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Full Length Research Paper

Effects of antioxidants, plant growth regulators and wounding on phenolic compound excretion during micropropagation of *Strelitzia reginae*

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The aim of this study was to determine the effects of antioxidant treatments, plant growth regulators (PGRs) and explants wounding in tissue culture involving *Strelitzia reginae* on total phenol exudation. Results showed that various 1-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) concentrations significantly affected phenolic exudation. The media containing the highest plant growth regulators concentration (that is, 0.5 mg L^{-1} NAA and 6 mg L^{-1} BAP) resulted in the highest phenol content. Whereas, the control (the treatment free of plant growth regulators), contained the lowest phenol content. Activated charcoal (AC) was found to significantly reduce the total phenol content of media by 53%, compared with ascorbic acid (AA). Furthermore, the wounding of explants significantly increased phenolic exudation. Interactions between the higher 1-naphthalene acetic acid and 6-benzylaminopurine concentrations and ascorbic acid significantly increased the total phenol content of media. A similar result was achieved in interactions between higher plant growth regulators concentrations and the wounding of explants. Interactions between antioxidants, wounding treatments and plant growth regulators concentrations resulted in activated charcoal significantly reducing the total phenol content in all plant growth regulators concentrations in both wounded and unwounded explants.

Key words: Total phenol exudation, browning, wounding, activated charcoal, ascorbic acid, 6-benzylaminopurine (BAP), NAA - 1-naphthalene acetic acid (NAA).

INTRODUCTION

The bird of paradise (*Strelitzia reginae*) is of significant commercial value (Paiva et al., 2004). *S. reginae* has been one of the most sought after cut flowers destined for exportation from developing countries (Criley, 1988). However, its success is limited by the slow conventional methods of propagation (North et al., 2010). Due to these constraints on propagation, there is considerable interest in the development of reliable tissue culture techniques for this plant. However, *S. reginae* has proven to be a difficult plant for *in vitro* culture. Tissue culture attempts of this plant have had limited success due to the oxidative browning of explants (Promtep, 1981; Ziv and Halevy, 1983; Paiva et al., 2004; Kantharaju et al., 2008). This crucial problem was also frequently encountered in genera related to *Strelitzia*, namely *Musa* and *Ensete* spp. (Zeweldu and Ludders, 1998; Birmeta and Welander, 2004; Diro and van Staden, 2004; Titov et al., 2006; Martin et al., 2007; Ko et al., 2009). The browning and subsequent death of cultured explants is a major problem that is usually dependent on the phenolic compounds and the quantity of total phenols (Ozyigit, 2008). Phenolic compounds occur as secondary metabolites in all plant species (Antolovich et al., 2000;

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Abbreviations: MS, Murashige and Skoog (1962); **AC,** activated charcoal; **AA,** ascorbic acid; BAP, 6-benzylaminopurine; **NAA,** 1-naphthalene acetic acid; **PGR,** plant growth regulator; **uL,** microliter; **rpm,** revolution per minute; **nm,** nanometer.

Kefeli et al., 2003). The phenols are synthesized by the plants and in many cases excreted and then oxidized (Ozyigit, 2008). In tissue culture studies, phenolic substances, especially oxidized phenols generally affect *in vitro* development negatively (Arnaldos et al., 2001). Oxidized phenolic compounds may inhibit enzyme activity and result in the darkening of the culture medium and subsequent lethal browning of explants (Compton and Preece, 1986; Laukkanen et al., 1999).

Activated charcoal is commonly used in tissue culture media to improve cell growth and development (Pan and van Staden, 1998; Thomas, 2008). The beneficial effects of AC may be attributed to its irreversible adsorption of inhibitory compounds in the culture medium and substantially reduce the toxic metabolites, phenolic exudation and exudate accumulation (Fridborg et al., 1978; Thomas, 2008). This high adsorptive capacity is due to the structure of AC. It has a very fine network of pores with a large inner surface area on which many substances can be adsorbed (Pan and van Staden, 1998; Dąbrowski et al., 2005; Thomas, 2008).

The antioxidant, ascorbic acid, was selected as it has been used successfully in the past to inhibit the exudation of phenols (Strosse et al., 2004) and to reduce oxidative browning in various plant species (Arditti and Ernst, 1993; George, 1996; Abdelwahd et al., 2008). AA is able to scavenge oxygen radicals produced when the plant tissue is wounded, therefore protecting the cells from oxidative injury. The oxidative browning of explant tissue is reduced by AA detoxifying these free radicals (Titov et al., 2006). Thus, AA is useful and effective in managing the problem of phenolics and improving plant growth *in vitro* (Abdelwahd et al., 2008).

Phenolic concentration is often affected by several internal and external factors (Zapprometov 1989). Some nutrients (Lux-Endrich et al., 2000) and some stress factors like drought, water, radiation and pathogen infection from injured surfaces effect concentrations of the phenolics in plants (Zapprometov 1989; Kefeli et al., 2003). The various PGR concentrations may affect phenolic exudation as phenols are reactive compounds (Lux-Endrich et al., 2000).

A study was carried out to determine the optimal antioxidant, PGR concentration and wounding treatment in efforts to stimulate axillary bud proliferation and overcome the problem of phenolic oxidation for the successful *in vitro* regeneration of *S. reginae*. AC and AA were incorporated in culture media for a comparative study to elucidate the most effective in reducing phenolic exudation. It is well-documented that apical dominance is under the control of various growth regulators (Wickson and Thimann, 1958; Woolley and Wareing, 1972; Cline, 1994). Thus, the proportions of PGRs in the media were manipulated in an effort to break dormancy and produce shoots (Razdan, 1993). In addition, meristem wounding was tested to stimulate the proliferation of axillary buds, which are otherwise suppressed by apical dominance.

In view of the above, the main objective of this study was (i) to determine the total amount of phenol excreted into the culture media within different treatments and (ii) to establish the relation between antioxidants, PGR concentrations and wounding on phenol exudation. This will provide insight into the processes contributing to the exudation of phenols and how these can be minimized as this is critical for successful *in vitro* culture of *S. reginae*.

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 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 BAP 0, 2, 3, 5, 6 mg L^{-1} and NAA 0, 0.1, 0.5 mg L^{-1} were added to the media. The antioxidants, 2.5 g L^{-1} activated charcoal and 0.05 g L^{-1} ascorbic acid, 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.

Chemical analysis

After 9 weeks of growth, the explants were removed and the total amounts of phenols in the culture media (for excreted phenols from explants to medium) were analyzed according to Folin-Ciocalteu method (Singleton and Rossi, 1965; Chandler and Dodds, 1983; Singleton et al., 1999) by using gallic acid as the standard and the results were given as gallic acid equivalents (Waterman and Mole, 1994).

Sample preparation for the determination of total phenols

In this study, ten replicates were used for each treatment. For each sample, 30 g of the culture media was extracted with 15 ml of methanol on a rotary mixer for 30 min. This was then centrifuged for 10 min at 4000 rpm (revolutions per minute). The supernatant was used in the analysis of the phenols. For the assay, 25 microliter (µL) of the supernatant was mixed with 125 µl Folin reagent (0.2 M), followed by 100 µl sodium carbonate (7.5%) in a 96-well clear plate. This was left to incubate for 2 h at room temperature. The plate was then read in a Multiskan plate reader (Thermo Electron Corporation, USA) at a wavelength of 765 nm (nanometer). Total phenols in the samples were expressed as gallic acid equivalents using a standard curve with a gallic acid concentration range of between 0 and 500 mg L^{-1} (Singleton and Rossi, 1965; Chandler and Dodds, 1983; Singleton et al., 1999).

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

Statistical analysis

Results obtained were analyzed for statistical significance using analysis of variance (ANOVA). These computations were done with the STATISTICA Software Programme version 2010 (StatSoft Inc., Tulsa, OK, USA). The Fisher's least significance difference was used to compare treatment means at $P = 0.05$ level of significance (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

Effects of various NAA and BAP concentrations, antioxidants and wounding treatments on phenolic exudation

The various NAA and BAP concentrations significantly affected phenolic exudation from explants into the culture media. The total phenol content (mg.L^{-1}) of culture media generally increased with the increasing concentrations of NAA and BAP (Table 2). The media with the highest PGR concentration (0.5 mg L^{-1} NAA and 6 mg L^{-1} BAP) contained the highest phenol content. This media contained 17.58 mg L^{-1} total phenols, as opposed to the control (the treatment free of plant hormones) which contained 12.9 mg L^{-1}, resulting in a 27% increase.

According to Lux-Endrich et al. (2000), many phenols are reactive compounds synthesized in plant tissues. Furthermore, Chamandoosti (2010) reported a relation between chemical composition of the media and phenolic exudation, media discoloration and explant browning and death. The results in this study are in agreement with Taviera et al. (2009) and Sayd et al. (2010) who found that media supplemented with increased NAA and BAP concentrations produced higher phenolic compound content. In other related studies, NAA and BAP are reported to have played an important role in the bio-synthesis of secondary metabolites in *in vitro* culture (Shilpashree and Rai, 2009). Therefore, total phenolic compounds in tissue culture can be minimized with the selection of suitable media constituents.

Activated charcoal significantly reduced the phenol content in culture media. A 53% reduction of phenols was recorded in media supplemented with AC, compared with those supplemented with AA (Table 2). Similar to our results, Birmeta and Welander (2004) reported AC as more effective than AA in reducing polyphenol exudation in *Ensete ventricosum* (Musaceae). Several researchers have also reported the success of AC in controlling the oxidative browning (which is associated with phenol production) of explants in tissue culture (Chang et al., 2001; Diro and van Staden, 2004; Wang et al., 2005; Guo et al., 2007; North et al., 2010; North et al., 2011). The incorporation of AC to media is an established practice that is most effective in controlling polyphenol exudation (Carlberg et al., 1983; Liu, 1993; Teixeira et al., 1994; Pan and van Staden, 1998; Chawla, 2002; Diro and van Staden, 2004; Kiong et al., 2007). The adsorption of phenols in the medium prevents the browning of tissues (Horner et al., 1977; Fridborg et al., 1978; Weatherhead et al., 1979; George and Sherrington, 1984; Madhusudhanan and Rahiman, 2000; Chawla, 2002).

Wounding treatments significantly affected the exudation of phenols into the culture medium. Wounded explants exuded 30% more phenols than unwounded explants (Table 2). These results indicate a strong relationship between total phenolics content and wound-ing. Tissue injury stimulates the production of phenols (Dodds and Roberts, 1995) and phenolic exudation is exaggerated in response to wounding (George, 1993; Zeweldu and Ludders, 1998; Strosse et al., 2009). The deposition of phenolic acids in plant cell walls is an important defense mechanism (Bolwell et al., 1985; Pan and van Staden, 1998; Ndakidemi and Dakora, 2003), which exerts an inhibitory growth function when they are excreted from the plant (Kefeli et al., 2003). When cells are damaged, like the wounding performed in this study, the sub-cellular compartmentation is lost, enabling the contents of cytoplasm and vacuoles to mix and phenolic

Table 2. Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on phenol exudation (mg l^{-1}) into culture media.

Parameter	Phenols mg L^{-1}
Concentration	
Control	12.90[b]
NAA 0.1 mg L^{-1} + BAP 2 mg L^{-1}	14.01[b]
NAA 0.1 mg L^{-1} + BAP 3 mg L^{-1}	13.86[b]
NAA 0.5 mg L^{-1} + BAP 5 mg L^{-1}	16.67[a]
NAA 0.5 mg L^{-1} + BAP 6 mg L^{-1}	17.58[a]
Antioxidant	
Activated charcoal	9.57[b]
Ascorbic acid	20.48[a]
Wounding	
Wounded	17.69[a]
Unwounded	12.35[b]
F value	
Concentration	8.5***
Antioxidant	322.3***
Wounding	77.3***
Interaction	
Concentration*antioxidants	9.4***
Concentration*wounding	3.9*
Antioxidants*wounding	5.9*
Concentration*antioxidants*wounding	3.8*

*: $P \leq 0.05$; ***: $P \leq 0.001$. Means followed by dissimilar letters in a column are significantly different by least significant difference test at $P=0.05$.

compounds readily become oxidized by air (Compton and Preece, 1986; Laukkanen et al., 1999). Phenol oxidation and exudation takes place in these scarred surface cells (Ozyigit, 2008). Oxidized phenolic compounds may inhibit enzyme activity and result in darkening of the culture medium and subsequent lethal browning of explants (Compton and Preece, 1986; Laukkanen et al., 1999).

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

The interactive effects of various NAA and BAP concentrations and antioxidant treatments significantly affected the severity of total phenolic compound excretion (Figure 1). In all NAA and BAP concentrations, AC was more effective than AA in reducing phenolic excretion. A significantly reduced level of total phenols was recorded in AC supplemented media. The lowest phenol content of media occurred in AC supplemented media with 0.1 mg L^{-1} NAA and 3 mg L^{-1} BAP. The highest amount of total phenols was recorded in AA supplemented media with the increased concentration of 0.5 mg L^{-1} NAA and 5 mg L^{-1} BAP. In AA treatments, the total phenol content of media increased with the increasing NAA and BAP concentrations.

As reported earlier in this study, the highest PGR concentration (0.5 mg L^{-1} NAA and 6 mg L^{-1} BAP) resulted in the highest phenolic content of culture media. In addition, AC was reported to be 53% more effective than AA in reducing the phenolic exudation. The interactive effects of the higher PGR concentrations and AA resulted in significantly increased exudation of phenols into culture media.

Interactions between NAA and BAP concentrations and wounding treatments significantly affected the amount of phenols explants excreted into the culture media (Figure 2). The increasing NAA and BAP concentrations increased the total phenol content of media in both wounded and unwounded treatments. Wounding treatments significantly increased the severity of phenolic compound

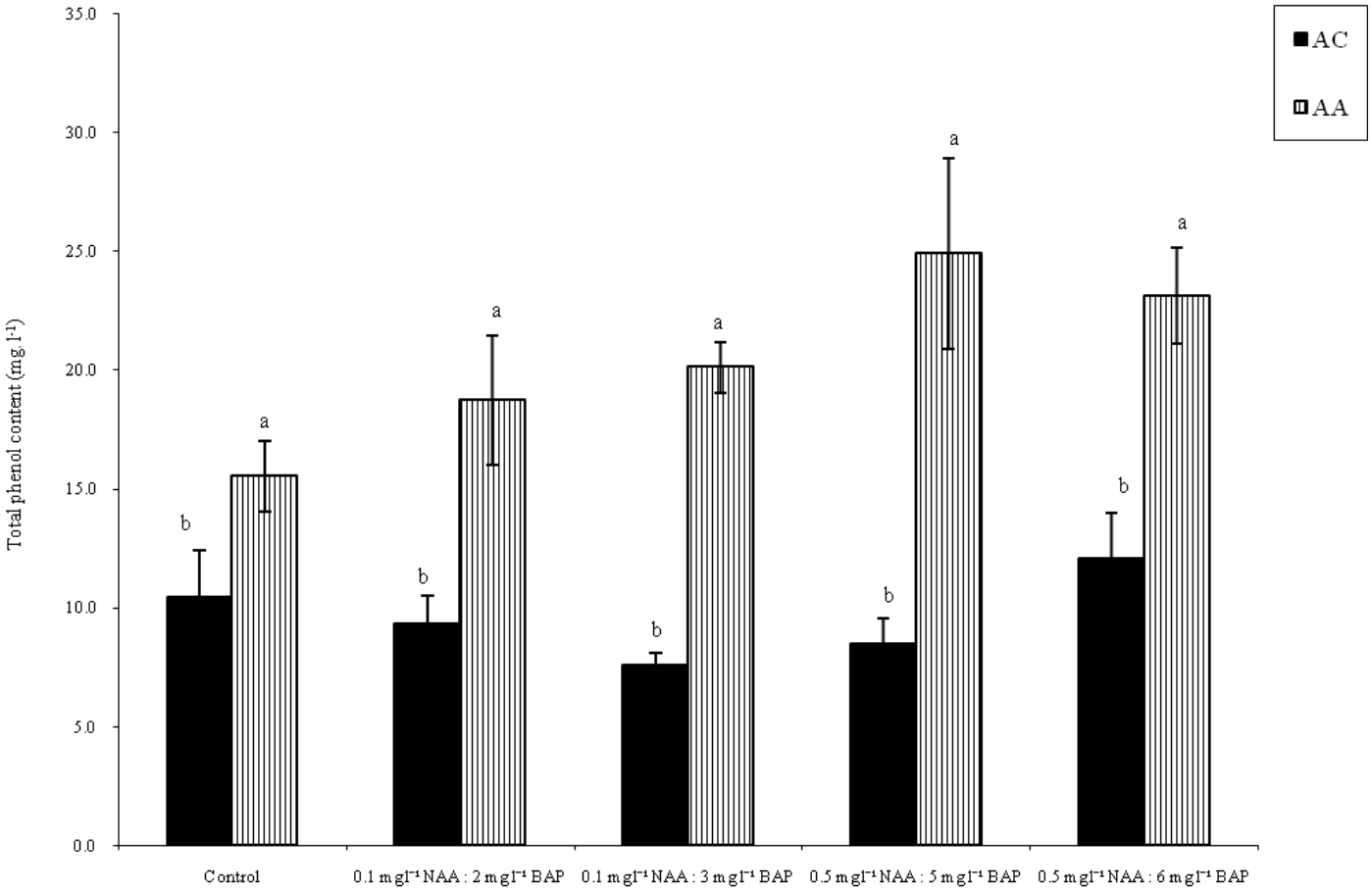

Figure 1. Interactive effects of NAA and BAP concentrations and antioxidants on total phenol content of culture media (mg L^{-1}). Bars followed by dissimilar letters are significantly different by Fischer's LSD test at $P = 0.05$.

excretion, compared with the unwounded explants. Wounding increased the phenol exudation in all NAA and BAP concentrations. However, the highest total phenol content was recorded in wounded explants in the highest PGR concentrations.

Wounding is reported to stimulate phenol production and exaggerate phenolic exudation (George, 1993; Dodds and Roberts, 1995; Zeweldu and Ludders, 1998; Strosse et al., 2009). The strong relationship between the total phenolic content and wounding has been demonstrated earlier in this study. Increased levels of phenolic exudation in response to higher PGR concentrations have also been reported earlier in this study. The interactions between these PGR and wounding treatments resulted in a significant increase in total phenolic content of culture media.

The significant interactive effects of antioxidant and wounding treatments on the exudation of phenols from explants to culture medium are indicated in Figure 3. The addition of AC to culture media resulted in a significantly reduced phenolic content, compared with AA supplemented treatments. With the lowest total phenol content recorded in AC supplemented media supporting unwounded explants. The most intense phenolic exudation was recorded in wounded explants in culture medium supplemented with AA. This was followed by the AA treatment with unwounded explants. Activated charcoal with wounded explants even proved to be more effective than AA with unwounded explants. The incorporation of AC to culture media is widely reported to be most effective in decreasing phenol oxidation and exudate accumulation (Carlberg et al., 1983; Liu, 1993; Teixeira et al., 1994; Pan and van Staden, 1998; Chawla, 2002; Diro and van Staden, 2004; Kiong et al., 2007; Thomas, 2008). Although AA is also widely reported to reduce the oxidative browning of explants (Wu and du Toit, 2004; Abeyaratne and Lathiff, 2002), it did not effectively control phenol exudation in either wounded or unwounded explant treatments, compared with AC.

The results in Figure 4 represent the significant interactive effects of NAA and BAP concentrations, antioxidant treatments and wounding on the total phenol content of culture media. The lowest phenol content occurred in unwounded explants sustained in AC

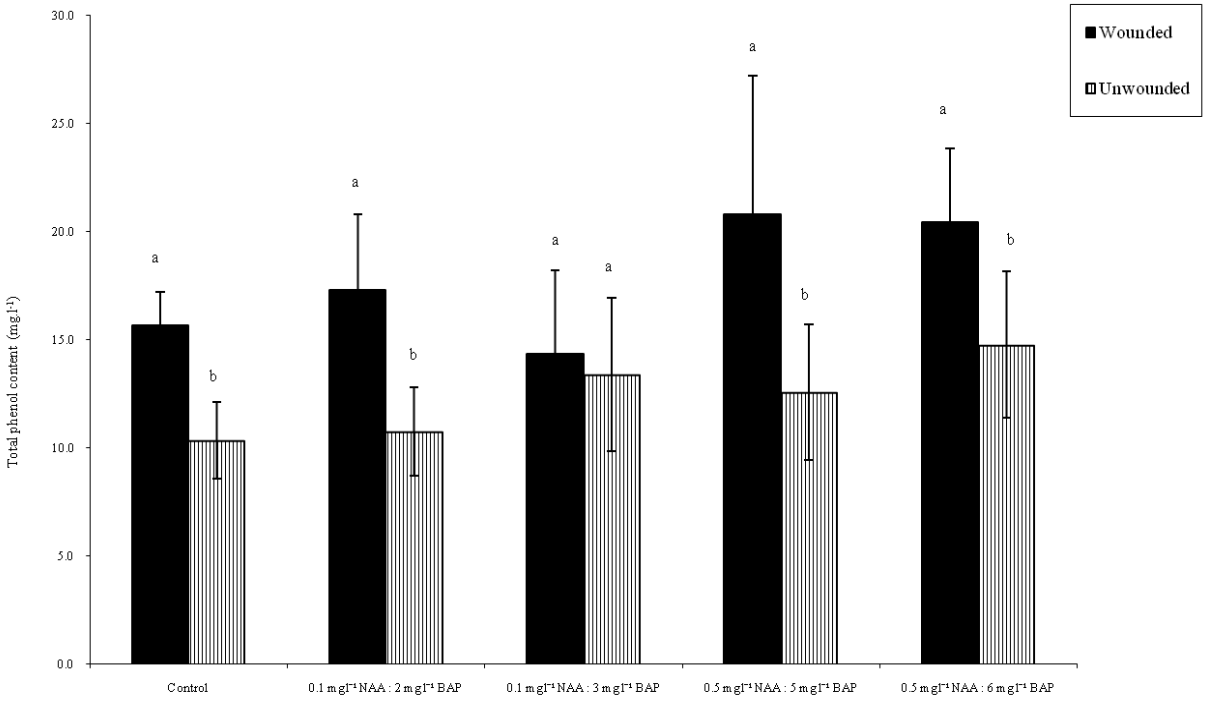

Figure 2. Interactive effects of NAA and BAP concentrations and wounding on total phenol content of culture media (mg L^{-1}). Bars followed by dissimilar letters are significantly different by Fischer's LSD test at $P = 0.05$.

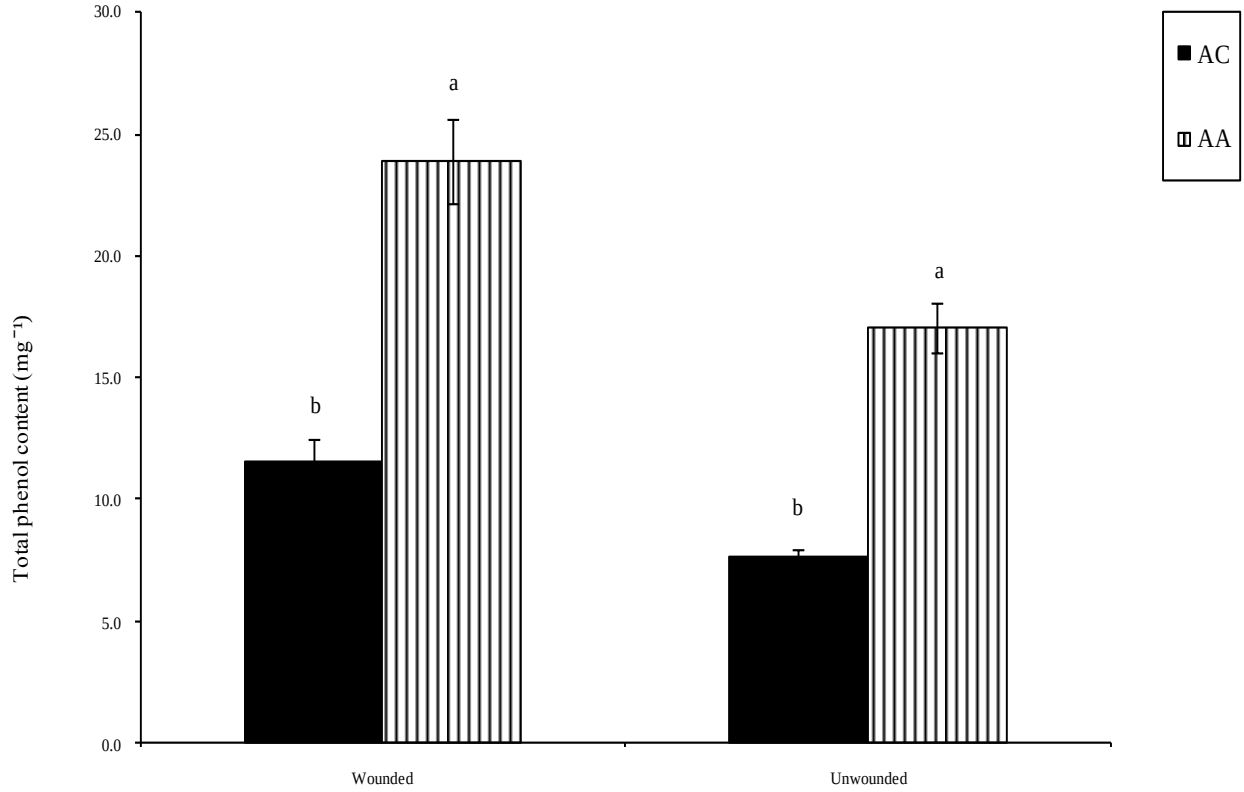

Figure 3. Interactive effects of antioxidants and wounding on total phenol content of culture media (mg L^{-1}). Bars followed by dissimilar letters are significantly different by Fischer's LSD test at $P = 0.05$.

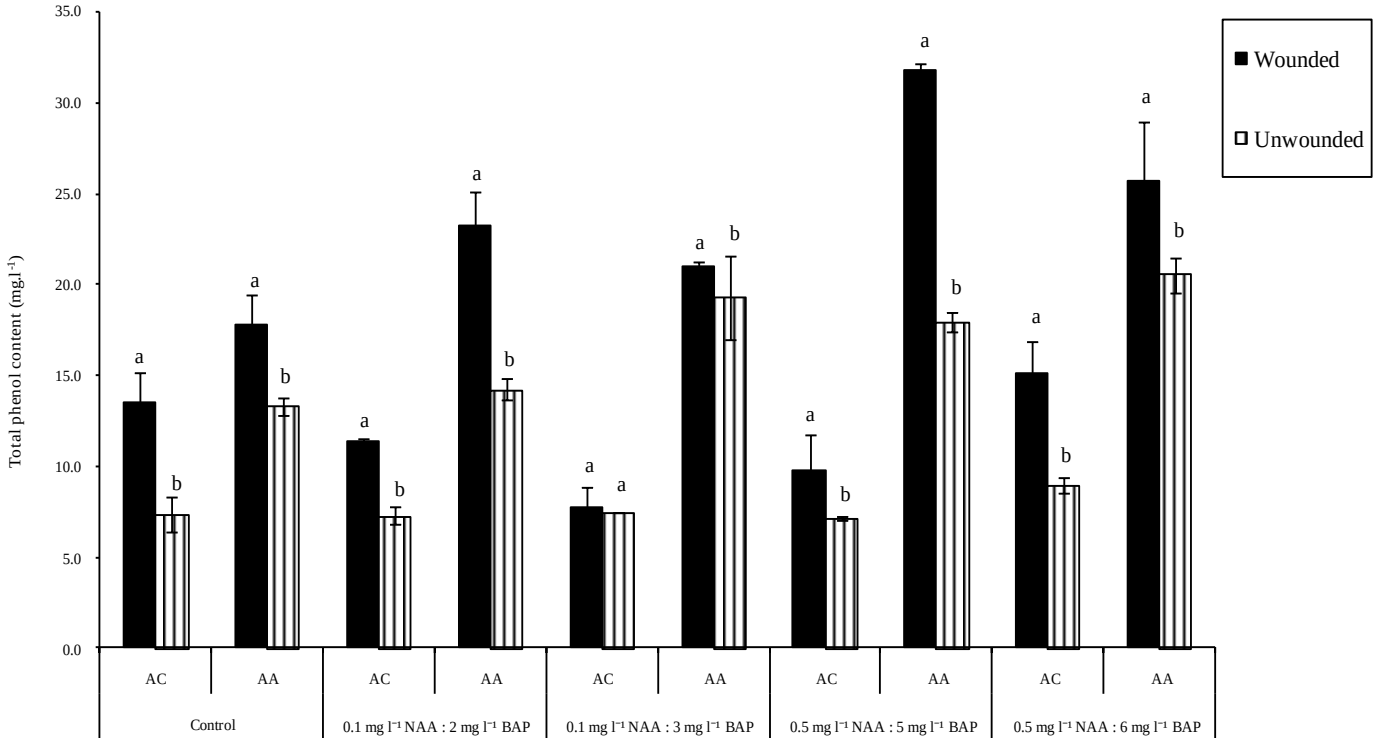

Figure 4. Interactive effects of NAA and BAP concentrations, antioxidants and wounding on total phenol content of culture media (mg L^{-1}). Bars followed by dissimilar letters are significantly different by Fischer's LSD test at $P = 0.05$.

supplemented media, across all NAA and BAP concentrations. This was followed by the wounded explants in the presence of AC. Despite damage to the tissue as a result of wounding techniques, AC still significantly reduced the phenolic exudation, in comparison with AA treatments. The most effective treatment in reducing phenol content in wounded explants was that of AC with 0.1 mg L^{-1} NAA and 3 mg L^{-1} BAP. In AA treatments, wounding increased the severity of phenolic exudation, with the highest phenol content recorded in 0.5 mg L^{-1} NAA and 5 mg L^{-1} BAP. With unwounded explants in AA, the phenol content generally increased with the increasing NAA and BAP concentrations.

Interactions between AA, higher PGR concentrations and wounding treatments resulted in highest total phenol content of culture media. In AC supplemented media, in both wounded and unwounded explants treatments, the concentration of PGRs did not significantly affect the total phenol content of media. The phenol content did not increase with the increasing PGR concentration. This may be due to AC adsorbing PGRs present in the media. AC has the characteristic property of high adsorptive power (Thomas, 2008). It is capable of adsorbing 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).

In conclusion, the reactive response of phenolic compounds to media composition and wounding is demonstrated in this study. The present work indicates the significant effects of PGR concentrations, antioxidants and wounding on the total exudation of phenolic compound. Furthermore, the interactive effects of these treatments on phenol exudation are exposed.

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