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Full Length Research Paper

In vitro sterilization protocol for micropropagation of *Achyranthes aspera* L. node

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Abstract

***Achyranthes aspera* L. (Apang locally) belongs to the family Amaranthaceae, is a highly significant medicinal herb found almost everywhere in Bangladesh. The herbs are produced by using traditional propagation and *in vitro* techniques. Micropropagation is a rapid propagation technique, but the greatest problem is contamination with fungi and bacteria. We analyzed the effects of Nistatin, Flugal, Bavistin, Ridomil gold, and $HgCl_2$ on surface sterilization to obtain desirable asepsis. However, among the different sterelants, Flugal 3% for 10min followed by 0.1% $HgCl_2$ for 2-3min was the most effective treatment when field-grown plants (node) were used as explants. At higher concentration, fungicides were showed maximum effects against contaminants although survival percentages were low. This is the first report of successful sterilization and reduced contamination of explants from naturally field grown *A. aspera* L. by different sterilizing agents.**

Keywords: *Achyranthes aspera* L.*,* surface sterilization, asepsis, sterilizing agents.

INTRODUCTION

Significant medicinal herb *Achyranthes aspera* L. which is commonly known as Apang (Bengali) and Rough Chaff tree (English). It is an erect or procumbent, annual or perennial, 1-2m in height, often with a woody base, commonly found as a weed of waysides, on roadsides of Bangladesh. It has diverse medicinal uses in the folk medicinal system. Along with the utilization in traditional medicine by local practitioners and healers, this plant also reportedly showed diverse pharmacological properties including lavicidal, immunostimulant, hypoglycaemic (Khandagle et al.,2011; Mali et al.,2006; Malarvili and Gomathi, 2009); also as anticancer, antipyretic, antioxidant, antiasthmatic antimicrobial, and antihelmintic agents (Kartik et al.,2010; Sutar et al.,2008; Suresh et al.,2008; Goyal and Mahajan, 2007; Londonkar et al.,2011; Sujitha et al.,2010).

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 through meristematic tissue culture technique, plant conservation and crop improvement through gene transfer (Sarasan et al.,2011). Contamination of plant tissue cultures by different sources, such as bacteria and fungi, reduces their productivity and can completely prevent their successful culture. A successful tissue culture protocol starts with effective explants sterilization (Dodds and Roberts, 1985). Several different methods are used to eliminate fungal and bacterial contamination, including the use of antibiotics, fungicides, and inactivation by heat and light (Kneifel and Leonhardt, 1992; Leifert et al., 1992; Haldeman et al., 1987).

There are several pathogens (microbial contaminants) which have been a major threat to *in vitro* cultures due to their rapid proliferation characteristics (Enjalric et al.,1998). Contaminants may be introduced with the explants, during manipulations in the laboratory, by micro-arthropod vectors (Leifert and Cassells, 2001) or endophytic bacteria (Pereira et al., 2003). Fungus may also arrive with explants, or airborne, or either a culture. Frequently encountered bacterial and fungal contami-

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nations especially in laboratories of commercial micropropagation pose a considerable problem (Reed et al., 1998). Obuekwe and Osagie (1989) reported that fungi such as *Aspergillus niger* and *Aspergillus flavus* produce oxalate and aflatoxin poisons respectively that can cause death to plant cultures.

Studies on the effect of antibiotics and fungicides on these kinds of contaminants have been carried out (George, 1993). Shields et al.,(1984) analyzed the effects of a number of fungicides against *in vitro* fungal contaminants and their toxicity in tobacco cultures. They recommend two fungicides, carbendazim and fenbendazole (30µgcm-3). In addition imizalil (20µgcm-3) and captofol (100µgcm-3) were alternative fungicides to prevent fungal contamination and a mixture of propiconazole plus carbendazim was effective to control fungal contaminants (George, 1993). But higher concentration of these disinfectants becomes toxic and responsible for lower growth and viability of explants.

To a great extent, asepsis has always been a key factor towards successful *in vitro* plant culturing and mass multiplication. However, there have been limited research studies undertaken to document the success or failure of asepsis in *in vitro* cultures of this plant. In this study, a simple and fast protocol for explants sterilization using Nistatin, Flugal, Bavistin, Ridomil gold, and $HgCl_2$ were evaluated. Nistatin, Flugal, Bavistin, and Ridomil gold are affordable and widely available in shops and supermarkets including in developing countries. Compared to $HgCl_2$, these are less toxic and do not require special handling and waste-disposal precautions, making it a safer option to minimize the use of $HgCl_2$ or at low amount for both researchers and the environment. In this study, optimization of this protocol was an important aspect to ensure that large numbers of clean explants survived after sterilization.

MATERIALS AND METHODS

Plant material and nutrition medium for micropropagation

The experiment was conducted at Plant Biotechnology Division (PBD) of National Institute of Biotechnology (NIB), Dhaka, Bangladesh, during 2011-2012. The nodal segments, these were healthy, free of symptoms of disease, pest problems and showed good biomass yield, collected from the experimental plot of PBD. Plant was taxonomically identified by taxonomist. Nodal segments were inoculated on MS medium (Murashige and Skoog, 1962) containing 3% sucrose and gelled with 0.8% agar supplemented with diverse concentrations of BAP and KIN. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20min at 121^0C for 15lbs pressure.

Surface sterilization of plant material

Nodal explants were washed for 10min under continuous stream of running tap water to remove iota of dist. After that they were again washed with liquid detergent (Vim or Trix). Explants were incubated in different concentration and durations of Nistatin, Flugal, Bavistin, Ridomil gold, and $HgCl_2$, again washed with running tap water to remove any traces of fungicides for 10min. Then the explants were treated with 70% ethyl alcohol for 45sec, dried, and then placed on media.

The conditions of culture room

The growth room conditions maintained for *in vitro* cultures were 25 ± 2^0C and 60-70% relative humidity, light intensity was 3000 lux with a photoperiod of 16hrs day light and 8hrs dark. Each experiment was conducted at least thrice and the development of shootlets from explants was observed in the culture room.

Data recording and analysis

The percentage of explants survival, the growth type and explants color were recorded. Contamination was evaluated 15days after the first incubation. Numbers of contaminated explants and shootelets were counted and the data was subjected to standard deviation using computer software.

RESULTS AND DISCUSSION

In this experiment, different concentrations of sterilizing agents were used (Table 1). Nistatin along with 0.1% solution of $HgCl_2$ for 2-3min were used for sterilization. After inoculation it was observed that, when explants were treated with 0.1% $HgCl_2$ and followed by washing with 25% Nistatin for 15min, 40% explants of *A. aspera* L. were found contamination free and showed vigorous growth also the color of explants were normal with 2.67±1.70 shoots (Figure 1C). At higher concentration it showed maximum effects against contamination but lower survival percentage. When Nistatin was used for relatively low concentrations, it failed to inhibit the microorganism and the activity of this sterilizing agent also supported by study of Altan et al. (2010).

When Flugal was used at different concentrations with 0.1% $HgCl_2$, it showed exciting results for eliminating, especially fungal contaminants from nodal segments. At the lowest concentration, 1% for 10min, half of the explants showed positive response where 2.67±1.70 shoots were produced. But when 7-9% was used, contamination was lowest but the explants conditions were about to death. Albeit at concentration 3-6%, the explants showed good and vigorous growth and up to 78% were contamination free and maximum number of

Table 1. Effects of different concentrations and combinations of surface sterilizers on nodal explants of *A. aspera* L.

Surface sterilizer	Concentration	Duration of exposure	Mercuric chloride treatment	Survival (%)	Contamination (%)	Shoot number	Explants color	Growth type
Nistatin	2%	15	2-3	---	100	---	Normal	---
	4%	15	2-3	---	100	---	Normal	---
	6%	15	2-3	---	100	---	Normal	---
	10%	15	2-3	---	100	---	Normal	---
	25%	15	2-3	40	60	2.67±1.70	Normal	Vigorous
	50%	15	2-3	70	30	1.33+0.47	Blackish	Corrugated
Flugal	1%	10	2-3	50	50	2.67±1.70	Normal	Vigorous
	3%	10	2-3	65	35	4.0±0.82	Normal	Vigorous
	4%	10	2-3	70	30	3.04±1.41	Normal	Vigorous
	5%	10	2-3	76	24	2.67±0.47	Normal	Vigorous
	6%	10	2-3	78	22	2.33±0.47	Normal	Vigorous
	7%	10	2-3	80	20	1.67±0.47	Blackish	Corrugated
	9%	10	2-3	94	6	1.33±0.47	Blackish	Morbid
Ridomil gold	0.5%	10	2-3	---	100	---	Normal	---
	0.7%	10	2-3	---	100	---	Normal	---
	1%	10	2-3	---	100	---	Normal	---
	1%	15	2-3	---	100	---	Normal	---
	1%	20	2-3	20	80	3.33±0.94	Normal	Vigorous
	2%	10	2-3	50	50	3.0±0.82	Normal	Vigorous
	3%	10	2-3	60	40	1.33±0.47	Blackish	Corrugated
	4%	10	2-3	80	40	1.33±0.47	Black	Morbid
	5%	10	2-3	80	20	---	Black	Dead
Bavistin	1%	10	2-3	20	80	3.67±1.25	Normal	Vigorous
	1%	12	2-3	60	40	3.0±0.82	Normal	Vigorous
	1%	14	2-3	60	40	2.33±0.47	Normal	Vigorous
	1%	15	2-3	70	30	2.33±1.25	Normal	Vigorous
	1%	16	2-3	70	30	2.0±0.82	Normal	Faded
	2%	10	2-3	80	20	1.67±0.47	Blackish	Faded
	2%	15	2-3	80	20	1.33±0.47	Black	Faded
	3%	15	2-3	80	20	1.67±0.47	Black	Dead
	4%	15	2-3	80	20	---	Black	Dead
$HgCl_2$	0.1%	3	---	---	100	---	Normal	---
	0.1%	4	---	---	100	---	Normal	---
	0.1%	5	---	10	90	2.67±0.12	Normal	Vigorous
	0.1%	6	---	30	70	2.0±0.82	Normal	Vigorous
	0.1%	7	---	40	60	2.33±0.47	Normal	Vigorous
	0.1%	8	---	50	50	1.33±0.47	Blackish	Faded
	0.1%	10	---	50	50	1.0±0	Black	Morbid
	0.1%	12	---	70	30	---	Black	Dead
	0.1%	14	---	70	30	---	Black	Dead
	0.1%	16	---	70	30	---	Black	Dead

*Each experiment was carried out as triplicate, Shoots number as mean ±SD.

shoots (4.0±0.82) produced from 3% Flugal treating explants (Figure 1E). Study on this fungicide also reported by Sohnle et al.,(1998).

It was observed that Ridomil gold was used at different concentrations with 0.1% $HgCl_2$, at lowest concentration, it was ineffective as a surface sterilizer. But at 2% for 10min, 50% of nodal segments were resistance against contaminants with high growth rate. If the concentration of this fungicide were higher (3-5%), causes no contamination but corrugated to dead condition of explants were observed (Figure 1B). Almost same results of the negative effects of Sodium hypochloride at high concentration were observed (Colgecen et al., 2011).

Bavistin, proved a good surface sterilizer by showing its excellence in good inhibiting power against fungal contaminants. At its lowest concentration, 1% for 10min, 20% of explants were viable against inhibitors and formation of shoots was noticed (3.67±1.25). But it showed its best result with vigorous growth at concentration 1% for 15min and only 30% was susceptible (Figure 1D). When the concentration and duration of time were increased then the resistances to fungus were increased but explants showed similar results alike at higher concentration of Ridomil gold and Flugal. No shoot growth was observed for 4% Bavistin treating nodes. The effectiveness of Bavistin was also reported by Garla et al. (2011).

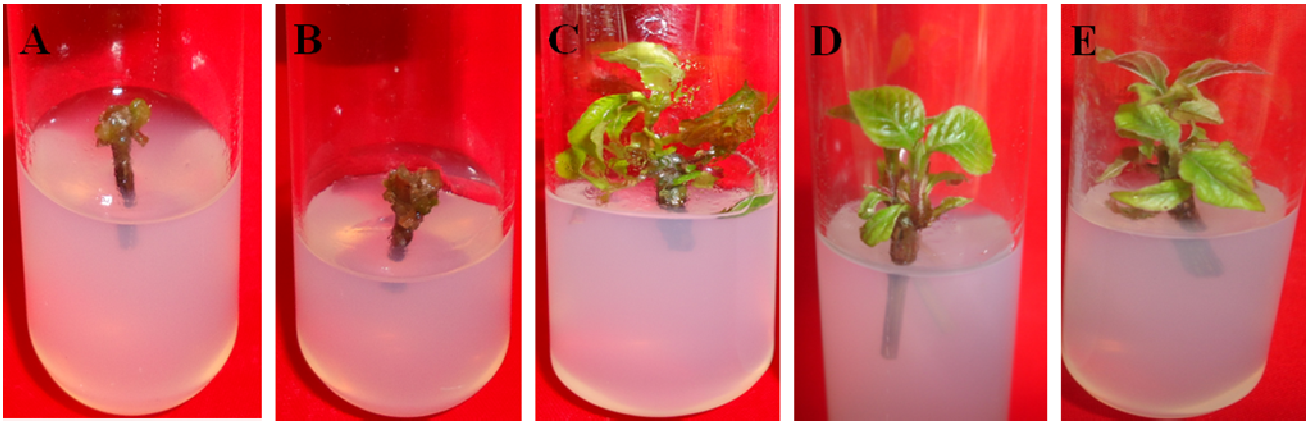

Figure 1. Growth morphology of explants after treatment with different surface sterilizers. A. $HgCl_2$ 0.1% for 12min. B. Ridomil gold 5% for 10min and $HgCl_2$ 0.1% for 2-3min. C. Nistatin 25% for 15min and $HgCl_2$ 0.1% for 2-3min. D. Bavistin1% for 15min and $HgCl_2$ 0.1% for 2-3min. E. Flugal 3% for 10min and $HgCl_2$ 0.1% for 2-3min.

0.1% $HgCl_2$ for 7min, 40% explants of *A. aspera* were found free of contamination and healthy. When $HgCl_2$ was used for relatively short duration (3 or 4min), it failed to kill the microorganisms attached to the explants. 5min treating explants showed maximum number of shoots (2.67±0.12). Explants treated for longer duration (8-16min), caused a few contamination but complete or partial tissue killing was observed (Figure 1A). There are many reports of surface sterilization using the most frequently used surface sterilizer $HgCl_2$ (Naika and Krishna, 2008; Preethi et al.,2011; Anburaj et al.,2011). But exposure of $HgCl_2$ had negative effects on survival rate of explants (Danso et al.,2011). High period of exposure with $HgCl_2$ leads the browning of explants and death. Our results were in tantamount to previous studies (Wesely et al., 2011; Johnson et al.,2011).

In this report, Flugal 3% for 10min followed by 0.1% $HgCl_2$ for 2-3min was the most effective treatment. However, selection of an appropriate fungicide or disinfectant and proper disposal of the used disinfectants in the plant culture laboratory are important as they may become corrosive to the water drainage systems, and hence dangerous to the environment. Many research studies are needed in order to increase our understanding with respect to various disinfectants used in the *in vitro* culture of *A. aspera*.

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REFERENCES

Altan F, Bürün B, Sahin N (2010). Fungal contaminants observed during micropropagation of *Lilium candidum* L. and the effect of chemotherapeutic substances applied after sterilization. Afr. J. Biotechnol.; 7: 991-995.

Anburaj J, Ravider Singh C, Kuberan T, Sundaravadivelan C, Kumar P (2011). Effects of plant growth regulators on callus induction from leaf explants of *Cleome viscose*. Res. J. Pharm. Biol. Chem. Sci.; 2: 576-583.

Colgecen H, Koca U and Toker G. Influence of different sterilization methods on callus initiation and production of pigmented callus in *Arnebia densiflora* Ledeb. Turk. J. Biol.; 35: 513-520.

Danso KE, Azu E, Elegba W, Asumeng A, Amoatey HM, Klu GYP (2011). Effective decontamination and subsequent plantlet regeneration of sugarcane (*Sacchrum officinarum* L.) *in vitro*. Int. J. Integra. Biol.; 11:90-96.

Dodds JH, Roberts LW (1985). Experiments in plant tissue culture (second edition). Cambridge University Press; 21-35.

Enjalric F, Carron MP, Lardet L (1998). Contamination of Primary cultures in tropical areas. The case of *Hevea brasiliensis*. Acta Horticult.; 223:57– 65

Goyal BR, Mahajan SG (2007). Beneficial effect of *Achyranthes apsera* Linn. in Toluene-di-isocyanate induced occupational asthma in rats. G. J. Pharmacol. 1: 06-12.

Garla M, Pratush A, Kumar S, Singh S, Shivani (2011). *In-vitro* callus induction and shoot regeneration in *Ephedra* – A medicinal Plant. Ann. Biol. Res.; 2:645-651.

George EF (1993). Plant Propagation by Tissue Culture, Part 1, Techonology, England: Exegetics Ltd; 121-145

Haldeman JH, Th omas RL, McKamy DL (1987). Use of benomyl and rifampicin for in vitro shoot tip culture of *Camellia sinensis* and *C. japonica*. Hort Science. 22:306-307.

Johnson M, Wesely EG, Kavitha MS, Uma V (2011). Antibacterial activity of leaves and inter-nodal callus extracts of *Mentha arvensis* L. Asian Pac. J. Trop. Med.*;* 4:196-200.

Khandagle AJ, Tare VS, Raut KD, Morey RA (2011).Bioactivity of essential oils of *Zingiber officinalis* and *Achyranthes aspera* against mosquitoes. Parasitol. Res.; 109: 339-343.

Kartik R, Rao CV, Trivedi SP, Pushpangadan P, Reddy GD (2010). Amelioration effects against N-nitrosodiethylamine and CCl4-induced hepatocarcinogenesis in Swiss albino rats by whole plant extract of *Achyranthes aspera*. Ind. J. Pharmacol. 42:370-375.

Kneifel W, Leonhardt W (1992). Testing of diff erent antibiotics against gram positive and gram negative bacteria isolated from plant tissue cultures. Plant Cell Tiss. Org. Cult. 29:139-144.

Leifert C, Camotta H, Waites WM (1992). Effect of combinations of antibiotics on micropropagated *Clematis, Delphinium, Hosta, Iris,* and *Photinia*. Plant Cell Tiss. Org. Cult. 29:153-160.

Londonkar R, Chinnappa RV, Abhay KK (2011). Potential antibacterial and antifungal activity of *Achyranthes aspera* L. Recent Res. Sci. Technol. 3:53-57.

Leifert C, Cassells AC (2001). Microbial hazards in plant tissue and cell cultures. In vitro: Cell Dev. Biol. Plant, 37:133-138.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15:473-497.
Mali RG, Hundiwale JC, Gavit RS, Patil KS, Kulkarni MV (2006). Effect of *Achyranthes aspera* extract on phagocytosis by human neutrophils. J. Nat. Remed. 6:115-119.
Malarvili T, Gomathi N (2009). Effect of *Achyranthes aspera* (Linn) seeds on redox and oxidative status in plasma and selected tissues of rats fed with high doses of fructose. Biosc.i Biotechnol. Res. Asia. 6:659-664.
Naika HR, Krishna V (2008).Plant Regeneration from Callus Culture of *Clematis gouriana* Roxb. – A Rare Medicinal Plant. Turk. J. of Biol. 32: 99-103.
Preethi D, Sridhar TM, Naidu CV (2011). Efficient Protocol for Indirect Shoot Regeneration from Leaf Explants of *Stevia rebaudiana* (Bert.) – An Important Calorie Free Biosweetner. J. Phytol. 3:56-60.
Pereira JES, Mattos MLT, Fortes GRD (2003). Identification and antibiotic control of endophytic bacteria contaminants in micropropagated potato explants. Pesquisa Agropecu. Bras.38:827-834.
Reed BM, Mentzer J, Tanprasert P, Yu X (1998). Internal bacterial contamination of micropropagated hazelnut : identification and antibiotic treatment. Plant Cell Tiss. Org. Cult. 52: 67-70.
Sutar NG, Sutar UN, Sharma YP, Shaikh IK, Kshirsagar SS (2008). Achyranthus aspera- An important medicinal plant. Biosci. Biotechnol. Res. Asia. 5:841-844.
Suresh KP, Sucheta S, Deepa VS, Selvamani P, Latha S (2008). Antioxidant activity in some selected Indian medicinal plants. Afr. J. Biotechnol. 7:1826-1828.
Sujitha K, Phani SA, Mohan RPM, Mahammed L, Srinivasarao K, Karuna SV (2010). Preliminary screening of *Syzygium cumini* and *Achyranthes aspera* for their anthelmintic activity. Res. J. Pharmacol. Phytochem. 2:441-445
Sarasan V, Kite GC, Sileshi GW, Stevenson PC (2011). Applications of phytochemical and *in vitro* techniques for reducing over-harvesting of medicinal and pesticidal plants and generating income for the rural poor. Plant Cell Reports. 30:1163-1172.
Sohnle PG, Hahn BL, Fassel TA and Kushnaryov VM (1998). Analysis of fluconazole effect on *Candida albicans* viability during extended incubations. Med. Mycol. 36: 29-36.
Wesely EG, Johnson M, Kavitha MS, Selvan N (2011). Micropropagation of *Alternanthera sessilis* (L.) using shoot tip and nodal segments. Iran. J. Biotechnol. 9: 206-212.