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RESEARCH NOTE

Effect of various sterilization procedures on the in vitro germination of cotton seeds

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Abstract In vitro manipulations of cotton often require high-quality sterile seedlings as a source of hypocotyl and cotyledon explants for initiation of embryogenic cultures or embryo apexes for shoot production. Unfortunately, in vitro seed germination is often hindered if cotton seeds were collected from the open field and stored under improper conditions. In our case a limited supply of field-grown cotton seeds was received, which necessitated the development of a more effective surface sterilization protocol. Seeds from two accessions, designated as I and II, with very high contamination levels and lowered germination rate were used in this study. These seeds were treated with the most commonly used sterilizing agents, which included commercial bleach, chlorine gas and hydrogen peroxide. Additional steps such as soap-water washes (SW), 70 % ethanol (ETH) and plant preservative mixture rinses were also included in the sterilization procedures to improve the efficiency of tested protocols. Surface sterilized seeds were germinated on Linsmaier and Skoog medium and the percentage of contamination free and well-germinated seeds were recorded for each treatment. Seeds treated with hydrogen peroxide showed significant improvement in germination rate and level of contamination when compared to identical seeds treated with chlorine gas and commercial bleach. The most effective sterilization protocol for all genotypes tested consisted of SW wash followed by ETH rinse and H_2O_2 sterilization for 7 h. This protocol was successfully used to sterilize seeds of 55 cotton lines.

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Introduction

Cotton is one of the most important sources of textile fiber and is valued at $500 billion worldwide. Cotton was one of the first genetically modified commercial crops to be produced globally (Rahman et al. 2012). To date the major traits targeted for genetic modification of cotton include yield, fiber quality, enhanced tolerance to biotic and abiotic stresses, and the introduction of resistance to pathogens. For in vitro gene manipulation studies, seeds and different parts of cotton seedlings (hypocotyl and cotyledon) have been used as explant material (Kumria et al. 2003). Hence, efficient seed sterilization is a prerequisite for successful cotton regeneration and transformation. However, the production of a high number of contamination free seedlings from a limited seed supply harvested from the open field remains a challenge. Seeds collected from the open field are often contaminated with exogenous and endogenous microbial contaminants that include fungi and bacteria (Halloin 1975; Klich 1986; Howell 2002; Ahmad et al. 2012).

Commonly used surface sterilization agents include ethanol, sodium hypochlorite, calcium hypochlorite, chlorine gas and mercuric chloride, which have been used for surface sterilization of plant and seed material of various species (Zhang et al. 2000; Talei et al. 2012; Daud et al. 2012). Unfortunately these agents often fail to efficiently remove contaminants, particularly when seeds are collected from the open field and stored under improper conditions. Mercuric chloride, one of the most dangerous chemicals used for sterilization, is highly toxic and requires safe

handling during the sterilization procedure, while the resulting hazardous waste requires special collection and disposal. Ethanol, sodium hypochlorite and calcium hypochlorite are the most often used agents for sterilization of plant material including seeds. Less commonly used agents such as chlorine gas and hydrogen peroxide have been successfully used for sterilization of *Arabidopsis* and soybean seeds (Clough and Bent 1998; Di et al. 1996) and other seeds and plant material (Amjad et al. 2004; Çavusoglu and Kabar 2010).

Our study compares different sterilization agents including sodium hypochlorite, hydrogen peroxide, and chlorine gas. Here we describe an efficient sterilization protocol that enables high in vitro cotton seed germination rate and provides an efficient production of high-quality sterile seedlings.

Materials and methods

Cotton seeds were provided by NC State University and the Research Breeder Testing Network (RBTN), North Carolina. All seeds were collected from the open field at various locations in the US and stored for an unknown duration and under unknown conditions. Among many cotton seeds received in our lab, two accessions (designated as I and II) were chosen for this study because they showed the highest degree of fungal and bacterial contamination and the lowest germination rate compared to other seeds tested in the preliminary studies, in addition to lacking a protective fungicide coating. Between these two accessions, the accession-II had a higher degree of contamination and lower germination rate compared to the accession-I.

All seeds were hand sorted to select for undamaged seeds before sterilization. Seeds of each accession were sterilized and germinated in vitro, and the seedlings were scored for contamination after 10 days. The seeds were subjected to three major seed sterilization treatments, which were based on chlorine gas (CHL), commercial Clorox bleach containing 5.25 % NaOCl (Bleach), and 3 % (v/v) hydrogen peroxide (H_2O_2). For each treatment 30 seeds were used. Seed sterilization was done in 50 mL Falcon tubes (Fischer Scientific, USA), which were mounted on a rotatory shaker and agitated at 150 rpm.

Chlorine gas

Chlorine gas was produced by combining 100 mL of bleach and 3 mL of concentrated hydrochloric acid in a small beaker placed in a glass desiccator under the fume hood. Cotton seeds were sterilized using four different treatments based on CHL. Treatment CHL (17 h): seeds were exposed to CHL for 17 h (overnight) prior to inoculation on the media. Treatment soapy water (SW) + CHL (17 h): seeds were washed with SW for 20 min, blotted on a paper towel, followed by exposure to CHL for 17 h prior to inoculation on the media. Treatment CHL (17 h) + ETH: seeds were exposed to CHL for 17 h followed by a 5 min wash with 70 % (v/v) ethanol (ETH), followed by 2–3 washes with sterile distilled water prior to inoculation on the media. Treatment CHL (17 h) + ETH + PPM: seeds were exposed to CHL for 17 h, then rinsed with 70 % (v/v) ETH for 5 min followed by 2–3 washes with sterile distilled water and a brief rinse with sterile ½ MS medium (Murashige and Skoog 1962) solution containing 1 % (v/v) plant preservative mixture (PPM^{TM}) without blotting or drying prior to inoculation on the media.

Bleach

The bleach (5.25 % sodium hypochlorite) sterilization treatments included two concentrations of commercial bleach (v/v) and two different time exposures - 25 % for 20 or 30 min and 50 % for 20 or 30 min. Before each treatment seeds were washed with SW for 20 min, rinsed 2–3 times with tap water, and washed with 70 % (v/v) ETH for 5 min. After each treatment seeds were rinsed 2–3 times with sterile distilled water followed by a brief rinse with sterile ½ MS medium solution containing 1 % (v/v) PPM^{TM}.

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) was purchased from the regular pharmacy where it is sold as 3 % solution. Cotton seeds were treated with 3 % hydrogen peroxide for 4 or 7 h. All H_2O_2 treatments were done either alone or combined with washes with SW, ETH, or PPM^{TM}. Two H_2O_2 treatments: seeds were treated with H_2O_2 alone for 4 or 7 h followed by 2–3 washes with sterile distilled water. Two SW + H_2O_2 treatments: seeds were washed with SW followed by 2–3 tap water rinses followed by H_2O_2 treatments for 4 or 7 h and 2–3 rinses with sterile distilled water. Two SW + ETH + H_2O_2 treatments: seeds were washed with SW followed by 2–3 tap water rinses, a 70 % (v/v) ETH wash for 5 min, followed by H_2O_2 treatments for 4 or 7 h and 2–3 rinses with sterile distilled water. Two SW + H_2O_2 + PPM treatments: seeds were washed with SW followed by 2–3 tap water rinses H_2O_2 treatments for 4 or 7 h, and 2–3 rinses with sterile distilled water followed by a brief rinse with sterile ½ MS medium solution containing 1 % (v/v) PPM. Two SW + ETH + H_2O_2 + PPM treatments: seeds were washed with SW followed by 2–3 tap water rinses, a 70 % (v/v) ETH wash for 5 min, H_2O_2 treatments for 4 or 7 h, and 2–3 rinses with sterile distilled water followed by a brief rinse with sterile ½ MS medium solution containing 1 % (v/v) PPM.

It should be noted that the 50 mL Falcon tubes used as vessels for hydrogen peroxide sterilization were checked for leakage every 3–4 h. Increased gas pressure can deform the plastic caps, which then must be replaced to prevent leakage. Following sterilization, seeds were placed on petri dishes containing LS (Linsmaier and Skoog 1965) medium with 6 g/L agar. The pH of the medium was adjusted to 5.8 ± 0.1 prior to autoclaving. All plates were incubated for 10 days in a growth chamber under a 16 h light/8 h dark photoperiod at a light intensity of 1,000 lux provided by cool white fluorescent tubes at 25 ± 1 °C and 65 % relative humidity. After 4 days the non-contaminated germinated seeds were transferred to magenta boxes for further development. At the 10th day the number of well-developed contamination free seedlings with elongated hypocotyls and expanded cotyledons was recorded.

Each of 18 experimental sterilization treatments was repeated three times, with 30 seeds for each replicate, for a total of 1,620 seeds. The GLIMMIX procedure (SAS Statistical software version 9.2) was used to fit a one-factor generalized linear model to these contamination and germination data, which were assumed to follow binomial distributions. For each of the four types of measurement made on seeds (contamination count under accession-I, contamination count under accession-II, germination count under accession-I, germination count under accession-II), we found a highly significant treatment effect ($p < 0.0001$). In a procedure akin to Fisher's (1935) protected least significant difference, pairwise comparisons among treatments were made without further adjustment for multiplicity.

Results and discussion

It has been reported that cotton seeds collected from the open field and stored improperly are often severely infected by various fungi (Halloin 1975; Klich 1986) in contrast to seeds stored in a controlled environment. Thus, it is a particular challenge when heavily contaminated seeds are germinated in vitro, particularly when the seed supply is limited. Use of a common surface sterilization agent, such as bleach, is not effective (Fig. 1) and results in poor germination. Of the seeds available in our cotton seed collection, accessions I and II were found to be the most highly contaminated with fungal hyphae and spores and had lower germination rates when compared to other seed accessions. Moreover, accession-II had a higher degree of contamination than accession-I (data not shown) in our initial sterilization attempts when we used 20 % bleach and 5 min of 70 % ethanol, a very common sterilization procedure used for cotton seeds (Ouma et al. 2004). Accession-II contamination levels were higher than accession-I, regardless of the sterilization agent used.

Chlorine gas has been successfully used for sterilizing *Arabidopsis* and soybean seeds (Clough and Bent 1998; Paz et al. 2004). We modified these protocols using a 17 h (overnight) CHL to treat our cotton seed. To improve the efficiency of this method, additional steps were included, such as a SW wash and rinses with ETH and PPM™. Our best CHL treatment results were obtained when accession-I seeds were treated with CHL (17 h) + ETH + PPM and accession-II seeds were treated with CHL (17 h) + ETH. Unfortunately, the CHL treatment of the highly contaminated accession-II seeds generated poor results, with only 28.88 % sterilization efficiency and a germination rate of only 7.7 % (Table 1). In contrast, CHL (17 h) + ETH + PPM treatment of less contaminated accession-I seeds increased the number of sterilized seeds to 81.11 % and the germination rate to 50 % (Table 2). The addition of SW wash or PPM rinse to the sterilization procedure failed to improve either the sterilization efficiency or germination rate

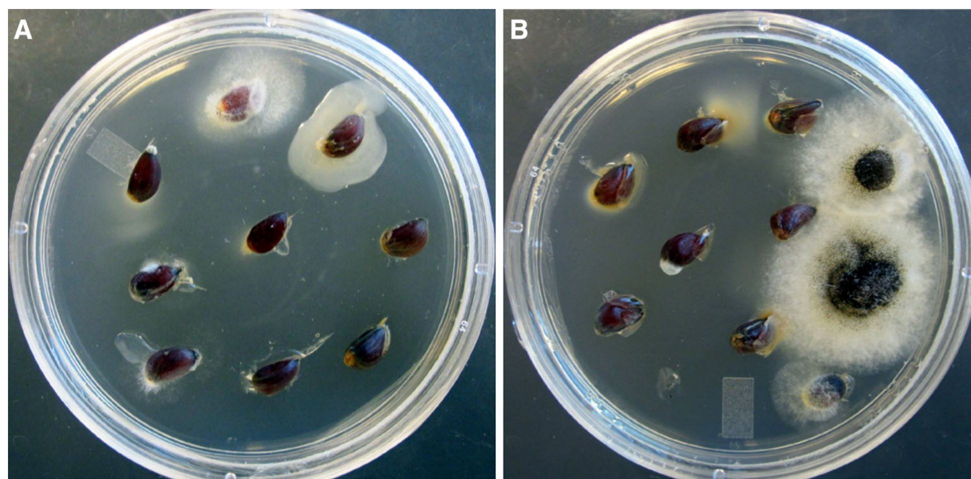

Fig. 1 Fungal and bacterial contamination observed in cotton seeds after using common sterilization protocol with 20 % bleach for 20 min. **A** accession-I; **B** accession-II

Table 1 Effect of different sterilization procedures on percentage of contamination free and germination rate in cotton seeds of accession-I

Sterilization treatment	Contamination free seeds (% mean ± SE)	Germinated seeds (% mean ± SE)
CHL (17 h)	57.77 ± 0.21g	37.77 ± 0.21fg
SW + CHL (17 h)	54.44 ± 0.21g	26.66 ± 0.23g
CHL (17 h) + ETH	77.77 ± 0.25f	40.00 ± 0.21fg
CHL (17 h) + ETH + PPM	81.11 ± 0.26ef	50.00 ± 0.21fe
SW + ETH + Bleach 25 (20 min)	77.77 ± 0.25f	56.66 ± 0.21de
SW + ETH + Bleach 25 (30 min)	80.00 ± 0.26ef	66.66 ± 0.22d
SW + ETH + Bleach 50 (20 min)	84.44 ± 0.29ef	66.66 ± 0.22d
SW + ETH + Bleach 50 (30 min)	85.55 ± 0.29def	71.11 ± 0.23dc
H_2O_2 (4 h)	87.77 ± 0.32def	81.11 ± 0.26bc
SW + H_2O_2 (4 h)	85.55 ± 0.32def	86.66 ± 0.29ab
SW + ETH + H_2O_2 (4 h)	90.00 ± 0.35bdec	87.77 ± 0.32ab
SW + H_2O_2 (4 h) + PPM	88.88 ± 0.33cdef	85.55 ± 0.29ab
SW + ETH + H_2O_2 (4 h) + PPM	94.44 ± 0.46bdac	93.33 ± 0.42a
H_2O_2 (7 h)	96.66 ± 0.58bac	93.33 ± 0.42a
SW + H_2O_2 (7 h)	98.88 ± 1.00a	94.44 ± 0.46a
SW + ETH + H_2O_2 (7 h)	97.77 ± 0.71ba	92.22 ± 0.39a
SW + H_2O_2 (7 h) + PPM	96.66 ± 0.58bac	92.22 ± 0.39a
SW + ETH + H_2O_2 (7 h) + PPM	96.66 ± 0.58bac	94.44 ± 0.46a

Values sharing the same letter in each column are not significantly different from each other (Fischer's least significance comparison test, $p < 0.0001$)

CHL chlorine gas, *SW* soapy water, *ETH* 70 % ethanol, *Bleach* 25 or 50 % commercial Clorox bleach containing 5.25 % NaOCl, H_2O_2 3 % hydrogen peroxide, *PPM* plant preservative mixture

for accession-II seeds. The only treatment that resulted in a significant difference in germination rate was CHL (17 h) + ETH.

Sodium hypochlorite (bleach) is the most common sterilization agent used for seed and explant sterilization in many plants. The concentration and exposure of bleach can vary from species to species. Bleach based sterilization protocols are often combined with 70 % ethanol rinse. We used this combined protocol to sterilize many cotton seeds until we received a limited quantity of heavily contaminated seeds. When accession-I seeds were sterilized using different bleach treatments, the highest germination rate of 71.11 and 85.55 % contamination free seeds were obtained after SW + ETH + Bleach 50 (30 min). All bleach treatments provided good results for accession-I seeds with a germination rate ranging from 56.66 to 71.11 % and sterilization efficiency of 77.77–85.55 %. In contrast, accession-II seeds, regardless of the bleach treatment, could not be sterilized efficiently. The percentage of contamination free accession-II seeds ranged from 20 to 24.44 %, and germination rates ranged from 2.22 to 4.44 %.

Hydrogen peroxide solution (3 %) has been previously used to sterilize seeds and improve germination of Douglas-fir, wax currant, and barley (Dumroese et al. 1988; Rosner et al. 2003; Çavusoglu and Kabar 2010). Higher concentration of hydrogen peroxide (5 %) was reported to negatively affect seed germination in sunflower and rape seed (Dolatabadian and Modarressanavy 2008). In preliminary experiments with heavily contaminated accession-II cotton seeds, overnight exposure with 3 % hydrogen peroxide resulted in sterilization efficiency of 85–90 % with nearly 100 % germination (data not shown). Unfortunately, the development of most of the germinated seeds in this experiment was arrested as the cotyledons failed to unfold and the hypocotyls failed to elongate. The remainder of the seedlings with properly unfolded cotyledons had burned areas on the cotyledons, while the hypocotyls were very short and stocky. Thus, these contamination free seedlings could not be used for cotyledon or hypocotyl explants. To reduce the detrimental effects, overnight exposure to hydrogen peroxide was reduced to 7 and/or 4 h. Sterilization of accession-I seeds with various combinations of hydrogen peroxide treatments for 4 h exposure resulted in a high percentage of contamination free seeds and germination rate (Table 1). Exposure of accession-I seeds to 7 h of hydrogen peroxide slightly improved the percentage of contamination free seeds and the germination rate. Two treatments, SW + ETH + H_2O_2 (7 h) and SW + H_2O_2 (7 h), resulted in a significant increase in the percentage of contamination free seeds in contrast to the

Table 2 Effect of different sterilization procedures on percentage of contamination free and germination rate in cotton seeds of accession-II

Sterilization treatment	Contamination free seeds (% mean ± SE)	Germinated seeds (% mean ± SE)
CHL (17 h)	18.88 ± 0.26d	3.33 ± 0.58c
SW + CHL (17 h)	18.88 ± 0.26d	4.44 ± 0.51c
CHL (17 h) + ETH	28.88 ± 0.23dc	7.77 ± 0.39bc
CHL (17 h) + ETH + PPM	25.55 ± 0.24d	5.55 ± 0.46c
SW + ETH + Bleach 25 (20 min)	20.00 ± 0.26d	4.44 ± 0.51c
SW + ETH + Bleach 25 (30 min)	20.00 ± 0.26d	2.22 ± 0.71c
SW + ETH + Bleach 50 (20 min)	24.44 ± 0.24d	3.33 ± 0.58c
SW + ETH + Bleach 50 (30 min)	22.22 ± 0.25d	2.22 ± 0.71c
H_2O_2 (4 h)	44.44 ± 0.21ba	20.00 ± 0.26a
SW + H_2O_2 (4 h)	44.44 ± 0.21ba	16.66 ± 0.28ba
SW + ETH + H_2O_2 (4 h)	45.55 ± 0.21ba	17.77 ± 0.27ba
SW + H_2O_2 (4 h) + PPM	47.77 ± 0.21ba	21.11 ± 0.25a
SW + ETH + H_2O_2 (4 h) + PPM	57.77 ± 0.21ba	22.22 ± 0.25a
H_2O_2 (7 h)	43.33 ± 0.21bc	17.77 ± 0.27ba
SW + H_2O_2 (7 h)	45.55 ± 0.21ba	21.11 ± 0.25a
SW + ETH + H_2O_2 (7 h)	58.88 ± 0.21a	21.11 ± 0.25a
SW + H_2O_2 (7 h) + PPM	54.44 ± 0.21ba	26.66 ± 0.23a
SW + ETH + H_2O_2 (7 h) + PPM	58.88 ± 0.21a	26.66 ± 0.23a

Values sharing the same letter in each column are not significantly different from each other (Fischer's Least significance comparison test, $p < 0.0001$)

CHL chlorine gas, *SW* soapy water, *ETH* 70 % ethanol, *Bleach* 25 or 50 % commercial Clorox bleach containing 5.25 % NaOCl, H_2O_2 3 % hydrogen peroxide, *PPM* plant preservative mixture

other 7 h treatments. However, there was no significant difference found in germination rate for the accession-II seeds regardless of which 7-h hydrogen peroxide sterilization treatment was used (Table 2). In contrast, the mean percentage of accession-I seed germination was >90 % following sterilization with the same combinations of hydrogen peroxide with 7-h exposure (Table 1). This suggests that the low rates of germination were specific to accession-II and could not be improved to the same level as accession-I seeds.

Hydrogen peroxide appears to be the superior surface sterilization agent, resulting in lower contamination levels and higher germination rates than the bleach and chlorine gas treatments. This finding is particularly important when a limited quantity of heavily contaminated seeds is used. The best results with the highest percentage of contamination free seeds were obtained when either the SW + ETH + H_2O_2 (7 h) or the SW + ETH + H_2O_2 (7 h) + PPM treatment was used, while seed germination rate in both treatments was the same (Table 2). Since the addition of PPM^{TM} did not improve sterilization efficiency or germination rate and is an additional cost, the SW + ETH + H_2O_2 (7 h) treatment is more practical and is recommended for sterilization of heavily contaminated cotton seeds.

We compared several modifications of three major surface sterilization methods based on the use of chlorine gas, sodium hypochlorite or hydrogen peroxide using cotton seed accessions with different degrees of contamination. However, in our study we did not include the mercuric chloride that is also frequently used for surface sterilization of seeds and plant material of numerous plant species (Shyamkumar et al. 2003; Verma et al. 2011; Daud et al. 2012). This chemical is very dangerous because of its high toxicity and more difficult to dispose as a hazardous waste (Li et al. 2005; Jean-Philippe et al. 2012). The surface sterilization protocol based on chlorine gas was the least effective resulting in the lowest seed germination rate. However, Paz et al. (2004) and others showed that chlorine gas treatment for 17 h was effective for surface sterilizing soybean seeds while retaining a high germination rate. Sodium hypochlorite, the most commonly used sterilization agent, was successfully used in our lab on good quality cotton seeds with low or medium levels of contamination. Interestingly, Baiyeri and Mbah (2006) used sodium hypochlorite for effective surface sterilization of *Treculia Africana* and reported an increased seed germination rate. However, we find that sodium hypochlorite is not effective for surface sterilization of heavily contaminated cotton seeds and their germination rate remains low, which is

similar to results from previous studies when sodium hypochlorite was used to surface sterilize wheat seeds (Sauer and Burroughs 1986).

Previous studies have shown that a hydrogen peroxide treatment is effective for surface sterilization and improves germination rates for both pine (Hoefnagels and Linderman 1999; Cram and Fraedrich 2009) and safflower seeds (Lizarraga-Paulin et al. 2013). The improved germination may be due to the oxidizing activity of hydrogen peroxide, which suppresses the germination inhibitors activity in the seed coat (Ogawa and Masaki 2001). In addition, hydrogen peroxide is more effective than other sterilization agents for plant tissue (Dolatabadian and Modarressanavy 2008). However, it should be noted that one study indicates that hydrogen peroxide was ineffective for surface sterilization of rice seeds (Miche and Balandreau 2001).

We find that hydrogen peroxide is the best of the three sterilization methods tested, with the lowest contamination and highest germination rates. Among all hydrogen peroxide modifications tested, the lowest contamination and best germination rates were achieved following treatment with SW + ETH + H_2O_2 (7 h). Currently, we are using this protocol for surface sterilization of all cotton seeds received from different locations. This optimized treatment has allowed us to successfully sterilize cotton seeds from 55 accessions, consistently resulting in high germination rates (80–95 %) and low levels of contamination (6–8 %).

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