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# Effects of virus infection on release of volatile organic compounds from insect-damaged bean, *Phaseolus vulgaris*

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## ABSTRACT

Insects can serve as important vectors of plant pathogens, especially viruses. Insect feeding on plants causes the systemic release of a wide range of plant volatile compounds that can serve as an indirect plant defense by attracting natural enemies of the herbivorous insect. Previous work suggests that the Mexican bean beetle (*Epilachna varivestis*) prefers to feed on plants infected by either of two viruses that it is known to transmit: Southern bean mosaic virus (SBMV) or Bean pod mottle virus (BPMV). A possible explanation for the preferred feeding on virus-infected tissues is that the beetles are attracted by volatile signals released from leaves. The purpose of this work was to determine whether volatile compounds from virus-infected plants are released differentially from those emitted by uninfected plants. To test the hypothesis, common bean plants (*Phaseolus vulgaris* cv. Black Valentine) were inoculated with either BPMV, SBMV, or a mixture of both viruses, and infected plants were compared to uninfected plants. An Ouchterlony assay was used with SBMV- and BPMV-specific antisera to confirm the presence of virus in inoculated plants. RNA blot analysis was performed on tissue from each plant and indicated that a well-characterized defense gene, encoding phenylalanine ammonia-lyase (PAL), was not induced in systemic tissue following virus infection. Plant volatiles were collected—and analyzed via gas chromatography (GC)—from plants that were either undamaged or beetle-damaged. In undamaged plants, there were no measurable differences in profiles or quantities of compounds released by uninfected and virus-infected plants. After Mexican bean beetles were allowed to feed on plants for 48 h, injured plants released several compounds that were not released from undamaged plants. Lower quantities of volatile compounds were released from virus-infected plants suggesting that enhanced release of plant-derived volatile organic compounds is not the cause for attraction of Mexican bean beetles to virus-infected plants.

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[‡‡] Gisela F. Erf, teacher and faculty mentor regarding the immunology aspects of this project, is an associate professor in the Department of Poultry Science .

## MEET THE STUDENT-AUTHORS

*Sarah Sossamon*

I graduated from Ozark High School in 2001. I am a sophomore poultry science major with a pre-vet emphasis. I have received many scholarships including a University scholarship, the Arkansas Game and Fish Commission scholarship, an FFA foundation scholarship, a Dale Bumpers College scholarship, and a poultry science scholarship. I am involved with many clubs and organizations on campus including Chi Alpha—of which I am the president—Collegiate 4-H/FFA, Pre-Vet Club, Poultry Science Club, and the equine program. I am also a member of the National Society of Collegiate Scholars.

As a student in the Poultry Science Department I have had many academic and hands-on opportunities, including this one. I started my research of plant pathology through a lab rotations class. Thanks to the help of Dr. Korth and Dr. Erf I was able to carry the research out further. Although this research is not directly related to my field of study, it has taught me a great deal about conducting a specific science experiment and the techniques used in experimentation. The skills I learned from the research will help me as I progress in the science field and have already helped me in some of the classes I am taking now.

Once I complete my undergraduate program I plan on attending veterinarian school and then practicing both large- and small-animal medicine.

*Britney K. Jackson*

I am a junior in the Poultry Science Department at the University of Arkansas. I grew up in Fayetteville and graduated from Fayetteville High School in the year 2000. I am working with the USDA in Agricultural Research Services concentrating on microbiological research. In this field I have continued to apply the research skills I acquired through this project.

*B. Alison Drumwright*

I am a senior poultry science major in the Poultry Science Department of the University of Arkansas. My hometown is Germantown, Tenn., and after my bachelor's degree is completed, I am hoping to pursue a career in veterinary medicine. I received a SURF grant to continue in this area of research.

## INTRODUCTION

Plants are continually threatened by a wide array of organisms including disease-causing microorganisms and herbivorous insects. In response to these attacks plants have developed a complex defense system in which specific mechanisms are triggered in response to specific pests (Karban and Baldwin, 1997). As part of a response to insect damage, plants release volatile compounds from leaves. These volatiles can serve as semiochemical attractants to natural enemies of the herbivore such as predatory arthropods (Dicke and Sabelis, 1988) and parasitoid wasps (Turlings et al., 1991), thereby serving an indirect defensive role (Kessler and Baldwin, 2001). The release of volatiles is systemic, occurring from both damaged and undamaged leaves of wounded plants (Turlings and Tumlinson, 1992). At least three chemical classes are represented in the profile of volatiles released following herbivory (Paré and Tumlinson, 1999). Green-leaf volatiles—namely C6 alcohols and aldehydes terpenes, and products of the shikimate pathway such as indole—are released following damage. The release of many of the terpenes and indole is often delayed by nearly a full day following initial damage, indicating that stimulation of plant biosynthetic pathways might be required.

Insects frequently serve as vectors of plant pathogens, especially viruses. By retaining viral particles in mouthparts and foregut, Mexican bean beetle (*Epilachna varivestis* Muls.) serves as an important vector of plant viruses that infect several species of beans. Viruses are spread when regurgitated on other plants during feeding. Two common viruses spread in this way, each with a narrow host range, are the Bean pod mottle virus (BPMV) and Southern bean mosaic virus (SBMV). Symptoms of BPMV include mild leaf mottling; BPMV has a single-stranded (ss) RNA genome and is a member of the Comoviridae. Bean leaves infected with SBMV have pale-yellow lesions and mosaic symptoms; SBMV also contains an ssRNA genome and is the type member of the genus Sobemovirus.

A previous study shows that Mexican bean beetles prefer feeding on virus-infected plants compared to feeding on non-infected plants, as measured by the amount of leaf material the beetles consumed (Musser, et al., 2003). We derived several hypotheses for the beetle preference for virus-infected tissue including the possibility that there are chemical antifeedants in uninfected plants. Another explanation is that volatile compounds released by virus-infected plants could be attracting the beetles. We attempted to determine whether common bean plants differentially release volatile compounds and so we collected and analyzed volatile organic compounds from beetle-damaged plants that were either virus-infected or uninfected.

## MATERIALS AND METHODS

*Plants and Insects.* Common bean plants (*Phaseolus vulgaris* L. cv. Black Valentine) were used for this study. Seeds were planted in LC-1 Sunshine Mix soil in 4.5-inch pots. Plants were watered daily and maintained in the greenhouse at 24°C under a 16:8 h light:dark regimen. Virus inoculation was conducted when plants were 10 d old. One leaf per plant was mechanically inoculated with either BPMV, SBMV, or a mixture of the viruses. Virus-containing plant sap was obtained from other previously infected bean leaves. Virus sap was prepared by grinding three to four infected leaves in a sterile mortar with 2 mL of ice-cold extraction buffer (50 mM potassium phosphate, pH 7.2). Leaf tissue was ground until no large pieces of tissue remained. The virus-containing sap was applied with a saturated cotton inoculation pad to a leaf that was lightly dusted with abrasive 600-mesh carborundum. Plants that were mock-inoculated with sap collected in the same manner from healthy plants served as controls throughout all experiments.

Mexican bean beetles and virus-infected leaves were graciously provided by Dr. Rose Gergerich and Sandy Wickizer, Department of Plant Pathology, University of Arkansas. Plants were exposed to 10 d after virus inoculation. A nylon net was placed over each pot containing two plants per pot to contain four beetles that were added and allowed to feed for 48 h.

*Virus Identification and Detection*

A standard Ouchterlony gel double-diffusion test in 1% agarose was used to identify and confirm viral infection of plants. Sap was extracted from virus-infected leaves by squeezing with a tissue-extractor. For this immunoassay, 35 µL of each virus extract was placed into labeled wells in the Ouchterlony gel plates. BPMV- and SBMV-specific antisera, diluted 1:10 in 50% glycerol, were placed into the center wells of duplicate Ouchterlony plates. Extracts from the mock-inoculated uninfected plants were used as negative controls for each test.

*RNA Analysis*

Bean plant RNA was isolated using TriReagent (Molecular Research, Inc., Cincinnati, Ohio) according to the manufacturer's instructions. Two leaves from each type of virus-infected plant were stored in liquid nitrogen until RNA isolation. For RNA isolation, leaves were ground to a fine powder using a sterile pestle and mortar containing liquid nitrogen. Final concentrations of RNA samples were determined spectrophotometrically by absorbance at 260 nm.

Total RNA was separated by electrophoresis in a 1% agarose formaldehyde gel and transferred to a nylon membrane via capillary transfer (Sambrook, et al., 1989). Membranes containing RNA were hybridized overnight (Church and Gilbert, 1984) with radiolabeled probes produced with $^{32}P$ in random-primer reactions (Sambrook, et al., 1989).

*Analysis of Plant Volatile Compounds*

Volatile compounds released from intact plants were collected by placing plants in 18-L glass chambers. Activated-carbon-filtered air was introduced at the top of each glass chamber at a rate of 1L/min. Gaseous contents of the chamber were removed by vacuum flow 0.8 L/min through a volatile-trap tube containing 75 mg SuperQ resin (Alltech, Inc., Deerfield, IL) near the bottom of each chamber. Excess introduced air was allowed to exit through the open bottom of each chamber. Volatile organic compounds bound to the resin were removed by washing with 250 μL of $CH_2Cl_2$ directly into glass gas-chromatography (GC) vials. GC was performed on a Hewlett Packard 5890 gas chromatograph equipped with Restek Rtx-1 column (30 m x 0.25 mm I.D.) essentially as described by Turlings, et al. (1991). Splitless injection of 2 μL per sample was performed and separation of compounds was monitored with a flame-ionization detector.

## RESULTS AND DISCUSSION

*Immunoassays Confirm Virus Infection*

An Ouchterlony assay is a simple method used to confirm presence of a specific compound through a binding interaction between specific antibodies and a corresponding antigen. Briefly, antiserum and test material are placed separately into an agar-gel matrix and allowed to diffuse through the medium until their paths cross. If antibodies in the serum bind to an antigen, a band of visible precipitant forms in the agar. Location of the band of precipitation can vary depending on the relative concentration of antibody and/or antigen in solution. Ouchterlony assays confirmed that BPMV and SBMV were present and replicating in the individually inoculated plants (Fig. 1). In addition, both viruses were able to replicate in a mixed-inoculation, based on precipitation patterns that appeared in the gel. The absence of a precipitation signal in the mock-inoculated sample confirmed that there was no cross-contamination between study plants. Therefore, uninfected healthy plants did not contain BPMV or SBMV and virus inoculations were successful.

*Defense Gene Induction in Response to Virus Infection and Beetle Herbivory*

Phenylalanine ammonia lyase (PAL) is a critical enzyme in the production of defense-related phenolic compounds in plants (Bate, et al., 1994). The accumulation of the PAL enzyme is known to be controlled at the level of RNA transcription that increases depending on the developmental stage of the plant (Liang, et al., 1989) and in response to fungal pathogens (Cuypers, et al., 1988). Accumulation of PAL transcripts was compared among mature leaves of uninfected healthy plants and those infected with BPMV, SBMV, or a mixture of both viruses. Virus-infected plants contained PAL transcript levels similar to those in uninfected plants (Fig. 2).

Essentially identical results were obtained when this experiment was repeated (data not shown). The study was complicated by a low level of unwanted insects, namely thrips, in the greenhouse, which could account for some of the PAL gene expression in uninfected plants. Nonetheless, it seems clear that expression of the gene encoding this enzyme is not strongly induced using the viral treatments described here. The RNA blots were also probed with a radiolabeled 18S rRNA to confirm equal loading of total RNA on the gel (Fig. 2). Although many studies have been performed showing increased PAL gene expression in response to bacterial or fungal pathogens, there are surprisingly few studies measuring PAL expression in response to viral infections in intact common bean plants.

*Volatile Compound Measurements*

Herbivore-damaged plants emit volatile organic compounds as part of a defense response. The emitted compounds can serve as an indirect defense against insect pests by attracting natural enemies of the herbivore or alternatively they might act as a repellant to herbivores (Kessler and Baldwin, 2001). Thus, possible explanations for beetle preference for infected plant material could be that virus-induced plant volatiles attract beetles, or that uninfected leaves emit deterrents to beetle feeding. To test these possibilities, we allowed Mexican bean beetles to damage leaves (Fig. 3A) and then analyzed volatile organic compounds released from leaves into a glass chamber (Fig. 3B).

Virus infection had little effect on the profile of volatiles collected from undamaged plants, as compared to uninfected, undamaged plants (Fig. 4). This suggests that volatile release prior to plant damage is not the primary reason for beetle feeding preferences.

The number and amounts of volatile compounds released from undamaged plants was much lower than was observed in insect-damaged plants. As expected,

beetle feeding resulted in more volatiles being released when compared to undamaged plants (Fig. 5A). Although there have been few reports of analysis of volatiles released from common bean leaves, the effects of mite damage on volatile release from the closely related lima bean, P. lunatus, have been studied in detail. Lima bean, along with virtually every other plant species examined to date, releases 11-carbon (4,8-dimethyl-1,3,7-nonatriene; DMNT) and 16-carbon (4,8,12-trimethyl-1,3,7,11-tridecatetraene; TMT) homoterpenes in response to arthropod herbivory (Boland, et al., 1992). In addition, lima bean is known to release other terpene compounds along with green-leaf volatiles and products of the shikimate pathway (Dicke, et al., 1990; 1999). We observed that the amounts of herbivory-induced volatiles released from bean leaves that were virus-infected were much lower than in uninfected leaves (Fig. 5). Collection of volatiles was carried out three times in independent experiments and the quantification of selected peaks is presented in Table 1. The results illustrate the general trend of reduced volatile release from virus-infected leaves and also the occasional variation observed in levels of individual compounds analyzed in these experiments. The volatile collection apparatus consists of four identical chambers used in parallel with forced air and vacuum lines that are equally distributed to each chamber. Control tests verified that the variation is due to natural plant differences and not to the experimental apparatus or the GC analysis.

We consistently observed that a mixed viral infection, which gave the most severe plant symptoms of any of the treatments, resulted in the greatest reduction of insect-induced volatiles. We did not observe unique GC peaks following any of the treatments. The putative identifications of compounds quantified in Table 1 are based on identical retention times of the unknown peaks with known standard compounds. In addition, the release of the homoterpenes DMNT and TMT, and the terpenes β-caryophyllene and α-bergamotene, is well documented in a lima bean-mite system (Takabayashi, et al., 1991). Absolute confirmation of the identification of the compounds would require an analysis such as mass spectrometry.

Because uninfected bean plants released higher levels of volatile organic compounds, this might suggest that Mexican bean beetles prefer to feed on virus-infected plants because some volatiles act as insect repellants. However, prior to insect damage we observed no difference in volatiles released by uninfected or virus-infected plants, so any repellant activity must be occurring well after the insects begin feeding.

A more plausible explanation might be that enhanced volatile release is an indicator that uninfected plants are better able to mount a defense response than are virus-infected plants with compromised defense systems. This reasoning would account for the insect feeding preference for pathogen-infected plants. The pathogen-induced defense signaling pathways of plants can be distinct from those induced by chewing insects. Although results vary with the experimental system, a growing body of literature suggests that antagonistic interactions exist between pathogen- and insect-defense pathways in plants (Felton and Korth, 2000; Kunkel and Brooks, 2002). Because the Mexican bean beetle serves as a vector of both BPMV and SBMV, enhanced feeding on infected plants would seem to increase the probability

**Table 1. Relative levels of insect-induced plant terpene volatiles released from virus-infected plants.**

| Retention time[w] | Putative I.D.[x] | Treatment[y] | Relative level +/- SD[z] |
|---|---|---|---|
| 13.82 min | DMNT | BPMV | 0.439 +/- 0.087 |
| | | SBMV | 0.992 +/- 0.519 |
| | | Mix | 0.599 +/- 0.054 |
| 22.08 min | β-caryophyllene | BPMV | 0.346 +/- 0.320 |
| | | SBMV | 0.712 +/- 0.534 |
| | | Mix | 0.086 +/- 0.010 |
| 22.88 min | α-bergamotene | BPMV | 0.329 +/- 0.288 |
| | | SBMV | 0.672 +/- 0.496 |
| | | Mix | 0.167 +/- 0.138 |
| 25.69 min | TMT | BPMV | 0.472 +/- 0.264 |
| | | SBMV | 0.779 +/- 0.570 |
| | | Mix | 0.291 +/- 0.049 |

[w] Retention time of compound as determined by gas chromatography described in Materials and Methods section.

[x] Putative identification of terpenes is based on identical retention time of known standard compounds. DMNT, 4,8-dimethyl-1,3,7-nonatriene. TMT, 4,8,12-trimethyl-1,3,7,11-tridecatetraene.

[y] Volatiles were analyzed after collection from beetle-damaged plants that were infected with bean pod mottle virus (BPMV), Southern bean mosaic virus (SBMV), or both viruses (Mix).

[z] Numbers represent average relative amount of compound as based on GC peak areas, compared to amount of compound released from an uninfected insect-damaged bean plant analyzed in parallel. Results are compiled from three independent replicated treatments, n=3. SD = standard deviation.

that viruses would be taken up and spread to other plants (Musser, et al., 2003). Likewise, if plant defenses against insects are weakened by virus infection then the fitness of beetle populations might increase as a result of feeding on these plants. Therefore, both the pathogen and its vector could be mutually aided in this interaction. Further studies are necessary to determine if these hypotheses hold true.

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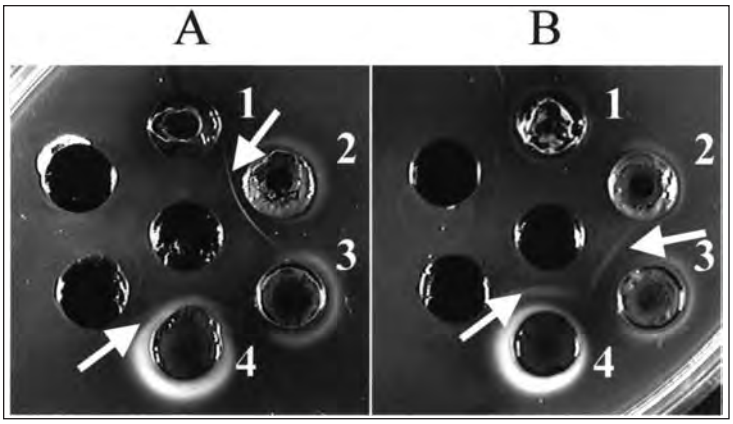


**Fig. 1.** Agar plates of Ouchterlony assays showing virus-antibody interactions. SBMV-specific (A) and BPMV-specific (B) antisera were added to the center well of each plate. Bean-leaf extracts added to the outside wells were 1) Uninoculated, 2) SBMV, 3) BPMV, and 4) Mixed infection. Arrow indicates antigen-antibody reaction near the well.

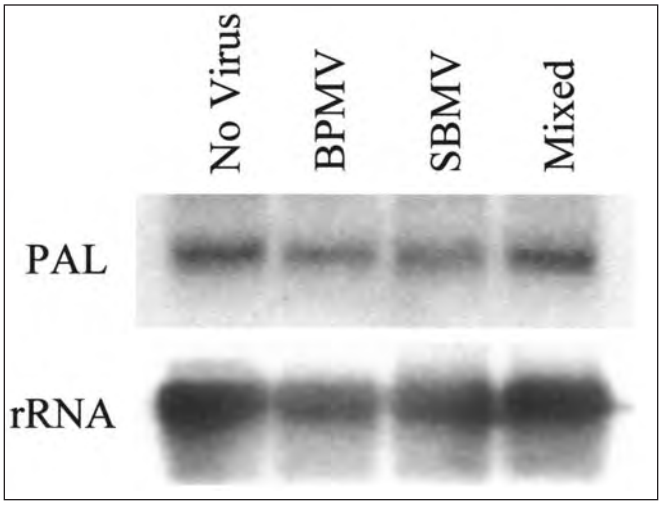


**Fig. 2.** Viral infection does not induce accumulation of transcripts for a defense gene. Autoradiographs showing presence and levels of mRNA encoding phenylalanine ammonia lyase (PAL) following gel electrophoresis and hybridization with a radiolabeled probe.

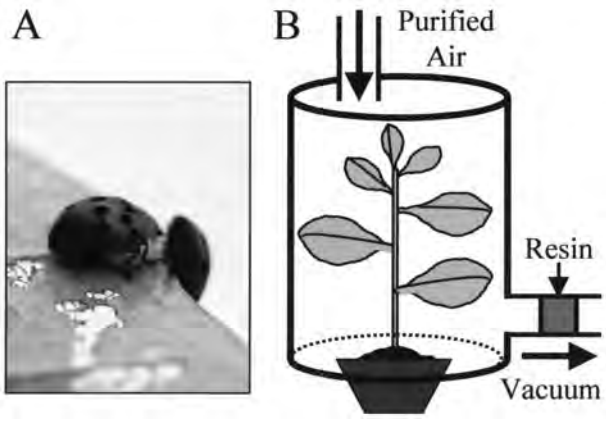


**Fig. 3.** A, Mexican bean beetle, *E. varivestis*, shown with feeding damage on a common bean, *P. vulgaris*, leaf. B. Schematic illustration of plant-volatile collection system described in the Materials and Methods section.

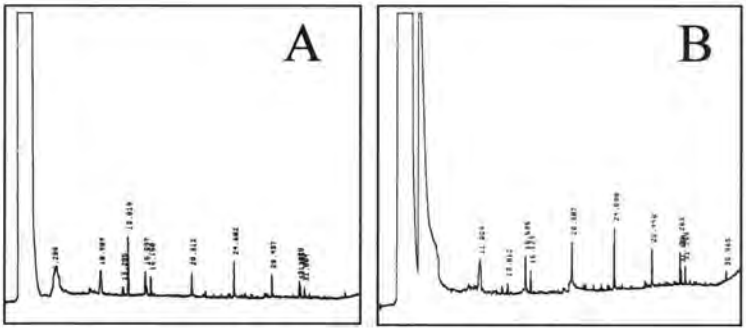


**Fig. 4.** Viral infection does not affect volatile release from undamaged plants. Gas chromatograph profiles are shown of separated compounds from undamaged bean plants that were either uninfected (A) or infected with a mixture of BPMV and SBMV (B).

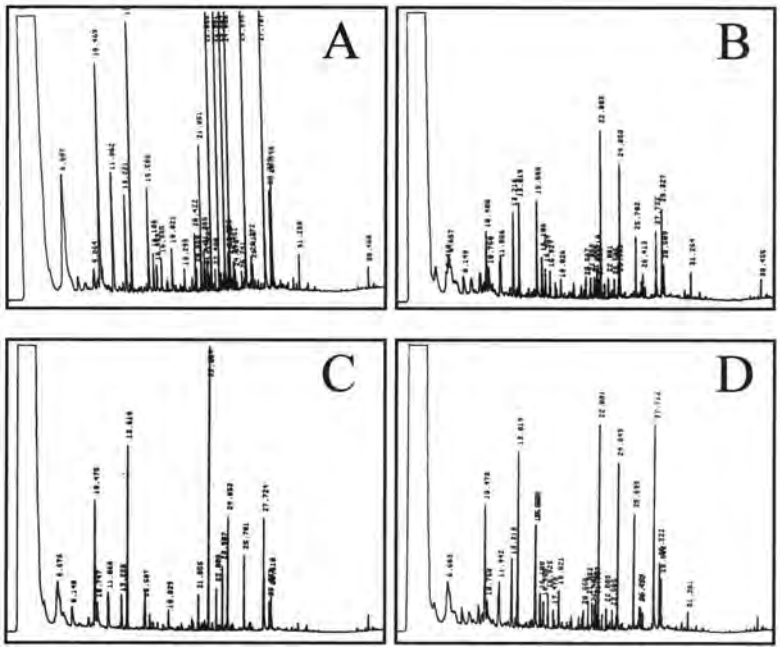


**Fig. 5.** Viral infection reduces the amount of volatile organic compounds released from bean leaves following beetle herbivory. Gas chromatograph profiles are shown of compounds from damaged bean plants that were either uninfected (A), infected with BPMV (B), SBMV (C), or a mixture of both viruses (D).