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Effects of *Azospirillum* spp. on Endogenous Gibberellin Content and Growth of Maize (*Zea mays* L.) Treated with Uniconazole

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Abstract

The growth of *Zea mays* L. plants was reduced by treatment with uniconazole, supplied 7 d prior to Gibberellin A_3 (GA_3) application or *Azospirillum* spp. inoculation. The effect of uniconazole was more remarkable in the aereal part than in the roots. Both *A. lipoferum* and *A. brasilense* increased height at similar levels with respect to application of 0.1 µg/plant of GA_3. Also, the stimulating effect on growth of the root system was expressed as an increase in fresh weight. This was correlated positively with the occurrence of *Azospirillum* spp. observed in roots and stems of maize plants. Gibberellin A_3 was characterized by capillary gas chromatography-selected ion monitoring in a free acid fraction from roots on the plants inoculated with *Azospirillum* spp. In the roots of the non-inoculated uniconazole-treated plants, GA_3 was not found. We conclude that production of GA_3 by *Azospirillum* spp. was involved in growth promotion of maize.

Keywords: *Zea mays*, *Azospirillum* spp., Gibberellin A_3, Uniconazole

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1. Introduction

Bacteria of the genus *Azospirillum* are N_2-fixing organisms living in close association with plants in the rhizosphere, particularly grasses, and have been repeatedly reported to stimulate the growth of cereals (Döbereiner and Pedrosa, 1987; Okon and Labanderas-Gonzáles, 1994). Inoculation of plants with *Azospirillum* spp. can result in a significant change in various plant growth parameters, which may or may not affect crop yield (Bashan and Levanony, 1990). The exact mechanism(s) of action of *Azospirillum* spp. on plants have not yet been fully elucidated.

Associative N_2-fixation by *Azospirillum* spp. is of less agronomic significance than initially expected; therefore, one of the principal mechanisms of growth promotion is related to the capability of *Azospirillum* spp. to produce plant growth promoting substances (Okon and Labanderas-Gonzáles, 1994).

Azospirillum spp. produce indole-3-acetic acid (IAA; Crozier et al., 1988). This capability is, however, widespread among bacteria. The production of IAA by *Azospirillum* spp. occurs only in the stationary growth phase in suspension cultures and is largely (or even strictly) dependent on tryptophan (Zimmer and Bothe, 1988).

With regard to gibberellins (GAs), Zimmer and Bothe (1988) failed to identify any GA-like activity by bioassay from crude culture supernatants of *A. brasilense* and *A. lipoferum*. However, analytical characterization of GA_1, GA_3 and iso-GA_3 in gnotobiotic cultures of *A. lipoferum* by capillary gas chromatography-mass spectrometry (GC-MS) with full scan (FS) or selected ion monitoring (SIM) has been reported (Bottini et al., 1989; Piccoli and Bottini, 1994; Piccoli et al., 1996). Also the GAs found in *A. brasilense* matched qualitatively and quantitatively those from *A. lipoferum* (Janzen et al., 1992).

Even though the levels of GAs produced by *Azospirillum* spp. in association with roots may be low, their significance should not be underestimated. In fact, Tanimoto (1987) showed that extremely low concentrations of GA_3 are involved in root growth promotion. These studies showed that in lettuce the amount of GA_3 required to promote recovery of growth in roots after GA synthesis had been inhibited by ancymidol application is 1 nM, while the amount necessary for shoots is 100 μM. Fulchieri et al. (1993) found that GA_3 had similar effects as *A. lipoferum* inoculation on promotion of root growth in 48 h-old maize seedlings, especially in increasing hair density in areas physiologically active for nutrient up-take and water absorption; moreover, inoculation had also substantial effects on the GA content of the maize seedling roots. Actually, *Azospirillum* spp. occurrence observed in roots, stems and leaves of maize and

rice dwarves was positively correlated with reversion of dwarfism in mutants of these species (Lucangeli and Bottini, 1996).

Uniconazole and other triazole-type plant growth regulators inhibit internode growth in a number of plant species (Izumi et al., 1985., Rademacher, 1991) by blocking the three oxidation steps from ent-kaurene to ent-kaurenoic acid in GA biosynthesis pathways, as has been proven in *Oryza sativa* L. seedlings and cell-free systems from *Lycopersicom esculentum* immature seeds and seedlings (Izumi et al., 1984 and 1985; Yamaji et al., 1991).

Based on the effects of inoculation with *A. lipoferum* and *A. brasilense* on the growth and endogenous GA content of the uniconazole treated plants of maize (*Zea mays* L.), the present study yielded evidence supporting the concept that GAs produced by *Azospirillum* spp. play an important role in the early stages of plant growth in Gramineae.

2. Material and Methods

Plant growth conditions

Seeds of maize (*Zea mays* L.) hybrid Dekalb 762 were surface-sterilized by soaking 3 min. in 1% sodium hypochlorite, and then washed with sterile distilled water to eliminate traces of sodium hypochlorite. Five seeds were planted (1 cm depth) in pots containing a perlite-sand (1:1) mixture. Thinning to three plants per pot was done after the full emergence of the first leaf. Plant pots were kept at field capacity by daily irrigation with sterile distilled water and once a week fertilized with 5 ml of half-strength Hoagland's solution. The growth conditions were 27±3°C under natural light in the greenhouse. When the plants reached the first leaf stage, the pots were treated as follows: a) control uniconazole; b) uniconazole + *A. lipoferum*; c) uniconazole + *A. brasilense* and d) control water. Five replicates per treatment were performed in a completely randomized statistical design. About 6 weeks later, the plants were carefully pulled out from the perlite/sand mixture and the root system rinsed for 5 sec in 0.06 M potassium phosphate buffer pH 7.0. Then, the plant height was measured, and fresh weight of roots and aerial parts were recorded and the whole plants immediately frozen in dry ice.

Bacterial inoculation

Azospirillum brasilense Cd (ATCC 29710) and *A. lipoferum* USA-5b (Dr. J. Döbereiner, EMBRAPA, Seropedica, Brazil) were grown in liquid LB (Luria Broth, Sigma Chem. Co.) medium at 30°C, 80 r.p.m. in an orbital rotary shaker to an OD (600 nm) of 1.0, corresponding to 3×10^8 bacteria/ml as determined by

plating. The cells were collected by centrifugation, washed twice in sterile 0.85% NaCl and resuspended in the original volume in MPCL (Marin-Prével-Charpentier-Lavigne) medium (Michiels et al., 1991). The plant pots were inoculated with 10^7 CFU/g of soil. Control plant pots were irrigated with an equivalent amount of MPCL medium alone at the time of inoculation. At the end of the experiments, the number of bacteria recovered from roots and aerial parts of the plants were estimated by using the most probable number technique with N-free semi-solid medium (NFb) (Baldani et al., 1986).

Application of chemicals

GA biosynthesis in maize seedlings was inhibited by supplying uniconazole [S-3307, (±), (E)-1-(4-chorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol; Sumitomo Chemical Co., Hyogo, Japan] to roots in subirrigation solution at a concentration of 80 µM (Nishijima et al., 1989). Treatment with uniconazole was initiated 7 d prior to GA_3 application or *Azospirillum* inoculation.

Plants were treated with GA_3 by applying 0.1 ml of an aqueous (10%, v/v, acetone, and 0.05%, v/v, Tween 20) solution containing 0.1, 1, 10 or 50 µg/plant of GA_3, to blades of leaves.

GA analysis in root extracts

The frozen plant material was homogenized and extracted overnight with methanol:water (4:1, v/v) at 4°C. After removal of the methanol in vacuo, 50 ng of 17,17-[$^2H_2$]-GA_3 (98% deuterium, Professor L.N. Mander, University of Adelaide, Australia) were added to the aqueous residues as internal standard and allowed 1 h equilibration. The aqueous residue was then partitioned as described by Fujioka et al. (1988). The ethyl acetate fraction (AE) was purified on a column of DEAE Sephadex A-25 as described by Gräbner et al. (1976). The column (25 ml) was eluted 6 times with 25 ml each of the following solvents: I) methanol, II) acetic acid 0.25 N in methanol, III) acetic acid 0.50 N in methanol, IV) acetic acid 0.75 N in methanol, V) acetic acid 1 N in methanol, and VI) acetic acid 3 N in methanol. Fractions II to VI were combined, concentrated and purified by HPLC on a Nucleosil 5 [$N(CH_3)_2$] column (Altech, 10 cm × 10 mm i.d.), eluted with 0.1 % (v/v) acetic acid in methanol at a flow rate of 1 ml/min; 15 fractions were collected every 2 min. The GA-like activity of the HPLC fractions was tested using the dwarf rice (Oryza sativa L.) cv. Tan-ginbozu microdrop assays modified by treatment with 80 µM uniconazole (Nishijima and Katsura, 1989). The resulting biological activity was mainly found in the fractions in which authentic [^{3}H]-GA_3 eluted. These fractions

were further analyzed by GC-MS-SIM as described by Piccoli and Bottini (1994). GA identification was made by comparison of the peak areas of the ions at a mass/charge (m/z) 506, 491, 447 for [$^2H_2$]-GA_3-MeTMSi and the ions at m/z 504, 489, 445 for [^{1}H]-GA_3-MeTMSi at the corresponding time, and quantification by comparison of the relative intensities of the respective molecular ions (506/504).

3. Results and Discussion

As expected, after treatment with uniconazole the growth of *Zea mays* L. plants was reduced (Table 1). The average height of plants treated with 80 µM uniconazole was 36% of that of untreated plants. The inhibition of growth was more remarkable in the aereal part (21% of fresh weight with respect to untreated) than in the roots (40%). There are similarities between our results and those of other workers who have examined the role of GAs through the use of inhibitors of their biosynthesis (Tanimoto, 1987; Yamaji et al., 1991). For instance, Tanimoto (1987) showed that ancymidol, a growth retardant which acts at the same point in the biosynthetic pathway as uniconazole (Rademacher, 1991), caused roots of lettuce seedlings to elongate more slowly and to swell behind the tip. The effects of ancymidol could be reversed by simultaneous application of GA_3.

Table 1. Plants of *Zea mays* L. treated with uniconazole 80 µM, then inoculated with *A. lipoferum* USA-5b, or *A. brasilense* Cd, or treated with GA_3

Treatment	Plant height (cm)	Root FW (g)	Shoot FW (g)
Uniconazole	10.8 a	1.28 a	0.6 a
Unic. + *A. lipoferum* USA-5b	14.6 b	1.70 b	1.2 b
Unic. + *A. brasilense* Cd	13.5 b	1.80 b	1.1 b
Unic. + GA_3 0.1 µg/plant	15.5 bc	1.70 b	0.9 ab
Unic. + GA_3 1 µg/plant	17.0 c	1.26 a	1.1 b
Unic. + GA_3 10 µg/plant	20.8 d	1.30 a	1.3 b
Unic. + GA_3 50 µg/plant	27.5 e	1.06 c	1.9 c
Control water	30.1 f	3.16 d	2.8 d

Values within columns followed by the same letter are not statistically different (Tukey 0.05).

Furthermore, inoculation with *A. lipoferum* and *A. brasilense* on plants treated with 80 μM uniconazole increased height at a similar level with respect to exogenous application of 0.1 μg/plant of GA_3. Also the stimulating effect on the growth of the root system was seen as an increase in fresh weight (Table 1). This was positively correlated with the occurrence of *A. lipoferum* and *A. brasilense* observed in roots and stems of plants (Table 2), confirming previous data on the presence of endophytic *Azospirillum* spp. (Baldani et al., 1986; Döbereiner, 1992, and 1993; Lucangeli and Bottini, 1996). The data suggest that uniconazole inhibited plant GA biosynthesis but probably not GA biosynthesis by *Azospirillum* spp. Although the effect of *Azospirillum* spp. might be metabolism of the uniconazole, GA biosynthesis by the bacteria is a well demonstrated fact (Bottini et al., 1989; Janzen et al., 1992). Moreover, our interpretation of the results are in agreement with dwarfism reversion previously found in *dwarf-1* maize (*Zea mays* L.) and *dwarf-x* rice (*Oryza sativa* L.) mutants by *Azospirillum* spp. (Lucangeli and Bottini, 1996).

Table 2. Number of cells/g FW, estimated by the most probable number technique (see Material and Methods), of *A. lipoferum* USA-5b and *A. brasilense* Cd in maize (*Zea mays* L.) plants treated with 80 μM uniconazole

	Roots	Shoots
A. lipoferum USA-5b	2×10^7	3×10^4
A. brasilense Cd	4×10^6	1×10^4
Control uniconazole	n.d	n.d

n.d = analyzed but not present or below a detectable concentration

Table 3. Quantification of GA_3 levels by GC-MS-SIM of their methyl ester trimethylsilyl derivatives in root extracts of *Zea mays* L. (see Material and Methods)

Treatment	Endogenous GA_3 in ng/g FW root
Control water	0.93
Control uniconazole	n.d
Uniconazole + *A. lipoferum*	0.70
Uniconazole + *A. brasilense*	0.88

n.d. = analyzed but not detected

Growth of the root system was inhibited by the highest GA concentration (50 μg/plant of GA_3) where significant promotion of growth of aerial parts took place (Table 1). This phenomenon may have resulted from the predominant consumption of nutrients by rapidly elongating hypocotyls and/or by the oversupply of auxin, the levels of which have been reported to be regulated by GAs (Law and Hamilton, 1984).

From the biologically active fractions, GA_3 was identified and quantified by GC-MS (Table 3). Gibberellin A_3 levels in plants treated with 80 μM uniconazole were obviously suppressed. However, fractions of root extracts of plants inoculated with either *A. lipoferum* or *A. brasilense* showed restoration of GA_3 levels similar to control water plant extracts. This observation suggests that uniconazole strongly inhibited steps of GA biosynthesis pathways between *ent*-Kaurene and *ent*-Kaurenoic acid in *Zea mays* L. plants as expected from the results of *Lycopersicom esculentum* Mill. seedlings (Yamaji et al., 1991). These results suggest that *Azospirillum* spp. inoculation affected GA status of *Zea mays* L. plant roots implying that production *per se* of GA_3 by *Azospirillum* is involved (Fulchieri et al., 1993; Lucangeli and Bottini, 1996).

The present results show direct evidence that GA_3 production by *Azospirillum* spp. was responsible for the promotion of growth.

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