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Sterilization factors affect seed germination and proliferation of *Achyranthes aspera* cultured *in vitro*

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Abstract

Achyranthes aspera (Amaranthaceae) is a highly significant medicinal herb that is common in Bangladesh. The effect of five sterilizing substances (ethanol, mercuric chloride, Flugal, Nystatin, and sodium hypochlorite) on germination and development of explants of *A. aspera* was evaluated. The percentage of contamination and germination, as well as seed color, growth pattern, and shootlet development were determined. Among the different sterilants, sodium hypochlorite solution application for 30 min was the most effective treatment. At higher concentration, all sterilizing agents showed maximum effect against microbiological contamination, although the survival percentage was low.

Key words: *Achyranthes aspera*, microbiological contamination, seeds, sterilizing substances, tissue culture.

Introduction

Achyranthes aspera L., commonly known as apang in Bengali and rough chaff in English, is a significant medicinal perennial herb native to Bangladesh. It is 1 to 2 m in height, often with a woody base, commonly found as a weed of waysides or roadsides of Bangladesh. It has diverse medicinal uses in traditional medicinal system (Dwivedi et al. 2008). Along with utilization in medicine by local practitioners and healers, this plant has also been reported to show diverse pharmacological properties (Dey 2011; Sen et al. 2012; Dash et al. 2013).

In vitro plant culture, which encompasses cell, tissue, organ and also embryo culture, has been a vital technique for mass multiplication of plants, elimination of plant diseases thorough meristematic tissue culture technique, plant conservation and crop improvement through gene transfer (Singh, Chand 2003; Sarasan et al. 2011).

Contamination of plant tissue cultures by different microorganisms, such as bacteria and fungi, reduces their productivity and can completely prevent their cultivation. Therefore, successful tissue culture protocols start with effective explant sterilization (Dodds, Roberts 1985; Sen et al. 2013). Several different methods are used to eliminate fungal and bacterial contamination, including the use of antibiotics and fungicides, as well as inactivation by heat and light (Kneifel, Leonhardt 1992; Leifert et al. 1992; Salehi, Khosh-Khui 1997; Haldeman et al. 1987; Reed, Tanprasert 1995; Seckinger 1995; Sen et al. 2013). Many sterilants are also toxic to the plant tissues, and hence

optimum concentrations of sterilants, duration of exposure of explants to sterilants, the sequences of sterilants used etc. need to be determined to minimize explant injury and to achieve better survival (CPRI 1992).

Sterilization of nodal explants for *in vitro* micropropagation of *A. aspera* has been achieved by using different surface sterilizing agents (Sen et al. 2013). However, there is no study yet documented on the success or failure of asepsis in *in vitro* seed germination of this plant. For sterilization and further germination of seed explants, a number of sterilants are widely used, including ethanol, sodium hypochlorite, mercuric chloride, Flugal, and Nystatin. The aim of the present study was to determine the best sterilization protocol for *in vitro* culture of *A. aspera*. Determination and optimization of a standard surface sterilizing process by using these different sterilants for seed explants was an important aspect in this study to obtain a large number of sterilized seed, as well as propagated explants for further experiments. Different seed varieties (dehusked or husked) were tested for germination and further proliferation.

Materials and methods

Explants and nutrient medium for micropropagation

The experiment was conducted at the Plant Biotechnology Division of the National Institute of Biotechnology, Dhaka, Bangladesh, with the objective to evaluate the effect of different sterilants on explants of *Achyranthes aspera* L. (apang) in conditions of *in vitro* culture. Healthy seeds free

of symptoms of disease and pest problems were collected from the experimental plot of Plant Biotechnology Division. Seeds were obtained from dehusked (without husk) varieties and husked (with husk) varieties. After sterilization by using different sterilants in an autoclaved beaker or conical flask, with different concentrations and duration, seeds were placed in a coffee jar, test tube, or conical flask on Murashige and Skoog medium (Duchefa, The Netherlands; Murashige, Skoog 1962) containing 3% sucrose (Merck, Germany), solidified with 0.8% agar (BDH Chemicals Ltd., England). The pH (Jenway 3520 pH Meter, Bibby Scientific Ltd., UK) of the medium was adjusted to 5.8 before autoclaving (ALP Co. Ltd., CL-40M, Japan) at 121 °C and 100 kPa for 20 min and gelling with agar. For each treatment, 12 seeds per container in five replications were used.

Surface sterilization of plant material

Seeds were dried for a few days to remove dust, and cleaned. Control seeds (dehusked De_c and husked Hu_c) were treated with 100% ethanol (Merck, Germany) for 1 min. Other seeds were washed with 75% ethanol for 45 s four to five times followed by treatment with mercuric chloride (Merck, Germany), Flugal (Square pharmaceutical, Bangladesh),

Nystatin (Square Pharmaceutical, Bangladesh), and sodium hypochlorite (Clotech, Bangladesh) containing a few drops of Tween 20 (Biabasic, Canada), at different concentrations and time periods (Table 1). Then seeds were washed with autoclaved distilled H₂O (except De_c and Hu_c samples) to remove small amount of chemicals. The seed explants were then dried and placed on media.

Cultivation conditions

The growth room for maintenance of *in vitro* cultures had 25 ± 2 °C temperature and 60 to 70% relative humidity, with a photoperiod of 16 h day light and 8 h dark. Illumination was provided with incandescent lamps (50 W, Philips Agro-Lite).

Data recording and analysis

Each experiment was conducted at least twice. Germination and proliferation were recorded. The degree of contamination was evaluated and the percentage of germination, seed color, and length of plantlets were recorded 10 days after sterilization. The number of contaminated explants was counted. The mean values and standard deviations were calculated using computer software (Microsoft Office Excel Worksheet).

Table 1. Effect of different seed sterilization procedures used on contamination, germination and growth of *Achyranthes aspera* explants. De, dehusked seed variety; Hu, husked seed variety; N, normal; BL, blakish; B, black; NG, no growth; V, vigorous; F, faded; M, morbid. Length data are means ± SD

Treatment	Sterilizant	Concentration (%)	Time (min)	Contamination (%)		Seed color	Germination (%)		Length (cm)		Growth pattern	
				De	Hu		De	Hu	De	Hu		
De _c	Hu _c	Ethanol	100	1	100	100	N	0	0	0.0	0.0	NG
De ₁	Hu ₁	HgCl ₂	0.1	1	77.81	96.43	N	90	70	5.67 ± 0.47	4.52 ± 0.80	V
De ₂	Hu ₂	HgCl ₂	0.1	3	64.38	76.57	N	90	70	5.67 ± 0.81	4.67 ± 0.50	V
De ₃	Hu ₃	HgCl ₂	0.1	5	43.17	63.14	N	90	70	4.67 ± 0.27	4.67 ± 0.50	V
De ₄	Hu ₄	HgCl ₂	0.1	8	35.93	68.86	BL	80	60	4.67 ± 0.22	4.67 ± 0.90	F
De ₅	Hu ₅	HgCl ₂	0.1	10	25.55	59.22	BL	80	60	4.76 ± 0.22	3.47 ± 0.50	F
De ₆	Hu ₆	HgCl ₂	0.1	13	22.06	36.48	B	30	50	4.94 ± 2.22	3.00 ± 0.82	M
De ₇	Hu ₇	HgCl ₂	0.1	16	21.32	33.12	B	20	30	4.00 ± 2.45	2.67 ± 0.90	M
De ₈	Hu ₈	HgCl ₂	0.1	20	17.13	29.19	B	20	30	4.00 ± 0.00	2.00 ± 1.41	M
De ₉	Hu ₉	HgCl ₂	0.2	5	15.00	26.75	B	20	30	2.33 ± 1.25	3.33 ± 0.50	M
De ₁₀	Hu ₁₀	HgCl ₂	0.3	5	8.00	21.75	B	20	30	3.67 ± 1.69	4.00 ± 1.41	M
De ₁₁	Hu ₁₁	NaClO	1	2 × 15	16.56	36.11	N	100	60	5.57 ± 0.47	4.52 ± 1.84	V
De ₁₂	Hu ₁₂	NaClO	1	20	30.00	51.08	N	90	60	4.00 ± 2.20	2.67 ± 2.36	V
De ₁₃	Hu ₁₃	NaClO	2	20	18.75	40.03	BL	80	50	4.33 ± 0.47	4.67 ± 0.47	V
De ₁₄	Hu ₁₄	NaClO	2	20	41.85	48.00	N	90	60	5.33 ± 0.47	3.33 ± 1.89	V
De ₁₅	Hu ₁₅	NaClO	3	20	17.94	33.66	BL	60	50	3.33 ± 0.47	2.33 ± 1.24	F
De ₁₆	Hu ₁₆	NaClO	3	25	60.00	69.77	N	90	60	5.00 ± 0.00	2.67 ± 1.25	V
De ₁₇	Hu ₁₇	Flugal	1	10	87.15	100.00	N	90	0	4.33 ± 1.70	0.00	V
De ₁₈	Hu ₁₈	Flugal	3	10	76.43	97.50	N	100	60	4.00 ± 1.63	3.00 ± 1.41	V
De ₁₉	Hu ₁₉	Flugal	5	10	55.62	91.67	N	80	60	6.00 ± 0.00	4.67 ± 0.50	V
De ₂₀	Hu ₂₀	Nystatin	50	15	84.86	85.81	N	90	50	3.60 ± 1.70	3.00 ± 2.16	V
De ₂₁	Hu ₂₁	Nystatin	50	20	77.85	84.21	N	90	50	5.00 ± 0.00	3.00 ± 1.41	V

Results

Various surface sterilizing agents were used at different concentrations and duration to determine the most efficient procedure for initiation of tissue culture of *A. aspera* using seeds as explants (Table 1).

Germination of *A. aspera* seeds started within three to four days of incubation, with dehusked seeds in general having better percentage germination in comparison to husked seeds (Fig. 1 A and B). Also, dehusked seeds had better survival and lower intensity of contamination for all sterilization treatments used (Table 1). However, growth of explants as indicated by plantlet length did not significantly differ between dehusked and husked seeds.

Simple treatment with 100% ethanol was ineffective, as there was 100% contamination and no growth of explants was evident (Table 1).

At minimum concentration (0.1%) with the shortest duration of treatment (1 min), HgCl_2 was efficient as sterilizing agent; 22.19% of dehusked seeds of *A. aspera* were found to be contamination-free (Table 1). With a germination rate of 90%, also vigorous growth with plantlets of 5.67 cm in length was recorded. For husked seeds, only 3.57% were resistant against contamination using this treatment and germination was only 60%. Increased sterilization time up to 20 min resulted in significant decrease of contamination percentage in both dehusked and husked seeds. However, the negative impact on germination percentage and development was evident starting from 8-min treatment, with significant growth-inhibitory effect on plantlets starting from 16 min. When seeds were sterilized for 5 min with increased concentration of HgCl_2 (0.2 and 0.3%), high efficiency of sterilization was achieved, together with negative consequences on seed germination, as well as on growth and development of explants. Consequently, the optimal results were recorded for seeds treated with 0.1% HgCl_2 for 5 min. After 10 days of cultivation, the survival rate was 56.83 and 36.85% for dehusked and husked varieties of seeds, respectively, with 90 and 70% germination and unaffected growth rate.

Sodium hypochlorite treatment for 15 + 15 min at 1% concentration was highly effective as a surface sterilizer with 83.44 and 63.88% survival of dehusked and husked seeds, respectively, accompanied by 100 and 60% germination rate (Table 1). In addition, growth and development of explants were not negatively affected by the treatment. Other procedures of NaClO treatment (both increased concentration and duration) were less efficient also in respect to sterilization, because of negative consequences on growth of plantlets.

Flugal showed relative low efficiency as a surface sterilizer at low concentration, with the best results achieved at 5% concentration, at which 44.38 and 8.33% of dehusked and husked seeds, respectively, were contamination-free (Table 1). No negative effect on explant growth and development was evident for this treatment.

When seeds were treated with Nystatin, sterilization efficiency was relatively low (Table 1). The best result was evident for 20 min treatment, when 22.15 and 15.79% of dehusked and husked seeds were free of contamination, respectively. Germination percentage and plantlet growth were negatively affected by Nystatin treatment only for husked seeds.

Discussion

Maintenance of an aseptic condition is a prerequisite for successful *in vitro* seed germination and proliferation. In the present study, several treatments showed good results both for dehusked and husked varieties of *A. aspera*. However, dehusked varieties consistently exhibited better results in respect to seed germination, survival of explants and contamination.

Ethanol is a strong, extremely phytotoxic sterilizing agent. For this reason when used the plant material should be exposed for a short period of time. To improve effectiveness in sterilization procedure, ethanol is generally used prior to treatment with other compounds. It has been reported that alcohols are rapidly bactericidal rather than bacteriostatic against vegetative forms of bacteria;

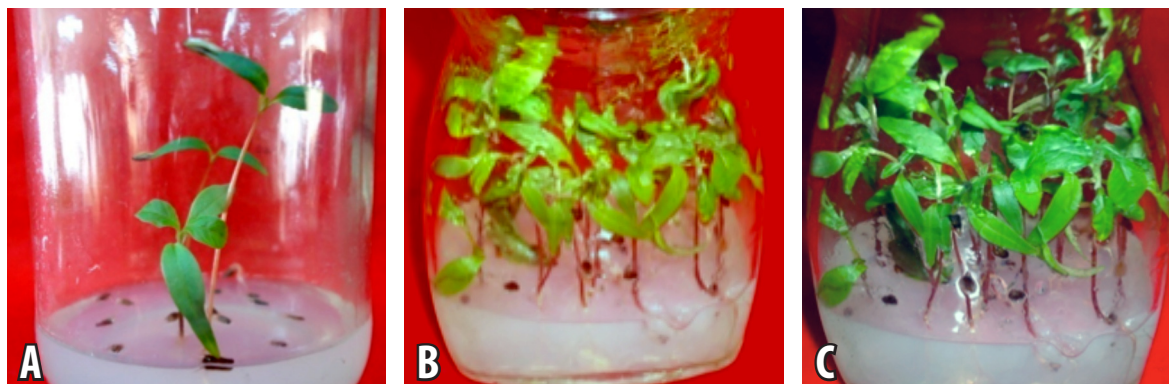


Fig. 1. Relatively low percentage of germination of husked seeds (A) in comparison to dehusked seeds (B). Highest percentage of germination and number of contamination free explants of De_{11} treatment (C; 1% NaClO for 2×15 min).

they also are tuberculocidal, fungicidal, and virucidal but do not destroy bacterial spores (Bloomfield 1978). Their cidal activity drops sharply when diluted below 50% concentration, and the optimum bactericidal concentration is 60 to 90% solution in water (Gross 1987).

There are many reports of surface sterilization in plant tissue culture using HgCl_2 (Naika, Krishna 2008; Preethi et al. 2011; Anburaj et al. 2011; Sen et al. 2013a). However, exposure to HgCl_2 may have negative effects on survival rate of explants (Danso et al. 2011). A long period of exposure with HgCl_2 leads to browning and death of explants. The present results showing deleterious effect of HgCl_2 at high concentration/long exposure is in agreement with other reports (Johnson et al. 2005; Wesely et al. 2011; Johnson et al. 2011; Sen et al. 2013).

Sodium hypochlorite has been reported to be very effective against many types of bacteria; even micromolar concentrations are enough to significantly reduce bacterial populations (Nakagawara et al. 1998). It has also been reported that, when diluted with water, the hypochlorite salts used [NaOCl , $\text{Ca}(\text{OCl})_2$, LiOCl , and KClO] lead to formation of HClO , the concentration of which is negatively correlated with bactericidal activity, perhaps in part due to lethal DNA damage (Wlodkowski, Rosenkranz 1975; Dukan et al. 1999). In this study, increase of sodium hypochlorite concentration showed negative effect on seeds, resulting in blackish color with lower germination rate. The use of sodium hypochlorite for surface sterilization of plant explants from different sources has been widely reported (Miche, Balandreau 2001; Vejsadova 2006; Badoni, Chauhan, 2010; Maina et al. 2010; Colgecen et al. 2011; Morla et al. 2011).

The fungicides Flugal and Nystatin are mainly used against fungal attack in humans. However, they are used also as surface sterilizants in plant tissue culture (Sohnle et al. 1998; Altan et al. 2010; Sen et al. 2013). In the present study, these sterilizants showed good results for cultivation of *A. aspera* and, therefore, may be used as a substitute for NaOCl and HgCl_2 .

Among the treatments used, sample De_{11} showed the best result with 100% germination rate, but the longest plantlets were observed in De_{20} . The treatments De_5 , Hu_5 , De_{13} , and Hu_{13} exhibited satisfactory results. Nystatin and Flugal are generally used for human treatment, but showed good results in the present study and may be used as a substitute for NaOCl and HgCl_2 . However, before final implementation of these results extensively, further research is needed to achieve better understanding of the actual mechanism underlying desirable aseptis.

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