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THE EFFECTIVENESS OF DISINFECTION METHODS ON GERMINATION OF GOJI SEEDS (*Lycium barbarum* L.) IN *IN VITRO* CULTURE

WPŁYW ZASTOSOWANYCH METOD DEZYNFEKCJI NA ZDOLNOŚĆ KIEŁKOWANIA NASION JAGODY GOJI (*Lycium barbarum* L.) W KULTURACH *IN VITRO*

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Streszczenie. Do skutecznego usuwania patogenów i mikroorganizmów z powierzchni materiału roślinnego w kulturach *in vitro* stosuje się odpowiednio dobrane metody dezynfekcji. Alternatywnym rozwiązaniem do powszechnie stosowanych środków chemicznych jest ozonowanie. Porównano efektywność dezynfekcji nasion dwóch odmian goi 'A' i 'New Big' powszechnie stosowanymi środkami chemicznymi: NaOCl (7%, 10% i 15%) oraz $HgCl_2$ (0,2%) z metodą ozonowania na sucho i mokro w czasie 5 i 15 min. Najwyższy procent skiełkowanych nasion odmian 'A' i 'New Big' (odpowiednio 62% i 78%), przy najmniejszej liczbie zakażeń (odpowiednio 8% i 14%), uzyskano po zastosowaniu 7% NaOCl. Mniej skuteczny był roztwór 0,2% $HgCl_2$, po zastosowaniu którego procent skiełkowanych nasion odmian 'A' i 'New Big' był niższy (odpowiednio 37% i 30%). Natomiast po zastosowaniu ozonowania eksplantatów 'A' i 'New Big' na mokro przez 15 min uzyskano najwyższy procent skiełkowanych nasion (w przypadku odmiany 'A' – 47%, a w przypadku odmiany 'New Big' – 53%); sterylność kultur u odmiany 'A' wynosiła 100%. Ponadto ozonowanie nasion wykazało podobną efektywność dezynfekcji jak zastosowanie 15-procentowego roztworu NaOCl. W związku z tym stosowanie ozonowania na sucho może stanowić alternatywną metodę dezynfekcji.

Key words: goji berry, $HgCl_2$, *Lycium barabarum* L., NaOCl, ozone, tissue culture.
Słowa kluczowe: $HgCl_2$, jagoda goji, kultury tkankowe, *Lycium barbarum* L., NaOCl, ozonowanie.

INTRODUCTION

Lycium barbarum L. is commonly known as goji berry. It is a spiked, perennial shrub belonging to the family of *Solanaceae*. It comes from the north-western parts of China (Osman et al. 2013). Fruits of goji berry are round or oval, red or orange with a large number of seeds. *L. barbarum* L. berries contain multiple mineral and organic compounds (vitamins B_1, B_6, A, C, E), with the potential of repairing epidermal damage and showing excellent effects on the cardiovascular and cholesterol levels (Dănăilă-Guidea et al. 2015).

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The tissue culture system allows the propagation of selected genotypes with high multiplication rate in an aseptic, temperature-controlled environment (Osman et al. 2013; Tarinejad 2013; Teixeira da Silva et al. 2016). Numerous scientific articles have reported using tissue culture techniques, with many researches successfully applying these to the goji shrub (Kairong et al. 1999; Hu et al. 2001, 2008; Osman et al. 2013; Dănăilă-Guidea et al. 2015). In these studies were used different sources of goji berry explants: shoot tips, nodal segments of leaves, stem axillary buds and roots. In most cases, shoot proliferation was achieved by axillary bud obtained from nodal explants. However, most of these works have no information regarding to the effectiveness of disinfection of the initial material. According to Amiri et al. (2013), Zeng (2014) and Teixeira da Silva et al. (2016) the establishment of an effective tissue culture from plant material derived from greenhouse or field up to the disinfection process. Well-chosen disinfectant allows to maintain a balance between highly effective in disinfecting explants and its high ability for survival and regeneration. In *in vitro* culture usually chemical disinfectants such as sodium hypochlorite, ethanol, calcium or mercuric chloride, hydrogen peroxide and silver nanoparticles or antibiotics have been used (Amiri et al. 2013; Tarinejad 2013). However, the chemicals used in too high concentrations may be toxic to the plant and to the environment. There is required a specialized utilization of these compounds. An alternative method of disinfection might be the ozonation. Ozone has relatively high solubility in water with a high redox potential and is recently declared Generally Recognized As Safe (GRAS) by an expert panel for use in food processing (Graham 1997). However, to date, it has been used in disinfection of potable water, processing, as well as in stored food (seeds, vegetables, fruits, meat). The aim of the study was to compare the effectiveness of seeds disinfection of two goji berries cultivars, ‘A’ and ‘New Big’ using different solution with disinfection methods used.

MATERIAL AND METHODS

Plant material

The experiment was conducted at tissue culture laboratory of the Department of Genetics, Plant Breeding and Biotechnology of West Pomeranian University of Technology in Szczecin.

Seeds of two cultivars ‘A’ and ‘New Big’ of goji berry (*Lycium barbarum* L.) were used as plant material. Goji fruits were obtained after harvest (November) from the experimental orchard of the Pomology Department of West Pomeranian University of Technology in Szczecin.

Disinfestations methods

Seeds were submerged in 70% ethanol for 30 s and after twice washing with sterile deionized water, they were submerged in different disinfecting treatments:

- sodium hypochlorite (NaOCl) in 7%, 10% and 15% for 15 min;
- 0.2% mercury chloride ($HgCl_2$) for 15 min;
- ozone gas in two times of exposure (5 and 15 min);
- ozone in water in two times of exposure (5 and 15 minutes).

Ozon was produced by a discharge generator (ZYH 135) for 3.5 $g \cdot h^{-1}$ capacity with the efficiency of the pump guaranteeing a flow of 15 liters of air within 1 minute.

After the above treatments, seeds were rinse three times with sterile and deionized water for 1, 2 and 5 minutes, respectively under a sterile laminar flow hood.

Medium and culture condition

Aseptic, sterilized seeds were placed individually in glass tube with capacity of 35 × 110 mm containing 15 mL of MS medium (Murashige and Skoog 1962) without plant growth regulators. Each combination included one hundred seeds (four replicates of 25 seeds).

Culture medium were supplemented with 8.0 g · dm^{-3} agar (Biocorp, Poland), 30 g · dm^{-3} sucrose and 100 mg · dm^{-3} myo-inositol, pH was adjusted to 5.7 by adding 0.1M of NaOH or HCl and autoclaved at 121ºC (0.1 MPa) for 19 minutes. The cultures were maintained in a growth room at a temperature of 24 ± 1ºC under 16h photoperiod under a fluorescent lamp (photosynthetic photon flux density 40 µmol · m^{-2} · s^{-1}). Contamination and germination rate were analyzed after 10 days for each combination.

Statistical analysis

The data were subjected to one-factor variance analysis (ANOVA). Mean comparisons were performed using Tukey's least significant difference (LSD) test; significance was set at $p < 0.05$. The percentage data of disinfection methods were transformed before analysis using the Bliss function $y = \arcsin\sqrt{x}$. To determine the relation between the disinfection method the results were subjected to an agglomerative cluster analysis and classified into groups in a hierarchical order by means of the Ward's method. The statistical analyses were performed using the Statistica 12.5 software (StatSoft, Polska).

RESULTS AND DISCUSSION

Adequately disinfection of components is one of most important ways of controlling contamination in *in vitro* culture (Amiri et al. 2013). Different explants require different types of compounds, concentrations and exposure periods for the disinfection process to be optimized (Teixeira da Silva et al. 2016). NaOCl is considered a very effective microbe killer and has been frequently used for surface sterilization of plants for *in vitro* culture (Bakhsh et al. 2016). While mercuric chloride ($HgCl_2$) is extremely toxic to plants and humans and must be carefully disposed (Talei et al. 2011). Ozone is reported to have 1.5 times the oxidizing potential of chlorine and 3000 times the potential of hypochlorus acid (HClO) – Suslow (1998).

In the current study the effectiveness of seeds sterilization depend on the disinfection solution used and its concentration. It was observed that goji seeds submerged in 15% NaOCl solution indicated the lowest number of contamination as well as germination ability (Table 1). However, when 7% solution of NaOCl was used for disinfection, the percentage of germinated goji 'A' and 'New Big' seeds was the highest (62% and 78%, respectively) (Table 2). As compared to NaOCl disinfection of seeds with 0.2% $HgCl_2$ was less effective mainly to 'New Big'. The percent of infected goji seeds of 'A' and 'New Big' was higher (17% and 10%, respectively) (Table 1). Furthermore, $HgCl_2$ solution was more toxic and inhibited the germination of goji seeds, which was 37% for cultivar 'A' and 30% for 'New Big' cultivars (Table 2). It was observed that O_3 in water and O_3 gas affected seeds ability to germination (Table 3). The highest number (87%) of uncontaminated explants that did not developed shoots was noticed after disinfection of goji 'New Big' explants using O_3 in water for 5 minutes and O_3 gas for 15 minutes. Comparing ozonation method for disinfection of goji seeds, it was observed that the best results were obtained when O_3 in water for 15 minutes was used when the percentage of germination was the highest ('A' – 47% and 'New Big' – 53%) (Table 2), and the efficiency of sterilization of goji seeds in 'A' cultivar was 100% (Table1).

Table 1. The percentage of contaminated goji ‘A’ and ‘New Big’ explants according to disinfection method used
Tabela 1. Procent zakażonych eksplantatów goji odmian ‘A’ i ‘New Big’ w zależności od zastosowanego środka dezynfekującego

Disinfection method Metody dezynfekcji		Cultivar – Odmiana		
		‘A’	‘New Big’	Mean Średnia
Ozone gas Ozonowanie na sucho	5 min	7 b	40 d	23.5 c
	15 min	7 b	0 a	3.5 a
Ozon in water Ozonowanie w wodzie	5 min	20 c	0 a	10 ab
	15 min	0 a	7 b	3.5 a
NaOCl	7%	8 b	14 c	11 b
	10%	7 b	3 a	5 a
	15%	4 ab	2 a	3 a
0.2% $HgCl_2$		17 c	10 b	13.5 b

Means in the same column followed by the same letter are not significantly different at $\alpha < 0.05$ according to Tukey test – Średnie w kolumnach oznaczone tymi samymi literami alfabetu nie różnią się według testu Tukeya na poziomie istotności $\alpha < 0,05$.

Table 2. The percentage of uncontaminated goji ‘A’ and ‘New Big’ explants that germinated according to disinfection method used
Tabela 2. Procent niezakażonych eksplantatów goji odmian ‘A’ i ‘New Big’, które skiełkowały, w zależności od zastosowanego środka dezynfekującego

Disinfection method Metody dezynfekcji		Cultivar – Odmiana		
		‘A’	‘New Big’	Mean Średnia
Ozone gas Ozonowanie na sucho	5 min	33 b	47 c	40 cd
	15 min	40 bc	7 a	23.5 ab
Ozon in water Ozonowanie w wodzie	5 min	7 a	13 a	10 a
	15 min	47 c	53 c	50 d
NaOCl	7%	62 d	78 d	70 e
	10%	47 c	47 c	47 d
	15%	38 b	36 b	37 c
0.2% $HgCl_2$		37 b	30 b	33.5 bc

Explanations see Table 1 – Objaśnienia zob. tab. 1.

Table 3. The percentage of uncontaminated goji ‘A’ and ‘New Big’ explants that did not germinated according to disinfection method used
Tabela 3. Procent niezakażonych eksplantatów goji odmian ‘A’ i ‘New Big’, które nie skiełkowały, w zależności od zastosowanego środka dezynfekującego

Disinfection method Metody dezynfekcji		Cultivar – Odmiana		
		‘A’	‘New Big’	Mean Średnia
Ozone gas Ozonowanie na sucho	5 min	53 b	0 a	26.5 a
	15 min	53 b	87 e	70 e
Ozon in water Ozonowanie w wodzie	5 min	67 d	87 e	77 e
	15 min	53 bc	33 cd	43 cd
NaOCl	7%	30 a	44 d	37 bc
	10%	47 b	17 b	32 b
	15%	60 cd	44 d	52 d
0.2% $HgCl_2$		47 b	30 c	38.5 bc

Explanations see Table 1 – Objaśnienia zob. tab. 1.

According to many authors (Teixeira et al. 2006; Tiwari et al. 2012; Amiri et al. 2013; Teixeira da Silva et al. 2016) disinfection solution of sodium hypochlorite and mercury chloride played an important role in the contamination control. Hu et al. (2001) applied 0.1% $HgCl_2$ for 6 minutes followed by three washes with sterile water for disinfection of *L. barbarum*. Then seeds were imbibed in sterile water for 6 h at room temperature and next were sterilized a second time for 8 minutes in 0.1% $HgCl_2$. While, seeds of *L. barbarum* cultivar 'Ningji No.1' were surface-sterilize in 70% ethanol (30–40 s) and after that they were submerged in 0.1% $HgCl_2$ for 8–10 minutes, followed by 5 rinses with sterile distilled water (Hu et al. 2008). Another disinfection method of goji seeds applied Dănăilă-Guidea et al. (2015) using commercial bleach (ACE) containing 4.85% sodium hypochlorite for 10 minutes and a dilute bleach product concentration trading 4.50% sodium hypochlorite for 20 minutes, followed by three washes with sterile distilled water. Similarly, Osman et al. (2013) for disinfection of goji seeds, obtained from dried fruits, applied sodium hypochlorite (no report of concentration) with two drops of Tween 20, followed by subsequent three time rinsing procedures with sterile distilled water. However, these work do not describe the effectiveness of disinfection method on the germination rate of goji seeds.

Alternative techniques have been appreciated because all of these disinfectants may be toxic for plant tissue and environment. One of an alternative method might be disinfection with ozone. However, there are a low number of reports about the effect of ozone disinfection in *in vitro* culture. Much more likely is described ozone to be used in food processing and for storage of vegetables and fruits. Tiwari et al. (2010) demonstrated that ozone which is natural agent, may offer unique advantage for grain processing along with addressing growing concerns over the use of harmful pesticides. Nowakowicz-Dębek et al. (2013) treated wheat grains with ozone produced by an ozone generator of 100 mg · h^{-1} capacity for 0, 0.5, 1, 3, 6 and 9 h. They observed that the exposure to ozone over longer period of time caused higher mold fungal reducibility. Krupa-Małkiewicz et al. (in press) compare the effectiveness of 7% and 10% NaOCl with ozone gas and ozone in water for disinfection of different seeds in *in vitro* culture. These have fund that ozone treatment of seeds gives good results, as in the case of using 7% NaOCl.

In the present study, goji seed contaminations under the different sterilization procedures varied from 0 to 40%. Analysis of the percentage of germinated seeds and their infections for both goji cultivars, carried out according to Ward's method, divided into four groups according to the similarity of their actions: A – 7% and 10% NaOCl, B – 0.2% $HgCl_2$, C – 15% NaOCl, 5 minutes O_3 gas and 15 minutes O_3 in water, D – 5 minutes O_3 in water and 15 minutes O_3 gas (Fig. 1). The results obtained in this study, demonstrated that the seeds cultured after O_3 treatment are characterized by a similar effectiveness of disinfection and germination as when applying 15% NaOCl. Therefore, ozone gas makes an alternative for commonly used disinfectants. Ozone rapidly attacks bacterial cell walls and is more effective against the thick-walled spores of plant pathogens than chlorine, at practical and safe concentrations (Suslow 1998).

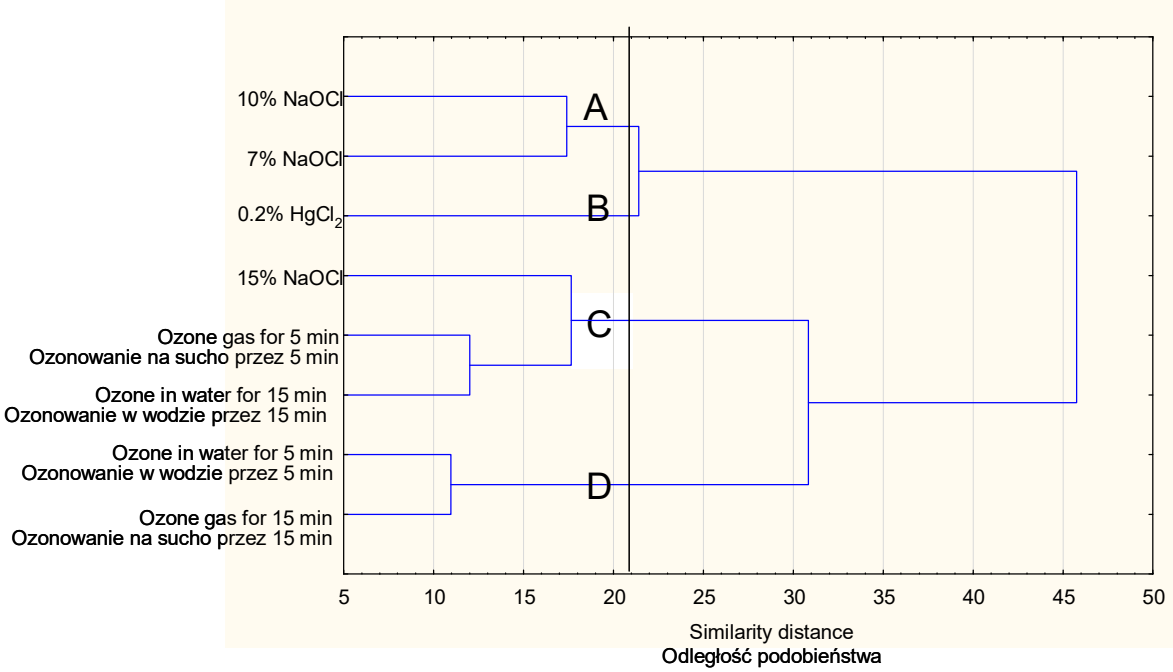

Fig. 1. Similarity distance between disinfection method use
Ryc. 1. Odległość podobieństwa pomiędzy zastosowanymi metodami dezynfekcji

CONCLUSIONS

The effectiveness of disinfection of explants in *in vitro* culture depended on many factors. One of them is properly chosen disinfection method. Ozone gas (O_3) makes an alternative for commonly used disinfectants. The efficacy of ozone was similar to sodium hypochlorite which it is less toxic to the plants and the environment. However, it requires further researches to evaluate the optimum conditions for disinfections of explants in *in vitro* culture. Therefore, use of ozone gas as a natural disinfectant is considered a very effective microbe killer and is used frequently for surface disinfection.

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Abstract. Using disinfectant components is one of main ways to control of fungal and bacterial contaminations in the *in vit*ro culture. Alternative to commonly used chemicals is ozonation. The present study was focused on the efficiency of seeds disinfection of two goji cultivars 'A' and 'New Big' using eight different treatments, 7%, 10% and 15% NaOCl and 0.2% $HgCl_2$ for 15 minutes each, O_3 gas and O_3 in water for 5 and 15 minutes. The highest percentage of germinated seeds of 'A' and 'New Big' (respectively 62% and 78%) with the lowest number of infections (respectively 8% and 14%) was obtained after using 7% NaOCl solution. Less effective was 0.2% $HgCl_2$ solution, where the percentage of germinated seeds of 'A' and 'New Big' was lower (respectively 37% and 30%). However, after O_3 in water treatment of goji 'A' and 'New Big' for 15 minutes the percentage of germinated seeds was the highest (respectively 47%, and 53%) and sterility of the cultures of goji 'A' was 100%. In addition to, the ozonation of the seeds showed similar disinfection efficiency, as with 15% NaOCl solution. Therefore, ozone gas makes an alternative for commonly used disinfectants.