

Full Length Research Paper

Rhizome derived callogenic plantlet production of *Mantisia wengeri* (Zingiberaceae), a rare and endemic medicinal plant of Mizoram, North-East India

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Mantisia wengeri Fischer is a rare and endemic zingiber of North-East India which has been traditionally used for curing bone fracture and gastro-intestinal ailments. However due to the extreme rarity of the germplasm, studies related to genetic diversity, phytochemical properties etc. in this plant has not been feasible. The study describes the development of high efficiency regeneration protocol for obtaining large- scale diverse plantlets from rhizomatous callus tissues in Murashige and Skoog medium incorporated with various concentration of growth regulators N6-benzyladenine (BA), α -Naphthalene acetic acid (NAA), 2, 4-dichlorophenoxyacetic acid (2,4-D) and sucrose at elevated concentrations. Bud forming capacity (BFC) to a maximum of 33.5 ± 3.7 was recorded from the organogenic callus tissues of *M. wengeri* within 8 - 10 weeks in MS medium supplemented with a combination of $10 \mu\text{M}$ of BA and $40 \mu\text{M}$ NAA at 10% sucrose. The callogenic plantlets are been transferred to the field for studying morphological and genetic variations.

Key words: - Conservation, regeneration, genetic variation, rhizomatous, callus

INTRODUCTION

Mantisia wengeri is a highly fascinating zingibers endemic to a single population in Lunglei, Mizoram, North-East India. The shape and posture of the flowers of *Mantisia* with the spread out arm-like staminodes and the skirt-like yellow coloured flared lip has earned these plants the popular name of 'dancing girls' and is suspected to be a hybrid of *M. spathulata* and *M. radicalis* (Dam et al., 1997). The rhizome of this plant has been used as a remedy for bone fracture and gastro-intestinal ailments in the past by local people. However, due its extreme rarity, studies on phyto-chemical properties of the rhizome have not been feasible. The rarity of *M. wengeri* has reached a critical level with only 60 - 70 plants and therefore been included in the national priority list for its recovery by Department of Biotechnology, New Delhi, India (Ganeshiah, 2005). In an effort to save the species from extinction, *in vitro*

techniques were used for high frequency plantlet regeneration following *ex situ* reintroduction in the Botanical Garden for recovery (Bhowmik et al., 2009). These plantlets were clonally derived from their mother plants without any significant genetic variations. However, successful germplasm conservation of endangered plants also requires genetic variations for long-term sustainability. Genetic variations under natural conditions in most of the zingibers including the species of *Mantisia* are sluggish because of their clonal propagation from rhizomes (Nadgauda et al., 1978) and lack of seed propagation (Sajina et al., 1997; Miceli et al., 2008; Bhowmik et al., 2010). Loss of genetic diversity results in disturbance of gene frequency in endangered species and thereby reduces evolutionary potential to adapt to changing environments (Tamaki et al., 2008). Under *in vitro* condition mature plant cells are able to revert to meristematic state and form undifferentiated callus tissues. These callus tissues can either redifferentiate into shoot or root organ and hence termed

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as organogenic callus or remain callogenic without redifferentiating into organs and hence termed as non-organogenic callus tissues. Regeneration of plantlets from undifferentiated callus tissues has been reported to be the potent source of producing soma-clonal variation in plants including many zingibers of medicinally importance for the improvement of species (Agrawal and Sardar, 2006). Owing to the need to sustain the genetic variability for the diminishing populations of *M. wengeri* the present study was undertaken to develop an efficient protocol for regenerating plantlets from the undifferentiated callus tissues.

MATERIAL AND METHODS

Initiation of aseptic primary cultures

Juvenile rhizomatous buds were selected as a starting material for raising the primary cultures from the limited plant material available. These were washed in running tap water for 1h along with few drops of detergent tween-20 as a measure to remove the dirt from the plant material. Rhizomatous shoot bud explants (~2 x 1.5 cm) were desiccated out and surface disinfected using 0.2% cetramide for 10min followed by surface sterilization using 0.1% mercuric chloride for 5mins. The explants were then inoculated in culture tubes containing 20ml of sterilised MS medium (Murashige and Skoog, 1962), supplemented singly with BA (0.0 - 22.2 μM) and Kinetin (KN, 0.0 - 23.2 μM) as well as in combination with NAA (0.0 - 26.8 μM). The pH of the media was adjusted to 5.8 prior to autoclaving. The culture tubes were incubated at 25 ± 2 °C under 12 h photoperiod of 54 $\mu\text{moles m}^{-2}\text{s}^{-1}$ light intensity. The medium responsible for shoot proliferation was considered as the initiation medium. The experiments were repeated thrice with ten replicates per treatment observations were made after 45 days of culture. Statistical analysis was done by ANOVA ($P < 0.05$) and means compared using Tukeys test (software Origin 7.0).

Induction of callus and proliferation of shoots

The miniature rhizomes from *in vitro* aseptic shoots (0.5 - 1.5 cm) were dissected out and subcultured in their respective initiation medium so as to obtain sufficient cultures for standardising the protocol for callus induction. Rhizomatous bud explants (~ 2.5 x 2.5 mm in size) from 4 weeks old *in vitro*-raised primary cultures were inoculated in MS medium supplemented with various combination of BA and NAA and BA and 2,4-D at 5, 10, 20, 40 μM for induction of callus. Sucrose at 3% and 10% were used in the medium for induction of callus and shoots regeneration. The cultures were kept in dark as well as in light with 12 h photoperiod of 54 $\mu\text{moles m}^{-2}\text{s}^{-1}$ light intensity. Observations were made on the percentage of explants forming organogenic as well as non-organogenic callus and frequency of shoot proliferation from the organogenic callus at weekly intervals and final record was made after 10 weeks of culture. Regeneration of shoot buds from callus mass was calculated as the bud forming capacity using the formula (Tandon et al., 2007).

$$\text{BFC} = (\text{average no of buds per callus mass}) \times (\% \text{ of callus mass forming buds}) \div 100$$

Experiments were repeated thrice with ten replicates per treatment and standard error of the mean value was calculated.

Rooting and hardening of plantlets

The shoots (1.5 - 2.0 cm) induced from the callus were separated from the mother tissues and transferred to $\frac{1}{2}$ MS medium

supplemented with different concentrations of IBA and indole-3-acetic acid (IAA) singly at 0.25, 0.5, 2.5, 5.0 μM . About 8 weeks-old *in vitro*-raised plantlets, measuring 2.5 - 3.0 cm 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 \pm 5% RH under glass-house conditions.

RESULTS AND DISCUSSION

Plants of *M. wengeri* flowered during the month of May, 2007 (Figure 1A) followed by the emergence of new vegetative shoots from the rhizome. Around 40% of the *in vivo* rhizomatous bud explants produced contamination-free rooted shoots under *in vitro* conditions. Around 2 - 3 axillary shoot buds sprouted within 3 - 4 weeks (Figure 1B) from the nodal portion of rhizomatous bud explants on initiation medium [MS supplemented with 4.4 μM BA + 2.7 μM NAA]. However, the explants remained non-responsive in the control as well as in MS medium incorporated with various concentrations of BA, KN and NAA singly and in combinations. The rhizomes from the aseptic shoots were multiplied in their initiation medium to raise sufficient explants for conducting experiments for callus induction.

The explants cultured in complete darkness failed to give any callus response. However, two types of callus were obtained from the rhizomatous explants within 4 weeks in MS medium supplemented with growth regulators BA, NAA and 2, 4-D at various combinations under 12 h photoperiod of 54 $\mu\text{molm}^{-2}\text{s}^{-1}$ light intensity. White friable callus that turns green and forms shoots organs were considered as organogenic callus (OC) while the whitish-brown callus that does not turn green for shoot formation was considered as non-organogenic callus (NOC). These types of organogenic callus (Figure 1C) were reported from the cotyledon derived explants of *Vigna radiata* (Amutha et al., 2003) and rhizome explants in zingibers like *Curcuma zedoaria* and *Zingiber officinale* (Miachir et al., 2004; Jamil et al., 2007). At lower concentrations of auxin viz. NAA and 2, 4-D combined with BA at different concentrations the explants failed to form these types of callus and either regenerated into complete shoots or remained non-responsive. However, the explants were recorded to differentiate into both types of callus tissues within 4 weeks in MS medium supplemented with BA at higher concentrations of auxins NAA and 2, 4-D (Table 1, 2). The overall frequency of explants forming callus in MS medium supplemented with a combination of 2, 4-D and BA was significantly higher than the frequency of callus formed in MS medium supplemented with a combination of NAA and BA (Table 2). However, the percentage of NOC was significantly higher with the combined effect of 2, 4-D and BA as compared to NAA and BA combinations. It was noted by enhancing the percentage of sucrose from 3 to 10% the conversion frequency of

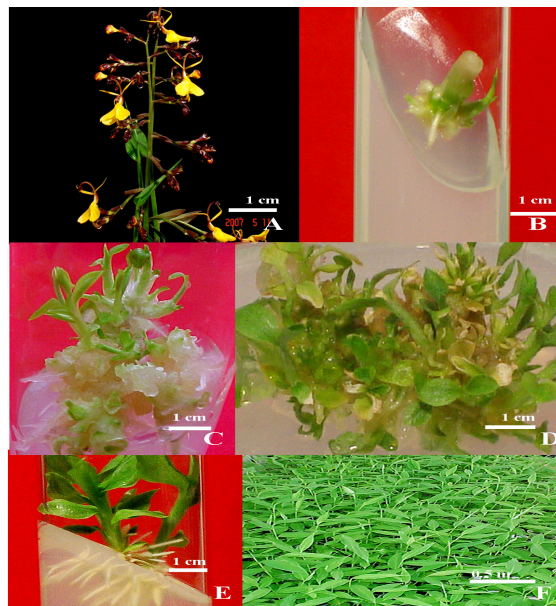


Fig 1.

Figure 1.

- A. Inflorescence of *M. wengeri*
- B. Initiation of aseptic shoots of *M. wengeri* within 3 - 4 weeks
- C. Induction of organogenic callus of *M. wengeri* within 4 weeks
- D. Proliferation of multiple shoots of *M. wengeri* from organogenic callus in MS medium supplemented with 10 μ M of BA and 40 μ M NAA at 10% sucrose
- E. Healthy rooting of *in vitro* shoots of *M. wengeri* within 2 weeks in $\frac{1}{2}$ MS medium supplemented with 0.5 μ M IBA
- F. *In vitro* plantlets of *M. wengeri* weaned in a mixture of soil and compost (1:1) within 4 weeks

shoots buds from the organogenic callus tissues gets significantly elevated. As compared to callus obtained in 3% sucrose, the friable whitish callus obtained at 10% sucrose initially formed greenish miniature rosette like globular structures after 2 weeks of callus induction and finally transformed into numerous small shoots. Shoot formation in tobacco callus cultures through osmotic inhibition using high levels of sucrose (10 - 15%) in the medium has been reported earlier (Narayanaswami, 2004). Similarly, sucrose mediated multiplication of shoot has been achieved in *Corylus avellana* (Yu et al., 1993). In nature, sugars are transported within the plants cells as sucrose and the tissue may have the inherent capacity for uptake, transport and utilization of sucrose (Sul and Korban, 2004). In the present study, a maximum of 33.5 \pm 3.7 BFC has been recorded from the organogenic callus tissues of *M. wengeri* within 8 - 10 weeks in MS medium supplemented with a combination of 10 μ M of BA and 40 μ M NAA at 10% sucrose (Table 1; Figure. 1D). Similar to our results, in most of the plants, a combination of high amount of auxins (2, 4-D or NAA) with lower cytokinins (BA or KN) have been widely used for the initiation of organogenic callus (Caboni et al., 2000; Ruguni and Muganu, 1998; Haliloglu, 2006; Rani et al.,

2006). In the present investigation, the combined effect of BA and NAA supplemented with elevated level of sucrose was recorded to be optimum for organogenic callus induction and simultaneous shoot proliferation without subculture. This result is in contradictory to the callus obtained in case of *Curcuma longa* where BA and NAA played a vital role for the induction of callus but the regeneration of shoots was achieved only by subculturing the callus in BA and TIBA incorporated MS medium (Salvi et al., 2001).

The number of roots formed from the *in vitro* shoots was significantly higher in $\frac{1}{2}$ MS medium supplemented with IBA as compared to IAA supplemented MS medium. A maximum of 11.6 \pm 0.3 healthy roots were regenerated from the *in vitro* shoots within 2 weeks in $\frac{1}{2}$ MS medium supplemented with 0.5 μ M IBA (Table 3; Figure 1E). The roots formed from the shoots in IAA supplemented $\frac{1}{2}$ MS medium was thin and less in number as compared to roots obtained in IBA supplemented medium. More than 90% of the *in vitro* plantlets were weaned in a mixture of soil and compost (1:1) within 4 weeks under glass house condition and finally transferred to the field (Figure 1F). Somaclonal variation has been suggested as a tool for increasing the genetic diversity in species with a very

Table 1. Synergistic effects of BA and NAA conjugated with sucrose in MS medium on callus induction and shoot regeneration of *M. Wengeri* [Values are mean \pm SE (*) of three experiments with ten replicates/experiment]

	BA+NAA (μ M)			Sucrose (3 %)			Sucrose (10 %)			
		% NOC*	% OC*	BFC*	% NOC*	% OC*	BFC*	% NOC*	% OC*	BFC*
Control	-	-	-	-	-	-	-	-	-	-
5.0+5.0	-	-	-	-	-	-	-	-	-	-
10.0+5.0	-	-	-	-	-	-	-	-	-	-
20.0+5.0	-	-	-	-	-	-	-	-	-	-
40.0+5.0	-	-	-	-	-	-	-	-	-	-
5.0+10.0	-	-	-	-	-	-	-	-	-	-
10.0+10.0	-	-	-	-	-	-	-	-	-	-
20.0+10.0	-	-	-	-	-	-	-	-	-	-
40.0+10.0	-	-	-	-	46.6 \pm 3.3 ^a	-	-	-	-	-
5.0+20.0	-	-	-	-	56.6 \pm 3.3 ^a	-	-	-	-	-
10.0+20.0	-	-	-	-	66.6 \pm 3.3 ^a	-	-	-	-	-
20.0+20.0	-	-	-	-	73.3 \pm 3.3 ^a	-	-	-	-	-
40.0+20.0	36.6 \pm 3.3 ^a	63.3 \pm 3.3 ^a	4.60 \pm 0.87 ^b	43.3 \pm 3.3 ^{ac}	56.6 \pm 3.3 ^b	7.30 \pm 0.5 ^{bc}				
5.0+40.0	46.6 \pm 3.3 ^a	53.3 \pm 3.3 ^a	10.6 \pm 1.35 ^b	33.3 \pm 3.3 ^{bc}	66.6 \pm 3.3 ^b	16.0 \pm 2.3 ^b				
10.0+40.0	76.6 \pm 6.6 ^a	23.3 \pm 6.6 ^a	13.0 \pm 1.50 ^b	3.30 \pm 3.3 ^b	96.6 \pm 3.3 ^a	33.5 \pm 3.7 ^a				
20.0+40.0	70.0 \pm 5.7 ^a	30.0 \pm 5.7 ^a	20.9 \pm 3.28 ^a	10.0 \pm 5.7 ^b	90.0 \pm 5.7 ^a	26.6 \pm 2.1 ^a				
40.0+40.0	63.3 \pm 3.3 ^a	36.6 \pm 3.3 ^a	17.0 \pm 1.53 ^a	23.3 \pm 3.3 ^b	76.6 \pm 3.3 ^{ab}	22.2 \pm 1.9 ^b				

Values followed by same letter are not significantly different according to ANOVA ($P \leq 0.05$) and Tukey's test; non callus response (-); NOC (Non Organogenic Callus); OC (Organogenic Callus)
Data recorded after 10 weeks of culture

Table 2. Synergistic effects of BA and 2, 4-D conjugated with sucrose in MS medium on callus induction and shoot regeneration of *M. Wengeri* [Values are mean \pm SE (*) of three experiments with ten replicates/experiment]

BA+2,4-D (μ M)	Sucrose (3 %)			Sucrose (10 %)		
	% NOC*	% OC*	BFC*	% NOC*	% OC*	BFC*
Control	-	-	-	-	-	-
5.0+5.0	-	-	-	-	-	-
10.0+5.0	-	-	-	-	-	-
20.0+5.0	-	-	-	-	-	-
40.0+5.0	-	-	-	-	-	-
5.0+10.0	50.0 \pm 5.7 ^b	-	-	-	-	-
10.0+10.0	53.3 \pm 3.3 ^b	-	-	-	-	-
20.0+10.0	63.3 \pm 3.3 ^a	-	-	-	-	-
40.0+10.0	76.6 \pm 3.3 ^a	-	-	-	-	-
5.0+20.0	80.0 \pm 0.0 ^a	-	-	73.3 \pm 3.3 ^a	26.6 \pm 3.3 ^b	18.5 \pm 0.96 ^{bc}
10.0+20.0	86.6 \pm 3.3 ^a	-	-	46.6 \pm 3.3 ^a	53.3 \pm 3.3 ^b	20.4 \pm 0.94 ^{ac}
20.0+20.0	90.0 \pm 0.0 ^a	10.0 \pm 0.0 ^a	5.6 \pm 0.86 ^a	30.0 \pm 0.0 ^b	70.0 \pm 0.0 ^a	22.8 \pm 2.2 ^{ac}
40.0+20.0	90.0 \pm 5.7 ^a	10.0 \pm 5.7 ^a	4.36 \pm 0.56 ^b	16.6 \pm 3.3 ^b	83.3 \pm 3.3 ^a	24.5 \pm 1.3 ^{ac}
5.0+40.0	43.3 \pm 3.3 ^b	56.6 \pm 3.3 ^a	5.2 \pm 0.72 ^a	26.6 \pm 3.3 ^b	56.6 \pm 3.3 ^a	6.20 \pm 0.5 ^b
10.0+40.0	53.3 \pm 3.3 ^b	46.6 \pm 3.3 ^a	7.73 \pm 0.63 ^a	43.3 \pm 3.3 ^b	73.3 \pm 3.3 ^a	9.53 \pm 1.1 ^b
20.0+40.0	86.6 \pm 3.3 ^a	10.0 \pm 0.0 ^a	3.53 \pm 0.75 ^b	16.6 \pm 3.3 ^b	90.0 \pm 5.7 ^a	16.63 \pm 0.9 ^b
40.0+40.0	83.3 \pm 3.3 ^a	16.6 \pm 3.3 ^a	1.6 \pm 0.80 ^b	10.0 \pm 5.7 ^b	83.3 \pm 3.3 ^a	13.3 \pm 0.76 ^b

Values followed by same letter are not significantly different according to ANOVA ($P \leq 0.05$) and Tukey's test; non callus response (-); NOC (Non Organogenic Callus); OC (Organogenic Callus)
Data recorded after 10 weeks of culture

narrow genetic base such as the Easter Island endemic, *Sophora toromiro* (Jacobsen and Dohmen, 1990). Thus,

the propagation of *M. wengeri* plants through callus tissues enhances the possibilities of maintaining genetic

Table 3. Effects of IBA and IAA in ½ MS medium on rooting of *in vitro* shoots of *M. Wengeri* [Values are mean ± SE (*) of three experiments with ten replicates/experiment]

IBA (µM)	Root no. *	IAA (µM)	Root no. *
Control	2.0±0.2 ^a	Control	1.6±0.82 ^a
0.25	7.8±0.92 ^b	0.25	3.8±0.72 ^a
0.50	11.6±0.3 ^b	0.50	5.6±2.2 ^{ab}
2.5	4.6±1.2 ^a	2.5	8.6±0.6 ^b
5.0	3.3±1.6 ^a	5.0	4.8±0.25 ^a

Values followed by same letter are not significantly different according to ANOVA ($P \leq 0.05$) and Tukey's test
Value Data recorded after 2 weeks of culture

diversity. Although, traditionally it has been used as a medicinally useful zingiber, scientific evaluation of its phyto-chemical constituents has not been reported till date. In this regard, induction of callus from the rhizome materials would help in exploring this rare zingiber for phyto-chemical and bio-chemical analysis under *in vitro* condition for its medicinal uses. In addition to this, the high efficiency *in vitro* regeneration protocol would be highly beneficial for carrying out *Agrobacterium* mediated genetic transformation for functional genomic studies on various novel medicinal genes from zingiberaceae species. The regenerated plantlets are currently being screened in the field for the studying morphological and genetic variations.

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