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A Novel Surface Sterilization Technique for in vitro Establishment of *Dianella tasmanica variegata* Nodal Explants

Malwattage, G., Ratnayake, R.D., Seneviratne K. L., Peiris, S.E* and Peiris, B.C.N.

Faculty of Humanities and Sciences, Sri Lanka Institute of Information Technology (SLIIT), New Kandy Road, Malabe, Sri Lanka
**sriyani.p@sliit.lk*

ABSTRACT

Dianella tasmanica 'variegata' is one of the popular species which is exported as ex-agar plants from Sri Lanka. In order to promote dianella exports, micropropagation should be increased to produce a high-quality large number required by the export markets. However, severe microbial contaminations in the in vitro establishment have become the bottle neck for large scale in vitro propagation of this species. Therefore, this study was undertaken with the objective of using sulfur nanoparticles (S-NP) to eliminate surface adhered fungal and bacterial contaminants to obtain a vast number of contamination-free cultures at the in vitro establishment stage. Apical parts of about 6 cm of *D. tasmanica* were used in this study. Effects of S-NP solution in 500 mg/L was used with the control of 10% Clorox™ for the surface sterilization of the nodal explants. Results revealed that 500 mg/L S-NP produced 80% and CloroxTM produced 40% contamination-free cultures after 4 weeks of establishment in vitro. The experiment was repeated twice. This study suggests that S-NP is a promising low-cost non-toxic material that can be used in the surface sterilization of dianella nodal explants.

Keywords: *Dianella tasmanica variegata; In vitro propagation; Sulfur nanoparticles (S-NP); Surface sterilization.*

INTRODUCTION

Plants from the Asphodelaceae family are popular as ornamental and medicinal plants. *Dianella* (also known as flax-lilies) is a well-known genus as an ornamental plant (Gilmour, 2006). As currently known, *Dianella* is a genus comprising 25–30 species ranging from southern Africa to South East Asia, Hawaii, the South Pacific islands, Australia, and New Zealand. The name of the genus is taken from the Roman goddess Diana (Chaudhuri et al., 2017). The Tasmanian flax-lily (*Dianella tasmanica variegata*) is a sedge-like, rhizomatous, herbaceous strappy perennial herb in the Asphodelaceae subfamily Hemerocallidoideae (Ahmed et al., 2021). Seed germination studies have been conducted to test the feasibility of the *Dianella tasmanica* micropropagation (Gilmour, 2006). Furthermore, it has been reported that they are afflicted with numerous fungal, bacterial, viral, and nematode damages, as reported from many areas of the world including India (Chaudhuri et al., 2017).

Plant micropropagation is a technique in which the cells, tissues or organs of chosen plants are separated, sterilized, and cultured in a growth-promoting aseptic environment to generate many clonal plantlets. Microbial contaminations in cultures, particularly during the in-vitro establishment stage, are one of the most severe issues in plant micropropagation. Surface sterilization eliminates epiphytic microbes from the explants. However, endophytic microbes in the explants cause contamination in the culture (Gupta et al., 2020). Therefore, explants must be disinfected before culturing to avoid this scenario.

Chemicals such as sodium hypochlorite (NaOCl), calcium hypochlorite ($Ca(OCl)_2$), ethanol, mercuric chloride ($HgCl_2$), benzalkonium chloride and hydrogen peroxide (H_2O_2) are the common disinfectants used for surface sterilization of explants (Peiris et al., 2020; Silva et al., 2015). However, most laboratories employ NaOCl or $Ca(OCl)_2$ different commercial bleaches (Mihaljevic et al., 2013) along with fungicides

and antibiotics (Parzymies, 2021). Contaminations must be eliminated without damaging plant cells since these chemicals are harmful to plant tissues (Mihaljevic et al., 2013). However, Nanoparticles (NP) have been shown a promising surface sterilization technique for various explants (Álvarez et al., 2019). For example, silver nanoparticles (Ag-NPs) have a strong capacity to remove microbial contaminants while having no detrimental impacts on plant growth and development (Sarmast et al., 2011). Our previous publication described the antibacterial properties of nanomaterials such as TiO_2, Fe_2O_3 and ZnO, and the capability of using nanoparticles for sterilization applications in the micropropagation technique (Seneviratne et al., 2021).

Sulfur nanoparticles (S-NP) were also shown extremely high antibacterial and antifungal properties (Choudhury et al., 2010; Gogoi, 2013; Rao and Paria, 2013; Roy Choudhury et al., 2011; Saedi et al., 2020; Suleiman et al., 2014; Yela et al., 2016). There were no previous investigations on the surface sterilization of explants using S-NP. Therefore, attempt of using S-NP for surface sterilization agent for plant tissues is a novel idea. Thus, current study was carried out to investigate the capability of S-NP as a surface sterilization agent on *Dianella tasmanica variegata* as an explant for micropropagation. Also, there were only a few publications about *Dianella* micropropergation. Hence, this study will help plant growers to initiate *Dianella* propagations and obtained high quality clones of mother plants without contaminations from microorganisms.

MATERIALS AND METHODS

Chemicals

The chemicals utilized in this investigation were sodium thiosulphate pentahydrate ($Na_2S_2O_3.5H_2O$) from Daejung Chemicals, Korea, 37% hydrochloric (HCl) acid from Sigma-Aldrich and commercially available sodium hypochlorite (CloroxTM) also used in this experiment.

Sulfur Nanoparticle (S-NP) Synthesizing Method

A $Na_2S_2O_3.5H_2O$ (1M) solution was prepared using 62.04 g of $Na_2S_2O_3.5H_2O$ solid crystals dissolved in 250 mL distilled water. The mixture was magnetically stirred at 500 rpm to dissolve crystals and heated (60 °C) the solution. The solution was then mixed with 125.0 mL of 1M HCl solution while being constantly stirring and heating. A white color turbidity was immediately formed with a few drops of 1M HCl and then turned into yellow particles, the reaction was terminated after 40 minutes. The yellow precipitate (S-NP) (Figure 1) was collected, washed with distilled water (3-5 times) and dried at 70 °C for 2 hours (Li et al., 2013; Suleiman et al., 2014). Ultimately ground the S-NP into a fine powder using mortar and pestle.

Figure 1: Synthesized S-NP

Characterization of S-NP

X-ray Diffractometer (XRD) analysis was carried out using Rigaku Ultima-IV with Cu Kα radiation (λ=1.5405 Å, 30 mA, 40 kV), the scanning rate of 4° min-1 within the range of 10-80° to determine the crystallographic characteristics of synthesized S-NP.

Surface Sterilization of Dianella Explant Experiment

This experiment focused on the effectiveness of S-NP in surface sterilization against fungal/bacterial species identified from recent publications and standards (Kumar et al., 2019), which severely damaged in-vitro grown explants. About 6-10 cm cuttings of the top part of dianella nodes were collected from the plants growing in a protected house. The stems were brought into the tissue culture laboratory after removing all the leaves. They were pre-washed with liquid soap (Dettol™ hand wash) and tap water by rubbing gently. These nodal stems were separated into single nodes to have

approximately 1 cm segment. Then they were divided into four groups to include 10 nodes per group. Nodes in all groups were thoroughly rewashed with tap water for 5 minutes and placed in a beaker with 400 mL deionized (DI) water with 1-2 drops of liquid soap and vigorously agitated for 30 minutes using an orbital shaker at 180 rpm, then soapy water was drained off and nodes were washed three times using DI water to remove excess soap from plant tissues. Finally, the 30 nodes were exposed to S-NP material in three-time durations as the treatments and 10 nodes were exposed to 10% Clorox as the control as described below.

The sterilization experiment was carried out with ten dianella nodes exposing to three-time durations, 10, 20 and 30minutes, separately in 100 ml of S-NP (500 mg/L) solution. The nodes in the S-NP solution were washed using a magnetic stirrer with a speed of 400 rpm. The nodes sterilized using S-NP were cultured without washing with sterilized water.

As the control experiment, 10% CloroxTM was used. Plant nodes were soaked for 15 min in 100 mL of 10% Clorox solution with two drops of liquid soap by agitating the mixture at 180 RPM on an orbital shaker. This washing mixture was decanted and repeated the washing step for nodal explants again with fresh 10% Clorox for 15 min and discarded the solution. Then the nodes were thoroughly rinsed three times with sterilized water.

Murashige & Skoog agar medium, (Murashige and Skoog, 1962) supplemented with 3% sugar, 100 mg/L myo inositol, 1 mg/L benzyl amino purine (BAP), 5 µL of fungicide (Folicur tebuconazole EW) was prepared, adjusted pH to 5.8. The medium was boiled for 2-3 minutes at 100 °C in a microwave oven to melt the agar. The culture tubes were sterilized using 5% Clorox solution, this sterilizing method is known as the CSUP technique (Peiris et al., 2012) and 6 mL of agar medium was poured into each tube and capped. Similarly, 240 mL of media was poured into 40 tubes. The tubes were kept at room temperature (25 °C) to solidify the media. This medium was used to culture the nodal explants of the S-NP and 10% Clorox surface sterilization treatments. The cultures were placed in the growth room at 25 °C and illuminated 14 h/d with 1500 Lux provided by LED (Light-emitting diode) lamps. This experiment was repeated twice. The number of cultures that survived without microbial contaminations was recorded weekly and final data was obtained after 4 weeks from culture establishment.

Statistical Analysis

The experiment samples were arranged using a complete randomized design. Chi-square ($\chi 2$) test was used for S-NP treatments to determine whether treatment is responsible or not for elimination of fungal/bacterial growth inhibition at the sterilization.

RESULTS AND DISCUSSION

Synthesis of S-NP

According to reaction 1, S-NP was produced by the redox reaction of $Na_2S_2O_3.5H_2O$ and 1M HCl. H^+ ion initiates the reaction by breaking down the thiosulfate molecule and generating S-NP and other by-products.

$$Na_2S_2O_3\,(aq) + HCl\,(aq) \xrightarrow{\Delta} S\,(s) + SO_2\,(g) + NaCl\,(aq) + H_2O\,(l)$$

Reaction (1)

After the reaction, particles were washed thoroughly to eliminate impurities (such as NaCl) and filtered. The S-NP were collected in high yield.

XRD Analysis of synthesized S-NP

The XRD examination of the synthesized S-NP diffraction peaks is shown in Figure 2. All peak positions (2-theta) were compared with XRD ICDD database PDF number 00-024-0733. According to the Debye–Scherrer formula (Seneviratne et al., 2021) the average crystallite size of the synthesized S-NP is around 5.9 nm. There were no impurity peaks apart from sulfur. It suggests that nanoparticle washing removes impurities like sodium chloride (NaCl) from the nanoparticle surface. We do not use ethanol-water for this. We use the gravity filtration method to wash sulfur particles. Therefore, our synthesis and washing method is low-cost and more effective than expensive chemical treatments.

Experiment on surface sterilization of nodal explants

Evaluation of the effects of S-NP solution in the in vitro initiation stage of *D. tasmanica 'variegata'* nodal explant showed that this material is an effective surface sterilization agent. The *D. tasmanica* nodal explants exposed to S-NP 500 mg/L solution for 10 minutes produced the highest survival percentage (80%). The nodes exposed to 20 minutes produced 60% uncontaminated cultures whereas the nodes exposed to 30 minutes had the survival rate of 50%. The control treatment, 10% Clorox, produced survival rate of 40% of *Dianella* nodes (Figure 3).

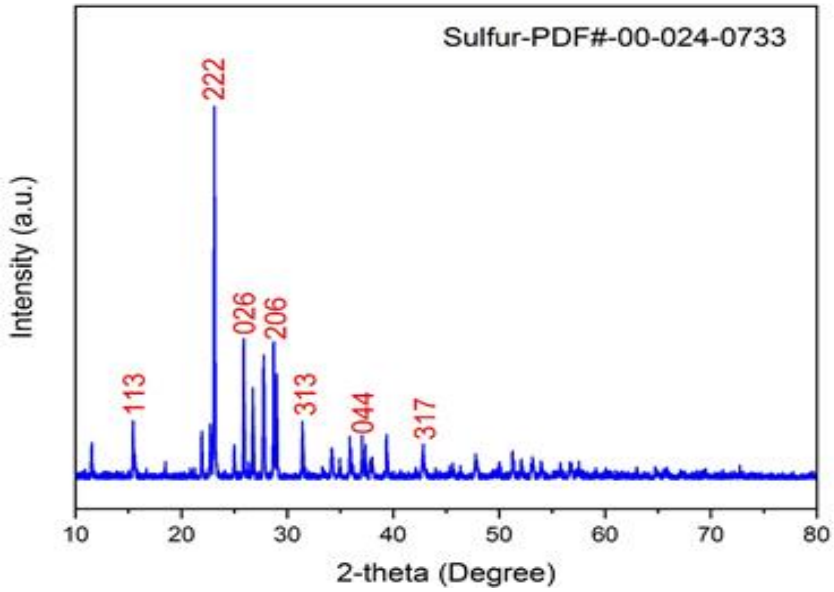

Figure 2: XRD Pattern of Synthesized S-NP.

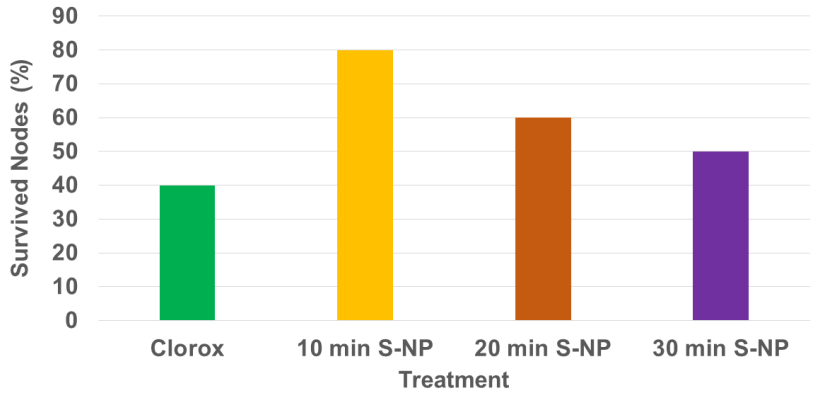

Figure 3: Survival Percentage of *Dianella* Explant within One Month Period (n=10).

Statistical Analysis of Surface Sterilization

SPSS analysis indicated critical value as 3.84. The calculated value was 4.44 in our experiment. According to the chi square table, the nodal culture survival rate and the treatments' chi-square significance value (p-value) was 0.0261. Thus, the results of the chi-square analysis showed that the p-value is more than our chosen significance level α = 0.05. Hence, the relationship between survival rate and treatments was statically significant. Therefore, we can reject the null hypothesis and conclude that there is an association between treatment and the nodal culture survival rate.

Figure 4: *Dianella* node after one month period from establishment.

There is little information regarding surface sterilization methods of *D. tasmanica 'variegata'* and none of them has used S-NP in tissue culture of Asphodelaceae family plants. In a study it is reported that dianella micropropagation using rhizome pieces, which were surface sterilized with 100% ethanol and 2% (w/v) NaOC1+ 0.1% (v/v) Tween 20 detergent. After one month, 30% of rhizome generated the shoots without any contaminations. However, they failed to achieve a high number of contamination-free cultures using the reported surface sterilization method (Gilmour, 2006). Our novel sterilizing method survived a comparatively high number of cultures (80%) by treating the explant nodes with 500 mg/L S-NP containing solution for 10 min washing (Figure 4). Therefore, our novel S-NP sterilization techniques comprehensively worked for *D. tasmonica* node sterilization in in-vitro culture establishment.

CONCLUSIONS

Sulfur, an antimicrobial agent is a promising chemical treatment that enables inhibition of microbial contamination in culturing nodes. We used 500 mg/L S-NP solutions to sterilize plant nodes and obtain a success percentage of 80% contamination-free cultures for 10 min sterilization, 60% for 20 min and 50% for 30 min in 4 weeks' time period. In comparison, Clorox only survived 40% of nodal cultures at the same duration. Thus, suggesting antimicrobial characteristics in sulfur is a promising phenomenon that can sterilize plant nodes. We utilized a very low amount (50 mg) of S-NP to sterilize 10 nodes, thus lowering the cost of plant production and the non-toxic effect can be beneficial in this experiment. Ultimately, we suggest that using S-NP to sterilize explants is more effective than the conventional Clorox techniques in the plant tissue culture industry.

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