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Leucojum aestivum L. *in vitro* bulbs induction and acclimatization

Research Article

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Abstract: The effects of growth retardants (paclobutrazol and ancymidol), sucrose, GA_3 (gibberellic acid) and physical state of the medium (solid and liquid - Rita® temporary immersion system) on *in vitro* induction of *Leucojum aestivum* bulbs and their acclimatization were studied. Paclobutrazol, regardless of the physical state of the medium, stimulated the formation of bulbs (99.3%). Under the influence 90 g L^{-1} of sucrose or paclobutrazol the bulbs with the highest fresh weight (FW) were formed (250 mg and 208.8 mg, respectively). However, the addition of ancymidol to the liquid medium led to obtaining the bulbs showing the highest number of leaves and roots (63.2% and 91.7%, respectively). The scanning microscopy study proved that plants obtained in the medium containing GA_3 produced the stomata which most closely resembled to the one observed in the mother plant. Cytometric analysis of all regenerants revealed absence of changes in the nuclear DNA content. The maximum survival rate (100%) was observed for plants derived from liquid medium containing 90 g L^{-1} of sucrose. Somewhat fewer plants were acclimatized after their cultivations in liquid medium enriched with paclobutrazol or ancymidol. The temporary immersion system led to perform successful *ex vitro* adaptation of *Leucojum aestivum* plants.

Keywords: *Growth retardants • Gibberellic acid • Sucrose • Bulbs formations • Ex vitro transfer*

Abbreviations:

ANC - ancymidol;
FW - fresh weight;
GA_3 - gibberellic acid;
LM - liquid medium;
MS - Murashige and Skoog basal medium;
PBZ - paclobutrazol;
SCR - sucrose;
SEM - scanning electron microscopy;
SM - solid medium.

1. Introduction

Leucojum aestivum L. (summer snowflake) is a bulbous member of the Amaryllidaceae family [1]. Plants of this family produce pharmacologically active alkaloids that have interesting pharmacological properties. The most important alkaloid is galanthamine (an acetylcholinesterase inhibitor used for treatment of Alzheimer's disease) [2]. Another valuable alkaloid is lycorine, a strong antiviral agent with antimitotic and cytotoxic activities [3,4]. Although the chemical synthesis of galanthamine has been successfully achieved, *Narccissus* and *Leucojum aestivum* plants remain the main sources of these alkaloids [5]. Considering the increasing demand of the pharmaceutical market and the limited availability of plant sources, galanthamine produced using *in vitro* cultures could be an alternative method [6,7]. Micropropagation ensures rapid multiplication of selected genotypes, allowing the useful metabolites to be collected in greater quantities. Several studies on *Leucojum aestivum* multiplication by organogenesis and somatic embryogenesis have been performed [5,8,9]. Species that naturally produce bulbs, like *Leucojum aestivum* can be induced to form bulbs in *in vitro* culture. *In vitro* bulbs can be planted or used to start new *in vitro* cultures [10]. Such bulbs can also be used for the extraction of medicinal compounds or for pharmacological studies [11].

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Acclimatization of *in vitro* propagated plants to the *ex vitro* environment is a critical step for successful propagation. It is necessary because *in vitro* plants usually show low photosynthetic activity and they can have poorly developed leaf cuticule or impaired stomata functioning. The abnormal morphology, anatomy and physiology of *in vitro* plantlets make it difficult for them to survive in the *ex vivo* environment. In the case of medicinal plants it is important to obtain high-quality plant material, *i.e.* the material that is morphologically, phytochemically and genetically homogeneous [12].

In this study the effects of chemical factors, such as growth retardants (paclobutrazol, ancymidol), sucrose, GA_3 and physical state of the medium (solid and liquid - Rita® temporary immersion system) on *in vitro* induction of *Leucojum aestivum* bulbs and their acclimatization were tested. The influence of these parameters on the quality of the obtained plants *via* somatic embryogenesis was also determined for the first time.

2. Experimental Procedures

2.1 Effect of *in vitro* culture conditions on the formation of *Leucojum aestivum* bulbs

In vitro plants of *Leucojum aestivum* were regenerated from somatic embryos obtained on a solid Murashige and Skoog (MS) [13] medium supplemented with 5 µM zeatin and 0.5 µM benzyladenine (BA) according to the procedure described by Ptak *et al.* [8,12]. For experiment 28-day-old plants (0.3-0.5 g FW each plant), were transferred to MS medium contained 30 g L^{-1} sucrose and 10 µM paclobutrazol, or 10 µM ancymidol, or 10 µM GA_3. The MS medium with different sucrose concentrations (30, 60 or 90 g L^{-1}) was also tested. The plants were cultivated either on solid media containing 6 g L^{-1} agar or in liquid media (200 mL of medium in bioreactor Rita® vessels, Vitropic, France). The immersion frequency was 5 minutes every 2 hours. The cultures were maintained at 25 ± 2°C under white fluorescent light with a 16 h photoperiod (Tungsram lamp, 40 WF, 90 µmol $m^{-2}s^{-1}$). The experiments were conducted five times, 75 plants for each treatments were used (25 plants x 5 repetitions). After 3 months of cultivation all plants (growing on solid media and in the bioreactor) were transferred onto fresh medium at 5°C for a month.

After that time the number of bulbs developed from plants on solid and in liquid media were counted and the percentage of formed bulbs and their growth parameters, *i.e.* the number of leaves, number of roots and fresh weight of bulbs, were calculated.

2.2 Effect of *in vitro* culture conditions on acclimatization of *Leucojum aestivum* plants

2.2.1 Ex vitro transfer

The plantlets obtained under various *in vitro* conditions were washed gently, their dead tissues were removed, and then they were transferred to Jiffy peat pots. The plants were covered with polytunnel and kept at 25 ± 2°C under white fluorescent light with a 16 h photoperiod (Tungsram lamp, 40 WF, 90 µmol $m^{-2}s^{-1}$) for 5 months. After 4 weeks of growth the percentage of acclimatized plants was determined, and cytometric DNA analysis was carried out. In addition, observation of stomata using a scanning electron microscope was made.

2.2.2 Flow cytometry analysis

The ploidy levels of the original mother plant and plants regenerated *in vitro* and growth *ex vitro* were estimated by flow cytometry. Leaves were chopped in buffer solution and stained with 4,6-diamidino-2-phenylindole (DAPI, DNA staining solution, Partec, Muster, Germany). Crude samples were passed through a 30 µm nylon mesh and analyzed in flow cytometry Partec CA II (Munster, Germany). For every sample, 1500-2000 cells were tested [14].

2.2.3 Scanning electron microscopy (SEM)

Leaves of *ex vitro* growing plants were fixed with 2.5% (w/v) glutaraldehyde in 0.1 mol L^{-1} phosphatic buffer at pH 7.2-7.4 for 15 min. They were dehydrated using a graded series of ethanol (30 – 100% v/v) and acetate (100%) and dried with liquid CO_2 in a critical point dryer Type E 3100 Industriel LADD (USA). After that, samples were coated with gold using a Coater Jeol JFC-1100E (Japan). Finally, samples were scanned using a scanning electron microscope Jeol model JSM 5410 (Japan) [15].

2.3 Statistical analysis

The results of the experiments were evaluated by an analysis of variance. The means that differed significantly were identified using Duncan's multiple test at the significance level of $P \leq 0.05$.

3. Results

3.1 Effect of *in vitro* culture conditions on the formation of *Leucojum aestivum* bulbs

After 3 months of cultivation, *Leucojum aestivum* plants were capable of forming bulbs under all the tested conditions. It is important, however, to indicate that some plants do not form bulbs. Moreover statistical analysis

has not shown any interactions between the tested components of the medium (paclobutrazol, ancymidol, sucrose, GA_3) and physical state of the medium (solid and liquid - Rita® temporary immersion system).

Irrespective of the physical state of the medium, the highest number of bulbs (99.3%) was noted in the case of plants growing on medium enriched with 10 µM paclobutrazol. Somewhat fewer bulbs (91%) were observed when the plants were grown on medium containing 10 µM ancymidol. While the lowest number of summer snowflake bulbs was developed under the influence of 30 g L^{-1} sucrose (49.5%), more bulbs (58.15-76.5%, respectively) were observed at higher sucrose concentrations (Figure 1).

In the presented study, a higher number of summer snowflake bulbs, irrespective of the components of the medium, was obtained in liquid cultures grown in the Rita® bioreactor (74.6%) as compared with solid culture media (71.7%) (Figure 1).

Irrespective of the physical state of the medium, the highest mass of *Leucojum asetivum* bulbs was obtained when the plants were cultivated on the medium enriched with 90 g L^{-1} sucrose or 10 µM paclobutrazol, (249.9 mg, 208.8 mg FW, respectively). In other cases bulbs with lower mass were formed (112 – 149 mg FW) (Figures 2,3).

It was also observed that the bulbs derivate from liquid medium (irrespective of the components of the

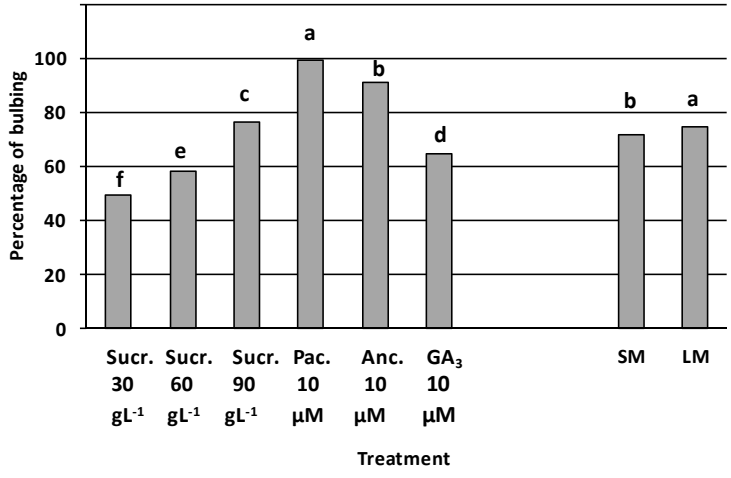

Figure 1. Effect of *in vitro* conditions on *Leucojum aestivum* bulb formations. The action of the factors: SCR- sucrose, PBZ- paclobutrazol, ANC-ancymidol and SM-solid medium, LM-liquid medium has been presented independently. Means followed by different letters are significantly different at P≤0.05, Duncan's test.

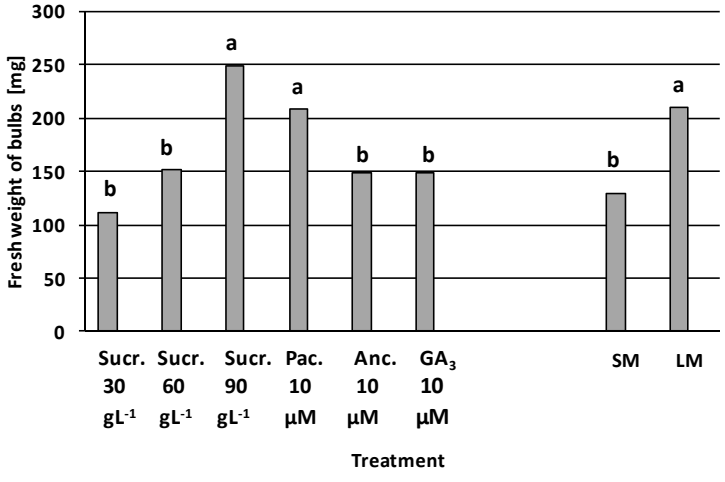

Figure 2. Effect of *in vitro* conditions on fresh weight of *Leucojum aestivum* bulbs. The action of the factors: SCR- sucrose, PBZ- paclobutrazol, ANC-ancymidol and SM-solid medium, LM-liquid medium has been presented independently. Means followed by different letters within a column are significantly different at P≤0.05, Duncan's test.

Figure 3. *Leucojum aestivum* bulb formations on solid media containing: a) 30 g L^{-1} sucrose, b) 60 g L^{-1} sucrose, c) 90 g L^{-1} sucrose, d) 10 μM paclobutrazol, e) 10 μM ancymidol, f) 10 μM GA_3 and on liquid media containing: g) 30 g L^{-1} sucrose, h) 60 g L^{-1} sucrose, i) 90 g L^{-1} sucrose, j) 10 μM paclobutrazol, k) 10 μM ancymidol, l) 10 μM GA_3 (*scale bar* = 7 mm).

medium) were characterized by 1.6 times higher FW than the bulbs obtained in solid medium (Figures 2,3).

3.2 Effect of *in vitro* culture conditions on the quality of *Leucojum aestivum* plants

In the presented experiment the addition of ancymidol to liquid media led to obtaining the bulbs with the highest number of leaves (63.2%) and roots (91.7%). Somewhat fewer leaves and roots were formed by bulbs growing on the liquid medium enriched with paclobutrazol (Table 1). While bulbs of *Leucojum aestivum* grown on solid medium enriched with 90 g L^{-1} sucrose produced least leaves and roots (18.2, 9.3%, respectively) (Table 1).

The scanning microscopy study showed that leaves of *Leucojum aestivum* plants had different stomata depending on cultivation conditions (Figure 4). Stomata which most closely resembled the stomata of mother plants, considering their shape and degree of stomata opening, were found in the leaves of plants regenerated under the influence of GA_3 (Figure 4f,l,m).

Leucojum aestivum plants regenerated on solid media developed larger stomata, some of them being almost as large as the stomata of mother plants (Figure 4a-f). On the contrary, abnormally small stomata of *Leucojum aestivum* were observed in plants derived from liquid media (Figure 4g-l). In presented experiment misshapen stomata, typical of hyperhydric leaves were also observed. This phenomenon occurred in some leaves derived from plants obtained in liquid media (Figure 4i,j).

To determine the quality of regenerated *Leucojum aestivum* plants cytometric analysis was carried out. The results obtained indicate a lack of detectable changes in the nuclear DNA content for all *in vitro* plants derived from liquid and solid media (Figure 5).

3.3 Effect of *in vitro* culture conditions on the *ex vitro* survival of plantlets

Two weeks after transferring into Jiffy peat pots, the chilled bulbs of *Leucojum aestivum* began to grow (Figure 6). Earlier observations have shown that bulbs of *Leucojum aestivum* should be chilled (5°C) during 4-week storage in the dark before acclimatization. Otherwise about 50% of bulbs can remain dormant (data not shown).

The carried out experiment has shown that *in vitro* conditions prior to acclimatization are important for the *ex vitro* growth of *Leucojum aestivum* plants. The maximum survival rate of *Leucojum aestivum* (100%) was observed for plantlets derived from liquid medium containing 90 g L^{-1} sucrose. A somewhat smaller survival rate was observed in the case of plantlets obtained in liquid medium containing paclobutrazol or ancymidol (92.8 and 86.95%, respectively). While the smallest number of acclimatized plants was noted for plantlets from solid medium containing 30 g L^{-1} sucrose (46.15%) (Figure 7). It was also observed that the larger bulbs derivated from the medium contained 90 g L^{-1} of sugar or 10 µM of paclobutrazol sprouting faster than the smaller bulbs (Figure 6).

Treatments	Bulbs with leaves [%]		Bulbs with roots [%]	
	SM	LM	SM	LM
30 g L^{-1} sucrose	42.2 de*	44.5 c	27.8 h	44.0 de
60 g L^{-1} sucrose	31.0 g	41.0 e	29.8 g	51.3 c
90 g L^{-1} sucrose	18.2 i	44.8 c	9.3 k	42.8 e
10 μM paclobutrazol	44.0 cd	52.0 b	45.3 d	81.3 b
10 μM ancymidol	50.8 b	63.2 a	35.0 f	91.7 a
10 μM GA_3	27.1 h	34.7 f	21.8 i	16.5 j

Table 1. Effect of *in vitro* conditions on *Leucojum aestivum* bulbs quality.

*SM - solid medium LM - liquid medium *Means followed by different letters within a column are significantly different at P≤0.05, Duncan's test*

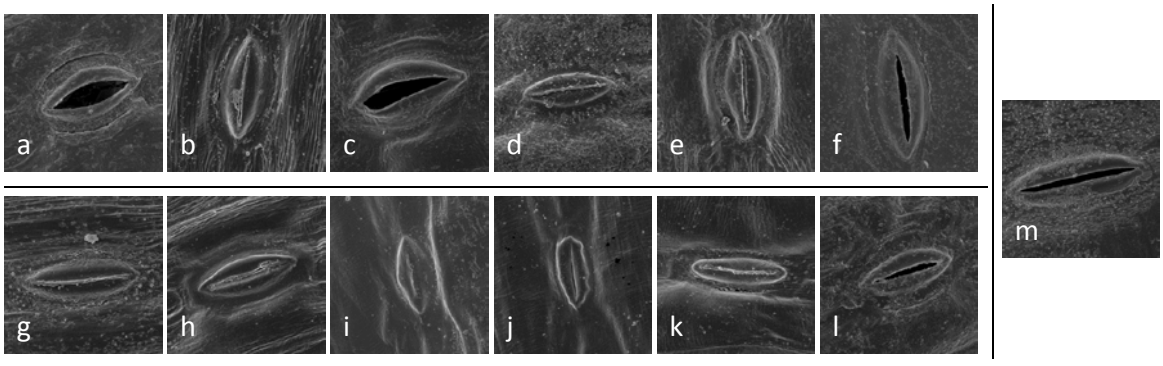

Figure 4. SEM micrograph of epiderms and stomata apparatus of *in vitro* grown *Leucojum aestivum* plants: solid media containing: a) 30 g L^{-1} sucrose, b) 60 g L^{-1} sucrose, c) 90 g L^{-1} sucrose, d) 10 μM paclobutrazol, e) 10 μM ancymidol, f) 10 μM GA_3 and liquid media containing: g) 30 g L^{-1} sucrose, h) 60 g L^{-1} sucrose, i) 90 g L^{-1} sucrose, j) 10 μM paclobutrazol, k) 10 μM ancymidol, l) 10 μM GA_3 and m) control - mother plants) (2000x).

Both well-rooted bulbs (obtained on medium enriched with paclobutrazol or ancymidol) and bulbs with a very small number of roots (derived from medium supplemented with 90 g L^{-1} sucrose) acclimatized very well (Table 1).

In conclusion, treatment of plantlets with 10 µM of paclobutrazol in bioreactor Rita® was found to be the most suitable for *Leucojum aestivum* bulbs formation and their successful acclimatization. The results of the current study provide, for the first time, information about these last steps of *Leucojum aestivum* plants propagation obtained by somatic embryogenesis.

4. Disscusion

This study presents, for the first time, the effects of chemical factors: retardants (paclobutrazol, ancymidol), sucrose, GA_3 and physical state of the medium (solid and liquid - Rita® temporary immersion system) on *in vitro* induction of *Leucojum aestivum* bulbs and their acclimatization. Results reported here show that paclobutrazol and ancymidol, regardless of the physical state of the medium, stimulate *Leucojum aestivum* bulb formation. It is known that growth retardants, such as paclobutrazol and ancymidol, inhibit gibberelin biosynthesis and stimulate bulb induction [16]. Ancymidol and paclobutrazol were found to be the best stimulators of *Gladiolus* cluster formation; they also enhance the formation of bulbs in *Allium cepa*, *Lilium* and *Tulipa* plants [16-19].

The lowest number of summer snowflake bulbs was developed under the influence of 30 g L^{-1} sucrose. While increasing the sugar concentration to 90 g L^{-1} meant that arose more bulbs. It is known that carbohydrates play an important role in the induction and growth of storage organs. Stimulation of the formation of these organs by increasing sucrose concentration in the culture medium has been reported for many species, including *Tulipa* [20], *Narcissus* [21], *Sparaxis* [22], *Leucojum vernum* [23]. In shoot and bulb cultures of *Leucojum aestivum,* Georgieva *et al.* [24] found that the concentration of carbon sources (glucose, fructose and sucrose) of 60 g L^{-1} was the optimum for the growth of the cultures. Lack of sugar, as well as lower (10 or 30 g L^{-1}) or higher (120 g L^{-1}) sugar concentrations had a negative effect on the development of the shoots.

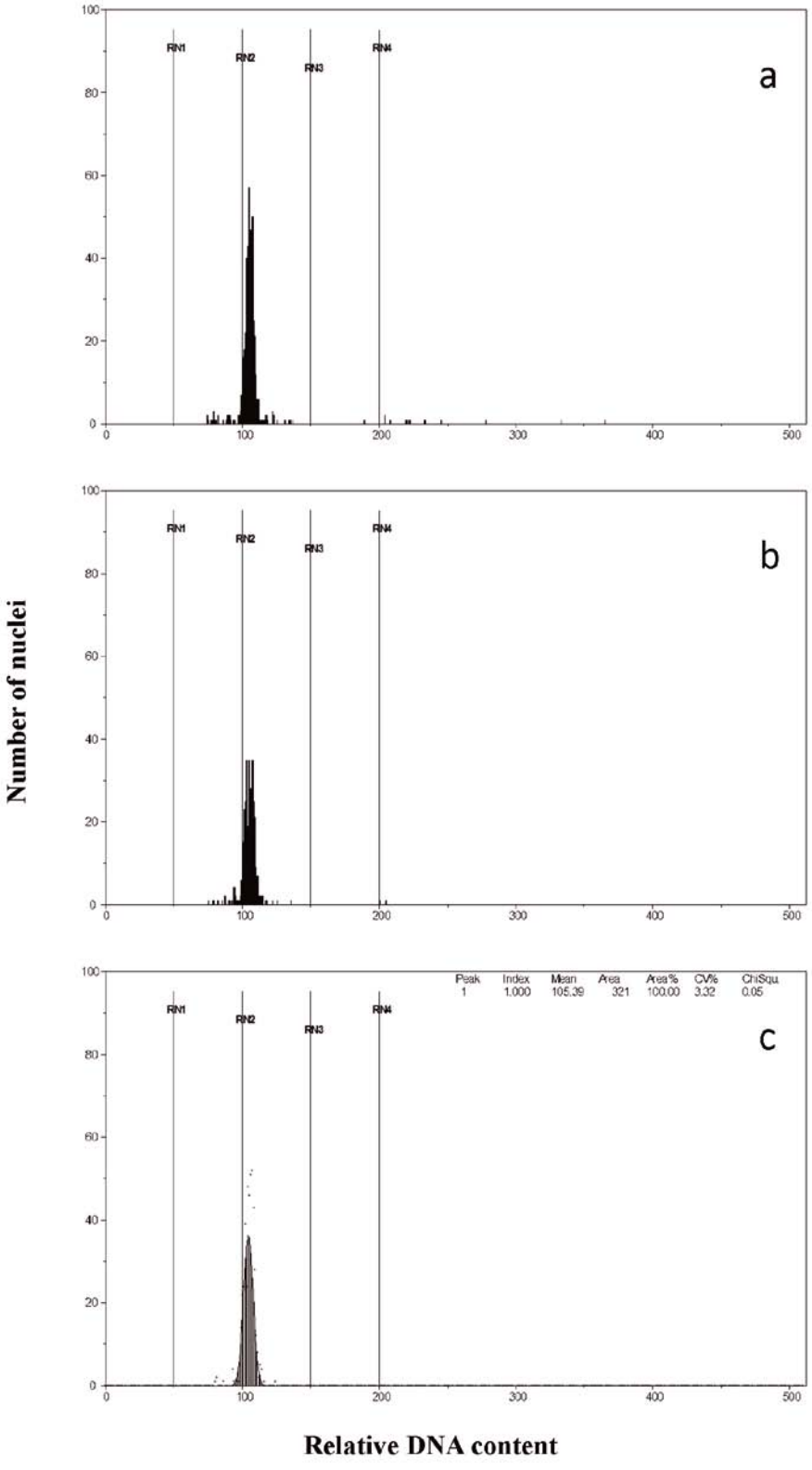

Figure 5. DNA - sample - histograms from nuclear preparations of *in vitro* cultured *Leucojum aestivum* plants a) solid medium: 10 μM L^{-1} paclobutrazol, b) liquid medium: 10 μM paclobutrazol and c) control - mother plants.

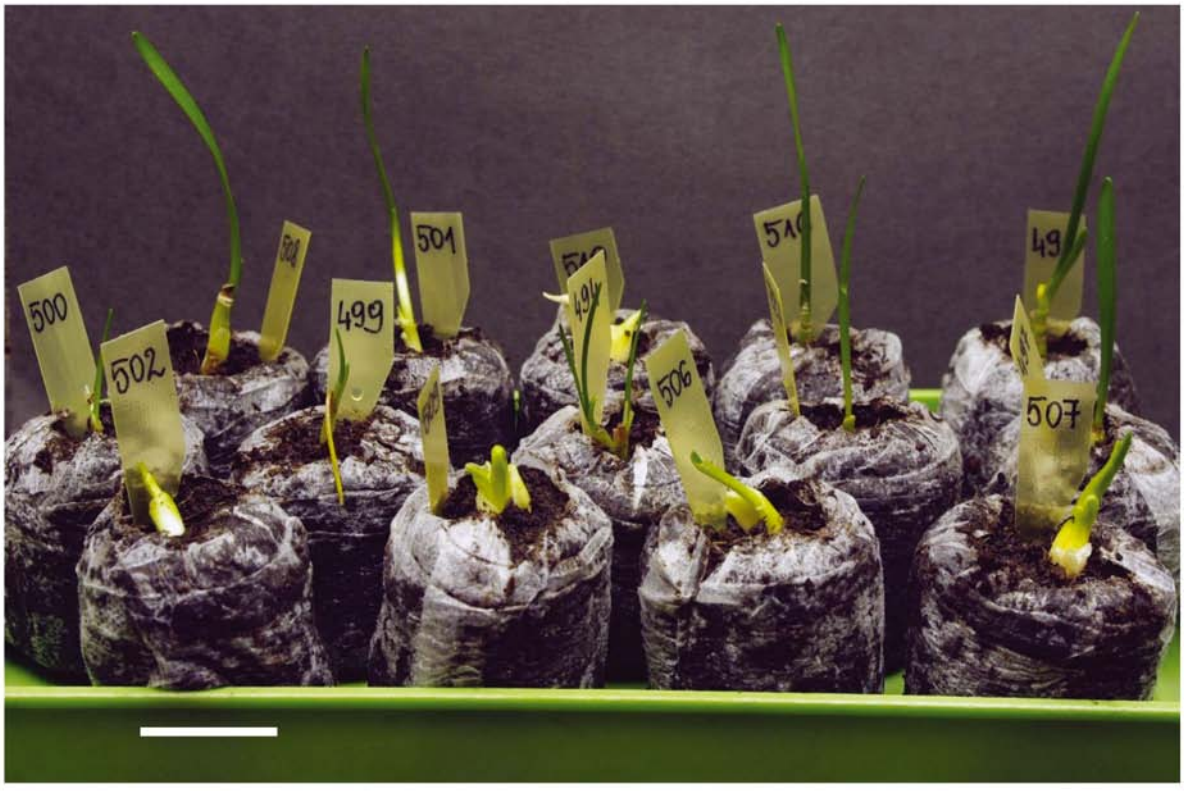

Figure 6. *Ex vitro* acclimatization of *Leucojum aestivum* plants (2 weeks after transfer) (*scale bar* = 25 mm).

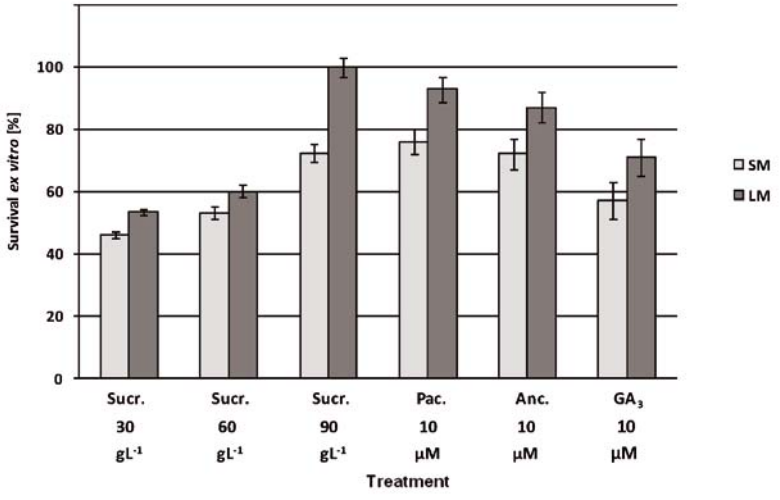

Figure 7. Frequency of survival of *Leucojum aestivum* plants during acclimatization. Data were taken 4 weeks in *ex vitro* conditions. Values represents the means (50 bulbs per treatment) ±S.D.

Addition to the medium 90 g L^{-1} of sugar and 10 µM of paclobutrazol affected positively increasing fresh weight of *Leucojum aestivum* bulbs. In *in vitro Gladiolus* cultures, like in the present study, a positive effect of paclobutrazol on cormel size was observed, but only when a higher sucrose concentration (120 g L^{-1}) was used [25].

Application of bioreactor Rita® stimulated bulbing of *Leucojum aestivum* plants (number of bulbs and FW). The production and quality of *in vitro* plants have been

improved for various species by temporary immersion system in bioreactor Rita® [26]. The temporary immersion system was reported for micropropagation of *Leucojum aestivum* somatic embryo and shoot cultures [12,27]. It was also observed that liquid medium affected an increase in the number and FW of bulbs, for example in tulip culture [20], while temporary immersion systems stimulated tuberization of potato [26].

Ancymidol added to the liquid medium induce formation of *Leucojum aestivum* leaves and roots. Somewhat fewer leaves and roots were formed by bulbs growing on the liquid medium enriched with paclobutrazol. Le Guen-Le Saos *et al.* [16] showed that the addition of ancymidol to the medium stimulated *Allium cepa* leaf and root growth. A stimulatory effect of ancymidol on rhizogenesis has also been reported by Chin [28] and Burkhart and Meyer [29]. The treatment of *Lilium* plants with ancymidol and paclobutrazol increased the number of roots per bulblets [18]. Quiala *et al.* [30] demonstrated that shoots of *Tectona grandis* derived from the temporary immersion system developed good root systems, as in the present study. It was observed that bulbs of *Leucojum aestivum* grown on solid medium enriched with 90 g L^{-1} sucrose produced least leaves and roots. High osmotic pressure in the medium also inhibited root and leaf growth in *Allium cepa* bulbs [16].

The scanning microscopy study indicates that the leaves of *Leucojum aestivum* plants regenerated under the influence of GA_3 produce stomata which most closely resembled the stomata of mother plants. Saibo *et al.* [31] show that gibberellin is the main signal inducing stomata formation in the *Arabidopsis* hypocotyls. Plant hormones generally affect not only the function but also the differentiation of the stomata [32]. Abnormally small stomata of *Leucojum aestivum* were observed in plants derived from liquid media. Stanilova *et al.* [33] also demonstrated abnormal *Leucojum aestivum* stomata in submerged leaves of *in vitro* liquid culture. In *in vitro* cultured plants, the guard cells in the leaf stomata do not always function properly [34]. It may be caused by changes in the structure of the stomata which may be raised, rounded and have damaged walls [35].

In the case of medicinal plants it is important to obtain genetically homogeneous plants [12]. There are many factors, like growth regulators or tissue age, which may induce variation. They may appear at the morphological, cytological and genetic levels [36]. Cytological analysis was recorded that the level of ploidity of *Leucojum aestivum* regenerated plants did not reveal changes. Flow cytometry is one of the methods to evaluate genetic stability. Currently this is not sufficient way to determine ploidy levels in plants but this is a popular, fast and easy technique [37-39]. Flow cytometry is widely used to establish DNA content and genome size in micropropagated medicinal plants [40].

Leucojum aestivum like *Lilium, Hippeastrum* and *Hemerocallis* bulbs cooling, resulted in their rapid growth after planting [41]. While better acclimatization capacity of *Leucojum aestivum* plants treated with 90 g L^{-1} sucrose and retardants might be due to larger bulb weight obtained under these conditions. The results of Georgieva *et al.* [24] indicated that the *ex vitro* adaptation of *Leucojum aestivum* plants was correlated with bulb size. Retardants, such as ancymidol, can also improve acclimatization and tolerance to desiccation in plantlets or rooted shoots [42]. Ziv [34] reported that the addition of paclobutrazol to the medium resulted in the formation of *Gladiolus* cormels with 100% survival after transfer to a greenhouse, whereas only a 58% rate was observed for the medium without paclobutrazol. Acclimatization of *Leucojum aestivum* was improved in plants obtained from Rita® systems. The results of these studies are similar to those obtained for *Eucalyptus* and apple roostock cultures [43,44]. Paclobutrazol and temporary immersion systems as in the present study stimulated also *Ananas comosus ex vitro* plants grown [45]. It is also worth noting that *Leucojum aestivum* bulbs, produced on the liquid medium, acclimatized better than plants derivate from solid media although they have defective stomata. This observation suggests that the plants which showed some morpho-physiological disorders during the multiplication stage were able to revert to a normally functioning plants after transfer to *ex vitro* conditions.

Rooting of shoots and bulbs can occur *in vitro* or after transplanting, during acclimatization to *ex vitro* conditions [46]. Plants which root well under *in vitro* conditions generally acclimatize faster and are more resistant to pathogens [47]. In the case of *Leucojum aestivum* there was no such correlations.

Acknowledgements

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