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HIGH FREQUENCY CLONAL PROPAGATION AND MICRORHIZOME INDUCTION OF *CURCUMA LONGA L.* (CV LAKADONG)- A RICH SOURCE OF CURCUMIN OF NORTH EAST INDIA

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ABSTRACT
A simple *in vitro* protocol has been developed for efficient plant regeneration and microrhizome induction from axillary buds of *Curcuma longa* L. The best response for shoot multiplication (19.40$\pm$0.51 shoots/ per explant) within 4 weeks was observed in MS medium supplemented with BAP (2.0 mgL^{-1}), NAA (1.0 mgL^{-1}) and 3.0% (w/v) sucrose. A maximum of (100.00$\pm$0.71%) root induction were obtained for the plant in MS medium supplemented with NAA (1.0 mgL^{-1}) with reduced level of sucrose (2%). The optimum concentration of sucrose was found to be 6% for the production of microrhizomes on ½ strength MS basal medium supplemented with BAP (5.0 mgL^{-1}) and NAA(0.5 mgL^{-1}) after 4 weeks of culture in microrhizome induction media with an average weight of 2.03g. Larger microrhizomes performed better under *in vivo* conditions and developed shoots readily, when they were transferred directly from *in vitro* to soil without acclimatization. Microrhizomes produced under *in vitro* conditions can be stored and transported easily which are advantageous for *in vitro* shoot multiplication.

INTRODUCTION

Curcuma longa L, also known as turmeric plant (*Zingiberaceae*) is a perennial rhizomatous herb (Naz *et al.*, 2009) and is widely distributed through out the tropics of Asia and extends to Africa and Australia (Govindarajan, 1980). Turmeric, the important colouring and aromatic ingredients of curry powders is enormously used in Asian cuisines (Apavatjrut *et al.*, 1999) and pharmaceutical industries (Majeed *et al.*, 1995). It is one of the most important spice crops of India. The processed underground rhizome used as spice, herbal medicines, dyeing agents and cosmetics since Vedic age (Shirgurkar *et al.*, 2001). The dried rhizome of *Curcuma longa* L. has been found to be a rich source of beneficial phenolic compounds known as the curcuminoids (Lechtenberg *et al.*, 2004). A number of different biologically active substances have identified by which demonstrate germicidal, aromatic, carminative, antihelmintic, antioxidant, anti-tumour, cholesterol lowering and neutroprotective activities (Sasaki *et al.*, 2004). *Curcuma longa* is one of the most important economic species produced in great quantities for international trade. India, Bangladesh, Taiwan and China are the major turmeric producing countries of the world. India enjoys a monopoly in production and export of turmeric with returning an income of Rs.140 million per year (Nayak and Naik, 2006), whereas, 85% production comes from Asian countries and 15% from Europe and North America (Luthra *et al.*, 2001). Higher curcumin content of turmeric fetches better price in international market. The turmeric cultivated in Meghalaya district (cv.Lakadong) is rich in curcumin content. Turmeric is a sterile triploid and is propagated vegetatively through underground rhizomes division and replanting with a low multiplication rate (6x - 8x per annum) with yield ranging from 15 to 25 ton/hectare (Balachandran *et al.*, 1990). However, some diploids and tetraploids have been reported which also do not produce or rarely produce fertile seeds due to incompatibility and high pollen sterility (Joseph *et al.*, 1999). Because of this, it is susceptible to accumulation and transmittance of pathogens from generation to generation, and amplification of particularly useful stocks is a slow process.

Turmeric rhizomes have a dormancy period and they sprout during monsoon only. A considerable amount of the edible part (rhizome) 10-20% of the total yield is stored for stock purpose for the next cropping year (Shirgurkar *et al.*, 2001). Maintenance of such a huge amount of seed-rhizomes for annual planting is expensive and labour intensive. Moreover, diseases such as rhizome rot, caused by *Phytium myriotylum*, *Phytium aphanider* and *Pseudomonas solanaraceum* (Balachandran *et al.*, 1990; Nayak, 2000; Salvi *et al.*, 2002; Shirgurkar *et al.*, 2001) and leaf spot, caused by species of *Taphrina* and *Colletoctrichum*, creates severe problem during storage and in the field, thereby causing shortage of healthy planting materials. The risk of transmittance of diseases from one generation to the next is great, and *Pseudomonas solancearum* (bacterial wilt disease), *Fusarium oxysporum* f. sp. Zingiberi (fusarium yellows disease), and *Pythium species* (soft/root rot) are transmitted through the rhizome which are conventionally used as planting material (they remain in the tissue used as seed) and lead to about 60% of crop losses

(Kuanar *et al.*, 2009) to growers. *In vitro* regeneration and microrhizomes induction is an ideal method for the production of disease-free planting material and also for conservation and exchange of germplasm in different species.

Micropropagation of different species of *Curcuma* viz., *C. zedoaria*, *C. aromatica* (Yasuda *et al.*, 1987), *C. aeruginosa* (Balachandran *et al.*, 1990), *C. amada* (Borthakur and Bordoloi, 1992; Prakash *et al.*, 2004), *C. haritha* (Bejoy *et al.*, 2006), *C. caesia* (Balachandran *et al.*, 1990; Tyagi *et al.*, 2004; Bharalee *et al.*, 2005) has been reported. Rapid multiplication of different cultivars of *C. longa* was reported earliar through rhizome bud proliferation as well as through other *in vitro* strategies by different workers (Balachandran *et al.*, 1990, Salvi *et al.*, 2002; Shirgurkar *et al.*, 2001; Sunitibala *et al.*, 2001; Nirmal Babu and Minoo, 2003; Praveen, 2005). Few reports are also available on microrhizome induction of *Curcuma longa* (Nirmal Babu and Minoo, 2003; Nayak, 2000; Sunitibala *et al.*, 2001; Shirgurkar *et al.*, 2001) and its work requires improvement for production of healthy, large sized microrhizome. However, reported studies of other cultivars of *Curcuma longa* are mostly dealt with the evaluation of plant growth regulators (PGRs) with a single nutrient formulation system. Here, we have studied and compared the effect of different PGRs on MS medium on *in vitro* multiplication and microrhizome induction of *Curcuma longa*. The present investigation was also carried out to see the effects of *in vitro* culture conditions such as sucrose concentration, strength of basal medium and light illumination on large scale healthy microrhizome induction in *C. longa* (cv. Lakadong).

MATERIALS AND METHODS

Freshly collected underground rhizomes of *Curcuma longa* were cleaned with running tap water and immature dormant rhizome axillary buds were then excised with sharp blade and washed with detergent (Tween-20, 0.1% v/v) for 10 min and subsequently rinsed with clean water , which were used as the source of explants. Explants were then surface sterilized in disinfectant (0.1% $HgCl_2$ to which two – three drops of Tween-20 were added) for 12 minutes and rinsed with sterile distilled water. The buds were trimmed into 3.0-4.0 mm size with rhizome axillary bud, which served as the primary explant. Initially explants were cultured on shoot induction medium in order to obtain contamination free cultures. MS (Murashige and Skoog, 1962) basal media supplemented with 3% sucrose and 2.2gL^{-1} Gelrite modified with various concentratinons of N6-Benzyl-aminopurine (BAP 0.05 to 3.0 mgL^{-1}) in combination with Kinetin (Kn; 0.5- 2.0 mgL^{-1}), Indole-3-acetic acid (IAA; 0.5-1.0 mgL^{-1}), β-naphthaleneacetic acid (NAA; 0.5-1.0 mgL^{-1}) and 2, 4-D (0.5-4.0 mgL^{-1}) were used for establishment of the culture. Laboratory reagent-grade sucrose was replaced by locally available commercial sugar (market sugar) as carbon source for reducing the cost of the media.

The pH of the media was adjusted to 5.8 prior to the addition of the gelling agent. The media was autoclaved at 121ºC with 15 lbs pressure for 15 minutes. Excised buds were inoculated into culture media under laminar air flow. Cultures were incubated at 25±2ºC under 16 hr of photoperiod from cool white fluorescent tube giving 12.5μmol m^{-2}s^{-1} at culture level. Most of the cultures sprouted within 5-15 days of inoculation and the emerged shoots (1.0-2.0 cm) were subcultured in the same media for further multiplication. A total of 20 explants were used for each of the treatments and the number of shoots per explant was recorded after four weeks of inoculation. Each set of experiment was repeated thrice.

Root induction and acclimatization of plants

Fully grown shoots were transferred to rooting media containing Indole-3-acetic acid (IAA; 0.005 – 2.5 mgL^{-1}), α - naphthaleneacetic acid (NAA; 0.005 – 2.5 mgL^{-1}), 2-3% sucrose (market sugar). Rooted shoots were transferred to ½ strength basal medium without growth regulator for 10 days. The plantlets were washed thoroughly with sterile distilled water and then transferred to potting mixture (soilrite and compost, 10: 1, v/v) and kept in the growth chamber for 7 days. The environment of the growth chamber was maintained initially at 80% R.H., 24ºC temperature, 12 hr photoperiod and gradually adjusted with the slight alteration of the environment to reach as similar to the outside. Finally *in vitro* acclimatized plantlets were transferred to the soil in the earthen pots and ultimately planted in the nursery.

Microrhizome induction

Aseptic shoots approximately 4-5 cm long, which were derived from the established culture of *C. longa*, were used as explants for induction of microrhizomes. After separating the fully grown shoots originated from the axillary rhizome bud the micro shoots were divided into small parts and again transferred to fresh medium devoid of growth hormones and cultured for another four weeks to avoid carryover effects of growth regulators and then cultured in freshly prepared medium for microrhizome induction. MS medium supplemented with BAP (0.5 to 5.0 mgL^{-1}), Kn (0.5-5.0 mgL^{-1}), NAA (0.5 to 1 mgL^{-1}) and IAA(0.5 to 1 mgL^{-1}l) along with sucrose (3%, 6% and 9%) was used for the study of microrhizome induction and cultures were incubated at 25±2ºC. Varying photoperiods (16 hr, 8 hr, 4 hr and 0 hr dark) were tried to test their effect on rhizome formation. 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 45 days of culture. The microrhizomes were harvested aseptically in a laminar flow hood and were repeatedly washed in running tap water and air-dried prior to storage. Microrhizomes were then stored in poly bags containing sterile sand and kept in a net house (30ºC) and growth initiation percentages of these microrhizomes were also recorded.

Statistical Analysis of Data

The recorded data was subjected to statistical analysis by using one way ANOVA using Duncan's Multiple Range Test (DMRT; Duncan, 1955) to determine the significant difference among various phytohormones, sucrose concentration, effect of photoperiod as well as the interaction among these factors on shoot multiplication, rooting and induction of microrhizomes in culture.

RESULTS AND DISCUSSION

Establishment of Culture

The nutrient formulation is critical in determining the success of *in vitro* propagation. In the present study, the morphogenetic responses of the rhizome axillary bud explants varied with respect to supplementation of different growth regulators. The culture medium with a suitable amount of cytokinin and auxins played an important role during the early stages of initiation and establishment of rhizome axillary buds of *Curcuma longa*. The synergistic effect of higher concentration of cytokinin with lower concentration of auxin induced better shoot organogenic response (Plate a) as reported by various workers in different plant species including other cultivar of *Curcuma longa* (Praveen, 2005, Misra *et al.*, 2004 and Vidya *et al.*, 2005).

The best response for shoot multiplication (86.20 ± 0.58) percent was obtained on MS medium containing BAP (2.0 mgL^{-1}) and NAA (1.0 mgL^{-1}) with highest number of shoots (19.60 ± 0.51) per explant within 4 weeks of culture (Table 1, Plate c). Similar results also reported by Sunitibala *et al.,* (2001) in other cultivar of *Curcuma longa*. This is followed by the MS media supplemented with BAP (1.0 mgL^{-1}) and 2,4-D(4.0 mgL^{-1}) showed (83.00 ± 1.41) percent response for shoot multiplication with (18.20 ± 1.93) number of shoots per explant (Table 1). But, higher concentrations of BAP reduced the frequency of shoot induction, which is in agreement with the studies in *Kaempferia rotunda* (Bejoy *et al.*, 2006).

However, Balachandran *et al.*, (1990) had reported BAP (3 mgL^{-1}) alone was optimum for maximum shoot formation in *Curcuma* species. But in the present study a comparatively lower response was recorded when BAP was added alone in the medium, which indicates that, the addition of NAA in the culture medium improves the response for *in vitro* shoot multiplication and growth of *Curcuma longa*. It was found that in higher cytokinin supplementation, shoots were recorded with stunted growth, reduced leaf number and crowded leaves, which is in conformity with the findings reported for few other medicinal plants (Rudra and Jewarkar, 2002). It has also been reported earlier that higher concentration of plant growth regulators was not suitable for *in vitro* culture of Zingiberaceae species (Stanly and Keng, 2007). Concentration of BAP above the optimal level (2.0 mgL^{-1}) and also Kn above the optimum level (1.0 mgL^{-1}) decreased the number of shoots per explants.

The positive effect of BAP on multiple shoot regeneration was also observed in ginger (*Zingiber officinale* Rosc.) (Balachandran *et al.*, 1990) and the present findings for *in vitro* propagation of lakadong cultivar of *Curcuma longa* was also confirm the same result. In MS media lower concentration of Kinetin was found to be responsive for *in vitro* establishment and shoot multiplication (3.80 ± 0.37 shoots/ explant) however, higher concentration inhibits shoot multiplication and growth. Balachandran *et al.*, (1990) also reported that higher concentration of Kinetin was not suitable for *Zingiber officinale*. In comparison to BAP, Kn did not show any promising result, which may be due to the root inducing factors, which are 'intrinsic' in the rhizomes of rhizomatous plants (Agretious *et al.*, 1996). The combined effect of BAP(1.0 mgL^{-1}) and IAA(0.5 mgL^{-1}) also showing the promising response with high percent (81.40 ± 0.51) of response and higher number (19.40 ± 0.51) of shoots per explant with a mean height of (5.80 ± 0.14)cm within 4 weeks of culture(Table 1, Plate b).

In the present study it was also recorded that, with the increase in the concentration of NAA above the optimum range (1.0 mgL^{-1}) callus induction was observed at the basal portion. Moreover, retention of the explants with proliferated shoots in the initiation medium for more than 8 weeks resulted in decline as indicated by shoot necrosis and premature leaf browning. It has been also observed from the present findings is that market sugar also effective for shoot multiplication of *C. longa*. Prakash *et al.*, (2002) also reported the same result for ginger and other cultivar of turmeric.

The extents of shoot multiplication in *Curcuima longa* could be increased to almost three-fold by simply modifying with favourable formulations of plant growth regulators. Nutrient composition, like PGR, plays a crucial role in achieving optimum explant response in *in vitro* and changing to a suitable formulation can have a favourable influence on survivality of explants, growth and development. Shoot tip necrosis was also observed in few treatments with higher concentrations of cytokinins. It was found that in high cytokinin medium, shoots were with stunted growth and crowded leaves. These responses are often related to nutritional, mainly calcium deficiencies in the immediate vicinity of the growing shoot as evidenced by Mc Crown and Sellmer (1987).

Spontaneous rooting was also recorded on the MS basal media supplemented with BAP along with IAA or NAA. Copious root formation along with the shoots was observed in many zingibers like, other cultivar of turmeric and ginger (Bharalee *et al.*, 2005; Balachandran *et al.*, 1990). The rhizome bud of *C. longa* produced shoots and roots simultaneously on most of the media tested for shoot multiplication. However, to develop a healthy root system, fully grown shoots (4-6 cm) after removing all the roots were transferred to MS medium supplemented with IAA or NAA. MS medium enriched with NAA(1.0 mgL^{-1}) and reduced level of sucrose (2%) responded for (100.00 ± 0.71) percent rooting in *Curcuma longa*, which produced a mean of (17.00 ± 0.32) number of roots per shoot within short period (9.00 ± 0.32 days) of culture (Table 2).

The rooted plantlets of *Curcuma longa* obtained from *in vitro* propagation were sequentially acclimatized (hardened). After hardening the plantlets were transferred in field conditions.

Microrhizome Induction

Fully-grown *in vitro* raised plantlets were trimed aseptically and transferred to microrhizome induction medium. After 20-25 days of incubation in microrhizome induction medium containing various phytohormones and different level of sucrose swelling of shoot bases was observed, followed by appearance of microrhizomes at the base within 30-45 days of incubation (Plate d). It was observed that sucrose plays a significant role in the size and number of microrhizomes in *Curcuma longa* and it was observed that 3% sucrose could not develop any microrhizomes even by increasing the concentration of BAP from the range of 1 to 5 mgL^{-1} or by increasing or decreasing the duration of the photoperiod. Present finding has in agreement with Nayak (2000) who also reported that 3% sucrose could not develop any microrhizomes of other cultivars of *C. longa* even by increasing

Table 1: *In vitro* response of *Curcuma longa* tested for multiple shoot induction from rhizome axillary buds. (after 4 weeks of culture in shoot multiplication media)

Concentration (mg/L) Media	% of explant with multiple shoots	Shoots/ explant MS Basal	Shoot length(Cm)
0.0	30.80±0.86f	1.40±0.24d	3.40±0.18c
BAP 0.05	40.00±1.41e	3.00±0.45c	3.70±0.35bc
BAP 0.5	46.00±1.14d	3.80±0.37c	4.20±0.29ab
BAP 1.0	50.40±0.93c	4.60±0.51b	4.30±0.30ab
BAP 1.5	56.80±0.97b	5.00±0.71b	4.30±0.28ab
BAP 2.0	63.20±1.39a	7.80±0.58a	4.80±0.18a
Kn 1.0	40.20±0.86c	3.80±0.37b	4.10±0.15b
Kn 1.5	56.20±0.73a	5.00±0.32a	5.20±0.17a
Kn 2.0	52.00±1.52b	3.40±0.24b	2.30±0.19d
BAP 1+Kn0.05	62.40±1.21a	11.20±0.37a	5.30±0.07a
BAP 1+Kn0.5	55.60±1.03b	9.00±0.45b	3.40±0.17d
BAP 1.5+Kn0.5	48.00±0.84c	8.80±0.37b	4.10±0.14c
BAP 2+Kn1.0	42.00±1.05d	6.20±0.66c	4.80±0.14ab
BAP 2.5+Kn1.0	39.60±0.51e	6.00±0.71c	3.30±0.24d
BAP 3.0+Kn1	30.60±0.40g	5.20±0.37c	3.00±0.16de
BAP 1.0+IAA0.5	81.40±0.51a	19.40±0.51a	5.80±0.14a
BAP 1.5+IAA0.5	66.20±1.11b	17.00±1.14b	5.10±0.10b
BAP 2+IAA0.5	62.00±0.71c	12.00±0.89c	4.50±0.11c
BAP 2.0+IAA1.0	57.00±0.63d	8.60±0.51d	4.00±0.18de
BAP1.0+2,4-D 0.5	60.40±2.11d	8.80±0.66d	5.00±0.17bc
BAP1.0+2,4-D 0.5	70.00±1.14c	12.00±0.71c	5.60±0.17b
BAP1.0+2,4-D 4.0	83.00±1.41a	18.20±1.93a	6.30±0.15a
BAP2.0+2,4-D 0.5	75.20±1.83b	15.00±0.89b	5.00±0.30bc
BAP2.0+2,4-D 1	58.00±2.70d	9.20±0.58d	4.20±0.20de
BAP2.5+2,4-D 1.5	44.00±1.52e	4.00±0.32e	3.60±0.19ef
BAP 1.0+ NAA 0.5	63.00±0.89d	14.20±0.86c	4.90±0.16b
BAP 1.5+ NAA 0.5	70.00±1.14c	14.00±1.00c	5.00±0.07b
BAP 2.0+ NAA 0.5	80.60±0.81b	16.60±0.68b	5.00±0.17b
BAP 2+ NAA 1.0	86.20±0.58a	19.60±0.51a	7.30±0.19a
BAP 2.5+ NAA 1.0	56.00±1.00e	8.60±0.51d	5.20±0.17b
Kn 1.0+ NAA 0.5	51.80±0.80bc	7.40±0.40c	4.10±0.14bc
Kn 1.5+ NAA 0.5	60.00±1.00a	11.00±0.32a	5.20±0.07a
Kn 2.0+ NAA 0.5	53.60±0.68b	10.40±0.51ab	4.30±0.09b
Kn 2.0+ NAA 1.0	50.00±0.71c	10.00±0.55ab	4.30±0.14b
Kn 2.5+ NAA 1.0	46.20±1.16d	9.60±0.51b	3.50±0.07d
Kn 1.0+ IAA 0.5	62.00±0.45a	10.20±0.58a	5.00±0.30a
Kn 1.0+ IAA 2.0	60.80±1.07a	9.60±0.51ab	4.00±0.17b
Kn 1.0+ IAA 4.0	60.00±0.71a	9.00±0.32b	4.10±0.10b
Kn 2.0+ IAA 0.5	52.60±0.51b	6.00±0.55c	3.30±0.10cd
Kn 2.0+ IAA 1.0	47.00±1.41c	6.00±0.45c	3.00±0.07d
Kn 2.5+ IAA 1.0	44.60±0.51d	4.20±0.37de	3.00±0.10d
Kn 1.0+2,4-D 0.5	52.00±0.71d	8.80±0.37d	4.70±0.13b
Kn 1.0+2,4-D 2.0	56.40±0.81c	10.20±0.37c	5.00±0.33b
Kn 1.0+2,4-D 4.0	67.20±0.58b	12.00±0.45b	5.00±0.18b
Kn 2.0+2,4-D 0.5	70.00±0.63a	15.40±0.51a	5.70±0.16a
Kn 2.0+2,4-D 1.0	56.00±0.71c	4.60±0.24e	4.20±0.16c
Kn 2.5+2,4-D 1.0	43.00±0.71f	3.00±0.00fg	3.40±0.10de
S.Ed.±	1.32	0.83	0.23
$CD_{0.05}$	2.66	1.68	0.47

the concentration of BA or by increasing the duration of the photoperiod.

In the present study 6% sucrose was found to be optimum for the production of microrhizomes on ½ strength MS basal medium supplemented with BAP (5.0 mgL^{-1}) and NAA(0.5 mgL^{-1}) after 4 weeks of culture. Similar results also reported earlier by Islam *et al.,* (2004) in full strength MS basal media. Healthy, large sized and maximum number (16.00±0.71) of microrhizomes with an average weight of (2.03±0.00)g were obtained in this combination under 16 hr of photoperiod (Table 3, Plate e). But incubation under complete darkness reduced the number and size of the microrhizomes significantly. Moreover, the basal media containing 9% sucrose also gave good result for the production of healthy and large sized microrhizomes (1.33±0.04)g on MS basal medium supplemented with Kn (1.0 mgL^{-1}), NAA (0.5 mgL^{-1}) and IAA (0.02 mgL^{-1}) after 4 weeks of culture condition. Shirgurkar *et al*., (2001), Sunitibala *et al*., (2001) and Nayak (2000) also confirms the same result in *Curcuma* sp. Shirgurkar *et al*., (2001) obtained the highest number of microrhizomes (5.6±0.8 - 7.0±1.1) at 6% sucrose, while at 8% sucrose they reported the reduction in microrhizome number (5.6±0.5-

Table 2: Rooting response of *Curcuma longa*

Sucrose concentration	Concentration (mg/L) IAA mg/L	NAA mg/L	No shoot of responded for rooting	Rooting %	Days to root induction	Root/shootlet	Root length(cm)
00+Sucrose 30 g/L	0	0	5.00±0.32gh	30.00±0.71m	28.00±0.71a	6.00±0.32h	2.00±0.00j
Sucrose 20 g/L	0.005	0	6.00±0.45fg	50.00±0.32j	17.00±0.55b	7.00±0.32gh	2.00±0.32j
Sucrose 20 g/L	0.05	0	7.00±0.45ef	58.00±0.32i	16.00±0.45bc	9.00±0.32ef	2.90±0.09ghi
Sucrose 20 g/L	0.5	0	8.00±0.32de	66.00±0.32h	13.00±0.45ef	9.40±0.24e	3.70±0.23bc
Sucrose 20 g/L	1.0	0	10.00±0.55bc	70.00±0.71g	11.00±0.71gh	10.00±0.71e	3.00±0.14fgh
Sucrose 20 g/L	1.5	0	11.00±0.45b	91.00±0.32b	10.00±0.32hi	12.80±0.37cd	3.20±0.10efg
Sucrose 20 g/L	2.0	0	9.00±0.32cd	75.00±0.71e	12.00±0.32fg	9.00±0.55ef	4.00±0.07ab
Sucrose 20 g/L	2.5	0	5.00±0.32gh	41.00±0.32k	15.00±0.32cd	7.00±0.32gh	3.70±0.10bc
Sucrose 20 g/L	0	0.005	6.00±0.32fg	50.00±0.71j	15.00±0.32cd	4.00±0.55i	3.60±0.13cd
Sucrose 20 g/L	0	0.05	7.00±0.32ef	58.00±0.84i	13.00±0.32ef	6.00±0.32h	2.70±0.07hi
Sucrose 20 g/L	0	0.5	9.00±0.32cd	75.00±0.45e	10.00±0.32hi	9.00±0.32ef	3.30±0.07def
Sucrose 20 g/L	0	1.0	12.00±0.32a	100.00±0.71a	9.00±0.32i	17.00±0.32a	4.20±0.07a
Sucrose 20 g/L	0	1.5	10.00±0.32bc	70.00±0.71g	12.00±0.32fg	13.00±0.45cd	3.70±0.07bc
Sucrose 20 g/L	0	2.0	8.00±0.32de	66.00±0.45h	13.00±0.45ef	8.00±0.32fg	3.00±0.07fgh
Sucrose 20 g/L	0	2.5	6.00±0.45fg	50.00±0.55j	14.00±0.32de	7.00±0.32gh	3.30±0.10def
S.Ed.±			0.49	0.80	0.56	0.58	0.16
CD0.05			0.97	1.58	1.12	1.15	0.31

5.8±1.1) but a slightly increase in their size. The enhanced rate of *in vitro* organ formation with increasing concentration of sucrose may be attributed to the presence of high carbon energy in the form of sucrose since storage organs mostly

Table 3: Effect of MS media modified with various concentration of sucrose, phytohormones and photoperiod on microrhizome formation in *Curcuma longa* (L) (after 4 weeks of culture in microrhizome induction media)

Media	Sucrose concen (%)	Photo period (hr)	Number of explant with micro-rhizome	% explant forming microrhizome	Weight of micro rhizome	Number of buds/rhizome
0	0	0	0	0	0	0
MS 0	30	16	-	-	-	-
½ MS 0	60	0	3.00±0.32pq	30.00±0.45 n	0.04±0.00pq	2.20±0.20ijk
½ MS 0+BAP 2.0 mg/L+NAA 0.5mg/L	90	8	3.00±0.32pq	20.00±0.32 p	0.07±0.00o	5.20±0.37d
½ MS 0+BAP 3.0mg/L+NAA0.5 mg/L	60	4	3.00±0.32pq	20.00±0.55 p	0.02±0.00r	6.40±0.51c
½ MS+BAP 5.0mg/L+NAA 0.5 mg/L	60	16	18.00±0.45a	80.00±0.32 a	2.03±0.00a	16.00±0.71a
½ MS+BAP 5.0mg/L+NAA 0.5 mg/L	90	16	16.00±0.32b	70.00±0.55 c	1.05±0.00c	8.20±0.37b
½ MS+BAP 5.0mg/L+NAA 1.0 mg/L	30	16	-	-	-	-
½ MS+BAP 0.5 mg/L	60	0	3.80±0.37op	10.00±0.32 s	0.15±0.00m	2.80±0.37ghijk
½ MS+ BAP 1.0 mg/L	60	0	4.20±0.37mno	26.00±0.32 o	0.21±0.00k	2.60±0.24ghij
½ MS+BAP 2.0 mg/L	60	0	15.60±0.24b	20.00±0.32 p	0.32±0.00h	8.80±0.37b
½ MS+BAP 3.0 mg/L	60	0	5.20±0.37klm	43.40±0.75 i	0.30±0.00hi	2.40±0.24hijk
MS+BAP 5.0 mg/L	30	16	-	-	-	-
MS+BAP 5.0 mg/L	60	16	5.00±0.32lmn	74.60±0.24 b	1.33±0.04b	8.20±0.37b
MS+ BAP 0.5 mg/L+NAA 0.05mg/L	30	8	-	-	-	-
MS+BAP3.0 mg/L+NAA 1.0mg/L	60	0	9.20±0.49g	48.00±0.32 g	0.24±0.00j	2.60±0.24ghij
MS+BAP5.0 mg/L+NAA 0.5 mg/L	60	0	11.20±0.37f	20.00±0.55 p	0.97±0.00d	3.00±0.00ghijk
MS+BAP 5.0 mg/L+NAA 1.0mg/L	60	0	9.00±0.32gh	16.60±0.51 q	0.29±0.01i	2.20±0.20ijk
MS+BAP 5.0 mg/L+NAA 1.0 mg/L	60	0	16.00±0.45b	40.00±0.71 j	0.80±0.01e	2.80±0.37ghijk
MS+Kn1.0 mg/L	60	4	-	-	-	-
MS+ Kn2.0 mg/L	60	0	13.00±0.45de	63.40±0.24 d	0.32±0.00h	2.60±0.24ghij
MS +Kn3.0 mg/L	60	4	5.60±0.40kl	20.20±0.37 p	0.11±0.00n	3.20±0.20ghi
MS+ Kn5.0 mg/L	60	0	-	-	-	-
MS+ Kn0.5 mg/L+NAA0.05 mg/L	90	16	6.20±0.37jk	40.00±0.71 j	0.14±0.00m	2.00±0.00jk
MS+ Kn1.0 mg/L+NAA 0.5 mg/L	90	8	8.00±0.45hi	33.40±0.24 l	0.11±0.00n	3.60±0.24fg
MS+ Kn2.0 mg/L+NAA1.0 mg/L	90	8	7.20±0.58ij	44.00±0.55 i	0.21±0.00k	2.60±0.24 ghij
MS+ Kn3.0 mg/L+NAA 1.0 mg/L	90	4	4.20±0.20mno	36.00±0.32 k	0.20±0.00kl	2.20±0.20 ijk
MS+ Kn5.0 mg/L+NAA 1.0 mg/L	90	4	4.00±0.32nop	13.60±0.24 r	0.20±0.00kl	2.00±0.32 jk
MS+ BAP1.0 mg/L +NAA 0.5 mg/L+IAA 0.02 mg/L	60	16	13.60±0.24cd	40.00±0.32 j	1.09±0.00c	3.00±0.00ghijk
MS +Kn1.0 mg/L+NAA 0.5 mg/L +IAA 0.02 mg/L	60	16	18.00±0.32a	60.00±0.32 e	1.33±0.04b	3.40±0.24fgh
MS+ Kn2.0 mg/L+NAA 1.0 mg/L	60	8	7.2±0.24i	44.00±0.32 h	0.20±0.00kl	2.6±0.24 ghij
MS+ Kn3.0 mg/L+NAA 1.0 mg/L	60	8	4.2±0.20mno	36.00±0.32 k	0.20±0.00kl	2.2±0.20 ijk
MS+ Kn5.0 mg/L+NAA 1.0 mg/L	60	4	4.0±0.32nop	13.60±0.24 r	0.1960±0.00l	2.0±0.32 jk
S.Ed.±			0.51	0.65	0.01	0.43
CD0.05			1.01	1.29	0.02	0.84

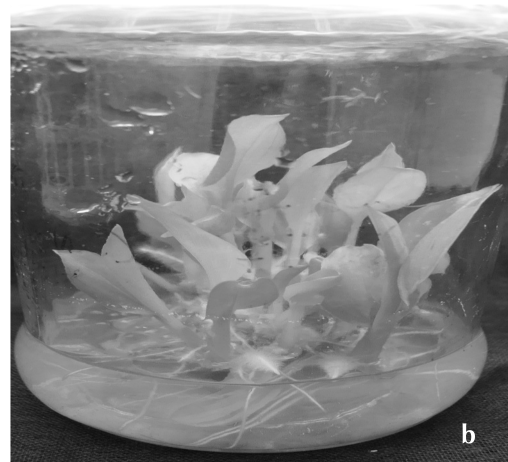

Plate 1: Micropropagation and microrhizome induction of *Curcuma longa* (cv. Lakadong) on MS medium; (a) Multiple shoots initiation with BAP (1.00mgL^{-1}) and 2, 4-D 4.0 mgL^{-1}) after 2 weeks of culture; (b) Shoot multiplication with BAP (1.0mgL^{-1}) and IAA (0.5mgL^{-1}) after 3 weeks of culture; (c) Shoot multiplication with BAP (2.0mgL^{-1}) and NAA 1.0mgL^{-1}) after 4 weeks of culture; (d) Microrhizomes bunch induced with half strength MS + BAP 5.0 mgL^{-1} + NAA 0.5 mgL^{-1}; (e) Separated microrhizomes; (f) Sprouted microrhizomes in poly beg;

Cont...Plate 1: Micropropagation and microrhizome induction of *Curcuma longa* (cv. Lakadong) on MS medium; (g) Microrhizome originated plantlets (from poly beg); (h) Separated plantlets transferred to earthen pots

Table 4: Morphological parameters of *Curcuma longa* (L) plants regenerated from different size of microrhizomes under *in vivo* condition (after 60 days of culture)

Parameters	Microrhizome Size cm (SE)			S.Ed. ±	$CD_{0.05}$
	0.5 – 1.0	1.1 -2.0	> 2.1		
Survival rate (%)	40.00±0.71c	80.00±0.55b	90.40±0.51a	0.58	1.83
Number of shoots	0.80±0.37b	1.60±0.24b	2.60±0.24a	0.29	0.91
Shoots length (cm)	7.20±0.20c	9.60±0.40b	16.60±0.51a	0.38	1.21
Number of roots	4.20±0.20c	7.00±0.32b	12.20±0.20a	0.24	0.75
Roots length (cm)	5.60±0.56c	8.80±0.49b	12.90±0.51a	0.51	1.61
Number of leaf	1.20±0.20b	2.80±0.37a	3.40±0.24a	0.27	0.87
Leaf length (cm)	3.40±0.31c	5.70±0.30ba	10.90±0.25	0.28	0.89
Leaf width (cm)	1.90±0.12b	3.70±0.31a	4.20±0.16a	0.20	0.65

store carbohydrates (Nayak, 2000).

The present study showed that BAP also influence on microrhizome induction in Lakadong turmeric. This is in conformity with Nayak (2000), Sharma and Singh (1995) in *Curcuma aromatica* Salisb and in ginger, respectively where BAP enhanced microrhizome production. In the present study, Kn alone or in presence of NAA did not show any promising result. However, Sunitibala *et al*. (2001) reported that Kn (1.0 mgL^{-1}) is suitable for *in vitro* rhizome induction in *C. longa* L. Effect of half strength MS medium for microrhizome induction is in conformity with Shirgurkar *et al*., (2001) in turmeric. Full strength MS basal medium also found to be suitable for microrhizome induction as reported earliar (Nayak, 2000; Sunitibala *et al.,* 2001; Islam *et al.*, 2004).

Development of plantlets and glasshouse evaluation: *In vitro* produced microrhizomes were isolated after 60 days. Harvested microrhizomes were categorized according to their size [0.5 – 1.0 cm (small), 1.1, 2.0 cm (medium) and > 2.0 cm (large)] and directly transferred to the soil for plantlet development. These potted rhizomes were maintained in the glasshouse at ambient temperature with 70 - 80% relative humidity and about 16 h photoperiod. After two months of establishment in soil, plants developed from three different sizes of microrhizomes were evaluated using various morphological characters. It was observed that germination percentage; survival rate and morphological characters varied depending upon the sizes of microrhizomes. Plants regenerated from bigger microrhizomes were found to be more vigorous in terms of their shoot, root and leaf growth parameters (Table 4, Plate e-g). Similarly, Shirgurkar *et al*., (2001) reported that bigger microrhizomes were more competent and vigorous in comparison to smaller microrhizomes. In the present study, a much higher survival rate was obtained because the average weight of microrhizomes was higher, which indicates that large sized microrhizomes is a key factor in achieving commercial success in microrhizome induction in *C. longa*. Microrhizomes produced in the present investigation were stored under moist conditions at room temperature and more than 90% of the sprouted microrhizomes developed shoots and roots. The microrhizome originated rooted sproutings had been successfully transferred to the field (Plate h).

CONCLUSION

It may be concluded from this study that the protocol developed will be useful for rapid *in vitro* propagation of the disease- free plants and commercially viable healthy microrhizome of *Curcuma longa* (cv. lakadong) in lesser time and also for the subsequent genetic manipulation studies. The procedure described here ensures 10 fold production of plantlets. The developed protocol also highlights the control of endopathogens as well as shoots multiplication that can be exploited for mass propagation to ensure the seasonal independent availability of material and also for germplasm conservation.

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