Indian Journal of Science and Technology, Vol 9(28), DOI: 10.17485/ijst/2016/v9i28/90896, July 2016
ISSN (Print) : 0974-6846
ISSN (Online) : 0974-5645

Optimization of Seed Surface Sterilization Method and *in vitro* Seed Germination in *Althaea officinalis* (L.) - An Important Medicinal Herb

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Abstract

Objectives: The species Althaea officinalis is belong to Malvaceae family; its common name is Marshmallow. In this study an efficient technique has been optimized for sterilization of seed surface and also in vitro germination of seeds. **Methods:** Effect of different sterilizing agents with various concentrations and duration times as well as different media with various concentrations of sucrose on MGT (mean of germination time), GP (germination percentage), GR (germination rate), NDFG (number of days to first germination), CP (contamination percentage) and length of root and shoot of seedlings were recorded. For each experiment 25 seeds were treated and it was repeated at least three times. **Findings:** The maximum GP (80.0%) was recorded in sterilized-distilled water for 20 min (control), whereas minimum GP (44.0%) was recorded in experiment using 0.3% mercuric chloride ($HgCl_2$) for 7 min. Between seed surface sterilization techniques used, maximum CP was achieved in control (85.33%) and minimum was found in 4% NaOCl (Sodium Hypochlorite), 10 min (13.33%), 0.3% $HgCl_2$ for 7 min (14.66%) and 4% NaOCl for 5 min (17.33%). We compared the measured seed germination values (NDFG, MGT, GR, GP and CP) and among seed surface sterilization methods tested, NaOCl, 5 min has been found to be the best in A. officinalis. In addition, presence of sucrose in germination medium has shown negative effect on seed germination measured values as well seedling development. The effect of different surface sterilization techniques have no significant effect ($p \leq 0.05$) on number of days to first germination (NDFG) but these techniques significantly affected ($p \leq 0.05$) the other characteristics of seed sterilization. According to ANOVA results we report for the first time the best sterilization method and medium for in vitro seed germination and seedling development in A. officinalis.

Keywords: Althaea officinalis, Seed Surface Sterilization, Seed Germination

1. Introduction

The species *Althaea officinalis* L. is an important medicinal herb and possesses antitussive, antiviral, antimicrobial and antifungal activities. The plant secondary metabolites viz. aspargine, althein, flavonol glycosides, pectin, quercetin and phenolic acids are isolated from its roots and leaves[1]. Germination of seeds and growth of embryo are key stages in the life cycle of a plant and it often controls population dynamics, with major practical implications[2]. Using sterilizing agents such as sodium hypochlorite (NaOCl), ethanol, mercuric chloride ($HgCl_2$) and Tween-20 are widely recommended[3,4] for seed surface sterilization. As there is no report on protocol for seed surface sterilization and seed germination we optimized methods for seed surface sterilization and germination by using different types of media and sucrose concentration for first of its kind in *A. officinalis*.

2. Materials and Methods

2.1 Seed Surface Sterilization Methods

An experiment was designed in order to establish an efficient method for seed-surface sterilization using

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different sterilizing agents (various concentrations and durations) in *A. officinalis* as follows:

1) Sterilized-distilled water — 20 minutes as Control;
2) 0.1, 0.2, 0.3% $HgCl_2$ (w/v) — 1, 3, 5, 7 minutes;
3) 70% ethanol + 100 µL tween- 20 — 5 and 10 minutes,
4) 4% NaOCl (w/v) — 5 and 10 minutes (see Table 1).

Paper boat technique was used for germination. Sterilized decoated seeds were inoculated on filter paper (Whatman No.1) moistened with sterilized-distilled water (Figure 4a,b). Twenty five decoated seeds for each experiment were used and all experiments were repeated for three times. The inoculated seeds were maintained in the culture room (25±2 °C) under a day photoperiod light (16 hours) using cool-white fluorescent tube lights (light intensity ~ 40 µmol m^{-2} s^{-1}).

2.3 Optimization of Medium for Seeds Germination

We made an attempt to optimize a medium for germination of seeds and development of seedlings in *A. officinalis*. An experiment was arranged to investigate the effect of presence as well as different concentrations of sucrose in germination medium on seed germination values and development of seedling. For this study, surface sterilized seeds (using 4% NaOCl, for 5 min method) were inoculated as follows:

i) Sterilized-mineral-water, Kinley, India;
ii) Sterilized-distilled-water,
iii) Sucrose free MS medium,
iv) MS medium containing 1, 2, and 3% sucrose (w/v);
v) MS medium containing 3% sucrose (w/v) solidified with 0.8% Agar-Agar (w/v),
vi) Free sucrose MS medium solidified with 0.8% Agar-Agar (w/v).

The medium was autoclaved (121°C, 15 ψ pressure, 15 minutes). All the cultures were maintained in the culture room (25 ± 2 °C) under a day photoperiod light (16 hours) using cool-white fluorescent tube lights.

2.4 Analysis of Data

The duration of radical emergence was measured to determine the kinetics of germination. Seedlings developed in same period have been chosen to determine the shoots and roots length. Twenty five surface sterilized and healthy decoated seeds were used for each experiment and all experiments were repeated at least three times. IBM SPSS (version 20) software was used to perform the ANOVA and means comparison analysis (DMRT). GP, MGT, GR and CP were recorded daily for 15 days. Calculation of the GP, MGT, number of days to first germination (NDFG), GR and CP have done within fifteen days as follows:[5]

For calculation of MFG the following formula was used

$$MGT = \sum \frac{D \cdot N}{n}$$

D) Number of certain day in each counting
N) Number of seeds that germinated in the certain counting day
n) Total number of grown seeds

The germination rate (GR) was calculated as follow:

$$GR = \frac{\text{Number of germinated seeds}}{\text{Number of days to first count}} + \ldots + \frac{\text{Number of germinated seeds}}{\text{Number of days to final count}}$$

In order to measure the length of shoots and roots, seedlings grown in the same period (15 days old) have been chosen.

3. Results and Discussion

3.1 Seed Surface Sterilization Technique

We made an attempt to optimize a method for seeds surface sterilization in *A. officinalis*. For this purpose, different techniques were used (Table 1). Initial sterilization and germination tests indicated that type, concentration and exposure time of sterilizing agent affected the GP, GR and CP. The maximum GP was recorded in sterilized-distilled water for 20 min (control) and 0.2 % $HgCl_2$ for 5 min, and the less percentage was recorded in 0.3 % $HgCl_2$, 7 min. Between different surface sterilization techniques used, the maximum contamination percentage was achieved by control and the minimum have been observed in NaOCl 4%, 10 min followed by $HgCl_2$ 0.3% for 7 min (Figure 1). The MGT was noted more in 0.1% (w/v) mercuric chloride, 5 min followed by 0.2% $HgCl_2$ for 3 min, whereas GR was found maximum at 70% ethanol + 100 µl Tween-20 for 5 min. According to results of ANOVA, treatments affected the GR, GP and CP as well as on MGT significantly ($p \leq 0.05$), while non significant effects were observed on number of days to first germination (NDFG) (Table 1). The correlation between

Table 1. Effect of various sterilization techniques on *in vitro* seed germination values in *A. officinalis*.

Conc. of Sterilizing agent (%) and Duration of Treatment	NDFG	GP	MGT	GR	CP
Sterilized Distilled Water, 20 min (control)	1.67±0.33^{a}	80.00±2.30^{a}	6.34±0.52^{a}	4.94±0.71^{de}	85.33±3.53^{a}
0.1% $HgCl_2$, 1 min	1.67±0.33^{ab}	77.33±3.53^{ab}	6.21±0.52^{a}	5.12±0.69^{cde}	69.33±4.81^{b}
0.1% $HgCl_2$, 3 min	1.67±0.33^{ab}	72.00±4.00^{abc}	5.70±0.32^{abc}	4.85±0.52^{de}	61.33±4.81^{bc}
0.1% $HgCl_2$, 5 min	2.33±0.33^{b}	65.33±1.33^{abcd}	7.19±0.56^{a}	3.20±0.08^{e}	57.33±2.67^{cd}
0.1% $HgCl_2$, 7 min	2.00±0.58^{ab}	66.66±6.66^{abcd}	6.81±0.30^{a}	3.69±0.47^{de}	50.66±3.53^{de}
0.2% $HgCl_2$, 1 min	1.67±0.33^{ab}	73.33±11.39^{abc}	6.68±0.29^{a}	4.65±1.21^{de}	58.66±5.81^{cd}
0.2% $HgCl_2$, 3 min	1.33±0.33^{a}	70.66±4.81^{abc}	7.09±0.46^{a}	3.98±0.26^{de}	58.66±3.53^{cd}
0.2% $HgCl_2$, 5 min	1.00±0.00^{a}	80.00±6.11^{a}	6.41±0.41^{a}	5.90±0.65^{bcde}	45.33±1.33^{ef}
0.2% $HgCl_2$, 7 min	1.33±0.33^{a}	72.00±2.31^{abc}	5.83±0.72^{ab}	5.58±1.16^{bcde}	37.33±3.53^{fg}
0.3% $HgCl_2$, 1 min	1.00±0.00^{a}	62.66±3.53^{bcd}	3.44±0.43^{d}	8.06±0.06^{abc}	49.33±3.53^{de}
0.3% $HgCl_2$, 3 min	1.33±0.33^{a}	61.33±2.67^{cd}	4.09±0.58^{cd}	6.36±0.99^{bcd}	38.00±1.15^{fg}
0.3% $HgCl_2$, 5 min	1.00±0.00^{a}	54.66±1.33^{de}	3.60±0.35^{d}	6.49±0.54^{bcd}	17.33±1.33^{h}
0.3% $HgCl_2$, 7 min	1.00±0.00^{a}	44.00±6.11^{e}	6.84±1.14^{a}	3.86±0.81^{de}	14.66±1.33^{h}
70% Ethanol + Tween-20 for 5 min	1.00±0.00^{a}	78.66±4.81^{a}	3.36±0.58^{d}	9.81±1.14^{a}	46.66±2.66^{ef}
70% Ethanol + Tween-20 for 10 min	1.33±0.33^{a}	58.66±3.53^{cde}	4.41±0.50^{bcd}	5.19±0.92^{cde}	33.33±1.33^{g}
4% NaOCl, 5 min	1.00±0.00^{a}	66.66±2.67^{abcd}	3.26±0.38^{d}	8.53±1.85^{ab}	17.33±3.53^{h}
4% NaOCl , 10 min	1.33±0.33^{a}	58.66±1.33^{cde}	4.25±0.68^{bcd}	6.02±1.51^{bcde}	13.33±1.33^{h}

In each columns means of measured trials sharing the same letter are not different significantly ($p \leq 0.05$) using DMRT.

Table 2. Coefficient of phenotypic correlation of seed germination values under various seed surface sterilization techniques in *A. officinalis*.

	MGT	GR	GP	CP
MGT	1.0			
GR	- 0.820*	1.0		
GP	0.141	0.222	1.0	
CP	0.434*	- 0.233	0.606*	1.0

*. At the level of 0.01 correlations are significant (2- tailed).

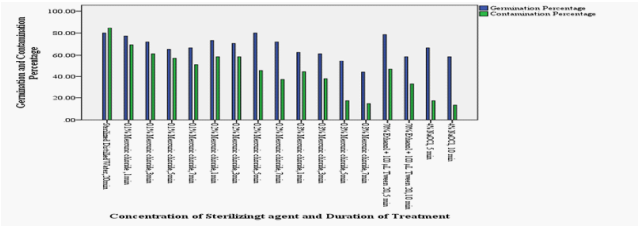

Figure 1. Comparison of germination and contamination percentages after using different surface sterilizing agents in *A. officinalis*.

CP-GP, CP-MGT, as well as germination rate GR were found significant and positive. While a negative correlation was recorded between MGT and GR (Table 2).

3.2 *In vitro* Seed Germination

In order to optimization of medium for *in vitro* seed germination, the sterilized (using 4% NaOCl, for 5 min) seeds of *A. officinalis* were placed on various medium used (Table 3). The highest germination percentage (GP) has been recorded in sterilized-mineral water and the lowest percentage was found in MS media containing 3% sucrose (w/v) (Figure 2). According to our experiments, mineral water followed by distilled water showed the best results in the case of length of shoot and root. Early germination (NDFG) was also recorded in sterilized mineral water followed by sterile distilled water. Rate of germination was observed more in sterilized mineral water as well as in sterile distilled water too. The results of ANOVA indicated that various medium types with different levels of sucrose was significantly affected the NDFG, MGT, GR and GP.

The ANOVA test results also indicated that different types of medium tested significantly affected ($p \leq 0.05$) the length of shoot and root of seedlings in *A. officinalis* (Table 3, Figure 3). The correlation between GP and GR was significant and positive but correlation was negatively significant between GP-MGT, GP-AGP and

Table 3. Effect of different media with various concentrations of sucrose on measured characteristics (Mean values ± Std. error) in *A. officinalis*.

Type of Medium	NDFG	GP	MGT	GR	Mean Shoot Length	Mean Root Length
Sterilized mineral Water	1.00±0.00^{e}	76.00±2.30^{a}	5.60±0.72^{d}	2.48±0.11^{a}	7.12±0.14^{a}	7.08±0.15^{a}
Sterilized Distilled Water	1.00±0.00^{e}	69.33±6.11^{a}	6.04±0.83^{d}	2.34±0.11^{a}	5.56±0.21^{ab}	6.63±0.22^{a}
MS without Sucrose	2.33±0.33^{d}	58.67±4.61^{b}	7.10±0.45^{cd}	1.57±0.09^{b}	5.08±0.27^{abc}	4.98±0.22^{b}
MS + 1% Sucrose	3.66±0.33^{c}	46.67±4.61^{c}	7.97±0.53^{bc}	1.19±0.06^{c}	4.20±0.27^{d}	4.76±0.26^{b}
MS + 2% Sucrose	5.00±0.58^{b}	41.33±6.11^{c}	9.41±0.34^{ab}	1.13±0.09^{cd}	4.32±0.24^{d}	4.03±0.20^{c}
MS + 3% Sucrose	6.33±0.67^{a}	29.33±6.11^{d}	9.98±0.67^{a}	0.90±0.09^{de}	2.23±0.23^{e}	3.39±0.22^{c}
MS + Agar + 3% Sucrose	7.33±0.33^{a}	28.00±4.00^{d}	10.89±0.62^{a}	0.84±0.04^{e}	1.92±0.15^{e}	1.17±0.18^{c}
MS + Agar without Sucrose	3.67±0.33^{c}	57.33±6.11^{b}	8.13±0.73^{bc}	1.44±0.03^{b}	4.56±0.21^{cd}	3.92±0.14^{c}

In each columns means of measured trials sharing the same letter are not different significantly ($p \leq 0.05$) using DMRT.

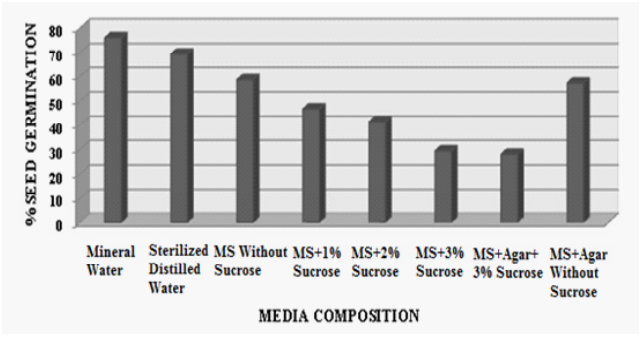

Figure 2. Effect of various medium on percentage of germination of seeds in *A. officinalis*.

GR-AGP (Table 4). Effect of medium composition on seed germination as well as seedling development have been reported with various researchers[6-,9]. It was reported that sucrose is more important role in most plants compare to other carbohydrates. Regulation of the respiration and photosynthesis and also osmotic pressure maintenance in the plant cytosol are the key roles of sucrose in the physiology of the plant[10]. Sucrose also involved in signaling pathway as a signaling molecule for seed germination regulation and development of seedling[11]. Similarly, effect of presence of sucrose as well as its concentration in the medium on *in vitro* germination of seeds and development of seedlings has been demonstrated in different plants[12-14]. Jang and Sheen (1997) have investigated the effect of concentration of sugar in media on seedlings development in higher plants. They reported that glucose in high concentration suppressed the seedling developmen[15]. High concentrations of glucose in media increase the production and accumulation of ABA, therefore seed germination is delayed. John *et al* reported that presence

Table 4. Coefficient of phenotypic correlation of seed germination values under various seed germination medium in *A. officinalis*.

	GP	MGT	GR
GP	1		
MGT	-0.846*	1	
GR	0.963*	-0.865*	1

*. At the level of 0.01 correlations are significant (2- tailed).

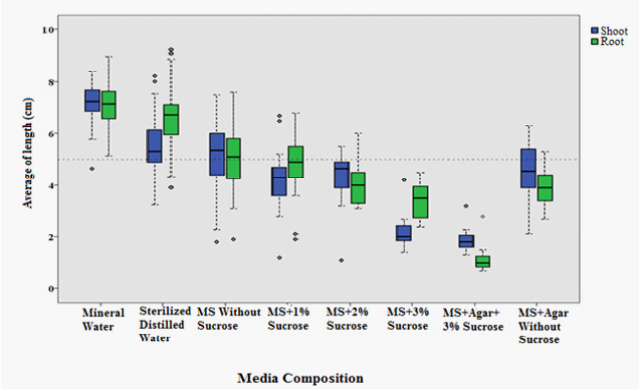

Figure 3. Effect of composition of germination medium on length of shoot and root of seedlings in *A. officinalis*.

of sucrose with high concentrations in medium also repressed development of seedlings in *Arabidopsis*[16]. According to our observations it was also revealed that using sucrose in germination media inhibited the development of seedlings in *A. officinalis*..

By comparison of ANOVA test results it was revealed that concentration of sucrose has negative effect on size of shoots and roots of seedlings in *A. officinalis*. In the other words, by increasing the concentration of sucrose in media

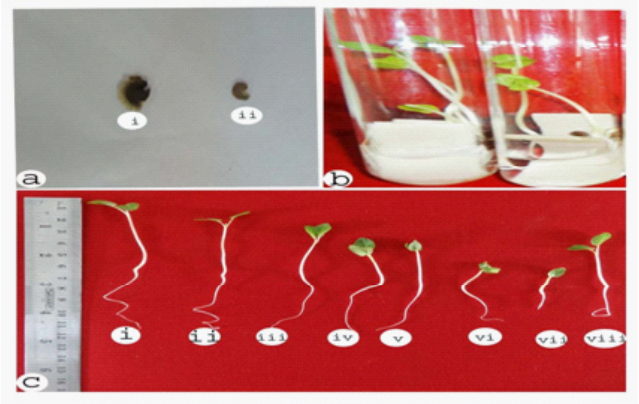

Figure 4. *In vitro* seed germination in *A. officinalis*. **a)** seeds: i) with coat, ii) decoated; **b)** *In vitro* seed germination using paper boat method; **c)** *In vitro* germination using various medium: i) Sterilized mineral-water, ii) Sterilized distilled-water, iii) Free sucrose MS, iv) MS + 1% sucrose, v) MS + 2% sucrose, vi) MS + 3% sucrose, vii) MS + agar + sucrose, viii) MS + agar without sucrose.

the size of seedlings (both the size of shoots and roots) has found to be decreased. Same results also observed in the case of negative effect of solidification of media with 0.8 agar on germination of seeds and seedlings growth in *A. officinalis* (Figure 4c). Initially seedlings used the exogenous source of sucrose and it decreases the utilization of internal source. Glucose, the decomposed products of sucrose, affects the expression of various genes involved in the metabolism of carbohydrates[16,17].

4. Conclusion

An efficient protocol has been optimized for seed surface sterilization and germination in this study. According to our observations (measurement of seed germination values, NDFG, MGT, GR, GP, CP), it was revealed that suitable technique for seed surface sterilization in *A. officinalis* was 4% NaOCl, 5 minutes. These techniques significantly affected ($p \leq 0.05$) other characteristics of seed sterilization. The sterilizing agent concentration and seed surface sterilization duration showed negative effect on germination values in *A. officinalis*, and it is due to harmful effect of ethanol, NaOCl and Hg on zygotic embryo. In addition, the type of medium had also shown effect on germination of seeds and development of seedling in *A. officinalis*. Thus, germination of seeds and development of seedling have shown negative response to presence of sucrose in medium (Table 3). Our recorded also indicated the negative effect of solidification of media with 0.8 agar on germination of seeds and seedlings growth in *A. officinalis*. According to ANOVA results of measured values we conclude that the sterilized mineral water is the best media for *in vitro* seed germination and also development of seedlings in *A. officinalis*.

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