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Effect of adenine, sucrose and plant growth regulators on the indirect organogenesis and on *in vitro* flowering in *Begonia* x *hiemalis* Fotsch.

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Abstract

Efficient shoot bud formation (94.5 ± 7.59%), *in vitro* regeneration and production of flowers were obtained from sterile plants of *Begonia* x *hiemalis* Fotsch. An *in vitro* regeneration was attempted using immature reproductive organs, which were not commonly used before, such as young inflorescences, peduncles and petals of flowers collected in the field. The flower segments were cultured on solid Murashige and Skoog (MS) medium, supplemented with various concentrations and combinations of plant growth regulators (PGRs) and adenine. Within 8 weeks of the culture period, the highest frequency of reproductive shoot regeneration (red calyx, 8.50%) was obtained from explants of immature inflorescence cultured on the MS medium supplemented with 1.0 mg L^{-1} benzyl amino purine (BA) and 1.0 mg L^{-1} 1-naphthalene acetic acid (NAA), added with 40 mg L^{-1} adenine and 3% sucrose. To attain further root growth and maturity, the clonal plantlets (with or without reproductive shoots) were excised and sub-cultured onto the MS medium fortified with 1.0 mg L^{-1} Gibberellic acid (GA_3). 87% of the regenerated plantlets were successfully acclimatized on 2:1:1 ratio of peat: soil: sand, under greenhouse conditions. All of them were capable of producing true-to-type flowers. The *in vitro* developed reproductive shoots, also further generated complete, small flowers, which were morphologically similar to the *in vivo* types, following an *ex vitro* acclimatization.
This study provides an alternative approach to generate early flowering in *Begonia* without undergoing the full growth cycle and hence can help overcome problems associated with flower growth and development in this species. The present study revealed that floral parts (inflorescence, peduncle and petals) could also be used as a source of explants besides the commonly used tissues such as leaf, stem, shoot and root segments.

Keywords: Acclimatization; adenine; *Begonia* x *hiemalis*; indirect organogenesis; *in vitro* flowering.
Abbreviations: MS - Murashige and Skoog; Kin/KN - Kinetin; NAA - 1-naphthalene acetic acid; BAP/BA - Benzyl adenine purine; 2,4-D -2,4-dichlorophenoxy acetic acid; IAA - Indole acetic acid; GA_3 - Gibberellic acid.

Introduction

In vitro fruiting offers a novel method for research on the physiology of fruits and seeds developed under an *in vitro* environment. Under natural growth conditions, the transition from the vegetative to the reproductive stage (flowering) in plant occurs after attaining maturity (Virupakshi et al., 2002). Flowering is generally considered as a complex process regulated by a combination of environmental and genetic factors, such as plant growth regulators, carbohydrates, light and pH of the culture medium (Heylen and Vendrig, 1988). Deliberate flowering in culture can serve as a tool for studying flower induction and development as well as to control breeding programs involving species with a long juvenile period (Lin et al., 2003). *In vitro* flowering has been reported for many plant species (Chang et al., 2010), however , *in vitro* fruiting and seed set are not readily encountered (Saritha and Naidu, 2007). In order to achieve *in vitro* fruiting, the plants must be able to produce flowers under *in vitro* conditions. Flowering and fruiting are two related processes and it is ideally studied together. Flowering is a complex process regulated by both internal plant factors and environmental signals. *In vitro* flowering serves as an important tool in studying flower induction, initiation and the floral developmental process by utilizing plant growth regulators such as cytokinins, gibberellins and auxins (Ziv and Noar, 2006). *In vitro* flowering can also reduce the influence of environmental factors and can clarify the key influences affecting the flowering process by controlling environmental factors and the application of plant growth regulators (Zhang et al., 2008). *Begonia* L. is a genus exceeding 1500 wild species throughout the tropical and subtropical regions of Asia, Africa and America. *Begonia* includes species with a variety of natural foliar variegation patterns, providing diverse examples of this phenomenon. This genus is one of the largest among the flowering plants and has a large morphological variation (Kiew, 2005). In recent years, many field surveys on begonias had been carried out in the world and new species continues to be discovered and described (Peng et al., 2010). Begonias are versatile temperate plants that belong to the family *Begoniaceae*, which consists of 5 genera and 920 species (Hickey and King, 1981). They are gaining popularity and commercially available as flowerbeds, in hanging baskets, pot plants and as indoor houseplants. *Begonia* x *hiemalis* Fotsch. cv. "Schwabenland red" or Elatior *Begonia* is developed from crosses between *B. socotrana* Hook and *B. tuberhybrida.* The inflorescences consist of single and double

Table 1. The responses of different explants of *Begonia* x *hiemalis* Fotsch cv. "Schwabenland red", cultured *in vitro* on MS solid media supplemented with different combinations of plant growth regulators, sucrose and adenine.

Explant type	MS media composition					Vegetative shoot buds (%)	Observations
	BA (mg L^{-1})	TDZ (mg L^{-1})	NAA (mg L^{-1})	Sucrose (%)	Adenine (mg L^{-1})		
Inflorescence	0.5	-	1.0	3.0	40.0	66.00 ± 8.83^{c}	NR
	1.0					94.50 ± 7.59^{a}	Red micro shoots
	1.2					79.00 ±7.18^{b}	Red micro shoots
	-	0.5	1.0	3.0	40.0	68.00 ± 1.52^{c}	Red micro shoots
		1.0				46.00 ± 2.31^{e}	Red micro shoots
		1.2				24.00 ± 9.40^{f}	NR
Peduncle	0.5	-	1.0	3.0	40.0	5.00 ± 0.00^{c}	Red micro shoots
	1.0					20.00 ±5.62^{a}	Red micro shoots
	1.2					38.00 ± 1.95^{a}	NR
	-	0.5	1.0	3.0	40.0	34.00 ± 1.95^{a}	NR
		1.0				24.00 ± 9.40^{b}	NR
		1.2				23.50 ± 8.75^{b}	NR
Petal	0.5	-	1.0	3.0	40.0	NR	NR
	1.0					NR	NR
	1.2					NR	NR
	-	0.5	1.0	3.0	40.0	NR	NR
		1.0				NR	NR
		1.2				NR	NR

* Mean values with different letters within a column (subject to explant type) differ significantly at $p < 0.05$, by one way ANOVA and Duncan's multiple range test. NR: no response.

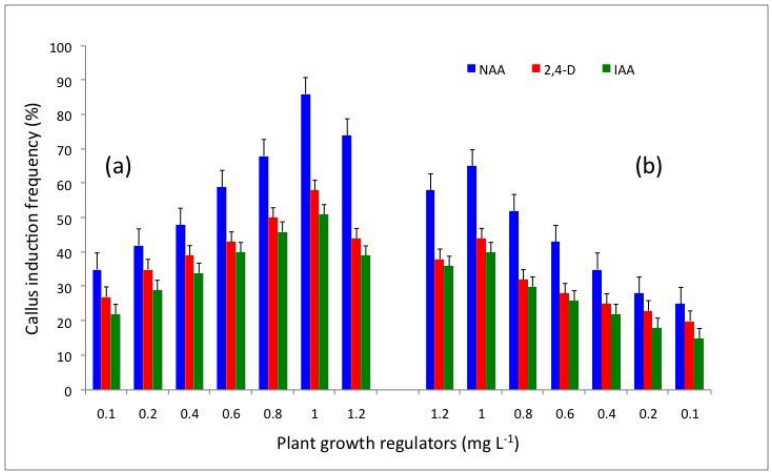

Fig 1. Induction of callus formation from different explant types of *Begonia* **x** *hiemalis* Fotsch. cv. "Schwabenland red", cultured on MS medium supplemented with 2,4-D , IAA and NAA after 45 days. **(a)** Inflorescence explants; **(b)** Peduncle explants. NAA was shown to perfom better than IAA and NAA in callus formation of *Begonia*.

flowers, usually red, yellow, pink or orange in color, arranged in loose clusters that rise above the foliage. Begonias are unique for their sheer beauty and variety of leaves. It is estimated that there are about 10,000 Begonia hybrids and cultivars worldwide. The *Begonia* x *hiemalis* Fotsch. plant normally has thick, shiny, dark green leaves.

Begonia is also a short-day plant and the time of bud appearance and flower initiation are strongly affected by the day length as well as night temperature (Heide, 1962). Previous studies revealed that exogenous cytokinins significantly induce *in vitro* flowering in many plant species (Chang and Chang, 2003; Lin et al., 2003). The commercial exploitation and production of medicinal plants via conventional propagation is hampered due to poor seed viability, low rate of germination and poor rooting capability of the vegetative cutting. Although begonias can be readily vegetatively propagated, they are susceptible to many pathogenic bacteria, fungi, and nematodes (Castillo and Smith, 1997). In recent years, tissue culture studies on *Begonia* x *hiemalis* Fotsch., especially on *in vitro* plant regeneration has become increasingly popular among scientists from different countries (Mikkelsen and Sink, 1978; Westerhof et al., 1984; Awal et al., 2008). However, plant regeneration of *Begonia* x *hiemalis* via somatic embryogenesis is new (Awal et al., 2008; 2010). The advances in cell and tissue culture techniques provide an excellent system for studying the physiological and molecular biological aspects of plants, including flowering. The present study is an advancement over the earlier protocol, because (1) It describes hormonal regulation, callus induction, regeneration, indirect organogenesis and clonal flower production obtained from young inflorescences, peduncles and petals of *in vivo* grown plants; (2) Three types of flower explants induced calluses under different parameters of sucrose and adenine; (3) Flower maturation and the subsequent transplantation of the plantlets to natural environmental conditions.

Table 2. Effect of MS medium supplemented with NAA (1.0 mg L^{-1}), BA (1.0 mg L^{-1}), adenine and sucrose on vegetative and reproductive shoot formation *in vitro* from inflorescence and peduncle explants of *Begonia* x *hiemalis* Fotsch. cv. "Schwabenland red".

Adenine (mg L^{-1})	Sucrose (mg L^{-1})	Vegetative green shoot / explants (± SE)		Vegetative red shoot / explants (± SE)	
		Inflorescence	Peduncle	Inflorescence	Peduncle
10	-	31.25 ± 1.04[b]	24.18 ± 4.14[b]	2.20 ± 0.85[b]	1.35 ± 1.50[b]
20		35.50 ± 1.09[a]	20.76 ± 7.60[b]	3.65 ± 0.82[a]	1.70 ± 1.25[a]
40		42.00 ± 6.16[a]	40.25 ± 9.46[a]	4.20 ± 1.32[a]	2.43 ± 0.84[a]
60		28.00 ± 5.13[b]	25.28 ± 6.45[b]	3.02 ± 0.95[a]	3.02 ± 0.95[a]
80		10.25 ± 3.79[b]	12.35 ± 3.79[c]	2.65 ± 1.42[b]	NR
100		5.21 ± 0.25[c]	4.28 ± 1.20[c]	2.05 ± 1.35[c]	NR
-	0	NR	NR	NR	NR
	1	11.50 ± 6.09[c]	26.00 ± 5.03[c]	NR	NR
	2	31.00 ± 6.94[b]	30.50 ± 8.87[bc]	8.13 ± 2.35[c]	4.32 ± 1.74[a]
	3	48.50 ± 9.98[a]	40.00 ± 5.72[a]	13.50 ± 2.35[a]	11.75 ± 2.04[a]
	4	36.50 ± 11.25[a]	37.00 ± 3.18[c]	9.55 ± 2.22[b]	6.00 ± 0.84[b]
	5	31.75 ± 8.15[b]	25.28 ± 8.43[c]	7.25 ± 1.32[c]	NR

* Mean values with different letters within a column (subject to explant type) differ significantly at $p < 0.05$, by one way ANOVA and Duncan's multiple range test. NR: no response

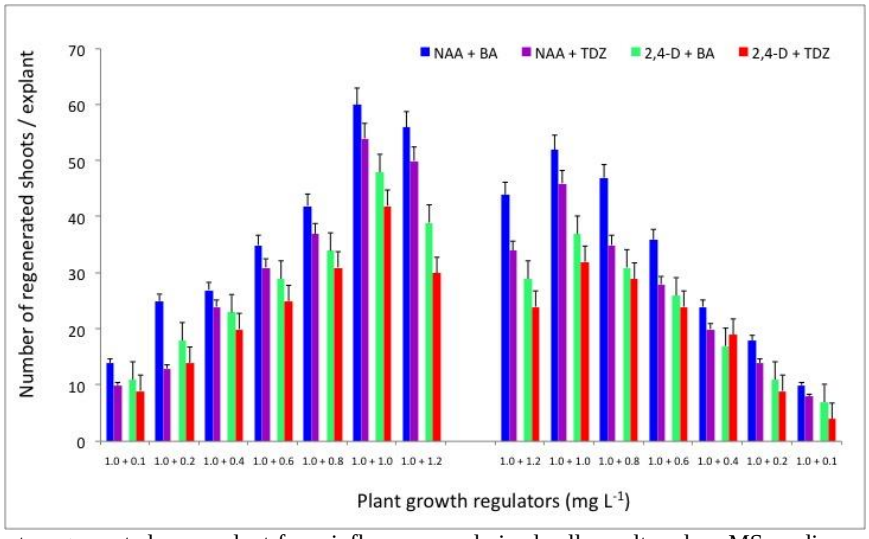

Fig 2. Number of shoots regenerated per explant from inflorescence-derived callus cultured on MS medium supplemented with 1.0 mg L^{-1} NAA and different concentrations (0.1 – 1.2 mg L^{-1}) of BA and TDZ.

Results and Discussion

The Explants role on callus induction

The present study mainly focuses on the effect of media components on callus formation and on *in vitro* flower induction process in *Begonia* x *hiemalis* Fotsch. *In vitro* callus formation failed to occur without addition of plant growth regulators to the MS medium. The callus performance of the three explant types, i.e. flower inflorescences, peduncles and petals were determined from *in vitro* cultures supplemented with different plant growth regulators fortified with different concentrations of sucrose and adenine. It was observed that petal explants did not show any callus formation and became necrotic (Table 1). A trial of auxins alone and combinations of auxins with cytokinins showed callus induction. Among the auxin concentrations used, optimum production of callus was obtained when 1.0 mg L^{-1} NAA was used (Fig 1). NAA was also better than 2,4-D and IAA in callus formation from both inflorescence and peduncle explants (Fig 1). For successful callus induction, factors such as type of explants, PGRs, culture media and culture conditions are very important (Yeoman and Yeoman, 1996; Ali et al., 2009). In this study, inflorescence and peduncle explants showed green compact regenerated mature callus. These results are well authenticated by previous reports depicting the suitability of NAA and 2,4-D for callus induction of *Gymnema sylvestre* (Ahmed et al., 2010; 2012a; 2012b). In addition, various combinations of BA and TDZ were also tested and analyzed for callus induction, which resulted in less shoot numbers and stunted shoot length compared to other PGR combinations (data not shown). During callus formation, the solid callus was continuously monitored and subcultured onto fresh media compositions at weekly intervals to prevent cell deaths and browning of media. BA was also reported to subsequently aid in formation of flowers, for example BA and KN were found to significantly increase the flower formation in regenerated shoots of *Rosa hybrida* cultivars (Kanchanpoom et al., 2010).

Shoot regeneration and the role of sucrose and adenine in flower bud formation

Within two weeks of culture, indirect organogenesis yielding the formation of green vegetative buds or microshoots was observed from callus derived from inflorescence and peduncle explants, under all treatment conditions (Table 1 and Table 2). On the other hand, no organogenesis (direct and indirect) was observed from petal cultures although the formation of callus

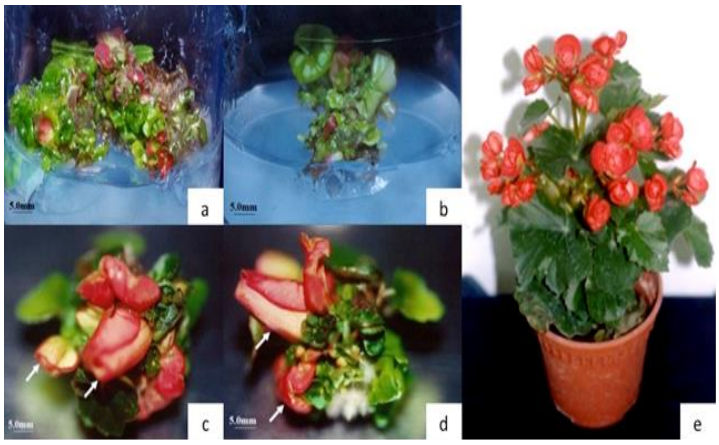

Fig 3. *In vitro* indirect organogenesis and flowering of *Begonia* ***x*** *hiemalis* Fotsch. cv. "Schwabenland red", from inflorescence explant cultured on MS medium supplemented with 1.0 mg L^{-1} NAA + 1.0 mg L^{-1} BA + 3% sucrose + 40 mg L^{-1} adenine. (a) Formation of vegetative and reproductive shoot (red calyx); (b) A close view of regenerated red calyx, taken at 7 weeks of culture; (c-d) *In vitro* flowering observed from inflorescence cultures; (e) *In vivo* grown *Begonia* (control). Arrows show the formation of red calyx and 'clustered' flower buds devoid of stamens and carpels.

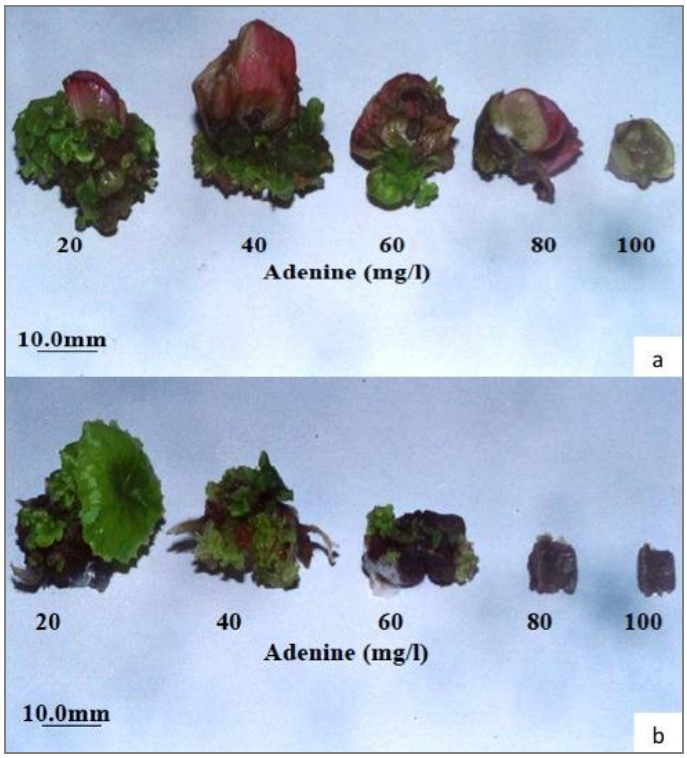

Fig 4. The effects of different concentrations of adenine on the induction of *in vitro* flowering from (a) inflorescence and (b) peduncle explants of *Begonia* x *hiemalis* Fotsch. cv. "Schwabenland red" after 8 weeks of culture. 40 mg L^{-1} adenine was clearly shown to be the most optimum concentration for *in vitro* flowering of *Begonia*.

was exhibited (Table 1). Both inflorescence and peduncle cultures showed that NAA (1.0 mg L^{-1}) combined with BA (1.0 mg L^{-1}) produced an optimum amount of vegetetive shoots (Table 1). NAA and BA combined also performed better than NAA and TDZ combined (Table 1 and Fig 2). Formation of callus and subsequent indicret shoot organogenesis are shown in Fig 3. Plant growth regulators have always been reported to influence many diverse development processess ranging from seed germination to root, shoot and flower formation (McCourt, 1999). Prior reseach has reported that NAA and BA combinations can successfully induce the regeneration of shoots in flowering plants (Ahmed et al., 2011a; 2011b). However, *in vitro* cultures of inflorescence and peduncle explants showed a significant reduction in multiple shoot formation when MS medium was supplemented with 1.0 mg L^{-1} NAA and 0.5 – 1.2 mg L^{-1} TDZ (Table 1). Inflorescence cultures showed formation of green callus which subsequently produced flower buds (Fig 3a) through indirect organogensis resulting from a series of continuous subcultures. When subcultured at 2-week intervals, *in vitro* floral buds derived from inflorescence explants started to bloom in treatments containing auxins and cytokinins (Fig 3b). However, auxins had also been reported as being either ineffective or inhibitory in the process of flower development (Rastogi and Sawhney, 1987). In the current study, the regenerated shoots developed into clonal plantlets, however the macro-morphological structure of the *in vitro* floral parts revealed the presence of only a red calyx (reproductive shoots) in a "clustered" manner but lacked stamens and carpels (Fig 3c and 3d). The incomplete flower did not mature when subcultured onto fresh MS medium with NAA and BA concentrations, even after a prolonged culture time. The present study also showed that additions of any cytokinin results in the stereotypic effect, such as formation of adventitious shoots with occasional *in vitro* bud formation. The flower buds developed earlier than the vegetative shoots in all observed materials and flowers developed to anthesis. *In vivo* grown *Begonia* (control) is shown in Fig 3e. Auxins are essential for flower induction and further development process, as reported in *Vigna radiata* (Avenido and Haulea, 1990), *Vigna mungo* (Ignacimuthu et al., 1997) and green pea (Franklin et al., 2000). *In vitro* plant regeneration from different flower explants of *Capparis spinosa* were achieved on the MS medium supplemented with BA, IBA and adenine treatment (Carra et al., 2012). Previous studies showed that exogenous cytokinins significantly influenced *in vitro* flowering of many plant species (Chang and Chang, 2003; Lin et al., 2003). A remarkable positive influence of BA on *in vitro* flowering and fruiting was observed in *Perilla frutescens*, an annual medicinal herb from the Lamiaceae family (Zhang et al., 2008). Several studies had shown the induction of *in vitro* flowering without the addition of flowering hormones, where flowering in these plants was found to be associated with endogenous auxin levels (Velayutham et al., 2006). Vila et al. (2004) produced *in vitro* flowers directly from the callus of *Melia azedarach*.

Additions of sucrose and adenine also helped to promote induction of *in vitro* flowering. Ringe and Nitsch (1968) reported that adenine played a positive role in inducing *in vitro* floral buds when used in low concentrations, but higher concentrations can inhibit bud formation. In this study, we observed that additions of 0 – 5% sucrose and 10 – 50 mg L^{-1} adenine to the culture media resulted in formation of green shoots which further developed into red shoots or flower buds (Table 1 and Fig 4). Within 8 weeks of the incubation period, profuse vegetative shoot formation (42.0%) and direct flower bud initiation (4.2%) were observed when inflorescences of

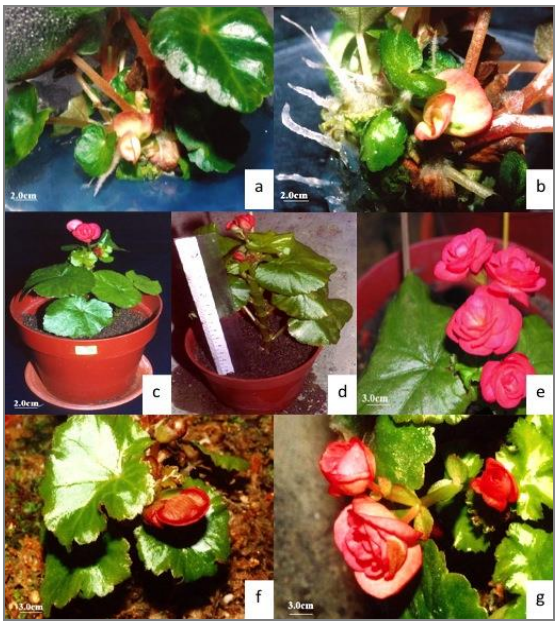

Fig 5. *In vitro* rooting and *ex vitro* flowering of *Begonia* x *hiemalis* Fotsch. cv. "Schwabenland red" clonal plants after acclimatization *ex vitro*. (a) Rooting was induced when *Begonia* plantlet was cultured on MS medium supplemented with 1.0 mg $L^{-1}GA_3$; (b) Further development of rooted *Begonia* plantlet, showing formation of *in vitro* flowers; (c-d) Successfully acclimatized *Begonia* plant (in the greenhouse), showing production of flowers; (e) *Ex vitro* plants maintained in the greenhouse showed development of inflorescences, peduncles and petals; (f) Successfully adapted *ex vitro* plants were transferred to from the greenhouse to natural environmental conditions; (g) Flower maturation showed by an *ex vitro* plant in the field.

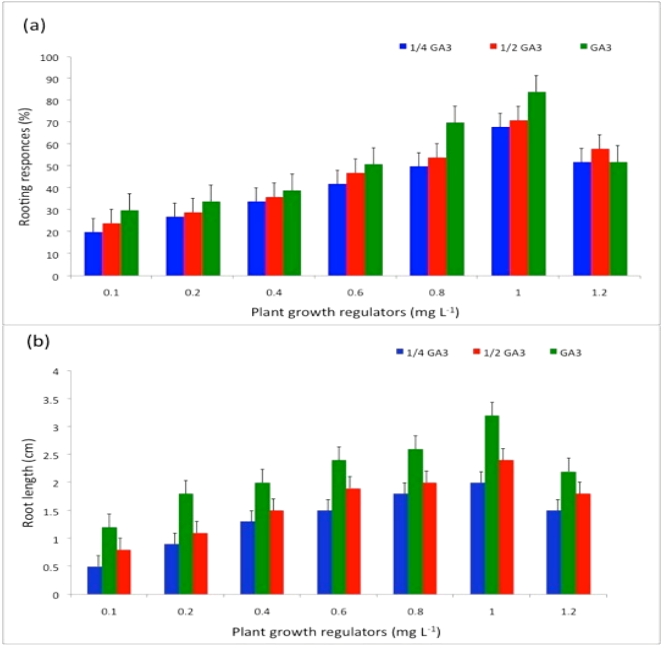

Fig 6. *In vitro* rooting of *Begonia* x *hiemalis* Fotsch. cv. "Schwabenland red" plantlets derived from inflorescence cultures. (a) Percentage of *Begonia* plantlets showing *in vitro* rooting; (b) Root length of *in vitro* grown *Begonia* roots (cm) after 45 days of culture.

the explants were cultured on the MS medium added with 40 mg L^{-1} adenine (Table 2). At the concentration of 10 mg L^{-1} adenine, the peduncle explants responded fairly well during the initiation of the vegetative shoots (24.18%) but only few resulted in the formation of red flower buds (1.35%) (Table 2). However, higher concentrations of adenine (ranging from 60 - 100 mgl^{-1}) gradually reduced the frequency of both vegetative and reproductive shoot formations in both the explant types (Table 2), in agreement with the findings by Ringe and Nitsch (1968). Samantaray and Maiti (2011) also reported on *in vitro* formation of flower buds in *Chlorophytum arundinaceum*, when the regenerated shoot buds showed further initiation of flower buds when these were subcultured onto ½ MS fortified with 3.0 mg L^{-1} BA, 0.1 mg L^{-1} NAA and 25 mg L^{-1} adenine sulfate at four monthly intervals. These suggests that adenine might be one of the key factors, without which, *in vitro* floral buds cannot be induced.

The effect of different concentrations of sucrose (0 - 5%) on the *in vitro* flowering process was also examined in the present study. Vegetetive production of green shoots were at a maximum when the inflorescence and peduncle explants were cultured on MS medium containing 3 mg L^{-1} sucrose, with shoot formation giving a percentage of 48.50 ± 9.98% and 40.00 ± 5.72%, respectively (Table 2). Similarly, vegetative production of red shoots was also at a maximum when both inflorescences and peduncle explants were cultured on the MS medium added with 3 mg L^{-1} sucrose, with shoot formation giving a percentage of 13.50 ± 2.35% and 11.75 ± 2.04%, respectively (Table 2). However, higher concentrations of sucrose gradually decreased the shoot formation frequency, but not at a significant level (Table 1). Moreover, it was observed that the lower concentration of sucrose (1.0 - 2.0%) was not sufficient to induce the *in vitro* flowering process, whereas higher concentration of sucrose (4.0 - 5.0%) caused the flowers to abscise. The effect of sucrose on shoot bearing floral buds were reported in a number of species such as *Fortunella hindsii* and *Fagopyrum esculentum* (Jumin and Nito, 1996; Kachonpadungkitti et al., 2001). However, BA, KN and sucrose were able to induce flowering in the *Begonia* system, perhaps due to cultivar differences. In the previous study, the highest percentage (45%) of *in vitro* flowering in rose (hybrid tea) was obtained on the MS medium supplemented with 3.0 mg L^{-1} BA, 0.1 mg L^{-1} NAA and 3.0 mg L^{-1} sucrose (Nguyen et al., 2006). Generally, sucrose is frequently used as a carbon source for *in vitro* flowering studies. Takimoto (1960) stated that plants that produced small amounts of chlorophyll could intiate formation of flowers independently of light conditions and that exogenous sucrose can substitute for the requirement of high intensity of light. Such data indicates that cultured plants receiving added agar might require a long dark incubation period for *in vitro* flower initiation. In the present investigation, the optimum frequency (13.50 ± 2.35%) of floral bud formation was obtained from inflorescence cultures when 3 mg L^{-1} sucrose were added to the culture medium (Table 2), similar with the findings by Zhang et al. (2008) in *Perilla frutescens*.

Rooting

After 8 weeks, all the regenerated plantlets with or without red floral shoots were further transferred onto fresh culture media containing GA_3, but devoid of any additional plant growth regulators. Within 2 weeks of transfer, initiation of rooting was observed (Fig 5a) and this then developed into individual plantlets with flower buds on maturation (Fig 5b). It was observed that all the regenerated adventitious shoots

(with or without red calyx) subsequently showed an increase in the percentage of rooting induction, after being transferred onto fresh, solid MS, at full, half and quarter strength medium supplemented with 1.0 mg L^{-1} GA_3 (Fig 6). In addition, the root length and root induction percentage was significantly reduced at half and quarter strength MS medium supplemented with 1.0 mg L^{-1} GA_3 (Fig 6).

Acclimatization and ex vitro flowering

Begonia plantlets were acclimatized and transferred *ex vitro* onto several growth substrates, however 2:1:1 ratio of peat : soil : sand was found to yield optimum results, with a 87% plantlet survival rate (data not shown). Fig 5c - 5e showed successfully acclimatized *Begonia* plants and generation of floral buds from red calyx (produced *in vitro*) that further developed into small red flowers with complete fertility. The greenhouse plantlets were maintained for 10 weeks, where the plantlets were observed to grow up to 15 cm with flower initiation (Fig 5d). Results showed that all regenerated potted plants could produce inflorescences from shoot tips within 3-4 months after undergoing the acclimatization process (Fig 5e). Almost 100% of the regenerated plantlets were able to produce true-to-type flowers, homogeneously without showing any detectable variant phenotypes (Fig 5f). Moreover, the abnormal floral parts that were developed under *in vitro* culture conditions were able to further develop normally, showing a similar morphology with the *in vivo* types, although these were phenotypically smaller (Fig 5g). All regenerated plantlets were clonal and no genetic variation was observed in this study. To complete the cycle, i.e. from *in vitro* regeneration to flowering of the acclimatized clonal plants, 6-7 months were needed, which is less than the required time taken by conventional propagation methods. The production of large numbers of genetically homogenous plants was very difficult when *Begonia* plants were propagated conventionally, due to the rapid occurrence of diseases coupled with the effect of day length as well as night temperatures on the induction of flowering (Rout et al., 2006). These studies, therefore provide an alternative way for producing early flowering without undergoing the full growth cycle of *Begonia*. However, further studies are necessary to discover the role of key factors to induce fertility on *in vitro* flowers. In this context, a possible strategy could be the over expression of some regulatory genes, such as *LEAFY (LFY)* or *APETALA 1 (AP 1)* which are also involved in the reproductive growth in flowering plant species (Shulga et al., 2009). These studies can be further extended to investigations on the molecular basis of plant flowering and the physiological factors relating to the transition from the vegetative to the reproductive state in *Begonia*.

Materials and methods

Plant collection and sterilization of explants

Three-month-old stock plants of *Begonia* x *hiemalis* were purchased from a local nursery in Sungai Buloh, Malaysia. The healthy plants were grown in earthen pots, maintained at the Institute of Biological Sciences, University of Malaya for the procurement of materials for the current research. The plant reproductive organs consisting of three types of explants such as inflorescences, flower peduncles and petals were collected from intact stock plants at the time of flowering. The inflorescences (basal part) were harvested 4 - 5 days prior to the opening of the first flower. All the explants were then washed thoroughly under running tap water for 3 to 5 times, washed with 2% (v/v) Teepol (Reckitt Benckiser, Malaysia) for 2 min; then soaked in 50% sodium hypochlorite (v/v) for 1 min followed by another wash with 70% ethanol (v/v) for 1 min. Prior to inoculation, explants were washed three times with sterile distilled water.

Preparation of explants and culture media

The surface sterilized explants were cut into segments (approx. 5 - 10 mm in length for flower peduncles and inflorescences and about 5 x 5 mm^2 pieces for petals). The explants were cultured on the MS (Murashige and Skoog, 1962) solid medium supplemented with or without (control) plant growth regulators such as 0.1 - 1.2 mg L^{-1} NAA (naphthalene acetic acid), 0.1 - 1.2 mg L^{-1} 2,4-D (2,4-dicholorophenoxy acetic acid), 0.1 - 1.2 mg L^{-1} IAA (Indole acetic acid), 0.1 - 1.2 mg L^{-1} BA (benzyl adenine), 0.1 - 1.2 mg L^{-1} TDZ (Thidiazuron), 0.1 - 1.2 mg L^{-1} GA_3 (Gibberellic acid), 20 - 50 mg L^{-1} adenine (Sigma-Aldrich, USA) and 1 - 5% sucrose (Duchefa Biochemie, Netherlands). During the preparation of the MS media, pH was adjusted to 5.8 (1N NaOH / HCl) and agar (0.8% w/v; Technical Oxoid Ltd, England) was added, prior to autoclaving at 121ºC (103 kpa) for 20 min. The cultures were maintained at 25 ± 2 ºC, 16/8 h (light/dark) of photoperiod at illumination of 1000 lux, with 90 – 100% relative humidity. The responses of the media on callus induction, regeneration and flower bud formation was studied in young inflorescences, peduncles and petals of *Begonia* x *hiemalis*.

Induction of flower bud formation and rooting

To achieve indirect organogenesis, peduncle-, inflorescence-, and petal-derived calluses were placed in the respective culture tubes containing the MS medium supplemented with different concentrations of BA (0.1 - 1.2 mg L^{-1}) and NAA (0.1 - 1.2 mg L^{-1}). During the experiment, to avoid cell deaths, the regenerated calluses were sub-cultured on a weekly basis until flower bud formation and maturation stage was reached. The *in vitro*-derived flowers were observed for 8 weeks of culture and the clonal plants were monitored for at least one full flowering cycle to identify true-to-type plants. After 8 weeks of culture, all types of clonal plantlets with or without red shoots (reproductive) were transferred onto the solid MS medium supplemented with 0.1 - 1.2 mg L^{-1} GA_3 and maintained for several weeks to induce a sufficient formation of roots. Cultures that exhibited successful callus formation, formation of shoots and flower buds and induction of rooting were monitored and data (percentage of callus, shoot and root formation, number of shoots and flower buds as well as root length) were subsequently recorded and analyzed.

Acclimatization

Rooted plantlets were removed from the culture tubes and thoroughly rinsed to get rid of any adhering agar. All growing plantlets were transferred from aseptic conditions to plastic pots (25 x 25 cm^2), containing 2:1:1 ratio of peat : garden soil : sand and maintained at 25 ± 2 ºC, 16 h day length (30 - 50 m EM^{-2} S^{-1}) and at 75 - 80% relative humidity. Subsequently, the grown plants were transplanted to the greenhouse containing natural soil, kept under shade for 2 weeks and finally moved to the botanical garden at Institute of Biological Sciences, University of Malaya.

Statistical analysis

Randomized complete block design (RCBD) with 30 replicates was used in the assessment of the results to improve accuracy and minimize error. All data and variables were statistically analyzed using the SPSS statistical package. Values were presented as mean ± SE. One-way ANOVA and multiple ranges were carried out on all data using Duncan's multiple range test (DMRT) at $p < 0.05$.

Conclusion

A quick method of reproductive shoot regeneration *in vitro* and early flowering from inflorescence explants of *Begonia* x *hiemalis* Fotsch. cv. "Schwabenland red" was established. Formation of reproductive shoot or flower bud (red calyx) was successfully obtained from the *in vitro* cultures of immature inflorescences after only 8 weeks of culture. The reproductive red calyx however, lacked stamens and carpels, and as a result, the immature flowers failed to develop into mature flowers when these were sub-cultured onto similar PGRs-fortified fresh MS medium and on MS supplemented with GA_3. Clonal *Begonia* plantlets sub-cultured onto the MS medium containing GA_3 showed successful formation of roots and were able to mature and further develop when acclimatized on *ex vitro*. All *ex vitro Begonia* plants were capable of producing true-to-type flowers. Similarly, the acclimatized plants with an *in vitro* grown under-developed reproductive shoots (red calyx) also further developed and matured to small, complete true-to-type flowers.

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