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# *IN VITRO* STERILIZATION PROCEDURES FOR MICROPROPAGATION OF 'OBLAČINSKA' SOUR CHERRY

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**Abstract:** Surface sterilization is the most important step in preparation of explants for micropropagation, because controlling fungal and bacterial contamination of woody plant from field sources is very difficult. Six sterilizing agents: sodium hypochlorite (NaOCl), calcium hypochlorite [$Ca(ClO)_2$], sodium dichloroisocyanurate (DICA), mercuric (II) chloride ($HgCl_2$), silver nitrate ($AgNO_3$) and hydrogen peroxide ($H_2O_2$) were tested for sterilization of 'Oblačinska' sour cherry buds, by varying their concentration and time of exposure. The aim of this study was to establish best surface sterilization for *in vitro* propagation of 'Oblačinska' sour cherry. Aseptic cultures of 'Oblačinska' sour cherry were established from axillary buds which were placed in nutrient medium, supplemented with plants hormones 6-benzylaminopurine (BA), 1-naphthaleneacetic acid (NAA) and gibberellic acid ($GA_3$). The results indicated that among these sterilizing agents silver nitrate ($AgNO_3$) at concentration of 1% for 20 minutes was the best for controlling the infection, whereas sterilization with sodium dichloroisocyanurate (DICA) at concentration of 1% for 10 minutes was not satisfactory.

**Key words:** explant, *in vitro* plant, contamination, 'Oblačinska' sour cherry, sterilization procedure.

## Introduction

'Oblačinska' sour cherry is an autochthonous and heterogeneous Serbian cultivar that was named after the small village Oblačina in south Serbia. The existing population was developed by the use of various types of propagation, both by suckers and by seeds. Also, it has been noted that intracultivar variability is caused by natural mutagenic factors (Mišić, 1989). 'Oblačinska' sour cherry represents a mixture of a great number of clones (genotypes) so problems with its

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reproduction and exploitation occur. Because of these reasons, considering breeding methods, special attention should be devoted to the clonal selection (Nikolić et al., 2005).

'Oblačinska' sour cherry is a leading cherry cultivar for the processing industry in Croatia because of its pomological characteristics, suitability for mechanical harvesting, precocity and good fertility (Jurković et al., 2008). Since fruits of 'Oblačinska' are significant source of antioxidants including anthocyannins and polyphenol compounds, involved in antioxidative defence against biotic and abiotic stressor, it has a positive influence on human health and it is very popular as a fruit crop (Viljevac et al., 2012).

Plant tissue culture (micropropagation) is a tool which allows the rapid production of many genetically identical plants using relatively small amounts of space, supplies and time (Odutayo et al., 2004). A method of micropropagation has been developed for the purpose of rapid multiplication. Conventional propagation of fruit trees, compared with micropropagation is very slow and expensive, so micropropagation is a good method for producing virus free plant material on its own roots in the short time. *In vitro* propagation consists of various stages: selection of explants, aseptic culture establishment, multiplication, rooting and acclimatization of plants. The most important step for aseptic culture establishment is sterilization of explants. Successful tissue culture of all plant species depends on the removal of exogenous and endogenous contaminating microorganisms (Constantine, 1986; Buckley and Reed, 1994). To eliminate contamination during *in vitro* propagation different methods have been developed (Barrett and Casselles, 1994; Husain et al., 1994; Herman, 1996). *In vitro* contamination by fungi, bacteria and yeast is one of the most serious problems of commercial and research plant tissue laboratories. Contaminated plants can reduce multiplication and rooting rates or may die. It is necessary to remove foreign contaminants including bacteria and fungi from explants and it is very difficult to obtain sterile plant material completely free from contamination. It becomes more problematic while dealing with woody plant material (Niedz and Bausher, 2002). The surfaces of living plant materials are naturally contaminated with microorganisms from the environment, so surface sterilization of explants in chemical solutions is a critical preparation step. The disinfectants usually used are sodium hypochlorite, calcium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide and silver nitrate. Most laboratories use sodium or calcium hypochlorite or various commercial bleaches for surface sterilization of explants. Since these sterilizing agents are toxic to the plant tissue, contamination must be removed without killing the plant cells. The presence of microbes in these plant cultures usually results in increased culture mortality. Different infections can influence variable growth, tissue necrosis, reduced shoot proliferation and rooting. Although the tissue culture techniques usually involve growing stock plants in ways that will minimize infection, treating the plant

material with disinfecting chemicals and sterilizing the tools used for dissection such as the vessels and media in which cultures are grown will kill superficial microbes (George, 1993). In control of contaminations in plant tissue cultures there are three main issues: preventing the introduction of microorganisms with the initial plant material, preventing their introduction from the environment during subculturing and reducing microbial contamination in the cultures at the stage of multiplication and rooting. The most effective way of preventing bacterial contamination *in vitro* is elimination of bacteria from the initial plant explants that are introduced into the culture. The methods for reducing contaminations include the use of explant of donor plants under a strict sanitary regime, efficient sterilization of the initial explants, and reduction of the size of the initial explants just to apical meristem.

The procedure of sterilization is various, depending on plant species and part (explant) taken from the plant for sterilization. Each plant material has variable surface contaminant levels, depending on the growth environment, age and part of the plant used for micropropagation.

It is difficult to determine standard sterilization procedures that apply to all plants. Therefore, the present study was aimed at standardizing the sterilization method for explants of 'Oblačinska' sour cherry for micropropagation, using different types of sterilizing agents by varying their concentration and duration of exposure.

## Material and Methods

The experiment was conducted at plant tissue culture laboratory, Agricultural Institute Osijek, Department for fruit growing, during December of 2010 and January of 2011. The shoots were taken from the orchard of Agricultural institute Osijek, from 'Oblačinska' sour cherry, clone OS. Before sterilization, shoots were washed and kept in laboratory and rinsed in water for few days. Shoots were cut into pieces containing axillary winter buds (1–2 cm). First, buds were sterilized with 70% ethanol for few seconds and after that were sterilized with sodium hypochlorite (NaOCl), calcium hypochlorite ($Ca(ClO)_2$), sodium dichloroisocyanurate (DICA), mercuric (II) chloride ($HgCl_2$), silver nitrate ($AgNO_3$) and hydrogen peroxide ($H_2O_2$) in duration from 2 to 30 minutes (Table 1).

For better sterilizing agent – explant contact, they were stirred with few drops of Tween 20 detergent while disinfecting. After the decontamination treatments, all explants were rinsed three times with sterilized water. Meristems with two or three primordial leaves were isolated and inoculated in culture medium containing the MS macro nutrients (Murashige and Skoog, 1962), SH micro nutrients (Schenk and Hildenbrandt, 1972), sucrose (30 g/l), MS vitamins, agar (6 g/l) and appropriate plant hormones such as: 6-benzylaminopurine (BA) (0.5 mg/l), 1-naphthaleneacetic

acid (NAA) (0.01 mg/l) and gibberellic acid ($GA_3$) (0.5 mg/l). The pH of the media was adjusted to 5.8 before autoclaving the media at 121°C and 1.5 atm for 20 min.

Table 1. Type of sterilizing agents used in a different concentration with varying time of sterilizing axillary buds.

| Treatment | Sterilizing agent | Concentrations %(w/v) | Time of exposure (min) |
|---|---|---|---|
| T1 | Sodium hypochlorite (NaOCl) | 1 | 20 |
| T2 | Sodium hypochlorite (NaOCl) | 2 | 15 |
| T3 | Sodium hypochlorite (NaOCl) | 3 | 10 |
| T4 | Calcium hypochlorite ($Ca(ClO)_2$) | 1 | 30 |
| T5 | Calcium hypochlorite ($Ca(ClO)_2$) | 3 | 15 |
| T6 | Calcium hypochlorite ($Ca(ClO)_2$) | 5 | 5 |
| T7 | Sodium dichloroisocyanurate (DICA) | 2 | 20 |
| T8 | Sodium dichloroisocyanurate (DICA) | 1.5 | 15 |
| T9 | Sodium dichloroisocyanurate (DICA) | 1 | 10 |
| T10 | Mercuric (II) chloride ($HgCl_2$) | 0.1 | 10 |
| T11 | Mercuric (II) chloride ($HgCl_2$) | 0.5 | 5 |
| T12 | Mercuric (II) chloride ($HgCl_2$) | 1 | 2 |
| T13 | Silver nitrate ($AgNO_3$) | 1 | 20 |
| T14 | Silver nitrate ($AgNO_3$) | 1 | 10 |
| T15 | Silver nitrate ($AgNO_3$) | 1 | 5 |
| T16 | Hydrogen peroxide ($H_2O_2$) | 10 | 15 |
| T17 | Hydrogen peroxide ($H_2O_2$) | 20 | 10 |
| T18 | Hydrogen peroxide ($H_2O_2$) | 30 | 5 |

The cultures were kept in a growth chamber for one month, at 25°C, with 16 hours photoperiod and 3500 lux of light intensity. After one month the percentage of contaminated, survived and dead buds was noted. Ten explants were used in each sterilization treatment, and each treatment was done in three replications. Data were analyzed by analysis of variance (ANOVA). The mean values were compared using the least significant difference (LSD) test. The differences between treatments were considered significant at $p \leq 0.05$ and designated by different letters. All statistical analyses were done with Statistica 7.1. software (StatSoft, Inc., USA).

## Results and Discussion

The results showed that among the disinfecting treatments for tissue culture explants, T13 with silver nitrate ($AgNO_3$) at concentration of 1% for 20 minutes was the best (Figure 1E). T13 treatment gave the 96.67% healthy explants (Figure 2) with 3.3% contaminated explants (Figure 3). Treatments with sodium hypochlorite (NaOCl) (Figure 1A), calcium hypochlorite [$Ca(ClO)_2$] (Figure 1B)

and mercuric (II) chloride ($HgCl_2$) (Figure 1D) also showed satisfactory results, while hydrogen peroxide ($H_2O_2$) (Figure 1F) and treatments with sodium dichloroisocyanurate (DICA) (Figure 1C) gave bad results.

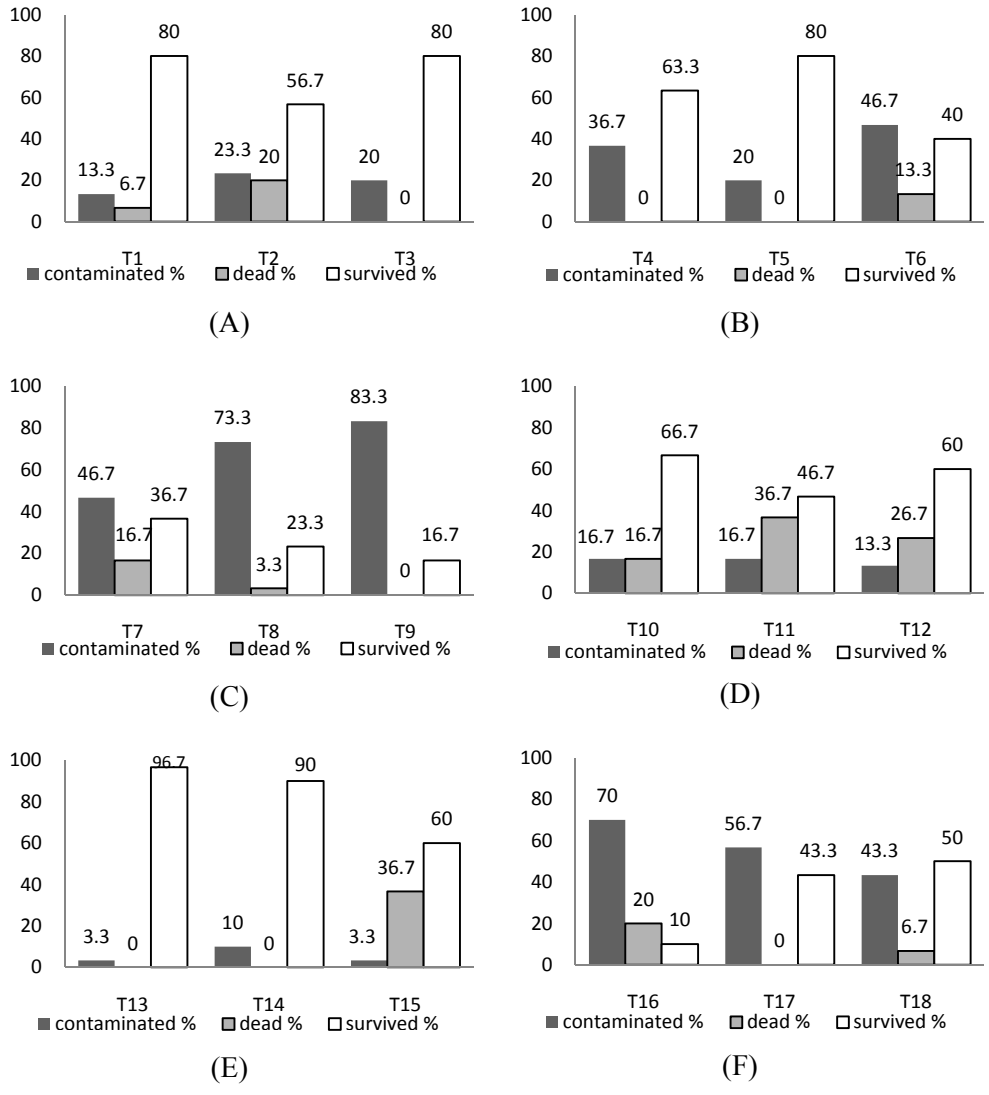


Figure 1. Influence of different sterilizing agents on 'Oblačinska' sour cherry explant sterilization: NaOCl (A), $Ca(ClO)_2$ (B), DICA (C), $HgCl_2$ (D), $AgNO_3$ (E) and $H_2O_2$ (F).

The less efficient sterilization procedure was with treatment T9 (DICA - sodium dichloroisocyanurate), with 83.3% contaminated explants and 16.7% survived explants. Analysis of variance showed significant difference between treatments at the 0.05 probability level. Data indicated that there was a significant difference in contamination percentage among the different treatments but there was no significant difference among dead explants. When comparing silver nitrate and sodium hypochlorite, silver nitrate showed to be better sterilizing agent which is not in accordance with Campbell and Tomes (1984). They showed that both sterilizing agents were effective in reducing contamination of red clover *Trifolium pratense* L. where sodium hypochlorite showed slightly better results. In our experiments, treatments with calcium hypochlorite [$Ca(ClO)_2$] were satisfactory for sterilization (Figure 2). Assareh and Sardabi (2005) reported that among treatments with $Ca(ClO)_2$, NaOCl and $HgCl_2$ for tissue culture explants sterilization of *Ziziphus spina-christi* (L.)Desf., $Ca(ClO)_2$ in concentration of 5% and duration of 20 minutes was the most efficient. Comparing the effect of $HgCl_2$ and NaOCl in our experiment, NaOCl was found to be better, with more survived explants (Figure 3). These results are in accordance with the experiments of Badoni and Chauhan (2010) where NaOCl was found to be better for controlling the infection of potato cv. 'Kufri Himalini'. Altaf (2006) reported that both sterilizing agents, $HgCl_2$ and NaOCl, were effective in making clean explants of Kinow tree.

The worst results in our experiment were achieved with sodium dichloroisocyanurate (DICA), with the biggest contamination of explants among all sterilizing agents used. Holobiuc et al. (2009) reported that DICA also was the least efficient sterilizing agent for *Dianthus nardiformis*, comparing it with $HgCl_2$, NaOCl and $H_2O_2$ On the contrary, Osterc et al. (2004) reported that reduction in contamination by using 16.6 g/L DICA for 15-min duration was very successful for vigorous cherry plants. Parkinson et al. (1996) reported that sodium dichloroisocyanurate was more effective than commercially available bleach for disinfection. The treatments with hydrogen peroxide ($H_2O_2$) showed unsatisfactory results, showing a high percent of contamination and a low percent of survived explants. The present findings are in agreement with the observation of Farooq et al. (2002) who showed that using sterilization with $H_2O_2$ resulted in 50% surface sterilization. When comparing three disinfectants by Tiefeng et al. (2005), $HgCl_2$ was better than NaClO and $H_2O_2$ for surface sterilization of *Pinellia ternata* (Thunb.) Breit.

Some explants in our experiment did not survive because of damages during sterilization procedure and isolation of meristem caused by human failure.

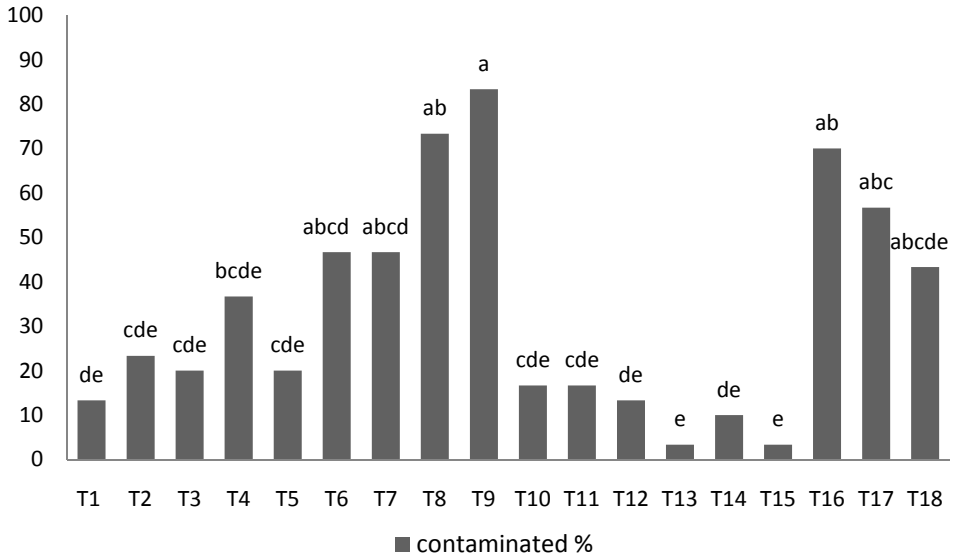


Figure 2. Percentage of contaminated buds after sterilization using various concentrations of sterilizing agents for different durations (differences between treatments were considered significant at $p \leq 0.05$ and designated by different letters).

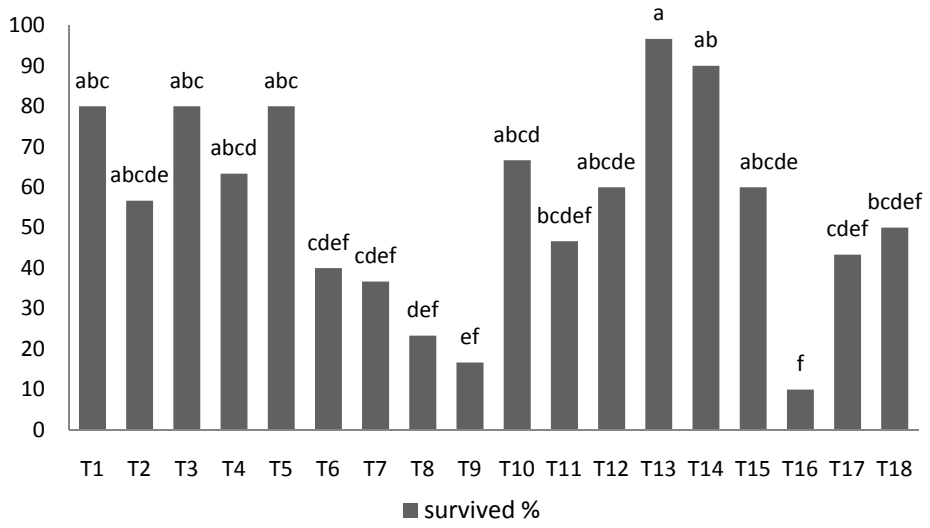


Figure 3. Percentage of survived buds after sterilization using various concentrations of sterilizing agents for different durations (differences between treatments were considered significant at $p \leq 0.05$ and designated by different letters).

## Conclusion

The most frequently used sterilization procedures for micropropagation are conducted with 70% ethanol and 1–3% NaOCl. Our results showed that during the sterilization procedure some other chemicals like $AgNO_3$, $Ca(ClO)_2$ and $HgCl_2$ showed also good results for the surface sterilization of 'Oblačinska' sour cherry buds. This can be explained by the fact that requirements for sterilization are different and depend on the tissue type and the nature of the explant used for micropropagation.

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# *IN VITRO* STERILIZACIJSKA PROCEDURA ZA MIKROPROPAGACIJU OBLAČINSKE VIŠNJE

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R e z i m e

Cilj ovog istraživanja bio je da se utvrdi najbolji postupak površinske sterilizacije pupoljaka za *in vitro* propagaciju Oblačinske višnje. Površinska sterilizacija je najvažniji korak u pripremi eksplantata za mikropropagaciju, budući da je za uspostavljanje aseptične kulture kod drvenastih kultura iz polja jako teško kontrolisati gljivičnu i bakterijsku kontaminaciju. Šest sredstava za sterilizaciju: natrijum hipohlorit (NaOCl), kalcijum hipohlorit [$Ca(ClO)_2$], natrijum dihlorizocijanurat (DICA), živa (II) hlorid ($HgCl_2$), srebro nitrat ($AgNO_3$) i vodonik peroksid ($H_2O_2$) testirani su za površinsku sterilizaciju pupoljaka Oblačinske višnje u različitim koncentracijama i različitom vremenu tretiranja. Aseptična kultura je uspostavljena izolacijom meristema iz aksilarnih pupoljaka koji su smešteni na hranjivu podlogu sa biljnim hormonima: benziladenin (BA), naftil sirćetna kiselina (NAA) i giberelinska kiselina ($GA_3$). Rezultati su pokazali da je od svih sredstava za sterilizaciju $AgNO_3$ u koncentraciji od 1% i trajanju sterilizacije 20 minuta pokazao najbolje rezultate u sprečavanju infekcije, dok sterilizacija sa DICA u koncentraciji od 1% i trajanju od 10 minuta nije bila zadovoljavajuća.

**Ključne reči**: eksplantat, *in vitro* biljka, kontaminacija, Oblačinska višnja, sterilizacijska procedura.

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