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Standardize the aseptic environment protocol for strawberry *in vitro* cloning (*Fragaria x ananassa* Duch.)

Vibhu Pandey, Arvind Kumar, Vipin Kumar, Satya Prakash, LK Gangwar, RS Sengar, Mukesh Kumar, Anuj Pal, Devendra Pal and Amit Kumar

Abstract
The successful application of *in vitro* propagation methods for strawberries leaned on the effective surface sterilisation of tissue culture explants. To reduce contamination and improve survival rates, a range of sterilizing chemicals were used. These treatments included various fungicides (Bavistin 0.4% + Carbendazim 1% and Tebuconazole 50% + Trifloxystrobin 25% w/w) as well as Mercuric Chloride (0.1%), $NaOCl_2$ (4%), Citric acid (0.45%), and Ascorbic acid (0.25%), followed by a 70% ethanol treatment. Upon examining different combinations and durations, the study revealed that the highest survival percentage (72.05±8.41%) was achieved using the combination of Tebuconazole 50% + Trifloxystrobin 25% w/w for 5 minutes, followed by a 1-minute treatment with 0.1% $HgCl_2$. This combination outperformed others significantly. Additionally, the treatments involving Tebuconazole 50% + Trifloxystrobin 25% w/w for 7 minutes and 0.1% $HgCl_2$ for 1.5 minutes, and Tebuconazole 50% + Trifloxystrobin 25% w/w for 3 minutes and 0.1% $HgCl_2$ for 0.5 minutes, resulted in survival rates of 63.52±5.78% and 57.79±4.93%, respectively. In conclusion, the most successful method for the *in vitro* propagation of strawberries involved treating the explants with Tebuconazole 50% + Trifloxystrobin 25% w/w for 5 minutes, followed by a 1-minute exposure to 0.1% $HgCl_2$. This combination offered the highest survival rate, presenting a promising technique to boost strawberry propagation through tissue culture. These findings hold significant implications for the agricultural industry, as they contribute to the advancement of strawberry cultivation methods and the availability of high-quality strawberry plants for commercial purposes.

Keywords: $HgCl_2$, $NaOCl_2$, sterilization, tebuconazole, trifloxystrobin, strawberry.

Introduction
A perennial herb of the dicotyledonous family, the cultivated strawberry (*Fragaria x ananassa* Duch.) is a product of a hybridization between the pistillate South American strawberry (*Fragaria chiloensis* L. Duch.) and the "scarlet" or "virginia" (*Fragaria virginiana* Duch.). It is widely cultivated in various arable regions across the globe (Debnath, 2013) [9]. There are around 20 species in the genus Fragaria, with ploidy levels ranging from diploid to decaploid (Lal & Singh, 2016) [21]. The cultivated strawberry (*Fragaria x ananassa* Duch.) possesses an allooctaploid genome, resulting from the chance hybridization of two other octoploid *Fragaria species*, *F. virginiana* and *F. chiloensis* (Karlund, 2016) [18]. It falls within the subfamily Rosoideae and the family Rosaceae. This herb is perennial and stoloniferous, emerging from a meristematic tissue crown, and it exhibits excellent adaptation to a wide array of climatic conditions. The growth of these plants is influenced by day length (i.e., long day, short day, or day-neutral) (Darnell *et al.*, 2003) [8]. During the 2019-20 period, the total area under strawberry cultivation in India reached 1,000 hectares, with a substantial production of 8,000 metric tons. The major strawberry-growing regions in India include Himachal Pradesh, Jammu and Kashmir, Uttar Pradesh, Maharashtra, West Bengal, Punjab, Haryana, Rajasthan, Delhi, and the Nilgiri hills. Maharashtra's Panchgani-Mahabaleshwar belt takes the lead in the country's strawberry production (NHB, 2019-20) [26]. Strawberries are a rich source of bioactive phytochemicals, including flavonoids and ellagic acid, which may have antioxidant properties. According to Wang *et al.*, (1996) [36] and Heinonen *et al.*, (1998) [15], these substances have been shown to lower the risk of cardiovascular events and carcinogenesis. Strawberries are cultivated worldwide not only for their digestive and tonic properties but also for their nutritional value.

Corresponding Author:
Vibhu Pandey
College of Horticulture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India

They are considered to be an important source of folate and vitamin C and are known for their high fiber content. Strawberries can improve human health when included in a regular diet (Hannum, 2004) [13] and as a result, their cultivation and production are witnessing a steady increase each year (Esitken *et al.,* 2010) [11]. Traditionally, strawberries have been propagated using runner seedlings, but this conventional approach is inadequate to meet the commercial demand (Ara *et al.,* 2013) [1]. This method has proven to be unsuitable due to the occurrence of various diseases, infections, and environmental hazards, leading to a gradual decline in the performance of cultivars (Biswas *et al.,* 2007) [3-4]. The significant development of *in vitro* production of strawberry plants dates back to the 1970s when it was first introduced in 1974 (Boxus, 1974; Boxus *et al.,* 1977) [5, 6], presenting an intriguing improvement over the rigid and slow conventional scheme. Karhu and Hakala (2002) [17] observed that micro-propagated strawberry plants exhibited superior characteristics (such as crown size, number of runners, flowering time, and berry yield) compared to conventionally propagated runner plants. The standardization of the protocol and procedure for micro-propagation of strawberries has been successfully attempted by various researchers (Kaur *et al.,* 2005; Sakila *et al.,* 2007; Gantait *et al.,* 2010) [19, 31, 12]. The initial step in micro-propagation is to establish plantlets that are free from contamination in a controlled environment (*in vitro*). Surface sterilization of the explants plays a crucial role in ensuring the success of *in vitro* propagation systems. The use of effective chemical sterilants guarantees the production of explants that are free from microorganisms and are clean. This process is vital for achieving successful *in vitro* propagation of plants (Jalil *et al.,* 2003; Molla *et al.,* 2004; Titov *et al.,* 2006; Rahman *et al.,* 2005; Madhulatha *et al.,* 2004) [16, 23, 35, 30, 22].

Materials and Methods

The research was carried out at the Tissue Culture Laboratory, Department of Horticulture, Sardar Vallabhbhai Patel University of Agriculture & Technology in Modipuram, Meerut, Uttar Pradesh. The primary aim of the study was to develop a standardized sterilization protocol for the *in vitro* production of strawberries (*Fragaria x ananassa* Duch). Nodal segments (3-5 cm) were collected from 6 to 8-month-old plants to examine the effects of different surface sterilization methods during the experimentation process. The sterilization process began with cleaning the explants using a Tween 20 cleaning solution from Hi media, followed by a 30-minute rinse with tap water. Three rounds of sterile double-distilled water washing and five minutes of rinsing were then performed on the explants. To ensure effective sterilization, the explants were subjected to different durations of treatment with various chemicals, including fungicides (Bavistin 0.4% + Carbendazim 1% and Tebuconazole 50% + Trifloxystrobin 25% w/w), Mercuric Chloride (0.1%), Sodium Hypochlorite ($NaOCl_2$ 4%), Citric acid (0.45%), and Ascorbic acid (0.25%). Subsequently, the explants underwent a treatment with 70% ethanol. After the sterilization process, the explants were cut, clipped, and placed into MS media, followed by three additional washes with sterile distilled water. All necessary glassware, equipment, and distilled water were autoclaved (25 minutes at 121.6 ºC and 15 psi) to ensure proper sterilization. Prior to starting the explant sterilisation, the inner surface of the laminar flow cabinet was washed with 70% ethanol and sterilised with UV light for 30 minutes (Pal *et al.,* 2020) [27]. Subsequently, every explant was injected onto basal MS media (Murashige and Skoog 1962) [25] and allowed to incubate in a culture chamber. With white fluorescent bulbs that produced 4000 lux of light, a 16-hour light and dark photoperiod, a temperature of 26 °C, and 60% humidity, the growth chamber was outfitted for optimal conditions. Any contaminated cultures were promptly discarded, and regular records were maintained to monitor explant survival rates and contamination percentages. The experiment was designed using a Complete Randomized Design (CRD) with ten repetitions (N=10) for each treatment.

Surface sterilization of explants: The surface sterilization of explants is the most crucial and delicate stage in plant tissue culture. Contamination of tissue-cultured plants mainly arises from inadequate aseptic techniques, insufficiently cleaned explants, and the presence of endogenous microorganisms within the explants. Additionally, improper sterilant concentrations hinder cell division and normal growth and development of the explants. The right use of sterilants at the right times and in the right amounts is essential for the success of *in vitro* cultures because it protects the cultures against pollutants.

Results and Discussion

In this study, a variety of sterilising chemicals were used to surface sterilise explants, or nodal segments. These agents included fungicide, Sodium hypochlorite, and Mercuric chloride used in combination with Tebuconazole 50% + Trifloxystrobin 25% w/w. Additionally, Citric acid and Ascorbic acid were used in combination with Bavistin 0.4% + Carbendazim 1% for different durations of exposure to these sterilizing agents. The findings shown in Table 1 and Figure 1 make it abundantly evident how varied survival rates are attained following various treatment periods with Bavistin 0.4% + Carbendazim 1% and citric acid. The highest survival rate (49.77±2.18%) was observed in treatment T_4 (Bavistin 0.4% + Carbendazim 1% for 15 minutes + 0.45% citric acid for 8 minutes), followed by a survival rate of (40.60±2.09%) in treatment T_3 (Bavistin 0.4% + Carbendazim 1% for 10 minutes + 0.45% citric acid for 5 minutes). Conversely, the lowest survival rate (27.76±3.55%) of explants was recorded in treatment T_1 (Bavistin 0.4% + Carbendazim 1% for 5 minutes + 0.45% citric acid for 2 minutes). These results are consistent with earlier research by Yildiz *et al.* (2012) [37] and Pal *et al.* (2022) [28], which suggests that extended explant exposure might increase the percentage of strawberries that survive. Shukla *et al.* (2019) [33] observed similar results in their study pertaining to bananas. The data presented in Table 2 and Figure 2 clearly depict the varying degrees of survival achieved after different durations of treatment with Bavistin 0.4% + Carbendazim 1% and Ascorbic acid 0.25%. The highest survival rate (52.59±2.30%) was observed in treatment T_4 (Bavistin 0.4% + Carbendazim 1% for 15 minutes + Ascorbic acid 0.25% for 6 minutes), followed by a survival rate of (44.44±2.30%) in treatment T_3 (Bavistin 0.4% + Carbendazim 1% for 10 minutes + Ascorbic acid 0.25% for 4.5 minutes). Conversely, the lowest survival rate (31.43±4.01%) of explants was recorded in treatment T_1 (Bavistin 0.4% + Carbendazim 1% for 5 minutes + Ascorbic acid 0.25% for 1.5 minutes). These results corroborate those of earlier research by Yildiz *et al.,* (2012) and Pal *et al.,*

(2022) [28], indicating that extended exposure of explants may increase the explant survival rate of strawberries. In their study on bananas, Shukla *et al.*, (2019) [33] obtained similar findings. Table 3 and Figure 3 clearly indicate that the combination T_4 (Tebuconazole 50% + Trifloxystrobin 25% w/w for 10 minutes + $NaOCl_2$ 4% for 10 minutes) resulted in the highest survival rate (49.11±2.15%), followed by (39.21±2.02%) under treatment T_3 (Tebuconazole 50% + Trifloxystrobin 25% w/w for 7 minutes + $NaOCl_2$ 4% for 7.5 minutes). In contrast, the lowest survival rate (32.33±4.13%) was recorded under treatment T_1 (Tebuconazole 50% + Trifloxystrobin 25% w/w for 3 minutes + $NaOCl_2$ 4% for 2.5 minutes). In order to obtain a high rate of survival, Koli *et al.*, (2014) [20] used two comparable step surface sterilisation methods on bananas. Table 4 and Figure 4 present the evaluation of explants exposed to different combinations of fungicide and 0.1% Mercuric chloride for varying durations. The maximum survival rate (72.05±8.41%) was recorded under T_2 (Tebuconazole 50% + Trifloxystrobin 25% w/w for 5 minutes + 0.1% $HgCl_2$ for 1 minute), followed by (63.52±5.78%) under T_3 (Tebuconazole 50% + Trifloxystrobin 25% w/w for 7 minutes + 0.1% $HgCl_2$ for 1.5 minutes). On the other hand, treatment T4 (Tebuconazole 50% + Trifloxystrobin 25% w/w for 10 minutes + 0.1% $HgCl_2$ for 2.0 minutes) resulted in the lowest survival rate (52.43±6.21%). These findings align with previous research by Singh *et al.* (2014) [34] in pomegranates, showing similar results. In the current study, various combinations of sterilants were employed to eliminate bacterial and fungal contaminants. This approach is supported by the findings of Moutia and Dookum (1999) [24], who suggested that neither of the sterilants (NaOCl and $HgCl_2$) used individually could effectively control the bacterial and fungal contaminants. The results of present study align with the research conducted by Biswas *et al.* (2007) [3-4]. They reported that treating with 0.1 per cent $HgCl_2$ for 5 minutes was the most effective method. This finding reinforces present own results, as we used 0.1 per cent $HgCl_2$ for 1 minutes. In contrast, Sharma *et al.* (2009) [32] reported that using 0.1 per cent $HgCl_2$ for 3 minutes had little impact in overcoming contamination. Present study indicated that $HgCl_2$ was a more effective sterilant than $NaOCl_2$, which is consistent with the findings of Biswas *et al.* (2007) [3-4], Hasan *et al.* (2010) [14], Ara *et al.* (2012) [2], Diengngan and Murthy (2014) [10], and Palei *et al.* (2017) [29]. These researchers also reported that $HgCl_2$ treatment outperformed $NaOCl_2$. However, it's worth noting that Chien-Yingko *et al.* (2009) [7] achieved successful surface sterilization of strawberries using $NaOCl_2$. The combination approach used in this study for sterilization showed promising results in eliminating bacterial and fungal contaminants. Findings of this study, together with those of previous research, suggest that $HgCl_2$ can be more effective than $NaOCl_2$ as a sterilant, but both may have specific applications in different contexts.

Table 1: Impact of fungicide and citric acid (%) exposed duration on survivality of *in vitro* inoculated plants

Treatment	Sterilizing agent				Survival %
	Fungicide	Duration (min)	Citric acid (%)	Duration (min)	
T_1	Bavistin 0.4% + Carbendazim 1%	5	0.45	2	27.76±3.55a
T_2	Bavistin 0.4% + Carbendazim 1%	7	0.45	3	37.76±1.82b
T_3	Bavistin 0.4% + Carbendazim 1%	10	0.45	5	40.60±2.09b
T_4	Bavistin 0.4% + Carbendazim 1%	15	0.45	8	49.77±2.18c
SE(m)					2.44
CD					1.94
C.V. %					2.44

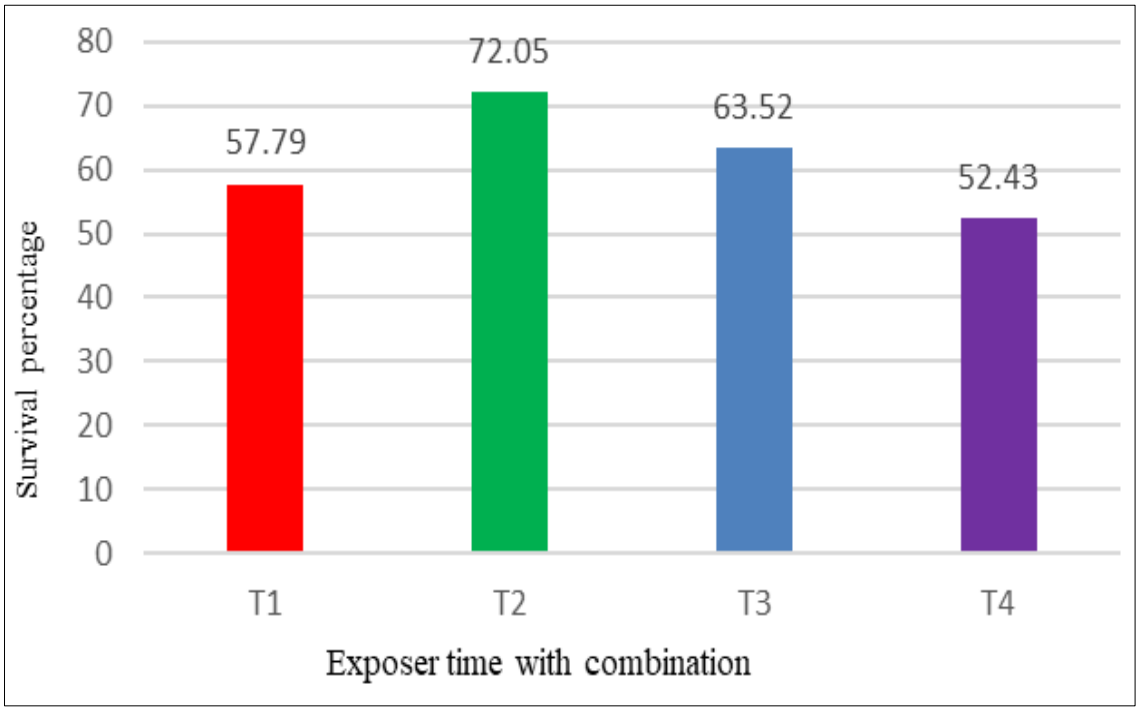

Fig 1: Fungicide and Citric acid (%) exposed duration on survivality of explant

Table 2: Impact of fungicide and ascorbic acid (%) exposed duration on survivality of *in vitro* inoculated plants

Treatment	Sterilizing agent				Survival %
	Fungiside	Duration (min)	Ascorbic acid (%)	Duration (min)	
T1	Bavistin 0.4% + Carbendazim 1%	5	0.25	1.5	31.43±4.01a
T2	Bavistin 0.4% + Carbendazim 1%	7	0.25	3	39.66±1.91b
T3	Bavistin 0.4% + Carbendazim 1%	10	0.25	4.5	44.44±2.30b
T4	Bavistin 0.4% + Carbendazim 1%	15	0.25	6	52.59±2.30c
SE(m)					2.40
CD					2.28
C.V. %					2.66

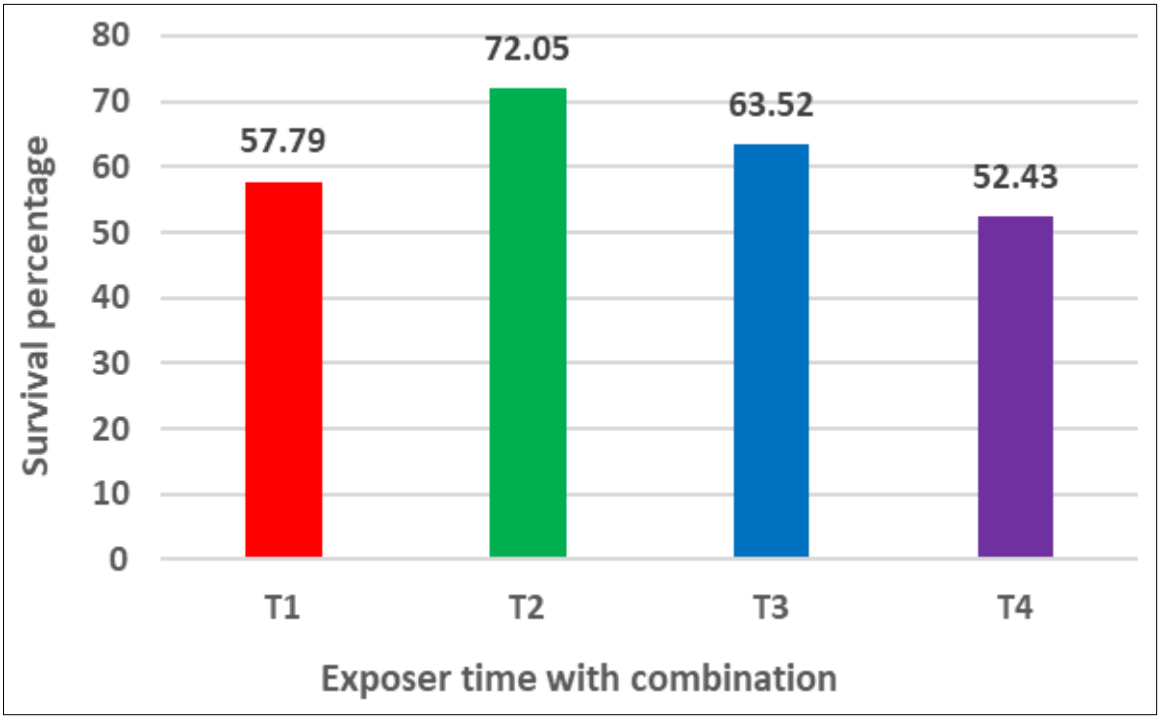

Fig 2: Fungicide and ascorbic acid (%) exposed duration on survivality of explant

Table 3: Impact of fungicide and sodium hypo-chloride (%) exposed duration on survivality of *in vitro* inoculated plants

Treatment	Sterilizing agent				Survival %
	Fungiside	Duration (min)	$NaOCl_2$ (%)	Duration (min)	
T_1	Tebuconazole 50% + Trifloxystrobin 25% w/w	3	4	2.5	32.33±4.13a
T_2	Tebuconazole 50% + Trifloxystrobin 25% w/w	5	4	5.0	37.65±1.82a
T_3	Tebuconazole 50% + Trifloxystrobin 25% w/w	7	4	7.5	39.21±2.02a
T_4	Tebuconazole 50% + Trifloxystrobin 25% w/w	10	4	10	49.11±2.15b
SE(m)					1.94
CD					2.49
C.V. %					3.08

Table 4: Impact of Fungicide and Mercuric chloride (%) exposed duration on survivality of *in vitro* inoculated plants

Treatment	Sterilizing agent				Survival %
	Fungiside	Duration (min)	$HgCl_2$ (%)	Duration (min)	
T1	Tebuconazole 50% + Trifloxystrobin 25% w/w	3	0.1	0.5	57.79±4.93ab
T2	Tebuconazole 50% + Trifloxystrobin 25% w/w	5	0.1	1.0	72.05±8.41b
T3	Tebuconazole 50% + Trifloxystrobin 25% w/w	7	0.1	1.5	63.52±5.78ab
T4	Tebuconazole 50% + Trifloxystrobin 25% w/w	10	0.1	2.0	52.43±6.21a
SE(m)					2.70
CD					12.37
C.V. %					9.88

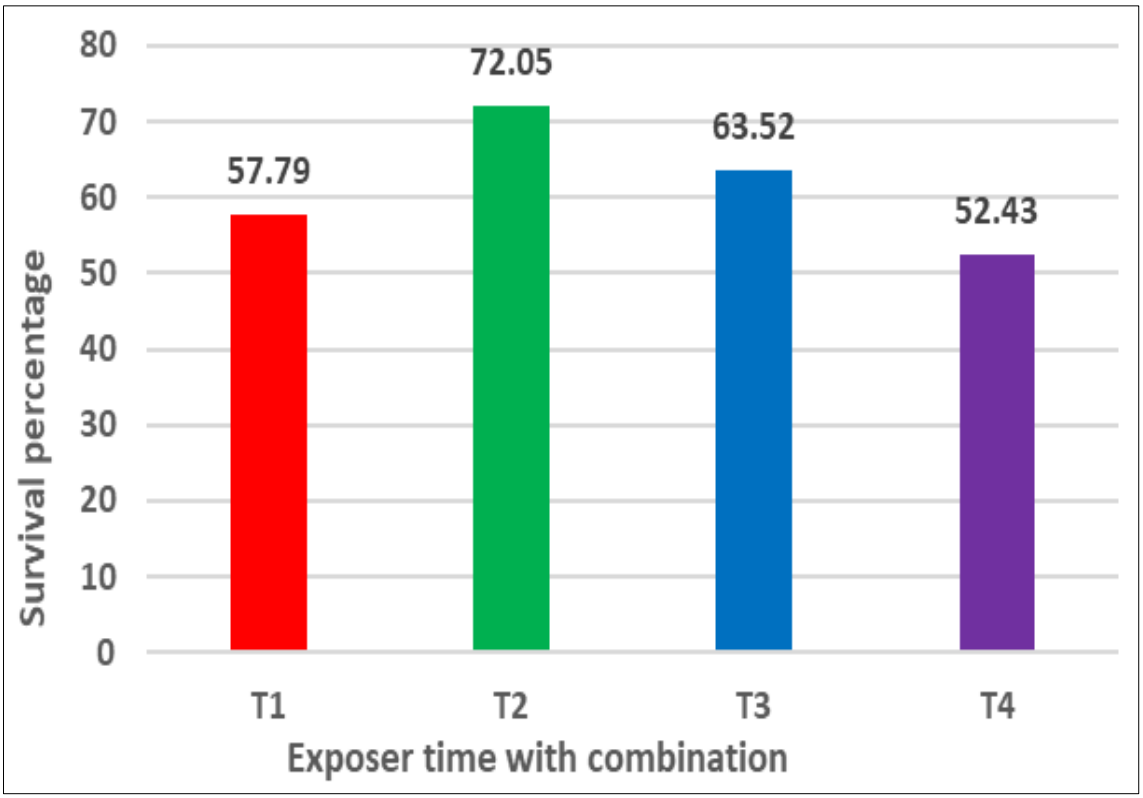

Fig 3: Fungicide and Sodium hypo-chloride (%) exposed duration on survivality of explant

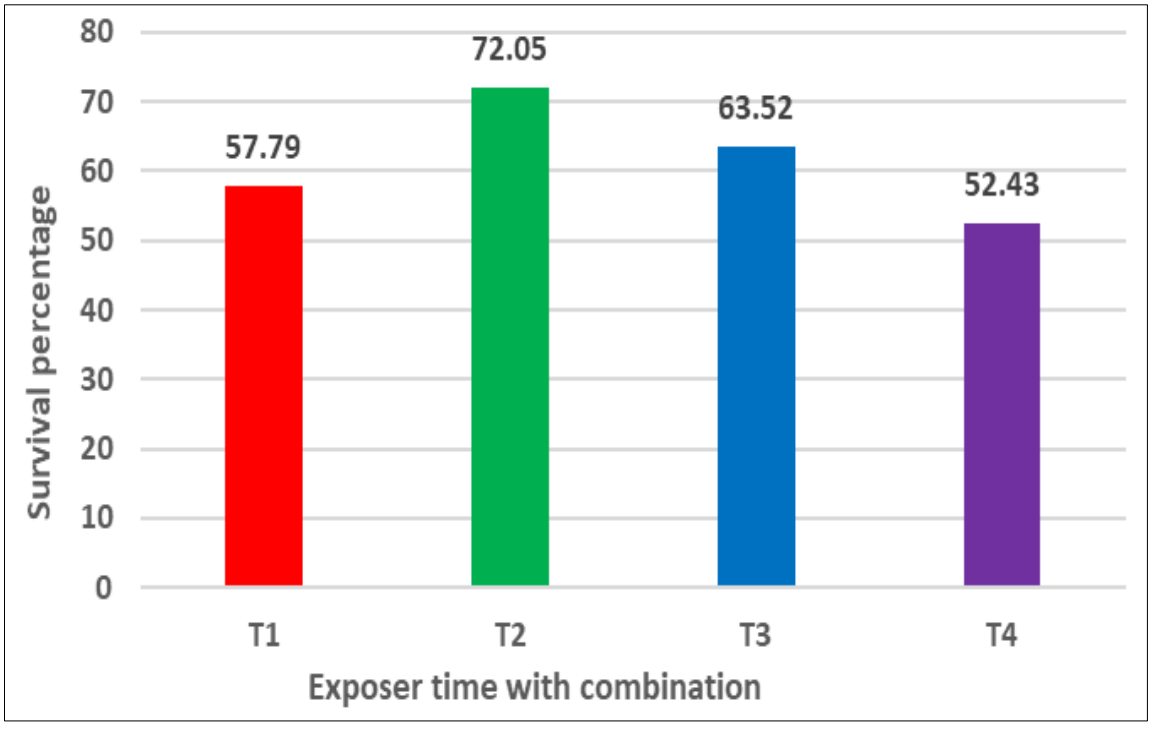

Fig 4: Fungicide and Mercuric chloride (%) exposed duration on survivality of explant

Conclusion

In conclusion, the standardized sterilization protocol using Tebuconzole 50% + Trifloxystrobin 25% w/w for 5 minutes, followed by a 1-minute exposure to 0.1% $HgCl_2$, offers the highest survival rate for *in vitro* propagation of strawberries. This protocol is a promising technique to boost strawberry propagation and contribute to the advancement of strawberry cultivation methods. By ensuring contamination-free plantlets, this protocol can enhance the availability of high-quality strawberry plants for commercial purposes. Further research can explore the long-term effects and scalability of this standardized protocol for large-scale strawberry production. The standardized protocol for strawberry *in vitro* propagation can revolutionize the strawberry cultivation industry by offering a more efficient and reliable method for mass production of high-quality plants. The ability to produce clean, disease-free plants can minimize the risk of crop loss and increase productivity. Implementing this protocol can also

ensure the availability of uniform and genetically consistent plants, which is crucial for commercial growers. Overall, the standardized aseptic environment protocol for strawberry *in vitro* cloning holds great potential for the future of strawberry cultivation.

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All Author's Names

Vibhu Pandey
College of Horticulture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India

Arvind Kumar
College of Horticulture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India

Vipin Kumar
College of Horticulture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India

Satya Prakash
College of Horticulture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India

LK Gangwar
College of Agriculture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India

RS Sengar
College of Agriculture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India

Mukesh Kumar
College of Horticulture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India

Anuj Pal
College of Agriculture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India

Devendra Pal
College of Agriculture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India

Amit Kumar
College of Agriculture, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut, Uttar Pradesh, India