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Influence of Thaxtomins in Different Combinations and Concentrations on Growth of Micropropagated Potato Shoot Cultures

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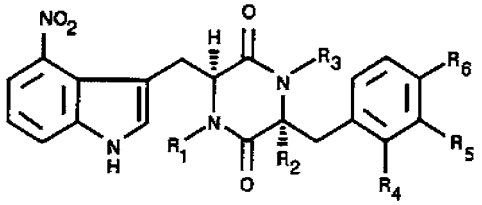
Plant-pathogenic *Streptomyces* species produce a variety of different phytotoxic 4-nitroindol-3-yl-containing 2,5-dioxopiperazines (thaxtomins) that induce scab symptoms on potato tubers (*Solanum tuberosum*). The possible mutual synergistic or antagonistic effects of thaxtomins are unknown. Modified methodology using column chromatography allowed the purification of thaxtomin A in large quantities (27 mg, HPLC purity of 97%). Thaxtomin A ortho isomer, thaxtomin B, and C-14 deoxythaxtomin B (thaxtomin D) were also purified. All four compounds induced similar symptoms of reduced root and shoot growth, root swelling (10−200 ppb), or necrosis (200−1000 ppb) on micropropagated in vitro cultures of potato. The scab-resistant potato cvs. Sabina and Nicola were more tolerant to thaxtomins than was the scab-susceptible cv. Matilda. Thaxtomins applied in combinations showed additive effects but no synergism, whereas thaxtomins A and B displayed antagonism with thaxtomin A ortho isomer.

KEYWORDS: Thaxtomin; phytotoxin; *Streptomyces scabies*; *Streptomyces turgidiscabies*; potato; *Solanum tuberosum*; HPLC; common scab

INTRODUCTION

Thaxtomins, a group of phytotoxins characterized as 4-nitroindol-3-yl-containing 2,5-dioxopiperazines, are produced by plant-pathogenic bacteria of the genus *Streptomyces* (*1*). The 4-nitroindole moiety of thaxtomins is unique among microbial compounds and essential for the physiological effects in symptom induction in plants (*2*−*4*). Thaxtomin A (**Figure 1**) is the predominant phytotoxin and major virulence factor produced by *S. scabies* (Thaxter) Lambert & Loria, *S. turgidiscabies* Miyajima, Tanaka, Takeuchi & Kuninaga, and *S. acidiscabies* Lambert & Loria (*1*, *5*−*9*). These bacteria are causal agents of common scab on potato tubers (*Solanum tuberosum* L.), a disease that causes yield losses in potato production worldwide (*10*−*13*). Application of purified thaxtomin A (M^+ 438.1708; *14*) on immature potato tubers causes similar superficial necrosis as observed following inoculation with the thaxtomin A-producing bacteria (*2*, *14*).



Compound	R_1	R_2	R_3	R_4	R_5	R_6
I Thaxtomin A (1)	Me	OH	Me	H	OH	H
II Thaxtomin A *o*-isomer (2)	Me	OH	Me	OH	H	H
III Thaxtomin B (4)	Me	OH	Me	H	H	H
IV C-14 deoxy thaxtomin B (5)	Me	H	Me	H	H	H

Figure 1. Structural formulas of the four thaxtomins included in this study. Compound numbers in parentheses are those used by King et al. (*4*).

The physiological effects of thaxtomins differ depending on concentration. Very low concentrations [0.05−1.0 μM, or 0.02−0.4 parts per billion (ppb)] are sufficient to cause a great increase in cell volume in hypocotyls of onion (*Allium cepa* L.) and

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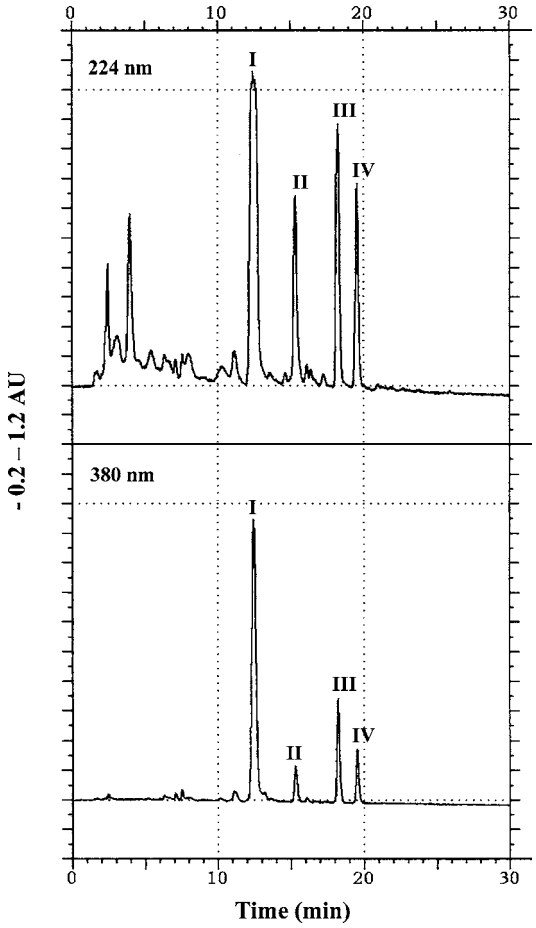

Figure 2. HPLC analysis of oatmeal broth inoculated with *Streptomyces* spp. causing potato common scab. Peaks were subsequently identified as **I**, thaxtomin A; **II**, thaxtomin A ortho isomer; **III**, thaxtomin B; and **IV**, C-14 deoxythaxtomin B.

radish seedlings (*Raphanus sativus* L.) and in tobacco suspension cells (*Nicotiana tabacum* L.) (*15*). Low concentrations of purified thaxtomin A (4−10 ppb) cause stunting and hypertrophy of roots and shoots of radish seedlings, whereas higher concentrations (20−40 ppb) cause tissue necrosis (*16*, *17*). The mode of action of thaxtomin A in plant cells is not fully known, but the symptoms resemble those caused by inhibitors of cellulose biosynthesis (*3*). Thus, symptoms may be associated with altered deposition or composition of plant cell wall due to interference with wall integrity and progression of cells through cytokinesis (*15*). Further evidence that thaxtomins inhibit cellulose biosynthesis was reported by Scheible et al. (*18*).

Besides thaxtomin A, more than 10 additional, related compounds have been isolated and characterized from bacterial cultures grown in different culture media or synthetic medium containing suberin (*3*, *4*, *7*, *9*, *19*−*21*). The amounts produced are dependent on culture media and bacterial strain and appear to be correlated with the level of virulence (*9*, *20*). Thaxtomins can be extracted from culture media using chloroform or ethyl acetate and fractionated by silica gel thin-layer chromatography (TLC) or reversed-phase TLC (*7*, *9*, *14*−*16*, *19*−*23*). The method allows only relatively small quantities of purified substances to be obtained. Protocols yielding larger amounts of thaxtomin for biological experiments would be useful.

There is little quantitative information on the biological effects of thaxtomins other than thaxtomin A. King et al. (*3*) compared the herbicidal activities of thaxtomin A, thaxtomin B, thaxtomin C, thaxtomin A ortho isomer (*o*-isomer), thaxtomin A para isomer, hydroxythaxtomin A, hydroxythaxtomin C, and des-*N*-methylthaxtomin C on several dicot and monocot species and found thaxtomin A to be physiologically the most active compound. It reduced the growth of *Arabidopsis thaliana* Holl. & Heynh. (dicot) and bentgrass (*Agrostis palustris* Huds.; monocot) by 50% (I_{50}) at the concentration of 10−25 ppb, respectively, whereas up to 400 and 600 ppb, respectively, of other thaxtomins were required for similar growth reduction. All compounds tested caused similar symptoms including stunting and root tip puffing (*3*).

Table 1. Characteristics of Isolated Thaxtomins Produced by *Streptomyces* Species in Oatmeal Broth

compound[a]	yield (μg mL^{-1} of growth medium)	HPLC purity (224 nm) (%)	mol wt
I: thaxtomin A (**1**)	5	97	438
II: thaxtomin A ortho isomer (**2**)	9×10^{-2}	62	438
III: thaxtomin B (**4**)	3×10^{-1}	94	422
IV: C-14 deoxythaxtomin B (**5**)	7×10^{-2}	97	406

[a] Numbers in parentheses indicate compound numbers used in King et al. (*4*).

Different thaxtomins are produced and accumulated at the same time during bacterial growth and infection. Some of them appear to be intermediates in the biosynthetic pathway of thaxtomin A (*6*, *19*). However, no study seems to have addressed their mutual putative additive, synergistic, or antagonistic effects on induction of the physiological effects. Therefore, the effects of four thaxtomins applied alone or in combinations were assessed and compared on potato growth in vitro. Furthermore, the possible potato genotype-specific differences were studied using three potato cultivars, of which one is sensitive to common scab and the two others express relatively high levels of resistance in the field. Methods for purification of thaxtomins in a larger scale were developed to facilitate the studies.

MATERIALS AND METHODS

Production of Thaxtomins. *S. scabies*, strain 364, and *S. turgidiscabies*, strain 300, were isolated from scab lesions of field-grown potato tubers in Finland (*8*). Oatmeal broth (OMB) was prepared by boiling 33 g of oatmeal in 1000 mL of distilled water for 20 min, filtering through muslin, diluting to 1000 mL, and adding the trace elements (0.1 μg L^{-1} $FeSO_4$, 0.1 μg L^{-1} $MnCl_2 \cdot 4H_2O$, and 0.1 μg L^{-1} $ZnSO_4 \cdot 7H_2O$). Seventeen flasks (1 L) with 300 mL of OMB were inoculated with sporal suspension of *S. scabies* or *S. turgidiscabies* grown on potato dextrose agar (PDA) (Difco, Becton Dicinson, Sparks, MO) to obtain 5 L of bacterial culture for thaxtomin purification. Cultures were incubated in a rotary shaker (180 rpm) at 28 °C for 2 weeks and centrifuged at 13000 rpm for 30 min (Sorvall RC5C centrifuge, Sorvall Instruments, Wilmington, DE). The supernatants were collected, pooled, and stored at −20 °C until thaxtomin extraction with an equal volume of ethyl acetate. The solvent phase containing the thaxtomins was evaporated to dryness under vacuum using a rotary evaporator (Büchi Rotavapor R-114, Büchi Labortechnik AG, Flawil, Switzerland) and stored under vacuum in the dark at 5 °C.

Column Chromatography (CC). Extracts (~2 g) containing thaxtomins were dissolved in ethyl acetate, combined, mixed with 4 g of silica gel, and, after drying, separated on a silica gel column (1600 g of the adsorbent, particle size = 0.2−0.5 mm, length = 1.2 m, 6 cm i.d., dead volume = 1.1 L). Elution of fractions was performed by chloroform/methanol with increasing methanol content: 0.2 L (1% MeOH), 1.5 L (2%), 0.5 L (3%), 0.5 L (4%), 1.5 L (5%), and 1.0 L (6%). Four yellow bands were visible in the main fractions and collected in 10 mL tubes. The occurrence of thaxtomins was confirmed by TLC (*21*) and HPLC, and the fractions with similar peak profiles were combined. The main constituent (thaxtomin A) in these fractions was crystallized from methanol.

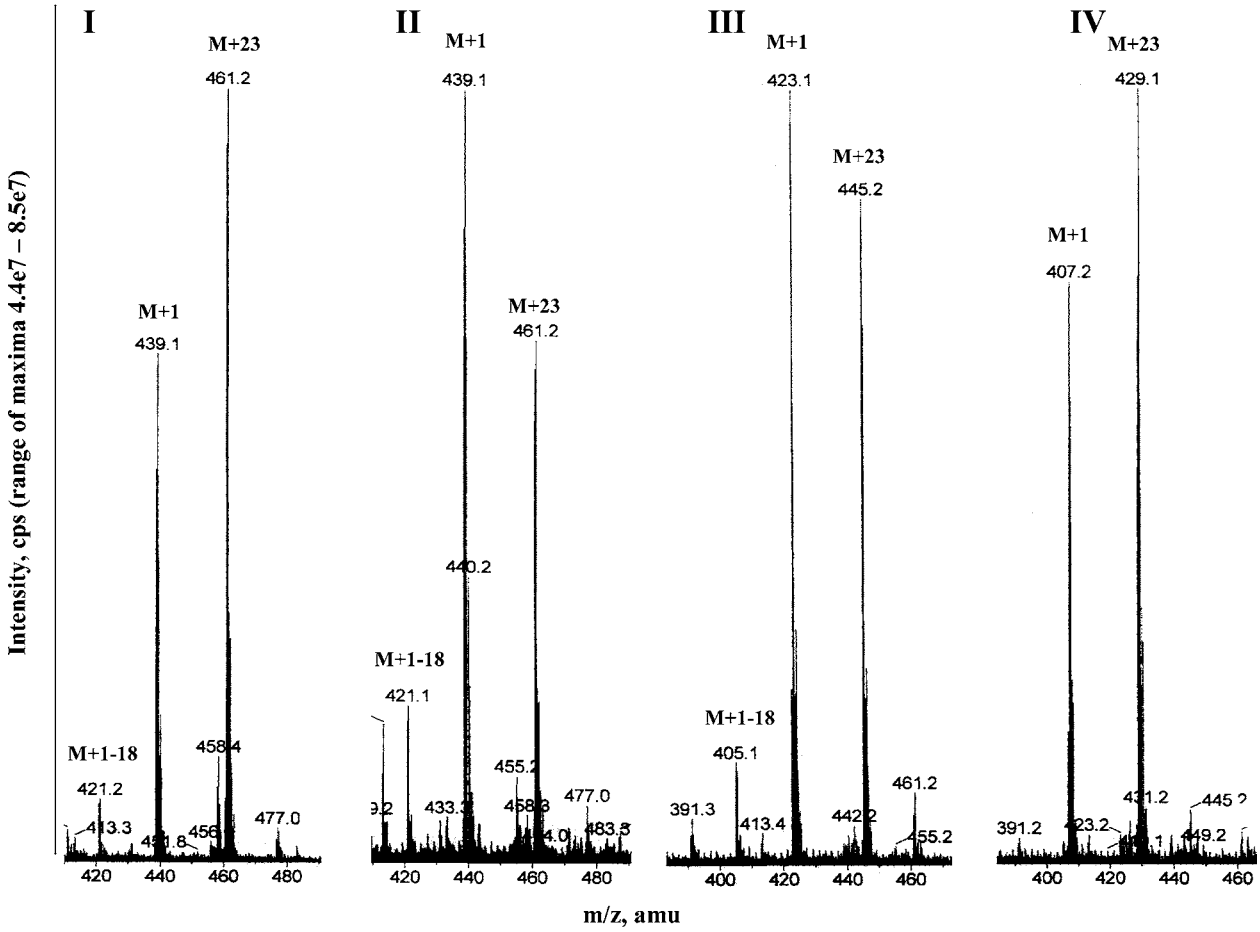

Figure 3. LC-MS fragmentation (positive ion mode) of isolated thaxtomins: **I**, thaxtomin A; **II**, thaxtomin A ortho isomer; **III**, thaxtomin B; **IV**, C-14 deoxythaxtomin B; M + 1, molecular ion; M + 1 + 22, sodium adduct; M + 1 − 18, loss of water.

Purification of the Fractions. Three minor thaxtomins were further purified by semipreparative HPLC using a Waters M45 chromatograph (Millipore Waters, Milford, MA) with a μBondapack C_{18} column (10 μm particle size, 7.8 × 300 mm). The compounds were eluted at 13, 18, and 20 min using 40% (v/v) acetonitrile at a flow rate of 2 mL min^{-1} (UV monitoring at 380 nm). The compounds were re-collected, acetonitrile was evaporated, and the thaxtomins were extracted from the aqueous phase using ethyl acetate. After evaporation, the resulting materials were stored in the dark at 5 °C.

HPLC Analysis. Thaxtomin composition of oatmeal broth and purity of isolated compounds were analyzed on a Waters 600E HPLC instrument (Millipore Waters) equipped with a Waters 991 photodiode array detector and using an Agilent Hypersil BDS C_{18} column (5 μm particle size, 4.6 × 150 mm). The eluting system was a 20−50% acetonitrile gradient over 20 min at a flow rate of 0.8 mL min^{-1}. The compounds were monitored at 240 and 380 nm, and the retention data and UV spectra of minor thaxtomins were compared with those of thaxtomin A. For the plant assays, the concentrations of the minor thaxtomins were determined by relating the peak areas measured at 380 nm to that of thaxtomin A obtained by using standard solution of 0.6 mg mL^{-1}. The concentrations of the minor thaxtomins stored as aqueous solutions were determined again after the experiments to confirm that they had remained constant.

Identification of Compounds. The molecular masses of the four isolated thaxtomins were obtained by liquid chromatography−mass spectrometry (LC-MS) using a Perkin-Elmer Sciex API 3000 triple-quadrupole tandem mass spectrometer (Sciex, Concord, ON, Canada) equipped with a turbo spray interface. The spectra were recorded in positive ion mode over the mass range of *m*/*z* 100−600.

Bioassays. Crystallized thaxtomin A was dissolved in ethanol (1.0 mg mL^{-1}, 99%). Stock solutions (10 μg mL^{-1}; 10000 ppb) of all four thaxtomins were made in sterile distilled water (SDW) and stored at 5 °C in the dark. These were further diluted 5- and 10-fold in SDW, and aliquots (0.3 mL) were added to sterile, cooled Murashige and Skoog (*24*) (MS) medium [MS powder (M0222, Duchefa Biochemie B.V., Haarlem, The Netherlands) 4.4 g L^{-1}, saccharose 30 g L^{-1}, and agar 8 g L^{-1}] (2.7 mL) that was dispensed in glass tubes. The final concentrations of the thaxtomins in the growth medium were 10, 20, 100, 200, and 1000 ppb. In the control treatments, SDW was added to the MS medium instead of thaxtomins.

Pathogen-free in vitro shoots of potato cvs. Matilda, Sabina, and Nicola were obtained from the Finnish Seed Potato Center, Tyrnävä, Finland. They were micropropagated on MS medium. Two-axillary-bud cuttings were transferred onto media amended with the different concentrations of the thaxtomins. Three cuttings were grown per cultivar and treatment at 20 °C under a 16 h photoperiod for 3 weeks. At the end of the experiment, shoot length was measured and the development of necrosis and formation of roots observed. Thaxtomins were tested also in combinations to determine their possible additive, synergistic, or antagonistic effects on growth of potato cvs. Matilda and Sabina. Here, each thaxtomin was tested in two concentrations in which growth reduction was <50%, as compared to untreated controls. All experiments were done twice.

Data Analysis. The data on shoot lengths were tested by analysis of variance. The least significant difference (LSD) of means was determined by the *t* test. The I_{50} value (thaxtomin concentration that reduced the shoot growth by 50%) was calculated by regression analysis of the dosage response curve for each thaxtomin on the three cultivars.

Synergistic or antagonistic interactions between different thaxtomins were tested by Limpel's formula: $E_e = x + y - (xy/100)$ (*25*). E_e is the expected effect from additive responses of two compounds, and x and y are the percentages of inhibition relative to each compound used alone. However, in this study, we used the symbol I_e (expected inhibition) instead of E_e. Synergism is indicated when the observed inhibition (I_o) for the combination of two thaxtomins is significantly greater than I_e. On the other hand, an I_o significantly smaller than I_e

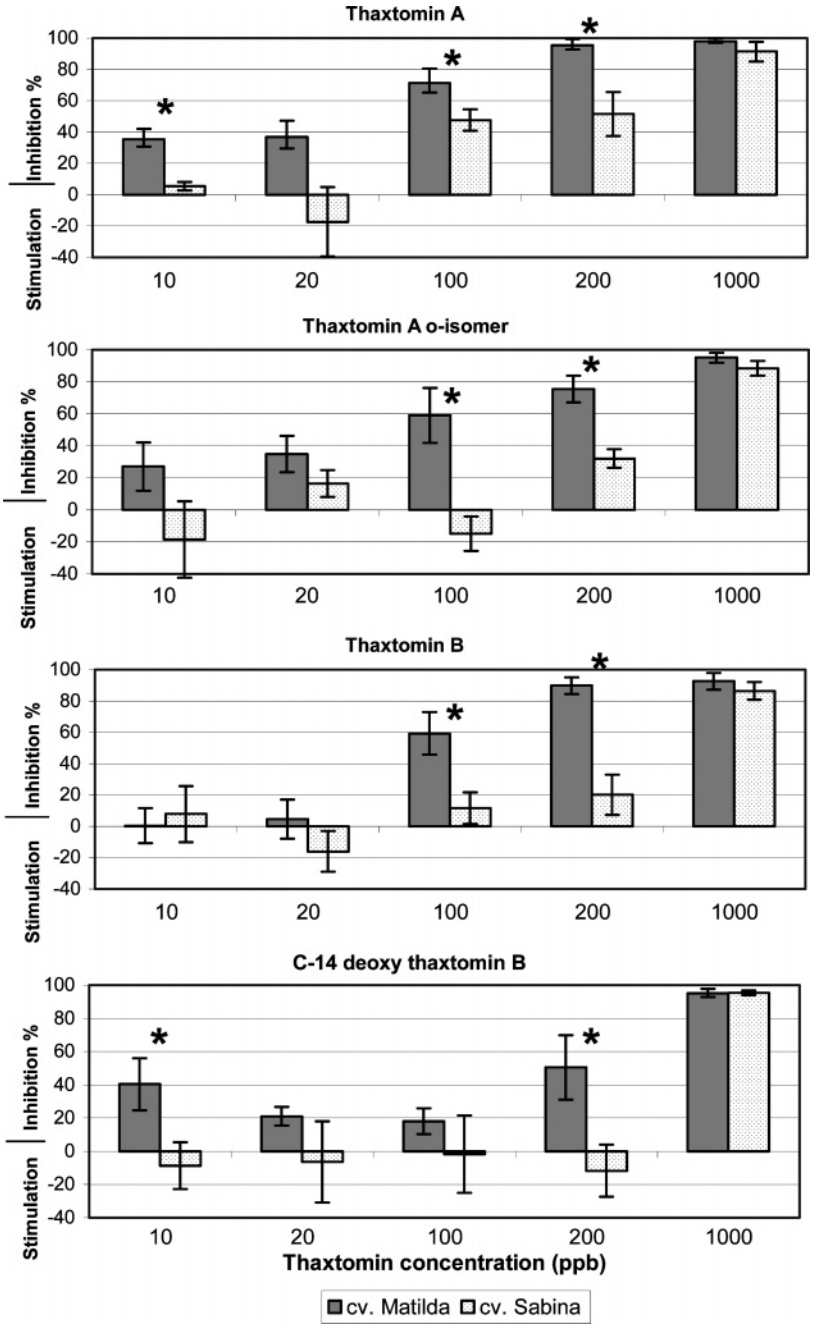

Figure 4. Inhibition or stimulation of the growth of micropropagated shoots of potato cvs. Matilda and Sabina in the presence of various concentrations of thaxtomins. Means of two experiments ($n = 6$) and a bar indicating the standard error are shown. An asterisk denotes a statistically significant difference of means ($p < 0.05$, t test) between the cultivars.

indicates antagonistic effects of the thaxtomins. No significant difference between I_o and I_e points to an additive effect. The significance of the differences between means of I_o and I_e was tested by t statistics.

Data analyses were done using SAS General Linear Models (SAS Institute Inc., Cary, NC) and Genstat 6 (VSN International Ltd., Hemel Hempstead, U.K.).

RESULTS

Purification of Thaxtomins. Thaxtomins were extracted from OMB with ethyl acetate 14 days after inoculation with the bacteria. HPLC analysis of the ethyl acetate extract revealed four major peaks (**Figure 2**). The wavelength 224 nm was used to detect major impurities in the extract, whereas quantitative determinations were performed at the more specific wavelength of 380 nm. The use of CC separation facilitated the fractionation of ethyl acetate extracts and yielded four components. One of them was collected in large quantities (total of 27 mg) in a relatively pure form (97% HPLC purity at 224 nm) with no need for further purification (**Table 1**). The other three compounds were collected in smaller quantities. They were further purified using semipreparative HPLC.

The UV spectrum of thaxtomin A showed maxima at 221 and 403 nm. The spectra of two of the three other compounds closely resembled that of thaxtomin A. LC-MS indicated molecular weights (**Table 1**; **Figure 3**), and TLC analysis provided data that were in agreement with those obtained previously (*7*, *19*, *21*) and allowed identification of the compounds as thaxtomin A (no. **I** in **Table 1**), thaxtomin A ortho isomer (no. **II**), thaxtomin B (no. **III**), and C-14 deoxythaxtomin B (no. **IV**), also known as thaxtomin D (*6*, *26*)

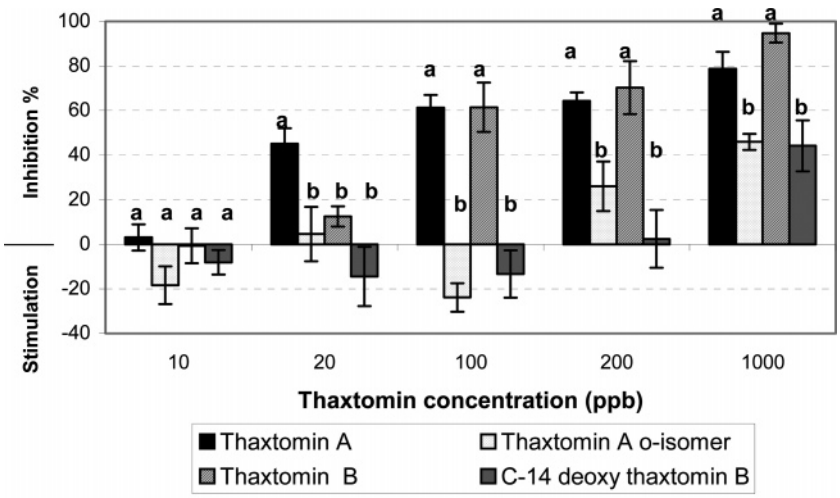

Figure 5. Inhibition or stimulation of the growth of micropropagated shoots of potato cv. Nicola in the presence of various concentrations of thaxtomins. Means of two experiments ($n = 6$) and a bar indicating the standard error are shown. Values at each concentration marked with a different letter are significantly different ($p < 0.05$, *t* tes
t).

(**Figure 1**). In addition to the molecular ions (M + 1), an abundance of sodium adducts (M + 23) were observed. The LC-MS spectra showed that the fragment representing loss of water (M + 1 − 18) is missing from compound **IV** due to the absence of a hydroxyl group (**Figure 1**).

HPLC-UV analysis suggested a relatively low purity (62%) of thaxtomin A ortho isomer (**II**) (**Table 1**), but this was not further supported by the LC-MS spectrum at the whole mass range of *m*/*z* 100−600, which indicated a purity close to 90% (data not shown). The six main fragments and their abundances, excluding those in **Figure 3**, were similar to those of thaxtomin A (**I**). Compound **II** had additionally four different fragments (*m*/*z* 195, 308, 391, and 413), but none of them more abundant than *m*/*z* 421 shown in **Figure 3**.

Effects of Thaxtomins on Potato Growth. All thaxtomins tested caused similar responses (physiological effects) on micropropagated potato. However, the concentrations at which visual responses appeared were dependent on the cultivar and the compound. The responses observed were classified as (i) enhanced shoot growth; (ii) shoot growth reduced by <50% as compared to nontreated controls; (iii) shoot growth reduced by >50%, root formation reduced, necrosis and swelling observed at the basal part of the stem and roots; and (iv) shoot and root growth completely inhibited followed by extensive necrotic symptoms and plant death.

Cultivars Matilda and Sabina were tested in the same experiments, allowing comparison of the relative differences of their responses to thaxtomins. Thaxtomin A, thaxtomin A ortho isomer, and C-14 deoxythaxtomin B had inhibitory effects on the shoot growth of potato cv. Matilda at all concentrations tested. Thaxtomin B was less effective at low concentrations because 10 and 20 ppb of thaxtomin B had little effect on the growth. However, higher concentrations of thaxtomin B (100, 200, and 1000 ppb) inhibited the growth by >50% (**Figure 4**). These results contrasted with those of cv. Sabina, for which shoot growth was unaffected, or slightly stimulated, at the two lowest concentrations of all thaxtomins (**Figure 5**). At 200 ppb concentrations, Sabina showed significantly higher levels of resistance to the deleterious effects of all thaxtomins, as compared to cv. Matilda. However, at the highest concentration of 1000 ppb, growth inhibition in both cultivars was >90% (**Figure 4**).

Table 2. Values for 50% Inhibition of Growth (I_{50}) for Different Thaxtomin Compounds on Three Cultivars of Micropropagated Potato

potato cv.	compound	I_{50}[a] (ppb)
Matilda	thaxtomin A	30
	thaxtomin A ortho isomer	40
	thaxtomin B	70
	C-14 deoxythaxtomin B	160
Sabina	thaxtomin A	90
	thaxtomin A ortho isomer	400
	thaxtomin B	550
	C-14 deoxythaxtomin B	590
Nicola	thaxtomin A	70
	thaxtomin A ortho isomer	>1000
	thaxtomin B	80
	C-14 deoxythaxtomin B	>1000

[a] I_{50} is the concentration that reduced shoot growth by 50% as compared to the control. ppb, parts per billion.

Cultivar Nicola was tested in experiments not including Matilda and Sabina. Thaxtomin A but not B had significant inhibitory effects on shoot growth already at the low concentration of 20 ppb, whereas both reduced shoot growth heavily at higher concentrations (**Figure 5**). In contrast, thaxtomin A ortho isomer and C-14 deoxythaxtomin B stimulated shoot growth at the low concentrations, and the high concentrations of 200 and 1000 ppb, respectively, caused only a subtle reduction by <50% in shoot growth (**Figure 5**).

The overall differences in the bioactivity of thaxtomins, depending on the potato cultivar tested, were reflected in their I_{50} values (**Table 2**). Thaxtomin A and C-14 deoxythaxtomin B exhibited the highest and lowest overall bioactivity on all three potato cultivars tested. On the other hand, results indicate differing sensitivities of the potato cultivars to the thaxtomins tested. I_{50} was <100 ppb for thaxtomin A in all three cultivars, indicating that all of them were most sensitive to it (**Table 2**). Matilda was also sensitive to the three other thaxtomins tested, in contrast to Sabina. Nicola was sensitive to thaxtomins A and B but remarkably tolerant to thaxtomin A ortho isomer (I_{50} > 1000) (**Table 2**). All cultivars exhibited the highest tolerance to C-14 deoxythaxtomin B.

According to Limpel's formula, no synergistic interaction was found between any of the compounds, but most interactions were

Table 3. Observed and Expected Inhibitions of Growth of Micropropagated Potato Cv. Matilda Obtained from Tests Using Binary Combinations of Thaxtomins and Calculations Using Single Compound Concentrations, Respectively

compd concn (ppb)		obsd inhibition (I_o)[a] (%)	expected inhibition (I_e)[b] (%)	difference between I_o and I_e[a] (%)	*t* value[c] (df = 10)
thaxtomin A	thaxtomin B				
10	20	−2.8 (± 14.1)	14.8 (± 14.5)	−17.6 (± 8.9)	−1.98
20	20	32.7 (± 16.7)	29.3 (± 22.0)	3.4 (± 29.6)	0.11
20	100	52.0 (± 19.3)	39.9 (± 16.6)	12.1 (± 23.6)	0.51
thaxtomin A	C-14 deoxythaxtomin B				
10	100	6.6 (± 9.5)	32.1 (± 16.7)	−25.5 (± 24.8)	−1.03
20	100	27.4 (± 11.4)	33.8 (± 19.4)	−6.4 (± 27.8)	−0.23
20	200	71.6 (± 13.1)	56.3 (± 17.6)	15.3 (± 25.1)	0.61
thaxtomin A	thaxtomin A ortho isomer				
10	20	14.7 (± 6.3)	8.8 (± 18.9)	5.9 (± 22.4)	0.26
20	10	18.9 (± 12.1)	46.9 (± 17.4)	−28.0 (± 18.5)	−1.51
20	20	3.5 (± 16.1)	31.4 (± 20.4)	−27.9 (± 23.9)	−1.17
thaxtomin B	C-14 deoxythaxtomin B				
20	200	37.4 (± 17.6)	41.8 (± 17.6)	−4.4 (± 22.5)	−0.20
20	100	28.3 (± 15.6)	31.6 (± 22.0)	−3.3 (± 13.7)	−0.24
100	100	57.4 (± 16.0)	41.7 (± 18.8)	15.7 (± 23.3)	0.67
thaxtomin B	thaxtomin A ortho isomer				
20	20	17.9 (± 14.1)	27.0 (± 14.7)	−9.1 (± 15.7)	−0.58
20	10	−10.2 (± 17.7)	33.6 (± 14.0)	−43.8 (± 17.2)	−2.55*
100	20	7.4 (± 9.3)	40.4 (± 9.9)	−33.0 (± 8.7)	−3.79*
C-14 deoxythaxtomin B	thaxtomin A ortho isomer				
100	20	16.9 (± 12.1)	40.5 (± 16.6)	−23.6 (± 14.6)	−1.62
100	10	37.6 (± 19.5)	50.4 (± 14.8)	−12.8 (± 25.0)	−0.51
200	20	63.2 (± 9.7)	46.8 (± 16.1)	16.4 (± 15.1)	1.09

[a] Value in parentheses is the standard error of the mean. [b] Expected inhibition was calculated using Limpel's formula: $I_e = x + y - (xy/100)$, in which x and y are percentages of inhibition relative to each compound used alone. Value in parentheses is the standard error of the mean. [c] If $|t| \geq t_{sign}$, the difference between the expected and the observed inhibition is statistically significant ($p < 0.05$) and is indicated with an asterisk.

Table 4. Observed and Expected Inhibitions of Growth of Micropropagated Potato Cv. Sabina Obtained from Tests Using Binary Combinations of Thaxtomins and Calculations Using Single Compound Concentrations, Respectively

compd concn (ppb)		obsd inhibition (I_o)[a] (%)	expected inhibition (I_e)[b] (%)	difference between I_o and I_e[a] (%)	*t* value[c] (df = 10)
thaxtomin A	thaxtomin B				
20	200	55.1 (± 10.0)	51.4 (± 11.3)	3.7 (± 18.8)	0.20
100	100	48.4 (± 12.2)	26.1 (± 16.1)	22.3 (± 15.8)	1.41
100	200	67.0 (± 6.8)	31.2 (± 25.0)	35.8 (± 21.9)	1.63
thaxtomin A	C-14 deoxythaxtomin B				
20	200	45.5 (± 5.1)	54.5 (± 11.6)	−9.0 (± 15.3)	−0.59
100	100	46.2 (± 13.0)	14.9 (± 35.0)[d]	31.3 (± 36.4)	0.86
100	200	70.6 (± 12.6)	48.0 (± 15.7)	22.6 (± 16.2)	1.40
thaxtomin A	thaxtomin A ortho isomer				
20	200	36.3 (± 9.8)	64.3 (± 13.8)	−28.0 (± 9.6)	−2.92*
100	100	46.0 (± 10.1)	12.9 (± 41.3)[e]	33.1 (± 40.7)	0.81
100	200	71.7 (± 7.8)	52.9 (± 23.9)	18.8 (± 20.3)	0.93
thaxtomin B	C-14 deoxythaxtomin B				
100	200	56.8 (± 7.7)	45.1 (± 11.1)	11.7 (± 7.8)	1.50
200	100	53.5 (± 11.3)	38.3 (± 17.4)	15.2 (± 8.2)	1.85
200	200	65.9 (± 7.9)	59.7 (± 9.1)	6.2 (± 14.8)	0.42
thaxtomin B	thaxtomin A ortho isomer				
100	200	47.3 (± 18.5)	46.9 (± 21.7)	0.5 (± 27.8)	0.02
200	100	47.1 (± 3.9)	39.0 (± 21.5)	8.1 (± 18.2)	0.45
200	200	77.3 (± 7.1)	56.7 (± 22.8)	20.6 (± 22.5)	0.92
C-14 deoxythaxtomin B	thaxtomin A ortho isomer				
100	200	58.9 (± 13.5)	61.7 (± 14.4)	−2.8 (± 10.1)	−0.28
200	100	48.3 (± 6.5)	48.1 (± 17.7)	0.1 (± 14.3)	0.01
200	200	52.0 (± 13.1)	71.2 (± 13.1)	−19.2 (± 18.5)	−1.04

[a] Value in parentheses is the standard error of the mean. [b] Expected inhibition was calculated using Limpel's formula: $I_e = x + y - (xy/100)$, in which x and y are percentages of inhibition relative to each compound used alone. Value in parentheses is the standard error of the mean. [c] If $|t| \geq t_{sign}$, the difference between the expected and the observed inhibition is statistically significant ($p < 0.05$) and is indicated with an asterisk. [d] A large SE for this I_e is due to one deviant shoot growth value for thaxtomin A at 100 ppb and one for C-14 thaxtomin B at 100 ppb. [e] A large SE for this I_e is due to one deviant shoot growth value for thaxtomin A at 100 ppb and one for thaxtomin A ortho isomer at 100 ppb.

additive on cvs. Matilda and Sabina (**Tables 3** and **4**). However, on cv. Matilda, thaxtomin B and thaxtomin A ortho isomer coapplied at the concentrations of 20 and 10 ppb, respectively, or 100 and 20 ppb, respectively, reduced the growth less than expected (**Table 3**). Similarly, thaxtomin A and thaxtomin A ortho isomer coapplied on cv. Sabina at concentrations of 20

and 200 ppb, respectively, reduced the growth less than expected (**Table 4**). These data were indicative of an antagonistic interaction between the coapplied thaxtomins. No other statistically significant deviations from I_e were observed.

DISCUSSION

Thaxtomins are unique, 4-nitroindole moiety-containing metabolites produced by plant-pathogenic *Streptomyces* spp. (*4*). Most of them have been purified from bacterial cultures and tested for physiological effects on plant cells and tissues (*1*, *3*, *4*, *15*, *18*, *27* and references cited therein). The C-14 deoxythaxtomin B (*4*) (or thaxtomin D; *6*, *26*) is converted to thaxtomin B, which in turn is a precursor for thaxtomin A (*6*). Our data showed that the deleterious bioactivity of the three thaxtomins on potato tissues increased respectively, as was also found in a recent study on lettuce seedlings (*26*). In high concentrations (1000 ppb), the deleterious effects of all thaxtomins studied were similarly severe on potato shoot and root tissues.

Hence, not only thaxtomin A, the predominant virulence factor of plant-pathogenic *Streptomyces* spp. (*1*, *5*, *7*, *9*, *15*, *16*), but also its biosynthetic precursors can be harmful. At least some of these different thaxtomin compounds accumulate in infected plant tissues simultaneously (*4*), consistent with their excretion to and accumulation in considerable concentrations in culture medium during bacterial growth. However, their putative interactive effects have received little attention. We applied four thaxtomins in combinations at different concentrations to detect possible deviation from the expected growth inhibition (I_e) caused by application of the single compounds. Limpel's formula used for calculation of I_e has been designed to establish synergism for agrochemical mixtures (*25*). It has also been used for studying synergistic interactions between enzymes and bacterial culture filtrates or membrane-affecting compounds (*28*, *29*). Data indicated that the overall physiological effects of different thaxtomins were similar and their mutual interactions were mostly additive, although the concentrations required for observable effects differed. These results suggest that the thaxtomins tested collectively interfere with potato cell homeostasis, which results in hypertrophy, developmental disturbance, altered cell wall synthesis, and, at high thaxtomin concentration, death of cells, as previously shown with other plant species (*3*, *15*−*17*).

However, in two cases, the interactions were regarded as antagonistic. On cv. Matilda, an antagonistic interaction was found between thaxtomin B and thaxtomin A ortho isomer, whereas in cv. Sabina, antagonism was observed between thaxtomin A and thaxtomin A ortho isomer. These interactions were detected at low thaxtomin A ortho isomer concentrations and might be indicative of competition for cellular receptors. There is little information about the cellular binding sites of thaxtomins (*18*), and further studies will be required to understand the molecular details of thaxtomin−plant cell interactions.

Closer comparison of potato cvs. Matilda, Sabina, and Nicola revealed differences in the overall sensitivity to thaxtomins. Whereas all cultivars were sensitive to <100 ppb concentrations of thaxtomin A, Sabina was quite tolerant to the three other thaxtomins tested. Nicola was remarkably tolerant to thaxtomin A ortho isomer and C-14 deoxythaxtomin B. In contrast, Matilda was sensitive to all four thaxtomins tested. Thaxtomin concentrations of 200 ppb and below were useful for detecting the differences, whereas the highest concentration of 1000 ppb caused severe symptoms and made cultivar differences less apparent. These results are consistent with the previously known relative differences in resistance or susceptibility of the cultivars to common scab. Sabina expresses significantly higher levels of resistance to common scab than Matilda (*10*, *30*), and resistance of Nicola to common scab in the field is reputed to be similar to that of Sabina (unpublished data). No potato cultivar is fully resistant to common scab (*12*), which is consistent with our results on sensitivity of the scab-resistant cultivars Sabina and Nicola to high concentrations of thaxtomins. Potato tubers will be exposed to high thaxtomin concentrations under conditions favoring efficient production of thaxtomins by the *Streptomyces* spp., which may partially explain why also some scab-resistant cultivars occasionally succumb to the disease in the field.

The results suggest that in vitro bioassays may be useful for initial screening of potato genotypes for their pronounced differences in sensitivity and tolerance to common scab, which is supported by other authors (*31*−*33*). This approach would allow large numbers of potato seedlings from crosses to be evaluated in a time- and cost-effective way and the most promising breeding lines to be selected for further evaluation by other methods. Inconsistent results due to an erratic distribution of the scab pathogen in the field and the varying effects of environmental factors could also be avoided. The in vitro bioassay has already been adopted for seedling selection in one potato breeding program (*32*). Selection of more scab-resistant clones among the somaclonal variants regenerated from calli of potato cultivars can also be based on a similar bioassay (*33*). Our results suggest that there are genetic differences in sensitivity to certain thaxtomins among potato genotypes, but the main problem is the predominant sensitivity to thaxtomin A. The overall physiological effects of thaxtomins seem to be additive and similar. Taken together, the results support using thaxtomin A for screening purposes in in vitro resistance bioassays.

Thaxtomin A was more abundantly produced in bacterial cultures and more feasible for large-scale purification than other thaxtomins, as also observed in the previous studies (*7*, *19*, *21*). Ethyl acetate extraction of thaxtomins from the growth medium, followed by column chromatographic separation, resulted in an abundance of thaxtomin A that was pure enough for use, whereas in previous studies extracts had to be fractionated by TLC and further purified (*9*, *16*, *19*−*22*). The methods and results of this study should be useful for future studies on the physiological effects of thaxtomins and development of potato varieties resistant to common scab.

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