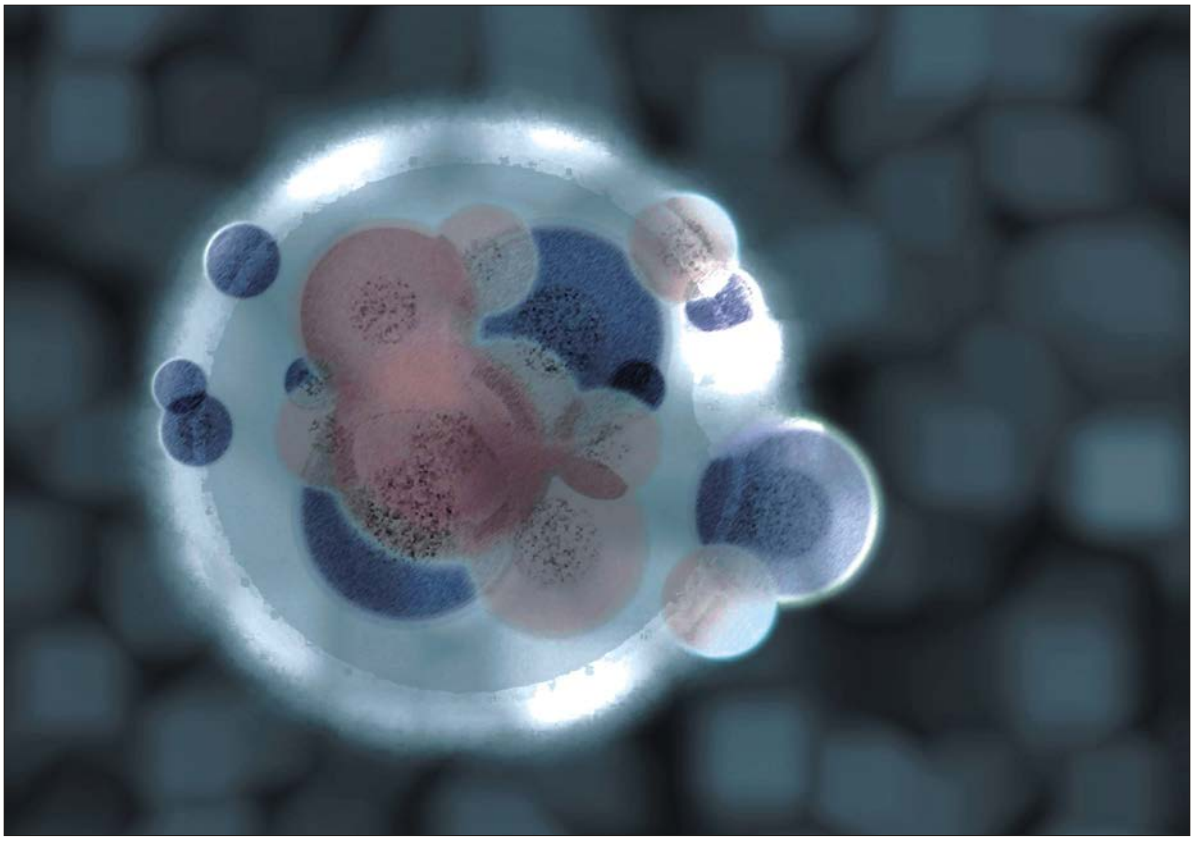

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Induction of Somatic Embryogenesis and Plant Regeneration in *Begonia* × *hiemalis* Fotsch. *in vitro*

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Abstract: Direct somatic embryogenesis induction of *Begonia* × *hiemalis* Fotsch. (Elatior Begonia) was initiated from two different explants i.e., leaves and petioles. Both explants were cultured on MS medium supplemented with different concentrations of Benzylaminopurine (BAP) and 2,4-Dichlorophenoxyacetic acid (2,4-D). The results showed that combinations of 0.5-1.0 mg L^{-1} BAP and 0.1 mg L^{-1} 2,4-D produced direct somatic embryogenesis from leaf and petiole explants. Different concentrations of casein hydrolysate were also tested to optimize somatic embryo induction. The results showed that 100 mg L^{-1} casein hydrolysate could produce 53.08% nodular callus and 24.16% green embryogenic callus, whereas 500 mg L^{-1} casein hydrolysate produced 30.83% nodular callus and 23.75% green embryogenic callus. The embryogenic callus were then transferred to MS medium supplemented with 0.5 mg L^{-1} Gibberelic Acid (GA_3) with 0.2 g L^{-1} activated charcoal for further embryogenesis development and further regeneration.

Key words: Direct somatic embryogenesis, casein hydrolysate, activated charcoal, *Begonia* × *hiemalis* Fotsch.

INTRODUCTION

Somatic embryogenesis is the process by which somatic cells differentiate into somatic embryos (Arnold *et al*., 2002) through characteristic embryological stages without fusion of gametes (Schumann *et al*., 1995). Somatic embryogenesis is one of the most important methods in plant propagation. Embryogenesis capacity is influenced by cultural conditions, genotype and their interaction (Petitprez *et al*., 2005). For the initiation of a tissue, capable of somatic embryogenesis, a basic requirement is the presence of an auxin (Gairi and Rashid, 2004). All plantlets obtained through somatic embryogenesis did not differ phenotypically from the parental clones (Stefaniak, 1994).

Begonias are normally grown as ornamental plants especially as decorative houseplants and for landscaping. Begonias are unique for their sheer beauty and variety of leaves. It is estimated that there are about 10 000 Begonias hybrids and cultivars worldwide. Other than great horticultural value, Begonias also has medicinal values. *Begonia* × *hiemalis* Fotsch. (Elatior Begonia) is a temperate plant, which is commercially used as flower potting plant and does not produce seeds.

Although *in vitro* regeneration systems have been established for Begonia, *Begonia* × *hiemalis* Fotsch. (Appelgren, 1985; Cassells and Morrish, 1985; Pierik and Tetteroo, 1987), somatic embryogenesis induction has not been defined. Direct somatic embryogenesis in Begonia was induced using leaf and petiole explants *in vitro*. The aim of this study was to identify the optimum media for direct somatic embryo induction of Begonia *in vitro* from two different types of explants. Apart from that, the effects of casein hydrolysate and dark treatment on somatic embryogenesis were also investigated. The development and germination of *in vitro* plantlets derived from somatic embryos were also discussed.

MATERIALS AND METHODS

Intact stock plants of *Begonia* × *hiemalis* Fotsch. var. *Schwabenland Red* obtained from local nursery were grown in the culture room under 16 h photoperiod at 25±1°C. Standard tissue culture methods were used. The healthy leaf explants were collected from the stock plants purchased from nursery, surface sterilized and cultured onto regeneration medium MS (Murashige and Skoog, 1962) medium supplemented with 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} NAA) to produce *in vitro* plantlets. The plantlets were maintained at 25±1°C and further subcultured every 6 weeks.

Two different explants including young leaves and petioles were selected from the *in vitro* plantlets. The leaf explants were cut approximately 0.5×0.5 cm whereas the

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petiole explants were cut into 0.5 cm long and then they were cultured onto MS medium supplemented with different concentrations of BAP (0.1, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}) and 2,4-D (0.1, 0.5, 1.0, 1.5 and 2.0 mg L^{-1}) for embryogenic callus induction.

The optimum medium for embryogenic callus induction was used to study the effect of different concentrations of casein hydrolysate on somatic embryo induction. The percentage of callus was recorded after 8 weeks in culture. Physical factors such as light and dark treatments were employed to develop different stages of somatic embryos *in vitro*. Leaf and petiole explants were incubated in the dark for 8 weeks. To promote regeneration, embryogenic callus were transferred to either MS basal medium or hormone free or supplemented with 0.5 mg L^{-1} gibberelic acid (GA_3) and 0.2% activated charcoal. After somatic embryos had developed to cotyledonary stage, the clusters of somatic embryos were transferred to maturation medium MS containing 0.5 mg L^{-1} GA_3 and charcoal. After 8 weeks, the plantlets were acclimatized in the greenhouse for hardening process.

The experiment was conducted for 20 weeks before acclimatization take place and the cultures were incubated under 16 h photoperiod at 25±1°C. All treatments consisted of 12 replicates and each replicate contained two explants. For comparison of response of the two explants (leaf and petiole) of *B.* × *hiemalis* Fotsch. var. *Schwabenland Red* to different combinations of BAP and 2, 4-D, different concentration of casein hydrolysate and the influence of light and dark treatment, statistical analysis were done. The data was subjected to ANOVA test and each treatment mean was compared by critical difference at 0.05% level of significance.

RESULTS AND DISCUSSION

Based on preliminary studies it was found that 2, 4-D induced only non-embryogenic callus and did not develop into embryo. From the present work, the results showed that combinations of BAP and 2, 4-D was found to be the best for somatic embryogenesis induction in Begonia. Leaf and petiole explants produced callus *in vitro* on MS media supplemented with combinations of BAP and 2, 4-D. The colour of the callus formed was more dependent on different concentrations of BAP and 2, 4-D than on the different types of explants used. Most of the callus was green, yellowish, compact and nodular in structure. The explants enlarged and callus tissues were initiated from the cut end of the petiole and leaf explants. The initiation of callus started 1-2 weeks after inoculation and 4-6 weeks after culture establishment, callus subsequently covered the entire surfaces of the explants. The cultures were maintained at 25±1°C with 16 h light and 8 h dark and were subcultured every 6 weeks.

The percentage of callus obtained from leaf and petiole explants were determined. The results revealed that different types of explants produced different amount of callus. The leaf explants produced significantly more callus than petiole explants. Mean percentage of callus was not significantly different when different concentrations of BAP and 2, 4-D were used (Table 1).

Table 1: Mean percentage of callus obtained from leaf and petiole explants of *Begonia* × *hiemalis* Fotsch. with different concentrations of BAP and 2,4-D

Combination of BAP and 2,4-D (mg L^{-1})		Mean percentage of callus (%)			
BAP	2,4-D	Leaf explant	Callus color	Petiole explant	Callus color
0.1	0.1	55.83±3.12^{d}	Yellowish, green	44.16±1.49^{b}	Yellowish, green
0.5		57.50±3.05^{d}		25.00±1.95^{d}	
1.0		77.50±2.50^{b}		25.00±1.51^{d}	
1.5		28.33±1.12^{e}		15.00±1.51^{e}	
2.0		23.33±1.42^{e}		7.92±0.74^{f}	
0.1	0.5	80.00±2.13^{b}	Yellowish, green	27.50±3.29^{d}	Yellowish
0.5		91.25±2.23^{a}		61.67±6.49^{d}	
1.0		80.00±0.00^{b}		38.33±6.13^{b}	
1.5		70.00±2.13^{c}		33.33±3.96^{c}	
2.0		91.25±2.62^{a}		13.33±1.42^{e}	
0.1	1.0	87.50±4.46^{a}	Yellowish	47.92±6.26^{b}	Yellowish
0.5		87.50±4.27^{a}		35.83±5.83^{c}	
1.0		65.00±7.33^{c}		45.00±3.37^{b}	
1.5		52.50±3.51^{d}		25.00±5.11^{d}	
2.0		80.00±5.77^{b}		24.58±4.32^{d}	
0.1	1.5	80.00±4.44^{b}	Yellowish	15.83±1.93^{e}	Yellowish
0.5		70.00±4.61^{c}		12.50±1.69^{e}	
1.0		80.00±4.92^{b}		7.92±1.30^{f}	
1.5		55.00±4.69^{d}		8.33±2.33^{f}	
2.0		79.16±5.70^{b}		5.42±0.42^{f}	
0.1	2.0	85.83±2.28^{a}	Yellowish	7.08±1.30^{f}	Yellowish
0.5		48.33±7.47^{d}		7.50±1.69^{f}	
1.0		69.16±6.33^{c}		5.83±0.56^{f}	
1.5		83.75±6.72^{b}		37.50±2.50^{f}	
2.0		71.66±5.48^{c}		7.50±1.69^{f}	

*Values followed by the same letter(s) in the columns are not significantly different at $p<0.05$

Fig. 1: A-E: Somatic embryos produced from leaf explants of Begonia x *hiemalis* Fotsch. var. *Schwabenland Red* cultured on MS medium supplemented with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} 2,4- D, 500 mg L^{-1} casein hydrolysate and incubated in the dark for 8 weeks. (A) Globular somatic embryos developed from leaf explants after 5-6 weeks on induction medium, (B) Different stages of somatic embryos after 2-3 weeks on development medium (G-globular, H-heart-shape, T-torpedo and C-cotyledonary stage), (C) SEM of different stages of somatic embryos, (D) Cotyledonary stage of somatic embryos cultured into maturation medium, (E) *In vitro* plantlets derived from somatic embryos and (F) Normal plant derived from somatic embryogenesis process was successfully acclimatized in the greenhouse

Table 2: Mean percentage of embryogenic callus from leaf and petiole explants of *Begonia* × *hiemalis* Fotsch. var. *Schwabenland Red* cultured on MS medium supplemented with 1.0 mg L^{-1} BAP and 0.1 mg L^{-1} 2,4-D with different concentrations of casein hydrolysate. Data were obtained after 8 weeks of inoculation

Casein hydrolysate (mg L^{-1})	Callus induction % (±SE)		Embryogenic callus % (±SE)	
	Leaf	Petiole	Leaf	Petiole
Control	45.00±2.30^{a}	49.17±3.58^{a}	10.44±1.79^{b}	25.00±2.61^{a}
100	53.08±4.87^{a}	40.00±0.00^{a}	24.16±3.79^{a}	20.00±0.00^{a}
200	40.00±4.60^{a}	23.33±1.42^{b}	8.33±3.66^{c}	10.83±0.56^{b}
300	28.33±2.71^{b}	25.83±1.49^{b}	1.67±1.12^{c}	11.67±0.71^{b}
400	29.16±3.53^{b}	29.17±0.83^{b}	16.67±3.96^{b}	11.25±0.65^{b}
500	30.83±2.88^{b}	25.00±1.50^{b}	23.75±3.20^{a}	12.27±0.79^{b}

*Values followed by the same superscript letter(s) in the columns are not significantly different at $p<0.05$

Table 3: The effect of different light treatment on the production of embryogenic callus from leaf and petiole explants of *Begonia* × *hiemalis* Fotsch. var. *Schwabenland Red* cultured on MS medium supplemented with 1.0 mg L^{-1} BAP, 0.1 mg L^{-1} 2,4-D and 0.5 g L^{-1} casein hydrolysate. Data were obtained after 8 weeks of inoculation

Light treatment	Observations		Embryogenic callus % (±SE)	
	Leaf	Petiole	Leaf	Petiole
16 h light 8 h dark	Green nodular callus	Green nodular callus	58.67±4.68^{a}	36.00±1.31^{a}
24 h dark	White nodular callus	White nodular callus	40.44±4.68^{a}	40.67±5.30^{a}

Values followed by the same superscript letter(s) in the columns are not significantly different at $p<0.05$

The best induction of direct somatic embryogenesis of *Begonia* × *hiemalis* Fotsch.var. *Schwabenland Red* was achieved on MS medium supplemented with 1.0 mg L^{-1} BAP, 0.1-0.5 mg L^{-1} 2,4-D, 3% sucrose and solidified with 0.2% phytagel. However, Castillo and Smith (1977) reported that 0.5 mg L^{-1} kinetin and 2% coconut water were effective in inducing direct somatic embryogenesis in *B.* × *gracilis* explants.

Addition of casein hydrolysate in the callus induction medium was found to be beneficial and different concentrations of casein hydrolysate were also identified to optimize somatic embryo induction. The mean percentage of callus formation was presented in Table 2. Several reports also have proved the use of casein hydrolysate as beneficial for the formation of somatic embryos *in vitro* (Augustine and D'Souza, 1997; Ling *et al*., 1983; Narayanaswamy, 1997).

The effects of different light treatment on the production of somatic embryogenesis were also investigated. The results showed that mean percentage of callus was not significantly different from different explants for different light treatment (Table 3). Dark incubation produced complete embryogenesis cycle compared with 16 h light 8 h dark incubation (Fig. 1A-D). Augustine and D'Souza (1997) also reported that callus incubation in the dark could give rise to a large number of immature embryos.

The embryogenic callus was successfully regenerated after being transferred to MS medium supplemented with 0.5 mg L^{-1} GA_3 and 0.2% activated charcoal. For development of somatic embryos into maturation, withdrawal of BAP and 2, 4-D from induction medium was necessary. The withdrawal of BAP and 2,4-D resulted in the growth of embryos into plantlets. In conclusion, the present research succeeded in inducing somatic embryos from leaf and petiole explants of Begonia and subsequently regeneration of embryogenic callus.

CONCLUSION

The formation of somatic embryo in *Begonia* × *hiemalis* Fotsch. var. *Schwabenland Red* was successfully induced in MS medium supplemented with 1.0 mg L^{-1} BAP, 0.1-0.5 mg L^{-1} 2,4-D, 500 mg L^{-1} casein hydrolysate, 3% sucrose, solidified with 0.2% phytagel and incubated in the dark. Subsequently, different stages of somatic embryo development were initiated to form globular, heart-shape, torpedo and cotyledonary stage before forming *in vitro* plantlets.

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