International Journal of Current Research and Academic Review
ISSN: 2347-3215 Volume 4 Number 2 (February-2016) pp. 335-339
Journal home page: http://www.ijcrar.com
doi: http://dx.doi.org/10.20546/ijcrar.2016.402.036

Standardization of Surface Sterilization Technique for *In-vitro* Propagation of Anthurium (*Anthurium andraeanum Lind*.) cv. Jewel

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KEYWORDS

Anthurium,
Contamination,
explant,
In vitro,
Sterilization
procedure

A B S T R A C T

An experiment was conducted to standardize the surface sterilization technique of *Anthurium andraeanum* cv. Jewel using immature bronze coloured leaves as explants for callus induction. Explants were collected, cleaned repeatedly using Tween–20 in fresh running water and then treated with 12 different combinations of Sodium hypochlorite (1.5%), Mercuric chloride (0.1%) and ethyl alcohol (70%) with varying exposure durations. Surface-sterilized explants were then inoculated in MS medium supplemented with various concentrations and combinations of 2,4-D, BAP and TDZ. Leaf explants when treated with mercuric chloride (0.1%) (3 minutes) + ethyl alcohol (70%) (30 seconds) resulted in lowest percentage (3.67) of contaminated cultures, lowest explant mortality (11.33%) as well as highest survivability (85.67%). Sodium hypochlorite (1.5%) (15 minutes) resulted in highest explant mortality (49.33%) while 5 minutes treatment resulted in lowest survivability (30.33%). Mercuric chloride (0.1%) (3 minutes) + ethyl alcohol (70%) (30 seconds) may be recommended for micropropagation of Anthurium through leaf explants.

Introduction

Anthurium andraeanum is one of the important potted and cut flower ornamentals in the world. They are highly prized for its attractive, long lasting flowers. In the global market, Anthurium stands second after orchid among the tropical cut flowers (Dufour and Guerin, 2003). Three conventional methods for Anthurium production are propagation by seeds, traditional propagation method and tissue culture. Conventional vegetative propagation of Anthurium by separating the newly developing plants is time consuming and it takes years to develop commercial quantities of elite clones (Harb *et al.*, 2010). The conventional propagation in the field has also faced some constraints due to disease attacks like involvement of *Erwinia*

caratovora and Dasheen Mosaic virus (DMV) (Budiarto, 2008). To produce disease free clones and rapid production of quality plantlets, *in vitro* propagation is the only best method (Martin *et. al*, 2003; Çimen and Ozge, 2009; Raad *et. al*, 2012). The successful *in vitro* propagation using young leaf explant to reduce contamination and oxidative browning of explants consists of various stages. The steps consist of selection of explants, aseptic culture establishment, multiplication, rooting and acclimatization of plants (Budiarto and Handayati, 2007). For a successful and efficient micro propagation method, it is efficient to start with an efficient plant material sterilization step (Traore *et al*., 2005). Successful tissue culture of all plant species depends on the removal of exogenous and endogenous contaminating microorganisms (Buckley and Reed, 1994). To eliminate contamination during *in vitro* propagation different methods have been developed (Hussain *et al*., 1994; Herman, 1996). *In vitro* contamination by fungi, bacteria and yeast is one of the most serious problems of commercial and research plant tissue laboratories. Contaminated plants can reduce multiplication and rooting rates or may die. It is necessary to remove foreign contaminants including bacteria and fungi from explants and it is very difficult to obtain sterile plant material completely free from contamination (Mihaljevic *et al*., 2013). Surface sterilization of the naturally contaminated living plant materials from the environment is a critical step since it involves the use of chemical solutions like sodium hypochlorite, ethanol, mercuric chloride etc. that are toxic to the plant tissues (George, 1993).

The procedure of sterilization depends on plant species and explant taken from the plant for sterilization. Each plant material has variable surface contaminant levels, depending on the growth environment, age and part of the plant used for micropropagation (Mihaljevic *et al.*, 2013). To avoid the problems of microbial contamination in *in-vitro* cultures, it is necessary to develop a protocol for disinfecting the field grown explants intended for *in vitro* culture. Keeping this in view, an efficient and simple disinfection protocol to increase survival of explants was developed.

Materials and Methods

The experiment was conducted at plant tissue culture laboratory, Department of Floriculture, Medicinal and Aromatic Plants, UBKV, Cooch Behar during January 2014 and February 2014. Immature bronze coloured leaf explants were collected from the net house of the department. Before sterilization, the explants were washed thoroughly under running tap water using 2-3 drops of Tween-20 detergent for about 20 minutes then rinsed with water for 4-5 times. After washing, the explants were reduced in size by removing tissues of size (0.5-1.0 cm) with the help of surgical blade and forceps before inoculation. The washed explants were then brought to laminar flow cabinet and were subjected to surface sterilization. The explants were subjected to different sterilants and their combinations for varying time durations as shown in Table 1, followed by a 5 minutes rinse in sterile distilled water under aseptic conditions in the laminar flow chamber. Before starting the work, the table surface of laminar flow cabinet was first swabbed with 95% ethanol and all the required materials except the excised explants were kept inside the chamber and exposed to UV light for 60 min. The laminar flow was switched on 10 min prior to inoculation. The explants were then put on Murashige and Skoog (MS) medium supplemented with 30g/l sucrose, vitamins, agar (7 g/l) and appropriate plant hormones such as 2,4-D (2 mg/l), BAP (0.5

mg/l) and TDZ (0.1 mg/l) with pH adjusted to 5.8 before autoclaving the media at 121^{o}C and 1.5 atm. for 15 minutes. The explants were placed in such a manner that conformed to the original polarity and exposed above the surface of growing medium.

The cultures were then kept in a growth chamber for about three weeks at 25±2^{o} C with 16 hours photoperiod and 3500 lux of light intensity. After three weeks of inoculation, the percentage of contaminated, dead and survived explants was recorded. Ten explants were used in each sterilization treatment and each treatment was done in 3 replications. The data generated was subjected to ANOVA in complete randomized design at 5% level of significance. To satisfy model, assumptions of experiments were subjected to square root transformations.

Results and Discussion

Anthurium explants (bronze coloured immature leaves) were subjected to 12 different sterilization regimes using MS (Murashige and Skoog, 1962) as the basal medium. Effect on various sterilants used on explants on contamination, death and survival of the explants (Table 2) was highly significant. Lowest contamination percentage (3.67) was obtained by treating the explants in mercuric chloride (0.1%) for 3 min + ethyl alcohol (70%) for 30 s as well as in mercuric chloride (0.1%) for 4 min + ethyl alcohol (70%) for 30 s. However, the lowest mortality percentage (11.33) and highest survivability percentage (85.67) was obtained when explants were surface sterilized with mercuric chloride (0.1%) for 3 min + ethyl alcohol (70%) for 30 s.

Though less percentage of contaminated cultures was obtained in mercuric chloride (0.1%) for 4 min + ethyl alcohol (70%) for 30 s, the surviving percent was less as a result of necrosis and tissue injury of explants (Jan *et al.*, 2013). Highest contamination percentage (39.33) and lowest survivability percentage (30.33) was obtained when the explants were treated with Sodium hypochlorite (1.5%) for 5 min.

Table.1 Different Sterilants and their Combination for Varying Time Duration

Sterilants and their combination	Time duration
Mercuric chloride (0.1%)	2 min
Mercuric chloride (0.1%)	3 min
Mercuric chloride (0.1%)	4 min
Sodium hypochlorite (1.5%)	5 min
Sodium hypochlorite (1.5%)	10 min
Sodium hypochlorite (1.5%)	15 min
Mercuric chloride (0.1%) + ethyl alcohol (70%)	2 min + 30 s
Mercuric chloride (0.1%) + ethyl alcohol (70%)	3 min + 30 s
Mercuric chloride (0.1%) + ethyl alcohol (70%)	4 min + 30 s
Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	5 min + 30 s
Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	10 min + 30 s
Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	15min + 30 s

Table.2 Influence of Different Sterilants on Percent Contaminated, Death and Survivability of Cultures in *Anthurium andraeanum* cv. Jewel

Time duration	Contaminated	Death	Survival
Mercuric chloride (0.1%) (2 min)	27.00 (31.30) ± 1.11	29.67 (33.00) ± 0.96	43.33 (41.16) ± 1.77
Mercuric chloride (0.1%) (3 min)	12.67 (20.78) ± 2.20	12.33 (20.54) ± 1.32	75.00 (60.04) ± 2.37
Mercuric chloride (0.1%) (4 min)	5.33 (13.27) ± 1.92	36.33 (37.07) ± 0.91	58.33 (49.80) ± 0.89
Sodium hypochlorite (1.5%) (5 min)	39.33 (38.84) ± 0.90	30.33 (33.41) ± 0.95	30.33 (33.41) ± 0.95
Sodium hypochlorite (1.5%) (10 min)	19.33 (26.07) ± 0.84	19.00 (25.82) ± 1.46	60.67 (51.16) ± 1.22
Sodium hypochlorite (1.5%) (15 min)	9.33 (17.69) ± 2.47	49.33 (44.62) ± 2.02	41.33 (40.01) ± 0.68
Mercuric chloride (0.1%) + ethyl alcohol (70%) (2 min + 30 s)	22.67 (26.76) ± 3.35	16.67 (24.08) ± 1.18	60.67 (51.16) ± 1.22
Mercuric chloride (0.1%) + ethyl alcohol (70%) (3 min + 30 s)	3.67 (10.95) ± 1.70	11.33 (19.64) ± 1.37	85.67 (67.77) ± 1.24
Mercuric chloride (0.1%) + ethyl alcohol (70%) (4 min + 30 s)	3.67 (11.02) ± 0.91	45.67 (42.51) ± 1.20	50.67 (45.38) ± 1.33
Sodium hypochlorite (1.5%) + ethyl alcohol (70%) (5 min + 30 s)	31.33 (34.03) ± 1.55	22.00 (27.95) ± 1.39	46.67 (43.09) ± 1.45
Sodium hypochlorite (1.5%) + ethyl alcohol (70%) (10 min + 30 s)	8.00 (16.35) ± 2.13	18.00 (25.05) ± 2.24	74.00 (59.36) ± 1.74
Sodium hypochlorite (1.5%) + ethyl alcohol (70%) (15min + 30 s)	7.33 (15.60) ± 2.38	21.33 (27.49) ± 1.47	71.00 (57.44) ± 2.17
S.Em±	1.00	0.73	0.61
CD at 5%	2.93	2.14	1.78

This might be the result of weaker chemical effect due to shorter exposure duration that unable to kill the pathogens present in the explant. Highest mortality percentage was obtained when the explants were treated with Sodium hypochlorite (1.5%) for 15 min. This may be due to the fact that longer exposure duration damages the explant tissues and ultimately dries out after becoming necrotic.

Conclusion

Among the several sterilization techniques followed, the lowest contamination percentage were achieved when the explants were treated with mercuric chloride (0.1%) for 3 min + ethyl alcohol (70%) for 30 s and as well as mercuric chloride (0.1%) for 4 min + ethyl alcohol (70%) for 30 s but the mortality percentage was much higher than the previous due to higher necrotic cultures. Overall, the highest explant survival and the lowest mortality percentage were observed in the previous treatment. So it is concluded from the above study that sterilization treatment of mercuric chloride (0.1%) for 3 min and ethyl alcohol (70%) for 30 s is effective for field grown anthurium leaf explants intended for *in vitro* culture.

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How to cite this article:

Rocky Thokchom and Soumen Maitra. 2016. Standardization of Surface Sterilization Technique for In-vitro Propagation of Anthurium (*Anthurium andraeanum* Lind.) cv. Jewel. *Int.J.Curr.Res.Aca.Rev.* 2016.4(2): 335-339.
doi: http://dx.doi.org/10.20546/ijcrar.2016.402.036