Viability in rice seeds obtained from plants developed *in vitro*

Viabilidad de semillas de arroz provenientes de plantas obtenidas *in vitro*

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Abstract

The viability of rice seeds (cv. IACuba-28) obtained from *in vitro* developed plants was evaluated. These seeds were stored for 0, 2, 5, 7, 9 and 12 years in a seed bank at 4ºC in dry and 34% relative humidity. For monitoring viability we conducted a germination test and an alpha-amylase activity assay in germinating seeds. In both experiments we set a positive control with new seeds obtained from plants grown in the field. It was observed a gradual decrease in germination percentage with increasing storage time. In the accession with 12 years of conservation the largest number of dead and contaminated seeds and abnormal plants was found. Accessions showed increasing in alpha amylase activity until the fifth day of germination and then decreased, but quantitative significant differences ($P<0.05$) among young and old accessions were found. The average of alpha amylase activity on the fifth day was 0.25 for young accessions, while for the oldest was 0.192. The highest difference was noted in the accession of 12 years, where the maximum activity only reached 0.0614. The present study demonstrated that from *in vitro* culture maintain their viability for 10 years of storage. After this time, we perceived adverse changes in germination parameters and in the alpha amylase activity.

Key words: Alpha amylase activity, germination test, *Oryza sativa*.

Resumen

Se evaluó la viabilidad de semillas de arroz índica (*Oryza sativa* L.) variedad IACuba-28 provenientes de plantas cultivadas *in vitro*. Dichas semillas fueron conservadas en bolsas de plástico durante 0, 2, 5, 7, 9 y 12 años en un banco de semillas a 4 ºC y 34% de humedad relativa. La prueba de germinación se realizó en una muestra aleatoria de las accesiones A2000, A2003, A2005, A2007, A2010, A2012 y como prueba bioquímica se determinó la actividad alfa-amilasa en las semillas en germinación. En ambas determinaciones se incluyeron controles positivos con semillas nuevas obtenidas de plantas cultivadas en campo. Se halló una reducción progresiva del porcentaje de germinación, a medida que aumentó el tiempo de conservación en banco de germoplasma, así, a los 12 años de conservación se dio el mayor número de semillas muertas, contaminadas y plantas anormales. En todas las accesiones se observó un aumento de la actividad alfa-amilasa hasta el quinto día de germinación para luego disminuir, encontrando diferencias ($P < 0.05$) entre accesiones jóvenes y antiguas. La actividad alfa-amilasa promedio en el quinto día de germinación fue de 0.25 en las accesiones con menor tiempo de almacenamiento, mientras que en las más antiguas fue de 0.192. La diferencia más marcada se observó en la accesión almacenada por 12 años, donde el pico de actividad sólo llegó a 0.0614. Este estudio demostró que las semillas provenientes de cultivo *in vitro* mantienen su viabilidad durante 10

años de almacenamiento en las condiciones del estudio; transcurrido este tiempo se perciben cambios desfavorables en los parámetros de germinación y en los niveles de la actividad alfa-amilasa.

Palabras clave: Actividad alfa-amilasa, cultivo *in vitro,* prueba de germinación.

Introduction

Banks are means to preserve the viability and purity of the seeds in a controlled way under low temperature and relative humidity. With that, the aim is to preserve them from harvesting till next sowing season, preserve species of high genetic value, and avoid the natural break of dormancy and the genetic erosion of the germplasm (Doria, 2010).

The viability is the measurement of the percentage of live seeds able to germinate and produce plants under suitable conditions. Low temperatures and low humidity percentages favor a slow metabolism in seeds, increasing their longevity (Doria, 2010). Stored seeds should have a high viability at the beginning and during storage (Bradford, 2004), however, this progressively diminishes over time; therefore, it is important to know the starting moment of that reduction in order to take appropriate actions to preserve the accession and reduce its excessive damaging and the loss of the material (Rao *et al.*, 2007).

There are several methods to determine seed viability, among them the germination test on paper (AOSA, 2005) is considered the most exact and trustable. This test is used on seed with < 2 mm diameter that are placed on a paper tissue used as substrate, with constant humidity and neutral pH. With this test is determined the proportion of seeds of a particular accession or lot that will germinate under favorable conditions producing normal seedlings with roots, buds and food reserves to grow as complete plants.

There are also fast biochemical tests that need special techniques to be performed; among them is the amylase test, a biochemical indicator of the viability of seeds with high starch content. In the rice (*Oryza sativa* L.) seeds amylase activity could be detected on early stages of germination and in the mobilization of reserve substances as an essential metabolic event. Amylases (alpha and beta) are enzymes synthesized in the aleuronic layer that catalyzes the starch hydrolysis to form monosaccharides aiming to cell respiration processes (Bernal and Martínez, 2006).

In the Seed Bank of the Genetic Engineer and Biotechnology Center of Sancti Spíritus, Cuba, rice seeds coming from *in vitro* seedlings are preserved. When these plants are 15 cm tall are transferred to the *ex vitro* environment, where the cultural cares are guarantee. For this study, the seeds obtained were dried, packed in plastic bags and conserved for several years at 4 ºC and 34% of relative humidity. The objective of this work was to evaluate the seed viability of different rice accessions coming from in vitro culture after 0, 2, 5, 7, 9 and 12 years of storage at the previously mention seed bank.

Materials and methods

Germination test

Seeds of the rice (*Oryza sativa* L.) accessions A2000, A2003, A2005, A2007, A2010, A2012 from the OACuba-28 variety were used, conserved at 4 ºC and relative humidity 34% during variable periods of 0, 2, 5, 7, 9 and 12 years, that correspond to the number of storage years. From each accession (treatments) were randomly selected 300 fresh seeds distributed in three replicates of 100 seeds each, plus a control

of 300 seeds that were harvested in the field in 2012. Before the germination test, all the seeds were subjected to 40 °C heat with circulating air during 5 days according to the AOSA regulation (2005).

Seeds were submerged for 10 min on a solution of sodium hypochlorite (1%) and washed five times with sterile water. Then they were placed on wet paper towels on petri dishes of 9 cm to incubate at 25 °C. Fourteen days after the starting of the test the counting of normal and abnormal seedlings and death and latent seeds was done and, the percentage of germination taking the normal plants versus the total of seeds, and discarding the contaminated seeds that were eliminated during the test.

Determination of alpha-amylase activity

To determine the alpha-amylase activity of each accession each 24 hours were taken 20 seeds from day 1 till 7 of germination-naked seeds were grinded on a mortar and homogenized with 1.5 ml of buffer 0.1 M Tris-HCl, pH 6.8 to extract total soluble proteins. The homogenized was centrifuged at 13000 x g for 20 min and supernatant was collected, this was used as sample for the assays. For the enzymatic reaction 50 µl of the sample and 50 µl of starch solution 0.5% were mixed. The colorimetric technique to determine reducing sugars was applied (Miller, 1959). Intervals to collect the sample were: 5, 10, 15 and 20 min. Four replicates of the reaction per interval were performed and incubated at 37 °C. In each interval 100 µl of dinitrosalicylic acid were added to each collected sample and heated on a water bath at 90 °C for 5 min.

The products of each reaction were poured on 96-well Costar plates using 200 µl per well to determine the absorbance at 550 nm on a plate reader PR-521 (SUMA, Cuba). A mixture of 0.5% starch and 0.1M buffer Tris-HCl 0.1M, pH 6.8 was used as blank. Alpha-amylase activity (Aαa) was determined by the following equation:

$$A\alpha a = \frac{\Delta Abs\ (550\ nm)}{At}$$

where, Abs is absorbance and t is time. $\Delta Abs = Abs2 - Abs1$, $\Delta t = t2 - t1$.

Statistical analysis

The data of percentage of germination and the alpha-amylase activity of the three replicates were analyzed by Anova on a completely randomized design. The mean comparison was done by the Tukey´s test ($P < 0.05$) with the statistical software SPSS version 11.5.

Results and discussion

Evaluation of germination

Seed germination is defined as the restart of embryo growth and the radicle protrusion from the seed coat (Rao *et al.,* 2007). The germination is not complete until the seedling is not qualified as normal according to the specific criteria for each species. The seedlings obtained during a germination test are classified as normal or abnormal. The normal ones have adequate structures of roots and essential buds for its later development; the abnormal ones do not have the capacity for development and suffer deficiency, decomposition or weakness in their roots and buds systems (AOSA, 2005). In Table 1 are shown the percentages of germination 14 days after the start of the germination test. Fungi contamination affected 3.85% of the total seeds, which were eliminated to avoid the propagation of the contaminant. The main deficiencies observed in the abnormal seedlings were the presence of less than two secondary roots and the emergence of short, thick and/or twisted buds.

The absence of germination in seeds is due, among other causes, to the dormancy and/or death of the embryo (Rao *et al.*,

Table 1. Results of the germination test on paper of rice seeds of accessions stored and coming from *in vitro*[a,b] plants.

Storage (years)	Seedlings			Seeds		Germinated
	Normals	Abnormals	Death	Hard/dormant	Contaminated	(%)[c]
12 years	180	18	57	0	45	70.58 a*
9 years	249	6	24	0	21	90.24 b
7 years	270	3	21	0	6	91.83 b
5 years	285	0	9	0	6	96.93 c
2 years	288	6	3	0	3	96.96 c
0 years	294	3	3	0	0	98.00 d
Control	294	0	0	6	0	98.00 d

a. Seed packed on plastic bags and stored at 4 °C and 34% of relative humidity.
b. Total counting of three replicates of 100 seeds each.
c. Percentage of germination was calculated with the number of normal seeds with respect to the total number of seeds (without the contaminated ones).

* Values followed by same letters in the same column do not significantly differ according to Tukey´s test (P < 0.05).

2007). In the last case, in general, the seeds soften during germination; meanwhile the dormant seeds are viable seeds that do not germinate even under favorable conditions. In this work, the seeds that do not germinate were inspected to check whether they were soft (death) or dormant. The results in the control treatment indicate that the conditions of germination were good since death seeds were not found and only two seeds that did not germinate stayed hard with potentially viable embryos; these were probably dormant seeds that are often present in recently harvested plots (Doria, 2010).

The rest of the non-germinated seeds were classified as death when soft to the touch and in some the embryos were observed to be dark. In the treatment of the A2012 accession there were no significant differences (P > 0.05) when compared to the control; both had equal storage times but, the seeds of the accession where coming from plants obtained by in vitro culture and the control ones were coming directly from plants in the field.

The percentage of germination decreased as the conservation time was increasing. In the treatments of 2 and 5 years of storage there were no differences (P > 0.05) in the percentage of germination but, there were differences in the number of death seeds (P < 0.05) (Table 1). The behavior of the seeds stored for 7 and 9 years was similar in all the parameters evaluated, showing that in both parcels of seeds there is a marked reduction in the percentage of germination in comparison to those stored for 2 and 5 years.

In the treatment of 12 years of storage there was a higher number of contaminated and death seeds and of abnormal seedlings, as well as lower germination percentage (75%). As this value was lower than 90%, a germination test was required using an additional sample according to recommendations of FAO/IPGRI (1994); the average of both tests was 69.33% showing that after 12 years of storage the seeds have lost their integrity and viability.

Measurements of water content, a critical factor that determines the longevity of seeds, showed a percentage of 4% for the accession 2012 which is a lower value than the one recommended for cereals under storage (Walters, 2003). Probert *et al.* (2003) considered that small changes in seed water content have a significant effect in the viability of them when stored. This is one of the causes of the reduction in the capacity

of germination of the seeds of the accession A2000. In the other accessions the water content was in the normal range (5.3%, approximately).

Alpha-amylase activity

The alpha-amylase enzyme catalyzes the hydrolysis of starch during germination and as products are sugars that reduce dinitrosalicylic acid in the presence of heat, showing a color change from yellow to dark red which intensity depends on the amount of sugars coming from the hydrolysis, this sugars can be quantified by colorimetric methods (Miller, 1959). In all the accessions there was an increase in the alpha-amylase activity till the fifth day of germination (Figure 1). From that day the activity was rapidly reduced, which is expected since the reserved substance in the seed are exhausted. In soybean seeds, Salinas *et al.* (2002) found that the alpha-amylase activity increased till the day 8 from the beginning of germination. On the other hand, Wilson (1987) observed that the starch reserve in the seeds is low at the beginning of germination, however after 5 days of imbibition there are picks of amylase activity.

In this study, despite the increasing and decreasing trends of the amylase activity, there were quantitative differences ($P < 0.05$) between younger and older accessions. The alpha-amylase activity average at the fifth day in the A2003 and A2007 accessions was 0.192, while for the accessions with shorter storage time (A2007, A2010 and A2012) the maximum activity was 0.25 with no differences with the control treatment. The most notable difference was observed in the A2000 accession, where the pick of activity was only 0.0614, four times lower than in the youngest seeds (Figure 1).

Due to the aging of the seeds there are noticeable changes in the enzymatic activities at the beginning of the germination. Salinas *et al.* (2002) found that the first stages of damage in the seeds are associated with the reduction of protein synthesis. Milanés and González (1999), on the other hand, found that the activity of the alpha-amylase enzyme decreases in seeds that have been exposed to stress conditions.

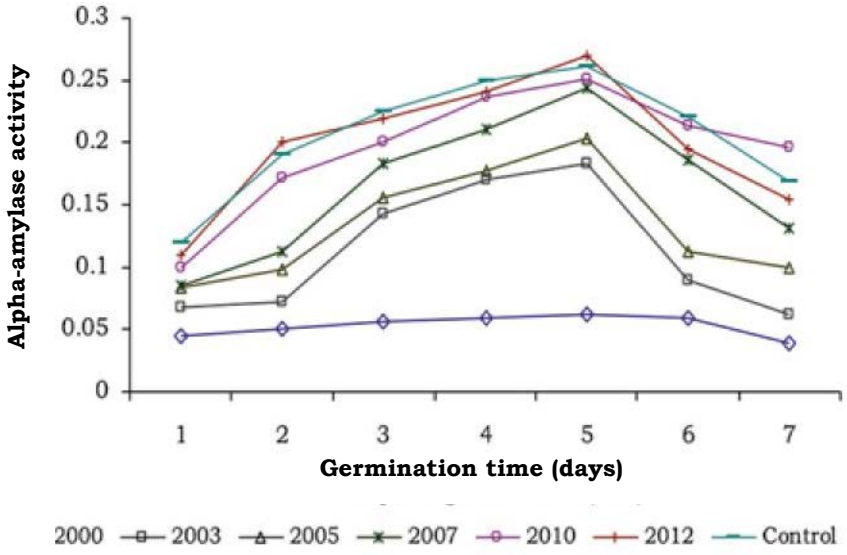

Figure 1. Alpha-amylase activity in total soluble protein extracts from rice seeds of the different accessions stored on conservation banks and germinated on paper for 7 days. Average of three replicates.

The poor results of the germination test and the low activity of alpha-amylase in the A2000 accession corroborate that the viability of seeds has decreased in the twelve days of storage. The other accessions remain normal in the germination parameters, including the A2003 accession that was about to get a decade of storage.

Conclusions

- As the storage time was increased there was a progressive reduction in the percentage of germination of the rice seeds coming from in vitro culture plants and preserved at 4 °C with 34% of humidity. However, the percentages stayed over 80% in the seeds stored for 10 years which means that the criterion for viability in this study was suitable.
- In the seeds preserved for more than 10 years adverse changes in germination parameters and alpha-amylase activity are noticeable.

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