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Plant growth regulators from ash strains of *Pseudomonas syringae* subsp. *savastanoi*

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Abstract. Indole-3-acetic acid and its methyl ester have been isolated from cultures of *Pseudomonas syringae* subsp. *savastanoi* isolated from ash. However, typical strains produced only small amounts of the auxins, whereas relatively high amounts of the above phytohormones accumulated in the culture of an atypical strain. No cytokinins were isolated from cultures of the typical ash strains. Conversely, four cytokinins, namely dihydrozeatin and its 9-β-riboside, *trans*-zeatin riboside and its 1″-methyl derivative, were found in an atypical strain culture.
Key words. *Pseudomonas syringae* subsp. *savastanoi*; ash; ash bacterial canker; phytohormones; auxins; cytokinins; indole-3-acetic acid methyl ester; dihydrozeatin riboside.

Pseudomonas syringae subsp. *savastanoi* infects oleander and several *Oleaceae* (olive, ash, jasmine, privet, *Phyllirea* sp.) on which it induces overgrowths at the infection sites. In particular, the pathogen causes knots (galls) on olive and oleander whereas canker, accompanied by wartlike excrescences, forms on ash. Other differences among strains isolated from different host species include pathogenic, genetic and biochemical features[1]. In particular, it is well established that olive and oleander strains produce relatively high amounts of indole-3-acetic acid (IAA) and several cytokinins in culture, and that knot formation on these host plants depends on the bacterial production of both classes of phytohormones[2]. Less definite is the ability of ash strains to produce plant growth substances and, conflicting findings have been reported[3,4]. Consequently, it is still unclear whether or not these growth substances play a role in the disease process on ash. Recently we have shown that ash strains, unlike olive and oleander strains, typically secrete relatively low amounts of indoles in culture and do not produce substances with cytokinin-like activity[5]. Only one out of twenty strains tested was atypical in this respect: in culture it produced levels of indoles and substances with cytokinin-like activity comparable to those produced by olive and oleander strains.

The present study has been undertaken to determine the chemical nature of indoles and cytokinin-like substances produced in culture by both typical and atypical ash strains of *P. s.* subsp. *savastanoi*.

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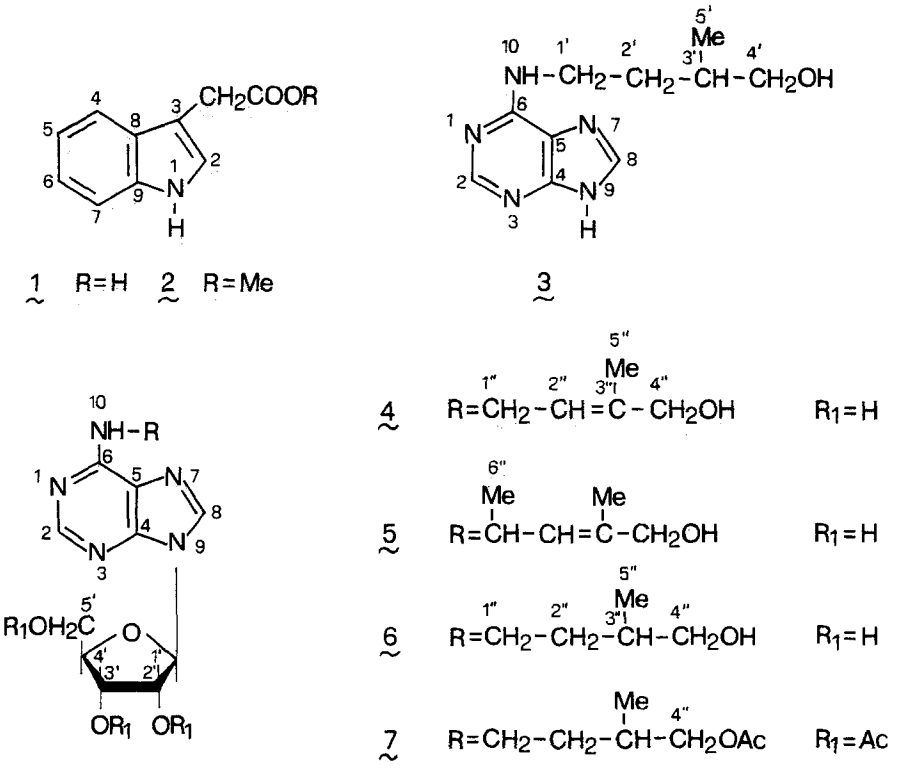
Materials and methods

HPLC. HPLC analyses were performed on a Perkin-Elmer Series 410 liquid chromatograph equipped with a Perkin-Elmer LC-90 variable wavelength UV-visible source. The elution and detection conditions were recently reported[2]. Authentic samples of IAA (Fluka, Buchs, Switzerland), dihydrozeatin, and its 9-β-D-riboside and the *trans*-zeatin riboside (diHZ, diHZR and *t*-ZR, Sigma, Chemical Co., St. Louis, USA) were used. The IAAMe and the 1″-methylzeatin riboside (1″MeZR), used as standard, were obtained by diazomethane esterification of IAA and by a stereospecific synthesis[6], respectively.

Spectroscopic analyses. The UV spectra were recorded on a Perkin-Elmer 550 S spectrophotometer in MeOH solution; ^{1}H- and ^{13}C-NMR spectra were recorded at 270 and 67.92 MHz, respectively, in CDOD solution on a AC Bruker spectrometer, using the same solvent as internal standard. EI MS spectra were recorded at 70 eV on a Fisons TRIO-2000 spectrometer. The MS analyses were performed on the natural auxins and on the total acetyl derivatives of cytokinins obtained as described below.

Production and purification of phytohormones. Three strains were used. Two of them (PD179 and F4) showed features of the majority of the strains isolated from ash; the third strain (NCPPB3474) was atypical[5]. Bacteria were grown for five days in shaken culture at 25 °C in minimal medium (glucose, 5 g; K_2HPO_4, 3 g; NH_4Cl, 1 g; $MgSO_4$, 0.3 g; sodium citrate, 0.5 g per litre) supplemented with L-tryptophan (0.5 mM) as previously reported[2]. The culture filtrates were extracted four times with an equal volume of ethyl acetate at pH 2.5 and



Scheme 1.

subsequently at pH 8.5. The combined organic extracts were dried (Na_2SO_4) and concentrated under reduced pressure. Crude acid extracts analyzed by TLC (silica gel, Merck, Kieselgel F_{254} 0.25 mm, eluent chloroform-methanol 6:4, v/v and chloroform-ethyl acetate-methanol 2:2:1, v/v/v) showed bands which reacted positively to staining reagents for indole and indole 3-substituted derivatives[7,8]. The most intensely stained bands corresponded to IAA and IAAMe. To purify the two indoles, crude extracts (50, 15 and 16 mg for strains NCPPB3474, F4 and PD179, respectively), corresponding to a 0.5 l culture, were fractionated by preparative TLC on silica gel plates (Merck, Kieselgel F_{254}, 0.50 mm) by using the above eluent systems. Pure oily compounds with the chromatographic behavior of IAA (**1**) and IAAMe (**2**) were obtained. Yields of compounds **1** and **2** were 35 and 3.8 mg/l (strain NCPPB3474), 0.3 and 0.024 mg/l (strain F4) and 0.096 and 0.076 mg/l (strain PD179), respectively. Alkaline extracts of the three strains were analyzed by TLC on silica gel (eluent *n*-butanol-acetic acid-water 60:15:25, v/v/v) and on reverse phase (Whatman, Stratocrom KC18 F_{254}, 0.20 mm, eluent water-ethanol 6:4, v/v) plates. Extract of strain NCPPB3474 showed four UV-absorbing bands with chromatographic behaviour of cytokinin-like substances. Conversely no bands with chromatographic behaviour of cytokinins were observed in the extracts of strains F4 and PD179. The extract residue of strain NCPPB3474 (57.8 mg, corresponding to 3.3 l culture) was purified by preparative TLC on silica gel followed by further purification of the bands of interest on reverse phase plates using the above eluent systems. Four pure substances with the chromatographic behaviours of diHZ (**3**, 0.094 mg/l), *t*-ZR (**4**, 0.178 mg/l) 1″MeZR (**5**, 0.070 mg/l) and diHZR (**6**, 0.474 mg/ml) were obtained. Compound **6** had: UV λ max nm (log ε): 264 (3.50); ^{1}H- and ^{13}C-NMR: tables 1 and 2, respectively.

Tetracetyl 6 (7). To prepare the peracetyl derivative of **6**, the natural cytokinin (0.2 mg) was dissolved in pyridine (50 µl) and treated with Ac_2O (50 µl) at room temperature for 24 h. The reaction was stopped by addition of methanol and the mixture evaporated, under reduced pressure, as azeotrope formed adding benzene. The residue, analyzed by EIMS, showed peaks at

Table 1. ^{1}H-NMR data of dihydrozeatin riboside (**6**). Chemical shifts are in δ-values (ppm) from TMS.

H-2*	8.22 *s*	H-1″	3.65 *m*
H-8*	8.24 *s*	H-2″A	1.79 *m*
H-1′	5.95 *d*	H-2″B	1.50 *m*
H-2′	4.74 *dd*	H-3″	1.79 *m*
H-3′	4.32 *dd*	H-4″A	3.48 *dd*
H-4′	4.17 *ddd*	H-4″B	3.45 *dd*
H-5′A	3.89 *dd*	H-5″	1.00 *d*
H-5′B	3.74 *dd*		

J (Hz): 1′,2′ = 6.5; 2′,3′ = 5.3; 3′,4′ = 4′,5′A = 4′,5′B = 2.5; 5′A, 5′B = 12.6; 3″,4″A = 3″,4″B = 5.8; 3″,5″ = 6.6; 4″A,4″B = 14.0
*Assigned in agreement with literature data[17].

Table 2. ^{13}C-NMR data of dihydrozeatin riboside (**6**). Chemical shifts are in δ-values (ppm) from TMS.

C-2	153.5 *d*	C-4'*	88.2 *d*
C-4*	145.7 *s*	C-5'*	63.5 *t*
C-5*	130.0 *s*	C-1''+	34.2 *t*
C-6*	155.3 *s*	C-2''+	34.7 *t*
C-8	141.3 *d*	C-3''	30.7 *d*
C-1'*	91.3 *d*	C-4''	68.2 *t*
C-2'*	72.7 *d*	C-5''	17.0 *q*
C-3'*	75.5 *d*		

*Assignments made in agreement with data reported for available reference compounds[18].
+These attributions may be reversed.

m/z (rel. int.): 462 [MH-AcOH]$^+$ (10), 420 [MH-AcOH-CH_2CO]$^+$ (2), 220 [B-H]$^+$ (15), 204 [B-OH]$^+$ (41), 162 [B-C_3H_7O]$^+$ (66), 148 [B-C_4H_9O]$^+$ (79), 135 [adenine]$^+$ (29), 97 (100).

Results and discussion

The pure compounds purified from the organic acid extract of the three ash strains (NCPPB3474, F4 and PD179), analyzed by TLC or by HPLC (see 'Materials and methods'), cochromatographed with authentic samples of IAA and IAAMe. These results were confirmed by ^{1}H-NMR and EI-MS investigation. In fact, the spectroscopic data obtained were consistent with those reported for the same auxins isolated from *P. amygdali*[9]. These findings represent the first report on IAA and IAAMe production by ash strains of *P. s.* subsp. *savastanoi* which confirm some indications previously reported[3]. However, the levels of IAA and IAAMe in the culture filtrates of the two typical strains (F4 and PD179) were dramatically lower in comparison to those of the atypical strain (NCPPB3474) as well as of the olive and oleander strains[2,10].

When the alkaline extracts were tested for cytokinin activity in the etiolated cucumber cotyledon bioassay[11], only the extract of strain NCPPB3474 strongly stimulated chlorophyll biosynthesis comparable to induction by extracts of cultures of olive and oleander strains[10]. Conversely no appreciable chlorophyll biosynthesis stimulation was induced by extracts from strains F4 and PD179.

These results were in agreement with the TLC analyses (see 'Materials and methods') that showed the presence of UV-absorbing bands with chromatographic behaviour of cytokinin-like substances only in the alkaline extract of strain NCPPB3474. The four pure substances obtained by purification of this latter, analyzed by TLC or by HPLC, cochromatographed with authentic samples of diHZ, *t*-ZR, diHZR and of 1''MeZR. The chemical nature of diHZ, *t*-ZR and 1''MeZR was confirmed by ^{1}H-NMR and EI-MS analysis of natural metabolites and the total acetyl derivatives, respectively, which were obtained by acetylation with acetic anhydride and pyridine. Spectroscopic data were consistent with those of diHZ, *t*-ZR and 1''MeZR previously isolated from *P. amygdali* and an oleander strain of *P. s.* subsp. *savastanoi*[12,13]. The fourth cytokinin diHZR has been reported to be produced by an olive strain of *P. s.* subsp. *savastanoi* but its identification was based on HPLC analysis[14]. Consequently it seemed worthwhile to define the chemical nature of **6** which was deduced by analysis of its ^{1}H- and ^{13}C-NMR spectra (tables 1 and 2, respectively). The structure of dihydrozeatin riboside was confirmed by EI-MS of its tetracetyl derivative **7**. This spectrum showed peaks at 462 and 420 *m/z*, due to the successive loss of AcOH and CH_2CO molecules from the molecular ion, together with other peaks characteristic of an N^{10}-substituted purine[15]. The UV spectrum of **6** also agreed with a diHZR structure, showing an absorption maximum at 264 nm.

The findings of this study demonstrate that indoles produced in culture by typical ash strains of *P. s.* subsp. *savastanoi* are the auxins, IAA and IAAMe. However, the levels of the two auxins in culture were dramatically lower than those produced by olive and oleander strains[2,10]. Furthermore, they confirm previous data obtained by TLC analysis, on the ability of ash strains to produce trace amounts of IAA in culture[3]. Finally, this is the first report of IAAMe as a metabolite of *P. s.* subsp. *savastanoi*.

From the available data the role of auxins in the disease process on ash is not clear but, as previously suggested[1], it cannot be excluded that they may be responsible for the formation of the wartlike excrescences. The extensive periderm formation in diseased tissues[16] is indicative in this regard and is possibly due to phellem activation in response to auxins produced *in planta* by the pathogen. The availability of mutants lacking the ability to produce the auxins might help to elucidate this matter.

The fact that no cytokinins have been isolated from cultures of typical ash strains of *P. s.* subsp. *savastanoi* confirm previous findings on the lack of cytokinin-like activity in their cultures[5] and, moreover, strongly indicate that typically cytokinins do not have a role in symptom expression in ash.

The atypical ash strain produced IAA and its methyl ester and their levels in the culture were comparable to those of olive and oleander strains of this pathogen[2,10]. Moreover, it produced high amounts of four cytokinins. One of them, the dihydrozeatin riboside, previously reported as a possible cytokinin produced by olive strain *P. s.* subsp. *savastanoi*[14], has been fully characterized for the first time in this study. The phytohormone production ability of this strain and its atypical pathological behaviour[5] suggest that it might be an olive strain occasionally spread on ash.

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SCIENTIFIC CORRESPONDENCE

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