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A Review :

Biotechnological tools for crop improvement in spices

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Spices constitute an important group of agricultural commodity considered indispensable for flavouring foods and beverages, pharmaceutical, perfumery and cosmetic industries. India, the land of spices continues to be the largest producer, consumer and exporter of spices in the world. Cultivation of spices in India started from immemorial and presently out of 109 spices available in the world, 63 are grown in the country. Indian spices are unique and valued for their high intrinsic qualities.

Crop improvement in majority of spice crops is a difficult and time consuming programme due to long pre-bearing age. The productivity of many spice crops is considerably low due to various factors such as inadequate availability of high yielding varieties, absence of genotypes resistant to pest and diseases and absence of variability in many of the introduced crops. Biotechnology with its apparently unlimited potential offers new and exciting opportunities to solve the crop specific problems. This paper addresses the various biotechnological tools in the following areas.

- Micro propagation
- Assessment of genetic diversity
- Protoplast isolation
- Haploid production
- Exploiting somaclonal variation
- Management of biotic and abiotic stress
- *In vitro* conservation of germplasm

Micro propagation :

Plant tissue culture and micro propagation techniques have been under development for the last 3 decades. Through these techniques one can produce a large number of plants in a shorter period than possible via other conventional methods. The advantages of this method are not only in gaining time and number but also in getting uniform population with better performance, use of less space for multiplication, *In vitro* storage and conservation of germplasm and also getting disease free plants Herbal spices are fragrant herbaceous plants which the whole plants, twigs, leaves, flowers, fruits, seeds etc., fresh or dried are used as flavouring agents. Once an elite genotype is identified it can be multiplied rapidly through tissue culture. The key developments made in this line of work in some spice crops are listed below:

Assessment of genetic diversity:

Black pepper :

Molecular markers effectively augment the phenotypic characters in generic characterization. RAPD protocols were standardized for black pepper varieties and related species and RAPD polymorphism was used to estimate the genetic distance between them. Of the 8 primers tested, only 2 primers OPA 4 and 14 amplified for all the genotypes. It indicated that the released verities, Panniyur 1, Sreekara, Subhakara, Panchami genetically differed from each other to larger extent. The higher similarity index was obtained between Panniyur 1 and Subhakara. The RAPD profiles also indicated that Sreekara and Subhakara differed from each other though it is difficult to distinguish between them morphologically.

Vanilla :

Isozyme profiles of 10 genotypes using native PAGE was carried out. The gel was stained for superoxide dismutase and peroxidase. Leaf samples were homogenized in tris extraction buffer (Bhat *et al.*, 1992). The PAI expressed as

Sr.No.	Crop and explant	Media	Response	Reference
1.	Cardamom			
	Vegetative buds	MS + 1.0 mg/l BAP, 0.2mg/l Kinetin, 0.2mg/l IAA, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 5% coconut milk	Multiple shoots	Nadgauda *et al.*, 1983
	Callus developed from vegetative buds	MS + 1mg/l, 2,4-D 0.1 mg/l, NAA 1mg/l, BAP 0.5mg/l	Organogenesis and regeneration of plantlets	Lukose, 1993
2 .	Ginger			
	vegetative buds, rhizome bits with axillary bud	MS + 1mg/l NAA	Multiple shoots with *In vitro* rooting	Nirmal babu et al. (1994)
	Stem base, leaf blade	MS + NAA 0.5mg/l + 5ppm BA	plantlets	Choi and Kim, 1991
	Meristem	¼ MS + 20% coconut milk + 100mg/l ascorbic acid, 400mg/l activated charcoal, 0.5 mg/l BAP, 00.4mg/l IBA	Shoots	Bhagyalkshmi and Singh, 1988
3.	Turmeric			
	Callus developed from vegetative buds	MS + 10% coconut milk + 2.5mg/l BAP	shoot	Shetty *et al*. 1982
4.	Black pepper			
	Shoot tip	MS + 1 mg/l IAA, 1mg/l IBA	Multiple shoot	Mathews and Rao, 1984
	Shoot tip	MS + 0.2 mg/l NAA	*In vitro* rooting	Mathews and Rao, 1984
	Shoot tip, node, leaf callus	MS + Kinetin + NAA	Callus organogenesis and plantlet regeneration	Geetha *et al.*, 1990 Nirmal bau *et al.*, 1994 Nazeem *et al.*, 1992
5.	Vanilla			
	Aerial root	MS + 1.5mg/l IBA	plantlets	Phillip and Nainar, 1986
	Cell suspension culture	MS + 0.1mg/l2,4-D, 0.5mg/l kinetin, 1mg/l BA	Secondary metabolites	Funk and Brodelios , 1990
6.	Cinnamon			
	Shoot tip	MS + 0.5 mg/l NAA, 0.5mg/l BAP	Multiple shoots	Ral and Jagdishchandra, 1987
7.	Clove			
	Shoot tip	MS + 0.5 mg/l NAA, 0.5mg/l BAP	Multiple shoots	Jagdishchandra and Ral, 1986
8.	Nutmeg			
	Shoot tip	WPM + 3mg/l BAP, 1mg/l kinetin	Bud break and proliferation	Nirmal babu *et al*., 1994
9.	Tamarind			
	Stem nodes	MS + 2mg/l BA + 0.2mg/l NAA	shoots	Rao *et al*., 1997
10.	Curry leaf			
	Tender shoot sections	MS + 2mg/l BA + 0.2mg/l NAA	shoots	Rao *et al.*, 1997

percentage indicated the similarity between any two seedling progenies. Paired Affinity Indices (PAI) were calculated by the formula

PAI = Number of similar bands / Total number of bands.

Protoplast isolation in black pepper :

In vitro raised plants and field leaves were tried for protoplast isolation. Leaf explants taken from the *in vitro* grown plants were found to be the best source. One gram of leaf tissue from axenic explants were digested in 10ml of enzyme mixture at 21^0 C and incubated in dark for 15 hrs (Rao and Gunasekaran, 1991). The enzyme mixture consisted of 1% cellulose and 0.5% pectinase. The osmoticum was maintained by mannitol and $CaCl_2$. After incubation the mixture was filtered through a stainless steel mesh of 100mm pore size, the filtrate was centrifuged at 750 rpm for *Piper nigrum* and 500 rpm for *P. colubrinum*.

The protoplasts were cultured initially in liquid medium. The mannitol was the carbon source. Auxin and cytokinin were added. The Petriplates were sealed with parafilm and incubated in dark at 21^0 C. the protoplasts were checked for their development periodically. In both the species, the leaf explants were found to be ideal and the yield were $2x10^2$ and $2x5^2$ / ml in *ex vitro* leaves in *P.*

nigrum and *P. colubrinum,* respectively. In *in vitro* leaves the yields were 5x10 /ml and 1x10 /ml in *P. nigrum* and *P. colubrinum,* respectively. In *P. nigrum* the isolated protoplast showed variability in size. The present results would help in future on the somatic hybridization of different genotypes in *P. nigrum* and *P. colubrinum* to develop resistant types.

Haploid plant production in ginger :

Ginger anthers were collected at uninucleate stage and were subjected to cold treatment for 7 days and induced profuse callus on MS medium + 2-3mg /l 2,4-D. plantlets were developed form calli on MS + BA 10 mg/ l and 0.2mg/l 2,4-D. the regenerated plantlets could be established in soil with 85% sucrose. (Samsudeen *et al.,* 2000)

Somaclonal variation :

Black pepper :

High amount of somaclonal variation was reported in callus cultures. The extent of variation is genotype dependent. A study was conducted for inducing somaclonal variation in black pepper in developing screening procedures for *Phytophthora* resistant lines. The toxins present in culture filtrate of *P. capsici* was found to be non specific and thermostable. Calli were induced on stem and leaf segments of *in vitro* seedlings and basal leaf segments of mature leaves of lass house plants of five black pepper cultivars *viz.*, Panniyur 1, Karimunda, Kalluvally, Cheriyakaniyakadan, Balankotta and *P. colubrinum* in MS + IAA 1.0 mg/l and BA 1.0 mg/l. Based on callus necrosis, 7.5% of concentrate culture filtrate was fixed as the level that can be withstood by the cultivars.

Direct selection of calli was not found to inhibit the regeneration potential and plantlet could be regenerated from calli screened against Phytophthora foot rot in all the cultivars, except Panniyur 1. The regeneration potential of the calli of different cultivars irradiated with gamma rays was found very low. Partial purification of the filtrate could be done using ion exchanges like Dowex 1 and Dowex 50. None of the regenerated calli clones were completely resistant to *P. capsici* in natural screening. In the artificial inoculation of culture disc of *P. capsici,* highly tolerant plants could be isolated.

In vitro selection

Biotic stress resistance in black pepper :

In vitro selection for resistance to Alternaria blight in cumin :

The fungal isolate of *Alternaria burnsii* was grown on PDA media at room temperature. The callus of GC 1 survived better and showed inhibited growth at 5% culture filtrate when exposed for a period of 24 hrs, thus 5% was selected as the test concentration for screening calli of other genotypes (Patel *et al.,* 1997).

Influence of toxic metabolites from *P. capsici* on callus induction and regeneration in Black pepper

Black pepper	Response of varieties (%)		
	Panniyur 1	Panniyur 4	Karimunda
Callus induction			
Callus induction medium (M_1)	76	94	89
M_1 + 50 ppm toxic matabolite	68	87	73
M_1+ 100 ppm toxic matabolite	54	82	65
Callus regeneration			
Callus regeneration medium(M_R)	46	78	55
M_R + 50 ppm toxic matabolite	20	48	32
M_R + 100 ppm toxic matabolite	5	16	9

Bacteria free rhizome production in ginger through meristem culture :

Ginger buds were sterilized and cultured in MS under dark for 1 month. The meristems were cut into 0.15, 0.30, 0.35, 0.55 and 0.75 mm in diameter and cultured in MS + 15% coconut water. The bacteria free shoots were multiplied on MS + 4 mg/l BA for 5 weeks. The ginger plantlets were transferred to *ex vitro* and produced the bacteria free rhizome. The lowest bacterial infection was observed in the shoots cut into 0.15 +0.5mm diameter. The shoots multiplied on MS + 4mg/l BA were 1.9 times higher compared with those multiplied supplemented with out. The vigorous growth, high survival percentage and high yield were observed in the ginger plants produced bacteria free rhizome.

Abiotic stress resistance :

Callus initiation and multiple shoot formation in shoot tip explants and cell growth in cultured callus pieces decreased with increasing NaCl concentration in the medium and were completely inhibited at 0.5% NaCl (for

***In vitro* selection for salt tolerance in**

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

shoot tip explant) and 1.25% NaCl (for callus growth).

Screening for drought tolerance in coriander through tissue culture :

From 15 days old seedlings, explants of hypocotyls, cotyledon and root segments were excised and cultured in MS + poly ethylene glycol (PEG) at 0, 0.25, 0.5, 0.75, 1.0, 1.25 and 1.50 % concentrations. The study revealed that the cotyledon callus was the best for *in vitro* selection experiments with high potential to proliferate under drought conditions.

In vitro hybridization :

Ginger :

In vitro crossing between selected cultivars showed that Rio de Janeiro as female parent can be crossed with Karuppampady, SG 66, Nadia and as male parent with Karuppampady, Nadia, SG 66 and Bajbai. Selfing studies showed that the cultivars Rio de janerio, Karuppampady can be selfed by the *In vitro* pollination and fertilization techniques. The testing of seed viability with tetrazolium salt showed that seeds of 40 and 80 DAP is viable.

Explant	Inhibitory concentration of PEG (%)	Concentration of PEG (%) in the selection medium	No. of explants or callus pieces cultured	Drought tolerance plants	
				Isolated (No.)	Stable (No.)
Hypocotyl callus	1.25	1.5	428	9	3
Cotyledon callus	0.50	0.75	1153	2	-

In vitro mutagenesis :

Ginger :

In vitro mutagenesis was attempted by subjecting the *in vitro* sprouts to gamma rays. The highest dose for survival was identified is 2.0 kr. The irradiated cultures were maintained in the medium identified for *in vitro* multiplication of ginger. The rate of multiplication was considerably reduced on increasing number of subcultures cycles. The culture recorded healthy growth up to third subculture. Increasing the level of cyotkinin at later subcultures favour the growth of cultures. The irradiated plants were planted out successfully.

Vanilla :

- Gamma irradiation doses above 50 kr and higher doses of chemical mutagen EMS were found to be lethal to vanilla
- Lower doses of gamma irradiation and EMS were found to enhance *in vitro* response

Methods for *in vitro* pollination

- ovules developed in placental pollination
- modified placental pollination
- ovule or test tube fertilization

In all three cases, pollen grains along with ME_3 medium were applied over the ovules.

Turmeric :

To tackle the problem of lack of seed set, *in vitro* pollination was tried. Basal medium of ½ MS + 3% sucrose + 0.5 mg/l NAA and 1 mg/l of each BAP and kinetin induced ovule swelling. *In vitro* pollination was done using pollen grains in modified ME_3 medium. Among the different pollination methods, seed development was observed in intra ovarian, placental and modified placental pollination techniques. These techniques are useful in conducting crosses involving short and medium duration cultivars provided medium duration cultivars are used as female

Production and storage of synthetic seeds in spices

Crop	Explant	Alginate Concentration (%)	Storage medium at 22.2 ^{o}C	Period (months)	Viability (%)
Black pepper	Shoot buds	5	Sterile water	9	65
Ginger	Somatic embryos	5	MS + BAP (1)+ IBA (0.5)	9	75
Cardamom	Callus	5	MS + BAP (1)+ IBA (0.5)	6	70
Turmeric	Adventious buds	5	MS + BAP (1)+ IBA (0.5)	7	60
Vanilla	Shoot buds	4	Sterile water	10	70
Cinnamon	Shoot buds	5	WPM + BAP (3) + K (1)	4	70
	Somatic embryos	5	WPM + BAP (3) + K (1)	4	60
Camphor	Shoot buds	5	Sterile water	9	80
Sage	Callus	5	MS + BAP (1)+ IBA (0.5)	6	65
Lavendor	Callus	5	MS + BAP (1)+ IBA (0.5)	6	60
Anise	Callus	5	MS + BAP (1)+ IBA (0.5)	6	60

parents, ovules / seeds developed after *In vitro* pollination were creamy white during the initial stage of development.

Vanilla :

More than 50 per cent pod set was observed in the inter specific hybridization between *V. planifolia and V. valslensis*. Seeds from these crosses were germinated *In vitro* and sub cultured for better proliferation.

Synthetic seed production :

Synthetic seeds are developed by coating a tissue or an organ which can grow into a complete plant, along with nutrients in an artificial film or covering. Synthetic seeds were successfully produced and stored up to 4 to 10 months in pepper, ginger, cardamom, turmeric, cinnamon, camphor, vanilla and in some herbal spices.

In vitro conservation :

Conservation of germplasm in vitro :

New species of pepper *P. peepuloids*- a high elevation species, *P. cebeba* - a medicinally important species and Vanilla andamanica, CCS 1, a high yielding variety of cardamom with high quality and RRI -1 a rhizome rot tolerant variety can be stored *In vitro*. The conserved germplasm which is under slow growth phase could be retrieved for multiplication medium to assess their regeneration capability. Cardamom cultures after 1 year of storage could be brought back to normal conditions with 90% success and are transferred to soil. The ginger and turmeric cultures after 10 months of storage were multiplied within 30 days. The rooted plantlets were established in the soil with 805 successes.

Micro rhizome formation in turmeric :

It was noticed under slow growth storage conditions and an experiment was conducted with the aim of enhancing the micro rhizome formation. MS with growth regulators with sucrose and mannitol at different levels were tried. The cross sections were taken and stained in 1% saffranin and observed under microscope and compared with normal rhizome activity.

Conservation of vanilla pollen :

Attempts to cryopreserve vanilla pollen, primarily to design a viable method for interspecific hybridization programmes. A pretreatment with Cryoprotectant (DMSO) was essential before plunging in liquid nitrogen, instead of direct desiccation and liquid nitrogen preservation.

Cryopreservation of ginger shoot buds :

Embryos were dehydrated with cryoprotectants like dimethyl sulfoxide, glycerol and sugar, singly as well as in combinations ranging from 5-15% for 30-60 min and kept in dark. The pretreated control (without liquid nitrogen) was also cultured on recovery medium. The survival percentage of encapsulated shoot buds after pregrowth and desiccation varied depending on the sucrose content in the preculture medium. Preculture for 1 day with MS as well as 0.75 N sucrose was determined to survival. Shoot buds could withstand 2 and 3 days preculture duration in both the sucrose concentrations with 60-705 survival. Shoot buds precultured for 3 days in 0.5 M and 0.75 M sucrose and desiccated for 4 hrs showed a post freeze viability of 30 % and 50%, respectively.

Establishment of DNA bank :

In vitro gene bank to store and conserve DNA for further studies and use in genetic engineering experiments, in making genomic libraries and in crop improvement

***In vitro* conservation of herbal spices**

Species	Medium	Duration (months	*survival %
Anise	½ MS + Sucrose 20g/l	6	60
Celery	½ MS + Sucrose 20g/l	5	70
Dill	½ MS + Sucrose 20g/l	4	60
Fennel	½ MS + Sucrose 20g/l	4	65
Lavender	½ MS + Sucrose 20g/l + Mannitol 10g/l	8	70
Marjoram	½ MS + Sucrose 20g/l	8	75
Oregano	½ MS + Sucrose 20g/l	8	70
Parsley	½ MS + Sucrose 20g/l	4	65
Peppermint	½ MS + Sucrose 20g/l	12	85
Sage	½ MS + Sucrose 20g/l + Mannitol 10g/l	6	65
Spearmint	½ MS + Sucrose 20g/l	6	65
Thyme	½ MS + Sucrose 20g/l	12	80

* Mean of 10 replications

programmes. At present, IISR have 17 accesions of pepper, 50 of cardamom, 40 of ginger and 2 of turmeric and 17 in vanilla.

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REFERENCES

Bhagyalakhsmi and Singh, N.S. (1988). Meristem culture and micropropagation of a variety of ginger with high yield and oleoresin. *J. Hort.Sci.*, **63** (2) : 321-27

Bhat, S.R., Kackar, A. and Chandel, K.P.S. (1992). plant regeneration from callus cultures of *Piper longum* by organogenesis. *Plant cell reports*, **11** : 525-28.

Choi, S.K. and Kim, D.C. (1991). The study on clonal multiplication of ginger through in vitro culture of shoot apex. Research report on thr urral development Admn., Biotechnol., **33** (1): 40-45

Funk, C. and Brodelios, P. (1990). Influence of growth regulators and an elicitor on phenyl propanoid metabolism in suspension cultures of vanilla. *Phytochemistry*, **29** : 845-48.

Nirmal Babu, K., Rema, J. and Ravindran, P.N. (1994). Biotechnology research in spice crops. *In Advances in Horticulture* , **9** : 632-649. Malhothra publishing house, New Delhi-64.

Phillip and Nainar (1986). Clonal propagation of vanilla. *J. Pl .Physiol*., **122** : 211-215.

Rao, K.S. and Gunasekaran, K. (1991). Control of morphogenesis in tobacco protoplast culture vs embryogenesis. *Indian J. biochemistry and biophysic*, **28** : 467-471.

Rao *et al*. (1997). Tissue culture studies in tree spices. *Biotech of spices, medicinal and aromatic plants*. 39-44.

Samsudeen, K., Babu, K.N. Divakaran, M. and Ravindran, P.N. (2000). Plant regeneration from anther derived callus cultures of ginger. **75** : 4: 447-450.

Settu, A., Ranjitha Kumari, B.D. and Jeyamary, R. (1997). In vitro selection for salt tolerance in fenugreek. *Biotech of spices, medicinal and aromatic plants*. 119-121.

Shetty, KM.S., Horidasan, P. and Iyer, R.D. (19.82). Proc. Natl. Seminar on ginger and turmeric, CPCRI, Kasarkod, Kerala.

Stephen *et al*. (1997). Screening for drought tolerance in coriander. *Biotech of spices, medicinal and aromatic plants*. 122-125

Shukla *et al.* (1997). In vitro selection for resistance to Altenaria blight in cumin. *Biotech of spices, medicinal and aromatic plants.* 126-28.

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