International Journal of Veterinary Science and Agriculture Research
Volume 8 Issue 1, January-February 2026
ISSN: 2582-4112, Available at www.ijvsar.com

A plant tissue culture protocol for the production of avocado (*Persea americana Mill.*) Var. Pollock planting material

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Abstract: *This study aimed to develop a protocol for producing avocado planting materials by refining surface sterilization for shoots of the Pollock avocado (Persea americana Mill.) variety and enhancing in vitro shoot multiplication and root induction. For surface sterilization, shoots were dipped in a Thiophanate Methyl fungicide solution for 24 hours, washed with running tap water for 60 minutes, and brushed three times with liquid Vim detergent. They were then treated with neem oil and Tween 20, followed by immersion in 0.06% Thiophanate Methyl fungicide for one hour, 70% Ethanol for one minute, and then immersed in 600 g L^{-1} ascorbic acid for three minutes. Finally, washed with 10% H_2O_2 for three minutes. The sterilized shoots were subjected to twelve different treatments with varying concentrations of 0.1% and 0.2% (w/v) $AgNO_3$ for 15 minutes, followed by 5% and 10% (v/v) NaOCl in 5, 10, and 15 minutes of exposure in hormone-free Murashige and Skoog (MS) medium. A survival rate of 90% was recorded with 0.2% $AgNO_3$ + 10% NaOCl in 10 minutes after three weeks. Shoot multiplication was conducted with six concentrations of 6-Benzyl Aminopurine (BAP), 0, 0.5, 1, 1.5, 2, and 2.5 mg/L, combined with 0.1 mg/L of Naphthalene Acetic Acid. In addition, root induction was conducted with six concentrations of indole-3-Butyric acid (IBA), 0, 0.5, 1, 1.5, 2, and 2.5 mg/L, supplemented with 0.1 mg/L of Naphthalene Acetic Acid. The significantly highest mean number of shoots (1.98), average shoot length (2.1 cm), and shooting percentage (70) were observed in MS medium with 1.5 mg/L BAP. The highest mean number of roots (2.3), average root length (70.6), and rooting percentage (60) were observed in MS medium with 2.0 mg/L IBA. In conclusion, the Pollock variety achieved the highest survival rate (87%) with 0.2% AgNO3 and 10% NaOCl for 10 minutes. The best shooting* and rooting *were observed in MS medium with 1.5 mg/L BAP and 2.0 mg/L IBA, respectively.*

Keywords: *6-Benzyl Aminopurine, Pollock, Shoot multiplication, Survival rate*

I. Introduction

The plant tissue culture method is especially valuable for crops like avocado, where traditional propagation through seeds or cuttings often leads to genetic variability and inconsistent plant performance. (Fassio, et al., 2016)

Tissue culture technology offers several advantages, including the production of high-quality, uniform planting materials under disease–free conditions throughout the year, irrespective of seasonal or weather variations. (Rout and Mohapatra 2010). This approach is particularly beneficial for crops like avocado, where conventional propagation methods often face limitations. For instance, avocados grown from seeds exhibit significant genetic variations, making them unsuitable for producing true-to-type planting materials (Saadat et al 2012). The performance of avocado plants propagated from cuttings or seeds can also vary significantly based on rootstock selection, resulting in a low rate of success, further complicating traditional propagation methods (Bandaralage et al., 2017). An additional advantage is that tissue-cultured plants can be produced within a short time period and can be used as rootstock.

In Sri Lanka, avocado cultivation is primarily concentrated in areas like Kegalle, Kandy, and Matale with expansion potential in other districts(Silva and Fernando, 1995). However, limited access to quality planting material hinders its wider cultivation (Pacheco et al., 2011). Tissue culture offers a solution by enabling rapid, cost-effective, and labour-efficient propagation of true-to-type avocado plants (Bommineni et al., 2001).

The ability to produce true–to–type avocado plants efficiently is crucial for meeting the growing demand for this fruit while addressing the challenges of traditional propagation methods (Sarker and Gomasta, 2023). By adopting tissue culture technology, Sri Lanka can overcome the limitations of seed-based propagation and ensure the sustainable expansion of avocado cultivation in suitable regions (Faber and Bender, 1999).

Hence, this study aims to identify the effective surface sterilization method for explants from field-grown avocado plants, and identify of best protocol for shoot multiplication and root induction of *in vitro* raised shoots, which helps to develop a suitable protocol for the production of true-to-type avocado planting materials(Bhojwani et al., 2013).

II. Materials and Methods

This research was conducted at the Tissue Culture Division of the Plant Virus Indexing Centre (PVIC), Homagama, Sri Lanka. The study aims to optimize a protocol for surface sterilization of shoots from field-grown plants and *in vitro* multiplication of *in vitro-raised* avocado shoots.

2.1 Preparation of stock solutions and culture media

Murashige and Skoog (MS) medium was used as the basal medium throughout the study, with both full and half-strength formulations. Plant growth regulators were added where appropriate. Stock solutions of IBA and NAA were prepared at 1 mg/mL by dissolving the powders in suitable solvents and diluting with double-autoclaved distilled water. Required volumes for 1 L of culture medium were measured and used during media preparation.

2.2 Preparation of the hormone-free culture media

To prepare hormone-free MS culture media, the required stock solutions were mixed in a beaker along with 30 g/L sugar, 0.1 mg/L Kinetin, 1 mg/L GA_3, and 0.1 g/L Myo-inositol. Distilled water was added, and the mixture was stirred until the sugar dissolved. The volume was adjusted to 1000 mL with distilled water, and the pH was set to

5.8. Agar (6.1 g/L) was added while heating until a clear solution formed. The medium was then dispensed into glass jars (35 mL per jar).

2.3 Selection of explants

Shoots were collected from the field-grown Pollock variety inPVIC. Shoots were cut into 5 cm lengths containing 2-3 axillary buds. The shoots were dipped in a beaker containing distilled water to prevent them from dehydration and to avoid damage. Then, transferred to the Tissue Culture Laboratory at PVIC.

2.4 Surface sterilization of explants

The explants were prepared by removing leaves. At first, explants were put into a beaker and subjected to running tap water for 60 minutes to eliminate surface pollutions. Explants were brushed three times using liquid Vim detergent solution. Then, explants were washed running tap water, adding 2-3 drops of Tween 20 for 15 minutes, followed by 3 washes in distilled water.

The explants were excised into convenient sizes before applying a disinfection procedure. The shoots were disinfected with 0.06% Topsin fungicide for1 hour and then subjected to 70% Ethanol for 1 minute. Subsequently, the shoots were immersed in 600 mg/L ascorbic acid for 3 minutes and washed with 10% H_2O_2 for 3 minutes. Explants were washed with distilled water after applying each disinfection step. After these steps, twelve treatments were conducted by treating shoots separately in two concentrations (w/v = 0.1%, 0.2%) of Silver nitrate ($AgNO_3$) for 15 minutes, followed by washing with distilled water.

Sterilized bottles were taken and filled with two different concentrations of (v/v = 5%, 10%) of Sodium hypochlorite (NaOCl). Then shoots were transferred separately, containing five samples to each bottle for replication. The bottles containing shoots were labelled for 12 treatments (T1, T2, T3, …T12) and placed on a shaker with three different time durations (5, 10, and 15 minutes) for surface sterilization, as shown in Table 1.

The bottles were removed from the shaker after the predetermined time intervals. Then, the NaOCl solution was removed from the bottles under the laminar air flow cabinet. Then the shoots were transferred into to antibacterial solution for 15 minutes. Finally, three times washed with double autoclaved distilled water. Already sterilized explants were taken out from the bottles and kept on paper under the laminar air flow cabinet, and proceeded to be excised into 2cm lengths. Then the explants were cultured on hormone-free media

Table 1: Treatment composition for the sterilization of Avocado explants

Treatments	**Silver Nitrate ($AgNo_3$) Concentration (%) for 20 minutes**	**NaOCl Concentration (%)**	**Exposure Time for NaOCl (minutes)**
T1	0.1%	5%	5
T2	0.1%	5%	10
T3	0.1%	5%	15
T4	0.1%	10%	5

T5	0.1%	10%	10
T6	0.1%	10%	15
T7	0.2%	5%	5
T8	0.2%	5%	10
T9	0.2%	5%	15
T10	0.2%	10%	5
T11	0.2%	10%	10
T12	0.2%	10%	15

2.5 Preparation of culture media for shoot initiation

As shown in Table 2, the culture media were prepared with six different concentrations (0, 0.5, 1, 1.5,2, and 2.5 mg/L) of 6 - 6-benzyl aminopurine (BAP) with 0.1 mg/L Naphthalene Acetic Acid (NAA) in full-strength MS media. The shoot multiplication medium was prepared by using full-strength M S medium. Under optimal sterilization conditions, shoots were carefully excised from the best sterilized treatment cultures. Each shoot was trimmed to an appropriate length and transferred to a multiplication medium to promote shoot proliferation. In this experiment, the average number of shoots per explant, the average length of the shoots (mm), and the shooting percentage were recorded after 10 weeks of culture establishment.

Table 2. Treatment composition for shoot initiation of Avocado explants

Treatments	**BAP Concentration (mg/L)**	**NAA Concentration (mg/L)**
CT	0	0.1
T1	0.5	0.1
T2	1.0	0.1
T3	1.5	0.1
T4	2.0	0.1
T5	2.5	0.1

2.6 Root induction of Avocado explants

The culture media were prepared with six different concentrations (0, 0.5, 1, 1.5,2, 2.5 mg/ L) of Indole-3-Butyric Acid (IBA) with 0.1 mg/L Naphthalene Acetic Acid (NAA) in MS media (Table 3). Under aseptic conditions,

selected multiplied shoots (2 cm height) were transferred to the rooting medium. And each treatment contained 3 replicates, and 5 explants were transferred for each replicate. Cultures were incubated at 23±1 ºC under 16 hours photoperiod using white fluorescent bulbs (1000 Lux) in a culture room.

Table 3. Treatment composition for shoot initiation of Avocado explants

Treatments	IBA Concentration (mg/L)	NAA Concentration (mg/L)
CT	0	0.1
T1	0.5	0.1
T2	1.0	0.1
T3	1.5	0.1
T4	2.0	0.1
T5	2.5	0.1

2.7 Experimental design, collection of experimental data, and data analysis

All experiments were arranged according to the CRD. All treatments were statistically evaluated for shooting/rooting performance (average length of shoots/roots (mm), average number of roots per explant/average number of roots per shoot, and shooting/rooting percentage). Data analysis was carried out using ANOVA using R Studio with a 95% confidence interval, and mean separation was performed using the Least Significant Difference (LSD) test. Percentage data were analyzed using the Chi-square test, while parametric data, such as shoot length, were analyzed using a one-way ANOVA procedure. In contrast, non-parametric data, including count data, were analyzed using the Kruskal-Wallis test.

III. Results and Discussion

3.1 Identification of the best surface sterilization protocol for explants from the Pollock variety.

As per Fig. 1, shoots from the pollock avocado variety were performed with the highest survival rate (87%) compared to the other treatments, resulting in 3% fungal contamination, 5% bacterial contamination, and 5% death culture in T11 (0.2% $AgNO_3$ + 10% NaOCl for 10 minutes). The lowest surviving explants resulted in T1 (0.1% $AgNO_3$ + 5% NaOCl for 5 minutes) with no surviving shoots after three weeks of culture. According to Ko *et al.* (2009), phenolic compounds are secreted to induce browning, leading the explants to the death. However, in this study, explant browning did not significantly affect to survival rate of explants.

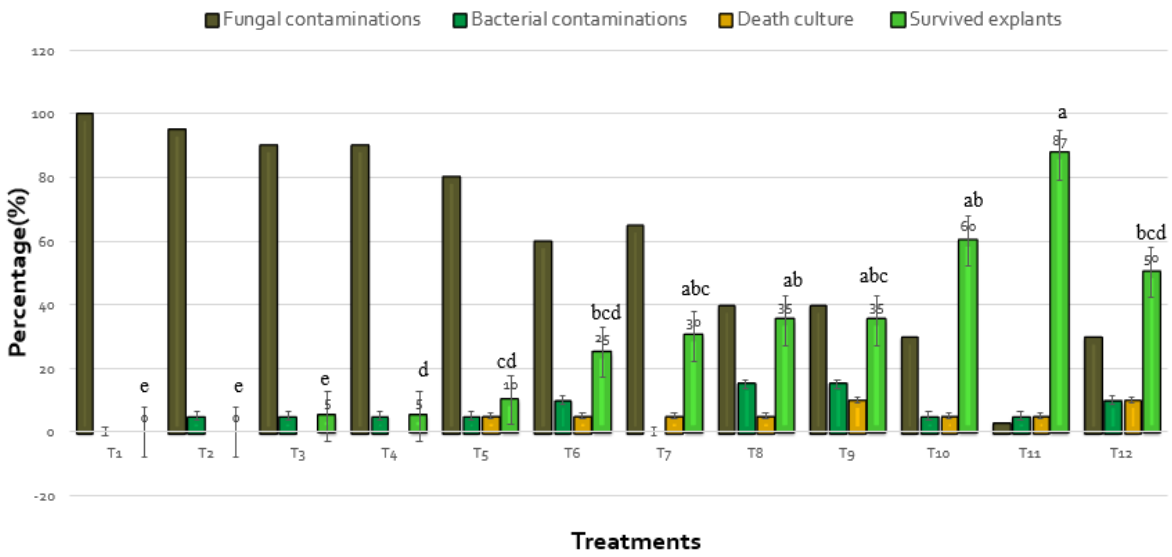

Figure 1. Effect of different treatments for surface sterilization of shoots from the Pollock variety

3.2 Identification of the best protocol for shoot initiation of *in vitro* raised Avocado

Shoot initiation commenced in 1.5mg/L BAP and 0.1 mg/L NAA treatments after the 4th week. Explants on 1.5 mg/L BAP and 0.1 mg/L NAA MS medium showed short and thin shoots. Further, callus formation was observed at the base of the shoot within the experimental period when the BAP concentration decreased. However, MS medium + 0 mg/L IBA (the control treatment) produced only a few numbers of shoots on some explants.

According to ANOVA results, the highest significant treatment effect for the average number of shoots per explant, the average length of the shoots (cm), and shooting percentage was observed in T3 (BAP 1.5 mg/L).

Figure 2. Shoot multiplication of Pollock Avocado 1.5 mg/L BAP

Figure 3. Shoot formation at 1.5 mg/L BAP after 8 weeks

3.2.1 Average number of shoots per explant

As shown in Figure 4, the highest average number of shoots per explant (1.98) was observed in T3 (Fig. 2). The plant had good shoot growth with large leaves (Fig. 3) in medium with 1.5 mg/L, and performed the optimum number of healthy shoots with optimum shoot growth. However, Zulfiqar et al. (2009) reported that a good response was observed in axillary buds with 1.0 mg/L BAP, which led to the best rate of shoot multiplication (4.80).

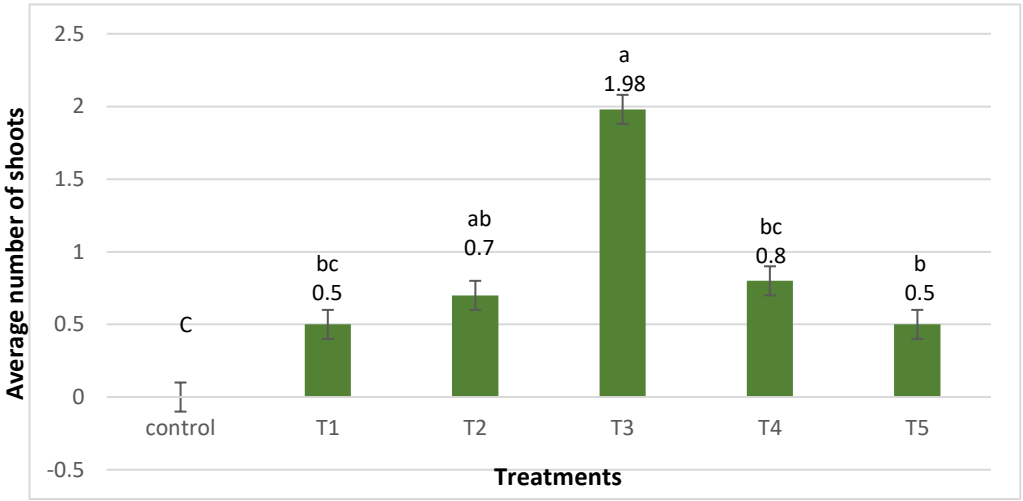

Figure 4. Effect of different treatments on the average number of shoots per explant

3.2.2 Effect of different treatments on the average length of shoots after 10 weeks of culture

MS medium supplemented with 1.5mg/L BAP resulted in 2.1 cm, the highest average shoot length, by producing fewer number of shoots per explant (Fig. 5). Accordingly, in a similar study, Sholi et al. (2022) reported *in vitro* regeneration of avocado (*Persea americana*) West Indian rootstock cv. Lula, achieving the highest shoot length of 3.2 cm at a BAP concentration of 1.5 mg/L. In addition, Zulfiqar, et al. (2009) reported that 2.91 was the highest shoot length in 1.5 mg/L BAP concentration in avocado.

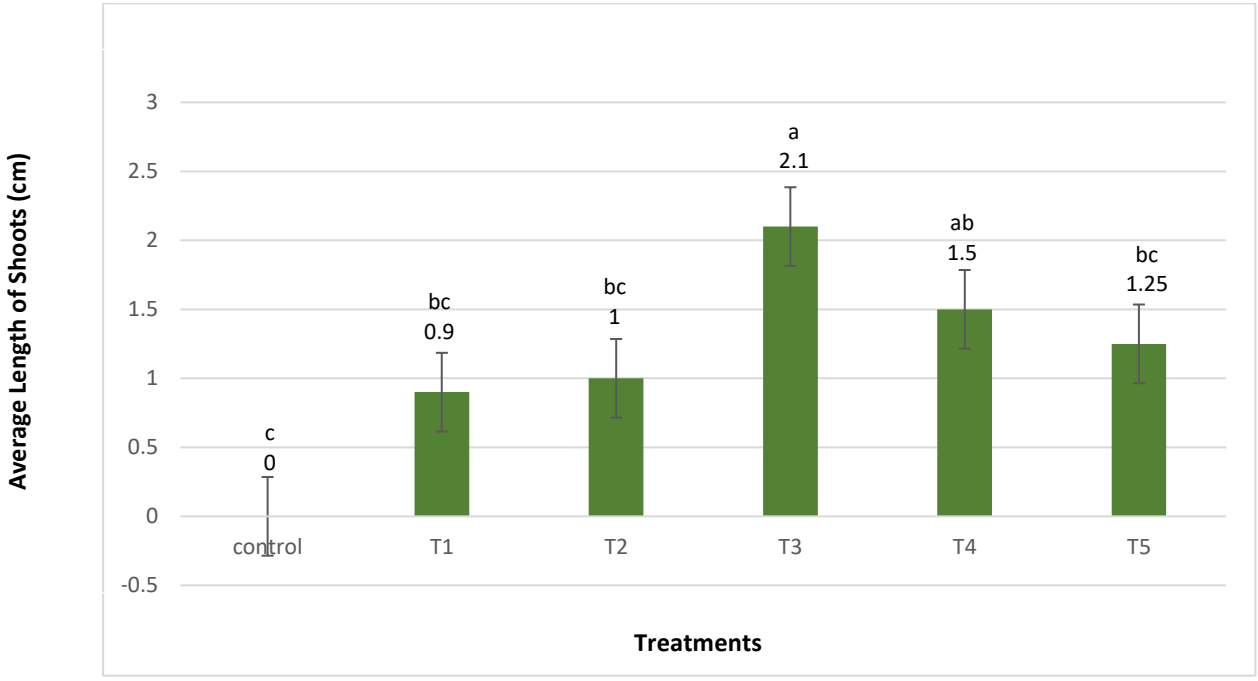

Figure 5. Effect of different treatments on the average length of shoots after 10 weeks of culture initiation

3.2.3 Shooting percentage

The highest shooting percentage observed was 70% in treatment T3, which contained 1.5 mg/L of BAP (Fig. 6). Similarly, a study by Afful et al. (2022) reported an 80% shooting percentage using MS medium with a BAP concentration of 1.5 mg/L. In contrast, lower shooting percentages were noted in treatments T1, T2, and T5, which had BAP concentrations of 0.5, 1, and 2.5 mg/L, respectively. The reduced shooting percentages in these treatments may be attributed to callus formation that occurred during the experimental period. The study conducted by Mansoor (2018) for the same species, in MS medium with either 0.5 or 1 mg/L BAP, also noted the observation of strong apical dominance and callus formation with the reduction of shooting percentage, and suggested that endogenous auxin levels might be higher than the cytokinin concentration used.

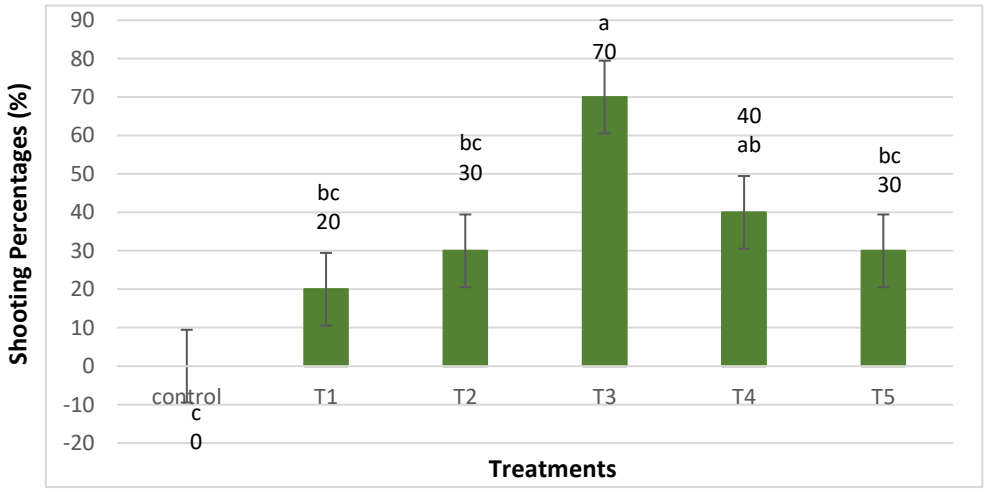

Figure 6. Effect of different treatments on shooting percentage after 10 weeks of culture initiation

3.3 Identification of the best protocol for root induction of *in vitro r*aised avocado shoots

In this experiment, root emergence began in the T4 treatment (2.0 mg/L IBA) after the fourth week of culture establishment (Fig. 7), while other treatments exhibited a delay in root emergence. Additionally, callus formation was noted at the base of the shoot during the experimental period at the lower concentrations of the IBA.

Based on the ANOVA results, the T4 treatment (IBA 2.0 mg/L) demonstrated the highest significant effects on the average number of roots per shoot, the average root length (mm), and the rooting percentage.

Figure 7. *In vitro* root induction of Avocado Pollock shoots

3.3.1 Average number of roots per shoot

According to statistical analysis, T4, which contained 2.0 mg/L IBA, exhibited the highest average number of roots per shoot at 2.3 (Fig. 8). Plants cultivated in MS medium enriched with 2.0 mg/L IBA demonstrated optimal root development and significant shoot growth. In a study by Zulfiqar et al. (2009), an average of 3.2 roots per explant was reported when employing the 1.5 mg/L concentration of IBA in MS medium for avocado cv. Fuerte.

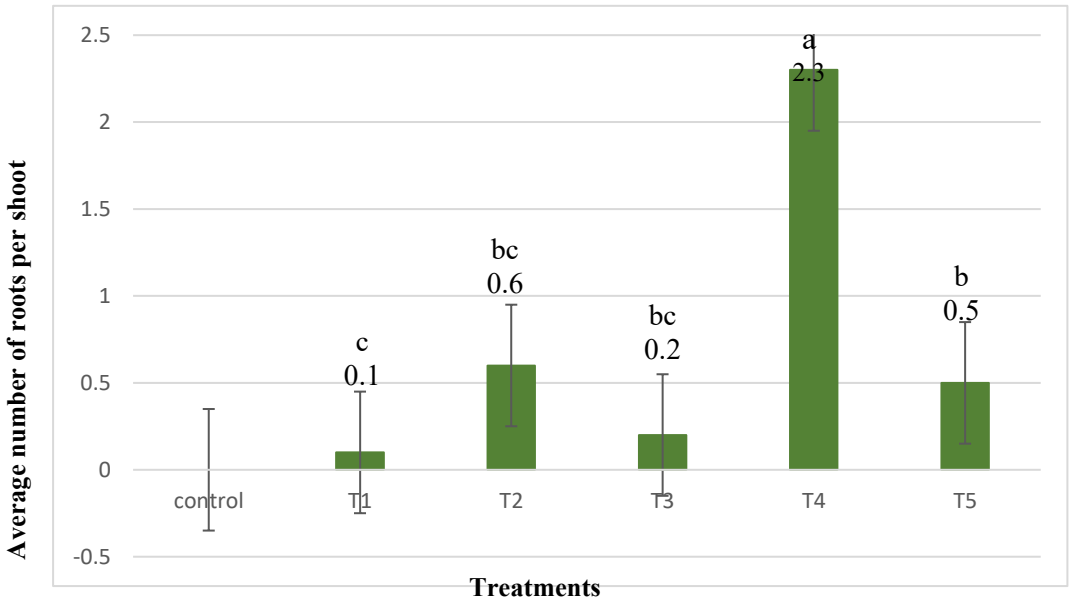

Figure 8. Effect of different treatments on the average number of roots per shoot

3.3.2 Aver

The highest average root length per shoot of 70.6 mm was observed in the medium supplemented with 2.0 mg/L of IBA (Fig. 9). However, in a related study, Zulfiqar et al. (2009) reported a maximum root length of 3.58 cm in avocado cv. Fuerte explants grown in MS medium supplemented with 1.5 mg/L of IBA. Also, a study conducted with *Morella pubescens*, by Borrero et. al. (2024) reported that 2 mg/L IBA resulted in the longest root length of 4.9 cm, and increasing IBA concentrations led to an increased number of roots.

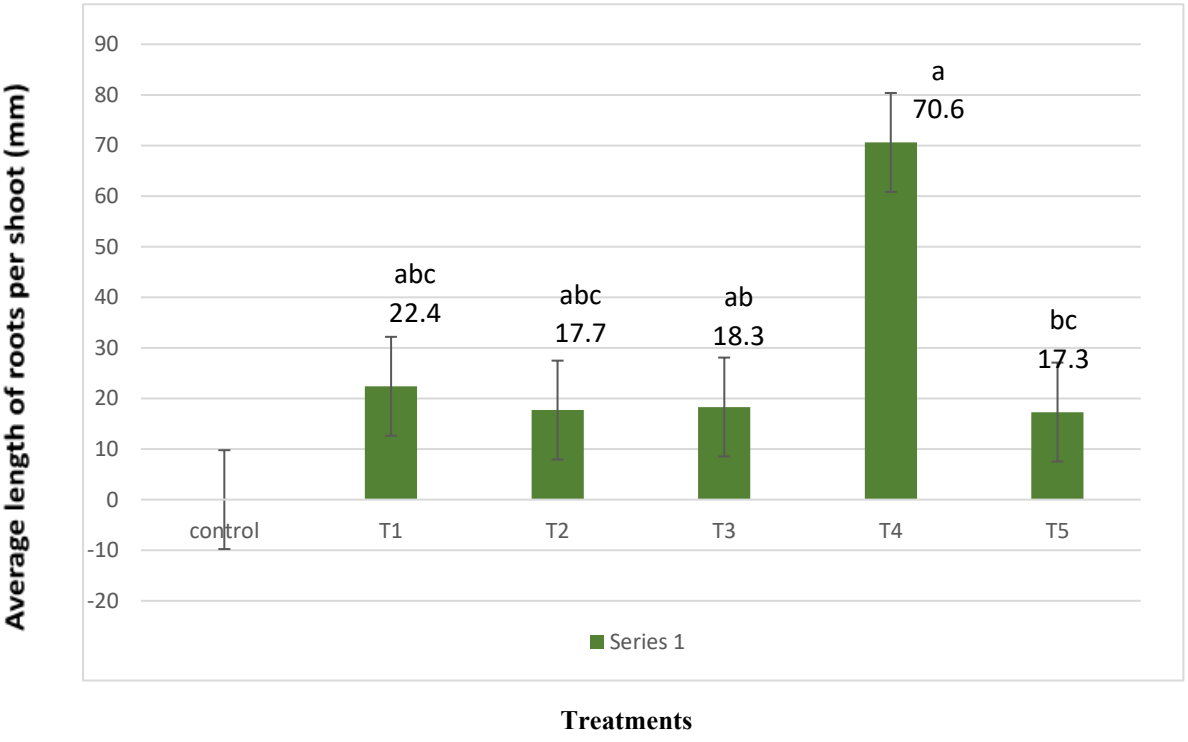

Figure 9. Effect of different treatments on the average length of roots after 10 weeks of culture initiation.

3.3.3 Rooting percentage

The highest rooting percentage observed in this study was 60%, which almost corresponds to the 53% rooting percentage found by Zulfiqar et. al (2009) in MS medium with 1.5 mg/L IBA for avocado cv. Fuerte. Some other studies also reported that the significance of Indole-3-butyric acid (IBA) in enhancing *in vitro* root development in avocado (Gracia-Gomez et al., 1994; Kahwas and Upadhyay, 2022; Hiti-Bandaralage et al., 2022).

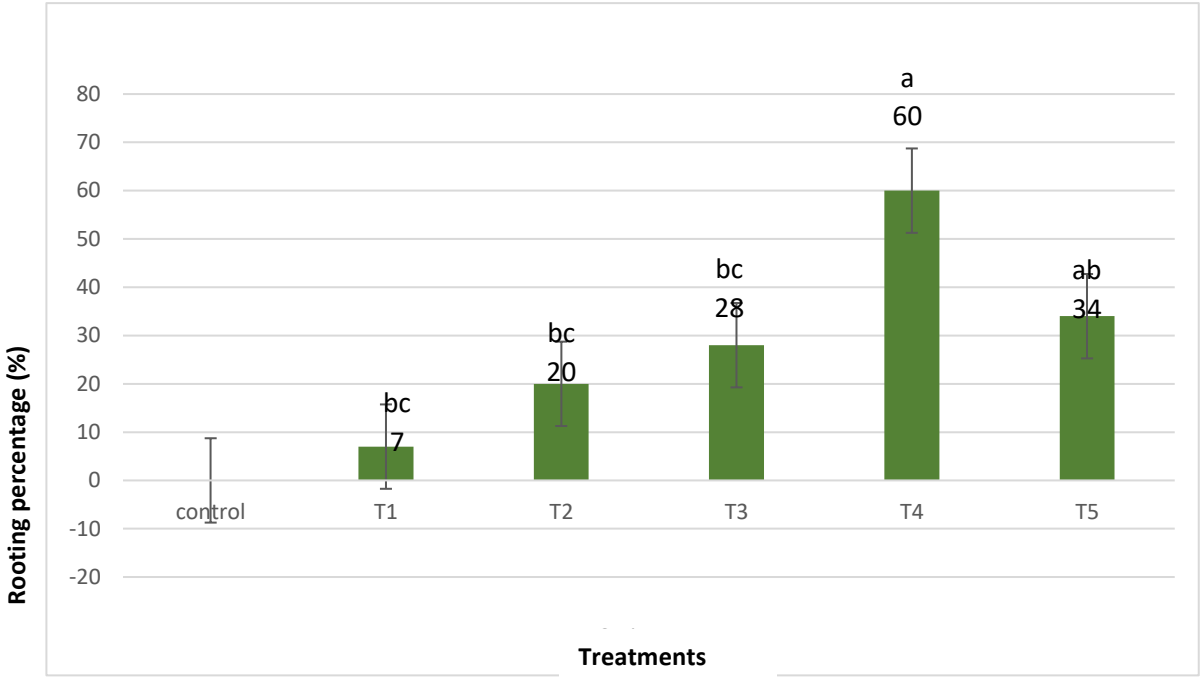

Figure 10. Effect of different treatments on the rooting percentage after 10 weeks of culture initiation

IV. Conclusion

It can be concluded that a treatment of 0.1% AgNO3 combined with 10% NaOCl for 10 minutes was the most effective method for surface sterilization of Avocado (*Persea americana* Mill.) var. Pollock shoots, resulting in a survival rate of 87%. For shoot initiation of *in vitro* raised avocado (*Persea americana*) explants, the optimal condition was found to be MS medium supplemented with 1.5 mg/L BAP. Furthermore, the highest root initiation occurred in MS medium supplemented with 2.0 mg/L IBA for the *in vitro* raised avocado (*Persea americana* Mill.) var. Pollock shoots. These results suggest the potential for mass production of avocado using locally sourced planting material through tissue culture technology.

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