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## **A simple standardized protocol to evaluate the reliability of seed rain estimates**

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HAL Authorization

1 **Running title: Reliability of seed rain estimates**

2

3

4 **A simple standardized protocol to evaluate the reliability of seed rain**

5 **estimates**

6

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15

16

17 **Abstract**

18 Seed dispersal has key implications for community dynamics and restoration ecology.  
19 However, estimating seed rain (the number and diversity of seeds arriving in a given area)  
20 is challenging, and the lack of standardization in measurement prevents cross-site  
21 comparisons. Seed trap effectiveness and accuracy of seed sorting methods are key  
22 components of seed rain estimates in need of standardization. We propose and describe  
23 a standardized protocol for evaluating the effectiveness of two seed trap types (sticky and  
24 funnel traps), and the accuracy of a seed sorting method. We used widely available seeds  
25 (arugula, quinoa, sesame and sunflower) to produce a gradient of seed size, weight and  
26 color. Proof-of-concept was tested in a tropical grassland, where traps were set for 30  
27 days. Our results suggest that we underestimate dispersal of small seeds (less than 2mm  
28 width) that can be easily mistaken for debris and soil particles or that fail to adhere to  
29 sticky traps. Seeds on sticky traps may be more vulnerable to removal by wind and rain,  
30 whereas seeds in funnel traps are more susceptible to decay. We found no evidence of  
31 observer bias on seed sorting for funnel trap samples. However, accuracy on seed sorting  
32 for funnel trap samples tended to decline for seeds with less than 2mm width, suggesting  
33 a size-dependence in seed retrieval success. Our standardized protocol addressing trap  
34 effectiveness and seed sorting methods will increase reliability of data obtained in seed  
35 rain studies in grasslands and allow more reliable comparisons between datasets.

36 **Keywords:** seed loss, seed rain, seeds sorting, seed trap, trap effectiveness

37

## 38 **Introduction**

39           Seed dispersal studies are vital to understanding plant distribution and community  
40 resilience, and they guide conservation and restoration activities (Török et al., 2018). A  
41 useful way of studying seed dispersal is to estimate seed rain —*i.e.* the number and  
42 diversity of new seeds reaching a given area (Baskin and Baskin 2014) — using seed  
43 traps to collect propagules at particular locations, then identifying and counting them.  
44 However, measuring seed rain is challenging, and a lack of methodological  
45 standardization persists, compromising the accuracy of seed rain estimates and impairing  
46 comparison of data between studies (Arruda et al., 2018; Wolfe et al., 2019). Seed rain  
47 has long been used by ecologists to address ecological succession and factors limiting  
48 regeneration in tropical forests (Holl 1999; Saulei and Swaine, 1988). In open  
49 environments, however, seed dispersal by wind and water run-off poses additional  
50 challenges to estimate seed rain. . For example, despite seed rain in temperate grasslands  
51 has been relatively well studied, we not only need more research in other grasslands types  
52 (e.g. tropical grasslands), but to improve many crucial aspects related to methods  
53 standardization and data reporting (Arruda et al. 2018). Determining the effectiveness of  
54 seed traps, and of seed sorting methods for samples, is crucial for improving  
55 reproducibility, but it is rarely tested in seed rain studies (Thompson and Mcginnes, 1963;  
56 Jackel and Poschlod, 1994; Kollmann and Goetze, 1998; Stevenson and Vargas, 2008).

57           Evaluation of seed trap effectiveness involves the evaluation of two processes:  
58 trap capacity to capture seeds (seed catch), and trap capacity to retain seeds (seed  
59 retention) and avoid seed loss (Box 1; Fig. 1). Additionally, the accuracy of seed sorting  
60 methods for trap samples depends not only on seed size—small seeds are harder to find—  
61 but also on the ability to separate seed material from debris, which can strongly affect  
62 seed retrieval rates (Cottrell, 2004). Knowing the seed retrieval rate of seed sorting is

63 important for determining the influence of seed size, of observer effect, and of sample  
64 composition, or more precisely, the color and size of soil particles, debris and litter often  
65 present in samples (Debussche and Isenmann, 1994).

66         Among other invertebrates, ants are known for their ability to collect large  
67 amounts of seeds and can have a major impact on seed trap effectiveness (Predavec,  
68 1997). Seed decay can vary greatly between seed types and is also modulated by other  
69 biotic and abiotic conditions such as pathogens, humidity and the amount of litter/soil  
70 accumulated within traps (Roberts, 1972; Box 1). Additionally, seed loss by wind or  
71 water run-off can vary greatly between trap types, seasons and plant communities, thereby  
72 influencing seed retrieval rates. Therefore, to maximize seed catch and minimize seed  
73 loss in open ecosystems, the use of complementary seed trap types is recommended  
74 (Chabrierie and Alard, 2005).

75         Funnel traps can be used to study local seed rain and the transportation of seeds  
76 by water run-off (Jackel and Poschlod, 1994). Funnel traps are effective in seed catch, but  
77 seed loss to predation and to decay caused by excessive moisture arise as potential  
78 problems (Schott, 1995; Kollmann and Goetze, 1998; Jensen, 1998). Sticky traps, in turn,  
79 are more suitable for studying wind-dispersed species (Jefferson and Usher 1989). While  
80 sticky traps carry a lower risk of seed predation, checking them is often hindered by  
81 trapped insects and debris (Kollmann and Goetze, 1998). Both sticky and funnel traps  
82 may also bias the seed catch towards larger seeds that are more easily detected by visual  
83 assessment, while soft seeds may easily rot, and smooth seeds may be lost through rain  
84 or wind action (Kollmann and Goetze, 1998; Cottrell, 2004). Despite the current state of  
85 knowledge, the influence of seed size and weight on the retention rate of seed traps is still  
86 overlooked.

87           Among the possible methods for sorting seeds in funnel-trap samples, the most  
88 effective is direct seed inspection after sieving to separate seeds from debris (Kollmann  
89 and Goetze, 1998; Cottrel, 2004). Seed identification requires training; however, one’s  
90 capacity to find and sort seeds also depends on seed traits, such as size and color (Martin  
91 and Barkley, 1961; Cottrell, 2004). Considering that results can be biased by differences  
92 in one’s capacity to find and sort seeds from the samples, it is important to obtain, prior  
93 to data interpretation in seed rain studies, an estimate of how many seeds are missed in  
94 the sorting procedure. Our goal is to propose and describe a simple standardized protocol  
95 to evaluate the effectiveness in seed retention of two types of seed trap (Box 1, Fig. 1),  
96 and the accuracy of a seed sorting method in assessing seed rain. The standardized  
97 protocol should be run along with every seed rain study using these traps to allow more  
98 reliable comparisons between studies. We tested these standardized protocols in a tropical  
99 grassland as a proof-of-concept.

100

## 101 **Description and implementation**

102

### 103 SEED TRAPS

104           We tested sticky and funnel traps (Fig. 2), which capture complementary processes  
105 of seed rain (Chabrierie and Alard, 2005) and are the most common traps used to estimate  
106 seed rain in grasslands (Arruda et al., 2018). We provide detailed instructions on how  
107 these two seed traps can be built using low-cost and readily available material  
108 (Appendices S1–S2).

109

### 110 STANDARDIZED PROTOCOL

111 We used four species: arugula (*Eruca sativa*), quinoa (*Chenopodium quinoa*),  
112 sesame (*Sesamum indicum*) and sunflower (*Helianthus annuus*). Here, the sunflower  
113 achenes are called seeds to facilitate the terminology throughout the manuscript. These  
114 seeds are available in most market or garden center worldwide and provide variation in  
115 size, weight and colors that can be tested across ecosystems (Table 1). To assess seed  
116 retention, the number of traps and the length of time that traps should be left in the field  
117 can be adapted to each study and grassland type. Killing the seeds before using them in  
118 the field is a mandatory step to 1) prevent the seeds from germinating on the seed traps,  
119 2) avoid contamination by pathogens that may be associated with these seeds (Godefroid  
120 et al., 2017), 3) prevent invasion of exotic species (Estévez et al., 2015). We placed all  
121 seeds in a drying oven at 120°C for an hour, as treatments heating seeds above 85°C for  
122 more than 8 min have shown to kill most seeds (Hess et al. 2018). We conducted all seed  
123 measurements (e.g. weight and size) after the heat treatment.

124 On each trap, we gently dropped 10 seeds of each species, totaling 40 seeds per  
125 trap (Table 1). On funnel traps, we put all seeds straight into the bag collectors. It is best  
126 to run the standardized protocol either during the same season(s) as the study or, if run  
127 over one year, during the season in which conditions are most challenging for  
128 preservation of the seeds on the traps. For the sticky traps, all samples collected from each  
129 trap are examined under magnification. For the funnel traps, bags are collected separately  
130 from each trap, and their content washed in a 250- $\mu$ m sieve to reduce the amount of fine  
131 soil particles, then examined under a magnifying glass to count and identify seeds. All  
132 seeds are counted and any signs of damage recorded.

133 To evaluate seed loss during the seed sorting of funnel traps samples, a second  
134 experiment examines the sorting accuracy with funnel traps samples. We chose to test the  
135 seed sorting accuracy with only funnel trap samples because the traps can accumulate

136 much litter and soil in the field, making it difficult to retrieve seeds. For this test, a given  
137 number of seeds of the four species are mixed in soil, in a proportion equal to that  
138 commonly found inside the funnel trap bags. The soil used must be taken from the study  
139 area to control for color, debris and litter composition. The number of seeds in each  
140 sample (with a minimum of 3 and maximum of 30 seeds per species) should be  
141 randomized and noted; this number is not known to the observers. A minimum of three  
142 previously trained observers then sort the samples, searching for, identifying and counting  
143 the seeds. For ethical aspects, observers' anonymity must be respected when collecting  
144 and reporting the data.

145         The proportion of seeds retrieved at the end of the experiments (retrieval success)  
146 is obtained by calculating the percentage of seeds retrieved by each observer. Both  
147 experiments are analyzed using generalized linear models that assume a quasibinomial  
148 distribution and use retrieval success as the response variable. For the seed trap  
149 effectiveness experiment, trap type and species are the categorical variables (interaction  
150 was tested). For the seed sorting experiment, species and experimenters are the  
151 categorical variables (interaction was tested). In both cases, pairwise contrast  
152 comparisons with a Tukey adjustment can be run. We here performed these analyses with  
153 R (R Core Team, 2018), packages *base* and *emmeans*.

154

## 155 PROOF OF CONCEPT

156         In order to proof-of-concept our protocol, we conducted fieldwork in the southern  
157 part of the Espinhaço mountain range, southeastern Brazil (43° 35' W, 19° 17' S). The  
158 annual precipitation averages around 1,400 mm, and climate is markedly seasonal, with  
159 most rainfall occurring in the hot summers (Silveira et al., 2016). We conducted the  
160 experiment in March 2017, at the end of the raining season, when high temperatures,

161 strong winds and rainy days prevail, creating the most challenging conditions in the study  
162 area. The main vegetation comprises the mountaintop *campo rupestre*, fire-prone open  
163 grasslands that establish on quartzite-derived rocks, with shallow and severely nutrient-  
164 poor sandy soils (Silveira et al., 2016).

165 We randomly placed six of each type of seed trap on a pristine site (50 m<sup>2</sup>), and  
166 retrieved seeds after a period of one month, as this the most common timeframe used to  
167 sample seed rain in grasslands (Arruda et al., 2018). For the second experiment, testing  
168 seed sorting accuracy with funnel trap samples, we ran the protocol with three previously  
169 trained observers in the laboratory.

170

### 171 **Results of the proof-of-concept**

172 In the proof-of-concept experiment, we found no difference between the two trap types  
173 in sunflower seed retention, with both performing well (98.6% for funnel trap and 100%  
174 for sticky trap) (Fig. 3). Funnel traps were ineffective in retaining quinoa seeds under the  
175 field conditions, while the sticky trap had a good retention rate for quinoa seeds (88.6%;  
176 LM quasibinomial,  $p < 0.001$ ; Fig. 3). We found no significant difference in the  
177 performance between seed traps for sesame seeds (Fig. 3). Funnel traps performed better  
178 than sticky traps in the retention of arugula seeds (41.4% more efficient than sticky traps)  
179 (Fig. 3). Quinoa and arugula seeds had the lowest retention rates, with only 1.4% and  
180 42.9% of the arugula seeds on sticky and funnel traps, respectively, and no quinoa seeds  
181 retrieved from funnel traps, after one month in the field. Most sesame seeds and sunflower  
182 seeds were retrieved from both sticky and funnel traps ( $> 88\%$ ) (Fig. 3).

183 We found no evidence to support an observer effect (GLM quasibinomial,  
184  $p > 0.05$ ), but, while finding most seeds during seed sorting, there was a slight difference  
185 in retrieval success between species, arugula being significantly lower: 88.6% of arugula

186 seeds, 97.9% of quinoa seeds, 96.9% of sesame seeds and 100% of sunflower seeds (Fig.  
187 4; GLM quasibinomial,  $p < 0.001$ ).

188

## 189 **Discussion**

190 Improving the accuracy and precision of seed rain estimates is necessary to further  
191 our understanding of both seed dispersal and seed limitation, and to support ecological  
192 restoration (Török et al., 2018). However, few studies test seed trap effectiveness in open  
193 ecosystems (Arruda et al., 2018), thus precluding attempts to understand these processes  
194 on a global scale. Our results show that, under the tested field conditions, both trap types  
195 underperformed for species having seeds smaller than 2mm width, and so that we are  
196 underestimating seed dispersal of small seeds. Despite finding no observer bias on seed  
197 sorting for funnel trap samples, we found that accuracy tended to decline for seeds under  
198 2mm width size, suggesting that size-dependence in seed retrieval success is more  
199 common than previously thought (Kollmann and Goetze, 1998).

200 Despite the lower risk of seed predation with sticky traps, due to the strong glue  
201 over the Plexiglas® plate, the seeds on sticky traps are more exposed, and thus more  
202 vulnerable to removal by wind and precipitation, than seeds in funnel traps. Sticky traps  
203 may also be problematic because insects, dust and litter can easily accumulate on the  
204 trap's glue, hindering the visual search for seeds. Traps near the ground are more  
205 vulnerable to contaminants, catching large quantities of dust and litter, especially during  
206 the rainy season. We believe that the height of our sticky traps (25 cm above the soil)  
207 greatly reduced their contamination by soil particles, but it did not prevent contamination  
208 by insects. Notably, many of the insects were mere incidental captures rather than active  
209 seed predators. In contrast, contamination by insects was negligible for funnel traps.

210           The high loss rates found for quinoa and arugula seeds in funnel traps indicate  
211 alarming losses for small seeds in general. The weak structure of the quinoa seed coat can  
212 make these seeds more vulnerable to mechanical stress, fluctuations in humidity and  
213 temperature, and growth of microorganisms (Mohamed-Yasseen et al., 1994). The loss  
214 of quinoa seeds are thus probably linked with humidity within the funnel traps and seed  
215 decay is likely the main cause of seed loss in funnel traps (seed removal by animals is  
216 unlikely due to the shape of the funnel trap). It shows that when reporting results, one  
217 should be aware that seed rain may presents diaspores vulnerable to decay resulting in  
218 underestimation on seed rain surveys, especially during data collection on rainy seasons.  
219 Seeds with hard seed coats, such as sesame and arugula, are generally long-lived and have  
220 high retention rates by funnel traps (Priestley, 1986). Hard seed coats may negative  
221 influence the retention capacity of small seeds on sticky traps, as most arugula seeds were  
222 probably washed off by rain or removed by strong winds from the glue.

223           Despite finding no observer bias on seed sorting for funnel trap samples, we found  
224 that accuracy tended to decline with decreasing seed size and for seeds of darker color.  
225 Soils with high content of organic particles, like where we tested the proof-of-concept,  
226 may directly impact the accuracy of seed sorting methods because soil particles are  
227 similar in color to some seeds; sorting methods should account for this similarity.

228

## 229 **Conclusion**

230 Our study clearly demonstrates that the traits of seeds influence their retrieval from seed  
231 traps used in seed rain studies. We provide a detailed standardized protocol that can be  
232 easily implemented in any seed rain study in grasslands using sticky and funnel traps that  
233 can be tested and used in globally distributed experiments (Borer et al. 2014). When  
234 discussing the results of a seed rain study using the proposed protocol, one must offer the

235 caveat that the methodology applied likely underestimates seed rain considering seed  
236 traits, traps types and environmental conditions. Under our field conditions, our seed rain  
237 study would not allow us to conclude that small, soft seeds cannot be dispersed by water  
238 run-off, as they may decay in funnel traps; nor could we infer anything regarding small,  
239 dark, smooth seeds, as they can be lost from sticky traps or not found by observers.  
240 Finally, we argue that our standardized protocol addressing trap effectiveness and seed  
241 sorting methods will increase reliability of data obtained in seed rain studies in grasslands  
242 and allow more reliable comparisons between datasets.

243

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249 no conflict of interest.

250

#### 251 **Authors' contributions**

252 All authors conceived the ideas and designed methodology; A.J.A. and E.B. collected the  
253 data; A.J.A. and E.B. analysed the data; A.J.A. led the writing of the manuscript. All  
254 authors contributed critically to the drafts and gave final approval for publication.

255

#### 256 **Supporting Information**

257 Additional Supporting Information may be found in the online version of this article.

258 **Data S1.** Sticky traps construction guidelines (Sticky\_trap.csv).

259 **Data S2.** Funnel traps construction guidelines (Funnel\_trap.csv).

260

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340 **Table 1:** Seed average width, length and weight for the four species that should be used in the protocol from measurements made from 15 seeds of  
341 each species after heat treatment at 120°C for an hour.

342

<b>Species and family</b>	<b>Weight (mg)</b>	<b>Width (mm)</b>	<b>Length (mm)</b>	<b>Color</b>
Arugula ( <i>Eruca sativa</i> , Brassicaceae)	2.2±0.0005	1.3±0.1	1.8±0.2	Dark brown
Quinoa ( <i>Chenopodium quinoa</i> , Amaranthaceae)	4.6±0.001	2.1±0.001	2.3±0.2	Whitish
Sesame ( <i>Sesamum indicum</i> , Pedaliaceae)	6.4±0.001	1.9±0.2	5.2±0.3	Pale beige
Sunflower ( <i>Helianthus annuus</i> , Asteraceae)	51.2±0.01	5.4±0.60	10.5±0.65	Pale grey

343

Box 1 – Definitions of terms related to seed rain estimates

**Seed dispersal** – the horizontal movement of diaspores away from the mother-plant

**Seed rain** – the number and diversity of seeds reaching a given area

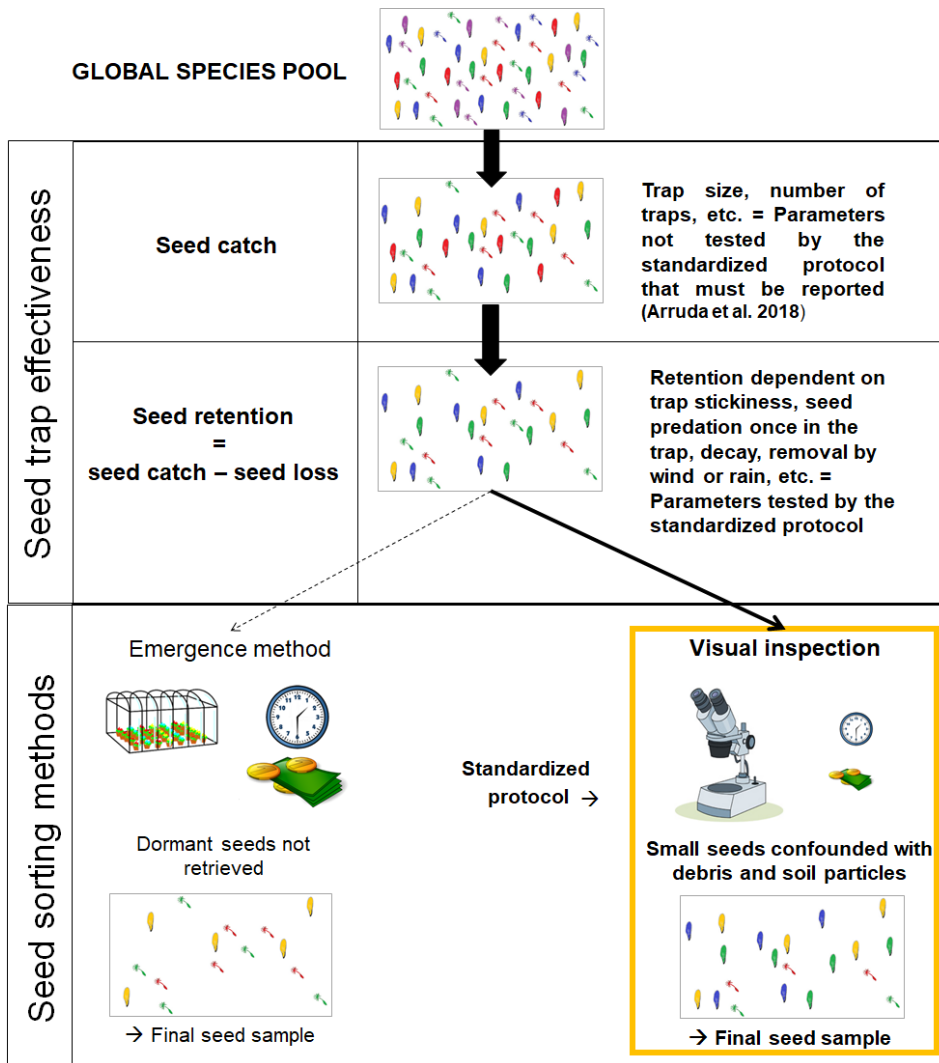
**Seed trap effectiveness** – the ability of seed traps to accurately and precisely estimate seed rain. Seed trap effectiveness is determined by seed catch and seed retention.

**Seed catch** – a property of seed traps that refers to its ability to trap seeds from the seed rain. The final number of seeds captured by a seed trap is affected by both seed retention and seed loss.

**Seed retention** – a property of seed traps that refers its capacity to maintain seeds on/in traps after seed catch until seed retrieval.

**Seed loss** – process caused by seed predators, pathogens and unknown causes that decreases seed trap effectiveness and produce the final trap sample

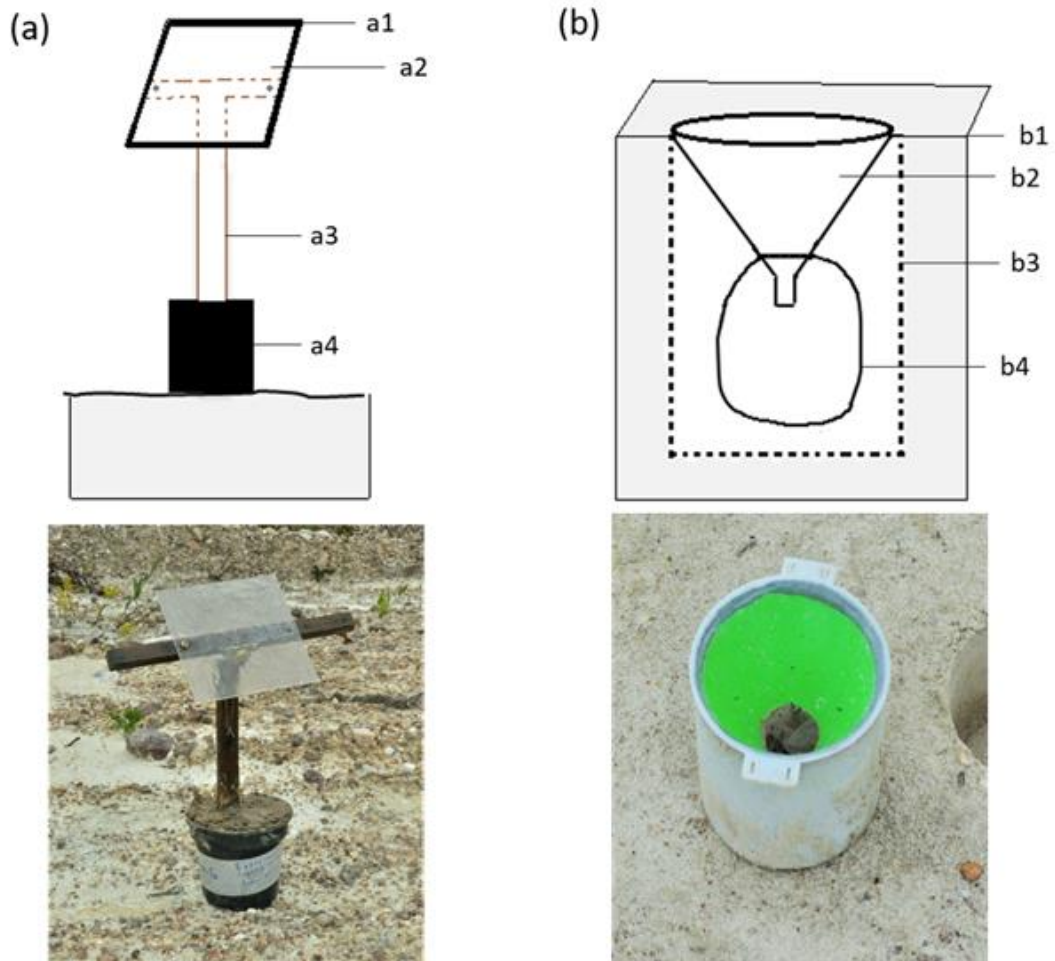
**Seed decay** - the progressive deterioration of the structures and functions of the seed over time, and which will ultimately leads to seed death



347

348 **Fig. 1.** Conceptual framework showing two key stages of seed rain measurements  
349 needing standardization. Each stage is composed of sequential steps in which the number  
350 and richness of seeds is potentially decreased (the direction of the black arrow, Box 1).  
351 There are two possible methods for seed sorting in trap samples. The grow-out method  
352 involves transferring the collected material to trays in greenhouses and identifying species  
353 from growing seedlings. This method is time- and labor-consuming, and underestimates  
354 dormant seeds. In the direct seed inspection method, each sample is processed for seed  
355 separation, and identification using a magnifying glass. Standardizing seed counting at  
356 the seed sorting stage is essential to decrease the likelihood of scoring bias.

357

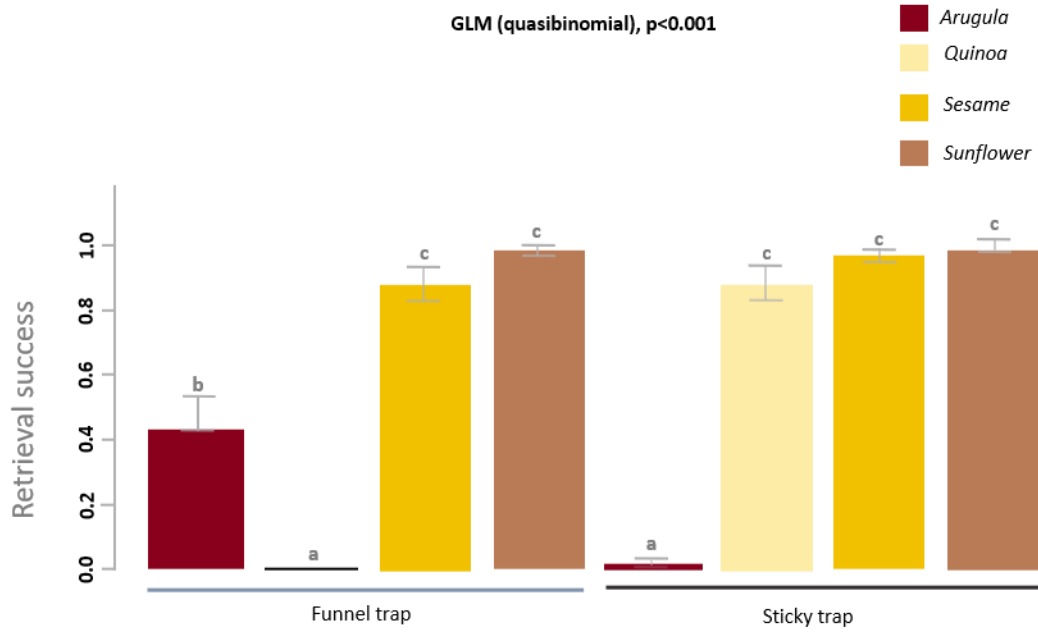


358

359 **Fig. 2.** (a) Sticky trap. a1: clear Plexiglas® plate (0.0225 m<sup>2</sup>); a2: sticky gel over a thin  
 360 plastic film placed over the Plexiglas plate; a3: wooden pole; a4: plastic pot filled with  
 361 concrete and (b) Funnel trap. b1: ground level; b2: PVC plastic funnel section (0.01 m<sup>2</sup>);  
 362 b3: white PVC pipe with bore holes 1 cm diameter; b4: bag of < 0.1mm mesh size.

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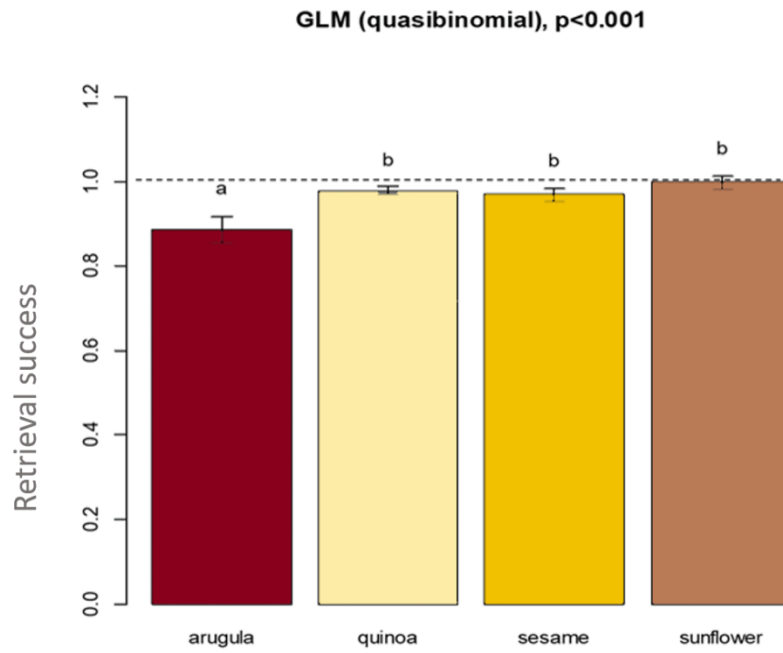
366 **Fig. 3.** Retrieval success for four species in two seed trap types over a 30-day period

367 (\*\*\*)= $p < 0.001$ ). For each species, 10 seeds were placed in each trap type (indicated by

368 the dashed line).

369

370



371

372 **Fig. 4.** Retrieval success on the sorting accuracy test of the funnel trap samples for the

373 four species.

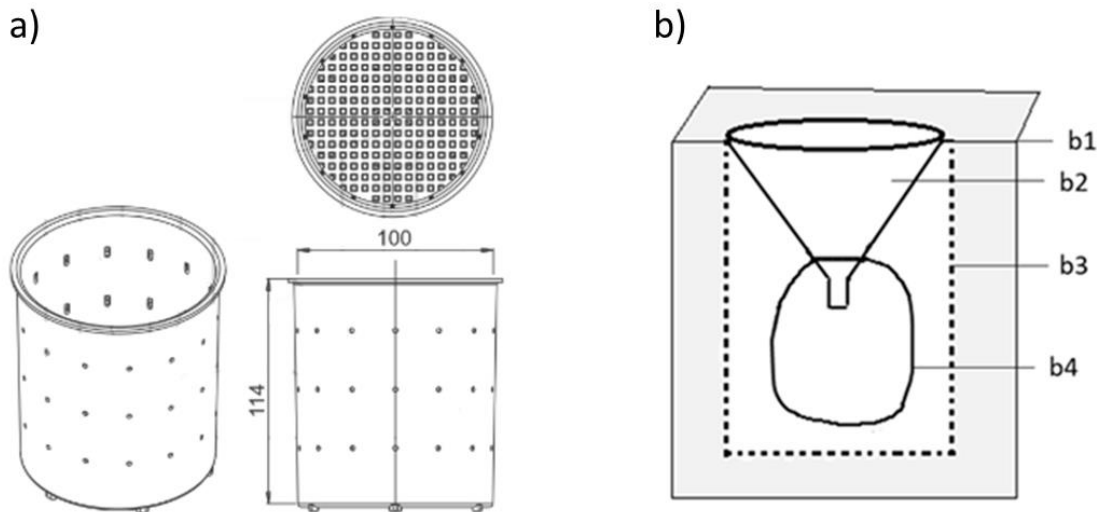
374

375

376 **Data S2. Funnel traps construction guidelines (Funnel\_trap.csv).**

377 Use a PVC pipe measuring 11.4 cm high  $\times$  15.0 cm wide (top)  $\times$  10 cm long, originally  
378 designed for cheese production. This cylindrical plastic structure should have holes, 1 cm  
379 in diameter, on the sides and bottom to allow drainage. Attach a conical PVC plastic  
380 funnel, 15 cm in diameter, to the top end of the cylindrical plastic structure  
381 (approximately 0.0177 m<sup>2</sup> in area). At the bottom of the funnel, the seeds are caught in a  
382 disposable polypropylene bag, with pores smaller than 0.1 mm, fixed with an elastic band  
383 to the base of the funnel. This polypropylene bag is originally produced as a disposable  
384 hygienic hair cap, and it retains its integrity over the period of exposure in the field,  
385 independent of weather conditions (TALGE, Santa Catarina, Brazil).

386



387

388 **Appendix S2:** a) External structure of the funnel trap before PVC funnel installation b)  
389 Internal structure of the funnel trap installed in the field; b1: ground level; b2: PVC plastic  
390 funnel (15 cm<sup>2</sup> upper end area); b3: PVC pipe with bore holes 1 cm diameter; b4: bag of  
391 < 0.1mm mesh size.