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Direct organogenesis and microrhizome production in ginger (*Zingiber officinale* Rosc.)

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Abstract
This study developed an effective protocol for *in vitro* shoot multiplication and microrhizome induction in ginger (*Zingiber officinale*). *In vitro* culture was established using sprouting shoot bud explants of ginger rhizome. A concentration of 1.0 mg/L BA and 0.5 mg/L NAA found to be optimum for shoot induction. Different plant growth regulators, photoperiod exposure level and sucrose concentration were investigated for microrhizome induction. The optimal response was observed in the MS medium containing 0.5 mg/L BA and 0.5 mg/L IAA. A healthy and maximum microrhizome production was obtained in the MS medium consisted of 8% sucrose under 8 hour photoperiod. This study can be used to develop protocols for mass production of pathogen-free microrhizome and conservation of ginger.

Keywords: Shoot multiplication, ginger, sucrose, microrhizome, photoperiod

Introduction
Ginger (*Zingiber officinale* Rosc.) belongs to the family Zingiberaceae, is a valuable medicinal and aromatic perennial herb distributed worldwide as a spice crop. It is cultivated on a commercial scale for medicinal and culinary preparations. Its rhizome possesses several medicinal properties such as a stimulant of gastrointestinal tracts, carminative, anti-inflammatory, diuretic, anti-oxidant and diaphoretic [1]. Ginger has also been shown to have potential action against stomach discomfort, tumours, asthma, cough, rheumatism and osteoporosis [2, 3].
Poor flowering and seed set is a major constraint in ginger breeding. Ginger is propagated exclusively through rhizomes and it's prone to several fungal, bacterial and viral diseases, which causes a significant crop loss [4]. *In vitro* culture of ginger offers the production of large quantity of disease-free clones in a short time period. There are many reports on the *in vitro* culture propagation in ginger [5-7]. In this study, we developed a direct regeneration protocol for ginger using sprouted shoot buds. This is a significant method to produce genetically stable propagation of ginger.
In vitro developed miniature rhizomes are known as microrhizomes. Microrhizome production is a very useful technique for germplasm storage and direct application in *in vivo* condition [8]. There are few reports on the microrhizome induction in ginger [1, 9]. The present study systematically investigated the microrhizome inducing factors such as growth regulators, sucrose concentration and photoperiod.

Materials and methods
Plant material
Mature and healthy disease-free rhizomes of *Z. officinale* were obtained from plants grown in Ramanattukara, Kerala and maintained in the campus garden of Jamal Mohamed College, Tiruchirappalli, Tamilnadu. Elongated sprouting shoot buds were excised from mother rhizome and used as the explant for *in vitro* culture.

Establishment of culture
Explants were washed with running tap water for 10 min to remove adhering soil and transferred to Tween-20 solution for 5 min. It was followed by a treatment with 1% (w/v) mercuric chloride ($HgCl_2$) for 2 min and thoroughly washed three times with sterilized distilled water. After removing outer scale, explants were transferred to MS medium [10] containing 3.0% (w/v) sucrose and solidified with 0.8% (w/v) agar. The culture medium was autoclaved at 121°C and 104 K pa for 20 min and the pH was adjusted to 5.7. Cultures were

maintained at a 25±1°C temperature under white fluorescent light with 40 µmol m^{-2} s^{-1} light intensity.

***In vitro* multiplication**

For shoot multiplication, 20-30 day old *in vitro* grown shoot buds were excised and sub cultured in a culture bottle containing 30 ml MS medium. Different treatments of BA alone or in combination with NAA, IAA and IBA were used in culture media. Mean number of shoots, shoot length and microrhizome biomass were recorded after 40 days of culture.

Induction of microrhizome

In vitro grown shootlets with 3-4 cm shoot length in MS medium containing 0.5 mg/L BA and 0.5 mg/L IAA were subjected to different concentration of sucrose (2-10%) for the induction of microrhizome. Cultures were examined under varying level of photoperiod (0 hr, 8 hr and 16 hr light). Growth characteristics were noted after 60 days of culture.

Statistical analysis

Experiments were carried out in triplicates with at least 12 explant. All data were subjected to one way analysis of variance (ANOVA) using SPSS software (Version 16). Means were compared by Duncan's multiple range test with a significance level of $P \geq 0.05$.

Results and discussion

Shoot induction and plant regeneration

Sprouted shoot bud explants of ginger were cultured on MS medium containing BA alone or with different combinations of NAA, IBA and IAA. Treatment with 1.0 mg/L BA and 0.5 mg/L NAA gave a maximum of shoots (12.33±0.33) per explant. A highest length of 10.66±0.44 cm shoots was also observed in the same media (Table 1). Influence of BA in shoot induction has been reported in many Zingiberaceae species [11, 12]. Sharma and Singh (1997) [5] reported the combined effect of 2.0 mg/L Kn and 2.0 mg/L NAA in ginger shoot formation. Ali *et al*. (2016) [13] reported the highest number of shoots on MS medium containing 4.5 mg/L BA in ginger. In our study, lower concentration of BA and NAA gave the optimum result (Table 1). This is in accordance with the previous findings of Chirangini and Sharma (2005) [14] in *Zingiber cassumunar*.

Effect of BA and IAA on microrhizome induction

The different treatments of BA, NAA and IAA showed variable size and fresh weight of rhizome (Table 1). Rhizome formation was started from the swelled shoot base. Nayak (2000) [15] reported the influence of BA (5.0 mg/L) on microrhizome induction in *Curcuma aromatica.* 5.0 mg/L BA in combination with 0.5 mg/L NAA was optimum for microrhizome production in *C. longa* [16]. In our study, among different combinations of plant growth regulators, 0.5 mg/L BA and 0.5 mg/L IAA recorded largest size (3.5±0.28cm) and maximum fresh weight (4.46±0.23g) of microrhizome formation. The same medium is used for further studies.

Effect of sucrose concentration on microrhizome induction

Sucrose provides carbon and energy for the shoot and microrhizome induction in ginger. So, the concentration of sucrose had a significant impact on *in vitro* microrhizome induction. No rhizomes were observed in 2% sucrose concentration under different photoperiod. But, the number of microrhizomes increased with higher sucrose concentration. 8% sucrose concentration was optimum for microrhizome induction (Table 2). The range of sucrose concentration varies for different species in Zingiberaceae. 9% in *C. longa* [17], 6% in *C. aromatic* [15], *C. zedoria* [18], 6-9% in *Hedychium stenopetalum* [19], 7-9% in *Z. cassumunar* [14].

Table 1: Effect of plant growth regulators on shoot and microrhizome induction in ginger

Plant growth regulators (mg/L)				Mean number of shoots per explant	Shoot length (cm)	Microrhizome size (cm)	Microrhizome Biomass (g)
BA	NAA	IAA	IBA				
0.5	0.00	0.00	0.00	2.66 ± 0.33^{i}	3.5 ± 1.0^{k}	0.0^{g}	0.0^{l}
	0.25			3.66 ± 0.88^{hi}	4.83 ± 0.6^{ijk}	0.83 ± 0.166^{defg}	0.4 ± 0.2^{kl}
	0.5			4.0 ± 0.577^{ghi}	6.43 ± 0.34^{fghi}	0.66 ± 0.44^{defg}	1.13 ± 0.2^{j}
	0.75			3.0 ± 1.15^{hi}	8.0 ± 0.28^{cdef}	0.33 ± 0.16^{fg}	0.8 ± 0.15^{jk}
		0.25		4.66 ± 0.33^{efghi}	9.83 ± 0.44^{ab}	1.6 ± 0.6^{bcd}	2.93 ± 0.12^{cd}
		0.5		6.66 ± 0.33^{de}	8.5 ± 0.28^{bcde}	3.5 ± 0.28^{a}	4.46 ± 0.23^{a}
		0.75		6.33 ± 0.33^{def}	7.33 ± 0.6^{defgh}	2.5 ± 0.28^{b}	3.93 ± 0.21^{b}
			0.25	3.0 ± 0.57^{hi}	5.16 ± 0.16^{ij}	0.66 ± 0.44^{defg}	1.3 ± 0.11^{hij}
			0.5	5.0 ± 0.0^{efgh}	5.83 ± 0.44^{hij}	1.5 ± 0.28^{bcde}	1.2 ± 0.35^{ij}
			0.75	4.33 ± 0.88^{fghi}	7.66 ± 0.44^{cdefg}	0.83 ± 0.16^{defg}	1.73 ± 0.06^{fghi}
1.0	0.25			11.33 ± 0.88^{a}	9.16 ± 0.44^{bc}	0.83 ± 0.16^{defg}	2.13 ± 0.08^{ef}
	0.5			12.33 ± 0.33^{a}	10.66 ± 0.44^{a}	1.33 ± 0.33^{cdef}	1.8 ± 0.17^{fgh}
	0.75			10.66 ± 0.66^{ab}	8.0 ± 0.57^{cdef}	0.66 ± 0.44^{defg}	1.36 ± 0.18^{ghij}
		0.25		6.0 ± 0.57^{defg}	7.16 ± 0.16^{efgh}	2.0 ± 0.28^{bc}	2.3 ± 0.15^{ef}
		0.5		7.33 ± 0.33^{cd}	7.5 ± 0.28^{defg}	2.16 ± 0.16^{bc}	3.43 ± 0.23^{bc}
		0.75		7.66 ± 0.33^{cd}	6.33 ± 0.6^{ghi}	2.16 ± 0.16^{bc}	3.63 ± 0.14^{b}
			0.25	7.66 ± 0.88^{cd}	8.16 ± 0.44^{cde}	0.66 ± 0.16^{defg}	1.16 ± 0.23^{ij}
			0.5	9.0 ± 0.57^{bc}	8.83 ± 0.44^{bcd}	1.16 ± 0.6^{cdef}	2.66 ± 0.18^{de}
			0.75	6.33 ± 0.88^{def}	7.0 ± 0.28^{efgh}	0.5 ± 0.28^{efg}	1.9 ± 0.15^{fg}

Values are expressed as the mean ± SE, taking ten explants in each experiment with three replications. Within each group, values with different letters indicate significant difference at $P \geq 0.05$ using Duncan's multiple range test (DMRT)

Table 2: Effect of sucrose concentration and photoperiod on microrhizome development in ginger

Sucrose (%)	Mean number of shoots per explant			Microrhizome Biomass (g)		
	0 h dark	8 h light	16 h light	0 h dark	8 h light	16 h light
2.0	0.0[b]	3.66±0.66b	1.66±1.2d	0.0c	0.0d	0.0d
4.0	3.0±0.57[a]	5.0±0.57ab	4.66±0.88cd	3.53±0.18b	5.43±0.28b	1.1±0.05c
6.0	3.0±1.0[a]	7.0±0.57a	12.33±0.88a	4.26±0.39b	5.83±0.2ab	2.3±0.15b
8.0	4.66±0.88[a]	7.0±1.0a	10.66±0.88ab	5.26±0.14a	6.26±0.12a	3.1±0.28a
10.0	2.66±0.33[a]	5.66±0.88ab	7.66±0.88bc	3.8±0.3b	3.33±0.14c	0.76±0.37c

Values are expressed as the mean ± SE, taking ten explants in each experiment with three replications. Within each group, values with different letters indicate significant difference at $P \geq 0.05$ using Duncan's multiple range test (DMRT)

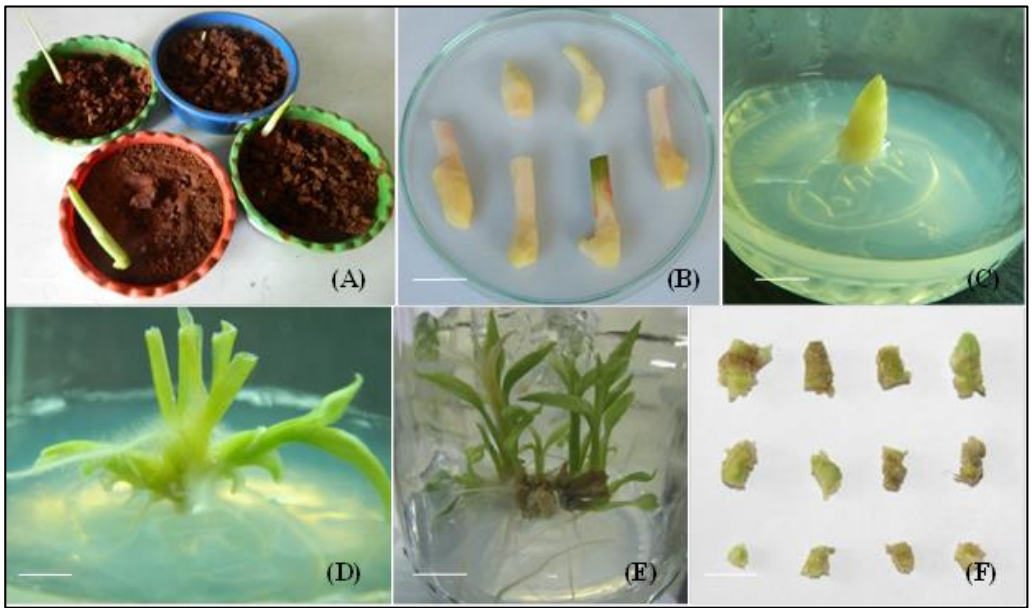

Fig 1: (A) Sprouted shoot buds of ginger (B) Excised explants after sterilization (C) Explant inoculated on culture medium (D) Axillary shoot induction on MS medium containing BA and NAA (E) Microrhizome formation on MS medium containing BA and IAA (F) Isolated microrhizomes. *Scale bars*: (A) 3 cm; (B) 2.5 cm; (C-E) 1.5 cm; (F) 2 cm

Effect of photoperiod on microrhizome induction

Microrhizome formation examined under 0, 8 and 16 hr photoperiod. It was observed that an intermediate light was significantly improved the microrhizome induction. The photoperiod of 8 hr light in 8% sucrose concentration gave the highest weight of 6.26±0.12g as compared to 3.1±0.28g in 16 hr light. However, the maximum number of shoots (12.33±0.88) recorded in the 16 hr photoperiod (Table 2). *In vitro* culture under complete darkness significantly reduced the number of shoots and biomass of microrhizome. Nayak (2000) [15] has made similar observations in *C. aromatica.*

Conclusion

In vitro clonal multiplication and microrhizome production of ginger is greatly influenced by plant growth hormones, sucrose concentration and photoperiod. MS medium supplemented with 0.5 mg/L BA, 0.5 mg/L IAA and 8% sucrose under 8 hr photoperiod is found to be an optimal condition for induction of microrhizome. Understanding the biosynthetic pathway of bioactive compounds in ginger rhizome is a future application of this study. The outcome of the present work can be adopted for large scale propagation and storage of disease free ginger microrhizome.

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