Indole-3-acetic acid (IAA) production by *Arthrobacter* species isolated from *Azolla*

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***Arthrobacter* species, isolated from the leaf cavities and the microsporocarps of the aquatic fern species *Azolla pinnata* and *Azolla filiculoides*, produced indole-3-acetic acid (IAA) in culture when the precursor tryptophan was added to the medium. No IAA production was detected in the absence of tryptophan. Maximum IAA formation was obtained in the first 2 d of incubation. Part of the tryptophan was transformed to Nα-acetyl-L-tryptophan.**

Introduction

Azolla Lam. is a genus of heterosporous aquatic fern which contains within the leaf cavities the N_2-fixing cyanobacterium *Anabaena azollae* Strasb. The host *Azolla* synthesizes and provides photosynthates essential for the nitrogenase activity of the cyanobiont, while *Anabaena azollae* supplies fixed nitrogen to the fern (Peters & Meeks, 1989).

The leaf cavities of the fern also contain heterotrophic eubacteria (Grilli Caiola *et al.*, 1988; Nierzwicki-Bauer & Aulfinger, 1990; Plazinski *et al.*, 1990) including high numbers of the genus *Arthrobacter* Conn and Dimmick (Forni *et al.*, 1989; Petro & Gates, 1987; Wallace & Gates, 1986). *Arthrobacter* spp. are ubiquitous in the fern being present not only in the leaf cavities of the *Azolla* species studied, but also in the sporocarps of *Azolla filiculoides* (Forni *et al.*, 1990). Therefore it may be possible that bacteria are a third component of the symbiosis (Forni *et al.*, 1989; Petro & Gates, 1987). It is not known if the bacteria are physiologically significant for the plant or what their role is in the association.

It is known that several micro-organisms exert a marked influence on the growth of plants by producing plant growth regulators. Preliminary data have shown that some of the bacterial strains isolated from *Azolla* produce the plant hormone indole-3-acetic acid (IAA) (Forni & Grilli Caiola, 1988). It is well-established that IAA regulates plant growth. Moreover, it has been suggested that IAA plays a role in bacteria–plant associations in the rhizosphere, such as *Azospirillum* and grass roots (Harari *et al.*, 1989) and in symbiosis, such as *Rhizobium*–legumes (Prinsen *et al.*, 1991). This work was undertaken to determine if IAA is produced by *Arthrobacter* species isolated from the leaf cavities and sporocarps of *Azolla*.

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Abbreviation: IAA, indole-3-acetic acid.

Methods

Bacterial strains. Arthrobacter globiformis Conn & Dimmick (strain A3) (Forni *et al.*, 1989) and *Arthrobacter nicotianae* Giovannozzi-Sermanni (strain MC8) (Forni *et al.*, 1990) were isolated respectively from the leaf cavity and from the microsporocarp of *Azolla filiculoides* Lam. *Arthrobacter crystallopoietes* Ensign and Rittenberg (strain E1) (Forni *et al.*, 1989) was isolated from the leaf cavity of *Azolla pinnata* Brown. All the strains belong to the collection of the Department of Biology, II University of Rome.

Growth of bacteria. The purity of the strains utilized for the study was routinely checked by streaking on TRN (Forni *et al.*, 1989) agar plates. Axenic cultures were grown in TRN medium for 4 d. The cells were harvested, washed three times with mineral medium M9 (Forni *et al.*, 1989) and inoculated in 250 ml flasks containing medium M9 supplemented with 10 mM-sucrose and different concentrations of L-tryptophan (Sigma). The cultures were incubated at 30 °C and 150 r.p.m. (New Brunswick orbital shaker). Samples for IAA determinations were taken every day for the first 4 d of the experiments. The data are means of three replicates.

Protein was determined by the Lowry method using bovine serum albumin as a standard.

IAA extraction and determination. The supernatant fraction, obtained after cell centrifugation for 20 min at 5500 *g*, was adjusted to pH 2·5. The solution was applied to a C-18 Sep-Pak cartridge (Sagee *et al.*, 1986). The cartridge was washed with distilled water and IAA was eluted with methanol containing 10 μg butylated hydroxytoluene ml^{-1}. The methanol solution containing IAA was evaporated under vacuum

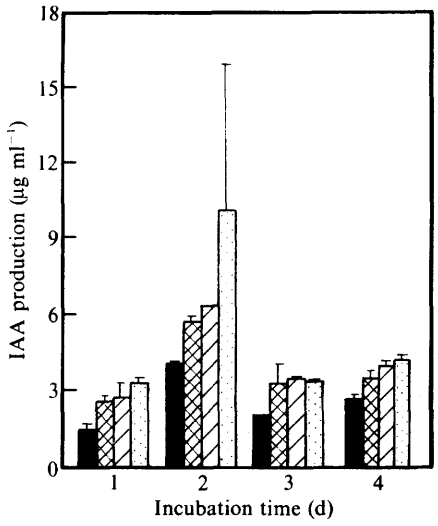

Fig. 1

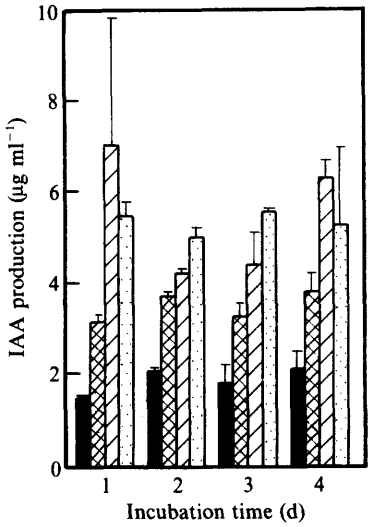

Fig. 2

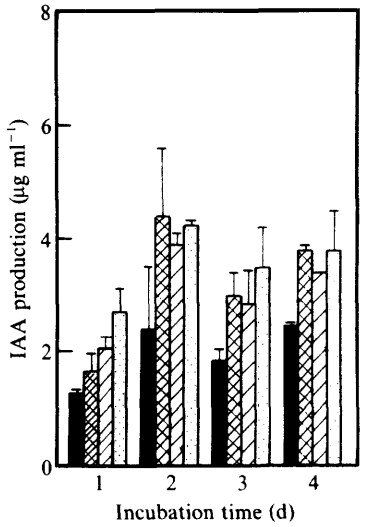

Fig. 3

Fig. 1. Production of IAA in M9 medium by a strain of *Arthrobacter globiformis*, isolated from the leaf cavity of *Azolla filiculoides*. The tryptophan concentration (μg ml^{-1}) in M9 medium was as follows: ■, 100; ▩, 200; ▨, 400; ⊡, 600.

Fig. 2. Production of IAA in M9 medium by a strain of *Arthrobacter crystallopoietes*, isolated from leaf cavity of *Azolla pinnata*. Symbols, as Fig. 1.

Fig. 3. Production of IAA in M9 medium by a strain of *Arthrobacter nicotianae*, isolated from the microsporocarp of *Azolla filiculoides*. Symbols, as Fig. 1.

and IAA was dissolved in 200 μl methanol. IAA was determined using Salkowski reagent (Ehmann, 1977).

Isolation of tryptophan metabolites. Cultures were centrifuged as above and the pH was adjusted to 2·5. Tryptophan metabolites were extracted with two equal volumes of ethyl acetate. The ethyl acetate was evaporated to dryness and the metabolites were methylated with diazomethane (Schlenk & Gellerman, 1960) for gas chromatography–mass spectrometry (GC–MS) analysis.

GC–MS. This was performed on a Finigan MAT 4600 mass spectrometer. Capillary GC was carried out on a 15 m × 0·32 mm i.d. DB-5 column (J & W Scientific) with a film thickness of 0·25 μm. The helium flow rate was 1·2 ml min^{-1} and the injector temperature was 250 °C. A temperature program of 120–220 °C at 3 °C min^{-1} was started upon injection. Mass spectra were recorded at an ionizing voltage of 70 eV with a source temperature of 270 °C.

Results

IAA production in culture

The growth rate of the bacteria in M9 medium supplemented with tryptophan was very low. The cell concentrations (10^7 cells ml^{-1}) and protein content did not increase during the 4 d of the experiments and fell within the range 1–3 mg protein ml^{-1}. Nevertheless, all the *Arthrobacter* species isolated from *Azolla* were able to produce IAA using tryptophan as a precursor (Figs 1, 2 and 3) since no IAA was produced in controls without tryptophan (data not shown).

The maximum production of IAA by *A. globiformis* (i.e. 10·1 μg IAA ml^{-1} in M9 containing 600 μg tryptophan ml^{-1}) and *A. nicotianae* (i.e 4·4 μg IAA ml^{-1} in M9 containing 400 μg tryptophan ml^{-1}) was obtained in the second day of the experiment (Figs 1 and 3), while *A. crystallopoietes* (Fig. 2) produced high levels of IAA during the 4 d of incubation. The IAA content in the growth medium varied according to the concentration of tryptophan added to the culture, generally being higher at 400 and 600 μg tryptophan ml^{-1} (Figs 1, 2 and 3). IAA production by the bacteria (from 0·5 to 2 μg ml^{-1}) was also detected at the lower tryptophan concentrations of 25 μg and 50 μg ml^{-1}.

IAA production was similar in TRN and M9 media containing 600 μg tryptophan ml^{-1}: after 3 d growth in TRN *A. nicotianae* produced 3·3 μg IAA ml^{-1} compared to 3·5 μg IAA ml^{-1} in M9, while *A. globiformis* produced 3·3 and 4·4 μg IAA ml^{-1} in TRN and in M9 respectively.

Identification of tryptophan metabolites.

Two major tryptophan metabolites were identified by GC–MS in all cultures analysed: IAA (Fig. 4) and Nα-acetyl-L-tryptophan (Fig. 5). IAA was the major meta-

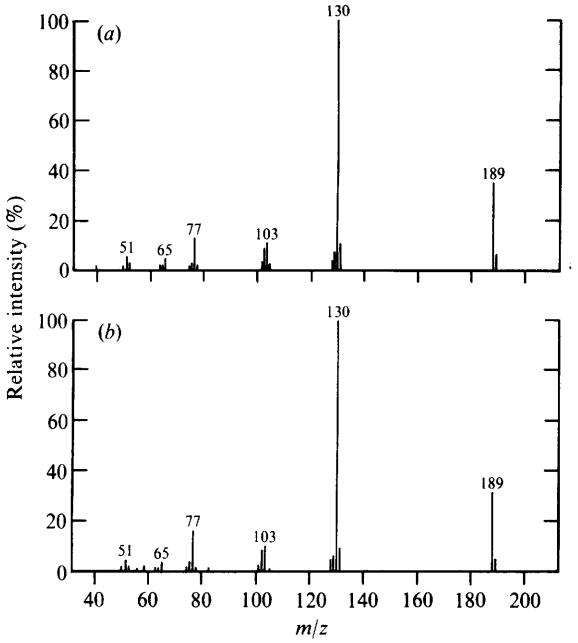

Fig. 4

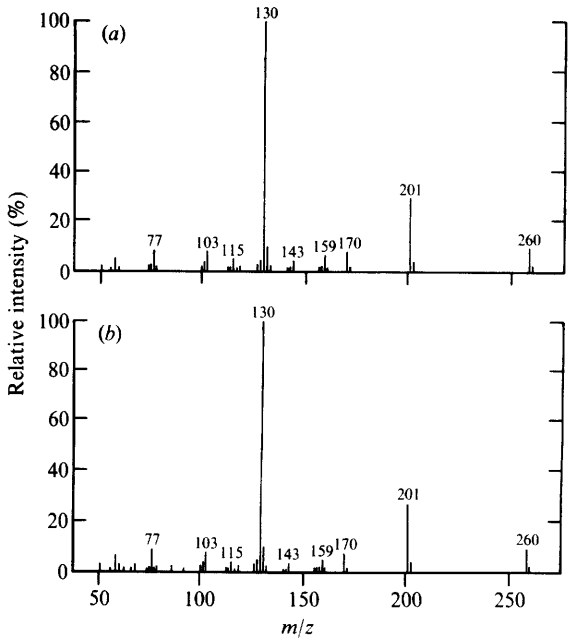

Fig. 5

Fig. 4. Electron impact (70 eV) mass spectra of (*a*) authentic methyl-IAA and (*b*) a methylated sample of putative IAA isolated from an *Arthrobacter nicotianae* culture grown on 100 μg L-tryptophan ml^{-1}. Ions characteristic of methyl-IAA are *m/z* 189 (M^+), 130 (base peak) and 103.

Fig. 5. Electron impact (70 eV) mass spectra of (*a*) authentic methyl-Nα-acetyl-L-tryptophan and (*b*) a methylated sample of putative Nα-acetyl-L-tryptophan isolated from an *Arthrobacter nicotianae* culture grown on 200 μg L-tryptophan ml^{-1}. Ions characteristic of methyl-Nα-acetyl-L-tryptophan are 260 (M^+), 201, 170 and 130 (base peak).

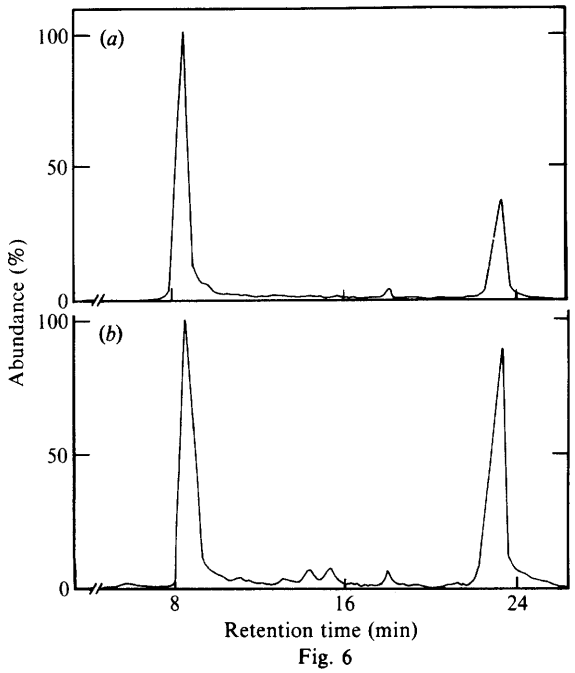

Fig. 6

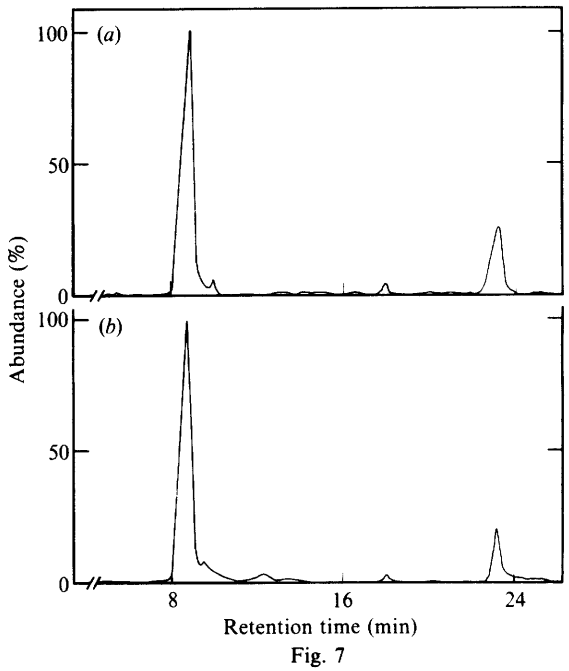

Fig. 7

Fig. 6. Relative abundance of L-tryptophan metabolites possessing the ion *m/z* 130 in cultures of *Arthrobacter nicotianae* grown on 100 μg L-tryptophan ml^{-1} (*a*) and 600 μg L-tryptophan ml^{-1} (*b*). Methyl-IAA is at R_t = 8·5 min and methyl-Nα-acetyl-L-tryptophan is at R_t = 23·8 min.

Fig. 7. Relative abundance of L-tryptophan metabolites possessing the ion *m/z* 130 in cultures of *Arthrobacter globiformis* grown on 100 μg L-tryptophan ml^{-1} (*a*) and 600 μg L-tryptophan ml^{-1} (*b*). Methyl-IAA is at R_t = 8·5 min and methyl-Nα-acetyl-L-tryptophan is at R_t = 23·8 min.

bolite when the cultures were grown on relatively low concentrations of L-tryptophan (up to 100 μg ml^{-1}) (Figs 6 and 7). In cultures of *A. globiformis* (Fig. 7) and *A. crystallopoietes* (data not shown) formation of Nα-acetyl-L-tryptophan was enhanced when increased L-tryptophan concentrations were supplied to the cultures. In *A. crystallopoietes*, Nα-acetyl-L-tryptophan was the major metabolite when 600 μg tryptophan ml^{-1} was supplied to the culture.

Two other minor metabolites, Nα-methyl-L-tryptophan and *N,N'*-dimethyl-L-tryptophan, were identified in most cultures (data not shown).

Discussion

The three species of *Arthrobacter* isolated from *Azolla* produced IAA in mineral medium, confirming previous observations (Forni & Grilli Caiola, 1988).

IAA production in non-growing (i.e. M9 medium) and in actively growing (i.e. TRN medium) cultures indicated the capability of this genus to produce IAA in different growth conditions. In medium M9 *Arthrobacter* produced IAA only when L-tryptophan was added as substrate and showed a significant increase in the presence of high concentrations of the amino acid. The amount of IAA produced by the bacteria was within the detection limits of the Salkowski reagent (Ehmann, 1977), which is specific for IAA and does not interact with L-tryptophan or Nα-acetyl-L-tryptophan.

The GC–MS analyses of the culture supernatants showed the presence of several tryptophan metabolites besides IAA, the most abundant of which was Nα-acetyl-L-tryptophan. The production of these compounds may be a mechanism by which the bacterial cell eliminates the possible toxic effects of high concentrations of tryptophan.

The leaf cavities of *Azolla imbricata* and of *Azolla* freed of *Anabaena azollae* contain respectively 0·09 and 0·29 mM-tryptophan (Yun-lu *et al.*, 1983); these tryptophan concentrations are within the range used in our experiments. It is not known what the effects of this amino acid are on *A. azollae*. However, Rawson (1985) demonstrated that a high concentration of tryptophan reduced nitrogenase activity and growth in *Anabaena cylindrica* PCC 7122. Thus in the association, *A. azollae* may benefit by the bacterial tryptophan utilization resulting in the synthesis of IAA.

It is likely that the bacteria excrete the hormone into the leaf cavity of the fern, where the hair cells might transfer it to the plant. The direct uptake by plants of IAA produced by bacteria has been observed in other plant–bacteria associations (Libbert & Silhengst, 1970). In addition, it has been demonstrated that epiphytic bacteria may increase the IAA content in plants (Libbert *et al.*, 1969).

The recognition that the growth and development of plants depends on growth regulators has led to the suggestion that they might benefit from the external supply of such substances. Moreover, it has been suggested that auxin may play a critical role in some symbioses, such as *Nostoc–Gunnera* (Towata, 1985) and in *Rhizobium*–legumes (Prinsen *et al.*, 1991) Therefore it can be postulated that IAA production by the bacteria might exert a marked influence on growth and morphogenesis of *Azolla*. Unfortunately, in view of the contradictory reports concerning the possible action of IAA on cyanobacteria (Ahmad & Winter, 1968; Leganes *et al.*, 1987) it is difficult at present to predict its effect on symbiotic *Anabaena azollae*.

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