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Scientific Article

A novel approach for begonias micropropagation by inflorescence explants

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Abstract

Begonias grown in greenhouses are susceptible to devastating disease caused by pathogenic bacteria and fungi, decreasing the quality of propagated material. Plant tissue culture provides an alternative for rapid propagation of healthy *Begonia* material. The present study was undertaken to develop the protocol of micropropagation of three *Begonia* species and one hybrid from inflorescence explants. Male flower buds with part of pedicel restricted to 1 mm have been cultured *in vitro* on 6 variants of modified N6 media. Adventitious shoot organogenesis has been shown to occur from both pedicel and receptacle tissues under the action of any type of cytokinin applied, whereas BA and 2-iP triggered mainly the direct organogenesis, while TDZ proceeding morphogenic events through the stage of callus formation. For the culture establishment *in vitro* the most effective was the medium, supplemented with 1.5 µM 2-iP + 0.54 µM NAA with the addition of 40 mg L^{-1} adenine sulfate, contributed to the highest shoot regeneration from floral explants of all begonias studied. Histological analysis of adventitious buds pathways approved that their induction occurs under the treatment directly from the subepidermal cells. Morphological analysis performed after plantlets adaptation to the greenhouse conditions showed no morphological or bloom variations in the progeny, derived from the begonias inflorescence. The suggested technique considered as a practical step toward obtaining the uniform planting material for the propagation of economically valuable Begonia plants.
Keywords: begonias, floral explants, morphogenesis *in vitro*.

Resumo

Uma nova abordagem para micropropagação de begônias por explantes de botões florais

Begônias cultivadas em estufas são susceptíveis a doenças causadas por bactérias e fungos patogênicos, diminuindo a qualidade do material propagado. A cultura de tecidos vegetais oferece uma alternativa para a rápida propagação de material saudável de Begônia. O presente estudo foi realizado para desenvolver o protocolo de micropropagação de três espécies de Begônia e um híbrido a partir de explantes de inflorescências. Botões florais masculinos com parte do pedicelo restrita a 1 mm foram cultivados *in vitro* em 6 variantes de meio N6 modificado. Demonstrou-se que a organogênese do broto adventício ocorreu tanto nos tecidos do pedicelo quanto no receptáculo sob a ação de qualquer tipo de citocinina aplicada. O uso de BA e 2-iP induziram principalmente a organogênese direta, enquanto o uso de TDZ induziu a eventos morfogênicos através do estágio de formação de calo. Para o estabelecimento do cultivo *in vitro* o mais eficaz foi o meio de cultura, suplementado com 1,5 µM 2-iP + 0,54 µM NAA com adição de 40 mg L^{-1} de sulfato de adenina, contribuindo para a maior regeneração de brotos de explantes florais de todas as begônias estudadas. A análise histológica das rotas de botões adventícios demonstrou que a sua indução ocorre sob tratamento direto das células subepidérmicas. A análise morfológica realizada após a adaptação das plântulas às condições de casa de vegetação não revelou variações morfológicas ou de florescimento na progênie, derivada da inflorescência das begônias. A técnica sugerida é considerada uma etapa prática para a obtenção de material de plantio uniforme para a propagação de plantas de Begônia economicamente valorizadas.
Palavras-chave: begônias, explantes florais, morfogênese in vitro.

Introduction

Begonia L. is one of the most species-rich genera of angiosperms, which comprises, according to different authors evaluations, from 2000 (Li et al., 2022) up to 2500 species (Tian, 2020). The majority of begonias appear to be valuable indoor and outdoor ornamental plants. Modern-day scientific interest in the Begonia's connected with the conservation of endangered species and applying new breeding techniques, including the advanced tissue culture methods.

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In the Central Siberian Botanical Garden the wide begonias collection represented by more than 350 species and cultivars belonging to 27 out of 63 sections according to Doorenbos et al. (1998) have been grown for 25 years. For conducting comprehensive *Begonia* studies, where morphophysiological, biochemical and microbiological methods are involved for identification promising representatives of the genus that are active against a wide range of test microorganisms due to their extended profiles of various phenolic compounds (Karpova et al., 2021), efficient methods of begonias micropropagation are highly demanded.

Through the methods of conventional propagation, starting mainly from stem and leaf cuttings, different pathogens inherent in the vegetative parts, have a significant negative impact on decorative value of these ornamental plants. Disease-free *Begonia* plants can be obtained with the use of cell culture techniques (Toma and Ahmed, 2019; Mehbub et al., 2022). Based on the literature data, the most common types of explants used in micropropagation of *Begonia* species are leaf and stem segments (Beiyi et al., 2021; Hosseinabadi et al., 2022). But a severe contamination in explants of vegetative origin (Correia et al., 2018), as well as a different gene-specific reaction to growth regulators application in the *Begonia in vitro* culture (Ismaini et al., 2021) create certain difficulties in developing methods for clonal micropropagation of *Begonia* representatives. The main problem with the establishment of *Begonia* flower explants *in vitro* is the lack of a strategy that provides regeneration of plantlets in the direct way. Adventitious shoots were yielding from immature floral buds predominantly via indirect organogenesis as exhibited in the work of Awal et al. (2013). Meanwhile, direct shoot organogenesis avoids the callus induced variation among tissue culture derived progeny, which has been described in detail by Bouman and De Klerk (2001). These aberrations were revealed by comparing the begonia plants regenerated under different outputs of exerted growth regulators and characterized either the epigenetic modifications (reversible changes in the original phenotype) or heritable genetic variations. Thus, direct shoot morphogenesis is preferred for *in vitro* propagation of both begonias in particular (Aswathy and Murugan, 2019) and all ornamental plants in general (Mehbub et al., 2022). Here, we tested such explants as begonia flower buds, which were rarely used before, including pedicel and receptacle tissues not cut from the perianth, and the hormonal needs of these explants, under which they differentiate shoot and root promeristem.

The aim of this work was to develop high efficiency micropropagation protocol of four Begonias from floral explants. For the establishment in *in vitro* culture of the inflorescences explants it should be proved that this micropropagation technique omitting the callus stage is suitable for mass propagation of true-to-type plants by histological and morphological analyses of obtained *Begonia* plantlets.

Material and Methods

Plant material source

The objects of the current research were three *Begonia* species from the scientific collection of the Botanical Garden and one hybrid taxon. The names of the species of the genus *Begonia* are updated on the website tropicos.org (TROPICOS, 2023).

B. grandis Dryand. (Sect. *Diploclinium)* – is the most cold-resistant representative of the genus Begonia, occurs on significant territory of China. This begonia is native to the subtropical biome and the warm temperate zone, where it hibernates when the temperature drops to -10°C (Li, 2014).

B. variegata Y.M. Shui & W.H. Chen (Sect *Coelocentrum*) – is a rhizomatous geophyte that grows in the subtropical biome, mainly in Vietnam (Shui and Chen, 2005).

B. sutherlandii Hook. f. (Sect. *Augustia*) – is the species of southern African origin, which occurs from Tanzania to South Africa. It is a tuberous geophyte, epiphytic or terrestrial. It grows mainly in the subtropical biome with a seasonal climate. By the breeders it is considered as the useful parental source in hybridization for creation orange/yellow colors of flower (Tebbitt, 2005).

B. 'Gluare de Loren**'** is the hybrid with abundant and persistent flowering in winter. It is resulted from crossing *Begonia socotrana* J.D. Hooker with *Begonia dregei* Otto and A. Dietrich., which grow in a seasonal climate in a subtropical biome.

In all of the listed taxa of begonia, reproduction is provided both by seed and vegetatively. However, *B. grandis* and *B. sutherlandii* do not propagate well from leaf cuttings. *B. variegata* and *B.* 'Gluare de Loren' give offspring through leafy cuttings, but this process is very slow.

Explants preparation

Begonia plants were grown to flowering in glasshouses. The inflorescences fragments were harvested 5-7 days prior to the opening of the first flower. There were closed immature male flower buds with receptacle and a small part of pedicel (1 mm) (Figure 1).

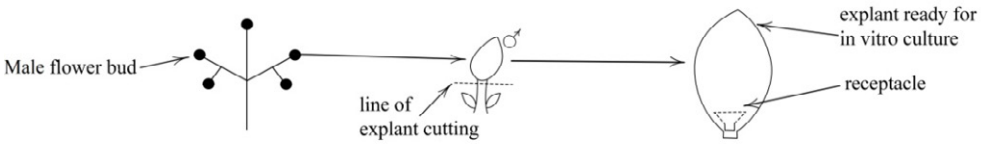

Figure 1. Scheme of explant excision from the inflorescence of begonias.

Male flower buds, isolated from inflorescence according to the following scheme (Figure 1) were sterilized with 70% C_2H_5OH (1 min) and 0.1% $HgCl_2$ solution (10 min).

***In vitro* culture conditions**

For each of the four different representatives of begonias, 6 variants of the modified N6 (mN6) medium were tested, the modification consisted in addition of 40 mg L^{-1} adenine sulfate to the original receipt of N6 medium (Chu et al., 1975). Variants of mN6 $N6_{1\text{-}5}$ were supplemented with plant growth regulators (PGRs): 0.54 µM α-Naphthaleneacetic acid (NAA), 4.4 µM 6-benzyladenine (BA), 1.5 µM 2-Isopentenyladenin (2-iP) or 1.0 µM Thidiazuron (TDZ), used in combination or separately. PGR-free medium $N6_0$ was used as the control (Table 1). The time of one passage was 4 weeks.

Table 1. The list of the different variants of modified N6 medium applied for regeneration of 4 *Begonia* taxa and content of growth regulators.

Medium	PGRs	Concentration of PGRs (µM)
$N6_0$	Without PGRs	0.0
$N6_1$	TDZ	1.0
$N6_2$	BA	4.4
$N6_3$	TDZ + NAA	1.0 + 0.54
$N6_4$	BA + NAA	4.4 + 0.54
$N6_5$	2-iP + NAA	1.5 + 0.54

Initial cultures were kept in the darkness for 1 month, and then transferred to light conditions at the temperature of 24 ± 2 °C. The cultivation of explants was carried out at the same temperature under artificial illumination of 40 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps and 16-hour photoperiod. From the clusters with numerous shoots 2-5 shoots were excised and placed on half-strength (macro- and micronutrients) MS medium (½MS), containing 30 mg L^{-1} adenine sulfate for elongation. Then the developed shoots were transferred to PGR-free ½MS for rooting.

Hardening and *ex vitro* adaptation

Plantlets with 5-6 leaves and roots, which height was not less than 3-4 cm, were transferred to containers with sterile sand and put in a chamber under a 16/8 h light/dark photoperiod, light intensity 40 µmol $m^{-2}s^{-1}$ and temperature 23 ± 2 °C, where they were kept partially shadowed under a film during the first 2 weeks under conditions of high humidity. After this period the plantlets were transplanted into plastic pots filled with mix substrate (soil mixture consisted of the rotted leaf litter and river sand in a ratio of 1:1), which was found universal for begonias with the different ecological requirements (Karpova et al., 2019). After acclimatization for 1 month in a chamber, developed plants were moved to the greenhouse (temperature 25 ± 2 °C, humidity 50-60%, and natural light intensity of 125-400 µmol $m^{-2}s^{-1}$).

Morphological and histological studies

The study of morphogenic reaction of flower buds excised from the begonias inflorescence was starting from 14 d of culture up to the transferring regenerants *ex vitro* with Stereomicroscope Stereo Discovery V 12 (Carl Zeiss, Germany). Histological analysis of the floral explants morphogenesis was conducted after the samples were collected and fixed at 20, 35, and 60 day from the beginning of the study. Then they were dehydrated and embedded in Paraplast, sectioned at 7 µm, using a microtome Microm HM 325 (Carl Zeiss) and stained with Ehrlich's hematoxylin (15 min) and 0.1% aniline blue (3 min). All the preparations were made according to standard cytological procedures (Pausheva, 1988). The number of adventitious shoots induced on the begonias floral explants were assessed after 60 d of culture. Histological observations of plant development were carried out using light microscope Axioskop40 (Carl Zeiss, Germany), equipped with a digital AxioCam MRc5 camera using AxioVision 4.8 software for acquisition, processing and analysis of images.

Statistical analysis

For all treatments the percentage of flower explants forming shoots (shoot induction efficiency) and the average number of shoots produced per explant (shoot organogenesis efficiency) were calculated, assessed at 30 and 60 d correspondingly from *in vitro* culture starting. The experiments were carried out in a completely randomized design and repeated thrice with 7 explants in every treatment. Data were subjected to an analysis of variance in Statistica, version 10.0 (StatSoft Inc., Tulsa, OK). For each begonia genotype and treatment, a single-factor analysis of variance was performed between means. The results are represented as the means ± standard errors (SE). Within diagrams, mean separation are indicated by different lowercase letters and determined by Duncan's new multiple range test (p = 0.05). Interaction between genotype × treatments was analyzed using two-way ANOVA. A difference of $p < 0.05$ was considered significant.

Results and Discussion

Adventitious bud induction and shoot proliferation

Adventitious shoots were substantially formed by the direct way from the explants cells of male begonias flowers, but on the control hormone-free medium shoot regeneration did not occurred and the perianth tissues of all representatives of begonias was subjected to necrosis. Preliminary tests using nutrient media supplemented with cytokinin and auxin made it possible to define N6 medium as the most suitable for obtaining regenerants from of *B. sutherlandii* female flower buds explants (Nabieva et al., 2019).

Frequencies of regeneration from begonias flower explants and shoot development were significantly affected by treatment, genotype and the genotype × treatment interaction ($p < 0.05$) (Table 2).

Table 2. The results of factorial analysis using two-way ANOVA.

Effect	Univariate Tests of Significance, Effect Sizes and Powers for Frequency of regeneration Sigma-restricted parameterization Effective hypothesis decomposition							
	SS	Degrees of freedom	MS	F	p	Partial eta-squared	Non-c entrality	Observed power (alpha=0.05)
Intercept	189675.0	1	189675.0	8603.307	0.00	0.975508	8603.307	1.000000
Genotype	24529.2	3	8176.4	370.867	0.00	0.837423	1112.601	1.000000
Nutrient medium	90272.3	5	18054.5	818.917	0.00	0.949891	4094.585	1.000000
Genotype x Nutrient medium	11806.3	15	787.1	36.701	0.00	0.712579	535.511	1.000000
Error	4762.1	216	22.0					

The regeneration frequency (number of explants formed adventitious shoots/all explants×100%) varied and was strongly depending on the genotype and growth regulators added to the modified N6 medium: combining BA or 2-iP with the low concentration of auxin (NAA) has promoted the formation of multiple shoots from male flower explants (Figure 2: $N6_4$ and $N6_5$ treatments; Figure 3).

Based on the fact that that the largest number of shoots was formed under 2-iP and NAA addition to $N6_5$ medium for all begonia studied (Figure 3), the combination of the mineral base N6 and growth regulators with a predominance of 2-iP was the best, thus contributing to the formation of 36.57 ± 4.03 adventitious shoots per explant on average among begonias genotypes; concurrently the efficiency of regeneration was 61.08% ± 5.66%.

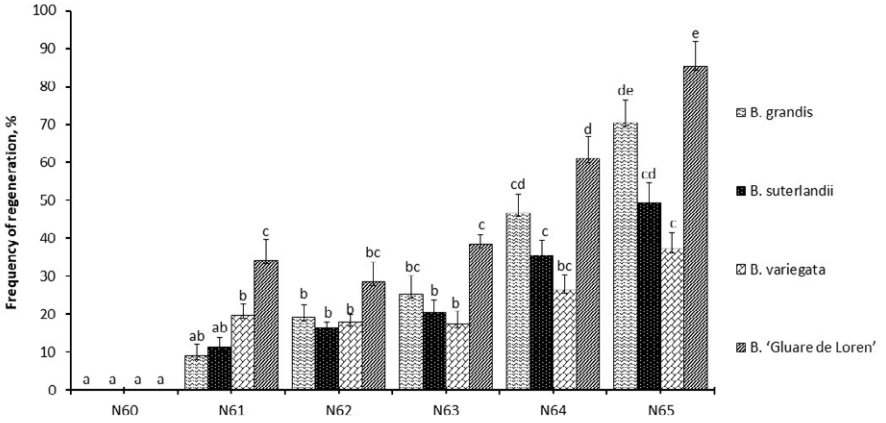

Figure 2. Effect of different plant growth regulators on the frequency of regeneration from the flower buds of *Begonia* representatives after 60-65 days of culture on different variants of modified N6 media: $N6_0$ – without PGRs; $N6_1$= N6+1µM TDZ; $N6_2$= N6+4.4µM BA; $N6_3$= N6+1µM TDZ+0.54µM NAA; $N6_4$= N6+4.4µM BA+0.54µM NAA; $N6_5$= N6+1.5µM 2-iP+0.54µM NAA. Means ± SE followed by the same letters indicate that the values are not significantly different according to Duncan's multiple test at $p < 0.05$

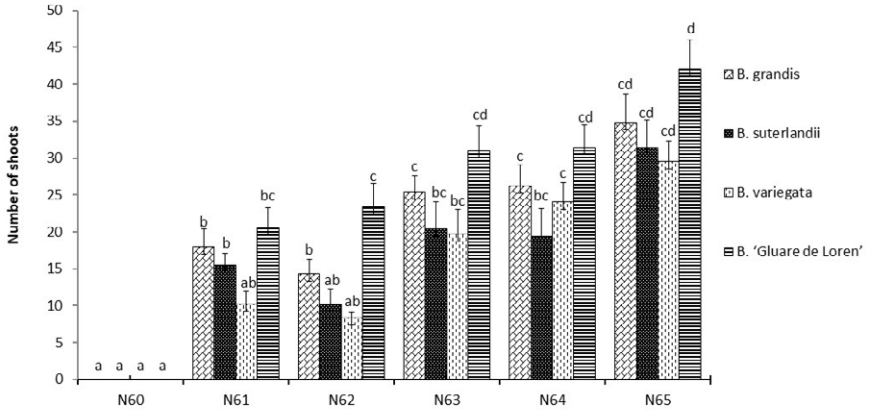

Figure 3. Effect of plant regulators on induction of adventitious shoots from begonias flower buds explants after 60-65 days on different variants of modified N6 media: $N6_0$ – without PGRs; $N6_1$= N6+1µM TDZ; $N6_2$= N6+4.4µM BA; $N6_3$= N6+1µM TDZ+0.54µM NAA; $N6_4$= N6+4.4µM BA+0.54µM NAA; $N6_5$= N6+1.5µM 2-iP+0.54µM NAA. Means ± SE followed by the same letters indicate that the values are not significantly different according to Duncan's multiple test at $p < 0.05$.

For begonias shoot regeneration the application of cytokinin in combination with a low level of auxin was usually more effective than cytokinin alone. This statement was confirmed in the work of Beiyi et al. (2021), when the highest number of *B. coptidifolia* shoots per explant was induced on MS medium with 5.0 µM BA and 1.0 µM NAA. A predominance of BA in relation to auxin in the medium is widely used in plant tissue culture for shoot multiplication either begonias (Aziz et al., 2021) or other plants (Shin et al., 2020; Raspor et al., 2021). The same trend was noted with 2-iP application, when the best direct organogenesis frequencies of *Scutellaria bornmuelleri* were achieved in half-MS medium, supplemented with a combination of 1 mg L^1 2-iP and 0.1 mg L^{-1} IAA (Gharari et al., 2021). Our results are consistent with Awal et al. (2013), who mentioned about the cytokine-like activity of adenine sulfate, which promoted the development of axillary shoots and efficient rooting of *Begonia elatior in vitro*. However, the results of the same work showed that the combination 1 mg·L^{-1} NAA and 1 mg·L^{-1} BA was appropriate for the induction of the extensive callus formation observed at the cut-off site of the young inflorescences and peduncles of the species. As a result, when the researchers have used high concentrations of these growth regulators, the emergence of somaclonal variability was revealed (Awal et al., 2013), thus the possibility of the occurrence of a somaclonal variation can be lowered by applying less PGR in the culture media. In our study the appearance of the first meristematic centers on the surface of receptacle was observed 25-35 days after introduction into tissue culture and occurred after the swelling of morphogenic tissues (Figure 4A; Figures 5A, B; Figure 6).

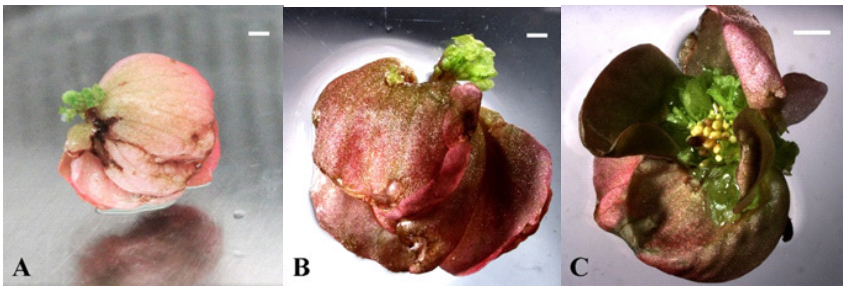

Figure 4. Stereomicroscopy observations of shoot organogenesis on the male flower explant of B. '*Gluare de Loren'*: A. Microshoots formed on the pedicels (A – 30 days of culture at $N6_5$, included 1.5 µM 2-iP + 0.54 µM NAA; B – 45 days) and receptacle (C – 55 days). Bars represent: (A, B) 1.0 mm; (C) 2.0 mm.

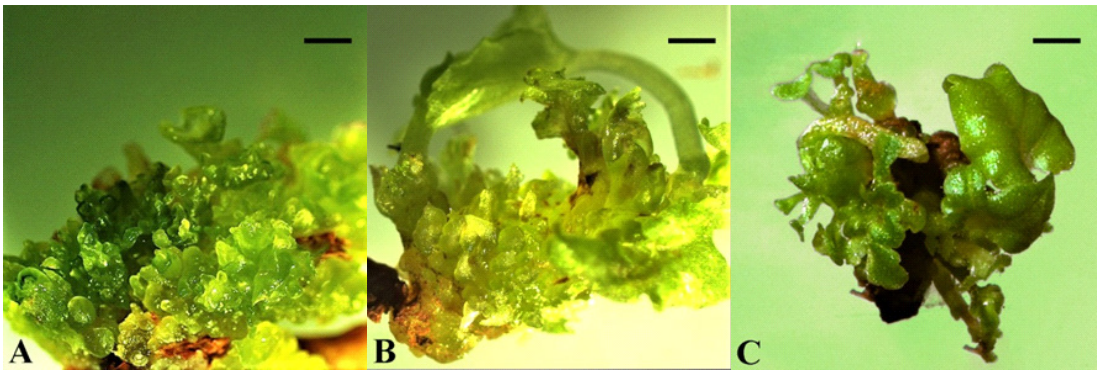

Figure 5. Adventitious shoot induction from the inflorescence of *B. grandis* explants in the regeneration medium $N6_4$, supplemented with 4.4 µM BA + 0.54 µM NAA: A – Swelling of receptacle tissue, protuberants formation; 30 day; B – Proliferation of adventitious buds and microshoots, 45 day; C – Mass proliferation of shoots formed de novo after 55 days of culture. Bar represents: (A, B, C) 1.0 mm.

Numerous protuberances developed directly on the surfaces of floral explants, except when indirect organogenesis was induced on the cut edge of the explants, treated with TDZ. It was obvious that numerous adventitious shoots were formed from callus when 1.0 µM TDZ was added to the both $N6_1$ and $N6_3$ media.

Histological analysis revealed that at the early stages of shoot morphogenesis *in vitro* numerous buds were arisen like the embryo-like protrusions directly from the subepidermal cells of the explant. Different developmental stages of primordia development included early swelling of explant tissues (Figures 4A, 5A, 6), formation of well-developed buds with numerous leaf primordia, which were observed after 30-35 d in the induction medium (Figures 4B, 5B), while completely formed shoots were noticed after 55-65 d for all *Begonia* genotypes studied (Figures 4C, 5C).

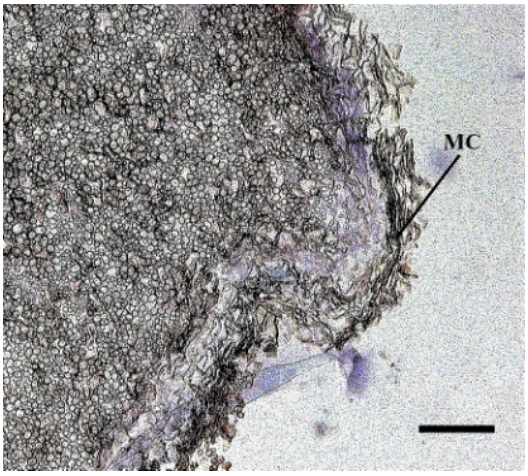

Figure 6. Longitudinal section through *B. sutherlandii* flower bud receptacle incubated on $N6_5$ medium supplemented with 1.5 µM 2-iP + 0.54 µM NAA. Pronounced cell division in the induced *de* novo meristematic centers (**MC**) and differentiation of bud originated from subepidermal tissue after 32 days of incubation. Bar represent: (A) 0.1mm.

The development of shoots and root apexes occurred directly under the BA and 2-iP action, while TDZ treatments led mainly to the indirect shoot organogenesis (variants $N6_1$ and $N6_3$). The callus phase also resulted in proliferation of the adventitious shoots, often incapable of developing roots. Adventitious shoots formed on induced inflorescence explants after 60 d, organized in clusters or microrosettes. Those microrosettes, which were less than 2 cm in diameter, were transferred on ½ MS medium supplemented with 30 mg L^{-1} adenine sulfate, which contributed to the shoot elongation *in vitro*, while more developed plantlets were placed on PGRs-free ½ MS medium for rooting (Figures 7 A, B, C, D, E).

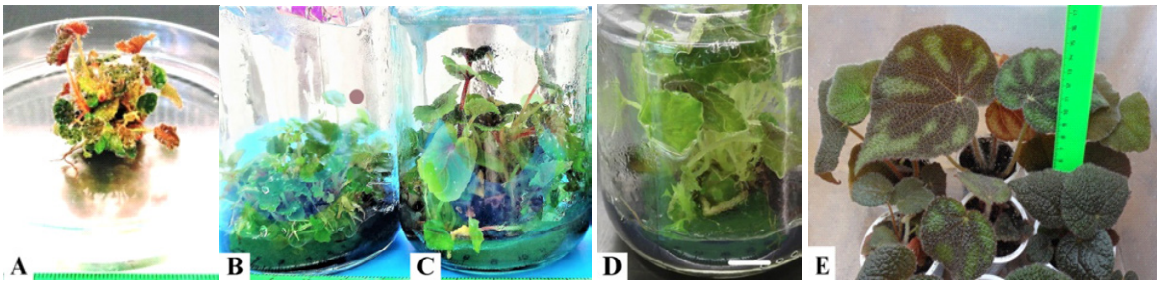

Figure 7. Begonias regenerants transferring to *ex vitro* conditions: A. *B. variegata*; *B. sutherlandii*; C. *B. grandis*; D. *B.* ***'Gluare de Loren'***; E. *B. variegata* plantlets during the adaptation period to the greenhouse conditions. Ruler divisions scale (A, B, C, E) and bar (D) represent 1.0 mm.

A similar differentiation pathway of receptacle epidermis played a crucial role in initial stages of *Allium altissimum* Regel shoot development *in vitro* culture from the immature inflorescences covered with the spathe (Poluboyarova et al., 2014). The ability to regenerate buds and shoots from subepidermal or epidermal layers was observed earlier in histological *Begonia* studies (Lai et al., 2018). The higher efficiency of shoot regeneration from flower explants reported by Awal et al. (2013) than in stem or leaf explants, may be associated with a preexisting population of stem cells in differentiated tissues, induced either by endogenous hormones or components of the regeneration medium for trans-differentiation into shoot primordia (Raspor et al, 2021). The amenability of the begonias studied to shoot regeneration from male flower bud explant varies: the highest shoot bud induction was achieved on $N6_5$ medium with 1.5 µM 2-iP and 0.5 µM NAA, giving rise to 46 ± 4.06 shoots per explant of *B. 'Gluare de Loren'* after 65 d from the inoculation. On the same medium, begonia species showed lower regenerative activity.

The application of TDZ to N6 medium led to the increase of the morphogenic potential of flower explants for the adventitious shoot proliferation mainly through callusogenesis, which may cause morphological and physiological aberrations. A similar observation about negative TDZ impact has been reported for the other *Begonia* species (Xiongwei et al., 2020), so we assumed that this PGR should be replaced by 2-iP or BA in the *Begonia in vitro* culture for cell determination to the proper tissue patterning.

Assessment of possible somaclonal variability

Successful adaptation to *ex vitro* conditions was carried out, providing 85%-90% survival of begonia plantlets of different genotype. During *in vitro* culture, changes in somatic cells could occasionally occur which led to the variability in progeny, mostly fixed in callus-derived plants (Bouman and De Klerk, 2001). In this study only well-developed rooted plantlets of 4 begonia genotypes were transferred to *ex vitro* conditions. These regenerants were obtained through direct shoot organogenesis. Phenotypic assay showed that in general, no morphological or bloom variations occurred in the acclimatized begonia plantlets after 12 months in the greenhouse growth conditions, when all regenerated plantlets produced morphologically true-to-type flowers without any detectable phenotype variance during maturation. Morphogenic tissues of receptacle and pedicel from begonia flower buds possessed the potency for direct shoot regeneration and no morphological aberrations were observed in tissue culture-derived begonia plantlets after one year.

Conclusions

As a result, we have developed a technology for obtaining regenerants from the somatic tissues of the flower of three *Begonia* species and one hybrid using morphohistological approaches. When inflorescence explants were used, the formation of shoots occurred mainly in a direct way, depending on the genotype and growth regulators applied into the suitable nutrient medium. The presence of adenine, 1.5 μM 2iP and 0.5 μM NAA in N6 medium was found necessary for the development of *de novo* shoots via direct morphogenesis. Histological observations approved that begonia inflorescence explants, including both receptacle and pedicel tissues, not excised from perianth, exhibited high potency for direct shoot regeneration, that made it possible to obtain uniform *Begonia* plantlets in *in vitro* culture. The proposed micropropagation technique seems to be an efficient tool for scientific study and commercial exploitation of valuable *Begonia* genotypes and beneficial for true-to-type plants production.

Author contribution

AYuN: proposed the research idea, carried out the experimental work in vitro. **TDF**: maintained the in vivo collection, and both authors drafted and approved the manuscript.

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