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High frequency plantlet regeneration from rhizomatous buds in *Mantisia spathulata* Schult. and *Mantisia wengeri* Fischer and analysis of genetic uniformity using RAPD markers

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A protocol has been devised for enhanced *in vitro* regeneration of critically endangered *Mantisia spathulata* Schult. and *Mantisia wengeri* Fischer. Highest Bud Forming Capacity (BFC) of 6.10±0.55 with an average of 19.93±3.19 roots was obtained for *M. spathulata* within 5-6 weeks in Murashige and Skoogs (MS) medium supplemented with a combination of 10.0μ*M* of N^6-benzyladenine (BA) and 2.5μ*M* of α-naphtalene acetic acid (NAA). For *M. wengeri*, BFC of 7.82±0.73 and 20.86±1.65 roots was achieved in MS media supplemented with a combination of 5.0μ*M* BA and 2.5μ*M* of NAA RAPD markers were used to evaluate the genetic stability of *in vitro* raised hardened plantlets. Similarity coefficient among the regenerated plants ranged between 0.85-0.98 for *M. spathulata* and 0.83-0.98 for *M. wengeri*. Maximum of 88 and 90% genetic similarity were obtained between *in vitro* raised hardened plantlets and mother stock of *M. spathulata* and *M. wengeri*, respectively through RAPD analysis. The hardened plantlets after RAPD analysis on being transferred to soil of experimental garden showed no marked phenotypic variations in vegetative or floral characteristics.

Keywords: *Mantisia spathulata*, *Mantisia wengeri*, Plant regeneration, RAPD analysis

Mantisia spathulata and *M. wengeri* are two critically endangered zingibers native to Mizoram, a North-eastern state of India. This area of India falls under the most poorly explored regions in Asia for plant diversity of all types[1] including many unique rare zingibers. Commonly known as 'dancing girl', *Mantisia* species are perennial herbs and therefore, floral spikes bearing numerous splendid flowers of *M. spathulata* (pale violet with yellow lip) and *M. wengeri* (yellow colour) appear before the onset of vegetative shoots during early monsoon. Due to natural calamities and human intervention, *M. spathulata* and *M. wengeri* have become critically endangered in the natural habitat [2] and are listed in the Red Data Sheet of rare and endangered Indian plants (www.envfor.nic.in/bsi/research.html). The rarity of *M. wengeri* has reached a critical level and has been included in the national priority list for its recovery by Department of Biotechnology, New Delhi, India [3]

For mass propagation and rapid recovery of rare and endangered zingibers, tissue culture techniques have been effectively used world wide [4-8]. However, the regenerants arising out of *in vitro* cultures are susceptible to genetic changes due to culture stress and mode of regeneration[9-11]. To minimize the risk of any cryptic variations arising out of *in vitro* cultures, it is crucial to assess the genetic uniformity of the *in vitro* plantlets with mother stock before their transfer to the field conditions. DNA markers offer a more attractive means for examining genetic similarity/dissimilarity since these markers are not developmentally regulated. Usefulness of Random Amplified Polymorphic DNA (RAPD) in detection of variation has been amply demonstrated in large array of *in vitro* plants by many workers[11-14]. Present report deals with the *in vitro* regeneration and RAPD analysis for finding genetic similarity of the *in vitro* regenerants of *M. spathulata* and *M. wengeri* with their respective mother stock.

Materials and Methods

Initiation of aseptic primary cultures—For initiating aseptic cultures, juvenile rhizomatous buds of *M. spathulata* and *M. wengeri* (collected from their natural habitats and maintained in the glass house of

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the Department) were washed in running tap water for 1h along with few drops of detergent (Tween-20, High Media, India). The buds were surface disinfected using 0.2% cetramide followed by surface sterilization with 0.1% $HgCl_2$ for 5min. Rhizomatous bud explants measuring 2.0 × 2.0cm were dissected out and cultured in tubes containing 20ml of Murashige and Skoogs (MS)[15] medium supplemented with 3% sucrose and BA (0.0, 4.4, 8.8, 13.2, 17.6, 22.2µ*M*) in combination with NAA (0.0, 2.7, 8.1, 16.2, 26.8µ*M*). The cultures were incubated at 25°±2°C under 12h photoperiod with a photosynthetic photon flux density (PPFD) of 40.5µmole $m^{-2}s^{-1}$ provided by cool white fluorescent lamps. Same cultural practices were followed for both the plants in all the experiments conducted. The miniature rhizomes from the *in vitro* aseptic shoots (0.5 - 1.5cm) were dissected out and sub cultured in their respective initiation medium so as to obtain sufficient cultures for standardizing the protocol for high frequency *in vitro* plantlet regeneration.

In vitro regeneration of plantlets—Rhizomatous bud explants (~ 2.5 × 2.5mm in size) from 4 weeks old *in vitro* - raised primary cultures were inoculated in MS medium supplemented with different concentrations of BA (2.5, 5.0, 10, 20 µ*M*) and NAA (2.5, 5.0, 10, 20µ*M*) in combination so as to optimize the best concentration for multiplication of shoot buds in the secondary cultures. Regeneration of shoot buds from each explant was calculated as the bud forming capacity using the formula, BFC = (average no of buds per explant) × (% of explants forming buds) ÷ 100 (Ref.8). Observation on percentage response, BFC and root number were made after 45 days of culture.

Statistical analysis—Ten replicates were maintained for each treatment and the experiment repeated thrice. Statistical analysis was done by Analysis of Variance (ANOVA) at 5% significant level and means compared using Tukeys test (PC version Origin 7.0. NORTHAMPTON, MA, USA).

Hardening and field transfer of plantlets—About 2 months-old *in vitro* raised plantlets, measuring 2.5-3.0cm in size were removed from culture vessels and washed with water to remove the agar containing medium. The plantlets were then potted to paper cups containing mixtures of soil and compost (1:1). The cups were covered with perforated polybags and sprinkled with water alternately at two days interval for the initial two weeks and acclimatized at 30°±2 °C and 70±5% RH under glass-house conditions. The established hardened plantlets were removed from the paper cups and transferred to the experimental garden after one year.

Random amplified polymorphic DNA (RAPD) analysis of the hardened plantlets—For RAPD analysis, five 6-month old hardened plantlets were randomly selected and compared for genetic similarity with mother stock of both the species. DNA was extracted with DNeasy Plant Mini Kit (QUAGEN) using 100mg of fresh leaves for each sample and dissolved in 100µl of buffer AE which yielded total DNA of ~50ng/µl DNA (1µl) was loaded in 1% (w/v) agarose gel and electrophoresed under 60V constant power supply for 3h and the amplification products were visualized in Geldoc (UVP biodoc-IT) system. In a pre-screen with 30 primers based on amplification of mother plant, 5 arbitrary decamer primers (Operon Technologies, USA) produced easily scorable distinct amplification profiles that were reproducible and hence selected for polymerase chain reaction (PCR). PCR was performed in a reaction volume of 25µl containing ~ 50ng template DNA, 1X Taq buffer A containing 1.5m*M* $MgCl_2$, 60µ*M* of each dNTP's, 0.05 units *Taq* DNA polymerase (Bangalore Genei Pvt. Ltd.) and 0.6ng primers (Operon Technologies, USA). Amplification was performed in a programmed thermal cycler (2700) supplied by Applied Biosystems (USA). After initial denaturation at 92°C for 3.30min., PCR was operated for 45 cycles consisting of a denaturing step (1min.), 35°C primer annealing step (1.30min.) and 72°C amplification (2min) step, at the end of the run final amplification period of 7min (72°C) was appended. Amplification products were separated in 1.5% agarose gels in 1X TBE buffers stained with ethidium bromide and photographed with UVP biodoc-IT system. The electrophoretogram was scored for the presence of a band (1) or its absence (0). The data were analysed using SIMQUAL (similarity for qualitative data) routine to generate Jaccard's similarity coefficient. These similarity coefficients were used to generate dendrograms using Unweighted Pair Group Methods with Arithmetic averages (UPGMA) employing the Sequential Agglomerative Hierarchic Non-overlapping (SAHN) programmes from NTSys PC version 2.02 k software. Similarity matrix was compared with dendrogram using MxComp and correlation coefficient of the association was obtained to determine the significance level.

Results

Initiation of aseptic primary cultures—Around 20 and 40% of rhizomatous bud explants of

M. spathulata and *M. wengeri* produced contamination-free rooted shoots, respectively. Axillary shoot buds (2-3) sprouted within 3-4 weeks (Fig. 1A, Fig. 2A) from the nodal portion of rhizomatous bud explants on initiation medium [MS supplemented with 8.8μ*M* BA+2.7μ*M* NAA for *M. spathulata* and BA (4.4μ*M*) + NAA (2.7μ*M*) for *M. wengeri*]. However, the explants of both the species remained non responsive even after 8 weeks of culture in MS medium incorporated with other concentrations of BA and NAA in combinations as well as in the control.

In vitro regeneration of plantlets—Bud regeneration potentiality was maximized by using the secondary explants obtained from primary cultures. Highest BFC of 6.10±0.55 with an average of 19.93±3.19 roots was obtained within 5-6 weeks for *M. spathulata*, in MS medium supplemented with BA (10.0μ*M*) and NAA (2.5μ*M*) (Fig. 1B, Table 1). A combination of BA at higher concentrations of NAA reduced the bud forming capacity and formed clumped roots. Similarly, BFC of 7.82±0.73 with an average of 20.86±1.65 roots was achieved within 5-6 weeks for *M. wengeri* in MS medium containing BA (5.0μ*M*) and NAA (2.5μ*M*) (Fig. 2B, Table 1). Although the BFC of *M. wengeri* got significantly reduced at higher concentrations of BA and NAA in combinations, the roots formed were normal. However, the roots of *M. spathulata* got clumped into globular mass at higher concentrations of BA and NAA. In both the species, the explants kept in MS control (devoid of any growth regulators) although showed more than 90% response, the BFC and root regeneration was however significantly less in comparison to medium supplemented with growth regulators.

Hardening and field transfer of plantlets—More than 90% plantlets of both the species got hardened and acclimatized within 8 weeks for both the species (Fig. 1C, 2C). So far all the plantlets transferred to field, established without any loss of plantlets and maintained for past two years.

RAPD analysis of hardened plantlets—Size of bands produced by the primers ranged from 500 bp with primers OPA 2, OPA 3, OPC2, OPC 8 and OPC 15 to 3250 bp with primer OPA 2 for *M. spathulata* (Fig. 3). On the other hand, the size of bands produced by the same primers ranged from ~ 800 bp

Fig. 1—(A)–Initiation of primary cultures of *M. spathulata* in MS medium containing BAP (8.8μ*M*) + NAA (2.7μ*M*) within 3 - 4 weeks; (B) Regeneration of *M. spathulata* in MS medium supplemented with BAP (10.0μ*M*) and NAA (2.5μ*M*) within 5 - 6 weeks; (C) Hardened plantlets of *M. spathulata* after 6 months in glass house; and (D) Re-established plantlets of *M. spathulata* in the experimental garden after two years

Fig. 2—(A)–Initiation of primary cultures of *M. wengeri* in MS medium containing BAP (4.4μ*M*) + NAA (2.7μ*M*) within 3 - 4 weeks; (B) Regeneration of *M. wengeri* in MS medium supplemented with BAP (5.0μ*M*) and of NAA (2.5μ*M*) within 5 - 6 weeks; (C) Hardened plantlets of *M. wengeri* after 6 months in glass house; and (D) Re-established plantlets of *M. wengeri* in the experimental garden after two years

with primers OPC 15 to ~ 3000 bp with primer OPA 2, OPA 3, OPC 2, OPC 8 and OPC 15 for *M. wengeri* (Fig. 4). From the analysis of the data the test for association using MxComp yielded the matrix correlation coefficient (r) as 0.917 and 0.778 for *M. spathulata* and *M. wengeri* respectively which was highly significant. From SIMQUAL generated matrix data it was observed that similarity coefficient ranged between 0.85-0.98 among regenerated plants of *M. spathulata* and 0.83-0.98 among regenerated plants of *M. wengeri* (Table 2). From the matrix generated dendrogram it was ampy clear that all the *in vitro* raised plants of both *M. spathulata* and *M. wengeri* had shown distant similarities with mother plant stock (Figs. 5, 6).

Discussion

In vitro induction and multiplication of shoots represents the first step in micropropagation[16]. Raising aseptic cultures from underground rhizomes in zingibers happen to be a challenging task due to excessive contamination of explants under *in vitro* conditions as reported in *Zingiber officinale* Rosc[17]. Once aseptic shoots buds are induced the successive multiplication rate gets maximized under *in vitro* conditions in most of zingibers such as *Zingiber officinale*, Rosc and *Curcuma longa*[18]. Efficiency of rhizomatous buds for high shoot bud regeneration has been reported in large number of other zingibers such

Table 2—Similarity coefficient among mother plant and *in vitro* raised hardened plantlets of *M. spathulata* and *M. wengeri* based on RAPD markers

	In vitro plantlets					
	1°	2	3	4	5	6
(*M. spathulata*)						
1°	1.00					
2	0.91	1.00				
3	0.85	0.89	1.00			
4	0.89	0.98	0.91	1.00		
5	0.87	0.96	0.93	0.98	1.00	
6	0.87	0.96	0.93	0.98	0.96	1.00
(*M. wengeri*)						
1°	1.00					
2	0.93	1.00				
3	0.98	0.91	1.00			
4	1.00	0.93	0.98	1.00		
5	0.90	0.83	0.88	0.90	1.00	
6	0.95	0.88	0.93	0.95	0.95	1.00

1°- Mother plant

Table 1—Effect of BA and NAA in MS medium on the regeneration of *M. spathulata* and *M. wengeri*
[Values are mean ± SE of three experiments with ten replicates/experiment]

BA+NAA (µ*M*)	Response of *M. spathulata* (%)	BFC	Root no.	Response of *M. wengeri* (%)	BFC	Root no.
Control	93.3±6.6^{a}	1.42±0.1^{a}	3.0±0.4^{a}	93.3±6.6^{a}	2.09±0.44^{a}	4.3±1.05^{a}
2.5+2.5	93.3±6.6^{a}	3.16±0.4^{a}	7.8±0.75^{a}	73.3±6.6^{a}	2.04±0.37^{a}	13.8±1.82^{bc}
5.0+2.5	93.3±6.6^{a}	4.25±0.6^{b}	11.0±0.90^{b}	93.3±6.6^{a}	7.82±0.73^{bc}	20.86±1.65^{bc}
10.0+2.5	93.3±6.6^{a}	6.10±0.5^{bc}	19.93±3.19^{bc}	93.3±6.6^{a}	5.73±0.58^{b}	13.46±0.89^{bc}
20.0+2.5	93.3±6.6^{a}	2.60±0.5^{a}	18.40±0.87^{bc}	93.3±6.6^{a}	3.36±0.50^{a}	8.03±1.33^{a}
2.5+5.0	66.6±6.6^{a}	1.33±0.3^{a}	11.73±1.09^{b}	60.0±11.5^{b}	1.32±0.56^{a}	6.86±1.43^{a}
5.0+5.0	73.3±6.6^{a}	2.25±0.4^{a}	15.83±1.85^{bc}	73.3±6.6^{a}	2.45±0.41^{a}	7.93±0.75^{a}
10.0+5.0	60.0±0.0^{a}	2.28±0.3^{a}	17.56±2.01^{bc}	86.6±6.6^{a}	2.46±0.60^{a}	10.9±0.86^{a}
20.0+5.0	53.3±6.6^{b}	1.32±0.1^{a}	12.9±1.86^{bc}	93.3±6.6^{a}	3.93±0.83^{a}	4.73±0.73^{a}
2.5+10.0	60.0±0.0^{b}	0.85±0.1^{a}	6.1±1.7^{a}	66.6±6.6^{a}	1.25±0.43^{a}	18.93±2.46^{bc}
5.0+10.0	40.0±0.0^{b}	1.32±0.1^{a}	CM	80.0±11.5^{a}	2.02±0.63^{a}	14.8±2.45^{bc}
10.0+10.0	40.0±0.0^{b}	0.32±0.0^{a}	CM	80.0±11.5^{a}	2.30±0.64^{a}	12.0±1.5^{a}
20.0+10.0	40.0±0.0^{b}	0.25±0.0^{a}	CM	73.3±13.3^{a}	1.49±0.66^{a}	7.86±0.78^{a}
2.5+20.0	46.6±6.6^{b}	0.24±0.0^{a}	CM	20.0±0.0^{b}	0.08±0.08^{a}	2.73±1.39^{a}
5.0+20.0	-	-	-	20.0±0.0^{b}	0.1±0.07^{a}	3.16±2.32^{a}
10.0+20.0	-	-	-	26.6±6.6^{b}	0.5±0.46^{a}	3.6±1.97^{a}
20.0+20.0	-	-	-	26.6±6.6^{b}	0.3±0.26^{a}	0.66±0.16^{a}

Values followed by the same letter are not significantly different according to ANOVA ($P \leq 0.05$) and Tukey's test; - no response; CM clump as *Zingiber officinale*[17], *Curcuma longa*[18], *Alipinia galanga*[5] etc. An optimum concentration of BA in combination with NAA has been found to be very effective in high shoot bud proliferation with simultaneous root formation in rare species like *Kaempferia galanga*[6] and *Zingiber petiolatum*[7]. Use of BA in combination with NAA has also been found to be effective in the enhancement of shoot buds and root formation in our investigation with the two species.

Genetic variation from *in vitro* cultures has been reported in some species. Variations to an extent of 26% have been reported in micropropagated plants of *Populus deltoides*[19]. Similarly, there are reports of genetic similarity coefficients in the range of 84-97% in field transferred plants of *Angelica acutiloba*[20] and 86-96% in shoot bud regenerated micropropagated plants of *Robinia pseudoacacia*[11]. The present estimations of about 88 and 90% similarity for *M. spathulata* and *M. wenger*, respectively with their mother plant are near to the acceptable level. By this

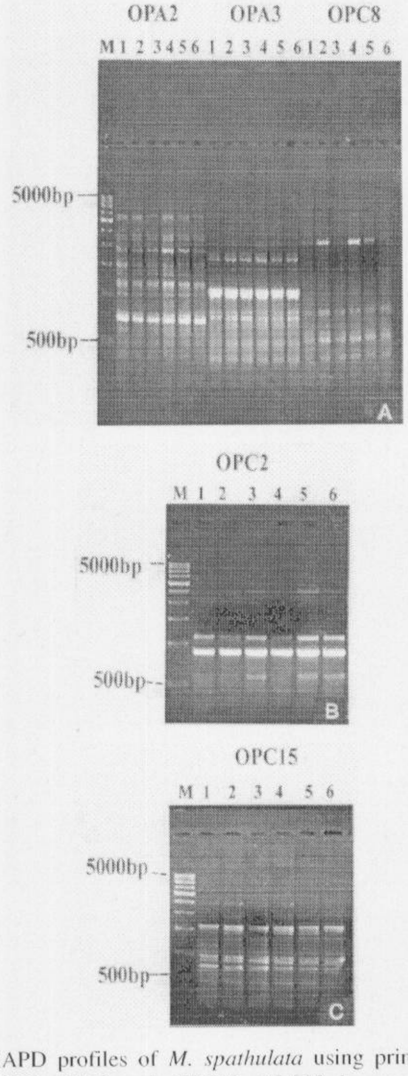

Fig. 3—RAPD profiles of *M. spathulata* using primers OPA2, OPA3, OPC8 OPC2 and OPC15, M-500 bp markers, Lane 1–mother plant, Lane 2, 3,4,5,6 – *in vitro* regenerated hardened plantlets

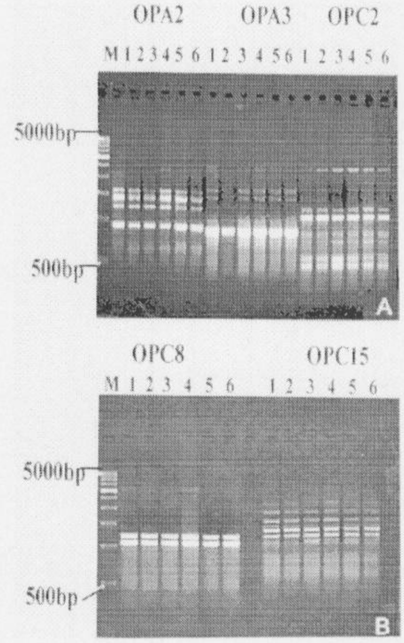

Fig 4—RAPD profiles of *M. wengeri* using primers OPA2, OPA3, OPC2, OPC8and OPC15, M- 500 bp markers, Lane 1–mother plant, Lane 2, 3,4,5,6–*in vitro* regenerated hardened plantlets

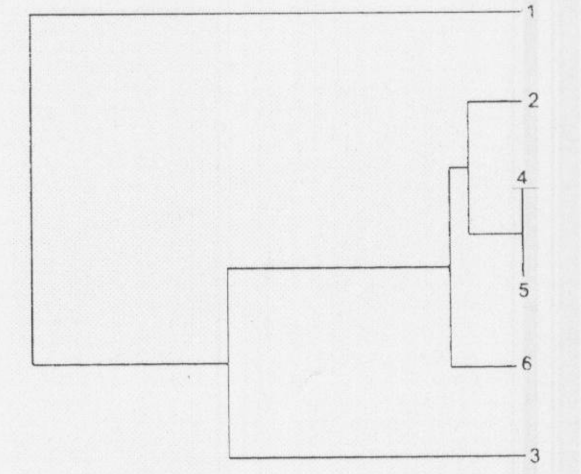

Fig. 5—Dendrogram showing the relationship of *in vitro* regenerated hardened plantlets of *M. spathulata* with their mother stock

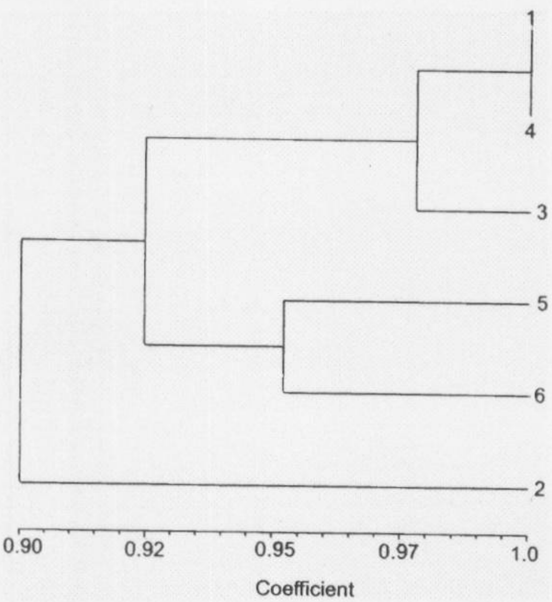

Fig. 6—Dendrogram showing the relationship of *in vitro* regenerated hardened plantlets of *M. wengeri* with their mother stock

parameter the regenerated plants produced presently for both *M. spathulata* and *M. wengeri* can be categorized as normal plants and equivalent with mother stock with regard to genetic make up.

Genetic variations under *in situ* conditions in most of zingibers including *Mantisia* are sluggish due to the clonal propagation through rhizomes[18] and lack of seed propagation[21, 22]. In this regard, minor genetic variations arising out of *in vitro* cultures without marked phenotypic alterations are expected to be beneficial for faster adaptability under varied climatic conditions and prolonged sustainability under biotic and abiotic stresses. Around 500 hardened plantlets of both the species after being transferred to the soil of experimental gardens got acclimatized under the identical climatic conditions of Lunglei, Mizoram which are prevalent in Shillong, Meghalaya of North-eastern India, where the experiments were conducted (Fig. 1D, 2D). Around 80% of the plantlets flowered after 2 years of field transfer without any marked phenotypic variations in floral or vegetative characteristics. Therefore, our novel strategy for large scale *ex situ* conservation of the two endemic plant species in the experimental garden is of paramount interest as it would lead to sustainability of the germplasm for many years to come.

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