THE EFFECT OF SOME ANTIOXIDANTS ON BLACKENING AND GROWTH OF *In Vitro* CULTURE OF BANANA (*Musa* spp.cv. GRAND NAINE)

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Bananas (*Musa* spp.) are one of the most valued fruit products. It belongs to the family *Musaceae* and section *Eumusa* (Dayarani *et al.*, 2013). It signifies variable benefits, both as a staple food as well as a major export commodity for many tropical and subtropical countries (Rahman *et al*., 2013). In general, banana cultivars are considered as good sources of carbohydrates, proteins, vitamins and minerals (Anbazhagan *et al*., 2014). Bananas are generally propagated vegetatively through suckers. Unfortunately, the traditional expansion methods limited the expansion of bananas production, due to a shortage of healthy plant material availability to farmers. The limitation is a result of the transmission of harmful insects, nematodes and viral diseases to field-grown suckers (Huq *et al*., 2012).

To overcome these issues and enable rapid multiplication of economically important commercial varieties, *in vitro* propagation is a preferred alternative method (Huq *et al*., 2012). Shoot tip culturing for bananas, provides second advantages that coincide with the farmers demands including, increased multiplication rate, physiological uniformity and the availability of disease-free materials all year round (Onuoha *et al*., 2011). The principle phenolic constituents in *Musa* spp. are dopamine, catechin, chlorogenic acid, cinnamic acid, hydroxyl benzoic, resorcinol, progallic acid, salicyclic acid, ferulic acid, vanillin coumurin, P-coumaric acid and phenol (Khalil *et al*., 2007). These constituents are oxidized during tissue damage to prevent the invasion of pathogens (Chikezie, 2012).

One of the pitfalls of *in vitro* culturing is that it reduces the uptake of nutrients (Chikezie, 2012). In the 3rd generation of tissue culture, phenolic compounds are also responsible for a high mortality rate (lethal blackening) which is initiated by the blackening of the surface of the plant tissues, resulting in the formation of quinones that are highly reactive and toxic to plant tissues (Titov *et al*., 2006). Different attempts have been made to alleviate this problem, including pretreatment of explants with antioxidants, incorporation of antioxidants into the culture medium, incubation of cultures in the dark and frequent subculture to fresh medium (Ahmad *et al*., 2013).

The antioxidant ascorbic acid, was selected as it has been successfully known

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to inhibit the exudation of phenols (Strosse *et al*., 2004) and to reduce the oxidative blackening in various plant species (Abdelwahd *et al*., 2008). Activated charcoal has effect on the morphogenesis by the irreversible adsorption of inhibitory compounds in the culture medium thus, substantially decreasing the toxic metabolites, phenolic exudation and accumulation of brown exudates (Thomas, 2008). Therefore, antioxidant growth regulators are considered as one of the most important factors in the development of a standard tissue culture protocol (Dayarani *et al*., 2013).

The objective of this study is to optimize rapid multiplication and rooting protocol for banana (*Musa* spp. cv. Grand Naine) from meristematic tips, using a medium supplemented with different treatments of ascorbic acid (150 mg/L), citric acid (150 mg/L) and activated charcoal (g/L), for the purpose of resolving the blackening phenomena. In addition, to examine the residual effect of the studied antioxidant compounds by the acclimatization of successful plantlets.

MATERIALS AND METHODS

The present study assesses the effect of some antioxidant compounds in inhibiting the blackening phenomena *in vitro* that occurs in banana (*Musa* spp. cv. Grand Naine). Experiments took place at the Biotechnology Lab., Central Laboratory for Date Palm, Agricultural Research Center, Giza, Egypt.

1. Explants material and preparation

The explant of choice for this study was the meristematic tips (suckers) of plantain plants. All biological materials were obtained by an agriculture development system project. The preparation of the sucker was accomplished through excision of the superfluous tissues, outer leaf sheaths, bases and corm. Approximately, 5-7 cm cube shaped shoot apexes were obtained. Upon retrieving the proper explant, a washing step occurred approximately for 1 hour under running tap water. Assurance of disinfection was carried out using a solution comprising of soaking for 30 min in commercial bleach (5.25% NaOCL) diluted to 30% (v/v) with two drops of Tween 20 per 100 ml. Since the prerequisite for any tissue culturing procedure are extreme sterile conditions, all procedures were aseptically carried out within a horizontal laminar flow hood. Autoclaved distilled water was used for subsequent washes (this step was repeated three times). The aseptic Murashige and Skoog (1962) MS culture media was used for multiplication. The media was supplemented with sucrose (20 g/L), vitamins glycine (2 mg/L), pyridoxin (0.5 mg/L), Nicotonic acid (0.5 mg/L), Thiamine HCL (0.1 mg/L) and Myoinositol (0.1 mg/L). The medium was also supplemented with 5 mg/L of benzylaminopurine (BAP).

Referring to the media in both the multiplication and rooting stages, the pH of medium was adjusted to 5.8 and 0.7% agar was added prior to autoclaving. Autoclaving was carried out at 121°C and 15

psi for 20 min. Ascorbic acid, citric acid and activated charcoal treatments were added after autoclaving, aseptically within the horizontal laminar flow hood. After inoculations, the cultures were maintained at a temperature of 25±2°C with a photoperiod of 16hrs per day. Lighting was supplied using fluorescent lamps with 1000 lux for multiplication stage and 3000 lux for rooting stage. The established cultures on shoot induction medium were routinely transferred every 3-4weeks.

2. Multiplication Stage

Small cultures of shoots 2-3 shoots, 0.3-0.5 cm in length were cultured on a multiplication medium supplemented with varying concentrations of ascorbic acid, citric acid and activated charcoal (Table 1). The assessment of the efficacy of the antioxidant treatments during the multiplication stage was conducted through recording the blackening degree, number and length of shoots after two subcultures.

3. Rooting Stage

Explants for rooting stage were *in vitro* propagated plantlets with shoot length of approximately 6.5 cm in length, 1-2 roots approximately 1.5 cm in length, without any developments of secondary roots. Shootlets were cultured on rooting medium comprising identical components to the multiplication medium, with the exception of supplementing 1.0 mg/L Naphthaleneacetic acid (NAA) instead of 5.0 mg/L BAP. The assessment of the efficacy of the antioxidant treatments during the rooting stage was conducted through recording the blackening degree, shoots length and the number and length of roots after two subcultures.

The blackening degree in both multiplication and rooting stages was scored visually according to (Pottino, 1981) as follows: 1- (-) Negative result, 2- (+) Below average result, 3- (++) Average result, 4- (+++) Good result, 5- (++++) Very good result.

4. Acclimatization Stage

Developed plantlets approximately (7 cm in length) received from the rooting stage were transformed to the greenhouse for proper hardening over a 4 week period. The adaptation and acclimatization occurred by placing the plantlets into plastic pots containing a 2:1 ratio of garden soil and compost with adequate hydration. Assessments of the residual effect of the antioxidant treatments on the growth during the acclimatization stage, was achieved by recording the shoot length as well as root number and length.

5. Statistical Analysis

The experiments were carried out using completely randomized design (Snedecor and Cochran, 1980). Treatments included of three replicates, each within a jar, and each jar contained 1 explant. The results were analyzed using analysis of variance (ANOVA) and the means were compared using least significant difference (LSD) at the 5% confidence interval ($p \leq 0.05$).

RESULTS

Browning phenomena is one of the most common problems associated with *in vitro* establishments of plantain micropropagation. In the present investigation, the effect of some antioxidants such as ascorbic acid (150 mg/L), citric acid (150 mg/L) and activated charcoal (1.5 g/L) on blackening and growth of banana (*Musa* spp. Grand Naine) were studied.

1. The effect of antioxidant treatments on the multiplication stage

The data demonstrated that when the multiplication medium was free from the studied antioxidants, the highest significant result of blackening was recorded (2.67) as in the control medium (Table 2). Generally, it was revealed that the blackening phenomena was inhibited with concentrations of (150 mg/L ascorbic acid + 1.5 g/L activated charcoal), (150 mg/L citric acid + 1.5 g/L activated charcoal) and (150 mg/L ascorbic acid + 150 mg/L citric acid + 1.5 g/L activated charcoal), were added to the medium, whether individually or in combination with each other, without significant differences between them (Fig. 1).

When examining the effect of antioxidant treatments on shoots, the data (Table 2), indicated that the different concentrations of antioxidants increase the number of shoots/explant. However, the number of the increased shoots varied according to the different treatments used. Among all the treatments used, the maximum number of shoots/explant were obtained by supplementing the medium with (150 mg/L ascorbic acid + 1.5 g/L activated charcoal), (5.00) and (150 mg/L ascorbic acid + 150 mg/L citric acid + 1.5 g/L activated charcoal), (5.00) (Table 2). When examining the effect of antioxidants on shoots, it was demonstrated that the addition of both (1.5 g/L activated charcoal + 150 mg/L ascorbic acid), significantly improved the shootlets length/explant and recording the highest significant result (1.50) (Fig. 2 and Table 2).

2. The effect of some antioxidant treatments on rooting stage

The highest appearance of blackening (1.33) during rooting stage was clear in control medium, due to the absence of antioxidants (Table 2 and Fig. 3). Adding ascorbic acid (150 mg/L), citric acid (150 mg/L), or activated charcoal (1.5 g/L) treatments to rooting medium, impressively decreased the formation of blackening, compared to the control medium (without significant differences among them). Moreover, treatments of combined antioxidants were successfully effective in inhibiting the development of blackening phenomena.

Among the different treatments used, Table (3) showed that citric acid (150 mg/L) and activated charcoal (1.5 g/L) gave the highest shootlets (12.33 cm in length) and root number/explant (8.00), respectively (Fig. 4). The addition of ascorbic acid (150 mg/L) and activated charcoal (1.5 g/L) treatments to the root-

ing medium, showed the highest significant result in roots (5.67 cm in length) and secondary roots/explant (10.67), respectively (Table 3). However, no secondary roots development were observed in the medium supplemented singly with 150 mg/L citric acid, 150 mg/L ascorbic acid or in the control medium (Fig. 5).

3. The effect of some antioxidant treatments on acclimatization stage

The effect of the treatments on the received rooting explants was studied during the acclimatization stage at the greenhouse. Table (4) indicates that when the rooting explants were treated using the treatments of 150 mg/L citric acid + 1.5 g/L activated charcoal during the rooting stage, the highest shootlets (13 cm in length) were witnessed. Plantlets received from these treatment posses a healthy morphology (Fig. 6).

Table (4) indicates that the root number/explant were very high (8.00) in the explants treated with 150 mg/L ascorbic acid + 150 mg/L citric acid + 1.5 g/L activated charcoal. As for the root length, the residual effect of ascorbic acid (150 mg/L) + activated charcoal (1.5 g/L) treatment seems to be very effective in the elongation of the roots (6.333 cm in length). Clearly, rooting explants from control treatment showed the lowest results in shootlets length/explant, root number/explant and root length/explant during the acclimatization stage.

DISCUSSION

Phenolic secretions and other exudates in plants tissue culture systems lessen the efficiency of explant initiation, growth and development (Kerns and Meyer, 1986). One of the major problems for several tissue culture system, is the lethal blackening which result in death of the cultured explants that depend on the rate of oxidation of phenolic compounds, as well as the quality of the total phenols (Ozyigit, 2008).

Phenolic compounds are secondary metabolites that are released from injured explants and are usually present in high amounts in response. The blackening phenomenon took place in response to oxidation process of released phenolic compounds from injured tissue by phenol oxidase and formation of quinones (Kefeli *et al*., 2003). Quinones negatively inhibit cell growth and can often result in death of cells (necrosis) (Ozyigit, 2008). Therefore, preconditioning of explants with media supplements such as, ascorbic acid (Ndakidemi *et al*., 2014), citric acid (Morfeine, 2013) and activated charcoal (Thomas, 2008), was necessary to limit the production of these harmful substances.

In the current investigation, a suitable and efficient treatment method to minimize and control the lethal blackening for banana (*Musa* spp. cv. Grand Naine) cultured on propagation medium was developed. The study established that the best results for controlling lethal browning were obtained when explants were cul-

tured on MS medium supplemented with activated charcoal (1.5 g/L) while adding ascorbic acid (150 mg/L) or citric acid (150 mg/L) or combination of both (Fig. 6).

Thomas (2008) reported that activated charcoal have a very fine network of pores with large inner surface areas, through which the absorbance of substances can occur. It is often used in tissue culture, to improve cell growth and development. Activated charcoal, plays a critical role in micropropagation, orchid seed germination, somatic embryogenesis, anther culture, synthetic seed production, protoplast culture, rooting, stem elongation, bulb formation etc... The positive effects of activated charcoal on morphogenesis might be, due to its irreversible adsorption of inhibitory compounds in the culture medium. As a result, it substantially decreases the toxic metabolites, phenolic exudation and brown exudates accumulation.

According to Kariyana & Nisyawati (2013) different concentrations of ascorbic acid (50 $mg.l^{-1}$, 100 $mg.l^{-1}$, 200 $mg.l^{-1}$) as well as activated charcoal (0.5 g/L, 1 g/L,2 g/L) successfully reduced explant blackening. Dibax *et al*. (2005) reported that, in addition to suppressing phenolics and subsequently blackening phenomena, the supplementation of activated charcoal to the culture media enhanced the elongation of explant, which is viably demonstrated in the present study. According to Table (2), the incorporation of 150 mg/L ascorbic acid + 1.5 g/L activated charcoal, inhibited blackening, caused the development of more shoots and enhanced the elongation of the explant.

A study done by Morfeine (2013) proved that when cysteine, citric acid and ascorbic acid were added to the MS media, blackening of medium was prevented and reduced. Moreover, Titov *et al*. (2006) illustrated the use of citric acid and ascorbic acid combinations in delaying the blackening phenomena. This results exclusively in agreement with the study conducted by Ndakidem *et al*. (2014), which found that the supplementation of ascorbic acid in basal woody plants medium significantly controlled the production of phenolic compounds of *Brahylaena huillensis* explants. The ascorbic acid is able to scavenge oxygen radicals produced when the plant tissue is wounded and hence protects the cells from oxidative injury. These results in agreement with with this study, which found that the supplementation of 150 mg/L ascorbic acid + 150 mg/L citric acid during the multiplication stage, reduced the blackening phenomena (Table 2). Whereas, the blackening phenomena with the same combinations and concentrations, was inhibited completely during the rooting stage (Table 3).

Thus, as observed in this study, the application of antioxidants supplemented in growth medium supported the multiplication and rooting stages. The further growth of acclimatization stage in greenhouse, as observed in the current investi-

gation and the residual effects of the stated antioxidant treatments, enhanced the growth even in the acclimatization. This statement coordinates with the study by Ahmad *et al*. (2013) which reported that the application of antioxidants is highly significant for different stages of organogenesis and high root formation for plant tissue culturing techniques.

CONCLUSION

Plantain explants are susceptible to tissue blackening, elimination or minimization of this process is an essential prerequisite to a successful culture establishment. Therefore, identification of a suitable treatment to minimize tissue blackening in the explants with particular emphasis on the use of antioxidants was the main objective of this study. The results concluded that using activated charcoal (1.5 g/L) in particular, either with ascorbic acid (150 mg/L) or citric acid (150 mg/L) or all of them combined, limited these lethal and costly phenomena. These antioxidants have demonstrated an overall improvement in growth, by obtaining healthy plantlets which could provide a high production in the propagation process. According to the literature, previous studies have demonstrated that using antioxidants can indeed eliminate the blackening phenomena in plants. However, the novelty presented in this study is shown in the choice of specific antioxidant treatments which have not been previously used.

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SUMMARY

The *in vitro* propagation of bananas plantain (*Musa* spp. cv. Grand Naine) is still faced with lots of challenges such as the blackening of tissues, due to the oxidation of phenolic compound by polyphenolic oxidase enzyme present in the tissue when excised. The objective of this study is to assess the inhibition of blackening phenomena efficacy using some antioxidant treatments specifically, ascorbic acid (150 mg/L), citric acid (150 mg/L) and activated charcoal (1.5 g/L). A secondary objective is to investigate the acclimatization of the explants *in vivo*. Results demonstrated that using activated charcoal (1.5 g/L) in particular, either with

ascorbic acid (150 mg/L) or citric acid (150 mg/L) or all of them combined, inhibited the blackening phenomena and enhanced the growth and the elongation of banana (*Musa* spp. cv. Grand Naine). Ultimately, healthy plantlets maybe employed for high yield developments.

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Table (1): Different concentrations of antioxidant treatments used for *in vitro* culture of banana *(Musa* spp. cv. Grand Naine).

Treatments	Ascorbic acid mg/L	Citric acid mg/L	Activated charcoal g/L
Control	0.0	0.0	0.0
1	150	0.0	0.0
2	0.0	150	0.0
3	0.0	0.0	1.5
4	150	150	0.0
5	150	0.0	1.5
6	0.0	150	1.5
7	150	150	1.5

Table (2): The effect of some antioxidant treatments on the multiplication stage for *in vitro* culture of banana (*Musa* spp. cv. Grand Naine).

Treatments	Blackening degree	Number of shoots/explant	Shoot length(cm)
Control	2.67 a	3.00 c	0.67 b
1	1.33 b	3.00 c	0.87b
2	1.00 bc	3.33 bc	0.87 b
3	0.67 bcd	4.00 abc	0.83 b
4	0.33 cd	3.33 bc	0.83 b
5	0.00 d	5.00 a	1.50 a
6	0.00 d	4.67 ab	0.50 b
7	0.00 d	5.00 a	0.50 b
LSD at 0.05%	0.763	1.547	0.502

Means with the same letters are not significant at 0.05 level of significant.

Table (3): The effect of some antioxidant treatments on rooting stage of *in vitro* culture of banana (*Musa* spp. cv. Grand Naine).

Treatments	Blackening degree	Shoot length (cm)	Number of roots/explant	Root length (cm)	Number of secondary roots/explant
Control	1.33 a	7.00 c	2.00 b	2.00 b	0.00 c
1	0.67 b	7.667 c	2.00 b	3.00 ab	0.00 c
2	0.67 b	8.00 bc	3.00 b	3.00 ab	0.00 c
3	0.67 b	10.00 ab	4.00 b	4.00 ab	10.00 a
4	0.00 c	8.00 bc	4.00 b	3.00 ab	10.33 a
5	0.00 c	9.33 bc	7.00 a	5.67 a	10.67 a
6	0.00 c	12.33 a	8.00 a	4.67 ab	8.33 a
7	0.00 c	10.00 ab	4.00 b	2.00 b	5.00 b
LSD at 0.05%	0.589	2.623	2.520	3.301	2.706

Means with the same letters are not significant at 0.05 level of significant.

Table (4): The effect of some antioxidant treatments on acclimatization stage of *in vitro* culture of *Musa* spp. cv. Grand Naine

Treatments	Shoot length (cm)	Number of roots/explant	Root length (cm)
Control	8.00 c	5.00 b	3.667 cd
1	10.67 ab	6.33 ab	4.333 bcd
2	10.67 ab	7.00 ab	4.333 bcd
3	12.00 ab	5.33 ab	5.00 abc
4	10.00 bc	7.33 ab	4.00 bcd
5	11.33 ab	7.33 ab	6.333 a
6	13.00 a	7.33 ab	5.333 ab
7	12.67 a	8.00 a	3.333 d
LSD at 0.05%	2.376	2.678	1.358

Means with the same letters are not significant at 0.05 level of significant.

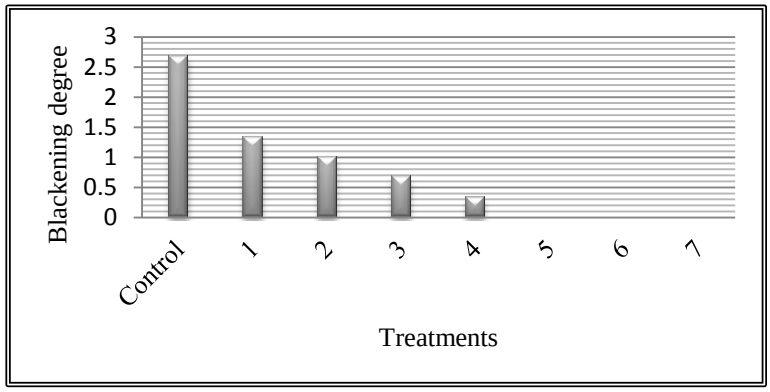

Fig. (1): The effect of different studied antioxidant treatments on the blackening degree during the multiplication stage of *in vitro* culture of banana (*Musa* spp.).

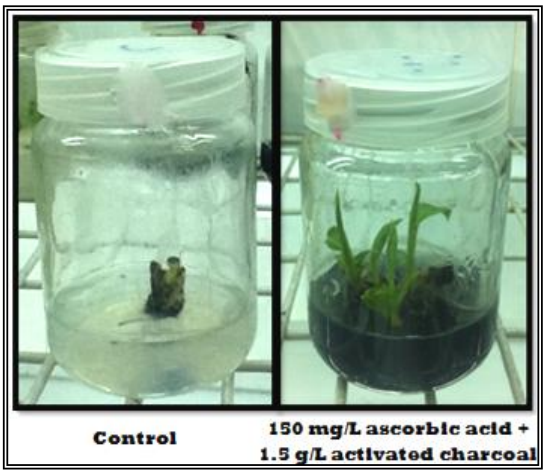

Fig. (2): The effect of ascorbic acid (150 mg/L) + activated charcoal (1.5 g/L) treatments on the number of shoots and shoots length during multiplication stage of *in vitro* culture of banana (*Musa* spp.). This treatment shows to be the best treatment to increase number and length of shoots.

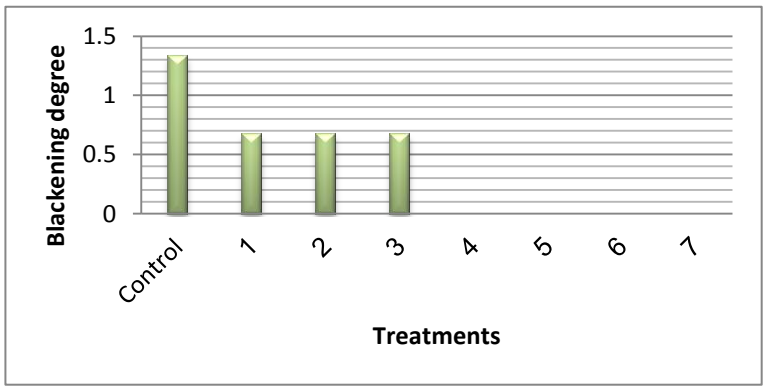

Fig. (3): The effect of the different studied antioxidant treatments on the blackening degree during the rooting stage of banana *in vitro* (*Musa* spp.).

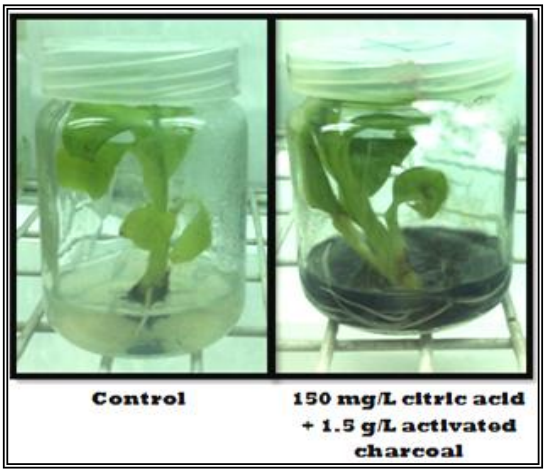

Fig. (4): The effect of citric acid (150 mg/L) + activated charcoal (1.5 g/L) treatments on shoot lets length and root number during rooting stage of *in vitro* culture of banana (*Musa* spp.). This treatment shows to be the best treatment to increase the shoots length and development of roots.

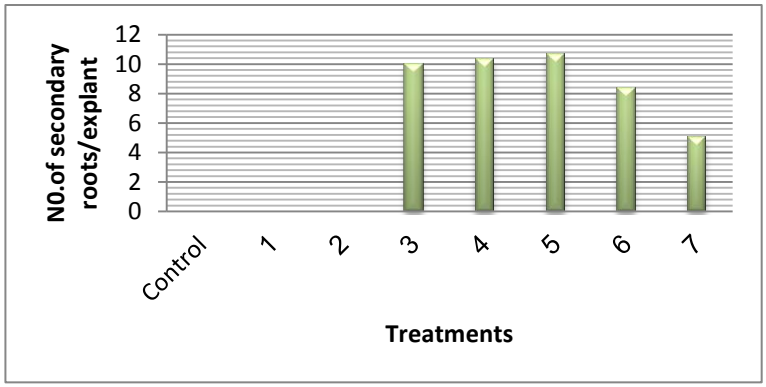

Fig. (5): The effect of different studied antioxidant treatments on the number of secondary roots during rooting stage of *in vitro* culture of banana (*Musa* spp.).

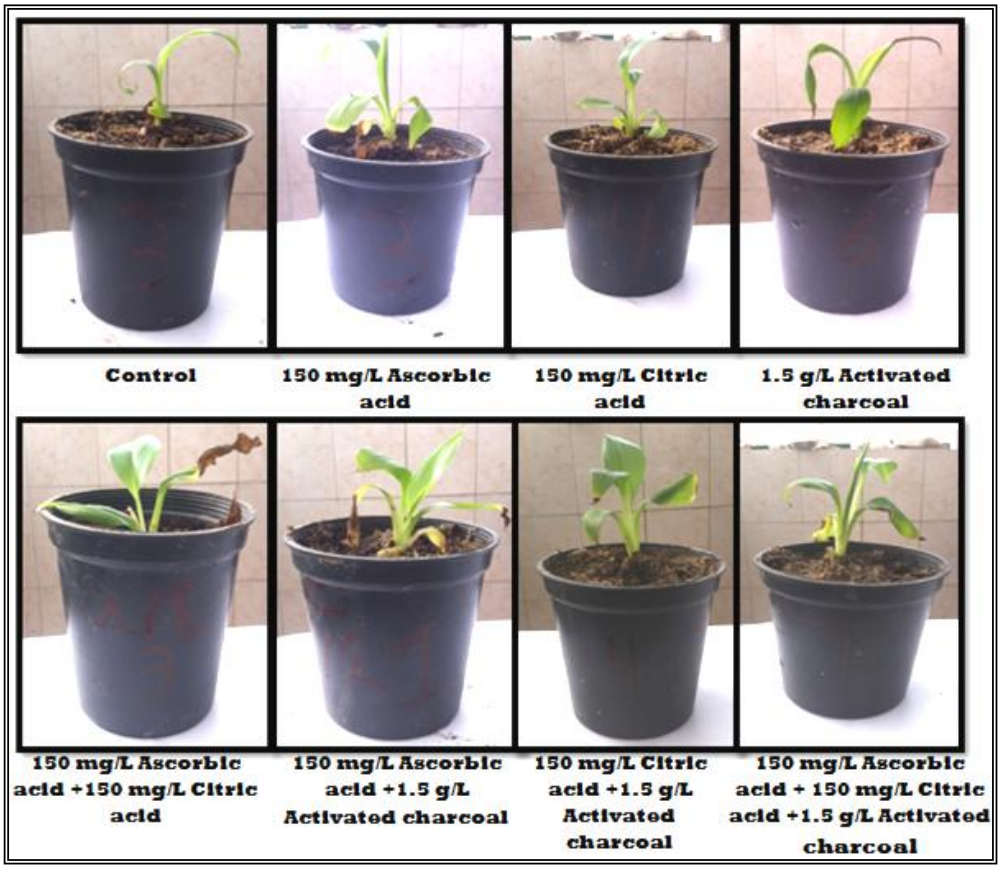

Fig. (6): The effect of different antioxidant treatments on plantlets growth during acclimatization stage of *in vitro* culture of banana (*Musa* spp.) in producing healthy plantlets for high production in field.