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Short Communication

AN EFFICIENT AND COST EFFECTIVE STERILIZING METHOD WITH LEAST MICROBIAL CONTAMINATION AND MAXIMUM GERMINATION RATIO FOR IN VITRO COTTON (*Gossypium hirsutum* L.) CULTURE

A. Bakhsh[1], E. Anayol[1], C. Sancak[2] and S. Özcan[3]

[1]Department of Agricultural Genetic Engineering. Faculty of Agricultural Sciences and Technologies Niğde University, 51240 Niğde, Turkey.
[2]Central Research Institute for Field Crops, Ministry of Food, Agriculture and Livestock, Ankara, Turkey
[3]Department of Field Crops, Faculty of Agriculture, University of Ankara, 06110, Dişkapi-Ankara, Turkey.
Corresponding author Email: abthebest@gmail.com; allah.bakhsh@nigde.edu.tr

ABSTRACT

Cotton (*Gossypium hirsutum* L.) is a highly significant crop because of its quality for textile industry of the world. With the advent of genetic engineering, cotton has gone through several genetic improvements that encode tolerance against biotic and abiotic stresses. The efficient seed sterilization method is essential for successful genetic improvement in cotton. The present study was conducted to compare different sterilizing agents employed for in vitro culture of cotton to find out an efficient, reproducible, cost effective and genotype independent in vitro seed sterilization procedure of cotton. The two cultivated cotton cultivars i.e. GSN-12 and Coker-312 were used for these studies that were further transformed with insecticidal genes under different promoters. The different sterilizing agents used (in separate or combination) were ethanol, commercial bleach (NaOCl), mercuric chloride ($HgCl_2$), Sodium Dodecyle (SDS), hydrogen peroxide (H_2O_2). The surface sterilized seeds of both cultivars were cultured on MS medium and data was recorded for contamination free seeds and germination ratio. The results showed that 5% n-Hexane in combination with 2% of H_2O_2 are the best sterilizing agents for both cultivars when compared to the efficacy of other chemicals. Seeds treated with n-hexane and hydrogen peroxide had minimum microbial contamination with improved germination rate. The sterilizing method was successfully used for surface sterilization of other cotton cultivars in our project. According to our knowledge, this is the first report of using n-hexane in combination with H_2O_2 for surface sterilization of cotton seed for in vitro culture studies.

Keyword: microbe contamination; cotton sterilization; germination; efficiency; tissue culture.

INTRODUCTION

Cotton is an economically important crop that is grown as a source of fiber, food and feed. The most important product lint provides a source of high quality fiber for the textile industry. The cotton seed is an important source of oil and a high protein product used as livestock feed (Bakhsh *et al.,* 2009; Keshmma *et al.,* 2008). Because of its high economic importance, considerable attention has been paid for improving cotton plants by conventional plant breeding methods (Agrawal *et al.,* 1997).

Cotton is one of the first crop that was commercialized with insect and herbicide traits back in 1996 (Rahman *et al.,* 2012). The seed, embryonic axes, hypocotyl and cotyledon explants have been used for in vitro gene manipulation studies (Gould *et al.,* 1998; Bakhsh *et al.,* 2012). An efficient seed sterilization protocol becomes a prerequisite for successful cotton regeneration and transformation in an order to introduce trait of interest.

Although aseptic conditions are usually practiced in plant tissue culture, microbes can be present in the explants (endophytic) or can be reintroduced from poor aseptic handling, unhygienic conditions in the laboratory or from laboratory instruments. Microbial contamination is a constant problem, which often compromises development of in vitro cultures (Webster *et al.,* 2003). These microbes compete adversely with plant tissue cultures for nutrients, and their presence often results in increased culture mortality or can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting (Oyebanji *et al.,* 2009).

It is evident that any crop seed collected from open field are heavily contaminated with microbes mainly fungi and bacteria (Odutayo *et al.,* 2007; Ahmad *et al.,* 2012). Thus the production of contamination free seedlings from such contaminated seed supply is considered a challenge. The earlier studies show that ethanol, sodium hypochlorite, mercuric chloride and SDS are the commonly used for surface sterilization of cotton seed (Rao *et al.,* 2011; Khan *et al.,* 2011; Daud *et al.,* 2012; Bajwa *et al.,* 2013). Mercuric chloride and SDS have also been used in combination to serve as surface disinfectants (Bakhsh *et al.,* 2012). Although hypochlorite, ethanol and mercuric chloride are good

sterilizing agents for other plant tissues but unfortunately these agents are unable to remove contaminants from cotton seed that is usually collected from the open field with insufficient storage conditions. Therefore, the present study was conducted to compare different sterilizing protocols employed for cotton in vitro culture and to find out the best, efficient and cost effective sterilization procedure that may result in least or no contamination in cotton seed cultures with improved germination rates.

MATERIALS AND METHODS

The seeds of two cotton cultivars (GSN-12 and Coker-312) were freshly collected from open field in Cotton growing zone of Turkey and sent to our laboratory. Approximately 100 sinker seeds of each cultivar were placed in a beaker. The seeds were delinted with commercial H_2SO_4 at a rate of 100 ml/kg of seed. While delinting, seeds were stirred slowly with spatula for 60 seconds. After removing the fuzz, seeds were again washed with tap water four times to remove the remaining acid from seeds and were further subjected to surface sterilization. Here on, all steps were performed in laminar flow cabinet to maintain aseptic conditions. Surface sterilization of seeds was performed in small bottles (500 ml) and 16 seeds of each replication were cultured in magenta box (Duchefa, Cat No. 1682). The cultures of disinfected seeds of both cultivars were placed in growth chamber at 26° C and 45 µMol photons m^{-2} s^{-1} light intensity in three replications. Following sterilization methods were employed:

Ethanol and Sodium Hypochlorite: Seeds were surface-sterilized by immersion in 70% ethanol for 10 min with continuous shaking, followed by 20% commercial bleach for 20 min. The surface-sterilized seeds were rinsed three times with sterile distilled water each washing for 5 min and cultured in magenta boxes containing MS medium without any plant growth regulator (MSO). The data for contamination and germination rates was recorded after six to seven days after surface sterilization.

Mercuric Chloride and Ethanol: Seeds of both cultivars were sterilized by stirring in 0.1 % (w/v) solution of mercuric acid ($HgCl_2$) for 10 min and washed subsequently three times with sterile distilled water, followed by one min soaking in 90% ethanol. The seeds were cultured on medium after proper washing with distilled autoclaved water.

Mercuric Chloride and Sodium Dodecyl: Using this procedure, the seeds were surface sterilized with 0.1% $HgCl_2$ and 0.1% SDS solution mixture for 30 minutes and rinsed with distilled autoclaved water three times. The surface sterilized seeds were further cultured on MS0 medium.

Hydrogen peroxide: Seeds of both cotton cultivars were surface-sterilized with 2% hydrogen peroxide (H_2O_2) for 30 mins and rinsed with distilled autoclaved water three for times. Each time few drops of Tween-20 were added in distilled autoclaved water. The seeds were further placed in magenta boxes to record microbial contamination and germination rate.

n-Hexane and Hydrogen peroxide: The commercially available n- hexane (5%) was used in combination with 2% hydrogen peroxide for 30 min to sterilize seed of both cultivars. The seeds were washed with distilled autoclaved water for three times and cultured on magenta boxes containing MS medium.

Following each sterilization, the seeds were placed on magenta boxes containing MS0 medium consisted of MS minerals and vitamins (Murashige and Skoog, 1962), 3% (w/v) sucrose and 0.3% (w/v) phytagel. The pH of the medium was adjusted to 5.8 prior to autoclaving. The magenta boxes were incubated for a week in a growth chamber under 16 hour light/8 hour dark photoperiod with light intensity of 45 µMol photons m-2 s-1 provided by cool white fluorescent tubes at 26^0C. The data was recorded and analysed statistically using one way analysis of variance. The fresh, healthy non contaminated seedlings were used to excise hypocotyl and cotyledonary nodes in further in vitro culture/genetic transformation experiments.

RESULTS AND DISCUSSION

The cotton seeds collected from field conditions for in vitro culture are heavily contaminated with microbes especially fungi (Klich *et al.*, 1986) compared to the seeds stored in a controlled environment that can lead to difficulties in proper surface sterilization of seeds and also results in poor germination. The present study was conducted to compare different sterilizing agents being used based on earlier available scientific data.

The commercial bleach is considered a very effective microbe killer and is used frequently for surface sterilization of plants for in vitro culture (Talei *et al.*, 2011). The concentration and exposure of bleach varies from species to species. Bleach based sterilization procedures are usually combined with 70 % ethanol rinse that is also considered a good sterilizing agent for many of the plants for in vitro culture (Aasim *et al.*, 2013). In our study, we found that surface sterilization performed with commercial bleach and ethanol is not enough to disinfect cotton seeds from microbes successfully as contamination was recorded in cultures and hence poor germination as well. Only 42.90 and 49.15% contamination free seeds were recorded in GSN-12 and Coker-312 respectively (Figure-1). The germination rate

of GSN-12 and Coker-312 was as 33.92 and 36.66 % of both cultivars respectively (Figure-2&3). These results are not in confirmatory with earlier reports which have reported sodium hypochlorite and ethanol as good sterilizing agents for cotton seed (Aragao *et al.,* 2005; Divya *et al.,* 2008; Ozygit *et al.,* 2009; Farahani *et al.,* 2010). The recent report from Barampuram *et al.,* (2014) also indicates that commercial bleach and ethanol are not good option for surface sterilization of cotton seeds.

The combination of mercuric chloride (0.1 %) and ethanol (90%) could not prevent our seed cultures from bacterial and fungal contamination. The percentage of contamination free seeds remained 45.39 and 52.62% in GSN-12 and Coker-312 respectively (Figure-1). The germination rate of GSN-12 and Coker-312 was as 35.70 and 41.77 % respectively. The contamination and poor germination was recorded (Figure-2&3). The results of the study are contrary to the reports available (Wang *et al.,* 2006; Daud *et al.,* 2009; Khan *et al.,* 2010; Bazargani *et al.,* 2013). However the results of mercuric chloride (0.1 %) in combination with SDS (0.1) were comparatively better as compared to bleach and ethanol but not the best. Many researchers have used these sterilizing agents successfully (Rashid *et al.,* 2008; Maqbool *et al.,* 2010; Bakhsh *et al.,* 2012) but we witnessed fungal contamination in our experiments using these sterilizing agents with less germination ratio of seeds. 66.12 and 68.44 % contamination free seeds were recorded in GSN-12 and Coker-312 respectively (Figure-1). The germination rate of GSN-12 and Coker-312 was noted as 44.37 and 49.14 % respectively (Figure-2&3).

Hydrogen peroxide solution as sterilizing agent has been reported for plants like Doug-las-fir, wax currant, pines, barley, safflower and cotton with improved germination (Dumroese *et al.,* 1988; Rosner *et al.,* 2003; Cram and Fraedrich, 2009; Çavusoglu and Kabar, 2010; Lizarraga-paulin *et al.,* 2013; Barampuram *et al.,* 2014). 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). However, higher concentration of hydrogen peroxide (5%) was reported to negatively affect seed germination in sunflower and rape seed (Dolatabadian and Modarressanavy, 2008), therefore, we decreased its concentration to 2% in our experiments. The contamination free cotton seed were recorded as 71.64 and 72.35 in GSN-12 and Coker-312 respectively (Figure-1) while the germination rate of seed cultures of GSN-12 and Coker-312 treated with hydrogen peroxide was calculated as 52.49 and 53.33% respectively (Figure-2 & 3). However, least contamination and maximum germination ratios of both cultivars were found in our seed cultures that were surface sterilized with n-hexane and H_2O_2 in combination (Figure-3). An appreciable percentage of contamination free seed was calculated as 90.25 and 92.55 in GSN and Coker-312 respectively while germination rate of GSN-12 and Coker-312 was recorded as 77.22 and 72.59 % respectively showing this combination as the most effective one (Figure-1 &2). The germinated seedlings were healthy, fresh and had robust rooting in medium.

In conclusion, we compared the competency of different surface sterilizing procedures for cotton seed available in literature. Based on our data, we conclude that n-hexane in combination with hydrogen peroxide is the best of all sterilization procedures tested, with the lowest contamination and highest germination rates. However the exact mechanism of n-hexane in combination with H_2O_2 needs to be fully understood.

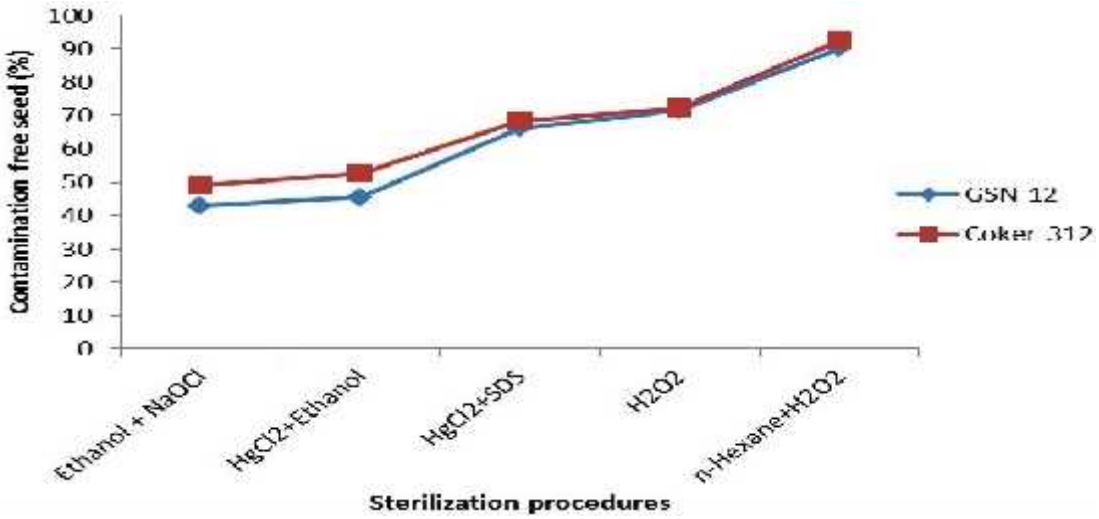

Figure-1. The percentage of contamination free seed in GSN-12 and Coker-312 subjected to different sterilization procedures.

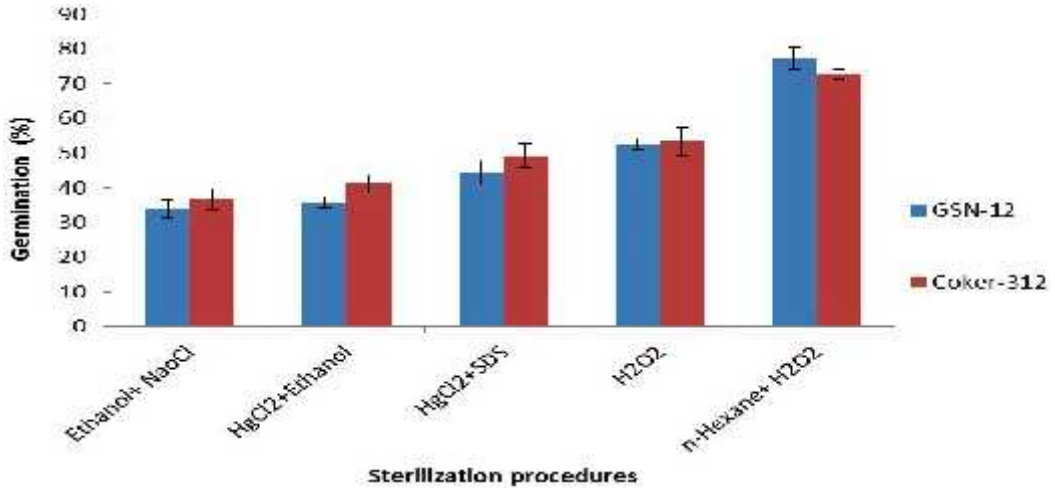

Figure-2. The germination ratio (%) of GSN-12 and Coker-312 cultivars subjected to different sterilization procedures

Figure-3: Germination of GSN-12 cotton seeds on MSO medium after 5-6 days later of seeds surface disinfection with different sterilizing agents. (A) Ethanol and Sodium hypochlorite, (B) Mercuric chloride and ethanol, (C) Mercuric chloride and SDS, (D) Hydrogen peroxide, (E) n-hexane and hydrogen peroxide. The data has been shown only of germination ratio (microbial contamination data has not been shown)

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