International Journal of Horticultural Science 2007, 13 (2): 67–71
Agroinform Publishing House, Budapest, Printed in Hungary
ISSN 1585-0404

Results with the establishment of *in vitro* culture of *Leucojum aestivum*

Kohut, E.[1], Ördögh, M.[1], Jámbor-Benczúr, E.[1] & Máthé, Á.[2]

[1]*Corvinus University of Budapest, Faculty of Horticultural Science, Department of Floriculture and Dendrology*
[2]*West-Hungarian University, Faculty of Agriculture and Food Science, Department of Botany*

Summary: *Leucojum aestivum* is a native, protected ornamental and medicinal plant in Hungary and in Ukraine too. The aim of our work was to establish in vitro cultures of this bulbous plant. Prior to surface sterilisation the old leaves and roots were dissected from the bulbs and they were stored in a refrigerator (2–3°C) for different periods (1 week for the first starting experiment and 5 weeks for the second one). After sterilisation, bulbs, bulb scales and leaves of the bulbs were placed on *Murashige and Skoog's* (1962) medium with 1 mg/l benzyl-adenine (BA) and 0,1 mg/l naphthalene acetic acid (NAA). At the first starting experiment 81,3%, and at the second one 92,3% of the explants turned to be sterile. Bulblets and roots were developed on the explants in the case of using bulb plates together with bulb scales and leaves as inoculua. The best result was achieved after 5 weeks chilling and it was possible to gain little bulbs from the bulb leaves too.

Key words: *Leucojum aestivum, in vitro culture, sterilisation, chilling*

Introduction

Leucojum aestivum (*Amaryllidaceae – Figure 1*) is a native and protected ornamental and medicinal plant in Hungary and in Ukraine too. It is an endangered bulbous plant because of its beauty *(Figure 1)* and active ingredients used for the treatment of neurological diseases.

Only few data are available in the literature concerning *in vitro* culture of this plant. A study of the morphogenetic potential of the basal and apical parts of bulbs, stems, leaves and ovaries revealed that the sheats of *L. aestivum* possessed the highest regenerative ability. MS medium + 1,0 mg/l benzyladenine (BA) + 1,0 mg/l kinetin, Linsmaier and Skoog (LS) medium + 0.5 mg/l NAA + 0.1 mg/l kinetin proved to be the most suitable for direct organogenesis from these explants (*Stanilova* et al., 1994).

Savona et al. (2004) used a different *Leucojum* species (*L. nicaense*) in their experiments. It was conducted to set up a preliminary protocol of *in vitro* culture, to evaluate the possibility to maintain *ex situ* germplasm and the reintroduction of micropropagated bulbs of *L. nicaense*. Seeds were surface-sterilized in a solution of sodium hypochlorite for 20 minutes and then rinsed twice with sterile distilled water. Thereafter, they were cultured on a semisolid medium (pH 5.7) consisting of MS macro- and microelements, vitamins, surose at 30 g/l, and agar at 8 g/l. The effects of IBA or BA, added to the basal medium at the same concentration (0,5 mg/l), were also tested. The best proliferation was achieved when the auxin-containing medium was used. Data of multiplication rates, fresh weights, height of the aerial parts and proportion of rooting were recorded after IBA treatment (0.5-1,0 mg/l), and compared to those ones measured on the growth regulator-free medium. Trials to propagate these bulbs using a temporary immersion system were also carried out. Micropropagated bulbs were transferred in a greenhouse for acclimatization and further evaluation.

Leucojum aestivum (summer snowflake) micropropagation was carried out using 2 and 4 bulb-scales explants and immature embryos. The highest number of bulblets (6.67 and 5.83) were achieved on MS medium containing 1 mg/l BA and 1 mg/l NAA or 2 mg/l BAP and 0.5 mg/l NAA from 4 and 2 bulb scale explants, respectively. In contrast, the highest numbers of bulblets (2.27) from immature embryos were obtained on MS medium containing 0.5 mg/l BA and 4 mg/l NAA. The best rooting of bulblets regenerated from bulb scales was obtained on MS medium containing 1 mg/l NAA. Rooted bulbs were finally transferred to compost and acclimatized to ambient conditions (*Karaoğlu*, 2004).

Jámborné Benczúr et al. (1989) successfully propagated *Narcissus,* that is a somewhat similar to *Leucojum* in appearance of the flower and in the structure of the bulb. They used the two-steps surface sterilisation procedure for the bulbs. It means, that bulbs were sterilised without cutting them into peaces and placed on hormone free medium at first. Later, following the period of incubation (1 week) the sterilisation was repeated in case if the bulbs were infected. After this the bulbs were cut into peaces, the explants were prepared. MS medium containing half concentration of macroelements plus 1 mg/l BA and 0.1 mg/l NAA was used for initiating of the cultures. The best proliferation rate was achieved on this medium too.

The aim of the present work was the establishment of *in vitro* cultures in order to micropropagate the plant, because the gathering from the natural living place causes the disappearing of it. We intended to observe the effect of the chilling – before starting the culture – on the propagation capacity.

Materials and methods

Bulbs were collected in Ukraine, Subcarpathia, Moorland Szernye, on 18 July 2006 (*Figure 2*). The experiments were carried out in the laboratory of the Department of Floriculture and Dendrology, Corvinus University, Budapest. Before surface sterilization the old leaves and the roots were dissected from the bulbs and they were stored in a refrigerator (2–3 °C) for different periods. The chilling period lasted one week for the first starting experiment and 5 weeks for the second one. The sterilization began with the surface cleaning of the bulbs and was followed by washing under tap water for 2 hours. Subsequently, the bulbs were sterilized with 70% ethanol for 10 minutes and 0.1% $HgCl_2$ for 15 minutes. Finally they were rinsed with sterile distilled water three times.

For the first experiment the sterile bulbs were cut up into bulb scales containing the bases of leaf blades. For the second experiment the sterile bulbs were chilled on hormone free medium *(Figure 3)* and the apical parts of bulbs with leaves were used. As a basic medium Murashige and Skoog formula (1962) was used (with half concentration of macro elements) with 1 mg/l benzyl-adenine (BA), 0,1 mg/l naphthalene acetic acid (NAA) and 30 g/l sucrose as supplements (E1). The pH was adjusted to 6.5 with KOH.

Explants were placed into 100 ml Erlenmeyer flasks (*Figure 4*) and the cultures were incubated at 20–24 °C in 8/16 hours dark/light photoperiod for 12 weeks. After 12 weeks, the number and length of shoots (bulblets), and the number and length of roots was measured. Tissue sections of the differentiated bulblets were examined by a scanning electronmicroscope.

Figure 1 *Leucojum aestivum* in its original, natural habitat (Subcarpathia)

Figure 2 Flora of Moorland Szernye (Ukraine, Subcarpathia), with *Leucojum aestivum and Fritillaria meleagris* plants

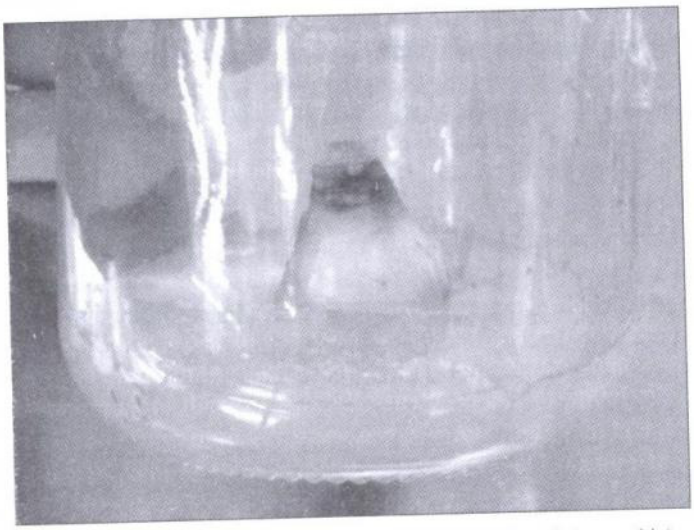

Figure 1 *Leucojum aestivum* in its original, natural habitat (Subcarpathia)

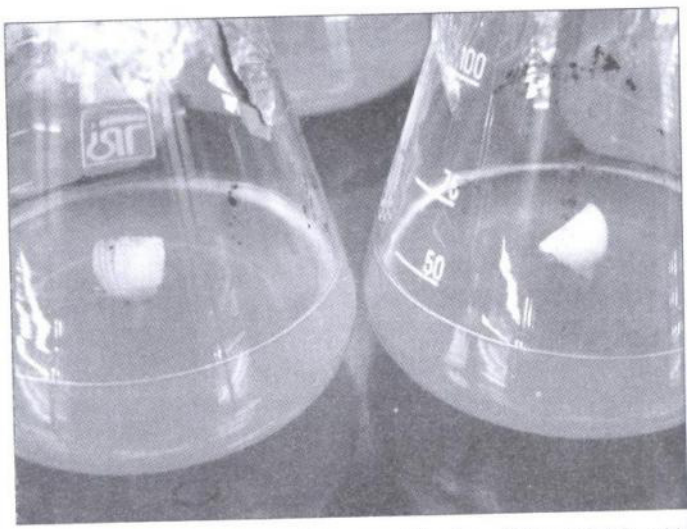

Figure 2 Flora of Moorland Szernye (Ukraine, Subcarpathia), with *Leucojum aestivum and Fritillaria meleagris* plants

Results and Discussion

At the first starting experiment 81.3% of the explants turned to be sterile. A subsequent amount of explants (69.2%) differentiated little bulbs (buds, bulblets). Roots were developed on 38.5% of the explants. The average number of the bulblets per explant was 12.44, and the length of the bulblets reached 2.8 mm. The average number of roots per explant was 1.8, with a length of 27.5 mm.

The second starting experiment resulted 92.3% of sterile explants. All of the explants differentiated small bulbs. Root formation occurred on 38.5% of the explants. The average number of bulblets per explant was 11.77 with the length of 3.7 mm. The average number of roots per explants was 1.2, and their length was 53.4 mm. The bulb scales and leaves originated from the apical part of the bulb become green and all of them differentiated small buds. The average number of bulblets was 7.2 and their length was 1.5 mm. It was very interesting, that root differentiation occurred on the leaf explants too. Results are presented in *Table 1* and *Figures 5–8, 13–14*. Bulb-differentiation from tissue of leaves was demonstrated by electronmicroscopic photos. (*Figures 9–12*).

Table 1 Data of *Leucojum aestivum* bulb's explants (first and second experiment)

Examined features	First starting from bulb scales with bulb plate	Second starting	
		From bulb scales with bulb plate	from bulb leaves
Sterility%	81.3	92.3	
Inocula with differentiated shoots%	69.2	100	
Number of shoots	12.44	11.77	7.2
Length of shoots (mm)	2.8	3.7	1.5
Number of roots	1.8	1.2	1.5
Length of roots (mm)	27.5	53.4	27.5
Rooting%	38.5	38.5	20

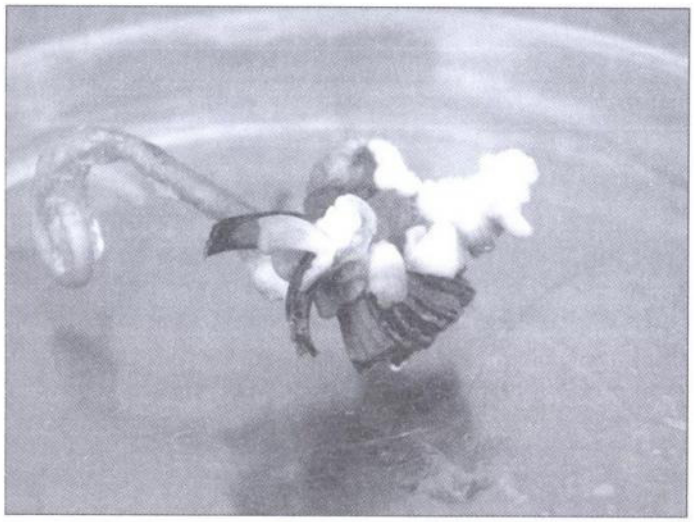

Figure 5 Shoot and root differentiation on bulb scales after 12 weeks in culture (medium E1)

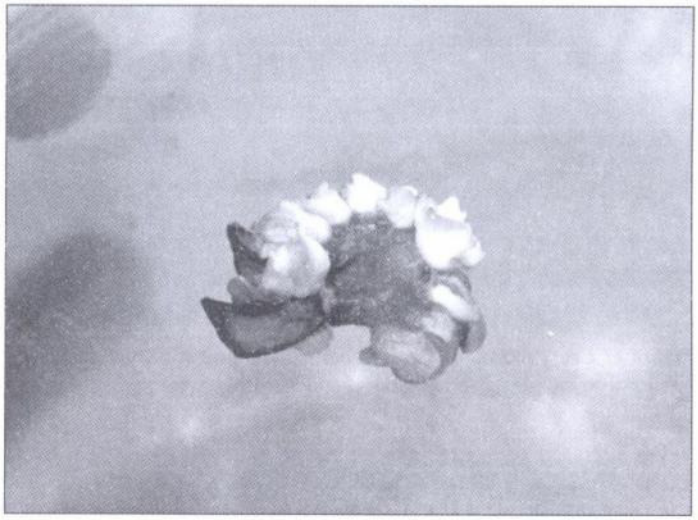

Figure 6 Shoot differentiation on bulb scales after 12 weeks in culture (medium E1)

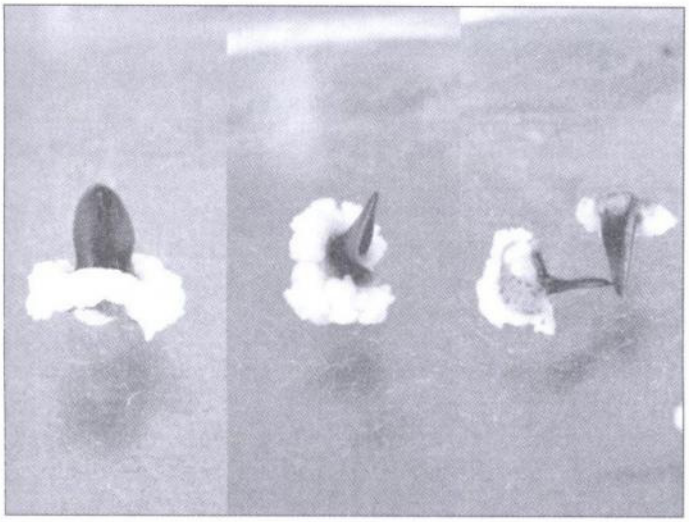

Figure 7 Shoot-cluster differentiation on 1 cm long bulb leaves after 12 weeks in culture (medium E1)

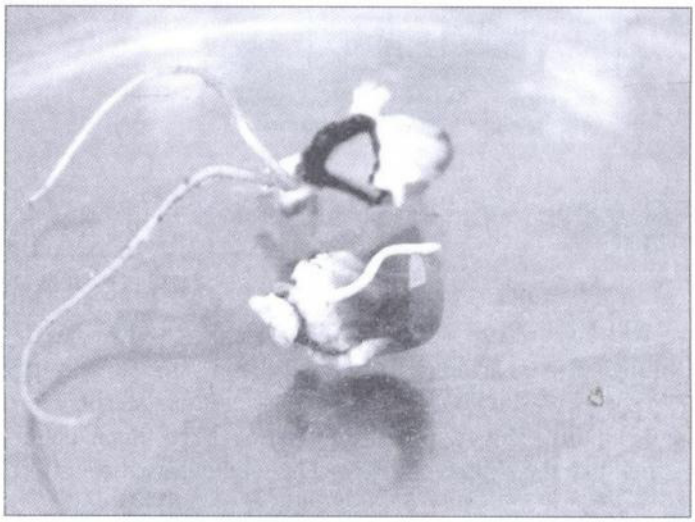

Figure 8 Shoot and root differentiation on leaf explant after 12 weeks in culture (medium E1)

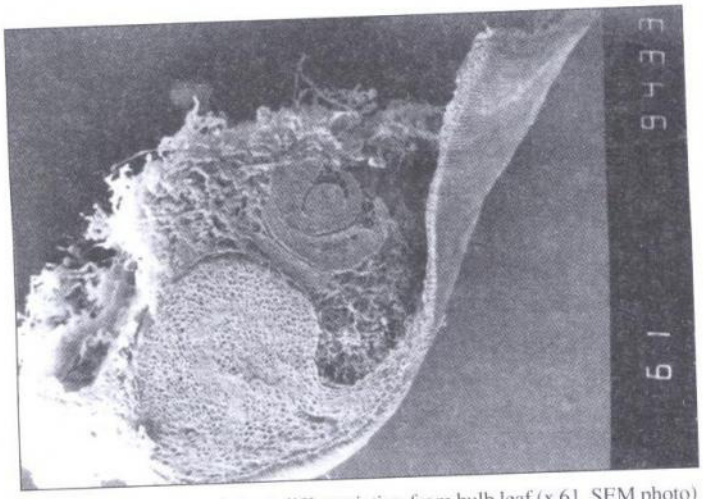

Figure 9 Meristem and shoot differentiation from bulb leaf (x 61, SEM photo)

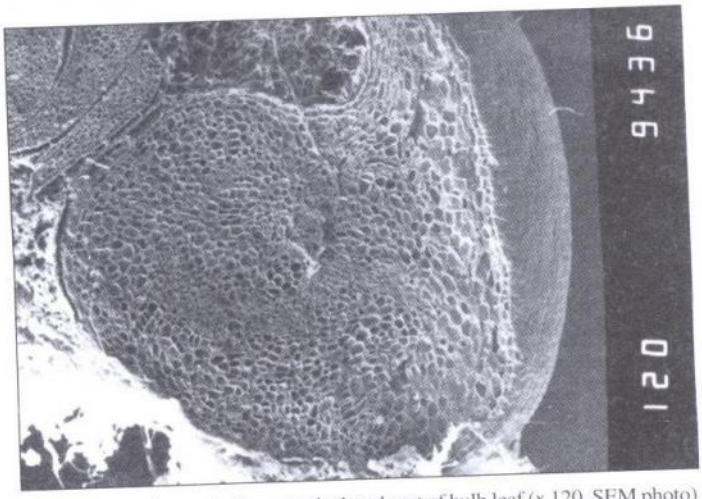

Figure 10 Meristematic tissue on the basal part of bulb leaf (x 120, SEM photo)

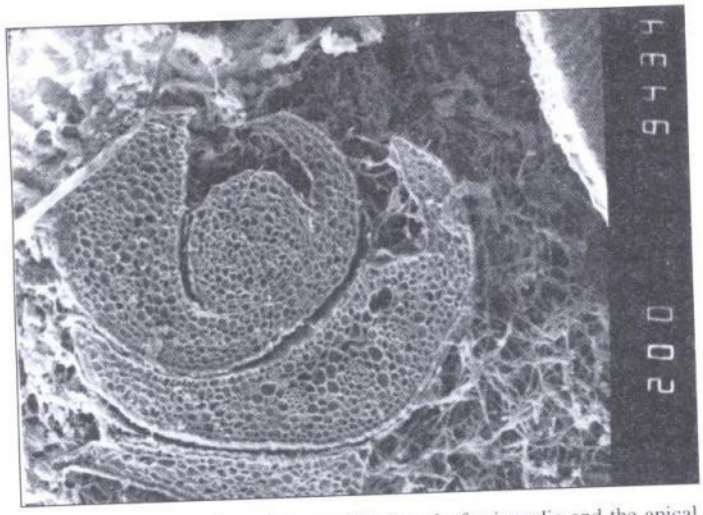

Figure 11 Cross section of shoot with three leaf primordia and the apical meristem on the basal part of bulb leaf (x 200, SEM photo)

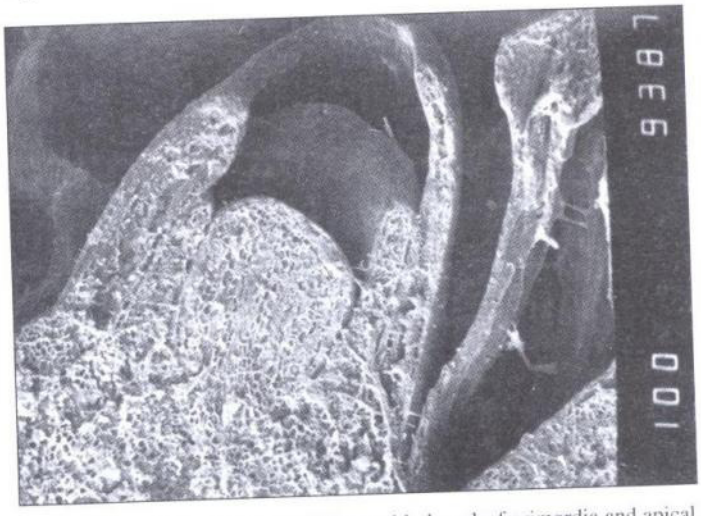

Figure 8 Longitudinal section of shoot with three leaf primordia and apical meristem on the basal part of bulb leaf (x 100, SEM photo)

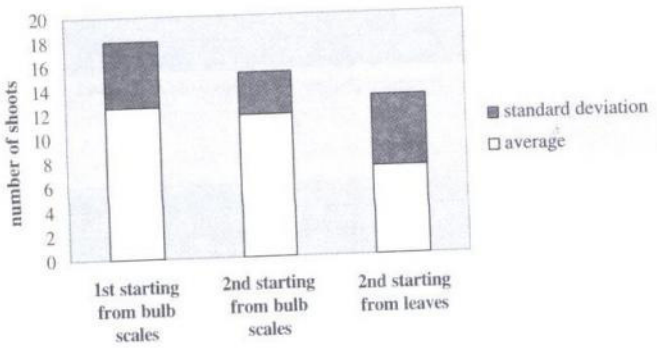

Figure 13 Average number of shoots on E1 medium after 12 weeks in case of both starting

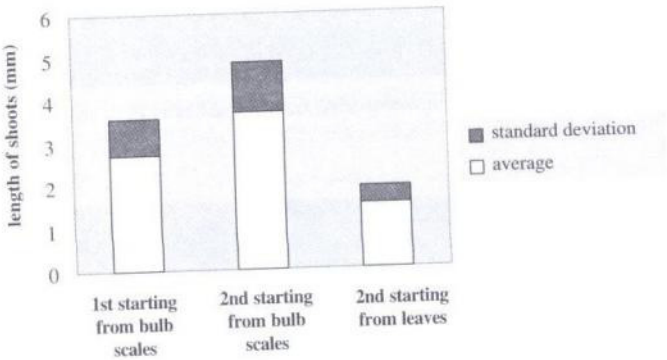

Figure 14 Average length of shoots on E1 medium after 12 weeks in case of both starting

The best result was achieved after five weeks sterile chilling. This is a novel method, because similar chilling period was used with the *Narcissus*, but without sterilisation of the bulbs. The two-step sterilisation gave much better results than the simple one-step. The medium used for starting was similar to that of *Stanilova* et al. (1994) with the difference, that they used kinetin too. For the initiation of the cultures the same medium that gave the best results with the *Narcissus* (*Jámborné* et al., 1989) was successfully used. Bulblet (shoot) differentiation was observed in the second experiment on the bulb scales and leaves too. Rooting was found on most of the explants, and even on some of the bulb leaves too. This phenomenon has not been mentioned in connection with starting of *Leucojum* cultures till now. Elektronmicroscopic photos proved the differentiation of the bulblets from bulb leaves too.

Acknowledgements

This work was supported by the OTKA (Hungarian Scientific Research Fund), project No. T-049642.

References

Jámborné Benczúr, E., Márta, K.-né & Peredi, A. (1989): A nárcisz mikroszaporitása. (Micropropagation of daffodils). Kertészeti és Élelmiszeripari Egyetem Közleményei, Budapest, LII, 101–107.

Karaoğlu, C. (2004): *In vitro* propagation of summer snowflake. Master Thesis, 2004, 38 pages, publicated in electronic form (Internet) (http://72.14.221.104/search?q=cache:X_xsbdsosl4J:papirus.ankara.edu.tr/tez/FenBilimleri/Yuksek_Lisans_Tezleri/2004/FY2004_184/Ozet.pdf+Leucojum+in+vitro&hl=hu&gl=hu&ct=clnk&cd=5)

Linsmaier, E.M. & Skoog, F. (1965): Organic growth factor requirements of tobacco tissue cultures. Physiologia Plantarum (18): 100–127.

Murashige, T. & Skoog, F. (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 155. 473–497.

Savona, M., Ruffoni, B., Minuto, L., Carli, S. & Profumo, P. (2004): *Leucojum nicaense* Ard.: application of the in vitro culture to preserve the biodiversity. Italus Hortus 11 (4): 138-140.

Stanilova, M.I., Ilcheva, V.P. & Zagorska, N.A. (1994): Morphogenetic potential and *in vitro* micropropagation of endangered plant species *Leucojum aestivum* L. and *Lilium rhodopaeum* Delip. Plant Cell Reports. 13 (8): 451–453.