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EFFECT OF THE RIPENING IN THE CRYOPRESERVATION OF THE *Nicotiana tabacum* L. SEEDS

Efecto de la madurez en la crioconservación de semillas de *Nicotiana tabacum* L.

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ABSTRACT. The main objective of this investigation was to determine the effect of the ripening in the cryopreservation of the *Nicotiana tabacum* L seeds. cv Sancti Spíritus 96 (SS-96). This study was done between the years 2012 and 2014, at the Tobacco experimental station located in Cabaiguan, Sancti Spiritus. SS-96 seeds were harvest at the 14, 21, 28, 35, 42, 49 and 56 days after the anthesis (DAA), determining the fresh and dry mass to over 1000 seeds, the humidity percentage (fresh mass) and germinating power (GP), as well as evaluate the tolerance of it to desiccation. At the same time, seeds collected every day, were conserved in 5 ºC or liquid nitrogen, and within 30 days, its GP was evaluated and put under accelerated aging and electrolyte leakage tests. During the research it was determined that the physiological ripeness of SS-96 seeds is reached at 29,6 DAA, with a 30,6 % of water and a dry mass of 1000 seeds of 81,2 mg of weight. 35 DAA were established as the optimum momentum for the harvest. The anticipation of the recollection process will end up in a diminishing the desiccation tolerance levels due to the lack of ripeness of seeds; meanwhile a late recollection ends up in lack of potency due to a larger exposure to the environment.

Key words: anthesis, conservation, desiccation, vigor

RESUMEN. La presente investigación tuvo como objetivo determinar el efecto de la madurez en la crioconservación de semillas de *Nicotiana tabacum* L. cv Sancti Spíritus 96 (SS-96). El estudio se realizó en la Estación experimental del tabaco de Cabaiguán, Sancti Spíritus, perteneciente al Instituto de Investigaciones del Tabaco, entre los años 2012 y 2014. Las semillas de SS-96 se colectaron a los 14, 21, 28, 35, 42, 49 y 56 días después de la antesis (DDA) y se les determinó masa fresca y masa seca de 1000 semillas, porcentaje de humedad (base masa fresca) y potencia germinativa (PG), además de evaluar su tolerancia a la desecación. Al mismo tiempo, semillas de cada día de colecta se conservaron a 5 ºC o en nitrógeno líquido y, transcurridos 30 días, les fue evaluada su PG y se sometieron a las pruebas de envejecimiento acelerado y pérdida de electrólitos. Durante el desarrollo de la investigación se determinó que la madurez fisiológica de las semillas de SS-96 se alcanza a los 29,6 DDA, con un contenido de agua de 30,6 % y masa seca de 1000 semillas de 81,2 mg. Se estableció 35 DDA como el momento idóneo para la colecta de semillas en este cultivar. Una colecta antes de esta fecha provoca una disminución en la tolerancia a la desecación producto de la inmadurez de las semillas; una recolección posterior conlleva a la pérdida de vigor debido a la mayor exposición de las semillas a las condiciones ambientales.

Palabras clave: antesis, conservación, desecación, vigor

INTRODUCTION

The Nicotiana collection, present at the germplasm bank of the Cuban Tobacco Research Institute, is constantly progressing with new varieties and species (1). Conserving long-term genetic variability of the genus is importance in

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order to introduce genes of value into commercial varieties (2) and to rebuild or reinforce *in situ* populations[A].

Since the 1980s, several investigations have been carried out worldwide in cryopreservation as an alternative to traditional seed storage methods (3, 4). Cryopreservation techniques normally use liquid nitrogen (-196 °C) because of their relatively low cost. The objective is to reach temperatures below -13 ^{0}C to achieve conditions of low molecular kinetic energy and extremely slow diffusion, so that the chemical reactions are practically paralyzed (5); under these conditions, extremely long longevities (3) are postulated.

Based on 10-year storage experiments and from the viability equation, Walters *et al*. (3) predicted a 3,400-year viability for lettuce seeds stored at -196 ° C. Therefore, cryopreservation can be very useful in the conservation of base collections and duplicate in germplasm seed banks, without neglecting the classical conditions, useful for short-term storage (active collection) (5).

It has been shown since the 1960s that genetic diversity can be preserved in germplasm banks. In the new century the question changes from "if" it is feasible to "how" to optimize the conservation process (6). The maturity with which seeds are collected and preserved is identified as a determinant of longevity (7, 8, 9). The objective of the research was to determine the effect of maturity on the germination and vigor of cryopreserved seeds of *Nicotiana tabacum* L.

MATERIALS AND METHODS

The study was conducted at the Tobacco Experiment Station from Cabaiguán, belonging to the Tobacco Research Institute of Cuba, during the tobacco campaigns 2012-2013 and 2013-2014. To carry out the research, the accession *Nicotiana tabacum* Linnaeus, cultivar Sancti Spíritus 96 (SS-96) was selected. This cultivar presents high productive potential (2 906 kg ha^{-1}), excellent quality (444 kg ha^{-1}, in upper classes) and excellent combustibility (> 20 s). It is one of the most planted cultivars in the central and eastern region of the country (10).

The seeds of SS-96 were seeded in expanded polyethylene trays with organic substrate with composition according to previous investigations (11), based on the technology of posture production in floating trays described (12). The transplantation of the postures to the field was performed at 45 days. The planting distance was 30 cm between plants and 180 cm between rows, and fertilization, irrigation and phytosanitary care were performed according to the technical instructions of the crop (13). The collection was performed at seven-day intervals at 14, 21, 28, 35, 42, 49 and 56 days after anthesis (DAA). For each day of collection, fresh mass and dry mass of 1000 seeds were determined.

Moisture tests were performed using the constant low temperature oven drying method (14), at a temperature of 103 °C for 4 h with three replicates of 0,5 g of seeds each. The moisture content was expressed as a percentage of fresh mass.

In the germination trials, four replicates of 100 seeds each were performed. The seeds were placed in 9 cm diameter Petri dishes on two filter paper disks moistened with 5 mL of distilled water. The incubation was performed at 27 ± 2 °C with a photoperiod of 12 h and an intensity of 350 µmol m^{-2}s^{-1}. In all the trials the emergence of the radicle was the criterion for considering that seed germination had taken place. The germination power (GP) was determined 14 days after the seeds were imbibed, counting the number of seeds germinated during that time interval among the total number of seeds (14).

In order to avoid imbibition damage, prior to germination tests, seeds with more than 35 DAA were humidified by dispersing them evenly in uncovered Petri dishes. They were then introduced into a glass desiccator with H_2O inside, avoiding direct contact of the seeds with water. The seeds were incubated under these conditions until reaching a moisture content higher than 14 %.

In order to illustrate the synthesis role of certain metabolites in the SS-96 seed desiccation tolerance, the values of GP measured only after dehydration of the seeds to 10,0 were evaluated for each day of collection; 7,5; 5,0 and 2,5 % humidity. Dehydration was carried out by exposure to the self-indicator desiccated silica gel agent in hermetically sealed glass desiccators at room temperature, in a ratio of 1: 3 (seed mass: self-indicator silica mass). The time of exposure to the drying agent was determined by the required drying intensity.

To determine the moment collection effect on the germination and vigor of the cryopreserved seeds of SS-96 samples of seeds collected from the different DAAand they were divided into two portions of equal mass. One portion was introduced directly into a cooling chamber at 5 °C

[A] Bacchetta, G.; Bueno, A.; Fenu, G.; Jiménez-Alfaro, B.; Mattana, E.; Piotto, B. y Virevaire, M. *Conservación ex situ de plantas silvestres*. edit. Principado de Asturias, La Caixa, 2008, 375 p.

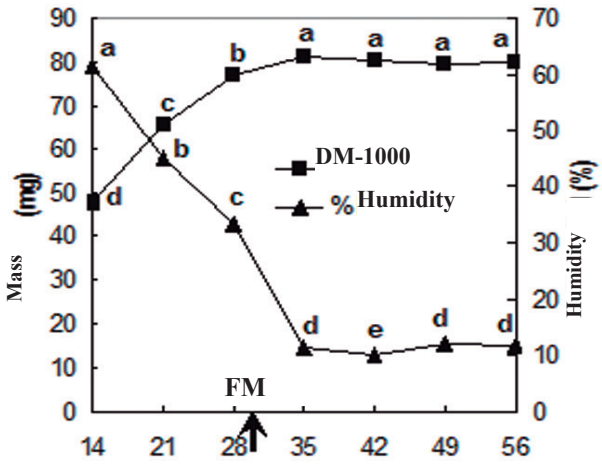
(non-cryopreserved) and the other into liquid nitrogen tanks (cryopreserved). After 30 days the cryovials were removed and the temperature was allowed to equilibrate to room temperature. Each sample was tested for GP and subjected to accelerated aging (AA) and electrolyte scape (EE) tests.

For the accomplishment of the AA and EE tests, the seeds were previously conditioned. According to the percentage of moisture, the seeds were dried or hydrated up to 14 % moisture. The AA test was performed according to the methodology described by Pérez-Rodríguez[B]. The assay was carried out until germination was reduced to below 50 % of the initial GP. Then, for each interval, the elapsed time was calculated for each sample to reach 50% of the initial germination (T_{50}) (15). The EE test was performed with some modifications (16). For each sample, four replicates were placed with 0,15 g of seeds each in 35 ml of deionized water at 30 ± 0,5 °C. The leachates were collected after 24 h of imbibition and the conductivity was measured with a conductivity meter.

The Statistical Package for Social Sciences (17) was used for the statistical processing of the data. The adjustment to the normal distribution of the data in each treatment (Kolmogorov-Smirnov) and the homogeneity of the variances (Levene) were checked. An Analysis of Variance (ANOVA) was performed to determine the possible significant differences among the means and also to discriminate the honestly significant difference method was used (HSD) of Tukey. In some cases it was necessary to transform the data to achieve the assumptions of the parametric tests performed. Each table and figure in the Results and Discussion section describes the specific statistical treatment performed.

RESULTS AND DISCUSSION

During the early development phase of SS-96 seeds, the moisture rapidly declines (Figure 1), from 61 % to 14 DAA to below 11 % at 35 DAA; although between the 28 DAA and 35 DAA this decrease becomes more accentuated. The dry mass of 1000 seeds (MS-1000) increases approximately linearly. However, since 35 DAA both MS-1000 and moisture tend to remain constant; Behavior that is maintained until the 56 DAA. The seeds reached physiological maturity (PM) at 30 DAA, determined according to the procedure described by Sanhew and Ellis (18), with humidity of 28 % and MS-1000 of 81 mg (Figure 1). From that moment the dry mass tends to remain practically constant until the 56 DAA.



The arrow points to the 30 AD as the physiological maturity (PM)
Unequal letters means, for each given parameter, have statistically significant differences
Simple ANOVA, p≤0.05, n = 9

Figure 1. Changes in the dry mass of 1000 seeds (DM-1000) and the moisture content of SS-96 seeds collected at 14, 21, 28, 35, 42, 49 and 56 days after anthesis (DAA)

These quantitative variables are correlated with the development status of dicotyledonous orthodox seeds in other species (19). There is a relationship among dry matter gain, moisture and GP with the stage or stage of development of seed C. Therefore, it is possible to relate with some approximation the morphological development of SS-96 seeds, with the values obtained of these variables, analyzed together.

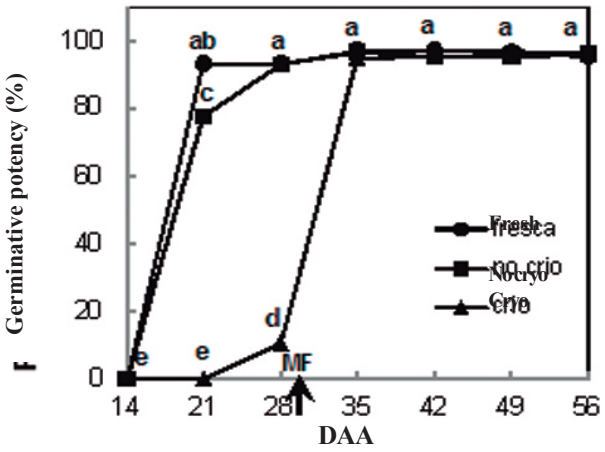
In the initial stage of development (up to 14 DAA), there is a progressive increase in dry mass, the product of embryo formation. However, the seeds do not have the necessary reserves to germinate (20), which is why the GP of fresh, non-cryopreserved and cryopreserved SS-96 seeds was zero (Figure 2).

As the study progresses the GP of fresh seeds increases significantly by 21 DAA. Similar results show the non-cryopreserved seeds, although in this case germination reaches values significantly higher only after 28 AD (Figure 2).

However, the cryopreserved seed presents a different behavior in the first part of the study when compared with the other treatments analyzed (Figure 2). While for fresh and non-cryopreserved seeds the GP increases rapidly from 14 DAA the germination of the cryopreserved barely reaches 10,5 % at 28 DAA and only takes values similar to the remaining treatments from the 35 DAA.

[B] Pérez, R. J. L. *Crioconservación de semillas de Nicotiana tabacum cv Sancti Spíritus 96*. [Tesis de Maestría], Universidad de Ciego de Ávila Máximo Gómez Báez, Ciego de Ávila, Cuba, 2014, 70 p.

[C] Zhu, H. *The Effect of Drying on Desiccation Tolerance and Late Embryogenesis Abundant Protein Gene Expression in Immature Seeds of Phalaenopsis amabilis* [en línea]. [Master These], Grand Valley State University, Michigan, USA, 2014, 714 p., [Consultado: 1 de febrero de 2016], Disponible en: <http://scholarworks.gvsu.edu/theses/714>.



Freshly collected (fresh), preserved for 30 days at 5 °C (non-cryopreserved) or in NL (cryopreserved)
Means with unequal letters have statistically significant differences. Bifactorial ANOVA, p≤0,05, n = 12
Only for statistical processing were the data transformed according to y = 2 arcsen ((and / 100) 0.5)

Figure 2. Germination power of SS-96 seeds collected at 14, 21, 28, 35, 42, 49 and 56 days after anthesis (DAA)

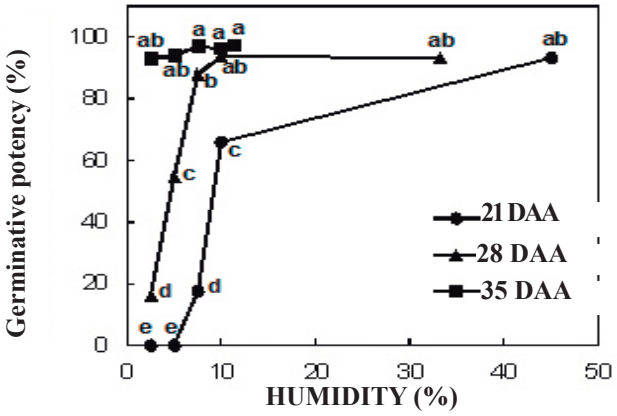
The immature seeds lack essential metabolites in their longevity during storage (Ex: LEA proteins) (21, 22), messenger ribonucleic acids (mRNA), required for the synthesis of proteins during the process of germination and sugars (9)). The low concentration of these metabolites at early stages of development of SS-96 seeds could be the source of the decrease in GP during the 30 days of storage of the non-cryopreserved seed when compared with fresh seed, both collected at 21 DAA.

Also, the main cause of the lower GP of the cryopreserved seeds could be related to the high percentage of humidity still present in them when reaching 28 DAA. The ice crystals, formed mainly during defrosting after exposure to NL (9), due to their spatial conformation, cause damage to cell membranes. In addition, when water displacement occurs for the formation of extracellular ice, the intracellular solute concentration rises gradually and jeopardizes functioning at the cellular level (7, 23).

The seeds of SS-96 shortly after reaching physiological maturity (30 DAA), suffer an accelerated loss of water. In this stage, the vascular connection with the mother plant decreases (24), with which the percentage of moisture in the seed drops rapidly until reaching equilibrium with the humidity of the environment (Figure 1). This tolerance to desiccation is correlated in other species with the presence of LEA proteins (21, 22) and sugars (9, 16). LEAs are a group of highly hydrophilic proteins that accumulate in seeds during the final stages of embryo formation (9, 25, 26) and that along with sugars such as raffinose and trehalose play an important role in the intracellular formation of vitreous state and in the protection of cell membranes (9, 21, 22). The vitreous state has characteristics similar to those of a solid, such as the decrease in molecular movement, but with the properties of a liquid (9). Because the viscosity of the cytoplasm when vitrifying is extremely high, fusion between membrane systems and conformational changes in proteins is avoided. In addition, the speed of aging reactions and the formation of ice crystals are considerably reduced (23, 25). This is the fundamental cause why the GP reaches significantly higher values for all treatments from the 35 DAA.

On the other hand, when the seed collected at 21 DAA is dried up to 10% humidity, it significantly decreases its GP, which descends abruptly when drying continues until it reaches values of 7.5 and 5% and becomes null when dried. extreme form at 2.5% (Figure 3). However, for the seed collected at 28 DAA the decrease in

GP is only significant when draining up to 5% and for the one collected at 35 DAA there were no significant differences in its GP, even at moisture values of 2.5%. Results similar to the 35 DAA are obtained by drying by the same procedure illustrated in Figure 3 to the seeds collected at 42, 49 and 56 DAA.



Means with unequal letters have statistically significant differences
Bifactorial ANOVA, p≤0,05, n = 4
Only for statistical processing were the data transformed according to y = 2 arcsen ((and / 100) 0.5)

Figure 3. Germination power of fresh seeds of SS96 collected at 21, 28 and 35 days after anthesis (DAA) and dried to 10,0, 7,5, 5,0 and 25 % moisture (base mass Fresh)

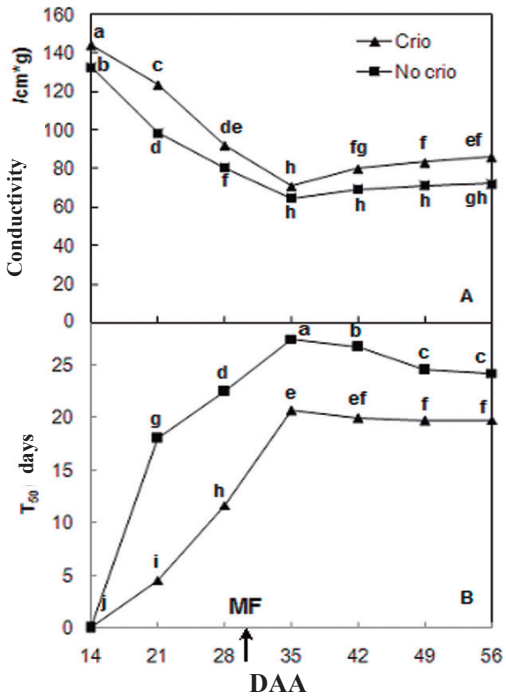
This result is closely related to the DM-1000 and moisture values shown in Figure 1. Several of the compounds synthesized in the seed during this stage (between 30 DAA and 35 DAA for SS-96 seeds), in addition to their correspondence with the acquisition of the desiccation tolerance, are linked with a greater longevity of the seeds during storage (15, 20). Thus, considering the treatments evaluated, it is not possible to collect adequate seed before 35 DAA, without taking an immature seed, which during the long-term storage loses its viability early.

In spite of the above, it is not possible to select the best collection time after the 35 DAA based on the results of MS-1000, humidity, GP and drying tolerance (Figures 1, 2 and 3). Indicators of vigor were used as discriminants in predicting the influence of conservation conditions on seed longevity (16, 18). In the present study vigor was evaluated using the EE and AA tests (16, 18).

Accelerated aging tests can provide valuable information on the most suitable storage conditions to achieve greater longevity in the seeds of a given species at short intervals (27). In Figure 4, a minimum in the conductivity of the leachate and an increase in the time to reach 50 % of the initial germination (T_{50}) of SS-96 seeds by 35 DDA. The 35 ADIs constitute the first measurement performed in the study to the seeds of SS-96 after acquiring this MF. In addition, upon reaching this stage of development the moisture of the seeds is reduced to values lower than 11 %. Both facts are in agreement with a greater integrity of the cellular membranes and, therefore, with the vigor shown (23).

Likewise, the increase in EE and in the sensitivity of the seeds to the AA test after 35 DAA, both cryopreserved and non-cryopreserved, should be noted. This behavior suggests a loss of vigor if the seeds are not collected immediately after the accelerated loss in water content, subsequent to PM. This behavior could be due to the effect of a greater exposure of the seeds to the environmental conditions (high temperature and humidity), which could accelerate the aging reactions (16).

In general, non-cryopreserved SS-96 seeds had greater vigor than cryopreserved seeds. No doubt, the high moisture content present in the seeds, well above the normalized (between 5 and 7 %) (14), causes an increase in the EE and in the sensitivity of the seeds to the AA test (24). It is necessary to check in future investigations the moisture percentage effect on the longevity of cryopreserved seeds, as a determinant factor (7, 16, 28).



Cryopreserved for 30 days (Cryo) and preserved at 5 °C (Non-cryo) for the same period and subjected to the accelerated aging test
Means with unequal letters have statistically significant differences
Bifactorial ANOVA, p≤0,05, n = 4

Figure 4. Leachate conductivity (A) and time to decrease to 50 % the initial germination potency (T_{50}) (B) of SS-96 seeds collected at 14, 21, 28, 35, 42, 49 and 56 after the anthesis (DAA)

There is no knowledge in the literature consulted of an influence maturity study on the cryopreservation of seeds. There was also no evidence of similar research, at least directed to fresh seed, in the genus *Nicotiana*. However, behavior similar to that shown by fresh SS-96 seeds during their development was reported in bean seeds (16, 18), Arabidopsis (29) and tomato (30). In all cases, there is a coincidence that the moment when the seed moisture reaches equilibrium with the environmental humidity, after the PM and the accelerated loss in water content (in the vicinity of the 35 DAA in this study), is the best time to collect.

CONCLUSIONS

- The collection of SS-96 seeds before 35 DAA causes a decrease in their tolerance to desiccation and, consequently, a decrease in longevity during cryopreservation.
- A collection after 35 DAA negatively affects the vigor of the cryopreserved seed, due to the greater exposure to environmental conditions.

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Note:

During the editing process it was not possible to access the work of retouching and improvement of images, so they have been inserted with the same quality as the ones sent by their authors.

The editorial

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