International Journal of Horticultural Science 2025, 31: 66-72.
https://doi.org/10.31421/ijhs/31/2025/15325

Optimizing disinfection protocols for yam explant regeneration in plant tissue culture

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Summary: Yam (*Dioscorea species*), does not produce commercially viable seeds, and asexual propagation is faced with challenges resulting from carried-over infections from previous generations. Contamination is a prevalent problem in Plant Tissue Culture (PTC), making the development of cost-effective and efficient disinfection protocols crucial for successful PTC. This study aimed to evaluate the effectiveness of yam explant disinfection protocols using various immersion timings, disinfectant concentrations (including ethanol (C_2H_5OH), sodium hypochlorite (NaOCl), and hydrogen peroxide (H_2O_2), and in single or combined disinfectants application. Twenty treatment combinations and one control were assessed on yam vines for Disinfection Efficiency (DE%), Negative Disinfection Effect (NDE%), and the regeneration of shoots and roots (SN & RN) from the culture after 21 days. The study showed that varying immersion times did not significantly impact the evaluated parameters. However, different concentrations of disinfectants resulted in diverse NDE responses. Surprisingly, higher concentrations of NaOCl led to reduced NDE, whereas lower concentrations increased NDE. On the contrary, higher concentrations of H_2O_2 increased NDE, while lower concentrations decreased it. Shoot and root regeneration rates were also significantly impacted by the choice of disinfection protocol. The research concluded that dual disinfection protocol, specifically 70% ethanol for 7 minutes followed by NaOCl, was most effective for eliminating surface-borne contaminants and achieving successful *in vitro* propagation of yam plantlets. This method offers a cost-effective solution for establishing microbe-free tissue culture yam plantlets and provides a basis for future research on other *Dioscorea* plants.

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Key words: *Dioscorea rotundata*, disinfection protocol, contamination control, disinfection efficiency, regeneration

Introduction

Yam (*Dioscorea* spp.) is a vital staple crop for over 300 million people in sub-Saharan Africa, particularly in West Africa, where it is primarily cultivated for its carbohydrate-rich tubers (Nweke, 2016). In addition to its nutritional value − contributing carbohydrates, proteins, lipids, vitamins, and minerals − yam also plays a prominent role in traditional ceremonies and cultural practices, symbolizing wealth and status. Medicinally, *Dioscorea* species have been associated with a variety of health benefits, including the treatment of inflammatory and cardiovascular diseases, aging disorders, menopausal symptoms, cancers, and osteoporosis (Obidiegwu et al., 2020). Its extracts are also used in cosmetics for pigmentation issues (Nashriyah et al., 2012), birth control, and treating skin infections (Jena et al., 2024).

Despite its agronomic and socio-economic importance, yam production faces serious challenges, particularly the scarcity and high cost of clean seed tubers. This constraint severely limits productivity, especially among smallholder farmers. Access to quality propagules is often hindered by technical, institutional, and economic barriers (Aighewi et al., 2015; Gildemacher et al., 2009; Poku et al., 2018). Mignouna et al. (2015) reported that planting materials alone can account for over 50% of total production costs, posing a significant burden to resource-limited growers. In addition, field multiplication is constrained by low tuber multiplication rates and vulnerability to pests and diseases.

To address these limitations, plant tissue culture (PTC) offers a promising solution for producing clean, virus-free planting materials through meristem culture, thermo- and chemotherapy, and mass propagation techniques (Negi et al., 2024). PTC also contributes to germplasm conservation and genetic improvement. However, one of the most persistent challenges in tissue culture is microbial contamination, which can delay culture initiation, reduce growth rates, and even result in the loss of valuable germplasm (Gammoudi et al., 2022).

Biological contaminants, particularly bacteria and fungi, can alter the chemical composition of culture media by releasing metabolites and degrading nutrients (Cassells, 2012). Contamination can originate from several sources, including the explant itself, laboratory air, instruments, operators, or inadequate sterilization (Cassells & Doyle-Prestwich, 2009; Okoroafor et al., 2022). Epiphytic bacteria may be embedded in plant crevices where surface disinfectants cannot reach

(Gunson & Spencer-Phillips, 1994), while endophytic microbes can reside intercellularly in tissues and resist standard disinfection methods (El-Banna et al., 2021; Singh, 2018).

Proper surface disinfection of explants is, therefore, a critical step in tissue culture protocols. Commonly used disinfectants include 70% ethanol, sodium hypochlorite (0.5–4.5%), and mercuric chloride (0.05–0.2%), either singly or in combination (Magaia, 2015; Demeke et al., 2014; Alla et al., 2013). Other agents such as silver nitrate, thiram, carbendazim, and fungicides are also used depending on species and explant type (Oyebanji et al., 2009; Yildiz et al., 2012). While combinations of disinfectants often improve decontamination rates, they can also compromise explant viability and regeneration due to phytotoxicity. Hence, the need for species-specific optimization is critical (Andrade et al., 2000; Chaves et al., 2005).

Furthermore, sodium hypochlorite and sodium dichloroisocyanurate have been used to sterilize culture media as an alternative to autoclaving (Ana et al., 2016; Pais et al., 2016; da Costa Urtiga et al., 2019). Fungicides, with their specific mechanisms of microbial inhibition − including disruption of protein and nucleic acid synthesis − are also gaining attention for integrated disinfection strategies (da Cruz et al., 2024; Vallieres & Avery, 2017).

Most tissue culture studies rely on multiple disinfectants to achieve sterilization (Twaij et al., 2020), which may not always be cost-effective or sustainable, particularly in low-resource settings. There is thus a growing need to develop simplified, effective disinfection protocols that balance microbial control with explant survival and regenerative capacity. Therefore, this study aims to optimize disinfection protocols for yam explants in plant tissue culture by evaluating the efficacy of single and combined disinfectants at varying concentrations and exposure durations. The goal is to reduce contamination without compromising explant viability, thereby supporting efficient micropropagation and contributing to food security and sustainable agricultural development in alignment with Sustainable Development Goals 1 and 2.

Materials and methods

The current investigation was carried out at the Biotechnology Department Plant Tissue Culture Unit Laboratory of the National Center of Genetic Resources and Biotechnology (NACGRAB), Ibadan, Oyo-state, Nigeria. The objective of this research was to assess the effectiveness of various disinfectants in reducing contamination and promoting the growth of yam explants while avoiding adverse effects. To achieve this, three different disinfectants were used to surface sterilize the explants, before their inoculation on growth-supporting culture media.

Source of plant material and explant collection

Yam vines (TDr8902665) were obtained from the Semi-Autotrophic Hydroponic (SAH) laboratory, Yam Breeding Department, IITA, Ibadan, Oyo-state, Nigeria. The yam plants were kept inside boxes (***Figure 1***) and transferred to NACGRAB for investigation. To obtain explants, the yam vines were carefully cut with a scalpel/surgical blade and kept inside a glass jar containing distilled water. Explants were rinsed under running water and washed with tween 20/liquid soap to remove dust and peat material.

Disinfection of explant

The effectiveness of three (3) disinfectants were compared (solely or combined) in this experiment, they include Ethanol (C_2H_5OH), Sodium hypochlorite (NaOCl), and Hydrogen peroxide (H_2O_2). Two (2) concentrations of each disinfectant were prepared and applied at two (2) different immersion timing durations. Yam vine explants treated with sterile distilled water for 20 min served as the control. ***Table 1*** describes the treatments applied to all the experimental units. All manipulations were carried out under the laminar airflow according to standard protocols.

***Table 1*:** Treatments (disinfectant, concentrations, immersion timing, and treatment code for labelling) applied to all experimental units.

Disinfectant	Concentration (%)	Immersion time (Min.)	Treatment code
Mock Control-Water	-	-	T1
C_2H_5OH	50	5	T2
		7	T3
	70	5	T4
		7	T5
NaOCl	1.26	10	T6
		20	T7
	1.62	10	T8
		20	T9
H_2O_2	5.25	10	T10
		20	T11
	12.25	10	T12
		20	T13
70% C_2H_5OH + NaOCl	1.26	10	T4 + T6
		20	T4 + T7
	1.62	10	T4 + T8
		20	T4 + T9
70% C_2H_5OH + H_2O_2	5.25	10	T4+ T10
		20	T4+ T11
	12.25	10	T4+ T12
		20	T4 + T13

Media preparation and growth conditions

Disinfected explants and control were inoculated in full-strength yam preparatory media, modified from Murashige and Skoog's (1962) plant tissue culture media protocol with vitamins, sucrose 30 g/L, purified agar at seven g/L, and activated charcoal. The pH of the media was adjusted to 5.8±0.1 before autoclaving at 121 °C for 20 min at 15 psi. A total of 63 yam vine explants were inoculated on the culture medium and incubated in the growth room under a controlled temperature of 25±1 °C, relative humidity (RH) 50-70%, and 16/8 h (light/dark) photoperiod (40µmol $m^{-2}s^{-1}$) for 30 days. Data for evaluation parameters were collected and recorded weekly.

Figure. 1*:* Yam plantlet from Semi-Autotropic Hydroponics, IITA.

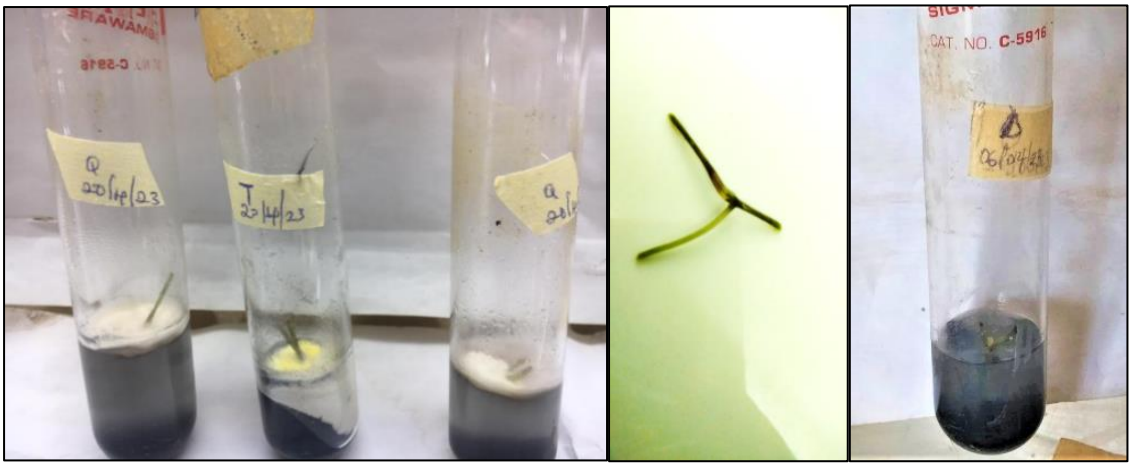

Figure 2: Contaminated culture with poor disinfection efficiency (**A**), Explant showing Negative Disinfection Effect (browning) (**B**), and Necrotic explant (**C**).

Observation of disinfection efficiency (DE%) and negative disinfection effect (NDE%)

Regular monitoring by visual examination for the presence of microbial contamination on inoculated explants or turbidity in the cultured media was carried out during the culture growth period to determine the effectiveness of disinfectants in eliminating contamination. Similarly, the potential negative disinfection effect (denoted by browning, chlorosis, and necrosis) was also determined only in the contamination−free cultures. Furthermore, the presence of emerging shoots and roots was monitored to evaluate the effect of disinfectants on organogenesis.

Data analysis

The data gathered were analyzed using a two-way analysis of variance (ANOVA) through a statistical software package for social sciences (SPSS version 20.0).

Results

Disinfection efficiency of sole and dual disinfectants

Disinfection efficiency (DE) was evaluated by observing the presence of microbial contamination in the culture. Our result showed that sole disinfectants had significantly higher DE (33-40%) in treatments T2, T9, T10 & T11 (representing 50% C_2H_5OH for 5 min, 1.62% NaOCl for 20 min, 5.25% H_2O_2 for both 10 and 20 min, respectively), while the control (mock – T1) and all other treatments (T3, T4, T5, T6, T7 & T8) showed 0% disinfection efficiency (***Figure 2A***).

Furthermore, using dual disinfectants (*i.e.* initial disinfection with 70% C_2H_5OH for 5 min (T4) followed by either NaOCl (T6 to T9) or H_2O_2 (T10 to T13)), 100% disinfection efficiency was observed in treatments T4 + T6 (1.26% NaOCl for 10 min) and T4 + T7 (1.62% NaOCl for 20 min) (***Figure 3A***). Eighty per cent (80%) disinfection efficiency were observed in treatments T4 +T8 (1.62% NaOCl for 10 min), T4 + T10 (5.25% H_2O_2 for 10 min), T4 + T11 (5.25% H_2O_2 for 20 min) & T4+ T12 (12.25% H_2O_2 for 10 min), but a significantly lower disinfection efficiency (40%) was recorded in treatment T4 + T13 (12.25% H_2O_2 for 20 min) (***Figure 4***).

Negative disinfection effect of sole and dual disinfectants

The Negative Disinfection Effect (NDE) measures the harmful impact of disinfectants on explants, such as chlorosis, browning, or necrosis. In this investigation, the results demonstrated how disinfectants can vary significantly in their detrimental effects on yam explants. Using sole disinfectants, significantly higher NDE (40%) was observed in treatment T2 (representing 50% C_2H_5OH for 5 min) while the control (mock – *i.e.* T1) and all other treatments (T3 to T13) showed 0% negative disinfection effect (***Figure 2 B&C***). Furthermore, dual disinfectants (*i.e.* initial disinfection with 70% C_2H_5OH for 5 min followed by either NaOCl or H_2O_2) showed a 100% negative disinfection effect in treatments T4 + T7, T4 + T12, & T4 + T13; 80% negative disinfection effect was observed in treatments T4 +T6, T4 + T10 & T4 + T11. A significantly lower negative disinfection effect (40%) was noted in treatments T4 +T8 & T4 + T9 (***Figure 5***).

Effects of sole and dual disinfectant on shoot regeneration

The regeneration of shoots is an important indicator of a successful disinfection protocol, as it reflects both the health of the tissue and the effectiveness of the protocol. In this study, shoot regeneration rates varied significantly depending on the

disinfectant used. Shoot Number (SN) was evaluated by observing the presence of shoots regenerated from 21-day-old cultures. Using sole disinfectants; 40% of cultures in treatment T2 (50% C_2H_5OH for 5 min) were observed to regenerate shoots from the cultured explants and 20% of cultures in treatments T9, T10 & T11 also regenerated shoots from the cultured explants (***Figure 3A&B***). In contrast, none of the cultures in control (mock *i.e.* T1) and other treatments T3, T4, T5, T6, T7, T8, T12, & T13 regenerated shoots from the cultured explants (***Figure 6***).

Figure 3: Contaminant-free culture with high disinfection efficiency (**A**) Regenerated shoot with well-expanded leaf (**B**) Plantlet with root and shoot

Using dual disinfectants (*i.e.* initial disinfection with 70% C_2H_5OH for 5 min followed by either NaOCl or H_2O_2); 60% of cultures in treatment T4 + T8, (representing T4 followed by 1.62% NaOCl for 10 min) was observed to regenerate shoots from the cultured explants and 20% of cultures in treatments T4 + T9, T4 + T10 & T4 + T11, was observed to regenerate shoots from the cultured explants. While control (mock *i.e* T1) and other treatments T4 + T6, T4 + T7, T4 + T12, & T4 + T13 showed no regenerated shoots from the cultured explants (***Figure 6***).

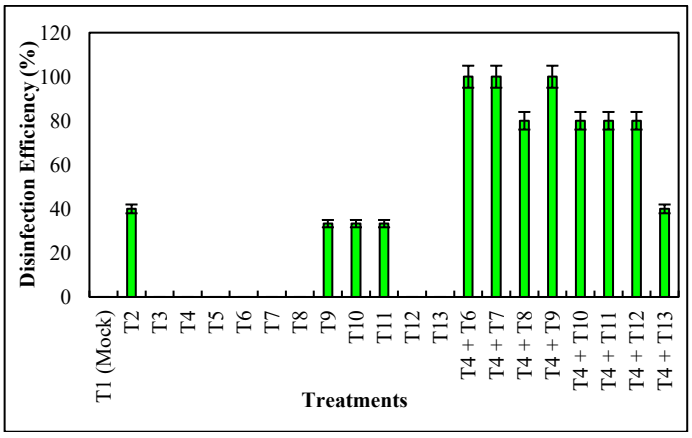

Figure 4: Disinfection Efficiency of *Dioscorea rotundata* using three disinfectants at different concentrations and immersion times after 30 days of culture. All treatments were replicated at least three times and raw data obtained were converted to percentages.

Effects of sole and dual disinfectant on root regeneration

The root regeneration capacity (RRC) of yam explants treated with different disinfectant protocols was found to vary significantly, reflecting the impact of both sole and dual disinfectant treatments on the overall health of the explants. The presence of regenerated roots was evaluated by observing the cultured plantlet 21 days after inoculation of explants for the presence of roots. Using sole disinfectants; 40% of cultures in treatment T2 (representing 50% C_2H_5OH for 5 min) were observed to regenerate roots from the cultured explants (***Figure 3B***), while 20% of cultures in treatments T5, T7, T10 & T11 were also noted to regenerate roots from the cultured explants. In contrast, none of the cultures in control (mock *i.e.* T1) and other treatments T3, T4, T6, T8 & T9 showed root regeneration from the cultured explants (***Figure 7***).

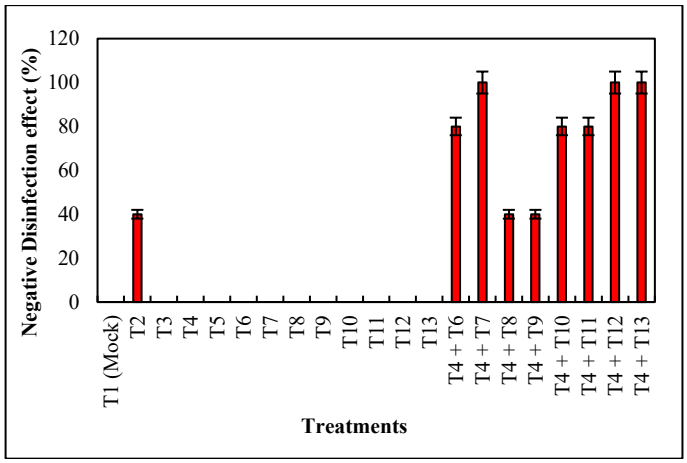

Figure 5: Negative Disinfection Effect of *Dioscorea rotundata* using three disinfectants at different concentrations and immersion times after 30 days of culture. All treatments were replicated at least three times and raw data obtained were converted to percentages.

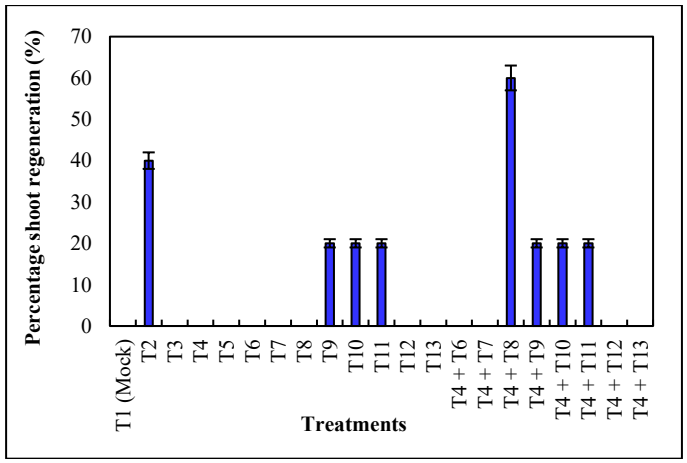

Figure 6: Shoot Number of *Dioscorea rotundata* using three disinfectants at different concentrations and immersion times after 30 days of culture.

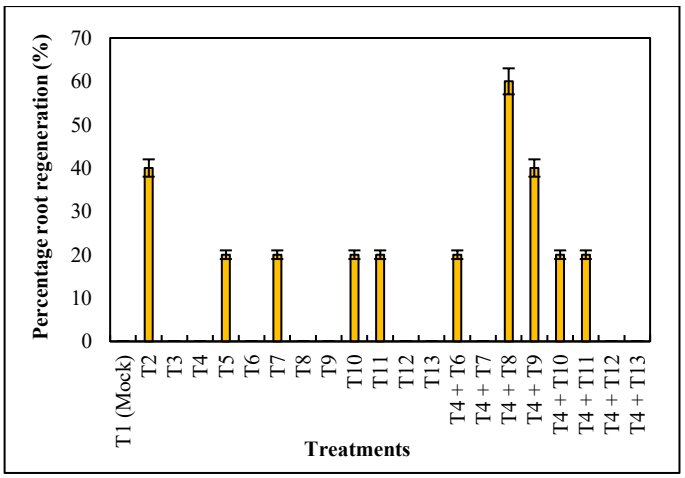

***Figure 7*:** Root Number of *Dioscorea rotundata* using three disinfectants at different concentrations and immersion time after 21 days of culture.

Similarly, using dual disinfectants (*i.e.* initial disinfection with 70% C_2H_5OH for 5 min followed by either NaOCl or H_2O_2), 60% of cultures in treatment T4 + T8 (representing T4 followed by 1.62% NaOCl for 10 min) was observed to regenerate roots from the cultured explants; 40% of cultures in treatment T4 + T9 (representing T4 followed by 1.62% NaOCl for 20 min) was observed to regenerate roots from the cultured explants and 20% of cultures in treatments T4 + T6, T4 + T10, T4 + T11 were noted to regenerate roots from the cultured explants. Control (mock i.e T1) and other treatments T4 + T7, T4 + T12 & T4 + T13 showed 0% regenerated roots from the cultured explants (***Figure 7***).

Discussion

Contamination is the most limiting challenge commonly encountered in PTC laboratories worldwide (Abdalla et al., 2022). Hence, the success of plant tissue culture and the elimination of contamination largely depends on the efficiency of sterilization processes (Teixeira da Silva et al., 2016). The optimized concentration of the disinfectant and required exposure time period will greatly influence the establishment of tissue culture systems (Altan et al., 2010; Teixeira da Silva et al., 2016; Hesami et al., 2018). This research aimed at evaluating the possibility of attaining successful disinfection of explants, using sole or dual disinfectant at specific concentrations and immersion time.

In this study the use of dual disinfectants (particularly T4 + NaOCl treatments) yielded the highest disinfection efficiency, optimizing the elimination of microbial contamination in yam explant cultures. This is in contrast to the report of Hesami et al., (2019), where the sole disinfectant of 1.5% NaOCl at 15 min immersion resulted in 100% explant viability (EV) and no contamination frequency (CF). It contradicts the report of Rodboot et al., (2024) that showed an effective reduction in microbial contamination in *Nymphaea colorata* Peter, a valuable waterlily species, resulting in a 10% contamination rate with a 90% survival rate when disinfected with 0.1% $HgCl_2$ (w/v) for 15 min. It is well-established that NaOCl could be highly effective against various kinds of viruses, fungi, and bacteria (Teixeira da Silva et al., 2016; Hesami et al., 2018), due to its strong oxidizing properties and high reactivity with amides, nucleic acids, amines, and amino acids (Mihaljevic et al., 2013).

Even though higher concentrations of disinfectants with longer exposure could result in better disinfection efficiency, nonetheless, the disinfectants can have a deleterious impact on explant viability, resulting in dehydrated-yellowish explants with low vitality. Hesami et al., (2018), demonstrated that an increase in the concentration of NaOCl and immersion time harmed the explant viability of *Chenopodium quinoa.* Similarly, our results suggest that while dual disinfectants can be effective at eliminating microbial contamination, they also increase the risk of phytotoxic effects, especially when higher concentrations or prolonged exposure times are used. The combination of NaOCl or H_2O_2 with ethanol shows a gradient of damage based on disinfectant strength and duration.

In other plant tissue culture studies, researchers have similarly reported the delicate balance between disinfection efficiency and tissue damage (Teixeira da Silva et al., 2016). Disinfectants like sodium hypochlorite and hydrogen peroxide are commonly used because of their ability to reduce contamination, but they can also cause oxidative stress or disrupt cellular function in plant tissues (López-Galindo et al., 2010; Shu-Hsien et al., 2005).

Our findings on the regeneration of shoots and roots are consistent with other research, such as a study on *Musa* spp. which found that appropriate disinfection protocols, particularly involving NaOCl, could significantly impact shoot regeneration rates without compromising explant health (Ukwueze, 2024). Furthermore, recent studies on the use of hydrogen peroxide have shown it to be effective at controlling contamination, but its oxidative nature can induce tissue stress, leading to reduced regeneration capacity(Benson, 2000). This is particularly important when working with sensitive plant species like yam, where balancing effective disinfection and explant viability is key to optimizing regeneration outcomes. Moreover, while the combination of ethanol and NaOCl are effective in promoting shoot regeneration, our study suggests that higher concentrations or prolonged exposure to disinfectants like NaOCl and H_2O_2 can negatively impact explant viability and limit regeneration potential.

The findings from this current investigation highlight the fine balance needed between effective sterilization and maintaining explant viability for root regeneration. Ethanol, when used in combination with NaOCl (particularly in treatment T4 + T8), was found to provide the best results for root formation. However, treatments with higher concentrations or prolonged exposure to NaOCl and H_2O_2 may lead to tissue stress or even toxicity, preventing root regeneration, as observed in the present study. Our results contribute valuable insights into the optimization of disinfection protocols for yam explants, especially by indicating that a balanced approach − using moderate concentrations and exposure times − can significantly improve root regeneration outcomes.

While the current study employed a minimum of three biological replicates per treatment − consistent with accepted statistical practices for preliminary experiments − we acknowledge that increasing the number of replicates can further enhance the reliability and reproducibility of experimental outcomes. Although scaling up to a larger sample size (e.g., 10 replicates per treatment) was not within the scope of the current experimental design, we recognize its value and consider it a worthwhile approach for future studies to strengthen data consistency and statistical power.

Similarly, an *in vitro* study by (Ditommaso & Nurse, 2004), reported that increased concentration of sodium hypochlorite (NaOCl) inhibited seed germination, seedling growth, and tissue viability at high doses, but was ineffective for tissue sterilizing at low concentrations. While Hesami et al., (2017) showed that an increase in sodium hypochlorite concentration and immersion time had a deleterious effect on the seeds of *Ficus religiosa*, resulting in a blackish colour and a reduced germination rate. Our study contributes to ongoing efforts to refine disinfection protocols by demonstrating that specific dual disinfectant combinations, especially T4 + T8, can support higher shoot regeneration rates. Future studies could investigate further optimization of concentration and exposure times to maximize shoot regeneration while minimizing tissue damage.

Conclusions

This study demonstrated that the optimization of disinfection protocols for yam explants in plant tissue culture is

crucial for maximizing disinfection efficiency (DE) while minimizing negative disinfection effects (NDE) and supporting regeneration potential. The use of dual disinfectants, specifically 70% ethanol followed by NaOCl or H_2O_2, significantly improved DE, achieving over 90% efficiency with NaOCl and 70% with H_2O_2. However, these dual treatments also led to substantial tissue damage, with NDEs reaching 90% and 60%, respectively.

In contrast, sole disinfectant treatments, particularly with C_2H_5OH alone, showed significantly lower DE and NDE, suggesting that while they are milder on explants, they may not be as effective at eliminating contaminants. The results highlight the delicate balance between effective disinfection and maintaining explant viability, where more aggressive disinfectants can compromise tissue health and regeneration outcomes.

Shoot and root regeneration rates were also significantly impacted by the choice of disinfection protocol. The T4 + T8 treatment (ethanol followed by 1.62% NaOCl for 10 min) enhanced the highest rates of both shoot and root regeneration, suggesting that this combination is optimal for maintaining explant viability while ensuring effective contamination control.

Overall, these findings underscore the importance of fine-tuning disinfection protocols to strike a balance between controlling contamination and preserving the regenerative capacity of yam explants. Future studies could focus on further refining disinfectant concentrations and exposure times to minimize NDE while maintaining high DE, offering broader applicability in plant tissue culture for other species.

Acknowledgements

We are grateful for the research materials received from the Semi-Autotrophic Hydroponic (SAH) laboratory, Yam Breeding Department, IITA, Ibadan, Oyo-state, Nigeria, and for infrastructure support at the Department of Biotechnology Plant Tissue Culture Unit Laboratory of the National Center of Genetic Resources and Biotechnology (NACGRAB), Ibadan, Oyo-state, Nigeria, O. O. is grateful to her academic sponsors for their financial support.

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