In vitro conservation of cardamom (*Elettaria cardamomum* Maton.) germplasm

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Summary

In vitro conservation of cardamom (*Elettaria cardamomum* Maton.) germplasm

Cardamom, *Elettaria cardamomum* Maton. is considered to be the queen of spices. As it is vegetatively propagated, cardamom germplasm is normally conserved in clonal field repositories. *In vitro* conservation would allow the medium-term conservation of cardamom germplasm and be a safe addition to the field genebank. Various parameters such as low temperature, reduction in carbon source and addition of mannitol, reduction in nutrient concentration and minimizing evaporation loss were tested for their efficiency in inducing slow growth in cardamom cultures in order to increase subculture intervals. The present study indicated that of the various parameters tested, the best method of inducing slow growth was half-strength MS medium without growth regulators, supplemented with 15 g/L each of sucrose and mannitol in screw-capped culture tubes and incubation at 22±2°C with a photoperiod of 12 h light/12 h dark and a light intensity of 2500 lux. Cultures maintained under these conditions can be conserved for a period of one year without subculture. Conserved plantlets multiplied normally and were planted in soil with an 85% success rate. Plants established in soil had morphological characters similar to the mother plants, thus indicating genetic stability.

Key words: Cardamom, *Elettaria cardamomum*, *in vitro* conservation, slow growth

Résumé

Conservation *in vitro* du germoplasme de cardamome (*Elettaria cardamomum* Maton.)

La cardamome (*Elettaria cardamomum* Maton.) est considérée comme la reine des épices. Comme il s'agit d'une plante à multiplication végétative, le germoplasme de cardamome est normalement conservé dans des collections en champ. La conservation *in vitro* devrait permettre la conservation à moyen terme du germoplasme de cardamome. Elle représente une mesure de protection supplémentaire, s'ajoutant aux collections en champ. On a évalué l'efficacité de différents paramètres, tels qu'une température basse, la réduction de la source carbonée et l'addition de mannitol, la réduction de la concentration en nutriments et la réduction de l'évapora-tion, dans l'induction d'une croissance ralentie des cultures de cardamome, afin d'allonger ainsi les intervalles entre subcultures. Cette étude montre que parmi les divers paramètres testés, la meilleure méthode pour induire une croissance lente consiste à utiliser un milieu MS semi-solide sans régulateurs de croissance, additionné de 15 g/l de saccharose et de mannitol dans des tubes avec bouchons à vis et une température d'incubation de 22 ± 2°C avec une photopériode de 12 h de lumière/12 h d'obscurité et une intensité lumineuse de 2500 lux. Les cultures maintenues dans ces conditions peuvent être conservées pendant un an sans subculture. Les plantules conservées se sont multipliées normalement et ont été plantées en terre avec un taux de succès de 85 %. Les plantes qui se sont développées en terre présentaient des caractéristiques morpho-logiques similaires à celles des plantes mères, indiquant donc qu'elles étaient génétiquement stables.

Resumen

Conservación *in vitro* de germoplasma de cardamomo (*Elettaria cardamomum* Maton.)

El cardamomo, *Elettaria cardamomum* Maton., se considera la reina de las especias. Como se propaga vegetativamente, el germoplasma de cardamomo se conserva normalmente en depósitos clónicos de campo. La conservación *in vitro* permitiría conservar el germoplasma de cardamomo a plazo medio y sería una valiosa adquisición para el banco de genes en el campo. Se pusieron a prueba varios parámetros como baja temperatura, reducción en la fuente de carbono y adición de manitol, reducción de la concentración de nutrientes y minimización de la pérdida por evaporación, para observar sus efectos en el retraso del crecimiento de los cultivos de cardamomo aumentando así los intervalos de subcultivo. Este estudio indicó que entre los varios parámetros ensayados, el mejor método para retardar el crecimiento era el cultivo de fuerza media sin reguladores de crecimiento, añadiendo 15 g/L de sucrosa y otro tanto de manitol en tubos de cultivo con tapón de rosca e incubación a 22±2°C con un fotoperíodo de 12 horas de luz y 12 de oscuridad y una intensidad luminosa de 2500 lux. Los cultivos mantenidos en estas condiciones pueden conservarse durante un año sin subcultivo. Los embriones de planta conservados se multiplicaron normalmente y se plantaron en tierra con un 85% de éxitos. Las plantas establecidas en tierra tenían caracteres morfológicos análogos a los de la planta madre, lo que indica estabilidad genética.

Introduction

Cardamom, *Elettaria cardamomum* Maton., is a major oriental spice which is known as the queen of spices. India is the major producer and exporter of this spice and an estimated 5000 tonnes of cardamom a year are produced in India in an area of 100 000 hectares (1 lakh) (Edison *et al.* 1991). Evergreen forests in the Western Ghats of south India and Sri Lanka, where cardamom is still to be found in its wild state, are considered to be the centre of diversity for cardamom (Purseglove *et al.* 1981). The Indian Institute of Spices Research (IISR), Calicut, India, is the national repository of cardamom germplasm and has over 265 accessions of cardamom and related species.

As cardamom is a vegetatively propagated crop, germplasm is presently maintained in clonal field repositories. Maintenance of cardamom germplasm in the field is labour-intensive and as it is a perennial crop, accessions are exposed to hazards such as outbreak of pests, diseases and drought. Field repositories of cardamom are also vulnerable to diseases such as katte (caused by katte

virus) and rhizome rot (*Pythium vexans*) which result in considerable loss. Therefore, conserving germplasm *in vitro*, in addition to field genebanks, would enhance the safety of germplasm collections.

In vitro conservation of germplasm has been reported for various crop species (Banerjee 1989; Dekkers *et al.* 1991; Engelman 1991; Agrawal *et al.* 1992; Bertrand-Desbrunais *et al.* 1992; Tay and Lin 1992; Bessembinder *et al.* 1993; Siddiqui *et al.* 1996). In addition, micropropagation protocols have been reported for cardamom (Nadgauda *et al.* 1983; Vatsya *et al.* 1987; Kumar *et al.* 1985), *in vitro* conservation of valuable genetic resources of spices have been reported by IISR (Geetha *et al.* 1995; Nirmal Babu *et al.* 1996), and the cryopreservation of cardamom seeds in the vapour phase of liquid nitrogen has been reported by Chaudhary and Chandel (1995). This paper gives the results of experiments to induce slow growth for *in vitro* conservation of cardamom germplasm.

Materials and methods

Culture initiation and multiplication

Rhizome pieces of cardamom with sprouting buds were collected and washed in running tap water. They were then surface-sterilized with 0.1% $HgCl_2$ solution for 7-10 minutes and rinsed in three to five changes of sterile distilled water. The outer scale leaves were removed aseptically and the shoot primordium was dissected out. Uniform-sized explants with shoot primordium were inoculated on MS (Murashige and Skoog 1962) basal medium with 3% sucrose, 0.5 mg/L kinetin and solidified with 0.7% agar. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C for 20 minutes. The cultures were incubated at 22±2°C with a 12 h light/12 h dark photoperiod and a light intensity of 2500 lux. Within 10 days the shoots started to emerge and were transferred to a fresh medium of the same composition but with the addition of 1.0 mg/L naphthalene acetic acid (NAA) for further multiplication and rooting. Rooted plantlets of approximately 2 cm were used for *in vitro* storage experiments.

In vitro *storage*

With the objective of finding suitable conditions for inducing minimal growth and substantially increasing subculture intervals, various parameters such as low temperature, reduction of carbon source, reduction of basal medium concentration, minimizing evaporation rate using closed culture vessels, use of mannitol, etc. were tried as given below.

i) Basal medium (MS): full and half strength
ii) Carbon source (sucrose)*: 30, 25, 20, 15, 10 and 5 g/L
iii) Osmoticum (mannitol)**: 5, 10 and 15 g/L
iv) Vessel closure: cotton plug, screw cap and polypropylene cap
v) Temperatures***: 5°C, 10°C, 15°C and 22±2°C

* Except for 30 g/L all other concentrations were tried in combination with mannitol.

** Tried in combination with sucrose.

*** For 5°C, 10°C and 15°C, BOD incubators were used.

There were 76 treatments and each treatment had 20 replicates. MS basal medium without growth regulators was used in this experiment. The amount of culture medium used per culture tube was 25 ml. Uniform-sized plantlets (approximately 2 cm) were inoculated in all the treatments tested. Data were recorded on the growth rate (increase in length over initial length and average number of multiple shoots produced), percentage survival of cultures and the period of storage. General health of the plantlets, symptoms of drying, yellowing and necrosis, exhaustion of medium, ability to multiply after storage, etc. were also monitored.

Planting out

The stored plantlets were transferred back to a multiplication medium (MS + 1.0 mg/L NAA) to stimulate growth before transplanting to soil. This took place when the plantlets reached normal size (6-8 cm long) and had a good rooting system. The soil mixture was garden soil, sand and perlite in equal proportions. For the first 20-25 days the transferred plantlets were kept in a humid chamber.

Results and discussion

Culture initiation and multiplication

Of the explants taken from the field-grown plants, 60% could be established *in vitro*. The shoot primordia turned green and developed into shoots within 10 days after inoculation on MS basal medium supplemented with 0.5 mg/L kinetin.

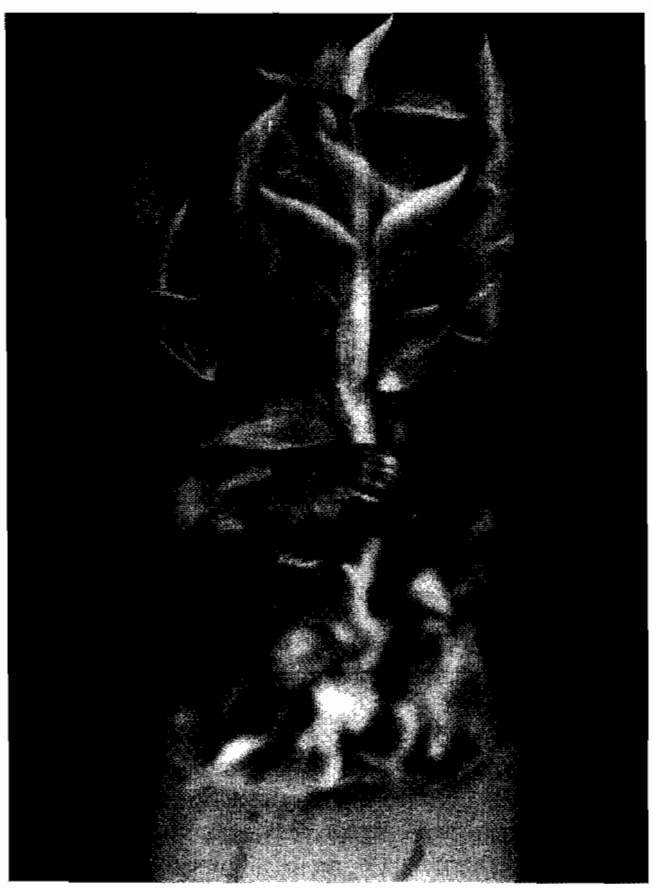


Fig. 1. Induction of multiple shoots in MS medium supplemented with 1.0 mg/L NAA.

At this stage, the cultures were transferred to a MS basal medium supplemented with 1.0 mg/L NAA which resulted in the production of multiple shoots (1:8) and good rooting (Fig. 1). In 2-3 months a good number of uniform-sized plantlets had been obtained for storage experiments.

In vitro *storage*

In total, four temperature regimes, three types of enclosures for culture tubes, the use of full-strength and half-strength basal medium, and sucrose alone or in combination with mannitol, etc. were tested for their efficiency in inducing slow growth and increasing subculture intervals in cardamom cultures. The results of the experiments are given in Tables 1, 2 and 3.

Effect of temperature. Of the four temperatures tested, only 22±2°C was found to be conducive for the growth of cardamom cultures. At 5°C and 10°C all the cultures turned white around the 15th day and lost viability within 20 days. Although cultures stored at 15°C could be stored for up to one year they exhibited an etiolated unhealthy nature (Fig. 2), therefore, this temperature was not suitable for storage (Table 1).

These results indicate that low temperatures are detrimental to cardamom cultures. There are many reports of successful storage at reduced temperature of 1-4°C for plant species such as apple, pear, plum, cherry, etc. (Lundergan and Janick 1979), although chilling sensitivity has been reported for some fruit trees (Wilkins *et al.* 1988). However, tropical crops such as banana (Banerjee 1989), coffee (Bertrand-Desbrunais *et al.* 1992), tuber crops and banana (NBPGR 1996), ginger (Dekkers *et al.* 1991; NBPGR 1996), and spices (Geetha *et al.* 1995; Nirmal Babu *et al.* 1996) respond better with *in vitro* storage at temperature ranges of between 15°C and 30°C. The present study indicated that a temperature of 22±2°C is adequate for *in vitro* storage of cardamom germplasm. This may certainly be due to the tropical nature of the crop.

Table 1. Effect of temperature on survival of cardamom tissue cultures

Temperature (°C)	Survival period (days)†	Growth rate
5	15 (100) - 20 (0)	No growth
10	15 (100) - 20 (0)	No growth
15	90 (100) - 360 (80)	Unhealthy growth
22±2	120 (100) - 360 (85)	Good growth

† Figures in parentheses indicate survival percentage of cultures.

Effect of medium constituents. The effect of various media components in inducing slow growth in cardamom cultures is given in Table 2. MS medium, when used at half strength, favoured the normal growth and development of plantlets during storage. With a full-strength culture medium the growth rate was much higher and hence not ideal for inducing slow growth.

High concentration of sucrose resulted in the faster growth of cultures, leading to the exhaustion of the culture medium. In a culture medium with 30 g/L sucrose, the cultures grew faster and filled the culture vessel within 120 days which resulted in a rapid drying up of cultures. When the concentration of sucrose was reduced to 20 g/L and the nutrient concentration to half, cultures could be maintained for the much longer period of 200 days with an 80% survival rate. However, the addition of mannitol and the further reduction of sucrose to lower levels induced slow growth and subsequently 80% of cultures could be maintained for the much longer period of between 200 to 360 days.

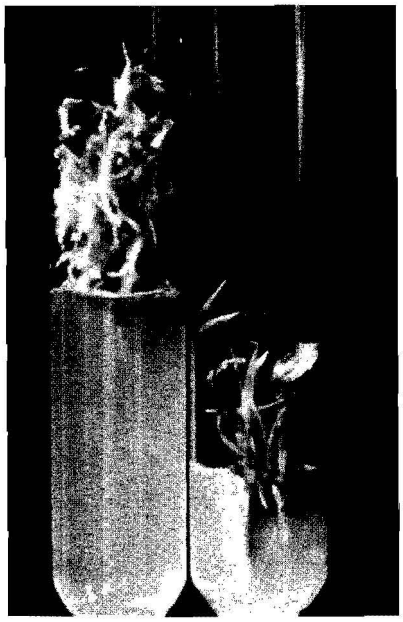


Fig. 2. One-year-old cultures of cardamom stored at 15°C.

Table 2. Effect of media constituents on inducing slow growth in cardamom cultures†

Media constituents		Growth		Duration of storage (days)	Survival (%)
Basal medium concentration	Sucrose/+ mannitol (g/L)	Increase in length (cm)	No. of multiple shoots produced		
MS full strength	30	9.3	5.0	180	80
	20	7.5	4.5	120	75
	25+5	2.4	6.0	250	80
	20+10	3.0	8.0	210	80
	15+15	3.0	8.0	360	80
	10+10	1.2	9.0	360	75
	5+15	2.0	5.0	360	65
MS half strength	30	3.1	6.0	360	55
	20	3.7	5.6	200	80
	25+5	2.4	6.0	250	80
	20+10	3.0	8.0	210	80
	15+15	3.3	9.0	360	85
	10+10	1.2	6.0	360	80
	5 + 15	2.0	5.0	250	90

† When screw-capped or polypropylene-capped culture tubes were used and incubated at 22±2°C.

The best results were obtained when mannitol and sucrose were given in an equal proportion of 15 g/L or 10 g/L. On this medium, 80-85% of the cultures could be maintained for a period of one year and the plantlets showed minimal growth. Of the two sucrose-mannitol combinations (15:15 and 10:10), that of 15 g/L each was more suitable where cultures were comparatively more healthy (Fig. 3). Although smaller in size these showed good multiplication and healthy appearance.

The induction of minimal growth may be due to the presence of mannitol in the culture medium. Various authors (Staritsky *et al.* 1985; Espinoza *et al.* 1986; Wilkins *et al.* 1988; Dekkers *et al.* 1991) have reported that the use of osmotically active compounds such as mannitol reduce growth and induce slow growth, thus extending subculture intervals.

Effect of enclosure on longevity of cultures. Cotton plugs, screw caps and polypropylene caps were used (Table 3). Although cotton plugs allowed better gaseous exchange they resulted in faster moisture loss, and the drying up of the culture medium and cultures. However, when screw-cap culture vessels were used, the moisture loss was minimal resulting in healthy cultures even after 360 days (Fig. 4). The use of polypropylene caps with ginger and turmeric cultures has shown similar results in minimizing evaporation loss as these can be stored for around one year without subculture (Balachandran *et al.* 1990).

Table 3. Effect of closure type on increasing subculture intervals† for tissue cultures of cardamom

Closure type	Survival period (days)	Survival (%)
Cotton plugs	200	60
Screw caps	360	85
Polypropylene caps	360	80

† When half-strength MS with 15 g/L sucrose and 15 g/L mannitol was used.

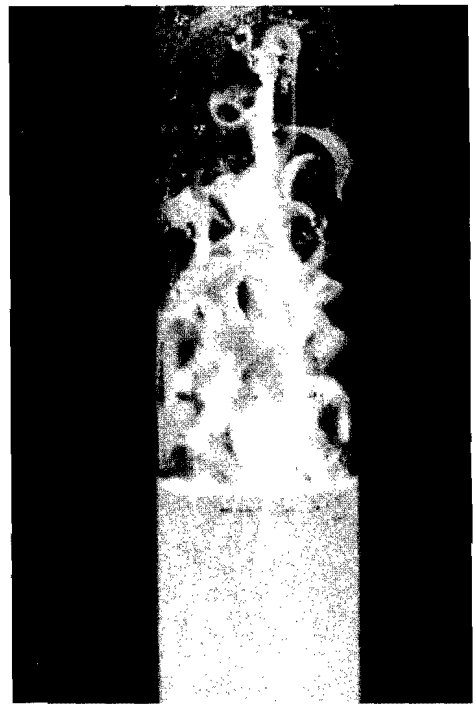


Fig. 3. One-year-old cultures of cardamom on MS medium containing 15 g/L mannitol and 15 g/L sucrose.

Recovery after storage and transfer to soil. Planting out is an important step if conserved material is to be of any use to plant breeders. The conserved material was transferred to the multiplication medium (MS basal medium + 30 g/L sucrose +1 mg/L NAA) for regrowth. Upon transfer the small plantlets kept in the conservation medium for nearly one year showed good regeneration and developed into normal-sized plantlets with good multiplication (1:8) and rooting. These plantlets, when transferred to soil (garden soil/sand/perlite in equal proportions) and kept in a humid chamber for 20 days, established easily with 85% success (Fig. 5). They later developed into normal plants with no abnormalities or deficiency symptoms and were similar to the mother plants. Throughout the whole culture process there were no abnormalities or deficiency symptoms in the cultured plantlets with regard to morphological features, except that plantlets were smaller on the slow-growth

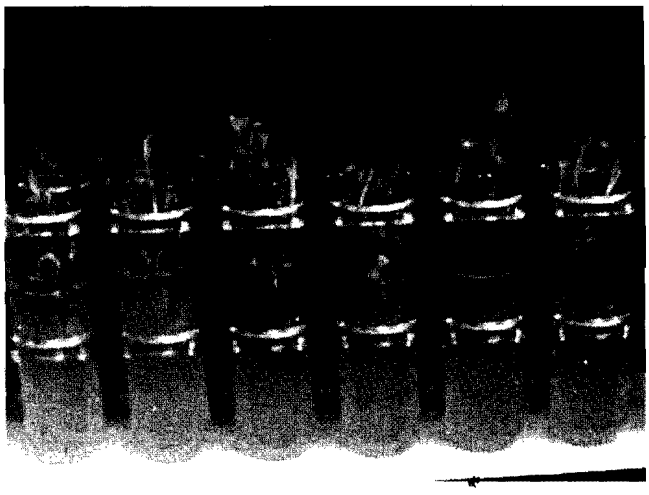
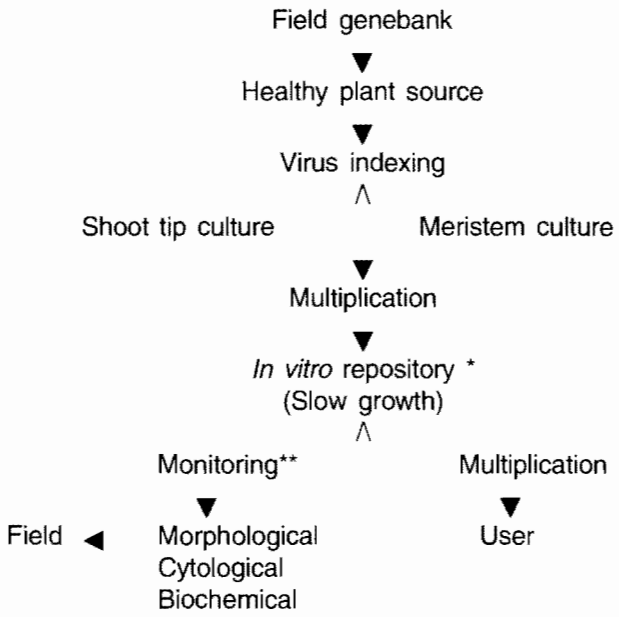


Fig. 4. One-year-old cultures of cardamom in screw-capped culture tubes.

Fig. 5. Planting out of material stored under slow-growth conditions.

medium. This indicates the apparent genetic stability of the cultures. However, cytological and other biochemical parameters, such as isozyme profiles and RAPDs, need to be tested for genetic stability analysis.

Thus cardamom genotypes can be conserved for up to one year when grown on a half-strength MS basal medium supplemented with 15 g/L each of sucrose and mannitol in polypropylene or screw-capped culture tubes, and incubated at 22 ±2°C with a 12 h light/12 h dark photoperiod of 2500 lux. Conservation of cardamom germplasm in an *in vitro* repository with yearly subculture is being attempted at IISR and at present there are 70 accessions of cardamom in the *in vitro* repository. The schematic protocol followed for *in vitro* conservation at IISR is given in Fig. 6.



* 20 replicates per accession in slow growth medium (½MS + 15 g/L mannitol + 15 g/L sucrose) maintained at 22±2°C with 12 h light/12 h dark and a light intensity photoperiod of 2500 lux, subcultured once in every 360 days.

** Yearly or once in two years.

Fig. 6. Schematic protocol for *in vitro* storage of cardamom germplasm at IISR.

Acknowledgements

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