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Gandamalla Madhuri
Ph.D., Research Scholar, Department of Floriculture and Landscaping, College of Agriculture, OUAT, Bhubaneswar, Odisha, India

Siddharth Kumar Palai
Floriculturist, All India Co-ordinated Research Project on Floriculture, College of Agriculture OUAT, Bhubaneswar, Odisha, India

Sashikala Beura
Professor and Head, Department of Floriculture and Landscaping, College of Agriculture OUAT, Bhubaneswar, Odisha, India

Corresponding Author:
Gandamalla Madhuri
Ph.D., Research Scholar, Department of Floriculture and Landscaping, College of Agriculture, OUAT, Bhubaneswar, Odisha, India

Effect of chemical sterilants on surface sterilization of flower stalk during *in vitro* propagation of *Phalaenopsis* hybrids cv. Shagan

Gandamalla Madhuri, Siddharth Kumar Palai and Sashikala Beura

Abstract
Phalaenopsis orchids have high economic value in the floriculture industry. *In vitro* techniques are being widely used for large scale multiplication of *Phalaenopsis.*An experiment was conducted at Biotechnology-cum-Tissue Culture Center, Department of Floriculture and Landscaping, CA, OUAT, Bhubaneswar during 2016-18 to standardize the method of sterilization of explants using $HgCl_2$ (0.1%) and NaOCl (0.5% and 1%) solution. The effect of treatment duration of $HgCl_2$ and NaOCl, either alone or in combinations, on flower stalk explants was studied. Results revealed that the explants treated with 0.1% $HgCl_2$ (3 min) followed by 1% NaOCl (2 min) resulted in minimum percentage of fungal infection (3.33%), and bacterial infection (0.00%). The same treatment recorded maximum percentage of aseptic culture (96.67%) and maximum survival percentage of explants (96.67%).

Keywords: Surface sterilization, *Phalaenopsis*, flower stalk

Introduction
Phalaenopsis orchids are highly priced in the floriculture industry for cut flowers as well as potted plants throughout the world. Plant tissue culture technology is being widely used for large scale plant multiplication of *Phalaenopsis* to feed into this industry. The flower stalk culture is an excellent method for rapid clonal propagation (Tanaka, 1992; Tokuhara and Mii, 1993) [14, 15] since the parent plant is not harmed and can grow normally after the stalks are harvested. Also, direct shoot regeneration without undesirable callus formation shortens the time period needed to produce plantlets and reduces the occurrence of somaclonal variants. However, reports of successful plant regeneration using flower stalks have been very limited (Arditti and Ernst, 1994; Tanaka, 1992; Tokuhara and Mii, 1993) [1, 14, 15]. The first condition for the success of a culture is asepsis. The maintenance of aseptic (free from all micro-organisms) or sterile conditions is essential for successful tissue culture procedures. To maintain an aseptic environment, all culture vessels, media and instruments used in handling tissues, as well as explant itself must be sterilized. The importance is to keep the air, surface and floor free of dust. All operations should be carried out in laminar airflow sterile cabinet Chawala, (2003) [5]. Sterilization is the process of making explants contamination free before establishment of cultures. Various sterilizing agents are used to decontaminate the tissues. These sterilizing chemicals are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposure to the sterilants, the sequences of using these sterilants has to be standardized to minimize explant injury and achieve better survival (CPRI, 1992) [4]. The most common products for disinfection are based on chlorine-derived commercial solutions, such as NaOCl or $HgCl_2$. The present research effort aims to elucidate standardization of sterilization process with $HgCl_2$ at 0.1% and NaOCl at 0.5% and 1% at different treatment duration in *Phalaenopsis* hybrids using flower stalk segments as explant.

Materials and Methods
Plant material and nutrition medium for Micropropagation
Phalaenopsis hybrid was chosen for the experiment as it is an important cut flower and potted plant. The plants are usually grown in earthen pots with media mixture of coconut husk chips and charcoal. The pots were kept on 2.5ft high iron benches inside a shade net house (50% shade) at Biotechnology-cum-Tissue Culture Centre, Department of FLS, CA, OUAT, Bhubaneswar.

The flower stalk segments were inoculated on MS medium (Murashige and Skoog, 1962[11] + 1ppm BAP + 0.5ppm NAA + 3% sucrose. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20min at 121^0C for 1.06Kg/cm^2 pressure.

Surface sterilization of explants

The explants were collected from healthy plants and washed thoroughly in running tap water for 30 minutes to remove all the adhering dust particles. These explants were soaked in liquid detergent, Tween 20 (Hi media laboratories, India) for 10 minutes with vigorous shaking and washed with running tap water 4-5 times to remove any traces of detergent. They were dipped in 0.2% w/v solution of Bavistin (BASF India Limited) for 30 min, followed by 3-4 times washing in distilled water. After treating the explants with Bavistin, they were brought to laminar flow cabinet and were subjected to treatment with sterilizing chemicals.

The flower stalk explants were surface sterilized with 0.1% (w/v) aqueous solution $HgCl_2$ for different duration according to treatment combinations**.** Subsequently, the explants were thoroughly washed with sterile distilled water 2-3 times to remove the traces of mercuric chloride. These explants were again treated with 0.5 and 1.0%sodium hypochlorite (NaOCl) for different duration according to treatment combination**.** Surface sterilized explants were washed with sterile double distilled water 2-3 times in the laminar air flow chamber, to remove the traces of sodium hypochlorite. This process was carried out under aseptic conditions, in a laminar air flow cabinet. After surface sterilization, the explants were kept in on autoclaved filter paper in sterilized petridishes and air dried. The air dried explants were cut into single node pieces with the help of surgical blade and forceps before inoculation. The explants were cultured in the medium maintaining polarity. The culture tubes/bottles were securely sealed with cotton plugs/plastic caps and labelled.

Incubation of cultures

The cultures were incubated in growth rooms at a temperature of 25 ± 2^0C and 60-70% relative humidity. A light intensity of 3000 lux and photoperiod of 16hrs day light and 8hrs dark was maintained in the culture rooms with cool, white, fluorescent light.

Data recording and analysis

This experiment was carried out in completely randomized design (CRD) with 25 treatments. Twenty explants were taken in each treatment combination and the experiment was replicated three times. The cultures were observed for contamination within 7 weeks of culture. The various observations *viz*., percentage of fungal infection, percentage of bacterial infection, percentage of aseptic culture, percentage of death of explants and survival percentage of explants was recorded and the data obtained were statistically analysed. The data generated were subjected to ANOVA following Complete Randomized Design at 5% level of significance. That data recorded in percentage were subjected to arc sine transformations for statistical analysis. The significant difference among treatments was compared by critical difference.

Results and Discussion

This study revealed that, significantly low percentage of fungal infection (3.33%) was observed in the flower stalk explants, surface sterilized with the treatment T_{20} (0.1% $HgCl_2$, 3 min followed by 1% NaOCl, 2 min). The percentage of infection was at par (8.33%) with the treatment T_{19} (0.1% $HgCl_2$, 3 min followed by 1% NaOCl, 1 min). All cultures were infected in the untreated control. Further increase in the duration of treatment over the best treatment, recorded (0.00%) fungal infection but led to death of explants and significantly declined survival percentage of explants. The percentage of bacterial infection was non-significant in this treatment. The percentage of aseptic culture was significantly maximum (96.67%) in T_{20} (0.1% $HgCl_2$, 3 min followed by 1% NaOCl, 2 min) and this was at par with the treatment T_{19} (0.1% $HgCl_2$, 3 min followed by 1% NaOCl, 1 min) (91.67%). There was no aseptic culture as all the explants were infected by fungus in treatment T_1 (control). Although the percentage of death of the explants was significantly low (0.00) in T_1 (0.1% $HgCl_2$, 1min) as compared to T_{20} (0.1% $HgCl_2$, 3 min followed by 1% NaOCl, 2 min) but T_{20} was considered as best treatment because it recorded minimum percentage of contamination (bacterial and fungal infection) and maximum percentage of aseptic culture and final survival of explants. Significantly highest number of dead explants were reported in T_{24} (0.1% $HgCl_2$, 4 min followed by 1% NaOCl, 2 min) (38.33%). Further increase in the treatment duration over the best treatment recorded increase in death of explants. It is known that, in order to reduce the rate of explant mortality during surface sterilization, the sterilizing agent concentration should be reduced and the exposure time is increased, and vice versa, to minimize the phytotoxic activity of the sterilizing agents (Sathyanarayana and Varghese, 2007) [12].

The percentage of survival of explants was significantly maximum (96.67%) in T_{20} (0.1% $HgCl_2$, 3 min followed by 1% NaOCl, 2 min) and it was at par with the treatment T_{19} (0.1% $HgCl_2$, 3 min followed by 1% NaOCl, 1 min) (91.67%). A single explant did not survive as all the explants recorded fungal infection in treatment T_1 (control). Further increase in the exposure time of sterilizing chemicals, over the best treatment, recorded declining trend of survival percentage of explants. Endress (1994) [7] reported that the main objective of any sterilization procedure is to obtain minimum contamination with maximum tissue survival. Seeni and Latha (2000) [13]reported 95-100 percent explants survival when the explants were surface sterilized using 70% ethanol for 20 seconds and 0.1% $HgCl_2$ for 3 minutes.

Standardization of the surface sterilization procedure is important as it varies with chemicals used as well as the crops. Holdgate (1977) [8] observed that, surface sterilization of explants using 70% alcohol and 0.1% $HgCl_2$, was suitable for orchids. Chen and Chang (2000) [6] reported successful regeneration of tissues after surface sterilizing the flower stalk explants of *Oncidium* with 0.25% mercuric chloride for 5 min. Begum *et al*. (2002) [2] used 0.1% mercuric chloride for 5 min to surface sterilize *Vanda pteris* axillary bud explants to obtain an efficient micropropagation protocol.

Sodium hypochlorite is widely used in orchids for disinfections because of its oxidizing nature and capability to kill the microorganisms. It is useful and reliable in plant sterilization as it is inexpensive and easily available (Canli and Kazaz, 2009) [3]. Sodium hypochlorite kills microbes by oxidizing biological molecules such as proteins and nucleic acids. Bacterial and fungal infections can be eliminated by proper immersion time of explants in the sterilization agents (Yildiz and Er, 2002) [16]. Further, Canli and Kazaz (2009) [3] reported that the time and concentration of NaOCl are equally important to eliminate contaminants from the plant surface. So, 0.1% $HgCl_2$, 3 min followed by 1% NaOCl, 2 min) was found to be the most effective surface disinfection treatment in case of flower stalk of *Phalaenopsis* hy brid, as $HgCl_2$ and

NaOCl killed the fungus and bacteria which are responsible for contamination. Similar, results have also been reported by Kumar *et al*. (1998) [9] and Misra and Singh (1999) [10] in ornamental crops.

Table 1: Effect of different surface sterilants and duration of treatment on survivality of flower stalk explants in *Phalaenopsis* hybrids cv. Shagan within 7 weeks of culture

Treatments		Fungal infection (%)*	Bacterial infection (%)*	Aseptic culture (%)*	Death (%)*	Survival (%)*
T_0	Distilled water (control)	100.00 (90.00)	0.00 (0.001)	0.00 (0.001)	0.00 (0.001)	0.00 (0.001)
T_1	0.1% $HgCl_2$ (1min)	86.67 (68.58)	5.00 (12.92)	8.33 (16.78)	0.00 (0.001)	8.33 (16.78)
T_2	0.1% $HgCl_2$ (2 min)	83.33 (65.91)	5.00 (12.92)	11.67 (19.97)	0.00 (0.001)	11.67 (19.97)
T_3	0.1% $HgCl_2$ (3 min)	81.67 (64.65)	5.00 (12.92)	13.33 (21.42)	0.00 (0.001)	13.33 (21.42)
T_4	0.1% $HgCl_2$ (4 min)	76.67 (61.12)	5.00 (12.92)	18.33 (24.09)	0.00 (0.001)	18.33 (24.09)
T_5	0.5% NaOCl (1min)	93.33 (75.04)	5.00 (24.09)	1.67 (7.42)	0.00 (0.001)	1.67 (7.42)
T_6	0.5% NaOCl (2 min)	90.00 (71.57)	5.00 (12.92)	5.00 (12.92)	0.00 (0.001)	5.00 (12.92)
T_7	1.0% NaOCl (1 min)	86.67 (68.58)	5.00 (12.92)	8.33 (16.78)	0.00 (0.001)	8.33 (16.78)
T_8	1.0% NaOCl (2 min)	85.00 (67.21)	5.00 (12.92)	10.00 (18.43)	0.00 (0.001)	10.00 (18.43)
T_9	0.1% $HgCl_2$ (1 min) + 0.5% NaOCl (1 min)	81.67 (64.65)	3.33 (10.52)	15.00 (22.79)	0.00 (0.001)	15.00 (22.79)
T_{10}	0.1% $HgCl_2$ (1 min) + 0.5% NaOCl (2 min)	73.33 (58.91)	3.33 (10.52)	23.33 (28.88)	0.00 (0.001)	23.33 (28.88)
T_{11}	0.1% $HgCl_2$ (1 min) + 1.0% NaOCl (1 min)	68.33 (55.76)	3.33 (10.52)	28.33 (32.16)	0.00 (0.001)	28.33 (32.16)
T_{12}	0.1% $HgCl_2$ (1 min) + 1.0% NaOCl (2 min)	65.00 (53.73)	3.33 (10.52)	31.67 (34.24)	0.00 (0.001)	31.67 (34.24)
T_{13}	0.1% $HgCl_2$ (2 min) + 0.5% NaOCl (1 min)	60.00 (50.77)	3.33 (10.52)	36.67 (37.27)	0.00 (0.001)	36.67 (37.27)
T_{14}	0.1% $HgCl_2$ (2 min) + 0.5% NaOCl (2 min)	53.33 (46.91)	3.33 (10.52)	43.33 (37.27)	0.00 (0.001)	43.33 (37.27)
T_{15}	0.1% $HgCl_2$ (2 min) + 1.0% NaOCl (1 min)	43.33 (41.17)	3.33 (10.52)	53.33 (46.91)	0.00 (0.001)	53.33 (46.91)
T_{16}	0.1% $HgCl_2$ (2 min) + 1.0% NaOCl (2 min)	38.33 (38.25)	3.33 (10.52)	58.33 (49.80)	0.00 (0.001)	58.33 (49.80)
T_{17}	0.1% $HgCl_2$ (3 min) + 0.5% NaOCl (1 min)	26.67 (31.09)	3.33 (10.52)	70.00 (56.79)	0.00 (0.001)	70.00 (56.79)
T_{18}	0.1% $HgCl_2$ (3 min) + 0.5% NaOCl (2 min)	18.33 (25.35)	3.33 (10.52)	78.33 (62.26)	0.00 (0.001)	78.33 (62.26)
T_{19}	0.1% $HgCl_2$ (3 min) + 1.0% NaOCl (1 min)	8.33 (16.78)	0.00 (0.001)	91.67 (73.22)	0.00 (0.001)	91.67 (73.22)
T_{20}	0.1% $HgCl_2$ (3 min) + 1.0% NaOCl (2 min)	3.33 (10.52)	0.00 (0.001)	96.67 (79.48)	0.00 (0.001)	96.67 (79.48)
T_{21}	0.1% $HgCl_2$ (4 min) + 0.5% NaOCl (1 min)	0.00 (0.001)	0.00 (0.001)	100.00 (90.00)	25.00 (30.00)	75.00 (60.00)
T_{22}	0.1% $HgCl_2$ (4 min) + 0.5% NaOCl (2 min)	0.00 (0.001)	0.00 (0.001)	100.00 (90.00)	30.00 (33.21)	70.00 (56.79)
T_{23}	0.1% $HgCl_2$ (4 min) + 1.0% NaOCl (1 min)	0.00 (0.001)	0.00 (0.001)	100.00 (90.00)	35.00 (36.27)	65.00 (53.72)
T_{24}	0.1% $HgCl_2$ (4 min) + 1.0% NaOCl (2 min)	0.00 (0.001)	0.00 (0.001)	100.00 (90.00)	38.33 (38.25)	61.67 (51.75)
SE(m) ±		2.92	NS	2.58	0.53	2.64
CD at (5%)		8.30		7.33	1.51	7.49

*****20 cultures per treatment; repeated thrice.
Values in the parenthesis are arc sine transformed data
NS- Not Significantly
MS medium (Murashige and Skoog, 1962) + 1ppm BAP + 0.5ppm NAA + 3% sucrose

Conclusion

The most frequently used sterilizing chemicals for *in vitro* propagation are $HgCl_2$ and NaOCl at different concentration and treatment time. The effect of $HgCl_2$ and NaOCl on surface sterilization of flower stalk explants was studied and the best treatment was 0.1% $HgCl_2$, 3 min followed by 1% NaOCl, 2 min), which resulted in minimum percentage of fungal infection (3.33%), bacterial infection (0.00%) and death of explants (0.00%). The same treatment recorded maximum percentage of aseptic culture (96.67%) and survival percentage of explants (96.67%).

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