Chiang Mai J. Sci. 2019; 46(6) : 1107-1112
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Improvement in Seed Surface Sterilization and *in vitro* Seed Germination of Ornamental and Medicinal Plant-*Catharanthus roseus* (L.)

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Received: 15 July 2018
Revised: 21 September 2019
Accepted: 23 September 2019

Abstract

Catharanthus roseus (L.) also known as Madagascar periwinkle is a valuable ornamental and medicinal plant mostly due to possessing two pharmaceutical important anticancer compounds; vincristine and vinblastine. Successful and efficient methods for seed surface sterilization and *in vitro* seed germination of *C. roseus* have been established in this study. Our analysis indicated that the effect of the sterilizing treatments were significant ($p \leq 0.05$) for all the germination parameters assessed, namely: NDFG (number of days of first germination), GP (germination percentage), MGT (mean germination time), GR (germination rate) and CP (contamination percentage). The maximum GP (94.66%) was observed in sterilized distilled water for 20 min as control whereas the lower GP (2.66%) was observed in the 0.3% mercuric chloride treatment. For the surface sterilization treatments, we observed the highest percentage of contamination (94.66% CP) in control seeds whereas the lower CP was achieved by 5% Sodium Hypochlorite for 10 min and 0.3% mercuric chloride for 6 min (14.66%, 13.33%, respectively). In addition, the effect of concentration of minerals and sugars of germination medium (sucrose and glucose) were significant ($p \leq 0.05$) for MGT, GR, GP, and NDFG. The maximum GP (74.66%) were achieved by mineral water medium while the minimum (48.0%) was observed in MS agar medium with 3% sucrose. The maximum length of shoot and root of *in vitro* developed seedlings was achieved with the mineral water treatment. According to our results, we established the best sterilization method (3% Sodium Hypochlorite for 5 min) and germination medium (mineral water, using paper boat method) for *in vitro* seed germination and seedling development in *C. roseus*.

Keywords: *Catharanthus roseus,* seed surface sterilization, seed germination

1. Introduction

Contamination caused by bacteria, fungi, viruses, yeast, etc. are considered as the main causes of loss of samples during *in vitro* plant tissue culture. Sterilizing agents used during sanitization processes should not affect the biological activity of the tissues while eliminating contaminants. Hence, it is important to apply these sterilizing agents at suitable concentrations for a specified period. In spite of the best concentration and exposure time, it is impossible to remove all the germs from plant tissue with a 3 to 15 percentage of contamination occurring over in plant propagated

under laboratory condition [1]. The common disinfectant agents which are widely recommended in plant tissue culture techniques are sodium hypochlorite (NaClO), calcium hypochlorite ($Ca(ClO)_2$), mercuric chloride ($HgCl_2$), silver nitrate ($AgNO_3$), ethanol (C_2H_6O) and hydrogen peroxide (H_2O_2) [2].

In order to increase the population size as well as preparation of sterilized tissue explants for further experiments, *in vitro* seeds germination can be considered as an alternative procedure. The main factors which affect the *in vitro* seed germination are selecting an appropriate germination medium, the concentrations of sugar, minerals and also additives, if required [3].

C. roseus (*Apocynaceae*) also known as Madagascar periwinkle is a valuable ornamental and medicinal plant mostly due to possessing two pharmaceutical important anticancer compounds; vincristine and vinblastine [4].

The seed, cotyledon and hypocotyl explants of Madagascar periwinkle have been used for *in vitro* secondary metabolites production and gene manipulation studies. Therefore an efficient seed surface sterilization and *in vitro* seed germination protocol becomes a prerequisite for successful callus induction, regeneration and genetic transformation in an order to introduce trait of interest.

Hence, in the present study we attempt to enhance the efficacy of seed surface sterilization protocol as well as *in vitro* seed germination medium for *Catharanthus roseus* (L.).

2. MATERIALS AND METHODS

2.1 Plant Material

The seeds of *C. roseus* procured from Pakan-Bazr Company, Esfahan, Iran (Figure 1A). The current experiments were attended at the Department of Plant Production, Torbat Heydarieh University, Iran with the objective to enhance the effectiveness of the sterilizing agents on seed surface sterilization with piddling side effect on seed germination values and optimization of *in vitro* seed germination medium composition in order to obtain maximum percentage of *in vitro* seed germination as well as seedling development.

2.2 Sterilization Treatments

Seeds of *C. roseus* were hand-sorted before sterilization. For each treatment 25 seeds were applicated and were soaked in distilled water (DW) for 12 hours. The soaked seeds were then treated with different concentrations of sterilizing agents and exposure times under sterile condition on a laminar flow cabinet as presented in table 1:

The seeds afterwards were rinsed three times with sterile DW and were inoculated in test tubes containing sterilized mineral water (MW) using paper boat method (Figure 1C). These tubes were shifted to the culture room with 25°C temperature and 16/8 hours day light provided by fluorescent tube lights (intensity of 40 μmol/ms).

2.3 Germination Medium Treatment

In order to optimize the *in vitro* seed germination, the seeds were surface sterilized with NaClO 3% (w/v) for 5 min (as the best result of section 2.2) and transferred aseptically on various fifteen mediums as showed in table 2.

The pH of culture medium was adjusted to 5.8 (by using either HCl or NaOH), distributed into vessels and autoclaved at 15 Psi, 15 min, 121°C.

2.4 Data Analysis

In order to determine the seeds germination parameters we measured the radical emergence duration. The *C. roseus* seedlings were developed in the same time have been chosen and picked up to measure the length of shoots and roots. Twenty five healthy seeds were used for each trial and all the trials were repeated at least three times. IBM SPSS software (version 20) was applied in order to carry out the ANOVA and Duncan Multiple Range Test (DMRT) ($P \leq 0.05$). GP (germnation percentage), MGT (mean germination time), GR (germination rate) and CP (contamination percentage) were recorded daily for 15 days. Calculation of the seeds germination values have

been done according to Younesikelaki et al. [5] description.

3. RESULTS AND DISCUSSION

3.1 Seed Surface Sterilization

Various sterilizing agents were applied at different concentrations and exposure times in order to determine the optimum protocol for seed surface sterilization and initiation of tissue culture of *C. roseus* (Table 1). Germination of seeds of *C. roseus* was started within one to three days of incubation. Treatment with absolute ethanol as well as sterilized DW was ineffective; more than 90% contamination in both and no growth of explants in the case of absolute ethanol has been observed. Whereas dilution of ethanol up to 70% increased the suitability of ethanol using as seed surface sterilizing agent.

Table 1. Effect of diverse sterilization methods on *in vitro* seeds germination characteristics ± std. error in *C. roseus*.

Conc. of Sterilizing agent (%) and Duration of Treatment	MGT±SE	GR±SE	GP±SE	CP±SE	NDFG±SE
Sterilized Distilled Water 30 min	3.68±0.82^{abc}	7.39±0.13^{g}	94.66±1.33^{m}	94.66±2.66^{l}	1.33±0.33^{a}
Mercuric Chloride 0.025 %, 2 min	5.58±0.00^{f}	2.40±0.15^{e}	53.33±1.33^{g}	33.33±1.33^{i}	2.67±0.33^{ab}
Mercuric Chloride 0.025 %, 4 min	5.76±0.87f^{fg}	2.00±0.01^{d}	44.00±2.39^{f}	30.67±1.33^{hi}	2.67±0.33^{ab}
Mercuric Chloride 0.025 %, 6 min	8.63±0.43^{k}	0.46±0.02^{b}	18.66±1.33^{d}	24.00±2.30^{efg}	5.33±0.33^{de}
Mercuric Chloride 0.05 %, 2 min	5.59±0.04^{f}	1.45±0.05^{c}	30.66±1.33^{e}	29.33±1.33^{ghi}	3.33±0.33^{bc}
Mercuric Chloride 0.05 %, 4 min	6.83±0.11^{h}	0.36±0.01^{ab}	10.66±1.33^{c}	20.00±2.30^{cde}	4.33±0.33^{cd}
Mercuric Chloride 0.05 %, 6 min	12.43±0.26^{m}	0.15±0.01^{ab}	6.66±1.33^{abc}	17.33±1.33^{bcd}	10.67±0.33^{i}
Mercuric Chloride 0.1 %, 2 min	6.17±0.12^{g}	0.22±0.01^{ab}	9.33±1.33^{bc}	26.67±1.33^{fgh}	5.33±0.33^{de}
Mercuric Chloride 0.1 %, 4 min	7.50±0.22^{i}	0.17±0.01^{ab}	5.33±1.33^{ab}	22.67±2.30^{def}	6.67±0.66^{ef}
Mercuric Chloride 0.1 %, 6 min	7.76±0.11^{ij}	0.14±0.01^{ab}	4.00±1.33^{a}	20.00±1.33^{cde}	8.00±0.57^{fg}
Mercuric Chloride 0.2 %, 2 min	8.08±0.01^{j}	0.13±0.00^{ab}	5.33±1.33^{ab}	18.67±0.66^{bcde}	8.33±0.33^{gh}
Mercuric Chloride 0.2 %, 4 min	9.86±0.00^{l}	0.09±0.03^{ab}	4.00±1.33^{a}	15.33±1.33^{abc}	9.67±0.33^{hi}
Mercuric Chloride 0.2 %, 6 min	12.65±0.14^{m}	0.07±0.01^{a}	2.66±1.33^{a}	13.33±1.33^{ab}	13.00±0.57^{j}
Mercuric Chloride 0.3 %, 2 min	13.51±0.29^{n}	0.26±0.21^{ab}	4.00±0.00^{a}	14.67±1.33^{abc}	13.00±1.15^{j}
Mercuric Chloride 0.3 %, 4 min	14.02±0.04^{o}	0.06±0.03^{a}	4.00±0.00^{a}	10.67±1.33^{a}	13.67±0.33^{j}
Mercuric Chloride 0.3 %, 6 min	14.13±0.03^{o}	0.04±0.03^{a}	2.66±0.1.33^{a}	13.33±1.33^{abc}	13.67±0.88^{j}
Sodium Hypochloride 1%, 5 min	3.31±0.10^{a}	8.30±0.11^{i}	85.33±1.33^{k}	33.33±2.30^{i}	1.67±0.33^{a}
Sodium Hypochloride 1%, 10 min	3.56±0.04^{ab}	8.16±0.14^{i}	74.33±2.66^{hi}	28.00±1.33fghi	2.67±0.33^{ab}
Sodium Hypochloride 3%, 5 min	3.26±0.12^{a}	7.80±0.30^{h}	77.33±1.33^{i}	30.66±1.33^{hi}	2.33±0.33^{ab}
Sodium Hypochloride 3%, 10 min	3.87±0.05^{bcd}	7.21±0.08^{g}	73.00±1.33^{hi}	26.66±1.33^{fgh}	2.00±0.57a^{ab}
Sodium Hypochloride 5%, 5 min	4.06±0.1^{cd}	7.10±0.065^{g}	76.00±2.30^{hi}	22.66±1.33^{def}	1.67±0.33^{a}
Sodium Hypochloride 5%, 10 min	4.21±0.13^{d}	6.60±0.22^{f}	72.00±0.00^{h}	14.66±0.66^{abc}	2.33±0.33^{ab}
Ethanol 70%, 2 min	3.33±0.11^{a}	10.32±0.06^{j}	81.33±1.33^{j}	68.00±2.30^{j}	2.00±0.00^{ab}
Ethanol 70%, 5 min	4.79±0.04^{e}	6.53±0.17^{f}	89.33±1.33^{l}	58.66±3.52^{k}	2.67±0.33^{ab}

Within each column, means with the same letter are not different ($p \leq 0.05$) significantly applying DMRT.

At minimum concentration (0.025%) with the shortest duration of exposure time (2 min), $HgCl_2$ was not efficient as a sterilizing agent where MGT, GR, GP, CP and NDFG were 5.58, 2.40, 53.33 and 2.67, respectively. By comparing the sterilization agents over the germination parameters (Table 1), it can be observed that $HgCl_2$ is not suitable sterilizing agent for seed surface sterilization of *C. roseus*. In addition, $HgCl_2$ also destroyed the seeds coat which may affect the seed germination values in *C. roseus* (Figure 1B). Application of $HgCl_2$ as surface sterilizing agent have been reported, but it was also proved that the application of $HgCl_2$ in the long exposure may leads to the destruction of plant tissues and seed mortality. Whereas short duration of application may not removes the germs sufficiently [6].

NaClO treatment for 5 and 10 min at 1%, 3% and 5% w/v concentrations showed high effectiveness as seed surface sterilizing agents in *C. roseus* and seeds germination values were not affected negatively and the best results were achieved in treatment of the seeds with 3% or 5% NaClO -5 min (Table-1).

Figure 1. *In vitro* seeds germination of *C. roseus*. **A)** intact seeds, **B)** seeds coat destroyed by $HgCl_2$, **C)** seeds germination applying paper boat technique, **D)** seeds germination on: **a-** DW, **b-** MW (note the length of shoot and root), **c-** ¼ MS media free sugar, **d-** ½ MS media free sugar, **e-** full MS media free sugar, **f-** full MS media 1% sucrose, **g-** full MS media 2% sucrose, **h-** full MS media 3% sucrose, **i-** full MS media 1% glucose, **j-** full MS media 2% glucose, **k-** full MS media 3% glucose, **l-** mineral water solidified with agar, **m-** full MS media free sugar solidified with agar, **n-** full MS media 3% sucrose solidified with agar, **o-** full MS media 3% glucose solidified with agar.

The potent effect of NaClO against many species of fungi and bacteria is because of formation of HClO during dilution in water and its concentration has negative correlation with growth and activity of fungi and bacteria due to its lethal damage on DNA [7].

3.2 *In vitro* Seeds Germination

The seeds were surface-sterilized (3% w/vNaClO with duration of 5 min) followed by inoculation on various germination medium in order to determine the optimum *in vitro* seed germination condition in *C. roseus*as described in table-2.

The ANOVA test indicated that various types of seeds germination medium affect significantly the seeds germination values ($p \leq 0.05$) (GR, GP, MGT, NDFG) and length of shoots and roots of seedlings in *C. roseus* (Table-2).

Based on our measurments, the highest GR-GP and lowest MGT-NDFG were found in sterilized MW. In addition, the length of shoots and roots were measured at the highest level in sterilized MW as well. Whereas, the lowest seeds germination values as well as length of shoots and roots were observed in MS medium solidified with agar and containing 3% sucrose.

According to our observations, the minerals presented in sterilized MW initiate and accelerate the seeds germination in *C. roseus*. While seed germination and seedling development values were decreased by using Murashige and Skooge (MS) minerals solution as germination media (Table-2).

Table 2. Effect of diverse media assayed on measured seeds germination characteristics ± std. error in *C. roseus*.

Type of Medium	NDFG	GP	MGT	GR	Mean Shoot Length	Mean Root Length
Distilled Wather	2.66 ± 0.33^{b}	70.33 ± 2.66^{g}	3.41 ± 0.12^{b}	5.80 ± 0.03^{g}	2.69 ± 0.10^{c}	3.51 ± 0.08^{g}
Mineral Wather	2.33 ± 0.33^{a}	74.66 ± 1.33^{h}	3.15 ± 0.02^{a}	8.02 ± 0.22^{i}	3.52 ± 0.04^{h}	3.41 ± 0.07^{g}
¼ MS Media Free Sugar	2.33 ± 0.33^{c}	67.00 ± 2.30^{f}	3.64 ± 0.22^{c}	7.37 ± 0.13^{h}	3.38 ± 0.05^{h}	1.03 ± 0.03^{e}
½ MS Media Free Sugar	3.00 ± 0.00^{de}	66.33 ± 1.33^{f}	4.14 ± 0.06^{de}	5.72 ± 0.16^{g}	3.18 ± 0.06^{g}	0.92 ± 0.04^{e}
Full MS Media Free Sugar	3.66 ± 0.33^{f}	64.66 ± 1.33^{e}	4.45 ± 0.13^{f}	4.60 ± 0.06^{f}	3.04 ± 0.05^{fg}	0.71 ± 0.02^{d}
Full MS Media 1% Sucrose	3.33 ± 0.33^{f}	62.33 ± 1.33^{de}	4.60 ± 0.42^{f}	3.98 ± 0.03^{cde}	2.98 ± 0.04^{ef}	0.67 ± 0.03^{cd}
Full MS Media 2% Sucrose	4.00 ± 0.00^{g}	58.66 ± 2.66^{cd}	5.05 ± 0.05^{g}	3.80 ± 0.18^{bc}	2.99 ± 0.06^{efg}	0.66 ± 0.03^{cd}
Full MS Media 3% Sucrose	4.33 ± 0.33^{h}	50.66 ± 1.33^{ab}	5.53 ± 0.20^{g}	3.51 ± 0.04^{ab}	2.75 ± 0.04^{cd}	0.55 ± 0.03^{abc}
Full MS Media 1% Glocuse	2.66 ± 0.33^{ef}	63.33 ± 1.33^{e}	4.32 ± 0.18^{ef}	4.23 ± 0.01^{de}	3.01 ± 0.05^{efg}	0.69 ± 0.03^{d}
Full MS Media 2% Glocuse	3.33 ± 0.33^{g}	59.33 ± 2.66^{cd}	4.97 ± 0.19^{g}	3.91 ± 0.05^{cd}	2.90 ± 0.05^{def}	0.67 ± 0.03^{cd}
Full MS Media 3% Glocuse	3.66 ± 0.33^{g}	54.66 ± 1.33^{bc}	5.16 ± 0.10^{h}	3.55 ± 0.10^{ab}	2.84 ± 0.05^{cde}	0.58 ± 0.03^{bcd}
Mineral Wather + Agar	2.33 ± 0.33^{d}	68.66 ± 1.33^{f}	3.93 ± 0.09^{d}	7.38 ± 0.08^{h}	3.33 ± 0.05^{b}	1.50 ± 0.04^{f}
Full MS Media + Agar Free Sugar	3.66 ± 0.33^{hi}	59.33 ± 1.33^{cd}	$5.65\pm0.^{06hi}$	4.27 ± 0.10^{e}	3.07 ± 0.04^{fg}	0.50 ± 0.02^{ab}
Full MS Media + Agar +3% Sucrose	4.66 ± 0.33^{i}	48.00 ± 2.30^{a}	5.84 ± 0.06^{i}	3.48 ± 0.10^{a}	1.95 ± 0.04^{a}	0.43 ± 0.01^{a}
Full MS Media + Agar +3%Glocuse	4.33 ± 0.33^{hi}	50.66 ± 1.33^{ab}	5.57 ± 0.09^{hi}	3.65 ± 0.10^{abc}	2.23 ± 0.06^{b}	0.51 ± 0.02^{ab}

Within each column, means with the same letter are not different ($p \leq 0.05$) significantly applying DMRT.

It must be noted that sterilized DW had insufficient minerals to activate or accelerate the mechanism of germination compare to sterilized MW. Interestingly, the solidification with agar and the presence of sugars in the media showed negative effects on *in vitro* seeds germination in *C. roseus* (Table-2, Figure 1D). Based on the ANOVA test, the concentrations of glucose and sucrose in the seed germination media affect the seeds germination values and seedlings development negatively. In the other hand, we observed that increasing the concentration of glucose and sucrose caused a significant decrease in the germination values and length of shoots and roots of seedlings in *C. roseus*. This is in agreement with previous reports for different plant species [8,9]. Sucrose involved in signaling pathway as a signaling molecule for seed germination regulation and development of seedling [10].

The effect of the composition of the seed germination media on *in vitro* seed germination efficiency and seedling properties have been reported in different plant species [5, 11].

4. Conclusions

The main aims of the present investigations were first to define a sterilizing agent and optimization of its concentration as well as exposure duration and secondly, the development of an *in vitro* seeds germination media via manipulation of the sugars and mineral concentrations to achieve the maximum seeds germination values and seedlings development. We report an optimized procedure of *in vitro* seeds germination and development of *C. roseus*. Based on our results, the 3% (w/v) NaClO with duration of 5 min was the best protocol for seed surface sterilization of *C. roseus*. In addition, because of the negative effect of sugars and high concentrations of minerals on the *in vitro* germination and plantlet development, the sterilized mineral water (using paper boat method) was the optimum germination medium for this species. Hence, for further tissue culture investigations we suggest to apply the mentioned optimal protocols used for *C. roseus* seeds.

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