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The Creation of Protected Ecosystems as the Conservation-Friendly Way to Save Genus Begonia from Extinction

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Abbreviations:
N6 medium; PGR - plant growth regulators; 2,4-D -2,4-dichlorophenoxy acetic acid; IBA- Indole-3-butyric acid , N-phenyl-N′-1,2,3-thiadiazol-5ylurea (TDZ; thidiazuron), 6-benzylaminopurine (BAP).

Abstract

The unique protected ecosystems created by the Central Siberian Botanical Garden (CSBG) researchers on the basis of ecological – geographical principle allowed the establishment of exact soil and microclimate conditions, favorable to the vigorous growth of many terrestrial *Begonias*. *Begonias* are successfully introduced in the CSBG as an understorey component of the pluristratal tropical forest and well adapted to the shady, humid conditions. Maintenance of rich *Begoniaceae* collection was based on the concept of imaging the natural habitat of the *Begonias* to determine environmental factors such as illuminance, temperature, humidity and substrate preference. *In vitro* regeneration of 4 Begonia genotypes was carried out starting from female flower segments and peduncles as explants. These regenerants have a better capability to grow than traditionally obtained ones and their flowers developed to anthesis *in vitro*. The techniques open a new way for an efficient micropropagation protocol for Begonia species conservation *in vitro*.

1. Introduction

Begonias are delicate plants that require undisturbed habitats in forests or on limestone hills to survive [1]. Many representatives of *Begoniaceae* family, which contains no less than 1500 species, are likely to become extinct in the wild. According to the IUCN Red List of Threatened Species, Version 2013.2 [2], there are 51 vulnerable species from the family *Begoniaceae*, most of them are local endemic to Malaysia. *Begoniaceae* green house collection in CSBG started in 1989 and now it is acknowledged as the biggest in Russia and all over the world. It contains 250 taxa, belonging to 27 sections from 63, by Doorenbos classification [3]. In common horticultural practice many *Begonias* can be vegetatively propagated, but propagation rate is often not very high. The collection establishment and propagation were conducted both in vivo and *in vitro*. The aim of the work was to develop the techniques of *Begonias* micropropagation from male and female flowers, starting from 2 types of explants - peduncles and flower buds. There was also a need to determine environmental and social factors for better propagation and acclimatization of *Begonias* under experiment.

2. Materials and Methods

In our study, an *in vitro* regeneration was attempted using immature reproductive organs, which were not commonly used before, such as young inflorescences. *Begonias* are monoecious, they produce male and female flowers on the same

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inflorescence, generally composed of many male flowers and few terminal female flowers. To reveal the influence of exogenous plant growth regulators, cytokinins TDZ and BAP and auxins 2,4-D and IBA were added to the composition of N6 medium in the modeling of the *in vitro* propagation process in 3 species and 1 cultivar of Begonia.

Table 1: Growth regulators and media used for regeneration of 4 *Begonia* representatives

Media	Growth regulators	Concentration (mg/l)
V0	Control	Without growth regulators
V1	Indole-3-butyric acid	1.0 mg/l
V2	2,4-dichlorophenoxy acetic acid	1.0 mg/l
V3	Thidiazuron	1.0 mg/l
V4	6-benzylaminopurine	1.0 mg/l
V5	Indole-3-butyric acid + Thidiazuron	1.0 mg/l +1.0 mg/l
V6	Indole-3-butyric acid + 6-benzylaminopurine	1.0 mg/l +1.0 mg/l
V7	2,4-dichlorophenoxy acetic acid+ Thidiazuron	1.0 mg/l +1.0 mg/l
V8	2,4-dichlorophenoxy acetic acid+ 6-benzylaminopurine	1.0 mg/l +1.0 mg/l

Table 2: The responses of different explants of 4 Begonia representatives cultured on different variants of N6 initiation media

Object/ explants	Morphogenetic responses N6 initial media variants Number of regenerated shoots / explants (± SE)							
	V1	V2	V3	V4	V5	V6	V7	V8
B. masoniana var. maculate/	NR	Ca	Ca	1.2±0.1	6.5±0.4	5.7±0.3	Ca	Ca
a. peduncle	Ca	Ca	5.3±	6.7±0.4	12.3±0.7	21.0±1.2	Ca	Ca
b. female flower fragments			0.3					
B. rockii/	NR	NR	Ca	2.3±0.1	7.8±0.4	9.7±2.3	Ca	Ca
a. peduncle	Ca	Ca	7.0±	9.7±0.5	15.5±0.9	20.3±1.1	Ca	Ca
b. female flower fragments			0.4					
B. hybrida 'Gluare de Loren'/	NR	Ca	Ca	NR	Ca	12.3±0.8	Ca	Ca
a. peduncle	Ca	Ca	Ca	8.5±0.5	23.4±1.3	32.0 ±1.8	Ca	12.3±0.
b. female flower fragments								7
B. sutherlandii/	Ca	NR	Ca	2.3±0.2	Ca	10.7±0.6	NR	Ca
a. peduncle	Ca	NR	Ca	6.5±0.4	12.8±0.7	22.3±1.3*	Ca	8.7±0.5
b. female flower fragments								

Notes: NR - no response; Ca – callusogenesis; *- *in vitro* flowering; Values are means of 10 replicates ± SE (standard error of Mean)

Parts of inflorescence – peduncles, young closed flowers and open flowers (Fig.1) of 1-2 years old plants of Begonia *masoniana* var. *maculate* S.K. Chen et R.X. Zheng ex D. Y. Xia, *B. rockii* Irmsch., *B. sutherlandii* Hook. f., *B. hybrida 'Gluare de Loren'* were surface sterilized as follows: 1 second in ethanol 70% (v/v), 10-12 min in 0,1% HgCl2 with 0,15 ml/l Tween 80 (on a magnetic stirrer), then rinsed 4 times in sterile tap water. The mineral basic medium N6 [4] culture was supplemented with 100 mg/l myo-inositol, 30 mg/l sucrose and 7 g/l Difco-Bacto agar. The pH of all media were adjusted to 5.8 with 0.1 M NaOH before autoclaving at 121 °C for 20 min. Plant material was exposed to white fluorescent light, with low intensity of 500 lx and normal intensity (1000-2000 lx) and a photoperiod of 16 hours light/8 hours darkness. The

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temperature varied at 23 ± 1°C. In each variant 30 explants were cultured, 5 explants per glass recipient. Morphogenic responses of female flower explants of *Begonias* were observed at 20, 40 and 60 days of culture.

3. Results and Discussion

In our experiment, *in vitro* response varied greatly in various explants, depending upon their age and sex. Different genotypes and explants type indicated different responses to type and concentration of PGR added to the culture media. No regeneration was obtained on the medium V0 without PGR. Male flowers did not show any regeneration, equally with the fragments of open female flowers of *Begonias*.

Some authors showed that young flower buds were capable of producing callus, in contrast to the leaf callus which was very organogenic [5]. Nowadays *in vitro* technologies facilitate conservation and propagation of rare species [6]. In most cases researchers aimed to develop an efficient *Begonias* micropropagation system, starting from leaf or caulinar explants [7,8], only a few studies were attempted using immature reproductive organs, such as young inflorescences [9,10]. In our previous experiments, contamination inherent in the vascular system of leaf explants of the same Begonia species and slow organogenesis, were found to be recurrent problems.

From all experimental variants studied, callusogenesis occurred on the variants V1, V2, V7 and V8. The formation of callus was probably due to the action of accumulated auxins which stimulates cell proliferation, especially in the presence of cytokinin. The use of peduncles as explants and 2,4-D (at 1.0 mg/l) with combination of TDZ (1.0 mg/l) or alone often led to the callus proliferation.

Our study revealed that the regenerative capability of female flowers fragments was different on each type of culture media used in this experiment, 6-BAP and TDZ can stimulate induction and multiplication of shoots either alone or in combination with other growth regulators. The stimulative effect of 6-BAP upon the shoot bud yield was higher than cytokinin TDZ but had the slight effect on *Begonias* shoot regeneration.

Figure 1: Young closed female (upper row) flowers and male flowers (bottom row) of *B. sutherlandii* from immature inflorescence.

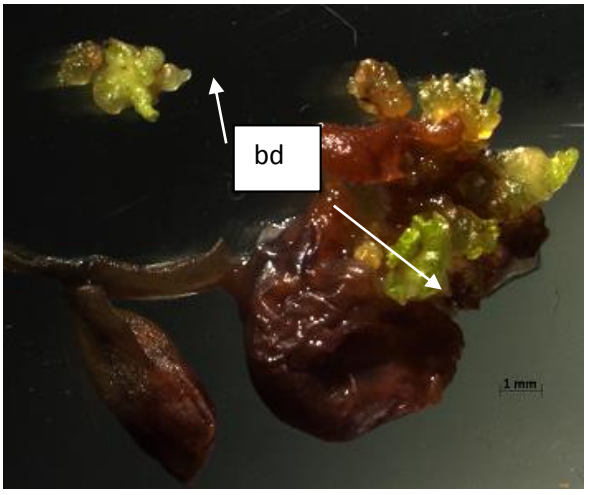

Figure 2: Image of *B. hybrida 'Gluare de Loren'* female flower explant (20 day of inoculation *in vitro*), on N6 medium culture supplemented with growth regulators TDZ + IBA (each 1.0 mg/l), where: bd – adventitious bud.

Our results are in concordance with other authors publications, which reported that without cytokinins – or in the presence of low cytokinin concentration – flowering of some species did not occur [11,12]. In our experiment, *in vitro* flowering has been observed only with several plantlets of *B. sutherlandii*, obtained from inflorescence cultures when 6-BAP + IBA (each in concentration 1.0 mg/l) was added to the culture medium (Table 2).

In this investigation, the highest shoot regeneration (32.0±6.6 shoots per explant) from immature female flowers of *B. hybrida 'Gluare de Loren'* was observed in V6 variant of N6 medium containing 1.0 mg/l 6-BAP and 1.0 mg/l IBA. The presence of growth regulators was stimulative for the morphogenetic capacity of female flowers of 4 *Begonias*, resulting in regeneration of both shoots and

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calluses. Rhizogenesis recorded the maximum values with the presence of IBA at 0.5 mg/l added to ½ MS medium.

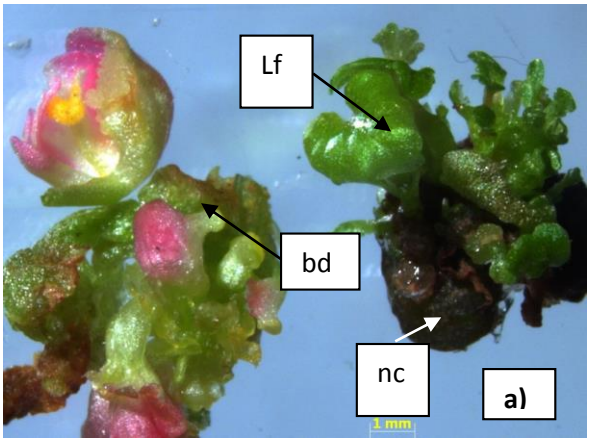

Figure 3: a) Image of *B. hybrida 'Gluare de Loren'* female flower explants (40 day of inoculation) on N6 medium culture supplemented with growth regulators TDZ + IBA (each 1.0 mg/l), where: Lf – new leaf; bd – bud; nc - necrosis.

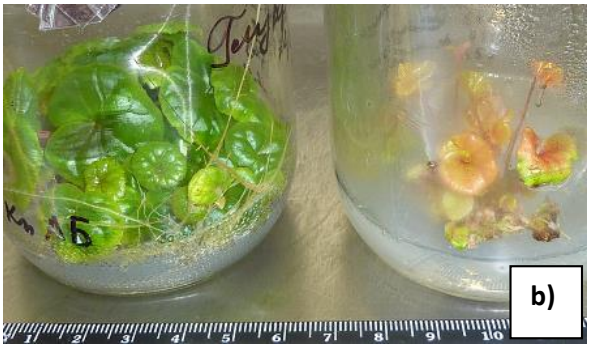

Figure 3: b) Shoot morphogenesis from B. hybrid 'Gluare de Loren' female flower explants (60 day of inoculation) on N6 medium supplemented with TDZ + IBA (each 0.5 mg/l)

Well-developed rooted shoots were removed from the culture vessels, washed gently under running tap water and planted in plastic cups containing sand and placed in net house under low illumination of 500 lx. Humidity (90 %) was maintained by sprinkling water at least 3 times throughout the day All cultures were visually examined periodically. Then plantlets were potted in mix substrate (6 soil: 2 sand: 1 peat dust) and kept in the greenhouse. After acclimatization for 1 month the plants were gradually exposed to the normal conditions (60-70% humidity and 1000 - 2000 lx). When transferred to the greenhouses assigned for excursions with the shady, humid conditions of the forest understorey, these *Begonias* produced morphologically normal flowers.

Figure 4: The male flower anthesis of micropropagated plant of *B. sutherlandii*

In this experiment we investigated the influence of IBA, 2,4-D, 6-BAP and TDZ upon the regenerative capability of 4 Begonia genotypes. *In vitro* regeneration of these *Begonias* was carried out starting from female flower segments and peduncles as the explants. Presence of auxin with cytokinin in regeneration medium increase regeneration efficiency. With increasing cell division, cytokinin causes plant growth and development especially when combined with auxin [5].

Our results suggested that the *in vitro* propagation of 4 Begonia genotypes, using addition of cytokinins like 6-BAP and TDZ was beneficial for the development of an efficient micropropagation system for obtaining Begonia plants with a better plant yield. These regenerants have a better capability to grow than traditionally obtained ones and their flowers developed to anthesis *in vitro* (Fig.4). The techniques open a new way for an efficient protocol for preserving and enlargement of Begonia species collection. Tissue-cultured *Begonias* were maintained in green houses conditions similar to their natural habitats where Begonia *masoniana* var. *maculate* , *B. rockii* and *B. hybrida 'Gluare de Loren'* – mesophytes – grow well in soil with leaf litter, while Begonia sutherlandii grows

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on rocks like a petrophyte. Many *Begonias* do not have the dormancy state and their development can be easily observed all year round. We have the unique opportunity to inform school children and students with different educational backgrounds about the features of the plants on the example of one genus – Begonia, to show them the different living forms, explain the influence of ecological and biochemical factors on the growth and development of living plant organism.

Figure 5: a) Acquiring skills of experimental work; b) Excursion: awakening the interest for studying plants directly in protected ecosystem; c) Hands-on training in advanced methods of *Begonias* propagation.

4. Conclusions

The techniques open a new way for an efficient protocol for preserving and enlargement of Begonia species collection.

We are working out the innovation project 'Begonia', which includes the investigations at different levels of the object organization: cell – organism – population and/or collection *in vitro* – ecosystem [10].

The unique protected ecosystems created by the CSBG researchers on the basis of ecological – geographical principle allowed us to introduce successfully more than 250 representatives of the family *Begoniaceae* as a component of the pluristratal tropical forest in green houses and used in the research as the model samples.

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