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Plant Biotechnology, **21** (3), 237-241 (2004)

# *In Vitro* Propagation and Storage of *Brugmansia versicolor* Lagerheim

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Received 1 April 2004; accepted 1 June 2004

**Abstract**

*Brugmansia versicolor* Lagerheim of the family Solanaceae was propagated through shoot tip culture and shoots were cold-stored *in vitro* at 5, 10 or 15℃ under light or dark condition. All the shoots died after cold storage for 6 months at 5℃ irrespective of light condition. When shoots were stored for 12 months at 15℃ under light illumination, the best 100% survival rate was obtained. The plants regenerated from shoots stored for 6 or 12 months retained the ability to accumulate scopolamine as much as the control plants which were raised from shoots maintained under normal culture condition without cold-storage. These findings show that slow growth storage of *in vitro B. versicolor* shoots at 15℃ can be used as a germplasm conservation system for short- or medium-term duration without deterioration of the ability to accumulate the secondary metabolites.

**Key words:** germplasm conservation, micropropagation, refrigeration, scopolamine, Solanaceae, tropane alkaloid.

## Abbreviations

BA, 6-benzyladenine; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; LS, Linsmaier and Skoog (1965) medium.

The genus *Brugmansia* (tree datura; angel's trumpet) is native to South America and widely distributed all over the world in the temperate and tropical regions. Several species are known within the genus and all are cultivated as ornamentals because of their decorative flowers (Preissel and Preissel, 2002). *Brugmansia* species exhibit much the same type of alkaloid spectrum including scopolamine and hyoscyamine as principal alkaloidal components. Since these alkaloids are of clinical importance, *Brugmansia* plants have been cultivated in South America for the production of the alkaloids. Tissue culture techniques were applied for the clonal propagation of useful cultivars (Griffin and Lin, 2000).

Although progress has been made on *in vitro* propagation technology as well as conservation methods (Fletcher, 1994; Hiraoka *et al.*, 1995; Bonnier and Tuyl, 1997; Bekheet, 2000; Reed *et al.*, 2003), biochemical stability of plants preserved for long time under refrigeration remains to be investigated. We previously reported successful slow growth storage of *Atractylodes lancea* (Hiraoka and Kagoshima, 1993), *A. ovata* (Hiraoka *et al.*, 2003), and *Scopolia japonica* (Hiraoka *et al.*, 2004) shoot cultures under refrigeration for two years without deterioration of the biochemical characteristics of regenerated plants. As a continuation of the investigation along this line, we now present the results on the clonal propagation through shoot tip culture and biochemical assessment of the plants regenerated from cold-stored shoot cultures of *B. versicolor*.

The plant material used in the present study has been maintained in greenhouse condition at the Medicinal Plant Garden, Niigata University of Pharmacy and Applied Life Sciences in Niigata City (plant registration number: #477). It is a small tree (4 m high) possessing elliptic to oblong leaves covered with short soft hairs. Pendulous flowers are about 30 cm long and have a spathe-like calyx with a single split. The corolla is trumpet-shaped and light green when emerged from a calyx and it turns first white then becomes light orange with time. The corolla tube is constricted near the calyx leaving a space between two parts of the flower. Corolla teeth are about 3 cm long and recurved. The flower has

strong scent in the evening. A fruit is fusiform and about 20 cm long. The plant was introduced to the Garden as *Datura arborea* L. These morphological features, however, suggest it to be *B. versicolor* Lagerheim. Its habitat is restricted to the tropical regions of Ecuador (Preissel and Preissel, 2002).

Throughout the present experiments, a basal medium was LS supplemented with 3% sucrose, 0.8% agar, and pH was adjusted to 5.6 prior to autoclaving at 121℃ for 20 min. A culture tube was capped with a silicone sponge plug. Cultures were maintained at 25℃ in culture room condition under a 16-h photoperiod of 50 $\mu$mol $m^{-2}$ $s^{-1}$ light provided by cool white fluorescent tubes whenever otherwise stated. A meristematic part of about 1 mm diameter was aseptically removed from each of three shoot tips and placed in a 13× 130 mm culture tube containing 20 ml medium supplemented with 1 $\mu$M 1-naphthaleneacetic acid and 10 $\mu$M BA. Out of three shoot tips, one initiated a shoot at the primary culture stage. Since initiation medium enhanced callus formation at the base of shoots, other hormonal combinations were tried to find out better conditions for shoot multiplication. Among tested combinations, 0.1, 1 or 10 $\mu$M BA/kinetin alone or in combination with 0.1 $\mu$M IAA, 1 $\mu$M kinetin alone gave the best result, 2.4 ± 1.3 shoots per culture. Therefore, shoots were routinely subcultured on shoot proliferation medium containing 1 $\mu$M kinetin at bimonthly intervals. In a later experiment, however, we have found that 1 $\mu$M BA in combination with 1-10 $\mu$M gibberellic acid gave a better result on shoot multiplication rate, 4 - 5 shoots per culture. Rooting medium included 1 $\mu$M IBA, since it was the best hormonal condition for rooting of shoots among hormone-free, IAA (1 $\mu$M), and IBA (0.1, 1.0 and 10 $\mu$M) by a preliminary experiment.

Experiments were designed to evaluate the response of shoots to cold storage and its effect on their viability after reculturing on freshly prepared medium. Shoots were precultured on shoot proliferation medium under normal culture condition for 18 days, and then 15 cultures were stored in each cold room condition of 5, 10 and 15℃ under darkness or of 5, 10 and 15℃ under a 16-h photoperiod of 10 $\mu$mol $m^{-2}$ $s^{-1}$ light provided by a cool white fluorescent tube. After 6, 12 and 18 months of cold storage, 5 shoots from each storage treatment were taken out and recultured on freshly prepared shoot proliferation medium. The control shoots were maintained on the same medium at bimonthly intervals under normal culture condition without cold storage. The criterion of the survival percentage was the initiation of new growth from recultured shoots maintained under normal culture condition. Data on shoot number and shoot length were recorded after the third passage of reculturing process. At the 4th passage of shoot reculturing, shoots from each group were transferred to rooting medium. *In vitro* plantlets were harvested after 30 days of incubation on rooting medium for biological and chemical assessment. To determine the appropriate growth stage for the assessment of scopolamine accumulation, plant materials at different growth stages were harvested and dried at 60℃ in a hot-air oven. Those included four cultures of *in vitro* shoots, leaves of each three in vitro plantlets grown on three kinds of media containing different concentrations of IBA, leaves of three potted plants, and six fully expanded leaves collected from a mature or mother plant. Two flowers were also harvested from the mother tree for alkaloid analysis. The scopolamine content was determined by gas chromatography following the procedure of Hiraoka *et al.* (1996). The percentage of scopolamine was expressed on a dry weight basis. The experiments were repeated twice excluding cold-storage under darkness. Values were expressed as mean ± standard deviation. The significance of the difference was assessed by applying Student's $t$-test or Duncan's multiple range test ($p<0.05$).

Shoots stored for 6 and 12 months at different light and dark conditions showed variation in their response. At low temperature (5℃) all the shoots died in both the light and dark conditions. However, 100% shoots survived at 10 and 15℃ in the light after 6 months storage. Shoots stored for 12 months showed 100% viability at 15℃ in the light. The reduction in percentage survival was observed in the dark at 10 and 15℃. In these conditions (cold storage for 6 and 12 months) 40 - 80% shoots could survive. The shoot number hardly increased during storage for 6 or 12 months under either condition. The shoot length increased nearly four times after 12 months of storage. Leaf color remained green at 6 months of storage at 10 and 15℃ in the light, but it became yellowish after 12 months of storage. Shoots partially died under dark conditions and remained healthy in the light at 10 and 15℃. Shoots recultured after 6 and 12 months of storage did not show any differences in their multiplication potential when compared to respective control (**Table 1**). On rooting medium, 80% or more shoots rooted in 6 months storage groups, but rooting ratio decreased to 50 - 70% in 12 months storage groups (**Table 2**). Regenerated plants did not show any apparent abnormality in morphology. Biomass production was not affected by cold storage for 6 months, but it decreased significantly by storage for 12 months

**Table 1** Multiplication potential of *Brugmansia versicolor* shoots after three passage of reculture

| Treatment[1)] | 6 months storage | | 12 months storage | |
|---|---|---|---|---|
| | No. of samples | Shoot No./culture[2)] | No. of samples | Shoot No./culture |
| Control | 5 | 2.4 ± 1.1 a | 17 | 1.7 ± 0.8 a |
| L10 | 10 | 2.7 ± 1.6 a | 14 | 1.6 ± 0.5 a |
| L15 | 8 | 3.5 ± 1.2 a | 17 | 1.7 ± 0.8 a |
| D10 | 2 | 3.5 ± 0.7 a | 9 | 1.5 ± 0.5 a |
| D15 | 4 | 3.8 ± 1.9 a | 13 | 1.4 ± 0.7 a |

[1)] L and D stand on light and dark conditions, respectively, and figures indicate temperature during storage.

[2)] Mean values with standard deviations. Means within a column followed by the same letter are not significantly different by Duncan's multiple range test ($p<0.05$).

**Table 2** Biomass and scopolamine production of *Brugmansia versicolor* plantlets regenerated from cold-stored shoots *in vitro*

| Duration and condition of storage | n[2)] | Fresh weight of aerial part (g) | No. of leaves | Fresh weight of leaf (g) | Scopolamine content (%) |
|---|---|---|---|---|---|
| **6 Months** | | | | | |
| Control | 20/20 | 1.26 ± 0.46 a[3)] | 8.0 ± 2.0 a | 0.72 ± 0.33 a | 0.21 ± 0.01 a |
| L10[1)] | 20/25 | 1.26 ± 0.22 a | 9.1 ± 1.1 ab | 0.63 ± 0.16 a | 0.22 ± 0.02 a |
| L15 | 17/19 | 1.25 ± 0.35 a | 9.2 ± 1.0 ab | 0.62 ± 0.27 a | 0.25 ± 0.03 a |
| D10 | 6/7 | 1.06 ± 0.35 a | 10.3 ± 1.4 b | 0.72 ± 0.25 a | 0.20 ± 0.07 a |
| D15 | 13/14 | 1.29 ± 0.45 a | 9.8 ± 1.7 b | 0.76 ± 0.32 a | 0.25 ± 0.03 a |
| **12 Months** | | | | | |
| Control | 10/16 | 1.35 ± 0.19 b | 14.8 ± 5.1 b | 0.79 ± 0.16 b | 0.10 ± 0.01 a |
| L10 | 7/14 | 0.88 ± 0.38 a | 11.7 ± 2.9 ab | 0.52 ± 0.21 a | 0.19 ± 0.01 c |
| L15 | 8/14 | 0.74 ± 0.29 a | 11.1 ± 1.9 a | 0.53 ± 0.14 a | 0.15 ± 0.03 bc |
| D10 | 7/14 | 0.85 ± 0.32 a | 11.6 ± 2.6 ab | 0.59 ± 0.17 a | 0.13 ± 0.01 ab |
| D15 | 10/14 | 0.86 ± 0.32 a | 11.0 ± 1.8 a | 0.54 ± 0.16 a | 0.20 ± 0.05 c |

[1)] See Table 1.

[2)] No. of rooted shoots/No. of inoculated shoots.

[3)] Mean values with standard deviations. Means within a column of each duration followed by the same letter are not significantly different by Duncan's multiple range test ($p<0.05$).

irrespective of storage condition compared with the control. No significant differences in biomass production were recorded for plantlets raised from shoot cultures that had been stored under 8 conditions differing in light illumination, temperature, and storage period. None of the shoots were alive when cold-stored for 18 months at 5 or 10℃ in both the light and dark conditions. However, 2 and 3 out of 5 cultures withstood the storage at 15℃ in the light and in the dark, respectively. The foliage of those shoots was mostly damaged, but the shoots grew on shoot multiplication medium under normal culture condition and rooted on rooting medium. The *in vitro* plantlets accumulated as much scopolamine as control ones.

In the present study, a simple shoot multiplication protocol and a medium term preservation method of *B. versicolor* plants have been developed. Shoot cultures could be stored in the light at 15℃ for 12 months. This is in agreement with data on other tropical species which could be stored at the same temperature for longer periods (Banerjee and de Langhe, 1985; Sharma and Chandel, 1992). All the *B. versicolor* shoots stored for 12 months at 15℃ in the light grew well when recultured under normal culture condition, but shoots stored at 5 ℃ could not survive. This could be attributed to the fact that the species growing in the tropical region resulted in the death of shoots at 5℃. It is likely that the highest survival percentage was obtained at 15℃ after 12 months of storage, considering the minimum of monthly average temperatures, 23℃, at the natural habitat regions of the species (Legates and Wilmott, 1990; Preissel and Preissel, 2002). Light seemed to

**Table 3** Relationship between growth stage and alkaloid accumulation in the leaf of *Brugmansia versicolor*

| Growth Stage | No. ofsamples | Hormone concentration | Scopolamine content (% of dry weight) |
|---|---|---|---|
| *In vitro* shoot | 4 | 1 $\mu$M kinetin | 0.032 ± 0.001[1)] |
| *In vitro* plantlet | 3 | 0.1 $\mu$M IBA | 0.16 ± 0.09 |
| | 3 | 1 $\mu$M IBA | 0.15 ± 0.08 |
| | 3 | 10 $\mu$M IBA | 0.20 ± 0.05 |
| Potted plant | 3 | - | 0.17 ± 0.02 |
| Mature plant | 6 | - | 0.11 ± 0.03 |

[1)] Mean ± standard deviation

affect the survival percentage of 6 months old stored shoots. All the shoots (100%) survived in the light condition at 10 and 15℃, but this value reduced to 40-80% in the dark. The shoot appearance and leaf color were also affected by light condition, green in the light and yellowish in the dark condition. Light affected shoot survival percentage, but scarcely seemed to affect post-storage vigor of shoots or *in vitro* plantlets. Irrespective of the conditions (light and dark), recultured shoots multiplied as vigorously as the control and did not show any variation in their multiplication potential. These results are in agreement on data obtained with other species such as poplar (Hausman *et al.*, 1994), *Glehnia littoralis* (Hiraoka *et al.*, 1995), *Atractylodes ovata* (Hiraoka *et al.*, 2003) where storage condition did not affect the proliferation rate of recultured shoots. Post storage vigor depends on the storage condition and may affect the morphological characteristics (Hausman *et al.*, 1994). In the case of *Brugmansia*, no significant differences were observed between *in vitro* plantlets derived from cold stored shoots and those of control set except the number of leaves. This could be attributed to the fact that the storage condition was favorable to this species and no carryover effect on the morphology of the plants was detected. As for the possible duration of cold storage, 12 months seemed to be the limit under the present storage conditions, because shoot survival percentage was low and shoot appearance was deteriorated to a considerable extent 18 moths after storage.

A whole plant of *B. versicolor* contains scopolamine as a major tropane alkaloid, but hyoscyamine is hardly detected in both the leaf and flower. Its quantity in the leaf varied with different developmental stages from an *in vitro* shoot to a mature plant (**Table 3**). Shoots in multiplication stage produced a lower level of scopolamine. The highest scopolamine content, 0.28%, was recorded in the flower. The scopolamine content in the *in vitro* plantlets is comparable to that of potted plants, demonstrating that assessment of alkaloid accumulation in plants regenerated from cold-stored shoots can be carried out at their *in vitro* plantlet stage instead of a pot or mature stage. The regenerated plants of all the shoot storage regimes accumulated scopolamine not less than the control (**Table 2**). Their alkaloid composition was same with the parent plant, comprising scopolamine as a sole major alkaloid and the amount of other alkaloids was negligible.

It is concluded that *B. versicolor* shoots *in vitro* can be preserved at 15℃ under the weak light illumination for 12 months and that the plantlets regenerated from cold-stored shoots show neither any detectable variation in their morphology nor any reduced potential in the scopolamine accumulation in the leaf.

## Acknowledgements

The authors wish to thank Ms.'s M. Hiro'oka, K. Utashiro and S. Kobayashi for their assistance in experiments. Funding for this study was provided by the Ministry of Education, Science, Culture, and Sports of Japan (grant No. 11672232 to NH and grant No. P-02820 to NH and IDB). IDB also thanks to the Japan Society for the Promotion of Science for being awarded the JSPS Postdoctoral Fellowship for Foreign Researchers (P-02820).

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