Morphogenetic pathways of floral and brood buds of *Begonia sutherlandii* Hook. F. induced in tissue culture

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This is the very first report on morphological studies of *B. sutherlandii* brood bud differentiation in tissue culture controlled conditions. Bulbils cool treatment at 2-4°C, followed by application of 0.2 mg/l 6-BAP was considered to have notable effect on their germination *in vitro*. *B. sutherlandii* bulbils induced by three-stage treatment formed well-developed numerous shoots after three months compared with intact bulbils germinated after five months of dormant period. In order to provide the efficient micropropagation system for *B. sutherlandii* as a non-persistent species to indoor conditions, immature floral buds were used. Proceeded from the suitable concentrations of IBA, BAP, TDZ, adenine sulfate, and the macroelement composition of modified MS and N6 media, different morphogenetic pathways of *B. sutherlandii* floral buds *in vitro* culture were revealed that included direct shoot organogenesis, flowering *in vitro*, and callusogenesis. The best multiple shoot induction (3.73±0.38 shoots/explant) was obtained from culturing flower buds on N6 modified medium supplemented with 50 mg/l adenine sulfate, 0.5 mg/l TDZ or 1.0 mg/l BAP in combination with 0.25 mg/l IBA. The transition from vegetative to flowering phase during *B. sutherlandii* micropropagation was induced when 0.2 mg/l BAP and 40 mg/l adenine sulfate were added to the N6 culture medium.

Keywords: Begonia L.; floral explants; shoot proliferation; flowering *in vitro*; brood buds induction.

Abbreviations: MS: Murashige & Skoog medium; N6: Chu medium; TDZ: thidiazuron; 6-BAP: 6-benzylaminopurine; IBA: indole-3-butyric acid; PGRs – plant growth regulators; CSBG: Central Siberian Botanical Garden.

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Introduction

The object of the investigation was *B. sutherlandii* from section *Augustia*. This species is presented within the relatively small group of seasonally adapted African Begonia, which comprises around 160 species [1]. It is deciduous tuberous plant which has adaptations to a drought season. After flowering of the species, the resting period is observed when all above-ground vegetative parts of the begonia die back, except brood buds, developing on the stem which begins to sprout after the cold dry season (or when stored at temperatures above freezing for a specific time period), as well as the mother tubers. The production of *B. sutherlandii* by conventional propagation in CSBG (Central Siberian Botanical Garden) is limited by poor seed viability and low rooting capability of the cuttings. As all tuberous begonias, *B. sutherlandii* is highly susceptible to pathogenic bacteria, fungi, and nematodes [2]. Furthermore, unsuitable environmental events in the course of its ontogenesis often cause the loss of the

species. In this way, tissue culture may act as a way of the species preservation and propagation.

Micropropagation has been applied as the successful strategy for large scale multiplication of *Begonia* from different explants of plant material [3]. The *in vitro* culture technique provides an excellent implement for studying the phenomena of flowering [4] and vivipary [5]. *In vitro* flowering was considered to be a complex process regulated by both endogenous and exogenous factors [6]. Transition to the generative phase (flowering) associates with the senescence and death of the only part of the individual [7], while viviparous propagule substitutes the whole plant during homophasic reproduction. *B. sutherlandii* is the important parental source in hybridization to create yellow/orange flower colors [8]. Developing the methods of inducing begonia flowering *in vitro* would facilitate the fast selection of plants with a desired phenotype for plant breeders.

This study explored the selected ways of *B. sutherlandii* multiplication, using immature floral explants and brood buds, in relation to applied exogenous plant growth regulators (PGRs), culture medium, and temperature fasting effects. As part of a wider study of reproduction in this species, the morphology of bulbil development was investigated. Histological analysis has allowed revealing the changes in the begonia dormant brood bud's development due to vernalization and cytokinin treatment.

The objectives of the present study were: (a) to analyze the effects of two media composition and plant growth regulators (PGRs) concentrations on morphogenetic pathways of *B. sutherlandii* floral bud's regeneration, (b) to reveal the early morphogenetic events during the begonia brood buds *in vitro* germination after three weeks chilling in the dark followed by the cytokinin application for one week at the room temperature.

Materials and methods

Plant material was excised from two-year old intact plants which were grown in glasshouse (figure 1 A, C).

The inflorescences segments were harvested 4-5 days prior to the opening of the first flower. Inflorescence of *B. sutherlandii* (figure 1B) was determined according to the typological approach of W. Troll [9], as "proliferating synflorescence". Usually it is bisexual or sometimes bisexual and male, protandrous, with rarely more than 3 female flowers. In the scheme of the flowering plant of *B. sutherlandii* (figure 1 B), the central flowers of the cyme are male (represented in black color), lateral flower(s) are female (in white).

Immature flower buds of *B. sutherlandii* with pedicels (figure 2), taken as explants, were surface sterilized as follows: 1 sec in 70% ethanol (v/v), 10-12 min in 0.1% $HgCl_2$ + 0.15 ml/l Tween 80, and rinsed 4 times with sterile water.

The explants were inoculated on the modified N6 (Chu medium) [10] and MS (Murashige & Skoog medium) [11] either without PGR or supplemented with 0.5 mg/l IBA (indole-3-butyric acid), 0.5-1.0 mg/l 6-BAP (6-benzylaminopurine) or TDZ (thidiazuron), in combination with 50 mg/l adenine sulfate. After four weeks of culture, proliferating structures were transferred to the same basal media containing 0.2 mg/l 6-BAP for elongation of shoots. The number of explants initiating shoots and the average number of shoots per explant were recorded after eight weeks.

Bulbil samples were obtained immediately before bud excision. For *B. sutherlandii* bulbil dormancy overcoming, the samples were exposed to cool treatment at 2-4°C in the dark for three weeks, followed by 0.2 mg/l 6-BAP application of brood buds on MS medium under 16 h photoperiod and 22°C. For studying the initial stages of organogenesis, induced brood buds of *B. sutherlandii* were collected at 2, 4, 6,

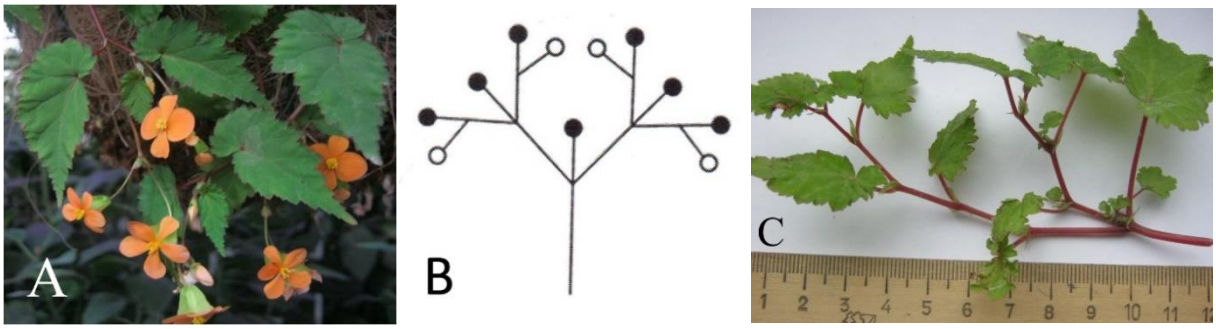

Figure 1. A: *B. sutherlandii* grown in the CSBG greenhouse. **B**: a scheme of inflorescence. **C**: fragment of shoot with germinated brood buds.

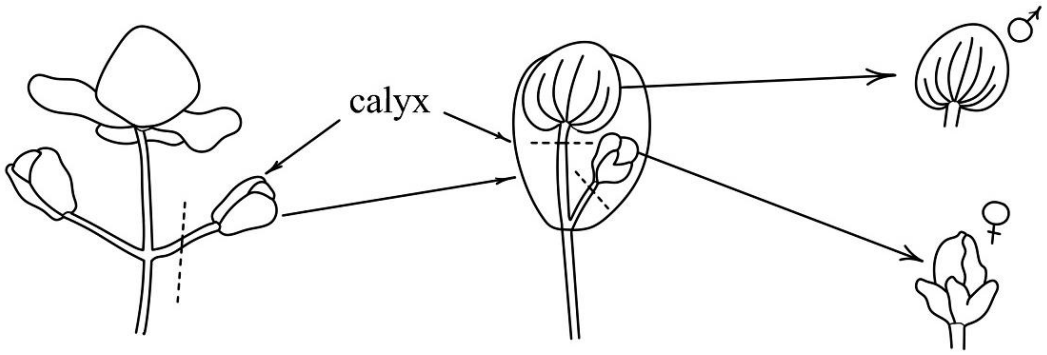

Figure 2. *B. sutherlandii* explants: female (lower row) and male (upper row) immature flower buds.

and 8 weeks after treatment and establishment on MS medium without PGRs. They were fixed for 48 h in the FAA solution (70% ethanol, formalin, and glacial acetic acid at the ratio of 100:7:7). Intact bulbils taken at the moment of separation from the plant were considered as the control. The permanent preparations for histological analysis were made according to standard cytological procedures [12]. Sections 8-10 µm in thickness were obtained using microtome Microm HM 325 (Carl Zeiss, Germany), stained with hematoxylin by Ehrlich (15 min) and 0.1% Alcian blue (3 min), and mounted with polyvinyl alcohol "Mowiol" (Fluka, Germany). The prepared slides were studied with the Axioplan 2 imaging (Carl Zeiss, Germany) microscope and photographed with the AxioCam MRc5 high-resolution digital camera, using AxioVision 4.8 digital image processing software.

In the experiments, each treatment consisted of 20 units. All experiments were repeated twice. The number of explants induced for regeneration was identified and the number of shoots produced per explant were determined. Values and means are followed by standard errors (± SE). The data was processed statistically using Statistica 6 [13], and the means were compared using Duncan's multiple range test [14] at the 5% level of probability.

Results and discussion

The analysis of morphogenetic reactions of *B. sutherlandii* floral explants *in vitro*

In our study an *in vitro* regeneration of *B. sutherlandii* was attempted using immature reproductive organs, which were not commonly used before, such as floral buds. Nowadays *in*

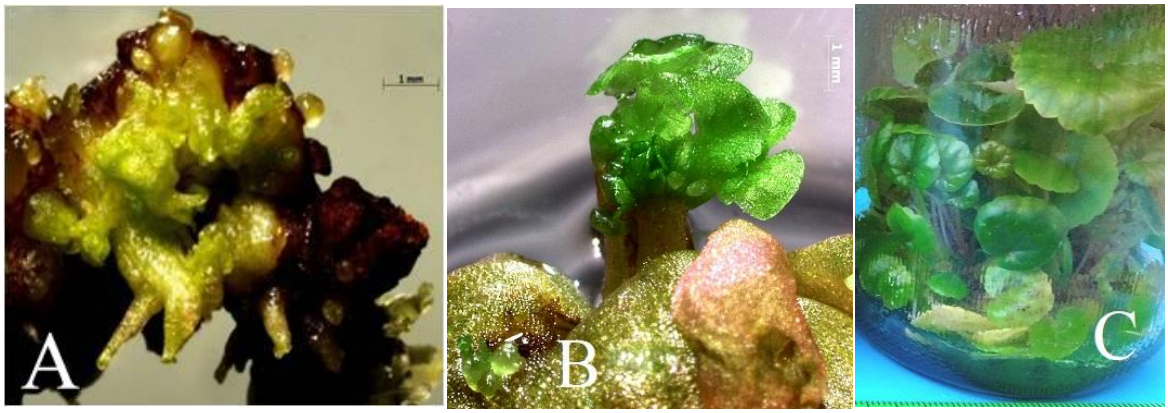

Figure 3. Direct shoots organogenesis from the female floral explants of *B. sutherlandii*. **A**: 4 weeks of culture. **B**: 8 weeks of culture. **C**: clusters of vegetative green shoots before *ex vitro* transplantation (12 weeks of culture).

Table 1. The influence of PGRs on *B. sutherlandii* shoot organogenesis on different variants of MS and N6 media.

Variants of media	Growth regulators	Shoot regeneration (%)*	Number of shoots per explant*
MS0	without PGRs	0.00±0.00^{c}	0.00±0.00^{c}
MS1	0.25 mg/l IBA+0.5 TDZ mg/l	24.00±0.33^{b}	0.77±0.20^{b}
MS2	0.25 mg/l IBA+1.0 mg/l 6-BAP	30.0±0.30^{b}	1.30±0.27^{b}
N60	without PGRs	0.00±0.00^{c}	0.00±0.00^{c}
N61	0.25 mg/l IBA+0.5 TDZ mg/l	48.00±0.62^{a}	3.57±0.55^{a}
N62	0.25 mg/l IBA+1.0 mg/l 6-BAP	54.00±2.17^{a}	3.73±0.38^{a}

*different letters denote a statistically significant difference at $P < 0.05$, as determined by Duncan's multiple range test.

vitro technologies facilitate conservation and propagation of many rare and valuable plants, as well as *Begonia* species [15]. In the most cases, researchers successfully developed begonias micropropagation techniques, starting from leaf or caulinar explants [16]. However, only in a few studies, immature reproductive organs, such as floral buds or inflorescences were used [17]. Our previous studies showed that contamination inherent in the vascular system of leaf explants of *B. sutherlandii*, along with slowed organogenesis, were found to be recurrent problems [18]. Addition of PGRs is the most important factor emerging the transition from vegetative to generative phase in plants and vice versa [19]. Regeneration occurred directly from the cells of *B. sutherlandii* floral buds or rarely the indirect type of organogenesis was presented.

As a result of 4 week of inoculation on N61 or N62 modified media supplemented with growth regulators 6-BAP or TDZ in combination with IBA, vigorous direct shoots organogenesis was induced from the floral explants of *B. sutherlandii*. Adventitious buds and shoots were formed on the flower base or on the tepals of female flower explants (figure 3 A, B, C; Table 1).

The shoot regenerants (1.5 - 2.0 cm) were separated from the clusters (figure 3 C) and transferred on ½ MS medium lacking growth regulators. In addition to the direct way of morphogenesis, two other pathways of morphogenetic reactions were observed in the

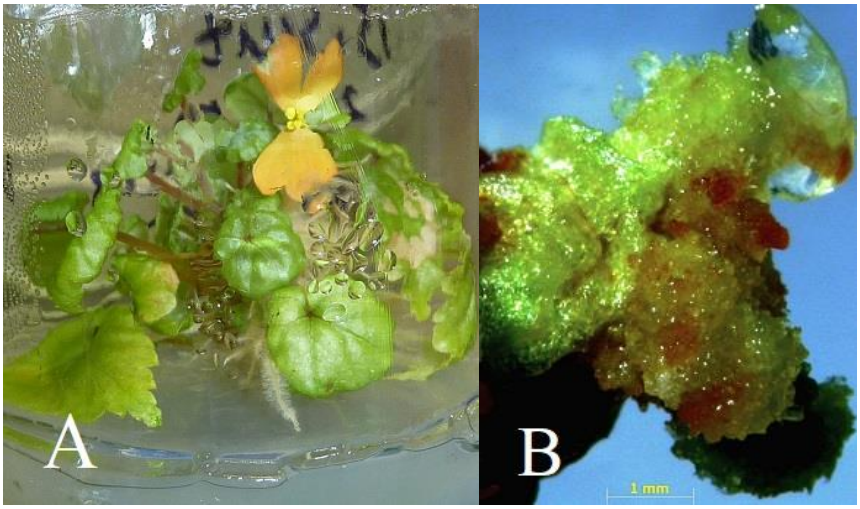

Figure 4. Different ways of morphogenetic reactions in the cultures of female flower buds of *B. sutherlandii*. **A**: *in vitro* flowering; **B**: callusogenesis.

cultures of *B. sutherlandii* female flower buds (figure 4 A, B).

Flowering *in vitro* of *B. sutherlandii* regenerants was obtained from floral explants with the frequency 7.50 ± 0.35% when 30 mg/l sucrose, 40 mg/l adenine sulfate together with 0.2 mg/l 6-BAP were added to the N6 culture medium. Plantlets initiated from floral buds bloomed after 8 weeks after the beginning of incubation period. The transition from vegetative to flowering phase *in vitro* has been induced by the application of exogenous PGRs in culture media [20, 21]. 6-BAP has been reported to be effective in floral induction of a number of plant species, such as orchids [22], *Chenopodium* [23], and others. Explant source is another factor determining success of *in vitro* flowering [24]. It has been reported to occur by the induction of floral buds of geophytes [4] while for *Begonia* x *hiemalis* Fotsch from immature reproductive organs such as young inflorescences and peduncles [17].

In our experiment, only male flowers on N6 medium supplemented with 0.2 mg/l 6-BAP were observed, while the production of female flowers wasn't revealed. Most of the flowers were malformed and had adnate tepals. Thus, the induction of flowering was observed when floral bud explants were incubated on modified N6 medium, containing a combination of 0.2 mg/l 6-BAP, 40 mg/l adenine sulfate, and 30 mg/l sucrose.

Callus was initiated from different zones including pedicels, the surface of the perianth segments, and the receptacle. Morphogenic callus was formed only at receptacle of the 20% flower buds. After subculturing onto the same medium, this callus differentiated into adventitious shoots for a longer time than direct shoots organogenesis occurred.

Regenerants obtained from the *B. sutherlandii* floral buds via direct shoots organogenesis were transferred to sterile mixture of perlite, peat, and sand in proportion of 1:1:1, and all of them survived after 3 weeks of acclimatization in greenhouse.

The analysis of the development of induced *B. sutherlandii* brood buds

Vivipary is known only for a hardy *Begonia* species (*B. sutherlandii*, *B. grandis* Dryander, and *B. emeiensis*). The reproductive strategies of *B. sutherlandii* comprise vegetative cauligenic type of vivipary (figure 5 A, B, C).

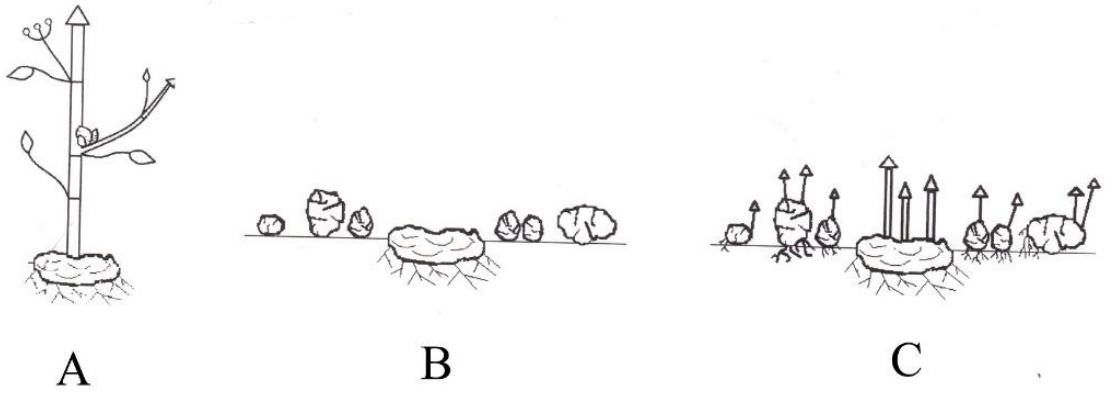

Figure 5. The details of *B. sutherlandii* fragmental ontogenesis. **A**: bulbils arising. **B**: tuber and bulbils undergoing dormant period. **C**: sprouting of propagules after dormancy breaking.

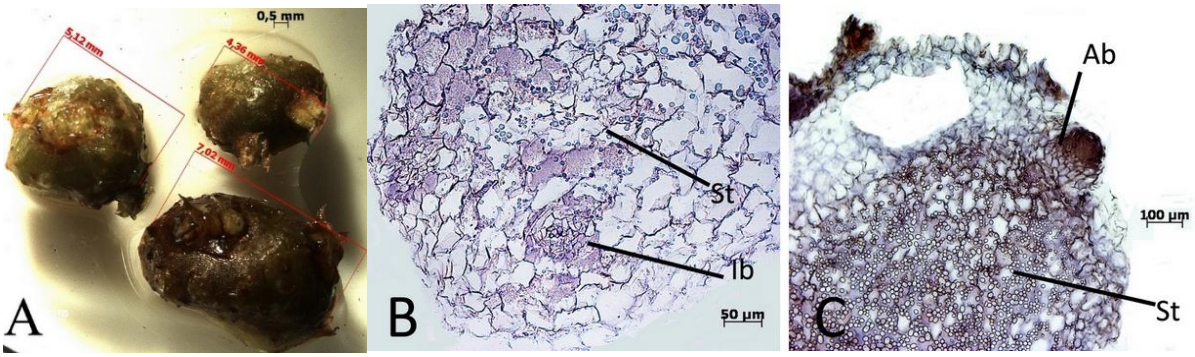

Figure 6. Histological observations of control and *B. sutherlandii* bulbils. **A**: dormant bulbils of *B. sutherlandii*. **B**: dormant phase of meristematic center of intact bulbil fallen from the maternal plant (0 day, control). **C**: adventitious bud (Ab) proliferation after 0.2 mg/l BAP exposure succeeded by 3 weeks' cool treatment at 2-4°C (0 day of maintenance on MS medium under 16 h photoperiod and 22°C). (**St**: storage tissue, **Ib**: initial meristematic bulbil center)

Viviparous propagules of *B. sutherlandii* are formed on the stem of the plant as bulbils of *Lilium tigrinum* and *Gagea bulbifera*. So that this form of vivipary should be called cauligenic [25]. Generally, vivipary is considered to be a special type of multiplication which is known in 281 species of flowering plants [26]. It is an intermediate form between typical seed reproduction and vegetative propagation and occurs as adaptation to seasonal climate. Vegetative propagules or bulbils which formed from the preestablished meristems at the lateral shoot axils of *B. sutherlandii* emerging with the beginning of the chilling period were abscised away from the flowering plant which becomes dormant.

Term bulbil is used to denote a propagule located in the aerial portion of a vegetative shoot or within an inflorescence [27]. For the development of *B. sutherlandii*, bulbils period of dormancy inherited from the maternal plant is common. It is interesting to note that not flowering *B. sutherlandii* plants usually do not produce bulbils. We observed only 5% of *B. sutherlandii* brood buds sprouting direct on the stem on the maternal plant produced shoots, without adventitious roots, and then established successfully. By no means all the other propagules grow out offspring after dormancy, which is about 60%. In contrast to embryos, brood buds of *B. sutherlandii* have monopolar structure, and their bipolarity was established when initiation of adventive roots occurred after

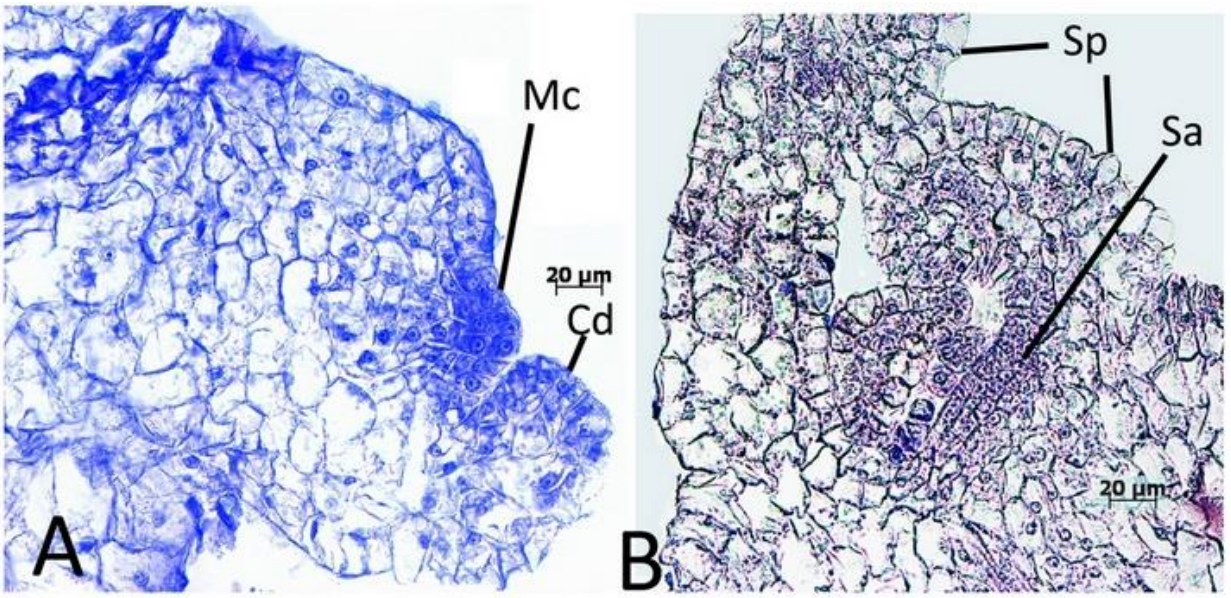

Figure 7. Longitudinal section through *B. sutherlandii* induced bulbil incubated on MS medium under 16 h photoperiod and 22°C. **A:** cell division and formation of meristematic centers *de novo* 2 weeks of maintenance. **B:** apical meristem initiates a pair of leaf primordial after 4 weeks of maintenance. (**Mc**: meristematic center, **Cd**: cell division, **Sp**: shoot primordium, **Sa**: shoot apex)

the dormancy breaking usually after 5 months in spring.

We conducted 3-stage vernalization of *B. sutherlandii* bulbils, which were (1) cold treatment under 2-4°C for 3 weeks in the dark; (2) 1-week application of bulbils with 0.2 mg/l 6-BAP under 16 h photoperiod and 22°C on MS medium; (3) bulbils were incubated for 0-8 weeks under 16 h photoperiod and 22°C on MS medium without PGRs.

Histological analysis facilitated the understanding of *B. sutherlandii* bulbils development promoted by vernalization. Intact brood buds have dormant initial meristematic center, while in induced propagules new meristematic centers were arising when subepidermal cells of brood buds were involved in *de novo* shoot regeneration (figure 6 A, B, C).

The tuber-like propagules including both control and induced ones were serially sectioned at 8 µm and were examined under cross-polarized light. Starch grains were easily identified by their distinctive pattern of birefringence and were observed within the cells of the mesophyll.

Light microscopy of longitudinal sections of *B. sutherlandii* induced bulbils demonstrated proliferation of meristematic cells that led to shoot organogenesis occurred through the formation of embryo-like protrusions of subepidermal origin. Under external protective tissue of bulbils, numerous adventitious buds were formed under the action of low temperatures, darkness, and cytokinin application (figure 7 A, B).

Vigorous shoots differentiation in *B. sutherlandii* bulbils after dormancy breaking were observed after 2-4 weeks of maintenance on MS medium and in bulbils at 6-8 weeks after dormancy breaking when well-developed buds with leaf primordia and elongated shoots were observed (figure 8 A, B).

The appearance of root apex was dropped back from the development of shoot apex, emerging from the base of the shoot after 8 weeks of bulbil incubation. With such exceptions as *Remusatia*

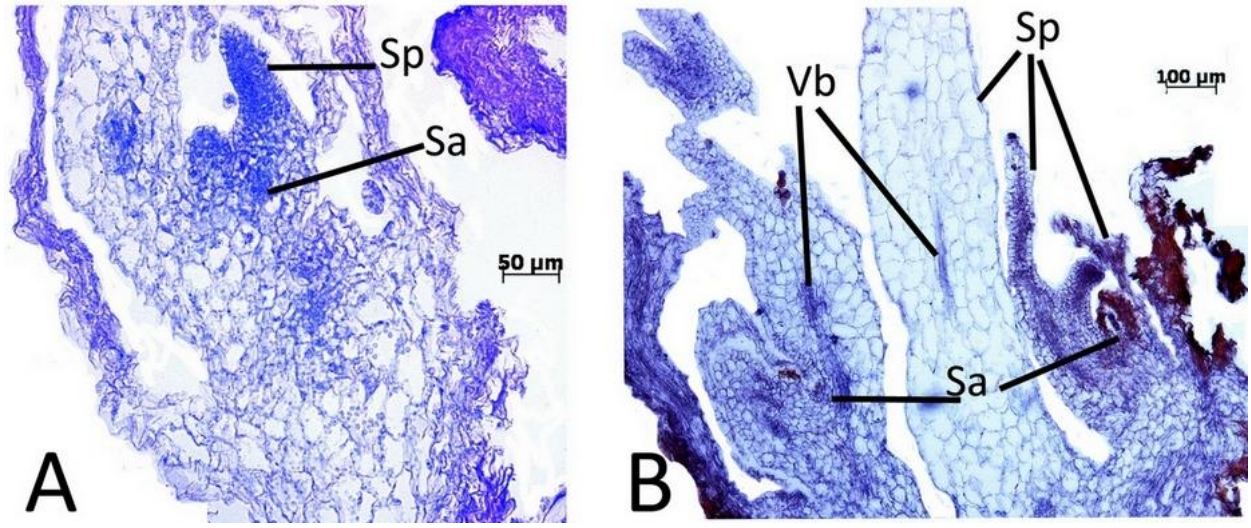

Figure 8. Longitudinal section of *B. sutherlandii* induced bud. **A**: differentiated bud with primordial leaf (6 weeks of incubation on MS medium under 16 h photoperiod and 22°C). **B**: formation of multiple primordial leaves and vascular system (8 weeks of bulbil germination after dormancy breaking). (**Sp**: shoot primordium, **Sa**: shoot apex, **Vb**: vascular bound)

vivipara Schott. and *Titanotrichum oldhamii* Soler., bulbils are unusual in tropical and subtropical plants [28]. It is very interesting in being a subtropical plant, *B. sutherlandii* produces bulbils as a result of its adaptive reproductive strategy. Almost every shoot axil initiates a bulbil resulting in massive bulbil production. We supposed that short days in September and the low temperatures in greenhouse (15°C maximum) played the triggers role for their production.

As it follows from anatomical observations of *B. sutherlandii* bulbils, the development of intact meristematic center of the bulbils was arrested during their incubation in the darkness on MS medium succeeded by 3 weeks' cool treatment at 2-4°C. Owing to the activity of the meristematic tissue raised under the treatment of 16 h photoperiod, 22°C, and application with 0.2 mg/l 6-BAP, the multiply shoot apices covered with a shoot primordium or a prophyll were originated along the periphery of the bulbils. The germination rate was very variable with about 65 % bulbils usually germinated in greenhouse taking 1 week and more for root development after 5 months of dormant period. Induced *B. sutherlandii* bulbils performed the fastest germination after 3 months when bulbils with the shoots being placed *ex vitro* in soil condition quickly emerged roots.

Conclusions

1. The highest frequency of regeneration (54%) and maximum number of shoots per explants (3.73±0.38) were obtained from immature floral buds of *B. sutherlandii* on the modified N6 media.

2. Direct shoot organogenesis of *B. sutherlandii* occurred on the both MS and N6 media, supplemented with TDZ, while 6-BAP was determined as the trigger of floral morphogenesis of *B. sutherlandii* on MS medium.

3. Histological examination revealed the early stages of shoot morphogenesis *in vitro* in sprouting bulbils after dormancy breaking. *B. sutherlandii* brood buds or bulbils were determined as derivates of dormant meristem cells arisen in the course of ontogenesis at the final stage of floral determinacy. The 3 weeks of cool treatment

at 2-4°C followed by application of 6-BAP in low concentrations was considered to have notable effect on germination of *B. sutherlandii* dormant bulbils *in vitro*.

Acknowledgments

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