COMMERCIAL PRODUCTION OF GINGER
(*Zingiber officinale* ROSC.) MICRORHIZOMES USING
TEMPORARY IMMERSION BIOREACTOR SYSTEM

By

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(2018-11-005)

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THESIS

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY
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2020

DECLARATION

I, hereby declare that this thesis entitled **'Commercial production of ginger (*Zingiber officinale* Rosc.) microrhizomes using temporary immersion bioreactor system'** is a bonafide record of research 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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Date: 23/10/2020

CERTIFICATE

Certified that this thesis entitled **'Commercial production of ginger (*Zingiber officinale* Rosc.) microrhizomes using temporary immersion bioreactor system'** is a record of research work done independently by **Ms. Rasha Fathima A. A.** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.
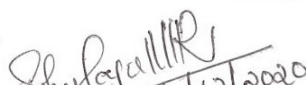
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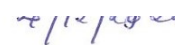(Member Advisory Committee)
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ABBREVIATIONS

%	Percentage
µg	Microgram
AFLP	Amplified Fragment Length Polymorphism
ABA	Abscisic Acid
Bp	Base pair
BAP	Benzyl amino purine
cm	Centimetre
CPBMB	Centre for Plant Biotechnology and Molecular Biology
CTAB	Cetyl Trimethyl Ammonium Bromide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribo Nucleoside Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
EtBr	Ethidium bromide
g	Gram
ha	Hectare
ISSR	Inter Simple Sequence Repeats
IAA	Indole acetic acid
IBA	Indole butyric acid
Kb	Kilo base pairs
L	Litre
M	Molar
mg	Milligram
ml	Millilitre
mM	Millimole
ng	Nanogram
NAA	1-Naphthalene acetic acid
ºC	Degree Celsius
OD	Optical Density

PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
PVP	Polyvinylpyrolidone
RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment LengthPolymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
Rpm	Revolutions per minute
SCoT	Start Codon Targeted Polymorphism
SSR	Simple Sequence Repeats
STS	Sequence Tagged Site
TAE	Tris Acetate EDTA
TE	Tris EDTA
TIB	Temporary immersion bioreactor
U	Unit
UV	Ultraviolet
V	Volts
β	Beta
μl	Microlitre

Introduction

1. INTRODUCTION

Ginger (*Zingiber officinale* Rosc.), is an important commercial spice crop grown in India. It is valued for its medicinal properties also. In India ginger is cultivated in an area of about 1,64,310 ha. with a production of 17,88,970 t. (www.indianspices.com). Ginger is vegetatively propagated through seed rhizomes. High seed rate of ginger (1500 kg/ha) and desiccation of seed rhizomes during storage are the problems faced by farmers in ginger cultivation. The tissue culture plants are not commercially distributed in ginger as they require an additional one more season for rhizome formation. Microrhizomes of ginger induced *in vitro* if used as planting materials, rhizome can be harvested in the same season as conventional seed rhizomes and year-round availability of seed material can be ensured. Hence, *in vitro* induced microrhizomes are included in the seed chain of ginger and there is high demand for microrhizomes for clean ginger production.

Induction of microrhizomes in ginger was reported by several researchers (Sharma and Singh, 1995; Zheng *et al.,* 2008; Singh *et al.,* 2014; Mehaboob *et al.,* 2019; An *et al.,* 2020; Manjusha, 2020). Kerala Agricultural University has released three high yielding, high quality ginger varieties (Shylaja *et al*., 2010; Shylaja *et al*., 2014). The protocol for *in vitro* induction of microrhizomes in the three KAU released ginger varieties was developed at CPBMB (Shylaja *et al*., 2016). The management practices for growing ginger microrhizomes in high tech polyhouses were also developed at CPBMB (Shylaja *et al*., 2018).

Temporary Immersion Bioreactor system (TIB) is a recent technique evolved in micropropagation which is semi automated and uses liquid medium. The system has many advantages over the conventional solid culture system as it incorporate the benefits of both solid and liquid system and the air exchange helps in proper uptake of nutrients (Albarran *et al.,* 2005; Park *et al.,* 2015) Temporary immersion bioreactor system was successfully employed for the micropropagation of commercially important crops like banana (Farahani and Majd, 2012), sugarcane (Estrada *et al.,* 2017), *Colocasia esculenta* (Arana-Avalos *et al*., 2019), *Capparis spinosa* L. (Gainguzzi *et al.* 2020). The use of TIB for microrhizome induction in

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ginger and similar crops was not yet reported. But many research reports were published on large scale production of potato microtubers using bioreactors (Akita and Takayama, 1994; Hulscher *et al*., 1996; Teisson and Alvard, 1999).

A new TIB named Plantform designed by Professor Margareta Welander and Dr A J Sayegh was purchased from Sweden (www.plantform.se.) was used for the present investigations. The system has many advantages over TIB developed earlier, which include greater interior bottom for cultures to grow and a suitable size for handling. Besides, these bioreactors could be placed above each other for saving culturing space, which is more effective for large scale production (Welander *et al*., 2014). The use of Plantform bioreactor for large scale micropropagation was reported in different crops by Welander *et al*. (2014), Welander *et al*.(2017), Nayyef *et al*. (2019), Clapa *et al*. (2019) and Gainguzzi *et al*. (2020)

Assessment of genetic stability of micropropagated plants is important for distribution of true-to-type plants. The occurrence of cryptic genetic defects arising due to somaclonal variation in the regenerants can seriously limit the utility of micropropagation (Rani and Raina, 2000). Therefore, it is very important to ensure the genetic stability of *in vitro* regenerated plants. Molecular marker technique is a widely used method to detect the somaclonal variation. PCR based molecular marker techniques such as RADP and ISSR are widely used for clonal fidelity analysis in many crops like *Swertia chirayita* (Joshi and Dhavan, 2007), *Alpinia galanga* (Parida *et al*., 2011), *Zingiber rubens* (Mohanty *et al*., 2011), *Zingiber officinale* Rosc. (Gavande, 2013), *Zingiber zerumbet* (Mohanty *et al*., 2012), *Moringa peregrine* (Purayil *et al*., 2018) and *Vanda* (Baby *et al*., 2019). ISSR marker system was reported to assess genetic stability in sugarcane plants produced using TIB system. Evaluation of somaclonal variation in sugarcane using ISSR markers showed that subculture passages up to eight can be recommended in TIB (Estrada *et al.,* 2017)

With this background, the present study on "Commercial production of ginger (*Zingiber officinale* Rosc.) microrhizomes using temporary immersion

bioreactor system" was carried out at Centre for Plant Biotechnology and Molecular Biology (CPBMB) to develop an efficient commercial production protocol for ginger microrhizomes using a temporary immersion bioreactor (TIB) system.

Review of literature

2. REVIEW OF LITERATURE

The present study on "Commercial production of ginger (*Zingiber officinale* Rosc.) microrhizomes using temporary immersion bioreactor system" was carried out in Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara. The objective of this study was to develop an efficient commercial production protocol for ginger microrhizomes using a temporary immersion bioreactor system. The significant literature dealing with various aspects of the study are reviewed in the chapter.

2.1 Botanical description of ginger

Ginger (*Zingiber officinale* Rosc.), belong to the family Zingiberaceae under the order Zingiberales and the tribe Zingibereae (Holtum, 1950). It is usually propagated vegetatively through the modified underground stem called rhizome. The anatomical features of ginger include xylem vessels with scalariform thickening, short lived functional cambium and absence of periderm (Ravindran *et al*., 1998). It is a perennial growing erect plant 1 - 3 feet in height with laterally compressed rhizomes, 7-15 cm length and 1- 1.5 cm breadth.

2.2 Chemistry of ginger

In ginger, Oleoresin content varies from 3 to 11 per cent depending on parameters like nature of rhizome whether fresh or dry, the drying process, geographical origin and the analytical methodology adopted (Ratnambal *et al*., 1987; Vernin and Parkanyl, 2005).

A study conducted by Natararajan *et al*. (1972) in 26 varieties of ginger showed that volatile oil, acetone extract, crude fibre and starch content varied from 1 to 2.7, 3.9 to 9.3, 4.7 to 9.8 and 40.4 to 59 per cent respectively. An analysis of fresh ginger conducted by Govindarajan *et al*. (1983), in the market sample of green ginger showed that it contains moisture (80.9 %), carbohydrates (12.3 %), fibre (2.4 %), protein (2.3 %), minerals (1.2 %) and fat (0.9 %). Along with starch the rhizome also contains 7.6 per cent of pentose on a dry weight basis. They also reported that, on a dry weight basis ginger was found to contain 1.6–2.4 per cent

nitrogen of which, one third part include non-protein nitrogen. The study on the content of gingerol and shogaol conducted by Zachariah *et al.* (1993) showed that in the exotic ginger, 'Oman', 8-shogaol was the main constituent while in the other accessions 6- gingerol was the predominant one. The cultivar, 'Angamali' (3.11%) recorded the highest level of 6-gingerol. In ginger rhizome starch constitute the major part of the dry weight (40%-60%), the rhizome also contains various constituents such as steam volatile oil, pungent compounds, fixed (fatty) oil, resin, cellulose, proteins, pentosans, and mineral elements (Parthasarathy *et al*., 2008). Characterization of 46 ginger accessions by Kizhakkayil and Sasikumar (2009), showed that the improved varieties have less level of essential oil and oleoresin content compared to some of the primitive ginger types such as 'Kozhikkalan', 'Kakakalan' and 'Sabarimala', and few landraces.

2.3 Pharmacological effects of ginger

In traditional medicine, ginger is recommended as a diaphoretic, antispasmodic, carminative, expectorant, astringent, peripheral circulatory stimulant, appetite stimulant, anti-inflammatory agent, digestive aid and diuretic (Warrier, 1989). The medicinal properties exhibited by ginger are due to its volatile and non-volatile compounds (Kizhakkayil and Sasikumar, 2011). According to Nugroho *et al.* (2006), curcumin (25.01 mM) has higher ES15 value when compared to dehydrozingerone. The result proved that the phenolic hydroxy moiety of dehydrozingerone effectively helps in antioxidant activity of dehydrozingerone.

Li *et al*. (2012) reported the preventive and protective properties of ginger in diabetes mellitus, diabetic complications, and associated lipid and other metabolic disorders. Investigations by Al Hroob *et al*. (2018) revealed that ginger can protect rats from STZ-induced diabetic nephropathy, mitigate hyperglycemia-induced oxidative stress, apoptosis and inflammation.

The cisplatin (a chemotherapeutic agent) along with ginger extract can induce beneficial effects such as delayed gastric emptying and emesis (Sharma *et al.*, 1997).Ginger is widely used to treat gastrointestinal disorders as it contains a

cholinergic type gastric stimulating factor, along with an antispasmodic component. It is mediated through the calcium antagonist action, which explains its wide range of uses (Ghayur and Gilani, 2005).

2.4 Ginger variety used in the study: Athira

Athira is a high yielding, high quality ginger variety released by Kerala Agricultural University suited for fresh and dry ginger. It is a single plant selection from somaclones of cultivar Maran. It has an average fresh yield of 21 t/ha and potential fresh yield of 30 t/ha. The clone has bold rhizomes with low crude fibre contents (3.4%). It has a dry recovery of 22.6 per cent, volatile oil content of 3.1 per cent and oleoresin content of 6.8 per cent. In the volatile oil 35.76 per cent is zingiberine and 1.31 per cent is citral. The gingerol content in the variety is 16.5 per cent of oleoresin. The variety is ideal for cultivation as pure and intercrop. It is tolerant to soft rot (*Pythium* sp.) and bacterial wilt (*Ralstonia solanacearum*) diseases as compared to the parent cultivar Maran (Shylaja *et al*., 2010).

2.5 Microrhizome technology in ginger

Microrhizome technology is gaining popularity in ginger due to several advantages. According to Shirgurkar *et al*., (2001) the increased sucrose concentration in liquid medium can cause *in vitro* induction of microrhizomes in many rhizomatous plants, these microrhizomes can survive in the field without any distinct acclimatization process. The *in vitro* developed miniature rhizomes are known as microrhizomes. Microrhizome production is a very useful technique for germplasm storage and direct application in *in vivo* condition (Devi *et al*., 2012). Lack of availability of disease free seeds, can be rectified using novel techniques such as *in vitro* induction of microrhizomes, which will produce disease free planting materials. The *in vitro* induced microrhizomes are highly accepted commercially due to its genetic stability and pathogen free nature (Archana *et al*., 2013).

The first report on microrhizome induction in ginger was by Sharma and Singh (1995). In the study they used liquid MS medium supplemented with 0.2

mgL^{-1} GA$_3$, 2 mgL^{-1} calcium pantothenate, 1 mgL^{-1} BAP, and 0.05 mgL^{-1} NAA for the shoot proliferation. The shoots proliferated after four weeks were then transferred to MS microrhizome induction medium with 75 mgL^{-1}sucrose supplemented with 8 mgL^{-1} BAP.

A study by Chuan-Hong *et al.* (2006) reported the effect of sucrose and paclobutrazol on *in vitro* microrhizome formation in ginger. The results showed that the rhizome production was best in tube culture on MS medium supplemented with 0.2 mgL^{-1} BA, 0.5 mgL^{-1} NAA, 2.5 mgL^{-1} Ca$_3$(PO$_4$)$_2$ and 2.5 mgL^{-1} paclobutrazol along with 8 per cent sucrose.

Zheng *et al.* (2008), reported the effects of various plant growth regulators such as gibberellic acid (GA), kinetin (KT), and naphthalene acetic acid (NAA) on microrhizome induction in ginger. The result showed that the NAA and KT have less effect on microrhizome induction than GA. The study also showed that the microrhizomes with weight exceeding 0.25 g can be produced with NAA, KT and GA at a concentration of 0.62, 0.49–0.66 and 1.33–2.35 respectively. A photoperiod of 24L:0D (light/dark) along MS medium with 80gL^{-1} of sucrose were found optimal for microrhizome induction.

In three high yielding varieties of ginger (cultivars IISR Rejatha, IISR Varada and IISR Mahima), Archana *et al*. (2013) tried microrhizome production using MS medium without NH$_4$NO$_3$ and with various concentrations of externally provided NH$_4$NO$_3$ and photoperiod. During *in vitro* culture period, IISR *Mahima* showed maximum growth responses in the medium with 0.413 mgL^{-1} of NH$_4$NO$_3$ during 1, 2 and 3 months of growth when incubated in light. Among these three varieties, response of IISR *Mahima* was good.

Singh *et al.* (2014) developed an improved and high frequency *in vitro* microrhizome induction system for two ginger varieties, *viz.,* 'Baishey' and 'Nadia'. The study emphasized the role of silver nitrate and different culture vessel for *in vitro* microrhizome induction within short period of time. The result showed that MS media supplemented with 2 mgL^{-1} BAP, 1mgL^{-1} NAA and 8 gL^{-1} sucrose with the presence of 11µM silver nitrate is suitable for high frequency *in vitro*

microrhizome production. Among different culture vessels examined, growtek gave the highest average production of microrhizomes in both the varieties of ginger with 35-45 days of incubation.

Mehaboob *et al.* (2019), developed an effective protocol for *in vitro* shoot multiplication and microrhizome induction in ginger (*Zingiber officinale*). *In vitro* culture was established using sprouting shoot bud explants of ginger rhizome. MS medium supplemented with 0.5 mg BA, 0.5 m gL^{-1} IAA and 8 per cent sucrose under 8 h. photoperiod is found to be an optimal condition for induction of microrhizome.

An *et al.* (2020), examined the effects of various parameters on microrhizome induction in ginger for developing a production protocol for the cultivar native to Hue, Vietnam. The study showed that IBA (indole-3-butyric acid), NAA (α- naphthalene acetic acid), BAP (6-benzyl amino purine), and a low ammonium nitrate concentration, with 0.478 g at 10.80 mm, 0.437 g at 9.73 mm, 0.433 g at 9.03 mm, and 0.449 g at 9.53 mm, respectively, increased the size and fresh weight of microrhizome. They also reported that kinetin has an inhibitory effect on the growth of microrhizome. The growth of microrhizomes was high in MS medium with optimal concentrations of, 80 gL^{-1} sucrose, 1.9 mgL^{-1} $AgNO_3$, 550 mgL^{-1} ammonium nitrate, 4 mgL^{-1} BAP, 6 mgL^{-1}NAA, and 4 mgL^{-1} IBA.

2.6 Bioreactor for micropropagation

A bioreactor is a semi-automated system which uses liquid media. It is designed for scaled-up cultures and also helps in monitoring and controlling of the micro-environmental conditions inside the system (Watt, 2012). The bioreactor culture offers various advantages such as, better control of the culture conditions, optimal supply of nutrients and growth regulators, the renewal of the culture atmosphere, changing of the medium during culture period based on developmental stage, filtration of the medium for exudates, contamination control and the production of clusters of buds or somatic embryos for automated handling of propagules (Ziv, 2005).

In the continuous immersion bioreactors, the continuous immersion of explants in the liquid medium causes vitrification or hyperhydricity of plant tissues due to limited exchange of gas in plant materials (Debergh and Maene, 1984). To avoid these issues associated with continuous immersion in liquid medium, different systems have been developed, *viz.* nutrient mist bioreactor, membrane raft system and temporary immersion bioreactor (Akita and Takayama, 1994). The high production rate with low physiological disorders of temporary immersion bioreactor increased its popularity (Afreen, 2008).

2.7 Temporary immersion bioreactor system

In temporary immersion system the entire culture is temporarily immersed in nutrient medium followed by draining away of the excess nutrient medium under gravity so that the plant tissue has access to air. As the system is with periodic wetting and drying cycle in a given period of time, it is termed as periodic/ temporary immersion (Afreen, 2008). The temporary immersion bioreactor, in which explants are periodically immersed in liquid medium following optimized cycles, was highly effective for micropropagation of pineapple, apple rootstocks, *Beta vulgaris* L. hairy root culture as compared to, conventional culture systems (Escalona *et al*.,1999, 2003; Zhu *et al*.,2005; Pavlov and Bley 2006; Welander *et al*., 2007; Fontanili *et al.,*2015).

2.7.1 Culture factors affecting the efficacy of micropropagation in temporary immersion bioreactor

Immersion time

In temporary immersion system, the immersion time is very important, since it determines nutrient uptake and control of hyperhydricity. Krueger *et al*. (1991) demonstrated the importance of immersion frequencies for the proliferation of serviceberry shoots. Hyperhydricity was observed with immersions of five minutes in every 30 minutes, but was not seen with immersions for five minutes in every 60 minutes. The immersions of five minutes every in 30 minutes combination were found better for the number of shoots obtained. Berthouly *et al*. (1995) with Coffee microcuttings showed that immersion time substantially affected the shoot

multiplication rate, as estimated by the number of micronodes produced after six weeks. The immersion times of one, five and 15 minutes applied every six hours gave multiplication rates of 3.5, 5.4 and 8.4, respectively. The optimum immersion time was 15 minutes every six hours for *Coffea arabica* microcuttings and only one minute in every six hours for *Coffea canephora* microcuttings.

Volume of liquid medium

Volume of liquid medium is a very important factor affecting the efficacy of temporary immersion bioreactor. Study conducted by Lorenzo *et al*. (1998) in *Saccharum* spp. in twin flask system showed that the optimum volume of medium for shoot proliferation was 50.0 ml per explant. An increase in multiplication rate from 8.3 to 23.9 shoots/30 days was observed when volume of standard medium was multiplied by 10 from 5.0 to 50.0 ml per explants. The work also suggested that the higher volumes are less efficient as it would dilute the extracellular chemicals that stimulate shoot proliferation. Using the same temporary immersion system, Escalona *et al*. (1999) reported that for pineapple shoot proliferation, the optimum volume for medium was 200 ml /explant.

Volume of culture container

According to Krueger *et al*. (1991) the micropropagation efficiency was increased by using large size culture vessel in serviceberry as it can prevent culture overcrowding and positively affect shoot elongation, compared to the 140-ml baby food jars. Use of larger containers positively affects growth and proliferation of plant materials as it can contain larger volumes of media, which can have a positive effect on plant material proliferation and growth (Berthouly and Etienne, 2002).

Aeration and forced ventilation

Work done by Alvard *et al*. (1993) in banana micropropagation showed that the growth of explant was decreased when air is limited in the liquid medium. In their study, the absence of liquid medium stirring led to asphyxia of the explant. They also showed that partial aeration did not provide sufficient aeration while bubble aeration improved the growth. According to Teisson and Alvard (1995),

temporary immersion system was most effective as in this system the gas exchanges primarily occur during immersion, by movement of the liquid and directly by the air pump.

2.7.2 Design and working of Temporary immersion bioreactor

Temporary Immersion Systems for plant micropropagation have been grouped into four categories according to operation; i) tilting and rocker machines, ii) complete immersion of plant material and renewal of nutrient medium, iii) partial immersion and a liquid nutrient renewal mechanism, iv) complete immersion by pneumatic driven transfer of liquid medium and without nutrient medium renewal (Berthouly and Etienne, 2002).

The hyperhydricity can be reduced in plant tissue by proper adjustment of the durations of the immersion and exposure period creating conditions for optimal humidity and nutrients supply with minimal liquid contact (Albarran *et al*., 2005). The direct exposure of the plant tissue to the gaseous environment significantly simplifies the interphase oxygen transport from the gas to the cultured cells, in contrast to the submerged culture, where the interphase oxygen transport faces resistance in a few boundary layers (gas–liquid and liquid–solid interfaces) (Curtis and Tuerk, 2006). Improved oxygen transport lowers physiological disorders such as asphyxia by helping better exchange of gas and reduced oxygen limitations. Increasing the level CO_2 improve multiplication, growth production of secondary metabolites and photosynthetic activity of plants (Aragon *et al*., 2010; Yang *et al*., 2010). The TIB can be designed for enriching the CO_2 during the period gas exchange. The technological design of some of the most commonly used TIB is discussed in more details.

Twin flask system

The Twin-Flask system (BIT[®]) of Escalona *et al*. (1999) for mass propagation of pineapple plants is one of the earliest developed TIB. The system mainly consists of two containers connected to each other by a U pipe or silicon tube. One of the container acts as the culture compartment while the other is used

as a media storage space (Aragon *et al.,* 2010). The system is considered too simple, easy to operate and can maintain sterile environment (Arencibia *et al.,* 2013). Forced ventilation, absence of media renewal system and complicated automation are major problems of Twin-Flask systems (Georgeiv *et al*., 2014).

Niemenak *et al*. (2008) developed an efficient protocol for somatic embryo multiplication in twin flask temporary immersion bioreactor in cacao. The system can be efficiently used for the mass production of somatic embryo which can be sown directly.

Topoonyanont *et al.,* (2011) investigated the efficiency of twin-flask temporary immersion bioreactor for *in vitro* culturing of *Curcuma alismatifolia*. The number shoots obtained per vessel showed that in TIB the 1263 to 1485 shoots were produced per vessel while in solid medium 285 shoots per vessel were produced.

Wawrosch (2015) worked with twin flask TIB for *in vitro* propagation of some important medicinal and aromatic plants. The plant material was only periodically immersed in the liquid medium and the results were compared with conventional micropropagation system. The result showed that the TIB system gave better multiplication rate than conventional system and individual optimisation of immersion period and frequency gave better results.

RITA® system

The RITA® system the works in such way that when pressure is applied to the lower compartment the medium is forced upward towards the plant materials in the upper compartment, this cause the immersion of plant material in the liquid media. When the pressure on lower compartment is removed the liquid media flows back to the media chamber (Teisson and Alvard, 1995). When the pressure is applied the tissue gets agitated gently as air is bubbled through the medium and when the pressure is removed through the outlet the atmosphere inside the culture chamber gets renewed (Etienne and Berthouly, 2002). The RITA® system shows high efficiency in mass propagation of somatic embryos (Etienne and Berthouly,

2005). It is a simple and reliable, compact apparatus where the propagule and liquid media are fully separated with sufficient level of relative humidity (Georgeiv *et al*., 2014).

McAlister *et al*. (2005) used RITA® temporary immersion bioreactor system for establishing micropropagation protocol for six Eucalyptus clones. The results indicated that in RITA® system the proliferation of axillary buds was increased four to six, within a less period of time compared to conventional system. The result also showed that plants grown in the RITA® system exhibited better growth and acclimatized better and there were more recovery of hardened plants.

The efficiency of different culture systems and culture medium composition were evaluated to optimize the efficiency of somatic embryogenesis in peach palm (Heringer *et al.,* 2014). The somatic embryos from RITA® bioreactor showed increased in level of starch, protein and ADH activity. The study also showed an enhancement in somatic embryogenesis. The RITA® bioreactor produced a greater number of plantlets for acclimatization compared to conventional system. The plantlets derived from the RITA® bioreactor showed better acclimatization.

Anguilar *et al*. (2019) reported that in teak, the production cost for micropropagation can be effectively reduced by using RITA® temporary immersion bioreactors. The study utilised the primary explants in several culture cycles in semi-solid medium to produce nodal explants for the multiplication phase in bioreactor.

Ebb and flow system

The ebb and flow system includes, a pair of containers, in which one is larger and functions as culture compartment and the smaller one functions as media storage compartment. The immersion frequency and immersion period were regulated by means of a programmable timer connected to three-way solenoid electrovalves (Akita and Takayama, 1994). The humidity during the immersion period is maintained by polyurethane support and also functions as an air sparger

during immersion (Ducos *et al*., 2007; Ducos *et al*., 2008). The system lack forced ventilation, uniform distribution of light inside the culture vessel and CO_2 enrichment were reported as the disadvantages of the system (Georgeiv *et al*., 2014).

Rocker system

Rocker systems consist of transparent culture boxes made of polycarbonate, which can be tilted on a mechanical platform at a particular angle to distribute the medium from one side of the culture vessel to the other side. The boxes are placed in tilting mechanical shelves, the tilting of the shelves cause small wave fronts, that can periodically immerse and aerate the cultured propagules (Adelberg and Toler, 2004; Kamarainen-Karppinen *et al*., 2010; Uchendu *et al.,* 2011). In the rocker system more numbers of culture vessels can be arranged on a single rack, without any additional aeration system. The system costs high initial investment as it requires an electromechanically driven tilting platform (Georgeiv *et al*., 2014).

Rotating drum system

The rotating drum system consists of an autoclavable plastic or glass bottle with stainless steel net or a mat of polyurethane foam for supporting the explant. The bottle is placed on a roller apparatus (Akita and Ohta, 1998). The rotating of the roller apparatus at low speed helps in periodical immersion and aeration of culture. The adventitious or hairy roots culture can get adsorbed to the walls of the bottle, due to which internal support is not necessary (Reis *et al.*, 2011). Independent and prolonged immersion period could not be set up in the system. High shear stress caused by mechanical mixing, and absence of external ventilation are other disadvantages (Georgeiv *et al*., 2014).

2.7.3 Plantform Bioreactor

Plantform bioreactor is a novel system which uses liquid medium. The culture is alternately exposed to wet and dry period with forced ventilation in the system to avoid gas accumulation inside the culture environment. When compared to other TIB systems it has relatively larger culture compartment for better growth

of culture. It can be effectively used for commercial purpose as it is small in size, easy to handle and can be placed one above the other saving large amount of culture space (Welander *et al.,* 2014). It is an environment friendly system with long shelf life and less production cost. The production is lower as it does not require agar, the positioning of shoots and more plants can be produced in less culture space. The system can also reduce labour cost as medium filling and replacing and assembly of bioreactor is simple and quick (Welander *et al*., 2017).

The new bioreactor consists of a culture vessel of $180 \times 160 \times 150$ mm in dimension made of transparent polycarbonate material. In the system it has a culture compartment with holes of 1 mm in size, which is placed above a chamber that controls the flow of medium, and holds the plant material. A frame with four legs is placed above the basket to assure the basket not to rise when air pressure is applied to the bioreactor. The construction and placement of the basket is made accurately so that the plants are only immersed into the liquid medium, only when the air pressure is applied to the bioreactor. Furthermore, the bioreactor will have three opening holes for medium supply, aeration and for the ventilation. The specially designed hollow screws which come with silicone seals are fitted tightly within the holes. Connected to these hollow screws are plastic Tygon tubes which is flexible, with an inner diameter of 3.2 mm, and 0.22 m polytetrafluoroethylene in order to make the airflow sterile inside the bioreactor filters are added. The bioreactors and filters were autoclaved separately at 121^0C for 20 min. The three holes were sealed using aluminium foil and the lid was only attached at two of the four sides in order to counteract pressure changes happening during autoclaving (Welander *et al.,* 2014).

Welander *et al*. (2014), used Plantform temporary immersion bioreactor for micropropagation of *Digitalis lutea × purpurea, Echinacea purpurea* and *Rubus idaeus*. The multiplication ratio and shoot quality in bioreactor were similar or sometimes better than those on agar medium. The shoot number was similar in both systems for *Digitalis* and *Rubus*, while *Echinacea* had a higher shoot number in bioreactor. *Digitalis* and *Echinacea* gained more fresh weights in bioreactor, while

more weight was observed for *Rubus* on agar medium. However, the dry weight was similar for all three species on comparing the two systems.

A study evaluating the new Plantform bioreactor for commercial micropropagation of various cultivars of *Vaccinium* which was conducted by Welander *et al*. (2017), showed that Plantform bioreactor can reduce the cost of production as it can eliminate agar, produce more number plants per unit, requires only less handling as positioning of explants in the bioreactor is not required and rooting in the same unit.

According to Nayyef *et al*. (2019), the Plantform bioreactor was excelled in the number of shoots, fresh weight of biomass, and its content of sugars compared with solid media in the micropropagation of date palm. Research showed that 'Duke'and hazelnut (*Corylus avellana* L.) 'Tonda di Giffoni' cultivars highbush blueberry (*Vaccinium corymbosum* L.) can be propagated successfully by using Plantform bioreactor systems (Clapa *et al*., 2019).

A study conducted by Gainguzzi *et al*. (2020) in *Capparis spinosa* L. showed that the explants coming from the Plantform bioreactors liquid medium supplemented with sucrose showed better rooting performances (up to double rooting percentages) than those which came from the solid medium.

2.7.4 Commercial micropropagation using temporary immersion bioreactor

Levin *et al.* (1997), reported some major advantages of bioreactors which include, less labour and laboratory space, reduction in medium cost and reduction in energy, faster growth and increased multiplication rate. The elimination of solidifying agents (e.g. agar) can reduce medium cost and filter sterilization can be adopted to avoid for autoclaving.

Gross and Levin (1999), tried production with mechanical separation of the multiplication cultures and multiplication and elongation in bioreactors. The study showed a significant reduction of labour space and energy requirements, which reduces the unit cost of production to $0.07 per unit.

According to Levin and Tanny (2002), in larger laboratories use bioreactors to reduce the cost by about $0.01. They also suggested that bioreactor simplify production management through the use of large culture batches and reduced labour.

Watt (2012), reviewed that bioreactor-based propagation of plants which can be utilised for the commercial micropropagation due to its efficiency in increasing the multiplication rate, growth of cultures, reduce space, energy and labour requirements. Escalona *et al*. (1999), reported successful large-scale propagation of pineapple using temporary immersion bioreactor. The result showed that the protocol can reduce 20 per cent of the production cost per pineapple plant in comparison with conventional micropropagation system.

Niemenak *et al*. (2013), established a temporary immersion technique for large-scale propagation of cocoyam (*Xanthosoma sagittifolium*). The study demonstrated that, the multiplication rate was 97-fold higher compared to regeneration in solid medium reported earlier.

Arana-Avalos *et al*. (2019) evaluated the temporary immersion technique for commercial micropropagation of *Colocasia esculenta*. In the study it was found that the TIB with immersion frequency of 2 minutes every 4 hour, and a volume of culture medium of 25ml and 50 ml per explant promote growth of shoots in comparison with the semi-solid medium and also its multiplication rate.

2.8 Clonal fidelity analysis

Clonal propagation through tissue culture can be achieved in short time and limited space (Razdan, 2002). Periodic monitoring of the degree of genetic stability of *in vitro* plants is of high importance for the commercial utilization of the technique for a large-scale production of true-to-type plants. The analysis of the genetic fidelity of *in vitro* derived regenerants at definite intervals can notably reduce or eradicate the chance of somaclonal variation (Larkins and Scowcroft, 1981). Somaclonal variation results from pre-existing genetic variation within the explant and variation induced amid the tissue culture phase (Evans *et al.*, 1984).

Clonal fidelity is the maintenance of the genetic constitution of a particular clone during throughout life span (Chaterjee and Prakash, 1996). The existence of cryptic genetic defects due to somaclonal variation in *in vitro* generated plants can limit the broader utility of micropropagation systems seriously (Rani and Raina, 2000). Genetic changes are associated with phenotypic variations which are reported in micropropagated plants of numerous species like Pelargonium, Phalaenopsis, Pineapple, and Musa (Cassels *et al*. 1997; Das and Bhowmik, 1997; Chen *et al*. 1998; Grajal-Martfn *et al*. 1998). A wide variety of analyses tests are available for the detection and characterization of somaclonal variants which are mainly based on the morphological traits and its differences, cytogenetical analysis for the determination of numerical and structural variation in the chromosomes and also the biochemical and molecular DNA markers (Mahdi *et al*., 2015).

2.8.1 Molecular marker based assessment of clonal fidelity

The molecular markers are classified on the basis of the techniques that are used for identifying polymorphism. Hybridisation based Restriction Fragment Length Polymorphism (RFLP) (Botstein *et al*.,1980) and Polymerase Chain Reaction (PCR) based molecular markers *viz*., Random Amplified Polymorphic DNA (RAPD) (Williams *et al*., 1990), Simple Sequence Repeats (SSR) (Hearne *et al*., 1992), Sequence-Tagged Sites (STS) (Fukuoka *et al*.,1994), Inter-Simple Sequence Repeat (ISSR) (Zietkiewicz *et al*.,1994) and Amplified Fragment Length Polymorphism (AFLP) (Vos *et al*., 1995) are the techniques available for detecting polymorphism.

The RFLP technique requires more preparatory work, is technically demanding, and it involves expensive reagents. Therefore, the efforts were made to develop simpler, and economically feasible and more appropriate marker systems. These actions led to the development of many Polymerase Chain Reaction (PCR) based marker systems during 1990s, which are called second-generation markers generally. PCR-based markers are titled as the second-generation of molecular markers and are based on the DNA sequence polymorphisms which are detected by PCR amplification of the DNA sample (Singh and Singh, 2015).

Random Amplified Polymorphic DNA (RAPD)

The RAPD is defined as a DNA polymorphism assay based on the amplification of DNA segments with single decamer primers of arbitrary nucleotide sequence selected randomly (William *et al*., 1990).The number of amplified fragments generated by PCR is depending up on the length of the primer and also the size of the target genome, and is based on the probability that the given DNA sequence (complementary to that of the primer) will appear in the genome, on opposite DNA strands, in opposite orientation within an amplifiable distance (Waugh and Powell, 1992). The RAPD markers was used in many applications which involve the detection of DNA sequence polymorphisms, populations mapping, isolation of the markers linked with various traits or specific targeted intervals and variety identification and analysis of parentage (Giovannoni *et al*., 1991).

The study conducted by Rout *et al*. (1998) used RAPD markers to evaluate the genetic stability of micropropagated plants of ginger (*Zingiber officinales*), the study utilised 15 arbitrary decamers to amplify DNA from *in vivo* and *in vitro* plant material to assess the genetic fidelity. The result showed that RAPD analysis can be used to determine the genetic fidelity of plants produced *in vitro*. This method also can be used for monitoring the stability of *in vitro* germplasm collections and also cryopreserved material.

Palombi and Damiano (2001) compared molecular markers in terms of efficiency of RAPD (Random Amplified Polymorphic DNA) and SSR (Simple Sequence Repeat), to evaluate the clonal stability of micropropagated kiwifruit (*Actinidia deliciosa*). Polymorphism, both in SSR and RAPD, was scored as either present (1) or absent (0) fragments and those readings were entered in a computer file as a binary matrix, one for each molecular marker. The results indicate that when the tissue culture procedure induces genetic variability, its detection is depending up on the choice of tools used for DNA analysis.

Gavande *et al*. (2018) evaluated 13 groups of somaclones (seven in Maran and six in Rio-de-Janeiro) using RADP. Out of the 35 RAPD primers screened,

twelve gave good amplification. Molecular analysis of micropropagated *Rauwolfia tetraphylla* L. was carried out by using ISSR, SCoT and RAPD primers. Ten RAPD primers gave a total of 42 bands with four as the average number of bands per primer, with band size (in bp) ranging from 300 to 2000. RAPD primer, OPA3, has given better amplification and produced six DNA bands which were clear, well-defined, and scorable, with the range (in bp) of 300-2200 (Rohela *et al.,* 2019).

Simple Sequence Repeat (SSR)

Microsatellites are tandem repeats of simple sequence that occur in plenty and randomly throughout the eukaryotic genomes. They are usually less than 100 bp long and are positioned between DNA with peculiar sequences. Microsatellites are easy to characterize and clone and they display considerable polymorphism because of the variation in the number of repeated units. The polymorphism is stable to use in genetic analyses (Hearne *et al*., 1992). In development of microsatellite marker, the efficiency depends on the abundance of repetitions in the target species and the ease with which these repetitions can be developed into informative markers. Microsatellites can function as highly sensitive markers for monitoring genetic variation that might signal the potential deleterious mutations during *in vitro* culture, because they reflect relatively high rate of mutation and high degree of genetic variability (Lopes *et al.,* 2006).

Nookaraju and Agrawal (2012) conducted the clonal fidelity analysis of *in vitro* cultured grapevine cultivar crimson seedless with Simple Sequence Repeat (SSR) markers and Inter Simple Sequence Repeat (ISSR). The study obtained 288 scorable bands from five selected SSR primers. The banding pattern of these five SSR primers did not show any polymorphism between 23 tissue culture derived plants and the source mother plant.

The assessment of clonal fidelity using RAPD and SSR markers in sugarcane plants regenerated through direct organogenesis, showed great genetic purity like the parent genotype (Pandey *et al*., 2012).

The Simple Sequence Repeats analysis were used to check the genetic stability of coconut plantlets produced by somatic embryogenesis of unfertilised ovaries in a study conducted by Bandupriya *et al*., (2017). The analysis showed that the plantlet does not show any polymorphism among each other and the source mother plant. The study utilised 13 largely polymorphic microsatellite primers.

Inter-Simple Sequence Repeat (ISSR)

A single primer marker system based on PCR, which utilises two similar microsatellites primers for amplifying DNA segments present between them. The primer may consist solely of a microsatellite sequence (non-anchored primers) or, more often, a microsatellite sequence plus a short (usually, two nucleotides long) arbitrary sequence either at the 30 or the 50 ends of the primer (anchored primers) (Singh and Singh, 2015).

The ISSR is a technique which combines most of the beneficial factors of AFLP and microsatellite analysis with universality of RAPD. It is highly reproducible with the use of longer primers (16- to 25 bp) when compared to RAPD primers (10 bp) which permit the subsequent use of high annealing temperature (45 to 60 ^{0}C) which leads to high stringency (Reddy *et al.*, 2002). No sequence data for the primer construction are required for ISSR assay. Because the analytical procedures only include PCR, and hence low quantities of template DNA are required (5–50 ng per reaction). Furthermore, ISSRs are randomly distributed throughout the genome. This can be dominant marker, though occasionally it exhibits codominance (Kumar *et al*., 2009). ISSR markers are easy to use, low-cost, and methodologically less demanding when compared to other dominant markers (NG and Tan, 2015).
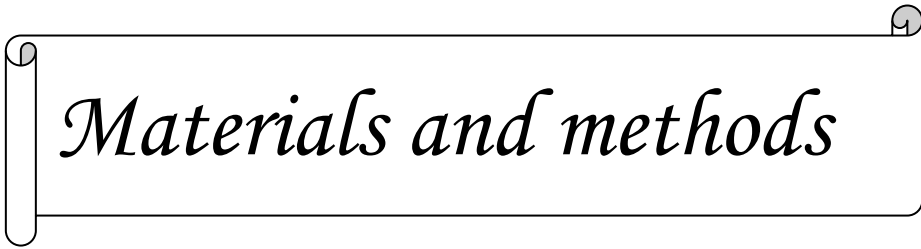
Leroy and Leon (2000) assessed the feasibility of microsatellite markers for genomic instability in cauliflower callus. The result showed that inter-simple sequence repeat assay can easily detect and measure the common genetical events underlying the plant genomic instability, which include deletions, amplifications, translocations, insertions and recombination or chemical alterations.

The genetic stability of *in vitro* derived *Swertia chirayita* plantlets obtained by multiplication of axillary buds were studied. The study utilised ISSR techniques to determine the genetic stability of plantlets produced upto 42 passages, the result showed that the technique was not suitable detect the variants (Joshi and Dhavan, 2007).

In galangal (*Alpinia galanga L.*) the *in vitro* derived plants were tested for genetic fidelity using RAPD and ISSR. Among 30 RAPD and 13 ISSR primers selected, only 12 RAPD and nine ISSR primers were suitable. They produced distinct and scorable bands to detect polymorphism. The study utilised these primers to effectively prove that the *in vitro* derived plants show no polymorphism (Parida *et al*., 2011).

From the third subculture of tissue culture derived regenerants of *Orthosiphon stamineus* ten random plants were selected for checking the genetic stability. Ali *et al*. (2019) utilised 20 inter-simple sequence repeat (ISSR) markers for this study. The study revealed that 10 primers studied were highly polymorphic with 7.11 amplicons per primer producing satisfactory amplification.

Baby *et al.*, (2019) used ISSR markers to study the genetic fidelity of tissue culture derived *Vanda* hybrid 'Dr. Anek'. The study showed no polymorphic amplicons when five highly efficient ISSR primers were used. Mother plants and micropropagated plants showed similar amplification pattern indicating the clonal fidelity of the tissue culture derived plantlets of *Vanda* hybrid.

Materials and methods

3. MATERIALS AND METHODS

The study entitled "Commercial production of ginger (*Zingiber officinale* Rosc.) microrhizomes using temporary immersion bioreactor system" was conducted at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture Vellanikkara during 2018 to 2020. The objective of the study was to develop an efficient commercial production protocol for ginger microrhizomes using a temporary immersion bioreactor (TIB) system. The study also focused on clonal fidelity analysis of microrhizomes derived from 8th subculture cycle using specific ISSR marker.

3.1 Materials

3.1.1 Plant material

The multiple shoot cultures of ginger in the 5th subculture stage of the variety Athira received from the Commercial micropropagation unit, of CPBMB, College of Horticulture, Vellanikkara were utilized for the study.

3.1.2 Laboratory chemicals, glasswares and plasticwares

Good quality chemicals procured from various agencies like Merck India Ltd., HIMEDIA and SISCO Research Laboratories were used for the study. ISSR primer for clonal fidelity analysis was purchased from Sigma Aldrich Chemicals Pvt. Ltd. The *Taq* DNA polymerase, dNTPs, *Taq* buffer and molecular marker (λDNA/*Hind*III + *Eco*RI double digest) were purchased from Bangalore Genei Ltd. Plasticwares used in the present study were supplied by Axygen and Tarson India Ltd. The glasswares used were purchased from borosil glasswares Ltd., Mumbai and vensil glassworks private limited, Bengaluru.

3.1.3 Laboratory equipment and machinery

The present study was carried out in the commercial micropropagation unit and molecular biology laboratory of CPBMB, College of Horticulture. Aseptic conditions for subculturing were ensured using laminar air flow from LABLINE INDUSTRIES and sterilization of media and equipment were carried out in

autoclave (Nat steel equipment Pvt. Ltd.). The temporary immersion bioreactor utilised in the study was supplied by Plantform, Sweden. Various equipment like electronic balance (Shimadzu), pH meter (Eutech Instruments PC 501), micropipettes (Eppendorf), icematic (F100 compact), high speed refrigerated centrifuge (KUBOTA 6500), thermocycler (ProFlex PCR System by Life Technologies) were used in the study. The quality and quantity of DNA were estimated using the NanoDrop® ND-1000 spectrophotometer. Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis system (BIO-RAD, USA). Gel documentation and imaging were done using the BIO-RAD Gel Doc system.

3.2 Methods

3.2.1 Culture Media

3.2.1.1 Preparation of MS medium supplemented with Benzyl Adenine

Murashige and Skoog medium (Murashige and Skoog, 1962) was prepared using standard procedure. Five nutrient stock solutions were prepared and stored in sterile bottles in refrigerator. Stock III was stored in amber coloured bottle. Stock solution for Benzyle Adenine (BA) was prepared by dissolving in 0.1N NaOH and the volume was made up with distilled water and stored in sterile bottle under refrigerated conditions.

Medium was prepared in a clean beaker rinsed with distilled water. All the five stock solutions were aliquoted into the beaker with 1/3rd volume of distilled water. For preparing full strength MS medium, 20 ml was pipetted from 50X stocks and 10 ml from 100X stocks. Required quantities of sucrose, myo-inositol and hormones were added and dissolved. The volume was then made up with distilled water. The pH of the medium was adjusted to 5.7 using 0.1N NaOH.

The liquid medium of 250 ml used in TIB was autoclaved without adding any solidifying agent in conical flask and plugged with non-absorbent cotton. Solid medium used for conventional method of micropropagation were prepared by adding required amount of agar and 50 ml of medium was poured into sterile bottles

and autoclaved.

3.2.1.2 Autoclaving

Media in the culture bottles and conical flasks were autoclaved in an autoclave at a pressure of 15 lbs/ sq.inch (121^0C) for 20 minutes. The autoclaved media was allowed to cool down. The media were poured into the bioreactor culture vessels only at the time of inoculation.

3.2.2 Installation of Bioreactor

3.2.2.1 Assembly and functioning of Temporary Immersion Bioreactor

Plantform temporary immersion bioreactor designed by Professor Margareta Welander and Dr A J Sayegh purchased from Sweden (www.plantform.se.) was used in the study. Twelve bioreactor culture vessels, each with a size of 180×150×150 mm was used. Plantform bioreactor consists of a polycarbonate culture vessel with a capacity of 500 ml. Three inlets/outlets anchored to the side by nuts and clamps through holes in the body are used for gas exchange. Gas passed into the culture vessel is kept sterile by passing through sterile filters connected by silicon tubes to the inlets. A basket containing the plant material is placed above the inner chamber. The small holes in the basket are placed in three rows which allow the nutrients to flow efficiently through the basket. A frame with four legs is placed at the bottom of the basket to press down the basket when a pressure is applied. The body is closed by a lid which is air tight using silicon seal inside a groove of the lid.

The filters were connected to two timers and two air pumps via silicon tubes to regulate gas exchange and immersion. The nutrient medium was forced upward towards the plant materials by applying pressure to the middle filter. When the pressure was released the nutrient solution was drained back through the holes in the basket to avoid hyperhydricity. The immersion time and frequency were set by the timers. The Plantform TIB setup is presented in Plate 1

3.2.2.2 Sterilization of bioreactor system

The bioreactor vessels and other parts were washed thoroughly, dried and sterilized by autoclaving at a pressure of 15 lbs/ sq.inch (121 ^{0}C) for 20 minutes. The filter and connection tubes were covered with aluminium foil during autoclaving.

3.2.3 Experimental Conditions

3.2.3.1 Transfer area and aseptic manipulation

All the aseptic manipulations such as subculturing for multiplication and microrhizome induction were carried out in the laminar airflow. The laminar airflow cabinet environment was made aseptic by exposing to the UV light for 20 minutes and the airflow was allowed to blow for 15 minutes before working in the laminar airflow.

Plate 1: Experimental setup with Plantform temporary immersion bioreactor

The working table of the laminar airflow was sterilized by wiping with 70 per cent ethanol.

3.2.3.2 Culture conditions.

The cultures were incubated at $26 \pm 2\ ^0C$ in an air-conditioned culture room with 16 hours photoperiod (1000 lux) from fluorescent tubes. In the bioreactor, the cultures were given one-minute immersion with the medium at an interval of three hours. The humidity in the culture room varied from 60 to 80 per cent according to the prevailing climate.

3.2.4 Induction of microrhizomes

The protocol for *in vitro* induction of microrhizomes in the KAU released ginger varieties was developed at CPBMB by Shylaja *et al*. (2016). Based on the preliminary optimization of TIB done at CPBMB, various treatments for the present study were fixed.

Development of large-scale production protocol in temporary immersion bioreactor.

3.2.4.1 Evaluationof clump size for multiple shoot production in Temporary Immersion Bioreactor and comparison with conventional systems.

Three clump sizes based on the size of the culture vessel *viz.,*10, 15 and 20 clumps/ 500ml of medium were used in the experiment. The medium used for the study was MS (Murashige and Skoog, 1962) supplemented with 3 mgL^{-1}of Benzyl Adenine (BA). The number of multiple shoots proliferated from each clump were recorded after 21 days of inoculation from TIB and conventional systems.

3.2.4.2 Evaluation of the multiplication medium in Temporary Immersion Bioreactor and comparison with conventional systems.

Murashige and Skoog (MS) medium supplemented with three different concentrations of BA *viz.*, 2, 3 and 4 mgL^{-1} were used as multiplication media. The clump size optimized in 3.2.4.1 was inoculated to three different media.   The

numbers of multiple shoots proliferated in each medium were recorded after 21 days of inoculation from TIB and conventional systems.

3.2.4.3. Evaluation of microrhizome induction medium in Temporary immersion bioreactor and comparison with conventional systems.

For evaluation of microrhizome induction medium, MS medium with different concentrations of sucrose (70, 80 and 90 gL^{-1}) were tried. The microrhizome induction in three media tried were recorded after two months of inoculation from TIB and conventional systems. Observations on days taken for microrhizome induction, number of microrhizomes produced, length of shoot and number of leaves, number of roots, root length in microrhizome plantlets and weight of microrhizomes were recorded.

3.2.5 Hardening and acclimatization

The *in vitro* regenerated ginger microrhizome plantlets from both bioreactor and conventional systems were taken out using forceps and soaked in water. Each microrhizome plantlet was then carefully separated by hand and washed in running tap water to remove the adhered solidified medium. The microrhizome plantlets were then planted out in protrays with 98 wells filled with peat, perlite and vermiculite in the proportion 3:1:1 and kept inside the hardening chamber for primary hardening. After one month of primary hardening, microrhizome plants were transferred to polythene bags containing sand, soil and cow dung at 1:1:1 ratio and were kept for secondary hardening for two months. The survival rate and growth parameters of the microrhizome plants such as height of the plant and number of leaves were recorded after two months of secondary hardening.

3.2.6 Clonal fidelity analysis using ISSR marker

Multiple shoot cultures of the ginger variety Athira received from CPBMB commercial micropropagation unit at the 5th subculture stage were subcultured in both TIB and conventional systems using protocol developed at CPBMB (Shylaja *et al*., 2016). Microrhizome plants derived from the eighth subculture cycle of both

bioreactor and conventional systems were used for clonal fidelity analysis. Ten random microrhizome plants were selected from both the systems and DNA was isolated along with source mother DNA. The clonal fidelity analysis was done using the ISSR (Inter Simple Sequence Repeats) marker (ISSR 05) as reported by Gavande (2013).

3.2.6.1 Genomic DNA isolation

Good quality DNA was isolated using CTAB procedure reported by Roger and Benedich (1994). Tender leaves from microrhizome plants obtained after eighth subculture stage were collected for genomic DNA isolation.

Reagents

1. 5X CTAB extraction buffer

- 5 per cent CTAB (w/v)

- 100 mM Tris (pH 8)

- 20 mM EDTA (pH 8)

- 1.4 M NaCl

- 1 per cent PVP

2. CTAB (10) per cent

- 10 per cent CTAB (w/v)

- 0.7 M NaCl

3. TE buffer

- 10 mM Tris HCl (pH 8)

- 1 mM EDTA (pH 8)

4. β- mercaptoethanol

5. Chloroform: isoamyl alcohol (24:1)

6. Isopropanol (chilled)

7. Ethanol (70 and 100 per cent)

8. Distilled water

9. RNase (1 per cent)

Procedure for DNA isolation

Tender leaves of microrhizome plants derived from eighth subculture stage were weighed (0.25 g) and ground in a pre chilled mortar and pestle to a fine powder using liquid nitrogen in presence of a pinch of PVP. The ground sample was mixed with 1 ml 5X CTAB extraction buffer. The homogenized samples were then transferred to 2 ml sterile centrifuge tubes and 50 µl of β-mercaptoethanol was added to it. The solution was mixed well and incubated for 30 minutes at 65 ^{0}C with occasional mixing by gentle inversion. The mixture was then incubated in the ice for 10 minutes. After the incubation, added equal volume of chloroform: isoamyl alcohol (24:1 v/v) to the mixture and inverted to emulsify. Centrifuged the content at 12,000 rpm for 15 minutes at 4 ^{0}C, for separation of the content into distinct phases. The top aqueous layer with DNA was carefully transferred to a clean centrifuge tube. The content was then incubated in 1 µl of RNase at 37 ^{0}C for 45 minutes. After the RNase treatment 1/10th volume of 10 per cent CTAB buffer and equal volume of chloroform: isoamyl alcohol (24:1 v/v) were added and mixed properly by inversion. The mixture was centrifuged at 12,000 rpm for 15 minutes at 4^{0}C. The aqueous phase was again separated carefully into a clean centrifuge tube and 0.6 volume of chilled isopropanol was added and mixed with gentle inversion for the DNA to precipitate. The mixture was incubated at $^-$20 ^{0}C for two hours for complete precipitation of genomic DNA. The mixture was then centrifuged at 10,000 rpm for 10 minutes at 4 ^{0}C, the supernatant was discarded and the pellet was washed with 70 per cent ethanol by centrifuging at 8000 rpm for three minutes at 4 ^{0}C. The DNA pellet was again washed with 100 per cent ethanol by spinning at 8000 rpm for three minutes at 4^{0}C and the ethanol was decanted. The

pellet was air dried for 30 minutes and dissolved in 50 µl of TE buffer. The DNA was then stored at –20 ^{0}C.

3.2.6.2. Assessing the quality of DNA by agarose gel electrophoresis

The quality of genomic DNA isolated can be assessed by agarose gel electrophoresis (Sambrook *et al.,* 1989).

Materials for agarose gel electrophoresis

1. Agarose

· 0.8 per cent for genomic DNA

· 1.8 per cent for ISSR assay

2. 50X TAE buffer (pH 8)

· Tris base

· Glacial acetic acid

· 0.5 M EDTA

3. 6X gel loading dye

· 0.25 per cent bromophenol blue

· 0.25 per cent xylene cyanol

· 30 per cent glycerol

4. Ethidium bromide (0.5 µg/ml)

5. Electrophoresis unit, power pack, gel casting tray, comb

Procedure for agarose gel electrophoresis

Prepared 1X TAE buffer from 50X TAE buffer for electrophoresis. The gel casting tray was prepared by sealing the ends using cello tape. The comb was placed about one inch away from the end of the gel tray and positioned vertically such that the teeth were about 1-2 mm above the surface of the tray. Agarose (0.8 % for genomic DNA and 1.8% for ISSR assay) was weighed and added to the beaker or flask with required volume of 1X TAE buffer. The mixture was melted till clear solution was obtained. The solution was allowed to cool, when the temperature was bearable, ethidium bromide (0.5 µg/ml) was added and mixed well. The solution was poured into the gel casting tray to a depth of about 5 mm. The gel was then allowed to solidify for about 30- 45 minutes at room temperature. Once the gel was set, the comb and the cello tape were removed carefully. The gel was then placed in the electrophoresis chamber and covered (just until wells are submerged) with 1X TAE buffer. The tray was kept in the electrophoresis unit with the well side directed towards the cathode. DNA sample (4 µl) along with a tracking dye (1 µl) was loaded into the wells. λDNA/ *Eco*RI+*Hind*III double digest was used as a molecular marker. After loading the samples the lid of the gel tank was closed and electric leads were attached for the migration DNA towards anode. The gel was run at a voltage of 80V. When the tracking dye reached about 2/3rd length of the gel, the power was turned off. The gel was taken out of the electrophoresis tank and examined under UV transilluminator to check the presence of DNA. The gel profile was examined to study the clarity and intactness of the DNA band. The documentation of the image was done using BIO-RAD gel doc system.

3.2.6.3 Assessing the quantity and quality of the DNA by NanoDrop method

The NanoDrop® ND-1000 is a full spectrum (220-750 nm) spectrophotometer that measures absorbance from 1 µl samples with high accuracy and reproducibility. The NanoDrop spectrophotometer was first connected to the computer and the operating software ND-1000 was opened. Before taking the sample reading, the blank value (zero) was set using the TE

buffer. Then 1 µl of the sample was placed in the pedestal to measure the absorbance at wavelengths of 260 nm and 280 nm. The A_{260}/A_{280} was recorded to assess the purity of DNA. Good quality DNA has an A_{260}/A_{280} value between 1.8 to 2.0. The quantity of DNA in the pure sample was calculated using the formula $A_{260}= 1$ is equivalent to 50 µg double stranded DNA/ µL sample.

1 Absorbance at 260 nm= 50 µg DNA/ml

Therefore, A_{260}x 50X dilution factor gives the quantity of DNA in µg/ml.

3.2.6.4 Molecular marker assay

The clonal fidelity analysis of the microrhizome plants derived from 8th subculture cycle in both TIB and conventional systems were done using specific ISSR marker ISSR 05 as reported by Gavande (2013) at CPBMB.

Inter Simple Sequence Repeat (ISSR) analysis

Inter Simple Sequence Repeat (ISSR) is a technique, which involves amplification of DNA segments present at an amplifiable distance in between two identical microsatellites repeat regions oriented in the opposite direction. The method uses a single primer related to the microsatellite region flanking the ISSR.

Good quality DNA (50 ng/µl) isolated from ginger variety Athira was subjected to ISSR assay. The ISSR 05 primer with good resolving power supplied by 'Sigma Aldrich Chemical Pvt. Ltd.' was used in the study. The amplification of DNA samples was carried out in ProFlex thermocycler using Polymerase Chain Reaction (PCR) technique. The reaction mixture was set to 20 µl volume.

Composition of reaction mixture for PCR

Materials	Quantity (µl)
a) Genomic DNA (50 ng/µl)	- 2.0
b) 10X *Taq* assay buffer B without $MgCl_2$	- 2.0
c) 25 mM $MgCl_2$	-2.0
d) dNTP mix (10 mM)	-1.5
d) *Taq* DNA polymerase (3U)	-0.4
e) Primer	-2.0
f) Autoclaved distilled water	-10.1
Total volume	**- 20.0**

PCR thermal cycling conditions followed:

Steps	Temperature (^{0}C)	Time (minutes)
Initial denaturation	94	4
Denaturation	94	45
Annealing	40	1
Extension	72	2
Final extension	72	8
Storage	4	Infinite

30 reaction cycles were performed.

The PCR for ISSR assay was carried out for 10 DNA samples each from TIB and conventional systems along with source mother DNA. The PCR products obtained from samples of both TIB and conventional systems were run separately in 1.8 per cent agarose gel along with the product from source mother DNA. The ladder (Low range ruler plus (100 bp- 3kb)) was used to identify the product size. The gel images were documented and saved in BIO-RAD gel doc system. The ISSR profile of samples were compared with the profile obtained from source mother plant to identify polymorphism if any in the microrhizome plants derived from 8th subculture cycle in TIB and conventional systems.

3.2.7 Statistical Analysis

Software OPSTAT developed by Chaudhary Charan Singh Haryana Agriculture University was used for statistical analysis. For comparing the responses of different media in TIB and conventional systems and growth of microrhizomes two factor analyses were performed.

Result

4. RESULT

The study entitled "Commercial production of ginger (*Zingiber officinale* Rosc.) microrhizomes using temporary immersion bioreactor system" was conducted at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture Vellanikkara during 2018 to 2020. The objective of the study was to develop an efficient commercial production protocol for ginger microrhizomes using a temporary immersion bioreactor (TIB) system. The study also focused on clonal fidelity analysis of microrhizomes derived from 8th subculture cycle using specific ISSR marker.

The multiple shoot cultures of ginger in the 5th subculture stage of the variety Athira received from the Commercial micropropagation unit, of CPBMB, College of Horticulture, Vellanikkara were utilized for the study. The result obtained from the study is presented in this chapter.

4.1 Development of large-scale production protocol of ginger microrhizomes in temporary immersion bioreactor.

4.1.1 Effect of clump size on shoot proliferation of ginger in different culture systems

Three clump sizes *viz.,* 10, 15 and 20 clumps/ 500 ml of media were used in the experiment. The medium used for the study was MS (Murashige and Skoog, 1962) supplemented with 3 mgL^{-1}of Benzyl Adenine (BA). Different number of clumps were inoculated to liquid medium in Temporary immersion bioreactor and to solid medium in conventional systems. The experiments were carried out separately for both the culture systems with four replications. The number of multiple shoots proliferated from each clump were recorded after 21 days of inoculation from TIB and conventional systems. The effect of clump size on multiple shoot production in different culture systems is presented in Table 1.

The multiple shoot production in TIB and conventional systems was found significantly different. The multiple shoot production in TIB was significantly higher (8.22 shoots/clump) than conventional system (7.96 shoots/clump). The

treatment with 15 clumps/ 500 ml of media showed higher shoot multiplication with 10.25 and 8.82 shoots/clump proliferated in bioreactor and conventional system respectively. In conventional system, shoot proliferation in 10 and 20 clumps/ 500 ml medium were on par, while in bioreactor, shoot multiplication in three clump sizes showed significant difference. Effect of clump size on shoot proliferation in temporary immersion bioreactor is presented in Plate 2 and in conventional microrhizome production is presented in Plate 3.

4.1.2 Effect of multiplication media on shoot proliferation of ginger in different culture systems

Murashige and Skoog (MS) medium supplemented with three different concentrations of BA *viz.*, 2, 3 and 4 mgL^{-1} were used as multiplication media. The clump size optimized in the previous experiment, 15 clumps/ 500 ml medium was inoculated to three different media. The number of multiple shoots proliferated in each medium was recorded after 21 days of inoculation from TIB and conventional systems. The effect of multiplication media on shoot proliferation in different culture systems is given in Table 2.

The observations showed that the shoot proliferation in bioreactor (7.71 shoots/clump) was significantly higher compared to shoot proliferation in conventional microrhizome production system (5.24 shoots/clump) regardless of the multiplication media used in the culture systems. The result showed that there is no significant effect of different media on shoot proliferation in both culture systems. Number of shoots proliferated were on par in the three-multiplication media tried and ranged from 7.33-8.11 shoots/clump in TIB and 4.48-5.85 shoots/clump in conventional system. The culture system and media interaction effects were also found significant with respect to shoot proliferation. Effect of multiplication media on shoot proliferation in TIB is presented in Plate 4 and in conventional microrhizome production is presented in Plate 5.

10 clumps/ 500ml medium
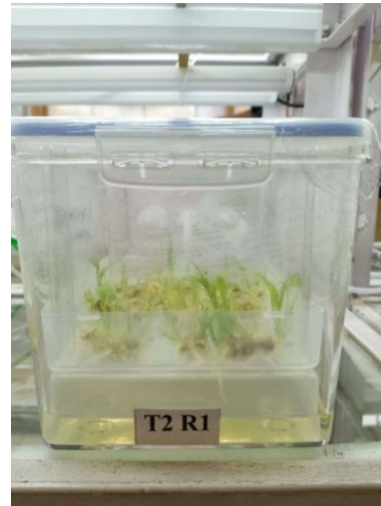
15 clumps/ 500ml medium
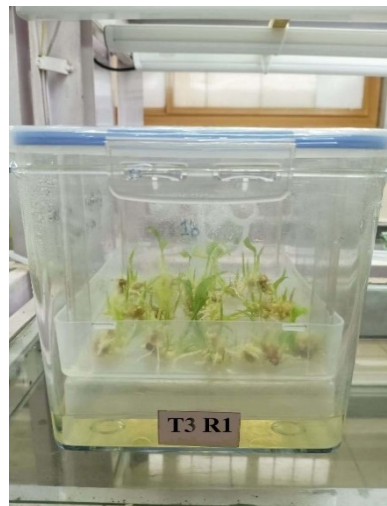
20 clumps/ 500ml medium

Plate 2: Effect of clump size on shoot proliferation in temporary immersion bioreactor.

10 clumps/ 500ml medium

15 clumps/ 500ml medium

20 clumps/ 500ml medium

Plate 3: Effect of clump size on shoot proliferation in conventional microrhizome production.

MS+2 mgL^{-1} BA

MS+3 mgL^{-1} BA
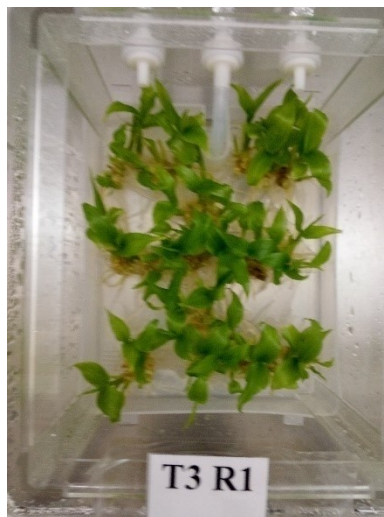
MS+4 mgL^{1} BA

Plate 4: Effect of multiplication media on shoot proliferation in temporary immersion bioreactor.

MS+2 mgL ^{1} BA

MS+3 mgL ^{1} BA

MS+4 mgL ^{1} BA

Plate 5: Effect of multiplication media on shoot proliferation in conventional microrhizome production

Table 1. Effect of clump size on shoot proliferation in different culture systems

Culture Systems	Number of clumps/ 500ml medium			Mean number of shoots proliferated/ clump/culture system
	10	**15**	**20**	
	*Mean number of shoots proliferated/ clump			
Temporary immersion bioreactor	8.42	10.25	5.99	8.22
Conventional microrhizome production	7.62	8.82	7.42	7.96
CD (0.05)	**Factor A (culture system)**	**Factor B (No. of clumps)**		**Factor A×B**
	0.205	0.251		0.356
SE (m)	0.069	0.084		0.119

*Mean of different clump sizes, No. of replications– 4

Table 2. Effect of multiplication media on shoot proliferation in different culture systems

Culture Systems	Multiplication media			Mean number of shoots proliferated/ clump/culture system
	MS+2 mgL^{-1} BA	MS+3 mgL^{-1} BA	MS+4 mgL^{-1} BA	
	*Mean Number of shoots proliferated/ clump			
Temporary immersion bioreactor	7.33	8.11	7.70	7.71
Conventional microrhizome production	5.85	5.38	4.48	5.24
CD (0.05)	Factor A (culture system)	Factor B (multiplication media)		Factor A×B
	0.559	NS		0.968
SE (m)	0.187	0.229		0.323

*Mean of 15 observations / replication, No. of replications– 4

4.1.3 Effect of microrhizome induction medium on various characters of ginger microrhizome in different culture systems

For induction of microrhizomes, MS medium with different concentrations of sucrose (70, 80 and 90 gL^{-1}) were tried. In each medium, 15 clumps were inoculated for microrhizome induction with four replications. The microrhizome induction in three different media tried were recorded after two months of incubation from TIB and conventional systems. In both the culture systems, microrhizome induction was observed along with shoot multiplication. Observations on days taken for microrhizome induction, number of microrhizomes

produced, length of shoot and number of leaves, number of roots, root length in microrhizome plantlets and weight of microrhizomes were recorded. Microrhizome plantlets produced in temporary immersion bioreactor is presented in Plate 6 and in conventional microrhizome production is presented in Plate 7. Effect of microrhizome induction media on various characters of ginger microrhizome in different culture systems is presented in Plate 8.

a. Days taken for microrhizome induction

The time taken for microrhizome induction in different microrhizome induction media in different culture systems is given in Table 3. Early induction of microrhizomes was observed in TIB system. In TIB, microrhizomes were induced 36.17 days after inoculation while in conventional system it took 44.33 days for microrhizome induction. In both the culture systems, microrhizome induction was faster in MS medium with 90 gL^{-1} sucrose followed by 80 gL^{-1} and 70 gL^{-1} of sucrose. The days taken for microrhizome induction showed significant difference in the three different media tried in both the culture systems, Murashige and Skoog (MS) media + 90 gL^{-1} sucrose took only 31 days for microrhizome induction in bioreactor while in conventional system it took 40.50 days for microrhizome induction. The interaction effects of culture system and media were not significant.

b. Number of microrhizomes produced per 500 ml of media

The effect of microrhizome induction media on number of microrhizome produced per 500 ml of media is shown in Table 4. The culture system, media and media× culture system interaction effects were not significant for number microrhizomes produced per 500 ml of media. The microrhizomes produced in TIB varied from 87.75 to 96.75 and in conventional system from 84 to 88.

c. Shoot growth in microrhizome plantlets

The effect of microrhizome induction media on length of shoot in microrhizome plantlets in different culture systems are given in Table 5. The culture system, media and media× culture system interaction effects were found

MS+70 gL^{-1} sucrose

MS+80 gL^{-1} sucrose

MS+90 gL^{1} sucrose

Plate 6: Microrhizome plantlets produced in temporary immersion bioreactor

MS+70 gL^{-1} sucrose

MS+80 gL^{-1} sucrose

MS+90 gL^{-1} sucrose

Plate 7: Microrhizome plantlets produced in conventional microrhizome production

MS+70 gL ^{1} sucrose (TIB)

MS+70 gL ^{1} sucrose (conventional micropropagation)

MS+80 gL ^{1} sucrose (TIB)

MS+80 gL ^{1} sucrose (conventional micropropagation)

MS+90 gL ^{1} sucrose (TIB)

MS+90 gL^{-1} sucrose (conventional micropropagation)

Plate 8: Effect of microrhizome induction media on various characters of ginger microrhizome in different culture systems

significant in shoot length. The observations showed that microrhizome plantlets obtained from TIB gave significantly higher shoot growth as compared to the microrhizome plantlets obtained from conventional systems. The shoot length obtained in TIB was 9.23 cm while it was 6.69 cm in conventional system. In bioreactor MS mediumwith70 gL^{-1} and 80 gL^{-1} of sucrose did not show any significant difference in shoot length in microrhizome plantlets. The treatment with 90 gL^{-1} of sucrose showed the lowest shoot length (8.01 cm) in bioreactor. In the conventional systems lower sucrose concentration exhibited higher shoot length 7.48 cm as in TIB. The length of shoots in MS medium with 80 gL^{-1} and 90 gL^{-1} of sucrose was found on par in conventional system and were 6.38 and 6.21 cm respectively.

Table 3. **Days taken for microrhizome induction in ginger in different microrhizome induction media in different culture systems**

Culture Systems	Microrhizome induction media			Days taken for microrhizome induction / culture system
	MS+70 gL^{-1} sucrose	MS+80 gL^{-1} sucrose	MS+90 gL^{-1} sucrose	
	* Mean number of days taken for microrhizome induction			
Temporary immersion bioreactor	40.75	36.75	31.00	36.17
Conventional microrhizome production	48.00	44.50	40.50	44.33
CD (0.05)	Factor A (culture system)	Factor B (Microrhizome induction media)		Factor A×B
	0.988	1.210		NS
SE (m)	0.330	0.404		0.571

*Mean of 20 observations / replication, No. of replications – 4

Table 4. Effect of microrhizome induction media on number of microrhizome per 500 ml of media in different culture systems

Culture Systems	Microrhizome induction media			No. of microrhizomes produced/ 500 ml medium/ culture system
	MS+70 gL^{-1} sucrose	MS+80 gL^{-1} sucrose	MS+90 gL^{-1} sucrose	
	No. of microrhizomes produced/ 500 ml medium			
Temporary immersion bioreactor	87.75	88.50	96.75	91.00
Conventional microrhizome production	84.00	88.00	84.75	85.58
CD (0.05)	Factor A (culture system)	Factor B (Microrhizome induction media)		Factor A×B
	NS	NS		NS
SE (m)	1.921	2.353		3.328

No of replications -4

Table 5. Effect of microrhizome induction media on length of shoot in ginger microrhizome plantlets in different culture systems

Culture Systems	Microrhizome induction media			Length of shoot in microrhizome plantlet / culture system (cm)
	MS+70 gL^{-1} sucrose	MS+80 gL^{-1} sucrose	MS+90 gL^{-1} sucrose	
	* Length of shoot in microrhizome plantlet (cm)			
Temporary immersion bioreactor	9.86	9.82	8.01	9.23
Conventional microrhizome production	7.47	6.38	6.21	6.69
CD (0.05)	Factor A (culture system)	Factor B (Microrhizome induction media)		Factor A×B
	0.297	0.363		0.514
SE (m)	0.099	0.121		0.172

*Mean of 20 observations / replication, No. of replications – 4

d. Number of leaves in microrhizome plantlets

The effect of microrhizome induction media on number of leaves in microrhizome plantlets in different culture systems is presented in Table 6.The culture system, media and media × culture systems interaction effects were found significant with respect to number of leaves. Microrhizome plantlets produced in TIB showed higher number of leaves (3.45) compared to conventional system (2.65) irrespective of the media used. In TIB, highest number of leaves was obtained in MS medium with 70 gL^{-1} of sucrose and number of leaves was significantly different in three media tried. In conventional system, the media with 70 gL^{-1} and 80 gL^{-1} of sucrose were found on par. In both the culture systems, lowest number of leaves was observed in the treatment with 90 $gL^{-1}$of sucrose.

e. Number of roots in microrhizome plantlets

The effect of microrhizome induction media on number of roots in microrhizome plantlet in different culture systems is given in Table 7. The TIB showed higher number of roots (8.9) compared to conventional systems (7.79) irrespective media tried. In TIB, the highest number of roots was recorded in the medium with 90 gL^{-1} sucrose (9.37). The treatments with 70 gL^{-1} and 80 gL^{-1} sucrose were found on par for number of roots in TIB and number of roots were 8.54 and 8.79 respectively. In conventional system, treatment with 80 gL^{-1} and 90 gL^{-1}sucrose did not show any significant difference in number of roots and were higher than medium with 70 gL^{-1} sucrose. Interaction effects of the culture system and media were not significant.

f. Length of roots in microrhizome plantlets

The effect of microrhizome induction media on root length in microrhizome plantlets in different culture systems is presented in Table 8. Culture system and various microrhizome induction media tried were found significant with respect to root length in microrhizome plantlets. But the interaction effect of culture systems and media tried were not significant. The root length in microrhizome plantlets from TIB was significantly higher (10.02 cm) than that from conventional system (6.08 cm). The root length in MS medium with 80 gL^{-1} and 90 gL^{-1} sucrose were

found on par and higher than root length in medium with 70 gL^{-1} sucrose in both the culture systems. The root length was found less in treatment with 70 gL^{-1} sucrose in both TIB (9.60 cm) and conventional system (5.57 cm). Higher sucrose concentration was found to give higher root length in both the culture systems.

g. Weight of microrhizomes

The effect of microrhizome induction media on weight of ginger microrhizomes in different culture systems is presented in Table 9. The culture system, media and media × culture system interaction effects were found significant with respect to weight of microrhizomes. The microrhizomes from bioreactor recorded higher weight (0.24 g) as compared to conventional system (0.18 g). In both the culture systems, the highest weights of microrhizomes was recorded in MS medium with 90 gL^{-1} sucrose and were 0.29 g in bioreactor and 0.21 g in conventional system. As the concentration of sucrose was reduced in the medium corresponding reduction in weight of microrhizomes was also noted.

Table 6. Effect of microrhizome induction media on number of leaves in microrhizome plantlet in different culture systems

Culture Systems	Microrhizome induction media			Number of leaves/ microrhizome plantlet/ culture system
	MS+70 gL⁻¹ sucrose	MS+80 gL⁻¹ sucrose	MS+90 gL⁻¹ sucrose	
	*Mean number of leaves/ microrhizome plantlet			
Temporary immersion bioreactor	4.16	3.64	2.54	3.45
Conventional microrhizome production	2.80	2.70	2.45	2.65
CD (0.05)	Factor A (culture system)	Factor B (Microrhizome induction media)		Factor A×B
	0.189	0.228		0.323
SE (m)	0.062	0.076		0.108

*Mean of 20 observations / replication, No. of replications – 4

Table 7. Effect of microrhizome induction media on number of roots per microrhizome plantlet in different culture systems

Culture Systems	Microrhizome induction media			Number of roots/ microrhizome plantlet/ culture system
	MS+70 gL^{-1} sucrose	MS+80 gL^{-1} sucrose	MS+90 gL^{-1} sucrose	
	*Mean number of roots/ microrhizome plantlet			
Temporary immersion bioreactor	8.54	8.79	9.37	8.90
Conventional microrhizome production	7.42	7.97	7.96	7.79
CD (0.05)	Factor A (culture system)	Factor B (Microrhizome induction media)		Factor A×B
	0.363	0.444		NS
SE (m)	0.121	0.148		0.210

*Mean of 20 observations / replication, No. of replications – 4

Table 8. Effect of microrhizome induction media on root length in ginger microrhizome plantlets in different culture systems

Culture Systems	Microrhizome induction media			Root length/ microrhizome plantlet/culture system (cm)
	MS+70 gL^{-1} sucrose	MS+80 gL^{-1} sucrose	MS+90 gL^{-1} sucrose	
	* Root length/ microrhizome plantlet (cm)			
Temporary immersion bioreactor	9.60	10.42	10.04	10.02
Conventional microrhizome production	5.57	6.29	6.39	6.08
CD (0.05)	Factor A (culture system)	Factor B (Microrhizome induction media)		Factor A×B
	0.398	0.487		NS
SE (m)	0.133	0.163		0.230

*Mean of 20 observations / replication, No. of replications – 4

Table 9. Effect of microrhizome induction media on weight of ginger microrhizomes in different culture systems

Culture Systems	Microrhizome induction media			Weight of microrhizomes/ culture system (g)
	MS+70 gL^{-1} sucrose	MS+80 gL^{-1} sucrose	MS+90 gL^{-1} sucrose	
	* Weight of microrhizomes (g)			
Temporary immersion bioreactor	0.16	0.27	0.29	0.24
Conventional microrhizome production	0.15	0.17	0.21	0.18
CD	Factor A (culture system)	Factor B (Microrhizome induction media)		Factor A×B
	0.006	0.007		0.010
SE (m)	0.002	0.002		0.003

*Mean of 20 observations / replication, No. of replications – 4

4.1.4 Hardening and Acclimatization

The *in vitro* induced ginger microrhizome plantlets were planted out in protrays with 98 wells filled with peat, perlite and vermiculite in the proportion 3:1:1 and kept inside the hardening chamber for primary hardening for one month. The microrhizome plantlets from different culture systems were planted out treatment wise separately. The survival rate was recorded after one month of primary hardening. After primary hardening, microrhizome plants were transferred to polythene bags containing sand, soil and cow dung at 1:1:1 ratio and were kept for secondary hardening for two months. The survival rate and growth parameters

of the microrhizome plants such as height of the plant and number of leaves were recorded after two months of secondary hardening. Microrhizome plants from temporary immersion bioreactor and after two months of secondary hardening is presented in Plate 9 and from conventional microrhizome production is presented in Plate 10.

The survival of microrhizome plants after primary hardening in different culture systems is presented in Table 10. The survival of microrhizome plants after primary hardening was 94.34 per cent in TIB and 93.85 per cent in conventional microrhizome production system irrespective of microrhizome induction media. The survival per cent of microrhizome plants in different induction media ranged from 93.57 to 94.90 per cent in TIB and 92.56 to 94.60 per cent in conventional system. The survival rate of microrhizome plants after secondary hardening was 99.29 per cent in TIB and 99.17 per cent in conventional microrhizome induction system. The survival of microrhizomes induced in different media was also above 99 per cent in both the culture systems. Survival of microrhizome plants after secondary hardening in different culture system is shown in Table 11.

MS+70 gL^{-1} sucrose

MS+80 gL^{-1} sucrose

MS+90 gL^{-1} sucrose

Plate 9: Microrhizome plants from temporary immersion bioreactor after two months of secondary hardening

MS+70 gL^{-1} sucrose

MS+80 gL^{-1} sucrose

MS+90 gL^{-1} sucrose

Plate 10: Microrhizome plants from conventional microrhizome production after two months of secondary hardening

Table 10. Survival of ginger microrhizome plants after primary hardening

Culture systems	Microrhizome induction media	No. of microrhizomes planted out	No. of micorhizomes survived after one month	Survival (%)	Survival (%) in different culture systems
Temporary immersion bioreactor	MS + 70 gL^{-1} Sucrose	311	291	93.57	94.34
	MS + 80 gL^{-1} Sucrose	353	335	94.90	
	MS + 90 gL^{-1} Sucrose	387	366	94.57	
Conventional microrhizome production	MS + 70 gL^{-1} Sucrose	336	311	92.56	93.85
	MS + 80 gL^{-1} Sucrose	352	333	94.60	
	MS + 90 gL^{-1} Sucrose	339	320	94.40	

Table 11. Survival of microrhizome plants after secondary hardening

Culture systems	Microrhizome induction media	No. of microrhizomes planted after primary hardening	No. of micorhizomes survived after two months	Survival (%)	Survival (%) in different culture systems
Temporary immersion bioreactor	MS + 70 gL^{-1} Sucrose	291	289	99.31	99.29
	MS + 80 gL^{-1} Sucrose	335	333	99.40	
	MS + 90 gL^{-1} Sucrose	366	363	99.18	
Conventional microrhizome production	MS + 70 gL^{-1} Sucrose	311	309	99.36	99.17
	MS + 80 gL^{-1} Sucrose	333	330	99.10	
	MS + 90 gL^{-1} Sucrose	320	317	99.06	

4.1.4.1 Growth parameters of ginger microrhizome plants after secondary hardening

Growth parameters such as, height and number of leaves of microrhizome plants produced from two culture systems were recorded sixty days after secondary hardening.

Height of microrhizome plants

The height of plants derived from different microrhizome induction media and culture systems are shown in Table 12. Microrhizome plants from bioreactor recorded significantly higher plant height (18.60 cm) compared to conventional system (14.80 cm). Microrhizomes induced in lowest sucrose concentration exhibited more height in bioreactor and height was found to decrease as the concentration of sucrose increased. In bioreactor, the plant height showed

significant difference in plants from three microrhizome induction media tried. The height recorded for microrhizome plants from MS medium with 70 gL^{-1} of sucrose was the highest (21.77 cm) in bioreactor followed by 80 gL^{-1} (17.92 cm) and 90 gL^{-1} (16.09 cm) of sucrose. In microrhizome plants of conventional system the height recorded for plants from MS medium with 80 gL^{-1} and 70 gL^{-1} of sucrose were on par and higher than plants from medium with 90 gL^{-1} of sucrose. The culture system, media and media × culture system interaction effects were found on par with respect to plant height.

Number of leaves in microrhizome plants

The number of leaves recorded in microrhizome plants of both the culture systems was on par and ranged from 6.97 to 7.18. In both TIB and conventional systems the number of leaves in the treatment with MS media with 70 gL^{-1} and 80 gL^{-1} sucrose was found on par. The interaction effects of media and culture system were non significant. The number of leaves in microrhizome plants derived from different microrhizome induction media and culture systems are given in Table 13.

Table 12. Height of microrhizome plants derived from different microrhizome induction media and culture systems (two months after secondary hardening)

Culture Systems	Microrhizome induction media			Plant height (cm)/ culture system
	MS+70 gL^{-1} sucrose	MS+80 gL^{-1} sucrose	MS+90 gL^{-1} sucrose	
	* Plant height (cm)			
Temporary immersion bioreactor	21.77	17.92	16.09	18.60
Conventional microrhizome production	16.12	16.24	12.02	14.80
CD (0.05)	Factor A (culture system)	Factor B (Microrhizome induction media)		Factor A×B
	0.495	0.606		0.857
SE (m)	0.165	0.202		0.286

*Mean of 20 observations / replication, No. of replications – 4

Table 13. Number of leaves in microrhizome plants derived from different microrhizome induction media and culture systems (two months after secondary hardening)

Culture Systems	Microrhizome induction media			No. of leaves/ plant / culture system
	MS+70 gL^{-1} sucrose	MS+80 gL^{-1} sucrose	MS+90 gL^{-1} sucrose	
	* Mean number of leaves/ microrhizome plant			
Temporary immersion bioreactor	7.60	7.54	5.77	6.97
Conventional microrhizome production	7.55	7.65	6.36	7.18
CD (0.05)	Factor A (culture system)	Factor B (Microrhizome induction media)		Factor A×B
	NS	0.303		NS
SE (m)	0.083	0.101		0.143

*Mean of 20 observations / replication, No. of replications – 4

4.2 Clonal fidelity analysis using ISSR marker

4.2.1 Genomic DNA isolation

Genomic DNA for clonal fidelity analysis was isolated from microrhizomes induced after the eighth subculture cycle from both bioreactor and conventional systems. Ten random microrhizome plants were selected from both the systems and tender leaves were used for DNA isolation. The source DNA of the mother plant was obtained from CPBMB.

Good quality DNA was isolated using modified version of CTAB procedure reported by Roger and Benedich (1994).

4.2.2 Assessing the quantity and quality of the DNA by NanoDrop method

The quality and quantity of DNA isolated were assessed using agarose gel electrophoresis and NanoDrop spectrophotometer respectively. The gel profile under the UV transilluminator showed clear and intact bands. The A_{260}/A_{280} was recorded to assess the purity of DNA. The DNA showed an A_{260}/A_{280} value between 1.8 to 2.0 which indicated good quality of DNA. The DNA isolated was diluted to 50 ng/µL for clonal fidelity analysis. The quantity analysis of isolated DNA is given in Table 14.

4.2.3 Clonal fidelity analysis using specific ISSR marker

The clonal fidelity analysis was done using the ISSR (Inter Simple Sequence Repeats) marker (ISSR 05) as reported by Gavande, (2013). The PCR for ISSR assay was carried out for 10 DNA samples of microrhizomes induced after 8th subculture cycle each from TIB and conventional systems along with source mother DNA. The PCR products obtained from samples of both TIB and conventional system were run separately in 1.8 per cent agarose gel along with the product from source mother DNA. The ISSR profile of samples was compared with the profile obtained from source mother plant to identify polymorphism. The DNA amplification pattern in microrhizome plants from temporary immersion bioreactor is presented in Plate 11 and from conventional microrhizome production is presented in Plate 12.

The gel profile was scored to observe number of amplicons generated, number of monomorphic and polymorphic amplicons formed. The amplification with ISSR 05 primer generated ten amplicons in the size range of 350-1100 bp. The analysis of amplification pattern showed that there were no polymorphic amplicons in the microrhizomes induced after the 8th subculture and microrhizome plants produced after 8th subculture cycle are true to type (Table 15).
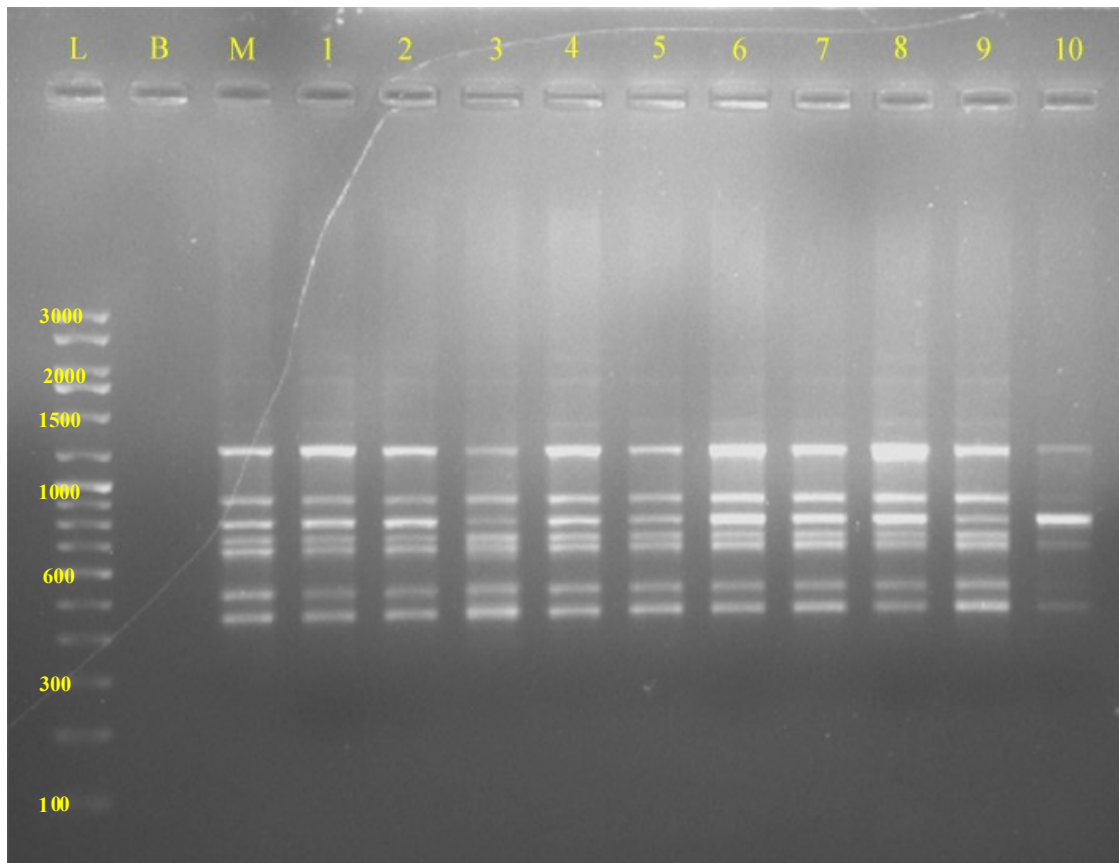
Plate 11: DNA amplification pattern in microrhizome plants from temporary immersion bioreactor

L - Molecular weight marker (3 kb), B - blank, M - mother plant, 1 to 10 - regenerants
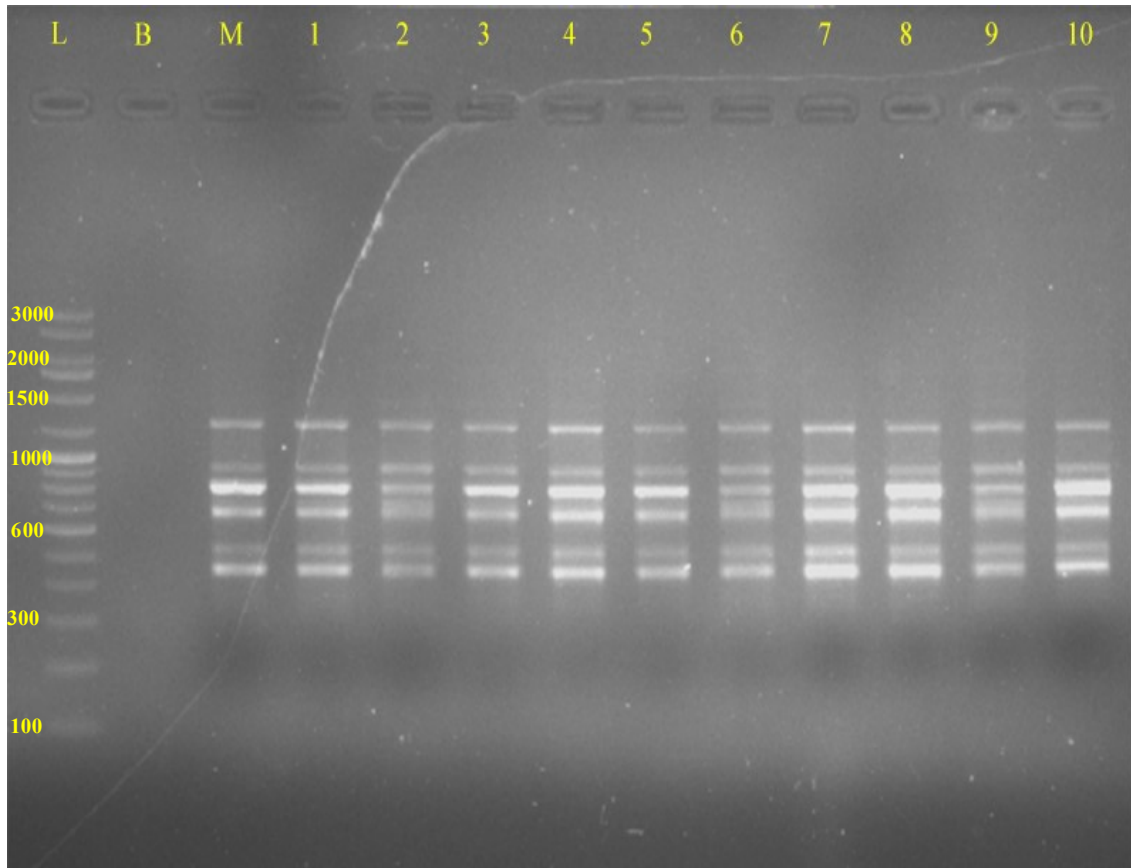
Plate 12: DNA amplification pattern in microrhizome plants from conventional microrhizome production

L   Molecular weight marker (3 kb), B   blank, M   mother plant, 1 to 10   regenerants

Table 14. Quantity analysis of DNA isolated from microrhizome plants

	Temporary immersion bioreactor			Conventional microrhizome production	
Sample No.	Concentration (ng/µL)	Absorbance ratio (A260/A280)	Sample No.	Concentration (ng/µL)	Absorbance ratio (A260/A280)
1	132.9	2.00	1	542.1	1.82
2	465.8	1.82	2	497.7	1.99
3	507.7	1.91	3	548.4	1.82
4	542.9	1.87	4	180.9	1.97
5	474.9	1.81	5	761.2	1.80
6	326.5	1.96	6	455.7	2.00
7	430.2	1.84	7	520.2	2.00
8	489.5	1.81	8	678.8	2.00
9	275.4	1.94	9	891.5	1.85
10	299.3	1.92	10	882.8	1.88

Table 15 (a). DNA amplification pattern in microrhizome plants from Temporary immersion bioreactor using ISSR 05 primer

Mother plant/ microrhizome plants	Total No. of amplicons	No. of monomorphic amplicons	No. of polymorphic amplicons
Source mother plant (Athira)	7	-	-
Microrhizome plants from temporary immersion bioreactor			
1	7	7	0
2	7	7	0
3	7	7	0
4	7	7	0
5	7	7	0
6	7	7	0
7	7	7	0
8	7	7	0
9	7	7	0
10	7	7	0

Table 15 (b). DNA amplification pattern in microrhizome plants from conventional microrhizome production system using ISSR 05 primer

Mother plant/ microrhizome plants	Total No. of amplicons	No. of monomorphic amplicons	No. of polymorphic amplicons
Source mother plant(Athira)	7	-	-
Microrhizome plants from conventional microrhizome production system			
1	7	7	0
2	7	7	0
3	7	7	0
4	7	7	0
5	7	7	0
6	7	7	0
7	7	7	0
8	7	7	0
9	7	7	0
10	7	7	0

Discussion

5. DISCUSSION

The study entitled "Commercial production of ginger (*Zingiber officinale* Rosc.) microrhizomes using temporary immersion bioreactor system" was to develop an efficient commercial production protocol for ginger microrhizomes using a temporary immersion bioreactor (TIB) system. The study also focused on clonal fidelity analysis of microrhizomes derived from 8th subculture cycle using specific ISSR marker.

The multiple shoot cultures of ginger in the 5th subculture stage of the variety Athira received from the Commercial micropropagation unit, of CPBMB, College of Horticulture, Vellanikkara were utilized for the study. The results obtained from the experiments are discussed in this chapter.

5.1 Development of large scale production protocol of ginger microrhizomes in temporary immersion bioreactor.

51.1 Effect of clump size on shoot proliferation of ginger in different culture systems

Three clump sizes *viz.,* 10, 15 and 20 clumps/ 500 ml of medium were inoculated to liquid medium in temporary immersion bioreactor and solid medium in conventional systems. The result showed that the multiple shoot production in TIB was significantly higher (8.22 shoots/clump) than conventional system (7.96 shoots/clump).

Mudoi *et al*. (2014) studied the effect of clump size on multiplication and growth of bamboo, the result showed that 4 shoots/clump was the best propagule size for *in vitro* shoot multiplication of bamboo with a maximum shoot numbers (11.40), shoot length (4.66 cm) and leaf numbers (18.60). They observed that apart from optimal medium composition the regeneration capacity of the *in vitro* shoot was found to be dependent upon the size and number of shoots/clump and time of sub-culturing.

The treatment with 15 clumps/ 500 ml of medium showed higher shoot multiplication with 10.25 and 8.82 shoots/clump proliferated in bioreactor and conventional system respectively. Volume of liquid medium is a very important factor affecting the efficacy of temporary immersion bioreactor for plant development (Etienne and Berthouly, 2002). This behaviour is probably due to the fact that a larger volume of medium per explants could dilute growth promoting components excreted by the explants (Ramos-Castella *et al*., 2014).

Lorenzo *et al*. (1998), suggested that an optimum volume of medium per explant (50.0 ml) is required for *Saccharum spp*. for shoot proliferation in the TIB. The result showed that increasing the volume from 5.0 to 50.0 ml per explant an increase in shoot proliferation was obtained. But when the volume per explants was increased further the efficiency was reduced. Similar to the present study, the work also demonstrated that the Twin Flasks system for temporary immersion clearly stimulated shoot formation and length. The study reported that multiplication rate (23.9 shoots/30 days) was found six times greater compared to standard protocol reported in other studies.

A study conducted in pineapple with different volume of culture media, *viz*., 50, 100, 200 and 250 ml per explant showed that higher multiplication rate was observed when a ratio of 200 ml per explant was used in TIB. The volume higher than 200 ml per explant showed decrease in multiplication rate and fresh and dry weight (Escalona *et al*., 1999).

Arano-Avalos *et al*. (2019) worked in *Colocasia esculenta* to show that, adding 25 or 50 ml of culture medium per explant favoured the number of shoots per explant, as well as the length and number of leaves per shoot, while a volume of 12.5 and 100 ml per explant caused a decrease in the evaluated variables.

5.1.2 Effect of multiplication media on shoot proliferation of ginger in different culture systems

Murashige and Skoog medium supplemented with three different concentrations of BA *viz.*, 2, 3 and 4 mgL^{-1} were used as multiplication media. The

clump size optimized in the previous experiment; 15 clumps/ 500 ml medium was used to inoculate to three different media. The number of multiple shoots proliferated in each medium was recorded after 21 days of inoculation in TIB and conventional systems.

The observations showed that the shoot proliferation in bioreactor (7.71 shoots/clump) was significantly higher compared to shoot proliferation in conventional microrhizome production system (5.24 shoots/clump) regardless of the multiplication media used in the culture systems. Number of shoots proliferated were on par in the three-multiplication media tried and ranged from 7.33-8.11 shoots/clump in TIB and 4.48-5.85 shoots/clump in conventional system. The reason for the rapid growth performance in TIB seemed partly due to high gas exchange capacity of the culture vessel (Hulscher *et al.*, 1996). The specific design of TIB has a direct effect on the *in vitro* development of plants, as it provides different physical culture conditions, which in turn have a direct impact on the *in vitro* environment, light availability, space, water, and nutrients (Georgiev *et al.*, 2014; Szopa *et al.*, 2017).

The effect of benzyle adenine (BA) on shoot proliferation has been reported earlier in ginger by Rout *et al.* (2001), Kavyashree (2009), Abbas *et al.* (2011), and Ayenew *et al.* (2012). The effect of BA may be due to its ability to induce the metabolism or production of the natural endogenous hormones for morphogenesis induction (Ahmed and Anis 2014). The study conducted by Miri (2020), showed that multiplication of shoot did not occur in the absence of cytokinin in the media and an increase in the BA concentration from 1 to 10 mgL^{-1} resulted in an increase in the induction of axillary shoots. The shoot proliferation was highest in the MS medium supplemented with 10 mgL^{-1} BA.

Alvard *et al.* (1993) showed that applying liquid medium strongly influenced the development and proliferation rate of micropropagated banana explants. The result showed a multiplication rate of 5.2 shoots/ explant in TIB while it was 2.2 and 3.1 shoots/explant in semi solid medium and partial immersion system.

Stanly *et al.* (2010) studied the response of shoot proliferation in TIB, shake flask and semi solid medium in *Curcuma zedoaria* and *Zingiber zerumbet*. The study revealed that shoot multiplication varied significantly in three different systems, with higher multiplication rate in TIB and shake flask system when compared to the conventional semi solid cultures. The result showed that in shake flask system 5.8 shoots/ explant was produced while in TIB it was 4.7 shoots/explants were produced. The study suggest that the reason for better shoot proliferation in liquid medium may be due to better uptake and availability of nutrients.

Highest multiplication rate and weight gain in TIB were also reported in banana by Farhani and Majd (2012). Those cultures grown in solid medium and subcultured in liquid medium with cotton substrate had multiplication rates of 2.7 to 3.5 shoots/ explant and explants grown in the TIB had the highest multiplication rate of 7 shoots/ explant.

Jo *et al.* (2007) investigated an efficient propagation method through airlift bioreactor system for *Zingiber officinale* Rosc., the effect of different factors and bioreactors on cultured plantlets were investigated. As in the case of present study they also observed that plant growth regulators did not affect the number of plantlets and mean fresh weight, but significant differences were observed with different culture vessel types. The Bottle Type Bubble Bioreactor (airlift type) culture was dramatically rapid and greater than the suspension culture on the number of plantlets, and fresh weight per plant.

5.1.3 Effect of microrhizome induction media on various characters of ginger microrhizome in different culture systems

For induction of microrhizomes, MS medium with different concentrations of sucrose (70, 80 and 90 gL^{-1}) were tried. Observations on days taken for microrhizome induction, number of microrhizomes produced, length of shoot, number of leaves and roots, root length in microrhizome plantlets and weight of microrhizomes were recorded in three different media tried after two months of incubation in TIB and conventional system.

a. Days taken for microrhizome induction

Early induction of microrhizomes was observed in TIB system. In TIB, microrhizomes were induced 36.17 days after inoculation while in conventional system it took 44.33 days for microrhizome induction (Fig. 1). The observations showed that number of days to induce microrhizome in TIB system was 22.5 per cent lesser compared to conventional system. In both the culture systems, microrhizome induction was faster in MS medium with 90 gL^{-1} sucrose followed by 80 gL^{-1} and 70 gL^{-1} of sucrose.

Arora *et al.* (1996) reported that the *in vitro* induction of storage organs such as bulbs, corms and tuber can be promoted with high concentration of sucrose. The increase in sucrose concentration has an enhancing effect on rate of *in vitro* microrhizome formation, which may be attributed due to the presence of high carbon energy in sucrose since rhizomes mostly contain carbohydrates and sucrose (Nayak, 2000). Abbas *et al.* (2014) reported that MS medium with 60-90 gL^{-1} sucrose were the best level of carbon source for induction of ginger microrhizomes.

Teisson and Alvard (1999), reported that in potato number and individual weight of microtuber developed in TIB, ten weeks after inoculation were better than those obtained from the semisolid cultures.
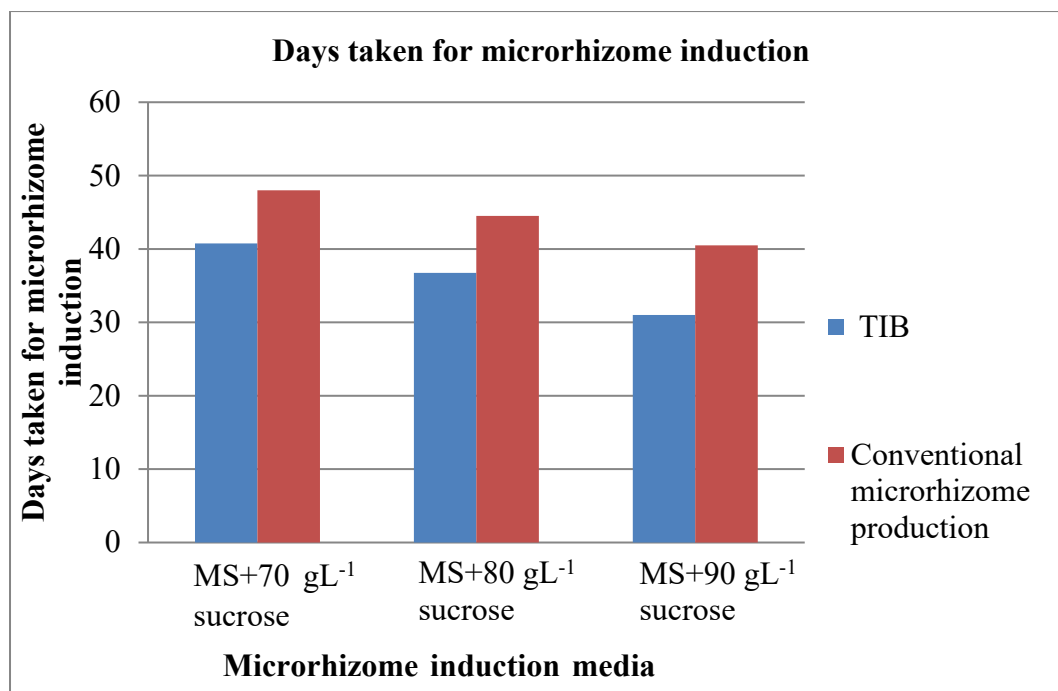
Fig. 1: Days taken for microrhizome induction in different microrhizome induction media in different culture systems

b. Number of microrhizomes produced per 500 ml of medium

The culture system, media and media× culture system interaction effects were not significant for number microrhizomes produced per 500 ml of media. The microrhizomes produced in TIB varied from 87.75 to 96.75 and in conventional system from 84 to 88.

Teisson and Alvard (1999) used twin flask based double RITA system for microtuber induction in potato. The study showed that temporary immersion bioreactors can efficiently induce microtuber *in vitro*. An incubation period of 10 weeks resulted in obtaining three microtubers per single node, with half of them weighing more than 0.5 g.

The study conducted by Young *et al.* (2000), evaluated the effect of two aeration levels during protocorm-like bodies proliferation. A low aeration rate was compared with a high rate of aeration. An identical growth was observed for both aeration rates, indicating that in the range of tested speed, aeration had no effect on biomass growth. The study indicated that aeration rate is one of the factors that may

affect the growth of propagules/explant in liquid cultures. Young *et al*. (2000) studied the *in vitro* multiplication of protocorm–like bodies in continuous immersion system (air-lift type) and temporally immersion system. The result indicates that all types of bioreactor system were suitable for protocorm–like bodies proliferation in liquid medium. However, temporary immersion bioreactor with activated charcoal filter attached was most suitable. In TIB 17 protocorm–like bodies per protocorm section was produced and biomass of protocorm–like bodies was also highest compared to other bioreactor systems.

A study conducted by Neimenak *et al*. (2013) evaluated that in cocoyam conventional system produced 15.2 microtubers, which is however 4.5 times lower than the proliferation rate achieved in TIB. The result showed that TIB is one step further in the process of large-scale propagation of cocoyam.

c. Shoot growth in microrhizome plantlet

The observations showed that microrhizome plantlets obtained from TIB gave significantly higher shoot growth as compared to the conventional system (Fig. 3). The shoot growth was 38 per cent more in TIB compared to conventional system. The shoot length obtained in TIB was 9.23 cm while it was 6.69 cm in conventional system. In bioreactor MS medium with 70 gL^{-1} and 80 gL^{-1} of sucrose did not show any significant difference in shoot length in microrhizome plantlets. The treatment with 90 gL^{-1} of sucrose showed the lowest shoot length (8.01 cm) in bioreactor. A similar result was obtained by Marfori and Cruz (2018) on effect of sucrose on *in vitro* grown ginger showed that shoot length was highest when ginger was cultured in a medium containing 60 gL^{-1} (14.7 cm). The shoot growth was inhibited when sucrose concentration was increased to 90 gL^{-1} and 120 gL^{-1}, with shoot lengths of 11.8 cm and 6.7 cm, respectively as in present study.

Aitken–Christie and Jones (1987) showed that better shoot growth could be obtained for *Pinus radiate* by replenishing liquid nutrient medium in contrast to monthly subculturing on semi solid medium. The study showed that without subculturing the system showed continuous shoot growth. The study also revealed

that shoots derived from partial and temporary immersion system exhibited better growth and quality compared semi solid medium.

Ramirez-Mosqueda *et al.* (2019), demonstrated that the highest shoot length was observed in TIB and Ebb-and-Flow, with 1.59 and 1.51 cm, respectively; the lowest shoot length was noted in RITA, with 1.39 cm in the *in vitro* propagation of anthurium.
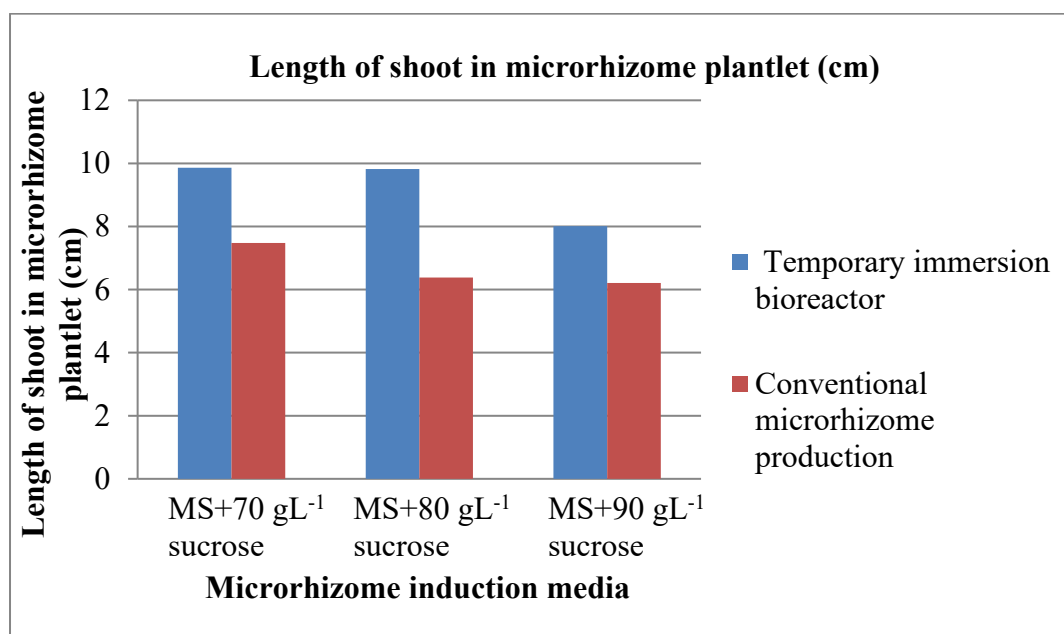
Fig. 2: Effect of microrhizome induction media on length of shoot in ginger microrhizome plantlets in different culture systems

d. Number of leaves in microrhizome plantlet

Microrhizome plantlets produced in TIB showed higher number of leaves (3.45) compared to conventional system (2.65) irrespective of the media used (Fig. 4). Number of leaves per microrhizome plantlets was 30.20 per cent more in TIB compared to conventional system. In TIB, highest number of leaves was obtained in MS medium with 70 gL^{-1} of sucrose. In both the culture systems, lowest number of leaves was observed in the treatment with 90 gL^{-1} of sucrose.

Similar to the result of the present study, Bello-Bello *et al.*, (2019) reported that plants from TIS had the highest number of leaves per explant, while those from

semi-solid medium and liquid medium in partial immersion had the lowest number of leaves.

Number of leaves/ microrhizome plantlet

Fig. 3: Effect of microrhizome induction media on number of leaves in microrhizome plantlet in different culture systems

e. Number of roots in microrhizome plantlet

The TIB showed higher number of roots (8.9) compared to conventional systems (7.79) irrespective media tried (Fig. 5). Number of roots per microrhizome plantlet was 14.25 per cent more in TIB compared to conventional system. In TIB, the highest number of roots was recorded in the medium with 90 gL^{-1} sucrose (9.37). Neimenak *et al*. (2013), suggested that in cocoyam the application of high sucrose concentration can promote root induction and elongation on completely differentiated shoots. The effect of sucrose on plant development may be due to its ability to acts as a sensor agent that triggers the activity of a group of genes that govern plant development (Sheen *et al*., 1999; Rolland *et al*., 2006).

Study conducted by Ayenew *et al.* (2013), showed that TIB gave an average root number of 16.33 which was higher compared to the semisolid culture.
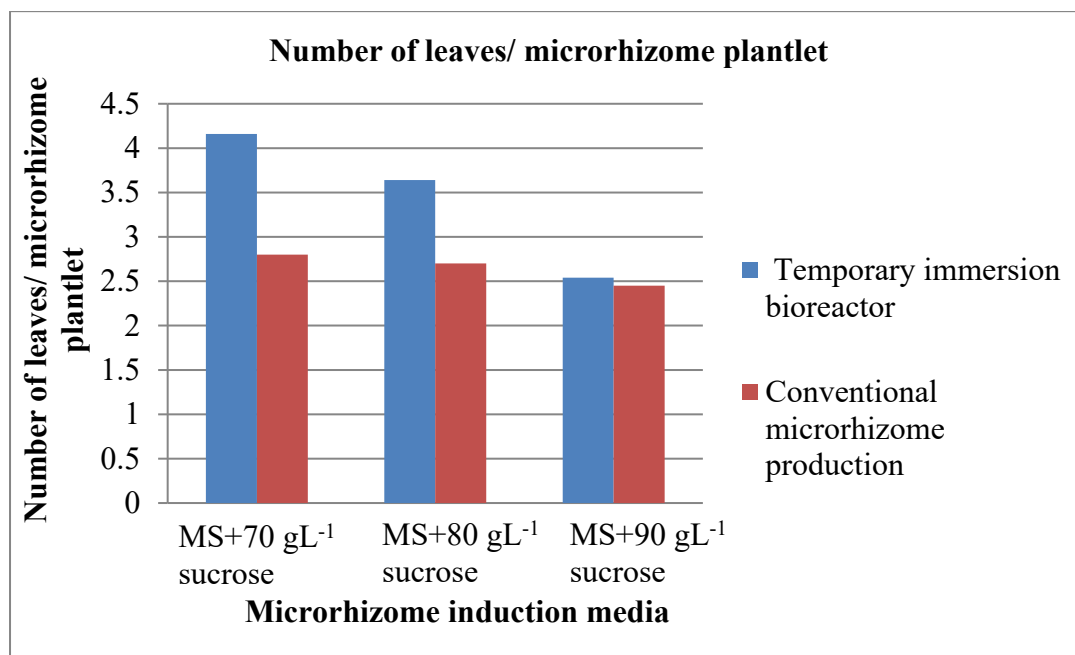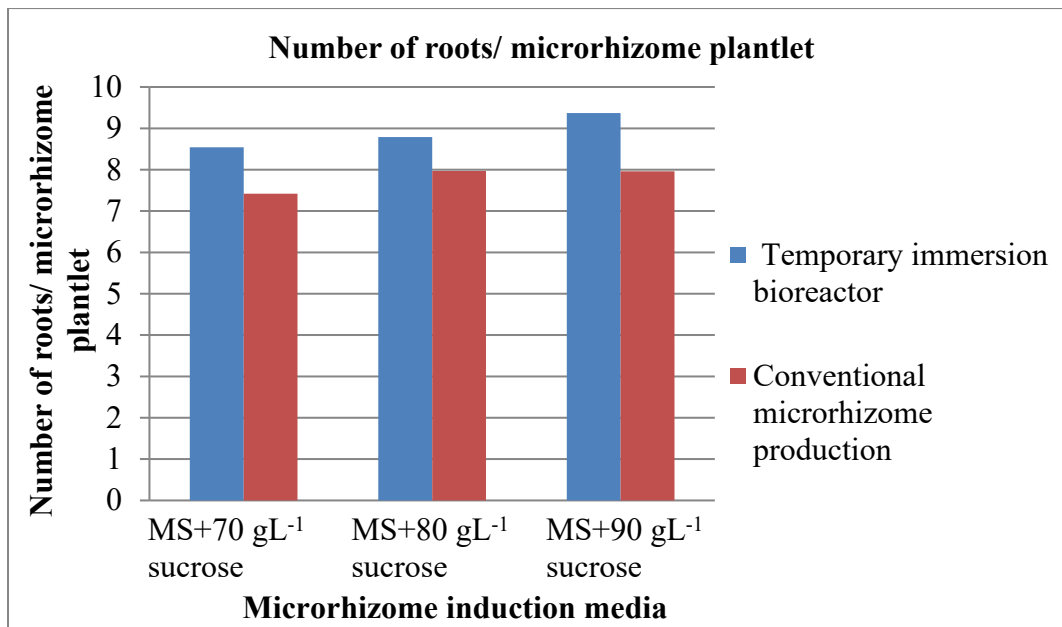
Fig. 4: Effect of microrhizome induction media on number of roots in microrhizome plantlet in different culture systems

f. Length of roots in microrhizome plantlets

The root length in microrhizome plantlets from TIB was significantly higher (10.02 cm) than that from conventional system (6.08 cm (Fig. 7). The root of microrhizome plantlet obtained from TIB were 64.80 per cent more longer compared to conventional system. The root length in MS medium with 80 gL^{-1} and 90 gL^{-1}sucrose were found on par and higher than root length in medium with 70 gL^{-1} sucrose in both the culture systems. The root length was found less in treatment with 70 gL^{-1} sucrose in both TIB (9.60 cm) and conventional system (5.57 cm).

In pineapple, TIB system produced significantly higher root length compared to semisolid culture system (Ayenew *et al*., 2013). The authors suggested that the TIB culture combines the advantages of both solid and liquid medium. Solid cultures allow aeration but not provide full contact with nutrient media while liquid medium permits an efficient nutrient uptake even though hyperhydricity was seen often (Smith and Spoomer 1995; Aitcken-Christie *et al*. 1995).
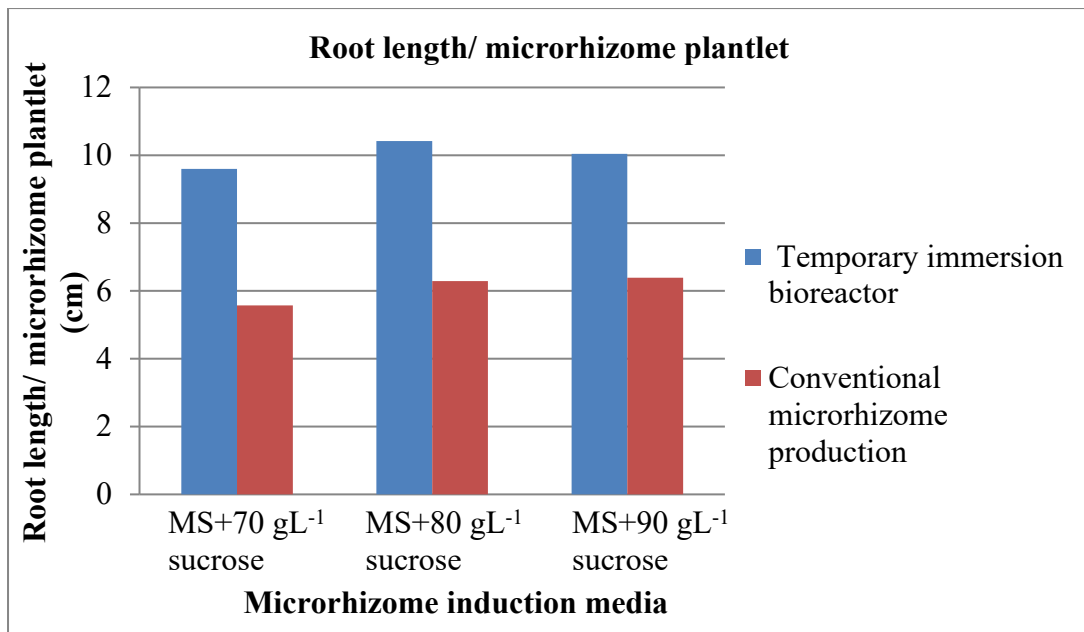
Fig. 5: Effect of microrhizome induction media on root length in ginger microrhizome plantlets in different culture systems

g. Weight of microrhizomes

The microrhizomes from bioreactor recorded higher weight (0.24 g) as compared to conventional system (0.18 g) (Fig. 9). The experiment showed that microrhizomes obtained from TIB has 33.33 per cent more weight compared to conventional system. In both the culture systems, the highest weight of microrhizomes was recorded in MS medium with 90 gL^{-1} sucrose and were 0.29 g in bioreactor and 0.21 g in conventional system. As the concentration of sucrose was reduced in the medium corresponding reduction in weight of microrhizomes was also noted. Sucrose provides carbon and energy for the shoot and microrhizome induction in ginger. So, the concentration of sucrose had a significant impact on *in vitro* microrhizome induction (Mehboob *et al.*, 2019).

Jamenez *et al.* (1999) suggested that, in temporary immersion bioreactor, the liquid medium was in contact with all parts of the plant for short periods, thus the tuber induction was more uniform among the axillary buds resulting in more tuber formation. The study also showed that in TIB, the size and weight of the tubers was greater than in solid cultures.

Teisson and Alvard (1999), evaluated that the weight of individual microtubers obtained, from a single node section in TIB and was better than those described in the study in semi-solid medium.
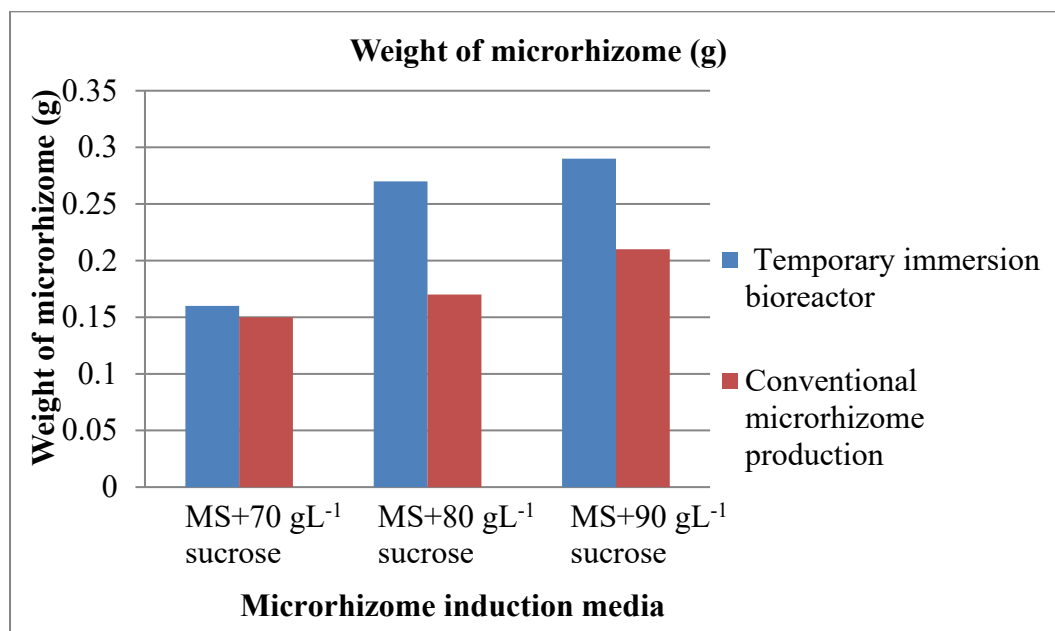
Fig. 6: Effect of microrhizome induction media on weight of ginger microrhizomes in different culture systems

5.1.4 Hardening and Acclimatization

The survival of microrhizome derived plants after primary hardening was 94.34 per cent in TIB and 93.85 per cent in conventional microrhizome production system irrespective of microrhizome induction media. The survival per cent of microrhizome derived plants after primary hardening in different induction media ranged from 93.57 to 94.90 per cent in TIB and 92.56 to 94.60 per cent in conventional system. The survival rate of microrhizome plants after secondary hardening was 99.29 per cent in TIB and 99.17 per cent in conventional microrhizome induction system. Growth parameters such as, height and number of leaves of microrhizome derived plants produced from two culture systems were recorded sixty days after secondary hardening. Microrhizome plants from bioreactor recorded significantly higher plant height (18.60 cm) compared to conventional system (14.80 cm). Microrhizome derived plants induced in lowest

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sucrose medium exhibited more height in bioreactor and height was found to decrease as the concentration of sucrose increased. The number of leaves recorded in microrhizome derived plants of both the culture systems was on par and ranged from 6.97 to 7.18.

Etienne and Berthouly (2002) and Debnath (2017) reported that temporary immersion technique has high *ex vitro* survival rate during acclimatization due to the hardening of *in vitro* plants caused by myxotrophic activity. Sugarcane plantlets obtained from solid culture medium and TIB showed similar growth pattern after acclimatization. Temporary immersion bioreactor derived plantlets of *Curcuma zedoaria* and *Zingiber zerumbet* from the three different micropropagation systems (agar-gelled medium cultures, shake flask system and temporary immersion system**)** grew normally as that of the mother plants after acclimatization. The study suggests that developed root system might be the major factor leading to proper acclimatization of plantlets from all the three systems tested (Stanly *et al.*, 2010).

Study conducted by Ayenew *et al.* (2013), reported that seedlings derived from TIB performed better and fast in acclimatization, this could be due to the availability of more number of roots and root hairs. Similar to the finding, higher number of roots and longer roots were observed in TIB in the present study.

5.2 Clonal fidelity analysis using ISSR marker

5.2.1 Genomic DNA isolation

Genomic DNA for clonal fidelity analysis was isolated from microrhizomes induced after the eighth subculture cycle from both bioreactor and conventional systems. Ten random microrhizome plants were selected from both the systems and tender leaves were used for DNA isolation. The source DNA of the mother plant was obtained from CPBMB. Good quality DNA was isolated using modified version of CTAB procedure reported by Roger and Benedich (1994).

The extraction buffer which contains CTAB detergent helps in disrupting the cell wall and release the nucleic acid into the buffer. EDTA present in Extraction buffer and TE buffer helps in chelating the Mg^{2+} ion which acts as the

cofactor for DNase enzyme and thus help in preserving the genomic DNA. The chloroform: isoamylalcohol helps in the separation of organic mixture and aqueous phase during DNA isolation (Sambrook *et al*., 1989). For proper extraction of genomic DNA, it is essential that the sample has undergone proper homogenization, pulverization and uniformity grinding. The liquid nitrogen helps in maintaining the tissue under frozen condition to prevent nucleic acid degradation and secondary metabolites oxidation that can form some complexes which could co-precipitate with the DNA. It also helps in mechanical disruption of the tissue in the mortar (Hernandez and Oyarzum, 2006). The β-mercaptoethanol helps in degradation of protein by disrupting the disulphide bonds. The polyvinylpyrrolidone (PVP) helps in removing polyphenols and inhibited the co-precipitation of polysaccharides (Matasyoh *et al*., 2008).

5.2.2 Assessing the quantity and quality of the DNA by NanoDrop method

The quality and quantity of DNA isolated were assessed using agarose gel electrophoresis and NanoDrop spectrophotometer respectively. The gel profile under the UV transilluminator showed clear and intact bands**.** The A_{260}/A_{280n} was recorded to assess the purity of DNA. The DNA showed an A_{260}/A_{280} value between 1.8 to 2.0 which indicated good quality of DNA.

5.2.3 Clonal fidelity analysis using specific ISSR marker

The clonal fidelity analysis was done using the ISSR (Inter Simple Sequence Repeats) marker (ISSR 05) as reported by Gavande, (2013). The PCR for ISSR assay was carried out for 10 DNA samples of microrhizomes induced after 8th subculture cycle each from TIB and conventional system along with source mother DNA. The ISSR profiles of samples were compared with the profile obtained from source mother plant to identify polymorphism. The analysis of amplification pattern showed that there were no polymorphic amplicon in the microrhizome plants produced after 8th subculture cycle are true to type.

The inter-simple sequence repeats (ISSR) are molecular marker that amplifies intermicrosatellite region at multiple loci throughout the genome by a 16-18 bp long single primer (Zietkiewicz *et al*., 1994). Each amplicon corresponds to a

unique DNA sequence between two inverted microsatellites, leading to multilocus and highly polymorphic patterns in which fragments often show polymorphism between different individuals (Nagaoka and Ogihara, 1997). The ISSR is a technique which combines the benefits of Amplified Fragment Length Polymorphism (AFLP) and microsatellite analysis with the universality of Randomly Amplified Polymorphic DNA (RAPD). It has high reproducibility due to the use of longer primers (16 to 25 bp) as compared to RAPD primers (10 bp) which permits the subsequent use of high annealing temperature (45 to 60 ^{0}C) leading to high stringency (Reddy *et al.*, 2002).

Somaclonal variation is one of the major issues associated with *in vitro* culture which arises due continuous subculturing of *in vitro* culture of plant cell tissues and organs (Larkins and Scowcroft 1981). Somaclonal variation is mainly caused due to rapid multiplication of a tissue or long-term cultures (Reuveni *et al.*, 1993). The ISSR marker system is are proved as an efficient system to assess the genetic integrity among micropropagated plants as reported by several workers in different species (Zietekiewicz *et al.*, 1994; Bhatia *et al.*, 2009; Mohanty *et al.*, 2011; Bhatia *et al.*, 2011).

Mohanty *et al.* (2011) studied, the genetic stability using RAPD and ISSR analysis in micropropagated *Zingiber rubens* from sprouted buds of rhizomes. They developed an efficient protocol for the micropropagation of *Z. rubens* and the evaluation clonal fidelity revealed that the clones were true to type, as the RAPD and ISSR marker assay showed no polymorphism.

Mohanty *et al.*, (2012), utilised RAPD and ISSR analysis for the assessment of micropropagated clones of *Zingiber zerumbet* for genetic stability. The result showed that the micropropagation protocol developed is effective for the rapid multiplication *Z. zerumbet*. The study confirmed that all the clones generated were genetically uniform.

The study conducted by Estrada *et al.* (2017), evaluated the somaclonal variation during micropropagation of sugarcane using temporary immersion bioreactor (TIB) using inter simple sequence repeat (ISSR) molecular markers. The

molecular analysis showed that the *in vitro* establishment and the number of subcultures are the factors that affected the frequency of somaclonal variation during the micropropagation of sugarcane. The result showed that after the first subculture, the variation between the intermediate subcultures (from two to eight) was stable and increased in the final ninth and tenth subcultures. Therefore, the determination of optimum number of subcultures that can be made from an explant for each species to be micropropagated is important.

Purayil *et al*. (2018), assessed the clonal fidelity using ISSR marker in the *in vitro* generated plantlets of *Moringa peregrine* developed through direct organogenesis. The progenies were highly similar to their mother plant as the similarity indices between the parental plants and their progenies were above 98.2 percent.

Baby *et al*. (2019), reported that ISSR markers can be utilized to confirm the genetic integrity of micropropagated plantlets of *Vanda* hybrid 'Dr.Anek'. Mother plants and their respective clones were subjected to clonal fidelity studies using ISSR assay with five ISSR primers reported for *Vanda* hybrids. The banding pattern was analysed to reveal the genetic stability thus validating the micropropagation protocol developed for the hybrid.

5.3 Development of large-scale production protocol for ginger microrhizomes in temporary immersion bioreactor

Fig 7: Production protocol for ginger microrhizomes using temporary immersion bioreactor

Culture establishment and subculturing upto 5th subculture cycle in conventional micropropagation system in MS + 3 mgL^{-1} BA (12 weeks)

⇩

Multiplication of ginger shoots in TIB for 6th, 7th and 8th subculture at an interval of 3 weeks in MS + 3 mgL^{-1} BA (9 weeks)

⇩

Ginger microrhizome induction in TIB after 8th subculture in MS + 90 gL^{-1} sucrose for two months (8 weeks)

⇩

Microrhizome plantlets are planted out for primary hardening for one month (4 weeks)

⇩

Secondary hardening of microrhizome plantlets for two months (8 weeks)

⇩

Clonal fidelity analysis

⇩

Tissue culture derived microrhizome plants ready for sale

Established cultures of ginger variety Athira in the 5th subculture cycle received from the commercial micropropagation unit of CPBMB were utilized for the study. The cultures were subcultured in TIB up to eighth subculture. The multiple shoots of ginger were proliferated in MS medium supplemented with 3 mgL^{-1} BA. The duration of each subculture was 21 days. After the eighth subculture the multiple shoots were transferred to microrhizome induction medium supplemented with 90 gL^{-1} sucrose and incubated for two months. The microrhizomes obtained from the TIB system was transferred to protrays filled with peat, perlite and vermiculite in the proportion 3:1:1 and kept inside the hardening chamber for primary hardening. After one month of primary

hardening, microrhizomes were transferred to polythene bags containing sand, soil and cowdung at 1:1:1 ratio and were kept for secondary hardening for two months. The clonal fidelity of microrhizome plants was checked using specific ISSR marker.

Fig 8: Possible multiplication of ginger microrhizomes in TIB and conventional microrhizome production system

Establishment of explant

⇩

1st subculture (one shoot)

⇩

2nd subculture (two shoots)

⇩

3rd subculture (four shoots)

⇩

4th subculture (eight shoots)

⇩

5th subculture (24 shoots)

Temporary immersion bioreactor	Conventional micropropagation
6th subculture ($15 \times 8 = 120$ shoots)	6th subculture ($15 \times 5 = 75$ shoots)
7th subculture ($120 \times 8 = 960$ shoots)	7th subculture ($75 \times 5 = 375$ shoots)
8th subculture ($960 \times 8 = 7680$ shoots)	8th subculture ($375 \times 5 = 1875$ shoots)
Microrhizome induction ($7680 \times 6 = 46080$ microrhizome plantlets)	Microrhizome induction ($1875 \times 6 = 11250$ microrhizome plantlets)
Microrhizomeplants (46080)	Microrhizome plants (11250)

The number of plants that can be produced in TIB and conventional system and the possible multiplication rates are compared in Fig. 8. The subculturing for shoot proliferation of the ginger variety Athira upto eighth subculture showed that more number of multiple shoots can be produced in TIB with starting clump size 15 clumps/ 500 ml of MS medium supplemented with 3 mgL^{-1} BA. The subculturing of multiple shoots up to eighth subculture resulted in 7680 shoots in TIB with a multiplication of 8 shoots/clump, while in conventional system only 1875 shoots can be produced with a multiplication of 5 shoots/clump. The multiple shoots obtained from the eighth subculture cycle can be transferred to MS medium with 90 gL^{-1} sucrose for the microrhizome induction in both the culture systems. Along with induction of microrhizomes, shoot multiplication is also observed. The shoot multiplication was similar in both the culture systems for microrhizome induction media (6 shoots/clump). Hence from TIB 46080 microrhizome plantlets and from conventional microrhizome production system 11250 microrhizome plantlets can be produced.

The possible shoot multiplication and production of plant show that approximately 46080 microrhizome plantlets from a single explant can be produced using TIB with in a culture period of 9.5 months. The number of microrhizome plants that can be produced using conventional microrhizome production system in the same culture period is 11250 plants from a single explant. The higher installation cost required for TIB can be compensated in the long run with the higher multiplication rate in TIB and less unit cost of production.

Table 16. Comparison of media cost in TIB and conventional microrhizome production system

Subculture	No. of multiples	Media (Litre)	Media cost (Rs)		Subculture	No. of multiples	Media (Litre)	Media cost (Rs)	
			Media cost/ microrhizome plantlet in TIB					Media cost/ microrhizome plantlet in conventional microrhizome production	
			Laboratory grade chemicals	Commercial grade chemicals				Laboratory grade chemicals	Commercial grade chemicals
5th	24	0.5	9.5	2.5	5th	24	0.5	43	11
6th	120	0.5	9.5	2.5	6th	75	0.5	43	11
7th	960	4.0	76	20	7th	375	2.5	215	60
8th	7680	32	608	160	8th	1875	12.5	1075	275
Microrhizome induction	46080	512	24576	4096	Microrhizome induction	11250	125	14500	3000
Total			25279	4281				15876	3357
Media cost/plantlet			0.55	0.09				1.41	0.30

Comparison of media cost in TIB and conventional microrhizome production system is presented in Table 16. The MS medium supplemented with 3 mgL^{-1} BA costs about Rs 19/L without laboratory grade agar while it cost Rs 86/L with laboratory grade agar. The cost of commercial grade MS medium supplemented with 3 mgL^{-1} without marine agar is about Rs 5/L and with marine agar is about Rs 22/L. The induction of microrhizome was done using MS media with 90 gL^{-1} of sucrose costs about Rs 48/L without laboratory grade agar while it cost Rs 116/L with laboratory grade agar. The commercial grade microrhizome induction media costs about Rs 8/L without marine agar while it cost Rs 24/L with marine agar. The media cost required to produce one microrhizome plantlet using laboratory grade chemicals in TIB without agar is about Rs 0.55 while it is Rs 1.41 per microrhizome plantlet in conventional microrhizome production system with agar. The cost of media required to produce one microrhizome plantlet in commercial grade chemicals in TIB without agar is about Rs 0.09 while it is Rs 0.30 per microrhizome plantlet in conventional microrhizome production system with agar. The observations show that induction of microrhizome in TIB is more cost effective compared to conventional microrhizome production system.

A commercially viable production protocol for true to type ginger microrhizomes using a Plantform temporary immersion bioreactor was developed in the present study. Early induction of microrhizomes, reduction in microrhizome induction period and better growth of microrhizome plants are the advantages of Plantform TIB system over conventional microrhizome production system.

The protocol developed in the present study can be further modified by early bulking of the clumps, employing TIB for multiple shoot production in early culture phase and reducing the number of culture cycles so that the entire protocol period can be reduced. Evaluation of TIB microrhizome plants in high tech poly house or field and comparison of yield and quality with microrhizome plants from conventional microrhizome production system are the research areas to be focused further.

Summary

6. SUMMARY

The study entitled "Commercial production of ginger (*Zingiber officinale* Rosc.) microrhizomes using temporary immersion bioreactor system" was conducted at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture Vellanikkara during 2018 to 2020. The objective of the study was to develop an efficient commercial production protocol for ginger microrhizomes using a Temporary Immersion Bioreactor (TIB) system.
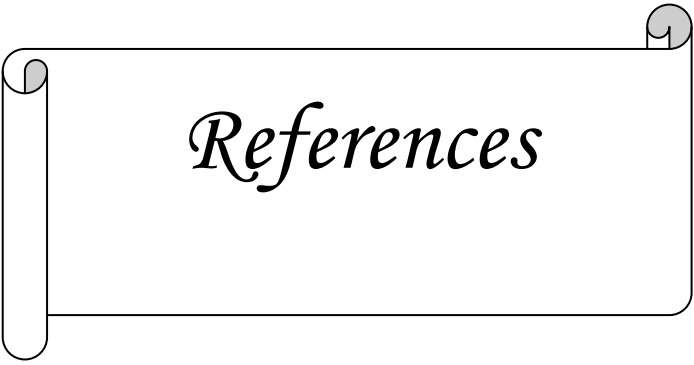
The multiple shoot cultures in the 5th subculture stage of the ginger variety Athira received from the Commercial micropropagation unit, of CPBMB, College of Horticulture, Vellanikkara were utilized for the study. The protocol for *in vitro* induction of microrhizomes in ginger developed at CPBMB by Shylaja *et al*. (2016) was followed for production of microrhizomes. The clonal fidelity analysis of microrhizome plants derived from 8th subculture cycle was done using specific ISSR marker as reported by Gavande, (2013). The Plantform TIB designed by Professor Margareta Welander and Dr A J Sayegh purchased from Sweden was used for the study.

Salient findings of the study are summarised below:

- Three clump sizes *viz.,* 10, 15 and 20 clumps/ 500 ml of medium were evaluated in TIB and conventional system. The treatment with 15 clumps/ 500 ml of medium showed higher shoot proliferation of 10.25 and 8.82 shoots/clump in bioreactor and conventional system respectively.

- Shoot proliferation in bioreactor (7.71 shoots/clump) was significantly higher compared to shoot proliferation in conventional microrhizome production system (5.24 shoots/clump).

- No significant effect of different media on shoot proliferation in both culture systems (MS+ 2, 3 and 4 mgL^{-1} BA).

- Early induction of microrhizome was observed in TIB. Microrhizomes were induced 36.17 days after inoculation in TIB, while in conventional system it took 44.33 days for microrhizome induction.

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- Murashige and Skoog medium with 90 gL^{-1} sucrose took only 31 days for microrhizome induction in bioreactor while in conventional system it took 40.50 days for microrhizome induction.

- The number of microrhizomes produced in TIB varied from 87.75 to 96.75 /500ml medium and in conventional system it varied from 84 to 88/500 ml medium.

- Microrhizome plantlets produced in TIB recorded significantly higher shoot growth as compared to the microrhizome plantlets produced in conventional systems.

- The Murashige and Skoog medium with 90 gL^{-1} of sucrose showed less shoot length both in bioreactor (8.01 cm) and conventional microrhizome production system (6.21 cm).

- Microrhizome plantlets produced in TIB showed higher number of leaves (3.45) than the conventional system (2.65) irrespective of the media used.

- In TIB, the highest number of leaves was obtained in MS medium with 70 gL^{-1} of sucrose. In conventional system, the MS media with 70 gL^{-1} and 80 gL^{-1} of sucrose were found on par in leaf production. In both the culture systems, lowest number of leaves was observed in MS medium with 90 gL^{-1} of sucrose.

- Microrhizome plantlets from TIB showed higher number of roots (8.9) compared to plantlets from conventional systems (7.79) irrespective media tried.

- The microrhizome plantlets from TIB recorded the highest number of roots in MS medium with 90 gL^{-1} sucrose (9.37).

- The root length in microrhizome plantlets produced in TIB was significantly higher (10.02 cm) than that from conventional system (6.08 cm).

- The root length in MS media with 80 gL^{-1} and 90 gL^{-1} sucrose were found on par and higher than root length in medium with 70 gL^{-1} sucrose in both the culture systems.

- The microrhizomes from bioreactor recorded higher weight (0.24 g) as compared to conventional system (0.18 g).

- In both the culture systems, the highest weight of microrhizomes was recorded in MS medium with 90 gL^{-1} sucrose and were 0.29 g in bioreactor and 0.21 g in conventional system.

- The survival of microrhizome plants after primary hardening was 94.34 per cent in TIB and 93.85 per cent in conventional microrhizome production system irrespective of microrhizome induction media.

- The survival rate of microrhizome plants after secondary hardening was 99.29 per cent in TIB and 99.17 per cent in conventional microrhizome induction system.

- Microrhizome plants from bioreactor recorded significantly higher plant height (18.60 cm) compared to conventional system (14.80 cm).

- Microrhizome plants induced in lowest sucrose concentration (70 gL^{-1}) exhibited more height (21.77 cm) in bioreactor and height of the plants was found to decrease as the concentration of sucrose increased in MS medium. The same trend was observed in conventional microrhizome production system also.

- The number of leaves recorded in microrhizome plants of both the culture systems was on par and ranged from 6.93 to 7.19.

- The clonal fidelity analyses of microrhizome plants produced after 8th subculture cycle in both the culture systems were done using specific ISSR marker (ISSR 05).

- There were no polymorphic amplicons in the microrhizome plants produced after the 8th subculture cycle and plants produced are thus true to type.

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Appendices

ANNEXURE I

List of laboratory equipments used for the study

Refrigerated centrifuge   : Kubota, Japan

Horizontal electrophoresis system   : Biorad, USA

Thermal cycler   : ProFlex PCR system
                                     (Life Technologies)

Gel documentation system   : Biorad, USA

Nanodrop® ND-1000 spectrophotometer   : Nanodrop® Technologies lnc.USA

ANNEXURE II

Chemical composition of the Murashige and Skoog medium

Stock	Chemical	mg/ Litre	Stock concentration	Stock
	KNO_3	1,900		95.0 g/l
	KH_2PO_4	170		8.5 g/l
	$MgSO_4.7H_2O$	370		18.5 g/l
II	$CaCl_2.2H_2O$	440	50 X	22.0 g/l

(Prepare the stock separately or it may precipitate)

Stock	Chemical	mg/ Litre	Stock concentration	Stock
III	Na_2EDTA	37.3	100 X	3.7 g/l
	$FeSO_4.7H_2O$	27.8		2.8 g/l

(Remember to prepare this as described under stock solution preparation)

Stock	Chemical	mg/ Litre	Stock concentration	Stock
IV	$MnSO_4.4H_2O$	22.3	100X	2.23 g/l
	$ZnSO_4.7H_2O$	8.6		860mg/l
	H_3BO_3	6.2		620 mg/l
	Kl	0.83		83.0 mg/l
	$Na_2MoO_4.2H_2O$	0.250		25.0 mg/l
	$CuSO_4.5H_2O$	0.025		2.5 mg/l
	$CoCl_2.6H_2O$	0.025		2.5 mg/l

Glycine	2.0	200 mg/l
Nicotinic acid	0.5	50 mg/l
Pyridoxine- HCL	0.5	50 mg/l
Thiamin- HCI	0.1	10 mg/l
Myo- inositol	100	
Agar	7.5 g/l	
sucrose	30 g/l	
pH	5.7- 5.8	

ANNEXURE III

Reagents required for DNA isolation

Reagents:

1. 5X CTAB extraction buffer (100 ml)

CTAB   : 5g

(Cetyl trimethyl ammonium bromide) Tris HCl   : 1.21 g

EDTA   : 0.745 g

NaCl   : 8.18 g

Adjusted the pH to 8 and made up final volume up to 100 ml.

2. CTAB (10 %, 100 ml)

CTAB   : 10 g

NaCl   : 4.09 g

3. Chloroform- Isoamyl alcohol (24:1 v/v)

To chloroform (24 parts), isoamyl alcohol (1 part) was added and mixed properly.

4. Chilled isopropanol

Isopropanol was stored in refrigerator at 0ºC and was used for the study.

5. Ethanol (70 %)

To the 70 parts of absolute ethanol (100 %), 30 parts of sterile distilled water was added to make 70 per cent ethanol.

6. TE buffer (pH 8, 100 ml) Tris HCl (10 mM) : 0.1576 g EDTA (1 mM)   : 0.0372 g

The solution was prepared, autoclaved and stored at room temperature

ANNEXURE IV

Composition of buffers and dyes used for gel electrophoresis

1. TAE Buffer 50X

Tris base   : 242 g

Glacial acetic acid   : 57.1 ml

0.5M EDTA (pH 8.0): 100 ml

2. Loading Dye (6X)

0.25% bromophenol blue

0.25% xylene cyanol

30% glycerol in water

3. Ethidium bromide

The dye was prepared as a stock solution of 10 mg/ml in water and was stored at room temperature in a dark bottle.

COMMERCIAL PRODUCTION OF GINGER (*Zingiber officinale* ROSC.) MICRORHIZOMES USING TEMPORARY IMMERSION BIOREACTOR SYSTEM

By,

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(2018-11-005)

ABSTRACT OF THE THESIS

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ABSTRACT

Ginger (*Zingiber officinale* Rosc.), is an important commercial spice crop grown in India from very ancient times. High seed rate of ginger (1500 kg/ha) and desiccation of seed rhizomes during storage are the problems faced by farmers in ginger cultivation. The tissue culture plants are not commercially distributed in ginger as they require an additional one more season for rhizome formation. Microrhizomes of ginger induced *in vitro* if used as planting materials, rhizomes can be harvested in the same season as conventional seed rhizomes and year round availability of seed material can be ensured. Hence, *in vitro* induced microrhizomes are included in the seed chain of ginger and there is high demand for microrhizomes for clean ginger production.

The study entitled "Commercial production of ginger (*Zingiber officinale* Rosc.) microrhizomes using Temporary Immersion Bioreactor (TIB) system" was conducted at Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara during 2018 to 2020. The objective of the study was to develop an efficient commercial production protocol for ginger microrhizomes using a TIB system. The study was conducted in the ginger variety Athira using Plantform TIB purchased from Sweden.

The multiple shoot cultures in the 5th subculture stage received from the commercial micropropagation unit, of CPBMB were used for the study. The protocol for *in vitro* induction of microrhizomes reported by Shylaja *et al*. (2016) was optimised for bioreactor production and at each stage it was compared with the conventional microrhizome production. The number of clumps/ 500ml of medium to initiate multiple shoot production, media for shoot multiplication and microrhizome induction were optimised for TIB and compared with conventional microrhizome production system. The microrhizome, root and shoot characters in microrhizome plantlets and growth of microrhizome plants after hardening were evaluated in the two culture systems. The clonal fidelity analyses of microrhizome plants derived from 8th subculture cycle were done using the specific ISSR marker as reported by Gavande, (2013).

The clump size of 15 clumps/ 500 ml of medium showed higher shoot multiplication in both TIB and conventional system. The shoot proliferation in bioreactor (7.71 shoots/clump) was significantly higher than the conventional microrhizome production system (5.24 shoots/clump). Early induction of microrhizomes was observed in TIB system. In both the culture systems, microrhizome induction was faster in MS medium with 90 gL^{-1} sucrose. The number of microrhizomes produced in the medium in TIB varied from 87.75 to 96.75/ 500ml medium and in conventional system it varied from 84 to 88/ 500 ml medium. The microrhizome plantlets produced in TIB recorded significantly higher weight of microrhizomes, better shoot and root growth and more number of leaves compared to the microrhizome plantlets produced in the conventional system. The weight of microrhizomes, number of roots and root length were higher in microrhizome plantlets in MS medium with 90 gL^{-1} sucrose. The mean weight of microrhizome in the medium was 0.29 g in bioreactor and 0.18 g in conventional system. The survival of microrhizome plants after primary hardening was 94.34 per cent and after secondary hardening was 99.29 per cent in TIB. Microrhizome plants from bioreactor recorded significantly higher plant height (18.60 cm) compared to conventional system (14.80 cm). The number of leaves recorded in microrhizome plants of both the culture systems were on par and ranged from 6.97 to 7.18.

Clonal fidelity analyses using specific ISSR marker revealed that there were no polymorphism in the ISSR amplification profiles in microrhizome plants produced after the 8th subculture cycle with the source mother plant and hence plants produced from both the culture systems are true to type.

The protocol developed in the present study can be further modified by early bulking of the clumps, employing TIB for multiple shoot production in early culture phase and reducing the number of culture cycles so that the entire protocol period can be reduced. Evaluation of TIB microrhizome plants in high tech poly house or field and comparison of yield and quality with microrhizome plants from conventional production system also can be focused for further research.