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Optimization of a Mercury-free Surface Sterilization Protocol for Dried Sorghum Grains (Sorghum bicolor [L.] Moench): Balancing Contamination Control and Explant Viability

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Abstract

Introduction: Aseptic culture establishment is essential for plant tissue culture and genetic transformation. In sorghum (*Sorghum bicolor* [L.] Moench), seed-associated microorganisms frequently compromise culture initiation and reduce regeneration efficiency. Sodium hypochlorite (NaOCl) is widely employed as a disinfectant, but optimal application conditions for dried sorghum grains remain poorly characterized.

Methods: This study investigated the interaction between NaOCl concentration and exposure duration on contamination and explant survival. Sixteen treatment combinations of four NaOCl concentrations (20%, 30%, 40%, and 50% commercial Clorox®) and four exposure durations (10, 15, 20, and 25 min) were tested in a factorial randomized complete block design with fifteen explants per treatment (240 total). Sterilized grains were cultured on hormone-free Murashige and Skoog medium and evaluated over two weeks using two-way ANOVA and binary logistic regression.
Results: Both concentration and exposure time had a statistically significant effect on contamination and viability, with a statistically significant interaction between them. 20% NaOCl for 25 min was the optimal condition with zero contamination (0.067 ± 0.064) and 100% viability (1.000 ± 0.000). High concentrations yielded greater inhibitory effects but decreased viability. The optimum conditions as estimated from logistic regression were near 21.6% NaOCl and 24.3 minutes, near the optimal empirical conditions.

Conclusion: The results indicate that the use of concentrated disinfectants or mercury-based compounds is not necessary for good sorghum grain disinfection. The use of moderate concentrations of sodium hypochlorite for relatively long periods offers acceptable antimicrobial activity and low phytotoxicity. The protocol outlined is viable and eco-friendly for sorghum tissue culture and could be useful for improving the future regeneration and transformation of this economically important crop.

Key message

Extended exposure (25 min) to moderate sodium hypochlorite concentration (20%) achieves optimal sterilization of dried sorghum grains with complete explant viability, providing an effective mercury-free alternative for tissue culture applications.

1. Introduction

Sorghum (*Sorghum bicolor* [L.] Moench) is one of the toughest cereals grown in the world's arid and semi-arid zones. Farmers in Africa, Asia, and the Americas keep planting it for a simple reason: it survives when other crops don't. Long dry spells, punishing heat, poor sandy soils, heavy waterlogged clays --- sorghum tolerates all of it reasonably well, if not always equally (Abreha et al. 2021). For generations it has fed both people and animals, and in many dryland communities it's still the crop a household's food security depends on.

Its role has expanded lately, though. Processors now turn sorghum grain into biofuel and bioplastic feedstocks, and agronomists increasingly point to it as a crop that can hold up as the climate shifts. This growing interest hasn't gone unnoticed by breeders. Sorghum breeding and biotechnology programs have picked up pace, with work underway to stabilize yields, boost iron and zinc content in the grain, and build tolerance to the kind of double stress; heat combined with low soil nitrogen, that is becoming more common in the field.

Plant tissue culture and genetic transformation systems now extensively adopted in plant improvement, and breakthroughs in application of these techniques in sorghum's functional genomics, gene editing and molecular breeding were demonstrated by Kumar et al. (2022) and Wang et al. (2023), despite both studies simultaneously confirm sorghum as one of the relatively difficult cereal crops for its transformation and tissue culture establishment, the latter also indicates limitations in establishment, efficiency and subsequent downstream transformation due to constraints of tissue in aseptic culture. Despite this, recently reported sorghum transformation techniques, i.e., establishment of genotype-independent protocols using seedling leaf whorls, have been innovated; however, tissue browning and accumulation of phenolic compounds are still serious roadblocks to high success, even with optimal sterilization and suboptimal culture of the explants (Lee and Wang 2025). Similarly, optimized protocols for embryogenic callus induction on immature inflorescences in some cultivars may achieve up to 96% embryogenic callus induction. Still, these protocols rely completely on clean and physiologically sound raw materials (Thaddi 2025).

The establishment of aseptic culture is one of the most critical steps in plant tissue culture because even the introduction of very small amounts of contaminants will negatively impact the entire downstream workflow, such as regeneration, transformation, and micropropagation. Microbes compete with explants for medium nutrients, change the contents of the medium through their metabolites, and secrete growth-inhibiting compounds or compounds that are, in fact, toxins, as described by Cassells (2001). Even if it is only for one single visit, large portions of experimental materials will be contaminated, and reproducibility will be affected.

Contamination in sorghum tissue culture traces back to diverse bacterial and fungal communities associated with seeds and vegetative tissues. Beacorn and Thiessen (2021) identified seed-borne fungi, particularly species within *Fusarium* and allied genera, as major obstacles to successful culture establishment, noting that these organisms may colonize external seed surfaces or internal tissues and persist despite standard sterilization. Surface-associated bacteria and fungi are generally introduced into the field at harvest, during processing, or during storage, and endophytes are internal bacteria and fungi that may not be detected until culture is started. Epiphytic microorganisms remain exposed to sterilizing agents and are generally susceptible to surface disinfection. Endophytes, which live in the spaces between cells and in vascular tissues, are well protected from many disinfectants. Contamination observed during culture may therefore reflect not sterilization failure, but the proliferation of latent populations triggered by the physiological stress imposed on the explants. Contamination levels are further modulated by cultivar, environmental conditions, storage duration, and seed-handling practices,

factors that collectively explain why protocols optimized in one setting often behave inconsistently elsewhere (Ahiakpa et al. 2020).

The sterilization step also poses a problem. Disinfectants must be able to kill contaminants in microbial pollution without harming the explant to a degree that weakens its regeneration capacity. Studies have shown that, in certain cases, a method effective against contamination is not effective for explant survival (Cassells and Curry 2001). Sodium hypochlorite is a commonly used surface sterilant due to its low cost, widespread availability, and excellent effectiveness against most microorganisms, as it disorganizes proteins, genetic material, and surfaces through oxidative action. Its mechanism of disinfection depends on the production of hypochlorous acid and reactive oxidative species (ROS), which denature proteins, nucleic acids, and cellular membranes (Reed et al. 2013). However, this substance is highly species-specific; its efficacy is proportional to concentration, and treatment efficiency in one crop may prove ineffective in another. The species dependency has been demonstrated by Reed et al. (2013), emphasizing the importance of each species-specific optimal condition. Thus, Oyebanji et al. (2009) found it efficient for contamination control in sorghum but point out one important fact: the impossibility of obtaining good yield at the high doses required to eliminate contaminants. More recently, Febyanti et al. (2025) carried out an appropriate sterilisation procedure for sorghum seed explants, achieving a contamination of 6.67% when using a combination of 70% alcohol, 25% Clorox, and 3% NaOCl. According to Bock et al. (2025), a very simple and effective protocol for sterilisation of explants using a two-step process in immature sorghum embryos has been successfully established using only ethanol and sodium hypochlorite.

The overall goal of tissue culture is the establishment of healthy explants that are fully regenerated. Sterilisation treatments can damage tissues, affect the entire process, and undermine the justification for applying tissue culture. This is because treatments applied effectively on bacteria can damage plant cell membranes, proteins, or nucleic acids, which may lead to poor germination or subsequent callusing, browning, or limited regeneration efficiencies, respectively (Cassells and Curry 2001). Numerous studies indicate that no correlation exists between sterilization concentrations and overall results. A reduction in contamination percentage did not result in increased explant germination or more efficient regeneration. For example, in sorghum, excessive sterilisation treatments often compromise embryogenic competence and plant development (Kumar et al. 2022). So, to accurately assess the efficiency of a given sterilisation treatment, the contaminant rate should be supplemented with an assessment of explant viability and potential for regeneration.

More people are interested in transforming biotechnology, and there have been increased efforts in optimizing the protocols to generate a large and homogeneous population of fertile plants; the transformation of sorghum has improved dramatically due to the development of morphogenic regulators, ternary vectors and plant regeneration techniques (Wang et al. 2023; Li et al. 2024). The major limiting step of transformation technology remains poor sterilisation rates; thus, providing contaminant-free and physiologically intact explants is an essential beginning of the research. Even with

major advances in transformation technology, it will remain a high-risk step that can determine whether the research succeeds or fails.

Sterilization has long been a subject of research for sorghum and other species, mainly employing immature embryos or tissues with specific physiology or nutritional needs. However, mature plant embryos stored long-term are preferable because they are easy to store and have low production costs. This makes mature seeds attractive because they are available year-round. More people have tried to carry out large-scale production of sterile mature seeds from *Sorghum* spp. Still, there has never been a systematic optimization study targeting the effects of combined treatments for sterilized dry sorghum seeds, especially. Another limitation is that most researchers did not test a broad range of combined treatments covering many concentration-time points, which limits the interpretation of possible synergistic effects between the two parameters.

Other environmentally sound treatments that have shown effective sterilization results include green alcohols, such as methanol or ethanol. Their properties vary according to the target plant, time of exposure to the sterilization agent, and sterilant concentrations, producing healthy plant tissues with high recovery. However, they can dehydrate delicate tissue for long periods (Ghanim et al. 2023). Among all safe alternatives, silver nanoparticles stand out, showing high activity at low doses (Ghanim et al. 2023). However, the cost and accessibility are not yet at the scale for use in some cases. Mercury compounds, traditionally used as potent sterilizing agents, have several drawbacks, including toxicity and environmental persistence; due to health and environmental concerns, they have been gradually abandoned for newer, more efficient chemicals that have often yielded comparable or even better results than mercuric chloride. Indeed, in raspberry tissue culture, sodium hypochlorite gave an improved response compared to $HgCl_2$, with a higher survival rate of micro-shoots (up to 79.3% aseptic cultures). Likewise, in the woody plant *Litsea cubeba*, application of 0.2% NaOCl solution to the seed explants for 15 minutes led to 6.76% contamination and 97.30% survival, while HgCl treatment yielded lower contamination (25%) but a lower survival rate of 2.26% (Anelić et al. 2024). Finally, advanced artificial intelligence techniques, including artificial neural networks, decision trees, etc., have also been demonstrated to enable prediction and optimization of treatment parameters by significantly reducing the number of experiments and avoiding the laborious trial-and-error method, which allows efficient derivation of a sterilisation protocol (Aasim et al. 2023).

It is well known from the literature that both concentrations and exposure times affect the sterility results of treated samples (Aasim et al. 2023; Anelić et al. 2024; Beacorn and Thiessen 2021; Bock et al. 2025; Febyanti et al. 2025; Ghanim et al. 2023; Karaca et al. 2024). However, relatively few studies have reported the effects of different sterilization treatments for dried sorghum grains (Ahiakpa et al. 2020; Kumar et al. 2022; Oloniruha et al. 2023; Wang et al. 2023). A wide range of the treatment space was unexplored in many previous studies, making it difficult to evaluate two-way interaction effects to select the best sterilization for dried sorghum grains. Most studies on sterilization for this purpose emphasize microbial control; there has been relatively little concern about the survival of plant explants or their regenerative ability after disinfection treatments (Cassells, 2001; Cassells & Curry, 2001; Gunawardhana

et al., 2022; Karaca et al., 2024; Reed et al., 2013). Thus, sterilization methods apparently suitable from a microbiological point of view might not be ideal from a plant physiology point of view.

Therefore, the effect of varying the concentrations and exposure times of sodium hypochlorite treatments was investigated to control contamination and the survival of dried sorghum grain explants. The purpose of this study was to identify a sterilized condition that adequately controls microorganisms for dried sorghum grain culturing with minimal injury to explant cells, using only sodium hypochlorite as the main sterilizing reagent throughout the experiment by testing a variety of concentrations and exposure times.

2. Materials and Methods

2.1 Plant Material and Experimental Site

Mature dried grains of sorghum (*Sorghum bicolor* [L.] Moench) were used as explants. The explants were procured from Legasi Z Agro, Kajang, Selangor, Malaysia. All experimental work was carried out between September and October 2025 at the Plant Biotechnology Laboratory, Faculty of Plantation and Agrotechnology, Universiti Teknologi MARA (UiTM), Malaysia.

No mercury-containing sterilizing agents were used at any stage. All sterilization treatments were based on sodium hypochlorite (commercial Clorox®).

2.2 Experimental Design

The study used a two-factor factorial arrangement within a Randomized Complete Block Design (RCBD). Factor one was sodium hypochlorite concentration at four levels (20%, 30%, 40%, and 50%); factor two was exposure duration at four levels (10, 15, 20, and 25 min), producing sixteen treatment combinations. Each treatment was applied to fifteen explants, with a single explant as the experimental unit, giving 240 explants in total. Treatment combinations are listed in Table 1.

Table 1
Treatment combinations of sodium hypochlorite concentration (%) and exposure duration (min) evaluated for surface sterilization of dried sorghum grains.

Treatment	Sodium Hypochlorite (%)	Exposure Duration (min)
T1	20	10
T2	20	15
T3	20	20
T4	20	25
T5	30	10
T6	30	15
T7	30	20
T8	30	25
T9	40	10
T10	40	15
T11	40	20
T12	40	25
T13	50	10
T14	50	15
T15	50	20
T16	50	25

2.3 Surface Sterilization Procedure

Sorghum grains were first washed under running tap water for approximately 20 min to remove dust and loosely attached surface material. They were then transferred to a laminar airflow cabinet, where all sterilization steps were performed under aseptic conditions.

Explants were immersed in sodium hypochlorite solutions at the assigned concentrations and durations (Table 1), then exposed to 70% ethanol for 1 min as a secondary disinfection step. Residual disinfectants were removed by five successive rinses with sterile distilled water. Explants were transferred immediately to culture media after the final rinse.

2.4 Culture Establishment and Growth Conditions

Sterilized grains were cultured on hormone-free Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962), dispensed into sterile 90-mm Petri dishes, and allowed to solidify before inoculation.

Petri dishes were sealed with Parafilm® to limit external contamination and moisture loss, then incubated under continuous illumination at 25 ± 2°C. Contamination symptoms and explant survival were monitored and documented over two weeks.

2.5 Data Collection

Two response variables were recorded. Contamination rate, defined as the visible appearance of fungal mycelia, bacterial colonies, or other microbial growth on the explant surface or culture medium, was calculated as the number of contaminated explants divided by the total number of explants per treatment.

Explant survival rate, determined from successful germination and maintenance of healthy tissue throughout the observation period, was calculated as the number of viable explants divided by the total number of explants. Both were expressed as proportions.

2.6 Statistical Analysis

Data were analyzed using SPSS version 27. Proportion data were subjected to an arcsine square-root transformation before analysis to meet assumptions of normality and homogeneity of variance. A two-way ANOVA was used to assess the effects of concentration, duration, and their interaction. Where significant treatment effects were detected, means were separated using Tukey's honest significant difference (HSD) test at $\alpha = 0.05$.

2.7 Logistic Regression Modeling

Binary logistic regression was conducted for both contamination and survival to characterize continuous relationships between the sterilization variables and biological responses. Each model included linear and quadratic terms for concentration and time, plus a concentration × time interaction. Quadratic terms allowed detection of nonlinear responses and identification of optimal sterilization conditions.

2.8 Calculation of Standard Deviations

Standard deviations for proportional responses were estimated using $SD = \sqrt{[p(1-p)/n]}$, where p is the observed proportion and n is the number of explants per treatment (15), consistent with a binomial variance structure.

3. Results

3.1 Overview of Treatment Responses

Across 240 explants and sixteen sterilization treatments, both contamination incidence and explant survival varied considerably. Treatments that most aggressively reduced microbial growth did not always yield the highest explant survival, confirming that neither variable can be assessed in isolation. Figure 1 provides photographic documentation of sorghum grains after treatment, illustrating the contrast between successful and failed sterilization. Panel A shows extensive fungal mycelial growth resulting

from sub-optimal sterilization (40% NaOCl, 10 min), where microbial contaminants proliferate on the culture medium. Panel B depicts a fully sterilized grain following the optimized treatment (20% NaOCl, 25 min), with healthy tissue and no visible microbial growth. This qualitative reference provides context for the quantitative treatment responses presented below.

3.2 Effects on Contamination

Two-way ANOVA showed significant main effects for concentration ($F_{3,224} = 3.84$, $p = 0.010$) and duration ($F_{3,224} = 3.29$, $p = 0.022$), as well as a significant concentration × duration interaction ($F_{9,224} = 2.17$, $p = 0.025$). The interaction indicates that how long an explant is exposed matters differently depending on the concentration used.

At 20% sodium hypochlorite, contamination was high at 10, 15, and 20 min exposures, ranging from 0.733 to 0.800. Extending exposure to 25 min produced a sharp drop to 0.067, the lowest contamination recorded across all treatments (Table 2). At 30%, contamination declined progressively with duration. At 40%, the minimum (0.200) occurred after 20 min. Responses at 50% were less consistent and showed no clear concentration-dependent pattern.

Exposure duration, rather than disinfectant strength alone, was the decisive factor in determining contamination control, particularly at the lower end of the concentration range tested.

3.3 Effects on Explant Survival

Considered independently, the main effects of concentration ($F_{3,224} = 1.58$, $p = 0.194$) and duration ($F_{3,224} = 1.53$, $p = 0.208$) were not statistically significant. Their interaction, however, significantly affected survival ($F_{9,224} = 3.45$, $p = 0.001$).

At 20% sodium hypochlorite, survival was low to moderate (0.400–0.467) at 10, 15, and 20 min but jumped to complete survival (1.000) at 25 min. At 30%, survival increased steadily with duration, reaching 0.867 at 25 min. Treatments at 40% and 50% were more variable; while certain combinations produced reasonably high survival, prolonged exposure at these concentrations often reduced viability.

The highest survival rates outside the identified optimum were 0.933 at 40%/20 min and 0.867 at 40%/10 min, neither of which achieved the combination of low contamination and full survival seen at T4. Figure 2 illustrates the interactive effects of NaOCl concentration and exposure time on contamination and survival rates across all treatments. The figure demonstrates that sterilization outcomes depend not on concentration or duration alone, but on their combination. The marked contamination reduction at 20% NaOCl with extended exposure, compared to the variable responses at higher concentrations, underscores the need for integrated evaluation. The survival panels further show that moderate concentrations applied for extended periods maintain explant viability, while intensive treatments compromise explant health.

3.4 Integrated Evaluation of Contamination and Explant Survival

Evaluating both variables together reveals the trade-off that runs through these data. Treatment T4, 20% sodium hypochlorite for 25 min, was the only combination to satisfy both criteria simultaneously: contamination of 6.7% (1 out of 15 explants) and 100% survival. Relative to the same concentration at 10 min, that represents a 73.3 percentage-point reduction in contamination, from 80% (12/15) to 6.7% (1/15).

Higher concentrations showed a different pattern. Applied briefly (10 or 15 min), 40% and 50% hypochlorite reduced contamination faster than 20% at the same short durations. For instance, 40% at 10 min yielded 26.7% contamination (4/15) versus 80% (12/15) for 20% at 10 min. The cost was paid in survival: 40% applied for 25 min, leaving only 53.3% of explants viable (8/15), against 100% for T4. Increasing sterilization intensity beyond an optimal point harms explant viability more than it helps contamination control. Oxidative damage to the explant accumulates faster than any additional gain in microbial eradication. Such an outcome makes a protocol that produces low contamination but high explant death functionally useless for tissue culture. T4 was the only treatment in this study that achieved both objectives.

The overall sterilization performance is summarized in a heatmap (Fig. 3), which combines contamination and survival results in a single composite measure. The higher the value, the better the performance, with lower contamination and higher explant survival. The heatmap clearly shows that the 20%/25 min treatment is the best combination of both and is clearly different from the nearby treatments, which allow for more contamination or lower survival. This visualization gives an easy at-a-glance summary of the treatment space and reinforces the conclusion that moderate concentration and long exposures are the optimum combination.

Table 2
Contamination and survival rates (mean ± SD, n = 15) of sorghum grain explants following surface sterilization with different sodium hypochlorite concentrations and exposure durations. Within each column, means followed by the same letter do not differ significantly (Tukey's HSD, α = 0.05). The optimal treatment (20%, 25 min) is highlighted in bold.

NaOCl (%)	Time (min)	Contamination (Mean ± SD)	Survival (Mean ± SD)
20	10	0.800 ± 0.103 (cd)	0.400 ± 0.126 (e)
20	15	0.733 ± 0.114 (cd)	0.467 ± 0.129 (de)
20	20	0.733 ± 0.114 (cd)	0.467 ± 0.129 (de)
20	**25**	**0.067 ± 0.064 (a)**	**1.000 ± 0.000 (a)**
30	10	0.467 ± 0.129 (bcd)	0.533 ± 0.129 (cde)
30	15	0.533 ± 0.129 (abcd)	0.800 ± 0.103 (abcd)
30	20	0.400 ± 0.126 (cd)	0.800 ± 0.103 (abcd)
30	25	0.333 ± 0.122 (cd)	0.867 ± 0.088 (abc)
40	10	0.267 ± 0.114 (cd)	0.867 ± 0.088 (abc)
40	15	0.400 ± 0.126 (bcd)	0.533 ± 0.129 (cde)
40	20	0.200 ± 0.103 (d)	0.933 ± 0.064 (ab)
40	25	0.400 ± 0.126 (bcd)	0.533 ± 0.129 (cde)
50	10	0.467 ± 0.129 (abcd)	0.600 ± 0.126 (bcde)
50	15	0.333 ± 0.122 (cd)	0.800 ± 0.103 (abcd)
50	20	0.333 ± 0.122 (cd)	0.733 ± 0.114 (abcde)
50	25	0.267 ± 0.114 (cd)	0.600 ± 0.126 (bcde)

Values are means ± standard deviations (n = 15). Means sharing a letter within a column do not differ significantly by Tukey's HSD test at α = 0.05.

3.5 Logistic Regression Analysis

Binary logistic regression confirmed the patterns emerging from the ANOVA (Table 3). For survival, the full model was significant ($\chi^2 = 58.4$, df = 5, $p < 0.001$). Exposure duration exerted a significant positive linear effect, and a significant negative quadratic term indicated an optimum beyond which additional exposure time is detrimental. A significant concentration × duration interaction confirmed that neither variable acts independently.

For contamination, the model was also significant ($\chi^2 = 49.7$, df = 5, $p < 0.001$). Both concentration and duration were negatively associated with contamination probability. The model identified an optimum

near 21.6% sodium hypochlorite and 24.3 min, at which it predicted 97.8% survival and 6.2% contamination, closely matching the experimentally determined optimum of T4.

Table 3
Logistic regression coefficients for the effects of sodium hypochlorite concentration and exposure duration on explant survival and contamination.

Predictor	Survival β	p-value	Contamination β	p-value
Concentration (linear)	0.087	0.183	-0.120	0.005
Concentration (quadratic)	-0.038	0.012	-0.009	0.412
Time (linear)	0.280	0.018	-0.210	0.008
Time (quadratic)	-0.008	0.024	-0.003	0.187
Concentration × Time	0.014	0.003	0.006	0.094
Model χ^2 (df = 5)	58.4	< 0.001	49.7	< 0.001

3.6 Summary of Key Findings

Concentration and duration interacted to shape both contamination control and explant survival. Among all treatments tested, 20% sodium hypochlorite for 25 min (T4) gave the most favorable balance, the lowest contamination rate, and complete explant survival. Agreement between empirical observations and logistic regression predictions supports this treatment as the robust optimum for dried sorghum grain sterilization.

4. Discussion

The success of plant tissue culture depends on establishing contamination-free explants that retain normal growth capacity. In this study, sodium hypochlorite concentration and exposure duration jointly and significantly shaped both contamination incidence and explant survival. Neither factor alone was a reliable predictor of culture establishment success, and this interaction is the central finding.

4.1 The role of sterilisation conditions in controlling contamination

The topography of the sorghum seed surface and variability in microflora present complicated attempts to overcome contamination of sorghum cultures. The sharp decline in contamination from about 55 per cent at 20% sodium hypochlorite when the duration is increased from 20 to 25 min demonstrates that it is possible to compensate for low chemical strength with length of contact. According to Reed et al. (2013), sodium hypochlorite kills microorganisms by chemically decomposing microcellular constituents through oxidation, and the longer the contact time, the more likely such compounds encounter microorganism components. It produces HOCl which oxidises proteins, nucleic acids, membrane lipids, and enzymes essential for cellular metabolism in all microorganisms. Such reactions involve interaction

between components of the oxidising agent and cellular parts, with the degree of killing a function of amounts of available active chlorine and exposure time. At lower concentrations of sodium hypochlorite, the coating on the surface of mature grain serves as a diffusion retarder. It limits the access of disinfectant to deeper tissues/crevices where micro-organisms may occur. Thus, they are probably still alive inside the cell layers when 20% sodium hypochlorite concentration is used for 20 minutes. When exposure was increased to 25 minutes, the cumulative amount of active chlorine that enters the cells would greatly increase, resulting in a sharper reduction in the number of cultivable contamination microbes without killing explants. Gunawardhana et al. (2022) and Karaca et al. (2024) also claimed time as at least as important as concentration in successful sterilisation of various types of plant tissues. At NaOCl concentrations exceeding 20%, contamination levels reached a plateau, a pattern consistent with other plant species in which increased chlorine intensity provides diminishing returns, concurrently elevating phytotoxicity and reducing the recovery of aseptic cultures.

4.2 Effect of sodium hypochlorite concentrations on explant survival

One observation from the results in the study was that there is no simple inverse relationship between antimicrobial effectiveness and recovery rate or the rate of increase of healthy explants surviving the culture medium. For several treatments with concentrations that were very good at controlling surface contaminants, there was substantial loss of recovered culture, at least partly due to damage incurred at higher concentrations and during longer contact times. According to Cassells and Curry (2001), too-long exposures cause such damage by mechanisms that probably operate through the generation of reactive oxygen species, which, on the one hand, disrupt microbial cell integrity whilst, on the other hand, compromise plasma-membrane structures and alter protein and nuclear integrity. Increased penetrance and reactivity of the chlorine oxidise the internal cell structures which not only damages contaminants but also can destabilise plant cell membranes which therefore caused damage of viability and loss of tissue. For most of cultures there was increased rate of surviving clean tissue as percentage or absolute value at most of the concentrations for short exposure times and at 20% NaOCl at prolonged exposure time for 25 mins and then started decreasing at high concentration(s); however, for all long exposure times the decrease was significant especially above 20%. This indicates that with a moderate-to-high range of chlorine intensity/extended prolonged time, adequate killing can be achieved without causing excessive damage to the cells. A similar pattern of lower-severity treatments resulting in more successful selection for regeneration (or increased survival percentage) in cultures has been reported for some other crops (Kumar et al. 2022).

4.3 Interaction between Concentration and Exposure Duration

This statistically significant interaction between concentrations and duration does not merely represent an artifact of the statistical method used; it demonstrates a real biological response. At lower chlorine concentrations, increasing duration allows ample time for interaction between corrosive compounds and

the microflora. At the same time, at higher levels this extension causes too much harm to plant cellular physiology, which begins to exhibit toxic damage. The observed significant linear-by-quadratic terms in the logistic regression model for survival demonstrate this, i.e., there is not a linear relationship between intensity of exposure and outcome.

4.4 Implications for Sorghum tissue culture and transformation systems

The establishment of reliable tissue culture methods are crucial for various sorghum applications including gene transformation and the protocols recently reported for generating high yields of gene transformants, such as with CRISPR/Cas9-based transformation via sorghum seedling's callus and leaf whorls system (Wang et al. 2023; Lee and Wang 2025) using a ternary vector system (Li et al. 2024), which rely on availability of clean healthy tissue resources to generate a reasonable transformation efficiency (28–70.9%). A simple, effective protocol, in which no specialized equipment and hazardous reagents are needed for decontamination with high efficiency, can promote the development of such advanced transformation technologies, especially since seed is stored throughout the year and is amenable to scale-up. The most serious limitations of sorghum transformation via explants are the occurrence of tissue browning, attributed largely to lipid degradation and consequent oxidative stress accumulation (Lee and Wang 2025), which would be expected to be diminished with a procedure that minimises the degree of overall damage imposed by oxidative and potentially chlorine stresses during explant preparation.

4.5 Environmental and Biosafety Considerations

For many years in many tissue culture facilities, mercuric chloride ($HgCl_2$) was the best readily available and effective antibacterial and antifungal compound. It became a ubiquitous component of tissue culture recipes. However, the increased concerns with the use and proper disposal of such compounds due to their toxicity and environmental effects have led to the discontinuation of most mercury-containing substances. The present study shows an effective approach to control contamination that does not utilize any mercury-based chemicals. This not only reduces the costs of handling chemicals but also increases the safety of the working environment. Supporting this finding, research into other systems revealed similar outcomes. Anelić et al. (2024) reported that among 0.1–0.5% concentrations of sodium hypochlorite (NaOCl) applied for between 5 and 15 min in raspberry tissue culture could result in up to 79.3% aseptic cultures while allowing better survival and lower toxicity as compared with 0.1% $HgCl_2$ treatment, which resulted in a comparable level of contamination (78.3%). Furthermore, 0.2% NaOCl for 15 min in *Litsea cubeba* tissue culture resulted in a 6.76% contamination rate and a 97.30% survival rate compared to mercury-based sterilization, which resulted in a lower contamination rate but a very low survival rate of up to 2.26% (Wang et al. 2017). These examples further justify replacing $HgCl_2$ with NaOCl in routine tissue cultures.

4.6 Physiological Implications for Regeneration

Oxidative stress induced by the sterilization agents is probably not fully resolved when explants are placed in culture; oxidative damage may influence subsequent developmental response. Degradation of cellular membranes and components of metabolic pathways could influence embryogenesis and callus formation capability of explants. Regarding the transformation process in sorghum, which usually requires healthy and actively responsive tissues, a procedure that minimizes excessive reactive oxygen species (ROS) production can affect the success of subsequent stages of culturing even more effectively than controlling microbial contaminants. An optimal tissue condition regarding the balance between tissue components and the impact of sterilization would result in better regenerative capability (Thaddi 2025). Thaddi (2025) described that up to 96% embryogenic induction capacity could be achieved in the selected sorghum varieties when healthy explants were used. Our study provided an effective method to minimize phytotoxicity at the sterilization stage, which should result in effective regeneration, as the plants introduced to the in vitro system will be healthy.

4.7 Limitations and Future Directions

Some limitations of this study exist. Our analysis of contamination was based only on visually identifying microbial or fungal growth in cultures, instead of molecular identification techniques that might have contributed to a more precise interpretation of contaminating agents on either endogenous (endophytes) or epiphytic microflora. Further analyses would involve molecular identification of all contaminants, which may allow optimization of treatment of endogenous contamination without harming seed tissues more significantly. Only a single commercial batch of sorghum seed was used. The viability of this method for sorghum germplasm could thus be reduced or increased as other cultivars differ in viability, susceptibility to contamination, age or origin. Studies comparing diverse sorghum varieties with different storage history/duration and a variety of contaminated sources could help generalize the application of this new method of contamination. This investigation does not fully describe the process on longer-term culture; therefore, investigation into how sterilization affected callus production, somatic embryogenesis and development, regeneration, and transformation success rate is required to have a fully developed method protocol.

5. Conclusion

The combined effects of disinfectant concentration and time on contamination of sorghum seed explants and explant survival are critically dependent on their interaction rather than on concentration or time per se.

According to our results, 20% NaOCl for 25 min was determined to be optimal for obtaining the highest percentage of sterilized cultures (contamination 0.067 ± 0.064) while ensuring full explant viability (1.000 ± 0.000). Logistic regression analysis predicted 97.8% viable explants and 6.2% contamination at approximately 21.6% and 24.3 min from concentration and time, respectively, within the optimal region.

Higher concentrations of disinfecting agents at reasonable times should be prioritized over prolonged contact of tissues with moderately stronger disinfecting agents, because it results in a higher

sterilization effect while minimizing any damaging effect on tissue viability and structure. The method developed here can be routinely carried out in a standard plant tissue culture laboratory; it avoids the use of hazardous equipment or extremely toxic mercury-based materials and provides efficient results at lower cost.

Thus, this work opens ways toward establishing effective guidelines for contamination control at the beginning of the culture cycle as first steps in genome engineering, transformation, and regeneration in the important cereal crop sorghum.

Abbreviations

Abbreviation	Full Term
ANOVA	Analysis of variance
HSD	Honest significant difference
MS	Murashige and Skoog
NaOCl	Sodium hypochlorite
RCBD	Randomized complete block design
SD	Standard deviation

Declarations

Competing Interests:

The authors have no relevant financial or non-financial interests to disclose.

Ethics declaration

Not applicable.

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Author Contribution

Author Contributions: Conceptualization: P.A.L., A.M.; Methodology: P.A.L., S.N.H., M.F.D.; Investigation: P.A.L.; Formal analysis: P.A.L., A.M.; Writing – original draft: P.A.L.; Writing – review & editing: S.N.H.,

M.F.D., A.M.; Visualization: P.A.L., A.M.; Supervision: S.N.H., M.F.D., A.M.; Project administration: M.F.D.; Resources: S.N.H. All authors have read and approved the final manuscript.

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Data Availability:

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

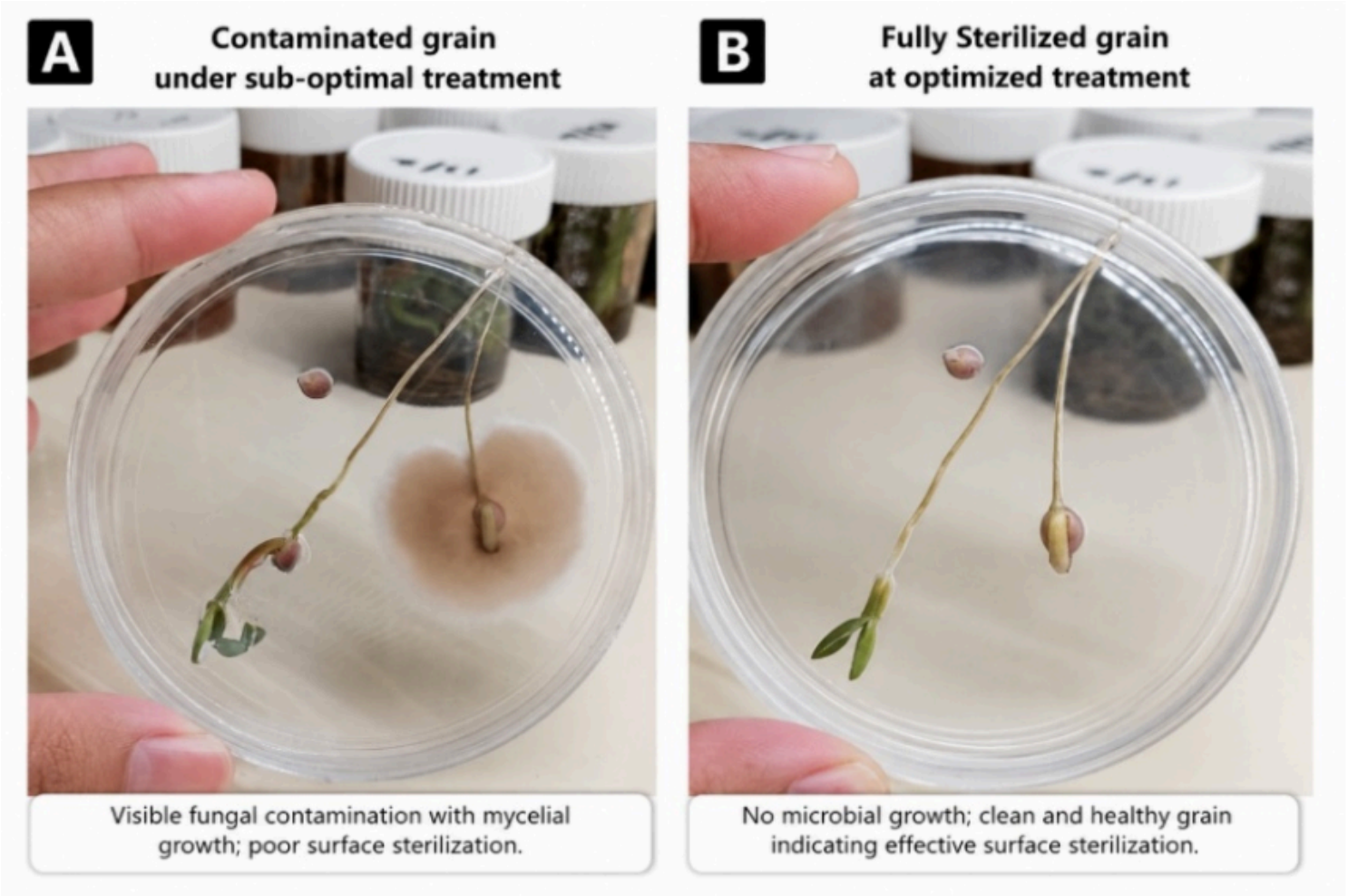


Figure 1

Sterilization outcomes of sorghum grains on hormone-free MS medium after 14 days of culture. (A) Contaminated grain showing fungal mycelial growth following sub-optimal sterilization (40% NaOCl, 10 min). (B) Sterilized grain with no visible microbial growth following optimal treatment (20% NaOCl, 25 min). Scale bar = 1 cm.

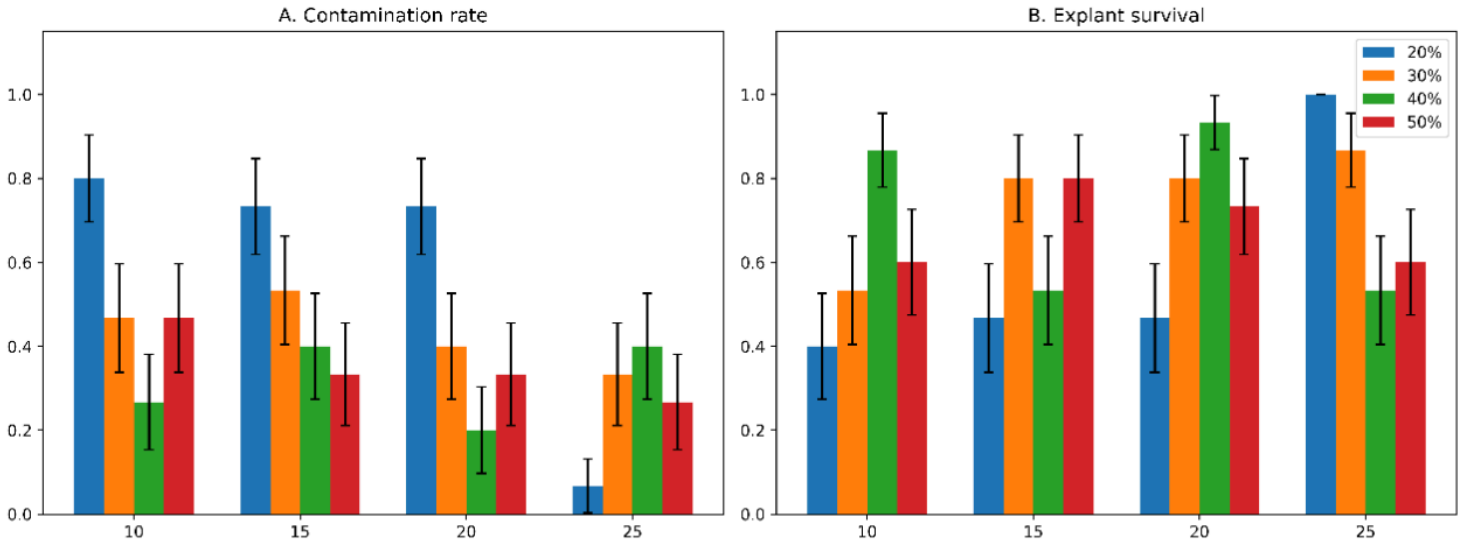


Figure 2

Interactive effects of sodium hypochlorite concentration (20–50%) and exposure duration (10–25 min) on (A) contamination rate and (B) explant survival of sorghum grains cultured on hormone-free MS medium for 14 days. Values are means ± SD (n = 15). The optimal treatment (20% NaOCl, 25 min) is indicated with an arrow.

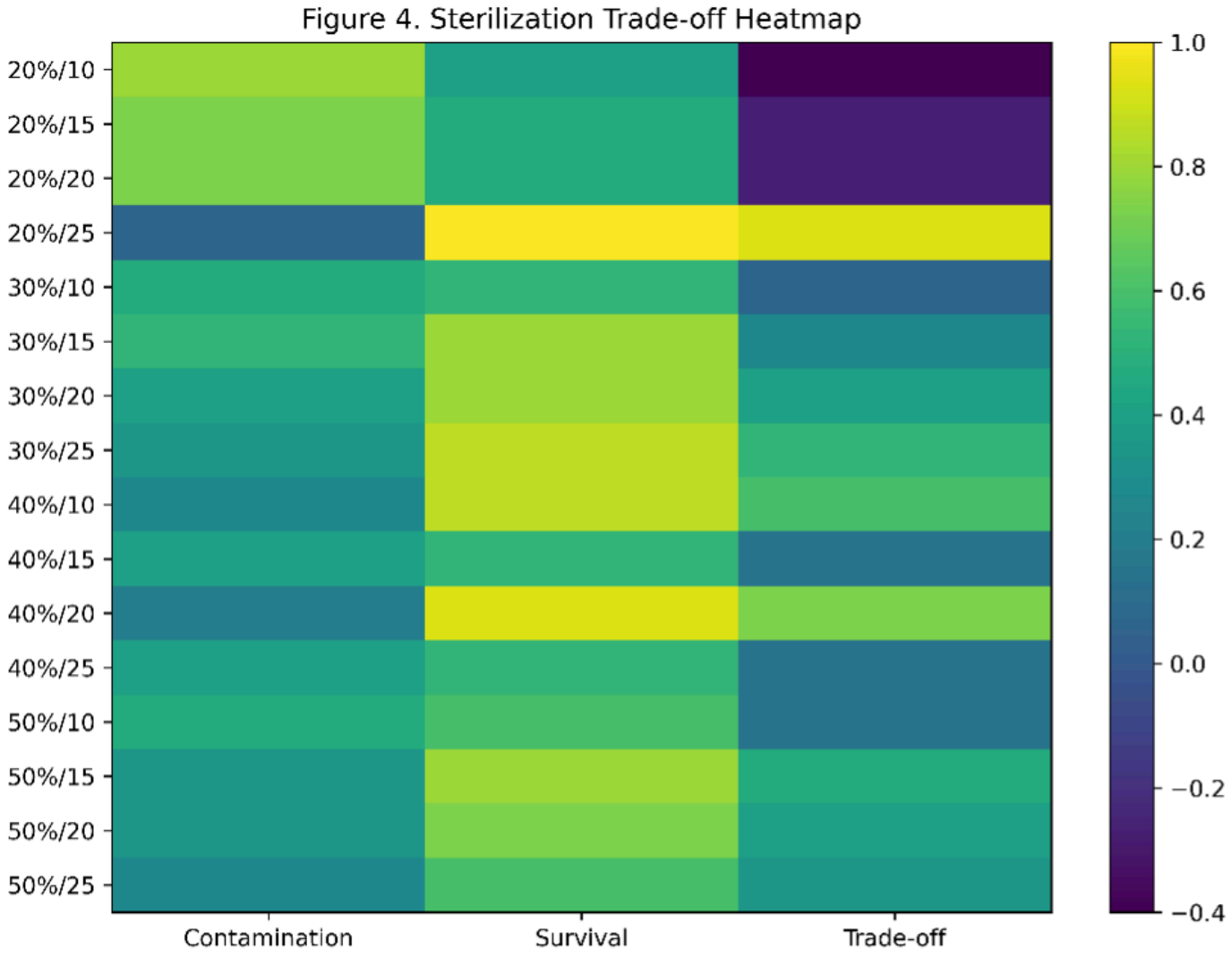


Figure 3

Heatmap of sterilization performance (composite score) across sodium hypochlorite concentration–duration combinations. The composite score was calculated as (1 – contamination rate) × survival rate, where higher values indicate better overall performance (range: 0–1). The optimal treatment (20% NaOCl, 25 min; composite score = 0.933) is highlighted with a red border. Values in cells represent the composite score.