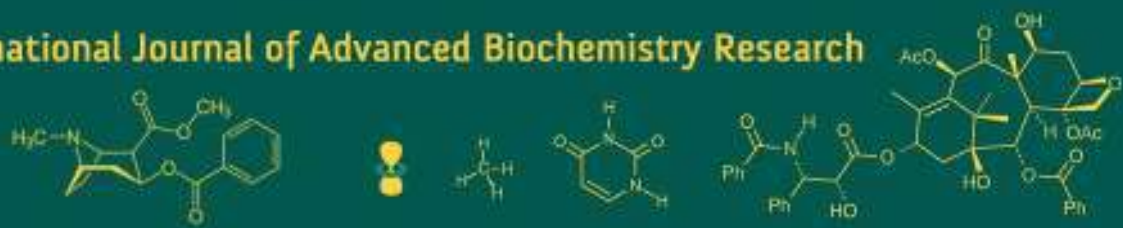


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Genotype-specific optimization of surface sterilization for axenic culture establishment in citrus micropropagation

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Abstract

Establishing contamination-free cultures remains a critical bottleneck in citrus micropropagation due to persistent microbial load and stress-induced explant browning. The present study aimed to optimize surface sterilization protocols by evaluating their efficiency in controlling microbial contamination while minimizing physiological damage across citrus genotypes. Shoot tips and nodal segments of three genotypes, Konkani Seedless (*Citrus limon*) (A1), Kaji Nemu (*Citrus limon*) (A2) and Sai Sharbati (*Citrus aurantifolia*) (A3), were subjected to four sterilization treatments (ST₁-ST₄) comprising graded concentrations of cefotaxime, streptomycin, kasugamycin, bavistin and HgCl₂ (0.1%) in a factorial completely randomized design. Explants were cultured on Murashige and Skoog (MS) basal medium and responses were assessed in terms of bacterial, fungal and total contamination, along with browning percentage. Significant genotype- and treatment-dependent variations were observed. ST₄ was most effective in reducing bacterial (6.829%) and total contamination (13.376%), whereas ST₃ showed superior control over fungal contamination (3.51%). However, increased sterilant intensity led to a marked rise in explant browning, indicating a trade-off between sterilization efficiency and tissue viability. Among the genotypes, Kaji Nemu exhibited the lowest contamination and browning, suggesting higher adaptability to *in vitro* conditions, while Sai Sharbati showed maximum browning, reflecting greater sensitivity to sterilization-induced stress. The observed browning is likely associated with enhanced phenolic oxidation triggered by chemical stress and tissue injury during sterilization. The findings demonstrate that successful axenic culture establishment in citrus depends on achieving a genotype-specific balance between microbial decontamination and physiological stability of explants. This study provides a refined framework for optimizing sterilization strategies to improve *in vitro* establishment efficiency in citrus micropropagation.

Keywords: Citrus micropropagation, axenic culture, surface sterilization, phenolic browning, genotype response, microbial contamination

1. Introduction

Citrus, a genus under the family Rutaceae, is one of the most economically important fruit crops cultivated worldwide. The centre of origin of Citrus is considered to be the tropical and subtropical regions of Southeast Asia, particularly northeastern India, Myanmar and the Yunnan province of China [1, 2]. Citrus fruits such as oranges, lemons, limes, mandarins, grapefruit and pomelo contribute significantly to global fruit production and human nutrition [3]. Among these, *Citrus limon* is widely cultivated due to its high economic and medicinal value [4]. India is one of the leading producers of Citrus fruits; however, productivity is often constrained by the limited availability of healthy and disease-free planting material [5].

Plant tissue culture offers an efficient alternative for the rapid multiplication of elite and disease-free planting material. *in vitro* propagation techniques enable the production of genetically uniform plants under controlled conditions, independent of seasonal limitations [6-8]. Recent studies have further emphasized that micropropagation allows large-scale multiplication of high-quality planting material with improved phytosanitary status and uniformity, making it a reliable tool in modern agriculture and horticulture [9, 10]. Plant tissue culture has gained importance for continuous, year-round production and for overcoming limitations associated with conventional propagation methods [10-13]. Since the pioneering concept of cellular totipotency, plant tissue culture has evolved into a vital tool

for crop improvement, conservation and large-scale propagation [13]. These approaches overcome several limitations of conventional propagation, including slow multiplication rate, heterozygosity and disease transmission [14, 7]. Developing optimized and reproducible *in vitro* protocols is therefore essential for large-scale commercial micropropagation and for ensuring the supply of pathogen-free planting material in citrus-based horticultural industries [15-17].

Despite the advantages of micropropagation, the successful establishment phase remains highly vulnerable to microbial contamination and physiological stress. In Citrus, the establishment of axenic cultures is a critical prerequisite for successful *in vitro* propagation, as explants derived from field-grown plants are frequently associated with diverse microbial contaminants, including bacteria and fungi [18, 19]. Techniques such as shoot tip culture, nucellar embryogenesis and micrografting not only facilitate clonal propagation but also play an important role in obtaining pathogen-free and aseptic cultures [10, 20, 21]. However, the success of these methods largely depends on the efficiency of surface sterilization and contamination control during culture initiation.

Various sterilants, including fungicides, antibiotics and disinfectants, have been employed to minimize both surface and endogenous contamination without compromising explant viability [22, 23]. Recent studies have emphasized the optimization of sterilization protocols involving combinations of chemical treatments and exposure durations to achieve higher aseptic culture establishment in Citrus [24, 25]. Furthermore, the use of antimicrobial agents and improved disinfection strategies has significantly enhanced the establishment of contamination-free cultures, thereby overcoming major limitations of conventional propagation such as disease transmission and low multiplication efficiency [26, 27]. However, increasing sterilant intensity often induces physiological stress in explants, leading to cellular injury and enhanced production of reactive oxygen species (ROS), resulting in oxidative stress and subsequent phenolic exudation and browning. Browning in explants is primarily associated with the oxidation of phenolic compounds mediated by enzymes such as polyphenol oxidase and peroxidase, ultimately impairing explant viability and culture establishment.

Despite considerable progress in Citrus tissue culture, a critical knowledge gap persists in understanding the trade-off between effective microbial decontamination and sterilization-induced physiological damage, particularly phenolic browning, across different genotypes [23, 28, 29]. Differences in endogenous microbial load, tissue sensitivity and phenolic metabolism among genotypes further complicate the development of universally applicable sterilization protocols. Efficient and reproducible sterilization strategies tailored to specific Citrus genotypes are still lacking, which limits large-scale application and commercial scalability. Such optimization is critical for improving the reproducibility and commercial scalability of citrus micropropagation systems. Therefore, the present study aimed to systematically evaluate different surface sterilization treatments to identify an optimal, genotype-specific balance between microbial decontamination and explant physiological stability, thereby improving the efficiency of axenic culture establishment in citrus.

2. Materials and methods.

The experiment was conducted at the Centre for Advanced Research in Plant Tissue Culture (CARPTC), Department of Agricultural Biotechnology, Anand Agricultural University, Anand, Gujarat, India, under controlled laboratory conditions.

2.1 Plant Material and Explant Preparation

Healthy, disease-free mother plants of three citrus genotypes, namely Konkan Seedless (A1), Kaji Nemu (A2) and Sai Sharbati (A3), were selected as the source of explants. Actively growing shoot tips and nodal segments were excised and transferred to the laboratory for further processing. Explants of approximately 1.0-1.5 cm in length were used to ensure uniformity across treatments. The explants were initially washed under running tap water for 15-20 min to remove surface debris, followed by treatment with a mild detergent solution (few drops of liquid detergent in distilled water) and subsequently rinsed thoroughly with distilled water.

2.2 Surface Sterilization Treatments

Surface sterilization of explants was carried out using different combinations of antibacterial and antifungal agents to establish axenic cultures. Four sterilization treatments (ST₁-ST₄) were evaluated, comprising varying concentrations of cefotaxime, streptomycin, kasugamycin, bavistin and mercuric chloride (HgCl₂, 0.1%). The overall workflow of surface sterilization and explant preparation is illustrated in (Figure 1).



Fig 1: Surface sterilization workflow and *in vitro* culture establishment of Citrus explants

(a) Preparation of sterilization solutions with varying concentrations of antibiotics and fungicide; (b) Sterilant treatment phase illustrating antimicrobial interaction and decontamination process; (c) Excised shoot tip and nodal explants following surface sterilization; (d) Inoculation of sterilized explants into culture vessels under aseptic conditions for initiation of *in vitro* cultures.

All sterilization procedures were carried out under aseptic conditions in a laminar airflow cabinet previously sterilized with 70% ethanol and UV exposure for 20-30 min. Following chemical treatment, explants were rinsed 3-4 times with sterile distilled water to remove residual sterilants prior to inoculation. The detailed composition and exposure duration of sterilization treatments for shoot tips and nodal segments are presented in (Table 1).

Table 1: Mechanistic composition and exposure profile of surface sterilization treatments (ST₁-ST₄) used for axenic culture establishment in Citrus explants

Treatment	Sterilant	Functional role	Target contaminant	Concentration	Exposure time (min)-Shoot tips	Exposure time (min)-Nodal segments
ST ₁	Cefotaxime	Antibiotic	Gram-negative bacteria	500 mg L ⁻¹	15	17
	Streptocycline	Antibiotic	Broad-spectrum bacteria	500 mg L ⁻¹	15	17
	Kasugamycin	Antibiotic	Protein synthesis inhibitor (bacteria)	2 mL L ⁻¹	15	17
	Bavistin	Systemic fungicide	Fungal pathogens	1000 mg L ⁻¹	18	20
	HgCl ₂	Surface disinfectant	Surface microbes	0.1%	2	4
ST ₂	Cefotaxime	Antibiotic	Gram-negative bacteria	750 mg L ⁻¹	13	15
	Streptocycline	Antibiotic	Broad-spectrum bacteria	750 mg L ⁻¹	13	15
	Kasugamycin	Antibiotic	Protein synthesis inhibitor (bacteria)	3 mL L ⁻¹	13	15
	Bavistin	Systemic fungicide	Fungal pathogens	1500 mg L ⁻¹	16	17
	HgCl ₂	Surface disinfectant	Surface microbes	0.1%	2	4
ST ₃	Cefotaxime	Antibiotic	Gram-negative bacteria	1000 mg L ⁻¹	12	13
	Streptocycline	Antibiotic	Broad-spectrum bacteria	1000 mg L ⁻¹	12	13
	Kasugamycin	Antibiotic	Protein synthesis inhibitor (bacteria)	4 mL L ⁻¹	12	13
	Bavistin	Systemic fungicide	Fungal pathogens	2000 mg L ⁻¹	15	15
	HgCl ₂	Surface disinfectant	Surface microbes	0.1%	2	4
ST ₄	Cefotaxime	Antibiotic	Gram-negative bacteria	1000 mg L ⁻¹	10	12
	Streptocycline	Antibiotic	Broad-spectrum bacteria	1000 mg L ⁻¹	10	12
	Kasugamycin	Antibiotic	Protein synthesis inhibitor (bacteria)	5 mL L ⁻¹	10	12
	Bavistin	Systemic fungicide	Fungal pathogens	2000 mg L ⁻¹	12	14
	HgCl ₂	Surface disinfectant	Surface microbes	0.1%	2	4

2.3 Culture Medium and Growth Conditions

Following surface sterilization, the explants (shoot tips and nodal segments) were aseptically inoculated onto Murashige and Skoog (MS) basal medium and maintained under controlled culture conditions for establishment. The culture medium was prepared using deionized distilled water by dissolving appropriate stock solutions and supplemented with 3% (w/v) sucrose as a carbon source. The pH of the medium was adjusted to 5.75 prior to the addition of 0.8% (w/v) agar for solidification. The medium was sterilized by autoclaving at 121 °C and 15 psi for 15 minutes. No plant growth regulators were added during the establishment phase. The cultures were incubated under controlled environmental conditions at 25±2 °C with a 16 h photoperiod and light intensity of approximately 40-50 μmol m⁻² s⁻¹ [30]. Cultures were monitored regularly for contamination and physiological responses.

2.4 Experimental Design and Data Recording

The experiment was laid out in a Factorial Completely Randomized Design (FCRD) with two factors: genotype (A1, A2, A3) and surface sterilization treatments (ST₁-ST₄), each replicated three times. Each replication consisted of 10-15 explants per treatment. Observations were recorded after 14 days of culture incubation to evaluate the effectiveness of sterilization treatments across the genotypes. The parameters assessed included percentage of bacterial contamination, fungal contamination, total contamination and browning percentage of explants. Contamination was identified based on visible microbial growth, including bacterial turbidity and fungal mycelial development, whereas browning was assessed based on

phenolic discoloration of explants and the surrounding medium.

2.5 Statistical Analysis

The recorded data were subjected to analysis of variance (ANOVA) appropriate for a factorial design to determine the significance of genotype, sterilization treatments and their interaction effects. Mean comparisons were performed using the critical difference (C.D.) test at the 5% significance level. Statistical analysis was carried out using standard statistical software suitable for factorial experimental designs.

3. Results and Discussion

The effectiveness of different surface sterilization treatments was evaluated across citrus genotypes based on microbial contamination and browning responses of explants cultured on MS medium. The results demonstrated significant variation among genotypes, treatments and their interactions, highlighting the importance of optimizing sterilization strategies for successful *in vitro* establishment.

3.1 Bacterial Contamination of Explants

Bacterial contamination was significantly influenced by both genotype and surface sterilization treatments. As shown in (Table 2), among the genotypes, A1 (Konkan Seedless) recorded the highest mean bacterial contamination (23.553%), followed by A3 (Sai Sharbati) (20.294%), whereas A2 (Kaji Nemu) exhibited the lowest contamination (16.16%). This variation suggests genotype-dependent differences in endogenous microbial load and tissue susceptibility.

Table 2. Effect of genotype and surface sterilization treatments on bacterial contamination (%) in Citrus explants

Genotype	ST ₁	ST ₂	ST ₃	ST ₄	Mean A
A1 (Konkan Seedless)	42.733	27.977	16.690	6.810	23.553
A2 (Kaji Nemu)	13.980	34.103	7.447	9.110	16.160
A3 (Sai Sharbati)	38.810	13.977	23.823	4.567	20.294
Mean B	31.841	25.352	15.987	6.829	—
Statistical parameters					
Source	C.D. ($p \leq 0.05$)		SE(d)	SE(m)	
Genotype (A)	1.218		0.587	0.415	
Treatment (B)	1.406		0.677	0.479	
A × B	2.436		1.173	0.830	

Note: Values represent mean percentage of bacterial contamination. A = Genotype; B = Sterilization treatment. C.D. = Critical difference at 5% significance level.

Among the sterilization treatments, ST₁ recorded the highest contamination (31.841%), while ST₄ was the most effective, exhibiting the lowest contamination (6.829%), followed by ST₃ (15.987%) and ST₂ (25.352%). The progressive reduction in contamination from ST₁ to ST₄ clearly indicates that increasing concentrations of antibacterial agents enhanced microbial suppression. A significant interaction effect between genotype and treatment was observed. The highest contamination was recorded in A1 under ST₁ (42.733%), whereas the lowest was observed in A3 under ST₄ (4.567%), followed by A1 under ST₄ (6.81%) and A2 under ST₃ (7.447%). The superior performance of ST₄ may be attributed to the synergistic action of high concentrations of broad-spectrum antibiotics, which effectively suppressed both surface-associated and endogenous bacterial populations. The statistical analysis further confirmed the significance of these differences, as indicated by the C.D.

values for genotype (1.218), treatment (1.406) and their interaction (2.436). These findings highlight the importance of genotype-specific optimization of sterilization protocols to minimize bacterial contamination during culture establishment.

3.2 Fungal Contamination of Explants

Fungal contamination was significantly affected by both genotype and surface sterilization treatments. As presented in (Table 3), fungal contamination among the genotypes, A1 (Konkan Seedless) exhibited the highest fungal contamination (14.087%), followed by A3 (Sai Sharbati) (11.199%), whereas A2 (Kaji Nemu) showed the lowest contamination (10.103%). These differences may be attributed to variation in surface microflora and tissue characteristics among genotypes.

Table 3: Effect of genotype and surface sterilization treatments on fungal contamination (%) in Citrus explants

Genotype	ST ₁	ST ₂	ST ₃	ST ₄	Mean A
A1 (Konkan Seedless)	29.233	18.540	5.560	3.013	14.087
A2 (Kaji Nemu)	16.863	15.393	0.887	7.267	10.103
A3 (Sai Sharbati)	14.120	17.233	4.083	9.360	11.199
Mean B	20.072	17.056	3.510	6.547	—
Statistical parameters					
Source	C.D. ($p \leq 0.05$)		SE(d)	SE(m)	
Genotype (A)	0.630		0.303	0.215	
Treatment (B)	0.727		0.350	0.248	
A × B	1.260		0.607	0.429	

Note: Values represent mean percentage of fungal contamination. A = Genotype; B = Sterilization treatment. C.D. = Critical difference at 5% significance level.

Among the sterilization treatments, ST₁ recorded the highest fungal contamination (20.072%), whereas ST₃ was the most effective, exhibiting the lowest contamination (3.51%), followed by ST₄ (6.547%) and ST₂ (17.056%). The superior performance of ST₃ can be attributed to higher concentrations of bavistin, a systemic fungicide that inhibits fungal cell division and spore germination. A significant interaction between genotype and treatment was observed. The highest contamination was recorded in A1 under ST₁ (29.233%), whereas the lowest was observed in A2 under ST₃ (0.887%). This indicates that antifungal efficiency depends on both treatment intensity and genotype-specific responses. The statistical analysis further confirmed the significance of these differences, as indicated by the C.D. values for genotype (0.63), treatment (0.727) and their

interaction (1.26). These findings emphasize that appropriate selection of sterilization treatments is critical for effective fungal contamination control during culture establishment.

3.3 Total Contamination of Explants

Total contamination, representing the combined effect of bacterial and fungal contamination, was significantly influenced by genotype and surface sterilization treatments. (Table 4) shows among the genotypes, A1 (Konkan Seedless) recorded the highest total contamination (37.639%), followed by A3 (Sai Sharbati) (31.493%), whereas A2 (Kaji Nemu) exhibited the lowest contamination (26.263%). These results indicate genotype-dependent differences in susceptibility to microbial contamination as shown in (Figure 2).

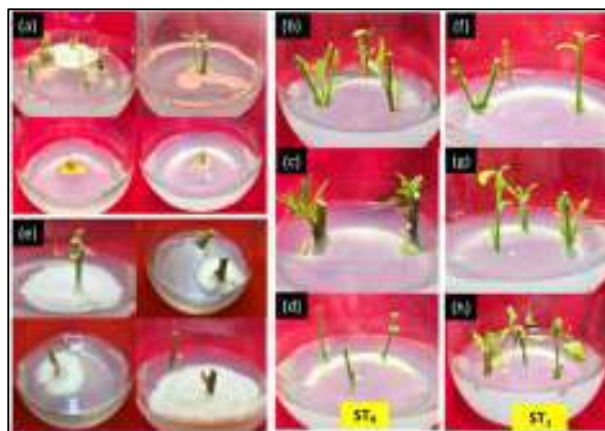
Table 4: Effect of genotype and surface sterilization treatments on total contamination (%) in Citrus explants

Genotype	ST ₁	ST ₂	ST ₃	ST ₄	Mean A
A1 (Konkan Seedless)	71.967	46.517	22.250	9.823	37.639
A2 (Kaji Nemu)	30.843	49.497	8.333	16.377	26.263
A3 (Sai Sharbati)	52.930	31.210	27.907	13.927	31.493
Mean B	51.913	42.408	19.497	13.376	—
Statistical parameters					
Source	C.D. ($p \leq 0.05$)	SE(d)	SE(m)		
Genotype (A)	1.531	0.737	0.521		
Treatment (B)	1.767	0.851	0.602		
A × B	3.061	1.474	1.043		

Note: Values represent mean percentage of total contamination. A = Genotype; B = Sterilization treatment. C.D. = Critical difference at 5% significance level.

Among the sterilization treatments, ST₁ exhibited the highest contamination (51.913%), whereas ST₄ was the most effective, exhibiting the lowest contamination (13.376%), followed by ST₃ (19.497%) and ST₂ (42.408%). The improved performance of ST₄ indicates its effectiveness in simultaneously controlling both bacterial and fungal contaminants. A significant interaction between genotype and treatment was observed. The highest contamination was recorded in A1 under ST₁ (71.967%), whereas the lowest was observed in A2 under ST₃ (8.333%) and A1 under ST₄ (9.823%). These results demonstrate that both genotype and treatment combinations play a crucial role in determining overall contamination levels. The statistical analysis further confirmed significant differences, as indicated by the C.D. values for genotype (1.531), treatment (1.767) and their interaction (3.061). Genotype-dependent variation in contamination has been reported due to differences in endogenous microbial load and tissue response to sterilants [25, 27, 31]. Additionally, the effectiveness of sterilization treatments depends on the type, concentration and duration of disinfectants, requiring careful optimization to balance

contamination control and explant viability (Cassells, 1991). These findings emphasize the need for optimized sterilization protocols during *in vitro* culture establishment.

**Fig 2:** Representative responses of Citrus explants under different surface sterilization treatments during *in vitro* culture establishment

a) Bacterial contamination observed in explants, (b-d) successful aseptic culture establishment under ST₄ in Konkan Seedless, Kaji Nemu, and Sai Sharbati, respectively, (e) fungal contamination, and (f-h) successful aseptic culture establishment under ST₃ in Konkan Seedless, Kaji Nemu, and Sai Sharbati, respectively.

3.4 Browning Response of Explants

The extent of browning varied significantly among genotypes and surface sterilization treatments. As shown in (Table 5), reflecting differences in physiological responses of explants under *in vitro* conditions. Among the genotypes, A3 (Sai Sharbati) exhibited the highest browning (23.792%), followed by A1 (Konkan Seedless) (14.117%), whereas A2 (Kaji Nemu) showed the lowest browning (7.292%). This variation may be attributed to differences in phenolic content and their oxidation behavior among genotypes.

Table 5: Effect of genotype and surface sterilization treatments on browning (%) of Citrus explants

Genotype	ST ₁	ST ₂	ST ₃	ST ₄	Mean A
A1 (Konkan Seedless)	5.633	8.133	19.233	23.467	14.117
A2 (Kaji Nemu)	0.667	2.433	14.900	11.167	7.292
A3 (Sai Sharbati)	17.467	13.100	29.333	35.267	23.792
Mean B	7.922	7.889	21.156	23.300	—
Statistical parameters					
Source	C.D. ($p \leq 0.05$)	SE(d)	SE(m)		
Genotype (A)	0.611	0.294	0.208		
Treatment (B)	0.706	0.340	0.240		
A × B	1.223	0.589	0.416		

Note: Values represent mean percentage of browning. A = Genotype; B = Sterilization treatment. C.D. = Critical difference at 5% significance level.

With respect to treatments, browning was lowest under ST₁ (7.922%) and ST₂ (7.889%), whereas it increased significantly under ST₃ (21.156%) and ST₄ (23.300%). This trend indicates that higher sterilization intensity, although effective in reducing contamination, induces physiological stress in explants, leading to increased phenolic exudation and subsequent browning. A significant interaction between genotype and treatment was observed. Maximum browning was recorded in A3 under ST₄ (35.267%), followed by A3

under ST₃ (29.333%), whereas the lowest browning was observed in A2 under ST₁ (0.667%). These results highlight genotype-specific sensitivity to sterilization-induced oxidative stress (Figure 3). The statistical analysis further confirmed the significance of these differences, as indicated by the C.D. values for genotype (0.611), treatment (0.706) and their interaction (1.223). These findings emphasize the need to balance sterilization efficiency with physiological stability to ensure successful culture establishment.

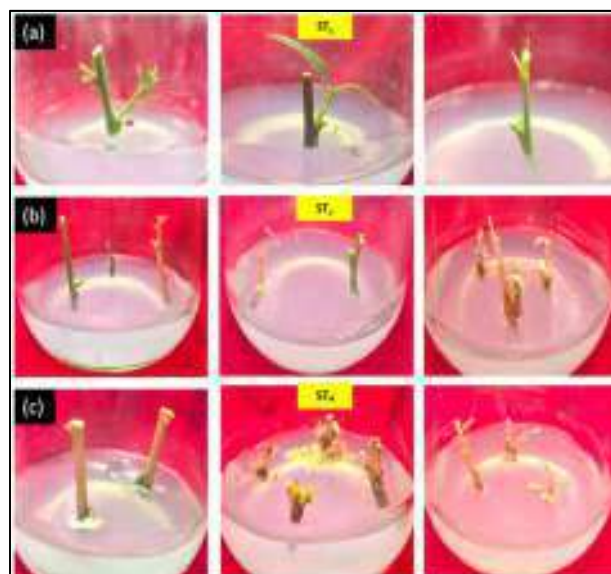


Fig 3: Browning response of Citrus explants under different surface sterilization treatments

(a) Low or negligible browning under ST₁, (b) moderate browning under ST₂, and (c) severe phenolic browning under ST₄ in Konkan Seedless, Kaji Nemu, and Sai Sharbati.

3.5 Integrated Interpretation: Trade-off Between Contamination Control and Explant Viability

The variation in contamination and browning observed in the present study can be directly linked to differences in sterilant concentration and exposure across treatments (Tables 2-5). Increasing concentrations of cefotaxime, streptomycin and kasugamycin from ST₁ to ST₄ enhanced bacterial control due to their broad-spectrum antibacterial activity, as reflected in reduced contamination levels (Table 2). Similarly, higher concentrations of bavistin in ST₃ and ST₄ effectively reduced fungal contamination by inhibiting fungal growth and spore germination (Table 3). However, increased sterilization intensity also resulted in higher browning (Table 5), likely due to chemical stress and enhanced production of reactive oxygen species, leading to oxidation of phenolic compounds. Although HgCl₂ was used at a constant concentration, its strong disinfectant nature, in combination with other sterilants, may have contributed to cellular injury and oxidative stress.

These results clearly demonstrate a trade-off between microbial decontamination and explant viability. While higher sterilization treatments (ST₃ and ST₄) were more effective in reducing contamination (Table 4), they simultaneously increased browning, thereby affecting explant survival. Similar trends have been reported in earlier studies, where excessive sterilization resulted in tissue damage and reduced regeneration efficiency [32, 33]. Furthermore, genotypic variation in response to sterilization treatments indicates that explant physiology, including phenolic metabolism and stress tolerance, plays a crucial role in determining culture establishment success. These findings are consistent with previous reports emphasizing genotype-specific responses in plant tissue culture [34]. Overall, the study highlights the importance of optimizing sterilization protocols to achieve a balance between effective contamination control and minimal physiological damage, which is essential for successful establishment of axenic cultures in citrus micropropagation.

3.6 Mechanistic Insights into Sterilization-Induced Responses

The differential response of explants to sterilization treatments can be explained by the mode of action of the sterilizing agents used. Cefotaxime and streptomycin are broad-spectrum antibiotics that inhibit bacterial cell wall synthesis and protein synthesis, respectively, thereby effectively reducing bacterial contamination [23, 35, 36]. Kasugamycin, a known inhibitor of protein synthesis in prokaryotes, further enhances antibacterial efficacy by suppressing microbial proliferation at early stages of culture establishment [37-39]. Similarly, bavistin (carbendazim), a systemic fungicide, disrupts fungal cell division by interfering with microtubule assembly, thereby preventing fungal growth and spore germination [40, 41]. The improved fungal control observed in higher treatments (ST₃ and ST₄) can therefore be attributed to increased fungicidal activity. However, the combined effect of these chemicals, particularly at higher concentrations, can lead to phytotoxicity. Mercuric chloride (HgCl₂), although highly effective as a surface disinfectant, is known to cause cellular toxicity by disrupting membrane integrity and enzyme activity [42, 43]. In combination with antibiotics and fungicides, this may result in cellular injury, triggering stress responses in plant tissues. One of the primary consequences of such stress is the enhanced production of reactive oxygen species (ROS), which leads to oxidative stress within explant tissues [44, 45]. This oxidative stress induces the activation of enzymes such as polyphenol oxidase (PPO) and peroxidase, resulting in the oxidation of phenolic compounds into quinones [46, 47]. These compounds subsequently polymerize to form brown pigments, leading to tissue browning and reduced explant viability.

3.7 Genotype-Specific Physiological Responses

The observed variation among genotypes indicates that explant response is strongly influenced by inherent physiological and biochemical characteristics. The lower contamination and browning observed in A2 (Kaji Nemu) suggest better tolerance to sterilization stress, possibly due to lower endogenous microbial load and reduced phenolic exudation. In contrast, A3 (Sai Sharbati), which exhibited higher browning, may possess higher levels of phenolic compounds or greater sensitivity to oxidative stress. Such genotype-dependent responses have been widely reported in plant tissue culture, where differences in phenolic metabolism, antioxidant capacity and tissue structure significantly influence *in vitro* establishment success.

3.8 Practical Implications for Citrus Micropropagation

The findings of the present study have important practical implications for large-scale citrus micropropagation. While higher sterilization treatments (ST₃ and ST₄) effectively reduce microbial contamination, they also increase the risk of explant browning and tissue damage. Therefore, selecting an appropriate sterilization treatment requires balancing microbial control with explant physiological stability. Based on the results, ST₃ may be considered optimal for fungal control with moderate browning, whereas ST₄ is most effective for bacterial control but associated with higher browning. Therefore, a combined or modified approach, including the use of antioxidants such as ascorbic acid or polyvinylpyrrolidone (PVP), may further improve culture establishment by reducing oxidative browning [48, 49].

3.9 Future Perspectives

Future studies should focus on integrating antioxidant treatments with optimized sterilization protocols to mitigate browning while maintaining effective contamination control. Additionally, exploring alternative, less phytotoxic sterilizing agents such as sodium hypochlorite or silver nanoparticles may provide improved outcomes. Molecular-level studies investigating oxidative stress markers, antioxidant enzyme activity and phenolic metabolism could further enhance understanding of genotype-specific responses and contribute to the development of more efficient and reproducible micropropagation protocols in Citrus.

4. Conclusion

The present study demonstrated that surface sterilization treatments significantly influence bacterial, fungal and total contamination, as well as browning responses of citrus explants, thereby playing a critical role in successful *in vitro* establishment. Higher concentrations of antibiotics (cefotaxime, streptocycline and kasugamycin) and fungicide (bavistin) effectively reduced microbial contamination, with ST₄ showing the lowest bacterial and total contamination and ST₃ being most effective against fungal contamination. However, increased sterilization intensity also resulted in enhanced browning due to stress-induced phenolic oxidation, indicating a clear trade-off between microbial decontamination and explant viability. Among the genotypes, Kaji Nemu (A2) exhibited lower contamination and browning, suggesting better adaptability for *in vitro* culture, whereas Konkani Seedless (A1) showed higher contamination and Sai Sharbati (A3) exhibited maximum browning, reflecting greater sensitivity to sterilization-induced stress.

Although HgCl₂ (0.1%) ensured effective surface disinfection, its interaction with other sterilants may have contributed to cellular injury and oxidative stress. Overall, the findings highlight that successful citrus micropropagation depends on genotype-specific optimization of sterilization protocols to achieve an appropriate balance between effective contamination control and minimal physiological damage. Future studies should focus on integrating antioxidant treatments and alternative, less phytotoxic sterilizing agents to further improve explant survival and enhance the efficiency of axenic culture establishment.

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6. Author Contributions

G.B.P. and S.M. conceptualized and supervised the study. K.C. conducted the experiments and collected the data. A.M.P. and A.S.P. performed data analysis, prepared the manuscript, and assisted in experimental work and data compilation. All authors reviewed and approved the final manuscript.

7. Conflict of Interest

The authors declare no conflict of interest.

8. Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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