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Development of Sanitation Protocol for Leaf Explants of *Ocimum gratissimum* for *in Vitro* Culture

Chaudhary Priyanka*[1] and Sharma Vikas[2]

[1]Department of Biotechnology and Microbiology,

Arni School of Basic Sciences, Arni University, Kathgarh, Himachal Pradesh INDIA

[2] Department of Biotechnology, DAV University, Jalandhar INDIA

ABSTRACT

Ocimum gratissimum is a multipurpose medicinal plant belonging to family Lamiaceae. Conventional methods of propagation of *O. gratissimum* have certain limitations. Therefore *in vitro* culture strategy offer splendid option for its mass propagation and conservation. During micropropagation surface sterilization is the most important step in the preparation of explants. The effect of three different sterilizing agents namely Mercuric chloride ($HgCl_2$), Sodium hypochlorite (NaOCl) and Hydrogen peroxide (H_2O_2) were evaluated for sterilization of nodal segments 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 the sterilization treatments 0.1% $HgCl_2$ was significantly reducing the contamination of explants and showed that necessity of sterilization may vary with the type tissue used for micropropagation. The described method has this potential to be successfully employed for micropropagation and *in vitro* conservation of *Ocimum gratissimum*.

Keywords: Conservation; Lamiaceae; Micropropagation; *Ocimum gratissimum*; Sterilization.

INTRODUCTION

O. gratissimum is a globally important economic crop because it has a trade value of US$ 15 million per year (Monga et al.; 2014) but Unfortunately with the quick increment in total populace, outrageous weight on the accessible cultivable land, and speedy disappearance of herbal habitats for restorative plants together with ecological and geopolitical hazards; it is exceptionally hard to secure plant-derived compounds (Mulabagal and Tsay, 2004). *Ocimum gratissimum* is routinely proliferated through seeds, seed viability is very poor, season dependence and low germination rate potential restricts its multiplication. Moreover, seed derived progenies are does not allow the production of homogeneous populations, due to cross pollination (Gopi et al.; 2006; Saha et al.; 2012). Therefore alternative techniques like micropropagation holds potential for producing large number of plantlets (Kataky and handique, 2010b).

The success of micropropagation depends on a number of factors which affect establishment of explants in the medium. The nutrient media in which the plant is cultivated is a good source of nutrient for microbial growth. These microbes compete adversely with plant tissue culture for nutrient. The presence of these microbes in these plant cultures usually results in increased culture mortality, variable growth, tissue necrosis and reduced rooting (Odutayo et al., 2007). These contaminants are not always seen during early culture stages; some internal contaminants become visible at later subcultures and are difficult to eliminate (Reed et al.; 1998). Utilizing a proper and precise sterilization procedure in tissue culture techniques can be vital to save time and effort.

Various sterilizing agents used to decontaminate plant tissues. Some of these agents are toxic to the plant tissues, therefore suitable concentration, exposure duration of the explants to the various sterility and the sequences of using these sterility have to be standardized to minimize explants injury and achieve better survival (Dhingra et al.; 1992). Therefore in the present study, an attempt has been made to establish an efficient surface sterilization protocol for the *in vitro* multiplication of *Ocimum gratissimum*, using different types of sterilizing agents and varying their concentrations and duration of exposure as a best alternative for conservation of this medicinally important plant.

MATERIALS AND METHODS

COLLECTION OF EXPLANTS

Healthy and profusely growing plantlets of *Ocimum gratissimum* were collected from Medicinal and Aromatic Plant Garden of Arni University, Himachal Pradesh. The plants were dried and prepared herbarium was submitted to The National Institute of Science Communication and Information Resources (NISCAIR), located at New Delhi, India for identification of species and plants were recognized as *Ocimum gratissimum.*

SURFACE STERILIZATION OF THE EXPLANTS

Nodal portions of *Ocimum gratissimum* were excised from plants of *Ocimum gratissimum*. These nodal portions were trimmed to approx 3 cm. in size and substantial beefy leaves were expelled. Shoot tips 2-3 cm long were placed under running tap water for thirty minutes to evacuate foreign contaminants. During washing surfactant (Tween-80) was added and explants in the solution were agitated constantly for 3 minutes. Thereafter, the detergent was completely drained out from the explants by washing it energetically under running tap water for 30 minutes to expel the microbial load and dust particles. Consequently the explants were taken to laminar airflow cabinet for further sterilization. Three different kinds of sterilizing agents viz., Mercuric Chloride ($HgCl_2$) (0.05%- 0.5%), Sodium Hypochlorite (NaOCl) (0.5%-2%) and hydrogen peroxide (H_2O_2) (3%-10%) were tested for explants sterilization by varying their concentration and time of exposure (1-15 minutes) simultaneously to ensure contaminant free culture. After this treatment, explants were given 4-5 thorough washings with autoclaved distilled water to evacuate any trace of the surface sterilants under aseptic conditions.

CULTURE MEDIUM AND CONDITIONS

The culture medium used for the shoot bud induction was Murashige and Skoog's (1962) basal medium supplemented with 3% (w/v) sucrose The pH of the medium was adjusted to 5.8 with 1N NaOH or HCl before gelling with 0.8% (w/v) agar and then sterilized by autoclaving for 20 min under 1.1kg/cm^2 pressure at 121^{0}C. All the cultures were incubated in a culture room under cool fluorescent light (16 h photo period 40 $\mu molm^{-2}s^{-1}$) maintained at constant temperature of 25±2^{0}C and 60%-70% relative humidity (RH).

INOCULATION

Murashige and Skoog basal medium supplemented with various concentrations BAP and IAA were used for inoculation. Medium was checked for the contamination before inoculation. The surface sterilized explants were trimmed on the either side using a sterile blade into 1.0-1.5cm pieces and explants were used for inoculation in culture medium. Regular and proper record for contamination was taken for four weeks.

RESULTS AND DISCUSSION

Plant tissue culture is a system of developing plant cells, tissue or organs that have been isolated from the mother plant (called explants) in synthetic medium under aseptic condition **(**Omamor et al.; 2007**).** The choice of an explant giving maximum response is vital stride for the accomplishment of any tissue culture

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programme. The utilization of field grown plants as direct sources of explants for the production of 'clean' *in vitro* plantlets, shows a major challenge because the surface of plants carries an extensive variety of microbial contaminants (Rout et al.; 2000; Odutayo et al.; 2007). To avoid source of infection, explants must be altogether surface-sterilized before inoculating them onto MS medium. Therefore to acquire clean *in vitro* cultures, sources of contamination other than surface contaminants need to be considered (Webster et al.; 2003). Torres, 1989 were accomplished best outcomes when explants were harvested during the active phase of growth. Hence for the present study, explants were reaped from actively growing field grown plants.

Following the selection of explants, the subsequent effort was to provoke maximum disease free cultures, which typically troublesome and problematic, because of high rate of contamination when the explants are taken from the field grown plants. Surface sterilization is a crucial step in preparation of healthy and viable explants in tissue culture because plants in the field are highly exposed to microbial contamination. Most surface contaminants, such as bacteria and fungi, can be expelled via surface sterilization with an appropriate sterilizing agent (Mahmoud et al.; 2016). Cleansing is the way toward influencing explants contamination free before establishment of cultures. Effective tissue culture of all plant species relies upon the evacuation of exogenous and endogenous contaminating microorganisms (Constantine DR. 1986; Buckley and Reed, 1994). Sterilization of plants is the prerequisite for micropropagation (Srivastava et al.; 2010), as entire plant or plant parts are specifically subjected to disinfection and utilized as explants. Subsequently, getting cleaned explants with no generous harm is vital and determination of sterilizing agent is of most extreme significance. Unsuccessful sterilization thwarts the micropropagation and getting reduced or contamination free explants (Falkiner FR. 1990).

Sterilization of explants can be done by utilizing different agents like sodium hypochlorite, calcium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide and silver nitrate to decontaminate the tissues (Mihaljevic et al., 2013). These sterilants are also lethal to the plant tissues, therefore proper concentration of sterilant and duration of exposing the explants to the various sterilants has to be standardized to minimize explant damage and accomplish better survival (CPRI, 1992). Mercury chloride is profoundly poisonous; because of this, its concentration and the span of treatment should be optimized to minimize tissue mortality of the explants caused by over sterilization (Kataky and Handique, 2010).

The present examination has been done to standardize the sterilization technique for explant of *Ocimum gratissimum* for *in-vitro* proliferation by utilizing distinctive types of sterilizing agents by varying their concentration and duration of exposure. Three different types of sterilizing agents (mercuric chloride, sodium hypochlorite and hydrogen peroxide) were used for the cleansing of *Ocimum gratissimum* explants. Persistent observing was carried out for fifteen days when the explants were in the culture media. Fungal contamination was observed as hyphal growth from the explants, and bacterial contamination was identified by observing colonies, seen as watery or slimy buildups on the agar surface. Our present outcome demonstrated that leaf explants of *Ocimum gratissimum* have indicated distinctive sterility reaction for various centralization of Mercuric Chloride ($HgCl_2$) (0.05%- 0.5%), Sodium Hypochlorite (NaOCl) (0.5%-2%) and hydrogen peroxide (H_2O_2) (3%-10%) by varying their concentration and time of exposure (1-15 minutes). Both $HgCl_2$ and NaOCl are toxic to the plant tissues, thus, proper concentrations of these sterilizing agents ought to be deliberately chosen. Here it observed that some nodal segments did not survive, it was probably due to damages occurred during sterilization procedure and/or due to damaged explants during excision. NaOCl, as weak/or mild sterilizing agent, should be used in higher percentage to control contamination. It has been discovered that among all the three sterilizing agents, 95% contamination free explants were obtained when explants were exposed with 0.1% $HgCl_2$ for 5 min showed significant reduction in bacterial as well as fungal contamination. Similarly highest rate of contamination free culture were obtained with 1% NaOCl for 5 minutes and 7.5% H_2O_2 for 9 minutes (Table1, Figure1). There are many reports of surface sterilization of explants using $HgCl_2$ (Naika and Krishna, 2008; Preethi et al., 2011) and NaOCl (Colgecen et al., 2011). It has been demonstrated that $HgCl_2$ showed better sterilization effect than NaOCL, which is in concurrence with our outcomes, though the duration of exposure was different (Mahmoud et al.; 2016).

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Table1: Types of sterilizing agents used in a different concentration with varying time of exposure for sterilizing explants of *Ocimum gratissimum*.

Sterilizing Agent	Concentration	Duration of Treatment	Rate of Contamination (%)	Uncontaminated cultures	
				Rate of Healthy Cultures (%)	Rate of Scorching (%)
Control	----	-----	90	5	5
$Hgcl_2$	0.05%	1	55	36	9
		3	41	51	8
		5	24	70	6
		7	36	55	9
		9	40	50	10
	0.1%	1	50	40	10
		3	27	65	8
		5	**5**	**95**	**5**
		7	33	60	7
		9	36	55	9
	0.2 %	1	43	45	12
		3	25	65	10
		5	20	72	8
		7	35	55	10
		9	45	47	8
	0.5%	1	46	45	9
		3	37	55	8
		5	40	50	10
		7	50	40	10
		9	59	30	11
NaOCl	0.5 %	3	40	50	10
		5	30	60	10
		9	42	49	9
		12	43	45	12
		15	47	44	9
	1%	3	31	62	7
		5	**14**	**80**	**6**
		9	33	60	7
		12	36	55	9
		15	50	40	10
	1.5%	3	41	50	9
		5	24	70	6
		9	35	57	8
		12	40	50	10
		15	47	44	9
NaOCl	2%	3	40	49	11
		5	35	55	10
		9	44	47	9
		12	46	42	12
		15	50	36	14
		3	40	51	9

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H_2O_2	3%	5	34	56	10
		9	28	60	12
		12	38	50	12
		15	42	45	13
	5%	3	32	55	13
		5	25	64	11
		9	20	68	12
		12	40	50	10
		15	44	43	13
	7.5%	3	27	60	13
		5	24	66	10
		9	**18**	**70**	**12**
		12	35	52	13
		15	40	46	14
	10 %	3	35	54	11
		5	30	57	13
		9	22	64	14
		12	42	45	13
		15	48	37	15

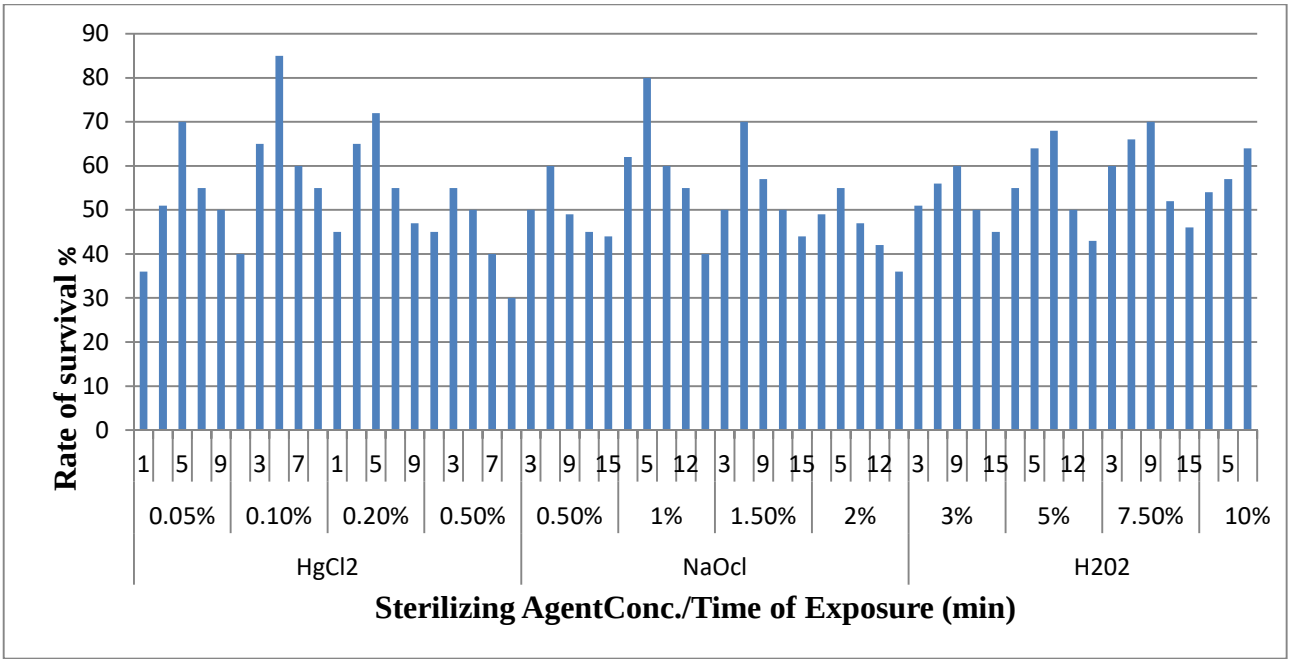

Fig1: Percentage of Healthy Plants (HP) obtained after sterilization of explants of *Ocimum gratissimum*

CONCLUSION

The use of appropriate disinfectant with optimum concentration and exposure time is an imperative factor for the establishment of *Ocimum gratissimum* aseptic culture from field grown plant. This investigation describes advanced protocol highlighting proficient method for surface sterilization of nodal explants which could be attainable to use for *in vitro* propagation of *Ocimum gratissimum.*

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REFERENCES

1. Buckley, P.M., and Reed, B.M. 1994. Antibiotic susceptibility of plant-associated bacteria. Hort. Science 29:434.
2. Central Potato Research Institute, Shimla 1992. Tissue Culture technique for potato health, conservation, micro propagation and improvement. CPRI, Shimla:1-23
3. Constantine, D.R. 1986. Micropropagation in the commercial environment. In: Withers L., Alderson PG (Eds) Plant Tissue Culture and its Agricultural Applications. Butterworth, London, 175-186.
4. Dhingra, M.K., Naik P.S., Chandra, R., and Randhwa G.J. 1992. Tissue culture technique for potato health, conservation, micropropagation and improvement. Tech. Bull. 39.
5. Falkiner, F.R. 1990. The criteria for choosing an antibiotic for control of bacteria in plant tissue culture. IAPT. Newsletter 60:13- 22.
6. Gopi, C., Sekhar, N., and Ponmurugan, P. 2000. *In vitro* multiplication of *Ocimum gratissimum* L. through direct regeneration. African Journal of Biotechnology. 5(9):723-726.
7. Kataky, A., and Handique, P.J. 2010. Standardization of sterilization techniques prior to in vitro propagation of *Andrographis paniculata* (Burm.f) Nees. Asian Journal of Science and Technology. 6: 119-122.
8. Mihaljevic, I., Dugalic, K., Tomas, V., Viljevac, M., Pranjic, A., Cmelik, Z., Puskar, B., and Jurkovic, Z. 2013. *In vitro* sterilization procedures for micropropagation of ‘Oblacinska’ sour cherry. J. Agric. Sci. 58(2): 117- 126.
9. Monga, S., Sethi, N., Kaura, S., Parle, M., and Lohan, S. 2014. Effect of 6-Benzyl Amino Purine hormone on the shooting growth of *Ocimum gratissimum*. Inernational Research Journal of Pharmacy. 5(2): 106-108.
10. Mulabagal, V., & Tsay, H.S. 2004. Plant cell cultures - An alternative and efficient source for the production of biologically important secondary metabolites. Int J Appl Sci Eng. 2 (1), 29-48.
11. Odutayo, O.I., Amusa, N.A., Okutade, O.O., and Ogunsanwo, Y.R. 2007. Sources of microbial contamination in tissue culture laboratories in southwestern Nigeria. Afr. J. Agric. Res. 2(3): 67-72.
12. Omamor, I.B., Asemota, A.O., Eke, C.R., and Eliashi, EI. 2007. Fungal Contamination of the Oil Palm Tissue Culture in Nigerian Institute for Oil Palm Research (NIFOR). African Journal of Agriculture Research. 2(10) 534-537. 54876197
13. Reed, B.M., Mentzer, J., Tanprasert, P., and Yu, X. 1998. Internal bacterial internal bacterial contamination of micropropogated hazelnut: identification and antibiotic treatment. Plant cell tissue and organ culture. 52(1): 67-70.
14. Rout, G.R.T., Samantaray, S., and Das, P. 2000. *In Vitro* Manipulation and Propagation of Medicinal Plants. Biotechnology Advances. 18(2): 91-120.
15. Saha, S., Kader, A., Sengupta, C., and Ghosh P. 2012 *In Vitro* Propagation of *Ocimum gratissimum* L. (Lamiaceae) and Its Evaluation of Genetic Fidelity Using RAPD Marker. American Journal of Plant Sciences. 3: 64-74.
16. Mahmoud, S.N., and Al-Ani, N.K. 2016. Effect of Different Sterilization Methods on Contamination and Viability of Nodal Segments of *Cestrum nocturnum L*. International Journal of Research Studies in Biosciences (IJRSB). 4(1): 4-9.
17. Srivastava, N., Kamal, B., Sharma, V., Negi, Y.K., Dobriyal, A.K., Gupta, S.; and Jadon, V.S. 2010. Standardization of Sterilization Protocol for Micropropagation of *Aconitum heterophyllum*- An Endangered Medicinal Herb Academic Arena 2(6): 37-42.
18. Torres, K.C. 1989 Tissue culture technique for horticulture crops. Van Nostrand , New York.
19. Webster, S., Mitchell, S.A., and Ahmad, M.H. 2003. A novel surface sterilization method for reducing fungal and bacterial contamination of field grown medicinal explants intended for in vitro culture. Proceedings of 17th SRC conference entitled ‘Science and Technology for Economic Development: technology driven Agriculture and Agro processing’ SRC, Jamia. http://ww.kitchenculturekit.com/surface sterilizationMitchell2003.pdf.