Research Article

Storage of embryos and *in vitro* development of embryonic axes in avocado[1]

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ABSTRACT

The *in vitro* establishment of *Persea americana* embryonic axes is critical for micropropagation and biotechnological applications, particularly following seed storage. This study aimed to evaluate the effects of seed storage duration and culture media composition on the *in vitro* development of *P. americana* ('Toro Canyon' and 'Duke 7' cultivars) embryonic axes in MS (Murashige & Skoog 1962), MSm (30 % of macronutrient reduction) or SHm (modified from Schenk & Hildebrandt 1972) media. Morphological parameters, including embryo and embryonic axis length, width and mass, were recorded. The *in vitro* assessments included survival, rooting and rosette formation. The experiment followed a completely randomized design, with four replicates. The 'Duke 7' seeds were longer and heavier than the 'Toro Canyon' seeds, but did not produce significantly larger embryos. Without storage, 'Duke 7' exhibited a higher survival in SHm and lower rooting in MS, while 'Toro Canyon' showed no significant differences in survival or development among the media, except for lower rooting in SHm. Storage duration and packaging type did not significantly affect survival or rooting percentages, but influenced the rosette explant formation, which was lower in MS. After six months of storage, the 'Duke 7' embryonic axes maintained 93 % of viability, with 31 % forming rosettes and 69 % rooting. These findings suggest that the storage viability depends more on culture medium composition than seed biometry.

KEYWORDS: *Persea americana* Mill., seed storage, embryo axis.

RESUMO

Armazenamento de embriões e desenvolvimento *in vitro* de eixos embrionários em abacateiro

O estabelecimento *in vitro* de eixos embrionários de *Persea americana* é fundamental para micropropagação e aplicações biotecnológicas, particularmente após o armazenamento de sementes. Objetivou-se avaliar os efeitos da duração de armazenamento de sementes e da composição dos meios de cultura sobre o desenvolvimento *in vitro* de eixos embrionários de *P. americana* (cultivares 'Toro Canyon' e 'Duke 7') em meio MS (Murashige & Skoog 1962), MSm (redução de 30 % de macronutrientes) ou SHm (modificado de Schenk & Hildebrandt 1972). Parâmetros morfológicos, incluindo comprimento, largura e massa do embrião e do eixo embrionário, foram registrados. As avaliações *in vitro* incluíram sobrevivência, enraizamento e formação de rosetas. O experimento seguiu delineamento inteiramente casualizado, com quatro repetições. As sementes de 'Duke 7' mostraram-se mais longas e pesadas do que as de 'Toro Canyon', mas não produziram embriões significativamente maiores. Sem armazenamento, 'Duke 7' exibiu maior sobrevivência em SHm e menor enraizamento em MS, enquanto 'Toro Canyon' não apresentou diferenças significativas na sobrevivência ou desenvolvimento entre os meios, exceto para menor enraizamento em SHm. A duração do armazenamento e o tipo de embalagem não afetaram significativamente a sobrevivência ou as porcentagens de enraizamento, mas influenciaram na formação do explante roseta, que foi menor no MS. Após seis meses de armazenamento, os eixos embrionários 'Duke 7' mantiveram 93 % de viabilidade, com 31 % formando rosetas e 69 % de enraizamento. Esses resultados sugerem que a viabilidade de armazenamento depende mais da composição do meio de cultura do que da biometria das sementes.

PALAVRAS-CHAVE: *Persea americana* Mill., armazenamento de sementes, eixo embrionário.

INTRODUCTION

Persea americana Miller, native to Central America, has been widely cultivated across various regions, including Latin America, the United States, Spain, South Africa, Haiti and Australia, with Brazil accounting for approximately 19,000 of the 890,000 ha planted worldwide (FAO 2022).

The commercial propagation of this perennial species is primarily achieved through grafting

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(Goldschmidt 2014, Williams et al. 2021). In this scenario, micropropagation techniques, including those using embryonic axes, facilitate seedling production and embryo rescue (Kaviani & Kulus 2022), enabling the generation of healthy, axenic seedlings for micrografting, physiological studies and germplasm conservation (Rai 2022)

Avocado is a single-embryonic species with recalcitrant seeds, what limits its genetic improvement due to low crossover rates and reduced viability after storage (Hazubska-Przybył & Bojarczuk 2016, Rendón-Anaya et al. 2019). Initial studies on *P. americana* embryonic axes suggest propagation potential. However, while *in vitro* techniques are increasingly employed to reduce costs associated with early-stage propagation, studies on the *in vitro* development of *P. americana* embryonic axes remain scarce, with limitations in structural development and culture media optimization for subsequent growth phases (Hiti-Bandaralage et al. 2017, Abo El-Fadl et al. 2022).

Certain pretreatments (e.g., partial seed desiccation) have been shown to enhance germination over time (Matilla 2021). However, no studies have investigated the *in vitro* development of previously stored seeds. Among cultivation media, MS (Murashige & Skoog 1962) is not recommended for avocados, due to its association with physiological disorders and altered explant development. Nevertheless, reducing MS macronutrient concentrations or using alternative media such as wood plant medium (Lloyd & McCown 1981) has demonstrated superior outcomes for avocado nodal shoots (Hiti-Bandaralage et al. 2017, Hiti-Bandaralage et al. 2018, Restrepo Osorio et al. 2018).

From this perspective, this study aimed to compare the variability of 'Toro Canyon' and 'Duke 7' avocado embryos and assess the *in vitro* development of avocado embryonic axes as influenced by culture media composition and seed storage conditions.

MATERIAL AND METHODS

The experiments were conducted at the Universidade Estadual Paulista (Jaboticabal, São Paulo state, Brazil) and the seeds extracted from mature fruits of 'Toro Canyon' and 'Duke 7' matrices obtained from its active germplasm bank, in 2020.

In order to evaluate morphological variability, fifty healthy, mature fruits from each cultivar were collected to assess the embryo mass and embryo-to-seed ratio in 'Toro Canyon' and 'Duke 7' avocados. The pulp was removed and the seeds washed with tap water to eliminate the epicarp before surface drying. To excise the embryonic axis, the seed was sectioned at the apical region along the cotyledon junction until complete separation. The axis was then excised from the base using a scalpel. Length (mm), width (mm) and mass (g) were measured, and correlations between seed size and embryonic axis dimensions analyzed.

For the embryo storage and viability, the seeds were prepared following the aforementioned procedure. After surface disinfestation in NaOCl solution (1.5 % active ingredient) for 30 min, the seeds were either stored or placed in the development medium, depending on the treatment.

Following each storage period, the seeds were disinfested again in NaOCl (1.5 % active ingredient) for 20 min and rinsed three times with autoclaved deionized water (30 min at 1.2 atm). For axis excision, the seed was separated at the cotyledon junction, and the embryonic axis was removed and placed in a 200-mL glass vial containing 25 mL of culture medium, sealed with polyethylene plastic film (0.910-0.940 g cm^{-3}).

The experimental conditions included irradiance of 11.4 (± 2.66) µmol m^{-2} s^{-1}. Inside the container, the temperature and relative humidity (RH), measured using an Instruterm® HT-500 thermo-hygrometer, averaged 22.0 ºC (± 0.7) and 95.43 % (± 0.7), respectively.

For the embryo storage and culture medium *in vitro* development of embryonic axes, embryonic axes of 'Duke 7' and 'Toro Canyon' were obtained following the methodology previously described to compare their *in vitro* development across three culture media: MS (Murashige & Skoog 1962), MSm (30 % of macronutrient reduction) or SHm (modified from Schenk & Hildebrandt 1972). The axes were either cultivated immediately or after two months of storage.

Storage was conducted in the dark within a growth chamber (10.3 ºC ± 0.5, 62.8 % ± 4.2 RH). Depending on the treatment, two seeds were placed in Kraft paper bags (100 g m^{-2}; 0.5 kg capacity), whereas ten seeds were stored in double plastic bags (1.10-1.35 g cm^{-3}), either hermetically sealed or perforated (~100 holes; 1 mm in diameter).

After 60 days, the survival rate, rooting, rosette formation and apical development were assessed (Figure 1). The experiment followed a completely randomized design, with ten vessels containing individual explants as the experimental unit. Variance and mean comparisons were performed using the Scott-Knott test ($p \leq 0.05$).

For the viability of embryonic axes of 'Duke 7' avocado embryos stored for six months, seed storage followed the general methodology previously described. Seeds were stored in sealed plastic boxes in the dark for six months (10 ºC; 63 % RH). After storage, the embryonic axes were extracted, placed in SHm culture medium under a laminar flow chamber, and maintained in a growth room under the aforementioned conditions.

The experiment followed a completely randomized design, with four replicates, using eight embryonic axes per experimental unit. The data were transformed (arcsin $\sqrt{x}/2$) to normalize residuals. Mean comparisons were conducted using an F-test, with regression analysis performed when significant ($p \leq 0.05$). The analyzed variables included survival rate, rooting and apical development after 65 days of culture establishment.

RESULTS AND DISCUSSION

The 'Duke 7' seeds were larger and heavier than the 'Toro Canyon' ones. However, whereas the embryonic axes of both cultivars were similar in size, those of 'Duke 7' were lighter, resulting in a lower seed mass-to-axis ratio, if compared to 'Toro Canyon' (Table 1). These physical characteristics are relevant, as they may contribute to physical dormancy, a germination-limiting factor commonly

Figure 1. Evaluation of survival and rooting in MS (Murashige & Skoog 1962), MSm (30 % of macronutrient reduction) or SHm (modified from Schenk & Hildebrandt 1972) culture media, after 60 days of *Persea americana* embryonic axes under *in vitro* conditions. a) Embryo axes excision; b) early-stage establishment; c) greening and establishing; d) apical and rooting differentiation; e) leaf differentiation; f) stem elongation and bud differentiation.

Table 1. Embryonic axis biometry of 'Duke 7' and 'Toro canyon' cultivars of *Persea americana*.

Cultivar	Seed			Embryonic axis			EM/EAR
	Height (mm)	Diameter (mm)	Mass (g)	Height (mm)	Diameter (mm)	Mass (g)	
'Toro Canyon'	43.03 b*	31.74 ns	25.20 b	10.03 ns	5.43 ns	0.10 a	0.40 a
'Duke 7'	49.66 a	32.34	30.47 a	10.3	5.68	0.08 b	0.27 b
P (0.05)	< 0.001	0.330	< 0.001	0.567	0.41	0.027	< 0.001

* Letters in each row indicate a significant difference by the Scott-Knott test ($p \leq 0.05$) at a significance level of 5 % by the F-test. ns: not significant; EM/EAR: embryo mass to embryonic axis ratio.

observed in recalcitrant seeds such as avocado (Gálvez-Cendegui et al. 2017, O'Brien et al. 2021, Shu'aibu Abubakar & Lawal Attanda 2022).

Seedling development is influenced by multiple factors, including seed size. It has been suggested that cotyledon size may also affect the protrusion of the embryonic axis (Penfield 2017, Steinbrecher & Leubner-Metzger 2018). The present study found variability in embryonic axis size among seed samples, with larger seeds not necessarily producing larger axes. The correlation between cotyledon size and embryonic axis size was not significant for 'Duke 7' (0.917), but was significant for 'Toro Canyon' ($p = 0.049$). The Pearson's correlation coefficients were 0.018 ($p > 0.050$) for 'Duke 7' and 0.335 for 'Toro Canyon'.

In tests without storage, neither 'Toro Canyon' nor 'Duke 7' exhibited interactions between factors over time. For 'Duke 7', survival ($p = 0.019$) and rooting ($p = 0.006$) differed significantly, whereas the number of rosette explants showed no difference ($p = 0.272$). For 'Toro Canyon', rooting varied among the culture media ($p = 0.020$), but survival ($p = 0.272$) and rosette explants ($p = 0.260$) did not. Means and adjusted regression models are presented in Table 2.

For 'Duke 7' axes stored for 60 days, the development rate of complete structures varied depending on the culture medium composition. However, no significant interactions were observed between storage methods and survival ($p = 0.145$), rooting ($p = 0.210$) or rosette explants ($p = 0.107$).

Among the simple effects of storage methods, there were no significant differences in rooting ($p = 0.104$), survival ($p = 0.116$) or the rosette phase ($p = 0.431$). However, the culture media influenced the percentage of rosette explants ($p = 0.001$), which was the lowest in MS (28 %). MSm and SHm showed no significant difference, with a mean of 52 %. The survival ($p = 0.620$) and rooting ($p = 0.085$) rates remained consistent, averaging 97 % (CV = 5.72 %) and 70 % (CV = 27.09 %), respectively.

The culture media composition directly influenced the morphogenic response of tissues under *in vitro* conditions, as reported in previous studies (Restrepo Osorio et al. 2018, Pasternak & Steinmacher 2024). According to Murashige (1973) and George et al. (2008), variations in macro and micronutrient concentrations, ionic forms, and the presence of vitamins and growth regulators significantly impact explant responses.

The primary difference between MS and MSm lies in the reduction of macronutrients. For MS, the main constituents were KNO_3 and NH_4NO_3, followed by $CaCl_2$ and $MgSO_4$ (Figure 2a). For SHm, KNO_3

Table 2. Average percentage of survival, rooting and *in vitro* development of *Persea americana* embryonic axes from 'Duke 7' and 'Toro Canyon' without storage in SHm (modified from Schenk & Hildebrandt 1972) medium after 60 days.

Cultivar	Average (%)	Adjusted model (Y =)	CV (%)
'Toro Canyon'			
Alive	99	-	4.52
Rooted	-	$0.485 + 0.027x - 0.0004X^2$	11.38
Rosette	-	$2.8225 - 0.3789x + 0.0199\ X^2$	25.32
'Duke 7'			
Alive	-		22.92
Rooted	-	$-0.0516 + 0.0134x + 0.0001\ X^2$	45.76
Rosette	57	$1.1712 + 0.0967 - 0.0011\ X^2$	20.97

Adjusted model, significant at 5 % by the F-test.

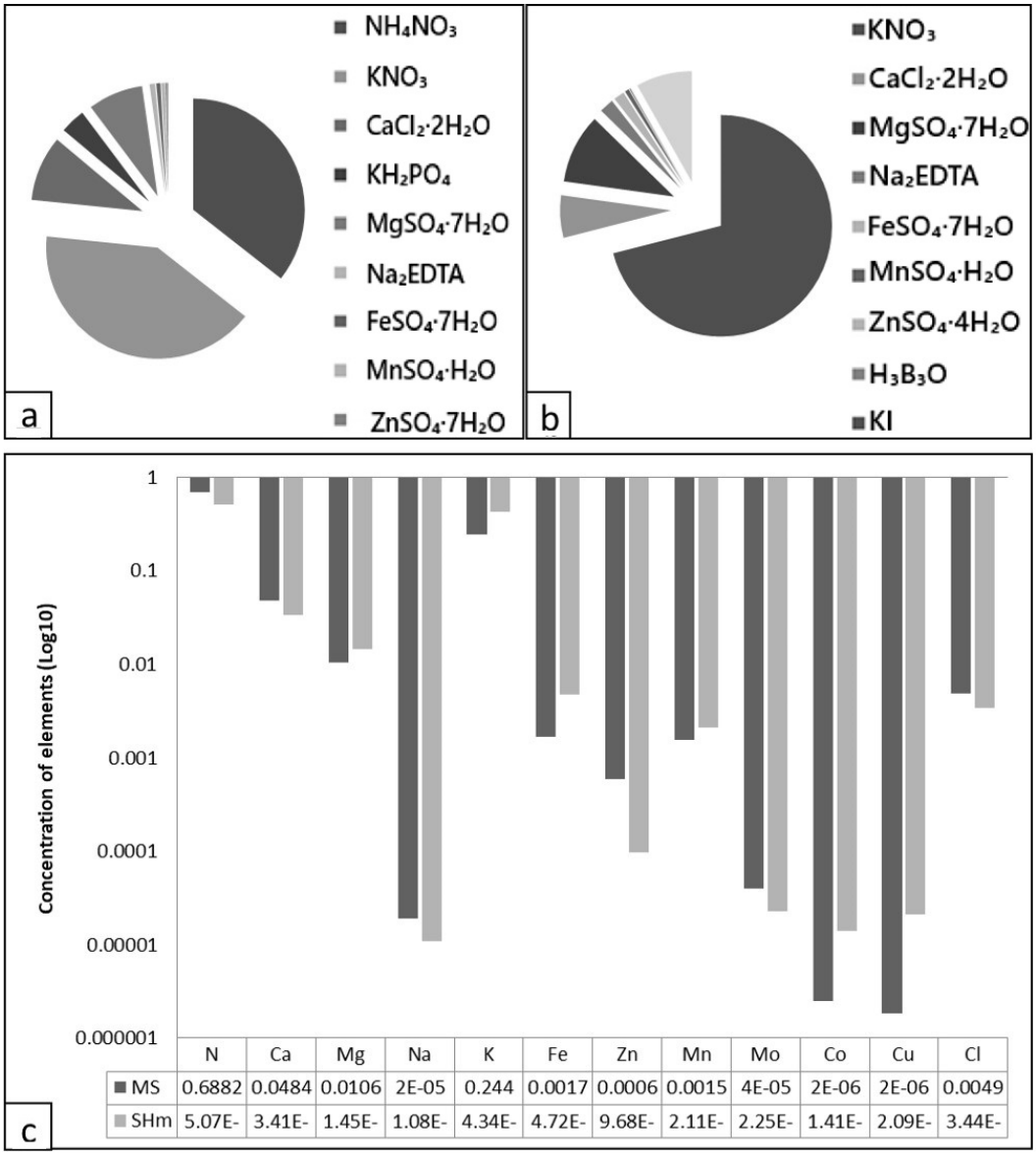

Figure 2. Comparison of significant nutrients between MS (Murashige and Skoog 1962) (a) and SHm (modified from Schenk & Hildebrandt 1972) (b), and constituent chemical elements in both (c).

was the primary component, followed by $MgSO_4$ and NH_2HPO_4 (Figure 2b). These differences suggest that variations in total salt concentration, NH_4/NO_3 ratio and Ca + Mg/K ratio (Figure 2c) are key factors influencing explant development.

For the MS medium, the NO_3 concentration was higher than that of NH_4, with N-to-Ca^{2+} and N-to-Mg^{2+} ratios of 40:1 and 20:1, respectively. These variations in ionic concentration and proportion relative to other culture medium components may influence the initiation, growth and differentiation of *in vitro* explants (Hajari et al. 2015, Wada et al. 2015).

Additionally, in avocado, factors such as seed maturity, cotyledon tissue presence and culture medium components - including activated charcoal and growth regulators - can impact the *in vitro* development (Pan & Staden 2000, Viñas & Jiménez 2011). Specifically, the MS medium has been associated with physiological disorders and phytotoxicity due to its composition. Conversely, media with reduced macronutrient concentrations and adjusted nitrogen ratios favor embryo dedifferentiation (Pliego-Alfaro 1988, Pasternak et al. 2003). Genetic factors must also be

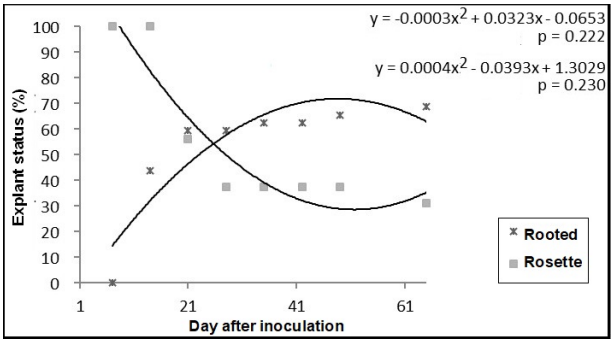

Figure 3. Regression variance analysis for *in vitro* development of the apical and root meristems of *Persea americana* cv. 'Duke 7' in SHm (modified from Schenk & Hildebrandt 1972) medium.

considered, as organ differentiation and development in various fruit species - including apple, coconut and citrus - are genotype-dependent (Thorpe & Yeung 2011, Wada et al. 2015).

Concerning the viability of 'Duke 7' embryo axes stored for six months, the survival rate remained stable throughout the evaluation period ($p > 0.050$), averaging 93 %. Over 65 days of incubation, root development ($p = 0.022$) and apical meristem growth ($p = 0.230$) followed a quadratic regression model (Figure 3).

Seed viability during storage depends on the species' physiological classification - whether recalcitrant, orthodox or intermediate (Von Teichman & Van Wyk 1994). In avocado with recalcitrant seeds, 'Esther' cultivar seeds stored in plastic bags (5 ± 1 ºC and 80 ± 5 % RH) for one to three months maintained a high stem length and diameter (Gálvez-Cendegui et al. 2017). However, seed physiology during storage is dynamic and influenced by factors such as seed size, parent plant nutritional status and reserve composition (Long et al. 2015, Pirredda et al. 2023).

Storage packaging also affects atmospheric gas composition - particularly nitrogen and oxygen levels (Bonner & Karrfalt 2008) - which can restrict metabolism and enhance seed longevity (Ratajczak et al. 2019).

CONCLUSIONS

1. The *in vitro* development of *Persea americana* embryonic axes varied among the evaluated genotypes;
2. The embryo-to-embryonic axis mass ratio differed between 'Duke 7' and 'Toro Canyon', with variation observed only for embryo mass;
3. Seed storage at 10.3 ± 0.5 ºC allows the use of embryonic axes for *in vitro* cultivation for up to two months, when stored in plastic bags, either perforated or sealed;
4. The 'Duke 7' embryonic axes remain viable for *in vitro* cultivation after six months of storage.

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