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Research article

STANDARDIZATION OF *IN VITRO* STERILIZATION PROCEDURES FOR MICROPROPAGATION OF GINGER (*ZINGIBER OFFICINALE* ROSC.)

M. M. Khatun[1]*, T. Tanny[1], Abdur. M. Razzak[2], Firoz Alam M[3], Ekhlas Uddin M[3], Ruhul Amin[3] and S. Yesmin[1]

[1]National Institute of Biotechnology, Ganakbari, Savar, Dhaka-1349, Bangladesh
[2]Pohang University of Science and Technology, Pohang 790–784, South Korea
[3]Department of Biotechnology and Genetic Engineering, Islamic University, Kushtia, Bangladesh

ABSTRACT: Ginger is one of the most important spices crop in all over the world. But, inadequate multiplication rate and disease transmittance are hampers ginger cultivation to meet the demand of high quality planting material for commercial cultivation. Therefore, an efficient *in vitro* regeneration protocol considers a best alternative to overcome this problem. During micropropagation surface sterilization is the most important step in preparation of explants. The effect of four different sterilizing agents: Mercuric (II) chloride ($HgCl_2$), Sodium hypochlorite (NaOCl), Hydrogen peroxide (H_2O_2) and Bavistin, were evaluated for sterilization of rhizome buds of *Zingiber officinale* Rosc. by varying their concentration and time of exposure. The percentage of contamination, tissue damage and survival of cultures were observed. The result showed that among all sterilization treatments 0.1% Mercuric (II) chloride ($HgCl_2$) was the most effective treatment. Highest rate of contamination free culture 86.66±0% was achieved with 0.1% ($HgCl_2$) for 15 minutes. Whereas, sterilization with Hydrogen peroxide, Sodium hypochlorite, Bavistin were not satisfactory.

Key Words: *Zingiber officinale*, contamination, surface sterilization, rhizome buds.

*Corresponding author: M. M. Khatun, [1]National Institute of Biotechnology, Ganakbari, Savar, Dhaka-1349, Bangladesh, muslimabtge@gmail.com Tel: +8801716280501

INTRODUCTION

Ginger (*Zingiber officinale* Rosc.), a herbaceous rhizomatous perennial herb belonging to the family Zingiberaceae is grown commercially in many tropical region and is native to tropical South East Asia (Sathyagowri and Seran, 2011). It is commonly used all over the world as spices and medicine. Ginger is commercially cultivated in India, China, Nepal, Nigeria, Thailand, Indonesia, Bangladesh and Japan. Ginger is one the most important spice crops in Bangladesh. Bangladesh is now seventh largest producer and third largest consumer of ginger. Bangladesh produces about 48,000 metric tons of ginger in an area of 6882 hectare of total land (Anon, 2004). In Bangladesh, yearly requirement is 122,000 metric tons. But the country can produce only 40% of its requirement. The rest 60% demand for home consumption totally depends on import. Breeding of ginger is handicapped by poor flowering and seed set therefore it is propagated vegetatively through rhizome (Kambaska and Santilata, 2009). Conventional propagation through seed rhizomes produces 10-15 lateral buds in a season of 8-10 months (Bhagya lakshmi and Singh, 1988). Vegetative propagation of ginger has the high risk of spreading systemic infections. Slow propagation rate and the risk of disease transmittance through division by sectioning of the rhizomes have hampered propagation by conventional means.

Therefore *in vitro* technique is considered the best alternatives method that may supply a large number of planting materials for commercial planting and further studies (Hamirah et al., 2010). During plant tissue cultures contamination may occur by different microorganisms that's reduces productivity and can completely prevent their cultivation. The contamination of explants may be due to fungi, bacteria, moulds etc., present on the surface or lodged in the cracks and scales (Hiremath, 2006). Many authors argued that the contamination of underground rhizomes was very high and the establishment of contamination frees cultures was difficult (Islam et al., 2004). Therefore, successful tissue culture protocols start with effective explant sterilization (Dodds and Roberts, 1985; Srivastava et al., 2010; Badoni and Chauhan, 2010; Mihaljević et al., 2013). It is difficult to determine standard sterilization procedures that apply to all plants. However, there is no satisfactory study to standardize the sterilization protocol for micropropagation of *Zingiber officinale* Rosc. Therefore, the present study was aimed at standardizing the sterilization method for explants of *Zingiber officinale* Rosc for micropropagation, using different types of sterilizing agents by varying their concentration and duration of exposure.

MATERIALS AND METHOD

Explants and nutrient medium: 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 *Zingiber officinale* Rosc. (Ginger) in conditions of *in vitro* culture. BARI ada-1 (ginger) was used in this experiment. Healthy rhizomes were kept in sand for sprouting. The stored sprouted rhizomes were used to get explants (Figure 2. A). After sterilization by using different sterilants in an autoclaved beaker or conical flask, with different concentrations and duration, rhizome buds about 1 to 2 cm long were placed in a coffee jar, test tube, or conical flask on MS medium (Duchefa, The Netherlands; Murashige and 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.

Explant sterilization: The sprouted rhizome buds were collected in beaker or conical flask and kept under running tap water prior to sterilization in the laminar airflow cabinet. For the experiment four different kinds of sterilizing agent's viz., Mercuric (II) chloride ($HgCl_2$), Sodium hypochlorite (NaOCl), Hydrogen peroxide (H_2O_2) and Bavistin, were tested for explant sterilization by varying their concentration and time of exposure.

Inoculation: MS basal medium supplemented with cytokinin (0.3mg/l BAP) and auxin (0.5mg/l NAA) were used for inoculation and subsequent micropropagation of *Zingiber officinale* Rosc. (Figure 2. B-E). Medium was checked for the contamination before inoculation. Sterilized explants were trimmed suitably to remove sterilizing agent affected parts/brown parts. Explants were then inoculated on the appropriate medium and labeled properly. Observation were recorded regularly till to 30 days for the contamination, tissue damage and survival cultures.

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

Statistical Analysis: Statistical analysis was done to find out the effect of different sterilizing agents its concentration and time of exposure on the asepsis of the said plant species. For each experiment, 12 rhizome buds and conducted at least twice. The mean values and standard deviations of contamination, tissue damage and survival cultures were calculated using computer software (Microsoft Office Excel Worksheet).

RESULTS AND DISCUSSION

Different surface sterilizing agents were used at different concentrations and duration to determine the most efficient procedure for initiation of tissue culture of *Zingiber officinale* Rosc. using rhizome bud as explants (Table 1). After observing the inoculated explants for 30 days on contamination, tissue damage and survival of cultures, it was found that increasing time and concentration significantly reduced contamination but showed adverse effect on explants. Among all the four sterilizing agents' viz., Mercuric (II) chloride ($HgCl_2$), Sodium hypochlorite (NaOCl), Hydrogen peroxide (H_2O_2) and Bavistin, the most effective surface sterilization treatments on rhizome buds were evaluated by using Mercuric (II) chloride ($HgCl_2$) solution (Figure 2).

The result showed that Mercuric (II) chloride treatments (0.1%) for 15 minutes was the best treatment given 11.11% contaminated, 8.88% tissue damaged and 86.66±0% survival cultures (Figure 1.A). Increasing the exposure time and sterilant concentration had reduced the contamination rate but high number of damage explants resulted. The effect of the sterilizing chemical alter or denature the shape and function of microbial enzymes (George et al., 2008). But the increasing concentration of sterilants and exposure time above certain optimum limit cause loss of explants because of the oxidant chemical ingredient killing the plant tissue as well (Danso et al., 2011). In our study, the explants exposed to long period with $HgCl_2$ leads to browning in color and cause death. Hence, the optimum treatment combination (concentration and time) for effective sterilization of explants should be determined. Suma et al., (2008) reported 0.1% (w/v) $HgCl_2$ for 10 min is an effective treatment to remove microbes for rhizome buds of ginger. Whereas, Kavyshree (2009) used 0.1% (w/v) $HgCl_2$ for 3 min followed by 0.1% streptomycine for 1 min to avoid bacterial contamination. There are many reports of surface sterilization in plant tissue culture using $HgCl_2$ (Naika and Krishna, 2008; Preethi et al., 2011; Anburaj et al., 2011).

Sodium hypochlorite has been reported to be very effective against different types of bacterial strains; even micro-molar concentrations are enough to significantly reduce bacterial populations (Nakagawara et al., 1998). In the present study the highest rate of contamination free culture with sodium hypochloride (3%) was obtained 51.11±2.22 (Figure 1.B) from treatment for 10 minutes. Similar results were obtained by Hamirah et al., (2010) in micropropagation of red ginger (*Zingiber montanum* Koenig). On the other hand, Sathyagowri and Seran, (2011) reported 20% of Clorox (sodium hypochloride, 5.25% active ingredient) with 2-3 drops of tween 20 for 20 minutes is an effective treatment to remove microbes. The use of sodium hypochlorite for surface sterilization of plant explants from different sources has been widely reported (Miche and Balandreau, 2001; Vejsadova, 2006; Maina et al., 2010; Colgecen et al., 2011; Morla et al., 2010).

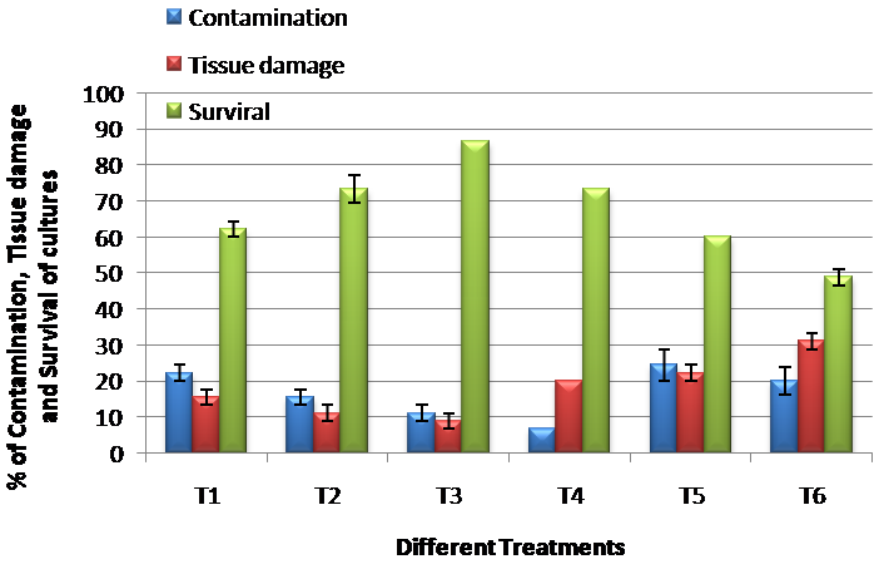
The treatments with hydrogen peroxide (H_2O_2) showed unsatisfactory results, showing a high percent of contamination and a low percent of survived explants. The highest rate of contaminated cultures 53.33±3.84 (Figure 1.C) was obtained with 5% H_2O_2 for 10 min. The present finding showed sterilization with hydrogen peroxide (H_2O_2) is less efficient comparing with other three disinfectants. Previously number of researchers used hydrogen peroxide (H_2O_2) for surface sterilization of their explants but they also didn't get satisfactory result (Sen et al., 2013; Himabindu et al., 2012).

Table 1. Type of sterilizing agents with different concentration and exposure time used for sterilizing rhizome buds of *Zingiber officinale* Rosc.

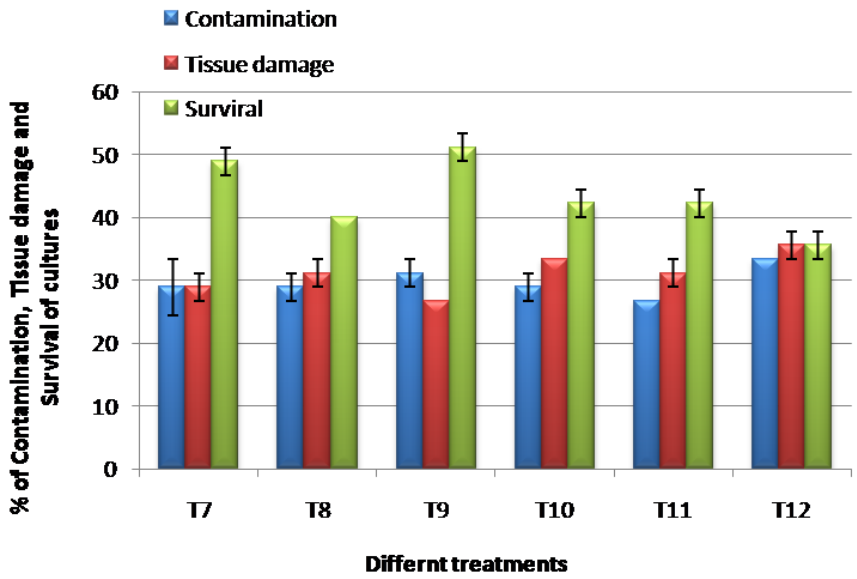
Treatment	Sterilizing agents	Concentration (%)	Time (min)
T1	Mercuric (II) chloride ($HgCl_2$)	0.1	5
T2	Mercuric (II) chloride ($HgCl_2$)	0.1	10
T3	Mercuric (II) chloride ($HgCl_2$)	0.1	15
T4	Mercuric (II) chloride ($HgCl_2$)	0.1	20
T5	Mercuric (II) chloride ($HgCl_2$)	0.5	5
T6	Mercuric (II) chloride ($HgCl_2$)	0.5	8
T7	Sodium hypochlorite (NaOCl)	2.0	10
T8	Sodium hypochlorite (NaOCl)	2.0	15
T9	Sodium hypochlorite (NaOCl)	3.0	10
T10	Sodium hypochlorite (NaOCl)	3.0	15
T11	Sodium hypochlorite (NaOCl)	5.0	8
T12	Sodium hypochlorite (NaOCl)	5.0	10
T13	Hydrogen peroxide (H_2O_2)	5.0	10
T14	Hydrogen peroxide (H_2O_2)	5.0	15
T15	Hydrogen peroxide (H_2O_2)	10.0	10
T16	Hydrogen peroxide (H_2O_2)	10.0	12
T17	Hydrogen peroxide (H_2O_2)	15.0	6
T18	Hydrogen peroxide (H_2O_2)	15.0	8
T19	Bavistin	1.0	12
T20	Bavistin	3.0	10
T21	Bavistin	5.0	8

The fungicides Bavistin is mainly used against fungal attack in humans. However, this is also used as surface sterilizants in plant tissue culture (Sohnle et al., 1998; Altan et al., 2010). In the present study, this sterilizant showed 51.11±2.22 contamination free cultures of *Zingiber officinale* Rosc (Figure 1.D).

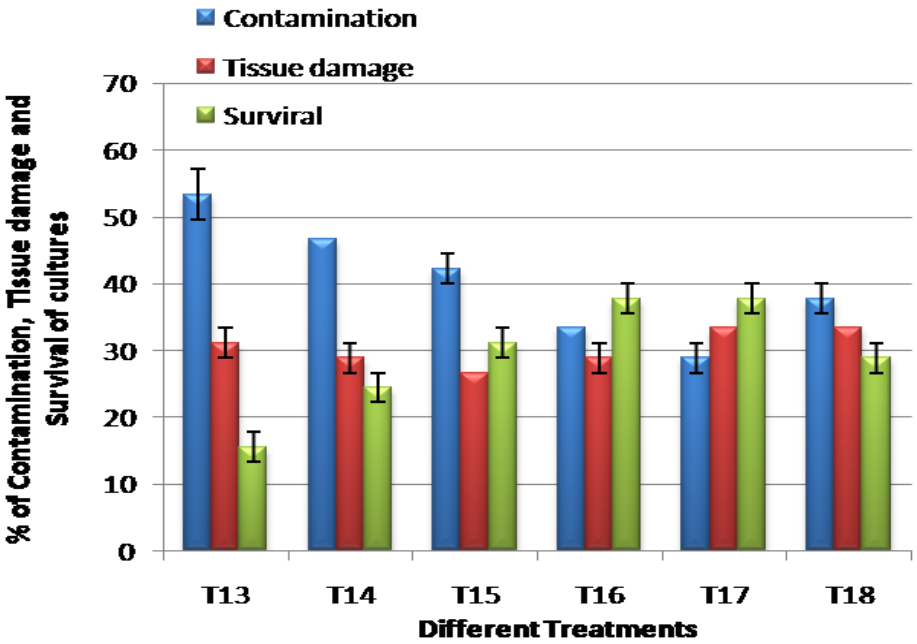
In conclusion, among all the treatment used in this experiment, 0.1% Mercuric (II) chloride for 15 minutes found better for controlling contamination and tissue damage of *Zingiber officinale* Rosc. It can be describe that requirements for sterilization are different and depend on the tissue type, age and the nature of the explant used for micropropagation. However, to achieve better understanding of the actual mechanism, further research is needed to exploit the development of quick regeneration and transformation protocol for ginger.



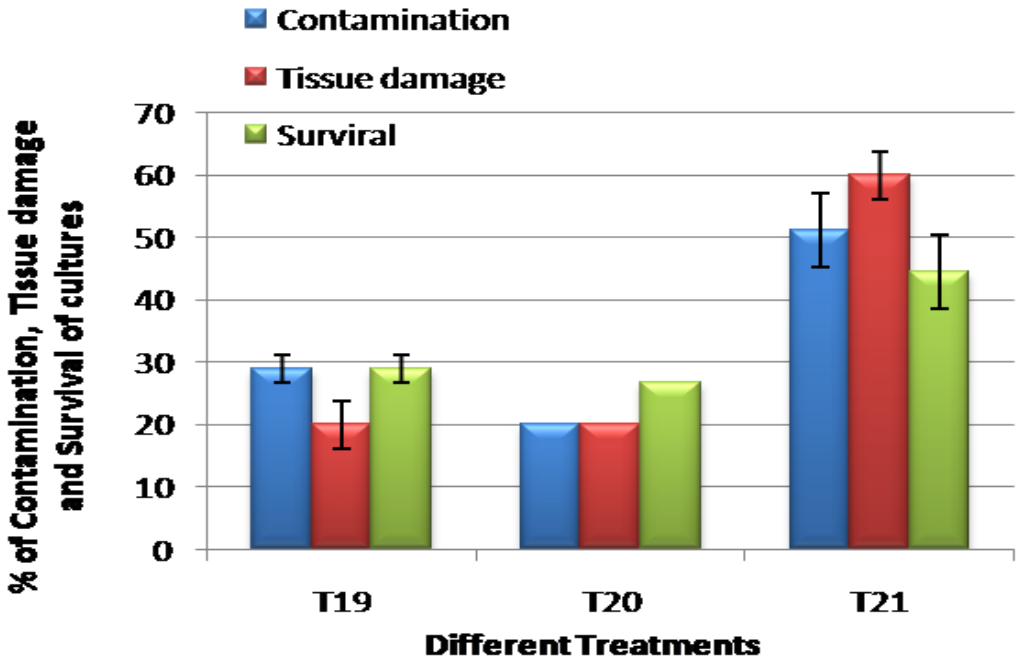
A. Mercuric (II) chloride ($HgCl_2$)



B. Sodium hypochlorite (NaOCl)

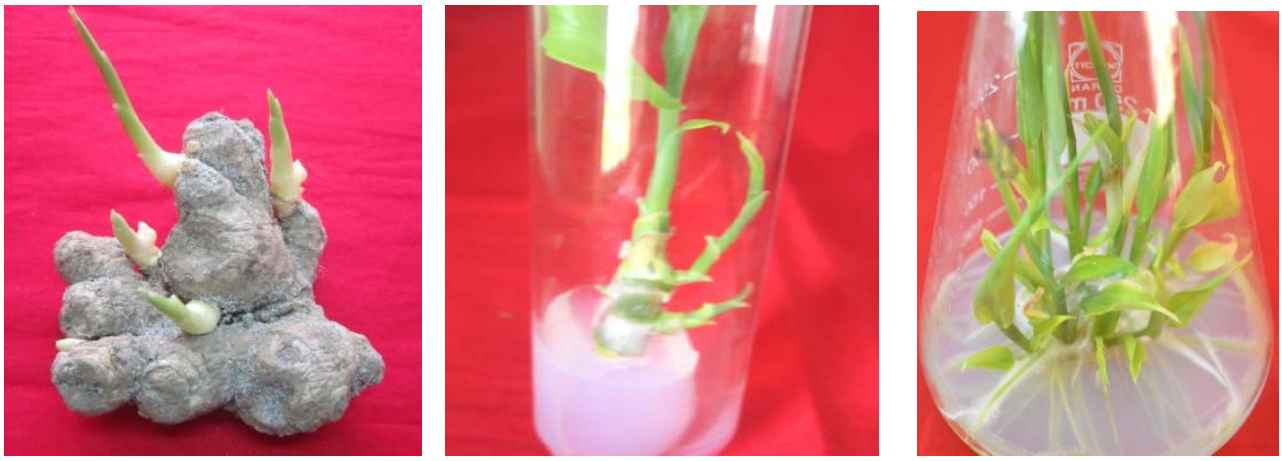


C. Hydrogen peroxide (H_2O_2)



D. Bavistin

Figure 1 (A-D): Effects of different sterilizing agents: A. Mercuric (II) chloride ($HgCl_2$), B. Sodium hypochlorite (NaOCl), C. Hydrogen peroxide (H_2O_2) and D. Bavistin with different concentration and exposure time on rhizome buds of *Zingiber officinale* Rosc. (Data were recorded after 4 weeks of culture on the MS medium using 3% w/v, sucrose). Values are the means ± standard



A. Sprouted rhizomes **B. *In vitro* shoot induction of *Gingiber officinale* Rosc.** **C. Shoot multiplication**

D. *In vitro* rooting **E. Acclimatization of *in vitro* plantlets in pot soil**

Figure 2. (A-E): **Different stages of plantlet's development from rhizome bud of *Gingiber officinale* Rosc. After sterilized with 0.1% Mercuric (II) chloride ($HgCl_2$) for 15 minutes**

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