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SHORT REPORTS

TRAPPING AND IDENTIFICATION OF NITROGEN OXIDES FROM SOYBEAN LEAVES

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Key Word Index—*Glycine max*; Leguminosae; Soybean; Mexican bean beetle; nitrogen oxides; volatile compounds; insect; toxicity.

Abstract—Volatile compounds were isolated from insect-resistant and insect-susceptible soybean leaves and from snap-bean leaves by dry vacuum distillation. Nitrogen oxides, methanol, acetaldehyde, and ethanol were identified. These volatiles were found in all three plants tested and exposure to these compounds was toxic to the Mexican bean beetle.

INTRODUCTION

It has been reported that nitrogen purging of soybean leaves contains nitric oxide and nitrogen dioxide [collectively nitrogen oxides NO(x)] [1]. However, Mulvaney and Hageman [2] have reported that NO(x) was not detected during *in vivo* nitrate reductase assays of soybean leaves. Interest in allelochemical properties of legume leaf volatiles led us to investigate the possible presence of NO(x) and to identify other volatile compounds. Consideration of the possible allelochemical role of these volatiles has been prompted by the observation that the adult Mexican bean beetle (Coleoptera: Coccinellidae) surgically deprived of its olfactory organs on antennae and maxillary palps failed to discriminate between insect-susceptible and insect-resistant soybean genotypes [3]. This paper details the isolation and identification of volatile compounds from insect resistant and insect-susceptible soybean leaves and also from snap-bean leaves.

RESULTS AND DISCUSSION

Gas samples isolated from soybean leaves were analysed directly by gas chromatography (GC). Nitrogen oxides, methanol, acetaldehyde, and ethanol were identified by comparing GC retention times with those of authentic samples. Table 1 lists the quantitative GC results for methanol, acetaldehyde, and ethanol. The quantitative GC determination of nitric oxide (NO) and nitrogen dioxides (NO_2) is very difficult because of their reactivities and strong irreversible adsorption on most materials [4]. Therefore, NO and NO_2 were measured spectrophotometrically and expressed as mol. NO_2^-/g fresh weight.

Because NO_2 reacts readily with water to form NO_2^- (5), $2NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+$, it is necessary to determine the amount of NO_2 converted to NO_2^- in the liquid fraction. The measurement of NO_2^- was performed as follows: The liquid fraction of the cold trap was treated with the Griess–Saltzman reagents [6]. NO_2^- being converted to a reddish-purpose azo-compound by the reagents. The concentration of the azo-compound was then measured on a spectrophotometer.

To determine the amount of nitrogen oxides in the gaseous fraction, oxygen was used to completely oxidize NO of the gas sample in the storage bulb. The gas sample immediately changed from colourless to brown according to the equation: $2NO$ (colourless) $+ O_2 \rightarrow 2NO_2$ (brown). This observation is additional evidence of the presence of nitrogen oxides (primarily as nitric oxide) in association with soybean leaves. The oxidized gas sample was then mixed with 10 ml Griess-Saltzman reagent in the storage bulb. The concentration of NO_2 was measured spectrophotometrically. The amounts of NO_2^- calculated from both gaseous and liquid fractions were listed in Table 1.

Amounts of NO_2^- in Table 1 are lower than those reported in ref. [1]. This difference is expected because of different extraction designs and operating conditions. In addition they included preoxidizer (glass beads coated with $Na_2Cr_2O_7$–H_2SO_4) in the trapping system. Those results do not exclude the possibility that the preoxidizer could oxidize a variety of chemical compounds, including NO. Consequently the results could be overestimated [2].

Mulvaney and Hageman [2] indicated that NO and NO_2 were not detected by their method. They also pointed out that only trace amounts of NO_2^- were detected by the Griess–Saltzman method when the preoxidizer was not used. These observations are not incompatible with our measured data (Table 1). Because only 0.2–3 g of soybean leaves was used in their studies, the total amount of NO(x) evolved was very small, probably below the detection limit of their instrumentation. Our results indicate that NO(x) can be detected if more than

Table 1. Amounts of NO_2^-, methanol, acetaldehyde and ethanol evolved from soybean leaves. (Values presented are means ± SD of three replications)

Bean varieties	NO_2^-	Methanol	Acetaldehyde	Ethanol
		10-7 mol/g	fresh wt	
229358	1.1 ± 0.1	10 ± 0.7	7.6 ± 0.9	13 ± 1
Williams	0.85 ± 0.12	5.1 ± 0.4	37 ± 6	25 ± 4
Snap	0.12 ± 0.02	3.9 ± 0.3	0.34 ± 0.05	trace

100 g of soybean leaves are used.

The mechanism of NO(x) evolution is unknown. Previous studies reported that the reaction sequence of nitrate reduction probably involves two unknown intermediates [7, 8].

$$NO_3^- \rightarrow NO_2^- \rightarrow X \rightarrow X \begin{cases} \nearrow NH_3 \\ \searrow N_2 \end{cases}$$

It has been postulated that NO is an intermediate and results from enzymatic reduction of NO_2^- [1]. Our study seems to support this hypothesis.

Gaseous samples isolated from leaves were also used for closed chamber tests on adult Mexican bean beetles. These adults were exposed in groups of three to the gaseous samples. It was found that exposure to these concentrated volatiles of the resistant variety was very toxic to the adults because all were dead after 8 hr exposure. On the contrary, exposure to concentrated volatiles of the common bean was not fatal to them. Nitrogen oxides and other volatile compounds are present in all three plants tested, but they are very low in foliage of common beans (Table 1). It appears that these compounds are probably important to insect olfaction. Further study on the volatile compounds by using electroantennagram is contemplated.

EXPERIMENTAL

Plant Materials. Seeds of common beans (*Phaseolus vulgaris* L.) and soybean [*Glycine max* (L.) Merrill] cultivar 'Williams' (insect susceptible) and plant introduction (PI) 229358 (insect resistant) were germinated in sand beds. One-week-old seedlings were transplanted to greenhouse benches filled with pea gravel, the gravel beds were flooded periodically with half strength Hoagland solution [9]. After plants exceeded the V5 growth stage, foliage was harvested [10].

Volatile Isolation. Leaves were dry-vacuum distilled by placing a 200 g sample in a 1 l flask that was wrapped in foil to exclude light. The flask was immersed in a 20° water bath and leaves were vacuum distilled for 5 hr. The total distillate was collected in a vacuum trap at liquid N_2 temperature. Using high vacuum techniques, the gas components were transferred to a storage bulb. The storage bulb was made from a 100 ml spherical Pyrex® flask fitted with a septum and Swagelok. The gas sample was withdrawn from the storage bulb and immediately analysed with a gas chromatograph. The remaining liquid fraction in the vacuum trap was stored at 2° for spectrophotometrical analyses.

Instrumentation. Compounds were identified with a Varian Model 2100 gas chromatograph equipped with an alkali flame ionization detector. A rubidium sulphate flame tip was used because it gives the best response to N_2-containing compounds, such as NO(x). Chromatography was performed with a glass column (2 mm × 180 cm) packed with Porapack Q. The temperature program started at 75° held for 1 min and then increased at 6° min to 180° and held at this temperature for 10 min. The nitrogen flow rate was 20 ml/min.

Chemical compounds were verified on a Varian MAT CH-7 mass spectrometer interfaced with a Varian Model 1700 gas chromatograph (11).

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REFERENCES

1. Harper, J. E. (1981) *Plant Physiol.* **68**, 1488.
2. Mulvaney, C. S. and Hageman, R. H. (1984) *Plant Physiol.* **76**, 118.
3. Fischer, D. C. and Kogan, M. (1985) *Entomol. Exp. Appl.* (in press).
4. Smith, K. A. (1977) In *Advances in Chromatography*, Vol. 15 (Giddings, J. C., ed.) p. 212–213. Marcel Dekker, New York.
5. Sisler, H. H. (1956) In *Comprehensive Inorganic Chemistry*, Vol. 5. Sneed, M. C. and Brasted, R. C., eds) pp. 11–105. D. Van Norstrand, Princeton.
6. Allen, J. D. (1973) *J. Inst. Fuel* **46**, 123.
7. Bandurski, R. S. (1965) In *Plant Biochemistry* (Bonner, J. and Varner, J. E., eds) pp. 479–488. Academic Press, New York.
8. Wharton, D. C. and Weintraub, S. T. (1980) *Biochem. Biophys. Commun.* **97**, 236–242.
9. Harper, J. E. (1971) *Crop Sci.* **11**, 347.
10. Fehr, W. R. and Caviness, C. E. (1977) *Iowa Coop. Serv. Spec. Rep.*
11. Wei, L. Y. and Felsot, A. S. (1982) *J. Assoc. Off. Anal. Chem.* **65**, 680.