SEMINAR REPORT

Temporary immersion bioreactor for commercial micropropagation

By

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2018-11-008

Presented on 01/11/2019

Submitted in partial fulfilment of requirement of the course

MBB 591 Masters' Seminar (0+1)

CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

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CERTIFICATE

This is to certify that the seminar report entitled **"Temporary immersion bioreactor for commercial micropropagation"** has been solely prepared by **Waghmare Vaibhav Gautam (2018-11-008),** under my guidance and has not been copied from seminar reports of any seniors, juniors or fellow students.

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I, Waghmare Vaibhav Gautam (2018-11-008) declare that the seminar entitled **"Temporary immersion bioreactor for commercial micropropagation"** has been prepared by me, after going through various references cited at the end and has not been copied from any of my fellow students.

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This is to certify that the seminar report entitled **"Temporary immersion bioreactor for commercial micropropagation"** is a record of seminar presented by **Waghmare Vaibhav Gautam (2018-11-008)** on 1st November, 2019 and is submitted for the partial requirement of the course MBB 591.

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1. Introduction

Conventional Plant Tissue Culture (CPTC) technology involves the culture/growth of small plant parts in laboratory containers such as test tubes in a nutrient medium to regenerate the complete plant (called plantlets). The technology is ideal for crops with a long growth cycle, those with hard to-germinate seeds (dormant), those with low propagation rates or those that lose viability easily (recalcitrant). The technology itself has the advantages of a controlled laboratory environment, not susceptible to changing weather conditions, so that production cycles can be planned. Clean, high quality and uniform plants are produced (Yam and Arditti 2009). The CPTC technology has found application in being capable of producing disease-free plantlets.

However, although CPTC was rapid, meristem cultures took between 6 and 24 months in this system. In addition, a long time is required for acclimatization and losses at transplanting are also high when environmental control is minimal. Frequent subculturing in conventional tissue culture increases labour costs while the small size of the culture container (hence the amount of nutrients) and insufficient aeration (Ziv, 1991) result in fragile plantlets (Ziv *et al*., 1998) and high losses at transplanting. The need to improve on these systems witnessed the emergence of the bioreactor technology.

Modern biotechnology has put the micropropagation industry on the verge of exciting new breakthroughs. It offers improvements in virtually every area of crop production and utilization, with potential benefits to agriculture, the food industry, consumers and the environment. As the world's population continues to grow, it is anticipated that there could be many mouths to feed in the next few decades. The advances made possible through micropropagation using temporary immersion bioreactor system (TIBs) will be essential to meet global food needs by increasing the yield, quality and quantity of crops available to farmers. TIBs offer further benefits in form of non-food crops. Through mass propagation of specific economic species, it will be possible to arrest desertification, soil erosion in affected areas and also increase industrial crop production as renewable sources of medicines, industrial chemicals, fuels *etc.* They offer potential benefits to the commercial farmers, industries, public, research scientists and students.

2. Bioreactors

Bioreactors are vessels designed for large-scale cell, tissue or organ culture in liquid media. The bioreactor is specialized technological equipment, designed for intensive culture by regulating various nutritional and/or physical factors (Afreen, 2006). Bioreactor systems usually consist of a culture vessel and an automated control block. The culture vessel is designed to accommodate the cultivated cells in aseptic environment and to ensure their maximal growth by providing opportunities for maintaining optimal micro environmental conditions, nutrients, and gaseous mass transfers. The automated control block is a computerized, fully automated or semi automated system, designed to monitor and control the cultivation conditions in the culture vessel, such as the agitation speed, temperature, dissolved oxygen and carbon dioxide (CO_2) concentrations, illumination regime, pH, composition of the overlay gaseous environment, and the level of the liquid medium.

Various bioreactor designs have been developed for a wide range of crops, culture types and stages. Functionally, plant culture bioreactors can be divided into two broad types: those in which the cultures are immersed partially or temporarily in the medium and those in which the cultures are continuously submerged.

2.1 Continuous immersion system

In liquid-phase bioreactors, the cultivated cultures are completely immersed in a liquid nutrient medium. Liquid-phase bioreactors (including mechanically agitated, pneumatically agitated, hydraulically agitated, and membrane bioreactors) are currently the best studied systems, revealing almost unlimited potential for application in growing undifferentiated plant cell suspension cultures (Georgiev and Weber, 2014). However, in most cases, liquid-phase bioreactor systems fail to secure satisfactory growth of differentiated plant *in vitro* systems. The complete immersion of plant tissue or organ cultures into the liquid medium often causes malformations and loss of material due to asphyxia and hyperhydricity (Debnath, 2011).

2.2 Temporary Immersion System (TIS)

TIS are simple automated systems, designed to provide optimal environment, improved nutrients and gas transfers, and lower mechanical stress in order to reduce physiological disorders, and to preserve the morphological integrity of the fast growing differentiated plant *in vitro* cultures. TIS provide the most natural environment for plant

tissue and organ *in vitro* cultures, where the cultivated propagules are periodically immersed into a liquid medium and then exposed to a gaseous environment. Different variations of TIS have been developed and are widely applied in commercial micropropagation of economically important plant species. Moreover, because of their simple design and flexible operation, TIS have been adapted in the research of secondary metabolite production, molecular farming and even in phytoremediation of toxic compounds (Steingroewer *et al*., 2013).

3. Temporary immersion system: An Overview

The development of TIS is closely related with the commercialization of plant micropropagation. TIS are periodic semi automated or fully automated cultivation systems, based on alternating cycles of temporary immersion of the cultured plant tissue into the liquid medium followed by draining and exposing the plant tissue to a gaseous environment.

3.1 Common features

TIS are usually constructed with transparent glass or plastic vessels, thus the light from external sources may be used to illuminate the cultivated plant materials. The use of interchangeable plastic materials for construction of vessels make this system simple to design, easy to handle, compact to store, autoclavable or ready for multiple use (Georgiev and Weber, 2014). Figure 1 showing the model set of a temporary immersion system.

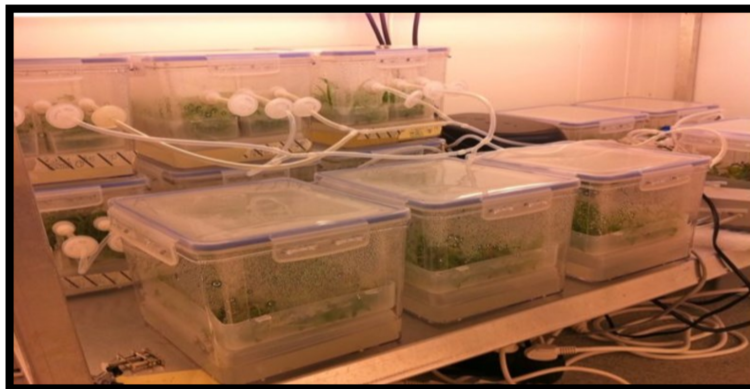


Fig. 1: Model set of temporary immersion bioreactor

3.2. Designs and types of bioreactor

The use of bioreactor was initially reported in microbiology world later it was directly applied in plant biotechnology with some minor modifications for plant suspension cultures and now a day it’s modified completely from their old structures. Some of the different designs and types of bioreactors are briefly described below.

3.2.1 Twin-Flask system

The Twin-Flask system is one of the earliest developed TIS (Watt, 2012). Basically, the Twin Flask system consists of two containers (wide-mouthflasks, bottles, or jars), connected together by a U shaped pipe (glass or plastic) or a silicone tube (Fig. 2) (Aragon *et al*., 2010). One of the containers has the function of a culture chamber, whereas the other

container is used as a medium storage tank. The culture chamber container may or may not be equipped with support material for explants (glass beads, polyurethane foam, metal or nylon sieves may be used) at its bottom (Liu *et al*., 2010). Each container is connected to its own pressurized-air line, controlled by two independent timer clocks, coupled with three way solenoid valves. The simple and reliable design makes Twin-Flask systems favourable for many laboratories. They are generally easy to operate and the construction can maintain sterility for long periods of cultivation. Some of the major disadvantages of Twin-Flask systems are the comprehensive automation and the lack of options for nutrient medium renewal and forced ventilation. Twin-Flask systems are also not equipped with a specialized port for external CO_2 supply during the exposure period. However, CO_2 enriched air may be used to ensure higher CO_2 concentrations in the gaseous environment of the culture chamber (Arencibia *et al*., 2013).

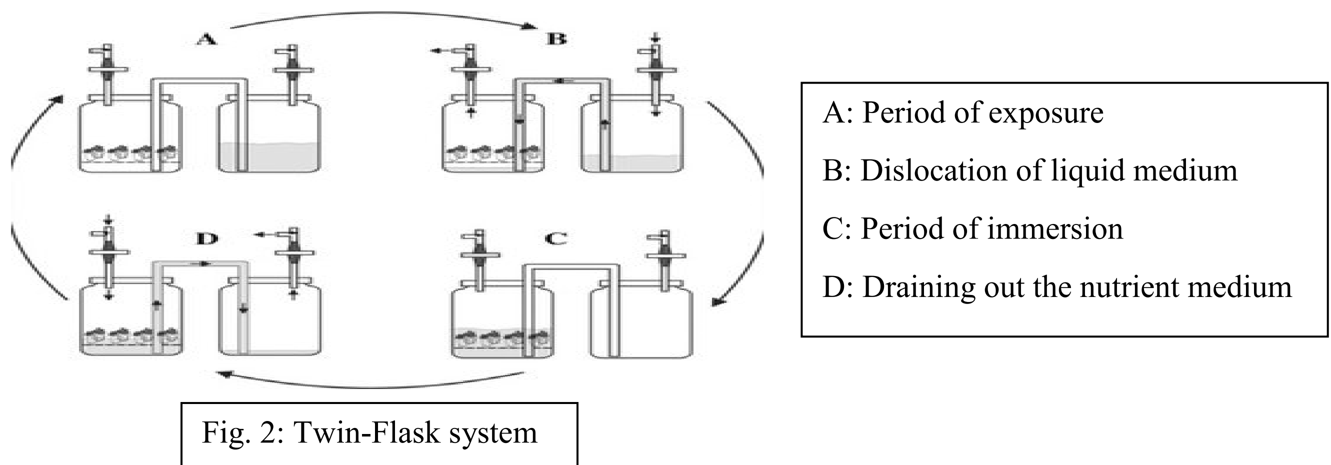


Fig. 2: Twin-Flask system

3.2.2 Ebb-and-Flow

Ebb-and-Flow systems could be described as a simplified modification of the Twin-Flask system. The system consists of two vessels one large wide mouth vessel functioning as a culture chamber and one smaller vessel functioning as a medium storage tank (Fig. 3). Both vessels are interconnected by external ports, mounted on the bottoms. The bigger vessel is the culture chamber, where the plant explants are placed on polyurethane foam support. The polyurethane support maintains sufficient humidity (85-90%) during the exposure period and has the function of an air sparger during the immersion phase (Ducos *et al*., 2007). The smaller vessel is the nutrient medium storage tank and is placed below the culture chamber vessel. The advantages of Ebb-and-Flow systems are the simple and reliable construction, simplified automation and lower energy input. The non uniform light distribution inside the

cultivation vessel and the lack of options for forced ventilation and CO_2 enrichment are the main disadvantages of this system.

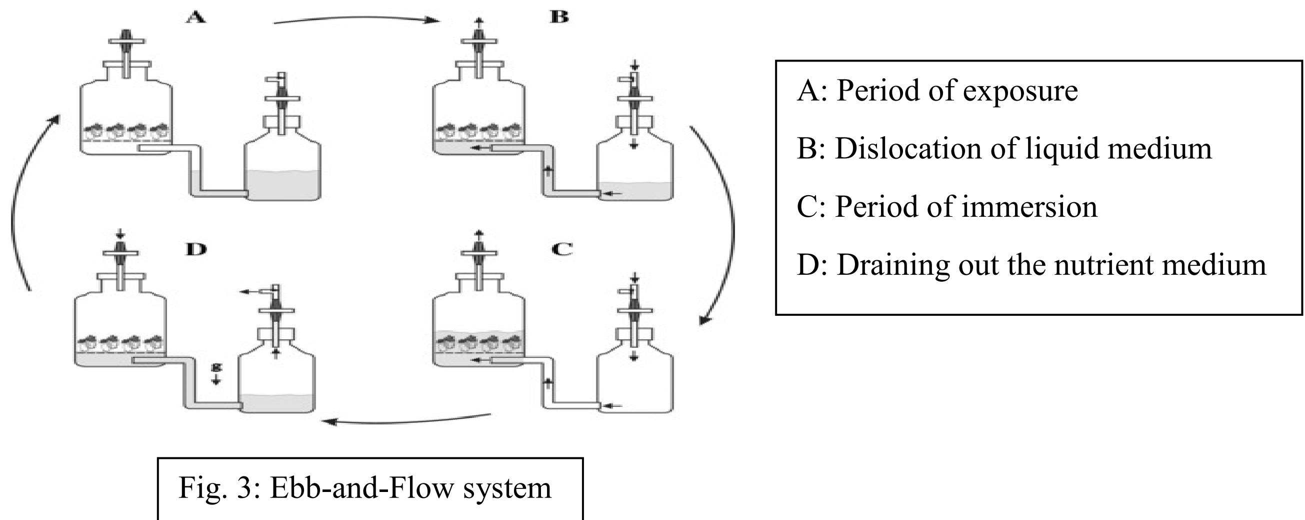


Fig. 3: Ebb-and-Flow system

3.2.3 Rocker system

Rocker system uses a mechanical platform to tilt the cultured boxes at a given angle, so that the medium can be dislocated from one end of the cultured box to the other and vice versa (Fig. 4). The cultivation boxes are made of autoclavable transparent polycarbonate and are rectangle shaped with a lateral wide mouth opening, inside it is closed by a wide screw cap with filter membrane. After inoculation, the boxes are placed on racks with mechanically tilted shelves. The tilts of the shelves create small wave fronts and alternately immerse and aerate the cultured propagules (Uchendu *et al*., 2011). The main advantage of the rocker system is that large numbers of cultivation boxes could be accommodated on one rack and no additional connection to an airline is necessary. The disadvantages of rocker system are related with the necessity of an electromechanically driven tilting platform that increases the investment and energy costs. Tilting platforms require more space to operate properly and this may reflect on the production cost per unit space in the growth chamber. The cultured boxes have no good air renewal and no options for forced ventilation or nutrient medium replacement exist.

3.2.4 Rotating drum system

The system consists of a roller apparatus and an autoclavable plastic or glass bottle lying on it (Fig. 5). A stainless steel net or a mat of polyurethane foam is placed inside the bottle to support the explants (Akita and Ohta, 1998). When the roller apparatus is rotating at low speed, the immobilized plants are periodically immersed and exposed to air environment.

In the case of adventitious or hairy roots cultivation, the installation of internal support is not necessary, since the roots are adsorbed onto the bottle walls by adhesion (Reis *et al*., 2011). The advantage of the rotating drum system is the simple construction. The main disadvantages are the inability to set up independent and prolonged times for immersion and exposure periods, higher shear stress due to mechanical mixing and the lack of options for ventilation and exchange of internal atmosphere.

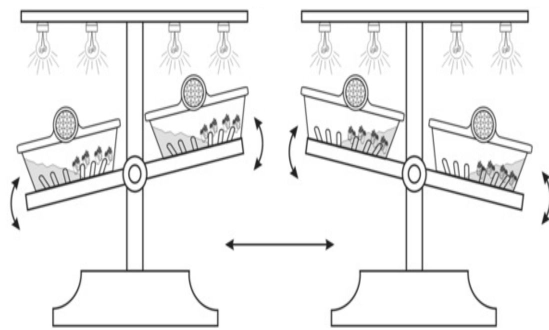


Fig. 4: Rocker system

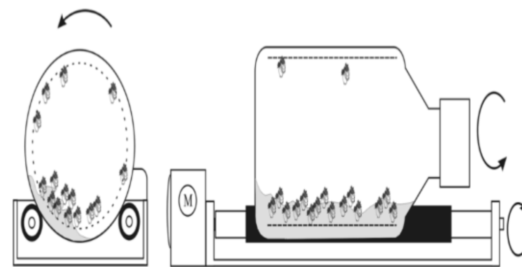


Fig. 5: Rotating drum system

3.2.5 Bioreactor of Immersion by Bubbles (BIB)

The bioreactor of immersion by bubbles utilizes a completely new cultivation strategy, based on temporary immersion of propagated explants in foam instead of liquid medium. The system consists of a single glass cylinder, transversely divided into two compartments by a microporous (170-220 µm pores) plate (Fig.6). The upper compartment is the growth chamber, in which a few stainless steel internal racks are stacked one upon another to support the cultured explants. The liquid nutrient medium with added detergent (Tween 20) is filled at the bottom of the culture chamber as well. The lower chamber is for uniform air distribution by the porous plate. BIB is commercially available in Brazil at 1.5 L scale. The presence of detergent in the nutrient medium, as well as the prolonged time for liquid drainage may restrict the application of BIB for propagation of some sensitive plant species.

3.2.6 BioMINT system

BioMINT bioreactor culture vessels utilizes rocker platform. The BioMINT is a mid-sized (1.2 L) bioreactor, consisting of two cylindrical autoclavable polycarbonate vessels that are joined together by a perforated adaptor with two female screw threads (Fig. 6 C and D). One vessel is for the plant tissues and the other for the liquid culture medium. The perforated

adaptor permits the free flow of the liquid medium while keeping the propagules in place when the bioreactors change position. The adaptor also has two external ports that allow the application of forced ventilation or CO_2 enrichment (Robert *et al*., 2006).

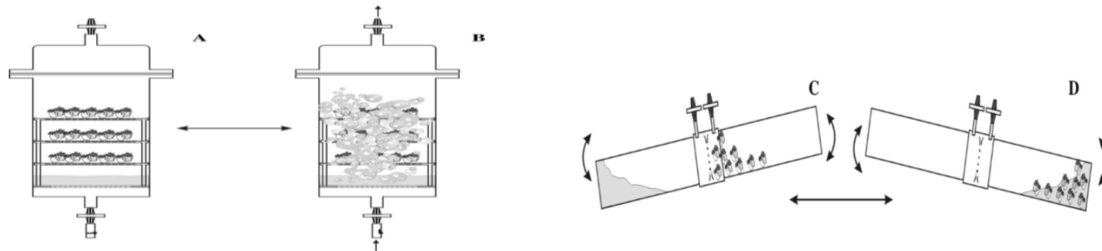


Fig. 6: (A, B) Immersion by bubbles and (C, D) BioMINT system

3.2.7 RALM bioreactor

Common characteristics of RALM bioreactor includes it is easy to handle, option for forced ventilation and CO_2 enrichment, option for nutrient medium renewal, low investment costs, complex automation, construction with several internal elements and low headspace humidity in growth chamber. Figure 7 showing the model of RALM vessel.

3.2.8 SETIS bioreactor

Common characteristics of SETIS bioreactor includes it is simple to construct, easy to handle, simplified automation, large illuminated area, improved drainage, low energy costs *etc*. The system is advantageous with optimal usage of growth room space and low investment costs. Forced ventilation and CO_2 enrichment or nutrient medium renewal is not possible. Figure 8 showing the model vessel of SETIS bioreactor.

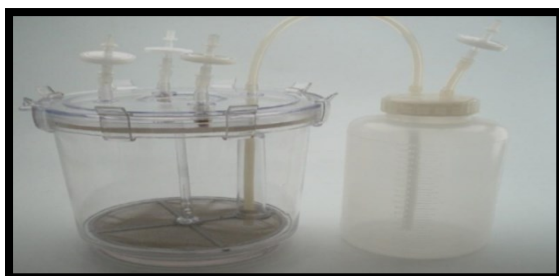


Fig. 7: RALM bioreactor

Fig. 8: SETIS bioreactor

3.2.9 RITA system

The RITA TIS (CIRAD, France) have been developed for intensive *in vitro* plant culture. The system consists of a single autoclavable polypropylene vessel (500 ml) with two

compartments, separated by an installed tray with a mesh support and a plastic pipe mounted to its center (Fig. 9). The vessel is closed by a wide screw lid, equipped with central and lateral external ports on the top. Both ports are secured with membrane filters and the central port is connected to an airline controlled by a timer clock and a three-way solenoid valve. The upper compartment of the vessel is the culture chamber, whereas the bottom compartment is the medium storage tank. The advantages of the RITA system are its simple and reliable operation, compact space for apparatus accommodation and the support of sufficient relative humidity level with full separation of the propagules and liquid medium. The main disadvantages of system are the inability for nutrient medium renewal and the lack of forced ventilation and CO_2 enrichment.

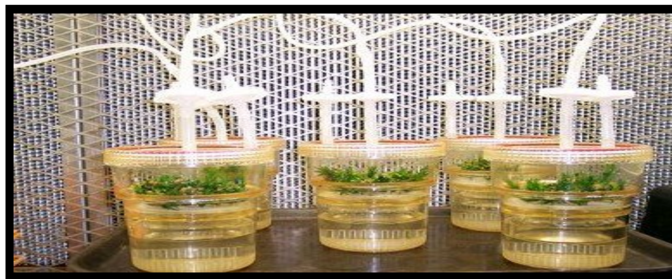


Fig. 9: RITA system

3.2.10 Plantform and Plantima bioreactor

The recently developed temporary immersion bioreactor systems with number of advantages include simple automation, reliable operation, easy to handle, high headspace humidity in growth chamber, apparatus may be stacked one on the other to save space and low investment costs (Georgiev *et al.,* 2014). Figure 10 showing the model vessel of Plantform and plantima bioreactors.

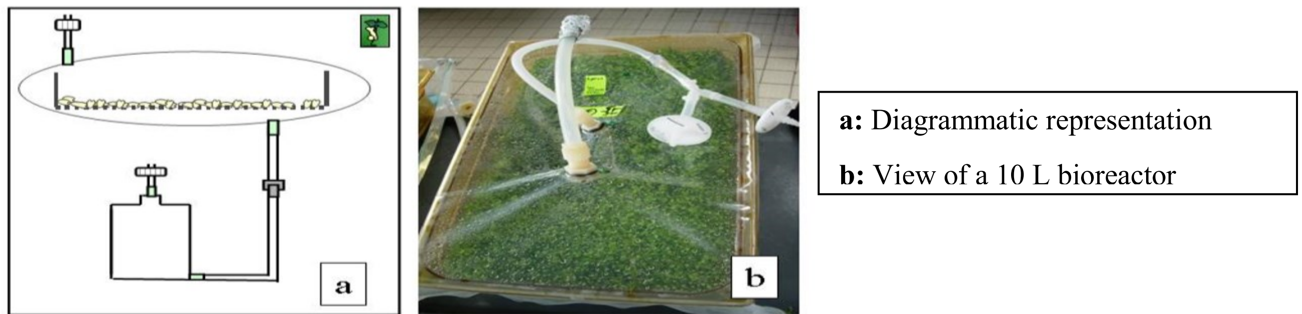
A. Plantform plabioreactor

B. Plantima bioreactor

Fig. 10: Plantform and Plantima bioreactor

3.2.11 Low-cost and disposable bioreactors

The "Box-in-Bag" disposable TIB (Fig.11) has more advantages provided by the two types of plastics, rigid and flexible. The rigid plastic box facilitates the manipulations, maintains a culture headspace between the immersion periods and allows a horizontal distribution of the biomass allowing better oxygenation and illumination. Moreover, the possibility of stacking several boxes one top of another makes this system easy for transportation and it is possible to send *in vitro* plants keeping them inside the bioreactor in which they have grown. The international exchanges of sterile plant material are therefore greatly facilitated. Both plastics can be used together as a mini-greenhouse for storage, shipment, hardening and probably even for *ex vitro* germination under microenvironment conditions.



a: Diagrammatic representation

b: View of a 10 L bioreactor

Fig. 11: Box-in-Bag bioreactor

3.3 Immersion time and immersion frequency

The frequency and time of immersion are the most decisive parameters for the efficiency of the system, since they determine the nutrients absorption and the control of Hyperhydricity (Etienne and Berthouly, 2002). Although such parameter varies considerably (probably due to the great variety of existing species, different micropropagation processes and different immersion systems used), a great part of the studies reported that in smaller immersion intervals there are greater growth in fresh mass and number of shoots, although hyperhydricity is sometimes more severe (Carvalho *et al*., 2019).

Figure 12 shows the assembly of the bioreactor and figure 13 describes the movement of the nutrient medium and aeration in the bioreactor (Welander *et al.,* 2017).

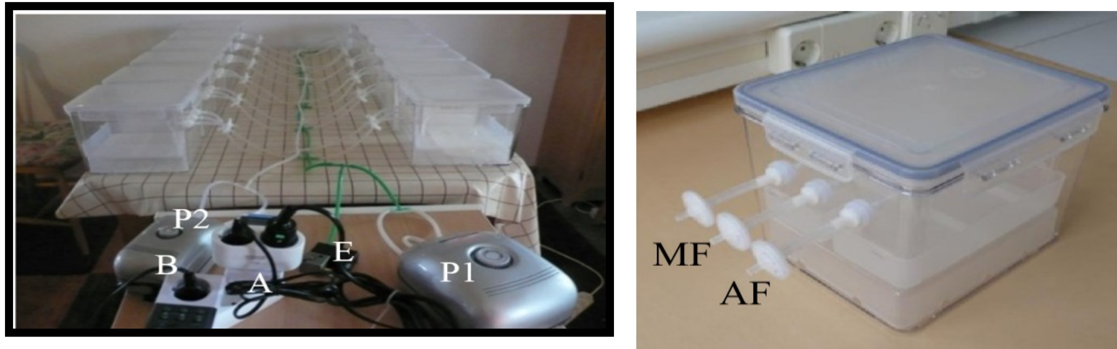


Fig.12: Assembly of 12 bioreactors. The green tubes are connected to the middle filter (MF) on the bioreactors. One end is connected to P1, the electric valve (E) and timer A, while at the other end there is a stopper. The white tubes are connected to one of the outer filters (AF). One end is connected to P2 and timer B and the other end to a stopper.

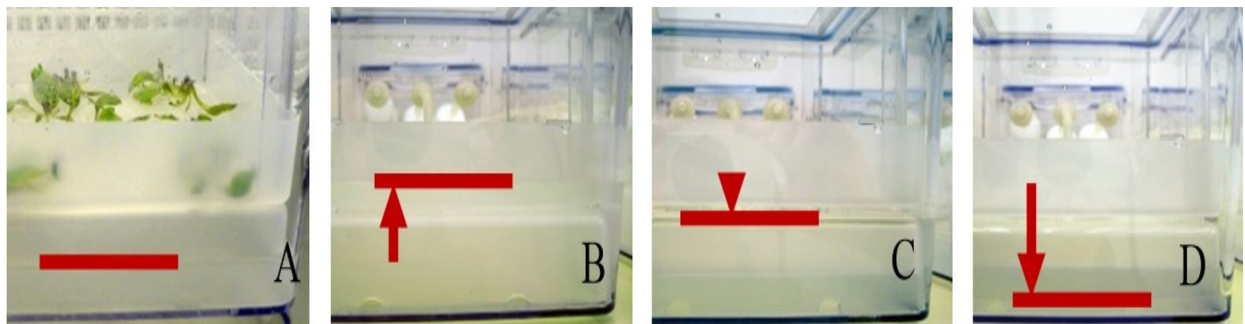


Fig. 13: Movement of the nutrient in the bioreactor and aeration.

A: when P1 and P2 are off, the medium is at the bottom and the electric valve is open.

B: when P1 is on, the medium floods the basket and the electric valve is closed.

C: when P1 is of and P2 is on, the electric valve opens and medium is drained back.

D: as long as P2 is on, the headspace is ventilated.

3.4 Advantages over conventional micropropagation

The advantages of bioreactors include increased culture multiplication rate, faster culture growth, reduction in medium cost and reduction in energy, labour and laboratory space. The increased rate of multiplication and growth primarily reflects the effect of liquid medium (Levin *et al.*, 1997). The elimination of gelling agents (*e.g.* agar) reduces medium cost, and filter sterilization of the medium eliminates the need for autoclaving. In bioreactors, the culture density in liquid media is much higher than in the conventional vessels with semisolid media. The conventional tissue culture vessels are typically kept on shelves with a

large space between the shelves. The use of bioreactors require much smaller space in the growth room, fewer clean work stations and less space for media preparation, vessel storage and washing than that used in the conventional micropropagation. The smaller size of the laboratory and the less number of people reduce air-conditioning needs that altimately affect reduction of energy costs. Reduced requirements for lighting the bioreactors and autoclaving and less labour, simplification of medium preparation, washing of vessels and simplified handling of the cultures, all lead to cost reduction.

3.5 Problems associated with TIS

There are many problems associated with the use of bioreactors in micropropagation. These includes contamination, lack of protocols and production procedures, increased hyperhydricity, problems of foaming or shear stress and release of growth inhibiting compounds by the cultures. Unfortunately, culture contamination, which is a major problem in conventional micropropagation (Leifert and Waites, 1990; Leifert and Woodward, 1998; Leifert, 2000), is even more acute in bioreactors. In conventional micropropagation, discarding a small number of the contaminated vessels is an acceptable loss; in bioreactors, even a single contaminated unit is a huge loss. However, despite these difficulties a number of commercial laboratories have developed effective procedures to control contamination in bioreactors.

3.5.1 Contamination

Microbial contamination by yeast, fungi, bacteria, viruses, mites and thrips is still the major problem that has hampered the establishment of truly aseptic plants and their successful micropropagation in bioreactors. Once established in the cultures, the microbes grow fast, depletes nutrient of the medium and produce toxic substances within the medium, which causes the mortality, tissue necrosis or variable growth of cultured plantlets resulting in partial or total loss of culture (Kane, 2003).

Control of Contamination

Prevention of microbial contamination is a very important issue since it is a major challenge in developing bioreactor systems for large scale production through micropropagation. It requires proper handling of the plant material, equipment and cultures during transfer and production. Only the surface sterilized explants, multiplied in small vessels and indexed for freedom from diseases are used to initiate cultures in bioreactors. The

contamination can be controlled with one or a combination of anti-microbial compounds, acidification of the media and micro-filtration of the medium (Schmidt *et al*., 2002).

Rahman *et al.* (2017) identified bacterial and fungal contaminants isolated during potato shoot growth in the TIB system. Evaluated four different antifungal agents and reported 0.15% Propiconazole given 100% inhibition (Table 1) against fungal contaminanats. They examined the effect of three antibacterial (Gentamycin, Vancomycin and Tetracycline) and four antifungal agents (Mencozeb, Propiconazole, Bavistin and Copper oxychloride) on the contaminants and on potato shoot growth. They found that Gentamycin (50 mg/L) and Propiconazole (0.15 %) were most effective against the isolated bacteria (35 mm inhibition zone) and fungi (100 %) respectively (Table 2), whereas Gentamycin in combination with Bavistin showed better performance on potato shoot and root development (Table 3).

Table 1: *In vitro* bioassay of fungal contaminants in potato micropropagation

Name of the antifungal	Strength (%)	Percentage of inhibition				
		Aspergillus	Penicillium	Mucor	Fusarium	Rhizopus
Mencozeb	0.05	75	86	56	67	74
	0.10	78	88	76	69	67
	0.15	79	89	78	75	73
Propiconazole	0.05	81	96	88	92	94
	0.10	91	93	93	95	97
	0.15	**100**	**100**	**100**	**100**	**100**
Bavistin	0.05	88	73	89	91	87
	0.10	91	77	91	94	88
	0.15	93	79	93	97	95
Copper oxychloride	0.05	16	19	14	28	32
	0.10	22	23	18	35	34
	0.15	26	31	24	38	39

Table 2: Effect of different combinations of antimicrobial agent

Name of antimicrobials	Duration of Treatments (days)	10 mg/L + 0.05%	30 mg/L + 0.10%	50 mg/L + 0.15%	75 mg/L + 0.20%	100 mg/L 0.25%
		Percentage of contamination				
Gentamycin + Bavistin	1	100[fb]	100[f]	100[fb]	75[f]	75[fb]
	3	100[fb]	25[fb]	50[f]	75[fb]	25[b]
	5	75[fb]	50[f]	0	0	0[m]
	7	50[f]	25[f]	0	0[m]	0[m]
Gentamycin + Propiconazole	1	75[fb]	100[fb]	25[f]	75[f]	50[f]
	3	50[f]	75[fb]	**0**	25[f]	75[fb]
	5	100[fb]	25[f]	**0**	0	25[f]
	7	75[fb]	25[f]	0[m]	0[m]	0[m]
Tetracycline + Propiconazole	1	75[fb]	100[fb]	75[fb]	50[fb]	75[b]
	3	75[fb]	25[f]	25[f]	0	0[m]
	5	50[f]	25[f]	0	25[b]	0[m]
	7	25[f]	50[f]	0[m]	0[m]	0[m]

b- bacterial contamination; f- fungal contamination; m- plantlet mortality.

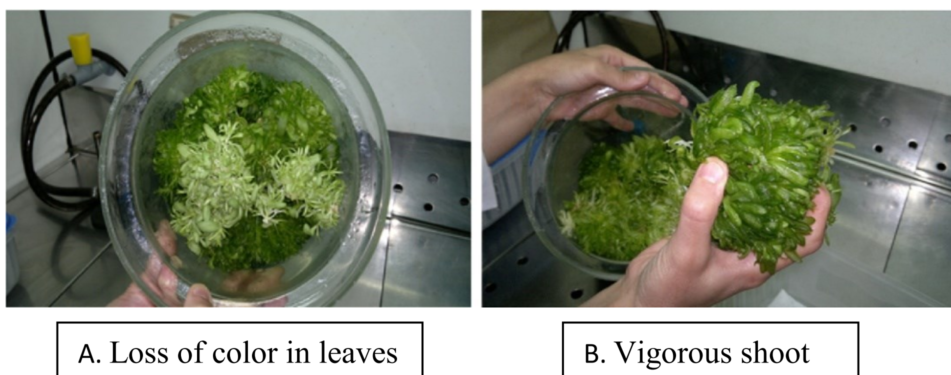
Table 3: Effect of antimicrobial agents on shoots and roots growth of potato plantlets

Antibacterial agent	Strength (cm)	Plantlet height	Number of nodes	Number of roots	Length of roots (cm)
Control	0	5.2±0.35	4.3±0.20	4.2±0.28	4.4±0.56
Gentamycin + Bavistin	50 mg/L + 0.15%	5.3±0.39	3.9±0.40	4.4±0.42	4.2±0.41
	75 mg/L + 0.20%	**6.1±0.35**	**4.9±0.76**	**5.3±0.56**	**4.8±0.28**
	100 mg/L + 0.25%	4.7±0.41	4.0±0.65	2.5±0.39	3.4±0.76

Gentamycin + Propiconazole	50 mg/L + 0.15%	5.4±0.65	4.1±0.42	4.3±0.29	4.0±0.29
	75 mg/L + 0.20%	4.9±0.56	3.8±0.41	4.2±0.31	3.8±0.40
	100 mg/L + 0.25%	5.1±0.29	4.3±0.76	4.4±0.29	3.3±0.42
Tetracycline + Bavistin	50 mg/L + 0.15%	5.9±0.28	5±0.65	4.2±0.42	3.9±0.76
	75 mg/L + 0.20%	5.5±0.39	4.3±0.35	3.3±0.40	4.1±0.28
	100 mg/L + 0.25%	nil	nil	nil	nil
	50 mg/L + 0.15%	4.2±0.56	3.2±0.41	4.1±0.35	3.9±0.28
	75 mg/L + 0.20%	nil	nil	nil	nil
	100 mg/L + 0.25%	nil	nil	nil	nil

3.5.2 Hyperhydricity

The major disadvantage encountered when plants are cultured in liquid media is the problem of shoot malformation. Plants tend to accumulate excess of water in their tissue resulting to anomalous morphogenesis, a phenomenon known as hyperhydricity. The plants that develop in liquid media are fragile and have a glassy appearance with succulent leaves or shoots and a poor root system (Fig. 14). They develop an unorganized mesophyll tissue that is made up mainly of spongy parenchyma tissue with large intercellular spaces (Etienne, *et al.,* 2006), a deformed vascular tissue and abnormal epidermis. Hyperhydricity affects plant survival after transplanting and causes loss of the *in vitro* developed leaves, or even whole plant, which often wilt and die.



A. Loss of color in leaves

B. Vigorous shoot

Fig. 14: Hyperhydric planlets

According to Marzieh *et al.* (2017) increasing aeration and reducing the immersion time helps to control the hyperhydricity. They evaluated three different numbers (2, 4 and 6) of immersion per day and found that 6 times immersion per day (every 4 h) caused the highest number of new shoots (17.33), although this treatments lead to highest rate of hyperhydricity (Table 4). The 4 times immersion per day via interval of 6 h (produced 14.33 new shoots) showed the most desirable treatment.

Table 4: Rate of hyperhydricity at different immersion frequency for carnation micropropagation

Number of immersions / day	No. of new shoots	Rate of hyperhydricity
2	7.33 c ± 0.12	1.66 c ± 0.12
4	14.33 b ± 0.14	2.20 b ± 0.11
6	17.83 a ± 0.14	4.00 a ± 0.10

4. Micropropagation in bioreactors

The first report on the use of a bioreactor for micropropagation was by Takayama and Misawa (1981) who multiplied *Begonia* using shake cultures. All of the available systems, those involving temporary immersion have a variety of characteristics that make them highly suitable for use in semi automated micropropagation. First and foremost, and as the name implies, the plant cultures are not constantly immersed in liquid media, which often affects plant growth and morphogenesis negatively. Temporary immersion systems allow for control of contamination, adequate nutrient and oxygen supply and mixing, relatively infrequent sub culturing, ease of medium changes and limited shear damage. For these reasons, Ziv (2000, 2005) and Arencibia *et al.* (2008), amongst others, have described TIS as the "most natural tissue culture approach".

A number of different TIS have been utilized successfully for the micropropagation of a variety of plant species of agricultural, medicinal and conservation value (Table 5).

Table 5: Micropropagation using temporary immersion bioreactor

Crops	**Bioreactor systems**	**References**
Grape	Rocker machines	Haris and Mason, (1983)
Banana	RITA®	Alvard *et al*. (1993)
Coffee	RITA®	Etiene *et al*. (1997)
Sugarcane	Twin flask (BIT®))	Lorenzo *et al*. (1998)
Pineapple	Twin flask (BIT®))	Escalona *et al.* (1999)
Apple	Bubble bioreactor	Chakrabarty *et al*. (2003)
Yam	Glass flask	Jova *et al*. (2005)
Eucalyptus	RITA®	Mc Alister *et al*. (2005)
Strawberry	RITA®	Hanhineva *et al.* (2005)
Ginseng	Rocker	Uchendu *et al*. (2011)
Chestnut	Twin flask, Plantform, RITA®	Vidal *et al.* (2015)
Stevia	BIT®	Vives *et al*. (2017)
Carnation	Glass containers	Marzieh *et al*. (2017)

Sugarcane	SETIS™	Distabanjong *et al.* (2018)
Anthurium andreanum	Ebb-and-Flow	Martínez-Estrada *et al.* (2019)
Capparis spinosa,	Plantform	Gianguzzi *et al.* (2019)
Date palm	Plantform	Nayyef *et al*. (2019)

Table 6: Effect of temporary immersion bioreactor on shoot multiplication (shoots/explant)

Crop	**Variety**	**Conventional micropropagation**	**Temporary immersion systems**
Pineapple	Smooth cayenne MD2	8.0 5.8	68.8 26.8
Sugarcane	C91-301 C1051-73 C120-78 C323-68 Cp-5243	3.7 4.1 3.9 4.3 4.0	34.1 58.0 30.2 39.5 32.5
Taro	INIVIT Mexico 1	3.0 2.8	10.4 7.7
Banana	FHIA-18 FHIA-01 Grand Nane	3.8 3.4 4.0	7.4 10.4 16.6
Plantain	CEMSA 3/4	2.5	7.8
Eucalyptus	Urograndis	2.7	11.6
Syngonium	W. Butterfly Pixle	7.3 2.2	28.0 18.4
Philodendron	Xanadu	2.0	8.8
Spathyphyllum	Sensation	3.7	17.6

(Paul *et al.,* 2012)

Case study

Temporary immersion improves *in vitro* multiplication and acclimatization of *Anthurium andreanum* Lind.

Martinez-Estrada *et al.* (2019) studied the effect of the culture system, immersion frequency, and culture medium volume per explant on shoot multiplication of *A. andreanum* cv. Rosa. Evaluated dfferent culture systems: semisolid medium, liquid medium with partial immersion and temporary immersion (TI) using an Ebb-and-Flow bioreactor for *in vitro* multiplication and acclimatization of *Anthurium andreanum*. They used nodal segments from *in vitro* derived adventitious shoots for different culture systems evaluation. The results showed significant dfferences in the variables evaluated among the dfferent culture systems (Fig. 15). The highest shoot production was obtained in TI with 31.50 ± 0.50 shoots per explant, followed by the partial immersion system and culture in semisolid medium, with 7.25 ± 0.16 and 4.50 ± 0.18 shoots per explant, respectively (Table 6). Immersion frequency and the amount of culture medium per explant did not show significant differences, which allows us to recommend the immersion frequency (Table 7) of every 12h and the culture medium volume (Table 8) of 25mL per explant. TI favored an increase in chlorophyll content, a low stomatal index and a high percentage of closed stomata, suggesting an increase in the functionality of the stomata and probably a higher photosynthetic rate. The survival rate during acclimatization increased when using TIS (Fig.16 and 17).

Table 7: Evaluation of different culture systems

Micropropagation system	No. shoots per explants	Shoot length (cm)	No. of leaves per shoot	Fresh weight per shoot (g)
Semisolid culture medium	4.50 ± 0.18[c]	1.15 ± 0.04[c]	3.00 ± 0.26[b]	0.048 ± 0.003[b]
Partial immersion	7.25 ± 0.16[b]	1.39 ± 0.04[b]	2.37 ± 0.18[b]	0.059 ± 0.005[b]
Temporary immersion	**31.50 ± 0.50[a]**	**1.76 ± 0.05[a]**	**4.25 ± 0.16[a]**	**0.082 ± 0.003[a]**

Table 8: Evaluation of the immersion frequency

Immersion frequency (hrs)	No. of shoots per explants	Shoot length (cm)	No. of leaves per shoot	Fresh weight per shoot (g)
4	31.50 ± 0.50[a]	1.76 ± 0.05[a]	4.25 ± 0.16[a]	0.082 ± 0.003[a]
8	31.62 ± 0.67[a]	1.49 ± 0.03[a]	**4.37 ± 0.18[a]**	0.085 ± 0.005[a]
12	**33.12 ± 0.97[a]**	**1.78 ± 0.04[a]**	4.25 ± 0.16[a]	**0.086 ± 0.003[a]**

Table 9: Evaluation of culture medium volume (ml) per explant

Volume of medium (ml) / explants	No. shoots per explants	Shoot length (cm)	No. of leaves per shoot	Fresh weight per shoot (g)
25	**32.84 ± 0.74[a]**	**1.76 ± 0.05[a]**	**4.37 ± 0.18[a]**	**0.074 ± 0.003[a]**
37.5	32.45 ± 0.82[a]	1.49 ± 0.03[a]	4.37 ± 0.32[a]	0.077 ± 0.005[a]
50	33.12 ± 0.97[a]	1.78 ± 0.04[a]	4.25 ± 0.32[a]	0.086 ± 0.003[a]

A: Semisolid

B: Partial immersion

C. D. E: Temporary immersion

Fig.15: Effect of different culture systems

Fig.16: Effect of different culture systems on acclimatization **a:** 30 days and **b:** 90 days of *ex vitro* culture

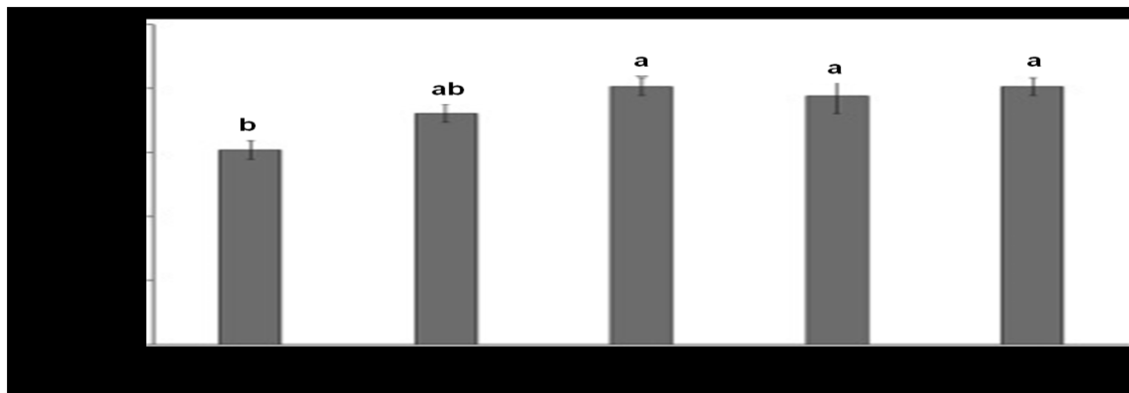


Fig. 17: Effect of culture system on survival percentage of *A. andreanum* cv. Rosa after 30 days of acclimatization

6. Cost-benefit analysis

Bioreactors can significantly reduce the production cost of micropropagation in developed countries from $0.16 to $0.07-$0.08 per unit (Levin and Tanny, 2004). This has recently been confirmed in commercial laboratories. For example, a major ornamental plant producer in the US is now propagating 40,000 units per month based on bioreactor multiplication and mechanical culture separation. The cost reduction (Table 9-11) is based on three situations. Model-1 is a conventional laboratory producing 20 million units per year. Model-2 is a laboratory using multiplication in bioreactors, mechanical separation of the culture and elongation on semi-solid medium. Model-3 is a laboratory using multiplication in bioreactors, mechanical separation of the culture and elongation in temporary immersion bioreactors (Levin and Tanny, 2004).

It is, however, unclear whether bioreactors will produce a commercially relevant cost reduction in developing countries. In countries, such as India, labour cost can be as low as US $0.20-0.60 per hour (Savangikar, 2002). Based on projections of Model 1 above, this would mean that in a developing country, a conventional laboratory with 20×106 units per year capacity could produce propagules at $0.06-$0.07/unit. This projection agrees with the available information on commercial laboratories in India with 6-10 million units capacity per year, and production costs of $0.06-$0.14/unit (Barathi, 2002). For larger laboratories, bioreactor micropropagation could probably further reduce this cost by about $0.01. In addition, bioreactor micropropagation would simplify production management through the use of large culture batches and reduced labour. Although cost savings alone might not justify the integration of the bioreactors into production, the simplification of production procedures and management might make bioreactor based micropropagation desirable for large-scale micropropagation concerns.

If the adoption of such technologies brings down the cost of production in developing countries, the cost reduction from bioreactor micropropagation would be indeed small. However, the simplification of production management could still make bioreactor micropropagation worthwhile for the large scale laboratories. Most laboratories in India (70%) produce less than 1 million units per year. It is likely that this also reflects the situation in many other developing countries. Since the use of bioreactors will not produce a large cost reduction or greatly increase efficiency of small laboratories, the integration of bioreactors

into production systems should only be attempted by facilities with skilled and experienced propagators.

Table 10. Estimated annual cost (US$) of major investment for the

Model	Building cost	Amortize years	Annual interest (%)	Annual cost of building	Annual labour cost	Annual energy cost	Total annual cost
1	26,96,000	20	4	1,40,190	21,63,600	2,09,160	25,12,950
2	20,59,000	20	4	1,07,070	6,34,800	13,74,520	8,79,390
3	16,41,000	20	4	85,332	5,30,400	48,960	6,64,683

Savings from Model 1-2=65%, from Model 1-3=73%

Table 11. Estimated annual cost (US$) of major equipment and furniture

Model	Equipment cost	Furniture cost	Amortize years	Annual interest (%)	Total annual cost
1	10,00,000	3,00,000	10	4	13,52,000
2	1,00,000	30,000	10	4	1,35,200
3	1,00,000	30,000	10	4	1,35,200

Table 12. Annual cost of production (US$) for each model (20×106 units/year)

Model	Total cost (A)	Total cost (B)	Cost/unit	Unit cost media	Unit cost vessels	Unit cost misc.	Total unit cost
1	25,12,950	13,52,000	0.13	0.01	0.01	0.01	0.16
2	8,79,390	1,35,200	0.05	0.01	0.01	0.01	0.08
3	6,64,683	1,35,200	0.04	0.01	0.01	0.01	0.07

A = Table 1, B = Table 2

7. Summary

- Common characteristics of bioreactor, different types and designs bioreactor
- Principle of temporary immersion system
- Advantages and disadvantages of temporary immersion system and their applications
- Commercial micropropagation in different crops using TIS
- Economical analysis of commercial micropropagation system

8. Future thrust

- Reduction of the risk of contamination
- Disposable bioreactors system with large size can be developed
- Commercialisation of micropropagation by automation

9. Discussion

1. What are the scopes of this technique in India?

Ans. Yes, this technique have a great scope in our country, because the conventional tissue culture strategies has number of limitations or these strategies are not fulfilling the current plantlets demand so for increasing production it is the need of commercial tissue culture.

2. Is there any other new techniques in tissue culture?

Ans. Yes there are number of new techniques in tissue culture but the current global research is going on the development of new bioreactor systems and standerdization of the micropropagation protocol for different crops by using bioreactor.

3. Does the micropropagation of cereal crops is possible with this techniques?

Ans. Micropropagation of any crop is possible by using bioreactor but as compared to horticulture or tree crop there are some limitations for cereal micropropagation. One another thing is the aim of using bioreactor is to produce large number of plantlets, but in case of cereals there is no need of production of plantlets, thus the use of bioreactor for micropropagation studied by all of the researchers are mainly on horticulture and tree crop species.

4. Does the indirect organogenesis is also possible by using bioreactor?

Ans. Yes, off course. Large scale micropropagation is a major application of bioreactor, but these are useful for indirect organogenesis that is callus induction and also for secondary metabolite production.

5. Whether this techniques is commercially applied anywhere in our country?

Ans. No, still it is not commercially applied in our country, but there are few reports by Indian authors and they described the micropropagation of some commercially produced tissue cultured plants, using bioreactor.

6. Amongst these which type of bioreactor is good? And why?

Ans. The each design of bioreactor has specific features. According to the objective the preference of the designs will be different. In case of micropropagation recently developed plantform^{TM} bioreactor and seties^{TM} bioreactor are more beneficial, because it is simple to construct and it's require less space.

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MBB 591- Master Seminar

Name	**:**	Waghmare Vaibhav Gautam	**Venue**	**:**	Seminar hall
Admission No.	**:**	2018-11-008	**Date**	**:**	01-11-2019
Major Advisor	**:**	Dr. M. R. Shylaja	**Time**	**:**	09.15 am.

Temporary immersion bioreactor for commercial micropropagation

Abstract

Micropropagation, popularly known for large-scale clonal propagation, is the first major and widely accepted practical application of plant biotechnology. The commercial utility of conventional micropropagation is limited due to high labour and media cost, low multiplication rate, high contamination risks and poor survival during acclimatization (Paul *et al*., 2012). Currently, new technologies are being developed for commercial micropropagation, among which temporary immersion bioreactor holds promise. A temporary immersion system (TIS) is a semi-automated micropropagation system where the culture are immersed in liquid medium for a certain period of time and then exposed to the gaseous environment. The technological designs of TIS include Twin-Flask, Ebb-and-Flow, immersion by bubbles, BioMINT, RALM, SETIS, RITA or Plantform bioreactor systems (Georgiev and Weber, 2014).

Bioreactor system offers many advantages including better control of the culture conditions, optimal supply of nutrients and growth regulators (Ziv, 2005). Despite these advantages, bioreactor systems possess a huge risk of culture loss due to microbial contamination (Rahman *et al*., 2017). Other problems associated with commercial micropropagation using bioreactors include increased hyperhydricity and release of growth inhibiting compounds by the cultures (Levin and Tanny, 2004). However, a number of commercial laboratories have developed effective micropropagation procedures to control contamination and hyperhydricity in bioreactors.

Temporary immersion bioreactor system has been successfully used in the micropropagation of several plants like apple, chrysanthemum, garlic, grape, lily, potato and ginseng (Peak *et al*., 2005), banana (Farahani and Majid, 2012) and sugarcane (Distabanjong *et al*., 2018).

Martinez-Estrada *et al*. (2019) reported that temporary immersion improved shoot proliferation and acclimatisation in *Anthurium andreanum* Lind. They studied different culture systems (semisolid medium, liquid medium with partial immersion and temporary immersion) for *in vitro* multiplication and acclimatization of *Anthurium andreanum* Lind. They observed high survival rate of plants from TIS during acclimatisation which was due to higher accumulation of photosynthates, lower stomatal index and higher percentage of closed stomata. The genetic stability of plantlets produced through TIS has to be ensured in the production protocol due to the high rate of multiplication.

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