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Research Paper

Can the Tetrazolium-staining test be used as an alternative to the Germination test in assessing Argan seed viability?

Mohamed Alouani[1,+] and Fouzia Bani-Aameur[2]

[1] *Laâyoune Higher School of Technology, Laboratory of Biotechnology and Valorization of Natural Resources, Ibn Zohr University, Morocco*

[2] *Faculty of Science Agadir, Laboratory of Biotechnology and Valorization of Natural Resources, Ibn Zohr University, Morocco*

Corresponding author: Mohamed Alouani

ABSTRACT: *Because argan (Argania spinosa (L.) Skeels) seed viability varies in great depending upon mother-tree genotypes, a rapid viability test is necessary for a better control of nursery productivity. Our objective in this experiment is to investigate the possibility of using tetrazolium-staining method to determine seed viability of different argan seed lots. Seeds from nine different mother-trees genotypes were cold stored at 4 °C, scarified before to be soaked in gibberellic acid (GA3) solution. Seeds were put to germinate at daylight. Germination percentage varied from 63.3% to 95% depending on tree genotype with a latent period to germination not exceeding four days. In parallel nuts from the same seed lots were soaked in a 1% tetrazolium solution for five hours at 40°C, expecting viable seed to show a red colour. Seed lots with embryos and cotyledons entirely or partially coloured in red represent 33% to 90% of the cases. We observed a highly significant linear regression and highly significant correlation (0.84) between percentage of seed coloured as described above and germination. Correlation not important when we consider percentage of seeds with coloured embryos only or totally or partially coloured cotyledons only. We also observed a negative significant correlation of germination percentage with non-coloured seeds. There for tetrazolium -staining test may be used as an alternative to the germination test in evaluating argan seed viability. This can save 2 to 3 week in testing time and deliver faster results to seed producers.*

KEYWORDS*: Argania spinosa; germination test; nursery; seed viability; tetrazolium test.*

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I. INTRODUCTION

Argan tree (*Argania spinosa* (L.) Skeels) is an endemic species of arid and semi-arid territories in the south-west of Morocco ([1], [2], [3], [4] and [5]). Adult argan trees are survived to drought periods and able to produce leaves, branches and fruits under as little as 100 mm rainfall ([6], [7], [8]). Argan, mainly used for the edible oil extracted from its fruit, has recently received increased attention as a candidate for domestication and as a non-timber producing tree for environment preservation and rural communities well being [2] and [9]. Moreover, Argan is threatened by large-scale destruction, over-use and the lack of natural regeneration ([10], [11], [8]). Germination and early seedling development stages are critical periods for the establishment of plant species [12]. Several studies have been carried out to improve the germination of argan seeds ([13], [14], [15], [16], [17], [18], [19], [20]). They concern the optimization of the germination temperature [20], stone characteristics such as weight, shape or age [17] and [18], the absence of hard smooth shell which seems to constitute a barrier [17] and [20]. The combination of stone scarification plus fungicide application, cold storage of seeds (4 ° C), application of gibberellic acid (GA3) and the presence of light during germination alleviate dormancy and increase germination rates [14] and [15].

However, even under these conditions, it has been shown that loss of argan seed viability was limiting factor of nursery productivity. It is important to know the argan seeds viability before their conservation and germination. Seed viability is the measure of how many seeds in a seed lot are alive and have the potential to germinate under favorable conditions ([21], [22], [23]). Several methods have been used to evaluate seed viability, but the germination test and the tetrazolium (2, 3, 5-triphenyl tetratzolium chloride (TTC)) staining test are the most used methods ([24], [25], [26], [27], [28], [29], [30]).

The germination test is the official test for evaluating seed viability in seed testing laboratories around the world. It directly gives the germination capacity of seed lots but this technique is typically performed under conditions that stimulate germination and may take several days or weeks and in some cases even many months. This test does not take into account seeds that can remain dormant even during favorable growing regimes [21]. Moreover, seeds that are viable at the beginning of the test may become nonviable at some point during the lengthy study period for reasons such as fungal infection.

The tetrazolium staining is a rapid test and provides a basis for assessing seed viability and only takes a few hours with minimal equipment ([31], [27], [22], [29]). This is a biochemical estimate of viability, determined by the red color of the living tissues of the seeds soaked in a diluted solution of triphenyl-tetrazolium-2,3,5 chloride at 0.1 - 1% ([26], [31], [29]). All living tissues, which respire, are capable of reducing a colourless chemical 2,3,5 triphenyl tetrazolium chloride into a red formazan by Hydrogen (H) transfer reactions catalyzed by the dehydrogenase enzymes. Seed viability can be interpreted according to the topographical staining pattern and the intensity of the coloration. However, this method is laborious and its application to small seeds requires much skill.

The objective of the present study is to evaluate argan seed viability of nine lots collected from different mother-trees genotypes by the germination and the tetrazolium tests.

II. MATERIALS AND METHODS

Argan fruit is a drupe containing a stone of one to five nuts ([32], [33], [34], Figure 1). The embryo carried by the nut is enclosed in sclerous seed coat which forms a hard shell.

Figure1. Argan fruits (A), stones (B) and nuts (C).

2.1. Germination test

Ripe Argan fruits were collected during June from nine Argan trees growing in Argana, a site located in the south-west of Morocco. The protocol for pre-treatment of Argan seeds set forth by [14] was followed. One hundred and twenty fruits randomly selected from each mother-tree were hulled and the stones maintained at 4 °C. The stones were soaked in 2% chlorine solution for 15 minutes followed by three fresh water rinses. They were then scarified by slightly cracking them and then immediately treated with the fungicide Thirame. Thereafter, the stones were soaked for 24 hours in a gibberellic acid solution (GA3) at 1000 PPM, and then rinsed twice with fresh water before seeding. Two replicates of 60 pre-treated seeds (120 seeds for each mother tree) were placed to germinate a plastic tray (40 x 30 x 10 cm3) filled with sterile sand and moistened with water. The main plot (plastic tray) placed under a clear plastic chamber to ensure good exposure to natural daylight [13]. The test is carried out during a period of the year when the minimum temperature was higher than 10 °C and maximum temperature was not higher than 25 °C, which is favorable to argan seed germination [20]. Humidity was maintained at field capacity using daily spraying. Germination, as radical emergence, was recorded daily for 15 days when it leveled off. Non-germinated seeds were analyzed to identify dormancy or contaminated seeds. The LSD test ($\alpha = 5\%$) of equality of means was used to compare significant factors means [35].

2.2. Tetrazolium test

One hundred and twenty stones of each seed lots used in the germination test were randomly taken from each mother-tree to determine their viability by tetrazolium staining.

Two replicates of 60 nuts extracted from stones (120 nuts for each mother-tree) were soaked in water to remove the envelope that surrounds to facilitate penetration of the tetrazolium. The pre-treated nuts were soaked in a 1% tetrazolium solution (pH 7.0) for five hours at 40 °C [26] and [36]. After staining, wash the nuts several times in distilled water to remove excess stain.

**Corresponding Author: Mohamed Alouani*

[1] Laâyoune Higher School of Technology, Laboratory of Biotechnology and Valorization of Natural Resources, Ibn Zohr University, Morocco

Staining of embryos and cotyledons with tetrazolium is observed using a low-powered (2 x) binocular microscope. Tetrazolium-treated seeds are classified into nine categories depending on staining pattern (Figure 2).

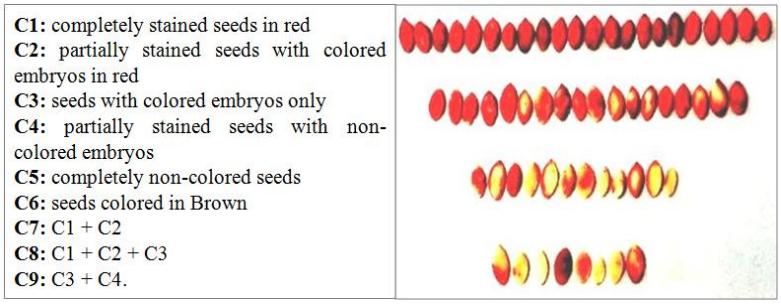

Figure 2. Argan seed viability according to tetrazolium staining.

Linear regression and correlation were used to study the association between the germination percentage and the percentage of each tetrazolium staining category. The square root transformation of (x + 0.5) is performed on the percentages. Statistix software was used for computation.

III. RESULTS

3.1. Germination test

Mother-tree genotype main effect was highly significant for germination and significant source of variation for contaminated seeds but none of dormant seeds (Table 1).

Table1. Analyses of variance of germination seeds (GS), contaminated seeds (CS) and dormant seeds (DS) for nine argan mother-trees.

Source of variation	DF	Mean squares		
		GS	CS	DS
Mother-tree genotype	8	97.06 **	79.76 *	1.51 ns
Error	9	17.33	15.28	0.83

DF: degrees of freedom; ** significant at 0.01; * significant at 0.05; ns: non significant.

Mean germination percentages varied between 63.3% for the genotype 3 and 95% for the genotypes 2 and 7 (Figure 3). The germination capacity of the seeds is therefore very variable according to the lots of the nine genotypes. The time of germination does not exceed four days for different genotypes, while the interval of germination varies between five and seven days.

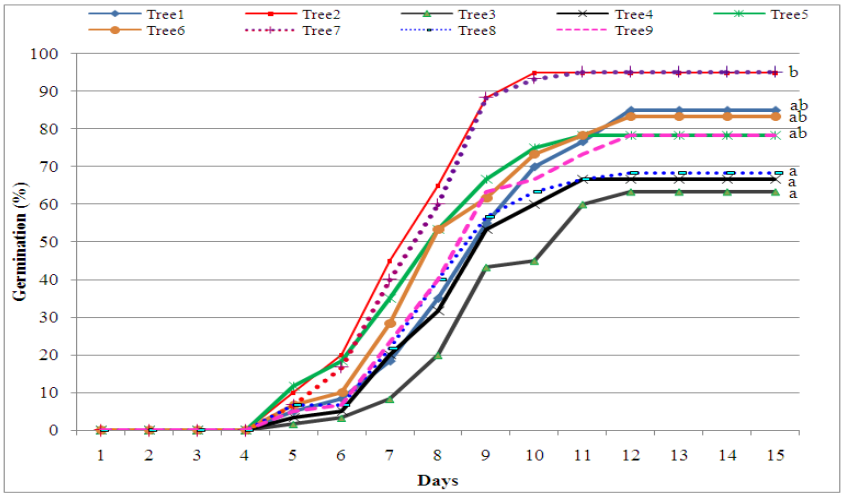

Figure3. Mean cumulative germination percentage of nine Argan mother-trees. Different letters note significant differences (LSD at 0.05).

Mean percentage of argan seed contamination is highly variable among genotypes and is the main cause of low germination percentages (Figure 4). It varies between 5% (genotypes 2 and 7) and 34.2% (genotype 3). However the seed dormancy remains very low, it does not exceed 4.2%.

**Corresponding Author: Mohamed Alouani*

[1] Laâyoune Higher School of Technology, Laboratory of Biotechnology and Valorization of Natural Resources, Ibn Zohr University, Morocco

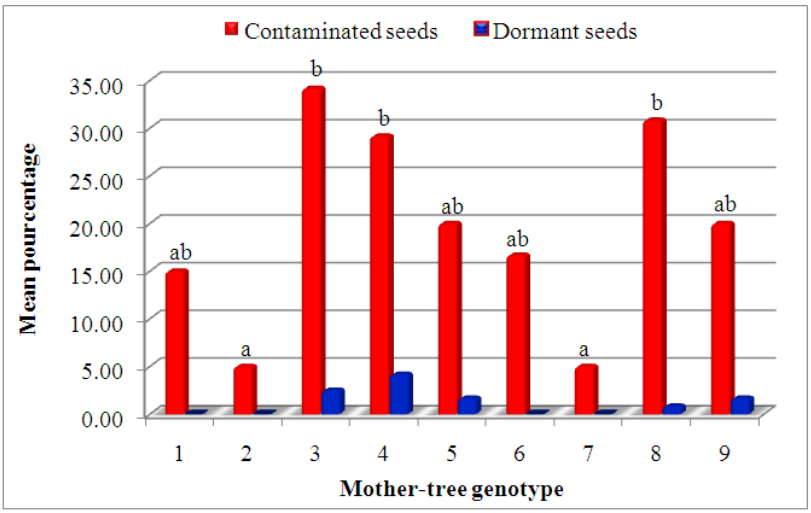

Figure4. Mean percentage of contaminated seeds (CS) and dormant seeds (DS) for nine argan mother-trees. Different letters note significant differences (LSD at 0.05).

3.2. Tetrazolium test

The results of the tetrazolium staining test show that there is a large variation between different categories seed coloration for the different genotypes (Table 2, Table 3).

Table 2. Linear regression analysis between the germination percentage and the percentage of each tetrazolium staining category (**C1:** completely stained seeds in red, **C2:** partially stained seeds with colored embryos in red, **C3:** seeds with colored embryos only, **C4:** partially stained seeds with non-colored embryos, **C5:** completely non-colored seeds, **C6:** seeds colored in Brown, **C7:** C1 + C2, **C8:** C1 + C2 + C3, **C9:** C3 + C4).

Source of variation	DF	Mean squares								
		C1	C2	C3	C4	C5	C6	C7	C8	C9
Regression	1	1.97*	3.13**	1.22ns	0.90ns	3.21**	1.26 ns	4.46**	4.04**	2.07*
Error	16	0.30	0.23	0.18	0.37	0.22	0.35	0.15	0.17	0.30

DF: degrees of freedom; ** significant at 0.01; * significant at 0.05; ns: non significant.

Table 3. Regression equations and correlation between the germination percentage and the percentage of each tetrazolium staining category (**C1**, **C2, C3**, **C4**, **C5**, **C6**, **C7**, **C8** et **C9**).

Tetrazolium staining category	Regression equations	Correlation
C1	Ger = 63.10 + 0.50 X	0.53 *
C2	Ger = 51.54 + 0.81 X	0.69 **
C3	Ger = 86.49 - 1.50 X	- 0.46 *
C4	Ger = 88.09 - 0.53 X	- 0.35 ns
C5	Ger = 86.86 - 0.64 X	- 0.68 **
C6	Ger = 75.25 + 3.08 X	0.37 ns
C7	Ger = 38.37 + 0.61 X	0.84 **
C8	Ger = 33.91 + 0.63 X	0.80 **
C9	Ger = 96.81 - 0.82 X	- 0.55 *

ns: non significatif, * : significatif à 5%, ** : significatif 1 %.

The percentage seed with embryos and cotyledons entirely or partially coloured in red (Category C7) represent 33% to 90% according to the genotypes (Figure 5). The regression line can give us an estimate of germination percentages from percentages of tetrazolium staining. Thus, the viability estimated by the tetrazolium test varies between 58.7% and 90.2% depending on the genotypes (Figure 5).

**Corresponding Author: Mohamed Alouani*

[1] Laâyoune Higher School of Technology, Laboratory of Biotechnology and Valorization of Natural Resources, Ibn Zohr University, Morocco

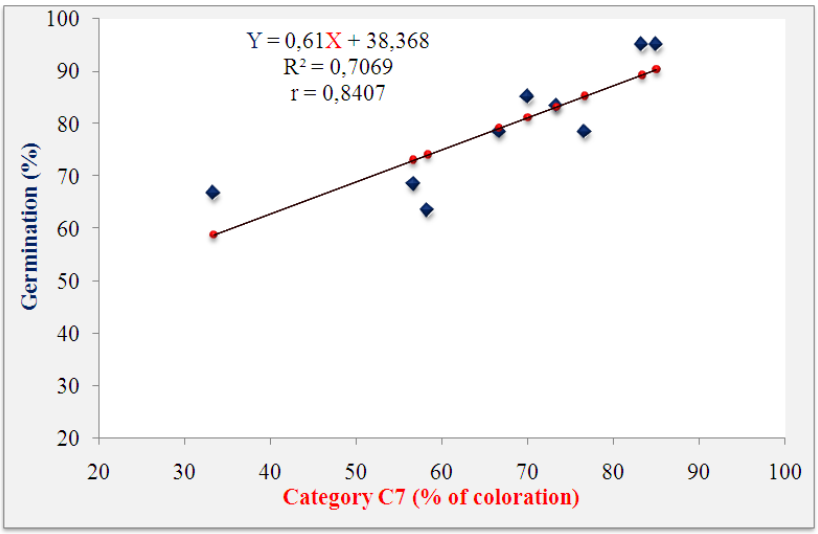

Figure5. Regression line of the germination percentage and the percentage of seed with embryos And cotyledons entirely or partially colored in red (C7) for nine genotypes.

A highly significant linear regression is observed between percentage of germination and seeds percentage of categories C2 (partially stained seeds with colored embryos in red), C5 (completely non-colored seeds), C7 (seed with embryos and cotyledons entirely or partially colored in red) et C8 (seed with embryos and cotyledons entirely or partially colored in red plus seeds with colored embryos only) (Table 2). It is not significant for seeds percentage of categories C3 (seeds with colored embryos only), C4 (partially stained seeds with non-colored embryos) and C6 (seeds colored in Brown). The correlation not important (r (C1) = 0.53 and r (C2) = 0.69) when we consider percentage of completely stained seeds in red (C1) or partially stained seeds with colored embryos in red (C2) (Table 3). A highly positive significant correlation (0.84) is observed for category C7 (The percentage seed with embryos and cotyledons entirely or partially colored in red). A negative significant correlation (-0.68) is also observed of germination percentage with non-colored seeds.

IV. DISCUSSION

In this study, a good correlation between viability tetrazolium-staining and germination tests results was found in the nine argan seed lots, proving the efficiency of the image scale established to evaluate viability in argan seeds. The germination test results ranged from 63.3% to 95%, and for the tetrazolium test the viability ranged from 58.7 to 90.2% according seed lots, which represents a wide range of samples with different seed qualities.

Germination test is a measure of viability based on the actual number of seeds germinated under a defined set of germination conditions [37]. The optimization of the germination conditions is a necessary step to carry out a germination test in a precise and reliable way. In this sense, seed scarified by slightly cracking, gibberellic acid (GA_3) treatment, seed cold storage and light treatment used in this study are a positive effects on argan seed germination and dormancy breaking independing from mother-tree genotype (Fig. 3 and 4). However, genotype has a direct effect on germination, which according to previous studies might be due to differential sensibilities to fungi contamination [35] and [14]. Genotype has also an indirect effect on germination because of differential trees precocity; ripe fruits may be more or less exposed to contamination and to unfavourable conditions to viability and germination [38] and [39]. Under the best germination conditions, our results show that the germination test can evaluating relative viability of the nine argan seeds lots in twelve days.

The tetrazolium-staining is a test that determines the percentage of viable seeds based on the activity of dehydrogenase enzymes in few hours, even for the most dormant seeds ([24]; [27]; [40]). Thus, it is considered one of the most significant advances in seed testing in the 20th century. However, this test requires specialized training and experience. The conduct and interpretation of the results of the tetrazolium-staining test are often dependent on the species [41]. In the present study, the best criteria to be used to associate tetrazolium staining of argan seeds to their germination capacity are based on the staining of the embryos and cotyledons entirely or partially in red [42] also found a positive correlation exceeding 94% between germination percentage and seed stained percentage on more than 75% of their surface. However, these authors did not specify whether the colored part (more than 75% of seed surface) including the embryo or not. Likewise, they used untreated seeds

**Corresponding Author: Mohamed Alouani*
[1] Laâyoune Higher School of Technology, Laboratory of Biotechnology and Valorization of Natural Resources, Ibn Zohr University, Morocco

to carry out the germination test, which influenced the germination rate obtained (63%) and consequently the correlation between the germination and colored seeds percentage. Germination and tetrazolium tests both provide information about the viability of a seed lot. However, dormancy and environmental conditions that may influence the results of a germination test do not usually affect the tetrazolium test ([36]; [30]).The common problem with using a germination test is that successful germination may take many months of stratification or conditioning, pretreatment structural or chemical to break or overcome dormancy. Argan seeds have both types of dormancy: embryonic and integumentary ([13]; [14]; [15]; [43]; [44]), so either germination times are often very long or the success of germination requires. Argan seed coat imposed dormancy can be removed by scarification whereas embryo dormancy can be removed by chilling for three months, GA3 soaking and by light ([13]; [14]). Thus, it is always interesting to have a rapid viability testing such as the tetrazolium test. This later test is also used to determine the viability of ungerminated seed at the end of a germination test ([23]). Moreover, Tetrazolium test can be employ at harvest before storage and during storage to quickly evaluate seed quality in a reliable way. It is worthy to note that one of the limitations of the Tetrazolium test is its inability to distinguish dormant from nondormant seeds ([40]).

Based on these results, it can be concluded that the Tetrazolium test can be used as an alternative viability test to the standard germination test. However, Tetrazolium-staining test of argan seed viability still needs to be improved. Thus, longitudinal cut through the embryo as a new preparation for staining method could be a solution to shorten and help in Tetrazolium testing, but still has to be investigated.

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**Corresponding Author: Mohamed Alouani*
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**Corresponding Author: Mohamed Alouani*
[1] Laâyoune Higher School of Technology, Laboratory of Biotechnology and Valorization of Natural Resources, Ibn Zohr University, Morocco