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# Investigation of Measures to Control Epiphytes and Endophytes Present in Nodal Explants of *Persea Americana* Mill in Its *in Vitro* Establishment

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## Abstract

Significant advancements in micropropagation methods facilitate the commercial cultivation of avocado (Persea americana Mill), a remarkable tropical and sub-tropical fruit, highly recommended for its nutritional value. Since high quality, true-to-type planting materials are required to promote the commercial production of avocado, this study was conducted with the objective of investigating an effective sterilization method to control both epiphytes and endophytes present in nodal explants of P. americana cv. 'Pollock' to optimize in vitro establishment measures which will lead to popularize its micropropagation. In this study, nodal segments of approximately 10 cm from softwood stem cuttings of P. americana cv 'Pollock' were used to obtain explants. The effects of 10%, 20%, and 30% concentrations of sodium hypochlorite (NaOCl) were evaluated, with exposure durations of 10 and 20 minutes, to determine their efficacy in eliminating epiphytes during vitro establishment. Additionally, the nodal explants were treated with 100 mg/L Augmentin™ for 20 - minutes and 30 - minutes periods respectively, as a final dip before culturing to eradicate endophytes. The effects of three different culture media, half-strength Murashige and Skoog (MS) medium, Woody Plant Medium (WPM), and Gamborg's medium (B5) were also investigated. The highest survival rate (83%) of nodal segments was achieved when explants were sterilized with 20% NaOCl for 20 minutes, followed by a final sterilization step involving a 30-minute dip in Augmentin™, and subsequently cultured in WPM medium.

***Keywords:*** Avocado; In vitro propagation; Nodal segments; Sodium Hypochlorite

## Introduction

Avocado, a leading tropical and sub-tropical fruit crop, is known for its nutritional value and economic influence. The global Gross Production Value of avocado in 2018 was about 5.812 billion USD, making it a major global crop (Bhore, et al., 2021). The avocado plant, P. americana Mill., is a member of the Lauraceae family. It is one of the finest salad fruits, the most nutrient-dense of all fruits, and can provide to the human diet (Purseglove (1991). Avocado's commercial significance, high nutritional value, energy, and monounsaturated oil content have sparked increased interest in its development due to global market demand and the need for sustainable propagation techniques (Ahmed, 2002). A viable alternative for the fast multiplication of avocado trees with desired characteristics while retaining genetic consistency is micropropagation. In vitro cultured plants are also promising root stocks to consider in plant propagation through grafting (Hiti- Bandaralage, et al., 2022). The capability of plant tissues to reproduce new shoots

and the growth of adventitious roots depend on the collaboration of several endogenous and exogenous variables (Ahmed, 2002). With limited shoot multiplication, elongation of current buds, and creation of scaly leaves, avocado shoot regeneration from shoot tips and nodal explants is quite challenging (Barringer et al., 1996). Plant growth regulators also have stimulating effects on the capability of plants to induce novel roots and shoot regeneration in addition to the explant source (Pospíšilová, 1999). This study was conducted to support sustainable production and expand in vitro propagation methods for P. americana with the objective of controlling epiphytes and endophytes present in nodal explants to achieve successful in vitro establishment.

**Materials and Methods**

**Collection of plant materials**

Healthy budded plants of P. americana cv. 'Pollock' were purchased from a reputed plant nursery for this study. The axillary and topmost buds were used as explants from approximately 10 cm in length of the growing shoot tips.

Explant surface sterilizationThe harvested shoots were soaked in a tebuconazole fungicide solution, with a concentration of 500 µl/L, for 2 hours. Then the leaves of the stem cuttings were removed, washed in tap water, and placed in a soap and fungicide (50 µl/L) solution. They were then agitated at 100 rpm on a shaker for 1 hour. The solution was then decanted, and the explants were sterilized using three different concentrations (10%, 20%, and 30%) of NaOCl (Clorox TM) solutions for two different time periods (10 minutes and 20 minutes) with agitation at 180 rpm on an orbital shaker. The solutions were agitated in two steps: first, each concentration was agitated for half the duration, the solution was decanted, and a fresh solution of the same concentration was added and agitated at the same speed again for the remaining time. These sterilized explants were rinsed three times with autoclaved distilled water inside the laminar airflow cabinet. The damaged edges were trimmed, and nodes were cut into 1-2 cm segments with axillary buds. The explants were divided into two groups and dipped in a solution of AugmentinTM (100 mg/L) for two different time periods: 20 minutes and 30 minutes.

***In vitro* establishment**

After dipping in the antibiotic solution, the explants were cultured as one nodal section per tube filled with 8 mL of semi-solid medium. There were three different media types: ½ MS medium (Murashige and Skoog, 1962), WPM (McCown and Lloyd, 1981), and Gamborg B5 medium (Gamborg et al., 1968). All media were supplemented with 1 mg/L benzyl amino purine (BAP), 3% sugar, 6 µL/L fungicide, 2 g/L charcoal, and solidified with 4.25 g/L agar, with a pH of 5.6-5.8. 1 mg/L BAP is commonly used in avocado in vitro propagation because it optimally promotes shoot multiplication while minimizing negative effects. The total number of samples for the initial establishment experiments depended on the amount of material that could be taken from the mother avocado plants. The medium was sterilized using the CSUP media sterilization method (Peiris et al., 2012). The cultures were kept in the growth room at 25±2°C with illumination provided by light-emitting diode (LED) lights at 2000 lux for a 14/8 h (day/night) photoperiod. The cultures were randomly placed using a completely randomized design. The investigation was conducted with 4 experiments using NaOCl (Clorox™) and antibiotic (Augmentin™) (Table 1).

***Table 1.*** The experiments that were conducted in the investigation

| Experiment | NaOCl Concentration | Time Period | Media |
|---|---|---|---|
| **Experiment 1** | 10% NaOCl | 10 min | ½ MS |
| | 10% NaOCl | 20 min | ½ MS |
| **Experiment 2** | 20% NaOCl | 10 min | ½ MS, WPM, B5 |
| | 20% NaOCl | 20 min | ½ MS, WPM, B5 |
| **Experiment 3** | 30% NaOCl | 10 min | ½ MS, WPM, B5 |
| | 30% NaOCl | 20 min | ½ MS, WPM, B5 |
| **Experiment 4** | 20% NaOCl | 10 min | WPM |
| | 20% NaOCl | 20 min | WPM |

The survival rate was calculated taking both the contaminated and dead cultures into consideration after 3 months of explant establishment. The data were not statistically analyzed as there was an obvious difference in the results generated.

**Results and Discussion**

**Experiment No. 1**

Sodium hypochlorite at 10% concentration showed 20% survival rate in the exposure time of 20 minutes and antibiotic exposure of 30 minutes. This is due to lack of strength in NaOCl which did not destroy the microorganisms present on the surface of nodal tissues. This may be due to woody explant nodes harboring spores of microorganisms which did not destroy with low concentration of NaOCl and may have grown in the nutrient rich culture medium under conducive in vitro environment (Table 2).

***Table 2.*** Survival rate of the Experiment 1 to check the suitable Sodium hypochlorite exposure

| Sodium Hypochlorite (%) | Sodium Hypochlorite exposure time (min) | Antibiotic Dipping time (min) | Survival Percentage (%) |
|---|---|---|---|
| 10 | 10 | 20 | 0 |
| 10 | 10 | 30 | 0 |
| 10 | 20 | 20 | 0 |
| 10 | 20 | 30 | 20 |

**Experiment No. 2**

In this experiment explants exposed to 20% NaOCl at 20 minutes time exposure and antibiotic dipping time of 30 minutes gave promising results having 83% survival rate in the WPM culture medium. In the same culture medium explants exposed to 20% NaOCl for 20 minutes with 20 minutes exposure to antibiotic produced 20% survival rate. Explants in Gamborg culture medium exposed to 20% NaOCl in 20-minute duration and in 30-minute antibiotic dipping time showed 10% explant survival. The survival rate was counted using healthy growing cultures free from microorganisms. Though some cultures were not contaminated they converted to brown in color and died within few weeks of establishment. This was highly observed in ½ MS medium as well as B5 medium. It is suspected that there had been toxic effects of nutrients which trigger death of explants. The WPM medium has been used for woody plants in vitro culturing successfully as the nutrients included in the medium were more suitable to their in vitro establishment and growth (Jeyaram,et al.,2022; Song et al, 2021b).

***Table 3.*** Survival rate of the nodal explants exposed to higher concentration using three different media at 20% Sodium hypochlorite

| Medium | Sodium Hypochlorite conc.% | Sodium Hypochlorite exposure time (min) | Antibiotic Dipping time(min) | Survival percentage (%) |
|---|---|---|---|---|
| ½ MS | 20 | 10 | 20 | 0 |
| | 20 | 10 | 30 | 0 |
| | 20 | 20 | 20 | 0 |
| | 20 | 20 | 30 | 0 |
| WPM | 20 | 10 | 20 | 0 |
| | 20 | 10 | 30 | 0 |
| | 20 | 20 | 20 | 20 |
| | 20 | 20 | 30 | 83.3 |
| B5 | 20 | 10 | 20 | 0 |
| | 20 | 10 | 30 | 0 |
| | 20 | 20 | 20 | 0 |
| | 20 | 20 | 30 | 25 |

**Experiment No. 3**

The results showed that the nodal explants cultured in WPM medium after exposing to 30 minutes in 20% NaOCl and dipped in Augmentin antibiotic for 30 minutes produced 50% survival. The ½ MS medium also showed 33% survival rate at NaoCL 30% with exposure time 20 minutes and antibiotic dipping time 30 minutes. When sodium hypochlorite concentration increased to 30% the survival rate differed, and WPM medium did not perform well. Also, the other two media used had varying results producing low rates of nodal explant survival (Table 4).

***Table 4.*** Survival rate of the nodal explants exposed to higher concentration using three different media at 30% Sodium hypochlorite.

| Medium | Sodium Hypochlorite conc. (%) | Sodium Hypochlorite time (min) | Antibiotic Dipping time(min) | Survival Percentage (%) |
|---|---|---|---|---|
| 1/2MS | 30 | 10 | 20 | 0 |
| | 30 | 10 | 30 | 0 |
| | 30 | 20 | 20 | 0 |
| | 30 | 20 | 30 | 33.3 |
| WPM | 30 | 10 | 20 | 0 |
| | 30 | 10 | 30 | 0 |
| | 30 | 20 | 20 | 0 |
| | 30 | 20 | 30 | 50 |
| B5 | 30 | 10 | 20 | 0 |
| | 30 | 10 | 30 | 0 |
| | 30 | 20 | 20 | 0 |
| | 30 | 20 | 30 | 0 |

**Experiment No. 4**

This experiment was conducted to confirm the results obtained in experiment 2. The results also showed a similar trend producing 80% survival rate with 20% NaOCl in 20 minutes and with the final dipping of 30 minutes in antibiotics (Table 5).

***Table 5:*** Survival rate of the nodal explants exposed to 20% NaOCl concentration with 20 minutes exposure time and 30 minutes antibiotic dip.

| Clorox Conc. % | Clorox Time | AB Time | Survival Percentage (%) |
|---|---|---|---|
| 20 | 20 | 30 | 80 |

As for the culture medium, the woody plant medium was found to be the most effective. The 4th experiment was conducted only on WPM medium with the combination which gave best results. It is possible that higher NaOCl concentrations caused toxicity, which had a negative effect on the explants' ability to survive. The results confirmed that while NaOCl can effectively reduce the rate of contamination, explants are severely damaged by its greater concentration (Nhut et al., 2008). The explants were dipped in an antibiotic solution, varying the dipping time (20 min and 30 min). There were less bacterial infected explants were found, indicating that the concentration of Augmentin, 100mg/L, was sufficient for effective sterilization. The dipping process effectively sterilized the explants due to antibiotic penetration. In some cases, both 20-minute and 30- minute dips produced bacterial contamination, with no significant benefit from the extra 10 minutes. Obtaining sterile plant material free of contaminants like bacteria and fungi is challenging, especially when dealing with woody plant material, making it essential to eliminate external contaminants (Niedz & Bausher, 2002). Woody plants often become highly contaminated with both endogenous and exogenous microorganisms, making it challenging to regulate in vitro due to their prolonged soil growth under ambient conditions (Ahmad

et al., 2003). The most essential step in setting up an in vitro culture is to discover a safe sterilizing agent that can eradicate microorganisms from the explant tissue (Ahmad et al., 2003). To efficiently sterilize surfaces of the explants, the concentration of NaOCl is an essential element. The highest number of surviving nodal segments was found in woody plant medium, followed by 1/2 MS and Gamburg's B5 medium, when avocado nodes were cultured in growth media with differing mineral compositions. It would be believed that there would have been an enormous amount of research done on the mechanisms of mineral uptake in vitro along with mineral nutrition, given the importance of the nutrient concentrations in the tissue culture media (Ahmed, 2002).

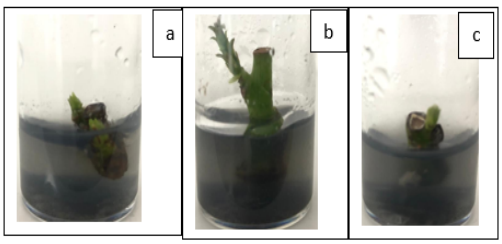


**Figure 1.** The growth of nodes after 4 weeks of culturing in different culture media a)1/2 Murashige & Skoog b) Woody plant medium c) Gamborg's B5

**Contamination**

In this experiment, explant contamination was observed two days after the explants were cultured. While the explants are exposed to a sterile environment after being established, it is normally expected that contamination will lessen with time in the culture (Greenwood, 1995) (Leifert et al., 1995). The high humidity in the culture container and the components in the tissue culture medium are two things that could stress out explants in an in vitro environment. The response of the nodal explants in this study revealed that endogenous contaminants were still present in the tissues (Leifert, 1995). Explants grown on different media with different sterilization treatments showed varying contamination responses. Gamburg's medium media was more contaminated, and avocado explants cultured with 10% and 30% NaOCl solutions had less survival than those with 20% NaOCl solution.

**Effects of activated charcoal in the medium**

Activated charcoal is used in avocado in vitro cultures for several reasons. It helps maintain the physiological and aesthetic qualities of avocado tissues by adsorbing phenolic compounds that can cause browning. It also neutralizes chemical contaminants in the culture media, including microbial byproducts and inhibitory complexes, creating a hygienic and optimal growth environment. Activated charcoal also influences the balance of plant hormones in the culture media, absorbing excess or inhibitory quantities of phytohormones. It can also reduce hazardous compounds in the explants or culture medium.

**Conclusions**

Efficient and well-standard regeneration protocol was successfully obtained from nodal segments of P. americana (Avocado) shoot tip nodal explants cultured in WPM medium implementing a sterilization method with 20% sodium hypochlorite in 20-minute exposure and 30-minute final dip in Augmentin produced 80 to 83% live growing cultures. In vitro survival is better in WPM medium than in ½ MS medium or in Gamburg's B5 medium for micropropagation.

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