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Factors Effecting *In Vitro* Microrhizome Formation and Growth in *Curcuma longa* L. and Improved Field Performance of Micropropagated Plants

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ABSTRACT: Microrhizomes were successfully produced from tissue culture derived shoots of *Curcuma longa* by transferring them to the liquid medium of Murashige and Skoog (MS) supplemented with 13.3mM BA (6-benzyladenine) and 6% sucrose, and culturing with a reduced photoperiod of 4 hours daily. Microrhizomes were formed at the base of the shoots grown on the medium after 30 days of incubation at 25 ± 1°C. The number of buds per microrhizome varied from 1-4 and the weight varied from 50 mg to 580 mg. Factors such as concentration of sucrose and BA in the medium, as well as photoperiod and their interaction, were found to have a significant effect in the induction of microrhizomes. Sucrose was most effective in rhizome formation, followed by photoperiod and BA in the medium. Microrhizomes were harvested after 120 days of culture. These microrhizomes could be stored in MS media with a low concentration of BA (0.04mM) and in moist sand at room temperature. Microrhizomes were produced *in vitro* independent of seasonal fluctuation, and sprouted with roots and shoots in the potting soil and were then transferred to the field. Rate of sprouting of microrhizomes was not affected by the amount of sucrose and BA in induction medium, but only by duration of photoperiod in culture. Comparison of field performance of conventionally seed propagated and micropropagated plants revealed relative improvement in rhizome yield of micropropagated plants of *Curcuma longa* through *in vitro* induction of microrhizome.

Abbreviations: BA,6 benzyladenine.

KEYWORDS: *Curcuma longa*, Microrhizome, Tissue culture, Sucrose, BA, Photoperiod.

INTRODUCTION

Turmeric (*Curcuma longa* L.), an important spice, is cultivated extensively in tropical regions of the world, from India to Indo-China, the East Indies and parts of China. It has been a well-known condiment and colouring agent since time immemorial. It is also highly valued for its medicinal properties[1]. It is widely used in traditional medicine in drugs of the digestive system, such as stomachics and cholagogues. Mucous stimulatory, antibacterial, antifungal, anti-inflammatory and antiarthritic activities of the essential oil of *Curcuma longa* have also been reported[1]. Various plant parts of turmeric are used for curing hazy vision, blindness, sores, rheumatism, indistinct speech, scabies etc. Curcumin, a major bioreactive secondary metabolite obtained from the rhizome of turmeric, which is anticarcinogenic[2], is now being used in anticancer drug development programmes. Leaves and stems of turmeric are also used as biofertilizer. In India, ethnologically, turmeric occupies an important position in our life, forming an integral part of rituals, ceremonies and cuisine.

India enjoys a monopoly in production and export of turmeric, returning an income of Rs.140 million per year. It is propagated vegetatively through rhizomes with a low multiplication rate (6x-8x per annum). Turmeric rhizomes have a dormancy period and only sprout during monsoon. A large amount of the edible part (rhizome) is stored for stock purpose for the next season. Maintenance of germ plasm by annual planting is expensive and labour intensive. Moreover, diseases such as rhizome rot, caused by *Pythium* sp., and leaf spot, caused by species of *Taphrina* and *Collectrichum,* take a heavy toll during storage and in the field, thereby causing a severe shortage of healthy planting materials. It is therefore highly desirable to resort to *in vitro* culture to provide a year round supply of disease-free seed rhizome and to maintain disease-free germplasm.

There are only a few reports on the use of tissue culture for *in vitro* multiplication of turmeric[3,4,5,6,7,8,9]. Among other species of *Curcuma*, microrhizome

formation has been reported for *C. aromatica* Salisb[10]. The present paper reports on successful *in vitro* formation of microrhizomes in *C. longa* and the different factors affecting induction and growth of rhizomes. Standardization of this protocol for *C. longa* was necessary because of its different genetic make up from *C. aromatica* and world wide market availability of this particular species for commercial production of turmeric. Moreover, this paper reports the role of *in vitro* formed microrhizomes in improved field establishment of micropropagated plants of *C. longa*, which was not included in recent work on microrhizome formation in this species[11] .

Materials and Methods

Establishment of Culture

Rhizomes of turmeric (*Curcuma longa*) were obtained from the market and maintained in the aromatic garden of the Regional Research Laboratory, Bhubaneswar, India. At the onset of the monsoon, when rhizomes sprouted, shoot buds of ca. 1 cm were collected and used as explants for establishment of tissue culture through multiple shoot bud induction on MS basal medium[12] supplemented with BA (4.4 - 22.2 μM).

Culture Conditions for Induction of Microrhizomes

Aseptic *in vitro* grown shoots approximately 4-5 cm long, which were derived from the established culture of turmeric, were used as explants for induction of microrhizomes. MS liquid medium supplemented with BA (4.4, 13.3 & 22.2 μM), sucrose (3, 6 & 9 g/l) was used and cultures were incubated at 25 ± 2°C. Varying photoperiods (16 hr, 8 hr, 4 hr and 0 hr (dark)) were tried to test their effect on rhizome formation. Culture tubes (25 x 150 mm) containing 20 mL liquid media were plugged with cotton and sterilized at 121°C and under 1.05kg/cm^2 pressure for 20 minutes. Cultures were grown under white fluorescent light with 55 μmol m^{-2} s^{-1} light intensity. The media pH was adjusted to 5.7. Each treatment was done on 15 replicates and repeated 3 times. Data on the percentage of rhizome formation in each replication, rhizome weight and number of buds (eyes) per rhizome were recorded after 90 days in culture.

Harvesting, Storage and Growth of Microrhizomes

The microrhizomes were harvested aseptically in a laminar flow hood and were stored either in media supplemented with 0.04 μM BA or in poly bags containing sterile sand. Prior to storage in soil

Table 1. Effect of different media on microrhizome formation in *Curcuma longa*.

	BA (mM) "A"	SU (g/l) "B"	Ph(hr.) "C"	No. of explant with rhizome (MEAN ± SD)	% explant forming rhizome	Wt. of rhizome (mg) (MEAN ± SD)	No. of buds /rhizome (MEAN ± SD)
	4.4	3.0	16	-	-	-	-
F	4.4	60	16	1.6 ± 0.4	8.0	50.6 ± 22.6	1.2 ± 0.4
	4.4	60	4	5.3 ± 0.8	26.5	108.5 ± 35.2	1.5 ± 0.6
A	13.3	30	4	-		-	-
	13.3	60	16	6.3 ± 0.6	31.5	225.5 ± 35.5	2.5 ± 0.4
C	13.3	60	8	14.0 ± 1.3	70.0	290.2 ± 32.6	2.3 ± 0.6
	13.3	60	4	16.0 ± 1.5	80.0	325.3 ± 58.5	2.7 ± 0.8
T	13.3	60	0	7.0 ± 0.5	35.0	105.6 ± 20.5	1.0 ± 0.5
	13.3	90	8	9.0 ± 0.8	45.0	210.6 ± 36.2	1.8 ± 0.3
O	22.2	90	4	9.3 ± 1.2	46.5	235.5 ± 40.6	2.0 ± 0.6
	22.2	30	4	-		-	-
R	22.2	60	16	3.6 ± 0.6	18.0	210.2 ± 26.5	2.2 ± 0.5
	22.2	60	8	11.3 ± 1.1	56.5	282.4 ± 42.3	2.3 ± 0.8
S	22.2	60	4	12.3 ± 1.2	61.5	315.6 ± 48.3	2.5 ± 0.8
	22.2	60	0	4.6 ± 0.8	23.0	102.2 ± 20.6	1.1 ± 0.4
	22.2	90	4	8.0 ± 1.4	40.0	180.3 ± 28.3	1.5 ± 0.6

F- Value of ANOVA	No. of explant with rhizome	Wt. of rhizome (mg)	No. of buds /rhizome
Factor-A	182.17**	165.25**	0.13*
Factor-B	644.75**	583.36**	5.22**
Factor-C	255.36**	226.71**	7.58**
Interaction of Ax B x C	6.93**	5.57**	0.07*

Data represent an average of 20 replicates per treatment. Experiments were repeated thrice. No response was obtained in media No.1,4 & 11 (-). 'SU' indicates sucrose and 'Ph' indicates photoperiod. Factors "A", "B" and "C" indicate BA, sucrose and photoperiod treatments.
* Not significant. ** Significant at P = 0.01.

microrhizomes were repeatedly washed in running tap water and air-dried. Poly bags were kept in a net house (ca. 30°C). Growth initiation percentages of rhizomes were recorded.

Field Analysis of Micropropagated Plants

Micropropagated plants with *in vitro* formed microrhizomes (TCM), plants grown in culture devoid of microrhizomes (TCO) and seed rhizomes (S) were planted at the onset of the monsoon season in a randomized block design. S, TCM and TCO plants were planted in 3 separate blocks. Each block consisted of a 3-row bed with 20 cm between plants along the row and 30 cm between rows. Observations were taken from 8 random plants per row. Data on shoot length and number were recorded after 4 months of growth in the field and data on rhizome yield/plant and number and weight of fingers were recorded at harvest during February, i.e. after 8 months of growth. Plants of the first generation were raised in the field from the rhizomes of culture-derived plants, whereas second generation plants were raised from field grown stock rhizomes of the first generation plants.

Statistical Analysis of Data

Data were subjected to analysis of variance to test the individual effect of photoperiod, BA and sucrose, as well as the interaction among these factors on induction of microrhizomes in culture. Field data were subjected to a 't' test. Experiments were repeated thrice.

Results

Establishment of Culture

The maximum number of shoots formed per bud (4.7 ± 0.3 shoots per explant) during culture initiation was obtained on the medium containing 13.3 μM BA within 15 days. With an increase in concentration of BA beyond 13.3 μM, the number of shoots per bud decreased, whereas an increase in the number of shoots formed was seen when BA was increased from 4.4 to 13.3 μM. Roots were simultaneously formed on shoots on the same medium and plantlets with well-developed roots and shoots were obtained after 4 weeks of incubation. Plantlets were maintained in MS liquid medium through repeated sub-culturing.

Microrhizome Induction

Results obtained for microrhizome formation on media differing in concentrations of sucrose, growth regulators as well as duration of photoperiod are tabulated in Table 1. All the media listed in Table 1 except S1, S4 and S11 were effective in stimulating microrhizome production *in vitro*. Growth under 4 hr photoperiod in medium (S7) containing 13.3 μM BA and 60g/L sucrose was found to be most effective in induction of microrhizome Table 1 and Fig.1. After 20-25 days of incubation in microrhizome induction, medium swelling of shoot bases was observed, followed by appearance of microrhizomes at the base within 40-45 days of incubation (Fig.2).

Fig 1. In vitro grown shoot of *Curcuma longa* showing induction of microrhizomes at its base after 40 days of culture.

Fig 2. Shoot base of *Curcuma longa* showing formation of microrhizomes.

Microrhizome induction medium was replaced with fresh medium at monthly intervals. Microrhizomes were then harvested after 120 days of incubation. Microrhizomes obtained from the different treatments varied considerably in average weight ranging from 50.6 to 325.3 (Table 1). The average number of buds per microrhizome also varied from 1.2 to 2.7 among different treatments (Table 1).

Effect of Sucrose

Considerable differences were observed in formation of *in vitro* microrhizomes in turmeric from the different concentrations of sucrose. Rhizome formation was induced *in vitro* at the basal region of each shoot when the concentration of sucrose was increased from 30 g/L to 60 g/L. Media (S7) with 60 g/L sucrose 13.3 μM BA and grown under a photoperiod of 4 hrs induced the best response. However, with further increase in concentration of sucrose from 60 g/L to 90 g/L there was a marked decrease in the percentage of response of explants showing rhizome formation (Table 1).

Effect of BA

Microrhizomes were formed in medium in the presence of BA (13.3 μM). However, the effect of BA in microrhizome formation was dependent on the presence of sucrose (60 g/L) in the medium in *C. longa*. It was also observed that microrhizome formation was more pronounced under 8 & 4 hr light than with 16 hr light.

Effect of Photoperiod

Microrhizomes *in vitro* were best initiated in a photoperiod of 4 hr and media supplemented with 60 g/L sucrose and 13.3 μM BA. With further reduction in the photoperiod to 0 hr (dark), cultures became etiolated with rudimentary leaves and thin upright stems. Microrhizomes produced in the dark (media S8 & S15) had a significantly lower number of buds (1.0 and 1.1 per microrhizome respectively) than those produced in presence of light in media with BA 13.3 μM and 60 g/L, sucrose (S5, S6, S7) and in media with BA 22.2 μM and sucrose 60 g/L (S12, S13, S14) (Table 1).

Interaction among Sucrose, BA and Photoperiod

It is evident from Table 1 that the interaction of BA, sucrose and photoperiod was significant for *in vitro* formation of rhizomes. Among these three factors, sucrose was most effective in formation of rhizomes followed by photoperiod and growth regulator, as evidenced by their respective 'F' values in Table 1. However, the effectiveness of sucrose concentration depended on the duration of photoperiod and concentration of BA in the medium, as evident from the best response obtained in the condition S7 with an enhanced concentration of sucrose (60 g/L) in the presence of 13.3 μM BA in the medium with a photoperiod of 4 hours. The interactions between BA and sucrose, sucrose and photoperiod, BA and photoperiod, and BA, sucrose and photoperiod taken together were highly significant, as shown in Table 1.

Harvesting

Microrhizomes were harvested after 120 days of growth in media. A comparison among treatments indicated that S7 produced the heaviest microrhizomes (average weight 325.3 mg) and S2 the lightest (average weight 50.6 mg). The range of variation in different media was from 50 mg to 580 mg. The average number of buds also varied in different media from 1.2 to 2.7 (Table 1). After harvest, microrhizomes were washed several times with running tap water and air dried in shade for 3-4 hrs. Since the microrhizomes were immersed in high sucrose concentrations, the chance of fungal attack could potentially be minimized by thorough washing.

Storage

Microrhizomes were formed year round *in vitro*, irrespective of any seasonal fluctuation. For *ex vitro* storage, microrhizomes were kept in polybags or pots filled with sand in a net house at room temperature. *In vitro* storage was done by sub-culturing microrhizomes to MS basal media with a low concentration of BA (0.04 μM). Microrhizomes could be stored in this medium up to 240 days, being subcultured once after 120 days. Microrhizomes produced *in vitro* could be of use to commercial growers as disease-free planting materials. *In vitro* storage also facilitates germplasm conservation and exchange at the regional, national and international levels. This technique can be useful for rapid and year round production of disease-free microrhizomes *in vitro*.

Growth Induction of Rhizomes

Microrhizomes sprouted on media and in potted soil producing shoots and roots. Microrhizomes stored in media with 0.04 μM BA sprouted in all seasons when the concentration of BA was changed to 13.3 μM. Microrhizomes, which were stored in soil sprouted (Fig.3) only at the onset of the monsoon in July. It was observed that rate of growth induction of microrhizomes in media was always relatively more than that obtained in soil. This higher rate of growth of microrhizomes on media may be due to the pathogen-free environment and perhaps also to ready nutrient availability *in vitro*. Work is in progress to minimize the rate of mortality in soil so as to increase the rate of germination in soil. While studying the

Table 2. Growth initiation of microrhizomes of *Curcuma* on media and soil.

Condition in which rhizome is induced	Rhizome growth on media (No.)	(%)	Rhizome growth in soil (No.)	(%)
MS+BA (4.4)+ SU (60) + Ph (4)	(15/21)	71.4	(12/21)	57.1
MS+BA (13.3)+ SU (60) + Ph (16)	(15/20)	75.0	(12/20)	60.0
MS+BA (13.3)+ SU (60) + Ph (8)	(16/18)	88.8	(12/18)	66.6
MS+BA (13.3)+ SU (60) + Ph (4)	(18/22)	81.8	(14/22)	63.6
MS+BA (13.3)+ SU (60) + Ph (0)	(11/24)	45.8	(8/24)	33.3
MS+BA (22.2)+ SU (90) + Ph (4)	(16/20)	80.0	(13/20)	65.0
MS+BA (22.2)+ SU (60) + Ph (4)	(14/18)	77.7	(10/18)	55.5
MS+BA (22.2)+ SU (60) + Ph (0)	(10/20)	50.0	(7/20)	35.0

effects of different factors such as BA, sucrose and photoperiod on rate of growth induction, no significant difference was obntained in the rate of induction of microrhizomes in media with different concentrations of sucrose (60 g/L and 90 g/L) or with different concentrations of BA (4.4, 13.3 & 22.2 μM) (Table 2). On the other hand, microrhizomes grown in media with different photoperiods (light and dark) showed different rates of induction, both *in vitro* and *ex vitro* (Table 2). Relatively lower rates of induction of microrhizomes on media grown in the dark could be attributed to the relatively lower number of buds formed on the microrhizomes in the dark. The freshly sprouted plantlets in pots (Fig. 4) were transferred to the field and showed normal growth.

Field Analysis of Micropropagated Plants

Comparative analysis was made among micropropagated turmeric plants with *in vitro* induced microrhizomes (TCM), without having microrhizome (TCO) and conventional seed propagated (S) plants. It was observed that the first generation of micropropated plants out of culture with or without *in vitro* induced microrhizome produced significantly greater (P=0.01) numbers of shoots than normal seed derived turmeric. The average numbers of shoot obtained were 6.1, 12.3 and 13.5, respectively, for S, TCO and TCM plants after four months of growth in the field. No marked difference was observed in length of the shoots among plants of any origin.

A comparison of rhizome characteristics

Fig 3. Sprouting microrhizomes with shoots and roots.

Fig 4. Microrhizome derived plants in pots.

Table 3. Comparative rhizome characteristics of micropropagated (TCM, TCO) and normal seeds derived parts in the field in the 1st generation (after 8 months of growth).

Character	TCM	TCO	S
Mean rhizome yield/plant (g)	85.2 *	43.3 **	123.5
Mean fingers/plant	13.5	7.3	9.8
Mean weight of fingers (g)	5.1	3.6	8.3
Mean weight of rhizome planted initially (g)	0.318	0	15.3

Data represents means of 24 plants.
TCM - Plant derived from culture with *in vitro* formed microrhizome.
TCO - Plant derived from culture without having preformed microrhizome.
**Significant as compared to S (P = 0.001).
*Significant as compared to TCO (P = 0.01).
(Student's 't' test was performed).

determined at harvest (i.e. after eight months of growth in the field) revealed marked differences. The rhizomes from conventional seed-derived turmeric (S) were significantly (P=0.001) heavier than tissue culture derived plants (TCO), which formed rhizomes after being planted in soil (Table 3). Similarly, plants (TCM) derived from culture with *in vitro* induced microrhizomes when planted in the field produced significantly (P=0.01) heavier rhizomes in comparison to micropropagated plants (TCO) (Table 3). The average rhizome yield per plant was 123.5 g, 85.2 g and 43.3 g, in S, TCM and TCO plants, respectively, whereas the average weights of rhizomes during plantation in the 1st generation was 15.3 g, 0.318 g and 0 g, respectively, for S, TCM and TCO plants. It was also observed that rhizome rot was less pronounced, with a 4% infection rate of rhizome derived from micropropagated plants in comparison to a 10% infection rate in seed derived plants. The difference in shoot number and rhizome size among conventional seed propagated and culture derived populations could only be differentiated clearly in the first generation of plants in the field. By the second generation, plants derived from culture (TCM) were indistinguishable from plants that had always been propagated from conventional seed-derived rhizomes.

Discussion

The result of the present study revealing relatively higher rate of shoot formation per bud with a relatively low concentration of BA is in agreement with that of the Sharma & Singh[13], who reported that a relatively lower concentration of growth regulator has a positive effect in *in vitro* shoot multiplication.

The role of growth regulators, photoperiod and sucrose concentration on *in vitro* induction of microrhizome in turmeric may be related to *in vitro* formation of tubers, bulbs and corms in other plants[14,15,16,17]. The promoting effect of sucrose on the formation of storage organs (corms and tubers) has been reported earlier[10,11,14,16]. The concentration of BA required for microrhizome formation *in vitro* for *C. longa* was different from that required for *C. aromatica*[10]. This difference in requirement of exogenous supply of BA to the medium could be attributed to the genotypic difference existing between *C. longa* and *C. aromatica*. The effectiveness of BA in microrhizome induction with relatively shorter photoperiods of 4-8 hours duration is in close agreement with the result of Hussey and Stackey[18], who reported that microtuber formation induction in potato by BA was more pronounced with short day length. The effect of photoperiod on microrhizome formation observed in the present study was comparable to the report of Gopal et al[19], who showed a relatively lower rate of bud formation in the dark during microtuberization of potato. However, the present observation differs from that obtained for *Zingiber officinalis*. In *Z. officinalis,* Sharma and Singh[20] reported formation of microrhizome in the dark (0 hr light), whereas Sakamura et al[21] reported requirement of continous light (24 hrs). Bhat et al[22] reported formation of microrhizome in *Z. officinalis* culture grown with a 16 hr photoperiod. The different photoperiod requirement in *Z. officinalis* may be potentially attributed to the different genetic make up of the species. The present findings with regards to the requirement of photoperiod on microrhizome induction in *C. longa* differ from that of *C. aromatica*, where 8 hr photoperiod is to be maintained[10].

Increased shoot formation in micropropagated plants in comparison with conventional seed (rhizomes) derived plants might be due to the carry over effect of BA supplied to the medium for shoot multiplication, as reported in *Z. officinalis*[23]. The higher rhizome yield of TCM micropropagated plant in the first generation in comparison to TCO micropropagated plants might be attributed to the pre-planting presence of rhizomes (seed reserve) in the former. The even higher rhizome yield in conventional seed propagated plants might also be due to the presence of pre-formed rhizomes. The observed yield difference in rhizomes of micropropagated and conventional seed propagated plants in *C. longa* is in agreement to the report of Bhagyalakshmi and Singh[24], who also found significantly lower yield with micro ginger harvested at 8 months compared with seed derived ginger, and also attributed their difference to micopropagated plants lacking a rhizome (seed reserve) when planted.

The protocol for *in vitro* microrhizome formation in *C. longa* and its role in improvement of rhizome yield of micropropagated plants can be utilized by commercial growers for production of disease-free

turmeric in large scale.

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