The Accumulation of β-Ionone and 3-Hydroxy Esters of β-Ionone in Tobacco Immunized by Foliar Inoculation with Tobacco Mosaic Virus

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ABSTRACT

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β-Ionone and several 3-hydroxy esters of β-ionone have been reported to have antifungal activity. Stem injection with β-ionone mimics the effects of stem injection with *Peronospora tabacina* sporangiospores. Both induce systemic resistance to blue mold and accelerate the growth and flowering of the plant. Systemic resistance induced by foliar inoculation with tobacco mosaic virus is associated with increases in the endogenous levels of β-ionone, 3-hydroxy-β-ionone, 3-n-butyroyl-β-ionone, and 3-acetoyl-β-ionone in uninfected stem tissue. Further increases in 3-n-butyroyl-β-ionone and 3-acetoyl-β-ionone occurred in protected plants in response to challenge inoculation with *P. tabacina*.

Additional keywords: *Nicotiana tabacum*.

In 1972, Leppik et al (1) reported the isolation of a compound (quiesone) from blue mold-infected tobacco leaves that strongly inhibited germination of sporangia of *Peronospora tabacina* D. B. Adams, $ED_{50} = 0.0001$ ppm. This compound was identified as 3-isobutyroyl-β-ionone. β-Ionone was also reported to be an inhibitor ($ED_{50} = 0.15$ ppm) of spore germination. In addition, other short-chain fatty acid esters of 3-hydroxy-β-ionone, including the 3-n-butyroyl and 3-n-valeroyl esters, were inhibitory to germination of *P. tabacina* sporangiospores with activity at concentrations as low as 6 and 25 parts per trillion, respectively (3). Sporulation and growth (as well as sporangial germination) of *P. tabacina* and *Aspergillus* sp. have also been reported as inhibited by β-ionone (5,6,7). Leppik was unable to detect the ionone compounds in uninfected tobacco. They appeared only

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after infection and increased with the spread of the pathogen. Because *P. tabacina* is an obligate parasite, he had no direct evidence as to whether the compounds were of host or fungal origin.

Physiological changes associated with resistance, induced by prior stem injection with *P. tabacina* sporangiospores, include a 50- to 600-fold increase in endogenous β-ionone (4). Additionally, Salt et al (4) demonstrated that stem injection with β-ionone mimicked the effects of stem injection with *P. tabacina* sporangiospores in both induced resistance to blue mold and accelerated growth and flowering. Foliar spray with β-ionone was less effective. However, in field tests in Mexico, foliar spray with 3-n-butyroyl-β-ionone increased growth and gave greater than 90% protection against metalaxyl-tolerant strains of *P. tabacina* (S. Tuzun and J. Kuc, *personal communication*).

The above reports prompted us to further investigate the accumulation of endogenous β-ionone and some of its 3-hydroxy esters in tobacco plants immunized by foliar inoculation with tobacco mosaic virus (TMV). The absence of the fungus in the inducing treatment would allow the separation of fungal and plant origin for the production of the compounds.

MATERIALS AND METHODS

Tobacco plants. Burley tobacco (*Nicotiana tabacum* L.) cv. Ky 14 seeds were sown in Metro Mix 250 (W. R. Grace Co., Fogelsville, PA) in small trays. After 2 wk, groups of 12 seedlings were transferred to trays in the same mix. The seedlings were then fertilized daily with Peter's 15-16-17 fertilizer (1.5 gl^{-1}) at half the recommended rate. After another 2 wk, individual seedlings were transplanted into 15-cm pots in Metro 260. At the six-leaf stage, the plants were transferred to a greenhouse with a 14-h photoperiod (daylight supplemented with sodium lights) and temperature range of 25–33°C and fertilized three times a week at half the recommended rate. Plants remained in the greenhouse until challenged.

***P. tabacina* and TMV.** Isolate 79 of *P. tabacina*, obtained in Kentucky in 1979, was maintained by weekly transfers of sporangiospores on young Ky 14 tobacco plants. Leaves with sporulation were frozen and stored at −20°C between wet paper towels in sealed paper bags. Purified TMV in sterile distilled water was provided by J. Shaw, Department of Plant Pathology, University of Kentucky.

Inducing inoculation with TMV. Upper surfaces of three lower leaves of plants at the 9- to 10-leaf stage (at about 2.5 mo old) were dusted with Carborundum, and the dusted surfaces were gently rubbed with 25 $\mu g\ ml^{-1}$ of TMV in distilled water. Control plants were sham-inoculated with water and Carborundum. At least one fully expanded leaf of each plant was always left uninoculated. The leaves were immediately washed with water after inoculation.

Challenge inoculation with *P. tabacina*. Plants were challenged 10–12 days after induction with TMV. Sporangiospores on frozen leaves were gently brushed into distilled water. The resulting suspension was filtered through four layers of cheese cloth, and the sporangiospores were washed three times with distilled water and collected on 8.0-μm membranes. They were then resuspended in distilled water, and the concentration was adjusted to 1×10^4 spores per milliliter. Plants were moved from the greenhouse to a growth room 24 h before challenge. The growth room was illuminated with white fluorescent and incandescent light with a 14-h photoperiod at 23°C. The upper surfaces of leaves were sprayed with a uniform fine mist of inoculum. Plants were then covered with black plastic bags sprayed on the inside with water and kept in the dark for 16 h.

Evaluation of disease. Resistance to blue mold was evaluated 7 days after challenge by estimating the percentage of leaf area covered by chlorotic lesions and determining sporangiospore production per cm^2 of leaf area (4). After estimation of percent chlorosis of total leaf area, the plants were sprayed with water and kept in moistened black plastic bags for 16 h in the dark. Sporangiospores were harvested and counted with the aid of a hemocytometer. Five expanded leaves from the top were assessed for chlorosis and sporulation in all plants.

Chemical analyses of tobacco tissues. Chemical analyses were performed using stem tissues of plants sampled at the time of induction with TMV, time of challenge with *P. tabacina*, and 4 days after challenge. Volatile constituents from tobacco were obtained by an 8-h steam distillation and continuous solvent extraction of 50 g of frozen stem tissue in 500 ml water per 75 ml hexane in a Kontes (Vineland, NJ) No. K-523010 codistillation and extraction apparatus. The hexane extracts were concentrated to dryness under reduced pressure, resuspended in 1 ml hexane, and analyzed by gas chromatography (GC). Appropriate standard chemical compounds dissolved in hexane were similarly analyzed. Gas chromatography was performed on samples of 0.5–1.0 μl (50:1 split injection) with a Perkin Elmer (Norwalk, CT) Sigma 2000 instrument equipped with an Alltech (Avondale, PA) OV 225 capillary column (10 m in length × 0.53 mm in internal diameter) 1.2-μm film. Operating conditions were: temperature programming, 140–180°C at 2°C min^{-1} and hold 5 min; carrier, N_2; flame ionization detection. Identification of unknown peaks was performed by comparison of retention times with those of standards and by coelution of samples with standards.

TABLE 1. Systemic protection against disease caused by *Peronospora tabacina* by foliar inoculation with tobacco mosaic virus (TMV)[a]

Treatment	Disease severity (0–4 scale)	Sporulation (spores/cm^2 leaf tissue)
TMV induced	0.26 ± 0.45	0
Control	3.87 ± 0.39	1.7×10^4

[a]Disease severity based on percent chlorosis of total leaf area (0 = no chlorosis, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%). Numbers are the mean of six experiments, 10 plants per treatment. The five expanded leaves from the top were evaluated for disease severity and sporulation.

RESULTS

Effects of induction with TMV against blue mold. Inoculation of three lower leaves of 2- to 3-mo-old tobacco plants with TMV

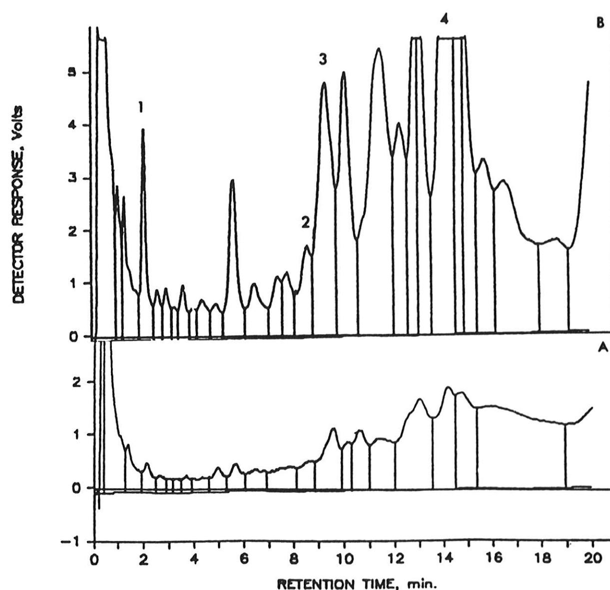

Fig. 1. Gas chromatograms of steam distillates from tobacco stem tissues at the time of challenge. **A**, stem tissue above mock-inoculated leaves 11 days after mock inoculation with distilled water, **B**, stem tissue above inoculated leaves from TMV-induced plants 11 days after inoculation with TMV. (1) βionone, (2) 3-hydroxy-β-ionone, (3) 3-acetoyl-β-ionone, (4) 3-n-butyroyl-β-ionone.

TABLE 2. The effects of induced systemic resistance with tobacco mosaic virus and challenge with *Peronospora tabacina* on levels of β-ionone, 3-hydroxy-β-ionone, and the 3-hydroxy esters of β-ionone[a]

	Compound (ng/g fresh weight)			
	Control[b]		Immunized[c]	
	0 DAC	4 DAC	0 DAC	4 DAC
β-ionone	12.8 ± 4.5	121.9 ± 19.1	96.5 ± 12.3	150.4 ± 20.2
3-hydroxyl-β-ionone	28.4 ± 6.8	176.2 ± 26.6	131.5 ± 21.4	185.7 ± 10.3
3-acetoyl-β-ionone	55.8 ± 10.3	477.3 ± 31.2	361.1 ± 28.4	224.8 ± 9.4
3-n-butyroyl-β-ionone	ND	2161.3 ± 137.2	219.6 ± 38.4	233.4 ± 29.2

[a]The data are representative of three experiments with four plants per time point and two replications per plant. DAC = days after challenge; ND = not detected.
[b]Tissue from plants not immunized by foliar inoculation with TMV.
[c]Tissue from plants immunized by foliar inoculation with TMV. 12 days before challenge, 96% protection was observed after subsequent challenge with *P. tabacina*, as compared to controls.

induced 95–99% protection of upper leaves against blue mold as demonstrated by fewer, more restricted chlorotic lesions and reduced sporulation (Table 1). On protected plants, *P. tabacina* often caused small chlorotic flecks on the upper surfaces of leaves, which appeared 2–3 days after challenge and remained very small. Sporulation of the fungus was not evident on systemically induced plants. Diffuse chlorotic lesions developed 3–4 days following challenge on control plants. On control plants, the lesions continued to enlarge throughout the test period, and infected leaves began to collapse on the seventh day.

Chemical analyses of steam distillates from tobacco tissues. Comparison of GC analyses of steam distillates of stem tissues from immunized and control tobacco plants revealed many qualitative and quantitative differences. The identities of many of the compounds found by GC are still under investigation. Several major peaks are detected in the stems of the immunized plants; these peaks are barely detectable or absent in the stems of control plants at the time of challenge (Fig. 1). One peak, at 2.07 min retention time, was identified as β-ionone by comparison with GC retention time and coelution with authentic β-ionone. Additionally, a small peak at 8.67 min and two larger peaks at 9.47 min and 14.17 min were identified as 3-hydroxy-β-ionone, 3-acetoyl-β-ionone, and 3-n-butyroyl-β-ionone, respectively. These peaks and several others are also detected in uninfected, green stem tissue of plants protected by stem injection with *P. tabacina* sporangiospores (data not shown). These peaks are absent or negligible in control plants at the time of challenge. By 4 days after challenge, as symptom expression begins, significant increases in 3-acetoyl-β-ionone, 3-n-butyroyl-β-ionone, and β-ionone occur in control plants as compared to control plants at the time of challenge (Table 2).

DISCUSSION

β-ionone and several of its 3-hydroxy esters are present in stem tissue of tobacco plants immunized by foliar inoculation with TMV. Although not excluding the possibility that *P. tabacina* can produce ionone-related compounds, the data clearly establish the plant's ability to synthesize ionone compounds in response to infection, both after induction and after challenge. This answers Leppik's concern (1) that the fungus was responsible for the increases in ionone compounds. The levels of β-ionone, 3-hydroxy-β-ionone, and 3-acetoyl-β-ionone in TMV-induced plants before challenge are often comparable to the control plants at 4 days after challenge when symptom expression has begun. This head start in induced plants may be sufficient to control pathogen development. The increases in β-ionone, 3-acetoyl-β-ionone, and 3-n-butyroyl-β-ionone in control plants by 4 days after challenge may be a response to the massive infection occurring in nonimmunized tissues at the time. Levels of resistance mechanisms are often higher in control plants as compared to immunized plants by the time symptom expression has occurred. Levels at the time of challenge and early in the infection process appear more important for controlling spread of the disease. Large increases after induction with TMV and challenge with *P. tabacina*, in a compound tentatively identified by thin-layer chromatography and GC as 3-isobutyroyl-β-ionone (retention time 11.07), were also detected. Identification of this compound is not confirmed, but the presence of the compound would be consistent with the report by Leppick (1).

Ionone compounds alone, however, may not be sufficient to restrict pathogen development. Induced systemic resistance is commonly seen as a general response to invasion and not a pathogen-specific response. Induction of systemic resistance in tobacco by inoculation with TMV results in induction of multiple responses, including increases in PR-proteins, chitinases, and β-1,3-glucanases (2,8,9). β-Ionone and its 3-hydroxy esters may be yet another component of this response. To better determine the role of the ionone compounds in resistance, the relative levels and temporal parameters of the ionones accumulating in leaf tissue (where infection occurs) and stem tissue must be assessed. β-Ionone, 3-hydroxy-β-ionone, and various 3-hydroxy esters may serve as precursors to more potent antifungal agents. The high levels of 3-n-butyroyl-β-ionone found in stems of infected plants and its high level of antifungal activity would suggest that 3-n-butyroyl-β-ionone may be an active compound for resistance. The association of the 3-hydroxy esters of β-ionone with induced resistance is evident, and investigation into the role of the esters in resistance is continuing.

LITERATURE CITED

1. Leppik, R. A., Holland, D. W., and Bottemley, W. 1972. Quiesone: An inhibitor of germination of *Peronospora tabacina*. Phytochemistry 11:2055-2063.
2. Pan, S. Q., Ye, X. S., and Kuc, J. 1989. Chitinase isozyme pattern and its coordinated induction with β-1,3-glucanase in tobacco plants immunized by *Peronospora tabacina* and tobacco mosaic virus. (Abstr.) Phytopathology 80:1024.
3. Salt, S., Reuveni, M., and Kuc, J. 1988. Inhibition of *Peronospora tabacina* (blue mold of tobacco) and related plant pathogens in vitro by esters of 3(R)-hydroxy-β-ionone. Page 22 in: Tob. Chem. Conf., 42nd. Proc. Am. Chem. Soc.
4. Salt, S. D., Tuzun, S. and Kuc, J. 1986. Effects of beta-ionone and abscisic acid on the growth of tobacco and resistance to blue mold. Physiol. Mol. Plant Pathol. 28:287-297.
5. Schiltz, P. 1974. Action inhibitrice de la β-ionone au cours du developpement de *Peronospora tabacina*. Ann. Tab. 11:207-216.
6. Wei, C. I., Tan, H., Fernando, S. Y., and Ko, N. J. 1986. Inhibitory effect of beta-ionone on growth and aflatoxin production by *Aspergillus parasiticus* on peanuts. J. Food Prot. 47:515-518.
7. Wilson, D. M., Gueldner, R. C., McKinney, J. K., Lievsay, R. H., Evans, B. D., and Hill, R. A. 1981. Effect of β-ionone on *Aspergillus flavus* and *Aspergillus parasiticus* growth, sporulation, morphology and aflatoxin production. J. Am. Oil Chem. Soc. 58:959-961.
8. Ye, X. S., Avdiushko, S., Pan, S. Q., Jarlfors, U., Tuzun, S., and Kuc, J. 1990. Cytological and biochemical changes of cell wall as related to systemic resistance to blue mold (*Peronospora tabacina*) induced by TMV in tobacco. (Abstr.) Phytopathology 80:1023-1024.
9. Ye, X. S., Pan, S. Q., and Kuc, J. 1989. Pathogenesis-related proteins and systemic resistance to blue mould and tobacco mosaic virus induced by tobacco mosaic virus, *Peronospora tabacina* and aspirin. Physiol. Mol. Plant Pathol. 35:161-175.