

Technical Note

## Trehalose drastically extends the *in vitro* vegetative culture period and facilitates maintenance of *Torenia fournieri* plants

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**Abstract** While *torenia* (*Torenia fournieri* Lind.) is a useful model flower for molecular biological studies of floral architecture, the maintenance of plant materials and resulting transgenic plants requires vegetative propagation due to its heterozygous nature. Reduction of labor and costs for maintaining thousands of *in vitro* *torenia* cultures is therefore a critical issue. We found that substituting trehalose for sucrose drastically extended the culture period to 70 days, which is more than twice as long as for the common, sucrose-based medium, without reduction in plant viability. Comparative measurement of the plant mass indicated that the increased survival benefit of the trehalose-based medium might be on account of improvement in the rhizosphere environment through reduction of root density in the culture, rather than by reduced plant growth. No harmful effects arising from the trehalose-based medium were observed in 1,800 laboratory lines during the bimonthly subculture for over 12 months, except for a wilting on the first transfer to the trehalose-based medium. In conjunction with the use of the commercial food additive, Okome-ni-TREHA<sup>®</sup> rather than reagent-grade trehalose, we have succeeded in reducing the costs and labor associated with the culture medium to less than one third of those for the sucrose-based system.

**Key words:** *In vitro* culture, rhizosphere environment, *Torenia fournieri*, trehalose.

Commercially important flowers such as chrysanthemums, carnations, and roses are heterozygotes; hence maintenance of stable varieties by seed propagation is impossible. Furthermore, inbreeding depression as a result of repeated selfing in some species causes difficulty in breeding pure lines. Therefore, maintenance of these flowers requires vegetative propagation.

Although *torenia* is a heterozygous plant, it is useful for genetic engineering because of its suitability for tissue culture, simplicity of transformation, and short generation time (Aida 2008). We have produced vast numbers of transgenic *torenia* (Narumi et al. 2008, 2011; Sasaki et al. 2010, 2011; Shikata et al. 2011) and ion beam-irradiated *torenia* mutants (Sasaki et al. 2008) in order to generate novel floral traits and thus study phenotypic behavior, while routinely maintaining 1,800 important lines by *in vitro* culture. Because the monthly subculture of these plants is costly and laborious, technical developments to extend the culture period have been awaited. The standard culture period for general, sucrose-based medium has been empirically determined to be between 25 and 30 days to keep all plants in a

healthy condition. *Torenia* plants at the end of the culture period usually reach to the top of the vessels, and continuous culture significantly decreases plant viability through rapid browning and wilting.

Sucrose is the most frequently used carbon source for *in vitro*-cultured plants, including *torenia* (Aida and Shibata 2001), but the effects of trehalose on *in vitro* culture have now been investigated in several plants. Liu et al. (2006) reported that trehalose was effective for propagation of *Phalaenopsis* and *Doritaenopsis* protocorm-like bodies. Furthermore, jojoba (*Simmondsia chinensis*) treated with trehalose exhibited greater adaptability to environmental conditions during acclimatization (Llorente et al. 2007). However, reports on the utilization of trehalose for extension of the culture period are absent. In this study, we report that the addition of trehalose to the culture medium rather than sucrose extends the culture period of *in vitro* *torenia* vegetative culture by more than twofold.

In this study, *Torenia fournieri* Lind. ‘Crown Violet’ was used as the plant material. The stem pieces 2–3 cm in height with some leaves and axillary buds were excised from *torenia* plants cultured for 25 days on 1/2

Abbreviations: MS; Murashige and Skoog.

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strength Murashige and Skoog medium (1/2 MS, pH 5.7) (Murashige and Skoog 1962) containing 3.0% (w/v) sucrose (Wako, Osaka, Japan) and 0.2% (w/v) gellan gum (Wako). Four pieces of each excised plant were then transferred to the culture vessels (CP-M-1, Iwaki, Tokyo, Japan), each containing 50 ml of 1/2 MS medium (pH 5.7) with 0.2% (w/v) gellan gum and either 3.0% (w/v) trehalose dihydrate (Wako) or 3.0% (w/v) sucrose. The plants were cultured in a growth chamber maintained at 25°C under fluorescent light (16 h light/8 h dark,  $85 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Seventy days after subculture, torenia plants grown on the sucrose-based medium turned brownish, in particular around the basal parts of the stems and roots (Figure 1A, left), and the culture medium exhibited browning, probably because of accumulation of waste products such as phenolic compounds (Figure 1B, left). In contrast, torenia plants subcultured on the trehalose-based medium remained healthy (Figure 1A, right), and no browning of the culture medium was observed (Figure 1B, right). Therefore, application of trehalose to the culture medium rather than sucrose extended the culture period more than twofold, thereby reducing the labor input required for subculturing.

After 30 days of culture, the root density in trehalose-cultured plants was lower than that in sucrose-cultured

plants (Figure 2). To understand the physiological role of trehalose in extending the culture period, we performed a comparative experiment on plant weight by focusing on the difference in root growth. The fresh weight of the aerial parts (leaves and stems) and roots at 30 days after subculture were measured for 15 torenia plants (Table 1). Different increments of plant growth were observed between sucrose- and trehalose-cultured plants, although this difference did not show any statistical significance. As observed in Table 1, the total average weight of trehalose-cultured plants (1146.5 mg) was 24% lower than that of sucrose-cultured plants (1503.4 mg). In addition, the roots:aerial parts ratio in the trehalose-grown plants (0.297) was 17% lower than that in the sucrose-grown plants (0.356). These differences reflected our observations on the 12-month maintenance of *in vitro*-cultured torenia, and this result indicates that reduction in total plant mass and relative root mass may synergistically extend the culture period of trehalose-cultured plants, probably by reducing root density and improving the rhizosphere environment. Similar inhibition of root elongation was observed in *Arabidopsis* when sucrose-grown plants were transferred to a trehalose-based medium (Bae et al. 2005; Wingler et al. 2000). Furthermore, Llorente et al. (2007) reported that addition of trehalose to a sucrose-based medium decreased root development in jojoba. Trehalose also reduced root mass in torenia in our study, and may have influenced growth inhibition in the aerial parts. In our

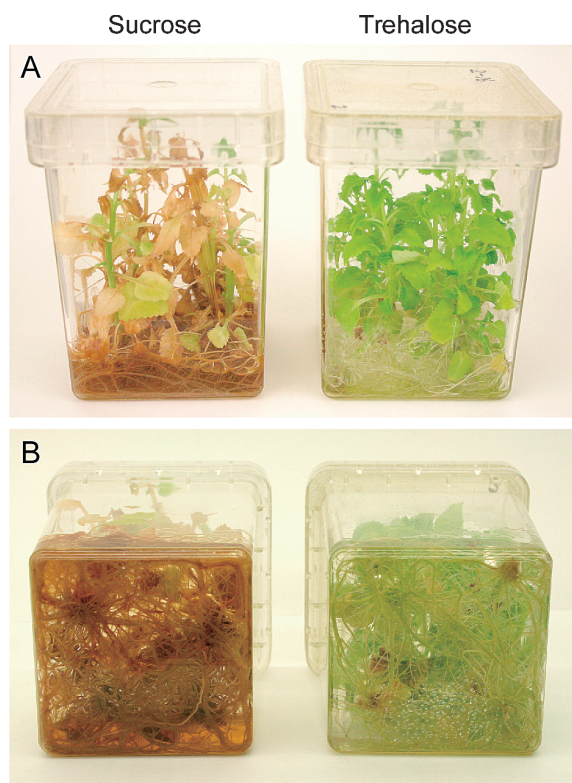


Figure 1. Effect of trehalose application on *in vitro*-cultured torenia. Excised stem pieces were cultured in either a sucrose-based medium (left) or a trehalose-based medium (right) for 70 days; (A) side view, (B) bottom view.

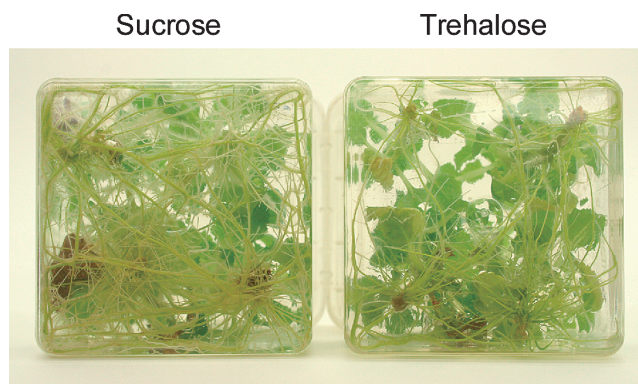


Figure 2. Effect of trehalose application on root density of cultured torenia. Roots after 30 days' subculture in media containing either sucrose (left) or trehalose (right).

Table 1. Differences in growth in *T. fournieri* on trehalose- and sucrose-based media

Carbon source	Fresh weight of leaf and stem <sup>a</sup> (mg)	Fresh weight of root <sup>a</sup> (mg)
Trehalose 3%	884.3 ± 114.4	262.2 ± 70.8
Sucrose 3%	1108.8 ± 103.6	394.6 ± 56.9

<sup>a</sup> Data are mean ± S.E. N=15

Fresh weights were investigated 30 days after planting on each medium.

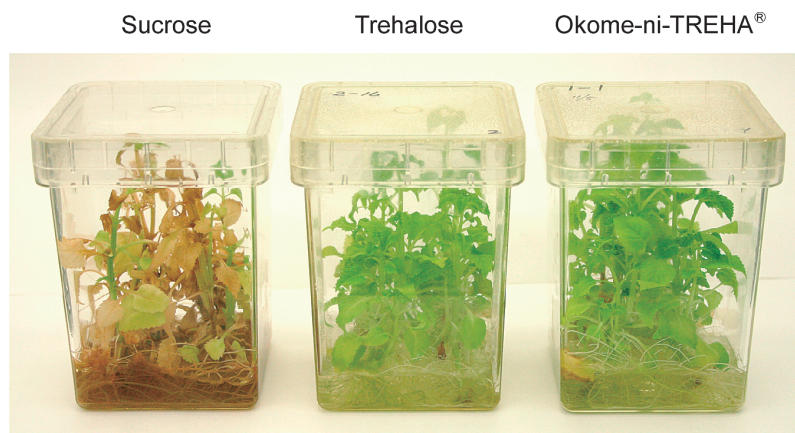


Figure 3. Comparison between the effects of trehalose reagent and Okome-ni-TREHA<sup>®</sup> on *in vitro*-cultured torenia. Excised stem pieces were cultured in a sucrose-based medium (left), a trehalose reagent-based medium (center), and a Okome-ni-TREHA<sup>®</sup>-based medium (right) for 70 days.

study, however, the reduction in total mass may not be a major factor for extension of the culture period because the trehalose-cultured plants maintained the same condition even after the total mass had been increased to the same level as the upper limit of sucrose-cultured plants, and they survived for over 70 days (data not shown). Trehalose not only inhibited root growth in cultured torenia, but it is also possible that it increased abiotic stress tolerance (Iordachescu and Imai 2008) and contributed to enhanced survival. In the future, we would undertake further study on the mechanisms of extension of the culture period and growth reduction in trehalose-based media.

As briefly mentioned above, wilting was observed in up to 25% of plants just after the first transfer to the trehalose-based medium (data not shown). At this stage, most of these plants do not grow well and wilt within 20 days after transfer. Because this is observed only after replacement of the carbon source from sucrose to trehalose and is never observed after the second transfer to the same trehalose medium, it is assumed that acclimatization to trehalose must have stressed the plants to some extent. This wilting phenomenon is seen to a greater extent in chrysanthemum (*Chrysanthemum morifolium* Ramat. ‘Sei-marine’, data not shown), but hardly observed in Japanese gentian (*Gentiana triflora* × *G. scabra* ‘Albireo’ and ‘Polano White’; M. Nishihara, personal communication). Estimation of adaptability and improvement through reduction of this transient stress will be a key factor in application of trehalose to other plant species. We are now investigating the cause of this phenomenon.

In terms of cost-effectiveness, reagent grade trehalose is too expensive and is unsuitable for routine culture; therefore, we examined the alternative product Okome-ni-TREHA<sup>®</sup> (H+B Life Science, Okayama, Japan), which consists of over 98% (dry matter) enzymatically synthesized trehalose and a small amount of unreacted

glucose. Okome-ni-TREHA<sup>®</sup> is available at less than half the cost of standard sucrose used in routine culture. After 2 years of verification experiments with bimonthly subculture, we confirmed that Okome-ni-TREHA<sup>®</sup> is equally effective in extension of the culture period as reagent grade trehalose (Figure 3), and we now apply Okome-ni-TREHA<sup>®</sup> to all our laboratory lines as a substitute for trehalose. Okome-ni-TREHA<sup>®</sup> contains a small amount of unreacted glucose; however, this foreign substance did not affect the maintenance of *in vitro*-cultured torenia. In fact, no harmful effects have been observed over the past 12 months, apart from wilting on the first transfer from sucrose- to trehalose-based medium, as described above. Furthermore, trehalose culture has no discernible impact on any of the events involved in *Agrobacterium*-mediated transformation, at least in torenia (Supplementary Table S1). Together with the extended culture period, cost of this culture medium is around 25% of that of the previous sucrose-based system.

Trehalose-based culture has an additional advantage in the facilitation of transgenic plant production over conventional, *Agrobacterium*-mediated method in torenia. In the previous sucrose-based system (Aida and Shibata 1995), monthly subculture of weekly isolated transgenic adventitious shoots was required for approximately 2 months to ensure that sufficient numbers of transgenic torenia grew enough size for naturalization. In addition, these shoots could not be naturalized simultaneously. In contrast, shoots in the trehalose-based medium can be continuously cultured until naturalization without the need for subculture, allowing naturalization *en masse* (Supplementary Figure S1). Thus, utilization of trehalose contributes not just to a reduction in labor and costs but also to the acceleration of research in torenia by simplifying transformation.



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