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S Swagath Kumar
Department of Horticulture, Dr. Y.S.R. Horticultural University, Venkataramannagudem, West Godavari, Andhra Pradesh, India

Anuradha Sane
ICAR-Indian Institute of Horticultural Research, Hessaraghatta, Bengaluru, Karnataka, India

M Lakshminarayana Reddy
Department of Horticulture, Dr. Y.S.R. Horticultural University, Venkataramannagudem, West Godavari, Andhra Pradesh, India

Leela Sahijram
ICAR-Indian Institute of Horticultural Research, Hessaraghatta, Bengaluru, Karnataka, India

AVD Dorajee Rao
Department of Horticulture, Dr. Y.S.R. Horticultural University, Venkataramannagudem, West Godavari, Andhra Pradesh, India

Corresponding Author:
S Swagath Kumar
Department of Horticulture, Dr. Y.S.R. Horticultural University, Venkataramannagudem, West Godavari, Andhra Pradesh, India

Effect of half-strength MS medium supplemented with plant growth regulators on *in vitro* response in 'Bird of Paradise' (*Strelitzia reginae* L.)

S Swagath Kumar, Anuradha Sane, M Lakshminarayana Reddy, Leela Sahijram and AVD Dorajee Rao

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Abstract
Propagation of Bird of Paradise (*Strelitzia reginae* L.) from seeds is a slow and complicated process due to the hard endocarp that encloses the seed and hinders germination. A study was undertaken to optimize *in vitro* protocol for mass multiplication of 'Bird of Paradise' (*Strelitzia reginae* L.). The experiment consisted of four treatments with growth regulators, *viz.*, GA_3 @1mg L^{-1}; GA_3 @3mg L^{-1}; GA_3 @5mg L^{-1}; and, GA_3 @1mg L^{-1} + BA @1mg L^{-1} with various medium components (½ MS + Sucrose @1% + Gelrite 3g L^{-1} + Inositol 100mg L^{-1} + Thiamine-HCl @ 1mg L^{-1} + Biotin 2mg L^{-1}; and, medium with ½ MS + Sucrose @1% + Gelrite 3g L^{-1} + Inositol 100mg L^{-1} + Thiamine- HCl @1mg L^{-1} + Biotin 2mg L^{-1} (Control). Results from the experiment showed the significant differences between the treatments. The maximum rate of contamination was found in control while, minimum was in GA_3 @ 5 mg L^{-1} and GA_3 @ 1mg L^{-1}+ BA @ 1 mg L^{-1} treatments. Higher rate and percentage of culture response (zygotic embryos germinated) were recorded in GA_3 @ 1 mg L^{-1}. Growth parameters such as shoot length (cm), root length (cm) and number of leaves per plant were maximum in ½ MS+ Sucrose @ 1% + Gelrite 3 g L^{-1} + Inositol 100 mg L^{-1} + Thiamine- HCl @ 1 mg L^{-1} + Biotin 2 mg L^{-1} + GA_3 @ 1 mg L^{-1}+BA @ 1 mg L^{-1} treatments. On the basis of overall results, our study confirmed that the media composed of ½ MS+ Sucrose @ 1% + Gelrite 3g L^{-1} + Inositol 100mg L^{-1}+ Thiamine-HCl @1mg L^{-1}+ Biotin 2mg L^{-1}+ GA_3 @1mg L^{-1} accelerated the germination rate and the combination of GA_3 @1mg L^{-1} + BA @1mg L^{-1} growth regulators showed the better growth performance in *in vitro* propagation of 'Bird of Paradises' (*Strelitzia reginae L.*).

Keywords: Bird of Paradise, germination, plant growth regulators, *in vitro*, zygotic embryos

Introduction
Bird of Paradise (*Strelitzia reginae* L.) is a perennial monocotyledonous plant of the family Strelitziaceae, and native to South Africa with a diploid chromosome number, 2n = 22. The genus is named after the duchy of Mecklenburg- Strelitz, birthplace of Queen Charlotte of the United Kingdom. A common name of the genus is bird of paradise flower or plant, because of a resemblance of its flowers to birds of paradise. In South Africa it is commonly known as crane flower and is featured on the reverse of the 50 cent coin. The plant can tolerate temperatures as low as -4.4 °C for a short time. However, freezing temperatures damage developing flower buds and flowers. To ensure the flower production in colder climes, bird of paradise can be grown in containers that can be moved indoors during freezes. The showy bloom is actually a combination of blue petals and orange sepals that emerge from a beak like bract (modified leaf). Blooms appear intermittently most of the year. Healthy, mature plants can produce as many as three dozen flower spikes a year, which can last up to two weeks when cut.

Due to slow growth and low multiplication rate, *in vitro* culture is an alternative method that can be applied to increase multiplication rate under controlled conditions. However, despite attempts made on *in vitro* propagation, no successful mass propagation has been achieved using tissue culture. Major constraints for *in vitro* culture of this species are (i) oxidative browning due to the presence of high levels of phenolic compounds in plant tissues (Ziv and Halevy, 1983) [26] and (ii) the recalcitrant nature of the seed. Therefore, there is a need to determine the effect of various limiting factors on seed germination and *in vitro* culture and optimize culture conditions

in this high value ornamental crop.

The present study explores possibilities of developing an *in vitro* method for successful propagation of *Strelitzia* spp. Tissue culture can be more promising than other conventional methods of multiplying *Strelitzia* (Promtep, 1981) [19]. A reliable and advanced propagation / cloning method would greatly contribute to overcoming limitations this species poses to the horticultural and landscaping industry worldwide (Ziv and Halevy, 1983) [26]. Despite the plant's commercial importance, a reliable method for micro propagation is not yet available (Chand, 2008). Several studies reported only partial success and a low rate of multiplication were obtained, indicating major problems with growing and multiplying this plant *in vitro*. Further, successful regeneration from zygotic embryo explants has not been reported, suggesting that protocols developed so far have not been efficient with respect to growth and large scale multiplication of this plant. Hence, the present investigation was carried out to standardize half strength MS media supplemented with plant growth regulators for *in vitro* responses of Bird of Paradise.

Materials and Methods

The present study was conducted during the year 2016-2017 at ICAR-Indian Institute of Horticultural Research, Bangalore. The experimental material used in the present investigation consisted of one year old seeds of *Strelitzia reginae* collected from plants grown in the field of Floriculture and Medicinal crops Division. The methodology used during this study is under following heads.

Sterilization

The healthy seeds were selected, counted and soaked in distilled water (Together with the orange aril) for 24 h. Seeds were surface-sterilized with 0.1 (w/v) mercury chloride ($HgCl_2$) for 25 minutes and rinsed three times with sterile distilled water (Bhattacharjee and Islam, 2014; Reshmi *et al.*, 2010) [3, 22]. The orange arils were removed from the seeds, since they are inhibiting the seed germination. Mature embryos were aseptically excised from the sterilized seeds and inoculated into various induction media. Explants were surface sterilized with various sterilants such as ethanol, sodium hypochlorite (NaOCl), calcium chlorite and mercury chloride (Badoni and Chauhan, 2010; Vasudevan and Van Staden, 2010) [2, 25].

Culture conditions and media preparation

pH of the medium was adjusted to 5.8 prior to autoclaving / adding Gelrite. Hot medium was poured into test tubes (25mm x 150mm) @15 ml and autoclaved at 121ºC (equivalent to 1.06 kg/cm^2 pressure) for 20 min. Zygotic embryos served as the starter explants. A total of eight test tubes were used per replication and each treatment comprised 33 test tubes. The experiment consisted of five types of media, *viz.*, T_1 (Control), with ½ MS + Sucrose @ 1% + Gelrite 3g L^{-1} + Inositol 100mg L^{-1} + Thiamine-HCl @ 1mg L^{-1} + Biotin 2mg L^{-1}; and, ½ MS + Sucrose @1% + Gelrite 3g L^{-1} + Inositol 100mg L^{-1} + Thiamine-HCl @1mg L^{-1} + Biotin 2mg L^{-1}), T_2 (medium with GA_3 @1mg L^{-1}); T_3 (medium with GA_3 @3mg L^{-1}), T_4 (medium with GA_3 @1mg L^{-1}) and T_5 medium with GA_3 @1mg L^{-1} + BA @1mg L^{-1}). Four replicates were used in each treatment. Inoculated cultures were incubated in a dark chamber at temperature of 27±2ºC for 7 days (Yam *et al.*, 1989) [34] after which, the culture tubes were shifted to a growth room at 25±2ºC under cool, white, fluorescent light (227µmol m^{-2} s^{-1}) under controlled photoperiod (16/8h).

Data recording and analysis

Data on rate of contaminated cultures, rate of healthy (Contamination-free) cultures, rate of culture response (plumule/ radicle emergence), type of culture response, callus induction, shoot development, shoot: Root ratio/ any other, number of shoots per culture, number of roots per culture, number of plantlets/culture (Multiplication rate) and *ex vitro* transfer (if any) of plantlets, were recorded at weekly intervals. Data collected on visual scoring/ observations were analyzed for statistical significance using Complete Randomized Design (CRD).

Special techniques

Seeds were sliced at their narrow end using a sharp blade, under a laminar air-flow hood/ work-station. Mechanical scarification requires that seeds be filed or nicked by hand using a knife, or sand paper, to remove the seed coat. The procedure generally works best for larger seeds, since, small seeds are difficult to handle.

Results and Discussion

Data on contamination rate in *Strelitzia reginae* cultures were recorded at 4th and 8th week from inoculation and are presented in Table 1. In the 4th week, lowest contamination percentage (2.9%) was observed in the Control and was on par with T_3. Highest contamination percentage (9%) was recorded in T_4 which was on par with T_1. In the 8th week of culture of *Strelitzia reginae* zygotic embryos, however, lowest contamination percentage (5.9%) was observed in T_4. Highest contamination percentage (17.9%) was recorded in the Control. Thus, Control showed highest percentage of contamination, compared to the other treatments. The contamination was due to fungi and bacteria, as also observed by Araujo (2012) [27]. These results were found to be also similar to findings of Souza *et al*. (2010) [28] where the authors encountered difficulty in micropropagation of many species of Heliconiaceae, mainly, due to the high frequency of endogenous contamination. The contaminants in *in vitro* establishment of explants in *Strelitzia reginae* may have been from endophytic communities in the stock plants, which rendered the contaminants resistant to surface-disinfection (Nakano, 2008) [29].

Rate of contamination-free cultures (%) was significantly influenced by various treatments seen at 4th week from culture initiation. Maximum number of contamination-free cultures (97.1%) was recorded in Control and was on par with T_3 (97%); whereas, lowest rate of contamination-free cultures (91.1%) was recorded in T_1, which was on par with T_4 (91%). The data also showed that per cent healthy cultures were significantly influenced by different treatments at 8th week too (Table 1). Maximum number of contamination-free cultures (91.1%) was recorded in T_4 and T_3 treatments. Minimum rate of contamination-free cultures (82.1%) was observed in the Control (Ziv and Halevy, 1983) [26].

Data on per cent responsive cultures in *Strelitzia reginae* were recorded at weekly intervals. Means in the data are shown in Table 2. Until the 4th week, no response was observed in *Strelitzia reginae* cultures when whole seeds were used. The seed coat was then removed partially and zygotic embryo were excised and transferred to test tubes in 15ml half-strength MS (Murashige and Skoog, 1962) [7] medium in the 5th week. Hensley *et al*. (1998) [6] and Burgess (2004) [4] opined that the outside of the seed (Seed coat) must be scraped before sowing the seeds. Prevention of germination may be caused by physical dormancy such as hard or impermeable seed-coat and internal

dormancy which includes an immature embryo and/or presence of an inhibitor or hormone such as abscisic acid (Yang *et al*., 2007; DuPont, 2011) [30, 31]. Araujo (2012) [27] also observed that propagation of *Heliconia* through seeds was a slow and complex process due to the hard endocarp enclosing the seed and hindering germination. The seed coat in dormant seeds poses a physical barrier that prevents water and oxygen permeability (Yang *et al*., 2007) [30]. Germination in such seeds can be induced by scarification which weakens the seed-coat, thereby allowing more water and oxygen to diffuse into the seed and promote germination.

At the 6th week, per cent responsive cultures differed significantly in half-strength MS medium supplemented with plant growth regulators. Germination started in the 6th week (Table 1) in all cultures due to the sliced or cut narrow-end of the seed. Maximum rate of germination (12.02%) was observed in T_1 (medium with GA_3 @1mg L^{-1}); whereas, minimum (3.00%) germination rate was seen in T_2, and was at par with Control (3.05%). The first germination response was observed under GA_3 @ 1 mg L^{-1} (T_1) treatment. The beginning of germination was observed after seven days (From 5th week to 6th week interval) of *in vitro* cultures. The same results were observed in *Heliconia* by Souza (2010) [28]. Cultured zygotic embryos, at seventh week exhibited significant difference was observed when the half strength MS media supplemented with plant growth regulator treatments. The greatest germination percentage (32.00%) was recorded in T_1, which, was at par with treatment T_4. However, the lowest percentage of germination (21.05%) was observed in T_2.

In the 8th week, a significant difference was noticed at the rate of responsive cultures in half strength MS media supplemented with plant growth regulator treatments. The highest rates of responsive (57%) siblings were observed in T_1. The lowest (45%) germination was recorded in control, T_2 and T_4. Above mentioned results under media with GA_3 @ 1 mg L^{-1} (T_1) treatment, highest germination was found compared to other treatments. Internal dormancy is caused by physiological conditions which delay germination and it is affected by internal factors such as an immature embryo and the presence of a plant growth hormone, notably abscisic acid (ABA). Gibberellic acid (GA_3) is a plant growth hormone that releases seed dormancy and its action is generally considered as antagonistic to ABA. Commercially, GA_3 is applied exogenously to release seed dormancy of many plant seeds (DuPont, 2011) [31]. Depending on the species the growth of immature embryos can be stimulated by the use of growth regulators like gibberellins.

The lowest percentage (45%) of results was recorded in the Control (C), T_2 Gibberellic acid (GA_3) @ 3 mg L^{-1}, T_4 GA_3 @ 1mg L^{-1} + Benzyladenine (BA) @ 1 mg L^{-1} treatments. The same result was obtained by Ulisses (2010) [32] in *Heliconia bihai* L. with use of media containing sucrose. Our results showed that a GA_3 and BA significantly improved *in vitro* germination of embryos from *S. reginae*. However, in the germination experiment of *Tagetus wallichiana* embryos, BA or GA_3 was non effective, which indicates that the effects of plant growth regulators on embryo culture differed from species to species.

The data related to shoot development in *S. reginae* as influenced by medium supplemented with plant growth regulators is presented in Table 2. The shooting (mm) was started at seventh week onwards in GA_3 @ 1 mg L^{-1} + Benzyladenine (BA) @ 1 mg L^{-1} (T_4) cultures. According to Williams and Maheswaran (1986) [33], BA acts strongly in inducing cell polarity and favors the differentiation process of embryogenic callus material.

In the 8th week, no significant difference was observed in the number of shoots per plant. Here, each zygotic embryo produced only one shoot. These results are in conformity with findings of Sayers (2007) [24] in *Strelitzia parvifolia* var. *juncea.* The average Shoot length (cm) was recorded in 8th week from inoculation and presented in Table 2. The highest shoot length recorded 5.53cm in T_4, whereas, lowest shoot length were recorded with 3.06 cm with Control.

The media supplemented with GA_3 @ 1 mg L^{-1} + BA @ 1 mg L^{-1} which shows the best shoot length. Similar results were also observed by Pant & Thapa (2012) [13] in orchids and souza (2010) [28] found that the best shoot formation was observed in a combination of BAP and GA_3. These same results were in conformity with findings of Ulisses (2010) [32] who observed that ½ MS medium promoted best shoot formation in *Heliconia*. Development of shoots was observed at 45 days after inoculation in *Heliconia* (Araujo, 2012) [27]. Longest shoots were observed with a combination treatment, BA (2.00mg L^{-1}) + NAA (0.2mg L^{-1}) in *Heliconia chartace* (Reshmi, 2008). BA @ 1 mg L^{-1} gave better shoot formation in *Paphiopedilum hangianum*. As mentioned above, T_4 was recorded highest shoot length and followed by GA_3 @ 1 mg L^{-1} (T_1) treatment second highest Shoot length (5.29 cm) was found that the Gibberellins are linked to activation of vegetative growth in plant embryos, to the weakening of the endosperm layer that surrounds the embryo thus restricting its growth, and to the mobilization of endosperm energy-reserves. Best shoot development was observed in *Heliconia* when sucrose was supplied (Ulisses *et al.*, 2010) [32]. Ledo *et al*. (2007) [35] observed that zygotic embryos isolated from mature fruits of *Cocus nucifera* L. also differentiated into complete and normal plants when sucrose was used as the *in vitro* carbohydrate-nutrient source in the culture medium. A three-fold increase in length and more than double increase in breadth of axillary buds in *Heliconia psittacorum* L. was successfully established in MS medium with 5.0-6.0mg L^{-1} BAP, 0.05-0.1mg L^{-1} 1AA and 1.50–2.00mg L^{-1} GA_3. Supplementation of 2.5mg L^{-1} BAP, 0.2mg L^{-1} IAA and 2.5mg L^{-1} GA_3 was ideal for initiation of multiple shoots and their further multiplication rate.

However, no multiple shoot induction was reported. To date, no reports are available for any *Strelitzia* species on *in vitro* propagation using seeds as the starting material,l with an attempt to induce multiple shoots from *in vitro* produced seedlings. Radicle emergence started sixth week onwards in all the treatments. The same results were observed by Araujo (2012) [27] in *Heliconia*. In the 8th week from culture initiation, number of roots showed no significant effect in the any of our treatments (C, T_1, T_2, T_3, T_4); only, a tap root was observed. However, in the 8th week, root length varied significantly. Longest roots (7.92cm) were recorded in T_4, while, shortest roots (3.36cm) were observed in T_1. Ulisses *et al.* (2010) [32] also observed best root development in *Heliconia* only when sucrose was supplied. Maximum root induction (6.1) and root-growth (4.0cm) in orchid was found on ½MS medium supplemented with 1.0mg L^{-1} IAA.

Whereas, the number of plantlets per culture had no significant effect in any of the treatments (C, T_1, T_2, T_3, T_4), only a single plantlet was produced in each culture. Sayers (2007) [24] also obtained one plant per seed in *Heliconia*. Plantlets obtained from zygotic embryos derived from mature fruits demonstrated better development on ½ MS medium than on full-strength medium (Ulisses *et al.*, 2010) [32]. Number of leaves/plant at 8th week from inoculation was significantly different between treatments.

Highest number of leaves (3.11) was recorded in T_4, which was at par with C (Control) and T_3. However, minimum number of leaves (2.75) was observed in T_1. As mentioned above, under GA_3 @1mg L^{-1} + benzyladenine (BA) @1mg L^{-1} (T_4), highest number of leaves/ plant were produced compared to that in other treatments. These results are in conformity with Ulisses *et al.* (2010) [32] in *Heliconia*. Likewise, maximum number of leaves was produced in *H. psittacorum* at 45 days of culture on full-strength MS with 2.25mg L^{-1} BA (Nathan *et al.*, 1992) [8]. Addition of BA increased the number of leaves regardless of the strength of MS medium used; and, low concentrations of BA used in this work reduced any risk of somaclonal variation, as, BA is one of the main agents for genotypic alteration.

***Ex vitro* transfer of *Strelitzia reginae* plantlets for hardening-off:** At 8th week from germination, plantlets were transferred from the culture chamber to an environment-controlled chamber and placed for a week; then, these were transferred to the glasshouse where 30% survival rate was achieved. These surviving plantlets showed superior growth and were in good condition, while the remaining showed wilting (70%). Generally, *in vitro* regenerated plants are removed from their culture media, transplanted into a mixture of sand, soil, vermiculite and compost, and gradually exposed to low humidity for acclimatization to the natural environment (Pant *et al.*, 2011) [12]. This is a crucial stage in plant tissue culture where plants are produced for commercial purposes (Reshmi *et al.*, 2010) [22].

Table 1: Rate of contaminated and healthy (Contamination-free) cultures recovered at 4th and 8th week of culture

Treatment	Rate (%) of contamination		Rate (%) of contamination-free cultures recovered	
	4th Week	8th Week	4th Week	8th Week
Control	2.90 (9.93)	17.90 (25.09)	97.10 (80.20)	82.10 (64.97)
T_1	8.90 (17.43)	11.90 (20.25)	91.10 (72.64)	88.10 (69.82)
T_2	6.10 (14.33)	12.00 (20.27)	93.90 (75.70)	88.00 (69.73)
T_3	3.00 (9.97)	8.90 (17.43)	97.00 (80.03)	91.10 (72.64)
T_4	9.00 (17.46)	5.90 (17.43)	91.00 (72.54)	91.10 (72.64)
CD (P=0.05)	0.06 (0.08)	0.06 (0.06)	0.11 (0.15)	0.19 (0.18)

Table 2: Rate (%) of responsive cultures (No. of embryos germinated) recovered at 6th, 7th and 8th week of culture, and, Type of culture response (shoot / root development) at 4thand 8th week from inoculation

Treatment	Per cent (%) responsive cultures (No. of embryos germinated)				No. of shoots/ plantlet		Shoot length (cm)		No. of roots/ plantlet		Root length (cm)		No. of leaves/ plant	
	4th Week	6th Week	7th Week	8th Week	4th Week	8th Week	4th Week	8th Week	4th Week	8th Week	4th Week	8th Week	4th Week	8th Week
Control	0.00	3.05 (10.06)	27.03 (31.32)	45.00 (42.13)	0.00	1.00	0.00	3.06	0.00	1.00	0.00	5.20	0.00	3.00
T_1	0.00	12.03 (20.29)	36.00 (36.87)	57.00 (49.02)	0.00	1.00	0.00	5.29	0.00	1.00	0.00	3.36	0.00	2.75
T_2	0.00	3.00 (9.97)	21.05 (27.31)	45.00 (42.13)	0.00	1.00	0.00	4.25	0.00	1.00	0.00	5.72	0.00	2.86
T_3	0.00	9.03 (17.78)	30.08 (33.26)	51.00 (45.57)	0.00	1.00	0.00	3.62	0.00	1.00	0.00	4.75	0.00	3.00
T_4	0.00	6.03 (14.21)	33.03 (35.08)	45.00 (42.13)	0.00	1.00	0.00	5.53	0.00	1.00	0.00	7.92	0.00	3.11
CD (P=0.05)	0.00	0.07 (0.09)	0.07 (0.05)	0.52 (0.29)	0.00	NS	0.00	0.01	0.00	NS	0.00	0.01	0.00	0.07

*NS = Non-significant

Conclusion

Results obtained in the present study show that ½ MS + Sucrose @1% + Gelrite 3g L^{-1} + Inositol 100mg L^{-1} + Thiamine-HCl @1mg L^{-1} + Biotin 2mg L^{-1} + GA_3 @1mg L^{-1} with slicing or cutting of micropylar-end give the best results. *Strelitzia reginae* seeds have a hard seed-coat which prevents seed germination. Hence, mechanical scarification, followed by tissue culture, is a superior propagation method for this ornamental monocot.

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