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Studies on the Effect of Various Sterilization Procedure for *in vitro* Seed Germination and Successful Micropropagation of *Cucumis sativus*

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ABSTRACT

Cucumber (Cucumis sativus L.) is one of the most economically important cucurbit which serve as a good source of nutrition. It plays a significant role in pharmaceutical sectors and also used as an Eco-Friendly Pesticides. An efficient in vitro protocol was developed for micro-propagation of C. sativus to meet the demand in tissue culture lab.The most effective treatment for sterilization of explant was evaluated with four different sterilizing agents i.e: Mercuric (II) chloride ($HgCl_2$), Sodium hypochlorite (NaOCl), Hydrogen peroxide (H_2O_2) and Bavistin. The percentage of contamination, survival rate, shoot length and growth pattern of cultures were studied. Among all experiment, 0.1% Mercuric (II) chloride at 4 min. was showed most effective treatment with highest rate of contamination free culture where the survival rate was 84.44%. Whereas, sterilization with Hydrogen peroxide, Sodium hypochlorite, Bavistin did not get any satisfactory result.

Key words: *Cucumber,Sterilization, in vitro, Explants, growth regulators.*

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is one of the most significant extremely nutritional and popular vegetable crop of the Cucurbitaceae family[27]. The crop is the fourth most significant vegetable after tomato, cabbage and onion in Asia, the second most important vegetable crop after tomato in Western Europe[26,19]. Cucumber have originated in Indian and has been cultivated for 3000 years and 2000 years in china, but now available in everywhere with new varieties and technologies[23,4].This fruitis commercially characterized by both pickling and fresh market cultivars all over the world[15]. The fruits are popular in Bangladesh as “salad” and are often consumed as cooked vegetables in various ways.The potential yield of cucumber in Bangladesh is 15t/ha[21].The edible fruit of *Cucumis sativus* contains 95% water; a 50-g portion provides 0.3 g of dietary fibre and supplies 5 kcal (20 kJ). It is very good for skin and contains anti-inflammatory properties[5]. The stems can expand the blood vessels and reduce blood pressure[12].

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Conventional propagation of fruit treesis very slow and expensive, so micro-propagation is an appropriate method for producing plant material in the short time. Establishment of proficient tissue cultures involves the surface sterilization of explants that carry a wide range of microbial contaminants. *In vitro* contamination by fungi, bacteria and yeast is the most serious problems of commercial and research plant tissue cultures[16]. The presence of microbes in these cultures usually results in increased culture mortality. Different infections lead to effect variable growth, tissue necrosis, reduced shoot proliferation and rooting. So eliminating contaminant from the explant is of prime concern.

The procedure of sterilization is various, depending on plant species, explant source, contamination levels, age and plant part (explant) taken from the plant for sterilization. Generally, the explants naturally carry different types of microbial contamination from the environment, so surface sterilization of explants in chemical solutions is a precarious preparation step. Sterilizing agents are also toxic to the plant tissue so contamination must be removed without killing the plant cells.In order to find an optimized protocol for sterilization of a specific tissue, three factors have to be taken into consideration *viz.*, sterilizing chemical, its concentration and the treatment duration[11]. The disinfectants usually used are sodium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide. These sterilants are also toxic to the plant tissue, hence the type, concentration, time of exposure and removal of traces elements becomes important in standardizing sterilization protocol.

MATERIALS AND METHODS

Seeds Collection: The experiment was conducted at Dept. of Biotechnology & Genetic Engineering of the Islamic University, Kushtia, Bangladesh. Seeds of Cucumber (*C. sativus*) were collected from horticulture center, Kushtia, Bangladesh.

Surface sterilization of seeds: Mature and vigorous seeds were separated carefully and washed with running tap water. The seeds were then washed in double distilled water repetitively. Inside the Laminar Air Flow cabinet, seeds were washed with 70% ethanol for 30s followed by treatment with different sterilizing agents. Different kind of sterilizing agents' *viz.*, Mercuric Chloride (Merck, Germany), Sodium Hypochlorite (Clotech, Bangladesh), Hydrogen Peroxide and Bavistin are tested for explant sterilization by varying their concentration and time of exposure (Table 1). The sterilized seeds were washed 4-5 times immediately with distilled water to eliminate all traces of sterilants and left to air dry.

Nutrient medium and growth condition: MS medium supplemented with appropriate growth hormones were used for explants growth[17]. Medium was checked for the contamination before inoculation. After inoculation, the culture tube was maintained for *in vitro* culture at 25 ± 2 °C temperature and 50 to 60% relative humidity, with a photoperiod of 16 h and 8 h light & dark respectively. Illumination was provided with incandescent lamps (50 W, Philips Agro Lite). Regular and proper record for contamination, germination (seeds) and growth were taken for 30 days.

Shoot development and Root induction: After an appropriate incubation, nodal explants were excised from 3-4 weeks old *in vitro* raised seedlings which serves as an explants. The nodal explants were placed vertically in each culture tube of MS medium containing BAP (1.5 mg/l) (Figure 2C). The medium pH was adjusted (5.8) after the addition of growth hormones. After 3weeks of incubation, isolated single shoots were inoculated in MS medium with NAA (0.5mg/l) for rooting (Figure 2D). The cultures were maintained as described above.

RESULTS AND DISCUSSION

For the primary establishment of *in vitro* culture, surface sterilization of explants was essential because the culture medium used in tissue culture techniques is most suitable for the growth of the microbes also.

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

Ethanol is a strong phytotoxic sterilizing agent. To improve effectiveness in sterilization procedure, ethanol is normally applied earlier to treatment with other compounds. Previous report defined that alcohols are rapidly bactericidal rather than bacteriostatic against bacterial vegetative cells; they are also tuberculocidal, fungicidal, and virucidal but do not destroy spores[7]. Simple treatment in our treatment, 70% ethanol was ineffective, as there was no growth and 100% of explants was contamination (data not shown).

There are many reports of surface sterilization in plant tissue culture using $HgCl_2$[3,20]. At minimum concentration (0.1%) with the shortest duration of treatment (2 min), 48.88% seeds were contaminated whereas 55.55% of cultures are survived. Such response might be due to bleaching action of two chloride atoms and also ions that combines strongly with proteins and causing the death of organisms. In contrast Bamel *et al.*,[6] and Singh *et al.*,[25] described seedling growth by using 0.1 % $HgCl_2$. Increased sterilization time up to 8 min resulted in significant decrease of contamination percentage (Fig1A)[6,25]. But the negative impact on germination percentage and development was evident starting from 5 min of treatment. Consequently, the optimal results were record 84.44% for seeds treated with 0.1% $HgCl_2$ for 4 min after 3 wk of inoculation (Figure 2B). However, exposure to $HgCl_2$ may leads to browning and death of explants[8]. The present results showing deleterious effect of $HgCl_2$ at high concentration/long exposure is agreement with other reports[13,28].

Sodium hypochlorite treatment for 20 min at 2% concentration was showed 60.00% survival of seeds and almost all the cultures were contaminated. But when the seeds were soaked with (2%) concentration of sodium hypochlorite but increased espouser duration of 25 min, resulted in comparatively higher germination rate (71.11 %) with uniform seedling growth and reduced levels of contamination but shown negative impact on germination (Fig 1B). It has also been reported that, when NaOCl diluted with water, the hypochlorite salts lead to formation of HClO, which is negatively correlated with bactericidal activity, perhaps in part due to lethal DNA damage[9,29]. The reports of Newman *et al.*,[18] used NaOCl 2.5% for 15 min and Reda *et al.* (2004) 3% (v/v) NaOCl sodium hypochlorite, also substantiate the role in determining sodium hypochlorite as an effective surface sterilant for sterilization[18,22].

Among different combinations of H_2O_2 lower concentrations i.e.10% for 15 min resulted 51.11% germination but 48.88% contamination of cultures (Fig 1C). Increased concentration of H_2O_2 i.e. 15% for10 min resulted 63.89%of contamination free cultures but seedling growth was not uniform. Previously number of researchers used hydrogen peroxide (H_2O_2) for surface sterilization of their explants but they also didn't get satisfactory result[10,11].

Fungicide Bavistin is widely used to control fungal attack. For surface sterilization during plant tissue culture, it is also used as a sterilizants[2,14]. In the present experiment, 3% of Bavistin for 12 min showed 57.77% of survive culture with less contamination (Fig 1D).

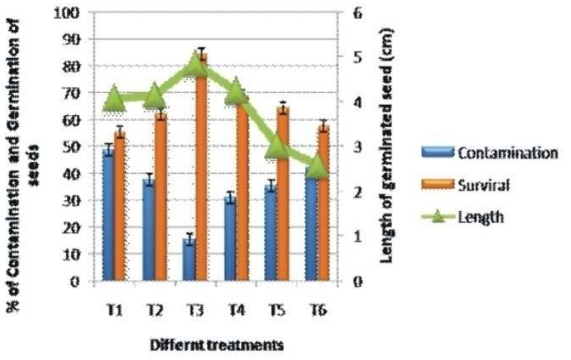
The morphogenetic responses of nodal segment of *C. sativus* depends on different types of growth regulators. Nodal explants cultured on growth regulator-free MS medium showed no significant bud break even after 30days. Addition of cytokinin was essential to induce bud break from the explants. Growth regulators and their concentration was optimized previously[1].

By and large, in the present experiment, surface sterilization of seeds with 0.1 % $HgCl_2$ for 4 minutes was found more effective ensuing in vigorous and high germination rate and contamination free cultures. The failure of above sterilants could be endorsed to resistance or tolerance of microbes present on the surface of explant or due to the high load of contaminants on the explant surface. This could be explained by the fact that requirements for sterilization are different and depend on the tissues and the type of the explants used for micro-propagation.

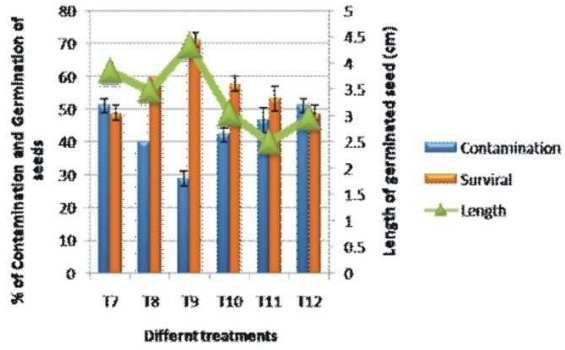
Table 1. Types of sterilizing agents used in adifferent concentration with varying time ofexposure and the morphology of growth (explants)

Treatment	Sterilants	Concentration (%)	Time (min.)	Length (cm)	Growth pattern
T1	$HgCl_2$	0.1	2	4.08	Medium, Non-uniform
T2			3	4.14	Healthy, Uniform
T3			**4**	**4.85**	Healthy, Uniform
T4			5	4.25	Healthy, Uniform
T5			6	3.03	Healthy, Uniform
T6			8	2.59	Medium, Uniform
T7	NaOCl	2.0	15	3.85	Medium, Contaminated
T8			20	3.48	Healthy, Uniform
T9			25	4.33	Medium, Uniform
T10		3.0	15	3.05	Healthy, Uniform
T11			20	2.51	Healthy, Non-uniform
T12			25	2.96	Healthy, Non-uniform
T13	H_2O_2	10	15	3.55	Medium, Contaminated
T14		10	12	3.61	Medium, Contaminated
T15		15	12	3.70	Healthy, Non-uniform
T16		15	10	3.75	Healthy, Non-uniform
T17		20	10	3.21	Medium, Contaminated
T18		20	8	2.96	Medium, Non-uniform
T19	Bavistin	1.0	12	2.89	Medium, Contaminated
T20		1.0	15	2.95	Medium, Contaminated
T21		3.0	10	3.10	Healthy, Non-uniform
T22		3.0	12	3.45	Healthy, Non-uniform
T23		5.0	10	2.90	Medium, Non-uniform
T24		5.0	8	2.88	Medium, Non-uniform

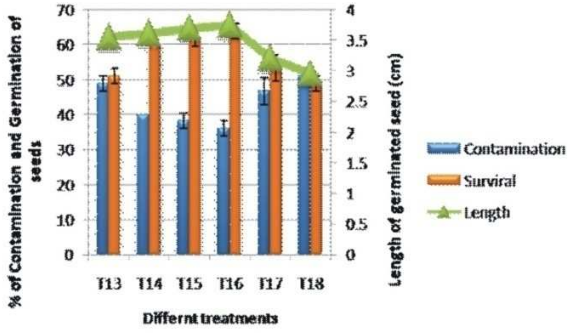
Fig. 1 (A-D): Effects of different sterilization procedures: contamination, survival rate & length of *C. sativus* explants. Values are the means ± standard



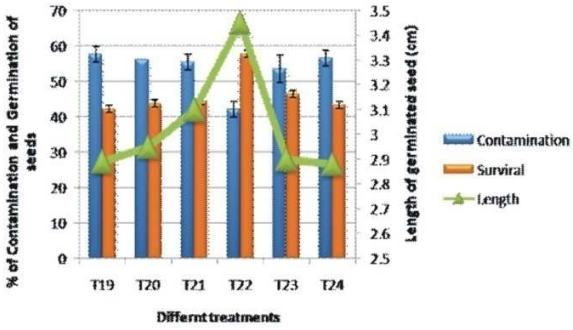
A. Mercuric (II) chloride ($HgCl_2$)



B. Sodium hypochlorite (NaOCl)



C. Hydrogen peroxide (H_2O_2)

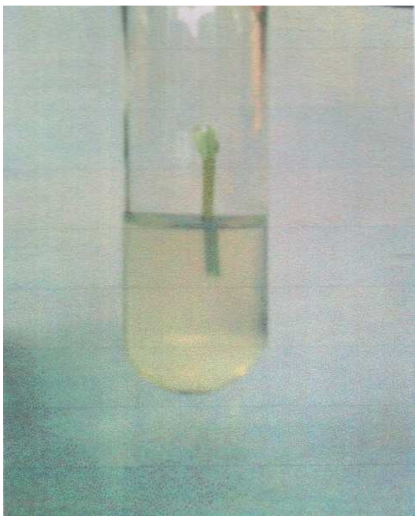


D. Bavistin

Fig. 2 (A-D): Different stages of plantlet's development of *Cucumis sativus* L. After sterilized with 0.1% Mercuric (II) chloride ($HgCl_2$) for 04 minutes

A. Inoculated seeds of *C. sativus*

B. Shoot formation from inoculated seed



C. *In vitro* shoot development

D. Root induction from regenerated shoot

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