



A Comparative Analysis among Different Surface Sterilisation Methods for Rice *Invitro* Culture

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Authors' contributions

This work was carried out in collaboration among all authors. Author MS designed the study, performed the research and statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SS, BKS, MNA and NM managed the analyses of the study and given guidance for the study. All authors read and approved the final manuscript.

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ABSTRACT

Invitro tissue culture establishment is the most important phase in rice (*Oryza sativa* L.) genetic improvement studies. But, invitro tissue growth demands a sterile condition conducive for unhampered nutrient supply to the explant which otherwise face competition from unwanted microbial contamination of explant origin. To eliminate such contaminants, there are several surface sterilisation techniques available for *Invitro* culture establishment of mature rice seed explants, which include the application of either mercuric chloride or sodium hypochlorite as sterilizing agents. Therefore, a comparative study was conducted to determine the effect of these sterilization techniques on decontaminating the rice seed explants without affecting their invitro germination potential. The most effective sterilisation was obtained with 0.2% mercuric chloride treatment for 8 minutes, which produced 93.62± 0.191% of contamination free seeds. But, the highest germination (94.53± 0.210%) was obtained with 4% of NaOCl treatment for 10 minutes. The results demonstrated that the level of contamination decreases with increasing concentration

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of sterilising agents and the duration of the treatment. The germination percentage decreased with increasing concentration of mercuric chloride. Sodium hypochlorite enhanced germination until a concentration increase of 4% and then the germination percentage decreased with increasing concentration and treatment duration. These findings may help in determination of most appropriate surface sterilisation technique in rice for in vitro culture.

Keywords: Explants sterilization; rice seeds; mercuric chloride; sodium hypochlorite.

1. INTRODUCTION

Tissue culture procedures are the most important part in production of genetically modified rice plants and various physiochemical and metabolomics studies [1,2]. It requires a totipotent explant for primary establishment of invitro tissue culture, which is mostly a matured seed in case of rice [3,4]. But, the seed explants often act as a source for microbial contamination in in vitro culture which is considered to be the single most important reason for losses during in vitro culture of plants. Microbial contamination with viruses, bacteria, yeast, fungi, etc. adversely affect plant tissue cultures due to competition for nutrients [5,6].

The presence of these microbes usually result in increased culture mortality but can also result in variable growth, tissue necrosis, reduced shoot proliferation and reduced rooting [7,8]. Despite the best timing and selection efforts, it is almost impossible to eliminate contamination from in vitro grown plants and losses due to contamination in vitro average between 3 and 15% at every subculture in the majority of commercial and scientific plant tissue culture laboratories [9]. The cumulative result is an abundant waste of time, effort and materials which if not mitigated can have severe economic consequences [10].

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 [11,12,13]. Unfortunately, these agents often fail to efficiently remove contaminants, particularly when seeds are collected from the open field and stored under improper conditions.

A variety of sterilization agents employed for decontaminating the explants, are also toxic to the plant tissues. Hence appropriate concentration of sterilisers, exposure time and the sequences of using them has to be optimized to reduce explants damage and attain better survival [14]. Mercuric chloride (HgCl_2) is a very strong sterilization agent. Applying 0.1 or 0.2%

HgCl_2 for 3 minutes resulted in a significant decrease in contamination of barley seeds [15]. Due to high toxicity of HgCl_2 , its concentration and the time of exposure of explants need to be optimized to decrease tissue mortality of the explants [16]. Prolonged treatment with HgCl_2 decrease contamination, but also brings about reasonable decline in seed germination [17]. Clorox bleach alone was not able to control bacterial and fungal contamination in explants [13]. In this context our study was focused to optimize a protocol for surface sterilization of matured rice seed.

2. MATERIALS AND METHODS

The dehusked mature seeds (approximately 100 for each treatment) of *indica* rice cultivar, Shatabdi (IET-4786) were surface sterilized with 70% alcohol for 2 min under laminar air flow cabinet and washed thrice with double distilled water. These seeds were then surface sterilized in either sodium hypochlorite or mercuric chloride solutions with Tween 20 (one drop per 50 ml volume). The concentrations of these sterilizers and time of sterilization were selected as different treatments (Table 2). After this, the seeds were rinsed in sterile water (3 min each) and dried on sterilized Whatman sheet No. 1. The seeds were then plated in MS (Murashige and Skoog) basal medium (15 seeds per plate) and incubated at a temperature of $25 \pm 2^\circ\text{C}$ and relative humidity of 50–60% with a photoperiod of 16 h day light and 8h dark. There were five replications for each treatment consisting twenty seeds per plate. After 10 days of culture, the plates were checked to take observations on occurrence of microbial contamination and germination. Data from the treatments were analyzed by the SPSS statistical software. The ANOVA and Duncan tests were used to compare treatment groups to find out whether they showed any statistically significant differences with significance level (α) set at 0.05.

3. RESULTS

It has been reported that the rice seeds collected from the open field and stored improperly are

often severely infected by a plethora of bacterial and fungal pathogens [7,18,19] 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. There are several established methods available to surface sterilise rice seeds for cultivation under *in vitro* condition. In this article, we compared the most commonly used methods including the use of $HgCl_2$ and NaOCl.

According to the analysis of variance (ANOVA), the effects of different surface sterilizing techniques (different concentrations and exposure time of sodium hypochlorite and mercuric chloride treatments) on contamination free seed production and germination percentage were significant (Table 1).

The data in Table 2 represents the effect of different surface sterilisation techniques consisting different strength of sterilising agents and duration of treatment. The effect was presented in terms of contamination free seed percentage as well as percentage of germination. There was significant variations among the different treatments. The highest contamination free seeds were obtained from 0.2% $HgCl_2$ treatment for 8 minutes as $93.62 \pm 0.191\%$. However, the highest seed germination was obtained from 4% NaOCl treatment for 10 minutes as $94.53 \pm 0.210\%$. The treatment control resulted in lowest contamination free seeds i.e. $28.19 \pm 0.233\%$. The germination percentage for this ($68.84 \pm 0.197\%$) was even higher than the 0.1% $HgCl_2$ treated seeds for 2hr ($65.99 \pm 0.268\%$).

When we only consider about the $HgCl_2$ treatments, highest germination was obtained from 0.1% $HgCl_2$ treated seeds for 10 min, i.e. $79.16 \pm 0.129\%$. The contamination free seed percentage gradually increased with increasing concentration and time duration. But the germination percentage was also decreased with this gradient.

In case of NaOCl treatments, the contamination free seed percentage was increased with higher concentration of NaOCl and time duration of NaOCl treatment. But the germination percentage gradually increased upto 4% NaOCl treatment for 10 min and then decreased with increasing concentration and duration of the treatment. Even though, the primary purpose of surface sterilisation is to eliminate all the microbial contaminants, the explants must retain their germination potential after the treatment.

4. DISCUSSION

We compared several rice seed surface sterilization methods used in rice tissue culture technique. These methods are based on the use of mercuric chloride or sodium hypochlorite with different degrees of concentration and treatment duration. During sterilization, the living materials should not lose their biological activity and only contaminants should be eliminated; therefore explants are surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period [7].

Mercuric chloride is a frequently used for surface sterilization of seeds and plant material of numerous plant species [29,30,13]. We observed highest percentage of contamination free seeds using $HgCl_2$ treatment. Mercury ions in solution work in sterilization by interfering with enzymes and protein in the cell membrane and cytoplasm of microorganisms [31]. Mercuric chloride has been found to be the best sterilizing agent for the seeds of alfalfa and white clover [17]. Maximum (90%) sterilization of seed has also been obtained by using 0.2% $HgCl_2$ for 12 minutes [13]. Lower sterilization (73.6%) was achieved for the rice seeds by using ethanol and Clorox in combination instead of $HgCl_2$ [32]. Some researchers have reduced contamination to about 5% by using 0.1% $HgCl_2$ [33]. However, in our study, the germination percentage of $HgCl_2$ treated seeds gradually decreased with increasing concentration of $HgCl_2$ and the duration of treatment. This chemical is very dangerous because of its high toxicity and more difficult to dispose as a hazardous waste [34,35].

Table 1. Analysis of variance of measured parameters

	Sum of squares	Degrees of freedom	Mean square	F
Contamination free seed (%)	16859.961	9	1873.329	8882.486
Germination (%) after heat treatments	6124.241	9	680.471	2759.889

*Significant at $P=0.05$ level

Table 2. Effect of surface sterilizer, various concentrations and time exposure on% of contamination and% of germination of mature rice seed explants after 10 days from culture (20 seeds/plate)

Variants	Adoption source	Surface sterilizer	Concentration	Exposure time	Contamination free seed (% mean \pm SE)	Germination (% mean \pm SE)
V1	Ahmad et al (2016) [20]	Mercuric Chloride (HgCl ₂)	0.2%	8 min	93.62A \pm 0.191	55.68J \pm 0.307
V2	Chun et al (1997) [21]		0.1%	2hr	90.38B \pm 0.178	65.99I \pm 0.268
V3	Raman et al (2018) [22]		0.1%	10 min	83.72D \pm 0.095	79.16E \pm 0.129
V4	Kumar et al (2005) [23]		0.1%	5 min	81.62F \pm 0.117	77.05F \pm 0.211
V5	Tran and Mishra (2015) [24]	Sodium hypochlorite (NaOCl)	6%	45 min	85.90C \pm 0.190	86.63C \pm 0.210
V6	Sahoo et al (2011) [25]		6%	30 min	83.05E \pm 0.286	90.11B \pm 0.197
V7	Yaqoob et al (2017) [26]		4%	10 min	74.76G \pm 0.301	94.53A \pm 0.210
V8	Hiei and Komari, (2008) [27]		2%	30 min	64.86H \pm 0.178	79.79D \pm 0.211
V9	Nishimura et al. (2006) [28]		1.5%	30 min	61.14I \pm 0.191	76.32G \pm 0.235
V10			Control	-----	-----	28.19J \pm 0.233

Values sharing the different letter in each column represent significantly different Duncan's multiple range test grades (significance at $P=0.05$)

Sodium hypochlorite (bleach) on the on the hand is the most common sterilization agent used for seed and explant sterilization in many plants. The concentration and exposure of bleach vary from species to species. It is known to be a very effective killer of bacteria; even micromolar concentrations are enough to reduce bacterial populations significantly. When diluted in water, the hypochlorite salts [NaOCl, Ca (OCl)₂] lead to the formation of HOCl whose concentration is correlated with bactericidal activity [36]. A balance between concentration and time must be determined empirically for each type of explants because of phytotoxicity [37]. We observed low to moderate level of contamination in NaOCl treated seeds. The highest contamination free seeds were obtained with 6% NaOCl treatment, but the highest germination was obtained with 4% NaOCl treatment. An effective surface sterilization and increased seed germination rate of *Treculia Africana* was also obtained with NaOCl treatment [38]. Our study points out that effective NaOCl treatment may decrease the level of contamination but the increase in germination are limited to a certain extent and do not follow an unlimited progression trend unlike lower contamination.

5. CONCLUSION

The results of the study indicate a differential response of sterilising agents on in vitro germination of rice seeds. Mercuric chloride proved to be the most effective agent for removal of microbial contaminants at 0.2% concentration for 8 minutes of treatment. However, it adversely affected the germination. Sodium hypochlorite proved to be lesser effective for seed sterilization in comparison with mercuric chloride. However, it produced 85.90± 0.190% of contamination free seeds after treatment with 6% concentration for 45 minutes. The germination percentage increased up to 94.53± 0.210% after treatment with 4% concentration for 10 minutes. This reflects the positive effect of sodium hypochlorite treatment on germination. Therefore, it is very important to standardize the concentration of sterilising agent and the duration of the treatment. In conclusion, we recommend the application of 4% sodium hypochlorite treatment for optimum sterilisation effect, which also enhances germination in return.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our

area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors. The authors gratefully acknowledge the DBT-JRF fellowship Fellow ID: DBT/2016/BCW/685, New Delhi, India to Monoj Sutradhar for financial support throughout the term.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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