

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

André J. Arruda, Fernando A.O. Silveira, Elise Buisson

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4	A simple standardized protocol to evaluate the reliability of seed rain			
5	estimates			
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7	André J. Arruda ^{1.2.3*} , Fernando A. O. Silveira ¹ , Elise Buisson ²			
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10	¹ Department of Botany, Federal University of Minas Gerais, Brazil;			
11	² Avignon Université, Institut Méditerranéen de Biodiversité et d'Ecologie, CNRS, IRD,			
12	Aix Marseille Université, IUT d'Avignon, AGROPARC, France			
13	³ University of Western Australia, School of Biological Sciences, Australia			
14	*Correspondence author. E-mail: ajarruda@gmail.com			
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17 Abstract

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

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

38 Introduction

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

Evaluation of seed trap effectiveness involves the evaluation of two processes: trap capacity to capture seeds (seed catch), and trap capacity to retain seeds (seed retention) and avoid seed loss (Box 1; Fig. 1). Additionally, the accuracy of seed sorting methods for trap samples depends not only on seed size—small seeds are harder to find but also on the ability to separate seed material from debris, which can strongly affect seed retrieval rates (Cottrell, 2004). Knowing the seed retrieval rate of seed sorting is important for determining the influence of seed size, of observer effect, and of sample
composition, or more precisely, the color and size of soil particles, debris and litter often
present in samples (Debussche and Isenmann, 1994).

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

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

Among the possible methods for sorting seeds in funnel-trap samples, the most 87 88 effective is direct seed inspection after sieving to separate seeds from debris (Kollmann and Goetze, 1998; Cottrel, 2004). Seed identification requires training; however, one's 89 90 capacity to find and sort seeds also depends on seed traits, such as size and color (Martin and Barkley, 1961; Cottrell, 2004). Considering that results can be biased by differences 91 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 the sorting procedure. Our goal is to propose and describe a simple standardized protocol 94 to evaluate the effectiveness in seed retention of two types of seed trap (Box 1, Fig. 1), 95 96 and the accuracy of a seed sorting method in assessing seed rain. The standardized protocol should be run along with every seed rain study using these traps to allow more 97 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

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

109

110 STANDARDIZED PROTOCOL

We used four species: arugula (Eruca sativa), quinoa (Chenopodium quinoa), 111 112 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 size, weight and colors that can be tested across ecosystems (Table 1). To assess seed 115 116 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 the field is a mandatory step to 1) prevent the seeds from germinating on the seed traps, 118 2) avoid contamination by pathogens that may be associated with these seeds (Godefroid 119 120 et al., 2017), 3) prevent invasion of exotic species (Estévez et al., 2015). We placed all seeds in a drying oven at 120°C for an hour, as treatments heating seeds above 85°C for 121 122 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 over one year, during the season in which conditions are most challenging for 127 preservation of the seeds on the traps. For the sticky traps, all samples collected from each 128 trap are examined under magnification. For the funnel traps, bags are collected separately 129 from each trap, and their content washed in a 250-µm sieve to reduce the amount of fine 130 131 soil particles, then examined under a magnifying glass to count and identify seeds. All 132 seeds are counted and any signs of damage recorded.

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

much litter and soil in the field, making it difficult to retrieve seeds. For this test, a given 136 137 number of seeds of the four species are mixed in soil, in a proportion equal to that commonly found inside the funnel trap bags. The soil used must be taken from the study 138 139 area to control for color, debris and litter composition. The number of seeds in each sample (with a minimum of 3 and maximum of 30 seeds per species) should be 140 141 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 the seeds. For ethical aspects, observers' anonymity must be respected when collecting 143 and reporting the data. 144

145 The proportion of seeds retrieved at the end of the experiments (retrieval success) is obtained by calculating the percentage of seeds retrieved by each observer. Both 146 experiments are analyzed using generalized linear models that assume a quasibinomial 147 148 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 comparisons with a Tukey adjustment can be run. We here performed these analyses with 152 153 R (R Core Team, 2018), packages *base* and *emmeans*.

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155 PROOF OF CONCEPT

In order to proof-of-concept our protocol, we conducted fieldwork in the southern part of the Espinhaço mountain range, southeastern Brazil (43° 35' W, 19° 17' S). The annual precipitation averages around 1,400 mm, and climate is markedly seasonal, with most rainfall occurring in the hot summers (Silveira et al., 2016). We conducted the experiment in March 2017, at the end of the raining season, when high temperatures, strong winds and rainy days prevail, creating the most challenging conditions in the study area. The main vegetation comprises the mountaintop *campo rupestre*, fire-prone open grasslands that establish on quartzite-derived rocks, with shallow and severely nutrientpoor sandy soils (Silveira et al., 2016).

We randomly placed six of each type of seed trap on a pristine site (50 m²), and retrieved seeds after a period of one month, as this the most common timeframe used to sample seed rain in grasslands (Arruda et al., 2018). For the second experiment, testing seed sorting accuracy with funnel trap samples, we ran the protocol with three previously 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 performance between seed traps for sesame seeds (Fig. 3). Funnel traps performed better 177 than sticky traps in the retention of arugula seeds (41.4% more efficient than sticky traps) 178 (Fig. 3). Quinoa and arugula seeds had the lowest retention rates, with only 1.4% and 179 42.9% of the arugula seeds on sticky and funnel traps, respectively, and no quinoa seeds 180 181 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).

We found no evidence to support an observer effect (GLM quasibinomial, p>0.05), but, while finding most seeds during seed sorting, there was a slight difference in retrieval success between species, arugula being significantly lower: 88.6% of arugula seeds, 97.9% of quinoa seeds, 96.9% of sesame seeds and 100% of sunflower seeds (Fig.
4; GLM quasibinomial, p<0.001).

188

189 **Discussion**

Improving the accuracy and precision of seed rain estimates is necessary to further 190 191 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 on a global scale. Our results show that, under the tested field conditions, both trap types 194 195 underperformed for species having seeds smaller than 2mm width, and so that we are underestimating seed dispersal of small seeds. Despite finding no observer bias on seed 196 197 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 may also be problematic because insects, dust and litter can easily accumulate on the 203 204 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 the rainy season. We believe that the height of our sticky traps (25 cm above the soil) 206 207 greatly reduced their contamination by soil particles, but it did not prevent contamination by insects. Notably, many of the insects were mere incidental captures rather than active 208 209 seed predators. In contrast, contamination by insects was negligible for funnel traps.

The high loss rates found for quinoa and arugula seeds in funnel traps indicate 210 211 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 of quinoa seeds are thus probably linked with humidity within the funnel traps and seed 214 215 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 underestimation on seed rain surveys, especially during data collection on rainy seasons. 218 219 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.

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

228

229 Conclusion

Our study clearly demonstrates that the traits of seeds influence their retrieval from seed traps used in seed rain studies. We provide a detailed standardized protocol that can be easily implemented in any seed rain study in grasslands using sticky and funnel traps that can be tested and used in globally distributed experiments (Borer et al. 2014). When 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 study would not allow us to conclude that small, soft seeds cannot be dispersed by water 237 238 run-off, as they may decay in funnel traps; nor could we infer anything regarding small, dark, smooth seeds, as they can be lost from sticky traps or not found by observers. 239 240 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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250

251 Authors' contributions

All authors conceived the ideas and designed methodology; A.J.A. and E.B. collected the

data; A.J.A. and E.B. analysed the data; A.J.A. led the writing of the manuscript. All

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.
- **Data S1.** Sticky traps construction guidelines (Sticky_trap.csv).
- **Data S2.** Funnel traps construction guidelines (Funnel_trap.csv).

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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
each species after heat treatment at 120°c for an hour.

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

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

345



Fig. 1. Conceptual framework showing two key stages of seed rain measurements 348 needing standardization. Each stage is composed of sequential steps in which the number 349 350 and richness of seeds is potentially decreased (the direction of the black arrow, Box 1). There are two possible methods for seed sorting in trap samples. The grow-out method 351 involves transferring the collected material to trays in greenhouses and identifying species 352 353 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 the seed sorting stage is essential to decrease the likelihood of scoring bias. 356



Fig. 2. (a) Sticky trap. a1: clear Plexiglas® plate (0.0225 m²); a2: sticky gel over a thin
plastic film placed over the Plexiglas plate; a3: wooden pole; a4: plastic pot filled with
concrete and (b) Funnel trap. b1: ground level; b2: PVC plastic funnel section (0.01 m²);
b3: white PVC pipe with bore holes 1 cm diameter; b4: bag of < 0.1mm mesh size.



Fig. 3. Retrieval success for four species in two seed trap types over a 30-day period
(***=p<0.001). For each species, 10 seeds were placed in each trap type (indicated by
the dashed line).

GLM (quasibinomial), p<0.001





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

- 373 four species.

Data S2. Funnel traps construction guidelines (Funnel_trap.csv).

Use a PVC pipe measuring 11.4 cm high \times 15.0 cm wide (top) \times 10 cm long, originally 377 378 designed for cheese production. This cylindrical plastic structure should have holes, 1 cm in diameter, on the sides and bottom to allow drainage. Attach a conical PVC plastic 379 380 funnel, 15 cm in diameter, to the top end of the cylindrical plastic structure 381 (approximately 0.0177 m² in area). At the bottom of the funnel, the seeds are caught in a disposable polypropylene bag, with pores smaller than 0.1 mm, fixed with an elastic band 382 to the base of the funnel. This polypropylene bag is originally produced as a disposable 383 hygienic hair cap, and it retains its integrity over the period of exposure in the field, 384 independent of weather conditions (TALGE, Santa Catarina, Brazil). 385

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Appendix S2: a) External structure of the funnel trap before PVC funnel installation b)
Internal structure of the funnel trap installed in the field; b1: ground level; b2: PVC plastic
funnel (15 cm² upper end area); b3: PVC pipe with bore holes 1 cm diameter; b4: bag of
< 0.1mm mesh size.