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Full Length Research Paper

Effects of various medium compositions and wounding treatments on *in vitro* growth and regeneration of bird of paradise(*Strelitzia reginae*)

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The aim of this study was to investigate the use of antioxidants, wounding treatments and hormone concentrations in efforts to overcome phenolic oxidation and stimulate axillary bud proliferation. Significant results were achieved with 1-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) concentrations on explants discoloration and callus formation. The antioxidant treatments, activated charcoal (AC) and ascorbic acid (AA) significantly affected explant discoloration, the induction of callus and the length of roots developed. Wounding treatments resulted in a reduction of plant height, an increase in both explants discoloration and callus formation. The most effective treatment in reducing explants discoloration at the media contact point was achieved in interactive effects of higher NAA and BAP concentrations (0.1 mg.l^{-1} NAA; 3 mg.l^{-1} BAP and 0.5 mg.l^{-1} NAA; 5 mg.l^{-1} BAP) without wounding. Interactions between antioxidants and wounding treatments resulted in the absolute absence of callus induction in all treatments involving ascorbic acid.

Key words: Ascorbic acid, activated charcoal, 6-benzylaminopurine (BAP), callus formation, discoloration, 1-naphthalene acetic acid (NAA).

INTRODUCTION

The bird of paradise (*Strelitzia reginae*) is a plant of significant commercial value (Paiva et al., 2004). However, its commercial exploitation and success is limited by its naturally low rate of multiplication (Ziv and Halevy, 1983). Micropropagation as an advanced propagation and cloning method could overcome the constraints posed by the slow conventional propagation methods, thus, allowing for the large scale production which is needed to exploit its horticultural potential. In most reported investigations (Promtep, 1981; Ziv and Halevy, 1983; Paiva et al., 2004; Kantharaju et al., 2008), only partial success and a low rate of multiplication were obtained, indicating major problems with growing and multiplying this plant *in vitro*. Furthermore, the successful regeneration from zygotic embryo explants has not been reported. There are no reports on success or attempts made in the stimulation of axillary bud proliferation from embryo-derived plantlets.

Axillary bud proliferation exploits the normal ontogenetic route for branch development by lateral meristems (Gamborg and Phillips, 2002). In *Strelitzia*, there is an absolute absence of branching from axillary buds *in vivo*. This may be a result of a strong apical dominance effect (van de Pol and van Hell, 1988). A method of eradicating apical dominance *in vitro* is required to promote branching and increase the multiplication rate of *Strelitzia*.

Since apical dominance has been proved to be under the control of various growth regulators (Wickson and Thimann, 1958; Woolley and Wareing, 1972; Cline, 1994),

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Abbreviations: MS, Murashige and Skoog (1962); **AC,** activated charcoal; **AA,** ascorbic acid; **BAP,** 6-benzylaminopurine; **NAA,** 1-naphthalene acetic acid.

the proportions of these substances in the media can be manipulated to break dormancy and produce shoots (Razdan, 1993). The concentration and combination of auxins and cytokinins is a key factor which determines successful plant regeneration (Razdan, 1993). In order to increase axillary bud development in *S. reginae*, the optimal balance between these two groups of growth regulators needs to be determined.

Axillary meristems are generally the source of buds that form when leaders are damaged (Burrows, 1989), thus, indicating the positive effects of meristem wounding and even apical bud removal on stimulating the growth of suppressed axillary buds. This method of eliminating apical dominance introduces branching to increase the multiplication rate. Similar to *in vivo* methods, an *in vitro* wounding method is needed to reduce apical dominance and promote axillary bud development. Significant effects of *in vitro* wounding on shoot and root induction have been reported for various species such as *Pyrus malus* (Korban and Skirvin, 1985; Browning et al., 1987; Welander, 1988) and *Yucca elephantipes* (Mauseth and Halperin, 1975; Bentz et al., 1988).

The failure of tissue culture attempts in the propagation of *S. reginae* is largely due to the oxidative browning of explants (Ziv and Halevy, 1983; Paiva et al., 2004, Kantharaju et al., 2008). The excessive production of polyphenols leads to the browning and eventual death of explants (Ziv and Halevy, 1983; Pan and van Staden, 1998; Zeweldu and Ludder, 1998; Birmeta and Welander, 2004; Diro and van Staden, 2004). Tissue injury stimulates the production of phenols (Dodds and Roberts, 1995). Thus, polyphenolic exudation will be exaggerated in response to the wounding techniques employed in this experiment, thus, making the need for antioxidants in the culture media even more evident. The addition of the antioxidant, activated charcoal (AC), to culture media to adsorb toxic substances is widely reported (Horner et al., 1977; Fridborg et al., 1978; Weatherhead et al., 1979; Theander and Nelson, 1988). However, the adsorption properties of AC are non-selective and capable of adsorbing high concentrations of various growth regulators (Pan and van Staden, 1998). As mentioned, the ratio and concentration of auxins and cytokinins in the media is a key factor in determining successful plant regeneration (Razdan, 1993). Thus, the addition of AC to shoot proliferation media may have adverse effects and inhibit growth and regeneration *in vitro* (Pan and van Staden, 1998); thus, highlighting the need to introduce another antioxidant to promote growth and regeneration. Ascorbic acid (AA) is an antioxidant used to control the oxidation of phenols (Chawla, 2002). A comparative study of these two antioxidants would gain further insight into the adsorption of the growth regulators and identify the most successful antioxidant for use in this stage of culture. The objective of this study was to assess the effects various auxin and cytokinin concentrations, antioxidant and wounding treatments have on shoot formation, explants height, explants discoloration (the entire explants and at the medium contact point only), callus formation and root length.

MATERIALS AND METHODS

Plant material

Embryo-derived *in vitro* seedlings of *S. reginae* were used in this experiment. Germinated plantlets were subjected to 2 wounding treatments; unwounded explants (control) and explants longitudinally sectioned through the apical meristem.

Culture conditions and media

Explants were transferred to different regeneration media. The basal medium comprised the Murashige and Skoog (1962) (MS) salts supplemented with 100 $mg.l^{-1}$ myo-inositol, 0.1 $mg.l^{-1}$ thiamine-HCl, 0.1 $mg.l^{-1}$ pyridoxine, 2 $mg.l^{-1}$ glycine and 30 $g.l^{-1}$ sucrose. Various concentrations of 6-benzylaminopurine (BAP) 0, 2, 3, 5 and 6 $mg.l^{-1}$ and 1-naphthalene acetic acid (NAA) 0, 0.1 and 0.5 $mg.l^{-1}$ were added to the media. The antioxidants of 2.5 $g.l^{-1}$ AC and 0.05 $g.l^{-1}$ AA were each separately added to the various media. The experiment consisted of ten medium types (Table 1). Ten replicates were used for each treatment. The media was solidified with 7 $g.l^{-1}$ agar. The pH of the media was adjusted to 5.95 prior to autoclaving at 121°C for 20 min. The unwounded cultures were incubated in a growth room with a 16 h light and 8 h dark cycle at 25±2°C.

Data collection and analysis

Data on number of shoots developed per explant, shoot length, root number and length, degree of plantlet discoloration and callus formation were collected at weekly intervals. Based on visual observations, the degree of plantlet discoloration (entire explant and at the media contact point only) was rated on a scale of 1 to 5 (1 = No discoloration and 5 = Extreme discoloration), modified from the rating scale given by Ziv and Halevy (1983). The degree of callus formation was rated as: 1 = none, 2 = low, 3 = medium and 4 = high. This rating scale was modified from that given by Ziv and Halevy (1983). Data collected were analyzed for statistical significance using unbalanced factorial analysis of variance (ANOVA) where 3 levels of NAA (0, 0.1 and 0.5 $mg.l^{-1}$) were split in 5 levels of BAP (0, 2, 3, 5, 6 $mg.l^{-1}$) and two antioxidants (2.5 $g.l^{-1}$ AC and 0.05 $g.l^{-1}$ AA) were added to each level of BAP making a total of ten treatments (Table 1). These computations were done with the STATISTICA Software Programme version 2010 (StatSoft Inc., Tulsa, OK, USA). The Fisher least significance difference was used to compare treatment means at P = 0.05 level of significance (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on explant height

The height of explants was not affected by variations of NAA and BAP concentrations. Similarly, the antioxidant treatments did not produce significant results. Although a

Table 1. Concentrations and combinations of auxin and cytokinin supplements and antioxidants tested in axillary bud proliferation of regenerated embryos.

Treatment	Auxin and cytokinin concentration ($mg.l^{-1}$)		Antioxidant ($g.l^{-1}$)
	NAA	BAP	
1	0	0	2.5 activated charcoal
2	0	0	0.05 ascorbic acid
3	0.1	2	2.5 activated charcoal
4	0.1	2	0.05 ascorbic acid
5	0.1	3	2.5 activated charcoal
6	0.1	3	0.05 ascorbic acid
7	0.5	5	2.5 activated charcoal
8	0.5	5	0.05 ascorbic acid
9	0.5	6	2.5 activated charcoal
10	0.5	6	0.05 ascorbic acid

slight increase in explants height was observed in the presence of AC. Wounding had a significant influence on explants height, with maximum height observed in entire, unwounded explants (Table 2). Wounded explants displayed a 28% reduction in height, compared with the unwounded. These results are in line with those reported by Bhatia et al. (2005), who found that shoot height in tomato was much lower in shoots regenerated from wounded explants compared with those that originated from intact cotyledons. Wounding induces stress in plant tissues and suppresses plant growth (Zhang and Turner, 2008).

Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on discoloration of the explants

The variations in NAA and BAP concentrations did not significantly affect explants discoloration in this experiment. Although a higher degree of discoloration was observed in the control (the treatment free of plant hormones). Antioxidant treatments had a significant ($P \leq 0.05$) effect on reducing explants discoloration (Table 3). The antioxidant, AA, was 20, 18 and 19% more effective in reducing entire explant discoloration than AC in weeks 6, 7 and 8, respectively. AA treatments have been widely reported to have positive effects on reducing the oxidative browning of explants (Wu and du Toit, 2004; Abeyaratne and Lathiff, 2002). A study showing parallel results with this study was achieved in Cavendish banana cv. Formosa (Ko et al., 2009) in which AC was not as effective as AA in reducing the incidence of lethal browning. Ko et al. (2009) suggested that AA may have been absorbed by the plantlets, translocated to leaves and prevented the oxidation of phenolic compounds on the target site. It is conceivable that this may also be the case in this study.

Throughout the experiment, a significantly higher ($P \leq 0.001$) degree of explants discoloration was observed in wounded explants (Table 3). A 37% higher level of discoloration was observed in wounded explants in week 2, which increased to 55% over the duration of the experiment, compared with the unwounded explants. Tissue injury stimulates the production of phenols (Dodds and Roberts, 1995), a defensive mechanism common in plants in response to any type of tissue damage (Pan and van Staden, 1998; Ndakidemi and Dakora, 2003). Thus, the production of polyphenolic compounds is exaggerated in response to wounding (George, 1993; Zeweldu and Ludders, 1998; Strosse et al., 2009). The excessive production of polyphenols results in browning and eventual death of tissues (Pan and van Staden, 1998).

Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on discoloration of explants at the media contact point

In week 2, the various NAA and BAP concentrations significantly ($P \leq 0.01$) affected explant discoloration at the medium contact point (Table 4). The treatment containing the highest level of NAA (0.5 $mg.l^{-1}$) and BAP (6 $mg.l^{-1}$) was the most effective in reducing discoloration. Whereas, the highest level of discoloration was observed in the control (the treatment without NAA and BAP). These results are in line with those of Xu et al. (2009) who reported increased levels of NAA and BAP to effectively reduce the discoloration of *Dioscorea opposita* explants.

Results revealed that AC was significantly ($P \leq 0.001$) more effective than AA in reducing explant discoloration at the medium contact point (Table 4). In weeks 2, 3 and 4, the respective reductions of 33, 34 and 31% were observed in AC treatments relative to AA treatments. It is

Table 2. Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on explant height (mm).

Treatment	Time (Weeks)								
	1	2	3	4	5	6	7	8	9
Concentration									
0 mg.l^{-1} NAA; 0 mg.l^{-1} BAP	13.45±1.26^{a}	16.60±1.69^{a}	18.50±1.85^{a}	18.80±1.87^{a}	19.00±1.91a	19.55±1.99^{a}	19.90±2.09^{a}	20.30±2.24^{a}	20.65±2.35^{a}
0.1 mg.l^{-1} NAA; 2 mg.l^{-1} BAP	13.70±1.29^{a}	16.55±1.89^{a}	20.40±1.94^{a}	20.95±1.96^{a}	21.20±2.17^{a}	21.80±2.21^{a}	22.25±2.28^{a}	22.95±2.32^{a}	23.25±2.39^{a}
0.1 mg.l^{-1} NAA; 3 mg.l^{-1} BAP	14.50±1.10^{a}	19.35±1.90^{a}	22.35±2.54^{a}	22.95±2.61^{a}	24.60±2.77^{a}	25.40±2.90^{a}	26.20±2.98^{a}	26.65±2.94^{a}	26.80±2.94^{a}
0.5 mg.l^{-1} NAA; 5 mg.l^{-1} BAP	16.20±1.26^{a}	19.25±1.40^{a}	21.20±1.74^{a}	21.75±1.59^{a}	21.70±1.46^{a}	22.30±1.49^{a}	22.90±1.64^{a}	23.50±1.78^{a}	23.60±1.83^{a}
0.5 mg.l^{-1} NAA; 6 mg.l^{-1} BAP	13.65±1.04^{a}	17.60±1.55^{a}	19.65±1.98^{a}	21.25±2.26^{a}	22.25±2.34^{a}	22.80±2.40^{a}	23.05±2.42^{a}	23.40±2.50^{a}	23.45±2.50^{a}
Antioxidants									
AA	14.32±0.73^{a}	16.68±0.93^{a}	18.90±1.06^{a}	19.62±1.14^{a}	20.24±1.19^{a}	20.94±1.23^{a}	21.46±1.27^{a}	22.32±1.34^{a}	22.50±1.38^{a}
AC	14.28±0.78^{a}	19.06±1.17^{a}	21.94±1.43^{a}	22.66±1.44^{a}	23.26±1.51^{a}	23.80±1.57^{a}	24.26±1.63^{a}	24.40±1.65^{a}	24.60±1.67^{a}
Wounding									
Wounded	13.56±0.58^{a}	15.92±0.92^{b}	18.06±1.18^{b}	18.34±1.16^{b}	18.66±1.22^{b}	19.12±1.31^{b}	19.24±1.35^{b}	19.60±1.43^{b}	19.72±1.47^{b}
Unwounded	15.04±0.89^{a}	19.82±1.14^{a}	22.78±1.28^{a}	23.94±1.34^{a}	24.84±1.39^{a}	25.62±1.38^{a}	26.48±1.41^{a}	27.12±1.40^{a}	27.38±1.40^{a}
3-way ANOVA (F-statistic)									
Main effects									
Concentration	0.8^{ns}	0.7^{ns}	0.6^{ns}	0.6^{ns}	0.9^{ns}	1.0^{ns}	1.1^{ns}	1.0^{ns}	0.9^{ns}
Antioxidant	0.0^{ns}	2.6^{ns}	3.0^{ns}	2.9^{ns}	2.7^{ns}	2.3^{ns}	2.0^{ns}	1.1^{ns}	1.1^{ns}
Wounding	1.7^{ns}	7.1**	7.2**	9.8**	11.2**	11.7***	13.6***	14.0***	14.1***
Interactions									
Concentration*Antioxidant	0.4^{ns}	1.1^{ns}	1.2^{ns}	1.3^{ns}	1.5^{ns}	1.5^{ns}	1.2^{ns}	1.2^{ns}	1.1^{ns}
Concentration*Wounding	0.4^{ns}	1.1^{ns}	0.8^{ns}	0.9^{ns}	0.7^{ns}	0.8^{ns}	0.7^{ns}	0.7^{ns}	0.8^{ns}
Antioxidant*Wounding	0.0^{ns}	0.2^{ns}	0.0^{ns}	0.0^{ns}	0.0^{ns}	0.2^{ns}	0.1^{ns}	0.2^{ns}	0.2^{ns}
Conc*Anto*Wounding	0.3^{ns}	0.8^{ns}	0.7^{ns}	0.5^{ns}	0.5^{ns}	0.8^{ns}	0.8^{ns}	1.1^{ns}	1.1^{ns}

**, P≤0.01; ***, P≤0.001; ns, not significantly different. Values (Mean ± MSE, n = 10) followed by dissimilar letters in a column are significantly different by least significant difference test at P=0.05.

at the point of contact between the plantlet and the culture medium that oxidative browning is exaggerated due to oxygen coming into contact with the tissue and the required nutrients (North et al., 2010). Results in this study indicate that AC played a key role, and was more effective than AA, in reducing plantlet discoloration at this point of contact. The browning and subsequent death of cultured explants is a major problem that usually occurs as a result of phenolic compounds (Ozyigit, 2008). Wounded tissues release these polyphenolic compounds, which diffuse into the medium (Strosse et al., 2009) and are detrimental to the further development of explants which become necrotic and die (Ziv and Halevy, 1983). The addition of AC to culture media has been widely reported to reduce tissue browning (Chang et al., 2001; Wang et al., 2005; Guo et al., 2007;

Table 3. Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on discoloration of the explants.

Treatment	Time (Weeks)								
	1	2	3	4	5	6	7	8	9
Concentration									
0 mg.l^{-1} NAA; 0 mg l^{-1} BAP	1.10±0.10^{a}	1.75±0.22^{a}	1.80±0.24^{a}	1.85±0.24^{a}	2.00±0.26^{a}	2.30±0.30^{a}	2.45±0.35^{a}	2.45±0.35^{a}	2.55±0.37^{a}
0.1 mg.l^{-1} NAA; 2 mg.l^{-1} BAP	1.00±0.00^{a}	1.45±0.15^{a}	1.60±0.18^{a}	1.60±0.18^{a}	1.75±0.23^{a}	1.85±0.24^{a}	2.10±0.26^{a}	2.25±0.26^{a}	2.30±0.27^{a}
0.1 mg.l^{-1} NAA; 3 mg.l^{-1} BAP	1.00±0.00^{a}	1.35±0.11^{a}	1.50±0.17^{a}	1.70±0.19^{a}	1.85±0.21^{a}	1.90±0.20^{a}	2.10±0.25^{a}	2.20±0.26^{a}	2.35±0.26^{a}
0.5 mg.l^{-1} NAA; 5 mg.l^{-1} BAP	1.00±0.00^{a}	1.40±0.11^{a}	1.50±0.15^{a}	1.55±0.18^{a}	1.75±0.25^{a}	1.95±0.32^{a}	1.95±0.32^{a}	2.00±0.32^{a}	2.10±0.33^{a}
0.5 mg.l^{-1} NAA; 6 mg.l^{-1} BAP	1.00±0.00^{a}	1.45±0.11^{a}	1.65±0.17^{a}	1.65±0.17^{a}	1.65±0.17^{a}	1.80±0.20^{a}	2.15±0.24^{a}	2.75±0.29^{a}	3.00±0.35^{a}
Antioxidants									
AA	1.04±0.04^{a}	1.54±0.08^{a}	1.60±0.10^{a}	1.70±0.11^{a}	1.70±0.11^{a}	1.74±0.12^{b}	1.94±0.14^{b}	2.08±0.15^{b}	2.26±0.18^{a}
AC	1.00±0.00^{a}	1.42±0.10^{a}	1.62±0.13^{a}	1.64±0.14^{a}	1.90±0.17^{a}	2.18±0.19^{a}	2.36±0.21^{a}	2.58±0.22^{a}	2.66±0.22^{a}
Wounding									
Wounded	1.00±0.00^{a}	1.82±0.07^{a}	2.06±0.09^{a}	2.12±0.10^{a}	2.28±0.12^{a}	2.56±0.15^{a}	2.90±0.15^{a}	3.14±0.15^{a}	3.40±0.16^{a}
Unwounded	1.04±0.04^{a}	1.14±0.09^{b}	1.16±0.10^{b}	1.22±0.11^{b}	1.32±0.12^{b}	1.36±0.13^{b}	1.40±0.14^{b}	1.52±0.15^{b}	1.52±0.15^{b}
3-way ANOVA (F-statistic)									
Main effects									
Concentration	1.0^{ns}	1.6^{ns}	0.7^{ns}	0.5^{ns}	0.5^{ns}	0.9^{ns}	0.6^{ns}	1.5^{ns}	2.0^{ns}
Antioxidant	1.0^{ns}	1.1^{ns}	0.0^{ns}	0.2^{ns}	1.3^{ns}	5.7*	4.0*	5.7*	3.3
Wounding	1.0^{ns}	36.7***	44.5***	37.5***	30.7***	42.1***	51.1***	59.4***	73.9***
Interactions									
Concentration*Antioxidant	1.0^{ns}	0.3^{ns}	0.8^{ns}	1.8^{ns}	1.1^{ns}	1.5^{ns}	0.4^{ns}	0.6^{ns}	0.2^{ns}
Concentration*Wounding	1.0^{ns}	1.1^{ns}	2.1^{ns}	1.7^{ns}	1.7^{ns}	1.7^{ns}	0.3^{ns}	0.3^{ns}	0.3^{ns}
Antioxidant*Wounding	1.0^{ns}	4.6*	0.2^{ns}	0.0^{ns}	0.1^{ns}	1.2^{ns}	4.0*	1.5^{ns}	0.5^{ns}
Conc*Anto*Wounding	1.0^{ns}	0.4^{ns}	1.4^{ns}	0.9^{ns}	1.4^{ns}	1.7^{ns}	0.4^{ns}	0.8^{ns}	0.9^{ns}

*, P≤0.05; ***, P≤0. 001; ns, not significantly different. Values (Mean ± MSE, n = 10) followed by dissimilar letters in a column are significantly different by Least significant difference test at P=0.05. Rating scale used is 1 to 5 (1, No discoloration; 5, extreme discoloration).

North et al., 2010). The promotary effects of AC may be attributed mainly to its irreversible adsorption of inhibitory compounds and decrease phenolic oxidation (Thomas, 2008). Phenols leached from the tissues may readily be absorbed by the AC, reducing the discoloration more effectively in those tissues coming into direct contact with the AC supplemented media.

Wounded explants displayed a significantly higher level of discoloration at the medium contact point throughout the duration of the experiment. In week 2, a 15% discolouration was observed which progressively increased to 57% in the final week. As mentioned, wounding stimulates the production of phenols (George, 1993; Dodds and

Table 4. Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on discoloration of explants at the media contact point.

Treatment	Time (Weeks)								
	1	2	3	4	5	6	7	8	9
Concentration									
0 mg.l^{-1} NAA; 0 mg l^{-1} BAP	1.30±0.22^{a}	1.75±0.22^{a}	1.75±0.27^{a}	1.75±0.27^{a}	1.75±0.27^{a}	1.95±0.28^{a}	2.35±0.36^{a}	2.40±0.36^{a}	2.45±0.37^{a}
0.1 mg.l^{-1} NAA; 2 mg.l^{-1} BAP	1.00±0.00^{a}	1.20±0.09^{b}	1.30±0.15^{a}	1.35±0.15^{a}	1.70±0.23^{a}	1.90±0.24^{a}	1.95±0.23^{a}	2.05±0.23^{a}	2.25±0.28^{a}
0.1 mg.l^{-1} NAA; 3 mg.l^{-1}BAP	1.00±0.00^{a}	1.40±0.15^{b}	1.40±0.15^{a}	1.45±0.17^{a}	1.50±0.18^{a}	1.70±0.21^{a}	2.05±0.25^{a}	2.05±0.25^{a}	2.30±0.32^{a}
0.5 mg.l^{-1} NAA; 5 mg.l^{-1} BAP	1.00±0.00^{a}	1.25±0.12^{b}	1.55±0.18^{a}	1.55±0.17^{a}	1.95±0.29^{a}	2.15±0.32^{a}	2.20±0.32^{a}	2.30±0.32^{a}	2.30±0.32^{a}
0.5 mg.l^{-1} NAA; 6 mg.l^{-1} BAP	1.00±0.00^{a}	1.15±0.08^{b}	1.30±0.13^{a}	1.40±0.13^{a}	1.55±0.17^{a}	1.80±0.25^{a}	2.05±0.29^{a}	2.35±0.30^{a}	2.70±0.37^{a}
Antioxidants									
AA	1.04±0.04^{a}	1.62±0.09^{a}	1.76±0.13^{a}	1.78±0.13^{a}	1.84±0.13^{a}	1.96±0.13^{a}	2.10±0.14^{a}	2.22±0.14^{a}	2.44±0.19^{a}
AC	1.08±0.08^{a}	1.08±0.08^{b}	1.16±0.09^{b}	1.22±0.09^{b}	1.54±0.16^{a}	1.84±0.19^{a}	2.14±0.22^{a}	2.24±0.22^{a}	2.36±0.23^{a}
Wounding									
Wounded	1.00±0.00^{a}	1.46±0.09^{a}	1.70±0.11^{a}	1.76±0.11^{a}	2.12±0.15^{a}	2.48±0.16^{a}	2.84±0.17^{a}	3.02±0.17^{a}	3.36±0.18^{a}
Unwounded	1.12±0.09^{a}	1.24±0.10^{b}	1.22±0.12^{b}	1.24±0.12^{b}	1.26±0.12^{b}	1.32±0.12^{b}	1.40±0.12^{b}	1.44±0.13^{b}	1.44±0.13^{b}
3-way ANOVA (F-statistic)									
Main effects									
Concentration	1.8^{ns}	4.5**	1.5^{ns}	1.0^{ns}	0.8^{ns}	0.6^{ns}	0.4^{ns}	0.5^{ns}	0.5^{ns}
Antioxidant	0.2^{ns}	28.0***	18.6***	15.7***	2.7^{ns}	0.4^{ns}	0.0^{ns}	0.0^{ns}	0.1^{ns}
Wounding	1.8^{ns}	4.7*	11.9***	13.5***	22.4***	33.6***	40.8***	53.1***	69.0***
Interactions									
Concentration*Antioxidant	0.2^{ns}	0.8^{ns}	0.7^{ns}	0.6^{ns}	1.3^{ns}	1.1^{ns}	0.7^{ns}	0.6^{ns}	0.9^{ns}
Concentration*Wounding	1.8^{ns}	3.6**	1.4ns	1.3ns	2.1^{ns}	1.7^{ns}	0.5^{ns}	0.5^{ns}	0.9^{ns}
Antioxidant*Wounding	0.2^{ns}	13.9***	11.9***	11.5**	1.0^{ns}	0.0^{ns}	1.5^{ns}	2.5^{ns}	1.1^{ns}
Conc*Anto*Wounding	0.2^{ns}	0.5^{ns}	0.3^{ns}	0.4^{ns}	1.2^{ns}	0.8^{ns}	0.2^{ns}	0.1^{ns}	0.3^{ns}

**, P≤0.01; ***, P≤0. 001; ns: not significantly different. Values (Mean ± MSE, n = 10) followed by dissimilar letters in a column are significantly different by least significant difference test at P=0.05. Rating scale used is 1 to 5 (1, No discoloration; 5, extreme discoloration).

Roberts, 1995; Zeweldu and Ludders, 1998; Strosse et al., 2009). Phenolic interactions expressed as oxidative browning of the explants can lead to the death of the plant material (Taji and Williams, 1996). Oxygen free radicals, generated by wounding (Salin and Bridges, 1981; Thompson et al., 1987), can also lead to the oxidative browning of explants. Reducing contact with oxygen reduces the rate of oxidation of

Table 5. Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on callus development.

Treatment	Time (Weeks)						
	3	4	5	6	7	8	9
Concentration							
0 mg.l^{-1} NAA; 0 mg l^{-1} BAP	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.05±0.05^{a}	0.10±0.10^{a}	0.15±0.15^{a}	0.15±0.15^{a}
0.1 mg.l^{-1} NAA; 2 mg.l^{-1} BAP	0.05±0.05^{b}	0.05±0.05^{b}	0.05±0.05^{b}	0.15±0.11^{a}	0.15±0.11^{a}	0.10±0.10^{a}	0.10±0.10^{a}
0.1 mg.l^{-1} NAA; 3 mg.l^{-1} BAP	0.10±0.07^{b}	0.10±0.07^{b}	0.15±0.11^{ab}	0.25±0.18^{a}	0.30±0.18^{a}	0.30±0.18^{a}	0.35±0.21^{a}
0.5 mg.l^{-1} NAA; 5 mg.l^{-1} BAP	0.30±0.11^{a}	0.30±0.11^{a}	0.30±0.11^{a}	0.40±0.15^{a}	0.60±0.23^{a}	0.60±0.23^{a}	0.60±0.23^{a}
0.5 mg.l^{-1} NAA; 6 mg.l^{-1} BAP	0.15±0.08^{ab}	0.15±0.08^{ab}	0.15±0.08^{ab}	0.20±0.12^{a}	0.30±0.18^{a}	0.30±0.18^{a}	0.30±0.18^{a}
Antioxidants							
AA	0.18±0.05^{a}	0.18±0.05^{a}	0.20±0.06^{a}	0.34±0.10^{a}	0.46±0.13^{a}	0.46±0.13^{a}	0.48±0.14^{a}
AC	0.06±0.03^{b}	0.06±0.03^{b}	0.06±0.03^{b}	0.08±0.05^{b}	0.12±0.07^{b}	0.12±0.07^{b}	0.12±0.07^{b}
Wounding							
Wounded	0.24±0.06^{a}	0.24±0.06^{a}	0.26±0.07^{a}	0.42±0.11^{a}	0.58±0.14^{a}	0.58±0.15^{a}	0.60±0.15^{a}
Unwounded	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}
3-way ANOVA (F-statistic)							
Main effects							
Concentration	3.79**	3.8**	2.8*	1.3^{ns}	1.8^{ns}	1.6^{ns}	1.6^{ns}
Antioxidant	5.14*	5.1*	5.2*	6.6*	6.9*	6.1*	6.5*
Wounding	20.57***	20.6***	17.8***	17.3***	20.0***	17.9***	18.0***
Interactions							
Concentration*Antioxidant	1.21^{ns}	1.2^{ns}	1.2^{ns}	0.8^{ns}	0.8^{ns}	0.7^{ns}	0.8^{ns}
Concentration*Wounding	3.79^{ns}	3.8^{ns}	2.8^{ns}	1.3^{ns}	1.8^{ns}	1.6^{ns}	1.5^{ns}
Antioxidant*Wounding	5.14*	5.1*	5.2*	6.6*	6.9*	6.1*	6.5*
Conc*Anto*Wounding	1.21^{ns}	1.2^{ns}	1.2^{ns}	0.8^{ns}	0.8^{ns}	0.7^{ns}	0.8^{ns}

*, P≤0.05; **, P≤0.01; ***, P≤0. 001; ns, not significantly different. Values (Mean ± MSE, n = 10) followed by dissimilar letters in a column are significantly different by least significant difference test at P=0.05. The degree of callus formation is rated as: 1, none; 2, low; 3, medium; 4, high.

phenols at the wounded site (Elmore et al., 1990). It is at the media contact point that an adequate supply of oxygen comes into contact with the plant material, resulting in an increased level of oxidative browning of these tissues.

Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on callus development

Callus formation from the explants was induced in NAA and BAP treatments. The highest level of callus was formed in 0.5 mg.l^{-1} NAA and 5 mg.l^{-1} BAP (Table 5). This significant increase was opposed to the control (the treatment without NAA and BAP), in which no callus was formed. Reports

Table 6. Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on the length of roots developed (mm).

Treatment	Root length (mm)
Concentration	
0 mg.l^{-1} NAA; 0 mg l^{-1} BAP	4.90±3.35^{a}
0.1 mg.l^{-1} NAA; 2 mg.l^{-1} BAP	6.55±3.52^{a}
0.1 mg.l^{-1} NAA; 3 mg.l^{-1} BAP	2.95±2.39^{a}
0.5 mg.l^{-1} NAA; 5 mg.l^{-1} BAP	9.10±5.15^{a}
0.5 mg.l^{-1} NAA; 6 mg.l^{-1} BAP	2.80±1.99^{a}
Antioxidants	
AA	1.98±1.15^{b}
AC	8.54±2.78^{a}
Wounding	
Wounded	3.48±1.59^{a}
Unwounded	7.04±2.62^{a}
3-way ANOVA (F-statistic)	
Main effects	0.59^{ns}
Concentration	4.54*
Antioxidant	1.34^{ns}
Wounding	
Interactions	0.23^{ns}
Concentration*Antioxidant	0.46^{ns}
Concentration*Wounding	1.19^{ns}
Antioxidant*Wounding	1.55^{ns}
Conc*Anto*Wounding	

*, P≤0.05; ns, not significantly different. Values (Mean ± MSE, n = 10) followed by dissimilar letters in a column are significantly different by least significant difference test at P = 0.05.

of NAA and BAP combinations supporting the development of callus have been well documented for several plant species (Koroch et al., 2002; Ray et al., 2011; Nurazah et al., 2009). Results presented here are in agreement with these studies, where it was observed that low concentrations of NAA in combination with increased rates of BAP resulted in the induction of callus. Similar to our study, Ray et al. (2011) reported that explants cultured on medium without hormones did not produce callus.

A significantly (P≤0.05) higher level of callus formation was observed in AA treatments than in AC treatments (Table 5). In week 1, callus formation was 66% greater in AA treatments, which gradually increased to 75% in the final week of the experiment, as compared with AC treatments. Several studies report the presence of AC to significantly reduce the formation of callus. The addition of AC prevented callus induction in sorghum and cotton (Zhang et el., 2000; Nguyen, et al., 2007) and reduced callus induction in black wattle and *Oxalis triangularis* (Teng and Ngai, 1999; Quoirin et al., 2001). There was no callus formation in unwounded explants. Whereas, wounding increased callus formation in wounded treatments increased from 0.24 in week 3 to 0.60 in week 9, resulting in a 60% increase (Table 5). The injury that explants experience in response to these wounding techniques may also influence the morphogenesis response in a way similar to that of plants in the natural environment, where wounding often stimulates callus formation (George, 1993).

Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on the length of roots developed

Root length was not significantly affected by NAA and BAP concentrations. Similarly, wounding treatments did not significantly affect root length, although a slight increase in length was observed in unwounded explants than in the wounded. In AC treatments, roots developed were 77% longer than roots developed in AA treatments (Table 6). The positive influence of AC on rooting is

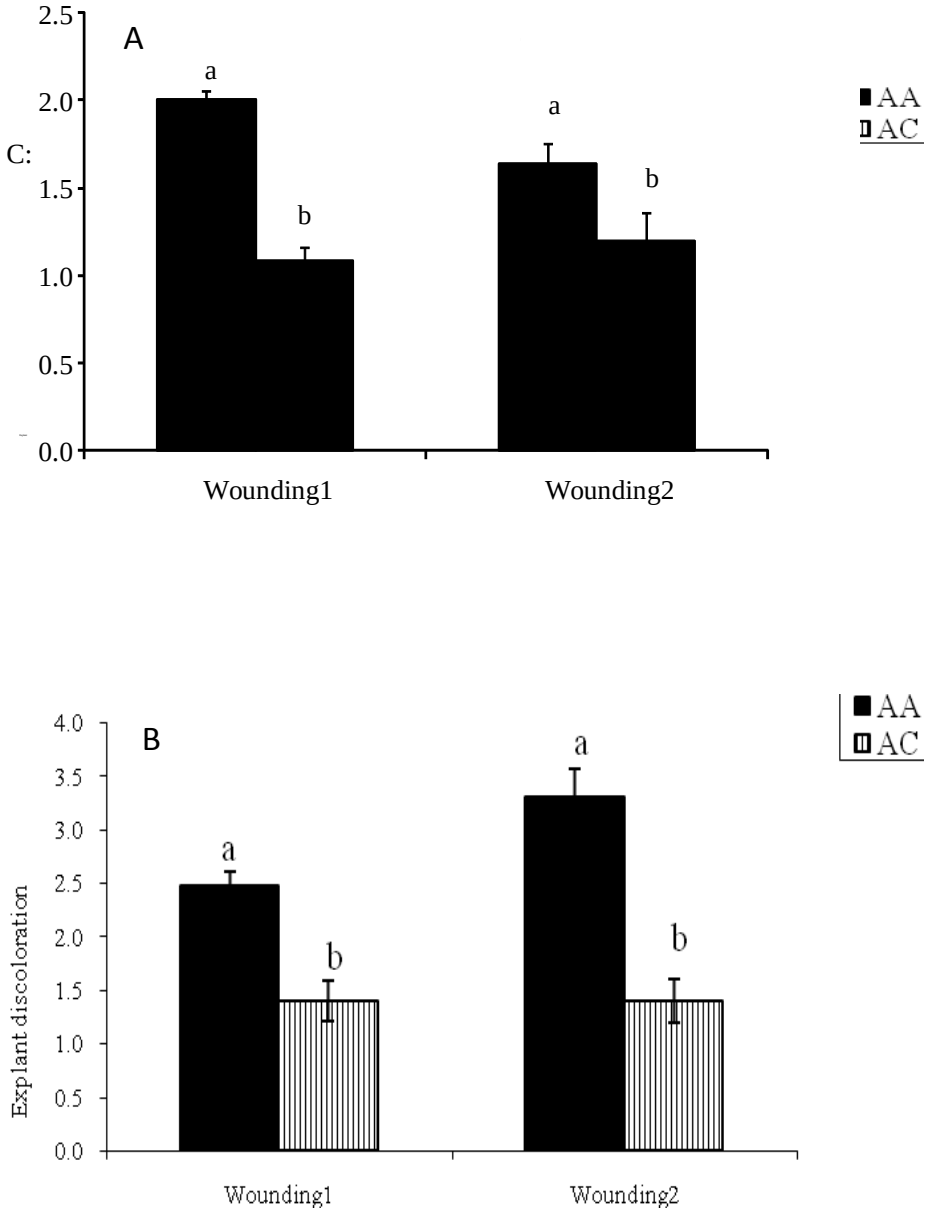

Figure 1. Interactive effects of antioxidants and wounding on entire explant discoloration in (A) week 2 and (B) week 7. Rating scale used is 1 to 5 (1, No discoloration; 5, extreme discoloration). Wounding 1= Wounded; Wounding 2= unwounded.

widely reported (Makunga et al., 2006; Mulwa and Bhalla, 2006; Yan et al., 2006; Agarwal and Kanwar, 2007; Xiao et al. 2007; Makunga and van Staden, 2008; Firoozabady et al., 2006; Feyissa et al., 2005; Loc et al., 2005). Enhanced root growth may be due to the ability of AC to adsorb the polyphenols produced through chemical processes within the media, which may act as growth inhibitors (Madhusudhanan and Rahiman, 2000). AC may also enhance rooting by eliminating light, providing a favourable physical environment to the rhizophere (Gantait et al., 2009).

Interactive effects of various NAA and BAP concentrations, antioxidants and wounding treatments

The results represent the significant (P≤0.05) interactive effects of antioxidant and wounding treatments on discoloration of the entire explants in week 2 (Figure 1A) and week 7 (Figure 1B). AC effectively reduced the level of discoloration in both wounded and unwounded treatments in both weeks. The highest level of explant discoloration was observed in AA treatments, either with

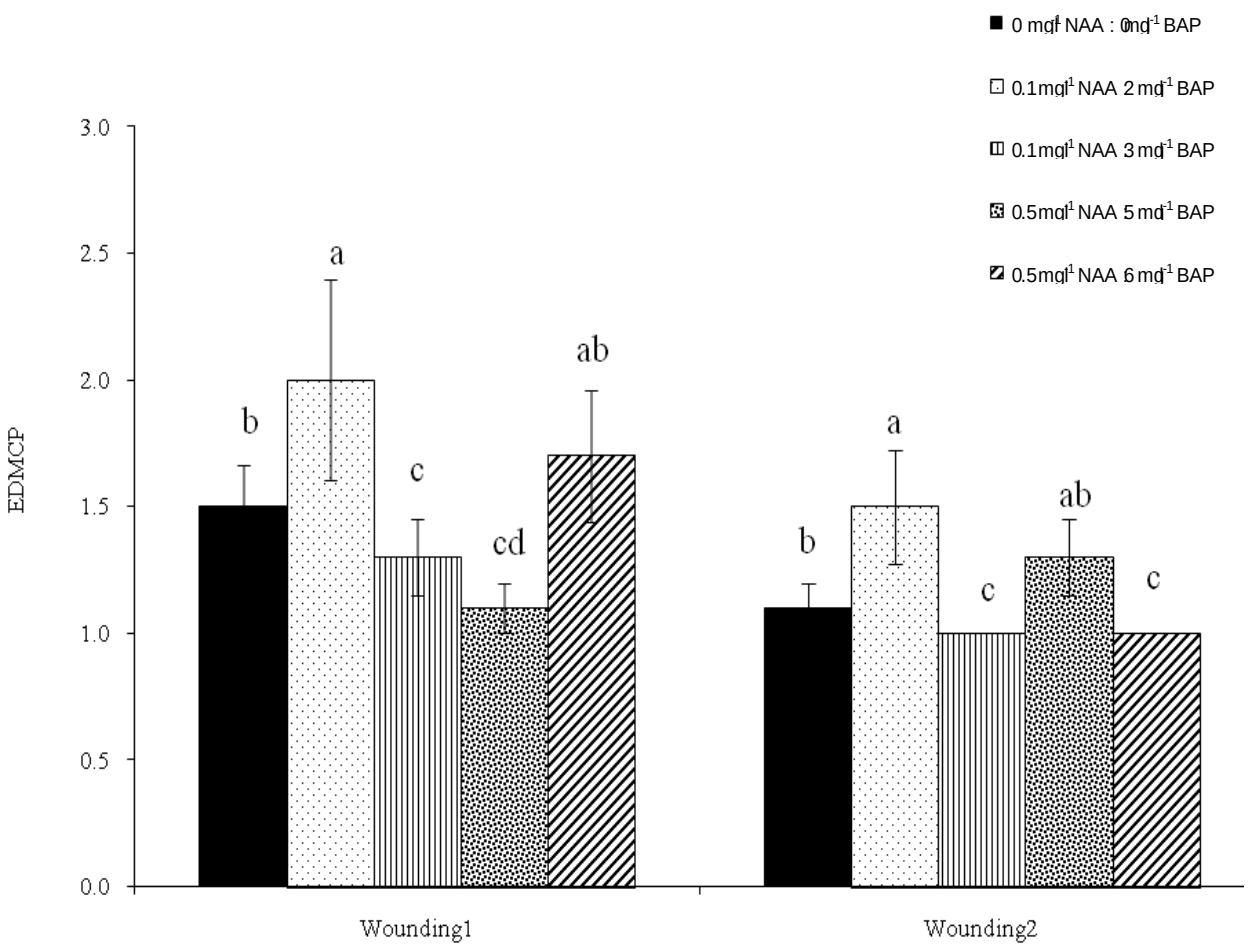

Figure 2. Interactive effects of wounding and concentration on explant discoloration at the medium contact point (EDMCP) in week 2, Rating scale used is 1 to 5 (1, No discoloration; 5, extreme discoloration). Wounding 1= Wounded; Wounding 2= unwounded.

wounding (week 2) or without wounding (week 7). In a study on *Ensete ventricosum*, which is related to *S. reginae* (Strosse et al.,2009), similar results were found. The addition of AA did not effectively reduce polyphenol exudation. However, the most effective treatment was AC, which prevented polyphenol exudation in wounded explants (Birmeta and Welander, 2004). The addition of AC to culture media is a recognized practice and its influence may be attributed to the adsorption of inhibitory substances in the medium (Horner et al., 1977; Fridborg et al., 1978; Weatherhead et al., 1979; Theander and Nelson, 1988) and a drastic decrease in the phenolic oxidation of tissues (Carlberg et al., 1983; Liu, 1993; Teixeira et al., 1994). AC has a very fine network of pores with large inner surface area on which many substances can be adsorbed (Thomas, 2008).

The interactive effects of wounding treatments and NAA and BAP concentrations on explants discoloration at the medium contact point produced significant results in weeks 2, (Figure 2). In this week, the highest level of discoloration was observed in 0.1 mg.l^{-1} NAA and 2 mg.l^{-1} BAP, in wounded and unwounded treatments. Whereas the lowest levels were observed in higher NAA and BAP concentrations (0.1 mg.l^{-1} NAA; 3 mg.l^{-1} BAP and 0.5 mg.l^{-1} NAA; 5 mg.l^{-1} BAP), in both unwounded and at a concentration of 0.5mg.l^{-1} NAA and 5mg.l^{-1} BAP in wounded explants. Similarly, in a study on *D. opposita* increased NAA and BAP concentrations effectively and reduced the discoloration of explants (Xu et al., 2009). In *Sorghum bicolor*, brown pigments completely inhibited shoot growth (Baskaran and Jayabalan, 2005). The addition of BAP to these cultures enabled the further growth of shoots. In a study on *Gossypium arboretum*, Smith et al. (1977) reported that both NAA and BAP alone did not support good growth or survival of explants. Various reports have revealed a strong synergistic effect in BAP and NAA interactions in banana (Novak et al., 1989; Okole and Schulz, 1996; Cote et al., 2000; Khalil et al., 2002; Srangsam and Kanchanapoom, 2007). The addition of these two plant growth regulators at an

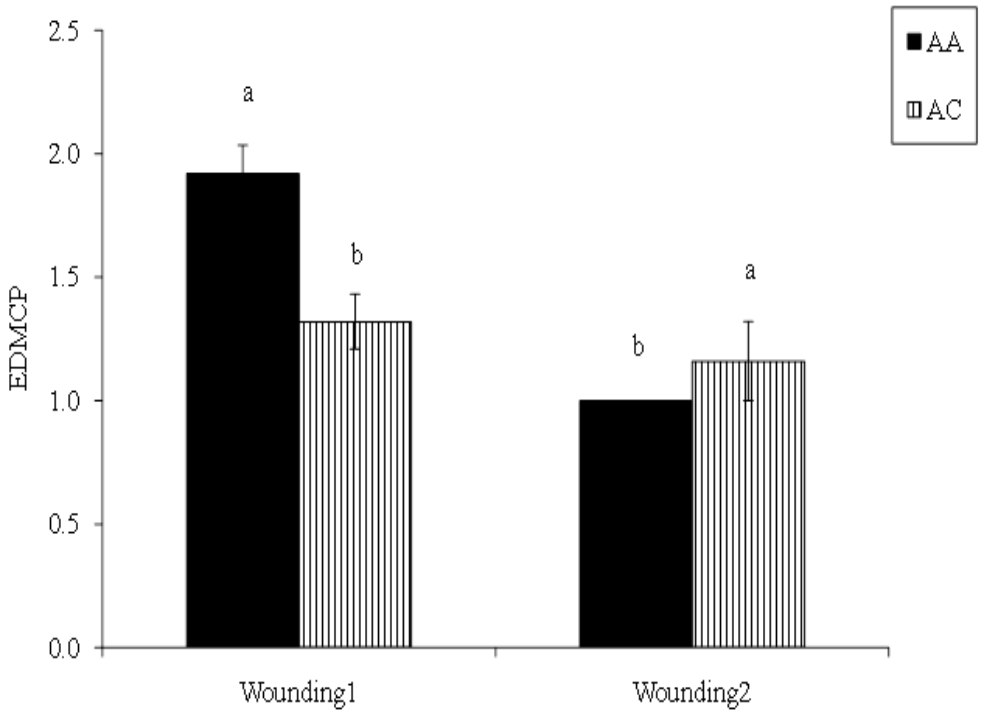

Figure 3. Interactive effects of antioxidants and wounding on explant discoloration at the medium contact point (EDMCP) in week 2. Rating scale used is 1 to 5 (1, No discoloration; 5, extreme discoloration). Wounding 1= Wounded; Wounding 2= unwounded.

increased concentration may reduce discoloration by enabling the establishment and further growth of plantlets

The interactive effects of antioxidants and wounding treatments gave rise to significant results on explants discoloration at the medium contact point in week 2 (Figure 3).

Both the lowest and highest levels of discoloration were observed in AA treatments. In wounded explants, AA was did not effectively reduce discoloration and resulted in the highest level. Whereas in unwounded explants, AA was the most effective antioxidant, resulting in the lowest level of discoloration, in wounding treatments AC significantly reduced explants discoloration at the medium contact point. As mentioned previously, wounded tissues produce an excess of polyphenolic compounds (George, 1993; Zeweldu and Ludders, 1998; Strosse et al., 2009), which result in an increased level of discoloration due to oxidative browning of explants. As mentioned previously, AC is most effective in reducing discoloration in wounded explants, by adsorbing phenols exuded. In complete, intact plantlets it is possible that AA may reduce discoloration by absorbing AA and prevent the oxidation of phenols on the target site (Ko et al., 2009).

The results in Figures 4 and 5 show the significant ($P \leq 0.05$) interactive effects of antioxidants and wounding on the formation of callus. The presence of AC completely inhibited the development of callus in both wounded and unwounded explants. An increased callus formation was observed in AA treatments, the highest level formed in wounded explants. This trend was observed throughout the duration of the experiment.

Although majority of reports confirm the positive role of AC in promoting the growth and development of plant tissues, AC inducing negative results is also reported in some cases (Thomas, 2008).

The difficulty in using AC is that its characteristic of high adsorptive power is non-selective. In addition to adsorbing inhibitory phenols, it is able to absorb high concentrations of growth regulators required by plant tissues (Fridborg et al., 1978; Ebert and Taylor, 1990; Nissen and Sutter, 1990; Ebert et al., 1993; Pan and van Staden, 1998; Thomas, 2008). As reported earlier, callus formation was induced by increased NAA and BAP concentrations, as opposed to no callus formation in the control (the treatment without NAA and BAP). Thus, it could be presumed that AC adsorbed the growth regulators required to induce callus formation.

In conclusion, although plant regeneration via axillary bud proliferation was not significantly increased, insight into the effects of NAA and BAP concentrations, antioxidants and wounding techniques on plant height, reducing explants discoloration, callus formation and root length was achieved. Future studies would be aimed at increasing the rate of axillary bud development and shoot regeneration from callus in efforts to improve the clone propagation in *S. reginae*.

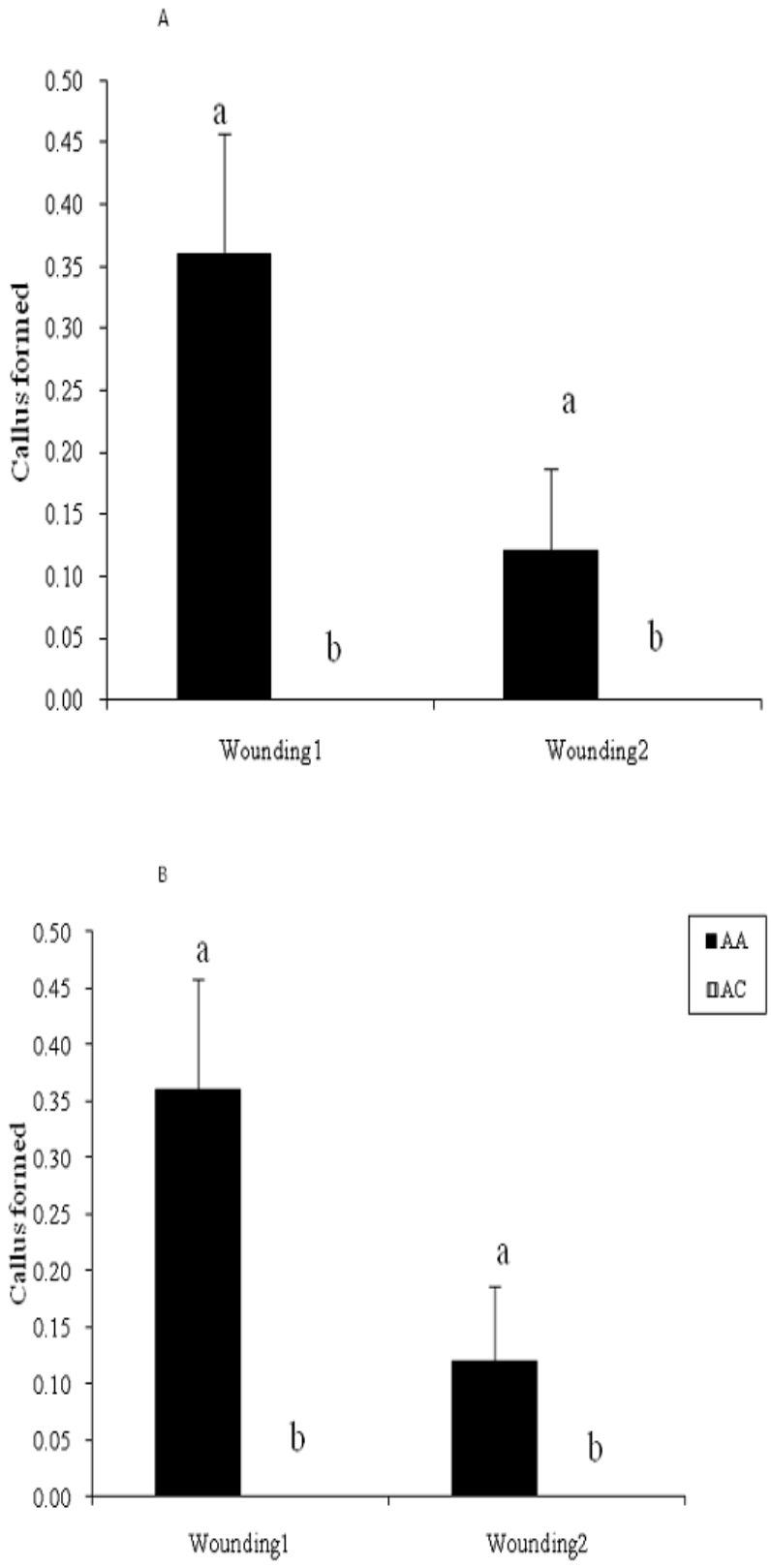

Figure 4. Interactive effects of antioxidants and wounding on callus formation in (A) WK3, (B) week 4, (C) week 5, (D) week 6. The degree of callus formation is rated as: 1, None; 2, low; 3, medium; 4, high. Wounding 1= Wounded; Wounding 2= unwounded.

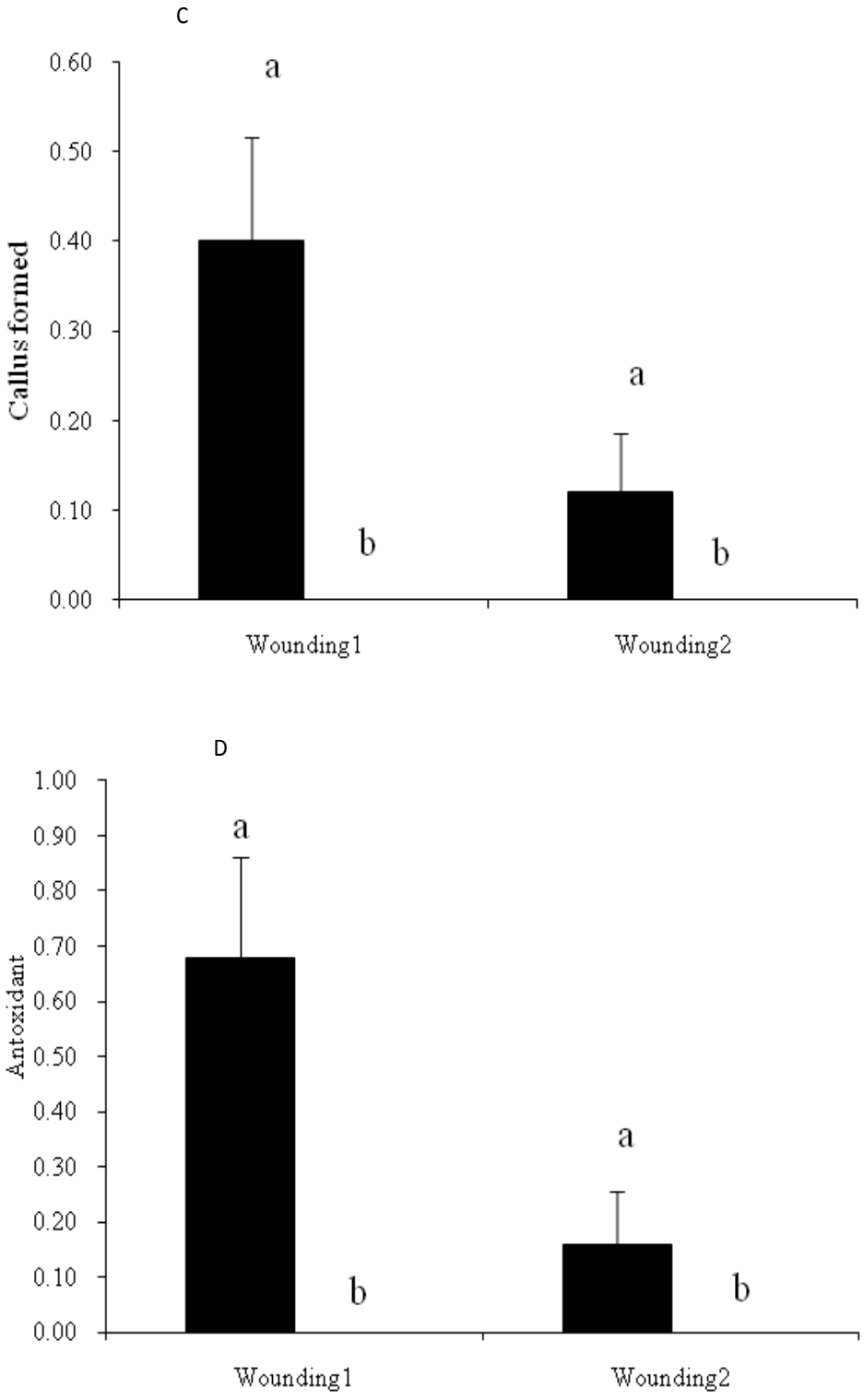

Figure 4. Contd.

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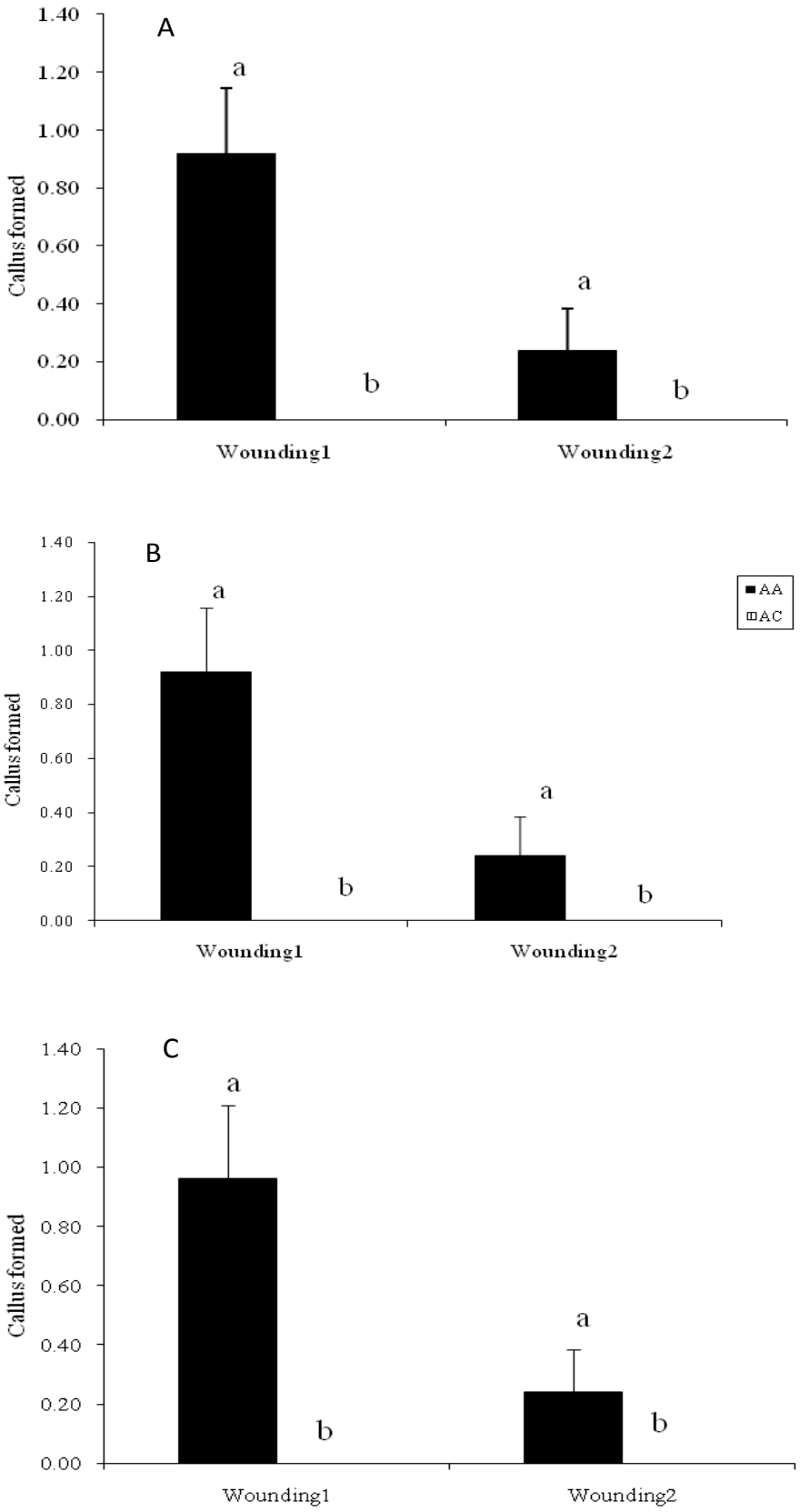

Figure 5, Interactive effects of antioxidants and wounding on callus formation in (A) WK7, (B) week 8 and (C) week 9. The degree of callus formation is rated as: 1, None; 2, low; 3, medium; 4, high. Wounding 1= Wounded; Wounding 2= unwoun

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