RESEARCH PAPER

Micropropagation of disease-free banana genotype 8818-william for field cultivation

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Key Message: Micropropagation emerges as a crucial strategy in addressing the challenges threatening banana cultivation, providing a pathway to produce disease-free banana plants. Our study on genotype 8818-William highlights the efficacy of this technique, emphasizing the variability in shoot multiplication, root induction, and survival rates.

Abstract

Banana is an economically important fruit crop that belongs to the genus Musa and is the main source of food in the world. The production of banana is affected by wide range of pathogenic organisms such as fungi, viruses, bacteria, insects, and nematodes. The conventional methods for the production of disease-free banana are hard to practice and time consuming. The micropropagation technique can be used to produce genetically same, disease free and pest resistant banana plants for commercial purposes. In the present study, the micropropagation technique has been used for the production of disease-free banana genotype 8818-William. During the micropropagation, evaluate the shoot multiplication, root induction and survival rate from the same explant source under similar physiological condition. The shoot multiplication rate of the explants was found to be significantly different with standard deviation of explant 1 (0.894), explant 2 (2.39), explant 3 (5.76), explant 4 (56.4) and explant 5 (108.9). The root induction frequency was also found to be different. The mean survival rate of about 83% was obtained for the plantlets grown under acclimatized conditions. The micropropagation technique can be successfully utilized for the production of disease-free banana plants with commercial applications.

Keywords: Banana 8818-william, Diseases free, Micropropagation

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Introduction

Bananas are a significant global crop, cultivated in 132 countries with an annual production of approximately 95 million tons (UNIDO, 2008). In developing nations, bananas rank as the fourth primary food source after rice, milk, and wheat. In terms of gross production value, bananas hold the fourth position, following rice, wheat, and maize. Bananas are grown on 5.6 million hectares of land in nearly 150 different countries, producing 117.9 million tonnes of fruit annually (FAO, 2018). In Pakistan annual banana production reached 10, 3000 tons, covering 30,000 hectares, with Sindh alone contributing 91% of the total cultivated area in the country (UNIDO, 2008).

Bananas and their plantains are highly susceptible to various pests and microorganisms, including fungi, viruses, bacteria, insects, and nematodes. One significant threat is the Sigatoka disease, particularly caused by the pathogenic fungi *Mycosphaerella fijiensis* (black sigatoka) and *M. musicola* (yellow sigatoka), leading to yield reductions of 30-50% in many cultivating regions (Sowmya et al., 2016). Another damaging pathogen is the soil-residing fungus *Fusarium oxysporum* f.sp. cubense, responsible for the Panama or banana wilt, causing a severe epidemic that destroyed around 40,000 hectares of plantations by 1960. In terms of viral diseases, the bunchy top disease, caused by the banana bunchy top virus (BBTV), is a major concern affecting banana cultivars (Shah et al., 2020). BBTV, a single-stranded DNA virus, is primarily transmitted by the aphid *Pentalonia nigronervosa* Coquerel and through vegetative propagation. This virus is widespread in the Asian-Pacific region (Thomas et al., 1995; Qazi, 2016). No BBTV-resistant source has been identified to date. Banana production worldwide is also impacted by the Banana streak virus, a badnavirus first reported in Morocco (Lockhart, 1986). This pararetrovirus, Banana streak virus, can integrate into the banana genome (Lockhart, 1996). Other viruses, such as cucumber mosaic virus, banana bract mosaic virus, and banana streak virus, are known to cause significant damage to bananas. In India and the Philippines, Banana bract mosaic virus poses a serious challenge due to the lack of proper diagnostic tests, identified as a potyvirus (Shaman et al., 2000). Cucumber mosaic virus, causing infectious chlorosis in bananas, is prevalent globally, and its control is challenging due to a wide range of hosts (Manjunatha et al., 2018).

To ensure an ample supply of planting material for newly introduced clones/hybrids, relying on suckers for propagation takes several years. Conventional breeding methods face limitations in achieving disease-free and pest-resistant bananas due to lengthy germination times and the triploid nature of most banana cultivars, making them highly sterile (Ortiz, 2013). Vegetative propagation can perpetuate pathogen transmission across generations, leading to widespread infection in a short time. To enhance plant propagation efficiency, there's a need to develop new high-value varieties that meet both domestic and export standards. Addressing these challenges involves utilizing plant tissue culture technology to produce disease-free banana plants and high-quality planting material under aseptic conditions (Shah et al., 2020). *In vitro* culture has been extensively used for rapid vegetative clone propagation of various genotypes (Vuylsteke & De Langhe, 1985; Das et al., 1998).

Micropropagation is an advanced technique used for mass-producing disease-free, high-yielding, and top-quality planting material. This method rapidly multiplies clonal plants, addressing issues like reduced vigor and yield due to infections. It is extensively employed for the quick and sterile production of disease-free banana plants, overcoming challenges posed by the traditional methods, which struggle with improving sterile and parthenocarpic banana plants. Conventional methods are time-consuming and difficult for large-scale, homogeneous plant production. *In vitro* micropropagation emerges as an effective alternative, providing a quicker and efficient way to produce disease-free banana plants for commercial purposes. Several studies have reported the *in vitro* propagation of banana using various explant sources and methods (Resmi & Nair 2007; Venkatachalam et al., 2007; Shirani et al., 2009). In the early 1970s, researchers from Taiwan demonstrated that shoot tips (explant) from banana could generate adventitious buds (Ma & Shii, 1972, 1974), sparking further investigations. A study revealed the potential to produce cucumber mosaic virus-free plants of banana cultivar Cavendish Group AAA by using meristem and lateral buds of virally infected plants, combined with heat treatment and aseptic culture (Tzean et al., 2019). Subsequent advancements in the procedure have allowed for the rapid production of numerous shoots within a short period, which can be rooted in approximately 3 to 4 days (Chakravarthy, 2013). Conventionally, various parts of the banana plant, such as suckers, lateral buds, axillary flower buds, and the apex of the inflorescence, containing shoot meristem, have been identified as suitable for initiating shoot cultures (Sivakumar & Visalakshi, 2021). Selecting mature plants as explants is crucial, considering their known qualitative traits influenced by genetic makeup and environmental factors. For in vitro multiplication of bananas, shoot tips from suckers sized 40-100 cm are commonly used. Larger explants are preferred for rapid multiplication due to more lateral buds, despite being more sensitive to blackening and contamination. Smaller explants, like meristem-tip culture, are ideal for virus and bacteria eradication, despite higher death rates and slower growth. The shoot tip culture method is significant for producing hybrids, disease-free plants, and maintaining germplasm stocks. Regeneration from shoot tips, when cut into 4 or more segments, is well-established. This technique is versatile for rapid multiplication of clones and hybrids with desired qualities, particularly for mass clonal propagation and germplasm conservation. Various studies differ in explant type, size, sterilization methods, and treatment parameters (Wong, 1986; Hamill et al., 1993).

Plant tissue culture media contain macro and micronutrients, amino acids, vitamins, carbon sources, organic nutrients, growth hormones, and solidifying agents (Ahmad et al., 2012; Shah et al., 2013; Shah et al., 2014a, b; Shah et al.,

2015; Jan et al., 2015; Ahmad et al., 2020). The use of liquid media is preferred for *in vitro* micropropagation due to cost-effectiveness, easy sterilization with ultrafiltration, short sub-culturing time, and no need for positioning (Alvard, 1993). Mechanization of micropropagation requires expertise in *in vitro* culture in liquid media (Loberant & Altman, 2010). Liquid media have been successful in promoting shoot proliferation, establishing embryogenic suspensions, and supporting growth in many plants (Ascough & Fennell, 2004; Bairwa et al., 2012). The mechanism behind this enhancement in liquid media is not precisely known (Alvard, 1993), but it lacks a solidifying agent, making water and dissolved substances readily available to the explant (Debergh, 1983). The main challenge with liquid media is oxygen deprivation, which can be mitigated by preventing complete immersion of the explant, ensuring oxygen availability by maintaining the media depth (Gomez kosky, 2002) Direct aeration of oxygen into the media is also employed in micropropagation (Curtis, 2005). Although liquid media are highly efficient for shoot multiplication (Adelberg, 2008; Swarnathilaka & Nilantha, 2012), solid media rounds are crucial for ex-vitro growth and high plant production. In banana *in vitro* propagation, experiments have utilized solid, semi-solid, and liquid media. Therefore, this research study aimed to optimize the growth conditions for the micro propagation of disease-free banana plants.

Materials and Methods

This research was conducted at Tissue Culture Laboratory, National Agricultural Research Centre (NARC), Islamabad during the year 2017.

Plant material

The banana variety named 8818-William was used to establish an *in-vitro* multiplication system. For initiation of culture, sword suckers were collected from the already grown banana field at NARC.

Preparation of MS media

In a 1 litter beaker, 500 ml distilled water was poured with a magnetic stirrer. Following the instruction from the manufacturer, MS mixture was added. Then, 30 g sucrose was dissolved in the media. The volume of the solution was adjusted to 990 ml while poured into graduated cylinder. Using 1N NaOH and HCl, the pH of the solution was adjusted to 5.8, which made up volume 1 liter. This 1 litter medium was divided into two halves of 500 ml and 2 g agar was added to 1 half and boiled it into the microwave oven. After boiling both the media mixed carefully, poured into glass jars with 30 ml each and covered the jars with autoclavable polyethylene sheets. Medium having jars were labeled finally, and preparation date was written.

Rooting medium

The banana rooting medium was prepared by the same method as that is used for the preparation of banana multiplication medium but only the difference was that in the banana rooting (BR) medium IAA was added twice that of used in banana multiplication medium and BAP was not added.

Sterilization of media

The medium jars were placed vertically in the autoclave, closed the top and locked it. Temperature, time and pressure were set 121 C for 15 minutes, at 15 psi pressure. After the given time, the lid of the autoclave was opened, and waited, till cooled down the medium. The medium jars were finally removed from the autoclave and stored.

Sucker collection

Healthy suckers were collected from the banana field for excising the shoot tips. 20 suckers were removed from the mother plants of banana verities 8818-William, using panga. The soils from the suckers were removed by cleaning them and the roots were cut off to uncover the corm. 30 cm above the base, the pseudo stems were cut, and the separated suckers were packed in clean nylon bags, labeled and carried to the tissue culture lab for further process.

Explant preparation

The collected suckers were washed to remove soil, mud and other debris using tap water. With the help of stainless-steel knife, root and outer leaves were removed in such a way preventing the shoot tips from damaging. Till the explant was 2 × 2 × 2 cm cube covering the shoot apex.

Explant sterilization and culturing

Before starting the work, the laminar flow hood was started before half an hour and then was cleaned with rectified spirit. Then in a sterile beaker 50%, Clorox solution was prepared with sterile water. The explants were placed in 50% Clorox, 2 drops of tween 20 were added to decrease the surface tension and shook in laminar flow hood for 10-15 minutes. Then for the removal of Clorox, it was washed with sterile water several times. Then jars having the medium were taken, uncovered and the explants were placed in them in such a manner that half of the shoot tips were in the medium. The jars after inoculation of the explants were covered immediately, sealed with rubber and properly labeled. The cultures were incubated at 26 ± 2 °C in dark room for four weeks.

Culture initiation

For the culture initiation media used was Murashige and Skoog (1962) salt mixture supplemented with 30 g/l sucrose and amino acid/ vitamin mixture, which include pyridoxine (0.5 mg/l), thiamine HCl (0.4 mg/l) Myo-inositol (100 mg/l),

glycine (2.0 mg/l) and L-tyrosine (100 mg/l). Various culture conditions, phytohormones and physical state of the media was tested to initiate the culture. The cultures were incubated at 26 ± 2 °C, 16 hours' photoperiod by using tube lights of Philips Electrical Co. After every two weeks, data were recorded on visual observation for physical changes and contamination, up to four weeks.

In vitro multiplication

Multiplication media used was MS salt mixture supplemented with different concentrations of phytohormones that includes MS+ BAP (5.0 mg/l) and IAA (2.0 mg/l). For adjustment of pH and autoclave, the previously described method was used. Explants from initiated cultures were shifted to banana multiplication media after four weeks. Fairly large size explants (3-10 mm) are known as desirable for rapid multiplication despite its higher susceptibility to getting contaminated and Blackening. The explants were processed further on a later time, reduced in size (0.5-1 mm length) having meristem dome with some leaf initials. The cultures were incubated at 26±2 ċ under 16-hour photoperiod. On the basis of the number of shoots/culture, the effects of different concentrations of PGRs were quantified.

In vitro rooting

Rooting media used was basal MS salt and vitamin mixture supplemented with (1.0 mg/l) IAA and solidified using (2.0 mg/l) agar. Basal MS without PGRs was used as the control. Media was poured in 200 ml jars with 30 ml each and sealed with polyethylene sheets. Autoclave and PH adjustment to 5.8 was done with the same method described previously. Shoots of 4-5 cm height were separated from the cluster and transferred to rooting media. Five shoots of equal size with leaves were cultured. Cultured jars were placed under controlled growth conditions, same as that used for the shoot multiplication in the growth room.

Primary hardening

Plantlets with roots were taken from the glass jars and washed using tap water in such a way that prevents the damage of the roots. Polythene bags filled with 1: 1 mixture of sand and clay were used to transfer the plantlets and labeled with the date of plantation and name of verity. ¾ of the bags were filled with peat moss. Single plantlet was transferred to each bag. For one week the plants were watered with the shower after every 2 hours, after one week these were watered every five hours for one month.

Secondary hardening

Polythene bags were half-filled with canal soil, the fertilizers (N, P, K) 2 g/ plant was mixed. The semi-hardened plants were removed from the bags and placed in the center of the half-filled bags with fertilizers mixed and filled the bags up to ¾ with soil. During the first week, plants were watered every day and after alternate day for one month.

Results

In vitro shoot regeneration

The external primordial of the explant becomes green, after thirty days of culture, which was primarily white at inoculation time. The increase in size of the explant was observed after a few days of incubation whereas blackening was observed at the base of the explant, which might be caused due to the secretion of the phenolic compounds in culture media. After the initial culturing of the explant, two shoots were observed on explant 2 whereas no shoots were found on the rest of the explant. The explants were sub-cultured and shoot multiplications were observed on all the explants. Furthermore, the shoots were sub-cultured four times for shoot multiplication. The results of explant inoculation, sub-culturing and the production of multiple shoots are given in Table 1.

Table 1 The number of shoots produced during each subculture from banana 8818-William

No of explant	Sub-culturing				
	Sub-culture 1	Sub-culture 2	Sub-culture 3	Sub-culture 4	Sub-culture 5
Explant No. 1	1	5	15	106	188
Explant No. 2	3	8	20	140	296
Explant No. 3	1	2	10	20	40
Explant No. 4	1	3	6	18	85
Explant No. 5	2	6	18	30	50
Total	8	24	79	314	659
Mean	1.6	4.8	13.8	62.8	131.8
S.D	0.894	2.39	5.76	56.4	108.9

Significant differences were found in the shoot multiplication from the same explant source cultured on the same medium and same physiological conditions (Table 1). The numbers of shoots produced were high from the explant no. 2 whereas lower numbers of shoots were produced by the explant no. 5.

In vitro root regeneration

Shoots from the same five explants of the *Musa spp*, genotype cv. 8818-william was rooted *in vitro*. Significant differences were found in terms of producing roots (Table 2). Maximum numbers of roots were observed in shoot originated from explant 2; following explant 1 and explant 3 produce least number of roots.

Acclimatization of plantlets

During the acclimatization, many plantlets failed to survive in *in vivo* conditions. Plantlets from the same five cultured explants were transferred from lab to the soil, and the survival rate was observed (Table 3). Plantlets from these cultured explants shows variability in term of survive and grow *in vivo*.

Table 2 Number of roots produced during *in vitro* culture of banana 8818-William

No. of explant	No. of shoots	Roots induction
1	188	146
2	296	243
3	40	29
4	85	67
5	50	41
Total	659	105.2
Mean	131.8	

Table 3 Number of plantlets produced during *in vitro* culture of banana 8818-William

No. of explant	Total explants cultured	No. of plantlets produced	Survival rate (%)
1	146	120	82
2	243	219	90
3	29	20	68
4	67	51	76
5	41	27	65
Total	526	437	83%
Mean	105.2	87.4	

Discussion

Bananas, part of the Musa genus, come in various sizes and colors. The name 'banana' originates from the Arabic word 'banan,' meaning finger. Initially introduced by Portuguese to West Africans, bananas are now a vital food source, particularly in the developing world. Classified by Simmonds and Shepherd (1955), dessert bananas have Musa acuminata genomes, while plantains result from crosses between *Musa acuminata* and *Musa balbisiana*. They are rich in carbohydrates, vitamin B, vitamin C, and potassium, with potential health benefits like lowering cholesterol levels. Embryo and shoot tips culture in bananas facilitate organized growth in aseptic conditions, crucial for breeding and overcoming low seed germination rates. Embryo culture aids in rescuing embryos, essential for traits like nematode resistance. The technique also enhances banana varieties through traditional breeding, addressing challenges like sterility and polyploidy. N6-benzyl amino purine (BAP) is a widely used cytokinin for micropropagation of banana cultivars due to its stability and effectiveness in inducing shoot formation. The concentration of BAP in the media significantly influences shoot proliferation, with 20 µM being recommended for banana micropropagation. Studies suggest that BAP's chemical stability and higher induction capability make it superior to other purine-based cytokinins. For root initiation in banana *in vitro* culture, commonly used auxins include indole acetic acid (IAA), naphthalene acetic acid (NAA), and indole butyric acid (IBA). Active charcoal may also influence root formation, but studies have shown no significant difference when combined with auxins.

Previously a lot of studies have been carried out for the production of disease-free banana plants through tissue culture technology. The conventional plant breeding approaches has been used with limited success for the production of disease-free banana plants due to long germination time, triploid nature of banana and the transfer of pathogen from one generation to another generation by conventional vegetative methods (Ortiz, 2013). The *in-vitro* propagation of banana offers several advantages and has been extensively used to efficiently propagate vegetative clones of many banana genotypes (Das et al., 1998). Numerous studies have been conducted to optimize the different parameters for the multiplication of banana through tissue culture system (Aman et al., 2018). The various parameters of interest include incubation temperature, pH, humidity, nutritional requirements, type of explant, and the use of hormonal concentration. We used the best and optimal condition chosen from the previous literature up till now and didn't interfere with the physiochemical parameters. In this research, we used the hormonal concentration, BAP 5.0 mg/l, IAA 2.0 mg/l, pH 5.8, and 26 ± 2 °C temperature for the micropropagation of banana. Results from this experiment showed that one cultured shoot tip can produce 87.4 plantlets on average. It was perceived that, in terms of *in vitro* multiplication, all explants did not respond similarly. Also, it was observed that culture No.2 produced a maximum number of plantlets (219), and culture No.3 produced the smallest number of plantlets, which were 20. Vuylsteke (1998) reported that different *Musa spp*, genotypes show differences in the rate of multiplication.

It was observed in this study that, explants of the same genotype behave differently in term of producing shoots and induction of roots, as shown in Table No 1 and 2. As the explants were of the same genotype, so the differences in the growth rate was due to their physiological response, and the difference in the physiological responses of explant depends on the age and health condition of the mother plant, from which the explants have been taken. The result of our studies also shows that in the initial subcultures the multiplication rate is higher. If the initial subcultures show a slow rate of multiplication and if commercially plant supply is of concern, then the initial subcultures should be discarded to save time. Good quality banana production is necessary for constant supplies of it for commercial use, which can be done using tissue culture technology for the clonal propagation of plant material, which provide a high rate of multiplication of genetically identical disease-free and pest resistant plants (Aman et al., 2018).

Conclusion

Bananas are the most widely used fruit in the world with economic, commercial and nutritional value. Because of having a good nutritive value, especially vitamin A, as discussed above in chapter first it is widely used by people living in tropical areas because their foods are deficient in vitamin A. But in some parts of the world, it is commonly used as a staple food. Even though its high demand, constant plant material supply is a challenge. The banana plant is very susceptible to many diseases, and many other problems make it hard for the farmers to fulfill these demands. Tissue culture is the only solution to overcome this challenge and to develop an efficient *in vitro* propagation for disease free banana and to fulfill the demand of this plant to the market. In the present study, we have developed efficient and quick in-vitro micropropagation techniques for the production of disease-free banana genotype 8818-Wiliam.

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