In vitro propagation of *Silene bolanthoides* Quézel, Contandr. & Pamukç. and assessment of genetic stability by flow cytometry

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Abstract: *Silene bolanthoides* Quézel, Contandr. & Pamukç. is an endemic species from Kazdagi (Mt. Ida), Canakkale-Balikesir, Turkey. In order to develop an efficient shoot regeneration protocol, the leaf, nodal and internodal explants of *S. bolanthoides* were cultured on Murashige and Skoog (MS) medium containing benzyladenine (BA) alone or in combination with α-naphthaleneacetic acid (NAA). The highest number of regenerated shoots (5.75±0.1) was obtained from nodal explants that were cultured on MS medium with 8.8 μM BA+0.54 μM NAA. Regenerated shoots were rooted on MS medium without plant growth regulators (PGRs). Rooted plants (2-3 cm) were separately transferred to pots containing a mixture of peat and perlite (3:1 v/v) and acclimatized successfully in a growth chamber. Genetic stability of the propagated plants was assessed by flow cytometry and cytological analysis. Flow cytometry analysis demonstrated that regenerated plants had 2.61±0.01 pg nuclear DNA (2C) and seed-derived plants had on average 2.58±0.02 pg/2C. Cytological analysis showed that the regenerated plants had the same chromosome number as seed-derived plants of *S. bolanthoides* (2n=24). It was determined that regenerated plants were uniform in chromosome number and had a similar DNA content to the seed-derived ones, indicating that the described efficient shoot regeneration protocol can be applied for *ex situ* conservation of this species.

Key words: catchfly; chromosome; endemic; genetic stability; regeneration

INTRODUCTION

The genus *Silene* (Caryophyllaceae) is represented by 187 taxa in Turkey, with 37% of these taxa being endemic [1-4]. This genus is divided into 31 sections. The *Spergulifoliae* section is represented by 11 taxa in Turkey and these species are *Silene arguta, S. cappadocica, S. montbretiana, S. oreophila, S. spergulifolia, S. stenobotrys, S. supina* with four endemic species, *S. bolanthoides* Quézel Contandr. & Pamukç., *S. muradica, S. sangaria* and *S. surculosa* [1,2].

   S. bolanthoides is an endemic species in the flora of Turkey and its natural habitats are alpine meadows and schistose rock. It is distributed on only one locality at an elevation of 1700 m in Kazdagi (Mt. Ida), Turkey. The conservation status of this species is declared as CR B1ab (ii, iii) according to IUCN criteria [5]. The

fact that this plant shows a specific distribution in only one locality can lead to a decline in the number of individuals in the population, which makes it potentially endangered. Thus, this plant species needs to be conserved in order to prevent the disappearance of genetic resources and inheritance in future generations.

   In vitro culture technologies have become increasingly important for the *ex situ* conservation of species such as rare, endemic and endangered plants [6,7]. *In vitro* propagated plants are expected to be genetically uniform and genetically equivalent as donor plants. However, many factors such as callus stage, number of subcultures, explant source, plant growth regulators or applied chemicals may cause environmental stress in culture and induce genetic or epigenetic variations, widely known as somaclonal variation, in propagated plants during the culture process [8-11]. As a result of

this unexpected and mostly undesired phenomenon in culture, propagated plants derived from organ cultures, calli and somatic embryos can be different from the donor plants with regard to phenotypic, physiological, cytological and molecular characteristics [12]. Therefore, it is necessary to analyze whether the propagated plants are genetically identical to the donor plants. Genetic uniformity of the plants can be detected using many techniques, such as cytogenetic analysis, flow cytometry and/or molecular markers such as amplified fragment length polymorphism (AFLP), inter simple sequence repeats (ISSR), isozymes, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphisms (RFLP) and simple sequence repeats (SSR) [13,14]. Among these techniques, flow cytometry has been shown to be an easy, rapid, accurate and economical method for screening the genetic stability of propagated plants [15-18]. Flow cytometry can be complemented by traditional cytological studies, including chromosome counting.

In recent years, many studies have been devoted to *in vitro* cultivation, propagation or the protection of genetic resources of rare, endemic and economically valuable plants [19-22]. *In vitro* culture of some *Silene* species such as micropropagation of *S. cretacea* [23], a *Silene* hybrid (*S. polypetala* x *S. virginica*) [24] and *S. sangaria* [25], suspension culture of *S. vulgaris* [26], shoot regeneration of *S. vulgaris* [27,28] and *S. thymifolia* [29] have been described previously. To date, there has been no report on the *in vitro* culture of *S. bolanthoides*.

The objectives of this study were to investigate the effect of plant growth regulators (PGRs) and to evaluate the regeneration capacity of the leaf, nodal and internodal tissues in order to establish an efficient shoot regeneration system for *S. bolanthoides*, as well as to assess the genetic stability of regenerated plants by cytological analysis and flow cytometry.

MATERIALS AND METHODS

Plant material

S. bolanthoides seeds were collected from alpine meadows at the Kartalcimen locality, Kazdagi, Canakkale-Balikesir, Turkey. Seeds were surface-disinfected by stirring in a 1% (v/v) solution of sodium hypochlorite containing two drops of 0.1% Tween 20 for 10 min under sterile conditions, followed by 5 rinses in sterile water. The surface-sterilized seeds were cultured on MS basal medium (MS: M0222, Duchefa Biochemie B.V., Haarlem, Netherlands) [30], containing 3% (w/v) sucrose and 0.8% (w/v) agar, and left in the dark for a week for germination. Germinated seedlings were subsequently transferred to a growth chamber at 25±2°C under a 16-h light and 8-h dark cycle with a light intensity of 72 μmol m^{-2}s^{-1} provided by cool-white fluorescent lamps.

Induction of shoots

Shoot cultures of *S. bolanthoides* were established *in vitro* using leaf (5x5 mm), nodal (ca. 5 mm long) and internodal (ca. 5 mm long) segments excised aseptically from 8-week-old *in vitro* germinated seedlings. The explants were cultured on MS medium supplemented with BA (0, 2.2, 4.4 or 8.8 μM) in combination with NAA (0, 0.54 or 2.69 μM) and 3% (w/v) sucrose (S0809, Duchefa). All media were gelled with 0.7% (w/v) agar (P1001, Duchefa) and the pH was adjusted to 5.75 before autoclaving. All cultures were maintained at 25±2°C under the 16/8 h photoperiod with a light intensity of 72 μmol m^{-2}s^{-1}. Ten explants were cultured per Petri dish for each type of explant, and at least five replicates were used for each treatment. The mean number of regenerated shoots per explant was recorded in each culture after eight weeks.

Shoot multiplication, rooting and transfer of plants to soil

In order to multiply the shoots, the well-developed shoots (ca. 1 cm in length) grown for 8 weeks on shoot induction medium were excised and transferred to fresh MS media containing the same PGRs as the shoot induction media. For rooting, the shoots (2-3 cm in length) produced after multiplication were separately transferred to basal MS medium with 3% (w/v) sucrose and 0.8% (w/v) agar. The rooting response to various treatments was recorded as a percentage of rooted plants after 4 weeks of culture. All of the plants with well-developed roots were taken out of the solid medium, gently washed in running water and shortly after transplanted into pots (5 mm in diameter) containing a mixture of peat and perlite (3:1 v/v). Plants were cov-

ered with plastic cups to maintain a high humidity and a few holes were opened on the cups. The diameter of the holes was gradually increased over the next 2 weeks. After one month, the plants were uncovered and were then transplanted to new pots (15 mm in diameter) containing the mixture of 3 peat:1 perlite.

Flow cytometry analysis

Fresh leaf samples were collected from randomly chosen healthy and fully developed plants. Flow cytometric analysis was carried out using a flow cytometer (Partec CyFlowR Space) in the Laboratory of Plant Genetics and Cytogenetics at Namik Kemal University. A Partec commercial kit (CyStain PI absolute P) was used for the isolation and staining of nuclei and propidium iodide served as the fluorescent dye. *Lycopersicon esculentum* Mill, which has 1.96 pg/2C DNA content [31], was used as the internal standard. Briefly, the procedure was as follows: about 20 mg of a fresh leaf sample (*S. bolanthoides*) and 40 mg of fresh leaf of the internal standard were placed into a Petri dish with 500 µL of extraction buffer. The tissues were chopped with a razor blade into small pieces for 30-40 s. The solution was transferred into a glass tube through a filter. Two mL of staining buffer was added to the glass tube and incubated for 1 h. The absolute DNA contents of *S. bolanthoides* plants were calculated according to the formula used for converting fluorescence values to the DNA content, based on ratios of the G1 peak means of sample and internal standard as follows: Sample DNA Content=[(sample G1 peak mean)/(standard G1 peak mean)] x Standard 2C DNA Content.

Three plants per sample (*n*=30) were analyzed to obtain the mean nuclear DNA content. The mean DNA content per plant was based on 2000 scanned nuclei.

Chromosome counts

Cytological analyses were performed on root tips of the plants used for flow cytometry. Chromosome counts were done on slides made according to the acetocarmine squash protocol as described by Tsuchiya and Nakamura [32] with some modifications. Root tips, approximately 1.5 cm in length, were cut and immediately pretreated with 8-hydroxyquinoline (Sigma, USA) for 4 h at room temperature and then fixed in

Farmer's solution (3:1 absolute ethanol:glacial acetic acid) and stored at 4°C for at least 24 h. For mitotic analysis, root tips were stained with 2% acetocarmine and kept at least 3-4 days at 4°C. The root cap of the stained root tip was removed before squashing on a glass slide. The chromosomes were observed under a light microscope to determine the ploidy level.

Statistical analysis

One-way analysis of variance (ANOVA) was used to evaluate significant differences in the mean values of different treatments, using the Statistical Package for the Social Sciences (SPSS), version 20.0. The differences between the mean values were compared by Tukey's test ($p \leq 0.05$).

RESULTS

***In vitro* culture**

In this study, different explant types (leaf, nodal and internodal segments) of *S. bolanthoides* were cultured on MS medium containing different concentrations of BA and NAA in order to achieve optimization of the shoot regeneration system. Axillary and *de novo* shoots directly appeared on the cut ends of the nodal and internodal explants on MS medium without PGRs. However, direct adventitious shoot induction from the leaf explants grown on the same medium was not achieved.

The effect of BA in combination with NAA on shoot regeneration from all explant types of *S. bolanthoides* was evaluated. The shoots were indirectly induced from all of the nodal explants except the ones grown on MS medium supplemented with 8.8 µM BA and 2.69 µM NAA. Although a large amount of yellow calli were induced on the leaf, nodal and internodal explants, shoot induction from any kind of explant was not achieved on MS medium containing 8.8 µM BA+2.69 µM NAA. Within 2 weeks in culture, compact green-yellow calli could be observed on the cut ends of the explants. During the subsequent week, dark green parts appeared on the calli surfaces. Small protuberances started to occur from these green parts and developed into *de novo* shoots after 4 weeks. The shoots were clearly visible for nodal and internodal

explants on MS media containing PGRs after 8 weeks in culture (Fig. 1A) and developed during culturing (Fig. 1B). Of the different concentrations of PGRs, the maximum number of shoots per explant was recorded on nodal explants cultured on MS medium containing 8.8 μM BA+0.54 μM NAA (5.75±0.11) with the highest regeneration frequency (100%) (Table 1).

When different explant types were compared with regard to their regeneration capacity, it was found that nodal explants had a higher shoot induction capacity than the leaf and internodal explants (Table 1). The induction of shoots on leaf explants could not be achieved on any of the used media. Shoot formation from leaf explants was only observed when the explants were cultured on MS medium supplemented with 2.2 μM BA and on MS medium with 8.8 μM BA+0.54 μM NAA. The highest number of regenerated adventitious shoots (0.85±0.02) was observed from the leaf explants cultured on MS medium with 8.8 μM BA+0.54 μM NAA (Table 1).

Shoot induction occurred on the internodal explants on MS media with PGRs except the media supplemented with 2.2 μM BA+0.54 μM NAA, 2.2 μM BA+2.69 μM NAA, 4.4 μM BA and 8.8 μM BA+2.69 μM NAA. However, the mean number of shoots per internodal explant was lower than the mean number of shoots per nodal explant on the media with the same growth regulators. The highest number of shoots from internodal explants occurred in the presence of 8.8 μM BA and 0.54 μM NAA (2.35±0.12), and is presented in Table 1.
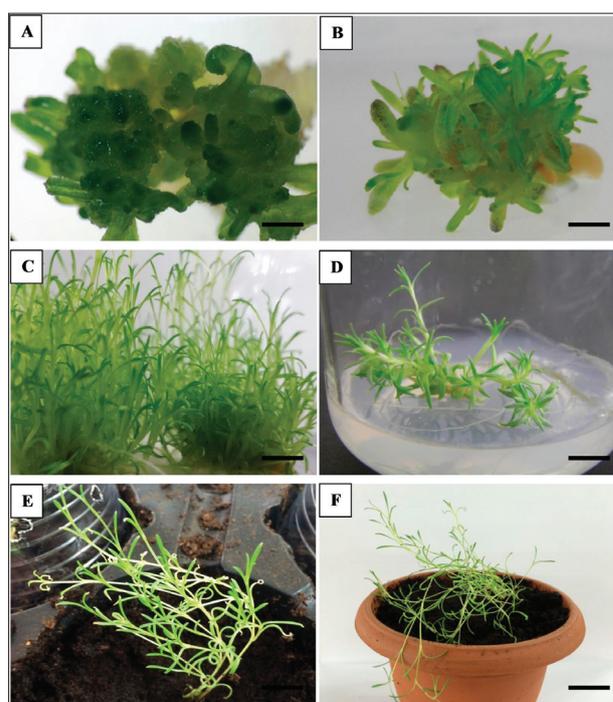

Fig 1. Indirect shoot organogenesis in *S. bolanthoides*. **A** – Shoot induction from explants cultured on MS medium with 8.8 μM BA+0.54 μM NAA after 4 weeks (*bar*=0.1 cm). **B** – Induced shoots on MS medium with 8.8 μM BA+0.54 μM NAA after 8 weeks (*bar*=0.2 cm). **C** – Propagated shoots after 12 weeks (*bar*=0.7 cm). **D** – Rooted plants on MS medium without PGRs (*bar*=0.7 cm). **E** – Plants immediately after *ex vitro* transfer (*bar*=1 cm). **F** – Plants in *ex vitro* condition (*bar*=3 cm).

The shoots were propagated on MS medium containing the same PGRs as the shoot-induction medium. When the shoots were subcultured, they displayed cluster-shaped growth and their lengths were not too long. Although the mean number of shoots per explant

Table 1. Effect of α-naphthaleneacetic acid (NAA) and benzyladenine (BA) on shoot regeneration from leaf, nodal and internodal explants of *S. bolanthoides*. Ten explants were cultured per Petri dish for each type of explant and at least five replicates were used for each treatment.

PGRs (μM)		Percentage of shoot induction (%)			Number of shoots per explants (±SE)			Rooting rates (%)
BA	NAA	Leaf	Internode	Node	Leaf	Internode	Node	
0	0	0 [a]	50±1.15 [d]	100±0.00 [g]	0 [a]	1.65±0.17 [bcd]	2.5±0.07 [de]	80
2.2	0	66.6±1.53 [e]	50±±2.45 [d]	100±0.00 [g]	0.33±0.05 [ab]	1.25±0.02 [abcd]	5.5±0.65 [g]	89.5
2.2	0.54	0 [a]	0 [a]	100±0.00 [g]	0 [a]	0 [a]	3.5±0.16 [ef]	97.83
2.2	2.69	0 [a]	0 [a]	100±0.00 [g]	0 [a]	0 [a]	0.5±0.02 [ab]	20
4.4	0	0 [a]	0 [a]	50±2.25 [d]	0 [a]	0 [a]	2.5±0.19 [de]	80.5
4.4	0.54	0 [a]	14.28±1.05 [b]	100±0.00 [g]	0 [a]	1.14±0.01 [abcd]	2.5±0.08 [de]	88.5
4.4	2.69	0 [a]	28.57±1.44 [c]	100±0.00 [g]	0 [a]	1.33±0.05 [abcd]	2.71±0.24 [de]	45.6
8.8	0	0 [a]	100±0.00 [g]	100±0.00 [g]	0 [a]	2.14±0.08 [cde]	4.5±0.02 [fg]	77.55
8.8	0.54	78.65±2.15 [f]	100±0.00 [g]	100±0.00 [g]	0.85±0.02 [ab]	2.35±0.12 [cde]	5.75±0.11 [g]	60.83
8.8	2.69	0 [a]	0 [a]	0 [a]	0 [a]	0 [a]	0 [a]	0

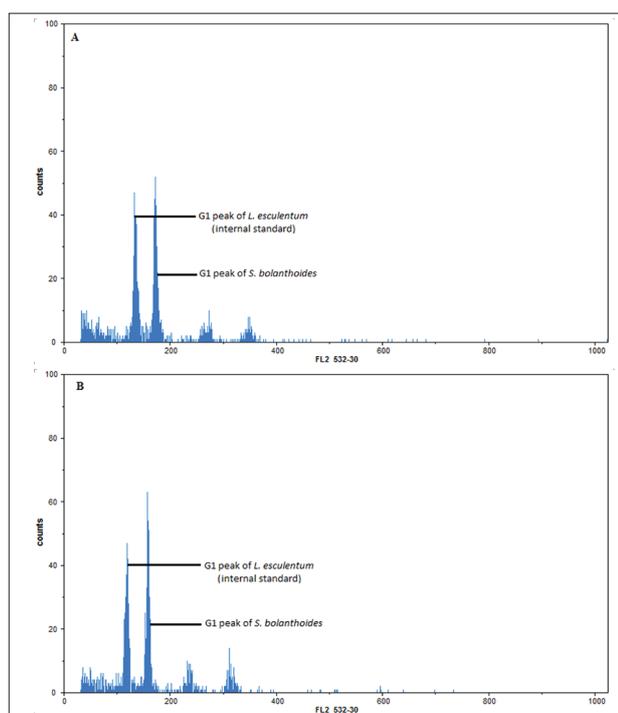*Means with the same letter in the columns are not significantly different at p ≤ 0.05

Fig 2. Flow cytometry histograms of *S. bolanthoides*. **A** – Seed-derived plants of *S. bolanthoides*. **B** – *In vitro*-propagated plants. The absolute DNA content of *S. bolanthoides* plants were calculated based on ratios of the G1 peak means of sample and internal standard (*L. esculentum*).
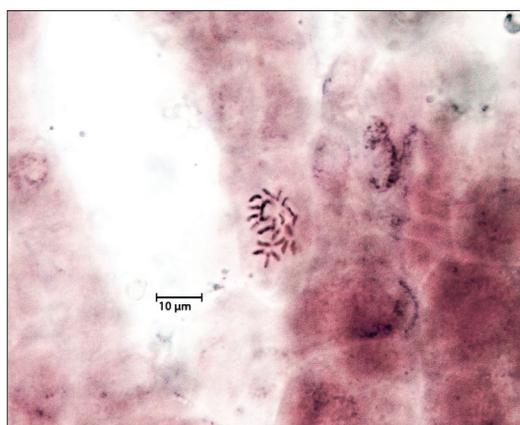

Fig 3. Mitotic chromosomes in root-tip cells of *in vitro* propagated plants of *S. bolanthoides* (2n=24).

was not too high in the shoot-induction medium, each shoot was multiplied to a large number in the propagation stage (Fig 1C). In contrast, a high multiplication rate was not observed in the shoots that regenerated from nodal explants cultured on MS medium containing 2.2 µM BA, even though the number of shoots per explant was statistically higher (5.5±0.65).

Single shoots were separated from the clusters and then transferred to basal MS medium for root induction (Table 1). Prolonged growth on basal MS led to root formation in all shoots (Fig 1D). The highest rooting rate was observed in MS with 2.2 µM BA+0.54 µM NAA. All regenerated plants exhibited normal growth and were fully developed by the end of the culture. When the regenerated plants were compared with control plants, no anatomical or morphological differences were observed. At the end of the experimental series of our research, the regenerated plants were successfully acclimatized to *ex vitro* conditions and then transplanted to 3 peat:1 perlite (Fig 1E). The plants were successfully transferred and grown in greenhouse conditions with an approximate 86% survival rate (Fig 1F).

Genome size and chromosome counting

Flow cytometric analyses and chromosome counting were used to assess the genetic stability of regenerated plants. Flow cytometric histograms indicated two peaks: the first peak representing nuclei in the G1 phase of the cell cycle belonging to the internal standard, with the second peak representing nuclei in the G1 phase of the *S. bolanthoides* samples (Fig. 2). It was determined that the nuclear DNA content of *in vitro*-propagated plants and seed-derived plants of *S. bolanthoides* were 2.61±0.01 pg/2C and 2.58±0.02 pg/2C, respectively. The mitotic metaphase chromosome of *in vitro*-propagated *S. bolanthoides* was counted in root-tip cells and compared with the seed-derived plants. The number of chromosomes of propagated plants was determined to be 2n=24, like the seed-derived plants (Fig 3). Flow cytometric analysis and chromosome counting revealed that the regenerated plants had a uniform chromosome number and similar DNA contents.

DISCUSSION

The leaf, nodal and internodal tissues of *S. bolanthoides* were evaluated in order to establish a shoot regeneration system. Furthermore, the effects of various concentrations of BA and NAA on shoot formation from these explants were investigated. The explant source is an important factor for successfully

establishing tissue culture techniques [33]. According to our results, the nodal explants were effective for shoot regeneration of *S. bolanthoides*. Our results indicated that the lowest number of regenerated adventitious shoots was observed from the leaf explants. Similarly, a study by other researchers demonstrated that the lowest callus formation was obtained with leaf explants of *S. sangaria* and shoot regeneration could not be obtained from leaf explants cultured on MS medium containing different combinations and concentrations of PGRs [25].

Although the induction of direct shoot regeneration was achieved on nodal and internodal explants cultured on MS medium without growth regulators, the number of shoots per explant was limited. It was also observed that the number of these shoots did not increase during the shoot multiplication phase. The induction of shoots on MS basal medium without growth regulators could be presumably due to endogenous hormone levels, especially cytokinin, of the nodal and internodal explants. There are a number of reports indicating that endogenous hormone levels in explants play an important role in shoot induction, initiation and differentiation [34-36]. On the other hand, we also observed that the supplementation of PGRs to MS medium promoted callus induction and indirect shoot regeneration. These shoots propagated in a large number in the propagation stage. Similarly, it has been reported on various *Silene* species that PGRs supplemented to the culture medium improve the number of shoots and shoot length [23-25].

According to our results, the 8.8 µM BA in combination with 0.54 µM NAA delivered the highest shoot regeneration from explants of *S. bolanthoides*. BA in combination with NAA has been the most widely used combination for promoting shoot induction and multiplication in *Silene* species [27,29].

Although the explants on MS medium containing 8.8 µM BA+2.69 µM NAA produced a high frequency of calli, their compact structure and yellow color failed to regenerate shoots. This result showed that the increase in the amount of NAA exogenously added to the MS medium inhibited shoot formation in *S. bolanthoides*. This response was similar to the previously published report on two ecotypes of *S. vulgaris*. In the study, it was reported that the high concentrations of

NAA acting in conjunction with BA eventually arrested growth and ultimately caused the callus to die over an 8-week period [27].

Our results showed that none of the regenerated plants had genomic alterations and different polyploidy levels compared to seed-derived plants of *S. bolanthoides*. There are a number of factors that induce somaclonal variation during *in vitro* culture. Based on the results, tissue culture conditions used in this study, such as explant tissue source, concentration and type of PGR, media components and the number of subcultures, could provide genomic stability of propagated plants of *S. bolanthoides*.

In this study, we carried out the propagation of *S. bolanthoides* via indirect shoot organogenesis. When unorganized cell mass growth occurs in culture, somaclonal variation may occur in these cells, and plants regenerated from the callus can be genetically different from the donor plant [37-40]. In this study, the callus stage was not prolonged, and this type of callus stage duration in culture could provide genetic stability of regenerated plants of *S. bolanthoides*. Additionally, a high rate of propagation of *S. bolanthoides* was achieved in relatively shorter periods with more frequent subculturing.

The results of this study confirmed that flow cytometry is a quick and effective method for estimating the nuclear DNA content of propagated plants. Flow cytometry has been used to determine genome size and ploidy levels of *in vitro* regenerated plants in various plant species. It was successfully used to assess genetic stability in micropropagated *Puya berteroniana* through adventitious shoots [41], in various tissue culture-derived plants of *Citrus limon* Burm. [13], in *Iris sibirica* plants regenerated by somatic embryogenesis and organogenesis [42], and in regenerated plants of *Eryngium planum* L. performed through axillary bud proliferation [17]. Cytogenetic studies and chromosome counts can provide information about abnormal mitosis or changes in ploidy levels of plants [43].

In conclusion, this is the first report on efficient shoot regeneration from nodal explants of *S. bolanthoides*. We showed that nodal explants can be cultured on MS medium with 8.8 µM BA+0.54 µM NAA with a satisfactory frequency of plant regeneration of *S. bolanthoides*. The results of both flow cytometry and

chromosome analysis show that the *in vitro* propagation protocol did not cause any genetic changes in the regenerated plants. Therefore, this efficient protocol can be applied for *ex situ* conservation of this species.

Author contributions: The authors make the following declarations regarding their contributions: conception and design of the experiments: NC; collection and identification of the plant samples of *S. bolanthoides*: OE; *in vitro* culture studies: NC; flow cytometry: GY and MT; karyotype analysis: NA. All authors contributed to the writing of the manuscript.

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