Journal of Chemical Ecology, Vol. 14, No. 4, 1988

DAMAGE-INDUCED ALKALOIDS IN TOBACCO: Pot-Bound Plants Are Not Inducible

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(Received April 2, 1987; accepted June 10, 1987)

Abstract—Field-grown wild tobacco plants (*Nicotiana sylvestris*) were subjected to a defoliation regime designed to mimic the rate and amount of leaf mass removed by one tobacco hornworm per plant. Undamaged leaves on these plants undergo a dramatic (457% for leaf position 5, 410% for leaf position 8) increase in total leaf alkaloids compared to same-age and positioned control leaves on undamaged control plants. However, potted greenhouse-grown plants fail to exhibit the same damage-induced increase in alkaloid content. The greenhouse environment differs from the field environment in factors known to influence leaf alkaloid content, particularly soil N, P, K, near-UV radiation, and relative humidity. However, altering these environmental factors does not make potted plants able to increase their leaf alkaloid levels in response to defoliation. Transplanting plants into larger pots with more soil does allow the plants to respond to defoliation. Thirty days after transplanting, the plants are again unresponsive to damage, probably as a result of becoming "pot-bound." This result suggests a mechanism for the induction response, specifically that leaf damage triggers synthesis of these alkaloids in the roots, and offers a potentially valuable experimental tool for the study of induced-plant defenses in tobacco and other plants that synthesize alkaloids in their root tissues.

Key Words—*Nicotiana sylvestris*, leaf alkaloids, defoliation, induced defense, pot-binding, nicotine.

INTRODUCTION

The herbivore-induced increases in plant secondary metabolites have received much attention in the ecological literature (Rhoades, 1979; Schultz and Baldwin, 1982; Haukioja, 1982) and have lent support to the hypothesis that plant

0098-0331/88/0400-1113$06.00/0

secondary metabolites are not just "waste products" of plant metabolism, but, rather, a component of plants' evolved defense responses against herbivory and pathogen attack. These responses have received little attention from plant physiologists. The mechanisms of only two herbivore-induced defense responses are understood in any detail: the induction of proteinase inhibitors in tomato leaves (Ryan, 1983) and quinolizidine alkaloid in lupine leaves (Wink, 1983). Understanding the mechanistic details of the induction response would greatly augment research into the ecological significance and evolutionary origins of such plant defensive responses. The first step in a mechanistic quest is to reproduce the response in the laboratory under controlled conditions.

Members of the genus *Nicotiana* produce pyridine-containing alkaloids that are toxic to most herbivores (Gordon, 1961; Hassall, 1969; Schmetz, 1971) and even, if present in adequate concentrations, deleterious to insects that feed primarily on these alkaloid-containing plants. In artificial diet experiments, Parr and Thurston (1972) showed that tobacco hornworms—despite their ability to efficiently excrete ingested tobacco alkaloids (Self et al., 1964)—reared on diets containing 2% or higher concentrations of nicotine had significantly reduced survivorship and larval performance.

Decapitation—topping—at the onset of flowering is standard practice in the production of cultivated tobacco (*N. tabacum*); it increases the size, weight, and alkaloid content of leaves (Woetz, 1955). Although it has long been known that damage to the flowering top of a tobacco plant increases the total alkaloid content of the plant (Reuter, 1957), the possible ecological significance of this response, namely, that a plant may increase its leaf alkaloid content after damage as a defensive response to herbivore attack, has gone unnoticed.

Here I demonstrate substantial increases in the alkaloid content of undamaged leaves of field-grown wild tobacco plants suffering damage not to their flowering tops, or apical or lateral buds, but only to their fully expanded leaves. However, plants grown under normal greenhouse conditions are not responsive to damage. Unrestrained root growth appears to be necessary for the response to occur.

METHODS AND MATERIALS

Alkaloid Quantification. The four principal alkaloids of *Nicotiana* species (Saitoh et al., 1985)—nicotine, anabasine, nornicotine, and anatabine—were separated by isocratic high-pressure liquid chromatography; detected by absorbance at 254 nm; quantified with a reporting integrator using the techniques of Saunders and Blume (1981); and modified by the use of (−)-scopolamine hydrochloride as an internal standard. The retention times (minutes) of standard alkaloids were: nornicotine (11.05), anabasine (12.30), anatabine (13.99), sco-

polamine (20.68), and nicotine (23.35). All samples were injected twice and the values expressed as a percentage of dry weight leaf (mean ± SD).

Alkaloid Extraction. Fresh leaf samples, rather than air-dried samples, were used to determine alkaloid contents. Replicate extractions ($N = 10$ per group; mean = ± SD % alkaloid content) of a pooled homogeneous leaf sample extracted after 24 hr (1.243 ± 0.188), 48 hr (1.115 ± 0.531), and 72 hr (0.969 ± 0.515) of air drying at 50°C produced lower ($P < 0.0001$) and more variable ($P < 0.0005$) results than an analysis of fresh leaf material (1.924 ± 0.011). Fresh leaf samples were weighed to 0.2 mg, frozen in liquid N_2, ground to a fine powder with a glass rod, and extracted in a single extraction step in 40% methanol 0.1% N HCl (Saunders and Blume, 1981) containing the internal standard at a concentration of 50 μg/ml. Four sequential extractions of 10 replicate leaf samples revealed that 97.86% ± 0.42 of the total alkaloids was removed in the first extraction when the concentration of leaf material in the extraction solvent was 10 mg fresh wt/ml extraction solution. The alkaloid extraction and quantification technique proved to be both precise—30 samples of tobacco leaves containing a wide range of alkaloid concentrations (0.03% minimum, 2.46% maximum) extracted in duplicate had a mean percentage diffference between replicates of 0.04—and accurate—when 10 leaf samples were spiked with known amounts of nicotine, better than 95% of the alkaloid was detected.

Plant Cultivation and Leaf Sampling for Alkaloids. Seeds of *Nicotiana sylvestris* Spegazzini and Comes were originally obtained from the USDA Beltsville Agricultural Research Center, Beltsville, Maryland. Seeds from one greenhouse plant (thus plants were full sibs) were germinated in Cornell Mix A (Boodley and Sheldrake, 1977) under greenhouse conditions and either planted in a field plot at Cornell University or under greenhouse conditions in 18-cm fiber pots with 3.5 g Osmocote 14-14-14 fertilizer. The field plot was a roto-tilled old field consisting of four rows of 10 plants with 61 cm between plants and rows. The plot was neither fertilized nor watered and was manually weeded only during the first two weeks after planting. In order to minimize possible edge effects, only the two middle rows were used in this study. All plants were grown to the buttoning stage before experimentation began. The eighth fully expanded leaf from the top was designated the sample leaf and labeled with a twist-tie. The fifth leaf of each field-grown plant was also designated a sample leaf. Leaf samples were removed in a 1-cm band (Rosa, 1973) one half the leaf length from the top, across the leaf but excluding the midrib; leaf samples were then immediately weighed and extracted. A comparable band adjacent to the sample band was removed, weighed, dried at 50°C for 48 hr, and reweighed to obtain a percent dry weight calculation.

Experimental Treatments. Plants were grouped by height (±5 cm to the developing flower button) and randomly assigned to a treatment with 10 plants per treatment. Plants were damaged in order to simulate the feeding of one

Manduca sexta larva, a common lepidopteran pest of cultivated tobacco. During its larval life, a caterpillar will eat approximately 2100 cm^2 of leaf area, of which 90% will be consumed in the last larval instar (Madden, 1945). Greenhouse-grown plants are smaller at the buttoning stage than are field-grown plants. For field-grown plants, 2000 cm^2 of leaf area represents approximately 50% of the shoot weight. Thus, instead of removing the same amount of leaf area as in the field-defoliation treatment, the same proportion of leaf area was removed. Fully expanded leaves from positions 3 through 12, excluding positions 5 and 8 (the sample positions), were cut daily with scissors over an eight-day period. Cuttings was placed in labeled bags, air-dried at 50°C, and weighed. Plants assigned to a fertilizer treatment were watered daily with 100 ml/pot of a fertilizer solution consisting of 1 kg of Peter's 20-20-20 per 400 liters of water for eight days prior to the initiation of the damage regime. Plants assigned to a transplant treatment were removed from their pots and placed in 30-cm fiber pots containing 4.2 times the volume of Cornell Mix A contained in the 18-cm pots. Control plants in the transplant experiment were removed from their 18-cm pots and returned to these pots.

Statistical Treatment. Alkaloid values were N-scored (Ryan et al., 1982) and found to be normally distributed. Alkaloid values were compared with the Student's *t* test modified for unequal variances; values from field-grown plants were compared with a paired *t* test for between-treatment comparisons (Sokal and Rohlf, 1981). Percentages were arcsin-transformed.

RESULTS AND DISCUSSION

The alkaloid composition of leaves in positions 5 and 8 of *N. sylvestris* was dominated by a single compound: nicotine. The percent composition of the alkaloid profile of the 130 leaves sampled in this study was 90.3 $\pm$ 8.6% nicotine, 9.2 $\pm$ 4.2% nornicotine, 0.6 $\pm$ 0.5% anabasine, and 0.1 $\pm$ 0.1 anatabine, which is consistent with the previous work on this species (Saitoh et al., 1985). Nicotine was the only alkaloid significantly ($P < 0.05$) influenced by any of the treatments.

Field-grown plants subjected to the eight-day defoliation regime lost 50.13 $\pm$ 13.34% of their shoot mass and had more than four times the total alkaloid content in their undamaged sample leaves (Table 1). The alkaloid content of sample leaves higher on the stalk (position 5) was not significantly different ($P = 0.60$) from that of leaves on the lower stalk position (8) on undamaged plants, but tended to be higher on damaged plants ($P = 0.057$).

Greenhouse-grown plants subjected to the defoliation treatment lost 45.2 $\pm$ 7.2% of their shoot mass over the eight-day cutting period. However, unlike the experiment with field plants, no difference ($P = 0.28$, $DF = 19$) was found

TABLE 1. TOTAL ALKALOIDS OF SAMPLE LEAVES IN TWO LEAF POSITIONS FROM DAMAGED AND UNDAMAGED FIELD-GROWN WILD TOBACCO PLANTS

	% of dry wt sample leaf total alkaloid			
	N	Mean	SD	*P*
Leaf position 5				
Damaged plant	10	2.527	1.210	0.00001
Undamaged plant	10	0.553	0.308	
Leaf position 8				
Damaged plant	10	1.985	0.791	0.00001
Undamaged plant	10	0.484	0.163	

between the alkaloid contents of undamaged sample leaves from damaged (0.189 $\pm$ 0.053) and undamaged (0.196 $\pm$ 0.049) plants.

Soil nutrients, particularly B, Ca, and N, are known to substantially affect leaf nicotine contents (Tso, 1972). Deficiency in soil B and Ca increases leaf nicotine contents but also destroys the plants' apical buds (Tso, 1972). Because the apical buds of greenhouse-grown plants appeared healthy, an experiment was conducted with an N-P-K fertilization treatment. Fertilizer increased the alkaloid contents of both damaged (0.578 $\pm$ 0.135) and undamaged (0.595 $\pm$ 0.151) plants, but the damage had no effect on leaf alkaloid contents ($P = 0.80$, $DF = 19$).

The greenhouse environment differs from the field environment in that the glass of the greenhouse substantially decreases the amount of near-UV radiation and the intensity of visible light. Both alterations of the light environment are known to decrease leaf alkaloid concentration (Anderson and Kasperbauer, 1973). Plotted plants placed outdoors for 10 days prior to the defoliation treatment had elevated alkaloid levels, but sample leaves did not differ ($P = 0.33$, $DF = 19$) between damaged (0.584 $\pm$ 0.216) and undamaged (0.492 $\pm$ 0.194) plants.

Potted plants transplanted into larger pots with more soil prior to a one-day defoliation treatment (removing 35.0 $\pm$ 6.0% of the total leaf dry weight with a single cutting) had substantially more nicotine in undamaged sample leaves of undamaged plants (Table 2). The transfer of a plotted plant into a larger pot with more soil apparently enables the plant to increase its leaf alkaloid level after leaf damage. The ability to respond to damage wanes 30 days after the transfer to the larger pot.

The increase in leaf alkaloid content in cultivated tobacco (*N. tabacum*) after topping is largely due to increased nicotine synthesis in the roots (Mizusaki et al., 1973). However, decreased leaf nicotine degradation rates in the leaves

TABLE 2. TOTAL ALKALOIDS OF SAMPLE LEAVES FROM DAMAGED AND CONTROL GREENHOUSE-GROWN WILD TOBACCO PLANTS

	Days after transplant		% of dry wt sample leaf total alkaloid			
Treatment	Damaged	Sampled	Mean	SD	*N*	*P*
Damage	7	10	0.669	0.091	10	0.012
Control		10	0.385	0.126	10	
Damage	18	20	0.985	0.076	10	0.00001
Control		20	0.266	0.057	10	
Damage	30	33	0.143	0.040	10	0.22
Control		33	0.171	0.055	10	

(Yoshida, 1962) and leaf nicotine synthesis (Bose et al., 1956) may play a role in the topping-induced increase in leaf alkaloid levels. Most nicotine synthesis is thought to occur in young dividing root tips (Dewey et al., 1955). Although the hypothesis needs to be experimentally demonstrated, the increased leaf alkaloid content after leaf damage is likely due to a similar physiological process. Pot-bound plants, those growing in a pot for 30 days or more, may be unable to initiate new root growth after leaf damage, and be hence unable to synthesize more alkaloids. This hypothesis is consistent with the observation that soil depth is positively correlated with constitutive leaf nicotine concentrations (Wolf and Bates, 1964).

The observation that pot-bound plants are not responsive to leaf damage may prove to be an important experimental tool for demonstrating the ecological consequences of altered leaf chemistry on herbivorous insects. Herbivore damage has dramatic effects on leaf secondary chemistry, as well as on primary metabolites such as protein (Wagner and Evans, 1985) and sugars (Valentine et al., 1983). In some studies leaf damage apparently increased the nutritional quality of the leaves to herbivores (Williams and Myers, 1984). Hence a plant cultivation technique that allows for manipulation of a damage-induced alteration in a secondary metabolite, against a background of damage-induced alterations in primary metabolities, would be an exciting experimental tool in the study of induced plant defenses. This phenomenon may also be applicable to other plant alkaloids that are synthesized in the roots, such as hyoscyamine in *Datura*, *Atropa*, and *Hyoscyamus*; and betaines in *Beta* (Mothes, 1960).

Acknowledgments—This work was supported by a grant from the Cornell Biotechnology Program, which is sponsored by the New York State Science and Technology Foundation and a consortium of industries, and by a grant from the National Institutes of Health (grant AI 02908 to T. Eisner). T. Dandurand, N. Figero, C. Sanchirico, C. Sims, E. Wheeler, and J. Wiley are thanked for their technical assistance; G. Pittarelli for supplying seeds; and T. Eisner, D. Harvell, E. Wheeler, and P. Wimberger for helpful comments.

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