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RAPD assessment for identification of clonal fidelity of microrhizome induced plants of Turmeric (*Curcuma longa* L.) cultivars

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Abstract

Curcuma longa L. (Zingiberaceae) is a tropical herbaceous plant cultivated as annual. Non-availability of quality planting materials of improved varieties is one of the important production constraints in turmeric. Large number of plants can be produced *in vitro* through microrhizome technology and it is advisable to check the clonal fidelity of these plants. In the present study microrhizome induction was carried out in three varieties of turmeric viz., *Alleppey Supreme*, *Prabha* and *Lakadong*. Most successful microrhizome induction was observed in the variety *Alleppey Supreme* in MS medium supplemented with $90gl^{-1}$ sucrose in Growtec culture vessels. The monomorphic pattern of RAPD profiles observed for the microrhizome derived plants in comparison with the mother plant confirmed the clonal fidelity. This recommends that planting material production through microrhizome technology is a safe method for multiplication of true-to-type plants in turmeric even after 30 subculture cycles. This is the first study that evaluates the applicability of RAPD markers in establishing clonal fidelity of turmeric plants raised through microrhizome technology.

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Introduction

Turmeric, the spice of commerce is the dried rhizomes of *Curcuma longa* L. (Zingiberaceae). India is the world's largest producer, consumer, and exporter of turmeric and Indian turmeric is regarded as the best in the world market because of its high curcumin content. Turmeric has innumerable uses as spice and in medicine (Ravindran *et al*., 2007). The use of turmeric in the treatment of Alzheimers disease has been reported earlier (Duke, 2003; Abascal and Yarnell, 2004). It is used to treat liver diseases, eye diseases, cancer and tumors and possess hypercholesterolemic, neuroprotective, immunomodulatory, anti HIV, nematicidal and mosquitocidal activities (Sarker and Nahar, 2007).

The productivity of turmeric is decreased due to many diseases like rhizome rot and foliar diseases that affect rhizome yield (Devasahayam and Koya, 2007; Dohroo, 2006). To overcome this production constraint, production of pathogen free seed rhizomes through biotechnological approaches is attempted. Protocols were developed for micropropagation of turmeric and also *in vitro* microrhizome production. In turmeric, micropropagation by *in vitro* microrhizomes is an ideal method for the production of disease free planting material and also for the conservation and exchange of germplasm (Babu *et al*., 2007). Micropropagation has been extensively used to propagate turmeric as reviewed by Babu *et al.* (2007). Microrhizome induction in turmeric was reported by Nayak (2000), Shirgurkar *et al.* (2001), Sunitibala *et al*. (2001), Islam *et al.* (2004), Adelberg and Cousins (2006; 2007), Nayak and Naik (2006), Cousins and Adelberg (2008), Chougule *et al.* (2011), Thingbaijam *et al.* (2012), Pistelli *et al.* (2012) etc.

Babu *et al.* (2003) reported the field evaluation of microrhizome derived plants of turmeric. Except morphological, biochemical and phytochemical markers, molecular markers are also being used for analyzing the genetic fidelity of micropropagated plants, as they are consistent, reproducible, independent of environment and fast to assay, and thus have been successfully employed to assess the genomic stability of regenerated plants (Rani and Raina, 2003; Agarwal *et al.*, 2008). There are a few reports on the assessment of genetic fidelity of micropropagated plants of turmeric. Lack of genetic fidelity (Salvi *et al*., 2001) and high degree of genetic fidelity (Salvi *et al*., 2002) of the micropropagated *C. longa* plantlets have been reported. Tyagi *et al*. (2007) confirmed the genetic stability of 12 months old *in vitro* conserved turmeric plantlets. Panda *et al*. (2007) revealed the genetic stability of micropropagated clones of turmeric using cytophotometry and RAPD analyses. The present study envisages assessment of microrhizome induction capacity of three varieties of turmeric in different types of culture vessels and

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genetic fidelity of long term microrhizome induced plantlets cultured for thirty sub-culture cycles using RAPD analysis.

Materials and Methods

Plant material

Three high yielding varieties of turmeric viz., *Alleppey supreme* and *Prabha* obtained from Indian Institute of Spices Research (IISR), Kozhikode, Kerala, India and a north east cultivar *Lakadong* obtained from The Energy and Resource Institute, North-Eastern Regional Centre, Guwahati, India were selected for the present study.

Microrhizome induction

Single shoots isolated from sixty-months old *in vitro* multiplied cultures of turmeric, after 30 subculture cycles, grown in MS medium (Murashige and Skoog, 1962) with 2 mgl^{-1} BA, 0.5 mgl^{-1} NAA and 25 mgl^{-1} Adenine sulphate; maintained in the Crop Improvement and Biotechnology Division of Centre for Medicinal Plants Research (CMPR), Kottakkal, Kerala, India was used as explants for microrhizome induction. Microrhizome induction experiment was carried out in two types of culture vessels such as Growtec (Tarsons, Mumbai, India) and 350 ml culture bottles (Excel Glass Works, Alleppey, Kerala, India) containing MS basal medium with 90 gl^{-1} sucrose. Cultures were maintained at 24 ± 2°C with 12 h photoperiod of 35–40 μmol $m^{-2}s^{-1}$ provided by white fluorescent tubes. The tender leaves collected from *in vitro* microrhizome induced plants after five months of culture and conventionally propagated plants (control) were subjected to DNA isolation using CTAB method with some modifications (Doyle and Doyle, 1987).

RAPD analysis

Twenty deca-nucleotide primers of Operon Technology Inc. (Alameda, CA, USA) were used for the RAPD analysis of turmeric cultivars. The polymerase chain reaction was carried out using 25 µL reaction mixture containing 5 µl of 5X *Taq* buffer, 2.5 mM $MgCl_2$, 2.5 mM dNTPs each, 3 µM of 10-mer oligo-nucleotide primers, 1 unit of *Taq* DNA polymerase and template DNA (25 ng).

RAPD amplification and electrophoresis

PCR amplification was carried out using the method of Williams *et al.* (1990) with some modifications of thermal cycles (94°C for 5.0 minutes; 39 cycles of 94°C for 1.0 minute; 38°C for 1.0 minute and 72°C for 2.0 minutes; 72°C for 10 minutes and 4°C at end). All the reactions were amplified in a mastercycler gradient (Eppendorf, Germany). The PCR products were separated on 1.5% agarose gel stained with ethidium bromide in 1X TBE buffer (pH 8.0). The size of the amplified fragments was determined using 1kb DNA ladder. The gels were documented using a gel documentation system (Alpha Innotech Corporation, USA).

Results and Discussion

Microrhizome induction

Successful microrhizome induction was achieved in all the cultivars of *C. longa* in MS medium supplemented with 90 gl^{-1} sucrose. Microrhizome induction was initiated within one month in all the cultivars. The cultivars showed specific differences in microrhizome induction with respect to the types of culture vessels used (Table 1). It was observed that the number of shoots and microrhizome formation in Growtec was higher when compared to the cultures grown in culture bottles (Figure 1). After 5 months of *in vitro* growth in Growtec culture vessel, var. *Alleppey Supreme* showed higher rate of shoot induction (12 ± 1.4) than that of *Lakadong* and *Prabha.* In the case of cultures grown in culture bottles, *Alleppey Supreme* produced less number of shoots (3.7 ± 1.7), but it was about one third of the rate in Growtec (Table 1).

Microrhizome production in Growtec culture vessel is very advantageous and economical as it guaranteed improved growth, the capacity of renewing fresh medium and less labor. The cost of media can be reduced in Growtec vessel, as liquid medium was used and it replaced agar, the most expensive component of the medium. This method and cultures has now being used as a source of pathogen free planting material production in turmeric, especially in elite superior varieties and cultivars.

RAPD fingerprinting

In order to confirm the genetic integrity, RAPD analysis of microrhizome induced plants of three turmeric varieties maintained in culture over a period of five years was carried out. A total of 12 plants from each variety were tested with 20 RAPD primers. RAPD profiles revealed that all the bands produced by microrhizome induced plants were monomorphic and similar to the mother plant for all the primers tested (Figure 2).

The presence or absence of variation depends upon the source of explant and method of regeneration or on the source of regenerants (Larkin *et al*., 1989). Salvi *et al.* (2001; 2002) reported both lack of genetic fidelity and high degree of genetic fidelity of the

Table 1. Culture responses of three cultivars of *Curcuma longa* during *in vitro* microrhizome induction in different types of culture vessels*

Cultivars	Number of shoots		Length of shoots (cm)		Number of leaves	
	GT	CB	GT	CB	GT	CB
Alleppey Supreme	12.0 ± 1.4	3.7 ± 1.7	6.94 ± 1.5	6.7 ± 2.1	3.04 ± 0.06	4.1 ± 0.6
Lakadong	2.5 ± 0.7	2.0 ± 1.0	16.42 ± 0.6	6.21 ± 1.9	6.0 ± 0.0	3.88 ± 1.17
Prabha	4.0 ± 0.0	2.7 ± 0.7	7.84 ± 0.5	7.3 ± 0.9	3.25 ±0.35	3.9 ± 1.2

GT – Growtec, CB – Culture bottles

*The experiment was repeated thrice in both Growtec and Culture bottles

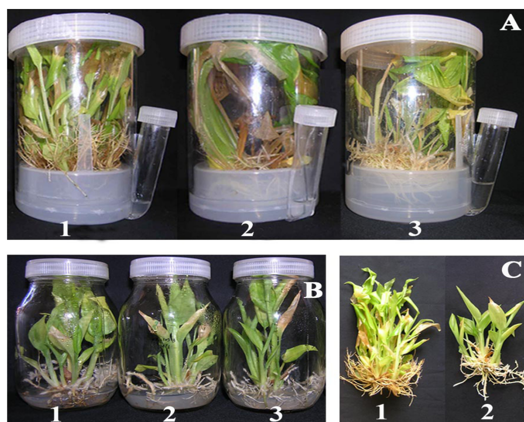

Figure 1. (A) Cultures grown in Growtec (1) *Alleppey Supreme*, (2) *Lakadong* and (3) *Prabha*; (B) Cultures grown in culture bottle (1) *Alleppey Supreme* (2) *Lakadong* and (3) *Prabha*; (C) *Alleppey Supreme* grown in (1) Growtec (2) Culture bottle

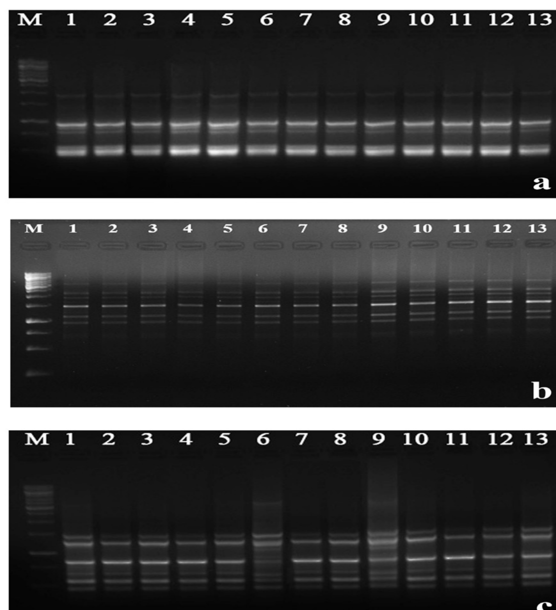

Figure 2. RAPD profiles of (a) *Alleppey Supreme* (OPP-18), (b) *Lakadong* (OPP-01) and (c) *Prabha* (OPP-04). M - 1kb ladder; Lane 1 - mother plant and Lane 2-13 represent microrhizome induced plants

micropropagated *C. longa* plantlets. Panda *et al.* (2007) reported genetic stability of micropropagated and conventionally propagated turmeric plants using RAPD technique. RAPD analysis of microrhizome derived turmeric plants after 30 subculture cycles showed profiles similar to that of the conventionally propagated mother plants (control) indicating that no genetic variation had occurred during the *in vitro* culture for five years. This is the first report on genetic integrity analysis of microrhizome induced plants in turmeric. We have used vegetative buds from the rhizome for micropropagation and subsequent microrhizome formation in *C. longa* so as to reduce the risk of genetic instability. In *C. longa*, the length of the culture period for more than 5 years with regular sub-culturing (once in two months) did not affect their genetic fidelity. Similar observations were recorded in long term micropropagated plants of apple rootstock MM 111 and Merton 793 after repeated subculture for two years (Pathak and Dhawan, 2010; 2012) using ISSR markers.

From the present investigation it was concluded that microrhizome technology can be used for the large scale commercial production and conservation of these species without affecting the problems of genetic instability even after 30 subculture cycles.

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