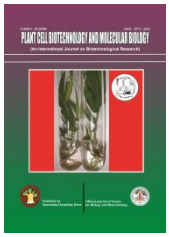

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Studies on *In vitro* Surface Sterilisation and Antioxidants on Pomegranate (*Punica granatum* L.) cv. Bhagwa

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Pomegranate, regarded as the "Fruit of Paradise", is one of the important fruit crops of tropical and subtropical regions. Pomegranate fruits are delicious and possess significant nutritional and medicinal benefits. Due to the increase in demand, the proliferation of pomegranate through tissue culture is essential to get high-quality planting material. However, microbial contamination in plant tissue culture is one of the bottlenecks for establishing aseptic cultures. Although multiple explant

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sterilisation methods have been developed for pomegranate, explant sterilisation methods can indeed vary from region to region due to the local environment and the mother plant. In the present study, 1-2 drops of tween 20 (20 min) + 500 mg/L carbendazim (30 min) + 500 mg/L streptomycin sulphate (10 min) + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (folicur) (30 min) + 0.1% $HgCl_2$ (1 min) + dipping explant after cutting the edges in sterile water (30 min) + dipping in 500 mg/L PVP (5 min) was found to be optimum in the prevention of microbial contamination with minimal cell death. Nodal segments-initiated callus at 54 days after culture, showing a callus induction percentage of 55.55% and a calli weight of 80 mg/explant.

Keywords: Bhagwa; fungicide; bactericide; antioxidants; callus.

1. INTRODUCTION

Pomegranate (*Punica granatum* L.) is a fruit-bearing shrub of the tropics and subtropics. It belongs to Lythraceae, with two species: *Punica granatum* L. and *Punica protopunica*. Pomegranate is a diploid with chromosome number 2n=2x=16. It is native to Central Asia (Georgia, Armenia, Azerbaijan, Iran and Turkey) and northern India [1].

Pomegranate is rich in nutrients and highly valued for its medicinal properties. The 100 g edible portion of raw pomegranate consists of 77.9% water, 18.7% carbohydrates, 1.67% protein, 1% fat, 10 mg calcium, 0.3 mg iron and 12 mg magnesium [2]. Pomegranate is a potent antioxidant attributed to its polyphenols, including punicalagin, ellagitannin and ellagic acid. Pomegranate can prevent and treat several types of cancer, cardiovascular disorders, diabetes, male infertility, Alzheimer's disease [3], rheumatoid arthritis [4], ageing, AIDS [5] and other diseases.

Pomegranate is a resilient crop, capable of thriving in poor and marginal soils, adapting to various climates, and exhibiting tolerance to salinity and drought [6;7]. The substantial returns per unit of land have fueled a continuous expansion in the cultivation area and production of pomegranates worldwide. The leading pomegranate-producing country is India followed by, Iran, Turkey, China, Afghanistan, the USA, Iraq, Pakistan, Syria and Spain. In India, pomegranate is cultivated in a 2.88 lakh ha area, producing 3.27 lakh metric tonnes [8].

Pomegranate cultivation has risen because of its nutritional richness, medicinal properties, climate resilience and high economic returns. By 2025, the pomegranate cultivation area is anticipated to rise to 7.50 lakh hectares in India alone [9]. Increased cultivation has increased the demand for planting material. Further, pomegranate is severely infested by bacterial blight and wilt, often leading to frequent replanting, raising the demand for plant material [10]. Conventionally, pomegranate is propagated by air layering [11], hard-wood [12] and soft-wood cuttings [13]. However, producing such planting material may require a year; it is season-dependent and does not ensure the production of pest- and disease-free plants [14]. Micropropagation of pomegranates ensures rapid and large-scale production of pests and disease-free planting material, round-the-year production, independent of the season [15]. Several pomegranate micropropagation protocols have been reported using different explants, including nodal cuttings [16;17], leaves [18;19;20], cotyledons [18;19;21] etc. Establishing thriving plant tissue culture in pomegranate also helps in the genetic transformation of pomegranate for developing transgenic plants and gene editing.

The contamination [16;22] of *in vitro* plants, explants and growth medium browning [18] are the significant obstacles to the micropropagation of pomegranate. Fungal and bacterial contamination is the most observed in pomegranate tissue cultures, which results from the microbes on the surface of the explants and the endophytic microbes in mother plants [23]. Several bacterial and fungal decontamination procedures, such as surface sterilisation with a fungicide [16], antibiotics [24;16;25], sodium hypochlorite [20;19;26], ethanol [19;16] and mercury chloride [16;27;25], have been reported. Further, successful micropropagation is also limited by explant and growth medium browning [18] due to the release of phenolic compounds by the excised explants. Preventing tissue and media browning in pomegranate tissue culture has been attempted by incubating explant in antioxidants such as citric acid [24], ascorbic acid [24] and adenine sulphate [16;25] into the MS media.

In this study, multiple explant sterilisation procedures were compared in the callus culture of the pomegranate variety 'Bhagwa' and their effects on the microbial decontamination and tissue/ growth medium browning were studied.

2. MATERIALS AND METHODS

Collection of explants: Nodal segments of the elite pomegranate variety 'Bhagwa' collected from the mother nursery maintained in a polyhouse at the College of Horticulture, Bengaluru, were used in this study. Two days before the explant collection, mother plants were sprayed with 0.5 g/L of carbendazim 50% WP (bavistin, Crystal, India) and streptomycin sulphate (streptocycline, Hindustan antibiotics Ltd.). Nodal segments were collected from the six-month-old pomegranate plants at 8.00 - 9.00 am.

Pre-sterilisation washing: Dust and other foreign particles adhered to the explants were thoroughly washed off by placing them in a beaker under slowly running water for an hour. Later, they were thoroughly washed in sterile water with 2-3 drops of Tween-20.

Sterilisation of explant: Explants were incubated in different combinations of bactericides, fungicides and antioxidants to minimise microbial contamination and cell death. Different combinations of explant sterilisation methods and antioxidants compared in the study are listed below.

- S_0: 1-2 drops of tween 20 (20 min) + 500 mg/L carbendazim (30 min) + 500 mg/L streptomycin sulphate (10 min)
- S_1: S_0 + 70% ethanol (2 min)
- S_2: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min)
- S_3: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 0.5% $HgCl_2$ (1 min) + Dipping explant after cutting the edges in 50 mg/L adenine sulphate (5 min)
- S_4: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + Dipping explant after cutting the edges in 100 mg/L citric acid (5 min)
- S_5: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 0.1% $HgCl_2$ (1 min) + Dipping explant after cutting the edges in 500 mg/L NaCl (5 min)
- S_6: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 0.1% $HgCl_2$ (1 min) + Dipping explant after cutting the edges in 500 mg/L PVP (5 min)
- S_7: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 1 % sodium hypochlorite (5 min) + 0.1% $HgCl_2$ (1 min) + dipping explant after cutting the edges in 500 mg/L PVP (5 min)
- S_8: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 0.1% $HgCl_2$ (1 min) + dipping explant after cutting the edges in sterile water (30 min) + dipping in 500 mg/L PVP (5 min)

After sterilisation, explants were blot-dried by pressing against a sterile blotting paper before inoculation onto the MS media.

Callus Induction: Sterilised nodal segments with 2-3 nodes were inoculated on MS medium supplemented with 3mg/L 2,4-D (Fig. 1) and incubated at 25±2^{0}C on the culture fitted with tube lights emitting white light at 3000 lux light intensity and approximately 70% relative humidity with 16 hours of light and 8 hours of dark periods.

Observation: Observations on the appearance of bacterial and fungal colonies and browning of either explant or the media were recorded at 3-day intervals from three days of post-inoculation until 15 days of post-inoculation.

3. RESULTS

Prevention of fungal contamination: After seven days of culture, fungal mycelial colonies were noticed with colonies similar to Aspergillus, Cladosporium and Penicillium in the explants sterilised by 1-2 drops of tween-20 (20 min) + 500 mg/L carbendazim (30 min) + 500 mg/L streptomycin sulphate (10 min) (S_0) (Table 1, Fig. 1). More than five colonies of fungi with varying mycelium colour and morphology were observed on the plates. Treating explants with tebuconazole minimised fungal contamination (S_2 to S_8). The growth of fungal colonies was not noticed either on the explants or in the culture media in sterilisation procedures S_4, S_7 and S_8, which involved treating explants with tebuconazole. However, moderate (browning of explant) to high (complete death of explant) cell death was noticed in the sterilisation procedures S_4 and S_7. Hence, sterilisation procedure S_8 [S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (folicur) (30 min) + 0.1% $HgCl_2$ (1 min) + dipping explant after cutting the edges in sterile water (30 min) + dipping in 500 mg/L PVP

(5 min)] was found to be optimum in the prevention of fungal contamination with minimal cell death.

Prevention of bacterial contamination: Contamination of explants and culture plates with bacteria was noticed three days post-inoculation (dpi) in the sterilisation procedure S_0 (Table 1, Fig. 1). Like fungal colonies, bacterial colonies also varied in colour and morphology. To prevent bacterial growth, extra measures were taken by treating explants: immersing them in 70% ethanol (S_1), one-minute treatment with 0.1% $HgCl_2$ (S_3, S_5, S_6, S_8) and five-minute exposure to 1% sodium hypochlorite (S_7). Bacterial contamination was prevented in all treatments where explants were treated with antibacterial agents such as 70% ethanol, $HgCl_2$ or sodium hypochlorite and streptomycin sulphate. However, a high cell death rate was noticed in the explants treated. Surprisingly, bacterial contamination was not observed in S_2, where explants were not treated with anti-bacterial agents other than streptomycin sulphate. Considering the absence of contamination with fungus or bacteria and minimal explant death, sterilisation procedure S_8 was considered the best.

Cell death: The relative cell damage in all the tested sterilisation procedures was considered apart from preventing bacterial and fungal contamination. Despite including antioxidant treatments, tissue browning was noticed in most treatments after five days of culture. Led to complete explant death by 7 to 10 days in the sterilisation procedures S_1 to S_5 and S_7 (Table 1). Cutting the edges of explants after sterilising with antibacterial and antifungal agents and treating them with sterile water for 30 minutes and PVP (500 mg/L) for 5 min (S_8) reduced tissue browning and exhibited cell division.

Callus Induction: Surface-sterilised nodal segments were inoculated on MS medium supplemented with 3 mg/L 2,4-D, resulting in callus induction 54 days after culture. The callus induction percentage was 55.55%, and the calli weight was 80 mg/explant (Fig. 2).

4. DISCUSSION

In the present study, S8: [1-2 drops of tween 20 (20 min) + 500 mg/L carbendazim (30 min) + 500 mg/L streptomycin sulphate (10 min) + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 0.1% $HgCl_2$ (1 min) + dipping the explant after cutting the edges in sterile water (30 min) + dipping in 500 mg/L PVP (5 min)] was found to be optimum with minimal microbial contamination and cell death.

Here, Carbendazim inhibits fungal contamination by reducing mycelial growth, germ tissue development, and appressoria formation and even interferes with fungal metabolism [28]. Similarly, fungal contamination was overcome in the tissue culture of pomegranate [16] and sugarcane variety Co 0118 [29]. Lakshmi et al. [29] used a higher concentration of carbendazim (300 mg/L) to curb fungal contamination

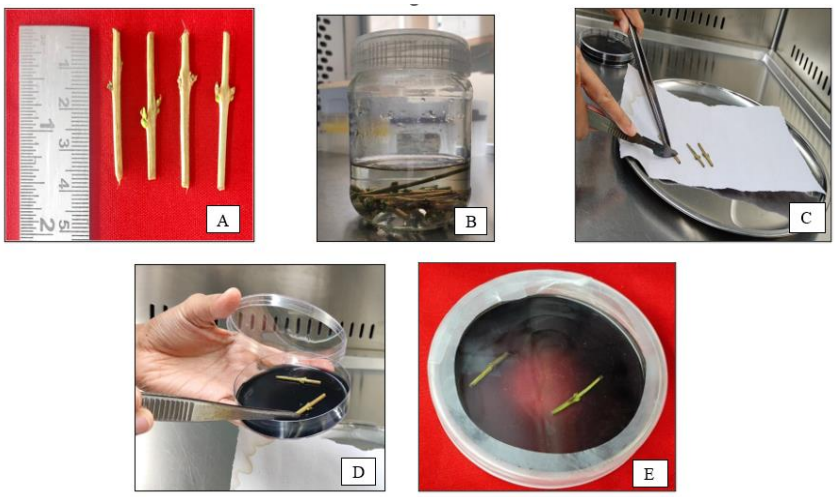

Fig. 1. Initiation of callus using nodal segments of pomegranate cultivar 'Bhagwa' Explant (3-4 cm long nodal segment) (B) Sterilisation of explants (C) Reducing the size of explant to 3 - 4 cm inside LAF (D) Placing the nodal segments inside callus induction media (E) Cultured plate

Table 1. Effect of different sterilisation procedures on microbial contamination and cell death of pomegranate cv. Bhagwa explants under *in vitro* culture

Sterilisation Protocol	Fungal contamination	Bacterial contamination	Cell death
S_0: 1-2 drops of tween 20 (20 min) + 500 mg/L carbendazim (30 min) + 500 mg/L streptomycin sulphate (10 min)	∗ ∗ ∗	∗ ∗ ∗	∗ ∗ ∗
S_1: S_0 + 70% ethanol (2 min)	∗ ∗ ∗	-	∗ ∗ ∗
S_2: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min)	-	-	∗ ∗ ∗
S_3: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 0.5% $HgCl_2$ (1 min) + Dipping explant after cutting the edges in 50 mg/L adenine sulphate (5 min)	∗ ∗	∗ ∗	∗ ∗ ∗
S_4: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + Dipping explant after cutting the edges in 100 mg/L citric acid (5 min)	-	∗ ∗	∗ ∗
S_5: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 0.1% $HgCl_2$ (1 min) + Dipping explant after cutting the edges in 0.5 g/L NaCl (5 min)	∗	-	∗ ∗ ∗
S_6: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 0.1% $HgCl_2$ (1 min) + Dipping explant after cutting the edges in 0.5 g/L PVP (5 min)	∗	-	∗
S_7: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 1% sodium hypochlorite (5 min) + 0.1% $HgCl_2$ (1 min) + dipping explant after cutting the edges in 0.5 g/L PVP (5 min)	-	-	∗ ∗
S_8: S_0 + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 0.1% $HgCl_2$ (1 min) + dipping explant after cutting the edges in sterile water (30min) + dipping in 500 mg/L PVP (5 min)	-	-	-

1 Contamination : ∗ : 1-2 colonies
∗ ∗ : 3-5 colonies
∗ ∗ ∗ : > 5 colonies

2 Cell death : ∗ : Browning of margins
∗ ∗ : Browning of explants
∗ ∗ ∗ : Blackening of explants

from 65 to 91 per cent with an explant survival rate of 54±00.0% during *in vitro* shoot regeneration of *Ruellia tuberosa* L. Additional treatment of explants with a systemic triazole fungicide, tebuconazole prevented fungal contamination throughout the culture period. It belongs to Demethylase inhibitors (DMI), which interfere in building the structure of fungal cell walls. Finally, it inhibits the reproduction and further growth of the fungus [30]. The use of tebuconazole as an effective systemic fungicide in explant surface sterilisation is rising [31;32]. The reduction in fungal contamination was also observed by Gerolino et al*.* [33] where they used 0.1% (w/v) tebuconazole for 15 min in the case of *Citrus sinensis*, and 100 ppm of tebuconazole found compelling as a sterilant in case of dragon fruit by Chongloi et al*.* [34].

Streptomycin sulphate reduces bacterial contamination since it is a broad-spectrum antibiotic, effective against most gram-negative and a few gram-positive bacteria [35]. It has been used as an antibacterial surface sterilisation agent in plant tissue culture [24;16;25]. Mercuric chloride is used as a disinfectant to remove the surface microorganisms. The result showed that the increasing concentration of mercuric chloride inhibits the efficient growth of bacteria with increasing contact time [36]. Findings of Pandey et al*.* [32], where they observed the minimum (21.76%) bacterial contamination under mercuric chloride ($HgCl_2$ 0.1%), Satheesh and Sridharan [16] used 0.1% $HgCl_2$ for 5 minutes for sterilisation in pomegranate which agrees with the result of Murkute et al*.* [26].

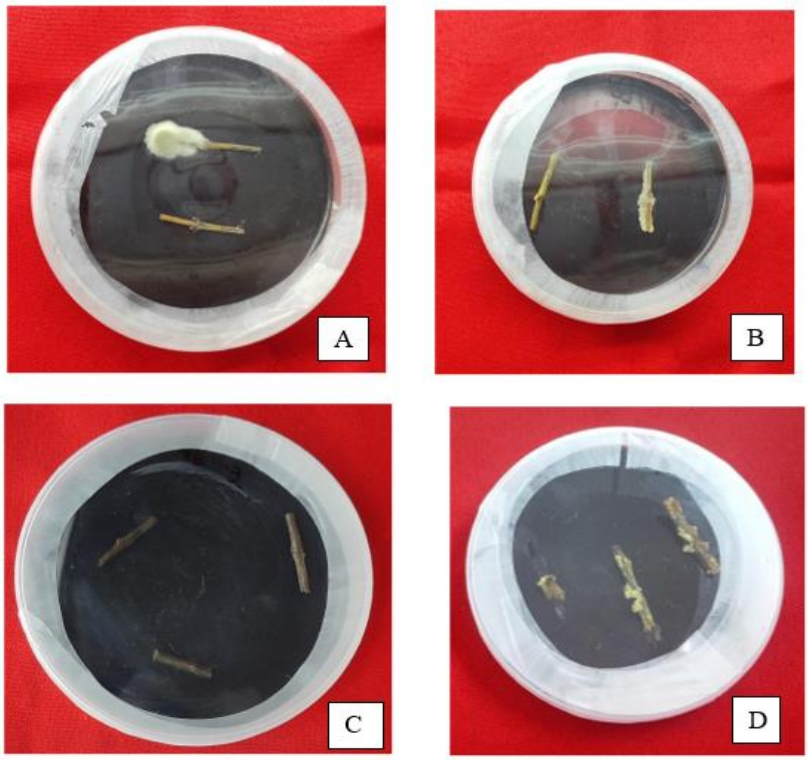

Fig. 2. Microbial contamination and tissue death in the culture plates of pomegranate (A) Fungal contamination (B) Bacterial contamination (C) Browning of explants (D) Nodal segment on MS_0 + 2,4-D 3 mg/L

Guranna et al. [25] also used $HgCl_2$ (100 mg/L) for 1 minute to avoid bacterial contamination.

Minimised contamination and increased survival percentage of pomegranate explants was recorded with the combination (4% $NaOCl_2$ for 7 min + 0.1% $HgCl_2$ for 2 min) [37].

The antioxidant citric acid effectively minimises cell death by reducing the oxidised substrate, preventing the media's and explants' browning. A similar result of minimum cell death was observed using citric acid (150 mg/ L) by Patil et al. [24], and 75mg/L citric acid in the case of guava resulted in a reduction of blackening by 45% [38]. Dipping the explant in 0.5 g/L PVP (5 min) considerably reduced the browning of explants. PVP use is related to the properties of this polymer, which can absorb organic compounds using hydrogen molecules, such as phenols, thereby reducing or avoiding oxidation [39]. The results follow Chavan et al. [40], who have reported that pre-soaking of apical and axillary buds in 0.5% polyvinyl pyrrolidone (PVP) along with 3% sucrose for 30 min found to be effective for browning control in mango. De Assis et al. [41] concluded that the use of PVP, regardless of the concentrations and when used in the presence of 2 g/L of activated charcoal, was observed to reduce the extent of oxidation *in vitro* more than L-cysteine did. Adding PVP significantly reduced the browning percentage to 29.6% in the case of *Paeonia lactiflora* [42].

MS media supplemented with 2,4-D 3 mg/L showed minimum time with higher callus induction and callus weight. This might be because of the potentiality of 2,4-D in increasing the rate of cell division resulting in early callus formation and increased callus induction. However, the response was decreased with an increase in 2,4-D concentration. This may be because auxin was added in high concentrations without cytokinin. Higher concentrations of 2,4-D prevented the growth and development of calli [43]. These results are concordant with the results of Karale et al. [44] in aonla, where white and friable callus was produced within 12 days in MS + 2, 4-D 5 mg/L. In turmeric, 2.5-3.0 mg/L of 2,4-D was found to be optimum for callus induction and callus was initiated within two weeks of culture [45]. Similarly, Savita et al. [46]

reported maximum callus induction of 98.66% from leaf segments on MS medium supplemented with four mg/L 2,4-D in *Citrus jambhiri* and *Citrus sinensis* [47].

5. CONCLUSION

By considering the absence of microbial contamination and reduced cell death through visual observation, S_8 [1-2 drops of tween 20 (20 min) + 500 mg/L carbendazim (30 min) + 500 mg/L streptomycin sulphate (10 min) + 100 mg/L citric acid (30 min) + 100 mg/L tebuconazole (30 min) + 0.1% $HgCl_2$ (1 min) + dipping the explant after cutting the edges in sterile water (30 min) + dipping in 500 mg/L PVP (5 min)] was considered to be optimum in sterilisation of pomegranate explants and MS_0 + 3 mg/L 2,4-D media for the development of yellowish green, friable calli from nodal segments.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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