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Viability of seeds of two varieties of *Coffea arabica* L. using different pretreatments in the tetrazolium test

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Viability of seeds of two varieties of *Coffea arabica* L. using different pretreatments in the tetrazolium test

Abstract: This research attempted to determine the efficacy of the tetrazolium test in the evaluation of the seed viability of two varieties of *Coffea arabica* L. ('Castillo' and 'Cenicafé'). The fruits were obtained from crops located in the municipalities of Salazar de las Palmas and Arboledas (Norte de Santander - Colombia). The test was carried out with embryos manually extracted from the seeds using tweezers. Three pretreatments were established: distilled water, sodium hypochlorite (2.5 %), sucrose (10 %), and a control (no pretreatment). Embryos were placed in a cysteine solution (0.5 %) to prevent oxidation, then immersed in tetrazolium solutions with concentrations of 0.035 %, 0.075 %, and 0.1 % for a period of 6, 9, and 12 hours in darkness. The results of the viability test were validated with seed germination, using the wet paper towel method in darkness. The best viability percentages were found with the application of sodium hypochlorite (NaClO 2.5 %), with a high correlation with the germination percentage. The use of pretreatments improved the efficiency of the viability test and allowed the use of low concentrations of the reagent (0.035 %), giving the farmer a quick and less expensive alternative to determine germination capacity.

Key words: coffee; germination; optimization; pretreatments

Viabilnost semen dveh sort kavovca (*Coffea arabica* L.) z uporabo različnih predobravnavanj pred tetrazolijevim testom

Izvleček: V raziskavi smo poskušali določiti učinkovitost tetrazolijevega testa za ovrednotenje viabilnosti semen dveh sort kavovca, *Coffea arabica* L. ('Castillo' in 'Cenicafé'). Plodovi kavovca so bili pridobljeni z območij Salazar de las Palmas in Arboledas (Norte de Santander - Colombia). Test je bil izveden na embrijih, ki smo jih s pinceto izolirali iz semen. Uporabljena so bila tri predobravnavanja in sicer: distilirana voda, natrijev hipoklorit (2,5 %), saharoza (10 %) in kontrola (brez predobravnavanja). Embriji so bili najprej vstavljeni v raztopino cisteina (0,5 %) za preprečitev oksidacije in nato potopljeni v različne koncentracije raztopin tetrazolija (0,035 %; 0,075 % in 0,1 %) za 6, 9, za 12 ur v temi. Rezulti viabilnosti embrijev so bili ovrednoteni s kalitvijo semen v temi na vlažnem filtrirnem papirju. Največji odstotek viabilnih semen je bil ugotovljen pri uporabi natrijevega hipoklorita (NaClO 2,5 %), z največjo korelacijo z odstotkom kalitve. Uporaba predobravnavanj je izboljšala učinkovitost testa viabilnosti in je dopuščala uporabo majnih koncentracij reagenta (0,035 %), kar daje kmetom hitro in manj drago alternativo določanja kalivosti.

Ključne besede: kavovec; kalitev; optimizacija; predobravnavanja

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1 INTRODUCTION

Coffea arabica represents one of the most important crops in the world (RAMÍREZ et al., 2016; Selmar et al., 2008). Global coffee consumption has been increasing due to its pleasant aroma and flavor, as well as its beneficial health effects, leading to a large amount of by-product generation (Khochapong et al., 2021). Coffee is one of the most traded beverages in the world and in 2016/17 more than 9 million tons were consumed (da Silva et al., 2021). The main coffee producers are Brazil, Colombia, Vietnam, India, and Indonesia which represents relevant economic importance for these countries (da Silva et al., 2021). 12.5 million households worldwide receive income from coffee cultivation (Montagnon et al., 2021). Commercially cultivated coffee species are *Coffea arabica* L. and *C. canephora* Pierre ex A.Froehner (Mishra & Slater, 2012). Tropical African countries such as Ethiopia, Sudan, Kenya, Guinea, or Mozambique are usually pointed out as possible centers of origin, although the most accepted is Ethiopia (Rojo, 2014).

The coffee plant belongs to the Rubiaceae family and there are more than 70 species of the *Coffea* L. genus (da Silva et al., 2021), it is a tropical evergreen tree, growing at altitudes between 700 and 2000 m.a.s.l. (Tinoco & Peña, 2019). The green seeds of *Coffea arabica* are very rich in secondary metabolites. These metabolites have antioxidant properties that reduce the incidence of cancer and diabetes (Aissaoui et al., 2020). The coffee seed is a nut, oblong, flat-convex, of variable size (10-18 mm long and 6.5-9.5 mm wide) and mostly constituted by a horny endosperm where, at one of its ends and very superficially, lies an embryo of 3.5 to 4.5 mm long, with a conical radicle and cordate cotyledons (Arcila et al., 2007). An undesirable feature of coffee seeds is that they have slow and asynchronous germination, which prevents obtaining seedlings of desirable quality; this type of germination hinders rapid evaluations of viability and/or vigor due to the excessive time required to obtain results (Da Rosa et al., 2010).

The use of quick methods to know the viability is important, to accelerate decision-making regarding the management of seed lots, (Medeiros et al., 2015). For this reason, the tetrazolium salt test has assumed a prominent place for some crops, mainly due to the large amount of information that the test provides. *Tetrazolium* (TZ) can be used regardless of the degree of seed dormancy, becoming very important for species with this problem. It is also a very useful tool for seed producers, graders, and traders as it can help in decisions that need to be made quickly. (França-Neto and Krzyzanowski, 2019). The tetrazolium test is based on the activity of dehydrogenase enzymes that reduce the tetrazolium salt in living seed tissues by generating triphenyl formazan, a non-diffusible red-colored compound, which indicates respiratory activity and viability of cells and tissues; in contrast, dead tissues show no coloration (Salazar et al., 2018; Salazar et al., 2020a). It is important to indicate that the effectiveness of the tetrazolium test is mediated by the development of preconditioning procedures, which facilitate the entry of the tetrazolium solution into the seed (Hosomi et al., 2017; Salazar et al., 2019).

Decreasing the concentration of tetrazolium and verifying its efficacy allows the evaluator a reasonable and optimal use of this reagent, reducing the total cost of the test. According to the above, this work aims to determine the efficacy of the tetrazolium test in the evaluation of seed viability of two varieties of *Coffea arabica* using different pretreatments.

2 MATERIAL AND METHODS

2.1 PLANT MATERIAL

The ripe fruits of the Castillo coffee variety (*C. arabica*) were collected in village Alto del Angulo in the municipality of Salazar de las Palmas. The Cenicafé variety drupes were collected in plantations in the village of San Onofre in the municipality of Arboledas, both belonging to Norte de Santander (Colombia). The material was placed in plastic containers with lids, lined with newspaper to avoid deterioration, and transported to the Biology laboratories of the Faculty of Basic Sciences at the Universidad Francisco de Paula Santander where the research study was carried out.

2.2 PRETREATMENT AND VIABILITY OF SEEDS

Solutions of 2,3,5-triphenyl tetrazolium chloride were prepared with concentrations of 0.035 %, 0.075 % and 0.1 %. To avoid embryo oxidation, a 50 mg l^{-1} cysteine solution was prepared (Azofeifa, 2009). The solutions were kept in dark bottles and stored in the refrigerator at 5 °C (Salazar et al., 2020b; Salazar et al., 2020c). Three pretreatments were established: 6-hour immersion in 2.5 % sodium hypochlorite (NaClO), 10 % sucrose (C12H22O11), and water (H2O). The fourth group of seeds corresponded to the control group.

The fleshy layers (protective skin, pulp, parchment) and the integument were removed from the fruits to obtain the endosperm containing the embryos. The embryos were extracted manually, using a dissecting case and forceps. One hundred embryos were placed in each Petri dish immersed in 5 ml of tetrazolium solution. Each test

had 5 replicates for a total of 500 embryos for each exposure time established (6 h, 9 h, 12 h) in complete darkness. An intense reddish coloration of the embryo was determined as positive for the tetrazolium test.

2.3 GERMINATION TEST

The germination test was performed to corroborate the data obtained in the viability test. Five replicates of 100 seeds were used for 500 seeds (Salazar et al., 2020b). For the test, the seeds were removed from the endosperm, washed with distilled water, and placed to germinate for 30 days in total darkness on paper towels moistened with distilled water in previously disinfected plastic containers. Germinated seeds were considered to be those that presented a root growth at least 4 mm long or that presented geotropic curvature and the result was expressed as germination percentage.

2.4 STATISTICAL ANALYSIS

For the viability test and germination test, data were randomly distributed with 5 replicates and 100 seeds per replicate. The experimental design consisted of a completely randomized block analysis. Data were analyzed using Infostat statistical software by analysis of variance (ANOVA), followed by Tukey's multiple range HSD (Honest Significant Difference) test, to compare averages and determine significant differences at a level of $p \leq 0.05$.

3 RESULTS AND DISCUSSION

3.1 PRETREATMENT AND VIABILITY OF SEEDS

The tetrazolium test evaluates the physical and physiological conditions of the embryonic structure of each seed (França-Neto & Krzyzanowski, 2019). Moreover, viable seeds are easily identified by their carmine red coloration (Figure 1), due to the reductive reaction of the tetrazolium solution under the action of dehydrogenase enzymes in cellular respiration (Salazar et al., 2020a).

In the results shown in Table 1, it can be observed that the pretreatment with chlorine (2.5 % 6 h) was the one that obtained the highest viability in all concentrations and exposure times with tetrazolium, values that ranged between 92 % and 100 % viability in *C. arabica* 'Castillo'. However, no significant differences were found between the control and sucrose pretreatment at the following concentrations and exposure time with tetrazolium: 0.035 % 6, 9, 12 h; 0.075 % 9, 12 h; 0.1 % 6, 9, 12 h (Table 1). The lowest viability values were found in the pretreatment with distilled water in most of the concentrations and exposure time with tetrazolium, unlike the pretreatments with sucrose (0.035 % 12 h, 0.075 % 6 h, and 0.1 % 6 h) where the viability using the tetrazolium test were lower.

The findings in the Cenicafe variety showed few significant differences among pretreatments, concentrations, and exposure times with tetrazolium (Table 2). However, the chlorine treatment had a better effect on seed viability. The lowest viability value (88 %) was found in the sucrose pretreatment, at the tetrazolium concentration and exposure time of 0.035 % 9 h (Table 2). In addition, the preconditioning with distilled water identified statistically homogeneous means with the chlorine pretreatment data, unlike the concentration and exposure period of 0.075 % 12 h of tetrazolium.

According to Lamarca and Barbedo (2014), the higher the concentration and exposure period to tetrazolium, the greater the intensity of embryo coloration, which facilitates the analysis and evaluation of viable and non-viable seeds. According to Tola et al. (2019), exposure times of 5, 16, and 24 h and concentrations of 0.1 %, 0.5 %, and 1 % tetrazolium are suitable for viability analysis in coffee seeds. However, in this research, it was possible to obtain reliable viability levels thanks to the use of pre-treatments with chlorine (2.5 % 6 h), in concentrations of 0.035 %, 0.075 %, 0.1 %, and exposure times of 6, 9, 12 h of tetrazolium. These parameters are decisive in the tetrazolium test since they improve the efficiency of the viability test. Likewise, Salazar et al. (2020b) increased the efficiency of the tetrazolium test on *S. lycopersicum* L. seeds, using 1 % sodium hypochlorite and

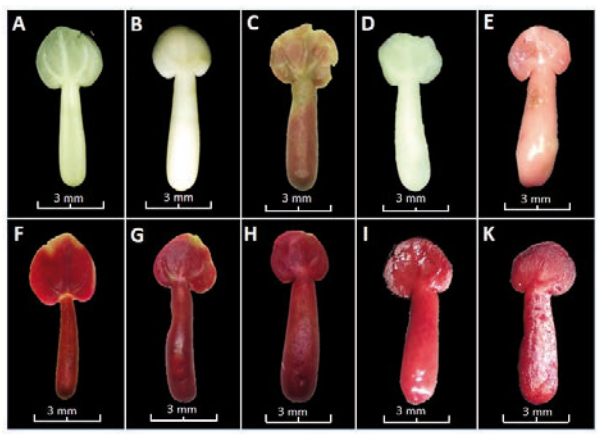

Figure 1: Viability of *Coffea arabica* seeds using the tetrazolium test. (A, B, C) non-viable seeds *C. arabica* Castilla' (D, E) non-viable seeds *C. arabica* 'Cenicafe'. (F, G, H): viable seed *C. arabica* 'Castilla' (I, K) viable seed *C. arabica* 'Cenicafe'

Table 1: Viability of *Coffea arabica* 'Castilla' seeds

	Tetrazolium concentration and exposure time								
Pretreatments	0.035 % 6 h	0.035 % 9 h	0.035 % 12 h	0.075 % 6 h	0.075 % 9 h	0.075 % 12 h	0.1 % 6 h	0.1 % 9 h	0.1 % 12 h
Control	90 ± 5.4 a	98 ± 4.4 a	96 ± 4.4 a	94 ± 8.9 a	98 ± 4.4 a	100 a	96 ± 5.4 ab	98 ± 4.4 a	100 a
H_2O 6h	72 ± 8.3 b	84 ± 8.9 b	96 ± 5 a	96 ± 5.4 a	88 ± 4.0 b	98 ± 4.4 a	92 ± 8.3 ab	98 ± 3.4 a	92 ± 5.4 b
Sucrose 10% 6h	92 ± 8.3 a	96 ± 54 a	94 ± 5.4 a	78 ± 7.8 b	98 ± 4.1 a	100 a	82 ± 7.8 a	98 ± 4.2 a	98 ± 1.9 a
Chlorine 2.5% 6h	92 ± 7.5 a	98 ± 4.4 a	96 ± 8.9 a	98 ± 4.4 a	98 ± 3.9 a	100 a	100 b	100 a	100 a

Different letters indicate significant differences ($p \leq 0.05$)
± Standard deviation

Table 2: Viability of *Coffea arabica* 'Cenicafe' seeds

	Tetrazolium concentration and exposure time								
Pretreatments	0.035 % 6 h	0.035 % 9 h	0.035 % 12 h	0.075 % 6 h	0.075 % 9 h	0.075 % 12 h	0.1 % 6 h	0.1 % 9 h	0.1 % 12 h
Control	89 ± 4.4 a	98 ± 4.4 a	98 ± 4.4 a	98 ± 4.4 a	96 ± 5.4 a	100 a	98 ± 4.4 a	98 ± 4.4 a	98 ± 3.5 a
H_2O 6h	96 ± 5.4 a	98 ± 4.2 a	94 ± 8.9 a	96 ± 5.4 a	98 ± 4.4 a	92 ± 8.3 b	98 ± 4.4 a	98 ± 4.4 a	98 ± 4.1 a
Sucrose 10% 6h	96 ± 5.4 a	88 ± 4.1 b	98 ± 4.4 a	98 ± 4.4 a	98 ± 4.4 a	100 a	92 ± 8.3 a	92 ± 8.4 a	98 ± 3 a
Chlorine 2.5% 6h	96 ± 5.2 a	98 ± 4.2 a	98 ± 4.2 a	98 ± 4.4 a	98 ± 4.4 a	100 a	98 ± 5.4 a	98 ± 4.4 a	100 ± 5 a

Different letters indicate significant differences ($p \leq 0.05$)
± Standard deviation

tetrazolium concentrations of 0.25% and 0.15% in 24h. However, in a study by Clemente et al. (2011) and Clemente et al. (2012), subjecting sodium hypochlorite (5 %, 6 %) for 6 h negatively impairs tetrazolium test results in *C. arabica* seeds with a moisture content below 25 %. Strobel et al. (2016) state that chlorine doses can generate embryo damage, leading to decreased seed viability. In addition, Salazar and Maldona, (2020) express, that chlorine generates toxic effects on cells at low doses, because it is a strong oxidant compound (Jiang et al., 2017).

In several studies sucrose solution (10 %) has had a positive effect on seed viability because sucrose is linked to the activation of metabolic processes related to dehydrogenase enzymes in cellular respiration, helping to maintain an osmotic balance in the seed, which prevents damage to the embryo (Mercado and Jaimes, 2022; Salazar and Botello, 2020; Salazar et al., 2019; Hosomi et al., 2017; Hosomi et al., 2012). Moreover, distilled water treatments resulted in low viability in the Castillo variety at several periods and tetrazolium concentrations. These results differ with Mercado et al. (2020), where pretreatment with distilled water on *Epidendrum microtum* Lindl seeds had 100% viability. According to Clemente et al. (2012), an imbibition time of 48 hours facilitates embryo extraction and does not affect the results of the tetrazolium test. According to Carvalho et al. (2014), hydration favors the absorption of tetrazolium and provides the activation of enzymatic metabolism.

3.2 COMPARISON OF GERMINATION AND VIABILITY

The viability test should be validated with the germination test, indicating the consistency of the findings concerning the behavior of the physiological quality of the seeds (Tola et al., 2019). In the Castillo variety, the average germination percentage was 95 %, comparing with the viability results, the same trend was maintained, where no statistically significant differences were found with the hypochlorite treatments. Similarly, in the cenicafe variety, the germination percentage was 96 %, which is positively correlated with the viability test. This reflects the efficacy of the pretreatments in improving seed viability. According to Salazar et al. (2020c), the germination test indicates whether the seeds did not germinate because they were dormant or because they showed embryo deterioration, being used to corroborate the vi-

ability results generated by the tetrazolium test. However, in most cases the viability test generates higher percentages concerning the germination test, agreeing with the studies conducted by Tola et al. (2019) on *Coffea arabica* seeds.

According to the results reported by Fantazzini et al. (2020), the tetrazolium test is not suitable for seeds with germination percentages lower than 60 %. Likewise, these authors report that for seeds with a germination value greater than 60 %, the results of the tetrazolium test were similar to those of the germination test on *C. arabica* seeds. Likewise, Figueiredo et al. (2017) point out, that several investigations have shown the discrepancy between the results of the tetrazolium and germination test in coffee seeds, specifically in those of lower quality.

Germination of *C. arabica* seeds occurs slowly and inconsistently. Therefore, the tetrazolium test is presented as a quick alternative to evaluate viability, provided that seed preparation and imbibition are established (Clemente 2012). In this research, germination percentages were higher than 93 % in the Castillo and Cenicafe varieties and indicated a close relationship with viability percentages with tetrazolium. According to the above, the use of pretreatments can improve the efficacy of the tetrazolium test on *C. arabica* seeds. In this case, the seeds exposed to sodium hypochlorite (2.5 % 6 h) generated high viability percentages, which allows reducing the concentrations and exposure periods of tetrazolium to 0.035 % and 6 h, respectively, which leads to a reduction in the cost of the reagent triphenyl tetrazolium chloride to determine the viability of *C. arabica* seeds.

4 CONCLUSIONS

Rapid identification of viability in a seed group is important because it allows decisions to be made about it. Coffee seed gives germination results in 25 to 30 days, but this can be extended up to 40 days. The use of tetrazolium determines effectively and with real results the viability of the seeds, considering the prolonged germination time. For the Castillo and Cenicafé varieties, the best viability percentages were found with the application of sodium hypochlorite (NaClO 2.5 %), with a high correlation with the germination percentage. The use of pretreatments improves the efficiency of the viability test, allowing the use of low concentrations, up to 0.035 %, giving the farmer a more economical alternative to the expenses generated by the use of the reagent.

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