# ESTIMATING POLLINATOR PERFORMANCE OF VISITORS TO THE SELF-INCOMPATIBLE CROP-PLANT *BRASSICA RAPA* BY SINGLE VISIT DEPOSITION AND POLLEN GERMINATION: A COMPARISON OF METHODS

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*Abstract*—Estimating the pollen-deposition effectiveness of flower visitors is fundamental to understanding their performance as pollinators. While estimates of visitation rates, pollen loads, and single visit deposition (SVD) are all useful proxies for performance, and so help to reveal the relative effectiveness of different visitors, none take into account the breeding system of the plants, or the quality of pollen deposited. Here we compare the performance of visitors to the self-incompatible plant *Brassica rapa* (turnip) using SVD and pollen germination. We also report the first use of the staining of *Brassica rapa* stigma papilla cells (known to reveal a specific reaction to self-pollen) to compare self-pollen deposition between insect visitors. We found that most of the pollen grains deposited by insect visitors (and therefore counted by SVD methods) were non-germinating self-pollen. A smaller proportion of grains were outcrossed and so germinated. There was also a significant positive relationship between environmental conditions (wind speed) and pollen deposition, but not pollen germination.

Both methods identified *Bombus* spp. as the best-performing visitors on turnip flowers, followed by *Eristalis* spp., whereas performance estimates for *Episyrphus balteatus* and 'other hoverflies' were no higher than controls for both methods. This study provides further insight into the methodology for estimating pollinator performance, especially in plants when only cross-pollen can germinate.

Keywords: Pollination, Pollinator effectiveness, Pollen deposition, Pollen germination, Brassicaceae

# INTRODUCTION

Understanding plant-pollinator interactions is vital, as pollinators play a key role in ecosystem services that maintain biodiversity. Thirty-five per cent of global food production relies on insect pollinators (Klein et al. 2007), and approximately 87% of flowering plant species globally are entomophilous (Ollerton et al. 2011). With many insect pollinator populations in decline (e.g. Potts et al. 2010) and a heavy dependency on a small number of pollinator species for crop pollination (Kleijn et al. 2015), a deeper quantitative insight into pollinator performance on crops is essential. Surveys of visitation patterns and rates can produce valuable large datasets quickly, but lack information on visit quality. More time-consuming surveys of single visit pollen deposition (SVD; sensu Ne'eman et al. 2010) provide a measure of visit quality and can distinguish conspecific deposition, but lack information on the viability of the pollen deposited (Ballantyne et al. 2015; Ballantyne et al. 2017). Here we compare SVD measures with counts of the number of pollen grains germinating after a single visit to a self-incompatible species, providing a direct measure of cross-pollen deposition. Outcrossed pollen is essential for fertilisation in self-incompatible species, and self-pollen deposition can be deleterious via stigma-clogging (Shore &

Barrett 1983; Galen et al. 1989; Gross 2005).

Counting the number of germinated pollen grains after a single visit is the ideal method for estimating pollinator performance in self-incompatible plants, but is only suitable for species where the reproductive systems are well-known. Wist & Davis (2013) aimed to compare SVD with pollen germination in the apparently self-incompatible *Echinacea angustifolia* (Asteraceae), but found it to be self-compatible, confounding pollen germination results. There are thus no studies to date that focus on a fully self-incompatible plant species.

Turnip, Brassica rapa, was chosen as an important crop species that is also fully self-incompatible through the sporophytic self-incompatibility (SSI) mechanism (Hiscock & McInnis 2003). Callose plugs are deposited by pollen tubes as they grow, and stigmatic papillae produce callose in response to the presence of self-pollen (Currier 1957). Callose can be stained with aniline blue and viewed with fluorescence microscopy (Kearns & Inouye 1993; Ästergaard et al. 2002). This allows pollen tubes of successfully germinated pollen grains, and also papilla cells that have reacted to self-pollen, to be identified and quantified. The average number of successfully germinated pollen grains after single visits from insect groups is then a measure of pollinator performance. For the first time ever, the papilla cell response can then be used to show the presence of selfpollen, so that self-pollen deposition can be compared between visitors.

Received 23 November 2016, accepted 9 August 2017

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This study asks the question: how do pollinator performance estimates compare between SVD and pollen germination methods? A positive relationship between SVD and pollen germination will indicate that SVD estimates are unlikely to be confounded by self-pollen deposition.

#### MATERIALS AND METHODS

#### Plants, visitors and study site

A row of *Brassica rapa* ssp. *rapa* (Brassicaceae) was grown from seed at Earlshall Castle garden, Fife (56 22.8' N, 2 52.1' W). Thirty-three plants were individually labelled for identification. The field site had a variety of flowering plant species in its vicinity, but the plot was maintained so that only *Brassica rapa* flowered within the experimental row. Data were collected through the main flowering period (28th August to 18th September 2015) between 10:00 and 15:00 on each suitable day, whenever dry and calm weather conditions permitted. Temperature, humidity and wind speed were recorded at 30-minute intervals throughout the sampling period.

Flower visitors were identified by photography or catch and release methods (Appendix I). *Bombus* were identified to species (although *B. terrestris* and *B. lucorum* are difficult to distinguish in the field (Falk 2015), so we hereafter refer to *B. terrestris/lucorum*); but later grouped by genus for analysis. For hoverflies, *Eristalis* were identified to genus, and *Episyrphus balteatus* to species. All others were small syrphids and grouped as 'other hoverflies'. Visits from *Apis mellifera*, a *Lasioglossum* species and a *Sphecodes* species were also recorded, but were too infrequent to include in the analysis.

## Measuring pollinator performance

Inflorescences were covered with mesh bags 24 hours prior to sampling, and a single petal was removed to identify flowers that were open prior to bagging. Flowers that opened and dehisced whilst in the bag were then used for SVD or pollen germination sampling. No data were collected from undehisced flowers or flowers greater than one day old based on preliminary tests that confirmed cross-pollen grains were best able to germinate on flowers that had dehisced and were <I day old.

For SVD measurements, individual open and virgin flowers were observed until their first insect visit. The time, visitor identity, visit duration and foraging behaviour (feeding on pollen or nectar) were recorded for each visit. After the visitor had left, the flower was carefully dissected using fine forceps, and the stigma's receptive surfaces were then dabbed onto a cube of fuchsin gel on a microscope slide, which was then melted under a coverslip. Stigmas were checked using a hand lens to ensure all pollen had been removed. Pollen grains were identified under a light microscope as either conspecific or heterospecific (Fig.I). This process was repeated (N= 39) on unvisited flowers to control for pollen deposition due to wind or to handling.

For pollen germination, the SVD method was followed until a visit was completed, but the stigma was not disturbed. Instead, the anthers were removed to prevent any additional





FIGURE I. Brassica rapa pollen grains in fuchsin gel viewed with light microscopy at (A)  $100 \times \text{magnification}$  and at (B)  $400 \times \text{magnification}$ .

pollen deposition and the inflorescences were re-bagged for 24 hours. Then bags were removed and the flowers dissected to access the pistils (stigma, style and ovaries), which were placed into Eppendorf tubes containing I.0 ml of FAA fixative (I part formalin: I part acetic acid: 18 parts 50% ethanol [Kearns & Inouye, 1993]). After 18 hours the pistils were transferred to new tubes containing I.0 ml of 70% ethanol, until they were counted. For microscopy, they were removed from the ethanol and softened in I.0 ml of I M NaOH for 6 hours at room temperature, then washed with distilled water, placed on a microscope slide with a drop of decolourised aniline blue (DAB) (0.0005% w/v) and squashed under a coverslip. The DAB stock solution mixed 0.01% w/v aniline blue (Sigma-Aldrich) with 0.1 M K2HPO4 buffer; this buffer is used to decolourise aniline blue, or it may interfere with the fluorescence (Kearns & Inouye 1993).



FIGURE 2. Fluorescence microscopy images of *Brassica rapa* stigmas ( $375 \times magnification$ ). (A) Ungerminated pollen grains (UPG), stained papilla cells (SPC) and a germinated pollen grain with a pollen tube that has not penetrated the stigma (circled in red). (B) Germinated pollen grains (GPG) with pollen tubes (PT) that have penetrated the stigma, and a callose deposit within a pollen tube (C). (C) Papilla cells on a virgin stigma. (D) Stained papilla cells after contact with self-pollen.

We used fluorescence microscopy (Leitz Ortholux II with a mercury vapour UV light source, and Leitz filter cube A;  $375 \times$  magnification with a water-immersion objective lens) to count germinated pollen grains (those producing pollen tubes that penetrate the stigma), non-germinated pollen grains, and stained papilla cells (see Fig. 2). Some pollen grains germinated but produced pollen tubes that failed to penetrate the stigma (Fig. 2A). These were easily identified: the tubes were very short and narrow compared to those from successfully germinated grains, and did not contain the callose deposits that were observed within the penetrating pollen tubes. Pollen grains that produced these failed tubes are typical of self-pollen deposition (Sulaman et al. 1997) and were not counted as having successfully germinated. This process was repeated (N = 27) with unvisited flowers (controls) as before, and with manual selfand cross-pollination tests to confirm the selfincompatibility of the plants.

#### Statistical analysis

Generalised linear models (GLMs) and mixed models (GLMMs) were used to analyse the data, with R version 3.2.1 (R Core Team 2015). The Lme4 package (Bates et al. 2015) was used for GLMMs. Random effects (intercept) considered in models were: Plant to account for between plant differences (e.g. position within the row, and possible biological differences between plants) and because each plant was used more than once for data collection; Individual, because in some cases the same insect would visit two or three flowers that were being watched for data collection so that more than one data point was collected from the same visitor; and Date was included to account for between-day variations in unmeasured abiotic conditions, which could affect pollen germination and tube growth. Fixed effects considered in models were: visitor, visit length, time of day, and wind speed. The interactions 'visitor\*visit length' and 'visitor\*wind' were not tested in GLMM's (i.e. alongside random effects) due to insufficient data. Instead, they were

tested in GLMs and found to be non-significant. The full models vary between analyses depending on what specification allowed for a robust model to be constructed. Stepwise model simplification was carried out by removing non-significant terms and comparing models using Akaike's information criterion (AIC) to produce minimum adequate models (Crawley 2007) (see Appendix II for details of full and minimum models).

The model validation procedure in Thomas et al. (2013) was followed, checking for over-dispersion and for patterns in the deviance residuals. In GLMs the dispersion parameter (theta) was calculated by dividing the residual deviance by the residual degrees of freedom. A theta value between 0.75 and 1.5 is deemed acceptable (Zuur et al. 2009; Thomas et al. 2013). Theta in GLMMs was calculated using the 'blmeco' package (Korner-Nievergelt et al. 2015). Negative binomial and Poisson distributions were used in the models (see Appendix II for detail on specific models). *Temperature* and *humidity* co-varied with *time of day* (Pearson correlation coefficients: 0.54, P < 0.001, and -0.45, P < 0.001 respectively) so both were omitted from the analyses.

Model statistics and sample sizes are reported in Tab. I, rather than in-text. Standard errors are back-transformed from GLMs, and are therefore asymmetrical.

## RESULTS

#### Single visit pollen deposition

A total of 101 SVD data points were collected from insect visits, plus 39 control data points. *Episyrphus* accounted for 31%, of observations, *Eristalis* 28%, *Bombus* 23% and 'other hoverflies' 18%. More than 99.9% of the pollen counted was conspecific. *Bombus* and *Eristalis* deposited significantly more pollen grains than found on control stigmas, whereas single visits from *Episyrphus* and 'other hoverflies' deposited no more pollen than on control stigmas (Fig. 3A; Tab. I). Wind speed had a marginally significant positive effect on pollen deposition (Tab. I).

#### Pollen germination

Manual cross- and self-pollination tests confirmed that the *Brassica rapa* plants were sporophytically selfincompatible; Fig. 4A shows that cross-pollen germinated and penetrated the stigma, whilst self-pollen did not. Selfpollination correlated with staining of papilla cells whereas cross-pollination did not (Fig. 4B). It is possible that some papilla cells did stain under the cross-pollination treatment, and that detection of these could have been masked by the staining of germinated pollen grains and pollen tubes. The control stigmas in Fig. 5 show a slightly higher number of stained papilla cells than those from cross-pollination in Fig. 4A, which is to be expected since some self-pollen is likely to end up on the stigmas of unvisited flowers.

A total of 7I pollen germination samples were collected from single visits (plus 27 control stigmas). Episyrphus accounted for 32%, of observations, Eristalis 28%, Bombus 24% and 'other hoverflies' 15% (i.e. giving similar proportions to the SVD study). Only one germinated pollen grain was found, on one control stigma; this is much lower



FIGURE 3. Pollinator performance of insect visitors on *Brassica rapa* (turnip) showing that *Bombus* and *Eristalis* were the most effective individual pollinators estimated by (A) single visit pollen deposition (SVD) and (B) pollen germination. Asterisks show significance in comparison to the controls: "\*\*\* = P < 0.001; "\*\* = P < 0.01; "\*\* = P < 0.05. Estimates (mean ± 1 SE) are produced from final model (see Tab. 1 for statistics and sample sizes).

than for SVD methods. This difference is most likely because ungerminated pollen is washed off stigmas during preparation for fluorescence microscopy. Only germinated pollen remains attached to the stigma. The numbers of germinated pollen grains detected after single visits from Bombus and Eristalis were also low, but significantly greater than on control stigmas, whereas the numbers found after single visits from *Episyrphus* and 'other hoverflies' were no different from controls (Fig. 3B; Tab. I). Single visits from *Bombus, Eristalis* and *Episyrphus* led to significantly greater numbers of stained papilla cells than found on control stigmas (Fig. 5; Tab. I).

TABLE 1. Pollinator performance final model results for SVD and pollen germination methods.

	Sample size	Estimate	SE	Z-value	P value
Field data					
<u>SVD</u>					
Control (Intercept)	39	3.533	0.246	14.361	<0.001 ***
Bombus	23	I.724	0.403	4.282	<0.001 ***
Episyrphus	31	0.047	0.373	0.125	0.9
Eristalis	28	I.249	0.381	3.275	<0.01 **
Other hoverflies	19	0.351	0.43	0.815	0.415
Wind (kph) (mean centred)		0.154	0.073	2.107	0.035 *
Pollen germination					
Control (Intercept)	27	-3.606	I.64I	-2.198	0.028 *
Bombus	17	4.495	1.672	2.688	<0.01 **
Episyrphus	23	1.983	1.727	I.I48	0.251
Eristalis	20	3.574	1.672	2.138	0.033 *
Other hoverflies	ΙI	2.119	1.777	1.193	0.233
<u>Papilla cells</u>					
Control (Intercept)	27	3.226	0.228	14.181	<0.001 ***
Bombus	17	I.49	0.317	4.707	<0.001 ***
Episyrphus	23	0.721	0.285	2.531	0.014 *
Eristalis	20	1.32	0.314	4.193	<0.01 **
Other hoverflies	II	0.655	0.347	I.887	0.059
Manual Pollination					
Germinated pollen					
Cross-pollination (Intercept)	7	5.348	0.44	12.16	<0.001 ***
Self-pollination	15	-6.195	0.676	-9.17	<0.001 ***
<u>Papilla cells</u>					
Cross-pollination (Intercept)	7	1.925	0.378	5.092	<0.001 ***
Self-pollination	15	3.358	0.452	7.427	<0.001 ***

# DISCUSSION

Both the SVD and pollen germination methods identified *Bombus* as the most effective pollinators of *Brassica rapa*, followed by *Eristalis*. This is not surprising, as Rader et al. (2009) found similar patterns for *Brassica rapa*, and Ali et al. (2011) reported that *Apis* (there were no *Bombus* in their geographical area) were more effective than *Eristalis*, which were more effective than *Episyrphus*. Jauker et al. (2012) found that hoverflies were individually less effective pollinators of *Brassica rapa* than bees, and in particular that hoverflies were poor at delivering crosspollen, which agrees with the pollen germination data in this study.

The self-pollen deposition in self-incompatible plants is an obvious concern in using SVD to assess pollinator performance, and analysing pollen germination helps to address this issue. Our study does not explicitly separate selfpollen and un-germinated cross-pollen; however it is likely that much of the pollen recorded on *Brassica rapa* by SVD is self-pollen, given the match of papilla cells staining in response to self-pollen deposition and the number of stained papilla cells found after single visits (e.g. single visits from *Bombus* had the greatest pollen deposition, but also the greatest number of stained papilla cells). But it is also likely that some of the pollen counted with SVD was low quality cross-pollen, since this included all deposition by insect visitors, by wind, and by handling, whereas counting germinated pollen on the stigma of self-incompatible plants included only viable cross-pollen. Here, the number of pollen grains that germinated was two orders of magnitude lower than the total number of grains recorded by SVD, highlighting the importance of including pollen quality as a component of PE.

SVD is a reasonable proxy for estimating performance in our study, even though it includes high numbers of pollen grains that do not germinate. However, SVD is unlikely be a good proxy in all situations, since the effect of pollen quality is likely to vary depending on the breeding system of the plant species, plant and flower density, visitor species and abiotic conditions. Heat stress reduces pollen viability for example, so the proportion of pollen grains germinating can vary with temperature (Orueta 2002; Galen & Stanton 2003; Cross et al. 2003).



FIGURE 4. Results of manual self- and cross-pollination tests showing that the *Brassica rapa* plants were self-incompatible, and showing that papilla cells of the stigma stain in response to self-pollen, but not cross-pollen. (A) Pollen germination response to self- and cross-pollen. Germinated pollen grains are those that have produced pollen tubes that penetrate the stigma (i.e. 'GPG' in Fig. 2B). (B) Papilla cell response to cross- and self-pollen. An example of the stained papilla cells is shown in Fig. 2D. Asterisks show significance in difference between self- and crosses: '\*\*\*' = P < 0.001. Estimates (mean  $\pm$  1 SE) are produced from final model (see Tab. I for statistics and sample sizes).

Frier et al. (2016) argued that using SVD to estimate PE on *Lonicera caerulea* (Caprifoliaceae) could incorrectly imply that some visitors were ineffective pollinators, since high variance in intra-floral self-pollen deposition would limit detection of pollen deposited by some visitor groups. The precise origin of the self-pollen in our study is unknown, and could either be intra- or inter-floral. It is likely that an insect visit leads to deposition from both sources (i.e. geitonogamous pollination from inter-floral but within-plant movements by insects, and intra-floral pollen transfer from anthers to stigma during a visit). However both sources of self-pollen are important in terms of sexual interference (Barrett 2002), so it may be unnecessary to know the source of self-pollen in the context of pollinator effectiveness, but simply to know whether it will germinate. In addition, the amount of high quality pollen required for seed production varies greatly between plant species (Cruden 2000), and ideally this should be considered when assessing pollinator performance.

SVD can quantify heterospecific pollen deposition, whilst adding on pollen germination analysis does not, since the preparative stages can remove some un-germinated conspecific and heterospecific grains. Heterospecific pollen deposition can have the same stigma-clogging effect in any plant as self-pollen has in self-incompatible plants (Traveset & Richardson 2006; Brown et al. 2013) and is therefore informative; for example it is often used in assessing the effect of invasive plants on native plant communities (Larson et al. 2006; Bartomeus et al. 2008). The low levels of heterospecific pollen deposition reported here have been observed elsewhere (e.g. Moragues & Traveset 2005; Bartomeus et al. 2008; Willmer et al. 2017), although levels can be rather variable (e.g. Montgomery & Rathcke 2012; Fang & Huang 2013). Choosing between simple SVD and the addition of pollen germination analysis therefore depends on the question being asked; knowing the proportion of heterospecific pollen grains deposited may be more useful than the number germinated in some circumstances.

Pollen deposition due to wind, as seen here (Tab. I), is not unexpected, but it is notable that other PE studies have not detected it. The effect of wind is likely to vary between plant species; for example, it might have a greater effect on self-pollen deposition in plant species with small pollen grains, or species that have anthers close to the stigma. The control stigmas in SVD studies take into account pollen deposition due to the wind and other factors, setting the baseline for comparisons after insect visits; control stigmas must therefore be taken regularly enough to account for the variation in wind speed throughout the data collection period so that the differences in pollen deposition between visitor species (or group) are not confounded by changing wind.

It is important not to over-generalise from this study, which concerns a single crop-plant species at one site, with data gathered during periods of peak visitation and good weather. Ideally it should be repeated on more SSI species to see if the results are typical, i.e. to determine if SVD and pollen germination regularly identify the same order of PE between visitors. It should also be carried out on plants with other self-incompatibility mechanisms, although this will be more difficult to achieve because both self- and cross-pollen germinate on the stigma in species with gametophytic (Newbiggin et al. 1993), cryptic (e.g. Jones 1994) or lateacting self-incompatibility (Gibbs 2014). Using the papilla cell callose response as a proxy for self-pollen deposition is restricted to species where the self-incompatibility mechanism acts at a papillate stigma, and even then callose deposition is not guaranteed and must be tested. There are relatively few studies that have observed the papilla cell



FIGURE 5. Results of papilla cell response to single insect visits, showing that insect visitors transfer self-pollen onto the stigma during a visit. Asterisks show significance in comparison to the control: '\*\*\*' = P < 0.001; '\*\*' = P < 0.01; '\*' = P < 0.05. Estimates (mean ± 1 SE) are produced from final model (see Tab. 1 for statistics and sample sizes).

response outside of the Brassicaceae; (see Friedman & Barrett (2008) for Asteraceae; Pontieri & Sage (1999) for Saururaceae; and Sedgley (1979) for Lauraceae). Nevertheless, using the papilla cell response in a suitable study system may prove valuable in researching the ecology of self-pollen deposition.

A number of variables that were not measured in this study could affect pollen deposition and pollen germination rates between flowers. The number of inflorescences on a plant, the number of open flowers in an inflorescence, variation in nectar production per flower and resultant flower density are all likely to affect the amount of selfpollen moved between individual plants, and between flowers of the same plant, and there is also likely to be within-plant variation in pollen germination rates. In addition, we did not record visitation rates in this study, but it is a key component in other measures of pollinator performance (e.g. in calculating Pollinator Importance in Ballantyne et al. (2015)). However, the main focus of this study, the methods comparison, is unlikely to be biased by these factors.

Future research should compare SVD and pollen germination methods across self-incompatibility mechanisms and should ideally include comparisons with a direct measure of fitness, such as fruit or seed set. Using the pollen germination method at the community-level is unlikely to be achievable, although it is an excellent technique for single species studies. A novel approach may be required to include pollen quality in community-level pollinator performance studies. SVD has already proven to be achievable at the community level, and this study helps to validate it as a suitable method for assessing pollinator performance.

#### ACKNOWLEDGEMENTS

Advice about fluorescence microscopy and callose staining was gratefully received from Dr Peter Gibbs.

#### **APPENDICES**

Additional supporting information may be found in the online version of this article:

APPENDIX I. Insect visitor sample sizes and taxonomic groupings.

APPENDIX II. Model selection analyses.

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