Cape Peninsula
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AN INVESTIGATION INTO THE POTENTIAL OF DEVELOPING AN *IN VITRO* METHOD FOR PROPAGATING STRELITZIACEAE.

by

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DECLARATION

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ABSTRACT

A study was conducted to investigate the effects of: i) various media compositions and wounding treatments on the *in vitro* germination, growth and regeneration of *Strelitzia reginae* plantlets derived from zygotic embryos, ii) antioxidants, plant growth regulator (PGR) concentrations and plant tissue wounding treatments on phenolic compound production.

One experiment consisted of 8 medium types including different combinations of Murashige and Skoog (MS) medium strength, activated charcoal and vitamin supplements. Twelve replicates were used for each treatment. In another experiment, germinated plantlets were subjected to 2 wounding treatments; (unwounded explants (control) and explants longitudinally sectioned through the apical meristem). The explants were transferred to ten different regeneration media consiting of different concentrations and combinations of auxin and cytokinin supplements and antioxidants. Ten replicates were used for each treatment.

Results indicated the positive role of activated charcoal (AC) in reducing oxidative browning of embryo explants. The highest germination rate of embryos was observed in media containing AC without vitamin supplementation. Germination significantly decreased with the addition of vitamins. With regard to effects of various media compositions and wounding treatments on *in vitro* growth and regeneration of *Strelitzia*, significant results were achieved with 1-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) concentrations on explant 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 affected plant height, increased explant height and callus formation. Interactions between higher NAA and BAP concentrations together with wounding resulted in the most effective treatment in reducing explant discoloration at the media contact point. Furthermore, results showed the various NAA and BAP concentrations to significantly affect phenolic exudation. The media containing the highest PGR concentration resulted in the highest phenol content. AC significantly reduced the total phenol content of media by 53%, compared with AA. Phenolic exudation was significantly increased as a result of explant wounding. Various interactions between the NAA and BAP concentrations, antioxidants and wounding affected phenolic exudation and the total phenol content of media.

This study provides insight into the contributing factors and methods of overcoming the major problem of phenolic oxidation and promoting the *in vitro* growth and regeneration of *Strelitzia*.

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DEDICATION

I dedicate this thesis to my

Grandmother, Joan Bursey,

who has always inspired and encouraged me.

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GLOSSARY

Terms/Acronyms/Abbreviations	Definition/Explanation
MS	Murashige and Skoog
Anova	Analysis of variance
AC	Activated Charcoal
AA	Ascorbic Acid
BAP	6-Benzylaminopurine
NAA	Naphthaleneacetic acid
PGR	Plant growth regulator
uL	Microliter
rpm	Revolution per minute
nm	Nanometer

CHAPTER ONE

LITERATURE REVIEW AND INTRODUCTION

Review

THE POTENTIAL OF DEVELOPING AN *IN VITRO* METHOD FOR PROPAGATING STRELITZIACEAE

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1.1 Abstract

Strelitzia spp. are highly valued as cut flowers and are of significant commercial value. Despite high demands, they have not been widely spread due to production constraints and are one of the few important cut flower plants for which no uniform cultivars are available. The conventional methods of propagation are very slow due to the plants low rate of multiplication. Large scale propagation and cloning is therefore needed to exploit their potential. Despite the plants commercial importance, a method for micropropagation has not yet been established. Attempts to propagate these species by tissue culture have failed due to the oxidative browning of explants. Wounded tissues release polyphenolic compounds which are detrimental to further development of explants. Only partial success and a low rate of multiplication have been obtained. This review explores the possibilities of developing an *in vitro* method for the successful propagation of *Strelitzia* spp.

Key words: *Strelitzia* spp., activated charcoal, antioxidants, auxins, cytokinins, dark incubation, immature embryos, media composition, wounding.

Abbreviations: BAP, 6-Benzylaminopurine; **NAA,** naphthaleneacetic acid; **MS,** Murashige and Skoog.

1.2 INTRODUCTION

The bird of paradise (S*trelitzia* spp.) is an important ornamental monocotyledonous plant of South African origin (Chand, 2008). This tropical perennial is a beautiful plant of significant commercial value (Paiva et al., 2004). The exotic features of its colourful flowers, the long length of the stem and the high post-harvest durability result in it being highly valued as a cut flower (Wood, 1995). *Strelitzia reginae* has been one of the most sought after cut flowers destined for exportation from developing countries (Criley, 1988). However, its commercial exploitation and success is limited by its low rate of multiplication (Ziv and Halevy, 1983). Propagation is achieved either by seed or vegetatively by division. Both of these conventional propagation methods are very slow (Karnataka, 2008).

Propagation by seed is undesirable as *Strelitzia* presents a slow development, requiring 4 to 7 years to start producing flowers (Ziv and Halevy, 1983). Furthermore, from pollination to seed harvesting, a further 5 to 6 months is required (Criley, 1988). However, the greatest difficulty in the propagation of *Strelitzia* spp. by seed is the dormancy of seeds (Garcia, 2006). A germination inhibitor is thought to occur within the seeds (van de Venter and Small, 1975), which prolongs the time and results in a low percentage of seed germination (Garcia, 2006). The limited production of seeds obtained per plant must be taken into consideration. There is also a great degree of genetic variation in plants developed from seed (van de Pol and van Hell, 1988).

For vegetative propagation by division, plants of at least 10 years old must be used (Ziv and Halevy, 1983). This method of dividing naturally developed branches is limited by a low rate of multiplication, which was determined to be 0.5 to 1.5 divisions per branch per year (Vonk Noordegraaf and van der Krogt, 1976).

Thus, both methods limit the large scale production which is needed to exploit the plants potential and to enhance its improvement by cultivar development. Due to these constraints on production, it is one of the few important cut flower plants for which no uniform cultivars are commercially available (Ziv and Halevy, 1983).

Tissue culture could be more promising than other conventional methods of multiplying *Strelitzia* (Promtep, 1981). A reliable and advanced propagation and cloning method would greatly contribute to overcoming the limitations this species poses to the horticultural and landscaping industry worldwide (Ziv and Halevy, 1983). Despite the plants commercial importance, a reliable method for micropropagation has not yet been developed (Chand, 2008).

The lack of successful tissue culture techniques for the propagation of *S. regina* was due to oxidative browning of the explants as reported earlier by Ziv and Halevy (1983). Wounded tissue releases polyphenolic compounds, which diffuse into the culture medium (Strosse et al., 2009). These undesirable exudates were found to be detrimental to the development of the explants, as they promoted the onset of necrosis (Ziv and Halevy, 1983). Terminal and axillary meristems of *S. reginae* were used by Ziv and Halevy (1983) but success depended on the use of antioxidants to prevent browning. This is a destructive method as plants are destroyed when their terminal and axillary buds are excised. Thus, it is an unsuitable method when starting material is limited or in the culture of rare plants. Paiva et al. (2004) reported the failure of *in vitro* development using axillary buds and leaf segments, irrespective of the applied treatments. Phenolic oxidation was identified as a crucial problem. Thus, it was not possible to reproduce the protocol developed by Ziv and Halevy (1983) for the *in vitro* propagation of *S. reginae* from axillary buds. However, the germination of immature embryos inoculated *in vitro* resulted in well-formed and complete plants (Paiva et al., 2004). There are no reports on success or attempts made in the stimulation of axillary bud proliferation from embryo-derived plantlets.

In several investigations, 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, suggesting that the protocols developed so far have not been efficient with respect to the growth and large scale multiplication of this plant.

The objective of this review is to discuss possible techniques for the rapid clonal propagation of *Strelitzia* spp., using embryos as the starting material. Zygotic embryo culture could induce faster growth and a higher germination rate (Chang and Yang, 1996; Bürün and Çoban Poyrazoğlu, 2002). The use of embryos as explants will allow the variation in seeds (Foolad and Jones, 1992; Larkin et al., 1984) to play an important role in the breeding cycle and development of cultivars, reducing seed dormancy. Furthermore, the limited wounding of the explants may remedy the production of phenolic exudates and the excessive need for antioxidants during the crucial initial stages of plant development. In addition, it is not a destructive method. There is no need to destroy plants in order to gain starting material for the culture.

Thus, indicating the possibility of developing an efficient method of clonal propagation and mass production of this kind of plant in the future.

1.3 POSSIBLE EFFECTS OF VARIOUS MEDIA COMPOSITIONS ON THE *IN VITRO* GERMINATION OF IMMATURE EMBRYOS OF *STRELITZIA* SPP.

The germination of excised embryos is influenced by the maturity of embryos at excision, the composition of the culture medium (Johri and Rao, 1984; Diro and van Staden, 2004) and genetic variation (Pierik, 1979).

Embryos excised from developing seed at or near maturity are completely autotrophic (Gamborg and Phillips, 2002), whereas, immature embryos require a far more critical medium composition compared with mature embryos (Pierik, 1979). The younger the embryo, the more complex is its nutritional requirements (Bajaj, 1977). The chances of success in this type of culture depend largely on the developmental stage of the excised embryo (Razdan, 1993). Paiva et al. (2004) reported that the best time for seed collection and embryo excision for *S. reginae* was 20 weeks after pollination. However, the most important aspect in culturing embryos is to develop and clearly define a culture medium that can sustain growth and development (Chawla, 2002). In less than optimum medium, the immature embryos may fail to survive, turn into undifferentiated callus, or germinate prematurely (Gamborg and Phillips, 2002).

1.3.1 A variation of macro- and micro-nutrients

The MS medium of Murashige and Skoog (1962) is a salt composition that supplies the needed macro- and micronutrients. To achieve growth differentiation, concentrations of inorganic nutrients must be optimized such that the medium meets the requirements of the cells or tissues used (Chawla, 2002). This is applicable to the nutritional requirements of an embryo and its developmental stage. The *in vivo* development of an embryo consists of two phases:

(1) The heterotrophic phase: An early phase wherein the embryo is nutritionally dependent on the endosperm and maternal tissues.
(2) The autotrophic phase: A later phase in which the embryo is metabolically capable of synthesising substances required for its growth, thus is fairly independent for nutrition.

Thus, while relatively mature embryos can grow on a simple inorganic salt medium supplemented with a carbohydrate source, the nutritional requirements of relatively immature embryos is complex (Bajaj, 1977). The critical stage at which the embryo passes from the heterotrophic phase into the autotrophic phase varies with the species (Razdan, 1993).

The developmental phase and nutritional requirements of *Strelitzia* embryos 20 weeks after pollination is uncertain. Therefore, it is necessary to evaluate a variation in inorganic nutrients (macro and micronutrients) and vitamins to obtain optimum embryo growth and development.

1.3.2 The use of activated charcoal as a phenolic adsorbent

The use of tissue culture for the propagation of *S. reginae* has invariably failed due to oxidative browning of the explants (Ziv and Halevy, 1983). During the initial stages of culture development, the production of polyphenols is excessive (Pan and van Staden, 1998). The polyphenol exudate that diffuses into the medium was found to be detrimental to the further development of the explants, which become necrotic and die (Ziv and Halevy, 1983).

The identified problem of phenolic oxidation has also been reported in *Musa* and *Ensete* spp. (Zeweldu and Ludders, 1998; Birmeta and Welander, 2004; Diro and van Staden, 2004), which are related to *Strelitzia* (Strosse et al., 2009). Activated charcoal, used in tissue culture media to adsorb inhibitory substances, has been used with success in Musaceae and Strelitziaceae (Diro and van Staden, 2004; Ziv and Halevy, 1983). In *Ensete*, a genus of Musaceae, the use of activated charcoal enabled zygotic embryos to regenerate healthy seedlings. The inclusion of activated charcoal into the media reduced oxidative browning, promoted germination of the embryos and improved the growth of seedlings (Diro and van Staden, 2004). In *S. reginae*, Ziv and Halevy (1983) found activated charcoal in combination with antioxidant treatments, effective in controlling oxidative browning of terminal and axillary buds. However, there are no reports on the use of activated charcoal on the zygotic embryos of *S. reginae* or other *Strelitzia* spp.

Strelitzia competence in responding to *in vitro* culture is dependent on reducing oxidative browning of the explants (Ziv and Halevy, 1983). Thus, an investigation into the addition of activated charcoal to culture media is of vital importance in the development of a suitable technique for the germination of excised embryos, as has been done in *Ensete*.

1.4 POSSIBLE EFFECTS OF AUXIN AND CYTOKININ CONCENTRATIONS ON INDUCING MULTIPLE SHOOTS FROM AXILLARY BUDS

This involves the use of plant growth regulators in an attempt to stimulate the development of axillary buds, which are usually present in the axil of each leaf (Chawla, 2002). Axillary bud proliferation exploits the normal ontogenetic route for branch development by lateral meristems (Gamborg and Phillips, 2002). However, many axillary meristems remain dormant

in vivo (in nature) if the type of branching for a particular species displays apical dominance (Razdan, 1993). Apical dominance suppresses the growth and development of axillary buds in the presence of a terminal bud (Chawla, 2002).

In *Strelitzia*, there is an absolute absence of branching from axillary buds *in vivo*. This may be as a result of a strong apical dominance effect (van de Pol and van Hell, 1988). Therefore, a method of eliminating apical dominance *in vitro* to promote branching, is required to increase the multiplication rate of *Strelitzia*.

Since the mechanism of apical dominance has been demonstrated to be under the control of various growth regulators, the proportions of these substances in the media can be manipulated to break dormancy and enhance shoot formation (Razdan, 1993). The growth regulators, auxins and cytokinins, are of particular significance in *in vitro* culture (Pierik, 1979). Cytokinins are concerned with the modification of apical dominance (Razdan, 1993). A high cytokinin concentration promotes axillary shoot formation by decreasing apical dominance (Pierik, 1979; Dodds and Roberts, 1995; Chawla, 2002). Although cytokinin induces the growth of buds, auxin is required in the culture media. Most cultures require a combination of these two compounds for growth regulation (Dodds and Roberts, 1995). Usually the manipulation and variation of auxin and cytokinin levels can successfully alter growth behaviour in plant cultures (Dixon and Gonzales, 1994). For axillary shoot formation, a low auxin concentration, together with a high cytokinin concentration is required (Pierik, 1979; Razdan, 1993).

The cytokinin treatment can be varied, that is, the selection of the type of cytokinin and concentration (Pierik, 1979). In *Musa,* a relative of *Strelitzia*, 6-benzylaminopurine (BAP) is said to be the preferred cytokinin (Banerjee and de Langhe, 1985). It has dramatically influenced axillary shoot formation in various *Musa* spp. (Wong, 1986; Arinaitwe et al., 2000; Srangsam and Kanchanapoom, 2007). Earlier reports revealed that there is a strong synergistic effect of BAP and 1-naphthaleneacetic acid (NAA) interactions (Novak et al., 1989; Okole and Schulz, 1996; Cote et al., 2000; Khalil et al., 2002; Srangsam and Kanchanapoom, 2007).

The concentration and combination of auxin and cytokinins in the nutrient medium is a key factor which determines successful plant regeneration (Razdan, 1993). To improve the success of axillary bud proliferation for *Strelitzia* spp., the optimal balance between these two groups of growth regulators needs to be determined. Thus, indicating the importance of a study into BAP-NAA interactions.

1.5 POSSIBLE EFFECTS OF WOUNDING ON SUPPRESSING APICAL DOMINANCE IN ORDER TO INDUCE MULTIPLE BUD DEVELOPMENT

Apical dominance inhibits the development of axillary meristems (Razdan, 1993). In nature, axillary meristems are generally the source of bud formation when leaders are damaged (Burrows, 1989). This indicates the positive effects of meristem wounding and even apical bud removal on stimulating the growth of axillary buds.

In *Strelitzia*, the absence of branching from axillary buds, may be due to a strong apical dominance effect. The introduction of *in vivo* branching can increase the multiplication rate of *Strelitzia.* This can be achieved by the removal of the apical dome. This method of eliminating apical dominance is practised in vegetative propagation by the division of branches known as fans (van de Pol and van Hell, 1988).

Similarly to *in vivo* methods, an *in vitro* method to reduce apical dominance and promote axillary bud development is needed. In *Musa* and *Ensete*, success has been achieved through wounding of the meristem region (Jarret et al., 1985; Gupta, 1986; Birmeta and Welander, 2004). There are no reports on the effects of *in vitro* wounding in Strelitziaceae. However, it is hopeful that axillary meristem development as observed in the *in vivo* methods as well as in the *in vitro* cultures of its relative Musaceae, can be applied for improving axillary bud development in *Strelitzia*. An investigation into the wounding effects on *Strelitzia* spp. may be significant in the clonal propagation of this plant.

1.6 POSSIBLE EFFECTS AN ADSORBENT AND ANTIOXIDANT MAY HAVE ON REDUCING OXIDATIVE BROWNING OF WOUNDED TISSUES

As previously mentioned, the excessive production of polyphenols is a problem frequently encountered during the initial stages of culture develoment (Ziv and Halevy, 1983; Pan and van Staden, 1998; Birmeta and Welander, 2004; Diro and van Staden, 2004). Tissue injury, during explant excision, stimulates the production of phenols (Dodds and Roberts, 1995) as a form of a defence mechanism (Pan and van Staden, 1998). The incorporation of activated charcoal to initiated cultures is most effective in controlling polyphenol oxidation (Ziv and Halevy, 1983; Pan and van Staden, 1998; Chawla, 2002; Birmeta and Welander, 2004; Kiong et al., 2007; Karnataka, 2008). The adsorption of toxic phenols prevents the browning and death of the tissues (Horner et al., 1977; Fridborg et al., 1978; Weatherhead et al., 1979; George and Sherrington, 1984; Madhusudhanan and Rahiman, 2000; Chawla, 2002).

In the (multiplication stage) of culture, wounding techniques will be employed in efforts to induce axillary bud proliferation. Polyphenol exudation is exaggerated in response to wounding (Birmeta and Welander, 2004). Thus, indicating the need for an adsorbent or the addition of an antioxidant in the culture media.

The beneficial use of activated charcoal as a culture component for the adsorption of toxic substances is established (Teixeira et al., 1994; Veramendi and Navarro, 1996; Pan and van Staden, 1998; Gallo-Meagher and Green, 2002). However, its addition to shoot proliferation media may have adverse effects on growth and development as activated charcoal is able to adsorb high concentrations of growth regulators (Fridborg et al., 1978; Ebert and Taylor, 1990; Nissen and Sutter, 1990; Ebert et al. 1993; Pan and van Staden, 1998; Thomas, 2008), thus reducing their effectiveness in tissue culture. The ratio and concentration of auxins and cytokinins in the medium is a key factor in determining successful plant regeneration (Razdan, 1993). The non-selective adsorption of these compounds by activated charcoal may result in inhibitory effects of growth *in vitro* (Pan and van Staden, 1998; Thomas, 2008).

Due to the instrumental role of auxin-cytokinin supplements on regulating plant growth, it is questionable whether to rely on activated charcoal. Although the complete effects of activated charcoal in the medium are unknown, its addition to the medium, may retard plant growth. This will require the need to evaluate an alternative treatment into experiments involving *Strelitzia* spp. The use of activated charcoal can make a difference in the success or failure of this culture attempt.

Ascorbic acid is an antioxidant used to control oxidation of phenols (Bharadwaj and Ramawat, 1993; Abeyaratne and Lathiff, 2002; Chawla, 2002). Its addition to the medium has reduced blackening of the medium to an acceptable level (Almaz et al., 2001). However, it has been reported not as effective as activated charcoal (Birmeta and Welander, 2004). A comparative study of these two phenolic reducing agents would gain further insight into the adsorption of the growth regulators, BAP and NAA, and identify the most successful way of reducing oxidative browning of wounded tissues. In determining the optimal agent, there is a trade-off between efficient control of browning and the instrumental role of auxin-cytokinin supplementation in the medium.

1.7 EFFECTS OF A DARK INCUBATION PERIOD ON REDUCING OXIDATIVE BROWNING OF WOUNDED TISSUES

The limitations of polyphenols on *in vitro* growth and development are evident as discussed previously. Tissues containing relatively high concentrations of phenolic compounds are difficult to culture (Scalbert et al., 1990; Dodds and Roberts, 1995; Khatri et al., 1997; Zweldu and Ludders, 1998; Abeyaratne and Lathiff, 2002; Titov et al., 2006). Success is often dependent upon the ability to reduce the phenolic oxidation reaction to wounding and cutting.

As discussed, adsorbents and antioxidants can be used as a method in reducing browning. Another useful technique is the incubation of cultures in darkness for the initial culture period (Bajaj, 1977; Durand-Cresswell and Nitsch, 1977; Birmeta and Welander, 2004; Titov et al., 2006; Kiong et al., 2007). Maintaining cultures in darkness suppresses the metabolic sequence as it is known that phenolic oxidation products are formed under illumination (Chawla, 2002).

In *Musa*, *Ensete* and *S. reginae*, the incubation of cultures in darkness is reported to be effective in reducing browning (Ziv and Halevy, 1983; Birmeta and Welander, 2004; Strosse et al., 2009). However, success in these cases was achieved when the dark incubation period was used together with an antioxidant treatment. Obtaining optimal techniques in reducing oxidative browning of *Strelitzia* spp., requires a study into the dark incubation period in conjunction with the use of an adsorbent or antioxidant and the variation of medium composition. The type of phenol reducing agent, its concentration and the specific species are of importance in reducing oxidative browning of wounded tissues. The optimal collaboration of a dark incubation period together with a phenol reducing agent would increase the potential of *Strelitzia* spp. *in vitro* (Ziv and Halevy, 1983; Birmeta and Welander, 2004; Strosse et al., 2009).

1.8 CONCLUSION

The *Strelitzia* is an ornamental plant of significant horticultural commercial value (Paiva et al., 2004). Despite high demands, it is not widely spread due to the constraints on its production and is one of the very few important cut flower plants for which no uniform cultivars are available. Its horticultural success is limited by the slow conventional propagation methods currently used (Ziv and Halevy, 1983). It is commonly propagated vegetatively by division or by seeds. Both these methods of propagation are slow (Karnataka, 2008). From this background an alternative propagation and cloning method is required for the large scale

production of *Strelitzia* to exploit its potential as an ornamental plant. The development of a reliable *in vitro* method for propagating *Strelitzia* spp., through the culture of excised embryos and multiple shoot formation, would overcome the constraints this plant poses to the horticultural industry worldwide, thus, greatly contributing to the commercial production of *Strelitzia* spp.

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CHAPTER TWO

EFFECTS OF VARIOUS MEDIA COMPOSITIONS ON THE *IN VITRO* GERMINATION AND DISCOLORATION OF IMMATURE EMBRYOS OF BIRD OF PARADISE (*STRELITZIA REGINAE*)

Full Length Research Paper

EFFECTS OF VARIOUS MEDIA COMPOSITIONS ON THE *IN VITRO* GERMINATION AND DISCOLORATION OF IMMATURE EMBRYOS OF BIRD OF PARADISE (*STRELITZIA REGINAE*)

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2.1 Abstract

The optimal media composition for the *in vitro* germination of isolated *Strelitzia reginae* embryos was investigated. Different media treatments were compared to determine the effects of MS medium strength, activated charcoal (AC) and vitamin supplementation on the germination and seedling development of immature *Strelitzia* embryos. Results indicate the positive role of AC in reducing oxidative browning. The addition of AC (0.2 gl^{-1}) to the culture medium significantly reduced the discoloration of embryo explants and the culture media. Similarly, the addition of the vitamins (100 mgl^{-1} Inositol, 0.1 mgl^{-1} Thiamine, 0.1 mgl^{-1} Pyridoxine, 2 mgl^{-1} Glycine) resulted in a significant reduction in embryo discoloration. Furthermore, the addition of vitamins significantly increased root formation. Interactions between these media components resulted in significant effects. In treatments of half strength MS with vitamin supplementation, both embryo and media discoloration were reduced. Interactions between vitamin and AC treatments presented a reduction in the embryo discoloration rate and an increased length of shoots, only when AC was absent in the media. When AC was added to vitamin supplemented media, the beneficial effects of vitamins were cancelled. The highest germination rate of embryos was observed in media containing AC without vitamin supplementation. A significant decrease in germination resulted with the addition of vitamins. The highest level of media discoloration was observed in half-strength MS media without activated charcoal and vitamin supplementation. Whilst the most effective media were; (half-strength MS without vitamins and with AC) or (half-srength MS with vitamins and without AC).

Keywords: oxidative browning, activated charcoal, MS medium strength, vitamins, media composition.

Abbreviations: MS – Murashige and Skoog (1962), **AC** – activated charcoal.

2.2 INTRODUCTION

The bird of paradise (*Strelitzia*) is a tropical perennial plant of significant commercial value (Paiva et al., 2004). Originating from South Africa, this important ornamental monocotyledonous plant (Chand, 2008) is highly valued as a cut flower. This is due to the strong exotic features of its colourful flowers, the long length of the stem and the high post-harvest durability (Wood, 1995). Despite it being one of the most sought after cut flowers destined for exportation from developing countries (Criley, 1988), its commercial exploitation and success is limited by its naturally low rate of multiplication (Ziv and Halevy, 1983). Propagation is either by seed or by division of naturally developed branches known as fans (Dyer, 1972). Both of these conventional propagation methods are very slow (Karnataka, 2008). Efforts have been made to increase and accelerate the propagation of this valuable plant, both by asexual (van dePol and van Hell, 1988) and sexual methods (Holley, 1970; Bekendam, 1974; van de Venter and Small, 1975; Besmer, 1976; Ishihata, 1976; Diaz-Perez, 1978; Ybema et al., 1984; Ndakidemi and Dakora, 2003). However, the commercial production of this plant has not been adequate to exploit its potential.

From this background an alternative propagation and cloning method is needed. Tissue culture is a reliable and advanced propagation method that could be more promising than the conventional propagation methods (Promtep, 1981). Despite the plants commercial importance, a reliable method for micropropagation has not yet been developed (Chand, 2008).

Reviews of the literature indicate the limited success in the application of tissue culture techniques in the propagation of *Strelitzia* (North et al., 2010). The failure of tissue culture techniques is reported to be due to oxidative browning of the wounded explants (Ziv and Halevy, 1983). However, with the extensive use of antioxidants to reduce browning, terminal and axillary buds were found to be capable of growth and further shoot proliferation (Ziv and Halevy, 1983). In efforts to reproduce the protocol developed by Ziv and Halevy (1983), Paiva et al. (2004) failed to establish axillary buds *in vitro.* Irrespective of applied treatments, phenolic oxidation was reported to be a crucial problem. In studies involving the use of immature *Strelitzia* embryos as explants, Paiva et al. (2004) reported the germination of embryos inoculated *in vitro*. Although attempts made to regenerate plants from this material were unsuccessful. In all investigations into the micropropagation of *Strelitzia*, only partial success and a low rate of multiplication were obtained, indicating major problems with growing and multiplying this plant *in vitro* (North et al., 2010).

Zygotic embryo culture is a useful tool that can be used for a variety of purposes. These include bypassing seed dormancy (Hu and Wang, 1986; Ho et al., 1987; Das et al., 1999; Bürün and Çoban Poyrazoğlu, 2002) and inducing a faster growth rate (Chang and Yang, 1996; Bürün and Çoban Poyrazoğlu, 2002). The variation in seeds (Larkin et al., 1984; Foolad and Jones, 1992) will allow the excised embryo explants to play an important role in the breeding cycle (Ho et al., 1987) and the development of cultivars. The limited wounding of embryo explants may reduce the production of phenolic exudates during the crucial initial stages of plant development. Furthermore, this non-destructive method of gaining starting material for the culture, may aid the production of rare species, as plants do not have to be destroyed (Bürün and Çoban Poyrazoğlu, 2002).

The most important aspect of culturing immature embryos is to develop and clearly define a culture media that can sustain growth and development (Chawla, 2002). Nutrients required by embryos vary depending on embryo age. Thus, while relatively mature embryos can grow on a simple inorganic medium, the nutritional requirements of relatively immature embryos are complex (Bajaj, 1977; Pierik, 1979; Monnier, 1990; Raghaven, 1994; Hu and Zanettini, 1995). Up to now, little is known of the *in vitro* culture factors affecting the germination of immature zygotic embryos of *Strelitzia* spp. The MS medium of Murashige and Skoog (1962) is a salt composition that supplies the needed macro- and micronutrients. The modification of MS medium according to the nutritional requirements of an embryo and its developmental stage is essential to attain the highest germination percentage and best morphological characteristics.

Although the basis of all nutrient media is a compostion of essential nutrients (Ramage and Williams, 2002), vitamins are required in trace amounts to serve catalytic functions in enzyme systems (Al-Khayri, 2001). Normal plants synthesize the vitamins required for growth and development (Chawla, 2002). Whereas plant cells grown *in vitro* are only capable of synthesizing essential vitamins in suboptimal quantities; thus culture media are often supplemented with vitamins to enhance growth (Al-Khayri, 2001). The role of vitamins on the germination of *Strelitzia* embryos and plant regeneration thereof, needs to be determined.

Phenolic oxidation is a crucial problem during the initial stages of culture as polyphenolic compounds are detrimental to the further development of explants (Ziv and Halevy, 1983; Pan and van Staden, 1998; Zeweldu and Ludders, 1998; Birmeta and Welander, 2004; Diro and van Staden, 2004). The use of activated charcoal can make a major difference in the success or failure of a given tissue culture attempt (Pan and van Staden, 1998). The incorporation of activated charcoal to tissue culture media may promote *in vitro* growth and alleviate this problem by adsorbing inhibitory substances (Ziv and Halevy, 1983; Tisserat,

1984; Teixeira et al., 1994; Veramendi and Navarro, 1996; Pan and van Staden, 1998; Diro and van Staden, 2004).

The aim of this study was to investigate the optimum media compositions for the *in vitro* germination of immature embryos of *Strelitzia reginae*. The specific objectives were; To determine if variations in the Murashige and Skoog (MS) medium (inorganic salt formulation) concentrations have an effect on the germination and development of embryos.To assess the effect of supplementing the plant tissue culture medium with vitamins.To evaluate the effects of activated charcoal in the culture media to control oxidative browning of embryo explants.

2.3 MATERIALS AND METHODS

2.3.1 Plant material

Immature seeds, 20 weeks after pollination, of *Strelitzia reginae* were collected from plants grown at Kirstenbosch National Botanical Gardens in Cape Town, South Africa.

2.3.2 Sterilization

Seeds were surface-sterilized with 70% ethanol for 30 sec, 1.5% solution of sodium hypochlorite (NaOCl) with 2 drops of Tween-20 for 15 min and then rinsed four times with sterile distilled water. Immature embryos were aseptically excised from the sterilized seeds and placed on various induction media.

2.3.3 Culture conditions and media

Embryos were placed in test tubes containing 10 ml of the culture media, supplemented with 30 g l^{-1} sucrose and solidified with 7 g l^{-1} agar. The pH was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. The experiment consisting of 8 medium types (Table 2.1) was set up to investigate the effects of MS medium strength, activatedcharcoal treatments and vitamin supplementation of the *in vitro* germination of embryos. Twelve replicates were used
for each treatment. Inoculated cultures were incubated in a growth room at 25 ± 2°C with a 16 h light and 8 h dark cycle.

2.3.4 Data collection and analysis

Data on embryo germination rates (radicle emergence), contamination, shoot length, root number and length, embryo size, degree of embryo discoloration and degree of media discoloration were collected at weekly intervals. Based on visual observations, the degree of media and embryo discoloration (entire explants and at the media contact point) was rated on a scale of 1-5 (1 = No discoloration and 5 = Extreme discoloration), modified from the rating scale given by Ziv and Halevy (1983). Data collected were analyzed for statistical significance using factorial analysis of variance (ANOVA). These computations were done with the software program STATISTICA Software Programme version 2010 (StatSoft Inc., Tulsa, OK, USA). The Fisher least significant test was used to compare treatment means at p = 0.05 level of significance (Steel and Torrie, 1980).

2.4 RESULTS AND DISCUSSION

2.4.1 Effect of MS medium strength, activated charcoal and vitamins on the discoloration of the embryo explants

In this experiment, MS medium strength had no significant effect on discoloration of the entire embryo explant (Table 2.2). However, slightly increased discoloration was observed in full-strength MS. The addition of activated charcoal in culture media significantly reduced embryo discoloration in week 1 ($P \leq 0.001$) and week 2 ($P \leq 0.05$). Based on the rating scale of 1-5, activated charcoal reduced embryo discoloration from 2.95 to 2.30 in week 1 and from 2.93 to 2.63 in week 2, which was equivalent to a 22% and 10% reduction respectively. In weeks that followed, activated charcoal only slightly reduced embryo discoloration. During the initial stages of culture, the excessive production of polyphenols often results in browning and eventual death of inoculated tissues. This is possibly due to the triggering of defense reactions (Pan and van Staden, 1998). The incorporation of activated charcoal to media is a recognized practise and its influence on culture establishment may be attributed to its adsorptive capability of inhibitory substances in the culture medium (Horner et al., 1977; Fridborg et al., 1978; Weatherhead et al., 1979; Theander and Nelson, 1988) and drastic decrease in phenolic oxidation (Carlberg et al., 1983; Liu, 1993; Teixeira et al., 1994). Similar to this study, the influence of activated charcoal on reducing explant browning has been reported in several plant species (Madhusudhanan and Rahiman, 2000; Chang et al., 2001; Birmeta and Welander, 2004; Wang et al., 2005; Thomas, 2008; Guo et al., 2007). The addition of vitamins significantly ($P \leq 0.05$) reduced embryo discoloration from 2.90 to 2.65 in week 2 of the experiment, which was equivalent to a 9% reduction. Thereafter, vitamins

showed no effect on reducing discoloration. These results are in accordance with previous studies that report the ability of vitamins to suppress the browning of tissues *in vitro* (Bergmann and Bergmann, 1968; Inoue and Maeda, 1980; Al-Khayri, 2001).

2.4.2 Effect of MS medium strength, activated charcoal and vitamins on the discoloration of the embryo explants at the media contact point

Half-strength MS medium significantly ($P \leq 0.05$) reduced embryo discoloration at the medium contact point. In week 2 (Table 2.2), the reduction of 3.38 to 3.15 resulted in a 7% decrease. A slight reduction was observed throughout the remainder of the experiment. Similarly, Abbasin et al. (2010) reported the browning of embryo explants in *Taxus baccata* when cultured in full-strength basal salts. In contrast, the half-strength MS was more effective in reducing necrosis and increasing the survival of explants. This may be due to the reduced salt concentration reducing the osmotic concentration of the medium. Activated charcoal significantly ($P \leq 0.05$) reduced discoloration in weeks 1, 2 and 4 by approximately 10%, 8% and 6% respectively. Activated charcoal resulted in a general reduction throughout the experiment. The addition of vitamins to the culture media significantly; $P \leq 0.001$, $P \leq 0.05$ and $P \leq 0.05$ reduced embryo discoloration at the media contact point in week 2, 3 and 5 of the experiment respectively. The values were reduced as follows; 3.53 to 3.00 in week 2, 3.70 to 3.40 in week 3 and 3.95 to 3.80 in week 5. Thus, a 15%, 8% and a 4% reduction was observed in the respective weeks. A general reduction in discoloration at the medium contact point was observed throughout the experiment in treatments containing vitamins. It is at the point of contact between the explant and the medium that oxidative browning is exaggerated due to an adequate supply of oxygen coming into contact with the growing tissue and the required nutrients. This study indicates that vitamins and activated charcoal both played a key role in reducing embryo discoloration at this point. In a study on *Brassica*, Tian et al. (2004) similarly reported that embryos grew more rapidly and browning rarely happened at the base of hypocotyls, when media was supplemented with vitamins. The ability of activated charcoal to reduce the browning of tissues is widely reported (Chang et al., 2001; Wang et al., 2005; Guo et al., 2007) and may be attributed to its high adsorptive capacity (Thomas, 2008). With the addition of activated charcoal to media, a drastic decrease in phenolic oxidation has been observed (Carlberg et al., 1983; Liu, 1993; Teixeira et al., 1994).

2.4.3 Effect of MS medium strength, vitamins and activated charcoal on the number of roots developed from germinated plantlets

MS medium strength had no significant effect on the formation of roots. However, half strength MS generally displayed a slightly higher rate of root formation (Table 2.3). The

addition of vitamins significantly ($P \leq 0.01$) increased root formation in week 1 of the experiment. Values increased from 0.35 to 0.60, resulting in a 42% increase. In the weeks that followed a slight increase was observed in media containing vitamins. Similarly, earlier studies confirmed that the vitamins thiamine and nicotinic acid affected cellular division in the pea root meristem (Bonner and Addicot, 1937; Addicot, 1941; Torrey, 1953). It was only in the presence of either or both vitamins, that root growth occurred (Torrey, 1953). A significantly ($P \leq 0.001$) higher number of roots were observed in the absence of activated charcoal in week 1 (Table 2.3). Root formation increased from 0.25 in charcoal treatments to 0.70 in treatments free of charcoal. Thus, a 64% increase was observed in treatments free of activated charcoal. Majority of reports confirm the positive effect of activated charcoal on rooting (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). However, in this study on *Strelitzia*, activated charcoal had a negative effect on rooting. These results are similar to those of Buendia-Gonzalez et al. (2007), who also reported activated charcoal to have a negative effect on rooting of the mesquite tree (*Prosopis laevigata*). Activated charcoal inducing negative results in the growth and development of plant tissues has also been reported in other micropropagation systems (Komalavalli and Rao, 2000; Kadota and Niimi, 2004; Wei et al., 2006; Motoike et al., 2007). This is possibly due to the adsorption of essential factors required for tissue growth (Komalavalli and Rao, 2000). In this study, it is established that vitamin supplemented media increased root formation. Whereas the addition of activated charcoal to this vitamin enriched media completely inhibited the root formation. This may be due to the activated charcoal adsorbing the vitamins promoting rooting. A crucial impact of adding activated charcoal to the culture media is that in addition to adsorbing unwanted substances, it may adsorb needed vitamins (Weatherhead et al., 1978; Weatherhead et al., 1979; Pan and van Staden, 1998).

2.4.4 Effect of MS medium strength, activated charcoal and vitamins on medium discoloration

The intensity of medium discoloration was not affected by the concentration of MS salts (Table 2.4). Only a slight reduction was observed in media containing full-strength MS. Based on the rating scale of 1-5, activated charcoal significantly ($P \leq 0.001$) reduced medium discoloration from 1.68 to 1.15 in week 1 of the experiment. Discoloration was 32% less severe in media containing activated charcoal, as opposed to media without it. In the weeks that followed, a slight reduction was observed in activated charcoal treatments (Figure 2.5). The incorporation of vitamins to culture media showed no significant effects. However, a slight reduction was observed in all treatments containing vitamins. Medium discoloration during initial stages of culture is due to tissues releasing polyphenolic compounds, which

diffuse into the medium (Strosse et al., 2009). The use of activated charcoal, in reducing these dark pigments, has been used with success in various plants (Das et al., 1999; Feyissa et al., 2005; Nguyen et al., 2007).

2.4.5 Interactive effects of MS medium strength, activated charcoal and vitamins

The results in Figure 2.2A indicate that there was a significant interaction between MS medium strength and vitamins on embryo discoloration at the medium contact point in week 3. In half strength MS with vitamins, embryo discoloration was significantly ($P \leq 0.05$) reduced. Whereas full-strength MS medium with vitamins showed no significant effects on reducing embryo discoloration at the media contact point. Similarly, Abbasin et al. (2010) reported half strength MS effective in reducing necrosis of the zygotic embryo explants of *Taxus baccata* and Tian et al. (2004) found vitamin supplementation effective in reducing browning at the base of hypocotyls in *Brassica*. Our study reports the interactive effects of MS medium strength and vitamins to have a significant influence on embryo discoloration at the media contact point. Media discoloration was significantly ($P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.05$) reduced by MS media strength and vitamin interactions in weeks 1, 3 and 4 respectively (Figure 2.1B, C and D). Half-strength MS supplemented with vitamins significantly reduced media discoloration. Whereas the addition of vitamins to full-strength MS had the opposite effect and resulted in an increase in media discoloration. The positive influence of vitamins in reducing oxidative browning has been established (Bergmann and Bergmann, 1968; Inoue and Maeda, 1980; Al-Khayri, 2001). Similarly, half-strength MS has been reported to be more effective in reducing necrosis (Abbasin et al., 2010). In this study the interactive effects of vitamins and half-strength MS were effective in reducing media discoloration. The increased media discoloration in full-strength MS supplemented with vitamins may due to an excess of mineral salts in the media. Therefore, modification of MS medium according to the requirements of tissues is essential to achieve optimal growth (Birmeta and Welander, 2004). Whereas, in treatments with vitamins, activated charcoal did not play a significant role in reducing media discoloration, irrespective of MS media strength. This may be due to the positive effect of the vitamins in the culture media. The only treatment showing a significantly higher level of media discoloration amongst those supplemented with vitamins, was that of full strength MS without activated charcoal. In the presence of full strength macro- and micronutrients and vitamins, phenols may be released more rapidly into the medium due to an excess of nutrients and vitamins causing an increased osmotic potential. The interactive effects of activated charcoal and vitamins showed significant results. Based on the rating scale of 1-5, treatments without activated charcoal and supplemented with vitamins significantly ($P \leq 0.05$) reduced embryo discoloration at the media contact point in week 3 (Figure 2.2A). However, when activated charcoal was present in the media, the addition of

vitamins did not play a significant role in reducing embryo discoloration at the medium contact point. As previously mentioned, this study indicates the significant reduction of embryo discoloration at the medium contact point, with the supplementation of media with vitamins. The adverse effect of this, with the addition of activated charcoal, may be due to the ability of activated charcoal to adsorb vitamins (Weatherhead et al., 1978; 1979; Pan and van Staden, 1998). Thus, the positive effects of vitamin supplementation cancelled. Results on the interactive effects of activated charcoal and vitamins showed that the number of roots developed was significantly affected in week 1 of the experiment. Treatments containing both activated charcoal and vitamins resulted in a significantly ($P \leq 0.01$) higher number of roots (Figure 2.2B). In treatments lacking activated charcoal, the addition or omission of vitamins showed no effects on the number of roots developed. Although this experiment previously reports a significantly lower number of roots developed in activated charcoal treatments, the interactive effects of activated charcoal with vitamins result in a significant increase in root formation. This increase in rooting is in conjunction with the reports on both the application of activated charcoal (Makunga et al., 2006; Agarwal and Kanwar, 2007; Xiao et al., 2007; Makunga and van Staden, 2008) and vitamins (Torrey, 1953). Shoot lengths from embryo derived plantlets were significantly affected by activated charcoal and vitamin interactions in week 5 of the experiment (Figure 2.2C). In treatments without activated charcoal, vitamins in the culture media significantly increased the length of shoots. However, when activated charcoal was present, vitamins inversely resulted in a significant reduction of shoot length (Figure 2.2C). Culture media are often supplemented with vitamins to enhance growth (Al Khayri, 2001). The reduction of shoot length with the addition of activated charcoal may be due to its high adsorptive characteristic (Thomas, 2008). As previously mentioned activated charcoal is known to adsorb beneficial vitamins (Weatherhead et al., 1978; 1979; Pan and van Staden, 1998), this may inversely affect growth. The results in Figures 2.3A, B and C indicate significant interactions in activated charcoal and vitamin treatments on the germination of immature *Strelitzia* embryos. In week 2, activated charcoal without vitamin supplementation resulted in the highest germination rate of embryos (Figure 2.3A). When activated charcoal was supplemented with vitamins, a significant decrease in germination was observed. In treatments without activated charcoal, vitamin supplementation did not significantly affect germination (Figure 2.3A), although a slight germination increase was observed in vitamin enriched media. In weeks 4 (Figure 2.3B) and 5 (Figure 2.3C), a similar trend continued. Activated charcoal has been widely reported to have positive effects on the *in vitro* germination of both embryos (Sarason et al., 2002; Shi et al., 2008; Fan et al., 2008) and seeds (Man et al., 2003; Kitsaki et al., 2004; Thompson et al., 2007). The significant reduction in embryo germination with vitamin supplementation may be due to an increase in osmotic potential. In the case of culturing zygotic embryos, only basic nutrients are necessary for germination (Thawaro and Techato, 2010). The results in Figure 2.4 represent

the significant interactive effects of MS media strength, activated charcoal and vitamins on the discoloration of the culture medium, based on the rating scale of 1-5. The poorest and most successful treatments in controlling media discoloration in week 1 (Figure 2.4A) were both observed in half strength MS media without vitamin supplementation. Activated charcoal caused significant ($P \leq 0.01$) interactive effects in the treatments. The addition of activated charcoal to half strength MS without the addition of vitamins, resulted in the most effective treatment in reducing media discoloration. Whilst the omission of activated charcoal from the same media of half strength MS without the addition of vitamins, displayed the highest level of media discoloration. In vitamin enriched media, the only treatment that varied was that of full-strength MS without activated charcoal. This treatment resulted in a significantly higher level of media discoloration, whereas other combinations of vitamins with either half- or full strength MS, with or without activated charcoal showed a constant reduced level of media discoloration in week 1. This trend was similarly observed for medium discoloration in weeks 3, 4 and 5 (Figures 2.4B, C and D, respectively). In treatments lacking vitamin supplementation, the promotary effects of activated charcoal were significantly evident in half-strength MS treatments. In this study, the addition of activated charcoal to the medium may have resulted in the adsorption of inhibitory compounds in the culture medium and decreased the accumulation of brown exudates from diffusing into the medium. Whereas, in treatments with vitamins, activated charcoal did not play a significant role in reducing media discoloration, irrespective of MS media strength. This may be due to the positive effect of the vitamins in the culture media. Similar to our results Tian et al. (2004) reported the influence of vitamin supplementation in reducing browning in *in vitro* propagation of *Brassica.* The only treatment showing a significantly higher level of media discoloration amongst those supplemented with vitamins, was that of full strength MS without activated charcoal. In the presence of full strength macro- and micronutrients and vitamins, phenols may be released more rapidly into the medium due to an excess of nutrients and vitamins. Therefore, the strength of MS medium together with the correct proportions of AC and vitamins are essential for optimum germination and growth of embryos. In conclusion, germinated plantlets were obtained from embryo explants and optimum nutrition and medium components as the first step towards the development of an efficient *in vitro* propagation system of *Strelitzia*, were determined. Initiation and *in vitro* propagation of *Strelitzia* is difficult due to oxidative browning. The present work, however, demonstrates the significant effects of MS medium strength, AC and vitamin supplementation on reducing the discoloration of explants and culture media. Furthermore, interactions between these media components significantly affected the rate of embryo germination, reducing explant and media discoloration, the length of shoots and the development of roots.

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Table 2.1: Media for the *in vitro* germination of immature *Strelitzia reginae* embryos

Media	Compositions
1	MS salts and vitamins*
2	½ MS salts and vitamins
3	MS salts and vitamins and 0.2 gl^{-1} activated charcoal
4	½ MS salts and vitamins and 0.2 gl^{-1} activated charcoal
5	MS salts
6	½ MS salts
7	MS salts and 0.2 gl^{-1} activated charcoal
8	½ MS salts and 0.2 gl^{-1} activated charcoal

*The medium was supplemented with vitamins including 100 mgl^{-1} Inositol, 0.1 mgl^{-1} Thiamine, 0.1 mgl^{-1} Pyridoxine, 2 mgl^{-1} Glycine.

Table 2.2: Effect of medium strength, activated charcoal and vitamins on the discoloration of the entire embryo explant and discoloration of embryo at media contact point. Rating was done on a scale of 1-5 (1 = No discoloration and 5 = Extreme discoloration)

Treatment	Time (Weeks)									
	1	2	3	4	5	1	2	3	4	5
	Embryo discoloration (entire explant)					Embryo discoloration (at media contact point)				
Medium strength										
Half	2.58±0.10a	2.75±0.09a	3.00±0.09a	3.03±0.09a	3.55±0.16a	2.83±0.11a	3.15±0.10b	3.58±0.09a	3.70±0.08a	3.83±0.06a
Full	2.68±0.10a	2.80±0.09a	2.85±0.09a	3.10±0.11a	3.83±0.16a	3.05±0.09a	3.38±0.10a	3.53±0.09a	3.78±0.08a	3.93±0.04a
Activated charcoal[a]										
-	2.95±0.08a	2.93±0.09a	3.03±0.08a	3.13±0.10a	3.58±0.16a	3.10±0.10a	3.40±0.09a	3.63±0.09a	3.85±0.06a	3.90±0.05a
+	2.30±0.09b	2.63±0.08b	2.83±0.10a	3.00±0.10a	3.80±0.16a	2.78±0.10b	3.13±0.10b	3.48±0.09a	3.63±0.09b	3.85±0.06a
Vitamins[b]										
-	2.70±0.08a	2.90±0.09a	2.98±0.10a	3.03±0.09a	3.53±0.17a	3.05±0.10a	3.53±0.09a	3.70±0.08a	3.83±0.07a	3.95±0.03a
+	2.55±0.11a	2.65±0.08b	2.88±0.09a	3.10±0.11a	3.85±0.15a	2.83±0.10a	3.00±0.09b	3.40±0.09b	3.65±0.08a	3.80±0.06b
3 - Way ANOVA (F-Statistic)										
MS	0.69	0.18	1.30	0.29	1.54	2.69	3.49*	0.16	0.49	1.85
AC	29.25***	6.35*	2.30	0.80	1.03	5.61*	5.21*	1.47	4.37*	0.46
VITAMINS	1.56	4.41*	0.58	0.29	2.15	2.69	18.99***	5.89*	2.64	4.15*
MS×AC	0.00	0.71	0.58	1.56	1.54	0.03	0.04	0.00	1.35	0.00
MS×VITAMIN	0.69	0.18	0.00	0.80	0.01	2.69	5.21*	0.16	1.35	0.00
AC×VITAMINS	1.56	0.00	1.30	0.80	1.54	0.30	0.04	4.09*	1.35	0.46
MS×AC×VITAMINS	0.69	0.71	1.30	1.56	0.62	0.83	3.49	0.65	0.49	1.85

*$P \leq 0.05$; ***: $P \leq 0.001$. Values (Mean ± SE, n = 12) followed by dissimilar letters in a column are significantly different by Least Significant Difference test at P=0.05. [a]0.2 gl^{-1} activated charcoal; [b]100 mgl^{-1} Inositol, 0.1 mgl^{-1} Thiamine, 0.1 mgl^{-1} Pyridoxine, 2 mgl^{-1} Glycine.

Table 2.3: Effect of medium strength, activated charcoal and vitamins on the number of roots developed by *Strelitzia reginae* during *in vitro* culture

Treatment	Time (Weeks)				
	1	2	3	4	5
Medium strength	Root Number				
Half	0.45±0.08a	3.43±0.43a	3.55±0.43a	3.88±0.51a	4.03±0.56a
Full	0.50±0.08a	2.95±0.42a	3.13±0.43a	3.30±0.49	3.35±0.50a
Activated charcoal[a]					
-	0.70±0.07a	3.10±0.40a	3.30±0.42a	3.60±0.50	3.68±0.55a
+	0.25±0.07b	3.28±0.45a	3.38±0.44a	3.58±0.50	3.70±0.52a
Vitamins[b]					
-	0.35±0.08b	3.45±0.40a	3.58±0.40a	3.73±0.41	3.75±0.41a
+	0.60±0.08a	2.93±0.45a	3.10±0.46a	3.45±0.57	3.63±0.63a
3 - Way ANOVA (F-Statistic)					
MS	0.28	0.60	0.46	0.64	0.77
AC	22.78***	0.08	0.01	0.00	0.00
VITAMINS	7.03**	0.73	0.58	0.15	0.03
MS×AC	0.28	0.13	0.01	0.35	0.38
MS×VITAMIN	0.28	0.00	0.01	0.00	0.05
AC×VITAMINS	7.03**	1.04	1.17	0.88	0.77
MS×AC×VITAMINS	2.53	0.48	0.46	1.16	1.01

**: $P \leq 0.01$; ***: $P \leq 0.001$. Values (Mean ± SE, n = 12) followed by dissimilar letters in a column are significantly different by Least Significant Difference test at P=0.05. [a]0.2 gl^{-1} activated charcoal; [b]100 mgl^{-1} Inositol, 0.1 mgl^{-1} Thiamine, 0.1 mgl^{-1} Pyridoxine, 2 mgl^{-1} Glycine.

Table 2.4: Effect of medium strength, activated charcoal and vitamins on root length of *Strelitzia reginae* and the discoloration of culture medium during *in vitro* culture. Rating was done on a scale of 1-5 (1 = No discoloration and 5 = Extreme discoloration)

Treatment	Time (Weeks)									
	1	2	3	4	5	1	2	3	4	5
	Root length (mm)					Discoloration of medium				
Medium strength										
Half	3.83±0.57a	15.73±2.05a	23.00±3.14a	26.80±3.80a	28.78±4.28a	1.40±0.11a	2.08±0.14a	2.33±0.16a	2.43±0.15a	2.58±0.18a
Full	3.90±0.51a	17.63±2.21a	22.95±3.08a	25.48±3.48a	26.65±3.67a	1.43±0.11a	2.05±0.14a	2.15±0.17a	2.40±0.16a	2.58±0.17a
Activated charcoal[a]										
-	4.00±0.55a	16.35±2.04a	22.93±3.03a	27.30±3.77a	29.45±4.26a	1.68±0.13a	2.15±0.14a	2.38±0.17a	2.53±0.16a	2.70±0.17a
+	3.73±0.54a	17.00±2.24a	23.03±3.18a	24.98±3.51a	25.98±3.67a	1.15±0.07b	1.98±0.15a	2.10±0.15a	2.30±0.15a	2.45±0.17a
Vitamins[b]										
-	4.03±0.54a	18.88±2.06a	25.13±2.86a	29.03±3.54a	30.35±3.73a	1.48±0.12a	2.23±0.14a	2.33±0.17a	2.45±0.15a	2.58±0.17a
+	3.70±0.55a	14.48±2.16a	20.83±3.29a	23.25±3.69a	25.08±4.18a	1.35±0.10a	1.90±0.14a	2.15±0.16a	2.38±0.16a	2.58±0.18a
3 - Way ANOVA (F-Statistic)										
MS	0.01	0.38	0.00	0.06	0.14	0.03	0.02	0.66	0.01	0.00
AC	0.13	0.05	0.00	0.19	0.36	15.21***	0.81	1.62	1.11	1.11
VITAMINS	0.18	2.06	0.91	1.20	0.84	0.86	2.80	0.66	0.12	0.00
MS×AC	5.34*	0.13	0.39	0.51	0.84	0.31	0.15	0.01	0.01	0.71
MS×VITAMIN	0.13	0.49	0.31	0.08	0.05	4.17*	2.80	7.10**	4.95*	2.18
AC×VITAMINS	0.31	0.67	0.81	0.57	0.60	2.79	0.81	1.62	1.66	0.18
MS×AC×VITAMINS	0.13	0.29	0.01	0.07	0.02	7.76**	3.73	4.84*	4.95*	5.39*

*$P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$. Values (Mean ± SE, n = 12) followed by dissimilar letters in a column are significantly different by Least Significant Difference test at P=0.05. [a]0.2 gl^{-1} activated charcoal; [b]100 mgl^{-1} Inositol, 0.1 mgl^{-1} Thiamine, 0.1 mgl^{-1} Pyridoxine, 2 mgl^{-1} Glycine.

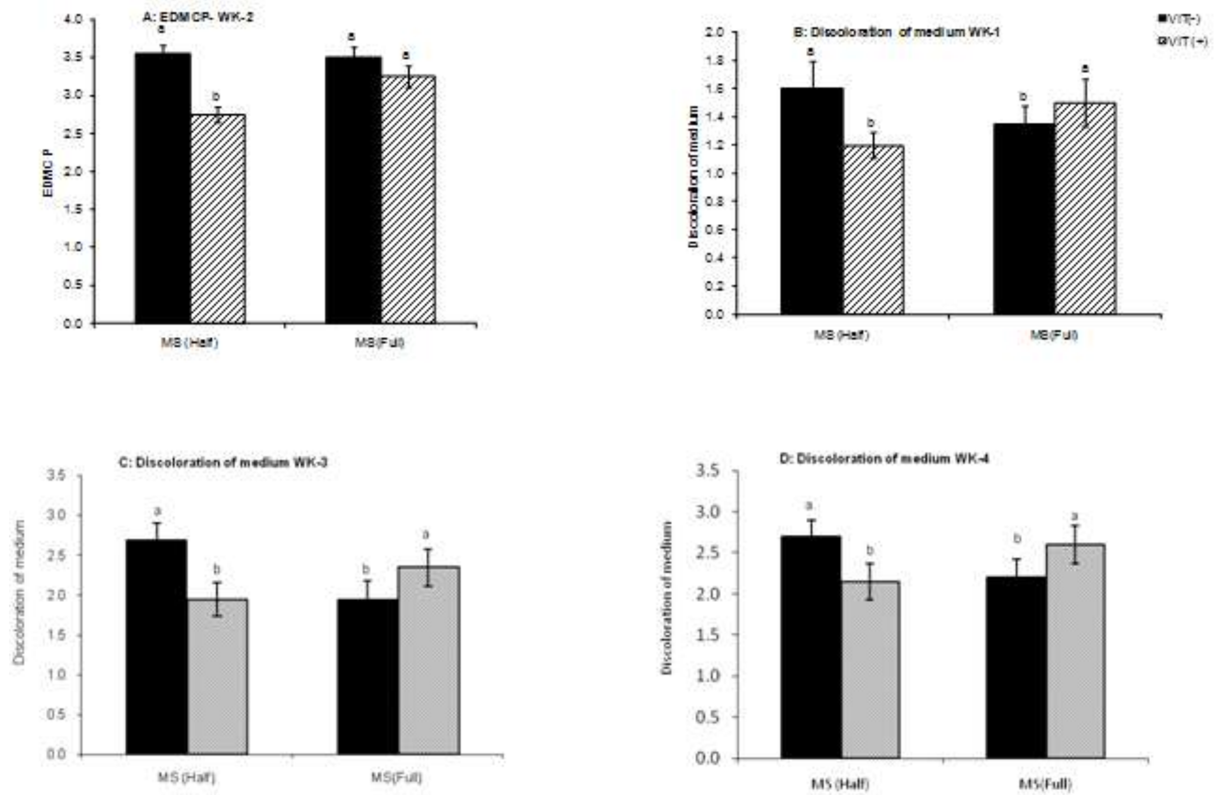

Figure 2.1: Interactive effects of Medium strength and vitamins on (A) Embryo discoloration at medium contact point (EDMCP) WK-2, (B) Discoloration of medium (WK-1), (C) Discoloration of medium (WK-3), (D) Discoloration of medium (WK-4). Rating scale used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration), MS (Half) = Half medium strength, MS (Full) = Full medium strength, VIT(-) = Without vitamin, VIT (+) = With vitamin

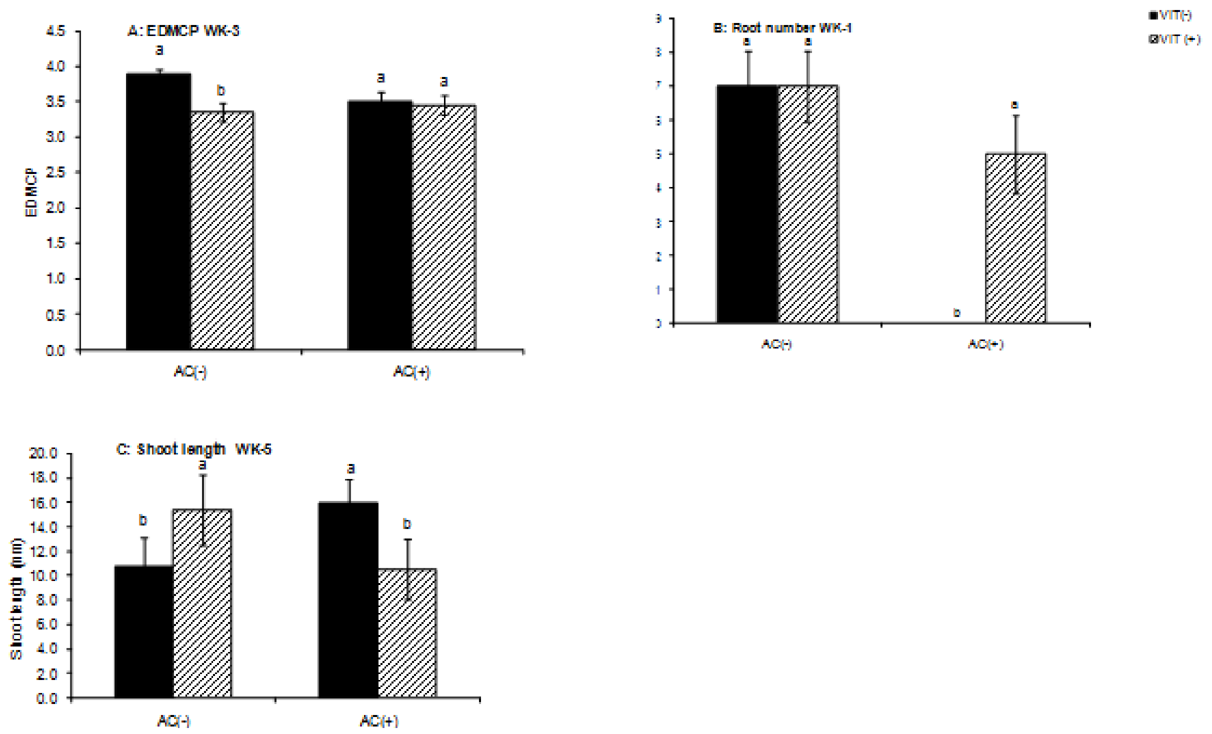

Figure 2.2: Interactive effects of Medium strength and vitamins on (A) Embryo discoloration at medium contact point (EDMCP) WK-3, (B) Root number WK-1, (C) Shoot length WK-5. Color code used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration), AC (-) = Without activated charcoal, AC (+) = With activated charcoal, VIT(-) = Without vitamin, VIT (+) = With vitamin

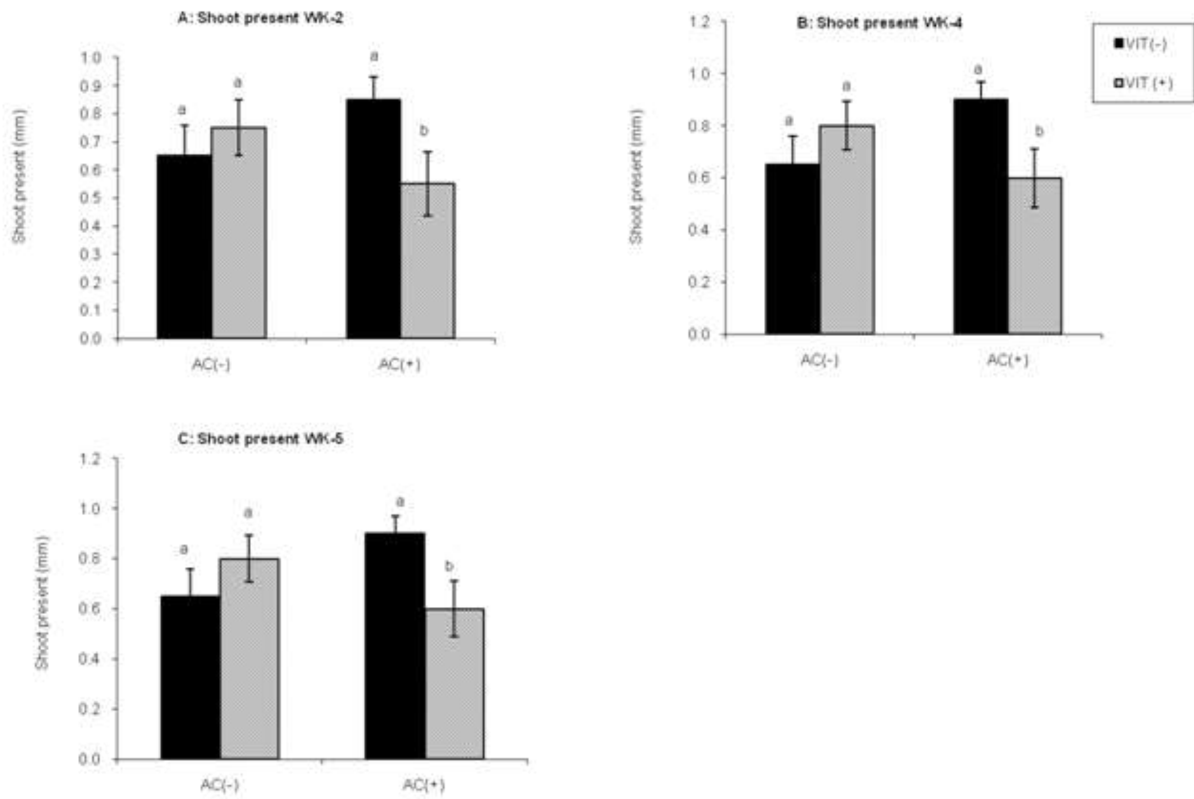

Figure 2.3: Interactive effects of activated charcoal and vitamins on (A) Shoot present WK-2, (B) Shoot present WK-4, (C) Shoot present WK-5. AC (-) = Without activated charcoal, AC (+) = With activated charcoal, VIT(-) = Without vitamin, VIT (+) = With vitamin

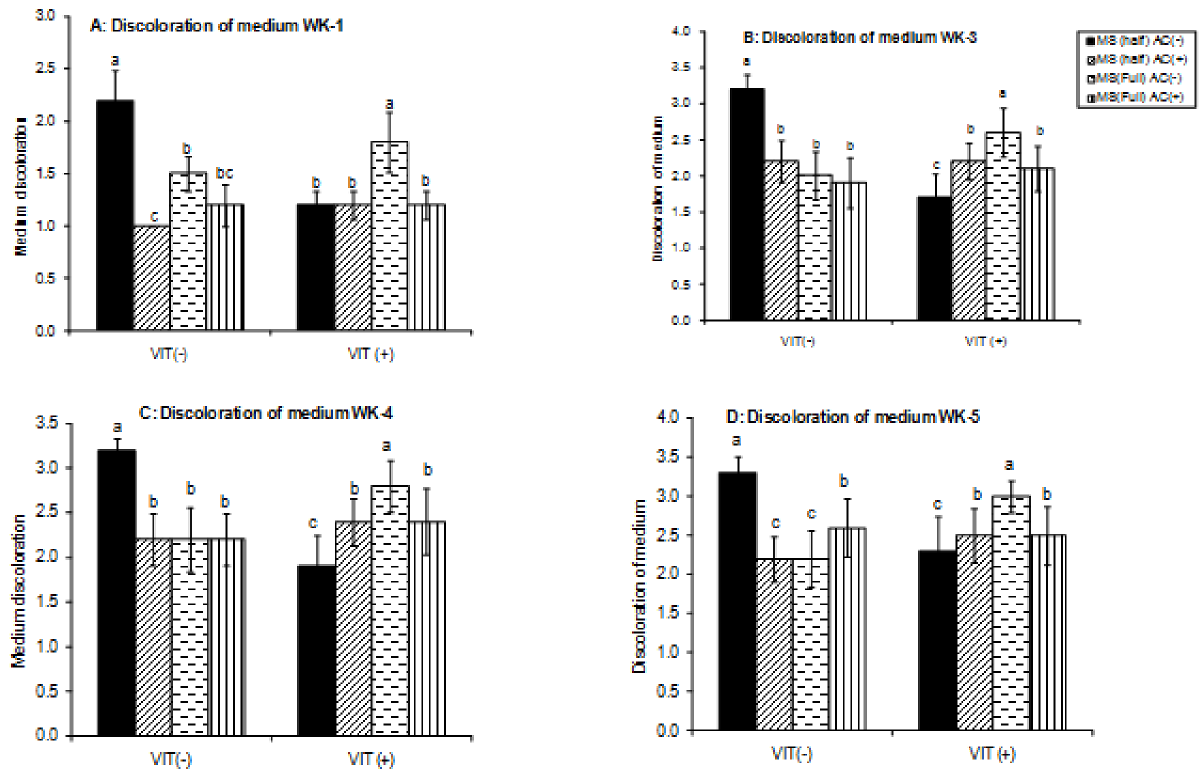

Figure 2.4: Interactive effects of Medium strength x activated charcoal x vitamins on discoloration of medium in: A) Week-1, B) Week-3, C) Week-4), D) (Week-5) in *Strelitzia reginae.* Color code used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration). MS (Half) = Half medium strength, MS (Full) = Full medium strength, AC (-) = Without activated charcoal, AC (+) = With activated charcoal, VIT(-) = Without vitamin, VIT (+) = With vitamin

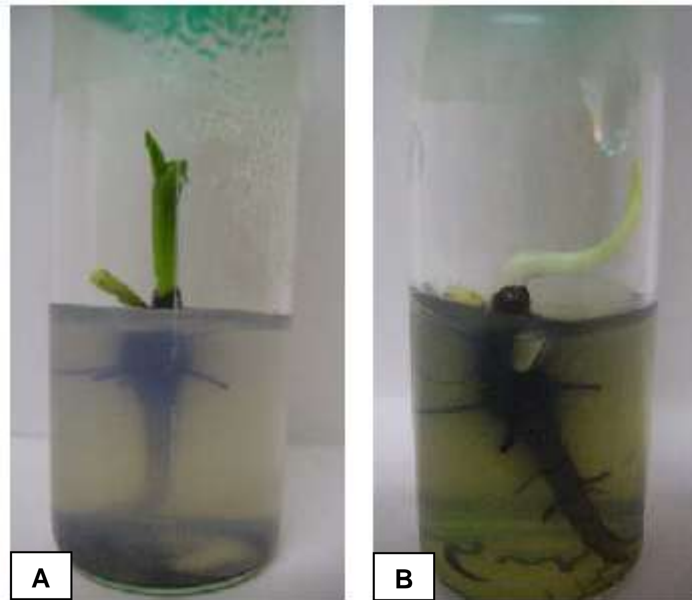

Figure 2.5: Effect of activated charcoal on medium discoloration. (A) Reduced media discoloration in the presence of activated charcoal and (B) an increased level of media discoloration in the absence of activated charcoal

CHAPTER THREE

EFFECTS OF VARIOUS MEDIUM COMPOSITIONS AND WOUNDING TREATMENTS ON *IN VITRO* GROWTH AND REGENERATION OF BIRD OF PARADISE (*STRELITZIA REGINAE*)

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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3.1 Abstract

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 NAA and BAP concentrations on explant discoloration and callus formation. The antioxidant treatments, activated charcoal and ascorbic acid 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 explant discoloration and callus formation. The most effective treatment in reducing explant discoloration at the media contact point was achieved in interactive effects of higher NAA and BAP concentrations (0.1 mg l^{-1} NAA : 3mg l^{-1} BAP and 0.5 mg l^{-1} NAA : 5mg l^{-1} BAP) with wounding. Interactions between antioxidants and wounding treatments resulted in the absolute absence of callus induction in activated charcoal treatments without explant wounding. Whereas, ascorbic acid with wounding induced the highest level of callus formation.

Key words: callus formation, discoloration, activated charcoal, ascorbic acid, BAP - 6-benzylaminopurine, NAA - 1-naphthalene acetic acid

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

3.2 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), 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 *Strelitzia 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. Similarly 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 *Strelitzia reginae* is largely due to the oxidative browing 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 activated charcoal 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, explant height, explant discoloration (the entire explant and at the medium contact point only), callus formation and root length.

3.3 MATERIALS AND METHODS

3.3.1 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.

3.3.2 Culture conditions and media

Explants were transferred to different regeneration media. The basal medium comprised the MS salts supplemented with 100mg l^{-1} myo-inositol, 0.1mg l^{-1} thiamine-HCl, 0.1 mg l^{-1} pyridoxine, 2mg l^{-1} glycine and 30g l^{-1} sucrose. Various concentrations of 6-benzylaminopurine (BAP) 0, 2, 3, 5, 6mg l^{-1} and 1-naphthalene acetic acid (NAA) 0, 0.1,

0.5mg l^{-1} were added to the media. The antioxidants, 2.5g l^{-1} activated charcoal and 0.05g l^{-1} ascorbic acid were each separately added to the various media. The experiment consisted of ten medium types (Table 3.1). Ten replicates were used for each treatment. The media was solidified with 7g l^{-1} agar. The pH of the media was adjusted to 5.95 prior to autoclaving at 121°C for 20 minutes. The unwounded cultures were incubated in a growth room with a 16h light and 8h dark cycle at 25 ± 2°C.

3.3.3 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 1-5 (1 = No discoloration and 5 = Extreme discoloration), modified from the rating scale given by Ziv and Halevy (1983). The degree of callus formation will be rated as: 1 = none, 2 = low, 3 = medium, 4 = high. This rating scale was modified from that given by Ziv and Halevy (1983). Data collected were analyzed for statistical significance using factorial analysis of variance (ANOVA). These computations were done with the software program STATISTICA Software Programme version 2010 (StatSoft Inc., Tulsa, OK, USA). The Fisher least significant difference test was used to compare treatment means at $p = 0.05$ level of significance (Steel and Torrie, 1980).

3.4 RESULTS AND DISCUSSION

3.4.1 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 slight increase in explant height was observed in the presence of AC. Wounding had a significant influence on explant height, with maximum height observed in entire, unwounded explants (Table 3.2). Wounded explants displayed a 28% reduction in height, comparative 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).

3.4.2 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 explant 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 explant discoloration (Table 3.3). The antioxidant, AA, was 20%, 18% and 19% more effective in reducing entire explant discoloration than AC in weeks 6, 7 and 8 respectively. Ascorbic acid treatments have been widely reported to have positive effects on reducing the oxidative browning of explants (Abeyaratne and Lathiff, 2002; Wu and du Toit, 2004). 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 explant discoloration was observed in wounded explants (Table 3.3). 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).

3.4.3 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 3.4). The treatment containing the highest level of NAA (0.5mg l^{-1}) and BAP (6mg 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 opposite* 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 3.4). 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 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; 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 adsorbed 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% was observed which progressively increased to 57% in the final week. As mentioned, wounding stimulates the production of phenols (George, 1993; Dodds and 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 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.

3.4.4 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 0.5mg l^{-1} NAA and 5mg l^{-1} BAP (Table 3.5). This significant increase was opposed to the control (the treatment without NAA and BAP), in which no callus was formed. Reports of NAA and BAP combinations supporting the development of callus have been well documented for several plant species (Koroch et al., 2002; Nurazah et

al., 2009; Ray et al., 2011). 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 ($P \leq 0.05$) higher level of callus formation was observed in AA treatments than in AC treatments (Table 3.5). 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 3.5). The injury that explants experience in response to these wounding techniques may also influence the morphogenic response in a way similar to that of plants in the natural environment, where wounding often stimulates callus formation (George, 1993).

3.4.5 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 3.6). The positive influence of AC on rooting is 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; Feyissa et al., 2005; Loc et al., 2005; Firoozabady et al., 2006). 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).

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

The results represent the significant ($P \leq 0.05$) interactive effects of antioxidant and wounding treatments on discoloration of the entire explants in week 2 (Figure 3.1A) and week 7 (Figure

3.1B). 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 wounding (week 2) or without wounding (week 7). In a study on *Ensete ventricosum*, which is related to *Strelitzia* (Strosse et al.,2009), similar results were found. The addition of AA did not effectively reduce polyphenol exudation. Whereas 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 explant discoloration at the medium contact point produced significant results in weeks 2, 3 and 4 (Figure 3.2). Throughout these weeks, the highest level of discoloration was observed in 0.1 mg l^{-1} NAA and 2mg l^{-1} BAP, in both wounded and unwounded treatments. Whereas the lowest levels were observed in higher NAA and BAP concentrations (0.1 mg l^{-1} NAA : 3mg l^{-1} BAP and 0.5 mg l^{-1} NAA : 5mg l^{-1} BAP), in both unwounded (week 2) and wounded explants (weeks 3 and 4). Similarly, in a study on *Dioscorea opposite* increased NAA and BAP concentrations effectively 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 reveal there to be a strong synergistic effect in BAP and NAA interactions (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 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 explant discoloration at the medium contact point in week 2 (Figure 3.3). 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 explant 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 AC may reduce discoloration by absorbing AA and prevent the oxidation of phenols on the target site (Ko et al., 2009).

The results in Figure 3.4.1 and 3.4.2 represent 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 adsorb 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 explant 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 clonal propagation in *Strelitzia reginae*.

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Table 3.1: Concentrations and combinations of auxin and cytokinin supplements and antioxidants tested in axillary bud proliferation of regenerated embryos.

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

Table 3.2: Plant 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±1.26a	16.60±1.69a	18.50±1.85a	18.80±1.87a	19.00±1.91a	19.55±1.99a	19.90±2.09a	20.30±2.24a	20.65±2.35a
0.1 mg l⁻¹ NAA : 2 mg l⁻¹ BAP	13.70±1.29a	16.55±1.89a	20.40±1.94a	20.95±1.96a	21.20±2.17a	21.80±2.21a	22.25±2.28a	22.95±2.32a	23.25±2.39a
0.1 mg l⁻¹ NAA : 3 mg l⁻¹ BAP	14.50±1.10a	19.35±1.90a	22.35±2.54a	22.95±2.61a	24.60±2.77a	25.40±2.90a	26.20±2.98a	26.65±2.94a	26.80±2.94a
0.5 mg l⁻¹ NAA : 5 mg l⁻¹ BAP	16.20±1.26a	19.25±1.40a	21.20±1.74a	21.75±1.59a	21.70±1.46a	22.30±1.49a	22.90±1.64a	23.50±1.78a	23.60±1.83a
0.5 mg l⁻¹ NAA : 6 mg l⁻¹ BAP	13.65±1.04a	17.60±1.55a	19.65±1.98a	21.25±2.26a	22.25±2.34a	22.80±2.40a	23.05±2.42a	23.40±2.50a	23.45±2.50a
Antioxidants									
AA	14.32±0.73a	16.68±0.93a	18.90±1.06a	19.62±1.14a	20.24±1.19a	20.94±1.23a	21.46±1.27a	22.32±1.34a	22.50±1.38a
AC	14.28±0.78a	19.06±1.17a	21.94±1.43a	22.66±1.44a	23.26±1.51a	23.80±1.57a	24.26±1.63a	24.40±1.65a	24.60±1.67a
Wounding									
Wounded	13.56±0.58a	**15.92±0.92b**	**18.06±1.18b**	**18.34±1.16b**	**18.66±1.22b**	**19.12±1.31b**	**19.24±1.35b**	**19.60±1.43b**	**19.72±1.47b**
Unwounded	15.04±0.89a	**19.82±1.14a**	**22.78±1.28a**	**23.94±1.34a**	**24.84±1.39a**	**25.62±1.38a**	**26.48±1.41a**	**27.12±1.40a**	**27.38±1.40a**
3 - Way ANOVA (F-Statistic)									
Main Effects									
Concentration	0.8ns	0.7ns	0.6ns	0.6ns	0.9ns	1.0ns	1.1ns	1.0ns	0.9ns
Antioxidant	0.0ns	2.6ns	3.0ns	2.9ns	2.7ns	2.3ns	2.0ns	1.1ns	1.1ns
Wounding	1.7ns	**7.1****	**7.2****	**9.8****	**11.2****	**11.7*****	**13.6*****	**14.0*****	**14.1*****
Interactions									
Concentration*Antioxidant	0.4ns	1.1ns	1.2ns	1.3ns	1.5ns	1.5ns	1.2ns	1.2ns	1.1ns
Concentration*Wounding	0.4ns	1.1ns	0.8ns	0.9ns	0.7ns	0.8ns	0.7ns	0.7ns	0.8ns
Antioxidant*Wounding	0.0ns	0.2ns	0.0ns	0.0ns	0.0ns	0.2ns	0.1ns	0.2ns	0.2ns
Conc*Anto*Wounding	0.3ns	0.8ns	0.7ns	0.5ns	0.5ns	0.8ns	0.8ns	1.1ns	1.1ns

**: $P \leq 0.01$; ***: $P \leq 0.001$; 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 $P=0.05$.

Table 3.3: Explant discoloration (entire explant)

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

*: $P \leq 0.05$; ***: $P \leq 0.001$; 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 - 5 (1 = No discoloration and 5 = Extreme discoloration).

Table 3.4: Explant discoloration (at medium 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±0.22a	**1.75±0.22a**	1.75±0.27a	1.75±0.27a	1.75±0.27a	1.95±0.28a	2.35±0.36a	2.40±0.36a	2.45±0.37a
0.1 mg l^{-1} NAA : 2 mg l^{-1} BAP	1.00±0.00a	**1.20±0.09b**	1.30±0.15a	1.35±0.15a	1.70±0.23a	1.90±0.24a	1.95±0.23a	2.05±0.23a	2.25±0.28a
0.1 mg l^{-1} NAA : 3 mg l^{-1} BAP	1.00±0.00a	**1.40±0.15b**	1.40±0.15a	1.45±0.17a	1.50±0.18a	1.70±0.21a	2.05±0.25a	2.05±0.25a	2.30±0.32a
0.5 mg l^{-1} NAA : 5 mg l^{-1} BAP	1.00±0.00a	**1.25±0.12b**	1.55±0.18a	1.55±0.17a	1.95±0.29a	2.15±0.32a	2.20±0.32a	2.30±0.32a	2.30±0.32a
0.5 mg l^{-1} NAA : 6 mg l^{-1} BAP	1.00±0.00a	**1.15±0.08b**	1.30±0.13a	1.40±0.13a	1.55±0.17a	1.80±0.25a	2.05±0.29a	2.35±0.30a	2.70±0.37a
Antioxidants									
AA	1.04±0.04a	**1.62±0.09a**	**1.76±0.13a**	**1.78±0.13a**	1.84±0.13a	1.96±0.13a	2.10±0.14a	2.22±0.14a	2.44±0.19a
AC	1.08±0.08a	**1.08±0.08b**	**1.16±0.09b**	**1.22±0.09b**	1.54±0.16a	1.84±0.19a	2.14±0.22a	2.24±0.22a	2.36±0.23a
Wounding									
Wounded	1.00±0.00a	**1.46±0.09a**	**1.70±0.11a**	**1.76±0.11a**	**2.12±0.15a**	**2.48±0.16a**	**2.84±0.17a**	**3.02±0.17a**	**3.36±0.18a**
Unwounded	1.12±0.09a	**1.24±0.10b**	**1.22±0.12b**	**1.24±0.12b**	**1.26±0.12b**	**1.32±0.12b**	**1.40±0.12b**	**1.44±0.13b**	**1.44±0.13b**
3 - Way ANOVA (F-Statistic)									
Main Effects									
Concentration	1.8ns	**4.5****	1.5ns	1.0ns	0.8ns	0.6ns	0.4ns	0.5ns	0.5ns
Antioxidant	0.2ns	**28.0*****	**18.6*****	**15.7*****	2.7ns	0.4ns	0.0ns	0.0ns	0.1ns
Wounding	1.8ns	**4.7***	**11.9*****	**13.5*****	**22.4*****	**33.6*****	**40.8*****	**53.1*****	**69.0*****
Interactions									
Concentration*Antioxidant	0.2ns	0.8ns	0.7ns	0.6ns	1.3ns	1.1ns	0.7ns	0.6ns	0.9ns
Concentration*Wounding	1.8ns	**3.6****	1.4ns	1.3ns	2.1ns	1.7ns	0.5ns	0.5ns	0.9ns
Antioxidant*Wounding	0.2ns	**13.9*****	**11.9*****	**11.5****	1.0ns	0.0ns	1.5ns	2.5ns	1.1ns
Conc*Anto*Wounding	0.2ns	0.5ns	0.3ns	0.4ns	1.2ns	0.8ns	0.2ns	0.1ns	0.3ns

**: 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 - 5 (1 = No discoloration and 5 = Extreme discoloration).

Table 3.5: Callus formation

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

*: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; 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.

Table 3.6: Root length (mm)

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

*: $P \leq 0.05$; 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

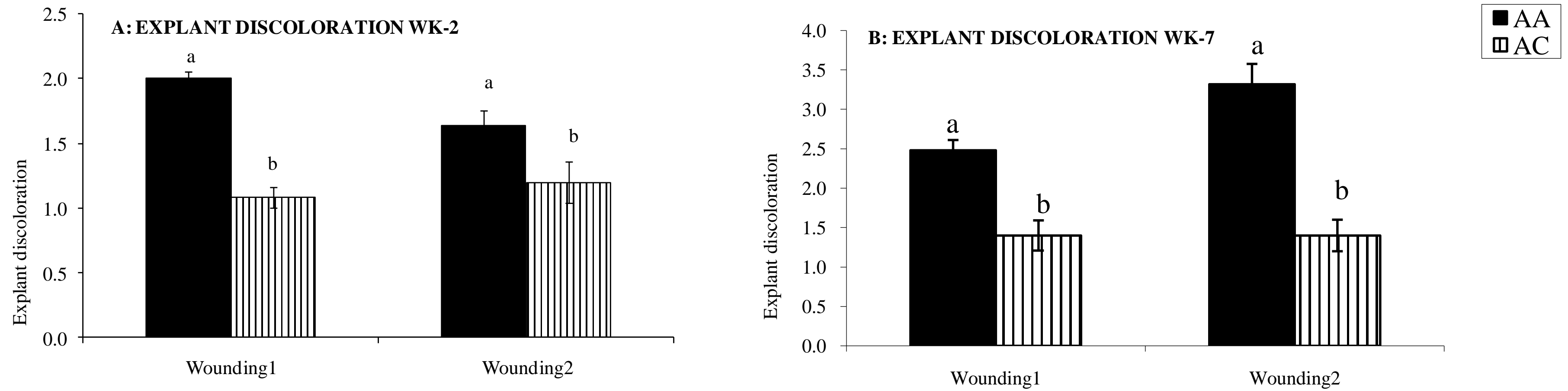

Figure 3.1: Interactive effects of antioxidants and wounding on entire explant discoloration in (A) WK2 and (B) WK7. Rating scale used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration). Wounding 1 = Wounded, Wounding 2 = Unwounded

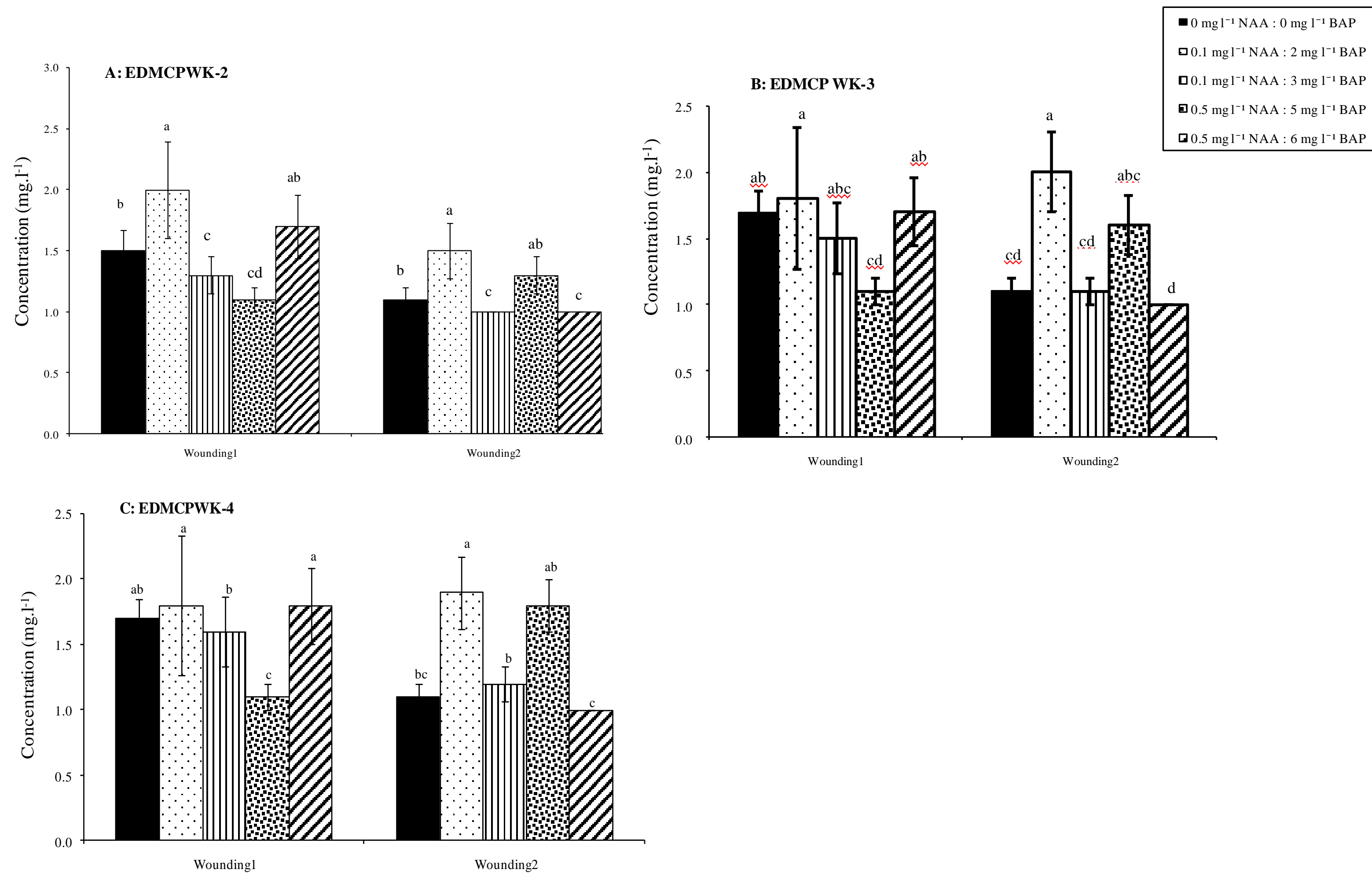

Figure 3.2: Interactive effects of wounding and concentration on explant discoloration at the medium contact point (EDMCP) in (A) WK2, (B) WK3, (C) WK4. Rating scale used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration). Wounding 1 = Wounded, Wounding 2 = Unwounded

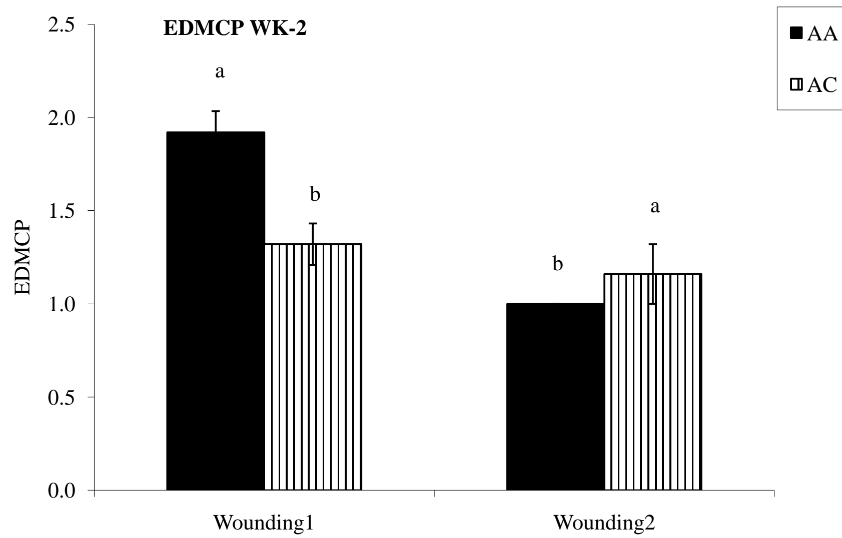

Figure 3.3: Interactive effects of antioxidants and wounding on explant discoloration at the medium contact point (EDMCP) in WK2. Rating scale used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration). Wounding 1 = Wounded, Wounding 2 = Unwounded

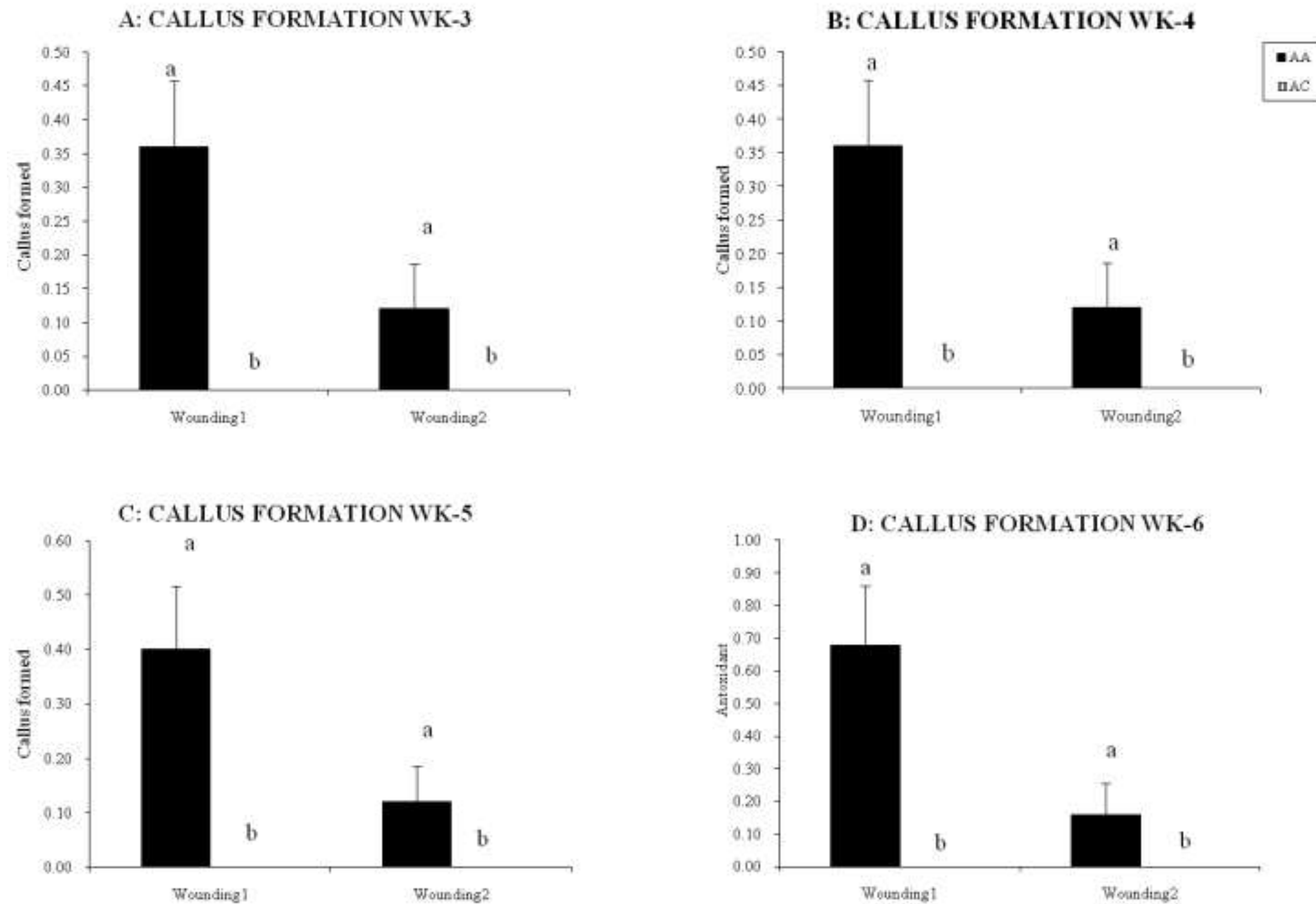

Figure 3.4.1: Interactive effects of antioxidants and wounding on callus formation in (A) WK3, (B) WK4, (C) WK5, (D) WK6. The degree of callus formation is rated as: 1 = none, 2 = low, 3 = medium, 4 = high. Wounding 1 = Wounded, Wounding 2 = Unwounded

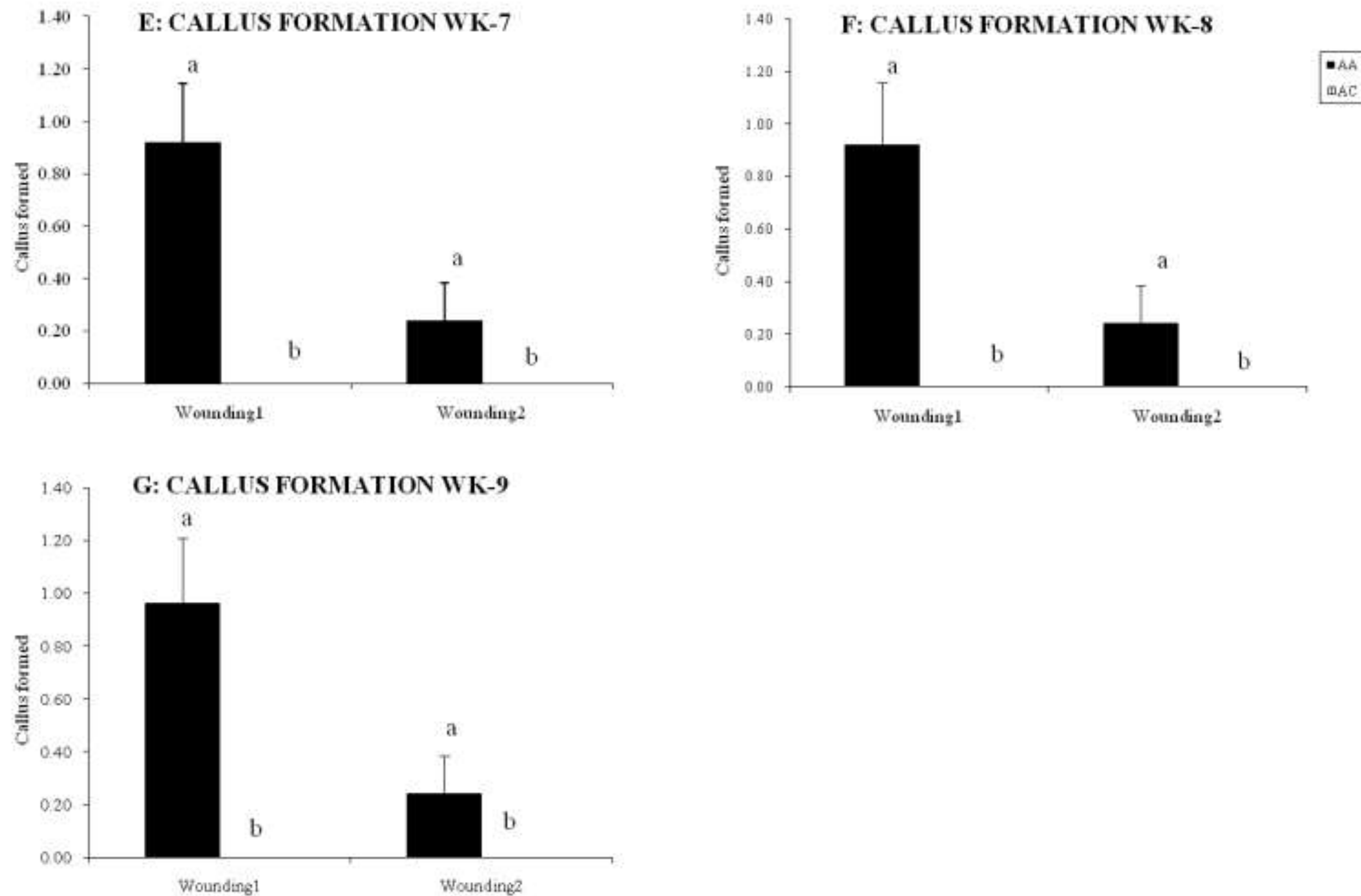

Figure 3.4.2: Interactive effects of antioxidants and wounding on callus formation in (E) WK7, (F) WK8 and (G) WK9. The degree of callus formation is rated as: 1 = none, 2 = low, 3 = medium, 4 = high. Wounding 1 = Wounded, Wounding 2 = Unwounded

CHAPTER FOUR

EFFECTS OF ANTIOXIDANTS, PLANT GROWTH REGULATORS AND WOUNDING ON PHENOLIC COMPOUND EXCRETION DURING MICROPROPAGATION OF *STRELITZIA REGINAE*.

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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4.1 Abstract

The aim of this study was to determine the effects of antioxidant treatments, plant growth regulators (PGRs) and explant wounding in tissue culture attempts involving *Strelitzia reginae* on total phenol exudation. Results showed that various NAA and BAP concentrations significantly affected phenolic exudation. The media containing the highest PGR concentration (0.5mg l^{-1} NAA and 6mg l^{-1} BAP) resulted in the highest phenol content. Whereas, the control (the treatment free of PGRs), contained the lowest phenol content. Activated charcoal (AC) significantly reduced 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 NAA and BAP concentrations and AA significantly increased the total phenol content of media. A similar result was achieved in interactions between higher PGR concentrations and the wounding of explants. Interactions between antioxidants, wounding treatments and PGR concentrations resulted in AC significantly reducing the total phenol content in all PGR concentrations in both wounded and unwounded explants.

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

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)

4.2 INTRODUCTION

The bird of paradise (*Strelitzia reginae*) is of significant commercial value (Paiva et al., 2004). It 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). For these reasons, 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 is also frequently encountered in genera related to *Strelitzia*, namely *Musa* and *Ensete* (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; 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 et al., 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 its structure, AC has a very fine network of pores with 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 et al., 1989). Phenols are reactive compounds (Lux-Endrich et al. (2000). Thus, the various PGR concentrations may affect phenolic exudation.

A study was carried out to determine the optimal antioxidant, plant growth regulator (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 into 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.

The main objective of the present study was to determine the total phenol amount excreted into the culture media within these different treatments and 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. This is critical for successful *in vitro* culture of *S. reginae*.

4.3 MATERIALS AND METHODS

4.3.1 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.

4.3.2 Culture conditions and media

Explants were transferred to different regeneration media. The basal medium comprised the MS salts supplemented with 100mg l^{-1} myo-inositol, 0.1mg l^{-1} thiamine-HCl, 0.1 mg l^{-1} pyridoxine, 2mg l^{-1} glycine and 30g l^{-1} sucrose. Various concentrations of 6-benzylaminopurine (BAP) 0, 2, 3, 5, 6mg l^{-1} and 1-naphthalene acetic acid (NAA) 0, 0.1,

0.5mg l^{-1} were added to the media. The antioxidants, 2.5g l^{-1} activated charcoal and 0.05g l^{-1} ascorbic acid, were each separately added to the various media. The experiment consisted of ten medium types (Table 4.1). Ten replicates were used for each treatment. The media was solidified with 7g l^{-1} agar. The pH of the media was adjusted to 5.95 prior to autoclaving at 121°C for 20 minutes. The unwounded cultures were incubated in a growth room with a 16h light and 8h dark cycle at 25 ± 2°C.

4.3.3 Chemical analysis

After 9 weeks of growth, the explants were removed and the total phenols amounts of the culture media (for excreted phenols from explants to medium) were analyzed according to Folin-Ciocalteu method (Singleton and Rossi, 1965; Chandler et al., 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).

4.3.4 Sample preparation for 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 15mL of methanol on a rotary mixer for 30 minutes. This was then centrifuged for 10 minutes at 4000 rpm (revolutions per minute). The supernatant was used in the analysis of the phenols. For the assay, 25 microliter (uL) of the supernatant was mixed with 125 uL Folin reagent (0.2M), followed by 100 uL sodium carbonate (7.5%) in a 96-well clear plate. This was left to incubate for 2 hours 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 (Singleton and Rossi, 1965; Chandler et al., 1983; Singleton et al., 1999).

4.3.5 Statistical analysis

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

4.4 RESULTS AND DISCUSSION

4.4.1 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 4.2). The media with the highest PGR concentration (0.5mg l^{-1} NAA and 6mg l^{-1} BAP) contained the highest phenol content. This media contained 17.58mg l^{-1} total phenols, as opposed to the control (the treatment free of plant hormones) which contained 12.9mg 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 biosynthesis 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.

The AC 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 4.2).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). AC has a very fine network of pores with large inner surface areas on which many substances can be adsorbed (Thomas, 2008). 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 that unwounded explants (Table 4.2). These results indicate a strong relationship between total phenolics content and wounding. 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 compounds readily become oxidized by air (Compton et al., 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 et al., 1986; Laukkanen et al., 1999).

4.4.2 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 4.1). In all NAA and BAP concentrations, AC was more effective than AA in reducing phenolic excretion. A significantly reduced level of total phenols were recorded in AC supplemented media. The lowest phenol content of media occurred in AC supplemented media with 0.1mg l^{-1} NAA and 3mg l^{-1} BAP. The highest amount of total phenols was recorded in AA supplemented media with the increased concentration of 0.5mg l^{-1} NAA and 5mg 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 (0.5mg l^{-1} NAA and 6mg 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 the 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 4.2). 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 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, has also been reported 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 4.3. 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. AC 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 4.4 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 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 0.1mg l^{-1} NAA and 3mg l^{-1} BAP. In AA treatments, wounding increased the severity of phenolic exudation, with the highest phenol content recorded in

0.5mg l^{-1} NAA and 5mg 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 explant treatments, the concentration of PGRs did not significantly affect he 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 total phenolic compound excretion. Furthermore, the interactive effects of these treatments on phenol exudation are exposed.

4.5 ACKNOWLEDGEMENTS

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Table 4.1: Concentrations and combinations of auxin and cytokinin supplements and antioxidants tested in axillary bud proliferation of regenerated embryos.

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

Table 4.2: Effect of various NAA and BAP concentrations, antioxidants and wounding treatments on phenol exudation (mg l^{-1}) into culture media.

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

*: $P \leq 0.05$; ***: $P \leq 0.001$. Values (Mean ± MSE, n = 10) followed by dissimilar letters in a column are significantly different by Least Significant Difference test at P=0.05.

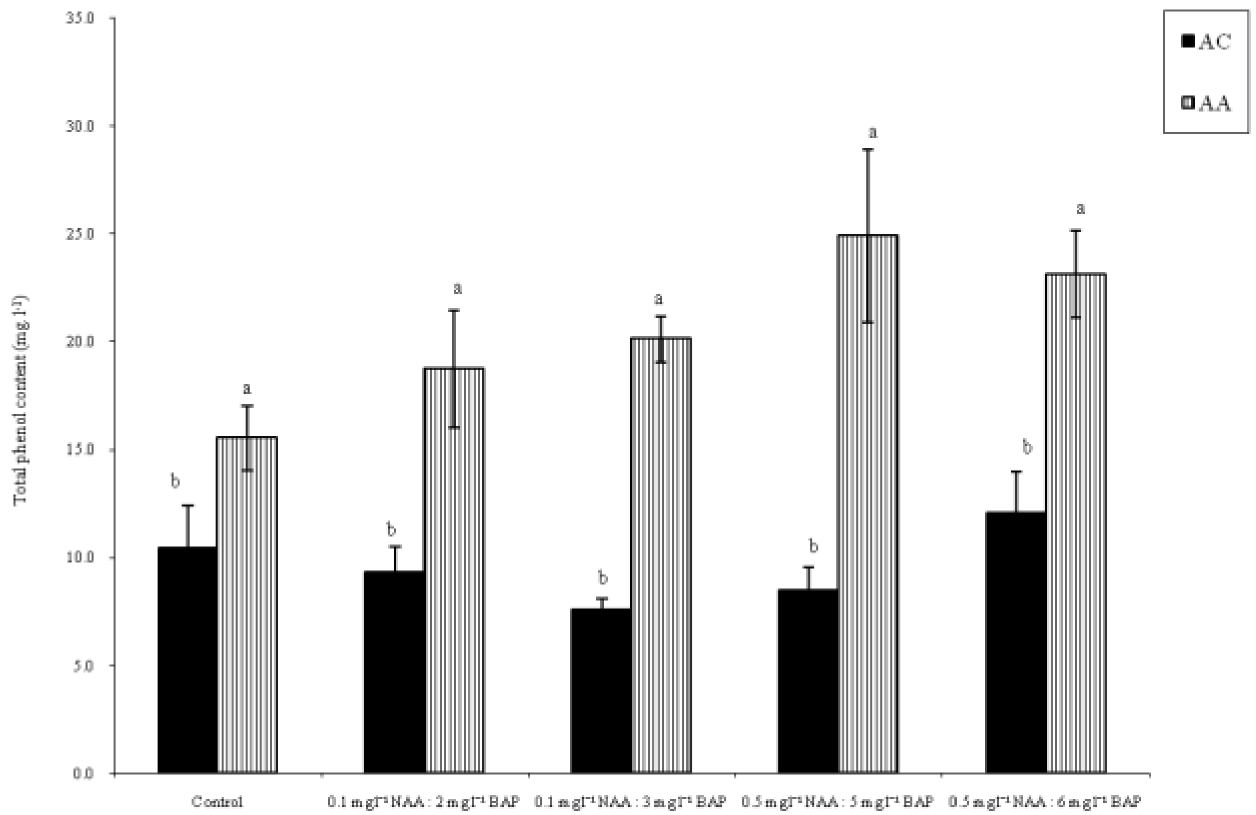

Figure 4.1: Interactive effects of NAA and BAP concentrations and antioxidants on total phenol content of culture media (mg l^{-1}).

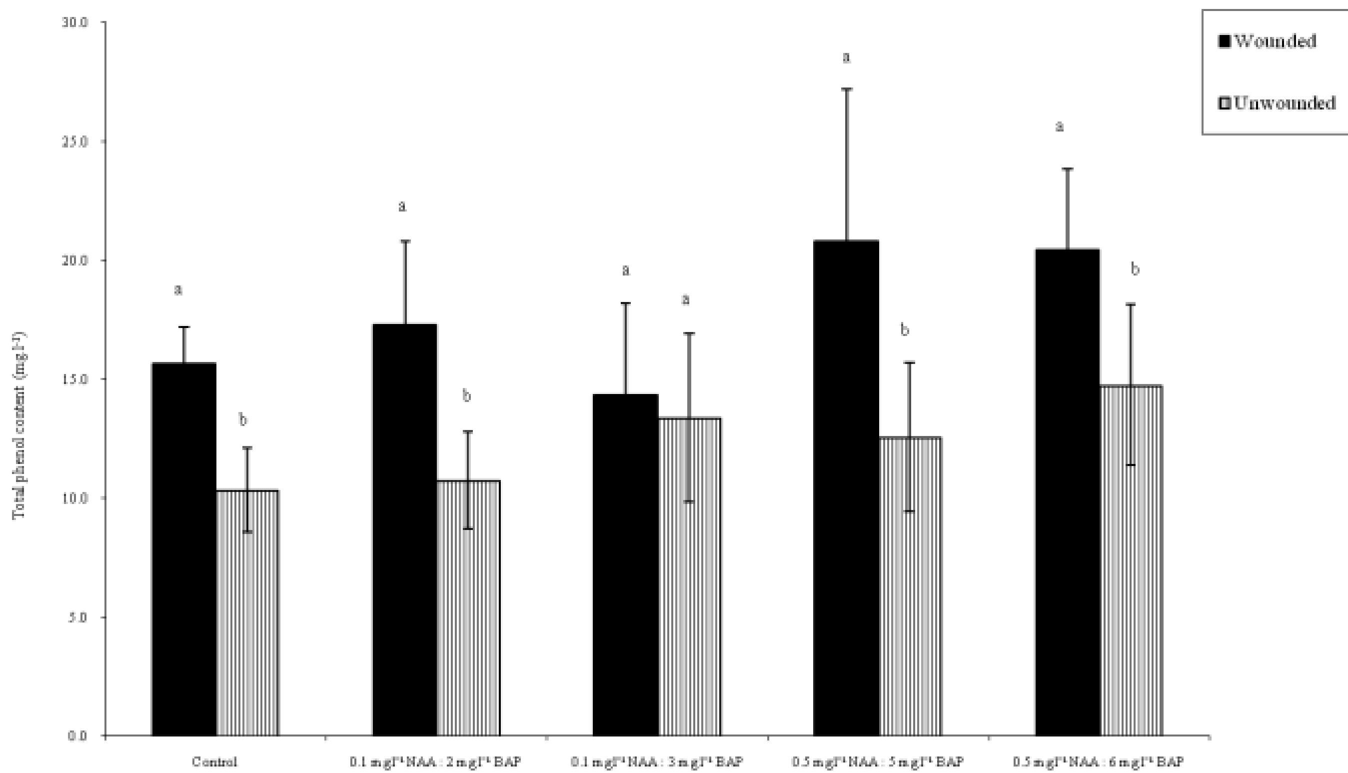

Figure 4.2: Interactive effects of NAA and BAP concentrations and wounding on total phenol content of culture media (mg l^{-1}).

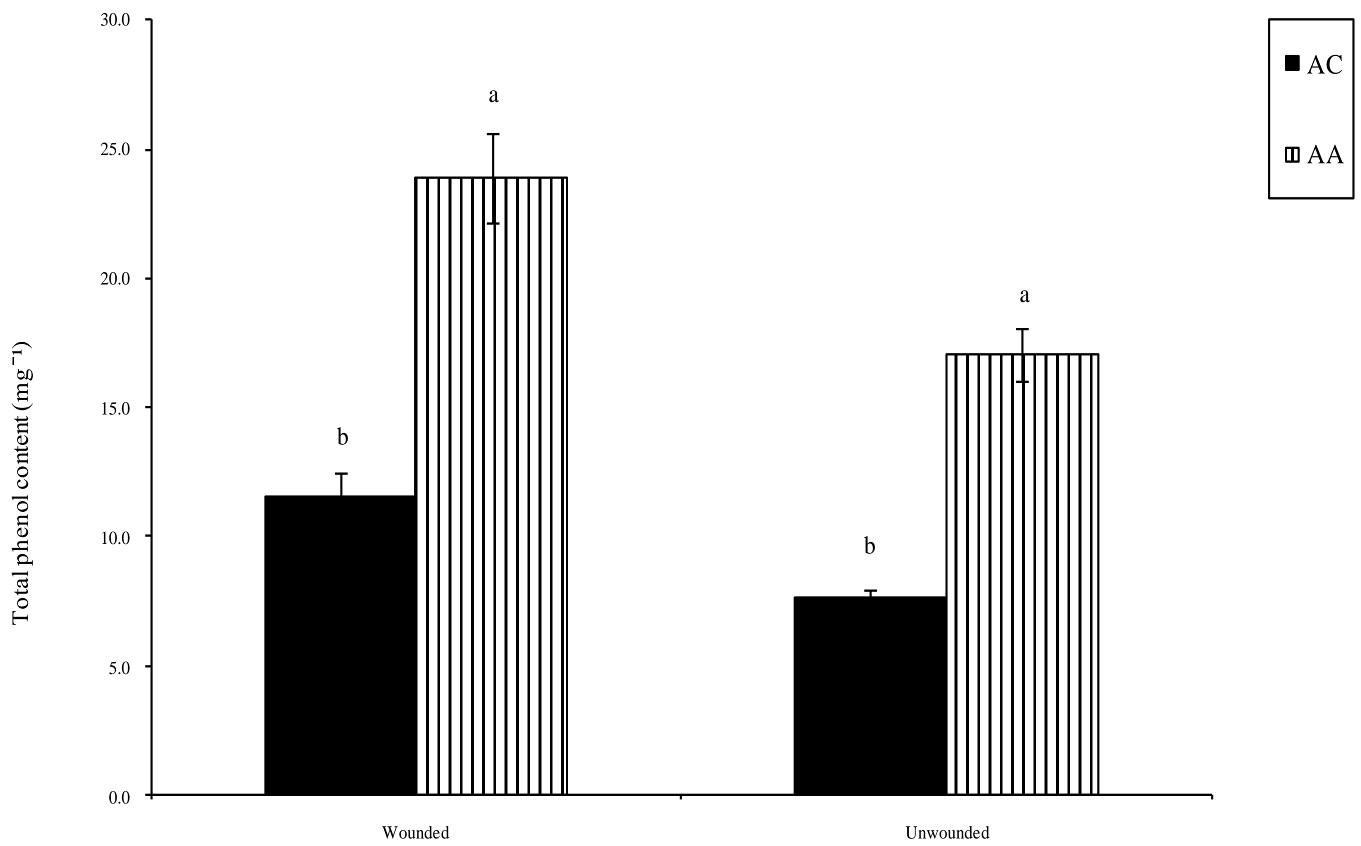

Figure 4.3: Interactive effects of antioxidants and wounding on total phenol content of culture media (mg l^{-1}).

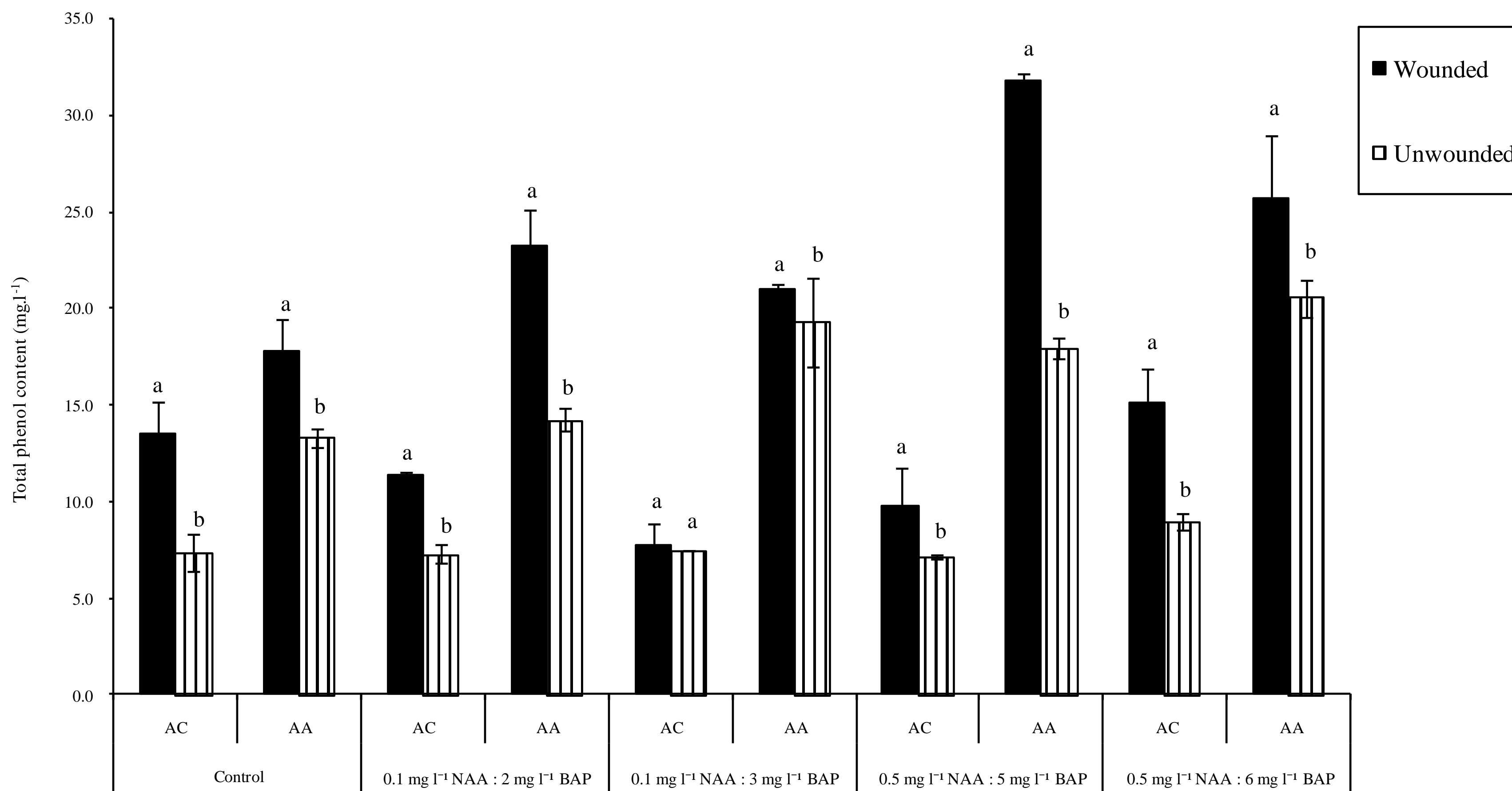

Figure 4.4: Interactive effects of NAA and BAP concentrations, antioxidants and wounding on total phenol content of culture media (mg l^{-1}).

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

The introductory chapter indicated the rationale for the study with a review of literature, highlighting the significant commercial value of *Strelitzia reginae*. Despite high demands it is not widely spread due to the constraints on its production. The slow and unfeasible conventional methods of propagation limit its success. This study identified the need for micropropagation to exploit the potential of this plant. The development of a reliable and advanced propagation method would overcome the constraints and enable the large scale, commercial production of *S. reginae*.

Despite high demands for this plant, a reliable method of micropropagation has not yet been developed. The limited success in the application of tissue culture techniques for *S. reginae* is mainly attributed to the oxidative browning of wounded explants. This study investigated the use of immature zygotic embryos as starting material. The reason for this being that the limited wounding needed to gain this material may reduce phenolic exudation during the crucial initial stages of culture and enable further explant development.

In order to define a media for the *in vitro* germination of embryos, macro- and micronutrient strengths and vitamin supplementation were investigated. The incorporation of activated charcoal (AC) to culture media was also tested in efforts to reduce phenolic oxidation. This study demonstrated that interactions on these media components significantly affected the rate of embryo germination. The highest germination rate of embryos was observed in media containing activated charcoal without vitamin supplementation. The addition of vitamins to culture media negatively affected germination. The concentration of macro- and micronutrients, vitamin supplemention and AC also significantly affected the discoloration of explants and culture media, the length of shoots and the development of roots. In order to gain the highest germination rate of *Strelitzia reginae*, it is important to supplement the culture media with a suitable level of AC without the addition of vitamins in either full or half strength MS medium.

In efforts to stimulate axillary bud proliferation, embryo derived seedlings were subjected to various media and wounding treatments. Media treatments were comprised of the plant growth regulators (NAA and BAP) at various ratios. The proportions of these hormones were manipulated to break dormancy and enhance shoot formation. Wounding treatments were aimed at eliminating the strong apical dominance effect present in *Strelitzia*, also in efforts to promote axillary bud development. A comparative study between the antioxidants, ascorbic acid (AA) and activated charcoal was carried out to determine the most effective in reducing phenolic oxidation of cultures. Insight was gained into the effects of the plant growth regulators (NAA and BAP), antioxidants (AC and AA) and wounding on *in vitro* growth and discoloration of plantlets. This study found the antioxidant AA more effective than AC in

reducing discoloration of the entire explant. Whereas, AC was more effective than AA in reducing explant discoloration at the point of contact. It is at the point of contact between the plantlet and the medium that oxidative browning is exaggerated. The levels of PGRs in culture media significantly affected entire explant discoloration, with the highest level (NAA (0.5mg l^{-1}) and BAP (6mg l^{-1}) being the most effective. The highest level of discoloration was observed in the control (the treatment without NAA and BAP). Callus formation was induced in NAA and BAP treatments. The highest level of callus was formed in 0.5mg l^{-1} NAA and 5mg l^{-1} BAP. Whereas, no callus was formed in the control (the treatment without NAA and BAP). AC was found to inhibit callus formation. Callus formation in AA treatments was 75% greater than that of AC. AC promoted root formation. Roots developed in AC treatments were 77% longer than roots in AA treatments. Plant regeneration via axillary bud proliferation was not significantly increased. In order to improve the clonal propagation of *S. reginae*, future studies should be aimed at increasing the rate of axillary bud development and shoot regeneration from callus.

Oxidative browning has been identified as a major problem in developing an efficient protocol for the micropropagation of *Strelitzia*. A study was carried out in efforts to identify factors contributing to and reducing the total phenolic exudation. Phenolic compounds were found to have a reactive response to media composition and wounding. The highest concentration of NAA and BAP resulted in the highest phenol content recorded in culture media. Wounding of explants significantly increased phenolic exudation. In a comparative study, AC was found to be 53% more effective in reducing the total phenol content of media than AA. In conclusion the ratio and concentration of NAA and BAP needs to be optimum in order to reduce phenolic exudation and enable successful *in vitro* culture of *Strelitzia reginae*. Although wounding is envisaged to promote axillary bud formation, but increases phenolic compound exudation into culture media.

Further studies must focus on optimising the multiplication rate of *Strelitzia reginae* propagules *in vitro*. Attention must be paid to inducing axillary bud formation and shoot regeneration via callus.

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APPENDICES

APPENDIX A:

North, J.J. Ndakidemi, P.A. & Laubscher, C.P. 2010. The potential of developing an *in vitro* method for propagating Strelitziaceae. *African Journal of Biotechnology*, 9(45): 7583-7588.

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Review

The potential of developing an *in vitro* method for propagating Strelitziaceae

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***Strelitzia* spp. are highly valued as cut flowers and are of significant commercial value. Despite high demands, they have not been widely spread due to production constraints and are one of the few important cut flower plants for which no uniform cultivars are available. The conventional methods of propagation are very slow due to the plants low rate of multiplication. Large scale propagation and cloning is therefore needed to exploit its potential. Despite the plants commercial importance, a method for micropropagation has not yet been established. Tissue culture attempts of this plant have failed due to the oxidative browning of explants. Wounded tissues release polyphenolic compounds which are detrimental to further development of explants. Only partial success and a low rate of multiplication have been obtained. This review explores the possibilities of developing an *in vitro* method for the successful propagation of *Strelitzia* spp.**

Key words: *Strelitzia* spp., activated charcoal, antioxidants, auxins, cytokinins, dark incubation, immature embryos, media composition, wounding.

INTRODUCTION

The bird of paradise (*Strelitzia*) is an important ornamental monocotyledonous plant of South African origin (Chand, 2008). This tropical perennial is a beautiful plant of significant commercial value (Paiva et al., 2004). The exotic features of its colourful flowers, the long length of the stem and the high post-harvest durability result in it being highly valued as a cut flower (Wood, 1995). *Strelitzia reginae* has been one of the most sought after cut flowers destined for exportation from developing countries (Criley, 1988). However, its commercial exploit-tation and success is limited by its low rate of multiplication (Ziv and Halevy, 1983). Propagation is achieved either by seed or vegetatively by division. Both of these conventional propagation methods are very slow (Karnataka, 2008).

Propagation by seed is undesirable as *Strelitzia* presents a slow development, requiring 4 to 7 years to start producing flowers (Ziv and Halevy, 1983). Furthermore, from pollination to seed harvesting, a further 5 to 6 months is required (Criley, 1988). However, the greatest difficulty in the propagation of *Strelitzia* spp. via seed is the dormancy of seeds (Garcia, 2006). A germination inhibitor is thought to occur within the seeds (van de Venter and Small, 1975), which prolongs the time and results in a low percentage of seed germination (Garcia, 2006). The limited production of seeds obtained per plant must be taken into consideration. There is also a great degree of genetic variation in plants developed from seed (van de Pol and van Hell, 1988).

For vegetative propagation by division, plants of at least 10 years old must be used (Ziv and Halevy, 1983). This method of dividing naturally developed branches is limited by a low rate of multiplication, which was determined to be 0.5 to 1.5 divisions per branch per year (Vonk Noordegraaf and van der Krogt, 1976).

Thus, both methods limit the large scale production which is needed to exploit the plants potential and to enhance its improvement by cultivar development. Due to these constraints on production, it is one of the few important cut flower plants for which no uniform cultivars are commercially available (Ziv and Halevy, 1983).

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Abbreviations: **BAP,** 6-Benzylaminopurine; **NAA,** naphthaleneacetic acid; **MS,** Murashige and Skoog.

Tissue culture could be more promising than other conventional methods of multiplying *Strelitzia* (Promtep, 1981). A reliable and advanced propagation and cloning method would greatly contribute to overcoming the limitations this species poses to the horticultural and landscaping industry worldwide (Ziv and Halevy, 1983). Despite the plants commercial importance, a reliable method for micropropagation has not yet been developed (Chand, 2008).

The lack of successful tissue culture techniques for the propagation of *S. regina* was due to oxidative browning of the explants as reported earlier by Ziv and Halevy (1983). Wounded tissue releases polyphenolic compounds, which diffuse into the culture medium (Strosse et al., 2009). These undesirable exudates were found to be detrimental to the development of the explants, as they promoted the onset of necrosis (Ziv and Halevy, 1983). Terminal and axillary meristems of *S. reginae* were used by Ziv and Halevy (1983) but success depended on the use of antioxidants to prevent browning. This is a destructive method as plants are destroyed when their terminal and axillary buds are excised. Thus, it is an unsuitable method when starting material is limited or in the culture of rare plants. Paiva et al. (2004) reported the failure of *in vitro* development using axillary buds and leaf segments, irrespective of the applied treatments. Phenolic oxidation was identified as a crucial problem. Thus, it was not possible to reproduce the protocol developed by Ziv and Halevy (1983) for the *in vitro* propagation of *S. reginae* from axillary buds. However, the germination of immature embryos inoculated *in vitro* resulted in well-formed and complete plants (Paiva et al., 2004). There are no reports on success or attempts made in the stimulation of axillary bud proliferation from embryo-derived plantlets.

In several investigations, 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, suggesting that the protocols developed so far have not been efficient with respect to the growth and large scale multiplication of this plant.

The objective of this review is to discuss possible techniques for the rapid clonal propagation of *Strelitzia* spp., using embryos as the starting material. Zygotic embryo culture could induce faster growth and a higher germination rate (Chang and Yang, 1996; Bürün and Çoban Poyrazoğlu, 2002). The use of embryos as explants will allow the variation in seeds (Foolad and Jones, 1992; Larkin et al., 1984) to play an important role in the breeding cycle and development of cultivars, reducing seed dormancy. Furthermore, the limited wounding of the explants may remedy the production of phenolic exudates and the excessive need for antioxidants during the crucial initial stages of plant development. In addition, it is not a destructive method. There is no need to destroy plants in order to gain starting material for the culture.

Thus, indicating the possibility of developing an efficient method of clonal propagation and mass production of this kind of plant in the future.

POSSIBLE EFFECTS OF VARIOUS MEDIA COMPOSITIONS ON THE *IN VITRO* GERMINATION OF IMMATURE EMBRYOS OF *STRELITZIA* SPP.

The germination of excised embryos is influenced by the maturity of embryos at excision, the composition of the culture medium (Johri and Rao, 1984; Diro and van Staden, 2004) and genetic variation (Pierik, 1979).

Embryos excised from developing seed at or near maturity are completely autotrophic (Gamborg and Phillips, 2002), whereas, immature embryos require a far more critical medium composition compared with mature embryos (Pierik, 1979). The younger the embryo, the more complex is its nutritional requirements (Bajaj, 1977). The chances of success in this type of culture depend largely on the developmental stage of the excised embryo (Razdan, 1993). Paiva et al. (2004) reported that the best time for seed collection and embryo excision for *S. reginae* was 20 weeks after pollination. However, the most important aspect in culturing embryos is to develop and clearly define a culture medium that can sustain growth and development (Chawla, 2002). In less than optimum medium, the immature embryos may fail to survive, turn into undifferentiated callus, or germinate prematurely (Gamborg and Phillips, 2002).

A variation of macro- and micro-nutrients

The MS medium of Murashige and Skoog (1962) is a salt composition that supplies the needed macro- and micronutrients. To achieve growth differentiation, concentrations of inorganic nutrients must be optomized such that the medium meets the requirements of the cells or tissues used (Chawla, 2002). This is applicable to the nutritional requirements of an embryo and its developmental stage. The *in vivo* development of an embryo consists of two phases:

(1) The heterotrophic phase: An early phase wherein the embryo is nutritionally dependent on the endosperm and maternal tissues.

(2) The autotrophic phase: A later phase in which the embryo is metabolically capable of synthesising substances required for its growth, thus is fairly independent for nutrition.

Thus, while relatively mature embryos can grow on a simple inorganic salt medium supplemented with a carbohydrate source, the nutritional requirements of relatively immature embryos is complex (Bajaj, 1977). The critical stage at which the embryo passes from the heterotrophic phase into the autotrophic phase varies with the species (Razdan, 1993).

The developmental phase and nutritional requirements

of *Strelitzia* embryos 20 weeks after pollination is uncertain. Therefore, it is necessary to evaluate a variation in inorganic nutrients (macro and micronutrients) and vitamins to obtain optimum embryo growth and development.

The use of activated charcoal as a phenolic adsorbent

The use of tissue culture for the propagation of *S. reginae* has invariably failed due to oxidative browning of the explants (Ziv and Halevy, 1983). During the initial stages of culture development, the production of polyphenols is excessive (Pan and van Staden, 1998). The polyphenol exudate that diffuses into the medium was found to be detrimental to the further development of the explants, which become necrotic and die (Ziv and Halevy, 1983).

The identified problem of phenolic oxidation has also been reported in *Musa* and *Ensete* spp. (Zeweldu and Ludders, 1998; Diro and van Staden, 2004; Birmeta and Welander, 2004), which are related to *Strelitzia* (Strosse et al., 2009). Activated charcoal, used in tissue culture media to adsorb inhibitory substances, has been used with success in Musaceae and Strelitziaceae (Diro and van Staden, 2004; Ziv and Halevy, 1983). In *Ensete*, a genus of Musaceae, the use of activated charcoal enabled zygotic embryos to regenerate healthy seedlings. The inclusion of activated charcoal into the media reduced oxidative browning, promoted germination of the embryos and improved the growth of seedlings (Diro and van Staden, 2004). In *S. reginae*, Ziv and Halevy (1983) found activated charcoal in combination with antioxidant treatments, effective in controlling oxidative browning of terminal and axillary buds. However, there are no reports on the use of activated charcoal on the zygotic embryos of *S. reginae* or other *Strelitzia* spp.

Strelitzia competence in responding to *in vitro* culture is dependent on reducing oxidative browning of the explants (Ziv and Halevy, 1983). Thus, an investigation into the addition of activated charcoal to culture media is of vital importance in the development of a suitable technique for the germination of excised embryos, as has been done in *Ensete*.

POSSIBLE EFFECTS OF AUXIN AND CYTOKININ CONCENTRATIONS ON INDUCING MULTIPLE SHOOTS FROM AXILLARY BUDS

This involves the use of plant growth regulators in an attempt to stimulate the development of axillary buds, which are usually present in the axil of each leaf (Chawla, 2002). Axillary bud proliferation exploits the normal ontogenetic route for branch development by lateral meristems (Gamborg and Phillips, 2002). However, many axillary meristems remain dormant *in vivo* (in nature) if the type of branching for a particular species displays apical dominance (Razdan, 1993). Apical dominance suppresses the growth and development of axillary buds in the presence of a terminal bud (Chawla, 2002).

In *Strelitzia*, there is an absolute absence of branching from axillary buds *in vivo*. This may be as a result of a strong apical dominance effect (van de Pol and van Hell, 1988). Therefore, a method of eliminating apical dominance *in vitro* to promote branching, is required to increase the multiplication rate of *Strelitzia*.

Since the mechanism of apical dominance has been demonstrated to be under the control of various growth regulators, the proportions of these substances in the media can be manipulated to break dormancy and enhance shoot formation (Razdan, 1993). The growth regulators, auxins and cytokinins, are of particular significance in *in vitro* culture (Pierik, 1979). Cytokinins are concerned with the modification of apical dominance (Razdan, 1993). A high cytokinin concentration promotes axillary shoot formation by decreasing apical dominance (Pierik, 1979; Dodds and Roberts, 1995; Chawla, 2002). Although cytokinin induces the growth of buds, auxin is required in the culture media. Most cultures require a combination of these two compounds for growth regulation (Dodds and Roberts, 1995). Usually the manipulation and variation of auxin and cytokinin levels can successfully alter growth behaviour in plant cultures (Dixon and Gonzales, 1994). For axillary shoot formation, a low auxin concentration, together with a high cytokinin concentration is required (Pierik, 1979; Razdan, 1993).

The cytokinin treatment can be varied, that is, the selection of the type of cytokinin and concentration (Pierik, 1979). In *Musa*, a relative of *Strelitzia*, 6-benzylaminopurine (BAP) is said to be the preferred cytokinin (Banerjee and de Langhe, 1985). It has dramatically influenced axillary shoot formation in various *Musa* spp. (Wong, 1986; Arinaitwe et al., 2000; Srangsam and Kanchanapoom, 2007). Earlier reports revealed that there is a strong synergistic effect of BAP-1-naphthaleneacetic acid (NAA) interactions (Novak et al., 1989; Okole and Schulz, 1996; Cote et al., 2000; Khalil et al., 2002; Srangsam and Kanchanapoom, 2007).

The concentration and combination of auxin and cytokinins in the nutrient medium is a key factor which determines successful plant regeneration (Razdan, 1993). To improve the success of axillary bud proliferation for *Strelitzia* spp., the optimal balance between these two groups of growth regulators needs to be determined. Thus, indicating the importance of a study into BAP-NAA interactions.

POSSIBLE EFFECTS OF WOUNDING ON SUPPRESSING APICAL DOMINANCE IN ORDER TO INDUCE MULTIPLE BUD DEVELOPMENT

Apical dominance inhibits the development of axillary meristems (Razdan, 1993). In nature, axillary meristems are generally the source of bud formation when leaders

are damaged (Burrows, 1989). This indicates the positive effects of meristem wounding and even apical bud removal on stimulating the growth of axillary buds.

In *Strelitzia*, the absence of branching from axillary buds, may be due to a strong apical dominance effect. The introduction of *in vivo* branching can increase the multiplication rate of *Strelitzia*. This can be achieved by the removal of the apical dome. This method of eliminating apical dominance is practised in vegetative propagation by the division of branches known as fans (van de Pol and van Hell, 1988).

Similarly to *in vivo* methods, an *in vitro* method to reduce apical dominance and promote axillary bud development is needed. In *Musa* and *Ensete*, success has been achieved through wounding of the meristem region (Jarret et al., 1985; Gupta, 1986; Birmeta and Welander, 2004). There are no reports on the effects of *in vitro* wounding in Strelitziaceae. However, it is hopeful that axillary meristem development as observed in the *in vivo* methods as well as in the *in vitro* cultures of its relative Musaceae, can be applied for improving axillary bud development in Strelitzia. An investigation into the wounding effects on *Strelitzia* spp. may be significant in the clonal propagation of this plant.

POSSIBLE EFFECTS AN ADSORBENT AND ANTIOXIDANT MAY HAVE ON REDUCING OXIDATIVE BROWNING OF WOUNDED TISSUES

As previously mentioned, the excessive production of polyphenols is a problem frequently encountered during the initial stages of culture develoment (Ziv and Halevy, 1983; Pan and van Staden, 1998; Birmeta and Welander, 2004; Diro and van Staden, 2004). Tissue injury, during explant excision, stimulates the production of phenols (Dodds and Roberts, 1995) as a form of a defence mechanism (Pan and van Staden, 1998). The incorporation of activated charcoal to initiated cultures is most effective in controlling polyphenol oxidation (Ziv and Halevy, 1983; Pan and van Staden, 1998; Chawla, 2002; Birmeta and Welander, 2004; Kiong et al., 2007; Karnataka, 2008). The adsorption of toxic phenols prevents the browning and death of the tissues (Horner et al., 1977; Fridborg et al., 1978; Weatherhead et al., 1979; George and Sherrington, 1984; Madhusudhanan and Rahiman, 2000; Chawla, 2002).

In the (multiplication stage) of culture, wounding techniques will be employed in efforts to induce axillary bud proliferation. Polyphenol exudation is exag-gerated in response to wounding (Birmeta and Welander, 2004). Thus, indicating the need for an adsorbent or the addition of an antioxidant in the culture media.

The beneficial use of activated charcoal as a culture component for the adsorption of toxic substances is established (Teixeira et al., 1994; Veramendi and Navarro, 1996; Pan and van Staden, 1998; Gallo-Meagher and Green, 2002). However, its addition to shoot proliferation media may have adverse effects on growth and development as activated charcoal is able to adsorb high concentrations of growth regulators (Fridborg et al., 1978; Ebert and Taylor, 1990; Nissen and Sutter, 1990; Ebert et al. 1993; Pan and van Staden, 1998; Thomas, 2008), thus reducing their effectiveness in tissue culture. The ratio and concentration of auxins and cytokinins in the medium is a key factor in determining successful plant regeneration (Razdan, 1993). The non-selective adsorption of these compounds by activated charcoal may result in inhibitory effects of growth *in vitro* (Pan and van Staden, 1998; Thomas, 2008).

Due to the instrumental role of auxin-cytokinin supplements on regulating plant growth, it is questionable whether to rely on activated charcoal. Although the complete effects of activated charcoal in the medium are unknown, its addition to the medium, may retard plant growth. This will require the need to evaluate an alternative treatment into experiments involving *Strelitzia* spp. The use of activated charcoal can make a difference in the success or failure of this culture attempt.

Ascorbic acid is an antioxidant used to control oxidation of phenols (Bharadwaj and Ramawat, 1993; Chawla, 2002; Abeyaratne and Lathiff, 2002). Its addition to the medium has reduced blackening of the medium to an acceptable level (Almaz et al., 2001). However, it has been reported not as effective as activated charcoal (Birmeta and Welander, 2004). A comparative study of these two phenolic reducing agents would gain further insight into the adsorption of the growth regulators, BAP and NAA, and identify the most successful way of reducing oxidative browning of wounded tissues. In determining the optimal agent, there is a trade-off between efficient control of browning and the instrumental role of auxin-cytokinin supplementation in the medium.

EFFECTS OF A DARK INCUBATION PERIOD ON REDUCING OXIDATIVE BROWNING OF WOUNDED TISSUES

The limitations of polyphenols on *in vitro* growth and development are evident as discussed previously. Tissues containing relatively high concentrations of phenolic compounds are difficult to culture (Scalbert et al., 1990; Dodds and Roberts, 1995; Khatri et al., 1997; Zweldu and Ludders, 1998; Abeyaratne and Lathiff, 2002; Titov et al., 2006). Success is often dependent upon the ability to reduce the phenolic oxidation reaction to wounding and cutting.

As discussed, adsorbents and antioxidants can be used as a method in reducing browning. Another useful technique is the incubation of cultures in darkness for the initial culture period (Bajaj, 1977; Durand-Cresswell and Nitsch, 1977; Birmeta and Welander, 2004; Titov et al., 2006; Kiong et al., 2007). Maintaining cultures in

darkness suppresses the metabolic sequence as it is known that phenolic oxidation products are formed under illumination (Chawla, 2002).

In *Musa*, *Ensete* and *S. reginae*, the incubation of cultures in darkness is reported to be effective in reducing browning (Ziv and Halevy, 1983; Birmeta and Welander, 2004; Strosse et al., 2009). However, success in these cases was achieved when the dark incubation period was used together with an antioxidant treatment. Obtaining optimal techniques in reducing oxidative browning of *Strelitzia* spp., requires a study into the dark incubation period in conjunction with the use of an adsorbent or antioxidant and the variation of medium composition. The type of phenol reducing agent, its concentration and the specific species are of importance in reducing oxidative browning of wounded tissues. The optimal collaboration of a dark incubation period together with a phenol reducing agent would increase the potential of *Strelitzia* spp. *in vitro* (Ziv and Halevy, 1983; Birmeta and Welander, 2004; Strosse et al., 2009).

CONCLUSION

The *Strelitzia* is an ornamental plant of significant horti-cultural commercial value (Paiva et al., 2004). Despite high demands, it is not widely spread due to the constraints on its production and is one of the very few important cut flower plants for which no uniform cultivars are available. Its horticultural success is limited by the slow conven-tional propagation methods currently used (Ziv and Halevy, 1983). It is commonly propagated vegetatively by division or by seeds. Both these methods of propagation are slow (Karnataka, 2008). From this background an alternative propagation and cloning method is required for the large scale production of *Strelitzia* to exploit its potential as an ornamental plant. The development of a reliable *in vitro* method for propagating *Strelitzia* spp., through the culture of excised embryos and multiple shoot formation, would overcome the constraints this plant poses to the horticultural industry worldwide, thus, greatly contributing to the commercial production of *Strelitzia* spp.

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APPENDIX B:

North, J.J. Ndakidemi, P.A. & Laubscher, C.P. 2011. Effects of various media compositions on the *in vitro* germination and discoloration of immature embryos of bird of paradise (*Strelitzia reginae*). *Plant Omics Journal*, 4(2): 100-113.

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Effects of various media compositions on the *in vitro* germination and discoloration of immature embryos of bird of paradise (*Strelitzia reginae*)

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Abstract

The optimal media composition for the *in vitro* germination of isolated *Strelitzia reginae* embryos was investigated. Different media treatments were compared to determine the effects of MS medium strength, activated charcoal (AC) and vitamin supplementation on the germination and seedling development of immature *Strelitzia* embryos. Results indicate the positive role of AC in reducing oxidative browning. The addition of AC (0.2 gl^{-1}) to the culture medium significantly reduced the discoloration of embryo explants and the culture media. Similarly, the addition of the vitamins (100 mgl^{-1} Inositol, 0.1 mgl^{-1} Thiamine, 0.1 mgl^{-1} Pyridoxine, 2 mgl^{-1} Glycine) resulted in a significant reduction in embryo discoloration. Furthermore, the addition of vitamins significantly increased root formation. Interactions between these media components resulted in significant effects. In treatments of half-strength MS with vitamin supplementation, both embryo and media discoloration were reduced. Interactions between vitamin and AC treatments presented a reduction in the embryo discoloration rate and an increased length of shoots, only when AC was absent in the media. When AC was added to vitamin supplemented media, the beneficial effects of vitamins were cancelled. The highest germination rate of embryos was observed in media containing AC without vitamin supplementation. A significant decrease in germination resulted with the addition of vitamins. The highest level of media discoloration was observed in half-strength MS media without activated charcoal and vitamin supplementation. Whilst the most effective media were: (half-strength MS without vitamins and with AC) or (half-srength MS with vitamins and without AC).

Keywords: oxidative browning, activated charcoal, MS medium strength, vitamins, media composition.
Abbreviations: MS – Murashige and Skoog (1962), AC – activated charcoal.

Introduction

The bird of paradise (*Strelitzia*) is a tropical perennial plant of significant commercial value (Paiva et al., 2004). Originating from South Africa, this important ornamental monocotyledonous plant (Chand, 2008) is highly valued as a cut flower. The strong exotic features of its colourful flowers, the long length of the stem and the high post-harvest durability attribute to this (Wood, 1995). Despite it being one of the most sought after cut flowers destined for exportation from developing countries (Criley, 1988), its commercial exploitation and success is limited by its naturally low rate of multiplication (Ziv and Halevy, 1983). Propagation is either by seed or by division of naturally developed branches known as fans (Dyer, 1972). Both of these conventional propagation methods are very slow (Karnataka, 2008). Efforts have been made to increase and accelerate the propagation of this valuable plant, both by asexual (van de Pol and van Hell, 1988) and sexual methods (Holley, 1970; Bekendam, 1974; van de Venter and Small, 1975; Besmer, 1976; Ishihata, 1976; Diaz-Perez, 1978; Ybema et al., 1984; Ndakidemi and Dakora, 2003). However, the commercial production of this plant has not been adequate to exploit its potential. From this background an alternative propagation and cloning method is needed. Tissue culture is a reliable and advanced propagation method that could be more promising than the conventional propagation methods (Promtep, 1981). Despite the plants commercial importance, a reliable method for micropropagation has not yet been developed (Chand, 2008). Reviews of the literature indicate the limited success in the application of tissue culture techniques in the propagation of *Strelitzia* (North et al., 2010). The failure of tissue culture techniques is reported to be due to oxidative browning of the wounded explants (Ziv and Halevy, 1983). However, with the extensive use of antioxidants to reduce browning, terminal and axillary buds were found to be capable of growth and further shoot proliferation (Ziv and Halevy, 1983). In efforts to reproduce the protocol developed by Ziv and Halevy (1983), Paiva et al. (2004) failed to establish axillary buds *in vitro*. Irrespective of applied treatments, phenolic oxidation was reported to be a crucial problem. In studies involving the use of immature *Strelitzia* embryos as explants, Paiva et al. (2004) reported the germination of embryos inoculated *in vitro*. Although attempts made to regenerate plants from this material were unsuccessful. In all investigations into the micropropagation of *Strelitzia*, only partial success and a low rate of multiplication were obtained, indicating major problems with growing and multiplying this plant *in vitro* (North et al., 2010). Zygotic embryo culture is a useful tool that can be used for a variety of purposes. These include bypassing seed dormancy (Hu and Wang, 1986; Ho et al., 1987; Das et al., 1999; Bürün and Çoban Poyrazoğlu, 2002) and inducing

Table 1. Media for the *in vitro* germination of immature *Strelitzia reginae* embryos

Media	Compositions
1	MS salts and vitamins*
2	½ MS salts and vitamins
3	MS salts and vitamins and 0.2 gl^{-1} activated charcoal
4	½ MS salts and vitamins and 0.2 gl^{-1} activated charcoal
5	MS salts
6	½ MS salts
7	MS salts and 0.2 gl^{-1} activated charcoal
8	½ MS salts and 0.2 gl^{-1} activated charcoal

*The medium was supplemented with vitamins including 100 mgl^{-1} Inositol, 0.1 mgl^{-1} Thiamine, 0.1 mgl^{-1} Pyridoxine, 2 mgl^{-1} Glycine.

a faster growth rate (Chang and Yang, 1996; Bürün and Çoban Poyrazoğlu, 2002). The variation in seeds (Larkin et al., 1984; Foolad and Jones, 1992) will allow the excised embryo explants to play an important role in the breeding cycle (Ho et al., 1987) and the development of cultivars. The limited wounding of embryo explants may reduce the production of phenolic exudates during the crucial initial stages of plant development. Furthermore, this non-destructive method of gaining starting material for the culture, may aid the production of rare species, as plants do not have to be destroyed (Bürün and Çoban Poyrazoğlu, 2002). The most important aspect of culturing immature embryos is to develop and clearly define a culture media that can sustain growth and development (Chawla, 2002). Nutrients required by embryos vary depending on embryo age. Thus, while relatively mature embryos can grow on a simple inorganic medium, the nutritional requirements of relatively immature embryos are complex (Bajaj, 1977; Pierik, 1979; Monnier, 1990; Raghaven, 1994; Hu and Zanettini, 1995). Up to now, little is known of the *in vitro* culture factors affecting the germination of immature zygotic embryos of *Strelitzia* spp. The MS medium of Murashige and Skoog (1962) is a salt composition that supplies the needed macro- and micronutrients. The modification of MS medium according to the nutritional requirements of an embryo and its developmental stage is essential to attain the highest germination percentage and best morphological characteristics. Although the basis of all nutrient media is a compostion of essential nutrients (Ramage and Williams, 2002), vitamins are required in trace amounts to serve catalytic functions in enzyme systems (Al-Khayri, 2001). Normal plants synthesize the vitamins required for growth and development (Chawla, 2002). Whereas plant cells grown *in vitro* are only capable of synthesizing essential vitamins in suboptimal quantities; thus culture media are often supplemented with vitamins to enhance growth (Al-Khayri, 2001). The role of vitamins on the germination of *Strelitzia* embryos and plant regeneration thereof, needs to be determined. Phenolic oxidation is a crucial problem during the initial stages of culture as polyphenolic compounds are detrimental to the further development of explants (Ziv and Halevy, 1983; Pan and van Staden, 1998; Zeweldu and Ludders, 1998; Birmeta and Welander, 2004; Diro and van Staden, 2004). The use of activated charcoal can make a major difference in the success or failure of a given tissue culture attempt (Pan and van Staden, 1998). The incorporation of activated charcoal to tissue culture media may promote *in vitro* growth and alleviate this problem by adsorbing inhibitory substances (Ziv and Halevy, 1983; Tisserat, 1984; Teixeira et al., 1994; Veramendi and Navarro, 1996; Pan and van Staden, 1998; Diro and van Staden, 2004).The aim of this study was to investigate the optimum media compositions for the *in vitro* germination of immature embryos of *Strelitzia reginae*. The specific objectives were; To determine if variations in the Murashige and Skoog (MS) medium (inorganic salt formulation) concentrations have an effect on the germination and development of embryos.To assess the effect of supplementing the plant tissue culture medium with vitamins.To evaluate the effects of activated charcoal in the culture media to control oxidative browning of embryo explants.

Materials and methods

Plant material

Immature seeds, 20 weeks after pollination, of *Strelitzia reginae* were collected from plants grown at Kirstenbosch National Botanical Gardens in Cape Town, South Africa.

Sterilization

Seeds were surface-sterilized with 70% ethanol for 30 sec, 1.5% solution of sodium hypochlorite (NaOCl) with 2 drops of Tween-20 for 15 min and then rinsed four times with sterile distilled water. Immature embryos were aseptically excised from the sterilized seeds and placed on various induction media.

Culture conditions and media

Embryos were placed in test tubes containing 10 ml of the culture media, supplemented with 30 g l^{-1} sucrose and solidified with 7 g l^{-1} agar. The pH was adjusted to 5.8 prior to autoclaving at 121°C for 20 min. The experiment consisting of 8 medium types (Table 1) was set up to investigate the effects of MS medium strength, activated charcoal treatments and vitamin supplementation of the *in vitro* germination of embryos. Twelve replicates were used for each treatment. Inoculated cultures were incubated in a growth room at 25 ± 2°C with a 16 h light and 8 h dark cycle.

Data collection and analysis

Data on embryo germination rates (radicle emergence), contamination, shoot length, root number and length, embryo size, degree of embryo discoloration and degree of media discoloration were collected at weekly intervals. Based on visual observations, the degree of media and embryo discoloration (entire explants and at the media contact point) was rated on a scale of 1-5 (1 = No discoloration and 5 = Extreme discoloration), modified from the rating scale given by Ziv and Halevy (1983). Data collected were analyzed for statistical significance using factorial analysis of variance (ANOVA). These computations were done with the software program STATISTICA Software Programme version 2010 (StatSoft Inc., Tulsa, OK, USA). The Fisher least significance

Table 2. Effect of medium strength, activated charcoal and vitamins on the discoloration of the entire embryo explant and discoloration of embryo at media contact point. Rating was done on a scale of 1-5 (1 = No discoloration and 5 = Extreme discoloration)

Treatment	Time (Weeks)									
	1	2	3	4	5	1	2	3	4	5
	Embryo discoloration (entire explant)					Embryo discoloration (at media contact point)				
Medium strength										
Half	2.58±0.10a	2.75±0.09a	3.00±0.09a	3.03±0.09a	3.55±0.16a	2.83±0.11a	3.15±0.10b	3.58±0.09a	3.70±0.08a	3.83±0.06a
Full	2.68±0.10a	2.80±0.09a	2.85±0.09a	3.10±0.11a	3.83±0.16a	3.05±0.09a	3.38±0.10a	3.53±0.09a	3.78±0.08a	3.93±0.04a
Activated charcoal[a]										
-	2.95±0.08a	2.93±0.09a	3.03±0.08a	3.13±0.10a	3.58±0.16a	3.10±0.10a	3.40±0.09a	3.63±0.09a	3.85±0.06a	3.90±0.05a
+	2.30±0.09b	2.63±0.08b	2.83±0.10a	3.00±0.10a	3.80±0.16a	2.78±0.10b	3.13±0.10b	3.48±0.09a	3.63±0.09b	3.85±0.06a
Vitamins[b]										
-	2.70±0.08a	2.90±0.09a	2.98±0.10a	3.03±0.09a	3.53±0.17a	3.05±0.10a	3.53±0.09a	3.70±0.08a	3.83±0.07a	3.95±0.03a
+	2.55±0.11a	2.65±0.08b	2.88±0.09a	3.10±0.11a	3.85±0.15a	2.83±0.10a	3.00±0.09b	3.40±0.09b	3.65±0.08a	3.80±0.06b
3 - Way ANOVA (F-Statistic)										
MS	0.69	0.18	1.30	0.29	1.54	2.69	3.49*	0.16	0.49	1.85
AC	29.25***	6.35*	2.30	0.80	1.03	5.61*	5.21*	1.47	4.37*	0.46
VITAMINS	1.56	4.41*	0.58	0.29	2.15	2.69	18.99***	5.89*	2.64	4.15*
MS×AC	0.00	0.71	0.58	1.56	1.54	0.03	0.04	0.00	1.35	0.00
MS×VITAMIN	0.69	0.18	0.00	0.80	0.01	2.69	5.21*	0.16	1.35	0.00
AC×VITAMINS	1.56	0.00	1.30	0.80	1.54	0.30	0.04	4.09*	1.35	0.46
MS×AC×VITAMINS	0.69	0.71	1.30	1.56	0.62	0.83	3.49	0.65	0.49	1.85

*$P \leq 0.05$; ***: $P \leq 0.001$. Values (Mean ± SE, n = 12) followed by dissimilar letters in a column are significantly different by Least Significant Difference test at P=0.05. [a]0.2 gl^{-1} activated charcoal; [b]100 mgl^{-1} Inositol, 0.1 mgl^{-1} Thiamine, 0.1 mgl^{-1} Pyridoxine, 2 mgl^{-1} Glycine.

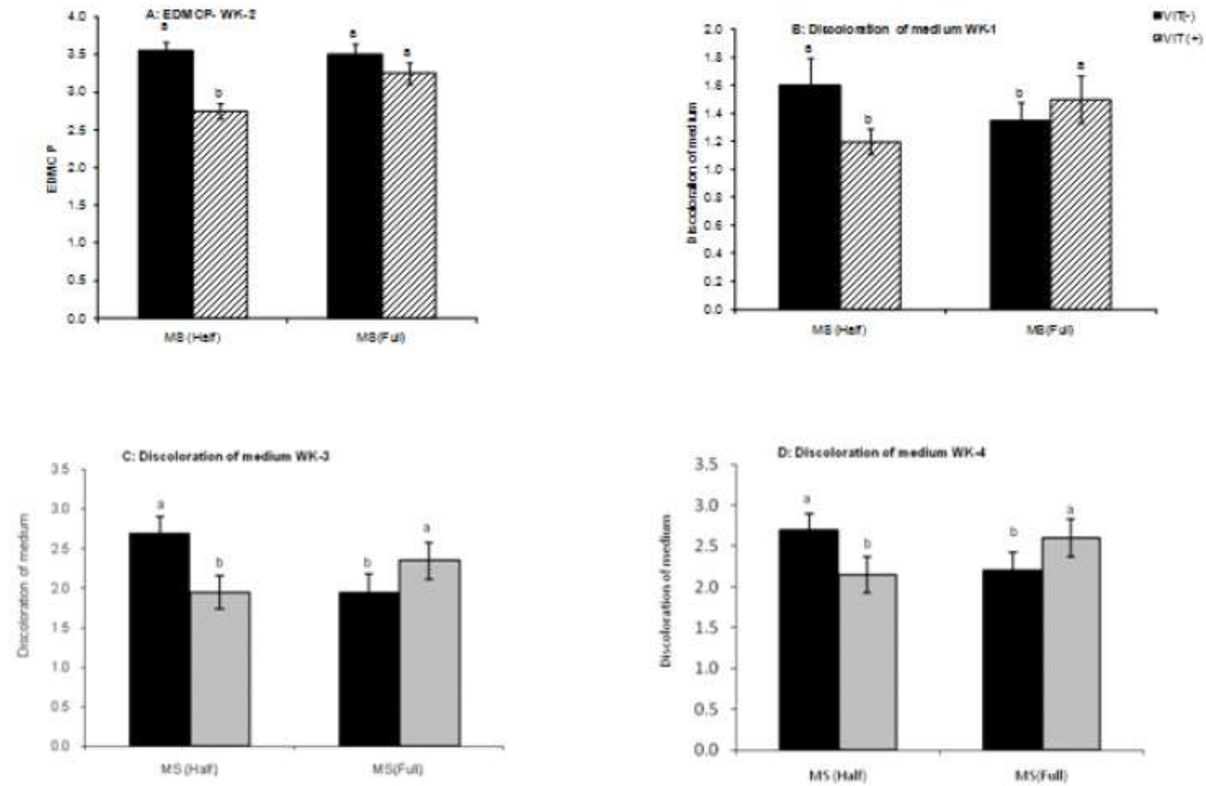

Fig 1. Interactive effects of Medium strength and vitamins on (A) Embryo discoloration at medium contact point (EDMCP) WK-2, (B) Discoloration of medium (WK-1), (C) Discoloration of medium (WK-3), (D) Discoloration of medium (WK-4). Rating scale used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration), MS (Half) = Half medium strength, MS (Full) = Full medium strength, VIT(-) = Without vitamin, VIT (+) = With vitamin.

difference was used to compare treatment means at p = 0.05 level of significance (Steel and Torrie, 1980).

Results and discussion

Effect of MS medium strength, activated charcoal and vitamins on the discoloration of the embryo explants

In this experiment, MS medium strength had no significant effect on discoloration of the entire embryo explant (Table 2). However, slightly increased discoloration was observed in full-strength MS. The addition of activated charcoal in culture media significantly reduced embryo discoloration in week 1 ($P \leq 0.001$) and week 2 ($P \leq 0.05$). Based on the rating scale of 1-5, activated charcoal reduced embryo discoloration from 2.95 to 2.30 in week 1 and from 2.93 to 2.63 in week 2, which was equivalent to a 22% and 10% reduction respectively. In weeks that followed, activated charcoal only slightly reduced embryo discoloration. During the initial stages of culture, the excessive production of polyphenols often results in browning and eventual death of inoculated tissues. This is possibly due to the triggering of defense reactions (Pan and van Staden, 1998). The incorporation of activated charcoal to media is a recognized practise and its influence on culture establishment may be attributed to its adsorptive capability of inhibitory substances in the culture medium (Horner et al., 1977; Fridborg et al., 1978; Weatherhead et al., 1979; Theander and Nelson, 1988) and drastic decrease in phenolic oxidation (Carlberg et al., 1983; Liu, 1993; Teixeira et al., 1994). Similar to this study, the influence of activated charcoal on reducing explant browning has been reported in several plant species (Madhusudhanan and Rahiman, 2000; Chang et al., 2001; Birmeta and Welander, 2004; Wang et al., 2005; Thomas, 2008; Guo et al., 2007). The addition of vitamins significantly ($P \leq 0.05$) reduced embryo discoloration from 2.90 to 2.65 in week 2 of the experiment, which was equivalent to a 9% reduction. Hereafter, vitamins showed no effect on reducing discoloration. These results are in accordance with previous studies that report the ability of vitamins to suppress the browning of tissues *in vitro* (Bergmann and Bergmann, 1968; Inoue and Maeda, 1980; Al-Khayri, 2001).

Effect of MS medium strength, activated charcoal and vitamins on the discoloration of the embryo explants at the media contact point

Half-strength MS medium significantly ($P \leq 0.05$) reduced embryo discoloration at the medium contact point. In week 2 (Table 2), the reduction of 3.38 to 3.15 resulted in a 7% decrease. A slight reduction was observed throughout the remainder of the experiment. Similarly, Abbasin et al. (2010) reported the browning of embryo explants in *Taxus baccata* when cultured in full-strength basal salts. In contrast, the half-strength MS was more effective in reducing necrosis and increasing the survival of explants. This may be due to the reduced salt concentration reducing the osmotic concentration of the medium. Activated charcoal significantly ($P \leq 0.05$) reduced discoloration in weeks 1, 2 and 4 by approximately 10%, 8% and 6% respectively. Activated charcoal resulted in a general reduction throughout the experiment. The addition of vitamins to the culture media significantly; $P \leq 0.001$, $P \leq 0.05$ and $P \leq 0.05$ reduced embryo discoloration at the media contact point in week 2, 3 and 5 of the experiment respectively. The values were reduced as follows; 3.53 to 3.00 in week 2, 3.70 to 3.40 in week 3 and 3.95 to 3.80 in week 5. Thus, a 15%, 8% and a 4% reduction was observed in the respective weeks. A general reduction in discoloration at the medium contact point was observed throughout the experiment in treatments containing vitamins. It is at the point of contact between the explant and the medium that oxidative browning is exaggerated due to an adequate supply of oxygen coming into contact with the growing tissue and the required nutrients. This study indicates that vitamins and activated charcoal both played a key role in reducing embryo discoloration at this point. In a study on *Brassica*, Tian et al. (2004) similarly reported that embryos grew more rapidly and browning rarely happened at the base of hypocotyls, when media was supplemented with vitamins. The ability of activated charcoal to reduce the browning of tissues is widely reported (Chang et al., 2001; Wang et al., 2005; Guo et al., 2007) and may be attributed to its high adsorptive capacity (Thomas, 2008). With the addition of activated charcoal to media, a drastic decrease in phenolic oxidation has been observed (Carlberg et al., 1983; Liu, 1993; Teixeira et al., 1994).

Effect of MS medium strength, vitamins and activated charcoal on the number of roots developed from germinated plantlets

MS medium strength had no significant effect on the formation of roots. However, half-strength MS generally displayed a slightly higher rate of root formation (Table 3). The addition of vitamins significantly ($P \leq 0.01$) increased root formation in week 1 of the experiment. Values increased from 0.35 to 0.60, resulting in a 42% increase. In the weeks that followed a slight increase was observed in media containing vitamins. Similarly, earlier studies confirm that the vitamins thiamine and nicotinic acid affected cellular division in the pea root meristem (Bonner and Addicot, 1937; Addicot, 1941; Torrey, 1953). It was only in the presence of either or both vitamins, that root growth occurred (Torrey, 1953). A significantly ($P \leq 0.001$) higher number of roots were observed in the absence of activated charcoal in week 1 (Table 3). Root formation increased from 0.25 in charcoal treatments to 0.70 in treatments free of charcoal. Thus, a 64% increase was observed in treatments free of activated charcoal. Majority of reports confirm the positive effect of activated charcoal on rooting (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). However, in this study on *Strelitzia*, activated charcoal had a negative effect on rooting. These results are similar to those of Buendia-Gonzalez et al. (2007), who also reported activated charcoal to have a negative effect on rooting of the mesquite tree (*Prosopis laevigata*). Activated charcoal inducing negative results in the growth and development of plant tissues has also been reported in other micropropagation systems (Komalavalli and Rao, 2000; Kadota and Niimi, 2004; Wei et al., 2006; Motoike et al., 2007). This is possibly due to the adsorption of essential factors required for tissue growth (Komalavalli and Rao, 2000). In this study, it is established that vitamin supplemented media increased root formation. Whereas the addition of activated charcoal to this vitamin enriched media completely inhibited the root formation. This may be due to the activated charcoal adsorbing the vitamins promoting rooting. A crucial impact of adding activated charcoal to the culture media is that in addition to adsorbing unwanted substances, it may adsorb needed vitamins (Weatherhead et al., 1978; Weatherhead et al., 1979; Pan and van Staden, 1998).

Table 3. Effect of medium strength, activated charcoal and vitamins on the number of roots developed by *Strelitzia reginae* during *in vitro* culture.

Treatment	Time (Weeks)				
	1	2	3	4	5
Medium strength			Root Number		
Half	0.45±0.08a	3.43±0.43a	3.55±0.43a	3.88±0.51a	4.03±0.56a
Full	0.50±0.08a	2.95±0.42a	3.13±0.43a	3.30±0.49	3.35±0.50a
Activated charcoal[a]					
-	0.70±0.07a	3.10±0.40a	3.30±0.42a	3.60±0.50	3.68±0.55a
+	0.25±0.07b	3.28±0.45a	3.38±0.44a	3.58±0.50	3.70±0.52a
Vitamins[b]					
-	0.35±0.08b	3.45±0.40a	3.58±0.40a	3.73±0.41	3.75±0.41a
+	0.60±0.08a	2.93±0.45a	3.10±0.46a	3.45±0.57	3.63±0.63a
3 - Way ANOVA (F-Statistic)					
MS	0.28	0.60	0.46	0.64	0.77
AC	22.78***	0.08	0.01	0.00	0.00
VITAMINS	7.03**	0.73	0.58	0.15	0.03
MS×AC	0.28	0.13	0.01	0.35	0.38
MS×VITAMIN	0.28	0.00	0.01	0.00	0.05
AC×VITAMINS	7.03**	1.04	1.17	0.88	0.77
MS×AC×VITAMINS	2.53	0.48	0.46	1.16	1.01

**: $P \leq 0.01$; ***: $P \leq 0.001$. Values (Mean ± SE, n = 12) followed by dissimilar letters in a column are significantly different by Least Significant Difference test at P=0.05. [a]0.2 gl^{-1} activated charcoal; [b]100 mgl^{-1} Inositol, 0.1 mgl^{-1} Thiamine, 0.1 mgl^{-1} Pyridoxine, 2 mgl^{-1} Glycine.

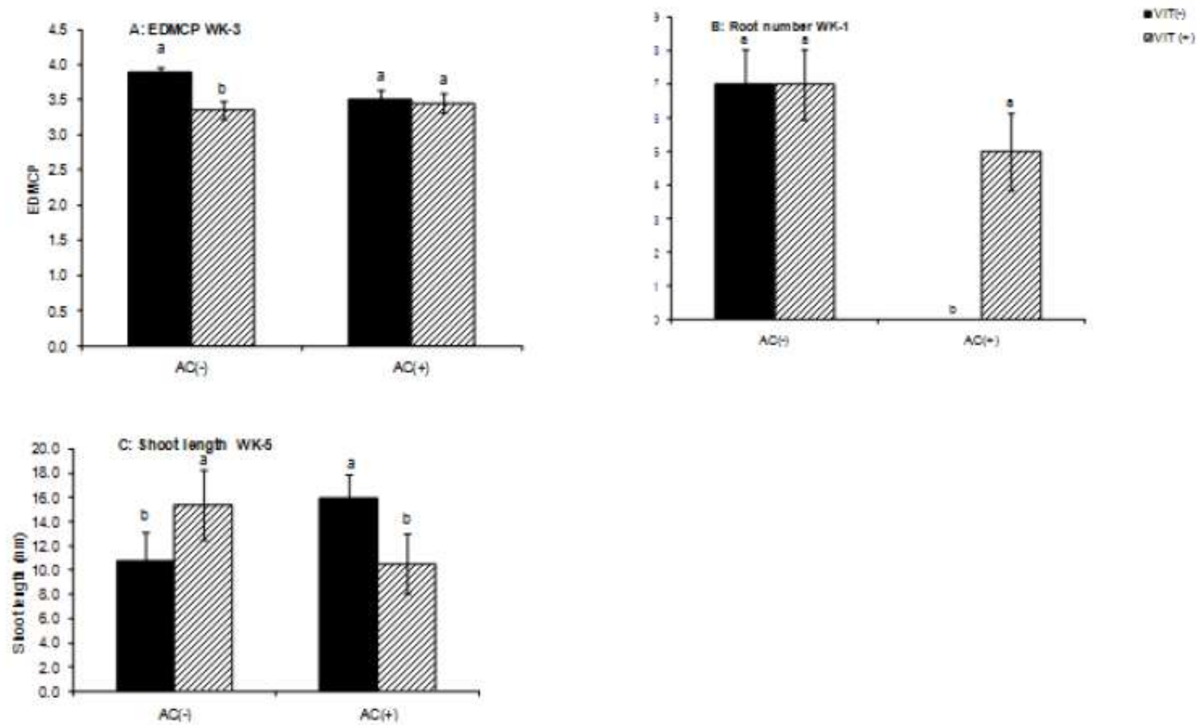

Fig 2. Interactive effects of Medium strength and vitamins on (A) Embryo discoloration at medium contact point (EDMCP) WK-3, (B) Root number WK-1, (C) Shoot length WK-5. Color code used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration), AC (-) = Without activated charcoal, AC (+) = With activated charcoal, VIT(-) = Without vitamin, VIT (+) = With vitamin.

Effect of MS medium strength, activated charcoal and vitamins on medium discoloration

The intensity of medium discoloration was not affected by the concentration of MS salts (Table 4). Only a slight reduction was observed in media containing full-strength MS. Based on the rating scale of 1-5, activated charcoal significantly ($P \leq 0.001$) reduced medium discoloration from 1.68 to 1.15 in week 1 of the experiment. Discoloration was 32% less severe in media containing activated charcoal, as opposed to media without it. In the weeks that followed, a slight reduction was observed in activated charcoal treatments (Figure 5). The incorporation of vitamins to culture media showed no significant effects. However, a slight reduction was observed in all treatments containing vitamins. Medium discoloration during initial stages of culture is due to tissues releasing polyphenolic compounds, which diffuse into the medium (Strosse et al., 2009). The use of activated charcoal, in reducing these dark pigments, has been used with success in various plants (Das et al., 1999; Feyissa et al., 2005; Nguyen et al., 2007).

Interactive effects of MS medium strength, activated charcoal and vitamins

The results in Figure 1A indicate that there was a significant interaction between MS medium strength and vitamins on embryo discoloration at the medium contact point in week 2. In half-strength MS with vitamins, embryo discoloration was significantly ($P \leq 0.05$) reduced. Whereas full-strength MS medium with vitamins showed no significant effects on reducing embryo discoloration at the media contact point. Similarly, Abbasin et al. (2010) reported half-strength MS effective in reducing necrosis of the zygotic embryo explants of *Taxus baccata* and Tian et al. (2004) found vitamin supplementation effective in reducing browning at the base of hypocotyls in *Brassica*. Our study reports the interactive effects of MS medium strength and vitamins to have a significant influence on embryo discoloration at the media contact point. Media discoloration was significantly ($P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.05$) reduced by MS media strength and vitamin interactions in weeks 1, 3 and 4 respectively (Figure 1B, C and D). Half-strength MS supplemented with vitamins significantly reduced media discoloration. Whereas the addition of vitamins to full-strength MS had the opposite effect and resulted in an increase in media discoloration. The positive influence of vitamins in reducing oxidative browning has been established (Bergmann and Bergmann, 1968; Inoue and Maeda, 1980; Al-Khayri, 2001). Similarly, half-strength MS has been reported to be more effective in reducing necrosis (Abbasin et al., 2010). In this study the interactive effects of vitamins and half-strength MS were effective in reducing media discoloration. The increased media discoloration in full-strength MS supplemented with vitamins may due to an excess of mineral salts in the media. Therefore, modification of MS medium according to the requirements of tissues is essential to achieve optimal growth (Birmeta and Welander, 2004). Whereas, in treatments with vitamins, activated charcoal did not play a significant role in reducing media discoloration, irrespective of MS media strength. This may be due to the positive effect of the vitamins in the culture media. The only treatment showing a significantly higher level of media discoloration amongst those supplemented with vitamins, was that of full strength MS without activated charcoal. In the presence of full strength macro- and micronutrients and vitamins, phenols may be released more rapidly into the medium due to an excess of nutrients and vitamins causing an increased osmotic potential. The interactive effects of activated charcoal and vitamins showed significant results. Based on the rating scale of 1-5, treatments without activated charcoal and supplemented with vitamins significantly ($P \leq 0.05$) reduced embryo discoloration at the media contact point in week 3 (Figure 2A). However, when activated charcoal was present in the media, the addition of vitamins did not play a significant role in reducing embryo discoloration at the medium contact point. As previously mentioned, this study indicates the significant reduction of embryo discoloration at the medium contact point, with the supplementation of media with vitamins. The adverse effect of this, with the addition of activated charcoal, may be due to the ability of activated charcoal to adsorb vitamins (Weatherhead et al., 1978; 1979; Pan and van Staden, 1998). Thus, the positive effects of vitamin supplementation cancelled. Results on the interactive effects of activated charcoal and vitamins showed that the number of roots developed was significantly affected in week 1 of the experiment. Treatments containing both activated charcoal and vitamins resulted in a significantly ($P \leq 0.01$) higher number of roots (Figure 2B). In treatments lacking activated charcoal, the addition or omission of vitamins showed no effects on the number of roots developed. Although this experiment previously reports a significantly lower number of roots developed in activated charcoal treatments, the interactive effects of activated charcoal with vitamins result in a significant increase in root formation. This increase in rooting is in conjunction with the reports on both the application of activated charcoal (Makunga et al., 2006; Agarwal and Kanwar, 2007; Xiao et al., 2007; Makunga and van Staden, 2008) and vitamins (Torrey, 1953). Shoot lengths from embryo-derived plantlets were significantly affected by activated charcoal and vitamin interactions in week 5 of the experiment (Figure 2C). In treatments without activated charcoal, vitamins in the culture media significantly increased the length of shoots. However, when activated charcoal was present, vitamins inversely resulted in a significant reduction of shoot length (Figure 2C). Culture media are often supplemented with vitamins to enhance growth (Al-Khayri, 2001). The reduction of shoot length with the addition of activated charcoal may be due to its high adsorptive characteristic (Thomas, 2008). As previously mentioned activated charcoal is known to adsorb beneficial vitamins (Weatherhead et al., 1978; 1979; Pan and van Staden, 1998), this may inversely affect growth. The results in Figures 3A, B and C indicate significant interactions in activated charcoal and vitamin treatments on the germination of immature *Strelitzia* embryos. In week 2, activated charcoal without vitamin supplementation resulted in the highest germination rate of embryos (Figure 3A). When activated charcoal was supplemented with vitamins, a significant decrease in germination was observed. In treatments without activated charcoal, vitamin supplementation did not significantly affect germination (Figure 3A), although a slight germination increase was observed in vitamin enriched media. In weeks 4 (Figure 3B) and 5 (Figure 3C), a similar trend continued. Activated charcoal has been widely reported to have positive effects on the *in vitro* germination of both embryos (Sarason et al., 2002; Shi et al., 2008; Fan et al., 2008) and seeds (Man et al., 2003; Kitsaki et al., 2004; Thompson et al., 2007). The significant reduction in embryo germination with vitamin supplementation may be due to an increase in osmotic potential. In the case of culturing zygotic embryos, only basic nutrients are necessary for germination (Thawaro and Te-chato, 2010). The results in Figure 4 represent the significant

Table 4. Effect of medium strength, activated charcoal and vitamins on root length of *Strelitzia reginae* and the discoloration of culture medium during *in vitro* culture. Rating was done on a scale of 1-5 (1 = No discoloration and 5 = Extreme discoloration)

Treatment	Time (Weeks)									
	1	2	3	4	5	1	2	3	4	5
	Root length (mm)					Discoloration of medium				
Medium strength										
Half	3.83±0.57a	15.73±2.05a	23.00±3.14a	26.80±3.80a	28.78±4.28a	1.40±0.11a	2.08±0.14a	2.33±0.16a	2.43±0.15a	2.58±0.18a
Full	3.90±0.51a	17.63±2.21a	22.95±3.08a	25.48±3.48a	26.65±3.67a	1.43±0.11a	2.05±0.14a	2.15±0.17a	2.40±0.16a	2.58±0.17a
Activated charcoal[a]										
-	4.00±0.55a	16.35±2.04a	22.93±3.03a	27.30±3.77a	29.45±4.26a	1.68±0.13a	2.15±0.14a	2.38±0.17a	2.53±0.16a	2.70±0.17a
+	3.73±0.54a	17.00±2.24a	23.03±3.18a	24.98±3.51a	25.98±3.67a	1.15±0.07b	1.98±0.15a	2.10±0.15a	2.30±0.15a	2.45±0.17a
Vitamins[b]										
-	4.03±0.54a	18.88±2.06a	25.13±2.86a	29.03±3.54a	30.35±3.73a	1.48±0.12a	2.23±0.14a	2.33±0.17a	2.45±0.15a	2.58±0.17a
+	3.70±0.55a	14.48±2.16a	20.83±3.29a	23.25±3.69a	25.08±4.18a	1.35±0.10a	1.90±0.14a	2.15±0.16a	2.38±0.16a	2.58±0.18a
3 - Way ANOVA (F-Statistic)										
MS	0.01	0.38	0.00	0.06	0.14	0.03	0.02	0.66	0.01	0.00
AC	0.13	0.05	0.00	0.19	0.36	15.21***	0.81	1.62	1.11	1.11
VITAMINS	0.18	2.06	0.91	1.20	0.84	0.86	2.80	0.66	0.12	0.00
MS×AC	5.34*	0.13	0.39	0.51	0.84	0.31	0.15	0.01	0.01	0.71
MS×VITAMIN	0.13	0.49	0.31	0.08	0.05	4.17*	2.80	7.10**	4.95*	2.18
AC×VITAMINS	0.31	0.67	0.81	0.57	0.60	2.79	0.81	1.62	1.66	0.18
MS×AC×VITAMINS	0.13	0.29	0.01	0.07	0.02	7.76**	3.73	4.84*	4.95*	5.39*

*$P≤0.05$; **: $P≤0.01$; ***: $P≤0.001$. Values (Mean ± SE, n = 12) followed by dissimilar letters in a column are significantly different by Least Significant Difference test at P=0.05. [a]0.2 gl^{-1} activated charcoal; [b]100 mgl^{-1} Inositol, 0.1 mgl^{-1} Thiamine, 0.1 mgl^{-1} Pyridoxine, 2 mgl^{-1} Glycine.

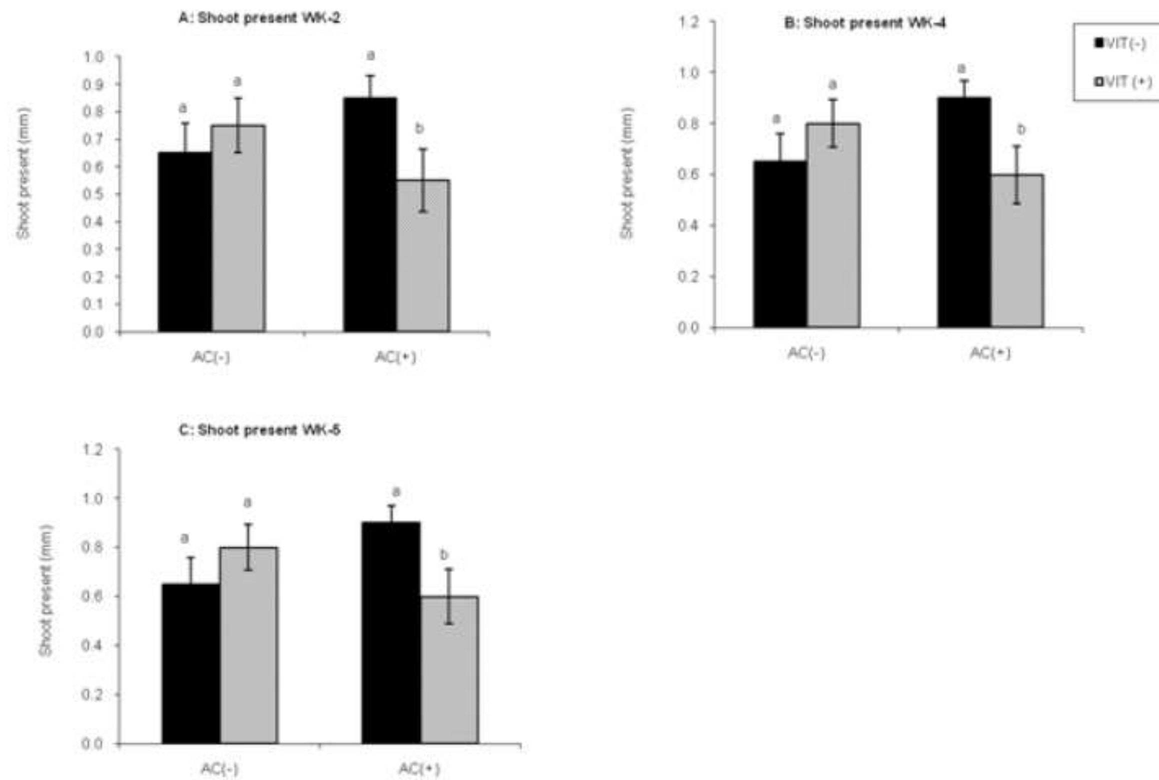

Fig 3. Interactive effects of activated charcoal and vitamins on (A) Shoot present WK-2, (B) Shoot present WK-4, (C) Shoot present WK-5. AC (-) = Without activated charcoal, AC (+) = With activated charcoal, VIT(-) = Without vitamin, VIT (+) = With vitamin.

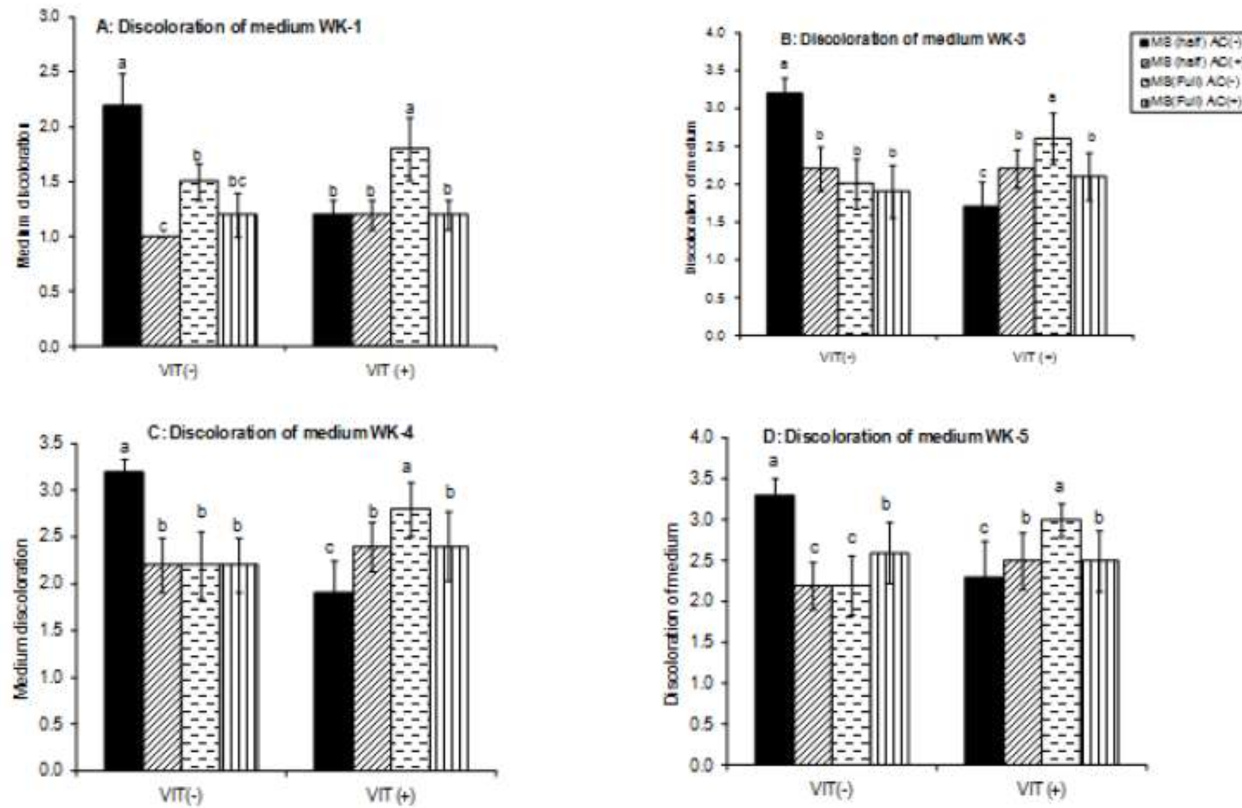

Fig 4. Interactive effects of Medium strength x activated charcoal x vitamins on discoloration of medium in: A) Week-1, B) Week-3, C) Week-4), D) (Week-5) in *Strelitzia reginae*. Color code used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration). MS (Half) = Half medium strength, MS (Full) = Full medium strength, AC (-) = Without activated charcoal, AC (+) = With activated charcoal, VIT(-) = Without vitamin, VIT (+) = With vitamin.

Fig 5. Effect of activated charcoal on medium discoloration. (A) Reduced media discoloration in the presence of activated charcoal and (B) an increased level of media discoloration in the absence of activated charcoal.

interactive effects of MS media strength, activated charcoal and vitamins on the discoloration of the culture medium, based on the rating scale of 1-5. The poorest and most successful treatments in controlling media discoloration in week 1 (Figure 4A) were both observed in half strength MS media without vitamin supplementation. Activated charcoal caused significant ($P \leq 0.01$) interactive effects in the treatments. The addition of activated charcoal to half strength MS without the addition of vitamins, resulted in the most effective treatment in reducing media discoloration. Whilst the omission of activated charcoal from the same media of half strength MS without the addition of vitamins, displayed the highest level of media discoloration. In vitamin enriched media, the only treatment that varied was that of full-strength MS without activated charcoal. This treatment resulted in a significantly higher level of media discoloration, whereas other combinations of vitamins with either half- or full-strength MS, with or without activated charcoal showed a constant reduced level of media discoloration in week 1. This trend was similarly observed for medium discoloration in weeks 3, 4 and 5 (Figures 4B, C and D respectively). In treatments lacking vitamin supplementation, the promotary effects of activated charcoal were significantly evident in half-strength MS treatments. In this study, the addition of activated charcoal to the medium may have resulted in the adsorption of inhibitory compounds in the culture medium and decreased the accumulation of brown exudates from diffusing into the medium. Whereas, in treatments with vitamins, activated charcoal did not play a significant role in reducing media discoloration, irrespective of MS media strength. This may be due to the positive effect of the vitamins in the culture media. Similar to our results Tian et al. (2004) reported the influence of vitamin supplementation in reducing browning in *in-vitro* propagation of *Brassica*. The only treatment showing a significantly higher level of media discoloration amongst those supplemented with vitamins, was that of full strength MS without activated charcoal. In the presence of full strength macro- and micronutrients and vitamins, phenols may be released more rapidly into the medium due to an excess of nutrients and vitamins. Therefore, the strength of MS medium together with the correct proportions of AC and vitamins are essential for optimum germination and growth of embryos. In conclusion, germinated plantlets were obtained from embryo explants and optimum nutrition and medium components as the first step towards the development of an efficient *in vitro* propagation system of *Strelitzia*, were determined. Initiation and *in vitro* propagation of *Strelitzia* is difficult due to oxidative browning. The present work, however, demonstrates the significant effects of MS medium strength, AC and vitamin supplementation on reducing the discoloration of explants and culture media. Furthermore, interactions between these media components significantly affected the rate of embryo germination, reducing explant and media discoloration, the length of shoots and the development of roots.

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APPENDIX C:

North, J.J. Ndakidemi, P.A. & Laubscher, C.P. 2012. Effects of various media compositions and wounding treatments on *in vitro* growth and regeneration of bird of paradise (*Strelitzia reginae*). *Scientific Research and Essays*, 7(10): 1118-1133.

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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}

**, $P\leq0.01$; ***, $P\leq0.001$; 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}

**, $P\leq0.01$; ***, $P\leq0.001$; 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

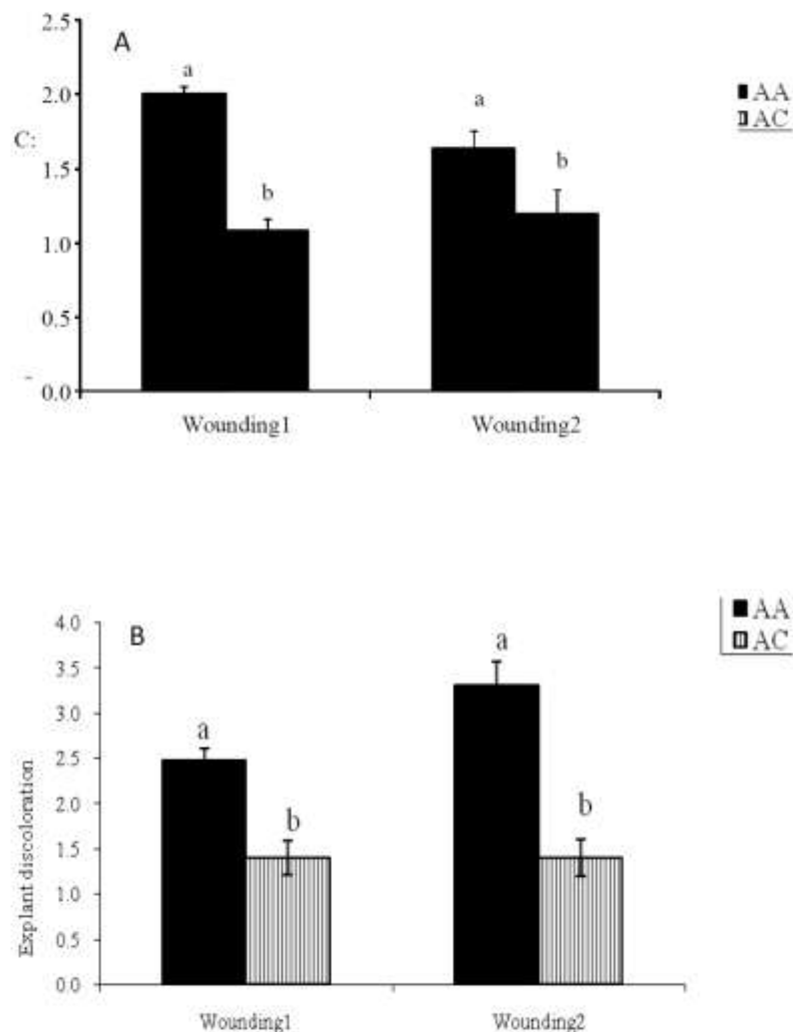

Figure 1. Interactive effects of antioxidants and wounding on entire explant discoloration in (A) week 2 and (B) week 7. Rating scale used is 1 to 5 (1, No discoloration; 5, extreme discoloration). Wounding 1= Wounded; Wounding 2= unwounded.

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 ($P \leq 0.05$) 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

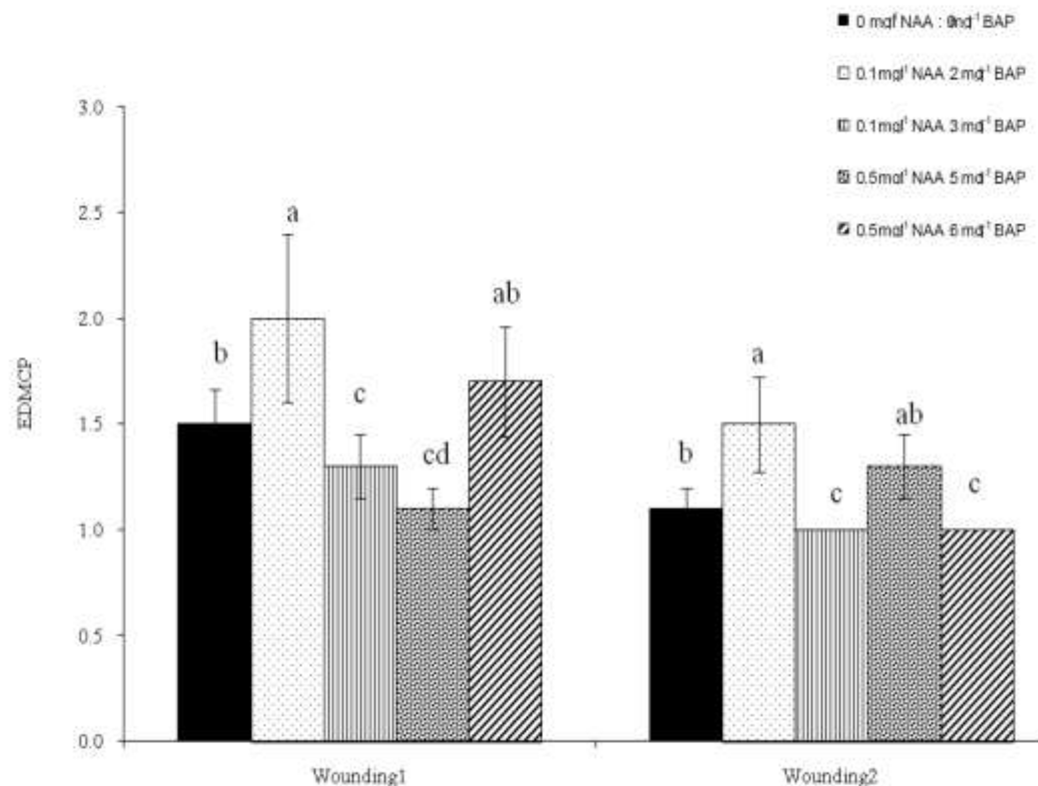

Figure 2. Interactive effects of wounding and concentration on explant discoloration at the medium contact point (EDMCP) in week 2. Rating scale used is 1 to 5 (1, No discoloration; 5, extreme discoloration). Wounding 1= Wounded; Wounding 2= unwounded.

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

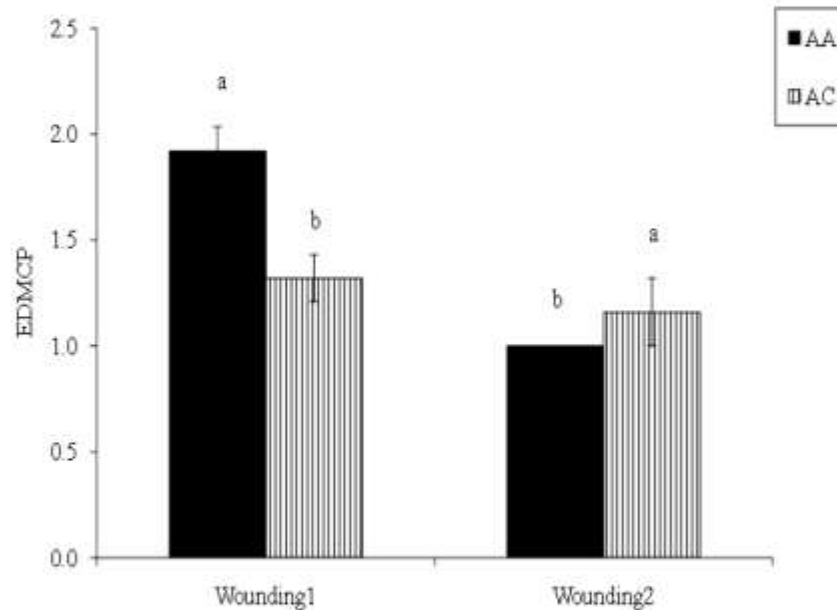

Figure 3. Interactive effects of antioxidants and wounding on explant discoloration at the medium contact point (EDMCP) in week 2. Rating scale used is 1 to 5 (1, No discoloration; 5, extreme discoloration). Wounding 1= Wounded; Wounding 2= unwounded.

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*.

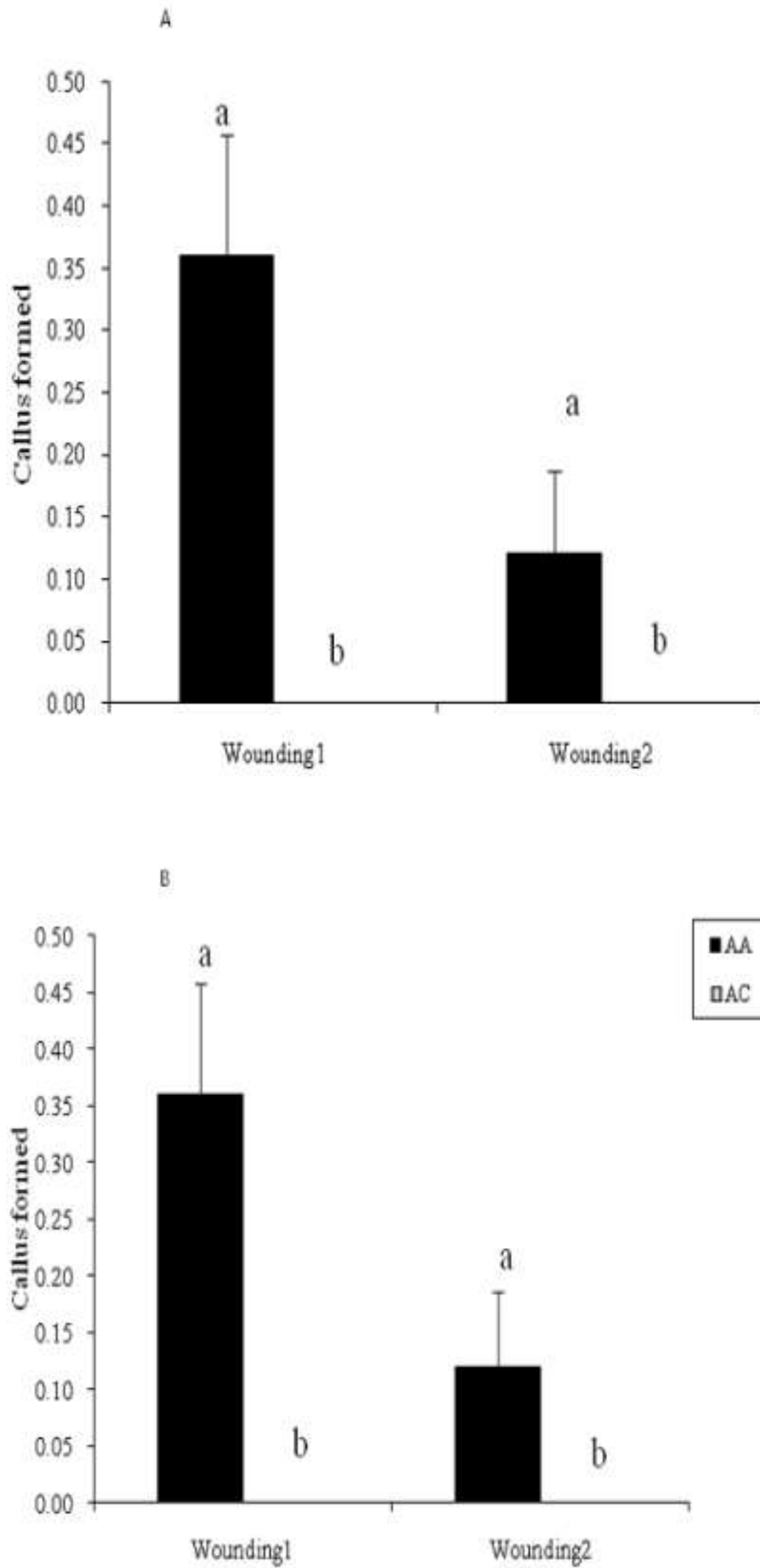

Figure 4. Interactive effects of antioxidants and wounding on callus formation in (A) WK3, (B) week 4, (C) week 5, (D) week 6. The degree of callus formation is rated as: 1, None; 2, low; 3, medium; 4, high. Wounding 1= Wounded; Wounding 2= unwounded.

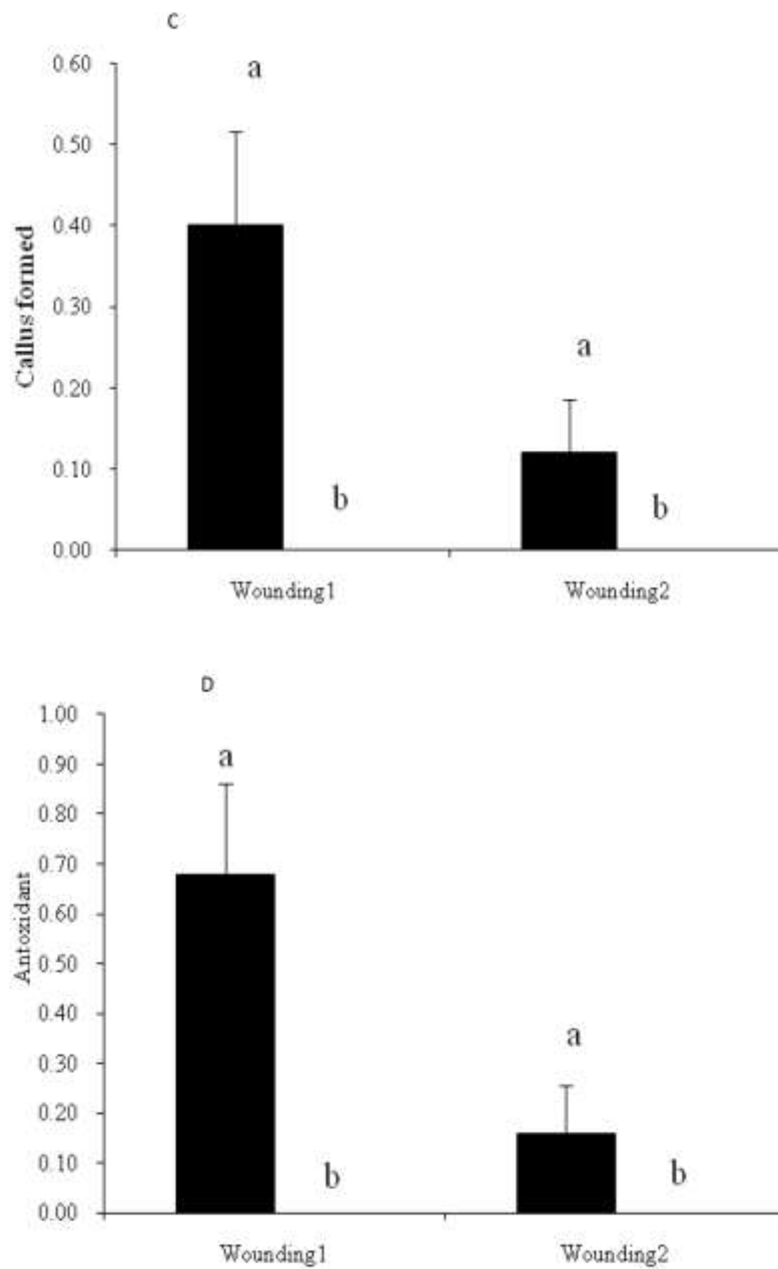

Figure 4. Contd.

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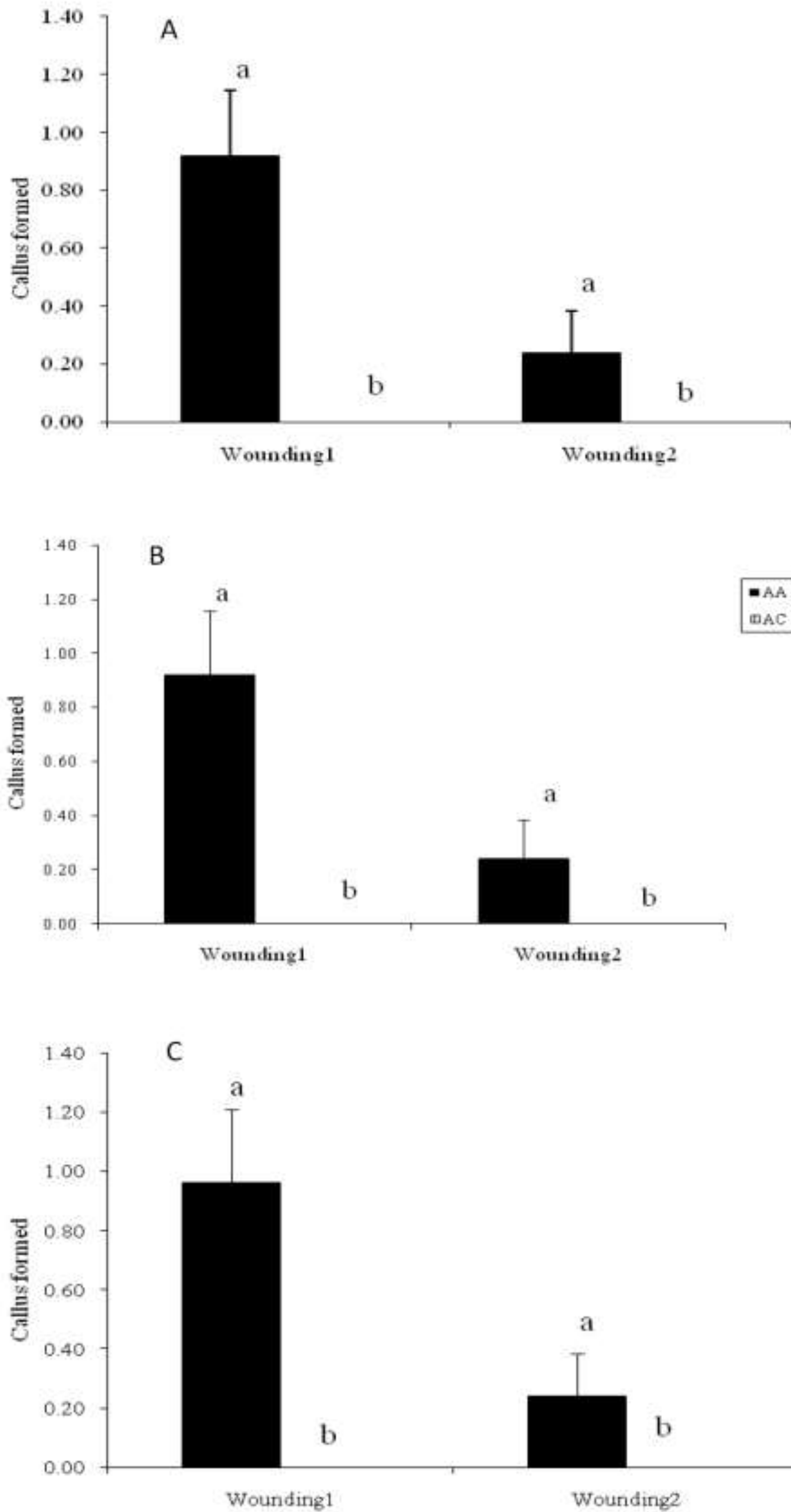

Figure 5, Interactive effects of antioxidants and wounding on callus formation in (A) WK7, (B) week 8 and (C) week 9. The degree of callus formation is rated as: 1, None; 2, low; 3, medium; 4, high. Wounding 1= Wounded; Wounding 2= unwoun

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APPENDIX D:

North, J.J. Ndakidemi, P.A. & Laubscher, C.P. 2012. Effects of antioxidants, plant growth regulators and wounding on phenolic compound excretion during micropropagation of *Strelitzia reginae*. *International Journal of the Physical Sciences*, 4(2): 100-113.

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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

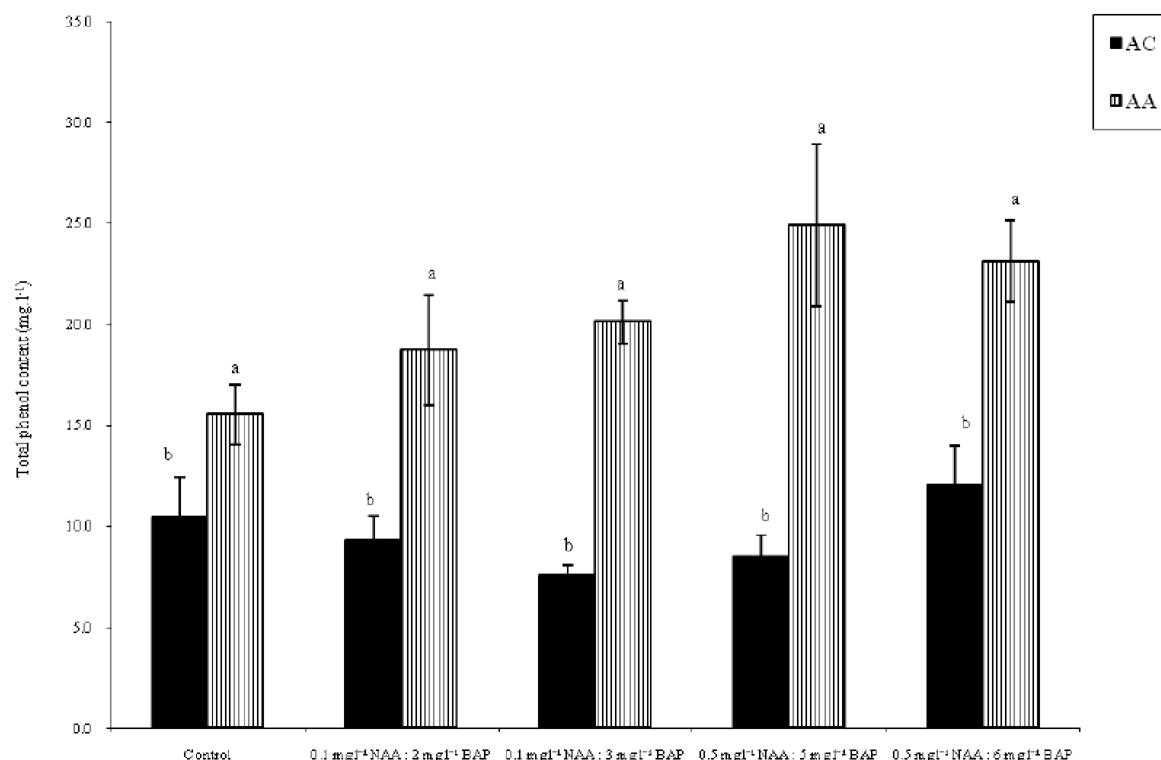

Figure 1. Interactive effects of NAA and BAP concentrations and antioxidants on total phenol content of culture media (mg L^{-1}). Bars followed by dissimilar letters are significantly different by Fischer's LSD test at *P* = 0.05.

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

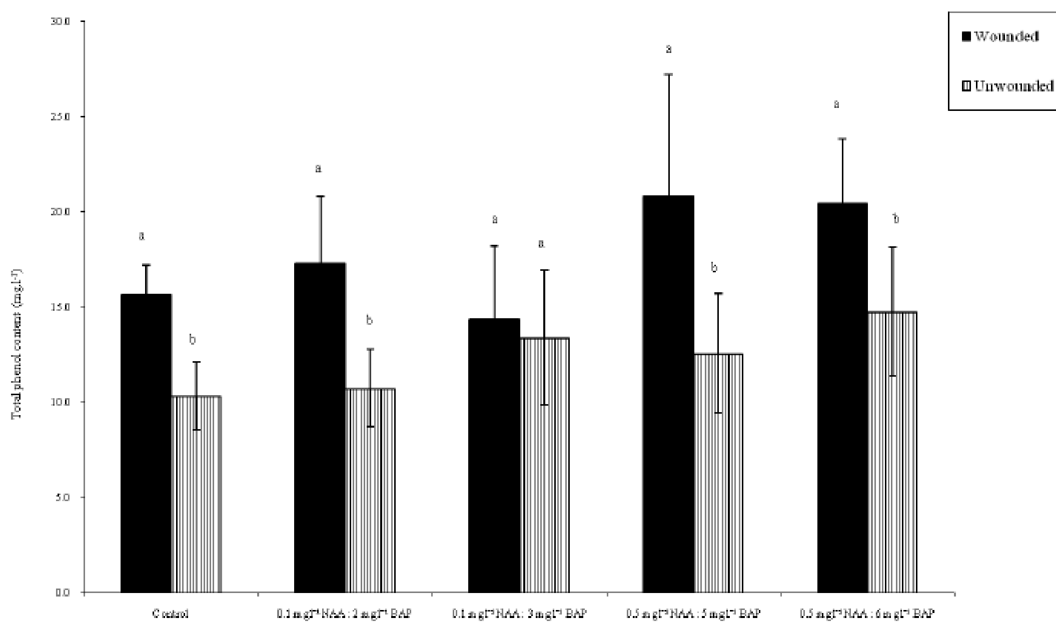

Figure 2. Interactive effects of NAA and BAP concentrations and wounding on total phenol content of culture media (mg L^{-1}). Bars followed by dissimilar letters are significantly different by Fischer's LSD test at $P = 0.05$.

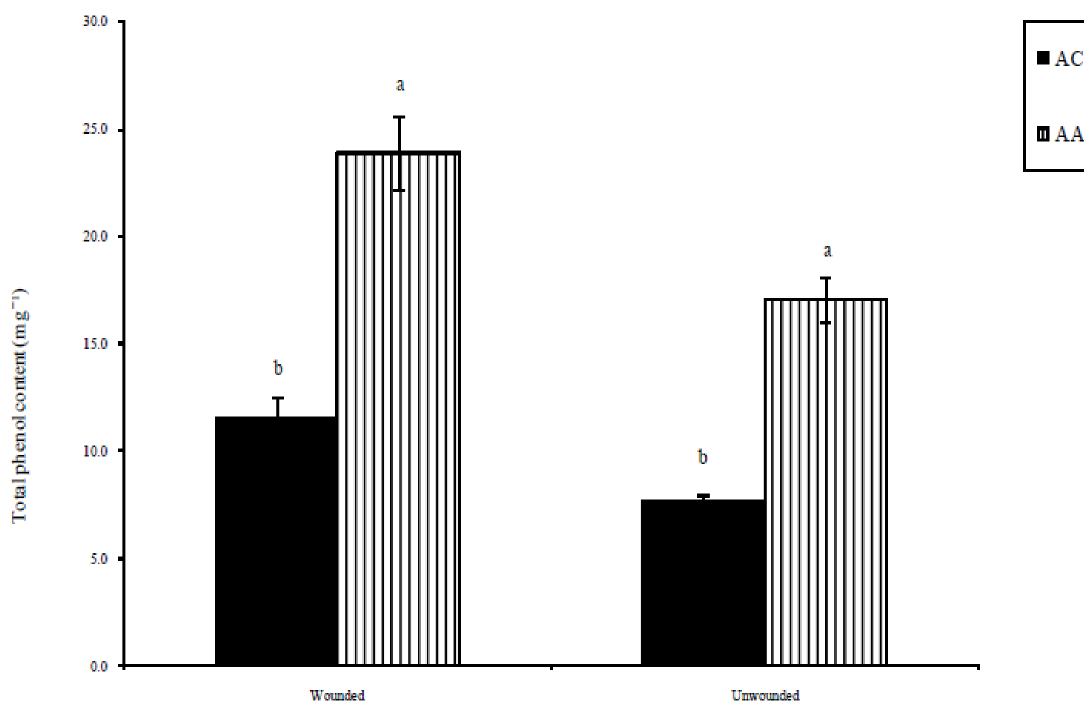

Figure 3. Interactive effects of antioxidants and wounding on total phenol content of culture media (mg L^{-1}). Bars followed by dissimilar letters are significantly different by Fischer's LSD test at $P = 0.05$.

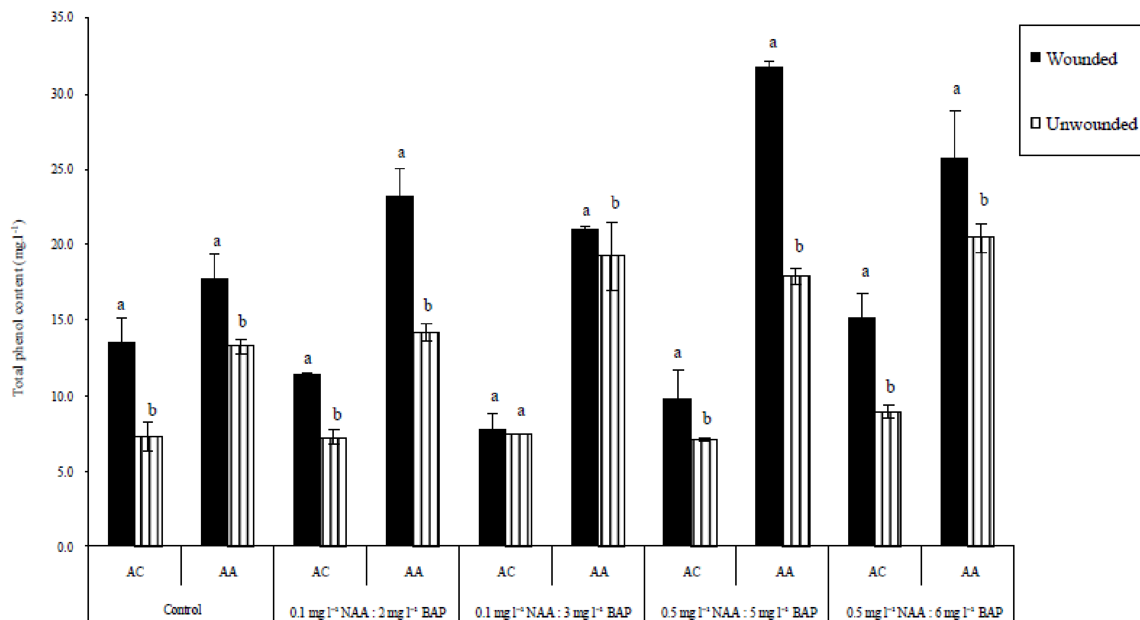

Figure 4. Interactive effects of NAA and BAP concentrations, antioxidants and wounding on total phenol content of culture media (mg L^{-1}). Bars followed by dissimilar letters are significantly different by Fischer's LSD test at *P* = 0.05.

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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