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Phytohormone production by strains of *Pantoea agglomerans* from knots on olive plants caused by *Pseudomonas savastanoi* pv. *savastanoi*

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Summary. *Pantoea agglomerans* is a common epiphyte of many plant species, and it is associated with *Pseudomonas savastanoi* pv. *savastanoi* in young and apparently intact olive knots. Strains of *P. agglomerans* collected from various olive groves in central Italy were studied for their ability to accumulate plant growth substances in culture. All the strains produced indole-3-aldehyde, indole-3-ethanol and indole-3-acetic acid (IAA), this last compound in amounts (average 8.7 mg l^{-1}) comparable to those produced *in vitro* by virulent strains of *P. savastanoi*. None of the olive strains produced cytokinins. It is suggested that the IAA produced by *P. agglomerans* may increase the size of the knots caused on olive by *P. savastanoi*.

Key words: auxins, indole-3-acetic acid, indole-3-aldehyde, indole-3-ethanol.

Introduction

In a survey of olive knot disease in central Italy, it was found that in 70% of the knots examined a yellow-pigmented bacterium, later identified as *Pantoea agglomerans*, was associated with *Pseudomonas savastanoi* pv. *savastanoi* (*P. savastanoi*), the causal agent of the disease (Marchi *et al.*, 2006). Pathogenicity tests in which these two bacteria were co-inoculated on the stems of 1-year-old olive plants at different ratios (1:1, 1:100 and 100:1) showed that the growth of *P. agglomerans* was apparently aided by the co-presence of an actively growing population of *P. savastanoi*. At the same time, however, a dominant population of *P. agglomerans* at the co-inoculation site tended to depress the growth of *P. savastanoi*, probably because of competition for space and nutrients between the two bacteria and because of antibiotic compounds produced by *P. agglomerans*. In some cases the association of *P. agglomerans* with *P. savastanoi* led to an increase in the size of the knots produced. This boosting effect of *P. agglomerans* on *P. savastanoi* knot size was attributed at least in part to the release of IAA by *P. agglomerans* at the inoculation sites since our strains when they were cultured accumulated IAA but not cytokinins. This part of the research has been published elsewhere (Marchi *et al.*, 2006). In the present paper we describe in greater detail the isolation and chemical

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identification of IAA and two other indole compounds belonging to the auxin group produced *in vitro* by *P. agglomerans* and we measure the production of IAA by four strains of the bacterium in culture filtrate using an optimized HPLC method.

Materials and methods

Bacterial strains

Strains SC1, FL1, CB2 and MM2 of *P. agglomerans* used in this study were isolated from young and apparently intact olive knots collected from four orchards located in the provinces of Florence (SC1) and Grosseto (FL1, CB2 and MM2) in Tuscany (Marchi *et al*., 2006). The strains were stored in 15% aqueous glycerol at -70°C and re-grown on nutrient sucrose agar plates prior to use.

Production, extraction and purification of plant growth substances

Erlenmeyer flasks containing 400 ml of minimal medium B (Surico *et al*., 1985) with 2.5 mM L-tryptophan were inoculated with 0.5 ml of 10^8 log-phase bacterial cells of *P. agglomerans* SC1 and incubated on shake culture at 26°C for 4 (or 9) days. The bacterial cells were then removed by centrifugation at 5000 *g* for 10 min. The supernatant (1.54 l) was lyophilized and when needed re-dissolved in one tenth (154 ml) of the original volume of distilled water. The resulting solutions were extracted four times with equal volumes of ethyl acetate at pH 2.5 and subsequently at pH 8.5. The combined extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Acidic and alkaline extracts of *P. agglomerans* SC1 (350 and 28 mg respectively) were analyzed for their auxin and cytokinin content by TLC as shown in Table 1 and the acidic extract was compared with IAA, and indole-3-aldhehyde (IAl), while the alkaline extract was compared with *trans*-zeatin (*t*-Z), *trans*-zeatin riboside (*t*-ZR), 1-methylzeatin (1'-MeZ) and 1"-methylzeatin riboside (1"-MeZR).

A portion of the acid organic extracts (180 mg) was purified by preparative TLC (eluent $CHCl_3$-MeOH, 8:2), yielding four main fractions (R_f 0.83, 13.4 mg; R_f 0.62, 5.9 mg; R_f 0.34, 13.4 mg; and R_f 0.20, 18 mg). The second polar fraction (5.9 mg, corresponding to 3.8 mg l^{-1}) appeared to be a homogeneous compound. The residue of the first fraction (13.4 mg) was further purified by preparative TLC on reverse phase plates (eluent EtOH-H_2O, 6:4 v:v) giving five fractions, of which the main fraction (7.4 mg) was further purified by preparative TLC on silica gel plates (eluent: petroleum ether-acetone, 7:3), giving two fractions (R_f 0.27, mg 1.8, corresponding to 1.2 mg l^{-1}; R_f 0.23, mg 2.0, corresponding to 1.3 mg l^{-1}) consisting of two pure amorphous solids. The remaining two polar fractions, R_f 0.34 and 0.20, were not further purified as they did not show an indolic nature.

The residue (28 mg) of the basic organic extract, when analyzed by TLC, showed on reverse phase (eluent: EtOH-H_2O, 4:6) metabolites with a R_f similar to the cytokinin standards mentioned above. It was therefore purified by preparative TLC in the same conditions.

Four-day-old culture filtrates of the other 3 strains were processed as described above and used to measure the amount of IAA produced in culture by *P. agglomerans*.

General chemical condition analysis

Plant-growth substances produced by SC1 were identified essentially with spectroscopic analysis. IR and UV spectra were determined with a Bio-Rad Wind FT-IR spectrometer in a neat solution, and with a Shimadzu UV-1601 visible spectropho-

Table 1. TLC methods for the analysis of indoles and cytocinins produced by *Pantoea agglomerans*.

Compound	Reference compound	Solvent	Detector
Auxins	IAA, indole-3-aldhehyde (IAl)	$CHCl_3$:MeOH, 8:2 (silica gel) EtOH:H_2O, 6:4 (reverse phase) Petroleum ether:acetone, 7:3 (silica gel)	UV light
Cytokinins	*trans*-zeatin (*t*-Z); *trans*-zeatin riboside (*t*-ZR); 1-methylzeatin (1'-MeZ); and 1"-methylzeatin riboside (1"-MeZR)	EtOH:H_2O, 4:6 (reverse phase)	UV light

tometer in an acetonitrile solution respectively. ^{1}H-NMR spectra were recorded at 400 MHz with Bruker spectrometers in CD_3OD. The same solvent was used as an internal standard. Electron ionization (EI) MS were taken on a Fison Trio-2000 at 70 eV. Electrospray Ionization (ESI) MS were recorded on a Perkin-Elmer API 100 LC-MS, with a probe voltage of 5300 V and a declustering potential of 50 V. Analytical and preparative TLC was performed on silica gel (Merck, Kieselgel 60, F254, 0.25 and 0.5 mm respectively) or on reverse-phase plates (Whatman, Stratocrom KC-18, 0.20 mm). The spots were visualized by exposure to UV radiation. Authentic samples of IAA, IAl, *t*-Z, *t*-ZR, 1'-MeZ and 1"-MeZR were used as standards.

HPLC analysis of the IAA content in strains of *P. agglomerans*

For IAA measurements, aliquots of the acid organic extract of isolates SC1, FL1, CB2 and MM2 were redissolved in methanol and analyzed by high performance liquid chromatography (HPLC) as described by Iacobellis *et al.* (1994) with modifications. The mobile phases employed were acetonitrile (eluent A); HPLC grade water (eluent B); and 1% dipotassium hydrogen phosphate in water adjusted to pH 7.35 with concentrated phosphoric acid (eluent C). Elution was initially with eluents A, B, and C, 14:26:60, which was transformed according to a linear gradient over 20 min. to A:B:C (74:26:0). The initial conditions were restored according to a linear gradient over 5 min., and the column was re-equilibrated under these conditions for 10 min. before the next run was commenced. The flow rate was 1 ml min^{-1}, and 20 μl aliquots of samples were injected for analysis. Detection was at 280 nm, where IAA shows maximum absorption. HPLC calibration curves (Table 2) for quantitative determination were obtained using a solution of a standard sample of IAA in methanol in the range 0.04–0.2 μg. HPLC was performed with a Shimadzu, HPLC LC-10 ADvp pump equipped with a spectrophotometric detector SPD-10AV at 280 nm. HPLC linear regression curves were obtained as lines calculated from the multiple injection of four different amounts of the standard in the range indicated above.

Results and discussion

Phytohormone isolation, identification and measurement

From the acid organic extract of isolate SC1 three homogeneous compounds were isolated. The first was found to be indole-3-acetic acid (IAA, Fig. 1, **1**) by comparing its chromatographic and spectroscopic properties (^{1}H-NMR and EI-MS) with those of an authentic sample and the properties previously reported by some members of our team (Evidente *et al.*, 1986)**.** Identification was confirmed with ESI-MS recorded in positive modality, which showed the following significant peaks *m*/*z*: 198 $[M+Na]^+$, 176 $[M+H]^+$; and with the same spectrum recorded in negative modality, which showed a peak at *m*/*z*: 174 $[M-H]^-$. Of the other two homogeneous compounds, one was found to be indole-3-ethanol (IEt, Fig. 1, **2**) by comparing its spectroscopic data (^{1}H-NMR and EI-MS) with those reported in the literature (Yue *et al.*, 2000). This identification was further supported by the following spectroscopic data: IR νmax: 3408 (OH), 1567, 1456 (indole ring) cm^{-1}; UV λmax nm log (ε): 281 (3.28), 221 (3.99); ESI MS, m/z: 184 $[M+Na]^+$, 162 $[M+H]^+$. The last homogeneous compound was found to be IAl (Fig. 1, **3**) by comparing its spectroscopic data (UV, IR, ^{1}H-NMR and MS) with those previously reported by some members of our team (Evidente and Surico, 1986). This identification was confirmed by the ESI-MS (+) spectrum, which showed significant peaks at *m*/*z*: 184 $[M+K]^+$, 168 $[M+Na]^+$, 146 $[M+H]^+$; and by the ESI-MS (-) spectrum, with a peak at *m*/*z*: 144 $[M-H]^-$.

The residue of the basic organic extract did not contain any cytokinins but only a further amount of indole-3-aldehyde (2.8 mg for a total of 4.6 mg, 3.0 mg l^{-1}).

IAA levels increased in the culture filtrates of strain SC1 grown for both 4 and 9 days, and in

Table 2. Characteristics of the calibration curves of indole-3-acetic acid (IAA).

Compound	Linear Range (μg)	Slope	Intercept	R^2	Number of data points	SDy
IAA	0.04–02	10137	-9.72	0.998	4	19.437

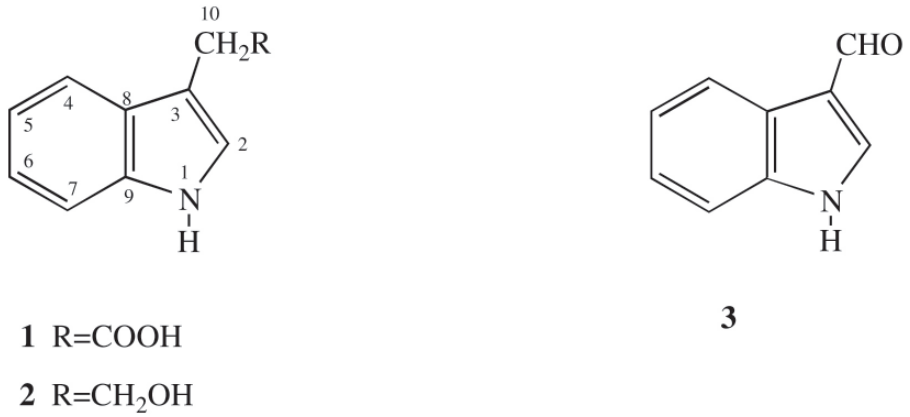

Fig. 1. The structural formulae of indole-3-acetic acid, indole-3-ethanol and indole-3-aldehyde (**1**, **2** and **3**) isolated from *Pantoea agglomerans*.

Table 3. IAA content in the culture filtrates of various *P. agglomerans* strains.

Strains	Duration of growth	Acid organic extract mg l^{-1}	IAA content[a] mg	IAA[a] mg l^{-1}
SC1-A	4	218	8.3±0.18	5.4±0.12
SC1-B	9	183	7.4±0.32	7.4±0.32
FL1	4	184	14.6±0.32	13.2±0.29
CB2	4	138	8.6±0.18	7.6±0.16
MM2	4	133	9.0±0.48	8.5±0.45

[a] Mean value obtained from three repetitions.

those of strains FL1, CB2 and MM2, and were measured by HPLC optimized as described in the Materials and methods section. The results obtained are shown in Table 3.

An average of 8.7 mg l^{-1} of IAA was found in the culture filtrates of the four strains of *P. agglomeran*s grown for 4 days. Specifically, after four days of growth in liquid culture IAA levels were 5.4 (SC1), 13.2 (FL1), 7.6 (CB2) and 8.5 (MM2) mg l^{-1}. The results of indole production in strain SC1 showed that a longer growth time increased the amount of IAA synthesised.

The three indoles isolated are bacterial metabolites and their biological activity is well known (Yue *et al.*, 2000; Evidente and Motta, 2002) but this is the first time that they are isolated and identified chemically as metabolites of *P. agglomerans*. Previously, these indoles were only identified in the ethyl acetate extract of *P. agglomerans* culture filtrates by HPLC (Scholz-Seidel and Ruppel, 1992; Brandl and Lindow, 1996; Höflich *et al.*, 2001).

Discussion

Pantoea agglomerans has shown to be able to accumulate in culture at least three indole compounds: indole-3-acetic acid, indole-3-aldehyde, and indole-3-ethanol also known as tryptophol. Indole-3-aldehyde is a product of the degradation metabolism of IAA; specifically, it is an alternate end-product of the oxidation of IAA by horseradish peroxidase with indolenine hydroperoxide and indolenine epoxide as intermediates (Himman and Lang, 1965). Indole-3-ethanol is a shunt product of the biosynthesis of IAA from tryptophan via indolpyruvic acid (Brandl and Lindow, 1996). This compound is produced by a number of micro-organisms, including bacteria, yeasts and fungi (Barroso *et al.*, 1986; Shin *et al.*, 1991; Lebuhn *et al.*, 1997), and its biological activity is varied, including both antimicrobial activity (Narayanan and Ramananda Rao, 1976) and plant-growth promoting activity (Selvadurai *et al.*, 1991). In the rhizo-

sphere, microbial indole-3-ethanol is easily absorbed by plants and serves as an IAA storage form that is then converted to active IAA by the plant enzyme TOL-oxydase (Persello-Cartieaux *et al*., 2003). In spite of this activity there are no indications at present that this compound is involved in the interaction between *P. agglomerans* and *P. savastanoi,* although such an interaction cannot be strictly ruled out. The third indole, IAA, is a compound produced by many bacterial species (Patten and Glick, 1996), usually through two biosynthetic pathways: the indole-3-acetamide (IAM) route, which also functions in *P. savastanoi* (Kosuge *et al*., 1966; Comai and Kosuge, 1982) as well as in other pathogenic bacteria (Yamada, 1993; Gaudin *et al*., 1994), and the indole-3-pyruvate (IPyA) route. In *P. agglomerans* pv. *gypsophilae*, the IAM mechanism is typically involved in gall formation and thus plays a primary role in disease symptoms development, while the IPyA pathway, which also occurs in non-pathogenic isolates of *P. agglomerans* (Manulis *et al*., 1991; Brandl and Lindow, 1996), seems to contribute significantly to the epiphytic fitness of the harbouring organism (Brandl and Lindow, 1998; Manulis *et al*., 1998), but not to gall formation (Manulis *et al*., 1998). In the phyllosphere the production of IAA by *P. agglomerans* epiphytic isolates through the IPyA pathway has been positively correlated with increased resistance to drought and increased nutrient leakage from the plant cells (Lindow and Brandl, 2003). Many researchers (Gaudin *et al*., 1994; Patten and Glick, 1996; Persello-Cartieaux *et al*., 2003) explain these two effects of IAA in the plant/bacterium interaction by postulating that, since the IAM pathway is rare in plants, those pathogenic bacteria that produce IAA through this pathway override the regulatory mechanism by which the host plant controls its IAA levels. By contrast, since the IPyA route is predominant in the higher plants, any IAA produced by bacteria through this pathway can be maintained at non-toxic or physiologically appropriate levels by the plant host itself. The data on the pathosystem *Gypsophila paniculata*/*Pantoea agglomerans* pv. *gypsophilae* seem to corroborate this hypothesis. Interestingly though, Yamada (1993) suggested that the overproduction of IAA through the IPyA route, although it cannot be directly correlated with host cell proliferation processes, might affect physiological conditions in the host in favour of the pathogen in other pathosystems. Moreover, Kawaguchi and Syono (1996) have raised several objections to the statement that indole-3-pyruvic acid pathway is common in many plant species. Consequently, the explanation that the plant host regulatory systems prevents the deleterious effects caused by IAA when it is produced through the IPyA pathway, is probably too generic. The data from our experiments suggest that the *P. agglomerans* olive knot strains produce IAA through the IPyA pathway, even though the other indolic compounds, indole-3-acetaldehyde and indole-3-pyruvic acid, which are intermediates of this biosynthetic route (Vandeputte *et al*., 2005) were not detected. This absence could however be explained by the fact that the *P. agglomerans* cultures from which the indolic compounds were extracted had reached the stationary-growth phase, since similar studies have found that these molecules can be detected in the filtrates only when the bacterial cultures are at the exponential phase of growth (El-Khawas and Adachi, 1999). A previous report (Marchi *et al*., 2006) found that the co-presence of *P. agglomerans* and *P. savastanoi* at the co-inoculation sites increases the size of olive knots, but the role played by the IAA produced *in vivo* by the former bacterium still remains to be clarified.

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