







# EFFECTS OF WATER RESTRICTION ON CARBOHYDRATES CONCENTRATION, STARCH GRANULES SIZE AND AMYLOLYTIC ACTIVITY IN SEEDS OF *PHASEOLUS VULGARIS* L. AND *P. ACUTIFOLIUS* A. GRAY

## EFFECTO DE LA RESTRICCIÓN DE HUMEDAD EN LA CONCENTRACIÓN DE CARBOHIDRATOS, TAMAÑO DE GRÁNULOS DE ALMIDÓN Y ACTIVIDAD AMILOLÍTICA EN SEMILLAS DE *PHASEOLUS VULGARIS* L. Y *P. ACUTIFOLIUS* A. GRAY

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### Abstract

**Background:** Seed mass is a key component of adaptation in plants that are strongly affected by development and maturity, and, at the same time, all is modulated by the environment of cultivation.

**Hypotheses:** The response to water deficit in seeds of *P. vulgaris* L. and *P. acutifolius* A. Gray is species-dependent and affects their biochemical and morphological characteristics.

**Studied species:** The studied cultivars were Rosa Bufo (*P. vulgaris*) and cv. 10017 (*P. acutifolius*). Seeds were obtained from plants grown at 100 % and 25 % soil field capacity during their pod development.

**Study site and dates:** The experiments were performed during May and August 2018 in a greenhouse at the Colegio de Postgraduados, Texcoco, State of Mexico (altitude 2,353 m).

**Methods:** The biochemical and morphological characteristics and the grain size of starch in seeds cotyledons were assessed.

**Results:** Water restriction had no significant effects on the seed thickness, width, or mass only length decreased in *P. acutifolius*. In both species, the axis size of the starch granules decreased due to the stress, glucose concentration increased, sucrose and starch were not altered. Water imbibition increased six times in *P. vulgaris* seeds with no effect on the germination. The  $\alpha$ -amylase activity was 25 - 35 % lower in both species due to the water restriction, particularly in *P. acutifolius* the activity was two-fold higher than in *P. vulgaris*.

**Conclusions:** Comparing the carbohydrate concentration in germinating seeds of common and Tepary beans gave insights on the nutrient reserves mobilization during seed maturation and germination.

**Keywords:** Enzymatic degradation, germination, imbibition, legume, photoassimilates, seed development.

### Resumen

**Antecedentes:** La acumulación de biomasa en la semilla es dependiente del desarrollo y la madurez, y responde al ambiente.

**Hipótesis:** Las semillas de *P. vulgaris* L. y *P. acutifolius* A. Gray responden a la deficiencia de agua con alteraciones bioquímicas y morfológicas en dependencia de la especie.

**Especies de estudio:** cv. Rosa Bufo (*P. vulgaris* L.) y cv. 10017 (*P. acutifolius*). Las semillas se obtuvieron de plantas en riego con 25 y 100 % de capacidad de campo durante el desarrollo de la vaina.

**Sitio y año de estudio:** Las semillas se obtuvieron en el Colegio de Postgraduados, Texcoco, Estado de México (altitud 2,353 m) entre mayo y agosto de 2018.

**Métodos:** Los análisis incluyeron las características bioquímicas y morfológicas de las semillas y el tamaño de los gránulos del almidón.

**Resultados:** La restricción de humedad no modificó significativamente el espesor, la anchura y la biomasa de la semilla, pero en *P. acutifolius* la longitud disminuyó. En ambas especies el tamaño del eje de los gránulos de almidón decreció, la concentración de glucosa incrementó, la concentración de sacarosa y almidón no cambió. Las semillas de *P. vulgaris* incrementaron seis veces su imbibición, sin afectar la germinación por el déficit de humedad. La actividad de  $\alpha$ -amilasas disminuyó entre 25 y 35 % pero en *P. acutifolius* fue el doble que en *P. vulgaris*.

**Conclusiones:** La comparación de la concentración de carbohidratos en semillas en germinación de *Phaseolus* aportó información sobre la movilización de nutrientes durante la maduración y la germinación.

**Palabras clave:** Degradación enzimática, desarrollo de semilla, fotoasimilados, germinación, imbibición, leguminosas.

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The seed's primary function is to protect the embryo, sensing favorable conditions for the germination and nourishment of the germinating seedling (Sehgal *et al.* 2018). Because of climate change, seed quality will be affected mainly due to seasonal droughts, fluctuations in soil moisture acting as a selective pressure for germination, seed size and yield (Beebe *et al.* 2013). This effect occurs when photoassimilates uptake and their remobilization from source tissues toward the seeds is modified and alter the grain composition (Farooq *et al.* 2018). In particular, common beans (*Phaseolus vulgaris* L.) grow in a range of habitats where it is exposed to seasonal droughts, with soil moisture fluctuations. This stress may act as a selective pressure on yield, seed size and quality (Beebe *et al.* 2013); however, the low yield is typical result in common beans grown at low water availability (Padilla-Chacón *et al.* 2019).

To achieve systematic progress in breeding, it is necessary to have a broad genetic base (Singh 2001). Most of the available diversity in wild and landraces of these species remains without use due to a 'bottleneck' effect that took place during the domestication process from a small number of wild populations, there is greater diversity in the wild forms of the species in the *Phaseolus* genus (Jiménez-Galindo & Acosta-Gallegos 2012).

Common bean is one of the most important legume crops, providing as much as 15 % of total daily caloric intake and 36 % of total daily protein in Africa and America (Schmutz *et al.* 2014; <http://faostat.fao.org/site/291/default.aspx>). The development of high yield varieties with increased drought tolerance and high grain quality are primary objectives of a bean genetic improvement program (Acosta *et al.* 2004). There are available genetic resources of *P. vulgaris* selected for drought tolerance developed at arid and semiarid areas (Beebe *et al.* 2013). In contrast to common bean, *Phaseolus acutifolius* A. Gray (Tepary bean) distributes in arid and semi-arid regions from northern Mexico to Southwestern USA; its yield under limited water availability is qualified as satisfactory, even outstanding (Gujaria-Verma *et al.* 2016, ILDIS 2018, Leal-Delgado *et al.* 2019); it is a pulse less consumed than common bean even its high nutritive value (Bhardwaj & Hamama 2004).

Carbohydrate content, as a key factor for plant growth, regulates synthesis and seed reserves accumulation during development, seed filling time and the overall metabolic activity (Sehgal *et al.* 2018). Starch is generally composed of amylose and amylopectin, about 98-99 % of the polysaccharide dry weight, organized as granules, can show

similar or heterogeneous size and shapes (Tayade *et al.* 2019). In cereals like wheat (*Triticum aestivum*), drought decreases the total starch concentration and amylose proportion, modifying the amylose-to-amylopectin ratio in the grain (Li *et al.* 2015, Yu *et al.* 2016).

The genetics and molecular details of starch synthesis and granules organization are described in cereal crops, yet, little is known of their synthesis in *Phaseolus*; even less to the effects water restriction has in the seeds, their sugars and starch accumulation and effects on the seed growth and germination (Lemmens *et al.* 2019). Though, in common beans, water restriction during plant growth modifies the amylose-amylopectin ratio, digestibility and pasting properties of isolated starch in the seeds (Ovando-Martínez *et al.* 2011).

The objective of this research was to evaluate the effect water restriction during on seed's development regard their carbohydrates concentration, starch granules size and amylolytic activity in seeds of *P. vulgaris* and *P. acutifolius*, which contrast in their drought tolerance. For this purpose, this study included common bean seeds from cv. "Rosa Bufo", from Chihuahua, improved for extremely dry environments at the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, INIFAP-Chihuahua, Mexico. This common bean cultivar has a reported yield of 376 kg ha<sup>-1</sup>, higher than that of other cultivars under the same stressing conditions (Jiménez-Galindo & Acosta-Gallegos 2012). The above contrasted with Tepary bean variant 10017 collected at Durango, Mexico, from highly dry habitats of northern Mexico. Tepary bean 10017 is registered in a core collection of the INIFAP (Cárdenas-Ramos *et al.* 1996). The outstanding tolerance to water restriction of the 10017 variants, during vegetative stage, was reported by Leal-Delgado *et al.* (2019). We hypothesized that the seed responses to water deficit in their physiology, carbohydrates concentration and starch grain morphology are species dependent.

## Materials and methods

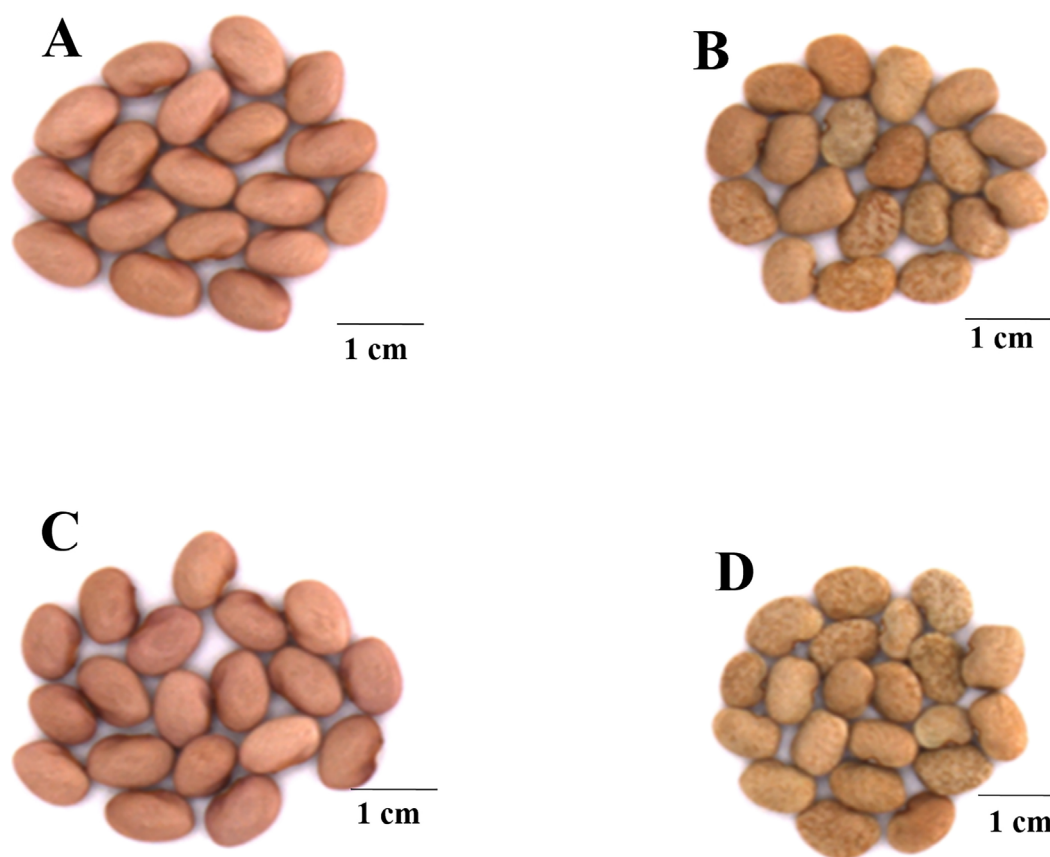
**Evaluated seeds.** Seeds of the Tepary bean cv. 10017 (*P. acutifolius* A. Gray) were donated by the INIFAP. The material was collected at Durango, Mexico, and is registered in the germplasm bank catalog for *Phaseolus* spp. of the INIFAP (Cárdenas-Ramos *et al.* 1996). The cv. Rosa Bufo (*P. vulgaris*) was also donated by INIFAP, Campo experimental Sierra Chihuahua (Jiménez-Galindo & Acosta Gallegos 2013). Plants from both species were cultivated

between May and August 2018, in a greenhouse at the Colegio de Postgraduados, Campus Montecillo, Texcoco, State of Mexico (19° 29' N, 98° 53' W, 2,240 m altitude). During plant development, the mean daily temperature was  $24 \pm 4$  °C, sunlight radiation of  $1,259.25 \mu\text{m m}^{-2} \text{s}^{-1}$  and the relative maximum and minimum humidity were 83.17 and 9.93 %. Each plant was grown in a plastic pot ( $15 \times 13 \times 11$  cm), in 5.5 kg of sandy crumb-type soil. Plants were daily irrigated ( $\sim 100$  % field capacity; FC) until the third trifoliate leaf was fully expanded ( $\sim 30$  days after sowing; DAS). Then, plants from each species were randomly separated into two groups. One group continued with regular irrigation to maintain soil near FC (control); the other group, was maintained at 25 % FC by irrigation restriction. Soil moisture was daily evaluated *via* the gravimetric method until harvest.

**Morphometric traits.** The mass of 100 randomly chosen seeds (Figure 1) was gravimetrically determined by weighing in an electronic balance (Hongzuan HZ-2003).

Seed size was determined by measuring the length, width and thickness using a vernier caliper (HER-411, Steren, China).

**Starch grain analysis.** Seeds were imbibed in water for 48 h and dehulled. Cotyledons were fixed in FAA solution (50 % ethanol, 5 % acetic acid, 10 % formol and 35 % distilled water volume ratios) for 72 h. Cotyledons were infiltrated and embedded into paraffin to obtain  $10 \mu\text{m}$  transverse sections in a microtome (Erma Inc. Japan). The samples were analyzed using periodic acid Schiff reaction and evaluated by field emission in light microscopy (Carl Zeiss, Model LG, Germany). Micrographs of each sample were taken with 400 times magnification. At least 12 mature grains from three independent plants of each treatment were imaged. The starch granule number per field was counted in eight micrographs of the same size. Grain total starch concentration was quantified using Image J (Java, JVM) software. Data were collected from at least eight cotyledons with four replicates per image.



**Figure 1.** Representative image of seeds harvested from plants of *P. vulgaris* (cv. Rosa Bufo) (A-C) and Tepary bean *P. acutifolius* (cv. 10017) (B-D) under 100 % FC (A-B) and 25 % FC (C-D). Barr represent 1cm.

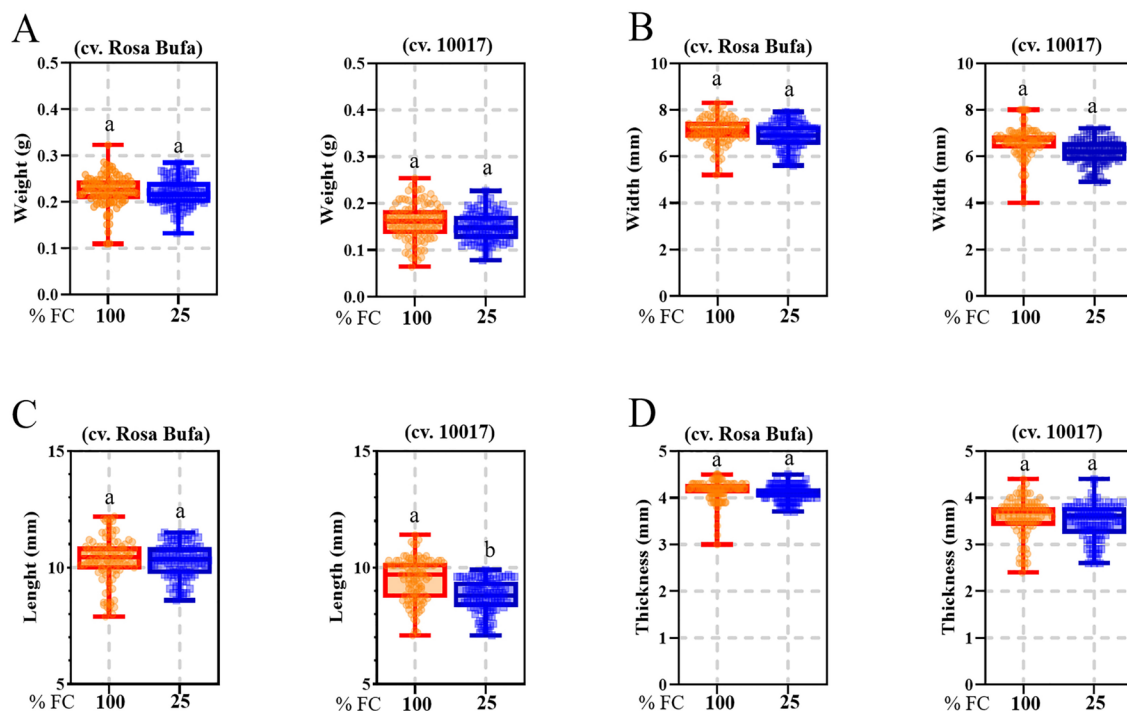
**Soluble sugars and starch.** Glucose, fructose, sucrose, and starch concentrations were determined in 12 h imbibed seeds. Fifty mg of triturated seed was mixed with 500  $\mu$ L of 80 % ethanol, heated at 80 °C for 30 min and centrifuged at  $10,000 \times g$  for 10 min. Glucose, fructose and sucrose were enzymatically quantified from the supernatants and starch in the insoluble material as described by Bernal *et al.* (2005) and Leal-Delgado *et al.* (2019).

**Water uptake and germination.** Twenty-five seeds of each treatment were germinated in Petri Dishes, for triplicated, for 96 hours, in total darkness and at 25 °C, following ISTA (International Seed Testing Association 2018) standards. The seeds were blotted and placed on paper towels to remove surface water. Seed imbibition was determined at 12, 24, 36, 48, 60 hours, or up to germination. A similar-seed set of each treatment was monitored every 12 h until reaching maximum germination. The criterion for germination was a radicle protrusion of  $> 1$  mm.

**Native PAGE and activity staining.** Native PAGE electrophoresis for starch amyolytic enzyme detection was

carried out with crude extracts from 12 h germinating seeds, as described by Zeeman *et al.* (1988) and Delatte *et al.* (2006). This method relies on the composition of a glucan substrate and amylopectin from potato (Sigma, Aldrich) incorporated into the gel and visualized by staining with an iodine solution. The native gels contained 6 % (w/v) polyacrylamide and 2 % amylopectin. After electrophoresis, gels were kept at 37 °C, for 1 h, in a medium containing buffer 50 mM sodium acetate, pH 5.5, 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 1 mM dithiothreitol. Clear bands developed in amylopectin-containing gels and were stained with Lugol solution.

**Amylase activity.** Amylase activity was assessed 12 h after germination started, in 100 mg of the cotyledon samples, using an amylase activity assay kit (Sigma Aldrich). Each sample was homogenized in 0.5 mL of the amylase assay buffer. The samples were centrifuged at  $13,000 \times g$  for 10 min to remove insoluble material. A standard curve was obtained using nitrophenol standards for colorimetric detection at 405 nm with 0, 2, 4, 6, 8, 10  $\mu$ L from the 2 mM nitrophenol standard, placed into a 96-well plate, generating a 0 (blank), 4, 8,



**Figure 2.** Seeds of *Phaseolus vulgaris* L. (cv. Rosa Bufo) and *P. acutifolius* (cv. 10017); seed weight (A), width (B), length (C) and thickness (D) at harvest. Plants were grown under two soil moisture treatments during the whole cycle, 100 % (red box) and 25 % of soil field capacity (blue box).  $n = 100$ . Different letters indicate a significant difference between moisture treatments. Tukey test ( $P \leq 0.05$ ).

**Table 1.** Comparison of the amount of water (g) imbibed by seeds of *P. vulgaris* cv. Rosa Bufo and *P. acutifolius* 10017 Tepary bean.

| Specie                | Cultivar  | Humidity (%) | DWS                  | g/12h                 | g/24h                | g/36h                | g/48h                | g/60h                | g/72h                | g/84h                | g/96h                |
|-----------------------|-----------|--------------|----------------------|-----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
| <i>P. vulgaris</i>    | Rosa Bufo | 100          | 0.24 ± 0.02 <i>a</i> | 0.02 ± 0.0 <i>a</i>   | 0.15 ± 0.04 <i>a</i> | 0.28 ± 0.08 <i>a</i> | 0.30 ± 0.06 <i>a</i> | 0.36 ± 0.07 <i>a</i> | 0.44 ± 0.08 <i>a</i> | 0.64 ± 0.11 <i>a</i> | 0.74 ± 0.13 <i>a</i> |
|                       |           | 25           | 0.22 ± 0.01 <i>b</i> | 0.12 ± 0.0 <i>b</i>   | 0.18 ± 0.03 <i>b</i> | 0.22 ± 0.07 <i>b</i> | 0.28 ± 0.07 <i>b</i> | 0.4 ± 0.12 <i>a</i>  | 0.47 ± 0.13 <i>a</i> | 0.65 ± 0.20 <i>a</i> | 0.77 ± 0.23 <i>a</i> |
| <i>P. acutifolius</i> | 10017     | 100          | 0.17 ± 0.02 <i>c</i> | 0.004 ± 0.02 <i>c</i> | 0.03 ± 0.06 <i>c</i> | 0.13 ± 0.03 <i>c</i> | 0.16 ± 0.03 <i>c</i> | 0.21 ± 0.05 <i>c</i> | 0.27 ± 0.06 <i>c</i> | 0.41 ± 0.09 <i>c</i> | 0.51 ± 0.11 <i>c</i> |
|                       |           | 25           | 0.16 ± 0.02 <i>d</i> | 0.002 ± 0.04 <i>c</i> | 0.03 ± 0.05 <i>c</i> | 0.12 ± 0.06 <i>b</i> | 0.15 ± 0.08 <i>d</i> | 0.21 ± 0.08 <i>c</i> | 0.27 ± 0.07 <i>c</i> | 0.41 ± 0.11 <i>c</i> | 0.47 ± 0.13 <i>c</i> |

Means (± ES) in the same column followed by the different lower case letters (a, b, c, d) are significantly different at ( $P < 0.05$ ) using Tukey test.  $n = 50$ . DWS = dry weight seed (g).

12, 16, and 20 nmol/well standards. Ten  $\mu\text{L}$  from each sample were added into the wells, plus a 40  $\mu\text{L}$  assay buffer and 100  $\mu\text{L}$  of the master reaction mix. A standard and positive control wells were included. The plate was mixed for 3 min using a horizontal shaker, their initial absorbance (T initial) was then measured at 405 nm (Abs405). The samples were kept at 25 °C the plate was protected from light during the incubation. Readings were made five times every 5 min. The results were calculated from the background by subtracting the final Abs405 blank absorbance from the final Abs405 measurement of the standards and those from the samples, calculating the change in absorbance from T initial to T final for the samples. The  $\Delta\text{Abs405}$  of each sample was compared to the standard curve to determine the amount of nitrophenol generated by the amylase between T initial to T final.

**Experimental design.** A completely randomized factorial design was used to analyze the effects of two water conditions (well-watered or 100 and 25 % FC) on two bean species. The experimental unit was a plant, five replicates were evaluated.

**Statistical analysis.** For the statistical comparison of treatments, the normality test (Shapiro-Wilk) was performed for each variable as well as the equal variance test. An ANOVA was performed for each response variable using the Sigma Stat 3.5 software, followed by a comparison of means using Tukey's multiple comparison test to found significant differences ( $P \leq 0.05$ ).

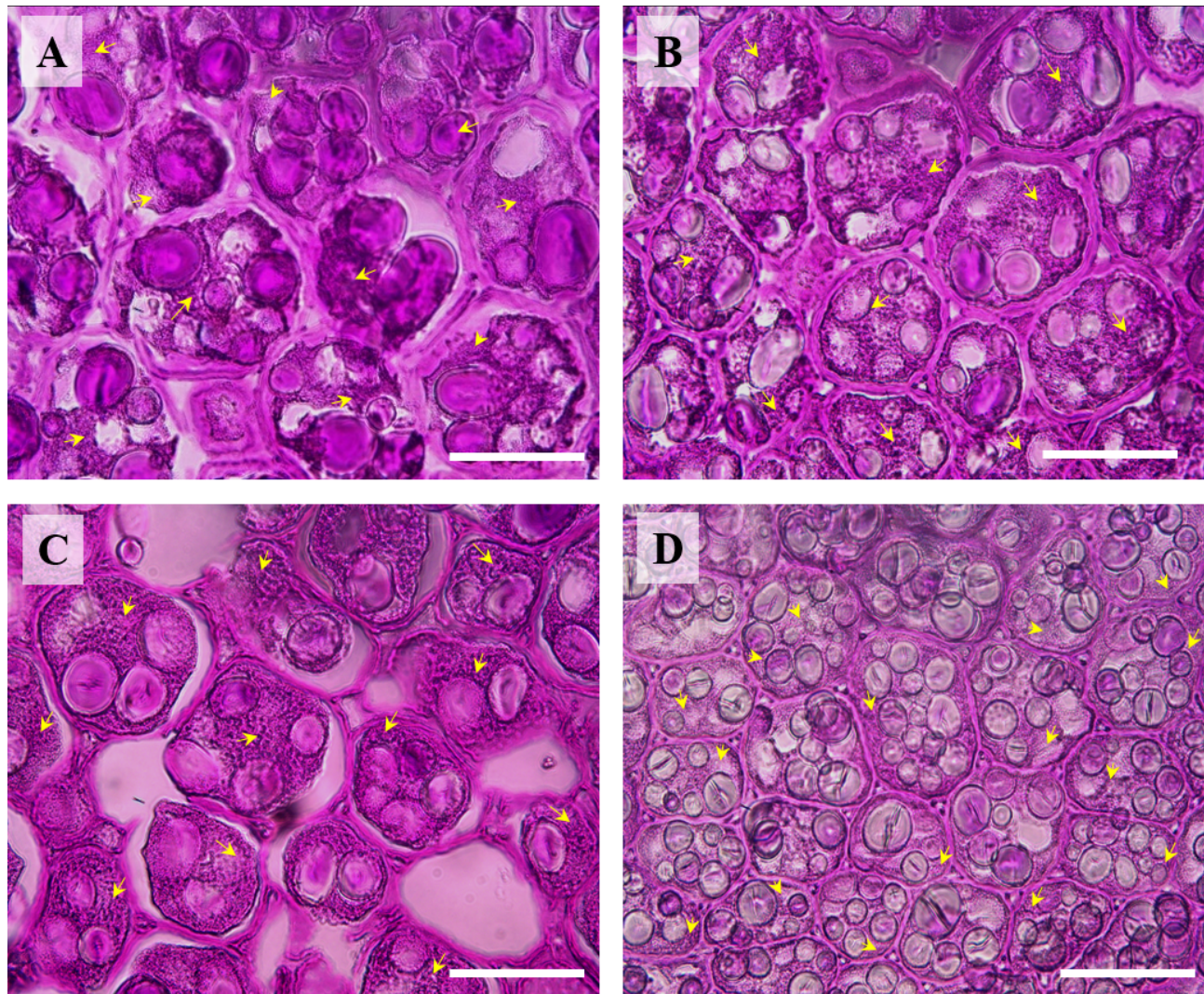
## Results

Seed weight, width, length, and thickness were significantly different between *P. vulgaris* (cv. Rosa Bufo) and *P. acutifolius* (10017) (Figure 2). Water restriction had no significant effect on *P. vulgaris* seed size or mass. In contrast, in *P. acutifolius* 10017 Tepary beans the seed length and width decreased 10 % each compared to its control (Figure 2). The starch granules of *P. vulgaris* were 27 % longer than those of *P. acutifolius* at 100 % FC (Figure 3). The starch granules and neutral carbohydrates were detected with periodic acid-Schiff's (Figure 3A-D). Interestingly, the major axis in *P. vulgaris* had no significant differences at 100 % FC while in *P. acutifolius* was decreased. However, in both species significantly decreased by 10 % the maximum and minimum axis length because of the water restriction. Regarding neutral carbohydrates no differences were detected (Figure 4A-B).

The glucose concentration ranged, in seeds of both species, between 180 to 230  $\mu\text{mol g}^{-1}$  at 100 % FC. The glucose concentration increased to 219-294  $\mu\text{mol g}^{-1}$  as an effect of 25 % FC (Figure 5A). The fructose concentration in both species and at both FC was less than 10 % than that of the glucose concentration, there were no significant differences between species and watering levels (Figure 5A). The sucrose and starch concentration had no difference between species or water treatment (Figure 5B).

Seed imbibition increased by the effect of water restriction (Table 1). Results show that *P. acutifolius* in both FCs had similar maximum imbibition. However, in the first 12 h, seeds grown at 25 % FC of cv. Rosa Bufo im-





**Figure 3.** Light micrographs (40X) showing starch granules and neutral carbohydrates (yellow arrows) of cotyledons of *Phaseolus vulgaris* (cv. Rosa Bufa) (A-C) and *P. acutifolius* (cv. 10017) seeds (B-D) growing at 100 % field capacity (FC) (A-B) or 25 % FC (C-D) during their development. Starch granules were analyzed with a Peryodic acid-Schiff reaction. The bars represent 30  $\mu$ m.

bibed water six-folds more than those grown at 100 % FC (Table 1).

The water restriction did not modify maximum germination in either species, both species began germination at 24 h (Figure 6A-B). However, *P. vulgaris* seeds from plants grow at 25 % FC reached 100 % germination at 36 h. In contrast, the seeds developed at 100 % FC (control), total germination was reached by 60 h. In *P. acutifolius* no changes were observed as an effect of FC (Figure 6B).

Amylolytic enzyme activity using semiquantitative native PAGE (zymogram) showed three major bands in crude extracts from both species. The clear colorless

bands were putative amylases (Figure 7A) represented as bands A1 and A2, the third one putative clear or pale blue, A3 leads, also suggested some activity (Zeeman *et al.* 1988) attributed to amylolytic enzymes. One minor band was visible when gels were incubated for a longer period (Figure 7A).

The enzymatic activity of the  $\alpha$ -amylases was quantified in crude extracts from both species (Figure 7B). Reduction between 15 and 20 % in their activity was observed as pNP-G7 production in seeds grown at 25 % FC (Figure 7B) in both species, but in Tepary beans the activity was twice as high compared to cv. Rosa Bufa.

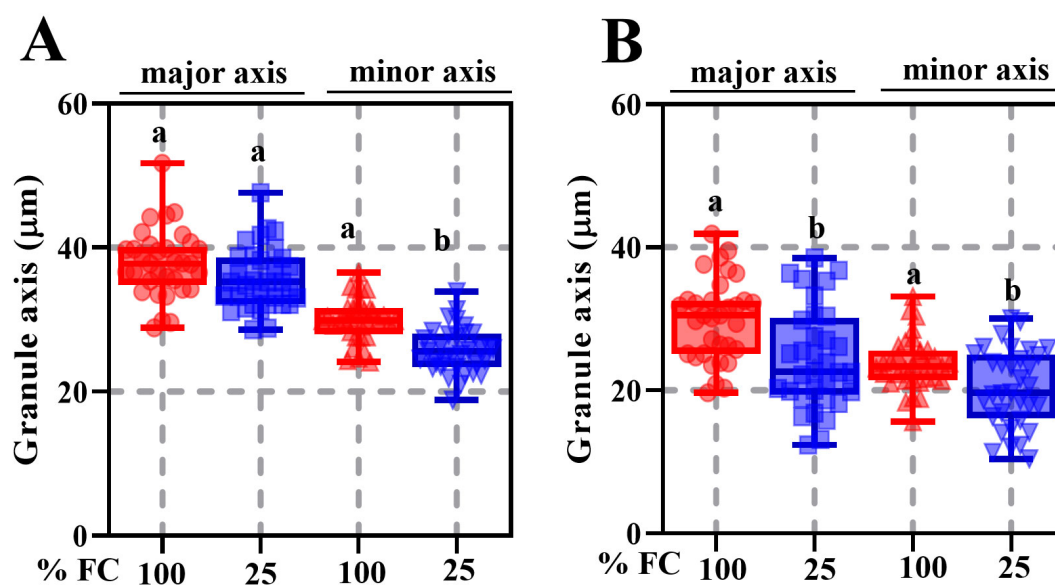
## Discussion

Starch is the most abundant nutrient in *Phaseolus* seeds, accounting for up to 70 to 80 % of their total carbohydrates. Nevertheless, little research has been focused on the impact drought has on the starch content of legume seeds. This research focuses on evaluating the water restriction effects on the seeds' developmental process. The seed weight of Tepary beans was ~40 % lower than *P. vulgaris*. These observations concur with previous reports of grain legumes with seed size variation between species (Blessing *et al.* 2018). Our results indicate that under water restriction (25 % FC), both species do not significantly modify their seed morphological traits (weight, thickness, and width) except for length in *P. acutifolius* (Figure 2). However, the number of seeds per plant decreased 40-50 %, due to the restriction, respect to the control (data not included) because of reproductive tissue abortion. Seed abortion can be a response to reduced resource availability because of low rates of carbohydrate remobilization from vegetative tissue and pod wall to seeds. Seed size increases under-water deficit before or at the early reproductive stage (Fang *et al.* 2011). However, on chickpeas (Pushpavalli *et al.* 2015), lentils (Shrestha *et al.* 2006), grass peas (Gusmao *et al.* 2012) and common beans (Sehgal *et al.* 2018), it is reported that seed size was not affected by water deficit despite yield decrease. This also points to a

relative increase in carbon allocation to reproductive tissues. Thus, our results indicate that 25 % FC has no effect on seed size in *P. vulgaris* and *P. acutifolius*.

At water restriction conditions and nitrogen deficiency cotyledons of maize (Zhou *et al.* 2018) and wheat (Zhang *et al.* 2017) alter their starch synthesis and grain weight. It is well documented that moisture regulates the grain's length and surface area, thus influencing grain yield. Starch granules of *P. vulgaris* are partially modified under moisture restrictions, but the seed morphology was not altered. In contrast to *P. acutifolius*, drought affects starch synthesis by regulating the enzymatic activity for starch biosyntheses, such as soluble starch synthase and ADP-glucose pyrophosphorylase (Lu *et al.* 2019). Our results indicate that 25 % FC in *P. vulgaris* could accelerate grain filling in a reduced number of seeds, maintaining grain weight. The observation would have great significance in achieving the dual goal of increasing crop yield and saving water for final product quality.

Information in the literature regards a differential role of starch granules in beans as a drought response is limited. The available data regard plants grown under optimal conditions indicate that enzymes as starch synthase and ADP-glucose pyrophosphorylase in rice grains contribute to establishing sink strength in seeds by distinctively participating in different stages of seed development (Prathap *et al.* 2019). The starch accumulation in common beans



**Figure 4.** Maximum and minimum starch granule length  $\pm$  SE in seeds of *Phaseolus vulgaris* (cv. Rosa Bufo) (A) and *P. acutifolius* (cv. 10017) (B) growing at 100 % (red box) and (25) % soil field capacity (blue box) during plant development;  $n = 8$ . Different letters indicate a significant difference between moisture treatments. Tukey test ( $P \leq 0.05$ ).



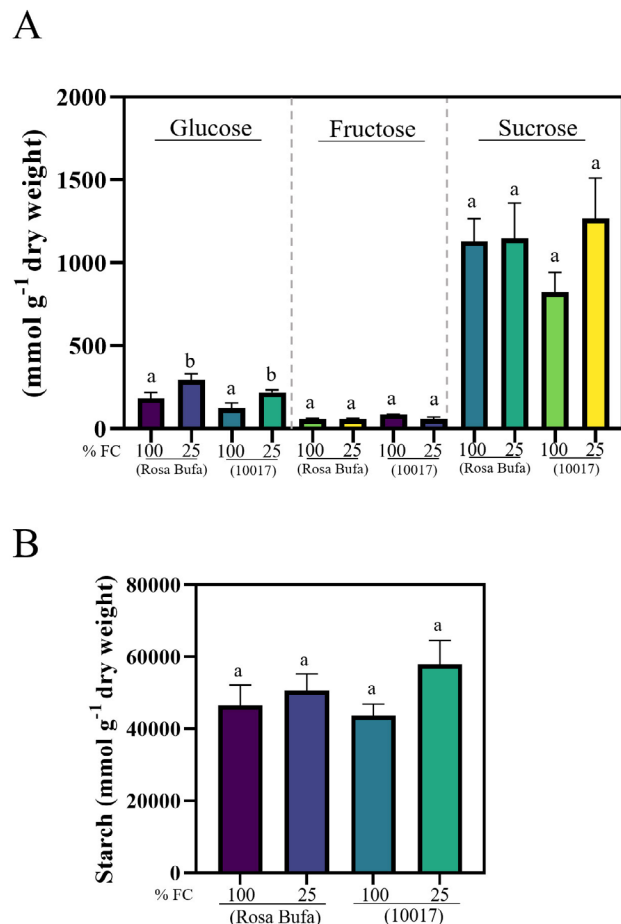
pre-filling pods analyzed in this research concurs with other crop data, where the AGPase activity increases as a response to drought treatments (Cuellar-Ortiz *et al.* 2008). However, results contrast with those obtained in storage organs, where a high sink strength is distinctive; as in stem rice, in which drought induces a decrease in starch accumulation and AGPase activity (Yang *et al.* 2001).

Although *P. acutifolius* yield was significantly reduced under water restrictions, the number of seeds per plant was three times higher than that in *P. vulgaris* cv. Rosa Bufo (data not published). This could be explained by the fact that Tepary bean plants have an indeterminate growth habit that may attenuate temporary drought effects, following deep rooting strategies which are more capable to withstand drought than shallow-rooted annuals (Ye *et al.*

*al.* 2018). Thus, the reduction of the maximum and minimum axis of starch granules in Tepary beans compared to *P. vulgaris* suggests that a high proportion of small starch granules in Tepary bean is a consequence of the enzymes involved in starch synthesis playing key roles in grain size, which could improve the starch molecular structure and functional properties in the grain. These results are consistent with analyses in two wheat varieties. On them, the authors report visual evidence of ultra-structural changes on granule surfaces induced by drought, with differential reduction of granule sizes, leading to an increased susceptibility of the starches to drought-induced hydrolytic enzymes (Li *et al.* 2015).

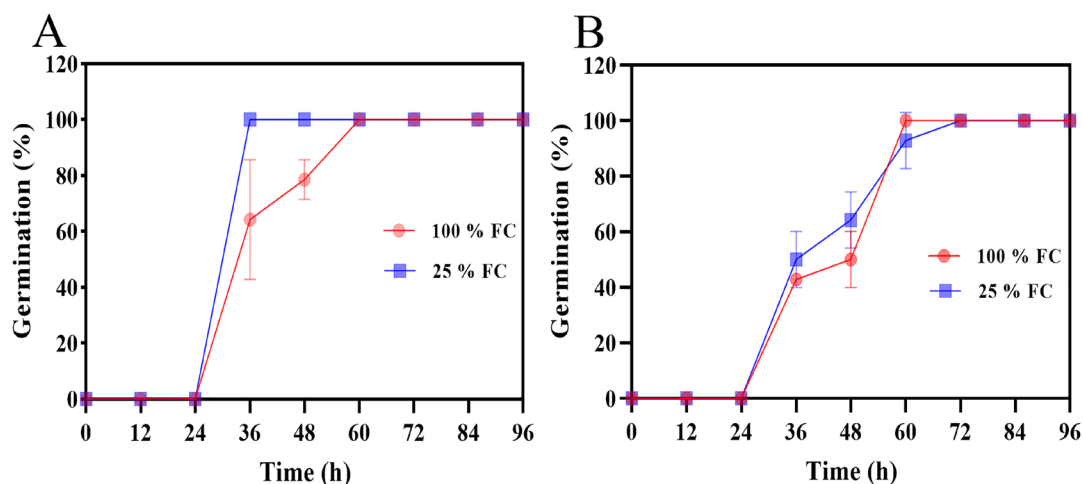
The differences between both bean species in the present study indicate the capacity of *P. acutifolius* seeds to buffer drought impact and maintain a similar nutrient concentration under both, irrigated and drought conditions. Nevertheless, this study supports the fact that genetic variation in bean species in response to drought exists not only for their yield, as previously detected but also for their nutrient concentration (Herrera *et al.* 2019).

Regarding their soluble sugars, glucose concentration in cotyledons increased as a response to water restriction. Fructose levels were maintained without changes in levels 10 to 20 times lower than glucose. These results contrast with those reported for wheat plants exposed to drought stress during grain filling, in which, glucose, fructose, and sucrose concentrations significantly declined in grains of a drought-sensitive genotype accompanied by a sharp reduction in cell wall invertase activity and soluble invertase activity (Koch 2004, Ruan 2012). Certain concentrations of soluble sugars in the cotyledons of cereals are associated with protection to stresses and drought response (Lotfi *et al.* 2010). Regarding sugar signaling in reproductive organs, it has been shown that hexoses stimulate cell division, while sucrose promotes cell endoreduplication and starch accumulation in the cotyledons in *Vicia faba* (Weber *et al.* 1996). These authors further propose that cell wall invertase (CWIN) in the seed coat of *V. faba* affects the developmental processes of seeds by regulating sugar signaling in the embryo. In maize, the mutation of a CWIN gene (*INCW2*) resulted in a miniature seed phenotype by blocking cell division in the endosperm (Vilhar *et al.* 2002). Our results indicate that a possible regulatory network modulates the sucrose metabolism and signaling in seeds set under abiotic stresses. In this study, we detected neutral carbohydrate in the light micrographs (Figure 3A-D). However, further work is needed to extend the method of



**Figure 5.** Glucose, fructose, sucrose (A) and starch concentration (B) ( $\pm$  SE) in seeds of *Phaseolus vulgaris* (cv. Rosa Bufo) and *P. acutifolius* (cv. 10017) grown at 100 and 25 % FC during seed development;  $n = 5$ . Different letters indicate a significant difference between moisture treatments. Tukey test ( $P \leq 0.05$ ).





**Figure 6.** Seed germination percentage of *P. vulgaris* cv. Rosa Bufo (A) and *P. acutifolius* (cv. 10017) (B) at 100 % (FC) red circle, and 25 % (FC) blue square during seed development;  $n = 50$ .

analysis to other oligosaccharides, currently underway in our laboratory.

Seed quality traits depend on the accumulation of various storage molecules during seed development. Drought stress during the initial stage of seed development reduces the capacity of seeds to sink strength, by decreasing the number of endosperm cells and formed amyloplasts (Sehgal *et al.* 2018), thus reducing grain weight with a decline in endosperm competence to gather starch, in terms of both, rate and duration. In the present study, a similar effect was observed in *P. acutifolius*.

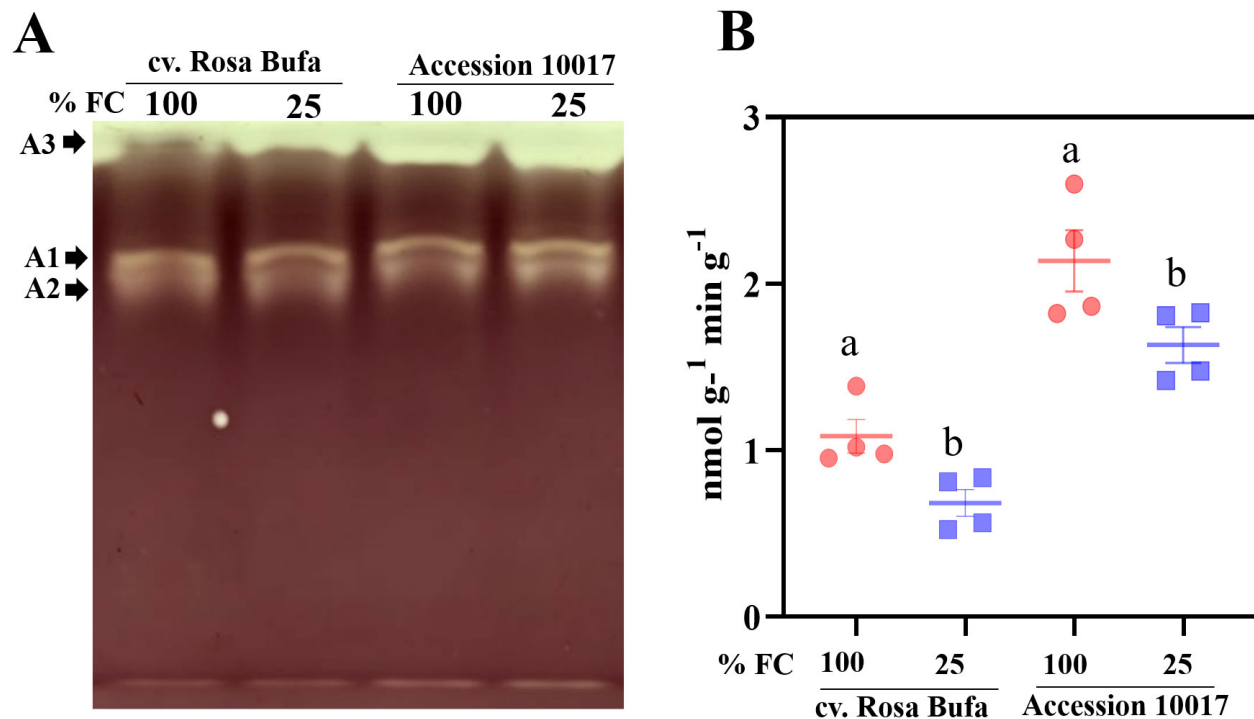
To test if water restriction affects the physiological seed quality of *Phaseolus* species, we tested the hypotheses that 25 % FC during seed development affects seed imbibition and germination percentage (Table 1). Results showed that in both cultivars, water absorption increased four-folds with respect to dry seeds at 72-96 h (Table 1). This result concurs with those reported by Aliu *et al.* (2016) where the time limit for the imbibition of seeds began to reduce after 72 h and even 48 h.

Surprisingly, in cv. Rosa Bufo at 25 % FC, imbibition significantly increased at 12 h respect to the 100 % FC (Table 1). These results correlate with the germination percentage reached at 36 h at 25 % FC while at 100 % FC was at 60 h. Some reports demonstrate that germination depends on water as the main factor that stimulates germination and the chemical composition of the seeds (Saux *et al.* 2020). We do not discard that in cv. Rosa Bufo the water restriction during seed development impacts the imbibition rate thus, germination as a consequence of alterations in the biochemical components associated with

the water adsorption. The differences in seeds imbibition among the species may relate to seed coat thickness, the number of seed coat pores and the size of the micropyle and hilum (Borji *et al.* 2007).

Several reports indicate that  $\alpha$ -amylases are key in the degradation of stored starch in the cotyledons (Ali & Elozeiri 2017, Damaris *et al.* 2019, Sehgal *et al.* 2018, Liu *et al.* 2018). These enzymes account for 40-60 % of *de novo* protein synthesis in grains (Liu *et al.* 2018). Although  $\alpha$ -amylase is necessary to initiate starch degradation in cereal grains (Beck & Ziegler 1989), little is known about the activity in legumes.

The water restriction effects during grain development on amylolytic activity and  $\alpha$ -amylase activity were investigated in this study (Figure 7). The native gels show three putative starch hydrolyzing enzymes involved in reserves remobilization during germination (Figure 7A). This indicates that bean extracts from *P. vulgaris* and *P. acutifolius* are capable of starch degradation and that endo amylases activity is present in the amyloplast. Therefore, we conducted a quantitative assay to assess  $\alpha$ -amylase activity on crude extracts from cotyledons. Results show that the enzymatic activity reduced ~10 to 15 % in seeds from plants grown at 25 % FC in both species, compared to seeds from plants in 100 % FC. However, a two-fold increase in activity was observed, twice as higher, in Tepary beans compared to *P. vulgaris* (Figure 7B). It might be possible that in Tepary beans an increase in the phosphorylase activity enables the phosphorylase of starch partly to accelerate the hydrolysis (Chen *et al.* 2017). Srivastava



**Figure 7.** Native PAGE of starch-degrading enzymes (A). A1, A2, A3 represent different band with amylolytic activity in the gel. Changes in enzymatic activities of  $\alpha$ -amylases (B). The experiments were carried out from crude homogenates of seeds at physiological maturity germinated for 24 h of *P.vulgaris* cv. Rosa Bufo and *P.acutifolius* (cv. 10017) at 100 % (FC), red circle and 25 % (FC) blue square. Different letters indicate a significant difference between moisture treatments. Tukey test ( $P \leq 0.05$ ).

& Kayastha (2014) report that in seeds of *Trigonella foenum-graecum*  $\beta$ -amylase was the major starch degrading enzyme, depending on the amount of enzyme present, compared to  $\alpha$ -amylase and on its localization at the periphery of amyloplasts. That might reflect their different roles among various amylolytic enzymes in a complex regulation. Further experiments are necessary to elucidate the role of each amylase in starch stored degradation in the cotyledons, particularly during seed germination, to facilitate crop breeding as an efficient biomarker in legumes.

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