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Standardization of Surface Sterilization for in Vitro Cloning of Pomegranate (*Punica granatum* L.) cv. Bhagwa

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ABSTRACT: Surface sterilization of explants in tissue culture determines the success or failure of any *in vitro* propagation system. In the present study, a number of sterilizing agents were used to minimize contamination and increase the survival percentage of pomegranate explants. Explants were alternately treated with fungicide (Tebuconzole 50% + Trifloxystorbin 25% w/w), mercuric chloride (0.1%), and 4% $NaOCl_2$ in combination with each other for different time intervals, followed by ethanol treatment (70%) as the final disinfectant. Every combination gives differential results when they are applied for various durations. Maximum survival (73.33±11.55%) was recorded with the combination (4% $NaOCl_2$ for 7 min + 0.1% $HgCl_2$ for 2 min), followed by (66.67± 5.777%) and 63.33± 11.555%) survival recorded under the treatment (Tebuconzole 50% + Trifloxystorbin 25% w/w for 15 min + 0.1% $HgCl_2$ for 2 min) and (4% $NaOCl_2$ for 5 min + 0.1% $HgCl_2$ for 1 min respectively. The combination of 4% $NaOCl_2$ for 7 minutesn and 0.1% $HgCl_2$ for 2minutesn was found to be the best among the combinations in the study, which gives the highest survival percentage *in vitro* propagation of pomegranate.

Keywords: combination; $HgCl_2$, $NaOCl_2$ sterilization, tebuconzole, trifloxystorbin, pomegranate.

INTRODUCTION

Pomegranate (*Punica granatum* L.) is one of the most important fruit crops belonging to the family Punicaceae. The family comprises only one genus (*Punica*) and two species: *P. granatum* and *P. protopunica*, having chromosomal numbers of 2n = 16 and 18, respectively. Pomegranate is also known as grenade in French and Granada in Spanish, and literally translates to "seed" (granatus) and "apple" (pomum) (Samir *et al.*, 2009). There has been an enormous increase in pomegranate area, production, and export over the past decades, owing to its immense medicinal and therapeutic values and high remuneration. It is grown in different countries, viz., Iran, Iraq, Afghanistan, Kazakhstan, Turkmenistan, Tajikistan, Armenia, Bangladesh, India, China, etc.

India is the world's leading country in pomegranate production. According to 2020-2021, the total area under pomegranate in India was 2,88,000 hectares with Maharashtra covering 90.0-thousand-hectares and Karnataka covering 16.6-thousand-hectares. Total production in India is 32,711 MT (NHB 2020–2021).

Pomegranate is a crop that is highly cross-pollinated, which results in heterozygosity among its progeny, which causes wide variations in tree and fruit characteristics raised through the seeds; as a result, commercial propagation through seeds is not a desirable method. To obtain planting material that is true to type, it is commercially propagated using vegetative methods using hard-wood cuttings, softwood cuttings, or air layering. For mass production of superior, strong, and healthy plantlets in a short time and with year-round availability, tissue culture techniques have recently been used more in pomegranates. Many researchers have reported pomegranate *in vitro* propagation techniques using different types of explants.

Tissue culture mainly refers to the proliferation and growth of tissues or organs in a sterile and controlled environment. It is not only essential for basic research but also has commercial application value (Bednarek and Orłowska 2020). Plant tissue culture is usually used as an *in vitro* biotechnology tool for the clonal propagation of plants with desirable traits (Gaur *et al.*., 2016; Hou *et al.*., 2020). In *in vitro* plant development process investigation, (Loyola-Vargas *et al.*., 2018) obtained plant materials without virus.

In commercial *in vitro propagation* of pomegranate, meristems, shoot tips, and nodal buds are mostly preferred. Because the explants have dormant vegetative meristematic buds, these are used to enhance the axillary branching. Juvenile explants were used in most *in vitro* propagation studies because, in the pomegranate, they have higher organo-genic

competence than mature explants (Kanwar *et al.,* 2008; Kanwar *et al.,* 2010). The basic step in micropropagation is the *in vitro* establishment of contamination-free plantlets. In any successful *in vitro* propagation system, surface sterilization of explants plays an important role. Microorganism-free and clean explants can be easily achieved using an effective chemical sterilant (Jalil *et al.,* 2003; Molla *et al.,* 2004; Titov *et al.,* 2006; Rahman *et al.,* 2005; Madhulatha *et al..,* 2004; Dharampal *et al.,* 2017). *In vitro* propagation of pomegranate involves the use of nodal segments of branches as explants, which are generally 2–3 months old. The woodiness of explants, along with the rich phenolic substances in pomegranate, increases the difficulty of sterilization. Therefore, this paper studied the effects of fungicides, Sodium Hypochloride, and Mercuric Chloride combinations in association with different durations of exposure for the evaluation of the contamination percentage. The data were successfully used to establish a high-efficiency surface sterilization system for pomegranate, which ensured efficient regeneration of pomegranate with lower contamination and higher explant survival percentages.

MATERIALS AND METHODS

This study was conducted in the Tissue Culture Laboratory, Department of Horticulture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Modipuram, Meerut, Uttar Pradesh, for the standardization of sterilization protocol *for in vitro* propagation of pomegranate (*Punica granatum* L.) Cv. Bhagwa. Nodal segments (4–5 cm) were taken from a 12-year-old plant as an explant to investigate the effects of different surface sterilization agents. The explants were washed with Tween 20 cleaning solution (Hi media), then rinsed with tap water for 30 minutes. The explants were washed with sterilized double-distilled water three times and rinsed for five minutes. The explants were alternately treated with fungicide (Tebuconzole 50% + Trifloxystorbin 25% w/w), mercuric chloride (0.1%), and 4% $NaOCl_2$ in combination with each other, followed by 70% ethanol, for different time intervals. In the final step, the explants were again washed with sterilized distilled water three times and were trimmed, cut, and cultured in MS media. All needed glassware, equipment, and distilled water were autoclaved at a pressure of 15 psi at 121.6°C for 25 minutes. The inside surface of the laminar flow was wiped with 70 percent ethanol and sterilized with ultra violet rays for 30 minutes before explant sterilization (Pal *et al.* 2020). Finally, all explants were inoculated onto basal MS medium (Murashige and Skoog 1962) and incubated in a culture room. During 16-hour light and dark photoperiods, temperature was kept at 26 °C and humidity at 60% under white fluorescent tubes with a light intensity of 4000 lux. The contamination percentage and explant survival rates were recorded at weekly intervals, and the contaminated cultures were immediately discarded. All the experiments were conducted in a Completely Randomized Design (CRD) with ten replicates (n = 10) per treatment and repeated three times.

Surface sterilization of explants. The crucial and most delicate step in plant tissue culture is the surface sterilization of the explant. Improper aseptic techniques, a lack of complete surface sterilization of the explant, and endogenous micro-flora present in the explants are the main contributors to contaminating tissue-cultured plants. Inappropriate sterilant concentrations also have a fatal effect on cell division by limiting the explant's ability to grow and develop. For *in vitro* cultures to survive, sterilants must be used at the right concentrations and at the right time because they shield them from various contaminants.

RESULTS AND DISCUSSION

Different sterilizing agents were used for the surface sterilization of explants (nodal segments), including fungicide, sodium hypochlorite, and mercuric chloride, in combinations of each other for different times of exposure to these sterilizing agents. The data in Table 1 and Fig. 1 clearly show the variable degree of survival after different durations of treatment with Tebuconzole 50% + Trifloxystorbin 25% w/w and Sodium Hypochloride. The maximum survival (50.00±10.00%) was recorded under the treatment T_3 (Tebuconzole 50% + Trifloxystorbin 25% w/w for 15 min + 4% $NaOCl_2$ for 7 min), followed by (40.00±0.00%) under the treatment T_2 (Tebuconzole 50% + Trifloxystorbin 25% w/w for 10 min + 4% $NaOCl_2$ for 5 min). However, the lowest survival (33.33±5.77%) of explants was observed under the treatment T_1 (Tebuconzole 50% + Trifloxystorbin 25% w/w for 5 min + 4% $NaOCl_2$ for 2 min). This might be due to the short period of exposure of the explant to Sodium Hypochloride. Similar observations were recorded by Yildiz *et al.* (2012). It indicates that the prolonged exposure of explants may increase the survival percentage of pomegranate. Similar observations were obtained by (Shukla *et al..,* 2019), while working on Banana. Pomegranate nodal explants exposed to 4% Sodium Hypochloride and 0.1% Mercuric Chloride for different durations showed variable results during the study. Table 2 and Fig. 2 clearly show that the combination T_3 (4% $NaOCl_2$ for 7 minutes + 0.1% $HgCl_2$ for 2 minutes) resulted in the highest survival (73.33±11.55%), followed by the treatment T_2 (63.33±11.55%). While, the minimum survival 60.00±10.000%) was recorded under the treatment T_1 (4% $NaOCl_2$ for 2 min + 0.1% $HgCl_2$ for 30sec). Koli *et al.* (2014) used two similar step surface sterilization protocols on Banana and achieved a high rate of survivability.

The exposure of explants to different combinations of fungicide and 0.1% muriatic chloride evaluated for different durations is presented in Table 3 and Fig. 3. Maximum survival (66.67±5.77%) was recorded under T_3 (Tebuconzole 50% + Trifloxystorbin 25% w/w for 15 min + 0.1% $HgCl_2$ for 2 min), followed by 60.00±10.00% under T_2 (Tebuconzole 50% + Trifloxystorbin 25% w/w for 10 min + 0.1% $HgCl_2$ for 1 min) while treatment T_1 (Tebuconzole 50% + Trifloxystorbin 25% w/w for 5 min + 0.1% HgCl2 for 30 sec) had the lowest minimum survival (46.67±5.77%). Similar results were earlier reported by Singh *et al.* (2014) in pomegranate.

Table 1: Effect of fungicides and $NaOCl_2$ (%) exposed duration on survivability of *in vitro* inoculated explants.

Treatments	Sterilizing agent				Survival %
	Fungicide	Duration Min/sec	$NaOCl_2$ (%)	Duration Min/sec	
T1	Tebuconzole 50% + Trifloxystorbin 25% w/w	5 min	4	2 min	33.33±5.77a
T2	Tebuconzole 50% + Trifloxystorbin 25% w/w	10 min	4	5 min	40.00±10.00a
T3	Tebuconzole 50% + Trifloxystorbin 25% w/w	15 min	4	7 min	50.00±10.00a
Se(m)					3.51

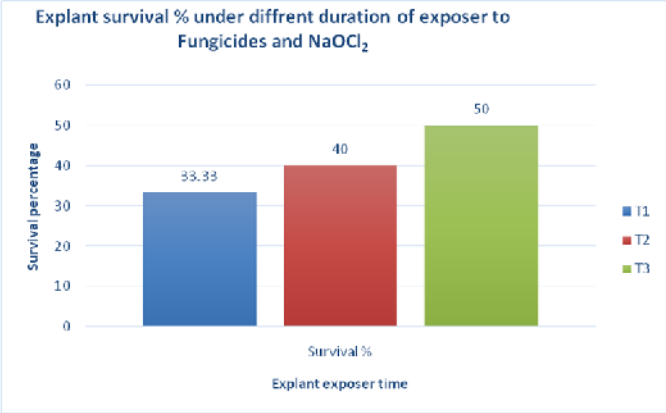

Fig. 1. Effect of fungicides and $NaOCl_2$ (%) exposed duration on survivability of *in vitro* inoculated explants.

Table 2: Effect of $NaOCl_2$(%) and $HgCl_2$(%) exposed duration on survivability of *in vitro* inoculated explants.

Treatments	Sterilizing agent				Survival %
	$NaOCl_2$(%)	Duration Min/sec	$HgCl_2$(%)	Duration Min/sec	
T1	4	2 min	0.1	30 sec	60.00±10.00a
T2	4	5 min	0.1	1min	63.33±11.55a
T3	4	7 min	0.1	2min	73.33±11.55a
Se(m)					3.76

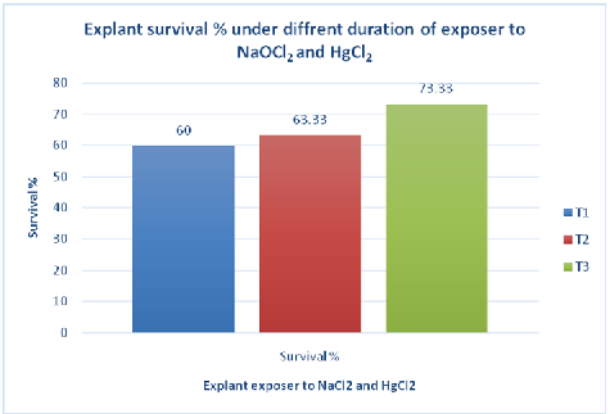

Fig. 2. Effect of $NaOCl_2$(%) and $HgCl_2$(%) exposed duration on survivability of *in vitro* inoculated explants.

Table 3: Effect of fungicide and $HgCl_2$ exposed duration on survivability of *in vitro* inoculated explants.

Treatments	Sterilizing agent				Survival %
	Fungicide	Duration Min/sec	$HgCl_2$ (%)	Duration Min/sec	
T1	Tebuconzole 50% + Trifloxystorbin 25% w/w	5 min	0.1	30 sec	46.67±5.77a
T2	Tebuconzole 50% + Trifloxystorbin 25% w/w	10 min	0.1	1min	60.00±10.00ab
T3	Tebuconzole 50% + Trifloxystorbin 25% w/w	15 min	0.1	2min	66.67±5.77b
Se(m)					3.64

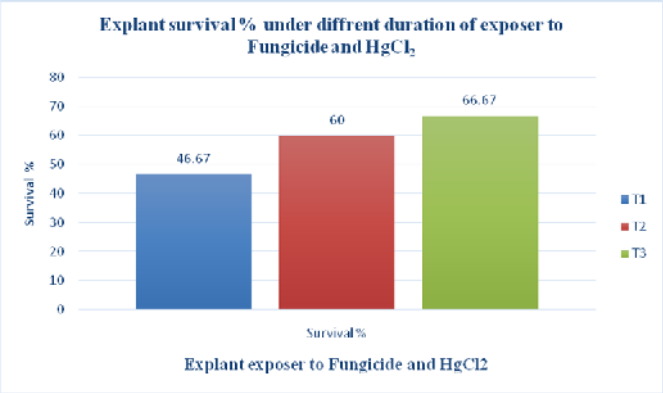

Fig. 3. Effect of fungicide and $HgCl_2$ exposed duration on survivability of *in vitro* inoculated explants.

CONCLUSION

In vitro propagation is the very sensitive system of plant propagation, which is positively correlated with the efficiency of sterilant. The exposure time of explants and different concentration of sterilizing agents may have a direct impact on the success rate as a result, the current investigation was carried out and resulted in the successful development of a complete sterilization package for the pomegranate.

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Conflicts of Interest. None.

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