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OPTIMIZATION OF THE PROCESSES OF STERILIZATION AND MICROPROPAGATION OF CUP PLANT (*Silphium perfoliatum* L.) FROM APICAL EXPLANTS OF SEEDLINGS IN *in vitro* CULTURES

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Abstract

Cup plant (*Silphium perfoliatum* L.) is a tall, reaching up to 2.5 m, perennial plant that represents the Asteraceae family. *Silphium perfoliatum* L can be applied in medicine, animal feeding, and as a decorative, honey-producing and energy production plant which proves its multi-functional features. Sowing material currently available in Poland is insufficient, which justifies the present attempts at propagating these plants in *in vitro* cultures. Therefore, *Silphium perfoliatum* L. seed sterilization and micropropagation processes were studied under controlled conditions in *in vitro* cultures. Among the tested methods, ACE proved to be the most effective for sterilization. The apical parts of seedlings were used as explants; they were placed onto MS media with growth regulators added. 4 growth regulator concentration variants were applied. The highest percentage of explants with lateral shoots (41.7%) and callus (90.3%) was obtained on MS medium with 5 mg x dm^{-3} BAP + 1 mg × dm^{-3} NAA added. Shoots were isolated and transferred onto MS rooting medium without growth regulators. The rooted plants were transferred to the greenhouse and acclimatised to *ex vitro* conditions.

Key words: apical explants, cup plant, growth regulators, *in vitro*, micropropagation, MS medium, regeneration, seed sterilization.

INTRODUCTION

The genus *Silphium* of the family Asteraceae comprises about 23 species (Rutkowski, 1998). The species occurs in the natural environment in the central and eastern parts of the United States of America and in the southern part of Canada (Podbielkowski, 1995). Due to its numerous functional traits, there has been a growing interest in cup plant over the recent years. Its herbage offers a potentially valuable material for pharmaceutical and foodstuffs industries since *Silphium perfoliatum* L. roots and rhizomes contain inulins (Kowalski and Wierciński, 2004), while its leaves, flowers, and rhizomes – biologically active substances of the isoprenoid group, phenol, and polyphenol compounds. Extracts from tissues of this species demonstrate analgesic, anti-inflammatory, sudorific, restorative, antibacterial, antifungal, and expectorant properties as well as they reduce the cholesterol level (El-Sayed et al. 2002; Kowalski, 2002; Kowalski and Wolski, 2003a; Kowalski and Wolski, 2003b; Kowalski and Wierciński, 2004; Kowalski and Kędzia, 2007). Similarly, this species is used as a fodder plant thanks to a high content of exogenous amino acids (Piłat et al. 2007).

Due to its long flowering period and production of high amounts of pollen grains as well as uneven development of flowers, the cup plant is grown to provide pollen and nectar for bees (Wróblewska, 1997). Since the species is a perennial on nutrient-poor soil and because it can adapt to different environments, it can also be used as a pioneer plant for land rehabilitation in areas destroyed by industry and municipal waste disposal (Woźniak and Góral, 1998; Klimont, 2007). *Silphium perfoliatum* plantations also provide considerable amounts of biomass used as energy-producing material (Majtkowski, 2007; Stolarski et al. 2008).

The common technology offering vegetative material for propagation purposes involves micropropagation of plants in *in vitro* cultures. Currently, the available literature seems to provide few reports on how to produce cup plants under controlled conditions (Figas et al. 2010).

The technology that allows regenerant plants to be obtained, preserving the traits of the genotype and virus-free plants, is the method using the apical parts of seedlings which can be applied once the factors favourable to plant organogenesis are determined; e.g. culture conditions, method and stages of isolation of the explant, or the medium composition. The aim of the present research was to optimise the process of sterilization and micropropagation of cup plants (*Silphium perfoliatum* L.) from apical explants of seedlings in *in vitro* cultures.

In the present experiment, the following were analyzed: methods of seed sterilization, number of seedlings obtained, number of shoots, and rooting efficiency.

MATERIALS AND METHODS

The initial research material comprised seeds of cup plant (*Silphium perfoliatum* L). Selected, surface-treated seeds of cup plants were placed in Petri dishes with filter paper soaked with bidistilled water. The plates were kept for 48 hours in the dark at 24°C. The seeds were sterilized according to different variants (Table 1 and 2). Swollen seeds were sterilized and defatted in 70% ethanol for 30 seconds, then sterilized in 5% calcium hypochlorite with 2-3 drops of Tween 20 for 20 minutes, 3% hydrogen peroxide for 5 min. or 30% of ACE (commercial bleach, which is a component of sodium hypochlorite (4.85%) and as well as other compounds containing chlorine, <5%) for 7 min., then the seeds were rinsed with sterile bidistilled water three times. In addition, another part of seeds was subjected to high temperature sterilization, by placing the seeds in 96% alcohol, and then in a flame for a few seconds (Table 1).

In another variant of the experiment, the seeds surface sterilized with 70% ethanol for 30 seconds were subjected to swelling. Therefore, they were placed in Petri dishes with filter paper soaked with bidistilled water and kept in the dark at 24°C for 48 hours. The embryos isolated with a preparation needle were sterilized in: 5% calcium hypochlorite with 2-3 drops of Tween 20 for 5 minutes, 3% hydrogen peroxide for 5 minutes, or 30% solution ACE for 7 and 8 minutes, and then rinsed with bidistilled water three times (Table 2).

Table 1.
Methods of sterilization of whole seeds of cup plant (*Silphium perfoliatum* L.)

Variant	Pre-sterilization		Proper sterilization		Final stage of sterilization
	Substance disinfectants	Exposure time (s)	Active ingredient	Exposure time (minutes)	
1/1	70% ethanol	30	5% calcium hypochlorite + 2-3 drops of Tween 20	20	sterile water
2/1	70% ethanol	30	3% hydrogen peroxide	5	sterile water
3/1	70% ethanol	30	30% ACE	7	sterile water
4/1			96% ethanol	1	flame

Table 2.
Methods of sterilization of embryos isolated from seeds of cup plants (*Silphium perfoliatum* L.)

Variant	Pre-sterilization		Proper sterilization of isolated embryos		Final stage of sterilization
	Substance disinfectants	Exposure time (s)	Active ingredient	Exposure time (minutes)	
1/2	70% ethanol	30	5% calcium hypochlorite + 2-3 drops of Tween 20	5	sterile water
2/2	70% ethanol	30	3% hydrogen peroxide	5	sterile water
3/2	70% ethanol	30	30% ACE	7	sterile water
4/2	70% ethanol	30	30% ACE	8	sterile water

To induce germination, the embryos were placed onto Murashige and Skoog medium (MS) (1962), diluted at a ratio of 1:1 and solidified with 0.7% Vitro Lab – Agar (Biocorp). The medium was brought up to pH 5.6-5.8, poured into tubes and autoclaved at a temperature of 121°C for 20 minutes. The embryos were placed on the medium so prepared by gentle pressing. Over the next weeks of culture, the sprouting process took place producing seedlings. Seedlings obtained from the seeds sterilized by ACE for 7 minutes were used for isolation of apical explants. The germination process occurred under controlled conditions: 16-hour photoperiod, light intensity of 2500 $\mu mol \times m^{-2} \times s^{-1}$, temperature of 25 +/-2°C, and air humidity of 70%. The apical parts of shoots, having been deprived of cotyledons and leaves, were isolated from 6-week sterile seedlings. The explants were put into test tubes on MS regeneration medium which, in addition to mineral compounds, contained organic substances and growth regulators: NAA (1-naphthaleneacetic acid), IAA (3-indoleacetic acid), BAP (6-benzylaminopurine), KIN (kinetin), and ABA (abscisic acid).

The experiment involved the use of four MS regeneration medium variants:

Variant 1: MS containing 2.5 mg × dm^{-3} BAP + 0.45 mg × dm^{-3} ABA;

Variant 2: MS containing 5 mg × dm^{-3} BAP + 1 mg × dm^{-3} NAA;

Variant 3: MS containing 2 mg × dm^{-3} BAP + 1 mg × dm^{-3} IAA;

Variant 4: MS containing 0.5 mg × dm^{-3} IAA + 4 mg × dm^{-3} KIN.

The medium was solidified with 0.7% Vitro Lab – Agar. The stands with the tubes were placed in the breeding room with the same conditions as during sprouting. The experiment was carried out in three replications. In each replication, 24 explants were placed on the medium. After 8 weeks of growth the regenerated shoots were excised and transferred from the regeneration medium to MS rooting medium without growth regulators. The culture was exposed to 16-hour photoperiod, light intensity of 2500 $\mu mol \times m^{-2} \times s^{-1}$, temperature of 25 +/-2°C, and air humidity of 70%. The rooted plants were transferred into the mixture of soil and perlite (1:1) and acclimatized under greenhouse conditions.

RESULTS AND DISCUSSION

Describing the proper method of sterilization of plant material is an essential step to achieve high efficiency in *in vitro* cultures of plant tissues. The effectiveness of sterilization may be dependent on the type, concentration and time of treatment with the sterilizing agent (Roxas et al., 1996). In this experiment, sterilization of *Silphium perfoliatum* seeds started with 5% calcium hypochlorite, as an appropriate disinfectant. However, in this experiment only 45.83% of sterile seeds were obtained and they sprouted in 8.33% (Fig. 1), while in the case of isolated embryos 33.3% of seedlings were obtained from 83.3% sterile embryos (Fig. 2). The seedlings were deformed, with their cotyledons curled, often brownish at the time of *in vitro* culture and with a single embryonic root or completely devoid of it. The low effectiveness of sterilization of cup plant seeds or embryos in 5% calcium hypochlorite and the deformation of germinated seedlings are explained by too long an action of the disinfectant or the phytotoxic properties of this species (Parkinson et al. 1996).

The best method used to sterilize the buds of yellow everlasting *Helichrysum aenarium* (L.) was to disinfect them in 70% ethanol for 1-2 seconds, and then in 9% calcium hypochlorite solution for 10 minutes. 63.33% of sterile seeds, capable of regenerating explants, are obtained with this method (Sawilska and Figas, 2006). The sterilization method in which the seeds were immersed in 96% ethanol and fired over a flame burner allowed 91.67% of sterile samples to be obtained, but further development took place in only 37.5% of all embryos (Fig. 1).

Seedlings in this experiment were characterized by deformed, fine and thick cotyledons. Due to the adverse effects of temperature directly on the tissue, this method is not used for sterilization of isolated embryos. Another known technique for disinfecting plant material is, for example, treatment of explants with solutions of mercuric chloride. During the sterilization of gerbera seeds, Altaf et al. (2009) used a 0.1% solution of mercuric chloride for 2 minutes at high efficiency. During the experiment, it was found that mercuric chloride caused the death of explants of some varieties, because in the case of those varieties a method was introduced using 5% and 10% hydrogen peroxide solution acting, respectively, for 5 and 10 minutes, which allowed one to obtain the maximum number of sterile explants (Roxas et al. 1996). In this experiment, the use of hydrogen peroxide for the disinfection of seeds also increased the effectiveness of sterilization, however, the percentage of germinated embryos was 31.25% in the variant 2/1 (Fig. 1) and 56% in the variant 2/2 (Fig. 2). The resulting seedlings were phenotypically different from the normal morphology of plants of this species. The seedlings were low and small and they had undeveloped cotyledons. An earlier study shows that hydrogen peroxide is typically used to disinfect seeds of gymnosperm plants (Wenny and Dumroese, 1987). The low efficiency of these methods induced us to seek alternative sterilization techniques. Therefore, seeds were

sterilized and embryos isolated using ACE. As a result of the use of ACE, as an appropriate disinfectant, 25% of germinated embryos and cultured seedlings were obtained from whole seeds (Fig. 1), but in the case of sterilization of embryos 100% of sterile embryos were obtained as well as 100% sterile and well-developed seedlings (Fig. 2). The seedlings obtained from seeds sterilized with ACE for 7 minutes had a better appearance and vigour, because it was used for isolation of apical explants. In a study on organogenesis of *Gerbera jamesonii* from seeds, Hasbullah et al. (2008) obtained the same result after using 40% sodium hypochlorite for 30 minutes, while Baiyeri and Mbah (2006) achieved optimal results by sterilizing the seeds of African breadfruit (*Treculia africana* Decne) with 3.5% sodium hypochlorite solution.

Shoot meristems set in the leaf corners have the same histological and genetic structure like shoot apical meristems; hence a possibility of producing plants genetically identical as the parent plant (Hu and Wang, 1983; Assim et al., 2008). As for the Asteraceae family plants, including the cup plant, growing tips used to initiate *in vitro* cultures guarantee a high genetic stability and enhance the potential of producing sterile tissue cultures (Kanwar and Kumar, 2008). The isolated cup plant apical explants, incubated on MS medium with growth regulators, kept on developing in the first weeks of culture and callus tissue formed on the cut surface, on which no adventitious bud growth was observed. Lateral shoots developed on the explants of lateral buds, located in the corners of leaves and stipules, during the successive weeks of culture on MS media with 2.5 mg × dm^{-3} BAP and 0.45 mg × dm^{-3} ABA, 5 mg × dm^{-3} BAP and 1 mg × dm^{-3} NAA as well as 2 mg × dm^{-3} BAP and 1mg × dm^{-3} IAA (Table 3, Fig. 3). Analyzing the results reported over the few weeks of culture, it can be observed that morphogenetic potential of cup plant apical explants depends on the ratio of the amount of auxin to cytokinin; the highest share of explants with lateral shoots (41.7%) and callus (90.3%) was recorded on MS medium with 5 mg × dm^{-3} BAP and 1 mg × dm^{-3} NAA. Figas et al. (2010), investigating cup plant micropropagation and applying the same BAP and NAA concentrations, reported 84.2% of explants with lateral shoots and 92.8 % with callus tissue, which coincides with the observations that a high content of cytokinins and an inconsiderable content of auxins enhance plant proliferation in *in vitro* cultures. Cytokinins originating from adenine (BAP, KIN) can tolerate the dominance of the apical bud, thus stimulating lateral shoots growing from the corner buds. Media of a similar composition as for the cup plant were reported by Hussein et al. (2008) for gerbera (*Gerbera jamesonii*). The highest regeneration efficiency (36.6%), however, was reported by the authors on the medium with BAP and ABA added. The application of ABA resulted in an effective induction of the processes of callus formation and, as a result of subsequent passages, also in successful shoot organogenesis.

Table 3.
Effect of growth regulators on the formation of callus and axillary shoots on cup plant (*Silphium perfoliatum* L.) shoot tip explants after 8 weeks of culture

Variant	Growth regulators [mg dm^{-3}]	Percentage of explants forming:			Number of shoots per explant
		Axillary shoots	Callus	Roots	
I	2,5 BAP + 0,45 ABA	18.0	26.4	0	0.5
II	5 BAP + 1 NAA	41.7	90.3	0	1.6
III	2 BAP + 1 IAA	29.2	31.9	0	1.3
IV	0.5 IAA + 4 KIN	0	0	73.6	0

On MS medium enriched with 0.5 mg × dm^{-3} IAA and 4 mg × dm^{-3} KIN on the cup plant explants, no formation of lateral shoots was observed. However, the growth of roots was reported, which accounted for 73.6%. Isolating shoot apexes of dwarf everlast (*Helichrysum arenarium* (L.) Moench), representing the Asteraceae family, and incubating them on MS medium of the same composition with growth regulators, Sawilska and Figas (2006), recorded 16.1 lateral shoots per explant. The results demonstrate that applying plant regulators of the same group in *in vitro* cultures can trigger a completely different response of the explants of the species taxonomically remote from each other and clones of the same species. It is suggested that genes conditioning the control of the process of synthesis of endogenous or using exogenous growth regulators can be responsible for the regeneration (Rakoczy-Trojanowska and Malepszy, 1993).

The induction of rhizogenesis in cup plant *in vitro* cultures was reported on MS media without growth regulators. In case of many plants representing the

Asteraceae family, the process occurred on MS media with a reduced content of mineral salts (˝ MS) and a lowered concentration of saccharose (1.5%) (W e b b and S t r e e t , 1977). Rooting of 90% of shoots in costus (*Saussurea lappa*) has been recorded on the media with 0.5-1.0 mg × dm^{-3} NAA (A r o r a and B h o j w a n i , 1989). Rooting gerbera shoots, R e y n o i r d et al. (1993) applied MS medium containing 0.25 µM NAA, and thus producing 100% of rooted shoots. Many plants form roots on media without growth regulators, however, the addition of IAA, IBA or NAA intensifies the process considerably (F a r i a and I l l g , 1995). In the present experiment, the application of MS medium without growth regulators, proposed by Aswath and C h o u d h a r y (2002) for cup plant rooting, resulted in 100% of rooted plants. The characteristic feature of the roots was the formation of compact and long systems and their short formation time (Fig. 4).

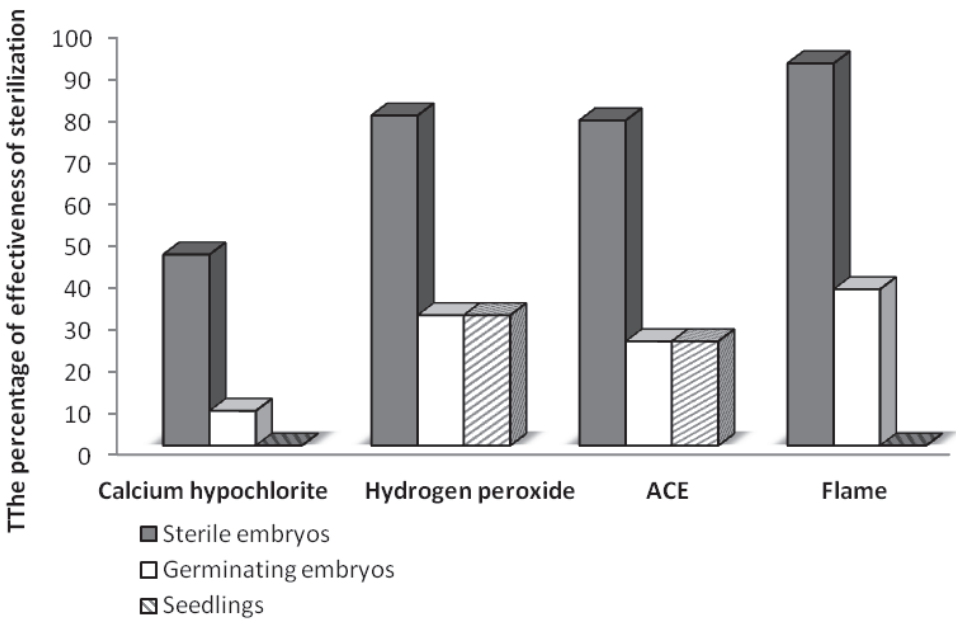

Fig. 1. Effect of sterilization methods used in whole seeds of cup plants (*Silphium perfoliatum* L.) to obtain sterile seedlings in *in vitro* cultures.

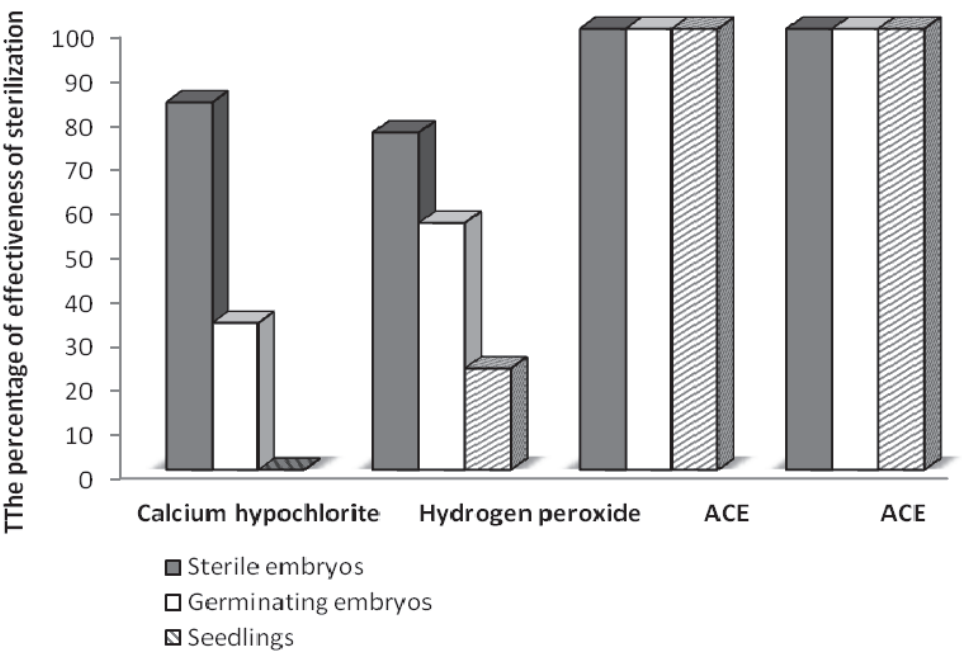

Fig. 2. Effect of sterilization methods used in embryos of cup plants (*Silphium perfoliatum* L.) to obtain sterile seedlings *in vitro* cultures.

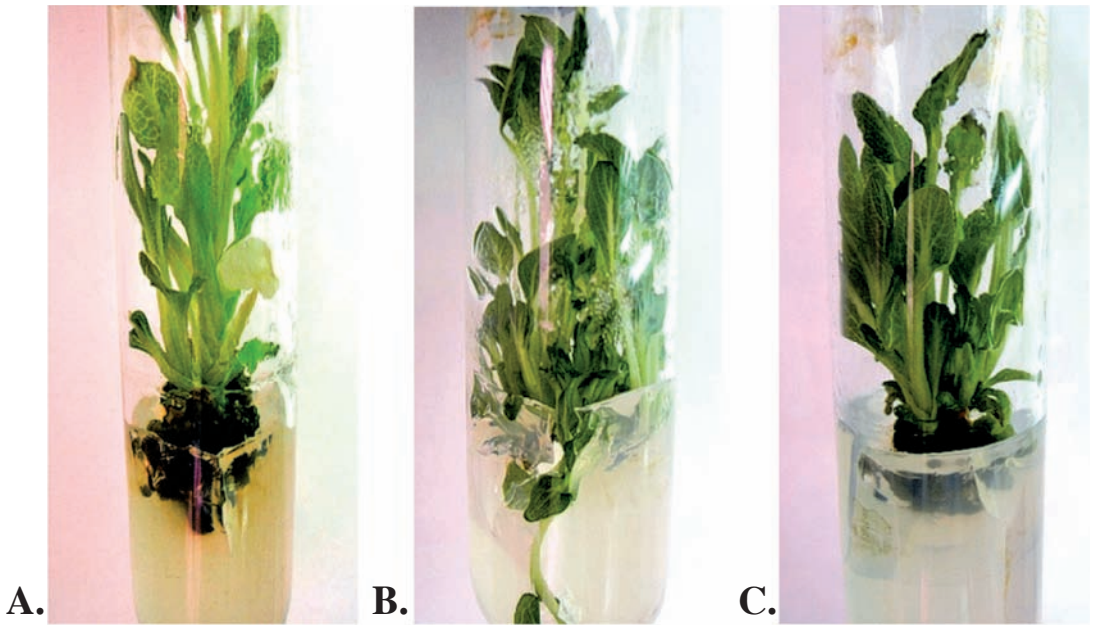

Fig. 3. Axillary shoots formed from shoot tip explants cultured on MS medium supplemented with 0.45 mg × dm^{-3} ABA and 2.5 mg × dm^{-3} BAP **(A)**, 1 mg × dm^{-3} NAA and 5 mg × dm^{-3} BAP **(B),** 1 mg × dm^{-3} IAA and 2 mg × dm^{-3} BAP **(C)** after 8 weeks of culture.

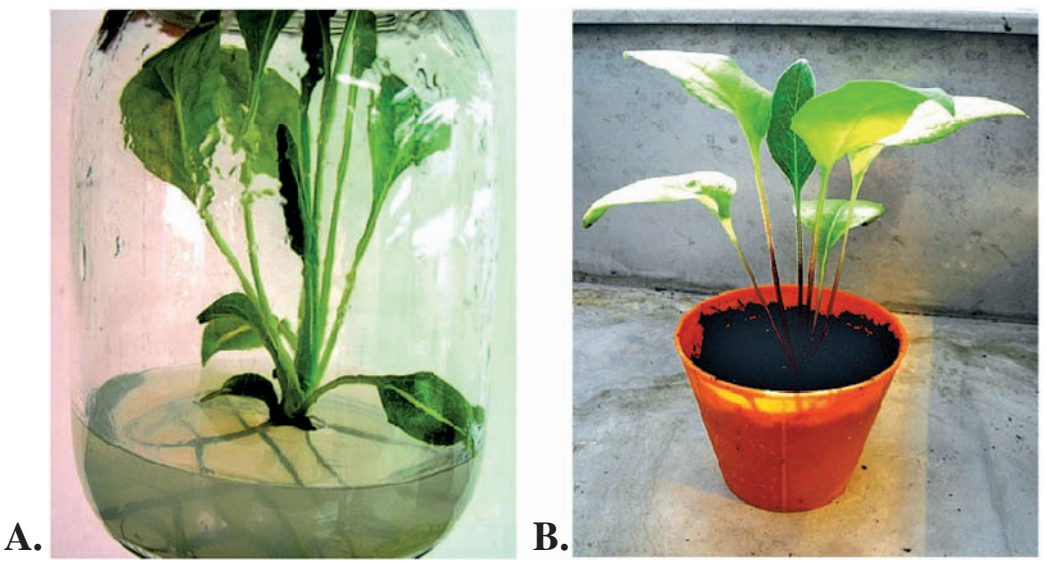

Fig. 4. Rooted plant of (*Silphium perfoliatum L.*) in *in vitro* cultures **(A)**; the cup plant after its transfer from *in vitro* to *ex vitro* conditions **(B)**. Phot. by Katarzyna Zadworna.

CONCLUSIONS

1. Numerous trials of sterilization indicate that the best method of obtaining sterile *in vitro* cultures is sterilization of isolated embryos of *Silphium perfoliatum* L. using ACE, which contains sodium hypochlorite as the active substance.
2. As a result of cup plant micropropagation in *in vitro* cultures from shoot apical explants, the highest number of shoots was produced, formed from lateral buds without adventitious organogenesis.
3. The applied method of micropropagation from shoot apical explants offers a potential of producing offspring plants which would be genetically stable and with the genotype identical as the parent plant**.**
4. The need of using growth regulators in the process of rhizogenesis was eliminated, since numerous and well-branched root systems were formed on MS medium without phytohormones. Using this method, 100% of shoots produced compact and long roots.

5. Considering many possible applications of cup plant (*Silphium perfoliatum* L.) in various industries, medicine and in agriculture, continued research to develop an optimal and efficient system of plant regeneration of that species is well justified.

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Optymalizacja procesów sterylizacji i mikrorozmnażania roślin rożnika przerośniętego (*Silphium perfoliatum* L.) z eksplantatów wierzchołkowych siewek w warunkach kultur *in vitro*

Streszczenie

Rożnik przerośnięty jest wysoką, osiągającą 2,5 metra byliną, należącą do rodziny Asteraceae. Gatunek ten może być uprawiany jako roślina lecznicza, paszowa, ozdobna, energetyczna i miododajna. W związku z tym jest wykorzystywana w wielu gałęziach przemysłu. Jednakże występujące w Polsce źródła materiału siewnego nie zaspokajają rosnącego zainteresowania producentów tym gatunkiem. Z tego powodu podjęto badania nad procesem sterylizacji i mikrorozmnażania *Silphium perfoliatum* L. w warunkach kultur *in vitro*. Spośród zastosowanych metod sterylizacji nasion najbardziej skuteczną okazało się zastosowanie ACE. Jako eksplantatów do procesu mikropropagacji użyto wierzchołkowych części siewek, które wykładano na pożywkę MS modyfikowaną poprzez dodanie regulatorów wzrostu i rozwoju roślin. Zastosowano 4 warianty pożywki różniące się składem i koncentracją fitohormonów. Najwyższy procent eksplantatów z pąkami przybyszowymi (41.7%) i kalusem (90.3%) zaobserwowano na pożywce MS zawierającej 5 mg × dm^{-3} BAP + 1 mg × dm^{-3} NAA. Uzyskane pędy izolowano i ukorzeniano na pożywce MS bez regulatorów wzrostu i rozwoju a ukorzenione rośliny przenoszono do szklarni i aklimatyzowano do warunków *ex vitro.*