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A NEW FORM OF ANTIBIOSIS IN *NICOTIANA*

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Key Word Index—*Nicotiana*; Solanaceae; *Manduca sexta*; tobacco hornworm; nicotine; alkaloids; trichome exudate.

Abstract—Species belonging to the genus *Nicotiana*, section *Repandae*, imparted high levels of mortality to *Manduca sexta*, the tobacco hornworm, a tobacco-associated insect which is not susceptible to the toxic effects of nicotine. One hundred per cent mortality was observed after 24 hr following topical application of 1 mg of crude exudate material. The nicotinoid antibiosis component of the leaf exudate was shown to be different from conventional nicotinoid alkaloids. Ten micrograms of the active alkaloidal material caused 87–100% mortality within 24 hr. The nicotine derivative produced by these plants is thus able to circumvent the insect's physiological defence against nicotine.

INTRODUCTION

A vast quantity of literature has been written on the toxicology and mode of action of conventional nicotinoid alkaloids as natural insecticides [1–3]. Workers in such diverse fields as insect physiology, plant genetics and pharmacology have long favoured the genus *Nicotiana* for the study of these alkaloids [4–6]. One reason the genus is so favoured is because it contains 66 species which differ not only morphologically, but also in the quality and quantity of their alkaloids [7]. The genus also contains a cultivated species, *Nicotiana tabacum* (tobacco), which has been the target of many cytogenetic, tissue culture and insect toxicology studies [8–12].

There has been much discussion concerning the function of secondary plant constituents, such as nicotine. Some authors believe that the compounds protect the plant from phytophagous insects, and indeed nicotine is a clear example of a secondary plant constituent which imparts antibiosis against phytophagous insects [13–15]. The resistance of *Nicotiana* spp. to insect feeding, including an insect that restricts itself to *Nicotiana* and related genera [tobacco hornworm, *Manduca sexta* (L.)], is generally thought to be due to nicotine, and to a lesser extent, the related alkaloids anabasine and nornicotine. Thurston [16], showed that the toxic compounds responsible for antibiosis towards *M. sexta* were contained in the leaf exudate, and also postulated that the above three alkaloids were responsible. Furthermore, he reported that species belonging to the section *Repandae*, namely, *N. nesophila*, *N. repanda* and *N. stocktonii*, imparted moderate to high levels of resistance to larvae of *M. sexta*.

However, subsequent researchers showed that larvae of *M. sexta* are able to excrete 90% of the nicotine fed to them within 2–4 hr of ingestion. Further, the CNS of *Manduca sexta* was found to be capable of metabolizing nicotine [17, 18]. Thus, nicotine is apparently not the compound responsible for the antibiosis effects of leaf exudate on *M. sexta* larvae, in some wild species of *Nicotiana*.

This situation presents a paradox in which a specialized insect herbivore is able to overcome the toxic effects of nicotine exposure, but yet it is susceptible to host plants whose resistance is attributed to nicotine. Therefore, we examined leaf exudate material from leaves of wild species in the section *Repandae* in an attempt to isolate the factor(s) responsible for imparting antibiosis to larvae of *M. sexta*.

RESULTS

Previous studies under field conditions verified that our material from the *Repandae* section used in the present study was highly toxic to *M. sexta* larvae [19]. Exudate of the species in section *Repandae* was obtained from greenhouse grown plants. Following application of 0.5 or 1.0 mg of exudate in 1 μl ethanol from *N. nesophila* to the dorsum of first instar *M. sexta* larvae, 100% mortality was observed within 48 hr (Table 1). Application of 1.0 mg of *N. repanda* exudate also caused complete mortality within 48 hr, while 80% mortality was observed after 48 hr for applications of 0.2 mg. In marked contrast, application of 0.5 mg of 99% pure nicotine base (the most insecticidal form), caused only 4.0% mortality within the same amount of time.

Previous investigators successfully employed TLC as a means of identifying and isolating for bioassay alkaloids which occur not only in cultivated as well as wild species of *Nicotiana*, but also in the excrement of phytophagous insects which have ingested alkaloids [2, 20–23]. We used these same techniques to compare our data with previous results. When either exudate of *N. tabacum* or pure nicotine base was chromatographed against the exudate of species from the section *Repandae*, two important results were obtained. Nicotine is only present at a low level in the leaf exudate of wild species of section *Repandae*. A second class of alkaloids is present in a much higher concentration. Exudate from species of the section *Repandae* gives rise to an intense red spot with an R_f of 0.7–0.8, when subjected to alkaloid staining. Slightly below this R_f was the lemon-yellow nicotine base region, with an R_f of 0.6–0.7. The nicotine spot in the wild species lanes is usually very faint. Further, the red staining region

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Table 1. Cumulative per cent mortality of *M. sexta* larvae following application of crude leaf exudate from *Repandae* species

Treatment†	Dose (mg)	n	Cumulative per cent mortality* 24 hr	48 hr
N. tabacum	1.0	10	0	0
N. repanda	1.0	10	100	100
	0.2	10	20	80
N. nesophila	1.0	10	100	100
	0.5	10	90	100
Nicotine base	0.5	25	4	4
EtOH	1.0‡	30	0	0

*Crude exudate material caused significantly greater mortality than either EtOH or nicotine base ($\chi^2, \alpha = 0.05$).

†Larvae were bioassayed late during the first stadium.

‡Ethanol was applied at a rate of 1 μl per insect as a control.

does not appear in the exudate from the *N. tabacum* control (Fig. 1).

Adjacent lanes of *Repandae* exudate were scraped in the R_f region of 0.7–0.8 and eluted. Spectrophotometric analysis showed that the concentration (w/w) of the alkaloid(s) in nicotine equivalents per mg of exudate is about 15%. When 50 μg of the alkaloid material, from either *N. repanda*, or *N. stocktonii*, was applied to first instar larvae, 100% mortality was observed within 24–48 hr (Table 2). In addition, when a lower amount (10 mg) of alkaloid from *N. stocktonii* was applied, 100% mortality was observed after 96 hr, while 87% mortality was observed after the same dose treatment of alkaloid from *N. nesophila*. Applications of 10- or 50-fold higher doses of nicotine base produced no mortality even after 96 hr (Table 2).

Fig. 1. TLC chromatogram of 1 mg of leaf exudate material from cultivated and wild species of *Nicotiana*. The plate has been stained with an alkaloid sensitive reagent (CNBr-PABA). (A) Nicotine base standard, (B) *N. tabacum*, (C) *N. repanda*, (D) *N. nesophila*, (E) *N. stocktonii*.

DISCUSSION

We have shown that species in the genus *Nicotiana*, section *Repandae*, possess an alkaloidal antibiosis component that is highly toxic to larvae of *M. sexta*, an insect which has been shown to be highly resistant to the toxic effects of nicotine. Second, the alkaloid content of the toxic, red-staining region on TLC is not nicotine, nornicotine or anabasine, or any other conventional nicotine alkaloid previously known from living *Nicotiana* tissue. Rather, it is a novel *N*-acylnornicotine found only in the *Repandae* section [24] and absent from the other 65 species [7, 24–27, Severson, R., personal communication]. Third, this insect has been shown to be highly resistant to the toxic effects of nicotine because of an efficient combination of nicotine detoxification and protective mechanisms found in no other examined insects [2, 17]. Therefore, this highly toxic, altered form of nicotine is not detoxifiable by the insect's nicotine detoxification mechanisms. Thus, these plant species have a form of nicotinoid antibiosis against which the specialized insect *M. sexta* has no suitable defence. It is interesting to note that at least one investigator has found that *M. sexta* is able to sequester atropine when fed on a diet of *Atropa belladona* L., indicating that this insect is able to tolerate plant toxins in addition to those found in its preferred host plant *N. tabacum* [28].

The conspicuous interaction that occurs between susceptible *Nicotiana* spp. and their specialized herbivore *M. sexta* is well established. The particular species in section *Repandae* also occur within the range of *M. sexta*. The section also occurs in the known range of another insect which has adapted to feeding on *N. tabacum*, the tobacco budworm (*Heliothis virescens*) [29, 30]. However, the *Repandae* species do not show resistance to this insect nor is the acylnornicotine in their exudate toxic to them, at least by several orders of magnitude [31]. This is also true for the fall armyworm *Spodoptera frugiperda* (Smith) [Jackson, M., personal communication]. Thus, one obvious interpretation of the data at hand is that the biosynthesis of *N*-acylnornicotine is selective toward *M. sexta* in its toxicity. It is pertinent that the biosynthesis of *N*-acylnornicotine arises as a further enzymatic addition to nornicotine [32] and nornicotine is found in all *Nicotiana* species. Either all members of the genus at one

Table 2. Cumulative per cent mortality of *M. sexta* larvae following application of TLC semipurified alkaloid from *Repandae* species

Treatment	Staining region from TLC	*n*	Amount alkaloid applied (μg)	Cumulative per cent mortality* 24	48	96†
N. nesophila						
	Red	30	10.0	60	77	87
	Red	15	1.0	33	33	33
	Remainder	10	All‡	0	0	0
N. stocktonii						
	Red	10	50.0	100	—	—
	Red	15	10.0	53	87	100
	Red	15	1.0	27	47	47
	Remainder	15	All‡	13	13	13
N. repanda						
	Red	5	50	80	100	—
	Remainder	5	All‡	0	0	0
Nicotine base	after TLC	22	115	6	6	6
Blank (EtOH)		35	1§	0	0	0
Nicotine base	(pure)	10	500	0	0	0

*Includes moribund larvae. Moribund larvae are larvae which are extensively paralysed but not yet dead.

†Non red-staining and nicotine base treatments caused significantly less mortality than that from treatment with material from the red-staining region (χ^2, $\alpha = 0.05$).

‡The regions which did not stain with the alkaloidal reagent were also bioassayed as a control. All refers to the fact that the material applied was obtained by pooling all eluted non-stained silica regions, on plates to which 1 mg was applied.

§Ethanol was applied at a rate of 1 μl per insect as a control.

time synthesized *N*-acylnornicotine, and then all those members around the world, except three in the section *Repandae*, lost the terminal enzymatic step, or acylation of the nornicotine substrate has developed only within the section *Repandae*. The latter interpretation is less complex and, following the law of parsimony, we favour it. Collectively, the data suggest that the *Manduca/Repandae* interaction should be investigated as a possible model for insect/plant coevolution, in which some species within *Nicotiana* may have successfully responded, evolutionarily, to the herbivores' defence against the original antibiosis factors which still culminate nicotinoid biosynthesis in related plant species.

In light of the voluminous literature on the mode of action of nicotine and its use as a pharmacological probe, our findings also have important implications for vertebrate and insect toxicology [3, 17, 34]. Much research has been performed on the nicotinoids as insecticides, but no new compounds have been forthcoming. Perhaps this new alkaloid, which selectively imparts such mortality to *M. sexta* and not to *H. virescens*, will open up new areas of applied investigation in nicotinoid toxicology.

EXPERIMENTAL

Extraction of crude exudate. Crude exudate from wild species in the section *Repandae* (*N. nesophila*, *N. repanda*, and *N. stocktonii*) was obtained by excising leaves and dipping them into acetonitrile for 6 sec. The resulting solution was dried under vacuum. The concentrated exudate was raised in EtOH, transferred to a 6 × 50 mm test tube and dried under N_2 for further use.

Separation of nicotinoid alkaloids. Concentrated exudate or pure nicotine base was dissolved in EtOH and spotted on silica TLC. The plates were developed in $CHCl_3$–MeOH (5:2). After drying, the plate, or lanes from it, was sprayed with a solution of 1% *p*-aminobenzoic acid (PABA), dried, and placed in CNBr vapor. The appropriate alkaloid region was eluted in EtOH, as were the nicotine spots from the standard. The remainder of each track which did not indicate any, or the appropriate, alkaloids was also eluted in a similar manner. The resulting samples were vortexed to insure thorough mixing and centrifuged. The supernatant was removed and transferred to 6 × 50 mm test tubes, and dried under mild heat and N_2.

Concentration of alkaloid. The concentration of alkaloid in exudate extracts was estimated spectrophotometrically. A UV absorbance scan on the TLC semipurified material in 0.1 N HCl produced a spectrum similar to that for nicotinoid-alkaloids (Fig. 2). Conveniently, there are no other compounds in *Repandae* exudate which give strong absorbance in the same region as nicotinoid alkaloids [27, 35]. Thus, it is valid to use the extinction coefficient of a nicotine chromatophore to estimate the concentration of alkaloid in the samples. Only 10% of the weight of material eluted from TLC was found to be alkaloid (in nicotine equivalents). In order to estimate the recovery of the toxic material from TLC, a known quantity of the alkaloid obtained from 1 mg of exudate on TLC was mixed with 1 mg of exudate from *N. tabacum* (which does not contain this alkaloid). Following reseparation and elution on TLC, an average of 30% of the toxic alkaloid material was recovered. Using these figures, we estimate the concentration (w/w) of the toxic alkaloid in 1 mg of *Repandae* exudate, in nicotine equivalents, to be approximately 15.0%. From these figures the amount applied to larvae in Table 2 was estimated.

Insects. Manduca sexta larvae were raised on an artificial wheat

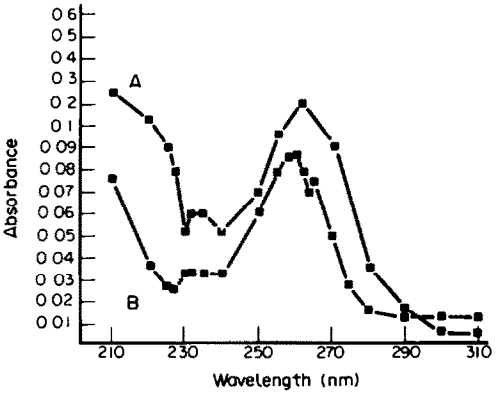

Fig. 2. UV absorbance scan of TLC purified alkaloidal material from section *Repandae* species (A) and nicotine base standards (B).

germ diet [36]. The larvae were taken from this culture at the appropriate stage and returned to the diet after treatment, as were the controls.

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REFERENCES

1. Gutherie, F. E., Ringler, R. L. and Bowery, T. G. (1957) *J. Econ. Entomol.* **50**, 821.
2. Self, L. S., Gutherie, F. E. and Hodgson, E. (1964) *Nature* **204**, 300.
3. Yamamoto, I., Kamimura, H. and Yamamoto, R. (1962) *Agric. Biol. Chem.* **26**, 709.
4. Campbell, F. L. and Sullivan, W. N. (1933) *J. Econ. Entomol.* **26**, 500.
5. East, E. M. (1935) *Genetics* **20**, 403.
6. Morris, C. E. (1984) *J. Exp. Zool.* **229**, 361.
7. Saitoh, F., Noma, M. and Kawashima, K. (1985) *Phytochemistry* **24**, 477.
8. Gerstel, D. V. (1976) in *Evolution of Crop Plants* (Simmonds, N. W., ed.) p. 273. Longman, London.
9. Yamamoto, I. (1965) in *Advances in Pest Control Research* (Metcalf, R. L., ed.) Vol. VI, p. 231. Interscience, New York.
10. Smith, H. H. and Abashian, P. V. (1963) *Am. J. Botany* **50**, 435.
11. Smith, H. H. and Mastrangelo, I. A. (1978) in *Plant Cells and Tissue Culture Principles and Application* (Larson, P. O., Paddock, E. F., Raghaven, V. and Sharp, W. R., eds). Ohio University Press, Columbus.
12. Schmeltz, I. (1971) in *Naturally Occurring Insecticides* (Jacobson, M. and Crosby, D. G., eds) p. 99. Marcel Dekker, New York.
13. Robinson, T. (1979) in *Herbivores: Their Interaction with Secondary Plant Metabolites* (Rosenthal, G. A. and Janzen, D. H., eds) p. 413. Academic Press, New York.
14. Thurston, R., Parr, J. C. and Smith, W. T. (1966) Proc. 4th Intern. Tob. Sci. Cong., Athens, p. 424.
15. Rothschild, M. (1972) in *Phytochemical Ecology* (Harborne, J. B., ed.) Academic Press. London.
16. Thurston, R. (1970) *J. Econ. Entomol.* **63**, 272.
17. Morris, C. E. (1983) *J. Insect Physiol.* **29**, 807.
18. Self, L. S., Gutherie, F. E. and Hodgson, E. (1964) *J. Insect Physiol.* **10**, 907.
19. Jones, D., Jones, G. A., Hagen, T. and Creech, E. (1985) *Entomol. Exp. Appl.* **38**, 157.
20. Schmuck, A. A. (1953) in *The Chemistry and Technology of Tobacco* (Gaurilov, N. I. ed.) Vol. III. Moscow.
21. Fejer-Kossey, O. (1967) *J. Chromatogr.* **31**, 592.
22. Tso, T. C. and Jeffrey, R. N. (1953) *Archiv. Biochem. Biophys.* **43**, 269.
23. Jeffrey, R. N. (1959) *Tob. Sci.* **3**, 89.
24. Severson, R. F., Arrendale, R. F., Snook, M. E. and Sisson, V. A. (1985) *Ga. J. Sci.* **43**, 21.
25. Miyano, M., Yasumatsu, N., Matsushita, H. and Nishida, K. (1981) *Agric. Biol. Chem.* **45**, 1029.
26. Bolt, A. J. N. (1972) *Phytochemistry* **11**, 2341.
27. Miyano, M., Matsushita, H., Yasumatsu, N. and Nichida, K. (1979) *Agric. Biol. Chem.* **43**, 1607.
28. Rothschild, M., Aplin, R., Baker, J. and Marsh, N. (1979) *Nature* **280**, 487.
29. Goodspeed, T. H. (1954) *The Genus* Nicotiana, Chronica Botanica, Waltham.
30. Holland, W. T. (1968) *The Moth Book, A Guide to the Moths of North America.* Dover, New York.
31. Jones, D., Huesing, J., Zador, E. and Heim, D. C. (1987) in *Molecular Entomology* (Law, J., ed.). Liss, New York. (in press).
32. Zador, E. and Jones, D. (1987) *Plant Physiol.* **82**, 479.
33. Yamamoto, I. (1965) in *Advances in Pest Control Research* (Metcalf, R. L., ed.) Vol. VI, p. 231. Interscience, New York.
34. Martineric, J., Izard, C. and Ramond, A. (1977) *Electroencephalogr. Clin. Neurophysiol.* **43**, 563.
35. Swain, M. L., Eisner, A., Woodward, C. F. and Brice, B. A. (1949) *J. Am. Chem. Soc.* **71**, 1341.
36. Yamamoto, R. T. (1969) *J. Econ. Entomol.* **62**, 1427.