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Determination of *in vitro* germination capacity of black pines seeds depending on the sterilizing agent

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Abstract Vegetative multiplication of pine can be established easily by mature embryos culture, realized by seed germination on *in vitro* conditions. Seeds inoculation on aseptic conditions must be preceded by seeds sterilization, using chemical agents. In this paper, three chemical sterilizing agents were used in different concentrations and $HgCl_2$ 0.1% was found to be the proper sterilizing agent both for a good germination percentage and low infectivity of cultures. Germination capacity depended highly by the genotype, Greek genotypes showing a lower germination capacity comparing with the Romanian ones, but also on the sterilizing agent. Cultures infection degree was not dependent on the genotype but on the sterilizing chemical agent used.

Key words

Black pine, germination capacity, infectivity, sterilizing agents

Germination is a physiological process of crossing a germ of life latent and active life that gives rise to a plant or plant organ. Inside the seed is the embryo, from which new plants arise (Raven et al., 2005). Seed store nutrients needed to grow embryo and new seedlings until they succeed alone to produce the necessary nutritive substance in the process of photosynthesis. To ensure optimal conditions, germination requires a well-hydrated culture medium (Tang and Newton, 2004). *In vitro* culture media for germination are strongly hydrated environments, in form as a gel, and the atmosphere of the growing rooms ensures constant moisture of 80% (De Diego et al., 2010). Passing the biological material from natural life conditions (*in vivo*) in an artificial environment (*in vitro*) of life requires a stage of biological material sterilization (Ishii et al., 2004). The culture medium used in the vitro culture for explants inoculated as nutritional support, is an environment rich in nutrients, optimal for growth and development of both the *in vitro* new formed plantlets and the many phytopathogens such as molds, fungi or bacteria (Alvard et al, 1993). These pathogens get into the culture medium by explants or by improper inoculation works. Once these pathogens enter in the environment consume the nutrients and eliminate toxins in the environment, which would make it unsuitable for cell culture and plant tissue (Ordas et al., 2007). Thus, every time we tried to develop protocols for sterilization, which however differ somewhat depending on the type of explant used, and the sterilizing agent used.

The most used sterilizing agents are of the class of chloride: mercuric chloride, calcium or sodium hypochlorite, etc., or in some cases - when cells grown on culture media are resistant to antibiotics, different antibiotics are used to maintain optimal health of crops and their sterilization (Skidmore et al., 1988).

Material and Method

Biologic material was constituted of one landrace of black pine (*Pinus nigra ssp nigra var nigra*) from Greece, in South of Ano Polydrossos village, in Mount Parnassos and two black pines (*Pinus nigra ssp nigra var Banatica*) landraces from Southern Banat in Băile Herculane from National Park Domogled-Valea Cernei and in Sviniţa village from National Parc Porţile de Fier. Data taken in consideration were represented by the average values obtained of all landraces of black pine studied. The average value and the standard deviation of 3-5 samples were calculated using the Microsoft Office Excel 2003 program.

Biological material sterilization for *in vitro* culture initiation was done with three sterilizing agent in different concentrations and with different periods of incubation according with literature (Rodriguez et al, 2004).

Table 1

Sterilizing agents in black pine seeds sterilization

Sterilizing agent	Concentration %	Time of sterilization
$HgCl_2$	0,1%	1.5-2 min
$HgCl_2$	0,5%	1.5-2 min
$Ca(OCl)_2$	5%	5.5- 6 min
$Ca(OCl)_2$	8%	3.5- 4 min
NaOCl	5%	6.5- 7 min
NaOCl	8%	4.5-5 min

Seeds were very well washed and soaked for 10 minutes in tap water. The seeds floating were eliminated and there were selected for *in vitro* initiation only the one that sinks. These were introduced in the sterilizing agent solution and incubated according with the specific period, than rinsed for tree times with sterile distilled water and inoculated on the solid media surface. Seed sterilization was done only after prolonged washing them under running water (4h). Seeds were immersed in 70% ethanol for 10 seconds, and then were sterilized for 1-2 minutes by immersion in mercuric chloride solution, 4-6 minutes in calcium hypochlorite solution or for 5-7 minutes in sodium hypochlorite solution. The sterilizing agents were added with a few drops of Tween (Polysorbate 20 - $C_{58}H_{114}O_{26}$), which is a detergent that determines the easier adhesion of the sterilizing agent to the surface of the biological material, favoring an efficient disinfecting of it. Post-sterilization in all variants of sterilization was achieved by performing successive rinses (3-5) in sterile distilled water.

Thus, we have used three types of sterilizing agent in different concentrations: two concentrations of mercuric chloride 0.1 and 0.5%; sodium hypochlorite solution and calcium hypochlorite solution that have also tested in two concentrations of 5% and 8% that are most commonly used to sterilize the biological material (table 1). Concentrations chosen were selected based on the bibliography (Attree şi Fowke, 1993; Find şi colab., 2003; Vookova şi Kormutak, 2002), trying to establish the most optimal methods for genotypes studied. Sterilization and inoculation of seeds on culture medium were made in sterile room at the sterile horizontal laminar flow hood. The room was first room sterilized by irradiation with UV light for 30 minutes and work surfaces were sterilized by wiping with alcohol.

Results and Discussions

As shown in table 2, optimal sterilizing agent in this case is mercuric chloride in a concentration of 0.1%, recording the lowest percentages of necrotic material correlated with the lowest rates of infection. Although the minimum percentage of infection was obtained when using 0.5% mercuric chloride; the concentration is not recommended because the percentage of necrotic embryo, revealed by a low percentage of germination is much higher.

Germination capacity was influenced by the sterilizing agent and the time of treatment, best results for all samples being obtained on the experimental variant HgCl (mercuric chloride) 0.1%. This variant involved the less period of sterilization of only 1.5 to 2 minutes comparing with the most aggressive agent NaOCl (sodium hipocloride) 8% which affected many of the embryos, variant that has got the lowest number of seeds that germinate.

Table 2

Experimental results regarding germination capacity of biological material depending on the sterilizing agent

Sterilizing agent	**Germination %**		
	Ano Polydrossos	**Herculane**	**Sviniţa**
$HgCl_2$ 0,1%	68.4±1.23	89.3±2.78	94.9±4.13
$HgCl_2$ 0,5%	59.9±5.4	79.7±5.45	84.2±6.45
$Ca(OCl)_2$ 5%	51.4±3.2	71.7±3.02	75.3±6.90
$Ca(OCl)_2$ 8%	50.8±2.31	62.3±4.06	72.1±5.32
NaOCl 5%	56±3.01	62±4.78	71.8±5.21
NaOCl 8%	48.9±2.03	58.5±4.88	69.3±2.20
$\overline{X}$	55.90	70.83	77.50

Because calcium hypochlorite does not dissolve completely in aqueous solution and this requires an appropriate filter (Danci et al., 2007), the percentage of necrosis is quite high for both concentrations used, being higher in concentration of 8%. This may be explained by the retention of many undissolved chloride particles, which burned the tissues reached with their high concentration. Although

a low level of infectivity was recorded on this experimental variant, when using calcium hypochlorite at a concentration of 8%, the percentage of necrosis is much too high to recommend this solution as a sterilizing agent for the biological material of pine. Attempts to reduce the concentration of calcium hypochlorite to 4% determined the achievement of a degree of infectivity of 94%.

Generally when in a vessel appears a growing infection the probability that infection will not be transmitted to the other inocula, in the next few days, is very low. Therefore, seedlings were carefully monitored and immediately subcultivated on fresh medium. But this is not desirable. An infection leads to significant losses in culture, the loss of time needed to subculturing, the loss of biological material and chemicals required for a new culture medium.

Sodium hypochlorite 8% would be the second best option to sterilize the biological material of pine (figure 1). Although dissolve well in water and filtering is not necessary, sodium hypochlorite generated higher percentages of necrosis, probably due to longer time required for the sterilizing agent to take effect, while penetrating inside the seed by imbibition, the sterilizing agent caused necrosis of the embryo and a much lower percentage germination of seeds. Sterilizing agents generally act on epidermal cells which have cell wall and thus must be resistant to their harmful action. It is therefore often necessary to optimize the sterilization time, but as the time of sterilization is reduced the chemical agent may not take effect, which would lead to a high degree of infectivity in culture (Verma et al, 2011).

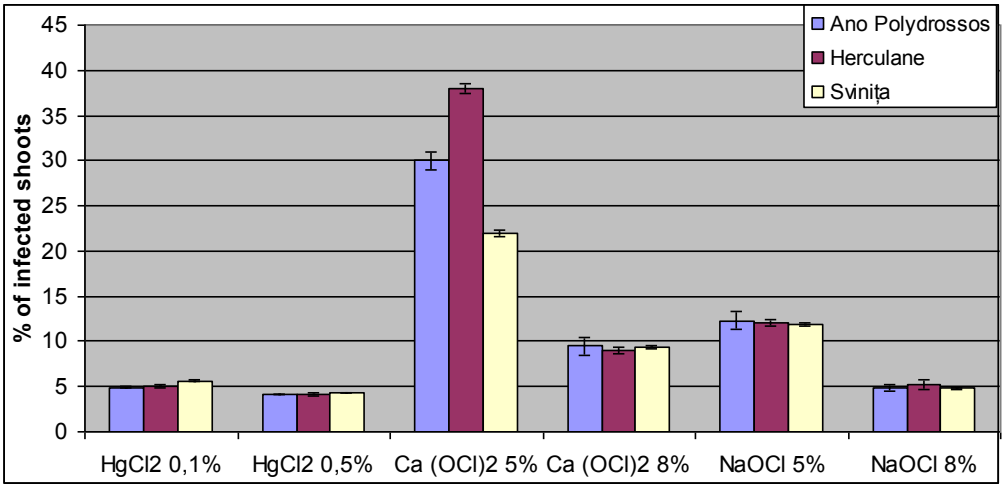

Fig. 1 Influence of sterilizing agent on infectivity degree of black pine seeds inoculated *in vitro*

In this respect, due to the observations made during experimentation and results obtained, we recommend as the second variant for pines seeds sterilization, the sodium hypochlorite 5%, that emphasized a medium level of infectivity (around 10%) but a high level of seeds germination (almost 80%), showing less harmful effect on seeds' embryos (table 2 and figure 1).

Greek seeds germination was significantly lower than Romanian seeds and one explanation might be also done by the fact that the period of cones collection was much earlier in Greece than in Romania and the Greek cones were almost green; their maturation was done mostly in the laboratory. This could be the reason why a great number of Greek black pine seeds did not presented mature embryos and gave lower percentages of germination (table 2).

a b c d

Fig. 2 a) Black pine seeds inoculated for germination; b) Pine cotyledon; c) Pine germinated seeds; d) Pine shoots from seeds

The highest infection level was emphasized by the culture with seeds of black pine population from Băile Herculane, after sterilizing the biological material with calcium hypochlorite 5% for 5.5-6 minutes (table 3.5) and the lowest level of infection was registered by the cultures of seeds from Sviniţa, marking an average of 9.68 %.

Table 3

Infection percentage registered in seeds cultures after sterilization with different chemical agents

Sterilizing agent	Initial infection %*		
	Ano Polydrossos	Herculane	Sviniţa
$HgCl_2$ 0,1%	4.9±0.1	5.1±0.22	5.6±0.09
$HgCl_2$ 0,5%	4.2±0.03	4.2±0.22	4.4±0.00
$Ca(OCl)_2$ 5%	30±0.9	38±0.64	22±0.38
$Ca(OCl)_2$ 8%	9.5±0.81	9±0.45	9.4±0.20
NaOCl 5%	12.3±0.97	12.1±0.39	11.9±0.25
NaOCl 8%	4.9±0.41	5.2±0.48	4.8±0.10
$\overline{X}$	10.97	12.27	9.68

*infection refers to bacteria and fungi and not to viral infection that can be identified only by special tests

It is thus seen from the table above that the fastest action on pathogens was shown by the mercuric chloride and due to its shorter time for action the percentage of regenerated plants from inoculated seed number it is much higher. The highest percentage of germination was obtained when seeds were collected from the Danube River, from city Sviniţa, a Serbian colony where the development of local populations of *Pinus nigra* var. *Banatica* was observed. This population is located in the area of at least 80 years, according to testimonies of one local women aged 82 years, who knows the pine forest as she recalls.

Influence of sterilizing agent on regeneration of healthy shoots was observed in the following subcultures (table 4).

Table 4

Infection percentage registered in seeds subcultures depending on the sterilization agent

Sterilizing agent	Subculture infection *%		
	Ano Polydrossos	Herculane	Sviniţa
$HgCl_2$ 0,1%	1.1	1.1	1.2
$HgCl_2$ 0,5%	0.7	1.2	0
$Ca(OCl)_2$ 5%	6.9	7.9	8
$Ca(OCl)_2$ 8%	4.5	4.3	5.1
NaOCl 5%	4.0	4.3	4.4
NaOCl 8%	3.3	3.6	3.2
$\overline{X}$	3.42	3.73	3.65

*infection refers to bacteria and fungi and not to viral infection that can be identified only by special tests

Not infected seeds were immediately subcultured on fresh media and another week later a new monitoring was done and the variant that emphasized the highest number of infected seeds was again from the seeds sterilized initially with $Ca(OCl)_2$ (calcium hypochlorite) 5% and the highest number of non infected cultures was obtained on the culture variant sterilized with $HgCl_2$ 0,5%.

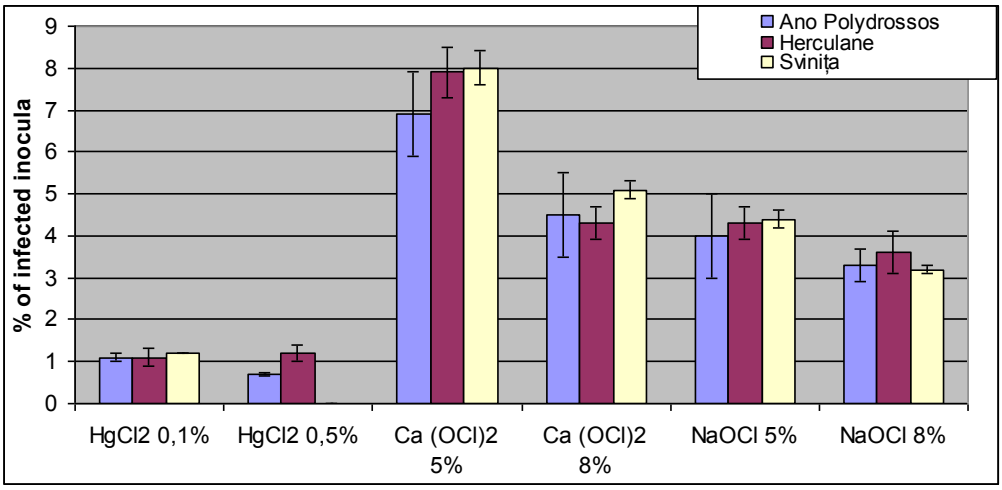

Fig. 3 Influence of sterilizing agent on infectivity degree of black pine seeds subcultured on fresh medium

Data from the above figure show that sterilizing agent used for seeds preparing for *in vitro* culture initiation has a still important influence; most infectivity was found on vessels cultured with seeds sterilized with calcium hypochlorite (5%). But in this case, the genotype influence is irrelevant, the lowest and the highest number of infected seeds/experimental variant was registered by the population from Sviniţa; none of the populations differentiate from the others in a meaningful way (figure 3).

Conclusions

1. Best germination capacity was observed when $HgCl_2$ 0,1% was used, the variants treated with Ca $(OCl)_2$ 8% emphasizing the lowest germination capacity for all genotypes studied.
2. Greek seeds germination was significantly lower than Romanian seeds and may be due to the period of cones collection that was much earlier in Greece than in Romania.
3. Infection degree depended in a greater measure on the sterilizing agent used than on the genotype; higher percentage of inocula that developed infection was shown on the variants sterilized with Ca $(OCl)_2$ 5% and the lowest infection percentage was obtained when $HgCl_2$ 0,5% was used. No statistically significant results were registered between genotypes on the infectivity parameter.
4. Due to the highest number of germinating seeds obtained and a high percentage of healthy regenerates it is recommended to use $HgCl_2$ 0,1% for pine seeds sterilization.

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