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RESEARCH ARTICLE

IN VITRO MICRORHIZOME FORMATION AND ITS REGENERATION IN *ZINGIBER OFFICINALE*

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ABSTRACT

Microrhizomes were successfully induced in *Zingiber officinale* and their *in vitro* and *ex vitro* conversion potential was investigated. The six weeks old axenic plants derived from vegetative bud culture induced microrhizomes with 78% response when transferred to LS liquid medium supplemented BAP (8.88µM) and Sucrose (7%). The average number, yield and average weight of microrhizome was found to be 5.4g, 2.65g and 0.49g respectively. The microrhizomes stored at 4°C for 12 months resulted in fairly good conversion response both *in vitro* and *ex vitro* conditions followed by development into complete plantlets without any significant loss of conversion potential. The protocol described in this paper is reproducible and shall definitely facilitate the utilization of this technique in propagation and conservation of *Zingiber officinale* Rosc.

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INTRODUCTION

Zingiber officinale Rosc. (Ginger) has been valued world over since ancient times for its flavour and medicinal properties. The rhizome of ginger has a volatile oil containing aromatic compounds such as Camphene, Phellandrene, Zingiberene and Zingirone with flavouring constituents like Cineole, Borneol, Geraniol, Linalool and Farmasceme (Farrell, 1985). These along with several other chemical constituents have made ginger one of the most popular medicinal spice used in folk medicine and for culinary purposes throughout the world (Dobelis,1990). It is exclusively propagated vegetatively using rhizome with a low multiplication rate. A considerable amount of the rhizome is stored as stock for next cropping year (Shirgurkar *et al.,* 2001). Maintenance of germplasm for annual planting is expensive and labour intensive (Islam *et al.,* 2004). Moreover, Ginger rhizomes degenerate after long-term propagation, and conventional breeding programmes have been severely hampered due to poor flowering and rare seed set. Further, ginger is also affected by various diseases of fungal, bacterial, viral and mycoplasmal origin particularly soft rot caused by *Pythiumsps.,* and *bacterial* wilt by *Ralstonia sps.,* resulting in considerable crop losses (Dake, 1995) threatening the germplasm of this important medicinal plant. *In vitro* culture techniques provide an alternative means of propagation that provides a disease free germplasm throughout the year. Studies related to *in vitro* formation of storage organs such as bulbs, corms, tubers and rhizomes have been considered as promising approach as these kind of propagules can be directly transferred to the field without hardening or acclimatization and can also be transported easily facilitating germplasm exchange across national borders. Although, several reports are available on *in vitro* formation of storage organs in plant systems like *Solanum tuberosum, Dioscoria composita, Buniumpersicum* and *Curcumalonga*, reports on microrhizome formation and its regeneration in ginger is very scanty (Islam *et al,*2004). Furthermore, adequate efforts have not been made to explore the possibility of storing microrhizomes at low temperature for long duration and testing its conversion potential. This study reports optimized parameters for the production, propagation and conservation of microrhizomes followed by its conversion potential into complete plantlets under *in vitro* and *ex vitro* conditions.

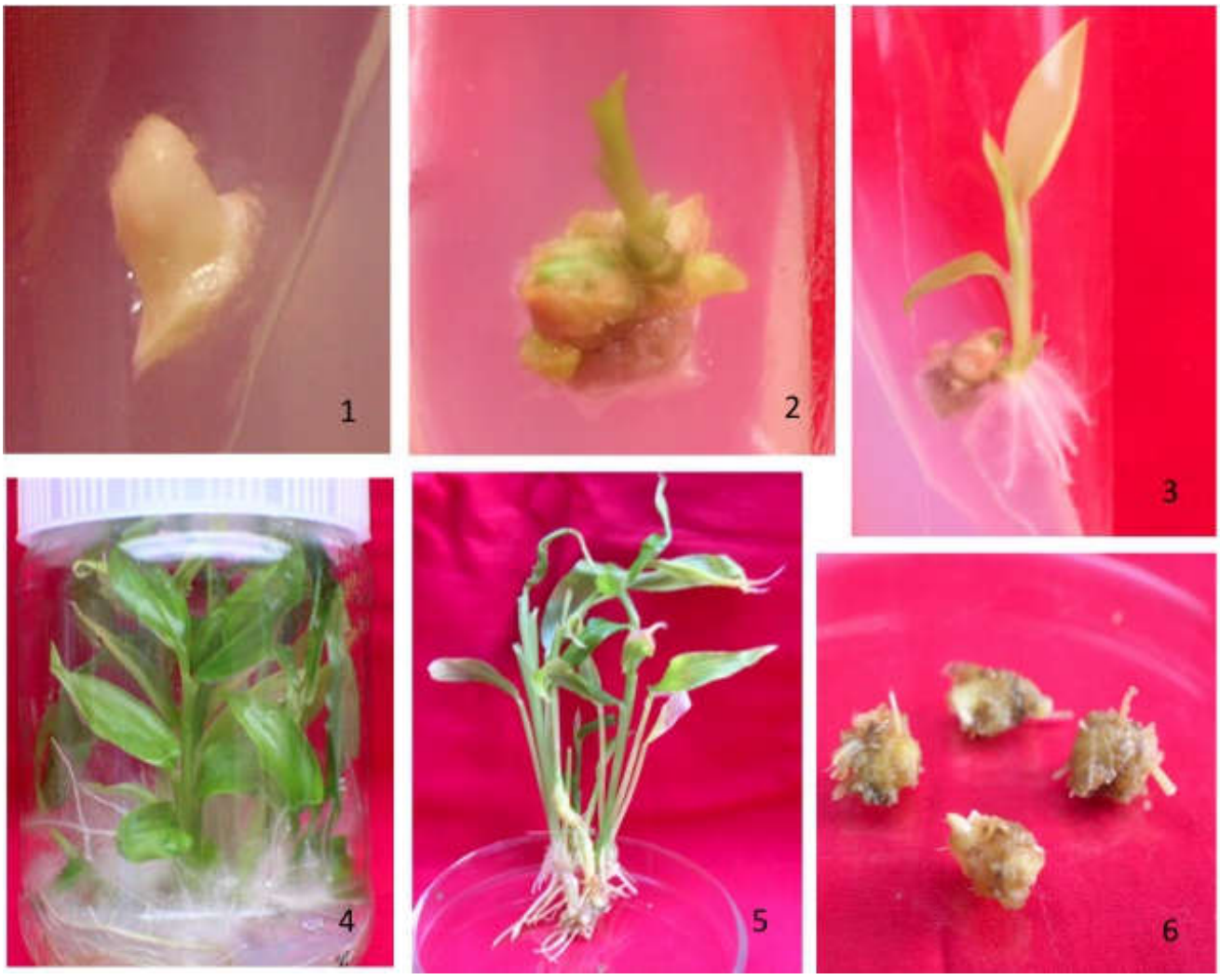
MATERIALS AND METHODS

Rhizomes of *Zingiber officinale* Rosc. (Ginger) collected from local market was chosen for the present study. The *in vivo* vegetative bud measuring 0.5 – 1.0 cm were dissociated from dried scales and washed thoroughly in running tap water, surface sterilized using liquid detergent labolene – 5% (v/v), rinsed in 70% ethanol for 15 s, disinfected in 0.1% Mercuric chloride ($HgCl_2$) for 3 min, treated with 0.1% Streptomycin for 1 min to avoid bacterial contamination and washed several times in sterile water to remove the traces of sterilant. The sterilized vegetative buds were inoculated on Linsmaier and

Skoog's basal medium (LSBM) fortified with BAP to obtain axenic plants. A clump of aseptic multiple shoots derived from vegetative bud were transferred to LS liquid medium supplemented with different concentrations of sucrose ranging from 3-11% for microrhizome formation. The culture bottles were maintained in the culture room at a temperature 25 ± 2°C under built-in white fluorescent light at a photon flux density of 30-50 $\mu Em^{-2}s^{-1}$ under different photoperiodic regime ranging from complete darkness to varying light/dark periods (Table 1). The microrhizomes thus obtained were harvested under aseptic conditions and tested for their conversion potential under *in vitro* and *ex vitro* conditions. 50 microrhizomes were cultured on LSBM supplemented with BAP (8.88µM) and a group of 50 microrhizomes were sown in petridishes containing horticultural grade soilrite mix –peat:perlite:vermiculite (1:1:1-v/v) supplemented with half strength LS nutrients, maintained in green house for a week, then transferred directly to soil and their survival frequency was recorded. For *in situ* conservation studies a group of 50 microrhizomes were washed thoroughly in running tap water, air dried and stored at 4°C in polybags containing soilrite mix for 1-12 months. The microrhizomes were tested for their conversion potential every month for a period of 12 months adopting both the *in vitro* and *ex vitro* methods described above. The experimental data was analyzed statistically, in each experiment six replications were used and each experiment was repeated three times.

RESULTS AND DISCUSSION

The vegetative bud explants (Fig. 1) of *Zingiber officinale* initiated shoot after 12 days of culture (Fig. 2 & 3) on LSBM supplemented BAP (8.88µM). A clump of multiple shoots was formed upon repeated subculture at 4-week intervals on the same medium (Fig. 4). These clumps also contained many small adventitious buds at various stages of emergence of multiple shoots. The clump of multiple shoots thus obtained was transferred to LS liquid medium supplemented with BAP (8.88µM) and different concentrations of sucrose ranging from 3-11% and maintained in different photoperiodic regimes to study the effect on microrhizome formation. The results on the effect of sucrose concentration and photoperiodic regime on *in vitro* microrhizome formation in Ginger are depicted in (Table1). It was observed that sucrose plays a significant role in the formation of microrhizomes. Sucrose concentration of 7% with photoperiodic regime of 8 hours light and 16 hours dark cycle was found to be optimum for the formation of more number of large healthy microrhizomes after 36 days of transfer (Fig. 5). However, at the lowest concentration of sucrose tried the microrhizomes formation was either not observed or was very low, whereas at higher concentration microrhizome formed were either smaller or less in number (Table 1). Further, complete darkness resulted in increased root formation and lesser yield.



Figs: 1-6 Formation of Microrhizome from vegetative bud of *Zingibe rofficinale*

Fig. 1. Vegetative explant at culture; Fig. 2. Initiation of shoot after 12 days of culture; Fig. 3. Formation of roots; Fig. 4. Clump of multiple shoots after subculture; Fig. 5. Formation of microrhizome; Fig. 6. Harvested microrhizomes

Table 1. Effect of different concentrations of sucrose, photoperiodic regime on *in vitro* microrhizome formation in *Zingiber officinale*

Concentration of Sucrose (%)	Photoperiodic regime (hr)	Response (%)	Average number of microrhizomes	Average yield of microrhizomes (g)	Average weight of microrhizomes (g)
3	0 hr light/24 hr dark	02	0.2 ± 1.2	0.03 ± 0.5	0.15 ± 0.04
5		02	0.2 ± 1.3	0.03 ± 0.7	0.15 ± 0.06
7		04	0.4 ± 0.9	0.04 ± 0.3	0.10 ± 0.05
9		02	0.2 ± 0.8	0.03 ± 0.5	0.15 ± 0.08
11		02	0.2 ± 1.1	0.02 ± 0.6	0.15 ± 0.06
3	16 hr light/8 hr dark	14	2.2 ± 1.2	0.09 ± 0.5	0.04 ± 0.04
5		22	2.6 ± 1.3	1.00 ± 0.7	0.38 ± 0.06
7		36	3.1 ± 0.9	1.12 ± 0.3	0.36 ± 0.05
9		31	2.8 ± 0.8	0.09 ± 0.5	0.03 ± 0.08
11		18	1.1 ± 1.1	0.04 ± 0.6	0.03 ± 0.06
3	8 hr light/16 hr dark	47	2.8 ± 1.2	1.08 ± 0.5	0.38 ± 0.04
5		62	4.2 ± 1.3	1.93 ± 0.7	0.45 ± 0.06
7		78	5.4 ± 0.9	2.65 ± 0.3	0.49 ± 0.05
9		31	3.6 ± 0.8	1.73 ± 0.5	0.43 ± 0.08
11		24	1.8 ± 1.1	0.75 ± 0.6	0.41 ± 0.06

Table 2. In vitro and ex vitro conversion response of microrhizomes of *Zingiber officinale*

	In vitro					*Ex vitro*			
Duration (months)	Initiation response (%)	Culture duration (days)	No. of shoots $\bar{x}$*±SE	No. of roots $\bar{x}$*±SE	Survival frequency (%)	Initiation response (%)	No. of shoots $\bar{x}$*±SE	No. of roots $\bar{x}$*±SE	Survival frequency (%)
0	80	28	13.8 ± 1.79	7.8 ± 1.22	94	74	12.5 ± 1.23	6.5 ± 0.66	98
2	78	28	14.6 ± 1.62	7.6 ± 1.29	92	72	12.5 ± 1.34	6.3 ± 0.60	96
4	78	35	12.3 ± 1.47	6.6 ± 1.08	86	68	11.1 ± 1.10	5.4 ± 0.89	95
6	71	32	12.8 ± 1.08	5.9 ± 0.82	87	68	11.9 ± 1.03	5.8 ± 1.12	89
8	70	35	11.5 ± 1.23	5.8 ± 0.93	79	67	10.7 ± 1.17	4.7 ± 0.90	84
10	66	42	12.4 ± 1.34	5.1 ± 0.87	72	64	10.9 ± 1.42	5.3 ± 0.89	79
12	68	40	10.1 ± 1.10	4.8 ± 1.04	66	65	9.4 ± 1.15	4.6 ± 1.02	75

$\bar{x}$: Mean of six replications; **SE:** Standard Error

Whereas, in the photoperiodic regime of 16 hours light and 8 hours dark cycle microrhizome formation was very poor. This does not coincide with the findings of Islam *et al*., (2004) who have reported that 7% sucrose reduced both number and size of microrhizomes in turmeric. However, their report on inhibitory effect of sucrose on microrhizome production at lower (0-5%) and higher (11%) concentration is in concurrence with our findings. Shirgurkar *et al*. (2001), Sanghamitra and Pradeep (2006) have reported formation of microrhizome in media containing 6% sucrose in 4 hr photoperiod. Zheng *et al.* (2008), have reported that sucrose concentration of 8-11% in culture medium is optimal for induction of rhizomes in ginger. The perusal of literature reveals that high concentration of sucrose (6-11%) is essential to promote *in vitro* formation of storage organs (Arora *et al*., 1996; Alizadeh *et al*., 1998, Slabbert and Niederwieser, 1999; Kim *et al*., 2003). This may be due to the fact that sucrose being a rich source of carbon, its presence in higher concentration might promote formation of storage organs as storage organs mostly store carbohydrates (Sanghamitra, 2000). Furthermore, the present study reports formation of microrhizome on LS liquid medium supplement with BAP (8.88µM) which is in contrary to the reports of Sanghamitra and Pradeep (2006) and Islam *et al.,* (2004) who have obtained microrhizomes on MS liquid medium fortified with BAP (13.3 µM) and BAP (12 µM) together with NAA (0.3µM) respectively. The photoperiodic regime of 8 hours light and 16 hours dark cycle depicted in the present study for formation of more number of large healthy microrhizomes differs with the findings of Sakamura *et al.* (1986), Sharma and Singh (1995), Islam *et al*., (2004) and Zheng *et al.* (2008) who have reported microrhizome formation at 24 hr, 0 hr, 4hr and 16-24hr photoperiod respectively. The different photoperiod requirement in ginger may be potentially attributed to the different genetic makeup of the species as suggested by Sanghamitra and Pradeep (2006). The microrhizome thus obtained were harvested (Fig. 6) under aseptic conditions and cultured on LSBM supplemented with BAP (8.88µM). The conversion response was found to be in the range of 68-80%. Maximum number of shoots (14.6+ 1.62) and roots (7.8 + 1.22) was obtained from 0-2 months stored microrhizomes on the same medium after 28 days of culture (Table 2). This is in concurrence with the findings of Shirgurkur*et al.,* (2001) who have reported 78.2% germination response. A group of microrhizomes sown directly into soil showed initiation response ranging from 65-74%. Maximum number of shoots (12.5 ± 1.23) and roots (6.5± 0.66) was obtained from 0-2 months stored microrhizomes. The plants retrieved from microrhizomes under *in vitro* conditions were successfully established in soil with varying percentage of survival frequency (66-94%). A fair percentage of survival frequency was recorded for a period of 0-12 months ranging from 74 – 98% with highest percentage of 98% survival frequency in the plant sown directly into soil. Further, it was noticed that in plants sown directly into soil, though conversion response was low (65-74%) the plants acclimatized faster with good survival frequency (75-98%) even after 12 month storage. Additionally, the microrhizomes produced fairly good number of shoots and roots on par with the microrhizomes cultured under *in vitro* conditions. The analysis of result reveals that the percentage of conversion response is high under *in vitro* conditions, which

may be due to the pathogen-free environment and ready nutrient availability as reported by Sanghamitra and Pradeep (2006). Furthermore, the conversion response is low under *in vivo* conditions, which may be due to sudden change in the culture vs soil conditions as these microrhizomes were acclimatized only for one week in soilrite mix. However, the survival frequency was high among these microrhizomes when compared to the plants grown under *in vitro* conditions indicating their stability after acclimatization. The protocol for *in vitro* microrhizome formation developed has tremendous application in commercial production of disease-free ginger in large scale. This study also provides a wholesome approach for propagation and conservation of ginger.

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Abbreviations

LSBM - Linsmaier and Skoog's Basal Medium
BAP - 6-Benzyl Amino Purine

Key Message: Microrhizomes were successfully induced, stored and regenerated using vegetative bud of *Zingibe rofficinale*. This has opened up new vistas for propagation, conservation and commercial production of disease-free ginger.

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