IN VITRO PRODUCTION OF MICRORHIZOMES IN *Curcuma aromatica* Salisb.

by

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(2009-12-121)

THESIS

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DECLARATION

I hereby declare that this thesis entitled **"*In vitro* production of microrhizomes in *Curcuma aromatica* Salisb."** is bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxy acetic acid
2 iP	2-isopentenyladine
BA	N^6-benzyl adenine
cm	Centimetre
g	Gram
H	Hour
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
Kn	Kinetic (6-furfurylaminopurine)
mg	Milligram
MS	Murashige and Skoog (1962)
NAA	α-naphthalene acetic acid
µM	Micromolar
Fig.	Figure
et al.	And others
CD (0.05)	Critical difference at 5 % level
X	Times
viz.	Namely

LIST OF SYMBOLS

°C	Degree Celsius
%	Per cent
±	Plus or minus

Introduction

1. INTRODUCTION

Curcuma aromatica Salisb. known as kasthuri turmeric (Kasthuri manjal in Malayalam), is a valued cosmetic cum medicinal plant. Kasthuri turmeric has been widely used as an effective skin care cosmetic and ayurvedic medicine (Sasikumar, 2000). It is also used externally in the treatment of various skin diseases like scabies owing to its antifungal and antimicrobial activity (Kirtikar and Basu, 1978). The essential oil obtained from kasthuri turmeric has been reported to have antifungal and antimicrobial activity and is used in the treatment of early stages of cervical cancer (Asolkar *et al*., 1992). Curcumin, the active principle of *C. aromatica* has been found to have anticarcinogenic properties and is now used in the formulation of an anticancer drug due to its inhibitory effect on the induction and growth of tumors (Hasmeda and Polya, 1996). It is also used for flavouring food as the rhizome is a rich source of aromatic volatile oils. The rhizome is also considered to be a tonic and carminative.

In spite of the multifarious applications and good export potential of the crop, its cultivation has not become wide spread; reasons for this are ignorance about the true identity of the crop, non availability of planting materials and low rate of propagation (ca.8x per annum by separation of rhizomes). The plants are also highly susceptible to rhizome rot/ soft rot disease caused by *Pythium* spp. and infection with *Ralstonia solonacerum* (formerly *Pseudomonas solanacerum*), and nematodes (*Meloidogyne* spp.) (Hosoki and Sagawa, 1977). Zhao (2002) reported that it is difficult to propagate through seed because of poor flowering and seed set.

To overcome these problems it has become necessary to develop methods for rapid propagation and long term conservation of the plant.

Biotechnology offers an array of techniques for rapid propagation and conservation of plants. *In vitro* tissue culture is an effective method for germplasm conservation particularly of endangered plant species. Also it is utilized in rapid mass propagation of plants and for their genetic manipulation

(Fay, 1992). Rapid *in vitro* propagation protocol has been standardized in several rhizomatous crops like ginger, turmeric, cardamom etc. (Balachandran *et al*. 1990, Reghunath and Bajaj, 1992).

Formation of storage organs such as tubers, corms and bulbs *in vitro* has been reported in a number of plant species (Abbot and Belcher, 1986). Induction of microrhizomes in *C. aromatica* was first reported by Nayak (2000). Microrhizomes are miniature rhizomes developed under rhizome inducing conditions *in vitro*. Production of microrhizomes *in vitro* has many advantages when compared to *in vitro* plantlet production. Microrhizomes can be produced year round irrespective of season and can be scheduled as per requirement. Micropropagated plants need acclimatization under controlled humidity and light conditions for a few day week and maintenance in a greenhouse till planted out. Transportation of plantlets to far off places is difficult and has to be done with great care. On the other hand, microrhizomes harvested from *in vitro* cultures can be directly transferred to the field without any acclimatization or hardening. As they are disease free seed rhizomes, they could be stored and transported easily for planting or further multiplication.

In this context, it becomes relevant to evolve protocols for rapid propagation and conservation of this medicinally valuable crop. The present study '*In vitro* production of microrhizomes in *Curcuma aromatica* Salisb.' was undertaken with the following objectives.

1. To standardize method(s) for rapid shoot multiplication *in vitro* in *Curcuma aromatica* Salisb.
2. To standardize method(s) for microrhizomes production *in vitro* in *Curcuma aromatica* Salisb., so as to utilize them for rapid propagation and germplasm conservation.

Review of literature

2. REVIEW OF LITERATURE

Curcuma aromatica Salisb., known as kasthuri turmeric (Kasthuri manjal in Malayalam), is a commercially valued cosmetic cum medicinal plant. Several cosmetics and ayurvedic preparations are commercially produced using kasthuri turmeric owing to its antifungal and antimicrobial activity. It is used externally in the treatment of various skin diseases like scabies (Kirtikar and Basu, 1978). It is also used for flavouring of food as the rhizome is a rich source of aromatic volatile oils. The rhizome is also considered to be a tonic and carminative. It is a good stomachic and an emmenogogue for skin diseases. Kojima *et al*. (1998) reported that *C. aromatica* is used as a nutraceutical in Japan.

The present study '*In vitro* production of microrhizomes in *Curcuma aromatica* Salisb.,' is comprised of standardization of method(s) for the production of microrhizomes in *Curcuma aromatica* Salisb. using *in vitro* techniques, so as to utilize them for rapid propagation and conservation of germplasm.

In this chapter, literature on rapid multiplication and microrhizome induction in *Curcuma aromatica* has been reviewed. Wherever literature on *Curcuma aromatica* is lacking, literature on related rhizomatous crops has been reviewed.

2.1 *IN VITRO* MULTIPLICATION

In vitro propagation is widely used for the propagation of a large number of plant species, including many medicinal plants (Rout *et al.*, 2000).

Turmeric is a sterile triploid plant that is vegetatively propagated by means of underground rhizomes. The rate of rhizome multiplication in this plant is very low (only 6x to 10x) with yield ranging from 15 to 25 t/ha (Balachandran *et al.*, 1990). Ten to twenty per cent of harvested rhizomes is required for raising the next crop (Shirgurkar *et al.*, 2001). Maintenance of such a huge amount of seed rhizomes for annual planting is expensive and labour intensive. Moreover,

many diseases and pests, particularly soft rot of turmeric caused by *Pythium aphanidermatum* and bacterial wilt are consistently threatening the germplasm of this important medicinal plant (Balachandran *et al.*,1990; Nayak, 2000; Salvi *et al.*, 2001; Shirgurkar *et al.*, 2001). Ali *et al.* (2004) suggested that to mass produce pathogen free uniform planting material, micropropagation can be utilized as an effective alternative method.

The first report on micropropagation on turmeric came from National Chemical Laboratory, Pune. Successful rapid clonal multiplication in turmeric was reported by Nadgauda *et al.* (1978).

In vitro multiplication in *Curcuma aromatica* was already reported by Yasuda *et al.,* 1988, Nirmal Babu *et al.* (1997) and Nayak (2000).

In vitro multiplication of ginger (Hosoki and Sagawa, 1977; Inden and Aashira, 1988), and mango-ginger (Borthakur and Bordoloi, 1992) belonging to family Zingiberaceae have also been reported.

2.1.1 Factors influencing shoot proliferation

The shoot proliferation is influenced by explant type, explant disinfection, culture medium, plant growth regulators and culture conditions.

2.1.1.1 Explant Type

The type of organs or explants chosen affects the successful establishment of the cultures and their subsequent growth. Not all the tissues or organs of a plant are equally capable of exhibiting morphogenesis (Hartmann *et al.,* 1997). So the success of any *in vitro* propagation system depends on the right choice of the explants, the kind of culture to be initiated, the purpose of the proposed culture and the plant species to be used. The response varies according to the type, stage and physiological age of the explants.

In Zingiberaceae, different plant organs have been used as explants for micropropagation; rhizome buds being the most successful for direct regeneration (Prakash *et al.*, 2004; Ma and David, 2006). Yasuda (1988) reported micropropagation in *Curcuma aromatica* using rhizome bud explants. Nirmal *et*

al. (1997) reported the successful rapid clonal multiplication in *C. aromatica* by using rhizome bud sprouts as explants. Nayak (2000) and Tyagi *et al.* (2004) also used rhizome buds as explants for culture establishment in *C. aromatica*.

Nadgauda *et al.* (1978) reported the successful rapid clonal multiplication in turmeric when buds were cultured on MS medium supplemented with coconut milk and BA. Successful micropropagation in turmeric was also achieved by Shetty *et al.* (1982) while culturing rhizome buds in the modified MS medium (pH 5.6) containing sucrose (40.0 g l^{-1} and Kn (0.5 mg l^{-1}). Mukhri and Yamaguchi (1986) reported that when rhizome buds turmeric were grown on the medium containing BA (1.0 mg l^{-1}) resulted in the formation of shoots and roots.

Kesavachandran and Khader (1989) cultured the rhizome buds (explants) of turmeric varieties CO- 1 and BSR- I on MS medium supplemented with Kn (1.0 mg l^{-1}), BA (1.0 mg l^{-1}) and sucrose (40.0 g l^{-1}). Balachandran *et al.* (1990) reported that multiple shoots were developed while culturing rhizome buds of *Curcuma* species on MS medium with varying levels of BA and Kn. The attempts made by other workers on the micropropagation of *Curcuma* species using rhizome buds as explants are given in Table1.

2.1.1.2 Explant Disinfection

The main objective in the establishment of a culture is to successfully place an explant into aseptic culture and then to provide an *in vitro* environment that promotes stable shoot production (Balachandran *et al.*, 1990). The type of sterilant, its concentration and time of sterilization depends on the nature of explant and species to be cultured (Razdan, 1993).

The establishment of contamination free *in vitro* culture from rhizome buds is a difficult and labourious process as the sterilization of underground rhizomes was very complex, due to the presence of different types of soil born pathogens. Although surface sterilization works initially, microbial contamination is observed later (7-15 days after inoculation) at the base of the explants. Huge number of turmeric explants was destroyed in the culture due to endogenous

Table 1. *In vitro* responses of *Curcuma* species

Sl. No.	Media Composition	Reference
	Curcuma longa	
1	MS + BA (4.0 mg l^{-1}), IAA (.01 mg l^{-1}) and adenine (10.0 mg l^{-1})	Rout *et al.* (1995)
2	MS + BA (1.0 mg l^{-1}) and NAA (0.5mg l^{-1})	Nirmal Babu *et al. (*1997)
3	MS + BA (2.0 mg l^{-1}) and NAA (1.0 mg 1^{-1})	Sunitibala *et al*. (2001)
4	MS + BA (10.0 μM) and NAA (1.0 μM)	Salvi *et al.* (2002)
5	MS + TDZ (18.17 mM)	Prathanturarug *et al.*, (2003)
6	MS + BA (6.0 μM) and NAA (0.3 μM)	Islam *et al.*, (2004)
7	MS + BA (2.0 mg l^{-1})	Rahman *et al.* (2004)
8	MS + TDZ (7264.0 μM)	Prathanturarug *et al.* (2005)
9	MS + BA (2.5 mg l^{-1}) and NAA (0.5 mg l^{-1})	Ma and David (2006)
10	MS + BA (3.0 mg l^{-1})	Naz *et al.* (2009)
11	MS + BA (2.0 mg l^{-1}) and Kn (2.0 mg l^{-1})	Pandey *et al.* (2009)
12	MS + BA (2.0 mg l^{-1}) and NAA (0.5 mg l^{-1})	Kambaska *et al.* (2010)
13	MS + BA (3.0 mg l^{-1}) and IAA (1.0 mg l^{-1})	Nayak *et al.* (2011)
14	MS + BA (2.0 mg l^{-1}) and NAA (1.0 mg l^{-1})	Jala (2012)
	Curcuma amada	
1	MS + BA (l.0 mg l^{-1}) and NAA (0.5 mg l^{-1})	Nirmal Babu *et al.*(1997)
2	MS + BA (8.8 μM) and NAA (2.7 μM)	Prakash *et al.* (2004)
	Curcuma aeruginosa	
1	MS + BA (3.0 mg l^{-1})	Balachandran *et al.*(1990)
2	MS + BA (11.1 μM)	Tyagi *et al.* (2004)
	Curcuma caesia	
1	MS + BA (22.2 μM)	Tyagi *et al.* (2004)
2	MS + BA (30 mg l^{-1})	Raju *et al.* (2005)
	Curcuma zedoaria	
1	MS + NAA (1.0 ppm) and Kn (0.1 ppm)	Yasuda (1988)
2	MS + BA (2.0 mg l^{-1}) and IBA (2.0 mg l^{-1})	Chan and Thong (2004)
3	MS + BA (2.0 mg l^{-1})	Miachir *et al.* (2004)
4	MS + BA (3.0 mg l^{-1}) and IBA (0.5 mg l^{-1})	Raju *et al.* (2005)
5	MS + BA (0.5 mg l^{-1}) and IBA (0.5 mg l^{-1})	Stanly and Keng (2007)

bacteria (Hadiuzzaman *et al.,* 2001). Hence surface sterilization is must and should be resorted prior to inoculation of explants. The use of Tween 20 (20 per cent) and sodium hypochlorite solution (5 per cent) effectively controls the exogenous contamination (Prakash *et al.,* 2004). Mukhri and Yamaguchi (1986) surface sterilized turmeric rhizome buds by immersing in 70 per cent ethanol for 2 minutes and in 0.5 per cent sodium hypochlorite for 10 minutes followed by washing with sterile distilled water several times.

Mukhri and Yamaguchi (1986) surface sterilized turmeric rhizome buds by immersing in 70 per cent ethanol for 2 minutes and in 0.5 per cent sodium hypochlorite for 10 minutes followed by washing with sterile distilled water several times. Balachandran *et al.* (1990) used mercuric chloride (0.1 per cent) solution for 15 minutes to sterilize the turmeric rhizome bud explants. Nayak (2000) disinfected *C. aromatica* rhizome buds using mercuric chloride (0.1 per cent for 10-12 minutes) followed by washing 5–6 times in sterile distilled water.

Sterilization technique in turmeric was standardized by Salvi *et al.* (2002) using 70 per cent ethyl alcohol for 30 seconds and 0.1 per cent mercuric chloride solution for 15 minutes followed by washing with sterile water six times. Islam *et al.* (2004) disinfected turmeric rhizome buds by incorporating Tween-20 with 0.1 per cent mercuric chloride. Immature buds were cleaned with running tap water and then washed with detergent (Tween-20) for 5 min and subsequently rinsed thoroughly under running tap water for 5 min. Explants were then immersed in 70 per cent ethanol for 30 - 40 s before incubation in disinfectant (0.1 per cent mercuric chloride to which 2-3 drops of Tween-20 were added). Under sterile conditions, Mercuric chloride solution was decanted and the explants were rinsed 5-6 times with sterile distilled water. Rahman *et al.* (2004) disinfected the explants of turmeric with a solution antiseptic (Savlon 5 per cent for 10 minutes), followed by repeated washing in distilled water and treated with mercuric chloride (0.1 per cent) for 15 minutes and again washed thoroughly with sterile distilled water 3 to 4 times to remove any traces of mercuric chloride.

Stanly and Keng, (2007) disinfected the explants of turmeric using mercuric chloride (0.1 per cent for 5 minutes) and Clorox (20 per cent) with few

drops of Tween 20 for 10 minutes, followed by washing with sterile water thrice. Naz *et al.* (2009) obtained 70 per cent contamination free cultures when turmeric explants were sterilized by immersing in ethanol (70 per cent) for 30 to 40 seconds and sodium hypochlorite (20 per cent) for 15 minutes and then washed three times with sterile distilled water Pandey *et al.* (2009) standardized a sterilization procedure for turmeric using labolene solution for 5 minutes, Bavistin solution (1.0 per cent) for 20 minutes, sodium hvpochlorite solution (2 per cent) 15 minutes, mercuric chloride (0.1 per cent) for 20 minutes and 70 per cent ethanol for two minutes followed by several washing with sterile water.

Decontamination of turmeric explants was reported by Kambaska *et al.* (2010) by surface sterilizing with bavistin (0.3 per cent) for 10 minutes, streptomycin (0.2 per cent) for 10 minutes and then washed with sterile distilled water. After transferring to laminar air flow cabinet, the explants were treated with 70 per cent alcohol for 30 seconds, 0.1 per cent mercuric chloride for 5 minutes and washed finally 3 to 4 times with sterile distilled water. Nayak *et al.* (2011) sterilized turmeric rhizome bud explants using 0.1 per cent mercuric chloride solution for 8 to 10 minutes followed by washing five times with sterile distilled water to obtain 83 per cent contamination free cultures.

Ahmad *et al.* (2011) developed an environmentally safe sterilization protocol for turmeric (by excluding mercuric chloride being a serious environmental pollutant), which comprises of hot water treatment of rhizomes at 50 ^{o}C (for 10 minutes, rinsing with autoclaved distilled water for five times, submerging in 10 per cent common bleach *(*pH 12.6) for 30 minutes, treating in 0.5 per cent plant preservative mixture (PPM) for 10 minutes followed by submerging in 70 per cent ethanol and rinsing with sterile distilled water finally. By using this method contamination rate was reduced to 11.76 per cent. Jala (2012) soaked turmeric rhizome buds in 70 per cent alcohol for 3 minutes, transferred to 10 per cent Clorox for 20 minutes, 5 per cent Clorox for 10 minutes and then rinsed finally with sterile distilled water for 3 minutes and thereby obtained 90 per cent contamination free cultures.

2.1.1.3 Culture Media

2.1.1.3.1 Nutrient composition

One of the most important factor governing the growth and morphogenesis of plant tissues *in vitro* is the composition of the medium. The selection of a particular culture medium depends upon the plant species and the purpose for which the cell, tissue or organ is cultured (Wang and Charles, 1991). The main components of most plant tissue culture media are mineral salts, sugar as carbon source and water. Other components may include organic supplements, growth regulators and a gelling agent (Gamborg *et al.,* 1986; Gamborg and Philips, 1995).

A wide variety of media has been devised for *in vitro* culture of plant tissues and organs. Murashige and Skoog (1962) medium (MS medium) is the widely used one for turmeric tissues culture. Many workers reported good results in MS medium for turmeric micropropagation (Balachandran *et al.,* 1990; Mogor *et al.,* 2003; Ali *et al.,* 2004; Tule *et al.,* 2005; Naz *et al.,* 2009; Nayak *et al.,* 2011). But, Shetty *et al.* (1982) suggested modified MS medium (pH 5.6) supplemented with sucrose (40.0 g l^{-1} and Kn (0.5 mg l^{-1}) for getting best response in turmeric clone 15B.

MS medium solidified with agar (0.4 and 0.6 per cent) was found to be superior to liquid medium in turmeric for the production of multiple shoots (Salvi *et al.,* 2002). Prathanturarug *et al.* (2005) preferred solid medium for the better response in turmeric.

Adelberg and Cousins (2006) compared both solid and liquid media by using large and small culture vessels and found that use of liquid media in large culture vessel with gentle tilting gave bigger plantlets, while liquid media in small vessels on a shaker gave the most vigorous plantlets. Increased biomass was also observed in liquid cultures as compared with solid (agar) cultures.

The aseptic shoot explants of *Curcuma zedoaria and Zingiber zerumbet* cultured in liquid MS medium supplemented with IBA (0.5 mg l^{-1}) produced significantly higher number of multiple shoots than those cultured on the solid

medium with the same formulation *C. zedoaria* produced 6.1 shoots per explant in liquid medium but only 2.4 shoots in solid medium While, *Z. zerumbet* produced 6.4 shoots per explant in the liquid medium and just 3.3 shoots in solid medium (Stanly and Keng, 2007).

2.1.1.3.2 Sucrose concentration

Plant cells and tissues in the culture medium lack autotrophic ability and therefore need external carbon for energy. Sucrose is the main carbon energy source in most tissue culture media. The concentration of sucrose varied from 2.0 to 30.0 g l^{-1} (Oka and Ohyama, 1982). Sucrose also acts as an osmoticum that can stimulate and regulate morphogenesis (Wetherell, 1984). Many other carbon sources are also found used, instead of sucrose. The use of alternative carbon sources like glucose, maltose, raffinose, fructose and galactose were found to be less effective and mannose and lactose being the least effective. Sucrose was found to be the best carbon source in the culture medium for *C. zedoaria* (Mello *et al.*, 2001).

2.1.1.3.3 Solidifying agents

Agar is the most commonly used gelling agent for preparing semi solid and solid plant tissue culture media. Compared to other gelling agents, agar possesses several advantages viz. when mixed with water it forms a gel that melts at a temperature above 60 ^{o}C and get solidified at 45 ^{o}C. Hence, agar gel is found to be stable at feasible incubation temperature. Further, there is no reaction with media constituents and are not digestible by plant enzymes. The brand of the agar used and its concentration (0.6 - 0.8 per cent) determines the firmness of' the culture media and pH (5.7 to 5.8) also contributes to it (Cronauer and Krikorian 1985; Doreswamy and Sahijram, 1989). During the growth of culture *in vitro* a marked acidification of the MS medium was noticed with the pH falling from 5.8 to 3.1 resulting in the hydrolysis of sucrose (Marchal, 1990).

Gelrite is a gelling agent which is commonly used for preparing tissue culture media and is available in the brand names as Applied Gel, Gellan and Phytagel. It is synthetic and should be used at a concentration of 1.25 to 2.5 g l^{-1}

which results in a clear gel that aids in detecting contaminations (Krikorian, 1989). Varying concentrations of Phytagel have been used in the tissue culture media for better responses (Salvi *et al.*, 2000; Ma and David, 2006, and Naz *et al.* (2009).

2.1.1.4 Plant Growth Regulators

The growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium and growth substance produced endogenously by the culture cell. Selection and addition of growth regulators at the optimum level are crucial for successful plant tissue culture (Krikorian, 1982).

Two principal classes of growth regulators namely auxins and cytokinins are used in tissue culture studies. Most commonly used auxins are NAA, IAA, IBA, 2,4-D, 2,4,5-T and PCPA. The most commonly used cytokinins are BA, Kn, adenine sulphate and isopentyl adenine (2ip). Auxins are widely used in micropropagation studies and are incorporated into nutrient media to promote the growth of callus, cell suspension or organs and to regulate morphogenesis. They are capable of initiating cell division and are involved in the origin of meristems giving rise to either unorganized tissue or defined organs. In organized tissue, auxins are responsible for the maintenance of apical dominance (Kunisaki, 1980).

In tissue culture, cytokinins have been utilized for axillary shoot proliferation by overcoming the apical dominance of shoots and by enhancing the branching of the lateral buds from leaf axils. In some cases, a kind of synergism between two cytokinins namely Kn and BA is noticed which results in the proliferation of *in vitro* axillary buds (Gupta *et al.*, 1981).

Krikorian (1982) reported that multiple shoots were induced when the cytokinin level is higher than auxin and when it is low rooting was induced. But at intermediate concentrations, unorganized callus was developed. Myoinositol present in coconut water had growth promoting activities which paved way for its inclusion in the plant tissue culture media (Kunisaki, 1980).

Although small quantity of cytokinin may be synthesized by apical rhizome buds grown *in vitro*; however, its exogenous supply also stimulates and

promotes shoot proliferation in most of the plants (Ammirato, 1986). It is found that cytokinin not only determined the regeneration response but also affected the mode of regeneration. Addition of BA alone in lower concentration in the medium supported poor regeneration response and produced single shoots (Short, 1986) but the higher concentration (Panda *et al.*, 2007) and combination with NAA stimulated high frequency of regeneration particularly the formation of multiple shoots (Nasirujjaman *et al.*, 2005).

2.1.1.4.1 Plant Growth Regulators for multiple shoot formation in Curcuma aromatica

Experiments conducted in *C. aromatica* by Yasuda *et al.*, 1988 revealed that higher number of shoots per culture was developed in MS media supplemented with NAA (1.0 mg 1^{-1}) and Kn (0.1 mg 1^{-1}).

Nirmal Babu *et al.*, (1997) reported a protocol for micropropagation by using young vegetative buds as explants in *C. aromatica* in MS medium supplemented with BA (1.0 mg l^{-1}**)** and NAA (0.5mg l^{-1}**)**.

Nayak (2000) reported plant regeneration in *C. aromatica* by using rhizome bud sprouts as explants in MS medium supplemented with 5.0 mg l^{-1} BA. These explants responded readily and resulted in the production of 2 to 5 multiple shoots with in 20–25 days of culture.

Tyagi *et al.*, (2004) reported that the maximum regeneration of 5.4 shootlets per culture and maximum shoot length of 4.8cm in *C. aromatica* in MS media with BA (11.1 µM).

2.1.1.4.1 Plant Growth Regulators for multiple shoot formation in Curcuma longa

Nadgauda *et al.*, (1978) reported a protocol for micropropagation in two varities (Duggirala and Tekkurpeta) of turmeric by using young vegetative buds as explants in MS medium supplemented with Kn, BA and coconut milk; and Smith's medium supplemented with Kn, BA, coconut milk and myoinositol).

Kesavachandran and Khader (1989) reported the production of 2.5 and 2.1 shoots in turmeric cv CO1 and BSR 1 respectively, when their rhizome buds

were cultured on MS medium supplemented with Kn **(**1.0 mg l^{-1}), BA (1.0 mg l^{-1}) and sucrose (40.0 mg l^{-1}). Balachandran *et al.,* (1990) obtained a proliferation rate of 3.43 shoots per explant when the rhizome buds were cultured on MS medium supplemented with BA (13.32 mM) for 4 weeks. Nirmal Nirmal *et al.,* (1997) reported micropropagation in turmeric by using young vegetative buds as explants in MS medium supplemented with BA (1.0 mg l^{-1}) and NAA (0.5 mg l^{-1}). These explants responded readily to culture conditions and resulted in the production of 8 to 10 multiple shoots in 40 days of culture.

In turmeric cv. Cuddapah, Meenakshi *et al.,* (2001) reported that the media combination of MS with BA (1.0 mg l^{-1}), GA (0.1 mg l^{-1}) and NAA (0.1 mg l^{-1}) were the best for initiation of *in vitro* cultures. Salvi *et al.,* (2001) produced the callus in the medium composition of MS with NAA (5.0 mg l^{-1}) and BA (0.5 mg l^{-1}) which on transferring to MS medium supplemented with Kn (1.0 mg l^{-1}) regenerated into12 shoots per tube. In turmeric Salvi *et al.,* (2002) also reported shoot multiplication rates of 4.2, 3.5 and 6.6, by culturing shot tip explants for 8 weeks in liquid medium supplemented with NAA (1 mM) and BA, Kn and 2 iP (10 mM each) respectively.

Prathanturarug *et al.,* (2003) induced 11 to 18 multiple shoots in turmeric, when its buds were cultured on MS medium supplemented with Thiaduzuron (18.17 to 72.64 μM). Zapata *et al.,* (2003) observed the development of two to three axillary buds from base of the main shoot after four weeks, in the *in vitro* cultures of turmeric having the media composition of MS with BA (0.2 mg l^{-1}) and NAA (0.1 mg l^{-1}). These buds grew vigorously and formed plantlets (8.0 to 10.0 per Magenta vessel) in the MS without plant growth regulators.

Experiments conducted in turmeric by Rahman *et al.,* (2004) revealed that higher number if shoots per culture (14.50 ± 1.82) was developed in MS media supplemented with BA (2.0 mg 1^{-1}). Nasirujjaman *et al.,* (2005) reported that when young shoots of turmeric were cultured on Woody Plant Medium supplemented with BA (4.0 mg 1^{-1}), about 6.25 new shoots were regenerated within 2 weeks.

Ma and David (2006) indicated that the combination of MS media with BA (2.5 mg 1^{-1}) and NAA (0.5 mg 1^{-1}) and NAA (0.5 mg 1^{-1}) were the most efficient in the production of maximum number of multiple shoots (10.85) in the *in vitro* cultures of turmeric. Nayak and Naik (2006) reported that the maximum number of turmeric shoots (4.7 ± 0.3 Shoots per explants) were obtained in the MS medium containing BA (13.3 µM) within 15 days of culture but on increasing the concentration of BA beyond 13.3 µM, the number of shoots formed per bud decreased.

Panda *et al.,* (2007) developed an *in vitro* propagation protocol for turmeric that enabled the production of 7.6 shoots by using MS medium with BA (3.0 mg 1^{-1}). A rapid propagation method for three different varieties of turmeric viz., Faisalabad, Kasur and Bannun were optimized by Naz *et al.,* (2009). In Faisalabad and Kasur varieties, MS medium containing BA (3.0 mg 1^{-1}) gave the best response in shoot induction (70 per cent and 60 per cent) whereas in the variety Bannun, MS medium with BA and NAA (2.0 mg 1^{-1} and 1.0 mg 1^{-1}) showed the maximum (75 per cent) response.

Among all the concentrations and combinations of the media tested in turmeric, Pandey *et al.,* (2009) reported that MS media with BA (2.0 mg 1^{-1}) and Kn (2.0 mg 1^{-1}) were found to be best for shoot initiation. Young sprouted buds produced large number of shoots (5.06 and 4.0) with good per cent of response (88.8 and 82.3 per cent) in the turmeric cultivar Local Raipur and Ama haldi respectively. In the *in vitro* cultures of *C. longa* cv. Ranga, Kambaska *et al.* (2010) evolved optimal response in the media combination of MS with BA (2.0 mg 1^{-1}) and NAA (0.5 mg 1^{-1}), by producing an average of 7.0 ± 0.18 shootlets with a mean shoot length if 5.4 ± 0.09 cm.

Nayak *et al.,* (2011) reported that of all the media combinations tried in the *in vitro* cultures of turmeric, MS medium containing BA (3.0 mg 1^{-1}) and IAA (1.0 mg 1^{-1}) were found to be optimum for sprouting of axillary buds (83 per cent) which then multiplied further producing eight shoots approximately within 30 days of explantation. Singh *et al.,* (2011) indicated that the combination of MS

media with BA (3.0 mg 1^{-1}) and IAA (1.0 mg 1^{-1}) produced maximum number of shoots per explants (6.5 ± 0.3) in *C. longa* cv Saroma.

Jala (2012) advocated that with the addition of BA (2.0 mg 1^{-1}) on to the MS medium, highest average number of shoots (2.6) and leaves (5.4) were produced in the *in vitro* cultures turmeric.

2.1.1.4.2 Plant Growth Regulars for multiple shoot formation in Zingberaceae.

In *C. xanthoria* (a medicinal plant), on culturing rhizome buds in MS medium containing BA (1.0 mg 1^{-1}) and NAA (1.0 mg 1^{-1}) roots and shoots were formed but on increasing the concentration of BA (10.0 mg 1^{-1}) and NAA (15.0 mg 1^{-1}) callus was formed (Mukhri and Yamaguchi, 1986).

In ginger, a linear relationship was found to exist between the rate of shoot multiplication and the concentration of BA, with the maximum multiplication rate at the concentration of 3.0 mg 1^{-1} BA producing an average number of 4.05 shoots per explants (Balachandran *et al.,* 1990). MS media supplemented with BA (3.0 mg 1^{-1}) could produce an average of 2.7 and 2.8 shoots per explants in *C. aeruginosa* and *C. caesia*, respectively (Balachandran *et al.,* 1990).

Sharma and Singh (1995) reported that culturing meristems of ginger in MS medium supplemented with Kn (2.0 mg 1^{-1}), NAA (2.0 mg 1^{-1}) and sucrose (20.0 mg 1^{-1}) leads to the generation of seven shootlets per rhizome bud. High frequency of shoot bud regeneration (82.8 percent) was reported in ginger by Rout and Das (1997) when rhizome buds were cultured on MS medium containing BA (5.0 mg 1^{-1}), IAA (1.0 mg 1^{-1}) and adenine sulphate (100.0 mg 1^{-1}).

Among all the *Curcuma* species taken for study, Tyagi *et al.,* (2004) reported that the maximum regeneration of shootlets viz., 3.0, 5.2, 3.6, 3.8 per culture were observed in *C. aeruginosa, C. brog, C. caesia,* and *C. raktakanta*, respectively in MS media with BA (11.1 µM). Whereas, the media composition of MS with zeatin (11.4 µM) produced highest number (7.2) of shoots in *C. malabarica*.

Jamil *et al.* (2007) reported that MS media amended with IAA (0.1 mg 1^{-1}) and BA (1.0 mg 1^{-1}) was the most suitable medium for *in vitro* multiplication of ginger. In *C. angustifolia* the optimum shoot bud initiation (80 per cent) and maximum number of shoots (6.9 ± 0.69) were produced on the MS medium containing BA (3.0 mg 1^{-1}) and adenine sulphate (25.0 mg 1^{-1}) (Shukla *et al.*, 2007). Kavyashree (2009) reported that in ginger cultivar Varada, the maximum number of shoots per culture (6.8) was observed on Linsmaier and Skoog's basal medium (Year?) incorporated with BA (17.76 µM) alone compared to Kn (4.64 µM) and NAA (5.36 µM). Lincy and Sasikumar (2010) reported that 11-22 shoots were produced in MS medium supplemented with TDZ and IBA (1:1 mg 1^{-1}) in the variety 'Jamaica' and 8-19 shoots in the variety 'Varada' of ginger.

In *C. mangga*, Abraham *et al.* (2011) obtained maximum number of multiple shoots (4.0) in the MS medium supplemented with BA (2.0 mg 1^{-1}) and NAA (0.5 mg 1^{-1}). In ginger, Sathyagowri and Thayamini (2011) reported the production of 5.25 shoots per explants in MS medium fortified with BA (5.0 mg 1^{-1}) and NAA (0.5 mg 1^{-1}). In *C. kwangsiensis* (subtropical ornamental plant), Zhang *et al.* (2011) reported that after 30 days of culture, MS medium supplemented with TDZ (1.4 µM) could produce on an average of 13.84 shoots per explants.

2.1.1.5 Culture conditions

The pH of the culture medium affects the growth and differentiation of tissues in culture. Plant cells in culture require an acidic pH of 5.5 to 5.8 (Gamborg and Shyluk, 1981).

Light has significant influence on the shoot proliferation. Murashige (1977) observed that the light intensity, quality and duration affect the growth of *in vitro* grown plant cultures. He found that the optimum light intensity for shoot formation in large number of herbaceous species to be around 1000 lux. Maintainance of cultures in 16 h light and 8 h dark cycle was reported by Sharma and Singh (1995) in ginger.

Yeoman (1986) reported that usual environment temperature of species should be taken into account for its better performance under *in vitro* conditions. However, most of *in vitro* cultures are grown successfully at temperatures around 25±1ºC. Maintenance of cultures at 25±1ºC with a photoperiod of 16 h at 2000-3000 lux light intensity of cool white fluorescent light was reported by Naz *et al.* (2009) in turmeric.

Air humidity in the culture room is most often set at 70.0 per cent (Hu and Wang, 1983). Relative humidity is also an important factor in hardening and planting out of *in vitro* raised plants.

2.1.2 Rooting

2.1.2.1 In vitro Rooting

The plantlet produced *in vitro* should have a strong and functional root system. The major cost of *in vitro* plant production is in the rooting and hardening stages. The process of rooting *in vitro* has been estimated to account for approximately 35 to 75 per cent of the total cost of micropropagation

Although number of *in vitro* developed shootlets root spontaneously in culture, shoots of most of the crop species multiplied *in vitro* lack root system. Roots are produced in the presence of a suitable auxin. IAA, IBA and NAA were found to initiate rooting in plants.

All cytokinins inhibit rooting phenomena and BA, which is widely used for shoot multiplication, does so particularly and strongly even after transferring to rooting medium. Web and street (1977) recommended that BA can be replaced by 2ip or Kn during the final stage of *in vitro* shoot multiplication so as to improve subsequent rooting. Since, auxin is essential for root initiation, majority of the root induction media contains it as a supplement .The concentration of the rooting hormone required is often critical so as to provide sufficient stimulus for root initiation and to prevent excess callus formation.

According to Hu and Wang (1983), three phases are involved in rhizogenesis namely induction, initiation and elongation. High concentration of auxin inhibits root elongation (Ancora *et al.*, 1981). Among the auxins, NAA is

found to be the most effective one for rooting. Ganapathi (1995) also supported the use of NAA in inducing roots and further growth of shoots.

Rooting of microshoots of turmeric appears to be quite easy and is comparable with earlier reports (Nadgauda *et al.*, 1978 and Balachandran *et al.*, 1990). Although in most of the cases regenerated shoots produced roots simultaneously, it was necessary to culture them in the particular rooting media for the better growth and development of roots. The MS medium with 1.0 mg 1^{-1} IBA seems to be more ideal than basal MS medium or MS medium with low auxin concentration (0.5mg 1^{-1} IBA) (Shetty *et al.*, 1982).

Several researches have shown that *in vitro* rooting can be successfully achieved by reducing the salt concentrations of the media, particularly in MS medium was reduced to one half, one third or one fourth of the standard strength (Gupta *et al.*, 1981). In turmeric, root proliferation was observed, when the concentration of the macro salts of the MSW medium was reduced to half (Rout *et al.* (1995).

In the *in vitro* regenerated shoots of *C. longa*, Nadgauda *et al.*, (1986) induced well developed roots in White's fresh liquid medium supplemented with 0.25 mg 1^{-1} of sodium molybdate and copper chloride, 10 per cent vitamins and without the addition of any of the plant growth regulators. Balachandran *et al.* (1990) observed that regenerated plants of *C. longa* produced profile roots in all the treatments regardless of the concentration of BA used in the medium.

In *C. longa*, Rout *et al.*, (1995) achieved with half strength MS medium supplemented with 0.25-0.50 mg 1^{-1} IBA and MS medium with 0.5-1.0 mg 1^{-1} IBA. However, all the media combinations differed in rooting per cent and number of roots produced. Maximum rooting (92.5 per cent) with highest number of roots per shoot (11.6) was obtained in the MS medium supplemented with IBA (1.0 mg 1^{-1}) and activated charcoal (5.0 mg 1^{-1}). They also reported nil rooting when the activated charcoal was not included in the basal medium. Rout and Das (1997) reported that regenerated shoots of ginger when cultured on half strength MS liquid medium supplemented with IAA (1.0 mg 1^{-1}) produced maximum number of roots (9.21 ± 0.7).

Ranjan *et al.*, (2001) obtained maximum (85) percentage of rooting in the regenerated shoots of turmeric when transferred to rooting media fortified with BA (0.1 mg 1^{-1}), IAA (0.02 mg 1^{-1}) and 0.25 percent w/v activated charcoal. Shirgurkar *et al.* (2001) confirmed that in *C. longa*, NAA is more effective than IBA on root induction as days required for rooting was less (8 to 10 days) in NAA as compared to that of IBA (10 to 15 days). Habiba *et al.*, (2002) reported that half strength MS with IBA (2.0 $mg1^{-1}$) was the best medium for root induction in the regenerated shoots of turmeric.

In *C. longa* cv 'Elite', Salvi *et al.*, (2002) noted that shoot multiplication was accompanied by rooting when cytokinins are included in the medium. Maximum number of roots per regenerated shoot (6.6) was obtained in MS liquid medium supplement with NAA (1 µ M) and Kn (10 µM1-1), where as the length of roots did not show any significant variation with the changes in the media composition. Prathanturarug *et al.* (2003) observed spontaneous rooting in *C. longa* with an average rooting frequency of 88.4 ± 2.6 per cent.

Rahman *et al.* (2004) induced rooting in the in viro proliferated shoots of *C. longa* by culturing on half strength MS medium with IBA (0.2 mg 1^{-1}) and produces 15.4 ± 1.23 mean number of roots per regenerated shoots with an average root length of 6.3 ± 0.92 cm . In turmeric, Ma and David (2006) indicated that the combination of M4 medium with BA (3.0 mg 1^{-1}) and NAA (0.5 $mg1^{-1}$) produced maximum number of *in vitro* roots (9.0) while, <4 medium fortified with BA (1.0 mg 1^{-1}) and NAA (1.0 mg 1^{-1}) induced maximum root length (5 cm).

In *C. angustifolia*, Shukla *et al.*, (2007) reported that MS medium supplemented with BA (3.0 mg 1^{-1}) and adenine sulphate (25.0 mg 1-1) produced the highest mean number of roots (16.0 ± 1.50). In ginger variety Varada, the highest mean number of roots per culture (12.3) were obtained on LSBM fortified with BA (17.76 µ M) and lowest number of roots (3.9) on LSBM containing BA (4.4 µ M) and NAA (5.36 µ M) as supplements (Kavyashree, 2009).

Naz *et al.* (2009) reported that in turmeric variety Faisalabad and Kasur, the maximum root length of 4.71 cm and 4.74 cm respectively were obtained in

MS medium supplemented with BA (3.0 mg 1^{-1}) and NAA (1.0 mg 1^{-1}) whereas in turmeric variety Bannum, highest root length (4.01 cm) was observed in MS medium supplemented with BA (1.0 mg 1^{-1}) and NAA (1.0 mg 1^{-1}). They also noted that addition of auxin is not required for the initiation of roots as the roots emerged simultaneously with shoots. Pandey *et al,* (2009) reported that 80 percent of the regenerated shoots of turmeric produced better response in half strength MS medium with 500.0 mg 1^{-1} activated charcoal and produced maximum (5.0) number of roots per regenerated shoot.

In *C. longa*, about 95 percentage of the cultures responded to the rooting media combination of half MS with NAA (2.0 mg 1^{-1}) and produced 7.3 ± 0.32 roots per plantlet with an average root length of 4.5 ± 0.12 cm (Kambaska *et al.*, 2010). In ginger variety Varada, Lincy and Sasikumar (2010) reported that highest number of roots (6.1) was formed in MS medium supplemented with TDZ and IBA (1:1 mg 1^{-1}). Nayak *et al.* (2011) reported that microshoots derived from *in vitro* sprouted axillary buds of *C. longa* rooted in MS medium supplemented with 2.0 mg 1^{-1} BA alone produced the highest average number of roots (2.6 root per shoot).

Rahaman *et al.* (2004) found that half strength MS medium with IBA 0.1-1.0 mgl^{-1} was best for rooting of shoots in *Curcuma longa* L.

2.1.2.2 Hardening and planting out

The benefit of any micropropagation system can be fully realized by the successful transfer of plantlets from tissue culture vessels to the *ex vitro* ambient conditions. Most species grown *in vitro* require acclimatization as *in vitro* plantlets are not adapted to *in vivo* conditions (Brainred and Fuchigami, 1981) and it enable plants to survive and grow vigorously when transferred to soil.

Light, temperature and humidity are the major factors to be controlled during acclimatization (Hu and Wang, 1983). The retardation in development of cuticle, epicuticular waxes and functionality of stomatal apparatus during *in vitro* culture cause high stomatal and cuticular transpiration rate in the leaves of plantlets taken out of culture vessels (Conner and Conner, 1984). The success of

acclimatization in micropropagated plants not only depends on the post transfer growth conditions but also on the pre transfer culture conditions (Ziv, 1986). According to Wainwright (1988), low relative humidity, higher light levels and more variable temperatures prevailing outside are the detrimental factors for tissue culture plants when taken *ex vitro*. Standardised rhizosphere environment is necessary for getting better growth of plants (Zimmerman and Fordham, 1985).

In order to maximize the survival of *in vitro* derived plantlets, it is routine practice to acclimatize them under high levels of relative humidity (Short, 1991). *Ex vitro* conditions produce wide variety of morphological abnormalities in the *in vitro* plants particularly of the stomata and cuticle, resulting in high mortality rate after transferring them to glass house or field conditions (Grout and Aston, 1977).

Balachandran *et al.* (1990) reported 100 per cent survival of *in vitro* grown plants of *C. longa under* field conditions after they were hardened in trays containing 1:1 mix of autoclaved sand and soil, and maintained at 27 ^{o} C with 16 h photoperiod. Rout and Das (1997) reported that the *in vitro* regenerated plantlets of ginger could be hardened in the green house and successfully established under field condition with 95-98 per cent success. Salvi *et al.* (2002) reported that in turmeric cv. 'Elite', on transferring the regenerated plants to paper cups containing sterilized soil, 50 out of 52 micropropagated plants survived and upon transferring to the field conditions after two months of hardening, the rate of survival was 100 per cent.

Prathanturarug *et al.* (2003) successfully hardened the regenerated plants of *C. longa* in small pots with sand and rice shell ash (1:1) as the medium for maintaining under greenhouse conditions for one month and then successfully transferred to field conditions. They could not observe any detectable variation in morphology or growth characteristics of *in vitro* regenerated plants as compared to their donor plant.

For the *ex vitro* acclimatization, a hydroponic system which allowed the adaptation of the *in vitro* regenerated plantlets of *C. longa* to the gradual decline in the relative humidity were developed by Zapata *et al.* (2003) and it enabled high percentage of survival and vigorous growth. Rahaman *et al.* (2004)

opinioned that, 70 per cent of the *in vitro* regenerated plantlets of *C. longa* could tolerate transplantation shock on transferring to the soil survived under *ex vitro* environment. As high as 96-100 per cent of the hardened plants of *C. aeruginosa*, *C. aromatica*, *C. cassia* and *C. malabarica* survived upon transplanting to the soil and resembled to their parents phenotypically (Tyagi *et al.*, 2004).

Ma and David (2006) observed 98 per cent of the *in vitro* propagated turmeric plants survived under green house conditions. Shukla *et al.* (2007) used cocoa peat substrate for the hardening of *in vitro* rooted *C. angustifolia* plantlets and obtained 83 per cent survival with normal growth. *In vitro* regenerated plantlets of *C. zedoaria* and *Z. zerumbet* were successfully acclimatized by Stanly and Keng (2007) and obtained 100 per cent survival rate under field condition.

In *C. longa* cv Faisalabad, Naz *et al.*(2009) observed that among the different combinations of potting mixture tried, the better response was observed in the hardening media of sand : soil: peat (1:1:1) combination. During the acclimatization of *in vitro* regenerated plantlets of ginger variety Varada, Kavyashree (2009) reported that the substrate containing soilrite mix peat, perlite and vermiculite (1:1:1) provided the best plant growth media.

Under controlled conditions of temparture (28 ^{o}C) and relative humidity (70-80 per cent) Kambaska *et al.* (2010) successfully established micropropagated plants of *C. longa* in vermiculture substrate and obtained survival rate of 95 per cent upon transplanting them to field conditions. In ginger variety Jamaica and Varada, Lincy and Sasikumar (2010) reported that the hardening media consisting of soil, sand, coir dust, cow dung and *Trichoderma harzianum* produced good results in terms of plant height, number of leaves and total chlorophyll content. Nayak *et al.* (2011) recorded survival rate of 96 per cent, when the *in vitro* grown plantlets of *C. longa* with well developed roots and shoots were transferred to pots containing soil, cow dung and sand mixture (1:1:1 ratio) and maintained under green house conditions for acclimatization. A survival rate of 95 per cent was achieved by Singh *et al.* (2011) in *C. longa* variety Suroma, when its *in vitro* derived plants were transferred to field conditions.

2.2 MICRORHIZOME PRODUCTION

Induction or formation of small rhizomes under *in vitro* condition has been observed in several rhizomatous plants. This is known as microrhizome production. Swelling of shoot base is observed initially when *in vitro* grown shoots are transferred to microrhizome induction medium. Later small sized rhizome is formed at the base of each shoot.

Nayak (2000) reported that microrhizomes initiation at the base of the shoot was seen after 30 days of inoculation and the size increased in culture until harvest. Microrhizome initiation was also accompanied by formation of 4-6 adventitious buds.

Sharma and Singh (1995) reported that the produced microrhizomes in ginger were of different size viz., small (0.1- 0.4 g), medium (0.41- 0.80 g) and big (> 0.81 g).

2.2.1 Factors influencing microrhizome production

Factors such as nutrient composition of basal medium especially sucrose concentration, plant growth regulators and culture conditions like photoperiod and temperature have been reported to play important roles in *in vitro* induction of storage organs such as rhizomes, tubers, bulbs and corms.

Bhat *et al.* (1994) reported that the production of microrhizomes was depended on the nutrient composition, growth regulators, and sucrose concentration of the medium.

2.2.1.1 Basal media

Murashige and Skoog (1962) is the most widely used medium for microtuber propagation. Growth regulators are usually omitted from culture medium. In general semisolid medium is used for initial inoculation. Agar is the most frequently used gelling agent for semisolid cultures.

Sharma and Singh, (1995) reported full strength MS basal medium for microrhizome induction in ginger. Shirgurkar *et al.* (2001) observed that half strength MS basal medium is suitable for microrhizome production in turmeric, obtaining an average number of 5.8 ± 0.7 with the biggest size being

0.55 ± 0.06 g, while, Nayak (2000) and Sunitibala *et al.* (2001) used full strength MS basal medium for microrhizome induction in *Curcuma aromatica* and *C. longa* respectively.

Islam *et al.*, (2004) reported that in turmeric full strength of MS medium was found to be most suitable for microrhizome induction in terms of number (8.3 ± 0.55) and size (0.81 ± 0.04 g) and lesser strength of MS medium produced a lower number of smaller microrhizomes.

Nayak and Naik (2006) reported that MS medium was used for microrhizome induction in turmeric. In *C. zedoaria,* Anisuzzaman *et al* (2008) also used full strength MS medium for microrhizome induction

Chougule *et al.* (2011) reported that the studies in microrhizome production in *C. longa* revealed that full strength MS medium was better than half strength or $1/4^{th}$ MS medium with regard to earliest induction of microrhizomes (45.11 days), weight of microrhizomes and nodes per microrhizomes.

2.2.1.2 Carbon source

Sucrose was reported to promote storage organ formation in potato and *Curcuma* spp. (Thorpe, 1982; Ross and Davies, 1992; Tyagi *et al.*, 1998). Sucrose might influence rhizome formation by being a carbon energy source or as an osmoticum (Bhat *et al.*, 1994). Tyagi *et al.* (1998) indicated that greater availability of a carbon energy source rather than the osmotic effect of sucrose was responsible for rhizome formation *in vitro*.

Sharma and Singh (1995) reported that sucrose concentration of 7.5 per cent was required for *in vitro* rhizome induction in ginger.

Nayak, (2000) opined that the level of sucrose in the medium significantly influence rhizome formation *in vitro* in *C. aromatica*. Microrhizome induction was observed in the media with enhanced level of sucrose (60 - 90 g l^{-1}). 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 store carbohydrates. He observed that at an obligatory concentration of sucrose (3 per cent), plants could not develop any microrhizomes even by increasing the concentration of BA (1.0 to 7.0 mg l^{-1}) or by increasing the duration of photoperiod.

Rout *et al.* (2001) reported that the rhizome induction in ginger is dependent on the concentration and the quantity of sucrose used in the cultures. The frequency of rhizome formation was the maximum in the medium containing 6 ± 8 per cent of sucrose. The medium supplemented with 1 ± 2 per cent sucrose did not produce rhizomes. At a higher concentration of sucrose (3 ± 8 per cent) there was an increase in the percentage of rhizome formation and number of rhizomes per plant.

Chirangini and Sharma (2005) reported that microrhizomes formed in 5 per cent sucrose medium in *Zingiber cassumanar* were larger in size with the highest fresh weight of 0.81 g and with 3 - 5 bud's'.

Among the concentrations of the sucrose tested in turmeric, Nayak and Naik (2006) reported that 6 per cent was most effective for in microrhizome induction.

In *C. zedoaria*, Anisuzzaman *et al* (2008) obtained highest frequency of microrhizome induction in the MS medium supplemented with BA (4.0 mg l^{-1}) and 60 g l^{-1} sucrose.

Chougule *et al.* (2011) reported that in turmeric full strength medium with 90 g sucrose per litre was found to be optimum for microrhizome induction and recorded highest number (5.43) of shoots producing microrhizomes.

2.2.1.3 Plant Growth Regulators

Results of study on influence of auxin and cytokinin, indicated that the BA, Kn and NAA had significant effects on *in vitro* microrhizome production. MS medium with BA, NAA and CCC seemed to be more suitable for mass tuberization in potato (Wang and Hu, 1982). Grewal (1996) reported that Kn was the more effective cytokinin for microtuber induction in *Bynium persicum* and microtuber induction of somatic embryos of *Bynium persicum* was enhanced by addition of Kn.

Sharma and Singh (1995) enhanced microrhizome production in ginger by using 8.0 mg l^{-1} BA. Nayak (2000) reported that 5.0 mg l^{-1} BA enhanced microrhizome production in *Curcuma aromatica* Salisb.

In contrast to it, Shirgurkar *et al.* (2001) stated that BA had an inhibitory effect on *in vitro* microrhizome production in turmeric, though at the lowest concentration of 4.4 µM, it did not show much adverse effects on the average number, weight and yield of microrhizomes. But at the maximum concentration of 35.2 µM BA microrhizome production was completely inhibited.

Sunitibala *et al.* (2001) reported that addition of Kn (1.0 mg l^{-1}) to media was suitable for *in vitro* rhizome induction in *Curcuma longa*.

Islam *et al.* (2004) reported that in *C. longa* BA (12.0 µM) and NAA (0.3 µM) were found suitable for the induction of microrhizomes and application of 12 µM BA together with 0.3 µM NAA exhibited a better response in terms of mean number (8.1 ± 0.36) and the weight (0.67 ± 0.03 g) of microrhizomes.

Nayak and Naik (2006) reported that in turmeric induction of microrhizome was found to be most effective in medium containing 13.3 µM BA.

In *C. zedoaria*, Anisuzzaman *et al* (2008) obtained highest frequency of mircorhizome induction in the MS medium supplemented with BA (4.0 mg l^{-1}). The maximum number of mircorhizomes per shoot (2.3 ± 0.3) was also obtained on BA (4.0 mg l^{-1}).

Chougule *et al.* (2011) reported that of all the media composition tried for in turmeric, MS medium with BA 1.0 mg l^{-1} was found to be optimum for microrhizome induction with respect to number of microrhizomes per shoot and weight of microrhizomes.

2.2.1.4 Culture conditions

Culture conditions like photoperiod and temperature have been reported to play an important role in *in vitro* microrhizome induction in addition to pH and relative humidity.

2.2.1.4.1 Photoperiod

The role of different lengths of photoperiod in microrhizome formation has not been elucidated in detail. There are contradictory reports on photoperiod requirement for *in vitro* microrhizome formation.

Wang and Hu (1982) found that an 8 h photoperiod was better than 16 h photoperiod in potato. Their study on the role of different lengths of photoperiod showed that short day length (8 h photoperiod) was optimum for formation of microtubers *in vitro*.

Sakamura *et al.* (1986) reported that in ginger, microrhizomes were induced in the presence of continuous light, whereas, Sharma and Singh (1995) reported formation of microrhizomes in ginger in complete darkness. This differential requirement of photoperiod for microrhizome induction in ginger may be attributed to genotypic differences.

Nayak (2000) found that the formation of microrhizomes in *C. aromatica* was better in cultures grown in 8 h photoperiod than in 16 h and 0 h photoperiod. Requirement of relatively reduced photoperiod (8 h) for microrhizome formation in *C. aromatica* can be compared with microtuberization in potato (Abbott and Belcher, 1986).

Rout *et al.* (2001) reported that the percentage of microrhizome formation and number of microrhizomes per plant were higher in the cultures incubated under continuous light. The number of microrhizomes declined when the photoperiods was shortened to 8 h. The cultures incubated under continuous dark for eight weeks showed inhibition of both shoot growth and production of microrhizomesrhizomes.

Chirangini and Sharma (2005) obtained microrhizomes in *Zingiber cassumanar*, when four months old microshoots were cultured under dark condition for eight weeks.

Nayak and Naik (2006) reported that in turmeric microrhizomes were best initiated in photoperiod of 4 h light condition, but microrhizomes produced in dark had significantly higher number of buds, than those produced in the presence of light.

Chougule *et al.* (2011) reported that in turmeric microrhizomes initiated in dark condition produced highest number of microrhizomes per shoot (7.0), when followed by 4 h light condition.

2.2.1.4.2 Temperature

For *in vitro* microrhizome formation, providing constant temperature was more effective than alternating day and night temperature. Chirangini and Sharma, (2005) reported that 25 ± 2°C temperature was found to be effective for microrhizome formation.

2.2.2 Dormancy and sprouting of microrhizomes

The microrhizomes transferred to culture initiation medium or shoot multiplication medium under aseptic conditions, showed about 78.2 ± 4 per cent regeneration. Under *in vitro* conditions most microrhizomes regenerated and regeneration of microrhizomes was independent of size and weight. Whereas, in *in vivo* conditions regeneration of plantlets from smaller (0.1 - 0.4 g) microrhizomes was very low (10.4 ± 0.5 per cent). Regeneration of medium sized (0.4 to 0.8 g) microrhizomes was higher (54.7 ± 3.7 per cent) and that of bigger (0.8 g) microrhizomes was highest (73.9 ± 4.3 per cent) (Shirgurkar *et al.*, 2001).

For *in vivo* storage, microrhizomes were washed thoroughly in running tap water after harvest and air dried under shade. Microrhizomes were then stored in moist sterile sand in small pots and kept in a net house, where they sprouted at the onset of the monsoon producing shoots and roots (Nayak, 2000).

For *ex vitro* storage, microrhizomes were kept in polybags or pots filled with sand in a net house at ambient 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 (Nayak and Naik, 2006).

Materials and methods

3. MATERIALS AND METHODS

The present study "*In vitro* production of microrhizomes in *Curcuma aromatica* Salisb." was carried out at the Department of Plantation Crops and Spices and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during the period 2009 - 2011.

The major objective of the study was to standardize method(s) for *in vitro* production of microrhizomes in *Curcuma aromatica* Salisb. The investigations were carried out in two phases viz., (i) *in vitro* shoot multiplication and (ii) microrhizome production.

The details of materials and methods adopted for the study are presented in this chapter.

3.1 PHASE I: *IN VITRO* MULTIPLICATION

3.1.1 Collection and Preparation

IISR Kozhikode accession of *Curcuma aromatica* maintained in the germplasm collection of the Department of Plantation Crops and Spices, College of Agriculture, Vellayani was used for the study. Details and description of the accession are given in Table 2.

The rhizomes of *Curcuma aromatica* Salisb. were planted in pots and harvested at maturity as indicated by drying of leaves. The fingers were separated from the mother rhizome and cleaned thoroughly by washing with water and were treated with Bavistin 0.2 per cent for 20 minutes and shade dried. They were then placed on moist sterilized sand for sprouting in a ventilated room. When rhizomes started sprouting, healthy green sprouts were carefully removed with a surgical blade and brought to the laboratory.

3.1.2 Surface sterilization

Rhizome sprouts (2 cm length) brought to the laboratory were washed thoroughly in running tap water for 20 minutes. They were then washed again with liquid detergent (Laboline) for 10 minutes with vigorous shaking, followed

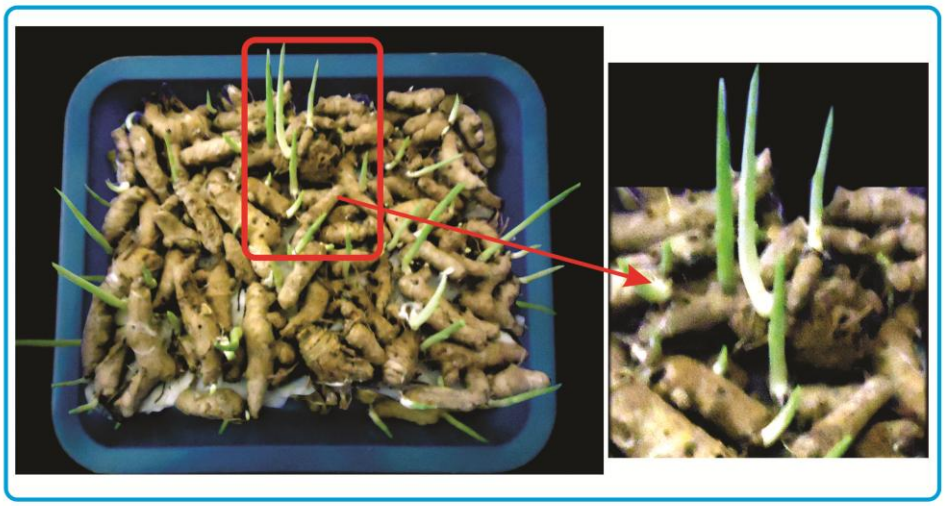
A. Curcuma aromatica plant

B. Rhizomes

Plate 1. Plant species selected for the study: *Curcuma aromatica* Salisb. (Kasthuri turmeric)

Table 2. Details and description of the accession of *Curcuma aromatica* Salisb. selected for the study

Plant species	Accession	Botanical description	Yield attributes
Curcuma aromatica Salisb. (Kasthuri manjal)	IISR Kozhikode accession	The plants are moderately tall and light green in colour. Leaves are characterized by the presence of hairs on lower side. The mid-rib of the leaf is green. The rhizomes are oblong and have a pale yellow or creamy colour with intense camphoraceous aroma.	Fresh rhizome yield = 257.49 g plant^{-1} Dry rhizome yield = 46.34 g plant^{-1} Essential oil content = 5.0 to 7.0 % Curcumin content = 0.05 to 0.10 % (Shamrao, 2012)



A. Sprouted rhizomes of *C. aromatica*

B. Prepared explants C. Explants seven days after inoculation

Plate 2. Explants of *Curcuma aromatica* Salisb.

by washing with double glass distilled water, to remove any traces of detergent, for 20 minutes.

Explants were then kept in 0.2 per cent w/v solution of Bavistin (BASF India Limited) for 30 minutes. After washing 2-3 times with sterile water explants were taken out and surface sterilized.

Surface sterilization of the plant material was carried out inside a laminar air flow chamber. After initial cleaning, explants were transferred to a sterile beaker and treated with freshly prepared 0.1 per cent w/v aqueous solution of mercuric chloride for 12 minutes. This was followed by thorough washing with 4-5 changes of sterile distilled water so as to remove any traces of mercuric chloride and then inoculated in the medium . Survival rate of explants were recorded 10 days after inoculation.

3.1.3 Culture vessels

The borosilicate glassware, Corning brand test tubes (25 x 150 mm) and Erlenmeyer flask (100 ml), jam bottles (300 ml) were used for the experiment. The glassware for the experiments were soaked in detergent solution (Laboline 0.1 per cent) over night, washed thoroughly in running tap water and rinsed twice in double glass distilled water. The glasswares were then dried and autoclaved at 121° C and 1.06 kg cm^{-2} for 45 minutes.

3.1.4 Culture Media

The basal medium suggested by Murashige and Skoog (1962) was used for the study. The chemicals used for the study were of analytical grade from Merck, Sisco Research Laboratory and Himedia.

Standard procedures were followed for the preparation of the basal medium (Thorpe, 1980). Stock solution of the major and minor nutrients, organics and plant growth substances were prepared by dissolving the required quantity of chemicals in specific volume of double glass distilled water/ ethyl alcohol/ 0.1N HCl or NaOH, depending on the chemical and were stored under refrigerated condition (4°C).

The medium was prepared by pipetting out specific quantities of all the ingredients into 1000 ml beaker. Sucrose and inositol were added fresh in required quantity, weighed using an electronic balance (Sartorius analytic A120S) and dissolved by constant stirring. The pH of the medium was adjusted to 5.7 by using 1N KOH or 1N HCl with the aid of an electronic pH meter (Philips, model PP9046). After adjusting the pH, agar was added to the medium at the rate of 0.65 per cent w/v and final volume made up to 1000 ml, using a volumetric flask. Agar was melted by placing the beaker in microwave oven till agar got fully dissolved in the medium. Melted medium was then dispensed into pre-sterilized culture vessels such as test tubes (25 x 150 mm) and Erlenmeyer flasks (100 ml)/ jam bottles (300 ml) which were rinsed with double glass distilled water. Measure of culture medium in test tube and flask/ bottle was 15 ml and 50 ml, respectively. After pouring media bottle caps were tightened. In case of test tubes, they were plugged tightly with non absorbent cotton and labelled properly. They were then autoclaved at 121° C and 1.06 $kgcm^{-2}$ pressure for 20 min using horizontal autoclave (NAT make).

3.1.5 Inoculation and incubation

All the inoculation operations were carried out under aseptic conditions in a laminar airflow chamber (Klenzaids, Model 1104); the laminar flow chamber was prepared for inoculation by swabbing the table with 80 per cent ethanol, after sterilization under UV light for 30 minutes.

Explants were transferred to large sterile glass petriplate and were further trimmed to suitable size (2 - 3 cm) by using sterile surgical scalpel and forceps. They were then transferred to culture bottles containing medium in front of flame in front of a spirit lamp. After vertically inoculating the explants in the culture bottle, the mouth of bottle/ test tube was quick flamed and bottles were tightly capped/ plugged and mouths of the bottles/ test tube were properly sealed with cling film. They were properly labeled and then incubated in a culture room maintained at 20 ± 2°C temperature and at a relative humidity of 60 per cent.

Table 3. Auxins and cytokinins tried for shoot multiplication *in vitro* from rhizome bud sprouts of *C. aromatica*

Treatments	Plant growth regulators (mg l^{-1})		
	BA	NAA	Kn
T1	1.0	-	-
T2	2.0	-	-
T3	3.0	-	-
T4	4.0	-	-
T5	5.0	-	-
T6	1.0	0.1	-
T7	2.0	0.1	-
T8	3.0	0.1	-
T9	4.0	0.1	-
T10	5.0	0.1	-
T11	-	-	0.5
T12	-	-	1.0
T13	-	-	2.0
T14	1.0	-	0.5
T15	3.0	-	0.5
T16	5.0	-	0.5
T17	1.0	-	1.0
T18	3.0	-	1.0
T19	5.0	-	1.0
T20	-	0.5	-
T21	-	1.0	-
T22	-	2.0	-

Culture medium: MS + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) + Agar (0.65 g $^{-1}$)

A light intensity of 3000 lux was maintained using white cool fluorescent tube lights for 16 h followed by a dark period of 8 h. The temperature of the room was regulated using an air conditioner.

3.1.6 Plant growth substances

3.1.6.1 Cytokinins and auxins

Explants were cultured in MS medium with different combinations of cytokinins (BA and Kn) and auxin (NAA). Treatments involved are given in Table 2. Treatments were replicated six times. Observations on number of cultures showing shoot proliferation, number of shoots produced per culture vessel, length of the longest shoot and shoot multiplication rate per subculture were recorded.

3.1.7 Rooting

3.1.7.1 *In vitro Rooting*

Well developed shoots (3-5 cm long) separated from *in vitro* shoot multiplication cultures were subjected to different rooting treatments. By inoculating into the medium containing varying levels of NAA (0.1 to 1.0 mg l^{-1}), IBA (0.1 to 1.0 mg l^{-1}), IAA (0.1 to 1.0 mg l^{-1}) (Table 3). Each treatment was replicated twelve times. Data were recorded after six weeks of culture. Observations were recorded on cultures initiating roots (in percentage), number of roots and length of roots (cm).

3.1. 7.2 *Planting out and acclimatization*

Plantlets produced *in vitro* were taken out from culture vessels with the help of forceps. The agar adhering to the roots was completely removed by thorough washing with running tap water. The plantlets were treated with Bavistin (1.0 per cent) solution for 30 min before planting out. Effect of potting medium on *ex vitro* establishment of plants was tried by providing different potting media (vermin compost, coir pith compost and mixture of vermi compost: coir pith compost (1:1 v/v). They were then kept in the mist chamber for primary hardening for two weeks and per cent survival of plantlets was recorded.

Table 4. Auxins tried for adventitious root formation in *in vitro* shoots of *C. aromatica*

Treatment	Concentration of auxin (mg l^{-1})		
	NAA	IBA	IAA
R1	0.1	-	-
R2	0.2	-	-
R3	0.5	-	-
R4	1.0	-	-
R5	-	0.1	-
R6	-	0.2	-
R7	-	0.5	-
R8	-	1.0	-
R9	-	-	0.1
R10	-	-	0.2
R11	-	-	0.5
R12	-	-	1.0

Culture medium: ½ MS + myoinositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) + Agar (0.65 g l^{-1})

Table 5. Different potting media tried for *ex vitro* establishment of plants

Treatment	Potting medium
PM1	Coir pith compost
PM2	Vermi compost
PM3	Coir pith compost : Vermi compost (1:1 v/v)

The plantlets were then planted in poly bags containing potting media (sand + top soil + vermi compost) and kept for secondary hardening for two weeks before planting in the field.

3.1.8 *Ex vitro* establishment

Twenty hardened TC plants were grown in the field. Per cent survival, shoot length, leaf length and number of leaves produced were observed two months after planting in the field.

3.1.10 Statistical analysis

Completely randomized design (CRD) was followed for statistical analysis. The data were subjected to ANOVA as suggested by Panse and Sukhatme (1985), wherever applicable. Critical difference values were tabulated at five per cent probability where F test was significant.

3.2 PHASE II: MICRORHIZOME INDUCTION

3.2.1 Explant

Well developed shoots (3 – 4 cm long) seperated from *in vitro* shoot multiplication cultures of *C. aromatica* were used as explants for microrhizome induction.

3.2.2 Culture vessel

Borosilicate glasswares, Corning brand test tubes (25 x 150 mm), Erlenmeyer flasks (100 ml) and jam bottles (300 ml) were used for the experiment.

3.2.3 Culture Medium

Culture medium consisting of MS basal medium with different levels of plant growth regulators, carbon source and photoperiod were tried for microrhizome induction.

3.2.3.1 Plant growth substances

Shoots were cultured in MS medium with different combinations of cytokinins (BA and Kn). Treatments involved different levels of BA (0 -7 mgl^{-1}) and Kn (0 -7.0 mg l^{-1}) and BA (1.0 -7.0 mg l^{-1}) at different concentrations in combination with Kn (0 - 7.0 mg l^{-1}) (Table 5). Treatments were replicated six times. Data were recorded after 90 days of culture. Observations on per cent of shoots initiating microrhizome, number induced per culture vessel, fresh weight of microrhizome, dry weight of microrhizome, cultures initiating callus and size of microrhizome were recorded.

3.2.3.2 Carbon source

Sucrose

Varying levels of sucrose (10, 20, 30, 40, 50, 60, 70, 80 and 90 g l^{-1}) were tried to study their effect on microrhizome induction in shoots obtained from shoot multiplication cultures Culture medium used was MS + Inositol (100 mg l^{-1}) + Agar (0.65 g l^{-1}) + Photoperiod (16 h) + plant growth regulators (best treatment from Table 5). Treatments were replicated six times. Data were recorded after 90 days of culture. Observations on cultures with microrhizome induction, time taken for induction of microrhizomes, number of microrhizomes per culture vessel and fresh and dry weight of microrhizomes were recorded.

3.2.3.3 Photoperiod

Photoperiod was tried at four levels (16 h, 8 h, 4 h and 0 h light) to assess its effect on microrhizome induction. Treatments were replicated six times. Culture medium used was MS + Inositol (100 mg l^{-1}) + Agar (0.65 g l^{-1}) + Sucrose (70 g l^{-1}) + plant growth regulators (best treatment from Table 5). Observations on number of cultures with microrhizome, number of microrhizome per culture vessel, fresh and dry weight of microrhizome were recorded.

3.2.4 Inoculation and incubation

The inoculated cultures were incubated in a culture room maintained at a light intensity of 3000 lux using cool white fluorescent tubes. The room

Table 6. Cytokinins tried for induction of microrhizomes *in vitro* in shoots of *C. aromatica*

Treatment	Plant growth regulator(s) (mg l^{-1})	
	BA	Kn
M1	-	1.0
M2	-	3.0
M3	-	5.0
M4	-	7.0
M5	1.0	1.0
M6	1.0	3.0
M7	1.0	5.0
M8	1.0	7.0
M9	3.0	1.0
M10	3.0	3.0
M11	3.0	5.0
M12	3.0	7.0
M13	5.0	1.0
M14	5.0	3.0
M15	5.0	5.0
M16	5.0	7.0
M17	7.0	1.0
M18	7.0	3.0
M19	7.0	5.0
M20	7.0	7.0
M21	1.0	-
M22	3.0	-
M23	5.0	-
M24	7.0	-
Control	-	-

Culture medium: MS+ Sucrose (30 g l^{-1}) + Agar (0.65 g l^{-1}) + Photoperiod 16 h

temperature was regulated using an air conditioner at 20 ± 2°C and the relative humidity was maintained at 60 per cent.

For microrhizome induction cultures were incubated under 4 h, 8 h, 16 h and 0 h light period. Darkness was provided by placing the cultures in culture racks completely covered with black paper. The culture vessels were also covered with aluminium foil.

3.2.5 Germination and survival of microrhizomes

For *in vitro* germination, harvested microrhizomes were transferred to MS medium containing 5.0 mg l^{-1} BA and 0.1 mg l^{-1} NAA. For *ex vitro* germination, harvested microrhizomes were washed repeatedly in running tap water, air dried and kept for germination in a net house in polybags filled with potting media (mixture of vermi compost and coir pith compost 1:1 v/v). The rate of germination and survival of microrhizomes in each set of experiment were recorded after 28 days of sowing. Observations on number of microrhizomes initiating shoot growth, length of shoot, rate of shoot growth, rate of survival of shoots and fresh and dry weight of the shoots were recorded.

3.2.5 Statistical analysis

Completely randomized design (CRD) was followed for statistical analysis. The data were subjected to ANOVA as suggested by Panse and Sukhatme (1985), wherever applicable.

Results

4. RESULTS

The present investigation entitled "*In vitro* production of microrhizomes in *Curcuma aromatica* Salisb." was carried out at the Department of Plantation Crops and Spices and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during the period from 2009 - 2011. The results of the investigation are presented in this chapter.

4.1 *IN VITRO* MULTIPLICATION

4.1.1 Explant(s)

Rhizome bud sprouts, used as explants gave good response with respect to *in vitro* culture establishment and shoot proliferation.

Surface sterilization was carried out using Bavistin (0.2 per cent for 30 minutes) and mercuric chloride (0.1 per cent for 12 minutes). Survival rate of explants was 87 per cent, rest of explants were discarded due to microbial contamination.

4.1.2 *Plant growth substances*

Cytokinins and auxin

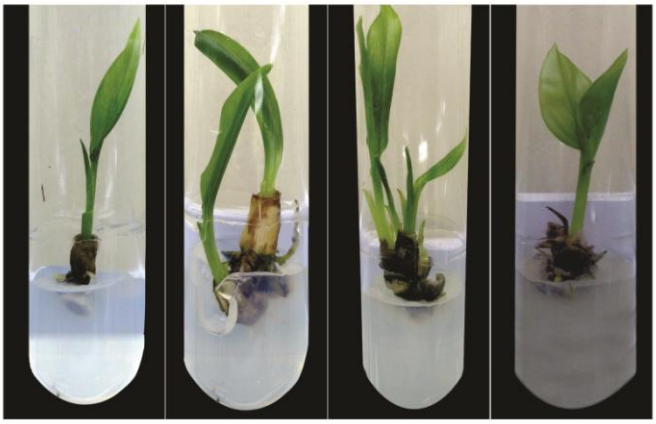
Twenty two treatments involving various combinations of plant growth substances were tried to study their effect on *in vitro* shoot multiplication in *Curcuma aromatica*. Results of the study are presented in the Table 7.

The results indicated significant difference among the treatments with regard to number of days taken for establishment of explants. The least number of days (11) for establishment of explants was recorded by T10 (BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1}) which was statistically on par with T5 (12 days). The next best treatment was T9 (13.7 days) (BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1}). These three treatments were significantly superior to other treatments. Establishment of explants was late (22.5 days) in T11 (Kn 0.5 mg l^{-1}) and T14 (BA 1.0 mg l^{-1}+ Kn 0.5 mg l^{-1}) and was on par with T1, T12, T13 and T17.

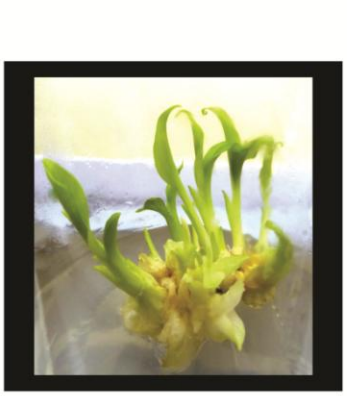
Table 7. Effect of auxins and cytokinin on *in vitro* shoot multiplication from rhizome bud sprouts of *C. aromatica*

Treatments	Plant growth regulator (mg l^{-1})			Culture establishment * (days)	Shoot proliferating cultures (%)	Shoot(s) / culture vessel	Longest shoot length (cm)
	BA	NAA	Kn				
T1	1.0	-	-	21.7	33	2.2	2.50
T2	2.0	-	-	18.5	50	3.2	2.82
T3	3.0	-	-	17.8	67	5.2	4.28
T4	4.0	-	-	14.5	83	7.2	4.95
T5	5.0	-	-	12.0	100	9.0	5.55
T6	1.0	0.1	-	19.0	42	4.6	2.80
T7	2.0	0.1	-	18.5	58	6.0	3.83
T8	3.0	0.1	-	15.5	75	8.0	4.77
T9	4.0	0.1	-	13.7	92	9.4	5.33
T10	5.0	0.1	-	11.0	100	12.6	6.03
T11	-	-	0.5	22.5	25	1.2	1.70
T12	-	-	1.0	21.5	42	1.4	1.77
T13	-	-	2.0	21.8	50	1.8	2.20
T14	1.0	-	0.5	22.5	33	1.6	2.50
T15	3.0	-	0.5	21.0	42	1.8	3.20
T16	5.0	-	0.5	20.0	75	2.4	3.80
T17	1.0	-	1.0	21.2	67	1.4	1.72
T18	3.0	-	1.0	19.5	75	1.4	1.95
T19	5.0	-	1.0	18.7	83	1.6	3.00
T20	-	0.5	-	19.0	-	-	-
T21	-	1.0	-	21.0	-	-	-
T22	-	2.0	-	18.5	-	-	-
Control	-	-	-	21.8	-	-	-
CD (5%)	-	-	-	1.2	-	0.6	0.14

The data represent mean of six replications; * The data represent mean of twelve replications; Culture medium: MS



A. Thirty days after inoculation



B. Fourty five days after subculture C. Seventy five days after subculture

Plate 3. Shoot proliferation in *Curcuma aromatica* in culture medium BA 5.0 mg l^{-1} + NAA 0.1 mg l^{-1}

Cent per cent of cultures showing shoot proliferation was obtained in the treatments T10 (BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1}) and T5 (BA 5.0 mg l^{-1}) followed by T9 (92 %). Treatment T11 (Kn 0.5 mg $l^{-1)}$ recorded minimum number (25 %) cultures showing shoot proliferation. There was no shoot proliferation in cultures of T20, T21, T22 and control (devoid of growth regulators) in spite of initial response.

Significant influence was noticed in the number of shoots per culture when varying levels of growth regulators were added to the culture medium. Treatment T10 (BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1}) produced significantly the highest number of shoots (12.6) followed by T9 which was on par with T5 (BA 5.0 mgl^{-1}). The least number of shoots (1.2) per culture was recorded by T11 (Kn 0.5 mg l^{-1}) which was on par with T12, T13, T14, T17, T18 and T19. Multiple shoot formation was more in BA compared to Kn. The addition of NAA along with BA had shown a positive influence on the number of shoots produced per culture.

Length of the longest shoot (6.03 cm) was maximum in T10 (BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1}) followed by T5. The length of the longest shoot was minimum in T11 (1.7 cm) which was on par with T12 and T17. There was progressive increase in shoot length when the concentration of BA was increased. The addition of NAA had positive influence on the shoot length. Medium containing NAA along with BA recorded increase in shoot length than that with BA alone.

Cultures were maintained by constant subculturing at an interval of four weeks. The effect of subculture on shoot multiplication was tested and the results indicated that the rate of multiplication over 24.4 shoots per culture was maintained in MS medium supplemented with BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1} during subsequent subcultures.

4.1.3 Rooting

4.1.3.1 *In vitro Rooting*

Twelve treatments with varying levels of auxins were tried for *in vitro* rooting. Result of the study is presented in the Table 8.

Cent per cent of cultures initiating roots was observed in treatments R1 (NAA 0.1 mg l^{-1}), R2 (NAA 0.2 mg l^{-1}), R5 (IBA 0.1 mg l^{-1}), R6 and R7(IBA 0.5 mg l^{-1}). R11 (IAA 0.5 mg l^{-1}) recorded least percent (42 %) of cultures initiating roots.

Maximum number of roots (15.4) per rooted shoot was obtained in R6 (IBA 0.2 mg l^{-1}) followed by R4 (NAA 1.0 mg l^{-1}) and minimum number of roots (2.3) per rooted shoots was obtained in R9 (IAA 0.1 mg l^{-1}). Mean length of roots was maximum (6.4 cm) in treatment R3 (NAA 0.5 mg l^{-1}) and R6 (IBA 0.2 mg l^{-1}) and minimum (3.0 cm) in R12 (IAA 1.0 mg l^{-1}).

4.1.3.2 *Planting out and acclimatization*

The *in vitro* rooted plants were carefully taken out from the culture vessels and planted out in different potting media (vermi compost, coir pith compost and mixture of vermi compost and coir pith compost 1:1 (v/v) to study their effect on *ex vitro* establishment. The per cent survival of plantlets was recorded after two weeks of planting out (Table 9).

PM3 (coir pith compost + vermi compost 1:1 (v/v) recorded maximum survival (100 %) and least survival rate (83 %) was recorded by PM1 (coir pith compost).

4.1.4.2.1 Field establishment

In the present study twenty hardened tissue culture plants of *C. aromatica* were planted in the field to study the field survival rate. In the field *in vitro* produced *C. aromatica* plants recorded 100 per cent survival. Plants recorded 55 cm mean plant height, 35 cm leaf length and produced a mean number of 5.5 leaves per plant two months after planting in the field. Plants were healthy and vigorous in growth and morphologically true to the parent variety.

Plate 4. Adventitious root formation in *in vitro* shoots of *Curcuma aromatica*

Table 8. Effect of auxins on adventitious root formation in *in vitro* shoots of *C. aromatica*

Treatment	Auxin (mgl^{-1})			Cultures initiating roots (%)	Roots/shoot	Mean root length (cm)
	NAA	IBA	IAA			
R1	0.1	-	-	100.0	7.5	6.0
R2	0.2	-	-	100.0	10.4	6.1
R3	0.5	-	-	92.0	10.8	6.4
R4	1.0	-	-	83.0	14.0	4.4
R5	-	0.1	-	100.0	5.5	5.4
R6	-	0.2	-	100.0	15.4	6.4
R7	-	0.5	-	100.0	10.4	5.0
R8	-	1.0	-	83.0	7.6	4.0
R9	-	-	0.1	67.0	2.3	3.2
R10	-	-	0.2	58.0	3.6	4.1
R11	-	-	0.5	42.0	5.8	3.3
R12	-	-	1.0	50.0	4.2	3.0

Data represent mean of 12 replication Culture medium: ½ MS

Table 9. Effect of different potting media on *ex vitro* establishment of *in vitro* generated plants of *C. aromatica*

Treatment	Potting media	Survival * (%)
PM1	Coir pith compost	83
PM2	Vermi compost	91
PM3	Coir pith compost : Vermi compost 1:1 (v/v)	100

Data represent mean of 12 replications; * After two weeks of planting out

Plate 5. Hardened tissue cultured plants of *Curcuma aromatica*

A. Thirty days after planting

B. Sixty days after planting

Plate 6. Planted out tissue cultured plants of *Curcuma aromatica* in the field

4.2 MICRORHIZOME INDUCTION

4.2.1 Explant(s)

Three to four cm long shoots generated from *in vitro* shoot multiplication cultures of *C. aromatica* were used as explants for microrhizome induction.

4.2.2 *Plant growth substances*

Cytokinins and auxin

Twenty four treatments involving various combinations of plant growth substances (BA and Kn) were used to study their effect on microrhizome induction from *in vitro* generated shoots in *Curcuma aromatica*. Results of the study arepresented in Table 10.

The results indicated significant difference among the treatments with regard to per cent of shoots initiating microrhizome, number of microrhizomes induced per culture vessel, fresh and dry weight of microrhizomes and size of microrhizomes.

Per cent of shoots initiating microrhizome varied among treatments. Treatment M23 (BA 5.0 mg l^{-1}) recorded cent per cent of shoots initiating microrhizome. Next best treatments was M13 (BA 5.0 mg l^{-1} + Kn 1.0 mg l^{-1}) and M18 (BA 7.0 mg l^{-1} + Kn 3.0 mg l^{-1}) which recorded 92 per cent. Least per cent (8 %) of shoots initiating microrhizome was observed in treatments M9 (BA 3.0 mg l^{-1} + Kn 1.0 mg l^{-1}) and M20 (BA 7.0 mg l^{-1} + Kn 7.0 mg l^{-1}). There was no microrhizome induction in control (devoid of growth regulator).

The number of microrhizomes induced per culture vessel was significantly influenced by the different concentration of growth regulator. Highest number of microrhizome induced per culture vessel (4.8) was recorded by treatment M23 (BA 5.0 mg l^{-1}) which was on par with M13 (BA 5.0 mg l^{-1} + Kn 1.0 mg l^{-}) which recorded 4.2 microrhizome per culture vessel. The least number of microrhizomes (1.2) per culture vessel was recorded by M1, M4, M9

Table 10. Effect of auxin and cytokinins on microrhizome induction in *in vitro* generated shoots of *C. aromatica*

Treatment	Plant growth regulator(s) (mg l^{-1})		Shoots initiating microrhizome* (%)	Number of microrhizomes/ culture vessel	Fresh weight of microrhizome (mg)**	Dry weight of microrhizome (mg)
	BA	Kn				
M1	-	1	17	1.2	38 (S)	18
M2	-	3	42	1.8	100 (M)	23
M3	-	5	67	2.8	130 (M)	37
M4	-	7	17	1.2	53 (S)	9
M5	1	1	25	1.3	82 (M)	35
M6	1	3	33	1.5	50 (S)	17
M7	1	5	42	1.8	80 (M)	26
M8	1	7	50	2.0	130 (M)	50
M9	3	1	8	1.2	82 (M)	27
M10	3	3	33	1.8	53 (S)	29
M11	3	5	50	2.2	57 (S)	30
M12	3	7	67	2.7	192 (L)	32
M13	5	1	92	4.2	83 (M)	16
M14	5	3	75	3.5	52 (S)	35
M15	5	5	83	3.2	55 (S)	31
M16	5	7	25	1.3	147 (M)	28
M17	7	1	58	2.5	33 (S)	12
M18	7	3	92	3.8	88 (M)	21
M19	7	5	67	3.0	80 (M)	9
M20	7	7	8	1.2	42 (S)	40
M21	1	-	58	2.3	62 (S)	24
M22	3	-	83	3.8	210 (L)	50
M23	5	-	100	4.8	247 (L)	65
M24	7	-	75	3.5	132 (M)	47
Control	-	-	-	-	-	-
CD (5%)	-	-	-	0.60	0.01	

The data represent mean of six replications;

Culture medium: MS + 16 h light period + sucrose 60 g l^{-1};

* The data represent mean of 12 replications

** Categorization of microrhizome based on their fresh weight:

S (small) = 0 - 75 mg; M (medium) = 75 - 150 mg; L (large) = >150 mg

Plate 7. Microrhizome formed at the base of *in vitro* derived *C. aromatica* plantlets of in culture medium BA 5.0 mg l^{-1} ninety days after incubation

and M20 which was on par with M5, M6 and M16. There was progressive increase in the number of microrhizomes produced per culture vessel when the concentration of BA was increased. Treatment with BA was found to be more effective than with Kn alone or Kn in combination with BA.

The fresh weight of microrhizome was significantly influenced by the different concentration of growth regulators. Treatment M23 (BA 5.0 mg l^{-1}) recorded highest fresh weight (247 mg) followed by M22 (BA 3.0 mg l^{-1}). The fresh weight of microrhizome was lowest (33 mg) in M17 (BA 7.0 mg l^{-1} + Kn 1.0 mg l^{-1}) which was on par with M1 (Kn 1.0 mg l^{-1}).

Microrhizomes produced were grouped into three sizes based on the fresh weight of microrhizomes; small (0 - 75 mg), medium (75 - 150 mg) and large (>150 mg). Larger microrhizomes (>150 mg) were obtained in the treatments M12, M22, M23 and medium microrhizomes (75 - 150 mg) in M2, M3, M5, M7, M8, M9, M13, M16, M18, M19 and M24. Treatments M1, M4, M6, M10, M11, M14, M15, M17 and M20 produced smaller microrhizomes (0 -75 mg).

With regard to dry weight of microrhizomes treatment M23 (BA 5.0 mg l^{-1}) recorded highest dry weight (65 mg) followed by M22 (BA 3.0 mg l^{-1}) and M8 (BA 1.0 mg l^{-1}+ Kn 7.0 mg l^{-1}) which recorded 50 mg. The dry weight of microrhizomes was lowest (9 mg) in M4 (Kn 7.0 mg l^{-1}) and M19 (BA 7.0 mg l^{-1}+ Kn 7.0 mg l^{-1}).

Callus formation was observed in treatments M7 (BA 1.0 mg l^{-1}+ Kn 5.0 mg l^{-1}), M10 (BA 3.0 mg l^{-1}+ Kn 3.0 mg l^{-1}), M14 (BA 5.0 mg l^{-1}+ Kn 3.0 mg l^{-1}) and in M20 (BA 7.0 mg l^{-1}+ Kn 7.0 mg l^{-1}).

4.2.3 Carbon source

Sucrose was tried at nine different levels (10, 20, 30, 40, 50, 60, 70, 80 and 90 g l^{-1}) to assess its effect on microrhizome induction. Results are presented in the Table 11.

There was significant variation among different levels of sucrose with regard to cultures with microrhizome induction, time taken for induction of

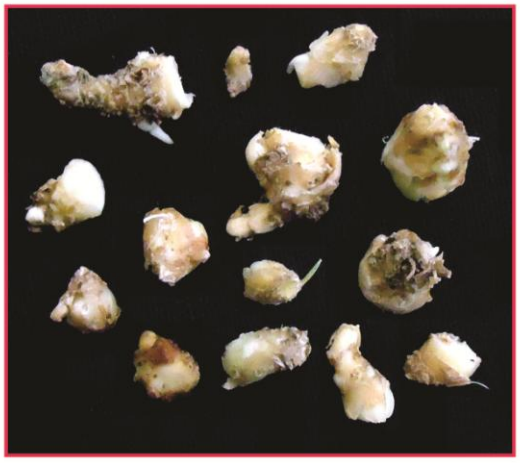


Plate 8. Microrhizomes harvested after 90 days of incubation of explants

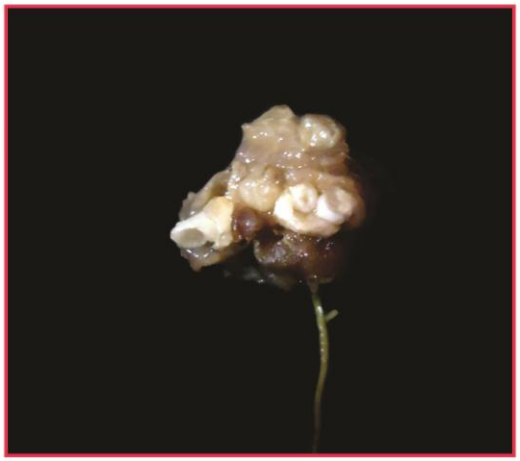


Plate 9. Callus obtained in culture medium BA 7.0 mg l^{-1}+ Kn 7.0 mg l^{-1}

Table 11. Effect of sucrose on microrhizome induction in *in vitro* generated shoots of *C. aromatica*

Treatment	Sucrose conc. (%)	Cultures with microrhizome induction * (%)	Time taken for induction of microrhizome (days)	Microrhizome(s)/ culture vessel	Fresh wt. of microrhizome (mg)	Dry wt. of microrhizome (mg)
S1	1	-	-	-	-	-
S2	2	-	-	-	-	-
S3	3	33	61	1.2	140	35
S4	4	50	54	3.0	160	38
S5	5	58	47	4.7	180	42
S6	6	83	43	4.8	210	50
S7	7	92	37	5.5	230	57
S8	8	67	51	4.0	260	70
S9	9	42	56	2.3	240	62
CD	-	-	-	0.60	0.01	-

Culture medium: MS + BA 5.0 mg l^{-1} + 16 h photo period (light).

The data represent mean of six replications. *The data represent mean of twelve replication

microrhizomes, number of microrhizomes per culture vessel and fresh and dry weight of microrhizomes.

With regard to the cultures with microrhizome induction, treatment S7 (70 g l^{-1}) recorded maximum percentage (92 %) of cultures with microrhizome and treatment S3 (10 g l^{-1}) recorded least (33 %). There was no microrhizome production in treatments S1 (10 g l^{-1}) and S2 (20 g l^{-1}).

Microrhizome initiation was earliest (37 days) when cultured in S7 (70 g l^{-1}) followed by 43 days in S6 (60 g l^{-1}) and late (61 days) in S3 (30 g l^{-1}).

Maximum number of microrhizomes per culture vessel (5.5) was recorded by S7 (70 g l^{-1}) followed by S6 (60 g l^{-1}). S3 (30 g l^{-1}) recorded least number of microrhizomes per culture vessel (1.2).

Sucrose concentration had remarkable effect on the fresh and dry weight of microrhizome produced. Fresh dry weight of microrhizome was maximum (260 mg) in treatment S8 (80 g l^{-1}) and minimum (140 mg) in treatment S3 (30 g l^{-1}). Dry wt of microrhizome also showed similar trend. Dry weight of microrhizome was maximum (70 mg) in treatment S8 (80 g l^{-1}) and minimum (35 mg) in treatment S3 (30 g l^{-1}). Both fresh and dry weight of microrhizome increased with increase of sucrose concentration up to 80 g l^{-1} and decreased thereafter.

4.2.4 Photoperiod

Four durations (16 h, 8 h, 4 h and 0 h light) of photoperiod (light period) was tried to assess their effect on microrhizome induction. Results of the study are presented in Table 12.

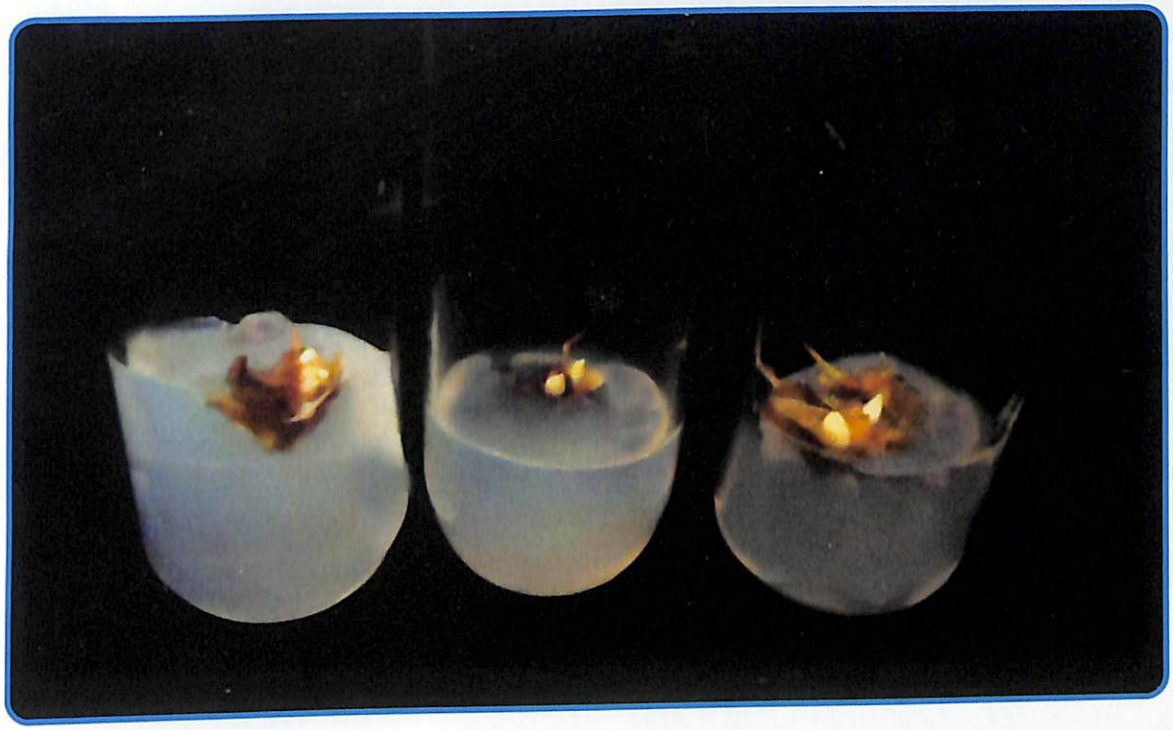
There was significant variation among the treatments with regard to cultures with microrhizome induction, time taken for initiation of microrhizome, number of microrhizome per culture vessel and fresh and dry weight of microrhizome.

Table 12. Effect of photoperiod on microrhizome induction in *in vitro* generated shoots of *C. aromatica*

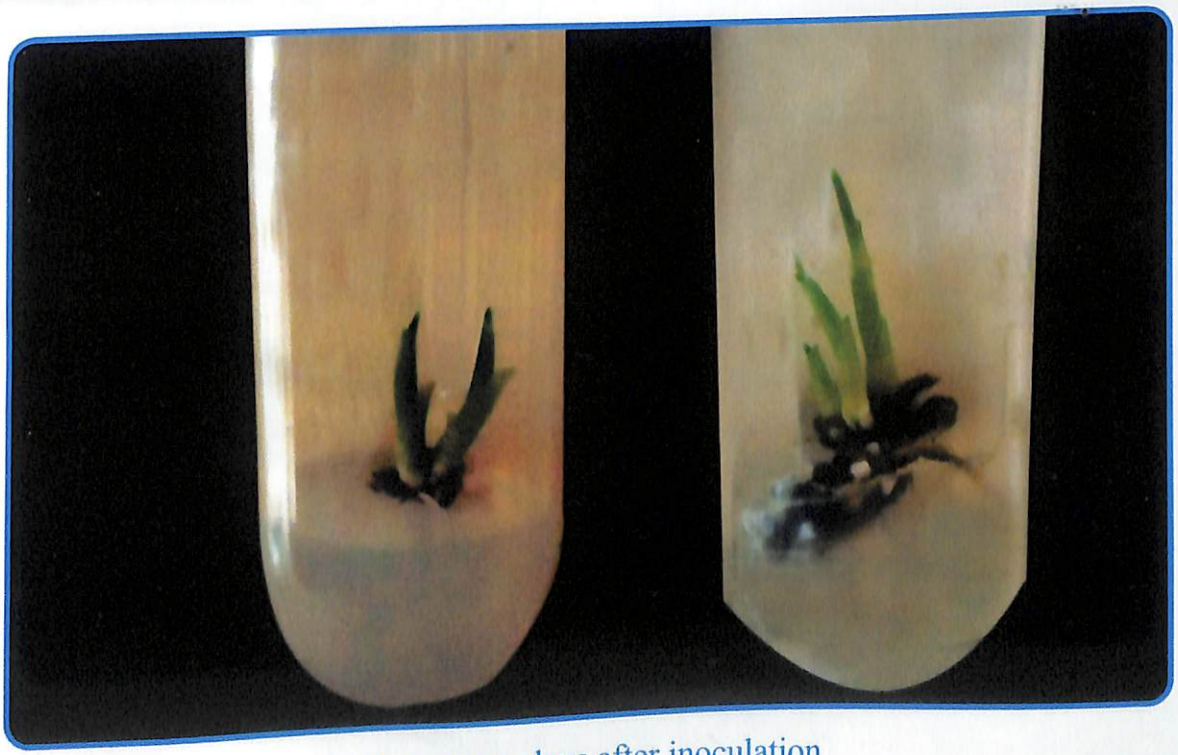
Treatment	Photoperiod (h light)	Cultures with microrhizome induction * (%)	Microrhizome(s)/ culture vessel	Time taken for induction of microrhizome (days)	Fresh wt. of microrhizome (mg)	Dry wt. of microrhizome (mg)
P1	16	75	4.2	47.6	190	42
P2	8	92	5.5	41.7	220	56
P3	4	33	1.2	54.9	160	38
P4	0	0	0	0	0	0

The data represent mean of six replications. Culture medium: MS + BA 5.0 mg l^{-1} + Sucrose 70 g l^{-1}.

* The data represent mean of twelve replication



A. Seven days after inoculation



B. Twenty one days after inoculation

Plate 10. Germinating microrhizomes under *in vitro* condition

Treatment P2 (8 h light) was superior to all other treatment with regard to number of cultures with microrhizome, number of microrhizome per culture vessel, fresh and dry weight of microrhizome. In P4 microrhizome production was completely inhibited. Moreover, plantlets grown in P4 (0 h light) developed etiolated and rudimentary leaves with upright thin stem.

Microrhizome initiation was earliest (41.7 days) when cultured in P2 (8 h light) and late (54.9 days) in P3 (4 h light).

Treatment P2 (8 h light) registered highest percentage (92%) of cultures with microrhizome induction and maximum number of microrhizome per culture (5.5). Minimum number of microrhizome per shoot (1.2) and lowest percentage (33%) of cultures with microrhizome was recorded by P3 (4 h light). Highest fresh weight (220 mg) and dry weight (56 mg) of microrhizome was recorded by P2 (8 h light) and lowest fresh weight (160 mg) and dry weight (38 mg) was recorded by P3 (4 h light). There was an increase in the number of cultures with microrhizome and number of microrhizome per culture vessel, fresh and dry weight of microrhizome and size of microrhizome with the increase in the photoperiod up to 8 h light and decreased thereafter.

4.5 Germination and survival of microrhizomes

Microrhizomes were germinated both *in vitro* and *ex vitro*. Three different sized viz. small (0 -75 mg), medium (75 -150 mg) and large (>150 mg) microrhizomes were used for germination. Germination and survival rate varied among the treatments. Results of the study are presented in Table13.

During *in vitro* germination, harvested microrhizomes transferred to shoot multiplication medium under aseptic conditions showed 83.3 per cent regeneration and survival.

Under *in vitro* conditions most of the microrhizomes regenerated, and regeneration of microrhizomes was independent of size and weight. Larger microrhizomes recorded maximum length of shoots (30 mm), highest rate of shoot growth (10 mm $week^{-1}$), maximum fresh weight (160 mg) and dry weight (40 mg) of shoot. Minimum length of shoots (5 mm), lowest rate of shoot growth

A. Seven days after sowing

B. Fourteen days after sowing

C. Twenty eight days after sowing

Plate 11. Germinating microrhizomes under *ex vitro* condition

Table 13. Morphological parameters of *C. aromatica* plants regenerated *in vitro* and *ex vitro* from different sized microrhizomes

Sl. No.	Parameters	Small microrhizome (0 -75 mg)		Medium microrhizome (75 -150 mg)		Large microrhizome (>150 mg)	
		in vitro	*ex vitro*	*in vitro*	*ex vitro*	*in vitro*	*ex vitro*
1	Regeneration of microrhizomes* (%)	83.3	58.3	83.3	83.3	83.3	91.6
2	Survival (%)	83.3	16.6	83.3	58.3	83.3	75.0
3	Number of shoots	2.0	1.0	3.1	1.3	6.0	2.0
4	Length of shoots (mm)	5.0	2.0	18.0	27.0	30.0	39.0
5	Rate of shoot growth (mm week^{-1})	1.7	0.5	6.0	7.0	10.0	10.0
6	Number of roots	-	1.3	1.0	2.3	2.0	4.0
5	Length of roots (mm)	-	18.0	9.0	27.0	15.0	4.1
6	Number of leaves	-	0.05	1.0	1.0	1.3	1.8
7	Fresh weight of shoot (mg)	20.0	80.0	70.0	130.0	160.0	200.0
8	Dry weight of shoot (mg)	5.0	20.0	17.0	30.0	40.0	50.0

The data represent mean of 12 replications.

Observations taken at 28 days after inoculation and 28 days after planting respectively.

*Regeneration of microrhizomes: Microrhizomes initiating shoot growth

Plate 12. Plants germinated from small sized, medium sized and bigger sized microrhizome

(1.7 mm week $^{-1}$), minimum fresh weight (20 mg) and dry weight (5 mg) of shoot was recorded by smaller sized microrhizomes.

Under *ex vitro* conditions regeneration of plantlets from smaller (0 -75 mg) microrhizomes was very low (58.3 %). Regeneration of medium sized (0 - 150 mg) microrhizomes was higher (83.3 %) and that of larger (>150 mg) microrhizomes was the highest (91.6 %). Moreover, the survival of regenerated plantlets was dependent on the size of the microrhizome. Only few plantlets regenerated from small microrhizomes survived (16.6 %), whereas most of the plantlets regenerated from medium-sized (58.3 %) and large (75 %) microrhizomes survived. During *ex vitro* germination also the plantlets regenerated from large microrhizomes grew faster and were more vigorous in terms of their shoot, root and leaf growth parameters. Maximum number of shoots (2.0), maximum length of shoots (39 mm), highest rate of shoot growth (10 mm week^{-1}), maximum number of roots (4.0), maximum length of roots (4.1 mm), maximum number of leaves (1.8), maximum leaf length (36 mm) maximum leaf width (15 mm), maximum fresh weight (200 mg) and dry weight (50mg) of shoot was recorded by large sized microrhizome

Discussion

DISCUSSION

The present project titled "*In vitro* production of microrhizomes in *Curcuma aromatica* Salisb." conducted during 2009 – 2011 at the Department of Plantation Crops and Spices and Department of Plant Biotechnology, College of Agriculture, Vellayani has resulted in standardization of rapid *in vitro* shoot multiplication methods and standardization of method(s) for *in vitro* production of microrhizomes in *Curcuma aromatica* Salisb. The results of the study are discussed in this chapter.

5.1 *IN VITRO* MULTIPLICATION

Curcuma aromatica Salisb. is conventionally propagated using seed rhizome bits having at least one viable bud. In this method of propagation, there is risk of transmitting soil borne diseases from one crop to the other crop and the seed rhizomes need proper preservation. Moreover they are prone to rhizomes rot caused by bacteria, fungi and infestation by pest. No alternative mode of multiplication is available to propagate and conserve genetic stock of this valuable crop. *In vitro* culture technique has been reported to be an effective tool not only for rapid and mass multiplication of plant species, but also for producing pathogen free planting materials.

5.1.1 Factors influencing shoot proliferation

The type of organs or explants chosen affects the successful establishment of the cultures and their subsequent growth. Not all tissues or organs of a plant are equally capable of exhibiting morphogenesis (Hartmann *et al.*, 1997).

In the present study, rhizome bud sprouts excised from sprouting rhizomes of *Curcuma aromatica* were used as explants. Micropropagation using rhizome bud explants has been reported as an ideal method in turmeric propagation (Nayak and Naik, 2006).

Murashige (1974) reported that shoot tips are highly regenerative. Excised apex when placed on a medium with high inorganic nutrient salt, further

development of the terminal meristem is inhibited resulting in axillary bud growth, proliferating into more number of small shoots (Hackett and Anderson, 1967).

The difficulty in establishing contamination free *in vitro* cultures from rhizome buds has been reported by many workers (Dekker *et al*., 1991; Nadgauda *et al*., 1978; Salvi *et al*., 2002; Shirgurkar *et al*., 2001; Sunitibala *et al*., 2001; Yasuda *et al*., 1988). Balachandran *et al*. (1990) also reported difficulty in establishment of contamination free cultures of explants taken from underground rhizomes. In the present investigation the survival percentage of *in vitro* culture from rhizome bud sprout was 87 per cent.

The growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators added in the medium and growth substance produced endogenously by the cultured cell. The ratio of auxin and cytokinin decides the morphogenesis or organ formation. High concentration of auxin promotes rooting, whereas, proportionately more amount of cytokinin promotes bud or shoot formation. This concept of hormonal control of organ formation was proposed by Skoog and Miller (1957). Proper combination of cytokinin and auxin formulations have shown to be critical for shoot growth and elongation in many other plant species for their *in vitro* propagation (Rout *et al*., 2000; Karim *et al*., 2003; Skala and Wysokińska, 2004; Nair and Reghunath, 2009).

Based on this theory, different combinations of cytokinins and auxin were tested to find out their combined effect on shoot proliferation. BA, Kn, IAA and NAA at various concentrations and combination were tried in the present study to standardize their optimum levels for shoot proliferation. In *C. aromatica*, the best hormone for shoot proliferation was found to be BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1} with respect to earliness in response, shoot number, shoot length and number of leaves per shoot.

The results revealed that multiple shoot formation was more with BA compared to Kn. This is in confirmation with the results of Zamora *et al.* (1986)

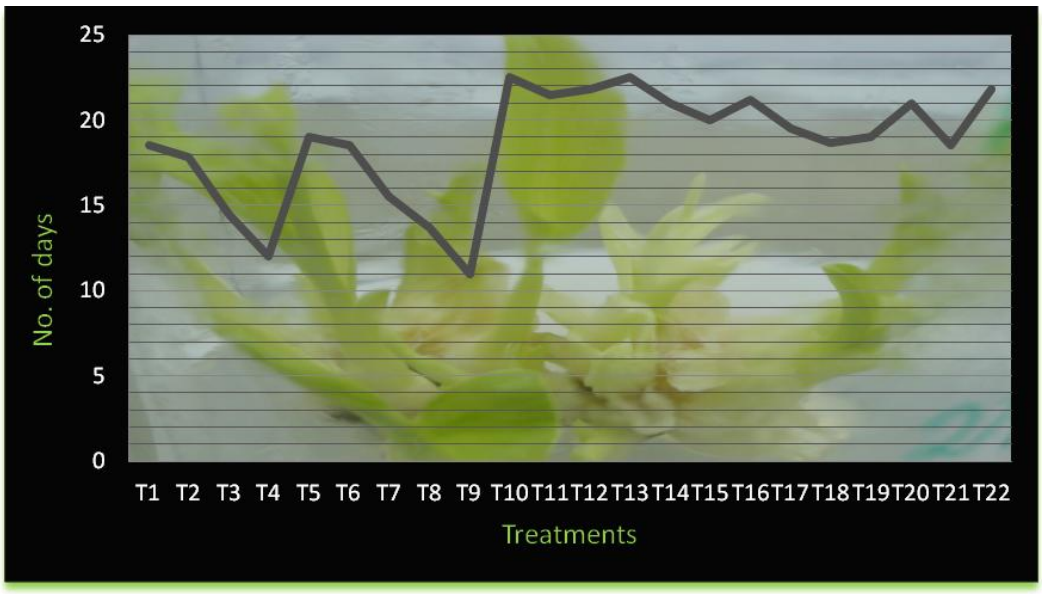


Fig 1. Effect of auxins and cytokinin on number of days taken for *in vitro* culture establishment in *C. aromatica*

Fig 2. Effect of auxins and cytokinin on shoots per culture vessel and length of the longest shoot during *in vitro* shoot proliferation in *C. aromatica*

who observed that in banana BA is the cytokinin of choice for induction of shoot bud proliferation *in vitro* and BA is superior to Kn.

In the present study, there was increase in shoot multiplication rate from 1.2 to 12.4 with increase in the concentration of BA from 1.0 mg l^{-1} to 5.0 mg l^{-1}. The highest concentration of cytokinin produced the highest number of shoots, which is in conformity with the results obtained by Nayak (2000) who reported that shoot multiplication and plant regeneration was achieved in *C. aromatica* on MS medium supplemented with BA alone (1.0 - 7.0 mg l^{-1}) or a combination of BA (1 - 5 mg l^{-1}) and Kn (0.5 mg l^{-1}). Similar, observations were made by Tule *et al.* (2005) in turmeric.

The addition of 0.1 mg l^{-1} NAA along with BA had shown a positive influence on the number of shoots produced per culture than the cultures with BA alone. This may be due to the reason that auxins at lower concentration have synergistic effect with cytokinin (Sunitibala *et al*. (2001) and Kambaska *et al.* (2010). A high concentration is inhibitory while a low concentration is stimulatory. Similar observations were made by Borthakur and Bordoloi (1992) who recorded synergistic effect of NAA in combination with BA on promotion of shoot multiplication of *Cucurma amada*. Hoang *et al.* (2005) also obtained more number of shoots of *Curcuma zedoaria* on MS medium with 3.0 mg l^{-1} BA and 0.5 mg l^{-1} IBA.

The length of micro shoots was also influenced significantly by the growth regulators used. Shoot length was higher in MS medium supplemented with BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1}. Similar results of greater length of shoot were obtained by Sharma and Singh (1995), Nayak (2000), Prakash *et al.* (2004) and Kambaska *et al*. (2010). Kambaska *et al*. (2010) reported that the quality of shoots and overall growth response in terms of average shoot length was better in growth regulator combination of BA 2.0 mg l^{-1}+ NAA 0.5 mg l^{-1} in *C. longa* cv Ranga. The increase in shoot length arises due to cell multiplication, cell elongation and cell division which is the outcome of the higher level of endogenous cytokinin developed in the micro shoots.

Besides producing more number of shoot and greater shoot length, MS medium supplemented with BA 5.0 mg l^{-1} + NAA 0.1 mg l^{-1} recorded more number of leaves indicating its superiority over other treatments in obtaining more number of quality shoots, which can be used either for producing plantlets or for microrhizome induction.

In the present investigation cultures were maintained by constant subculturing at an interval of 4 weeks and the rate of multiplication increased after the first subculture and 24.4 shoots per culture was obtained during subsequent subcultures in BA 5.0 mg l^{-1} + NAA 0.1 mg l^{-1}. The increase in the capacity for shoot formation after the first subculture and subculturing at an interval of four weeks was also reported by Nadgauda *et al* (1978).

The basal medium requirement depends upon the plant species and the purpose of culturing cell, tissue or organ. In the present study full MS was used. The beneficial effect of MS medium fortified with BA for multiple shoot production from fresh rhizome sprouts of *C. aromatica* was already reported by Nayak (2000). This is in agreement with the findings of Balachandran *et al*. (1990), Winnaar *et al*.(1989), Mogor *et al*.(2003), Ali *et al*.(2004) and Tule *et al*.(2005) in turmeric.

Plant cells and tissues in the culture medium lack autotrophic ability and therefore need external carbon for energy. In the present study 3 per cent sucrose was used as carbon source. Nayak (2000) also reported use of 3 per cent sucrose as carbon source for shoot multiplication in *C. aromatica.* This is in agreement with the findings of Mello *et al.* (2001) in *C. zedoaria*.

5.1.2 Rooting

5.1.2.1 In vitro Rooting

Efficient rooting of *in vitro* regenerated plants and subsequent field establishment is the last and crucial step of rapid clonal propagation. An efficient rooting treatment yields a high percentage of rooted shoots and a high quality root system. The latter involves number of roots per shoot and length of roots

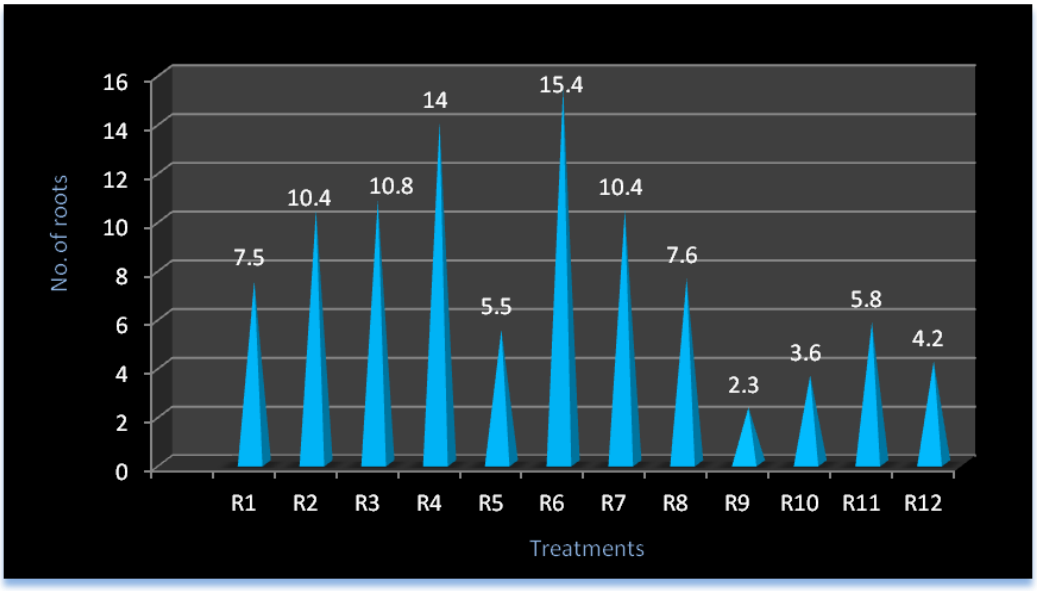


Fig 3. Effect of auxins on the production of roots per shootlet in *C. aromatica*

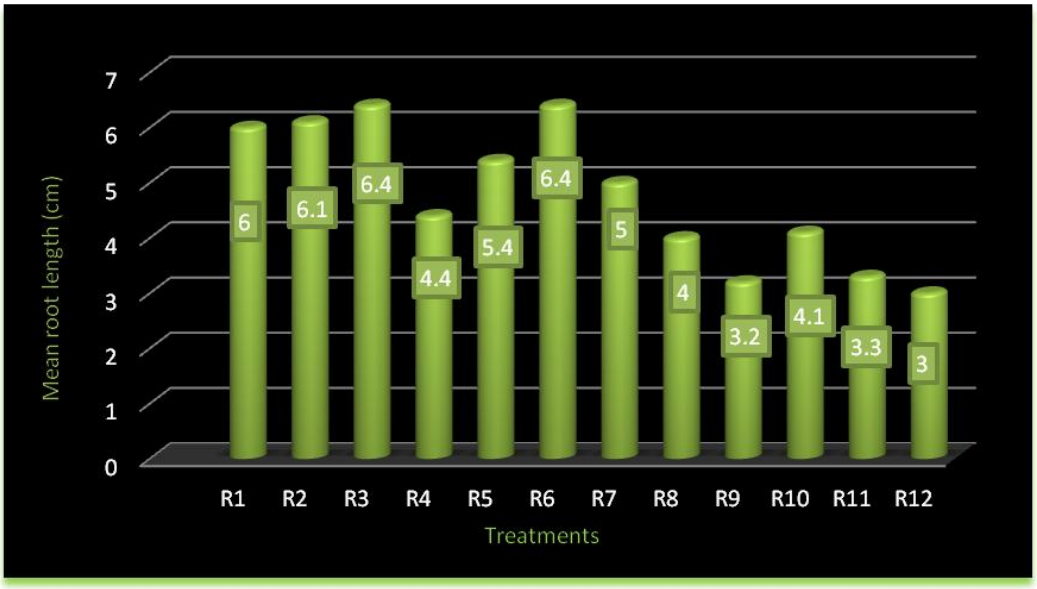


Fig 4. Effect of auxins on the mean root length in *C. aromatica*

which determines the successful establishment of regenerated plants in soil. In general, *in vitro* root formation is inhibited by cytokinin and therefore auxin has to be supplemented for better and easy rooting (George and Sherrington, 1984).

Roots are produced in the presence of a suitable auxin. Various auxins differ in their physioiogical activity and the extent to which they move within tissues or bound within cells or metabolized (Razdan, 2004). Auxins frequently used for rooting are IAA, IBA and NAA (George and Sherrington, 1984).

In the present study, varying levels of NAA (0.1 to1.0 mg l^{-1}), IBA (0.1 to 1.0 mg l^{-1}) and IAA (0.1 to1.0 mg l^{-1}) were tried. Half strength MS medium with IBA 0.2 mg l^{-1} favoured best rooting with regard to number of roots and root length. Rahman *et al.,* (2004) also reported that half strength MS medium with IBA 0.1-1.0 mgl^{-1} was best for rooting of shoots in *Curcuma longa*. The findings are in agreement with those observed in other rhizomatous plant species such as ginger (Hague *et al.,* 1999) and *Alpinia calcarata* (Amin *et al.,* 2001). The quick rooting and production of more number of roots in the regenerated shoots might be due to the quick absorbance of IAA and quicker enzymatic degradation in the plant tissues.

5.1.2.2 Planting out and acclimatization

The benefit of any micropropagation system can be fully realized by the successful transfer of plantlet from tissue culture vessels to the ambient conditions found *ex vitro*.

The significance of a gradual acclimatization process of *in vitro* plants is to enable favourable structural and physiological changes to take place which make them fit for *ex vitro* survival as emphasized by Rout *et al.* (2006). Most species grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil (Hazarika, 2003). Mortality is mainly because of the inability of *in vitro* derived plantlets to get adapted to the outside environment. Wainwright (1988) reported that low relative humidity, higher light levels and more variable temperatures

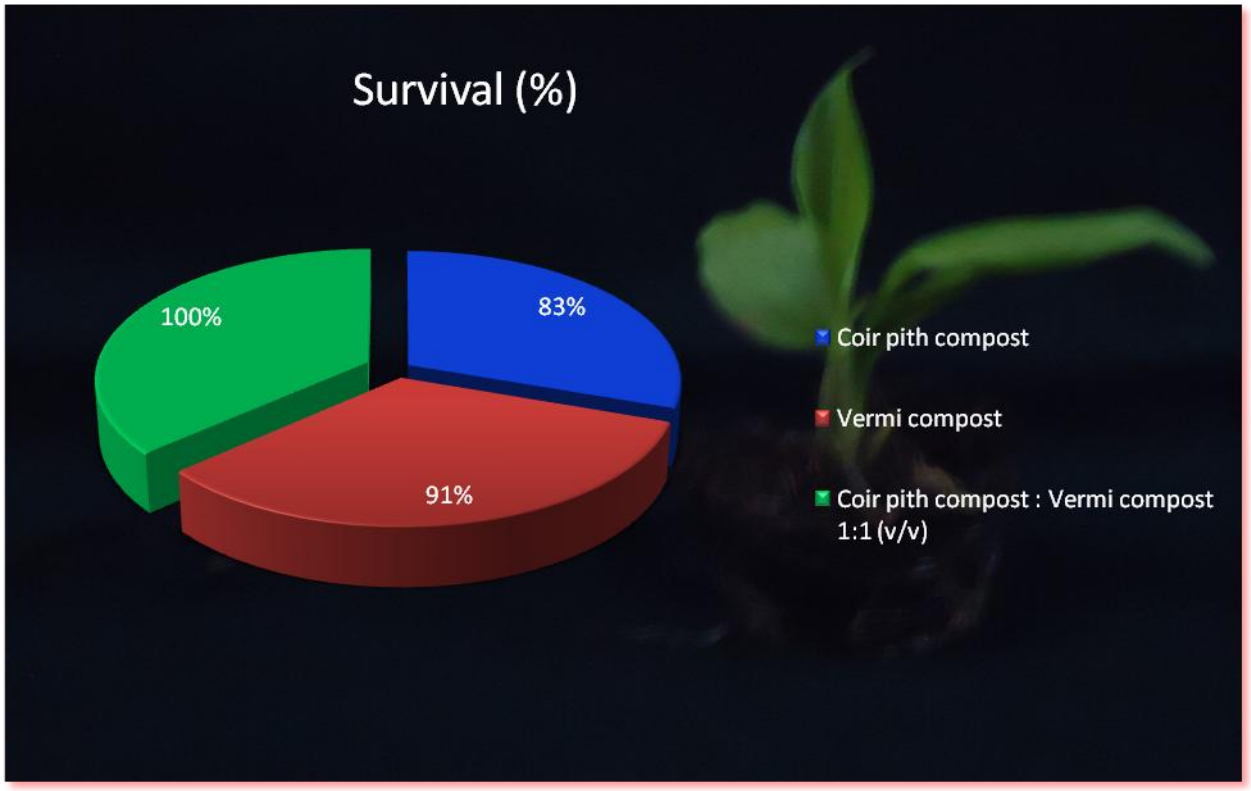


Fig 5. Effect of different potting media on *ex vitro* establishment of *in vitro* generated plants of *C. aromatica*

prevailing outside are the detrimental factors for tissue culture plants when taken *ex vitro*. Mortality is also attributed to heavy water loss (Conner and Thomas, 1981) and reduction or absence of epicuticular wax on the leaves (Sutter and Langhans, 1982). Since both the anatomy and morphology of leaves and the physiology of the *in vitro* derived plantlets are different from those of normal plants, it is important to consider these differences while acclimatizing them.

In order to maximize the survival of *in vitro* derived plants, it is routine practice to acclimatize them under high levels of relative humidity (Short, 1991). A better hardening condition can increase survival, quality of plantlets and reduces the number of days required for hardening.

In the present investigation to study the effect acclimatization on survival of *in vitro* raised plants. Mixture of coir pith compost and vermi compost 1:1(v/v) was the ideal medium registering cent per cent survival rate. The good water holding capacity coupled with optimal porosity could have helped the plantlets to establish better in this medium. Also, vermicompost in the potting media might have stimulated the biochemical activity and nutrient cycling and thereby increase in the growth of vegetative characters. These findings are in line with that of Yuvaraj (2008) who reported highest per cent survival of plantlets of banana when hardened on sterile soil in combination with coir compost and vermicompost (1:1:1).

5.1.2.3 Field establishment

In the present investigation twenty hardened tissue culture plants of *C. aromatica* were planted in the field to assess the field survival rate and record their morphological characters. *In vitro* produced *C. aromatica* plants recorded 100 per cent field survival. Cent per cent survival of *in vitro* produced plants of *Curcuma* spp. and ginger in *ex vitro* situation was previously reported by Balachandran *et al.* (1990). Kambaska *et al.* (2010) and Singh *et al.* (2011) reported 95 per cent survival during field transplantation in *C. longa*. The *in vitro* multiplied *C. aromatica* plants were healthy, vigorous and morphologically uniform resembling its parent variety.

In this study, methods for rapid *in vitro* multiplication of shoots in *Curcuma aromatica* Salisb have been standardized. This has made possible the availability of pathogen free and quality planting materials for year round planting for mass multiplication. The rapid *in vitro* multiplication methods standardized in this study can be effectively made use for microrhizome induction, genetic transformation and biotechnological methods of crop improvement.

5.2 MICRORHIZOME INDUCTION

Microrhizomes are miniature rhizomes developed at the base of shoots under rhizome inducing conditions *in vitro*. Production of microrhizomes *in vitro* has many advantages when compared to *in vitro* plantlet production. Microrhizomes can be produced year round irrespective of season and can be scheduled as per requirement. Micropropagated plants need acclimatization under controlled humidity and light conditions for few days and maintenance in a greenhouse till planted out. Transportation of plantlets to far off places is difficult. On the other hand, microrhizomes harvested from *in vitro* conditions can be directly transferred to the field without any acclimatization or hardening. They are disease free seed rhizomes; it could be stored and transported easily for planting/ further multiplication. Microrhizome induction has been reported to be an effective tool not only for production of good quality pathogen free planting material but also for the production of miniature rhizomes which can be stored and transported and hence can be utilized for germplasm conservation.

5.2.1 Factors influencing microrhizome induction

Factors such as nutrient composition of basal medium especially sucrose concentration, plant growth regulators and culture conditions like photoperiod and temperature have been reported to play important roles in *in vitro* induction of storage organs such as rhizomes, tubers, bulbs and corms. In the present investigation effect of cytokinins (BA and Kn), sucrose concentration and photo period has been explored.

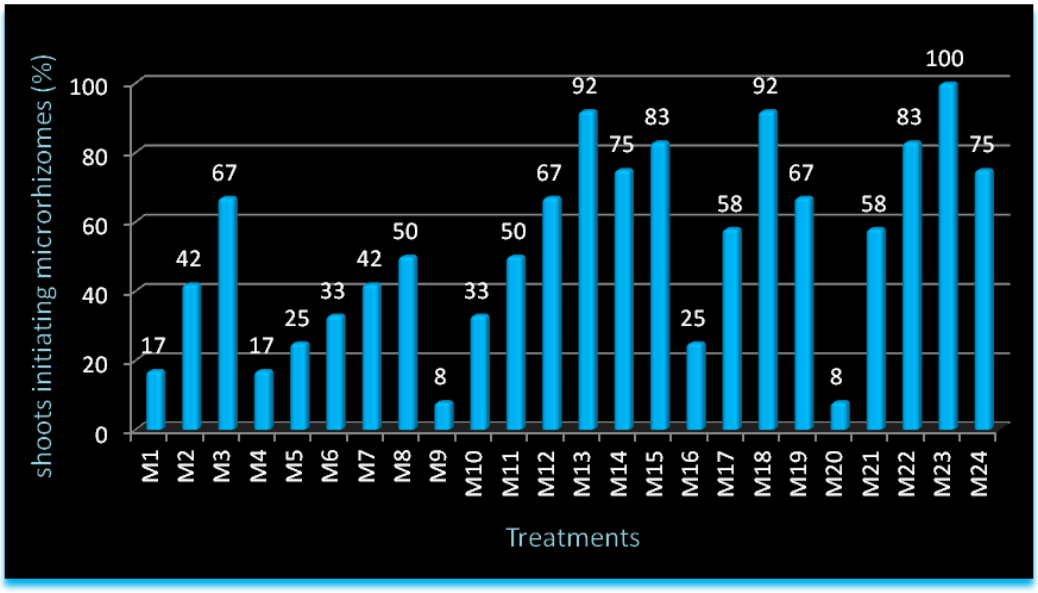


Fig 6. Effect of cytokinins on per cent of shoots initiating microrhizome in *in vitro* generated shoots of *C. aromatica*

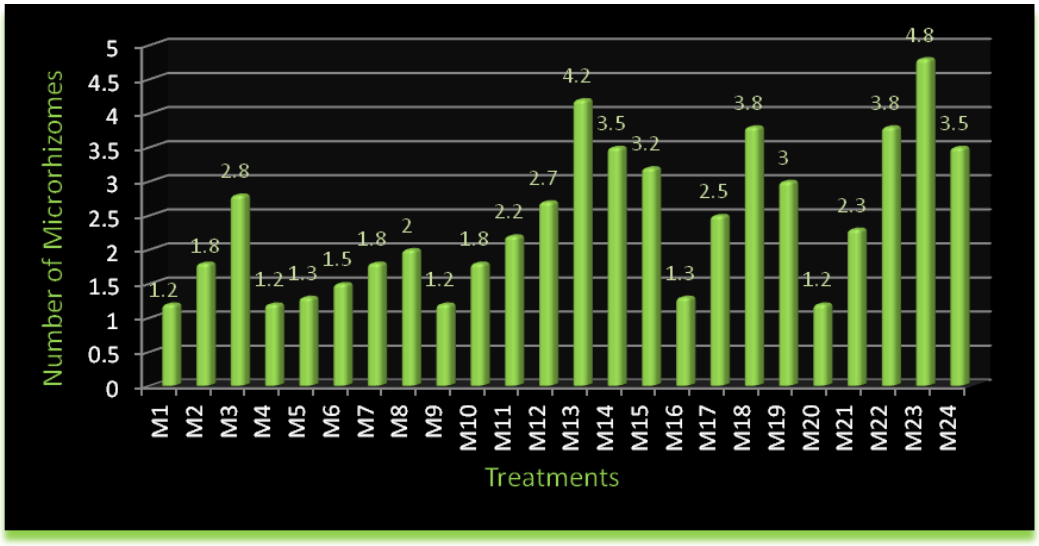


Fig 7. Effect of cytokinins on number of microrhizomes/ culture vessel in *in vitro* generated shoots of *C. aromatica*

5.2.1.1 *Plant growth substances*

The results indicate that growth regulators play an important role in microrhizome induction; auxin and cytokinin regulate not only vegetative growth, but also facilitate cell division and sprouting. Cytokinin influence a multitude of morphological and physiological processes, cell division and cell elongation, control of apical dominance, delayed senescence and morphology of cultured tissues. This is due to influence of cytokinin in mobilization of carbohydrates.

In the present study, BA was found to be more effective than Kn alone or Kn in combination with BA for microrhizome induction. The highest number of microrhizome per culture vessel (4.8), maximum fresh weight (247 mg) and dry weight (65 mg) of microrhizome and larger rhizomes were noticed in MS media containing 5.0 mg l^{-1} BA. This is in agreement with Nayak (2000), who reported 5.0 mg l^{-1} BA was the best hormone for microrhizome induction in *C.aromatica* and in *C. amada*. However, Shirgurkar *et al.* (2001) stated that BA had an inhibitory effect on *in vitro* microrhizome production in turmeric. Anisuzzaman *et al.* (2008) and Sharma and Singh (1995) also reported that 4.0 mg l^{-1} BA in *C. zedoaria* and 8.0 mg l^{-1} BA in ginger, respectively enhanced mircorhizome production.

5.2.1.2 *Sucrose*

Sucrose has been found to promote formation of various storage organs (bulbs, croms and tuber) in most of the cases investigated by Abbott and Belcher (1986), Arora *et al.* (1996) and Grewal (1996). The enhanced rate of *in vitro* microrhizome formation with increasing concentration of sucrose may be attributed to the presence of high carbon energy in sucrose, since rhizomes mostly contain carbohydrates and sucrose. It was observed that sucrose plays a significant role in the induction of microrhizomes in *C. aromatica.*

In the present investigation, 70 g l^{-1} sucrose recorded 92 per cent of cultures induced with microrhizome and highest number (5.5) of microrhizomes

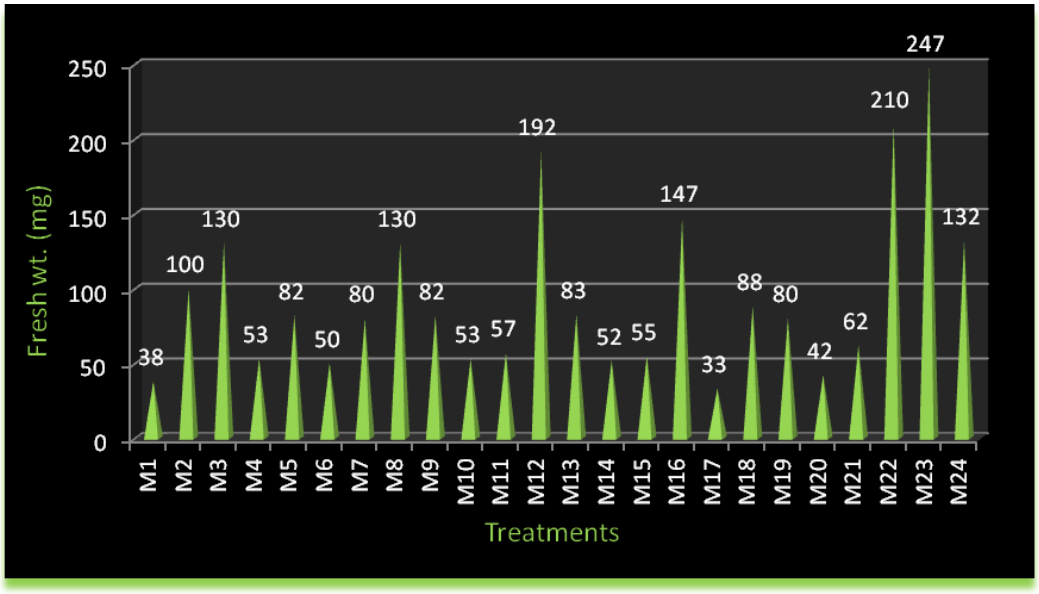


Fig 8. Effect of cytokinins on fresh weight of microrhizomes in *in vitro* generated shoots of *C. aromatica*

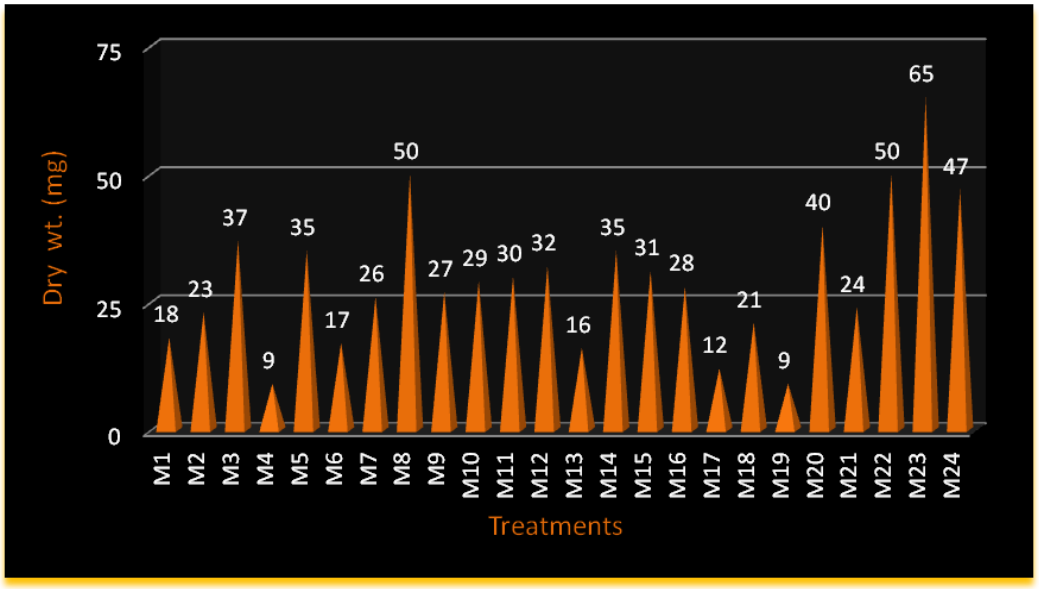


Fig 9. Effect of cytokinins on dry weight of microrhizomes in *in vitro* generated shoots of *C. aromatica*

per culture vessel. The microrhizome production was also earliest (37 days) in the same media. Even though maximum fresh weight (260 mg) and dry weight (140 mg) of microrhizome was observed in media supplemented with 80 g l^{-1} sucrose there was a decrease in their number. The lowest number of rhizomes were induced *in vitro* in the MS medium containing a normal amount of sucrose *i.e.* 30 g l^{-1}. However, both lower (0 – 2 %) and higher concentrations of sucrose had inhibitory effects on microrhizome production.

The results of the present investigation support the reports of Shirgurkar *et al.* (2001), Sunitibala *et al.* (2001) and Nayak (2000) who obtained optimum micro-rhizome induction in *Curcuma* using 6 – 9 per cent sucrose. They also stated that a lower concentration of sucrose decreased the size and number of rhizomes or even completely prevented the induction of any microrhizomes. Shirgurkar *et al.* (2001) obtained the highest number of microrhizomes (5.6 ± 0.8 - 7.0 ± 1.1) at 6 per cent sucrose, while at 8 % sucrose they found reduction in the number (5.6 ± 0.5 – 5.8 ± 1.1) but a slight increase in their size. Nayak (2000) observed that at an obligatory concentration of sucrose (3 %), plants could not develop any microrhizomes even by increasing the concentration of BA from 1.0 to 7.0 mg l^{-1} or by increasing the duration of the photoperiod. Sharma and Singh (1995) reported that a concentration of 7.5 % was required for *in vitro* rhizome induction in ginger. Sedigeh *et al.* (1998) reported that 6 - 8 per cent sucrose (w/v) was the single most significant constituent for *in vitro* microtuber formation in yam, *Diascoria composita.* As a carbon and energy source, sucrose provides a carbon skeleton and energy for shoot and microrhizome induction in turmeric Nayak (2000).

5.2.1.3 *Photoperiod*

In the present investigation of the effect of various durations of photoperiod i.e. 16 h, 8 h, 4 h and 0 h (dark) on the formation of microrhizomes revealed that microrhizome formation in *C. aromatica* was better when relatively a reduced photoperiod i.e. (8 – 0 h) was provided along with an elevated level of sucrose 60 g l^{-1} in the presence of BA in the medium. Overall response in the

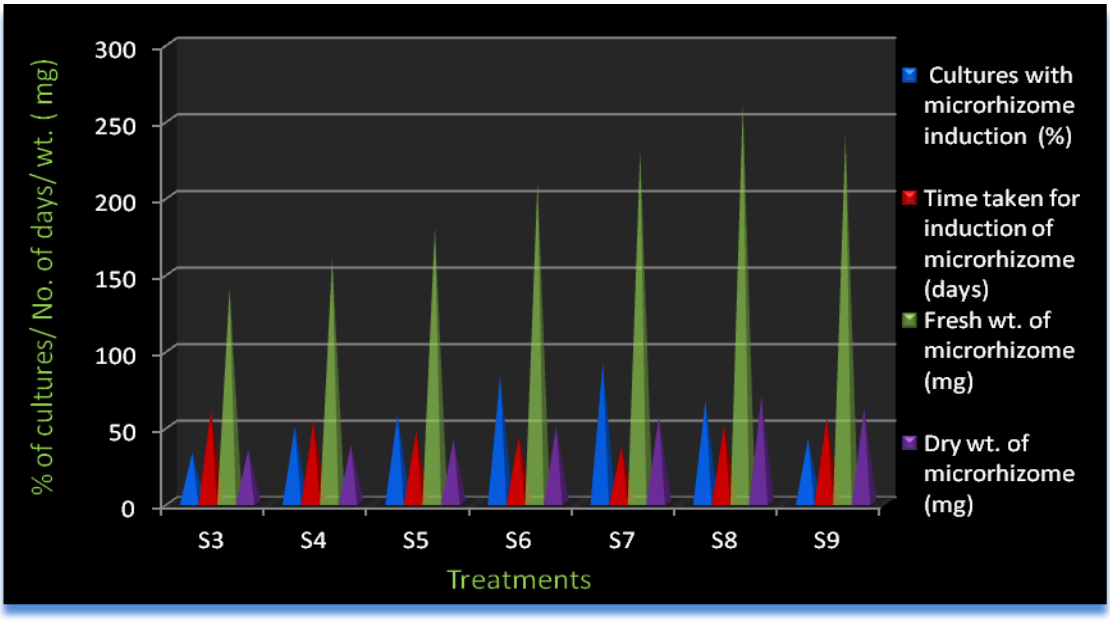


Fig 10. Effect of sucrose on cultures with microrhizome induction, time taken for induction of microrhizomes and fresh and dry weight of microrhizomes

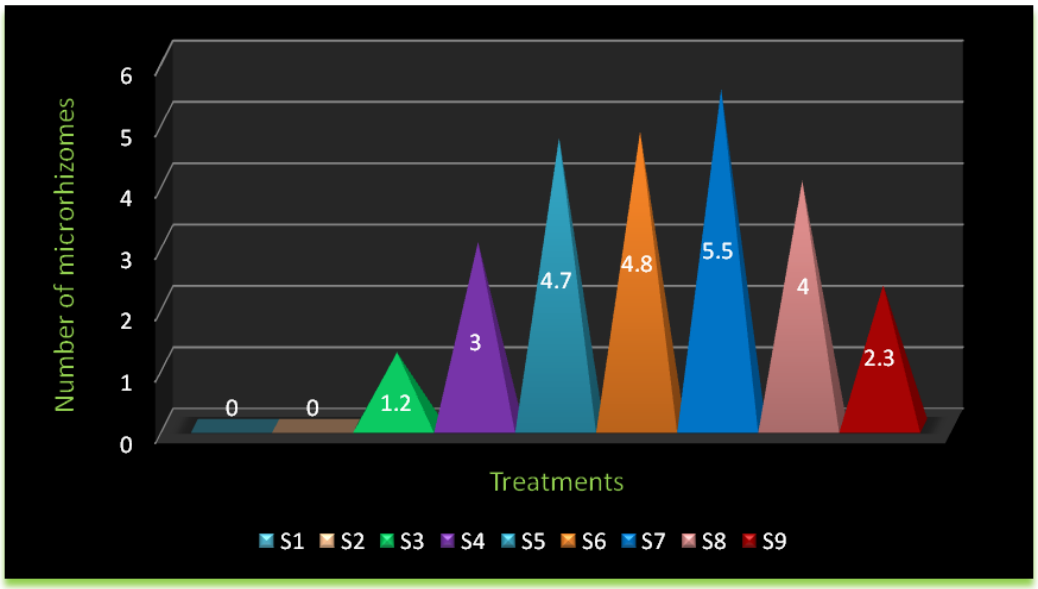


Fig 11. Effect of sucrose on number of microrhizomes induced per culture vessel

formation of microrhizomes was, however, better in cultures grown in 8 h photoperiod than in 0 h photoperiod. Moreover, plantlets grown in the dark developed etiolated and rudimentary leaves with upright thin stems. This result showing optimum formation of microrhizomes in *C. aromatica* with relatively lesser photoperiod (8 h) is contradictory to that reported in ginger. Though the role of different lengths of photoperiod in microrhizome formation in ginger has not been elucidated in detail, Sakamura *et al.* (1986) reported that microrhizomes were induced in the presence of continuous light whereas Sharma and Singh (1995) reported formation of microrhizomes in ginger in complete darkness. This differential requirement of photoperiod for microrhizome induction in ginger may be attributed to genotypic differences. The present result ie., requirement of relatively lesser photoperiod (8 h) for microrhizome formation in *C. aromatica,* can be compared with that obtained for microtuberisation in potato by Abbott and Belcher (1986).Their study on the role of different lengths of photoperiod showed that short day length (8 h photoperiod) was optimum for formation of microtubers *in vitro*.

5.2.2 Germination and survival of microrhizomes

Microrhizomes were germinated both *in vitro* and *ex vitro*. Plants germinated from microrhizomes were morphologically alike. Germinating rhizomes with freshly sprouted leaves were found to have a pleasant aroma characteristic to the species. Plants regenerated from larger microrhizomes (>150 mg) both *in vitro* and *ex vitro* were found to be more vigorous in terms of their shoot, root and leaf growth parameters. During *in vitro* germination 83.3 per cent regeneration and survival was obtained. Regeneration of microrhizomes was independent of their size and weight. During *ex vitro* germination, large (>150 mg) microrhizomes showed highest (91.6 per cent) regeneration. Similarly, Shirgurkar *et al.* (2001) reported that bigger microrhizomes were more competent and vigorous in the field and grew faster. However, they obtained a survival rate of 10.4, 54.7 and 73.9 per cent, respectively from small (0.1 - 0.4 g), medium (0.41 - 0.8 g) and large (> 0.8 g) microrhizomes. Sharma and Singh (1995)

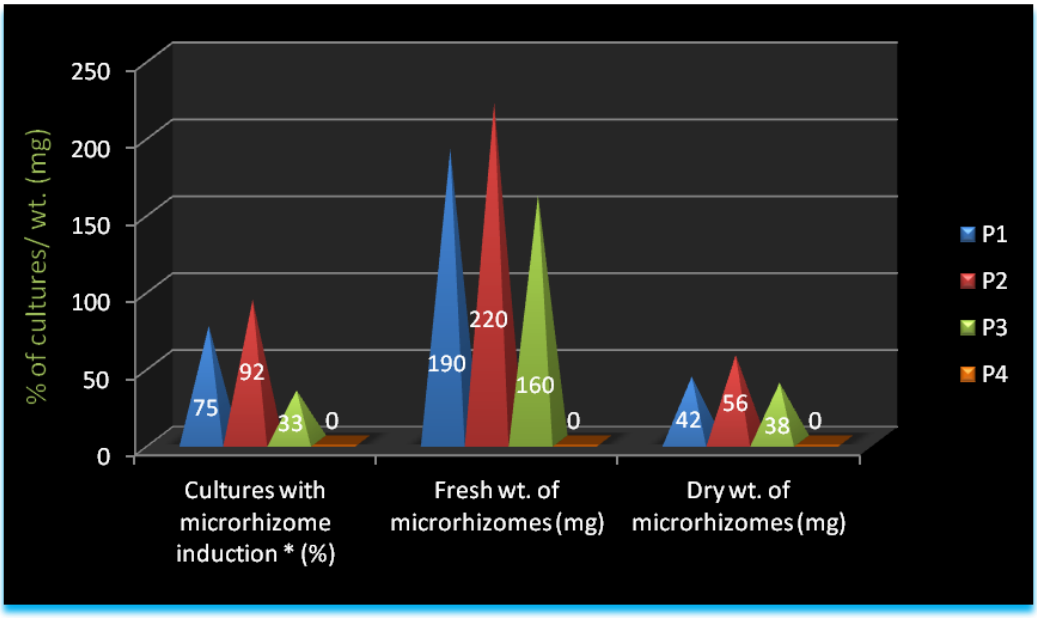


Fig 12. Effect of photoperiod on cultures with microrhizome induction, fresh and dry weight of microrhizomes

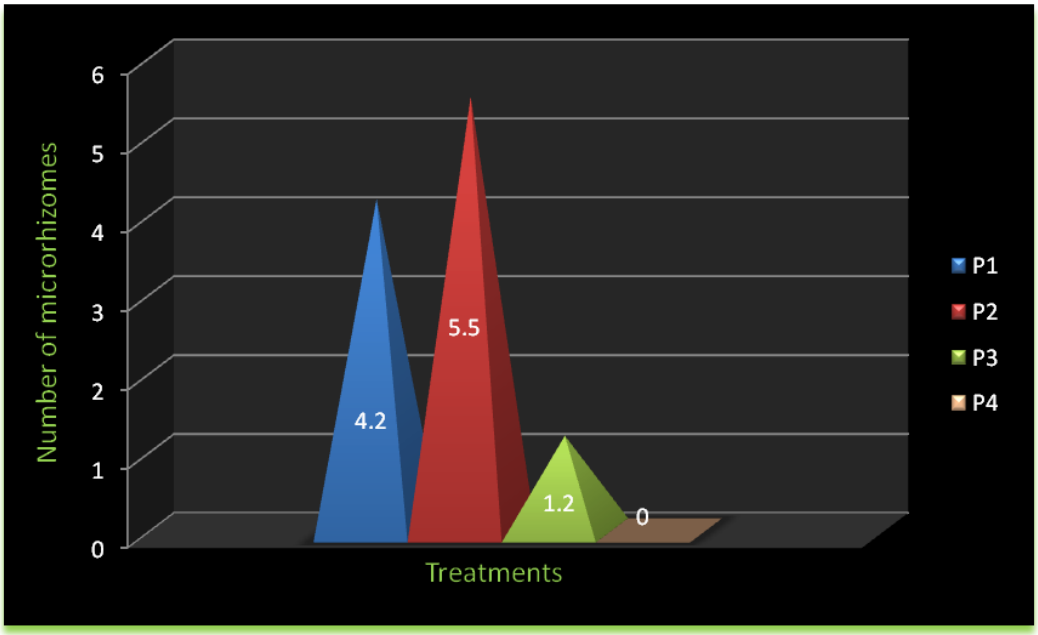


Fig 13. Effect of photoperiod on number of microrhizomes induced per culture vessel

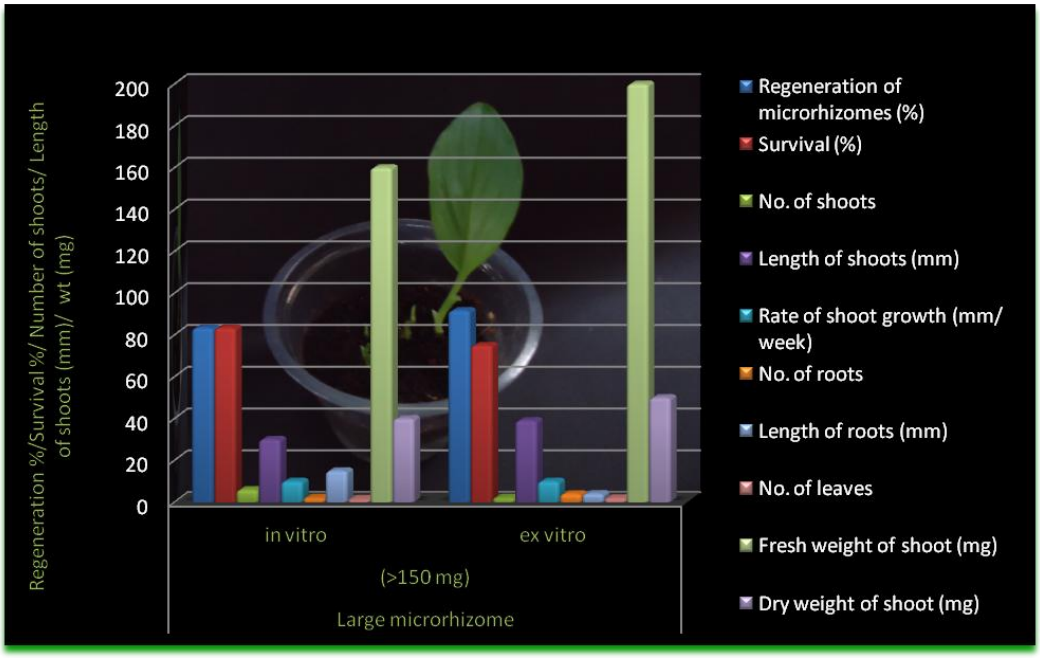


Fig 14. Morphological parameters of *C. aromatica* plants regenerated *in vitro* and *exvitro* from different sized microrhizomes

reported that microrhizomes can be stored under moist conditions at room temperature and that more than 80 per cent of the sprouted microrhizomes developed shoots and roots two months after they had been successfully transferred to the field.

In this study, methods for *in vitro* production of microrhizomes in *Curcuma aromatica* Salisb. have been standardized. This has made possible the availability of pathogen free, quality planting materials which can be used without acclimatization and which can be stored and transported easily. The methods for *in vitro* production of microrhizomes standardized in this study can be effectively utilized for biotechnological methods of crop improvement, germplasm exchange, transportation and germplasm conservation.

Summary

6. SUMMARY

The study, "*In vitro* production of microrhizomes in *Curcuma aromatica* Salisb." was carried out at the Department of Plantation Crops and Spices and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during the period from 2009 - 2011. The investigations were carried out in two phases' viz., *in vitro* shoot multiplication and microrhizome production phase in *Curcuma aromatica* Salisb. The results of the investigations are summarized below.

6.1 *IN VITRO* MULTIPLICATION

6.1.1 Factors influencing shoot proliferation

Rhizome bud sprouts were used as explants, gave good response with respect to *in vitro* culture establishment and shoot proliferation.

For *in vitro* multiplication studies, Bavistin (0.2 per cent for 30 minutes) and mercuric chloride (0.1 per cent for 12 minutes) were used for establishing contamination free cultures.

Among the cytokinin - auxin combinations tried, BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1} in MS medium recorded maximum shoot proliferation. Earliness in response, maximum shoot number, shoot length and number of leaves per shoot was obtained in full MS basal medium with BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1} and 3 per cent sucrose. Cultures were maintained by constant subculturing at an interval of four weeks. The effect of subculture on shoot multiplication was tested and the results indicated that the rate of multiplication over 24.4 shoots per culture was maintained in MS medium supplemented with BA 5.0 mg l^{-1}+ NAA 0.1 mg l^{-1}during subsequent subcultures.

6.1.2 Rooting

In the present study, varying levels of auxins, NAA (0.1 to 1.0 mg l^{-1}), IBA (0.1 to 1.0 mg l^{-1}) and IAA (0.1 to 1.0 mg l^{-1}) were tried for *in vitro*

rooting. Half strength MS medium with IBA 0.2 mg l^{-1} favoured best rooting with regard to percent of cultures initiating roots, number of roots and root length

Mixture of coir pith compost and vermi compost (1:1 v/v) was identified to be the best potting medium for planting out and acclimatization, registering 100 per cent survival rate.

The *in vitro* multiplied tissue culture plants of *C. aromatica* recorded 100 per cent field survival. The produced *C. aromatica* plants were healthy, vigorous and morphologically uniform.

6.2 MICRORHIZOME INDUCTION

6.2.1 Factors influencing microrhizome induction

Three to four cm long shoots generated from *in vitro* multiplication of *Curcuma aromatica* was used as explants gave good response for microrhizome induction.

Among the various combinations of plant growth substances (BA and Kn) used to study their effect on microrhizome induction in *Curcuma aromatica*, 5.0 mg l^{-1} BA was the best hormone. Full MS media containing 5.0 mg l^{-1} BA and 60g l^{-1} sucrose incubated at 16 h photo period was found significantly superior to all other media with regard to number of microrhizome per culture vessel, fresh weight and dry weight and size of microrhizomes.

With regard to different levels of sucrose influencing microrhizome induction, 70 g l^{-1} sucrose was ideal registering earliest induction, cultures with microrhizome induction and highest number of microrhizome per culture vessel. Maximum fresh wt and dry wt microrhizome was noticed at higher sucrose concentration of 80 g l^{-1}.

Out of the various durations of photo period tried, 8 h light was found better than others with respect to cultures with microrhizome induction, time taken for initiation of microrhizome, number of microrhizome per culture vessel. Maximum fresh wt and dry wt of microrhizome was also recorded by 8 h light.

6.2.2 Germination and survival of microrhizomes

Harvested microrhizomes were germinated both under *in vitro* as well as *ex vitro* conditions. Plants regenerated were morphologically alike and freshly sprouted leaves have a pleasant aroma characteristic to the species.

Out of the plants germinated *in vitro* from different sized microrhizomes, regeneration was independent of microrhizome weight. With respect to length of shoots, rate of shoot growth, fresh and dry weight of shoots, larger microrhizomes registered maximum value. The regenerated plantlets can be used for further *in vitro* multiplication in *Curcuma aromatica*

Among the plants germinated *ex vitro* from different sized microrhizomes, larger microrhizomes registered highest regeneration and survival per cent and were more vigorous in terms of their shoot, root and leaf growth parameters. The regenerated plantlets from *ex vitro* germinated microrhizomes can be used for field planting.

Future lines of work

The microrhizomes may be tested for their field performance, vis-a-vis micropropagated plantlets and conventional rhizome raised plants. The storability and dormancy of microrhizomes need to be studied.

Cryopreservation and slow growth techniques have to be standardized for conservation of germplasm. The possibility of encapsulated microrhizomes for propagation like a normal seed and long term conservation need to be explored.

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Abstract

IN VITRO PRODUCTION OF MICRORHIZOMES IN *Curcuma aromatica* Salisb.

by

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8. ABSTRACT

Investigations on "*In vitro* production of microrhizomes in *Curcuma aromatica* Salisb." was carried out at the Department of Plantation Crops and Spices and Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2009-2011. The objective of the study was to standardize the method(s) for *in vitro* production of microrhizomes in *Curcuma aromatica* Salisb. so as to utilize them for rapid propagation and conservation of germplasm.

The investigations were carried out in two phases viz., (i) *In vitro* shoot multiplication and (ii) Microrhizome production. IISR accession of *Curcuma aromatica* from the germplasm collection of the Department of Plantation crops and Spices, College of Agriculture, Vellayani was used for the study.

For *in vitro* shoot multiplication rhizome bud sprouts was used as explants. Sprouted rhizhome bud of *C. aromatica* treated with Bavistin 0.2 per cent for 30 minutes and mercuric chloride 0.1 per cent for 12 minutes registered the highest survival percentage (87%). Murashige and Skoog's medium supplemented with BA and NAA and agar 6.5 per cent was optimum for shoot multiplication. Maximum number of multiple shoots (12.4), and the longest shoot (6.03 cm) was obtained in 5 mgl^{-1} BA and 0.10 mg l^{-1} NAA.

For *in vitro* rooting, the shootlets produced *in vitro* were transferred to rooting media where half strength MS medium with IBA 0.2 mg l^{-1} favoured best rooting with regard to percent of cultures initiating roots (100%), number of roots (15.4) and root length (6.4 cm).

Mixture of coir pith compost and vermi compost (1:1 v/v) was identified to be the best potting medium for planting out and acclimatization, registering 100 per cent survival rate.

The *in vitro* multiplied tissue cultured plants were successfully established in the field. The plants were healthy and vigorous with cent per cent field survival and were morphologically uniform.

For microrhizome induction, three to four cm long shoots generated from *in vitro* shoot multiplication cultures were used as explants. Microrhizome formation was found to be controlled by the concentrations of cytokinins and sucrose as well as photoperiod during the culture.

BA 5.0 mg l^{-1} was identified as the best hormone for microrhizome induction with regard to number of microrhizome per culture vessel (4.8), fresh weight (247 mg) and dry weight (65 mg) of microrhizomes produced. Different concentration of growth regulators on microrhizome production showed that the number of shoots producing microrhizomes range between two to five.

Among the different levels of sucrose, 70 g l^{-1} was most effective for microrhizome induction as indicated by earliness in induction (37 days), maximum percentage (92 %) of cultures with microrhizome and highest number (5.5) of microrhizome per culture vessel. But maximum fresh weight (260 mg) and dry weight (70 mg) microrhizome was noticed at higher concentration of 80 g l^{-1}.

With regard to various durations of photo period used for microrhizome induction, eight hours light was found better than others with respect to percentage (92 %) of cultures with microrhizome, number (5.5) of microrhizome per culture and fresh (220 mg) and dry weight (56 mg) of microrhizome.

Harvested microrhizomes from *in vitro* culture were germinated both *in vitro* and *ex vitro*. During *in vitro* germination, regeneration of microrhizomes was independent of size and weight and registered 83.3 per cent regeneration and survival. But in *ex vitro* germination, regeneration of microrhizomes was dependent of size and weight and larger microrhizomes (>150 mg) registered highest regeneration percentage (91.6 %) and survival percentage (75 %). With regard to growth parameters larger microrhizomes (>150 mg) performed better both under *in vitro* as well as *ex vitro* conditions. They recorded maximum shoot length (30 mm), highest rate of shoot growth (10 mm $week^{-1}$), maximum fresh weight (160 mg) of shoot and dry weight (40 mg) of shoot during *in vitro* germination and highest rate of shoot growth (10 mm $week^{-1}$), maximum shoot number (20 mm), maximum root number (4.0), maximum shoot length (39 mm), maximum root length (4.1 cm), maximum fresh weight (200 mg) of shoot and dry weight (50 mg) of shoot during *ex vitro* germination.