Repellency to the Potato Leafhopper (Homoptera: Cicadellidae) by Erect Glandular Trichomes on Alfalfa

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ABSTRACT We sought to gain insights into the mechanism of resistance of glandular-haired alfalfa to the potato leafhopper, by testing the trichomes of proprietary genotypes for chemical versus morphological differences. Two-choice, noncontact tests were conducted using four glandular-haired and one glabrous genotype of alfalfa, representing a spectrum of resistance, to determine whether adult female leafhoppers could detect and be repelled by putative volatiles released from the trichomes. Settling patterns roughly agreed with the degree of resistance reported by industry scientists who provided the plants. Repellency was strong for the most resistant genotypes, and absent with the most susceptible genotype. The remaining, intermediate-to-low-resistance genotypes exhibited variable repellency. A no-choice, plant-contact preference test showed no significant differences in settling among stems left intact or denuded, neither within nor among all genotypes. In all cases, adult leafhoppers settled on glandular-haired alfalfa if given no alternative. Environmental scanning electron microscopy of erect, glandular trichomes of eight proprietary genotypes (including the four used above) showed no obvious morphological differences among genotypes, or between stems and leaves within each genotype, although morphological comparisons were not quantified due to the fragility of the specimens. Taken together, our results suggest that volatile compounds contribute to variable levels of repellency to potato leafhoppers by glandular-haired alfalfa, but that such compounds cannot strongly prevent feeding at all costs.

KEY WORDS *Empoasca fabae*, *Medicago sativa*, plant volatiles, exudates, host plant resistance

THROUGHOUT THE EASTERN and central United States, the potato leafhopper, *Empoasca fabae* (Harris), is one of the most economically important pests on alfalfa, *Medicago sativa* L. (Lamp et al. 1991, Elden and Elgin 1992). The primary damage caused by the leafhopper is from hopperburn, a severe yellowing and stunting condition that results from a plant response cascade triggered by the feeding of the leafhopper (Ecale and Backus 1995a, 1995b; Ecale Zhou and Backus 1999). In years of high infestation, hopperburn can be so severe that it can reduce yield and even destroy entire stands (Hower and Flinn 1986, Hutchins and Pedigo 1989). Thus far, early cuttings and insecticides have been the only two effective tactics for potato leafhopper control. However, overuse of pesticides and development of insecticide resistance have stimulated a search for better host plant resistance (Schalk and Ratcliffe 1976).

Alfalfa breeding companies have used traditional, field-based screening to select genotypes that appear to be resistant to hopperburn. These companies are limited in their use of genetic engineering technology because of alfalfa's complex tetraploid genome, although efforts to adapt this technology are progressing. Still, traditional breeding programs have met with some success over the years, the most important being the registration in 1985 of the first line of glandular-haired alfalfa (Sorensen et al. 1985). For the last 16 yr, industry and academia alike have worked to breed this trait into modern agronomically elite lines. However, the trait is difficult to maintain through multiple generations. Also, many offspring of crosses have reduced numbers of trichomes, while some lack trichomes entirely (Lenssen et al. 1999).

Recent work has demonstrated that variation in levels of resistance of some alfalfa genotypes cannot be explained by trichome density alone (Hogg and McCaslin 1994, Elden and McCaslin 1997, Lenssen et al. 1999, Shockley et al. 2002). Glandular trichomes in alfalfa consist of two basic types: erect and procumbent (Ranger and Hower 2000a). The erect trichomes stand perpendicular to the plant surface and consist of a ball-like gland atop a multicellular, tiered stalk (Ranger and Hower 2000a). In contrast, the procumbent trichomes have glands atop a one- or two-cell stalk that lies parallel to the plant surface (Ranger and

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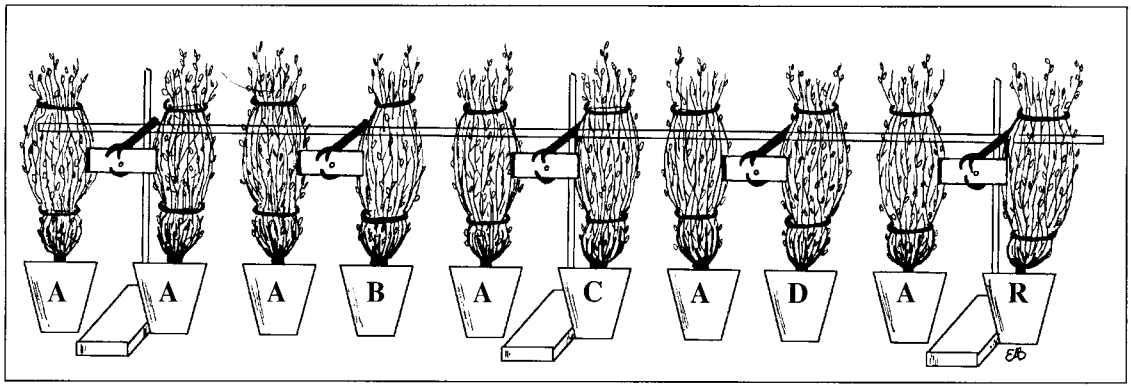

Fig. 1. Testing apparatus for two-choice repellency tests, using G98A as the example base genotype. Letter on plant pots correspond to genotype designation (A, G98A; B, G98B; C, G98C; D, G98D; and R, Ranger).

Hower 2000a). Both types of trichomes have been shown to produce a sticky exudate that can be an effective mechanical and chemical impediment to nymphs. However, against adults the trichome chemistry probably acts as a nonmechanical resistance mechanism (Ranger and Hower 2000b). Thus, hypothesized mechanisms have been both physical (entrapment of nymphs) and chemical (antibiosis or antixenosis of adults) (Hogg and McCaslin 1994, Elden and McCaslin 1997, Ranger and Hower 2000b). However, virtually no studies comparing among several genotypes for resistance mechanisms had been done before our work.

In this study, we continued the work described in Shockley et al. (2002) by comparing among proprietary, variably resistant genotypes of glandular-haired alfalfa for leafhopper settling preferences (five genotypes) and trichome morphology (eight genotypes). Our objectives were to gain insights into the mechanisms of resistance by testing the roles of putative repellency via volatile chemicals, and chemistry versus morphology of the hairs.

Materials and Methods

Insect and Plant Rearing. Insects were reared on greenhouse-grown broad beans, *Vicia faba* L. 'Windsor', in a growth chamber (25 ± 2°C, 16:8 [L:D] h). Ten-day old, conditioned, adult females, treated as in Shockley et al. (2002), were used. All leafhoppers were starved for 1 h before use. The same eight genotypes of glandular-haired alfalfa in Shockley et al. (2002) were used (Cal/West (Woodland, CA): G98A, G98B, G98C, G98D; Forage Genetics (W. Salem, WI): 1–27–1, 1–27–4, 1–27–7, and 3–73–1). The genotypes were selected as representing two spectra of resistance to hopperburn, one from each company. Due to a shortage of plants from Forage Genetics, genotypes from that company were only used in the environmental scanning electron microscope (ESEM) study. All other studies were limited to the genotypes from Cal/West. Alfalfa plants were vegetatively propagated and grown in growth chambers, according to the methods of Shockley et al. (2002) and Kabrick and Backus (1990). Plants were cut once and allowed to regrow until they reached 10–12 internodes in height.

Two-Choice Repellency Test. To determine whether the leafhoppers could detect volatile chemicals without apparent visual cues or making plant surface contact, a two-choice repellency test was performed using the four glandular Cal/West genotypes plus the highly susceptible variety Ranger. All possible pairings (a total of 15 combinations) were performed during each replicate, with 10 total replicates performed. All replicates were performed under laboratory conditions approximating the plant growing conditions (i.e., 25 ± 2°C, 16:8 [L:D] h).

Three testing apparatuses were constructed (Fig. 1), consisting of three Fisher chemistry ring stands, three hook connectors, three long aluminum rods (length = 365.76 cm, diameter = 1.27 cm), and five 3-prong adjustable angle clamps. Ring stands were placed along the length of each aluminum rod at 30.48, 182.88, and 335.28 cm and fixed at those points 30.48 cm perpendicular to the table surface using the hook connectors. The five 3-prong angle clamps were placed equidistant along the rod. Horizontal, small tube cages were constructed from 50-ml Fisher centrifuge tubes cut to ≈9 cm in length. A small hole (0.5 cm) was drilled directly in the center of the tube to allow insects to be dropped equidistant from both ends of the tube. The ends were covered with fine mesh organza cloth double-sided-taped to the tube. Each tube was divided into two sectors, labeled with replicate and pairing information, and then randomly clamped into position in the apparatus. Plants were placed at either end of the tube, with foliage nested close to but not touching the organza cloth. Each plant's stems were loosely tied into a bundle 2.54 cm in diameter, to concentrate the amount of stem and leaf surface at the end of the tube and maximize the surface area from which the volatiles were being released. Once plants were placed, 10 adult females were introduced into the center of each tube. After allowing 1 h for insect orientation, each cage was examined and

numbers of insects located in each sector were documented.

Intact Versus Denuded Stem Settling Test. We tested the level of trichome resistance when adults were forced to feed under a no-choice situation. Settling behavior of potato leafhopper adults was compared for stems (minus leaves) with intact glandular trichomes versus stems (minus leaves) whose trichomes had been denuded. Stem trichomes were removed by wrapping each with a new Kleenex brand tissue (Kimberly-Clark, Neenah, WI) and rubbing down each side of the quadrate stem with a sharp edge. Alcohol washes were not used because we suspected that stems would be damaged or chemical compounds in the epidermis would be extracted. Preliminary tests showed that the Kleenex procedure removed 90–100% of the erect, glandular trichomes from the stems. Leaf trichomes could not be similarly removed, nor by any other method tried. Therefore, leaves were excised at the petiole base from test and control plants. Tube cages were constructed, as in Shockley et al. (2002), and plants were held for 2 d before each test so that any volatile compounds that had been involuntarily spread during trichome removal could dissipate. Each replicate consisted of one control cage with an alfalfa stem with intact glandular trichomes, and one test cage with an alfalfa stem that had been denuded. Only the Cal/West genotypes (G98A, G98B, G98C, and G98D) plus Ranger were used in this test. Ten replicates were simultaneously performed with 20 plants of each genotype, for a total of 100 plants tested. Observations were made every 4 h for 36 h, or until all three insects in a cage were dead.

Statistical Analysis. The Statistical Analysis System (SAS) (SAS Institute 1985) was used for all tests, with type I error $\alpha = 0.05$. The two-choice repellency tests were recorded and analyzed as proportions (the percentage of insects choosing a genotype). The same number of insects was used for each pairing and replicate. Since the range of proportions was primarily between 0.3 and 0.7, the arcsine square-root transformation was not used, as suggested by Snedecor and Cochran (1989). Within each type of pairing (e.g., A-B) across the replicates, one chosen genotype (the "base genotype," e.g., A) was compared against the other genotype, including when the second genotype was the same as the base (e.g., A-A). This resulted in five sets of comparisons using each of the genotypes as the base, for a total of 25 pairings. Significant differences among genotypes within each set were determined using Fischer's least significant difference (LSD) tests. Paired *t*-tests were used to compare genotypes within each individual pairing. Because insects were not given a choice among all five genotypes simultaneously, the analysis had to be divided into five sets of base genotypes among which direct comparisons could not be made.

For the intact versus denuded stem preference test, we used a completely randomized analysis of variance (ANOVA), 5×2 factorial (five genotypes, two stem conditions [denuded, intact]) design. Mean pairwise differences were tested using Fischer's protected LSD tests.

Scanning Electron Microscopy. Alfalfa trichomes from healthy samples of all eight genotypes were examined using an ESEM (XL30 ESEM-FEG from FEI/Philips, Peabody, MA), in "wet" mode with water vapor pressure in the chamber of 5.5–6.0 Torr. The scan voltage was 10 kV, and specimens were mounted on a cooling stage with a constant temperature of 5°C. The stem and leaf sections were mounted and viewed on a chilled stage to avoid desiccation or beam damage. Erect trichomes were assessed visually along 1-mm sections of the stem and within 1-mm^2 sections of the leaves for differences in morphology. Virtually no tissue preparation before examination is required for ESEM, allowing the preservation of delicate plant structures like trichomes. Unfortunately, accelerated tissue destruction at higher magnifications precluded prolonged observation. Therefore, no specific measurements could be taken. However, micrographs include scale bars produced by the ESEM for visual comparison of trichomes within each genotype and between genotypes.

Results

Two-Choice Repellency Test. For all genotypes, there was no significant difference when the base genotype was compared with itself. G98A, the genotype deemed most resistant by the Cal/West scientists and by our previous work (Shockley et al. 2002), was clearly the most repellent, and gave the most consistent results. Significantly fewer insects settled on the G98A side of the tube than on the opposite side, for all other genotypes with which G98A was paired. This was the case both for the pairwise comparisons in the G98A base genotype set (Fig. 2a), as well as in most cases when G98A was compared in sets with other base genotypes (Fig. 2 b–e). The only exception was the pairing with G98C as the base genotype (Fig. 2c). In that case, the mean number of insects on G98A was numerically lower than on G98C, but not significantly, because the standard errors varied in relation to which genotype was used as the base. When G98C was used as the base, the variance was great enough that the mean number of insects on G98A did not differ significantly from that on G98C ($P = 0.07$). It was common for a pairing to show significant differences when one genotype was used as the base but not when the other was the base. Similar lack of statistical significance, but possible biological importance, could be inferred for the G98C-Ranger comparison ($P = 0.09$) (Fig. 2c), as well as the G98D-G98B ($P = 0.16$) (Fig. 2d) and Ranger-G98C ($P = 0.12$) (Fig. 2e) comparisons. Given the reciprocal nature of the comparison in each case, it is possible that the lack of significance in one of the pair of comparisons is an artifact of small sample sizes.

Therefore, the degrees of repellency exhibited by the remaining three glandular-haired genotypes (G98B, G98C and G98D) and Ranger are less clear-cut and more variable, as measured both by the pairwise

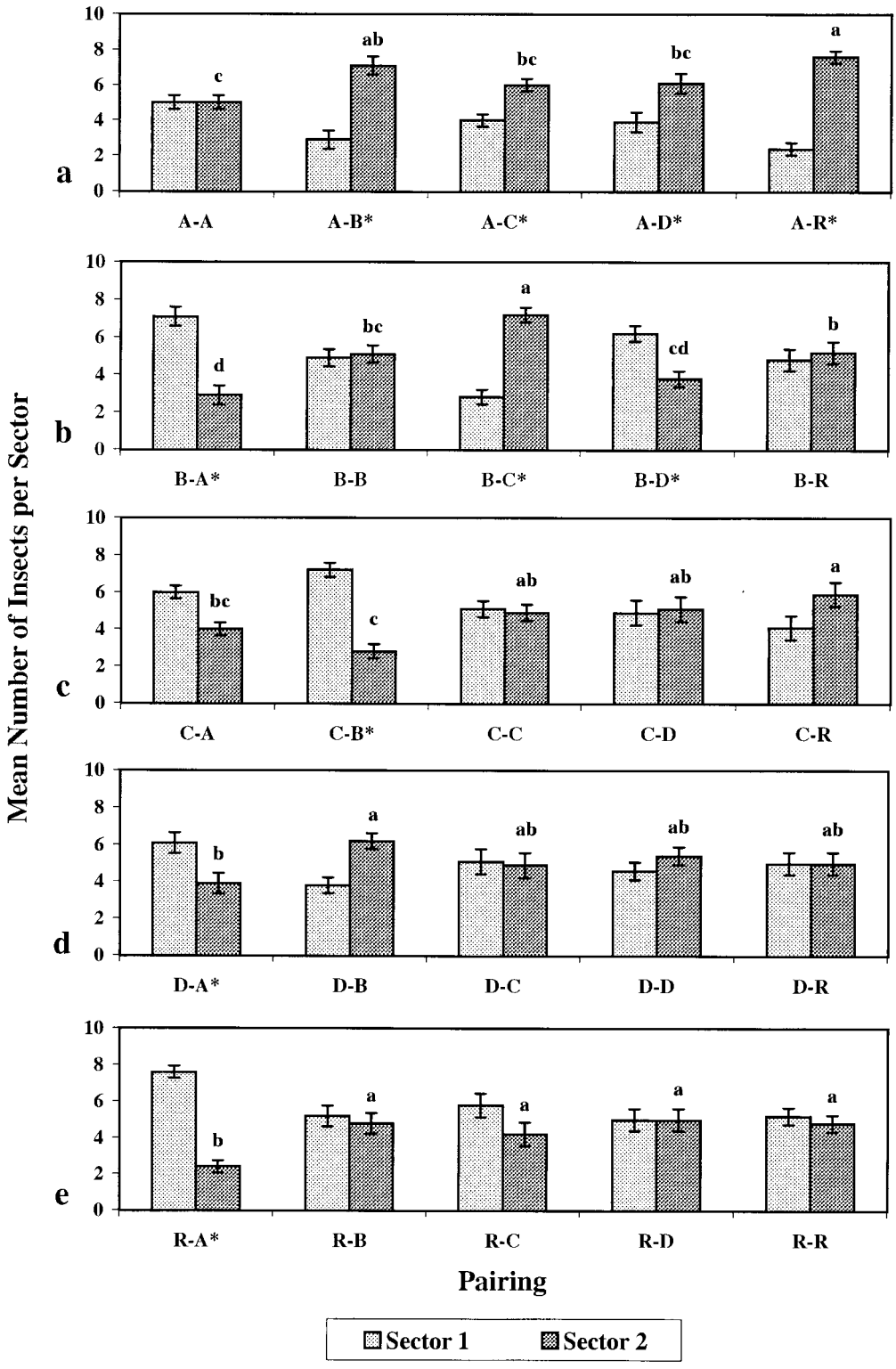

Fig. 2. Mean numbers of insects located in sector 1 (left side of tube) versus sector 2 (right side of tube) for each of the two-choice repellency test tubes. * Designates a significant difference between the base genotype and its paired genotype, within each comparison set. Lowercase letters denote significant differences among genotypes within each base genotype comparison set.

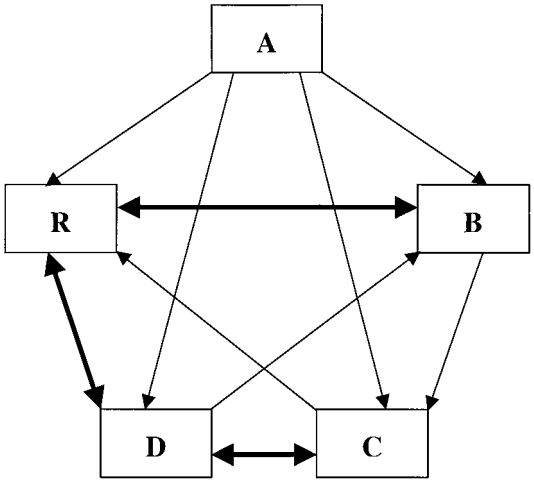

Fig. 3. Relationship in repellency among genotypes of glandular-haired alfalfa. Larger, thicker arrows represent relationships that show no significant differences in repellent attributes. Smaller, thinner arrows represent relationships that show significant differences among genotypes (the genotype near the arrowhead represents the more preferred [less repellent] one). Genotype designation as in Fig. 1.

comparisons and the among-genotype comparisons within each base genotype set (Fig. 2). Although the others are all less repellent than G98A, comparisons among them reveal that their relationship is nonlinear and potentially complex. For example, G98C, G98D, and Ranger are not significantly different from one another when any are paired (either within or among pairs; Fig. 2 d and e). However, when each is paired with the somewhat more resistant G98B, differences are revealed among the other three. Fig. 2b shows that G98C is preferred when paired with G98B, yet G98D is not preferred when paired with G98B, and Ranger is not significantly different from G98B. The results of this test, and the repellency relationship among the genotypes, are summarized graphically in Fig. 3 and numerically in Table 1. The repellency attributes of each genotype generally corresponded to its reputed resistance status, as indicated by the code letters A through D designated by Cal/West scientists (Table 1).

Table 2. Results of the intact vs. denuded stem test, showing the mean number of insects (mean ± SE) found on the plant, off of the plant (alive), and off of the plant (dead) at each observation

Genotype	Sample	Mean no. of insects		
		On stem	Off (alive)	Off (dead)
G98A	Denuded	2.43 ± 0.04	0.57 ± 0.04	0.00 ± 0.00
	Intact	2.32 ± 0.09	0.62 ± 0.08	0.06 ± 0.04
G98B	Denuded	2.50 ± 0.07	0.50 ± 0.07	0.00 ± 0.00
	Intact	2.40 ± 0.13	0.60 ± 0.13	0.00 ± 0.00
G98C	Denuded	2.38 ± 0.43	0.43 ± 0.08	0.19 ± 0.13
	Intact	2.53 ± 0.13	0.27 ± 0.08	0.20 ± 0.13
G98D	Denuded	2.39 ± 0.16	0.41 ± 0.14	0.20 ± 0.13
	Intact	2.27 ± 0.20	0.42 ± 0.08	0.31 ± 0.21
Ranger	Denuded	2.43 ± 0.12	0.37 ± 0.08	0.20 ± 0.13
	Intact	2.40 ± 0.19	0.30 ± 0.07	0.30 ± 0.21

Intact Versus Denuded Stem Settling Test. For all genotypes including the susceptible Ranger, approximately five times as many insects settled on the stems as off, regardless of whether the stems were intact or denuded. There were no significant differences between denuded and intact stems within each genotype, nor among genotypes for either settling site (Table 2).

ESEM of Intact Glandular Hairs. All erect, glandular trichomes on the examined plants from all eight genotypes had the same apparent morphology, i.e., a simple spherical gland atop a multi-segmented stalk (Fig. 4). There were no obvious differences in morphology, at any magnification, among genotypes or between leaves and stems within each genotype. No external difference in trichome structure was expected since both companies' glandular genotypes share a common origin for these genetic lines. However, upon strong manual rupturing, all examined genotypes' trichomes exuded a viscous substance (arrow; Fig. 4d). Representative trichomes from three of the test genotypes are shown in Fig. 4. Views of trichomes from all other examined genotypes can be found in Shockley (2000).

Discussion

The Nature of Repellency and Deterrency in the Alfalfa-Potato Leafhopper System. This is the first published study to use several differentially resistant ge-

Table 1. Results of the two-choice repellency test, showing the number of pairings in which the base genotype was preferred, repellent or not significantly different from the paired genotype

Base genotype	Base is preferred (no.)	Paired genotype designation[a]	Base is repellent (no.)	Paired genotype designation[b]	Not significantly different (no.)	Paired genotype designation[c]
G98A	0	—	4	all	0	—
G98B	2	A, D	1	C	1	R
G98C	2	A, B	1	R	1	D
G98D	1	A	1	B	2	R, C
Ranger	2	A, C	0	—	2	D, B

[a] Defined as the genotype(s) that, when paired with the base, was less preferred than the base.
[b] Defined as the genotype(s) that, when paired with the base, was less repellent than the base.
[c] Defined as the genotype(s) that, when paired with the base, was not significantly different than the base.

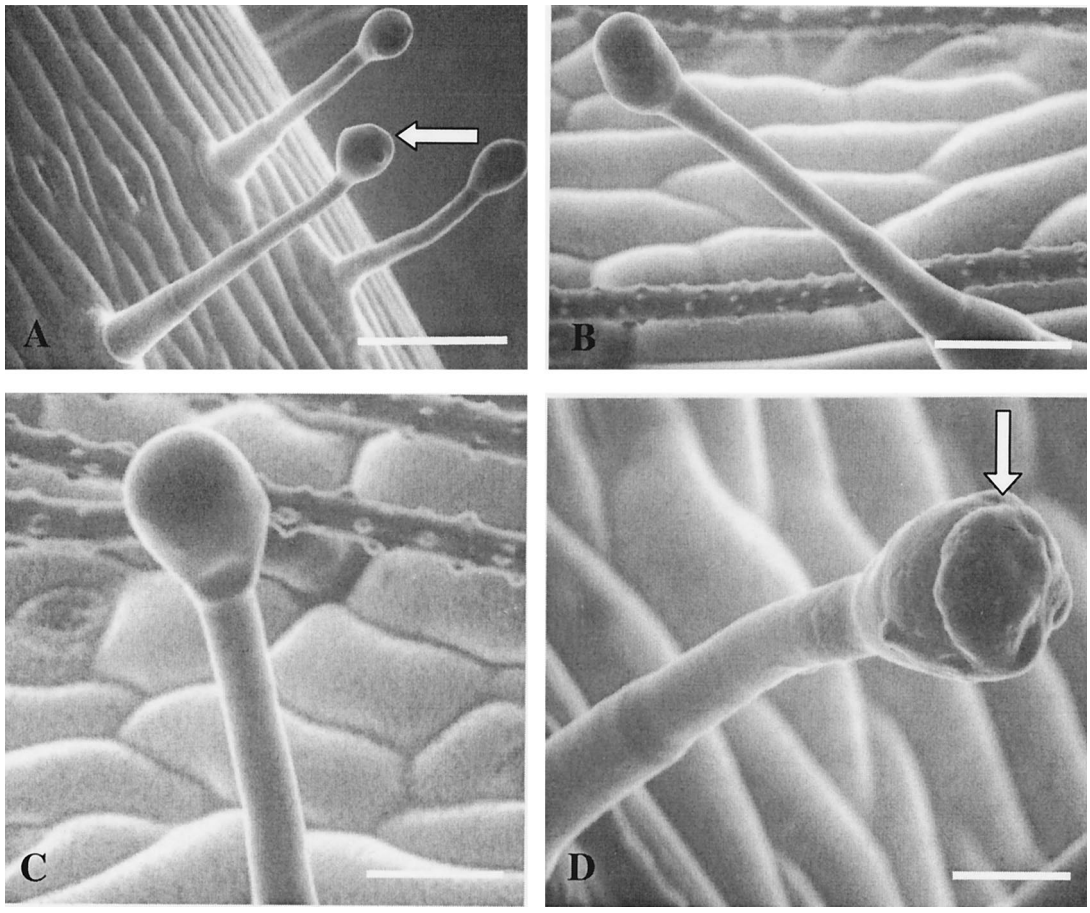

Fig. 4. Environmental scanning electron micrographs of stem and leaf glandular trichomes of glandular-haired genotypes of alfalfa. (A) G98A, stem trichome. Scale bar = 100 μm (magnification, 312×). Note the trichome gland consists of a simple spherical gland (arrow) atop a multicellular stalk. (B and C) G98C, leaf trichomes. Scale bar = 50 μm (600×). (D) Ruptured 1–27–4 leaf glandular trichome with exudate (arrow). Scale bar = 20 μm. (1,000×).

notypes of glandular-haired alfalfa to compare the effects of a whole plant's short-distance, noncontact sensory cues on host finding by the potato leafhopper. Short-distance, noncontact avoidance of a plant is most likely to be mediated by perception of volatile, repellent chemical compounds (i.e., those that trigger oriented movement of an insect away from the source [Bernays and Chapman 1994, Schoonhoven et al. 1998]) in experiments such as ours, which remove visual cues that could differentiate among genotypes. Therefore, we define the term "repellency" to imply the ability of alfalfa's (presumably) glandular trichomes to produce volatile, repellant compounds (Saxena and Okech 1985). It is likely that overall glandular-hair alfalfa resistance also involves other, nonvolatile, contact cues used by the insect for host plant acceptance after landing on the plant. These cues are detected by gustatory sensilla (usually involving deterrent chemical compounds [Schoonhoven et al. 1998]) or tactile sensilla. However, our experiment cannot test the role of contact cues because it was designed to test noncontact cues.

Implications for Resistance to the Potato Leafhopper. Potato leafhopper settling patterns in two-choice, noncontact tests roughly agreed with the degree of resistance reported by Cal/West scientists for these genotypes. Repellency was especially apparent with the most resistant genotype, G98A, and absent with the most susceptible genotype, Ranger. However, with the remaining, intermediate- (G98B and G98C) to low-resistance (G98D) genotypes the levels of repellency were variable and did not segregate the genotypes clearly. Such variable levels of repellency may support the hypothesis that additional, contact sensory information must be assessed before the insect makes a final decision on acceptance or rejection of the plant.

Our results also show that even the strongest glandular-hair resistance can be overcome, if the leafhopper is offered no alternative feeding sites, is allowed to contact the plant, and it becomes sufficiently hungry. No-choice, contact tests with either intact or trichome-denuded alfalfa stems showed that settling occurs on all genotypes, regardless of presence or

absence of trichomes. Overall, these results suggest that the trichomes will not prevent feeding at all costs, but can be variably repellent when the insect has a choice.

The differences in repellency among genotypes probably are not based on differences in erect trichome morphology, since all genotypes' trichomes were visually similar at the level of detail we could investigate. This finding supports the conclusions of Ranger and Hower (2000a, 2000b) for the smaller, procumbent trichomes. Volatile chemical compounds are well documented to cause repellency in many insect-plant interactions, and glandular trichomes are known to have such an effect (Saxena and Okech 1985, Avé et al. 1987).

Differences in repellency among genotypes also apparently are not directly related to trichome density (at least during the first 24 h of plant contact), because a previous study (Shockley et al. 2002) showed no significant differences in trichome densities among these same genotypes, including G98B, G98C, and G98D. Two possible, overlapping conclusions can be made about the nature of the volatile compounds: (1) their effect is quantitative, i.e., one or two key chemical compounds are found in varying concentrations among genotypes and the insects are sensitive to certain threshold concentrations; and/or (2) their effect is qualitative, i.e., the volatiles are actually blends of numerous compounds, and the insects are able to distinguish susceptible versus resistant genotypes based on exactly which chemicals are present (perhaps in what concentrations).

Thus, potato leafhoppers are not only repelled by glandular-haired alfalfa, but are able to distinguish subtle differences in resistance levels among variably resistant genotypes, probably by detection and identification of volatile chemical compounds. New studies to examine the chemistry of the trichome exudates and their mode of action on the leafhopper are currently underway.

Implications for Resistance to Hopperburn. Results presented elsewhere (Shockley et al. 2002) demonstrated that, when potato leafhopper mortality is compared across the eight genotypes of glandular-haired alfalfa, it is directly associated with decreased feeding, and such decreases may have a minor effect on the onset of hopperburn. However, this effect may not predict ultimate hopperburn symptom severity. This is because on all of the intermediate-resistance genotypes, mortality is low-to-moderate, leafhoppers can feed to at least some extent, and hopperburn severity is not correlated with amount of mortality or feeding (Shockley et al. 2002). Only the most resistant genotype (e.g., G98A) is sufficiently lethal to prevent most leafhopper feeding, and only such a strong decrease in feeding is correlated with decreased hopperburn. Thus, unless feeding is almost eliminated, decreased feeding does not lead to proportionally decreased hopperburn. Therefore, variable resistance to the leafhopper (i.e., variable amounts of mortality) causes variable (and not usually proportional) resistance to hopperburn (i.e., decrease in hopperburn severity).

We suspect that two other mechanisms of reduced hopperburn are probably also occurring to a variable extent in all resistant genotypes: (1) additional, contact chemical compounds in the plant may prevent feeding by the leafhopper, or cause the insect to alter its feeding to a less hopperburn-causing type (Serrano et al. 2000), and/or (2) physiological tolerance mechanisms of the plant may reduce the plant response (a "saliva-enhanced wound response" [Ecale and Backus 1995b, Ecale Zhou and Backus 1999]) that initiates hopperburn. Both of these types of antixenosis and tolerance, respectively, already have been demonstrated in common bean varieties exposed to *Empoasca* leafhoppers (Serrano et al. 2000), and also seem likely in alfalfa.

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