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Assessment of Biomass Accumulation, Total Phenolic and Flavonoid Contents and Antioxidant Activity of Callus Culture of Avocado (*Persea americana*. Mill) cv. Hass

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ABSTRACT

The cultivation of avocado (*Persea Americana* Mill.) cv. Hass via callus culture presents a sustainable approach for producing secondary metabolites. This study was conducted out to investigate the potential of callus culture in the production of bioactive compounds of avocado. Callus cultures were initiated from zygotic embryos on Murashige and Skoog (MS) medium supplemented with different concentrations of auxins 2, 4-dichlorophenoxyacetic acid (2, 4-D) and naphthalene acetic acid (NAA). Callus growth (fresh and dry weights), total phenolic and flavonoid contents, and antioxidant activity were evaluated four weeks after culture establishment. The results indicated that callus cultures were successfully established on all growth regulators combinations. In this regard, treatment of 5.0 mg/L 2,4-D exhibited the highest biomass accumulation. Total phenolic and flavonoid contents varied among treatments, with the highest values observed in callus grown on medium containing either 2.5 mg/L 2,4-D or 5.0 mg/L 2,4-D. The same trend was also observed with the antioxidant activity where 2.5 mg/L and 5.0 mg/L 2,4-D proved to demonstrate the highest antioxidant capacity (88.7% and 88.9%, respectively). Further investigation is needed to optimize culture conditions for enhanced production of bioactive compounds in avocado callus cultures. This study provides preliminary evidence for the potential of 'Hass' avocado callus culture as a source of natural antioxidants.

Keywords: Avocado, Callus culture, Biomass accumulation, Phenolics, Flavonoids.

1. Introduction

Free radicals molecules are linked to the development and worsening of many chronic and degenerative diseases, including cancer, stroke, anemia, arthritis, heart disease, lung disease, autoimmune disorders, and conditions affecting the brain and cardiovascular system (Zehiroglu & Ozturk Sarikaya, 2019; Chaudhary *et al*., 2023). When the body struggles to eliminate these free radicals, a state called oxidative stress occurs. This stress can damage all types of important molecules in the body, including DNA, proteins, carbohydrates, and fats (Amer & Aly, 2019; Mancini *et al*., 2020; Zhang *et al*., 2021). To combat the harmful effects of free radicals, our bodies rely on antioxidants. These are special molecules that can neutralize free radicals, preventing them from damaging important cellular components. While some antioxidants are produced naturally within the body, we must obtain

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essential ones like vitamin E, vitamin C, and beta-carotene through our diet. Plants are a treasure trove of naturally occurring antioxidants. In fact, an estimated two-thirds of all plant species have medicinal properties, and nearly all possess significant antioxidant potential (Kasote *et al*., 2015; Liao *et al*., 2022). This has led to a renewed interest in traditional medicine around the world, with researchers exploring the potential of numerous plant species. One such plant with promising properties is the avocado.

Avocado (*Persea americana* Mill.) is a significant fruit crop belonging to the Lauraceae family. This trendy and delicious fruit originates from Central America. Avocado trees thrive in diverse environments, adapting to both humid tropics and drier, Mediterranean-like regions around the world (Tremocoldi *et al*., 2018; Tamayo-Ramos *et al*., 2022). Embraced by health-conscious consumers, avocado consumption has skyrocketed in recent years. Global production has tripled (increased by 300%) in the past two decades (2000-2018), with no signs of slowing down (Food and Agriculture Organization of the United Nations Statistical Databases, FAOSTAT, 2018). This exceptional growth is evident not only in overall production but also in the expanding area of land dedicated to avocado cultivation. Avocados are nutrient powerhouses**,** containing high levels of minerals, vitamins, protein, fiber, and unsaturated fatty acids (Mardigan *et al*., 2018; Waly *et al*., 2023; Wang *et al*., 2023; Sina *et al*., 2024). They are also rich in bioactive compounds like phenolic acids, flavonoids, and tannins (Figueroa *et al*., 2018; Cárdenas-Castro *et al*., 2023). These bioactive compounds are known for their strong antioxidant properties and various health benefits. For these reasons, avocados are widely used in the food industry, as well as nutraceutical, pharmaceutical, and cosmetic applications (Bhuyan *et al*., 2019; Vidales-Paz *et al*., 2021).

Despite the growing interest in extracting antioxidants and other beneficial compounds from avocados, the process faces several challenges. Fresh plant tissues, like avocados, can deteriorate quickly, are only available seasonally, and most importantly, can yield inconsistent quality extracts. (Irshad *et al*., 2018; Ochatt *et al*., 2022; Wu *et al*., 2024). Additionally, even avocado grown from the same parent tree using seedlings exhibit genetic variations, leading to differences in the amount and type of these bioactive compounds. This variability makes it difficult to obtain consistent results when extracting antioxidants from avocados.

Plant tissue culture technology offers a potential solution to the challenges of extracting antioxidants from avocados. This technology allows for year-round production of these valuable compounds in a controlled environment, using minimal space. Additionally, it provides a way to optimize the production of these bioactive compounds and ensure consistent quality through mass cloning of specific avocado plants or tissues. From an industrial standpoint, this consistency is crucial. (Amer and Omar., 2019; Shasmita *et al*., 2023).

However, despite initial research on avocado tissue culture starting decades ago, the technology for avocados is still considered underdeveloped. Studies have shown that avocados are difficult to propagate using this method, often resulting in low yields of regenerated plant material. (Hiti-Bandaralage *et al*., 2017). Despite persistent efforts to improve regeneration and transformation protocols (methods for introducing new genes, success has been limited). (Catalina *et al*., 2018; Palomo-Ríos *et al*., 2017).

Although, avocado has been found to be a potential source of various phenolic compounds with high antioxidant capacities, to our knowledge, research on *in vitro* induction of phenolic antioxidants, using callus cultures, is non-existent. Hence, the present study aims to investigate the effect of different growth regulators on biomass accumulation, total phenolic and flavonoid contents and antioxidant activity of callus cultures of Hass avocado.

2. Materials and Methods

2.1. Plant material and surface disinfestation

Mature fruits of the 'Hass' avocado cultivar were obtained from a local orchard. Fruits with no blemishes or signs of disease were selected. Avocado fruits were washed with running tap water and soap followed by rinse in water for 1 hour, and ethanol 70% soaking for 15 minutes. Fruit were rinsed with sterile purified water and then sectioned for seed extraction.

2.2. Seed sterilization procedures

Inside a laminar flow hood, fruit and seeds were disinfested using 10 % sodium hypochlorite solution with 2-3 drops of Tween −20 for 20 minutes, then final washing 2-3 times with sterile distilled water. Seeds were rinsed with sterile purified water to eliminate remaining parts of the mesocarp (flesh).

The clean seeds were placed in 70% alcohol for 15 minutes and then placed in a laminar flow hood where they were flamed superficially. Clean seeds were sectioned and the zygotic embryos extracted.

2.3. Medium preparation

The basal medium used for callus induction was MS medium (Murashige and Skoog., 1962) with full-strength macro and microelements and vitamins.

2.4. Plant growth regulators (PGRs):

The medium was supplemented with two different PGRs at different concentrations

Treatment 1 (T1): 2, 4-dichlorophenoxyacetic acid (2, 4-D) at 0.5 mg/L.
Treatment 2 (T2): 2, 4-dichlorophenoxyacetic acid (2, 4-D) at 2.5 mg/L.
Treatment 3 (T3): 2, 4-dichlorophenoxyacetic acid (2, 4-D) at 5.0 mg/L.
Treatment 4 (T4): 2, 4-dichlorophenoxyacetic acid (2, 4-D) at 7.0 mg/L.
Treatment 5 (T5): Naphthalene Acetic Acid (NAA) at 0.5 mg/L.
Treatment 6 (T6): Naphthalene Acetic Acid (NAA) at 2.5 mg/L.
Treatment 7 (T7): Naphthalene Acetic Acid (NAA) at 5.0 mg/L.
Treatment 8 (T8): Naphthalene Acetic Acid (NAA) at 7.0 mg/L.

The culture medium consisted of basal salts and vitamins supplemented with 100 mg/L Myo-inositol, 30 g/L sucrose, and 7 g/L agar. The pH was adjusted to 5.8 before adding filter-sterilized plant growth regulators (PGRs).The medium was autoclaved at 121°C and 1.05 bar pressure for 20 minutes. After autoclaving, the medium was allowed to cool to approximately 45°C before adding the filter-sterilized PGRs and other supplements. The autoclaved medium was then dispensed into sterile Petri dishes (90 mm diameter) at 20 ml per dish and allowed to solidify.

2.5. Callus culture establishment

Isolated zygotic embryos were placed onto the semi-solid callus induction medium in Petri dishes under aseptic conditions in the laminar flow hood. Seven explants were placed per Petri dish.

2.6. Culture conditions: The inoculated Petri dishes were sealed with Parafilm and incubated in a growth chamber under controlled environmental conditions with temperature of 25 ± 1°C, in darkness conditions, and a relative humidity of 60%.

2.7. Sub-culturing

Callus was sub-cultured every four weeks onto fresh callus induction medium. Approximately half of the actively growing callus tissue was transferred to new Petri dishes using sterile techniques.

2.8. Callus characterization

2.8.1. Morphology characteristics

The visual characteristics of the developing callus tissue were documented throughout the experiment. Observations included color, texture, and growth pattern. Digital photographs were taken at regular intervals to monitor callus development.

2.8.2. Growth measurement

Callus growth was quantified by measuring the fresh and dry weight of the callus tissue every four weeks during sub-culturing. The callus tissue was carefully separated from the medium and weighed using an analytical balance.

2.9. Analysis of secondary metabolite production

2.9.1. Total phenolic content (TPC) (mg/g DW)

Phenolic compounds were extracted from callus tissue using 80% methanol. The TPC was determined using the Folin-Ciocalteu reagent method, with gallic acid as the standard. Sample was prepared using a mixture of water and ethanol 80%. Then the sample extract is mixed with the Folin-Ciocalteu reagent and allowed to react for 20 min under controlled conditions (65°C). Phenolic

compounds in the sample reduce the F-C reagent, causing a color change (from yellow to blue). The absorbance of the reaction mixture is measured at a specific wavelength (765 nm) using a spectrophotometer. Using the absorbance values of the gallic acid standard solutions, a standard curve is generated. The absorbance of the sample solution is compared to this curve to estimate its total phenol content, expressed as gallic acid equivalents (GAE), (Kupina *et al*., 2017; Geremu *et al*., 2016).

2.9.2. Total Flavonoid Content (TFC) (mg QE/g DW)

Flavonoids were extracted from callus tissue using methanol 80%. The powdered callus tissue is mixed with the solvent and subjected to extraction procedures shaking. This process repeated several times to ensure maximum extraction of flavonoids. The TFC was measured using a colorimetric assay, with quercetin as the standard. The absorbance of the flavonoid extract is measured using a spectrophotometer, typically at a wavelength of (415 nm) where the color change is most pronounced (Sen *et al*., 2020).

2.10. Antioxidant Capacity (%)

2.10.1. DPPH Radical Scavenging Activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the avocado callus extract was evaluated as five hundred microliters (µL) of the fresh methanolic callus extract were combined with 2 mL of a 0.004% DPPH solution in 80% methanol. The reaction mixture was thoroughly mixed and incubated in the dark at room temperature for 30 minutes. As described by Abo El-Fadl *et al*. (2022), the wavelength of maximum absorption (λmax) of the mixture was determined using a spectrophotometer, and a value of (517 nm) was obtained. The percentage of DPPH radical scavenging (%RSC) was calculated using the following formula: %RSC = [(Ablank – Asample) / Ablank] x 100. Where, %RSC; DPPH radical scavenging activity (%), Ablank; absorbance of the blank control (2 mL DPPH solution + 0.5 mL methanol), and Asample; absorbance of the reaction mixture containing the avocado extract.

2.11. Statistical Analysis

All experiments were conducted in triplicate. Statistical analysis was performed using one-way ANOVA followed by LSD test using SPSS software.

3. Results

3.1. Callus induction frequency

Various concentrations of 2,4-D or NAA were evaluated for the maximum frequency of callus induction. The results demonstrate that both of them were efficient for callus induction Table (1). Moreover, callus induction frequency was 100% for all treatment regardless of the type (2,4-D, NAA) or the concentration (0.5, 2.5, 5.0, 7.0 mg/L) of plant growth regulator.

3.2. Fresh weight

Table (1) showed that the callus fresh weight ranged from 5.48 g to 11.0 g. The highest fresh weight was observed for callus under 2,4-D at a concentration of 5.0 mg/L followed by NAA at a concentration of 5.0 mg/L and 2.5 mg of both 2,4-D and NAA with no significant differences between these groups whereas 0.5 mg/L of NAA exhibited the lowest fresh weight. It is noticeable that all treatments with 2,4-D were higher than the corresponding NAA treatments from statistical point of view. The highest fresh weight for NAA supplementations were recoded for 5.0 mg/L and 7.0 mg/L treatment with no significant difference between them (8.83 g, 8.80 g).

3.3. Dry weight

Dry weights of callus cultures treated with 2,4-D ranged from 0.11 g to 0.43 g (Table 1). Maximum efficiency of dry weight accumulation was recorded for the concentration of 5 mg/L followed by 2.5 mg/L treatment whereas both treatments with either 0.5 mg/L or 7.0 mg/L showed the lowest dry weights with no significant differences between them. In contrast, callus treated with NAA ranged between 0.06 g/L and 0.28 g/L (Table 1). The highest dry weight was recorded for both 5.0 mg/L and 7.0 mg/L treatments (0.28 g and 0.27 g, respectively) with no significant difference between them whereas the lowest dry weight was recorded for treatment with 0.5 g/L. Overall, 5.0 mg/L 2,4-D showed the highest

dry weight whereas 0.5 mg/L showed the lowest dry weight. Similar to fresh weight results, all treatments with 2,4-D were higher than the corresponding NAA treatments.

Table 1: Effect of different concentrations of 2, 4-D and NAA on growth of Hass avocado callus after 4 weeks of culture

Auxin type	Concentration (mg/L)	Callus induction frequency (%)	Fresh weight (g)	Dry weight (g)
2,4-D	0.5	100	6.65 c	0.11 c
	2.5	100	8.92 b	0.26 b
	5.0	100	11.00 a	0.43 a
	7.0	100	6.77 c	0.25 b
NAA	0.5	100	5.84 d	0.06 d
	2.5	100	6.72 c	0.12 c
	5.0	100	8.83 b	0.28 b
	7.0	100	8.80 b	0.27 b

3.4. Callus morphology

At 2.5 mg/L 2,4-D, callus showed a white color, compact in texture, and exhibited a smooth growth pattern (Table 2). Increasing the concentration to 5.0 mg/L 2,4-D maintained the white color and compact texture but introduced a nodular aspect to the otherwise smooth growth pattern. At 7.0 mg/L 2,4-D, the callus turned yellow, became friable (crumbly), and continued to show a nodular growth pattern (Fig 1). The change in color indicate a physiological response to the higher concentration of 2,4-D, while the friable texture indicates less cohesive growth, which can be useful for certain types of studies or propagation methods. NAA concentration significantly influenced avocado callus morphology. Callus color shifted from white to yellow-brown with increasing NAA, potentially due to altered phenolic compounds or stress responses (Fig 2). Higher NAA promoted callus compaction, indicating effects on cell wall metabolism. Growth patterns were mixed, with higher NAA leading to nodular structures alongside smooth areas, reflecting localized differentiation and hormonal regulation.

Table 2. Callus initiation from Hass avocado seeds on MS medium supplemented with different concentrations of 2, 4-D and NAA.

Auxin type	Concentration (mg/L)	Color	Texture	Growth Pattern
2,4-D	0.5	White	Friable	Nodular
	2.5	White-Yellow	Compact	Smooth
	5.0	White - Yellow	Compact	Nodular, Smooth
	7.0	Yellow - Brown	Friable	Nodular
NAA	0.5	White	Friable	Smooth
	2.5	White	Friable-Compact	Smooth
	5.0	White- Yellow	Friable	Nodular
	7.0	White- Brown	Compact	Nodular

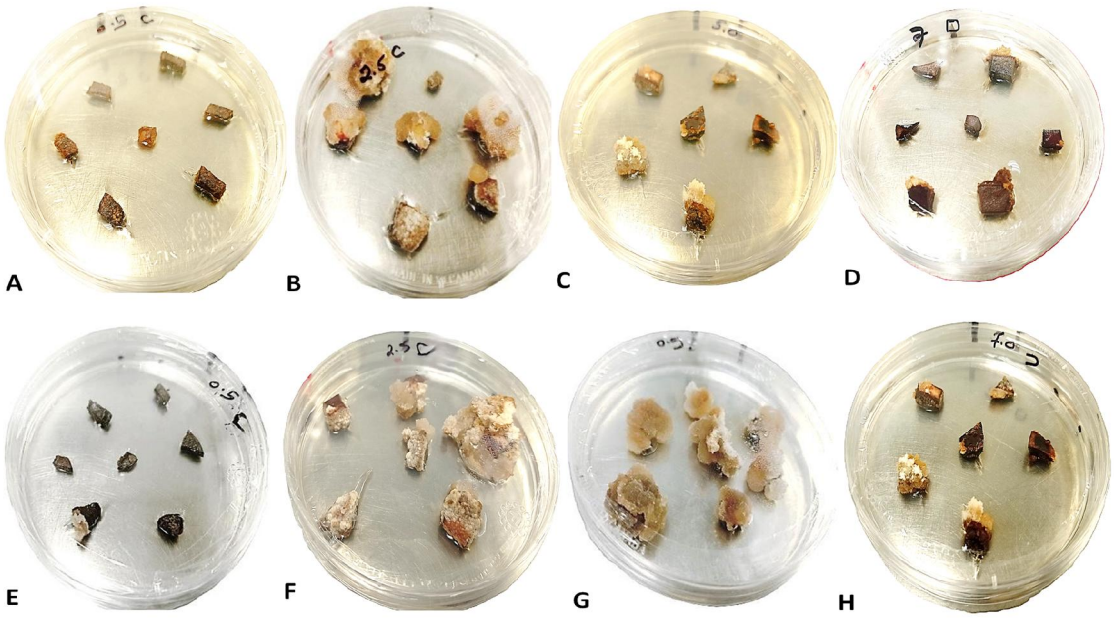

Fig. 1: Callus induction from Hass avocado seed explants after 4 weeks of culture on MS medium containing: (A)- 0.5 mg/l 2,4-D, (B)- 2.5 mg/l 2,4-D, (C)- 5.0 mg/l 2,4-D, (D)- 7.0 mg/l 2,4-D. (E)- 0.5 mg/l 2,4-D white, friable and nodular, (F)- 2.5 mg/l 2,4-D white, compact and smooth, (G)- 5.0 mg/l 2,4-D white, compact and nodular smooth, (H)- 7.0 mg/l 2,4-D yellow, friable and nodular.

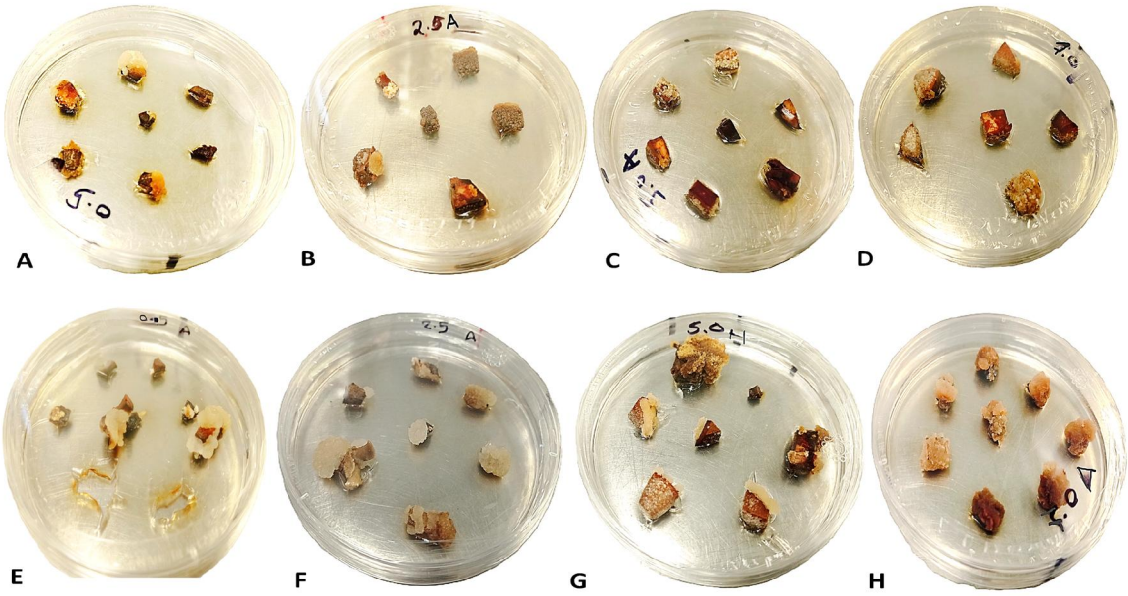

Fig. 2: Callus induction from Hass avocado seed explants after 4 weeks of culture on MS medium containing: (A)- 0.5 mg/l NAA, (B)- 2.5 mg/l NAA, (C)- 5.0 mg/l NAA, (D)- 7.0 mg/l NAA. (E)- 0.5 mg/l NAA white, friable and nodular, (F)- 2.5 mg/l NAA white, compact and smooth, (G)- 5.0 mg/l NAA white, compact and nodular smooth, (H)- 7.0 mg/l NAA yellow, friable and nodular.

3.5. Total phenolic content (TPC)

The phenolic content of callus cultures treated with 2,4-D ranged from 26.16 mg/g to 44.15 mg/g dry weight (Table 3). The highest total phenolic content was observed in the concentrations of 2.5 mg/L and 5 mg/L (43.76 mg/g DW and 44.15 mg/g DW, respectively) with no significant difference between them. The lowest total phenolic content was recorded in the concentrations of 0.5 mg/L and 7.0 mg/L (26.26 mg/g DW and 27.03 mg/g DW, respectively) with no significant difference between them. The phenolic content of callus cultures treated with NAA ranged between 13.82 mg/g DW and 37.14 mg/g DW (Table 3). Maximum amount of total phenolic content was obtained with the treatment of NAA at a concentration of 5.0 mg/L and 7.0 mg/L (36.69 mg/g DW and 37.14 mg/g DW, respectively) with no significant difference between them followed by the treatment of NAA at a concentration of 2.5 mg/L (19.96 mg/g DW) whereas the lowest total phenolic content was recorded for the 0.5 mg/L NAA (13.82 mg/g DW). The best treatment for the accumulation of phenolics in callus culture of Has avocado is 2,3-D at a concentration of 2.5 mg/L and 5.0 mg/L with no significant difference between them whereas NAA at a concentration of 0.5 mg/L demonstrated the lowest level of total phenolic content.

Total Flavonoid Content (TFC)

Total flavonoid content of callus cultures treated with 2,4-D ranged between 4.68 mg/g DW and 5.54 mg/g DW (Table 3). The highest content was recorded for both treatments, i.e., 2.5 mg/L and 5 mg/L (5.39 mg/g DW and 5.54 mg/g DW, respectively) whereas the lowest content was recorded for both treatments, i.e., 0.5 mg/L and 7 mg/L (5.39 mg/g DW and 5.54 mg/g DW, respectively). Total flavonoid content of callus cultures treated with NAA ranged from 2.05 mg/g DW to 4.89 mg/g DW (Table 3). Maximum amount of total flavonoid was obtained with the supplementation of NAA at a concentration of 5.0 mg/L and 7.0 mg/L (4.76 mg/g DW and 4.89 mg/g DW, respectively) with no significant difference between them followed by NAA at a concentration of 2.5 mg/L (2.84 mg/g DW) whereas 0.5 mg/L NAA showed the lowest level of total flavonoid content (2.05 mg/g DW). In general, maximum amount of flavonoid content was obtained when 2,4-D at a concentration of 2.5 mg/L and 5.0 mg/L was used whereas the lowest flavonoid content was recorded for NAA at a concentration of 0.5 mg/L).

Antioxidant Capacity

The results in Table (3) indicate that all sample exhibited antioxidant activity with varying degrees. Similar to the results of total phenolic and flavonoid contents, 2,4-D proved to be superior over NAA. All treatments with 2,4-D showed either higher or equal performance compared with corresponding treatments with NAA. The highest antioxidant activity was recorded for 2,4-D at a concentration of 2.5 mg/L and 5 mg/L (88.7 %, 88.9 %, respectively) with no significant difference between them followed by 2,4-D at a concentration of 0.5 mg/L and 7.0 mg/L and NAA at a concentration of 5 mg/L and 7 mg/L with no significant differences between these groups. The lowest antioxidant power was recorded for callus cultures treated with NAA at a concentration of 0.5 mg/L.

Table 3: Effect of different concentrations of 2,4-D and NAA on total phenolic and flavonoid contents and antioxidant capacity of avocado callus.

Auxin type	Concentration (mg/L)	Total phenolic content (mg/g)	Total flavonoid content (mg/g)	Antioxidant capacity (%)
2,4-D	0.5	26.16 c	4.68b	80.2d
	2.5	43.76 a	5.39a	88.7a
	5.0	44.15 a	5.54a	88.9a
	7.0	27.03 c	4.73b	79.8b
NAA	0.5	13.82e	2.05d	61.5d
	2.5	19.9dd	2.84c	70.9c
	5.0	36.69b	4.76b	80.1b
	7.0	37.14b	4.89b	80.2b

4. Discussion

Although shoot multiplication techniques are common in plant tissue culture, research on woody plants like avocado prioritizes somatic embryogenesis (Germanà & Lambardi, 2016). This method is particularly valuable for long-lived, difficult-to-propagate woody plants (Isah, 2016). Somatic embryogenesis offers advantages over other methods: it can potentially generate plantlets from single cells, ensuring genetic uniformity, and these embryos hold promise for mass production in bioreactors, similar to synthetic seeds, for efficient planting (Giri *et al*., 2004). While previous research on avocado somatic embryogenesis explored using immature embryos or fruit (Pliego-Alfaro., 1981; Marquez-Martin *et al*., 2012), this study successfully induced embryogenic callus cultures in the Hass variety using mature avocado seeds. This approach, known as indirect embryogenesis, has also been reported for other avocado varieties, with some studies exploring both direct and indirect methods (Encina *et al*., 2014; Fehér, 2019; Quintero-Jiménez *et al*., 2020). In essence, somatic embryogenesis holds promise for efficient and consistent propagation of avocados, particularly the Hass variety, offering advantages in terms o genetic uniformity, potential for mass production, and simplified plant regeneration.

The results in (Table 1) of callus induction indicated that all explants treated with different concentrations of either 2,4-D or NAA induced callus with the maximum efficiency (100%). This confirms the well-established knowledge that callogenesis (callus formation) is influenced by multiple factors, primarily the balance between the plant's natural hormones and those introduced in the culture medium (endogenous vs. exogenous growth regulators). These findings suggest the suitability of both 2,4-D and NAA for inducing callus from zygotic embryos of Hass avocado, further highlighting the high callogenic potential of this particular variety. It's important to remember that plant tissue culture success depends heavily on the specific plant species and even genotype. Some plants, like rice, regenerate easily in vitro, while others, like faba bean and wheat, are more challenging (Long *et al*., 2022). Even within a species, variations can exist, with Japonica rice varieties demonstrating a higher capacity for callus formation compared to Indica varieties (Mohamed *et al*., 2021). However, the optimal type and concentration of auxin for efficient callus formation can vary depending on the specific plant species or even cultivar. In this study, 2,4-D at a concentration of 5 mg/L resulted in callus cultures with the highest fresh and dry weights.

Several studies support the effectiveness of 2,4-D for callus induction in various plants, including avocado. Abo El-Fadl *et al*. (2022) found that 3 mg/L of 2,4-D was optimal for initiating callus from gazania leaf segments. Similarly, Tahereh *et al*. (2018) reported the highest callus fresh weight in Stevia rebaudiana using 2.0 mg/L of 2,4-D on leaf explants. Research by Zheng and Konzak, (1999) suggests that the optimal concentration and duration of 2,4-D exposure can vary depending on the plant genotype. Our findings align with these studies, as 2,4-D demonstrated superiority over other auxins for callus induction in this investigation. This advantage of 2,4-D for callus formation has also been documented in other plant species, such as those studied by Deng *et al*. (2020) and Liu *et al*. (2018).

Our study found that all the avocado explants contained measurable amounts of total phenolics and flavonoids. These findings are consistent with previous research highlighting avocados as a rich source of beneficial bioactive compounds like phenolics, flavonoids, and alkaloids (Rahman *et al*., 2022; Lyu *et al*., 2023). However, the quantities measured in our study differed from those reported in prior investigations. This variation is most likely due to the type of plant tissue used. In our experiment, we analyzed avocado callus, whereas (Lyu *et al*., 2023) focused on avocado peel, and Rahman *et al*. (2022) studied the pulp and seed.

Plants are loaded with natural chemicals called phytochemicals, known for their diverse health benefits. These bioactive compounds play a multifaceted role within the body, acting as both inhibitors and cofactors in enzyme reactions, scavenging harmful free radicals, enhancing nutrient metabolism, and even influencing a plant's taste and color. Among phytochemicals, phenolic compounds are hydrogen donors capable of directly scavenging free radicals and reducing oxidative damage (Kesharwani *et al*., 2012), which makes them potent antioxidants. In this research, the highest antioxidant activities were recorded for 2.5 mg/L and 5 mg/L 2,4-D treatments which is in accordance with our previous observation that these two treatments showed the highest total phenolic and flavonoid contents. This high correlation suggests that phenolic compounds were the main contributors to the antioxidant activity measured in avocados. The relationship between phenolic compounds content and the radical scavenging capacities in avocados was consistent with (Dudonné *et al*., 2009) Several studies have shown the link between high total phenol content and strong antioxidant activity (Zhang, 2015).

Additionally, (Zhang *et al*., 2011) found a positive correlation between total phenols and flavonoids, and their ability to inhibit DPPH free radicals, a common method for measuring antioxidant capacity. However, the complex nature of bioactive compounds in plants means a single test, like the DPPH assay, isn't enough to fully capture their antioxidant potential.

5. Conclusion

This study successfully established callus cultures from 'Hass' avocado zygotic embryos, demonstrating their potential as a viable source of valuable bioactive compounds. The observed differential growth and compound accumulation in response to various media with different PGRs concentrations highlight the promise of optimizing callus culture conditions for enhanced production. The treatment with 5 mg/L 2,4-D yielded the highest biomass and significantly influenced the accumulation of phenolic content, flavonoids, and antioxidant activity. These findings underscore the importance of further research to refine culture parameters and maximize the production of these bioactive compounds. This research paves a solid foundation for establishing a sustainable and eco-friendly approach to producing natural antioxidants from 'Hass' avocado, leveraging the advantages of callus culture technology. Ultimately, this approach not only contributes to advancements in the agricultural and biotechnological fields but also offers a potential pathway for enhancing the nutritional value and health benefits of avocado products.

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Conflict of Interests

Authors do not have any conflict of interest.

References

Abo El-Fadl, R., A. Mahdi, A. E. Abdelhamid, M. R. Abdel Magid and A. H. Hassan, (2022). Enhancing the biochemical constituents in avocado callus using encapsulated chitosan nanoparticles. Egyptian Journal of Chemistry, 65(12): 769-782.

Amer, A. and H. Omar, 2019. *In-vitro* propagation of the multipurpose Egyptian medicinal plant *Pimpinella anisum*. Egypt Pharmaceut J., 18:254-62.

Amer, A.M. and Aly U.I., 2019. Antioxidant and antibacterial properties of anise (*Pimpinella anisum* L.). Egypt Pharmaceut J. 2019; 18:68-73. DOI: 10.4103/epj.epj_44_18.

Bhuyan, D.J., M.A. Alsherbiny, S. Perera, M. Low, A. Basu, O.A. Devi, M.S. Barooah, C.G. Li and K. Papoutsis, 2019. The Odyssey of Bioactive Compounds in Avocado (*Persea americana*) and Their Health Benefits. Antioxidants, 8, 426.

Catalina, R. O., F. A. Gómez Velásquez, A. Gil Correal, J. M. T. Bonilla and A. I. Urrea Trujillo, 2018. *In vitro* propagation of avocado (*Persea americana* Mill.) cv. Hass through morphogenesis. Acta Agronómica, 67(1), 160-167. https://dx.doi.org/10.1s446/acag.v67n1.61474.

Cárdenas-Castro, A.P., Á. Fernández-Ochoa, M.D.L.L. Cádiz-Gurrea, A. Segura Carretero and S. G.Sáyago-Ayerdi, 2023. Bioactive phytochemicals from avocado oil processing by- products. In Bioactive phytochemicals from vegetable oil and oilseed processing by- products (pp. 403-430). Cham: Springer International Publishing.

Chaudhary, P., P. Janmeda, A. O. Docea, B. Yeskaliyeva, A. F.Abdull Razis, , B. Modu and J. Sharifi-Rad, 2023. Oxidative stress, free radicals and antioxidants: Potential crosstalk in the pathophysiology of human diseases. Frontiers in chemistry, 11, 1158198.

Deng, X., Y. Xiong, J. Li, D. Yang, J. Liu, H. Sun, H. Song, Y. Wang, J. Ma, Y. Liu and M. Yang, 2020. The Establishment of an Efficient Callus Induction System for Lotus (*Nelumbo nucifera*). Plants (Basel). 25;9(11):1436. doi: 10.3390/plants9111436. PMID: 33113801; PMCID: PMC7693671.

Dudonné S., X. Vitrac, P. Coutière, M. Woillez and J.M. Mérillon, 2009. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. J. Agric. Food Chem., 57:1768–1774. doi: 10.1021/jf803011r.

Encina, C. L., A. Parisi, C. O'Brien, and N. Mitter, 2014. Enhancing somatic embryogenesis in avocado (*Persea americana* Mill.) using a two-step culture system and including glutamine in the culture medium. Sci. Hortic., 165:44-50. Doi: 10.1016/j.scienta.2013.10.019.

Fehér, A. 2019. Callus, dedifferentiation, totipotency, somatic embryogenesis: what these terms mean in the era of molecular plant biology? Front. Plant Sci., 10:536. Doi: 10.3389/fpls.2019.00536.

Figueroa, J.G., I. Borra´s-Linares, J. Lozano-Sanchez and A. Segura Carretero, 2018. Comprehensive characterization of phenolic and other polar compounds in the seed and seed coat of avocado by HPLC-DAD-ESI-QTOF-MS. Food Res Int., 105:752–763.

Food and Agriculture Organization of the United Nations Statistical Databases, FAOSTAT. 2018. Food and Agricultural Organization, United Nations, Rome. http://www.fao.org/faostat/en/#data/QC/visualize. [Accessed August 18, 2020].

Geremu, M., Y.B. Tola and A. Sualeh, 2016. Extraction and determination of total polyphenols and antioxidant capacity of red coffee (*Coffea arabica* L.) pulp of wet processing plants. Chem. Biol. Technol. Agric. 3, 25. https://doi.org/10.1186/s40538-016-0077-1.

Germanà, M. A., and M. Lambardi, 2016. *In vitro* Embryogenesis in Higher Plants. New York, NY: Springer Science Business Media.

Giri, C., B. Shyamkumar and C. Anjaneyulu, 2004. Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. Trees 18, 115–135. doi: 10.1007/s00468-003-0287-6.

Hiti-Bandaralage, J., A. Hayward, and N. Mitter, 2017. Micropropagation of Avocado (*Persea Americana* Mill.). American Journal of Plant Sciences, **8**, 2898-2921.

doi:10.4236/ajps.2017.811197.https://pdfs.semanticscholar.org/c7ab/7ac9e59447af95a8c765a33c8f065d4a3c51.pdf.

Irshad, M., B. Debnath, S. Mitra, *et al., 2018.* Accumulation of anthocyanin in callus cultures of red-pod okra (*Abelmoschus esculentus* (L.) Hongjiao) in response to light and nitrogen levels. Plant Cell Tiss Organ Cult., 134, 29–39. https://doi.org/10.1007/s11240-018-1397-6.

Isah, T., 2016. Induction of somatic embryogenesis in woody plants. Acta Physiol. Plant 38, 1–22. doi: 10.1007/s11738-016-2134-6.

Vidales-Paz, J.E., L. D. Medina-Salas, P. U. Bautista-Rosales and P. P. C. Junco, 2021. Hass Avocado: A Potential in the Industry For Its Bioactive Compounds and Benefits for Health. J. Bio. Innov.,10(5): 1265-1273. https://doi.org/10.46344/JBINO.2021.v10i05.08.

Kasote, D. M., S.S. Katyare, M.V. Hegde and H. Bae, 2015. Significance of antioxidant potential of plants and its relevance to therapeutic applications. International journal of Biological Sciences, 11(8): 982–991. https://doi.org/10.7150/ijbs.12096.

Kesharwani, R.K., D.V. Singh, K. Misra and S.I. Rizvi, 2012. Plant polyphenols as electrondonors for erythrocyte plasma membrane redox system: validation throughin silico approach. Org Med Chem Lett., 2:12.

Liao, S., S.O. Omage, L. Börmel, S. Kluge, M. Schubert, M. Wallert and S. Lorkowski, 2022. Vitamin E and metabolic health: Relevance of interactions with other micronutrients. Antioxidants, 11(9): 1785.

Liu, J., H. Feng, Y. Ma, L. Zhang, H. Han and X. Huang, 2018. Effects of different planthormones on callus induction and plant regeneration of miniature roses (*Rosa hybrida* L.). Horticult Int J., 2:201–206. doi: 10.15406/hij.2018.02.00053.

Lyu, X., O.T. Agar, C.J. Barrow, F.R. Dunshea and H.A.R. Suleria, 2023. Phenolic Compounds Profiling and Their Antioxidant Capacity in the Peel, Pulp, and Seed of Australian Grown Avocado. Antioxidants (Basel). 2023 Jan 12;12(1):185. doi: 10.3390/antiox12010185. PMID: 36671046; PMCID: PMC9855119.

Mancini, A., C. Bruno, E. Vergani, F. Guidi, F. Angelini, E. Meucci and A. Silvestrini, 2020. Evaluation of oxidative stress effects on different macromolecules in adult growth hormone deficiency. PLoS One, 15(7), e0236357.

Márquez-Martín, B., A. Barceló-Muñoz, F. Pliego-Alfaro and C. Sánchez-Romero, 2012. Somatic embryogenesis and plant regeneration in avocado (*Persea americana* Mill.): influence of embryogenic culture type. Journal of plant biochemistry and biotechnology, 21, 180-188.

Mohamed, G. M., A. M. Amer, N. H. Osman, M. Z. Sedikc and M. H. Hussein, 2021. Effects of different gelling agents on the different stages of rice regeneration in two rice cultivars. Saudi Journal of Biological Sciences, 28(10):5738-5744.

Murashige, T. and F.Skoog, 1962. A revised medium for rapid growth requirements of tobacco tissues cultures. Physiol Plant., 15:473-497.

Ochatt, S. and A.R. Alan, 2022, Bhattacharya, A. et al. Secondary metabolites: a boon from plants, the best chemist in nature: preface from the editors. Plant Cell Tiss Organ Cult., 149, 1–6 (2022). https://doi.org/10.1007/s11240-022-02289-2.

Palomo-Ríos, E., Cerezo, S., J.A. Mercado, *et al.* 2017. *Agrobacterium*-mediated transformation of avocado (*Persea americana* Mill.) somatic embryos with fluorescent marker genes and optimization of transgenic plant recovery. Plant Cell Tiss Organ Cult., 128, 447–455. https://doi.org/10.1007/s11240-016-1122-2.

Pliego-Alfaro F., 1981. A Morphogenetic Study of the Avocado (*Persea americana* Mill.) in vitro: I. Development of a Rooting Bioassay and its Application to Studying Restoration by Grafting of Rooting Competence in Adult. II. Somatic Embryogenesis in Callus. PhD Thesis. University of California, Riverside, California.

Quintero-Jiménez, A., S. Espinosa-Zaragoza, A. Guerrero-Rangel and I. J. L. González-Durán, 2020. Embryogenesis and organogenesis of Mexican creole avocado. Revista Mexicana de Ciencias Agrícolas, 11(7): 1525-1536.

Rahman, N., S.M. Sabang, R. Abdullah and B. Bohari, 2022. Antioxidant properties of the methanolic extract of avocado fruit peel (*Persea americana* Mill.) from Indonesia. J Adv Pharm Technol Res., 13(3):166-170. doi: 10.4103/japtr.japtr_22_22. Epub 2022 Jul 5. PMID: 35935697; PMCID: PMC9355060.

Sen, A.K., D.B. Sen and R.A. Maheshwari, 2020. Extraction, Isolation, and Quantitative Determination of Flavonoids by HPLC. In: Sen, S., Chakraborty, R. (eds) Herbal Medicine in India. Springer, Singapore. https://doi.org/10.1007/978-981-13-7248-3_21.

Shasmita, S. Behera, P. Mishra, *et al.,* 2023. Recent advances in tissue culture and secondary metabolite production in *Hypericum perforatum* L.. Plant Cell Tiss Organ Cult., 154, 13–28 (2023). https://doi.org/10.1007/s11240-023-02525-3.

Sina, B., H. Demissie, and Y. Rezene, 2024. Assessment of the Constraints and Challenges in Avocado (*Persea americana* Mill.) Production and Marketing in Southern Ethiopia. International Journal of Fruit Science, 24(1): 60-72.

Kupina, S., C. Fields, M. C Roman, S. L. Brunelle, 2018. Determination of Total Phenolic Content Using the Folin-C Assay: Single-Laboratory Validation, First Action 2017.13, Journal of AOAC International, 101(5): 1466–1472. https://doi.org/10.5740/jaoacint.18-0031.

Tahereh, K., N. Abdollah, K. Danial and Z. Alireza, 2018. Callus induction and somatic embryogenesis in Stevia rebaudiana Bertoni as a medicinal plant. Cell. Mol. Biol., 64(2): 46-49.

Tamayo-Ramos, D. I., J.A. Salazar-González, S.A. Casson and R. Urrea-López, 2022. Old and new horizons on Persea americana transformation techniques and applications. Plant Cell, Tissue and Organ Culture (PCTOC), 150(2): 253-266.https://doi.org/10.1007/s11240-022-02268-7.

Tremocoldi, M. A., P. L. Rosalen, M. Franchin, A.P. Massarioli, C. Denny, É. R. Daiuto, J. Paschoal, P. S. Melo and S. M. Alencar, 2018. Exploration of avocado by-products as natural sources of bioactive compounds. PloS one, 13(2), e0192577. https://doi.org/10.1371/journal.pone.0192577.

Waly, D. A., A. H. A. Zeid, H. N. Attia, K. A. Ahmed, E. S. A. El-Kashoury, A. M. El Halawany and R. S. Mohammed, 2023. Comprehensive phytochemical characterization of Persea americana Mill. Fruit via UPLC/HR-ESI–MS/MS and anti-arthritic evaluation using adjuvant- induced arthritis model. Inflammopharmacology, 31(6): 3243-3262.

Wang, J., H. Yang, P. Wu, J. Zhang, W. Ma, Y. Li and J. Liu, 2023. Effect of Predry- treatment on the bioactive constituents and quality of avocado (*Persea americana* Mill.) oil from three cultivars growing in China. Frontiers in Nutrition, 10. https://doi.org/10.3389/fnut.2023.1230204

Wu, Y., Zhang, L., Zhang, Y. et al., 2024. Roles of Antioxidant Enzymes, Secondary Metabolites, and Lipids in Light Adaption of Tea-Oil Plant (*Camellia oleifera* Abel). J Plant Growth Regul., https://doi.org/10.1007/s00344-024-11256-4.

Zehiroglu, C. and S.B. Ozturk Sarikaya, 2019. The importance of antioxidants and place in today's scientific and technological studies. Journal of Food Science and Technology, 56(11):4757- 4774. DOI: 10.1007/s13197-019-03952-x.

Zhang, L., A.S. Ravipati, S.R. Koyyalamudi, S.C. Jeong, N. Reddy, P.T. Smith, et al., 2011. Antioxidant and anti-inflammatory activities of selected medicinal plants containing phenolic and flavonoid compounds. J Agric Food Chem., 59:12361–7.

Zhang, Y.J., R.Y. Gan, S. Li, Y. Zhou, A.N. Li, D.P. Xu, et al., 2015. Antioxidant phytochemicals for the prevention and treatment of chronic diseases. Molecules, 20:21138–56.

Zhang, Y., Y. Chen, Y. Wan, Y. Zhao, Q. Wen, X. Tang and F. Du, 2021. Circular RNAs in the regulation of oxidative stress. Frontiers in Pharmacology, 12, 697903.

Zheng, M.Y. and C.F. Konzak, 1999. Effect of 2,4-dichlorophenoxyacetic acid on callus induction and plant regeneration in another culture of wheat (*Triticum aestivum* L.). Plant Cell Rep., 19(1):69-73. doi: 10.1007/s002990050712. PMID: 30754762.