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Acclimatization of coffee seedlings obtained from zygotic embryos of aged seeds

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Abstract

Coffee seeds rapidly lose viability during storage, which hinders the development of vigorous seedlings for crop establishment. There are reports that seed endosperm is more sensitive to deterioration than embryos, which can be excised and cultivated *in vitro*. However, a substantial number of plants grown *in vitro* do not survive during transfer to a greenhouse or field environment. The objective of this study was to evaluate the acclimatization of coffee seedlings of cultivar Catuaí Amarelo IAC 62, developed from zygotic embryos obtained from aged seeds in different substrates and environments, for the production of well-developed seedlings suitable for planting. For this purpose, seedlings were obtained from the *in vitro* cultivation of embryos obtained from seeds of two quality levels: freshly harvested seeds and artificially aged seeds. Zygotic embryos were extracted from the seeds and cultivated in MS medium. At 60 days, the percentages of normal and abnormal seedlings and dead seeds were evaluated. The good-quality seedlings grown *in vitro* for 60 days were transplanted into two different substrates (Tropstrato and coconut fiber) and acclimatized in two environments (growth room and greenhouse with a misting system). The plants were evaluated for height, stem diameter, number of leaves, chlorophyll content, and growth rate. The greenhouse environment was better for seedling growth, possibly due to its higher sunlight and temperature. The best substrate was coconut fiber, as it ensured better development of plants from freshly harvested seeds and those from aged seeds. It is possible to develop healthy seedlings from seeds with low viability.

Keywords: *Coffea arabica* L.; *In vitro* culture; Greenhouse; Growth room; Substrates.
Abbreviations: CECAFE_Conselho dos Exportadores de Café do Brasil**,** RH_relative humidity**,** BOD_biochemical oxygen demand, CV_coefficient of variation.

Introduction

Coffee is the second-most consumed beverage in the world, and Brazil is its largest producer. According to CECAFE (2021), Arabica coffee exports from Brazil from January to November 2020 totaled more than US$4 billion, making it one of the most important crops in both the domestic and foreign markets. In 2020, the total planted area was approximately 2.16 million hectares, representing an increase of 1.4% over the previous year (Conab, 2020).

There is concern about the rapid loss of viability of coffee seeds (*Coffea arabica* L.). Stored seeds lose quality due to deterioration, which is a natural process, but it can be intensified in the postharvest period. Processing, drying, and storage are examples of activities that can accelerate seed degradation due to the formation of reactive oxygen species, which cause damage to plant tissues (Fantazzini *et al*, 2018). In addition, the short longevity of coffee seeds is a limiting factor of the conservation of their long-term genetic variability (Dussert *et al*., 2012).

Some studies have shown that in coffee seeds, the endosperm may be more sensitive to deterioration than embryos, which can germinate and generate normal seedlings when excised (Figueiredo *et al*, 2017; Coelho *et al*., 2015). Zygotic embryos are a good explant for *in vitro* cultivation because they germinate faster and more uniformly than seeds. In the case of crops such as coffee, this process can be used to adapt them to the best planting time (Maciel *et al*., 2016), in addition to enabling the recovery of hybrids from incompatible crosses, micropropagation, breaking dormancy, and overcoming the sterility of seeds (Campos *et al*., 2017). There is also the possibility of cultivating embryos from stored seeds that have low viability. In this sense, the *in vitro* cultivation of coffee embryos can assist in the conservation of the germplasm of the species (Bramel *et al*., 2017).

However, a substantial number of plants grown *in vitro* do not survive transfer to a greenhouse or field environment. The seedlings are grown in test tubes under low light, aseptic conditions, in a medium containing sugars and nutrients to allow heterotrophic growth and in an atmosphere with a high level of moisture (Maciel *et al*., 2016). These conditions make it difficult for seedlings to adapt to new environmental conditions. To assist in the adaptation of seedlings during acclimatization, the climatic conditions need to be changed progressively to reduce stress, prevent damage, and prevent seedling senescence. Conditions such as relative humidity, temperature, light intensity, and a substrate that aids in the autotrophic

process are important characteristics to be established for adaptation (Maciel *et al*., 2016)).

Several studies have been conducted on the acclimatization of *in vitro* coffee seedlings (Almeida *et al*., 2011; Carvalho *et al.,* 2011; Maciel *et al.,* 2016; Santos *et al*., 2014) and seedling establishment (Assis *et al*., 2019; Dardengo, 2013). However, studies related to the acclimatization of seedlings from coffee zygotic embryos grown *in vitro* are scarce. Thus, the objective of this study was to evaluate the acclimatization of seedlings from coffee zygotic embryos taken from aged or freshly harvested seeds in different substrates and environments for the production of well-developed seedlings suitable for planting.

Results and discussion

Seed quality of the lots used

According to the analysis of variance for the seed germination data, there was a significant effect of seed quality level, i.e., a significant difference between the seeds that underwent the accelerated aging method and the newly harvested seeds. The aged seeds showed worse physiological performance in all analyzed variables, demonstrating that the accelerated aging process caused loss of seed quality compared with fresh harvesting (Table 1).

According to Carvalho and Nakagawa (2012), longer seed exposure to the conditions of the artificial aging test causes increased moisture and thus increases the temperature of the seeds and respiratory processes, thereby also favoring greater activity of microorganisms. This phenomenon results in greater deterioration in aged seeds than in nonaged seeds. Fantazzini *et al*. (2018) correlated the accelerated aging time with the storage time of coffee seeds and observed a positive correlation. The germination of seeds that underwent accelerated aging for 4 days was statistically equal to the germination of seeds stored for 2 months in multilayer bags under uncontrolled conditions.

In the present study, there was a 43% reduction in germination with the accelerated aging of coffee seeds, i.e., from 95% germination before to 52% after the procedure. This finding demonstrated the efficiency of the technique in yielding seeds with a lower quality standard than those that did not undergo this process and was therefore established as a method for efficiently simulating the natural viability loss that occurs in stored coffee seeds.

***In vitro* germination**

According to the analysis of variance of the *in vitro* germination of zygotic embryos removed from coffee seeds, there was no significant difference between the batches of freshly harvested and aged seeds in the percentage of normal, abnormal, or dead seedlings. The percentage of normal seedlings was greater than 80%, abnormal seedlings were less than 5%, and dead seedlings were less than 2% under both treatments.

Seedling growth data, such as height, diameter, and number of leaves, also showed no significant differences. These results corroborate studies by Figueiredo *et al.* (2017) and Coelho *et al.* (2015), in which they suggest that the endosperm is more sensitive to degradation than the seed embryo. Therefore, when the zygotic embryos of aged seeds are isolated and cultivated *in vitro,* they can generate healthy seedlings as vigorously as those of newly harvested seeds with high vigor. Thus, the coffee zygotic embryo cultivation technique can be used for the recovery of seeds that have gone through storage periods, which may have lost some viability, as occurs in storage in germplasm banks or in the shipping and transport of seeds to distant places, which involves long transport times under conditions that are not always adequate.

Acclimatization of coffee seedlings

According to the results of the analysis of variance, there was a triple interaction between the environment, seed quality, and substrate type on the variables shoot dry matter, root dry matter, and shoot/root ratio. For the variables stem diameter, number of leaves, and leaf area, there was a significant effect of the environmental factor, while for the variable plant height, there were isolated effects of the factors environment and seed quality. The greenhouse environment in general provided greater accumulation of dry matter in both the shoot and the root in seedlings from newly harvested and aged seeds (Tables 2 and 3).

In general, the greenhouse environment provided higher mean shoot dry matter and root dry matter than the growth room, both for seeds with different levels of quality and for seeds in different substrates (Tables 2 and 3). However, when comparing the values obtained for seedlings produced from seeds (Araújo *et al*., 2020; Almeida *et al* 2011), the present study showed lower means than those obtained in other studies. However, the measurements were performed at 150 days, which was 30 days sooner than in the cited studies. The best shoot dry matter result was 0.41 g, and the best root dry matter result was 0.735 g in freshly harvested seeds in the coconut fiber substrate in a greenhouse. The worst result reached 0.13 g in aged seeds in the Tropstrato© substrate in a growth room. The root dry matter reached the lowest mean of 0.145 g in aged seeds in the coconut fiber substrate in the growth room.

The mean root/shoot ratio was highest in seedlings from aged seed embryos grown in the coconut fiber substrate in a growth room (Table 4). This treatment showed a higher mean shoot dry matter than root dry matter. This imbalance can be detrimental in terms of adaptation after planting at the definitive site, since seedlings with a well-developed root system have greater chances of survival in the field, especially under water limitation (Maciel *et al*., 2016). In the greenhouse environment, this variable was not affected by seed quality or substrate.

Plant height was greater in the seedlings grown from embryos of newly harvested seeds (3.74 cm) than aged seeds (2.99 cm). Regarding the type of environment, the seedlings placed in the growth room environment, with less sunlight, reached a higher mean (3.65 cm) height than those placed in the greenhouse environment (3.07 cm).

In environments with less sunlight, such as in the growth room, seedlings tend to grow taller as an effect of plant etiolation, since the plant grows in search of light (Campa *et al*., 2017). This phenomenon was observed in the present study, as the height and stem diameter of the seedlings were greater under conditions with less sunlight. The stem diameter was also larger in the growth room (2 mm) than in the greenhouse (1.66 mm).

According to DaMatta *et al.* (2018), plants that grow under low sunlight conditions usually fix less carbon, transpire less, and therefore allocate more biomass to the stem.

Table 1. Percentage of root protrusion, germination, seedlings with expanded cotyledons (EC), and dry matter of seedlings from freshly harvested seeds and seeds that underwent accelerated aging.

Batch of seeds	Root protrusion (%)	Germination (%)	EC (%)	Shoot dry matter (mg)	Root dry matter (mg)
Freshly harvested	98 a	95 a	90 a	0.167 a	0.144 a
Aged	75 b	52 b	48 b	0.078 b	0.066 b
CV	7.73	16.74	21.3	23.17	25

*Means followed by the same letter in a column are not significantly different according to Tukey's test at 5% probability.

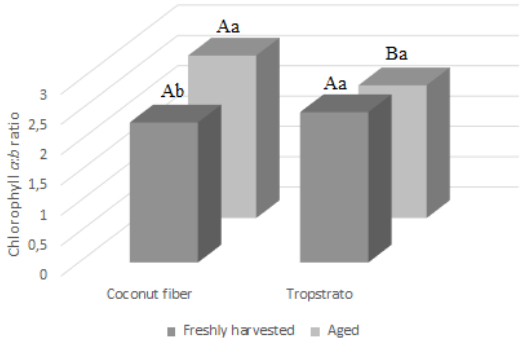

Figure 1. Chlorophyll *a*:*b* ratio of coffee seedlings from *in vitro* cultivation of zygotic embryos of seeds of different qualities acclimatized to different substrates. *Means followed by the same lowercase letter in a column and uppercase letter in a row are not significantly different according to Tukey's test at 5% probability.

Table 2. Shoot dry matter (mg) of coffee seedlings from *in vitro* cultivation of zygotic embryos of seeds of different quality levels acclimatized in two environments and two substrates.

Shoot dry matter (mg)			
Environment	Substrate	Seed quality	
		Freshly harvested	Aged
Greenhouse	Tropstrato©	290 Ab	360 Aa
	Coconut fiber	410 Aa	205 Bb
Growth room	Tropstrato©	210 Aa	130 Ab
	Coconut fiber	135 Ba	355 Aa

Shoot dry matter (mg)			
Seed quality	Substrate	Environment	
		Greenhouse	Growth room
Freshly harvested	Tropstrato©	290 A	210 A
	Coconut fiber	410 A	135 B
Aged	Tropstrato©	360 A	130 B
	Coconut fiber	205 B	355 A
CV	28.92		

*Means followed by the same uppercase letter in a row and lowercase letter in a column do not differ by Tukey's test at 5% probability.

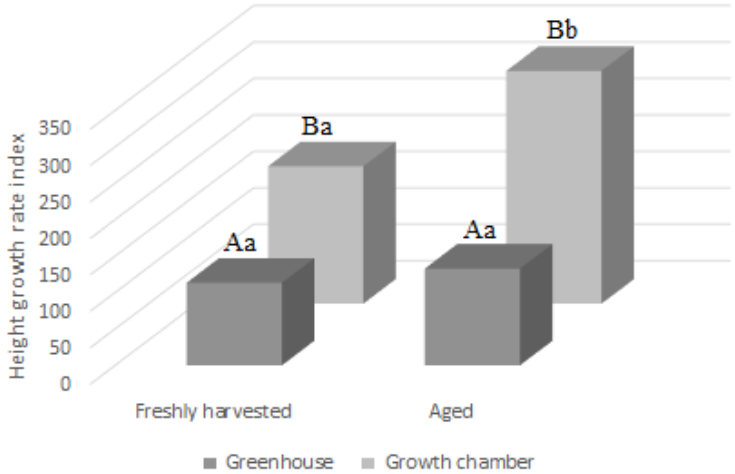

Figure 2. Height growth rate index of coffee seedlings from *in vitro* cultivation of zygotic embryos from seeds of two quality levels grown in two environments. *Means followed by the same uppercase letters in the column and lowercase letters of the row are not significantly different according to Tukey's test at 5% probability.

Table 3. Root dry matter (mg) of coffee seedlings from *in vitro* cultivation of zygotic embryos of seeds of different quality levels acclimatized in two environments and two substrates.

Root dry matter (mg)			
Environment	Substrate	Seed quality	
		Freshly harvested	Aged
Greenhouse	Tropstrato©	415 Bb	650 Aa
	Coconut fiber	735 Aa	245 Bb
Growth room	Tropstrato©	210 Aa	165 Aa
	Coconut fiber	255 Aa	145 Aa
Root dry matter (mg)			
Seed quality	Substrate	Environment	
		Greenhouse	Growth room
Freshly harvested	Tropstrato©	415 A	210 B
	Coconut fiber	735 A	255 B
Aged	Tropstrato©	650 A	165 B
	Coconut fiber	245 A	145 A
CV	32,75		

*Means followed by the same uppercase letter in a row and lowercase letter in a column are not significantly different according to Tukey's test at 5% probability.

Table 4. Shoot/root ratio of coffee seedlings from *in vitro* cultivation of zygotic embryos of seeds with different quality levels acclimatized in two environments and grown in two substrates.

Shoot/root ratio			
Environment	Substrate	Seed quality	
		Freshly harvested	Aged
Greenhouse	Tropstrato©	0.70 Aa	0.57 Aa
	Coconut fiber	0.58 Aa	0.92 Aa
Growth room	Tropstrato©	1.00 Aa	0.81 Aa
	Coconut fiber	0.53 Aa	2.76 Bb
Shoot/root ratio			
Seed quality	Substrate	Environment	
		Greenhouse	Growth room
Freshly harvested	Tropstrato©	0.70 A	1.00 A
	Coconut fiber	0.58 A	0.53 A
Aged	Tropstrato©	0.57 A	0.81 A
	Coconut fiber	0.92 A	2.76 B
CV	58.84		

*Means followed by the same uppercase letter in a row and lowercase letter in a column are not significantly different according to Tukey's test at 5% probability.

Table 5. Chlorophyll *a* levels of coffee seedlings from *in vitro* cultivation of zygotic embryos of seeds with different quality levels acclimatized in two environments and two substrates.

Chlorophyll *a*			
Environment	Substrate	Seed quality	
		Freshly harvested	Aged
Greenhouse	Tropstrato©	184.20 Aa	191.61 Aa
	Coconut fiber	177.82 Aa	151.87 Ab
Growth room	Tropstrato©	186.60 Aa	143.81 Ba
	Coconut fiber	158.62 Aa	155.06 Aa
Chlorophyll *a*			
Seed quality	Substrate	Environment	
		Greenhouse	Growth room
Freshly harvested	Tropstrato©	184.20 A	186.60 A
	Coconut fiber	177.82 A	158.62 A
Aged	Tropstrato©	191.61 A	143.81 B
	Coconut fiber	151.87 A	155.06 A
CV	13,92		

*Means followed by the same uppercase letter in a row and lowercase letter in a column are not significantly different according to Tukey's test at 5% probability.

This trend was also evidenced by Maciel (2016) in coffee somaclones obtained from a temporary immersion bioreactor system (RITA). The number of leaves and leaf area did not differ by seed quality or substrate, only by environment. Higher values were observed in seedlings produced in a greenhouse with seven leaves and a leaf area of 8.33 while in the growth room, the average was six leaves and a leaf area of 4.02. Another factor that may have

favored more growth in the greenhouse is the higher temperature, which was on average 30 °C, in contrast to 25 °C in the growth room. Temperature affects all biochemical reactions of photosynthesis, such as the membrane integrity of chloroplasts, and respiration rates increase as a function of temperature (Slattery & Ort, 2019). According to Maciel *et al*. (2016), the number of leaves per explant is one of the most important variables to be considered during acclimatization because a sufficient number provides a sufficient leaf area for photosynthesis. According to Rodríguez-López *et al.* (2014), coffee seedlings spend on average of 6 months developing and can be taken to the field when they have three to four pairs of true leaves. In the present study, at the time the seedlings were evaluated, i.e., at 150 days of transplantation, all groups had an average of more than six pairs of leaves. The treatments that stood out showed an average of up to eight pairs of leaves.

The lower performance of seedlings grown in the growth room than in the greenhouse can be explained by the lower light intensity available in that environment, even with the greenhouse being covered with 75% shade. Individuals exposed to high light intensity tend to have greater photosynthetic capacity and therefore greater mass accumulation. Excessive shading reduces the quality of transmitted sunlight, which affects plant physiological processes such as photosynthesis and growth (DaMatta *et al.,* 2018).

In general, *in vitro* seedlings require low relative luminosity and, when subjected to increased light, undergo a process of destruction of their chlorophyll molecules, becoming chlorotic and burned. This phenomenon occurs due to the reduced development of plants *in vitro* and the low photosynthetic activity (Vieira *et al*., 2020). Therefore, growth rooms are widely used for acclimatization. However, according to the results of this study, the coffee zygotic embryos grown *in vitro* did not exhibit high sensitivity to a higher light intensity, as in the greenhouse, during acclimatization.

Coffee plants can be grown in low-light environments because they have a low saturation radiance, ranging from 300 to 600 mmol m^{-2} s^{-1} (Dubberstein *et al*., 2020). Thus, they can be grown in more shaded systems, where there is a predominance of low sunlight. Regardless, a minimum amount of sunlight is necessary for efficient functioning of the photosynthetic apparatus. In the growth rooms in this study, a mean photon radiance of 36 µmol m^{-2} s^{-1} was limiting to the growth of the seedlings, even though they were potentially more sensitive to higher sunlight radiation.

According to the results of the analysis of variance of the chlorophyll *a* indices found in the different treatments, there was a triple interaction between the environment (greenhouse or growth room), seed quality (freshly harvested or aged), and substrate (coconut fiber or Tropstrato©). For total chlorophyll, there was no interaction between the factors, but there was significance in all the isolated factors. For the chlorophyll *a* and *b* ratio, there was a double interaction between the quality level and substrate. In turn, analysis of variance for chlorophyll *b* showed statistical significance for the environment and substrate individually. Chlorophyll *a* differed little between the treatments, although in general, the lowest means were found in seedlings from embryos of aged seeds (Table 5).

Total chlorophyll was greater in coffee seedlings that grew from embryos of freshly harvested seeds (251.43) than aged seeds (228.54), in a greenhouse (254.36) than in a growth room (225.61), and in Tropstrato© (252.65) than in coconut fiber substrate (227.31).

Acclimatization to different sunlight radiation levels results in changes that maximize the metabolic processes and ensure the growth and development of the individual. Such changes involve adjustments of photosynthetic organs and organelles, such as the composition of photosynthetic pigments (Rodríguez-López *et al.* 2014) and, consequently, the photosynthetic capacity (DaMatta *et al.,* 2018). Therefore, the higher amounts of chlorophyll *a* and total chlorophyll found in seedlings from the cultivation of embryos of freshly harvested seeds provide evidence of greater adaptability of these plants to the new environment with greater sunlight radiation.

Evaluating the chlorophyll *b* indices in the different locations, the growth room environment favored higher means (77.80) than the greenhouse environment (64.58). Chlorophyll *b* increases the light range used by photosynthesis and is considered an accessory pigment. By absorbing light, chlorophyll *b* transfers energy to a chlorophyll *a* molecule, which will use it to perform photosynthesis (DaMatta *et al*., 2018).

The higher amount of chlorophyll *b* in the leaves of seedlings grown in the growth room, which has less sunlight, may favor the absorption of photons, since this pigment absorbs energy at different wavelengths compared to chlorophyll *a* (DaMatta *et al*., 2018), as reflected by the lower chlorophyll *a*:*b* ratio commonly found in the shade (Dubberstein *et al*., 2020). Simultaneously, the seedlings grown with the Tropstrato© substrate reached higher chlorophyll *b* values (76.09) than those grown in coconut fiber substrate (66.47). The Tropstrato© substrate has slow-release macro- and micronutrients, which may have enabled greater synthesis of chlorophyll compounds, but this phenomenon could not be evidenced in other growth parameters.

The seedlings from embryos of aged seeds cultivated in coconut fiber were also observed to have a higher chlorophyll *a* and *b* ratio than those from embryos of freshly harvested seeds (Figure 1). In the Tropstrato© substrate, the embryo seedlings of newly harvested seeds had higher values.

The ratio between chlorophyll *a* and *b* is widely used to evaluate the amount of light absorbed by light-harvesting complexes (Dubberstein *et al*., 2020). According to DaMatta *et al.* (2018), this parameter is related to an adaptation of the photosynthetic apparatus to be more efficient at capturing sunlight. Normally, this ratio is 3:1 in plant species. It is usually lower in leaves with low irradiance, as described by Rodríguez-López *et al.* (2014). Therefore, a reduction in the chlorophyll *a*:*b* ratio is expected to be one of the main responses observed in plants grown in environments with lower light incidence (Campa *et al*., 2017), which can be observed under all treatments performed in this study.

According to the results of the analysis of variance of the growth rate index proposed by Edmond & Drapala (1958), there was a dual interaction between environment and seed quality. There was a significant effect of substrate alone.

The lower the germination or emergence speed index (Edmond & Drapala, 1958), the more seeds there are with greater physiological potential. Since the growth rate is based on the principle that the faster the seed germinates, the greater is its vigor, it can be inferred that the seedlings in the greenhouse environment showed greater vigor (Figure 2). Seed quality in the growth room was poorer in the newly harvested group, whereas in the greenhouse, there were no differences between seedlings from aged seeds and freshly harvested seeds. Thus, there is potential to obtain seedlings with a better or equal pattern when they come from

embryos extracted from freshly harvested seeds or aged seeds.

Acclimatization of the seedlings in the Tropstrato© substrate provided a higher growth rate index (210.877), indicating that the seedlings grown in this substrate needed more time to reach their highest height. A faster height increase, as seen in the coconut fiber substrate (166.135), can improve the performance of seedlings, increasing their vigor.

Materials and methods

Location and plant material

The study was conducted at the Seed Laboratory of the Department of Agriculture and the Plant Tissue Culture Laboratory of the Department of Biology, Plant Physiology Sector, Federal University of Lavras (*Universidade Federal de Lavras*). Seeds from the 2017/2018 harvest of the species *Coffea arabica* L., cultivar Catuaí Amarelo IAC 62 were used.

Seed collection, selection, and processing

The coffee berries were harvested at the Procafé Foundation Experimental Farm in Varginha, Minas Gerais state, Brazil, at the coffee berry maturation stage, selectively chosen from the middle branches of the plants and from the middle parts of the branches. After harvesting, the berries were washed to separate malformed and insect-damaged fruits, in addition to impurities, before being mechanically pulped. The seeds were demucilated by fermentation in water at a temperature of 30 °C for 24 hours, washed in running water, and dried in sieves placed in the shade to remove surface moisture. After predrying, the seeds were dried in a fixed-bed dryer at 25 °C for approximately 10 hours per day. The temperature of the seed mass was monitored with a digital thermometer, and the seeds were turned over every 1 hour until reaching a water content of 12%. The water content was determined by the oven method at 105 °C for 24 hours (Brasil, 2009), with two replicates of 10 seeds. The results are expressed as percentages based on the wet weight of the seeds.

The seeds were stored in a cold chamber at a temperature of 10 °C and RH of 55% in plastic bags until testing, when they were homogenized and had the parchments removed manually.

Obtaining seed lots

Two lots of seeds of different levels of physiological quality were used, one of better quality, consisting of freshly harvested seeds, and the other of lower quality, obtained from the same freshly harvested seeds but subjected to the accelerated aging process.

To perform accelerated aging, the seeds were placed in a single uniform layer on a screen inside plastic germination boxes (Gerbox) containing 40 mL of water and kept at 42 °C in a biochemical oxygen demand (BOD) incubator for 6 days. The freshly harvested seeds were also placed on the roofs of Gerbox containing 40 mL of distilled water in a BOD incubator at a temperature of 25 °C for 6 days to moisten and standardize the moisture with the artificially aged seeds.

Seed germination test

The seeds from lots of different quality levels, recently harvested and aged, were evaluated using the germination test. For this purpose, four replicates of 25 seeds of each treatment were sown on germination paper moistened with distilled water that weighed 2.5 times the dry paper weight. The germination rolls were placed in a germinator set at 30 °C in the presence of light (Brasil, 2009). Evaluations were performed at 15, 30, and 45 days.

At 15 days, the percentage of root protrusion was determined. At 30 days after sowing, the percentage of normal seedlings was determined, and those with primary roots and at least two healthy and well-formed lateral roots were considered normal seedlings. At 45 days after sowing, the percentage of seedlings with expanded cotyledons was calculated. For the evaluation of dry matter, the aerial parts of the normal seedlings at 45 days were separated from the roots and dried in a forced-air oven at 60 °C for 5 days until reaching constant weight. The dry matter was weighed on a precision scale, and the results are expressed in grams/seedling.

***In vitro* culture of embryos: antisepsis of seeds and embryo extraction**

Before extracting the embryos, the seeds underwent antisepsis by being soaked in 1.6% formaldehyde for 20 minutes under agitation, followed by three washes with distilled, autoclaved water and another soak of 72 hours in boric acid at 0.5%. Before embryo extraction, the seeds were washed three times in distilled, autoclaved water.

After antisepsis of the seeds, the embryos were extracted under a laminar flow hood with the aid of tweezers and inoculated in MS culture medium (Murashige & Skoog, 1962) supplemented with 30 g L^{-1} sucrose, solidified with 2.5 g L^{-1} Phytagel®, and pH-adjusted to 5.8 before autoclaving, which was performed at 121 °C for 20 minutes. Eight blocks of 40 embryos were inoculated in test tubes with 10 mL of culture medium, which were kept in the BOD incubators under a photoperiod of 16 hours and a temperature of 25 ± 2 °C for 60 days.

***In vitro* germination**

After 60 days of embryo inoculation, the percentage of germination of the embryonic axes of normal seedlings, the percentage of abnormal seedlings, and the percentage of dead embryos were evaluated. Normal seedlings were those with embryonic axes having cotyledons and at least one main root. Those considered abnormal were embryos that did not show root protrusion, cotyledon formation, malformation, or inverted geotropism (roots up and cotyledons down). Seedlings and embryos that were brown in color were considered dead.

Stem diameter (mm), height (cm), and number of leaves were also evaluated at 60 days. The diameter was measured where the stem met the roots (stem collar) using a caliper (0.05 cm precision), and the height was measured with a graduated ruler using the terminal bud (apical meristem) as a standard. To count the number of leaves, only fully expanded leaves were counted.

Acclimatization and evaluation of seedlings

Seedlings at 60 days *in vitro* were taken for acclimatization because they already had all the necessary characteristics to start the acclimatization process, such as true leaves and main roots. The acclimatization process began by transferring the seedlings to plastic bags containing two different substrates, Tropstrato© coffee or coconut fiber. There were 20 bags per treatment, divided into four blocks of five bags. To the bags with the seedlings, 5 g of Osmocote® slow-release fertilizer was added every 2 months. They were then placed in two different environments: in a plant growth room with 12 hours of light/day at 25 °C and in a greenhouse with approximately 90% relative air humidity with an automatic fogging system,

in which the average temperature was close to 30 °C and covered with 75% natural-light shade.

Five months after the beginning of seedling acclimatization, growth evaluations were performed. To evaluate shoot, root, and total dry matter, the aerial part and root were separated and then dried in an oven at 60 °C for 5 days (until they reached a constant weight). The dry matter weight was determined using a precision digital scale to three decimal places. With the data from these evaluations, we calculated the shoot/root ratio. The stem diameter (mm), height (cm), and number of leaves were also evaluated. The diameter of the stem collar at ground height was measured with a caliper (0.05 cm precision), and the height was measured with a graduated ruler using the terminal bud (apical meristem) as a standard. To count the number of leaves, only fully expanded leaves were counted. The leaf area was also calculated using the following formula:

$$LA = 0.667\ x\ L\ x\ W$$

where 0.667 = correction factor; L = leaf length; W= leaf width.

Chlorophyll *a*, *b*, and total contents were obtained using a ClorofiLOG® CFL 1030 digital chlorophyll meter (Falker). The measurements were performed on fully expanded leaves located in the third pair of leaves from the apex of the branch. With these data, the chlorophyll *a* and *b* ratios were also calculated.

From the height of the seedlings measured every 30 days, the growth velocity index in height was determined using the formula of Edmond and Drapala (1958):

$$I = \frac{(N1 * G1) + (Nn * Gn)}{(G1 + Gn)}$$

Where N = day of measurement, and G = seedling height on the day measurement.

Experimental design and statistical analysis

In the seed germination test, a completely randomized design was used, with four replicates of 25 seeds. *In vitro* culture of embryos from the two different seed lots (freshly harvested and aged) was also performed in a completely randomized design, divided into eight replicates of 40 embryos each.

The process of acclimatizing seedlings from zygotic embryos cultivated *in vitro* was performed in a randomized block design, with four blocks of five seedlings in a 2 × 2 × 2 factorial scheme, with two levels of seed quality (newly harvested and aged), two substrates (Tropstrato© and coconut fiber), and two environments (growth room and greenhouse).

All data were subjected to analysis of variance using the statistical software SISVAR (Ferreira, 2019), and means were evaluated by Tukey's test at 5% probability.

Conclusions

Coffee seedlings from the *in vitro* cultivation of zygotic embryos acclimatize well in a greenhouse with fogging. The coconut fiber substrate is more efficient at acclimatizing seedlings originating from either zygotic embryos of freshly harvested seeds or from aged seeds. It is possible to produce healthy coffee seedlings obtained from zygotic embryos of aged seeds with a minimum quality standard.

References

Almeida GRR, Carvalho CHS de, Guimarães RJ, Teixeira JB, Padilha L (2011) Performance of somatic embryogenesis propagated coffee plants under different levels of soil water. Coffee Sci. 6(2): 114-119.

Araújo FHV, Cruz RS, Porto DWB, Machado CMM, França AC (2020) Effects of mycorrhizal association and phosphate fertilization on the initial growth of coffee plants. Pesqui Agropecu Trop. 50.

Assis BP, Gross E, Pereira NE, Mielke MS, Gomes Junior GA (2019) Growth response of four conilon coffee varieties (Coffea canephora Pierre ex a. froehner) to different shading levels. J Agric Sci 11(7)

Bramel P, Krishna NS, Horna D, Lainoff B, Montagnon C (2017) Global conservation strategy for coffee genetic resources. Crop Trust, World Coffee Research. 72 p.

Brasil. (2009) Ministério da Agricultura, Pecuária e Abastecimento. Regras para Análise de Sementes. Brasília: MAPA/ACS, 399 p.

Campa C, Urban L, Mondolot L, Fabre D, Roques S, Lizza Y, Aarrouf J, Doulbeau S, Breitler J, Letrez C, Toniutti L, Bertrand B, La Fisca P, Bidel L P R, Etienne H (2017) Juvenile Coffee Leaves Acclimated to Low Light Are Unable to Cope with a Moderate Light Increase. Front. Plant Sci. 8.

Campos NA, Panis B, Carpentier SC (2017) Somatic Embryogenesis in Coffee: The Evolution of Biotechnology and the Integration of Omics Technologies Offer Great Opportunities. Front. Plant Sci. 8,1460.

Carvalho CHS, Rezende JC, Almeida GRR, Teixeira JB, Padilha L (2011) Características agronômicas e morfológicas de cafeeiro 'Catuaí Vermelho' propagado por embriogênese somática. Pesqui Agropecu Bras. 46:378-383.

Carvalho NM, Nakagawa J (2012) Sementes: ciência, tecnologia e produção. 4. ed. Jaboticabal: Funep. 588 p.

CECAFÉ, (2021) Conselho dos Exportadores de Café do Brasil. Exportações brasileiras de café, Relatório Mensal x Tipo de Café. Disponível em: <https://www.cecafe.com.br/dados-estatisticos/exportacoes-brasileiras/> Acesso em: 15 jan. 2021.

Coelho SVB, Figueiredo MA, Clemente ACS, Coelho LFS, Rosa SDVF (2015) Alterações fisiológicas e bioquímicas em sementes de café secas em sílica gel e soluções salinas saturadas. Pesqui Agropecu Bras. 50:483-491.

Conab. (2020) Companhia Nacional de Abastecimento**.** Acompanhamento da safra brasileira de café, Safra 2020 – Quarto levantamento, Dezembro 2020. Brasília: CONAB, 5:1-45.

DaMatta FM, Avila RT, Cardoso AA, Martins SCV, Ramalho JC (2018) Physiological and Agronomic Performance of the Coffee Crop in the Context of Climate Change and Global Warming: A Review. J Agric Food Chem. 66:5264−5274.

Dardengo MCJD, Sousa EF, Reis EF, Gravina GA. (2013) Crescimento e qualidade de mudas café conilon produzidas em diferentes recipientes e níveis de sombreamento. Coffee Science, 8:500-509.

Dubberstein D, Lindon FC, Rodrigues AP, Semedo JN, Marques I, Rodrigues WP.... Ramalho JC (2020) Resilient and Sensitive Key Points of the Photosynthetic Machinery of Coffea spp. to the Single and Superimposed Exposure to Severe Drought and Heat Stresses. Front Plant Sci. 11.

Dussert S, Couturon E, Engelmann F, Joët T (2012) Biologie de la conservation des semences de caféiers : aspects fondamentaux et conséquences pratiques. Une revue. [Coffee seed conservation biology**:** Fundamental aspects and practical implications. A review]. Cah Agric. 21:106-114.

Edmond JB & Drapala WJ (1958) The effects of temperature, sand and soil, and acetone on germination of okra seeds. Proceedings American Society Hort Science. 74: 601-606.

Fantazzini TB, Rosa SDVF, Pereira CC, Pereira DS, Cirillo MA, Ossani PC **(**2018) Association between the artificial aging test and the natural storage of coffee seeds**.** J Seed Sci. 40:164-172.

Ferreira DF (2019) SISVAR: A Computer Analysis System to Fixed Effects Split Plot Type Designs. Rev Bras Biom. 37: 529-535.

Figueiredo MA, Coelho SVB, Rosa SDVF, Vilela AL, Silva, LC (2017) Exploratory studies for cryopreservation of Coffea arabica L. seeds. J Seed Sci. 39: 150-158.

Maciel ALR, Rodrigues FA, Pasqual M, Carvalho CHS (2016) Acclimatization of coffee ('Coffea racemosa'x'Coffea arabica') somaclones obtained from temporary immersion bioreactor system (RITA). Aust J Crop Sci. 10: 169.

Murasshige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol Plant. 15: 473-497.

Rodríguez-López N, Martins SCV, Cavatte PC, Silva PEM, Morais LE, Pereira LF, Reis JV, Avila R, Godoy AG, Lavinski AO, DaMatta FM (2014) Morphological and physiological acclimations of coffee seedlings to growth over a range of fixed or changing light supplies. Environ Exp Bot. 102, 1–10.

Santos MRA, Lima RA, Ferreira MGR, Rocha RF, Espindula MC, Alves EA (2014) Aclimatização de mudas micropropagadas de Coffea canephora. J Biotechnol Biol. 5: 12-19.

Slattery RA & Ort DR (2019) Carbon assimilation in crops at high temperatures. Plant Cell Environ. 1–9.

Vieira CF, Carvalho FEL, Lima-Meloa CY, Carvalho CPS, Lima Neto MC, Martinsa M O, Silveira JAG (2020) Integrative approach reveals new insights into photosynthetic and redox protection in ex vitro tobacco plantlets acclimatization to increasing light intensity. Biotechnol Res Innov. 3:59-72.