Physiological response of *Cratylia argentea* (Desvaux) O. Kuntze seeds to storage and cryo-preservation conditions

Respuesta fisiológica de semillas de C*ratylia argentea* (Desvaux) O. Kuntze a condiciones de almacenamiento y crioconservación

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Abstract

The impact of cryopreservation conditions on seed germination of *Cratylia argentea* seeds (cv. Veranera) were evaluated in the cryopreservation facilities of the National University of Colombia, the Colombian Humboldt Institute, and the International Center for Tropical Agriculture (CIAT). A completely randomized design, with four repetitions, evaluated the effect of three levels of humidity (6%, 8% and 10%), the ultra-rapid freezing method using immersion in liquid nitrogen (LN), and two storage conditions. The results showed high initial physiological quality of seeds and a lack of any type of physical or physiological dormancy, although germination was drastically reduced under non-controlled storage conditions. Germination was significantly reduced when moisture content was reduced to 6%, indicating possible recalcitrant behavior of these *Cratylia* seeds, with seeds losing viability on dehydration. For seeds cryo-preserved by immersion in LN for one month, the adequate moisture content was 8%, although no differences were detected in germination when the seeds at all three levels of humidity were cryo-preserved in LN for one hour.

Key words: C*ratylia argentea***,** legume, seeds, physiological quality, cryopreservation, germination.

Resumen

En la Universidad Nacional de Colombia y en los cuartos de crioconservación del Instituto Humboldt del Centro Internacional de Agricultura Tropical (CIAT) (Palmira, Colombia) en un diseño completamente al azar, con cuatro repeticiones se evaluaron los efectos de tres contenidos de humedad (10%, 8% y 6 %), el congelamiento ultrarápido a través de inmersión en nitrógeno líquido (NL) y dos condiciones de almacenamiento en la germinación de las semillas de C*ratylia argentea* (Desvaux) O. Kuntze cultivar Veranera. Los resultados mostraron la alta calidad fisiológica inicial de las semillas de esta leguminosa forrajera y la ausencia de latencia física o fisiológica, aunque la germinación se reduce drásticamente en condiciones no controladas de almacenamiento. La germinación disminuyó significativamente cuando el contenido de humedad se redujo a 6%, indicando el posible comportamiento recalcitrante, o sea semillas que pierden su viabilidad por deshidratación producida por el medio donde se

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encuentren, sea éste de almacenamiento o natural (Vieira et al., 1994). Para la crioconservación por inmersión en nitrógeno líquido durante 1 mes, el contenido adecuado de humedad fue de 8%, aunque no se detectaron diferencias en la germinación cuando las semillas en los tres niveles de humedad se crioconservaron durante 1 hora en nitrógeno líquido.

Palabras clave: C*ratylia argentea,* leguminosae, leguminosas forrajeras, semillas, almacenamiento de semillas, calidad fisiológica, crioconservación, germinación

Introduction

Cratylia argentea (Desv.) Kuntze is a forage leguminous shrub native to Amazonia and the central part of Brazil, Peru and Bolivia incorporated into programs of forage evaluation in tropical latin America. This species is a shrub reaching 1.5 to 3 m of height. It adapts well to different types of well-drained soils. The species grows at altitudes from sea level to 1200 m.a.s.l., with rainfall between 1000 and 4000 mm. An important characteristic of this species is its foliage retention and its capacity to reshoot during the dry season, due to the development of vigorous roots that reach between 1.3 and 1.80 m, and favor drought tolerance, even in poor and acid soils (Peters et al., 2003; Pizarro et al., 1995).

Although seed production is high, of good quality and without any marked physical (hardness) or physiological latency, preservation problems have been detected in that, under certain storage conditions, drastic losses in physiological quality occur (viability, vigor and germination) (Lascano, 1998).

A number of studies (Hong & Ellis, 1992b; Ellis et al., 1995; Vertucci et al., 1994, Cromarty et al., 1982; Delouche, 1971) have demonstrated that the seeds are orthodox – i.e. that they do not suffer reduced viability under dehydration, and they tend to maintain high quality over long periods (longevity, viability, and germination) if stored at low temperature and with low relative humidity. In contrast, recalcitrant seeds are those that lose viability with the dehydration resulting from the environment in which they are located, either under storage or natural conditions, and rapidly lose their capacity for germination when exposed to low humidity conditions (Kermode & Finch-Savage, 2002). Although drying techniques are sufficiently well known, some studies have been carried out using silica gel for germplasm preservation. Zhan and Tao (1989) were able to treat seeds using low ratios of silica gel: seed, without affecting their viability. Arce et al. (2007) found positive results with cryo-preserved seeds of *Sapindus saponaria*. When drying is rapid or brusque the viability of the seeds is compromised, particularly when these have not reached the levels of humidity or maturity necessary to bear rapid reductions in humidity.

One of the most important aspects in the preservation of seeds of forage species is storage, and its duration depends on the humidity content, degree of latency, scarification treatments, bottling and commercialization, amongst others (Ferguson, 1994). Ultra-rapid freezing through immersion in liquid nitrogen is one of the available methods for long term conservation of genetic resources, particularly of species that have recalcitrant seeds.

Cryo-preservation technology allows the conservation of cells, tissues and vegetal organs for an indefinite period, without loss of viability. The vegetative material is conserved at very low temperatures (between -150 ºC and -196 ºC) with LN, or its vapor, used as the refrigerant. At these temperatures, the cellular metabolism is completely stopped, impeding the processes that could cause irreparable damage to the material. This technique has been evaluated in more than 70 species and in many cases, although the resistance of cellular suspensions, calluses, protoplasts, meristems and embryos to LN freezing has been proved, it does not necessarily signify that the technique may be used successfully for the general storage of plant species (Engelmann, 2000; Villa et al., 2007). Cardoso et al. (2000) studied the effect of cryo-preservation on the germination of seeds from ten species of forage legumes, and found no negative effects on viability. This shows that this technique is an adequate method for the preservation of seeds from these species.

The present study was carried out with the aim to evaluate the physiological response of seeds of *Cratylia argentea* (Desv.) O. Kuntze cultivar Veranera to cryo-preservation and to various storage conditions.

Materials and methods

Localization

The study was carried out in the Plant Physiology Laboratory of the National University of Colombia, Palmira campus, and in the cryo-preservation facilities of the Colombian Humboldt Institute and the International Center for Tropical Agriculture (CIAT), located in the municipality of Palmira (3º 31' N, 76° 81' W, at 980 m.a.s.l., 1100 mm precipitation, relative humidity of 78% , and average annual temperature of 24.5 °C), Valle del Cauca, Colombia. The seed was obtained from harvested lots in April of 2004, in CIAT, by the Program for Improved Multiple Purpose Forages for the Developing World.

The seeds were separated by size in large, medium and small fractions, using a General blower. Humidity content was determined following the recommendations of ISTA (2005), and was calculated using the following formula:

$$H(\%) = \frac{(M_2 - M_3)}{(M_2 - M_1)} * 100$$

where,

M_1 = weight of the pot with lid (g), M_2 = weight of the pot with lid + seed content (5 ± 0.2 g) before drying in an electric oven at 130 ˚C for 8 h, M_3 = weight of pot with lid + content after drying (g). Measurements were taken with three repeats.

Seed drying

The seeds were dried using a ratio silica gel : seed of 2:1. For the construction of the drying curve, the humidity levels of 10%, 8% and 6% were established, and the formula of Hong and Ellis (1996) used to determine humidity content:

$$Pi(100 - Hi) = Pf(100 - Hf)$$

from which the final humidity is obtained:

$$Hf = 100 - \frac{Pi(100 - Hi)}{Pf}$$

where, Hi = Initial humidity content (%) of the seed, determined with the gravimetric method; Hf = humidity content determined (%); Pi = initial weight and Pf = final weight of the seeds. The measurements were performed every 30 minutes for 6 hours, avoiding, as much as possible, humidity gain by the seeds from the environment. The drying curves were constructed based on the weight lost when placed in the silica gel. The samples of seeds of *C. argentea,* with humidity content of 10%, 8% y 6% were vacuum packed in hermetically-sealed aluminum bags, and submitted directly to LN without cryo-protectors. The effect on germination of two periods of LN cryo-preservation was evaluated: 1 month and 1 hour. Additional seed samples were stored at room temperature in the Palmira conditions (relative humidity between 50% and 70%, and average temperature of 24 ºC) and in refrigerated conditions at 10 ºC in the Plant Physiology Laboratory of the National University of Colombia, Palmira campus.

Germination tests.

The cryo-preserved seeds were defrosted at ambient temperature for 12 h, eliminating those that showed signs of damage. The germination tests were performed with four repetitions, with 25 seeds each, under ambient conditions n the Plant Physiology Laboratory. Sand was used as the germination substrate. The sand was passed through a sieve to eliminate foreign elements, washed

with water, disinfected with sodium hypochlorite at 5% for 12 h, dried and sterilized in the oven at 105 °C for 6 h. Depending on the humidity of the sand in the seed trays, watering was performed using distilled water. The evaluations included counts of normal, abnormal seedlings and dead seeds (ISTA, 2005).

Test of seed viability

This test is used to determine the germination potential and the vigor of the seeds, evaluating the degree of staining of the embryo and the cotyledons on immersion into a solution of 0.5% triphenyltetrazolium chloride. This test was performed in two repetitions of 25 seeds each. The seeds were washed with water and dried with paper towel, before determining the number of viable and unviable seeds, using the interpretation protocols for soya seeds (Delouche et al., 1971). The results were analyzed using S.A.S (Statistical Analysis System). Version 8.2 of 2002.

Results and discussion

Seed Characteristics

The unit weight of the seeds varied between 32 and 43 g/100 seeds, reflecting the effect of the variable climatic conditions during flowering and seed formation of *C. argentea*, which for this species may extend up to three months (Peters et al, 2003). The seeds began to germinate at 3 days post planting (d.p.p), although most of the germination occurred between 7 and 21 d.p.p. Initial viability and germination were high (97% and 95%, respectively) (Box 1), indicating a high physiological quality, and an absence of physical and / or physiological latency in the seeds.

Drying curves

The seeds placed in silica gel presented an accelerated loss in humidity, decreasing from 10.9% to 6.2% in 120 min. A subsequent equal duration passed for the humidity content to drop from 6.2% to 4%, and then stay approximately constant (Figure 1). The rate of water loss is related to the thickness of the seed coat (Chacón & Bustamante, 2001), and recalcitrant seeds with broken coats dry more rapidly and in a higher percentage (Mwang'Ingo et al., 2004).

Box 1. Initial characterization of seeds of *Cratylia argentea* cv. Veranera.

Measure	Unit	Value
Unit weight		
Large seeds	g/100 seeds	43
Medium seeds	g/100 seeds	35
Small seeds	g/100 seeds	32
Humidity	%	11 (10.9-11.2)
Germination	%	95
Viability in tetrazolium	%	97

Seed germination without cryo-preservation

At the three humidity levels, the seeds began germinating at 5 d.p.p. At 7 d.p.p germination was 69% in seeds with 10% humidity, and 18% and 30% in seeds with 8% and 6% humidity, respectively. At 20 d.p.p. an important reduction in germination was seen at low humidity levels, indicating a probably non-orthodox behavior in the seeds of this species. Although recalcitrant seeds normally lose their germination ability rapidly on exposure to low humidity conditions (Kermode y Finch-Savage, 2002), at the end of the study (28 d.p.p.) germinations of 95%, 86% and 76% were obtained for the humidity levels of 10%, 8% and 6%, respectively. Box 2 shows the germination results obtained at the different levels of humidity.

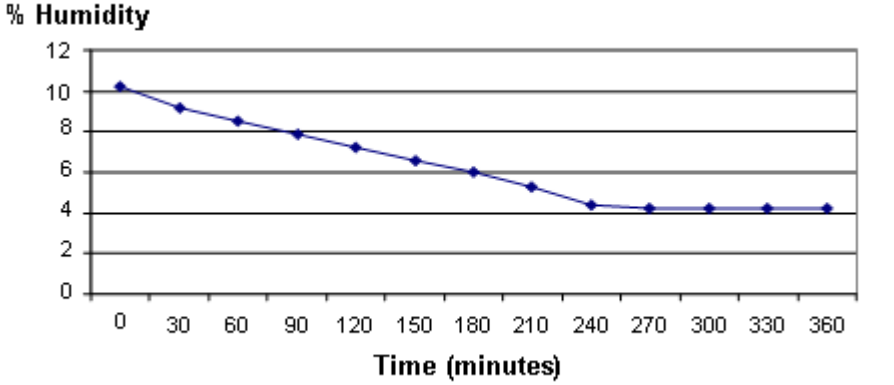

Figure 1. Humidity content with time of seeds of *Cratylia argentea* cv. Veranera.

Cryo-preservation tests

Cryo-preservation in LN for 1 month. After one month in LN, the highest percentage of germination was observed in those seeds with 8% humidity, which presented 12% germination at 5 d.p.p., and 86% at 10 d.p.p (Box 3). At the end of the test, 5% of abnormal seedlings, and 9% of dead seeds were registered. Seeds with 6% humidity presented the lowest germination (73% at 21 d.p.p.). This was the treatment that also presented the highest proportions of abnormal seedlings

(15%) and dead seeds (27%). Seeds with 10% humidity began germinating at 5 d.p.p., and reached 79% germination at 21 d.p.p.. From this proportion 71% corresponded to normal seedlings. The proportion of dead seeds was 21%. Thus, seeds cryo-preserved with 6% humidity presented the highest proportion of abnormal seedlings and seed seeds.

Box 2. Germination of seeds of *Cratylia argentea* with different humidity contents and days post planting

Day post planting	Humidity content (%)		
	10	8	6
		Germination (%)[a]	
5	9	12	5
6	15	15	10
7	69	18	30
8	73	60	50
9	76	65	58
10	76	72	69
15	86	85	72
18	90	86	76
20	95a*	86b*	76c*

a. Normal seedlings

*.Significantly different at P<0.05, according to the Dumcan test.

If one considers that the drying treatment (without cryo-preservation) obtained germination frequencies of 95%, 86%, and 76% for the humidity treatments of 10%, 8% and 6%, respectively, and it is assumed that these values represent the maximum germination potential that seeds suspended in LN can achieve, then the total relative germination (TRG), calculated as the ratio between total seedlings obtained in the cryo-preserved seed germination test and the maximum germination potential, of the cryo-preserved seeds would be 83%, 100% and 96% at the three level of humidity . The normal relative germination (NRG), calculated as the ratio between normal seedlings obtained in the cryo-preserved seed germination test and the maximum germination potential, would be 75%, 94% and 76% respectively. These results indicate that the adequate humidity content for cryo-preserved seeds of *C. argentea* is 8%.

The seeds damaged by the cryo-preservation treatment did not present statistically significant differences, and varied between 2% and 4% in the humidity levels evaluated. The optimum level of humidity for the cryo-preservation in LN of seeds of *Arachis pintoi* (Leguminosae) was 4% (Rey & Mroginski, 2009)

Box 3. Germination dynamic of seeds of *Cratylia argentea* with different humidity content after one month of storage in liquid nitrogen.

Days post planting	Humidity content (%)		
	10	8	6
		Germination (%)	
5	5	12	7
6	6	28	15
7	7	56	22
8	9	70	35
9	9	75	45
10	22	86	52
12	38	86	70
18	79b	86a	73c
21	79	86	73
Summary			
Total Germination (%)	79	86	73
Normal seedlings (%)	71	81	58
Abnormal seedlings (%)	8	5	15
Dead seeds (%)	21	9	27
Total germination without cryo-preservation	95a*	86b*	76c*
Total relative germination (TRG) with cryo-preservation	83.1	100	96
Relative normal germination (RNG) with cryo-preservation	74.7	94.1	76.3

*.Significantly different at P<0.05, according to the Dumcan test.

Cryo-preservation in LN for 1 hour. Seeds with 10% humidity presented the greatest total germination (88%), while seeds with 6% humidity had the lowest germination rate (75%). The TRG of the seeds exposed to LN for one hour was 93%, 94% and 99% respectively, for humidity contents of 10%, 8%, and 6% (P > 0.05), indicating that the exposure of seeds to LN for one hour did not affect germination in the humidity levels evaluated. In an evaluation of the effect of cryo-preservation in seeds of various legume species, results indicated the enormous potential for cryo-preservation for seed conservation in these species (Cardoso et al, 2000).

Storage. After one month of storage in cold room storage conditions (R.H.: 55% & T:18ºC), seeds with 10%, 8% and 6% humidity reduced their germination rate by 20%, 6% and 14%, respectively. Seeds stored at ambient conditions suffered drastic reductions in germination success (40%, 34% y 34%) for the humidity levels of 10%, 8% y 6 %, respectively (Box 4), indicating the sensitivity of the seeds of this species to storage conditions.

Box 4. Initial germination and germination after one month of seeds of *Cratylia argentea* under different storage and humidity conditions.

Condition	Humidity Content (%)		
	10	8	6
		Germination (%)	
Initial Germination	95	86	76
Storage (1 month):			
In the cold room (R.H.: 55%, T:18ºC)	76	81	65
Reduction	19	5	11
In ambient conditions for Palmira	55	52	42
Reduction	40	34	34

Conclusions

From the results of this study it is possible to conclude the following:

- Seeds of C*ratylia argentea* (Desvaux) O. Kuntze present a high initial physiological quality, and absence of physiological or physical latency, but their germination success is drastically reduced under uncontrolled storage conditions.
- The seeds lose water rapidly, reducing humidity content from 11% to 6%, in the first four hours of storage.
- The seeds present an important reduction in germination success at low levels of humidity (<8%) indicating a probably non-orthodox behavior in this species.
- Optimum storage conditions were found when seeds with 8% humidity content were cryo-preserved in LN for one month.

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