Running title: Reliability of seed rain estimates

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

André J. Arruda^{1-2,3*}, Fernando A. O. Silveira¹, Elise Buisson²

¹ Department of Botany, Federal University of Minas Gerais, Brazil;

²Avignon Université, Institut Méditerranéen de Biodiversité et d'Ecologie, CNRS, IRD,
Aix Marseille Université, IUT d'Avignon, AGROPARC, France

³ University of Western Australia, School of Biological Sciences, Australia

*Correspondence author. E-mail: ajarruda@gmail.com

Abstract

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) is challenging, and the lack of standardization in measurement prevents cross-site comparisons. Seed trap effectiveness and accuracy of seed sorting methods are key components of seed rain estimates in need of standardization. We propose and describe 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 (arugula, quinoa, sesame and sunflower) to produce a gradient of seed size, weight and 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 width) that can be easily mistaken for debris and soil particles or that fail to adhere to 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 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 a size-dependence in seed retrieval success. Our standardized protocol addressing trap effectiveness and seed sorting methods will increase reliability of data obtained in seed rain studies in grasslands and allow more reliable comparisons between datasets.

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

Introduction

Seed dispersal studies are vital to understanding plant distribution and community resilience, and they guide conservation and restoration activities (Török et al., 2018). A 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 traps to collect propagules at particular locations, then identifying and counting them. However, measuring seed rain is challenging, and a lack of methodological standardization persists, compromising the accuracy of seed rain estimates and impairing comparison of data between studies (Arruda et al., 2018; Wolfe et al., 2019). Seed rain 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 environments, however, seed dispersal by wind and water run-off poses additional 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 (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 seed traps, and of seed sorting methods for samples, is crucial for improving 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).

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).

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, 1997). Seed decay can vary greatly between seed types and is also modulated by other 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 water run-off can vary greatly between trap types, seasons and plant communities, thereby influencing seed retrieval rates. Therefore, to maximize seed catch and minimize seed loss in open ecosystems, the use of complementary seed trap types is recommended (Chabrierie and Alard, 2005).

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 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, are more suitable for studying wind-dispersed species (Jefferson and Usher 1989). While 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 may also bias the seed catch towards larger seeds that are more easily detected by visual assessment, while soft seeds may easily rot, and smooth seeds may be lost through rain or wind action (Kollmann and Goetze, 1998; Cottrell, 2004). Despite the current state of knowledge, the influence of seed size and weight on the retention rate of seed traps is still overlooked.

Among the possible methods for sorting seeds in funnel-trap samples, the most effective is direct seed inspection after sieving to separate seeds from debris (Kollmann and Goetze, 1998; Cottrell, 2004). Seed identification requires training; however, one's 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 in one's capacity to find and sort seeds from the samples, it is important to obtain, prior 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 to evaluate the effectiveness in seed retention of two types of seed trap (Box 1, Fig. 1), 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 reliable comparisons between studies. We tested these standardized protocols in a tropical grassland as a proof-of-concept.

Description and implementation

SEED TRAPS

We tested sticky and funnel traps (Fig. 2), which capture complementary processes of seed rain (Chabrierie 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).

STANDARDIZED PROTOCOL

We used four species: arugula (*Eruca sativa*), quinoa (*Chenopodium quinoa*), sesame (*Sesamum indicum*) and sunflower (*Helianthus annuus*). Here, the sunflower achenes are called seeds to facilitate the terminology throughout the manuscript. These 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 retention, the number of traps and the length of time that traps should be left in the field 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, 2) avoid contamination by pathogens that may be associated with these seeds (Godefroid 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 more than 8 min have shown to kill most seeds (Hess et al. 2018). We conducted all seed measurements (e.g. weight and size) after the heat treatment.

On each trap, we gently dropped 10 seeds of each species, totaling 40 seeds per trap (Table 1). On funnel traps, we put all seeds straight into the bag collectors. It is best 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 preservation of the seeds on the traps. For the sticky traps, all samples collected from each trap are examined under magnification. For the funnel traps, bags are collected separately from each trap, and their content washed in a 250-µm sieve to reduce the amount of fine soil particles, then examined under a magnifying glass to count and identify seeds. All 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 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 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 randomized and noted; this number is not known to the observers. A minimum of three previously trained observers then sort the samples, searching for, identifying and counting the seeds. For ethical aspects, observers' anonymity must be respected when collecting and reporting the data.

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 experiments are analyzed using generalized linear models that assume a quasibinomial distribution and use retrieval success as the response variable. For the seed trap effectiveness experiment, trap type and species are the categorical variables (interaction was tested). For the seed sorting experiment, species and experimenters are the categorical variables (interaction was tested). In both cases, pairwise contrast comparisons with a Tukey adjustment can be run. We here performed these analyses with R (R Core Team, 2018), packages *base* and *emmeans*.

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 nutrient-poor 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.

Results of the proof-of-concept

In the proof-of-concept experiment, we found no difference between the two trap types in sunflower seed retention, with both performing well (98.6% for funnel trap and 100% for sticky trap) (Fig. 3). Funnel traps were ineffective in retaining quinoa seeds under the field conditions, while the sticky trap had a good retention rate for quinoa seeds (88.6%; 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 than sticky traps in the retention of arugula seeds (41.4% more efficient than sticky traps) (Fig. 3). Quinoa and arugula seeds had the lowest retention rates, with only 1.4% and 42.9% of the arugula seeds on sticky and funnel traps, respectively, and no quinoa seeds retrieved from funnel traps, after one month in the field. Most sesame seeds and sunflower 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, $p < 0.001$).

Discussion

Improving the accuracy and precision of seed rain estimates is necessary to further our understanding of both seed dispersal and seed limitation, and to support ecological restoration (Török et al., 2018). However, few studies test seed trap effectiveness in open 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 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 sorting for funnel trap samples, we found that accuracy tended to decline for seeds under 2mm width size, suggesting that size-dependence in seed retrieval success is more common than previously thought (Kollmann and Goetze, 1998).

Despite the lower risk of seed predation with sticky traps, due to the strong glue over the Plexiglas® plate, the seeds on sticky traps are more exposed, and thus more 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 trap's glue, hindering the visual search for seeds. Traps near the ground are more 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) 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 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 alarming losses for small seeds in general. The weak structure of the quinoa seed coat can make these seeds more vulnerable to mechanical stress, fluctuations in humidity and 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 decay is likely the main cause of seed loss in funnel traps (seed removal by animals is unlikely due to the shape of the funnel trap). It shows that when reporting results, one 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. Seeds with hard seed coats, such as sesame and arugula, are generally long-lived and have high retention rates by funnel traps (Priestley, 1986). Hard seed coats may negative influence the retention capacity of small seeds on sticky traps, as most arugula seeds were 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.

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

caveat that the methodology applied likely underestimates seed rain considering seed 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 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. Finally, we argue that our standardized protocol addressing trap effectiveness and seed sorting methods will increase reliability of data obtained in seed rain studies in grasslands and allow more reliable comparisons between datasets.

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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.

Supporting Information

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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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.

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Species and family	Weight (mg)	Width (mm)	Length (mm)	Color
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

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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

Fig. 1. Conceptual framework showing two key stages of seed rain measurements needing standardization. Each stage is composed of sequential steps in which the number 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 involves transferring the collected material to trays in greenhouses and identifying species from growing seedlings. This method is time- and labor-consuming, and underestimates dormant seeds. In the direct seed inspection method, each sample is processed for seed separation, and identification using a magnifying glass. Standardizing seed counting at the seed sorting stage is essential to decrease the likelihood of scoring bias.

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).

Fig. 4. Retrieval success on the sorting accuracy test of the funnel trap samples for the 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 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 funnel, 15 cm in diameter, to the top end of the cylindrical plastic structure (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 to the base of the funnel. This polypropylene bag is originally produced as a disposable hygienic hair cap, and it retains its integrity over the period of exposure in the field, independent of weather conditions (TALGE, Santa Catarina, Brazil).

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.