Propagation of *Zephyra elegans* D. Don by the Use of *In Vitro* Techniques

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Contents

Chapter 1

General Introduction

Background of this study

International flower market is in constant demand for new crops in order to stimulate economic growth and establish new fashions and tendencies. Chile is recognized for having native species with ornamental potential (Schiappacasse *et al.,* 2003; Bridgen *et al.,* 2002; Bridgen, 2001; Kim and Ohkawa, 1997).

Chile is can be considered an ecological island because sea, mountains and desert act as geographic barriers that isolate its biological communities from the rest of the continent. Because of this, endemism rate goes to 52%. For geophytes this percentage is even higher, and rises to 90% (Hoffmann *et. al,* 1998).

In years in which "El Niño" phenomenon is present it is very probable that in the coastal areas of northern Chile (Atacama Desert) rain reaches 50 mm during the months from May to September. This makes possible the sprouting and flowering of various species (including *Zephyra elegans*) in an event called "Desierto Florido" or "Flowering Desert" (Vidiella *et al.,* 1999).

In 1989, Hoffmann stands out the ornamental potential of several desert flowering geophytes, including *Zephyra elegans, Leontochir ovallei* (Garra de León), *Pasithea coerulea* (Azulillo), *Conanthera, Herbertia, Calydorea xyphioides* y *Tigridia philippiana* (Flor de un Día). Kim *et. al*, in 2001 and 1997, specially points out the potential as a cut flower for *Zephyra elegans* because of its prolonged vase life, "fresh" colors, tidy architecture, beautiful flower and adequate stem length.

Zephyra elegans D. Don (Edinburgh New Philos. J. 13 (oct): 236, 1832) belongs to Liliopsida class (Monocotyledons), Tecophilaceae family, and the endemic genera *Zephyra*, with only one specie. It is native to the coastal sandy and rocky areas of the Atacama Desert, in northern Chile (Muñoz, 1985). Distribution locations of *Zephyra elegans* are described in figure 1. In 2001, Ehrhart proposes *Z.*

compacta as a new species belonging to the *Zephyra* genera. But this study was based only on morphological characteristics and has not been corroborated by any other authors.

At first, this species was classified as belonging to the Liliaceae family, but further studies, made it be classified into the Tecophilaceae family, due to its cormous structure and partially inferior ovary (Simpson and Rudall, 1998). Tecophilaceae family has 8 genera, and near 23 species in Chile, South Africa, Tropical Africa, Madagascar and California (Simpson and Rudall, 1998).

In Chile generas *Conanthera, Tecophilaea* and *Zephyra* exist and are endemic (Muñoz and Moreira, 2000; Hoffman, 1989).

The zone of most abundance of *Z. elegans* corresponds to a limited zone in the route leading towards Playa Brava (fig. 1), south of Huasco (28°29'12"S, 71°14'25"W). Here is also located the most abundance in color variation. Colors observed here go from whole plane white flower petal flowers to a white center and deep blue en the distal area of the petal flower (fig. 2). Light blue and white colors are easy to observe in other zones, but deep blue tones are only seen in the area near Playa Brava (fig. 1). Morphological variability is also more abundant in this area (fig. 3). These phenotypes are interesting material for the generation of new products for international flower market. It has potential as cut flower, flower pot and direct *in vitro* sell. The size of an adult flowering corm allows having a flowering *in vitro* plant, which may be attractive to sophisticated consumers (fig. 4).

In the zone of most variability, on the way to Playa Brava, south of Huasco, a steel refinery (Compañía Minera del Pacífico, 28°28'48"S, 71°14'50"W) and a thermoelectrical power plant (Termoeléctrica Guacolda, 28°27'54"S, 71°15'23"W) can be seen generating a cloud of contaminating particles originated from its chimneys (fig. 5). During collection of plant material of this zone it was very clear the accumulation of this contaminating material on the surface of soil and plants (fig. 6). This may affect wild life in this area, so some kind of protection and conservation of genetic material should be done as soon as possible.

In order to be able to use this vegetal material it is necessary to know more about its agronomic features. Among these, one of the most important ones is multiplication. (Kim *et al*, 1997).

Domestication, propagation and growing of Zephyra elegans

Mother corm degrades throughout growing season, while a new daughter corm is being formed. This corm grows and transforms into the mother corm for next season. In this process a secondary corm is rarely formed. This structure has to fulfill certain thermal requirements in order to sprout and flower when environmental conditions are adequate.

Thermal requirements in nature are complied after a dry warm period of 7 to 10 months. Growing season corresponds to a soft, short and rainy winter, as it occurs during "El Niño" phenomenon and subsequent "Desierto Florido" (Flowering Desert) (Vidiella *et al.,* 1999; Kim *et al.*, 1997). In nature, if "El Niño" phenomenon does not occur, rain quantity will not be enough and plants will not sprout even thermal requirements were previously fulfilled (Kim *et al.*, 1997).

Artificially, dormancy can be broken by placing the corms at constant 25°C for 22 weeks in a dry environment. This way, a sprout and flowering rate of up to 100% can be achieved. Temperatures inferior or equal to 20°C do not break dormancy, and above 30°C make the flowering rate drop down to 62,5% (Kim *et al.*, 1997).

In the prefecture of Shizuoka, Japan, *Z. elegans* is a geophyte planted in autumn, with requirements similar to Fresia. Corms go into deep dormancy after completing their cycle. Corms planted around 3 to 5 cm deep will generate contractile roots, which deepen the corm to around 10 cm (Kim *et al.*, 1996).

Time needed for obtaining a flowering corm has not been directly determined. From several several studies it can be extrapolated that it takes at least 4 growing seasons to obtain a flowering corm when starting from seeds (Yanez *et al.,* 2005; Kim and Ohkawa, 2001; Kim *et al.*, 1998; Kim and Ohkawa, 1997; Kim *et al.,*

1997; Kim *et al.*, 1996). Natural vegetative reproduction of *Zephyra elegans* is by generation of corms, reproduction rate by this method is not viable due to its low rate (Kim *et al.*, 1997).

Aim of the present study and outline of this thesis

The general objective of this study is to create protocols that allow the *in vitro* propagation by seeds and reduce time needed from seed to flowering corm; and achieve vegetative propagation of *Zephyra elegans.* These *in vitro* cultivated plantlets have to be able to later grow and flower under greenhouse conditions.

Specific objectives for seeds include achieving adequate levels of disinfection, achieve *in vitro* germination, induction of enhanced corm weight gain and obtain flowering corms in a reduced amount of time when compared to green house conditions. Plants have to later be acclimatized to greenhouse growing conditions and flower.

Specific objectives vegetative propagation include determination of disinfection method, determination of most suitable plant material for vegetative initiation, determination of adequate mediums for vegetative initiation and later propagation.

When data analysis was performed, data was subjected to analysis of variance using one way ANOVA at $p \leq 0.05$. Graphpad Prism 5 software (Graphpad Software Inc., La Jolla, CA 92037 USA) was used for the analysis.

Chapter 2

Preliminary experiments that justify *in vitro* propagation of *Zephyra elegans*

Introduction

Introduction

Most of previous recorded work with *Zephyra elegans* has been performed in greenhouses in Shizuoka University (Yanez *et al.,* 2005; Kim and Ohkawa, 2001; Kim *et al.*, 1998; Kim and Ohkawa, 1997; Kim *et al.,* 1997; Kim *et al.*, 1996). Not much detail is given in these about specific growing conditions in these studies, so it was important to study if this specie adapted to grow in coastal desert climate (Hoffman, 1989) would also be able top adapt to Niigata greenhouse growing conditions. Ability to germinate was studied and also sprouting and flowering was tested under different soils.

Greenhouse experiments were performed in Niigata University campus non heated greenhouse facilities (37°52'12"N, 138°56'38"E).

Chapter 2

Preliminary experiments that justify *in vitro* propagation of *Zephyra elegans*

Experiment 1

Planting season

Introduction

Corms that were collected from nature (in the zone of most abundance of *Zephyra elegans,* in the route leading towards Playa Brava (fig. 1), south of Huasco, 28°29'12"S, 71°14'25"W), had to be tested under Niigata greenhouse growing conditions. Previous experiments performed in Shizuoka prefecture (Yanez *et al.,* 2005; Kim *et al.*, 1998; Kim and Ohkawa, 1997; Kim *et al.,* 1997) served as basis for these experiments.

Temperature regime has been demonstrated as being crucial for greenhouse growing of *Zephyra elegans* (Yanez *et al*, 2005), and under Shizuoka conditions, mid-November to mid-December is the recommended planting season in these previously performed experiments.

Results from this experiment will be used as a standard in future experiments involving greenhouse acclimatization and growing of *Zephyra elegans*.

Materials and methods

Plant material for this experiment was corms collected from nature in Playa Brava, Huasco (fig. 7). Corms weighing between 1.0 and 1.5 g were selected for this experiment. Corms collected from nature were exposed to 25°C for 24 weeks in order to break dormancy (Yanez, *et al.,* 2005).

Corms were planted in the middle of September, November and January. Soil selected for these experiments was Akadama commercial soil in individual pots 25 cm diameter by 30 cm deep. Corms were planted 5 cm deep.

Twelve corms per treatment were planted and 3 repetitions were made. Sprout rate and flowering rate were recorded.

Results and discussion

Highest results in sprout rate and flowering rate were seen on plants planted in November, followed by September, but with no statistical difference between them (table 1). When corm planting was done in January, sprouting and flowering decreased significantly. It seems increasing temperatures at the beginning of spring (March-April) affect flowering negatively. Similar results were seen in previous studies (Kim and Ohkawa, 1997).

Chapter 2

Preliminary experiments that justify *in vitro* propagation of *Zephyra elegans*

Experiment 2

Soil for growing under greenhouse conditions

Introduction

In nature, *Z. elegans* usually grows in sandy soils in coastal areas (Yanez *et al.*, 2005; Hoffman, 1989). Many Chilean geophytes have shown selectiveness towards growing substrate (Schiappacasse *et al.*, 2003) so this experiment was performed in order to determine the best growing soil for greenhouse conditions. Results from this experiment will be used as a standard in future experiments involving greenhouse growing *Zephyra elegans*.

Materials and methods

Corms collected from nature weighing from 1 to 1.5 g were planted in mid November. Corms were planted 5 cm deep.

As soil in Niigata University fields fit visually with the soil seen during collection of samples of *Zephyra elegans* in its natural habitat, it was selected as a soil to be tested. Akadama commercial or perlite were also selected to be tested as growing substrate.

Twelve corms per treatment were planted and 3 repetitions were made. Sprout rate and flowering rate were recorded.

Results and discussion

Highest sprout rate and flowering rate were seen in plants growing on Akadama or perlite soils (table 2). Sandy soil had lower sprout rate and flowering rate. This could be due to hardening of the surface of the soil, constituting a physical obstacle to sprouting (fig. 8). Plants that were able to sprout and flower were similar in

height to plants growing on other soils. Also it was noted that perlite offered little support for the plant, which tended to fall when flowering occurred (fig. 9).

According to commercial source, Akadama soil pH varies from 6.6 to 7.0, which is considered suitable for *Z. elegans* growth (Yanez *et al.,* 2005). Though it is not part of this experiment, it is important to mention that Yanez *et al.* (2005) mention pH of 6.5 to 6.7 in natural habitat. Analysis of soil sampled during collection of *Zephyra elegans* in the same region (Huasco), showed pH of 8.86 (table 3). Future studies on methods of the determination of pH are necessary in order to explain the over two whole points difference in pH measurements, and this way determine best growing conditions.

Chapter 2

Preliminary experiments that justify *in vitro* propagation of *Zephyra elegans*

Experiment 3

Phenotype difference observed under same growing conditions

Introduction

Vegetative propagation would be justified if phenotypes are determined to some extent to the genotype of the plant and not only due to environmental conditions. Plants with different genotypes, collected from nature were grown under standard greenhouse conditions, avoiding the effect of different growing conditions having different effects on plant growth.

Materials and methods

Fifty corms between 0.3 and 1.8 g were planted in individual pots 25 cm diameter by 30 cm deep with Akadama soil. Planting was done in mid-November. Corm weight at planting and plant height at full blossom was recorded. Flower colors were also compared to check for flower color variance under the same environmental conditions.

Results and discussion.

Plant height was not correlated to corm weight at planting (fig. 10). As environmental conditions were the same for all individuals, this evidence suggests that the difference in plant height is not only related to corm weight as suggested by Kim and Ohkawa in 1997, but could be also caused by different genotypes of these individuals. A variety of flower colors similar to the ones observed in nature were also observed. As growing conditions were the same for all plants, the evidence would suggest that plant height (growth habit) and flower color variability is determined by also by genotype.

Results of this experiment would suggest that vegetative propagation would be a useful tool in order to multiply individuals with desirable treats. As traditional vegetative propagation methods have been unsuccessful, *in vitro* techniques are tested with the objective of achieving vegetative propagation.

Chapter 2

Preliminary experiments that justify *in vitro* propagation of *Zephyra elegans*

Experiment 4

Time needed from seed to flowering corm under greenhouse conditions

Introduction

When seeds germinate they produce a small cormlet, which starts to gain in weight in every growing season. Corm weight has been reported as influencing flowering capability, Corms under 0.3 g will not flower (Kim *et al.*, 1998; Yanez *et al.*, 2005) and corms of 0.8 g are required for commercial flowering capability (Kim *et al.*, 1998; Yanez *et al.*, 2005). Though it has not been directly studied, by cross reference of previous studies it can be estimated that at least growing seasons are needed in order to achieve mature flowering corms. In nature corms only grow during rainy years, so this time could extend to over 20 years depending on the occurrence of the "El Nino" phenomenon (Munoz and Moreira, 2000; Hoffman, 1989). In this study the time needed to obtain a mature flowering corm from seed will be determined.

Materials and methods

Plant material for this experiment *Zephyra elegans* collected from nature in Playa Brava, Huasco (fig. 11).

Three hundred seeds were sown in early November of 2005 in seedling containers with Akadama soil and watered as surface got dry. Seedlings were transferred to individual pots 25 cm diameter by 30 cm deep with Akadama commercial soil at the end of the first growing season.

Corms were lifted in late May, when corms became dormant. Dormancy break treatment of 25°C for 24 weeks was applied and corms were again planted in mid-November. This process was repeated every year.

Growth seasons needed for the generation of flowering corms were recorded.

Results and discussion

After 4 growing seasons no flowering corms were observed. As of January 2011, during the fifth growing season it is yet to be seen if flowering corms will be obtained. All 23 corms planted during mid-November 2010 were over 0.3 g and 12 of them are over 0.8 g so according to previous literature, flowering should occur in late February. It should be noted that from the initial 300 seeds, which generated 218 seedlings, only 23 are left. Main losses were seen during the second and third growing seasons due to corm rotting. Corm death has been mentioned as common within small cormlets and corms of *Zephyra elegans* (Kim *et al*, 1998).

Chapter 3

Production of flowering corm *via in vitro* culture of seeds

Introduction

Introduction

With the use of modern techniques, a larger number of genetic improvement programs include *in vitro* steps. These techniques make possible embryo rescue in traditional genetic improvement programs and genetic engineering. These techniques are also used to multiply vegetal material (Bridgen, 2001).

In vitro techniques are based in aseptic conditions of the plant material, growth medium and aseptic controlled working conditions so disinfection process is an important initial step to start an *in vitro* protocol. A balance between successful disinfection and explant survival must be achieved.

One of the most used techniques for disinfection of vegetal material is the use of sodium hypochlorite (NaClO) in different concentrations and time of exposure. Combination with other techniques have also been reported (Padilla and Encina, 2003; Hodson de Jaramillo *et al.*, 2008).

It has been described as having high potential as an ornamental crop due to its blue flowers (figure 5) and growth habit (Kim and Ohkawa, 2001; Bridegen *et al.*, 2002). Yanez *et al.*, 2004 reported that *Z. elegans* produces only one daughter corm after the growing season (Kim and Ohkawa, 1997), making natural vegetative propagation difficult to achieve. Although seed propagation may cause variability, at the present, it is the only method to obtain large number of plants. There is no information regarding its massive vegetative propagation. Seed propagation produces seedlings that may take four years or more in order to produce a flowering plant (Yanez *et al.,* 2005; Kim and Ohkawa, 2001; Kim *et al.*, 1998; Kim and Ohkawa, 1997; Kim *et al.,* 1997; Kim *et al.*, 1996; and experiments performed in Niigata University greenhouses, described in chapter 2). Not many experiments have been done with respect to complete the life cycle of *Zephyra elegans.* According to Yanez *et al.* (2005), in nature, *Zephyra elegans* usually grows in sandy soil with pH 6.7 but analysis performed with soil sampled during collection of plant material showed pH of 8.86. Seedlings produce small cormlets,

which have difficulty in surviving to the next growing season (Kim *et al.*, 1998). Decreasing time from seed to flower may also be a very interesting aid to genetic improvement programs. Corm weight at planting is not only related to sprout rate (plant survival) but also to flowering rate. Corms under 0.3 g will not flower (Yanez *et al.*, 2005; Kim *et al.*, 1998) and corms of 0.8 g are required for commercial flowering capability (Yanez *et al.*, 2005; Kim *et al.*, 1998).

The main objective of experiments performed in this chapter is to achieve flowering corms in a reduced period of time as compared to nature, field or greenhouse conditions.

The initial seed material for these experiments was collected from nature. Collection was performed in Playa Brava, South of Huasco, Chile (28°29'12"S, 71°14'25"W). Near here is placed industrial port Huasco, and thermo electrical plant Guacolda, who could post a thread to the presence to wildlife in this area, including *Zephyra elegans*.

Chapter 3

Production of flowering corm *via in vitro* culture of seeds

Experiment 1

Seed disinfection with sodium hypochlorite

Introduction

As disinfection method becomes more aggressive it can decrease survival and a balance has to be achieved between them. In this experiment, plant survival will be measured as seed germination. Seed germination can be influenced by the medium in which the seeds are germinated, so in this experiment, growth medium for germination will also be analyzed. In preliminary works, seeds have shown to be difficult to disinfect so several studies were performed in order to achieve satisfactory levels of decontamination for *in vitro* sowing. Difficulty for disinfection may be due to irregular surface of the testa (fig. 12).

Materials and methods

Seeds were collected from its natural habitat in Playa Brava, Huasco. Seeds had been exposed to natural conditions *in situ* for about half a year before its collection. Germination tests done previous to this experiment showed they had germination capability. After seeds germinate, in disinfection and medium selection experiments, these same plantlets will be used as the plant material for following experiments on corm growth and later acclimatization to greenhouse conditions.

Best seed disinfection protocol was aimed. For all experiments, seeds were pre-disinfected by dipping in ethanol 70%. Whenever sodium hypochlorite was used, Tween 20 at 0.5% was also added. Seeds were rinsed 3 times with sterile distilled water after each disinfection. Best result of previous experiment was used as standard for the following experiment. Seeds were initiated in individual test tubes with 10 mL of full strength MS basal medium supplemented with vitamins and 3% sucrose. Solidifying agent used was agar at concentration of 0.7%. Medium pH was adjusted to 5.8 prior to addition of gelling agent. Autoclaving was performed

at 121°C for 15 minutes. *In vitro* growth chamber conditions were 25°C and 24 hour photoperiod under a light strength of 2000 lux.

Sodium hypochlorite (NaClO) concentrations of 1, 2, 4 and 6% w/v of active ingredient were tested for 15 minutes. Disinfectant solution was obtained by diluting sodium hypochlorite at 6% commercial solution in distilled water. Once the most adequate concentration of NaClO was determined, different disinfection times were tested in order to lower contamination levels. The best result obtained was again tested for 15, 30, 60 and 120 minutes.

For each treatment, 25 seeds were used and 4 repetitions were made. Seeds were initiated in individual test tube containers with 10 mL of medium each. Contamination and germination was measured 3 weeks after initiation. Seeds that gave origin to abnormal plantlets were not considered as germinated.

Results and discussion

Contamination level was lowest when sodium hypochlorite concentrations of 4 and 6% were used (table 4). These higher concentrations promoted germination instead of reducing germination percentage, probably due to testa softening, effect also seen in other Chilean geophyte species (Schiappacasse *et al.,* 2003). Though there was no statistical difference between 4 and 6% concentration, due to slightly better results, 6% sodium hypochlorite concentration was assumed as the best result at this stage.

When length of disinfection time was increased in order to achieve lower contamination rates, abnormal plantlets could be seen when time was extended to 30 minutes or more (fig 13). Lower germination rate could be attributed to this cause, especially as disinfection time got longer (table 5). Although with disinfection times of 60 minutes and 120 minutes contamination was low, due to abnormal plant appearance (not considered as germinated due to its lack of survival

afterwards), 15 minutes was still considered as the best disinfection time when NaClO at 6% is used. Extending disinfection time was not a viable option for lowering contamination rate.

Later it was noted when 6% sodium hypochlorite was used for 15 minutes, initially contamination was reduced to levels near 12% but when seeds were cultured beyond the observation period of 3 weeks, later appearing contamination was present in around 90% of the cases. This contamination could be seen after on month from germination or when plantlets were transferred to fresh medium. Rough seed surface or the pathogen inocule being present on parts other than the surface of the seed could be the cause for this. Further experiments extending this observation period were done in order to achieve a protocol to lower this later appearing contamination.

Chapter 3

Production of flowering corm *via in vitro* culture of seeds

Experiment 2

Seed disinfection with methods complementary to sodium hypochlorite

Introduction

Though initially, disinfection with sodium hypochlorite at 6% for 15 minutes may seem successful, around one month after germination or after transfer to fresh medium, a later appearing contamination could be seen in around 90% of the cases. This was not initially considered and became a mayor issue in *in vitro* growing of seeds. Complementary disinfection methods had to be tested in order to overcome this later contamination.

Materials and methods

The effect of Benzalkonium at 1% w/v of active ingredient for 15 minutes and the effect of Benomyl at 0.5% w/v of active ingredient for 20 minutes (modified from Hodson de Jaramillo *et al.,* 2008), performed prior to the disinfection with sodium hypochlorite were studied.

Also, the effect of double disinfection was tested by disinfecting with sodium hypochlorite at 6% w/v of active ingredient for 15 minutes, soaking in sterile distilled water for 24 hours, followed by a second disinfection with NaClO at 3% for 15 minutes (modified from Padilla and Encina, 2003).

For each treatment, 25 seeds were used and 3 repetitions were made. Seeds were initiated in test tubes with 10 mL of full strength MS basal medium supplemented with vitamins and 3% sucrose. Solidifying agent used was agar at concentration of 0.7%. Medium pH was adjusted to 5.8 prior to addition of gelling agent. Autoclaving was performed at 121°C for 15 minutes.

In vitro growth chamber conditions were 25°C and 24 hour photoperiod under a light strength of 2000 lux.

Contamination and germination was measured 6 weeks after initiation. Seeds that gave origin to abnormal plantlets were not considered as germinated.

Results and discussion

Contamination levels were lowest when Benomyl was used (modified protocol from Hodson de Jaramillo *et al.,* 2008). Although Benzalkonium reduced contamination, this reduction was not statistically significant. Also no significant effect on germination levels was observed.

Double disinfection also had a significant effect in reducing contamination. Double disinfection not only reduced contamination, but also increased germination rate (Table 6).

Best disinfection protocol is as follows:

Dipping in Ethanol (70%) for 5 seconds; Benomyl 0.5% for 20 minutes; NaClO 6% for 15 minutes; Soaking in sterile distilled water for 24 hours; NaClO 3% for 15 minutes, with rinses with sterile distilled water after every disinfection.

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Production of flowering corm *via in vitro* culture of seeds

Experiment 3

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

Introduction

Germination rate is usually diminished when disinfection protocols are applied because plant material is also affected by this disinfection treatment. The effect of disinfection will be tested by comparing germination of disinfected and non disinfected seeds, and this cannot be done under *in vitro* conditions. Germination in a medium providing less osmotic stress may increase germination (Song *et al.*, 2008), so it was also tested in with the aim to increase the near 60% germination obtained in the previous experiment.

Materials and methods

Seeds were germinated in Agar water medium or MS medium supplemented with vitamins and 3% sucrose. Parallel to this, seeds were initiated in soil under greenhouse conditions. Seeds sown in soil were divided into two groups, disinfected or non-disinfected in order to see the direct effect of disinfection protocol on germination capability. Seed disinfection was performed by dipping in Ethanol (70%) for 5 seconds; Benomyl 0.5% for 20 minutes; NaClO 6% for 15 minutes; Soaking in sterile distilled water for 24 hours; NaClO 3% for 15 minutes, with rinses with sterile distilled water after every disinfection. For each treatment, 25 seeds were used and 3 repetitions were made. Seeds were initiated in individual containers with 10 mL of medium for *in vitro* experiments, and 5 seeds per 100mL pots with Akadama soil for greenhouse germination. Germination was measured 6 weeks after initiation. Contaminated seedlings or seeds that gave origin to abnormal plantlets were not considered as germinated.

Result and discussion

Seeds germinated in agar water medium had the highest germination rate (fig. 14), but plantlets germinated in this medium, when transferred, were incapable of adapting to MS medium with sucrose concentration levels of 3% or more. Moreover, contamination on agar water medium could only be determined once transfer was done, making the process inefficient, so MS medium with 3% sucrose was selected as germination medium.

MS medium with 3% sucrose could also be acting as a selection method (Song *et al.,* 2008), allowing only vigorous seeds to germinate, so plain medium would be recommended only if seed quantity is limiting.

Seeds germinated on soil represent germination where the effect of *in vitro* conditions is avoided. Here it is also possible to see that disinfection itself had no adverse effect on germination (fig. 15). Though the difference is not significant, slightly higher germination could be seen after seed disinfection was done, probably due to testa chemical scarification during this process (Msanga and Maghembe, 1998).

Chapter 3

Production of flowering corm *via in vitro* culture of seeds

Experiment 4

Effect of light intensity and pH on germination

Introduction

Some species are sensitive to light when it comes to germination. Depending on the specie, seed may be promoted or reduced (Huanga *et al.*, 2003; Jager *et al.*, 1996).

Yanez *et al.* (2005) mention pH of 6.5 to 6.7 in natural habitat for *Zephyra elegans*. Analysis of soil sampled during collection of *Zephyra elegans* in the same region (Huasco), showed pH of 8.86 (table 3). Although it is not part of this study to compare such differences and discuss the different results, higher germination medium pH seems an interesting factor to be tested in order to increase germination of near 60% obtained in previous experiments.

Materials and methods

In the present research, a medium pH similar to this specie's natural growing conditions and pH 6.7 (Yanez *et al.*, 2005) was chosen in order to be tested for in vitro germination. Effect of light intensity was also tested on in vitro germination of *Zephyra elegans*.

Seed germination was performed in MS medium with pH adjusted to 5.7 or 6.7 before agar addition; and light conditions were adjusted to dark, 2000 lux or 9500 lux.

Seed disinfection was performed by dipping in Ethanol (70%) for 5 seconds; Benomyl 0.5% for 20 minutes; NaClO 6% for 15 minutes; Soaking in sterile distilled water for 24 hours; NaClO 3% for 15 minutes, with rinses with sterile distilled water after every disinfection. In each of the treatments, 25 seeds were sown and 5 repetitions were made. Germination was recorded every 2 weeks for 10 weeks. Contaminated seedlings or seeds that gave origin to non viable seedlings were not considered as germinated.

Results and discussion

Highest germination rate was observed with conditions of 9500 lux and medium pH adjusted to 6.7 (figure 16). Light intensity had a significant effect on germination, with 9500 lux condition having over 90% germination for both pH conditions, 2000 lux slightly over 50% and dark conditions under 40% germination at the end of the 10 week evaluation period. Light intensity also had a significant effect on faster and more uniform germination. Maximum germination is reached between 4 to 6 weeks for 9500 lux, between 6 to 10 weeks for 2000 lux and maximum germination was not reached for seeds germinating under dark condition during the 10 week study. As light intensity gets stronger, pH has less effect on germination, with significant effect of pH on final germination rates only for dark conditions. At 9500 lux, higher pH has a significant effect on germination only during the second week after sowing (Figure 16).

By increasing light intensity and adjusting to higher pH, germination in MS medium can be increased from around 55% to nearly 95%.

Chapter 3

Production of flowering corm *via in vitro* culture of seeds

Experiment 5

Determination of sucrose range adequate for enhanced corm growth

Introduction

Corm weight has been reported as influencing flowering capability. Corms under 0.3 g will not flower (Kim *et al.*, 1998; Yanez *et al.*, 2005) and corms of 0.8 g or more are required for commercial flowering capability in corms (Kim *et al.*, 1998; Yanez *et al.*, 2005).

Enhanced corm weight gain of plantlets is the main objective of this chapter. This experiment aims to determine adequate range of sucrose concentration in MS medium in order to promote faster corm weight gain when compared to greenhouse conditions. Future experiments will be performed in order to determine specific concentration.

Materials and methods

After seed germination, uniform 8 week old seedlings obtained in the previous experiment were selected as plant material for this experiment. Seeds were initiated in MS medium supplemented with 3% sucrose and 8 weeks later transferred to MS basal medium with vitamins, supplemented with 0, 3, 6, 9 and 12% sucrose. Seedlings were cultivated for 16 weeks in individual containers with 20 mL of this medium. One transfer to fresh medium with the same corresponding sucrose concentration was made after 8 weeks. *In vitro* growing conditions were 25°C with a 24 hour photoperiod at 2000 lux.

Seeds were also sown in akadama commercial soil under greenhouse conditions. Greenhouse experiments were performed in Niigata University campus non heated greenhouse facilities (37°52'12"N, 138°56'38"E). Size of used pots was 25 x 25 x 30 cm. Eighteen seedlings (or seeds for the greenhouse experiments) per treatment were used and 3 repetitions were made. Corm weight was measured 24 weeks after seeds were sown.

Results and discussion

Seedlings cultivated in MS medium with sucrose concentrations of 60 and 90 $g \cdot L^{-1}$ sucrose concentration medium showed highest corm weight gain after the 16 weeks (figure 17). Corm weight gain was significantly higher than under greenhouse conditions. Seedlings growing in medium with no sucrose experimented lowest growth probably from lack of nutrients. Early entrance into dormancy could be observed in those growing in 12% sucrose concentration medium. Shortening of the growing period is probably affecting corm growth negatively. Optimal sucrose concentration for stimulating corm growth was 6%, though there was no statistical difference with 9% concentration (fig. 17). At the end of this period no growth could be observed in corms, so it was assumed that corms became dormant.

When corms were transferred to greenhouse conditions, corm sprout rate (survival) was higher for corms that were cultured in these same mediums (fig. 18). From figure 19, it can be deduced that the higher sprout rate is may be due to higher corm weight achieved in these mediums.

Concentrations around the range of 6 and 9 % sucrose are to be tested in a more detailed experiment in order to determine the best sucrose concentration that must be supplied with MS medium in order to have the highest corm weight gain.

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Production of flowering corm *via in vitro* culture of seeds

Experiment 6

Determination of exact sucrose concentration in MS medium for accelerated corm growth

Introduction

Corm weight has been reported as influencing flowering capability, and this has been the main objective of the experiments in this chapter. Corms under 0.3 g will not flower (Kim *et al.*, 1998; Yanez *et al.*, 2005) and corms of 0.8 g are required for commercial flowering capability (Kim *et al.*, 1998; Yanez *et al.*, 2005).

In the previous experiment it was determined that concentrations around of sucrose around 6 and 9% supplemented to MS medium are adequate for corm weight gain of Z. elegans. In this experiment the aim is to attain a more precise sucrose concentration, optimal for *in vitro* accelerated corm weight gain.

As pH more similar to natural growing conditions had a significant effect on germination on the previous experiment, the effect of pH also was tested on *in vitro* corm growth.

Materials and methods

Eight week old seedlings were selected as plant material for this experiment. Seeds were germinated in MS medium supplemented with 30 $g \cdot L^{-1}$ sucrose and 8 weeks later, seedlings were transferred to MS medium supplemented with 45, 60, 75, 90 or 105 $g \cdot L^{-1}$ sucrose in two groups of pH 5.7 or 6.7. Then seeds were cultured in this medium for 16 weeks, with one transfer to fresh medium at the 8th week. Individual test tubes (2.5 cm diameter by 12 cm height) with 20 mL were used. Parallel to the *in vitro* experiments, seeds were also sown under greenhouse conditions.

Fifteen seedlings per treatment were used and 5 repetitions were made. Corm weight was recorded 24 weeks after seeds were initially sown.

The medium showing highest corm weight gain was selected and supplemented with ABA concentrations of 0, 0.01, 0.1, 1 or 10 $mg \cdot L^{-1}$, to study its effect on corm

growth. The rest of culture conditions and data recording was with the same method as in the previous experiment.

Results and discussion

Seedlings cultivated in 75 $g \cdot L^{-1}$ sucrose concentration pH 6.7 medium showed highest corm weight gain (fig. 20).

For 5.7 pH conditions, 60 and 75 $g \cdot L^{-1}$ sucrose concentrations had the highest corm weight gains. For pH 6.7, corm weight gain in medium with 75 $g \cdot L^{-1}$ sucrose was significantly higher than in 60 $g \cdot L^{-1}$ sucrose. For all sucrose concentrations, except in 105 $g \cdot L^{-1}$ sucrose concentration, pH increase form 5.7 to 6.7 had a significant effect on increasing corm weight gain (fig. 20). Early entrance into dormancy was observed in plants growing in 105 $g \cdot L^{-1}$ sucrose medium. High sucrose concentration could be affecting corm weight by shortening the growth period by promoting ABA senescence pathways (Finkelstein and Gibson, 2001).

MS medium with 75 $g \cdot L^{-1}$ sucrose and pH 6.7 was selected for ABA concentration testing. ABA has been described as stimulate reserve structure growth by promoting reserve polysaccharide pathways (Sharma et al., 2004; Finkelstein and Gibson, 2001), but no boost effect of ABA on corm weight gain could be observed (fig. 21). It was noticed that as ABA concentration increased, plantlets entered into earlier dormancy and corm growth was decreased. Instead of the desired effect of promoting reserve structure growth, ABA acted as dormancy inducing agent (Finkelstein and Gibson, 2001). Medium concentrations of ABA between 0 and 0.01 mg•L-1 have to be studied in order to find an adequate dose that stimulates reserve organ growth but does not accelerate senescence and early entrance into dormancy. At the end of the observation period no growth could be observed, so it was assumed that plants became dormant.

Chapter 3

Production of flowering corm *via in vitro* culture of seeds

Experiment 7

Acclimatization and transplanting of corms generated *via in vitro* culture

Introduction

The main objective of the experiments in this chapter was to obtain a flowering corm in a reduced amount of time when compared to time needed under greenhouse conditions. Part of this objective consists on achieving corms that have the highest weight possible, as corms with higher weight have higher flowering capability (Yanez *et al.,* 2005; Kim *et al.,* 1998). As already corms that have higher weight gains than their greenhouse counterparts has been achieved, now these *in vitro* grown corms have to be tested under greenhouse conditions for their flowering capability. A first step to this is to acclimate and be able to transfer these corms from *in vitro* conditions to greenhouse conditions.

Materials and methods

After the corm weight gain trial, plantlets became dormant corms. Dormancy period has been recommended as the appropriate moment for bringing out geophytes from their *in vitro* conditions (Dantu and Bhojwani, 1995). Newly formed corms were taken out from the culture medium during their dormant stage. Corms were washed with tap water until medium remains were removed. A dormancy break treatment was done at 25°C for 22 weeks (Yanez *et al.* 2005; Kim *et al.,* 1998; Kim *et al.,* 1996). After breaking dormancy, corms were planted in individual pots and grown under greenhouse conditions. Watering was done when soil surface got dry. Fifteen corms per treatment were used and 5 repetitions were made. Survival rate was recorded 2 months after planting by counting corms that sprouted.

Results and discussion

Corms sprouted within 2 months after planting. Corms that did not sprout after this period, decayed or desiccated in soil towards the end of the growing season.

Sprout rate was significantly higher in corms generated in pH 6.7 MS basal medium, supplemented with 60, 75 and 90 $g \cdot L^{-1}$sucrose (fig. 22). Higher sprout rates may be due to higher corm weights seen on corms grown under 60, 75 and 90 $g \cdot L^{-1}$ medium sucrose concentrations, and not solely to the composition of the growing medium. In order to isolate the effect of medium source, corms were grouped according to weight at planting, independent of their in vitro origin in figure 23.

Corms under 0.12g did not survive acclimatization and did not sprout, while all corms over 0.3g had sprouting capability (fig. 23). Sprout rate increased as corm weight at planting increased. No flowering could be seen in these 1 year old plants, even though according to corm weight at planting, corms over 0.3 g would potentially have flowering capability (Yanez *et al.*, 2005; Kim *et al.*, 1998).

The fact that no flowering was observed in 1 year old plants with corms over 0.3g, potentially having flowering capability (Yanez *et al.*, 2005; Kim *et al.*, 1998), could mean that corm weight is not the only cause influencing flowering. Development of the plant could also be involved (Ishimori *et al.*, 2007). Further studies are necessary to clarify this aspect.

Chapter 3

Production of flowering corm *via in vitro* culture of seeds

Experiment 8

Flowering capability of corms generated *via in vitro* culture

Introduction

The fact that no flowering was seen in the first season on greenhouse cultivation, even though corms would have flowering potential according to their weight over 0.3 g (Yanez *et al.,* 2005; Kim *et al.,* 1998) may indicate that there are other factors influencing flowering as well. Factors like plant maturity and phase transition could be the cause (Ishimori *et al.,* 2005).

Materials and methods

After the first growing season, corms that survived were collected and submitted to 25°C for 22 weeks to break dormancy. Subsequently corms were planted again in individual pots and grown under greenhouse conditions to test flowering capability on the following growing season. Number of flowering plants was recorded three months after sprouting.

Results and discussion

In the second growing season, highest flowering frequency was observed in plants that had been grown in pH 6.7 MS medium supplemented with 75 $g \cdot L^{-1}$ during their in vitro stage, with significant difference when compared to their pH 5.7 counterpart. For plants growing under 45 and 105 $g \cdot L^{-1}$ sucrose, no flowering could be observed when pH 5.7 medium was used, but plants growing under 6.7 pH, flowering could be observed in some cases (fig. 24). No flowering was observed in plants germinated and grown only in soil under greenhouse conditions. All corms over 0.4 g after the in vitro stage, gave origin to flowering corms of over

0.8g after the first year of culture under greenhouse conditions and flowered in the second season (fig 25).

Flowering plants had seeding capability. No difference could be observed between flowers generated from corms obtained during this experiment and flowers from corms obtained from natural environment collection, indicating that *in vitro* culture itself had no effect on plant morphology (fig. 26). Decreasing time from seed to flowering plant is an interesting aid to genetic improvement programs as well as massive propagation method for producers.

Chapter 3

Production of flowering corm *via in vitro* culture of seeds

Conclusion

Conclusion

Germination in MS medium at full strength decreases germination rate as compared to germination in agar-water medium, but this can be overcome by increasing light intensity and pH of the medium.

Even flowering could not be achieved in the first year, time from seed to flowering plant was reduced when compared to greenhouse cultivation only. In vitro enhanced corm weight gain is an interesting process for achieving commercially flowering corms of over 0.8 g from seeds in 2 growing seasons.

By using this technique, desired phenotypes like deep 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, flowering corms are still not obtained even after 4 years of cultivation in Niigata University greenhouses.

Chapter 4

Introduction

In vitro vegetative propagation of *Zephyra elegans*

Introduction

Vegetative propagation or asexual propagation results in progeny that are identical to their single parent. The main difference with sexual propagation, where the genetic qualities of the plant originated from seed are visually unknown until maturity and flowering is reached (Raven *et al.,* 2003). Vegetative reproduction allows the exact replication of individuals with desirable characteristics, in *Zephyra elegans* being blue flowers and plant height (Kim *et al.*, 1998; Kim and Ohkawa, 1997; Kim *et al.,* 1997).

Zhephyra elegans has shown incapability to satisfactory reproduce asexually. Natural vegetative propagation is low and only 1 corm divided into 2 during the first year where in a preliminary study involving 148 plants grown in pots. Induced artificial vegetative propagation under greenhouse conditions, like corm division, has shown that the corm produces a gel substance (fig. 27) and decays, not allowing traditional multiplication by reserve organ division.

In vitro techniques are tested in the search of a protocol that allows vegetative propagation of *Zephyra elegans*.

Chapter 4

In vitro vegetative propagation of *Zephyra elegans*

Experiment 1

Determination of plant material for *in vitro* propagation with conservation of mother plant

Introduction

Different plant organs have different responses to *in vitro* hormone concentrations with the objective of vegetative propagation. Different responses may or may not allow vegetative propagation. Development stage also may influence this response (Niimi and Onozawa, 1979).

Responsiveness of different organs at different development stages will be tested, but corms will not be used in order to conserve the mother plant. This would be interesting in order to not affect natural populations during collection of individuals from nature. If corm is not collected it would not affect natural population.

Materials and Methods

Flower bud, upper leaf, lower leaf, upper internode, lower internode and node were collected from adult plants grown under greenhouse conditions were used as explants. Time of collection was as soon as stem emerged from the leaves and was visible for early stage (fig. 28), or as soon as flower color could be determined by sight for late stage (fig. 29). Advantage of late stage would be to be able to know flower color at time of collection, but early stages of plant development may show better response to vegetative propagation (Niimi and Onozawa, 1979).

Explants were initiated in MS medium complemented with 1 $mg \cdot L^{-1}$ NAA and 0.1 $mg \cdot L^{-1}$ BA in MS basal medium supplemented with vitamins and 30 $g \cdot L^{-1}$ sucrose, as these concentrations were recommended for other geophytes (Niimi, 1986; Niimi and Onozawa, 1979). Growing conditions were 25°C under dark conditions. For each repetition, 6 plants were divided into flower, upper leaf, lower leaf, upper internode, lower internode and node, in both early and advanced stage, and 3 repetitions were made. Data of responsive tissue was collected at 8th week

after initiation. Tissue responsiveness was considered when tissue swelling (fig. 30) occurred.

Results and discussion

Significantly higher tissue responsiveness in the form of tissue swelling (fig. 30) or callus formation (fig. 31). was observed in lower internodes obtained from plants at early stage (table 7). Though significantly lower, some response was also present in flower buds, lower leafs, upper internodes and nodes collected at early stage. In explants collected at late stage, tissue responsiveness was observed only in lower internodes.

In *Lilium* species, with 1 $mg \cdot L^{-1}$ NAA and 0.1 $mg \cdot L^{-1}$ BA vegetative multiplication is possible (Niimi, 1986; Niimi and Onozawa, 1979), but in *Z. elegans* vegetative multiplication could not be achieved under such conditions. Lower internode collected at early stage was the most responsive explant, and will be used in future experiments with different hormone concentrations in order to achieve vegetative propagation. When reactive lower internodes were transferred to fresh medium with the same hormone concentration, after 16 weeks, callus formation occurred on 17% of the cases.

Chapter 4

In vitro vegetative propagation of *Zephyra elegans*

Experiment 2

Sprout induction from callus formed from lower internodes collected at early stage

Introduction

As lower internodes collected at early stage showed highest responsiveness, it was selected as plant material and tested under different hormone concentrations in order to observe responses that would facilitate vegetative propagation. From cultivating in the same culture medium it was noticed that callus formation occurred.

Materials and methods

Callus formed from lower internodes collected at early stage from adult plants grown under greenhouse conditions were used as plant material for this experiment (fig. 31). Six callus clusters from 0.5 to 0.8 cm diameter were used for every hormone concentration treatment and 3 repetitions were made. A transfer to fresh medium was done every 8 weeks.

Hormone concentrations tested for sprout induction in callus were combinations of NAA 0, 0.1 or 1 $mg \cdot L^{-1}$, and BA 0, 0.1 or 1 $mg \cdot L^{-1}$ in MS basal medium supplemented with vitamins and 30 $g \cdot L^{-1}$ sucrose. Growing conditions were 25°C with 24h photoperiod at 2000 lux. Sprout induction was measured after 16 weeks.

Results and discussion

Sprout induction was only observed in callus grown in medium supplemented with BA 1 $mg \cdot L^{-1}$ and NAA 0.1 $mg \cdot L^{-1}$, and sprout induction rate was significantly higher at 11%. Once sprouts were induced in a callus, 2 to 5 sprouts can be collected during the 12 weeks period of this study (fig. 32).

After this period the callus starts to grow but does not produce sprouts. Sprouts that do not produce sprouts grow at twice to three times the rate of non sprout producing callus clusters. After a period varying from 12 to 36 weeks the callus turns brown and necrotic.

Chapter 4

In vitro vegetative propagation of *Zephyra elegans*

Experiment 3

Sprout rooting, corm formation and survival

Introduction

Sprouts that were formed in the previous experiment need to be rooted in order to be able to later transfer to greenhouse conditions. Satisfactory rooting is necessary in order to successfully achieve vegetative propagation.

Materials and methods

Sprouts formed with the protocol determined in previous experiments were transferred to MS basal medium supplemented with vitamins and 30 $g \cdot L^{-1}$ sucrose, and hormone levels of NAA 0, 0.1 or 1 $mg \cdot L^{-1}$, and BA 0, 0.1 or 1 $mg \cdot L^{-1}$. Two to 3 sprouts were tested in each hormone concentration. Lack of number of sprouts available did not allow statistical analysis with repetitions. Culture of sprouts was done for 16 weeks.

Results and discussion

No rooting was observed in sprouts. In medium with no hormone, 2 of the sprouts became slightly swollen at base after 4 weeks of culture (fig. 33). The shape was similar corm formation, but later became necrotic anyway.

In future experiments, different sucrose concentrations and medium concentrations could be tested. Though rooting or corm formation was unsuccessful, MS medium with no hormone would seem the way to test future protocols. Changing sucrose concentration or medium nutrient concentration should be tested in further experiments for rooting of sprouts.

Chapter 4

In vitro vegetative propagation of *Zephyra elegans*

Experiment 4

Direct sprout induction from lower nodes and internodes collected at early stage

Introduction

In previous experiments, BA concentrations of up to 0.1 $mg \cdot L^{-1}$ were tested and callus formation was obtained when combined with NAA. Though higher concentrations of NAA are suggested in comparison to BA for multiplication and shoot induction (Niimi, 1986; Niimi and Onozawa, 1979), other authors suggest higher BA concentration in comparison to NAA (Ulrich *et al.*, 1999). In this experiment concentration of 1 $mg \cdot L^{-1}$ BA will be tested in combinations of NAA in order to produce direct sprout induction.

Materials and methods

As lower internodes collected at early stage and internodes collected at early stage showed the highest responsiveness in previous experiments, they were selected as plant material to be tested under different hormone concentrations in order to induce shoot formation. Hormone concentrations of 1 $mg \cdot L^{-1}$ BA with NAA concentrations of 0, 0.1 or 1 $mg \cdot L^{-1}$ were tested in MS basal medium supplemented with vitamins and 30 $g \cdot L^{-1}$ sucrose concentration. Growing conditions were 25°C with 24h photoperiod at 2000 lux. Sprouting was measured 24 weeks after initiation.

Results and discussion

No sprouting could be observed after the 24 week growing period, after this period, explants became necrotic. Some response was seen on medium supplemented with BA 1 $mg \cdot L^{-1}$ and NAA 0.1 $mg \cdot L^{-1}$ on both lower internodes collected at early stage and nodes collected at early stage (fig. 34) but explants

finally became necrotic without producing any sprout. When the medium was supplemented with 1 $mg \cdot L^{-1}$ BA and 1 $mg \cdot L^{-1}$ NAA callus formation was observed in one observation, but no sprouting occurred.

Plant material tested was not satisfactory for vegetative multiplication, so corms or roots (Zhou *et al.,* 2010; Ebrahim, 2004; Kantharajah *et al.,* 1992; Sim *et al.,* 1989) would be a source of plant material for vegetative propagation.

Chapter 4

In vitro vegetative propagation of *Zephyra elegans*

Experiment 5

Corm disinfection for *in vitro* culture

Introduction

Aerial explants had been tested but propagation had been unsuccessful. In order to achieve vegetative propagation, corm and root tips were used as explants. The inconvenience for this is that the mother plant is not conserved. The first step for *in* vitro culture is to achieve satisfactory disinfection without damaging the plant material.

Materials and methods

Dormancy break has shown to be necessary for corms to sprout under greenhouse conditions (Yanez *et al.,* 2005), so in order to avoid the effect of dormancy, corms used for this experiment would be subject to dormancy break treatment (25°C for 24 weeks) before initiation.

Corms of *Zephyra elegans* between 0.8 and 1.2 g were selected for this experiment. Corm tunic was carefully removed (fig. 35) with extreme care in order not to damage the apical bud (fig. 36). For disinfection, corms were immersed in sodium hypochlorite at 0.5 or 1% w/v of active ingredient for 15 minutes. After the disinfection treatment, corms were used as a whole or divided in 2 at the equator, leaving an upper half and a lower half (fig.37), which were initiated in individual containers. Each corm or corm section was initiated in MS basal medium with vitamins and 30 g/L sucrose. For each treatment, 6 corms or corm halves were used and 3 repetitions were made. Contamination and sprout rate were recorded 8 weeks after initiation.

Results and discussion

With sodium hypochlorite at 0.5% it was unable to achieve satisfactory disinfection. All plant material disinfected with 0.5% sodium hypochlorite was contaminated after 8 weeks if incubation so it was not considered as part of the statistical analysis. Contamination was lowest when sodium hypochlorite was used at 1% concentration and only upper half of the corm was used. When the whole corm was used, contamination was as high as when lower half was used (table 8). Highest sprout rate was also observed when upper half only was used. When the whole corm was used, contamination seemed to decrease sprout capability.

Chapter 4

In vitro vegetative propagation of *Zephyra elegans*

Experiment 6

Corms and root tips as explant for vegetative propagation *via* direct shoot induction

Intorduction

Mother plant is not conserved when corms are used as explants, so it was avoided until now. But as in previous experiments it was not possible to achieve vegetative propagation using other type of explants, corms and root tips will be used as plant material for vegetative propagation. As in corms, buds are located in the corm, it is recommended as plant material for species that have this kind of organ, like gladiolus (Shina and Roy, 2002; Bajaj *et al.,* 1983). Root tip has also been suggested as plant material for vegetative propagation (Ebrahim, 2004; Kantharajah *et al.,* 1992) so they will be used as plant material for this experiment.

Materials and methods

As with the previous experiment, in order to avoid the effect of dormancy, corms were subject to dormancy break treatment (25°C for 24 weeks) before initiation.

Corms of *Zephyra elegans* between 0.8 and 1.2 g were selected for this experiment. For disinfection, corms were immersed in sodium hypochlorite at 1% w/v of active ingredient for 15 minutes. Corms were divided at the equator, leaving an upper half and a lower half (fig.37), that were initiated in different containers.

Upper corms were initiated in MS basal medium with vitamins and 30 $g \cdot L^{-1}$ sucrose, supplemented with BA 1 $mg \cdot L^{-1}$ and NAA 0, 0.1 or 1 $mg \cdot L^{-1}$ and cultured for 16 weeks. Lower halves were initiated in MS basal medium with vitamins and 30 $g \cdot L^{-1}$ without hormones. After rooting occurred, roots were harvested when they reached 2 cm. Roots were collected till no more roots were generated or contamination appeared. Collected roots were transferred to MS basal medium with vitamins and 30 $g \cdot L^{-1}$ sucrose, supplemented with BA 1 $mg \cdot L^{-1}$ and NAA 0,

0.1 or 1 $mg \cdot L^{-1}$ and cultured for 16 weeks. For every treatment, 6 corm halves or root tips were used and 3 repetitions were made.

Results and discussion

Multiple shoot induction (over 3 shoots per explant) was achieved in 22% of the explants when upper halves were grown in MS basal medium with vitamins and 30 $g \cdot L^{-1}$ sucrose, supplemented with BA 1 $mg \cdot L^{-1}$ and NAA 0.1 $mg \cdot L^{-1}$ (fig. 39). At later stages of culture period, callus formation was observed. NAA was necessary in order to induce multiple shoots. Without NAA, a maximum of 3 shoots were produced by any individual explant, corresponding to what seemed previously existing buds (fig. 38). With NAA at 1 $mg \cdot L^{-1}$, callus formation was observed and could be the cause to low multiple shoot formation. Root tips were not responsive so only observations including upper halves of corms were considered in the statistical analysis.

Shoots were collected when they formed a small corm-like structure at the base (fig. 39). Collected shoots were transferred to MS basal medium with vitamins and 30 g/L sucrose, without hormones. Shoots rooted, developed a corm and became dormant 10 to 16 weeks after the transfer to hormone free medium. Currently, these corms are under dormancy break treatment.

Experiments combining *in vitro* enhanced corm weight used for seeds at the beginning of this thesis may be interesting way to achieve necessary weight for corms to be able to flower (Yanez *et al,* 2005). Acclimatization to greenhouse conditions and flowering experiments are also needed in the future.

Chapter 5

General Discussion

General discussion

In this work, *in vitro* techniques were a major tool in the aid of successfully propagating *Zephyra elegans*. For seed propagation, germination in MS medium at full strength decreases germination rate as compared to germination in agar-water medium, but this can be overcome by increasing light intensity and pH of the medium. If *in vitro* growing conditions are adjusted to be more similar to the ones found in its natural habitat, germination and growth was higher, probably because it caused less stress to the plant.

Even flowering could not be achieved in the first year, time from seed to flowering plant was reduced when compared to greenhouse cultivation only. In vitro enhanced corm weight gain is an interesting process for achieving commercially flowering corms of over 0.8 g from seeds in 2 growing seasons.

By using this technique, desired phenotypes like deep blue flowers, could be selected in a reduced period of time of 3 years. When seeds are germinated and grown only under greenhouse conditions, flowering corms are still not obtained even after 5 years of cultivation in Niigata University greenhouses.

Even though vegetative propagation using aerial explants (which would allow conserving the mother corm) was not successful, it gave the guidelines for achieving vegetative propagation using the corm as explant for vegetative propagation. Only callus and sprouting stage was achieved, but vitrification occurred. Here testing pH variations and light intensity could also be helpful in order to solve these problems, as with seed experiments.

Using the only the upper half of the corm, contamination was reduced significantly to levels that allowed initiation of this explant. Multiple shoot induction was achieved when they were grown in MS basal medium with vitamins and 30 g/L sucrose, supplemented with BA 1 $mg \cdot L^{-1}$ and NAA 0.1 $mg \cdot L^{-1}$. The

number of reactive corms was limited to around 30%, so further studies would have to be made in this area in the future.

Combining enhanced corm weight gain results would also be interesting for fast production of clonal specimens. Other area that would be interesting to research in the future would be the effect of pH in facilitating vegetative propagation protocols, because of its significant effect on seed germination and enhanced corm weight gain. Higher pH could mean increasing propagation rates and reactiveness to propagation treatments. This could mean in achieving large number of flowering clonal plants in a reduced period of time. Research is needed in order to determine whether vegetative propagation originated corms weighing over 0.3 g would have flowering capability and corms over 0.8 g would have commercial flowering capability. If this is reached in the second year it would mean that inability to flower in the first year of greenhouse cultivation after the *in vitro* cultivation of seeds could be due to young phase to adult phase transition.

Tables

Table 1. Sprouting and flowering of *Zephyra elegans* growing under greenhouse conditions in Niigata University greenhouses.

Planting season	Sprouting (%)	Flowering (%)
September	91.7± 8.3 a[1]	75.0±13.0 a
November	100.0± 0.0 a	91.7± 8.3 a
January	66.7±14.2 b	41.7±14.9 b

[1] Values associated to the same letter in a same column were not statistically different at trust level of $P<0.05$. Values ± S.E.

Table 2. Sprouting and flowering of *Zephyra elegans* in different soils under greenhouse conditions.

Type of soil	Sprouting (%)	Flowering (%)
Akadama	100.0± 0.0 a[1]	100.0± 0.0 a
Perlite	100.0± 0.0 a	91.7± 8.3 a
Sandy soil	58.3±14.9 b	50.0±15.1 b

[1] Values associated to the same letter in a same column were not statistically different at trust level of $p<0.05$. Values ± S.E.

Table 3. Soil analysis results of soil sampled in Playa Brava, Huasco, Chile, in growth habitat of *Zephyra ekegans*.

pH in water	8,86
Organic mater (%)	0,50
Suspension salinity (mmhos/cm)	0,36
Extract salinity (mmhos/cm)	2,36
Available N (ppm)	3,00
Available P (ppm)	20,00
Available K (ppm)	66,00
Available Cu (ppm)	1,22
Available Fe (ppm)	3,75
Available Mn (ppm)	1,89
Available Zn (ppm)	0,08
Available B (ppm)	0,71
Available S (ppm)	25,0

Table 4. Germination and contamination levels when different concentrations of NaOCl were used for disinfection for 15 minutes, measurements performed 3 weeks after initiation.

Concentration of NaOCl (%)	Contaminated in the total[2] (%)	Germinated in the total[3] (%)	Non contaminated germinated in the total[4] (%)	Germination in non contaminated only[5] (%)
1	43.0 ± 3.0 c[1]	18.0 ± 2.0 b	10.0 ± 2.3 b	17.4 ± 4.2 b
2	26.0 ± 4.8 b	14.0 ± 2.6 b	11.0 ± 2.3 b	17.7 ± 5.2 b
4	19.0 ± 3.4 ab	28.0 ± 2.2 a	22.0 ± 2.8 a	27.9 ± 4.2 a
6	12.0 ± 2.7 a	30.0 ± 3.4 a	25.0 ± 3.2 a	28.9 ± 3.7 a

[1] Values associated to the same letter in a same column were not statistically different at trust level of $p<0.05$. Values ± S.E.

[2] Values represent total contamination seen.

[3] Values represent germination, regardless of contamination.

[4] Germination accounted for, only if no contamination was seen (usable seedlings).

[5] Values represent germination only in the non contaminated universe.

Table 5. Contamination and germination levels when NaOCl at 6% was used at different disinfection time lengths, 3 weeks after initiation.

Time of NaOCl disinfection (minutes)	Contaminated in the total[2] (%)	Germinated in the total[3] (%)	Non contaminated germinated in the total[4] (%)	Germination in non contaminated only[5] (%)
15	16.0 ± 2.8 b[1]	32.0 ± 4.0 a	28.0 ± 3.7 a	32.0 ± 6.7 a
30	12.0 ± 3.0 b	27.0 ± 4.7 a	24.0 ± 4.3 a	28.5 ± 4.8 a
60	1.0 ± 1.0 a	11.0 ± 3.1 b	11.0 ± 3.1 b	11.0 ± 2.2 b
120	2.0 ± 1.4 a	4.0 ± 1.8 b	4.0 ± 1.8 b	4.0 ± 1.5 b

[1] Values associated to the same letter in a same column were not statistically different at trust level of $p<0.05$. Values ± S.E.

[2] Values represent total contamination seen.

[3] Values represent germination, regardless of contamination.

[4] Germination accounted for, only if no contamination was seen (usable seedlings).

[5] Values represent germination only in the non contaminated universe.

Table 6. Contamination and germination levels when NaOCl at 6% was used at different disinfection time lengths, 3 weeks after initiation.

Disinfection method	Contaminated in the total[2] (%)	Germinated in the total[2] (%)	Non contaminated germinated in the total[3] (%)	Germination in non contaminated only[4] (%)
NaOCl at 6% for 15 minutes	85.3 ± 2.1 c[1]	34.7 ± 6.0 b	5.3 ± 3.1 c	31.6 ± 16.2 b
Double disinfection	54.6 ± 1.3 b	72.0 ± 5.8 a	30.7 ± 5.4 b	66.7 ± 9.2 a
Benzalkonium + Double disinfection	41.3 ± 5.0 b	58.7 ± 6.3 a	37.3 ± 5.4 b	68.3 ± 9.4 a
Benomyl + Benzalkonium + Double disinf.	1.3 ± 5.7 a	62.7 ± 5.8 a	60.0 ± 5.5 a	62.0 ± 5.9 a
Benomyl + Double disinfection	4.0 ± 5.3 a	64.0 ± 5.6 a	61.3 ± 6.0 a	63.3 ± 5.8 a

[1] Values associated to the same letter in a same column were not statistically different at trust level of $p<0.05$. Values ± S.E.

[2] Values represent total contamination seen.

[3] Values represent germination, regardless of contamination.

[4] Germination accounted for, only if no contamination was seen (usable seedlings).

[5] Values represent germination only in the non contaminated universe.

Table 7. Response of 6 different organs collected at 2 different development stages, after 8 weeks of culture in MS medium supplemented with 1 $mg \cdot L^{-1}$ NAA and 0.1 $mg \cdot L^{-1}$ BA.

	Organ response (%)	
Organ	Early stage	Advanced stage
Flower bud	11.1 ± 7.6 bcd[1]	0.0 ± 0.0 d
Upper leaf	0.0 ± 0.0 d	0.0 ± 0.0 d
Lower leaf	16.7 ± 9.0 bc	0.0 ± 0.0 d
Upper Internode	5.6 ± 5.6 cd	0.0 ± 0.0 d
Lower Internode	38.9 ±11.8 a	11.1 ± 7.6 bcd
Node	22.2 ±10.1 b	0.0 ± 0.0 d

[1] Values associated to the same letter in a same column were not statistically different at trust level of $p<0.05$. Values ± S.E.

Table 8. Contamination and sprout rate when corms were used as plant material for *in vitro* initiation in hormone free medium.

	Whole corm	Upper half of corm	Lower half of corm
Contamination (%)	77.8 ± 10.1 b[1]	5.6 ± 5.6 a	83.3 ± 9.0 b
Sprout (%)	16.7 ± 9.0 b	83.3 ± 9.0 a	0.0 ± 0.0 c

[1]Values associated to a same letter in a same row were not statistically different at a trust level of $p<0.05$. Values ± S.E.

Figures

Figure 1. Locations of *Zephyra elegans* natural habitat. Playa Brava location is shown in red arrow, as the place where it is most common and most variability is present. Adapted from Munoz and Moreira, 2000.

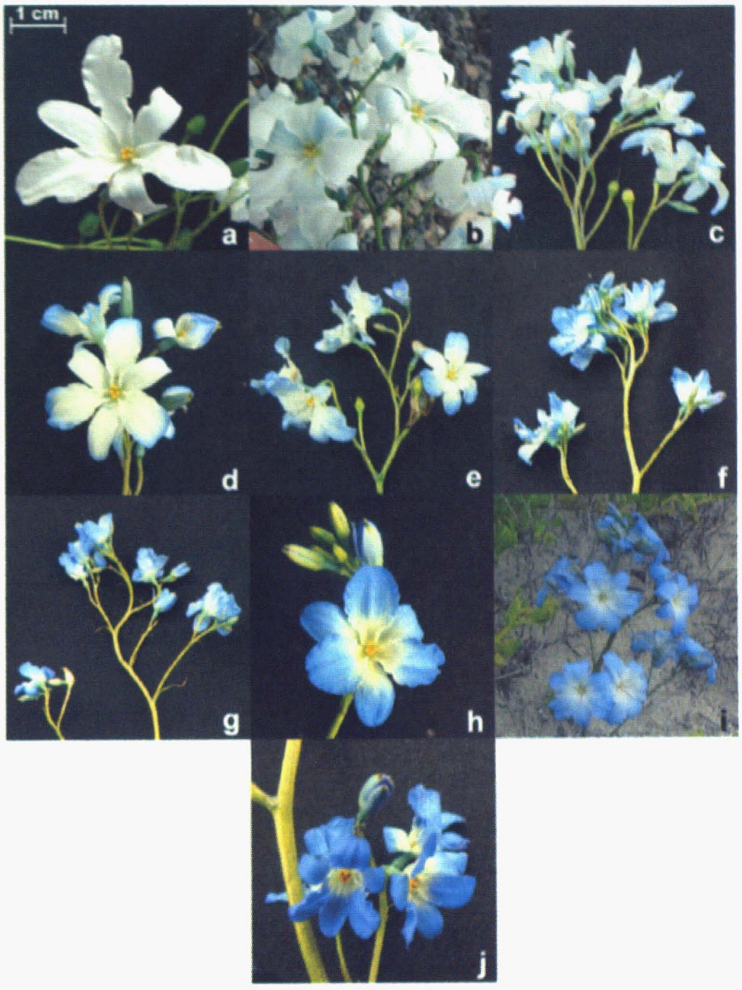

Figure 2. Colors of the flower of *Zephyra elegans*, from plane white to deep blue. Colors associated to letters h, i, j were only found in the area near Playa Brava.

Figure 3. Variability in growth habit of plants of *Zephyra elegans* collected near Playa Brava, Huasco, Chile.

Figure 4. Formats of *in vitro* plants sold to end consumers in Japan.

a. Tokyo flower market, 2004.

b. Tokyo flower market, 2004.

c. Department store in Kyoto, 2005.

Figure 5. Steel refinery and thermoelectric plant in the zone near Playa Brava. Contamination residues can be seen on the surface of the soil, threatening nearby wildlife.

Figure 6. Residues existing on the surface of plants and soil, which are originated by industries nearby the collection area of *Zephyra elegans*, in the zone of Playa Brava, south of Huasco.

Figure 7. Corms of *Zephyra elegans* at collection from nature in Playa Brava, Huasco (28°29'52"S, 71°15'18"S) in 2005.

Figure 8. Surface soil hardening when *Zephyra elegans* was planted in sandy soil.

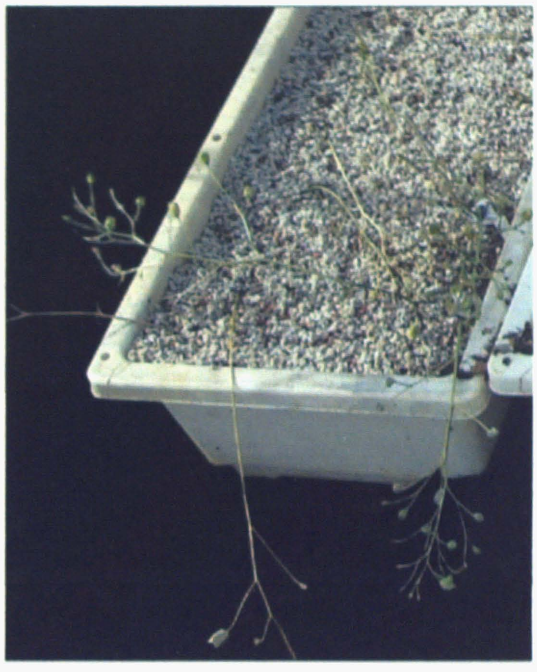

Figure 9. Plants of *Zephyra elegans* falling probably due to lack of sustention provided by the soil when grown in perlite.

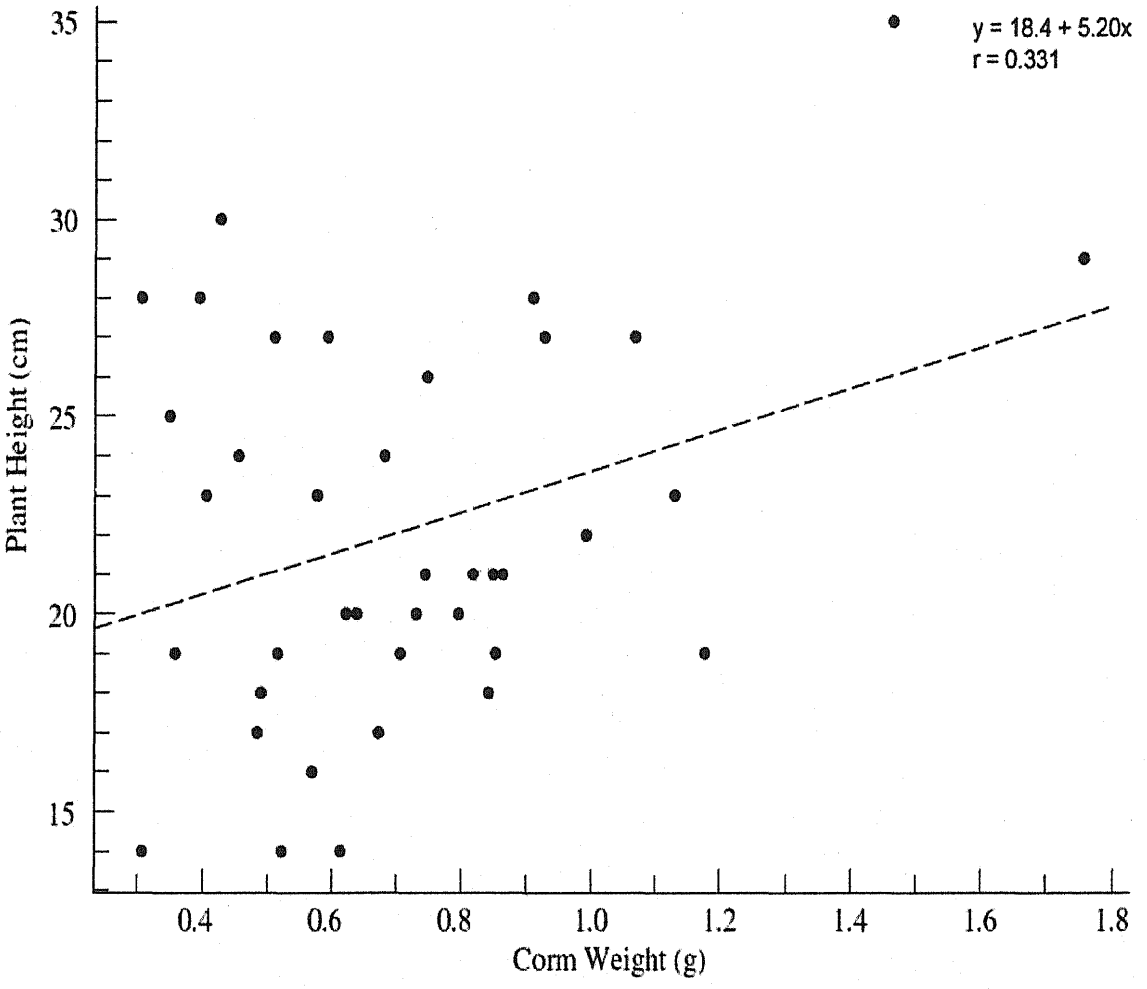

Figure 10. Correlation of 0.331 (extremely low) between corm weight at planting with plant height at flowering.

Figure 11. Seeds at the moment of collection in Playa Brava, Huasco (28°29'52"S, 71°15'18"S) in 2005, laying over the soil. Identified seeds of *Zephyra elegans* are circled in red.

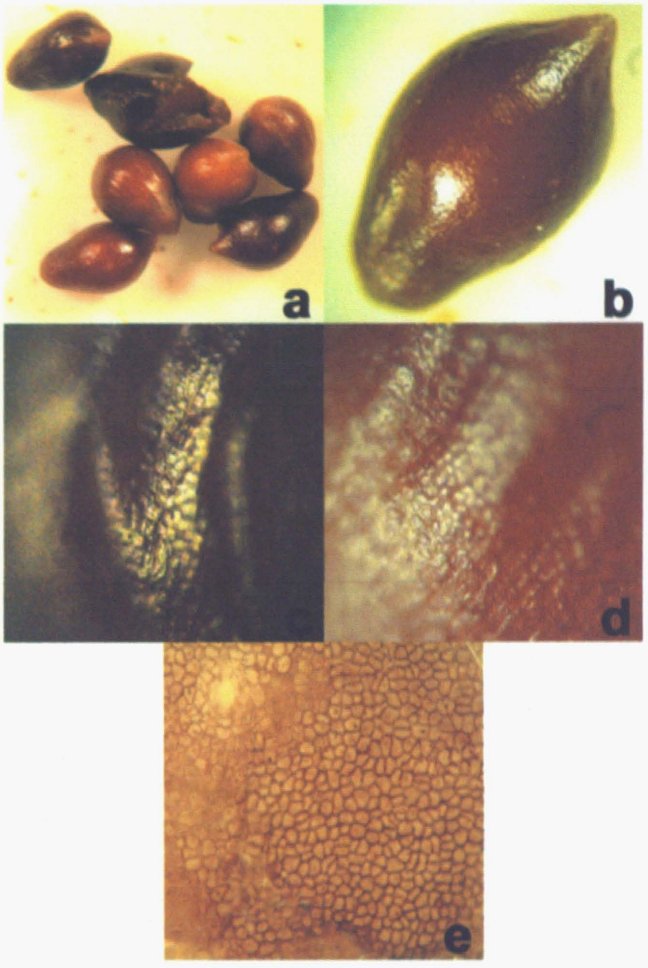

Figure 12. Irregular surface of the seeds of *Zephyra elegans*.

a. 8X (with Nikon® microscope, model SMZ-1B).

b. 40X (with Nikon® microscope, model SMZ-1B).

c, d. 200X (with Nikon® microscope, model YS2-H).

e. 300X (with Nikon® microscope, model YS2-H).

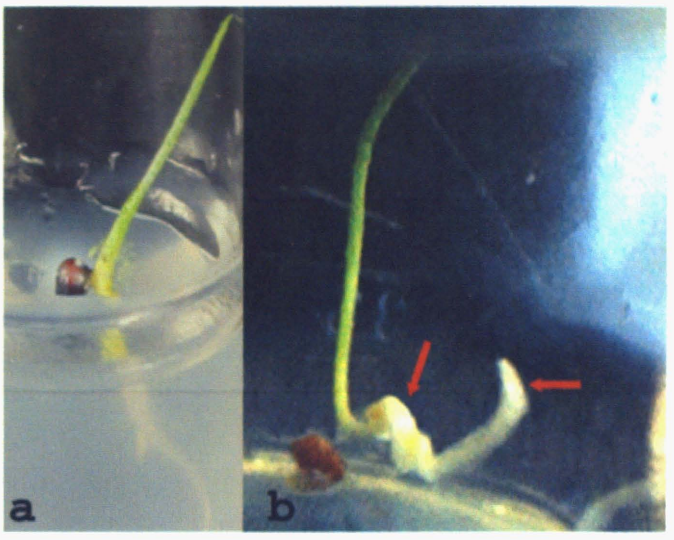

Figure 13. Germination in *Zephyra elegans*. Normal germination (a), compared to abnormal germination (b, arrows show root curling and irregular geotropism in roots), the later seen increasingly as disinfection times were 30 minutes or longer.

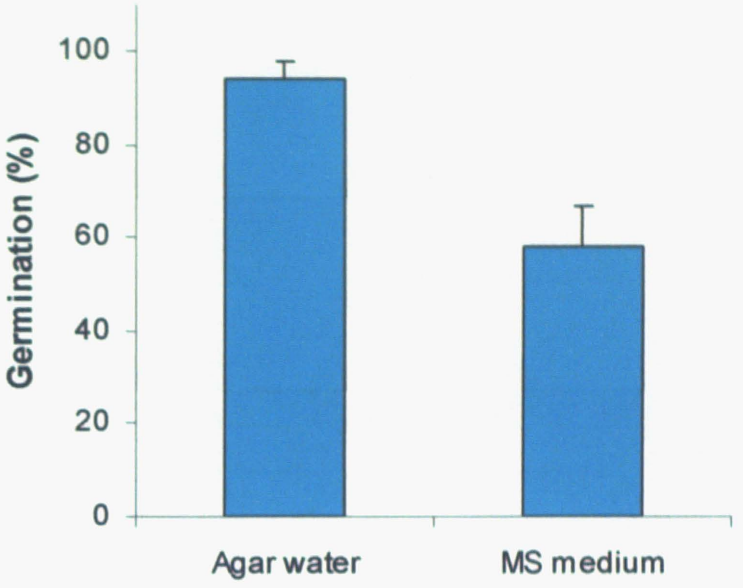

Figure 14. Germination rate 8 weeks after *in vitro* sowing in agar-water medium and MS medium. Bars represent S.E.

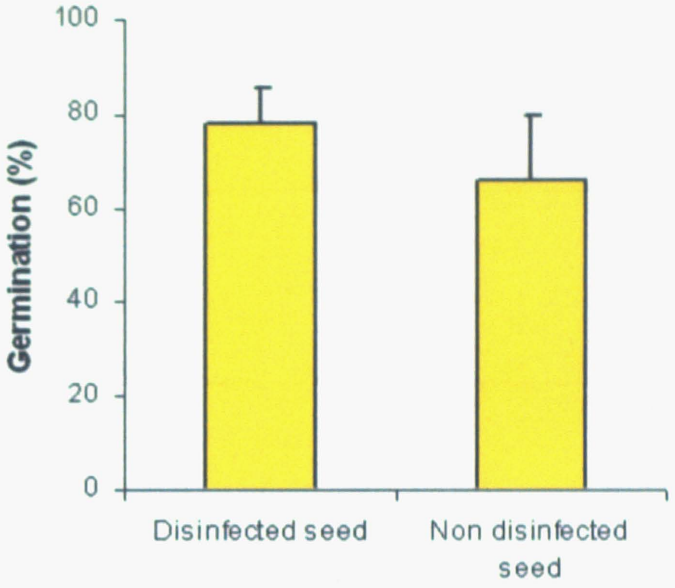

Figure 15. Effect of disinfection on germination rate 8 weeks after sowing in soil and kept for under greenhouse conditions. Bars represent S.E.

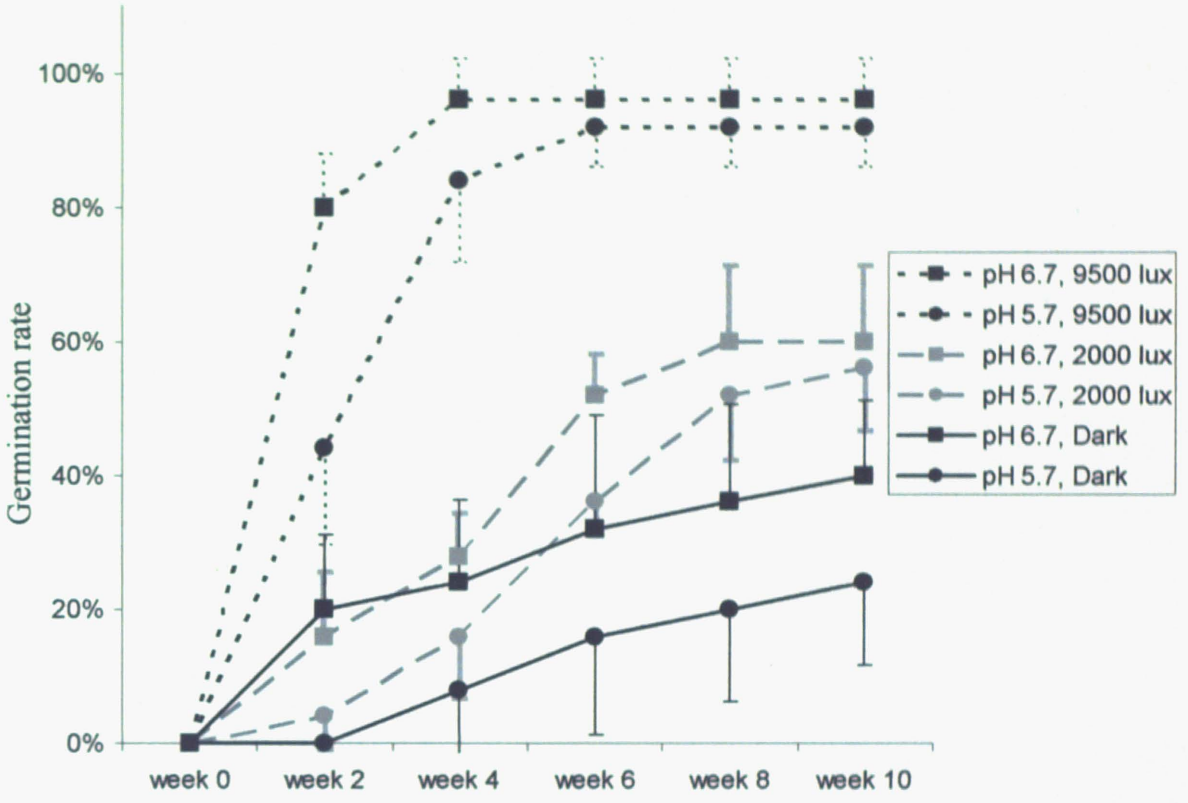

Figure 16. *In vitro* germination rate in MS medium adjusted to pH 5.7 and 6.7, at light intensities of 9500 lux, 2000 lux and dark conditions. Bars represent S.E.

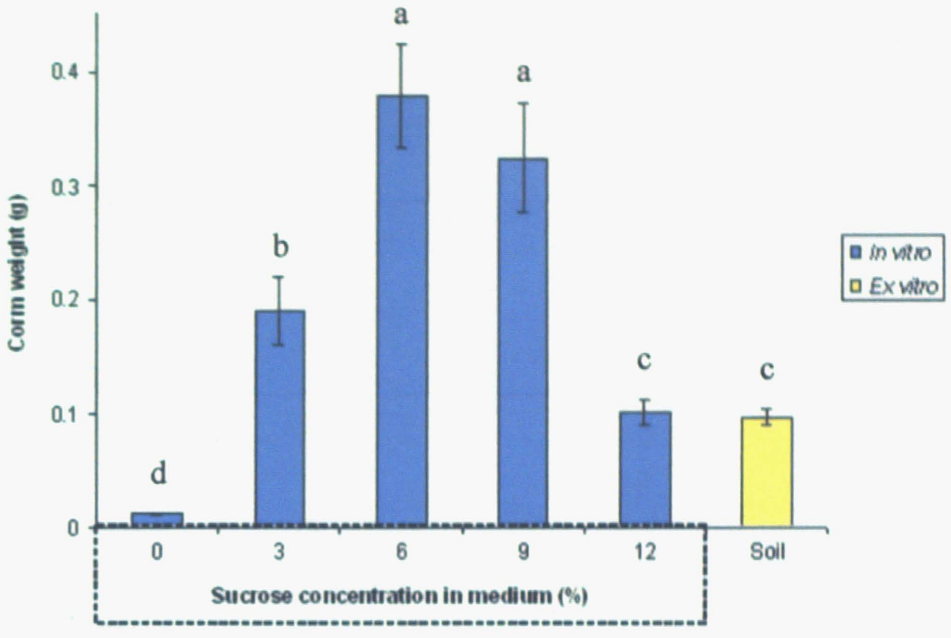

Figure 17. Effect of sucrose concentration in growth medium on corm final weight at the end of one growing season, for 8 weeks seedlings, after growing for 16 weeks in this medium; compared to a soil control performed under greenhouse conditions. Values associated to the same letter were not statistically different at trust level of $P<0.05$. Values ± S.E.

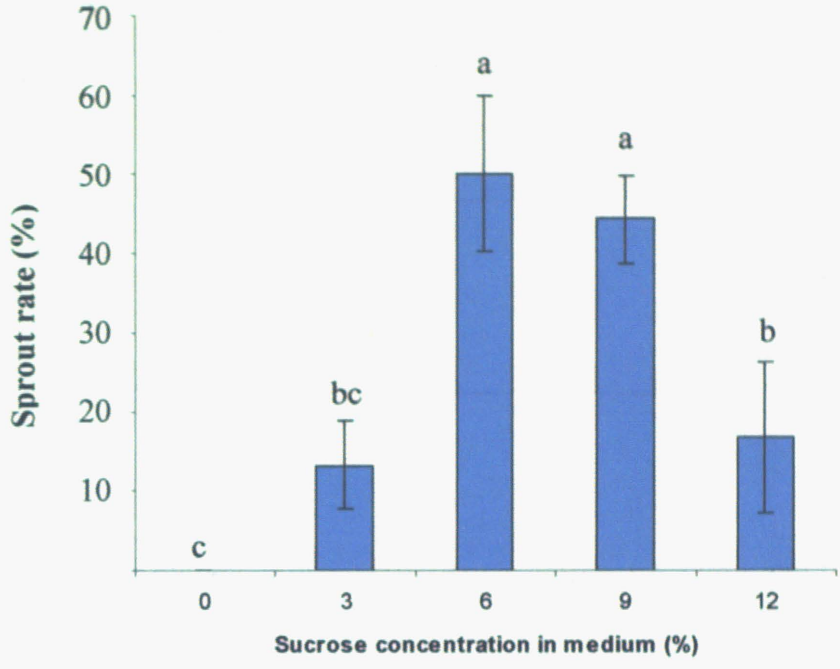

Figure 18. Sprout rate in corms grown in MS mediums supplemented with different concentrations of sucrose from the 8th week to the 24th week after germination. Values associated to the same letter were not statistically different with by means separation at 5% level (LSD). Bars represent S.E.

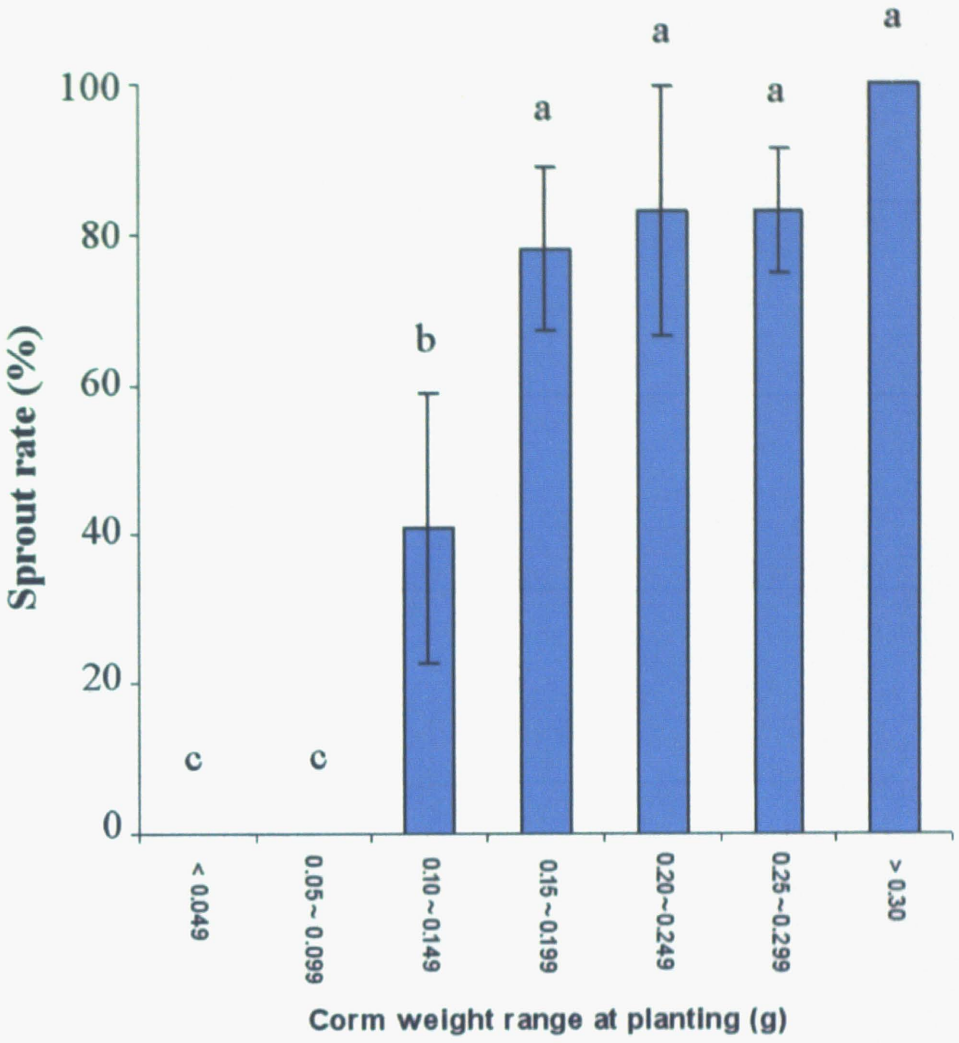

Figure 19. Sprout rate in different weight range categories after *in vitro* cultivation from the 8th week to the 24th week after germination. Values associated to the same letter were not statistically different with by means separation at 5% level (LSD). Bars represent S.E.

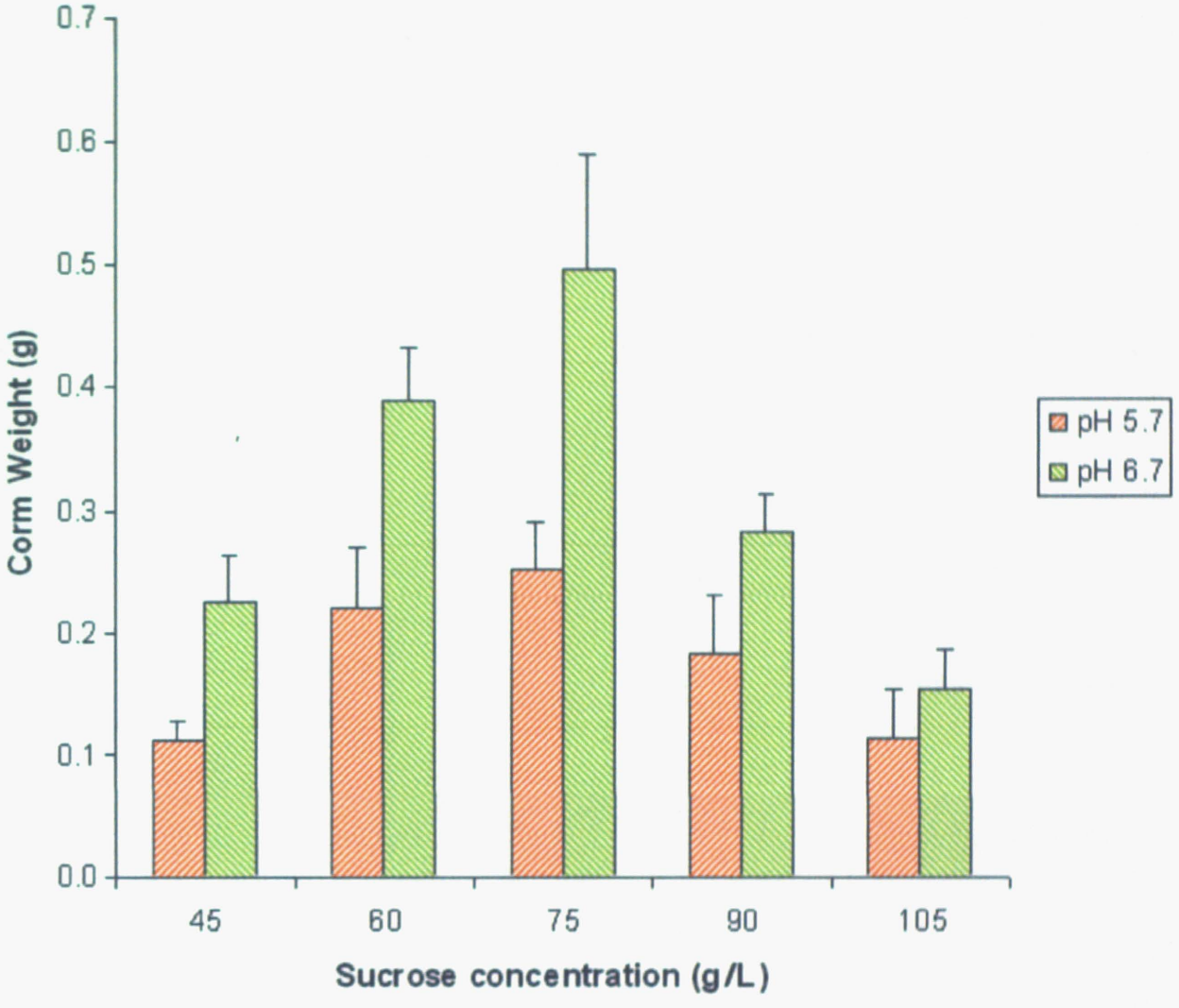

Figure 20. Corm weight of plantlets grown from the 8th to the 24th week in MS media supplemented with different concentrations of sucrose ranging from 45 to 105 g•L^{-1} with pH adjusted to 5.7 or 6.7. Bars represent S.E.

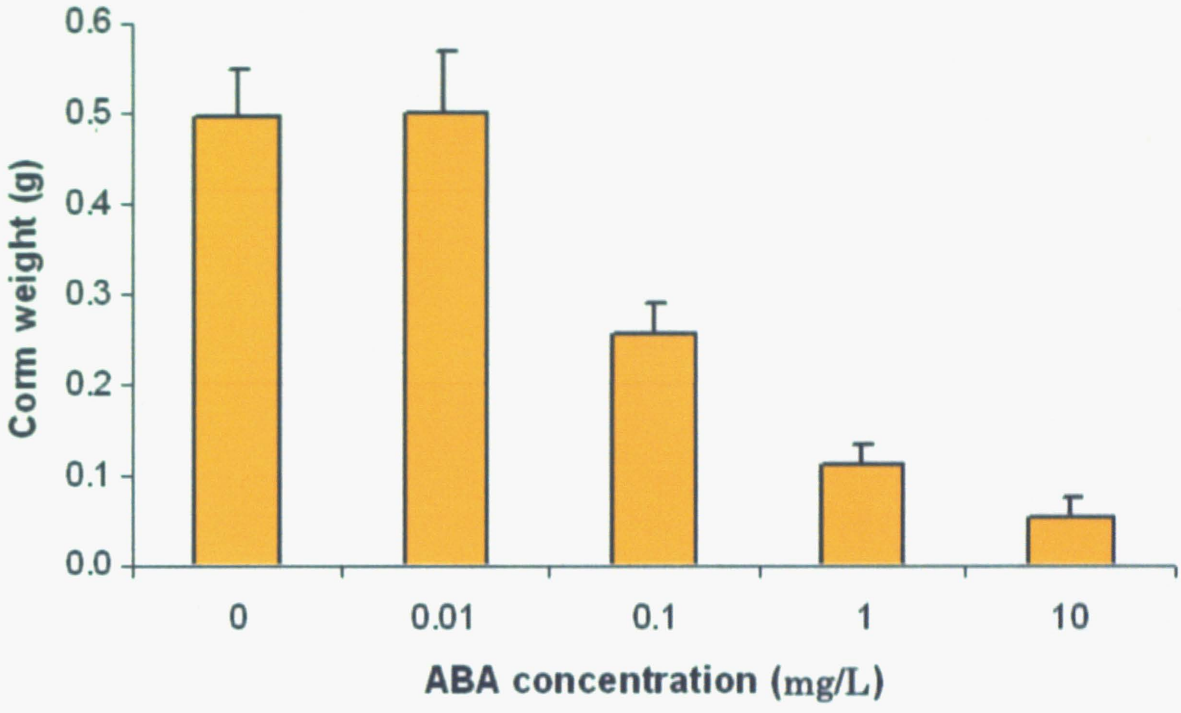

Figure 21. Effect of ABA concentration in culture medium on corm weight gain when 8 week old plantlets are cultivated for 16 weeks in MS medium pH 6.7 supplemented with 75 $g \cdot L^{-1}$ sucrose. Bars represent S.E.

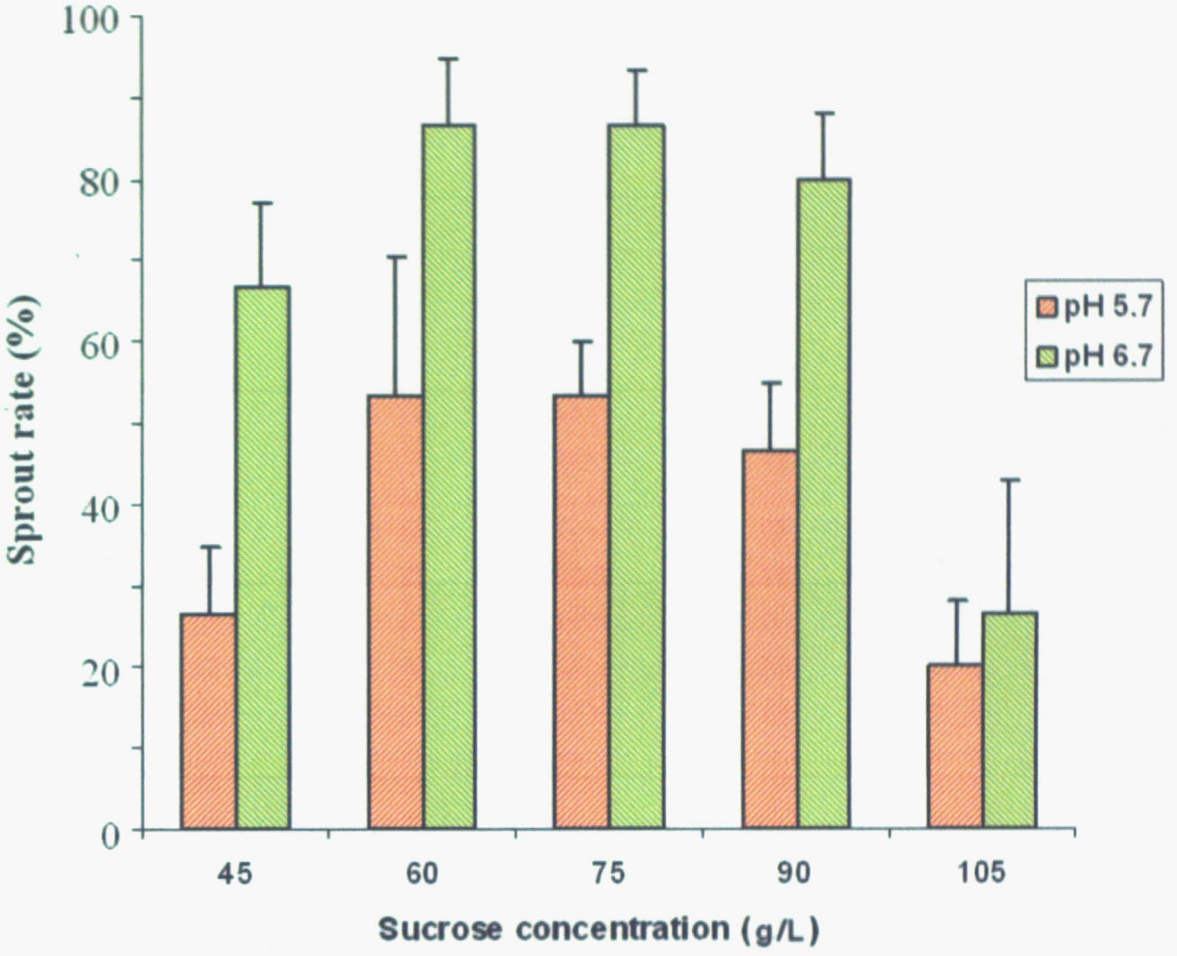

Figure 22. Sprout rate of corms grown from the 8th to the 24th week in MS media supplemented with different concentrations of sucrose ranging from 45 to 105 $g \cdot L^{-1}$ with pH adjusted to 5.7 or 6.7, when planted under greenhouse conditions after the *in vitro* stage. Bars represent S.E.

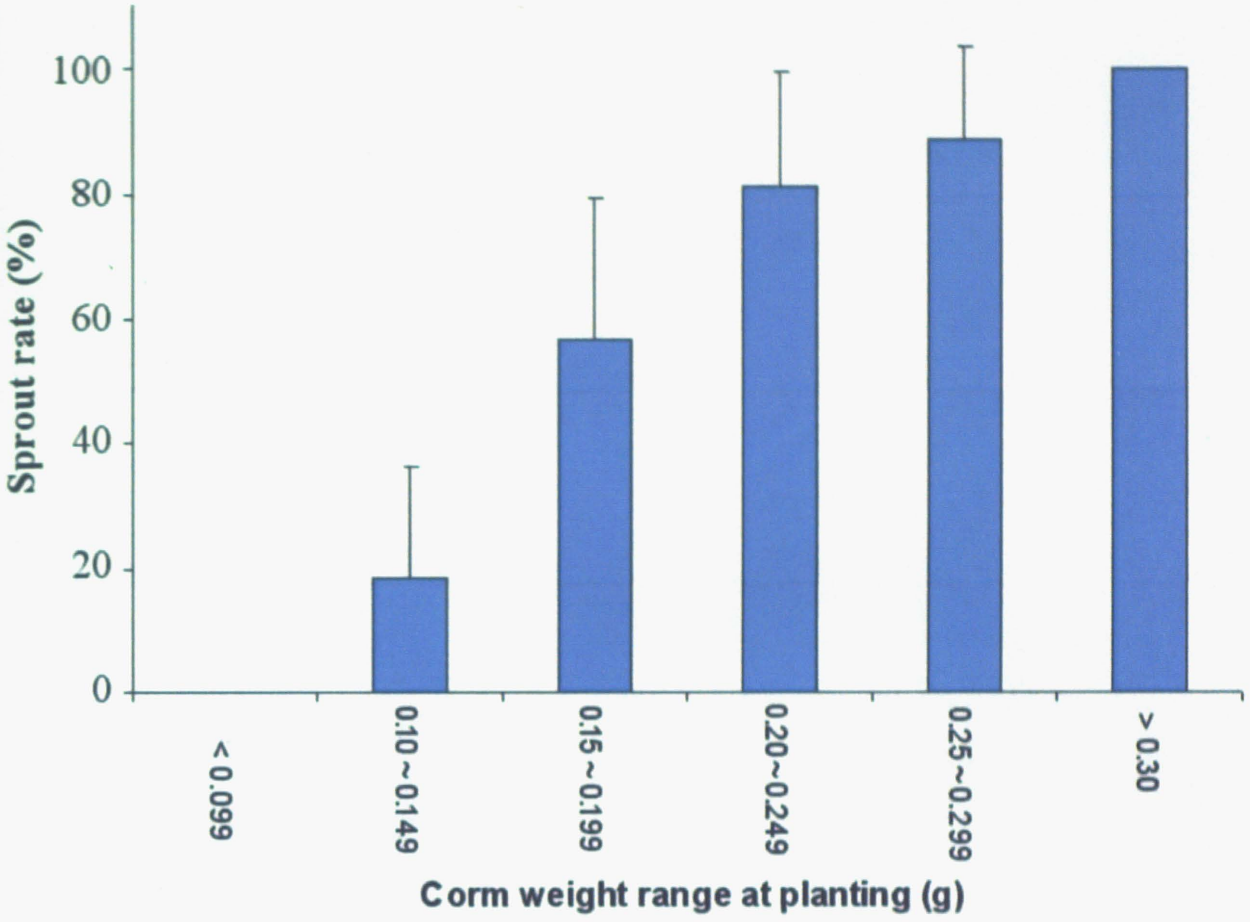

Figure 23. Sprout rate of corms when transferred to greenhouse conditions, grouped by corm weight after the *in vitro* stage. Bars represent S.E.

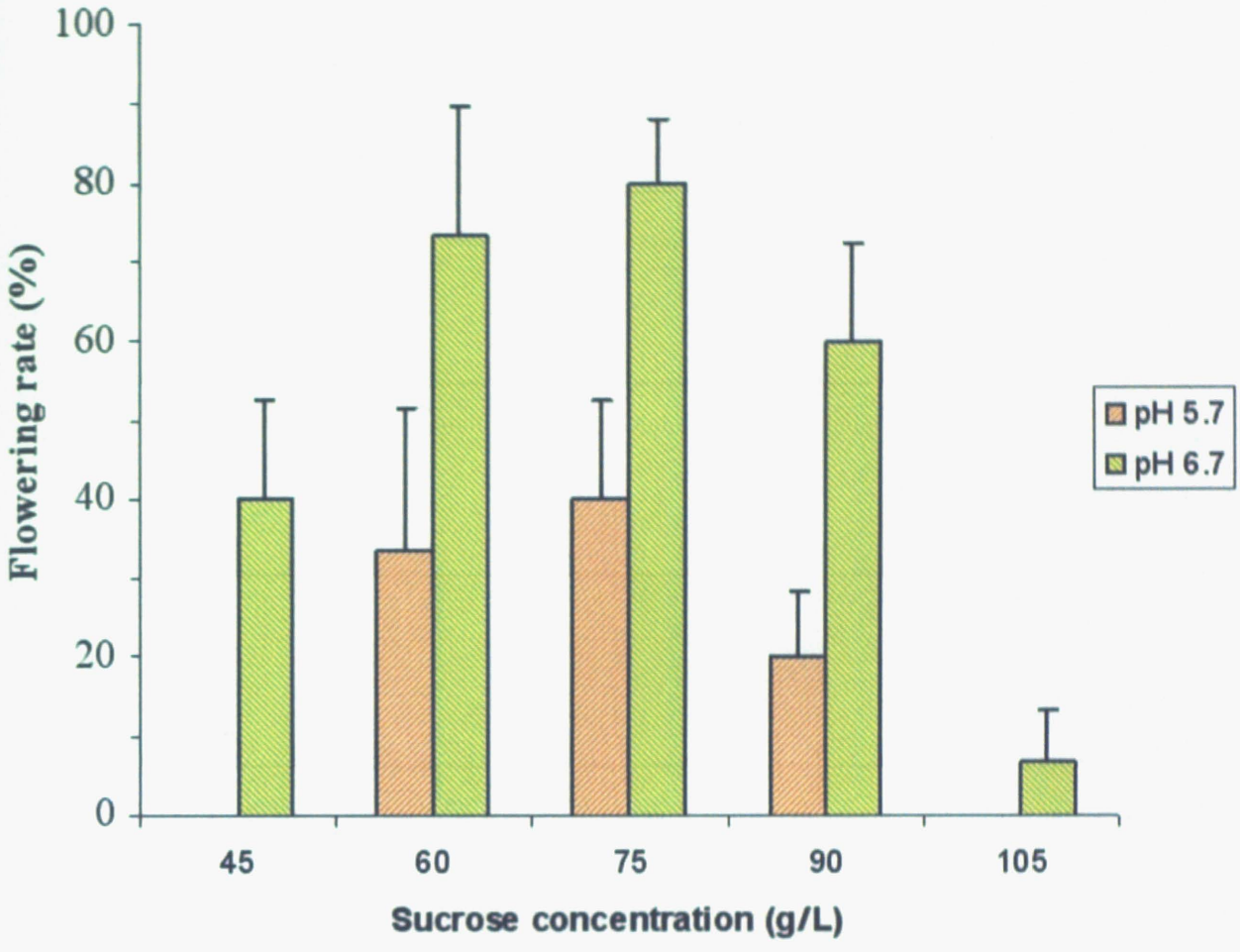

Figure 24. Flowering rate of corms were grown from the 8th to the 24th week in MS media supplemented with different concentrations of sucrose ranging from 45 to 105 g•L^{-1} with pH adjusted to 5.7 or 6.7. Bars represent S.E.

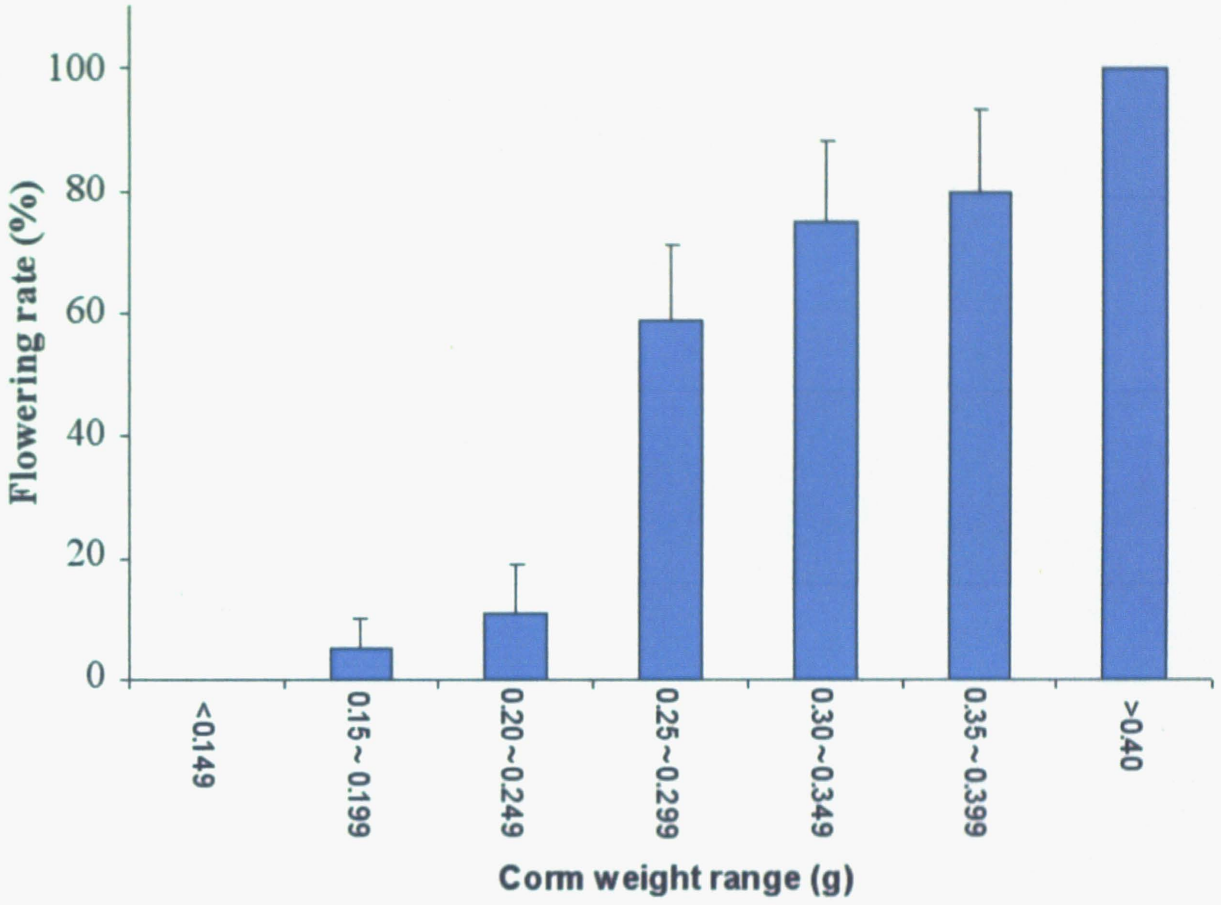

Figure 25. Flowering rate in the second year of cultivation under greenhouse conditions according to corm weight after the *in vitro* stage. Bars represent S.E.

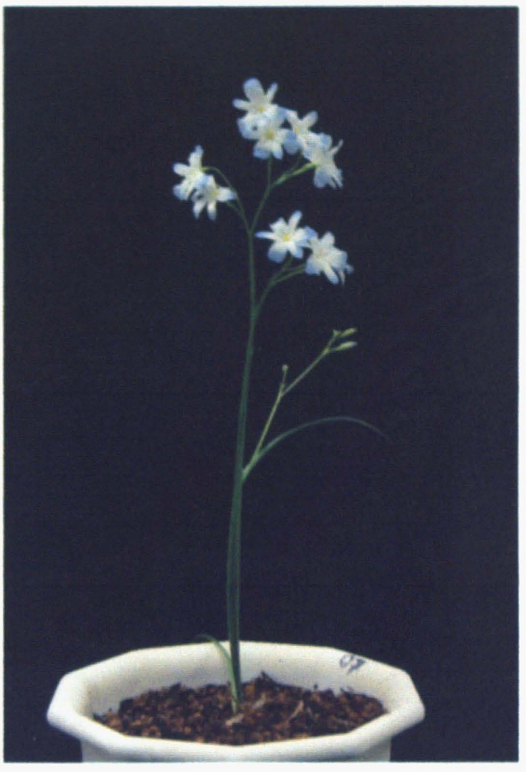

Figure 26. Flowering adult plant of *Zephyra elegans*, generated *via* enhanced *in vitro* corm enlargement.

Figure 27. Transparent jelly substance generated on wounded zones of corms of *Zephyra elegans*. Corms are very delicate and easily wounded after the tunic is removed.

Figure 28. Plant at early stage for collection of explants to be tested for vegetative propagation.

Figure 29. Plant at advanced stage for collection of explants to be tested for vegetative propagation.

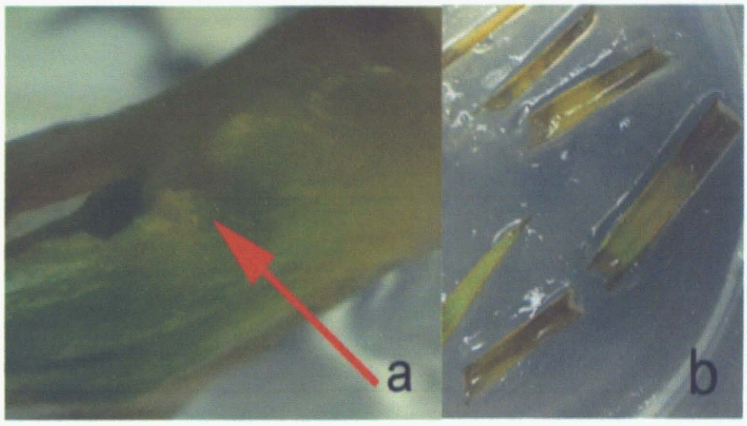

Figure 30. (a) Responsive tissue with detail of responsive lower leaf segment (red arrow). (b) Non responsive tissue.

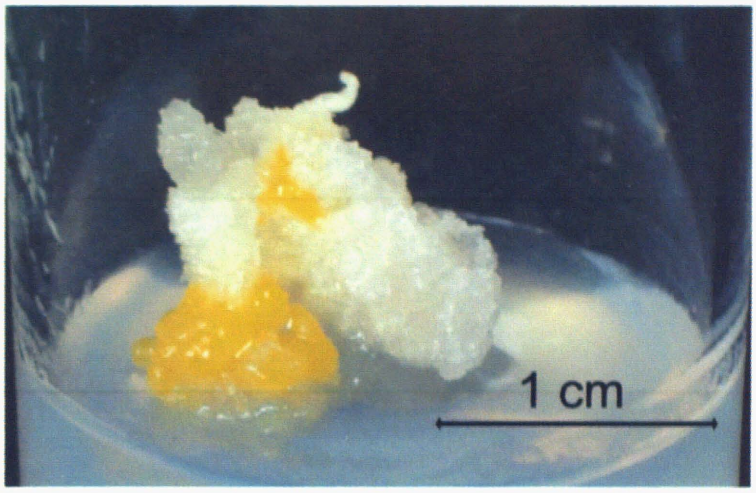

Figure 31. Callus cluster after 16 weeks of *in vitro* culture.

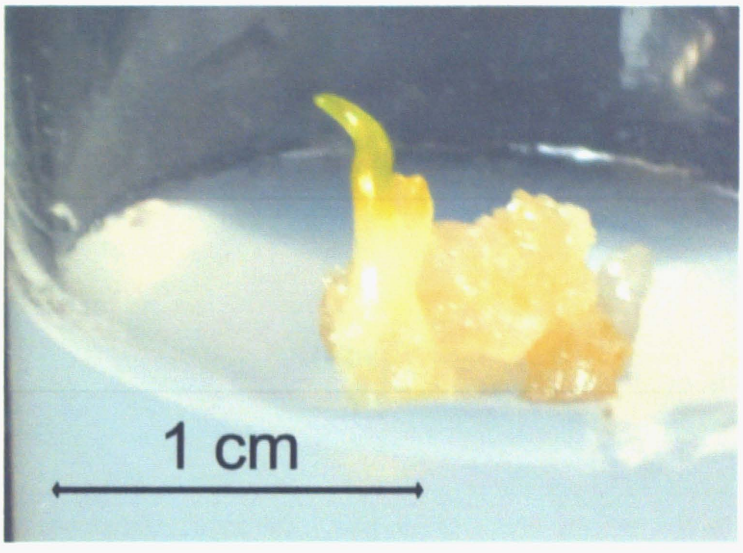

Figure 32. Sprout forming from callus after 16 weeks of *in vitro* culture.

Figure 33. Sprout separated from the callus and grown in hormone free medium for 4 weeks.

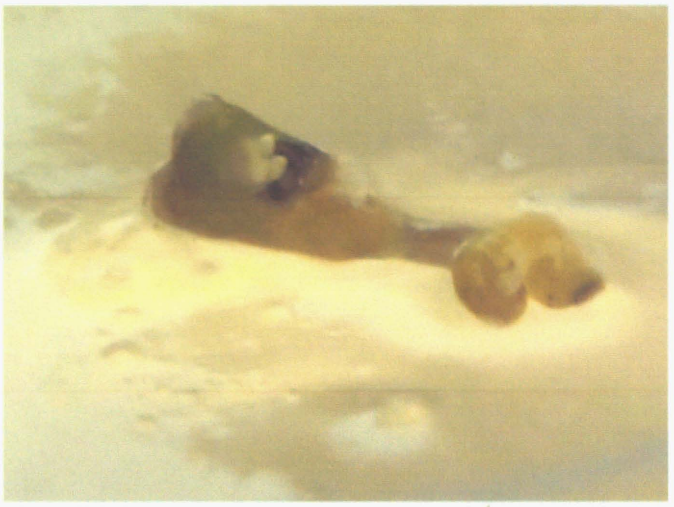

Figure 34. Response of nodes to direct sprout formation treatment with BA 1 $mg \cdot L^{-1}$ and NAA 0.1 $mg \cdot L^{-1}$, but no successful sprout formation.

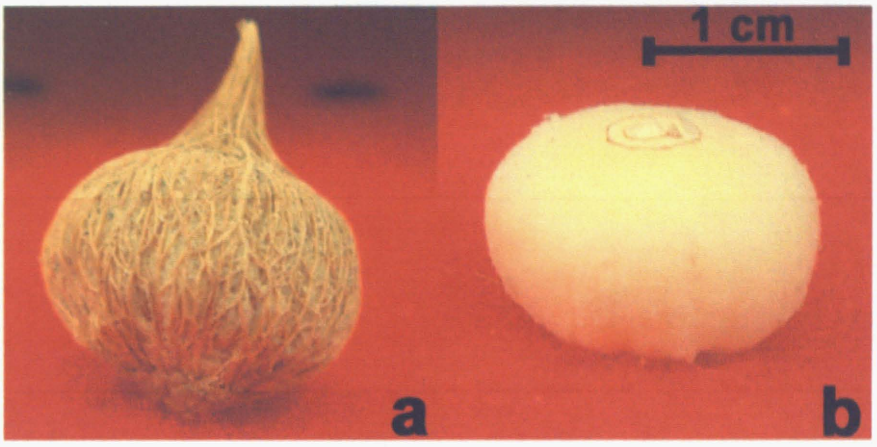

Figure 35. Tunic removal corm of *Zephyra elegans* after collection.

a. Corm before removal of tunic. b. Corm after removal of tunic.

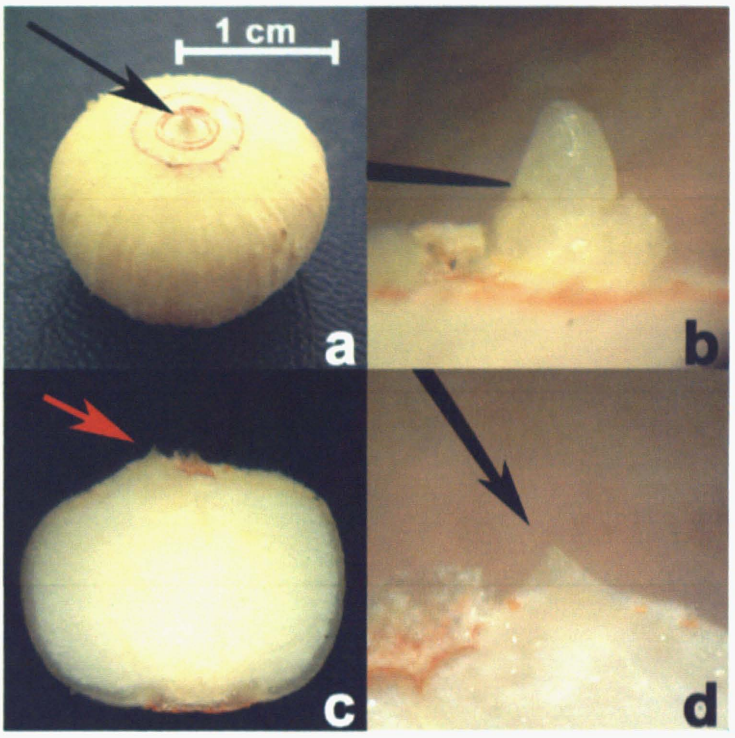

Figure 36. Apical bud of corm of *Zephyra elegans*. a. General view of the corm and its apical bud after tunic removal. b. Detail of apical bud under magnifying glass (40X under SMZ-1B model Nikon® magnifying glass). c. Dissection of the corm and its apical bud. d. Dissection of the corm and its apical bud under a magnifying glass (40X under SMZ-1B model Nikon® magnifying glass).

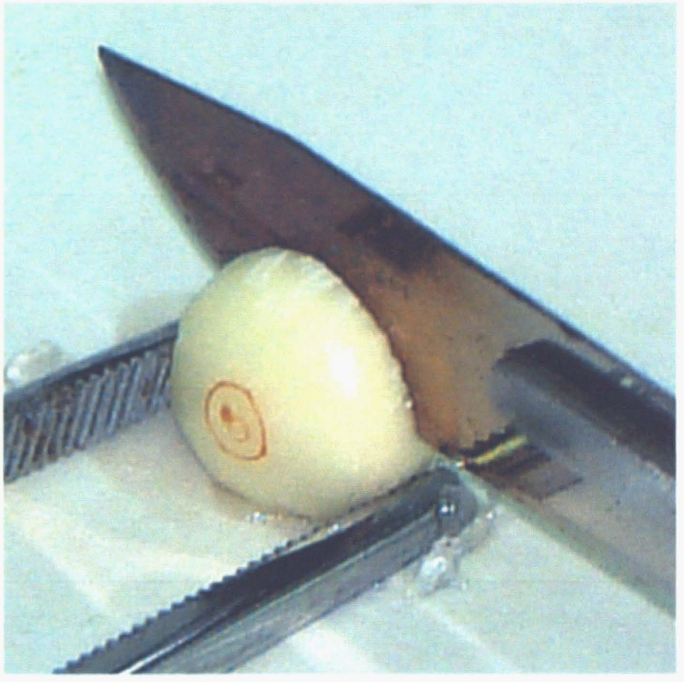

Figure 37. Equatorial division of the corm of *Z. elegans* under aseptic conditions.

Figure 38. Sprouts formed from upper half corm alter *in vitro* culture for 8 weeks in hormone free medium.

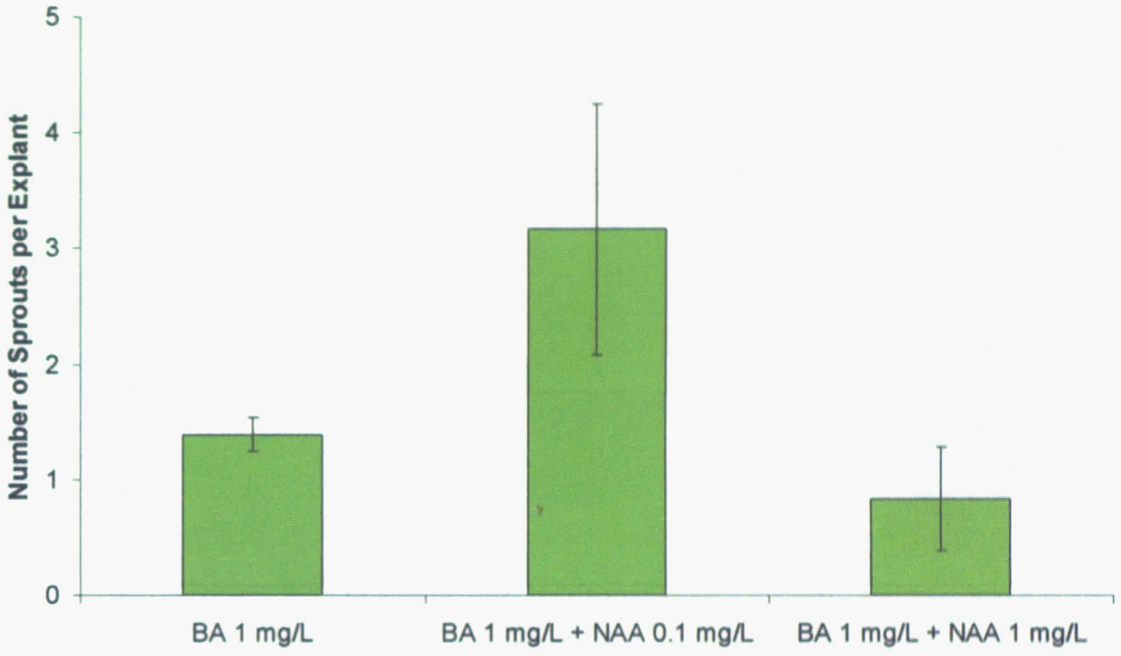

Figure 39. Number of sprouts formed per upper half corm after *in vitro* culture for 16 weeks in MS medium supplemented with hormones.

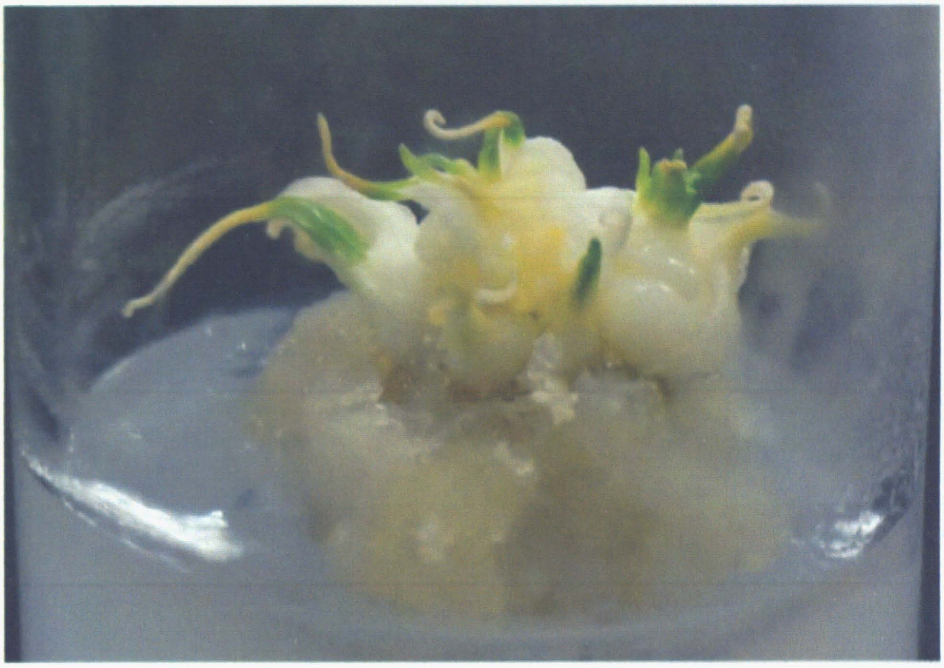

Figure 40. Multiple shoot formation from *in vitro* cultured upper half of corm of *Zephyra elegans* after 16 weeks of culture in MS basal medium with vitamins and 30 $g \cdot L^{-1}$ sucrose, supplemented with BA 1 $mg \cdot L^{-1}$ and NAA 0.1 $mg \cdot L^{-1}$.

References

Bridgen, M., 2001. A non destructive harvesting technique for the collection of native geophyte plant species. Herbetia, Journal of the Bulb Society. 56:51-60.

Bridgen, M., Olate, E. and Schiappacasse, F., 2002. Flowering geophytes from Chile. Acta Hort. 570:75-80.

Dantu, P. and Bhojwani, S., 1995. *In vitro* corm formation and field evaluation of corm-derived plants of *Gladiolus*. Sci. Hort., 61: 115-129.

Ebrahim, M., 2004. Comparison, determination and optimizing the conditions required for rhizome and shoot formation, and flowering of *in vitro* cultured calla explants. Sci. Hort., 101: 305-313.

Ehrhart, C., 2001 *Zephyra compacta* (Tecophilaeaceae): eine neue Art aus Chile. Sendtnera 7:47-52.

Finkelstein, R. and Gibson, S., 2001. ABA and sugar interactions regulating development: cross-talk or voices in a crowd?. Plant Biology, 5: 26-32.

Hodson de Jaramillo, E., Forero, A., Cancino, G., Moreno., A., Monsalve, L. and Acero, W., 2008. *In vitro* regeneration of three Chrysanthemum (*Dendrathema grandiflora*) varieties "*via*" organogenesis and somatic embryogenesis. Universitas scientiarum, 13: 118-127.

Hoffmann, A., 1989. Chilean monocotyledoneous geophytes taxonomic considerations and their state of conservation. Herbetia, 45:13-29.

Huanga, Z., Zhanga, X., Zhengb, G. and Gutterman, Y., 2003. Influence of light,temperature, salinity and storage on seed germination of *Haloxylon ammodendron*. Journal of Arid Environments 55:453-464.

Ishimori, T., Niimi, Y. and Han, D-S., 2007. Benzyladenine and low temperature promote phase transition from juvenile to vegetative adult in bulblets of *Lilium* × *formolongi* 'White Aga' cultured *in vitro*. Plant Cell Tiss. Organ Cult., 88: 313-318.

Jager A., Light, M. and Van Staden, J., 1996. Effects of source of plant material and temperature on the production of smoke extracts that promote germination of light-sensitive lettuce seeds. Environmental and Experimental Botany, 36:421-429.

Kantharajah, A., Richards, G. and Dodd, W., 1992. Roots as a source of explants for the successful micropropagation of carambola (*Averrhoa carambola* L.). Sci. Hort., 51:169-177.

Kim, H. and Ohkawa, K., 2001. Introduction of two chilean geophytes, *Leucocoryne coquimbensis* F. Phil. and *Zephyra elegans* D. Don as new ornamentals. Acta Hort. 552:179-184.

Kim, H., Ohkawa, K. and Sakaguchi, K., 1996. Effects of storage temperature and duration on flower bud development, emergence and flowering of Zephyra elegans D. Don. Scientia Horticulturae, 67: 55-63.

Kim, H., Ohkawa, K. and Sakaguchi, K.,1998. Effects on corm weight on the growth and flowering of Zephyra elegans D. Don. Acta Horticulturae, 454: 335-340.

Kim, H., Ohkawa, K., 1997. Zephyra elegans D.Don as a cut flower. Herbertia, 52:26–30.

Kim, H., Sakaguchi, K. and Ohkawa, K., 1997. *Zephyra elegans* D. Don., A potential new cormous crop. Acta hort. (ishs) 430:133-138

Msanga, H., Maghembe, J., 1986. Effect of hot water and chemical treatments on the germination of *Albizia schimperana* seed. Forest Ecology and Management, 17: 137–146.

Muñoz, M., 1985. Flores del norte chico. Second edition. Dirección de bibliotecas, archivos y museos, Chile Publishers. 95 p.

Munoz, M. and Moreira, A., 2000. Los géneros endémicos de monocotiledóneas de Chile continental, Chile, available at http://www.chlorischile.cl/Monocotiledoneas/zephyra_gen.htm

Murashige, T. and Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant, 15: 473-497.

Niimi, Y., 1986. Application of leaf segment culture to in vitro bulblets production of six *Lilium* species. Acta Bot., 35: 189-194.

Niimi, Y. and Onozawa, T., 1979. In vitro bulblet formation from leaf segments of lilies, especially *Lilium rubellum* Baker. Sci. Hort., 11:379-389.

Nomizu, T., Niimi, Y., Kasahara, S., 2003. In vitro micropropagation of 'Yukiwariso' (*Hepatica nobilis* Schreber var. japonica f. magna) by leaf segment culture. J. Japan. Hort. Sci., 3:205-211.

Padilla, I. and Encina, C., 2003. *In vitro* germination of cherimoya (*Annona cherimola* Mill.) seeds, Sci. Hort., 97: 219-227.

Raven, P., Evert, R. and Eichhorn, S., 2003. Biology of Plants. Sixth edition. Freeman-Worth Publishers, 994 p.

Schiappacasse, F., Peñailillo, P. and Yánez, P., 2003. Propagación de bulbosas chilenas ornamentales. Editorial Universidad de Talca Publishers, Chile, 70 p.

Sharma, P., Pandey, S., Bhattacharya, A., Nagar, P. K. and Ahuja P., 2004. ABA associated biochemical changes during somatic embryo development in *Camellia sinensis* (L.) O. Kuntze. Plant Physiol., 161: 1269-1276.

Sim, G., Goh, C. and Loh C., 1989. Micropropagation of *Citrus mitis* blanco-multiple bud formation from shoot and root explants in the presence of 6-Benzylaminopurine. Plant Sci., 59:203-210.

Simpson, M. and Rudall, P., 1998. Tecophilaeaceae. In: Families and Genera of Flowering Plants. Monocotyledons. Kubitski Publishers, 3:429-436.

Song, J., Fan, H., Zhao, Y., Jia, Y., Du, X. and Wang, B., 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.

Ulrich, M., Davies, F., Cheong Y., Duray, S. and Egilla, J., 1999. Micropropagation of *Crinum* 'Ellen Bosanquet' by tri-scales. Sci. Hort., 82:95-102.

Vidal, A. K. and Niimi Y., 2008. *In vitro* initiation of seeds and further corm weight gain in *Zephyra elegans*. Act. Hort., 865:301-304.

Vidiella, E., Armesto J. and Gutierrez J., 1999. Vegetation changes and sequential flowering after rain in the southern Atacama Desert. Journal of Arid Environments, 43: 449-458.

Yanez, P., Ohno, H., Ohkawa, K., 2005. Temperature effects on corm dormancy and growth of *Zephyra elegans* D.Don. Sci. Hort., 105:127-138.

Zhou, Q., Jiang, Z., Huang, T., Li, W., Sun, A., Dai, X. and Li, Z., 2010. Plant regeneration *via* somatic embryogenesis from root explants of *Hevea brasiliensis*. African Journal of Biotechnology. 9:8168-8173.

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Summary

Zephyra elegans is endemic specie belonging to the Tecophilaceae family, from the coastal desert in northern Chile. It has been described as a specie with high potential as an ornamental crop. One of the steps in order to have this new crop is the ability to commercially propagate this specie. This work focuses on solving this problem by the propagation of *Zephyra elegans via in vitro* culture of seeds and vegetal material. Greenhouse experiments suggest that *Zephyra elegans* should be treated as a winter flowering specie under greenhouse conditions. Under greenhouse conditions, commercial vegetative propagation of *Zephyra elegans* is not possible due to low propagation rate. Propagation by seeds takes time, and seeds will take more than 5 years in order to produce flowering comrs. As a solution for these propagation problems, *in vitro* protocols were created. There are very few works regarding propagation of *Zephyra elegans*.

Experiments were performed in order to establish a method to reduce time from seed to flowering in *Zephyra elegans*. Nonetheless germination of seeds was highest in water-agar medium, due to plant necrosis occurring when plants were later transferred, MS medium, with a germination rate of 59%, was selected as the medium for germination. Increasing light intensity from dark conditions to 2000 lux to 9500 lux had a significant effect on increasing germination. Only under dark conditions increasing pH from 5.7 to 6.7 had a significant effect on increasing germination rate. With the objective of achieving the highest corm weight gain, 8 weeks old seedlings were cultured for 16 weeks in MS mediums with sucrose concentrations from 0 to 120 $g \cdot L^{-1}$ and pH 5.7 or 6.7. Maximum weight gain was observed in MS medium with 75 $g \cdot L^{-1}$ sucrose and pH 6.7. Corms were later transferred to pots and grown under greenhouse conditions. Corms weighing under 0.12 g did not sprout, while all corms over 0.3g sprouted. All corms that were over 0.4 g at the end of the *in vitro* culture stage flowered in the second year of greenhouse cultivation but those under 0.15 g did not flower.

Vegetative propagation was initially tested on flower buds, leaves, internodes and nodes. Callus was achieved with lower internodes in MS medium with 0.1 $mg \cdot L^{-1}$ BA and 1 $mg \cdot L^{-1}$ NAA. Sprouts were achieved when callus clusters were later transferred to MS medium with 1 $mg \cdot L^{-1}$ BA and 0.1 $mg \cdot L^{-1}$ NAA, but no survival was achieved, mainly due to vitrification. Vegetative propagation was possible *via* multiple shoot formation only when upper halves of corms were cultured for 16 weeks in MS basal semisolid medium with vitamins and 30 $g \cdot L^{-1}$ sucrose, supplemented with 1 $mg \cdot L^{-1}$ BA and 0.1 $mg \cdot L^{-1}$. Rooting was achieved when sprouts were transferred to hormone free MS medium.

Time required from seeds to corms with flowering capability was reduced to 3 years by the use of the *in vitro* protocol described in this work. This may be helpful in genetic improvement programs as well as large scale propagation of homogenetic lines. Though *in vitro* vegetative propagation protocol achieved in this work does not allow for conservation of the mother plant, it may be useful in commercial production and conservation of genetic material. Future experiments are needed in order to perform acclimation and greenhouse flowering stages for vegetative propagation generated plantlets, as with plantlets from seed experiments. Mass vegetative propagation was achieved for the first time in *Zephyra elegans*.

要旨

Zephyra elegans は，チリ北部海岸沿いの乾燥地域に自生する Tecophilaceae 科球根植物の一種であり，観賞植物として高い潜在的な利用価値がある．*Z. elegans* を花き園芸植物として利用するためには，効率的な繁殖と栽培技術の確立が不可欠である．しかし，自生地での成長は気象条件に大きく左右され，数年おきに萌芽して開花することや，人工栽培条件下でも種子発芽から開花まで通常 5 年以上かかるため，繁殖や栽培に関する研究が非常に少ない．これまで，*Z. elegans* の球根の休眠打破と開花についての研究結果が若干報告されているが，増殖に関する研究はほとんど行われていない．そこで，本研究は *Z. elegans* における効率的な増殖技術の確立を目的として，*in vitro* での種子繁殖および栄養繁殖について研究を行った．

In vitro での種子繁殖に関しては，①発芽培地を検討した結果，寒天培地での種子発芽率が高かったが，MS 培地に移植した後，実生の発育は停止した．一方，MS 培地では，発芽率が 59%であったが，実生は球根を形成した．②種子発芽に及ぼす光条件（暗黒，2000 lux，9500 lux）および pH（5.7，6.7）の影響を検討した結果，9500 lux の光条件では種子発芽率が 97%で最も高かった．また，pH 6.7 では発芽が約 2 週間程度促進された．③球根の発達に及ぼすショ糖濃度（0～120 g/L）および pH（5.7，6.7）の影響を検討した結果，75 g/L ショ糖と pH 6.7 の条件が球根の発達に最も適していた．④球根の重さが順化後の活着率に影響を及ぼした．0.12 g 以下の球根は萌芽しなかったが，0.3 g 以上の球根はほぼすべて萌芽した．順化後 2 年目に開花が確認された．*In vitro* での球根の重さは開花率にも影響し，0.15 g 以下の球根は開花しなかったが，0.4 g 以上のものはすべて開花した．

組織培養による栄養繁殖においては，①花蕾，葉片，節間および節など外植体を，0.1 mg/L BA および 1 mg/L NAA を含む MS 培地に置床して培養した．カルスが下部の節間外植体から誘導された．形成されたカルスを 1 mg/L BA および 0.1 mg/L NAA を含む MS 培地に移植したところ，少数のシュートが形成された．しかし，それらのシュートはガラス化状態となり，球根に発達しなかった．②芽を付けた球根の上の半分を 1 mg/L BA および 0.1 mg/L NAA を含む MS 培地で培養した結果，外植体から複数のシュートが誘導された．さらに，これらのシュートから小球根が形成され，それらの小球根を植物成長調節物質無添加の MS 培地に移植したところ，根の形成が観察された．

本研究の結果より，*in vitro* での種子培養による *Z. elegans* の球根生産が可能であることが明らかとなった．また，種子発芽から開花までに要する期間が 3 年間に短縮された．一方，節間や球根外植体からシュートが誘導され，小球根を形成したことから，組織培養による *Z. elegans* の栄養繁殖の可能性が示された．これらの結果は，*Z. elegans* の増殖に関する新しい知見を提供し，効率的な繁殖技術の確立に役立つものであると考えられる．

Abbreviations

ABA: Abscisic acid

NAA: 1-naphtaleneacetic acid

MS: Murashige and Skoog (1962) (growth medium)

BA: 6-benzylaminopurine

NaClO: Sodium hypochlorite