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Optimization of a Surface Sterilization Protocol and Determination of Hormonal Combination for *In vitro* Propagation of the Aquatic Ornamental Plant *Echinodorus palifolius*

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Abstract

The *in vitro* propagation of *Echinodorus palifolius*, an aquatic plant highly valued in aquascaping, is crucial for meeting its growing demand in the global market. This study aimed to optimize the surface sterilization protocol and hormonal combination to establish *in vitro* propagation of *Echinodorus palifolius* under the specific conditions of Sri Lanka. Various sterilants, including Clorox, ethanol, and hydrogen peroxide, and different time durations were tested for leaves and roots to identify the most effective sterilization method. The study also evaluated the impact of different hormone treatments on root and leaf growth. The findings indicate that a combination of 10% Clorox and 70% ethanol is most effective for root sterilization, while TDZ and 2,4-D hormones show significant promise in inducing root growth. These optimized protocols offer a practical solution for large-scale production, providing a sustainable and efficient method to cultivate high-quality plants for both commercial and research purposes.

Keywords: Echinodorus palifolius; Aquatic; Ornamental; Surface sterilization; In vitro propagation

Introduction

Echinodorus palifolius, commonly known as the Amazon Sword plant, is a popular aquatic plant widely used in aquascaping and as an ornamental species in freshwater aquariums. Native to South America, particularly Brazil, this species is highly regarded for its aesthetic appeal and adaptability to various aquatic environments. As global interest in aquascaping grows, traditional propagation methods, such as seed germination and vegetative cuttings, have proven insufficient to meet the rising commercial demand for high-quality, uniform plants. *In vitro* propagation offers a promising alternative, enabling rapid multiplication under controlled conditions, reducing disease transmission risks, and ensuring plant uniformity. This technique's success, however, relies on optimizing surface sterilization protocols and hormonal treatments, which are critical to preventing contamination and inducing proper plant growth responses. Despite its potential, research on optimizing these protocols for *E. palifolius* is limited, particularly in regions like Sri Lanka, where unique climatic and environmental conditions may require specific adjustments. This study seeks to develop tailored sterilization and hormonal protocols for the successful *in vitro* propagation of *E. palifolius* in Sri Lanka, contributing to the sustainable and efficient production of this valuable aquatic plant.

Materials and Methods

Healthy *Echinodorus palifolius* plants (Figure 1) were selected, focusing on vibrant green specimens. Leaves were cut, washed with tap water, dish soap,

and rinsed. Roots were removed, cleaned with dish soap, rinsed with distilled water, and dried with sterile cotton wool. The leaves were further washed with Teepol, agitated for 10 minutes, and rinsed with distilled water. In a sterile laminar flow hood, leaves were sectioned into 1 cm x 1 cm pieces, including the midrib, and roots were trimmed to uniform sections for *in vitro* propagation.

Figure 1. Echinodorus palifolius mother plant

The MS media preparation for *Echinodorus palifolius* involved two tailored formulations for leaf and root explants. A half-strength MS medium was prepared for leaf explants to prevent osmotic stress, using 50 mL of distilled water, 3 g of sugar, and precise volumes of macro and micro solutions, Fe source, organic solution, and myoinositol. The pH was adjusted to 5.5-5.8, and 0.425 g of agar was added before microwaving for dissolution. The medium was then poured into sterilized tubes and autoclaved at 121°C for 45 minutes. For root explants, a full-strength MS medium with higher nutrient concentrations was prepared using a similar process. Each tube for both media types received 6-8 mL of the prepared medium.

Surface Sterilization

The sterilization of *Echinodorus palifolius* leaf and root pieces involved various methods to assess their effectiveness for *in vitro* growth. In Procedure 1, samples were immersed in 70% ethanol for 1 minute, rinsed with autoclaved distilled water, dipped in fungicide, and placed in growth media tubes. Procedure 2 used a 10% Clorox solution for 10 minutes, while Procedure 3 used 20% hydrogen peroxide for 2 minutes. Procedure 4 combined ethanol and Clorox treatments, Procedure 5 combined ethanol and hydrogen peroxide, and Procedure 6 combined Clorox and hydrogen peroxide. Procedure 7 involved ethanol, Clorox, and hydrogen peroxide treatments sequentially. All samples were rinsed, dipped in fungicide, and placed in media tubes before being stored under appropriate conditions for 28 days to evaluate the effectiveness of each sterilization method in promoting growth.

To optimize the sterilization of *Echinodorus palifolius*, leaves and roots were first cleaned under running tap water to remove superficial contaminants. They were then immersed in a beaker with water and two drops of Teepol, agitated on an orbital shaker for 10 minutes. The leaves were treated with a 10% Clorox solution containing Tween 20, agitated for another 10 minutes, and rinsed thoroughly with distilled water to eliminate any residual chemicals. In a sterile laminar flow hood, the leaves were cut into 1 cm^2 pieces, immersed in 70% ethanol for 1 minute, rinsed three times with autoclaved distilled water, dipped in a fungicide solution, and transferred to labeled growth media tubes. For root sterilization, a similar protocol was followed: the cleaned roots were treated with a 10% Clorox solution with Tween 20, rinsed, sectioned under sterile conditions, treated with 70% ethanol, rinsed, dipped in fungicide, and placed in tubes containing full-strength MS medium. The labeled tubes with treated leaf and root samples were stored in the dark to encourage growth and minimize contamination, with observations made over 28 days to evaluate the effectiveness of the sterilization procedures.

To assess the effectiveness of various Clorox concentrations for sterilizing *Echinodorus palifolius* leaves, four concentrations—5%, 10%, 20%, and 40%—were tested, each supplemented with three drops of Tween 20 to enhance penetration. The leaves were immersed in each Clorox solution, agitated on an orbital shaker for 10 minutes, then

rinsed with distilled water and sectioned into 1 cm x 1 cm pieces under sterile conditions in a laminar flow hood. Following this, the sections were treated with 70% ethanol for 1 minute, rinsed three times with autoclaved distilled water, dipped in a fungicide solution, and placed into labeled growth media tubes. The procedure was identical for all Clorox concentrations, with the sections then stored in a dark environment to encourage growth and minimize contamination. The effectiveness of each sterilization treatment was evaluated over a 28-day period by monitoring the growth and sterility of the samples.

Selecting Suitable Hormones for *Echinodorus palifolius In vitro* Propagation

Three formulations of 100 mL half-strength MS medium were prepared for specific hormonal treatments. For the first formulation, 3 g of sugar was dissolved in distilled water, then 2.5 mL Macro solution, 0.25 mL each of Micro I, Micro II, Fe Source, and Organic solution, 0.5 mL NAA, 2 mL BAP, and 10 mg Myoinositol were added. The pH was adjusted to 5.5-5.8, and the medium was completed with 0.425 g agar before microwaving for dissolution. The second formulation substituted NAA and BAP with 0.1 mL 2,4-D and 2 mL BAP, while the third used 0.1 mL 2,4-D and 0.2 mL TDZ. Each medium was dispensed into tubes (6-8 mL per tube) and autoclaved at 121°C for 45 minutes. After identifying the optimal sterilization method, leaf samples were treated with a 20% Clorox solution and three drops of Tween 20, agitated for 10 minutes, rinsed with distilled water, and sectioned under sterile conditions. Following a 1-minute immersion in 70% ethanol and a triple rinse, the leaves were treated with fungicide and placed in labeled media tubes for a 28-day observation period.

Three full-strength MS media formulations were prepared as follows: First, 3 g of sugar was dissolved in distilled water at 400 rpm. For the first medium, 5 mL of Macro solution, 0.5 mL each of Micro I, Micro II, Fe Source, and Organic solution, 0.5 mL NAA, 2 mL BAP, and 10 mg Myoinositol were added. The pH was adjusted to 5.5-5.8, and the volume of the solution was adjusted to 100 mL with distilled water before adding 0.425 g agar. After microwaving to dissolve the agar, the medium was dispensed into tubes (6-8 mL per tube) and autoclaved at 121°C for 45 minutes. The second medium substituted NAA with 0.1 mL 2,4-D, while the third medium replaced NAA and BAP with 0.1 mL 2,4-D and 0.2 mL TDZ, following the same preparation and sterilization protocol. Root samples were treated with a 10% Clorox solution containing three drops of Tween 20, agitated for 10 minutes, rinsed with distilled water, and sectioned under sterile conditions. After a 1-minute immersion in 70% ethanol and a triple rinse, the roots were treated with fungicide and placed in labeled media tubes for a 28-day observation period.

Results and Discussion

Surface Sterilization

Leaves: As seen in Figure 2, the combination of 10% Clorox followed by 70% ethanol was the most effective in reducing contamination, with a contamination rate of only 5%. This protocol also maintained a high survival rate of 90%. In contrast, treatments using only ethanol or hydrogen peroxide resulted in higher contamination rates, with the hydrogen peroxide treatment showing a 25% contamination rate and a lower survival rate of 70%.

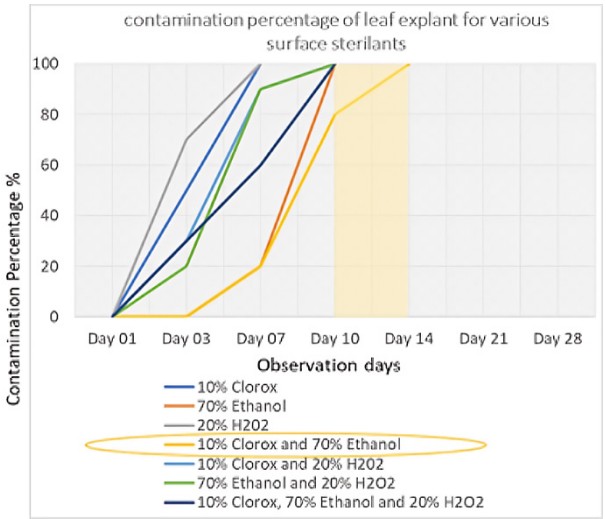

Figure 2. Contamination percentage of leaf explant for various surface sterilants

Roots: As seen in Figure 3, similar to the leaves, the root explants showed the best results when treated with 10% Clorox followed by 70% ethanol. The contamination rate was 8%, and the survival rate was 85%. Other treatments, especially those using only Clorox or hydrogen peroxide, were less effective, with contamination rates reaching up to 30% and survival rates dropping to 60%.

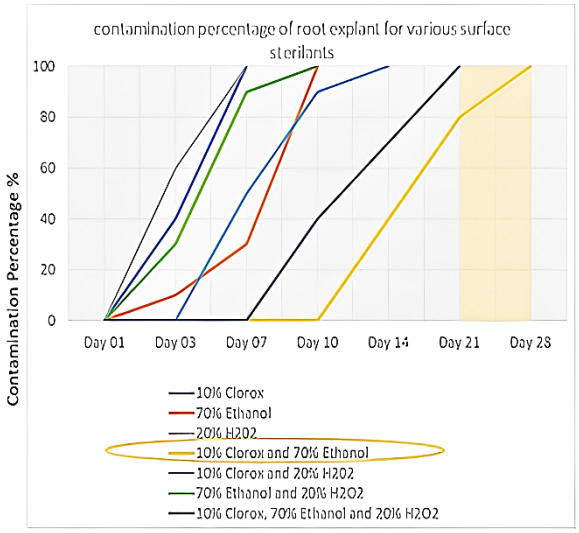

Figure 3. Contamination percentage of root explant for various surface sterilants

Optimizing Sterilization of *Echinodorus Palifolius*

The refined sterilization protocol using a combination of 10% Clorox and 70% ethanol effectively extended the sterility of *Echinodorus palifolius* cultures. Initially, both leaf and root explants remained sterile up to the seventh day. However, by day seven, contamination appeared in three leaf tubes, while root cultures stayed uncontaminated. Contamination in leaf cultures increased by day ten, with additional tubes affected, while root cultures continued to be free of contamination. By day fourteen, five leaf tubes were contaminated, but no further contamination occurred beyond this point, and root cultures remained unaffected throughout the 28-day period (Figure 4). This optimized protocol notably enhanced sterility for roots, though leaves still experienced some contamination, indicating a need for further refinement.

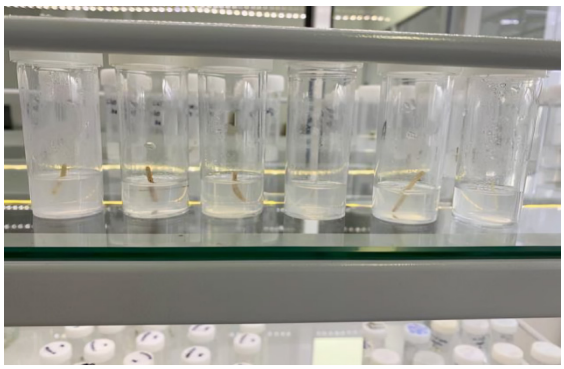

Figure 4. Survived root explant after 28 days

Concentration Refinement for Sterilization of Leaves

To optimize the sterilization process for *Echinodorus palifolius* leaves, various Clorox concentrations (5%, 10%, 20%, and 40%) combined with 70% ethanol were tested over 28 days. As seen in figure 5; the 5% Clorox concentration led to contamination in five tubes by day three and worsened by day seven. The 10% Clorox also proved inadequate, with contamination in two tubes by day seven and five by day fourteen. Although the 40% Clorox showed initial promise, it caused contamination in two tubes by day fourteen and phytotoxicity, indicated by darkened leaves. The 20% Clorox concentration, despite resulting in six contaminated tubes by day twenty-eight, effectively maintained sterility in four tubes without adverse color changes throughout. This suggests that while lower Clorox concentrations are insufficient and higher concentrations are phytotoxic, (Figure 6) 20% Clorox offers a balance between effective sterilization and reduced phytotoxicity.

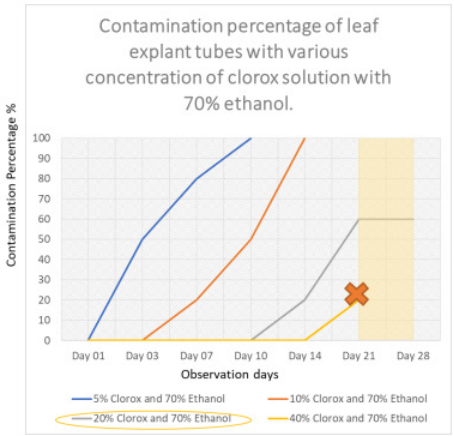

Figure 5. Contamination percentage of leaf explant tubes with various concentration of clorox solution with 70% ethanol.

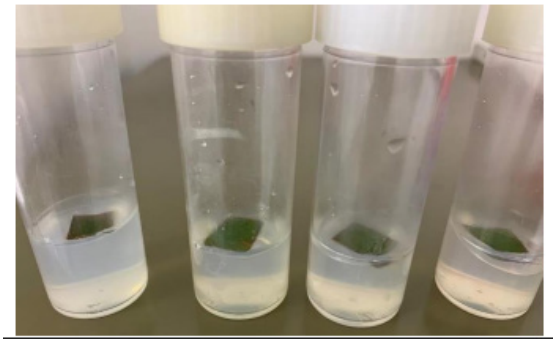

Figure 6. Survived leaf explant under 20% Clorox and 70% ethano

Hormonal Treatments and Growth Response

The final stage of the research evaluated hormone combinations for inducing root growth in Echinodorus palifolius in vitro. Three combinations were tested: NAA with BAP, TDZ with 2,4-D, and 2,4-D with BAP. After 28 days, the NAA and BAP combination resulted in four tubes with green roots (Figure 7), indicating successful root development.

The TDZ and 2,4-D combination was most effective (Figure 8), producing green roots in six tubes, likely due to TDZ's strong cytokinin-like activity enhancing cell division and differentiation more than BAP. The 2,4-D and BAP combination showed moderate success with three tubes exhibiting green roots (Figure 9). In a similar evaluation of leaf culture survival, five uncontaminated tubes remained at the end of the period: two with NAA and BAP (Figure 10), and three with TDZ and 2,4-D (Figure 11).

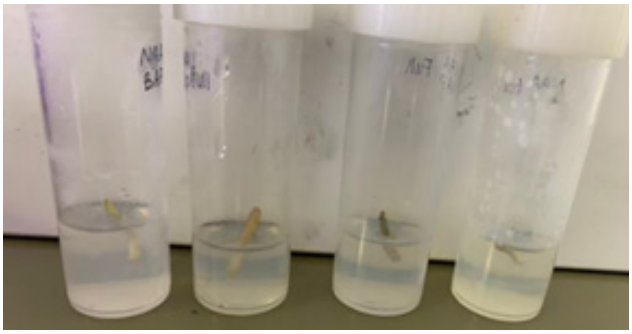

Figure 7. Lightly green colored roots with NAA and BAP

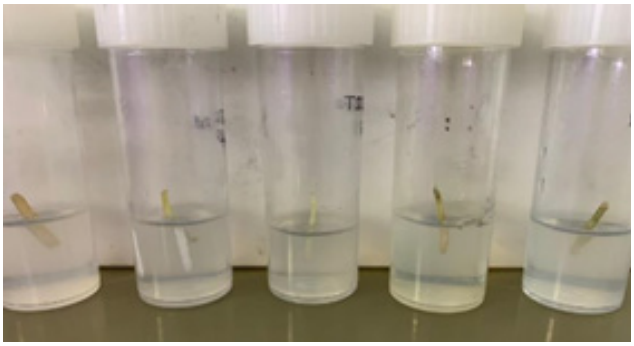

Figure 8. Lightly green colored roots with 2,4-D and TDZ

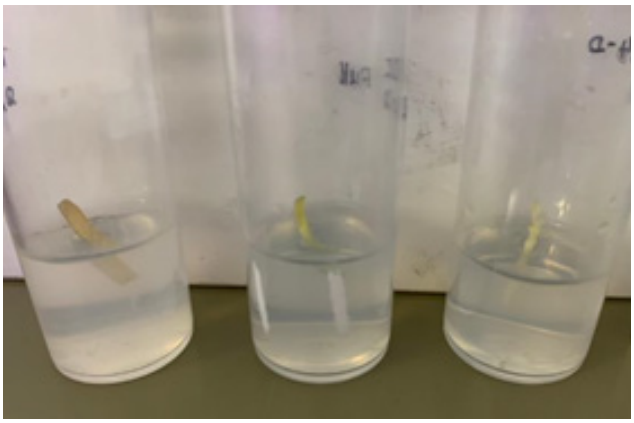

Figure 9. lighlty greeny colored rootS with BAP and 2,4-D

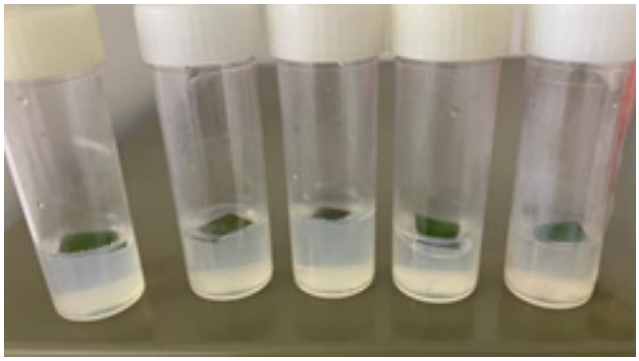

Figure 10. Survived leaf explants with NAA and BAP

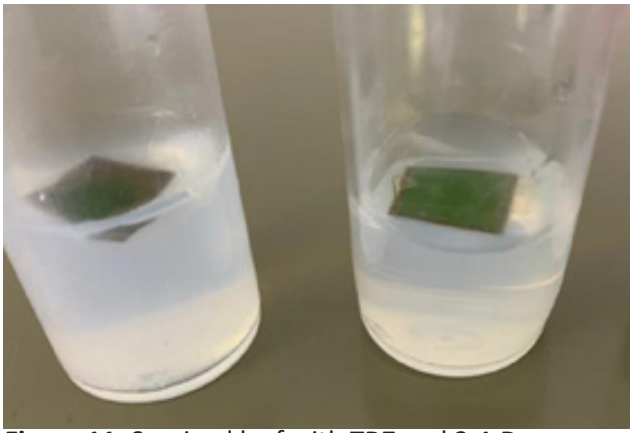

Figure 11. Survived leaf with TDZ and 2,4-D

Discussion

This research aimed to develop an effective in vitro culture protocol for Echinodorus palifolius by optimizing sterilization techniques and hormone treatments. Initial trials focused on evaluating different sterilants, including 70% ethanol, 10% Clorox, and 20% hydrogen peroxide, to determine their efficacy in maintaining sterility while preserving plant tissue viability. The combination of 10% Clorox and 70% ethanol was particularly successful for root cultures, which remained uncontaminated for the entire 28-day observation period. However, leaf tissues proved more challenging to sterilize, exhibiting a higher susceptibility to contamination.

To address this, the study refined the sterilization protocol, especially for leaves, by adjusting Clorox concentrations. A 20% Clorox solution emerged as a promising option, striking a balance between antimicrobial efficacy and phytotoxicity, though it did not completely eliminate contamination. The research highlighted the inherent difficulties in sterilizing leaf tissues compared to roots, suggesting that further optimization is necessary to achieve consistent sterility for all plant parts.

Hormonal studies were also conducted to identify the most effective combinations for inducing root and leaf growth. The combination of TDZ with 2,4-D was the most successful for root induction, while leaf cultures remained problematic due to contamination. These findings underscore the need for tailored approaches in plant tissue culture, considering the specific requirements of different tissues to achieve reliable and contamination-free propagation of Echinodorus palifolius.

Conclusion

This study comprehensively investigated the in vitro propagation of Echinodorus palifolius, focusing on the intricate processes of sterilization, tissue induction, and hormonal influence. The research advanced sterilization protocols by demonstrating that while a combination of 10% Clorox and 70% ethanol effectively maintained sterility in root cultures, a 20% Clorox and 70% ethanol mix showed better results for leaves. The study also identified effective hormonal combinations, such as TDZ and 2,4-D, which promoted healthy root growth, emphasizing the importance of hormonal balance in tissue culture. Additionally, the exploration of various Clorox concentrations for leaf sterilization revealed a delicate balance between antimicrobial efficacy and plant tissue tolerance, providing critical insights for future protocols. Overall, the findings not only contribute valuable knowledge to plant tissue culture but also underscore the complexities of aquatic plant culture, paving the way for further innovation in the field.

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