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RESEARCH ARTICLE

Pineapple stem-derived bromelain based priming improves pepper seed protein reserve mobilization, germination, emergence and plant growth

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Abstract Pepper seeds are slow to germinate and emergence is often non-uniform and incomplete, reducing gains from this cash crop. This study investigated the effects of pineapple stem- derived protease (stem bromelain) based priming on pepper seed germination in relation to reserve mobilization (specifically, proteins and amino acids), germination, emergence and plant growth. These parameters were compared across two controls, (1) unsoaked seeds and (2) seeds soaked in deionized water, and seeds soaked in pineapple stem bromelain crude extract (treatment). Seeds were soaked in bromelain crude extract possessing a proteolytic activity of 6.25 tU or deionized water (first control) for 3 h at 35 °C. Light microscopy revealed an abundance of protein bodies in the endosperm of the seeds prior to imbibition. When observed for a period of 96 h, these bodies were progressively degraded, with the rate of this degradation being fastest in bromelain-treated seeds. Quantitative analysis of protein levels confirmed this observation: 17.2 mg proteins/g FW at 120 h after priming in bromelain-treated seeds compared with 22.1 mg/g FW in controls (average). The bromelain treatment also increased levels of free amino acids from 3.9 mg/g FW in the controls to 4.6 mg/g FW after 120 h of imbibition. Germination and emergence percentages were initially higher in bromelain-treated seeds: 92.0% germination in bromelain-treated seeds vs. ~ 52.2% in the controls at 18 d; 100% emergence in protease-treated seeds vs. ~ 72.2% in the controls at 18 d. However, these parameters were comparable across the treatment and the controls at 28 d. Importantly, plant fresh and dry weights were significantly higher when seeds were primed with bromelain. The results suggest the use of bromelain extracts for priming pepper seeds based on their proteolytic activity, since germination is dependent on the availability of crude protein and essential amino acids. The benefits of bromelain seed priming appear to translate into improved seedling growth as well.

Keywords *Ananas comosus* (L.) Merr · *Capsicum annuum* L. · Plant natural products · Proteolytic activity

Introduction

Pepper (*Capsicum annuum* L.) fruit has a great agronomic, nutritional and commercial importance based on its high levels of antioxidant compounds such as carotenes, and vitamins C and E (Chávez-Mendoza et al. 2015). According to the Food and Agriculture Organization (FAO), the world´s production of pepper reached 4 164 594 t in 2018 (FAOSTAT 2018). However, seedling establishment in this cash crop is often not maximized owing to the low speed and uniformity in the germination exhibited by pepper seeds (Demir et al. 2018).

As in other vegetable species (Delian et al. 2017; Anwar et al. 2020), efforts have been made to ameliorate these undesirable seed traits in pepper seeds (Bradford

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et al. 1990; Andreoli and Khan 1999). For example, Dutta et al. (2015) reported that K_2SO_4 (1%) priming exerted a positive influence via increasing pepper seed germination percentage by 31.29% compared to non primed seeds. Siri et al. (2013) reported that seeds primed in a polyethylene glycol (PEG 6000) solution with the osmotic potential of −1.5 MPa for 6 days improved germination compared to control seeds. The disadvantage of using these chemical substances as priming agents is that the cost of osmotic agents such as PEG is high, and they also pose obstacles to their commercialization due to their relative complexity. In addition, they must be removed from the seeds before they dry out. Studies on the priming of peppers seeds with plant extracts are rare (Barchenger and Bosland 2016). However, organic priming has been used to increase the speed and uniformity of pepper seed germination (Ozbay 2018), e.g. leaf extracts of moringa (Hala and Nabila 2017), tea (Mavi 2018), *Melia azedarach* L., *eucalyptus*, and *Allium sativum* L. (Mehta et al. 2010). The benefits are, however, often limited; for example, priming with moringa leaf extract (4%, 6 h) stimulated germination by 13.6% compared with control seeds (Hala and Nabila 2017). To the best of our knowledge, bromelain extracts from pineapple stems have not been used so far to prime pepper seeds.

Proteases play an important role in mobilizing protein reserves in seeds (Zhao et al. 2018). Cysteine proteases are the most abundant group of proteases responsible for the degradation and mobilization of reserve proteins to promote seed germination (Grudkowska and Zagdańska 2004). Bromelain is a cysteine protease (Heinicke and Gortner 1957) with several applications in fields such as medicine and biotechnology (Mohri and Matsushita 1984; Carvajal et al. 2010; Zhao et al. 2013; Kwatra 2019). In 1997, the Bioplant Centre patented a simple and efficient new technology to obtain bromelain from pineapple stems (harvest remains) (Hernández et al. 1997). Reference to the literature indicated only one study, published in 1994, where the effect of exogenous bromelain application on germination was assessed. This study, which was conducted on the seeds of *Rosa multiflora* Thunb. (Kuska 1994), used reagent grade bromelain from the stem of pineapple and increased seed germination percentage by three times compared with unprimed seeds. However, studies on bromelain's potential mechanism(s) of action in terms of its effects on the biochemical processes that influence germination were not conducted. This motivated the present study on the use of pineapple stem- derived bromelain for pepper seed priming. Seed germination, emergence and plant growth (fresh and dry weight) were related to levels of proteins and amino acids to identify bromelain's potential mechanism of action in this species. Light microscopy was also used to assess levels of protein degradation during imbibition.

Materials and methods

Seed treatments

Sweet pepper seeds cv. Lamuyo from mature fruits were obtained from the Ceballos Agro-Industrial Company, Ciego de Ávila, Cuba. The experiment involved two controls, (1) unsoaked seeds and (2) seeds soaked in deionized water, and one treatment, seeds soaked in pineapple stem bromelain crude extract. Bromelain crude extract was obtained from pineapple cv. MD-2 stems from harvest residues. Extraction was performed using the procedure described by Hernández et al. (1997) with 2 mmol/L sodium sulphite to stabilize the active centers of the enzyme.

Seeds were soaked in bromelain crude extract with a proteolytic activity of 6.25 tU or deionized water for 3 h at 35 °C in 100 mL-Erlenmeyer without shaking in the dark. One total U is defined as the amount of enzyme, in the total volume of extract used, that liberates 1 µmol of soluble TCA. The optimal duration of pepper seed priming was previously determined in our laboratory (unpublished data). One hundred pepper seeds (4 replicates of 25 seeds) were used per treatment/control. Later seeds were dried at 25 °C for 48 h until reaching their initial mass (8.5 mg ± 0.61 mg per seed). For germination, all treatments were placed in Petri dishes on filter paper moistened with 10 mL of deionized water. A germination chamber (RTOP-D Series, China) was used at 30 ± 2 °C for 28 d (Baskin and Baskin 2014; Pittenger 2014). During the first five days they were kept in the dark and later in a photoperiod of 16 h of light/8 h of darkness.

Histochemical evaluation of protein bodies in pepper seeds

A separate batch of 125 pepper seeds (5 replicates of 25 seeds) per treatment/control was set to germinate as described above and 25 seeds per treatment/control were randomly sampled (4 seeds/replicate) at 0, 24, 48, 72 and 96 h for histochemical analysis (Johansen 1940). Samples were fixed in FAA, dehydrated in an ethanol series, and embedded in paraffin. Cross Sects. (5 µm thick) were cut with a hand rotary microtome KD-202A and collected on glass slides covered with a gelatin solution. Sections were stained with solutions of mercury (1%) and blue bromophenol (0.05%) according to Pearse (1985). Observations were carried out using a Carl Zeiss MicroImaging

(GmbH 37,081) microscope at 20 × , and images captured with a Cannon (EOS 600D) digital camera.

Levels of soluble proteins and free amino acids in pepper seeds

Another separate batch of 125 pepper seeds (5 replicates of 25 seeds) was used per treatment/control for measuring levels of soluble proteins and free amino acids. Twenty seeds per treatment were randomly sampled (4 seeds/replicate) at 0, 24, 48, 72, 96 and 120 h for protein and free amino acids evaluation. Protein extraction was carried out with Tris–HCl 0.1 mol/L buffer, pH = 8.5—8.8, 5 mmol/L EDTA and 20 mmol/L β-mercaptoethanol (Isaacson et al. 2006). The total protein content was determined according to Bradford (1976). The aminoacid extraction of each sample was carried out twice with ethanol (80%, v: v) and both supernatants were combined. Quantification of the total aminoacid content was carried out using the ninhydrin reaction according to Moore and Stein (1948). Biochemical assays of each sample were replicated three times.

Evaluation of plant fresh and dry weights

Another separate batch of 125 pepper seeds per treatment/control were planted in pots (500 mL with one seed per pot) with Ferralytic-red soil and filter-cake-sugarcane ash (1:1, v:v). There were five replicates of 25 pots each per treatment. At 28 d of growth, plant fresh and dry weights were measured.

Data analysis

The quantitative data collected were analyzed using SPSS (Version 8.0 for Windows, SPSS Inc., New York, NY). Data were subjected to ANOVA and Tukey tests ($p = 0.05$) after normality was established (Shapiro–Wilk test). The complete experiment was repeated twice.

Results and discussion

The mobilization of reserved proteins, carbohydrates and lipids is crucial for germination efficiency and establishment of seedlings (Thompson 2018). Abud et al. (2017) pointed out that the proteins constitute one of the main reserve components in *C. annuum* seeds. Galão et al. (2007) observed in the seeds of *Prosopis juliflora* after 48 h under germination conditions, that the globular protein bodies were hydrolyzed and the protein material became more soluble. The hydrolysis of reserve proteins to their constituent amino acids is carried out by proteases, both during germination and in post-germination processes (Zhao et al. 2018).

In the present study, bromophenol blue staining developed a strong blue coloration of the cytoplasmic granules suggesting the presence of protein bodies in vacuoles of the endosperm of seeds in both controls and the treatment (Fig. 1). Numerous protein bodies were observed in pepper seed endosperm prior to imbibition (Fig. 1A). Their number was reduced progressively during the first 96 h of germination in the treatment and controls, although protein degradation appeared to be markedly faster (more extensive) in bromelain-primed seeds (Fig. 1B). More specifically, during the 96 h of observation, higher protein disintegration (less intense coloration) was observed in bromelain-primed compared with untreated and hydro-primed seeds (Fig. 1B). After 48 h, a greater spacing and dispersion of the protein bodies of the seeds conditioned with crude bromelain extract was observed (Fig. 1).

Quantitative analysis of protein levels confirmed this observation (Fig. 2A). At 120 h after priming, bromelain-treated seeds contained 17.2 mg proteins/g FW while controls showed 22.1 mg/g FW (Fig. 2A). The bromelain treatment also increased the levels of free amino acids from 3.9 mg/g FW in the controls to 4.6 mg/g FW in the treatment (120 h; Fig. 2B).

Percentages of germination (Fig. 3A) and emergence (Fig. 3B) were initially higher in bromelain-primed seeds. For instance, germination at 18 d reached 92.0% in bromelain-primed seeds while germination in the controls was about 52.2% (Fig. 3A). Emergence at 18 d reached 100% in bromelain-primed seeds but was about 72.2% in the controls (Fig. 3B). However, these parameters were comparable across the controls and the treatment at 28 d (Fig. 3A, B). Plant fresh (Fig. 4A) and dry (Fig. 4B) weights were, however, significantly higher when seeds were primed with bromelain: 2.6 g fresh weight versus. 2.3 g fresh weight in the controls (Fig. 4A); and 0.18 g dry weight versus. 0.14 g dry weight in the controls (Fig. 4B).

Several authors have reported the predominance of cysteine-proteases during the degradation processes of reserve proteins (Szewińska et al. 2016; Liu et al. 2018). Tozzi and Takaki (2011) observed in seeds of *Passiflora edulis* the degradation of the protein bodies, the loss of which began creating spaces as a consequence of reserve mobilization from the sixth day of being placed under germination conditions. The biochemical analyses carried out here indicate that the content of protein reserves decreased fastest in the bromelain-primed seeds, most likely as a result of hydrolysis by the action of the proteolytic activity of bromelain (cysteine-protease) (Kwatra 2019; Mendes et al. 2019) and other proteases present in the seed.

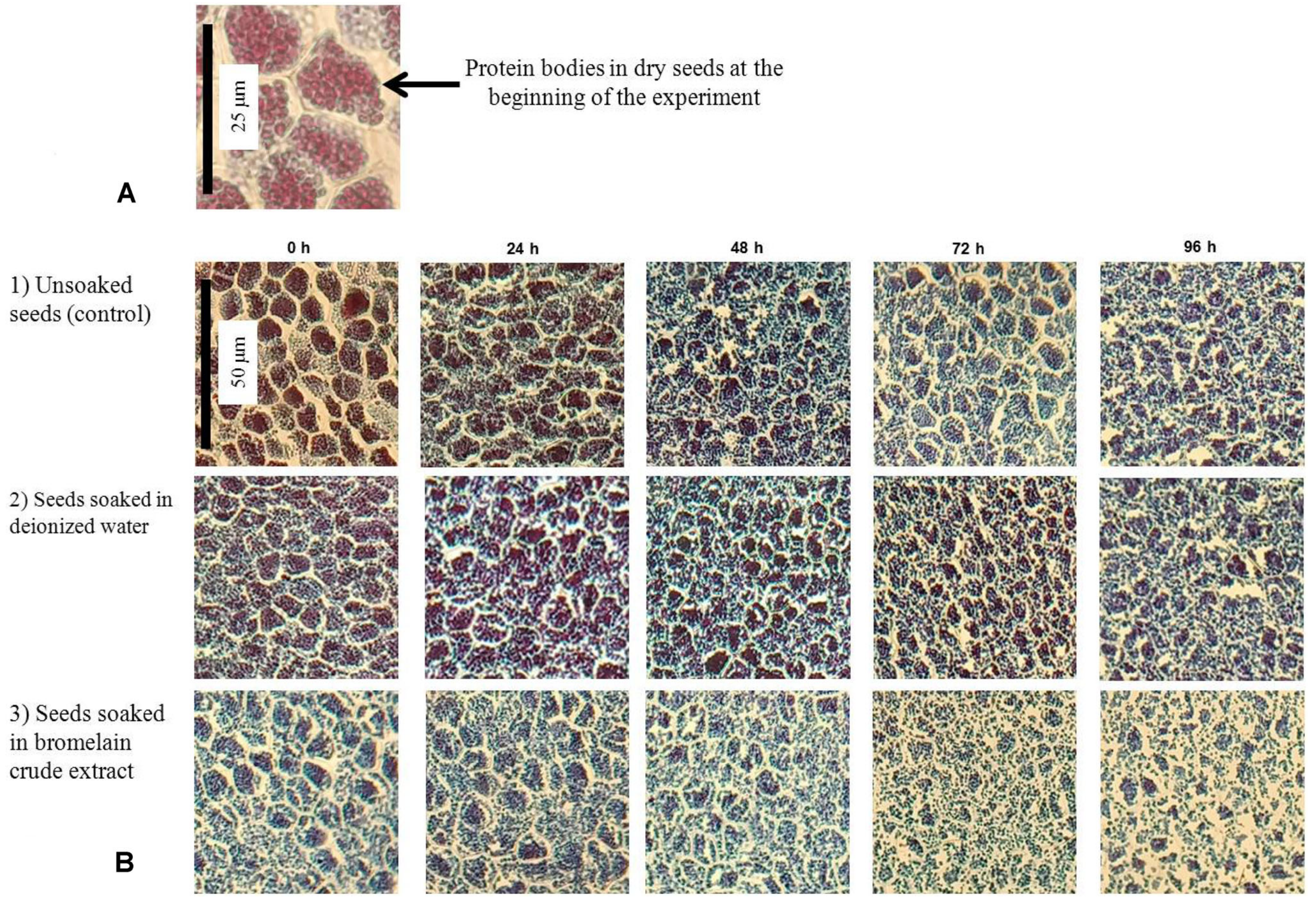

Fig. 1 Protein levels in *Capsicum annuum* L. endosperm during germination in Petri dishes after priming. **A** Protein bodies observed in the endosperm at the beginning of the experiment. **B** Progressive degradation of protein bodies during germination (0–96 h) which was faster in seeds treated with bromelain

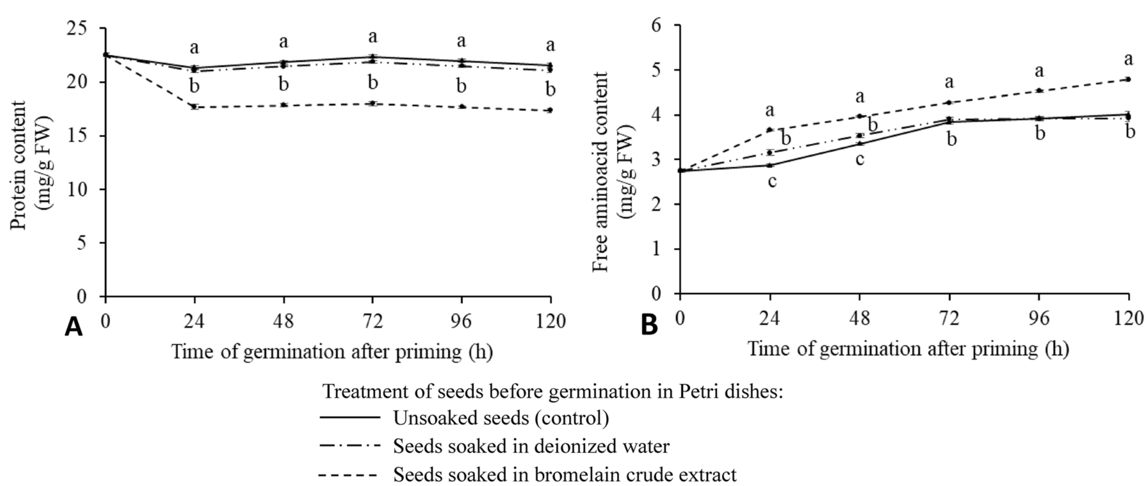

Fig. 2 Soluble protein **A** and free aminoacid **B** levels in *Capsicum annuum* L. seeds during germination in Petri dishes after priming. Results with the same *letter* are not statistically different when compared within time intervals (One-Way ANOVA, Tukey, $p > 0{,}05$). Vertical bars represent $\pm$ SE

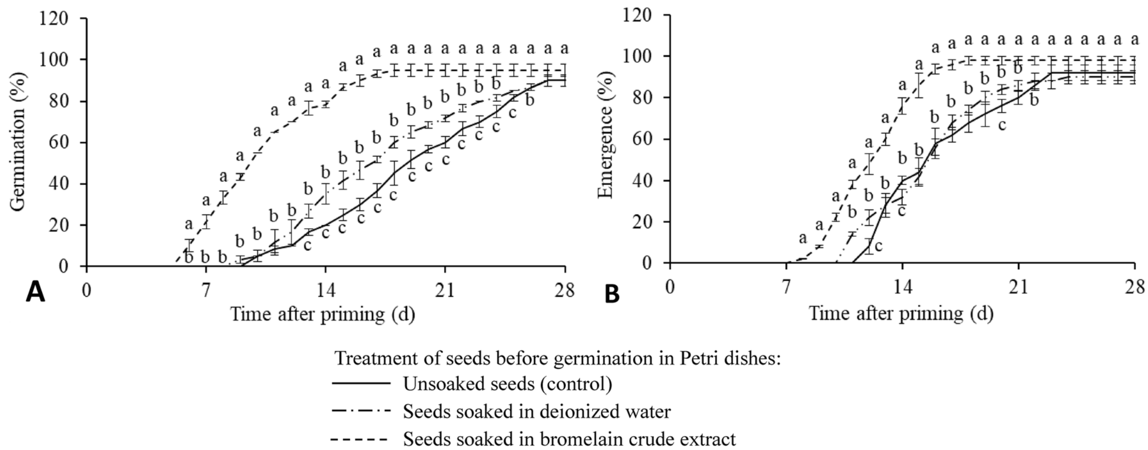

Fig. 3 Germination **A** and emergence **B** after *Capsicum annuum* L. seed priming. Results with the same *letter* are not statistically different when compared within time intervals (One-Way ANOVA, Tukey, $p > 0,05$). Vertical bars represent ± SE

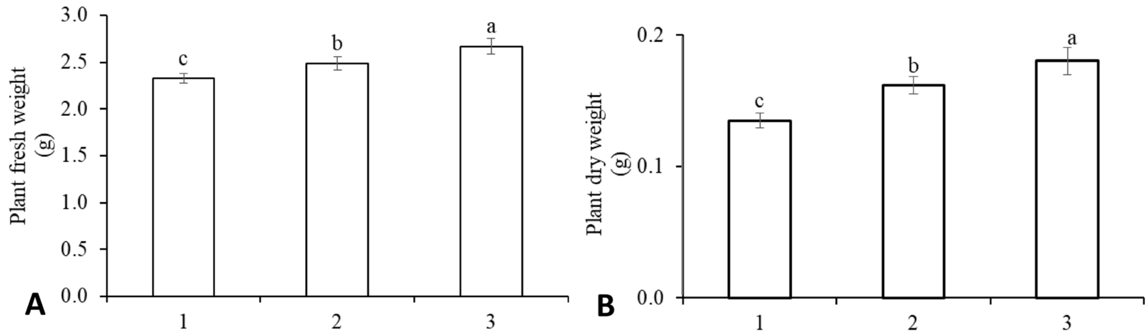

Fig. 4 *Capsicum annuum* L. plant fresh **A** and dry **B** weights at 28 d of growth in soil. Legend: (1) Unsoaked seeds (control); (2) Seeds soaked in deionized water; (3) Seeds soaked in bromelain crude extract. Results with the same *letter* are not statistically different (One-Way ANOVA, Tukey, $p > 0,05$). Vertical bars represent ± SE

The contents of free amino acids in the treatment and control seeds were in agreement with these observations (Fig. 2B). The relationship observed between the content of soluble proteins and free amino acids has been described in seeds of different plants during the mobilization of reserves. Satyanarayana et al. (2011) observed a decrease in the content of soluble proteins in the cotyledons of *Sterculia urens* Roxb. seeds and an accompanying increase in the content of free amino acids over a 9 d germination period. Aragão et al. (2015) also observed a significant decrease in ethanol-soluble proteins and an increase in the total content of free amino acids during germination (5–7 days) in *Cedrela fissilis* Vellozo seeds, which suggests that amino acids could be made available by the mobilization of proteins stored in mature seeds.

The results of the present study indicate that the priming of the seeds with crude bromelain extract has a beneficial effect on the pepper seeds, increasing their initial germination percentage. The results collectively suggest that bromelain-priming enhanced the rate of hydrolysis of storage proteins promoting the mobilization of protein reserves and therefore the generation of amino acids. The amino acids obtained can then be used in the biosynthesis of enzymes, hormones, proteins, pyrimidines and purine bases, which contribute to the germinative development of the embryo (Aragão et al. 2015). This may explain the superior germination observed in bromelain-primed seeds. In addition, the crude bromelain extract contains other compounds such as minerals, sugars, vitamins and amino acids that may have accelerated the development of the embryo and contributed to increasing the speed of germination, which coincides with that indicated by Mavi and Atak (2016). The findings suggest for the use of bromelain extracts for priming pepper seeds, since the hastening of germination through increased reserve mobilization during the early stages of germinative development can promote the seedling establishment and subsequent growth.

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Author contribution LP, YA, LN, CC, CL, S, JCL and AP designed the research; LP, YA, LN, CC and CL conducted the experiments; LP, YA, S, JCL and AP analyzed the data; LP, YA, S, JC and AP wrote the paper; S, JCL and AP had primary responsibility for the final content. All authors have read and approved the final manuscript.

Declaration

Conflict of interest Authors do not have any conflict of interests.

Human and animal rights This research did not involve experiments with human or animal participants.

Informed consent Informed consent was obtained from all individual participants included in the study. Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

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