

Article

Status of the Ex Situ and In Situ Conservation of Brazilian Crop Wild Relatives of Rice, Potato, Sweet Potato, and Finger Millet: Filling the Gaps of Germplasm Collections

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Abstract: This study presents the status of ex situ and in situ conservation for the crop wild relatives of rice, potato, sweet potato, and finger millet in Brazil, and the subsequent germplasm collection expeditions. This research is part of a global initiative entitled “Adapting Agriculture to Climate Change: Collecting, Protecting, and Preparing Crop Wild Relatives” supported by the Global Crop Diversity Trust. Species of the primary, secondary, and tertiary gene pools with occurrences reported in Brazil were included: *Oryza alta* Swallen, *O. grandiglumis* (Döll) Prod., *O. latifolia* Desv., *O. glumaepatula* Steud., *Eleusine tristachya* (Lam.) Lam., *E. indica* (L.) Gaertn., *Solanum commersonii* Dunal, *S. chacoense* Bitter, *Ipomoea grandifolia* (Dammer) O'Donell, *I. ramosissima* (Poir.) Choisy, *I. tiliacea* (Willd.) Choisy, *I. triloba* L., and *I. cynanchifolia* Meisn. The status of the ex situ and in situ conservation of each taxon was assessed using the gap analysis methodology, and the results were used to plan 16 germplasm collection expeditions. Seeds of the collected material were evaluated for viability, and the protocols for seed germination and cryopreservation were tested. The final conservation score, resulting from the gap analysis and including the average of the ex situ and in situ scores, resulted in a classification of medium priority of conservation for all the species, with the exception of *I. grandifolia* (high priority). The total accessions collected (174) almost doubled the total accessions of these crop wild relatives incorporated in Embrapa's ex situ conservation system prior to 2015. In addition, accessions for practically absent species were collected for the ex situ conservation system, such as *Ipomoea* species, *Eleusine indica*, and *Solanum chacoense*. The methods used for dormancy breaking and low temperature conservation for the *Oryza*, *Eleusine*, and *Ipomoea* species were promising for the incorporation of accessions in the respective gene banks. The results show the importance of efforts to collect and conserve ex situ crop wild relatives in Brazil based on previous gap analysis. The complementarity with the in situ strategy also appears to be very promising in the country.

Keywords: crop wild relatives; gap analysis; gene banks; ex situ conservation; in situ conservation; germplasm accessions

1. Introduction

Crop wild relatives (CWRs) are an important source of variability for food security, and they have been used in the genetic improvement of various crops for multiple purposes [1]. Despite their importance, the preservation of these genetic resources is highly threatened worldwide. The expansion of agriculture and anthropogenic pressures has led to the

disturbance of natural habitats, and the persistence of CWR populations growing in situ is becoming less and less likely.

A recent study found that 83 CWR species are predicted to lose more than 50% of their current range by 2070, and 39 CWRs are expected to lose over 50% of their genetic diversity, which is currently passively conserved in protected areas, due to climate change worldwide [2]. Additionally, the representativeness of this kind of resource in the germplasm collections maintained ex situ is still low [3], and the conservation of this genetic resource remains a global challenge [1].

To address the issue of the low representation of CWRs in ex situ germplasm collections, a global initiative entitled “Adapting Agriculture to Climate Change: Collecting, Protecting, and Preparing Crop Wild Relatives” supported by the Government of Norway and managed by the Global Crop Diversity Trust and the Millennium Seed Bank of the Royal Botanic Gardens, Kew, was established. Projects funded by this initiative, carried out by national or international gene banks, aimed to evaluate the gap of representativeness of CWRs in ex situ collections, to collect germplasm in the prioritized areas, to deposit the germplasm in national collections, and to duplicate the germplasm at the Millennium Seed Bank [4].

Initiatives related to the in situ conservation of CWRs have also been established in many countries [5], and some researchers call for the establishment of a global CWR in situ conservation network to maximize the conservation of these resources, considered extremely important for future food security worldwide [2], although such a global network initiative will substantially depend on the peculiarities of national legislations.

In Brazil, efforts have been put into the collection and ex situ conservation of the germplasm of native species related to important crops predominantly since the second half of the last century [6]. After the creation of Embrapa Genetic Resources and Biotechnology in the 1970s, a program for botanical exploration and the collection of germplasm was established, and many collecting expeditions were carried out for native species related to multiple crops, such as cassava [6,7], pineapples, cashew nuts, peanuts, chili-peppers, rubber trees, forest species, medicinal plants, forage legumes, ornamental plants, palm trees, rice [6], and potatoes [8], among others.

Despite the efforts of the Brazilian research institutions to collect and conserve ex situ genetic resources for the major crops with wild relatives in Brazil, the country still harbors hotspot areas for further collecting activities of high-priority crop wild relatives, that are still poorly represented in gene banks [3]. The gap analysis performed by these authors identified priority areas for collecting the CWRs of finger millet (*Eleusine*, Poaceae), sweet potatoes (*Ipomoea*, Convolvulaceae), rice (*Oryza*, Poaceae), and potatoes (*Solanum*, Solanaceae) within Brazilian territory, among other lower-priority crops (<http://www.cwrdiversity.org/distribution-map/>, accessed on 22 April 2019).

Potato and rice CWRs collected in Brazil have been conserved in Embrapa’s gene banks since 1985 and 1992, respectively. In 2015, for instance, the Rice Genebank, located at the Embrapa Rice and Beans, conserved 177 accessions of *Oryza alta* Swallen, *O. grandiglumis* (Döll) Prod., *O. latifolia* Desv., and *O. glumaepatula* Steud. collected in Brazil, while the Potato Genebank, located at the Embrapa Temperate Climate, conserved 86 accessions of *Solanum commersonii* Dunal and *S. chacoense* Bitter (in the “Alelo” information system [9]).

For finger millet, 67 accessions of *Eleusine tristachya* (Lam.) Lam. and *E. indica* (L.) Gaertn. collected in Brazil were incorporated in the Millet Genebank located at the Embrapa Maize and Sorghum. An earlier study did not access the information on these CWRs conserved in the Brazilian collections [3], because, at that time, this information was not publicly available in the Embrapa information system for genetic resources, known as Alelo. Therefore, the estimated gap of ex situ representativeness of these CWRs obtained by this research [3] may be somewhat overestimated. The number of accessions of these wild relatives conserved in these Brazilian collections was small, and there is no doubt that there is still a gap of ex situ representativeness for these taxa.

In addition to the ex situ approach, the Convention on Biological Diversity (CBD) and the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) both stress the desirability of in situ conservation; primarily due to the overall need to maintain ecosystem health, but also because it has the advantage that it maintains the dynamic evolution of CWR diversity itself, in relation to parallel environmental changes [10]. In this sense, Brazil is one of the countries with the greatest potential for in situ conservation in a climate change scenario, covering a wide diversity of CWRs in unique ecosystems and a network of diversified protected areas [2].

Here, we present the results of the aforementioned project conducted in Brazil as part of the global initiative for collecting and conserving ex situ CWRs [4]. This project, conducted from 2015 to 2018, focused on the CWRs of four important crops for food security worldwide: rice, potato, sweet potato, and finger millet. We describe the following steps covered by the project: The status of the ex situ and in situ conservation for the CWRs, the collection expeditions after this analysis, the germplasm collected and taxonomic identification, the monitoring of seed viability, and the development of protocols for seed germination and cryopreservation of the collected material.

2. Material and Methods

2.1. Species Selection

For this study, the wild relatives of rice, potato, sweet potato, and finger millet were selected based on the gene pool concept [11]. Species of the primary, secondary, and tertiary gene pools (Table 1) with occurrences reported in Brazil were included. The selected species have hybridization capacity (the primary and secondary gene pools) and confirmed evidence regarding potential use in breeding (the tertiary gene pool).

Table 1. List of species evaluated, gene pool, number of georeferenced records for herbarium occurrence and germplasm accessions conserved ex situ.

Species	Genepool	No. of Germplasm Accessions	No. of Herbarium Records
<i>Eleusine indica</i> (L.) Gaertn.	Primary	1	835
<i>Eleusine tristachya</i> (Lam.) Lam.	Secondary	26	175
<i>Oryza alta</i> Swallen	Secondary	17	7
<i>Oryza glumaepatula</i> Steud.	Primary	73	24
<i>Oryza grandiglumis</i> (Döll) Prod.	Secondary	25	22
<i>Oryza latifolia</i> Desv.	Secondary	15	59
<i>Solanum chacoense</i> Bitter	Secondary	3	54
<i>Solanum commersonii</i> Dunal	Tertiary	37	191
<i>Ipomoea cynanchifolia</i> Meisn.	Tertiary	0	49
<i>Ipomoea grandifolia</i> (Dammer) O'Donell	Tertiary	0	230
<i>Ipomoea ramosissima</i> (Poir.) Choisy	Tertiary	0	187
<i>Ipomoea tiliacea</i> (Willd.) Choisy	Tertiary	0	190
<i>Ipomoea triloba</i> L.	Tertiary	0	154
Total		204	2184

Brazil is one of the few countries that still presents extensive populations of wild rice growing in natural conditions, isolated from the commercial cultivation of the crop and, therefore, possibly without the introgression of alleles from the cultivated gene pool of *Oryza sativa* L. [12]. Four wild rice species occur in Brazil: *O. alta* Swallen, *O. grandiglumis*, *O. latifolia* (all of these, $2n = 48$, allotetraploid CCDD, secondary gene pool), and *O. glumaepatula* ($2n = 24$, AA; primary gene pool) [13]. As *O. glumaepatula* is self-pollinated and diploid, and presents a similar genome to the cultivated species, it is the one with the greatest potential for breeding programs among the Brazilian species [14].

Two wild potato species geographically distributed in Brazil are traditionally recognized as *Solanum commersonii* (with the two subspecies *S. commersonii* subsp. *commersonii*

and *S. commersonii* subsp. *malmeanum*) and *S. chacoense* (tertiary and secondary gene pools, respectively) [15]. The species *S. commersonii* and *S. chacoense* are distributed in southern and southeastern Brazil, Paraguay, Uruguay, and Argentina, with the latter also occurring in Bolivia and Peru.

Two relatives of finger millet (*Eleusine coracana* L.) occur in Brazil: *E. indica*, widespread in ruderal and otherwise open vegetation areas under anthropic influence in the Brazilian territory, and *E. tristachya*, native to the Paraguay-Paraná-La Plata river basin, typically concentrated in southern Brazil, Uruguay, and the adjacent areas of Argentina. *E. indica* is seen as the diploid A genome donor of *E. coracana*, while its B genome donor is still a matter of investigation.

Five *Ipomoea* species that pertain to the tertiary gene pool of sweet potato (*Ipomoea batatas* L. (Lam.)) occur in different regions within the country: *I. grandifolia* (Dammer) O'Donell, *I. ramosissima* (Poir.) Choisy, *I. tiliacea* (Willd.) Choisy, *I. triloba* L., and *I. cynanchifolia* Meisn.) [15]. *I. pintoii* O'Donell, *I. regnellii* Meisn., and *I. rupestris* Sim.-Bianch. and Pirani are other native species bearing tuberous roots that are edible and that are used as local sources of food in traditional cuisine in Bahia state, where they are known locally as “batatinha-da-serra” [16,17].

2.2. Species Distribution Models and the Status of Ex Situ and In Situ Conservation

Species occurrence data for the entire Brazilian territory were obtained from the Global Biodiversity Information Facility [18] and SpeciesLink [19] database. The occurrence data of germplasms conserved ex situ in gene banks were obtained from the Alelo database of the Brazilian Agricultural Research Corporation-Embrapa. The occurrence data were checked with the spOutlier tool [19] to eliminate possible errors (coordinates outside the area of occurrence), and locations without coordinates were georeferenced with the geoLoc tool [19]. The numbers of records for georeferenced herbarium occurrence and germplasm accessions for the Brazilian territory used in our analyses are presented in Table 1.

Species distribution models (SDMs) were generated using the MaxEnt algorithm [20]. This algorithm was used because it showed the highest performance [21,22], even with scarce occurrence data [23]. Nineteen bioclimatic variables from the Worldclim database [24] were used, in the spatial resolution of 5 min. To avoid collinearity and generating over-fitting models, a selection based on principal component analysis (PCA) was used [25], considering correlation values ≥ 0.70 for eliminating variables and above 70% of the variation in axes of the PCA.

After this procedure, nine bioclimatic variables (2, 4, 5, 7, 11, 14, 15, 18 and 19) were used. The models used 70% of the data for training and 30% for tests, with 10 replications (bootstrap, $k = 10$). The SDM performance was assessed using the area under the Receiver Operating Characteristic -ROC curve (AUC) statistic and the standard deviation of the AUC across replicates (SDAUC), in accordance with some studies [3,26,27]. The SDMs with values of $AUC \geq 0.7$ and $SDAUC < 0.15$ were considered robust.

The status of the ex situ and in situ conservation of each taxon was assessed using the gap analysis methodology. For ex situ conservation, scores were generated for four metrics [3,26,27]. For in situ conservation, four metrics were used [28]:

1. SRSex = Sampling Representativeness Score ex situ, an indicator of the existing gaps in the ex situ collections, which compares the total records in the gene banks (G) in relation to the total herbarium records (H). Values range from 0 to 100, with 0 indicating no ex situ conservation and 100 indicating full conservation status.
2. GRSex = Geographical Representativeness Score ex situ. For this score, buffers of a 50 km radius (CA50) are created for each collection location as an estimate of the area covered by the gene banks considering the SDM.
3. ERSex = Ecological Representativeness Score ex situ. This score compares the ecological diversity included in the ex situ collections with the ecological diversity represented in the SDMs. Ecological diversity is defined here by the concept of ecoregions as land units containing distinct biological communities, within borders that

- are close to the original extent (biogeographic realms and biomes) of these communities, prior to changes in land use patterns [29]. The CA50 buffer for germplasm collections is used here as an area to calculate the number of ecoregions represented in the collections in relation to the total number of ecoregions within the SDM [3,27].
4. FCSEX = Final Conservation Score for ex situ, considering the average of the three scores (SRSEX, GRSEX and ERSEX).
 5. GRSIN = Geographical Representativeness Score in situ, which compares the area of the SDM within protected areas in relation to the total area of the SDM. The protected areas were generated from the World Database of Protected Areas (WDPA), International Union for Conservation of Nature [30] using information from terrestrial and coastal areas designated as proposed and/or established.
 6. ERSIN = Ecological Representativeness Score in situ for the potential area of distribution of species within protected areas in relation to the total area of the SDM. This score considers the number of ecoregions as a proxy for the ecological representativeness (variation) of the species.
 7. FCSIN = Final Conservation Score for in situ, considering the average of the two scores (GRSIN and ERSIN).
 8. FCSC-mean = Combined Final Conservation Score, considering the average of FCSEX and FCSIN scores.

The species were categorized based on their conservation score, considering a numerical range between 0 and 100: species of high priority conservation, $FCS < 25$; medium priority, $25 \leq FCS < 50$; low priority, $50 \leq FCS < 75$; and sufficiently maintained, $FCS \geq 75$.

The gap analysis was performed with the packages 'GapAnalysis' [31], 'raster' [32], 'rgdal' [33], 'vegan' [34], 'knitr' [35], 'rgeos' [36], 'kableExtra' [37], and 'DT' [38] for the R statistical environment [39]. Maps were produced with the software QGIS v. 3.10.0 [40].

Based on the results of the species distribution models and the gap analysis, germplasm collection expeditions were planned for the different Brazilian biomes in the period from 2016 to 2018.

2.3. Collecting Expeditions

Germplasm expeditions were performed according to standard procedures [6]. Herbarium voucher specimens were deposited at the Cenargen Herbarium (CEN), with duplicates at Embrapa Clima Temperado Herbarium (ECT) (*Solanum*) and Kew Herbarium (K) (all genera) (acronyms as per Thiers, 2021, continuously updated).

For *Oryza* species, collection expeditions took place during the wild species' fruiting period, from April to July. Five collection expeditions were conducted for rice: one in the Pantanal and four in the Amazon region. The Amazon biome covers a huge area of Brazil's territory (49.3%) [41], and one single site would not fill the sampling gap. As the Pantanal's area corresponds to 1.8% [41] of Brazil's territory, there was only one collection site in this biome.

For planning the *Eleusine* species collecting expeditions, the road trips followed routes suggested by the gap analysis and the previous review of each *Eleusine* specimen from regional herbaria, as well as the Species Link [19] and Re flora databases [42]. Three expeditions were carried out to collect germplasm with a priority focus on *E. tristachya*. Live plants were also collected from populations with little or no availability of mature seeds, for subsequent transplantation and plant growth in greenhouses.

The *Ipomoea* CWRs were sampled in the Cerrado Biome in 2016, 2017, and 2018. The Cerrado biome covers about two million square kilometers or 21% of Brazilian territory. Most of this biome is located in the Central Plateau, and its vegetation consists of a mosaic with herbaceous, savanna, and forest formations. Two expeditions were carried out for collecting *Ipomoea* native species in the Cerrado region, including the highlands of the Diamantina Plateau (Bahia state) and the *cerrados* in Minas Gerais state. Two expeditions in 2018 also explored the Atlantic Forest biomes from Minas Gerais to the Rio Grande do Sul states.

The Atlantic Forest and the Pampas region were also the object of four expeditions to collect species of *Solanum* species, from March to August. The natural vegetation in Southern Brazil is a mosaic of grassland, shrubland, and different forest types. *Araucaria* forest is physiognomically dominated by the species *Araucaria angustifolia* (Bertol.) Kuntze in the upper stratum is found mostly on the plateau in the Paraná, Santa Catarina, and Rio Grande do Sul states, forming mosaics with natural *Campos* (grasslands), the area of the latter increasing toward the south. We searched for remaining wild *Solanum* populations known from historical records deposited in herbaria that were lying in priority areas and were not sampled in gene banks. Secondly, we investigated areas highlighted by the climate niche modelling as potential sources that were within the priority areas and with no record known from herbaria nor gene banks.

Herbarium vouchers were checked by experts in each botanical family—Convolvulaceae (André Luís Costa Moreira), Poaceae (José Francisco Montenegro Valls and Mayco Werllén dos Santos Sousa), and Solanaceae (Gustavo Heiden and Suelma Ribeiro Silva). Tubers were collected for wild potatoes (*Solanum*) and *Ipomoea* and kept in paper bags. Seeds were placed in paper and cloth bags and dried using absorbent papers at room temperature after harvesting. After that, seeds were dried in a drying chamber at 20 °C and 15% humidity. The relative humidity of seeds reached <7% before storage.

2.4. Seed Evaluation

To evaluate the effects of different substrates and methods of sowing on the germination potential of finger millet CWR seeds, an experiment was carried out at the Laboratory of Seed Analysis at Embrapa Maize and Sorghum. Seeds of two accessions of *Eleusine tristachya* (BGE 39 and BGE 55) and one accession of *E. indica* (BGE 57), as species with the highest density of seed science studies, were evaluated using substrates and methods of sowing in paper (rolls, above, and between paper) and sand. A completely randomized design with four replicates was used for the accessions × substrates and methods of sowing by factorial arrangement.

The *Eleusine* seeds were previously processed using sieves and air, and manually selected by transillumination with an adapted diaphanoscope. Subsequently, the selected pure seeds were sanitized with a 0.25% sodium hypochlorite solution for two minutes, rinsed under running water, and dried on filter paper. Fifty selected pure seeds were placed in an individual Gerbox box (length 115 mm, width 110 mm, height 35 mm on top of two blotter paper sheets or between them. The same number of seeds were used in “Germitest” paper towels and over-sand.

The substrates were initially moistened with potassium nitrate solution (0.2% *m/m*), considering 2.5 times the mass of the dry paper and 60% of the field capacity for the sand substrate. The water was checked daily and replenished when necessary. The experiment was carried out in a germination chamber at 25 °C (± 2 °C) and a 12-h photoperiod. On the seventh day, germination was determined by seedling evaluations.

The results are expressed as a percentage of normal seedlings, using, as criteria, the protrusion of the radicle (3 mm) and the elevation of the seedlings on the substrate surfaces. Statistical analysis was performed using the ExpDes package in R [43]. The data were transformed by box-cox, and after verifying the normality of the residues by the Shapiro–Wilk test, analysis of variance was performed, and the means were compared using the Scott–Knott test ($p < 0.05$).

Seeds of all the accessions of the collected rice CWRs were evaluated for physiological quality (germination and dormancy) in the Seed Laboratory of Embrapa Genetic Resources and Biotechnology. During seed processing, the removal of the inert material, including all parts and structures not defined as seed, was carried out with the aid of sieves and gloves for the separation of straw, soil particles, stones, and other small particles.

When possible, the edges were removed from the seeds. After cleaning, the seeds were dried in an air-conditioned chamber (15% relative humidity and 20 °C), and the water

content of the seeds was determined with the oven method at 105 ± 3 °C/24 h [44]. The average water content was 6.2%.

For the germination test, seeds were sown between “Germitest” paper towels (neutral pH, width 28 cm, length 38 cm, moistened with distilled water in the proportion of $2.5 \text{ mL} \cdot \text{g}^{-1}$ of paper). The seeds remained in a germination chamber regulated at 25 °C, and the evaluations of normal seedlings were performed at 21 days after sowing [44]. Seedlings were considered normal if shown as a straight coleoptile with a green leaf (plumule), which extended at least up to half its length and, if presented, a primary root.

Physiological evaluation (germination and dormancy) and freezing tests were performed on seeds of *Ipomoea cynanchifolia*. to investigate the seed viability, germinability, and freezing at -18 °C and cryopreservation responses. Seeds of a single accession (ISG 17) were used in the tests, carried out at the Laboratory of Plant Cryobiology at Embrapa Genetic Resources and Biotechnology. Upon arrival at the laboratory, the seeds were carefully inspected, and empty, malformed, or perforated seeds were discarded.

After inspection, seven seed samples were separated for testing, each consisting of four reps of 20 seeds. The first sample was used to determine the water content by the oven method, at 103 ± 2 °C/24 h [44] and the results were expressed as mean percentages of the fresh weight. The other six samples were transferred to labeled cryovials and stored at three different temperatures for seven days. The control sample was stored at 25 °C, in the dark, in a seed germinator.

To test the seeds’ freezing tolerance, samples were stored at -18 °C, in a freezer, and at -196 °C, in a cryotank, submerged in liquid nitrogen. Seeds stored at -18 °C and -196 °C were thawed out rapidly by submerging the cryovials in a water bath at 40 ± 2 °C, with manual shaking for 2–3 min. After storage in each temperature and before they were sown, seeds of one of the two sub-samples underwent scarification treatment (SC+), and seeds of the other sub-sample did not receive scarification (SC–).

Seed decontamination consisted of washing with a sodium hypochlorite solution (NaOCl, 2.5% active chlorine, *v/v*), containing 1 mL of commercial detergent plus 1 mL of Tween 20 for 15 min., under agitation on a rotary shaker at 190 rpm. After 15 min., the seeds were transferred to a laminar flow hood and rinsed three times with distilled sterilized water, with manual shaking during each rinse. After rinsing, scarification was performed with a cut made on the extremity opposite to the seed hilum, using the tip of a small plier sterilized by autoclaving (15 min, 121 °C, 1.5 atm).

Seeds with and without scarification were sown in Petri dishes (9 mm diameter) between filter paper discs soaked to the point of saturation with 5 mL of distilled-sterilized water. After sowing, Petri dishes were transferred to a growth room at 25 ± 2 °C, with a photoperiod of 12 h provided by LED lights. The Petri dishes were assessed daily for up to seven days after sowing (DAS) to detect germination and contamination by microorganisms.

After that, assessment was done weekly up to 30 DAS, with germination percentages recorded at 3, 7, 15, and 30 DAS. Radicle protrusion (botanical criterion) or the presence of a green hypocotyl and main root (technological criterion) were used as the criteria to determine the occurrence of germination. The number of seeds showing radicle protrusion or a green hypocotyl and a main root were subjected to analysis of variance (two-way ANOVA) followed by Bonferroni’s post-test ($p < 0.001$) using the statistical program GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. CWR Species Distribution Models and the Status of Ex Situ and In Situ Conservation

Our analysis resulted in adequate distribution models for all species, considering the statistical values $\text{AUC} \geq 0.7$ and $\text{SDAUC} < 0.15$. These models were used in the subsequent gap analysis for ex situ and in situ conservation.

Our results indicated gaps of representativeness in the ex situ collections; all species of *Ipomoea* and *Eleusine indica* were classified as high priority, while *E. tristachya*,

Oryza alta, *O. latifolia*, and *Solanum chacoense* were classified as medium priority (Table 2). The Sampling Representativeness scores ex situ (SRSex) were particularly high (100) for *O. alta*, *O. glumaepaula*, and *O. grandiglumis*, while they presented lower values—below 10—for the *Ipomoea* species *E. indica* and *S. chacoense*. The Ecological Representativeness score ex situ (ERSex) showed high values—over 60—for the two species of *Solanum*.

Regarding the representativeness for in situ conservation, nine species were classified as medium priority for in situ conservation. The Ecological Representativeness score in situ (ERSin) had a strong influence on the final score, with values above 50 for all species and above 90 for *O. alta*, *S. chacoense*, and *S. commersonii* (Table 2).

Taking into account the combined ex situ and in situ final score, among the 13 evaluated species, 12 were classified as a medium conservation priority, including species with a broader distribution, such as *E. indica* and *O. latifolia*, as well as species with a more restricted distribution, such as the genus *Ipomoea* and *S. chacoense* (Table 2). Only *I. grandifolia* was classified as high priority.

3.2. Collection Expeditions

In total, 16 expeditions were carried out to collect CWRs during the period covered by this project (Table 3). The expeditions covered broad areas of the Brazilian biomes and were guided by the maps generated in the gap analysis (geographic and ecological representativeness ex situ). Figure 1 shows, as an example, these maps of non-conserved ex situ potential distribution areas and non-conserved ex situ ecoregions for CWR rice.

In total, the expeditions collected 174 herbarium samples and 174 accessions (Table 4, Figure 2). For species of *Oryza*, *Eleusine*, and *Ipomoea cynanchifolia*, the number of seeds per accession was, on average, more than 1000, reaching values of up to 29,900 seeds from a single accession of *O. alta*. For *I. tiliacea* and *I. ramosissima*, the number of seeds per accession was between 100 and 400.

Filling the ex situ conservation gaps, the main objective of the project, was achieved for many target species, with emphasis on *Eleusine*, *Oryza*, and *Solanum* species. An exception was the *Solanum commersonii* subsp. *malmeanum*, with only one accession collected. The species *Oryza alta* is currently considered a synonym for *O. latifolia*, and *I. grandifolia* is a synonym for *I. triloba* [15]. Thus, the accessions of *O. alta* are considered *O. latifolia* and accessions of *I. grandifolia* are considered *I. triloba*.

Rice CWR: populations of rice CWRs formed clusters of plants on the riverbanks of smaller streams and lakes. On the main streams, plants were not observed, or only a few detached individuals. Generally, the populations of *O. alta* (*O. latifolia*) and *O. glumaepatula* were large and sometimes presented more than one species co-occurring. *O. glumaepatula* was found in all the expedition sites, *O. alta* was found in three expedition sites (Marajó, Tapajós, and Pantanal), and *O. grandiglumis*, only in Amazonas state. Seeds were collected from different parts of the population edges, as it was impossible to reach the inner parts.

During the Tapajós expedition, we identified certain *O. glumaepatula* plants infected with blast (*Magnaporthe oryzae* B. Couch), the most important disease in rice. Leaves from these plants were sampled and the fungus was isolated at Embrapa Rice and Beans, and included in the Microorganism gene bank (BRM 63182, BRM 63183 and BRM 63184).

Finger millet CWR: *E. tristachya* is a typical heliophite grass, and thus collections were made successfully, particularly on overgrazed natural pastures, as well as along rural dirt roads, farm entrance roads, or rocky outcrops. It also occurs on open land in urban areas but not as abundantly as the ubiquitous *E. indica*. While *E. indica* occurs with very high frequency throughout the Brazilian territory, mainly in anthropic conditions, *E. tristachya* is more frequent in Rio Grande do Sul, decreasing over the more northern states.

Table 2. Conservation gap analysis scores per taxon. sampling representativeness score ex situ = SRSex; geographical representativeness score ex situ = GRSex; ecological representativeness score ex situ = ERSex; sampling representativeness score in situ = SRSin; geographical representativeness score in situ = GRSin; ecological representativeness score in situ = ERSin; combined final conservation score = FCSc-mean; high priority (HP, FCSc-mean < 25), medium priority (MP, $25 \leq$ FCSc-mean < 50), and low priority (LP, $50 \leq$ FCSc-mean < 7.5) [28].

Species	SRSex	GRSex	ERSex	FCSex	FCSex Class	SRSin	GRSin	ERSin	FCSin	FCSin Class	FCSc Mean	FCSc Mean Class
<i>Eleusine indica</i> (L.) Gaertn.	0.2	0.6	12.5	4.5	HP	7.1	9.1	81.3	45.2	MP	29.4	MP
<i>Eleusine tristachya</i> (Lam.) Lam.	14.9	34.3	42.9	30.7	MP	0.0	4.1	85.7	44.9	MP	33.1	MP
<i>Oryza alta</i> Swallen	100.0	6.9	23.8	43.6	MP	0.0	28.6	90.5	59.5	LP	44.4	MP
<i>Oryza glumaepatula</i> Steud.	100.0	27.5	37.5	55.0	LP	24.6	25.2	62.5	43.8	MP	42.2	MP
<i>Oryza grandiglumis</i> (Döll) Prod.	100.0	18.9	35.3	51.4	LP	3.4	29.3	64.7	47.0	MP	39.2	MP
<i>Oryza latifolia</i> Desv.	25.4	23.7	28.6	25.9	MP	0.0	11.1	57.1	34.1	MP	25.6	MP
<i>Solanum chacoense</i> Bitter	5.6	10.9	66.7	27.7	MP	6.5	4.9	100.0	52.5	LP	38.3	MP
<i>Solanum commersonii</i> Dunal	19.4	71.1	80.0	56.8	LP	6.5	4.9	100.0	52.5	LP	44.1	MP
<i>Ipomoea cynanchifolia</i> Meisn.	2.5	0.9	15.0	6.1	HP	37.9	9.6	90.0	49.8	MP	38.7	MP
<i>Ipomoea grandifolia</i> (Dammer) O'Donell	1.1	1.3	17.6	6.7	HP	5.4	7.0	52.9	30.0	MP	20.4	HP
<i>Ipomoea ramosissima</i> (Poir.) Choisy	0.7	1.0	13.0	4.9	HP	40.6	11.9	73.9	42.9	MP	34.8	MP
<i>Ipomoea tiliacea</i> (Willd.) Choisy	0.8	2.4	21.4	8.2	HP	12.7	16.3	85.7	51.0	LP	34.8	MP
<i>Ipomoea triloba</i> L.	0.8	1.0	6.7	2.8	HP	13.9	11.3	86.7	49.0	MP	32.7	MP

Table 3. Expeditions and sites for collecting germplasm from crop wild relatives of rice, potato, sweet potato, and finger millet.

Genus	Surveyed Sites
<i>Oryza</i>	Five expeditions: Marajó Island (state of Pará), Pantanal (state of Mato Grosso do Sul), Tapajós river (state of Pará), Solimões, Amazonas, and Negro rivers (state of Amazonas)
<i>Eleusine</i>	Three expeditions: Pantanal biome (state of Mato Grosso do Sul), Atlantic Forest biome (Santa Catarina, Paraná and Rio Grande do Sul states)
<i>Solanum</i>	Four expeditions: Atlantic Forest biome (Minas Gerais, Paraná, Santa Catarina and Rio Grande do Sul states), and Pampas
<i>Ipomoea</i>	Four expeditions: Cerrado and Atlantic Forest biomes, from Minas Gerais to Rio Grande do Sul states

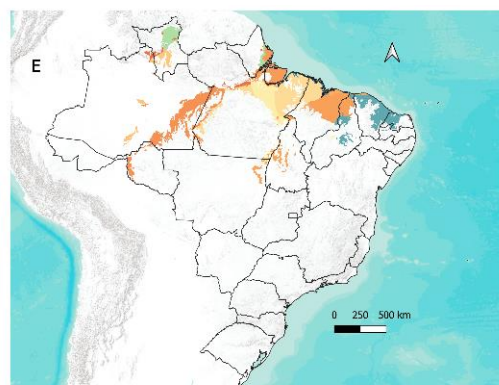
Table 4. Collected accessions of *Oryza*, *Solanum*, *Eleusine*, and *Ipomoea* wild species.

Project Target Taxa	No. of Accessions
<i>Eleusine indica</i> (L.) Gaertn.	25
<i>Eleusine tristachya</i> (Lam.) Lam.	40
<i>Ipomoea grandifolia</i> (Dammer) O'Donell *	2
<i>Ipomoea ramosissima</i> (Poir.) Choisy	6
<i>Ipomoea tiliacea</i> (Willd.) Choisy	21
<i>Ipomoea cynanchifolia</i> Meisn.	5
<i>Ipomoea triloba</i> L.	4
<i>Oryza glumaepatula</i> Steud.	19
<i>Oryza alta</i> Swallen *	20
<i>Oryza grandiglumis</i> (Döll) Prod.	8
<i>Oryza latifolia</i> Desv.	0
<i>Solanum chacoense</i> Bitter	13
<i>Solanum commersonii</i> subsp. <i>commersonii</i> Dunal	10
<i>Solanum commersonii</i> Bitter subsp. <i>malmeanum</i>	1
Total	174

* *O. alta* is a synonym for *O. latifolia*; *I. grandifolia* is a synonym for *I. triloba*.

Therefore, it was quite difficult to locate populations of this species in Mato Grosso do Sul, and especially in São Paulo, where previous herbarium collections were rare, anyway. Since field expeditions to collect the germplasm of *E. tristachya* were concentrated in May or November, most of the difficulties in acquiring new accessions were related to the sparse distribution of its populations in the northernmost areas of documented occurrence.

Sweet potato CWR: despite the extension of the areas sampled at different times of the year, only the collections of *I. tiliacea* and *I. cynanchifolia* resulted in a significant number of germplasm seeds per accession. The rapid release of *Ipomoea* seeds was a limiting factor for the collections of *I. ramosissima* and *I. triloba*, in addition to the difficulty in locating individuals.

**Figure 1.** Cont.

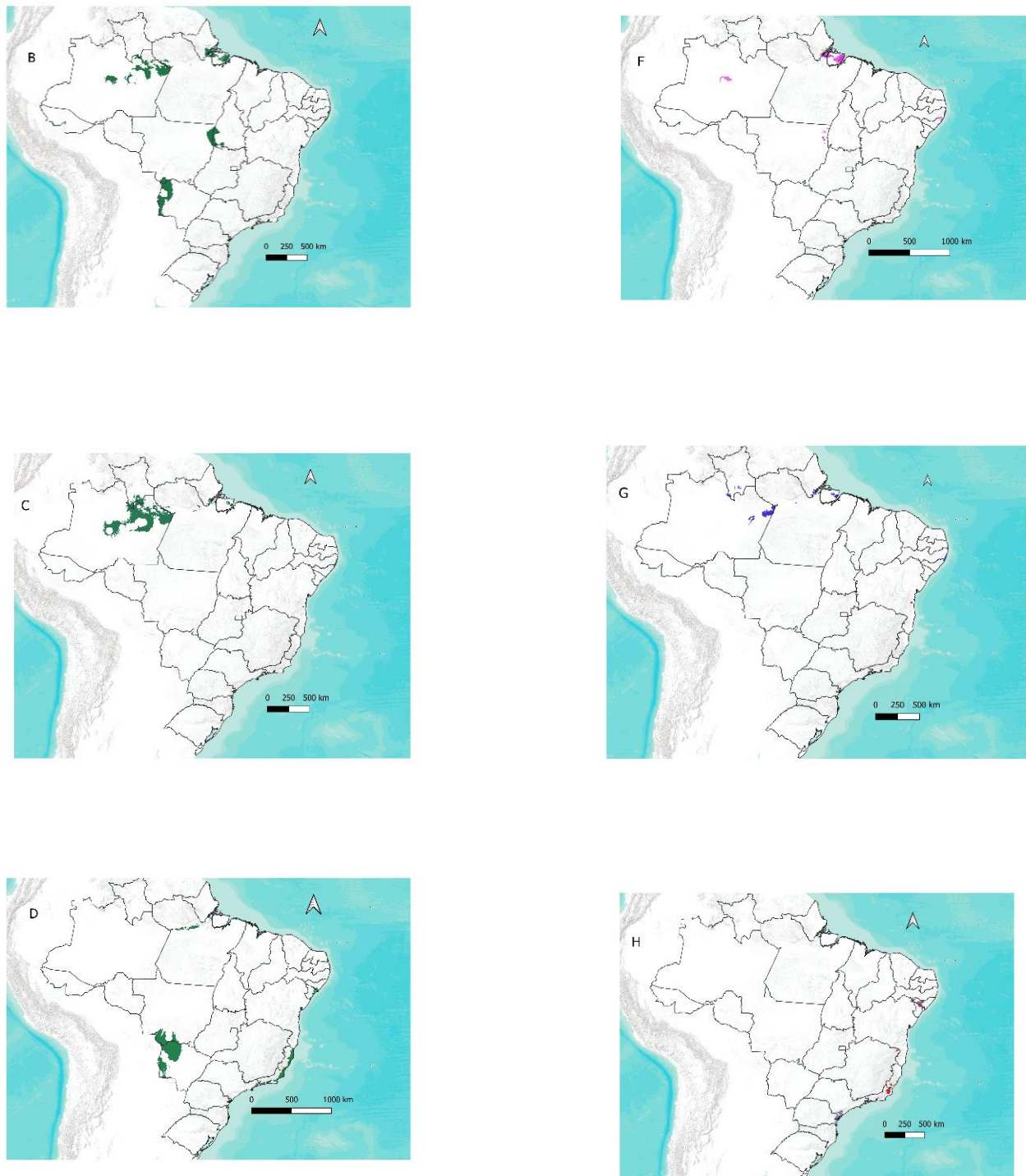


Figure 1. In green, areas of potential distribution not conserved ex situ for *Oryza alta* (A), *O. glumaepatula* (B), *O. grandiglumis* (C), and *O. latifolia* (D). In color, non-conserved ex situ ecoregions for *Oryza alta* (E), *O. glumaepatula* (F), *O. grandiglumis* (G), and *O. latifolia* (H).

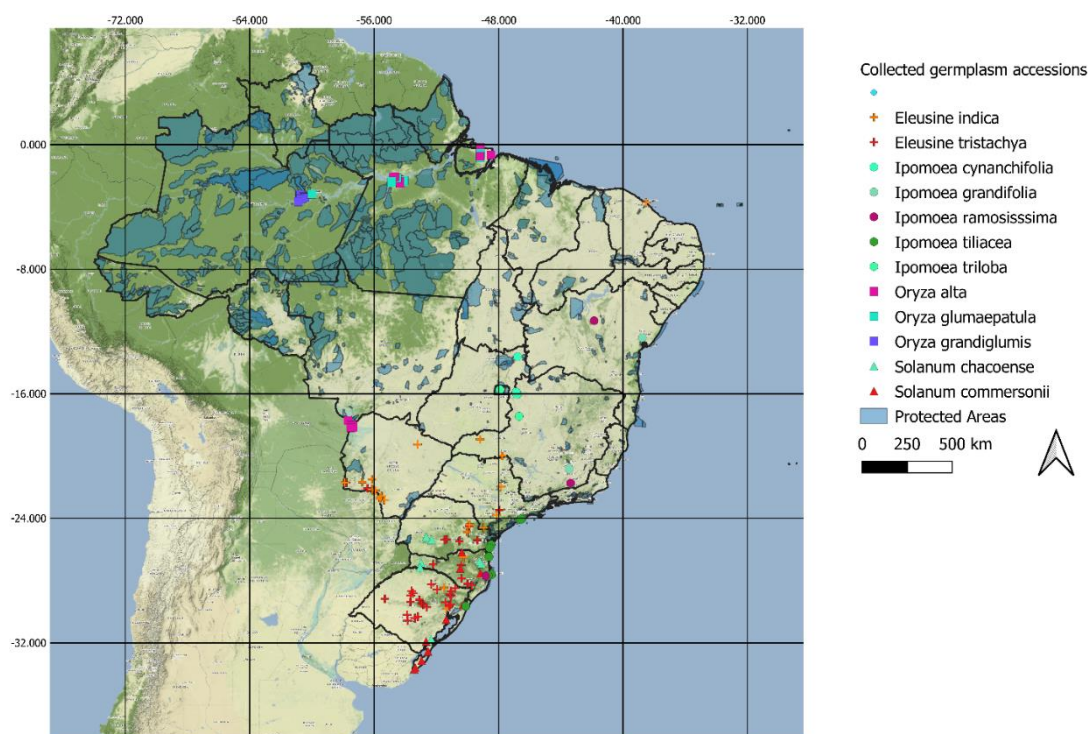


Figure 2. Geographic distribution of germplasm collection for *Oryza*, *Ipomoea*, *Eleusine*, and *Solanum* species from 2016 to 2018.

Potato CWR: four collection expeditions were performed in south and southeastern Brazil. The Brazilian wild potato species generally grow gregariously forming stable perennial populations. They establish from seeds or tubers and then flower and fruit generally twice a year in spring and fall, while during summer and winter they generally cannot be easily spotted because they are in vegetative development or hidden underground as tubers.

During flowering, these populations can easily be spotted due to the showy white, purplish or purple flowers. However, when fruiting, they are harder to find due to the green color of the fruit even when ripe. Added to the difficulty of finding these populations in the right season, several of them are clonally propagated and due to self-incompatibility and habitat fragmentation, most of the remaining Brazilian populations of wild potatoes do not set fruit, making the digging of tubers or living plants the only strategy possible for germplasm collection with later trials of greenhouse multiplication of seeds via controlled pollination.

3.3. Seed Evaluation

Sweet potato CWR: the water content of *I. cynanchifolia* seeds was 7.8%, on a fresh weight basis. This water content is compatible with storage at sub-zero temperatures, and thus no adjustments in the water content of the seeds were made before storage at -18 and -196 °C. Regardless of the storage temperature, seeds of *I. cynanchifolia* without scarification (SC) had lower germination percentages than scarified seeds. The highest germination percentage of non-scarified seeds (15%) was obtained for seeds stored in liquid nitrogen, at -196 °C.

In contrast, the germination percentages for seeds with scarification (SC+) ranged from 97% to 100% (Figure 3). Apart from achieving higher germination levels at all three storage temperatures tested, seeds with scarification also presented more uniform germination, with only slight variations in radicle protrusion and seedling growth speed, as a function of the storage temperature.

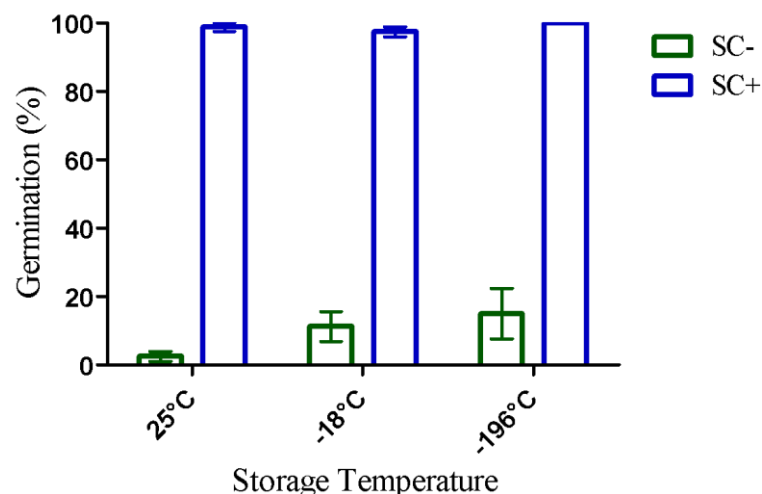


Figure 3. Germination percentages of *Ipomoea cynanchifolia* Meisn. seeds with (SC+) and without scarification (SC-), stored at three different temperatures: 25, −18, and −196 °C for seven days.

Regardless of the scarification treatment, there was no statistically significant effect of the sub-zero temperatures on the seed germination percentages. The seedlings obtained had green hypocotyls, obdeltoid cotyledons with a concave–convex base, adherent to the integument and well-developed roots.

Rice CWR: Among the 37 accessions of wild rice species evaluated for physiological seed quality (*O. alta*, *O. glumaepatula*, and *O. grandiglumis*), only two accessions of *O. alta* presented less intense dormancy, with average germination values of 89% and 74% in the control treatment (without application of methods to break dormancy). For the other accessions, there was a differentiated response to the different treatments for overcoming dormancy, and these variations occurred between accessions within species and between species.

For the species *O. grandiglumis*, treatment with sodium hypochlorite improved the seed germination in most accessions. In the case of *O. alta*, the treatment containing gibberellins and cytokinin improved the performance of the seeds of most accessions, although the treatment using sodium hypochlorite also had positive effects, since it did not cause damage to the seeds, and the germination rates were superior to those of the seeds in the control treatment. In *O. glumaepatula*, the treatment with sodium hypochlorite and growth regulator provided better seed germination performance for most accessions. The immersion of the seeds in hot water was not enough to overcome the seeds dormancy.

Finger millet CWR: the substrates and sowing methods influenced the seed germination rates of the evaluated *Eleusine* genotypes. The overall mean was 72.2%, reflecting the high potential of the genus to develop and establish itself in the field. The genotypes diverged from each other in all substrates, except in the Germitest paper, which presented the lowest germination values. The mean germination rate for *E. tristachya* seeds was 74.8%, a value 11.5% higher than the one observed for *E. indica* seeds at 67.1% (Figure 4).

The germination responses of the seeds demonstrated no dormancy attributes in the evaluated seed lots. In our study, the lowest levels of seed germination in *E. tristachya* were obtained using sand and Germitest roll paper. The genotype BGE 39 (*E. tristachya*) showed the highest germination average among the evaluated substrates, with better results achieved using the between paper (83.3%) and on blotter paper (90.0%) substrates.

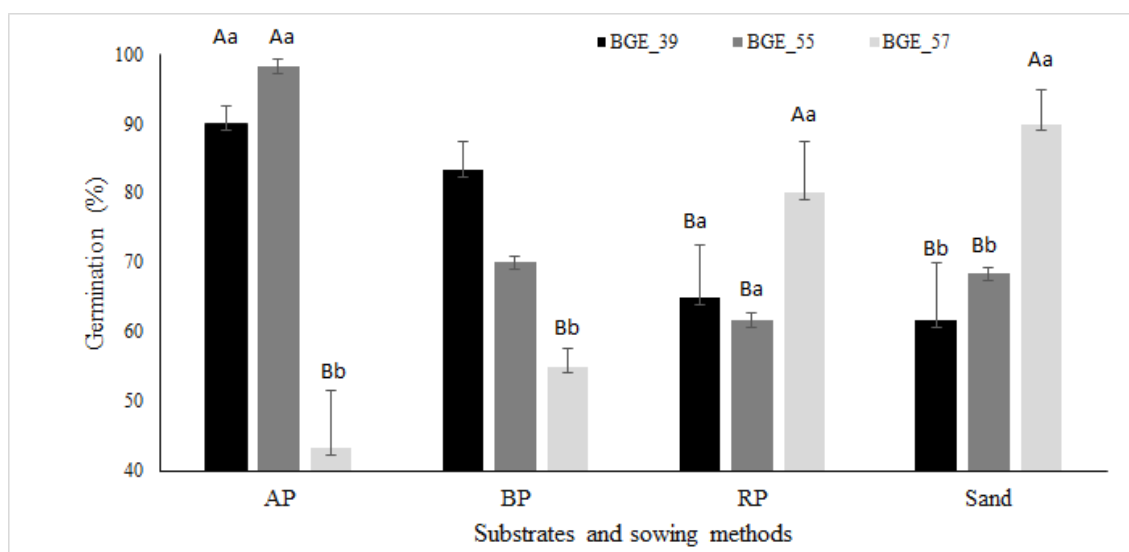


Figure 4. The germination rates of *Eleusine indica* (BGE 57) and *E. tristachya* (BGE 39 and BGE 55) seeds in sand, between papers (BP), Germitest rolled paper towel (RP) and over blotter paper (AP). Bars represented by the same upper-case letters, between the different substrates, and same lower-case letters, among *Eleusine* accessions, are not different by the Scott–Knott test and F test ($p < 0.05$), respectively. Vertical lines represent the mean standard error.

On the same hand, the genotype BGE 55 (*E. tristachya*) showed the highest general value at 98.3% when the seeds were set above blotter paper sheets. *E. indica* yielded the highest amplitude in germination rates on different substrates, with a variation of 108% for sand (90%) in relation to sowing on the top paper (43%).

4. Discussion

4.1. Previous Status of Ex Situ Conservation

Among the 13 species evaluated, six were classified as high priority and four as medium priority for ex situ conservation. These results, which included 76.9% of the analyzed species, corroborate a study which posited that Brazil would be one of the priority global hotspots for the collection and conservation of germplasm from CWRs [3].

The species classified as high priority for germplasm collection, in addition to *E. indica*, included all five *Ipomoea* species, due to the complete absence of accessions in the national gene banks, or with only one germplasm accession, as in the case of *E. indica*. Despite the early stages in the use of CWRs in sweet potato and finger millet breeding programs, accessions of native species constitute a source of potential traits [1].

For the three species classified as low priority for ex situ conservation, the results for *O. grandiglumis* and *O. glumaepatula* were likely influenced by previous germplasm collecting. The native species of *Oryza* already have a history of collection and use in rice breeding programs worldwide [45]. However, despite germplasm collection efforts in several countries, rice CWR are still considered a medium priority for ex situ conservation, with significant collection gaps that still need to be filled in many countries [3].

The classification of *S. commersonii* as a low ex situ conservation priority was also influenced by the higher number of germplasm accessions before collections of this project. Classified as medium priority for ex situ conservation, *S. chacoense* presented this same classification in a study that considered gap analysis for ex situ conservation of all potato CWRs in the American continent [46].

For the five *Ipomoea* species with distribution throughout the American continent, with the exception of *I. grandifolia*, classified as medium priority for collection, all the other species were also classified as high priority for ex situ conservation [47].

One limitation of gap analysis for germplasm collection is in the data source in calculating the ecological representativeness score ex situ—ERSex. This score uses the

ecological diversity represented by the number of ecoregions in the ex situ collections and in the species distribution models. In the database of ecoregions [29] used for the ERSex calculation, some Brazilian biomes, such as the Cerrado, the Caatinga, and the Atlantic Forest are poorly represented in terms of ecoregion diversity when compared to the Amazon.

Two studies [48,49] identified, respectively, 19 different ecoregions for the Cerrado biome, and 55 biogeographic sub-regions for the Atlantic Forest, showing a significant underestimation of ecological diversity for the calculation of the ecological representativeness score in these two biomes. The ERSex score was particularly high for *S. commersonii* and *S. chacoense*, two species from southern Brazil, with the classification of low and medium ex situ conservation priority, respectively.

The potential distribution areas and the germplasm collection areas of *S. commersonii* were restricted to the Uruguayan Savanna ecoregion, a formerly extensive area of natural grasslands recognized as the Pampa biome in Brazil. The potential distribution area and the germplasm collection areas of *S. chacoense* were restricted to the ecoregions Alto Paraná Atlantic Forest and Araucaria Moist Forests, and the high ERSex score was decisive for the classification of this species as a medium ex situ conservation priority. Considering the low geographic and sampling representativeness scores for *S. chacoense*, the final score of the gap analysis likely underestimated the collection priority for this species due to the high ERSex.

4.2. Status of In Situ Conservation

The final score for the in situ conservation for all species was strongly influenced by the high values of the ecological representativeness score (ERSin), with values above 50, and also underestimated the conservation priorities considering the classification bias of ecoregions. The bias caused by the ERSin was noticeable when observing the geographic representativeness values, which were generally much lower, such as for the species of *Solanum*, *Ipomoea*, and *Eleusine*.

In addition, many ecosystems in the areas of potential distribution of these species are characterized by a low representativeness of protected areas and a high degree of anthropogenic disturbances as in the two global diversity hotspots, Cerrado [50] and the Atlantic Forest [49]. The Cerrado biome, important for the conservation of at least five species (*E. indica*, *I. cynanchifolia*, *I. triloba*, *I. ramosissima*, and *O. latifolia*), has a low percentage of protected areas, with only 8.31% of the represented biome.

A total of 85% of the Cerrado's protected areas are Environmental Protection Areas, a category of sustainable use conservation unit that allows greater flexibility in the use of natural resources and is also much less effective in preventing deforestation in relation to areas of comprehensive protection, such as parks, ecological stations, and biological reserves [51].

The Atlantic Forest biome, the habitat of many species, such as *I. tiliacea*, *I. triloba*, *I. ramosissima*, *E. indica*, *E. tristachya*, *S. chacoense*, and *S. commersonii*, has a very high degree of human interference in the landscape, with only 12% of the natural remnants of its original coverage and only 1.05% of that coverage in protected areas [48]. When "looking" into the Pantanal ecoregion and the habitat of *E. indica*, *O. latifolia*, and *O. glumaepatula*, only 5.37% of the territory is protected [52].

While most of these protected areas are included in the Environmental Protection Areas conservation unit category—which is not very effective in containing the deforestation process—these areas are still subject to direct and indirect threats, such as invasion by exotic species as well as river damming and mining, which can compromise the in situ conservation of these species in the long term [52].

A recent study noted the importance of indigenous territories for the conservation of CWRs in Brazil, particularly in the Cerrado biome, selected as one of the top 10 priority sites in the world for the in situ conservation of CWRs within a network of protected areas [2] and 75 species of *Manihot* occur in the Cerrado, including 59 endemic ones [7].

Among the endemic taxa, 24 are at some level of threat according to the International Union for Conservation of Nature (IUCN) classification (14% Vulnerable, 22% Endangered, and 5% Critically endangered), mostly owing to a narrow geographic range. The results of the final score for in situ conservation corroborate the need to expand and consolidate a network of protected areas for the maintenance of populations of CWR with genetic variability and the maintenance of ecological and evolutionary processes.

The final conservation score, including the average of the final scores ex situ and in situ, resulted in a classification of medium priority of collection for all species, with the exception of *I. grandifolia* (high priority). The results show the importance of the complementarity of in situ and ex situ conservation strategies for the conservation of CWR. A national CWR strategy to establish key national CWR protected areas should be associated with a safety back-up to ensure the conservation of the germplasm; therefore, population samples should be collected and deposited in appropriate ex situ collections [9].

4.3. Collection Expeditions: Filling the Gaps

The total accessions collected (174) almost doubled the total accessions collected until 2015 for Embrapa's ex situ conservation system. In addition, accessions of absent or practically absent species were collected for the ex situ conservation system, such as *Ipomoea* species, in addition to *E. indica* and *S. chacoense*. The areas of potential distribution not conserved ex situ, generated by the score of geographic representativeness (GRSex), were fundamental in guiding collections to priority areas.

For ecoregions not conserved ex situ, the limitations in relation to the diversity of ecoregions for biomes, such as the Cerrado and Atlantic Forest, previously mentioned, limited the use of this metric to define priority ecoregions for germplasm collection. Despite the likely underestimations in relation to the indications of priority species for ex situ conservation scores generated by the gap analysis, this method may be used mainly when the limitations are identified in the calculation of the scores, as was the case with ecological representativeness—ERSex. Thus, in general, our previous gap analysis provided valuable support for filling ex situ conservation gaps in the Brazilian national gene banks.

4.4. Seed Evaluation

Understanding the determining factors in the early stages of plant development in *Eleusine* genotypes is of extreme importance for the development of knowledge that supports alternative strategies of germplasm conservation and breeding programs for this species [53]. Germination for *E. indica*, an important weed in the genus, is reported to be dependent on the sowing substrates and methods.

For *E. indica*, research reported that mechanical scarification was able to increase germination rates and speed [54], as well as light and KNO₃ use [55,56], demonstrating an important ecological dormancy mechanism, commonly found in other seeds of weed species. We found in previous studies that the use of potassium nitrate and light in *E. tristachya* was not necessary to promote seed dormancy breaking and germination in this species [57]. We found that for *Eleusine tristachya*, it is recommended to use blotter paper, setting the seed above the paper, as a standard substrate and sowing method for the evaluation of the germinative power of seed lots.

The results obtained in the present work demonstrate the existence of dormancy in seeds of *I. cynanchifolia* and corroborate those obtained in previous studies, which reported that the seeds of several wild species of the genus *Ipomoea* present dormancy caused by impermeability of the integument to water, attributed to the layer of palisade cells of the external integument [58–60].

Mechanical scarification using sandpaper, vegetable peelers, or sharp instruments (pliers, scissors, or scalpel) to make cuts or perforations, or to wear out the integument, is an efficient method for overcoming dormancy, and has been used to break the dormancy of seeds of *I. obscura*, *I. aquatica*, *I. hederifolia*, and *I. involucrata* [61].

In the present study, mechanical scarification, which consisted of making a cut on the tip opposite the hilum with fine-tipped pliers, was efficient to break the dormancy of *I. cynanchifolia* seeds and to cause a significant increase in the germination percentages. This work also showed that there was no statistically significant difference in the germination percentages for seeds stored at both sub-zero temperatures tested. This is a very important result since it suggests that long-term conservation of *I. cynanchifolia* germplasm could be successfully achieved either by storage of seed samples in cold rooms at $-18\text{ }^{\circ}\text{C}$ or in liquid nitrogen ($-196\text{ }^{\circ}\text{C}$).

The response to treatments for overcoming seed dormancy varied among *Oryza* species and among accessions of the same species according to seed dormancy intensity. Seeds of wild species of *Oryza* present strong dormancy, and dormancy-breaking treatments are needed. This aspect has to be carefully considered when managing gene bank collections, including by sowing a higher number of seeds per container in greenhouse multiplication. Accessions and species may respond differently to treatments to break dormancy; however, it is possible to establish treatments that function well enough for collection purposes.

In general, seeds of *O. glumaepatula* showed low germination when not treated, suggesting more intense dormancy. The use of a growth regulator, although not described in official procedures, was a promising treatment for these species. The growth regulator acts in the germination process, that is, in the events involved in root protrusion [62,63]. The use of sodium hypochlorite and growth regulator in the same treatment is a promising alternative when the type of dormancy involved is unknown and if the seed availability is a limitation.

5. Conclusions

The methods used for dormancy breaking and low-temperature conservation for the *Oryza*, *Eleusine*, and *Ipomoea* species were promising for the incorporation of accessions in the respective gene banks. The participation of gene banks and seed laboratories in the ex situ conservation process through studies related to breaking dormancy and long-term storage was a fundamental part of the process.

Despite the bias in the results of ecological representativeness, by using an unbalanced number of ecoregions that influenced the results of the gap analysis, the method for ex situ conservation was very useful for targeting germplasm collections (species and locations). Important gaps were filled for most of the target species, and the total number of accessions collected in this project almost doubled the total number of accessions collected by 2015 in Embrapa's ex situ conservation system.

The results related to in situ conservation provided unprecedented data and indicate the broad potential to complement the ex situ conservation that protected areas can play in maintaining populations of CWRs. In addition to rice, potatoes, sweet potatoes, and finger millet, Brazil harbors high species richness in the numbers of CWRs from other important crops, such as manioc, pineapple, cashew, peppers, and peanuts, which require continuous efforts to collect germplasm and implement conservation measures through projects such as this one.

Author Contributions: M.B.M. and M.L.B. designed the study. M.B.M. produced the Formal Analysis with input from G.H., A.G.A., S.R.-S., J.F.M.V., S.C.B.R.J., I.R.I.S., and A.M.A.P.; M.B.M. and M.L.B. wrote the Original Draft, with input from G.H., A.G.A., S.R.-S., J.F.M.V., S.C.B.R.J., I.R.I.S. and A.M.A.P.; M.L.B. provided Funding Acquisition; and M.B.M. performed Project Administration. All authors have read and agreed to the published version of the manuscript.

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References

- Dempewolf, H.; Baute, G.; Anderson, J.; Kilian, B.; Smith, C.; Guarino, L. Past and future of wild relatives in crop breeding. *Crop. Sci.* **2017**, *57*, 1070–1082. [CrossRef]
- Vincent, H.; Amri, A.; Castañeda-Álvarez, N.P.; Dempewolf, H.; Duloo, E.; Guarino, L.; Hole, D.; Mba, C.; Toledo, A.; Maxted, N. Modeling crop wild relative species identifies areas globally for in situ conservation. *Commun. Biol.* **2019**, *2*, 136. [CrossRef]
- Castañeda-Álvarez, N.P.; Khoury, C.K.; Achicanoy, H.A.; Bernau, V.; Dempewolf, H.; Eastwood, R.J.; Guarino, L.; Harker, R.H.; Jarvis, A.; Maxted, N.; et al. Global conservation priorities for crop wild relatives. *Nat. Plants* **2016**, *2*, 16022. [CrossRef]
- Dempewolf, H.; Eastwood, R.J.; Guarino, L.; Khoury, C.K.; Muller, J.V.; Toll, J. Adapting agriculture to climate change: A global initiative to collect, conserve and use crop wild relatives. *Agroecol. Sustain. Food Syst.* **2014**, *38*, 369–377. [CrossRef]
- FAO. *The Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture*; FAO: Rome, Italy, 2010.
- Walter, B.M.T.; Cavalcanti, T.B. *Fundamentos Para a Coleta de Germoplasma Vegetal*, 1st ed.; Embrapa Recursos Genéticos e Biotecnologia: Brasília, Brazil, 2005; p. 778.
- Simon, M.F.; Reis, T.S.; Mendoza, F.J.M.; Arquelão, T.K.M.; Bringel, J.B.A., Jr.; Noronha, S.E.; Martins, M.L.L.; Ledo, C.A.S.; Silva, M.J.; Sampaio, A.B.; et al. Conservation assessment of cassava wild relatives in central Brazil. *Biodivers. Conserv.* **2020**, *29*, 1589–1612. [CrossRef]
- Castro, C.M.; Pereira, A.S.; Costa, D.M.; Choer, E.; Augusti, E.; Gomes, C.B.; Campos, A.D.; Pedroso, R.; Garrastazú, M.C.; Barbieri, R.L.; et al. Wild potato genetic resources conserved in southern Brazil: Current knowledge and future perspectives. *Acta Hortic.* **2007**, *745*, 323–330. [CrossRef]
- Alelo. Available online: <http://alelobag.cenargen.embrapa.br/AleloConsultas/Home/index.do> (accessed on 26 February 2021).
- Maxted, N.; Kell, S. *Establishment of a Global Network for the In Situ Conservation of Crop Wild Relatives: Status and Needs*; FAO: Rome, Italy, 2009.
- Harlan, J.; de Wet, J. Towards a rational classification of cultivated plants. *Taxon* **1971**, *20*, 509–517. [CrossRef]
- Rangel, P.H.N.; Buso, G.S.C.; Brondani, C.; Guimarães, E.P.; Rangel, P.N.; Ferreira, M.E. Coleta, caracterização e uso de germoplasma silvestre de arroz diplóide e tetraplóide (*Oryza* spp.) nativo do Brasil no melhoramento genético. In *Fundamentos Para a Coleta de Germoplasma Vegetal*, 1st ed.; Walter, B.M.T., Cavalcanti, T.B., Eds.; Embrapa Recursos Genéticos e Biotecnologia: Brasília, Brazil, 2005; pp. 585–631.
- Vaughan, D.A.; Morishima, H.; Kadowaki, K. Diversity in the genus *Oryza*. *Curr. Opin. Plant Biol.* **2003**, *6*, 139–146. [CrossRef]
- Brondani, C.; Rangel, P.H.N.; Brondani, R.P.V.; Ferreira, M.E. QTL mapping and introgression of yield-related traits from *O. glumaepatula* to cultivated rice (*Oryza sativa*) using microsatellite markers. *Theor. Appl. Genet.* **2002**, *104*, 1192–1203. [CrossRef]
- Brazil Flora Group. *Brazilian Flora 2020 Project*; Instituto de Pesquisas Jardim Botânico do Rio de Janeiro: Rio de Janeiro, Brazil, 2021. [CrossRef]
- de Oliveira, G.T. Diversidade e Estrutura Genética de Populações de Batata da Serra (*Ipomoea serrana* Sim.-Bianch. & L.V. Vasconcelos) da Chapada Diamantina, Bahia, Utilizando Marcadores ISSR. Master's Thesis, Escola Superior de Agricultura Luiz de Queiroz—Universidade de São Paulo, São Paulo, Piracicaba, 2016. [CrossRef]
- Gonçalves, C.N.; Azevedo-Gonçalves, C.F. Batata da serra: Uma espécie nativa que fazia parte da dieta garimpeira. In *Aspectos Botânicos e Ecológicos em Comunidades da Chapada Diamantina*; Gonçalves, C.N., Azevedo-Gonçalves, C.F., Eds.; Novas Edições Acadêmicas: Saarbrücken, Germany, 2016.
- GBIF: Global Biodiversity Information Facility. Available online: <https://www.gbif.org/> (accessed on 26 February 2021).
- Species Link. Available online: <http://www.splink.org.br/index> (accessed on 26 February 2021).
- Phillips, S.J.; Anderson, R.P.; Schapire, R.E. Maximum entropy modeling of species geographic distributions. *Ecol. Model.* **2006**, *190*, 231–259. [CrossRef]

21. Elith, J.; Graham, C.H.; Anderson, R.P.; Dudik, M.; Ferrier, S.; Guisan, A.; Hijmans, R.J.; Huettmann, F.; Leathwick, J.R.; Lehmann, A.; et al. Novel methods improve prediction of species' distributions from occurrence data. *Ecography* **2006**, *29*, 129–151. [CrossRef]
22. Costa, G.C.; Nogueira, C.; Machado, R.B.; Colli, G.R. Sampling bias and the use of ecological niche modeling in conservation planning: A field evaluation in a biodiversity hotspot. *Biodivers. Conserv.* **2010**, *19*, 883–899. [CrossRef]
23. Pearson, R.G.; Raxworthy, C.J.; Nakamura, M.; Peterson, A.T. Predicting species distributions from small numbers of occurrence records: A test case using cryptic geckos in Madagascar. *J. Biogeogr.* **2007**, *34*, 102–117. [CrossRef]
24. Hijmans, R.J.; Cameron, S.; Parra, J.; Jones, P.G.; Jarvis, A. *WorldClim*, version 1.3; University of California: Berkeley, CA, USA, 2005.
25. Warren, D.L.; Wright, A.N.; Seifert, S.N.; Shaffer, H.B. Incorporating model complexity and spatial sampling bias into ecological niche models of climate change risks faced by 90 California vertebrate species of concern. *Divers. Distrib.* **2014**, *20*, 334–343. [CrossRef]
26. Ramirez-Villegas, J.; Khoury, C.K.; Jarvis, A.; Debouck, D.G.; Guarino, L. A gap analysis methodology for collecting crop gene pools: A case study with *Phaseolus* beans. *PLoS ONE* **2010**, *5*, e13497. [CrossRef]
27. Khoury, C.K.; Castañeda-Alvarez, N.P.; Achicanoy, H.A.; Sosa, C.C.; Bernau, V.M.T.; Kassa, M.T. Crop wild relatives of pigeonpea [*Cajanus cajan* (L.) Millsp.]: Distributions, ex situ conservation status, and potential genetic resources for abiotic stress tolerance. *Biol. Conserv.* **2015**, *184*, 259–270. [CrossRef]
28. Khoury, C.K.; Amariles, D.; Soto, J.S.; Diaz, M.V.; Sotelo, S.; Sosa, C.C.; Ramírez-Villegas, J.; Achicanoy, H.A.; Velásquez-Tibatá, J.; Guarino, L.; et al. Comprehensiveness of conservation of useful wild plants: An operational indicator for biodiversity and sustainable development targets. *Ecol. Indic.* **2019**, *98*, 420–429. [CrossRef]
29. Olson, D.M.; Dinerstein, E.; Wikramanayake, E.D.; Burgess, N.D.; Powell, G.V.N.; Underwood, E.C. Terrestrial ecoregions of the world: A new map of life on earth. *BioScience* **2001**, *51*, 933–938. [CrossRef]
30. International Union for Conservation of Nature. World Database on Protected Areas. Protected Planet. 2014. Available online: <https://protectedplanet.net/> (accessed on 26 February 2021).
31. CIAT. *GapAnalysis: An R Package to Calculate Conservation Indicators Using Spatial Information*. R Package 1.0.1; CIAT: Cali, Colombia, 2020. Available online: <https://cran.r-project.org/web/packages/GapAnalysis/index.html> (accessed on 15 January 2020).
32. Hijmans, R.J.; van Etten, J. Raster: Geographic Data Analysis and Modeling. R Package. Version 3.4-5. 2014. Available online: <https://cran.r-project.org/web/packages/raster/index.html> (accessed on 26 February 2020).
33. Bivand, R.; Keitt, T.; Rowlingson, B.; Pebesma, E. Rgdal: Bindings for the Geospatial Data Abstraction LIBRARY. R package. Version 1.5-9. Available online: <https://cran.r-project.org/web/packages/rgdal/index.html> (accessed on 26 February 2020).
34. Oksanen, J.; Blanchet, F.G.; Kindt, R.; Legendre, P.; Minchin, P.R. Vegan: Community Ecology Package. R package Version 2.0-8. 2013. Available online: <http://CRAN.R-project.org/package=vegan> (accessed on 2 March 2020).
35. Xie, Y. *knitr: A General-Purpose Package for Dynamic Report Generation in R*. R Package Version 1.30; 2020. Available online: <https://cran.r-project.org/web/packages/knitr/index.html> (accessed on 9 August 2020).
36. Bivand, R.; Rundel, C. *Rgeos: Interface to Geometry Engine-Open Source (GEOS)*; R package Version 0.2-2. Available online: <https://rdr.io/cran/rgeos/> (accessed on 23 August 2020).
37. Zhu, H. *kableExtra: Construct Complex Table with 'kable' and Pipe Syntax*. R Package; 2020. Available online: <https://rdr.io/cran/kableExtra/> (accessed on 23 August 2020).
38. Xie, Y. *DT: A Wrapper of the JavaScript Library 'DataTables'*, R Package Version 0.17; 2020. Available online: <https://rdr.io/cran/DT/> (accessed on 15 October 2020).
39. R Development Core Team. *R: A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2012.
40. QGIS.org. QGIS Geographic Information System. QGIS Association. v. 3.10.0. 2019. Available online: <http://www.qgis.org> (accessed on 9 September 2019).
41. Instituto Brasileiro de Geografia e Estatística—IBGE. *Mapa de Biomas do Brasil: Primeira Aproximação (escala 1:5,000,000)*; IBGE: Rio de Janeiro, Brazil, 2004. Available online: [Ftp://ftp.ibge.gov.br/Cartas_e_Mapas/Mapas_Murais/biomas_pdf.zip](ftp://ftp.ibge.gov.br/Cartas_e_Mapas/Mapas_Murais/biomas_pdf.zip) (accessed on 26 February 2021).
42. Re flora—Herbário Virtual. Available online: <http://reflora.jbrj.gov.br/reflora/herbarioVirtual/> (accessed on 26 February 2021).
43. Ferreira, B.E.; Cavalcanti, P.P.; Nogueira, D.A. ExpDes: Experimental Designs. R Package. Available online: <https://cran.r-project.org/web/packages/ExpDes/index.html> (accessed on 26 February 2019).
44. Brasil—Ministério da Agricultura, Pecuária e Abastecimento. *Regras Para Análises de Sementes*; MAPA/SDA/ACS: Brasília, Brazil, 2009; p. 399.
45. Xiao, J.; Grandillo, S.; Ahn, S.N.; McCouch, S.R.; Tanksley, S.D. Genes from wild rice improve yield. *Nature* **1996**, *384*, 356–358. [CrossRef]
46. Castaneda-Alvarez, N.P.; de Haan, S.; Juarez, H.; Khoury, C.K.; Achicanoy, H.A.; Sosa, C.C.; Bernau, V.; Salas, A.; Heider, B.; Simon, R.; et al. Ex situ conservation priorities for the wild relatives of potato (*Solanum* L. Section *petota*). *PLoS ONE* **2015**. [CrossRef]
47. Khoury, C.K.; Heider, B.; Castaneda-Alvarez, N.P.; Achicanoy, H.A.; Sosa, C.C.R.E.; Miller, R.E. Distributions, ex situ conservation priorities, and genetic resource potential of crop wild relatives of sweetpotato [*Ipomoea batatas* (L.) Lam., I. series *Batatas*]. *Front. Plant Sci.* **2015**, *6*. [CrossRef] [PubMed]

48. Sano, E.E.; Rodrigues, A.A.; Martins, E.S.; Bettiol, G.M.; Bustamante, M.M.C.; Bezerra, A.S.; Couto, A.F., Jr.; Vasconcelos, V.; Schuler, J.; Bolfe, E.L. Cerrado ecoregions: A spatial framework to assess and prioritize Brazilian savanna environmental diversity for conservation. *J. Environ. Manag.* **2019**, *232*, 818–828. [[CrossRef](#)]
49. Ribeiro, M.C.; Martensen, A.C.; Metzger, J.P.; Tabarelli, M.; Scarano, F.; Fortin, M. The Brazilian Atlantic Forest: A Shrinking Biodiversity Hotspot. In *Biodiversity Hotspots. Distribution and Protection of Conservation Priority Areas*; Zachos, F.E., Habel, J.C., Eds.; Springer: New York, NY, USA, 2011; pp. 405–434.
50. Carranza, C.T.; Balmford, A.; Kapos, V.; Manica, A. Protected area effectiveness in reducing conversion in a rapidly vanishing ecosystem: The Brazilian Cerrado. *Cons. Lett.* **2013**, *7*, 1–8. [[CrossRef](#)]
51. Françoso, R.D.; Brandão, R.; Nogueira, C.C.; Salmona, Y.B.; Machado, R.B.; Colli, G.R. Habitat loss and the effectiveness of protected areas in the Cerrado Biodiversity Hotspot. *Nat. Conserv.* **2015**, *13*, 35–40. [[CrossRef](#)]
52. Irigaray, C.T.G.H.; Braun, A. Marco Regulatório. In *Pantanal à Margem da Lei: Panorama das Ameaças e Perspectivas à Conservação*; Cunha, C.N., Junk, W.J., Eds.; Wetlands International: Cuiabá, Brazil, 2020.
53. Kamenya, S.N.; Mikwa, E.O.; Song, B.; Odeny, D.A. Genetics and breeding for climate change in Orphan crops. *Theor. Appl. Genet.* **2021**, *3*. [[CrossRef](#)]
54. Ismail, B.S.; Chuah, T.S.; Salmijah, S.; Teng, Y.T.; Schumacher, R.W. Germination and seedling emergence of glyphosate-resistant and susceptible biotypes of goosegrass (*Eleusine indica* [L.] Gaertn.). *Weed Biol. Manag.* **2002**, *2*, 177–185. [[CrossRef](#)]
55. Nishimoto, R.K.; McCarty, L.B. Fluctuating Temperature and Light Influence Seed Germination of Goosegrass (*Eleusine indica*). *Weed Sci.* **1997**, *45*, 426–429. [[CrossRef](#)]
56. Kalimashe, M. Germination on the grass weed *Eleusine indica* (L.) Gaertn Population as Affected by Temperature, Light, and Its Response to Glyphosate. Master's Thesis, University Pretoria, Pretoria, South Africa, 2019.
57. Amaral, R.S.S.; Pereira, L.L.; Guimarães, V.R.; Antunes Neto, A.; Passos, A.M.A. *Germinação de Sementes de Eleusine Indica e E. tristachya: Fotoblastia*; Embrapa Milho e Sorgo: Sete Lagoas, Brazil, 2020; p. 10. Available online: <https://www.infoteca.cnptia.embrapa.br/infoteca/bitstream/doc/1126608/1/Circ-Tec-269.pdf> (accessed on 26 February 2021).
58. Azania, A.A.P.M.; Azania, C.A.M.; Pavani, M.C.M.D.; Cunha, M.C.S. Métodos de superação de dormência em sementes de Ipomoea e Merremia. *Planta Daninha* **2003**, *21*, 203–209. [[CrossRef](#)]
59. Chaves, I.S.; Silva, N.C.Q.; Ribeiro, D.M. Effect of the seed coat on dormancy ena Germination in *Stylosanthes humilis* H. B. K. seeds. *J. Seed Sci.* **2017**, *39*, 114–122. [[CrossRef](#)]
60. Pazuch, D.; Trezzi, M.M.; Diesel, F.; Barancelli, M.V.J.; Batistel, S.C.; Pasini, R. Superação de dormência em sementes de três espécies de *Ipomoea*. *Ciência Rural*. **2015**, *45*, 192–199. [[CrossRef](#)]
61. Ogunwenmo, K.; Ugborogho, R.E. Effects of chemical and mechanical scarification on seeds germination of five species of *Ipomoea* (Convolvulaceae). *Boletim Soc. Broteriana* **1999**, *69*, 147–162.
62. Cardoso, V.J.M. Dormência: Estabelecimento do processo. In *Germinação: Do básico ao Aplicado*; Ferreira, A.G., Borghetti, F., Eds.; Artmed Editora: Porto Alegre, Brazil, 2004.
63. Taiz, L.; Zeiger, E. *Fisiologia Vegetal*, 3rd ed.; Artmed: Porto Alegre, Brazil, 2004; p. 719.