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Research Paper

Seed-sterilization of *Rhododendron wardii* for micropropagation

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Abstract: Success in tissue culture of all plant species depends on the removal of exogenous and endogenous contaminating microorganisms. *Rhododendron wardii* is a parent of many of the best yellow hybrids and it has been identified as a Least Concern spp. An efficient protocol was developed for sterilization of seeds of *R. wardii* for *in vitro* cultivation for mass propagation and conservation. Three sterilizing agents, namely, $HgCl_2$ (w/v), NaOCl (v/v) + Tween20 and H_2O_2 (v/v) at varying their concentration and time of exposure were tested. Treatment with 25% H_2O_2 for 20 min was the most effective with no contaminated cultures, where the germination percentage was 92.6% (*ex vitro* germination percentage was 58.61%) and produced 100% healthy plants. Even though the exposure to 15% NaOCl + Tween 20 for 20 min produced 100% contamination-free cultures, the percentage seed germination was 51%. Seeds could be sterilized with 5% NaOCl, for 20 min and 15% for 10 min for maximum germination (64%), but contamination rate was 1:2. Sterilization with $HgCl_2$ did not produce any satisfactory result (maximum germination percentage = 18%).

Keywords: *Rhododendron wardii,* conservation, micropropagation, seeds, sterilization

Introduction

Rhododendron wardii is native to Sichuan, Xizang and Yunnan areas in China and southeastern Tibet. It has been evaluated as a Least Concern spp. (Gibbs *et al*., 2011, Oregon State University, 2015). Flowers are bowl or cup shaped, clear lemon to bright yellow, occasionally flushed with green or with a crimson blotch, in loose clusters of 5 to 14. The plant is a parent of many of the best yellow hybrids (Forster, 2000). The regeneration status in the form of available seedlings/saplings is poor for many of the rhododendrons. Tissue culture is the only method to maintain and propagate the genetically-identical clone rapidly in large numbers and in long term culture (Singh *et al*., 2013). The fruit of the *Rhododendron* is a five to ten-celled capsules. Each capsule normally contains many seeds. In the case of true species, each viable seed will reproduce substantially the same characteristics as the parent plant. Grown from seeds may be in an effort to secure new and better varieties (Fillmore, 1949). Use of seeds as starting material, rather than propagating new plants from pre-existing individual plants, is a more advantageous strategy for the species

conservation, as the former method preserves the genetic diversity of the populations (Cantos *et al*., 2007).

In vitro propagation involves several steps such as selection of explants, culture establishment, multiplication, rooting and acclimatization. Regarding the *Rhododendron*, the most challenging step is the sterilization of explants for proper culture establishment. Use of proper sterilization procedure leads to save time, effort and material, which if not mitigated, can have serious economic problems. Since *Rhododendron* is a slow-growing annual bearing woody plant, save planting materials and time is important.

Commonly used surface sterilization agents include Ethanol, Sodium Hypochlorite (NaOCl), Calcium Hypochlorite, and Mercuric Chloride ($HgCl_2$), which have been used for surface sterilization of plant and seed material of various species. Unfortunately these agents often fail to remove contaminants efficiently, particularly when seeds are collected from the open field and stored under improper conditions. Less commonly used agents such as hydrogen peroxide (H_2O_2) have been successfully used for sterilization of seeds and plant material (Barampuram *et al*., 2014). Disinfection agents used for surface sterilization of explants can also be toxic to plant tissues and therefore, a balance between the level of contamination and explant survival should always be considered when using disinfection agents (Silva *et al*., 2015). Unfortunately, literature does not reveal information on influence of sterilizing compounds on establishment of explants belonging to *Rhododendron* species.

A high yield of viable explants should be determined experimentally (Kutas and Ogorodnik, 2011) in order to identify the optimum regime of sterilization. The procedure of sterilization varies depending on plant species and explant taken for sterilization. It is difficult to determine standard sterilization procedures that apply to all plants and all explants. There is no study yet documented on the success or failure of *in vitro* seed germination of *R. wardii*. Therefore, an experiment as conducted to find the most suitable sterilization procedure to reduce the contamination and achieve efficient micropropagation of *Rhododendron* seeds.

Materials and Methods

The experiment was conducted at the Flower Research Institute of Yunnan Academy for Agricultural Sciences, Kunming, China. Healthy mature seed capsules (fruits) of *R. wardii* were collected from the native populations in Xianggelila, Yunnan. Seeds in all treatments were first dipped in 70% alcohol for one min and subsequently washed with double distilled (2D) water for two times. Then seeds were surface sterilized with three different concentrations of $HgCl_2$ (w/v), NaOCl (v/v) + Tween20 and H_2O_2 (v/v) for different time periods (Table 1).

Table 1. Sterilization treatments

Chemicals	Concentrations	Different time durations of seed exposure (min)	Washing with double distilled (2D) water after sterilization
Mercuric chloride ($HgCl_2$)	0.05%, 0.1%, 0.05% (w/v)	2, 4 and 6	Six times
Sodium hypochlorite (NaOCl) + Tween20	5% , 10% , 15 % (v/v)	10,15 and 20	Three times
Hydrogen peroxide (H_2O_2)	20%, 25% and 30% (v/v)	10,15 and 20	None

Seed establishment and nutrient medium:
After sterilizing in each treatment, seeds were kept for 20 min within the laminar flow cabinet for air drying. Then the treated seeds were transferred into Petri dishes with Woody Plant Medium (WPM) nutrient medium. Around 50 seeds were used in one treatment and each was replicated three times. The WPM was prepared without hormones and the pH of the medium was adjusted to 5.4. The WPM was sterilized at 121 °C for 20 min at 1.06 Kg/cm^2 pressure and 15 ml of this medium was used in 6 cm diameter sterilized Petri dishes. The Petri dishes with transplanted explants were placed in a growth room (temperature 24±2 °C, illumination 4000 lux, RH 70%; photoperiod 16h). Seedlings were raised up to four-leaf stage to observe their healthiness. At the same time, formal *ex vitro* seed viability test was done in Petri dishes.

Data recording and analysis:
Each experiment was conducted at least twice. The degree of contamination and percentage of germination were recorded 30 days after sterilization (DAS). Calculations were done using MS Excel Worksheet.

Results and Discussion

Surface sterilization of seeds is an essential pre-requisite for the primary establishment of *in vitro* culture and production of seedlings in any tissue culture experiment. This is to minimize the contamination as the culture medium used in tissue culture techniques is generally suitable for the growth of the microbes. The data generated on the efficacy of the *in vitro* seed germination of *R. wardii* with the three sterilants, namely, Mercuric Chloride, Hydrogen Peroxide and Sodium Hypochlorite at different concentrations and for different exposure duration are shown in Table 2.

The *ex vitro* seed germination test resulted in an average seed germination of 58.61% (Figure 1a). The results presented in Table 2 revealed that the *R. wardii* seeds treated with $HgCl_2$ had a low percentage of germination. All $HgCl_2$ treatments, except that where seeds were sterilized with 0.05% $HgCl_2$ for 2 min, produced contamination-free cultures. However, at 30 days after seedling growth, increasing exposure time and concentration of $HgCl_2$ significantly reduced contamination but had an adverse effect on germination and further growth of plants with seedlings developing abnormalities (Figure 1b). This result contradicted those reported by Kutas and Ogorodnik (2011) who reported that sterilization with 0.1% solution of $HgCl_2$ for 5 min was the most effective in seed sterilization of eight *Rhododendron* species. Alam *et al*. (2016) found that surface sterilization of *Cucumis sativus* seeds with $HgCl_2$ was more effective, ensuring vigorous and high germination rate and contamination-free cultures than H_2O_2 and NaOCl. Alam *et al.* (2016) explained that such responses may be due to the bleaching action of two chloride atoms and also ions that combine strongly with proteins and causing the death of organisms. Himabindu *et al*. (2012) reported that the seed exposure to $HgCl_2$ led to browning and death of *Solanum lycopersicum* seeds and there is a deleterious effect of $HgCl_2$ at higher concentration and longer exposure. Alam *et al*. (2016) further reported that the requirements for sterilization are different and depend on the tssues and the type of the explants used for micro-propagation (Alam *et al.*, 2016).

Furthermore, NaOCl being a mild sterilizing agent resulted in a higher percentage of contamination. When the seeds were soaked at 5% concentration of NaOCl for 20 min and 15% for 10 min, recorded more than 60% germination and healthy plants (Figure 1c), however, the treatment resulted in a contamination level os 1:2 contamination level. The NaOCl treatment for 20 min at 15% concentration showed a germination of 51% and zero level of contamination (Table 2). When NaOCl is diluted with water, the hypochlorite ions could lead to formation of HClO, which is negatively correlated with bactericidal activity, perhaps in part due to lethal DNA damage (Alam *et al*., 2016). Sterilization using NaOCl also resulted in an increase of the time taken to germination, while the increased concentration of the chemical decreased the contamination and the germination percentage. Sen *et al*. (2013) also reported similar result with *Achyranthes aspera* seeds. This could be explained

with the studies done by Abdul-Baki (1974) adequate quantities of NaOCl would remain on the seeds even after repeated washings and that this situation could alter the pattern of amino acid metabolism. The residual NaOCI would react with the amino acids and reduce their concentrations in the incubation media. This reaction also could result in higher production of CO_2 and low uptake of amino acids by the seeds. Abdul-Baker (1974) further stated that lower concentrations of NaOCl were less effective.

Table 2. Effect of sterilization with $HgCl_2$, NaOCl + Tween20 and H_2O_2 at three different concentrations for three different time periods on contamination and germination of *Rhododendron wardii* seeds

Sterilizing Agent	Concentration %	Time of Exposure (min)	Contamination Ratio*	Germination %
$HgCl_2$ (w/v)	0.05	2	1:2	18.00
		4	0:3	11.70
		6	0:3	6.38
	0.1	2	0:3	2.83
		4	0:3	7.78
		6	0:3	0.00
	0.15	2	0:3	1.82
		4	0:3	0.00
		6	0:3	0.00
NaOCl (v/v) + Tween 20	05	10	2:1	54.55
		15	1:2	36.69
		20	1:2	63.82
	10	10	2:1	38.46
		15	2:1	32.00
		20	1:2	42.29
	15	10	1:2	63.69
		15	1:2	59.84
		20	0:3	51.41
H_2O_2 (v/v)	20	10	1:2	91.44
		15	0:3	91.22
		20	0:3	91.11
	25	10	1:2	86.92
		15	0:3	88.45
		20	0:3	92.60
	30	10	1:2	55.89
		15	0:3	72.10
		20	0:3	51.99

* Contamination Ratio = Contamination : Non-contamination)

Seeds treated with H_2O_2 yielded the best results on seed germination (Table 2). When compared to the seeds germinated *ex vitro*, those treated with H_2O_2 showed a higher germination percentage (Table 2). Treatment with 25% H_2O_2 for 20 min had the highest percentage of germination (92.6%) with zero level of contamination. At the lowest concentration of H_2O_2 (20%) coupled with the shortest duration of seed exposure (10 min), the *Rhododendron* seeds showed 91.4% germination. At the exposure time of 10 min, the three concentrations used showed a contamination level of 1:2. However, all the other treatments with H_2O_2, produced contamination-free cultures.

Similar to other two chemicals, the increase in concentration of H_2O_2 and duration of seed exposure have decreased the germination percentage. This may be due to the tissue damage caused by these chemicals. Curvetto *et al*. (2006) reported that addition of H_2O_2 as a chemical sterilizer to the culture medium and during explant

manipulation following aseptic techniques might preserve the medium and lead to explants free from contamination. Curvetto *et al*. (2006) further explained that H_2O_2 has germicidal and fungicidal activities at low concentrations, without affecting *in vitro* seed germination and seedling growth. Lack of cellular and tissue damage has been attributed to the activity of plant peroxidases that act against H_2O_2 by transforming it into water and oxygen. Many plant cells contain enzymes, including catalases and peroxidases that decompose H_2O_2, thus providing a protection mechanism to preserve cells from the deleterious effects of peroxides produced either by their own metabolism or from an external source. By 45 days and 90 days after sowing, the seedlings from H_2O_2 treatment were (Figure 1d-g) appeared with full size cotyledons.

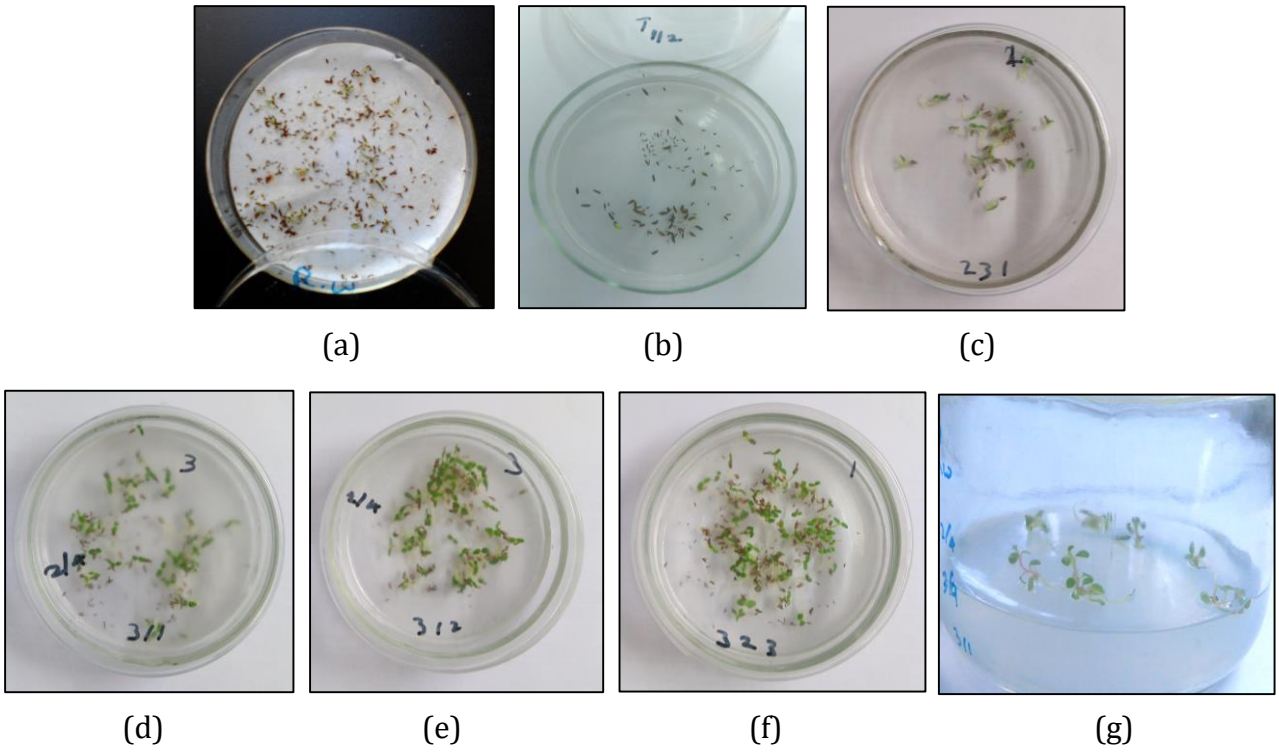

Figure 1: *Ex vitro* and *in vitro* germinated seeds of *Rhododendron wardii* to evaluate the effective sterilization procedure. (a) *ex vitro* germinated seeds in Petri-dish method after 15days, (b-f) *in vitro* grown seedlings at 45 days after sowing, (b) abnormal seedlings from the treatment 0.05% $HgCl_2$ for 4 min., (c) seedlings from 15% NaOCl for 10 min., (d) seedlings from 20% H_2O_2 for 10min., (e) seedlings from 20% H_2O_2 for 15 min., (f) seedlings from 25% H_2O_2 for 20min., and (g) 90-days old seedlings from H_2O_2 treatment

Even though NaClO + Tween 20 increased the percentage germination of *Rhododendron* seeds up to some extent, it also inhibited the seed germination at higher concentrations and longer seed exposure to those concentrations. Overall, the most effective chemical was the H_2O_2 where seed sterilizations with the chemical produced contamination-free environment and increase of seed germination. Similar results were observed by Srivastava *et al.* (2010) with *Aconitum heterophyllum*, Barampuram *et al*. (2014) with cotton seeds, and Miche and Balandreau (2001) for rice seeds.

Conclusion

Surface sterilization of *R. wardii* seeds with 25% H_2O_2 for 20 min was effective to produce higher germination rate and contamination-free cultures. Use of H_2O_2 for seed-sterilization is a safe and efficient method to obtain healthy plants *in vitro* for its mass propagation and conservation

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