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EDICIONES COMPLUTENSE

Lights and shadows in the application of the resazurin test on Macaronesian flora

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Abstract. Seed viability tests are essential for seed bank management and the *ex situ* conservation of genetic biodiversity. Although there are different methods for determining the viability of the seeds, none of them is 100% effective, many require a considerable number of resources, some are not entirely reliable, others are time-consuming, they destroy the seeds, and/or can even be dangerous for the researcher/laboratory technician. However, a new simple, quick and non-destructive seed viability test has been recently described. This method is based on the reduction of resazurin to highly fluorescent resorufin by the respiration of a yeast activated by solute leak from non-viable seeds. In the present study, we tested this method in 53 taxa from 28 families from the Macaronesian region. We did not find a significant correlation between the germination and the resazurin test. Although there were several taxa that showed a high positive correlation, many other taxa exhibited a low positive correlation. Besides, we did not detect standard absorbance values from the resazurin test that determined seed health conditions. Though the resazurin viability method could be a good viability test, this should be standardized for each taxon.

Keywords. Macaronesian flora, resazurin test, seed viability.

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Introduction

Seed banks are essential for *ex situ* conservation of genetic biodiversity and are especially relevant for threatened species, since seeds are the main tool for plant reintroduce (Broadhurst *et al*., 2008). In this way, over recent years, an increasing number of germplasm banks, more than 1.000 seed banks around the word, have been established to support the future recovery of species that might become extinct in the near future (Myers *et al*., 2000; Ray & Bordolui, 2021). In these seed banks, the maintenance of *ex situ* seed viability over long periods under storage controlled conditions (humidity and temperature) must be optimized to conserve plant genetic resources (Harrington, 1972; Bacchetta *et al*., 2008; Fu *et al*., 2015). In addition, germination is a decisive stage in the plant life cycle, and its study is fundamental for the conservation of species (Melo *et al*., 2004). Therefore, germination studies are essential for reintroducing threatened species (Udayangani *et al*., 2020; González-Pérez & Cabrera-García 2021a). Consequently, the germination test is the most widely used method to determine seed viability.

However, there are certain aspects of the germination test, as a seed viability test, that do not make it foolproof. Normally, when a germination test does not work, we cannot ensure that the seeds have not germinated because they are inviable, since those seeds may be dormant and we have not been able to get them out of the dormant state (Bradford & Nonogaki, 2007). Therefore, in order to ensure the germination of those viable seeds of a seedlot from a species, we must have a germination protocol that ensures the breaking of dormancy and the activation of the germination process (Udayangani *et al*., 2020; González-Pérez & García-Cabrera, 2021a). Instead, germination tests require at least one month of testing, although there are species in which the process can take longer periods, with the susceptible problems of contamination by fungi (Luna *et al*., 2014; González-Pérez & Cabrera-García, 2021a). Also, it must be taken into account that the germination test, as a measure of viability, is a destructive test, since the seeds that we use to carry out that test cannot be used in the conservation of biodiversity. Although there are other methods for determining the viability of seeds, none of them is 100% effective, many of them require a considerable resource (infrared thermography), some are not entirely reliable (tetrazolium staining), others are time consuming (tetrazolium staining, cutting test, etc), destroy the seeds (cutting test), and can even be dangerous for the researcher/laboratory technician (X-ray tomography).

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However, a relatively new and simple, quick and non-destructive seed viability test has been described for Brassicaceae species (Min & Kang, 2011). This method is based on the redox adjuvant, resazurin. Resazurin is a non-toxic, water-soluble dye that is reduced by electron-transfer reactions associated with respiration to an easily measured water-soluble fluorescent product resorufin (O´Brien *et al*., 2000). The idea is to observe the reduction of the resazurin, detected by increases in absorbance (A) at 570 nm, due to the respiration of yeast that would be activated by a solute from nonviable seeds. In this study, seeds from three Brassicaceae species were tested and a high positive prediction accuracy of 98.2% was achieved (Min & Kang, 2011).

Later, this test was carried out in seeds of 10 weed species (Schutte *et al*., 2019) from six families, with prediction were relatively low (66–86%) for lightly damaged seeds. Nevertheless, prediction that were high (91–99%) for severely damaged seeds. Mohammed *et al*. (2019) used this method in seeds from 15 families detecting a high positive correlation between absorbance and germination percentage. In addition, authors described standard absorbance values for healthy (A=4–6), aging (A=2–4), and dead/damaged seeds (A= 0–2).

Macaronesia, which comprises Azores, Canaries, Cape Verde, Madeira and Selvagems, harbour a high plant biodiversity, and its flora is included into the Mediterranean basin biodiversity hotspot (Myers *et al*., 2000). There are 4.500 species described in the Macaronesian archipelagos, five of which are endemic (Bramwell & Caujapé-Castells, 2011), hosting over a quarter of the plant species listed in Annex II of the Habitats Directive (Sundseth, 2009). Although there are several institutions (Jardín Botánico Viera y Clavijo, Jardín de Aclimatación de La Orotava, Jardim Botânico da Madeira, Faial's Botanic Garden and Instituto Nacional de Investigação e Desenvolvimento Agrário) that host germplasm of these endemic endangered species, resazurin method has been barely tested in the Macaronesian flora.

Nowadays, there are only two studies where resazurin test has been carried out in Macaronesian endemic species. González-Pérez & Cabrera-García (2021b) found a high positive correlation (r=0.884) between absorbance at 570 nm and germination percentage in the Canarian endangered endemic species *Solanum lidii*. However, these authors only found a relatively low positive correlation (r=0.687) in other endangered endemic specie from the Canary Islands, *Isoplexis isabelliana* (González-Pérez *et al*., 2021). Those same authors suggest that this weak correlation could be attributed to small seed size, due to it not leak enough ethanol or sugar. In that regard, not one of the previous works on resazurin test related absorbance and seed size, weight, impermeability of the coat or storage time. Therefore, increasing the number of tests on endemic species is relevant in order to determine the effectivity of the resazurin method as a seed viability test in the Macaronesian flora, as well as in seedbank management.

The general aims of this study are: i) to determine the effectivity of the resazurin method as a viability test in the Macaronesian flora; ii) to test any relationship between absorbance and seed size, weight, or storage time; iii) to test standard absorbance values described for healthy or dead/damage seeds in Macaronesian flora; iv) to determine the effectivity of the resazurin method as a seedbank management tool.

Material and methods

Sample selection

In order to test resazurin reagent as an efficient seed viability test, 87 accessions belonging to 53 taxa (42 endemics from the Canary Islands, and 11 endemics from the Macaronesian region) from 28 families were selected to carry out tests (Table 1). All selected material was deposited in the seed bank from different periods of time range from 1 to 35 years (Table 1).

We differentiate two samples subset to address the different questions that we aim to respond. On the one hand, a sample subset with significant number of seeds of each accession (72–192 seeds) were analysed in order to determine the correlation between absorbance and germination percentage in 10 taxa (Table 1). On the other hand, a seed subset (12–48 seeds from each taxon) of a wide range of taxa (43 taxa) were analysed in order to obtain a standard absorbance value for healthy, aging and dead/damaged seeds (Table 1). In both sample subset, seeds used in resazurin analysis were not used in the germination test (see explain in results section).

Resazurin Viability test

Single seed were placed into each well of a 96-well PCR plate containing 150 µl of resazurin reagent (50 µl/ml resazurin, Sigma-Aldrich, St. Luis, USA and 400 µl/ml yeast Saccharomyces cerevisiae, Sigma-Aldrich, St. Luis, USA) in each well (Min & Kang, 2011). The plate was incubated at 35 °C for 4 hours. Absorbance was measured at 570 nm with a spectrophotometer plate reader (Heales, MB-580, Shenzhen Huisong Technology Development Co., Ltd, Shenzhen, China).).

Germination tests

Germination tests were carried out following germination protocols described by González-Pérez & Cabrera-Garcia (2021a) or those protocols recorded in the database of the Seed Bank of the Botanical Garden "Viera y Clavijo" (JBCVC). Overall, germination was carried out on 1% water agar in Petri dishes with different pre-treatments and temperatures in order to breaking seed dormancy and avoid fungi contamination (Table S1, Supplementary Material). Seeds were incubated under a photoperiod of 16 hours of light (cool white fluorescent tubes, 750–1250 lux) and 8 hours of darkness. Seeds were considered to have completed germination when the radicle was 1–2 mm long. Seeds with an emerged radicle were counted every day, removed from the Petri dishes. The final germination percentage was recorded after 30 days of incubation.

Data analysis

The germination percentage (%G) was calculated according to Coolbear *et al*. (1984) and Gavassi *et al*. (2014). Seed area was measured using a stereoscopic microscope (Olympus SZX12, Tokyo, Japan). Pictures were taken with a digital camera (Nikon DSFi2, Tokyo, Japan) and resulting images were analysed using NIS Elements v 4.0 software to calculate the seeds' area. Seed weight was determined during this study by weighing four replicates of 100 seeds on an analytical balance with 0.001 g-precision (Sartorius Quintix 65-1S, Goettingen, Germany).

Pearson correlation between absorbance (570nm) and germination percentage was calculated using XLSTAT ver. 3.02, 2008. These analyses were carried out hierarchically at the family and taxon level. In order test the capacity of resazurin test to differentiated between dead/damaged seeds (0% germination) and healthy seeds (germination over 90%) a t-student test was carried out (Microsfot Office Standard 2016, New York, USA). In addition, a correlation analysis between absorbance values and seed area, weight, and storage time was performed to determine the role of these factors in the absorbance detected.

Principal component analysis was carried out in order to determine the relationship among all these variables (absorbance, percentage of germination, time of storage, seed area and seed weight) as well as, relationship among taxa analysed.

Table 1. Taxa, time of storage (years) of the different accessions essayed and sample size (seeds). In bold taxa with a significant number of samples that were used in order to test the correlation between germination percentage and absorbance.

Taxon	**Family**	**Time of storage** (years)	**Sample size** (seeds)
Adenocarpus foliolosus	Fabaceae	10	12
Adenocarpus ombriosus	**Fabaceae**	**26**	**72**
Aeonium percarneum	Crassulaceae	4	12
Anagyris latifolia	**Fabaceae**	**15,22,27,32**	**102**
Arbutus canariensis	Ericaceae	4,21	36
Argyranthemum broussonetii	Asteraceae	7	17
Argyrolobium armindae	Fabaceae	4	36
Bencomia caudata	Rosaceae	4,23	35
Bosea yervamora	Amaranthaceae	13	24
Bryonia verrucosa	Cucurbitaceae	-	12
Campylanthus salsoloides	Plantaginaceae	3	12
Canarina canariensis	Campanulaceae	7	11
Ceballosia fruticosa	Boraginaceae	4	12
Chamaecytisus proliferus	Magnoliopsida	6,11	24
Cheirolophus arboreus	**Asteraceae**	**27**	**72**
Cistus grancanariae	Cistaceae	6	24
Clethra arborea	Clethraceae	20	12
Convolvulus loridus	Convolvulaceae	11	12
Crambe sventenii	Brassicaceae	12	12
Dendriopoterium pulidoi	Rosaceae	5	12
Descurainia preauxiana	Brassicaceae	24	12
Dorycnium spectabile	**Fabaceae**	**21**	**72**
Dracaena draco	Asparagaceae	23	12
Erica arborea	Ericaceae	11	23
Erysimum albescens	Brassicaceae	19,24	36
Erysimum bicolor	Brassicaceae	2	24
Geranium palmatum	Geraniaceae	20	12
Gonospermum oshanahanii	**Asteraceae**	**5,20,24**	**103**
Ilex canariensis	Aquifoliaceae	17	12
Isoplexis chalcantha	Plantaginaceae	6	23
Isoplexis isabelliana	**Plantaginaceae**	**3,19,25,34**	**96**
Ixanthus viscosus	Gentianaceae	6	12
Justicia hyssopifolia	Acanthaceae	36	12

Taxon	Family	Time of storage (years)	Sample size (seeds)
Kleinia neriifolia	**Asteraceae**	**1**	**96**
Limonium preauxii	Plumbaginaceae	20	8
Lotus kunkelii	Fabaceae	6	13
Lotus lancerottensis	Fabaceae	29	22
Navaea phoenicea	Malvaceae	33	12
Parolinia aridanae	**Brassicaceae**	**10,22,26**	**192**
Pericallis appendiculata	Asteraceae	4	24
Pericallis hadrosoma	Asteraceae	1	23
Periploca laevigata	**Apocynaceae**	**9,22,27,35**	**174**
Plantago ovata	Plantaginaceae	16	24
Ranunculus cortusifolius	Ranunculaceae	-	12
Retama rhodorhizoides	Fabaceae	4	12
Salvia canariensis	Lamiaceae	2,13	20
Schizogyne sericea	Asteraceae	-	24
Scrophularia calliantha	Scrophulariaceae	7	12
Semele gayae	Liliopsida	4	12
Sideritis amagroi	**Lamiaceae**	**4**	**96**
Sideritis discolor	Lamiaceae	7	24
Solanum lidii	Solanaceae	22,23,37	48
Sventenia bupleuroides	Magnoliopsida	1	12

Results

In the first trials, the same seeds that were treated with resazurin reagent were used for germination tests following Min & Kang (2011) and Mohammed *et al*. (2019) protocol. However, we detected germination and contamination problems with these seeds (germination percentage: *Salvia canariensis*: 0%, *Sideritis amagroi*: 1%, *Isoplexis isabelliana*: 40%). Whereas when seeds from the same accession that had not been exposed to resazurin reagent were used for germination tests, a high germination percentage and no contamination problems were detected (germination percentage: *Salvia canariensis*: 48%, *Sideritis amagroi*: 68%, *Isoplexis isabelliana*: 80%). Therefore, in subsequent analyses, different sample of seeds from those used in the resazurin test were used to assess the completion of germination.

Absorbance ranged between 0.017 and 1.911, while germination percentage oscillated between 0–100% (Figure 1; Table S2, Supplementary Material). Absorbance values from the different taxon studies did not show significant positive correlation with germination percentage (r=0.071).

At the family level, there were some families that showed a more positive correlation between absorbance and germination than others (e.g., Lamiaceae, r=0.823; Rosaceae, r=0.895; Solanaceae, r=0.878; Plantaginaceae, r=0.446; Asteraceae, r=0.232; Brassicaceae, r=0.090; Magnoliopsida, r=-0.450; Fabaceae, r=-0.234; Figure 1). At species level, there were taxa that exhibited a high positive correlation between absorbance and germination percentage (e.g., *Solanum lidi*, r = 0.878; *Dorycnium spectabile*, r =0.735), and others that did not show this correlation (e.g., *Sideritis amagroi*, r = 0.190; *Anagyris latifolia*, r = 0.052).

Significant differences ($p<0.01$) were detected in the absorbance values between dead/damaged seeds (0% germination) and healthy seeds (over 90% germination). But not significant positive correlation was found between both variables (absorbance value and percentage of germination).

No correlation was detected between absorbance (570 nm) and seed area, weight, or storage time. Besides, no correlation was detected between storage time (from 1 to 37 years) and germination percentage (Table S3, Supplementary Material). The only correlation detected among the variables studied was between seed area and seed size (r = 0.899)

Principal component analysis (Figure 2), in which the first two eigenvectors accounted for 63.39% of the total variance, revealed a scarce correlation between absorbance and germination percentage. Whereas seed weight and area vectors showed a positive correlation, since two vectors were close and formed a small angle. Biplot analysis indicated that germination percentage vector is which less contributed to the principal component. PCA analysis did not showed clustered of the taxa by families based on the variables analysed (Figure 2).

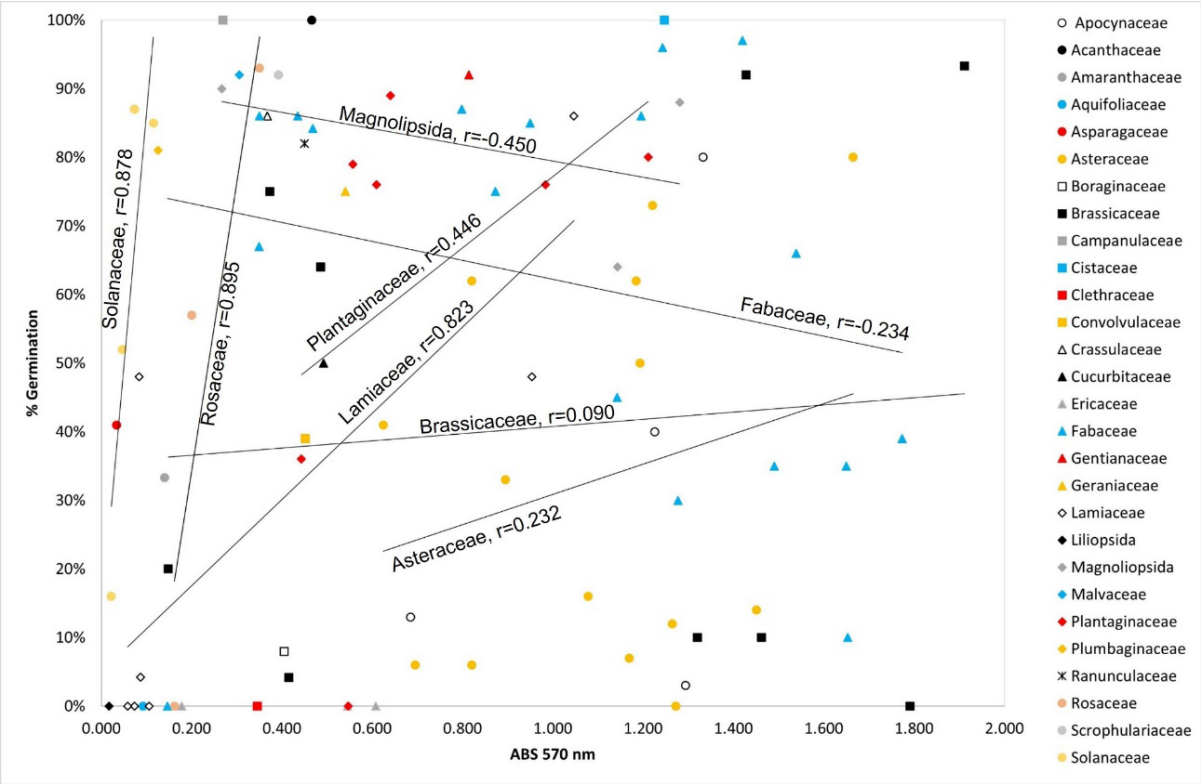

Figure 1. Representation of the correlation detected between the absorbance at 570 nm and germination percentage of all taxon analysed. The linear trend line is shown, as well as Pearson´s correlation value of those families with higher taxa representation in the analysis (Asteraceae, Brassicaceae, Fabaceae, Lamiaceae, Magnolipsida, Plantaginaceae, Rosaceae and Solanaceae).

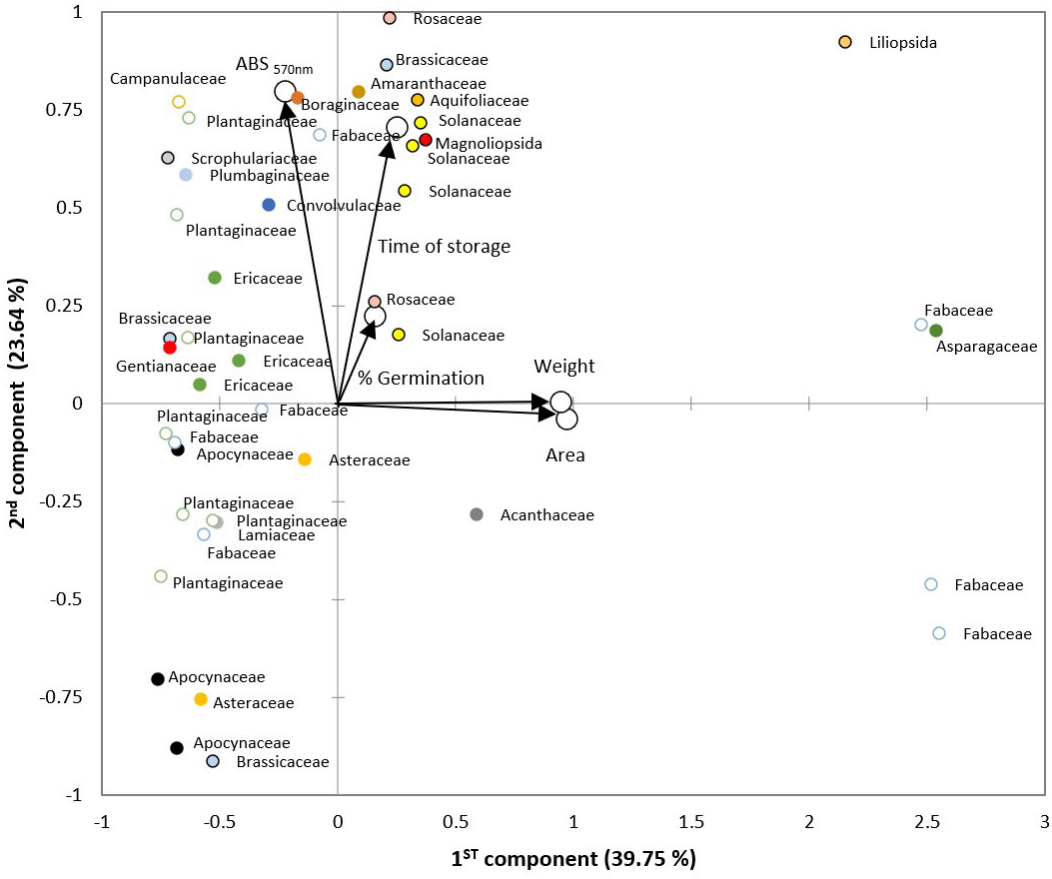

Figure 2. Principal component analysis biplot of the taxon tested with resazurin method (plots) and the variables (arrows) analysed. The percentage of explained variance of each axis is given in parenthesis.

Discussion

Although the resazurin method is a non-destructive test according to the literature, our results show that it incapacitated seeds preventing them from completing germination subsequently, since the seeds end up covered with a fungus (yeast), inducing contamination problems during the germination process if the seeds from the resazurin test are not surface sterilized before being tested for the completion of germination.

Pearson´s correlation analysis, as well as, PCA biplot did not show a direct positive correlation with the absorbance at 570 nm as a result of the resazurin test in opposition to previous studies. In addition, absorbance values did not follow standards described by Mohammed *et al*. (2019), since there were accessions with absorbance values lower than 2.0, putative dead/damaged seeds, but with a high percentage of germination (e.g. *Justicia hyssopifolia*, ABS_{570nm} = 0.466, %G =100%). In fact, according to these authors all seeds analysed that had absorbance values less than 2.0 should be dead or damaged. However, we found a mean germination value of 48%, and a large number showed germination percentage between 80–100%. In this sense, we detected absorbance ranging from 0.017 to 1.791 for accessions that showed a 0% of germination. However, accessions with a germination over 90% showed absorbance values ranging from 0.267 to 1.911. Therefore, we cannot establish an absorbance standard value related to the health of the seed in the Macaronesian flora.

Min & Kang (2011) working with Brassicaceae species, obtained absorbance values ranging from 0.058 to 2.031, even for healthy seeds, which is in agreement with the results obtained in the present study for Macaronesian flora (ABS_{570nm} = 0.148 to 1.911).

The lack of response to resazurin in aged seeds could be attributed to the fact that seeds do not leak enough ethanol or sugar, perhaps due to a seed coat impermeability or small seed size (Min and Kang, 2011; González-Pérez *et al*., 2021a).

We did not find a positive correlation between absorbance values and seed area or seed weight for those accessions that exhibited a low germination percentage, revealing aging of seeds. Therefore, results suggest that we cannot standardize absorbance values for all taxa since there is a high seed diversity with different seed shapes, sizes, weights and impermeability coat that produce the more or less leak of solutes in the nonviable seeds. This implies higher or lower yeast respiration and therefore a higher or lower reduction of resazurin reagent, and different absorbance values for seeds in the same health condition. For instance, the solutes that could be released by an *Isoplexis isabelliana* seed (area = 0.372 mm^2) are not the same than those from an *Anagyris latifolia* seed (area = 43.31 mm^2).

In this way, seeds from the same taxon have the same characteristics; shape, size, weight, coat impermeability (Šerá & Šerý, 2004) and under the same state (healthy, aging or dead/damaged) leak the same solutes amount and therefore showed the same absorbance values at 570 nm. Therefore, the present study recommends standardizing the resazurin method for each taxon in order to identify the absorbance values where a seed is healthy, aging and dead/damaged and, ultimately, determine if this method is, in fact, suitable for determining seed viability within the species of interest. Another relevant factor is that we should not forget that seeds could carry fungal spores on their coats. In fact, most germination protocols include seed disinfection pre-treatment with hypochlorite or other substances in order to eliminate these fungi (Table S1, Supplementary Material) (Ferrer-Gallego *et al*., 2013; González-Pérez & García-Cabrera, 2021a). These fungi could produce the resazurin reagent reduction through their respiration and therefore decrease absorbance value. In fact, we have detected significant differences in absorbance values when a seed disinfection pre-treatment is carried out (ABS_{570nm} = 0.105) and when it is not (ABS_{570nm} = 0.057). So, fungal contamination of seed coat could affect absorbance measurement and therefore, the determination of the health status of the seeds.

Absence of correlation between storage time and germination percentage highlight the good state of conservation of the material deposited in the Seed Bank of the Botanical Garden “Viera y Clavijo” and the reliability of the temperature and humidity conditions in which the seeds have been stored

The present study has shown that there are no standard absorbance values for dead/damage or healthy seeds for all Macaronesian taxa. On the whole, resazurin method could be an alternative viability test although this should be tested and standardized for each taxon. So, the future direction in this research is test and standarized the resazurin method in other Macaronesian taxa.

Author contribution

MAG-P: Conceptualization, data curation, formal analysis, Management of the project, writing; NC-G: Conceptualization, research; CS-S: Research; RS-H: Conceptualization, research, writing.

Conflict of interest

None.

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Supplementary Material

Table S1. Taxa, germination temperature (ºC) and seed pre-treatment of the different taxa analysed. GA: gibberellic acid, HP: hydrogen peroxide, ME: mechanical scarification, PN: potassium nitrate, SH: sodium hypochlorite.

Table S2. Absorbance at 570 nm and germination percentage of the different accessions analysed.

Table S3. Pearson correlation among absorbance and the different variables analysed.