Contamination control of microbe *Ziziphus spina* [christti] seed in vitro culture

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Abstract *Ziziphus spina* [christti] is a naturally distributed tree in subtropical, arid and semi-arid parts of Iran. It is ecologically and economically important due to its tolerance to drought and salinity. Most tree seeds are infected with parasitic and saprophytic microorganisms which decrease the seed germination and seedling establishment. The goal of this paper was to evaluate the ability of selected chemical solutions to inhibit the growth of variety of microbial contaminants in *Z. spina* [christti] seeds and to enhance the seed germination. Different chemical treatments were used in surface sterilization of seeds: (Treatment 1) sodium hypochlorite (NaOCl) in concentrations of 1, 2 and 4% for 20 min. (Treatment 2) hydrogen peroxide (H₂O₂) in concentration of 4, 8, 12% treated for 10 min. (Treatment 3) 1% mercuric chloride (HgCl₂) at duration 10, 15, 20, 25 min. Seeds were scarified and aseptically, planted on agar Murashige and Skoog (MS) medium. Contaminants were identified according to their morphological and cultural characteristics. Bacterial contaminants included *Xanthomonas* sp. While Fungal isolates were *Fusarium*, *Penicillium*, *Alternaria*, *Rhizopus*, and *Aspergillus*. Our experiment reveals that 4% NaOCl followed by benomyl is the best sterilization treatment for *Z. spina* [christti] seeds, since the highest number of germination

and highest number of sterilized seeds was observed after this treatment.

Keywords Microbial contamination · Surface sterilization · Chemical solution · *Ziziphus spina* [christti]

Introduction

The genus *Ziziphus* belongs to the family Rhamnaceae, consisting of about 100 deciduous or evergreen tree and shrub species distributed in the tropical and subtropical regions (Dweck 2005). *Ziziphus spina* [christti] (L.) Willd is a species distributed in the West Africa to North East Africa, Ethiopia and Eastern Africa, especially in the drier tropical areas. It is wild in the Middle East (especially Iran), Saudi Arabia and also farther west in Turkey (Azam-Ali et al. 2006). The species is ecologically and economically important due to its tolerance to drought and salinity besides the high value of non-woody products; nutritious fruit and leaves and shoots as a source of saponins and tannin (Al-Sulaiman and Barakat 2010). It is suitable for re-vegetation of degraded lands, but little is known about the physiology of seed germination and seedling establishment (Sohail et al. 2009). All the parts of the plant are used in folklore medicine for the curing of various diseases (Motamedi et al. 2009). In a study conducted by Farrar et al. (2008) during 2001–2007 at different areas in South Iran, they showed that there were 28 insects and 3 mites that damaged different parts of *Z. spina* [christti] trees. Tree, leaves, spines, seeds and fruits of the *Z. spina* [christti] are shown in Fig. 1. Since *Ziziphus spina* [christti] is a cross-pollinated plant, a wide range of genetic variability exists in the nature. Vegetative propagation, particularly in vitro propagation, assumes importance for mass

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and clonal propagation (Assareh and Sardabi 2005). Since seeds of *Z. spina* [christti] require scarification treatments to germinate (Moustafa et al. 1998), vegetative propagation plays a significant role in its improvement in breeding programs. However, in vitro *Ziziphus* seed germination; low multiplication rates and high contamination in the seeds are some of the difficulties in *Ziziphus* micropropagation. In vitro contamination by fungi, bacteria, or yeast is one of the most serious problems of commercial and research plant tissue culture laboratories. The inability to control contamination levels adequately is the primary reason for failures in commercial laboratories (Niedz and Bausher 2002). The establishment of an in vitro culture requires the removal of fungal and bacterial contaminants in the planting material. Some previous studies have shown that sodium hypochlorite solution at different concentrations was effective in seed disinfection of various species. Do Rego et al. (2009) showed that a 0.5% sodium hypochlorite solution was effective in *Cereus jamacaru* seed disinfestation. De Moraes et al. (2010) found that the disinfection of artichoke seeds can be done by immersing them in 70% alcohol for 30 min followed by immersion in 2% active chlorine solution for 10 min, before removing the tegument. Oyebanji et al. (2009) showed that a treatment with locally produced bleaching solution, containing 3.5% sodium hypochlorite for 20–45 min could be used to surface sterilize cowpea, rice and sorghum seeds for seed culture. Golle et al. (2010) found that the best disinfection treatment for *Pinus taeda* seeds was 30 s in 70% ethanol + 5 min in 3% sodium hypochlorite treatment. Ksenija and Dragana (2005) carried out the experiments to investigate the infection of fungi or bacteria on seeds at germination and hypocotyls and growth in sunflower. The best way to control fungi or bacteria according to their results was the combination of 5% commercial bleach for 60 min and dry heating at 45°C for 60 min. According to the scientific literature, the most effective treatment against fungal contaminations was benomyl treatment. Altan et al. (2010) showed that the benomyl (100 mg dm^{-3}) + nystatin (100 mg dm^{-3}) treatment combination was the most effective treatment against fungal contaminants. Similar results were obtained by Barnett and McGilvary (2001) and Allen et al. (2004). Barnett and Varela (2004) tested many chemicals to reduce large populations of microorganisms on longleaf pine (*Pinus palustris*). However, hydrogen peroxide, thiophanate methyl, and thiram provided the best amount of disinfection. Numerous techniques have been examined with varying degrees of success for increasing seed germination and eliminating contaminations of microbial and other seed borne fungi. These treatments included fungicides (Runion and Bruck 1988), surface sterilization agents (Barnett 1976; Wenny and Dumroese

1987) and hot water and microwave treatments (James et al. 1988).

Seed treatments to control seed borne inoculums can be based on the chemical, physical, mechanical, and biological practices (Fraedrich 1996). Chemical and physical methods are primary considerations for quarantine issues. Chemical seed treatments (like the application of fungicides) have been used routinely to control seed borne pathogens and are often the cheapest and most effective means for control. Fungicides are used to kill or to inhibit growth of seed borne fungi and can be systemic or non-systemic in their action (Allen et al. 2004). Highly selective systemic fungicides have proved to be most useful for the eradication of inoculum in seeds. Techniques for the infusion of fungicides into seeds have been developed for numerous agricultural crops (Fraedrich 1996), but experiences with seeds of forest tree species are limited. Fungicides, such as benomyl and thiobendazole have been used to control seed borne pathogens on conifers with mixed results. Disinfectants, such as sodium hypochlorite and hydrogen peroxide have proved useful for elimination of inoculum associated with the seed coats of conifer species (Allen et al. 2004). Combining this practice with the use of selective fungicides may provide effective control of pathogens internally and externally on forest tree seeds. This study was undertaken to evaluate the effect of fungicide and other chemical solutions on sterilizing of *Ziziphus* seeds. We used benomyl to enhance germination and hydrogen peroxide, sodium hypochlorite and mercuric chloride as seed disinfectants. The aim of this experiment was removal of contamination of *Ziziphus* seed under laboratory conditions and producing plants free of pathogens from seeds by the application of a simple, efficient and economical method in which we could obtain results in short time. Seeds free of contamination and also healthy plants free of pathogens could be applied in natural field and extension of this species.

Materials and methods

Collection and identification of plant material

Ziziphus spina [christti] seeds were collected from research institute of forests and rangelands, Ahwaz, Iran in 2010. The laboratory tests were conducted at the tissue culture laboratory at Department of Forestry, Faculty of Natural Resources, Sari Agricultural Science and tissue culture laboratory at Natural Resources University, Sari, Iran. Murashinge and Skoog (1962) agar medium was used as the tissue culture medium and sterilization of the medium was done by autoclaving at 121°C for 20 min.

Fig. 1 Physiognomy of the tree (a) and morphology of leaves, spines, seeds and fruits (b) of *Ziziphus spina* [christii]. Scale (cm)

Sterilization and incubation of plant cultures

The seeds were surface sterilized by rinsing with tap water and then dipping them in 0.1% HgCl_2 solution for 15 min. The seeds were dipped in 70% ethanol for 40 s followed by thorough rinsing in sterile distilled water. The excised seeds were then aseptically transferred to the culture medium, labeled and incubated at $23 \pm 1^\circ\text{C}$ during the day and $19 \pm 1^\circ\text{C}$ at night for 3 weeks.

Laboratory analysis of isolation of microbial contaminants

From the contaminated plant seed culture tubes, emerging microbes were isolated by inoculating them on acidified potato dextrose agar (APDA) and incubated for 6 days at 26°C under 12 h photoperiod in the case of fungi and on nutrient agar incubated for 3 days at 30°C under 12 h photoperiod. Pure isolates obtained from repeated sub-culturing of the isolates were placed in an agar slant in McCartney bottles and stored at 4°C in a refrigerator.

Characterization and identification of isolates

The fungal isolates were identified using cultural characters and morphology by comparison with standard descriptions (Barnett and Hunter 1972). In case of bacteria, beside the morphological characteristics, a number of biochemical and physiological tests were carried out on the isolates. The biochemical tests includes Gram staining, spore staining, motility test, catalase production, oxidase test, indole production, citrate utilization, urease activity, hydrogen sulfide

production, gelatin hydrolysis, starch hydrolysis and carbohydrate utilization.

Effect of different treatments on seed sterilization and seed germination

Different surface sterilization treatments included: (T1) sodium hypochlorite (NaOCl) in concentrations of 1, 2, 4% for 20 min, (T2) hydrogen peroxide (H_2O_2) in concentration of 4, 8, 12% for 10 min, (T3) 1% mercuric chloride (HgCl_2) for 10, 15, 20 or 25 min. Seeds were scarified with sulfuric acid 98% for 4 h and then washed thoroughly with tap water and distilled water and then immersed in the above-mentioned solutions and then dipped seeds in 2% benomyl for 5 min and rinsed with distilled water. Finally, seeds were dipped in 70% ethanol for 90 s followed by thorough rinsing in sterile distilled water. The seeds were aseptically planted on agar-solidified Murashige and Skoog (MS) (1962) in culture tubes (25 mm $\times$ 200 mm) containing cotton plugs. The cultures were incubated under 1,000 lux light intensity provided by white fluorescent lamps at 27°C . Number of germinated seeds and number of uninfected seeds (sterilized seeds) in each tube was observed weekly for calculation and statistically analyzed. Final germination percentage (FG) and sterilization of seed (S) was calculated (Panwar and Bhardwaj 2005).

$$\text{FG} = n/N \times 100$$

$$S = n/N \times 100$$

where n are the number of germinated seeds or n number of uninfected of seed, N is the total number of seeds.

Statistical analysis

A randomized complete design was used with five replications and five seeds in each replicate. Data were statistically analyzed in SPSS program. Duncan's test ($p < 0.05$) was used to compare means within and among treatments.

Results

Microbial contaminants of identification of isolates

Four microbial contaminants (1 bacteria and 3 fungi) were found associated with the seed cultures. The bacterial contaminant was *Xanthomonas* sp., while fungal isolates were *Fusarium*, *Penicillium*, *Alternaria*, *Rhizopus*, and *Aspergillus*. The rate of occurrence of bacteria was higher than that of fungi in the plant tissue cultures.

Effect of chemical treatments on seed germination and seed sterilization

The present study clearly demonstrated significant differences in seed germination and sterilization percentages were observed among different chemical treatments ($p < 0.0001$). Both the duration of the treatment, chemicals used and their strength affected the seed germination ($F = 77.75$, $p < 0.001$ and $F = 64.57$, $p < 0.001$) and sterilization ($F = 34.15$, $p < 0.0001$ and $F = 13.42$, $p < 0.001$) significantly. However, the mean total number of sterilized seed parameter was not affected significantly by the interaction between time and sterile treatment. Figure 2 presents the maximum effect of sterilization and

germination of seeds by 4% NaOCl and inadequate ability of H_2O_2 for controlling the contamination and germination.

Effect of chemical treatments and time factors on germination parameters

Mean final germination in interaction between time and sterile treatment in NaOCl 4%, NaOCl 2% solution and third, fourth and fifth weeks and $HgCl_2$ 10 min in fifth week were significantly higher when compared with those of other treatments. NaOCl 1%, H_2O_2 12%, $HgCl_2$ 15 min, $HgCl_2$ 20 min solution in first week and H_2O_2 8% in first, second and third week and H_2O_2 4% solution in all 4 weeks revealed the lowest treatments in final germination. $HgCl_2$ 25 min, 20 min treatment in third, fourth and fifth weeks was more than that of this treatments. Final germination parameter in NaOCl 4% treatment was significantly greater than that of other treatments and the maximum final germination was recorded in this treatments. This treatment was followed by $HgCl_2$ 20 min and 10 min and NaOCl 2% treatments. H_2O_2 4% and H_2O_2 8% solution treatments recorded the lowest germination parameter as compared to other treatments in final germination.

Effect of germination

Sterilization percentage of seeds in NaOCl 4%, $HgCl_2$ 15, 20 and 25 min treatments were significantly greater than that of other treatments. H_2O_2 4%, H_2O_2 8% solutions were the least effective treatments for sterilizing seeds. Other treatments could be almost placed in a similar means range (Fig. 3).

Fig. 2 Effect of treatments on germination and sterilization of seeds (correct NaClO and $HgCl_2$ in legends to figure) **a** Germination of the seeds sterilized and plants free of microbial contamination by 4% NaOCl + benomyl (right tube). Seeds sterilized by 4% NaOCl + benomyl (left tube). **b** Germination of the seeds sterilized and

plants free of microbial contamination by $HgCl_2$ 15 min + benomyl (right tube). Seeds sterilized by $HgCl_2$ 15 min + benomyl (left tube). **c** Seeds sterilized by 4% H_2O_2 + benomyl (right tube) inadequate ability of H_2O_2 + benomyl for controlling the contamination and germination

Fig. 3 Comparison of means obtained for parameters on sterilization of seeds parameter in different sterilization treatments (H₂O₂ 4, 8, 12%; NaOCl 1, 2, 4%; HgCl₂ 10, 15, 20, 25 min)

Discussion

Seed germination failure in *Ziziphus* was observed in our study to be due to seed pathogenic bacteria and fungi. Since permanent treatment of seed is the most expensive and time-consuming method, we have used surface sterilization. Surface sterilization can be performed by exposure of seeds to UV light, heat or to chemicals. However, UV irradiation can damage DNA and heat can induce death of embryo. Thus, we tried to develop sterilization technique using chemical solutions. In the current study, benomyl showed some efficacy against fungi in *Ziziphus* seed. Barnett et al. (1999) and Runion and Bruck (1988) have shown that benomyl and thiabendazole improved germination of longleaf pine seed in previous studies. Based on the obtained data, germination of *Ziziphus* seeds was significantly lower after sterilization by 4% H₂O₂ and 8% H₂O₂ (Fig. 3). Significantly lower germination of seeds was also found after sterilization by 15 min HgCl₂ 12% H₂O₂ and 1% NaOCl treatment when compared with the seeds sterilized by 2 or 4%, NaOCl and 10, 20 or 25 min HgCl₂ treatments (Fig. 4). The infection of seeds was significantly reduced by 4% NaOCl, 15 min, 20 min and

Fig. 4 Comparison of means of final germination parameter in different sterile treatments (H₂O₂ 4, 8, 12%; NaOCl 1, 2, 4; HgCl₂ 10, 15, 20, 25 min)

25 min HgCl₂ sterilization methods (Fig. 3). These results are in agreement with the finding of other investigators (Do Rego et al. 2009; Oyebanji et al. 2009; Golle et al. 2010; Ksenija and Dragana 2005); 4 and 8% H₂O₂ treatments were less effective than that of other treatments in sterilization of seeds.

Barnett and Varela (2004) have reported that the reduction in large populations of microorganisms on longleaf pine (*Pinus palustris*) with hydrogen peroxide seemed to provide the best result. Allen et al. (2004) found that Mancozeb, a protectant, and hydrogen dioxide, a disinfectant, also exhibited some efficacy in longleaf pine seed. Our results contrasted with the classical responses to sterilization by hydrogen peroxide because, it has not acted as a phytotoxic chemical for *Ziziphus* seeds and actually has not acted as stimulator for germination of seed. This may be because of the hard seed coat and or is it because that due to high pathogenic attack. Thus, H₂O₂ can be used as a powerful and cheap sterile solution. Our experiment reveals that 4% NaOCl followed by benomyl is the best sterilization treatment for *Ziziphus spina* [christti] seeds, since the highest number of germination and highest number of sterilized seeds was observed after this treatment. Thus, it could represent a good method to obtain plants free of microbial contamination for seed culture. Sterilization technique using chemical solutions can be costly and potentially hazardous when used at a high concentration for large amounts of seeds (Fraedrich 1996). In addition, chemical solution is not registered as a pesticide, although some believe that it may be used legally to stratify seeds. Studies are needed to identify the concentrations of chemical solution and soaking times required to disinfect seeds efficiently. Further research is needed for the development of additional chemical and non-chemical procedures that could be used to control diseases caused by pathogens associated with *Ziziphus* seeds.

Conclusion

The results of our experiment showed that sterilization by 4% NaOCl + benomyl is the best method to be used in surface sterilization of *Ziziphus spina* [christti] seeds. Seeds treated with this treatment showed the highest final germination and highest number of sterilized seeds. Fungicidal and sterile solution is an inexpensive means of disease control that can help prevent seedling losses by a variety of fungal pathogens. These treatments could increase the overall number of marketable seedlings.

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