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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 (NaOCI) 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.

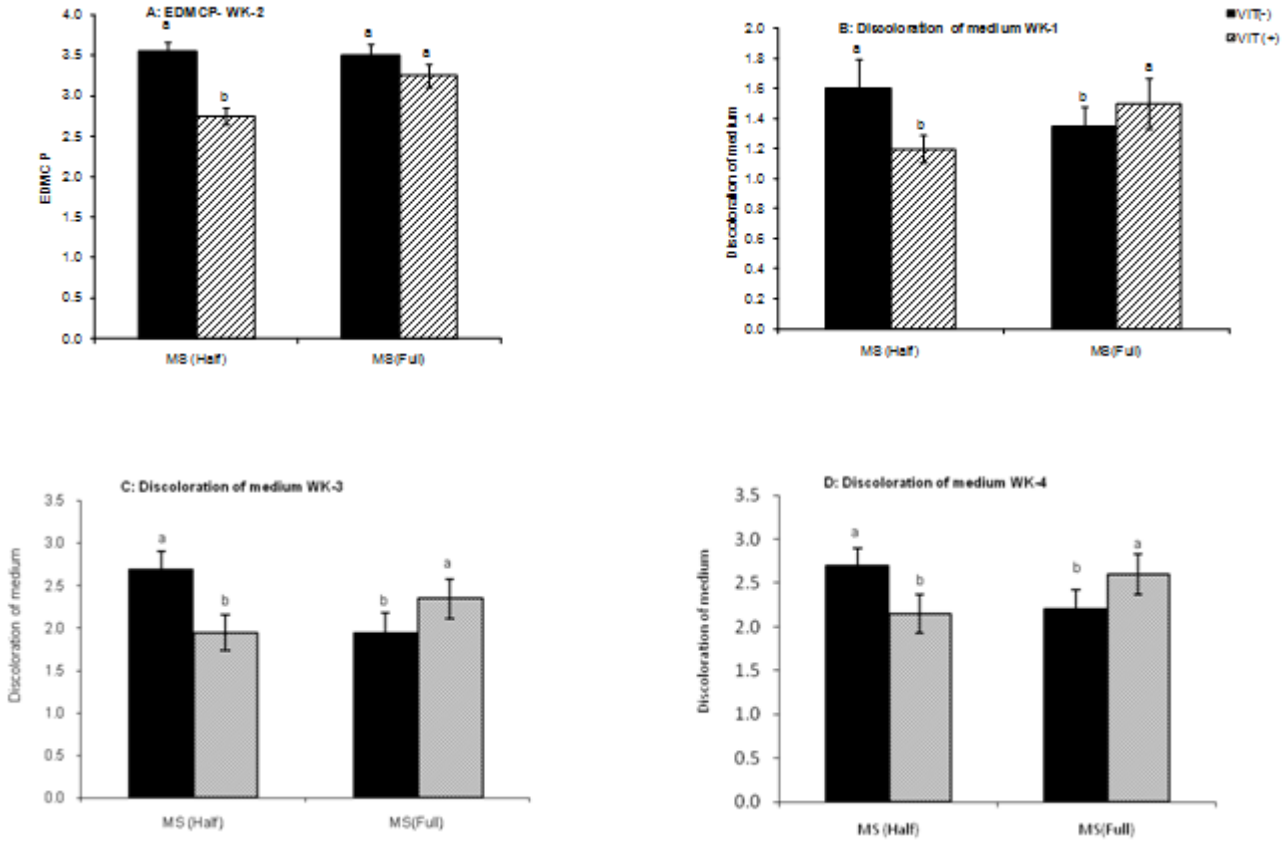

Fig 1. Interactive effects of Medium strength and vitamins on (A) Embryo discoloration at medium contact point (EDMCP) WK-2, (B) Discoloration of medium (WK-1), (C) Discoloration of medium (WK-3), (D) Discoloration of medium (WK-4). Rating scale used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration), 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.

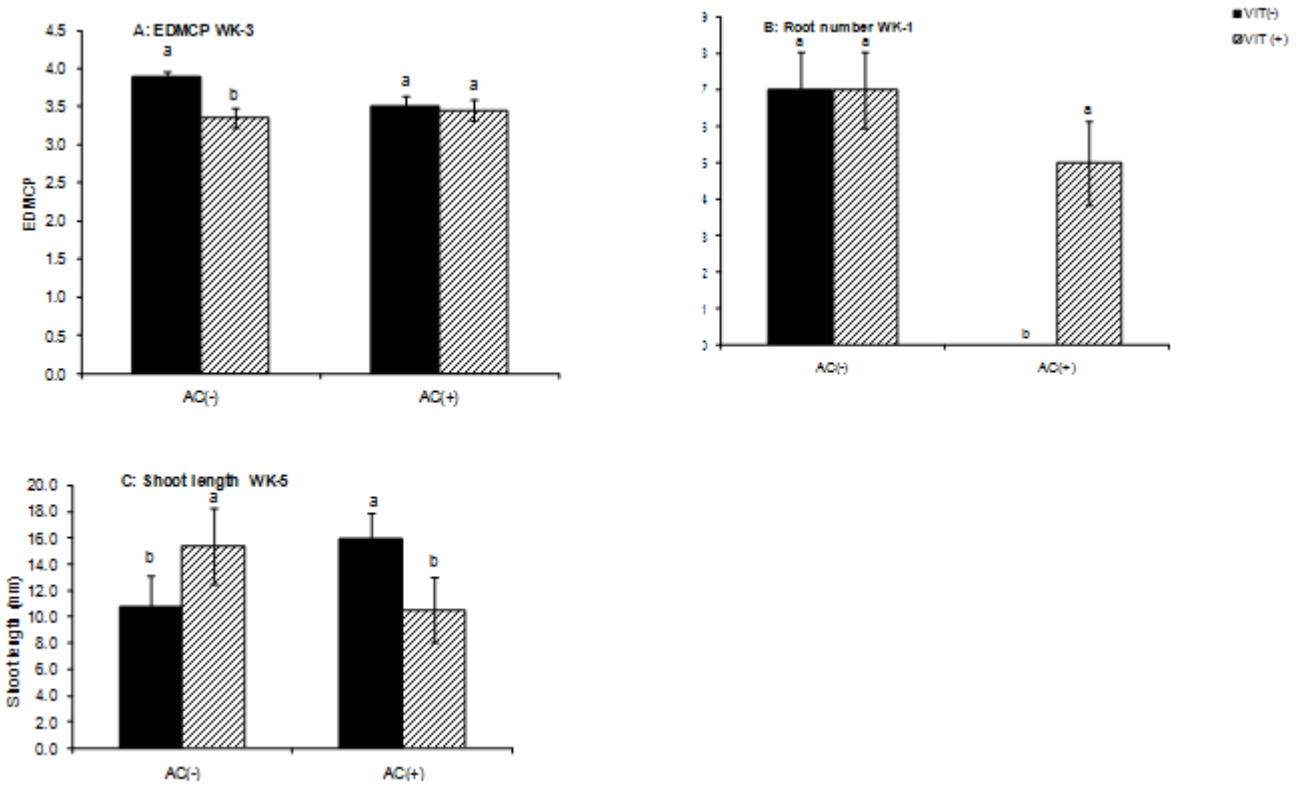

Fig 2. Interactive effects of Medium strength and vitamins on (A) Embryo discoloration at medium contact point (EDMCP) WK-3, (B) Root number WK-1, (C) Shoot length WK-5. Color code used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration), AC (-) = Without activated charcoal, AC (+) = With activated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

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

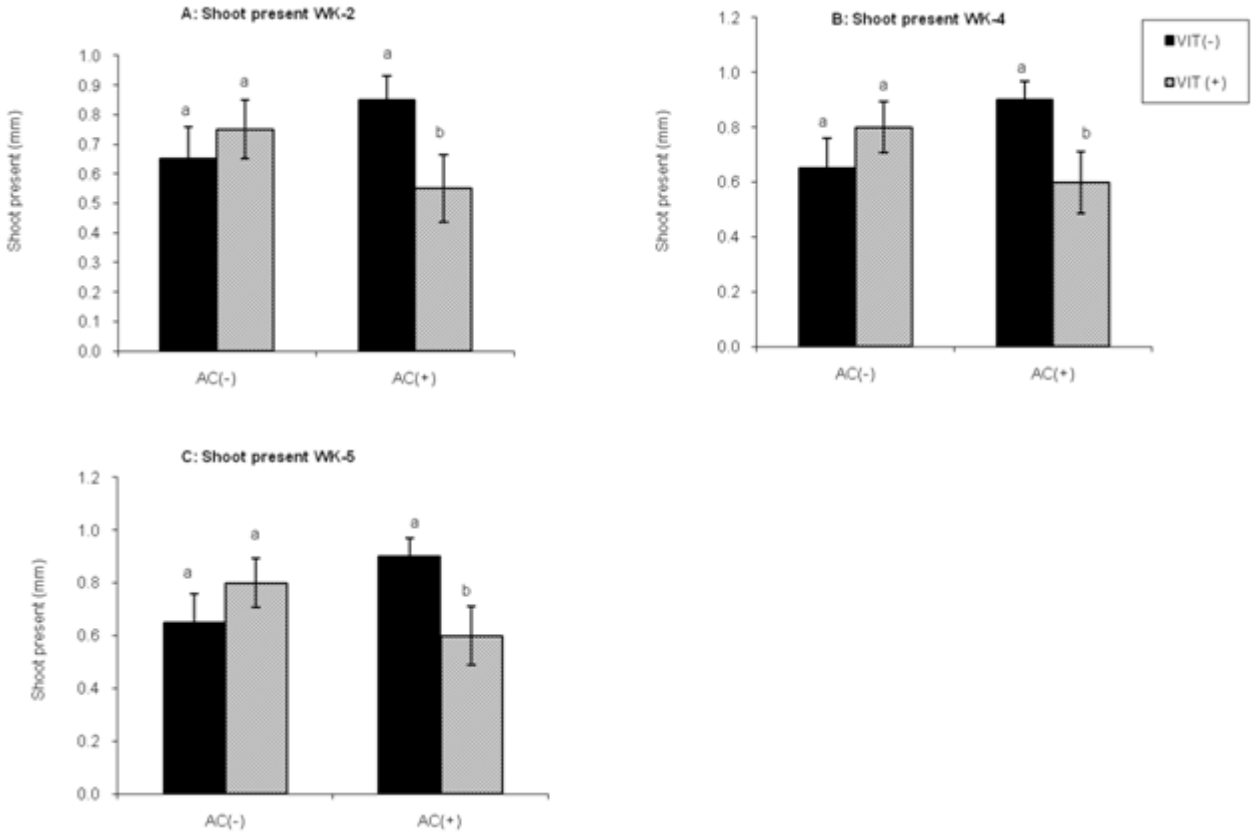

Fig 3. Interactive effects of activated charcoal and vitamins on (A) Shoot present WK-2, (B) Shoot present WK-4, (C) Shoot present WK-5. AC (-) = Without activated charcoal, AC (+) = With activated charcoal, VIT(-) = Without vitamin, VIT (+) = With vitamin.

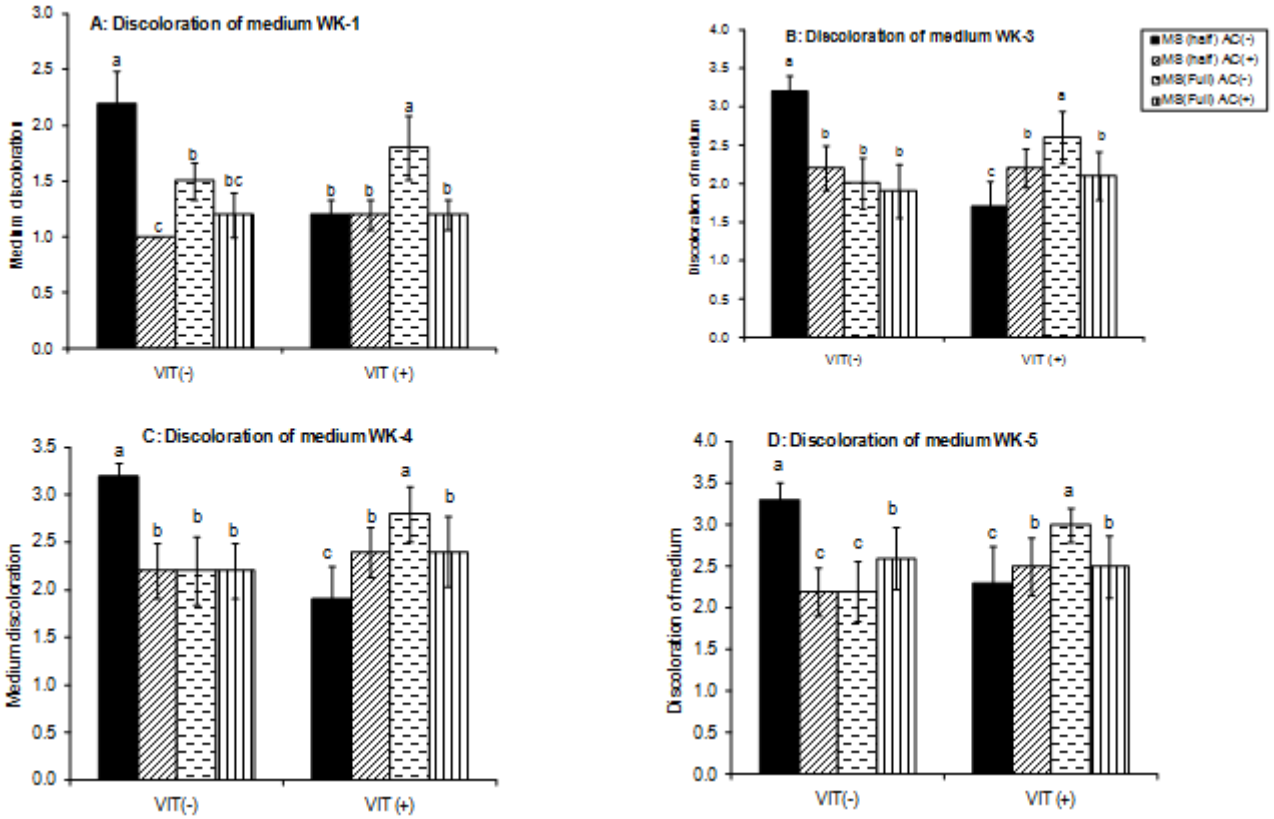

Fig 4. Interactive effects of Medium strength x activated charcoal x vitamins on discoloration of medium in: A) Week-1, B) Week-3, C) Week-4), D) (Week-5) in *Strelitzia reginae.* Color code used is 1 - 5 (1 = No discoloration and 5 = Extreme discoloration). MS (Half) = Half medium strength, MS (Full) = 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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