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Studies on the effect of polyamines supplementation on growth and biochemical parameters in rose micropropagation

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ABSTRACT

Rose (*Rosa hybrida* L. cv. bush rose) micropropagules were cultivated on MS medium supplemented with polyamines (PAs) [Cadaverine (Cad), Spermidine (Spd), and Putrescine (Put)] to investigate its effects on growth and multiplication under *in vitro* conditions during 2023. Polyamines were added to the medium at different concentrations of 10 mM, 30 mM, and 50 mM via filter sterilization before autoclaving, to assess their impact on *in vitro* growth parameters, which indicate that a lower concentration of polyamines, specifically at 30 mM, significantly enhances biomass accumulation and overall plant growth, whereas higher concentrations (50 mM) tend to exert a diminishing effect. Biochemical parameters revealed that polyamines at 30 mM notably increased the levels of key biomolecules, including carbohydrates content, proteins content, and chlorophylls content, and also shows significant level of phenol content in the rose micropropagules. Furthermore, antioxidant activities, as measured by superoxide dismutase and peroxidase activities, was markedly higher in micropropagules grown on medium supplemented with 30 mM polyamines These findings suggest that low concentrations of polyamines can serve as effective growth regulators, promoting enhanced growth and biochemical responses in rose micropropagation. Therefore, incorporating polyamines, particularly at optimal concentrations, could improve the efficiency and quality of rose micropropagation protocols, benefiting commercial production and conservation efforts.

Key words: Biochemical parameters, growth parameters, peroxidase, polyamines, rose micropropagation, superoxide dismutase

INTRODUCTION

Roses are one of the world's most loved ornamental flowers for quite a while and considered as the most vital horticulture crop in the world. They are cultivated throughout the world as cut blossoms, potted plants and most importantly in home gardens. Rose (*Rosa hybrida* L.) is a perennial flower shrub of the genus *Rosa*, inside the family Rosaceae that contains more than 100 species and found in multiple colours (Aggarwal *et al.*, 2020). It is said that *in vitro* propagation of roses could multiply rapidly cultivars with desirable traits and production of healthy and disease-free plants. During the last several years, researchers are witnessing several approaches for rose micropropagation. However, it is always challenging to find a suitable protocol and refinements with a high rate of shoot multiplication and a cost-effective method for a valuable variety (Huong *et al.*, 2021). Polyamines (PAs) are water-soluble, low molecular weight, polycationic, aliphatic nitrogenous compounds containing more than two amino groups, and are present in all living organisms. They can exist freely or be associated with other molecules, such as phenolic acids and macromolecules like nucleic acids and proteins (Rakesh *et al.*, 2021). In plants, polyamines play a crucial role in regulating various physiological processes, including flower development, embryogenesis, organogenesis, senescence, and fruit maturation. Additionally, they are involved in the plant's response to biotic and abiotic stresses (Chen *et al.*, 2019). Recent studies have examined the role of polyamines in plant

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development and their mechanisms of action through the use of exogenous PAs, PA synthesis inhibitors, and transgenic approaches. Studies has demonstrated that polyamines (PAs) play a significant role in plant growth, stabilizing nucleic acids and membrane structures, enhancing stress resistance, and even supporting plant survival (Sequera-Mutiozabal *et al.*, 2016). For instance, studies on *Pinus virginiana* have shown that the individual application of putrescine, spermidine, and spermine can help recover brown tissues into normal callus, achieving a recovery rate of 1.4%, along with increased antioxidant enzyme activity. However, combining these polyamines resulted in lower recovery rates than when each was applied alone (Tang *et al.*, 2004). Polyamines have also been found to enhance the quality of *in vitro* cultures of *Bacopa monnieri*, as they were shown to increase phenolic and flavonoid content, as well as antioxidant activities (Dey *et al.*, 2019). In *Citrullus lanatus* (watermelon), putrescine (Put) has shown the most favourable effects on rooting, root number, and root length. In contrast, spermidine (Spd) yielded the best results for the number of responding explants, shoot number, and shoot length. At this concentration of Spd, chlorophyll and carotenoid content were also higher compared to other Spd concentrations and other polyamines (Vasudevan *et al.*, 2017). Rajpal and Tomar (2020) described the presence of cadaverine (Cad) in corn coleoptiles, pea, tomato, and *Datura*, demonstrating that cadaverine levels depend on plant age and progressively decrease as the plant ages. This study aims to explore the enhancing effects of polyamines on rose micropropagules under *in vitro* conditions, with the goal of improving their proliferation and multiplication efficiency.

MATERIALS AND METHODS

Shoot cultures of *Rosa hybrida* L. cv. bush rose were initiated following the method of Makarov *et al.* (2024). Mature nodal segments were collected from greenhouse-grown plants during 2023, washed, and sterilized using ethanol and sodium hypochlorite. The explants were then inoculated on MS medium with 3.0 mg/L BAP, 0.01 mg/L NAA, 0.8% agar, and 3.0% sucrose, and maintained under controlled conditions (28±2°C, 16-hour light cycle). Various concentrations of polyamines (10 mM-50 mM) were added to the medium pre-autoclaving with filter sterilization to assess their effects on shoot multiplication. Cultures were sub-cultured every three weeks over six cycles, totalling 126 days. Growth parameters, biochemical analyses, and antioxidant assays were conducted at the experiment's end, with each treatment replicated three times.

Measurement of Growth Parameters

The total number of shoots, average shoot length, and biomass production in terms of both fresh and dry weights were determined. To measure biomass (fresh and dry weight), propagules from were removed and their fresh weight was measured using an electronic top pan balance. For calculating dry weight, after measuring the fresh weight, the shoots were placed in an oven at 62°C for 48 hours to dry.

Biochemical Analyses

Chlorophyll Content

Chlorophyll content was determined following the method outlined by Shaikh *et al.* (2023). For this, 500 mg of shoots (grown Cn PAs-containing medium) were weighed and ground in a mortar with 80% acetone under dark conditions. The extracts were then centrifuged at 10,000 rpm, and the supernatant was used to measure absorbance using a spectrophotometer (UV-Vis Shimadzu, Japan) at three wavelengths (663 nm, 652 nm and 645 nm). Concentrations of chlorophyll a, chlorophyll b, and total chlorophyll were calculated using the following formulas:

$$\text{Total Cholophyll (mg/g)} = \frac{20.2 \times A645 + 8.02 \times A663}{a \times 1000 \times w} \times V$$

$$\text{Cholophyll A (mg/g)} = \frac{12.7 \times A663 - 2.69 \times A645}{a \times 1000 \times w} \times V$$

$$\text{Cholophyll B (mg/g)} = \frac{22.9 \times A645 - 4.68 \times A663}{a \times 1000 \times w} \times V$$

Where, V = Volume of the extract in ml, W = Fresh weight of the sample (leaf) in g and a = Length of light path in cell (1 cm).

Total Phenol Content

The phenol content was measured using the Folin-Ciocalteu method (Sharma and Kumar, 2020). Shoots (500 mg) cultivated on polyamines-containing medium were ground in 70% methanol, and the extract was centrifuged at 10,000 rpm for 15 minutes. For analysis, 500 µl of the methanolic extract was mixed with 1.0 ml of diluted Folin-Ciocalteu's reagent and 2.0 ml of 20% Na_2CO_2 solution, then heated in a boiling water bath. After cooling, the solution was diluted to 25 ml with distilled deionized water, and percent transmittance was measured at 650 nm using a UV-Vis spectrophotometer. Total phenol concentration was determined from a standard curve of caffeic acid (10-100 µg).

Total Carbohydrates Content

The total carbohydrate content was estimated using method of Tandon (1976). *In vitro*-derived propagules treated with PAs were homogenized in 0.1 M phosphate buffer (pH 7.0) and centrifuged at 10,000 rpm for 15 minutes. For each reaction, 15 µl of supernatant was mixed with 4.0 ml of 0.2% Anthrone reagent in concentrated H_2SO_4 and incubated for 5 minutes in a water bath. Absorbance was measured at 610 nm, and total carbohydrate content was determined using a glucose standard curve.

Total Protein Content

Total protein content was estimated using method of Karimi (2022). 1ml of diluted crude tissue extract was mixed with 5.0 ml of Coomassie Brilliant Blue G-250 dye. The absorbance of the resulting complex was measured at 595 nm using a UV-Vis spectrophotometer. Protein concentration was determined from a standard curve prepared with various concentrations of albumin.

Enzyme Assay

Peroxidase (POD)

POD activity was measured by monitoring absorbance changes at 470 nm using guaiacol and H_2O_2 as substrates, following the method by Jiao *et al.* (2021). The substrate solution contained 0.5% guaiacol in 0.1M K_2HPO_2 (pH 6.0) and was stirred for 30 minutes before adding 0.008% H_2O_2. A cuvette with 2.5 mL of the substrate received 50 µL of the enzyme solution, and absorbance was recorded.

Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) activity was assessed using a modified NBT method (Alam *et al.*, 2021). In a 2 mL reaction mixture, 50 mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 9.9 mM L-methionine, 55 µM NBT, and 0.025% Triton- x100 was prepared. Then, 40 µL of diluted sample and 20 µL of 1 mM riboflavin were added. The reaction was initiated by illuminating the samples under a 15 W fluorescent tube for 10 minutes. Duplicate tubes without light served as blanks. After the reaction, absorbance was measured at 560 nm, and enzyme activity was calculated using a standard curve derived from pure SOD.

All analyses of data were conducted in triplicate with three replicates for each experiment, including appropriate blanks. The data was checked for variance using a one-way analysis of variance (ANOVA). In addition to ANOVA, data analysis was performed by using Duncan's multiple range test (DMRT) to compare multiple treatment and identify significant differences between them. Statistical validation of the data was performed using XLSTAT software.

RESULTS AND DISCUSSION

In this study, incorporating different concentrations of polyamines (PAs) into the standard rose multiplication medium produced varied responses. Adding PAs to the MS medium before autoclaving resulted in significant differences in growth parameters, including shoot length, shoot number, fresh weight, and dry weight. Additionally, there were notable increases in biochemical parameters, such as carbohydrate, protein, and phenol content. Enhanced activity of antioxidant enzymes like peroxidase (POD) and superoxide dismutase (SOD) was also observed. For comparison, rose cultures at the multiplication stage grown on the standard MS medium supplemented with the recommended plant growth regulators (PGRs) were used as controls.

At a low concentration of polyamines (10 mM), fewer shoots and shorter shoot lengths were observed. As PA concentrations increased to 30 mM, both shoot number and length showed a proportional increase, exceeding those of the control plantlets. However, at 50 mM, there was a decline in both shoot number and length compared to the 30 mM concentration (Table 1). Polyamines interact with phytohormones, functioning as plant growth regulators, hormonal secondary messengers, and sources of carbon and nitrogen in cultured tissues (Sivanandhan *et al.*, 2011). Vasudevan *et al.* (2008) suggested that polyamine application could significantly improve regeneration and differentiation in *Cucumis sativus* L. A similar trend was observed for total biomass (fresh and dry weight), a low concentration of PAs (10 mM) led to a significant increase in biomass compared to the control. Biomass continued to increase at 30 mM and 50 mM PAs, though at 50 mM, it decreased slightly relative to the 30 mM concentration (Table 1). An upward trend in shoot fresh and dry weight was observed with 30 mM Spd, achieving values of 3.24 g and 0.36 g, respectively, while similar effects were noted with Put and Cad.

Polyamines have been shown to promote shoot regeneration from *Passiflora* leaves, *Brassica campestris* cotyledons, and cucumber shoot tips (*Cucumis sativus*) (Shankar *et al.*, 2011). In rose explants, Spd and Put did not differ significantly in shoot length compared to Cad, although all three polyamines enhanced shoot fresh and dry weight over the control (Table 1). Previous studies have reported that exogenous Put application increased shoot length in various species, including flax, *Linum usitatissimum* L. (El-Lethy *et al.*, 2010), myrtle, *Catharanthus roseus* L. (Talaat *et al.*, 2005), onion, *Allium cepa* L. cv. 'Giza 20' (Amin *et al.*, 2011), artichoke, *Cynara scolymus* L. (El-Abagy *et al.*, 2010), and bean seedlings, *Phaseolus vulgaris* L. cv. Giza (Zeid, 2004). The positive effects of polyamines on vegetative growth are likely due to their role in strengthening cell division and expansion (Yang *et al.*, 2024). In rose explants, polyamines increased the total carbohydrate content in shoots. The highest carbohydrate levels were recorded with 30 mM concentrations of exogenous Spd and Cad (65.04 and 65.03, respectively), followed closely by Put at 30 mM (64.62). All concentrations resulted in higher carbohydrate content compared to the control (56.93) (Table 2). Similar findings have been reported in wheat, *T. aestivum* var. Giza 168 (El-Bassiouny *et al.*, 2008). In this study, polyamines also enhanced the total protein content in rose plantlets compared to the control, with the 30 mM concentration yielding the highest protein levels (Spd - 78.47, Put - 77.87, Cad - 73.87) relative to 10 mM and 50 mM (Table 2). Comparable results were observed in barley, where polyamines promoted protein accumulation under stress conditions (Özmen *et al.*, 2022). In contrast, polyamines reduced phenolic content in plantlets compared to the control level of 2.4, with the lowest phenolic levels observed at 30 mM for all three polyamines (Spd - 2.12, Put - 2.13, Cad - 2.09), and Cad showing the lowest phenolic content across all three concentrations (10 mM, 30 mM, and 50 mM) (Table 2). Similar work in

Table 1. Effect of different polyamines concentration on *in vitro* growth of rose micropropagules

Polyamines	Polyamines concentration (mM)	No. of shoots (mean)	Length of shoots (cm)	Fresh weight (g)	Dry weight (g)
Control	0	8.667 a	3.407 a	3.188 a	0.318 a
Spermidine	10	13.667 b	3.453 bc	3.218 bc	0.343 d
	30	18.000 c	3.573 d	3.248 e	0.369 g
	50	12.667 b	3.493 c	3.238 de	0.348 e
Putrescine	10	11.667 b	3.463 bc	3.208 abc	0.343 d
	30	18.333 c	3.547 d	3.228 cd	0.369 g
	50	13.000 b	3.433 ab	3.208 abc	0.348 e
Cadaverine	10	11.333 b	3.463 bc	3.198 ab	0.328 b
	30	17.333 c	3.493 c	3.218 bc	0.358 f
	50	12.667 b	3.397 a	3.198 ab	0.335 c

Means in the same columns followed by different letters are significantly different (P≤0.05) according to Duncan's Multiple Range Test.

Table 2. Effect of different polyamines concentration on biochemical parameters in rose micropropagules grown under *in vitro* conditions

Polyamines	Polyamines concentration (mM)	Total carbohydrate (mg/g fwt)	Total phenol (mg/g fwt)	Total protein (mg/g fwt)
Control	0	56.933 a	2.400 a	69.370 a
Spermidine	10	61.973 bc	2.210 a	75.870 f
	30	65.043 d	2.120 a	78.470 g
	50	63.973 cd	2.163 a	71.070 b
Putrescine	10	61.733 bc	2.207 a	74.870 ef
	30	64.623 d	2.130 a	77.870 g
	50	63.523 cd	2.173 a	72.080 bc
Cadaverine	10	60.833 b	2.180 a	72.870 cd
	30	65.033 d	2.093 a	73.870 de
	50	63.733 cd	2.117 a	71.970 bc

Means in the same columns followed by different letters are significantly different (P≤0.05) according to Duncan's Multiple Range Test.

rose has shown that foliar applications of polyamines like Put, Spd, and spermine can reduce phenolic content under stress conditions (Yousefi *et al.*, 2021).

We found that adding polyamines (PAs) resulted in a gradual increase in chlorophyll a, chlorophyll b, and total chlorophyll content. However, further increases in PAs concentration led to a decline in chlorophyll levels, although these values remained higher than those of the corresponding control propagules (Fig. 1). Similar findings have been reported in previous studies, where the application of polyamines like spermidine (Spd) and spermine (Spm) increased chlorophyll content in *Calendula officinalis* L. (Baniasadi *et al.*, 2018).

POD activity was significantly enhanced with increasing concentrations of polyamines (PAs). Different concentrations of PAs resulted in increased POD activity compared to the control. Notably, 30 mM of PAs exhibited the highest POD activity compared to both 10 mM and 50 mM concentrations (Fig. 2). Interestingly, cadaverine (Cad) demonstrated higher POD activity than spermidine (Spd) and putrescine (Put). Similar results were observed with superoxide dismutase (SOD) activity, where the 30 mM concentration displayed the highest activity compared to 10 mM and 50 mM, with Cad again showing greater activity than Spd and Put (Fig. 3). Polyamines play a complex role in plant oxidative stress, as they can enhance the function of the enzymatic antioxidant defense system, aiding in the efficient regulation of oxidative stress in

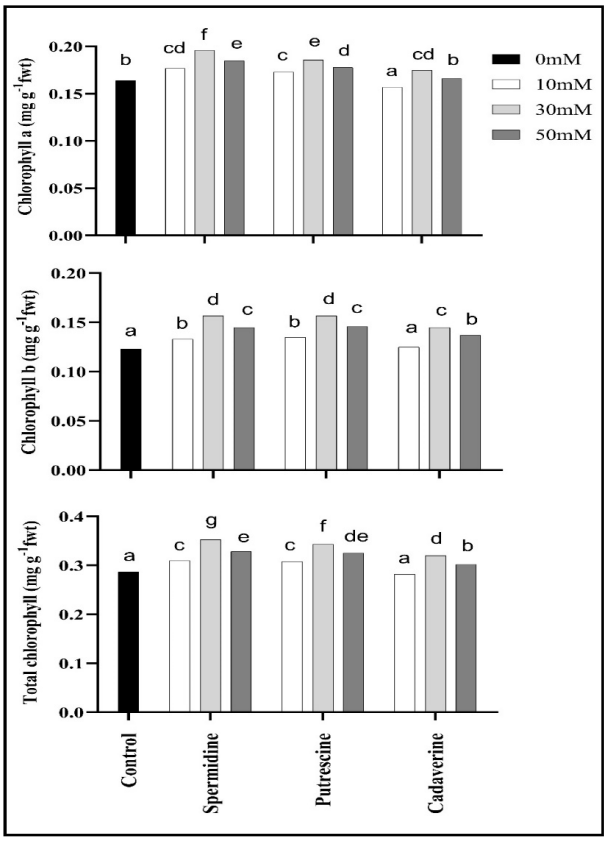

Fig. 1. Effect of polyamine (PAs) on chlorophyll contents in rose micropropagules grown under *in vitro* conditions.

plants exposed to environmental challenges (Wang *et al.*, 2020). Exogenously applied spermidine (Spd) increased levels of Spd and spermine (Spm) while reducing putrescine (Put) levels in cucumber roots under hypoxic stress. This effect was attributed to enhanced enzymatic antioxidant activity, greater reactive oxygen species (ROS) detoxification,

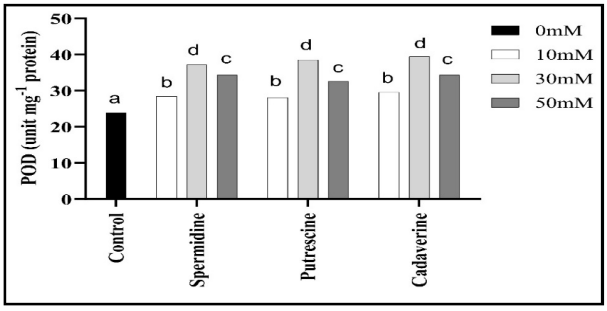

Fig. 2. Effect of polyamine (PAs) on antioxidant enzyme like peroxidase (POD) in rose micropropagules grown under *in vitro* conditions.

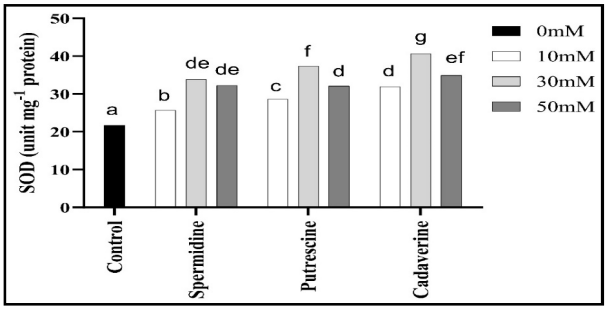

Fig. 3. Effect of polyamine (PAs) on antioxidant enzyme like superoxide dismutase (SOD) in rose micropropagules grown under *in vitro* conditions.

ultimately improving stress resistance (Wu *et al.*, 2018).

However, polyamines can also produce ROS due to their catabolism, which generates strong oxidizers such as hydrogen peroxide (H_2O_2) and acrolein, potentially leading to cellular breakdown under stress. At the same time, H_2O_2 acts as a signalling molecule in the stress signal transduction pathway and triggers an antioxidant defense response. Therefore, polyamines appear to be regulators of redox homeostasis, exhibiting a dual role in plant oxidative stress (Shao *et al.*, 2022).

CONCLUSION

This study demonstrates the varied effects of polyamines (PAs) on rose micropropagules during the multiplication stage. The results indicate that the inclusion of PAs in the culture medium significantly influences both growth and biochemical parameters. Specifically, moderate PAs concentrations (30 mM) were most effective in enhancing shoot proliferation, biomass, carbohydrate, protein content, and antioxidant enzyme activity, while maintaining lower phenolic content, which collectively support improved plant growth and stress resilience. The reduction in growth parameters at higher PAs concentrations (50 mM) suggests an optimal range for PAs application to maximize benefits in rose tissue cultures. This study highlights the role of PAs not only as plant growth promoters but also as modulators of oxidative stress responses through enhanced POD and SOD enzyme activities. These findings are consistent with earlier studies on other plant species, where PAs were shown to promote cell division, expand biomass, and regulate oxidative stress. The observed interactions between PAs and phytohormones, along with their influence on carbohydrate, protein, and phenol content, underline the potential of PAs to act as multifaceted growth regulators in tissue culture applications.

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Liquid Culture System: An Efficient Approach for Sustainable Micropropagation

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Abstract

Micropropagation of important forestry, horticultural and medicinal plants have made revolutionary changes in terms of research and commercialization. However, there are a variety of factors which influence the scaling-up and commercialization aspects, which decide whether mass propagation will be effective and lucrative. Low rates of shoot multiplication, increased costs of media components, loss of cultures due to contamination, and difficulties with hardening and acclimation are the key obstacles to scaling up micropropagation technology. These restrictions have forced a large number of *in vitro* technologies developed for a range of plant species to be used only under research laboratories settings. To apply tissue culture technology to large-scale propagation, it is required to develop techniques that are relatively simple to adopt, have high multiplication rate with high levels of reproducibility, and exhibit higher survival of plantlets when transferred to *ex vitro* conditions. Efficient techniques include utilization of liquid culture systems and replacement of agar with other gelling agents. These techniques allow development of micropropagules that not only function better in *post-vitro* soil conditions and are comparatively less expensive, but will also help develop a workable micropropagation technique that can be applied to the mass production of desirable plant species. The current review describes liquid culture system as an efficient approach to produce large number of plants at low production cost.

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Introduction

In the last decade, plant tissue culture has proved its significance in several areas of research and commercialization. These include: a) CRISPR-CAS-9 mediated improvement of crops, b) commercial production of horticulture and medicinal plants, c) transgenic plant development, c) *in vitro* production of important secondary metabolites, d) production of novel varieties through embryo rescue and haploid culture, e) germplasm conservation.[1] Tissue culture methods are generally used to improvise the plants which do not produce seeds or have stubborn

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seeds that can't be stored in seed gene banks under normal capacity settings. In order to maintain quality of plantlets, *in vitro* procedures have been proved to be very useful in various roots and tubers, ornamental plants, medicinal plants, and several other tropical fruit plants. Plant tissue culture was initially utilized as a research method with a primary goal of cultivating and studying the development of tiny, isolated plant tissue pieces or isolated cells.[1] Plant tissue culture has gone through numerous stages of progress, including logical curiosity, a research tool and innovative applications, similar to other advanced methods. Micropropagation technology is a technique for *in vitro* propagation of plants by using principles of biotechnology. The plants are derived from taking initiation material like stem part, root part or leaf tissues and the technique developed guides in large scale production of economically important crops varieties. Singh *et al.* (2016) has enlisted key characteristics of micropropagation technique, which include a regulated environment, managed plant growth, and product (micro-propagules) that are free of many pests and diseases.[2] Due to the propagated plants' compact size, nursery space and plant transportation expenses are reduced. The biggest drawback of tissue culture plants is how expensive they are to produce. A variety of plants intended for commercial tissue culture propagation are constrained by this challenge.

Plant organs and tissues are cultured *in vitro* on artificial media, which supply the nutrients vital for development. The progress of micropropagation as a method for plant propagation is enormously impacted by the type and concentration of the culture medium components used. Generally, in any tissue culture medium, the components are majorly classified in four groups excluding sucrose (carbon source) and agar (solidifying agent). These groups are: a) Macronutrients (Nitrogen, Potassium, Phosphorus, Magnesium, Sulphur), b) Micronutrients (Manganese, Boron, Zinc, Cobalt, Copper), c) Iron and chelating agents ($FeSO_4$ and EDTA) and d) organic supplements (vitamins and amino acids). The most commonly utilized medium is the one described by Murashige and Skoog (1962). This medium was ideally developed for growth of tobacco callus and later on it was proved beneficial for wide range of species with slight modifications.[3]

In addition to these inorganic supplements, plant tissue culture medium often provides a carbohydrate (sucrose is typically standard) to substitute the carbon that a plant normally fixes from the air through photosynthesis. As mentioned above, numerous media also include various organic substances, vitamins, and plant growth regulators to promote development. In early trials of research in development of growth media, undefined components like natural plant products, yeast extracts, protein hydrolysates *etc*. were utilized instead of defined nutrients or amino acids, or even as additional supplements. Coconut milk, for example, is still frequently utilized, and banana homogenate has been a famous expansion to media for orchid culture. There are certain precautions, which are needed to be followed while designing and development of medium. For example, there should not be any changes in laboratory conditions and type of inorganic or organic salts (for instance the hydration of compounds). Table 1 shows general composition of plant tissue culture medium, which is followed by most of the researchers.

Table 1: List of inorganic/organic salts, carbon source, vitamins, and solidifying agents used in generalized plant tissue culture media

Sr. No.	Components	Elements	Inorganic/Organic salts
1	Macro-elements	Nitrogen (N)	NH_4NO_3
			$(NH_4)_2SO_4$
		Potassium (K)	KNO_3
			KCl
			KH_2PO_4
			K_2SO_4
		Calcium (Ca)	$Ca(NO_3)_{2.}4H_2O$

			$CaCl_2.2H_2O$
		Magnesium (Mg)	$MgSO_4.7H_2O$
		Sodium (Na)	Na_2- EDTA
			$NaH_2PO_4.2H_2O$
			Na_2SO_4
		Ferrous (Fe)	$FeSO_4.7H_2O$
2	Micro-elements	Potassium (K)	KI
		Boron (B)	H_3BO_3
		Ferrous (Fe)	$Fe_2(SO_4)_3$
		Manganese (Mn)	$MnSO_4.4H_2O$
		Zinc (Zn)	$ZnSO_4.7H_2O$
		Sodium (Na)	$Na_2MoO_4.2H_2O$
		Copper (Cu)	$CuSO_4.5H_2O$
		Cobalt (Co)	$CoCl_2.6H_2O$
3	Vitamins	Calcium pantothenate	
		Thiamine HCl	
		Inositol	
		Nicotinic acid	
		Pyridoxine HCl	
4	Amino acids	Glycine	
		Cysteine HCl	
		Glutamine	
5	Carbon Source	Sucrose	
6	Solidifying agents	Agar	

Liquid Medium as an Efficient Approach for Tissue Culture

In plant tissue culture, generally semi-solid medium is used for regeneration and other purposes. However, there are several constrains in using semi-solid medium for example, high production cost, less protocol efficiency and multiplication rate, high contamination rate and somaclonal variations, which arise during culture conditions.[4] The high prices of media have restricted the widespread adoption of the plant tissue culture application.[5] Low plantlet production rates, high labour costs, and increased space requirements continue to be the barriers in adoption of semisolid media for commercial production.[6] In order to produce valuable and affordable *in vitro* plantlets, the proper selection of media components should be taken into account. Liquid culture media have been employed as an effective way to address the problems which arise during the use of semisolid medium and also enable the researchers or commercial producers in development of automation and cut down both time and cost.[5,7] Uniform culture conditions, quick media replacement without changing the container, sterilisation with ultra-filtration, and simpler container cleaning after use are all benefits of liquid culture systems. Agar culture media require surface culturing of tissues, whereas liquid culture media allow for the use of containers of various capacities.[8,9] Faster growth rates, efficient nutrient absorption by tissues, and dilution of secreted growth inhibitors, such as phenolics produced by explants, all represent potential advantages of liquid culture systems over solid cultures.[10]

Plant tissues/ explants of various species have shown improved performance in liquid medium as opposed to solid or semi-solid medium.[11] *Acacia nilotica* shoot numbers were around ten times higher in liquid culture than in gelled culture.[12] However, liquid culture is characterised by excessive humidity and a restricted exchange of gases between the interior atmosphere of the culture vessel and its surroundings. These circumstances might lead to physiological illnesses such as hyperhydricity. If liquid culture is defined as the growing of explants on a nonsolid media, then other changes to the fundamental system are feasible. Explants are placed in a static liquid solution in the most basic liquid culture methods. For instance, thousands

of embryos may be produced from androgenic pollen grains of wheat in a static liquid culture. Modifications include aeration, which involves bubbling air through the medium, use of a support for the explants (such as cellulose substrate), shaking the culture to maximize contact between the medium and explants. Temporary immersion system, in which the explants are submerged and removed in the medium for varying lengths of time are also very efficient methods apart from use of bioreactors (closed or open). Scaling up and utilization of bioreactors for commercial production, as well as the development of organ-genic propagules like bulblets, have all been studied in detail.[13,14] While it is outside the purview of this work to evaluate all occurrences and implications described in liquid culture, the process by which liquid culture can control plant growth and development is covered. Growth rates and morphogenetic patterns are used to demonstrate the advantages of liquid culture over traditional gelled medium, while the drawbacks are underlined. The role of certain chemical variables in coordinating growth and development is also discussed.

Explants cultivated in solid media will display polarity in their response and cells that are not in direct touch with the medium must absorb nutrients and process regulatory signals by diffusion from nearby cells. This may result in the peripherally placed cells sensing a different signal. Since the entire surface of the explant is immersed in the medium and may thus sense chemical signals, this condition is avoided in liquid culture. The decrease in the harmful effects of toxins is another benefit of adopting liquid systems for culturing. Any metabolite that the explant releases into the media may have harmful or inhibiting effects on subsequent growth and/or development. Toxins quickly dilute in liquid systems, as opposed to solidified media, where released compounds stay near to the explant, lessening any possible inhibitory impact. Liquid cultures often have higher rates of multiplication and proliferation than traditional gelled cultures. According to Kim *et al* (2003), garlic shoots on solidified cultures showed a decrease in the growth rate and fresh weight of shoots.[15] Explants produced on liquid medium for potato micropropagation showed faster shoot development rates than explants cultured on solidified media.[16] Sugarcane meristems were cultivated in both liquid and solid medium as well as in a transient immersion system, and[17] compared the growth rates of these meristems.[15] In some cases, strangely, the growth rates of the liquid and solid media did not differ much, whereas the temporary immersion system yielded a growth rate that was double than that of the other two techniques.[18]

In liquid culture, shoots of *Pinus radiata*, tea, wild pear, and *Calotropis gigantea* have all shown better rates of multiplication, when compared with explants grown on agar-solidified media.[19-21] When cultivated in a liquid-shake culture, aspen root cultures quickly increased in biomass.[22] But faster development rate in a liquid environment is not a general occurrence. Suspension culture offers a lot of promise for reproduction and the generation of synthetic seeds since it can result in the formation of many somatic embryos. After just eight weeks in culture, one gram of embryogenic callus from coffee leaves may generate 1.2×10^5 somatic embryos under ideal development circumstances. According to Gawel *et al* (1990), liquid cultures generate more cotton somatic embryos than gelled cultures do.[23] A number of factors have been put forth as the causes of this improved growth rate, including better nutrient availability,[16,24] increased water availability,[25] a less pronounced gradients in nutrients and endogenous hormones, and a more gradual pH shift throughout culture,[23] removal of polarity, and a lessened impact of toxins. According to Singha (1982), decreased diffusion resistance and tighter contact between the explant and culture media lead to greater availability of nutrients and water.[24] However, there was no appreciable difference in the water content of micropropagated potato shoots between liquid and solid cultures. Increased carbohydrate and organic nitrogen build up led to an increase in shoot fresh weight, indicating that liquid culture favours nutrient digestion.[16] Depending on the species, kind of explant, and particular culture circumstances, growth rate increases may be caused by increased carbohydrate build up, increased water intake, or a combination of the two.

In contrast to gelled cultures, where depletion zones (gradients) do form around the explant, agitation of a liquid culture enables uniform dispersion of nutrients and growth-promoting agents. This is helpful because in agitated liquid cultures, the concentrations can be maintained uniformly, whereas the action of exogenously administered growth regulators frequently fluctuates with

concentration. Additionally, culture agitation results in higher explant aeration and, thus, increased growth rates26. Microspores are grown in a liquid media with developing ovaries to produce haploid wheat plants. As a "nurse culture," the ovaries release substances needed by growing microspores to finish androgenic development and form a haploid embryo.[27] Nevertheless, an extract made from immature ovaries did not promote androgenesis, indicating that ovaries actively create the necessary factor(s) in response to the physical environment of the liquid media. In order to ensure that all elements and regulators are dispersed uniformly throughout the media, androgenic differentiation will profit from the diminished barrier to diffusion.

Regeneration in Liquid Culture System

Somatic embryogenesis is the process through which a non-zygotic cell grows into a bipolar structure that resembles a zygotic embryo without having a vascular link to the original tissue.[28] In order to research different facets of embryogenesis, somatic embryos are employed as a model system. The possibility for extensive vegetative reproduction is perhaps the biggest benefit of creating somatic embryos. This method enables the production of genetically homogeneous plants from a superior parent as well as the multiplication of plants that are thought to be challenging to replicate. Furthermore, research involving genetic transformation benefit from the generation of a lot of embryogenic calli in liquid culture.[29] Establishing the proper kind of suspension culture is necessary for somatic embryogenesis to take place in liquid culture. Large vacuolated cells in suspension are frequently produced by undifferentiated callus and perish after two weeks. When the callus is triggered on a media that contains an auxin like 2,4-Dichlorophenoxyacetic acid (2,4-D) however, an embryogenic suspension can be produced; these cells are typically smaller and have dense cytoplasm. The development of embryogenic callus occurs in asparagus when the kind and ratio of the hormones alter. When the hormone combination of Indole Acetic acid (IAA), Benzyl Adinine (BA), and 6-(γ,γ-Dimethylallylamino)purine(2-iP) was substituted with kinetin and 2,4-D, globular callus clumps were seen. In contrast, *Ophiopogon japonicus* suspension cultures do not need plant growth regulators to produce somatic embryos.[30]

For the formation of somatic embryos and subsequent plant regeneration in some species, huge numbers of embryogenic cells or cell clumps are produced in suspension cultures, filtered, and then plated on solid media.[31] In these situations, the liquid phase of the culture serves just to promote cell multiplication, while keeping the individual cells and cell clumps in an embryogenic condition. In their study, Jayashankar *et al.* (2003), examined somatic embryos produced in both solid and liquid environments. These authors noted that although embryos derived from a solid media had big cotyledons, a poorly formed suspensor, and a relatively underdeveloped concave apical meristem, those obtained from a liquid medium had smaller cotyledons, a distinct suspensor, and a flat-to-convex shoot apical meristem. Embryos from the liquid media did not demonstrate dormancy, in contrast to those from the solidified medium, and they had high rates of plant regeneration.[32] What characteristic of the liquid medium is able to keep cell clumping in an embryogenic state is still a mystery? It is most likely a result of several variables working together. For instance, increased availability of metabolites and growth-regulating compounds that can be absorbed by all areas of the explant (owing to closer contact with the medium) along with lowered nutritional gradients all likely help to preserving the embryogenic potential of the culture.

Secondary Metabolites Production in Liquid Culture System

Plants create a wide range of organic substances known as secondary metabolites to help them interact with their biotic environment and develop defensive mechanisms.[33,34] The majority of secondary metabolites, including terpenes, phenolics, and alkaloids, are categorised based on their biosynthetic origin, exhibit a variety of biological activities, and are employed as biopesticides, agrochemicals, medicines, flavouring agents, perfumes, colours, and food additives. Field cultivation for the purpose of producing secondary metabolites has a number of drawbacks, such as poor yields and concentration swings resulting from environmental, seasonal, and geographic differences. In order to produce secondary metabolites, plant cells and cultures have therefore become appealing alternatives (Table 2).

Table 2: Reported plants species producing secondary metabolites under in vitro conditions

Sr. No.	Plant Species	Secondary metabolites reported	References
1.	*Capsicum chinense*	Capsaicin	[35]
2.	*Salvia castanea*	Tanshinone	[36]
3.	*Papavar orientale*	Morphine	[37]
4.	*Astragalus membranaceus*	Isoflvonoid	[38]
5.	*Psoralea corylifolia*	Daidzin	[39]
6.	*Bacopa monnieri*	Bacoside	[40]
7.	*Catharanthus roseus*	Vinblastine, vincristine	[41]
8.	*Chlorophytum borivilianum*	Saponin	[42]
9.	*Camptotheca acuminata*	Camptothecin	[43]
10.	*Isoplexis canariensis*	Canarigenin, uzarigenin, digitoxigenin, xysmalogenin	[44]
11.	*Ruta graveolens*	Psoralen, bergapten, xanthotoxin, isopimpinellin, imperatorin, umbelliferon	[45]
12.	*Salvia officinalis*	Carnosol, carnosic acid, rosmarinic acid	[46]
13.	*Tripterygium wilfordii*	Triptolide, wilforgine, wilforine	[47]
14.	*Rosa hybrida*	Anthocyanin	[48]
15.	*Panax ginseng*	Ginsenoside	[49]
16.	*Genista tinctoria*	Isoflavones	[50]
17.	*Nothapodytes nimmoniana*	Camptothecin	[51]
18.	*Ruta graveolens*	Psoralen, bergapten, xanthotoxin, isopimpinellin	[52]
19.	*Securinega suffruticosa*	Securinine, allosecurinine	[53]
20.	*Withania somnifera*	Withanolides	[54]

Bioreactors

Plant tissue, cell, and organ cultures have been acknowledged as potent tools for the clonal propagation of commercially significant crops (micropropagation), the production of valuable secondary metabolites, the expression of complex foreign proteins (molecular farming), and phytoremediation of waste waters (Phyto transformation and phytoextraction). It is possible to cultivate plant cultures on a large scale using liquid media *in vitro* under controlled environmental conditions in bioreactor systems. These plant cultures can be differentiated (embryos, shoots, seedlings, transformed or adventitious roots), or dedifferentiated (suspended cells). The main goal of the strategy is to produce as much plant biomass as is economically viable, ready for immediate use or for later separation of valuable products.

The bioreactor is a piece of specialised technology that controls numerous physical and/or nutritional parameters to enable intense culture. Systems using bioreactors typically include a culture vessel and an automated control block. The culture vessel is made to hold the grown cells in an aseptic environment while enabling options for maintaining ideal micro-environmental conditions, nutrients, and gaseous mass transfers to ensure their maximum development. The automated control block is a computerised, fully automated or semi-automated system that is intended to monitor and regulate the cultivation conditions in the culture vessel, including 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. Existing bioreactors may be divided into four major categories based on the makeup of the environment in which the grown cells are housed: liquid-phase bioreactors, gas-phase bioreactors, temporary immersion systems (TIS), and hybrid bioreactors. The cultured cells/ tissues are fully submerged in a liquid nutrient solution in liquid-phase bioreactors. The best researched systems

at the moment are liquid-phase bioreactors, which include mechanically agitated, pneumatically agitated, hydraulically agitated, and membrane bioreactors. These systems have nearly infinite potential for use in generating undifferentiated plant cell suspension cultures.[55]

However, liquid-phase bioreactor methods often are unable to guarantee adequate development of differentiated plant *in vitro* systems. Because of hypoxia and hyper-hydricity, total submersion of plant tissue or organ cultures in the liquid media frequently results in deformities and material loss. Asphyxia and hyper-hydria are unfavourable physiological states that are solely brought on by the culture media's low oxygen concentration and water potential.[56] The creation of bioreactors with a sophisticated design, capable of supplying a specialised microenvironment in order to ensure the growth and physiological integrity of the cultures, is necessitated by the complex morphology of differentiated plant tissue and organs.[57] Gas-phase bioreactors TIS, and hybrid bioreactors[58,59] have been created to solve the problems that currently exist. The goal of TIS is to decrease physiological problems and retain the morphological integrity of fast-growing differentiated plant *in vitro* cultures by creating an ideal environment, improving nutrition and gas exchanges, and lowering mechanical stress. In TIS, explants are regularly submerged in a liquid media and subsequently exposed to a gaseous atmosphere, providing the most natural environment for plant tissue and organ *in vitro* cultures[57] TIS has been developed in many forms and is often used in the commercial micropropagation of commercially significant plant species. TIS have also been used in the study of secondary metabolite synthesis, molecular farming, and even phytoremediation of hazardous substances[57] because to its straight forward design and adaptable functioning.

Temporary Immersion System (TIS): A Modification In Liquid Culture System

The original idea for TIS was developed by scientists in 1983, when they created a device called "auxophyton" that could combine aeration and liquid media cultivation.[60] Auxophyton rotated the culture containers on a wheel, alternating exposing the test plants to air or submerging them in liquid. The carrot tissue was 2.6 times heavier after 20 days than the tissue grown on an agar medium.[5] Earlier attempts in growing carrot tissue cultures completely immersed in water failed, probably for lack of oxygen.[61] TIS-based bioreactors have experienced several developments since that time. However, every device complies with the specifications given by Teisson *et al.,*[62] including: (a) no continuous immersion, (b) sufficient mixing and OTR, (c) consecutive medium changes and automation, (d) low shear stress, contamination and costs. Different plant species have indicated that TIS has good impacts on shoot proliferation,[63] shoot vigor,[64] SE,[65] plant material quality.[66] as well as micro cuttings and microtuberization.[67,68] The most important factors determining the effectiveness of TIS are hyperhydricity and adjusting the immersion time.[69,70]

Hyperhydricity: a Disadvantage in Liquid Culture System

Although liquid culture techniques often promote greater, more rapid multiplication and biomass build up, there are several species that are not suited for liquid cultures because they are more likely to exhibit physiological abnormalities called hyperhydric syndrome.[71] The normal soil environment is produced by solid medium for terrestrial plants. Usually, explants that are totally submerged in growing media have the morphological alterations common to plants from liquid environments. Hyperhydricity, a condition where plants store too much water in their tissues, can occur in these situations.[72] The stems of hyperhydric shoots are transparent and brittle, contain a lot of water, and have a severe lack of chlorophyll, among other physiological abnormalities. Shoots with excessive water content frequently have thick, elongated, curled, and wrinkled leaves.[73] They have fewer layers of palisade cells, wide intercellular gaps, chloroplast degeneration, uneven stomata, and a very thin cuticle with less cellulose when seen anatomically. Because there is so much water in the apoplastic gaps of hyperhydric tissues, they have reduced dry biomass.[74]

The plant material may occasionally still be unable to develop even after the transfer of the hyperhydrated tissues to the solid medium to restore the plant to a normal state.[75] Later phases of plant growth may experience ramifications from the vitreous effect. Additionally, proliferating hyperhydric shoots have trouble establishing roots, as seen in the cases

of *Salvia officinalis*[46] and *Centaurium erythraea*.[75] This is not always the case and can be observed as exception as we can see in other plants, like in case of *Catharanthus roseus* liquid culture system did not prevent the subsequent formation of roots on the shoots but actually aided in the process.[41] The shoots also rooted more quickly, showed higher percentages of rooted shoots and numbers of roots on a single shoot, and were longer than the plants grown in the solid medium. Most of the time, the process of acclimatising rooted shoots is independent of the consistency of the growing medium and has no impact on the survival rate. Even more has been said about how rooting in liquid may forecast a later stage of micropropagation since there is less chance of root system injury during soil transplanting.[41] A large decrease in the generation of bioactive metabolites in the morphologically altered organs may be another unfavourable effect of hyperhydricity.[76] This phenomenon manifests as a result of several stressful circumstances, such as extreme humidity. A gaseous restricted environment with low oxygen concentration may cause hyperhydricity. Hypoxia can result from the extra water in the tissues reaching low saturation levels.[19] Free radical-induced oxidative stress can harm tissues and cells and interfere with their metabolic processes. Normal development is disturbed as a result of all these variables. Agitation might produce aeration, however certain species are delicate to the shear stress and mechanical damage brought on by shaking the culture. Hyperhydricity may be markedly increased by exogenously applying cytokinin to a liquid media, especially at high doses.[77] In the meantime, the medium is frequently supplemented with cytokinin to promote the development and proliferation of *in vitro* shoots.

Role of Support Matrix in Liquid Culture System

Many methods for supporting plants over stationary liquid to lessen hyperhydricity have recently been investigated. Support matrix facilitates continuous and simple nutrient absorption, while permitting shoot development at very high levels of aeration. It enables dangerous phenolic exudates to spread throughout the media. Furthermore, the shear stress and mechanical damage brought on by the aeration and agitation associated with shake flask cultures are eliminated by the supports' static nature.[78] For most plant systems to multiply, root well, and anchor better in various types of culture containers, some sort of solid matrix is also fundamentally necessary. The addition of expensive gelling agents, as well as the cost of washing and cleaning, are avoided when support matrices are used. The likelihood of contamination can be decreased during the maintenance of these types of cultures since subculturing is only possible with the addition of sterile liquid medium.[79,80] However, when employed, a mechanical support should be porous, inert, non-toxic, resistant to plant digestion enzymes, and autoclavable. There are currently several mechanical supports available, and many people have successfully used them in various industrial systems. The majority of the time, a significant decrease in manufacturing costs favoured overall growth. For instance, cotton fibre costs around $2/kg, whereas agar costs between $100 and $200/kg. Similar to that, apple rootstock was rooted using a matrix made of sugarcane bagasse.[81] A high-quality plant cultivated on sugarcane bagasse was significantly (13.4%) less expensive than one grown on agar-gelled media. A cost reduction of roughly 35% was made possible when the quantity of high-quality rooted plants exceeded 1000. In order to cultivate ginger and turmeric at a lower cost than agar,[82] successfully employed glass beads. In his testing, there was a 94% decrease in the cost of the media. He also showed that only 15 to 18 ml of media were needed per culture container (an Erlenmeyer flask with a 100 ml capacity) when glass beads were employed as support matrices. As a result of this technique, the price of medium was significantly reduced because one litter of media created 50 cultured vessels (only 30 containers are filled in the case of agar-gelled semi-solid medium).

Plants of ginger and turmeric proliferated as well on liquid glass bead media as they did on agar-based medium. For vanilla, a similar kind of reaction was seen. Even with a modest vitrification, *Ficus cv.* "Mini lucii" had a greater rate of multiplication. On glass bead liquid-medium, *Saintpaulia, Syngonium, Philodendron*, and *Spathiphyllum* also showed faster multiplication rates and better growth.[82] Glass beads were employed by McLeod and Nowak (1990) to propagate raspberry and white clove plants, and they claimed a 60 percent media cost savings as a result. Glass beads were effective in maintaining callus and shoot organogenesis in *Rhododendron.* After being washed with acid, the beads can be utilised again.

An effective strategy for the speedy and inexpensive *in vitro* multiplication of certain commercially relevant plant species was glass bead-supported liquid media, such as *Celastrus paniculatus*, *Chlorophytum borivilianum, Terminalia bellerica*, and *Boswellia serrata.* In all of these plants, liquid medium encouraged shoot multiplication, shoot elongation, and accumulation of total fresh and dry weight. The shoots raised in this medium had a greater number of leaves, each with a bigger surface area and thicker laminae. For *C. paniculatus* and *B. serrata* shoot cultures, an increase in chlorophyll a, b, and total chlorophyll content was seen. The use of glass beads was very successful and did not result in any degradation due to hyperhydricity in liquid culture. Plantlets may be easily removed from the media due to use of glass beads. The support matrix used by different researchers has been listed in Table 3.

Table 3: Different mechanical support types are utilized at various phases of micropropagation of various plant species.

Sr. No.	Different supports systems	Micropropagation stages	Plants	References
1	Cotton fibre	Callus organogenesis	Artemisia annua	[83]
2	Filter paper bridges	Multiplication	Chrysanthemum and potato	[84]
3	Luffa sponge	Multiplication and rooting	Philodendron spp.	[79]
4	Paddy straw, jute, coir	Rooting	Nicotiana, Beta, Chenopodium, Tectona, Musa,	[80]
5	Coir	Microcorm production	Gladiolus	[85]
6	Sugarcane baggase	Rooting	Apple	[81]
7	Peat pellets	Rooting	Sunflower	[86]
		Multiplication	Terminalia, Celastrus, Feronia, Boswellia, Chlorophytum	[87]
8	Glass wool	Multiplication	Chrysanthemum	[84]
9	Rock wool	Shoot development	Eucalyptus citriodora	[88],[89]
			Spathiphyllum	[90]
10	Nylon cloth	Multiplication	Chrysanthemum	[84]
11	Polyurethane foams	Multiplication	Nicotiana and Vitis	[91]
			Gladiolus	[78]
12	Foam plastics	Adventitious root development	Rhododendron	[92]
13	Polyester squares	Multiplication	Musa	[93]
14	Polyester rafts	Multiplication	Anthurium	[94]
15	Florailite and vermiculite	Multiplication	Ipomoea batatas (sweet potato)	[95]
16	Polypropylene Membrane Rafts	Multiplication	Gladiolus	[78]

Conclusion

The main challenge with commercial tissue culture technology is its high manufacturing costs. The development of micropropagation procedures in the laboratory as a component of R&D programs should result in a viable technology suited for the mass production of desired clones. The established methodology that is accessible

for a species and the advantages and risks attached to it are key factors in determining whether commercialization is successful. In numerous economic plant species, *in vitro* propagation is limited by lack of contemporary techniques to overcome rigorous labour manipulation. Scaled-up unit cost of micropropagules can be decreased by employing creative and more affordable options mentioned above. In order to determine the efficacy of such methods, pilot-scale testing is required.

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Conflict of Interest
The authors declare no conflict of interest personal or financial.

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Studies on the effect of natural extract of some marine algae on *in vitro* growth and development of banana micropropagules

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Abstract

The current work aims to provide a cost-effective and reliable micropropagation method of banana using some natural extract of unexplored potential seaweeds. We tried natural extract of eight different seaweeds (*Caulerpa racemosa, Gracilaria edalis, Caulerpa paspaloides, Ulva lactuca, Sargassum tenerrimum., Caulerpa sertularioides, Sargassum wightii, Gracilaria corticata*.) to check their role in *in vitro* growth and development of micropropagules and their potential in replacing commercial plant growth regulators (PGRs) in banana micropropagation. The *in vitro* developed shoots were allowed to grow on modified MS medium supplemented with different seaweed extracts and regularly subcultured on the same medium throughout the experiment. The growth and biochemical parameters were recorded and compared with micropropagules growing in a controlled environment (*i.e.*, MS medium supplemented with/ without standard PGRs). We found a significant increase in shoot length (highest in *S. tenerrimum, i.e.* double as compared to negative control), shoot number (highest in *G. edalis*, *i.e.,* three fold as compared to negative control) and fresh weight (*S. tenerrimum*) of the banana micropropagules when grown under the influence of Liquid Seaweed Extract (LSE). This is comparable to growth observed in standard control conditions (*i.e.* positive control). However, addition of LSE of *G. corticate, C. paspaloides* and *S. wightii* did not show any noteworthy effect on shoot length, shoot number and fresh weight. Total chlorophyll and other biomolecule accumulation in test propagules varied with types and concentrations of LSE. *S. tenerrimum* proved to be overall good growth promoter, as it caused maximum accumulation of chlorophyll (0.202±0.02 mg g^{-1}) and protein (105±0.45 mg g^{-1}) and significant and encouraging results in phenol, carbohydrates and proline. Our results show that seaweed can be potential source of beneficial natural compounds and it can be a cheaper option in banana micropropagation technology.

Key words: Seaweed, micropropagation, banana, low-cost alternatives

Introduction

Banana (*Musa sp*.) is one of humankind's important and oldest fruits and is valued for its nutritional properties and economic role. Based on its gross value, it is the world's fourth most widely consumed food crop after rice, wheat, and corn. It is commonly grown in the tropics and subtropics in all agricultural systems. In India, bananas account for 32.39% (2020-2021) of total fruit production. Multiplication of bananas by traditional methods increases the chance of disease in plants, and these methods are also very tedious and time-consuming. These problems in banana cultivation can be overcome by tissue culture technology, which is an exclusive tool for producing banana plants in less time.

Moreover, micropropagation also helps to develop pathogen-free and healthy plants that can be directly planted to fields (Joshi and Purohit, 2012). In the last two decades, the commercial tissue culture has witnessed a revolution, and the technique has been converted into a successful industry-oriented method, especially for ornamental and horticultural plants (Patil *et al.*, 2021). However, there are a few limitations of this technology when compared to the traditional methods of plant propagation. For example, commercial micropropagation is a capital-intensive sector since it requires various expertise, infrastructure, sophisticated equipment, and controlled environmental conditions. Consequently, the unit cost per plant can be excessive in some cases (Teraiya *et al.*, 2023). Therefore, business growth in developing countries like India has been limited, with only well-funded institutions and large corporations thriving, while smaller units exit the market. Studies have aimed to reduce production costs in large-scale conditions to address this issue. They've explored replacing expensive medium ingredients, such as agar-agar, a major contributor to costs, with alternatives like guar gum, cotton fiber, xanthan gum, isabgol, and corn/potato starch (Nirmal *et al.*, 2023; Chauhan *et al.*, 2018). Similarly, high-grade analytical salts have been swapped with low-grade raw salts, and pure sucrose (as a carbon source) with cheaper sugars. Reducing the costs of agar-agar and sucrose, which constitute 49.61% and 38.49% of standard MS medium preparation, has been a primary research focus (Patil *et al.*, 2021). Synthetic plant growth regulators (PGRs), such as 6-Benzylaminopurine (6-BA), have been overlooked in the cost analysis of standard MS medium production. These PGRs contribute significantly to production costs, with 6-BA alone accounting for 7.78% of costs and other components totaling 4.12% (Chauhan *et al.*, 2018).

Marine microalgae, commonly known as seaweeds, have been used by humans since ancient times for many important applications. Earlier, the Romans, Chinese and Japanese were using seaweeds as a biofertilizer and manure in agriculture and other industries. Seaweeds as a biofertilizer, promote plant growth, provide better growth under stress conditions and help in nutrient uptake from soil (Yadav *et al.*, 2016). Seaweed also contains some essential minerals (like micronutrients,

macronutrients, amino acids, and vitamins), which help the plant to grow in adverse conditions and act as a source of nutrients for crops (Yadav *et al.*, 2016). Seaweeds have garnered significant attention in plant growth due to their potential as biofertilizers. Maxicrop, in the 1940s, was among the pioneers to introduce seaweed-based liquid biofertilizers to the market, igniting interest in the growth-promoting properties of seaweeds. Researchers have identified nine different types of plant growth-promoting hormones in seaweeds, including auxins (IBA, NAA, IAA), cytokinins (BAP, Kin, trans-zeatin, 2iP), and abscisic acid (ABA) (Mori *et al.*, 2017). Studies have also detected indole-3-pyruvic acid (IPA), a key auxin biosynthesis regulator in seaweeds like *Sargassum tennerrimun*, *Kappaphcus alvarezii*, and *Gracilaria edulis*. Cytokinins such as iPR, tZR, tZ, and aromatic cytokinins like 5 topolins have been reported in species like *L. japonica*, *G. edulis*, and *S. tenerrimum*. Additionally, gibberellic acid (GA_3) was found in *M. oxyspermum*, *G. edulis*, *S. tenerrimum*, and various *Ulva* species (Gupta *et al.*, 2011). Many studies have utilized natural seaweed extracts to create liquid biofertilizers, leading to increased shoot growth, longer roots, and improved overall plant development *in vivo*. However, the application of these extracts *in vitro* remains relatively unexplored, except for some promising reports, particularly in tomato cultivation. Seaweed-based biofertilizers hold the potential for enhancing plant growth and productivity.

In plant tissue culture, synthetic MS media typically contain various essential components, but specific nutritional requirements vary among plant species and even within plant cells. It is hypothesized that incorporating natural Liquid Seaweed Extracts (LSE) into the growth medium can potentially reduce the need for synthetic plant growth regulators (PGRs). This approach lowers production costs and serves as a natural growth enhancer in cultured conditions. This innovation could make banana micropropagation more economically viable, representing a cost-effective method for its production.

Materials and methods

Explant preparation and culture establishment: Banana (*Musa sp.*) shoot culture was initiated using healthy plant suckers collected from Sokhda farm near Vadodara (Gujarat). Suckers were initially washed with tap water to remove dirt and then sterilized in the lab with 2% Bavistin (fungicide) for 20 minutes. Afterward, they were rinsed twice with autoclaved distilled water. Further, explant sterilization was performed in a laminar airflow (LAF) bench using 1% sodium hypochlorite (NaOCl) for 10 minutes, followed by three to four washes with sterile distilled water. The explants, cut into 3-4 cm pieces, were aseptically placed on Murashige and Skoog's (1962) medium supplemented with 5.0 mg L^{-1} 6-Benzylaminopurine (BAP), 0.01 mg L^{-1} 1-Naphthaleneacetic acid (NAA), 0.8% agar, and 3.0% sucrose. Routine sub-culturing was performed every three weeks under controlled conditions: 28.0 °C temperature, 16-hour light/8-hour dark cycle, 85% humidity, and a photon flux density of 45 µmol $m^{-2}s^{-1}$ (Ahmed *et al.*, 2014).

Seaweed extraction preparation: Eight seaweed species from Bayt Dwarka, Gujarat (22° 28' 39.9" N, 69° 08' 10.8" E) were collected, cleaned, and dried at 60 °C for 72 hours. They were then ground into a powder using an electric mill. To prepare liquid seaweed extracts (LSEs), 500 g of each sample was boiled in 1L of distilled water with constant stirring for 20 minutes. The resulting solutions were filtered through muslin cloth and Whatman No. 40 filter paper and stored at 4 °C as stock solutions for experiments (Vinoth *et al.*, 2014).

Experimental design: In our experiment, we assessed the impact of seaweed extracts on banana micropropagule growth. We selected eight seaweed species (*C. racemosa*, *Gracilaria edulis*, *Caulerpa paspaloides*, *Ulva Lactuca*, *Sargassum tenerrimum*, *Caulerpa sertularioides*, *Sargassum wightii*, and *G. corticata*) and tested various concentrations of liquid seaweed extract (LSE) ranging from 10% to 50% (v/v). We included positive and negative controls (standard MS medium with and without plant growth regulators) for comparison. Each experiment was conducted in 200 mL culture bottles containing 50 mL of medium with a pH of 5.8. After autoclaving and solidification, five aseptically inoculated shoots were placed in each bottle. The bottles were transferred to a growth room for 126 days, involving six subcultures. We maintained six replicates per experiment, with three repetitions of each treatment. At the experiment's end, micropropagules were evaluated using various growth factors and biochemical analyses.

Measurement of growth parameters: We measured shoot length, total shoot count, and biomass (fresh and dry weight) of propagules. Fresh weight was assessed with an electronic balance, and shoots were dried at 62 °C until a constant weight was reached to determine dry weight.

Chlorophyll contents: The chlorophyll contents in the micropropagules of banana were calculated as per the protocol of Arnon (1949). Green shoots (500 mg) were crushed in 80% acetone under darkness, and the extract was centrifuged at 10,000 rpm. The supernatant was then used for absorbance measurements using a Shimadzu UV-1800 spectrophotometer (Japan).

Total phenols: Phenol content was assessed following Mahadevan's (1975) method. 500 mg of shoots were crushed in 70% methanol, centrifuged at 10,000 rpm for 15 minutes, and the clear supernatant was used. In a test tube, it was mixed with diluted Folin-Ciocalteu reagent and sodium carbonate. After brief heating and cooling, the blue product was diluted, and phenol concentration was measured at 650 nm using a Shimadzu UV-1800 spectrophotometer with a caffeic acid standard.

Total carbohydrates: Total carbohydrate estimation followed Tandon's (1976) method. One-gram fresh shoot samples were crushed in 0.1 M phosphate buffer (pH 7.0) centrifuged at 10,000 rpm for 15 min. Then, 15 µL supernatant was mixed with 4.0 mL of 0.2% anthrone reagent in concentrated H_2SO_4 and boiled in a water bath for 5 min. Absorbance at 610 nm was measured after green colour development, with a standard curve prepared using varying glucose concentrations.

Total protein: To estimate total protein, we followed the method described by Bradford (1976). One mL of supernatant (methanolic extract suitably diluted and centrifuged) was taken and mix with 5.0 mL Bradford reagent (Coomassie Brilliant Blue G-250 dye) and absorbance was taken at 595 nm. The protein concentration was calculated by using a standard curve of bovine serum albumin protein.

Total proline: Total proline was determined following Bates *et al.*'s (1973) method. One gram of fresh tissue was crushed in 10 mL of 3.0% aqueous sulphosalicylic acid and centrifuged at 10,000 rpm for 15 minutes. Two mL of appropriately diluted supernatant was mixed with 2.0 mL of glacial acetic acid and 2.0 mL of freshly prepared ninhydrin. The mixture was boiled for 1 hour, then cooled on ice. After adding 4.0 mL of toluene, the pink toluene layer was separated, and transmittance was measured at 520 nm using a Shimadzu UV-1800 spectrophotometer with a standard curve prepared using varying concentrations of L-proline.

Result and discussion

India's agri-biotechnology industry, focusing on horticulture, plays a significant role in the economy by supplying plantlets to over 100 countries. The Department of Biotechnology certified about 100 plant tissue culture-based industries under the "National Certification System for Tissue Culture Raised Plants (NCS-TCP)" in 2016, a significant increase from just one in 1985. However, this number has declined to 69 in the last decade (source: https://dbtncstcp.nic.in/Recognised-TCPUs). Notably, bananas, guavas, date palms, and ornamental plants are preferred for commercial production *via* tissue culture. Improving micropropagation protocols, especially for bananas, is crucial, although the higher production cost remains a challenge compared to traditional methods. Researchers have often tried to cut production costs in tissue culture by altering gelling agents and carbon sources, while the expense of synthetic plant growth regulators (PGRs) has been overlooked. This study investigated the potential of seaweed extracts as more economical alternatives to synthetic PGRs.

The number of shoots was found to be highest (9.32±1.83) when *Gracilaria edalis* extract was added at a 30% (v/v) concentration range (Table 1). Among the different concentrations of various other LSE tested, 30% concentration was found to be best in terms of increase in shoot number except in the case of *Caulerpa racemose* and *Sargassum tenerrimum* where 50% concentration (9.00±0.82) was found to be best. In contrast, in the case of *C. sertularioides,* 10% concentration was found to be best for the increase in shoot number (7.60±0.50). It was recorded that a higher concentration (50% v/v) of LSE always resulted in a decrease in shoot number and this can be explained by the fact that with increasing concentrations, the number of inhibitory molecules present in the LSE also increases, which subsequently interfere in growth (Chauhan *et al.*, 2018). In addition to the growth regulators, the addition of LSE also increases various other important nutrients like minerals, amino acids and vitamins in the medium, which indirectly help in the increase of shoot number (Niedz *et al.*, 2015). The shoot numbers in LSE-treated plants were comparable to those obtained in control plants, where standard PGRs were used (Table 1).

Similarly, a significant increase in shoot length was also observed with the addition of LSE however it is not comparable to control conditions where standard PGRs where added. The highest increase in shoot length was recorded in *S. tenerrimum* extract (4.97±0.42 cm @ 50% v/v) treated propagules, which was more than control. In general,

Table 1. Effect of LSE on shoot length, shoot number and biomass of banana micropropagules grown *in vitro* conditions

Liquid Seaweed Extract (LSE)	Concentration of LSE (% v/v)	Shoot length (cm)	Shoot number (mean)	Fresh weight (g)	Dry weight (g)
C. racemosa	10	1.900^{b}	5.000^{abcde}	4.310^{cde}	0.980^{def}
	30	2.700^{def}	5.600^{cdef}	3.580^{bc}	0.800^{bcd}
	50	3.530^{gh}	6.800^{efg}	5.780^{ghi}	1.280^{hi}
G. corticata	10	2.800^{ef}	4.000^{abc}	4.370^{cde}	0.970^{cdef}
	30	2.850^{f}	4.800^{abcd}	3.720^{bcd}	0.820^{bcd}
	50	2.870^{f}	4.600^{abcd}	2.480^{a}	0.560^{a}
C. paspaloides	10	2.800^{ef}	4.600^{abcd}	6.240^{hi}	1.410^{ij}
	30	2.370^{cd}	5.800^{cdefg}	6.460^{i}	1.460^{j}
	50	2.770^{ef}	3.200^{a}	4.370^{cde}	0.970^{cdef}
U. lactuca	10	2.670^{def}	4.400^{abcd}	3.930^{bcd}	0.880^{bcde}
	30	2.530^{cdef}	5.000^{abcde}	5.850^{ghi}	1.310^{hij}
	50	2.770^{ef}	4.000^{abc}	3.410^{bc}	0.780^{bc}
S. tenerrimum	10	3.270^{g}	5.400^{cdef}	5.400^{fgh}	1.220^{gh}
	30	4.370^{j}	6.200^{defg}	5.160^{efg}	1.160^{gh}
	50	4.970^{k}	9.000^{h}	11.610^{k}	2.710^{l}
C. sertularioides	10	2.470^{cde}	7.600^{gh}	4.040^{bcd}	0.870^{bcd}
	30	3.470^{g}	5.200^{bcde}	4.590^{def}	1.060^{efg}
	50	3.800^{hi}	4.400^{abcd}	5.070^{efg}	1.100^{fg}
Sargassum wightii	10	1.600^{ab}	6.200^{defg}	3.830^{bcd}	0.850^{bcd}
	30	2.300^{c}	7.600^{gh}	6.410^{i}	1.470^{j}
	50	1.370^{a}	4.200^{abc}	4.360^{cde}	0.970^{cdef}
Gracilaria edalis	10	2.800^{ef}	7.200^{fg}	6.410^{i}	1.470^{j}
	30	3.870^{i}	9.200^{h}	8.060^{j}	1.800^{k}
	50				
MS medium with standard PGR		4.500^{j}	11.000^{i}	16.010^{l}	3.410^{m}
MS medium without standard PGR		2.300^{c}	3.300^{ab}	3.230^{ab}	0.750^{b}
CV		11.90	15.35	6.91	6.23
SEM		0.17	0.40	0.18	0.04
CD 5%		0.50	1.12	0.50	0.12

SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation; SD - Standard Deviation; Means in the same columns followed by different letters are significantly different ($P \leq 0.05$) using the Duncan's Multiple Range Test

the 50 % concentration, positively affect the increase in shoot length except in case of *S. wightii* where 30 % concentration was found to be best. The least increase in shoot length was observed with *G. corticata*. This may be due to the higher concentration of zinc reported in this species, which hampers the shoot length (Rosemary *et al.*, 2019). When evaluating the growth performance of *in vitro* grown plants, fresh weight (FW) and dry weight (DW) gives us a better idea. The total biomass of micropropagules can be directly connected to plant performance as a response to photosynthetic ability, nutrition, environmental conditions, and more. In our study, a significant increase in biomass was recorded when LSE of *Sargassum tenerrimum* was used @50% concentration (v/v) (FW 11.61±0.89 g and DW 2.71±0.01 g) (Table 1). Overall increase in biomass was observed @ 30% concentration in all LSE tested. LSE of *G. corticata* have a detrimental effect on biomass accumulation and this was recorded 2.48±0.13 g FW, and 0.56±0.04 g DW, almost equivalent to the data recorded for plant grown on medium without synthetic PGRs. No morphological abnormalities like callus formation, curling of shoots, stunted growth, or rooting were observed in LSE treated plant at this multiplication stage.

Regarding biochemical parameters, different LSE at different concentrations evoked varied responses (Table 2). We observed changes in terms of biomolecule accumulation. Phenolic compounds are a vast collection of secondary metabolites formed by plants, the common

component of which is the aromatic ring linked to at least one hydroxyl group. Phenolic-based secondary metabolites are required and fulfill many important roles in the plant system. These compounds protect the plants from UV radiation, interact with the environment, and offer a defense mechanism against biotic and abiotic stresses. It is also evident that the phenolics give colour to the plant and cooperate with hormones (*e.g.,* auxins) (Kołton *et al.*, 2022). When LSE was added to the medium, it showed a significant increase in the total phenol compared to the shoots growing on synthetic PGRs (Table 2). In all cases, the highest accumulation of phenolics was recorded on the maximum concentration of LSE (*i.e.,* 50% concentration). Although all seaweed extracts showed a similar response, a slightly higher accumulation of phenolics was observed in *C. paspaloides* (1.31±0.03 mg g^{-1} fresh tissue @ 10% concentration).

The increased accumulation of phenols in tissue culture propagules can be attributed to external glucose in the growth medium, which is facilitated by LSE and is responsible for this effect. A similar observation was recorded in Scarlet Rose by Amorim (1977), where an exogenous supply of glucose in culture medium resulted in an increased accumulation of phenols. Another important reason for increased phenol accumulation is due to increased proline biosynthesis. It is clearly observed in Table 2 that the accumulation of phenol and proline are correlated and directly proportionate. Our results are in accordance with the observation of Shetty (2004) that the proline-linked pentose phosphate pathway stimulate shikimate and phenylpropanoid pathwa, leading to the stimulation of phenol biosynthesis in cell. There are two views on role of phenols under *in vitro* growth and development of plants. One opinion states that the phenols depress plants' *in vitro* proliferation and growth while others talk about the opposite (Arnaldos *et al.*, 2001). Role of phenols in controlling the interaction between PGRs and averting the abscisic acid promoted cell senescence under *in vitro* conditions has also been reported (Feucht and Treutter 1996), which are in agreement with our observation and hypothesis that an increase in phenol is not hampering growth of propagules.

Table 2. Effect of Liquid Seaweeds Extracts (LSE) on accumulation of biomolecules in *in vitro* grown micropropagules of banana

Name of different LSE	Concentration of LSE (% v/v)	Total phenol content (mg g^{-1} fresh tissue)	Total carbohydrate content (mg g^{-1} fresh tissue)	Total protein content (mg g^{-1} fresh tissue)	Total proline content (mg g^{-1} fresh tissue)
C. racemosa	10	1.030^{abc}	111.300^{abcdef}	61.000^{ab}	18.000^{de}
	30	1.230^{defg}	121.300^{efgh}	63.000^{abc}	19.000^{ef}
	50	1.300^{fgh}	135.770^{gh}	70.000^{bcd}	21.000^{gh}
G. corticata	10	1.230^{defg}	116.470^{cdef}	75.000^{def}	15.000^{b}
	30	1.450^{hi}	117.270^{defg}	77.000^{def}	21.000^{gh}
	50	1.530^{i}	120.370^{efgh}	78.000^{defg}	28.000^{j}
C. paspaloides	10	1.300^{fgh}	95.570^{ab}	80.000^{defgh}	17.000^{cd}
	30	1.470^{hi}	97.720^{abc}	82.000^{efgh}	20.000^{fg}
	50	1.520_{i}	109.700^{abcdef}	89.000^{gh}	27.000^{j}
U. lactuca	10	1.030^{abc}	94.470^{a}	72.000^{cde}	18.000^{de}
	30	1.120^{bcdef}	99.290^{abcd}	75.000^{def}	22.000^{h}
	50	1.250^{efg}	126.600^{fgh}	77.000^{def}	25.000^{i}
S. tenerrimum	10	1.240^{defg}	95.500_{ab}	83.000^{efgh}	13.000^{a}
	30	1.360^{ghi}	115.570^{cdef}	89.000^{gh}	15.000^{b}
	50	1.500^{i}	136.630^{h}	105.000^{i}	18.000^{de}
C. sertularioides	10	1.050^{abcd}	92.300^{a}	77.000^{def}	19.000^{ef}
	30	1.110^{bcdef}	111.470^{abcdef}	79.000^{defgh}	25.000^{i}
	50	1.360^{ghi}	115.780^{cdef}	80.000^{defgh}	30.000^{k}
S. wightii	10	1.140^{cdef}	111.500^{abcdef}	78.000^{defg}	16.000^{bc}
	30	1.190^{cdefg}	119.230^{efgh}	82.000^{efgh}	18.000^{de}
	50	1.250^{efg}	124.670^{fgh}	83.000^{efgh}	21.000^{gh}
G. edalis	10	1.060^{abcde}	102.800^{abcde}	79.000^{defgh}	15.000^{b}
	30	1.160^{cdef}	114.230^{bcdef}	85.000^{fgh}	17.000^{cd}
	50				
MS medium (+standard PGR)		0.900^{a}	125.370^{fgh}	90.400^{h}	15.000^{b}
MS medium (-standard PGR)		0.950^{ab}	92.000^{a}	58.400^{a}	25.000^{i}
CV		3.53	2.97	13.47	19.96
SEM		0.02	0.19	0.16	0.13
CD 5%		0.06	0.56	0.47	0.39

SEM - Standard Error Mean; CD - Critical Difference; CV - Coefficient of variation; SD - Standard Deviation; Means in the same columns followed by different letters are significantly different ($P \leq 0.05$) using the Duncan's Multiple Range Test

Carbohydrates are not only a source of energy for plant cells but also play many important roles in signaling pathways, photosynthesis and also in cell differentiation. In our study, we have observed a varied response in the accumulation of carbohydrates in various treated shoots. In general, the gradual increase in LSE resulted in a higher accumulation of carbohydrates, which was highest in 50% concentration in all tested LSEs. In the case of *S. tenerrimum* extract, we observed 136.63±0.31 mg g^{-1} of carbohydrate accumulation at 50% concentration, which was even higher than positive control plants (Table 2). This can be explained on the basis that LSE contain some level of sugars that contribute to hexose feeding in the medium and hence more carbohydrate is accumulated. Our results are in accordance with the results obtained by Chauhan *et al.* (2018), where the addition of some natural extracts significantly increased carbohydrates in rose micropropagules.

Studies on the protein contents in *in vitro* grown shoots give us an idea of protein metabolism and its role in cell differentiation. We observed a steady increase in protein accumulation regarding increased concentrations of different LSE. The highest protein accumulation was observed @ 50% concentration in all LSE samples, although it is slightly lesser than the shoots grown on synthetic medium (90.4±0.25 mg g^{-1} of fresh tissue), except the shoots grown on *Sargassum tenerrimum* extract (105±0.45 mg g^{-1} of fresh tissue). The probable reason for decreased protein accumulation is the presence of protein synthesis inhibitors in crude seaweed extracts that interfere with protein biosynthesis. Moreover, the shoots growing in a positive control environment, where purified synthetic PGR are present, stimulate the cells to grow and divide; hence more protein will be synthesized and accumulated.

A similar tendency in change of proline accumulation was observed when different LSE were tried. Proline is an important amino acid considered as a highly beneficial compound that protects against various stress conditions. Proline is considered an excellent osmolyte that protects in three ways: i) it acts as a metal chelator, ii) it is a proven antioxidative defense molecule and iii) it is an important signaling molecule. As a general observation, adding more LSE resulted in more proline accumulation. In all cases 50 % concentration of LSE

resulted in highest accumulation of proline which was higher than positive control plants (15±0.10 mg g^{-1} fresh tissue) (Table 2). The highest proline content was recorded in propagules treated with *C. sertularioides* extract @ 50 % concentration, which was almost double compared to positive control. The possible reason behind increased proline synthesis under LSE treatment is due to several inorganic salt, acidic compounds, secondary metabolites and phenols present in seaweed extracts. Sometimes adaptive response of micropropagules in culture conditions also induce proline accumulation. Similar results were also observed by Putnik-Delić *et al.* (2012) in *in vitro* culture of sugar beet where increased salt concentration resulted in increased proline accumulation.

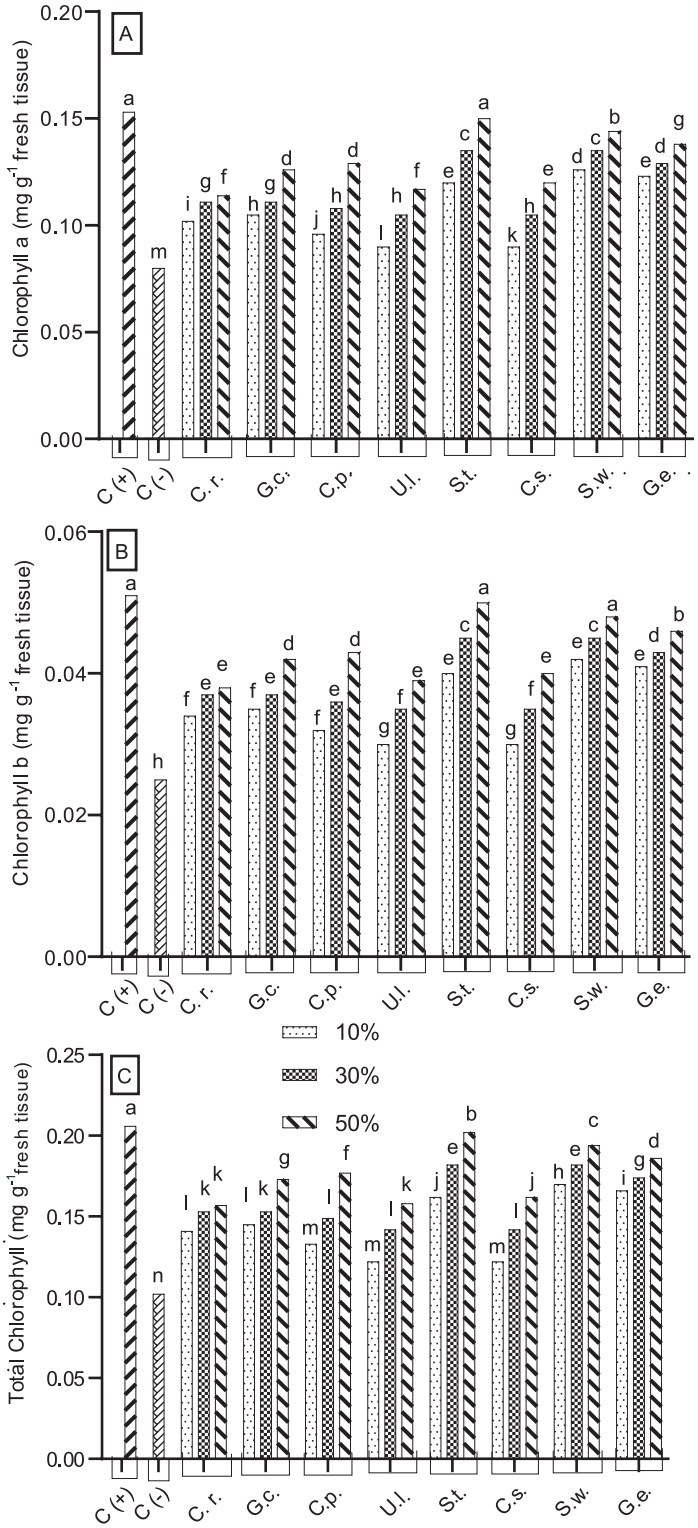

Fig. 1. Effect of different LSE on accumulation of Chlorophyll a (A), Chlorophyll b (B) and total chlorophyll (C) in banana micropropagules grown under *in vitro* conditions. C (+) represent – positive control plant grown on a standard MS medium with PGRs, C (-) represent – negative control plant grown under standard MS medium without PGRs; *C.r., G.c., C.p., U.l., S.t., C.s., S.w.* and *G.e.* represent *C. racemosa, G. corticata, C. paspaloides , U. lactuca, S. tenerrimum, C. sertularioides, S. wightii and G. edalis,* respectively.

Chlorophyll is an important photosynthetic pigment in all autotrophic organisms like algae, photosynthetic bacteria and plants, which can convert light energy into chemical energy. The chlorophyll content reflects the plant's health condition and ability to fix atmospheric carbon dioxide. In our experiment, adding LSE does not significantly affect the increase in chlorophyll content (Fig. 1). In all the cases, the increasing concentration of LSE also resulted in a steady increase in chlorophyll, which was recorded highest in *Sargassum tenerrimum* @ 50 % concentration. However, this value did not surpass that of the positive control plants. The probable reason for slightly low chlorophyll content may be due to the alkaline nature of LSE, which does not support chlorophyll synthesis. Moreover, the presence of elements in extract, like calcium, zinc, manganese, phosphorus, or copper, can bind with iron, making it available for chlorophyll synthesis (Li *et al.*, 2018).

Numerous studies have explored the use of natural extracts to enhance the *in vitro* growth of plantlets in tissue culture. Plant tissue culture media are often enriched with various natural extracts, such as protein hydrolysates, coconut milk, yeast, malt extract, ground banana, orange juice, potato extract, and tomato juice. Several natural cytokinins, like zeatin and zeatin riboside, along with cell division-promoting activity, have been identified in sweet corn extract. In the case of *Anthurium cubense*, substituting cytokinins with the cost-effective citrus fruit rind-derived Pectimorf showed improved *in vitro* growth and 90% plantlet survival during acclimatization (Montes *et al.*, 2000). Similar results were observed with *Spathiphyllum* using Pectimorf compared to the synthetic cytokinin BA (Hernandez *et al.*, 2009). Orange juice has also been successfully applied in culturing explants of various citrus species. Coconut water, known for containing ribosyl-zeatin similar to maize zeatin, has promoted embryo culture in multiple species. Other extracts from sweet lime juice and tomato fruit have also demonstrated growth-promoting properties (Maria *et al.*, 2012). While natural plant extracts have been extensively studied, the use of seaweed extracts containing growth-promoting substances like auxins, cytokinins, and betaines in tissue culture remains relatively unexplored. Some reports have highlighted seaweed extracts, including *U. lactuca*, *C. sertularioides*, *P. gymnospora*, and *Sargassum liebmannii*, as cost-effective options for tomato seedling growth under laboratory conditions. Similar seaweed-based growth studies have been conducted with tomato, wheat, soybean, and maize, but these were *ex vitro* studies. Notably, *in vitro* applications of seaweed extracts in *Lycopersicon esculentum* showed a significant increase in shoot length. Our findings align with these observations, suggesting that seaweed-derived growth promoters may positively influence *in vitro* growth of banana micropropagules, potentially serving as economical alternatives to synthetic plant growth regulators (PGRs). However, further testing is necessary to evaluate the suitability of seaweed extracts

for *in vitro* culture in a broader range of plant species, including various horticultural plants.

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Chapter 13

Potential scope and prospects of plant growth-promoting microbes (PGPMs) in micropropagation technology

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1 Introduction

Plant tissue culture technology utilizes the ability of a single cell or a group of plant cells to transform into a whole plant when grown under controlled environmental conditions. This interesting idea of in vitro culturing of the plant cell was put forward by Gottlieb Haberlandt in 1902 in the form of a postulate "totipotentiality," which later on led to significant discoveries in biology. One important aspect of plant tissue culture is micropropagation, which is being exploited by a large number of researchers and business firms. The primary use of micropropagation is large-scale production of plants, ranging from nursery stock species (like rhododendron or rose) to ornamentals (like gerbera or carnation), fruits (like banana or raspberries), and vegetables and crops (like cauliflower, potato, or pointed gourd). In the last two decades, there has been a significant growth in micropropagation-based industries, and these industries have been internationally acknowledged as one of the significant tools for the direct application of this technology in the agriculture field. Other important applications of tissue culture technology are conservation of endangered plants, in vitro production of secondary metabolite, crop improvement, and development of new varieties through transgenic approach. Besides its several advantages, this technique has many challenges. For example, any micropropagation system must produce large numbers of genetically uniform plants that maintain the genetic truthfulness (i.e., genetic fidelity). Moreover, the technique involves the use of certain chemical sterilizers, plant growth regulators (PGRs), and sometimes antifungal agents and antibiotics to control the contamination. Most of these chemicals are very costly and therefore limit the profitability to end

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users. Similarly, it requires certain costly instruments and a sophisticated setup and skilled manpower, which further increase the production cost of plants. Other important challenges in micropropagation are the low in vitro multiplication rate, loss of plantlets due to contamination, increased susceptibility toward pathogens pre- and postfield transfer, mixotrophic behavior of plantlets during culture conditions, and low survival of plantlets during the hardening and acclimatization stage.

To address these challenges and cut the production cost, plant growth-promoting microbes (PGPMs) can be used as an effective tool. PGPMs could be an effective agent for the promotion of growth, uptake of nutrients from soil, and sometimes can be an alternative source of nitrogen fertilizer for plants. After confirmation of the role of microbes in soil fertility and plant growth, PGPMs have gained a lot of attention from many soil scientists and agriculture biotechnologists. PGPMs promote plant growth in many ways, for example, they may produce plant hormones [1–3] or growth-stimulating biomolecules, viz., vitamins and related products [4], by suppressing the growth of pathogens by different mechanisms [5]. Nowadays, PGPMs have received a lot of attention, particularly in the field of crop improvement, and many related articles got published in the last two decades. However, research into the application of PGPMs in plant tissue culture has not gained much popularity just because it is a general notion that the presence of microbes in the tissue culture growth medium is deleterious and is considered as a can of worms, which not only limit the establishment of culture but also leads to further obstacles in subsequent stages [6]. Hence, most of the focus in tissue culture is on how to get rid of microbes despite the fact that many PGPMs can be beneficial at different stages of tissue culture. However, many PGPMs can be beneficial at different stages of tissue culture, viz., at the stage of in vitro rooting, in vitro shoot multiplication and elongation, and the acclimatization stage. Moreover, they can provide a defense against biotic (pathogens) and abiotic (temperature, salinity, heavy metals, etc.) stress that arises during the hardening and acclimatization stage. PGPMs can act as a nostrum for sustainable agriculture, if used judiciously, and therefore promoting the use of PGPMs in tissue culture is advantageous as well as challenging. This chapter is mainly focused on the potential possibilities of PGPMs in the advancement of micropropagation technology.

2 Plant tissue culture

Plant tissue culture system is a method in which a whole plant, a plant part (generally a 1–2 cm portion of a leaf/node/internode/cotyledon or any other suitable plant part), or even sometime a single cell is taken and allowed to grow under controlled aseptic environmental conditions. The tissue culture setup is optimized to provide all macro- and micronutrients, carbon as a source of energy, phytohormones for division and differentiation and, of course, water, which is necessary for the growth of plants. All these requirements are provided in the

form of a basal growth medium. In addition, environmental factors, viz., light, temperature, and humidity are also maintained optimally in a way that supports better in vitro growth and multiplication. Further, the plant development and differentiation can then be controlled by providing plant growth regulators, viz., auxin, cytokinin, gibberellins, etc. Regeneration of a plant or plant part (often regarded as an explant) under in vitro conditions relies on the concept of totipotency, originally proposed by Haberlandt in 1902. The explant is any plant part (generally 1–2 cm in size, viz., a nodal segment, an internode, a leaf segment, an immature embryo, a pollen grain, a seed, an ovule, an anther, etc.), which is used as an initial material for establishment purpose. Micropropagation may also be regarded as the method of taking explants, putting aseptically this explant on a suitable growth medium and allowing it to undergo differentiation and develop into a whole new plant [7]. The plant part (i.e., explant) is allowed to grow in a culture vessel filled with synthetic growth medium under aseptic conditions in a chamber where all the environmental conditions are kept at the optimum level. In addition to large-scale production of plants, micropropagation technology is also a key step in transgenic plant development in which the regeneration of novel plants from genetically engineered cells is carried out. Micropropagation of plants can be achieved by four different pathways, namely: (a) enhanced axillary branching; (b) adventitious shoot bud differentiation; (c) callus organogenesis; and (d) somatic embryogenesis. In the case of enhanced axillary branching, the explant contains preexisting axillary shoot buds, while in callus organogenesis and adventitious shoot bud formation, the shoots are formed de novo by the process of organogenesis. During the process of somatic embryogenesis, bipolar somatic embryos are formed that have the competency to develop into a complete plant. In any chosen pathway of micropropagation, a sequence of events is involved to achieve success (Fig. 1). Micropropagation, in contrast to conventional propagation methods, is a multistage process in which every stage is important to realize the goal of producing plants in culture.

Notwithstanding the advantage or disadvantage of various methods of micropropagation, each method involves five different stages to achieve the goal. These stages are as follows:

Stage 0:. Management of donor plant/s (source of explant)
Stage 1:. Aseptic establishment and initiation of cultures
Stage 2:. Shoot multiplication and/or elongation
Stage 3:. In vitro rooting of shoots
Stage 4:. Hardening, acclimatization, and transplantation in soil.

The first four stages described above are carried out in a highly controlled manner where the main concern is to avoid any kind of microbial contamination; hence, a high levels of aseptic conditions is maintained which results in zero contact of regenerated micropropagules with the common microbiota of the environment. The outcome of this is that the regenerated plants become more

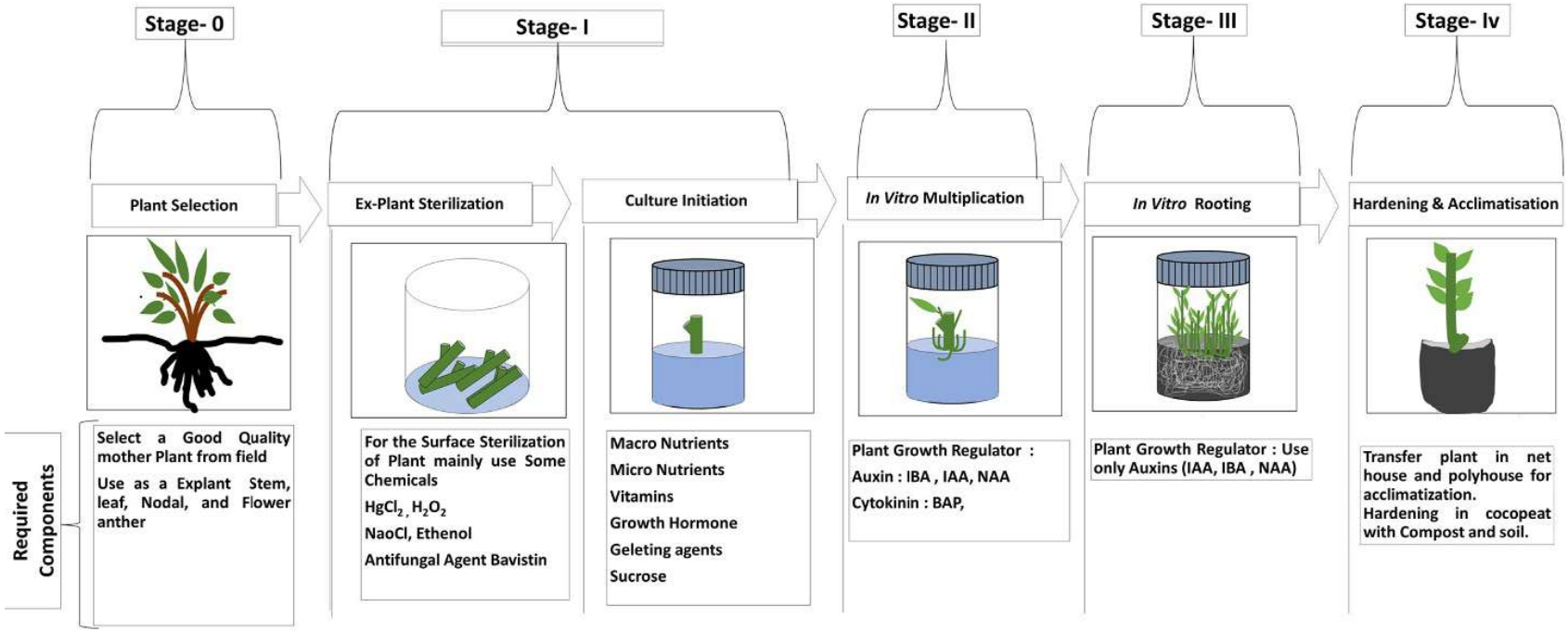

FIG. 1 Different steps involved in successful establishment of tissue culture protocol for a plant.

vulnerable and, when transferred to the soil, become more sensitive to infections as well as the harsh environmental conditions. This makes it imperative that the controlled exposure of some beneficial microorganism may positively affect the in vitro and ex vitro growth of plants during tissue culture. Although there have been few reports where the beneficial effects of these PGPMs during in vitro culture conditions have been reported, a thorough study needs to be done to explore the potential possibilities of PGPMs in micropropagation technology.

3 Challenges in plant tissue culture

Plant tissue culture technology sometimes fails to translate at the commercial level for large-scale production due to certain limitations. Some of the problems that are encountered during large-scale micropropagation are discussed here:

Higher production cost: Due to the requirement of sophisticated instruments, production setup and skilled labor, the production cost of plants increases in tissue culture. Moreover, raw materials like glassware and chemicals (viz., agar, sucrose, plant hormones, and other media components) make this technology a costly affair. In some cases, the unit cost per plant becomes exorbitant. This has restricted the growth of these industries in developing countries like India [8].

Low multiplication rate: In some plant species, the multiplication rate in tissue culture is less than threefold, which makes it nonviable technology. The high multiplication rate is an important primary concern, particularly during the commercialization phase. The high multiplication rate lessens the number of cycles required for subculturing in mass cloning and thus cuts the labor cost. The high multiplication rate also partially compensates for the loss that occurs due to the contamination at different culture stages.

Loss of culture due to contamination: In tissue culture, contamination is a major problem, which sometimes wipes out the hard work of months. The major contaminants in tissue culture are bacteria and fungi, which are either present in explants or may arise due to handling error. Whatever the reason, the contaminants are responsible for the huge loss of plantlets which ultimately result in further economic loss.

Hyperhydricity: Shoots grown in vitro are exposed to a unique microenvironment which is nutrient rich and has high humid conditions. Sometimes these cultural conditions induce morphological, anatomical, and physiological abnormalities in micropropagules. Hyperhydricity or vitrification is a physiological deformity that results in excessive hydration, low lignification, nonfunctional stomata, and poor mechanical strength in shoots. The result of this is poor regeneration abilities in such plants which require intensive care and hardening and acclimatization before soil transfer.

Susceptibility to diseases: The tissue culture grown plants are more susceptible to the soil microflora and do not show sufficient resistance against

bacterial and fungal pathogen. One of the reasons behind this response is their sudden exposure (mainly the roots) to the microbes present in the soil and outer environment. Under natural conditions, plants are continuously exposed to various microflora which directly or indirectly induce various defense mechanisms in plants, which finally leads to the development of resistance against pathogens. If the natural defense mechanism of plantlets is induced during the culture conditions against different pathogens, at least for the time when they are most susceptible, the problem of quick susceptibility to the infection can be reduced to a great extent [9,10].

Acclimatization of micropropagated plants: During tissue culture, there is a loss of a significant number of plants when transferred to field conditions. The shoots grown in tissue culture are continuously exposed to a unique and lavish microenvironment where there are minimal stress conditions. Moreover, there is a continuous supply of sucrose in the medium which makes the plants partially heterotrophic in nature. All these conditions contribute to a physiological and anatomical transformation in the plants like poor development of cuticles, raised and nonfunctional stomata, poorly developed internal anatomy, less efficient photosystem, etc. Finally, when the plant is transferred to the field, it fails to tolerate the sudden shock of outer stressful conditions and strives to survive [11].

4 Plant growth-promoting microbes

Plant growth-promoting microbes (PGPMs) are a special heterogenous group of microbes which are considered advantageous for the plants in terms of being not only a growth promoter but also a savior against biotic and abiotic stress. PGPMs are generally found near the rhizospheric zone of the roots of plants or inside the plant tissue (in the case of certain endophytes) and exert their beneficial effects through several mechanisms. Some of these mechanisms include biological fixation of nitrogen, solubilization of phosphate, alleviation of stress through modulation of ACC deaminase expression, production of siderophore, synthesis of plant growth regulators, etc. Moreover, they also act as biocontrol agents against several pathogens. PGPMs are further classified into three categories on the basis of their mode of action:

(a) **Biofertilizers**: This group of PGPMs acts through the direct mechanism of PGP and contributes to plant growth through solubilization of minerals (like phosphate, potassium, and zinc) and also through the biological fixation of nitrogen.
(b) **Biostimulants**: This group includes PGPMs which enhance plant growth through the biosynthesis of phytohormones, organic compounds, and certain enzymes. This class of microbes may act either through a direct mechanism or through an indirect mechanism.

(c) **Biocontrol agents**: The PGPMs of this group provide protection to the plants against pathogens by synthesizing certain antimicrobial compounds or by challenging the pathogens for available space and nutrients.

Few of the PGPMs exhibit more than two mechanisms of growth promotion and hence may be categorized in two groups in the above classification [12]. Furthermore, on the basis of the type of microorganisms, PGPMs can be of two types:

(a) **Plant growth-promoting fungi (PGPF)**: The growth-promoting effect of several rhizospheric fungi has been reported. These PGPF include a number of species belonging to different genera of fungi. These fungi mostly belong to the arbuscular mycorrhizal fungi (AMF) family. The life cycle of these AMF cannot complete without the plant host; hence, they are termed as obligate biotrophs and are grouped in the phylum Glomeromycota. The phylum Glomeromycota includes 10 important families and the most prominent genera of this phylum include Glomus, Acaulospora, and Gigaspora. Besides this, the other PGPF include species of the genera *Trichoderma*, *Aspergillus*, *Penicillium*, *Fusarium*, *Piriformospora*, *Phoma*, and *Rhizoctonia*, which have the innate ability to enhance the growth of plants [13]. These PGPF are found in soil as well as other natural habitats and exert their beneficial effects on plants by improving the plant nutrition, soil fertility and providing resistance against pathogens.

(b) **Plant growth-promoting (rhizo) bacteria (PGPB or PGPR)**: PGPB represent 2%–5% of the rhizospheric bacteria, classified mainly into four groups: (a) free-living bacteria, (b) associative bacteria, (c) endophytic bacteria, and (d) nodule-forming bacteria (symbiotic). Similar to PGPF, these bacteria also have proved their potentiality as biofertilizers, biostimulants, and/or biocontrol agents. On the basis of their location in the host plant, PGPB can be classified into two groups: (i) extracellular plant growth-promoting rhizobacteria (ePGPR) and (ii) intracellular plant growth-promoting rhizobacteria (iPGPR). The ePGPRs may acquire the space on the surface of the root/or on the rhizoplane/or in the intercellular space of the root cortex. In contrast, iPGPRs are commonly found inside the cells of the nodule (a specific compacted tissue found in roots). Examples of ePGPR include *Azotobacter*, *Erwinia*, *Arthrobacter*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Chromobacterium*, *Flavobacterium*, *Caulobacter*, *Micrococcus*, *Serratia*, *Pseudomonas*, etc. iPGPR mainly include certain endophytes and species of *Frankia*, both of which can fix environmental nitrogen symbiotically with the higher plants. The examples of some potent endophytes are *Azorhizobium*, *Mesorhizobium*, *Allorhizobium*, *Bradyrhizobium*, and *Rhizobium* of the family *Rhizobiaceae*. The members of this family invade the roots of plants, particularly the members of the Leguminosae family, form nodules, and fix the atmospheric nitrogen. PGPB are generally used for the promotion of growth, uptake of nutrient from soil

and sometimes as a substitute of N-fertilizers of nonleguminous crops. PGPR have also proved to be an effective tool against several plant pathogen, they act as a biocontrol agent by secreting some important antibiotics [4].

Recently, research on PGPF and PGPB for crop improvement is gaining importance and many researchers are getting attracted toward this fascinating area. However, the application of these microbes in micropropagation technology is limited. Nevertheless, encouraging results from various research findings suggest that these PGPM strains can successfully be used in micropropagation technology to produce more vigor and resistant plants.

5 Application of PGPM in micropropagation technology

In tissue culture, sterilization of explants is carried out to remove all microbes during the establishment stage. Moreover, strict aseptic conditions are maintained throughout all growth room conditions considering the microbes as a potential enemy. After establishing the advantageous role of PGPM in plant growth and protection, the perspective of complete removal of microbes from tissue culture has shifted and is restricted to only harmful microbes. In fact, more attention is paid to the right utilization of PGPM at different culture stages during in vitro growth conditions. This idea of using beneficial microbes during in vitro conditions was conceptualized by Nowak in 1998 and was termed as "Biotization." As stated by Gosal et al. [14], "Biotization is the metabolic response of in vitro grown plant material to a microbial inoculum(s), leading to development and physiological changes enhancing biotic and abiotic resistance of the derived propagules." The process of biotization can be carried out at any stage of tissue culture which can generally be contingent on the objective of the researcher or the nature of the problem. For example, at the establishment stage (stage I), multiplication stage (stage II), and rooting stage (stage III), those PGPMs are added which act as biostimulants and stimulate overall growth, multiplication, rooting or competency in propagules, while at stage IV (hardening and acclimatization stage), the main choice is those PGPMs that stimulate the resistance and photosynthetic efficiency of plantlets. Besides being potential biostimulator and biocontrol agents, certain PGPMs (e.g., *Frankia*, *Bradyrhizobium*, *Rhizobium*, *Azofobacler*, *Bacillus*, and *Xanthomonas*) play an important role in improving the physical properties of soil [15]. The schematic representation of the Biotization approach and its advantages during tissue culture is presented in Fig. 2.

5.1 Biotization with plant growth-promoting fungi

It has been observed that prolonged exposure of plantlets to in vitro conditions makes their roots unresponsive to water absorption, which leads to water stress

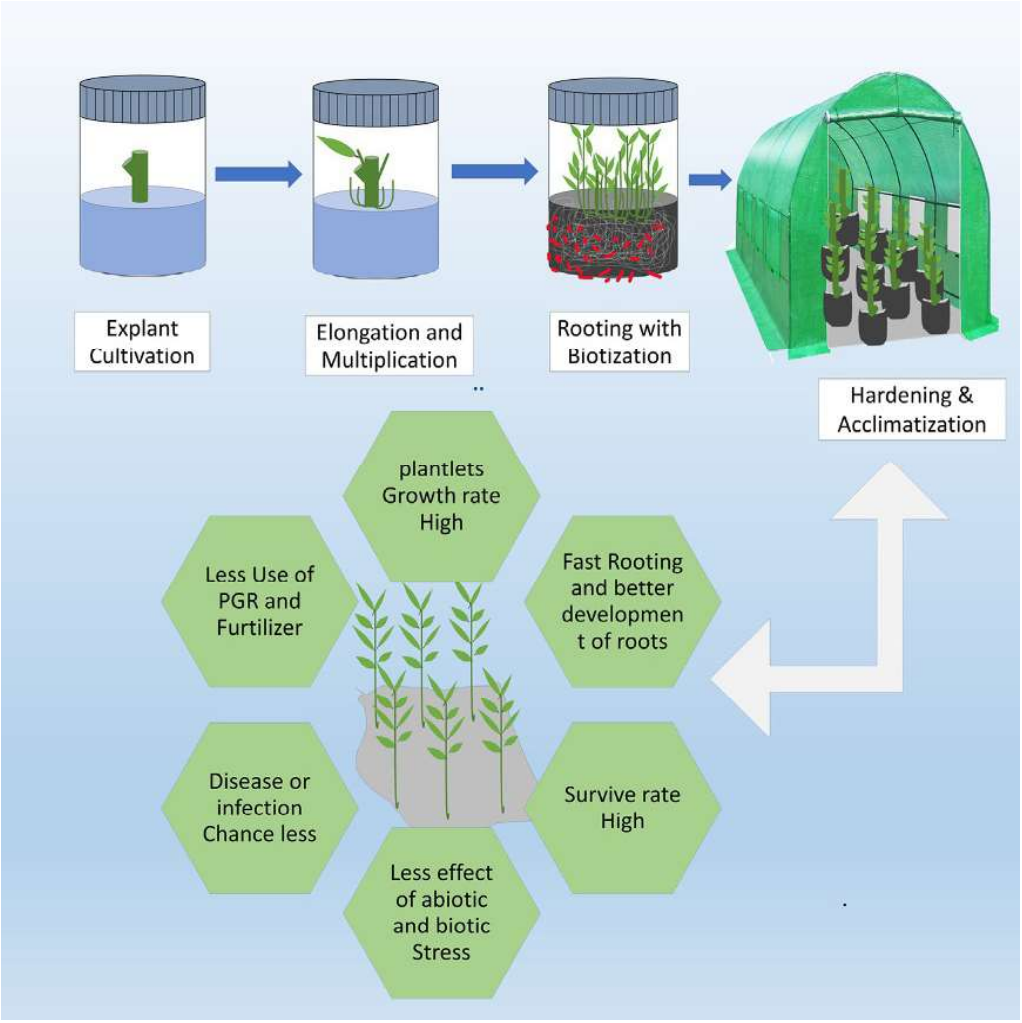

FIG. 2 Schematic representation of the Biotization approach and its advantages during tissue culture.

at a later stage. Inoculation of such plants with AM fungi during tissue culture may be beneficial to overcome this problem [16]. It has also been testified that inoculation of AM fungi during culture conditions helps the plants in nutrient availability, increased growth and resistance to pathogens after transplantation to the soil [17]. The other experimentally proved advantages of AM fungi under in vivo conditions include the ability to utilize the available Phosphate present in the soil through their hyphae [18]. Moreover, the PGPF help in better growth of plants [19] as well as higher production of secondary metabolites and related compounds such as alkaloids, phenolics, plant-based sterols, vitamins, lignans, terpenes, etc. These compounds are valued from the human health perspective as well as provide tolerance to the plants against various biotic and abiotic stresses [20]. Moreover, the PGPF also play a significant role in enhancing the production of several enzymes [21], stimulating the photosynthesis process [22], and improving the fertility of the soil [23]. According to Streletskii et al.

[24], fungi produce plant hormones, and these hormones regulate the development of plants by activating signaling pathways throughout the biotic and/or abiotic stresses. Considering the above properties, PGPFs have been tried during tissue culture for overcoming the existing problem of post vitro survival. Tissue culture raised plantlets of the wood-apple were allowed to get colonized with the root fungus *Piriformospora indica* during stage III (in vitro rooting stage) and stage IV (hardening and acclimatization stage), and significant growth was observed in terms of shoot number, shoot length, root length, leaf number, leaf area, and fresh weight. Moreover, the survival percentage and performance of plants after field transfer were also significantly increased [25]. Similar results were also observed in tissue culture raised *Terminalia bellerica* by Suthar and Purohit [26]. Since the AM fungi significantly upsurge mineral uptake, their role is specifically important during stage IV (i.e., hardening and acclimatization stage). AMF can be an effective tool to address a common problem in tissue culture derived plants, i.e., mineral absorption, since the AM fungi have very well-developed arbuscules and hyphae which can easily transfer nutrients (particularly the phosphate) from the soil to the plant [27]. The main reason behind the poor survival of micropropagated plants, post transfer to the soil, is absence of their microsymbiont partner, and this can be mitigated by inoculating the plantlets with PGPG at the hardening stage. This was proved in the case of in vitro grown hydrangea plants where the post survival rate was attained up to 100% when inoculated with AMF *Glomus intraradices* at the hardening stage [28]. Similar results were observed in *Quercus suber*, where inoculation at the hardening stage with *Pisolithus tinctorius* and *Scleroderma polyrhizum* resulted in better growth and performance of plants [29]. Likewise, inoculation with *Piriformospora indica* showed better results in tobacco and brinjal [30]. Besides the biostimulatory effect, the biocontrol action of certain fungi has also been reported in tissue cultured plants [31]. A comprehensive list of some successful biotization with PGPFs is presented in Table 1.

Apart from having many advantages, there are also some challenges in using these PGPFs during micropropagation. The major challenge is to prepare a pure fungal inoculum without any contamination. When such contaminated mix culture is exposed to plants, it may cause significant damage to the plants. Secondly, sometimes germination and growth of fungal spores on the Murashige and Skoog medium is quite difficult as it is not a favorable choice to grow [45]. To overcome this problem, the modification and optimization of the MS medium, suitable for coculture of the plant cell and PGPF, can be done.

5.2 Biotization with plant growth-promoting (rhizo)bacteria (PGPB or PGPR)

The beneficial effect and role of certain bacteria that can enhance plant growth and add to productivity were known for over a century. Over time, their application in plant tissue culture was first proved by Digat et al. [46] in Primrose.

TABLE 1 Effect of different PGPF (plant growth-promoting fungi) on plant propagules when used as Biotization agents during tissue culture process.

Name of fungi	Investigational plant	Micropropagation stage at which fungi was inoculated	Observed effect	References
Piriformospora indica	*Chlorophytum* sp.	Rooting	Increased root length, shoot length, fresh and dry weight, and leaf length and area	[14]
AM fungus *Gigaspora rosea*	*Pyrus* sp. clone HW 609	Rooting	Increased shoot length	[32]
Piriformospora indica	*Boswellia serrata* Roxb	Acclimatization	Increased shoot length, increase root length, increased leaf no., increased fresh weight, and dry weight	[33]
Trichoderma viride	*Albizia amara*	Acclimatization	Increased shoot length, root length, and leaf number	[34]
VAM fungi *Glomus fasciculatim*	*Banana*	Acclimatization	Increased shoot length, root biomass (fresh and dry weight)	[35]
Endomycorrhizal Fungi	*Helleborus niger* L.	Acclimatization	Increased dry weight of plant, dry weight of root	[36]
Piriformospora indica	*Vernonia divergens*	Rooting	Increased shoot-root length, shoot-root dry weight, leaf numbers. Increased anticancerous properties of plant extract	[37]
Piriformospora indica	*Tinospora cordifolia, Vernonia divergens* and *Mucuna pruriens*	Rooting	Increased shoot length, shoot numbers	[38]

Continued

TABLE 1 Effect of different PGPF (plant growth-promoting fungi) on plant propagules when used as Biotization agents during tissue culture process—cont'd

Name of fungi	Investigational plant	Micropropagation stage at which fungi was inoculated	Observed effect	References
Piriformospora indica	*Terminalia bellerica* Roxb.	Acclimatization	Increased biomass and root system	[26]
Serendipita indica	*Hordeum vulgare* L.	Acclimatization	Increased photosynthesis	[39]
Gigaspora margarita, Glomus etunicatumus	*Scutellaria integrifolia*	Acclimatization	Increase plant growth and survival	[40]
Glomus intraradice	*Prunus* sp.	Rooting	Increased shoot length and root number	[41]
Glomus mosseae	*Juglans* sp.	Rooting	Increase plant survive rate	[42]
Glomus intraradices	*Fragaria x ananassa*	Rooting	Extensive root systems and better shoot growth	[43]
Glomus coronatum	*Prunus cerasifera*	Acclimatization	Better plant growth and survival rate	[44]

They observed that when microshoots of Primrose (at the rooting stage and hardening stage) were exposed to *Pseudomonas putida* and *Pseudomonas fluorescens*, they exerted positive effects on growth and survival. A few years later, Elmeskaoui et al. [43] proved that biotization also improves the photosynthetic efficiency in in vitro grown plants, which leads to increased biomass accumulation. The biostimulatory action of PGPR is generally through the production of phytohormones. The production of auxins and cytokinins is a common phenomenon in PGPRs which is reported in more than 80% of rhizobacteria. The phytohormones produced by PGPBs mitigate the insufficient endogenous quantity of these hormones in microshoots during culture conditions [47]. The effects of phytohormones produced by a variety of PGPBs (viz., *Bradyrhizobium*, *Rhizobium*, *Bacillus*, *Microbacterium*, *Rhodococcus*, etc.) during tissue culture have been studied by Spaepen and Vanderleyden [48]. Rodríguez-Romero [49] studied the combined effect of PGPF and PGPR during the hardening phase of micropropagation of banana. They took *Glomus manihotis* (AMF) and the rhizobacteria consortium of *Bacillus* spp. and inoculated them with stage III plantlets, alone and in combination, and observed that the combined application of fungi and bacteria proved to be an effective inoculant and the resultant plants showed better growth in terms of more fresh weight, dry weight, shoot length, leaf area and required less time to become ready to get transplanted in the soil. Both the fungal and bacterial partners did not show any antagonistic effect toward each other. The positive effect of biotization was also reported in the banana plantlets at the rooting stage. In a study carried out by Mia [50], rhizobacteria were inoculated at the in vitro rooting stage of banana and the results were encouraging as a significant increase in the root length, root number, and root biomass was observed. Moreover, if exposure to PGPR continues during the subsequent steps of hardening and soil transfer, it results in the early attainment of the reproductive stage. Flowering takes place 3 weeks before compared to normal noninoculated plants and the yield also increases up to 51%. Similar results were also reported in potato micropropagation. When potato microcuttings were inoculated with *Azospirillum brasilense* during the in vitro rooting stage on a hormone-free medium, it significantly increased the IAA production, which ultimately led to the development of a solid root system and better survival in post vitro transfer [51]. Moreover, in another study, the *Azospirillum brasilense* strain Sp245 was inoculated at the hardening stage of potato, which not only results in an increase in the post vitro survival rate of plantlets (1.5 times) but also increases the weight of tubers by 30% [52]. The inoculation of rhizobacteria on a modified MS medium was studied on in vitro grown banana plantlets by Mahmood et al. [53]. The modification was done in terms of addition of salt (0.2% Sodium chloride), and it resulted not only in better growth and performance but also increased the synthesis of protein and chlorophyll. Likewise, biotization with *Azorhizobium caulinodans* in the rice plant gave better performance of plants in terms of biomass accumulation and grain yield as compared to uninoculated plants [54]. Other successful

biotization trials were reported in many food crops such as wheat, where inoculation of *Azotobacter chroococcum* resulted in better root length [55]; potato and strawberry, where *Pseudomonas aureofaciens* was used which resulted in better growth [56]; watermelon, where the pseudomonas strain resulted in increased root length [57]; and maize, where the use of *Streptomyces griseorubens* and *Norcardiopsis alba* resulted in better growth of plantlets under phosphorus-deficient soil [58]. The addition of PGPR also resulted in the uptake of phosphorus in banana and rapeseed [59]. In banana, besides nutrient uptake, several physiological processes such as photosynthesis, stomatal conductance, and proline accumulation are also positively got affected by PGPR [60]. More recently, Lim et al. [61] testified that in the palm, biotized with *Herbaspirillum seropedicae* induces embryogenic callus formation and proliferation.

Biotization with PGPR have also proved to be an effective method to induce resistance against pathogens. The defense mechanism in plants are greatly affected by ethylene. Moreover, during culture conditions, attributes of growth and senescence are controlled by ethylene production, which is indirectly modulated through other growth hormones like auxins and cytokinins [62,63]. Ethylene induces defense in plants by activating several complex pathways, which ends in production of important defense molecules like jasmonic acid (JA), salicylic acid (SA), and abscisic acid [9,10]. Similarly, phenolic compounds and other secondary metabolites also play a significant role in pigmentation and provide protection against pathogens [64]. Biotization with *Pseudomonas* spp in oregano cultures resulted in an elevated level of phenolic compounds and chlorophyll [57]. Certain toxic compounds, antibiotics, and hydrolytic enzymes have also shown a negative effect on the growth of pathogens. These compounds act by either degrading the cell wall of pathogens or by suppressing pathogenic molecules [65]. Many PGPB synthesize the ACC (1-aminocyclopropane-1-carboxylate) deaminase enzyme. This bacterial enzyme promotes plant growth by decreasing the plant ethylene concentration. This enzyme converts amino cyclopropane carboxylate (ACC) into ammonia and α-ketobutyrate (α-KB), which leads to the scarcity of ACC, the prime precursor of ethylene in the cell. The role of ACC deaminase producing PGPR in alleviating stress and inducing resistance through reduced ethylene production has already been reported [66]. Beside this, many PGPB also synthesize certain volatile compounds which can promote callus organogenesis [67], enhance the photosynthesis efficiency [68], and offer better defense against abiotic stresses [69]. PGPMs also play a critical role in Induced Systemic Resistance (ISR) and hence provide protection against pathogens as well as insects. Many bacteria and fungi (like *Bacillus*, *Pseudomonas*, and *Trichoderma*) prepare the plant for any future attack of pathogen and save the energy of the plant which may get wasted unnecessarily otherwise during infection [70]. A comprehensive list of some successful biotization with PGPBs is presented in Table 2.

TABLE 2 Effect of different PGPBs (plant growth-promoting bacteria) on plant propagules when used as Biotization agents during tissue culture process.

Name of bacteria	Investigational plant	Micropropagation stage at which bacteria was inoculated	Observed effect	References
Methylobacterium salsuginis	*Banana*	Acclimatization	Increased plant height, girth and number of leaves, root length, lateral root, and biomass	[71]
Bacillus megaterium MiR-4	*Vigna radiata* (L.) *R. Wilczek*	Rooting	Increased shoot length and root length	[72]
Pseudomonas putida	*Mentha piperita*	Multiplication	Increased biomass and increased essential oil production	[73]
Pseudomonas putida	*Pennisetum glaucum* and *Zea mays*	Multiplication	Increased plant resistance against stress condition	[74]
Azospirillum brasilense Sp245	*Prunus cerasifera*	Acclimatization	Increased stem length and node number	[75]
Acinetobacter lwofii Acinetobacter haemoliticus Pseudomonas sp.	*Crocus sativus* L.	Seed germination	Increased seed germination rate and decrease germination time	[76]
Azospirillum brasilense (Sp245, S27, and SR8)	*Solanum tuberosum* L.	Multiplication and acclimatization	Increased shoot length and shoot number in in vitro and ex vitro condition	[51]

Continued

TABLE 2 Effect of different PGPBs (plant growth-promoting bacteria) on plant propagules when used as Biotization agents during tissue culture process—cont'd

Name of bacteria	Investigational plant	Micropropagation stage at which bacteria was inoculated	Observed effect	References
Bacillus subtilis M3 *Trichoderma harzianum Gliocladium catenulatum*	*Fragaria* × *ananssa*	Acclimatization	Overall growth promotion and disease resistance	[77]
Bacillus consortium (INR7, T4 and INR 937b)	Banana "Grande Naine"	Hardening	Increase all over growth of plant	[78]
Pseudomonas putida strains G2-8 and Gl l-32	*Glycine mux* L.	Callus	Increased biomass of callus	[79]
Bacillus amyloliquifaciens UCMB5113	*Arabidopsis thaliana* (L.)	Rooting	Increased lateral root outgrowth and elongation and root hair formation	[80]
Bacillus	*Platycladus orientalis*	Multiplication	Increased biomass in water stress Condition	[81]
Pseudomonas putida	*Arabidopsis thaliana*	Rooting	Increased shoot number and root number	[82]

Herbaspirillum seropedicae strain Z78	*Elaeis guineensis* Jacq	Calluse Multiplication	Increased biomass of callus	[61]
Pseudomonas fluorescens	*Chlorophytum* sp.	Rooting	Increase root length shoot dry weight, and leaf length	[14]
Agrobacterium rhizogene	*Arnebia hispidissima*	Rootsing	Increase root and enhance alkannin and shikonin derivatives in hairy roots	[83]
Trichoderma viride, P. fluorescens	*Albizia amara*	Acclimatization	Enhanced stress tolerance, increased root length and number, increased shoot length and higher leaf number and biomass	[34]
Pseudomonas fluorescens isolates	*Arachis hypogaea* L.	Acclimatization	Increased resistance and vigourness	[84]
Bacillus pumilus	*Alnus glutinosa*	Seed germination and acclimatization	Stem elongation	[85]
Methylobacterium salsuginis TNMB03	Banana "Grande Naine"	Acclimatization	Increased plant growth	[86]
Bacillus sp.	*Sorghum bicolor* (L.)	Acclimatization	Increased plant growth in stress condition and better survival	[87]
Serratia marcescens Brevibacillus parabrevis	*Guadua chacoensis*	Multiplication	Overall growth and better survival	[88]
Rhizophagus irregularis MUCL 41833	*Medicago truncatula*	Rooting	Increased root number and root length	[89]

5.3 Biotization to elevate in vitro secondary metabolite production

Higher plants synthesize a variety of secondary metabolites (SM) such as alkaloids, flavonoids, steroids, terpenoids, quinones, lignans, and anthocyanins. These SM are immensely valued products, generally used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives. For plants, these SM have no significant role in vital metabolic pathways for survival, but play an important role in the interaction of the plant with its environment and also act as defense chemicals [90]. Generally, these SM are accumulated in plants in a very low amount (less than 1%). Moreover, their synthesis is dependent on the physiological conditions (particularly stress conditions) and developmental stage of the plant [91]. Considering their immense economical values, their production through tissue culture methods was promoted. However, since in tissue culture, plant cells are grown under lavish environmental conditions, which do not favor SM synthesis, their accumulation further decreases. Several biotechnological approaches have been applied to increase SM production under in vitro conditions, but elicitation is recognized as the most viable technique for increasing the production of desirable SM from cell, organ, and plant culture [92,93]. The strategy through which SM production is stimulated through the involvement of any biotic or abiotic factor is called "elicitation" and the factor is called the "elicitor." Elicitors may be formed inside or outside plant cells and can be endogenous or exogenous in nature. Depending on their origin, they are classified as biotic or abiotic elicitors. Abiotic elicitors include UV irradiation, salts of heavy metals, and some other chemicals (like jasmonic acid, salicylic acid, etc.), while biotic elicitors may include chitin, chitosan, or glucans present in fungal cell wall materials, glycoprotein present in bacteria, and low molecular weight organic acids. Sometimes the entire microorganism (which may be a pathogen also) can act as elicitors. Several PGPB and PGPF have also been proved to be potential elicitors and their role in increasing SM production has been established. Inoculation of plant with PGPM (biotic elicitors) may significantly induce higher production of SM during tissue culture conditions. Several studies on the effect of PGPRs on higher SM production were carried out and it was found that PGPRs induce SM production through the ISR (induced systemic resistance) mechanism [94]. PGPR act as a potent activator of the key enzymes that are involved in the biosynthetic pathways of secondary metabolites [95].

PGPR also induce biosynthesis of certain other chemicals (like jasmonic acid and salicylic acid) in plants which acts as a transducer for elicitor signaling pathways and ultimately leads to the accumulation of secondary metabolites in plants [96]. Similarly, several PGPFs (mostly AM fungi) also induce increased production of SM when inoculated during culture conditions. The effect of different PGPMs on secondary metabolite production is depicted in Table 3.

TABLE 3 Effect of different PGPMs (plant growth-promoting microbes) on secondary metabolite production.

Name of PGPMs (bacteria/fungi)	Investigational plant	Compounds	References
Phaseolus vulgaris (F)	*Colletotrichum lindemuthianum*	Krevitone	[97]
Coriolus versicolor (F)	*Rhodiola sachalinensis*	Salidroside	[98]
Trichoderma viride (F)	*Catharanthus roseus*	Ajmalicine	[93]
Fusarium oxysporum (F)	*Hypericum perforatum*	Gymnemic acid	[99]
Trichoderma atroviride (B)	*Salvia miltiorrhiza*	Tanshinone	[100]
Bacillus polymyxa (B)	*Stevia rebaudiana*	Stevioside	[23]
Bacillus subtilis (B)	*Ocimum basilicum*	Eugeno	[101]
Pseudomonas fluorescens (B)	*Catharanthus roseus*	Ajmalicine	[93]
Azospirillum brasilense (B)	*Origanum* × *majoricum*	Thymol	[102]
Bradyrhizobium sp. (B)	*Origanum majorana* L.	Trans-sabinene hydrate	[103]
Datura stramonium (F)	*Penicillium chrysogenum*	Lubimin	[104]
Pseudomonas aeruginosa (B)	*Scopolia parviflora*	Scopolamine.	[105]
Pseudomonas fluorescens (B)	*Rubus fruticosus*	Phenolic compounds, flavonoids and anthocyanins	[106]
Pseudomonas fluorescens (B)	*Glycine max*	Isoflavone	[107]
Agrobacterium rhizogenes (B)	*Althaea officinalis*	Phenolics and flavonoids	[108]

Continued

TABLE 3 Effect of different PGPMs (plant growth-promoting microbes) on secondary metabolite production—cont'd

Name of PGPMs (bacteria/fungi)	Investigational plant	Compounds	References
Rhizobium radiobacter (B)	*Hypericum perforatum*	Xanthon	[109]
Glomus mosseae (F)	*Andrographispaniculata*	Andrographolide	[110]
Pythium aphanidermatum (F)	*Coleus blume*	Rosmarinic acid	[111]
Pythium aphanidermatum (F)	*Daucus carota*	*p*-Hydroxybenzoic acid	[112]
Yeast (F)	*Hypericum perforatum*	Hypericin	[113]
Fusarium sp. (F)	*Euphorbia pekinensis*	Euphol	[114]

6 Future prospects of biotization

Despite their immense potential, the application of PGPMs in tissue culture has not been exploited thoroughly. One of the reasons behind this is the response of PGPM, which varies not only from plant to plant but also at the explant level (e.g., root, stem, leaf, etc.) [12]. Further research is required to select efficient PGPMs as well as the development of an efficient protocol so that these organisms can be effectively used in tissue culture. Understand the signal recognition and transduction during natural conditions and culture conditions, which leads to association between the plant partner and microbial partner, is also a challenge. The combined use of more than two organisms can also be a viable option for better results [115]. Moreover, an efficient technique for the inoculation of PGPMs at different stages of tissue culture and a mechanism to control the population of microbes without affecting plant growth as well as potency of microbes should also be developed. Bio-nanotechnology can be used to address this problem and ready-to-use effective formulation of PGPMs can be developed [116]. Currently, very few reports are there on the use of bio-nanotechnology in tissue culture; hence, it will be a bright field to investigate and surely will add new development in the biotization process. Another important challenge in biotization is the low potency, specificity, and neutral response toward certain plant species. In this respect, the transgenic approach to develop a highly vigorous strain can be adopted in order to achieve a specific objective. The recent advancement taking place in biotechnology (such as functional genomics, bioinformatics, signaling in the rhizosphere, etc.) can be used as an effective tool in the development of transgenic microorganisms to confer better utilization of these PGPMs as biotization agents in tissue culture.

7 Conclusions

Micropropagation is an important tool for the large-scale production of elite germplasm of many economically important plants. However, due to certain limitations (like the high production cost and loss of plants during the acclimatization phase), the technique has not reached the planned success point. PGPMs are considered as potentially advantageous microbes for the plants in terms of better growth and providing protection against biotic and abiotic stress. The biofertilizer, biostimulation, and biocontrol properties of these PGPMs can be exploited to overcome the existing problems in tissue culture. An efficient consortium of PGPMs and the inoculation method can be developed which will not only decrease the production cost (by replacing the costly synthetic phytohormones) but also increase the survival percentage of plants after field transfer. However, intensive care should be taken to ensure that any vigorous plant/human pathogen should not contaminate the culture, particularly when the plant is used as raw food, as certain pathogens can stably survive in the tissue for a prolonged period both under in vitro as well as ex vitro conditions. In addition, a

lot of research needs to be carried out in order to identify suitable PGPMs and to develop formulations for appropriate application in plant tissue culture. Moreover, the molecular understanding of relationship between plants and PGPMs will open new avenues in this prospective field of biotization.

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