Leaf Extracts from Cytokinin-Overproducing Transgenic Plants Kill Sugarbeet Root Maggot (*Tetanops myopaeformis*) Larvae

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ABSTRACT

The sugarbeet root maggot (SBRM), *Tetanops myopaeformis* von Röder (Diptera: Otitidae), is the major insect pest of sugarbeet in the central and western United States and Canada and is capable of inflicting losses ranging from 10 to 100%. Currently, chemical insecticides are the only available measure for control of the maggot and a strong impetus exists for development of alternative control measures. Insecticidal leaf extracts prepared from *N. plumbaginifolia* plants transformed with the cytokinin biosynthesis gene *ipt* (PI-II-*ipt*) were tested on the sugarbeet root maggot larvae. Exposure of first instar maggots to a 1% suspension of the compounds in surface extracts of PI-II-*ipt* plants induced an almost immediate, repetitive twitching and thrashing movement that was not detected when the larvae were placed in similarly prepared extracts from

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control plants. After 24 hr, 68% of the larvae in the transgenic extract were twitching compared to none in the control extract. After 5 days, 92% (55 of 60) of the larvae were dead and 2 of the remaining 5 larvae were twitching. Similarly, a 0.1% suspension killed more than 80% of the larvae. No twitching or thrashing was observed in the control extract for up to 3 days and only 5% (2 of 40) of the larvae died. Normal movement of about half and all of the larvae exposed to the control extract and saline, respectively, was observed after 5 days. These results suggest that cytokinin-mediated insect resistance can potentially be exploited for development of effective control strategies for the sugarbeet root maggot.

The sugarbeet root maggot (SBRM), *Tetanops myopaeformis* von Röder (Diptera: Otitidae), is the major insect pest of sugarbeet in the central and western United States and Canada and is capable of inflicting losses ranging from 10 to 100% (Cooke 1993). Nearly half of the 1.4 million acres of sugarbeet grown annually are infested with root maggot. During late May and June, adult females lay as many as 200 eggs each around the base of sugarbeet seedlings. Following egg hatch, the developing larvae feed on tap and feeder roots inducing significant crop damage either by severing the taproots of seedlings or badly scarring the surface of larger roots (Figure 1). Due to the mobility of adult flies, cultural control practices, such as crop rotation, are difficult, and the existence of several weed species as alternate hosts hinders population control (Callenbach et al. 1957; Mahrt and Blickenstaff, 1979). Currently, insecticides are the only available measure for control of the maggot in infested fields (Yun 1986). However, the regulatory review mandated by the Food Quality Protection Act could have negative implications for continued registration of chemical insecticides effective against the root maggot as well as development of new conventional insecticides labeled for use on sugarbeet. Therefore, a strong impetus exists for development of alternative control measures.

The subject of this report is based on the use of plant-derived insecticidal compounds for pest control and on our reports that *Nicotiana plumbaginifolia* cv. Viviani plants transformed with the cytokinin biosynthesis gene *ipt* had elevated levels of cytokinins that were correlated
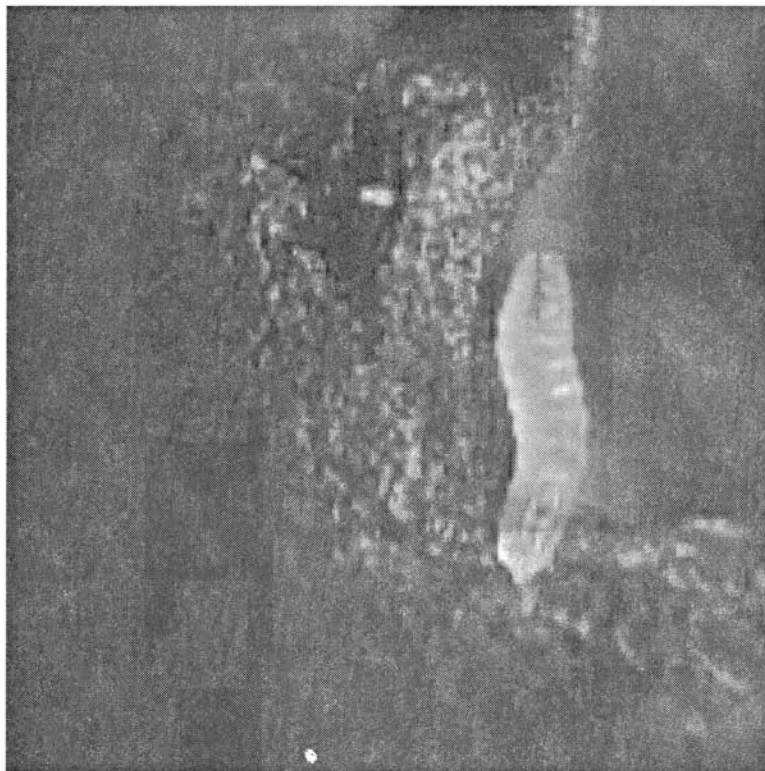
Figure 1. A first instar root maggot feeding on a taproot of a sugar beet seedling.

with an acquired insect resistance (Smigocki et al. 1993). Cytokinins are plant growth regulators and are known to modulate numerous physiological and biochemical processes associated with normal plant growth and development that also influence disease responses. A number of studies have correlated exogenous cytokinin applications with the subsequent accumulation of secondary metabolites, many of which have insecticidal properties (Decendit et al. 1992; Hallahan et al. 1992; Chilton 1997). In field trials using commercial formulations of natural cytokinins, an overall increase in yield was attributed in part to reduced insect populations and was correlated with an increase in the concentration of four secondary metabolites, all shown to be insecticidal (Hedin et al., 1988; Hedin and McCarty 1994). Due to economic considerations, cytokinin instability and uptake and metabolism issues, cytokinin action has been studied in transgenic plants that express the *ipt* gene (Smigocki

and Owens 1988; Martineau et al. 1994; Smigocki 1995; Gan and Amasino 1995). To specifically focus on effects of elevated cytokinin levels on insect resistance, the *ipt* gene was fused to a wound-inducible promoter (PI-II-*ipt*) for expression in transgenic plants (Johnson et al. 1989; Smigocki et al. 1993; Smigocki 1995). Cytokinin levels were about 70-fold higher in late-flowering transgenic plants than in untransformed controls and the transgenic plants were resistant to a number of insects (Smigocki et al. 1993). Tobacco hornworm (*Manduca sexta*) larvae consumed only 10% of the transgenic *ipt* leaves in comparison to 100% of the control leaves (Smigocki et al. 1993). Normal development and survival of newly hatched green peach aphid (*Myzus persicae*) nymphs were reduced by more than 60% on the transgenic leaves (Smigocki et al. 1993). Similar antifeedent and toxic effects were associated with cytokinin overproduction in transgenic *N. tabacum* cvs. Xanthi and MD609 (Smigocki et al. 2000). Host plant resistance was speculated to be correlated with the production or secretion of insecticidal secondary metabolites induced by the high cytokinin concentrations (Smigocki et al. 1993). Extracts prepared from leaves of the transgenic plants were demonstrated to be lethal to *M. sexta* larvae (Smigocki et al. 2000). Chemical analyses of the partially purified extracts revealed compounds suggestive of oxygen-containing aliphatic molecules in the molecular weight range of diterpenes (Smigocki, et al. 1997; Smigocki et al. 2000).

In this report, the effects of *N. plumbaginifolia* leaf surface extracts on first instar SBRM are summarized. An almost immediate twitching and thrashing behavioral response of the larvae to the transgenic extract prepared from PI-II-*ipt* plants was observed. More than 90% of the larvae died after 5 days of exposure to an aqueous suspension of the extract suggesting that cytokinin-mediated insect resistance can potentially be exploited for development of effective control strategies for the sugarbeet root maggot.

MATERIALS AND METHODS

Sugarbeet root maggot bioassay.

Sugarbeet root maggot, *T. myopaeformis*, eggs were collected from commercial sugarbeet fields near St. Thomas, ND (Pembina County) and stored at 4°C for up to 72 hr. Eggs were surface disinfected in 4% (v/v) commercial bleach (0.2% hypochlorite) for 5 min, washed in phosphate-buffered saline (10 mM Na_2HPO_4, 10 mM NaCl, pH 7.2; PBS) and sterile water, and placed on 0.3% (w/v) gelrite containing PBS and 1 mM $CaCl_2$, at 21 to 23°C for 3 days. Ten newly hatched first instars were transferred into a Costar 24-well culture plate in sterile 0.85% (w/v)

saline in a final volume of 150 µl per well. Plant extracts were resuspended in sterile saline at 2 or 20 mg/ml by vortexing (1 min) and sonicating (2 min) and added in equal volume (150 µl) to individual wells containing ten newly hatched larvae. Plates were incubated at 22±1°C in the dark and examined daily to determine behavioral response and mortality.

Mortality data were analyzed as a set of four 5 X 2 contingency tables. Each table included the number of larvae dead and alive for each of the five treatments (plant extract X concentration combinations) for days 2 to 5. Day one was not included in the analysis because no dead larvae were observed. Behavioral response (twitching) data were analyzed in the same way, except observations from day 1 were included. Mantel-Haenszel Chi-square tests were used to identify significant patterns of association (Stokes et al. 2000).

Source of leaf extracts.

Leaf extracts used for the sugarbeet root maggot bioassay were prepared from *N. plumbaginifolia* PI-II-*ipt* and control plants as previously described (Buta et al. 1993; Neal et al. 1994; Smigocki et al. 1997; Smigocki et al. 2000). Briefly, compounds on the surfaces of fully expanded leaves were solubilized with methylene dichloride, concentrated by rotary evaporation at 30°C, dried under a stream of N_2, and stored in the dark at 4°C. To confirm that all fresh extracts prepared from the leaves of the transgenic PI-II-*ipt* plants were insecticidal, they were bioassayed using tobacco hornworm (*Manduca sexta*) larvae (Carolina Biological Supply Company, Burlington, NC) as reported previously (Smigocki et al. 1993). Leaf disks excised from untransformed *Nicotiana* plants were coated with freshly resuspended, aqueous suspensions of the prepared extracts and placed on water-moistened filter paper with a single second instar hornworm.

RESULTS AND DISCUSSION

We tested the effects of insecticidal leaf extracts derived from *N. plumbaginifolia* plants transformed with the cytokinin biosynthesis gene *ipt* (Smigocki et al. 1993; 1997) on the most devastating insect pest of sugarbeet, the sugarbeet root maggot. Exposure of root maggot first instars to a 0.1 or 1% suspension of the compounds in surface extracts induced an almost immediate, repetitive twitching and thrashing movement that was not detected in the control extracts (Figure 2). After 24 hr, 68% of the larvae in the 1% suspension of the transgenic extract were twitching as compared to none in the control extract (Table 1). In general, twitching and thrashing was the first response of the larvae to
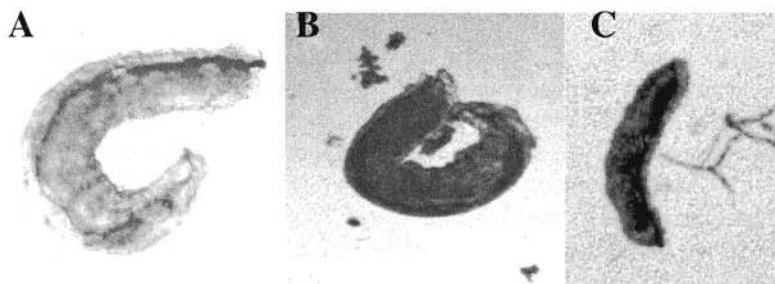
Figure 2. A) and B) Twitching and thrashing response of a first instar sugar beet root maggot exposed to a partially purified insecticidal fraction of transformed *N. plumbaginifolia* surface extract. C) Control larva in saline.

the transgenic extract that preceded the death of the larvae. After 4 days, more than two thirds (41 out of 60) of the larvae treated with the 1% suspension were dead and, of the remaining larvae, 62% (12 out of 19 that were still alive) were twitching (Table 1). After 5 days, 92% (55 of 60) of the larvae were dead and 2 of the remaining 5 were twitching. A ten-fold dilution of the surface extract suspension (0.1%) killed more than 80% of the larvae at 5 days and, of those that were still alive, 40% were twitching (Table 1). In the 1% control extract prepared from untransformed plants, no twitching was observed for up to 3 days, at which point only 5% (2 out of 40) of the larvae died and only 4% of the remaining larvae were twitching. At 5 days, almost half of the larvae in the control extract still exhibited synchronous contraction of circular and longitudinal muscle tissues associated with normal locomotion. Some sensitivity of the larvae to the extracts from the untransformed plants was anticipated as *Nicotiana* species has been shown to produce low concentrations of insecticidal secondary metabolites (Buta et al. 1993; Neal et al. 1994).

Biological insecticides and plants that have been genetically modified with insect-resistance genes are desirable alternatives for combating insect pests without the aid of externally applied chemicals. Natural plant defense mechanisms have been under-exploited, in part due to a lack of a thorough understanding of the numerous complex and integrated defense responses. Production of toxic compounds that are mainly products of secondary metabolic pathways is part of that response (Graham and Graham 1991). Several reports document the correlation between cytokinin application and accumulation of secondary metabolites

Table 1. Effects of surface extracts from transgenic *ipt* and control (untransformed) *N. plumbaginifolia* plants on first instar *T. myopaeformis*. Plates were scored for mortality and a twitching response at 1, 2, 3, 4 and 5 days.

		Mortality[1]					Twitching[2]				
				Day					Day		
		1	2	3	4	5	1	2	3	4	5
							%				
Extract	Concentration										
Transgenic *ipt*	1%	0	5	30	68	92	68	68	75	62	42
	0.1%	0	10	30	50	83	10	44	48	40	40
untransformed	1%	0	2	5	12	23	0	0	4	12	19
	0.1%	0	0	20	30	53	0	10	0	0	20
Saline		0	0	6	17	26	0	0	0	0	0
Chi-square		--	6.8	18.3*	45.3*	76.7*	73.4*	71.4*	70.3*	37.9*	11.5*

[1]At 1%, 60 or 40 larvae were used in transgenic *ipt* and the control, respectively. For saline treatments, 50 larvae were used. At 0.1%, 30 and 20 larvae were used for the *ipt* and control, respectively.

[2]Percent of live larvae that were twitching.

* Indicates association between frequency of mortality or twitching and treatment within a column is significant ($\forall = 0.05$), based on Mantel-Haenszel Chi-square test.

(Binns et al. 1987; Teutonico et al. 1991; Orr and Lynn 1992). Cytokinins applied to cotton fields increased yields and the increases were correlated with elevated levels of four insecticidal metabolites and reduced insect populations (Hedin and McCarty 1994).

Our studies also suggested the involvement of cytokinins in modulation of secondary metabolic pathways as most of the insecticidal activity was recovered in methylene dichloride surface extracts of transgenic *ipt* plants (Smigocki et al. 1997; Smigocki et al. 2000). Whether this beneficial effect of cytokinin will be applicable to other plant species and will contribute to a more universal effect of defense responses in plants is yet to be determined. The demonstration of resistance in both transgenic *N. plumbaginifolia* and *N. tabacum* PI-II-*ipt* plants supports that possibility (Smigocki et al. 1993; Smigocki et al. 2000). In addition, insects from three different orders, Lepidoptera (tobacco hornworm), Homoptera (green peach aphid) and Diptera (sugarbeet root maggot), were sensitive to the insecticidal activity (Smigocki et al. 1993; this study). Preliminary studies with two coleopteran insects, the alfalfa weevil and Colorado potato beetle, however, suggested that the larvae were not sensitive to the insecticidal compounds in the transgenic extracts (Smigocki and Elden, unpublished).

Purification of *N. plumbaginifolia* insecticidal extracts has not proceeded to where biological activity could be ascribed to any one (or more) of the compounds but partial chemical analyses yielded an active fraction that suggests the compounds are diterpenes (Smigocki et al. 1997; Smigocki et al. 2000). Terpenoid compounds play diverse functional roles in plants as attractants for pollinators and seed dispersers, competitive phytotoxins, antibiotics, and herbivore repellents (Chappel 1995; McGarvey and Croteau 1995). A diterpene-type compound would fit well with the numerous examples of natural products that are light sensitive and involved in insect resistance. Exposure of a few SBRM larvae to a suspension of the active fraction induced a similar twitching and thrashing behavioral response as was observed with the unfractionated extract (Figure 2A, B). Bioassays were not done because the yields of the active fraction were minute, and quantities needed for the bioassays far exceeded the recovered amount.

Although the pathways for the synthesis of secondary metabolites are complex and involve numerous enzymatic reactions, fortuitously, the pathways appear to be similar in all crop plants so strategies are being devised that will require only limited modifications for increased production of a potentially useful metabolite (Hallahan et al. 1992; Rhodes 1994; Chilton 1997). Identification of the active compound(s) in the *Nicotiana* surface extracts will help define how

cytokinins modulate their production, secretion or availability and lead to additional biotechnological approaches for environmentally friendly insect control.

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