IN VITRO DARKENING AGENTS, PHLOROGLUCINOL AND DARK TREATMENT INFLUENCE ROOTING PERCENTAGE IN AVOCADO (PERSEA *AMERICANA* MILL.)

Elsa S. du Toit and Demi Tait

Department of Plant and Soil Sciences,
Faculty of Natural and Agricultural Sciences,
University of Pretoria, Pretoria 0002, SOUTH AFRICA

Corresponding author: elsa.dutoit@up.ac.za

ABSTRACT

Avocado (*Persea americana* Mill.) micropropagation faces significant challenges in root induction, particularly for clonal rootstocks like 'Dusa' and 'Merensky 6 (Leola™)'. This study explored the effects of darkening agents (activated charcoal and black food colouring), phloroglucinol, and dark treatments on root development in tissue-cultured avocado micro shoots. Micro shoots were cultured in half-strength Murashige and Skoog medium supplemented with indole-butyric acid (IBA), naphthalene-acetic acid (NAA), sucrose, agar, vitamins, and either activated charcoal or black food colouring, with or without phloroglucinol. Half of the treatments received a three-week dark treatment, while the others were kept under full light. Results indicated that 'Dusa' micro shoots rooted more effectively in media containing activated charcoal and phloroglucinol, with reduced tissue browning. Conversely, black food colouring was less effective, leading to poor rooting. For 'Merensky 6', the highest rooting percentages were observed in black food-coloured media combined with phloroglucinol and dark treatment, demonstrating the beneficial effects of etiolation on root induction. However, oxidative browning was common in treatments with black food colouring that lacked phloroglucinol and dark treatment. This study highlights that activated charcoal and phloroglucinol are effective additives for promoting rooting, while dark treatment enhances rooting in challenging micro shoots. Further research is essential to refine these protocols and optimise tissue culture conditions for various avocado genotypes, addressing the increasing demand for high-quality avocado plants in commercial production.

INTRODUCTION

Darkening agents, dark treatments, and phloroglucinol have been recognised for their roles in enhancing root development across various plant species. This literature review explores these components and their effects on rooting during *in vitro* propagation.

Darkening agents

While the use of food colouring as a media darkening agent for promoting rooting has not been extensively studied, the general principles suggest potential benefits. The primary mechanism behind media darkening involves protecting auxins from light degradation, thereby maintaining their efficacy in promoting root formation (Hangarter and Stasinopoulos, 1991).

Activated charcoal is particularly significant in tissue culture for inducing a beneficial role in root formation and helping to prevent tissue browning. Numerous studies indicate that activated charcoal facilitates rooting by adsorbing inhibitory substances from the culture medium, such as phenolic exudates, ethylene, and other toxic metabolites that can impede root development. By removing these compounds, activated charcoal creates a more favourable environment for root induction and growth (Thomas, 2008). Furthermore, while activated charcoal can adsorb various compounds, including plant hormones, it has been shown to enhance auxin stability. Auxins are crucial for root induction, and activated charcoal may protect these from degradation, thus preserving their efficacy (Martins, 2024). Experimental evidence supports improved rooting success in various plant species when activated charcoal is incorporated into the culture medium, as demonstrated in *in vitro*-derived *Acacia leucophloea* shoots (Sharma *et al*., 2012).

Phloroglucinol

Phloroglucinol, a phenolic compound, effectively promotes rooting in tissue-cultured plants through several mechanisms. It acts synergistically with auxins, enhancing their effectiveness in root formation (Teixeira da Silva *et al*., 2013). For instance, the addition of phloroglucinol positively affected rooting in wild cherry shoots propagated *in vitro* (Hammatt, 1994), a species known for its rooting difficulties, similar to avocado. Additionally, phloroglucinol has been shown to increase rooting percentages in apple cuttings during the rooting stage (Caboni *et al*., 1992).

Phloroglucinol protects auxins from oxidative degradation and decarboxylation, thereby maintaining their potency in promoting root development (Petti, 2020). Studies have also indicated that phloroglucinol exhibits hormone-like properties similar to cytokinins. In experiments with *Ornithogalum dubium*, it induced callus formation and plant regeneration, mimicking the effects of essential plant hormones. Moreover, phloroglucinol has been found to reduce hyperhydricity in tissue-cultured plants, a common issue that affects plantlet quality and rooting ability. By improving overall plantlet quality, phloroglucinol indirectly supports better root formation (Petti, 2020). Its antioxidant properties further protect plant tissues from oxidative stress during rooting, maintaining cell integrity, and supporting root development (Liu, 2022). Notably, phloroglucinol is resistant to autoclaving, ensuring its activity remains intact during tissue culture processes (Teixeira da Silva *et al*., 2013).

Dark treatment

Although a dark treatment is a critical step for avocado grafting during conventional propagation methods, it is also a critical step in the tissue culture, primarily aimed at mitigating browning caused by the oxidation of phenolic compounds. This process involves placing explants or micro shoots in darkness for a specific period after transplantation to enhance survival rates and promote successful growth. Studies have reported varying durations for dark treatment, typically ranging from a few days to several weeks, depending on the specific protocol and the stage of tissue culture. For example, one study suggested a dark treatment period of 7 to 10 days after avocado (*Persea americana* Mill.) culture initiation to effectively address browning issues (Hiti-Bandaralage *et al*., 2017).

The aim of this research is therefore to determine the influence of *in vitro* darkening agents, phloroglucinol, and dark treatment on the rooting percentage in avocados (*Persea americana* Mill.).

MATERIALS AND METHODS

This trial was a preliminary investigation to determine whether rooting can occur using the aforementioned media components for each cultivar, as well as to establish a timeline for the Stage 3 rooting phase during the *in vitro* propagation of avocado.

'Dusa' and 'Merensky 6' rootstocks, among other important cultivars, play a crucial role in enhancing the sustainability and productivity of avocado orchards, particularly in regions where disease pressure and environmental challenges are prevalent. Their development represents a significant advancement in the avocado industry, helping growers achieve better yields and healthier trees. Consequently, the above cultivars were used for this trial. Both 'Dusa' and 'Merensky 6' micro shoots, which were grown from nodal micro cuttings, and taken from stage two multiplication media, were used for this trial.

The micro shoots for each cultivar were placed in rooting media to determine which media and treatment were most effective in inducing rooting. All rooting media were prepared with half-strength Murashige and Skoog basal medium supplemented with 4 mg/l indole-butyric acid (IBA), 0.5 mg/l naphthalene-acetic acid (NAA) (Mohamad *et al*., 2022), sucrose, agar, and vitamins. The treatment rooting media were either darkened with activated charcoal (1 g/l) or black food colouring (0.1 mg/l). The control rooting media contained no phloroglucinol, while the different treatment media contained 160 mg/l phloroglucinol. The rooting media were autoclaved for 15 minutes at 121 °C.

Half of the treatments underwent a dark treatment period of three weeks, while the remaining treatments were kept under full light conditions in the growth room.

RESULTS

Each cultivar responded differently to different treatments. Overall, within two to three weeks root nodule formation began. The results of cultivars will be discussed separately:

Table 1: Summary of rooting trial treatments

Growth media	Darkening agent	Phloroglucinol	Dark treatment
Treatment 1.1	Activated charcoal	Yes	No
Treatment 1.2	Activated charcoal	No	No
Treatment 1.3	Activated charcoal	Yes	Yes
Treatment 1.4	Activated charcoal	No	Yes
Treatment 2.1	Black Food Colouring	Yes	No
Treatment 2.2	Black Food Colouring	No	No
Treatment 2.3	Black Food Colouring	Yes	Yes
Treatment 2.4	Black Food Colouring	No	Yes

'Dusa'

This preliminary trial found that no 'Dusa' micro shoots formed roots without phloroglucinol in the activated charcoal media. Visible root formation of micro shoots was observed during the third week of treatment in media 1.1 and 1.3. However, micro shoots placed in treatment media 1.2 and 1.4 did not form any roots over the five-week trial period. Based on the rooting that occurred in micro shoots, regardless of whether they underwent dark treatment, we could not conclude that this treatment had any effect on rooting.

Overall, 'Dusa' micro shoots rooted better in rooting media containing activated charcoal and phloroglucinol. We also observed that rooting media supplemented with activated charcoal not only supported root induction but also resulted in lower levels of tissue browning, which could be attributed to the antioxidant properties of activated charcoal. As shown in Table 2, 'Dusa' micro shoots rooted poorly in rooting media supplemented with black food colouring. Additionally, we noted a high percentage of micro shoot death in the black food colouring rooting media at the end of the five-week trial period.

'Merensky 6'

Table 3 shows that treatment media 2.3 and 2.4 resulted in the highest percentage of rooted 'Merensky 6' micro shoots, which were derived from the food-coloured rooting media. However, these micro shoots had worse tissue browning. Treatments 1.1, 1.3, 2.1, and 2.3 which contained phloroglucinol, also demonstrated good rooting percentage.

It is interesting to note that treatments 2.3 and 2.4 had a significantly higher percentage of rooted micro shoots compared to those in treatments 2.1 and 2.2. This indicates that the dark treatment positively affected micro shoot rooting in the black food-coloured growing media.

Unfortunately, we observed a high level of oxidative browning in micro shoots placed in rooting media containing black food colouring without phloroglucinol and without dark treatment, as illustrated in Table 3.

Overall, 'Merensky 6' performed better than 'Dusa' in the different treatment media.

Figure 1: Rooted micro shoot in treatment media 2.1 (left). Rooted micro shoot with leaf browning (right).

DISCUSSION

More targeted research is needed to fully understand the specific effects of food colouring on root development in tissue culture. Future studies comparing different darkening agents at various concentrations

Table 2: Rooting percentages of 'Dusa' micro shoots on different treatment media

'Dusa' micro shoots				
Growth media	**Darkening agent**	**Phloroglucinol**	**Dark treatment**	**Rooting %**
Treatment 1.1	Activated charcoal	Yes	No	43
Treatment 1.2	Activated charcoal	No	No	0
Treatment 1.3	Activated charcoal	Yes	Yes	33
Treatment 1.4	Activated charcoal	No	Yes	0
Treatment 2.1	Black Food Colouring	Yes	No	17
Treatment 2.2	Black Food Colouring	No	No	14
Treatment 2.3	Black Food Colouring	Yes	Yes	0
Treatment 2.4	Black Food Colouring	No	Yes	14

Table 3: Rooting percentages (%) of 'Merensky 6' micro shoots on different treatment media

Merensky micro shoots				
Growth media	**Darkening agent**	**Phloroglucinol**	**Dark treatment**	**Rooting %**
Treatment 1.1	Activated charcoal	Yes	No	38
Treatment 1.2	Activated charcoal	No	No	25
Treatment 1.3	Activated charcoal	Yes	Yes	33
Treatment 1.4	Activated charcoal	No	Yes	38
Treatment 2.1	Black Food Colouring	Yes	No	33
Treatment 2.2	Black Food Colouring	No	No	10
Treatment 2.3	Black Food Colouring	Yes	Yes	40
Treatment 2.4	Black Food Colouring	No	Yes	40

could provide more conclusive evidence and optimise tissue culture conditions for enhanced root growth across different cultivars.

It is important to note that while activated charcoal, used in this trial as a darkening agent for rooting media, offers many benefits for root induction, its application must be carefully managed. The high adsorption capacity of activated charcoal can also lead to the removal of beneficial substances from the medium, including essential nutrients and growth regulators. Therefore, the concentration of activated charcoal must be optimised for each specific cultivar and culture condition to balance its positive effects on root induction with potential drawbacks.

Lastly, the dark treatment, a form of etiolation, also promoted micro shoot rooting in 'Dusa' and 'Merensky 6' by potentially altering the physiological, biochemical, and morphological conditions of the micro shoots. This technique has proven to be a valuable tool in overcoming the propagation challenges associated with avocados, particularly for difficult-to-root clonal rootstocks. Consequently, we need to refine this treatment protocol to assess its effectiveness, as it may vary depending on the genotype of the avocado and the specific conditions of the tissue culture environment.

CONCLUSION

Activated charcoal aids root formation in tissue culture primarily by creating a more favourable environment through the adsorption of inhibitory compounds. Its use has been shown to significantly improve rooting success in 'Dusa' and 'Merensky 6', making it a valuable tool in plant tissue culture and micropropagation techniques.

The efficacy of phloroglucinol in promoting rooting in tissue-cultured avocado micro shoots has been demonstrated in this trial, establishing it as a valuable additive in plant tissue culture protocols. Its synergistic effect with auxins, combined with its hormone-like and protective properties, significantly enhances the rooting process.

The successful application of dark treatment (etiolation) in avocado propagation highlights its potential to revolutionise the industry by improving the efficiency and success rates of *in vitro* propagation methods.

As research continues, further refinements in dark treatment protocols and even darkening agents may lead to even more effective propagation techniques, addressing the growing demand for high-quality avocado plants in commercial production.

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