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Alleviation of Tissue Browning During Clonal Propagation of Banana cv. Grand Naine

A. M. Hassanein, J. M. Salem*, B. A. El-Deep, and Z. E. Farghal

Central Laboratory of Genetic Engineering, Botany and Microbiology Department, Faculty of Science, Sohag University, Sohag 82524, Egypt.
**E-mail: gehan.salim1@science.sohag.edu.eg*

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Abstract: Banana micropropagation is commercially established by shoot tips obtained from intact plants on a shoot multiplication medium under optimal culture conditions. However, this is somewhat associated with tissue browning, which may be associated with reduced efficiency of the shoot multiplication process. It was found that changes in culture conditions such as increase or decrease of incubation temperature around 27 ºC, change in the texture of the media from the solid or liquid to semisolid state, addition of ascorbic acid, and substitution of optimal concentration of benzyl amino purine (5 mg/l BAP) with 2-isopentyl adenine (4 mg/l 2ip), could reduce tissue browning but decreased shoot multiplication and growth. The present results indicated that the chemical composition of the MS medium containing 5 mg/l BAP was ideal for banana shoot multiplication. Adding some natural ingredients rich in many important components (yeast extract, coconut endosperm milk, or moringa leaf extract) was unsuitable for in vitro multiplication of bananas. The observed exception was that the growth parameters of shoots formed on MS medium of 4 mg/l 2ip were better than those formed on MS of 5 mg/l BAP. In addition, shoot multiplication on 2-isopentenyl adenine (2ip) containing media showed root formation, adaptation, and transfer of plants to soil.
Keywords: Phenolic compounds, micropropagation, cytokinins, natural substances, Musa sp.

1. Introduction

Musa spp, are vegetatively propagated crops of major importance worldwide. Since bananas are a rich source of carbohydrates, vitamins, potassium, calcium, magnesium, and phosphorus, they provide healthy nutrition to people of all ages in all countries of the World [1]. In Egypt, bananas are widely cultivated, where 71901 faddans were planted by bananas according to Ministry of Agriculture data in 2014, and the Egyptian production of bananas reached 1283644 tons with an average of about 19.595 tons/faddan.

During the last 30 years, an increase in banana production in Egypt has been associated with micropropagation by tissue culture in different laboratories in universities, research institutions, and private sectors. The difficulties of vegetative propagation of many plants, including bananas are avoided using the tissue culture technique [2], where plants are faster-growing, uniform, and obtained in mass quantity in a short time and free from harmful pathogens [3]. Consequently, banana planters recommend seedlings obtained from tissue culture compared to suckers of previously cultivated plants [4]. On the other side, the cost of plants produced from tissue culture laboratories is still high for small banana farmers, so any attempt to reduce these expenses has a positive return for farmers and national production. Large-scale micropropagation commercialized plants production, but a somaclonal variation, hyperhydricity, tissue browning, and shoot necrosis in banana and other plants [5 - 7] should be avoided by changing some of the chemical and physical culture conditions [8, 9].

For banana *in vitro* multiplication, different cytokinins were applied, but benzyl amino purine (BAP) stimulated multiplication to a higher extent than kinetin (Kin), 2-isopentenyl adenine (2ip), and zeatin [10]. Regardless of the appearance of the tissue browning, the application of 5 mg/l BAP was recommended [11,12]. These growth regulators should be applied at optimal concentrations where their sub- or supra-optimal levels are associated with somaclonal variation [8]. In bananas, the optimum media texture was semisolid and the optimum temperature was 27°C, but they depended on genotype [11, 13].

The addition of natural compounds provides the tissue culture media with many components, some of which are not specified. Some of these additives may have a positive effect on *in vitro* plant multiplication and may have an opposite action. Coconut endosperm milk, malt extract, yeast extract, tomato or orange juice, and casein hydrolysate have been added to plant multiplication artificial media [14]. Coconut water supplements the medium with phytohormones such as cytokinines, indole-3-acetic acid [15], and gibberellin [16]. Moringa leaf extract has been applied as an exogenous plant growth enhancer, which contains cytokinin [17], ascorbates, carotenoids, phenols, potassium, and calcium [18]. Yeast extract was used in vitro due to its ability to stimulate phenolics biosynthesis [19].

Sometimes the base of explants and surrounding medium-colored brown due to the oxidation of phenolics by phenol oxidase leading to the release of quinone [20]. The emergence of the “tissue browning” phenomenon [21] may be due to the

application of specific physical or chemical culture conditions [22]. The beneficial effect of phenolic compounds on cell division and cell elongation during morphogenesis cannot be neglected [23, 24], where the antioxidant effects of phenolics act synergistically with growth regulators. Phenolic compounds protect plants from oxidative stress [25]. However, tissue browning was correlated with excessive accumulation of phenolic compounds leading to retardation of morphogenesis and ends with necrosis of cultured plant materials and diminishing the initiation of growth and development of plant materials [26, 27]. Consequently, any culture conditions, especially growth regulators and elicitors, that may act as stress agents should be avoided [28]. Changes in some culture conditions and the application of antioxidants in the culture medium may control the excessive appearance or prevent the oxidation of phenolic compounds [29]. Ascorbic acid or natural compounds as antioxidants were successfully used to inhibit the exudation of phenols [18, 30] and browning [5, 30].

Among the banana varieties, the cultivar Grande Naine has the acceptance the satisfaction of producers and consumers; it accounts for > 40% of the world's banana production [31]. Consequently, investigating the effect of several culture conditions that decrease tissue browning during multiplication of banana cv. Grand Naine using shoot tip explants was the aim of the present work.

2. Materials and methods

2.1. Plant material, explant preparation, and culture initiation

Field-grown banana (Grand Naine cultivar) suckers (about 50 cm high) were obtained from Agriculture Research Center at Sohag Governorate and used as a source of shoot tip explants. Successive overlapping leaf sheaths were removed, and four angled cuts were made to obtain shoot tip explants (5 x 1.5 cm cylinder). These explants were extensively surface sterilized in sodium hypochlorite solution (5.25%) for 20 min and in 0.2% (w/v) mercuric chloride ($HgCl_2$) for 5 min. Then, the explants were rinsed three times in sterile deionized water. The excessive tissues were removed to obtain shoot tip explants of approximately 10 x 7 mm, including the corm tissues. Each explant was cut one time vertically while keeping the rhizome (corm) base intact and cultured on a solid MS [32] medium supplemented with 5 mg/l of benzyl amino purine (BAP). Explants were cultured in glass jars and incubated at 25±2 ºC, 16 h photoperiod with 100 µmol $m^{-2}s^{-1}$ and 70% relative humidity for two months. They were aseptically transferred to fresh media three times at intervals of two weeks to prevent phenolic exudate accumulation around the cultured explants.

In all the applied treatments, six replicates with five explants (4-5 mm height each) were done. The number of shoots/explant, length of shoot (cm), the number of leaves/shoot, and fresh weight (g)/shoot cluster were determined for shoot tips cultured under the influence of any specific treatment for one month. In addition, the intensity of the brown color on the base of the explants and their surrounding media under the influence of any factor was compared to control plants (planted on semisolid MS medium containing 5 mg/l BAP and incubated for a month at 27°C).

2.2. Shoot proliferation and growth under the effect of isopentenyl adenine (2ip)

In vitro obtained shoot tip explants were cultured on semisolid (with 4 g/l agar) MS medium containing different concentrations of 2ip (1. 2, 3, 4, or 5 mg/l). Cultures were incubated for one month under the applied culture conditions.

2.3. Shoot proliferation and growth as influenced by temperatures

Shoot tip explants were obtained from *in vitro* grown shoots and cultured on solid (with 8 g/l agar) MS medium with 5 mg/l BAP. Cultures were incubated for one month at three temperatures; 24, 27, or 30 ºC.

2.4. Effect of medium solidification on shoot proliferation and growth

In vitro obtained shoot tip explants were cultured on liquid, semisolid, or solid MS media containing 5 mg/l BAP. Cultures were incubated at 27 ºC, 16 h photoperiod with 100 µmol m-2s-1 and 70% relative humidity (culture room conditions) for one month.

2.5. Shoot proliferation and growth under different natural substances treatments

In vitro, shoot tip explants were cultured on semisolid MS media containing 5 mg/l BAP and different concentrations of yeast extract (0, 1, 5, 10, or 20 g/l), coconut endosperm milk (0, 25, 50, 100, or 200 ml/l) or moringa leaf aqueous extract (0, 5, 10, 15 or 20 ml/l). Moringa leaf extract was prepared by grinding equal grams of leaves in equal volumes of deionized water, the extract was then centrifuged, and the supernatant was used to be added to the previous media. Different concentrations of coconut endosperm milk and those of moringa leaf extract were added to the media and then the media were autoclaved. Cultures were incubated for one month under established culture conditions.

2.6. Shoot proliferation and growth under the effect of ascorbic acid (ASA)

In vitro obtained shoot tip explants were cultured on semisolid MS media containing 5 mg/l BAP and different concentrations of ascorbic acid (0, 100, 200, or 300 mg/l) for one month.

2.7. Transfer of plantlets to the soil

Microshoots with extensive root systems obtained on semisolid half-strength MS medium supplemented with 1 mg/l IBA or 4 mg/l 2ip were washed to remove the remaining agar. They were transferred to plastic pots containing a mixture of peat moss and sand [2: 1 (v/v)]. For plantlet's acclimatization, the pots were covered by plastic bags (25 x 40 cm) to maintain high humidity around the newly transferred plantlets. For a gradual decrease of humidity, every five days, an opening was made in the plastic bag, allowing the smaller finger of the hand to pass through it for four weeks, and then the bag was removed after another five days.

2.8. Statistical analysis

Method modified by Snedecor and Cochran [33] was used to calculate the means ± standard deviation (SD). Analysis of variance (ANOVA) was accomplished using the software SPSS 16. The significance level was measured by running a Tukey test; $P < 0.05$ was considered significant.

3. Results and Discussion

3.1. Effects of cytokinin on proliferation, growth, and tissue browning of banana shoots tip explants

Shoot tip explants cultured on basal MS medium did not show any growth. The inclusion of growth regulators in the shoot multiplication medium (MS) was obligatory to induce shoot multiplication. The exogenous application of BAP was enough to induce shoot multiplication on shoot tip explants of banana, cv Grand Naine. Shoot tips cultured on semisolid MS medium gave the highest mean number of shoots per explant (8-10) when 5 mg/l BAP was used. To study the effect of another cytokinin, the MS medium was supplemented with different concentrations of 2ip. All the used concentrations of 2ip induced shoot multiplication, but the recorded values were lower than that of BAP. The number of obtained shoots varied for each 2ip treatment, but the medium with 4 mg/l of 2ip was the best one. Growth parameters including shoot length, number of leaves, and fresh weight of shoot cluster formed on MS with 4 mg/l 2ip were better than those formed on MS with 5 mg/l BAP. In addition, shoots multiplied on medium with 2ip formed an extensive root system (Figs. 1 and 2), a phenomenon that was never observed when the BAP was used to stimulate shoot multiplication. The values presented in Fig. 1 showed that shoot growth increased with increasing 2ip concentration until the optimal one (4 mg/l). Upon transfer of shoots from a multiplication medium containing 5 mg/l BAP to a new one with 4 mg/l 2ip, simultaneous improvement of shoot growth and formation of adventitious roots were detected, allowing a greater opportunity for the success of the adaptation and transfer of the produced seedlings to the open environment.

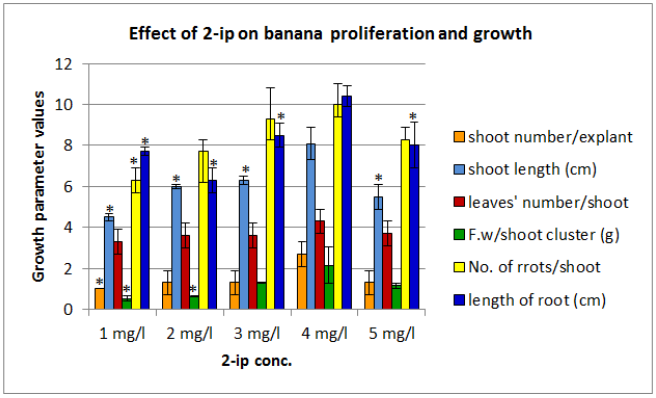

Fig. 1: Proliferation and growth of banana shoots on shoot tip explants cultured on semisolid MS medium supplemented with different 2-isopentenyl adenine (2iP) concentrations for one month. * indicates significant differences compared to medium supplemented with 4 mg/l 2-iP, at $P < 0.05$.

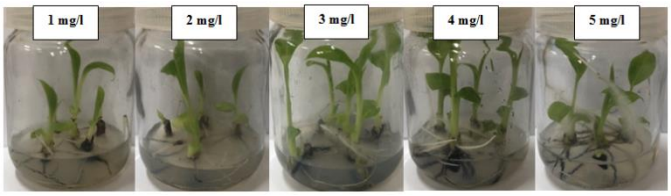

Fig. 2: Banana shoots derived from shoot tip explants on semisolid MS medium supplemented with different concentrations of 2-ip for one month.

3.2. Effect of temperature on proliferation, growth, and tissue browning of banana shoot tip explants

Effects of three temperatures (24, 27, or 30 ºC) on banana shoot multiplication were investigated (Fig. 3) when shoot tips were incubated on a solid MS medium supplemented with 5 mg/l BAP. Among the three temperatures tested, 27 ºC was the best for banana micropropagation. It was noted that raising the temperature from 24 ºC to 27 ºC increased the ability of the plant to proliferate and increased the capacity of the resulting shoots to grow. In comparison to plants grown at 27 ºC, tissue browning was reduced under the influence of low temperature, but it was increased when shoots were incubated to multiply at high temperature (30 ºC). While tissue browning disappeared under low temperatures, it was associated with a reduction in shoot multiplication and growth.

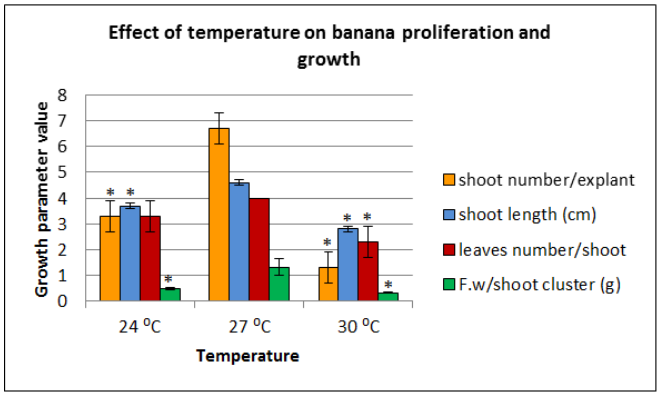

Fig. 3: Proliferation and growth of banana shoots formed on shoot tip explants cultured on MS medium supplemented with 5 mg/l BAP for one month under the influence of three different temperature degrees. * indicates significant differences compared to 27 ºC, at $P < 0.05$.

3.3. Effect of gelling agent on proliferation, growth, and tissue browning of banana shoots

Shoot multiplication was detected when solidified MS medium with 8 g/l agar (0.8%) and 5 mg/l BAP was used. The fresh weight of the shoot cluster and shoot length were observed to improve with a reduction in the agar concentration of the shoot multiplication medium from 0.8 to 0.4%. On the other side, shoot multiplication disappeared when a liquid medium was used (Figs. 4 and 5). Also, liquid media did not induce the growth of cultivated explants, and it expressed the lowest value of shoot formation frequency (92%) in comparison to solid and semisolid ones (100%). Browning was decreased by reducing agar concentrations in a semisolid medium, and it completely disappeared when banana shoots were cultured in liquid media. Under liquid culture conditions,

the disappearance of browning was associated with the disappearance of the ability of shoots to multiply and grow.

3.4. Effect of added natural compounds on proliferation, growth, and tissue browning of banana shoots

When yeast extract was used in low or high concentrations, shoot multiplication and shoot growth decreased significantly (Fig. 6A). Under the influence of a low concentration of yeast extract (1 g/l), while the frequency of shoot formation was not influenced, the number of leaves was non-significantly reduced. Adding the yeast extract at high concentrations (5-20 g/l) resulted in an increase in the tissue browning not only around the cultivated plant but also throughout the media within the culture jars, noting that the intensity of the tissue browning increased with increasing the concentration of yeast extract in media (Fig. 7A). In association with the tissue browning due to the application of relatively high concentrations of yeast extract (10-20 g/l), several abnormalities were recorded, where shoot multiplication, shoot growth and leaf formation were retarded.

The effect of coconut endosperm milk and moringa leaf extract on shoot proliferation of banana shoot tips was investigated. The obtained results indicated that as the concentration of both substances increased, the number of formed shoots and their growth decreased (Figs. 6B and C, respectively). On the other side, the application of both substances reduced tissue browning (Figs. 7B and C). While the growth of shoots was strongly decreased when yeast extract was applied in any concentration, while it was only reduced when a higher concentration of coconut milk or Moringa plant extract was used. Generally, the frequency of shoot multiplication remained relatively high upon coconut endosperm milk or moringa leaf extract application. Banana shoot multiplication on semisolid MS media containing 5 mg/l BAP (9.3 ± 0.6 shoots/shoot tip) was higher than that on semisolid MS medium containing 5 mg/l BAP and 10 ml/l moringa leaf extract (3.7 ± 0.6 shoots/shoot tip) or 10 ml/l coconut endosperm milk (2.7 ± 0.6 shoots/shoot tip).

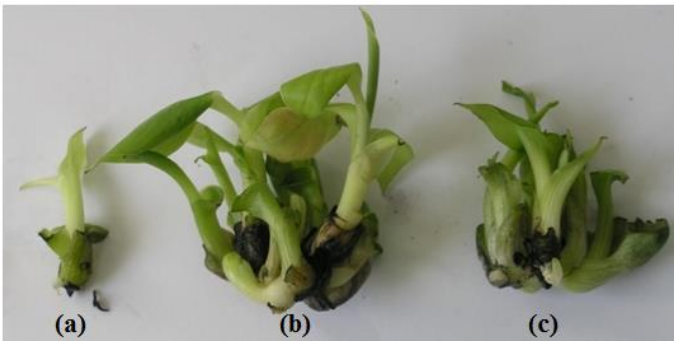

Fig. 5: Banana shoots derived from shoot tip explants after one month on MS medium supplemented with 5 mg/l BAP in different textures: (a) liquid; (b) semisolid; and (c) solid.

3.5. Effect of ascorbic acid on proliferation, growth, and tissue browning of banana shoots

Shoot multiplication medium supplemented with 5 mg/l BAP stimulated shoot formation and shoot growth, but the base of explants and their surrounding became brown. Shoots that formed on the cultured explant were exited from their bases, where the brown exudates were accumulated over time. Different concentrations of ascorbic acid were used to control the accumulation of brown exudates on the surface of cultured shoots and in the surrounding area (Fig. 8). Tissue browning decreased with an increase in the concentration of ascorbic acid (ASA) in the media, but this was not accompanied by an increase in the shoot multiplication (Fig. 9). On the contrary, the number of shoots and their growth decreased significantly when the medium was fortified with ASA for shoot multiplication. Application of 200 mg/l ASA in the shoot multiplication medium resulted in the formation of a valuable number of healthy shoots (4 ± 1 shoots/shoot tip) and reduced tissue browning.

3.4. Root formation and acclimatization

The proliferated shoots were subjected to root induction on a semisolid half-strength MS medium containing 1 mg/l IBA for one month. Shoots subjected to 1 mg/l IBA showed an extensive root system (Fig. 10). Also, extensive root systems were formed on shoots multiplicated on a semisolid medium containing 2ip. They were transferred for acclimatization in plastic pots containing peat moss and sand (2:1 v/v). All the transferred shoots continued growth in normal phenotype (**Fig. 11**).

4. Discussion

Natural exudates in tissue culture have been used to avoid any circumstances that prevent achieving the desired results [34]. Different trials including the addition of antioxidants, dark incubation of culture, and shortened subculture period, were investigated to avoid the toxic effect of excreted phenolic compounds on the shoot multiplication of bananas or other species [35].

While banana shoot multiplication depended on the type and concentration of cytokinin as well as genotype [36], 5 mg/l BAP was the most efficient concentration for shoot multiplication of many banana cultivars, including cv. Grand Naine [11]. To avoid oxidative browning during banana shoot tip proliferation, different culture conditions were investigated. Application of 5 mg/l BAP as optimal concentration for true-to-type clonal fidelity was associated with the accumulation of oxidative browning exudates on multiplied shoots. In addition, sub- and supra-optimal levels of BAP were avoided to avoid low multiplication and somaclonal variation, respectively [8]. Waman et al. [12] reported that BAP was found to be the most conventional cytokinin for shoot multiplication in bananas. When another cytokinin (2ip) was used for shoot tip multiplication of the Grand Naine cultivar, browning disappeared. All the used concentrations of 2ip induced shoot multiplication; 4 mg/l 2ip was optimal one. The number of shoots on MS medium with 4 mg/l 2ip was lower than that with the optimal concentration of BAP (5 mg/l). When the optimum concentration of 2ip was applied, the growth of the formed shoots was better than the growth of shoots at the ideal concentration of BAP. In addition, the use of 2ip also outperformed the use of BAP, where better growth of shoots

was accompanied by the formation of an extensive root system without the need to transfer shoots from the shoot multiplication medium to the root formation medium.

Browning on the base of multiplied banana (cv. Grand Naine) shoots was expected as banana tissue of all cultivars contains large amounts of latex and phenolic compounds [37] but it depends on culture conditions. On MS medium supplemented with 5 mg/l BAP, cultured banana showed darkening of the medium around the cultured multiplied plant materials due to exudation and oxidation of phenolic compounds leading to the formation of quinones [38]. These oxidation products exerted highly reactive and toxic effects on cultured plant tissue [38]. In bananas and other plant species, tissue browning of *in vitro* cultured shoots was positively correlated with phenolic content [39]. Accumulation of oxidative browning exudates may lead to sealing up and prevent leaching of phenolics from the base of explants [35] and diminish nutrient supply from medium to cultured explants and this may explain the decrease in growth in the present investigation. Shoot growth was improved when BAP was substituted with 2ip as cytokinin content of the shoot multiplication medium. In other reports, phenols improved root formation in certain shoot cultures, which protect IAA from oxidative decarboxylation [40] and stimulated embryo differentiation under absolutely black tissue conditions [41].

While the optimal temperature for adventitious root formation was 25 ± 2°C, the optimal temperatures for shoot or callus formation ranged between 21 and 30 °C [42, 43]. Changes in membrane fluidity, protein folding, protein binding, and enzyme kinetics were positive when the incubation temperature of the banana shoot tips was raised from 24 to 27, and this led to increased shoot tip multiplication and growth. Under optimal temperature, stem elongation may be due to an increase of endogenous GA_3 [44]. On the other hand, no correlation between growth and endogenous GA_3 levels was reported by [45] and [46]. In the current study, elevated temperature up to 30 ºC retarded shoot proliferation and growth. Relatively extreme temperatures diminished growth due to increased ethylene evolution [47] and abscisic acid biosynthesis [48]. These responses were tightly regulated by complex signaling pathways [49, 50].

Reduction of Grand Naine shoot multiplication and growth under the influence of relatively high temperature may be due to reducing the efficacy of cytokinins, even if this enhanced the activity of auxins [51]. Consequently, the requirement for cytokinin increased with increased temperature [52]. The Grand Naine shoot tips also suffered from heat stress when incubated for multiplication at 30 ºC leading to the production of dark phenolic substances. Increasing the intensity of browning due to an increase in incubation temperature reduced the ability of Grand Naine shoot tips to multiply and grow because it limited the efficiency of nutrient transport from the medium to the cultured plant materials. Production of phenolics is a natural process in higher plants; their accumulation during *in vitro* culture conditions adversely affected the growth and survival of explants [53, 54].

Efficient shoot multiplication on shoot tips of the Grand Naine cultivar was detected on semisolid MS medium [4 g/l agar (0.4%)] containing 5 mg/l BAP. Under these conditions, milder browning on shoot bases was detected. The development of an intermediate browning does not seem harmful for *in vitro* cultured plant tissues, but deterioration or retardation of cultured plant materials was detected during dark tissue browning [23]. Consequently, semisolid medium with low concentrations of agar facilitated adequate diffusion of medium constituents to cultured plant tissue resulting in better differentiation and growth in bananas and other plants [55, 46]. On the other side, decreased shoot multiplication and growth caused by higher agar concentration in the medium was accompanied by increased tissue browning. High gel strength was accompanied by reduced water availability from the medium to the cultured plant materials [56- 58]. The resulting stress increased exudative browning exudates that magnified the unavailability of water and nutrients to the cultured plant materials leading to reduce proliferation and growth.

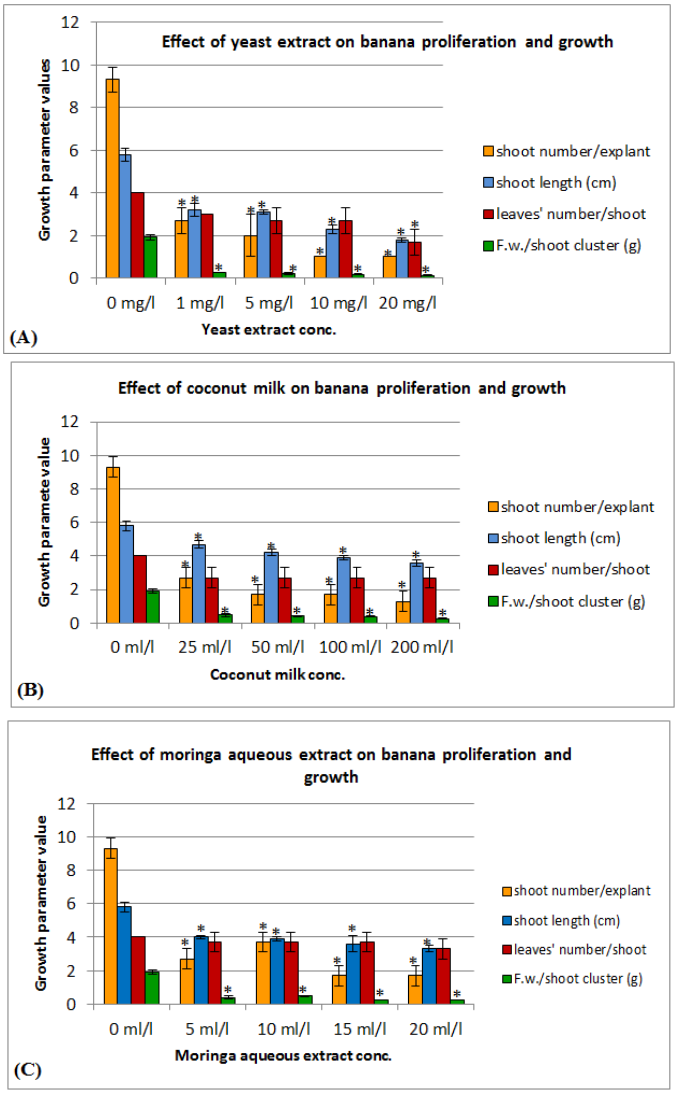

Fig. 6: Proliferation and growth of banana shoots on shoot tip explants cultured on semisolid MS medium supplemented with 5 mg/l BAP without or with different concentrations of (A) yeast extract, (B) coconut milk, or (C) Moringa extract for one month. * indicates significant differences compared to the control, at $P < 0.05$.

The presence of yeast extract in the shoot multiplication medium reduced shoot proliferation and growth in bananas, but the effect of yeast extract on plant growth varies according to the used concentration. Yeast extract was used as a nutrient [59 - 61] and an abiotic elicitor to enhance secondary metabolite production [19]. Our results support the assumption of [19], which stated that yeast's adjective as a nutrient was excluded where its use did not increase shoot growth but rather increased abnormalities. These effects resulted from stress due to the presence of yeast extract in the media leading to the accumulation of phenolic compounds [62]. Consequently, browning around the cultured banana shoot tips increased with increased yeast extract concentration in the media. As was detected in this work, media supplemented with 5 g/l yeast extract or more acted as elicitors for phenolics production and retard growth. This also indicated that the production of secondary metabolites by cultured banana shoot tips may act as an antioxidant defense action to control the stress caused by the presence of yeast extract in the media [19]. Accumulation of toxic substances associated with tissue browning was not the main reason for tissue death, where amendment of the plant culture medium with adsorptive materials, such as activated charcoal and polyvinylpyrrolidone, did not alleviate the problem [63].

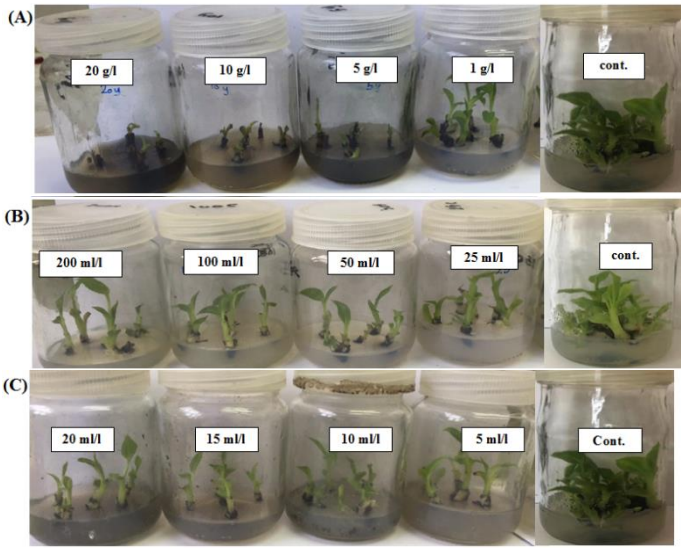

Fig. 7: Banana shoots derived from shoot tip explants after one month on semisolid MS medium supplemented with 5 mg/l BAP and different concentrations of natural substances: (A) yeast extract; (B) coconut milk and (C) moringa aqueous solution.

The effect of coconut endosperm milk and BAP in the shoot multiplication medium was evaluated, but no synergistic effect was detected. In contrast to our result, coconut endosperm milk resulted in increased shoot numbers and lengths in other plants [64, 65]. While moringa leaf extracts increased total phenolic, flavonoid, and tannins [66], no tissue browning was observed when banana shoot tips were cultured on the MS medium with BAP and moringa leaf extract. This means that moringa leaf extracts significantly increased antioxidant activities due to an increase in some antioxidant components such as carotenoids and total phenolic [66]. These components might control phenolics oxidation and prevent the accumulation of tissue browning. In comparison to a semisolid MS medium containing 5 mg/l BAP, the same medium supplemented with different concentrations of moringa leaf extract decreased the number of shoots/explant. The retardation effect of moringa leaf explants was detected by Amer et al. [67]. Antioxidants such as ascorbic acid were detected at high concentrations in moringa chloroplasts and other cellular components [68]. In addition, moringa leaf extract has plant growth promoters, mineral nutrients, and vitamins that promote plant growth, leading to increased plant yield [69]. The results of this study indicated that the chemical composition of the MS medium containing 5 mg/l BAP was the ideal composition. The addition of any natural ingredients, even if they were rich in many important components, made the media less suitable for *in vitro* multiplication of bananas.

In the current study, browning was reduced by the application of ASA and decreased with the increase of ASA concentration in the medium. At 200 mg/l ASA, mild browning was detected, and a valuable number of healthy shoots were formed. In *Brahylaena huillensis*, the production of phenolic compounds by explants was significantly controlled by ASA, and the best control was achieved by the application of 200 - 250 mg/l ASA in a medium containing BAP [30]. Oxidative browning of explant tissue was reduced through detoxifying of free radicals by ascorbic acid [30] or inhibiting of oxidation process [70]. In another banana cv Cavendish, ASA not only reduced death due to the browning of explants but also increased the number of shoots/explants [5].

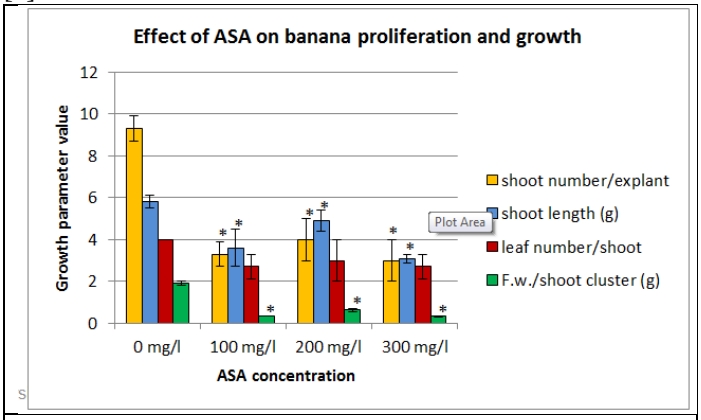

Fig. 8: Proliferation and growth of banana shoots on shoot tip cultured for one month on semisolid MS medium supplemented with 5 mg/l BAP and different concentrations of ASA. * indicates significant differences compared to control, at $P < 0.05$

Regarding root formation and acclimatization, 2ip was better than BAP, as 2ip gave stronger plantlets that coincided with the formation of a root system. Consequently, using 2ip for banana multiplication saved effort, money, and the need to transfer plants from the multiplication medium to the root formation medium. Contrary to these results, in healthy plantlets with extensive root systems, shoot multiplication was carried out on a medium containing auxins in combination with cytokinins [71]. Shoots were successfully rooted on media containing 2iP (1 mg/l) in combination with naphthaleneacetic acid (0.5 mg/l) and casein hydrolysate or $AgNO_3$ [72, 73]. In the current study, acclimatized plants did not show any

morphological abnormalities when they were grown in the field. It's worth mentioning that banana survival was detected even without root induction before the acclimatization stage [74].

Healthy shoots with extensive root systems are essential prerequisites for the transfer of micro-propagated plantlets from *in vitro* to *ex-vitro* culture conditions. The growth of banana plants obtained on media containing 4 mg/l 2ip was better than the others obtained on other media containing 5 mg/l BAP. In addition, the healthy growth of multiplied banana shoots on 2ip-containing media was accompanied by the formation of strong root systems. Lakshmanan et al. [75] reported that 5 mg/l BAP was efficient for banana shoots multiplication but obtained bud clumps were very delicate upon repeated subcultures for further multiplication. Consequently, different sources of cytokinins were investigated to obtain more healthy shoots for successful rooting and acclimatization [12]. The obtained results confirmed that BAP-containing media are suitable for banana *in vitro* multiplication, as the resulting shoots were suitable for multiplication for no more than seven subcultures. This agreed with the work of Hassanein et al. [9]. After any subculture, the transfer of the resulting plants from the shoot multiplication medium to the root formation medium resulted in the formation of a strong root system that paved the way for the acclimatization and transfer of plants to the field.

Fig. 9: Banana shoots derived from shoot tips explant after one month culture on semisolid MS with 5 mg/l BAP and 200 mg/l ASA.

Fig. 10: Banana plantlet with root system formed after one month on semisolid half-strength MS medium with 1 mg/l BAP and ready for acclimatization.

Fig. 10: Banana with well-developed roots transplanted in soil containing peat and sand (2: 1 v/v) for four weeks.

5. Conclusion

For the best multiplication of banana (cv. Grand Naine), semisolid MS medium supplemented with 5 mg/l BAP and incubation of cultures at 27 °C should be achieved. The inclusion of some natural nutrients such as yeast extract, coconut endosperm milk, or moringa leaf extract induced the execration of phenolic compounds that were photo-oxidized causing tissue browning and darkness of multiplication medium which consequently decreased banana shoot multiplication. The addition of ASA and substitution of optimal concentration of BAP (5 mg/l) with 2-ip (4 mg/l), reduced tissue browning and decreased shoot multiplication and growth of banana.

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