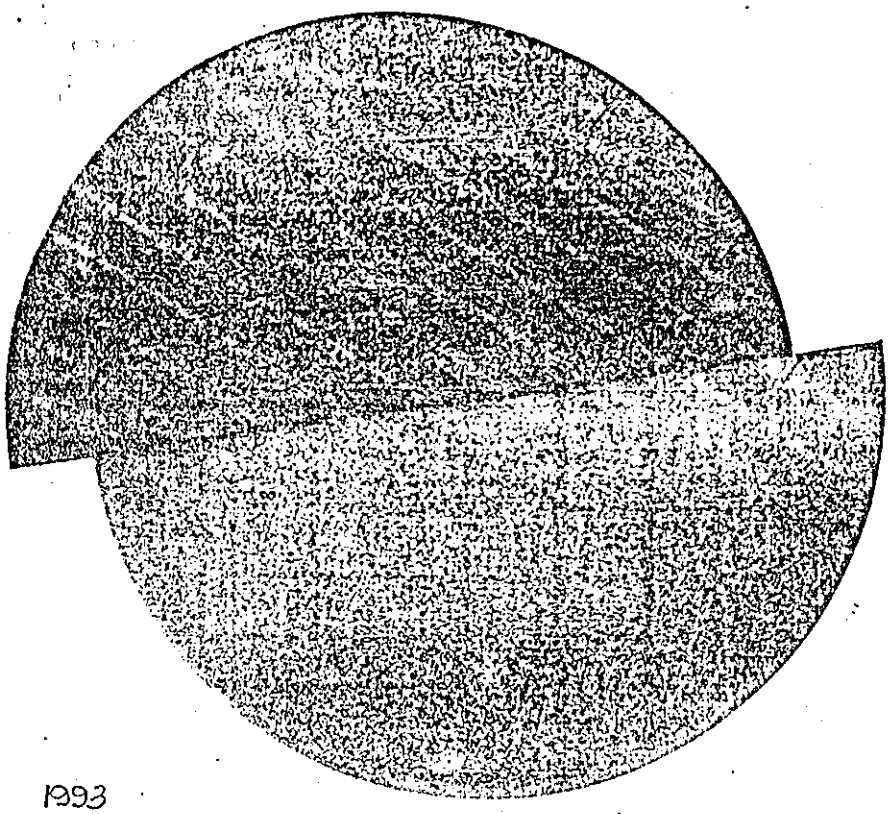
GENETIC ENGINEERING

MOLECULAR BIOLOGY AND TISSUE CULTURE FOR CROP PEST AND DISEASE MANAGEMENT



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TISSUE CULTURE OF TROPICAL SPICES

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Introduction

Black pepper, cardamom, ginger, turmeric and tree spices (clove, nutmeg, cinnamon) are the tropical spices important in the context of Indian economy. The production and trading in these crops are to the tune of 4,200 crores annually, out of which approximately 300 crores are in the form of foreign currency. In recent times much attention has been given to improve the production and productivity of these spice crops. To achieve this aim tissue culture technology is also being made use of. This paper reviews the achievements made in this field of tissue culture with special reference to the work done at the National Research Centre for Spices (NRCS).

The major bottlenecks in increasing production and productivity of the spices are the inadequate supply of disease free planting materials of elite lines and the absence of variability with regard to pest and disease resistance. Tissue culture offers a rapid and excellent method for the production of good quality planting materials in spice crops. It can also solve the sex segregation problem in nutmeg. In cardamom the rampant 'katte virus disease' is the major constraint in production and tissue culture can provide disease free planting materials of elite clones. In ginger the rhizome rot and bacterial wilt diseases are major hurdles, and here conventional breeding methods cannot be employed due to the absence of seed set. Induction and utilisation of somaclonal variations through tissue culture offers potential for the improvement of ginger. Here even the maintenance of the germplasm is threatened by the rhizome rot and bacterial wilt. Tissue culture technology also offers an easy way to conserve the germplasm in such cases, and also for the exchange of disease free planting materials. This is especially important in tree spices where the genetic base available in India is very narrow and the seeds of which are recalcitrant and cannot be stored for long.

In vitro Clonal Propagation

During recent years plant tissue culture propagation methods have been developed for several plant species, allowing rapid multiplication and in many cases recovery of disease free plants [14]. The work done so far on tropical spices are summarised in Table 1.

Table 1: Micro-propagation of tropical spices - response of different explants to various hormonal combinations

Crop and explant used	*Media composition*	*Morphological response*	*Refrence*
1. *Cardamom*			
Vegetative bud	**MS + 1.0 mg/1 BAP, 0.2 mg/1 kinetin, 0.2 mg/1 IBA, 0.5 mg/1 NAA, 200 ml/1 coconut milk	Multiple shoots	NRCS*
	MS + 0.5 mg/1 BAP, 0.5 mg/1 kinetin, 2 mg/1 IAA, 0.1 mg/1 biotin, 0.1 mg/1 calcium pantothenate, 5% coconut milk	Multiple shoots	Nadgauda *et al.* (32)
Shoot tips	*** White's medium + 0.5 mg/1 NAA (liquid)	*In vitro* rooting	NRCS*
Immature panicles	MS + 0.5 mg/1 NAA, 0.5 mg/1 kinetin, 1.0 mg/1 BAP, 0.1 mg/1 Calcium pantothenate, 0.1 mg/1 folic acid, 10% coconut milk	Floral premordia transformed into vegetative buds	Kumar *et al.* (22)
2. *Ginger*			
Vegetative bud rhizome bits with axillary bud	MS + 1mg/1 NAA (liquid medium from second sub-culture)	Multiple Shoots and *in vitro* rooting	NRCS*
Vegetative buds	MS major elements + Ringe-Nitsch minor elements, vitamins, 1 ppm BAP, 2% sucrose	Multiple shoots with roots	Hosoki and Sagawa (13)
Vegetative buds, rhizome, ovary, leaf sheath (pseudostem)	MS + 2 mg/1 2, 4-D	Callus	NRCS*
Shoot tip, shoot bud	+B5 + BAP (0.2 mg/1) MS + BAP (0.2 mg/1) B5 + (0.2 mg/1) NAA (2 mg/1)	Plantlets Plantlets Plantlets	Sakamura and Suga (38)
Shoot tip	White's LS + BAP (2 mg/1)	Shoot & Plants Callus & P Roots	Mori (27) Mori (27)
Meristem	+ MS + 20% coconut milk + 100 mg/1 ascorbic acid, 400 mg/1 glutamine, 250 mg/1 activated charcoal, 0.5 mg/1 BAP, 0.4 mg/1 IBA.	Production of shoots	Bhagyalakshmi & Singh (2)

3. *Turmeric*			
Vegetative buds, rhizome sets with axillary buds	MS + 1 mg/1 NAA (liquid medium from second sub-culture)	Multiple shoots and *in vitro* rooting	NRCS*
Vegetative buds	MS + 10% coconut milk, 0.1 mg/1 kinetin, 0.2 mg/1 BAP	Plantlet formation	Nadgouda *et al.* (31)
Vegetative buds, rhizome sections pseudostem tissues	MS + 2 mg/1 2, 4-D	Callus	NRCS*
4. *Black pepper*			
Shoot tip	MS + 1 mg/1 IAA, 1 mg/1 BA	Multiple shoot formation	Mathews and Rao (26)
Shoot tip	MS + 0.2 mg/1 NAA	*in vitro* rooting	Mathews and Rao (26)
Shoot tip	MS + 1 mg/1 NAA	*In vitro* rooting	NRCS*
Hypocotyl, lateral bud, stem segments	MS + 1 mg/1 BA	Callus	Mathews & Rao (26)
Leaf discs	MS + 2 mg/1 2, 4-D	Callus	NRCS*
5. *Cinnamon*			
Shoot tip from seedlings	MS + 0.5 mg/1 NAA, 0.5 mg/1 BAP	Multiple shoots	Rai and Jagdishchandra (36)
Hypocotyl	MS + 0.5 mg/1 kinetin, 0.5 mg/1 BAP	Multiple shoots	-do-
Shoor tips	White's + IAA, IBA, IPA 0.1 mg/1 each.	*In vitro* rooting	-do-
Shoot tips	MS + 2 mg/1 2, 4-D	Callus	NRCS*
Shoot tips	MS + 1 mg/1 2, 4-D	Callus	Rai & Jagdishchandra (36)
6. *Clove*			
Shoot tips, hypocotyl	MS + NAA and BAP	Multiple shoots	Jagdishchandra & Rai (16)
Shoot tips	MS + 2.5 mg/1 NAA, 10 mg/1 BAP	Callus	Mary Mathew *et al.* (24)

* NRCS - Unpublished data

** MS basal medium (Murashige and Skoog, 1962)

*** White's basal medium (White, 1963)

+ B5 basal medium (Gamborg *et al.*, 1968)

Cardamom (*Elettaria cardamomum Ma ton)*

Studies carried out at the Cardamom Research Centre of NRCS at Appangala (previously under the CPCRI) resulted in developing protocols for clonal multiplication of cardamom by vegetative bud and inflorescence cultures. Explants from vegetative buds have produced multiple shoots when cultured for 90-120 days in MS (28) basal medium supplemented with 0.5 ml/1 NAA; 1.0 mg/1 BAP; 0.2 mg/1 IBA; 0.2 mg/1 kinetin and 200 ml/1 coconut milk (cm). The cultures were initially established in solid medium and then transferred to liquid medium for subsequent cultures. The separated shoots were rooted in White's basal medium (45), supplemented with 0.5 mg/1 of NAA. The rooted plants were transferred to soil and the establishment in the soil was about 70%.

When the immature panicles of cardamom were cultured on MS basal medium with 0.5 mg/1 NAA, 0.5 mg/1 kinetin, 1.0 mg/1 BAP, 1.0 mg/1 calcium pantothenate, 0.1 mg/1 folic acid and 10% coconut milk, the floral primordia transformed into vegetative buds which could be rooted on MS + 2 mg/1 NAA and 0.05 mg/1 kinetin (22). Rapid multiplication of cardamom by tissue culture was also reported earlier by Nadgouda *et al.* (32). A field trial laid out at NRCS to compare the tissue cultured plantlets to those from suckers indicated that both were on par with regard to yield attributes and morphological characters.

Ginger (*Zingiber officinale Roscoe.*) and Turmeric (*Curcuma longa* L.)

The protocols for ginger has been reported by many previous workers (2, 13, 15, 27, 30, 34, 35). Clonal multiplication in ginger was achieved by culturing vegetative buds or rhizome bits on MS liquid medium with 1 mg/1 NAA. This not only resulted in excellent production of roots but also in proliferation of tillers. These tillers on separation can be used for further multiplication or can be directly transferred to soil. Subculturing can be done successfully in MS liquid medium with 0.5 mg/1 NAA and 0.5 mg/1 BAP which resulted in an increased production of multiple shoots, though the root development decreased relatively. The establishment of the plantlets in soil is above 80% and these are morphologically similar to the parent plants. Culture of meristems with or without leaf primordia and development of plantlets was reported by Bhagyalakshmi and Singh (2). Using tissue culture technique nematode free planting material could be recovered from highly nematode infested rhizomes of ginger (4).

Sakamura and Suga (38) have developed a rotating liquid culturing system for the shoot tip culture of ginger. The media used were B5 (7) and MS, supplemented with NAA and BAP. This method is reported to induce precocious branching and multiple shoots. The volatile oil composition of ginger rhizomes grown by tissue culture were qualitatively similar to the parent material but much quantitative variations were reported (38).

In turmeric protocols for *in vitro* multiplications have been reported (21, 31, 46). Nadgouda *et al.* (29) have also reported the isolation of high curcumin lines from tissue cultured plantlets of turmeric.

Black Pepper (*Piper nigrum* L.)

In vitro studies on black pepper have been taken up only recently (6, 26). Here the progress has been hampered by the high rate of contamination probably due to endogenous microbes (6). Pre-treatments with antibiotics and fungicides could not be of much use. Only a few shoot tip cultures could be established and they were made to root on MS medium with 1.0 mg/1 of NAA. Callus could be induced when the shoot tips and leaf tissues were cultured on MS medium with 3 mg/1 of 2, 4-D in dark. The problem of contamination could be eliminated when leaf tissues were used. Earlier reports suggested that multiple shoots could be induced from shoot tip cultures using MS medium supplemented with 1 mg/1 each of IAA and BA (26). These shoots were made to root on MS medium with 0.2 mg/1 of NAA.

Tree Spices

Studies on *in vitro* propagation of tree spices are limited. In cinnamon (*Cinnamomum verum* Berch & Presl.) multiple shoots could be induced when shoot tips were cultured on MS + 0.5 mg/1 NAA and 1 mg/1 BAP and the shoot tips could be rooted easily in White's liquid medium with 0.1 mg/1 each of IAA, IBA and IPA (16, 36). Callus could be easily induced when shoot tips were cultured on MS medium + 2 mg/1 2, 4-D. Multiple shoots were induced in clove *Eugenia caryophyllus* (Sprengel Bullocke & Harrison) also (10). Production of callus from shoot tip explants of clove using MS medium with 2.5 mg/1 NAA and 10 mg/1 BAP were reported (24). There is no report on the micropropagation of nutmeg (*Myristica fragrans* Houtt.). The shoot tip, leaf explants etc. did not show any response even after 3-4 months when cultured on MS basal medium with varying levels of growth factors either alone or in combination. This work is still continuing.

Regeneration of Plantlets from Tissue Culture and Somaclonal Variation

Plant tissue and cell culture are potential adjuncts to plant improvement (37). This is usually seen in terms of applicability of cellular selection for recovering useful genetic mutants, anther culture to speed up the attainment of homozygosity, somatic hybridisation for recombining genomes of sexually incompatible species and the possibility of specific gene addition or modification by recombinant DNA technique (23). In sugarcane somaclones with increased resistance to both Fiji disease and downy mildew were obtained (11, 18, 19). In potato, protoclones having significant stable variations were observed with regard to growth habit, maturity date, tuber uniformity, photoperiod requirements etc. Somaclones with increased resistance to *Alternaria solani* toxin and potato blight disease (*Phytophthora infestans*) were isolated (25, 40).

In ginger, where there is no seed set, exploitation of somaclonal variations becomes all the more important especially with regard to resistance to *Pythium aphanidermatum* and *Pseudomonas solanacearum*, the causative organisms of soft rot and bacterial wilt. Procedures for production of somaclones through intermediary callus state have been standardised at this centre using tissues from leaf, vegetative bud, rhizomes and ovary (Table 2). Callus could be easily induced and multiplied

Table 2: Organogenesis in Tropical Spices

Crop	*Explant origin*	*Medium composition*	*Morphogenic response*	*Reference*
1. Ginger	Callus derived from pseudostem, vegetative bud ovary	MS** + 10 mg/l BAP, 0.2 mg/l 2, 4-D for 3-4 cycles and later in hormone free MS medium	Organogenesis and plantlet formation	NRCS*
	Callus derived from vegetative buds	MS + 0.1 mg/l kinetin, 0.2 mg/l BAP, 10% coconut milk	Shoot regeneration	Nadgouda *et al.* (30)
2. Turmeric	Callus derived from vegetative bud	MS + 0.4 mg/l BAP, 0.2 mg/l kinetin, 0.01 mg/l GA_3	Shoot regeneration	Shetty *et al.* (21)
3. Cardamom	Callus derived from vegetative buds	MS + 10% coconut milk, 2-5 mg/l BAP	Regeneration of plantlets	Srinivasa Rao *et al.* (43)

*NRCS - Unpublished data.

** MS basal medium (Murashige & Skoog, 1962).

from these tissues by culturing them on MS medium with 2 mg/l 2, 4-D. This callus differentiated into shoot spices when the hormonal level is altered with 10 mg/l of BAP and 0.2 mg/l 2, 4-D in the next 4-5 subcultures. The rate of organogenesis and plantlet formation further increased when the hormones were completely removed from the medium. The calliclones were being studied for the spectrum of somaclonal variations and their reaction to *Pythium* as well as *Pseudomonas* both *in vivo* and *in vitro*. Isolation of *Pythium* tolerant lines from cell suspension cultures in ginger was reported by Kulkarni *et al.* (20).

In tumeric organogenesis and plantlet formation was reported when the callus was cultured on MS medium with 0.2 mg/l kinetin, 0.4 mg/l BAP and 0.01 mg/l Gibberlic acid (GA_3) (21). It was also possible to isolate lines with high curcumin from tissue cultured plantlet after mutagenic treatments (29).

In cardamom regeneration of plantlets from callus cultures was also reported (43), when callus was cultured on MS medium with 10% coconut milk and 2-5 mg/l of BAP. In both black pepper and cinnamon, our efforts to induce organogenesis proved not successful so far.

Conclusion

During the past three decades great technological advancements have been achieved in the field of plant tissue culture. Meristem culture techniques have found their place in clonal multiplication, disease elimination and in germplasm preservation. In tropical spices the most challenging aims involve the development of cultivars resistant to virus ('katte' virus of cardamom), fungus (foot rot of black pepper and rhizome rot of ginger), bacteria (bacterial wilt of ginger) and nematode (pepper yellows caused by the burrowing nematode). These major diseases are probably conditioned by multiple genes. The inheritance of these genes may be therefore, similar to that of the quantitatively inherited traits such as yield. If plant tissue culture can generate wider genetic variations for quantitative traits such as yield, alteration or modification should also be possible in complex disease resistance characters (41). Heinz and Mee (12) have given a classical example of such a phenomenon in sugarcane, where clones derived through tissue culture possessed higher levels of resistance than the parents to downy mildew (*Sclerospora sacchari*), eye spot (*Dreschlera sacchari*) the Fiji disease and smut (*Ustilago scitaminae*). One such clone (Pindar 70-31) was resistant to both Fiji and downy mildew disease while maintaining the yield potential of the donor parent (33). Similarly resistance to fungal pathogens such as *Alternaria solani* and *Phytophthora infestens* had been observed in plants regenerated from mesophyll protoplasts of potato cultivar Russet (40, 41). Way back in 1977 Gengenbach *et al.* (8) used the pathotoxin from *Helminthosporium maydis* race T1, the Southern corn leaf blight organism prevalent in USA, to select cell lines resistant to the toxin from a susceptible population. The plants regenerated from the surviving cells were not only resistant to the pathotoxin, but also to the disease organism. Such studies are mostly confined to diseases caused by either fungi or bacteria, where a relationship could be established between the host, the pathogen and the toxin. The absence of

specific toxin makes the extension of these *in vitro* selection system for disease resistance difficult.

Fungal resistance by means of genetic engineering is not yet achieved but it could be possible by the synthesis of transgenic plants carrying genes coding for chitinase (39) glucan endo-D. 3-glucosinidase (42) or phytoalexin synthesis (9). On the other hand the protection against viral diseases makes use of the phenomenon of cross protection, the underlying mechanism of which is not clearly understood. The viral coat proteins seems to play a pivotal role in this and the studies undertaken so far are based on this hypothesis [1].

In cell and tissue culture technology the unit of selection is the cell or bits of tissues as against the whole plant in the case of conventional breeding procedures. The achievements in this field will depend, to a very large extent on the technological improvements in manipulating single cells and tissues, which will involve the ability to effectively regenerate plants from protoplast cultures or single cell suspensions. Development of highly efficient selection procedures will be the essential prerequisite in making cell and tissue culture technology a routine breeding programme in any crop. At present the achievements in tropical spices is far away from realising these aims.

In black pepper, total resistance to both *Phytophthora capsici* and the burrowing nematode *(Radopholus similis)* are present in the Central American species *P. colubrinum*. No doubt, this is an ideal source of resistance, but unfortunately, this species is so distant from the black pepper that conventional breeding technique cannot be used. It is worthwhile to explore the possibility of transferring the resistance through the *Agrobacterium* mediated gene transfer. The basic prerequisite for this is the perfection of the cell and tissue culture techniques in black pepper.

In ginger, induction and exploitation of somaclonal variations may prove useful in developing clones resistant to rhizome rot caused by *Pythium* sp. and bacterial wilt caused by *Pseudomonas solanacearum*. Efforts in this direction are in progress. On the other hand the leaf spot of ginger (*Phyllosticta zingiberi*) and leaf blotch of turmeric (*Taphrina maculens*) though less important, should be more amenable for *in vitro* selection using specific toxins, or even by employing crude culture filtrate. Behnke (3) was able to select for potato callus resistant to the crude culture filtrate of *Phytophthora infestens*. Similarly Hartman *et al.* (10) selected resistance to *Fusarium* wilt of alfalfa in cultures of alfalfa using culture filtrate of *Fusarium oxysporum* f. sp. *melicagenis*. These works suggest that the purity of the toxic material may not limit its use in selecting for resistance in a tissue culture (5). At the same time the results also carry a warning: Most of Behnke's late blight plants were killed by virus and *Erwinia* infestation, indicating that increased resistance to *Phytophthora infestens* was associated with an increased susceptibility to other pathogens (44). We are not aware of the existence of a pathogen derived toxin associated with symptom expression in the case of foot rot of *Piper* or the rhizome rot of ginger. But an approach in this line may well prove useful.

Sure, tissue and cell culture technology have open up an entirely new horizon and has given considerable hope to the plant breeders and plant pathologists.

The present decade may witness the flowering and fruiting of this technology in association with the more recent techniques such as PCR, antisense technology etc. that have come to the stage of molecular biology, and may well prove a bonanza for plant biologists for incorporating any desirable trait into any crop plant.

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