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Original Article

Germination of *Crotalaria* and *Lupinus* (Fabaceae) seeds submitted to different pre-germination treatments and their effect on enzymatic activity during early germination

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(With 4 figures)

Abstract

Most of the wild and native legume seeds has a hard and impermeable testa, which causes physical dormancy and prevents them from germinating even when environmental conditions are favorable. The study evaluated the effect of scarification treatments on germination and enzymatic activity of *Crotalaria longirostrata* (*Cl*) and *Lupinus exaltatus* (*Le*) seeds. After scarification treatments, germination percentage (GP) and rate (GR) were assessed during 30 days after seeding (DAS); and water absorption (WA) and specific enzymatic activity (SEA) during early germination (0, 6, 18, 36, 72, 120 h) in a growing chamber at 25 °C and photoperiod of 12 h. Scarification with 98% H_2SO_4 15 min increased GP and GR in both species. At 30 DAS, GP and GR of *Le* seeds were 34% and 0.97 seeds day^{-1}, respectively. In *Cl* seeds, GP was 64% and GR 0.90 seeds day^{-1}. Scarification with H_2O at 80 °C 1 min also promoted germination in *Cl* (52%). At 120 h after seeding, *Le* and *Cl* seeds showed already a high GP with acid scarification (31% and 48%, respectively). In seeds of both species, scarification treatments affected WA and SEA during early germination. During this period, scarification treatments that increased GP also showed a higher α-D-galactosidase activity. The maximum enzyme activity was observed 72 h after hot water scarification in *Cl* (82.6 U/mg total protein), followed by acid scarification (54.5 U/mg total protein). In *Le*, the activity peak was 36 h after acid scarification (9.5 U/mg total protein). No relationship was observed between β-glucosidase activity and GP in both species. In conclusion, during early germination of both species, the increase in GP is accompanied by a rise in α-D-galactosidase activity between 36 and 72 h after seeding; and in *Cl* seeds, an alternative scarification treatment to increase GP may be the use of hot water.

Keywords: scarification, native legumes, enzymes.

Germinação de sementes de *Crotalaria* e *Lupinus* (Fabaceae) submetidas a diferentes tratamentos pré-germinativos e o efeito da atividade enzimática na fase inicial da germinação

Resumo

A maioria das sementes de leguminosas nativas e selvagens têm um tegumento rígido e impermeável, o que causa dormência física e impede a germinação, mesmo se as condições ambientais forem favoráveis. O estudo avaliou o efeito de tratamentos de escarificação sobre a germinação e a atividade enzimática de sementes de *Crotalaria longirostrata* (*Cl*) e *Lupinus exaltatus* (*Le*). Após os tratamentos de escarificação, a percentagem (PG) e a velocidade de germinação (VG) foram avaliadas durante 30 dias após a semeadura (DAS); absorção de água (AA) e atividade enzimática específica (AEE) na fase inicial da germinação (0, 6, 18, 36, 72 e 120 h) em uma câmara de crescimento a 25 °C e fotoperíodo de 12 h. A escarificação com 98% de H_2SO_4, durante 15 min aumentou PG e VG nas duas espécies. Aos 30 DDS, PG e VG de sementes de *Le* foram de 34% e 0,97 sementes dia^{-1}, respectivamente. Em sementes de *Cl*, PG foi de 64% e VG 0,90 sementes dias^{-1}. A escarificação com H_2O a 80 °C 1 min também promoveu a germinação em *Cl* (52%). A 120 h após a semeadura, as sementes de *Cl* e *Le* já tinha atingido uma alta PG com escarificação ácida (31% e 48%, respectivamente). Nas sementes das duas espécies, os tratamentos de escarificação afetaram a AEE e a AA nafase inicial da germinação. Durante este período, os tratamentos de escarificação que aumentaram PG, também mostraram a atividade mais elevada de α-D-galactosidase. A atividade enzimática máxima foi observada 72 h após o tratamento com água quente em *Cl* (82,6 U/mg de proteína total), seguido por escarificação ácida (54,5 U/mg de proteína total).

Em *Le*, o pico de atividade foi de 36 h após a aplicação do tratamento ácido (9,5 U/mg de proteína total). Em contraste, não foi observado nenhuma relação entre a actividade β-glicosidase e PG. Em conclusão, durante a germinação precoce das duas espécies, o aumento da GP é acompanhado por um aumento da atividade da α-D-galactosidase entre 36 e 72 h após a semeadura; e em sementes de *Cl*, um tratamento de escarificação alternativo para aumentar GP pode ser o uso de água quente.

Palavras-chave: escarificação, leguminosas nativas, enzimas.

1. Introduction

Legumes are essential components of ecosystems and crop systems because of the biological capacity to fix atmospheric nitrogen. They have a vital role in food security and in resilience from effects of climate change (Liew et al., 2014). In Mexico, they constitute the second largest family of Phanerogamae (Sousa and Delgado, 1998), and a high percentage are semi-domesticated or are still wild (Fraile et al., 2007).

A limitation for the agronomic use or ecological restoration of most of the legume seeds is that they do not germinate even when environmental conditions are favorable because of their hard, impermeable testa, which causes physical dormancy (Jeller et al., 2003; Smýkal et al., 2014). In breaking dormancy in this type of seeds, scarification treatments applied affect seed imbibition (Bewley et al., 2013). Application of acid or heat treatments can release physical dormancy in legumes seeds; and effectivity of these scarification treatments to promote germination range from 12 to 100% in *Lupinus and Crotalaria* species (Elliott et al., 2011; Alderete-Chávez et al., 2010a, b; Ayala-Herrada et al., 2010; Tiryaki and Topu, 2014).

Scarification treatments that break physical dormancy could have an effect on the composition, distribution of nutrients and enzymatic activity during dormancy release. In dormant seeds, the water content and metabolic rate is low, but a few enzymes are active, as α-galactosidase (Guimarães et al., 2001; Fialho et al., 2008). The activity of α-galactosidase and other hydrolases increases during the polysaccharides hydrolysis in the early stages of seed germination (Minic, 2008; Buckeridge, 2010; Bewley et al., 2013). The application of scarification treatments, weaken the cell walls, allowing to water enter; the seed hydrated increases metabolic activity, particularly enzyme in the endosperm or cotyledons for synthesis of new materials and embryo growth (Bewley et al., 2013; Kamithi et al., 2016). In *Euphorbia heterophylla* L. seeds, high levels of α-D-galactosidase are detected during the period of imbibition, related to reduction of endosperm resistance to cotyledon expansion (Suda et al., 2003). In *L. angustifolius* this enzyme catalyzes hydrolysis of galactans in the cell walls of cotyledons during early stages of germination (Buckeridge et al., 2005), and in *Arabidopsis* seeds ß-glucosidase, seems to be involved in the embryo cell wall loosening needed for cell elongation and radicle extension (Gallardo et al., 2002).

Species from the *Lupinus* and *Crotalaria* genus are abundant in ecosystem of Mexico (Acosta-Percástegui and Rodríguez-Trejo, 2005; Ayala-Herrada et al., 2010; Lagunes-Espinoza et al., 2012; Soto-Correa et al., 2015). Seed dormancy of these legumes can be broken by physical (Pablo-Pérez et al., 2013) or chemical treatments (Ayala-Herrada et al., 2010; Acosta-Percástegui and Rodríguez-Trejo, 2005). Water absorption by seeds after scarification treatments enhances mobilization of nutrients and enzyme activity, required for rapid seed germination, so that scarification treatments could have a beneficial effect on enzyme activities involved in early germination (Kamithi et al., 2016). In *Lupinus* and *Crotalaria*, few studies have focused on the effect of scarification treatments in the increase of enzymes activity during early germination that can be relationship with germination. In the present study, the effect of scarification treatments on percentage and rate of germination during time and specific enzyme activity of β-glucosidase and α-D-galactosidase in early germination were studied in *C. longirostrata* and *L. exaltatus* seeds.

2. Material and Methods

Dry fruits of *L. exaltatus* L. were collected in August 2014 (summer season), and those of *Crotalaria longirostrata* Hook. & Arn. fruits in September 2013 (autumn season), from plants grown in agroecosystems of the states of Puebla and Tabasco, Mexico, respectively. The seeds were separated from the dry fruits and conserved at 4 °C for later analysis.

Before application of scarification treatments, seeds were disinfected 2 min with a 3% solution of sodium hypochlorite, and washed three times with sterile distilled water. After seed disinfection, seeds of both species were tested for viability. 20 seeds in triplicate per species were disinfected and mechanically scarified to separate the testa from the cotyledons. The cotyledons were submerged in 5 mL of a 1% tetrazolium chloride solution and left in darkness for 24 h. The embryos dyed red (viable seeds) were counted. Seed viability was 93±5.7% and 90±5.0% for *C. longirostrata* and *L. exaltatus*, respectively.

After this step, other seeds were used for the scarification treatments. The scarification treatments applied to the seeds of *L. exaltatus* were: I) Immersion in H_2SO_4 98% for 15 min followed by three washes with sterile distilled water; II) Immersion in wet sand for 8 h at 35 °C, followed by 16 h at room temperature (23-25 °C); III) Drying seeds at 80 °C for 7 min, then allowed to cool to room temperature; IV) Drying seeds in dry sand at 150 °C for 1 min; V) Without scarification (control). In *C. longirostrata* seeds the treatments applied were: I) Immersion in H_2SO_4 98% for 15 min followed by three washes with sterile

distilled water; II) Soaking in distilled water at 80 °C for 1 min; III) Without scarification (control).

After treatments, groups of 50 seeds in triplicate were sown in Petri dishes disinfected between sterile paper and placed in a bioclimatic chamber (Thermo Scientific) at 25 °C and 12 h light photoperiod (Acosta-Percástegui and Rodriguez-Trejo, 2005; Gutierrez-Nava et al., 2010). All lots of seeds were watered with sterile distilled water during the evaluation period. In a separate group of 50 seeds in triplicate, an imbibition test was performed after scarification treatments during 0, 6, 18, 36, 72, 120 and 148 h.

Germination percentage (GP) was determined with the formula of Maguire (1962): $GP(\%) = \frac{NGS}{TNSS} \times 100$, where *NGS* = number of germinated seeds and *TNSS* = total number of sown seeds. For the germination test, germinated seeds were counted daily during 30 days or 0, 6, 18, 36, 72, and 120 h after seeding; a seed was considered germinated when the radicle was ≥ 2 mm long. Germination rate (GR) is defined as the measure of the number of seeds germinated relative to the time of germination, determined with the formula GR = ∑ (NGSi)/t, where NGSi = number of germinated seeds on day i; t = germination time from sowing to germination of the last seed.

To detect the peak of maximum enzyme activity during the first hours of seed imbibition, evaluation of enzymatic activity was at 0, 6, 18, 36, 72, and 120 h after seeding. To β-glucosidase enzyme activity, samples were powdered homogeneously in liquid N_2; de-fatted to obtain acetone-dry powders (ADP) by addition of 95% hexane (1:10 w/v), with constant shaking (150 rpm) for two hours at 4 °C and centrifuged (10 000 rpm) for five min at 4 °C. The supernatant was decanted and the precipitate was left to stand until the solvent evaporated completely. Immediately, three extractions were done successively with 80% acetone (1:3 w/v); between each extraction, the sample was centrifuged (5,000 rpm) for 5 min at 4 °C. The supernatant was discarded. Finally, one extraction was done with 100% acetone (1:3 w/v) at 10,000 rpm for 5 min at 4 °C, discarding the supernatant; the residue was left at room temperature until the solvent evaporated completely. The resulting paste (ADP) was powdered and conserved at -67 °C. Thirty mg of polyvinylpolypyrrolidone (PVPP) and 900 µL of 1% Triton X100 solution (dissolved in 100 mM pH 7.0 of Tris ultrapure buffer) were added to 15 mg ADP and incubated at 37 °C in a water bath for 30 min with constant shaking at 150 rpm. The solution was then centrifuged (10,000 rpm) for 10 min at 4 °C. The supernatant was used to determine β-glucosidase enzyme activity. β-glucosidase activity was determined following Ross et al. (1993) and Gerardi et al. (2001): 200 µL of the enzyme extract was mixed with 200 µL of 125 mM pH 4.0 sodium acetate buffer containing 200 mM 2-β-mercaptoethanol, 50 mL of 40 mM p-nitrophenyl β-D-glucopyranoside and 350 µL sterile deionized water. All was mixed in a vortex and placed in incubation at 30 °C for 60 min. The reaction was stopped by the addition of 200 µL 1 M Na_2CO_3. In the blank samples, enzyme reaction was ended before incubation. Absorbance was determined at 405 nm in a Thermo Scientific model Multiskan Go spectrophotometer. The type curve was constructed using p-nitrophenol as standard at a concentration of 0 to 100 µM. One unit of β-glucosidase was defined as the quantity of enzyme capable of releasing one µM p-nitrophenol per minute.

To α-D-galactosidase enzyme activity, samples were powdered with liquid N_2, and homogenized with 20 mL 15 mM trihydrated sodium acetate buffer pH 5.5 solution. The homogenized solution was centrifuged (10,000 ppm) at 4 °C for five min (Fialho et al., 2008). Finally, the supernatant was decanted and stored at -67 °C. Activity was determined following Fialho et al. (2008), using 100 µL enzyme extract, 600 µL 200 mM sodium acetate buffer pH 5, and 50 µL 20 mM p-nitrophenol α-D- galactopyranoside substrate. The reaction was run for 15 min at 40 °C and stopped with the addition of 250 µL 2 M sodium carbonate (Na_2CO_3), centrifuged (10,000 rpm) for 5 min at room temperature. For the blanks, the enzyme reaction was stopped before incubation. The amount of p-nitrophenol released was determined at 410 nm. A standard curve of p-nitrophenol of 0 to 100 µM was constructed to interpolate the data. One unit of α-D-galactosidase was defined as the amount of enzyme capable of releasing one µM p-nitrophenol per minute.

The concentration of total protein in the enzymatic extracts was determined by the micro Markwell method (Markwell et al., 1978). To 20 µL of extract, 280 µL sterile deionized water was added. While shaking, 1,000 µL of a solution composed of **A** [2% solution of anhydrous sodium carbonate (Na_2CO_3), 0.4% sodium hydroxide (NaOH), 0.16% sodium tartrate and potassium ($C_4H_4KNaO_6 \cdot 4H_2O$), 1% sodium dodecyl sulfate ($CH_3(CH_2)_{11}OSO_3Na$)] was mixed with solution **B** [1:100 w/v of cuprous sulfate ($CuSO_4 \cdot 5H_2O$)]. The mixture was incubated for 15 min at room temperature, and then while shaking 100 µL Folin & Ciocalteu's reagent (1:1, reagent:water) were added and incubated for 5 min at 37 °C. The peptides released during the reaction were determined at 660 nm using bovine serum albumin (0 to 100 µg/µL).

Previous statistical analysis, data on germination percentage were transformed to the arcsine square root to have an approximated normal distribution. All data were subjected, by species, to analysis of variance under a completely randomized design in a factorial array with three replications. The first factor were the scarification treatments, and the second factor was time in days (30 days) or hours (0, 6, 18, 36, 72 and 120 h). The Tukey means test at 5% of probability was used to determine significant differences between treatments with SAS 9.3 software.

3. Results

The scarification pre-treatments affected germination percentage (GP) and rate (GR) of *L. exaltatus* and *C. longirostrata* seeds. Acid scarification pre-treatment increase germination percentage and rate in both species

(Figure 1). At 30 DAS of *C. longirostrata* seeds had germinated and only 34% in *L. exaltatus.* In both species, germination rate decreased with germination time, the highest was five DAS after acid pre-treatment (4.8 seeds day^{-1} in *C. longirostrata* and 3.8 in *L. exaltatus*). In *C. longirostrata*, the pre-treatment with moist heat also increased significantly GP and GR (52% and 3.4 seeds day^{-1}, respectively), in relation to control five DAS.

Regarding the GP and GR during early germination, dry heat shock (80 °C and 150 °C) inhibited the germination process almost entirely in *L. exaltatus* (Figure 2A and 2B). In contrast, pre-treatment of sulfuric acid 98% 15 min increased GP and GR in *L. exaltatus* seeds. At the end of 120 h, the seeds had already reached 31% germination. In contrast to *L. exaltatus*, the response to germination of *C. longirostrata* seeds to scarification pre-treatment

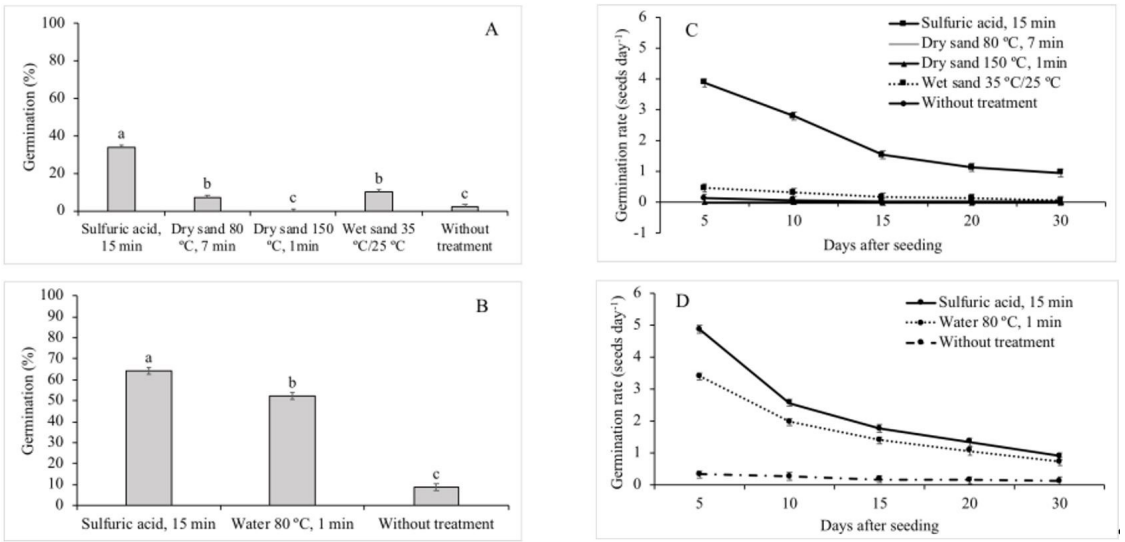

Figure 1. Germination percentage of *L. exaltatus* (A) and *C. longirostrata* (B) seeds at 30 days after application of scarification treatments. Germination rate of *L. exaltatus* (C) and *C. longirostrata* (D) seeds during germination time after application of scarification treatments. Different letters by treatments are statistically different (Tukey, p≤0.05). Error bars represent ± s.e. (n=3).

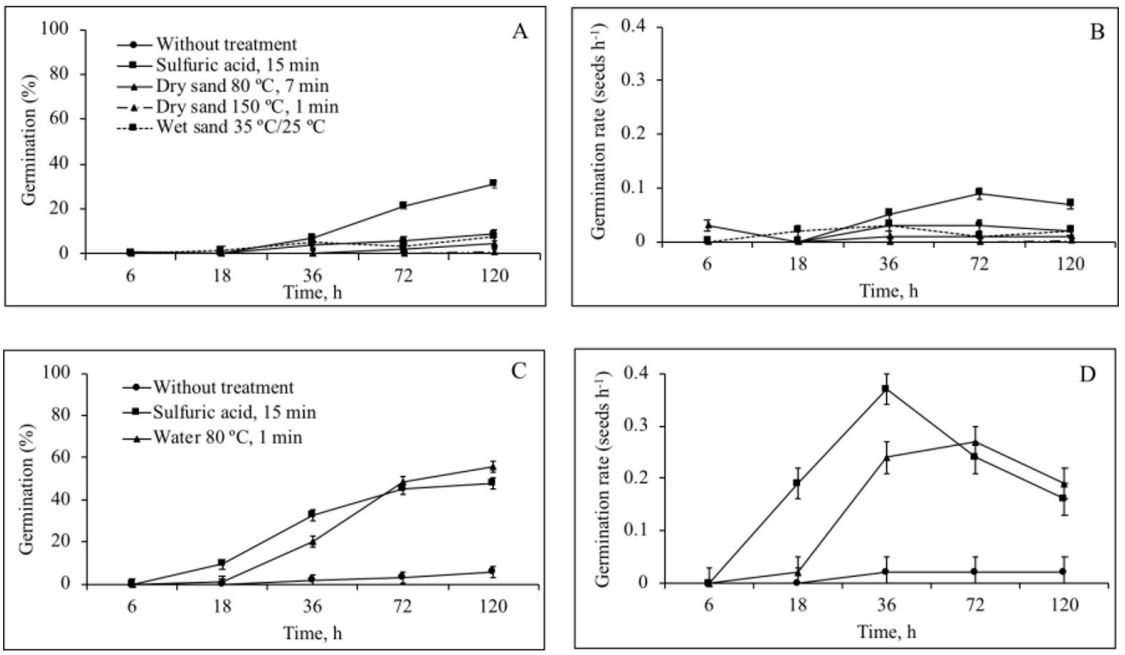

Figure 2. Germination percentage and rate of *L. exaltatus* (A, B) and *C. longirostrata* (C, D) seed seed during early germination time (0, 6, 18, 36, 72 and 120 h after application of scarification treatments). Different letters by treatments are statistically different (Tukey, p≤0.05). Error bars represent ± s.e. (n=3).

was higher (Figure 2C). In this species, no significant differences ($p \leq 0.0001$) were observed between treatments with sulfuric acid 98% and water at 80 °C on the GR at 120 h. The higher GR was observed with sulfuric acid (0.37 seeds h^{-1}) followed by treatment of immersion in water at 80 °C for 1 min (0.27 seeds h^{-1}) at 36 h after seeding (Figure 2D). In GP, at 120 h, 48 and 55% of the seeds germinated after sulfuric acid 98% and water at 80 °C treatment, respectively.

Water absorption was faster in *L. exaltatus* seeds after application of sulfuric acid 98% for 15 min (Figure 3A), than in the other scarification pre-treatments during early germination. No seed weight gain was observed after application of dry heat at 150 °C treatment. In *C. longirostrata*, from 72 h after imbibition, water absorption increased after application of water at 80 °C for 1 min followed by sulfuric acid 98% for 15 min pre-treatment (Figure 3B). At this time, the increase in the growth of the embryo and radicle emergence affected seed weight due to as more than 50% of seeds began germination.

During early germination, in *L exaltatus*, heat shock pre-treatment (dry sand 150 °C) that completely inhibited GP, also inhibited enzyme activity (Figure 4A and 4B). The α-D-galactosidase activity increased after acid pre-treatment to 36 h, and then declined (Figure 4A). The maximum peak activity of this enzyme was found at 36 h (55.6 U/mg total protein). Enzymatic activity of β-glucosidase was no significantly different ($p \geq 0.05$) between the pre-treatments (Figure 4B). In *C. longirostrata*, the highest α-D-galactosidase activity was observed at 72 h with treatment of immersion in water at 80 °C 1 min (Figure 4C), followed by those of sulfuric acid 98% pre-treatment. At time 120 h, both pre-treatments showed close to or more than 50% germination (Figure 2C), just as the enzyme activity decreased significantly. Indicating that once most of seeds germinated, the activity of α-D-galactosidase decreased. The highest concentration of β-glucosidase activity was found in the seeds treated with sulfuric acid at time 0 (3.77 U/mg total protein) (Figure 4D).

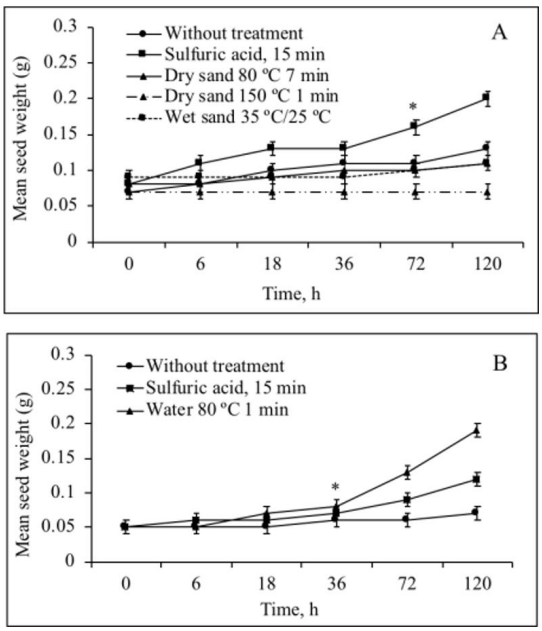

Figure 3. Accumulated seed weight (water absorption) of *L. exaltatus* (A) and *C. longirostrata* (B) after application of scarification treatments over time. *Start of germination. Error bars represent ± s.e. (n=3).

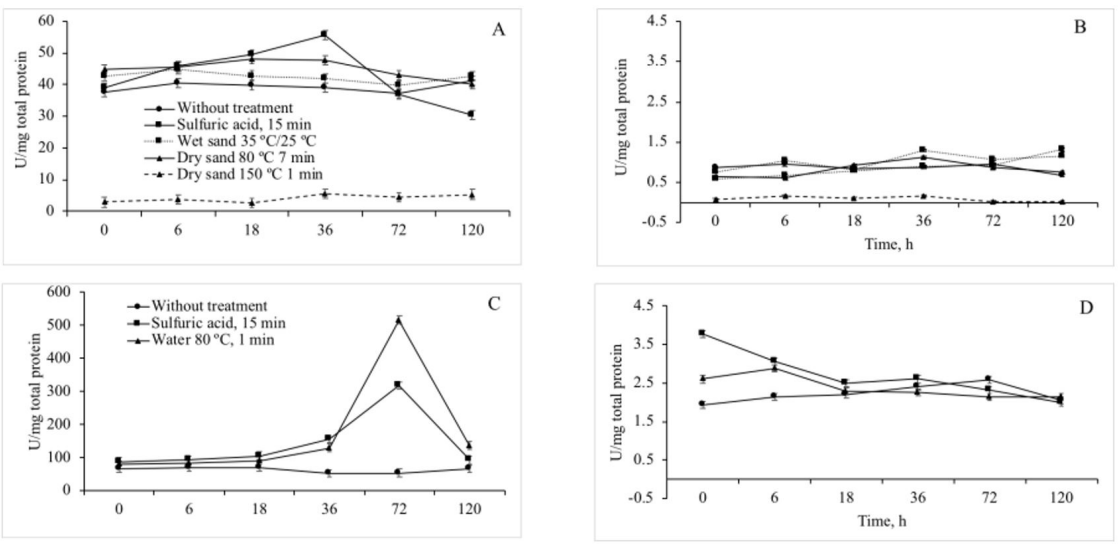

Figure 4. Specific activity of α-D-galactosidase and β-glucosidase in *L. exaltatus* (A, B) and *C. longirostrata* (C, D) seeds during early germination time, after subjected to scarification treatments. Different letters by enzyme are statistically different (Tukey, $p \leq 0.05$). Error bars represent ± s.e. (n=3).

4. Discussion

Seeds of *C. longirostrata* and *L. exaltatus* need scarification to increase water absorption (Ayala-Herrada et al., 2010; Pablo-Pérez et al., 2013) and initiate metabolic changes to carry out the germination process. Scarification with sulfuric acid 98% to soften the seed coat had a significant effect on the germination percentage and rate in the two species. In *L. exaltatus* GP was 31% at 120 h after seeding and 34% at 30 DAS. In studies with *L. leptophyllus* the application of this acid promoted 15% of germination at 120 h (Alderete-Chávez et al., 2010a). A higher germination percentage has been observed in other *Lupinus* species. In *L. campestris* seeds, sulfuric acid also broke dormancy, but with 90 min of exposure, peaking at 50% at 30 DAS (Gutierrez-Nava et al., 2010). Dry heat treatment did not promote germination in *L. exaltatus* from study area after 30 DAS. Nevertheless, *L. exaltatus* growing in areas prone to accidental fires in the state of Jalisco, Mexico, shows 93% of germination seven weeks after seeding, when exposed to heat shock at 150 °C 1 min (Zuloaga-Aguilar et al., 2010). The results obtained suggest that a longer germination time could be necessary to evaluate these pre-treatments of dry heat in this species from Puebla, Mexico, although in the genus *Lupinus* has been observed a high variation in germination, in response to pre-germination treatments (Elliott et al., 2011).

Different studies in *Crotalaria* spp. have shown a positive effect of using acid sulfuric to release dormancy and promote germination. In *C. longirostrata* from Oaxaca, Mexico, application of sulfuric acid 98% for 60 min resulted in 99% of germination at seven days (Ayala-Herrada et al., 2010), but in *C. retusa* with 20 min of acid exposure was only 20% at 5 DAS (Alderete-Chávez et al., 2010b). In *Crotalaria* seeds from Tabasco, Mexico it was observed 48% of germination at 5 DAS with sulfuric acid for 15 min. But using water 80 °C 1 min, the germination percentage was increased to 55% 5 DAS and >60% 30 DAS. Muñoz et al. (2009) observed a similar response in seeds of *Crotalaria* cv. right to acid treatment, despite seeds had more than 12 years of storage.

High specific activity of α-D-galactosidase was observed in seeds of both species as of five DAS. Kadlec et al. (2008) observed that in soybeans, peas and lupine seeds levels of α-galactosides decrease during the first two days of imbibition, and hydrolysis takes 4 to 5 days. In *Tachigali multijuga* (Fialho et al., 2008) an increase in α-D-galactosidase activity was detected the first 108 h of imbibition. This enzyme catalyzes hydrolysis of galactans in the cell walls of *L. angustifolius* cotyledons during early stages of germination (Buckeridge et al., 2005). In this study, changes in the activity of enzymes tested occurred during the first hours of imbibition. Acid scarification treatment modified enzymatic activity of α-D-galactosidase during the first 120 h, and this increase was when the germination percentage was high. In *C. longirostrata*, the enzyme showed higher activity at 72 h after application of treatments. In *L. exaltatus*, at 36 h after sulfuric acid treatment. As previously indicated, this enzyme has the ability to hydrolyze both stachyose and raffinose sugars and galactomannans in the cell walls of cotyledons to support radicle emergence (Bewley et al., 2013; Buckeridge et al., 2005). As increase α-galactosidase activity, raffinose and stachyose content decrease (Fialho et al., 2008). The β-glucosidase activity did not show relation with germination percentage although several studies mentioned their involvement in germination process (Singh et al., 2016; Minic, 2008).

In conclusion, sulfuric acid treatment increases GP and GR in both species. In seeds of *C. longirostrata,* scarification treatment using hot water may be an alternative scarification treatment as it also showed a high GP. During early germination of the seeds of both species, between 36 and 76 h after seeding the increase in GP is accompanied by a rise in α-D-galactosidase activity.

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