Int. J. Plant Sci. 170(1):12–20. 2009.

1058-5893/2009/17001-0002$15.00 DOI: 10.1086/593039

EFFECTS OF GENETIC VARIATION AND INBREEDING ON VOLATILE PRODUCTION IN A FIELD POPULATION OF HORSENETTLE

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Plant volatiles mediate numerous interactions between plants and insects, yet few studies have examined variation in volatile production within plant populations or the genetic and environmental causes of this variation. Here we document the effects of inbreeding and maternal family on volatile production by horsenettle *Solanum carolinense* L. (Solanaceae). We collected volatiles from ramets (clones) of each of 12 genets (genotypes) of horsenettle grown in four agricultural fields with natural levels of herbivory. The 12 genets included self- and cross-pollinated progeny from six maternal plants. We found that inbreeding reduced total volatile production relative to that of outcrossed plants. We also found a breeding-by-family interaction for the total amount and blend of volatiles, indicating genetic variation among families for inbreeding depression. Analysis of outcrossed plants alone (a random sample from the population) revealed a genet effect on the total amount and blend of volatiles released, indicating broad-sense heritability of volatile traits. Our findings offer insight into the consequences of inbreeding on volatile production and the variation in volatile cues available to foraging insects in a wild plant system. Moreover, we believe this to be the first study demonstrating genetic variation for plant volatiles in a noncultivated species under field conditions.

Keywords: inbreeding, plant volatiles, genetic variation, *Solanum carolinense*, horsenettle, Solanaceae.

Online enhancement: appendix table.

Introduction

Plant volatiles mediate many important ecological interactions among plants and insects, serving, for example, as foraging cues for insect pollinators, herbivores, and predators (Pichersky and Gershenzon 2002; Bruce et al. 2005; Dudareva et al. 2006). The individual compounds released by plants are numerous and chemically diverse, and the overall makeup of the volatile blend emitted exhibits significant variation in composition and amount across taxa and over time (Turlings et al. 1998; D'Alessandro and Turlings 2006; Pichersky et al. 2006). While some volatiles are released in the course of plants' normal physiological activities, herbivory and other environmental stimuli can induce the release of additional compounds and alter the blend of volatiles released (Paré and Tumlinson 1997; Dicke et al. 1998).

Numerous studies have shown that even subtle differences in plant volatile profiles can provide important information to foraging insects. For example, female *Heliothis virescens* moths searching for oviposition sites use herbivore-induced changes in the nighttime volatile profiles of their host plants (tobacco) to distinguish between infested and uninfested plants (De Moraes et al. 2001). Similar effects have been demonstrated for other herbivores (Visser and Avé 1978; Dethier 1982; Bernays and Chapman 1994; Bruce et al. 2005; Schoonhoven et al. 2005). Predatory and parasitic insects also utilize herbivore-induced volatiles to locate their hosts (Vet and Dicke 1992; Turlings et al. 1995; Du et al. 1998; Dicke 1999; Paré and Tumlinson 1999; Kessler and Baldwin 2001) and can exploit these induced volatiles in quite sophisticated ways. For example, the specialist parasitoid wasp *Cardiochiles nigriceps* distinguishes volatile profiles induced by its host *H. virescens* from those induced by the closely related nonhost *Helicoverpa zea* and preferentially responds to the former (De Moraes et al. 1998). Herbivore-induced volatiles are thought to function in nature as an indirect mode of plant defense because of their role in attracting natural enemies (Turlings et al. 1995; Paré and Tumlinson 1999; Kessler and Baldwin 2001). Several studies have shown that the release of herbivore-induced volatiles can enhance parasitism rates (Thaler 1999; Kessler and Baldwin 2001; Lou et al. 2006) and that increased parasitism enhances plant fitness (seed production) by reducing herbivory (van Loon et al. 2000; Chattopadhayay et al. 2001; Hoballah and Turlings 2001).

The hypothesis that induced volatile release is an adaptive defense response by plants rests on the assumption that there is heritable variation in volatile production within natural plant populations. Several studies of cultivated species have demonstrated varietal or genotypic differences in induced volatile production (Takabayashi et al. 1991; Loughrin et al. 1995; Lou et al. 2006). Gouinguené et al. (2001) examined 11 maize cultivars and found variation in the total amounts and composition of volatile profiles among cultivars. Similarly, Degen et al.

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Manuscript received January 2008; revised manuscript received May 2008.

(2004) assessed the genetic variability of induced volatiles among 31 lines of maize and found significant variation in the total amount of volatiles released and in the emission profiles, which were distinct for each genotype. Few studies have examined variation for volatile production in non-cultivated species (Halitschke et al. 2000; Gouinguené et al. 2001; Glawe et al. 2003; Hare 2007) and fewer still have assessed the heritability of plant volatiles (Degen et al. 2004; Hare 2007). To the best of our knowledge, no previous study has tested whether genotypic variation of plant volatiles in the field is heritable and therefore subject to natural selection.

Recently, researchers have begun to examine the effects of inbreeding on plant-insect interactions (Nunez-Farfan et al. 1996; Carr and Eubanks 2002; Hayes et al. 2004; Stephenson et al. 2004; Hull-Sanders and Eubanks 2005; Du et al. 2008). These studies have shown that the net effect of inbreeding on plant-herbivore interactions may be either positive or negative because inbreeding affects both the quality of plants as hosts and their resistance to herbivores (Carr and Eubanks 2002; Stephenson et al. 2004; Du et al. 2008). Carr and Eubanks (2002) found that inbreeding in the yellow monkeyflower *Mimulus guttatus* affected the growth rate of spittlebug nymphs *Philaenus spumarius*, although the direction of the effects varied between host plant populations. Hull-Sanders and Eubanks (2005) found that two species of tortoise beetle and beet armyworm caterpillars performed better on outcrossed plants of the entire-leaf morning glory *Ipomoea hederacea*, while cotton aphids performed better on selfed (inbred) plants. In the wild gourd *Cucurbita pepo* ssp. *texana*, selfed plants exhibited higher levels of leaf herbivory by cucumber beetles and harbored larger populations of aphids than outcrossed plants (Stephenson et al. 2004); the amount of beetle damage sustained by selfed plants increased with the levels of inbreeding (Hayes et al. 2004). Using the same plant, Ferrari et al. (2006) demonstrated that inbreeding reduced total floral volatile production and altered floral volatile profiles, with potential implications for plant-insect interactions mediated by floral volatiles (e.g., pollination). To our knowledge, this is the only previous study that examined the effects of inbreeding on volatile production.

In our study, we examined the volatile production of ramets (clones) of horsenettle *Solanum carolinense* L. exposed to natural levels of herbivory under field conditions. The ramets included selfed and outcrossed progeny of six maternal plants (i.e., 12 genotypes) and were grown in four agricultural plots that differed in their cultivation history and surrounding vegetation. We examined (1) whether there were quantitative or qualitative differences in the volatile blends of selfed and outcrossed plants and (2) whether there was variation in volatile profiles among outcrossed progeny from different maternal families.

Material and Methods

Study System

Solanum carolinense L. is an herbaceous perennial that inhabits early successional habitats, waste places, crop fields, and pastures. It is listed as a noxious weed by both the USDA (2002) and the Seeds Act and Regulations of Canada (Basset and Munro 1986) and is considered to be invasive in each of the 43 states in which it has been reported. Once established, it spreads via horizontal rhizomes that can extend >1 m from the parent stem (Ilnicki et al. 1962). The aboveground parts die with the first frost in the autumn. The belowground parts overwinter, and new shoots emerge in the spring. Both growth and reproduction are indeterminate. The white to violet flowers are similar in shape to those of tomato and potato except that the pistil is exerted.

As with other Solanaceae, the flowers of horsenettle possess a ribonuclease-mediated gametophytic self-incompatibility system (Harden et al. 1972; Richman et al. 1995). Self-incompatibility is uncommon in weedy and invasive species (Baker 1955; Byers and Meagher 1992), which often rely on populations of only one or two initial plants to colonize new habitats (Stebbins 1957; Baker 1965). Previous studies by our research group have investigated this apparent anomaly (i.e., a highly successful weed that is self-incompatible) and found that the ability of most horsenettle genotypes to set selfed seed increases with the age of unpollinated flowers (Stephenson et al. 2003) and under conditions of low fruit production (Travers et al. 2004). Poor fruit set on prior inflorescences and the presence of older unpollinated flowers are conditions expected when pollinator activity is low and/or when outcross pollen sources (mates with suitable S-alleles) are scarce. Breakdown of self-incompatibility with floral age is even more pronounced in plants possessing the common S_9 allele (Mena-Alí and Stephenson 2007).

Plants

Rhizome cuttings of *S. carolinense* were collected in the field from 16 plants from a large population located near State College, Pennsylvania. Cuttings were taken from plants located at least 5 m apart to decrease the possibility of taking rhizomes from the same genet. These cuttings were brought to the greenhouse, planted in 4-L pots, and allowed to resprout, grow, and flower. After flowering, the stems were cut off, and the pots were moved to a cold room at 4°C to vernalize for 6–8 wk. After the cold treatment, the plants were returned to the greenhouse and allowed to acclimate for 1 wk. Ramets (clones) were then created from each of the 16 plants (genets) by dividing the rhizome into five or six pieces of similar size. Each rhizome cutting was replanted in a 1-gal pot and allowed to resprout and grow. Flowers produced on one ramet from each of the original 16 field-collected plants were outcrossed until a total of 40 flowers per ramet were pollinated (for details, see Mena-Alí 2006; Mena-Alí and Stephenson 2007). Flowers from a second ramet from each of the 16 original genets were self-pollinated until a total of 40 flowers per ramet were pollinated. Outcross pollinations were performed by collecting pollen in a microcentrifuge tube from at least five different genets (of the 16 growing in the greenhouse) using a buzz-pollination device (an electric toothbrush modified with a wire loop on the end), vibrating the tube to thoroughly mix the pollen, and then touching the mixture of pollen to a stigma by guiding the stigma into the tube. Self-pollinations were performed similarly. A sample of the resulting seeds from self- and cross-pollinations were germinated and grown in the greenhouse. The purpose of this rather complicated design was to minimize the impact of cross-

generational environmental maternal effects on our experimental genets and the ramets produced from them.

Because of logistical constraints associated with collecting volatiles in the field, progeny from a subset of six of the original 16 genets were used for this study (a relatively small sample size compared to most studies of heritability and inbreeding depression). We randomly selected one self-pollinated and one cross-pollinated progeny from each of the six maternal families for a total of 12 genets (i.e., genotypes). Four ramets were created from each of the 12 genets by dividing the rhizomes into four similar-sized pieces (12 genets $\times$ 4 replicates = 48 plants total). Rhizomes were grown in 4-L pots in a peat-based, general-purpose potting soil (Pro-Mix, Premier Horticulture, Quakertown, PA) in a growth chamber (16L : 8D; day/night temperatures 25°/22°C; 65% relative humidity) and watered daily. At ~4 wk of age, plants received an application of fertilizer (50 ppm 8-45-14 N-P-K, plus micronutrients; Scotts, Marysville, OH) and iron chelate (Sprint 138 at 6%; Becker Underwood, Ames, IA). At ~5 wk of age, two complete sets of ramets that included each of the 12 genets were transplanted into two separate field plots at the Russell E. Larson Agricultural Research Farm at Rock Springs, Pennsylvania. The following week, the remaining two sets of ramets were planted into two additional field plots at Rock Springs. All plants received one additional application of fertilizer (200 ppm) 2 wk after transplanting. Each 8 $\times$ 8-m plot contained one ramet from each of the 12 genets randomly assigned to one of 12 evenly spaced (1.5 m apart) planting positions. Two of the field plots were separated from each another by 10 m, the third plot was located ~133 m from the first two plots, and the fourth plot was located ~117 m from the third plot. The fields differed in their recent cultivation history (either alfalfa/grass or soybean) and in the crop types in adjacent fields (peppers, sweet corn, field corn, or soybeans). Plants were subject to natural levels of herbivory.

Volatile Collection and Analysis

Volatile collections began 1 wk after plants were transplanted to the field and continued every other week until all genets in each of the four plots had been sampled three times. Because only one plot could be sampled per day and we could only sample twice a week, volatile collections took place over a 2-d period each week.

Volatiles were collected from plants using portable volatile collecting systems comprising two automated vacuum pumps enclosed in a waterproof case (Bryan Banks, Lemont, PA). A 30-cm^2 Teflon film (gauge 200A) with a hole for the plant stem rested on the soil and acted as a base. A wire cage (15 cm tall $\times$ 16 cm diameter) enclosed the plant and rested on this base. A Teflon bag (50 cm wide $\times$ 60 cm tall) was placed over the cage (American Durafilm, Holliston, MA). Two lay-flat tubes at either end of the top of the bag allowed filters to be inserted. A filter was placed in one lay-flat tube and attached to the vacuum via Teflon tubing. Air was then pulled by the vacuum at 0.8–0.9 L/min through the filter and across beds of adsorbent Super-Q (25 mg; Alltech, Deerfield, IL). A charcoal filter, placed in the second lay-flat tube, allowed clean air to pass into the bag during the collection. Because of the limited number of volatile collection systems available, plant volatiles were collected for 45 min/plant, either from 1100 to 1145 hours or from 1215 to 1300 hours, allowing each pump to be used twice. This collection period was chosen to minimize potential stress on the plants while obtaining adequate recovery of volatiles. As a control, volatiles were collected from cages and bags that were left empty. The Super-Q traps were rinsed with 150 μL of dichloromethane, and 200 ng of *n*-octane and 400 ng of *n*-nonyl acetate (80 ng/μL) were added as internal standards. Samples were immediately analyzed by gas chromatography and then mass spectrometry as described by Delphia et al. (2007), except that the column was maintained at 35°C for 0.5 min and then increased by 5°C/min to 180°C. After analysis, samples were stored at −80°C.

Before each volatile collection, the number of open flowers, fruits, adults and larvae of the false Colorado potato beetle (*Leptinotarsa juncta*), and other herbivores present on each plant (primarily flea beetles, *Epitrix* spp.) were recorded, and herbivore-damage levels were assessed. Because all plants appeared equally damaged by flea beetle herbivory, herbivore damage was assessed based on the approximate leaf area removed by all other herbivores, which caused feeding damage that was distinctly different from that caused by flea beetles. The majority of this damage likely reflects that caused by adults and larvae of the false Colorado potato beetle, which, apart from flea beetles, was the predominant herbivore found on plants. After final volatile collections were completed, all aboveground plant material was brought back to the laboratory. Fruits were removed from plants, weighed, and counted, and the remaining plant material was dried in a large crop dryer at 150°F for 1 wk and then weighed.

Statistical Analyses

Volatile production may be influenced by a variety of factors. For instance, there may be trade-offs between volatile production and plant growth, reproduction, or other defenses against herbivory. Consequently, it is critical to account for these factors when examining the effects of inbreeding on volatile production and the heritability of volatiles. While a single measure of plant growth (plant dry weight) was obtained, three correlated measures of plant reproductive effort (number of flowers, number of fruits, and fruit weight) and two correlated measures of plant herbivory (number of flea beetles and amount of leaf damage) were assessed. A factor analysis was conducted on each of the two sets of correlated variables, and factor loadings on the first axis were used to generate a single estimate for the hypothetical variables "reproductive effort" and "herbivory" (an approach recommended by McCune and Grace [2002]). These analyses and all subsequent analyses were conducted using the mean of the three collecting periods from each plot as the replicate. Spatial blocks (plots), plant size, and factor scores for reproduction and herbivory were used as covariates for all analyses.

Total Volatiles

To determine whether inbreeding and maternal family influence volatile production, we tested for the effects of family and

breeding and their interaction on total volatile production (square-root transformed) for the 12 genets (six selfed and six outcrossed), using the general linear model. Comparisons between all pairs of families were conducted using Tukey's honestly significant difference (HSD) test, and comparisons of selfed versus outcrossed plants within each family were conducted using a Bonferroni correction ($\alpha = 0.0083$).

To assess whether volatile production was heritable, we tested for the effects of genet (a random factor) on total volatile production (square-root transformed) for only the outcrossed plants from the six maternal families, using the general linear model. Only the outcrossed plants were used for this analysis because they represented a random sample from the population, whereas selfed plants do not (because they were chosen to examine the effects of inbreeding on each of the six maternal families). Comparisons between all pairs of genets were conducted using Tukey's HSD test. Broad-sense heritability was calculated as $H^2 = V_g/V_p$ (genetic variance over phenotypic variance), where $V_p = V_g + V_e$ (V_g is the among-genet variance component, V_e is the within-genet variance component, and V_p is the phenotypic variance). All statistical analyses for total volatiles were conducted using Statistica, version 6 (StatSoft, Tulsa, OK).

Volatile Blend

Because the composition of volatile blends has been shown to be important for insect attraction (De Moraes et al. 1998), the effects of inbreeding and genetic variation on volatile composition was also examined. The relative concentration of each volatile compound was calculated by dividing its amount by the total amount of volatiles produced by each plant. Two principal coordinate analyses (PCoAs) were then conducted on the square-root, Bray-Curtis-transformed volatile concentrations, with a correction for negative eigenvalues and inter-sample scaling—one included all 12 genets (six selfed and six outcrossed), and one included only the outcrossed genets (treated as a random factor; Canoco 4.5, Microcomputer Power, Ithaca, NY). To determine whether family, breeding, and their interaction could account for significant portions of the dispersion of samples in the first PCoA (all 12 genets) and whether genet could account for significant portions of the dispersion of samples in the second PCoA (six outcrossed genets), the program PERMANOVA (Anderson 2005) was used to conduct a permutation-based, multivariate ANOVA (999 iterations) on the sample scores from the first two axes of each of the PCoAs. Comparisons between all pairs of families, all pairs of outcrossed genets, and selfed and outcrossed plants within each family were conducted post hoc using PERMANOVA. A Bonferroni adjustment was used to ensure an overall α of 0.05. Heritability estimates for volatile blend were calculated in the same manner as for total volatiles.

Results

Herbivores

The dominant herbivores observed on *Solanum carolinense* were flea beetles (*Epitrix* spp.), and adults and larvae of the false Colorado potato beetle (*Leptinotarsa juncta*). Other herbivores included Colorado potato beetles (*Leptinotarsa decemlineata*), the tobacco hornworm (*Manduca sexta*), a flower weevil (*Anthonomus* sp.), and larvae of the fruit-infesting moth *Frumenta nundinella*.

Factor Analysis

The factor analysis confirmed that fruit weight, number of fruits, and number of flowers all loaded heavily on the first axis (all 12 genets: 0.934, 0.953, 0.843 for the three factors, respectively; outcrossed genets only: 0.913, 0.968, 0.850), justifying the use of the factor scores on the first axis in subsequent analyses. Likewise, number of flea beetles and leaf damage had similar covariance structures, with both loading heavily on the first axis of the herbivory factor analysis (all 12 genets: 0.768 for both factors; outcrossed genets only: 0.766 for both factors).

Total Volatiles

A total of 28 detectable volatile compounds were released from the six maternal families (12 genets) that we examined (table A1 in the online edition of the *International Journal of Plant Sciences*). Outcrossed plants released significantly more volatiles (1853 ± 286 ng/45 min) than selfed plants (1258 ± 168 ng/45 min; $F_{1,30} = 8.82$, $P < 0.01$; fig. 1). We also found a significant effect of family on total volatile production ($F_{5,30} = 18.19$, $P < 0.001$), with families 1–3 releasing significantly more volatiles than families 4–6 (fig. 1). There was

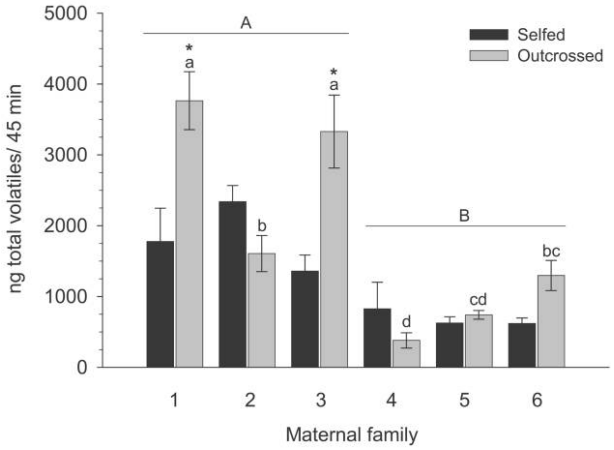

Fig. 1 Total volatiles (ng/45 min; mean ± SE) released by selfed and outcrossed horsenettle plants from six maternal families ($n = 4$). Asterisks indicate significant differences between selfed and outcrossed plants within families (ANOVA corrected for multiple comparisons, $\alpha < 0.0083$). Capital letters indicate significant differences between families; families under the same line are not significantly different from one another. Lowercase letters indicate significant differences between outcrossed genets only (Tukey's HSD test on square-root transformed data, $\alpha = 0.05$). Data are presented untransformed.

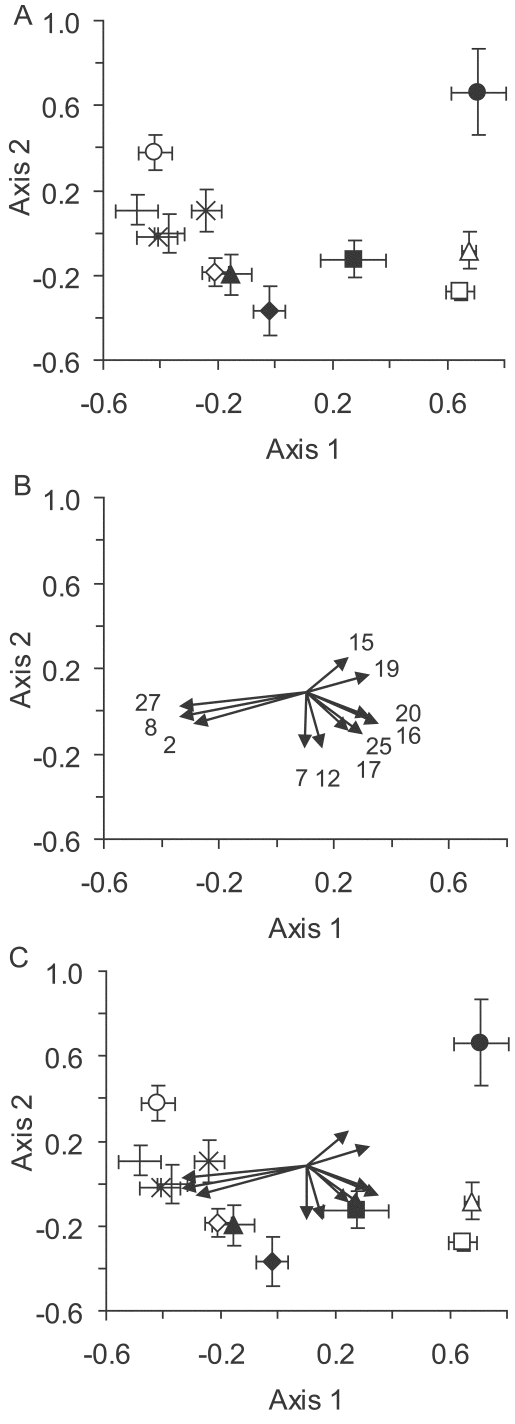

Fig. 2 Results of a principal coordinate analysis displaying (*A*) the differences in volatile blend among selfed and outcrossed plants from six maternal families of horsenettle, (*B*) the volatiles with significant relationships ($\alpha < 0.001786$) with at least one of the two axes, and (*C*) the results in *A* and *B* overlaid. Means and standard errors ($n = 4$ plants) are presented in *A*. Axes 1 and 2, which seem to account for differences in volatile composition at the family and breeding-by-family levels, accounted for 20.5% and 10.5% of the variation, respectively. Open symbols = outcrossed, filled symbols = inbred; squares = family 1, diamonds = family 2, triangles = family 3, circles = family 4, crosses = family 5, asterisks = family 6. Volatiles are labeled as follows: *2* = (*Z*)-3-hexen-1-ol, *7* = (*E*)-β-ocimene, *8* = (3*E*)-4,8-dimethyl-1,3,7-nonatriene, *12* = α-copaene, *15* = β-caryophyllene, *16* = α-caryophyllene, *17* = β-farnescene, *19* = unknown sesquiterpene, *20* = unknown sesquiterpene, *25* = caryophyllene oxide, *27* = unknown.

a significant breeding-by-family effect on volatile production ($F_{5,30} = 5.36$, $P = 0.001$), with outcrossed plants from families 1 and 3 releasing significantly more volatiles than selfed plants from these same families (fig. 1). This trend—outcrossed plants releasing more total volatiles—was reversed for two families, in which selfed plants produced more volatiles, although not significantly so (fig. 1).

Analysis of only the outcrossed genets revealed a significant main effect of genet on total volatile production ($F_{5,12} = 67.93$, $P < 0.001$) and represented a large portion of the total variation among ramets ($H^2 = 0.86$). Outcrossed genets 1 and 3 released more volatiles than outcrossed genets 2, 4, 5, and 6 ($P < 0.01$; fig. 1), outcrossed genet 2 released more volatiles than outcrossed genets 4 and 5 ($P < 0.01$; fig. 1), and outcrossed genet 6 released more volatiles than genet 4 ($P < 0.001$; fig. 1). Field plot was the only significant covariable ($F_{3,12} = 6.61$, $P < 0.01$), indicating that volatile production was also influenced by environmental differences.

Volatile Blend

In the first PCoA, which included all 12 genets, the covariables accounted for 14% of the variance in volatile composition (blend), whereas axes 1 and 2, which seem to account for family and breeding-by-family-level differences in volatile blend, accounted for 20.5% and 10.5% of the variation, respectively (fig. 2*a*).

In the biplot (fig. 3*a*), the distance between the 12 genets indicates the degree of dissimilarity in volatile composition. Volatiles with significant relationships to at least one of the two displayed axes ($\alpha < 0.001786$ because of multiple pairwise comparisons) are shown (fig. 2*b*). The origin from which the arrows extend indicates the grand mean (fig. 2*b*), and the length of each arrow shows how strongly that volatile is related to the displayed ordination (fig. 2*a*). When overlaid (fig. 2*c*), the position of the 12 genets relative to the arrows provides an estimate of the proportion of a given volatile for a given treatment (i.e., genet). For example, extending the arrow for β-caryophyllene in both directions through the origin and drawing perpendicular lines from each of the 12 genets to that arrow reveals that selfed family 4 (4s) has a greater than average proportion of β-caryophyllene and selfed family 2 (2s) has a lower than average proportion of β-caryophyllene in the volatile blend (fig. 2*c*). This is because 4s projects onto the positive side of the β-caryophyllene arrow/axis (greater than the grand mean) and 2s projects on the negative side of the β-caryophyllene arrow/axis (less than the grand mean). Additionally, looking at the mean production of β-caryophyllene for 4s and 2s (table A1) and determining its relative concentration in the blends shows that β-caryophyllene constitutes a greater proportion of the blend in 4s than it does in 2s (4% vs. 0.38%, respectively). This approach can be used to estimate the relative abundance

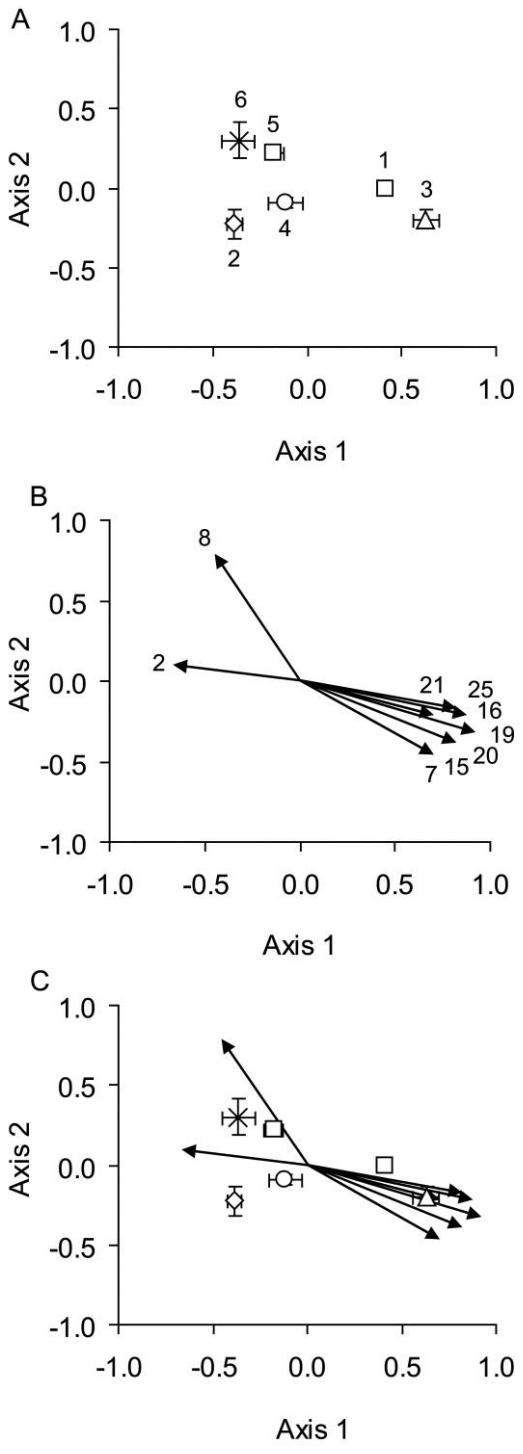

Fig. 3 Results of a principal coordinate analysis (PCoA) displaying (*A*) the differences in volatile blend among six outcrossed genets of horsenettle, (*B*) the interset correlations for volatiles with significant relationships ($\alpha < 0.001786$) with at least one of the two axes, and (*C*) *A* and *B* overlaid. Means and standard errors ($n = 4$ plants) are presented in *A*. Axes 1 and 2 accounted for 27% and 10% of the variation, respectively, and appear to represent genet-level differences in volatile blends. Squares = family 1, diamonds = family 2, triangles = family 3, circles = family 4, crosses = family 5, asterisks = family 6. Volatiles are labeled as follows: *2* = (*Z*)-3-hexen-1-ol, *7* = (*E*)-β-ocimene, *8* = (3*E*)-4,8-dimethyl-1,3,7-nonatriene, *15* = β-caryophyllene, *16* = α-caryophyllene, *19* = unknown sesquiterpene, *20* = unknown sesquiterpene, *21* = unknown sesquiterpene, *25* = caryophyllene oxide.

of other volatiles for each treatment. The biplot (fig. 2*c*) provides a visual display of the data that is easier to interpret than a table. It shows that the volatile blends of some treatments (e.g., 1s, 1x, 3x) contain larger proportions of certain volatiles (caryophyllene oxide, α-caryophyllene, β-farnescene, etc.) than the grand mean and are more similar to one another than to other treatments (e.g., 4x, 5s, 5x, 6s, 6x) whose blends contain larger proportions of other volatiles ([*Z*]-3-hexen-1-ol and [3*E*]-4,8-dimethyl-1,3,7-nonatriene).

The PERMANOVA analysis verified the pattern displayed in the PCoA biplot (fig. 2). There was a significant main effect of family on the volatile blend released ($F_{5,47} = 28.68$, $P = 0.001$). The volatile blend released by family 1 was significantly different from those released by families 2, 5, and 6; family 2 released a volatile blend significantly different than those of families 4–6; and the volatile blend released by family 3 was different from that released by family 5. While we did not find a significant breeding effect, this cannot be generalized to all families because we did find a breeding-by-family effect on the volatile blend released by selfed versus outcrossed plants within families 3 and 4 ($F_{5,47} = 16.47$, $P = 0.001$; fig. 2), indicating that for certain families there is a significant difference between the blends of selfed and outcrossed plants.

In the second PCoA, which included the six outcrossed genets only, axes 1 and 2 accounted for 27% and 10% of the variation in volatile composition, respectively, and appear to represent genet-level differences in volatile blends because the genets strongly separate on both axes (fig. 3*a*; distance indicates the degree of dissimilarity in volatile composition). The volatiles with significant relationships to at least one of the two displayed axes ($\alpha < 0.001786$ because of multiple pairwise comparisons) are shown (fig. 3*b*). The PCoA (fig. 3*c*) is interpreted as above and demonstrates that the volatile blends of some genets (e.g., 1x and 3x) contain larger proportions of certain volatiles ([*E*]-β-ocimene, β-caryophyllene, caryophyllene oxide, etc.) than the grand mean and are more similar to one another than to other genets (e.g., 5x and 6x) whose blends contain larger proportions of other volatiles ([*Z*]-3-hexen-1-ol and [3*E*]-4,8-dimethyl-1,3,7-nonatriene).

The PERMANOVA analysis verified the pattern displayed in the PCoA biplot (fig. 3). There was a significant main effect of genet on the volatile blend released ($F_{5,18} = 25.30$, $P = 0.001$). Similar to total volatile production, most of the variation in the volatile blends among genets was due to genetic differences with a broad-sense heritability of $H^2 = 0.96$. However, it should be noted that this measure is likely to be an overestimation. While the PCoA did control for all of the covariates in the model, the PERMANOVA analysis was conducted using the sample scores from the PCoA and did not separate the variance among all of the factors. The multiple comparisons test revealed that the volatile blend released by outcrossed genet 1 was different from that released by outcrossed genets 2, 4, 5, and 6, that genet 2 released a different volatile blend than outcrossed genet 3, and that the blend released

by outcrossed genet 3 was different from those released by outcrossed genets 4–6, ($P < 0.002$ for all comparisons; fig. 3).

Discussion

The organic volatile compounds released by plants have been shown in a variety of systems to serve as important foraging cues for insect pollinators, herbivores, and the natural enemies of herbivores (Pichersky and Gershenzon 2002; Bruce et al. 2005; Dudareva et al. 2006). It is therefore reasonable to speculate that the amount and composition of these volatiles is subject to natural selection, but this possibility rests on the availability of genetic variation for volatile traits. Several greenhouse and growth chamber studies have demonstrated differences in volatile production among inbred lines and varieties of cultivated species (Takabayashi et al. 1991; Loughrin et al. 1995; Gouinguené et al. 2001; Degen et al. 2004; Lou et al. 2006), and a few studies have found variation for volatile production in noncultivated species under greenhouse conditions (Halitschke et al. 2000; Gouinguené et al. 2001; Glawe et al. 2003; Hare 2007). Although our study is relatively small because of logistical constraints involved in collecting volatiles in the field, it is one of the few studies to examine variation in volatile production by a noncultivated species and, so far as we know, the first study to investigate genetic variation for volatile production under field conditions. We found significant differences in both the total amount and composition of volatiles released by the ramets of six outcrossed genets. Moreover, these differences among genets are consistent across plots differing in cultivation history and neighboring vegetation. Furthermore, our estimates of heritability in the broad sense are large ($H^2 = 0.86$ and $H^2 = 0.96$, respectively) and suggest that both the amount and the composition of the volatile blend could respond to selection. Interestingly, our estimate is similar to the heritability estimate reported for total volatile production among inbred maize lines (Degen et al. 2004). Because the parents of the plants used in this study were collected from a single population, the genets used represent a portion of the genetic variation for volatile production present in a single population. Because the ramets (rhizome cuttings) used in this field study were produced from plants that were germinated and grown under relatively uniform greenhouse conditions, we were able to minimize the environmental (nongenetic) maternal effects that plague many studies using clonal replicates.

Our results further found that inbreeding significantly reduced total volatile production among the six families of horsenettle examined. Moreover, there was a significant breeding-by-family interaction for total volatile production and the blend of volatiles released, suggesting that there is genetic variation among maternal families for inbreeding depression affecting volatile traits. A previous study using six selfed and six outcrossed progeny from each of 16 maternal families of horsenettle from the same population as the plants in our study found that selfed progeny produced significantly fewer flowers, set fewer fruits, and experienced reduced rhizome vigor relative to outcrossed plants (Mena-Alí et al. 2008). Consequently, our smaller study extends these previous findings to include inbreeding depression for volatile production. Because inbreeding increases homozygosity, thereby simultaneously exposing deleterious recessives to selection and reducing the contribution of overdominance to fitness, inbreeding may have both indirect and direct effects on volatile production. Because inbreeding typically results in general decreases in plant vigor (Charlesworth and Charlesworth 1987; Husband and Schemske 1996), it may indirectly decrease the resources available for the synthesis of volatiles. Inbreeding may also directly affect any number of the many genes in the pathways leading to volatile synthesis and emission; several mutations are known that alter the quantity and composition of the volatiles released by plants (Arimura et al. 2000; Mercke et al. 2004; Schnee et al. 2006).

Because volatiles are known in many species, including several in the Solanaceae, to serve as foraging cues for both herbivores and their natural enemies, it is reasonable to predict that the considerable variation that we observed among the 12 horsenettle genets (six selfed and six outcrossed) in both total volatile production and the composition of volatiles released will have important ecological consequences for tritrophic interactions (Hoballah et al. 2002; Bruce et al. 2005; Lou et al. 2006). Several of the compounds released by horsenettle, including (*Z*)-3-hexen-1-ol, (*E*)-β-ocimene, and β-caryophyllene, have previously been identified as important cues for natural enemies in other plant species (Flint et al. 1979; De Moraes et al. 1998; Weissbecker et al. 1999; Rasmann et al. 2005; D'Alessandro and Turlings 2006). Some genets in our study released larger proportions of (*Z*)-3-hexen-1-ol, while others released greater proportions of (*E*)-β-ocimene or β-caryophyllene. If certain compounds or blends are more attractive to particular natural enemies than others, it is likely that plants within this population will vary in their attractiveness. Additionally, if the total amount of volatiles released plays a role in the attraction of specialist herbivores, then the reduction in total volatiles that we observed for selfed plants may lead to less herbivore damage and thereby mitigate the effects of inbreeding on growth and reproduction. Alternatively, if the total amount of volatiles released is important for natural-enemy attraction, the reduction in total volatiles that we observed for selfed plants may render them less attractive to foraging predators and parasitoids, and potentially more attractive to foraging herbivores by providing enemy-free space, resulting in increased herbivory and greater inbreeding depression relative to outcrossed plants. However, at this time it is difficult to determine how changes in volatile production due to inbreeding might impact interactions with other organisms or how these interactions could further affect plant fitness. Future studies will be needed to determine the response of generalist and specialist herbivores and their natural enemies to the variation in volatile production that we observed and to determine the impact of these differences on the relative fitness of selfed and outcrossed plants in natural populations.

Documentation of heritable variation in the volatile profiles from a rather small, wild population of horsenettle highlights the need for further investigations of genetic variation for volatile production in plant populations as well as for a better understanding of the factors that influence this variation (e.g., inbreeding). Several studies have stressed the importance of incorporating genotypic diversity in conservation efforts (Ellstrand and Elam 1993; Crutsinger et al. 2006). Variation in plant volatile profiles seems particularly likely to have ecological implications because of the key role of volatiles in mediating interactions between plants and insects, including tritrophic interactions. The measurement of volatile production under

field conditions and the responses of insects to these volatiles are also important for crop plants, which often exhibit greatly reduced genetic variability compared to their wild progenitors. For example, Rasmann et al. (2005) found that North American maize lines lack an insect-induced volatile response—involving the belowground release of (*E*)-β-caryophyllene—that is present in European lines and in the wild ancestor, teosinte, which strongly attracts an entomopathogenic nematode. Further documentation of genetic variation of plant volatiles under field conditions may facilitate efforts to conserve or enhance plant defenses in agricultural crops, as well as wild species of conservation concern, and thereby reduce the use of pesticides and facilitate organic cultivation.

Acknowledgments

We thank J. Saunders for logistical support, E. Bogus for technical assistance, J. Mena-Alí for preparation of plants, A. Conrad for field and laboratory assistance, and S. Smiles and V. Houck for farm assistance. The project was supported by USDA National Research Initiative grants 2005-35320-15351 and 2006-01370 to A. G. Stephenson and J. R. Rohr, respectively; an NSF grant (DEB-0516227) to J. R. Rohr; an NSF Doctoral Dissertation Improvement grant (0608347) to C. M. Delphia; a DuPont Young Investigator grant to C. M. De Moraes; and the David and Lucile Packard Foundation and the Arnold and Mabel Beckman Foundation.

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