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APPLICATION

Individual chambers for controlling crosses in wind-pollinated plants

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Summary

1. Controlled plant crosses can be an important component of studies ranging from applied artificial selection research to evolutionary investigations of heritability. Controlling pollen flow is especially challenging for wind-pollinated species.

2. We developed a system capable of housing hundreds of ragweed (*Ambrosia artemisiifolia*) plants simultaneously in individual chambers through the reproductive portion of their life cycles.

3. We confirmed that our chambers allowed us to control pollen movement and paternity of offspring using unpollinated isolated plants and microsatellite markers for parents and their putative offspring. Our system had per plant costs and efficacy superior to pollen bags used in past studies of wind-pollinated plants.

4. Our chamber system is flexible, affordable and widely applicable to other wind-pollinated plants, or applications in which the distribution of highly mobile species, individuals, spores or gametes must be controlled.

Key-words: additive genetic variance, *Ambrosia artemisiifolia*, controlled crosses, greenhouse, pollen containment, wind pollination

Introduction

Controlled matings between individuals are an important step in many basic and applied studies in ecology and evolution. For plants, controlling pollen dispersal and flow is often critical, especially when the timing of pollination and sire identity are imperative to the experimental design. Wind-pollinated plants produce prodigious amounts of pollen that can easily be dispersed even in controlled indoor settings, presenting a special challenge to researchers. Here, we describe the development and application of a system that effectively controls pollen dispersal in wind-pollinated plants, thus facilitating research and applications requiring controlled crosses.

The impacts and effectiveness of typical control crossing methods, such as pollination bags (enclosures made of various materials that cover a flower, inflorescence or entire plant to exclude unwanted pollen), are seldom tested (Cruden & Hermann 1983; Neal & Anderson 2004). Pollination bags must meet two criteria: first, they must restrict the movement of pollen, and second, not interfere with the health and development of the plant. A trade-off develops between having a material with small enough pores to prevent pollen escape, but not so small that air flow becomes too limited.

For wind-pollinated plants, pollination bags either fundamentally alter the temperature and moisture environment, are inadequate in terms of controlling pollen movement, or are very expensive on a per plant basis. Recorded temperature within bags have ranged between 3 and 20 °C above ambient depending on the bag material (Cruden & Hermann 1983; Gitz *et al.* 2015), while Wyatt, Broyles & Derda (1992) found the less porous materials were associated with higher levels of humidity within bags. These microenvironmental differences impact plant development, such as nectar concentrations and volumes (Wyatt, Broyles & Derda 1992) and seed production (Hayes & Virk 2016), and can increase the likelihood of fungal infection (Neal & Anderson 2004; Gitz *et al.* 2015). When the success of pollen bags and other methods have been tested with molecular markers (to confirm paternity), the results show high failure rates. For example, reported contamination rates range from 22% in eucalyptus (Cupertino *et al.* 2009), 30% in fir and pine trees (Adams, Neale & Loopstra 1988), 64% in olive trees (de la Rosa, James & Tobutt 2004), and between 39 and 80% in switchgrass using traditional pollination bag materials (Todd 2011). To improve these high failure rates, Vogel, Sarath & Mitchell (2014) developed micromesh bags to prevent pollen contamination in switchgrass, which are effective but cost $15USD per bag. Likewise very impermeable materials, such as 5 μm Nitex® (Shannon & Holsinger 2007) and duraweb®

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bags (Hayes & Virk 2016), have also been used successfully with wind-pollinated species, but are costly (c. \$189USD m^{-2}, c. \$20USD m^{-2}). As a consequence, with existing methods, researchers must either fundamentally alter the temperature and humidity environment of developing flowers, accept high contamination rates that would invalidate most genetic studies of heritability, paternity and targeted breeding, or incur costly per plant expenses.

As part of a larger project on the adaptive potential of native and introduced *Ambrosia artemisiifolia* (ragweed) populations, we developed an individual chamber system for use in greenhouse settings that would allow us to control pollen flow, and hence paternity of progeny. *Ambrosia artemisiifolia* is a self-incompatible annual herb (Friedman & Barrett 2008). Because our research goals required a nested paternal half-sibling design (Conner & Hartl 2003), we designed a system that allowed us to collect pollen from individual plants, and prevent uncontrolled pollination within the greenhouse.

Performing controlled crosses on ragweed plants presented several challenges. Ragweed produces around 1·2 billion grains of pollen per individual (Fumanal, Chauvel & Bretagnolle 2007). Female heads are sessile, single-flowered and distributed throughout the plant (Bassett & Crompton 1975), making bagging individual flowers or branches difficult. Almost all ragweed plants are monoecious. Consequently, dam (pollen recipient) plants produce pollen continuously while flowering, requiring that their pollen be controlled to prevent inadvertent pollination of other dams during crosses. For these reasons, we decided to construct chambers which would contain pollen for each plant individually.

As we discuss below, the system is flexible, and could be widely adapted for applications in which the distribution of highly mobile and small organisms, spores or gametes is required.

Materials and methods

We collected seeds in 2012 from three North American and three French populations, and germinated and grew individuals from these collections for crossing. For each population, we crossed 50 sires to three dams each (for a total of 200 plants). We treated all plants the same, but staggered the germination times by population for logistical reasons. Using methods adapted from Willemsen (1975), we placed seeds in clear plastic bags with damp silica. We then stored the bags in the dark at 4 °C for 3 months. After rinsing the seeds with distilled water, we placed them on a filter paper in Petri dishes, which we dampened with distilled water. We used diluted No Damp (contains oxine benzoate and isopropyl alcohol) to prevent fungal growth. No Damp is no longer commercially available but where damping off is a concern, salicylic acid or hydrogen peroxide could be used.

We placed the Petri dishes in 14-hour light in the greenhouses in the Earth Sciences Centre at the University of Toronto. As cotyledons appeared, we planted seeds into flat trays. After 3–4 weeks of growing in trays, we transplanted seedlings to 4-inch pots. After 8 weeks, we switched to short days to induce flowering. We only moved plants into individual chambers when inflorescences had begun to develop and flowering was imminent. Once the plants were on the air-flow system, they were bottom-watered every day to avoid desiccation.

INDIVIDUAL CHAMBERS

All our materials were purchased at consumer hardware stores. Each individual chamber was composed of a foam disk and an impermeable plastic bag (Fig. 1). We cut the disks into rings which fit snugly around round 4-inch pots. Each ring had two smaller holes on opposite sides, one for air inflow (1·5 cm diameter) and one for outflow (2·2 cm diameter). We glued high efficiency particle arresting (HEPA) filter media to both openings. Our filters posed minimal resistance to air flow and moisture exchange while removing 99·97% of particles greater than 0·3 μm in size. We cut the outer edge of the foam ring with a groove along its circumference, around which we secured an elastic band to hold the plastic bag.

FILTERED AIR SUPPLY

We used two different air supply methods to inflate the bags with slightly higher air pressure than ambient while avoiding pollen contamination. Each was constructed to fit on greenhouse benches.

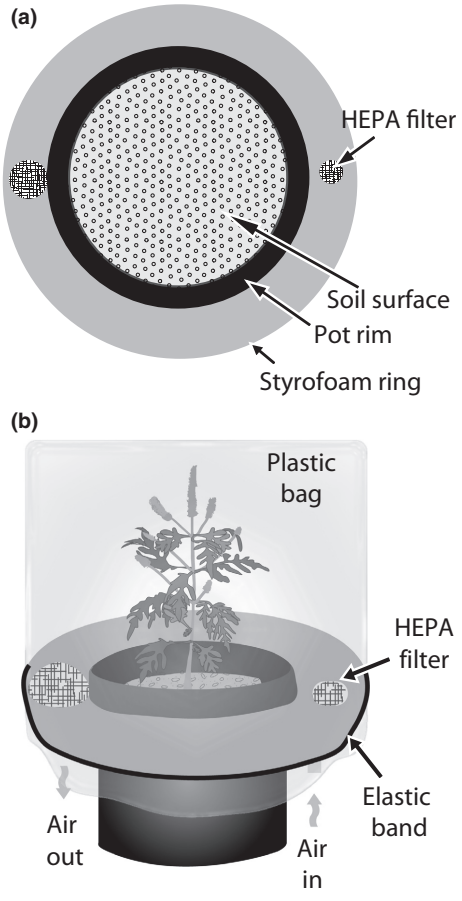

Fig. 1. Diagram of chambers used to house *Ambrosia artemisiifolia* plants. Styrofoam rings were fitted tightly around round pots with four inch diameters. Plastic bags were secured with elastic bands over the rings. Both the inflow and outflow holes were covered with high-efficiency particulate arrestance (HEPA) filter material. Air was actively pumped in, and passively flowed out, leading to a slightly higher internal air pressure which inflated the bags. (a) Overhead view of empty individual chamber. (b) Front view of individual chamber with plant inside.

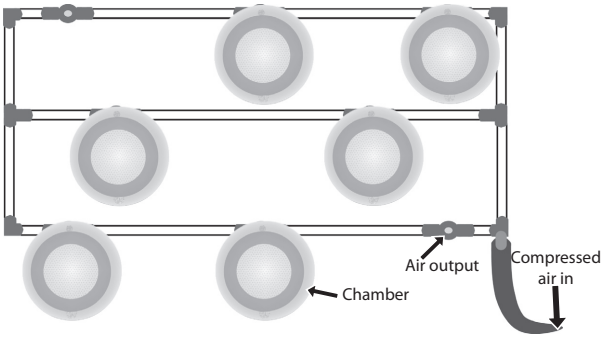

Fig. 2. Overhead view of airflow system 1 used to pump clean air to *Ambrosia artemisiifolia* chambers. The grid was made of pex tubing using brass connectors to connect each piece. The site of each t-connector was fitted with a piece of tygon tubing, which fit tightly inside the input hole of the chamber disks. The diagram shows eight sites where chambers could connect, our grid had 80 such sites.

Design 1

We took advantage of compressed airlines in our greenhouse to supply filtered air to a manifold (grid of pipes) that distributed air to each of the chambers. We made the manifold out of PEX tubing using brass t- and elbow connectors to form rows (Fig. 2). The site of each t-connector was fitted with a piece of tygon tubing, which fit tightly inside the input hole of the disks, allowing for airflow through the filter and into the bags without pressure loss. Since this air was not coming from within the greenhouse space, and the plants were grown out of season, there was no chance of it being contaminated with ambient *Ambrosia* pollen. The tubing was all airtight to prevent leaks and loss of pressure. This system was easy to implement as the greenhouse was already equipped with a high pressure air supply, but was limited in the number of plants that could be placed on the grid. In our greenhouse, a maximum of about 100 plants could be used on this set-up at once, with the optimal number being 80.

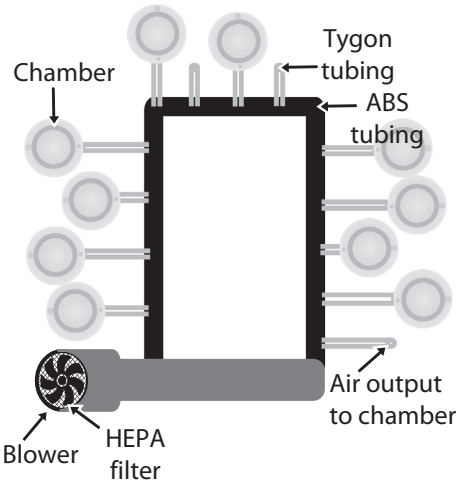

Fig. 3. Overhead partial view of airflow system 2 used to pump clean air to *Ambrosia artemisiifolia* chambers. Small holes drilled into ABS tubes allowed air to flow into tygon tubes which connected to individual chambers. Air was pulled through a HEPA filter using an industrial blower.

Design 2

To overcome the limited high pressure air supply, our second design employed a high-volume low-pressure blower attached to a grid of pipes with larger diameters. We fitted acrylonitrile butadiene styrene (ABS) pipe (3-inch diameter) to run the length of the greenhouse bench, one on each side (Fig. 3). We connected them using 90° elbow connectors and more ABS piping at the far end of the bench. To complete this rectangle, we used a flexible 4-inch heating duct tubing for the fourth side. We drilled small (3 cm) holes along the ABS tubes which were fitted with short pieces of tygon tubing in each hole. These led to the input holes of each chamber disk. All these connections were snug and required no adhesive. The advantages of this system were that it was less expensive, easily scalable, easier to construct, and enabled more plants to be accommodated on each bench.

To prevent pollen contamination with our second design, we took several steps. First, because the air was coming from within the greenhouse, we had a stand-alone air-purifier (Honeywell 17000, Honeywell Inc., Southborough, MA, USA) running constantly in the room, which would mitigate contamination from any leaks. We also added a HEPA filter to the input, so that filtered air was drawn into the pipes. Lastly, as stated above, each disk had a filter before the air passed into the bag. The heating duct tubing allowed us to connect ABS tubes on one bench to the next, allowing more plants to be grown at once. Using both systems, a total of 600 plants could be grown simultaneously.

CONTROLLED CROSSES

One advantage of individual chambers is that plants could be removed from the air grid without releasing any pollen, or exposing them to the pollen of others. We removed four plants at a time. Crosses were performed in either a fumehood or in another separated room. We cut a small opening in the plastic bag of the plant designated as a pollen donor, and another in the bag of the first pollen recipient. We used a paintbrush to transfer pollen from one to the other. After we distributed pollen on the female flowers of the dam plant, we taped over the holes in its plastic bag with electrical tape. We repeated these steps one dam at a time, to minimize potential contamination. We then returned the plants to the grid. Brushes were left in ethanol overnight to destroy any pollen that might remain viable, and then rinsed thoroughly with water before reuse.

VALIDATION AND EFFICACY TESTS

Control plants

To test that our system was effective in preventing uncontrolled pollination, we used five control plants. These plants were bagged and watered as all the experimental plants but we never pollinated them. We searched these individuals for seeds regularly.

Paternity testing

To confirm that our pollen-donor plants were indeed actual sires, we validated our crosses with microsatellite markers. During growth, we sampled leaves from sires and dams and dried them with silica; we collected leaf samples from offspring in the same manner. For 96 offspring, we extracted DNA using Qiagen DNeasy kits (Germantown, MD, USA). We used four of the microsatellite markers identified by Genton, Jonot & Thevenet (2005) (AMB12, AMB16, AMB30 and AMB82) to genotype each of these plants. PCR products were sent to

the Centre for the Analysis of Genome Evolution and Function at the University of Toronto where they were read using a ThermoFisher Scientific 3730 DNA Analyzer. We used both GeneMarker (SoftGenetics 2012) and R scripts (R Development Core Team 2004; Smith *et al.* 2015) to score chromatogram peaks.

We tested for cases where an offspring individual possessed an allele found in neither the known dam nor the presumed sire using R scripts. Our scripts assigned maternal alleles based on the known mother's genotype. The remaining alleles were compared to the putative father's genotype to ensure a match.

Results and discussion

All crossed plants produced seeds with seed production ranging from 3 seeds to 27 seeds, although we could have obtained more by performing more crosses between a given sire and dam. In subsequent uses of this system, we have been able to generate 7000 seeds of known parentage for quantitative genetic studies, indicating that our system can be scaled up to generate more biological material as needed. None of the control plants produced any seeds. We gathered microsatellite data from offspring individuals representing 36 different sire and dam combinations (4% of the total number of crosses). In all cases, the microsatellite markers were consistent with the presumed sire. The combined results of the paternity analysis and the control plants failing to set seed indicate that the system was successful at controlling pollen flow and paternity of experimental seeds. The system we have developed (Fig. 4) is easy to construct, more affordable and effective than other options, and could be easily adapted for other plant species or situations.

PRACTICAL CONSIDERATIONS

The most important part of the chambers is the delivery of air to each individual plant. The best method will depend on what is available at a given greenhouse and the size of the plants and associated plastic bags. For 100 or fewer chambers, pressurized air worked well. However, this depends on having air inputs into the greenhouse, took up more space, and was more labour intensive and expensive. The advantage of the second air supply is that it is much faster and cheaper to set-up, and more plants can be accommodated. The biggest disadvantage is the need to pre-filter the air before it went to the tubing system, in case of ambient air contamination. HEPA filters are very effective; however they create more resistance, requiring a stronger blower to force the air through the filter. Ragweed is very phenotypically plastic, and progressed to flowering and seed-set in 4-inch pots; larger plants that require larger plastic bags would entail fewer chambers in use at a time, while smaller plants (and smaller bags) would allow more chambers to be used for a given air supply. In general, the upper limit on the size of individual chambers will be the difficulty of maintaining air pressure in bags. The blower needs to be on at almost all times and have sufficient force; when our blower was off, moisture quickly built up in the bags. There is not an increased risk of contamination if a blower is shut off, but it could lead to the bags sticking to, and potentially damaging the plants.

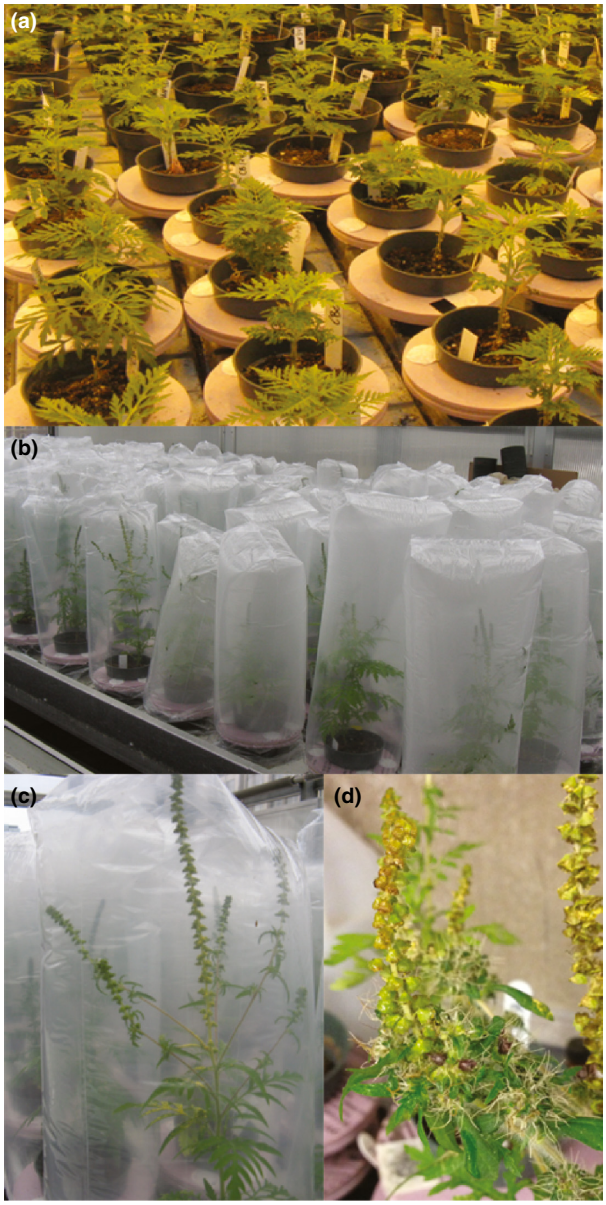

Fig. 4. *Ambrosia artemisiifolia* plants in a greenhouse. (a) *Ambrosia artemisiifolia* plants in the vegetative stage before they were covered with plastic bags and placed on airflow system. (b) Plants growing in individual chambers. (c) Reproductively mature *A. artemisiifolia* plant. (d) Seeds on *A. artemisiifolia* plant.

ADAPTING FOR OTHER EXPERIMENTS

The main advantage of our system over currently available pollination bags is that the continuous air flow allows for ambient temperature and humidity while not risking contamination. Depending on the material used for pollination bags, our system is also less expensive. We constructed our chambers and grids for <$3USD per plant, whereas very impermeable pollination bags used for wind-pollinated plants can cost c. $15–20. Our chambers and manifolds can be reused, the costs of replacing the plastic bag component is trivial. Plants of various sizes and growth habits could be accommodated using different sizes of pots, disks and plastic bags.

Some crossing designs for monoecious plants involve reciprocal crosses where both individuals act as pollen donor and recipient. Our chambers would be ideal, and would require very minimal effort: two self-incompatible plants could simply be placed in the same pot at the seedling stage, grow in the same chamber, and cross without risk of contamination once they are mature. Likewise, for dioecious plants, the pollen donor plant could be grown in the same chamber as the dam (s). Although full siblings are not the optimal crossing design for our purposes (estimating additive genetic variance), they are still commonly used in ecology and evolutionary research (e.g. Lynch & Walsh 1998) as well as for applied purposes. For example, controlled crosses have been critical for the development of hemp (Salentijn *et al*. 2015). *Cannabis sativa*, is dioecious and wind-pollinated, and the difficulty in controlling crosses may be why reported ancestry of many strains is unreliable (Sawler *et al*. 2015). Growing *Cannabis*, an increasingly legal venture in many jurisdictions, is also plagued by problems of controlling humidity, allergens and providing enough CO_2, all of which could be remedied using an air delivery system like ours.

Our system could be adapted for any plant study where controlling the movement of small individual organisms, gametes or spores is required. We took inspiration from Coyier (1973) and Züst *et al*. (2012). Coyier designed chambers to contain single isolates of a fungus growing on a plant, while Züst *et al*. constructed chambers to contain aphids. With slight adjustments, our chambers could be used for both these purposes, and have the advantage of being simpler, and much less expensive. With our cost-effective design, it would be easy to construct and use many chambers if necessitated by an experiment.

Authors' contributions

B.V.M. conceived and implemented the air delivery system and molecular work; R.J. assisted with design and construction of the isolation chambers; J.R.S. advised on the experimental set-up; B.V.M. and J.R.S. wrote the manuscript.

Acknowledgements

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Data accessibility

The microsatellite results are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.6p1j0 (McGoey, Janik & Stinchcombe 2016).

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