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An efficient Multiple shoots induction from Zygotic embryos and rhizomes of *Zingiber roseum* Rosc, an endangered medicinal plant

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ABSTRACT

A protocol of high efficient multiple shoot induction and plant regeneration from immature embryo and rhizome has been developed for Zingiber roseum – an endangered medicinal plant. The optimum (94%) of multiple shoots was obtained on woody medium supplemented with 3.21 µM NAA in combination with 17.68 µM BAP at 7.4 shoots per explants. In vitro shoots developed optimally (95.4%) rooted upon transferring onto the rooting medium with 2.68 µM NAA with 6.4 roots per shoot. The in vitro raised plantlets with well developed shoot and roots were successfully established in moistened sterile mixture of garden soil: sand: vermicompost in 1:1:1 ratio and grown in greenhouse for acclimatization at 23 ± 2 ^{0}C temperature and 18 h photoperiod with 30 µmol m^{-2} s^{-1}at light intensity.

Keywords: *In vitro* propagation, Embryo culture, *Zingiber roseum* Rosc., Zygotic Embryo, Multiple shoots.

INTRODUCTION

Zingiber roseum Rosc. (Zingiberaceae) is an important endangered medicinal herb and the plant is grown rarely, scattered and widely in himalayan region [1]. The ethanobotanical data from tribal and other Indian ethnic groups documented about 31 species of *Zingiberaceae* used in Indian and oriental medicines and as spices and food flavouring agents [2]. The rhizomes of this species are used among tribal communities in Central India for fever and rheumatism [3-5]. Antifertility and myorelaxant activities of stem extract of *Zingiber roseum* has been reported [6-8]. *Zingiber roseum* seed oil (ZRSEO) has been found to possess a dual response (contraction and relaxation) and spasmolytic effect in rat duodenal smooth muscle. The seeds of *Zingiber roseum* contain 60 compounds of which 47 were identified as oil (96.2%) and the major compounds are α-pinene, β-pinene, limonene, p-cymene, α-terpineol and verticiole. The unique

feature is the dominant presence of mono- and sesquiterpene hydrocarbons which make about 82% of the oil [9]. According to CAMP/IUCN reports, now it is under endangered condition now [10]. Due to the over-expoitation of *Zingiber roseum* from the nature and inadequate efforts for its cultivation resulted in marked decline in the population of this species. Therefore, it is necessary to develop *in vitro* propagation and conservation of this endangered medicinal plant. The present communication is the first report on an efficient *in vitro* regeneration from immature embryos of *Zingiber roseum* through direct regeneration.

MATERIALS AND METHODS

Plant material and surface sterilization

Mature seeds of *Zingiber roseum* were collected from Rampachodavaram region, East Godavary district, India. The herbarium was deposited in Botany department, Andhra university, Visakhapatnam, India, and the number is A.U. (B.D.H.) 20376.

These seeds and rhizomes were washed under running tap water for 45 min and then soaked in 2% (*v/v*) Tween-20 for 15 min. After through washing, their surfaces were sterilized with 70% (*v/v*) ethanol for 3 min followed by 0.1% (*w/v*) $Hgcl_2$ for 5 min and finally the seeds were rinsed 5 times with sterile deionized water. The seed coat was removed with scalpel and forceps under a laminar flow hood and inoculated on sterile woody plant medium.

Culture media and culture conditions

The immature embryos and rhizomes were inoculated on a sterile woody medium containing basal salts with 3% sucrose and 0.8% agar. The pH of the medium was adjusted to 5.8 by 1 N NaOH or 1 N HCl before being autoclaved at 121^0C for 15 min. All of the inoculated cultures were incubated under 50 µmol m^{-2} s^{-1} light provided by cool white fluorescent lamp for a photoperiod of 16 h at 23 ± 2^0C.

Multiple shoot induction

The surface sterilised zygotic embryos and rhizomes were cultured on Woody medium supplemented with various plant growth regulators α-napthaleneacetic acid (NAA) [1.07 to 8.04 µM] in combination with 6-benzylaminopurine (BAP) [2.21 to 17.68 µM] for multiple shoot induction. The data on shoot induction response were collected after 6 weeks of culture on regeneration medium and the cultures were sub cultured onto the fresh medium after 8 weeks. The frequency with which the explants produced shoots, the number of shoots per explants was recorded after six weeks of culture (Table 1).

Rooting of regenerated shoots

The shoots induced directly from embryos and rhizomes were transferred to woody medium supplemented with IBA or NAA (2.46 to 21.44 µM). The medium was solidified with 0.25% (*w/v*) agar. Data were recorded for the percentage of rooting, the mean number of roots per shoot after four weeks of transfer onto the rooting medium (Table 2).

Acclimatization

The *in vitro* regenerated plantlets with well developed shoots and roots were washed with distilled water and transferred to pots containing 1:1:1 ratio of garden soil: sand: vermicompost

at 23 ± 2^{0}C temperature and 16 h photoperiod with 30 µmol m^{-2} s^{-1}at light intensity under sterile conditions. Potted plants were covered with transparent polythene membrane to ensure 80% relative humidity, and watered every three days with half strength Woody salt solution free of sucrose for 2 weeks. After initial acclimatization, plants were transferred to greenhouse at 28–30^{0}C and watered with tap water. At an approximate height, plants were transferred to the field for evaluation.

RESULTS

Effect of growth regulators on multiple shoot induction

Embryo and rhizome explants were inoculated on woody medium supplemented with different NAA (1.07 to 8.04 µM) & BAP (2.21 to 17.68 µM) concentration combinations in order to study the effect of growth regulators on shoot induction. The optimal (94%) shoot induction of embryo explants was obtained on woody media supplemented with 3.21 µM NAA + 17.68 µM BAP at 7.4 number of shoots per explant (Figure 1a & 1b). The second optimal (90.5%) shoot induction was found in 3.21 µM NAA + 13.26 µM BAP and NAA (1.07 µM) in combination with BAP (2.21 µM) showed relatively poor performance for shoot induction. The rhizome explant shown relatively less response than embryo explants (Figure 4). The data was recorded after six weeks of incubation (Table 1). All the cultures were maintained at 23±2^{0}C, 16 hrs photoperiod responded well. Regenerated shoots 1 cm or longer were used for rooting while shorter shoots were transferred and sub-cultured on woody medium supplemented with 4.42 µM BAP for further growth.

Root induction and regenerating whole plantlets

The *in vitro* regenerated shoots were transferred to the woody medium supplemented with IBA (2.46 to 19.68 µM) & NAA (2.68 to 21.44 µM) alone. Data was recorded on the percentage of rooting, the mean number of roots per shoot after four weeks of transfer onto the rooting medium. The optimal (6.4) number of roots per shoot was obtained on the medium supplemented with 2.68 µM NAA with an efficiency of 95.4% (Figure 2 & 3). A decrease in an average means number of roots per shoot with increase in the concentration of NAA (5.32 to 21.44 µM) (Table 2). Roots grew rapidly and reached 1–2 cm in length after 10 days of incubation. The rooted shoots were transferred to the poly cups for hardening.

Acclimatization

The shoots rooted successfully transferred to the pots containing 1:1:1 ratio of garden soil: sand: vermicompost at 23 ± 2^{0}C temperature and 16 h photoperiod with 30 µmol m^{-2} s^{-1} at light intensity under sterile condition with a survival rate of 100% (Figure 5). Roots had elongated, and lateral roots had emerged in the sand 1 wk after plantlets were transferred to pots. After 2 wk, roots grew down to the soil layer.

DISCUSSION

The main objective of this study was to establish a reproducible regeneration system in *Zingiber roseum* using immature embryo and rhizome cultures. Immature embryos are the most responsive source to regenerate plantlets among other explants in culture. In this study, it was

found that the optimum shoot regeneration efficiency 94% with 3.21 µM NAA + 17.68 µM BAP [11].

Table 1 : Effect of plant growth regulators NAA & BAP combination on shoot regeneration from immature embryos of *Zingiber roseum* after 6 weeks of culture

Plant growth regulators (µM)		% of response of an explant ± SE		Mean number of shoots per explants ±SE
NAA	BAP	Embryo explants	Rhizome explant	
1.07	2.21	10.6 ± 1.06 ^{j}	-	1.2 ± 0.20 ^{g}
	4.42	21.3 ± 0.74 ^{i}	-	1.6 ± 0.58 ^{g}
	8.84	39.9 ± 071 ^{gh}	-	2.2 ± 0.40 ^{f}
	13.26	63.5 ± 1.20 ^{de}	23.1± 0.20 ^{j}	2.1 ± 0.58 ^{f}
	17.68	58.8 ± 0.68 ^{e}	30.8 ± 0.81 ^{i}	2.2 ± 0.50 ^{f}
2.14	2.21	29.0 ± 1.26 ^{h}	-	1.6 ± 0.40 ^{g}
	4.42	60.9 ± 1.24 ^{de}	-	3.2 ± 0.50 ^{e}
	8.84	82.7 ± 0.90 ^{c}	15.2 ± 0.19 ^{c}	3.4 ± 0.73 ^{e}
	13.26	60.1 ± 2.75 ^{de}	22.1 ± 1.27 ^{j}	3.5 ± 0.92 ^{e}
	17.68	41.3 ± 1.37 ^{gh}	29.8 ± 0.17 ^{i}	3.2 ± 1.66 ^{e}
3.21	2.21	55.3 ± 1.14 ^{ef}	11.3 ± 0.94 ^{k}	4.4 ± 0.50 ^{d}
	4.42	71.6 ± 3.69 ^{d}	26.9 ± 1.69 ^{ij}	5.2 ± 0.67 ^{c}
	8.84	79.0 ± 0.70 ^{cd}	33.1 ± 0.40 ^{gh}	5.4 ± 0.67 ^{c}
	13.26	90.5 ± 0.53 ^{b}	62.2 ± 0.30^{d}	6.4 ± 1.63 ^{b}
	17.68	94.0 ± 0.65 ^{a}	70.6 ± 0.26^{c}	7.4 ± 0.50 ^{a}
4.28	2.21	30.0 ± 1.38 ^{gh}	22.0 ± 0.32 ^{j}	4.1 ± 1.20 ^{d}
	4.42	45.4 ± 1.70 ^{g}	35.3 ± 1.40 ^{gh}	4.4 ± 0.71 ^{d}
	8.84	53.3 ± 1.70 ^{ef}	42.2 ± 1.60 ^{fg}	3.0 ± 0.96 ^{e}
	13.26	70.1 ± 0.71 ^{d}	74.3 ± 0.32^{b}	2.7 ± 1.11 ^{f}
	17.68	89.8 ± 1.41 ^{b}	79.8 ± 1.40^{a}	1.4 ± 0.23 ^{g}
5.36	2.21	21.0 ± 1.72 ^{i}	19.1 ± 1.40 ^{k}	1.8 ± 0.58 ^{g}
	4.42	31.9 ± 1.83 ^{h}	26.7 ± 1.40^{ij}	2.2 ± 0.76 ^{f}
	8.84	35.5 ± 0.75 ^{h}	32.0 ± 0.51 ^{gh}	2.0 ± 6.41 ^{f}
	13.26	50.0 ± 2.49 ^{efg}	44.2 ± 0.51 ^{f}	3.6 ± 0.50 ^{e}
	17.68	55.3 ± 1.60 ^{ef}	50.1 ± 1.05 ^{e}	2.4 ± 0.41 ^{f}
8.04	2.21	31.3 ± 0.98 ^{h}	16.1 ± 1.05 ^{k}	2.1 ± 0.74 ^{f}
	4.42	50.7 ± 0.88 ^{efg}	22.4 ± 0.98 ^{j}	4.2 ± 0.86 ^{d}
	8.84	59.6 ± 1.36 ^{e}	34.3 ± 0.61 ^{gh}	3.2 ± 1.35 ^{e}
	13.26	41.9 ± 1.26 ^{gh}	39.9 ± 1.06 ^{g}	3.0 ± 0.70 ^{e}
	17.68	24.1 ± 0.91 ^{i}	21.0 ± 0.81 ^{j}	2.0 ± 0.65 ^{f}

The values represent the mean (±SE) of three indepe ndent experiments. Mean values within a column followed by different letters are significantly different by Duncan's multiple range test (P<0.05).

Plant growth regulators, especially cytokinins and auxins alone and in combination, are known to play a very important role in the process of regeneration. However, the optimum concentrations for shoot induction differ according to the type of auxin, genotype, and explant source. BAP has been reported to be more beneficial than other cytokinins for micropropagation of various members of the Euphorbiaceae [13,14]. In *Jatropha integerrima*, a high frequency of response was reported in media containing IBA +BAP in hypocotyls, stem, peduncle, and leaf explants [14]. Similarly, in *J. curcas*, BAP promoted higher regeneration from hypocotyl explants than Kn [15]. The favorable influence of BAP on the morphogenic capacity of the explants has also

been reported in other species: castor, *Euphorbia peplus* [16]. The protocols for plant regeneration are reported with varying efficiency in *J. curcas*. with 33–35% shoot regeneration on cotyledon explants [17] while, 71.2% adventitious shoot regeneration from leaf explants [18]. Our data on immature embryo cultures of *Zingiber roseum* showed very similar observations of the favorable influence of BAP whereas rhizomes show relatively shown poor response. The present results indicate the most favourable combination for shoot induction reported NAA–BAP (Table 1). The differential response of cytokinins, i.e. in this case, BA and Kn, may be attributed to differences in uptake, levels of endogenous growth regulators, and recognition by cells. In this study, it was found that the regeneration time can significantly shorten.

Table 2: Effect of Woody medium with various concentrations of IBA & NAA on induction of roots from *in vitro* regenerated shoots

Plant growth regulators (µM)		% of rooting ± SE	Mean number of roots per shoot ± SE
IBA	NAA		
2.46	--	61.8 ± 1.15 ^{e}	1.6 ± 0.41 ^{e}
4.92	--	70.0 ± 0.70 ^{d}	2.3 ± 0.58 ^{d}
9.84	--	80.8 ± 0.81 ^{c}	3.4 ± 0.50 ^{c}
14.76	--	63.2 ± 1.56 ^{e}	3.8 ± 0.39 ^{c}
19.68	--	48.6 ± 0.93 ^{g}	4.2 ± 0.86 ^{b}
--	2.68	95.4 ± 0.87 ^{a}	6.4 ± 0.73 ^{a}
--	5.36	89.8 ± 0.76 ^{b}	4.4 ± 0.46 ^{b}
--	10.72	68.2 ± 1.28 ^{d}	3.8 ± 0.67 ^{c}
--	16.08	59.4 ± 0.92 ^{f}	3.2 ± 0.58 ^{c}
--	21.44	43.6 ± 1.20 ^{gh}	3.2 ± 1.11 ^{c}

The values represent the mean (±SE) of three indepe ndent experiments. Mean values within a column followed by different letters are significantly different by Duncan's multiple range test (P<0.05).

BAP was required for optimal regeneration of *Zingiber embryo* explants, and the number of regenerated shoots and regeneration efficiencies reached an optimum level when the culture medium contained the concentration of 4mg/l of this cytokinin. The necessity of BA for citrus regeneration has been reported previously [19-22] so, *Zingiber* responded in a manner similar to these other *Citrus* species. In addition, NAA promotes bud differentiation when explants were incubated under the light for 16 hrs. The present observation is similar to some previous reports in which NAA was essential on shoot regeneration for several citrus genotypes [22-24].

Considering regeneration frequency and average shoot number per explant of *Zingiber roseum*, A significant effect of light on shoot organogenesis was observed especially when the incubation at 8 hrs in darkness followed by 16 hrs in light conditions was superior to the incubation performed under the light continuously. Transgenic shoots was recovered from four sweet oranges only when the explants were cultured under a 16/8-h (day/night) photoperiod with Escherichia coli phosphomannose–isomerase gene as the selectable marker gene [25].

It was observed that the regeneration medium (woody medium + 17.68 µM BAP) favored the development of long, thin, and weak roots that did not support the plant growth. These weak roots were removed and shoots were used for root induction study. The presence of 2.68 µM NAA in woody medium was found to result in the best root formation, with strong multiple roots (average of 6.4 roots per shoot) developed within 4 wk (Table 2). Fully developed plantlets with

well-developed root and shoot systems were transferred to field conditions with 100% survival rate [26,27] whereas 90% plant regeneration in immature embryo explants of *Jatropa* [16].

Figure 1 a : Multiple shoots from embryo explant at 3.21 µM NAA + 17.68 µM BAP after 2 weeks of inoculation

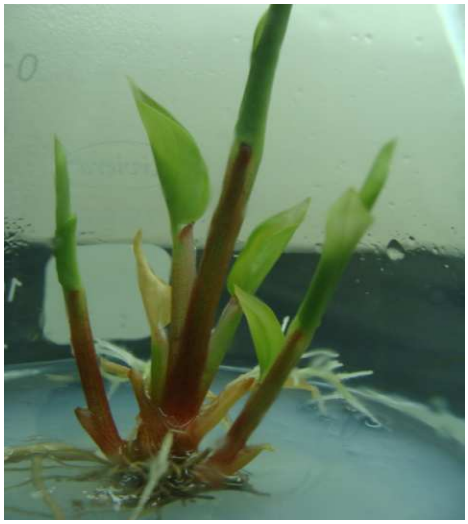

Figure 1b: Multiple shoots from rhizome bud at 3.21 µM NAA + 17.68 µM BAP after 2 weeks of inoculation.

Figure 2: Root induction at 2.68 µM NAA with an efficiency of 95.4%

Figure 3: Rooting of germinated shoots at 2.68 µM NAA

Figure 4 : Rhizome bud germination at a concentration of 3.21 µM NAA + 17.68 µM BAP

Figure 5 : Acclimatization

CONCLUSION

The present study reports a protocol for 100% direct *in vitro* plant regeneration from immature embryos of *Zingiber roseum.* Cotyledons are the best explants source for direct organogenesis has an advantage over callus-mediated embryogenesis in terms of less time consumed and a decrease in somaclonal variation.

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