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DIFFERENT STERILIZATION METHODS FOR OVERCOMING INTERNAL BACTERIAL INFECTION IN SUNFLOWER SEEDS

ABSTRACT: During culture of protoplasts in agarose droplets, permanent problem was bacterial infection. It was assumed that the seeds are the origin of infection, so different sterilization methods were tested in order to overcome this problem. Germination, infection of seeds and hypocotyls and their growth were examined. Based on these parameters, the best result was obtained with the combined use of 5% commercial bleach and dry heating at 45°C.

KEY WORDS: bacterial infection, seeds, sterilization, sunflower, tissue culture

INTRODUCTION

A critical stage in the introduction of plants to tissue culture is to obtain cultures free of microbial contamination. In spite of the surface sterilization process carried out for explants before culture, microbial growth inside the plant cannot be eliminated (Hennerty at al., 1988), particularly when explants are excised from field grown plants (Savela and Uosukainen, 1994) and transferred to *in vitro* culture.

Contaminants in the xylem vessel, which are protected from surface sterilization are endophytic bacteria (Hallman et al., 1997). Endophytic bacteria have probably evolved a close relationship with their host plant through co--evolutionary processes and may influence plant physiology in ways that have not yet been elucidated (Misaghi and Donndelinger, 1990). Inside the plant they have very little microbial competition (Misaghi and Donndelinger, 1990) and usually do not cause visible symptoms to the plant (Hallman et al., 1997; Peñalver et al., 1994). The bacteria may stay latent or symptomless (Peñalver et al., 1994) up to several months after

the initiation of culture and may not survive outside the plant tissue (Reed et al., 1995). Endophytic bacteria may even promote beneficial effects for field grown crops, but in stress conditions such as *in vitro* culture, latent endophytic bacteria may become pathogenic and detrimental to the growth and development of the plantlets (Leifert et al., 1989).

During culture of protoplasts in agarose droplets, permanent problem was bacterial infection. It was assumed that seeds are the origin of infection, so different sterilization methods were tested in order to overcome this problem.

MATERIAL AND METHODS

Plant material

Seeds of inbred line PH-BC_2-91A and Ha-74A of cultivated sunflower were obtained from Institute of Field and Vegetable Crops.

Sterilization methods

Different sterilization methods were tested:

1. soaking seeds in 70% ethanol for one minute followed by soaking in 14% commercial bleach for 20 minutes; rinsed tree times in distilled water; removing the seed coats; soaking seeds in 70% ethanol for one minute followed by soaking in 14% commercial bleach for 15 minutes; rinsed tree times in sterile distilled water
2. soaking seeds in 14% commercial bleach for 20 minutes; rinsed tree times in distilled water; removing the seed coats; soaking seeds in 5% commercial bleach for 60 minute; rinsed tree times in sterile distilled water
3. soaking seeds in 14% commercial bleach for 20 minutes; rinsed tree times in distilled water; removing the seed coats; soaking seeds in 14% commercial bleach for 15 minutes; rinsed tree times in sterile distilled water; heat sterilization at 45°C during 60 minutes
4. soaking seeds in 14% commercial bleach for 20 minutes; rinsed tree times in distilled water; removing the seed coats; soaking seeds in 5% commercial bleach for 60 minutes; rinsed tree times in sterile distilled water; heat sterilization at 45°C during 60 minutes
5. soaking seeds in 14% commercial bleach for 20 minutes; rinsed tree times in distilled water; removing the seed coats; soaking seeds in 5% commercial bleach for 60 minutes; rinsed tree times in sterile distilled water; heat sterilization in water bath at 45°C during 60 minutes.

The experiments were set in 6 repetitions with 6 seeds. The seeds were germinated for 2 days in the dark at 25°C. Germination of seeds and infection were followed.

Germinated seeds without infection were placed on a MS medium (Murashige and Skoog, 1962) and cultured in the dark at 25°C. After 7 days of culture infection of hypocotyls and their growth were examined.

All results were expressed as mean ± standard error (SE). Statistical analysis was performed by the analysis of variance (ANOVA), and posthoc comparisons between means were made by Duncan's multiple range test. Statistical significance was defined as being at the level $p < 0.05$.

RESULTS AND DISCUSION

During culture of protoplasts in agarose droplets, a permanent problem was internal bacterial infection, different methods were tested in order to overcome this problem. Other authors also report problems with internal bacterial infection in plant tissue culture (Hennerty at al., 1988; Misaghi and Donndelinger, 1990).

Besides sterilization of seeds with chemicals, the surface sterilization can be performed by exposure of seeds to UV light or heat. Since UV irradiation can damage DNA, seeds were sterilized according to 5 different protocols with commercial bleach and dry and moist heating. *Percent age* of germinated seeds (Fig. 1) and *percent age* of seed infection (Fig. 2) were followed, as well as growth (Fig. 3) and infection of hypocotyls (Fig. 4).

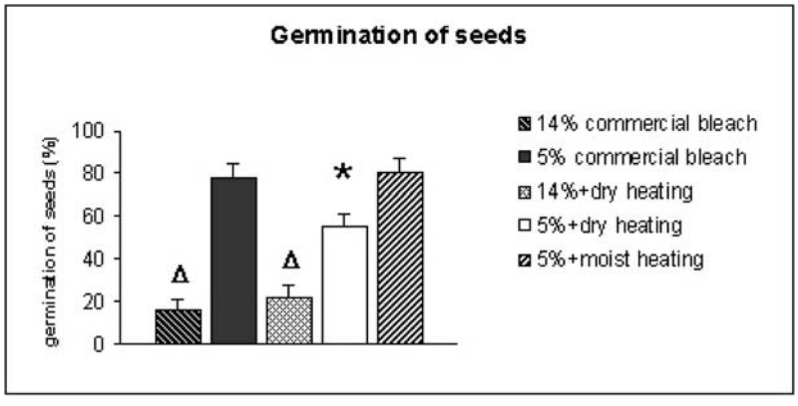

Figure 1. Germination of the seeds. Significance: *$p < 0.05$ vs. protocol 2, 4 and 5; $^{\Delta}p < 0.05$ vs. protocol 2 and 5

Based on the obtained data, germination of sunflower seeds was significantly lower after sterilization by 14% commercial bleach (Fig. 5). Significantly lower germination of seeds was also found after sterilization by combination of 5% commercial bleach and dry heating, when compared to the seeds sterilized by 5% commercial bleach and combination of 5% commercial bleach and dry heating (Fig. 1).

Seeds that were sterilized by dry heating (5% commercial bleach + dry heating and 14% commercial bleach and dry heating) were not infected. The infection of seeds was significantly reduced with these sterilization methods, when compared to the sterilization by 14% commercial bleach and 5% commercial bleach + moist heating (Fig 2.).

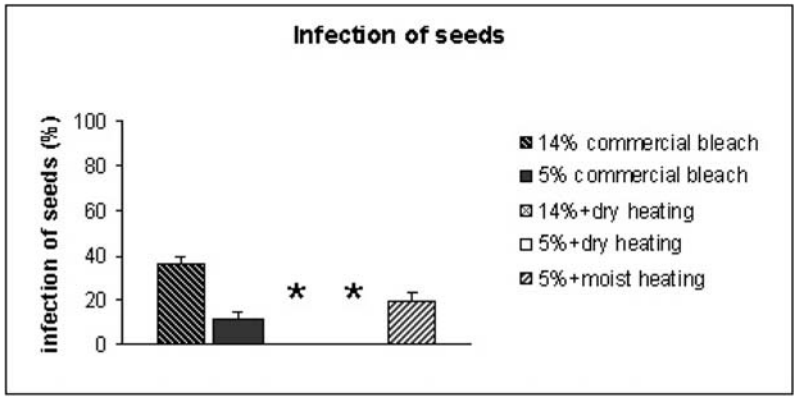

Figure 2. Infection of the seeds. Significance: *p < 0.05 vs. protocol 1 and 5

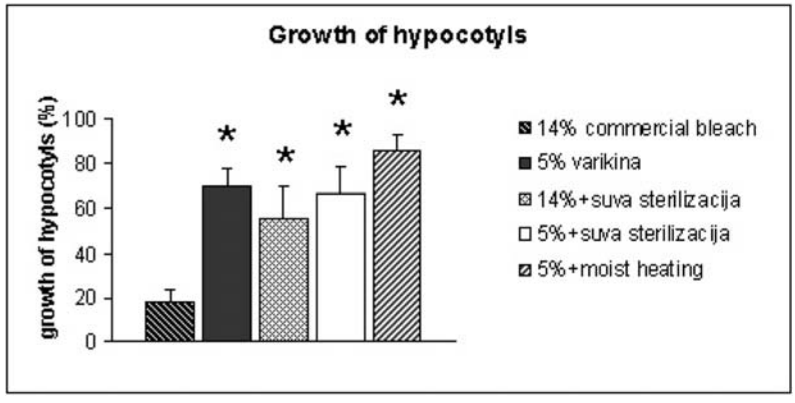

Figure 3. Growth of the hypocotyls. Significance: *p < 0.05 vs. protocol 1

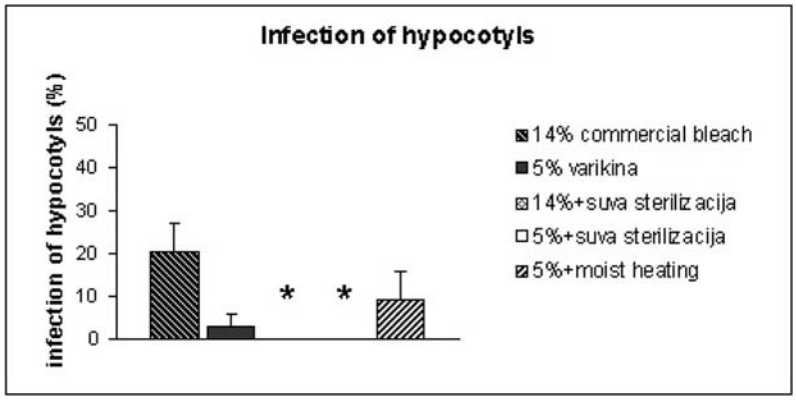

Figure 4. Infection of the hypocotyls. Significance: *p < 0.05 vs. protocol 1

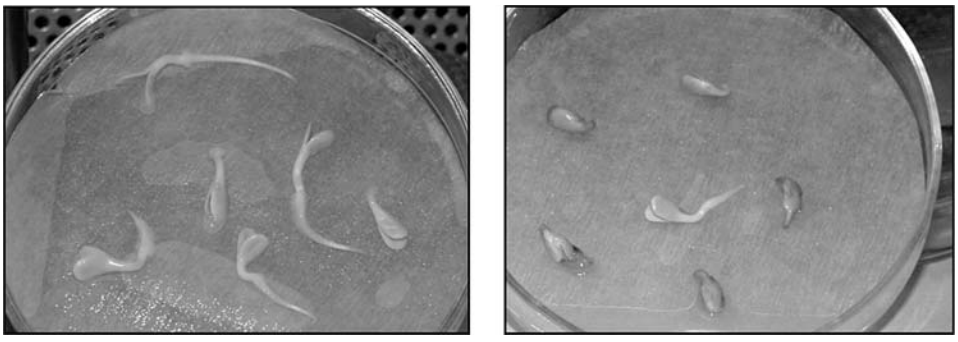

Figure 5. Germination of the seeds sterilized by 5% commercial bleach (left) and 14% commercial bleach (right)

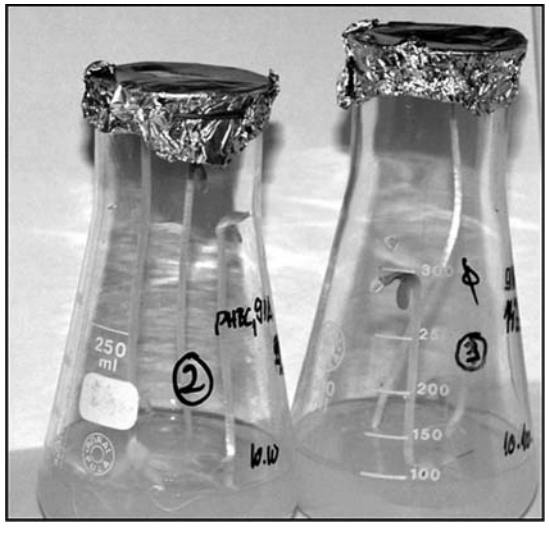

Figure 6. Growth of the hypocotyls after seed sterilization by 14% commercial bleach (right) and combination of sterilization by 5% commercial bleach and dry heating (left)

Growth of hypocotyls after the sterilization of seeds by 14% commercial bleach was significantly lower, when compared to the other protocols (Fig 6.), and also when compared to the protocols with combination of sterilization by 14% commercial bleach + dry heating (Fig 3.).

After seed sterilization according to the protocols with dry heating (5% commercial bleach + dry heating and 14% commercial bleach and dry heating) hypocotyls were not infected (Fig 4.). However, with those methods infection of the hypocotyls was significantly reduced when compared only to the sterilization of seeds by 14% commercial bleach.

Similar results were obtained by inbred line Ha-74A.

The obtained results showed that combination of sterilization by 5% commercial bleach and dry heating gives the best results in overcoming problems

with internal bacterial infection. Thus it could represent a good method to obtain plants free of microbial contamination for tissue culture.

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УПОТРЕБА РАЗЛИЧИТИХ МЕТОДА СТЕРИЛИЗАЦИЈЕ СЕМЕНА СУНЦОКРЕТА У ПРЕВАЗИЛАЖЕЊУ ЕНДОГЕНЕ БАКТЕРИЈСКЕ ИНФЕКЦИЈЕ

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Резиме

Приликом култивације протопласта гајеног сунцокрета у капљицама агарозе сталан проблем је била бактеријска инфекција. Како је претпостављено да је семе извор ове инфекције, испробане су различите методе његове стерилизације да би се покушао превазићи овај проблем. Праћени су клијавост семена, број инфекција семена и хипокотила, као и њихов раст. На основу ових параметара најбољи резултат је добијен након комбиноване употребе 5% варикине и суве стерилизације семена на 45°C.