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Original article

Storage methods For *Cinchona Pubescens* Vahl. Seeds, Imbabura, Ecuador

Métodos de almacenamiento de semillas *Cinchona Pubescens* Vahl., Imbabura, Ecuador

Métodos de armazenamento de sementess *Cinchona Pubescens* Vahl., Imbabura, Equador

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RESUMEN

El manejo de semillas forestales en Ecuador, en relación al almacenamiento de las mismas, es todavía insuficiente, en particular la *Cinchona pubescens* Vahl. El objetivo fue determinar la calidad de las semillas y los mejores métodos de almacenamiento de semillas de *C. pubescens*, con el empleo de diferentes tiempos, envases y condiciones. La recolección de semillas fue en Intag, Imbabura. Los factores en estudio fueron: tiempo de almacenamiento, tipos de envase y condiciones de almacenamiento. Se determinó la calidad de las semillas al momento de la cosecha y en cada tratamiento. La pureza alcanzó un 76,5 %, el peso de 1 000 semillas fue 0,31 g y el contenido de humedad un 14 %. En el primer ensayo de almacenamiento no se manifestó la germinación en ningún tratamiento desde el primer mes. En el segundo ensayo, el mejor método de almacenamiento fue funda transparente almacenada en refrigeración durante una semana, el que mostró 9,25 % de poder germinativo. Las semillas de *C. pubescens*, pierden de manera rápida su viabilidad, que se hace nula al mes de almacenamiento.

Palabras clave: Refrigeración; Envases; Germinación; Pureza; Contenido de humedad; Vigor germinativo.

ABSTRACT

The management of forest seeds in Ecuador, in relation to their storage, is still insufficient, in particular the *Cinchona pubescens* Vahl. The objective was to determine the quality of the seeds and the best methods of seed storage of *C. pubescens*, using different times, containers and conditions. Seed collection was in Intag, Imbabura. The factors under study were: storage time, types of packaging and storage conditions. The quality of the seeds was determined at the time of harvest and in each treatment. Purity reached 76.5 %, the weight of 1,000 seeds was 0.31 g and the humidity content was 14 %. In the first storage trial, no germination was observed in any treatment from the first month. In the second trial, the best storage method was transparent cover stored in refrigeration for one week, which showed 9.25 % germination power. *Cinchona pubescens* seeds lose their viability quickly, which becomes null after one month of storage.

Keywords: Refrigeration; Containers; Germination; Purity; Humidity content; Germinative vigor.

RESUMO

O manejo de sementes florestais em Equador, em relação a armazenagem das mesmas, é ainda insuficiente, particularmente, a *Cinchona pubescens* Vahl. O objetivo foi determinar a calidade das sementes e os melhores métodos de armazenagem delas, com o emprego de diferentes tempos, envases e condições. A recoleita de sementes realizou se em Intag, Imbabura. Os fatores em estudo foram: tempo de armazenagem, tipos de envases e condições de armazenagem. Se determinou a calidade das sementes no momento de semear e em cada tratamento. A pureza alcanzou um 76,5 %; A pesagem de 1 000 sementes foi 0,31g e o conteúdo da umidade, um 14 %. No primeiro ensaio de armazenagem não foi manifesto em refrigeração tratamento nenhum do primeiro mês, no caso da germinação, mas num segundo ensaio, a funda transparente foi o melhor método do ensaio, armazenadas durante uma semana, o que mostrou 9.25 % de poder germinativo. As sementes de *C. pubescens*, perdem com rapidez sua viabilidade a que se faz nula ao mês da armazenagem.

Palavras clave: Refrigeração; Envases; Germinação; Pureza; Conteúdo de umidade; Vigor germinativo.

INTRODUCTION

The genus *Cinchona,* of the botanical family Rubiaceae, known as "cascarilla or cinchona tree" is made up of 23 species (Andersson and Taylor, 1994). It is native to the South American Andes (Garmendia, 2005). It is distributed along the tropical and equatorial zone of the Andes, from 10° North latitude to 20° South latitude. In Ecuador there are 12 of these species, (Ulloa and Jørgensen, 1995), where four are endemic and eight are native. Generally, they are medium to small size trees or shrubs with bitter bark, they can reach a height that can reach 25 meters (Mahecha, *et al.,* 2016). In natural conditions the genus *Cinchona* presents low rate of germination and regeneration, found only in remote places and in small groups (Buddenhagen, *et al.,* 2004).

The species *C. pubescens* (quinine tree, red quina, cascarilla, quina) is an evergreen tree, 10-25 m high, with a diameter at breast height (DBH) of 20-80 cm in Ecuador (Jugar, 2015). It has the largest geographical distribution area within its genus in the Americas and is found from northern Bolivia to Costa Rica. It is introduced in Tahiti and Hawaii, Asia and in Tanzania, Africa. This species grows at altitudes between 300 and 3300 m (Jäger, 2015), very robust so it was used as a grafting pattern. The total alkaloid content is 3.8 %, of which less than 50 % is quinine. This species is considered rare and endangered in its native range in Ecuador (Günter *et al.*, 2004), while (Jäger and Kowarik, 2010) and (Jäger, 2015), it is considered invasive in the insular conditions of Galapagos.

In the zone of Intag, Imbabura Ecuador, the species *C. pubescens* is located in relict forests, secondary forests and forming part of silvopastoral systems, at altitudes higher than 1 600 and up to 3 000 m above sea level with a scarce abundance since, according to the inhabitants of the place, its trees are cut down for timber, coinciding with (Gómez, 2016), which poses a similar situation for the District of Kañaris, Lambayeque Region, in Peru.

In the study of forest germplasm supply chains in Ecuador (Prado, Samaniego and Ugarte, 2010), it is stated that there is little availability of quality forest seed in quantity, timely supply to meet producer demand. They also state that there is limited technical and scientific information on the techniques for producing, processing and storing forest seeds of many native species to ensure their viability.

The techniques for the collection, processing and storage of seeds, is a basic condition for designing programs of reforestation, ecological restoration and agroforestry development. Ceballos and López (2007) state that there are few studies on native species, such as the case of the C. pubescens species, on phenological calendars and on the collection and processing (cleaning, drying, humidity content and storage) of forest seeds in Colombia, which is the case in Ecuador. Given the adaptability of this species to the conditions of Intag and other areas of the country, it can be used for various purposes within the country's forestry programs.

Stored seeds are a primary means of production in a country's plant production programs; however, seeds cannot retain their germination capacity indefinitely. The maintenance of their viability depends very much on the storage conditions (Doria, 2010).

In general, the heterogeneity of forest seeds does not allow the same storage technique to be approved, since many show good storage behaviour, while others, on the contrary, deteriorate rapidly under the same conditions. The management of the relative humidity of the air and the temperature of the environment are two key factors to achieve the best results in the storage of the seeds, since they directly influence the speed of breathing of the same ones (Blanco, Durañona and Acosta, 2016).

The cascararilla had a use in the past for its contribution with the quinine alkaloid to act on malaria. The most used species was *C. officinalis*, while the first species used was *C. pubescens*. On this basis of knowledge, chemical was synthesized. The virus that causes malaria has mutated and therefore new synthetic products are required, but it is also necessary to know the chemical compounds of other species of *Cinchona*, so the study of *C. pubescens*. It is necessary then, to know different aspects of its physiology, among others: time of fructification, viability of the seeds and the conditions of storage that allow to have a successful germination of the same ones.

The objective was to determine the quality of the seeds and the best method(s) to store the seeds of *C. pubescens*, collected in the area of Intag, Northwest of the Ecuadorian Andes.

MATERIALS AND METHODS

The collection of fruits was carried out in the town of Pucará Alto, Intag, Imbabura, Ecuador, where 30 individuals were selected at random as candidate trees in a silvopastoral system, which were marked and georeferenced. The phenology of the species was carried out through direct observation in the field and the most adequate moment for the collection of the fruits was determined, which was carried out in the month of September, taking into account the relationship of the color of these with their state of ripeness, which corresponded to the color from maroon to brown.

The drying was done in ambient conditions under shade, during three days, for which they were placed on a cloth and constantly removed to obtain a homogeneous drying. The process of extracting the seeds was done manually from fruits selected according to the best phytosanitary condition and of greater length and width. The extracted seeds were dried, by a process similar to the fruits in environmental conditions on a laboratory table. The seeds were partially cleaned, by means of a manual procedure, eliminating the remains of larger fruits.

The ISTA Standards (2016) were used to determine the quality of the seeds in terms of purity, weight of 1000 seeds, humidity content and germination power.

Three factors were studied: type of containers, storage medium and time. The levels by factors were:

- Types of containers: FT translucent cover; FC dark plastic cover; CT translucent glass; CC amber glass.
- Storage medium: N natural to the environment and R refrigerated 6-8ºC.
- Time. First test: T1 one month; T2 two months; T3 three months; T4 four months; T5 five months and T6 six months.
- Second trial T1 for one week; T2 for two weeks; T3 for three weeks and T4 for four weeks.

There were 48 treatments evaluated for the first trial and 32 treatments for the second trial, using an unrestricted randomized design, under laboratory conditions.

The observations on germination were made during 40 days, at 10:00 a.m.

Germination power (Equation 1).

$$PG = (\Sigma Sg * Ts) * 100 \quad (1)$$

Where:

PG: Germination power;
Sg: Sprouted seeds;
Ts: Total number of seeds sown

Germinative vigor (Equation 2).

$$VG = VM * GDM \qquad (2)$$

Where:

VM: maximum or top value that is presented between the values product of the division of the accumulated percentage of germination and the number of days that it took to obtain it.
GDM: is the average daily germination, calculated as the ratio between the final percentage of germination (PG) and the number of days it took to reach that value.

The assumptions for carrying out the analysis of trifactor variance were verified, which were not fulfilled, and a non-parametric Kruskal-Wallis test was carried out.

RESULTS

The collected seed showed a purity of 75.8 %, which can be given by the type of fruit that is a dehiscent capsule, reducing impurities from the process of drying the fruit and at the time of extraction. It was obtained a weight of 0.315 g for 1 000 seeds of *C. pubescens*, while the percentage of humidity content of the seeds was 13.6 %. The germinative power was very low, 12 %, the beginning of germination at 29 days and culminated at 36 days, while the value of 0.43 for germinative vigor was negligible.

Storage tests

In the first germination test on *C. pubescens* seeds, after one month of storage, zero percentage of germination was obtained, for the environments and types of containers used.

For the second test with weekly evaluation periods it is obtained that, the assumptions of homogeneity and normality for the results of the germination power are not fulfilled (Figure 1 and Figure 2), which shows a fan shape in the distribution of the residuals of germination.

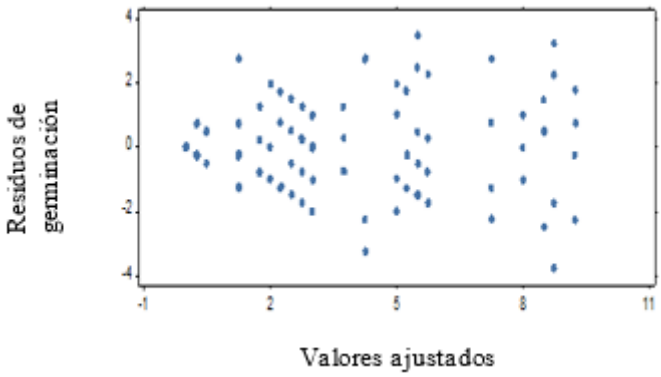

Figure 1. - Diagram of homogeneity of the germination power of *C. pubescens* seeds

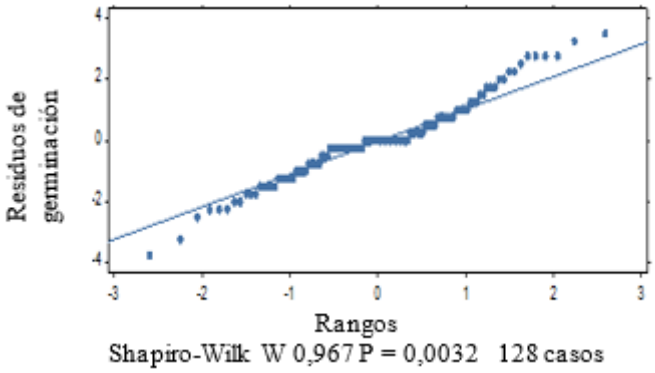

Figure 2. - Normal probability diagram for *C. pubescens* seed germination

The normality graph of the germination power data (Figure 2), plus the results of the Shapiro-Wilk test at $P < 0.05$, indicate the non-fulfillment of the normality assumption. The Kruskal-Wallis test (Table 1) shows significant differences between treatments ($P = 0.002$).

Table 1. - Germination power of treatments

Tratamientos	Medias	Tratamientos	Medias
FTRT1	9,25 a	FTNT4	0,00 j
FTNT1	8,75 ab	CCRT4	0,00 j
CTRT1	8,50 abc	CTNT4	0,00 j
CCNT1	8,00 abc	FCNT4	0,00 j
CTNT1	7,25 abcd	FCRT4	0,00 j

Types of containers: CT- translucent glass; CC- amber glass; FT- translucent plastic cover; FC- black plastic cover; R- refrigeration; N- ambient; T1- one week and T4- four weeks
Different letters in the rows and columns indicate significant differences for $P < 0.05$

The treatment with transparent sleeves in refrigeration for one week, with 9.25 % germination, was the best treatment (Table 1), while those with the worst behavior correspond to the treatments of week four, regardless of the medium and the type of container, since they show zero percentages of germination.

By eliminating the treatments of week four of storage, the assumptions of homogeneity and normality for the results of the germination power are not fulfilled (Figure 3 and Figure 4), which shows a fan shape in the distribution of the residuals of germination.

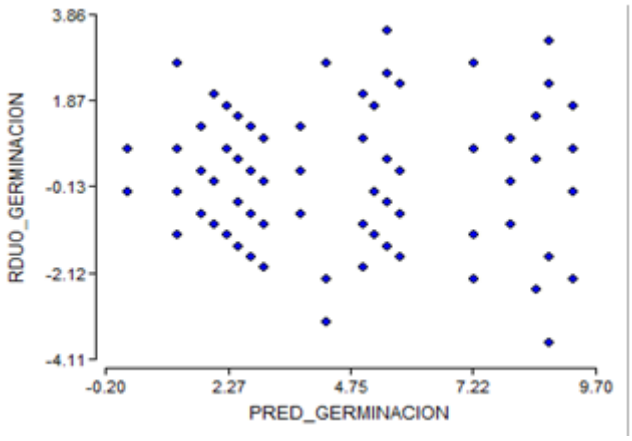

Figure 3. - Diagram of germination power homogeneity of *C. pubescens* seeds for three weeks of storage

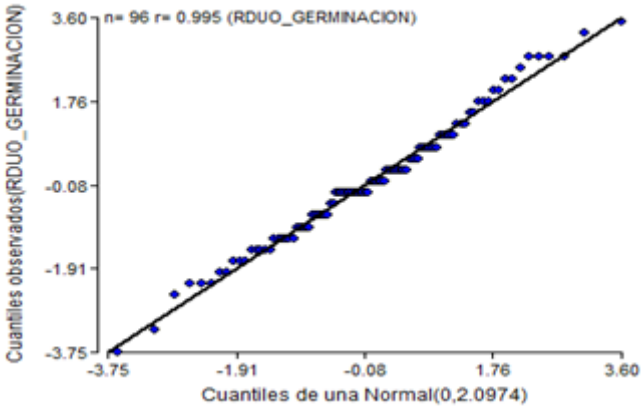

Figure 4. - Normal probability diagram of the germination power of *C. pubescens* seeds for three weeks of storage

The normality graph of the germination power data at three weeks (Figure 4), plus the results of the Shapiro- Wilk test at $P < 0.05$, indicate that the normality assumption was not fulfilled.

The Kruskal-Wallis test shows significant statistical differences between the treatments for the three weeks of storage with respect to the variable germination power. The mean comparison test (Table 2) shows differences between treatments. The treatment of transparent sheath in refrigeration is ratified at week one within the best group, to which the treatments of transparent sheath in natural medium and translucent glass in refrigeration are also incorporated, both also for the first week.

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Table 2. - Power and germinative vigor of treatments for three weeks' storage

Treatments	Germinative power germinativo (%)	Germinative vigor germinativo	Treatments	Germinative power germinativo (%)	Vigor germinativo	Treatments	Poder germinativo (%)	Germinative vigor germinativo
FTRT1	9,25 a	0,35 a	FTNT2	0,03 de	0,03 de	FTRT3	2,25 cdef	0,01 e
FTNT1	8,75 a	0,27 ab	FCRT1	0,08 cde	0,08 cde	CCRT2	2,25 cdefg	0,01 e
CTRT1	8,50 a	0,25 ab	CTNT2	0,04 de	0,04 de	FCRT3	2,00 defg	0,01 e
CCNT1	8,00 ab	0,15 bcde	CCNT2	0,04 de	0,01 e	FTNT3	1,75 defg	0,001 e
CTNT1	7,25 abc	0,21 abcd	CTRT3	3,00 bcdef	0,01 e	CCRT3	1,25 efg	0,001 e
CTRT2	5,75 abcd	0,04 de	FCRT2	3,00 bcdef	0,01 e	CTNT3	1,25 efg	0,01 e
FTRT2	5,50 abcd	0,03 de	FCNT1	0,02 e	0,02 e	FCNT2	1,25 fg	0,001 e
CCRT1	5,50 abcd	0,13 bcde	CCNT3	2,75 cdef	0.01 e	FCNT3	0,025 g	0,001 e

Types of Legend: Containers: CT- translucent glass; CC- amber glass; FT- translucent plastic cover; FC- black plastic cover; R- refrigeration; N- ambient; T1- one week; T2- two weeks; T4- three weeks; T4- four weeks
Kruskal Wallis at P <0.01.
Different letters in the rows and columns indicate significant differences for P < 0.05

The results of the normality and homogeneity tests for germinative vigour showed a similar tendency to germination power. Both tests are not fulfilled (Figure 5 and Figure 6), where it is observed with typical cone for homogeneity and normality ratifies its non fulfilment according to the Shapiro-Wilk test at P < 0.01.

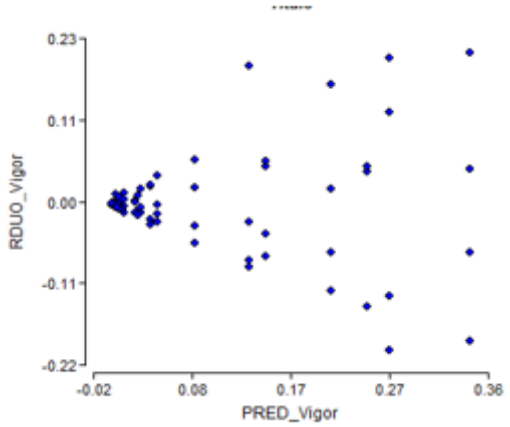

Figure 5. - Diagram of germinative vigor homogeneity of *C. pubescens* seeds for three weeks of storage

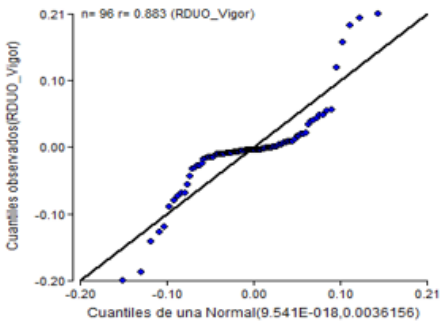

Figure 6. - Normal probability diagram of germinative vigor of *C. pubescens* seeds for three weeks of storage

The results of the comparison of means for germinative vigor (Table 2) show that there is no biunivocal relationship between the order of treatments with respect to germinative power. The best treatment is the refrigerated translucent sheath treatment after one week, but the group in which the treatments are located changes with respect to the grouping of the averages for germination power.

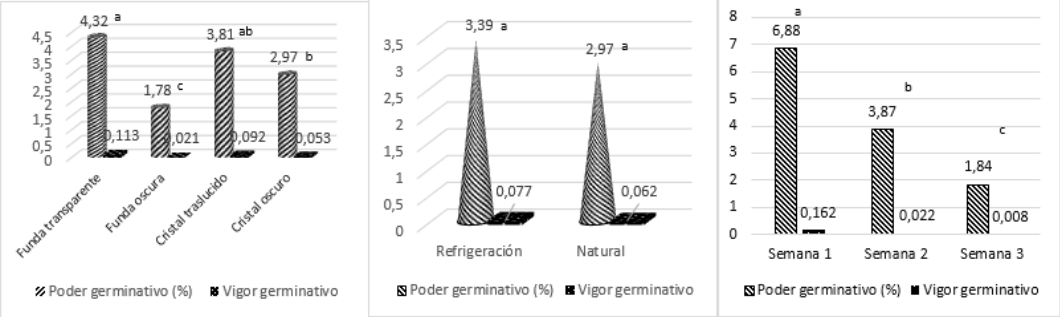

Figure 7. - Power behavior and germinative vigor of the factors under study
Different letters indicate significant differences for P < 0.05

When comparing the averages of the study factors for germination power and vigor, Figure 7, it is observed that the translucent containers are superior to the dark colored ones where the transparent cover stands out, while there are no significant differences between the averages and week one reaches a higher average than the rest, being week three the one with the worst behavior.

DISCUSSION

In the experience, 77.1 % purity was obtained in *C. officinalis*, while (Caraguay *et al*., 2016), it reached 38.04%, in the same species. This result is given by the benefit process carried out from the selection of the fruits of greater size and better sanitary state, plus the handling of the seeds from the extraction of the fruits and its initial cleaning, all of which allowed to have a seed with less impurities.

The weight of 1,000 seeds obtained, is greater than that posed (Campos *et al.,* 2014) of 0.024 g. We agree with these authors that this behavior could be one of the disadvantages of germination, because when the seeds are smaller they have minimum reserves (Pascualides and Ateca, 2013; Alvarado *et al.,* 2015; Rodríguez and Pompa, 2016; Ruíz, *et al.,* 2018), which can make use of it quickly after the harvest, so it contributes little to the growth of the new plant. This constitutes a risk for the non-occurrence of germination, since this so complex process can be initiated from the phase of imbibition, without necessarily occurring the activation of the synthesis and degradation, leading to the division and cellular elongation, so that the rupture of the seminal cover by the embryo occurs.

The result of humidity in the seeds, 13.6 %, is lower than that found by (Campos *et al*., 2014), with a value of 16.67% in the same species. The initial humidity content can influence the period of imbibition of the seed (Hernández *et al*., 2018), which implies a greater speed in the absorption of water for seeds with lower humidity content. This greater speed of absorption at the beginning of the imbibition does not imply a superior behavior in the germination of the seeds (Vargas *et al*., 2015), that when studying two contents of humidity of the seed in four species (*S. saman*, 11.5 and 6.1 %; *P. dulce,* 13.8 and 5.5 %; *J. caucana*, 8.4 and 3.5 %; *T. rosea*, 8.3 and 3.6 %) does not obtain significant differences for the germination between the levels of initial humidity of the seeds for each species. On the other hand (Lines *et al*.,

2006), they evaluated five moisture contents (4.8 %, 10.5 %, 21.3 %, 26.3 % and 40.3 %) and found that the seed germinates in less time and greater percentage with the highest humidity content and that the decrease in the germination percentage is proportional to the decrease in the humidity content.

A greater time of drying of the seeds in reason of obtaining a smaller content of humidity in these, can affect its germinative capacity since, the absorption of humidity by the seeds to reach its hygroscopic balance during the process of imbibition, causes the deterioration of the plasmatic membrane of the seeds and diminishes its physiological quality (Crivelari, *et al*., 2019).

The behavior of the germinative power, 12 % was very low, which can be given by different causes, such as: undeveloped embryos, dead embryos, very small seeds and low germinative reserve, among others. The seeds of *C. pubescens* present difficulties in their germination, (Campos *et al*.,2014), since it is directly influenced by the physiological maturation of the seeds. A cause to be considered would also be the level of humidity of the seeds once the drying process is done, since a low percentage of humidity implies that the seed has a negative matric potential, so it tends to soak very quickly (phase I), regardless of whether the seed is dormant or viable (Matilla, 2008).

Small seeds with low reserves and low humidity, make a rapid absorption of water, which causes temporary alterations in the differential permeability of the membranes of the seed and, therefore, a loss to the surrounding environment of solutes and different metabolites of low molecular weight (sugars, organic acids, ions, amino acids, peptides, etc.), corroborated in the results of (Ribeiro *et al.,* 2015). The results make it possible to assume as low the metabolic activation of the seeds, which prevents them from developing phase II (plateau), a period of delayed water absorption, without reaching the activation of the embryo. Dead and dormant seeds maintain this level of typical hydration of phase II, but unlike seeds germinating they do not enter phase III, which is associated with the protrusion of the radicle. Everything agrees with what was concluded by (Marler, 2019), since the speed of imbibition during the physical phase does not necessarily correlate directly with the speed of final germination.

In imbibition, seeds are transformed from an inactive dry state (without translation) to a fully active metabolic state, and selectively translate subsets of these stored RNAm. Therefore, the seeds provide a unique on/off switch (Sajeev *et al.*, 2019), regulated by the development for translation. The results of the very low germination power of *C. pubescens* seeds in the study suggest that their activation could be reduced in seeds that failed to germinate, even when they developed phase I imbibition.

In relation to the maximum time of germination, 36 days, do not coincide with that obtained by (Bargali and Singh, 2007) which was 22 days, nor with Campos *et al*., (2014), *Cinchona sp*. from the town of La Cascarilla-Jaén, with original soil substrates, which fluctuated between 12 and 24 days, independently of the origin of the different places of the mentioned town.

The germinative vigour was negligible, 0.43, in accordance with the low germination power and the duration of germination. Those species that distribute the energy they devote to fructification in producing a large number of small seeds, as is the case of *C. pubences*, this capacity to be widely distributed because it has greater opportunities for the seeds to find a favorable place to grow (Rosseto *et al*., 2000). Its small size contributes little to the growth of the new plant, which has a high

probability of dying because it depends quickly on the resources available in the environment. In addition, these plants are sensitive to damage from biotic and abiotic agents, and their survival is minimal, which is compensated by the large number of seeds that individuals of this species produce.

Storage tests

The non-germination of the seeds after one month of storage coincides with what was stated by Acosta (1945), referring to the fact that when the seeds are fresh they germinate between a period of 11 and 20 days and if they are old, depending on the storage period, the germination percentage decreases, therefore the seeds of this species when they are subjected to storage for more than one month, lose their viability.

The results show that the time factor influences the germination since, the seeds of *C. pubescens* when being submitted to each one of the treatments of conservation in times from 1 to 4 weeks, the percentage of germination diminishes as the time of conservation is increased until arriving at a value of zero to the fourth week, independently of the means of storage and the type of used package.

The treatments under refrigeration conditions for the different types of containers in the first week of storage showed a better trend in the germination behavior. Ortiz *et al*., (2004) highlight the advantage of air-conditioned chambers with domestic air conditioning, confirming the favorable effect of low temperatures in seed storage. On the other hand, Ruíz *et al.,* (2017), Valverde *et al*., (2019), ratify that the refrigerated treatments showed significance with respect to the non-refrigerated treatments.

When studying the storage of *Dypsis lutescens* seeds, Doria *et al*., (2012), found that the best container was black polyethylene followed by translucent plastic bottles, while the best treatments were black polyethylene in refrigeration and environmental conditions. These results differ from those obtained in this research because the translucent polyethylene sleeves were the best behaved, but they coincide as they are the best containers in both storage conditions.

In relation to the light requirement, most tree species behave as indifferent (Flores *et al.,* 2017), germinating under both light and dark conditions. Based on the fact that, ecologically, the perception of light by the seed can act as an indicator of the amount of light available to the seedling, it can indicate planting depth, canopy shading and soil disturbance. This behavior is supported by the fact that the presence or absence of light indicates to the seeds if they are close to the surface or buried, on the other hand the red/distant red ratio (R/RL) is an indicator of the presence and size of forest clearings, that is to say, of the density of the canopy according to what Escobar and Cardoso have exposed, (2015). In the experience, four of the five treatments with the best behavior have the characteristic of a translucent type of container, which allows us to assume that the seeds of *C. pubencens* could better manifest their germination, with certain levels of presence of light during storage, either artificial or natural in the soil seed bank.

By using airtight storage, containers that prevent air and humidity from entering the product. In these conditions, the breathing of the seed and the insects (when there are any) deplete the existing oxygen, causing the death of the latter and the reduction of the activity of the seed, so the storage can last a long time without deterioration.

When opening the containers to extract the seeds at the time of the germination assemblies, a gaseous exchange with the local environment is carried out. Once the container is closed, an exchange of humidity between the seeds and the environment inside the container is restored, which generally causes an increase in the humidity content of the seeds. This aspect is more relevant for storage in environmental conditions, which can accelerate the deterioration of the seeds, unlike the cold environment since the humidity in the microenvironment of the container is lower, since the relative humidity in the refrigerator is also lower (Ruíz *et al.*, 2017).

The germinative vigour of the treatments shows a similar tendency to that of the germinative power, but it is distinguished that there are some increases for some cases, which is given by the reduction of the number of days in manifesting the maximum accumulated germinative power. In experience, values of less than one to zero are obtained, while Campos *et al. (*2014) achieved values from zero to 13.5 of germinative vigor.

CONCLUSIONS

The quality of the seeds of *C. pubescens* is low in relation to the percentage of germination, of low weight and average physical purity.

The germination of the seeds of *C. pubescens* does not occur after a month of storage, with a significant reduction from the first week, in both transparent covers and packaging is the best option, without any difference between the means of refrigeration and natural environment.

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