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Finding the Optimum Ratio of 6-Benzyl Amino Purine and Indole-3 Acetic Acid for *In vitro* Shoot Proliferation in the Ethiopian Banana Variety, "AsmaraLong Type"

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Abstract

Bananas (*Musa* spp.) are important fruit crops cultivated in more than 120 countries throughout the tropics and subtropics, which have a significant contribution to food security and income. Tissue culture of banana can solve the problem of shortage in quality planting material of banana. This study aimed to find the optimum ratio of 6-bezyl amino purine and indole-3 acetic acid for *in vitro* shoot proliferation in the Ethiopian banana variety, "Asmara-long type". The plant is a widely cultivated variety in Arba Minch. Murashige and Skoog medium was used in the present study as it is the most commonly used plant tissue culture medium. This study covered three experiments. Firstly, it compared three surface sterilization procedures to control contamination. Secondly, it examined the effect of ascorbic acid in controlling lethal browning of shoot tip. Lastly, it investigated the impact of four different hormonal ratios on shoot tip proliferation. Based on the results it has been found that both sterilizations (NaOCl, NaOCl+0.1% $HgCl_2$ and 0.1% $HgCl_2$) methods were effective for surface sterilization of shoot tips of banana in preventing contamination. When there was 10mgl^{-1} ascorbic acid in the media, there was no lethal browning of explants and media colour did not change. Among the four hormonal ratios tried, the ratio I i.e., 4 mgl^{-1} 6-benzyl amino purine and 0 mgl^{-1} indole 3-acetic acid has been found to induce shoot growth in 50% of the explants. The culture protocols developed in this study provide a baseline for future work on micro propagation and genetic improvement of Ethiopian banana cultivars.

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Keywords

Banana, 6-benzyl amino purine, indole-3 acetic acid, in vitro and sterilization.

Introduction

Bananas (*Musa spp*.) are important fruit crops cultivated in more than 120 nations all through the tropics and subtropics, where they make a critical commitment to nourishment security and pay (Ghosh *et al.*, 2009). Banana (*Musa* spp.) is one of the most punctual edit plants domesticated by people. Bananas are devoured as ready natural products. Initially, it could be trim from muggy tropics. In any case, it is presently acclimatized to a wide extend of climatic conditions. Bananas possess the status of a tall esteem commercial edit. Independent of its commercial status, banana is alluded as 'Poor man's apple' (Singh *et al.*, 2011).

Banana is all inclusive positioned fourth, following to rice, wheat and maize in terms of net esteem of generation. It may be a major staple nourishment trim for millions of individuals as well as gives pay through nearby and universal exchange. Among the boring staple

nourishment crops, banana positions third with regard to the overall generation. In spite of the fact that cassava and sweet potato are situated as to begin with and moment, banana has nearly risen to significance in all the tropical locales of the world. Conventional bananas and other species of family *Musaceae* have been the major calorie source of numerous ethnic tribes of Africa and Pacific Islands.

High utilization of bananas has been detailed in little nations of Pacific Islands like Samoa (132 Kcal) and Vanuatu (92 Kcal). Bananas too discover significance within the slim down of Caribbean (Haiti and Dominican Republic) and Latin American nations (Ecuador and Brazil) (Singh *et al.*, 2011).

The time banana was presented to Ethiopia is sometime recently 1980s. Until the early 1980s, maize, cotton and sweet potato were imperative crops delivered by agriculturists in Arba Minch Zuria and Mirab Abaya areas of the Gamo-Gofa zone in Southern nation nationalities and people region. Amid that period, the Arba Minch state cultivate had 62 hectares of arrive secured by predominate Cavendish banana (Natnael and Mamuye, 2016).

The agro ecology of Arba Minch was great for Cavendish banana; most of the suckers at the pilot ranches bore bunches effortlessly and gave great yields a few 10 months after planting. The primary gather was transported and showcased in Addis Ababa.

The cultivate door cost for a kilo of banana was 15 Ethiopian birr. Seeing this, the farmer's understood the financial good thing about locks in an inundated banana generation. Almost five a long time afterward, most cultivate lands that had a simple get to water system in Arba Minch were secured by overshadow Cavendish.

Currently banana in Ethiopia covers around 59.64% (53,956.16 hectares) of the whole fruit zone, around 68.00% (478,251.04 tones) of the whole natural products created, and approximately 38.30% (2,574,035) of the whole natural product creating agriculturists.

On the other hand, almost 68.72% (37,076.85 hectares) hectares of arrive secured by banana, approximately 77.53% (370,784.17 tones) of the banana created and 22.38% (1,504,207) of the banana makers in Ethiopia are found within the Southern Nation Nationalities and Peoples' Regional State (SNNPRS) (CSA, 2014) (CSA, 2014). Gamo-Gofa, Bnech Maji and Sheka zones are among the major banana creating zones of the SNNPRS, of which Gamo-Gofa zone alone covers over 70% of the whole banana showcased over the major showcase outlets in Ethiopia (CFC, 2004).

Banana is defenseless to a number of biotic and abiotic stresses which constrain its generation especially among little and negligible agriculturists with constrained assets. BBTV (Banana bunchy best infection), CMV (Cucumber mosaic infection), BSV (Banana streak infection) and BBMV (Banana bract mosaic infection) are the four most imperative viral illnesses influencing bananas (Singh *et al.*, 2011).

Improvement of tissue culture innovation has been the establishment of large quality, disease free planting material generation at a mass scale, especially in vegetative proliferated crops. Micro-propagation of banana makes a difference in malady disposal and generation of quality banana planting material.

The foremost critical arrange in micro-propagation is shoot increase which can be accomplished by adjusting the hormone levels within the plant tissue culture medium.

Auxins and cytokines are the two most imperative classes of hormones utilized in plant tissue culture. Therefore the aim of this study is to investigate the optimum ratio of 6-bezyl amino purine and indole-3 acetic acid for *in vitro* shoot proliferation in the Ethiopian banana variety, "Asmara".

Materials and Methods

Sampling Area

The raw material used in this research was banana. The banana variety that was used for the *in vitro* culture in this study was "Asmara-long type". It is a Cavendish type. It is the widely cultivated variety in Arba Minch. It was collected from surrounding of Arba Minch town and district southern Ethiopia.

Study Design

Laboratory based experimental study was conducted at Arba Minch University (AMU) in Plant Biotechnology Laboratory, College of Natural and Computational Sciences (CNS), AMU. Asmara-long type banana sample were collected from Arba Minch town and district.

Explant collection

Shoot tips were extracted from the sword suckers (Fig. 1c) which contains shoot meristems. Suckers are preferred because of their ease of handling and the minimum damage caused to the parent during their removal.

Culture medium

Murashige and Skoog medium were used in the present study as it is the most commonly used plant tissue culture medium (Murashige and Skoog, 1962).

Culture initiation

The sword suckers of 2-3 months were removed from healthy disease free mother plants for shoot tip culture. The suckers were cut to expose the shoot tip of $10cm^3$ and cut further to about 3cm diameter and 5cm length. The explant was carefully cut to avoid injury to the growing meristem. The shoot tips were washed in tap water and then, subjected to surface sterilization.

Comparing three surface sterilization procedures to control contamination

Surface sterilization with sodium hypochlorite solution

The shoot tips were kept in commercial sodium hypochlorite solution for 15 minutes. Then, two outer juvenile leaves and the corm base were trimmed out using a sterile scalpel. And then, washed in sterile distilled water for 5 minutes before inoculating on the surface of four sterilized MS mediums under aseptic conditions. Then, the cultures were incubated at room temperature, in natural light intensity and natural photoperiod inside the Plant Biotechnology Laboratory, CNS, AMU. Finally, the cultures were observed regularly for the appearance of contamination up to four weeks.

Surface sterilization with combination of commercial sodium hypochlorite solution and 0.1 % (w/v) mercuric chloride solution

Initially the shoot tip was kept in commercial sodium hypochlorite solution for 15 minutes and then, the outer surface of the explant exposed to NaOCl solution was trimmed out. And then, the shoot tip was kept in 0.1% $HgCl_2$ solution for 5 minutes. Using a sharp sterile blade, one or two outer juvenile leaves and the corm base were then trimmed out. The shoot meristem was thereafter washed in sterilized distilled water 5times before inoculating on the surface of our sterilized MS medium under aseptic conditions. Then, the cultures were incubated at room temperature, natural light intensity and natural photoperiod inside the Plant Biotechnology Laboratory, CNS, AMU. Finally, the cultures were observed regularly for the appearance of contamination up to four weeks.

Surface sterilization with 0.1 % (w/v) mercuric chloride solution

The shoot tips were kept in 0.1% (w/v) mercuric chloride solution for 5 minutes. Then, two outer juvenile leaves and the corm base were trimmed out using a sterile scalpel. And then, washed in sterile distilled water for 5 minutes before inoculating on the surface of four sterilized MS medium under aseptic conditions. Then, the cultures were incubated at room temperature, in natural light intensity and natural photoperiod inside the Plant Biotechnology Laboratory, CNS, AMU. Finally, the cultures were observed regularly for the appearance of contamination up to four weeks.

Effect of ascorbic acid in controlling lethal browning of shoot tip

An attempt was made to investigate the effect of different quantities of L-ascorbic acid on the lethal browning of plantlets. The concentrations tried were $0mgl^{-1}$, $10mgl^{-1}$ and $25mgl^{-1}$. Four replications were used for each L-ascorbic acid concentration. The cultures were incubated at room temperature, natural light intensity and natural photoperiod inside the Plant Biotechnology Laboratory, CNS, AMU. The cultures were observed regularly for lethal browning of explants up to four weeks.

Studying the effect of four different hormonal ratios on shoot tip proliferation

MS medium with various combinations of BAP and IAAwas used to identify the optimum hormonal ratio for shoot induction *in vitro*. The explants were surface sterilized only using NaOCl. Four replications were used for each hormonal ratio. The cultures were incubated at room temperature, natural light intensity and natural photoperiod inside the Plant Biotechnology Laboratory, CNS, AMU. The cultures were observed regularly for shoot induction up to four weeks.

Results and Discussion

Comparing three surface sterilization procedures to control contamination

In the present study, it has been noticed that all methods of surface sterilization are equally effective in controlling contamination. Results are presented in Table 2.

Based on the results of the first experiment, it has been found that sodium hypochlorite can be used for surface sterilization of shoot tips of bananas to prevent contamination. Sodium hypochlorite is the most commonly used disinfectant for surface sterilization of different types of explants (Zinabu *et al.,* 2018).

An increase in the NaOCl concentration can decrease in contamination and explants survival rate (Zinabu *et al.*, 2018). Similarly previous findings showed that explants contamination rate depends on several plants and environmental related factors such as species, age, explant source or size, level of aseptic work, prevailing weather condition and time of exposure to the disinfectants. Moreover, sodium hypochlorite produced the highest reduction in bacterial and fungal contamination at time intervals between 20-45 minutes (Zinabu *et al.,* 2018). Contamination in tissue cultures may be caused by endogenous bacteria that escape initial disinfection or by microorganisms introduced during tissue culture manipulations. Both types of contaminants may survive in the plant material for several subculture cycles and over extended periods of time without expressing symptoms in the tissue or visible signs in the medium. Plant cells growing *in vitro* are considered to be under some degree of stress and maybe predisposed to direct infection, even by bacteria not normally pathogenic to them (Bradbury, 1970). The medium may contain many different bacterial nutrients, both original constituents of the medium and exudates from the plant cells. Thus pathogens, endophytes, epiphytes and incidental contaminants may all occur and may interfere with growth of the plant tissue (Bradbury, 1988).

Based on the results of the second experiment, it has been also found that NaOCl+$HgCl_2$ can be used for surface sterilization of shoot tips of bananas to prevent contamination. This method was also effective in controlling contamination. The use of fungicide, NaOCl and $HgCl_2$ were effective to eradicate microorganism contamination on sprout explants of oil palm (Neliyati *et al.,* 2019). NaOCl and $HgCl_2$ were used in sterilizing Kinow tree explants (Altaf, 2006).

Based on the results of the third experiment, it has been also found that NaOCl+$HgCl_2$ can be used for surface sterilization of shoot tips of bananas to prevent contamination. For *in vitro* propagation of banana cv. Kanthali, huge numbers of explants die due to microbial contamination; it studies that contamination free cultures were established by $HgCl_2$ for 6 min followed by several washes in sterile water and removed the need to develop extensive and complicated surface sterilization protocol (Titov *et al.,* 2006). Also, *in vitro* propagation of banana also found the treatment of explant with $HgCl_2$ (0.1%) for 6 min most effective surface sterilization procedure registering maximum culture establishment with minimum contamination (Jaisy and Gahi, 2011).

Moreover, it is known that $HgCl_2$ is highly toxic and its use is not recommended generally. Besides, $HgCl_2$ can be difficult to remove from the surface of explants and this chemical can also inhibit plant cell growth. Due to high toxicity of $HgCl_2$, its concentration and the time of exposure of explants need to be optimized to decrease tissue mortality of the explants (Kataky and Handique, 2010). Prolonged treatment with $HgCl_2$ decreases contamination, but also brings about a reasonable decline in seed germination (Anolles *et al.,* 1990).

Effect of ascorbic acid in controlling lethal browning of shoot tip

The role of ascorbic acid in preventing the lethal browning of explants in the present study has been presented in Table 4.

When ascorbic acid was not added to the media, in all the four replicates, the explant changed into brown to black colour and the media also has changed to black colour. This occurs due to phenol oxidation by the explants which thereby, led to lethal browning. Such explants with lethal browning did not show any signs of growth.

When $10mgl^{-1}$ ascorbic acid was added to the media, there was no lethal browning of explants and media colour did not change. Such explants initially turned into green colour and started to proliferate. Ascorbic acid is an antioxidant that inhibits phenol exudation by the shoot tips of banana, thereby helps in preventing lethal browning. Using low concentration of ascorbic acid (0.0005%) applied directly on the surface of the media after autoclaving was able to reduce the number of diseased plantlets and increased the number of healthy plantlets than without ascorbic acid (Ko *et al.,* 2008).

Table.1 Hormonal ratios tried in the present study

Hormones	Ratio I	Ratio II	Ratio III	Ratio IV
BAB	4.00 mgl^{-1}	4.00 mgl^{-1}	8.00 mgl^{-1}	8.00 mgl^{-1}
IAA	0.00 mgl^{-1}	1.00 mgl^{-1}	2.00 mgl^{-1}	4.00 mgl^{-1}

Table.2 Influence of sterilization methods on preventing contamination

Method	% of survival rate	% of bottles with contamination
NaOCl	100.00	0.00
NaOCl+$HgCl_2$	100.00	0.00
$HgCl_2$	100.00	0.00

Table.3 Role of ascorbic acid in MS medium in preventing lethal browning of explants

Concentration of L-ascorbic acid (mgl^{-1})	Observation
0.00	Lethal browning & media turns black
10.00	No lethal browning & media colour does not change
25.00	Media does not solidify

Table.4 Effect of certain hormonal ratios on *in vitro* shoot tip culture of banana

Hormonal ratios	Number of bottles Showing shoot growth	Remarks
Ratio I (4.00 mgl^{-1}BAP; 0.00 mgl^{-1}IAA)	2 out of 4 (50% growth)	No colour change in media
Ratio II (4.00 mgl^{-1}BAP; 1.00 mgl^{-1}IAA)	0 out of 4 (0% growth)	Media slightly turned into brown; explants did not change the colour
Ratio III (8.00mgl^{-1}BAP; 2.00 mgl^{-1}IAA)	0 out of 4 (0% growth)	Lethal browning of explants
Ratio IV (8.00mgl^{-1}BAP; 4.00mgl^{-1}IAA)	0 out of 4 (0% growth)	Media turned into dark brown colour; lethal browning of explants

Fig.1a Fruit part of sample

Fig.1b Plant material

Fig.1c Sword suckers

When the concentration of ascorbic acid was raised to 25mgl^{-1}, the media did not solidify as ascorbic acid could have lowered the media pH. When the media is too acidic, it does not solidify.

Banana tissue cultures often suffer from excessive blackening caused by oxidation of polyphenolic compounds released from wounded tissues. These undesirable exudates form a barrier round the tissue, preventing nutrient uptake and hindering growth. Antioxidants such as ascorbic acid or citric acid in concentrations ranging from 10-150mgl^{-1} are added to the growth medium to reduce blackening, or the explants are dipped in antioxidant solution (cysteine 50mgl^{-1}) before their transfer to the culture medium (Jarret *et al.*, 1985).

Studying the effect of four different hormonal ratios on shoot tip proliferation

Among the four hormonal ratios tried, the ratio I i.e., 4.00 mgl^{-1}BAP has been found to induce shoot growth in 50% of the explants as given in Table 5. The other three hormonal ratios (i.e., ratio II, III and IV) failed to induce any growth of explants.

Similar results were obtained which state that MS Medium enriched with 4.00 mgl^{-1} showed maximum response as compared to other BAP concentrations assessed (Shiv *et al.*, 2014). Cytokine concentration has been several times reported to be decisive for shoot proliferation and elongation of many medicinal plant species (Rout and Jain, 2004). However, (Sipen and Davey, 2012) found on the contrary that BAP was effective when combined with IAA, for optimum shoot multiplication from shoot tips of the banana cultivars although the most effective BAP concentration for shoot multiplication differed among the banana cultivars.

Conclusion and Recommendation

Based on the results it was found that both sterilization methods can be used for surface sterilization of shoot tips of banana to prevent contamination. Using 10.00 mgl^{-1} ascorbic acid in the MS media is important since it will help not to produce lethal browning of explant and not form colour change of the media. Among the four hormonal ratios tried, the ratio i.e., 4.00 mgl^{-1} BPA has been found to induce shoot growth in 50% of the explants. Further studies on standardizing rooting of shooted explants should be carried out in the near future. Besides, studies on micropropagation of different varieties of banana using various hormones and hormonal ratios are necessary to develop an economical protocol for micropropagation of banana to produce quality planting material which can be supplied to local farmers in Arba Minch.

Author contributions

The author conducted practical work in laboratory, prepared the research article and approved the final manuscript.

Competing interests

The author has declared no conflict of interest.

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