**Effect of Carbendazim on *in vitro* conservation and genetic stability**

**assessment in *Curcuma longa*, L. and *Zingiber officinale*, Rosc.**

**Abstract**

An efficient *in vitro* multiplication and conservation protocol for *Curcuma longa and Zingiber officinale* has been standardized by adding fungicide carbendazim (Bavistin) in multiplication medium. For micropropagation of *Curcuma* and ginger cultures optimum concentration of carbendazim was tested by adding 25, 50, 75 and 100 mg.L-1 in MS basal and MS hormone medium (supplemented with 2.5 mg.L-1 BAP), 3% (w/v) sucrose and 0.45% (w/v) Clarigar. Enhancement in shoot proliferation from 4.33 to 8.01 shoots per explant in *Z. officinale*, 3.67 to 6.32 shoots per explant in *C. longa* with *in vitro* conservation period from 6 to 12 months was recorded in MS hormone medium supplemented with carbendazim @ 25 mg l-l. Survival of 85% cultures was recorded along with rooting on the same medium. One year old field grown tissue culture derived turmeric plants showed normal vegetative growth and rhizomes formation. After three years of *in vitro* conservation, agro – morphological and drug yielding potential of curcuma rhizomes was assessed, no significant differences were recorded in all quantitative and qualitative parameters of conserved plants compared with previous observation. No genetic variation was observed among plants conserved on BB25 medium and control as revealed by SRAP (Sequence-Related Amplified Polymorphism) profiles of curcuma and RAPD (Random Amplified Polymorphic DNA) profiles of ginger after three years of *in vitro* conservation. The protocol is cost-effective for pest free long-term *in vitro* conservation of curcuma and ginger germplasm.

**Key words** Bavistin, Ginger, Turmeric, Micropropagation, RAPD, SRAP

**Introduction**

Ginger (*Zingiber officinale* Rosc.) and turmeric (*Curcuma longa* L.) are commercially important spice crops of family *Zingiberaceae*. India is the global leader in production and export of turmeric and ginger in many forms. Rhizomes of these crops are widely used for medicinal purpose and as nutritional supplement. The major limitation in production of sufficient disease-free planting material of high yielding varieties is lack of adequate healthy elite planting material and high susceptibility of these commercially important crops to diseases like rhizome rot, leaf spot and bacterial wilt (28). However, the maintenance of germplasm by annual planting in field is expensive, labour intensive and prone to loss due to natural calamities (5). Tissue culture techniques offer a viable tool for rapid multiplication of pathogen-free planting material and germplasm conservation of many vegetatively propagated crops including curcuma and ginger (22). To conserve genetic diversity of vegetatively propagated crops for any crop improvement programme, in-*vitro* conservation considered as an alternative to field gene bank conservation. However, bacterial and fungal contaminations pose challenges for long term maintenance. Fungal outbreaks with loss of cultures/ accessions have been reported (1). Antimicrobial agents (fungicides and antibiotics) are generally used in plant tissue culture media to eliminate microorganisms that are present in explants or arise as laboratory contaminants. Though, due to toxicity, some antimicrobial agents are undesirable for long-term use. Several antimicrobial agents like carbendazim, fenbendazole *etc.* which are found to be least toxic to plant cells and simultaneously enhance the growth of cultures will be most desirable for long term maintenance of *in* *vitro* cultures. Carbendazim have a broad spectrum fungicidal activity (13) and primarily used for pre-teatment of explants. However it is also reported for cytokinin like action when used in tissue culture media and resulted in more number of shoots. (6, 32). A number of protocols have been reported by different workers for *in vitro* propagation and conservation of turmeric (35, 5, 28, 24, 4, 10) etc. and on ginger by (8, 5, 12, 29) etc.

*In vitro* cultures are always associated with possible occurrence of somaclonal variation among the sub clones of one parental line. Besides, morphological and drug yielding traits, the assessment of genetic fidelity of *in vitro* conserved cultures at regular interval is important to eliminate the chance of occurrence of somaclonal variation during long term conservation of cultures (25). Molecular markers are being used for genetic stability assessment of *in vitro* conserved plantlets as they are constant, reproducible and quick to assay. Many reports are available on genetic stability assessment of micropropagated plants of curcuma (22, 20, 9) and ginger (26, 9) using various molecular markers like RAPD, micro satellite and ISSR.

There is no report on utilizing carbendazim for micropropagation and conservation of *Curcuma longa* *and Zingiber officinale* on preventing the contamination and for genetic stability assessment of tissueculture derived plantlets of Curcuma using qualitative, quantitative parameters and molecular markers, SRAP. The main objective of the present study was to optimise the concentration of carbendazim on i) affectivity in controlling out breaks of fungal contamination ii) its effect on growth of culture iii) enhancing the conservation period iv) cost effective *in vitro* conservation protocol and genetic stability assessment through morphological, drug yielding potential and molecular marker (RAPD and SRAP) analysis.

**Materials and Methods**

*In vitro* cultures of *Curcuma longa* cv. NDH-98 (IC564498) and *Zingiber officinale* cv. HP Pullapally (IC248817) used for the experiment had been maintained for 5-10 years at *in vitro* genebank of ICAR-NBPGR, New Delhi, India through periodic subculturing at 6 - 8 months interval.*In vitro* shoot tip (basal 1-2 cm swollen part of shoot) excised from 4 week-old cultures were used asexplant, leaves and roots were removed. Turmeric and ginger explants were inoculated on to MS (20) basal medium and MS hormone medium supplemented with 2.5 mg.L-1 (BAP) 6-benzyl amino purine, 3% (w/v) sucrose (Himedia, India) and 0.45% (w/v) Clarigar, pH 5.8. Different concentrations 25-100 mg.L-1 of carbendazim, (BavistinTM, M/S BASF India Pvt. Ltd., India) was added in the medium. MS hormone medium without added carbendazim was used as control. The following treatments were tested:

Control - MS + 2.5 mg.L-1 BAP + 3% Sucrose + 0.45% Clarigar

B25 - MS + 25 mg.L-1 Carbendazim + 3% Sucrose + 0.45% Clarigar

B50 - MS + 50 mg.L-1 Carbendazim + 3% Sucrose + 0.45% Clarigar

B75 - MS +75 mg.L-1 Carbendazim + 3% Sucrose + 0.45% Clarigar

B100 - MS +100 mg.L-1 Carbendazim+ 3% Sucrose + 0.45% Clarigar

BB25 - MS + 2.5 mg.L-1 BAP + 25 mg.L-1 Carbendazim + 3% Sucrose + 0.45% Clarigar

BB50 - MS + 2.5 mg.L-1 BAP + 50 mg.L-1 Carbendazim + 3% Sucrose + 0.45% Clarigar

BB75 - MS + 2.5 mg.L-1 BAP +75 mg.L-1 Carbendazim + 3% Sucrose + 0.45% Clarigar

BB100 - MS + 2.5 mg.L-1 BAP + 100 mg.L-1 Carbendazim + 3% Sucrose + 0.45% Clarigar

***In vitro* Culture Conditions**

The pH of all the media was adjusted to 5.8 with 0.1N NaOH or 0.1N HCl prior to addition of gelling agent and approximately 20 ml of the medium was dispensed into each culture tube (25×150 mm, Borosil). The medium was autoclaved at 15 lbs/cm3 pressure and 121°C temperature for 15 min. Each culture tube received one explant. The cultures were incubated at 25 ± 2°C and a light irradiance of 40 µmol m−2 s−1 provided by cool white fluorescent lamps (Philips, Mumbai, India) under 16-h light and 8h dark conditions and 60-70% relative humidity. After raising cultures aseptically, culture tubes were enclosed with polypropylene caps (Tarsons, India) and sealed with parafilm to prevent dehydration of media and cultures.

**Hardening**

Eight-weeks-old *in vitro* derived plantlets were hardened for 2 weeks in plastic pots containing a mixture of soil rite: soil: sawdust (2:1:1) moistened with ¼ MS major salt solution and kept at 25 ± 2°C with high humidity (70-80%) followed by transfer to soil in earthen pots. healthy rhizomes were harvested after maturity of crop and were subsequently used for estimation of curcuminoides profile and percent oleoresin.

**Quantitative**

Observations for plant regeneration were recorded for the number of shoots per explant, shoot length (cm), number of roots per explant, root length (cm), number of leaves and responses to carbendazim on *in vitro* shoot multiplication as well as conservation period in the tested cultures at two weeks interval up to eight weeks after inoculation. The period from the initiation of culture to next subculture was considered as conservation period for a given culture.

**Qualitative**

# Total Curcumin Content

Sample was finely grinded and sieved through ASTM 40 mesh test sieve. Curcumin was extracted by pressurised liquid extraction using acetone as solvent till the washings did not give absorbance at 425nm against solvent blank. Total curcumin content was estimated spectrophotometricaly at 425nm as per (2, 3) against standard Curcumin (Accros organics).

1. **Curcuminoides Profile**

Acetone extract as used in total curcumin content estimation was injected in HPLC for profiling curcuminoides. Separation of curcuminoides was done on waters atlantis dc 18 column using gradient mobile phase constituted by 0.1% glacial acetic acid aqueous solution and acetonitrile (23). Separated peaks were detected at 425 nm on waters 2487 UV-Vis dual wavelength detector. Quantization was done based on linear standard curve of curcumin, demethoxy curcumin and bisdemethoxy curcumin.

**c. Oleoresin Content**

The extraction of oil from the curcuma sample was done using cold percolation method where grinded sample was mixed with anhydrous sodium sulphate and extracted with petroleum ether (40-60ºC) as per (16).

**Genetic Stability Assessment**

To study whether carbendazim used in media (BB25) have any effect on the genetic stability of *in vitro* conserved plantlets, the genetic stability analysis using agro morphological parameters, biochemical traits andmolecular markers viz., RAPD (for ginger) and SRAP (for Curcuma) was done after one year and three year of *in vitro* conservation, respectively.

**DNA Extraction**

Total genomic DNA was extracted using 1g leaves from mother plants and from three to five *in* *vitro* conserved individual cultures and using method followed by (15). For RAPD analysis, the protocol by (34) was followed. A total of 50 primers (Operon Technologies, USA) were screened for RAPD analysis. Out of these, some 8 primers— OPC 4, OPC 5, OPC 7, OPC 8, OPC 11, OPC 12, OPD 5 and OPD 7 were found to be polymorphic and used for further analyses. The amplification products of *in vitro*-conserved plantlets derived from conservation medium were scored across the lanes, and they were compared with that of mother plants. Each band was treated as one RAPD marker and scored as present or absent from the photographs. Amplifications were repeated twice.

A total of five samples including controls were analysed using 10 RAPD primers. The PCR conditions were as follows: initial extended step of denaturation at 94oC for 5 min followed by 35 cycles of denaturation at 94oC for 1 min, primer annealing at 40-45oC for 1 min, primer elongation at 72oC for 2 min, followed by an extended elongation step at 72oC for 10 min. Reaction products were electrophoresed on 1.4% agarose gel at 90 volts followed by staining with ethidium bromide and photographed under ultraviolet light using a Digital Imaging System. The amplification products of the plants conserved on different media were scored across the lanes comparing with that of control plant.

**Sequence-Related Amplified Polymorphism (SRAP) Analysis**

For sequence-related amplified polymorphism (SRAP) analysis the protocol by (19) was followed. PCR reaction was carried out in a DNA Thermal Cycler. Each 12.5 µL reaction mix contained 1x PCR reaction buffer (10 mM Tris-HCl, 50 mM KCl and pH 8.3), 3 mM MgCl2, 0.5 U of Taq DNA polymerase; 200 µM each of dATP, dTTP, dCTP and dGTP; 0.6 µM of primer (Operon Technologies, USA) and approximately 50ng of template DNA. Each 15 µl reaction mix contained 10 x buffers (1.5µl), Mgcl2 (1.0µl), dNTPs (0.3µl), Taq polymerase (2µl), Primers (RandF) (0.5µl), DNA (2µL (50ng), Distt. Water (6.5µl) and 6 X Orange Dye (2 µL). About 50 primers (Operon Technologies, USA) screened initially, 26 primers producing clear and reproducible bands were used for SRAP analysis. Out of these, 8 primers - ME2/EM2, ME2/EM8, ME3/EM2, ME3/EM3, ME4/EM4, ME5/EM4, ME5/EM12 and ME5/EM11 were used for further analyses.

The PCR conditions were as follows: initial extended step of denaturation at 94oC for 5 min followed by 35 cycles of denaturation at 94oC for 1 min, primer annealing at 46-51oC for 1 min, primer elongation at 72oC for 1 min, followed by an extended elongation step at 72oC for 10 min. Reaction products were mixed with 2.0 µL of 6 x orange DNA loading dye using a microfuge. The mixture was electrophoresed on 1.6% agarose gel at 60 volts followed by staining with ethidium bromide and photographed under ultraviolet light using a Digital Imaging System. The amplification products of the plants conserved on different media were scored across the lanes comparing with that of control plant. Each band was treated as one SRAP marker and scored as present or absent from the photographs. Amplifications repeated twice. The scoring was done in samples where the bands were clearly visible and amplified products were reproducible over two repeated amplifications. The details of the primers and their annealing temperature are presented in following table.

**Statistical Analysis**

Each treatment consisted of 24 cultures and was replicated at least thrice in CRD. Data for all the replicates of shoots and roots per explant were calculated and analysed using one-way ANOVA (SPSS Version 16.0, 2008) and significant differences between treatment means were assessed using Duncan’s multiple range test (DMRT) at a level of *P* < 0.05.

**Results**

In the present investigation, in addition to its antifungal activity carbendazim was used to study the effect on bud proliferation and *in vitro* conservation. Shoot multiplication was found to occur by shoot buds, which is ideal for maintaining genetic stability. The rate of multiplication in both ginger and turmeric cultures was significantly different in the treatments with carbendazim in comparison to the ones without carbendazim. Maximum number of shoots per explant in turmeric were 6.32 on BB25 as compared to control 4.51 . While in ginger increase in maximum number of shoots per explant were 8.01 on the same medium (BB25) compared to control 3.67 (Table 1, Fig. 1A and 2A). Enhancement in number of shoots per plant and *in vitro* conservation period was also recorded in the cultures grown in medium with 50 mg.L-1 carbendazim (BB50). Reduction in number of shoots was recorded in treatment BB75 and BB100 indicates that the higher concentration of carbendazim has negative effect on shoot multiplication of both the crops tested. After eight weeks of inoculation, the explants grown in all the treatments did not show growth of any bacterial cells or fungal colony.

The plants regenerated from the cultures grown in medium supplemented with BAP @ 2.5 mg.L-1 and Carbendazim @ 25 mg.L-1 showed normal growth and multiplication in subsequent subculture of the explants. After acclimatization in plastic pots, *in vitro* derived plantlets produced healthy rhizomes. More than 90% survival was recorded after two months of hardening.

No significant variation was observed in shoot length among all the cultures of turmeric and ginger grown in various treatments, ranged between 0.5-9.0 cm. Rooting occurred simultaneously on the same medium (BB25) with the highest 7.15 roots/ explant in turmeric and 7.97 in ginger*.* No difference was recorded in root length of cultures grown in all the treatments, ranged between 0.5 - 3.0 cm in turmeric and 0.5 - 4.0 in ginger. Maximum leaves per explant (7.19 in turmeric and 9.11 in ginger) were recorded in the cultures inoculated in BB25. The *in vitro* conservation period of curcuma and ginger cultures could be extended significantly by addition of 25 mg.L-1 carbendazim in MS hormone medium (BB25), up to 12 months in turmeric (Fig.1B) and up to 13 months in ginger (Fig. 2B) with 85% survival in comparison with control (6-7 months), respectively. After hardening process, the in vitro derived plants showed 100% survival with uniformity in all morphological parameters.

**Genetic stability analysis**

Genetic stability assessment of *in vitro* conserved curcuma cultures was done using morphological characters, curcumin and oleoresin content and molecular markers including RAPD. As shown in Table 4, non-significant difference was recorded in all the qualitative and quantitative parameters of cultures conserved for three years in MS medium supplemented with 2.5mg.L-1 BAP and 25 mg.L-1 carbendazim with 3% sucrose (w/v) and 0.45% clarigar, pH 5.8. A total of 414 scorable bands, ranging from 0.35 to 2.0 kb with an average of 7.08 bands per primer, were obtained using eight primers (Table-2). The total number of bands produced by an individual primer ranged from four in OPC 7 to 13 in OPC8. Out of 26 primers, eight polymorphic markers were used to assess genetic stability of *in vitro* cultures of ginger conserved on BB25 (Table. 3). A total of 211 alleles at 63 loci were amplified. Amplified products exhibited monomorphic pattern across all the plants of ginger conserved on medium BB25 and on control medium Fig. 3. On the basis of available data, no major genetic variation was observed in RAPD and SRAP profiles of plantlets regenerated on BB25 medium and their controls (Fig. 4a and 4b).

**Discussion**

Carbendazim has been reported as alternate plant growth regulator in micropropagation of *Cordyline terminalis* and *Prunus avium* without any phytotoxic effect up to use of 160 mg.L-1(11). The results confirm the reports on cytokinin-like physiological functions of carbendazim, which has been well documented by several workers, In *Daucas carota* (33). The effect of carbendazim was attributed to its interaction with general mechanism of cytokinin like action was reported by (32). The benzimidazole carbamate groups of compound can disrupt microtubule function *in vitro* and *in vivo*. The carbendazim has been found to promote axillary shoot induction and multiple branching in *Bacopa monniera* (30, 31). Out of two additives, carbendazim appeared to have much stronger cytokinins-like activity than adenine sulphate on shoot multiplication of *Picrorhiza scriophulariiflora* (7)*.* Further, Carbendazim has been found to be thermostable and can be autoclaved without any loss of activity and degradation over long period of conservation in tissue culture system (11). Studies conducted in some other plant system also shown that bavistin promotes shoot regeneration (13). The present study confirms the beneficial effect of carbendazim on physiology of plant by inducing high frequency shoot bud proliferation and enhancement of conservation period both on ginger and turmeric.

In addition to its effect as growth promoter, carbendazim inhibits the breakdown of chlorophyll as well as RNA and protein. It also protects the leaves by preventing the flow of ions responsible for the degradation of cell membrane (14). RAPD technique, being simple and cost effective, has been used to assess the genetic variations of tissue culture plants in numerous studies and references therein, including *C. longa* (17, 27). RAPD polymorphism was reported in callus-derived plants of *C. longa* (27). However, RAPD and SRAP analysis showed no evidence of polymorphism between mother plants and the *in vitro* conserved cultures of *C. longa* and *Z. officinale* conserved for 3 years on BB25 media, which may be due to the fact that plantlets were regenerated directly from organized shoot tip explants.

Results indicate that the fungicide, carbendazim does not show any harmful effect on shoot regeneration, *in* *vitro* conservation and genetic stability of curcuma and ginger cultures even after three years of *in vitro* conservation.. Instead its use in culture media enabled fungal infestation free multiplication with more number of shoots per explants and longer conservation period. Thus it has high potential for use in *in vitro* multiplication and conservation protocols and work on other plant species has to be attempted.

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