Indole-3-butyric acid (IBA) production in culture medium by wild strain *Azospirillum brasilense*

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Abstract

Some microorganisms found in the soil are able to produce substances which regulate plant growth. In this study, we show the presence of a substance associated with auxin activity, identified as indole-3-butyric acid (IBA), in *Azospirillum brasilense* UAP 154 growth medium. *A. brasilense* was grown and indolic compounds were extracted from the supernatant. These were then analyzed by high performance liquid chromatography (HPLC), gas chromatography and gas chromatography mass spectrometry. The retention time was similar to those of the authentic IBA standard. The compound obtained from HPLC was collected and applied to maize seedlings (*Zea mays*), inducing biological activity along the roots, similar to that induced by an authentic IBA standard.

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1. Introduction

Plant growth regulators (phytohormones) are organic substances, which at low concentrations (less than 1 mM), promote, inhibit, or modify the growth and development of plants. Commonly six major groups of phytohormones are recognized: gibberellins, cytokinins, abscisic acid, ethylene, brassinosteroids and auxins [1,2]. The plant hormone (auxin, indole-3-acetic acid (IAA)), is an organic compound synthesized in one part of the plant and translocated to another, where in very low concentrations it causes a physiological response as a curvature of oat coleoptiles towards light [2].

Among auxin-like compounds, there exist indole-3-propionic acid (IPA), indole butyric acid and naphthalene acetic acid. Some of these, such as indole-3-butyric acid (IBA) occur naturally in more plants [1].

Blommaert [3] found greater amounts of IBA than of IAA at sprouting onset. Likewise, Bayer [4] also found that *Nicotiana* tumors have more IBA than normal tissues. *IBA* has also been identified in peas, maize and carrot tissues inoculated with *Agrobacterium rhizogenes* [4].

One of the factors that dramatically limits grain production is the capacity of plants to take in nitrogen from the soil. The need to increase grain production has been achieved with the addition of nitrogen fertilizers, among other agricultural practices; however, when used in excess these are harmful to world ecology, and besides their manufacture is quite expensive. A substitute for chemist nitrogen fertilizers is biofertilization with certain bacteria, cyanobacteria and actinomycetes, that when applied to plants, may provide beneficial effects through the supply of fixed nitrogen and the production of phytohormones [5]. One of these nitrogen-fixing bacteria is *Azospirillum* spp., which has been isolated from grain plants, forage grass, and cacti [6-8]. The *Azospirillum* genus appears in certain species:
A. brasilense [10], A. halopreferens [12], A. lipoferum [13], A. larginobile [14], A. lipoforum [10] and others (http://www.ncbi.nlm.nih.gov/Taxonomy). A. brasilense has great potential as a growth phytohormone. Inoculation of grain crops of agronomical importance with these bacteria causes an increase in grain production [15].

Besides fixing nitrogen, A. brasilense produces auxins, such as IAA which cause an increase in root hair production [16], thus improving nutrient uptake from the soil [17]. Another auxin associated with A. brasilense inoculation is IBA, which has been found in the roots of maize seedlings inoculated with this microorganism [18].

Several reports indicate the existence of microorganisms associated with plant roots, or colonizing internal tissues in which the production of plant growth-regulating substances has been shown. Auxins have been identified in Azotobacter chroococum and Azotobacter vinelandii [19], in Rhizobium spp. [20], Agrobacterium tumefaciens [21,22], A. rhizogenes [23], Bradyrhizobium spp. [24], Azospirillum spp. [16,25], Acetobacter diazotrophicus [26, 27].

Although IBA has been detected in the root tissues of maize inoculated with A. brasilense, it has never been found in the supernatant of a culture grown from this microorganism [18].

Previous studies in our laboratory have determined several intermediates participating in the IAA biosynthetic pathways, in wild and mutant strains of A. brasilense. When an IBA standard was introduced into a thin layer chromatography (TLC) assay, where a cell-free culture of A. brasilense had been run, the presence of a very weak signal of IBA was identified (unpublished data).

In this study we identified IBA in the wild strain A. brasilense UAP 154 by high performance liquid chromatography (HPLC), gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS); furthermore, its biological activity was assayed on maize plantlets.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used in this work are listed in Table 1.

2.2. Media and growth conditions

A. brasilense was grown at 32°C on Jain and Patriquin medium [17] supplemented with tryptophan (Trp) and antibiotics Rif or Amp, during 72 h for indolic compounds production. As a negative control, Escherichia coli was grown at 37°C in Luria–Bertani (LB) medium and supplemented with antibiotic when required.

Table 1

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>E. coli C600</td>
<td>Smr supE hsdR</td>
<td>[39]</td>
</tr>
<tr>
<td>A. brasilense UAP 154</td>
<td>Ap^ [40]</td>
<td>Velásquez, M., personal communication</td>
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</tbody>
</table>

2.3. TLC

Wild strain A. brasilense UAP 154 was grown at 32°C in a 250-ml Erlenmeyer flask on a rotary shaker at 160 rpm for 72 h. The flask contained 60 ml of Jain and Patriquin medium [17] (supplemented with 100 μg ml⁻¹ Trp). The cell-free culture supernatant was adjusted to pH = 2.7 with HCl 1 N and extracted with 1 volume of ethyl acetate at room temperature, vacuum dried at 37°C, and dissolved in 2 ml of methanol. The assay was run in 2D TLC, by using a mixture of eluents: acetone:chloroform:acetic acid (96%) (v:v:v) in the first dimension and chloroform:acetic acid (96%) (v:v) in the second dimension, and then developed with the Salkowski reagent.

2.4. IBA detection by HPLC

Analytical assay: extracted samples were dissolved in methanol and analyzed by HPLC (Ultrosphere ODS-Beckman, programmable solvent module 125 with diode array detector module 168), and eluted with 1% water-acetic acid/methanol gradient for 32 min in the following program: at the moment of initiation, 60% acetic acid and 40% methanol for 13 min; at 13.01 min, 0% acetic acid and 100% methanol for 10 min; at 23.01 min, 60% acetic acid and 40% methanol for 9 min. Detection was performed at 280 nm.

Preparative assay method for IBA isolation: in order to increase the amount of IBA from wild strain, an analysis by HPLC in preparative column (Ultrosphere ODS-Beckman) was performed. Conditions were as those described above and samples were taken after column fractioning purification every 2 min, and subsequently each fraction was assayed in analytical column under the same conditions.

2.5. GC

The material obtained and dissolved in methanol was derived with bistrimethylsilylfluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS). The derivation reaction was as follows: 75 μl of pure indolic compounds and that extracted from the methanol (obtained previously), were completely evaporated, dissolved in 30 μl of pyridine and 45 μl of BSTFA and then 1% of TMCS was added. The mixture was incubated at 60°C in a double boiler for 2 h.
From each sample, 1 µl was injected into a silica cast capillary column (J&W Scientific DB-17 of 30 m×0.55 mm of i.d. and 1.0 mm of film thickness, Varian star 3400 CX). The running parameters were: 2 min at 173°C, followed by increments of 10°C min⁻¹ in the column temperature until it reached 250°C, which was maintained for a further 3 min. The injector was adjusted to 250°C and the carrier gas flow (N₂) was 10 ml min⁻¹ [28–30].

2.6. GC-MS

With the aim of obtaining a more accurate identification of IBA from A. brasilense UAP 154, GC-MS was performed. From samples obtained by HPLC, 1 µl was injected in the split-splitless mode in a GC-MS system (HP 5890 Series II GC), with operation and data analysis, using Chemstation software under the following conditions: column: link cross methylsilicone, 12 m×0.25 mm of o.d. method: temperature 40–270°C, flux 1 ml min⁻¹, split 1:25, scale 15–350.

Fig. 1. 2D TLC of a cell-free supernatant of wild strain A. brasilense UAP 154 and authentic tryptamine (TAM), IAN, indole-3-pyruvic acid (IPyA), ILA, indole acetamide (IAm), IAA, indole-3-ethanol (TOL) and IBA as standards. The arrows refer to the presence of IBA. In sample 2, IBA; 3, TOL; 4, IAm; 5, ILA; 6, IPyA; 7, TAM (Rf 4.5) IAA (Rf 9); 8, Trp (Rf 4) IAN (Rf 9.5); 13, UAP 154.

Fig. 2. Reverse-phase HPLC profiles. A: Authentic indolic compound standards. Retention times: ILA, 8.47; IAA, 10.82; IPyA, 18.99; and IBA, 21.18 min. B: Indolic compounds produced by wild strain A. brasilense UAP 154, retention time IBA is 21.19 min, culture in minimal medium with 100 µg ml⁻¹ Trp added. The column was eluted in mobile phase methanol-acetic acid 1%.
2.7. Biological assay

Creole maize (Zea mays) seeds were surface sterilized with chloramine T (10%) for 10 min, then vigorously washed three times with sterile water, and subsequently germinated on Petri dishes lined with filter paper and kept in a humid chamber in the dark. 24 h after germination, the seedlings were put into a hydroponic system and the standard and purified IBA (12 μM) were applied. Controls consisted in the addition of culture medium (15 μl) and methanol (15 μl) into the hydroponic solution. Longitudinal growth of the root and root hair production was measured after 72 h. After that, the mean root dry weight was determined in tree plants, for each treatment four times over.

3. Results

3.1. Detection of IBA in A. brasilense

The first evidence for the presence of IBA in the supernatant of A. brasilense was obtained using 2D TLC where a weak signal co-migrated with the standard IBA (Fig. 1).

3.2. Detection of indolic compounds by HPLC in A. brasilense

The detection of standard indolic compounds showed retention times as follows: indole lactic acid (ILA), 8.47 min; indole acetic acid, 10.82 min; indole propionic acid, 18.99 min; and indole butyric acid, 21.18 min (Fig. 2A).

Indolic compounds related production from A. brasilense.

Fig. 2B shows the HPLC chromatogram of the wild strain A. brasilense as UAP 154 (IBA 1.158 μg ml⁻¹ growth medium, S.D. = 0.006149, S.E.M. = 0.003550) where a peak appears with a retention time of 21.18 min similar to the one presented by the authentic IBA standard (Fig. 2A). Another indolic compound with retention time of 10.82 min was the IAA (59 μg ml⁻¹ growth medium, S.D. = 1.143, S.E.M. = 0.8083).

Fig. 2A shows the HPLC chromatogram of authentic indolic compound standards. Together with the production of some indolic compounds in A. brasilense the presence of IBA could be assessed with the corresponding standards.

3.3. Detection of IBA from A. brasilense by GC

With the aim of detecting IBA by GC, samples were derived (as described in Section 2) and subjected to chromatography. Fig. 3A shows GC chromatogram with retention times of the authentic indolic compound standards: retention time of IBA: 14.5 min; of IPA: 10.6 min; of IAA: 9.85 min.

Fig. 3B shows a GC chromatogram of wild strain A. brasilense UAP 154 (IBA 1.158 μg ml⁻¹ growth medium)
with the appearance of a peak with a retention time (14.5 min) similar to the one presented by the authentic IBA standard (Fig. 3A). IAA was present with retention time similar to IAA standard.

3.4. Detection of IBA in *A. brasilense* by GC-MS

Fig. 4 shows the comparison between two GC-MS chromatograms: the IBA standard control (Fig. 4A) and the IBA produced by wild strain *A. brasilense* UAP 154 (Fig. 4B) (IBA 1.158 μg ml⁻¹ growth medium), with a retention time similar to the authentic IBA standard retention time 9.71 min (Fig. 4A). The presence of characteristic ions of IBA was similar in both cases.

3.5. IBA biological assay

Plant biological assays showed that IBA (12 μM) produced by *A. brasilense* acts as an auxin in maize (*Z. mays*), promoting lateral root formation and inhibiting root elongation (Fig. 5). The root dry weight with the addition of IBA standard was 0.0379 g (S.D. = 0.014349, S.E.M. = 0.007175). IBA produced by *A. brasilense* was 0.0658 g (S.D. = 0.011045, S.E.M. = 0.005523). medium growth was 0.0483 g (S.D. = 0.017115, S.E.M. = 0.008558). IAA standard and methanol data are not shown.

The present observations on bioassays in maize seedlings bound to proofs in chromatography prove the existence of IBA in the supernatant of the *A. brasilense* culture.

4. Discussion

It is known that many nitrogen-fixing bacteria are often referred to as plant growth-promoting rhizobacteria (PGPR) because of their capacity to produce plant growth
regulators such as indole acetic acid and gibberellins, among other features.

Although IBA is an auxin produced synthetically for application as a rooting agent, Blommaert [3] indicated that it has been identified as a natural product of potato skin, using paper chromatography and bioassays. In this work we report the presence of IBA in the supernatant of A. brasilense UAP 154 growth culture by TLC, HPLC, GC, GC-MS and bioassay in maize seedlings. Because of this, when referring to A. brasilense as a PGPR bacteria, the IBA production should be mentioned.

The linear gradient conditions used by Alvarez [31] in HPLC determination assays for IAA, were similar to those presented in this work for IAA and IBA. In his work, Alvarez shows the presence of these compounds to be present in apple shoots cultured in vitro through HPLC analysis [31], but does not develop this further with a more complete physical-chemical study.

The IBA from wild strain A. brasilense UAP 154 used in this work for GC, GC-MS and biological assays, was obtained from HPLC using acetic acid 1%/water (v/v) and methanol as eluent. Fractions were collected at 2-min intervals in the preparative column. These conditions were chosen in order to permit the obtaining of a better chromatogram resolution, showing clear differences between indolic compounds and permitting that more IBA be obtained for subsequent assays. The differences found in Alvarez’s method are: the use of acetonitrile/water gradient, collected at 1-min intervals [31].

We have some A. brasilense UAP 154 transconjugants with Tn5 inserted, that were analyzed using HPLC. Some transconjugants displayed a variant, producing IAA and IBA simultaneously: IBA overproducing and IAA under-producing or vice versa, suggesting a probable genetic regulation of these indolic compounds (data not shown).

In GC-MS assays, our conditions were similar to those shown by Bastian et al. [32] to detect A3 gibberellin (GA3) in A. diazotrophicus and Herbaspirillum seropedicae cultures, but with some differences: we did not use isotopes, the cells were not sonicated, we only took the supernatant, and we obtained 59 μg ml⁻¹ IAA when A. brasilense was grown in minimal medium with Trp added, versus 32 ng ml⁻¹ IAA from A. diazotrophicus in LGIP medium, with 10% of sucrose added, and 7 ng ml⁻¹ IAA from H. seropedicae in NFb medium. This indicates a less complicated approach for analyzing the production of auxins by GC-MS.

Auxin IBA is very effective in the promotion of adventitious root formation, as demonstrated in apple plants [31]. It was shown that IBA is more effective than IAA when inducing the formation of adventitious roots [33,34], possibly due to the following considerations: (i) IBA seems to be less susceptible to enzymatic degradation than IAA; (ii) IBA can slowly be transformed into IAA, thus providing a secure IAA supplement; (iii) studies by Nordström et al. [35] have shown that the great effective-

ness of IBA could be due to its extensive presence in plant tissues; and (iv) IBA can induce tissue-specific responses due to differential sensitivity among cell types.

The IBA biological activity observed in this work on maize plants showed promotion of lateral root formation and inhibition of root elongation (Fig. 5). Similar data were shown by Zolman et al. [36] for Arabidopsis thaliana with IBA standard. Furthermore, the beneficial biological activity of IBA on maize plantlets was supported by the root dry weight values obtained, being higher when adding IBA produced by wild strain A. brasilense UAP 154 (0.0658 g), as compared with IBA standard (0.0379 g) and the controls. Bhalerao et al. [37] showed de novo IAA synthesis in Arabidopsis seeding, being low at early seedling development (4%) and increasing through time (e.g. 37%, 10–11 days after germination). When considering this, it must be remembered that our assays were carried out during the first 4 days after germination with the purpose of avoiding IAA and IBA endogenous activity. IBA production under the natural conditions of plant-bacterium association is perhaps caused by the presence of Trp in root exudates [19].

The production of IBA and other plant growth regulators by A. brasilense, together with the production of major outer membrane proteins [38], are two processes that can help in the understanding of the positive effects observed when there is plant–microbe association.

The findings of this study provide significant evidence that IBA was produced by A. brasilense and that it causes clear biological activity in root plants.

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