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Tissue Culture and RAPD Analysis of *Cinnamomum camphora* and *Cinnamomum verum*

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Abstract: Molecular techniques and tissue culture were used to validate the morphological differences in two species of the Lauraceae family, namely *Cinnamomum camphora* and *Cinnamimum verum*. Shoot tip and leaf explants of *Cinnamomum camphora* and *Cinnamomum verum* obtained from field grown trees were cultured on MS medium supplemented with various levels of BAP and TDZ. Hundred percent multiple shoot formation was observed in shoot tip explants of *C. camphora* cultured on MS medium containing 1.0 mg L^{-1} BAP supplemented with 2.5 mg L^{-1} TDZ. Leaf explants of *C. camphora* responded only to MS medium containing 1.0 mg L^{-1} BAP supplemented with TDZ (0.005-5.0 mg L^{-1}) by forming compact callus. *C. verum* explants reacted to the treatments by sprouting. All shoots (100%) produced roots two weeks after transfer on the basic MS medium and the plantlets were successfully transplanted to pots containing topsoil plus compost (2:1) after acclimatization. Random Amplified Polymorphic DNA (RAPD) was used as a preliminary technique to evaluate differences between *C. camphora* and *C. verum*. All the eleven primers used in this study produced polymorphic bands in both *Cinnamomum* species. Results are promising and there is scope for developing (Sequence Characterized Amplified Regions) SCAR primers for identification of these two species as well as the other species in the same genus.

Key words: *Cinnamomum*, RAPD, micropropagation, TDZ, BAP

INTRODUCTION

Cinnamomum Schaeffer is a genus of evergreen trees and shrubs consisting of over 300 species which have aromatic oils in their leaves and barks. *Cinnamomum camphora* Siebold and *Cinnamomum verum* J. Presl species within this genus are cultivated in many regions of the world for their aromatic and medicinal attributes. *C. camphora*, native of China, is extensively used for landscaping and used in the folk medicine of Mauritius to treat fever, rheumatism and broncho-pulmonary infections. The bark of the other species, *C. verum* originating from Sri Lanka, is widely exploited for its culinary properties and it is also utilized to treat digestive problems (Gurib-Fakim, 2002).

The *Cinnamomum* species have not been exploited commercially in Mauritius because conventional methods of propagation by cuttings, seeds and layering are very slow and do not guarantee homogeneity. Moreover, the few existing plants cannot be exploited locally owing to the poor quality of their bark. Consequently, Mauritius imports about 14 tons of bark of these species worth Rs. 300,000 annually. Owing to their commercial importance and extensive use in the Mauritian folk medicine, there is an urgent need to develop rapid and reliable methods of propagating these species whilst safeguarding their genetic base.

Plant micropropagation by tissue culture represents the ideal pathway to achieve these objectives. Although micropropagation of woody species has been previously reported (Huetteeman and Preece, 1993; Kane, 2004) information on the tissue culture of *Cinnamomum* species is scanty. A micropropagation protocol for *C. camphora* has been described with the occurrence of hyperhydricity in regenerated shoots (Huang *et al*., 1988). Other literature relates to the micropropagation of less exploited *Cinnamomum* species such as *C. zeylanicum* (Rai and Chandra, 1987) and *C. cassia* (Inamoto and Kitani, 1989). Plant regeneration using protoplasts isolated from embryogenic suspension cultured cells of *C. camphora* has recently been described (Li and Manchu, 2005). Hence, this study was devised to assess the response of locally existing *C. camphora* and *C. verum* to tissue culture and to establish an efficient protocol for the clonal propagation of these species using different media and explant types. The two *Cinnamomum* species are very different from each other morphologically more specifically in their types of leaves, flower and fruit

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structures. Genetic difference between these two species was assessed to evaluate whether the morphological differences are also reflected at the molecular level. DNA-based techniques are widely used to assess genetic diversity within a species or to find genetic differences between species. These techniques are generally based either on the use of restriction enzyme which recognise and cut DNA sequences at specific sites (RFLP) or amplification of target sequences using short oligonucleotides (PCR). PCR-based techniques used for assessing genetic diversity are Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeats (SSRs). The appropriateness of each one of these techniques varies depending on the objectives of the study (Karp *et al*., 1997). RAPD (Williams *et al*., 1990; Welsch and McClelland, 1990) uses random primers to reveal nucleotide sequence variation through the amplification of unknown DNA sequences using single, short, random oligonucleotide primers. The RAPD system has been used in linkage map construction (Grattapaglia and Sedroff, 1994), resistance gene localization (Dweikat *et al*., 1997), hybrid origin identification (Friesen *et al*., 1997) and assessment of genetic variation (Liu and Furnier, 1993; Nesbitt *et al*., 1995). RAPDs have been widely used in woody species including eucalyptus (Keil and Griffin, 1994), citrus species (Kijas *et al*., 1997), guava (Prakash *et al*., 2002) and mango species (Ravishankar *et al*., 2000). No molecular studies have yet been reported in *Cinnamomum,* therefore an assessment of the genetic difference between the two species *Cinnamomum* was carried out using the RAPD technique which will pave the way for further work on the phylogeny of this genus or aid commercial expoitation of these species.

As it is well known that tissue culture is genotype dependent (Bhojwani *et al*., 1996), we decided to investigate whether *C. camphora* and *C. verum* respond differently to the micropropagation protocol as reflected by their morphological differences or whether they have similar responses because they belong to the same genus. Consequently we aim at evaluating the potential of tissue culture and molecular tools to assist the exploitation of these species in agriculture or for the pharmaceutical industry.

MATERIALS AND METHODS

Micropropagation studies: For tissue culture work, both well-established and young seedling trees were used as source plant material for clonal propagation of *C. verum* and *C. camphora*. Explants were taken from field-grown trees in the University of Mauritius farm and the forestry department of the Ministry of Agriculture in January 2005 and brought to the tissue culture laboratory of the Faculty of Agriculture, The University of Mauritius. The trees were identified at the herbarium of the Mauritius Sugar Industry Research Institute and were given accession numbers as follows: *Cinnamomum camphora* (Accession No. 23892) and *C. verum* (Accession No. 23893). The source plant of *C. verum* from the Forestry department was given the following Accession No. 24010. Two types of explants from both species were used for initiation of tissue cultures. Shoot tips and leaf segments were washed under running tap water for 2 h. They were then disinfested by overnight immersion in 2% bleach containing 1 drop of Tween 20 and rinsed 5 times in sterile distilled water. The explants were again surface sterilized for 15 min in Vircon S (2 g/100 mL) and rinsed 5 times in sterile distilled water. The last disinfection stage consisted of 8 min immersion in 0.1% $HgCl_2$. After 5 rinses in sterile distilled water, the cleansed explants were inoculated on basic Murashige and Skoog (1962) (MS) medium containing 4% sucrose, solidified with 0.6% agar and adjusted to pH 5.7 before autoclaving. All explants were inoculated on 25 mL standard MS medium as follows:

Inoculation conditions and assessments: Cultures were incubated at 26±1°C, illuminated under white fluorescent tubes (2500-3000 Lux photon flux density) with 14/10 h (light/dark) photoperiod. Hormones were filter sterilized with a 0.5 μm filter before addition to the medium. All treatments were performed in 15 replicates and explants were sub cultured to fresh medium every four weeks.

Molecular studies: All molecular work was carried out in the molecular biology laboratory of the Faculty of Agriculture, the University of Mauritius.

DNA extraction: Genomic DNA extraction was carried out using a modification of the method described for the extraction of DNA from fungi (Ranghoo *et al*., 1999). The yield was poor and ranged from 10 to 20 μg of DNA per extraction.

RAPD analysis: Isolates were screened by Random Amplified Polymorphic DNA (RAPD) to find genetic variation between the two *Cinnamomum* species. Each 50 μL of reaction consisted of 2 U of *Taq* polymerase, 50 mM KCl, 10 mM Tris-HCl pH 8.8, 4 mM $MgCl_2$, 200 μM dNTP, 0.22 μM primer and approximately 50 ng (≈10 μL) of template DNA. Forty amplification cycles were performed on a MJ Research minicycler. Each cycle consisted of denaturation at 94°C for 3 min, annealing at

Table 1: List of the operon primers used in this study and their sequence

Primer	Sequence
OPA 07	GAAACGGGTG
OPB 19	ACCCCCGAAG
OPB 2	GGACCCTTAC
OPC01	TTCGAGCCAG
OPC08	TGGACCGGTC
OPC10	TGTCTGGGTG
OPC13	AAGCCTCGTC
OPC16	CACACTCCAG
OPD04	TCTGGTGAGG
OPD09	CTCTGGAGAC
OPL07	AGGCGGGAAC

35°C for 1 min, followed by a 2 min rise to 72°C for primer elongation. The final primer elongation segment of the run was extended to 7 min. Approximately, 15 µL of the amplified RAPD products was loaded onto a 2% agarose gel at 4 V/cm for 2 h and separated by electrophoresis in TAE (Tris 1.6 M, acetic acid 0.8 M, EDTA 40 mM) buffer at 100 volts. Gels were stained with ethidium bromide and products visualized by UV light. PCR products were photographed with Polaroid film. Marker II Hyperladder (Bioline) was included in all gels as a molecular weight standard. Primers which were screened to identify those which produced maximum polymorphisms include OPC 08, OPL 07, OPC 01, OPC 16, OPC 10, OPB 19, OPC 13, OPD 04, OPB 20, OPD 09, OPA 07 (Table 1).

RESULTS

Micropropagation studies: All shoot tip explants of *C. camphora* (100%) produced multiple shoots when they were cultured on MS medium containing 1.0 mg L^{-1} BAP supplemented with TDZ (0.005-5.0 mg L^{-1} (Table 2). An average of 6 shoots per explant was observed with media containing 2.5 mg L^{-1} TDZ after 6 weeks (Table 2). The number of shoots induced from each shoot tip increased by an average of 1 as the TDZ concentration in the medium increased. The best response was noted with shoot tip explants of *C. camphora* on MS medium containing 1.0 mg L^{-1} BAP supplemented with 2.5 mg L^{-1} TDZ (Table 2). No multiple shoots formation was observed from *C. camphora* shoot tips cultured on media containing (0-3.0 mg L^{-1}) BAP only.

However, the response of *C. verum* shoot tips to the same treatments was different and only bud break was observed after 8 weeks on MS medium containing 1.0 mg L^{-1} BAP supplemented with 2.5 mg L^{-1} TDZ (Fig. 1).

Leaf explants of *C. camphora* responded only to MS medium containing 1.0 mg L^{-1} BAP supplemented with TDZ (0.005-5.0 mg L^{-1} by forming compact callus (Fig. 2) while no response was obtained with leaf explants of *C. verum* on the same media or on MS medium containing 0-3.0 mg L^{-1} BAP only.

Table 2: Effect of TDZ at different concentrations on shoot formation from shoot tip explants of *C. camphora* and *C. verum* after 6 weeks of culture

	No. of shoots per shoot tip explant	
MS medium + 1.0 mg L^{-1} BAP + TDZ (mg L^{-1})	*C. camphora*	*C. verum*
0	0	0
0.005	0	0
0.05	1[c]	0
0.5	3[b]	0
1.0	4[ab]	0
2.5	6[a]	1
5.0	0	0

C. camphora

C. verum

Fig. 1: Multiple shoots observed with shoot tip explants of *C. camphora* and budbreak in *C. verum* on MS medium supplemented with BAP (1.0 mg L^{-1}) and TDZ (2.5 mg L^{-1})

All shoots (100%) produced roots two weeks after transfer on the basic MS medium. After two weeks hardening in a container covered with perforated transparent plastic, the plantlets were successfully transplanted to pots containing topsoil plus compost (2:1).

Fig. 2: Callus formation on leaf explants of *C. camphora*

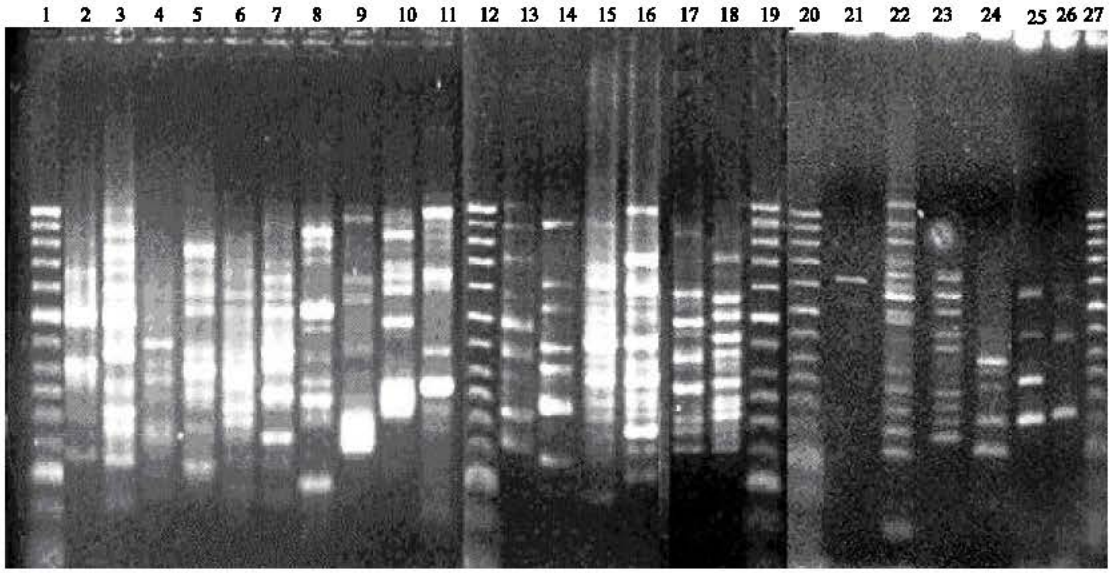

Fig. 3: RAPD profiles obtained with different primers loaded on 1.5% agarose gel. Lanes 1, 12, 19, 20 and 27: Marker II Hyperladder (Bioline); Lane 2 and Lane 3: Amplification of *C. verum* and *C. camphora* with OPC 08; Lane 4 and Lane 5: Amplification of *C. verum* and *C. camphora* with OPC 01; Lane 6 and Lane 7: Amplification of *C. verum* and *C. camphora* with OPC 10; Lane 8 and Lane 9: Amplification of *C. verum* and *C. camphora* with OPC 13; Lane 10 and Lane 11: Amplification of *C. verum* and *C. camphora* with OPB 20; Lane 13 and Lane 14: Amplification of *C. verum* and *C. camphora* with OPA 07; Lane 15 and Lane 16: Amplification of *C. verum* and *C. camphora* with OPL 07; Lane 17 and Lane 18: Amplification of *C. verum* and *C. camphora* with OPC 16; Lane 21 and Lane 22: Amplification of *C. verum* and *C. camphora* with OPB 19; Lane 23 and Lane 24: Amplification of *C. verum* and *C. camphora* with OPD 04; Lane 25 and Lane 26: Amplification of *C. verum* and *C. camphora* with OPD 09

Molecular studies

RAPD analysis: Eleven RAPD primers were used in DNA amplifications and all of them gave scorable PCR bands/loci. Band sizes ranged from 0.4 to 3.8 kb and the number of scorable bands per primer ranged from 1 to 14 with an average of 8. All the primers utilized were highly informative because they either amplified more than 5 polymorphic or monomorphic bands or amplified a polymorphic band which could differentiate between the two plant species (Fig. 3)

DISCUSSION

The observations and interpretations of this investigation are interesting as a preliminary exploratory

analysis of the genetic variation and micropropagation of the two *Cinnamomum* species.

The response of plant species to tissue culture is known to be affected by many factors including genotype, explant and media composition (Bhojwani *et al*., 1996; Tran Thanh Van, 1981; Murashige, 1990). This study has shown that the response of *C. camphora* to micropropagation seems to be dependent on the explant source as well as on the media more precisely with respect to the type and balance of plant growth regulators. Consequently the shoot tip explants of *C. camphora* responded to MS + cytokinins by forming multiple shoots whereas the leaf explants responded by forming compact callus. Moreover the effect of the cytokinins BAP and TDZ on multiple shoot formation of *C. camphora* seemed to be a function of the concentration of one hormone with respect to the other. As such shoot tip explants cultured on BAP only did not respond whereas the addition of TDZ (0.05-2.5 mg L^{-1}) resulted in multiple shoot formation in *C. camphora* shoot tip explants. This confirmed the fact that growth regulators work in conjunction with each other and have synergistic effects. Besides TDZ was more powerful than BAP in inducing adventitious shoot formation from shoot tip explants of *C. camphora*. TDZ is known to be more effective than BAP in shoot regeneration as reported in literature (Malik and Saxena, 1992). This could also be the reason why shoot tip explants cultured on BAP + TDZ produced much bigger shoots with broader leaves than those on TDZ only. The findings in our study differ from others (Huang *et al*., 1998) in that BAP stimulated multiple shoot development in shoot tip explants *C. camphora* whereas similar concentrations of TDZ did not induce multiple shoot formation but callus in the same explants. In fact callus was obtained from different explants namely leaf explants of *C. camphora*. This difference in response might reveal the existence of a Mauritian ecotype of *C. camphora*. In addition we did not observe the phenomenon of hyperhydricity in our regenerated plantlets. The difference in response of *C. verum* to the same media could be explained by morphological differences between these two species (Gurib-Fakim, 2002). In fact although both are woody evergreen species of the same genus, they differ in size at maturity and with respect to the type of trunk. *C. camphora* is an evergreen tree with vertical cracks in its bark and grows to 20 m height when mature whereas *C. verum* has a thick smooth bark and the adult tree does not exceed 10 m.

The present study is the first report on molecular work being carried on *Cinnamomum* species and in fact provides the first information on the genetic differences which exists between *C. verum* and *C. camphora* and shows that the large morphological differences between these species are actually reflected at the molecular level. The uniqueness of certain profiles and bands indicates that the RAPD analysis approach is directly applicable to assessing the genetic diversity between both species. RAPD markers seems to be a promising technique to assess the genetic variation for the 300 species within the *Cinnamomum* genus which can be used to generate a matrix of genetic distances with dissimilarity indices which can later be used in cluster analysis for the inference of genetic relationships.

In addition, RAPD markers may also provide a rapid technique for identifying and marking individual genotypes as elite genotypes. These could then be used for clonal propagation, *in vitro* selection and identification of high yielding essential oil producing strains. RAPD can be considered to be one of the techniques commonly used in germplasm characterization and similar studies have been carried out in crops including maize (Stojsin *et al*., 1996), potato (Hosaka *et al*., 1994; Demeke *et al*., 1996; Sosinski and Douches, 1996; McGregor *et al*., 2000), Brassica (Geraci *et al*., 2001) species and cassava collections (Zacarias *et al*., 2004). It can be concluded that RAPD analysis is a rapid and reliable technique for assessing genetic diversity between C. *verum* and *C. camphora* as a large degree of polymorphism has been obtained with the use of only 11 primers. The possibility of designing SCAR primers for each *Cinnamomum* species is very promising since most of the DNA amplification products are polymorphic.

Moreover, this study has confirmed the fact that the difference in response of *C. camphorum* and *C. verum* to tissue culture is due to their genetic difference, which is reflected at the morphological level (Bhojwani *et al*., 1996).

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