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Isolation of plant growth regulators from *Pseudomonas amygdali*

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Summary. Trans-zeatin and indole-3-acetic acid were isolated, as the main components of the cytokinin and indole mixtures respectively, from culture filtrates of *Pseudomonas amygdali,* the causal agent of hyperplastic bacterial canker of almond.
Key words. Pseudomonas amygdali; hyperplasia; almond; plant growth regulators; cytokinins; auxins; *trans*-zeatin; indole-3-acetic acid.

Several plant diseases are characterized by growth abnormalities of the infected tissues. Such effects are in many cases determined by an alteration of the physiological hormone balance in the plant[1]. Among phytopathogenic bacteria *Pseudomonas syringae* pv. *savastanoi* (Smith) Young, Dye & Wilkie, *Agrobacterium tumefaciens* (Smith and Towsend) and *Corynebacterium fascians* (Tilford) Dowson produce indole-3-acetic acid (IAA)[2,3] and/or cytokinins[4-7], which have been shown to have an important role in plant pathogenesis[8-10].

Pseudomonas amygdali Psallidas & Panagopoulos is the cause of hyperplastic bacterial canker of almond (*Prunus communis* Arc.). The most characteristic symptoms of the disease, which is present in Greece[11], Turkey[12] and Afghanistan[13], is the formation of perennial cankers on trunks, branches, twigs and shoots. The cankers, which are slow in their development, begin as swellings of the bark, which crack open and become surrounded by swollen cortical tissue.

The development of the symptoms and the final appearance of the cankers suggest the possible involvement of phytohormones in the disease process of *P. amygdali.*

The present study has been undertaken to investigate the ability of *P. amygdali* to produce plant growth substances in vitro. Here we report on the isolation and characterization of 6-(4-hydroxy-3-methylbut-2-enylamino)purine (*trans*-zeatin) and indole-3-acetic acid from cultures of *P. amygdali. P. amygdali* strain NCPPB 2610 was grown at 20 °C in Woolley's[14] medium supplemented with 1.5% peptone (Difco). After 5 days of incubation in shake culture the cells were removed by centrifugation (5000 g, 10 min) and filtration (Millipore, 0.45 µm). The culture filtrate (6.5 l) was lyophilized and the residue redissolved in one tenth of the original volume of distilled water. The pH of the solution was adjusted to 2.5 using HCl 1 N and extracted four times with an equal volume of ethyl acetate. The combined organic extracts were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure; the final product was an oily residue. The crude extract analyzed by TLC (silica gel Merck, Kieselgel F_{254}, 0.25 mm, chloroform-methanol, 6:4; chloroform-ethyl acetate-methanol, 2:2:1, v:v:v) showed several bands which reacted positively to indole and indole-3-substituted derivative staining reagent[15,16]. The most intensely stained zone correspond to IAA.

To purify the free IAA the residue was fractioned on a Sephadex LH-20 (Pharmacia, 25-100 µm) column, using chloroform-methanol (6:4, v:v) as an eluent system. The fractions containing IAA (**1**) were pooled and evaporated under reduced pressure, then further purified by TLC (silica gel, chloroform-ethyl acetate-methanol, 2:2:1, v:v:v). The R_f region from the chromatogram corresponding to IAA was scraped off, eluted with methanol and evaporated to dryness under reduced pressure. The purified compound **1** obtained as an oily residue (54 mg corresponding to 8.3 mg/l), when analyzed by TLC (silica gel) and High Performance Thin Layer Chromatography (HPTLC) (silica gel Merck, Kieselgel F_{254} 0.20 mm) using chloroform-methanol (6:4, v:v); chloroform-ethyl acetate-methanol (2:2:1, v:v:v) or *n*-butanol-acetic acid-water (60:15:25, v:v:v) as solvent systems, co-chromatographed with authentic IAA (Fluka A.G.).

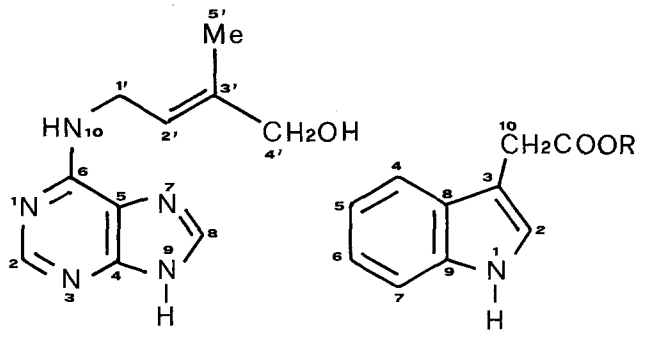
The ^{1}H-NMR (table) and UV (MeOH) spectra of **1** were also identical to those of IAA used as a reference compound and consistent with the data reported for indole-3-substituted derivatives[17,18].

The chemical nature of **1** was confirmed by its conversion into the corresponding methyl ester (**2**), by treatment of **1** with ethereal diazomethane. The derivative **2** when analyzed by TLC (silica gel, chloroform-*iso*-propanol, 95:5, v:v), exhibited the same R_f value also by co-chromatography of the methyl ester prepared performing the same reaction on an

^{1}H-NMR of indole-3-acetic acid (**1**, CD_3OD at 250 MHz) and *trans*-zeatin (**3**, CD_3OD at 300 MHz). Chemical shifts are in δ-values (ppm) from TMS.

	1		**3**
H-2	7.12 *s*	H-2*	8.18 *s*
H-4$^+$	7.59 *dd*	H-8*	7.98 *s*
H-5$^+$	6.95 *ddd*	2H-1′	4.23 *br d*
H-6$^+$	7.04 *ddd*	H-2′	5.65 *br t*
H-7$^+$	7.28 *dd*	2H-4′	3.97 *br s*
2H-10	3.60 *s*	3H-5′	1.78 *br s*

J (Hz) **1**: 4, 5=5, 6=6, 7=8.0; 4, 6=5, 7=1.5; **3**: 1′,2′=6.6; 2′,4′=1.5; 2′,5′=1.1; $^+$ Assigned also by comparison with data reported for derivatives of indole[17]. * Attributions made in agreement with literature data[23].



3

1 R=H

2 R=Me

Indole-3- acetic (IAA): **1**; methyl ester of indole-3-acetic acid: **2**; *trans*-zeatin: **3**.

authentic sample of IAA. In addition, when analyzed by electron impact mass spectrometry (70 eV), **2** gave a molecular ion at *m/z* 189 and the fragment peak characteristic of an indole-3-substituted derivative at *m/z* 130[19]; the remaining peak pattern was identical to that of the methyl ester of IAA. Finally a purified IAA sample was bioassayed in comparison to authentic IAA for auxin activity. Seeds of *Triticum vulgare* L. were soaked in tap water and allowed to germinate at 25 °C for 72 h in the dark. Then, 3-mm-long sections from 2–3-cm-long coleoptiles were isolated 4 mm below the tip and transferred into the test solutions. Lengths of coleoptile sections were measured after 24 h of incubation in the dark. Both **1** and authentic IAA induced comparable stimulation of wheat coleoptile elongation at 1 mg/l.

The determination of the chemical nature of the other acid metabolites, including other indole derivatives, is now in progress.

For the extraction and purification of cytokinins, the procedure of Iacobellis et al.[5] was followed. After adjusting the pH to 8.5 with NaOH 1 N, the culture filtrate, resulting from the acidic extraction, was extracted four times with an equal volume of ethyl acetate. The combined organic extracts were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure; the product was an oily residue. The fractionation of this mixture by analytical TLC (silica gel, *n*-butanol-acetic acid-water, 60:15:25, v:v:v) yielded three UV absorbing bands. The zone with a zeatin-like R_f was scraped off, eluted with ethanol and dried under reduced pressure. The further purification of this fraction by reverse phase TLC (Whatman, Stratocrom SIF_{254}, C18, 0.20 mm, water-ethanol, 6:4, v:v) resulted in two components A and B (**3**). By TLC (normal and reverse phase using the eluents cited before) or by HPTLC (silica gel, *n*-butanol-acetic acid-water, 60:15:25, v:v:v), substance A exhibited R_f values different from those of the cytokinins used as references (zeatin, zeatin riboside, dihydrozeatin, iP, iPA, 2-methylthiozeatin, 2-methylthio-iP, 2-methylthio-iPA, 1′-methylzeatin and 1″-methylzeatin riboside), whereas the R_f value of **3**, obtained as pure compound (7.8 mg corresponding to about 1.2 mg/l), corresponded to that of authentic *trans*-zeatin (Sigma chemicals), also by co-chromatography.

The ^{1}H-NMR spectrum (table 1) of **3** showed a signal pattern coinciding with that of authentic *trans*-zeatin and in agreement with the data for zeatin picrate, reported in the literature[20]. Also, the UV-spectrum (ethanol) of **3** was the one for *trans*-zeatin and consistent with the data already reported[20].

Finally, the chemical ionization mass spectrum (ammonia as reagent gas) of **3** gave a pseudomolecular ion at *m/z* 220 (MH^+) and fragmentation peaks at *m/z* 202, 188, 160, 148, 136 and 135, as observed in electron impact spectrum of zeatin[20, 21].

A purified sample of *trans*-zeatin was tested for cytokinin activity in the etiolated cucumber cotyledon bioassay[22]. The result of the assay indicated that the sample tested exerted a high stimulation of chlorophyll in etiolated cotyledons.

The above results indicate that *P. amygdali* is able to accumulate a significant amout of IAA and *trans*-zeatin in culture, but this does not necessarily mean that the bacteria, multiplying in almond tissues, induce an alteration of the physiological auxin-cytokinin ratio causing the development of hyperplastic cankers. In oleander isolates of *P. syringae* pv. *savastanoi*, the causal agent of olive and oleander knot, auxin biosynthetic genes have been associated with a plasmid of variable molecular weight[24, 25]. Mutants that had lost the plasmid, either by treatment with acridine orange or by selection for resistance to α-methyltryptophan, were neither IAA producers nor pathogenic. Reintroduction of the plasmid into *P. syringae* pv. *savastanoi* mutants restored IAA biosynthesis and pathogenicity[26], demonstrating that IAA is a limiting factor in knot induction on olive and oleander plants. Moreover, it has been recently demonstrated that in *P. syringae* pv. *savastanoi* cytokinin biosynthesis is also plasmid determined[27].

Since *P. amygdali* isolates have a complement of 3–4 plasmids and as a result are sensitive to α-methyltryptophan (data not shown), it should be possible to select by this means mutants defective in IAA biosynthesis, as in *P. syringae* pv. *savastanoi*. However they were obtained, the availability of *P. amygdali* mutants lacking the ability to synthesize IAA and /or cytokinins might help to elucidate the role of these phytohormones in the development of hyperplastic cankers on almond stems.

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Shoot regeneration from *Agrobacterium tumefaciens*-induced tumors of a tropical timber tree, *Fagraea fragrans*

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Summary. Agrobacterium tumefaciens A208 with nopaline plasmid pTiT37 was used to obtain stem tumors on plantlets of *Fagraea fragrans* grown in vitro. Bacterial elimination and tissue proliferation were simultaneously achieved by growing tumors on cefatoxime medium. After some tissue growth the shoots regenerated. An examination of these showed the presence of nopaline, indicating genetic transformation by T-DNA.
Key words. Agrobacterium tumefaciens; nopaline plasmid; tropical timber tree; *Fagraea fragrans;* genetic transformation.

The bacterium, *Agrobacterium tumefaciens*, transfers a particular DNA segment, the T-DNA, from its large Ti plasmid into the cells of host plants. The T-DNA is integrated into the plant genome. This system is a natural example of genetic transformation. It is suggested that it might be applicable for tree improvement in certain cases. If the transformations were found to be beneficial in such modified tree tissues, they could be used for many practical purposes[8,12]. Genetic transformation of woody tree tissues was reported in poplar[11] and in loblolly pine[12]. But in both these cases, only the genetically transformed tissues were obtained, and there was no shoot or root regeneration from them. Here, we describe the regeneration of shoots from *Agrobacterium tumefaciens*-induced tumors of a tropical timber tree. As far as we know this is the first report on shoot regeneration from such tumors of a tree species.

Fagraea fragrans Roxb. (local name – Tembusu; family – Loganiaceae) is a slow growing indigenous species that attains a height of 50–60 m when mature and yields[9] heavy hardwood timber of commercial value. Multiple shoots of *F. fragrans* were initiated and maintained in in vitro culture according to the methods described earlier[10]. The shoots, approximately 4–5 cm tall with 8–10 leaves, were subcultured onto Murashige and Skoog (MS) medium[2] supplemented with sucrose (2%) and solidified with gelrite (0.2%) but without the addition of any plant growth regulators. pH value was adjusted to 5.8 before the gelrite was added, then the medium was autoclaved for 20 min at 122 °C. Two shoots were cultured in each container with 50 ml of the medium. All the cultures were incubated under white fluorescent light 136 uEm^{2}s^2, 16-h photoperiod, at 26 ± 2 °C.

Roots developed in three or four weeks after subculturing and the plantlets were ready for use in further experiments. Both the nodal and internodal regions were punctured with a sharp needle and simultaneously the bacteria were carefully smeared onto the wounded surface without touching any other parts of the plant. The bacteria were obtained from 3-day-old agar cultures of *A. tumefaciens* A208 containing nopaline plasmid pTiT37. Three to four weeks after inoculation with *A. tumefaciens*, tumors developed on the inoculated sites of *F. fragrans* plantlets. These tumors grew rapidly and were creamy yellow in color, reaching 0.4–0.6 cm in diameter seven weeks after inoculation (fig. 1).

They were detached from the stems and cultured on MS medium supplemented and sterilised with cefotaxime (0.5 g/l) in the first three passages. This was to eliminate the bacteria from the growing tissue system. After obtaining some good growth the callus cultures developed from tumors were transferred to MS medium with no antibiotics. The tissues grew vigorously on the fresh media and turned green (fig. 2). To induce shoot regeneration some of the tissues were transferred to MS + benzylamino purine (BA) medium (0.5, 1.0 and 5.0 mg/l). There was no shoot development in any of them. On the other hand, the tissues maintained on growth regulator-free medium grew well for another 6–9 months and spontaneous bud formation was observed in many of the cultures. Some of these buds developed into shoots (figs 3 and 4). Others dedifferentiated into callus again after subculturing onto fresh medium. Individual regenerated shoots cultured on MS medium did not produce any roots and again they produced callus at basal ends.

Both the axenic callus (about 100 µg) and leaf tissues (50–100 µg) from spontaneously regenerated shoots were examined for the presence of nopaline. They were incubated overnight in MS liquid medium supplemented with sucrose (2%) and arginine (0.1 M). The tissues were then blotted dry on a piece of filter paper and macerated in an Eppendorf tube by using glass rod. After centrifugation at 15,000 × g for 2 min, 20-µl aliquots of the supernatant were applied to Whatman 3-mm paper for electrophoresis at 400 V in formic