EXPERIMENTAL PAPER

Establishing an efficient explant superficial sterilization protocol for *in vitro* micropropagation of bear's garlic (*Allium ursinum* L.)

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Summary

Introduction: *Allium ursinum* L. has a commercial value due to its high contents of bio-active compounds and mild, garlic-like taste. *In vitro* culture played an important role in obtaining *Allium* species with the desired characteristics and in the production of healthy reproductive material. **Objective:** Developing an effective method of sterilization bear's garlic bulbs. **Methods:** To obtain sterile shoots of garlic several methods of sterilization involving such factors as ACE, H₂O₂, HgCl₂, and UV-C were tested. **Results:** In order to obtain sterile shoots of bear's garlic, several sterilization procedures were tested. The best procedure was based on a two-step disinfection, where the whole onions were treated with ethanol and H₂O₂ for 20 min. Thereafter, the isolated apical buds were sterilized in ACE for 10 min, rinsed in double-distilled water and transferred onto MS medium for growing. Up to 95% of the inoculated explants formed shoots, which were sub-cultured on MS with 4 mg dm⁻³ BAP in order to enable further propagation. **Conclusion:** After optimization and stabilization, this procedure may become the basic concept of a proper and reliable propagation method of bear's garlic on commercial scale.

Key words: bear's garlic, *Allium ursinum* L., *in vitro* cultures, sterilization procedures, UV-C irradiation

INTRODUCTION

Bear's garlic (*Allium ursinum* L.) is a plant of the family *Amaryllidaceae* (sub-family *Allioideae*) and in Poland is one of partially protected species. The natural habitat of bear's garlic in Poland are the foothills of the Carpathian and Sudeten mountains and the Eastern regions of the country rich in humus, moist and shaded forests, where, due to the allelopathic properties of *A. ursinum*, wide communities are developed [1]. The health promoting properties of *A. ursinum* were already known by our forefathers. The first information about this genus along with a botanical description appeared in the herbarium of Hieronymus Bock in 1539 [2]. *A. ursinum* is characterized by antifungal, antibacterial, anticancer, cardioprotective, antihypertensive and diuretic properties due to the presence of large contents of flavonoids, carotenoids, and chlorophyll in the tissues [3]. The health promoting properties of bear's garlic are also bound with the contents of allin, allicin and other sulphuric compounds. There is evidence that the therapeutic effects of *A. ursinum* are stronger than those of *A. sativum* L. with respect to improving blood pressure and its composition [4]. The entire herb or only the leaves of *A. ursinum* are used in the natural medicine. The strongest antioxidant properties are found in the leaves of bear's garlic, which are the most commonly used part of this plant. They are delicate and fragile and should be collected during the first phase of blooming [5]. Pollen of bear's garlic are also valuable due to its high protein content, therefore, it is very valuable for honey-bee and wild pollinating insects [6].

Leaves and onions of wild garlic can also be used as a spice and as an aromatic addition to salads [7-10]. The culinary advantage of this species compared to *A. sativum* represents the fact that every part is edible and that the sharp garlic smell after consumption disappears in a short time.

Despite the great potential of *A. ursinum*, this species is not yet cultivated in Poland and in Europe on a commercial scale. Most of available plant material is obtained from natural habitats, which may lead to the risk of excessive exploitation of natural resources. Another limiting factor represents its short availability, which is only three months in the year, due to the above-ground developmental cycle of bear's garlic. In addition, since 2004 this plant is partially protected in Poland and may be harvested only to a rather limited extent for its utilization as a herbal medicine [10]. Therefore, cultivation of *A. ursinum* is highly desirable, but limited by low germinating efficiency of seeds. As a consequence, *in vitro* culture of bear's garlic may be an alternative to the standard propagation of bear's garlic by seeds or onions. In addition, the plant material used for medicinal purposes should be produced from standardized raw material with a high genetic stability and uniformity. This can be assured by the application of *in vitro* culture techniques, the method providing homogeneous and high-quality propagation material in sufficient amounts [11].

In case of the genus *Allium*, the regeneration of plants from such explants as onions, flower stems, and roots occurs in tissue culture by direct organogenesis. Several publications [e.g. 12, 13] indicate the influence of the type of explant, plant growth regulators, and culture conditions on *in vitro* regeneration and propagation of *Allium*, but information concerning *in vitro* cultures of *A. ursinum* is still not available. The optimization of techniques related to the micropropagation of

different members of the *Allioideae* subfamily, in combination with the improvement of genetic transformation methods, may contribute to progress in their proliferation and use on the industrial scale and research [14].

In order to spread the cultivation of *A. ursinum*, the sterilization procedures, which would enable further development of sufficient amounts of explants in short time and, as a consequence, the micropropagation of *A. ursinum*, should be optimized. The aim of the present study was to test several disinfectants and physical methods, such as the application of a UV-C radiation, to maintain healthy shoots able to grow in *in vitro* culture. The introduction of appropriate sterilization procedures will allow efficient initiation of tissue culture of bear's garlic and results in the production of healthy plants with the desired properties.

MATERIAL AND METHODS

Studies were conducted on onions of bear's garlic (*Allium ursinum* L.) obtained in April and May from the forest area in the north-western part of Vienna (Austria). Onions were cleaned and the roots were stripped off (fig. 1). In the presented study, chemical and physical methods were applied, in order to optimize the sterilization process. Four sterilization procedures were tested for obtaining sterile reproductive material: sterilization of onions (tab. 1, fig.1), sterilization of apical buds (tab. 2), a two-stage sterilization procedure (tab. 3), and UV-C sterilization (tab. 4).

Figure 1.
Onions of bear's garlic (*A. ursinum*) before and after cleaning (A) and stripping (B)

Table 1.
Sterilization procedures of bear's garlic onions (*A. ursinum* L.)

Combination	Pre-sterilization		Main sterilization		Final stage of sterilization
	Disinfectant	Exposure time [s]	Active compound	Exposure time [min]	Rinsing
1A	70% ethanol	60	HgCl ₂ 0.1%	20	bi-distilled water
1B	70% ethanol	60	ACE 30%	20	bi-distilled water
1C	70% ethanol	60	ACE 30%	7	bi-distilled water

Table 2.

Sterilization procedures of apical buds of bear's garlic (*A. ursinum* L.)

Combination	Pre-sterilization		Main sterilization		Final stage of sterilization
	Disinfectant	Exposure time [s]	Active compound	Exposure time [min]	Rinsing
2A	70% ethanol	60	ACE 30%	5	bi-distilled water
2B	70% ethanol	60	ACE 30%	7	bi-distilled water
2C	70% ethanol	60	ACE 30%	10	bi-distilled water

Table 3.

Two-stage sterilization procedures of onions of bear's garlic (*A. ursinum* L.)

Combination	Plant material	Pre-sterilization		Main sterilization		Final stage of sterilization
		Disinfectants	Exposure time [s]	Active compound	Exposure time [min]	Rinsing
3A	Onions	70% ethanol	60	30% H ₂ O ₂	20	
	Apical buds isolated from sterile onions			ACE 30%	5	bi-distilled water
3B	Onions	70% ethanol	60	30% H ₂ O ₂	20	
	Apical buds isolated from sterile onions			ACE 30%	10	bi-distilled water

Table 4.

Sterilization procedures of bear's garlic onions by application of UV-C

Combination	Plant material	Pre-sterilization		Main sterilization		Final stage of sterilization
		Disinfectants	Exposure time [min]	Active compound	Exposure time [min]	Rinsing
4A	Onions	UV-C	20			
	Apical buds			ACE 30%	10	bi-distilled water
4B	Onions	UV-C	40			
	Apical buds			ACE 30%	10	bi-distilled water
4C	Onions	UV-C	40			
	Apical buds					

Sterilization of onions – combination No. 1

Purified onions of bear's garlic were pre-sterilized using 70% ethanol for 60 s and then used for different disinfection procedures. In the first case, the onions were treated with a 0.1% HgCl_2 solution, in the second and third with a 30% solution of ACE (a commercial bleach, which 4.85% of sodium hypochlorite and further chlorine-containing compounds) for 20 and 7 min, respectively. Thereafter, they were rinsed three times with sterile bi-distilled water. Subsequently, the apical buds of the disinfected onions were isolated using tweezers and scalpels under sterile conditions and were placed on MS medium solidified with 0.7% agar (tab. 1).

Sterilization of the apical buds – combination No. 2

In this method the explants were isolated from previously cleaned and unrooted garlic onions. The isolated apical buds were superficially disinfected in 70% ethanol for 60 s and then immersed in a solution of 30% ACE (active chlorine water solution, v/v) for 5, 7, and 10 min, respectively. Finally, the buds were rinsed three times in sterile, bi-distilled water and placed on solidified MS medium (tab. 2).

Two-stage procedure of sterilization – combination No. 3

A third sterilization procedure consisted of a two-stage decontamination of whole onions followed by the isolation of apical buds. After washing and removal of roots, the onions were pre-sterilized in 70% ethanol for 60 s and then properly with 30% H_2O_2 for 20 min. Apical buds were isolated from the disinfected plant material, treated with 30% ACE for 5 or 10 min (tab. 3) and then placed on MS medium solidified with 0.7% agar.

Sterilization with UV-C radiation – combination No. 4

Disinfection of the plant material was performed using UV-C in combination with 30% ACE (active chlorine water solution v/v). The application of UV-C was carried out in a closed chamber that enabled irradiation of the plant material from above and below using 4 UV-C lamps (Philips TUV 36W LL) supplying a radiation efficiency of 14.6 W with a maximum emission at a wavelength of 253.7 nm. Samples were irradiated for 20 or 40 min. After irradiation of whole onions, the apical buds were isolated. In two of the combinations the explants were kept for 10 min in 30% ACE, then rinsed three times with bi-distilled water and transferred on MS medium solidified with 0.7% agar. In one of the cases, when onions were

irradiated for 40 min, the isolated apical buds were transferred immediately to the culture media (tab. 4).

After sterilization procedures, 20 explants of each combination were inoculated on solid MS medium enriched with 4 mg dm⁻³ BAP [15]. Before inoculation the medium was adjusted to pH 5.6-5.8 and autoclaved at 121°C for 20 minutes. The flasks were placed in a culture room under a 16 h day and 8 h night photoperiod, where light intensity was approx. 40 μmol m⁻² s⁻¹ and the temperature set to 25±2°C. The whole experiment were repeated three times. The efficacy of the sterilization procedures was evaluated by the statistical FR-ANALWAR software program based on Microsoft Excel table sheets. Results were evaluated statistically by the application of one-way and multiple ANOVA, where homogeneous groups were built after Tukey test at $p \leq 0.05$.

Ethical approval: The conducted research is not related to either human or animal use.

RESULTS AND DISCUSSION

A number of experiments aimed at the regeneration of *Allium* species by direct organogenesis from different kinds of explants, such as onions, flower stems, roots, etc. Studies comprised the influence of the type of explant, the effect of plant growth regulators and of culture conditions on *in vitro* regeneration and propagation of *Allium* spp. [12, 13]. The improvement of techniques related to the micropropagation of different members of the *Allioideae* sub-family in combination with the continuous improvement of methods for genetic transformation using *Agrobacterium tumefaciens* were performed. These methods allow higher yields or achievement of herbicide resistant cultivars and may contribute to the progress of large-scale production for commercial purposes [13, 14]. Already successful are transformed cultivars of *Allium cepa* L. [16], *A. sativum* [17, 18] and *Allium ampeloprasum* L. [17], whereas information on recent achievements in the *in vitro* cultivation of *A. ursinum* L. are not available. The reproduction of *Allium* spp. faces some limitations, e.g. achieving completely formed onions, dormancy, tissue vitrification and difficulties in the regeneration of the obtained plants [12, 19-22]. Thus, conducting conventional breeding of *Allium* spp. in combination with genetic engineering and plant regeneration in *in vitro* cultures represents a forward-looking strategy. It allows to obtain a high yield of regenerated plants and cultivars resistant or tolerant to biotic or abiotic stress. In addition, it gives the possibility to cultivate plants on a large, commercial scale.

The basic element in the initiation of any kind of tissue culture represents the disinfection of the plant material. The effectiveness of the surficial sterilization procedure has a significant impact on the development and growth of the explants. In the presented experiments, a new, innovative disinfection method was tested. In addition, information on tissue culture of bear's garlic is presented for

the first time. The use of a two-stage disinfection procedure and involving the irradiation of plant material with UV-C is proposed for the *Allioideae* sub-family for the first time and tested as a representative example on *A. ursinum*. Standard disinfection procedures for initiating a tissue culture of members of the *Allioideae* sub-family involve the application of alcohol, sodium hypochlorite, antibiotics or mercury chloride [14, 23, 24]. The type of the disinfecting substances, the time of exposure of explants to them, and the selection of an appropriate substrate varies depending on authors. However, there is not any well established and generally accepted disinfection procedure that ensures a high efficiency.

In the presented experiments, 11 sterilization procedures were compared, which differed with respect to the procedure and active substances applied. The obtained results of the initial stage of tissue culture are interpreted in order to identify the most effective method for sterilizing the onions of bear's garlic (fig. 2).

Figure 2.
Mean number of sterile explants of *A. ursinum* as affected by the method of disinfection (LSD – the lowest significant difference, Tukey's confidence half-interval, at $P=95\%$ and $\alpha=0.05$)

Evaluation of the degree of contamination of explants performed seven days after the initiation of the culture revealed that the best sterilization procedure was the two-step disinfection procedure involving treatment of entire onions with 70% ethanol in order to clean, degrease and pre-disinfect the plant material, followed by a 20 min immersion into 30% H_2O_2 (water solution v/v) and the subsequent isolation of the apical buds, which were disinfected for 10 min with 30% ACE (combination 3B). This technique was most effective, since a purification level of 100% was achieved. A similarly high efficiency of 95% explants free of pathogens were reported by Seif El *et al.* [14], who sterilized onions of garlic *A. sativum* during a preliminary stage using ethanol, followed by the main sterilization with a commercially available substance containing sodium hypochlorite. In the present study, the apical buds isolated from sterile onions were additionally sterilized with ACE and then transferred onto MS medium, whereas the Egyptian researchers

transferred the isolated explants immediately onto a modified MS medium without any additional sterilization procedure.

According to the literature [20, 25, 26] sodium hypochlorite as a source of active chlorine is used for the disinfection of onions (*A. sativum*) at various concentrations ranging from 0.5% to 2.4%. Another method being characterized by a high efficiency in eliminating viruses in cultures of *A. sativum* represents the thermotherapy used by Uzman *et al.* [27]. In order to increase the performance of the method, the effectiveness of the active substance can also be increased by adding the non-ionic detergent Tween 20 to the sodium hypochlorite solution. Haque *et al.* [25] used Tween for the sterilization of garlic onions, which reduced the surface tension and thus facilitated the penetration of the active substance into deeper parts of the tissue, thus resulting in an efficiency of 95.6% for sterilization. Procedures, where buds were isolated from non-sterile onions and buds were immersed for 10 min into 30% ACE, proved less efficient. Applying this procedure, 80% of the explants were free of pathogens (combination 2C). Application of 0.1% HgCl_2 (combination 1A) as a disinfectant did not improve the performance of the sterilization procedure. The obtained sterile explants (40%) continued to grow only to a limited extent or decayed. This response might be explained by a too long-lasting treatment with this sterilization agent. However, its use has been proven as effective in another study on somatic embryogenesis of garlic *A. sativum* [24] and also for the disinfection of onion tissue of *A. cepa* [19]. Because of the high degree of contamination of their garlic onions, Robledo-Paz *et al.* [23] treated their explants in the initial stage of the sterilization procedure with antibiotics and antifungal preparations such as Radomil Bravo or Benlate. The so-prepared onions were then disinfected with commercially available bleachers containing active chlorine. The sterile explants isolated from germinating onion were transferred onto a medium in order to regenerate the garlic plants [23].

A high sterilization efficiency of plant material can be also achieved by the application of UV-C irradiation. Physical methods, such as UV-C irradiation at a wavelength between 100 and 280 nm are highly promising because of their low labor input as well as their easy and cheap mode of operation. UV-C irradiation represents an effective procedure for the inactivation of bio-contamination. It eliminates viruses and bacteria, and it is frequently used for the disinfection of materials sensitive to high temperatures or materials that cannot be treated with chemically active substances [28]. This method has been used successfully for superficial sterilization in the biopharmaceutical industry [29] or in post-harvest crop protection [30, 31]. In order to obtain an aseptic culture, an UV-C dose of low intensity was applied that might also induce the hormesis phenomenon, which is the appearance of beneficial effects due to low doses of e.g. ionizing radiation on living organisms. The mechanism of this phenomenon is based on the stimulation of cellular repair mechanisms for small doses, resulting in a decrease in the number of DNA damage [32, 33]. In the experiment presented here, as much as 90% of the explants, which developed from onions exposed to UV-C for 40 min, followed by an exposure to ACE for 10 min, were free from infections (combination 4B). However, most losses due to the low decontamination efficiency of the procedure

were observed for combination 4C, where only 20% of the apical buds isolated from onions and irradiated for 40 min remained sterile after transferring onto a medium. The comparison of combination of 4C with 4B indicates rather significant influence of the treatment with 30% ACE followed by rinsing with bi-distilled water. In addition, also the period of exposure to UV-C irradiation is an important factor. In the case of an exposure longer than 40 min, dark spots appeared on the surface of onions, which probably indicate cellular decay processes induced by the irradiation (results of a pilot experiment).

After six weeks of culture on MS medium a total of 121 sterile explants formed shoots. The obtained sterile shoots were sub-cultured on MS medium supplemented with 4 mg dm⁻³ BAP in order to induce amplification and elongation (fig. 3).

Figure 3.

Shoots of bear's garlic (*A. ursinum*) developed during *in vitro* culture

CONCLUSIONS

The basis for the success of *in vitro* cultures is an effective sterilization procedure that allows proper development of explants in sterile conditions. The protocols used in the present study led to the development of effective procedures for the initiation of the culture of *Allium ursinum* with onion explants. The development of an effective sterilization method of onions of bear's garlic enables further work aiming at the optimization of regeneration systems, as well as at the selection and propagation of genotypes suitable for pharmaceutical or culinary use. In addition, micropropagation of bear's garlic may contribute to the protection of natural stands of *Allium ursinum* and, in the ideal case, represents a prerequisite for the market supply with bear's garlic during an extended period of the year.

Conflict of interest: Authors declare no conflict of interest.

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OPRACOWANIE WYDAJNEGO PROTOKOŁU STERYLIZACJI POWIERZCHNIOWEJ EKSPLANTATÓW W KULTURACH *IN VITRO* CZOSNKU NIEDŹWIEDZIEGO (*ALLIUM URSINUM* L.)

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Streszczenie

Wstęp: *Allium ursinum* L. wykazuje wysoką wartość handlową dzięki temu, iż charakteryzuje się dużą zawartością substancji czynnych oraz łagodnym, czosnkowym smakiem. Kultury *in vitro* odegrały istotną rolę w uzyskaniu gatunków *Allium* o pożądanych cechach

i produkcji zdrowego materiału rozmnożeniowego. **Cel:** Opracowanie efektywnej metody sterylizacji cebul czosnku niedźwiedziego. **Metody:** W celu pozyskania sterylnych pędów czosnku przetestowano kilka metod sterylizacji z udziałem takich czynników jak ACE, H_2O_2 , $HgCl_2$ i UV-C. **Wyniki:** Procedura, która przyniosła najlepsze efekty, polegała na dwustopniowej dezynfekcji, w której całe cebule poddane zostały działaniu alkoholu etylowego i H_2O_2 przez 20 min. Wyizolowane z cebul eksplantaty sterylizowano w ACE przez 10 min, a po przepłukaniu w wodzie bidestylowanej wyjałowione paki inokulowano na pożywkę MS, na której kontynuowały wzrost. Spośród wyłożonych na pożywkę eksplantatów aż 95% formowało pędy. Uzyskane w ten sposób pędy pasażowano na pożywkę MS z dodatkiem 4 mg dm^{-3} BAP w celu ich dalszego namnożenia. **Wnioski:** Po przeprowadzeniu procesu optymalizacji i stabilizacji opisana metoda sterylizacji może być odpowiednim i niezawodnym sposobem rozmnażania czosnku niedźwiedziego na skalę przemysłową.

Słowa kluczowe: czosnek niedźwiedzi, *Allium ursinum* L., kultury *in vitro*, sterylizacja, promieniowanie UV-C