Regeneration of summer snowflake (*Leucojum aestivum* L.) in *in vitro* cultures

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Summary

Slices of scales and leaves isolated from *Leucojum aestivum* L. bulbs were cultured in the presence of auxins (NAA, 2,4-D, Picloram) and cytokinin, BA at different proportions and concentrations. The medium free of growth regulators was also used. Regeneration occurred as a result of somatic embryogenesis and organogenesis. Explants cultivated on the media containing 2,4-D and Picloram showed a capacity of somatic embryogenesis, viz. formation of embryogenic callus and somatic embryos. On the other hand, direct organogenesis (induction of bulblets and roots) occurred in explants grown on the medium devoid of growth regulators and enriched in NAA.

Key words:
Leucojum aestivum, somatic embryogenesis, organogenesis, galanthamine.

1. Introduction

Leucojum sp. is an ornamental bulbous plant belonging to *Amaryllidaceae* family. Plants of this family are well-known not only for their ornamental value, but also for the alkaloids they produce. *Amaryllidaceae* alkaloids have been used for centuries in the treatments of a variety of diseases. As early as in the times of Hippocrates, species of *Narcissus* genus were used in neoplastic diseases (1). Some of these plants were used as an arrow poison. Species of genus *Crinum* were used to treat leprosy (2).

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The genera *Leucojum* from which more than 20 alkaloids were isolated is also of great interest in the present phytochemistry. Galanthamine (GAL), a common alkaloid in this family, has shown cholinesterase inhibitory activity and is currently undergoing clinical trials in the treatment of Alzheimer's disease (3).

For medical application, galanthamine is isolated from plant material, especially from *Leucojum aestivum,* since the total chemical synthesis of galanthamine on an indrustial scale is presently not economical (4).

A biotechnological approach has been considered to be an alternative way for the production of this alkaloid. One of the most important issues in plant biotechnology is production of secondary metabolites under *in vitro* conditions (5). *In vitro* technique offers numerous options for high quality plant reproduction. Moreover, under *in vitro* conditions, production of secondary metabolites can be intensified, since biosynthetic processes can be strictly controlled and optimized (6). The data on galanthamine production in *Leucojum* sp. *in vitro* cultures are lacking. Such studies have been carried out only on *Narcissus* sp. (7). There is also little information about micropropagation of *Leucojum* sp. in *in vitro* cultures. The aim of the present study was to develop a method of *Leucojum aestivum* regeneration under *in vitro* conditions.

2. Materials and methods

Thin slices of scales and leaves isolated from the *Leucojum aestivum* L. bulbs not chilled and chilled were used as a plant material for 12 weeks at 5°C. Initial explants were cultivated on the Murashige and Skoog (8) medium without growth regulators or supplemented with auxins: 2,4-D, Picloram, NAA (5-25 μM) and cytokinin, BA (0,5-10 μM) at varying proportions: more auxin than cytokinin and balanced amounts of auxin and cytokinin. The cultures were maintained at 25°C in the darkness. Embryogenic callus was multiplied on initial medium. One gram of callus was placed on each of the media, and multiplication rate was calculated after 4-weeks growth. A number of somatic embryos developed from the callus was determined. The roots and bulblets developed in the process of organogenesis were also counted. The results of the observations on induction of caulogenesis, regeneration of embryos, bulblets and roots were evaluated by analysis of variance.

3. Results and discussion

3.1. Somatic embryogenesis

Regeneration in *Leucojum aestivum in vitro* cultures resulted from somatic embryogenesis and organogenesis and depended on the applied growth regulators

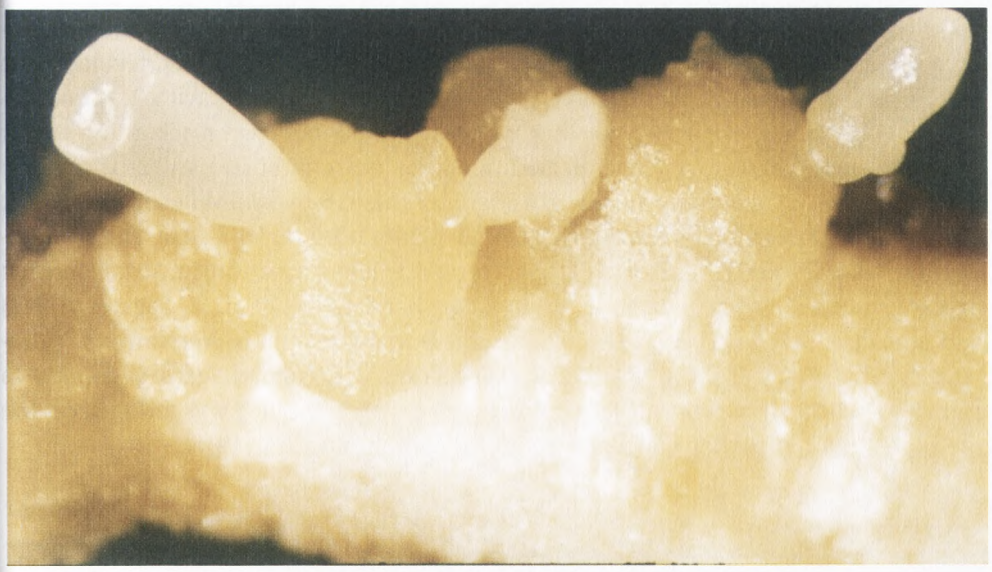

Photo 1. Somatic embryogenesis: induction of callus and somatic embryos.

Photo 2. Organogenesis: induction of bulbs and roots.

and their proportion in the medium (Photo 1, 2). After about 6 weeks, nodular embryogenic callus developed on the cut surface of the explants. Callus development was observed only on explants cultivated on media supplemented with 2,4-D or Picloram. This phenomenon was not observed when medium was enriched in NAA or free of growth regulators (control medium). The most intensive callus formation occurred when the medium contained 25 μM 2,4-D and 5 or 10 μM Picloram (tab. 1). Ziv et al. (9) induced callus development on explants from *Nerine* x Mansellii by addition of 2,4-D, while Fereol et al. (10) observed callus formation in *Allium sativum* explants under similar conditions. On the other hand, Selles et al. (7) Sage et al. (11) and Malik and Bach (12) used two auxins, 2,4-D and Picloram in *Narcissus* sp. cultures. These auxins were also applied in experiments with somatic embryogenesis in tulip (13), while in *Galanthus elwessi* cultures, embryogenic callus was induced on the media containing higher concentrations of Picloram and BA (14).

Table 1

Induction of callus formation in *Leucojum aestivum* cultures

Growth regulators (μM)	Percent of explants forming callus	Calllus formation
Control (0)	0,0 a*	–**
2,4-D 5 + BA 0,5	93,7 c	++
2,4-D 10 + BA 0,5	90,6 c	+
2,4-D 25 + BA 0,5	100,0 c	+++
Picloram 5 + BA 0,5	93,7 c	+++
Picloram 10 + BA 0,5	100,0 c	+++
Picloram 25 + BA 0,5	100,0 c	++
NAA 5 + BA 0,5	0,0 a	–
NAA 10 +BA 0,5	0,0 a	–
NAA 25 + BA 0,5	0,0 a	–
2,4-D 5 + BA 5	95,8 c	++
2,4-D 10 + BA 10	73,1 b	++

* means followed by the same letters are not significantly different

** callus formation: – no callus; + poor; ++ good; +++ very good

The efficiency of induction of nodular callus depended also on chilling of the starting material. More intensive callus formation was observed when explants were isolated from the chilled bulbs (tab. 2), which resulted directly from the changes in the level of endogenous growth regulators. Saniewski and Kawa-Miszczak (15) reported that chilling of tulip bulbs caused an increase in the contents of endogenous gibberellins and cytokinins, while the level of abscisic acid dropped.

Table 2

The effect of explants isolation term on callus formation

Term of isolation	Percent of explants forming callus	Calllus formation
I. unchilled bulbs	50,5 a	++
II. 12-weeks chilled bulbs	64,7 a	+++

Explanation: see Table 1.

In the present studies, the callus was obtained on slices of scales and leaves. However, there were differences in intensity of callus formation. More callus developed on scale explants (tab. 3).

Table 3

The effect of explants type on callus induction

Explants type	Percent of explants forming callus	Callus formation
Leaves	80,8 a	++
Scales	85,2 a	+++

Explanation: see Table 1.

Exogenous growth regulators had decisive effect on multiplication of embryogenic callus and on induction of somatic embryos. The highest multiplication rate was noted on the medium supplemented with 10 μM 2,4-D and 0,5 μM BA, whereas the largest number of somatic embryos developed on the medium containing 10 μM 2,4-D and 10 μM BA (tab. 4). Sage et al. (11) and Malik and Bach (12) demonstrated that rising cytokinin content in relation to auxin had beneficial effect on induction of somatic embryos in *Narcissus* cultures.

Table 4

The effect of medium on callus multiplication rate and somatic embryos induction

Growth regulators (μM)	Rate of callus multiplication	Number of embryos
2,4-D 5 + BA 0,5	1,5 abc	1,6 abc
2,4-D 10 + BA 0,5	1,8 e	1,3 ab
2,4-D 25 + BA 0,5	1,5 abc	0,1 a
Picloram 5 + BA 0,5	1,5 abc	1,1 a
Picloram 10 + BA 0,5	1,7 cde	1,0 a
Picloram 25 + BA 0,5	1,7 cde	2,8 bcd
2,4-D 5 + BA 5	1,6 bcd	2,9 cd
2,4-D 10 + BA 10	1,4 a	4,3 d

Explanation: see Table 1.

3.2. Organogenesis

Direct organogenesis was observed from leaf and scale explants. The formation of bulblets and roots was most effective in the case of medium containing 10 μM NAA and 0,5 μM BA (tab. 5). Stanilova et al. (16) also used NAA-containing medium to directly induce organogenesis in *Leucojum aestivum* cultures.

Table 5

The effect of growth regulators on bulbs and roots formation

Growth regulators (μM)	Number of bulbs per 1 explant	Number of roots per 1 explant
Control (0)	0,4 a	0,0 a
NAA 5 + BA 0,5	0,6 b	2,1 b
NAA 10 + BA 0,5	1,5 c	5,6 d
NAA 25 + BA 0,5	0,7 b	3,8 c

Explanation: see Table 1.

4. Conclusions

The explants derived from fragments of bulb scales and leaves were characterized by capability of induction of somatic embryogenesis and organogenesis. Morphogenetic response was directly connected with the type of initial explants, chilling of starting material and composition of growth regulators in the medium.

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