequency (%)	Average no. of shoots/ explant		Average no. of roots/ explant	
Kin	BAP	NAA	IBA		Mean	SD	Mean	SD
0.5	-	-	-	60	1.7	1.1	1.5	1.1
1.0	-	-	-	60	1.6	1.1	1.5	1.1
-	0.5	-	-	60	1.7	1.1	1.6	1.1
-	1.0	-	-	70	1.7	1.1	1.5	1.1
-	-	0.5	-	70	3.4	0.7	3.3	1.0
-	-	1.0	-	90	5.2	0.8	5.6	0.8
-	-	-	0.5	60	1.3	0.9	3.9	0.7
-	-	-	1.0	30	2.0	0.8	4.4	1.2
0.5	0.5	-	-	60	2.1	1.2	1.6	1.1
0.5	1.0	-	-	70	3.5	0.7	2.5	1.1
1.0	0.5	-	-	70	2.5	1.6	1.6	0.7
1.0	1.0	-	-	60	1.9	1.4	1.7	1.1
0.5	-	0.5	-	70	4.0	0.7	3.8	0.8
0.5	-	1.0	-	70	4.4	1.7	3.4	0.8
1.0	-	0.5	-	60	3.6	0.7	1.7	0.8
1.0	-	1.0	-	60	4.1	1.7	2.7	0.7
0.5	-	-	0.5	50	2.2	1.3	4.1	1.9
0.5	-	-	1.0	70	2.7	1.5	3.8	1.3
1.0	-	-	0.5	70	2.3	1.4	3.0	0.8
1.0	-	-	1.0	70	3.5	0.7	3.6	0.7
-	0.5	0.5	-	80	4.8	0.9	4.8	1.7
-	0.5	1.0	-	80	3.8	0.8	4.4	0.7
-	1.0	0.5	-	90	5.8	0.9	6.2	1.1
-	1.0	1.0	-	60	4.5	0.7	5.6	0.7
-	0.5	-	0.5	50	3.1	0.7	4.1	0.9
-	0.5	-	1.0	60	3.6	0.8	4.0	0.8
-	1.0	-	0.5	60	4.0	0.7	3.0	0.8
-	1.0	-	1.0	70	4.2	0.6	4.8	0.8
-	-	0.5	0.5	70	2.1	0.7	3.7	1.6
-	-	0.5	1.0	70	2.2	1.7	4.6	0.8
-	-	1.0	0.5	60	4.4	1.0	3.6	0.5
-	-	1.0	1.0	60	3.9	0.7	4.8	0.9
CD					**3.45**		**3.90**	
SE					**0.34**		**0.32**	
F-value					**12.36**		**17.40**	

Among the auxin-cytokinin combinations, MS with 0.5 mgl^{-1} Kin and 0.5mgl^{-1} IBA and another with 1 mgl^{-1} Kin and 0.5 mgl^{-1} NAA gave significant difference in terms of multiple shoot production and rooting respectively. All other treatments did not show much significant difference from the combination, which gave best result.

The mean number of multiple shoots ranged from 1.3 to 5.8 in different treatments. MS medium supplemented with 1 mgl^{-1} IBA gave the lowest number of multiple shoots (1.3) while that supplemented with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA gave the highest number (5.8) of multiple shoots (Fig. 2f) followed by 1.0 mgl^{-1} NAA (5.2). There were no significant differences between these two treatments.

The mean number of roots ranged from 1.5 to 6.2 in different treatments. MS medium supplemented with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA gave the highest number (6.2) of roots followed by the one with BAP and NAA at 1 mgl^{-1} each and NAA at 1 mgl^{-1}. These treatments did not show any significant difference between them. MS media supplemented with 0.5 or 1 mgl^{-1} Kin and with 1 mgl^{-1} BAP gave the least number (1.5) of roots.

The combinations that gave better responses i.e., MS with 1 mgl^{-1} NAA and MS in combination with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA, were repeated and the results were reproducible. The medium combination having MS + 1 mgl^{-1} BAP + 0.5 mgl^{-1} NAA was used for the multiplication of cardamom throughout this study. The plantlets from the vegetative bud cultures were healthy, robust, 8–12 cm tall, with 5–6 roots. These plantlets were hardened in humid chamber for 20–25 days with 80% establishment (fig. 2g) and were then transplanted to polybags (Fig. 2h) with about 80% survival.

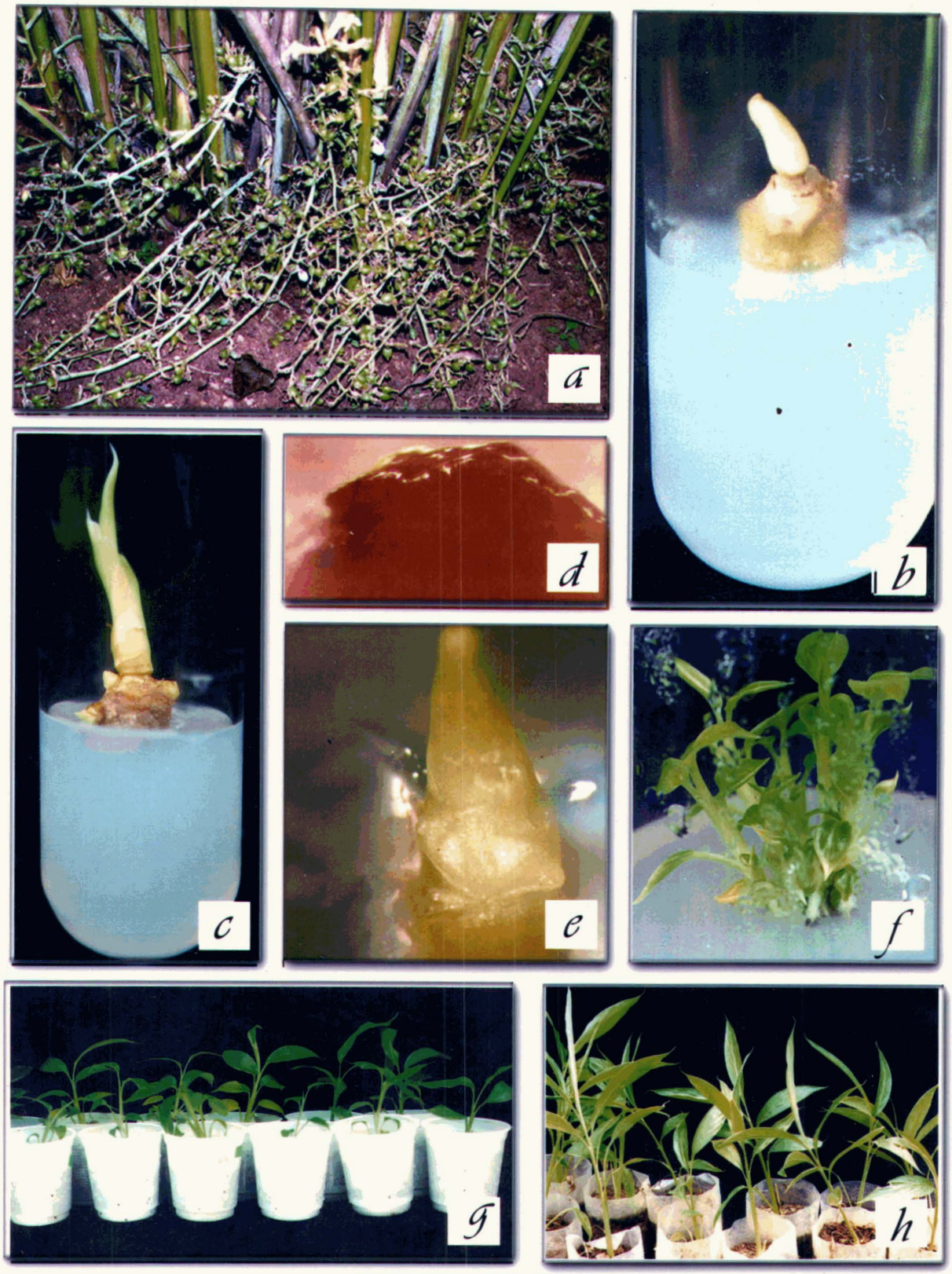


Fig. 2. Micropropagation in cardamom (*Elettaria cardamomum* Maton)

(a) Cardamom plant, (b) Culture initiation from vegetative bud explant in MS + 0.5 mgl^{-1} Kin, (c) Apical bud growth and initiation of axillary buds, (d) Freshly isolated meristem, (e) Growing meristem after 3 months of culture, (f) Development of multiple shoots and roots in MS + 1 mgl^{-1} BAP + 0.5 mgl^{-1} NAA, (g) Micropropagated plants after hardening, (h) Hardened plantlets transplanted to polybags.

Slow (minimal) growth storage

An experiment was conducted to standardize slow growth storage methods for medium term conservation of *in vitro* plantlets of cardamom. Various parameters such as low temperatures, composition of culture medium and prevention of evaporation loss by using sealed culture tubes were tried in various combinations. The effect of these factors on growth and survival of cardamom *in vitro* cultures is given in Table 20. The effect of closure types, temperatures and media components on induction of minimal growth and storage of cultures, when other parameters kept constant, is represented in Figs. 3, 4 and 5. While adopting suitable method for *in vitro* conservation emphasis was given for the extension of subculture interval up to a minimum period of 12 months with about 80% survival.

Effect of different types of culture tube closures

Cotton plugs allowed better gaseous exchange resulting in faster growth of the plantlets. But more rapid moisture loss coupled with the faster growth of plantlets resulted in early exhaustion of medium leading to drying up of cultures. Use of screw-capped, aluminium foil-covered or polypropylene capped culture tubes, minimized the moisture loss and resulted in availability of nutrients for a longer period. Only 25–50% of the medium was exhausted even after 12 months of culture in some of the media combinations (Fig. 3 and 6a).

In vitro cultures of cardamom kept in cotton-plugged tubes could be maintained up to a maximum of 3 months with 100% survival and drying up of cultures was noticed thereafter (Fig 6a). Cultures in cotton plugged tubes could be maintained up to a maximum period of 7 months with 80% survival in the slow growth inducing medium. The cultures perished completely after 7 months and further

storage was not possible. Cultures in screw-capped tubes exhibited high longevity and 100% of them survived up to 9 months and 80% of them up to 12 months in some of the media combinations. The survival of the cultures continued up to 15 months though the survival percentage was dropped to 50%. Cultures in both polypropylene capped tubes and aluminium foil-covered tubes performed similar to those in screw-capped tubes (Fig. 3).

Effect of various temperatures

Various temperature regimes (5^0C, 10^0C, 15^0C and 22 ± 2^0C) were tried for induction of slow growth in cardamom cultures. The effect of various levels of temperatures on growth and storage of *in vitro* cultures is given in Table 19 and Fig. 4. Among the temperature regimes tested, a temperature of 22 ± 2^0C was better for *in vitro* storage of healthy cardamom cultures. Survival of cultures was 80% after 12 months in some of the media combinations. At 15^0C the cultures could be stored up to a maximum period of 12 months with 70% survival, but the cultures showed etiolated and necrotic shoots (Fig. 6b). Lower temperatures of 5^0C and 10^0C resulted in cultures turning pale and their subsequent mortality within 20 days (Fig. 4).

Effect of different media constituents

Two concentrations of MS basal medium, at full and half strength, with sucrose and/mannitol in various concentrations and combinations were tried to induce slow growth in cardamom cultures (Fig. 5). Growth rate was measured in terms of increase in plant height over the height of the initial explant (2 cm) and number of shoots per culture. In general, full strength of the MS basal medium with 20–30 gl^{-1} sucrose supported higher growth rate and hence was not suitable for *in vitro* storage. The plants over grow the culture tube within 2 to 4 months. When the concentration

of the MS basal medium was reduced to half strength, the cultures could be stored up to a longer period of 4–7 months with 80% survival. The average increase in plant height was 9.4 cm with 4.6 shoots per culture on full strength MS and 30 gl^{-1} sucrose. This was reduced to 3.1 and 5.2 respectively when half strength MS medium was used. When the sucrose concentration was reduced to 20 gl^{-1}, the mean increase in plant height was 7.4 cm and 3.7 cm in full MS and half MS, respectively, while the mean number of shoots in the respective media was 4.6 and 3.8 per culture

However, further reduction of sucrose (10–20 gl^{-1}) coupled with addition of mannitol (10–15 gl^{-1}) induced slow growth in cultures. Gradual reduction in growth rate was noticed in different combinations of media (Fig. 6c). Mannitol and sucrose at 10 gl-1 or 15 gl^{-1} each, with half strength MS medium induced slow growth. In this media 80% of cultures survived up to 12 months (Fig 6d) and 50% of cultures survived even after 15 months of storage.

Addition of mannitol in the medium significantly reduced the growth rate in terms of increase in height, but there was an increase in the number of shoots produced per culture. In full strength MS supplemented with 20 gl^{-1} sucrose and 10gl^{-1} mannitol, the increase in height was 3.1 cm and the mean number of shoots per culture was 8.1, whereas in half strength MS it was 2.7 cm and 6.6 shoots per culture. In full and half strength of the medium supplemented with 15 gl^{-1} each of sucrose and mannitol the plantlets achieved a height of 3.8 cm and 3.3 cm respectively, and the number of shoots per culture was 8.4 and 5.5. Further reduction in sucrose and mannitol (10gl^{-1} each) gave further reduction in growth rate, as the increase in plant height was 1.4 cm and 1.2 cm and the mean number of shoots per culture was 6.8 and 5.7 in full and half strength MS medium respectively.

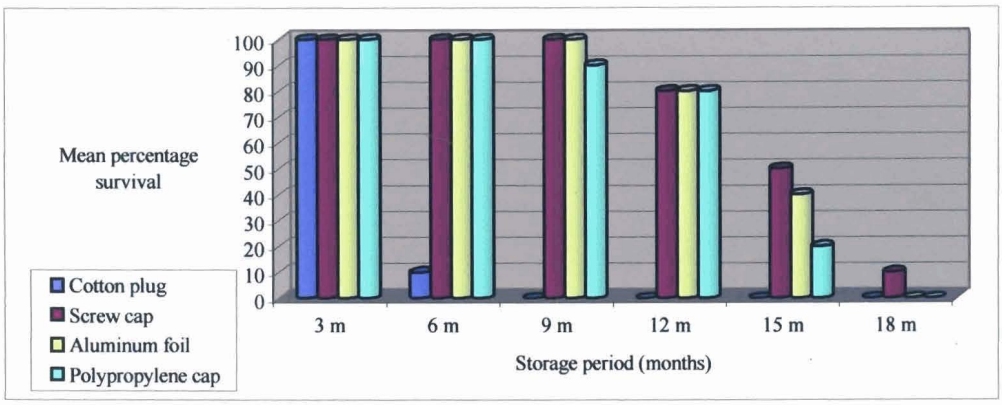
Table 20. Effect of media components, temperature and closure types on induction of minimal growth in cardamom cultures

Treatment				Growth rate				Storage period (months) with 80% survival of cultures*
Basal medium (MS) concentration	Sucrose (S) + Mannitol (M)	Temp. (^{0}C)	Closure type	Increase in height (cm)*		Average no. of shoots /culture		
				Mean	SD	Mean	SD	
Full strength	30S + 0M	15	CP	4.4	0.5	3.0	0.7	4
Full strength	30S + 0M	15	SC	6.4	0.5	4.3	0.7	6
Full strength	30S + 0M	22	CP	5.5	0.7	4.2	0.8	2
Full strength	30S + 0M	22	SC	9.4	0.9	4.6	1.1	6
Full strength	20S + 0M	15	CP	4.2	0.6	3.0	0.7	4
Full strength	20S + 0M	15	SC	5.5	0.8	3.6	0.8	6
Full strength	20S + 0M	22	CP	6.0	0.9	4.0	0.8	3
Full strength	20S + 0M	22	SC	7.4	0.7	4.6	0.7	6
Full strength	20S + 10M	15	CP	2.5	0.8	2.2	0.6	6
Full strength	20S + 10M	15	SC	3.3	0.9	1.4	0.5	8
Full strength	20S + 10M	22	CP	2.4	0.9	5.7	1.1	4
Full strength	20S + 10M	22	SC	3.1	0.7	8.1	0.7	9
Full strength	15S + 15M	15	CP	1.3	0.3	1.4	0.5	5
Full strength	15S + 15M	15	SC	2.2	0.7	1.3	0.5	10
Full strength	15S + 15M	22	CP	2.5	0.4	7.4	1.0	4
Full strength	15S + 15M	22	SC	3.8	0.6	8.4	0.7	10
Full strength	10S + 10M	15	CP	5.3	0.7	2.1	0.7	7
Full strength	10S + 10M	15	SC	6.9	0.7	2.0	0.8	10
Full strength	10S + 10M	22	CP	0.1	0.1	2.8	0.8	6
Full strength	10S + 10M	22	SC	1.4	0.4	6.8	1.7	10
Half strength	30S + 0M	15	CP	CP	0.5	2.1	0.7	4
Half strength	30S + 0M	15	SC	SC	0.4	2.0	0.8	7
Half strength	30S + 0M	22	CP	CP	0.4	3.2	0.8	4
Half strength	30S + 0M	22	SC	SC	0.5	5.2	0.8	6
Half strength	20S + 0M	15	CP	CP	0.3	2.2	0.6	4
Half strength	20S + 0M	15	SC	SC	0.4	2.2	0.8	6
Half strength	20S + 0M	22	CP	CP	0.5	2.9	0.9	4
Half strength	20S + 0M	22	SC	SC	0.3	3.8	1.4	7
Half strength	20S + 10M	15	CP	CP	0.2	1.4	0.5	6
Half strength	20S + 10M	15	SC	SC	0.4	2.1	0.6	10
Half strength	20S + 10M	22	CP	CP	0.4	5.7	0.9	7
Half strength	20S + 10M	22	SC	SC	0.2	6.6	1.3	9
Half strength	15S + 15M	15	CP	CP	0.1	6.9	0.8	6
Half strength	15S + 15M	15	SC	SC	0.2	7.3	1.6	12
Half strength	15S + 15M	22	CP	CP	0.4	7.6	1.2	6
Half strength	15S + 15M	22	SC	SC	0.6	5.5	1.8	12
Half strength	10S + 10M	15	CP	CP	0.3	1.9	0.9	7
Half strength	10S + 10M	15	SC	SC	0.3	1.8	0.6	12
Half strength	10S + 10M	22	CP	CP	0.2	4.7	1.1	7
Half strength	10S + 10M	22	SC	SC	0.4	5.7	0.9	12
CD					**5.69**		**6.10**	
SE					**0.17**		**0.26**	
F-value					**130.323**		**52.33**	

MS- Murashige and Skoog medium; S–Sucrose; M–Mannitol; SC–Screw cap; CP–Cotton plug

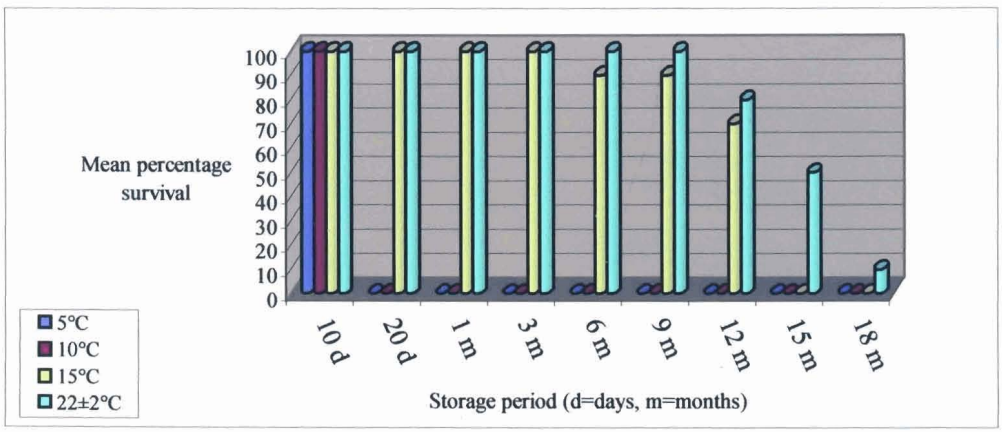
* Mean of 10 replications (Data collected after the respective storage period with 80% survival)

Fig. 3. Survival percentage of cardamom cultures at various time periods in different closure types*



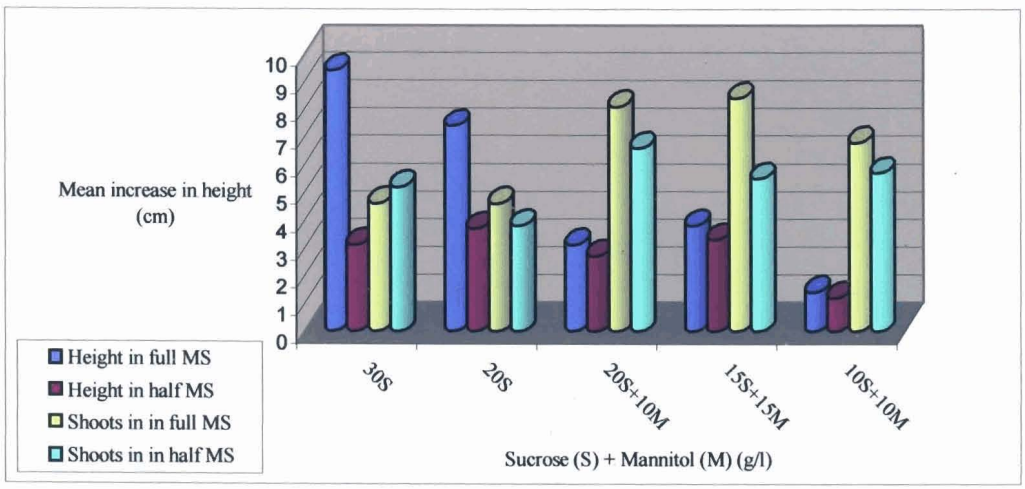
* In half MS medium with 15 gl^{-1} sucrose and 15 gl^{-1} mannitol at 22±2°C

Fig. 4. Survival percentage of cardamom cultures under different temperature regimes at various time periods*



* In half MS medium with 15 gl^{-1} sucrose and 15 gl^{-1} mannitol at 22±2°C in sealed culture tubes

Fig. 5. Growth rate and survival percentage of cardamom cultures in different media combinations*



* In sealed culture tubes at 22±2°C

Fig. 6. Induction of slow growth in cardamom

(a) Effect of cotton plug and screw cap closures on growth of cultures and media exhaustion (arrow indicates exhaustion of medium), (b) Growth of cultures at 15°C in half MS + 15 gl^{-1} sucrose + 15 gl^{-1} mannitol after 8 months of storage, (c) Difference in growth rate in various combinations of media (d) One year old culture in half MS + 15 gl^{-1} mannitol + 15 gl^{-1} sucrose, (e) Cultures under minimal growth storage (f) *In vitro* multiplication after minimal growth storage.

Thus in cardamom, minimal growth could be induced and cultures could be stored with 80% survival up to 12 months without subculture in half strength MS medium supplemented with 15 gl^{-1} each of sucrose and mannitol in sealed culture tubes at 22 ± 2^0C. Under these conditions cultures exhibited a miniature growth pattern (Fig. 6e). These cultures could be maintained up to 15 months with 50% survival and sudden deterioration of cultures was observed beyond that period.

After minimal growth storage, 12 months old cultures were transferred either to the same medium for the second cycle of *in vitro* storage or to multiplication medium for normal growth and planting out. *In vitro* cultures after 12 months of storage on transfer to multiplication medium (MS + 1 mgl^{-1} BAP + 0.5 mgl^{-1} NAA) resumed growth and the miniature-sized plantlets grew and multiplied normally in 1 month of culture (Fig. 6f).

GINGER

Micropropagation of ginger (Fig. 7a) from vegetative bud explants was attempted in MS basal medium supplemented with various concentrations and combinations of cytokinins (BAP and Kin) and auxins (NAA and IBA).

***In vitro* culture initiation**

The explants were initiated to grow on MS medium supplemented with 0.5 mgl^{-1} Kin to establish initial aseptic cultures. The percentage of culture establishment in ginger was 50–60, when explants of 1–2 cm size were used. The loss of cultures was due to fungal and bacterial contamination, which appeared within 5–15 days of culture initiation. When the explant size was more than 2 cm, contamination loss increased (>80%). Smaller explants (<5mm) were mostly free from contamination, but exhibited more than 50% mortality of the aseptic cultures. The shoot tips turned green

and started to grow within 7–15 days of inoculation (Fig. 7b and 7c). New shoot emergence was noticed in 50–60 percent of established cultures (Fig.7d). Explants of 1–2 cm were used for the multiplication of sufficient stock material for further trials. Contamination free cultures were used for further experimentation.

***In vitro* multiplication**

Aseptic cultures were transferred to media with different combinations of growth regulators (BAP, Kin, NAA and IBA at 0.5 and 1.0 mgl^{-1}). Multiple shoots as well as roots were produced in all the growth regulator concentrations tried. However, the cultures responded differently in various treatments (Table 21).

With respect to culture response, MS medium supplemented with 1.0 mgl^{-1} each of NAA and IBA gave lowest culture response (50%) in terms of production of multiple shoots or roots while that supplemented with 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA gave the highest culture response (90%).

Cytokinins (BAP and Kin) when used alone has induced approximately equal amount of shoots and roots. Use of auxins (IBA and NAA) alone resulted in a significant increase in the number of roots than shoots. Among these NAA at 1 mgl^{-1} has induced more shoots (5.1) and roots (7.9). When the growth regulators were used in combination NAA gave better response than IBA in combination with either Kin or BAP, the latter gave the best response.

The mean number of multiple shoots ranged from 1.4 to 7.5 in different treatments. MS medium supplemented with 1.0 mgl^{-1} IBA and that with 0.5 mgl^{-1} IBA in combination with 0.5 mgl^{-1} NAA gave the lowest number of multiple shoots (1.4) while that with 0.5 mgl^{-1} NAA and 1.0 mgl^{-1} BAP gave the highest number (7.5) of multiple shoots (Fig. 7e).

Table 21: Effect of growth regulators on multiple shoot and root induction from vegetative bud explant in ginger on MS basal medium

Growth regulators (mgl^{-1})				Response frequency (%)	Average no. of shoots/ explant		Average no. of roots/ culture	
Kin	BAP	NAA	IBA		Mean	SD	Mean	SD
0.5	-	-	-	80	2.2	0.9	2.6	1.0
1.0	-	-	-	70	2.4	0.7	1.6	0.7
-	0.5	-	-	80	2.8	1.2	2.9	1.0
-	1.0	-	-	60	3.5	0.7	2.2	1.4
-	-	0.5	-	80	3.8	0.9	6.8	1.5
-	-	1.0	-	80	5.1	2.0	7.9	1.1
-	-	-	0.5	60	2.2	1.2	6.1	1.4
-	-	-	1.0	60	1.4	0.7	4.4	1.5
0.5	0.5	-	-	70	3.5	1.3	3.9	1.4
0.5	1.0	-	-	70	4.2	0.9	2.1	0.7
1.0	0.5	-	-	80	3.7	0.9	2.2	1.2
1.0	1.0	-	-	60	3.0	0.8	1.9	0.9
0.5	-	0.5	-	80	3.2	1.5	3.1	0.7
0.5	-	1.0	-	80	3.6	1.6	5.0	1.0
1.0	-	0.5	-	70	3.5	0.7	3.7	1.8
1.0	-	1.0	-	70	4.7	1.8	5.0	1.1
0.5	-	-	0.5	70	2.2	0.9	3.4	0.7
0.5	-	-	1.0	60	2.0	1.2	3.7	1.7
1.0	-	-	0.5	60	3.3	1.8	3.1	1.7
1.0	-	-	1.0	70	3.2	0.8	4.1	1.1
-	0.5	0.5	-	80	4.6	0.7	6.7	1.6
-	0.5	1.0	-	80	5.5	0.7	7.3	0.8
-	1.0	0.5	-	90	7.5	0.7	9.1	1.5
-	1.0	1.0	-	80	5.3	2.0	8.2	0.9
-	0.5	-	0.5	70	4.5	0.7	4.4	0.8
-	0.5	-	1.0	80	3.5	0.7	5.7	0.8
-	1.0	-	0.5	80	4.9	1.6	4.2	1.1
-	1.0	-	1.0	70	3.8	0.8	4.9	1.0
-	-	0.5	0.5	60	1.4	0.8	3.2	0.8
-	-	0.5	1.0	60	1.6	1.0	3.3	1.8
-	-	1.0	0.5	70	3.1	1.7	3.5	1.1
-	-	1.0	1.0	50	1.5	0.8	1.9	1.0
CD					**3.9**		**5.7**	
SE					**0.37**		**0.38**	
F-value					**13.51**		**27.89**	

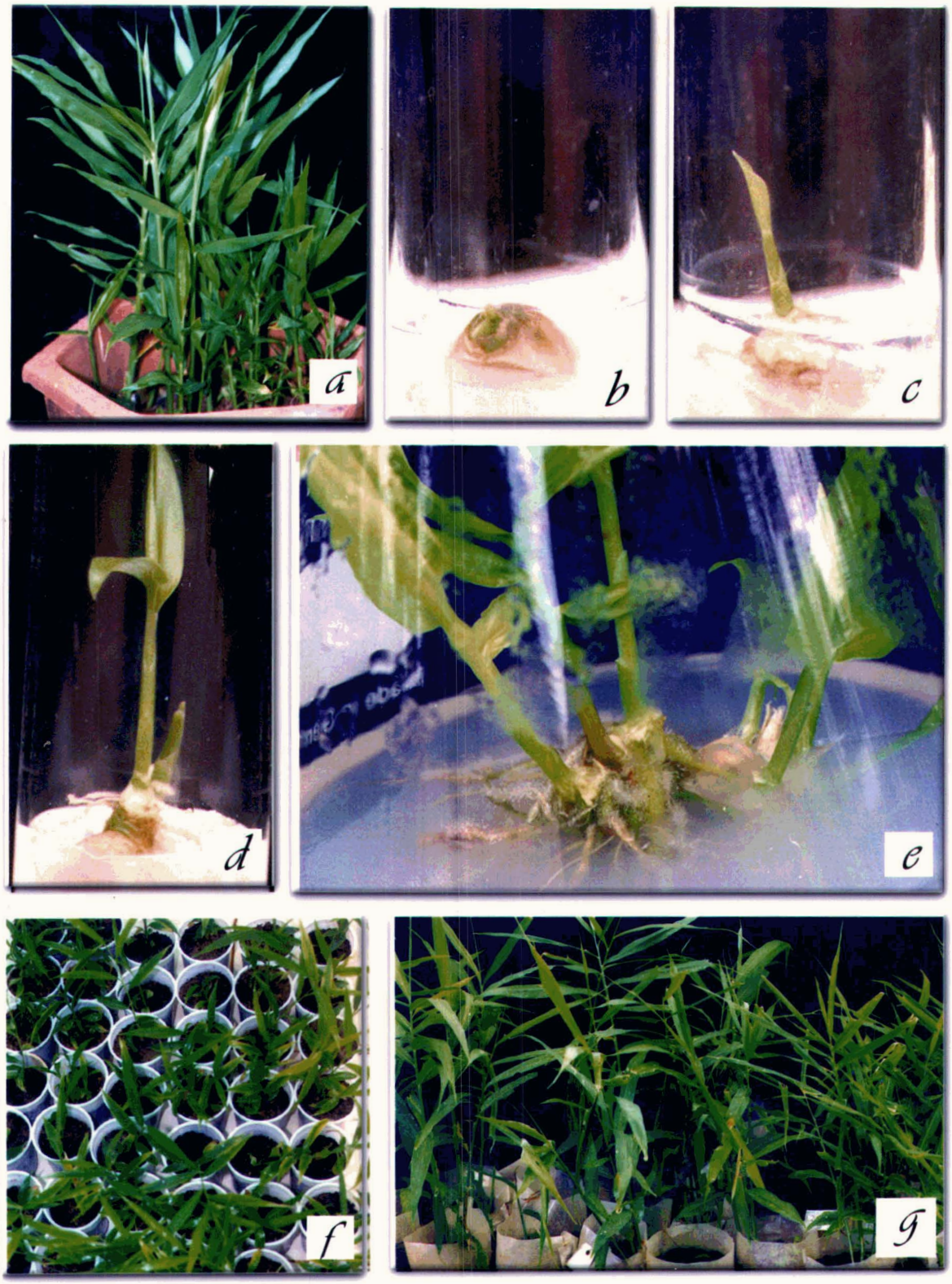


Fig. 7. Micropropagation in ginger (*Zingiber officinale* Rosc.)

a) Ginger plant, (b) & (c) Vegetative bud explant and culture initiation in MS + 0.5 mgl^{-1} Kin, (d) Axillary bud growth, (e) Development of multiple shoots and roots in MS + 1 mgl^{-1} BAP + 0.5 mgl^{-1} NAA, (f) Micropropagated plants after hardening, (g) Hardened plantlets transplanted to polybags.

The mean number of roots ranged from 1.6 to 9.1 in different treatments. MS media supplemented with 1.0 mgl^{-1} NAA gave the highest number (9.1) of roots and MS media supplemented with 1.0 mgl^{-1} Kin gave the least number (1.6) of roots.

MS medium supplemented with 0.5 mgl^{-1} NAA and 1 mgl^{-1} BAP gave the best response with an average of 7.5 multiple shoots and 9.1 roots in 90% of the cultures tested. This treatment was repeated and the results were reproducible and were used for *in vitro* conservation experiments.

The plantlets were healthy, 9–12 cm tall, with 7–10 roots. These plantlets were planted out and hardened in humid chamber for 20–25 days with 80% establishment (Fig. 7f) and further transplanted to polybags (Fig. 7g).

Slow (minimal) growth storage

An experiment was conducted in ginger also to study the effectiveness of slow growth storage regimes for medium term conservation of *in vitro* plantlets. The treatments were similar to those tried in cardamom. Different parameters such as reduced temperatures (15^0C, 22 ± 2^0C), modifications in media and prevention of evaporation loss were tried in combination. Attempts were made to increase the subculture interval up to 12 months with 80% survival of cultures. The effect of various parameters (closure types, temperatures and media components) on growth and survival of cultures is given in Table 22 and Figs. 8, 9 and 10.

Effect of different types of culture tube closures

As in cardamom, use of cotton plugs resulted in moisture loss and faster exhaustion of medium leading to drying up of cultures within 4–7 months (Fig. 8). In screw-capped, aluminium foil-covered or polypropylene-capped cultures, the moisture loss was minimized, and the nutrients were available to the cultures for

longer period. The cultures remained healthy up to 12 months in some of the media combinations tested. About 50–75% of the medium was exhausted after 12 months.

Table 22. Effect of media components, temperature and closure types on induction of minimal growth in ginger cultures

Treatment			Growth rate				Storage period (months) with 80% survival of cultures*
Basal medium (MS) concentration	Sucrose (S) + Mannitol (M)	Closure type	Increase in height (cm)*		Average no. of shoots /culture		
			Mean	SD	Mean	SD	
Full strength	30S + 0M	CP	7.2	7.2	4.6	0.7	4
Full strength	30S + 0M	SC	10.4	10.4	4.9	0.9	5
Full strength	20S + 0M	CP	5.6	5.6	2.6	0.8	3
Full strength	20S + 0M	SC	8.6	8.6	3.3	1.1	5
Full strength	20S + 10M	CP	4.7	4.7	2.8	0.9	3
Full strength	20S + 10M	SC	6.8	6.8	2.6	1.0	7
Full strength	15S + 15M	CP	6.3	6.3	2.7	0.9	3
Full strength	15S + 15M	SC	6.6	6.6	3	0.8	8
Full strength	10S + 10M	CP	3.7	3.7	1.5	0.7	4
Full strength	10S + 10M	SC	4.8	4.8	2	0.8	8
Half strength	30S + 0M	CP	6.4	0.8	3.4	1.0	3
Half strength	30S + 0M	SC	6.9	0.6	4	0.8	6
Half strength	20S + 0M	CP	5.4	0.4	1.9	1.0	4
Half strength	20S + 0M	SC	6.9	0.4	1.7	0.8	7
Half strength	20S + 10M	CP	5.1	0.4	1.7	0.7	4
Half strength	20S + 10M	SC	6.3	0.4	1.9	0.7	7
Half strength	15S + 15M	CP	4.8	0.5	2.3	0.8	5
Half strength	15S + 15M	SC	5.7	0.5	3.4	0.8	12
Half strength	10S + 10M	CP	5.4	0.8	2.2	0.6	5
Half strength	10S + 10M	SC	5.9	0.3	2.8	0.8	12
CD				**4.4**		**2.8**	
SE				**0.19**		**0.27**	
F-value				**57.9**		**12.84**	

MS - Murashige and Skoog medium; S – Sucrose; M – Mannitol; SC – Screw cap; CP – Cotton plug
* Mean of 10 replications ((Data collected after the respective storage period with 80% survival)

In vitro cultures in cotton-plugged tubes could be stored only up to 4 months with 100% survival. The cultures dried up completely within 4–6 months. Whereas, 90% of cultures in screw-capped tubes survived up to 9 months and 80% up to 12 months in the minimal growth medium. Cultures in both polypropylene-capped tubes and aluminium foil-covered tubes performed similar to that in screw-capped tubes in which retention of medium was high and the cultures could be stored for longer periods (Fig. 11a and 11b).

Effect of various temperatures

In ginger, incubation temperature of 22 ± 2^0C was better for growth and storage of *in vitro* cultures compared to 15^0C in which complete loss of cultures was observed within 15 days (Fig. 9).

Effect of different media constituents

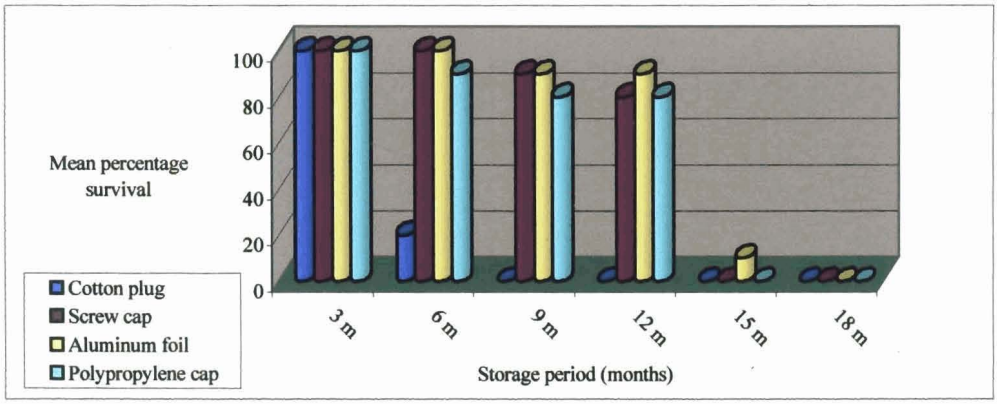
Full strength of MS basal medium with $30gl^{-1}$ sucrose supported faster and high growth rate with an average of 10.4 cm increase in plant height and 4.9 shoots per culture, hence was not suitable for *in vitro* storage in ginger, as the cultures fill the vessel within 2–3 months. When the nutrient concentration alone was reduced to half, the growth rate could be reduced slightly (8.6 cm increase in plant height and 3.3 shoots per culture), but storage beyond 6 months was not possible. But with the reduction in sucrose concentration (20 gl^{-1}) along with reduction in nutrient level (half strength MS), the culture storage could be increased up to 7 months with 80% survival. In this medium growth rate was also significantly reduced with respect to number of shoots per culture. Further reduction of sucrose (20–10 gl^{-1}) coupled with addition of mannitol (10 and 15 gl^{-1}) with full or half strength of the basal medium,

helped in inducing slow growth with 80% survival of cultures up to 12 months. The gradual reduction in growth of cultures was observed in these cultures (Figs. 10 and 11c). In full strength MS addition of mannitol did not give any significant reduction in growth rate.

Mannitol and sucrose at 10 gl^{-1} or 15 gl^{-1} each with half strength MS basal medium, helped in inducing slow growth in cultures. There was no significant difference between these treatments in terms of number of shoots and roots and both medium gave a survival of 80% after 12 months. In half strength MS medium with 10 gl^{-1} each of sucrose and mannitol, increase in height was 5.9 cm and the mean number of shoots per culture was 3.4. (Fig. 11d), whereas the medium with 15 gl^{-1} each of sucrose and mannitol, it was 5.7 and 3.4 respectively. The cultures in the latter medium were comparatively healthy and this medium was used for *in vitro* storage of ginger cultures.

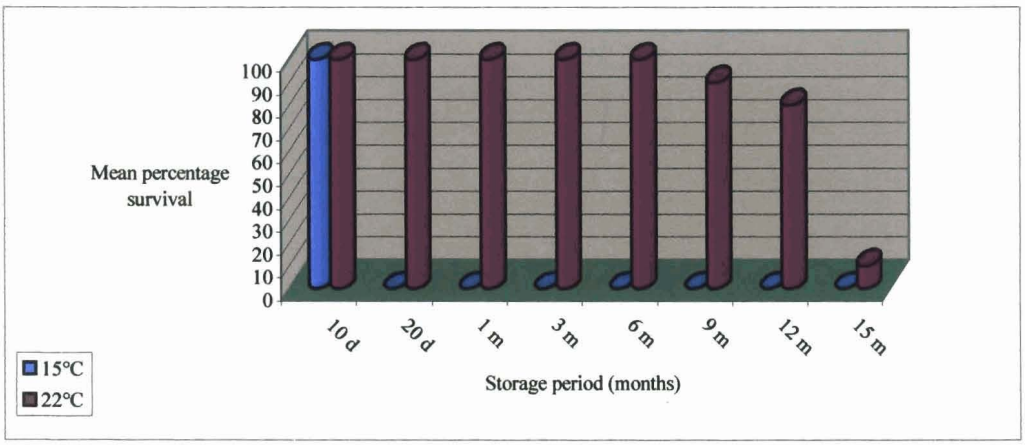
Thus in ginger, the cultures could be stored up to 12 months with 80% survival in half strength MS medium supplemented with 15 gl^{-1} each of sucrose and mannitol in sealed culture tubes (Fig. 11e). After 12 months of storage these cultures were transferred to multiplication medium (MS with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA), where they multiplied normally in 3 weeks. Multiple shoots and roots were produced in the same medium (Fig. 11f). Beyond 12 months of storage most of the cultures lost their capacity to regenerate new shoots. The rooted plantlets were transferred to plastic cups for hardening (Fig. 11g) and established with 80–90% survival.

Fig. 8. Survival percentage of ginger cultures at various time periods in different closure types*



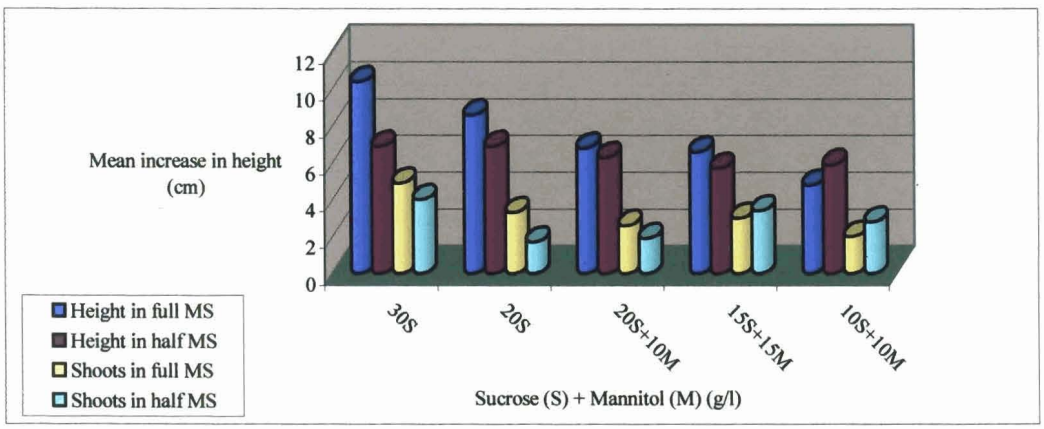
* In half MS with 15 gl^{-1} sucrose and 15 gl^{-1} mannitol at 22±2°C

Fig. 9. Survival percentage of ginger cultures under different temperature regimes at various time periods*



* In half MS with 15 gl^{-1} sucrose and 15 gl^{-1} mannitol at 22±2°C in sealed culture tubes

Fig. 10. Growth rate and survival percentage of ginger cultures in various media combinations*



* In half MS with 15 gl^{-1} sucrose and 15 gl^{-1} mannitol at 22±2°C in sealed culture tubes

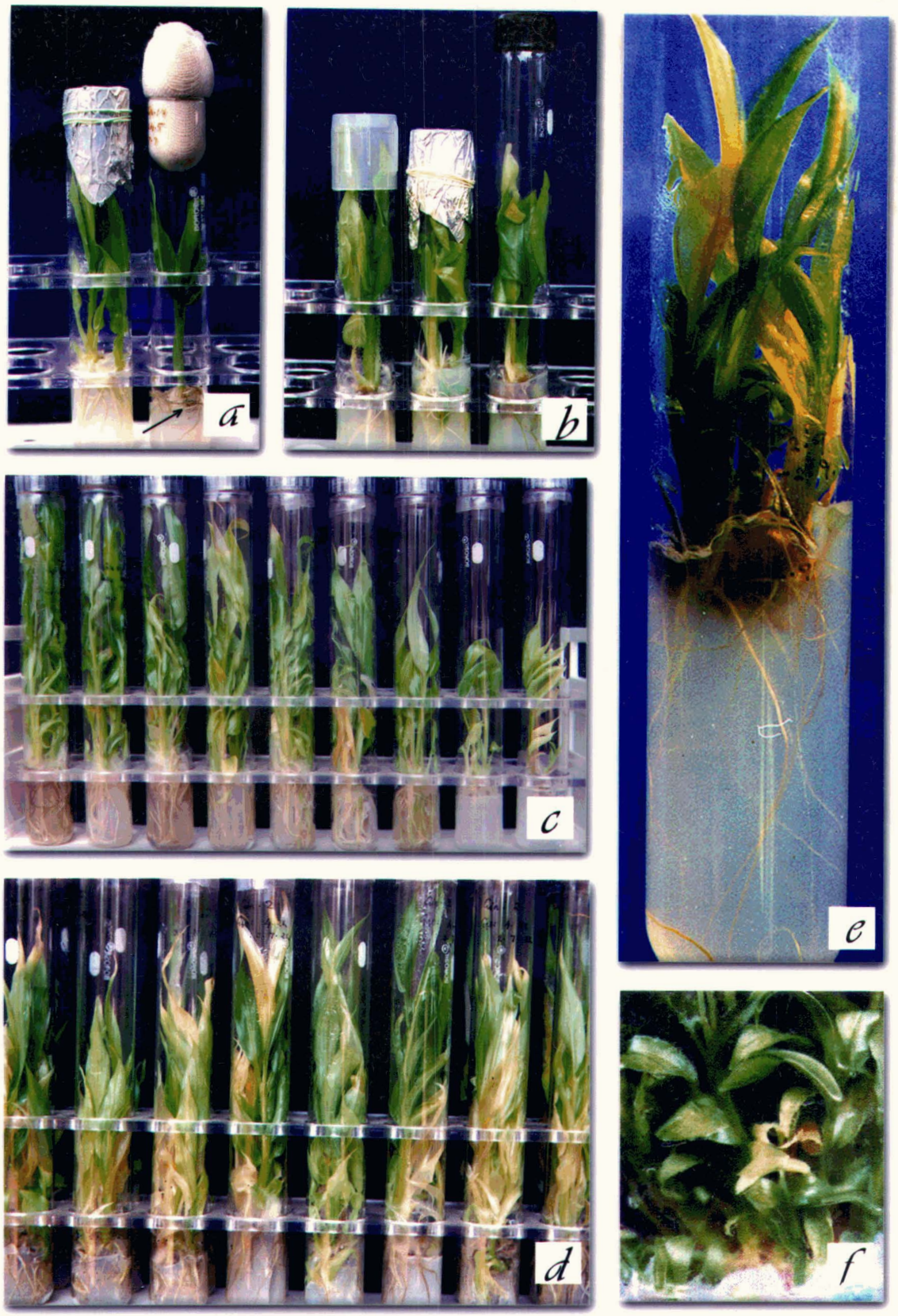


Fig. 11. Induction of slow growth in ginger

(a) Effect of cotton plug and aluminium foil closures on growth of cultures and exhaustion of medium (arrow indicates exhaustion of medium), (b) Effect of polypropylene cap, aluminium foil and screw cap closures on growth of cultures (1 year old culture), (c) Effect of various combinations of media on growth of cultures and induction of slow growth, (d) Cultures in half MS + 15 gl^{-1} sucrose + 15 gl^{-1} mannitol after I year of minimal growth storage, (e) Close up of one year old culture under minimal growth (f) *In vitro* multiplication after minimal growth storage.

TURMERIC

Micropropagation of turmeric (Fig. 12a) from vegetative bud explants using MS basal medium supplemented with various concentrations of cytokinins (BAP and Kin) and auxins (NAA and IBA) was attempted in the presernt study.

***In vitro* culture initiation**

Aseptic cultures were established using rhizome bud explants on MS medium with 0.5 mgl^{-1} Kin. The response was almost similar to that in ginger. Sixty to seventy per cent of the cultures could be established while the rest were lost due to contamination when explants of 1–2 cm size were used. Within 10 days the explants turned green and showed bud growth (Fig. 12b). In 50–60% of the established cultures emergence of new shoots (Fig. 12c) and roots noticed. When the explant size was more than 2 cm, culture loss due to contamination was over 80%, but response was 100%. Smaller explants (<5mm) resulted more than 80% contamination free cultures, but more than 60% of the established cultures exhibited mortality and response was slow.

***In vitro* multiplication**

Explants from established aseptic cultures were transferred to media with different combinations of growth regulators (BAP, Kin, NAA and IBA at 0.5 and 1.0 mgl^{-1}) to standardize efficient micropropagation protocols (Table 23). All the treatments tried have induced multiple shoots and roots, but the degree of response differed in different treatments.

MS medium supplemented with 1 mgl^{-1} each of IBA and NAA gave lowest response (40%) in terms of production of multiple shoots or roots while that supplemented with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA gave highest culture response

(90%). Culture response in the other treatments ranged from 50–80% but there was no significant difference between the treatments.

As in ginger and cardamom NAA at 1 mgl^{-1} gave the best response with an average of 4 shoots and 5.1 roots when the growth regulators were tested individually. Among the combinations tested BAP at 1 mgl^{-1} and NAA 0.5 mgl^{-1} gave 7.2 shoots and 5.8 roots.

The mean number of multiple shoots ranged from 1.3 to 7.2 in different treatments after 90 days of culture. MS medium supplemented with 1.0 mgl^{-1} IBA gave the lowest number of multiple shoots (1.3) while that supplemented with 0.5 mgl^{-1} NAA and 1.0 mgl^{-1} BAP gave significantly high number (7.2) of multiple shoots (Fig. 12d).

Though there was no significant difference between any of the treatment with regard to number of roots, the mean number of roots ranged from 2.2 to 5.8 in different treatments. MS media supplemented with 0.5 mgl^{-1} NAA and 1 mgl^{-1} BAP gave the highest number (5.8) of roots and MS media supplemented with 0.5 mgl^{-1} Kin gave the least number (2.2) of roots.

The medium combination that gave best response (MS + 0.5 mgl^{-1} NAA + 1.0 mgl^{-1} BAP) was repeated and consistency in results was observed and hence this combination was used for the production of stock material for *in vitro* conservation experiments. The plantlets from the vegetative bud cultures were healthy, 9–13cm tall, with 5–7 roots. These plantlets were hardened in humid chamber for 20–25 days with 80% establishment (Fig. 12e) and further transplanted to polythene bags (Fig. 12f).

Table 23. Effect of growth regulators on multiple shoot and root induction from vegetative bud explant in turmeric on MS basal medium

Growth regulator (mgl^{-1})				Response frequency (%)	Average no. of shoots/ explant		Average no. of roots/ culture	
Kin	BAP	NAA	IBA		Mean	SD	Mean	SD
0.5	-	-	-	70	1.9	0.7	2.2	1.5
1.0	-	-	-	80	2.0	0.8	2.6	1.0
-	0.5	-	-	70	3.1	1.0	2.8	1.0
-	1.0	-	-	70	3.2	1.0	3.4	0.8
-	-	0.5	-	50	1.5	1.1	3.9	0.7
-	-	1.0	-	70	4.0	1.6	5.1	1.1
-	-	-	0.5	70	1.4	0.8	3.3	0.8
-	-	-	1.0	70	1.3	0.7	4.3	1.1
0.5	0.5	-	-	80	4.2	1.7	3.6	0.8
0.5	1.0	-	-	80	3.4	1.4	3.6	0.7
1.0	0.5	-	-	70	2.4	0.7	3.4	0.7
1.0	1.0	-	-	60	2.9	0.9	2.7	0.8
0.5	-	0.5	-	80	4.1	0.7	5.6	1.0
0.5	-	1.0	-	70	5.0	1.5	6.2	1.1
1.0	-	0.5	-	80	3.4	1.3	5.4	1.2
1.0	-	1.0	-	60	3.3	1.1	5.0	1.0
0.5	-	-	0.5	60	2.4	0.7	3.9	0.7
0.5	-	-	1.0	60	3.8	1.0	5.5	1.3
1.0	-	-	0.5	60	3.7	1.5	3.8	0.7
1.0	-	-	1.0	50	3.7	0.8	3.6	1.1
-	0.5	0.5	-	80	4.7	0.9	5.5	1.0
-	0.5	1.0	-	80	4.0	1.6	5.2	0.8
-	1.0	0.5	-	90	7.2	1.1	5.8	1.3
-	1.0	1.0	-	70	4.3	1.6	4.9	0.9
-	0.5	-	0.5	60	3.1	0.7	3.7	1.4
-	0.5	-	1.0	70	3.0	1.6	4.4	0.8
-	1.0	-	0.5	70	4.1	0.7	5.2	1.0
-	1.0	-	1.0	60	3.3	1.5	3.8	1.6
-	-	0.5	0.5	60	1.6	1.0	2.6	1.3
-	-	0.5	1.0	50	2.8	1.1	3.4	0.8
-	-	1.0	0.5	50	3.2	0.9	2.5	0.7
-	-	1.0	1.0	40	1.5	0.7	2.5	0.8
CD					**3.57**		**3.28**	
SE					**0.36**		**0.32**	
F-value					**11.94**		**12.40**	

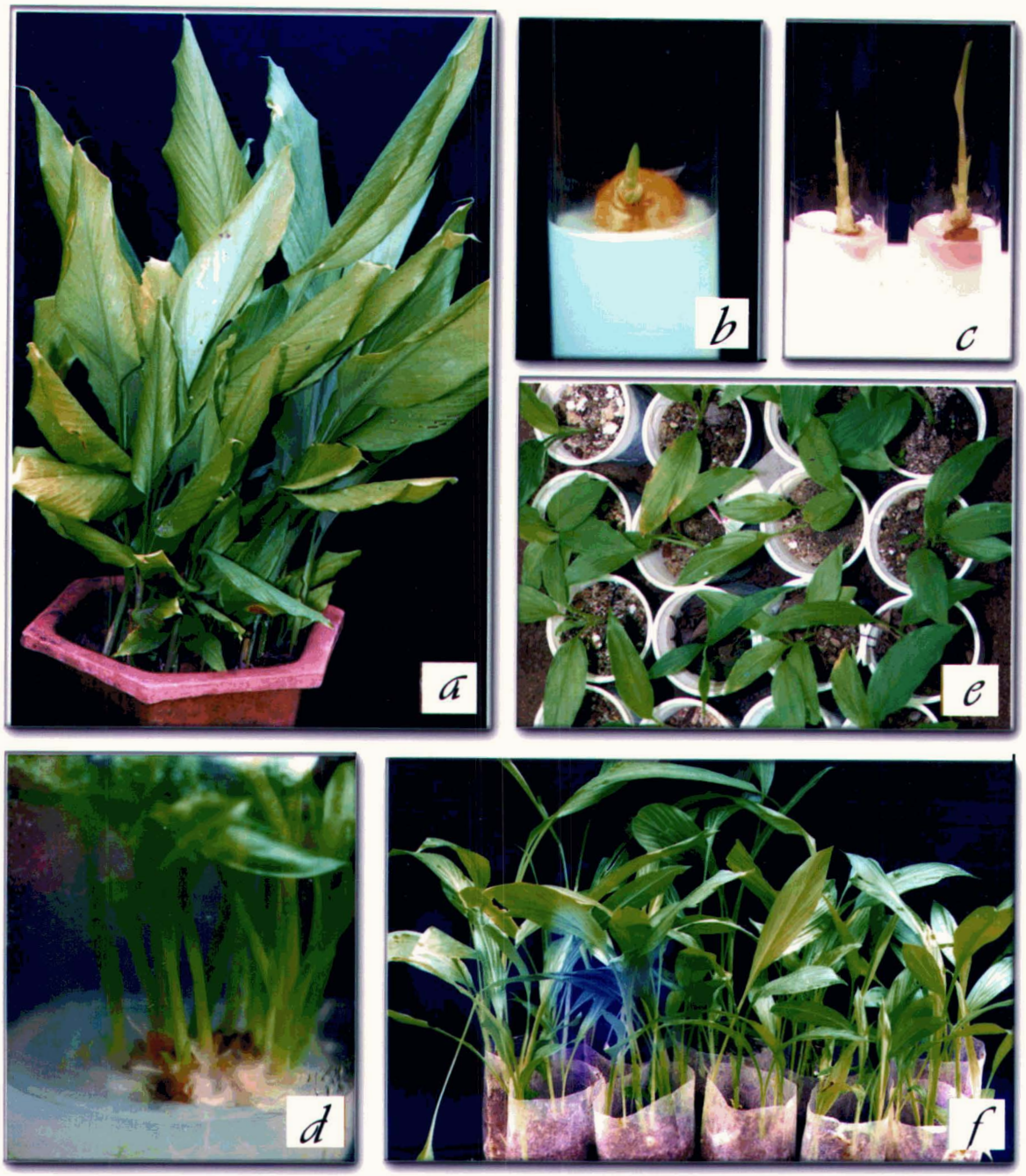


Fig. 12. Micropropagation in turmeric (*Curcuma longa* L.)

(a) Turmeric plant, (b) Culture initiation from vegetative bud explant in MS + 0.5 mgl^{-1} Kin, (c) Apical bud growth and initiation of axillary buds, (d) Development of multiple shoots and roots in MS + 1 mgl^{-1} BAP + 0.5 mgl^{-1} NAA, (g) Micropropagated plants after hardening, (h) Hardened plantlets transplanted to polybags.

Slow (minimal) growth storage

Various parameters like closure types, temperatures and media components were tested for their efficiency to induce slow growth in turmeric cultures also. The effect of these factors on the growth and survival of cultures are presented in Table 24 and in Figs. 13, 14 and 15.

Effect of different types of culture tube closures

The effect of different closure types on growth and survival of cultures in turmeric is given in Fig. 13. As in cardamom and ginger use of cotton plugs resulted in moisture loss and faster exhaustion of the medium leading to drying up of cultures within 5–6 months.

Turmeric cultures in cotton-plugged tubes could be stored up to 3, 4 and 6 months with 100%, 80% and 20% survival respectively in certain media combinations (Fig. 13). Drying up of cultures was noticed after 6 months. The cultures dried up completely by 7 months. But use of screw-capped, aluminium foil-closed or polypropylene-capped culture vessels minimized the moisture loss and resulted in availability of nutrients for a longer period and the cultures were healthy (Figs. 16a and 16b). When sealed culture tubes were used the cultures could be stored up to 12 months with 80% survival. Cultures in screw-capped tubes, polypropylene-capped tubes and aluminium foil-covered tubes performed more or less similar in response. In these cases the retention of medium was high and the cultures could be stored for longer periods of time (Fig. 16c). In turmeric only 25% of the medium was exhausted even after 12 months of storage.

Table 24. Effect of media components, temperature and closure types on induction of minimal growth in turmeric cultures

Treatment			Growth rate				Storage period (months) with 80% survival of cultures*
Basal medium (MS) concentration	Sucrose (S) + Mannitol (M)	Closure type	Increase in height (cm)*		Average no. of shoots /culture		
			Mean	SD	Mean	SD	
Full strength	30S + 0M	CP	6.7	0.5	5.0	1.0	3
Full strength	30S + 0M	SC	10.4	1.1	4.1	0.7	4
Full strength	20S + 0M	CP	6.2	0.4	3.4	0.8	3
Full strength	20S + 0M	SC	6.8	2.5	4.1	1.1	5
Full strength	20S + 10M	CP	6.0	0.6	3.3	0.7	5
Full strength	20S + 10M	SC	6.5	0.4	3.0	0.7	7
Full strength	15S + 15M	CP	5.7	0.4	2.8	0.8	5
Full strength	15S + 15M	SC	7.1	0.4	3.2	0.8	8
Full strength	10S + 10M	CP	4.8	0.5	1.9	0.9	5
Full strength	10S + 10M	SC	5.3	0.5	2.3	0.9	7
Half strength	30S + 0M	CP	6.4	0.4	4.1	0.9	3
Half strength	30S + 0M	SC	6.6	1.2	4.9	1.0	4
Half strength	20S + 0M	CP	5.6	0.2	3.9	1.2	3
Half strength	20S + 0M	SC	6.9	0.5	3.1	1.4	5
Half strength	20S + 10M	CP	5.4	0.4	1.7	0.7	5
Half strength	20S + 10M	SC	6.1	2.0	2.1	0.9	8
Half strength	15S + 15M	CP	5.4	0.4	2.1	0.9	5
Half strength	15S + 15M	SC	5.7	0.3	2.9	1.2	12
Half strength	10S + 10M	CP	5.2	0.4	2.0	0.8	6
Half strength	10S + 10M	SC	5.0	0.5	2.1	0.7	12
CD				**3.56**		**2.96**	
SE				**0.30**		**0.29**	
F-value				**15.65**		**11.76**	

MS- Murashige and Skoog medium; S – Sucrose; M – Mannitol; SC – Screw cap; CP – Cotton plug

* Mean of 10 replications (Data collected after the respective storage period with 80% survival)

Effect of temperature

In turmeric also incubation temperature of 22 ± 2^{0}C was suitable for growth and storage of *in vitro* cultures and 15^{0}C was deleterious to the cultures and complete loss of viability was observed within 15 days (Fig. 14).

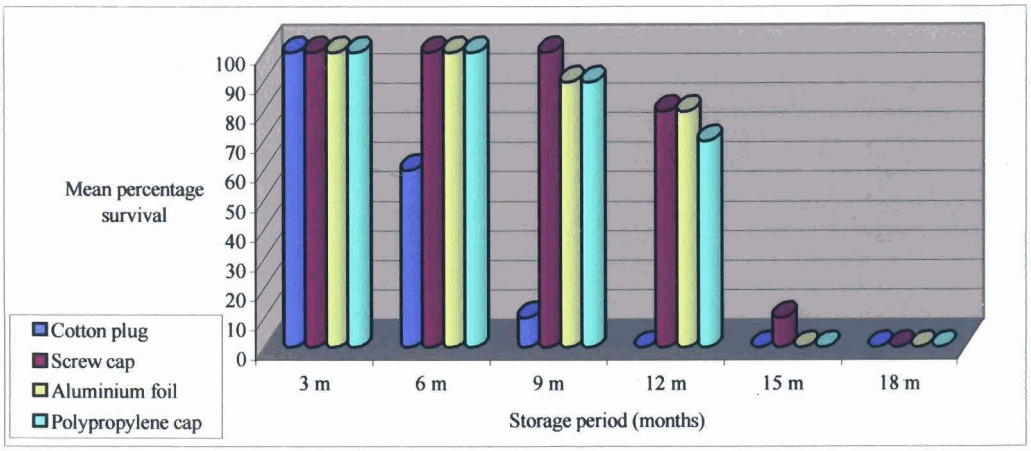
Effect of different media constituents

The effect of different media combinations on growth rate and survival of *in vitro* cultures of turmeric is presented in Fig. 15. High growth rate was observed in full strength MS basal medium with 30 gl^{-1} sucrose, and hence was not suitable for *in vitro* storage. The cultures over grew the culture tube within 3–4 months. When the nutrient concentration alone was reduced to half, there was slight reduction in growth rate. When sucrose concentration was also reduced along with reduction in nutrient level, no significant reduction in growth was observed.

Reduction in sucrose coupled with addition of mannitol, either with full or half strength of the basal medium, helped in inducing slow growth. Mannitol and sucrose at 10 gl^{-1} each or 15 gl^{-1} each, with MS at half strength was suitable for inducing slow growth in cultures with more than 80% survival after 12 months (Fig. 16d). A gradual reduction in growth of cultures was noticed by the manipulation of culture medium.

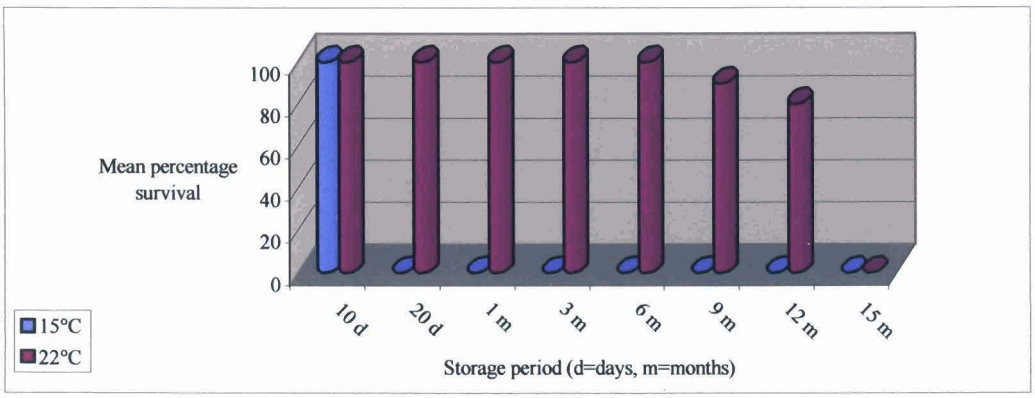
Turmeric plantlets grown in medium supplemented with full MS and 30 gl^{-1} sucrose achieved an average of 10.4 cm increase in height and had 4.1 shoots per culture. When the nutrient concentration was reduced to half, the increase in height was 6.6 cm and with a mean number of 4.9 shoots per culture. When sucrose concentration was reduced to 20 gl^{-1}, the mean increase in height was 6.8 cm and 6.9 cm in full MS and half MS, respectively. The mean number of shoots in the respective media was 4.1 and 3.1 per culture.

Fig. 13. Survival percentage of turmeric cultures at various time periods in different closure types*



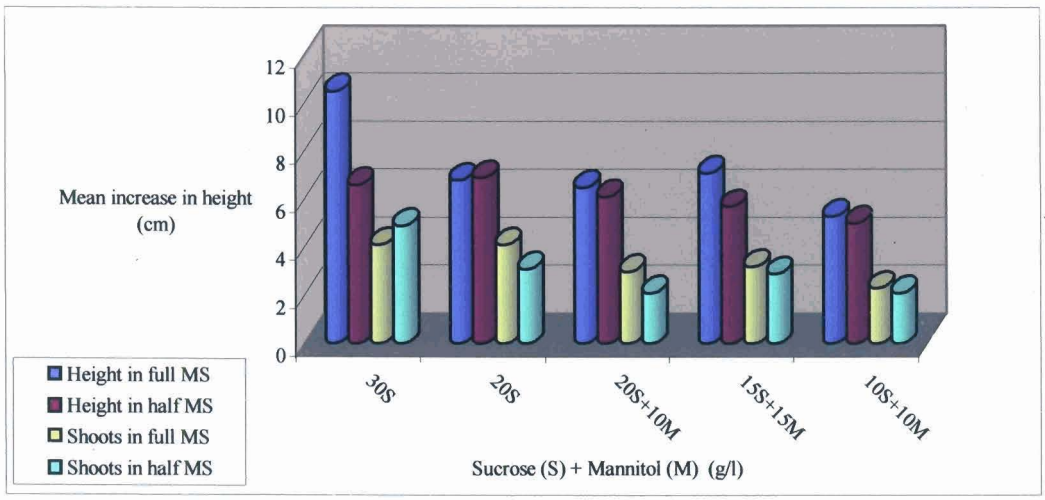
* In half MS medium with 15 gl^{-1} sucrose and 15 gl^{-1} mannitol at 22±2°C

Fig. 14. Survival percentage of turmeric cultures under different temperature regimes at various time periods*



* In half MS medium with 15 gl^{-1} sucrose and 15 gl^{-1} mannitol at 22±2°C in sealed culture tubes

Fig. 15. Growth rate and survival percentage of turmeric cultures in various media combinations*



* In sealed culture tubes at 22±2°C

Fig. 16. Induction of slow growth in turmeric

(a) Effect of cotton plug and Aluminium foil closures on growth of cultures and exhaustion of medium (arrow indicates exhaustion of medium), (b) Effect of cotton plug and polypropylene cap closures on growth of cultures and exhaustion of medium, (c) Effect of polypropylene cap, aluminium foil and screw cap closures on growth of cultures (1 ear old culture), (d) One year old culture in minimal growth medium (half MS + 15 gl^{-1} sucrose + 15 gl^{-1} mannitol), (e) Cultures under minimal growth storage, (f) *In vitro* multiplication after minimal growth storage.

In full strength MS supplemented with 20 gl^{-1} sucrose and 10 gl^{-1} mannitol, the increase in height was 6.5 cm and the mean number of shoots per culture was 3.0, whereas in half strength MS it was 6.1 cm increase in height and 2.1 shoots per culture. In full and half strength of the medium supplemented with 15 gl^{-1} each of sucrose and mannitol, the plantlets achieved a height of 7.1 cm and 5.7 cm respectively, and the number of shoots per culture was 3.2 and 2.9. Further reduction in sucrose and mannitol (10 gl^{-1}each) gave an increase in height of 5.3 cm and 5.0 cm and the mean number of shoots per culture was 2.3 and 2.1 in full and half MS, respectively. In full MS, the maximum period of storage with 80% survival was 7–8 months, whereas in half MS it was possible to store up to 12 months with 80% survival when sucrose and mannitol was used in equal proportions (10 gl^{-1} each / 15 gl^{-1} each) in sealed culture tubes. In this media growth reduction was also achieved.

Thus in turmeric, cultures could be stored up to 12 months with 80% survival in half strength MS medium supplemented with 10 gl^{-1} or 15 gl^{-1} each of sucrose and mannitol in sealed culture tubes ((Fig. 16c). Storage of these cultures beyond 12 months resulted in yellowing of aerial shoots and gradually the cultures failed to regenerate new axillary shoots and died. These *in vitro* stored cultures, on transfer to multiplication medium (MS with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA) multiplied to normal plants in 3 weeks of culture (Fig. 16f). The rooted plantlets were transferred to cups and established with 80–90% survival.

KAEMPFERIA SPECIES (*K. GALANGA* and *K. ROTUNDA*)

MS medium with cytokinins (BAP and Kin) and auxins (NAA and IBA) were tested for standardizing micropropagation protocols for clonal multiplication in *K. galanga* (Fig. 17a) and *K. rotunda* (Fig. 18a) using vegetative bud explants.

In vitro culture initiation

Contamination free cultures were established on MS medium supplemented with 0.5 mgl^{-1} Kin. The culture establishment was 70% and 60% in *K. galanga* and *K. rotunda* respectively. The explant size of 1–2 cm was ideal for culture initiation. Rhizome buds of both the species turned green and sprouted within 10 days (Figs. 17b and 18b) and within 2 weeks shoot growth and root initiation was observed in the initiation medium (Fig. 18c).

In vitro multiplication

The established contamination-free cultures were used as source for explants in micropropagation experiment. MS medium with different combinations of growth regulators (BAP, Kin, NAA and IBA at 0.5 and 1.0 mgl^{-1}) were tested for induction of micropropagation. The culture responses in various combinations of growth regulators on Ms basal medium in *K. galanga* are given in Table 25 and those of *K. rotunda* are presented in Table 26.

MS medium supplemented with 1 mgl^{-1} IBA gave lowest response (30%) in terms of production of multiple shoots or roots while that supplemented with 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA or 0.5 mgl^{-1} BAP and 1 mgl^{-1} NAA gave highest culture response (100%). In *K. galanga*, the mean number of shoots produced in various media combinations ranged from 1.1 to 8.2 after 90 days of culture. MS medium supplemented with 1.0 mgl^{-1} IBA and medium with 0.5 mgl^{-1} kinetin and 1 mgl^{-1} IBA gave the lowest number of multiple shoots (1.1) while that supplemented with 0.5 mgl^{-1} NAA and 1 mgl^{-1} BAP gave the highest number (8.2) of multiple shoots. Medium with 0.5 mgl^{-1} each of NAA and BAP also gave almost same response in terms of multiple shoot production (8.1).

Table 25. Effect of growth regulators on multiple shoot and root induction from vegetative bud explant in *Kaempferia galanga* on MS basal medium

Growth regulators				Response frequency (%)	Average no. of shoots/ explant		Average no. of roots/ shoot	
Kinetin	BAP	NAA	IBA		Mean	SD	Mean	SD
0.5	-	-	-	70	1.2	1.1	11.6	1.5
1.0	-	-	-	80	5.0	1.7	4.1	0.7
-	0.5	-	-	80	2.1	1.3	6.0	0.9
-	1.0	-	-	90	3.0	1.7	6.3	1.1
-	-	0.5	-	90	4.0	1.6	4.9	1.0
-	-	1.0	-	100	6.5	0.7	7.7	1.5
-	-	-	0.5	60	3.0	0.8	2.0	0.8
-	-	-	1.0	50	1.1	0.8	2.9	0.9
0.5	0.5	-	-	90	5.0	1.4	6.5	0.8
0.5	1.0	-	-	90	3.3	0.8	5.4	1.1
1.0	0.5	-	-	80	3.3	0.8	4.1	0.7
1.0	1.0	-	-	70	2.3	1.1	2.9	0.9
0.5	-	0.5	-	70	3.9	0.7	4.1	0.9
0.5	-	1.0	-	80	5.0	1.4	5.8	0.8
1.0	-	0.5	-	80	4.0	1.6	4.0	1.3
1.0	-	1.0	-	60	3.1	1.7	3.5	1.0
0.5	-	-	0.5	60	3.0	1.6	2.6	0.7
0.5	-	-	1.0	70	1.1	0.6	5.5	0.7
1.0	-	-	0.5	60	2.3	1.4	3.7	0.8
1.0	-	-	1.0	70	2.3	1.3	5.1	0.9
-	0.5	0.5	-	90	8.1	1.0	7.5	1.0
-	0.5	1.0	-	100	5.2	1.1	7.1	0.9
-	1.0	0.5	-	100	8.2	0.8	10.4	1.7
-	1.0	1.0	-	80	5.2	1.7	5.2	1.2
-	0.5	-	0.5	80	3.1	0.7	4.0	0.9
-	0.5	-	1.0	60	1.3	1.1	3.3	1.3
-	1.0	-	0.5	70	4.9	0.7	4.7	0.8
-	1.0	-	1.0	60	3.5	0.7	5.4	0.8
-	-	0.5	0.5	70	3.9	0.9	3.3	1.2
-	-	0.5	1.0	80	3.1	1.6	4.3	0.8
-	-	1.0	0.5	80	4.8	1.5	4.5	0.7
-	-	1.0	1.0	60	3.3	1.5	8.2	1.0
CD						**5.0**		**6.2**
SE						**0.40**		**0.32**
F-value						**10.78**		**44.81**

Table 26. Effect of growth regulators on multiple shoot and root induction from vegetative bud explant in *Kaempferia rotunda* on MS basal medium

Growth regulators				Response frequency (%)	Average no. of shoots/ explant		Average no. of roots/ culture	
Kin	BAP	NAA	IBA		Mean	SD	Mean	SD
0.5	-	-	-	60	1.4	0.8	7.3	0.8
1.0	-	-	-	80	3.1	1.4	4.9	1.0
-	0.5	-	-	70	1.4	1.0	8.0	1.0
-	1.0	-	-	80	3.4	0.7	3.8	0.9
-	-	0.5	-	70	3.3	1.6	4.1	1.0
-	-	1.0	-	70	3.9	1.6	4.4	1.0
-	-	-	0.5	40	1.2	0.4	2.5	1.3
-	-	-	1.0	50	1.2	0.6	4.0	1.6
0.5	0.5	-	-	70	5.0	0.9	5.4	1.3
0.5	1.0	-	-	80	3.2	1.1	5.9	1.0
1.0	0.5	-	-	70	3.2	0.8	4.4	1.0
1.0	1.0	-	-	80	3.9	1.0	2.9	1.0
0.5	-	0.5	-	70	4.2	0.8	5.7	0.8
0.5	-	1.0	-	60	4.3	0.8	6.6	0.7
1.0	-	0.5	-	60	3.2	1.5	4.3	0.8
1.0	-	1.0	-	70	3.8	0.8	3.7	1.4
0.5	-	-	0.5	60	1.4	0.8	3.2	1.6
0.5	-	-	1.0	50	1.9	1.2	3.2	0.9
1.0	-	-	0.5	70	2.2	1.2	4.0	1.7
1.0	-	-	1.0	60	2.7	0.8	3.7	1.1
-	0.5	0.5	-	80	4.1	0.9	3.3	0.8
-	0.5	1.0	-	90	3.5	0.8	4.5	1.0
-	1.0	0.5	-	90	6.1	1.0	8.4	0.8
-	1.0	1.0	-	70	3.8	0.8	7.4	0.8
-	0.5	-	0.5	60	3.6	0.7	3.8	0.8
-	0.5	-	1.0	70	1.2	0.6	4.2	1.0
-	1.0	-	0.5	50	3.9	0.7	4.0	1.3
-	1.0	-	1.0	60	3.2	0.8	4.8	0.9
-	-	0.5	0.5	60	1.4	0.8	3.5	0.7
-	-	0.5	1.0	60	1.2	0.6	3.9	1.8
-	-	1.0	0.5	60	1.6	1.0	3.4	0.8
-	-	1.0	1.0	50	2.3	0.9	4.0	0.8
CD					**3.7**		**3.9**	
SE					**0.31**		**0.35**	
F-value					**17.17**		**18.68**	

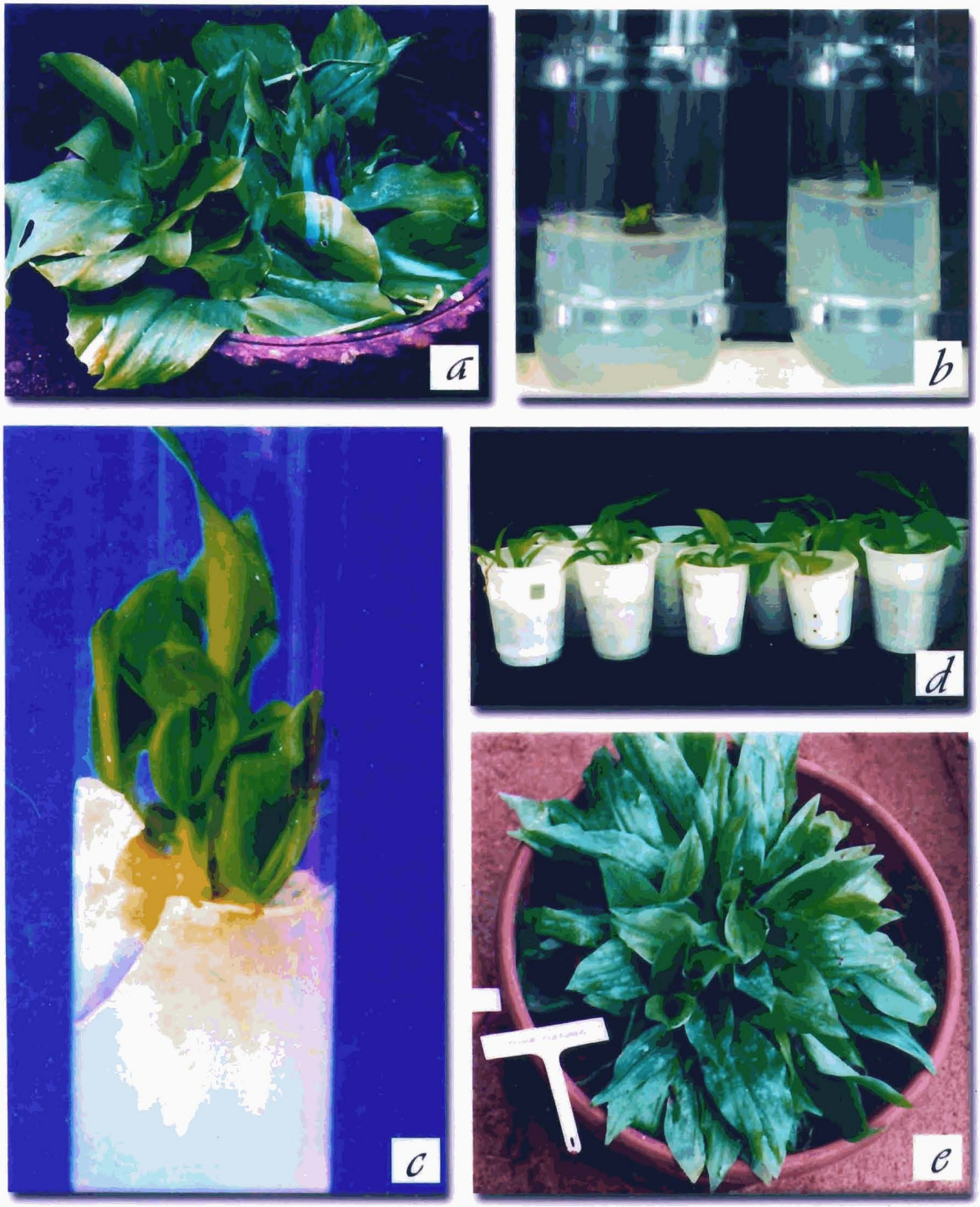


Fig. 17. Micropropagation in *Kaempferia galanga* L.

(a) K. *galanga* plant, (b) Culture initiation from vegetative bud explant in MS + 0.5 mgl^{-1} Kin, (c) Development of multiple shoots and roots in MS + 1 mgl^{-1} BAP + 0.5 mgl^{-1} NAA, (d) Micropropagated plants after hardening, (h) Micropropagated plant transplanted to pot.

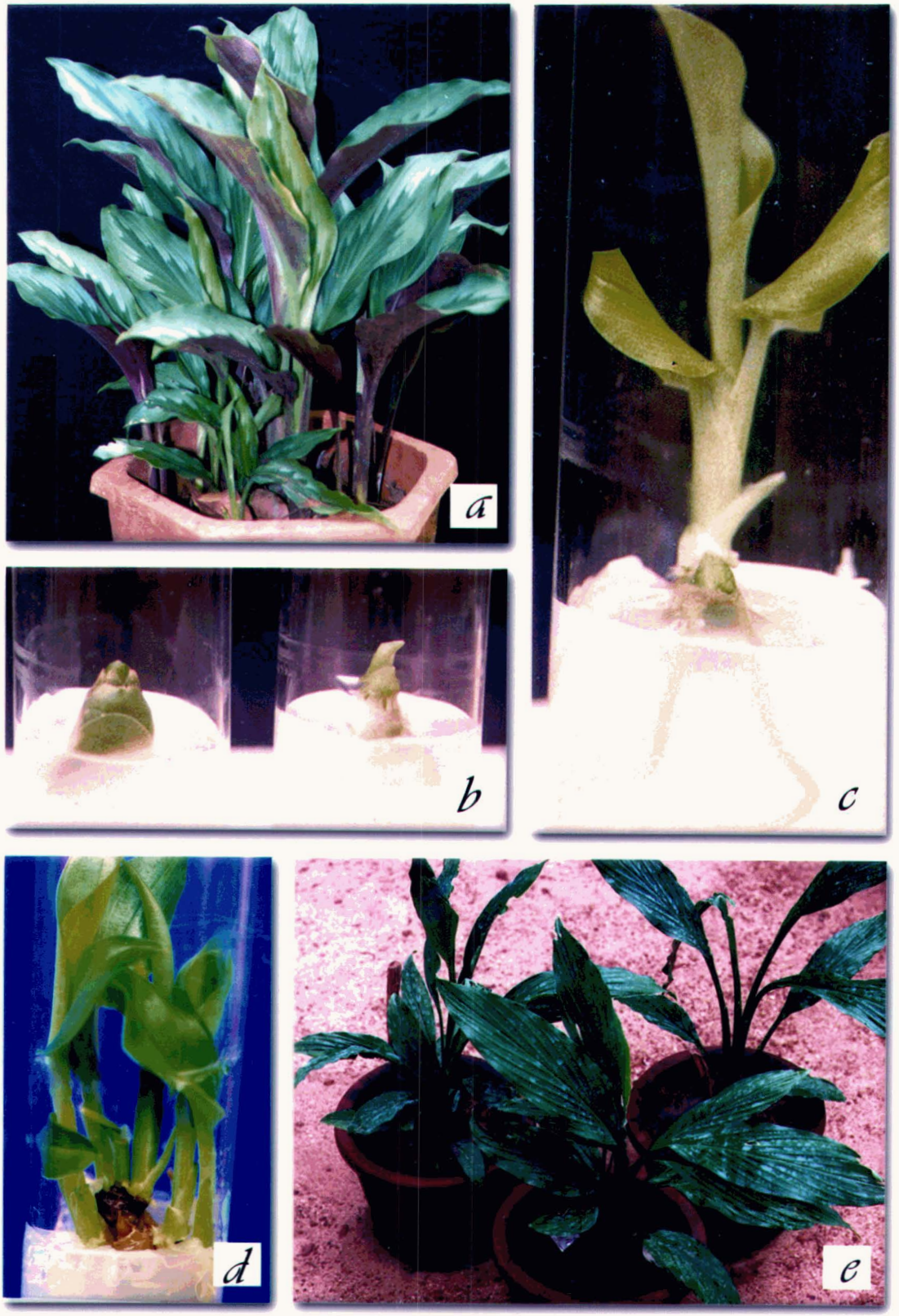


Fig. 18. Micropropagation in *Kaempferia rotunda* L.

(a) *K. rotunda* plant, (b) Culture initiation from vegetative bud explant in MS + 0.5 mgl^{-1} Kin, (c) Apical bud growth, axillary bud initiation and rooting (d) Development of multiple shoots and roots in MS + 1 mgl^{-1} BAP + 0.5 mgl^{-1} NAA, (e) Hardened plantlets transplanted to pots.

The mean number of roots ranged from 2.0 to 11.6 in different treatments. MS media supplemented with 0.5 mgl^{-1} Kin gave the highest number (11.6) of roots and MS media supplemented with 0.5 mgl^{-1} IBA gave the least number (2.0) of roots. Considering both shoot formation and root formation, the medium supplemented with 0.5 mgl^{-1} NAA and 1 mgl^{-1} BAP was superior to others.

In *K. rotunda*, MS medium supplemented with 0.5 mgl^{-1} IBA gave lowest response (40%) in terms shoot elongation and production of roots while that supplemented with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA or 0.5 mgl^{-1} BAP and 1 mgl^{-1} NAA gave highest culture response (90%). The mean number of multiple shoots ranged from 1.2 to 6.1 in different treatments. Multiple shoots were rarely produced in four media combinations (IBA at 0.5 and 1 mgl^{-1} levels, 1 mgl^{-1} IBA in combination with 0.5 mgl^{-1} NAA or 0.5 mgl^{-1} BAP), in which mostly single shoot growth and rooting was observed. MS medium supplemented with 0.5 mgl^{-1} NAA and 1 mgl^{-1} BAP gave the highest number (6.1) of multiple shoots. Medium with 0.5 mgl^{-1} each of NAA and BAP showed no significant difference in response in terms of multiple shoot production (6.1).

The mean number of roots ranged from 2.9 to 8.4 in different treatments. MS media supplemented with 0.5 mgl^{-1} NAA and 1.0 mgl^{-1} BAP gave the highest number (8.4) of roots and MS media supplemented with 0.5 mgl^{-1} IBA gave the least number (2.9) of roots. Considering both shoot formation and root formation the medium supplemented with 0.5 mgl^{-1} NAA and 1.0 mgl^{-1} BAP was superior to others.

When cytokinins (BAP and Kin) were used alone, kinetin at 1.0 mgl^{-1} level produced more multiple shoots (5.0) in the case of *K. galanga*, while profuse rooting was observed in the medium with 0.5 mgl^{-1} Kin (11.6). Medium containing either

BAP or Kin at 1 mgl^{-1} was good for *K. rotunda*, in which 3.4 and 3.1 multiple shoots were produced respectively.

When cytokinins were used in combination, the medium supplemented with 0.5 mgl^{-1} each of kin and BAP could produce an average of 5.0 harvestable shoots in both the species, with good rooting (6.5 in *K. galanga* and 5.4 in *K. rotunda*).

The effect of auxins (NAA and IBA) alone and in combination was studied and IBA alone did not give any favourable responses. The root system produced was generally unhealthy. In the medium with NAA at 1 mgl^{-1} up to 6.5 and 4.0 harvestable shoots with very good root system was produced in *K. galanga* and *K. rotunda* respectively. In combination of IBA at 0.5 mgl^{-1} and NAA at and 1 mgl^{-1}, an average of 4–5 shoots and good rooting was observed in *K. galanga,* whereas in all other auxin combinations the root system was short and stout without secondary roots.

Among the cytokinin-auxin combinations, the one with NAA at 0.5 mgl^{-1} and BAP at 1 mgl^{-1} levels was suitable for both the species. In this medium, *K. galanga* produced 8–10 shoots (Fig. 17c) and *K. rotunda* produced 6–7 shoots (Fig. 18d). Rooting of 1:10 and 1:6 was achieved in the same medium. *In vitro* plantlets with 7–13 cm height and 7–12 roots could be successfully hardened (Fig. 17d) and transplanted to polythene bags or pots (Fig. 17e and 18e).

Slow (minimal) growth storage

Different slow growth inducing parameters such as nutrient media at various levels, prevention of evaporation loss using different culture tube enclosures were tried in various combinations in *K. galanga* and *K. rotunda.* Only one incubation temperature ($22\pm2^{0}C$) was used, since this temperature was found suitable for maintaining cultures of cardamom, ginger and turmeric.

The effect of various factors on growth and survival of *K. galanga* and *K. rotunda* cultures are given in Tables 27 and 28 respectively. The effect of different closure types and medium constitution on the growth and survival of cultures in both species are presented in Figures 19–22.

Effect of different type of culture tube closures

As in cardamom ginger and turmeric use of cotton plugs resulted in moisture loss, faster exhaustion of the medium and drying up of cultures within 4–5 months in both the species. The other enclosures minimized moisture loss resulting in healthy cultures up to 12 months. In *K. galanga* the cultures could be maintained even up to 15 months (Figs. 19 and 20). *In vitro* cultures in screw-capped tubes survived up to 12 months with 90% and 80% survival in *K. galanga* and *K. rotunda* respectively (Fig. 23a).

Effect of different media constituents

Full strength MS basal medium with 30 gl^{-1}sucrose induced higher growth rate and over grew the culture tube within 3–4 months hence not suitable for *in vitro* storage of *K. galanga* and *K. rotunda.* In this medium *K. galanga* plantlets achieved an average of 10.6 cm increase in height and 2.7 shoots per culture and *K. rotunda* plantlets achieved an average of 11.4 cm increase in height and 1.8 shoots per culture. When the basal medium concentration alone was reduced to half no significant growth reduction was observed. In this medium the increase in height was 6.1 cm and 9.8 cm with a mean number of 2.4 and 1.8 shoots per culture in *K. galanga* and in *K. rotunda* respectively. When sucrose concentration was reduced to 20 gl^{-1}, the mean increase in height was 9.9 cm and 5.8 cm in full MS and half MS, respectively and the mean number of shoots in the respective media was 2.3 and 2.0 per culture in *K.*

galanga. In *K.* rotunda it was 9.9 cm and 5.6 cm in full MS and half MS respectively and the mean numbers of shoots in the both the media were 1.4 per culture. In half strength MS medium with 20 gl^{-1} sucrose the cultures of both these species could be stored up to a longer period of 6–7 months with 80% viability (Figs. 21 and 22).

Table 27. Effect of media components, temperature and closure types on induction of minimal growth in *Kaempferia galanga* cultures

Treatment			Growth rate				Storage period (months) with 80% survival of cultures*
Basal medium (MS) concentration	Sucrose (S) + Mannitol (M)	Closure type	Increase in height (cm)*		Average no. of shoots /culture		
			Mean	SD	Mean	SD	
Full strength	30S + 0M	CP	7.5	1.9	3.1	0.7	4
Full strength	30S + 0M	SC	10.6	0.7	2.7	0.9	3
Full strength	20S + 0M	CP	6.3	1,1	3.0	1.0	3
Full strength	20S + 0M	SC	9.9	0.6	2.3	0.8	4
Full strength	20S + 10M	CP	5.9	0.8	2.0	0.8	5
Full strength	20S + 10M	SC	6.9	0.6	1.9	0.7	6
Full strength	15S + 15M	CP	4.1	1.1	1.8	0.6	7
Full strength	15S + 15M	SC	4.5	0.7	2.3	0.8	8
Full strength	10S + 10M	CP	3.8	0.8	2.0	1,1	6
Full strength	10S + 10M	SC	4.2	0.4	2.2	0.8	8
Half strength	30S + 0M	CP	5.3	0.8	2.1	1.1	4
Half strength	30S + 0M	SC	6.1	0.4	2.4	1.0	6
Half strength	20S + 0M	CP	5.0	1.0	2.2	1.0	3
Half strength	20S + 0M	SC	5.8	0.4	1.9	0.7	6
Half strength	20S + 10M	CP	3.5	0.5	1.5	0.7	7
Half strength	20S + 10M	SC	4.1	0.3	1.9	0.9	9
Half strength	15S + 15M	CP	2.3	0.7	1.8	0.8	7
Half strength	15S + 15M	SC	2.1	0.6	2.0	0.7	12
Half strength	10S + 10M	CP	2.1	0.8	2.0	0.9	7
Half strength	10S + 10M	SC	1.8	0.4	1.7	0.7	12
CD			**7.13**		**1.21**		
SE			**0.26**		**0.27**		
F-value			**8.33**		**2.25**		

MS- Murashige and Skoog medium; S – Sucrose; M – Mannitol; SC – Screw cap; CP – Cotton plug

* Mean of 10 replications ((Data collected after the respective storage period with 80% survival)

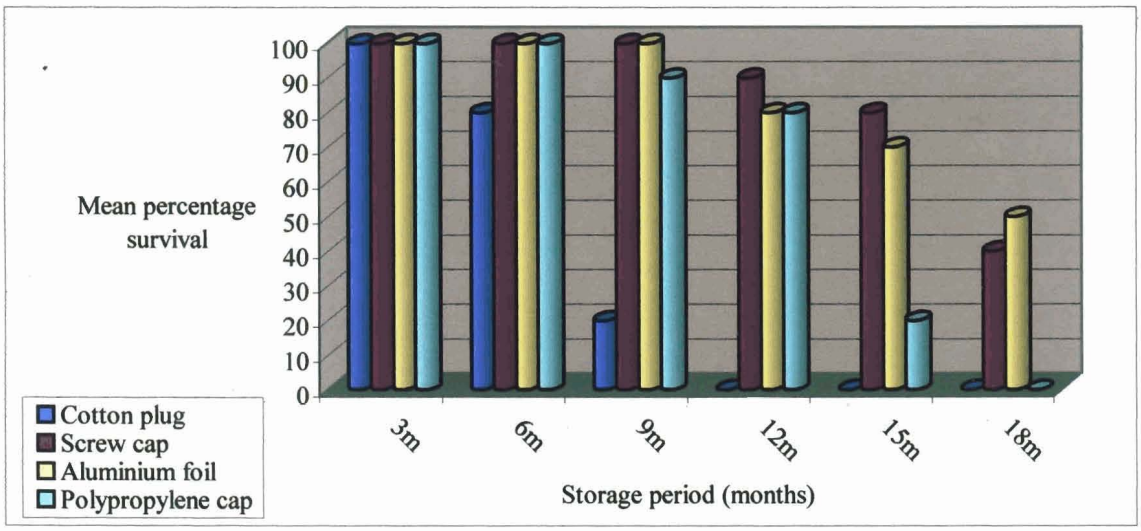
Table 28. Effect of media components, temperature and closure types on induction of minimal growth in *Kaempferia rotunda* cultures

Treatment			Growth rate				Storage period (months) with 80% survival of cultures*
Basal medium (MS) concentration	Sucrose (S) + Mannitol (M)	Closure type	Increase in height (cm)*		Average no. of shoots /culture		
			Mean	SD	Mean	SD	
Full strength	30S + 0M	CP	9.2	1,21	2.0	0.82	3
Full strength	30S + 0M	SC	11.4	0.84	1.8	0.79	3
Full strength	20S + 0M	CP	9.6	1.00	2.1	0.88	3
Full strength	20S + 0M	SC	9.9	0.71	1.4	0.52	2
Full strength	20S + 10M	CP	8.1	0.94	1.4	0.52	3
Full strength	20S + 10M	SC	7.3	0.65	1.2	0.42	4
Full strength	15S + 15M	CP	5.3	0.51	1.6	0.84	5
Full strength	15S + 15M	SC	5.6	0.25	1.6	0.70	6
Full strength	10S + 10M	CP	4.2	0.56	1.5	0.71	7
Full strength	10S + 10M	SC	4.9	0.41	1.2	0.42	8
Half strength	30S + 0M	CP	8.6	0.90	2.1	0.88	3
Half strength	30S + 0M	SC	9.8	0.27	1.8	0.79	4
Half strength	20S + 0M	CP	5.3	0.55	1.6	0.70	2
Half strength	20S + 0M	SC	5.6	0.43	1.4	0.52	3
Half strength	20S + 10M	CP	4.2	0.59	1.8	0.79	4
Half strength	20S + 10M	SC	5.9	0.38	1.4	0.52	8
Half strength	15S + 15M	CP	4.4	0.58	1.5	0.71	7
Half strength	15S + 15M	SC	6.3	0.52	1.2	0.42	12
Half strength	10S + 10M	CP	5.6	0.60	1.5	0.71	7
Half strength	10S + 10M	SC	4.4	0.40	1.1	0.32	12
CD					**4.62**		**3.58**
SE					**0.38**		**0.29**
F-value					**15.17**		**2.68**

MS- Murashige and Skoog medium; S – Sucrose; M – Mannitol; SC – Screw cap; CP – Cotton plug
* Mean of 10 replications ((Data collected after the respective storage period with 80% survival)

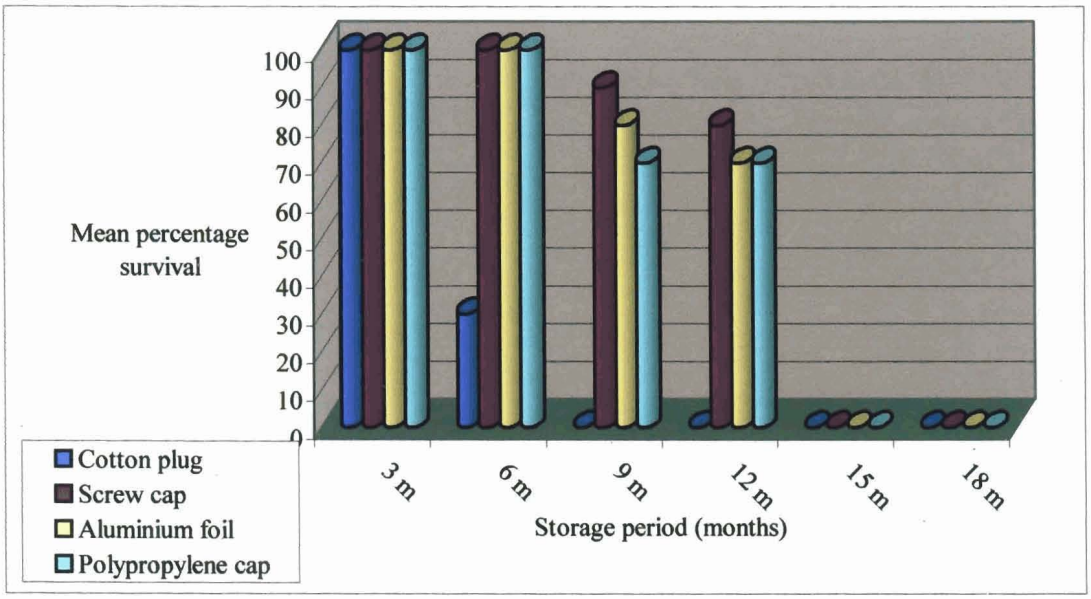
Further reduction of sucrose and addition of mannitol with full or half MS medium induced slow growth. In full strength MS with $20gl^{-1}$ sucrose and $10gl^{-1}$ mannitol, increase in height was 6.9 cm and the mean number of shoots per culture was 1.9, whereas in half strength MS it was 4.1 cm and 1.9 shoots per culture in *K. galanga*. The same medium gave 7.5 cm and 5.9 cm increase in height and 1.2 and 1.4 shoots per culture in full and half strength MS medium respectively in *K. rotunda*.

Fig. 19. Survival percentage of *Kaempferia galanga* cultures at various time periods in different closure types*



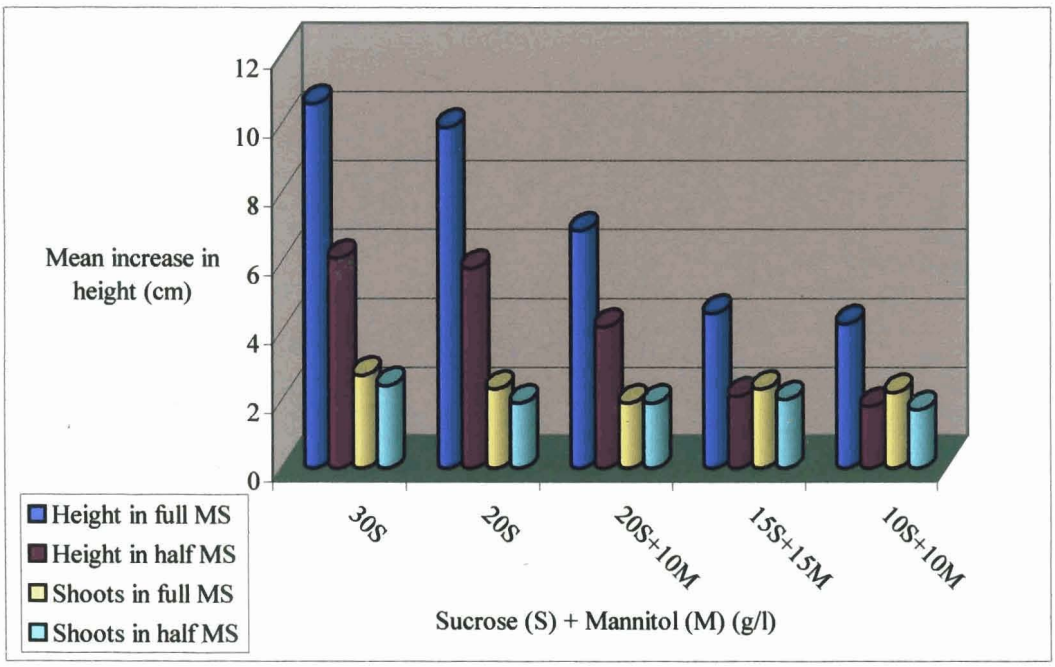
* In half MS medium with 15 gl^{-1} sucrose and 15 gl^{-1} mannitol at 22±2°C

Fig. 20. Survival percentage of *Kaempferia rotunda* cultures at various time periods in different closure types*



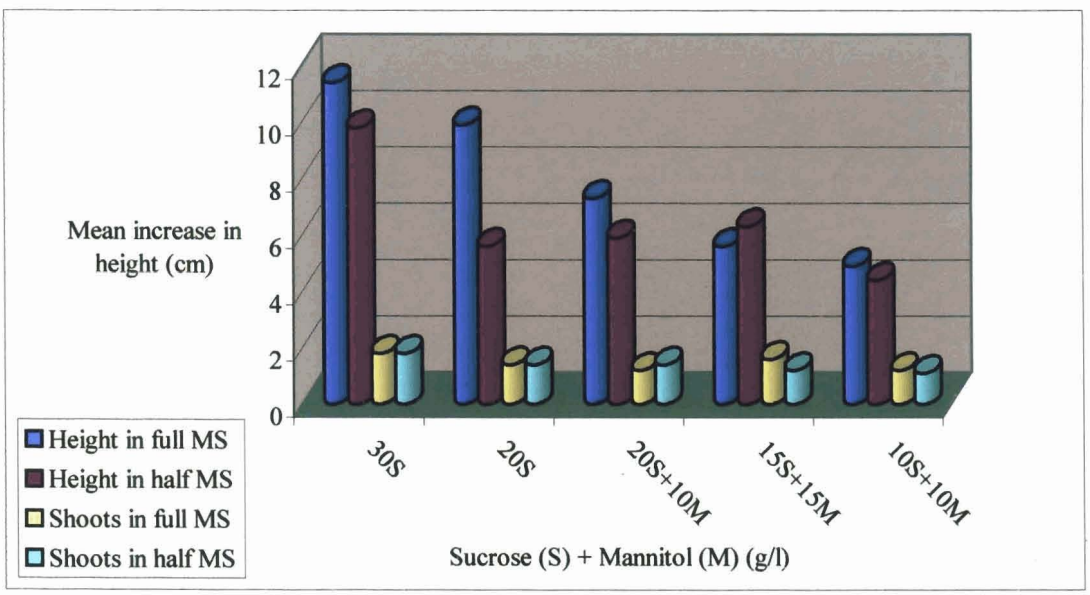
* In half MS medium with 15 gl^{-1} sucrose and 15 gl^{-1} mannitol at 22±2°C

Fig. 21. Growth rate and survival percentage of *Kaempferia galanga* cultures in different media combinations*



* In sealed culture tubes at 22±2°C

Fig. 22. Growth rate and survival percentage of *Kaempferia rotunda* cultures in different media combinations*



* In sealed culture tubes at 22±2°C

Fig 23. Induction of slow growth in *Kaempferia galanga* and *K. rotunda*

(a) Effect of aluminium foil and screw cap closures in the storage of *K. rotunda* (1 year old) cultures , (b) One year old cultures of *K. galanga* in minimal growth medium (half MS + 15 gl^{-1} sucrose + 15 gl^{-1} mannitol) (c) One year old culture of *K. rotunda* in half MS + 15 gl^{-1} sucrose + 15 gl^{-1} mannitol (d) *In vitro* multiplication after minimal growth storage in *K. galanga* (e) *In vitro* multiplication after minimal growth storage in *K. rotunda*

Though the reduction in growth rate was not statistically significant between the treatments, mannitol and sucrose at 10 gl^{-1} each or 15 gl^{-1} each, with MS at half strength showed comparative growth reduction in both the species and helped in the maintenance of cultures up to 12 months with 80% survival (Fig. 23b and 23c). In *K. galanga* the cultures could be maintained up to 16 months with 70% survival. In *K. galanga* full and half strength of the medium supplemented with 15 gl^{-1} each of sucrose and mannitol, the plantlets achieved a height of 4.5 cm and 2.1 cm respectively, and the number of shoots per culture was 2.3 and 2.0. Further reduction in sucrose and mannitol (10 gl^{-1} each) gave an increase in height of 4.2 cm and 1.9 cm and the mean number of shoots per culture was 2.2 and 1.7 in full and half MS , respectively (Fig. 23b). In *K. rotunda* full and half strength of the medium supplemented with 15 gl^{-1} each of sucrose and mannitol the plantlets achieved a height of 5.6 cm and 6.3 cm respectively, and the number of shoots per culture was 1.6 and 1.2. Further reduction in sucrose and mannitol (10gl^{-1} each) gave an increase in height of 4.9 cm and 4.4 cm and the number of shoots per culture was 1.2 and 1.1 in full and half MS, respectively (Fig. 23c).

Thus in *K. galanga* and *K. rotunda*, the cultures could be stored up to 12 months with 80–90% survival in half strength MS medium with 10gl^{-1} each of sucrose and mannitol in sealed culture tubes (Fig. 23b and 23c). These *in vitro* stored cultures, multiplied normally in 3 weeks of culture (Figs. 23d and 23e). The rooted plantlets were transferred to thermo-coal cups and established with 80–90% survival.

Cryopreservation

Since all the zingiberaceous crops tried responded more or less similarly in *in vitro* conservation experiments, cryopreservation experiments were conducted only in

two crops namely ginger and cardamom with an objective to supplement the *in vitro* conservation programme. Attempts were made to standardize cryopreservation protocols for long-term conservation of shoot buds in ginger and pollen in cardamom, which is the only zingiberaceous crop in which the fruit is the economically useful part. Among the species studied only cardamom and rarely turmeric reproduces sexually.

Cryopreservation of ginger shoot buds

Cryoprotective dehydration

The excised shoot buds were taken from pre-established *in vitro* cultures and desiccated for 0–4 h in laminar airflow after three days of pre-treatment by immersing in MS medium supplemented with high concentrations of sucrose. These dehydrated shoot buds were then plunged in to liquid nitrogen for cryo-storage. The shoot buds were cultured on recovery medium (MS with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA) after thawing. Viability of shoot tips after cryoprotective dehydration and cryopreservation as expressed by post-thaw recovery and growth are given in Table 29.

Pre-treatment of shoot tips with 0.5 M sucrose for 3 d gave higher viability of 90%–0% after 1-4 h desiccation (Fig. 24a) compared to pretreatment at 0.75 M sucrose where the viability drastically decreased after 2 h of desiccation. In both these pre-culture media the viability was 90% without desiccation as well as 1 h desiccation. However when these pre-treated propagules were cryopreserved they lost viability in all treatments except for those, which were desiccated for 1 h. At 1 h desiccation followed by cryopreservation only 20% of the cultures survived and upon recovery and growth developed only callus (Fig. 24b). But cultures pre-cultured on

0.75 M sucrose the post thaw recovery was much higher at 40% and half of these cultures developed only callus while the others developed in to plantlets.

Table 29. Percentage survival of ginger shoot buds on cryopreservation by cryoprotective dehydration*

Treatment		Viability (%)		Type of response	
Sucrose conc. (M)	Desiccation time (h)	Control	Cryopreserved	Control	Cryopreserved
0.50	0	90	0	Plantlet formation	No viability
0.50	0	90	0	Plantlet formation	No viability
0.50	1	90	20	Plantlet formation	Callus growth in 20% of cultures
0.50	2	50	0	Plantlet formation	No viability
0.50	3	10	0	Callus formation	No viability
0.50	4	0	0	No viability	No viability
0.75	0	90	0	Plantlet formation	No viability
0.75	1	70	40	Plantlet formation	Callus growth in 20% and plantlet growth in 20% of cultures
0.75	2	30	0	Plantlet formation	No viability
0.75	3	0	0	No viability	No viability
0.75	4	0	0	No viability	No viability

*** Average of 20 replications**

Thus ginger shoot tips could be successfully cryopreserved with limited success (40% of the cultures) after pre-culture on 0.75 M sucrose for three days and desiccated for 1 h on laminar air flow (Fig. 24c). Plantlets could be regenerated from fifty percent of these viable cultures after cryopreservation.

Encapsulation and dehydration

An alternate strategy of cryopreservation of encapsulated shoot tips (synthetic seeds) of ginger was also tried to study the effect of encapsulation on their viability after cryopreservation. The other treatments tried were similar to cryoprotective dehydration. Viability and growth response of encapsulated shoot buds in various treatments are presented in Table 30.

Table 30. Percentage survival of ginger shoot buds 3 days after pre-culture by encapsulation/dehydration method*

Treatments		Viability (%)		Type of response	
Sucrose conc. (M)	Desiccation time (h)	Control	Cryopreserved	Control	Cryopreserved
0.50	0	90	0	No viability	No viability
0.50	1	90	0	No viability	No viability
0.50	2	80	0	Plantlet formation	No viability
0.50	3	60	0	Plantlet formation	No viability
0.50	4	70	30	Plantlet formation	Callus formation in 50%; slight growth
0.75	0	90	0	No viability	No viability
0.75	1	90	0	No viability	No viability
0.75	2	60	0	Plantlet formation	No viability
0.75	3	70	20	Plantlet formation	Callus growth
0.75	4	70	50	Plantlet formation	Callus growth in 20%; Plantlet formation in 30% of cultures

*** Average of 20 replications**

Pre-treatment of encapsulated shoot tips with 0.5 M and 0.75 M sucrose for 3 d gave a viability of 90%–70% after 1-4 h desiccation (Fig. 24d). However when these pre-treated synseeds were cryopreserved, they lost their viability in all treatments except for those, which were desiccated for 3 and 4 h. Cryopreservation after pre-treatment with 0.5 M sucrose followed by 3 h desiccation only 20% of the cultures survived and upon recovery and growth they developed in to callus. Cultures pre-cultured on 0.75 M sucrose and desiccated for 4 h gave maximum post thaw recovery of 50% (Fig. 24e) and 20% of these cultures developed only callus while the rest 30% developed in to plantlets.

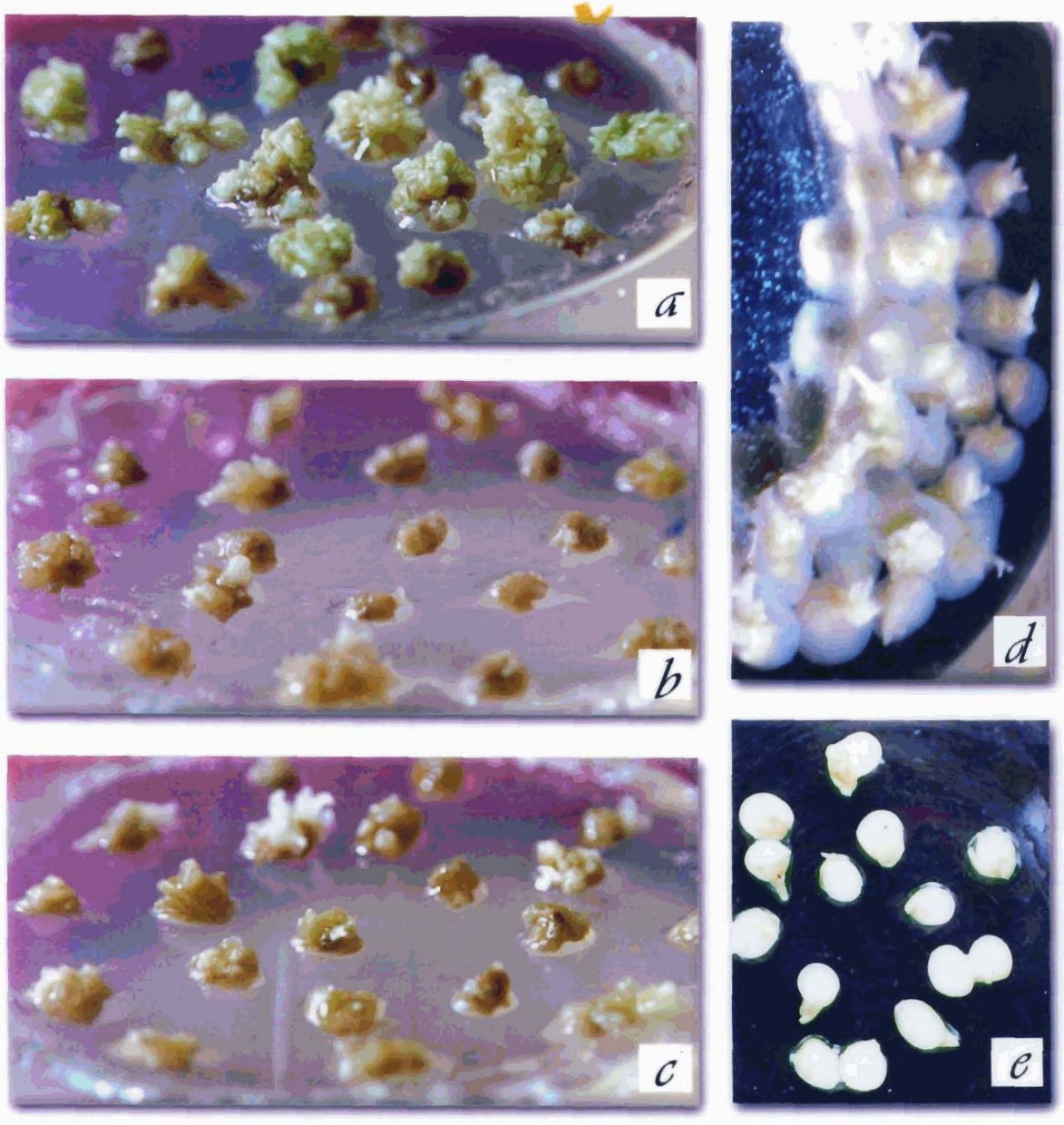


Fig. 24. Cryopreservation of shoot buds and encapsulated shoot buds in ginger

(a) Shoot buds pre-treated with 0.5 M sucrose and desiccated for 1 h prior to cryopreservation (control),
(b) Recovery and re-growth of shoot buds pretreated with 0.5 M sucrose, desiccated for 1 h and cryopreserved for 2 h,
(c) Recovery and re-growth of shoot buds pretreated with 0.75 M sucrose, desiccated for 1 h and cryopreserved for 2 h,
(d) Encapsulated shoot buds pre-cultured for 3 days in 0.75 M sucrose and desiccated for 4 h (control),
(e) Recovery and re-growth of encapsulated shoot buds precultured for 3 days in 0.75 M sucrose and desiccated for 4 h after exposure to liquid nitrogen.

Thus encapsulated shoot buds of ginger could be successfully cryopreserved with better success (50% of the cultures) after pre-culture on 0.75 M sucrose for three days and desiccated for 4 h on laminar airflow. Plantlets could be regenerated from 20 percent of the cultures after cryopreservation. The plantlets looked healthy more sturdy and grew well.

Use of encapsulated shoot buds or synthetic seeds increased the post-thaw recovery and the development of plants by 10%. However they need a longer period (4 h) of desiccation in laminar airflow compared to naked shoot tips, which required only 1 h desiccation. The non-encapsulated shoot buds lost their viability when cryopreserved after 2 h desiccation.

Cryopreservation of cardamom pollen

Cardamom is cross-pollinated and among zingiberaceous species conservation of pollen assumes importance only in cardamom. Hence cryopreservation of cardamom pollen was attempted. Pollen was used fresh, after desiccation and after treatment with cryoprotectant DMSO for cryo-storage. The viability of pollen was assessed in various treatments using acetocarmine staining, FDA staining and *in vitro* germination studies (Table 31). Fresh pollen showed 60–65% viability on *in vitro* germination in Brewbaker and Kwack's medium with 10% sucrose. Acetocarmine staining, FDA staining or *in vitro* germination studies showed more or less the same viability in both control and cryopreserved samples.

Fresh pollen showed 60–65 % viability in control (Fig. 25a) and its cryopreserved sample showed 15–23% viability on staining with acetocarmine and FDA, while no *in vitro* germination was observed. The percentage viability decreased with increase in time of desiccation. After 1 h desiccation the viability was almost nil.

The samples desiccated for 10 min showed 45–53% viability (Fig. 25b). Its respective cryopreserved samples exhibited a viability of 15–20% in the pollen desiccated for 10 min (Fig. 25c).

Table 31. Viability of cardamom pollen in cryopreservation studies

Treatment	Viability (%)					
	Acetocarmine staining		FDA staining		*In vitro* germination	
	C	Cr	C	Cr	C	Cr
Fresh pollen	65	15	60	23	63	0
Dessication 10 min	53	15	50	20	45	15
20 min	23	12	15	18	35	0
30 min	15	5	20	8	9	0
1h	0	0	8	0	0	0
Dessication (10 min) + DMSO (10%) for 10 min	68	62	72	75	61	58
20 min	43	21	38	0	42	0
30 min	48	0	42	0	12	0
1 h	12	0	15	0	2	0

C= Control; Cr= Cryopreserved

Desiccation coupled with DMSO treatment could increase the viability of fresh pollen as well as cryopreserved samples. The samples desiccated for 10 min and treated with 10% DMSO for 10 min gave the maximum viability in control (60–72%) (Fig. 25d) and after cryopreservation (58–75%) (Fig. 25e). Increase of treatment time in DMSO did not increase pollen germination in control as well as in cryopreserved samples. There was reduction in the viability of control pollen (from 72%–2%) and pre treatment with DMSO beyond 20 min was deleterious as there was no viable pollen.

Thus a method was developed for cryopreservation of cardamom pollen after 10 min. pre treatment with DMSO. In this treatment 62 % of the pollen was viable.

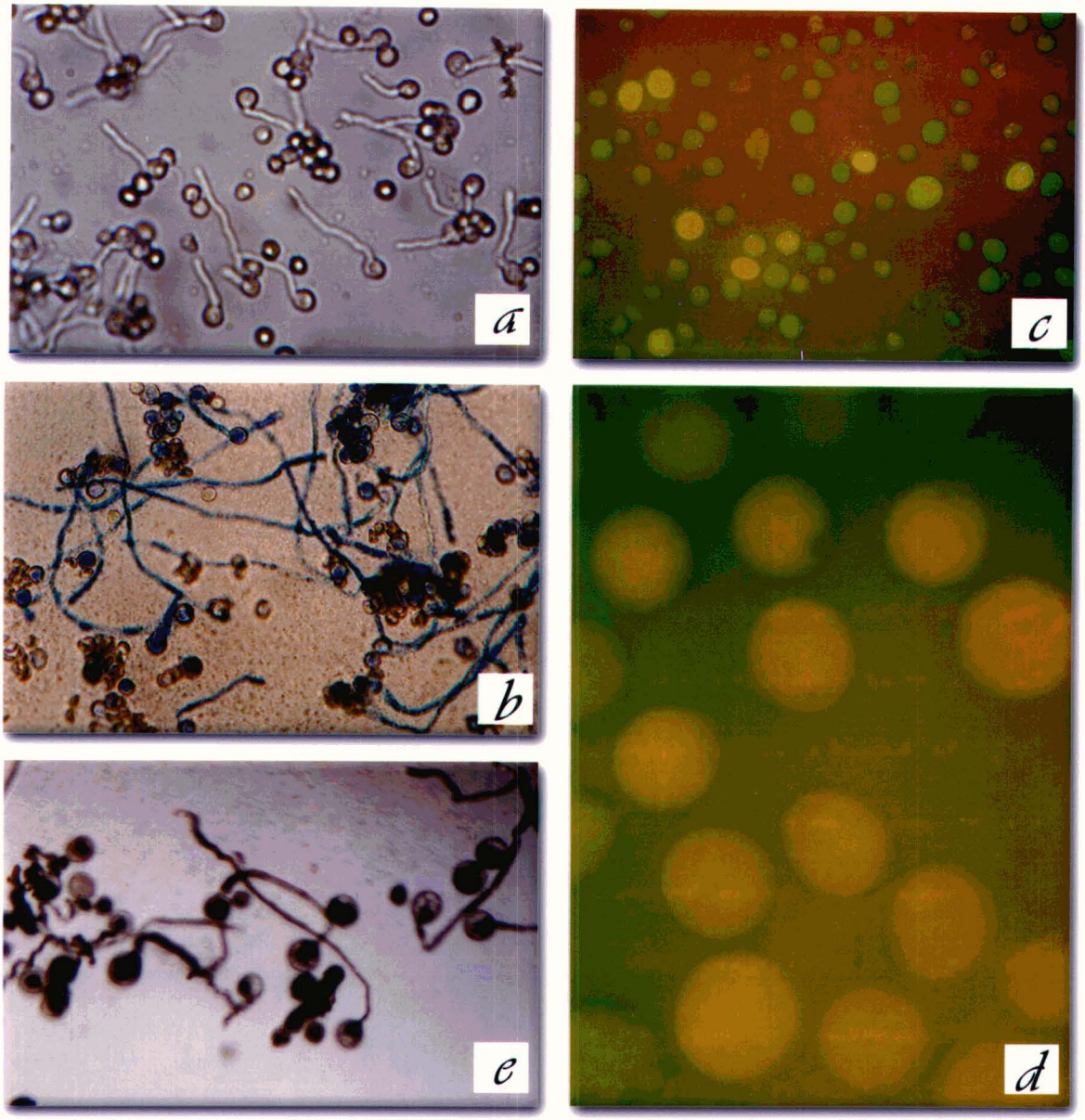


Fig. 25. Cryopreservation of cardamom pollen

(a) *In vitro* germination of fresh pollen, (b) *In vitro* germination of pollen desiccated for10 min, (c) Viability of pollen desiccated for 10 min after liquid nitrogen storage (FDA staining - fluorescence of viable pollen), (d) Viability of pollen desiccated for 10 min and treated with 10% dimethyl sulfoxide (DMSO) for10 min (FDA staining), (e) *In vitro* germination of pollen desiccated for 10 min and treated with 10% DMSO for 10 min after liquid nitrogen exposure.

SYNTHETIC SEEDS

In vitro regenerated shoot buds of cardamom, ginger and turmeric, were successfully encapsulated in 3% calcium alginate matrix (Figs. 26a, 26b, 26c). The beads were sufficiently strong for easy handling. Encapsulated synthetic seeds were stored at 5^0C, 10^0C and 22^0C to study the effect of low temperatures on storage and viability (Table 32). Low temperatures were not suitable for synthetic seed storage as they lost their viability within 30 days. But at 22 ± 2^0C, the synthetic seeds could be stored up to 6 to 9 months in sterile environment, when incubated in MS basal medium. When cultured on MS medium supplemented with BAP (1.0 mgl^{-1}) and NAA (0.5 mgl^{-1}), maximum germination (80%) was observed after 2 weeks. The plants derived from these encapsulated buds were healthy and developed into normal plantlets. Synthetic seeds form an ideal source of material for disease free germplasm conservation and exchange in zingeberaceous crops, especially in ginger, where most of the diseases are spread through infected seed rhizomes.

Table 32. Effect of temperature and duration of storage on viability of synthetic seeds in cardamom, ginger and turmeric

Temperature	Duration of storage (%Viability)		
	Cardamom	Ginger	Turmeric
5°C	10 d (100)	10 d (100)	10 d (100)
	30 d (0)	30 d (10)	30 d (10)
10°C	10 d (100)	10 d (100)	10 d (100)
	30 d (10)	30 d (10)	30 d (10)
$22\pm2^{\circ}$C	4 m (100)	6 m (100)	5 m (100)
	8 m (80)	10 m (80)	9 m (90)

D=days; m=months
Figures in parenthesis indicate percentage of viability

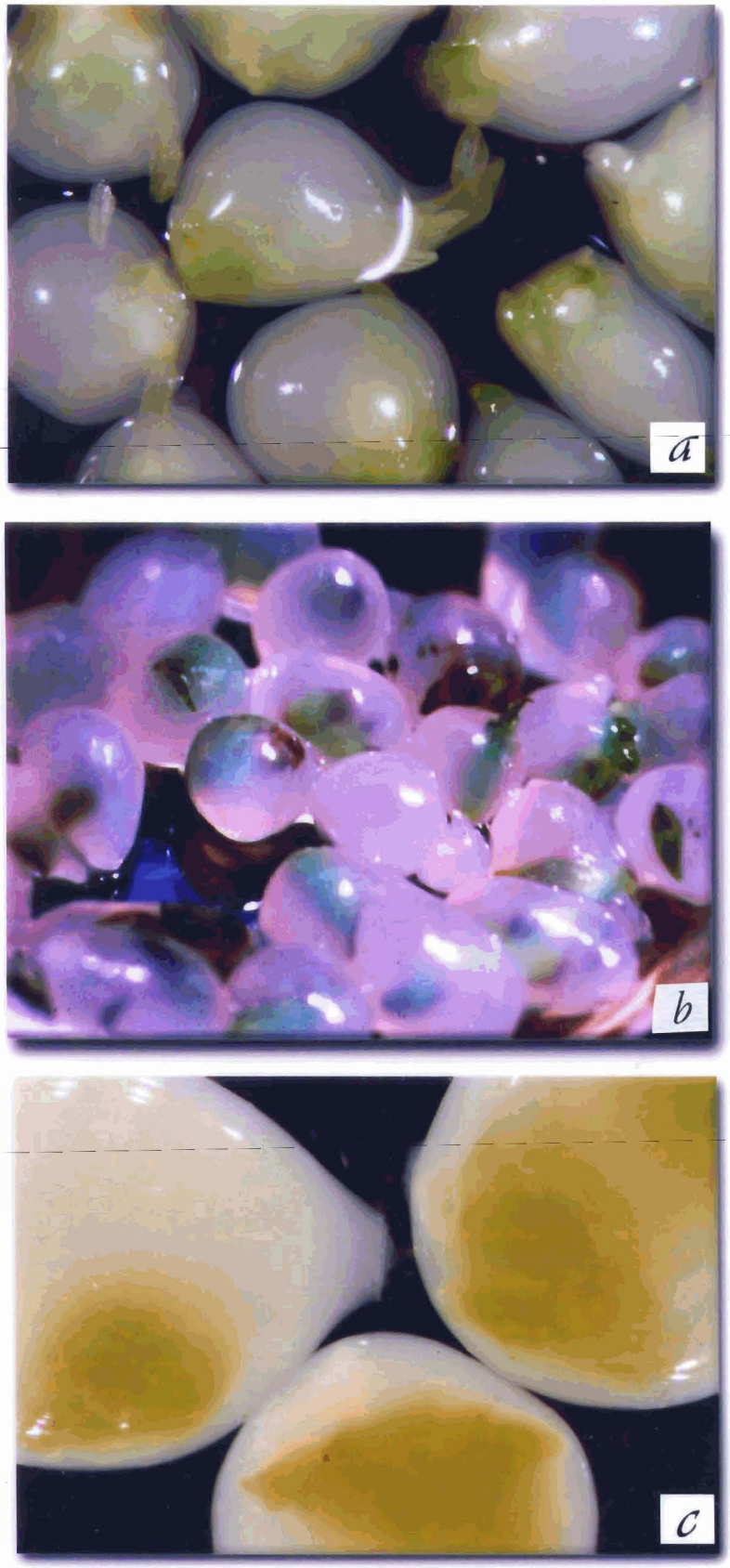


Fig. 26. Synthetic seeds
Shoot tips of (a) Cardamom, (b) Ginger and (c) Turmeric shoot buds encapsulated in sodium alginate beads.

IN VITRO MICRORHIZOME FORMATION

Micro rhizomes were induced in *in vitro* cultures of ginger and turmeric by using high concentrations of sucrose and mannitol.

Ginger

Various concentrations of sucrose with or with out mannitol in different combinations were tried for their ability to induce micro rhizomes in ginger (Table 33). Microrhizomes of 0.05–15 g fresh weight per explant were induced in ginger tissue cultures in 1–12 months on MS basal medium supplemented with higher levels of carbon source. In 3% sucrose microrhizome formation was observed in 30–40% cultures only after 12 months in culture. In the medium with 1% or 1.5% each of sucrose and mannitol, time taken for microrhizome formation was reduced to 8 months with 50–60% of the cultures responding. In this combination comparatively smaller microrhizomes, weighed up to 0.05–1.2g, were produced. Microrhizomes were produced in 80–100% of cultures when sucrose concentrations were increased to 9, 10 and 12%. The microrhizomes from these cultures were larger with a fresh weight of 3–15 g. The other combinations of sucrose and mannitol did not induce microrhizomes

Table 33. Microrhizome formation in ginger at different sucrose concentrations

Sucrose (%)	Mannitol (%)	Time period for induction and production of microrhizome	Percentage response*	Fresh weight (g)*
3.0	-	12m	30–40	0.05–1.0
9.0	-	1m	80–100	5–15
10.0	-	1m	80–100	5–10
12.0	-	1m	80–100	3–6
1.0	1.0	8m	50–60	0.06 – 1.2
1.5	1.5	8m	50–60	0.1 – 1.2
2.0	1.0	8m	30–40	0.02 – 0.5
3.0	3.0	No induction	-	-
3.0	6.0	No induction	-	-
5.0	5.0	No induction	-	-
6.0	6.0	No induction	-	-

*** Average of 20 treatments**

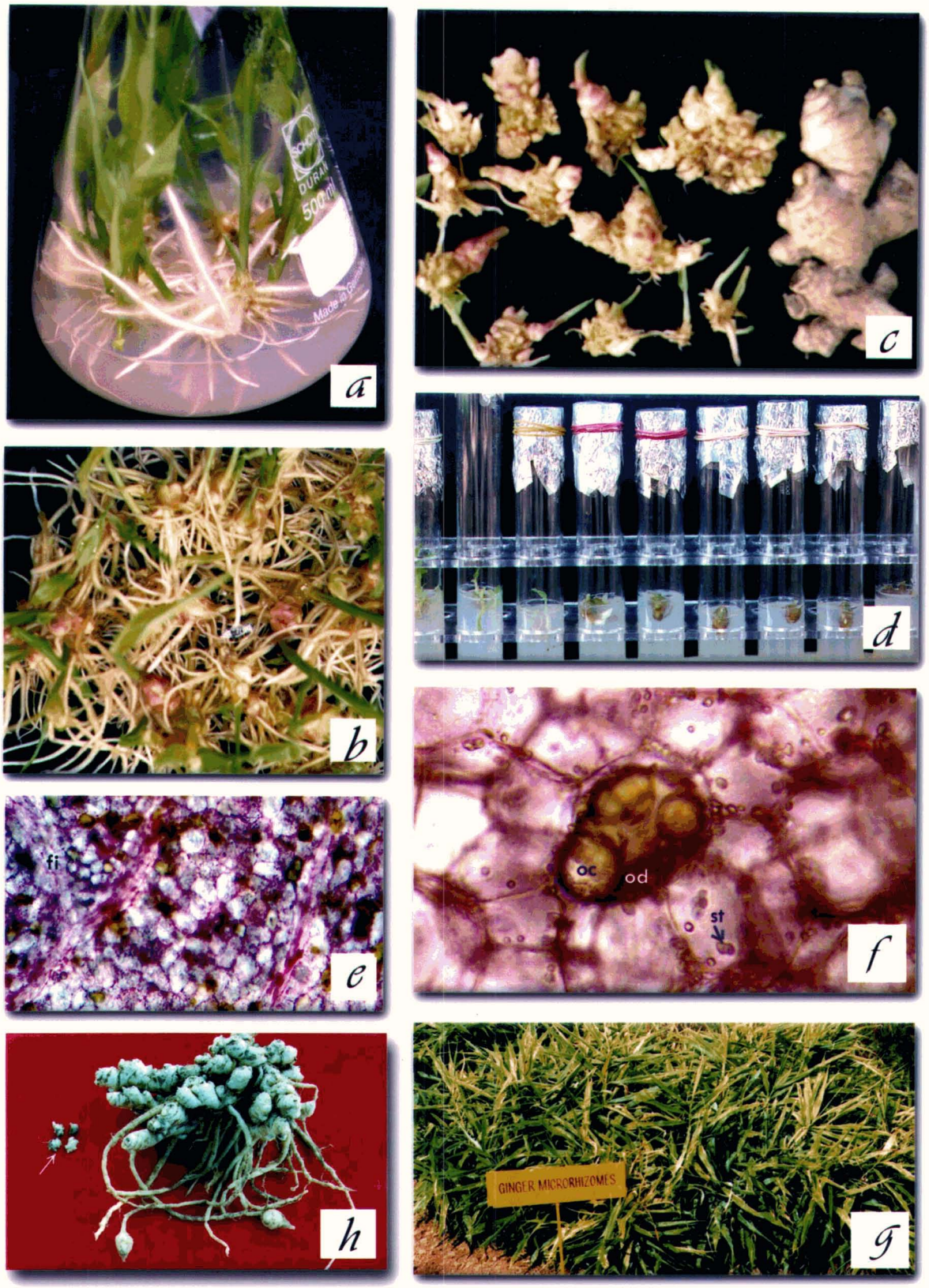


Fig. 27. *In vitro* microrhizomes in ginger

(a) Induction of microrhizomes in MS medium with 9% sucrose, (b) Microrhizomes harvested after 6 months in culture, (c) Microrhizomes ready for field transfer (in comparison with normal rhizome), (d) *In vitro* germination of microrhizomes, (e) Microrhizome macerated to show fibre (fi) and starch grains (st), (f) Cross section of microrhizome showing oil duct (od) and oil cells (oc), (g) Microrhizome derived plants in the field. (h) Rhizomes harvested from microrhizome derived plants (arrow indicates microrhizome used as planting material)

Cultures producing microrhizomes exhibited a peculiar pattern of growth with profuse rooting and less aerial shoots (Fig. 27a, 27b). Microrhizomes resembled the normal rhizomes in all respects, except for their small size. The microrhizomes consisted of 2 to 4 nodes and 1 to 6 buds (Figs. 27c). The fresh weight of the microrhizome per explant ranged from 3 g to 15 g in 6 months of culture. The rhizomes germinated *in vitro* as well as *ex vitro*. The microrhizomes on transfer to MS medium without any growth regulators, germinated within 5 to 7 days with 100% success (Fig. 27d). The microrhizomes had the aromatic flavour of ginger and they resembled the normal rhizome in anatomical features. Presence of well-developed oil cells, fibres, starch grains and curcumin cells was also observed (Figs. 27e and 27f).

The microrhizomes could be directly planted in the field with out hardening and established with 90–100% success (Fig. 27g). Field trials showed that the microrhizomes gave commercially viable yields ie 200–500 g of fresh rhizomes per plant with an estimated 12–20 kg of fresh rhizomes per 3 m^2 bed (Fig. 27h). The microrhizomes had more tillers per plant though the plant height was less compared to normal seed rhizomes. The seed rate requirement per 3 m^2 bed is about 800 g (@ 40 plants per bed) for the normal seed rhizomes and in case of microrhizomes it is about half that amount.

Turmeric

Microrhizomes were induced in turmeric also under *in vitro* conditions, when the medium is supplemented with higher levels of carbon source (Table 34). The results were similar to those of ginger except that the size if the microrhizomes were smaller in turmeric.

Table 34. Microrhizome formation in turmeric at different sucrose concentrations

Sucrose (%)	Mannitol (%)	Time period for induction and production of microrhizome	Percentage response*	Fresh weight (g)*
3.0	-	12 m	30–40	0.05–1.0
9.0	-	1 m	80–100	1.0–2.5
10.0	-	1 m	80–100	1.0–2.5
12.0	-	1 m	80–100	1.0–2.5
1.0	1.0	12 m	50–60	0.02 – 0.7
1.5	1.5	12 m	50–60	0.1 – 1.0
2.0	1.0	12 m	30–40	0.02 – 0.5
3.0	3.0	No induction	-	-
3.0	6.0	No induction	-	-
5.0	5.0	No induction	-	-
6.0	6.0	No induction	-	-

*** Average of 20 treatments**

Sucrose at 3–12 % induced microrhizomes in turmeric but the percentage of culture response, time taken for microrhizome formation and the size of microrhizome varied in different treatments. In culture medium with 9–12% sucrose microrhizomes were induced in one month in 80–100% of the cultures (Fig. 28a). The weight of the microrhizomes ranged from 1.0–2.5 g. In MS medium with 30 gl^{-1} sucrose microrhizome formation was observed after 10 months of culture. Microrhizomes were small when compared to that produced in ginger, but had the typical yellow colour of normal rhizomes (Fig. 28b and 28c). Anatomically the microrhizomes resembled the normal rhizomes and a large number of curcumin cells were noticed (Figs. 28d and 28e). These microrhizomes could be directly planted in the field and established with 90–100% survival (Fig. 28f).

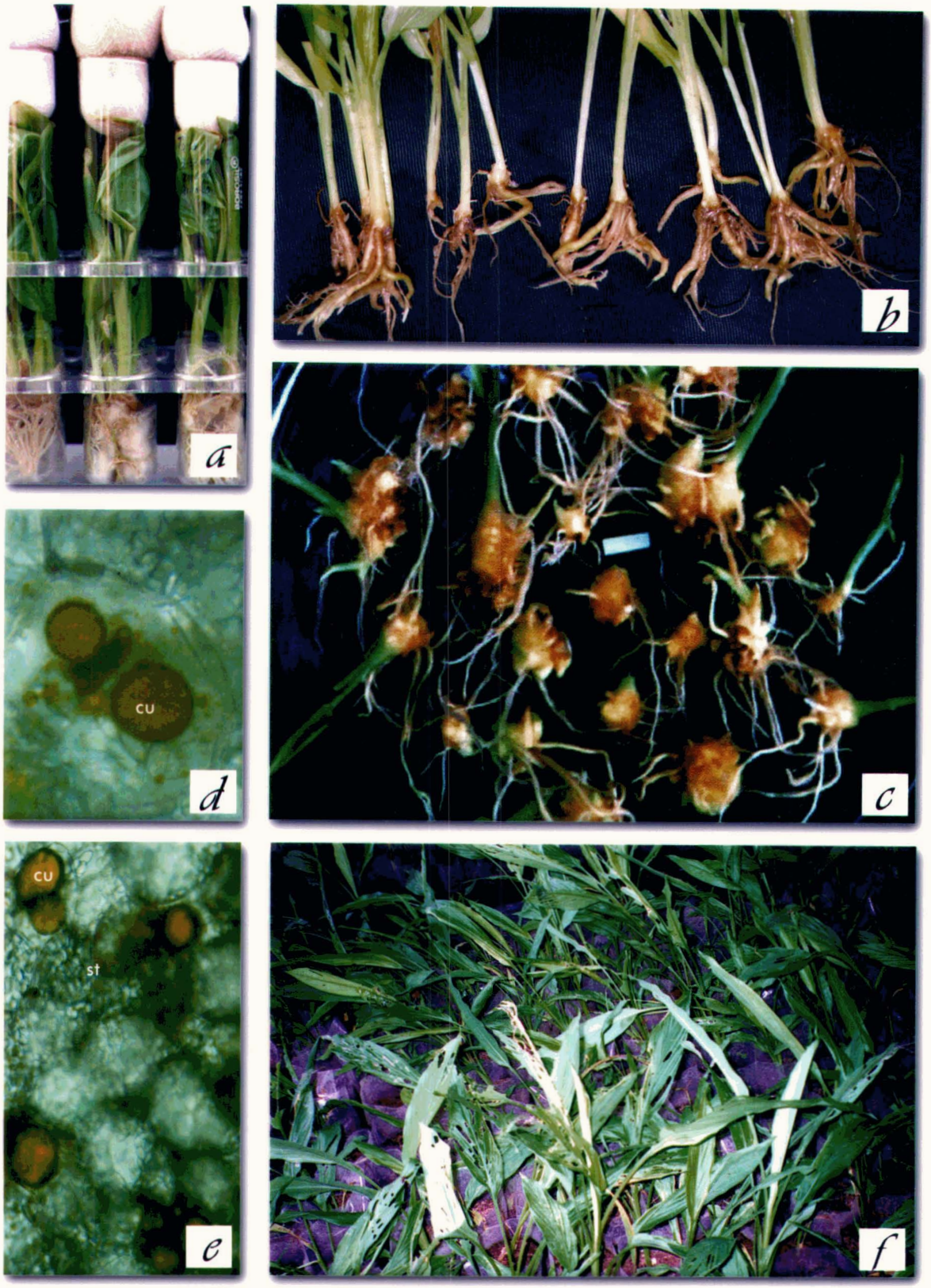


Fig. 28. *In vitro* microrhizomes in turmeric

(a) Induction of microrhizomes in MS medium with 12% sucrose, (b) Microrhizomes harvested after 6 months in culture, (c) Microrhizomes ready for field transfer (d) & (e) Transverse section of microrhizome showing curcumin cells (cu) and starch cells (st), (f) Microrhizome derived plants in the polybags.

GENETIC STABILITY ANALYSIS OF CONSERVED MATERIAL

Genetic fidelity is an important factor in any conservation programme. In the present study the genetic fidelity of conserved genotypes was assessed by morphological, biochemical and molecular characterization.

Morphological characterization

The conserved plants were observed for differences and deficiencies during recovery and growth after *in vitro* and cryoconservation, during hardening and planting out and their performance in pots and field.

Recovery and growth

A random sample of *in vitro* conserved cultures of cardamom, ginger, turmeric, *K. galanga* and *K. rotunda*, were transferred to multiplication medium (MS with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA). The age of the cultures ranged from 2-5 years with yearly subculture. The miniature-sized plantlets of cardamom under minimal growth storage grew, multiplied and rooted normally within 1 month (Fig. 6f). In ginger, turmeric, *K. galanga* and *K. rotunda* the cultures multiplied and rooted normally within 2–3 weeks (Fig. 11f, 16f, 23d and 23e). These *in vitro* multiplied cultures did not exhibit any deficiency symptoms or deformities and were similar to the control in its appearance and growth.

Planting out and field establishment

The plantlets were hardened by maintaining a humidity of around 90% by covering with polybags for 20–30 days. There was no reduction in establishment percentage after years of *in vitro* conservation when compared with normal micropropagated plantlets. All the plantlets were established with 80–90% survival. The established plantlets of cardamom, ginger, turmeric, *K. galanga* and *K. rotunda* were planted in

pots or field after hardening (Figs. 29a–29e). The morphological features of these plantlets in the first year during hardening and field transfer are given in Table 35.

These plantlets were developed as normal plants without any deformities or deficiency symptoms and they exhibited apparent morphological similarities with the controls. In *K. galanga* the plants showed narrower leaves in the initial stages of their growth and gradually the leaves become same as that in the control. *K. rotunda* plantlets were lacking the pink colouration in *in vitro*, but after planting out and establishment the plants gradually developed the colouration. The rhizomes of tissue-cultured plantlets of ginger, turmeric and *Kaempferia* spp. were very small (0.7–3.3 g) to harvest after the first season and mortality was high if harvested.

Table 35. Morphological features of *in vitro* conserved plantlets (recovered from minimal growth storage) during hardening and field establishment*

Parameter	Species				
	Cardamom	**Ginger**	**Turmeric**	***K. galanga***	***K. rotunda***
No. of plantlets/culture	5.8	7.5	7.2	8.2	6.1
Plant height (cm)	10.2	11.5	11.8	10.9	11.3
No. of roots/plant	6.2	9.1	5.8	10.4	8.4
No. of leaves/plant	6.8	5.3	6.1	6.5	4.3
Leaf length (mm)	4.5	5.6	6.3	5.3	7.1
Leaf breadth (cm)	0.5	0.7	0.6	0.7	1.0
Time taken for hardening (days)	30.0	20.0	25.0	20.0	25.0
Establishment rate (%)	80.0	90.0	90.0	90.0	80.0

- **Mean of 10 replications**

The micropropagated plantlets of ginger, turmeric and *Kaempferia* spp behaved like seedlings of zingiberaceous crops except when microrhizomes were used as planting material. The size of the rhizome increased over the years and developed into normal size comparable to that of mother plants only after the third year.

Table 36. Morphological characters of *in vitro* conserved plants of cardamom, ginger, turmeric, *K. galanga* and *K. rotunda* in pots*

Plant species	Plant height (cm)	No. of tillers	No. leaves /plant	Width of rhizome (cm)	No. nodes/finger	Internode distance (cm)	Yield/plant (g)
Cardamom**	65.0	5.0	14.0	-	-	-	-
	(72.0)	(3.0)	(12)				
Ginger	80.5	15.5	14.0	2.5	8.2	5.5	350
	(90.0)	(12.5)	(12.0)	(2.0)	(6.8)	(6.3)	(378)
Turmeric	105.4	8.5	9.0	3.4	10.4	4.4	480
	(125.5)	(6.8)	(14.0)	(4.6)	(8.6)	(6.4)	(450)
K. galanga	15.0	15.0	12.0	3.2	3.6	2.3	350
	(15.5)	(13.5)	(10.8)	(4.8)	(3.2)	(2.7)	345
K. rotunda	58.0	9.0	9.0	4.6	4.2	3.2	450
	(61.2)	(10.6)	(11.0)	(5.2)	(3.8	(2.5)	440

*** Mean of 10 replicates; ** Data was taken after 1 year after planting out in cardamom, in others it was taken after third year; Figures in parenthesis denotes the value obtained in control plants**

The morphological characters such as plant height, number of tillers, number of leaves per plant, width of rhizome, number of nodes per finger, internode distance and yield per plant in these species did not reveal any distinguishable difference between the control and *in vitro* conserved lines (Table 36).

In cardamom the tissue cultured plants performed equal to those of seedlings and hence can be directly used for commercial planting within six months of hardening. But in ginger, turmeric and *Kaempferia* spp. where the commercially useful part is the rhizome, tissue cultured plantlets of these species cannot be directly used for commercial cultivation and needs at least three crop seasons in the nursery or field before they develop rhizomes of normal size suitable for commercial planting. Only microrhizomes can directly be used as planting material in commercial scale. These microrhizomes can be planted in the field without hardening.

Fig. 29. Field evaluation of *in vitro* conserved plants (under minimal growth storage for 3 years) after recovery and normal growth

In vitro conserved plants of (a) Cardamom, (b) Ginger, (c) Turmeric, (d) *Kaempferia galanga* and (e) *K. rotunda* in pots after recovery and regrowth.

Biochemical characterization

Morphological characterization is insufficient assess minute changes in the genotype of the plant and hence biochemical markers (isozymes) were used for further genetic fidelity analysis of *in vitro* conserved lines.

Isozyme profiles

Isozyme profiles were studied in six randomly selected conserved plants of two species namely, cardamom and *K. galanga.* They were compared with the profiles of control mother plants to estimate their genetic uniformity. It was observed that, in all the four isozymes tested, such as polyphenol oxidase (PPO), esterase, peroxidase and superoxide dismutase (SOD) (Figs. 30a–30e), the conserved material showed similar banding pattern to that of control. The peroxidase and SOD profiles of *in vitro* conserved plants of cardamom are given in Fig. 30a and 30b. The bands are monomorphic and no polymorphism was detected. The isozyme profiles of *K. galanga* indicated, SOD gave 5 monomorphic bands (Fig. 30c), PPO gave 4 monomophic bands (Fig. 30d) and esterase gave 7 monomorphic bands (Fig. 30e).

Thus in general the isozyme profiles indicated that the *in vitro* plants of cardamom and *Kaempferia galanga* are genetically stable even after 3 years in slow growth.

Molecular characterization using RAPD

DNA markers can detect even minute differences in nucleic acid sequences which were otherwise difficult to detect in both morphological and biochemical characterization. In the present study an attempt was made to use RAPD polymorphism as an index for genetic stability of *in vitro* conserved genotypes after 3 years in *in vitro* repository with yearly subculture on minimal growth medium. DNA

was isolated and RAPD profiles were developed from six randomly selected replicates of one of the genotypes of cardamom, ginger, *K. galanga* and *K. rotunda.*

Isolation and purification of genomic DNA

Genomic DNA was successfully isolated using, CTAB method from young fresh leaves of 6 different plantlets of *in vitro* conserved materials. The extracted DNA samples were dissolved in TE buffer and its quality was tested on 0.8% agarose gels. The DNA obtained was of reasonably good quality. The extracted samples were further purified by RNase treatment, followed by phenol:chloroform extraction and precipitation of DNA with absolute alcohol to remove RNA contamination.

Quantification of DNA

The amount of DNA in the purified samples was calculated by comparing with standard λ DNA marker on agarose gels as given in Table 37. The cardamom samples contained 75–150 ng/µl of DNA. The ginger samples contained 50–200 ng/µl DNA whereas the *K. galanga* and *K. rotunda* samples contained 100–200 ng/µl of DNA.

Optimization of Polymerase Chain Reaction (PCR)

Three levels of template DNA concentrations namely, 25, 50 and 75 ng/µl were tested in cardamom, ginger, *K. galanga* and *K. rotunda* to find out the optimum concentration of DNA for best amplification. The results based on amplified products indicated that 25 ng/µl of template DNA concentration was optimum in all the four species.

Three concentrations of dNTPs namely, 125, 150 and 175 µM were used for standardization and the best amplification was obtained from 125 µM in all the genera. Good amplification products were obtained at enzyme (*Taq* polymerase)

concentration of 1.0 U/µl, primer concentration of 10 picomoles/µl, 0.5 mM $MgCl_2$ were suitable for best amplification.

Table 37. Concentration of DNA in cardamom, ginger and *Kaempferia* species used for RAPD analysis samples

Accession/line	Sample	Concentration of DNA (ng/µl)
Cardamom		
Clone 37	1 (Control)	150
Clone 37	2 (Ivc line)	100
Clone 37	3 (Ivc line)	100
Clone 37	4 (Ivc line)	100
Clone 37	5 (Ivc line)	100
Clone 37	6 (Ivc line)	100
Clone 37	7 (Ivc line)	125
Ginger		
Maran	1 (Control)	150
Maran	2 (Ivc line)	100
Maran	3 (Ivc line)	125
Maran	4 (Ivc line)	100
Maran	5 (Ivc line)	125
Maran	6 (Ivc line)	125
Maran	7 (Ivc line)	100
K. galanga		
K. galanga 1	1 (Control)	200
K. galanga 2	2 (Ivc line)	175
K. galanga 3	3 (Ivc line)	100
K. galanga 4	4 (Ivc line)	100
K. galanga 5	5 (Ivc line)	100
K. galanga 6	6 (Ivc line)	200
K. galanga 7	7 (Ivc line)	100
K. rotunda		
K. rotunda 1	1 (Control)	250
K. rotunda 2	2 (Ivc line)	150
K. rotunda 3	3 (Ivc line)	150
K. rotunda 4	4 (Ivc line)	100
K. rotunda 5	5 (Ivc line)	100
K. rotunda 6	6 (Ivc line)	100
K. rotunda 7	7 (Ivc line)	100

Ivc = *In vitro* conserved lines

The PCR cycles and the temperature regimes used for best amplification were given below.

Reaction I Cycles: 3
Ist cycle: 94°C for 2 min
37 °C for 1 min
72 °C for 2 min Cycle repeats: 1

IInd cycle:	94 °C for 1 min	
	37 °C for 1 min	
	72 °C for 2 min	Cycle repeats: 38
IIIrd cycle :	94 °C for 1 min	
	37 °C for 1 min	
	72 °C for 15 min	Cycle repeats: 1

Primer screening

In cardamom, genomic DNA of CL-37 and APG 50 were used as template with 25 ng/µl concentration. Nineteen OPERON primers tested and 13 of them namely, OPA 01, OPA 03, OPA 05, OPA 06, OPA 12, OPC 02, OPC 06, OPC 07, OPC 19, OPD 02, OPE 02, OPF 01 and OPF 02 gave good amplification. Only three of the primers namely OPA 12, OPC 19, OPE 02 were polymorphic between genotypes.

In ginger, two cultivars namely, Maran and Varada were used for primer screening. Eleven OPERON primers were tested and 8 of them namely, OPA 12, OPB 2, OPC 4, OPC 5, OPC 07, OPD 01, OPE 02 and OPF 01 gave good amplification were poymorphic between the genotypes.

In *Kaempferia* spp. six primers (OPA 04, OPA 06, OPA 08, OPA 15, OPB 02 and OPE 01) were tested and 3 of them namely, OPA 06, OPA 08 and OPE 01 were poymorphic between the two species.

RAPD profiles of cardamom* in vitro *conserved lines

In cardamom three primers, OPA 12, OPC 19 and OPE 02 were used to develop RAPD profiles of *in vitro* conserved lines. These primers gave 3, 9 and 8 bands respectively (Fig. 31a & b). No polymorphism was detected between the conserved lines in any of the primers tested. Thus in general the patterns did not indicate any genetic variability within the replicates tested.

RAPD profiles of ginger* in vitro *conserved lines

Four primers, viz, OPB 2, OPC 5, OPE 02 and OPF 01 were selected to develop RAPD profiles of ginger in vitro conserved lines. Though OPF 01 gave some non-specific bands, no polymorphism was detected between the conserved lines in any of the primers tested (Fig. 31c & d). Thus RAPD profiles did not indicate any genetic variability within the replicates of ginger *in vitro* conserved lines.

***RAPD profiles of* in vitro *conserved lines in* K. galanga *and* K. rotunda**

Two primers, OPA 06 and OPE 01 were used to develop RAPD profiles (Fig, 32 a-d). Primer OPE 01 gave many non-specific bands indicating some amount of 'polymorphism'. The other primers did not detect any polymorphism between the lines in both the species.

DNA BANK

High molecular weight DNA was isolated from 50 lines each of cardamom, ginger, and turmeric and 20 lines each of *K. galanga* and *K. rotunda*. DNA was dissolved in 500 µl TE buffer and stored at $-20^{O}C$. The quality of the stored DNA samples was analyzed. DNA was of good quality as seen in agarose gel electrophoresis even after 2 years of storage. The concentration of DNA in the samples ranged from 100–200 ng/µl.

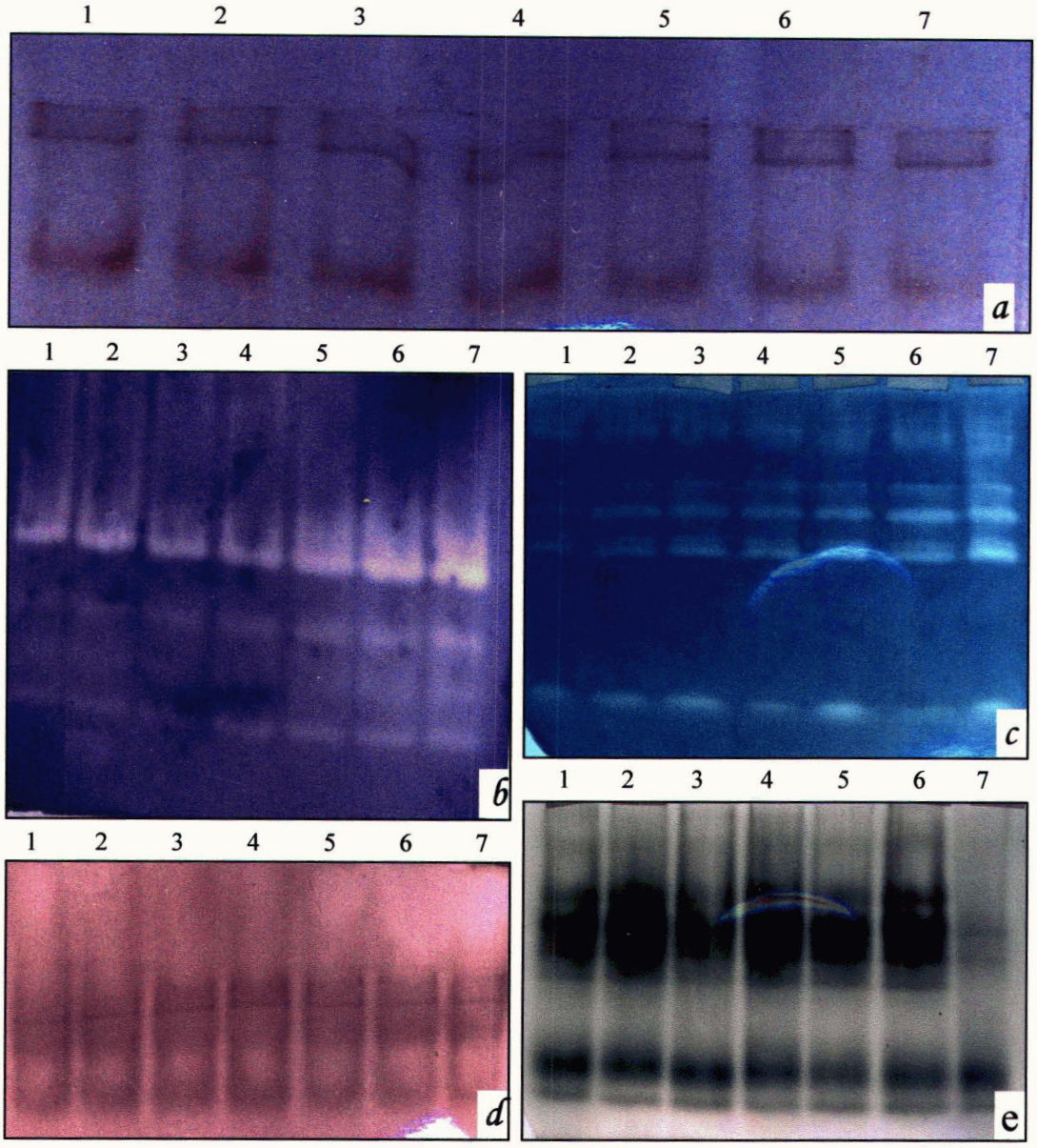


Fig. 30. Isoenzyme profiles of *in vitro* conserved lines of cardamom and *Kaempferia galanga* showing genetic uniformity

a. Cardamom (Peroxidase), b. Cardamom (Superoxide dismutase), c. *K.galanga* (Superoxide dismutase), d. *K. galanga* (Polyphenol oxidase), e. *K. galanga* (Esterase).

1=parent plant (control), 2–7=*In vitro* conserved lines

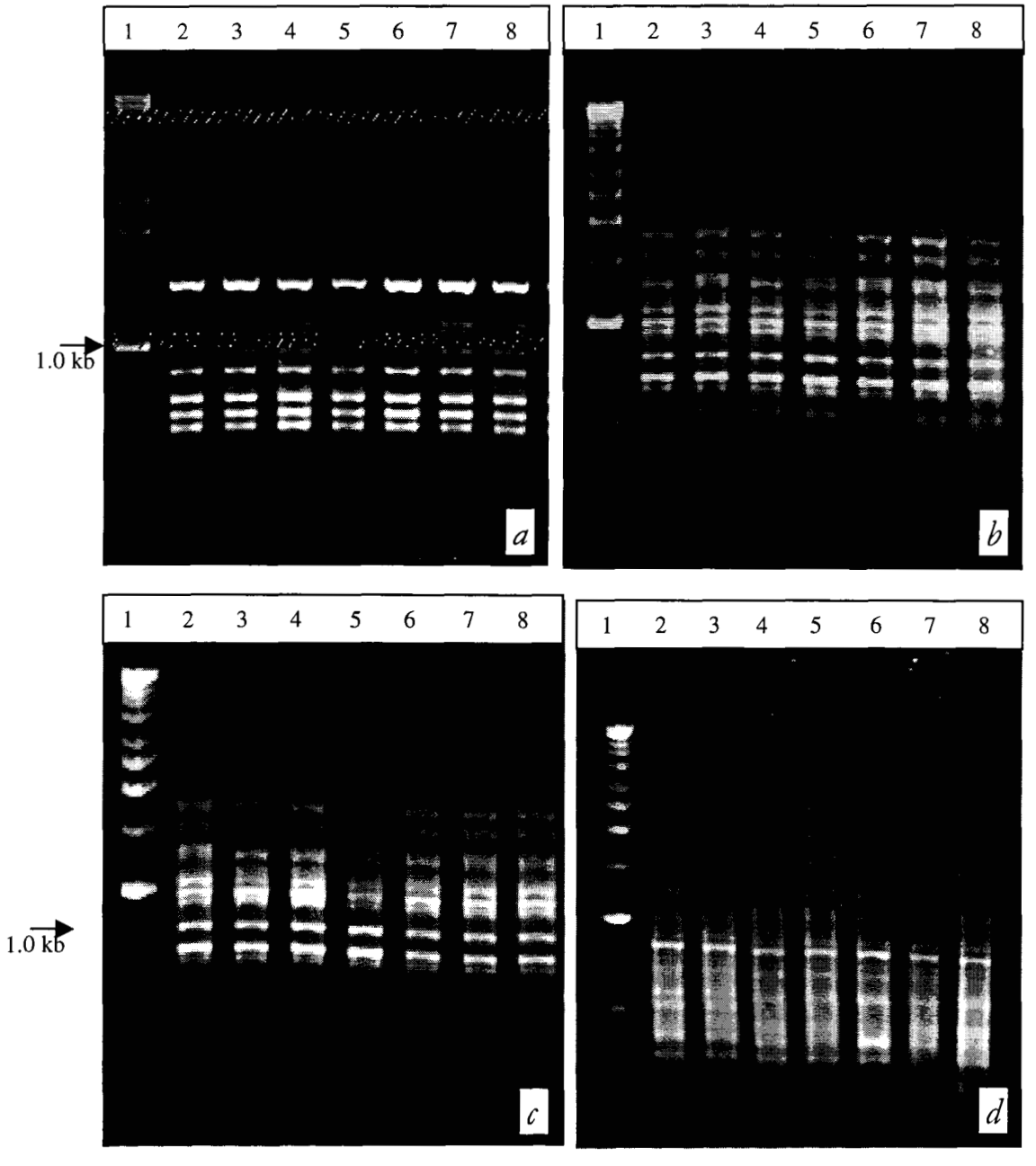


Fig. 31. Genetic fidelity analysis of *in vitro* conserved plants using RAPD

a. RAPD profiles of randomly selected *in vitro* conserved cardamom plants as amplified by OPERON primer OPC 19.

b. RAPD profiles of randomly selected *in vitro* conserved cardamom plants as amplified by OPERON primer OPE 02.

c. RAPD profiles of randomly selected *in vitro* conserved ginger plants as amplified by OPERON primer OPC 05.

d. RAPD profiles of randomly selected *in vitro* conserved ginger plants as amplified by OPERON primer OPF 01

(1=DNA marker, 2=Control plant, 3–8=*In vitro* conserved lines).

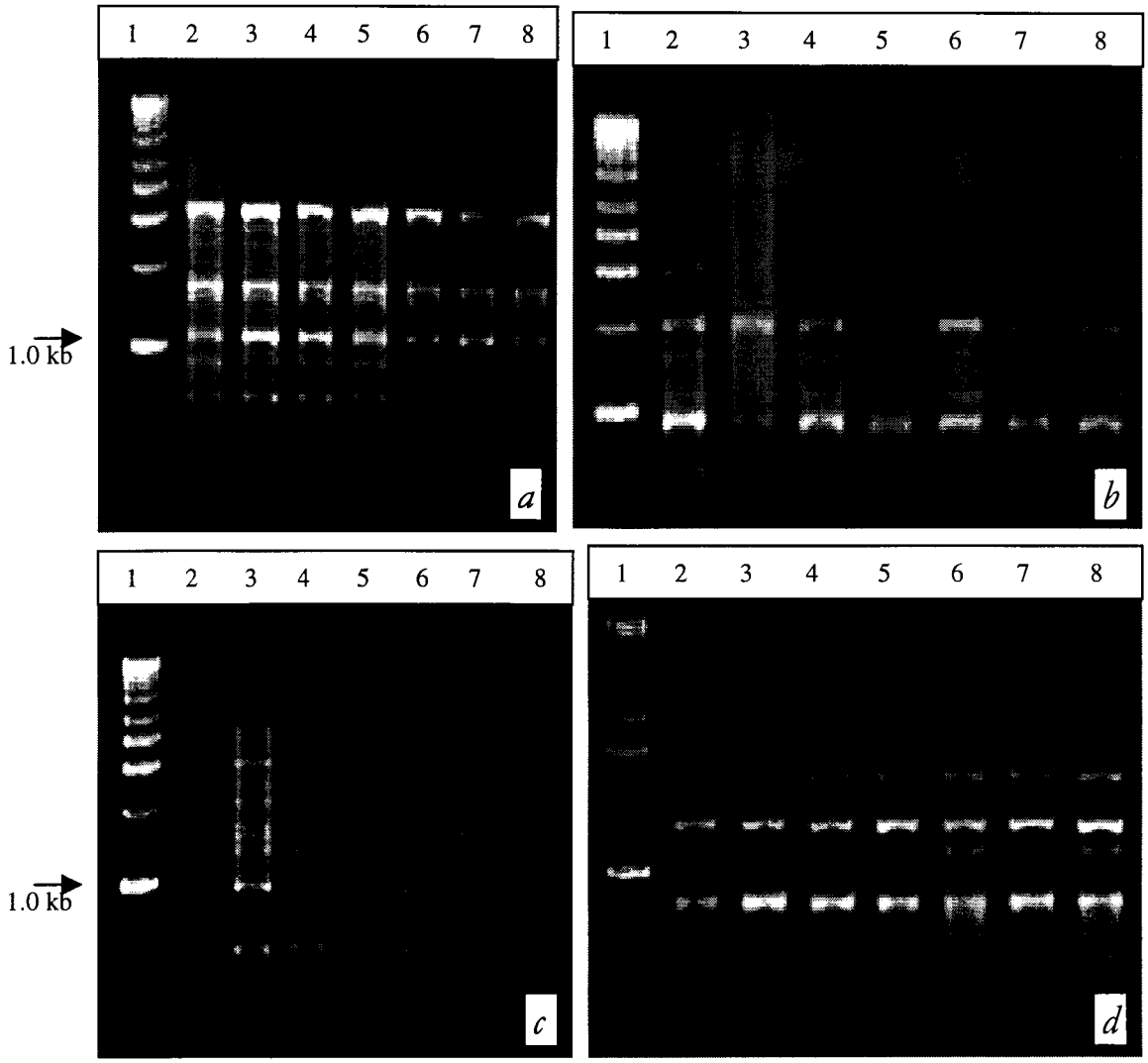


Fig. 32. Genetic fidelity analysis of *in vitro* conserved plants of *Kaempferia* spp. using RAPD

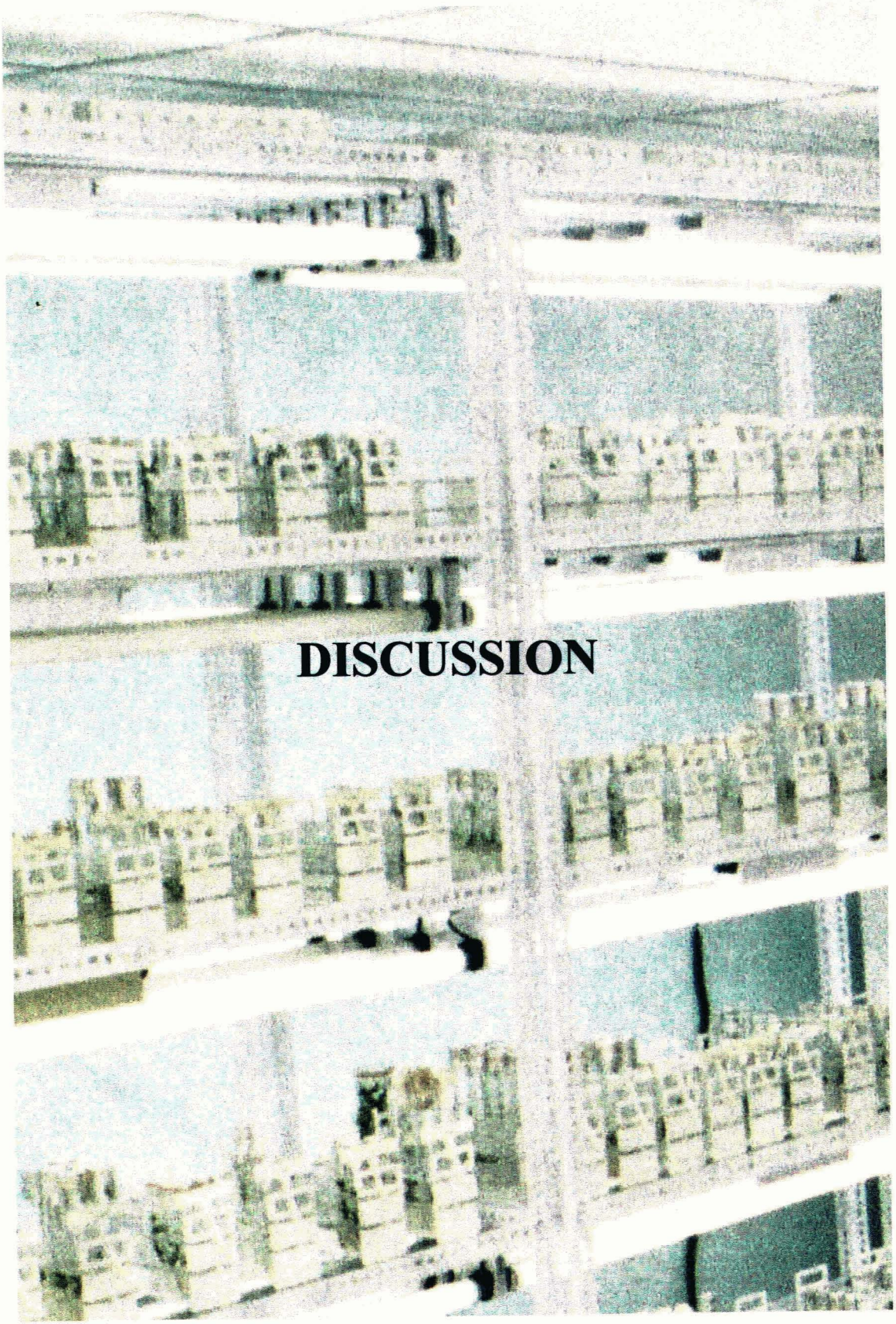
a. RAPD profiles of randomly selected *in vitro* conserved *K. galanga* plants as amplified by OPERON primer OPE 01.

b. RAPD profiles of randomly selected *in vitro* conserved *K. galanga* plants as amplified by OPERON primer OPA 06.

c. RAPD profiles of randomly selected *in vitro* conserved *K. rotunda* plants as amplified by OPERON primer OPC 05.

d. RAPD profiles of randomly selected *in vitro* conserved *K. rotunda* plants as amplified by OPERON primer OPF 01

(1=DNA marker, 2=Control plant, 3–8=*In vitro* conserved lines).



DISCUSSION

1988

In vitro techniques may be defined as those, which utilize tissue culture methods in the maintenance, production or modification of plant material. This involves growth of tissue on sterile medium in sterile culture vessels. The cultured material is generally maintained in temperature controlled incubators or culture rooms with artificial light provided by fluorescent tubes. The range of tissue culture techniques include undifferentiated and differentiated cultures. The former category include callus cultures and cell suspension cultures. In differentiated cultures, plant structures are maintained, and are derived either from existing meristems or from adventitious meristems arising in culture. These techniques include micropropagation in the form of shoot cultures, root cultures, microtubers or other plant organs.

Micropropagation encourages rapid multiplication of plant material to produce nuclear stock, free from pathogens and viruses. Tissue culture techniques offer the opportunity for *in vitro* collection, rapid propagation, medium and long term storage of germplasm and its distribution. Other more specialized tissue culture techniques include the use of meristem tip culture for the elimination of viruses, *in vitro* fertilization and embryo rescue, production of haploid plants, genetic transformation for the production of modified plants, somatic hybridization using protoplast fusion and the production of synthetic seeds. The full range of plant tissue culture techniques are described in detail by Bhojwani and Razdan (1983); Withers and Alderson (1986); Bhojwani (1990); Debergh and Zimmerman (1991); Vasil and Thorpe (1994) and George (1993; 1996).

In vitro techniques may be utilized at various stages in the conservation and use of plant germplasm. These techniques are invaluable to complement other plant health and conservation strategies, particularly for vegetatively propagated species

and species with recalcitrant seeds. Zingiberaceous species such as cardamom, ginger, turmeric, *Kaempferia galanga* and *K. rotunda* are no exception. The germplasm of these economically important crops are conserved in clonal field repositories because they are propagated vegetatively. Seeds of cardamom are recalcitrant and in ginger there is no seed set. Turmeric and *Kaempferia* rarely sets seed. All these crops are threatened by serious soil-borne diseases of fungal, bacterial and viral origin and outbreak of any of these diseases can wipe out the germplasm completely. There is also high risk of mixing up of germplasm due to planting in the same area year after year through the leftover rhizome pieces in the case of ginger, turmeric, *K. galanga* and *K. rotunda*. Hence it is important to formulate *in vitro* conservation strategy as a complement to the field genebank.

Plant cell and tissue culture is the science of growing plant cells, tissues or organs isolated from the mother plant on artificial media. Rapid clonal propagation was the first major practical application of plant tissue culture (George and Sherington, 1984). One of the other important applications is the eradication of diseases and the conservation and exchange of germplasm (De Langhe, 1984; Withers and Williams, 1985).

In the present study, the advantages of *in vitro* techniques were utilized for micropropagation and *in vitro* conservation of some of the zingiberaceous species such as cardamom, ginger, turmeric, *K. galanga* and *K. rotunda* with the ultimate purpose of germplasm conservation.

MICROPROPAGATION FROM VEGETATIVE BUD CULTURES

The term 'shoot culture' denotes the cultures which were initiated from explants with shoot meristem (eg. vegetative bud). In such explants shoot multiplication is by formation of axillary buds. The newly formed shoots further serve

as explants for repeated proliferation. Detached shoots are rooted to produce plantlets, which can be grown *in vivo*. The growth and proliferation of axillary shoots is usually produced by incorporation of growth regulators in the culture medium, which in turn effectively remove apical dominance and result in axillary shoot proliferation. This is the most widely used method for cloning any given genotype. Moreover, the plantlets obtained from shoot apices are genetically stable (George, 1993). Micropropagation techniques are now available for a large number of plant species, and in many cases the propagation of plants using tissue culture is on a commercial scale (Lindsey and Jones, 1989; Debergh and Zimmerman, 1991; George, 1993).

Vegetative bud culture in cardamom, ginger, turmeric and *Kaempferia* spp.

It was well established that the size of the explant is an important factor in the successful shoot tip culture of many crop species, especially in bananas and plantains (Krikorian and Cronauer, 1984; Vuylsteke and De Langhe, 1985; Gupta, 1986; Jarret, 1986a; 1986b; Novak *et al.*, 1986). In the present study, vegetative bud explants with shoot tip or axillary buds were initiated in MS medium containing 0.5 mgl^{-1} Kin. It was observed that, the size of the explant played important role in the successful shoot tip culture of these zingiberaceous crops. Freshly sprouting vegetative buds (1–2 cm long) from the rhizome gave vigorous and fast growing cultures and hence were used for culture initiation. Very small explants consisting only of the apical meristematic dome (< 1 mm in height), increased the chances of producing disease free plants, although they had a high mortality rate and grew very slowly. Dore Swamy (1983) reported more rapid growth and multiplication from larger explants of banana because of the presence of more lateral buds in the explant. The present study supports this view. Explants that were too large (> 3 cm in height) were also unsatisfactory because they were prone to more contamination and thus

resulted in lower survival rates, than smaller explants. Contamination of cultures is unavoidable and generally occurred at 20–50% of the cultures were affected. Fungal and bacterial contamination usually appeared within 5– 10 days in culture.

Earlier reports are available on the tissue culture studies in cardamom. Micropropagation protocols for cardamom are discussed in detail by Reghunath and Bajaj (1992). *In vitro* methods for clonal propagation of cardamom from vegetative buds have been standardized (Nadgauda *et al.*, 1983; Priyadarsan and Zachariah 1986, Vatsy *et al.*, 1987, Reghunath and Gopalakrishnan 1991). 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 successful conversion of immature floral buds into vegetative buds and subsequently into plantlets. Nadgauda *et al.* (1983) initiated cardamom cultures from young sprouting buds, on MS medium supplemented with BAP, Kin, IAA and additives like coconut water (5%), calcium pantothenate (0.1 mgl^{-1}) and biotin (0.1 mgl^{-1}). They obtained a multiplication ratio of 1:3 and the shoots were induced to root in White's liquid medium. Reghunath and Bajaj (1992) reported that SH (Schenk and Hildebrandt, 1972) medium supplemented with 4.0 mg/l BAP and 0.5 mg/l NAA gave better growth and higher shoot proliferation than MS medium. They also reported that shoot explants cultured in liquid medium in conical flasks under gyratory shaking produced 57% more axillary branches than those cultured in semi-solid medium, when maintained at 23±2°C under a light intensity of 3000 lux and 16 h photoperiod.

Micropropagation of ginger through tissue culture was reported by various authors (Hosoki and Sagawa, 1977; Nadgauda *et al,* 1980; Pillai and Kumar, 1982; Sato *et al,* 1987; Ilahi and Jabeen, 1987; Bhagyalaksmi and Singh, 1988; Inden *et al.*, 1988; Noguchi and Yamakawa, 1988; Sakamura and Suga, 1989, Wang, 1989;

Saradha and Padmanabhan, 1989; Balachandran *et al.*, 1990; Huang, 1995; Sarma and Singh, 1997). Various explants like young sprouts, dormant buds, rhizome bits, meristem, pseudostem, etc. were used as explants. The nutrient medium used was mostly MS (Murashige and Skoog, 1962), though other media like Gamborg's B5 (Gamborg *et al.*, 1968), White's (White, 1963), Smith's (Kassanis 1967), Schenk and Hildebrandt's (Schenk and Hildebrandt, 1972) were also used by some workers. The most commonly used growth regulators were cytokinins (BAP and Kin) and auxins (2.4-D, IAA and IBA and in one case Decamba). Sharma and Singh (1997) reported high frequency *in vitro* multiplication of disease free clones by culturing small and active buds of ginger on MS medium supplemented with $2mgl^{-1}$ Kin and plantlet regeneration was obtained in MS with 2 mgl^{-1} Kin and 2 mgl^{-1} NAA.

Successful micropropagation of turmeric has been reported by several workers (Nadgauda *et al.*, 1978; Shetty *et al.*, 1982; Yasuda *et al.*, 1988; Babu *et al.*, 1997). This technique could be used for production of disease-free planting material of elite plants. Organogenesis and plantlet formation was achieved from callus cultures of turmeric (Shetty *et al.*, 1982). Variants with high curcumin content were isolated from tissue-cultured plantlets (Nadgauda *et al.*, 1982). Nadgauda *et al.*, (1978), have reported the rapid multiplication of turmeric vegetative buds when cultured on MS medium supplemented with coconut milk, Kin and BAP and the plantlets were hardened on liquid medium.

Economically and medicinally important zingiberaceous species like *K. galanga* could be micropropagated using vegetative bud in MS medium supplemented with BAP and NAA (Vincent *et al.*, 1992). Micropropagation of both the species were reported by Geetha *et al.*, (1997). Successful plant regeneration and variations among regenerated plants were reported in *K. galanga* (Kumar and Seeni, 1995).

In the present study, actively growing vegetative buds of cardamom, ginger, turmeric, *K. galanga* and *K. rotunda* were cultured on MS basal medium supplemented with auxins (NAA and IBA) and cytokinins (BAP and Kin) at 0.5 and 1 mgl^{-1} levels singly and in combination. All the treatments gave positive response in inducing multiple shoots and roots. Some of the treatments such as MS with 1 mgl^{-1} NAA alone and MS with 1mgl^{-1} BAP and 0.5 mgl^{-1} NAA were significantly different from the rest in both root and shoot induction. This indicates the presence of NAA at low concentrations (1 mgl^{-1}) resulted in good growth of culture, root induction and shoot multiplication and addition of BAP at 1mgl^{-1} increased the multiple shoot induction and root induction slightly, though not significantly. Earlier workers also reported that BAP and NAA combinations were best for shoot multiplication in some of the zingiberaceous crops such as ginger, turmeric and *Kaempferia* species (Sakamura *et al.*, 1986; Charlwood *et al.*, 1988; Sakamura and Suga, 1989, Vincent *et al.*, 1992).

In general, cytokinins are used for axillary shoot proliferation to overcome apical dominance of shoots and auxins for induction of roots (Hu and Wang, 1983; Vuylsteke and De Langhe, 1985; Zamora, *et al.,* 1986; George, 1993). However in the present study the results indicated that NAA alone or NAA in combination with BAP gave the best response for induction of multiple shoots as well as roots in the same medium.

The role of NAA in root induction is a well-known phenomenon. The development of multiple shoots in medium containing NAA may be due to its interaction with residual cytokinin (Kin), which is used in the culture initiation medium or due to endogenous cytokinin formation combined with physiological state of the explant. It was suggested that shoot and root apices are the sites of cytokinin

biosynthesis as demonstrated in *Asparagus* shoot apex cultures (Koda and Okazawa, 1980). It was found that cultured shoot apices of *Asparagus* released a small and constant amount of cytokinin into the medium. The root tip of *Asparagus* produced more cytokinins than shoot apex. In zingiberaceous genera like cardamom, ginger, turmeric and *Kaempferia* spp. also, a similar mechanism may be in operation, thus giving rise to both multiple shoot as well as root development in the same medium. In addition the inherent characters of rhizomes of these species and the requirement of these rhizomes to branch in the soil may necessitate endogenous production of cytokinins.

The induction of both roots and shoots in the same medium considerably reduces the time taken for cloning. In these zingiberaceous genera it takes about 80–90 days for the explant to develop into well developed plantlets in the first cycle using the explant from the source plant grown under protected conditions. During the multiplication from the *in vitro* developed axillary shoots, the rate of proliferation was further increased and the time taken for plantlet development was reduced to 30–45 days per cycle. Similar results were reported in various crops like rose and cherry (Hutchinson, 1985; George, 1993).

Thus simple protocols were standardized for micropropagation of cardamom, ginger, turmeric and two species of *Kaempferia*. These protocols are efficient and use low levels of growth regulators, which will ensure genetic stability, which is a norm in *in vitro* conservation of plant genetic resources.

Meristem isolation and culture in cardamom

Virus infection in plant tissue is very difficult to eliminate. Viruses can be transferred during generative as well as vegetative propagation. Viruses can result in

the loss of crop production. So it is extremely important to use virus free planting material in vegetative propagation. (Pierik, 1987).

Meristem culture is considered an efficient method for the elimination of viruses from plants. Virus elimination from *Dahlia* stocks through shoot apex culture was demonstrated by Morel (1960) for the first time.

In the present study meristem culture was tried in cardamom alone because, cardamom cultivation is threatened due to the severe incidence of *katte* disease caused by Car MV that is an aphid transmitted poty virus. Apical and axillary meristems of about 0.2–0.5 mm sizes could be isolated from *in vitro* grown cultures of cardamom and established with 30–40% survival. Meristem developed in to whole plants when cultured in MS medium containing 0.5 gl^{-1} Kin.

The size of the isolated meristem and the number of leaf primordia present in the meristem are important while intending a virus free plant. The chance of getting a virus free plant increases with decrease in size of the meristem, but the chance of a meristem surviving without leaf primordia is very less (Pierik, 1987).

The average time needed to regenerate plantlets from meristems varies with genotype, explant size, culture media and culture conditions. Usually explants of about 0.1 mm should take more than 3–5 months to regenerate in to a plant (Love *et al.*, 1987). In the present study the whole plants were developed within 6 months.

The percentage of isolated meristems that develop in to virus free plants is generally very less. The high mortality rate is due to infection, damage during excision, drying out and browning, media which is not optimal for growth, dormancy problems etc. One virus free plant is enough to produce a virus free clone using efficient methods of micropropagation (Pierik, 1987).

This is the first report of meristem isolation and culture from cardamom, but in the present study no assay was carried out to test the virus free nature of the plants derived through meristem culture. The technique needs to be refined to isolate smallest possible meristem to ensure a virus free clone and the micropropagation protocols standardized in this crop can be used to clone the virus free plant if at all one is obtained by the present method.

Virus elimination was achieved after successful meristem culture in many crop species like garlic, pineapple, *Brassica*, Caladium, Colocassia, chrysanthemum, dahlia, carnation, *Fragaria*, Freesia, *Gladiolus*, apple, cassava, banana, geranium, *Phaseolus*, sugarcane, potato etc (Quak, 1977; Love *et al.*, 1987).

IN VITRO CONSERVATION OF PLANT GENETIC RESOURCES

The germplasm of all organisms manifests differing degrees of variability in the characteristics or traits that they possess as a result of heritable changes called mutations. It is this variability that is designated as biodiversity. Biodiversity is a fundamental property of life and without it no evolutionary changes are possible (Solbrig, 1991; Okigbo, 1994).

Conservation of genetic resources is of prime importance and the aim is to ensure the availability of useful germplasm at any given time. The most widely used method for conserving plant genetic resources is seed storage. However, many categories of crops face problems with regard to seed storage. At present, the most common method to preserve the genetic resources of these problem crop species is as whole plants in the field, which are called field genebanks. There are however serious problems with field genebanks especially when there is disease and pest out break (Withers and Engels, 1990). The development of biotechnology has led to the production of a new category of germplasm, including clones obtained from elite

genotypes, cell lines with special attributes, protoplasts, DNA and genetically transformed material (Withers, 1985b; Engelmann, 1994). This new germplasm is of high added value and often very difficult to produce. These can be conserved only in *in vitro* or cryo gene banks.

***In vitro* conservation by slow (minimal) growth**

In vitro cultures can be used to maintain genotypes over long periods by reducing the growth rate of cultures. The length of time during which the cultures can be stored is species specific and periods between 6 and 24 months are reported. Single shoot cultures, unrooted shoot clusters; somatic embryos and /or rooted plantlets are suitable for storage.

There are different ways by which the growth rate of *in vitro* cultures can be reduced, so that the life of the cultured tissue is extended. The techniques, which have been used to maintain collections in slow growth include the use of reduced temperature, reduced light conditions, modifications in media, particularly the addition of osmotic inhibitors or growth retardants, dehydration of tissue, or modification of gaseous environment (Engelmann, 1997). Combinations of these techniques are used for many species, such as cassava, potato, *Musa*, coffee, etc. (Ashmore, 1997).

One of the factors, which causes tissue death, or drying of *in vitro* cultures is moisture loss due to evaporation both from the tissue and from the culture medium. This can be prevented or minimized by the use of suitable culture vessel closures. Instead of closures, which allow more evaporation, caps or films which allows gaseous exchange but prevent moisture loss should be used. By closing the culture tube with screw caps, the storage period of *Mentha* spp., blackberry, *Rubus, Vaccinium, Pistachio* and *Coffea arabica* cultures could be extended for 12 months

under normal culture conditions (Kartha *et al.*, 1981; Barghchi, 1986; Gunning and Lagerstedt, 1986). White spruce embryogenic tissues withstood a 1 year storage period in hermetically sealed serum capped flasks (Joy *et al.*, 1991). Replacing cotton plugs by polypropelene caps, thus reducing the evaporation of the culture medium increased the survival rate of *Rauvolfia serpentina* during storage (Sharma and Chandel, 1992). The use of heat-sealable polypropylene bags instead of glass test tubes or plastic boxes was beneficial for the storage of several strawberry varieties (Reed, 1991; 1992). Sharma (2001) reported storage of *in vitro* cultures of a medicinal plant, *Gentiana kuroo* up to 7 months at 25°C by replacing the cotton plugs with polypropylene caps as culture tube closures.

Shoots and plantlets of many plant species have been stored successfully at low temperatures. Optimal conditions usually vary from one genus to another. Temperatures in the range of 0–5°C have been found to be suitable for conservation of *in vitro* cultures of cold tolerant species. Temperate woody species like apple, pear, *Pinus*, etc. could be stored at 0–5°C (Lundergan and Janik, 1979; Chun and Hall, 1986; Aitken-Christie and Singh, 1987). Apple and *Prunus* shoots survived 52 weeks at 2°C (Druart, 1985). Strawberry (*Fragaria* x *ananassa*) plantlets have been stored at 4°C in the dark and kept viable for 6 years with regular addition of a few drops of liquid medium (Mullin and Schegel, 1976). Tropical species are often cold sensitive and have to be stored at higher temperatures, which depend on the cold sensitivity of the species. For many tropical and subtropical species in which low temperature storage reduces the viability of shoot cultures, a temperature of 14–20°C is suitable for inducing minimal growth (Henshaw *et al.*, 1980). Kiwi fruit shoots could be conserved at 8°C (Monette, 1986) and taro tolerates 3 years of storage at 9°C (Staritsky *et al.*, 1986). Banerjee and De Langhe (1985) maintained proliferating

cultures of seven cultivars of *Musa* for more than 1 year at 15°C and low light level (1000 lux). Jarret *et al.*, (1986) stored 38 *Musa* accessions for up to 18 months at 18°C under low light intensity in liquid media on filter paper bridges. Cultures of cassava deteriorated if stored at temperatures lower than 20°C (Roca *et al.*, 1982; 1984). Shoot cultures of *Actinidia chinensis*, *Vitis* species, sugarbeet and potato are reported to be stored at 12°C (Hussey and Hepher, 1978; Westcott, 1981; Henshaw, 1982; Monette, 1986, Galzy and Compan, 1988). Oil palm, another tropical species, is more cold sensitive and the somatic embryos do not withstand even a short exposure to temperatures lower than 18°C (Corbineau *et al.*, 1990).

An alternative but less common approach is the modification of culture media by reducing the nutrient concentration of the basal medium or by adding an osmoticum to the culture medium or by removal of growth regulators from the culture medium or by adding growth retardants to the medium. Embryogenic cultures of carrot could be conserved on a medium without sucrose for 2 years, and proliferated if a sucrose solution was supplied (Jones, 1974). Zamora *et al.* (1986; 1987) reported storage of *Musa* cultures up to 4 months on media containing sorbitol or mannitol. Replacement of sucrose by ribose allowed the conservation of banana plantlets for 24 months (Ko *et al.*, 1991). Addition of osmotic growth inhibitors such as mannitol or hormonal growth inhibitors (abscisic acid) is also employed successfully to reduce growth (Westcott, 1981a, b; Staritsky *et al.*, 1986; Ng and Ng, 1991; Viterbo and Rabinowitch, 1994; Vysotskaya, 1994). Induction of slow growth by altering the constituents of the culture media was reported in many crop species like *Cinchona*, cassava, coffee, strawberry, tomato, potato, *Vitis*, etc. (Henshaw *et al.*, 1980; Kartha *et al.*, 1981; Gunning and Lagerstedt, 1986; Hunter *et al.*, 1986; Schnapp and Preece, 1986; Moriguchi and Yamaki, 1989). Kartha *et al.*, (1981) could conserve coffee

plantlets on a medium devoid of sugar and with only half of the mineral elements of the standard medium. Other factors like storage in reduced light, desiccation combined with cold treatment, reduced oxygen tension and use of growth regulators like abscisic acid were also successful in reducing growth rate and thereby storage (Ammirato, 1974; Roca *et al.*, 1982; Nitzsche, 1983; McCulloch, 1988; Moriguchi *et al.*, 1988; Mathur, 1991).

The type of explant used can influence the duration of storage achieved. In chrysanthemum nodal segments showed higher survival rates than apical buds (Roxas *et al.*, 1995). The presence of a root system generally increases the storage capacities, as observed by Kartha *et al.* (1981).

The so far published reports on slow growth protocols indicate that a variety of techniques are still being utilized, with no obvious optimal techniques emerging. Shoots of *Coffea* spp. are maintained at 27°C on reduced cytokinin levels (Dussert *et al.*, 1997), shoots of *Ananas* spp. are maintained at 25°C with quarter strength salts (Zee and Munekata, 1992), shoots and microtubers of *Dioscorea* spp. are stored at 28°C on minimal medium with no plant growth substances (Malaurie *et al.* 1993). Low temperature regimes are used for the storage of sugarcane shoots at 18°C on reduced mineral salts (Paulet and Glaszmann 1994); potato microtubers at 10°C (Kwiatkowski *et al.*, 1988); shoots of *Rauwolfia serpentina* Benth. X Kurz. at 15°C (Sharma and Chandel 1992), strawberry and *Rubus* shoots at 4°C in the presence of plant growth substances (Reed, 1992), and banana shoots at 16°C in the presence of plant growth substances (Van den houwe *et al.*, 1995). The subculture interval ranged from every 6 months for *Coffea* spp. to every 21 months for potato microtubers.

In the present study, cardamom cultures could be stored with 80% survival up to 12 months without subculture in half strength MS medium supplemented with 15gl^{-}

^{1} each of sucrose and mannitol in culture tubes sealed with screw caps, aluminium foil or polypropylene caps and maintained at 22±2°C. These cultures could be stored up to a maximum period of 15 months with 70% survival and beyond that there is sudden deterioration of the cultures. Under minimal growth conditions, the cultures exhibited a miniature growth pattern.

In ginger and turmeric, the cultures could be stored up to 12 months with 70–80% survival in half strength MS medium supplemented with 15 gl^{-1} each of sucrose and mannitol in sealed culture tubes. Storage of these cultures beyond 12 months was impossible as the aerial shoot exhibited yellowing and symptoms of deficiency by then.

In *K. galanga* and *K. rotunda*, the cultures could be stored up to 12 months with 80–90% survival in half strength MS medium supplemented with 15 gl^{-1} each of sucrose and mannitol in sealed culture tubes. In *K. galanga*, the cultures could be stored up to a maximum period of 18–19 months with 60% survival, whereas in *K. rotunda* further storage was not possible as the culture growth was comparatively high and the cultures exhibited drying.

In all these species minimal growth could be induced and cultures were maintained up to 12 months without subculture in half strength MS medium supplemented with 10 gl^{-1} each of sucrose and mannitol also. But in this medium cultures were not very healthy and showed symptoms of deficiency, hence the medium with 15 gl^{-1} each of sucrose and mannitol was used for *in vitro* conservation of these species.

Thus in the present study minimal growth was induced in the zingiberaceous crops namely, cardamom, ginger, turmeric, *K. galanga* and *K. rotunda,* by minimizing the evaporation loss using screw cap, aluminium foil or polypropylene caps to seal the

culture vessel and by the reduction of basal medium concentration as well as sucrose concentration to half and by addition of mannitol as an osmoticum. In all the species studied the cultures could be maintained only at 22±°C and lower temperatures of 5°C, 10°C and 15°C were not suitable for growth of *in vitro* cultures. This observation is supported by the earlier reports of Galzy (1969), Henshaw *et al.*, (1980), Roca *et al.*, (1982), Banarjee and De Langhe (1985) and Monette (1986). Since these zingiberaceous crops are tropical, low temperature storage is not suitable and a temperature of 22±°C is ideal for both micropropagation and conservation.

Few attempts have been made earlier to use tissue culture for conservation of ginger germplasm. The subculture interval could be extended up to 7–12 months by using mannitol as osmoticum and polypropylene caps to minimize evaporation loss (Balachandran *et al.*, 1990). Dekkers *et al.* (1991) reported that ginger shoot cultures could be maintained over 1 year at ambient temperatures of 24–29°C in a medium containing 25 gl^{-1} mannitol with an overlay of mineral oil. Babu (1997) reported medium term storage of *in vitro* cultures of ginger in mannitol and sucrose containing medium in culture tubes closed with aluminium foil and he reported the deterioration of ginger cultures under low temperatures of 5°C and 10°C. The present study is in agreement with these reports.

Many of the literature on minimal growth storage reports the use of protocols on a large number of accessions or genotypes, indicating that single protocols may be applicable across the genetic diversity for a given species. The suitability of the protocol standardized in the present study supports this view.

CRYOPRESERVATION

Cryopreservation of plant material is the only viable option for long-term storage of germplasm of vegetatively propagated species and species with recalcitrant

seed. This involves storage at ultra low temperatures, usually that of liquid nitrogen (-196°C). At this temperature, cell division and metabolic processes stop, and the plant material can thus be stored without modification or alteration for unlimited periods of time. Cryopreserved material also requires very limited space, is protected from contamination and needs very little maintenance.

The choice of material for storage can include shoot apices, pollen, somatic embryos, seed or excised zygotic embryos. Cryopreservation protocols, with a few exceptions, are still in the development stages for most of the crops. Crop species for which cryopreservation is routinely used across a range of genotypes include *Rubus*, *Pyrus*, *Solanum* spp. and *Elaeis guineensis* (Ashmore, 1997).

The techniques currently being used for cryopreservation are quite varied, and include the older, classical techniques, which are based on freeze-induced dehydration of cells (Kartha 1985), as well as a series of newer techniques, which are mostly based on vitrification (Kartha and Engelmann, 1994; Engelmann, 1997).

In classical techniques, the tissue is initially cooled slowly and at a controlled rate, down to about –40°C, followed by rapid cooling in liquid nitrogen. It is apparent that classical techniques have mainly been used on cell cultures, and are more difficult to apply to larger cellular units such as shoot apices or embryos.

The new techniques based on vitrification have been developed over the past 10 years. Included among these are encapsulation-dehydration, vitrification, encapsulation-vitrification, desiccation, pregrowth, pregrowth-desiccation and droplet freezing. In all these methods a very rapid freezing process is used, with samples being plunged directly into liquid nitrogen once the pretreatment stages have been completed and which offer practical advantages in comparison to classical ones (Steponkus *et al.*, 1992; Sakai, 1995; Engelmann, 1997).

The encapsulation-dehydration technique is based on the technology for the production of synthetic seeds where somatic embryos or apices are encapsulated in a bead of hydro soluble gel (Redenbaugh *et al.*, 1991). This technique has been applied mostly to apices of more than 10 species of temperate and tropical origin, and to somatic embryos of several crop species as well as to *Catheranthus* cell suspension. The specimens are usually encapsulated in 3% calcium alginate gel. They are then subjected to pregrowth, desiccation, freezing, thawing and recovery. Pregrowth is performed in liquid medium enriched with sucrose (0.3–1.5 M) for periods varying between 16 hours (Niino and Sakai, 1992) and 10 days (Fabre and Dereuddre, 1990). Desiccation is performed by using either the air current of laminar airflow cabinet or silica gel. If pregrowth conditions have been well defined, only limited loss is observed after desiccation with most species. However, banana apices were found to be highly sensitive to both sucrose pregrowth treatment and to even limited desiccation, which induced a drastic reduction in survival (Panis, 1995a; b).

Desiccated samples are usually frozen by direct immersion in liquid nitrogen. However, modifications in the freezing rate are reported to have different consequences on the survival of different materials. Survival of encapsulated grape apices was higher after slow cooling down to 100°C (Plessis *et al.*, 1991). In contrast, survival of sugarcane apices was higher after rapid freezing than after slow controlled cooling (Gonzalez-Arnao *et al.*, 1993). Freezing encapsulated carnation apices at different cooling rates had no effect on survival (Tannoury *et al.*, 1994).

Samples are usually stored in liquid nitrogen at -196°C. The thawed samples are placed directly under standard conditions for recovery. There is a need to modify the conditions to enhance recovery in some cases. Sugarcane apices are placed in the

dark for 1 week on a medium supplemented with growth hormones (Paulet *et al.*, 1993). Addition of antioxidant ascorbic acid significantly improved the recovery of sugar beet apices, which are extremely sensitive to oxidation (Vandenbussche and De Proft, 1995). Extraction of apices from beads was necessary to allow regrowth of pear and grape apices (Plessis *et al.*, 1991; Scottez *et al.*, 1992). Regrowth of material frozen using encapsulation-dehydration technique is usually direct and rapid, without callus formation. This is due to the fact that this method preserves the structural integrity of most cells and regrowth usually originates from the whole meristematic zone, as reported in the case of sugarcane (Gonzalez-Arnao *et al.*, 1993).

In the present study naked shoot tips frozen in liquid nitrogen, pretreated with 0.75 M sucrose and desiccated for 1 h by air drying showed 40% viability and 20% of these cultures developed into plantlets. Desiccation for more than 1 h was deleterious resulting in complete loss of viability. In case of encapsulated shoot buds pre-cultured for 3 days in 0.5 M and 0.75 M sucrose and desiccated for 4 h air drying showed a post freeze viability of 30% and 50% respectively against their unfrozen control with 70% viability. Higher desiccation time was required for encapsulated shoot buds. Callus formation was observed in 20% of the cultures, the others developed into complete plantlets. This may be due to freeze injury to many of the cells, which may in turn affect the regeneration capacity of cryopreserved tissue. Viability could be observed only in the apices extracted out of the beads and similar observations were reported in grapes and pear (Plessis *et al.*, 1991; Scottez *et al.*, 1992). Cryopreservation of shoot apices by encapsulation-dehydration method was reported in *Solanum* spp. (Fabre and Deruedre, 1990), grapes (Plessis *et al.*, 1991, 1993), *Malus* spp., *Pyrus* spp. and *Morus* spp. (Niino and Sakai, 1992), chicory (Vandenbussche *et al.*, 1993), coffee (Engelmann *et al.*, 1994b), *Dianthus* (Fukai *et*

al., 1994a), Sugarcane (Engelmann *et al.*, 1994b), *Beta vulgaris* (Vandenbussche and De Proft, 1995), *Musa* spp. (Panis, 1995a; b), etc. The encapsulation-dehydration technique has been successfully extended to several genotypes or varieties in four crops namely, pear, apple, sugarcane and potato.

Present study reports successful cryopreservation of ginger shoot buds for the first time and with little refinement can be used for cryopreservation of base germplasm in this crop.

Pollen cryopreservation

Pollen banks, where pollen is cryopreserved, may make pollen easily and readily available for any use (Bajaj, 1987). Pollen is stored for facilitating crosses in breeding programmes, distributing and exchanging germplasm among locations, preserving nuclear genes of germplasm, studies in basic physiology, biochemistry and fertility and studies on biotechnology involving gene expression, transformation and *in vitro* fertilization. Pollen preservation supplements seed or clone preservation for germplasm banks and is not intended to replace them. Storage for use in haploid generation through pollen embryogenesis has also been advocated and for this pollen at uninucleate stage is more responsive. This indicates the need for long-term storage of uninucleate pollen, but such storage has not been addressed yet (Towil and Walters, 2000).

The ultimate goal of any pollen storage is to retain viability and functionality in a large percentage of stored pollen grains. Measurement of viability is crucial for any storage study. Pollen viability may be measured by metabolic activity, membrane semipermiability, germination and seed set. Metabolic activity is rarely

used as a method, probably because it is difficult to quantify. Membrane semipermiability can be measured using different tests and the most common test is by using fluorescein diacetate (FDA), which is permeable and cleaved within the cell by nonspecific esterases to form fluorescein (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison *et al.*, 1984). Fluorescein is impermeable and can be detected by cellular fluorescence. *In vitro* germination is commonly used for assessing the viability. Pollen germination on the stigma and growth in the style are also used as viability tests. Fertility is an absolute measurement of the ability of the pollen grain to germinate and set seed. The FDA test is generally considered the most rapid and accurate staining test for viability.

Palynological studies in cardamom are very much limited. Panchaksharappa (1966) conducted some studies, and he pointed out that the pollen grains are two celled at the time of dehiscence. Pollen fertility is reported to be maximum at full bloom stage and low at the beginning and end of the flowering periods (Venugopal and Parameswar, 1974). Krishnamoorthi *et al.* (1989) reported that the pollen grains loose their viability quickly and only 6.5% remained viable upto 2 h and none after 6 h of storage. *In vitro* pollen germination studies were reported by Kuruvila and Madhusoodanan (1988). Pollen germinates in 15% sucrose solution and addition of 150 ppm boric acid improves germination and tube growth and that the ideal temperature is 15–20°C.

In the present study cryopreservation of cardamom pollen was attempted. Cardamom pollen was collected from flowers, which are about to open, dehydrated in the air current of laminar airflow before cryopreservation. DMSO at 5–10% was used as a cryoprotectant. Cryopreservation of fresh pollen without any pre-treatment

resulted in complete loss of viability. Pretreatment increased the viability significantly in certain treatments and the pollen germinated *in vitro*. Pollen air dried for 10 minutes combined with 10% DMSO treatment for 10 minutes gave highest pollen viability of 50 % after cryopreservation. This is the first report of cryopreservation of cardamom pollen. Further studies are needed to increase the pollen viability and to test the *in vivo* germination and fruit set in cardamom.

SYNTHETIC SEEDS

Murashige (1978) was the first to point out about the application of artificial seeds. Earlier reports on synthetic seeds are available in crops like alfalfa (Bingham *et al*, 1975), celery, lettuce (Lawrence, 1981), carrot (Drew, 1979; Kitto and Janick, 1985a; b).

Synthetic seeds consist of somatic embryos or other propagules individually encapsulated in hydrogel such as sodium alginate or polyoxyethylene glycol. Synthetic seeds made of hydrated gels are more difficult to store because of the requirement of propagule respiration and these capsules dry out quickly unless kept in humid environment (Redenbaugh *et al.*, 1986; 1987a). Recently, there are many reports on the use of hydrogel coating such as sodium alginate for producing synthetic seeds and have been documented in the reviews of Fujii *et al.* (1987) and Redenbaugh *et al.* (1988; 1991). Various hydrated gels like sodium alginate, sodium alginate with gelatin, carrageenan with locust bean gum and gelrite were used for the encapsulation of plant propagules and several were found to produce sufficiently hard capsules while maintaining embryo viability (Redenbaugh *et al.* 1987a; 1987b). The coating material must be mild enough to protect the propagule but sufficiently durable for rough handling during production, storage, transportation and planting and should not

damage the propagules. The coating must contain nutrients and other substances necessary for growth and germination of the propagules.

In the present study *in vitro* regenerated shoot buds of cardamom, ginger and turmeric were successfully encapsulated in 3% sodium alginate matrix. The beads were sufficiently strong for easy handling. The encapsulated synthetic seeds were stored at 5°C, 10°C and 22°C to study the effect of low temperatures on their storage and viability. Low temperatures were not suitable for synthetic seed storage as they lost their viability within 30 days. But at 22±2°C, synthetic seeds could be stored up to 6–9 months in sterile environment, when incubated in MS basal medium. When cultured on MS medium supplemented with BAP (1.0 mgl^{-1}) and NAA (0.5 mgl^{-1}), maximum germination (80%) was observed after two weeks. The plants derived from these encapsulated buds were healthy and developed into normal plantlets. Synthetic seeds form an ideal source of material for germplasm conservation and exchange, especially in ginger, where there is no natural seed set. Production of disease-free encapsulated shoot buds were reported in ginger by Sharma *et al.*, (1994) and Babu (1997) and in some of the spices (Sajina *et al.*, 1997). There is considerable potential for the use of synthetic seeds for germplasm exchange, and for storage of plant germplasm (Senaratna and McKersie, 1989).

A few of the other reports on synthetic seeds were in carrot, sandalwood, groundnut, mulberry, *Dioscorea*, *Eleusine coracana*, citrus, babnana etc., (Mitra and Chaturvedi, 1972; Kitto and Janick, 1985a; b; Bapat and Rao, 1990; Fernandes *et al.* 1994; George and Eapen, 1995; Hassan and Takagi, 1995; Padmaja *et al.* 1995).

IN VITRO RHIZOME FORMATION

Storage organs such as bulbs, tubers, corms, etc., can be induced grow in *in vitro* cultures of those species which normally produce these structures. Induction of

characteristic, small, storage structures in several plant species like *Amaryllis*, hyacinth, lily, onion, narcissus, gladiolus, orchids, potato, yams etc., have been reported (Ziv *et al.*, 1970; Ziv and Halevy, 1972; Hussey, 1978; George, 1993; 1996). Under appropriate environmental conditions plants that normally produce tubers and can be induced to produce miniature versions of the storage organs in a medium containing high cytokinin levels (Hussey and Stacey, 1981; Ng, 1988).

There are several reports on *in vitro* microtuber formation and their application in medium-term to long-term conservation of potato germplasm (Sylvestre, 1983, Tovar *et al*, 1985, Bohec and Miller, 1988, Kwiatkowshi *et al*, 1988, Mitten *et al.*, 1988). Miniature storage organs like microtubers in potato have great advantage as they can be readily removed from culture flask in a dormant condition and stored *ex vitro*. When planted in soil they behave as normal tubers and produce plants from axillary shoots. If they are produced *in vitro* from virus free stocks, the microtubers provide an ideal method for propagating and distributing virus free planting material to farmers.

In the present study, microrhizomes were induced in both ginger and turmeric. In ginger microrhizomes were produced in 80–100% of cultures when higher sucrose concentrations (9,10 and 12%) were used in culture medium. Microrhizomes from these cultures were larger with a fresh weight of 3-15 g. Lower concentrations of sucrose resulted in smaller microrhizomes, that too after a longer period of incubation.

Microrhizomes resembled the normal rhizomes in all respects, except for their small size. Microrhizomes consisted of 2 to 4 nodes and 1 to 6 buds. The rhizomes germinated *in vitro* as well as *ex vitro*. Microrhizomes on transfer to MS medium without any growth regulators, germinated within 5 to 7 days with 100% success. Microrhizomes had the aromatic flavour of ginger and they resembled the normal

rhizome in anatomical features. Presence of well-developed oil cells, fibres, starch grains and curcumin cells was also observed.

Microrhizomes were directly planted in the field with out hardening. Their field establishment is between 90–100%. Field trials showed that microrhizomes gave commercially viable yields ie 200–500 g of fresh rhizomes per plant with an estimated 12–20 kg of fresh rhizomes per 3 m^2 bed. Microrhizome derived plants had more tillers per plant though the plant height was less compared to normal seed rhizomes. The seed rate requirement per 3 m^2 bed is about 800 g (@ 40 plants per bed) for the normal seed rhizomes and in case of microrhizomes it is about half that amount.

Microrhizomes were induced in turmeric also under *in vitro* conditions, In culture medium with 9–12% sucrose microrhizoms were induced in one month in 80–100% of the cultures The weight of the microrhizomes ranged from 1.0–2.5 g. Microrhizomes of turmeric have typical yellow colour but were small when compared to those produced in ginger. Anatomically microrhizomes resembled the normal rhizomes and a large number of curcumin cells were noticed. These microrhizomes also were directly planted in the field with out hardening and their establishment was 90–100%.

In vitro rhizome formation and their germination in ginger was reported by various workers also (Sakamura *et al.*, 1986; Sakamura and Suga, 1989; Bhat *et al.*, 1994, Babu, 1997). Bhat *et al.* (1994) reported *in vitro* induction of rhizomes in ginger at higher sucrose concentrations (9–12%). Quality analysis of *in vitro* developed rhizomes indicated that they contain the same constituents as the original rhizome but with quantitative differences. The composition of basal medium seems to affect the composition of oil (Sakamura *et al.*, 1986; Sakamura and Suga, 1989; Charlwood *et al.*, 1988).

In vitro rhizome formation and their germination in turmeric was also reported. Reghuarajan (1997) reported formation of microrhizomes in a medium containing ancymidol and Nayak (2000) could induce microrhizomes in turmeric in a medium containing BAP and higher levels of sucrose. In the present study sucrose at higher levels alone could induce microrhizomes in turmeric.

Thus a highly repeatable and efficient protocol for microrhizome induction was developed in ginger and turmeric. This technology has immense potential in disease free planting material production since most of the disease spread in these crops is through infected rhizomes.

GENETIC STABILITY OF CONSERVED MATERIAL

MORPHOLOGICAL CHARACTERISATION

Recovery and growth

In vitro germplasm storage currently uses slow growth techniques rather than cryopreservation. Minimal growth conditions put germplasm integrity at serious risk because of directional genetic change in response to selection. There is a chance of enhanced somaclonal variation, where regeneration is adventitious. Cryopreservation of meristem and shoot tips provides greatest safety and ensure least genetic instability during *in vitro* germplasm conservation (Scowcroft, 1984). Measures such as use of highly organized culture systems and careful monitoring of cultures can be taken care to mnimize the risk of genetic instability. Some regular assessment must be made of plants during culture as well as in field conditions. Ideally, simple monitoring systems need to be developed for each crop species (Ashmore, 1997).

The ultimate aim of any conservation strategy will be retention of morphogenic potential of the stored germplasm and plant regeneration from that material. Under *in vitro* storage conditions the morphological features, which help in

defining a plant in question are likely to be either absent or modified. Therefore alternative methods of characterization and evaluation are particularly available (Withers, 1980).

Variation in culture morphology and growth rate has often been described elsewhere (Blakely and Steward, 1964; Seivert and Hildebrandt, 1965; Davey *et al.*, 1971; Snijman *et al.*, 1977). Careful adjustments of the culture conditions and medium constituents may be required to reveal morphogenic potential during prolonged culture (Asuwa, 1972; Drew, 1979; Koblitz and Schumann, 1976).

In the present study *in vitro* cultures of cardamom, ginger, turmeric, *K. galanga* and *K. rotunda,* after 12 months of storage under minimal growth conditions were multiplied normally in 3–4 weeks of culture. The rooted plantlets were planted out and established with 80–90% survival. The multiplied cultures did not exhibit any visible variations from that of the control.

Planting out and field establishment

Substantial number of micropropagated plants does not survive transfer from *in vitro* conditions to green house or field environments, due to exposure to substantially lower relative humidity, higher light and septic environment. The 'delicate' nature of plants raised *in vitro* results in high mortality if they are transferred directly from the culture vessel to the natural environment. Most plant species grown *in vitro* require a gradual acclimatization and hardening for survival and growth in natural environment (Preece and Sutter, 1991; George, 1996). This is more so in *in vitro* conserved plantlets, which are exposed to higher levels of osmotic stress. Shoots and plants in culture are grown in conditions that provide little physiological stress since a carbon source is provided, reducing the need for photosynthesis. Due to high relative humidity and low light in *in vitro* conditions, the

anatomy and physiology of tissues are different to those of plants grown in green house conditions. In addition, the aseptic environment *in vitro*, reduces the stress of pathogens. These conditions lead to production of plants unsuited for survival in greenhouse and field conditions. The survival of *in vitro* plants depends upon their ability to withstand water loss and carry out photosynthesis. Other factors like development of epicuticular wax, functional stomata, root system and increased photosynthetic ability etc. also influence the acclimatization. In most species, the leaves of *in vitro* developed plants have less epicuticular wax than those grown *in vivo* (Sutter and Langhams, 1982, Sutter, 1984). The failure of leaves to develop normal amount of epicuticular wax seems to be mainly due to high humidity in the *in vitro* environment (Grout and Aston, 1977) and thus these plants tend to loose more water through transpiration when transferred to the external environment (Shackel *et al.*, 1990). Epicuticular wax formation can be induced upon leaves *in vitro* or during hardening, by gradually reducing the relative humidity during leaf formation (Grout, 1975; Wardle et al, 1979).

Deficiency in stomatal structure and their function has been implicated in the severe water loss exhibited by micropropagated plants when planted out. Many researchers have reported that stomata of cultured plants show the characteristic inability to close when first moved out from culture (Brainerd et al, 1981; Brainerd and Fuchigami, 1982, Ziv et al, 1987; Sutter, 1988; Sallanon et al, 1993). These stomata were able to revert to a functional state during the process of acclimatization by gradual reduction in relative humidity (Wetzstein and Sommer, 1983).

The ability of shoots to withstand water loss ultimately depends upon the presence of roots capable of absorbing water from soil. The death of *in vitro* induced roots was observed in many plants during hardening. The possible reason for this may

be due to an intercalary callus phase between roots and shoots and adventitious roots produced *in vitro* are from primary vascular system with poor conductance. Under these circumstances plant survive the acclimatization process only by the development of new roots *ex vitro*. Plants with good root system only can be more easily acclimatized.

Pre-transfer treatments like decreasing the water potential of the medium by addition of non toxic osmoticums such as mannitol and PEG, increasing sucrose content and concentration of agar, reduce the occurrence of hyperhydric shoots, enhance the formation of wax deposits on leaves which increases the survival of *in vitro* plants during hardening.

As the plantlets are progressively acclimatized, the rate of water loss from their leaves decreases and the photosynthetic ability of the plant increases especially in the leaves newly produced after transfer resulting in higher rate of establishment.

In the present study, micropropagated plants were transplanted in a porous soil mixture of vermiculite, sand and garden soil in equal proportions. High humidity was maintained for the initial 20-30 days by keeping them in humid chamber. The humidity was reduced gradually for hardening and establishment. The survival of the transplanted plantlets ranged from 80–100 percent in different plant species studied. Among the species studied cardamom has the lowest establishment rate (80%) and *K. galanga* has the highest (100%). Turmeric and ginger showed 90% establishment. The time taken for hardening ranged from 20 days to 35 days being highest in cardamom and lowest in ginger and turmeric. *K. galanga* and *K. rotunda* took 25–30 days for hardening. The establishment percentage depends upon the size of the plantlets and the number of healthy and younger roots. The bigger the plant size, higher the percentage of establishment during hardening and field transfer.

Field performance of micropropagated plants

Tissue cultured plantlets of cardamom, ginger, turmeric, K. galanga and K. rotunda could be hardened and transferred to field conditions without any difficulty. Field evaluation of tissue cultured plants of cardamom showed that the micropropagated plants performed on par with suckers (Lukose *et al.*, 1993). Sudharshan *et al.* (1997) and Chandrappa *et al.* (1997) have also reported the performance of tissue-cultured plants. Reghunath and Priyadarsan (1992) reported occurrence of somaclonal variation in cardamom derived from aseptic cultures of juvenile shoot primordia during their large-scale production. Somaclonal variation in micropropagated and callus regenerated plants of ginger was reported by Samsudeen (1996) and Babu (1997).

Thus tissue-cultured plants of zingiberaceous taxa could be hardened and acclimatized to the field conditions with relative ease. This may be due to genetic nature of the zingiberaceous crops, which are conventionally propagated through vegetative means. Earlier studies in ginger (Hosoki and Sagawa, 1977; Bhagyalakshmi and Singh, 1988; Samsudeen, 1996; Smith and Hamill, 1996; Babu, 1997), turmeric (Nadgauda *et al*, 1983) *K. galanga* (Vincent *et al.*, 1992; Geetha *et al.*, 1997) and K. rotunda (Geetha *et al.*, 1997) supports this view. It was found that some species like orchids, *Asparagus*, begonia, potato, *Digitalis, Phyllodendron, Cryptanthus* etc. which often produce roots spontaneously *ex vitro* also develop good root system *in vitro* (Thakur, 1975; Debergh and Maene, 1981; Hussey and Stacey, 1981; Chin, 1982; Herrera *et al.*, 1990).

Morphological characterization

The success of any *in vitro* culture technique depends either on the ability to clone the genotypes for production of uniform planting material, or the ability to bring

about variations, which can be exploited in crop improvement programmes. The genetic uniformity of plants multiplied by tissue culture depends on a number of factors, the two most important being the method of multiplication and the genotype. Accumulated information now shows that plants propagated by precocious shoots show no more spontaneous mutation than those propagated by conventional means. The evidence for this comes mainly from the successful large-scale operation in commercial tissue culture laboratories throughout the world. In contrast, plants regenerated from callus or cell suspension cultures may include a varying proportion showing structural or physiological abnormalities depending upon the species, origin and the age of culture (Yeoman, 1986), various other factors like growth regulators (Singh and Harvey, 1975; D'Amato, 1978; Zakhlenyuk and Kunakh, 1987), composition of the culture medium (Bayliss, 1977; Feng and Quyang, 1988), culture conditions (Cerutti, 1985; Jackson and Dale, 1988) and culture method (Wilson *et al.*, 1976) influence somaclonal variation. The reasons for variations in micropropagated plants can be due to the variation that existed in the source plant (pre-existing variation), epigenetic or physiological effects and genetic changes (Swartz, 1991; George, 1996). Extensive studies conducted during the last decade have shown that the cell and callus cultures especially on periodical subculture undergo various morphological and genetic changes i.e., polyploidy, aneuploidy, chromosome breakage, deletions, translocations, gene amplifications, inversions, and mutations (Nagl, 1972, D'Amato, 1985). In addition, there are changes at the molecular and biochemical level including changes in the DNA, rearrangement of genes, somatic crossing over, altered nucleotide methylation, perturbation of DNA replication by altered nucleotide pools, slicing or activation of genes by mutations in associated non

coding regions and transposons (Scowcroft, 1984) and enzymes (Cullis, 1983; Day and Ellis, 1984; Ball and Seilleur, 1986; Brettel *et al*, 1986).

In the present study, tissue cultured plants of zingiberaceous crops except cardamom maintained in pots developed small (0.7-3.3g) rhizomes in the first season. The size of the rhizomes increased over the years when transplanted into pots and only after third year they developed to normal size comparable to those of controls. The micropropagated plants behaved like seedlings of similar zingiberaceous crops in that they have miniature plant type and rhizome size. Thus tissue-cultured plants could not be used directly for commercial planting. They need to grow over the seasons and develop rhizomes of normal size before the rhizomes are used for commercial planting because the size of the rhizome used as a propagule is directly correlated with yield (Aiyadurai, 1966).

Morphological characterization at the second year after field transfer, of micropropagated plants and *in vitro* conserved plants as separate groups and compared with conventionally propagated plants (controls) revealed that the amount of variation with regard to plant height, number of tillers and of leaves per plant, width of rhizome, number of nodes per finger, internodal distance and yield per plant is almost nil in the case of cardamom, turmeric, *K. galanga* and *K. rotunda,* whereas comparatively high in the case of ginger.

Intra-clonal variations are known in many vegetatively propagated plants like potato, sweet potato, bamboo, ginger, turmeric *etc.*, and many promising lines and varieties were identified after clonal selection (Strange and Blackmore, 1989; Rajeevan and Mohanakumaran, 1993; Khader *et al*, 1994; Rattan *et al*, 1994; Huang and Huang, 1995; Villordon and Labonte, 1995, Samsudeen, 1996; Babu, 1997). In the case of ginger this accounts for variations observed in conventionally propagated

plants. These pre-existing variations are also reflected in micropropagated and *in vitro* conserved plants.

Most of the somaclonal variations observed among tissue cultured plants are either genetic or epigenetic in nature. According to Phillips *et al.* (1990), most of the mutational events occurring in tissue culture are directly or indirectly related to alterations in the state of DNA methylation. When DNA is highly methylated, gene activity is suppressed. A decrease in methylation correlates with increased gene activity. An increase or decrease in DNA methylation, might account for quantitative mutations such as those controlled by single recessive genes, for increased transposable element activity for simultaneous changes in quantitative characters and for the mutations caused by chromosome breakage. It is also suggested that increased transposable element activity could be responsible for the genetic changes in callus cell culture, which occur during prolonged incubation. DNA methylation was also found to affect DNA replication (George, 1993).

BIOCHEMICAL CHARACTERIZATION USING ISOZYME PROFILES

Isozyme profiles were used as tool for assessing the variability in many crop plants. Development of isozymes profiles is relatively simple and less expensive but the availability of limited number of enzyme loci, and developmental and season dependent expression of activity is the major disadvantage. IPGRI has used this technique for cocao, taro and *Musa* sp. (Withers, 1985d, Simpson and Withers 1986).

Isozyme profiles were studied in six randomly selected *in vitro* conserved plants of two species namely, cardamom and *K. galanga.* They were compared with the profiles of control mother plants to estimate their genetic uniformity. It was observed that, in all the four isozymes tested, such as Polyphenol Oxidase (PPO), esterase, peroxidase and Superoxide dismutase (SOD) the conserved material showed

similar banding pattern to that of control. Thus the isozyme profiles indicated that the *n vitro* plants of cardamom and *Kaempferia galanga* are genetically stable even after 5 years in slow growth.

Isozyme technology offers a unique opportunity of estimating the degree of genetic stability in cultures stored by methods of slow growth and cryopreservation (Withers and Williams, 1985). Very little attention has been given to isozyme studies in *in vitro* culture and the studies revealed that the cell suspensions and callus cultures tend to differ considerably from the intact plant (Simpson and Withers, 1986). Isozymes are used in species like *Hordeum* spp., wheat, maize, banana and plantains (Jarret and Litz, 1986); *Saccharum* spp., *Phoenix* spp., soybean etc. (Orton, 1980a; b; Larkin, *et al.*, 1984; Zeleneva and Khavkin, 1980; Heinz and Mee, 1971; Finkle, *et al.*, 1983; Stejskal and Griga, 1992).

MOLECULAR CHARACTERIZATION USING RAPD

DNA markers can detect even minute differences in nucleic acid sequences which were otherwise difficult to detect in both morphological and biochemical characterization. In the present study an attempt was made to use RAPD polymorphism as an index for genetic stability of *in vitro* conserved genotypes after 3 years in *in vitro* repository with yearly subculture on minimal growth medium. DNA was isolated and RAPD profiles were developed from six randomly selected replicates of one of the genotypes of cardamom, ginger, *K. galanga* and *K. rotunda.*

Isolation of genomic DNA

Genomic DNA was successfully isolated using, CTAB method from young fresh leaves of 6 different plantlets of *in vitro* conserved materials. The extracted DNA samples were dissolved in TE buffer and its quality was tested on 0.8% agarose gels. The DNA obtained was of reasonably good quality. The extracted samples were

further purified by RNase treatment, followed by phenol:chloroform extraction and precipitation of DNA with absolute alcohol to remove RNA contamination.

Many protocols are available for isolation of DNA from plant tissues (Draper and Scott, 1988; Ausubel *et al*, 1995). In the present study, genomic DNA was successfully isolated from leaves of cardamom, ginger and *Kaempferia* species using modified CTAB method described by Ausubel *et al* (1995). The DNA when visualized on 0.8 percent agarose gel gave a clear band of high molecular weight DNA. The yield of DNA ranged from 75–150 µg/ml in cardamom, 50–200 µg/ml in ginger and 100- 250 µg/ml in *Kaempferia* species indicating good yield.

Thus efficient protocol for isolation of high molecular weight DNA from leaf tissues was standardized in cardamom, ginger and *Kaempferia* species.

The CTAB method of DNA isolation is widely used in plants because of its versatility. The total genomic DNA has been isolated from many genera of monocotyledons and dicotyledons adopting these techniques and with certain modifications (Murray and Thompson 1980; Rogers and Benedicht, 1985; Guillemaut and Drouard, 1992; Rether *et al.*, 1993; de la Cruz *et al.*, 1997; Porebski, *et al.*, 1997; Barnwell *et al.*, 1998)

Optimization of Polymerase Chain Reaction (PCR)

In the present study PCR reaction components and conditions were standardized for optimum amplification. Amplification reactions were performed with 2 µml of 10 x buffer (Boehringer Mannheim), 125 µM dNTPs picomoles/µl of primer, enzyme (*Taq* polymerase) concentration of 1.0 U/µl, 0.5 mM $MgCl_2$ and 25 ng/µl of template DNA.

The PCR cycle and the temperature regimes were standardized and DNA amplification was performed for 40 cycles as follows: 1st cycle of 2 min at 94°C

followed by 1 min at 37°C, 2 min at 72°C; followed by 38 cycles each of 1 min at 94°C, 1 min at 37°C 2 min at 72°C; followed by final cycle of 1 min at 94°C, 1 min at 37°C and one final extension cycle of 15 min 72°C.

Primer screening

In cardamom, genomic DNA of CL-37 and APG 50 were used as template for primer screening. Twenty OPERON primers were tested and only three of the primers namely OPA 12, OPC 19, OPE 02 were polymorphic between the two genotypes.

In ginger, two cultivars namely, Maran and Varada were used for primer screening. Eleven OPERON primers were tested and 8 of them namely, OPA 12, OPB 2, OPC 4, OPC 5, OPC 07, OPD 01, OPE 02 and OPF 01 gave good amplification and were polymorphic between the genotypes.

In *Kaempferia* spp. six primers were tested and 3 of them namely, OPA 06, OPA 08 and OPE 01 were highly polymorphic between the two species.

RAPD profiles of cardamom *in vitro* conserved lines

The primers which were polymorphic between the genotypes and genera were used to develop RAPD profiles in *in vitro* conserved lines. No polymorphism was detected between the conserved lines in any of the primers tested. Thus in general the patterns did not indicate any genetic variability within the replicates tested in *in vitro* conserved lines of cardamom, ginger and *Kaempferia* species.

There are also reports on the use of RAPD markers to detect somaclonal variation in different species such as *Lolium* and *Festuca* (Valles *et al.,* 1993); wheat (Brown *et al.*, 1993); *Picea* (Isabel *et al.,* 1993); sugarbeet (Munthali *et al.*, 1996); ginger (Rout *et al.,* 1998), *Allium* sp. (Al Zahim *et al.*, 1999), *Lilium* sp. (Varshney *et al.*, 2001), turmeric (Salvi *et al.*, 2001) etc.

But reports on use of molecular markers for estimating the genetic stability of *in vitro* conserved genotypes are limited. The reports available are in crop species such as sugarcane (Chaudhary and Vasil, 1993); navel orange; (Kobayashi *et al.*, 1994); potato (Harding, 1991, Harding and Benson, 2000, Harding and Benson, 2001) and cassava (Angel *et al.*, 1996).

Thus the present study is the first report in using RAPD profiles for estimating the genetic stability of *in vitro* conserved genotypes of spice crops. The study indicated genetic uniformity within the replicates tested in *in vitro* conserved lines of cardamom, ginger and *Kaempferia* species. RAPD marker information can be obtained very quickly and that the amount of starting material required is very small.

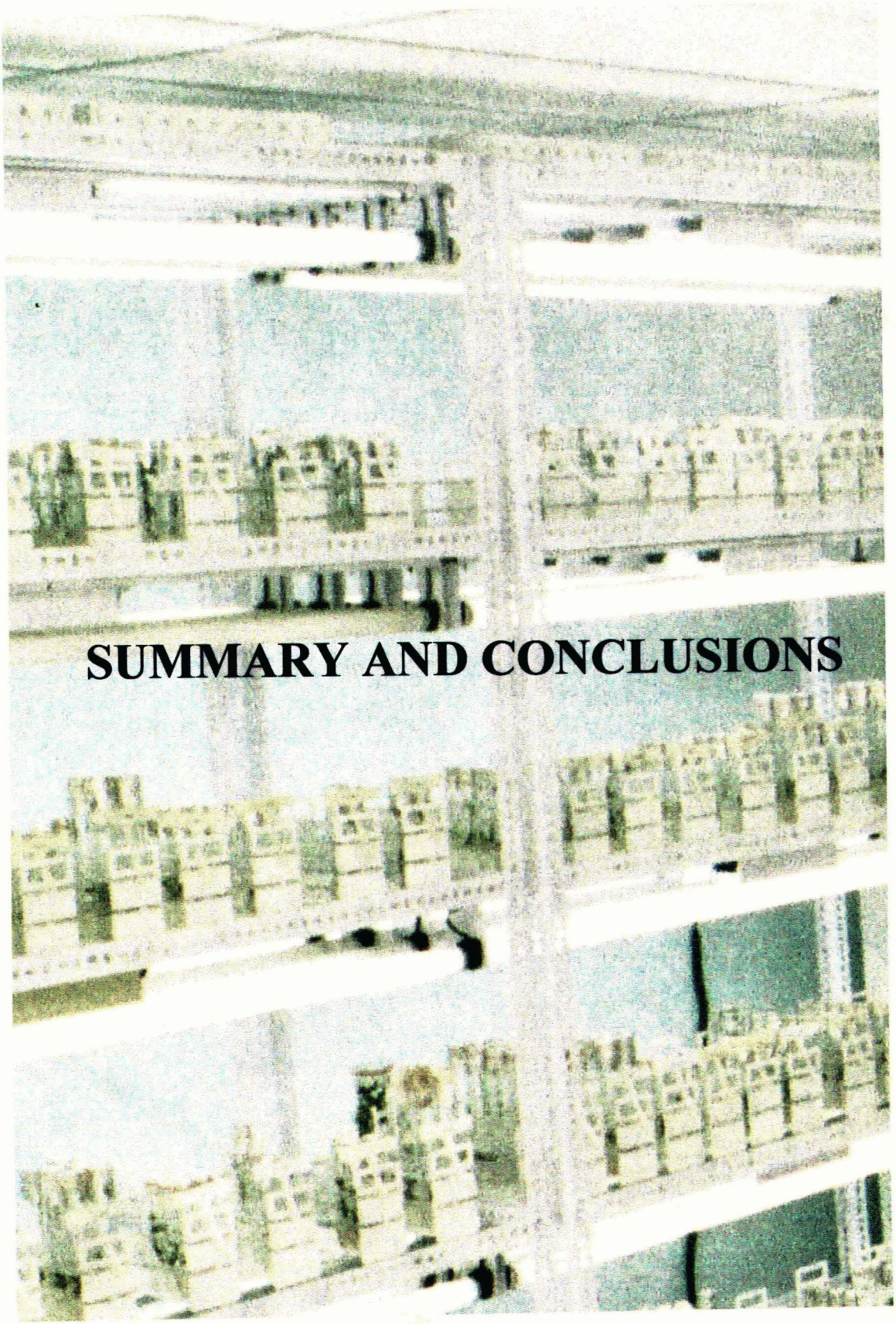
DNA BANK

High molecular weight DNA was isolated from 50 lines each of cardamom, ginger, and turmeric and 20 lines each of *K. galanga* and *K. rotunda*. DNA was dissolved in 500 µl TE buffer and stored at –20°C. The quality of the stored DNA samples was analyzed. DNA was of good quality as seen in agarose gel electrophoresis even after 2 years of storage. The concentration of DNA in the samples ranged from 100–200 ng/µl.

There are a few reports on establishment of DNA bank. DNA is the basis of all genetic information contained in a plant and hence DNA storage can be used for germplasm conservation. This option has to be considered where no other alternatives are available. Cryostorage of DNA and DNA rich materials is currently being used in 40 institutions representing 25 nations that have expressed interest in DNA bank (Adams, 1988, 1990; Adams and Adams, 1991; Giannasi, 1991; Mattich *et al.*, 1991; Adams *et al.*, 1994; Adams, 1997).

The conserved DNA can be used for studying the molecular phylogenetics and systematics of extant and extinct taxa, production of previously characterized secondary compounds in transgenic cell cultures, production of transgenic plants using genes from gene families, *in vitro* expression and study of enzyme structure and function and genomic probes for research laboratories (Adams, 1997).

Thus the technologies developed during the present investigation can be effectively used for *in vitro* and cryoconservation of genetic recourses of zingeberaceous crops such as cardamom, ginger, turmeric and *Kaempferia* spp. These techniques, especially of synthetic seeds and *in vitro* microrhizomes helps in the disease free exchange of genotypes and their planting material. These technologies form an integral and important part of overall conservation strategy in genetic recourses management of these crop species.



SUMMARY AND CONCLUSIONS

Zingiberaceae is a monocotyledonous family of perennial rhizomatous herbs, consisting of about 50 genera and 1400 species. It is an important group with plants of considerable economic potential, such as *Elettaria cardamomum* Maton (cardamom), *Zingiber officinale* Rosc. (ginger), *Curcuma longa* L. (turmeric), *Kaempferia galanga* L. and *K. rotunda* L. These plants have been used in ayurvedic and other native systems of medicine from time immemorial and are also widely used as spice.

The germplasm of these economically important crops are conserved in clonal field repositories. All these crops are threatened by serious viral and soil-borne diseases that can wipe out the germplasm completely. There is also a high risk of mixing up of germplasm by the left over rhizome pieces due to planting in the same area year after year. Hence it is important to formulate *in vitro* conservation strategies to complement the field genebank and an insurance against natural calamities.

The present investigation was undertaken to develop an efficient *in vitro* conservation strategy as a safe alternative to the field genebank and also for germplasm exchange in zingiberaceous plants such as cardamom (clone-37), Ginger (Maran), turmeric (Sudarsana), *K. galanga* and *K. rotunda*. The study includes standardization of protocols for micropropagation; *in vitro* conservation using minimal growth and cryopreservation techniques; *in vitro* production of microrhizomes and synthetic seeds as a propagule for conservation and exchange of germplasm and assessment of genetic fidelity of the conserved material using morphological, biochemical and molecular approaches.

MICROPROPAGATION AND *IN VITRO* CONSERVATION

Micropropagation

Experiments were carried to standardize micropropagation protocols in the species studied, as it forms the backbone of any *in vitro* conservation scheme. Vegetative buds from rhizome were used as explants in all the plant species studied. Cultures were initiated on MS medium supplemented with 0.5 mgl^{-1} Kin using vegetative buds and apical meristem. Fifty to seventy per cent of cultures could be established. The aseptic cultures were cultured in MS medium with different combinations of growth regulators (BAP, Kin, NAA and IBA at 0.5 and 1.0 mgl^{-1}) to standardize optimum conditions for clonal propagation. Multiple shoots as well as roots were produced in all the growth regulator concentrations tried.

MS medium supplemented with 1.0 mgl^{-1} BAP and 0.5 mgl^{-1} NAA gave the best response (90%) in all the species. In cardamom, the number of multiple shoots ranged from 1.3–5.8 and the number of roots ranged from 1.5–6.2. In ginger, the same medium gave the best response with an average of 7.5 multiple shoots and 9.1 roots in 90% of the cultures tested. In turmeric also, MS medium with 0.5 mgl^{-1} NAA and 1.0 mgl^{-1} BAP gave best response with an average of 7.2 shoots and 5.8 roots. The same medium was most suitable for micropropagation of both *K. galanga* and *K. rotunda.* In this medium *K. galanga* produced 8–10 shoots and *K. rotunda* produced 6–7 shoots. Good rooting of 1:10 and 1:6 was achieved in the same medium in these species respectively.

The plantlets from the vegetative bud cultures were healthy, about 10–12 cm tall, with an average of 6–10 roots. These plantlets were hardened in humid

chamber for 20–30 days with 80–100% establishment. The hardened plantlets were successfully transplanted to pots/field.

Slow (minimal) growth storage

Parameters such as low temperatures, various types of culture tube closures, half and full strength MS basal medium and various concentrations of carbon source (sucrose) and osmoticum (mannitol) were tried in various combinations to induce slow growth. Importance was given for the extension of subculture interval up to12 months with about 80% survival.

Cotton plugs allowed better gaseous exchange resulting in faster growth of the plantlets. But quicker moisture loss coupled with the faster growth of the plant resulted in early exhaustion of the medium leading to drying up of cultures. Use of screw-capped, aluminium foil-covered or polypropylene capped culture tubes, minimized the moisture loss and resulted in availability of nutrients for a longer period.

An incubation temperature of 22 ± 2^0C was better for *in vitro* storage of healthy cultures of cardamom, ginger, turmeric, *K. galanga* and *K. rotunda*.

In general, full strength of the MS basal medium with 20–30 gl^{-1} sucrose supported higher growth rate and hence was not suitable for *in vitro* storage. When the concentration of the basal medium was reduced to half strength, the cultures could be stored up to a longer period. Reduction of basal medium concentration to half and sucrose to 10–15gl^{-1} and addition of mannitol (10–15 gl^{-1}) induced slow growth in the cultures. In cardamom, ginger, turmeric, *K. galanga* and *K. rotunda*, minimal growth could be induced and the cultures could be stored with 80–90% survival up to 12 months without subculture in half strength MS basal medium supplemented with 10 or 15 gl^{-1} each of sucrose and mannitol in sealed culture tubes at 22 ± 2°C.

These *in vitro* cultures after 12 months of storage, multiplied normally in 3–4 weeks of culture. The rooted plantlets were transferred to pots and established with 80–90% survival.

The present study indicated that the entire genus studied responded more or less similarly to the set of conditions and medium. The micropropagation as well as minimal growth protocols standardized are applicable to all the genera studied.

Cryopreservation

Since all the zingiberaceous crops tried responded more or less similarly in *in vitro* conservation experiments, cryopreservation experiments were conducted only in two crops namely, ginger and cardamom with an objective to supplement the *in vitro* conservation programme. Attempts were made to standardize cryopreservation protocols for long-term conservation of shoot buds in ginger and pollen in cardamom.

Cryopreservation of ginger shoot buds

Two methods namely, cryoprotective dehydration and encapsulation dehydration were tried in ginger shoot buds. In cryoprotetive dehydration ginger shoot tips could be successfully cryopreserved with limited success (40% of the cultures) after pre-culture on 0.75 M sucrose for 3 days and desiccated for 1 h on laminar airflow. Plantlets could be regenerated from 20% of these viable cultures after cryopreservation.

An alternate strategy of cryopreservation of encapsulated shoot tips (synthetic seeds) of ginger was also tried to study the effect of encapsulation on their viability after cryopreservation. Encapsulated shoot buds of ginger could be successfully cryopreserved with better success (50% of the cultures) after pre-culture on 0.75 M sucrose for 3 days and desiccated for 4 h on laminar airflow. Plantlets could be regenerated from 30% of the cultures after cryopreservation. Use of encapsulated

shoot buds or synthetic seeds increased the post-thaw recovery and the development of plants by 10%. However they need a longer period (4 h) of desiccation in laminar airflow compared to naked shoot tips, which required only 1 h desiccation. The non-encapsulated shoot buds lost their viability when cryopreserved after 2 h desiccation.

Cryopreservation of cardamom pollen

Cardamom is cross-pollinated and among zingiberaceous species, conservation of pollen assumes importance only in cardamom. Desiccation coupled with DMSO treatment could increase the viability of fresh pollen as well as cryopreserved samples. Pollen desiccated for 10 min and treated with 10% DMSO for 10 min gave the maximum viability in control (60–72%) and after cryopreservation (58–75%). Increase of treatment time in DMSO did not increase pollen germination in control as well as in cryopreserved samples. This is the first report of successful cryopreservation of cardamom pollen.

SYNTHETIC SEEDS

In vitro regenerated shoot buds of cardamom, ginger and turmeric, were successfully encapsulated in 3% calcium alginate matrix. The beads were sufficiently strong for easy handling. Encapsulated synthetic seeds were stored up to 6 to 9 months in sterile environment at 22 ± 2^0C. When cultured on MS medium supplemented with 1.0 mgl^{-1} BAP and 0.5 mgl^{-1}NAA, maximum germination (80%) of synseeds was observed after 2 weeks. The plants derived from these encapsulated buds were healthy and developed into normal plantlets. Synthetic seeds form an ideal source of material for disease free germplasm conservation and exchange in zingeberaceous crops, especially in ginger, where most of the diseases are spread through infected seed rhizomes.

IN VITRO MICRORHIZOME FORMATION

Microrhizomes were induced in *in vitro* cultures of ginger and turmeric by using high concentrations of sucrose and mannitol. In ginger, microrhizomes were produced in 80–100% of cultures when sucrose concentrations were increased to 9, 10 and 12%. Microrhizomes from these cultures were larger with a fresh weight of 3–15 g. Microrhizomes resembled the normal rhizomes in all respects, except for their small size. Microrhizomes consisted of 2 to 4 nodes and 1 to 6 buds. The microrhizomes on transfer to MS medium without any growth regulators, germinated within 5 to 7 days with 100% success. The microrhizomes had the aromatic flavour of ginger and they resembled the normal rhizome in anatomical features. Presence of well-developed oil cells, fibres, starch grains and curcumin cells was also observed. Microrhizomes could be directly planted in the field without hardening and established with 90–100% success. Field trials showed that the microrhizomes gave commercially viable yields ie, 200–500 g of fresh rhizomes per plant with an estimated 12–20 kg of fresh rhizomes per 3 m^2 bed. The microrhizomes had more tillers per plant though the plant height was less compared to normal seed rhizomes. The seed rate requirement per 3 m^2 bed is about 800 g (@ 40 plants per bed) for the normal seed rhizomes and in case of microrhizomes it is about half that amount.

Microrhizomes were induced in turmeric also in MS medium with sucrose at 12%. Microrhizomes were induced in 80–100% of the cultures in 1 month. Fresh weight of the microrhizomes ranged from 1.0–2.5 g. The microrhizomes were small when compared to that produced in ginger, but had the typical yellow colour of normal rhizomes. Anatomically the microrhizomes resembled the normal rhizomes and a large number of curcumin cells were noticed. These microrhizomes could be directly planted in the field and established with 90–100% survival.

GENETIC STABILITY ANALYSIS OF CONSERVED MATERIAL

Genetic fidelity is an important factor in any conservation programme. In the present study the genetic fidelity of conserved genotypes was assessed by morphological, biochemical and molecular characterization.

Morphological characterization

The conserved plants were observed for differences and deficiencies during recovery and growth after *in vitro* and cryoconservation, during hardening and planting out and their performance in pots and field. These *in vitro* multiplied cultures did not exhibit any deficiency symptoms or deformities and were similar to the control in their appearance and growth. There was no reduction in establishment percentage after 3 years of *in vitro* conservation when compared with normal micropropagated plantlets. All the plantlets were established with 80–90% success. The morphological features of these plantlets in the first year after hardening showed no deformities or deficiency symptoms and they exhibited apparent morphological similarities with the parents. The rhizomes of tissue-cultured plantlets of ginger, turmeric and *Kaempferia* spp. were very small (0.7–3.3 g) to harvest after the first season. The micropropagated plantlets behaved like seedlings of zingiberaceous crops except when microrhizomes were used as planting material. The size of the rhizome increased over the years and developed into normal size comparable to that of mother plants only after the third year. In cardamom, the tissue cultured plants performed similar to those of seedlings and hence can be directly used for commercial planting within 6 months of hardening. But in ginger, turmeric and *Kaempferia* spp. where the commercially useful part is the rhizome, tissue cultured plantlets cannot be directly used for commercial cultivation and needs at least three crop seasons in the nursery or

field before they develop rhizomes of normal size suitable for commercial planting. Only microrhizomes can directly be used as planting material in commercial scale.

Biochemical characterization using isozyme profiles

Isozyme profiles were studied in six randomly selected conserved plants of two species namely, cardamom and *K. galanga.* They were compared with the profiles of control mother plants to estimate their genetic uniformity. It was observed that, in all the four isozymes tested, such as polyphenol oxidase (PPO), esterase, peroxidase and superoxide dismutase (SOD), the conserved material showed similar banding pattern to that of control. Thus in general the isozyme profiles indicated that the *in vitro* plants of cardamom and *K. galanga* are genetically stable even after 3–5 years in slow growth.

Molecular characterization using RAPD

DNA markers can detect even minute differences in nucleic acid sequences which are otherwise difficult to detect by morphological and biochemical characterization. In the present study an attempt was made to use RAPD polymorphism as an index for genetic stability of *in vitro* conserved genotypes after 3 years in *in vitro* repository with yearly subculture on minimal growth medium. DNA was isolated and RAPD profiles were developed from six randomly selected replicates of one of the genotypes of cardamom, ginger, *K. galanga* and *K. rotunda.*

Genomic DNA was successfully isolated from leaves of cardamom, ginger and *Kaempferia* species using modified CTAB method. The DNA when visualized on 1% agarose gel gave a clear band of high molecular weight DNA. The yield of DNA ranged from 75–150 µg/ml in cardamom, 50–200 µg/ml in ginger and 100–250 µg/ml in *Kaempferia* species indicating good yield. Thus an efficient protocol for

isolation of high molecular weight DNA from leaf tissues was standardized in cardamom, ginger and *Kaempferia* species.

Polymerase Chain Reaction (PCR) components and conditions were standardized for optimum amplification. Amplification reactions were performed with 2 µml of 10 x buffer, 125 µM dNTPs, 10 picomoles/µl of primer, enzyme (*Taq* polymerase) concentration of 1.0 U/µl, 0.5 mM $MgCl_2$ and 25 ng/µl of template DNA. The PCR cycle and the temperature regimes were standardized and DNA amplification was performed for 40 cycles as follows: 1st cycle of 2 min at 94°C followed by 1 min at 37°C, 2 min at 72°C; followed by 38 cycles each of 1 min at 94°C, 1 min at 37°C 2 min at 72°C; followed by final cycle of 1 min at 94°C, 1 min at 37°C and one final extension cycle of 15 min 72°C.

Various primers were tried to find out polymorphism between genotypes in different species. In cardamom, genomic DNA of Cl-37 and APG 50 were used as template. Twenty OPERON primers were tested and only three of the primers namely OPA 12, OPC 19, OPE 02 were polymorphic between genotypes viz. Cl-37 and APG 50.

In ginger, two cultivars namely, Maran and Varada were used for primer screening. Eleven OPERON primers were tested and 8 of them namely, OPA 12, OPB 2, OPC 4, OPC 5, OPC 07, OPD 01, OPE 02 and OPF 01 gave good amplification and were polymorphic between the genotypes.

In *Kaempferia* spp., six primers were tested and 3 of them namely, OPA 06, OPA 08 and OPE 01 were polymorphic between the two species.

RAPD profiles of* in vitro *conserved lines

The primers which were polymorphic between the genotypes were used to develop RAPD profiles in *in vitro* conserved lines. No polymorphism was detected

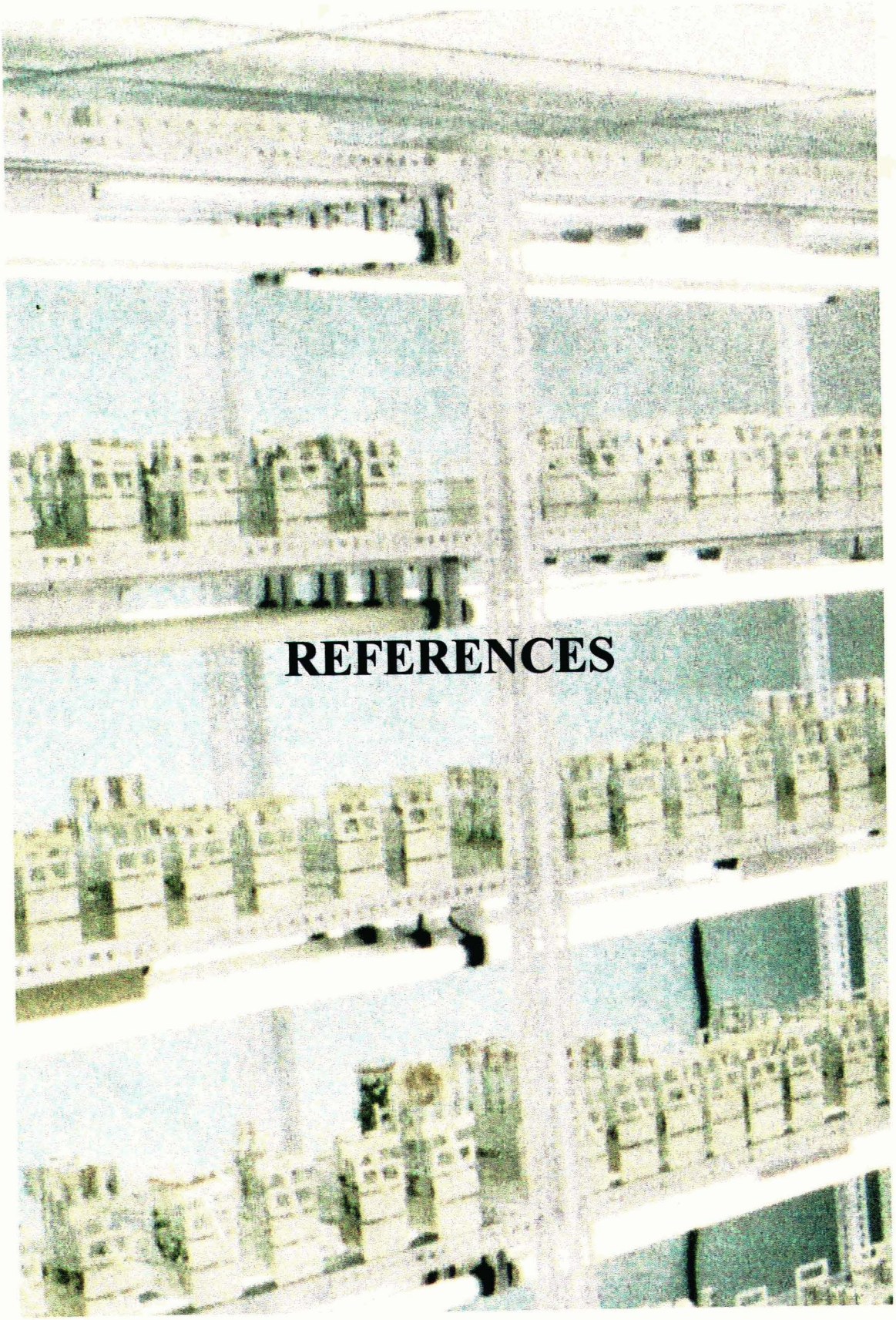
between the conserved lines in any of the primers tested. Thus in general the patterns did not indicate any genetic variability within the replicates tested in *in vitro* conserved lines of cardamom, ginger and *Kaempferia* species.

Thus the present study is the first report in using RAPD profiles for estimating the genetic stability of *in vitro* conserved genotypes of spice crops. The study indicated genetic uniformity within the replicates tested in *in vitro* conserved lines of cardamom, ginger and *Kaempferia* species.

DNA BANK

High molecular weight DNA was isolated from 50 lines each of cardamom, ginger, and turmeric and 20 lines each of *K. galanga* and *K. rotunda*. DNA was dissolved in 500 µl TE buffer and stored at $-20^{O}C$. The quality of the stored DNA samples was analyzed. DNA was of good quality as seen in agarose gel electrophoresis even after 2 years of storage. The concentration of DNA in the samples ranged from 100–200 ng/µl.

Thus, many technologies were developed for *in vitro* and cryoconservation of genetic recourses of zingiberaceous crops and disease free exchange of genotypes and their planting material. These technologies form an integral and important part of overall conservation strategy in genetic resources management of these crop species.



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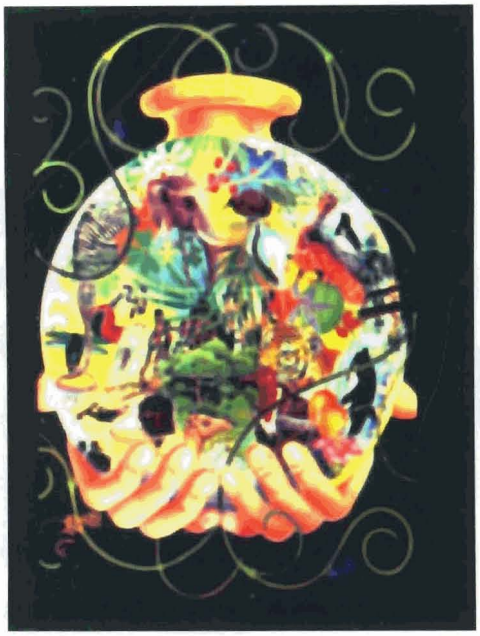

PURNA-GHATA

A symbolic representation of the earth, Purna-Ghata,

In ancient Indian art, epitomizes abundance and procreation.

The human hands indicate mankind's vital role in safeguarding natural wealth,

While the endless green vine represents life

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