RESEARCH ARTICLE

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Silver nitrate and chlorhexidine gluconate – effective surface sterilization agents in disinfection procedures at the initiation of woody shoot tip and embryo culture

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ABSTRACT
The surface sterilization of explants in chemical solutions is an important step to remove contaminants with minimal damage to plant cells. Woody and mature plants growing in the open field are known to harbor a large amount of microflora and are very difficult to sterilize. The routinely used surface sterilization procedures with the solution of $Ca(OCl)_2$ had proven to be relatively unsuccessful with *Taxus baccata* and some other woody fruit and ornamental species. The objective of the present study was to propose and to verify new procedures of surface-sterilization of *Ginkgo* and plum shoot tip explants from old trees as well as sweet cherry embryos employing silver nitrate or chlorhexidine gluconate in different concentration and periods of exposure. According to the results obtained in the present experiments we could recommend silver nitrate and chlorhexidine gluconate as effective surface sterilization agents in disinfection procedures at the initiation of woody shoot tip and embryo culture.

Key words: plant tissue culture, micropropagation

Introduction

The surface sterilization of explants in chemical solutions is an important step to remove contaminants with minimal damage to plant cells. Woody and mature plants growing in the open field are known to harbor a large amount of microflora and surface sterilization of explants in chemical solutions is a critical step in the establishment of in vitro culture. For example, *Taxus* plants have been found to be associated in vivo with a large number of endophytic microorganisms, which includes more than 300 fungal and bacterial genera (Strobel et al., 2001) and this is the main reason why several reviews have highlighted the difficulties in *in vitro* establishment of the culture of Taxus species (Kulkarni, 2007).

Different methods have been developed to eliminate contamination during *in vitro* propagation (Herman, 1996). The disinfectants widely used in plant tissue culture are sodium hypochlorite, calcium hypochlorite [$Ca(OCl)_2$], ethanol, mercuric chloride, hydrogen peroxide, silver nitrate [$AgNO_3$]. Since these sterilizing agents are toxic to the plant tissue, contamination must be removed without killing the plant cells. It is not possible to determine standard sterilization procedures applicable to all plants. For the disinfection of tissues of woody plants, it is important to develop specific methods depending on plant species and the type of the explants. Silver and its compounds have long been used as antimicrobial agents. Silver ions possess different modes of action. They interact with a wide range of molecular processes within microorganisms resulting in a range of effects from inhibition of growth, loss of infectivity through cell death (Russell & Hugo, 1994; Herman 1996).

Chlorhexidine digluconate (CHG) possess broad-spectrum antimicrobial activity against many Gram-positive and Gram-negative bacteria, as well as *C. albicans* (Hope & Wilson, 2004) and used in the medicine as a disinfectant. But in plant tissue cultures the solution of CHG, is not used frequently. Some authors used Savlon antiseptic solution (Johnson & Johnson, USA, active ingredients: chlorhexidine gluconate and cetrimide) for pretreatment of cuttings or seeds before disinfection with other chemical agents (Thomas et al., 1999; Chand & Sing, 2004).

The objective of the present study was to propose and to verify new procedures of surface-sterilization of *Ginkgo* and plum shoot tip explants from old trees as well as sweet cherry embryos employing silver nitrate or chlorhexidine gluconate in different concentration and periods of exposure.

Materials and Methods

Plant material

The experiments were carried out with actively growing shoot explants from *Ginkgo biloba* L., plum rootstock

'Docera 6' (*Prunus domestica* x *Prunus cerasifera*) trees, as well as sweet cherry embryos (*Prunus avium* 'Rosalina').

Initial explants used in the experiment were obtained from the corresponding plant early in the morning and kept in distilled water-filled jars inside icebox to keep them vital. Explants were washed with liquid soap solution and rinsed with running water for one hour. After washing, different disinfection treatments were given. The routine surface sterilization procedure used in our laboratory - 5% solution of $Ca(OCl)_2$ for 5 minutes was employed as a control. After that solution was rinsed three times with sterilized distilled water, 10 minutes each, under aseptic conditions. The explants were then dissected on sterilized paper cutting pads and inoculated on the corresponding media.

***Ginkgo biloba* L.**

Ginkgo biloba L. shoot tip explants were collected in April from a single male 20-year-old tree located at the park of the Agricultural University, Plovdiv. The leaves were trimmed and then 1.5-2.5 cm long shoot apices were treated by immersion in disinfection solutions.

Variants of treatment:

GC - Control - 5% $Ca(OCl)_2$ for 7 min;

GV2 - 2% $AgNO_3$ for 5 min;

GV3 - 2%$AgNO_3$ for 3 min, a rinse with sterilized distilled water for 5 min and then exposed to 5 minutes immersion in 5% $Ca(OCl)_2$;

GV4 - 5% $Ca(OCl)_2$ for 3 min, a rinse with sterilized distilled water for 5 min and then exposed to 5 minutes immersion in 2%$AgNO_3$.

Plum rootstock 'Docera 6' (Prunus domestica x Prunus cerasifera 'Docera 6')

Explants were collected at the end of April from six-years-old tree. Shoot apices (1.5-2.5 cm long) were treated by immersion in disinfection solutions. Commercial disinfectant GLUCO CHeX (Cerkamed, Poland) containing 2% chlorhexidine gluconate (CHG) was used as an active ingredient.

Variants of treatment:

DC - Control - 5% $Ca(OCl)_2$ for 5 min;

DV2 - 1% chlorhexidine gluconate - 3 min;

DV3 - 1% chlorhexidine gluconate - 5 min;

DV4 - 2% chlorhexidine gluconate - 3 min;

DV5 - 2% chlorhexidine gluconate - 5 min;

DV6 - 2% $AgNO_3$ - 5 min;

DV7 - 5% $Ca(OCl)_2$ for 3 min, a rinse with sterilized distilled water for 10 min and then exposed to 3 minutes immersion in 2% chlorhexidine gluconate.

Sweet cherry embryos (Prunus avium 'Rosalina')

The fruits of sweet cherry were collected at the beginning of May and washed with tap water for 30 min and the fruit flesh was removed. Endocarps were cracked and the seeds were surface disinfected on laminar-box by immersion in CHG (commercial disinfectant GLUCO CHeX) for three exposure durations (1, 3 and 5 minutes). The routine surface sterilization procedure - 5% solution of $Ca(OCl)_2$ for 5 minutes was employed as a control. The seeds received three rinses with sterilized distilled water, 10 minutes each and remained imbibed in the final rinsing water until the embryos were excised and then were cultured on the corresponding nutrient medium.

All cultures were kept at 22±2°C under 16-h photoperiod (fluorescent tubes OSRAM 40 W, 40 µmol m^{-2} s^{-1} PPFD).

For each treatment of ginkgo and 'Docera', ten explants were used in each sterilization treatment, and each treatment was done in three replications. A single explant in each culture tube was inoculated. The treatment with sweet cherry embryos was performed with 50 embryos each (five replications with ten embryos in each treatment). The experiment repeated twice in the spring of 2014 and 2015 year. Data on contamination and explant performance were recorded on the 30th day.

Results and Discussion

Ginkgo biloba shoot tip explants

The lowest percentage of clean (non-contaminated) ginkgo shoot tip explants was observed in control treatment (GC, 37%) (Figure1).

Of the different treatments tested, in the variant with 2% $AgNO_3$ (GV2) for 5 minutes, maximum clean and viable

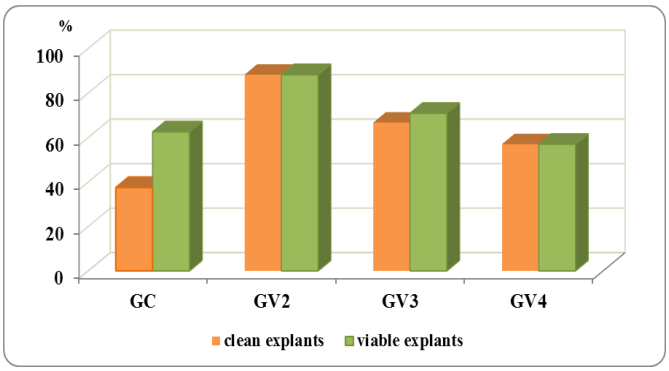

Figure 1. *Clean explants of Ginkgo biloba L. shoot tip culture (%) and viable explants (% of all explants) after different surface disinfection protocols. Treatments: GC (Control) - 5% $Ca(OCl)_2$ for 7 min; GV2 - 2% $AgNO_3$ for 5 min; GV3 - 2% $AgNO_3$ for 3 min, a rinse with sterilized distilled water for 5 min and then 5% $Ca(OCl)_2$ 5 minutes; GV4 - 5% $Ca(OCl)_2$ for 3 min, a rinse with sterilized distilled water for 5 min and then 5 minutes 2% $AgNO_3$.*

explants (88%) were recorded. The combination of the two disinfectants [$AgNO_3$ and $Ca(OCl)_2$] did not have the expected effect. We received a higher percentage of pure explants compared to the control but not sufficiently viable.

The commonly practiced surface sterilization procedure in our laboratory (a 5 min immersion in a 5% solution of $Ca(OCl)_2$ - control treatment in this study) had proven to be unsuccessful with *Taxus* too. Our earlier studies showed that pretreatment with 1.0% $AgNO_3$ for 3min and 2.5% $Ca(OCl)_2$ for 5 minutes led to high viability of *Taxus* apical shoot explants (65%) (Ibrahim et. al, 2011). This combination, however, proved to be unsuitable for ginkgo shoot tip explants.

Plum rootstock 'Docera 6' (Prunus domestica x Prunus cerasifera)

There was a significant effect of treatment with 5% solution of $Ca(OCl)_2$ – control treatment, to the percentage of non-contaminated (clear) and viable shoot tip explants – 90% (Figure 2).

The treatment with CHG at the tested concentrations provided at least 62% non-contaminated shoots and was suitable for the disinfection procedure with plum shoots. The disinfection effect depended on the concentration of CHG and the time of exposure. The less efficient sterilization procedure was the treatment with the lower concentration of CHG (1%) with 62-72% clean explants. Data indicated that there was a significant difference in the percentage of the dead explants between 3 and 5 minutes treatments (variants DV2 and DV3).

Treatments with 2% CHG (DV4 and DV5) provided at least 92 % of non-contaminated explants, but longer exposure (5 min, variant DV5) resulted in dramatically decrease of viable explants from 90% for 3 min (DV4) to 40% for 5 minutes treatment (DV5). The worst result in our experiment with plum shoot explants was achieved with 2% $AgNO_3$ for 5 min (DV6) with the highest percentage of contaminated explants (48%). These results were different from our results with *Ginkgo* explants where the treatment with silver nitrate (2%) was the best (Figure 1). The presented results with 'Docera' also not in accordance with Mihaljevic et al. (2013) where silver nitrate (1% for 20 min treatment) was found to be better for controlling the infection of sour cherry explants.

When combining $Ca(OCl)_2$ and CHG (variants DV6 and DV7), again we had a low percentage of non-infected plants and also a lower percentage of vital explants (60-72%) than the control and the other variants, except for variant DV5. Having in mind low percent of contaminated explants and high viability of 'Docera 6' shoot tip explants we could recommend disinfection procedure with 2% chlorhexidine gluconate (CHG) for 3 minutes.

However, the treatments of sweet cherry embryos 'Rosalina' with 2% CHG in our experiments did not show satisfactory results (Figure 3). In these variants, we received a higher percentage of infected embryos and a lower percentage of surviving embryos compared to the control treatment with 5% $Ca(OCl)_2$ for 5 min.

The present findings are in agreement with the observation of Assareh & Sardabi (2005) who reported that $Ca(OCl)_2$ in concentration 5% was the most efficient for disinfection of *Ziziphus* explants. They applied the same concentration $Ca(OCl)_2$ – 5%, but for a longer duration of 20 minutes. The sterilization of explants is the most important step for aseptic culture establishment. *In vitro* contamination

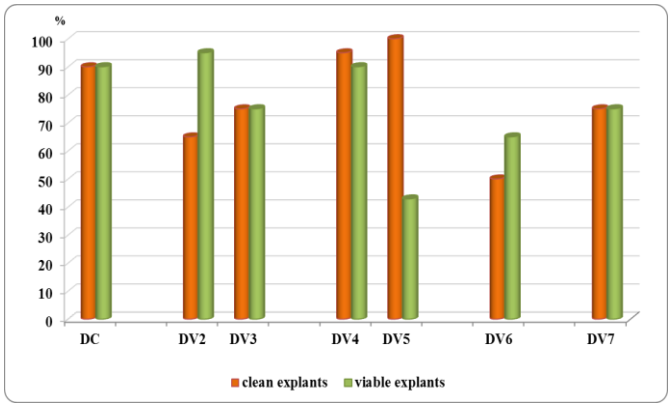

Figure 2. *Effect of different protocols of surface disinfection on the percentage of clean explants of 'Docera 6' shoot tip culture (%) and viable explants (% of all explants). Treatments: DC – (Control)- 5% $Ca(OCl)_2$ for 5 min; DV2 and DV3 - 1% chlorhexidine gluconate (CHG) for 3 min or 5 minutes respectively; DV4 and DV5- 2% CHG for 3 or 5 minutes respectively; DV6 - 2% $AgNO_3$ for 5 min; DV7 - 5% $Ca(OCl)_2$ for 3 min, a rinse with sterilized distilled water for 10 min and then exposed to 3 minutes immersion in 2% CHG.*

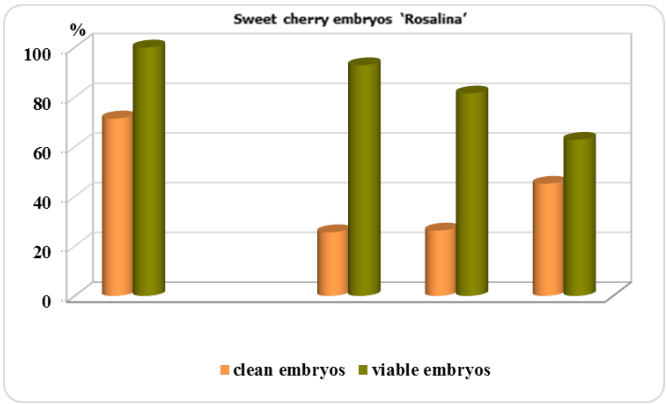

Figure 3. *Effect of different surface disinfection protocols with 2% chlorhexidine gluconate (CHG) on the percentage of non-contamined (clean) sweet cherry embryos 'Rosalina' (%) and viable embryos (% of total number). Control - 5% $Ca(OCl)_2$ for 5 minutes.*

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. There is no universal sterilization procedure that applies to all explants of all species. The disinfection procedures are various, depending on plant species, the growth environment, age and part of the plant (explant) used for establishment of plant tissue culture. The most frequently used sterilization procedures for micropropagation are conducted with sodium hypochlorite (NaOCl) in concentrations 1-3%. But our results showed that other chemical agents like silver nitrate [$AgNO_3$], calcium hypochlorite [$Ca(OCl)_2$] and chlorhexidine gluconate (CHG) are more effective for disinfection, especially for the disinfection of tissues of woody plants and it is important to develop specific methods depending on plant species and the type of the explants

Conclusion

According to the results obtained in the present experiment we could summarize that silver nitrate ($AgNO_3$) and chlorhexidine gluconate (CHG) are highly effective in surface disinfection of woody plants at the initiation of *in vitro* cultures.

Of the different treatments tested, the variant with 2% $AgNO_3$ (GV2) for 5 minutes was found to be optimum for the disinfection of shoot tip explants of *Ginkgo biloba* L.

Having in mind low percent of contaminated explants and high viability of 'Docera 6' shoot tip explants we could recommend disinfection procedure with 2% chlorhexidine gluconate (CHG) for 3 minutes. The routine surface sterilization procedure used in our laboratory – with 5% calcium hypochlorite [$Ca(OCl)_2$] for 5 minutes (employed as a control) was the best for surface disinfection of sweet cherry embryos 'Rozalina'.

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