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IN VITRO STERILIZATION PROTOCOL OF *VANILLA PLANIFOLIA* EXPLANTS FOR MICROPROPAGATION

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ABSTRACT

Microbial contamination is one of the most serious problems of plant tissue culture. It is a major challenge to use field grown plants as a direct source of explant for the production of contamination free *in vitro* plantlets, because controlling fungal and bacterial contamination from field sources is very difficult. Therefore, surface sterilization is the most important step in preparation of explants for micropropagation. In the present investigation, an attempt was made to develop an effective surface sterilization protocol with an enhanced survival rate of nodal and shoot tip explants of vanilla by treating with various combination of mercuric chloride ($HgCl_2$), fungicides (carbendazim 'Derosal' 0.10%) and alcohol (70% ethanol) for varying time periods in order to establish maximum contaminant free cultures. Results showed that the highest percentage of explant response without any toxicity (death of the tissue) was obtained when the explants were exposed to 0.10 % $HgCl_2$ for 5 minutes (90.00 %) which was proved to be more effective on contamination control.

KEYWORDS: Contamination, Explant, *in Vitro*, Surface Sterilization, Vanilla

INTRODUCTION

Vanilla (*Vanilla planifolia*) is an herbaceous climbing orchid and a tropical commercial spice crop suitable for the warm humid tropics. There are about 110 species of vanilla in the Orchidaceae family. This exotic orchid, the beans of which on processing yields the popular, commercial flavouring agent "vanillin" which rules the world of edible flavours.

Vanilla is usually propagated vegetatively through stem cuttings from a mother plant which has not been allowed to flower. But, growing the plant in this manner is labour-intensive, time-consuming (Janarthanam and Seshadri 2008; Mengesha *et al.* 2012), tedious and economically inviable, since the numbers of seedlings that are produced by cuttings are few and collection of stem cuttings (approx 1 -1.5 m long cutting) from mother plants can cause retardation of growth of mother plant, rendering this method inefficient as it concerns meeting the national demand for quality planting materials. This is where the exploitation of the potentials of tissue culture for efficient multiplication and supply of required planting materials for large scale cultivation of vanilla. By employing tissue culture techniques, vanilla has been propagated both through direct organogenesis (Philip and Nainar, 1986) and indirect organogenesis (Davidonis and Knorr, 1991; Gu *et al.*, 1987). Different explant sources have been utilized for *in vitro* propagation of vanilla through direct organogenesis. Geetha and Sheety (2000) and Kononowicz and Janick (1984) reported the use of shoot tip and nodal segments for efficient micro-propagation protocol, whereas George and Ravishanker (1997) used axillary buds for *in vitro* multiplication of vanilla. In most cases, shoot proliferation was achieved by axillary bud growth or protocorm formation. However, commercial level of mass propagation demands simple, economical, high multiplication rate and highly reproducible protocol without an intervening callus or protocorm phase so as to give true-to-type clones.

In vitro propagation consists of various stages: selection of explants, aseptic culture establishment, multiplication, rooting and acclimatization of plants. The most important step for aseptic culture establishment is sterilization of explants. Successful tissue culture of all plant species depends on the removal of exogenous and endogenous contaminating microorganisms (Constantine, 1986; Buckley and Reed, 1994). 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 (Chawla, 2003).

Plant tissues inherently have various bacteria and fungi on their surfaces. It is important that the explants be devoid of any surface contaminants prior to tissue culture since contaminants can grow in the culture medium, rendering the culture non sterile. In addition, they compete with the plant tissue for nutrition, thus depriving the plant tissue of nutrients. Bacteria and especially fungi can rapidly overtake plant tissue. Sterilization is the process of making explants contamination free before establishment of cultures. Various sterilization agents are used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explant to the various sterilants, the sequences of using these sterilants has to be standardized to minimize explant injury and achieve better survival (CPRI, 1992). The surface sterility chosen for an experiment typically depend on the type of explants and also plant species (Rezadost *et al*.,2013).Explants are commonly surface-sterilized using sodium hypochlorite (household bleach), ethanol, and fungicides when using field grown tissues. The time of sterilization is dependent on the type of tissue; for example, leaf tissue will require a shorter sterilization time than will seeds with a tough seed coat (Funguomali *et al*.,2013, Sharma and Nautiya,2009). Wetting agents such as Tween added to the sterilant can improve surface contact with the tissue. Although surface contamination can be eliminated by sterilization, it is very difficult to remove contaminants that are present inside the explants that may show up at a later stage in culture. This internal contamination can be controlled to a certain extent by frequent transfer to fresh medium or by the use of a low concentration of antibiotics in the medium. Overexposing tissues to decontaminating chemicals can also kill tissues, so there is a balancing act between sterilizing explants and killing the explants themselves (Qin *et al*., 2012 and Olew *et al.*, 2014).

It is difficult to determine standard sterilization procedures that apply to all plants. Therefore, the present study was aimed at standardizing the sterilization method for different explants (nodal segment and shoot tip) of *Vanilla planifolia* for micropropagation, using different types of sterilizing agents (mercuric chloride, fungicide and alcohol) by varying their concentration and duration of exposure to increase the overall efficiency in culture initiation by minimizing the undesirable contamination.

MATERIALS AND METHODS

Plant Material Collection

The experiment was conducted at commercial plant tissue culture laboratory, Assam Agricultural University, Jorhat, with the objective to evaluate the effect of different sterilizing agents on different explants of vanilla for *in vitro* culture. The explants were taken from the Horticultural orchard of Assam Agricultural University, Jorhat.

Media Preparation

The Murashige and Skoog (1962) media was used for the experiment. Media was prepared by dissolving the

organic and inorganic components in distilled water. The solution was stirred until dissolved and made up to final volume. The media pH was adjusted between 5.7 and 5.8 by using either 1N HCl or 1N NaOH before the gelling agent was added. Media was then heated on a gas stove with continuous stirring using a magnetic stirrer until agar is dissolved and media dispensed in the culture vessels. The culture vessels were capped with lids and placed in trays and autoclaved. Autoclave was set at a temperature of 121^{o}C and a pressure of 15 psi for 20 minutes. All media was autoclaved within 12 hours of preparation and when possible freshly autoclaved media was used. However, when it was not possible to use the media immediately it was stored in a refrigerator at 4ºC for no longer than two weeks before use.

Aseptic Techniques

The process of sterilization and dissection of plant materials was carried out under sterile conditions in lamina flow cabinet. The cabinet was switched on and swabbed down with 70% ethanol using cotton wool or sterile towel and kept running for about 15 minutes before the work in the cabinet starts. All the plant materials were dissected on the sterile Petri plate. The lamina flow cabinet was frequently swabbed down with 70% alcohol. Hands were sprayed with 70% ethanol at suitable intervals while working for protracted periods in front of the cabinets. Personal hygienic precautions were observed by wearing a clean lab coat and gloves while working in the lamina flow cabinet.

Dissecting Tools

All tools were placed in an aluminum foil and sterilized in an autoclave. During their use in the cabinet, tools were dipped in 70% ethanol followed by heat sterilization in steribead sterilizer maintained at 250oC. In between operations, the tools were frequently sterilized by dipping them in ethanol and in the steribead sterilizer for 30 seconds.

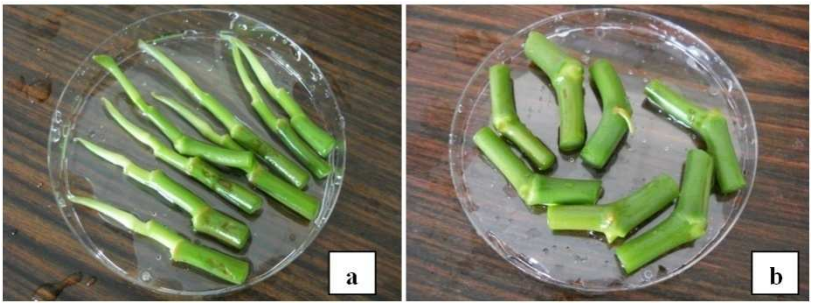
Washing of Glassware and Vessels

All glassware and vessels were washed in hot water to which few drops of liquid detergent had been added. The glassware were then rinsed in cold water three times followed by a final rinse in distilled water with a few drops of commercial bleach (JIK®). All this was carried out in a clean dust free washing room. The glassware was then dried in the oven at 60^{o}C in a clean dust free place.

Surface Sterilization of Explants

Vanilla shoot tip and nodal explants were harvested and transported from the greenhouse in a beaker containing tap water to the laboratory. Once in the laboratory, the explants were cut into 2 cm long sections (Fig.1) and then were kept under running tap water for 30 Minutes and rinsed 3-4 times with distilled water followed by washing in 0.50% teepol solution (wetting agent) for 5 minutes. To remove any traces of the detergent, the explants were rinsed 4-5 times with distilled water and blotted dry. In case of treatment ST5 and ST6, before using teepol solution, fungicide pre-treatment was done by dipping explants in 0.5% carbendazim solution (Derosal 50WP) for 2 to 3 hours (Table 1). Mercuric chloride solution diluted to concentration of 0.1% (w/v), were tested to decontaminate all the explants and subjected to an exposure time ranging from 5 to 10 min at 5-min intervals (**Table 1**). After mercuric chloride treatment, the explants were thoroughly washed 3 times for 10 minutes each with sterile deionized water. The above step was followed by transferring the explants to the laminar hood. Inside the laminar hood, the explants of ST3, ST4 immersed in 70% ethanol each for 30 seconds. After decontamination, all explants were rinsed manually three times with sterilized water in the laminar hood. The wounded sites exposed to sterilizing agents were trimmed properly and subsequently inoculated in culture medium

containing the MS macro nutrients (Murashige and Skoog 1962), sucrose (30 g/l), MS vitamins, agar (8 g/l) and plant hormones such as: 6-benzylaminopurine (BA) (2.0 mg/l), 1-naphthaleneacetic acid (NAA) (1.0 mg/l). The pH of the media was adjusted to 5.8 before autoclaving the media at 121°C and 15 psi for 20 min.



(a) Vanilla Shoot Tips as Explants

(b) Vanilla Nodal Segments as Explants

Figure 1: Different Explants Used in the Study

The cultures were kept in a growth chamber for one month, at 25°C, with 16 hours photoperiod and 2000 lux of light intensity. After one month the percentage of contaminated, survived and dead explants was noted. Twenty explants were used in each sterilization treatment, and each treatment was done in three replications.

Statistical Analysis

The experiments were performed in a completely randomized design (CRD). Data were subjected to one-way analysis of variance (ANOVA) Multiple comparisons among means were performed using Duncan's multiple range test with the level of significance at $p = 0.05$.

RESULTS

Among the treatments, the highest rate of contamination was recorded in control (100.00%). The highest percentage (90.00%) of healthy and contaminant free explants were established when they were exposed to 0.10 per cent mercuric chloride for a duration of 5 minutes. This was followed by establishment of 55 per cent of the explants when treated with the same surface sterilant for 10 minutes. When explants were exposed to 70 per cent alcohol for 30 sec followed by 0.10 per cent $HgCl_2$ for 5 minutes and 0.10 per cent $HgCl_2$ for 10 minutes, subsequently 70 per cent and 50 per cent contaminant free cultures were obtained but, out of those 80 per cent explants were died. As per results highest percentage of explant response without any toxicity (death of the tissue) was obtained when they were exposed to 0.10 % $HgCl_2$ for 5 minutes (90.00 %) which proved to be the best treatment and was significantly superior to other treatments in case of vanilla explants (Table II).The lower concentration resulted in contamination due to microbial load in the explants. The higher duration/concentration caused toxicity in the explants leading to death of tissues.

DISCUSSIONS

The explants *viz*., nodal segments and shoot-tips were excised from the field grown plant showed a very high percentage of bacterial and fungal contamination. In current investigation the shoot-tips and nodal explants of the healthy vanilla plants were subjected to various surface sterilization treatments to establish the contamination free cultures. Explants which were surface sterilized by agitation in 0.10% $HgCl_2$ for 5 minutes gave the highest survival rate (90.00%)

with minimum microbial contamination (Table II) and proved to be the best treatment and significantly superior to other treatments. This is because the most useful radical in $HgCl_2$ is probably the chlorite, commonly present as bi-chloride of mercury. The efficacy of mercuric chloride might be due to its extreme poisonous nature because of high bleaching action of two chloride atoms and tendency of mercuric ions to combine strongly with protein causing death of organism (Pauling 1955). The higher treatment duration of $HgCl_2$ at 0.10 per cent for 10 minutes, observed death of the explants. This is due to the high bleaching activity of chlorine which killed the cells. When explants were treated less than 5 min, contamination was high and when treatment duration was high then browning due to death of tissue occurred. The results were found in close conformity with the finding of Janarthanam & Seshadri (2008), who surface sterilized the explants by cleaning thoroughly under running tap water for 20 min, washed with a solution of Tween 20 (two drops in 100 ml water) for 3 min, finally treated with $HgCl_2$ (0.10%) for 8 min under aseptic conditions and George and Ravishankar (1997), who excised stem node sections measuring 1.5-2.0 cm each with one dormant axillary buds and were surface sterilized with 0.15% (wt/vol) mercuric chloride for 5 min followed by three rinses in sterile distilled water to control the microbial contamination in the explants of vanilla.

CONCLUSIONS

The most frequently used sterilization procedures for micropropagation are conducted with 70% ethanol and 1–3% NaOCl. Our results showed that during the sterilization procedure chemicals like $HgCl_2$ showed also good results for the surface sterilization of vanilla explants. This can be explained by the fact that requirements for sterilization are different and depend on the tissue type and the nature of the explant used for micropropagation.

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APPENDICES

Table 1: **Methods of Sterilization of Nodal Segment and Shoot Tip Explants of Vanilla**

Treatments	Mercuric Chloride ($HgCl_2$) (%)	Exposure Time of Mercuric Chloride (Min)	Alcohol (%)	Exposure Time of Alcohol (sec)	Fungicide Carbendazim (Derosal) (%)	Exposure Time of Fungicide Pre-treatment
ST_1	0.10	5.00	-	-	-	-
ST_2	0.10	10.00	-	-	-	-
ST_3	0.10	5.00	70.00	30.00	-	-
ST_4	0.10	10.00	70.00	30.00	-	-
ST_5	0.10	5.00	-	-	0.30	overnight
ST_6	0.10	10.00	-	-	0.30	overnight
ST_0	-	-	-	-	-	-

ST_0 = Control (without any chemicals and pre-treatment)
ST = Surface Sterilization Treatment

Table 2: Effect of Surface Disinfectants on Per Cent Contamination and Number of Healthy Cultured Established in Vanilla Explants

[a]Medium	[*]No. of Healthy Cultures Established	Per Cent Survival	*No. of Explants Contaminated	Per Cent Contamination
ST_1	18^{a}	90 (71.56)	2	10 (18.44)
ST_2	11^{c}	55 (47.87)	9	45 (42.13)
ST_3	14^{b}	70 (56.78)	6	30 (33.21)
ST_4	10^{cd}	50 (45.00)	10	50 (45.00)
ST_5	8^{d}	40 (39.23)	12	60 (50.77)
ST_6	2^{e}	10 (18.44)	18	90 (71.56)
ST_0	0	0 (0.00)	20	100 (90)
SEd±	**0.873**		**0.976**	
CD at 5 %	**2.418**		**2.703**	

[*]No. of explants inoculated = 20
[a]for media composition see Table 1.
Figures in parentheses are arcsine transformation values.
Means within columns separated by Duncan's multiple range test P = 0.05
Means followed by same letter shown in superscript(s) are not significantly different.