

Liquid Nitrogen as promotor of seeds germination and seedling growth in tropical legumes

El nitrógeno líquido como promotor de la germinación de las semillas y del crecimiento de las plántulas en las leguminosas tropicales

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Abstract

Introduction— The hard seed is the main cause of dormancy in most of the Leguminosae species. Seed scarification methods, where physical damage is sought to break hard seed coat without diminishing quality, have been modified over time to make them more effective. The most commonly used seed scarification methods include heat, mechanical scarification, and freeze-thaw. Some methods for freeze-thaw scarification include ultra-low temperature immersion in Liquid Nitrogen (LN, -196°C).

Objective— Determine the effectiveness use of Liquid Nitrogen (LN) as a scarification method to overcome dormancy in seeds of species of the *Leguminosae* family.

Methodology— The physiological quality of all freshly harvested seeds was determined and scarified by direct immersion in LN for 30 minutes. Total germination was determined under laboratory conditions, as well as the time required for the seeds to reach 50% germination (T50) and the total number of seeds that remained hard at the end of the experiment. The percentage of emerged seedlings and their vegetative growth was evaluated for 21 days after sowing.

Results— The seeds of all species evaluated showed a high physiological quality at the time of harvest. Scarification with LN improved germination, emergence and vegetative growth in the species *Desmodium scorpiorus*, *Teramnus labialis*, *Neonotonia wigthii* and *Phueraria phaseoloides*.

Conclusions— Dormancy was effectively overcome in the seeds of the species *D. scorpiorus*, *T. labialis*, *N. wigthii* and *P. phaseoloides*. It was possible to increase the percentage and speed of germination and emergence, managing to obtain plants with greater vegetative growth during the first 21 days after sowing.

Keywords— Legumes; seed dormancy; germination; liquid nitrogen

Resumen

Introducción— La semilla dura es la principal causa de latencia en la mayoría de las especies de Leguminosae. Los métodos de escarificación de la semilla, en los que se busca un daño físico para romper la cubierta dura de la semilla sin disminuir su calidad, se han modificado a lo largo del tiempo para hacerlos más efectivos. Los métodos de escarificación de semillas más utilizados son el calor, la escarificación mecánica y la congelación-descongelación. Algunos métodos de escarificación por congelación-descongelación incluyen la inmersión a muy baja temperatura en Nitrógeno Líquido (LN, -196°C).

Objetivo— Determinar la efectividad del uso de Nitrógeno Líquido (LN) como método de escarificación para superar la latencia en semillas de especies de la familia *Leguminosae*.

Metodología— Se determinó la calidad fisiológica de todas las semillas recién cosechadas y se escarificaron por inmersión directa en LN durante 30 minutos. Se determinó la germinación total en condiciones de laboratorio, así como el tiempo necesario para que las semillas alcanzaran el 50% de germinación (T50) y el número total de semillas que permanecieron duras al final del experimento. Se evaluó el porcentaje de plántulas emergidas y su crecimiento vegetativo durante 21 días después de la siembra.

Resultados— Las semillas de todas las especies evaluadas mostraron una alta calidad fisiológica en el momento de la cosecha. La escarificación con LN mejoró la germinación, emergencia y crecimiento vegetativo en las especies *Desmodium scorpiorus*, *Teramnus labialis*, *Neonotonia wigthii* y *Phueraria phaseoloides*.

Conclusiones— La dormancia fue superada efectivamente en las semillas de las especies *D. scorpiorus*, *T. labialis*, *N. wigthii* y *P. phaseoloides*. Se logró aumentar el porcentaje y la velocidad de germinación y emergencia, logrando obtener plantas con mayor crecimiento vegetativo durante los primeros 21 días después de la siembra.

Palabras clave— Leguminosas; latencia de semillas; germinación; nitrógeno líquido

I. INTRODUCTION

The legume family (*Leguminosae*) is one of the most numerous within the flowering plants and has been one of the most economically important for humans for 3000 years [1]. This taxa, is made up of 700 genera and approximately 20000 species [2]. It is found throughout the planet, except polar regions and deserts with extreme temperatures, and in the tropical region there is a wide biodiversity, where the genera *Neonotonia*, *Macroptilium*, *Clitoria*, *Desmodium*, *Phueraria* and *Teramnus* stand out [3].

Legumes are important in animal nutrition, both for the nutritional value of fruits and seeds, and for the rest of the plant used as forage or grazing. In this sense, the biomass of trees, shrubs and especially creeping legumes have played a leading role due to their considerable protein content and acceptable nutritional value [4]. In addition, legumes have a generalized characteristic that is to harbor atmospheric nitrogen-fixing bacteria in their roots to provide it to the plant when necessary [5]. However, the use and management of the different species is affected, almost entirely, by the low percentages of germination of their seeds [6].

Hard seed (that is, the presence of a hard seed coat that blocks the germination process by not allowing water to pass to embryo) is the main cause of dormancy in most species of *Leguminosae* [7]. There is considerable scientific and technical literature on scarification methods to overcome dormancy in the seeds of legume species [1]. Seed scarification methods, where physical damage is sought to break the hard seed coat without diminishing quality, have been modified over time to make them more effective.

The most commonly used seed scarification methods include heat, mechanical scarification, and freeze-thaw [1]. Freeze-thaw scarification methods allow breaking the seed coat by exposing them to alternating low and high temperatures. Some methods for freeze-thaw scarification include ultra-low temperature by immersion in Liquid Nitrogen (LN, -196°C) [8].

II. THEORETICAL FOUNDATION

LN is mainly used for long-term safe storage in seeds of species of agricultural and forestry interest [9]. However, since 1930 the first works began where LN was used to overcome dormancy in seeds [10]. Some researchers [11] compiled the results obtained in seeds of *Melilotus alba*, *Lotus corniculata*, *Trifolium repens*, *Medicago sativa*, *Trifolium hybridum*, and *Coronilla varia*, when the exposure time, as well as the number of occasions that the seeds are introduced into the LN were the elements that were the object of experimentation.

Rapid temperature changes cause tensions between the different tissues that make up the seeds, which generate expansion and contraction that cause the appearance of cracks in the seed coat [10]. Other studies [12] found cracks in the coat and lens of *T. arvense* seeds after immersion in LN. These cracks, in the seeds of other species, are associated with the entry of water and with this the beginning of germination [13].

For their part, publications from University Cambridge (USA) [14] considered that the size of the seed has a marked influence on the number of cracks and the place that these appear in the coat, while Institute of Biology and Soil Science (Russia) [15] demonstrated that small seeds present a greater germination after immersion in LN compared to larger seeds. These authors remarked that the species and the hardness of the seed are elements that determine whether or not germination is promoted after immersion in LN.

Studies carried out to date have shown that the use of LN as a seed scarification method can be effective for many species but not for others [11]. For this reason, this research aims to determine the effectiveness use of LN as a scarification method to overcome dormancy in seeds of species of the *Leguminosae* family.

III. MATERIALS AND METHODS

A. Species and seeds material

The species under study in this research (*Desmodium scorpiurus* (Sw.) Desv., *Macroptilium atropurpureum* (DC.) Urb., *Teramnus labialis* (Lf) Spreng., *Neonotonia wigthii* (cv.) Copper, *Clitoria ternatea*, *Phueraria phaseoloides*) were found in the germplasm bank belong-

ing to the Bioplant Center of the University of Ciego de Ávila Máximo Gómez Báez, located at (21°53'05.63" N, 78°41'30.35" W), in Cuba. The seeds were manually separated from the ripe fruits harvested in 2018, and were placed in an amber glass container in a conventional refrigerator at 4°C until use.

B. *Seeds moisture content (% , H₂O mass: fresh mass)*

To completely remove the internal moisture from the seeds, the oven-drying method was used, together with dielectric water content sensors using the LCR (HP.2358) equipment [16]. Three samples of 50 seeds were taken for each species, placed in a porcelain container and weighed on an analytical balance (SARTORIUS, BL 1500). The containers were placed in an oven (HS62A) at a temperature of 130°C for up to constant mass. Subsequently, the containers were placed in a desiccator with silica gel and allowed to cool for 45 min. Next, the samples were weighed again and the internal moisture content of the seeds was calculated based on the fresh mass, using the following formula (1):

$$\text{SMC}(\% \text{ fm}) = \frac{\text{Fresh mass} - \text{Dry mass}}{\text{Fresh mass}} * 100 \quad (1)$$

Where:

SMC: Seeds Moisture Content.

Fresh mass: Mass of the seeds before placing in the oven.

Dry mass: Mass of the seeds after drying in the oven.

C. *Seed viability*

Three samples of 50 seeds were taken for each species to determine viability by means of the topographic tetrazolium test [17]. In the coat of all the seeds a small cut with a scalpel was made in the region opposite the thread to facilitate imbibition. Each sample was placed in a Petri dish with felt paper previously moistened with 10 mL of distilled water for 24 h. After that, the coat of each seed was removed to expose the embryo. The embryos were placed back in the Petri dishes, but this time 10 mL of a 1% solution of 2, 3, 5 Triphenyl-2H-Tetrazolium Chloride (TTC) was added. After 6h in the dark, the embryos were classified according to their coloration as: 1) Viable, when they were totally stained deep red, pale red or with discolored sections; and 2) Non-viable, when they remained with their original color; and the result was expressed as a percentage of live embryos out of the total evaluated [18].

D. *Scarification by immersion in LN*

Two hundred seeds of each species were taken and placed in cryo-vials that were placed directly in a tank with LN (-196°C) for 30 minutes [19]. The cryo-vials were recovered and placed in a tray in the open air until the seeds reached equilibrium with room temperature [20].

E. *Germination*

Five samples were taken from 20 seeds previously scarified with LN and 5 samples from 20 seeds without scarification (control). Each seed sample was placed in a Petri dish with felt paper previously moistened with 5 mL of distilled water. The Petri dishes were placed in a germination chamber (Model, RTOP-D series) for 21 days at 30°C, 80% relative humidity and a photoperiod of 14 h light / 10 h darkness, under fluorescent light tubes with a flow of photosynthetic photons (FFF) of 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

Germinated seeds were counted daily taking the emergence of the radicle (≥ 2 mm) as a criterion. On the last day (day 21) the ungerminated seeds which that were apparently viable, were counted and designated hard seeds. With the total number of germinated seeds, the time required for reaching 50% of germinated seeds (T_{50}) was calculated [21].

F. Emergence and plant development

Five samples were taken from 20 seeds previously scarified with LN and 5 samples from 20 seeds without scarification (control). The seeds were placed to germinate in polyethylene trays 60 cm long and 30 cm wide, with 20 alveoli each. The trays were filled with a substrate based on typical Fersialytic Brown-Reddish soil (50%) and humus (50%) and one seed was sown for each socket at a depth of 2.0 cm. The trays were placed in a controlled environment pre-germination chamber (Model, RTOP-D Series) for 21 days. Simulating natural conditions, the temperature 30°C/25°C and the photoperiod 14 h light/10 h darkness was alternated, under fluorescent light tubes with a Photosynthetic Photons Flux (PPF) of 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

At 21 days, the total number of plants was counted and 10 plants were taken for each species and treatment (control and LN) and the length of the stem (cm), the length of the radicle (cm) and the total of true leaves were measured per plant.

G. Statistical analysis of the results

The Statistical Package for Social Sciences utility [40] was used for the statistical processing of the data. The adjustment to the normal distribution of the data of each treatment (Kolmogorov-Smirnov) and the homogeneity of the variances (Levene) were checked. The analysis for the different variables were carried out through the t-Student test, $p \leq 0.05$). In some cases, it was necessary to transform the data to achieve the assumptions of the parametric tests carried out using the formula:

III. RESULTS AND DISCUSSION

A. Seed moisture content (% , H_2O mass: fresh mass)

The internal moisture content of the seeds was less than 10% in all the species studied with the exception of *D. scorpiorus*, which was $10.08 \pm 0.01\%$ (Table 1). The moisture content of the seed at the time of harvest depends considerably on the environmental conditions experienced during the last phase of its development [22], and it is essential that it be as low as possible (2% - 12%) to guarantee a high survival of the seed after scarification with LN [23], [24]. The moisture contents of all studied seeds correspond to a safe degree of hydration that allows survival at the low temperatures caused by the scarification with LN.

TABLE 1. PHYSIOLOGICAL SEEDS QUALITY.

Species	SMC (%)	Viability (%)
<i>D. scorpiorus</i>	10.08 ± 0.01	98.33 ± 3.88
<i>M. atropurpureum</i>	8.75 ± 0.12	94 ± 2.35
<i>T. labialis</i>	7.71 ± 0.07	95 ± 1.52
<i>N. wigthii</i>	8.28 ± 0.12	95.33 ± 1.66
<i>C. ternatea</i>	6.55 ± 0.16	91 ± 3.15
<i>P. phaseoloides</i>	9.51 ± 0.02	95.66 ± 3.22

SMC (Seeds Moisture contents).
Source: Authors.

B. Seed viability

Seed viability was greater than 90% in all species and in *D. scorpiorus*, *T. labialis*, *N. wigthii* and *P. phaseoloides* it exceeded 95% (Table 1). This result is a sample of the good physiological quality of the seeds of these species harvested in the edaphoclimatic conditions of Ciego de Ávila (Cuba). The high viability of a seed translates into its ability to complete germination and generate a new plant, as long as it does not present any kind of dormancy [11].

C. Germination

The seed germination percentage without scarifying (control), in all species, was less than 50%, only *N. wigthii* exceeded 45% with $46 \pm 3.8\%$ (Fig. 1). The lowest values were collected for *M. atropurpureum* ($24 \pm 1.54\%$), *P. phaseoloides* ($23 \pm 1.39\%$) and *D. scorpiurus* ($7 \pm 0.52\%$). The results achieved in germination show a strong dormant state in all studied species, which was previously reported by several authors.

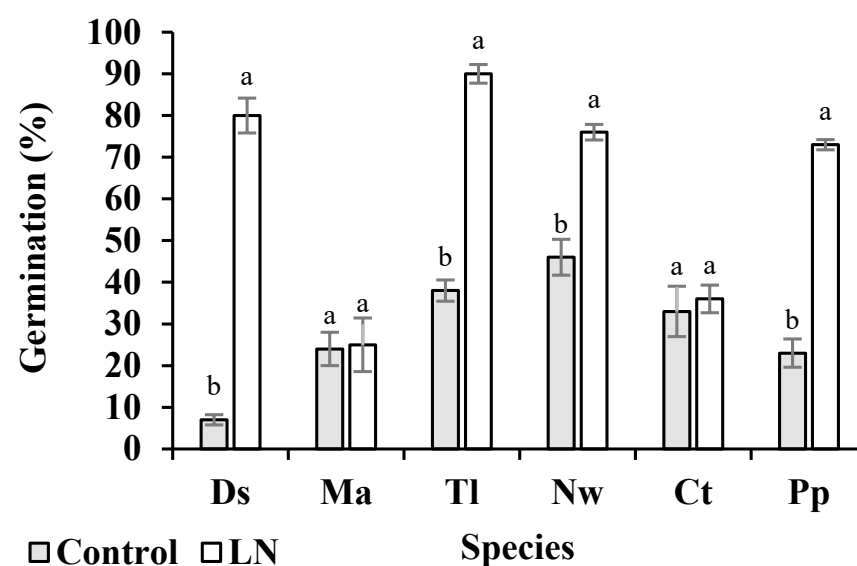


Fig. 1. Seeds germinated at 21 days. **Ds)** *Desmodium scorpiurus* (Sw.) Desv., **Ma)** *Macroptilium atropurpureum* (DC.) Urb., **Tl)** *Teramnus labialis* (L.f.) Spreng., **Nw)** *Neonotonia wigthii* (cv.) Copper, **Ct)** *Clitoria ternatea*, **Pp)** *Phueraria phaseoloides*. Seeds without scarifying (**Control**), Seeds scarifying with Liquid Nitrogen (**LN**). Results with same letter, for each species, are not statistically different; *t*-Student, $p > 0.05$, $n = 5$. Source: Authors.

Dormancy has a hereditary component and it has been shown that it can be controlled by the maternal and paternal genotypes [25]. However, the environment in which the seed develops also has an important influence on the acquisition of dormancy [26]. In the seeds of many species of the *Leguminosae* family, various authors have systematically reported the presence of dormancy [11].

Scarification with LN allowed to effectively overcome the dormancy present in the seeds of the species *D. scorpiurus*, *T. labialis*, *N. wigthii* and *P. phaseoloides* (Fig. 1), showing statistically significant differences in relation to the control treatment. Scarification with LN causes an increase in the rate of seed imbibition, which translates into increased germination [14]. The increase in the capacity for imbibition in the seeds is due to the occurrence of morphological changes in the seeds coat with the appearance of cracks [27].

In dry seeds, the reserve tissues are made up of organic substances such as proteins, lipids, carbohydrates and sugars that are in a very flexible glassy or rubbery state and presumably very heat resistant [28]. On the contrary, the seed coat is made up of tissues with dead cells and thick lignified secondary walls, generally impregnated with hydrophobic organic substances such as cutin, lignin, quinones, suberin and wax. The stresses caused by changes in temperatures in the different tissues of the seeds cause the coat to break or crack [9], [27].

A similar result to that achieved in this research was described in seeds with physical dormancy of some species of the *Leguminosae* family by [11]. Other results, where scarification with LN allowed to overcome dormancy, was described for seeds of *Medicago orbicularis*, *Medicago truncatula* and *Phillyrea angustifolia* [8], [29]. Also in the species *Lotus mascaensis* [30] and *Robinia pseudoacacia* [31] the dormancy present in the seeds after direct exposure to LN was exceeded.

For the species *M. atropurpureum* and *C. ternatea*, the scarification treatment with LN was not enough to overcome the dormancy present in the seeds (Fig. 1). The loss of impermeability of the seeds when they are scarified with LN occurs mainly during the cooling process [32]. Factors such as coat thickness, seed size, chemical composition and internal seed moisture content provoke different responses to scarification with LN [33]. The size of the seeds, larger in these two species than in the rest of those evaluated, was presumably the factor that caused the used scarification treatment to be ineffective.

Two indicators associated with germination, such as the time required to reach 50% of the germinated seeds (T_{50}) (Table 2) and the percentage of seeds that remained hard at the end of the experiment (Fig. 2) were statistically higher in the species where LN favored overcoming dormancy (*D. scorpiurus*, *T. labialis*, *N. wigthii* and *P. phaseoloides*).

TABLE 2. TIME REQUIRED FOR REACHING 50% OF GERMINATED SEEDS (T_{50}) (DAYS).

Species	Control	LN
<i>D. scorpiurus</i>	11 ± 0.91 b	6.8 ± 0.12 a
<i>M. atropurpureum</i>	11.2 ± 0.66 b	8 ± 0.31 a
<i>T. labialis</i>	7 ± 0.44 b	4.4 ± 0.24 a
<i>N. wigthii</i>	4.8 ± 0.2 b	3.1 ± 0.19 a
<i>C. ternatea</i>	5 ± 0.25 a	5.1 ± 0.33 a
<i>P. phaseoloides</i>	5.2 ± 0.31 b	3.1 ± 0.2 a

Results with same letter, for each species, are not statistically different; t-Student, $p > 0.05$, $n = 5$.
Source: Authors.

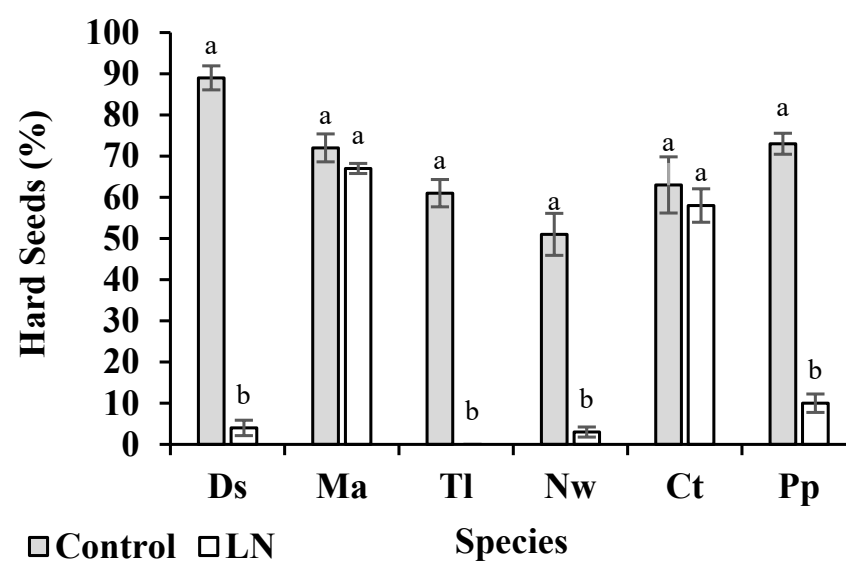


Fig. 2. Hard seeds at 21 days. **Ds**) *Desmodium scorpiurus* (Sw.) Desv., **Ma**) *Macroptilium atropurpureum* (DC.) Urb., **Tl**) *Teramnus labialis* (L.f.) Spreng., **Nw**) *Neonotonia wigthii* (cv.) Copper, **Ct**) *Clitoria ternatea*, **Pp**) *Phueraria phaseoloides*. Seeds without scarifying (**Control**), Seeds scarifying with Liquid Nitrogen (**LN**). Results with same letter, for each species, are not statistically different; t-Student, $p > 0.05$, $n = 5$.
Source: Authors.

In these species, not only was the final germination percentage improved, but homogenization and its speed were also favored. In addition, it was possible to significantly reduce the percentage of seeds that remain hard at the end of the experiment. The results achieved in these species are similar to those obtained previously for other members of the *Leguminosae* family [34].

D. Emergence and plant development

The percentage of emerged plants was significantly higher in the seeds scarified with LN of the species *D. scorpiurus*, *T. labialis*, *N. wigthii* and *P. phaseoloides* (Fig. 3), which corresponds to the germination results obtained for these same species.

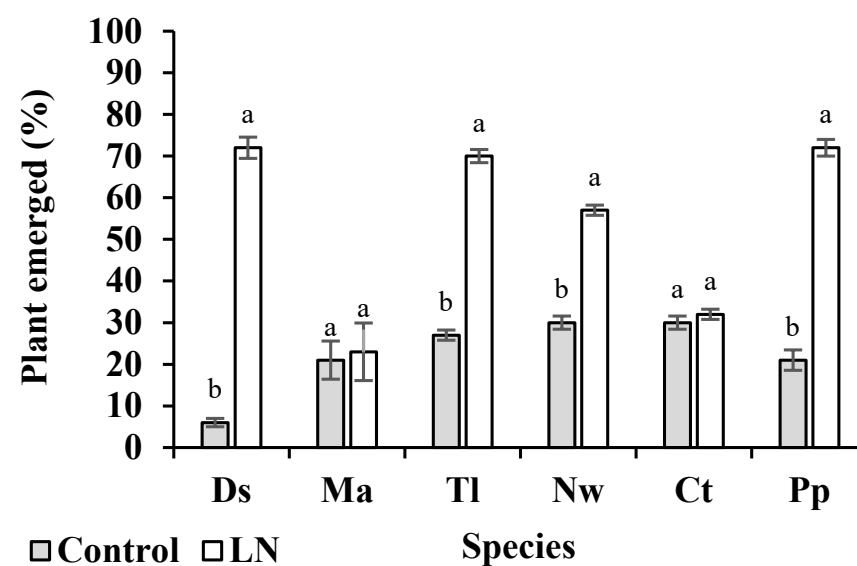


Fig. 3. Emerged plants at 21 days. **Ds)** *Desmodium scorpiurus* (Sw.) Desv., **Ma)** *Macroptilium atropurpureum* (DC.) Urb., **Tl)** *Teramnus labialis* (L.f.) Spreng., **Nw)** *Neonotonia wightii* (cv.) Copper, **Ct)** *Clitoria ternatea*, **Pp)** *Phueraria phaseoloides*. Seeds without scarifying (**Control**), Seeds scarifying with Liquid Nitrogen (**LN**). Results with same letter, for each species, are not statistically different; t-Student, $p > 0.05$, $n = 5$. Source: Authors.

T. labialis and *P. phaseoloides* obtained the highest percentages of emergence with 70 ± 2.63 and 72 ± 3.53 respectively. As in germination, the species *M. atropurpureum* and *C. ternatea* did not find statistical differences between the seeds of the control treatment and those scarified with LN.

Emergence is probably the most important phenological event that influences the success of a plantation and represents the moment in which a plant begins photosynthetic autotrophism [35].

The results achieved in the emergence of plants obtained from seeds scarified with LN show that this treatment not only favors germination under laboratory conditions, but also emergence under field conditions. Previous results, similar to those obtained in this research, were published for the species *T. labialis* and *N. wightii* [20], [36].

The plants that emerged and developed for 21 days showed greater growth (Table 3) in those obtained from seeds scarified with LN for the species *D. scorpiurus*, *T. labialis*, *N. wightii* and *P. phaseoloides* (Fig. 4). This result is due to the overcoming of the dormancy present in these seeds through scarification with LN, which allowed a higher emergence speed.

The rapid emergence in the field is related to the development of plants of many species; and it is an advantage for the establishment of the culture [37]. Some Cuban studies [38] define the speed of germination and emergence as one of the most important properties for the establishment of a species.

The time of emergence many times determines whether a plant can compete successfully with its neighbors, flowers, reproduces and matures properly at the end of its growth stage, or is consumed by herbivores [39].

TABLE 3. INDICATORS EVALUATED IN PLANT AT 21 DAYS OF GROWTH.

Species	Stems length (cm)		Roots length (cm)		Leaves per plant	
	Control	NL	Control	NL	Control	NL
<i>D. scorpiurus</i>	2.6 ± 0.10 b	5.5 ± 0.15 a	2.2 ± 0.12 b	2.9 ± 0.18 a	2 ± 0.0 a	2.2 ± 0.2 a
<i>M. atropurpureum</i>	10 ± 0.87 a	11.2 ± 0.71 a	4.3 ± 0.12 a	4.3 ± 0.2 a	3 ± 0.21 a	3.4 ± 0.24 a
<i>T. labialis</i>	3 ± 0.15 b	3.3 ± 0.11 a	2.1 ± 0.1 a	2.2 ± 0.12 a	2 ± 0.1 b	3 ± 0.1 a
<i>N. wightii</i>	3.2 ± 0.2 b	5.3 ± 0.2 a	2.3 ± 0.12 b	4.8 ± 0.12 a	2.4 ± 0.24 b	3 ± 0.1 a
<i>C. ternatea</i>	5.3 ± 0.35 a	5.7 ± 0.21 a	3.1 ± 0.18 a	3.4 ± 0.15 a	2.4 ± 0.24 a	2.6 ± 0.24 a
<i>P. phaseoloides</i>	5.7 ± 0.3 b	10 ± 0.35 a	3.1 ± 0.24 b	6 ± 0.15 a	2 ± 0.1 b	2.8 ± 0.2 a

Results with same letter, for each species and indicator, are not statistically different; t-Student, $p > 0.05$, $n = 10$. Source: Authors.

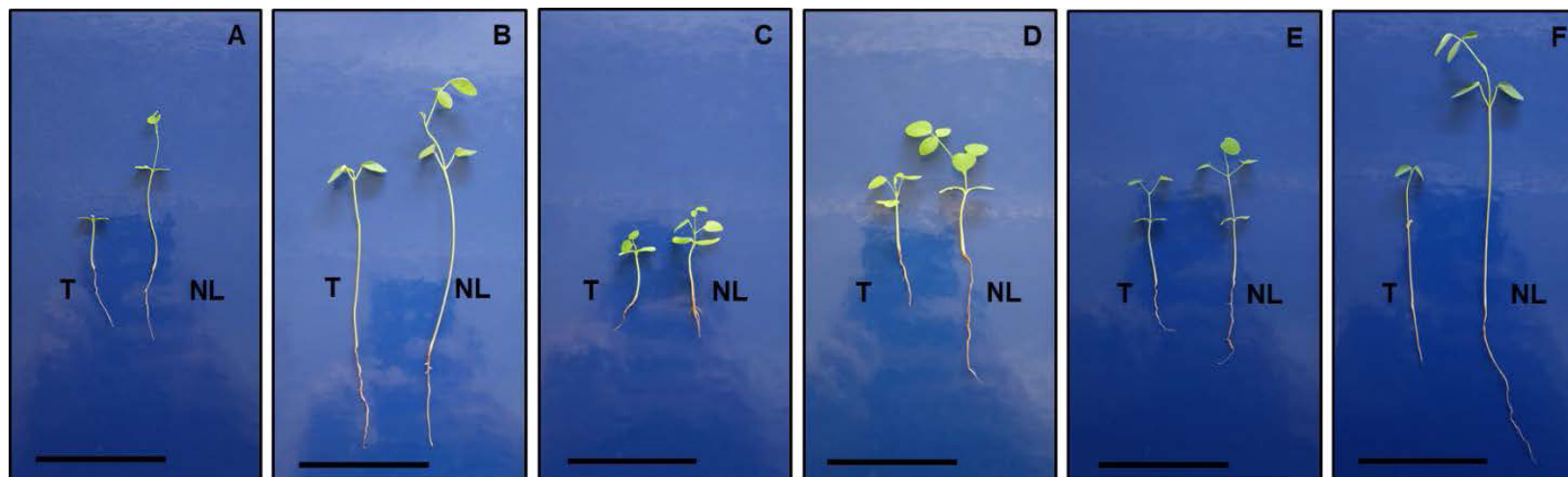


Fig. 4. Plant size at 21 days after shown. A) *Desmodium scorpiurus* (Sw.) Desv., B) *Macroptilium atropurpureum* (DC.) Urb., C) *Teramnus labialis* (L.f.) Spreng., D) *Neonotonia wightii* (cv.) Copper, E) *Clitoria ternatea*, F) *Pueraria phaseoloides*. T) (Control); NL) Liquid Nitrogen. Bar represents 5 cm.
Source: Authors.

IV. CONCLUSIONS

The results obtained in this investigation demonstrate the potential of the use of LN as a scarification method for some species of the *Leguminosae* family. Dormancy was exceeded in the seeds of the species *D. scorpiurus*, *T. labialis*, *N. wightii* and *P. phaseoloides*. It was possible to increase the percentage and speed of germination and emergence, achieving plants with greater vegetative development during the first 21 days after sowing. Only in the species *M. atropurpureum* and *C. ternatea* it was not possible to overcome the existing dormancy in the seeds.

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