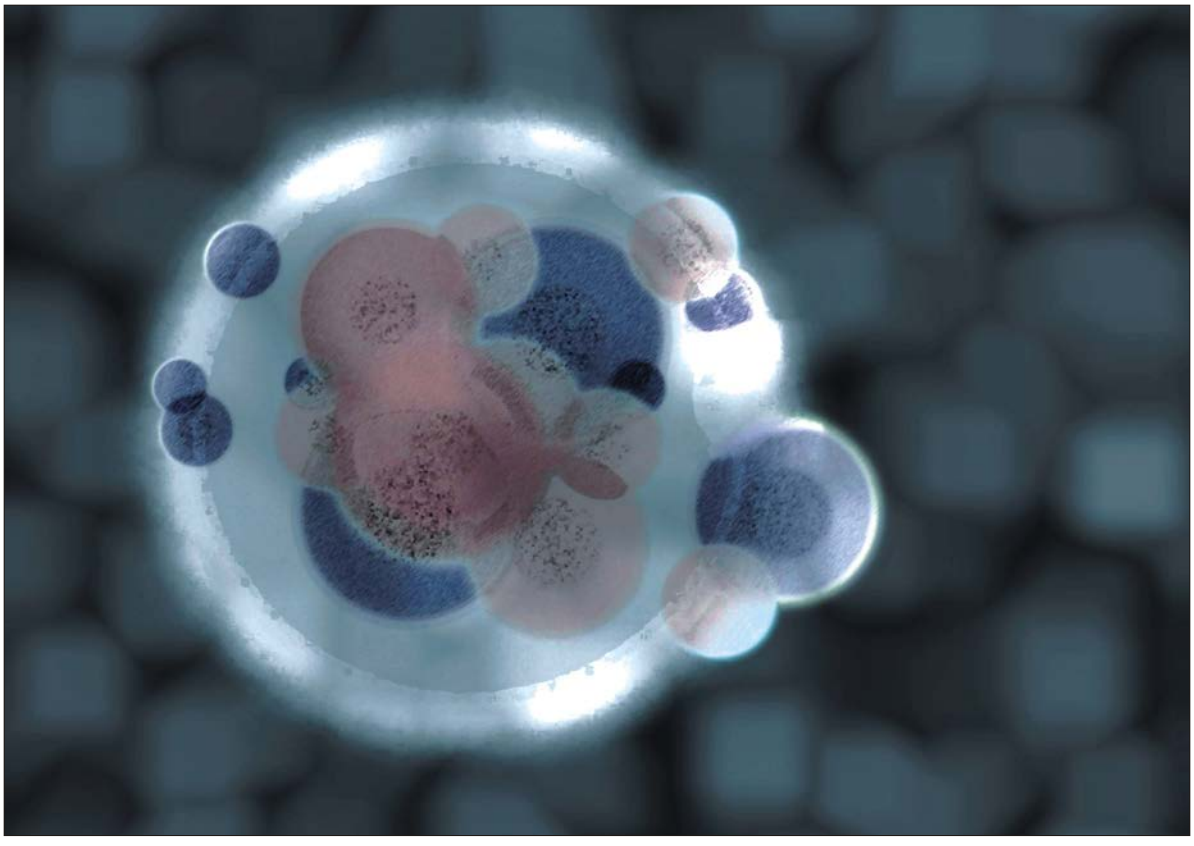

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In vitro Microrhizome Induction in *Curcuma zedoaria* (Christm.) Roscoe-A Conservation Prioritized Medicinal Plant

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Abstract: *In vitro* microrhizome was successfully produced in *Curcuma zedoaria*-a valuable but critically threatened medicinal plant. Ten to twelve weeks old *in vitro* multiplied shoots were cultured in MS medium supplemented with different level of BAP and NAA. Effect of different carbon sources has also been evaluated. Microrhizome formation started within 7-9 weeks of culture. The best result was obtained in 4.0 mg L^{-1} BAP along with 6% sucrose in terms of frequency and number of rhizome. Around 4 microrhizomes with an average weight of 2.5 g were harvested after 11-12 weeks. Microrhizomes were readily germinated in the growth regulator free MS medium. With the best of our knowledge this is the first report on *in vitro* microrhizome production in *Curcuma zedoaria*.

Key words: *Curcuma zedoaria*, critically threatened, microrhizome, carbon source

INTRODUCTION

Curcuma zedoaria (Christm.) Roscoe (Zingiberaceae), also known as 'zedoary' is a perennial herb with fleshy and branching rhizomes. The species is native to Bangladesh, India and some other South East Asian countries (Maciel and Criley, 2003). It has traditionally been used as spice, tonic and perfume for centuries in these regions. The constituents of *Curcuma zedoaria* rhizome oil has been investigated extensively and recognized as a rich source of terpenoids (Haque and Rashid, 1973; Shibuya *et al*., 1986; Liu, 1996). Many herbal drugs derived from this plant have attracted attention because of their anti-inflammatory (Yoshioka *et al*., 1998), antihepatotoxic (Matsuda *et al*., 1998), neuroprotective (Sasaki *et al*., 2002) antimicrobial and antimutagenic activity against benzo(a)pyrene-induced mutations in the *Salmonella*/microsomal system (Syu *et al*., 1998) and cytotoxic effect against human ovarian cancer cells (Syu *et al*.,1998). Zedoary not only inhibits cancer but also helps to prevent leukopenia due to cancer therapies (Minyi, 1992). Zedoary rhizome is a rich source of easily digestible starch which is used for the preparation of baby foods. Beautiful inflorescence and luxurious foliage that has an immense commercial value in floriculture (Maciel and Criley, 2003). Like many other medicinal plants, injudicious use together with habitat destruction has critically threatened *C. zedoaria*, especially in the densely populated non hilly areas of plain and plateau lands of Bangladesh and India where agricultural activities is more intensive. The decreasing number of natural populations and the rapid fragmentation of natural habitats have a severe impact on genetic diversity of the genus *Curcuma* (Paisookasantivatana *et al*., 2001). Accordingly, *C. zedoaria* is now considered as a critically threatened species in Bangladesh (Islam *et al*., 2005) and in India (Chhetri *et al*., 2005).

In vitro culture techniques provide an alternative means of propagation and a tool for crop improvement. Advanced biotechnological methods of culturing plant cells and tissues would provide new means of conserving and rapidly propagating valuable, rare and endangered medicinal plants (Nalawade *et al*., 2003). Conventionally, zedoary rhizomes are used planting material which encounter several disadvantages such as: low multiplication rate by rhizome separation method (annually is 5-10-fold), requirement of large amount of rhizome for propagation in the next season (10-20%) and senescence and degeneration due to intrinsic pathogens and disease incidence in field and storage. These common disadvantages encourage scientist to develop *in vitro* microrhizome in zingibers. Even though micropropagated plantlets overcome some of these, application of *in vitro* microrhizomes have a number of advantages over *in vitro*

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multiplied plantlets. Microrhizomes can be planted directly in the soil without acclimatization. Storage and transport of microrhizomes are also easier, facilitating germplasm exchange across national borders. Besides these, *in vitro* microrhizome can be a useful tool to understand the fundamental physiological mechanism of rhizome development. Moreover, transgene expression in rhizome can be studied *in vitro* using the system. There are few reports are available on micropropagation of *C. zedoaria* (Bharalee *et al*., 2005; Loc *et al*., 2005). Reports are available on successful induction of microrhizome in *C. aromatica* (Nayak, 2000), turmeric (Shirgurkar *et al*., 2001), *Curcuma amada* (Nayak, 2002), Zinger (Rout *et al*., 2001; Zheng *et al*., 2008), *Zingiber cassumunar* (Chirangini and Sharma, 2005). However, with the best of our knowledge, no reports have been published on the induction of microrhizome of *C. zedoaria*. In this study, a simple protocol has been described for *in vitro* microrhizome production in *C. zedoaria*.

MATERIALS AND METHODS

Plant materials and culture conditions: We have developed a protocol for rapid *in vitro* shoot multiplication of *Curcuma zedoaria* in Plant Biotechnology Laboratory, Rajshahi University, Bangladesh (Alam *et al*., unpublished). Mature rhizomes were collected from field grown healthy and disease-free plants and cleaned thoroughly by repeated washings and maintained in the laboratory for sprouting. Young buds were used as explants for shoot multiplication. The explants were surface sterilized with mercuric chloride and cultured on MS medium (Murashige and Skoog, 1962) containing different concentrations of TDZ for shoot induction. The induced shoots were multiplied and maintained by periodic subculturing on fresh nutrient medium (Fig. 1a). For microrhizome induction, 4-6 months old *in vitro* shoots were cultured in MS medium containing different concentration of BAP alone or

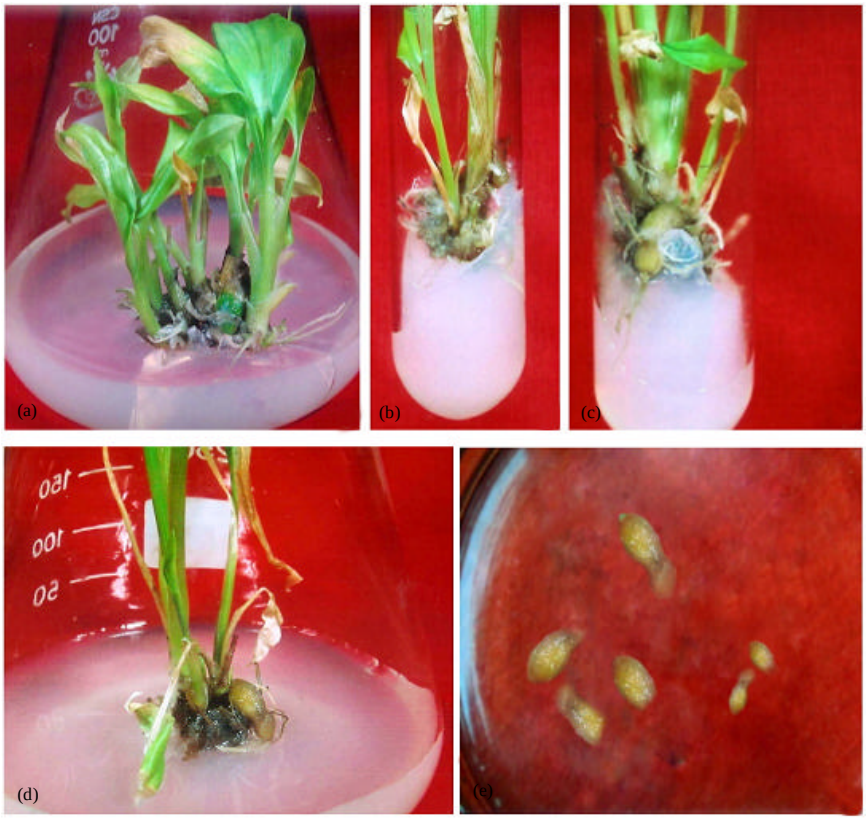

Fig. 1: *In vitro* microrhizome induction in *C. zedoaria* (a) Twelve-week old *in vitro* stock of *C. zedoaria* plant ready for microrhizome induction (b) Microrhizome initiation after 8 weeks of incubation on microrhizome induction medium (c, d) Development of the microrhizome and (e) Harvested microrhizome after 15 weeks of culture taken for germination

Table 1: Effect of PGR on *in vitro* microrhizome induction of *C. zedoaria*. In all cases, MS basal medium contained 30 g L^{-1} sucrose. Data were recorded after 12 weeks of culture

Growth regulator (mg L^{-1})		Weeks to micro-rhizome induction	*In vitro* microrhizome formation (%)	No. of microrhizome/ shoot ($\bar{x} \pm SE$)	Weight of microrhizome/ shoot (g) ($\bar{x} \pm SE$)
	2	8-9	35	1.5±0.02	0.55±0.05
	3	7-8	42	2.2±0.02	0.65±0.03
	4	7-8	48	2.3±0.03	0.71±0.06
	5	8-9	40	2.1±0.04	0.60±0.03
	6	8-9	37	1.6±0.05	2.54±0.04
	7	9-10	30	1.2±0.03	0.42±0.05
BAP	3.0+0.5	8-9	37	1.2±0.05	0.37±0.08
+	3.0+1.0	8-9	35	1.0±0.04	0.34±0.02
NAA	4.0+0.5	8-9	39	1.4±0.01	0.45±0.03
	4.0+1.0	8-9	36	1.2±0.02	0.39±0.01
	4.0+0.5	8-9	30	1.1±0.03	0.32±0.06
	5.0+1.0	9-10	26	0.8±0.01	0.25±0.05
MS_0		11-12	10	0.4±0.07	0.10±0.02

Table 2: Effect of PGR on *in vitro* microrhizome induction of *C. zedoaria*. In addition of different saccharides, MS medium supplemented with 4.0 mg L^{-1} BAP. Data were recorded after 12 weeks of culture

Sources of carbon (g L^{-1})		Microrhizome induction (%)	Microrhizome No./shoot ($\bar{x} \pm SE$)	Weight of microhizome/shoot (g) ($\bar{x} \pm SE$)
Commercial sugar	30	50	2.3±0.02	0.90±0.03
(Local Origin)	40	52	2.4±0.03	1.0±0.05
	50	55	2.9±0.02	1.2±0.01
	60	58	2.5±0.04	1.5±0.03
	70	60	3.2±0.01	1.5±0.03
	80	65	3.0±0.02	1.7±0.04
Sucrose	30	63	2.8±0.03	1.7±0.04
(Phytotech)	40	64	3.4±0.03	2.0±0.04
	50	67	3.8±0.01	2.2±0.03
	60	70	4.2±0.02	2.5±0.05
	70	64	3.6±0.03	2.1±0.07
	80	60	3.2±0.01	1.5±0.02
Glucose	30	47	2.3±0.01	0.8±0.02
(Phytotech)	40	50	2.4±0.04	1.2±0.05
	50	54	2.6±0.03	1.5±0.01
	60	55	2.8±0.02	1.4±0.02
	70	50	2.6±0.01	1.6±0.04
	80	48	2.5±0.04	1.4±0.04
Maltose	30	46	2.3±0.05	0.7±0.03
(Phytotech)	40	48	2.5±0.04	1.0±0.04
	50	50	2.5±0.01	1.1±0.02
	60	51	2.6±0.02	1.5±0.03
	70	52	2.8±0.02	1.7±0.05
	80	50	2.5±0.03	1.5±0.06

combined with NAA, along with different carbon sources, such as sucrose, maltose, glucose and table sugar in various concentrations (Table 1, 2).

In all cases, after adjusting the pH to 5.7±0.01 prior to gelled with 0.8% agar (w/v) (BHD, England), the media were sterilized by autoclaving at 121°C for 20 min (1.06 kg cm^{-2}). All cultures were maintained in a growth chamber at 25±1°C under a 16/8 h (light/dark) photoperiod with a light intensity of 28-30 mol m^{-2} sec^{-1} (supplied by cool-white fluorescent lamps).

Data recording: To test the efficiency of microrhizome induction medium, data were collected for the following parameters: percentage of cultures induced rhizome, number of microrhizome per shoot and mean weight of microrhizome. Mean and standard error was calculated for the parameters. Each experiment contained at least 12 replications and the whole experiment was repeated three times.

RESULTS AND DISCUSSION

Effect of PGR: BAP singly and BAP in six different combination with NAA were used in this experiment to find out a suitable culture media for microrhizome induction (Table 1). Thirty gram per litter sucrose was used constantly in this experiment. The cultured shoots induced microrhizome within 7-10 weeks upon culturing on different media (Fig. 1c). Highest frequency of rhizome induction was recorded in 4.0 mg L^{-1} BAP containing medium. The maximum number (2.3±0.03) of microrhizomes per shoot was noticed on the same hormonal treatment.

Increased level of BAP showed the lower percentage of microrhizome induction. On the other hand, addition of NAA increase neither induction rate nor the number of microrhizome compared with the single use of BAP. Rhizome induction in PGR-free medium is rare. The effects of BAP as well as the NAA were tested as they were described as effective growth regulators for the genus *Curcuma* (Balachandran *et al*., 1990; Salvi *et al*., 2002; Sunitibala *et al*., 2001). Present result is in agreement with Nayak (2000) in *C. aromatica*, however, Shirgurkar *et al*. (2001), found BAP as inhibitory to microrhizome induction in turmeric.

Effect of carbon sources: Four different kinds of carbon sources viz., commercial sugar, sucrose, glucose and maltose were used in different concentrations (30, 40, 50, 60, 70 and 80 g L^{-1}) keeping constant BAP level (4.0 mg L^{-1}). Among the four different kinds of carbon sources, maximum parentage (70%) of shoot induced microrhizome when it was cultured on 60 g L^{-1} sucrose containing medium (Fig. 1c, d), whereas maltose showed the worst performance. From this investigation it could concluded that sucrose was most suitable and effective as a carbon sources and 60 g L^{-1} concentration with 4 mg L^{-1} BAP was best for microhizome induction in *C. zedoaria*. Being a sink organ, microrhizome formation and growth is obviously influenced by carbon sources. Large body of literature support the requirement of high sucrose level in inducing *in vitro* sink organ such as potato microtuber (Alam *et al*., 2003) sweetpotato tuber (Egnin *et al*., 2003) and zingiber microrhizomes (Sharma and Singh, 1995; Nayak, 2000; Shirgurkar *et al*., 2001). In the present study, the small difference between sucrose and commercial table sugar offers the possibility of using table sugar as a low cost alternative for large scale microrhizome production, which is remained to be explored.

Germination: Finally, the microrhizomes were transferred to PGR-free germination medium for shoot bud induction under aseptic conditions (Fig. 1e). About 45% rhizomes were germinated in this condition regardless of size and weight. Currently, we are evaluating the *ex vitro* germination and survival of the germinated plantlets.

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