# Fungal and Plant Extracts Improve Germination and Early Development of Urochloa brizantha

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# Fungal and Plant Extracts Improve Germination and Early Development of *Urochloa brizantha*

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**Abstract**

*Urochloa brizantha* is widely used in tropical livestock systems, but its seeds often germinate slowly and unevenly because external structures impose a physical barrier. This study evaluated seed scarification and biomolecule-rich extracts from *Trichoderma orientale* and *Pereskia aculeata* Mill. as strategies to improve germination and early seedling development of *U. brizantha* cv. MG-5. The experiment used a 5 × 2 factorial design comprising five seed treatments and two seed conditions (scarified and non-scarified). Fungal extracts were produced by solid-state fermentation of *T. orientale* on orange peel or maize cob, whereas the plant extract was obtained from *P. aculeata* leaves. Proteolytic properties of the extracts and seed germination, seedling growth, and biomass accumulation were evaluated. The orange-peel extract had the highest proteolytic activity (200 U $mL^{-1}$). The orange-peel and maize-cob extracts exhibited similarly high specific proteolytic activities, whereas the *P. aculeata* extract showed the highest protein concentration (0.502 mg $mL^{-1}$). Scarification increased final germination from 50% to 69%, a gain of 19 percentage points, and improved total seedling length and biomass accumulation. All seed treatments increased germination speed, first germination count, and final germination relative to the water control. The *P. aculeata* extract also promoted greater shoot growth and biomass production. These findings indicate that removing external seed structures effectively reduces the physical constraint on germination and that biomolecule-rich extracts can further support early seedling development. Seed scarification and bioactive extracts therefore represent promising complementary strategies for improving the physiological performance of *U. brizantha* seeds.

**Keywords:** forage, germination, dormancy, biomass, enzymatic extracts, biomolecules.

## 1. INTRODUCTION

Species of *Urochloa* (syn. *Brachiaria*) are among the most widely cultivated tropical forage grasses and constitute a major feed source for ruminants throughout tropical and subtropical regions worldwide [1]. In addition to their use as forage, these grasses are grown as cover crops, helping to mitigate environmental impacts by reducing nutrient losses through leaching [2]. They can also alleviate soil compaction and enhance carbon sequestration, thereby improving the physical, chemical, and biological properties of the soil and contributing to more sustainable production systems [3].

Most tropical forage grasses, including *Urochloa* spp., are propagated from seed [1]. Seed quality is therefore critical to successful pasture establishment, highlighting the need for strategies that improve germination performance [4]. However, *Urochloa* species often show poor germination because of uneven inflorescence emergence, asynchronous flowering within racemes, a low proportion of fertile seeds, pronounced natural seed shattering, and dormancy [5]. Consequently, commercial seed lots may vary considerably in physiological quality and dormancy level. Dormancy in *Urochloa* spp. is thought to be imposed by seed-covering structures, with its intensity varying among cultivars [6–10]. Such variability can impair germination, compromise uniform pasture establishment, and restrict seed marketing [8].

Given these challenges, several strategies have been investigated to improve germination in *Urochloa* species. Among these, the application of biomolecule-rich extracts has received increasing attention because such extracts may contain enzymes involved in metabolic pathways that regulate the synthesis and degradation of compounds associated with seed germination [4]. This approach may provide a promising and sustainable alternative for developing biofertilizer-based seed treatments [11]. In addition, the controlled removal of seed-covering structures without damaging the embryo may further enhance germination.

Microorganisms, particularly fungi, are major sources of enzymes with biotechnological potential. Although ecotones such as the transition zone between the Cerrado and Caatinga biomes harbor substantial fungal diversity, many fungal species remain underexplored for

biotechnological applications [12, 13]. Among these fungi, *Trichoderma* species are particularly noteworthy because of their well-documented beneficial effects in agriculture [14–17].

Fungal enzymes can be produced through fermentation using agro-industrial residues and by-products as cultivation substrates [18, 19]. Maize cobs are an abundant agro-industrial residue, with approximately 18 kg generated for every 100 kg of maize harvested [20]. Accordingly, using these materials as fermentation substrates is economically and environmentally promising because it adds value to agro-industrial residues, reduces their environmental impact, and may generate additional revenue for the industries involved [13].

Plants are another promising source of enzymes with biotechnological potential. One notable example is *Pereskia aculeata* Mill., commonly known in Brazil as ora-pro-nóbis [21]. Although its fruits and flowers are also edible, the leaves are the most widely marketed and consumed part of the plant [22], largely because of their high nutritional value. On a dry-matter basis, the leaves contain 23% protein, 31% carbohydrates, 14% minerals, 8% lipids, and 4% soluble dietary fiber [23].

Accordingly, this study evaluated seed scarification and treatment with biomolecule-rich extracts as strategies to improve seed germination and early seedling development in *Urochloa brizantha* cv. MG-5. The extracts were obtained from cultures of *Trichoderma orientale* grown by fermentation using orange peel and maize cobs as substrates and from leaves of *Pereskia aculeata* Mill.

## 2. MATERIALS AND METHODS

### 2.1 Location

The experiment was conducted at the Genetics Laboratory of the Federal University of Piauí (UFPI), Professora Cinobelina Elvas Campus (CPCE), located in Bom Jesus, Piauí, Brazil (09°04′28″ S, 44°21′31″ W).

The seeds of *Urochloa brizantha* cv. MG-5 used in this study were obtained from Riza Sementes, located in Unaí, Minas Gerais, Brazil. The seeds belonged to the 2022/2023 crop year and were supplied as a commercial seed lot.

The leaves of *Pereskia aculeata* used for extract preparation were obtained from UFPI-CPCE, located in Bom Jesus, Piauí, Brazil (09°04′28″ S, 44°21′31″ W). The plant material was collected from cultivated plants maintained under local agricultural conditions.

The orange peel of *Citrus sinensis* L. Osbeck and maize cobs of *Zea mays* L. used as substrates for solid-state fermentation were obtained from UFPI-CPCE, located in Bom Jesus, Piauí, Brazil. These materials consisted of agro-industrial residues and were used only as fermentation substrates after drying, grinding, sieving, and sterilization.

## 2.2 Evaluation of the physiological characteristics of the seeds

In the laboratory, *Urochloa brizantha* seeds were stored in a cold chamber at 10 °C and 50% relative humidity (RH) to maintain seed quality throughout the experimental period. The seeds were processed to remove impurities and subsequently homogenized to obtain the working sample, according to the Rules for Seed Testing [24]. Prior to treatment application, the moisture content of the seed lot was determined by the oven-drying method at 105 ± 3 °C for 24 h, according to the Rules for Seed Testing [24] . The seed lot had a moisture content of 10.65%. For seed lot characterization, the following determinations and tests were performed: viability by the tetrazolium test (TZ), germination percentage (G), first germination count (FGC), and germination speed index (GSI), according to the Rules for Seed Testing [24].

### *2.2.1 Tetrazolium test (TZ)*

In the laboratory, *U. brizantha* seeds were stored in a cold chamber at 10 °C and 50% relative humidity (RH) to maintain seed quality throughout the experimental period. To facilitate the absorption of the tetrazolium solution, the seeds were pre-moistened for 18 hours at 30 °C. Then, they were longitudinally sectioned with the help of tweezers and a scalpel and immersed in a 0.5% solution of 2,3,5-triphenyl tetrazolium chloride in dark bottles, and subsequently kept in a BOD germination chamber at 30 °C for three hours [24] . After this period, the seeds were evaluated as viable or non-viable based on the location and intensity of the coloration of their structures (Fig. 1).

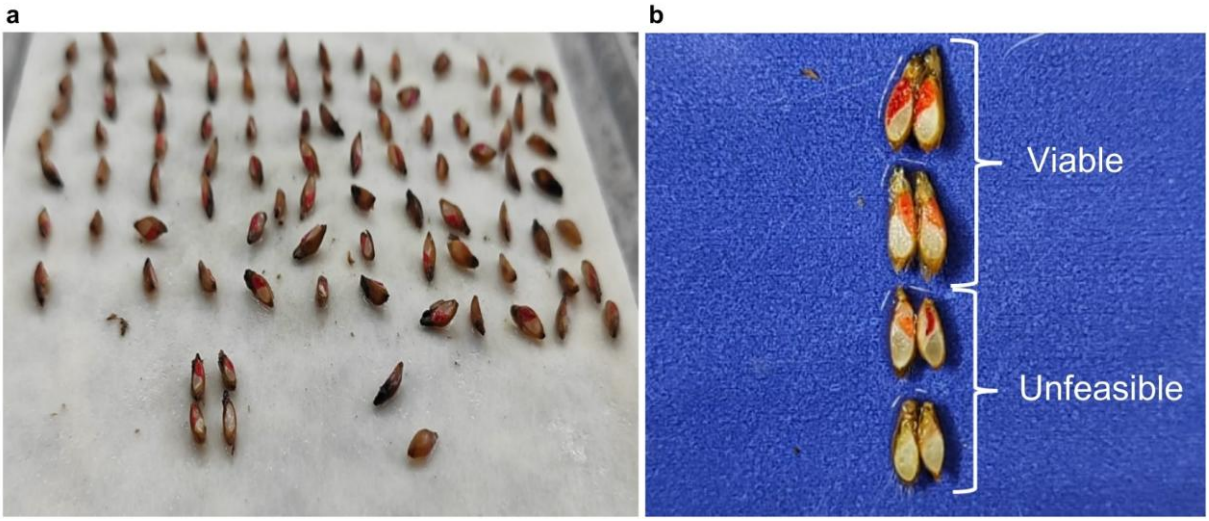



**Fig. 1** Assessment of *Urochloa brizantha* cv. MG-5 seed viability using the tetrazolium test. (A) Seeds after immersion for 3 h in a 2,3,5-triphenyl tetrazolium chloride solution. (B) Classification of seeds as viable or nonviable based on the location and intensity of embryo staining. Red staining, resulting from the formation of triphenyl formazan, indicates living, metabolically active tissues, whereas the absence of staining indicates dead tissues and nonviable seeds.

*2.2.2 Germination (G)*

The germination test was conducted with four repetitions of 100 seeds, sown on two sheets of white blotting paper, previously moistened with distilled water in a volume corresponding to 2.5 times the dry weight of the paper. The seeds were placed in acrylic Gerbox boxes and kept in a BOD (Biochemical Oxygen Demand) chamber under an alternating temperature regime of 20 to 35 °C, with a photoperiod of 12 hours of darkness and 12 hours of light. The germination test was conducted for 21 days after sowing.

At the end of the germination test, seedlings were classified as normal or abnormal according to the criteria established in the Rules for Seed Testing [24]. Normal seedlings were those with all essential structures sufficiently developed to ensure continued growth into normal plants. Abnormal seedlings were those presenting missing, damaged, deformed, or deteriorated essential structures that impaired their normal development. Seeds that did not

produce seedlings by the end of the evaluation period were classified as ungerminated. Germination percentage was calculated as the proportion of normal seedlings relative to the total number of seeds sown.

*2.2.3 First germination count (FGC)*

The test was conducted in conjunction with the germination test, with the evaluation being performed on the seventh day, according to the guidelines established by RAS [24] for the species under study.

*2.2.4 Germination speed index (GSI)*

The GSI was determined by daily counts of germinated seeds, according to the methodology described by Maguire [25]. The calculation was performed using the following formula:

$$GSI = \left(\frac{G1}{N1}\right) + \left(\frac{G2}{N2}\right) + \cdots \left(\frac{Gn}{Nn}\right)$$

Where: GSI=germination speed index; G = number of seedlings counted on the day of the count; N = number of days after sowing when the count was made.

**2.3 Production of fungal metabolites by Solid State Fermentation (SSF)**

*2.3.1 Cultivation and origin of Trichoderma*

The fungal isolate of *Trichoderma orientale* (UFPI14) was obtained from the biocontrol collection of the Phytopathology Laboratory at the UFPI-CPCE. This isolate was collected from an ecotone between the Caatinga and Cerrado biomes in the state of Piauí, Brazil, located at the geographical coordinates 8°51'7.48" S latitude and 44°11'39.95" W longitude. The reactivation of *Trichoderma* was carried out on potato dextrose agar (PDA) medium in Petri dishes, with incubation for seven days at a temperature of 28 °C.

*2.3.2 Inoculation of Trichoderma in substrates from agro-industrial waste*

To obtain the enzymatic extracts, a fungal suspension was prepared from the isolate, with spores quantified using a Neubauer chamber and adjusted to a final concentration of $1\times10^7$ spores/mL. The suspension was inoculated onto the substrates along with a sterile solution containing 0.5% (w/v) yeast extract and 1% (w/v) glucose. Each 125 mL Erlenmeyer flask contained 3 g of substrates. The substrates used were agro-industrial residues of orange peel (*Citrus sinensis* (L.) Osbeck) and maize cobs (*Zea mays* L.), standardized using sieves with particle sizes ranging from 1.0 to 2.0 mm, all of which were previously autoclaved at 121 °C for 20 minutes. After inoculation, the substrate moisture was adjusted to 40%, followed by incubation of the flasks in an oven at 25 °C.

*2.3.3 Metabolic extract from the fermentation process*

The metabolic extract was obtained after 72 hours of fermentation, with 7.5 mL of phosphate buffer (pH 7, 245 mM) added for each gram of substrate, according to Nascimento et al. [26]. Subsequently, the Erlenmeyer flasks were placed on an orbital shaker at 150 rpm for 60 minutes at room temperature. The content was then filtered through gauze and centrifuged at 8,000 rpm for 10 minutes. The resulting supernatant was referred to as the metabolic extract and was stored in a freezer for future analyses.

**2.4 Metabolic extract of *Pereskia aculeata***

*Pereskia aculeata* leaves were washed with distilled water, dried at 33 °C for 3 days, and subsequently ground in a knife mill for extract preparation. Metabolites were extracted using a 0.15 M NaCl buffer adjusted to pH 7.0. Plant material was added at a concentration of 10% (w/v), and the mixture was agitated for 2 h at room temperature (25 °C). The solution was then filtered through gauze and centrifuged at 3000 x g for 15 minutes to obtain the metabolic extract.

## 2.5 Enzymatic quantification

### *2.5.1 Proteolytic activity*

Proteolytic activity was determined according to the method of Ginther [27]. For the assay, 1 mL aliquots containing 0.2 M Tris-HCl buffer (pH 7.2), $10^{-3}$ M $CaCl_2$, 1.0% (w/w) azocasein, and 150 µL of the crude enzymatic extract were incubated at 28 °C for 1 hour. The reaction was then stopped by the addition of 1.0 mL of 10% (w/v) trichloroacetic acid solution.

After centrifugation at 8000 rpm for 15 minutes, 800 µL of the supernatant were transferred to a second tube containing 200 µL of 1.8 N NaOH. The samples were homogenized in a vortex mixer, and the absorbance was measured at 420 nm. Enzymatic activity was defined as the amount of enzyme required to produce a 0.1 increase in absorbance per hour.

### *2.5.2 Protein quantification*

The protein quantification was performed using the method described by Bradford [28], with bovine serum albumin (BSA) as the standard for constructing the calibration curve, with concentrations ranging from 0 to 1,000 µg $mL^{-1}$. The analyses were carried out in triplicate, and the average values were calculated after correcting for the corresponding blank.

### *2.5.3 Specific activity*

The specific activity of the protease was expressed in U $mg^{-1}$ of protein, determined by the ratio between the enzymatic activity of the extract and the protein content (mg) quantified in the sample.

## 2.6 Seed treatment

The experiment was conducted in a completely randomized design using a 5 × 2 factorial arrangement, consisting of five seed treatments and two seed conditions. The treatments were: distilled water as the control (T1), a commercial mineral fertilizer-based product (T2), a metabolic extract of *T. orientale* grown on orange peel (T3), a metabolic extract

of *T. orientale* grown on maize cob (T4), and an extract obtained from *Pereskia aculeata* leaves (T5). The two seed conditions were scarified and non-scarified seeds of *U. brizantha* cv. MG-5.

For scarification, the glumes, lemmas, and paleas were manually removed from the seeds (Fig. 2). Each treatment combination comprised four replicates of 50 seeds, totaling 200 seeds per treatment combination.

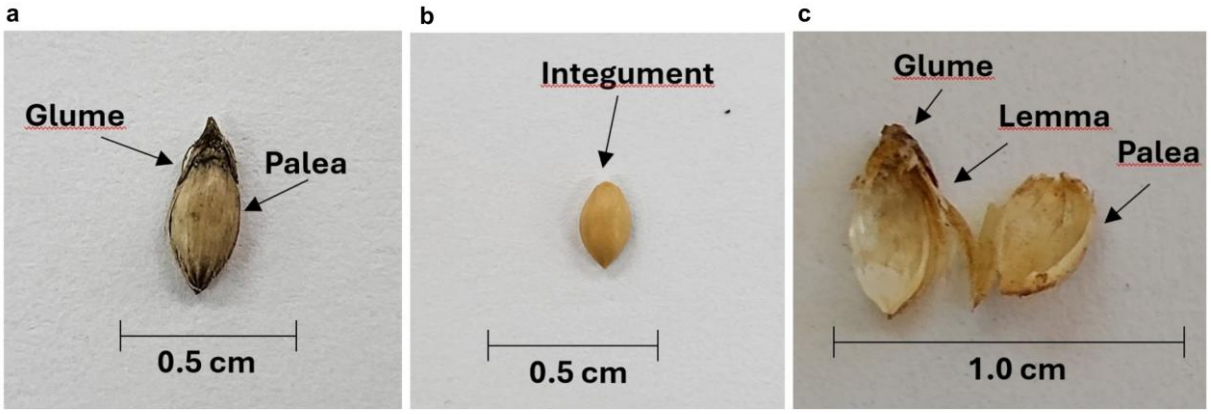



**Fig. 2** Structure of *U. brizantha* cv. MG-5; whole seed (a), naked seed (without glumes, lemmas, and paleas) (b), glumes, lemmas, and paleas (c)

For each treatment, the seeds were immersed in the respective solution for 1 h. The following variables were evaluated according to the criteria established in the Rules for Seed Testing (Brasil, 2009): germination speed index (GSI), first germination count (FGC), and germination percentage (G). Initial seedling development was also assessed based on shoot fresh mass (SFM), root fresh mass (RFM), total fresh mass (TFM), shoot dry mass (SDM), root dry mass (RDM), total dry mass (TDM), shoot length (SL), root length (RL), and total seedling length (TSL). For the variables GSI, FGC, and G, the same methodology used in the test for evaluating the physiological characteristics of the seeds was adopted.

*2.6.1 Shoot fresh mass, root fresh mass and total fresh mass.*

The same ten seedlings selected for the length analysis were used for the determination of fresh mass. For this, each seedling was carefully removed, and excess

surface moisture was eliminated with paper. Then, the weighing was performed using an analytical precision balance to ensure the accuracy of the measurements.

*2.6.2 Shoot dry mass, root dry mass and total dry mass.*

After obtaining the fresh mass, the seedlings were placed in properly labeled paper bags and transferred to an oven with forced air circulation, maintained at a constant temperature of 60 °C for a period of 72 hours. At the end of this period, the dry mass of each replication was determined using an analytical precision balance. The values obtained were expressed in grams, considering the average of the measurements.

*2.6.3 Shoot length, root length and total seedling length.*

At the end of the germination test, the ten most vigorous seedlings from each replication within each treatment were selected, prioritizing those that best represented the characteristics influenced by the respective treatments. To avoid bias in the selection, atypical or non-representative seedlings were excluded. The length measurements were made using a millimeter ruler.

*2.6.4 Seedling Vigor Index I (SVI I)*

Seedling vigor index I was calculated as the product of the final germination percentage and the mean total seedling length, according to the following equation:

$$SVI\ I = G \times TSL$$

where $G$ is the final germination percentage (%) and $TSL$ is the mean total seedling length, corresponding to the sum of shoot and root lengths (cm).

*2.6.5 Seedling vigor index II (SVI II)*

Seedling vigor index II was calculated as the product of the final germination percentage and the mean total seedling dry mass:

$$SVI\ II = G \times TDM$$

where $G$ is the final germination percentage (%) and $TDM$ is the mean total seedling dry mass, corresponding to the sum of shoot and root dry masses (g).

*2.6.6 Seedling water content (WC)*

Seedling water content was calculated on a fresh-mass basis from the total fresh and dry masses determined for each experimental unit:

$$WC(\%) = \frac{TFM - TDM}{TFM} \times 100$$

where $TFM$ and $TDM$ are the total fresh and dry masses of the seedlings, respectively. The results were expressed as percentages.

**2.7 Statistical analysis**

The data was subjected to analysis of variance (ANOVA). Residual normality and homogeneity of variances were assessed using the Shapiro–Wilk and Bartlett tests, respectively. When significant effects were detected ($P \leq 0.05$), treatment means were compared using the Scott–Knott test at the 5% significance level. All univariate analyses were performed in R software (version 4.4.0). Pearson's correlation coefficients were subsequently calculated among the evaluated variables, and a correlation network was generated to graphically represent these relationships, with the proximity between nodes proportional to the absolute correlation coefficients. Correlation analyses and network construction were performed using the RBio package [29].

## 3. RESULTS

### 3.1 Enzymatic quantification

Proteolytic activity differed among the extracts, with T3 showing the highest value (200 U $mL^{-1}$), followed by T4 (75.07 U $mL^{-1}$) and T5 (27.83 U $mL^{-1}$) (Fig. 3a). Protein concentration was highest in T5 (0.502 mg $mL^{-1}$), intermediate in T3 (0.340 mg $mL^{-1}$), and lowest in T4 (0.120 mg $mL^{-1}$) (Fig. 3b). Specific proteolytic activity did not differ between T3 and T4 (588.24 and

625.58 U $mg^{-1}$, respectively), and both extracts showed higher values than T5 (55.43 U $mg^{-1}$) (Fig. 3c).

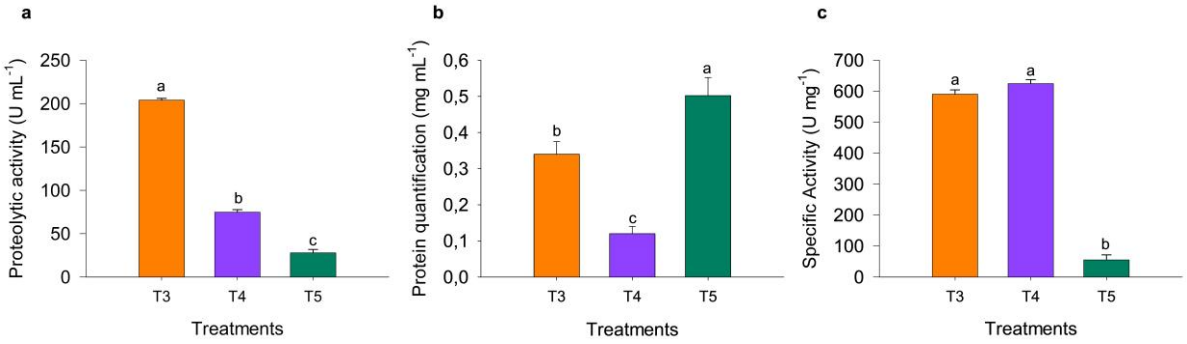



**Fig. 3** Proteolytic activity (a), protein concentration (b), and specific activity (c) of extracts obtained from *Trichoderma orientale* cultivated on orange peel (T3) or maize cob (T4) and from *Pereskia aculeata* leaves (T5). Bars represent means ± standard deviation. Means followed by the same letter within each panel do not differ according to the Scott-Knott test at the 5% significance level

### 3.2 Physiological characteristics of seeds

Based on the results of the initial seed physiological quality assessment, it was found that the seeds showed 86% viability in the tetrazolium test, meeting the minimum standards required for commercialization. This method has been widely used in physiological quality assessment because it provides rapid results, allowing differentiation between viable and non-viable seeds even before germination begins. However, the germination test analysis revealed a low germination speed index (GSI = 7.19), resulting in a first germination count of only 27.00%, and a germination percentage of 55%.

### 3.3 Seed treatment

Analysis of variance revealed a significant interaction between seed treatment and seed scarification only for root length (RL) (Table S1). Seed treatment and scarification both significantly affected germination speed index (GSI), first germination count (FGC),

germination percentage (G), total dry mass (TDM), and seedling vigor index I (SVI I). Seed treatment alone affected shoot fresh mass (SFM), shoot dry mass (SDM), shoot length (SL), and seedling vigor index II (SVI II), whereas scarification alone affected root fresh mass (RFM), total fresh mass (TFM), root dry mass (RDM), and total seedling length (TSL). Seedling water content (WC) was not significantly affected by seed treatment, scarification, or their interaction ($p > 0.05$).

*3.3.1 Germination test*

All seed treatments significantly improved the germination speed index, first germination count , and final germination percentage compared with the control (Table 1). The GSI was 5.82 in T1 and ranged from 8.30 in T3 to 9.42 in T2 among the treated seeds, with no significant differences among these treatments. Similarly, FGC increased from 40.00% in T1 to values ranging from 56% in T4 to 62% in T2, while final germination increased from 44% to values ranging from 61% in T4 to 67% in T2. All treated seeds therefore exceeded the minimum germination standard of 60% required for the commercial sale of *Urochloa brizantha* seed.

**Table 1** Average values of the germination speed index (GSI), first germination count (FGC), and germination (G) of *U. brizantha* cv. MG-5 seeds subjected to seed treatment

| Treatments | Variables | | |
|---|---|---|---|
| | GSI | FGC (%) | G (%) |
| T1 | 5.82 b | 40.00 b | 44.00 b |
| T2 | 9.42 a | 62.00 a | 67.75 a |
| T3 | 8.30 a | 58.00 a | 63.00 a |
| T4 | 8.32 a | 56.00 a | 61.00 a |
| T5 | 8.51 a | 57.00 a | 63.00 a |

Means followed by the same letter within a column do not differ according to the Scott–Knott test at the 5% significance level. T1: control; T2: commercial product; T3: fungal enzymatic extract: orange peel; T4: fungal enzymatic extract: maize cob and T5: enzymatic extract: Pereskia aculeata leaves.

The results indicate that dormancy in *U. brizantha* cv. MG-5 seeds is at least partly associated with the physical barrier imposed by seed-covering structures. This interpretation is supported by the significantly higher germination speed index, first germination count, and final germination percentage of scarified seeds compared with non-scarified seeds (Table 2).

Scarified seeds had a GSI of 10.25, which was 73.7% higher than the value of 5.90 recorded for non-scarified seeds. Similarly, FGC increased from 43% in non-scarified seeds to 66% in scarified seeds, a difference of 23 percentage points. Final germination increased from 50% to 69%, corresponding to a difference of 19 percentage points.

**Table 2** Average values of the germination speed index (GSI), first germination count (FGC), and germination (G) of *U. brizantha* cv. MG-5 seeds subjected to the scarification process

| Seed | Variables | | |
|---|---|---|---|
| | GSI | FGC (%) | G (%) |
| Scarified | 10.25 a | 66.00 a | 69.00 a |
| Not scarified | 5.9 b | 43.00 b | 50.00 b |

Means followed by the same letter within a column do not differ according to the Scott–Knott test at the 5% significance level.

The results suggest that seed-covering structures, including the glumes, lemmas, and paleae, act as physical barriers that may restrict or delay germination. The effects of this physical constraint are illustrated in Supplementary Figs. S1-S5, which compare the germination of scarified and non-scarified seeds across treatments.

*3.3.2 Seedling Development*

Seed treatments significantly affected seedling growth (Table 3). Shoot fresh mass was significantly higher in T3, T4, and T5 than in T1 and T2, which did not differ from each other. T5 produced the highest shoot dry mass and total dry mass, exceeding all other treatments. Shoot length was also significantly greater in T4 and T5 than in the remaining treatments.

**Table 3** Mean shoot fresh mass (SFM), shoot dry mass (SDM), total dry mass (TDM), and shoot length (SL) of *Urochloa brizantha* cv. MG-5 seedlings as affected by seed treatment.

| Treatments | Variables | | | |
|---|---|---|---|---|
| | SFM (g) | SDM (g) | TDM (g) | SL (cm) |
| T1 | 0.22 b | 0.025 b | 0.064 b | 7.16 b |
| T2 | 0.19 b | 0.024 b | 0.064 b | 6.54 b |
| T3 | 0.24 a | 0.025 b | 0.067 b | 6.80 b |
| T4 | 0.27 a | 0.026 b | 0.067 b | 7.55 a |
| T5 | 0.26 a | 0.029 a | 0.072 a | 8.28 a |

Means followed by the same letter within a column do not differ according to the Scott–Knott test at the 5% significance level. T1: control; T2: commercial product; T3: fungal enzymatic extract: orange peel; T4: fungal enzymatic extract: maize cob; and T5: enzymatic extract: *Pereskia aculeata* leaves.

The results also showed that seed scarification positively influenced seedling development (Table 4). Scarified seeds had significantly higher values for RFM, TFM, RDM, TDM, and TSL than non-scarified seeds. These findings suggest that scarification enhances germination and early seedling growth, likely by facilitating water uptake and accelerating imbibition. Improved water uptake may promote faster and more uniform germination, thereby contributing to seedling establishment. Consequently, scarified seeds accumulated more biomass and exhibited greater structural development, highlighting scarification as an effective strategy for improving plant establishment during the early growth stages.

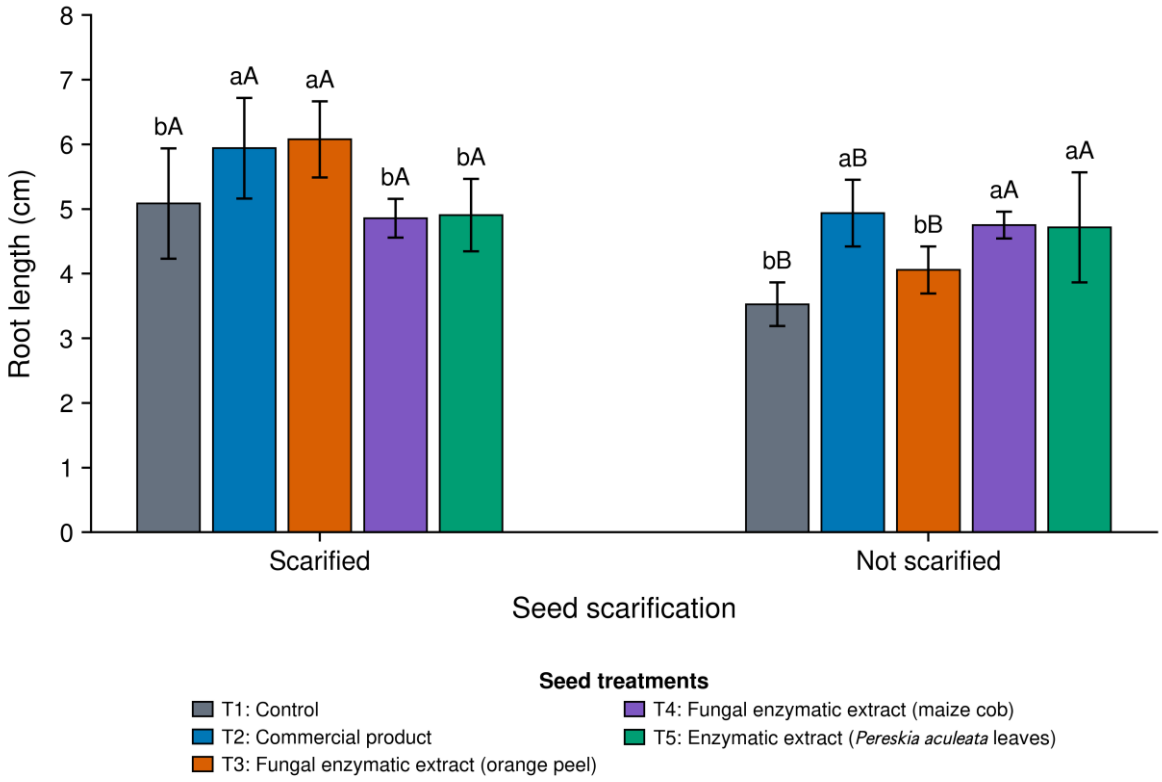
**Table 4** Average values of root fresh mass (RFM), total fresh mass (TFM), root dry mass (RDM), total dry mass (TDM), and total seedling length (TSL) of *Urochloa brizantha* cv. MG-5 subjected to seed scarification.

| Seed | Variables | | | | |
|---|---|---|---|---|---|
| | RFM (g) | TFM (g) | RDM (g) | TDM (g) | TSL (cm) |
| Scarified | 0.15 a | 0.40 a | 0.046 a | 0.072 a | 12.93 a |
| Not scarified | 0.12 b | 0.35 b | 0.036 b | 0.061 b | 11.38 b |

Means followed by the same letter within a column do not differ according to the Scott-Knott test at the 5% significance level.

The interaction between seed treatment and scarification for seedling root length is shown in Figure 4. Among scarified seeds, T2 and T3 produced significantly longer roots than the other treatments. Among non-scarified seeds, T2, T4, and T5 produced significantly longer roots than the remaining treatments.

Within T1, T2, and T3, scarified seeds had significantly longer roots than non-scarified seeds. In T4 and T5, however, root length did not differ significantly between scarified and non-scarified seeds. These results indicate that the effect of scarification on root development depended on the seed treatment, highlighting the importance of considering the interaction between these factors when selecting strategies to improve early seedling growth.




415

416 **Fig. 4** Root length of *Urochloa brizantha* cv. MG-5 seedlings as affected by seed scarification
417 and seed treatment. Bars represent means ± standard deviation. Lowercase letters compare
418 seed treatments within each scarification condition, whereas uppercase letters compare
419 scarification conditions within each seed treatment. Means followed by the same letter do not
420 differ according to the Scott-Knott test at the 5% significance level.

421

422 Across scarification conditions, the commercial product and enzymatic extracts did not
423 differ from one another and produced higher vigor indices than the control (Fig. 5a). SVI I
424 ranged from 756.89 to 817.62 under T2-T5, compared with 512.55 under T1, corresponding to
425 increases of 47.7-59.5%. Similarly, SVI II ranged from 3.97 to 4.46 under T2-T5, compared
426 with 2.77 under T1, representing increases of 43.3-61.1%.

427 Scarification increased SVI I by 53.5%, from 579.36 in non-scarified seeds to 889.10 in
428 scarified seeds. In contrast, scarification did not affect SVI II. Seedling water content was
429 unaffected by seed treatment ($p = 0.163$), scarification ($p = 0.704$). Treatment means ranged

from 79.61 to 83.90%, while the marginal means for scarified and non-scarified seeds were 81.68 and 81.26%, respectively.

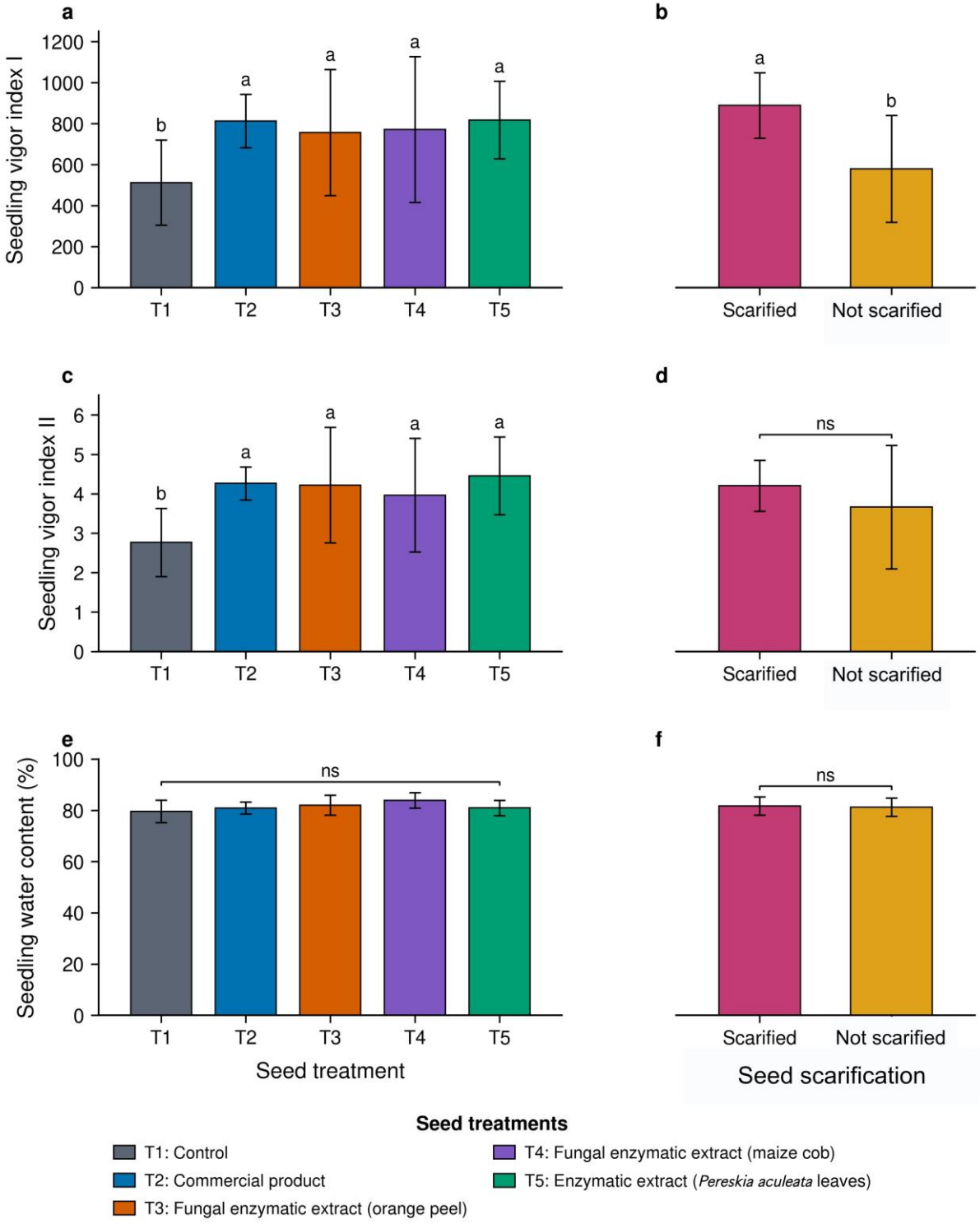



**Fig. 5** Seedling vigor indices I (a, b) and II (c, d) and seedling water content (e, f) of *Urochloa brizantha* cv. MG-5 seedlings as affected by seed treatment and seed scarification. Bars represent means ± standard deviation. Within each panel, means followed by different

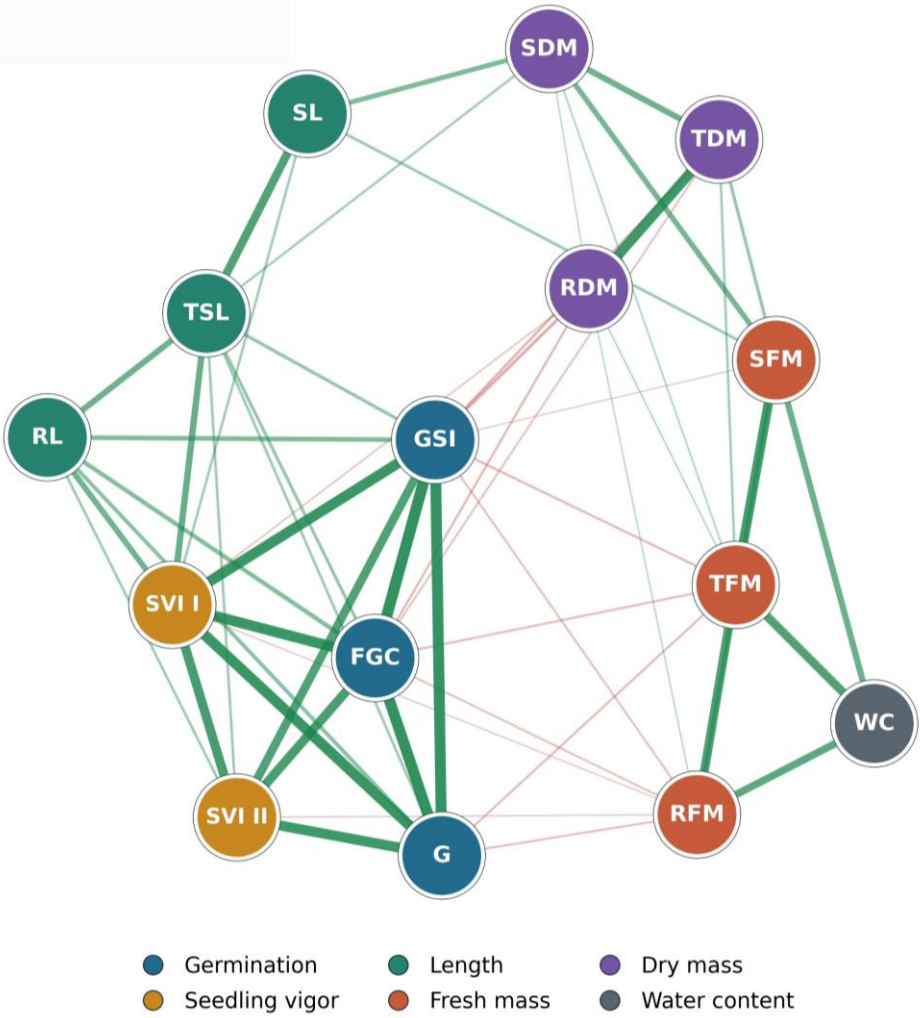
lowercase letters differ according to the Scott-Knott test at the 5% significance level, whereas ns= indicates no significant difference.

The correlation network revealed distinct patterns of association among the evaluated morphophysiological traits (Fig. 6). All correlations described below were significant ($p < 0.05$). The germination variables were strongly interconnected: GSI was correlated with FGC ($r$ = 0.99) and final germination ($r$ = 0.97), while FGC was correlated with final germination ($r$ = 0.98). SVI I showed strong correlations with GSI, FGC, and final germination (all $r$ = 0.94). Similarly, SVI II was correlated with final germination ($r$ = 0.92), FGC ($r$ = 0.86), and GSI ($r$ = 0.83). The two vigor indices were also strongly correlated ($r$ = 0.87).

Germination and seedling vigor were positively associated with seedling length. RL was correlated with GSI ($r$ = 0.58), FGC ($r$ = 0.55), and final germination ($r$ = 0.52), whereas TSL was correlated with GSI ($r$ = 0.47), FGC ($r$ = 0.47), and final germination ($r$ = 0.43). TSL was strongly correlated with SL ($r$ = 0.82) and RL ($r$ = 0.67). SVI I was also correlated with TSL ($r$ = 0.69), RL ($r$ = 0.65), and SL ($r$ = 0.42), while SVI II was correlated with both RL and TSL ($r$ = 0.43).

The fresh-mass variables formed another strongly interconnected group. TFM was positively correlated with SFM ($r$ = 0.89), RFM ($r$ = 0.84), and WC ($r$ = 0.83). WC was also correlated with RFM ($r$ = 0.76) and SFM ($r$ = 0.68). In addition, TFM showed weaker positive correlations with TDM ($r$ = 0.41), SDM ($r$ = 0.35), and RDM ($r$ = 0.34).

Among the dry-mass variables, TDM was more strongly correlated with RDM ($r$ = 0.92) than with SDM ($r$ = 0.67). The main negative associations connected germination performance with biomass traits. GSI was negatively correlated with RDM ($r$ = −0.45), TDM ($r$ = −0.42), and TFM ($r$ = −0.39). FGC was negatively correlated with RDM ($r$ = −0.39) and TFM ($r$ = −0.38), while final germination was negatively correlated with TFM ($r$ = −0.38) and RFM ($r$ = −0.37). Overall, greater germination speed and percentage were associated with higher vigor indices and longer seedlings, but with lower values for some fresh- and dry-mass traits.




464

465 **Fig. 6** Pearson correlation network among germination speed index (GSI), first germination
466 count (FGC), final germination percentage (G), shoot fresh mass (SFM), root fresh mass
467 (RFM), total fresh mass (TFM), shoot dry mass (SDM), root dry mass (RDM), total dry mass
468 (TDM), shoot length (SL), root length (RL), total seedling length (TSL), seedling vigor indices I
469 and II (SVI I and SVI II), and seedling water content (WC) in *Urochloa brizantha* cv. MG-5
470 seedlings across seed treatments and scarification conditions. Only significant correlations ($p$
471 < 0.05) are shown. Green and red edges represent positive and negative correlations,
472 respectively, and edge thickness is proportional to the absolute Pearson correlation coefficient.
473 Node colors distinguish variables related to germination, seedling vigor, length, fresh mass,
474 dry mass, and water content

475

## 4. DISCUSSION

### 4.1 Enzymatic quantification

The results demonstrate that *T. orientale* has considerable potential for producing proteolytic enzymes, and that the composition of the culture medium plays a determining role in modulating both enzyme production and catalytic activity. This impact highlights the importance of carefully selecting the culture medium for optimizing enzymatic production. The activity of the proteolytic enzymes produced by *T. orientale* in the present study was higher than that reported in previous research with other fungi. Lopes et al. [30] observed an activity of 10.27 U $mL^{-1}$ in *Aspergillus niger*, while Novelli, Barros, and Fleuri [31] reported 11.89 U $mL^{-1}$ for *Aspergillus brasiliensis*, 5.10 U $mL^{-1}$ for *Aspergillus oryzae*, and 27.78 U $mL^{-1}$ for *Aspergillus flavipes.* Ortega et al. [32] evaluated 11 strains of *Fusarium graminearum*, with activities ranging between 1 and 11 U $mL^{-1}$. Prado et al. [33] reported values of up to 32.00 U $mL^{-1}$ for different species of *Aspergillus*, while Santos et al. [34] analyzed 23 strains of *Fusarium*, finding activities between 1.67 and 22.03 U $mL^{-1}$.

The production of proteolytic enzymes by fungal strains can be enhanced through the optimization of substrates [34]. In the present study, the medium containing orange peel resulted in the highest absolute proteolytic activity, which may be related to its chemical composition. Orange peel is rich in insoluble polysaccharides, such as pectin, cellulose, and hemicellulose [35, 36], in addition to having a high concentration of soluble carbohydrates, such as glucose, sucrose, and fructose. These compounds can act as easily metabolizable carbon sources, stimulating the synthesis and secretion of proteases by *T. orientale*.

On the other hand, maize cobs are predominantly composed of holocellulose (cellulose and hemicellulose) and lignin, with over 70% of its structure made up of holocellulose [37], whose monomers include hexose and pentose sugars that can be converted into valuable metabolic products through various biochemical pathways [38]. Although the absolute proteolytic activity of the *T. orientale* extract grown on maize cob (T4) was lower than that observed in the medium containing orange peel (T3), the specific activity of the enzymes was higher in this treatment. This result suggests that, despite the lower total amount of

504 extracellular proteins, the enzymatic fraction produced on the maize cob exhibited higher
505 catalytic efficiency, indicating a possible selective induction of enzymes with high specific
506 activity.

507 The results obtained in the present study highlight the potential of the fungus *T.*
508 *orientale* for the production of proteolytic enzymes from orange peel and maize cob substrates,
509 which are considered agroindustrial residues. The use of these substrates not only promotes
510 the valorization of agricultural waste, contributing to environmental sustainability, but also
511 significantly influences enzyme production and activity. These findings emphasize the
512 feasibility of using alternative, low-cost substrates to optimize enzyme production, with
513 potential applications in various industrial sectors, including the agricultural sector, such as in
514 seed treatment.

515 With regard to the extract obtained from the leaves of *Pereskia aculeata* (T5), although
516 it exhibited low enzymatic activity compared to the other treatments, it has a high protein
517 concentration. This high protein content is associated with the characteristics of the species,
518 whose protein content ranges from 17.4% to 28.4% [21, 22, 39]. The leaves of this species
519 have a higher protein content compared to other plant sources, such as corn (9-12%), wheat
520 (8-15%), and sorghum (9-17%) [40]. In addition, its proteins have high in vitro digestibility,
521 reaching approximately 85%, which enhances its significant nutritional value [22, 41].

522

523 **4.2 Physiological characteristics of seeds**

524 The results obtained from the comparison between the tetrazolium viability test and the
525 germination data showed discrepancies, indicating that the viability estimated by the
526 tetrazolium test did not directly reflect the actual germination of the seeds. These findings
527 highlight the need for strategies that improve the germination of the species, especially
528 considering the minimum germination requirement of 60% for the commercialization of
529 *Urochloa brizantha* seed lots, as established by Normative Instruction nº 30 [42].

530 The low values observed in the germination test may be associated with the presence
531 of dormancy mechanisms in the seeds of the *Urochloa* genus [8, 9, 43], as the germination

test was conducted under ideal conditions, as established by the International Seed Testing Association [44]. Previous studies indicate that *U. brizantha* presents challenges to germination due to irregular maturation and the presence of dormancy. The nature, intensity, and persistence of this phenomenon are still not fully understood. However, most studies on dormancy in this species suggest that the main cause is of a physical origin, possibly associated with the restrictions imposed by the structures surrounding the seed [6–10], such as glumes, paleas, lemma, and seed coat, which act as barriers to water absorption and oxygen diffusion, essential for germination [45], directly impacting its development in the field [7, 9, 46].

**4.3 Seed treatment**

During germination, seeds undergo a series of physiological, biochemical, and molecular processes essential for metabolic activation and the development of the embryonic axis [47, 48]. These transformations are triggered by the synthesis and activation of enzymes in the aleurone layer, a process that begins at the moment of imbibition [49, 50]. The enzymes mobilize the reserves stored in the seeds, promoting the release of compounds essential for the growth and development of the embryo [51, 52].

In the present study, it was observed that the removal of covering structures (glumes, palea, and lemma) in *U. brizantha* cv. MG-5 seeds may have favored the imbibition process and oxygen diffusion, promoting more efficient germination. Additionally, the application of the crude extracts resulted in a significant increase in germination and the initial development of seedlings. These findings highlight the importance of interventions that facilitate the mobilization of reserves and overcome the physical barriers imposed by the seed's external structures, aiming to optimize the germinative performance of this species.

All the treatments applied in the present study improved the seed germination process compared to the control treatment, promoting faster germination, measured through the germination speed index and the first count, as well as a higher final germination percentage, reaching the minimum percentage required for the commercialization of *U. brizantha*. These

results highlight the effectiveness of the treatments in improving the physiological quality of the seeds.

Seed treatments also influenced early seedling development, although the response pattern depended on the trait evaluated. T5 promoted shoot fresh mass, shoot dry mass, total dry mass, and shoot length. Root length followed a different pattern and depended on the interaction between seed treatment and scarification: T2 and T3 produced the longest roots among scarified seeds, whereas T2, T4, and T5 produced the longest roots among non-scarified seeds. Thus, the contribution of each extract was trait-specific and conditioned by the presence of the external covering structures.

The contrasting performance of T5, which promoted shoot growth and dry-mass accumulation despite its comparatively low proteolytic activity, indicates that biological effectiveness was not determined by proteolysis alone. Although hydrolytic enzymes contribute to reserve mobilization during germination [53–58], crude extracts comprise enzymatic and nonenzymatic constituents that may act jointly. Accordingly, the observed responses are best interpreted as the integrated bioactivity of each extract rather than as the isolated action of specific enzymes.

The results obtained demonstrate that, regardless of the treatment applied to the seeds, scarification is an effective technique for overcoming the physical barriers imposed by the seed coat structures of *U. brizantha* cv. MG-5. This method favors water absorption and the solutions used in the treatment, as well as optimizes gas exchange, resulting in a significant increase in the germination rate [9]. It is important to note that, despite the scarification, no changes were made to the seed coat structure, which, in species of the *Urochloa* genus, is known for its rigidity and impermeability [59]. Thus, the procedure adopted in the present study confirms its viability as a strategy to accelerate and uniformize germination, without the risk of damaging the embryo.

### 4.4 Integration of extract properties and seed responses

The biological responses of the seeds did not reproduce the ranking observed for the biochemical properties of the extracts. The orange-peel extract showed the highest volumetric proteolytic activity, whereas the orange-peel and maize-cob extracts exhibited similarly high specific activities. However, T2–T5 produced comparable germination speed, first germination count, and final germination. Moreover, the *Pereskia aculeata* extract, despite its comparatively lower proteolytic activity, promoted shoot growth and dry-mass accumulation. This contrasting response indicates that extract performance was associated with broader biochemical functionality rather than with proteolytic activity alone.

Because the treatments comprised crude extracts, their effects likely reflected the combined contribution of enzymatic and nonenzymatic constituents. Proteins, peptides, amino acids, minerals, soluble carbohydrates, and other metabolites potentially present in these matrices may have contributed to the observed responses. Thus, the response profiles obtained here provide a basis for targeted fractionation and heat-inactivation assays aimed at distinguishing the contribution of catalytic activity from that of accompanying compounds and identifying biochemical markers associated with treatment efficiency.

The correlation network reinforced this integrative interpretation by linking germination performance primarily to early-germination traits and seedling length, whereas fresh- and dry-mass variables formed partially distinct associations. The moderate inverse relationships between germination and some biomass traits suggest that faster establishment was not necessarily accompanied by greater individual biomass, possibly reflecting differences in biomass allocation or developmental stage at evaluation. These relationships are associative, and those involving SVI I, SVI II, and WC should also be considered in light of the component variables used in their calculation. The stability of final seedling WC further indicates that the treatments influenced growth and biomass accumulation without substantially altering tissue hydration at the end of the germination test.

### 4.5 Practical implications and perspectives for seed technology

Under laboratory conditions, immersion in the evaluated extracts for 1 h increased final germination from 44% in the water control to 61-68%, allowing the treated seed lot to reach the minimum germination standard for commercialization. Scarification increased final germination from 50% to 69% and improved seedling length, dry-mass accumulation, and SVI I. Together, these responses identify two complementary intervention points for improving seed performance: biochemical conditioning with bioactive crude extracts and physical reduction of the restriction imposed by the external covering structures.

The interpretation of these findings applies to the evaluated cultivar, seed lot, application conditions, and controlled germination environment. From a technological perspective, the manual removal of the covering structures provides a proof of concept for developing controlled and scalable mechanical scarification procedures. Similarly, the performance of the crude extracts supports further optimization of application rate, exposure time, formulation stability, and compatibility with seed storage. Validation across seed lots and field-emergence conditions, combined with refined biochemical characterization and dose-response assessments, may establish the robustness of these responses and support their translation into practical seed-treatment protocols.

## 5. CONCLUSION

This study demonstrates the complementary potential of scarification and crude bioactive extracts to improve the physiological performance of *Urochloa brizantha* cv. MG-5 seeds. The fungal and plant extracts produced responses comparable to the commercial product, supporting their use as alternative seed-conditioning agents. Importantly, treatment effectiveness did not follow the proteolytic-activity profile: extracts with distinct biochemical properties produced similar germination responses but differed in seedling growth and biomass allocation. This functional dissociation indicates that seed responses depend on the integrated bioactivity of the extracts and highlights *Trichoderma orientale* fermentation products from agro-industrial residues and *Pereskia aculeata* leaf extract as promising

resources for forage-seed technology. Future studies should identify the bioactive fractions responsible for these responses, optimize application conditions, and validate treatment performance across seed lots, storage periods, and field-emergence environments.

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**DECLARATIONS**

**Ethical approval**

Not applicable.

**Consent for publication**

Not applicable.

**Conflict of interest statement**

The authors declare that they have no conflict of interest.

**Permissions to collect the plants**

The collection and use of the plant materials evaluated in this study complied with applicable local and national regulations. *Pereskia aculeata* Mill. leaves were collected from cultivated plants maintained at the experimental area of the Federal University of Piauí (UFPI), Professora Cinobelina Elvas Campus, Bom Jesus, Piauí, Brazil. Orange peel and maize cobs

were obtained from commercially available agricultural products. No wild plant material was collected, and no endangered or protected species were involved in this study. Therefore, no specific collection permits or licenses were required. All procedures were conducted in accordance with institutional regulations and applicable Brazilian legislation.

## CRediT authorship contribution statement

**TdeOS**: Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **TIP**: Investigation, Conceptualization, Methodology, Formal analysis, Supervision, Resources, Validation, Writing - review & editing. **TBC**: Investigation, Formal analysis, Data curation, Visualization. **AdeSM**: Investigation. **SOG**: Investigation, Resources. **AMGS**: Investigation, Resources. **TPN**: Supervision, Investigation, Writing - review & editing.

## Funding

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## Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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