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Silver Nitrate and Different Culture Vessels Influence High Frequency Microrhizome Induction *In Vitro* and Enhancement Growth of Turmeric Plantlet During *Ex Vitro* Acclimatization

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Abstract

Eleven cultivars of *C. longa* var. Lakadong were collected from Manipur having different topography. Curcumin content in different cultivars has been analyzed by using UV-Visible Spectrophotometer (100 Bio-Carry Spectrophotometer). The curcuminoids content were analyzed and quantified for identification of best quality cultivar. Thoubal Cultivar with highest curcumin content (9.44%) was subjected for tissue culture technique using different culture vessels and silver nitrate for rapid multiplication and scaling up of microrhizome production. High multiplication rate of 27.40±0.47 were obtained in Murashige and Skoog's medium supplemented with 3% sucrose + 1 mg L^{-1} α-napthalene acetic acid, 4 mg L^{-1} 6-benzyl-amino-purine and 11 µM silver nitrate. Effect of different culture vessels and silver nitrate were studied for microrhizome and multiple shoots formation. Relatively higher rate of shoots along with microrhizome (17.5±0.32) can be seen in Growtek which was grown without any plant growth regulator. Growtek was used for scaling up of microrhizome production *in vitro* and utmost microrhizome was produced in liquid Murashige and Skoog's medium supplemented with 8% sucrose, 1 mg L^{-1} α-napthalene acetic acid, 4 mg L^{-1} 6-benzyl-amino-purine and 11 µM silver nitrate (36.25±0.27). Addition of silver nitrate in the medium resulted in improvement of microrhizome induction *in vitro*. Higher concentration of silver nitrate (33, 44, 66, 88 µM) negatively affected the microrhizome and shoot multiplication and shows inhibition of tissue response completely. Analysis of *in vitro* derived plantlets during acclimatization shows that the exogenous applied of silver nitrate shows superior growth as compared to control. 90-95% of plantlets with and 75-80% plantlets without silver nitrate treatment were successfully established under *ex vitro* acclimatization. The protocol could be utilized for large scale production of true-to-type plantlets and as alternative method to step forward towards an improved commercial propagation system for more efficient and productivity.

Keywords: culture vessels, *Curcuma longa*, Curcumin, rapid microrhizome production, silver nitrate, sucrose

Introduction

Turmeric (*C. longa* L.) var. Lakadong is a plant of family Zingiberaceae commonly known as the ginger family and comprises about 70 species (Smart and Simmonds, 1992). Turmeric is found throughout south and South-east Asia (Pieris, 1982) and used as common condiment for various foods and beverages and has a long history of medicinal uses dating back 2500 years (Shukla and Singh, 2007). Turmeric is known for its aromatic, stimulant, carminative and antihelmintic properties (Satyavati *et al*., 1976). A yellow-pigmented fraction isolated from the rhizomes of *Curcuma* contains curcuminoids belonging to the desmethoxycurcumin and bis-desmethoxycurcxumin. It is an important active ingredient responsible for the biological activity of *Curcuma*. Though the major activity is anti-inflammatory, it has also been reported to possess antioxidant, anti allergic, wound healing, anti-bacterial, anti-fungal and anti-tumor activity (Chattopadhay *et al*., 2004). A high consumption of curcumin in the diet is considered safe; it is commonly believed that the cancer chemopreventive and therapeutic properties of curcumin may be accompanied by a lack of toxicity (Lopez-Lazaro, 2008). Structure of curcumin is showing in Fig. 1.

Turmeric is exclusively propagated vegetatively using rhizomes due to poor flowering and seed set. Thus conventional propagation through seed rhizome produces only 10-15 lateral buds in a season of 8-10 months (Bhagyalakshmi and Singh, 1988). Preservation of rhizome seeds is a hard job and required much attention, time and space. Besides they are prone to damage due to different factors such as adverse environment condition, bacterial wilt (*Pseudomonas solanacearum*), fungal attack (*Fusarium oxysporum f.* sp. *zingiberi*) and root knot nematode (*Melaidoryne incognita)* (Dohroo, 1989). Recently, this potent medicinal herb has been propagated by tissue culture techniques as it remains an indispensible tool for rapid multiplication and sustainable growth of slow propagating

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species. Propagation through rhizome bud multiplication is an easy and safe method for obtaining uniformity and assures the consistent production of true-to-type plants within a short span of time. Therefore very intensive research and development programmed are needed to overcome the chronic issue of production, yield and quality.

In vitro multiplication of plant such as ginger (Chang and Chiley, 1993; Hosoki and Sagawa, 1977; Inden *et al*., 1988), *Curcuma amada* (Borthakur and Bordoloi, 1992), *Alpinia galanga* (Borthakur *et al*., 1999) and *Curcuma longa* (George, 1993; Misra *et al*., 2004; Nasirujjaman *et al*,. 2005; Panda *et al*,. 2007; Prathanturarug *et al*., 2003; Praveen, 2005; Rahman *et al*., 2004; Roy and Raychandhari, 2004; Sato *et al*., 1987; Sunitibala *et al*., 2001; Vidya *et al*., 2005) belonging to Zingiberaceae has already been reported. However, there is scant information on methods to induce microrhizome development in *C. longa* using MS (Murashige and Skoog's, 1962) medium modified with Benzyladenine (BA), other plant growth regulators, increased sucrose concentration and modified environmental conditions. Raghu (1997) produce microrhizome on liquid MS medium supplemented with 1.33 μM BA, 0.54 μM NAA (α-napthalene acetic acid), 1.95 μM ancymidol and 10% sucrose. Sanghamitra and Nayak (2000) produced microrhizome on liquid MS medium with 13.32 μM BA, 6% sucrose in 4 h photoperiod. Shirgurkar *et al*. (2001) found that 4.4 μM BA allowed microrhizome production while Sunitibala *et al*. (2001) induced microrhizome in MS medium supplemented with 0.54 μM NAA, 4.65 μM Kinetin and 8% sucrose.

In recent years, used of silver ions in the form of nitrate, such as silver nitrate ($AgNO_3$), play a major role in influencing somatic embryogenesis, shoot formation and efficient root formation which are prerequisites for successful genetic transformation (Bais *et al*., 1999; 2000 a; 2000 b; 2001 a; 2001 b; 2001 c). $AgNO_3$ has been known to inhibit ethylene actions (Beyer, 1976 a) where, silver ions is capable of specifically blocking the action of exogenously applied ethylene in classical responses such as abscission, senescence and growth retardation (Beyer, 1976 b). These observations led to its application in tissue culture. However, addition of $AgNO_3$ to the culture medium improved the regeneration of both dicots and monocots plant tissue cultures (Bais *et al*., 2000 a; Beyer, 1976 b; Chi and Pua, 1989; Chithra *et al*., 2005, Davies, 1987; Duncan *et al*., 1985; Giridhar *et al*., 2003; Purnhauser *et al*., 1987; Songstad *et al*., 1988). The exact mechanism of $AgNO_3$ actions on plant is still unclear, except, few existing evidences suggest its interference in ethylene perception mechanism (Beyer, 1976 a).

Induction of *in vitro* microrhizome is an alternative process to produce diseases free plants for commercialization and conservation of germplasm. The onset of rhizome formation is under the control of various environmental, sucrose concentrations and hormonal culture regimes. However, in the present study, *in vitro* techniques for rapid clonal propagation of turmeric plant from rhizome buds were undertaken at lower cost. Although effect of plant growth regulators, sucrose concentration and different culture vessels on micropropagation and *in vitro* formation of microrhizome has been extensively investigated. However, the impact of different culture vessels and used of $AgNO_3$ in scaling up of *in vitro* microrhizome in turmeric has not been reported so far. The objective of present study were undertaken to enhance the production of *in vitro* microrhizome by employing tissue culture technique using different hormone combinations, culture vessels, sucrose concentrations and $AgNO_3$ in *Curcuma longa* L. var. Lakadong.

Materials and methods

Rhizome of *C. longa* L. var. Lakadong was collected from different locations of Manipur such as Imphal, Bishnupur, Ukhrul Churachandpur, senapati, Tamenglong and Thoubal in 2010. Standard curcumin and ethyl alcohol were purchased from Sigma Aldrich and Merck respectively. Rhizomes were cleaned thoroughly by repeated washings, air dried, stocked loosely in large utility tray (360 × 310 × 130 mm, Tarson, Kolkata, India) and maintained in the BOD incubator (Narang Scientific Works Pvt. Ltd., New Delhi, India) at ambient temperature (27±2°C) under long day condition (16 h light -8 h dark). For curcumin estimation, weigh accurately 0.1 g turmeric extract or 0.005 g to 0.1 g curcuminoids in 25 ml ethyl alcohol. Warm it if necessary for the complete dissolution, filter through wattman No. 41, wash the filter paper with little alcohol and transfer it to 100 ml volumetric flask. Make up the volume with ethyl alcohol and pipette 10 ml to another 100 ml volumetric flask, make up the volume with alcohol. Measure the absorbance at 425 nm in 1 cm cell against an alcohol blank. Curcumin content was calculated by using the formula (Anonymous, 1984).

Calculations: a standard solution of curcumin 0.0025 g/l gives absorbance value of 0.42 at 425 nm.

$$\text{Absorptivity(A)} = \frac{0.42}{1\text{x}0.0025}$$

$$\%\text{of curcuminin extract} = \frac{\text{a x } 100}{1 \text{ x A x W}}$$

a=absorbance at 425 nm
I=cell length in cm
A=absorptivity
W=Wt of sample of gram
Colour value=Absorbance at 425 nm × 1000

After selection of best turmeric in terms of its curcumin content, sprouting rhizome buds after two weeks of incubation were cut apart from rhizomes keeping the leaf sheath intact. The redundant roots and other residuals

were removed from the rhizome buds. The explants were washed scrupulously under running tap water for 15-20 minutes to remove the loose contaminants. The excised rhizome buds were then washed and soaked it for 10-15 minutes with labklin detergent (SD Fine-Chem Limited, Mumbai, India). Subsequently, rinsed thoroughly under running tap water to remove the detergent trace. The clean explants were then treated with gentamycin (HiMedia, Mumbai, India) overnight (antibiotic treatment, 8 mgL^{-1}). They were washed 5-10 times with distilled water to remove the remaining antibiotic trace. Final surface sterilization was taken out in laminar air-flow cabinet with 0.2% mercuric chloride ($HgCl_2$) (HiMedia, Mumbai, India) for 15 minutes. Then the materials were washed 5 times with sterilized double distilled water to removed $HgCl_2$. After surface sterilization, the outer leaf sheaths were excised with a sterilized surgical scalpel. The explants were then deep in 70% ethanol and the upper damage tissue were excised with care without damaging the inner portions.

Prepared explants were inoculated aseptically on semi solid Murashige and Skoog's basal medium (MS) supplemented with plant growth hormones (Sigma, St. Louis, USA) 0.5-12.0 mg L^{-1} BAP (6-benzyl-amino-purine), 0.5-4.0 mg L^{-1} NAA (α-napthalene acetic acid), 0.5-8.0 mg L^{-1} Kn (Kinetin) (HiMedia, Mumbai, India), 0.5-2.0 mg L^{-1} Zn(Zeatin) (HiMedia, Mumbai, India), 30 g L^{-1} sucrose (HiMedia, Mumbai, India), 11 µM silver nitrate (Qualigen, Mumbai, India) and 8 g L^{-1} agar (HiMedia, Mumbai, India). pH of the medium was attuned to 5.8 prior to adding agar and medium was autoclaved (All American, Manitowoc, West Indies) at 15 lbs psi pressure for 20 minutes at 121°C. For shoot multiplication experiment, 250 ml phytajars (HiMedia, Mumbai, India,) were used. The cultures were then kept in a growth chamber under 16 hours light photoperiod at 25±2°C. Microshoots were initiated after 5-6 weeks. The survived explants were transferred to semi solid MS medium supplemented with 2.0 mg L^{-1} BAP and 1 mg L^{-1} NAA for the *in vitro* plant regeneration. Multiple shoot production was observed along with the roots.

The regenerated *in vitro* plantlets were taken out of the culture vessels and transferred on semi solid MS medium containing varying combination of cytokinins and auxin to get optimum medium for *C. longa* var. Lakadong. The effects of different culture vessels *viz*. Growtek (Tarson, Kolkata, India, 100 × 1500 mm), planton (Tarson, Kolkata, India, 7.5 × 10 cm), phytajar (HiMedia Mumbai, India, 250 ml), saveer biotech culture bottle (Saveer Biotech, New Delhi, India, 190 ml and 290 ml), and culture tube (Borosil, Mumbai, India, 32 × 200 mm) on *in vitro* microrhizome production were tested. Among the different culture vessels, the best was selected for further experiments using different concentration and combination of plant growth regulators, higher sucrose concentrations with the presence of $AgNO_3$ to enhance the number of microrhizome production.

Anatomical studies

In vitro and *ex vitro* derived leaf of *C. longa* var. Lakadong were used for anatomical studies. Fine T.S (Transverse section) were cut with the help of fine razor, stained with safranin and fast green, and mounted on glass slides with distyrene plastisizer xylene (DPX). Photographs were taken with a camera (SZ-PT, Olympus, Japan) attached to the Stereo Zoom microscope (SZ-PT, Olympus, Japan).

Data analysis

All the cultures were examined periodically and the number of shoot, height of plantlet, number of microrhizome and weight of microrhizome were recorded every after 3 weeks of initial culture. All the experiments were repeated 3 times, and each treatment contained 20 replicates. Data are expressed as means of three independent experiments. All statistical analyses were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to compare means.

Results and discussion

Determination of curcumin content

Curcuminoids constitutes the major coloring matter in turmeric. The three major constituents were identified as desmethoxycurcumin, bis-desmethoxycurcumin and curcumin. A mixture of curcuminoid in the ethanol extract of powdered samples of *Curcuma* were analyze by UV-Vis spectrophotometer (100 Bio-Carry UV-Visible Spectrophotometer) and detected at a wavelength of 425 nm. Among the collected cultivars, curcumin content variation was recorded from 1.92% to 9.44% in Manipur while 3% - 4% in other regions of India (Spice Board of India, 2010). The maximum curcumin content was found to be 9.44% (Hilly area) and (8.42%) in Thoubal cultivar having 93.970 longitude, 24.564 latitude followed by Senapati (6.16 %) and Churchandpur (4.62%) cultivar respectively (Tab. 1). However, the present study suggested that the Lakadong from Thoubal has more curcumin content than that of others which are found in Manipur. Cultivars with highest curcumin content were than subjected for mass clonal production using simple tissue culture technique.

Culture initiation and multiple shoot production

Surface sterilized rhizome buds were cultured in semi solid MS medium supplemented with different combination and concentration of plant growth hormones and 11 µM $AgNO_3$. Of the 760 (20 × 38) rhizome buds inoculated, 97% survived and developed multiple shoot along with roots within 4-5 weeks of initial culture. Highest average numbers of shoots along with roots were obtained in MS medium supplemented with BAP (4 mg L^{-1} BAP),

Tab. 1. Curcumin content in different cultivars of Lakadong variety found in different locations of Manipur

Cultivar	Place	Longitude	Latitude	Curcumin %
Lakadong variety	Imphal east	93.982	24.916	1.92
	Senapati	93.911	25.204	3.28
	Senapati	93.976	25.169	6.16
	Bishnupur	24.6333	93.7667	4.47
	Ukhrul	94.52501	25.2797	4.05
	Churchanpur	93.57644	24.4528	4.33
	Churchanpur	93.80644	23.3128	4.62
	Thoubal	93.998	24.680	8.42
	Thoubal	93.970	24.564	9.44
	Tamenglong	93.49864	24.97583	3.55
	Tamenglong	91.3298	22.4583	3.24

Tab. 2. Effects of different hormones and $AgNO_3$ in *in vitro* multiple shoots formation (n = 20)

Plant growth regulators concentration (mg/L) with 11μM $AgNO_3$			No. of shoots
NAA	BAP	Kinetin	(mean±SE)[a]
0	0	0	0.00
0.5	1		11.40±0.48m
0.5	1.5		13.80±0.31n
0.5	2		15.80±0.45o
1	1		15.30±0.36o
1	1.5		17.25±0.50s
1	2		22.25±0.42p
1	4		27.40±0.47q
1	6		22.50±0.44p
1	8		6.8±0.11f
1	10		5.30±0.25e
1	12		1.95±0.63d
0.5		1	8.15±0.50j
0.5		2	10.65±0.32l
0.5		4	13.70±0.33n
0.5		6	7.90±0.19i
1		1	6.05±0.19f
1		2	9.25±0.38k
1		4	7.80±0.28i
1		6	2.70±0.14a

[a] Means followed by same letters are not significantly different at $p < 0.05$, according to Tukey's comparison test

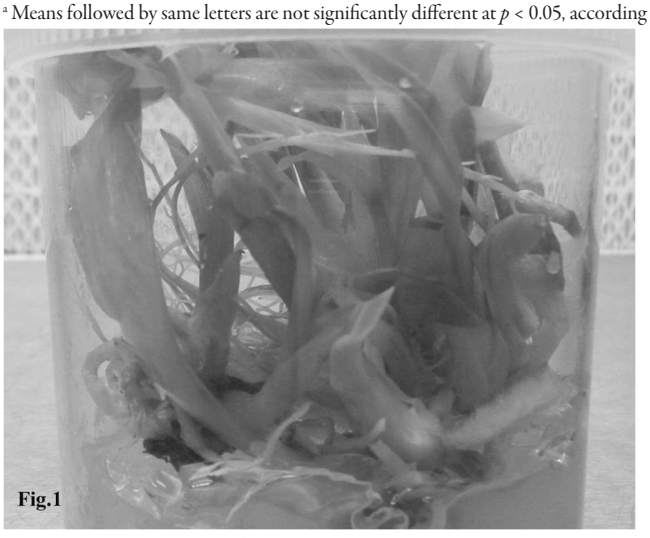

Fig. 1. Multiple shoots formation in 4 mg L^{-1} BAP + 1.0 mg L^{-1} NAA using 250 ml phytajar

NAA (1 mg L^{-1}) and 11 μM $AgNO_3$ (27.40±0.47) within 4 weeks of culture (Tab. 2, Fig. 1).

The medium having BAP, NAA, Kn or Zn alone had no effect on shoot multiplication or growth. Interestingly, apart from NAA and BAP combination, NAA in combination with Kn showed a significant number of multiple shoots (8.15±0.50 to 13.70±0.33) as compared with NAA and Zn combination which shows shoots multiplication of only 1.60±0.18 to 2.65±0.10. The increase of BAP concentration higher than 8 mg L^{-1} or Kinetin higher than 4 mg L^{-1} suppressed the rate of shoot multiplication and shows stunted growth. In the present study, the number of shoots produced was much higher than previous reports by various workers (George, 1993; Misra *et al*., 2004; Nasirujjaman *et al*., 2005; Panda *et al*., 2007; Prathanturarug *et al*., 2003; Praveen, 2005; Rahman *et al*., 2004; Roy and Raychandhari, 2004; Sato *et al*., 1987;

Sunitibala *et al*., 2001; Vidya *et al*., 2005). During the present investigation, highest number of shoot multiplication (27.40±0.47) in MS medium supplemented with the same plant growth regulators combination i.e., 4.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA + 11 μM $AgNO_3$ using 250 ml phytajar (Fig. 1) which is parallel with the finding of Nasirujjaman *et al*. (2005) reported 6.7 multiple shoots of turmeric on WP medium supplemented with 4.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA in culture tube. The present findings suggest a high frequency shoot production from a single rhizome bud by manipulating the different plant growth regulators with the presence of $AgNO_3$. Thus, culture medium with a suitable combination and concentration of auxin and cytokinins plays an important role in *in vitr*o shoots multiplication. Misra *et al*. (2004); Praveen (2005); Vidya *et al*. (2005) had reported that, higher cytokinin concentration with lower auxin concentration is suitable for shoots multiplication and rooting in *C. longa*, which is analogous with the present finding. The effect of BAP alone or combination with NAA was also documented by Debergh and Zimmerman (1991) and suggested that herbaceous plants are highly responsive and most cultured herbaceous species produce robust, well-formed shoots suitable for further shoot proliferation to BAP treatments. The plantlets developed in the present investigation were then used for the further experiments.

Effects of different culture vessels on microrhizome production

The objective of the present study was to achieve high-frequency microrhizome formation by manipulating the culture vessel type. MS liquid medium supplemented with 8% sucrose was prepared in different culture vessels. The results on the effect of different culture vessels in *C. longa* var. Lakadong are presented in Tab. 3 (Fig. 2).

Different culture vessels shows different multiplication rate after 35-45 days of incubation. In general, higher sucrose concentration had remarkable effect on the number and size of microrhizome and the ability of 8% sucrose in induction of *in vitro* microrhizome was reported by different workers (Garner and Blake, 1989; Shirgurkar *et al*., 2001; Sunitibala *et al*., 2001; Vreugdenhil *et al*., 1998). Thus, highest multiplication rate (17.50±0.34) with average height of (13.02±0.27) was seen in Growtek (Fig. 2 a) and minimum multiplication (3.20±0.15) with average height of (11.30±0.32) in culture tube (Fig. 2 e). Inspite of producing minimum average number of microrhizome, culture tubes show much larger microrhizome size (1.64±0.16) as compared to other vessels (growtek- 1.01±0.23, Fig. 2 a; saveer 290 ml- 0.29±0.06, Fig. 2 d; saveer 190 ml- 0.81±0.23, Fig. 2 c; phytajar- 0.26±0.05, Fig. 2 b and planton- 0.79±0.22, Fig. 2 f).

Fig. 2. Effect of different culture vessels on microrhizomes production in (a) Growtek, (b) Phytajar, (c) Saveer culture bottle 190 ml, (d) Saveer culture bottle 290 ml, (e) Culture tube and (f) Planton

Shoot length, proliferation rate, and weight of *in vitro* microrhizome found to have varied according to culture vessel type. Mackay and Kitto (1988) demonstrated the ratio of explants number to volume of medium, which could cause differences in fresh weight and proliferation rates. The present study examines the effects of different culture vessel type on *in vitro* microshoot prolifiration and microrhizome development for a range of commer-

Tab. 3. Effect of different culture vessels in plant height, number and weight of microrhizome in *in vitro* (n = 20)

Vessel type	Plant height (cm) (mean±SE)[a]	Average no. of microrhizome per vessel (mean±SE)[a]	Average weight of microrhizome(g) (mean±SE)[a]
Growth-Tek (100×1500mm)	13.02±0.27wvz	17.50±0.34drt	1.01±0.23z
Saveer biotech culture bottle (290ml)	12.65±0.35wvz	12.70±0.21abc	0.29±0.06z
Phytajar (250ml)	9.77±0.28pqr	10.10±0.23prt	0.26±0.05z
Saveer biotech Culture bottle (190ml)	6.40±0.23yrm	8.80±0.13uvw	0.81±0.23z
Planton (7.5×10cm)	5.96±0.25yrm	7.0±0.29lm	0.79±0.22z
Culture tube (32×200mm)	11.30±0.32swm	3.20±0.15pq	1.64±0.16trm

[a] Means followed by same letters are not significantly different at $p < 0.05$, according to Tukey's comparison test

cially micropropagated turmeric plant, using production methods that parallel commercial standards. Number of microrhizome was significantly greater in all the culture replicate grown in larger vessels i.e. Growtek, than in the culture tube or any other vessels. Response of shoot length, microrhizome number and weight of microrhizome as a function of vessel type, varied from one vessel to another (Tab. 3). Number of microrhizome production increased as vessel-type growing area increased. Growtek not only produced highest number of microrhizome but substantially greater shoot height and microrhizome weight (except culture tube) than explants in any other vessels type. The most striking factor affecting *in vitro* microrhizome production during the experiment was different vessel type employed. In previous reports, the role of different culture vessels on growth parameters and micro environment of plantlets by the interface between inside and outside environments have been demonstrated by Kacar *et al*. (2010) and Huang and Chen (2005). Hence, it can be inferred that rapid clonal multiplication and induction of microrhizome depends on culture vessel, apart from high sucrose concentration. Consequently, larger and taller the culture vessel (Growtek) relatively higher rate of microrhizome formation was observed (Fig. 2 a).

Scaling up of microrhizome production

The most effective culture vessel (Growtek) was used to scale up the production of *in vitro* microrhizome production. The average number of microrhizome production in liquid MS medium supplemented with different plant growth regulators and sucrose concentration with 11 µM $AgNO_3$ are indicated in Tab. 4.

Higher level of sucrose with 11 µM $AgNO_3$ in the medium significantly influenced microrhizome formation *in vitro*. No microrhizome could be induced *in vitro* in the liquid MS medium containing normal concentration of sucrose i.e. 3%, instead multiplication of shoot was observed. However on liquid MS medium supplemented with 6-10% sucrose and different combination and concentration of plant growth regulators, swelling up of shoot bases followed by induction of microhizome was observed. The overall response was always better in medium supplemented with 8% sucrose. Maximum number of microrhizome formation (36.25±0.27) was seen in liquid MS medium supplemented with 8% sucrose, 1 mg L^{-1} NAA, 4 mg L^{-1} BAP and 11 µM $AgNO_3$ (Tab. 4, Fig. 3).

Fig. 3: High frequency microrhizome production using growtek in MS liquid medium supplemented with 8% sucrose, 1 mg L^{-1} NAA and 4 mg L^{-1} BAP

Between the two cytokinins-BAP and Kn tested, 4 mg L^{-1} BAP in combination with 80 g L^{-1} sucrose and 1 mg L^{-1} NAA was found to be most competent for microrhizome production. This is in conformity with Sharma and Singh (1995), Nayak (2000), Shirgurkar *et al*. (2001) and Sunitibala *et al*. (2001). In the present study, the highest number of microrhizome (36.25±0.27) was in 80 g L^{-1} sucrose and is much higher than those reported previously by Nayak (2000), Shirgurkar *et al*. (2001) and Sunitibala *et al*. (2001). Sharma and Singh (1995) found that Kn was better cytokinin for vegetative bud multiplication than BAP. However, in the present investigation, inclusion of Kn in combination with NAA does not show mark response on number of microrhizome differentiation but shows larger microrhizome size (0.5 mg L^{-1} NAA + 2 mg L^{-1} Kn) (1.34±0.06) (Tab. 4) as compared with medium supplemented with BAP and NAA. This is in agreement with the finding of Sunitibala *et al*. (2001) who reported that Kn is suitable for *in vitro* rhizome formation in *C. longa* L. Further, higher concentrations of sucrose i.e. 10% had an inhibitory effect on *in vitro* microrhizome production and show a marked decrease in the percentage of explants response with rhizome formation (Tab. 4). However, 10% sucrose decreases the number of *in vitro* microrhizome production in all the culture tested even by increasing the concentration of BAP or Kn.

Plant growth regulators and their interaction with sugar play an important role in the storage organ induction of many plants propagated *in vitro*. The increase of the sucrose concentration in the medium stimulated the lily and onion bulb formation (Gerrits and De Klerk, 1992; Kastner *et al*., 2001; Keller, 1993; Takayama and Misawa, 1980), the corm induction of *Watsonia vanderspuyiae* (Ascough *et al*,. 2008), the microtubers formation of *Solanum tuberosum* (Gopal *et al*., 2001), *Xanthosoma sagittifolium* (Omokolo *et al*., 2003), *Dioscorea cayenensis – D. rotundata* complex (Ovono *et al*., 2009) and the microrhizome production of *Zingiber officinale* (Sharma *et al*., 1997). Sugar also act as signaling molecules whose transduction pathways influence developmental and metabolic processes. Some of the effect of sugar on plant growth and development suggest an interaction of sugar signals with hormonal regulation (Rolland *et al*., 2006; Smeekens, 2000). However, a high level of sucrose in the medium can overcome the cytoki-

Tab. 4. Effect of different sucrose level, plant growth regulators and with and without silver nitrate in *in vitro* microrhizome production (n = 20)

Plant growth regulators concentration (mg/L)			Sucrose conc. (%)	No. of explants with microrhizome (mean±SE)[a] With 11µM $AgNO_3$	Average weight of microrhizome (g) (mean±SE)[a] With 11µM $AgNO_3$	No. of explants with microrhizome (mean±SE)[a] Without $AgNO_3$	Average weight of microrhizome (g) (mean±SE)[a] Without $AgNO_3$	No. of explants with microrhizome (mean±SE)[a] With (33-88 µM) $AgNO_3$	Average weight of microrhizome (g) (mean±SE)[a] With (33-88 µM) $AgNO_3$
NAA	BAP	Kn							
0	0	0	30	0.00	0.00	0.00	0.00	0.00	0.00
1	1		60	17.45±0.32rs	0.78±0.03g	----	----	----	----
1	1		80	18.20±0.37rs	1.10±0.03h	9.60±0.25 xyzz	0.36±0.01 plkz	0.00	0.00
1	1		100	6.50±0.32yrl	0.67±0.008e	----	----	----	----
1	2		60	21.90±0.39ab	0.75±0.06f	----	----	----	----
1	2		80	24.80±0.26y	1.12±0.015h	15.8±0.35 xyrr	0.63±0.01 pptt	0.00	0.00
1	2		100	7.50±0.18yrl	0.49±0.01c	----	----	----	----
1	4		60	25.45±0.26y	0.6±0.04d	----	----	----	----
1	4		80	36.25±0.27pqrt	1.24±0.03j	21.10±0.31 xyyy	0.84±0.11 ppll	0.00	0.00
1	4		100	6.40±0.13yrl	0.72±0.02e	----	----	----	----
1	6		60	22.25±0.39ab	0.68±0.01e	----	----	----	----
1	6		80	25.8±0.31y	1.19±0.01i	16.25±0.09xymm	0.56±0.56 pytz	0.00	0.00
1	6		100	9.30±0.30yrl	0.40±0.02c	----	----	----	----
1		1	60	17.30±0.37pxz	0.80±0.02g	14.50±0.18 xyll	0.85±0.85 ppll	0.00	0.00
1		1	80	21.90±0.41ab	1.03±0.004h	----	----	----	----
1		1	100	8.10±0.28yrl	0.61±0.02d	----	----	----	----
1		2	60	19.25±0.34px	0.80±0.02g	----	----	----	----
1		2	80	22.30±0.46ab	1.31±0.02e	16.20±0.09xymm	0.93±0.13 pqrt	0.00	0.00
1		2	100	6.80±0.23yrl	0.51±0.02c	----	----	----	----
1		4	60	18.45±0.26rs	0.69±0.2e	----	----	----	----
1		4	80	22.05±0.48abx	1.25±0.015k	16.50±0.08xymm	0.83±0.07 ppll	0.00	0.00
1		4	100	8.40±0.15yrl	0.37±0.2c	----	----	----	----
1		6	60	23.10±0.17rx	0.77±0.2g	----	----	----	----
1		6	80	23.85±0.15pt	1.22±0.04i	11.45±0.16xypp	0.76± .2 pstv	0.00	0.00
1		6	100	8.2±0.15yrl	0.58±0.3d	----	----	----	----
0.5		2	60	17.25±0.25pxz	0.68±0.2e	----	----	----	----
0.5		2	80	19.1±0.16px	1.34±0.06m	----	----	----	----
0.5		2	100	7.0±0.19yrl	0.49±0.02c	----	----	----	----
0.5	4		60	17.90±21rs	0.71±0.02e	----	----	----	----
0.5	4		80	18.2±0.29px	1.08±0.007h	----	----	----	----
0.5	4		100	6.3±0.19yrl	0.43±0.01c	----	----	----	----

[a] Means followed by same letters are not significantly different at $p < 0.05$, according to Tukey's comparison test

nin-derived inhibition and stimulate the tuber formation (Lauzer *et al*., 1995).

Interaction of all plant growth hormones employed in the present investigation (Kn, BAP and NAA) with high sucrose level and 11 µM $AgNO_3$ induced renewal bud formation along with microrhizome on the turmeric explants. The present results indicate that, *in vitro* microrhizome formation due to higher sucrose concentration may be due to presence of high carbon energy source in the form of sucrose. Since storage organ mostly store carbohydrates, high concentration of sucrose and BAP may have enhanced microrhizome production in Zingiberaceae (Nayak, 2000; Sharma and Singh, 1995). The tendency of the larger vessels lead to the production of high frequency microrhizome may be important to employ and can be another way of avoiding the damage caused by different diseases and will thus improve turmeric propagation with positive consequences in any micropropagation program.

Effect of silver nitrate on in vitro microrhizome production

The addition of 11 µM $AgNO_3$ in the medium improved the regeneration frequency in all the culture tested (Tab. 4). The highest number of microrhizome production *in vitro* was seen in medium containing 11 µM $AgNO_3$ (36.25±0.27) and are much efficient than medium devoid of $AgNO_3$ (Tab. 4), which is in conformity with Citra *et al*. (2005). Many reports have shown the positive effect of $AgNO_3$ on plant tissue culture viz. cucumber (Mohiuddin *et al*., 1997); apple (Ma *et al*., 1998); Chinese cabbage (Zhang *et al*., 1998); cassava (Zang *et al*., 2001); pearl millet and sorghum (Oldach *et al*., 2001); date palm (Al-Khayri and Al-Bahrany, 2004) and rapeseed (Akasaka-Kennedy, 2005). $AgNO_3$ at the concentration of 11 µM was very beneficial to shoot regeneration along with the number of microrhizome production in *C. longa* var. Lakadong. The mode of action of $AgNO_3$ in plant tissue culture is assumed to be associated with physiological effects of ethylene, silver ions acting as a competitive inhibitor of ethylene action rather than inhibiting ethylene synthesis. However, in during the present investigation, the observed effects of $AgNO_3$ on microrhizome production may support the hypothesis that this compound acts as a direct inhibitor of the ethylene action, which in turn regulates the availability of ethylene in the culture vessel during specific stages of organ development.

In control experiment (Tab. 4), without $AgNO_3$ (P2), *in vitro* derived plantlets showed low frequency microrhizome formation as compared with medium supplemented with $AgNO_3$ (P1) at different concentration and combination of auxin (1 mg L^{-1} NAA) and cytokinins (1, 2, 4, 6 mg L^{-1} BAP and 1, 2, 4, 6 mg L^{-1} Kn) with 8% sucrose. Highest microrhizome were produced in medium supplemented with 1 mg L^{-1} NAA and 4 mg L^{-1} BAP (21.10±0.31) which was much lower as compared with medium supplemented with $AgNO_3$ (36.25±0.27). However, this observation suggested that the poor regeneration response found in control experiment (without $AgNO_3$) of *C. longa* may be associated with ethylene production by *in vitro* cultured cells or tissues.

Inhibitory effect of $AgNO_3$ in higher conc. were studied by preparing the medium supplemented with 8% sucrose, 1 mg L^{-1} NAA, 4 mg L^{-1} BAP, 2 mg L^{-1} Kn and 33-88 µM $AgNO_3$ in 250 ml phytajar (Tab. 5).

Is been observed that high conc. of $AgNO_3$ (33, 44, 66, 88 µM) have inhibitory effect rather than stimulating effect in microrhizome formation which was contrast with the finding of Zhu *et al*. (1998), which shows increased positive response in 16 mg L^{-1} $AgNO_3$.

Analysis of in vitro developed plantlets with and without AgNO3 treatment during acclimatization

Micropropagation of many plants is achieved through the establishment of explants, their initial growth *in vitro* being followed by transplanting into *ex vitro* condition (shade house). Most often, used of rapid multiplication of many plant species are restricted by the often high frequency of plant lost or damage during field transfer. Transfer of plantlets, from tissue culture environment to the shade house cause tissue stress and is often associated with slow growth and significant plant losses. To overcome such chronic issue, acclimatization units have been developed with temperature, humidity, irradiance, CO_2 conc. and air flow rate controlled by computer (Hayashi *et al*., 1988).

Tab. 5. Effects of higher conc. of $AgNO_3$ in *in vitro* microrhizome production (n = 20)

Plant growth regulators (mg/L)			Sucrose conc. (g/L)	Silver nitrate conc (µM)	No. of microrhizomes per vessels
NAA	BAP	Kn			
1	4		80	33	0.00
				44	0.00
				66	0.00
				88	0.00
1		2	80	33	0.00
				44	0.00
				66	0.00
				88	0.00

[a] Means followed by same letters are not significantly different at $p < 0.05$, according to Tukey's comparison test

During the present investigation, *in vitro* developed plantlets with microrhizome are monitored directly under shade house without primary hardening stage or any acclimatization units. P1 and P2 was transfer separately to sterilized soil and were maintained in shade house (80% relative humidity and about 16 h photoperiod). Different parameter such as plant height, no. of leave, leaf length, no. of root, rhizome weight, and survival rate were recorded every after 2 weeks of initial establishment (Tab. 6). 90-95% (P1, Fig. 4) and 75-80% (P2, Fig. 5) survival rates were documented during field establishment.

Among P1 and P2, P1 shows much higher plant height (31.00±0.22 cm) then P2 (17.59±0.08 cm) (Fig. 6).

The optimum growth rate of deflasked plantlets frequency does not occur until new leaf and root developed in *ex vitro* environment. No. of leave (7.70±0.17) as well as no. of root (15.75±0.18) were developed more in P1 as compared with P2, which shows leave and root multiplication of only 5.70±0.19 and 8.30±0.10 respectively. Since, rhizome is the key aspect in commercial exploitation of *C. longa,* larger minirhizome (5.37±0.11 g) were achieved from P1 then P2 (2.53±0.13 g). However, from the overall observation P1 shows superior quality over P2 accordance to different parameter tested. This may be due to ethylene inhibitory effect of $AgNO_3$, which in lower concentration cause delayed senescence resulting in improved growth of the shoots (Fuentes *et al*., 2000). The influence of exogenously applied of silver ions in the form of $AgNO_3$ significantly regulates the ethylene activity in the plant systems. The major morphological effect of $AgNO_3$ treatment was achieved during *ex vitro* conditions in turmeric via the above observation. The mode of action of $AgNO_3$ in plant tissue culture assumed to be associated with the physiological effect of ethylene, silver ions acting as a competitive inhibitor of ethylene action rather than inhibiting ethylene synthesis per se. Ethylene production infact, increased in plant cultures treated with $AgNO_3$ (Lee *et al*., 1997; Pua and Chi, 1993; Zhang *et al*., 1998). Even though, the mechanism of ethylene action on plant tissue culture has not been elucidated, from the present investigation, during field analysis (Tab. 6) higher transformation frequencies, more efficient and faster growth (P1) of *C. longa* may be due to the above hypothesis.

Fig. 4. Established plants with the presence of Silver nitrate in shade house

Fig. 5. Established plants without Silver nitrate in shade house

Comparative leaf anatomy of in vitro and ex vitro derived leaf

T.S of *in vitro* (Fig. 7 a) and *ex vitro* (Fig. 7 b) derived leaf of *C. longa* var. Lakadong indicate isobilateral types of leaf with single raw of abaxial and adaxial epidermis (Fig. 7).

The mesophyll is not differentiated but consists solely of spongy mesophyll tissue. Sunken stomata are observed which is kidney-shaped in surface view. A few cells in the lower epidermis are enlarged to form mother cells called bulliform cells. In both the section of *in vitro* (Fig. 7 a) and *ex vitro* (Fig. 7 b) derived leaf, vascular bundle surrounded by single layer of compactly arranged cells. Xylem

Fig. 6. Comparative morphology analysis of *in vitro* developed plantlets with and without $AgNO_3$ treatment during acclimatization

Tab. 6. Morphological analysis of *in vitro* plantlets treated with or without $AgNO_3$ during *ex vitro* acclimatization after 60 days of initial establishment (n = 20)

Parameter	Plantlets with $AgNO_3$ treatment (P1)	Plantlets with $AgNO_3$ treatment (P2)
Plant height (cm)	31.00±0.22	17.59±0.08
Number of leave	7.70±0.17	5.70±0.19
Leaf length (cm)	15.45±0.10	9.83±0.15
Number of root	15.75±0.18	8.30±0.10
Rhizome size (g)	5.37±0.11	2.53±0.13
Survival rate (%)	90-95	75-80

[a] Means followed by same letters are not significantly different at $p < 0.05$, according to Tukey's comparison test

was found towards upper and phloem towards lower epidermis. Oldest and the largest vascular bundle (ovb) were found in the centre and known as midrib vein. As the veins typically all parallel to each other in the blade, all appear in a cross sectional view in a cross section of the leaf. Two large stoma are present on both side of midrib vein called air space or air canals (ac). 3rd to 6th layer of upper mesophyll cells (uc) are larger and more regularly arranged than the central cells of the mesophyll. However, anatomical comparisons of *in vitro* and *ex vitro* derived leaf does not show any variability and the structures of cells of different tissues including the shunken stomata, mesophyll cells, large stoma, large vascular bundle, bulliform cells etc. were found to be similar in both the section studied.

Anatomical study of in vitro derived microrhizome

The cross-section of in vitro microrhizome showed the epidermis, the cortex and the vascular bundles comprising typical 'scattered' bundles of monocotyledons, distributed randomly in the fundamental tissue. The parenchyma cells contain a great quantity of starch grains. In the T.S

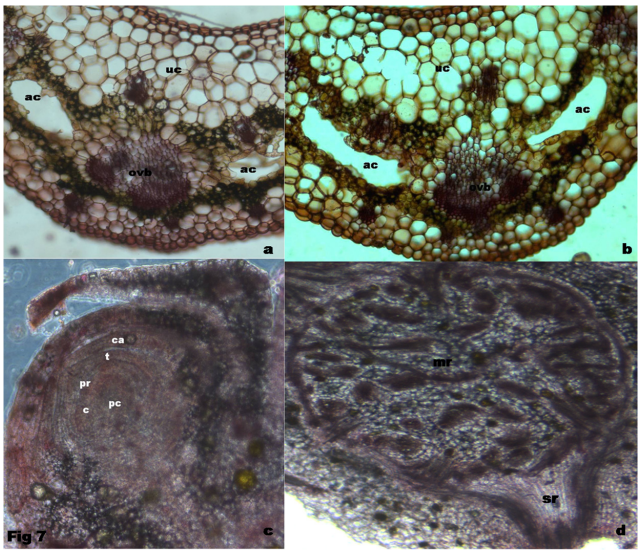

Fig. 7. a) T. S of *In vitro* derived leaf; b) T. S of *ex vitro* derived leaf; c) and d) T. S. of *in vitro* derived microrhizome

the promeristematic region (pr) showed a tunica-corpus organization, with a layer of tunica (t) and two or three layers of corpus (c) (Fig. 7 c). In the apical meristems, procambium strands (ps) were observed in the inner region, and also surrounding the vascular cylinder. The stem apex is protected by cataphylls (ca) (Fig. 7 d).

In conclusion, the present study indicates a competent protocol for large scale production of microrhizome within a short period of time. Unnecessary expenditure for storage and transport of seed rhizomes for annual plantation can be avoided completely and ensured the availability of quality planting material throughout the year. It is imperative to produce disease free quality planting material in large scale simply by manipulating the *in vitro* requirement such as different growth vessels and plant growth regulators, sucrose concentration, and $AgNO_3$. These studies provide a platform for large scale propagation of turmeric which is difficult to multiply by conventional methods, and a better strategy to conserve the germplasm.

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