

RESEARCH NOTE

## Decreased time from seed to flowering corm size in *Zephyra elegans* via *in vitro* cultivation

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### Abstract

**A. K. Vidal, D.-S. Han, M. Nakano and Y. Niimi. 2012. Decreased time from seed to flowering corm size in *Zephyra elegans* via *in vitro* cultivation. Cien. Inv. Agr. 39(3): 577-584.** Experiments were performed to establish a method to reduce the time from seed to flowering in *Zephyra elegans*. Seedlings took at least four years to produce a flowering corm. Although germination was highest in a water-agar medium, plant necrosis occurred when plants were later transferred; therefore, MS (Murashige and Skoog) medium was selected as the medium for germination. An increase in the light intensity to 9500 lux and in the pH to 6.7, significantly increased the germination rate. Eight weeks after seed germination, seedlings were transferred to MS media with sucrose concentrations of 45, 60, 75, 90 or 105 g L<sup>-1</sup> and a pH 5.7 or 6.7, with the aim of achieving the greatest corm weight gain. After 16 weeks, the best weight gain was obtained in the MS medium with 75 g L<sup>-1</sup> of sucrose and a pH of 6.7. The resulting corms were transferred to pots and grown under greenhouse conditions. Corms weighing under 0.12 g did not sprout, whereas all corms over 0.3 g sprouted and all those over 0.4 g at the end of the *in vitro* culture stage bloomed in the second year of greenhouse cultivation. The time required for development from seedling to flowering corm was reduced to two years.

**Key words:** Chilean geophyte, corm weight gain, flowering, seed propagation, *Zephyra elegans*.

### Introduction

*Zephyra elegans* D. Don is a Chilean endemic geophyte species from the Tecophilaceae family, endemic to the coastal desert areas of northern Chile (Hoffmann, 1989; Muñoz and Moreira, 2000). In nature, *Z. elegans* usually grows in sandy soil (Yáñez *et al.*, 2005). It has been described as having

a high potential as an ornamental crop because of its light blue to blue flowers and growth habit (Bridgen *et al.*, 2002; Kim and Ohkawa, 2001; Kim *et al.*, 1997). *Z. elegans* produces only one daughter corm after the growing season, making natural vegetative propagation difficult to achieve (Yáñez, 2005). Although seed propagation may cause variability, it is currently the only method to obtain a large number of plants. There is no information regarding its massive vegetative propagation. Seed propagation produces seedlings that may require at least four years before produc-

ing a flowering corm (Kim *et al.*, 1996; Kim *et al.*, 1998 and experiments conducted previously in Niigata University Greenhouses).

In the present study, a medium with a pH of 6.7, a value typical of growing conditions natural to *Z. elegans*, (Yáñez *et al.*, 2005), was tested in this species for *in vitro* germination and growth. The effect of light intensity on the *in vitro* germination of *Z. elegans* was also tested. Previous studies showed that seedlings produce a considerable number of small cormlets, which have difficulty surviving to the next growing season under greenhouse conditions (Kim *et al.*, 1998). Corms under 0.3 g will not bloom, and corms of 0.8 g are required for commercial flowering capability (Kim *et al.*, 1998; Yáñez *et al.*, 2005).

Sucrose concentrations of approximately 60 and 90 g L<sup>-1</sup> have been reported as suitable for *in vitro* corm weight gain (Vidal and Niimi, 2008). ABA has been described as increasing the growth of reserve organs by promoting the accumulation of starch (Rook *et al.*, 2006; Sharma *et al.*, 2004). The present study focuses on more precisely defining the optimal sucrose concentrations for *Z. elegans* corm weight gain and also examines the effects of pH and ABA concentration on this weight gain. In addition, the acclimatization of *in vitro*-obtained plants and their subsequent flowering under greenhouse conditions were investigated.

The aim of this research is to reduce the time required for *Z. elegans* to develop from seed to flowering plant by using *in vitro* techniques. Such reduction in developmental time would aid genetic improvement programs and producer efforts to achieve plant propagation on a massive scale.

## Materials and methods

### *Plant material and growing conditions*

*Z. elegans* seeds were collected from their natural habitat in Playa Brava, Huasco, Chile and seeds

had been exposed to natural conditions *in situ* for about six months before collection.

The seeds were disinfected by dipping in ethanol (70%); and then soaking on 0.5% Benlate (DuPont, Delaware, USA) for 20 minutes followed by 6% sodium hypochlorite for 15 minutes, sterile distilled water for 24 hours and, finally, 3% sodium hypochlorite for 15 minutes. Three rinses with sterile distilled water were conducted between each of the disinfection steps (modified from Vidal and Niimi, 2008).

After seed germination, uniform seedlings were selected and used for the following experiments on corm growth, plant acclimatization to greenhouse conditions and flowering.

Unless stated otherwise, the medium used was the MS medium (Murashige and Skoog, 1962) supplemented with vitamins and containing 30 g L<sup>-1</sup> of sucrose. *In vitro* conditions were 25°C with a 24 hour photoperiod supplied by white fluorescent light at intensity of 2000 lux.

The greenhouse experiments were performed in unheated greenhouse facilities on the Niigata University campus (37°52'12"N, 138°56'38"E). The greenhouse temperature was 20±5 °C during the day and 15±5 °C at night. Commercial pelletized red clay soil (Nihonsen© Akadama soil) was placed in pots 20 cm in diameter by 25 cm deep was used because this soil showed the best results for growth and germination when compared in previous experiments with other available soils (data not shown). Water was supplied every two days for the germination experiments and when the surface became dry during the corm cultivation experiments.

The greenhouse planting was performed in late November as recommended by Yáñez *et al.* (2005) for conditions in Shizuoka (Japan). The *in vitro* experiments and the breaking of dormancy were synchronized in order to meet this planting requirement.

### *Determination of preferred germination medium and effect of disinfection on germination capability*

Some of the collected seeds were sown in either agar-water or the MS medium to determine the better medium for germination. Concurrently, two additional group of the seeds, only one of which was disinfected to elucidate the direct effect of the disinfection protocol on germination capability, were sown in soil under the greenhouse conditions. Each of the four treatments consisted of 25 seeds and was replicated five times. The seeds were sown in individual containers with 10 mL of medium for the *in vitro* experiments or 5 seeds per pot for the treatments conducted in soil in the greenhouse. Germination was recorded 8 weeks after sowing. Any seed that produced contaminated seedlings or nonviable seedlings were considered not to have germinated.

### *Effect of light intensity and pH on germination*

For this experiment conducted at light intensities of darkness, 2000 lux and 9,500 lux, seed germination was performed in the MS medium with the pH adjusted to 5.7 or 6.7 before agar addition. For each of the six treatments, 25 seeds were sown and 5 replications were conducted. Germination was recorded every 2 weeks for 10 weeks and was considered not to have occurred if the seedlings were contaminated or nonviable.

### *Enhanced corm growth*

For this experiment, seeds were germinated in MS medium containing with 30 g L<sup>-1</sup> of sucrose. Eight weeks later, the seedlings were transferred to MS medium containing 45, 60, 75, 90 or 105 g L<sup>-1</sup> of sucrose and cultured for 16 weeks with one transfer to fresh medium during week 8 of this period. The plants were germinated and grown in individual test tubes containing 20 mL of the specified medium. Concurrently with the *in vitro*

experiment, seeds were also sown in soil under the greenhouse conditions.

Fifteen seedlings per treatment and five replications were used. The corm weight was recorded 24 weeks after the seeds were sown.

The MS medium with the sucrose concentration that produced the greatest corm weight gain was selected and further supplemented with ABA concentrations of 0, 0.01, 0.1, 1 or 10 mg L<sup>-1</sup> to study the ABA effect on corm growth. Otherwise, the culture conditions and data recording procedures were the same as those in the previous experiment.

### *Acclimatization and transplanting*

After the corm weight gain treatment, the plantlets became dormant corms. Because the dormancy period has been recommended as the appropriate time to release geophytes from their *in vitro* conditions (Dantu and Bhojwani, 1995) the newly formed corms were removed from the culture medium during their dormant stage. The corms were washed with tap water to remove medium and then held at 25 °C for 22 weeks to break the dormancy (Yáñez *et al.* 2005; Kim *et al.*, 1998; Kim *et al.*, 1996). After breaking their dormancy, the corms were planted in individual pots of soil and grown under greenhouse conditions. The survival rate was determined two months after planting by counting the number of corms that did and did not sprout.

### *Flowering capability*

After the first growing season, the corms that survived were collected and again exposed to 25 °C for 22 weeks to break dormancy. Then, the corms were replanted in individual pots of soil and grown under greenhouse conditions to test their flowering capability in the following growing season.

The numbers of flowering and nonflowering plants were recorded three months after sprouting.

### *Statistical analysis of data*

The data were subjected to analysis of variance using a one-way ANOVA model and GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA).

## **Results and discussion**

### *Determination of preferred germination medium and effect of disinfection on germination capability*

Eight weeks after *in vitro* sowing, the seeds germinated in the agar-water medium showed a higher germination rate (98%) than did those germinated in the MS medium (56%). Nevertheless, the seedlings germinated in the former medium were incapable of adapting when later transferred to the MS medium. Plantlet weakness from a lack of nutrients and a consequent inability to overcome the osmotic stress associated with the transfer to the MS medium could have caused the inability to adapt. Moreover, contamination of the agar-water medium could only be detected after the transfer. Based on these inconveniences, the MS medium was selected as the *in vitro* germination medium even though it produced less germination. The MS medium could also be acting as a selection method (Song *et al.*, 2008).

Seeds germinated on soil represent germination where the effect of *in vitro* conditions is avoided. A comparison of the percentage of the disinfected (76%) and non-disinfected seeds (68%) that germinated indicated that the disinfection itself had no adverse effect on the germination rate. Although the difference was not statistically significant, a slightly higher germination rate was observed with the seed disinfection, likely due to chemical scarification of the testa by the

sodium hypochlorite used during the disinfection process (Schiappacasse *et al.*, 2003; Msanga and Maghembe, 1986).

### *Effect of light intensity and pH on germination*

The highest germination rate was observed at light intensity of 9,500 lux and a culture medium pH adjusted to 6.7.

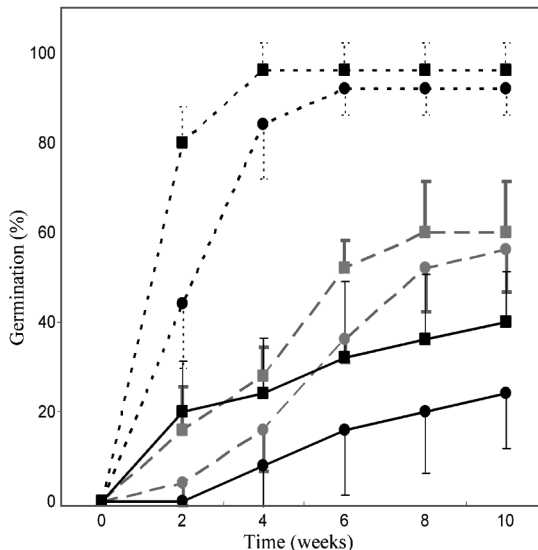
The light intensity significantly affected germination, with germination at the end of the 10-week evaluation period over 90% for the 9,500-lux treatments at the two pH levels, slightly over 50% for the 2,000-lux treatments and, for darkness, less than 40%. Higher light intensity also resulted in significantly faster and more uniform germination. Maximum germination was reached between 4 and 6 weeks for 9,500-lux, 6 and 10 weeks for 2,000-lux and after the 10-week study ended, for the darkness treatment. As the light intensity increased, the pH showed less effect on germination, with a significant effect of pH on final germination rates only for the darkness treatment. At 9,500 lux, the higher pH significant affected only during the second week after sowing (Figure 1).

By increasing the light intensity and adjusting to a higher pH, germination in the MS medium can be increased to nearly 95%.

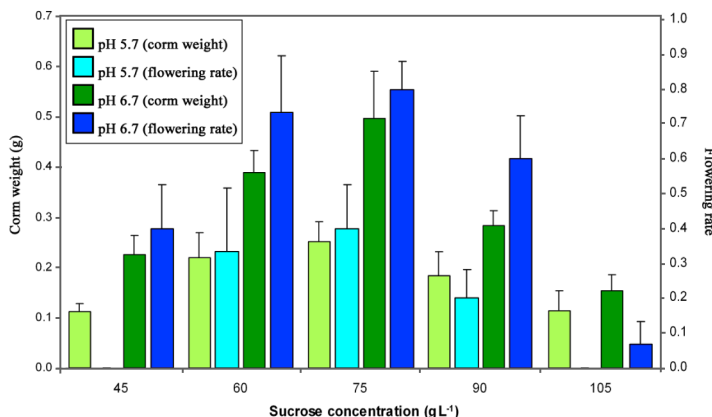
### *Enhanced corm growth*

The seedlings cultivated in a medium containing 75 g L<sup>-1</sup> of sucrose at a pH of 6.7 showed the greatest corm weight gain (Figure 2).

For the 5.7 pH treatment, the 60 and 75 g L<sup>-1</sup> sucrose concentrations produced the greatest corm weight gains. For pH 6.7, the corm weight gain in medium containing 75 g L<sup>-1</sup> of sucrose was significantly greater than that in the medium containing 60 g L<sup>-1</sup> of sucrose. Except for the 105



**Figure 1.** *In vitro* germination rate of *Zephyra elegans* in MS medium adjusted to pH 5.7 or 6.7, at light intensities of 9500 lux, 2000 lux or darkness. Bars = standard errors. -■- pH 6.7, 9,500 lux; -●- pH 5.7, 9,500 lux; -■- pH 6.7, 2,000 lux; -●- pH 5.7; 2,000 lux; -■- pH 6.7, Dark; -●- pH 5.7, Dark.



**Figure 2.** Corm weight and flowering rate of *Zephyra elegans* grown from week 8 to 24 in MS medium containing concentrations of sucrose ranging from 45 to 105 g L<sup>-1</sup> and adjusted to pH 5.7 or 6.7. The flowering rate was determined during year two according to the medium in which the plants were cultivated during their *in vitro* stage during the year one. Bars = standard errors.

g L<sup>-1</sup> sucrose concentration, the pH increase from 5.7 to 6.7 significantly increased the corm weight gain (Figure 2). An early entrance into dormancy was observed in plants growing in the 105 g L<sup>-1</sup> sucrose medium. This shortening of the growing period most likely affected the final corm weight of the plant grown in the latter medium.

High intracellular sucrose concentrations promote the conversion of sucrose to starch (Rook *et al.*,

2006), and this conversion could be the cause of the greater corm weight gains in the media with higher sucrose concentrations, excepting the 105 g L<sup>-1</sup> concentration.

The MS medium with 75 g L<sup>-1</sup> of sucrose and a pH of 6.7 was selected for the ABA test, but no ABA concentration boosted the corm weight. Instead, the weight gain was significantly decreased compared to the control by the addition to the culture

medium of ABA concentrations of 0.1 mg L<sup>-1</sup> or higher. As the ABA concentration increased, the plantlets were observed to enter into dormancy earlier; therefore, the corm growth was likely decreased because of the shortened growth period. It was assumed that plants became dormant at the end of the observation period because no growth could be observed.

### Acclimatization and transplanting

The corms sprouted within two months after planting. Those corms that did not sprout after this period were found to be rotten or desiccated when the soil in the corresponding pot was searched at the end of the growing season.

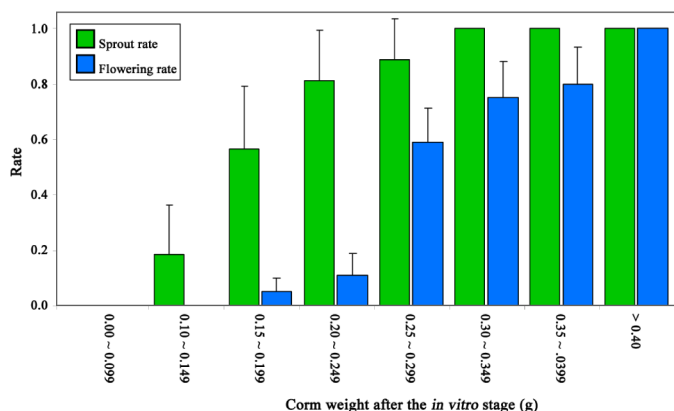
The sprouting rate was significantly higher in the corms generated at pH 6.7 compared with 5.7. The sprouting rate of corms previously grown at pH 6.7 in MS medium containing 60, 75 or 90 g L<sup>-1</sup> of sucrose showed the highest sprouting rates by a statistically significant amount. The higher sprouting rates may be explained by the higher corm weights observed on corms grown under these concentrations and not solely by the composition of the growing medium. To isolate the effect of medium source, the corms were grouped in Figure 3 according to their weight at planting, independent of their *in vitro* origin.

Corms under 0.12 g did not survive acclimatization and did not sprout, while all corms over 0.3 g were capable of sprouting (Figure 3). The sprouting rate was observed to increase as the corm weight at planting increased. No flowering was observed in these one-year-old plants, even though according to the corm weight at planting, corms over 0.3 g have the potential to flower (Kim *et al.*, 1998; Yáñez *et al.*, 2005).

Flowering could be influenced not only by corm weight but also by plant maturity. Further studies are necessary to investigate this possibility.

### Flowering capability

In the second growing season, the greatest flowering frequency was observed in plants that had been grown at pH 6.7 in the MS medium containing with 75 g L<sup>-1</sup> of sucrose during their *in vitro* stage, and this frequency was significantly greater than that observed in their pH 5.7 counterparts. For plants growing on culture media containing 45 or 105 g L<sup>-1</sup> of sucrose, no flowering could be observed at pH 5.7, but some plants flowered for the 6.7 pH treatment (Figure 2). No flowering was observed in plants germinated and grown only in soil under greenhouse conditions.



**Figure 3.** Sprouting rate of *Zephyra elegans* corms transferred to greenhouse conditions and *Z. elegans* flowering rate under greenhouse conditions in year two of cultivation grouped by corm weight after the *in vitro* stage. Bars = standard errors.



All corms over 0.4 g after the *in vitro* stage, produced flowering corms of over 0.8g after the first year of culture under greenhouse conditions and bloomed in the second season (Figure 3). The reduction in the time required for flowering could be due to the increased corm weights at optimum sucrose concentrations (Dantu and Bhojwani, 1995; Le Guen-Le Saos *et al.*, 2002). This effect was increased when the growth medium pH was similar to the natural soil pH and thus suitable for extending the growing period (Yáñez *et al.*, 2005).

The flowering plants were capable of seeding. No difference could be observed between the flowers generated from corms obtained during this experiment and flowers from corms collected from the natural environment, which indicates that the *in vitro* culture itself had no discernible effect on plant morphology.

The above results support the following conclusions. Germination in MS medium at full strength instead of in agar-water medium decreases the germination rate, but this disadvantage of the MS medium can be overcome by increasing the light intensity and pH of the medium.

The fact that no flowering was observed in one-year-old plants with corms over 0.3 g, which are potentially capable of flowering (Kim *et al.*,

1998; Yáñez *et al.*, 2005), could mean that corm weight is not the only influence on flowering. The development of the plant could also be involved. Further studies would be necessary to address this matter.

Even though flowering could not be achieved in the first year, the time from seed to flowering was reduced when compared with greenhouse cultivation only. Enhancement of the corm weight gain by *in vitro* cultivation is a potentially viable process for achieving commercially flowering corms of over 0.8 g from seeds in two growing seasons.

By using this technique, desired phenotypes such as blue flowers (Bridgen *et al.*, 2002; Kim and Ohkawa, 2001) could be selected in a reduced period of time. When seeds are germinated and grown only under greenhouse conditions, no flowering corms are obtained even after four years of cultivation in Niigata University greenhouses.

The reduced time needed to obtain flowering could be due to an increased corm weight gain during the *in vitro* stage. During this stage, a culture medium with an optimum sucrose concentration that was readily available for uptake (Le Guen-Le Saos *et al.*, 2002), and a pH that simulated natural conditions favored an extended growing period (Yáñez *et al.*, 2005).

## Resumen

**A. K. Vidal, D.-S. Han, M. Nakano y Y. Niimi. 2010. Disminución del tiempo requerido desde semilla a cormos con peso adecuado para la floración en *Zephyra elegans* mediante el uso de técnicas *in vitro*. Cien. Inv. Agr. 39(3): 577-584.** El objetivo de este trabajo fue reducir el tiempo requerido desde semilla a floración en *Zephyra elegans*. Plántulas de *Z. elegans* tardaron más de cuatro años en producir un cormo floral. El porcentaje de germinación fue mayor en medio agar-agua, pero las plántulas resultantes no fueron capaces de sobrevivir cuando eran transferidas a medio MS, por lo que el medio MS fue elegido como medio de germinación. Incrementar la intensidad lumínica a 9500 lux y el pH del medio a 6,7 tuvo un efecto significativo en incrementar el porcentaje de germinación. Ocho semanas después de la germinación, las plántulas fueron transferidas a medio MS con concentraciones de sacarosa de 45, 60, 75, 90 o 105 g L<sup>-1</sup> y pH ajustado a 5,7 o 6,7, donde el objetivo era incentivar el aumento el peso de los cormos de las plántulas. Luego de 16 semanas de crecimiento en este medio, se observó que el mayor aumento de peso ocurrió en medio MS con pH 6,7 y 75 g L<sup>-1</sup>

de sacarosa. Los cormos formados en la fase *in vitro* fueron transferidos a macetas y cultivados en invernaderos. Cormos con peso menor a 0,12 g no sobrevivieron, mientras que la totalidad de los cormos sobre 0,3 g emergieron. Todos los cormos con peso superior a 0,4 g al final de la fase *in vitro* florecieron en la segunda temporada de cultivo en invernadero. El tiempo requerido desde plántula a corno floral se redujo a dos años.

**Palabras clave:** Aumento de peso en cormos, floración, geófitas chilenas, propagación por semillas, *Zephyra elegans*.

## References

- Bridgen, M., E. Olate, and F. Schiappacasse. 2002. Flowering geophytes from Chile. *Acta Horticulturae* 570:75-80.
- Dantu, P. and S. Bhojwani. 1995. *In vitro* corm formation and field evaluation of corm-derived plants of *Gladiolus*. *Sci. Hort.* 61: 115-129.
- Hoffmann, A.E. 1989. Chilean monocotyledonous geophytes taxonomic considerations and their state of conservation. *Herbetia* 45: 13-29.
- Kim, H., K. Ohkawa, and K. Sakaguchi. 1996. Effects of storage temperature and duration on flower bud development, emergence and flowering of *Zephyra elegans* D. Don. *Sci. Hort.* 67: 55-63.
- Kim, H., K. Ohkawa, and K. Sakaguchi. 1998. Effects on corm weight on the growth and flowering of *Zephyra elegans* D. Don. *Acta Horticulturae* 454: 335-340.
- Kim, H., K. Sakaguchi, and K. Ohkawa. 1997. *Zephyra elegans* D. Don., a potential new cormous crop. *Act. Hort.* 430: 133-137.
- Kim, H. and K. Ohkawa. 2001. Introduction of two Chilean geophytes, *Leucocoryne coquimbensis* F. Phil. and *Zephyra elegans* D. Don. as new ornamentals. *Acta Horticulturae* 552: 179-183.
- Le Guen-Le Saos, F., A. Hourmant, F. Esnault, and J. Chauvin. 2002. *In vitro* bulb development in Shallot (*Allium cepa* L. Aggregatum Group): Effects of anti-gibberellins, sucrose and light. *Annals of Botany* 89: 419-425.
- Msanga, H.P., and J.A. Maghembe. 1986. Effect of hot water and chemical treatments on the germination of *Albizia schimperana* seed. *Forest Ecology and Management* 17: 137-146.
- Muñoz, M., and A. Moreira. 2000. Géneros Endémicos de Monocotiledóneas de Chile Continental. *Actas Museo Nacional de Historia Natural*. Available at [www.chlorischile.cl/Monocotiledoneas/zephyra\\_gen.htm](http://www.chlorischile.cl/Monocotiledoneas/zephyra_gen.htm). In Spanish.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol. Plant* 15: 473-497.
- Rook, F., S. Hadingham, Y. Li, and M. Bevan. 2006. Sugar and ABA response pathways and the control of gene expression. *Plant, Cell & Environment* 29: 426-434.
- Schiappacasse, F., P. Peñailillo, and P. Yáñez. 2003. Propagación de Bulbosas Chilenas Ornamentales. Editorial Universidad de Talca Publishers, Chile. 21 pp.
- Sharma, P., S. Pandey, A. Bhattacharya, P.K. Nagar, and P. Ahuja. 2004. ABA associated biochemical changes during somatic embryo development in *Camellia sinensis* (L.) O. Kuntze. *Plant Physiol.* 161: 1269-1276.
- Song, J., H. Fan, Y. Zhao, Y. Jia, X. Du, and B. Wang. 2008. Effect of salinity on germination, seedling emergence, seedling growth and ion accumulation of a euhalophyte *Suaeda salsa* in an intertidal zone and on saline inland. *Aquat. Bot.* 88: 31-37
- Vidal, A. K. and Y. Niimi. 2008. *In vitro* initiation of seeds and further corm weight gain in *Zephyra elegans*. *Acta Horticulturae* 865:301-304.
- Yáñez, P., H. Ohno, and K. Ohkawa. 2005. Temperature effects on corm dormancy and growth of *Zephyra elegans* D. Don. *Sci. Hort.* 105: 127-136.