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Azospirillum spp. metabolize [17,17-$^2H_2$]gibberellin A_{20} to [17,17-$^2H_2$]gibberellin A_1 in vivo in *dy* rice mutant seedlings

Fabricio D. Cassán [1], Carlos D. Lucangeli [1, 2], Rubén Bottini [1, 3] and Patricia N. Piccoli [1]

[1] *Laboratorio de Fisiología Vegetal, Departamento de Ciencias Naturales, Universidad Nacional de Río Cuarto, Campus Universitario, 5800 Río Cuarto, Argentina*

***Azospirillum* spp. are endophytic bacteria with beneficial effects on cereals — effects partially attributed to gibberellin production by the microorganisms. *Azospirillum lipoferum* and *Azospirillum brasilense* inoculated to rice *dy* mutant reversed dwarfism in seedlings incubated with [17,17-$^2H_2$]GA_{20} with formation of [17,17-$^2H_2$]GA_1, showing the in vivo capacity to perform the 3β-hydroxylation. When Prohexadione-Ca, an inhibitor of late steps in gibberellin biosynthesis, was added to the culture medium, no complementation was observed and no [17,17-$^2H_2$]GA_1 was produced. The latter suggests that the bacterial operating enzyme may be a 2-oxoglutarate-dependent dioxygenase, similar to those of plants.**

Key words: *Azospirillum* spp. — Dwarf Rice (*Oryza sativa* L.) *dy* mutant — Gibberellin 3β-hydroxylation.

Abbreviations: CFU, colony forming units; Me-TMSi, methyl ester-trimethylsilyl ether; NFb, nitrogen free biotin-based medium; OD_{540}, optical density at 540 nm.

Introduction

Although there are more than 130 gibberellins known, works with mutants and inhibitors of the gibberellin biosynthesis led to the conclusion that few are active per se, the others being precursors or byproducts (Ingram et al. 1984, Spray et al. 1984, Phinney 1985, Fujioka et al. 1990, Nakayama et al. 1991). In fact, a deficiency in concentration of GA_1, GA_3 and/or GA_4 is correlated with proportional decreases of length in plant internodes. While the increment in the levels of any of these gibberellins is expressed in bigger and more vigorous shoot elongation (Reid 1993, Zeevaart et al. 1993). In the fungus *Gibberella fujikuroi* GA_1 and GA_3 are formed from GA_4 in a metabolic pathway known as early-3-hydroxylation (Crozier 1982). In maize, however, GA_{20} is the immediate precursor of GA_1 and of GA_3 via GA_5 (Smith et al. 1991) in the early-13-hydroxylation pathway. Williams et al. (1998) suggested that cDNA corresponding *Arabidopsis GA4* gene (Chiang et al. 1995) encodes a 3β-hydroxylase able to catalyze the conversion of GA_{20} to GA_1. Similar results have been obtained for the gene *LE* of *Pisum sativum* (Lester et al. 1997). Fungal 3β-hydroxylation is catalyzed by cytochrome P450 monoxigenases while plant gibberellin 3β-hydroxylases are 2-oxoglutarate-dependent dioxygenases (Hedden 1999).

Azospirillum spp. are rhizospheric and/or endophytic bacteria that improve growth and yield of several cereals (Okon and Labandera-González 1994). The bacteria penetrate the roots and allocate in intercellular spaces of both roots and leaves, as well as in the vascular system of the infected plants (Okon et al. 1977, Patriquin et al. 1983). Phytohormone production by the microorganism (reviewed in Glick et al. 1999) is among the proposed factors in order to explain such improvement, although most of the attention has been focussed on auxin, cytokinin and ethylene. However, *Azospirillum* spp. produce GA_1 and GA_3 in vitro in chemically defined media (Bottini et al. 1989, Jansen et al. 1992). By working with *Azospirillum* spp. and gibberellin deficient plants, either dwarf mutants of maize and rice (Lucangeli and Bottini 1996) or maize individuals treated with Uniconazole (Izumi et al. 1985), the inhibitor of GA synthesis (Lucangeli and Bottini 1997), it has been shown that gibberellins produced by these bacteria affects the plant growth. Based on metabolic studies in vitro, Piccoli et al. (1996) suggested that in *Azospirillum* spp. GA_1 and GA_3 could be produced from different metabolic precursors, GA_1 being the result of the 3β-hydroxylation of GA_{20} (Piccoli and Bottini 1994) while GA_3 could come from GA_9 in a early non-hydroxylation pathway (Piccoli et al. 1996). This is also sustained because the production of GA_1 and GA_3 in cultures of *Azospirillum lipoferum* is differentially stimulated by blue light (Piccoli and Bottini 1996). However, actual evidence of the in vivo occurrence of such processes has to be demonstrated.

The rice (*Oryza sativa* L. cv. Waito-C) *dy* dwarf mutant possesses a genetic blockage in the *dy* gene that controls the 3β-hydroxylation of GA_{20} to GA_1 (Kobayashi et al. 1989, Kobayashi et al. 1994). This mutant allowed designing an experimental model valid to demonstrate the in vivo metabolic capacity of the microorganism to transform biologically inactive gibberellin precursors to bioactive gibberellins. The present work provides evidence related to the 3β-hydroxylation of GA_{20} by *Azospirillum* spp. in seedlings of *dy* rice inoculated with these bacteria, and the possible mechanism is proposed in the response of the plant to bacterial inoculation.

[2] Present address: Estación Experimental Agropecuaria San Pedro, Instituto Nacional de Tecnología Agropecuaria, 3290 San Pedro, Argentina.
[3] Corresponding author: E-mail, rbottini@exa.unrc.edu.ar; Fax, +54-358-4676230.

Materials and Methods

Biological material

The bacterial strains used were *A. lipoferum* strain USA 5b (a gift from Dr. V. Baldani, EMBRAPA Brasil) and *Azospirillum brasilense* strain Cd (ATCC 29710). The rice (*Oryza sativa* L. cv. Waito-C) dwarf *dy* mutant used was a gift from Dr. M. Koshioka, National Research Institute of Vegetables, Ornamental Plants and Tea.

Bacterial growth

Both *Azospirillum* spp. strains were grown in nitrogen free biotin-based (NFb) medium with malic acid (5 g liter^{-1}) and NH_4Cl (1.25 g liter^{-1}) as described by Piccoli et al. (1996), in a water bath with orbital shaking (80 rpm) at 30°C, until reaching an OD_{540} of 1.0 and corresponding to a concentration of approximately 10^8 colony forming units (CFU) ml^{-1} as determined by growth in NFb agar plates. Bacteria were harvested by centrifugation at 8,000 rpm for 15 min at 4°C. Cell pellet was washed twice with 0.85% NaCl and re-suspended in the same volume of 0.05 M phosphate buffer, in order to obtain a titer of 3×10^6 CFU ml^{-1} for further inoculation.

Labeled gibberellins

[17,17-$^2H_2$]GA_{20} was provided by Prof. L. Mander, University of Adelaide, Australia, and [17-^{3}H]GA_{20} was a gift from Prof. R. P. Pharis, University of Calgary, Canada.

Seedling growth

Seeds of rice were surface-sterilized by immersion in 70% (v/v) ethanol for 20 s, then in 2% (v/v) of Na hipochloryde for 20 min, and finally washed thoroughly with sterile distilled water. Seeds were pregerminated in Uniconazole (S-3307D, Sumitomo Chem. Co., Nagoya, Japan) 80 μM for 48–72 h at 30°C. The use of Uniconazole (an inhibitor of *ent*-Kaurene oxidation) increases the seedling sensitivity to exogenous gibberellins, since the low gibberellin content in the mutants is depleted even more. Selected germinated seedlings were then washed extensively with sterile distilled water and sowed in 20×200 mm glass tubes (one seed per tube, five tubes per treatment) containing Fahraëus (1957) solution. Some of the treatments included the addition of 20 mg liter^{-1} of Prohexadione-Ca (BX-112, a gift from W. Rademacher, BASF, Linburgerof, Germany) in the medium. The seedlings were then incubated for 48 h at 30°C under continuous cold-fluorescent light (2,000–3,000 lux) and 100% RH.

Treatments

After 72 h of growing under the conditions described above, the seedlings were inoculated via the roots with *A. lipoferum* strain USA 5b or *A. brasilense* strain Cd, in 100 μl of 0.05 M phosphate buffer containing a bacterial titer of 3×10^6 CFU plant^{-1}. After another 72 h, 1 μg of [17,17-$^2H_2$]GA_{20} plus 6,000 dpm of [17-^{3}H]GA_{20} were added dissolved in 1 μl 95% ethanol to the first leaf of each seedling with the aid of a microsyringe. The treatments were done with and without BX-112 in the medium. Controls were carried out with: (i) only phosphate buffer and ethanol (absolute control); (ii) hormone alone; (iii) inoculated with *A. lipoferum* USA 5b; (iv) inoculated with *A. brasilense* Cd; (v) BX-112 in the medium; (vi) hormone plus BX-112 in the medium. All the treatments were done in triplicate and under aseptic conditions.

Growth data and statistical analysis

Seventy-two h after application of the growth regulators, the growth parameters second leaf length and root length of seedlings were evaluated. Values were analyzed by a posteriori Tuckey Test with $p \leq 0.05$.

Bacterial count in stems and roots

Bacterial count was carried out in stems and roots from one seedling from each treatment in agar plates with NFb medium. Tissues were macerated in a mortar with buffer phosphate 0.05 M in a dilution 1/10. Decimal dilutions in the same buffer were sowed in duplicate at 0.1 ml plate^{-1} containing NFb agar medium. After incubation for 72 h at 30°C the bacterial count was carried out based on colony morphology.

Gibberellin purification

Five seedlings from each treatment were taken for gibberellin analysis and freeze dried. The frozen material was homogenized with 0.5 liter of methanol : H_2O (4 : 1) at 4°C. After 24 h the homogenized material was filtered and the residue re-extracted. Filtrates were combined and the methanol evaporated. Then, aqueous solution was adjusted to pH 2.5 and partitioned four times with the same volume of water (pH 2.5) saturated ethyl acetate. Ethyl acetate was evaporated and redissolved in 1 ml of acetic acid : methanol : H_2O (0.1 : 10 : 89.9), filtered through 0.45 μm membranes and injected in a HPLC Konik Model KNK-500 (Konic Inc.) with a C_{18} reverse phase (μBondapack, 300×3.9 mm, Waters Associates) column. The elution was done at 2 ml min^{-1} with a gradient of 10–73% methanol in 1% of acetic acid. Fractions at 19–32 min showing radioactivity were collected, grouped, evaporated, re-suspended in 1 ml of 0.1% acetic acid in methanol and injected in a second HPLC with a column of Nucleosil 5[$N(CH_3)_2$] (15 cm × 4.6 mm, Altech). Elution was carried out with 0.1% acetic acid in methanol, with a flow of 1 ml min^{-1}. Twenty fractions of 2 ml were collected, evaporated and re-suspended in 80% methanol.

Radio counting

To quantify radioactivity, aliquots of 100 μl from each HPLC fraction were dissolved in 4 ml of scintillation cocktail and radioactivity was measured with a Beckman Instruments, Model LS 6000 IC, radio counter.

Gibberellin determination by GC-MS

Radioactive fractions from the Nucleosil HPLC were grouped, evaporated with N_2 and derivatized to methyl ester-trimethylsilyl ether (Me-TMSi). Derivatization was done first with 10–20 ml methanol and 50–100 μl of fresh CH_2N_2 and left for 30 min at room temperature; after solvents had been eliminated under N_2 and freeze dried, the samples were treated with 10 μl of dry pyridine and 50–100 μl *N,O-bis* trimethylsilyl trifluroacetamide plus 1% trimethylchlorosilane and heated for 30 min at 80°C. After solvent evaporation extracts were dissolved in 5 μl hexane, and 1 μl was injected split-splitless in a HP-1 cross-linked methyl silicone capillary column (25 m length × 0.25 mm internal diameter × 0.22 μm film thickness) fitted in a HP 5890 Series II GC with a capillary direct interface to a 5970B mass selective detector. The GC temperature program was 100–195°C at 15°C min^{-1}, then to 260°C at 4°C min^{-1}. In each case full scan spectra of [17,17-$^2H_2$]GA_{20} and [17,17-$^2H_2$]GA_1 were obtained or characteristic ions were monitored for [17,17-$^2H_2$]GA_3, [17,17-$^2H_2$]GA_8 and [17,17-$^2H_2$]GA_{29} at the proper retention times. Tentative quantification was done by calculation of the area for the respective $[M]^+$.

Results and Discussion

The growth response of rice *dy* mutant seedlings treated with [17,17-$^2H_2$]GA_{20} and/or inoculated with *A. brasilense* Cd and *A. lipoferum* USA 5b is observed in Fig. 1. Growth of second leaf sheath was slightly improved with respect to the con-

Table 1 Estimation of deutero gibberellins in *dy* mutant seedlings fed with [17,17-$^2H_2$]GA_{20}, control and inoculated with *A. lipoferum* USA 5b or *A. brasilense* Cd, or treated with Prohexadione-Ca (BX-112)

Treatments	M+ 420 area × 10^3 [$^2H_2$]GA_{20}	M+ 508 area × 10^3 [$^2H_2$]GA_1
[17,17-$^2H_2$]GA_{20} control	1,680	30
[17,17-$^2H_2$]GA_{20} control + BX-112	1,830	nd
[17,17-$^2H_2$]GA_{20} + *A. lipoferum* USA5b	292	3,426
[17,17-$^2H_2$]GA_{20} + *A. lipoferum* USA5b + BX-112	560	nd
[17,17-$^2H_2$]GA_{20} +*A. brasilense* Cd	221	2,095
[17,17-$^2H_2$]GA_{20} +*A. brasilense* Cd + BX-112	2,220	nd

nd, non-detected.

trol seedlings (treatment 1, Fig. 1) by the inoculation with *Azospirillum* spp. (treatments 9 and 10, Fig. 1). This promotion, although not significant, may be due to gibberellin production by *Azospirillum*, as it has been demonstrated both in vitro in chemical-defined media (Bottini et al. 1989) and in vivo (Lucangeli and Bottini 1996, Lucangeli and Bottini 1997). In addition, the root length of seedlings was larger (data not shown).

When [17,17-$^2H_2$]GA_{20} was applied alone (treatment 3, Fig. 1) it also stimulated shoot internode elongation. Even though the *dy* mutant possesses a blockage in the conversion of GA_{20} to GA_1 (Kobayashi et al. 1989), the mutant is leaky and produces some GA_1. The original bioassay showed that GA_{20} is 2% as active as GA_1 in promoting shoot elongation in the rice (Murakami 1972, Nishijima and Katsura 1989).

Notwithstanding, those seedlings treated with [17,17-$^2H_2$]GA_{20} and inoculated with *A. brasilense* Cd or *A. lipoferum* USA 5b showed the highest significant increases (treatments 5 and 7, Fig. 1). *Azospirillum brasilense* showed to be more effective, and this was correlated with a higher number of bacteria in the infected plants (Table 2).

However, when the inoculated and GA_{20}-fed seedlings were cultured in the presence of Prohexadione-Ca (Nakayama et al. 1990a, Nakayama et al. 1990b), an inhibitor of 3β-hydroxylation, they showed less growth (treatments 6 and 8, Fig. 1) similar to the control (treatment 1, Fig. 1).

After incubation the rice seedlings treated with [17,17-$^2H_2$]GA_{20} and inoculated with *A. brasilense* Cd and *A. lipoferum* USA 5b were extracted and the gibberellin fraction was analyzed by, either full scan GC-MS or selected ion current monitoring, [17,17-$^2H_2$]GA_1 (*m/z* 508, 493, 450, 378, 315, 239, 225, 209) were found at the same retention times and matching the abundance of those of authentic standard. No ions corresponding to [17,17-$^2H_2$]GA_3 (*m/z* 506, 491, 447) or [17,17-$^2H_2$]GA_{29} (*m/z* 508, 493, 377) were found at the expected reten-

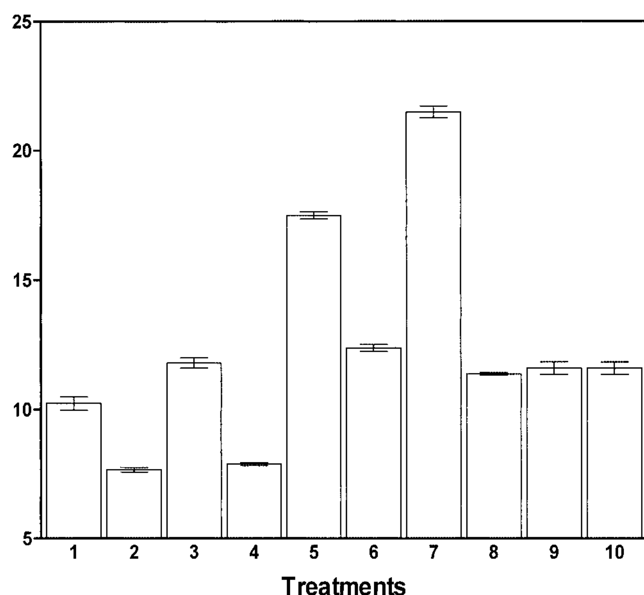

Fig. 1 Second leaf growth (in mm) of *dy* mutant seedlings pretreated with Uniconazole (for details see Materials and Methods). 1, Control (buffer phosphate 0.05 M + 1 µl ethanol 95%); 2, BX-112 20 mg liter^{-1} added to the Faraëhus medium; 3, 1 µg [17,17-$^2H_2$]GA_{20} plant^{-1}; 4, 1 µg [17,17-$^2H_2$]GA_{20} plant^{-1} + BX-112 20 mg liter^{-1}; 5, 1 µg [17,17-$^2H_2$]GA_{20} plant^{-1} + *A. lipoferum* USA 5b; 6, 1 µg [17,17-$^2H_2$]GA_{20} plant^{-1} + *A. lipoferum* USA 5b + BX-112 20 mg liter^{-1}; 7, 1 µg [17,17-$^2H_2$]GA_{20} plant^{-1} + *A. brasilense* Cd; 8, 1 µg [17,17-$^2H_2$]GA_{20} plant^{-1} + *A. brasilense* Cd + BX-112 20 mg liter^{-1}; 9, *A. brasilense* Cd; 10, *A. lipoferum* USA 5b.

Table 2 Bacterial assessment 72 h after inoculation in *dy* mutant seedlings

Strain	Fraction	CFU g^{-1}
A. lipoferum USA 5b	Root	8×10^6
A. lipoferum USA 5b	Stem and leaves	1×10^4
A. brasilense Cd	Root	3×10^7
A. brasilense Cd	Stem and leaves	7×10^5
Buffer 0.05 M	Root, stem and leaves	nd

nd, non-detected.

tion times. Characteristic ions for [17,17-$^2H_2$]GA_8 (e.g. *m/z* 596, 581, 450) were found, although their abundance was low. A rough estimation of the presence for [17,17-$^2H_2$]GA_{20} and [17,17-$^2H_2$]GA_1 is shown in Table 1, based on the calculation of areas for the respective parent ions from injection of sample aliquots. In those plants inoculated with *Azospirillum* spp., a relatively abundant amount of [17,17-$^2H_2$]GA_1 was found. However, the presence of Prohexadione-Ca (BX-112) in the medium completely abolished the $GA_{20} \Rightarrow GA_1$ conversion.

In Table 2 the number of bacteria alive in root and aerial parts of the seedlings is shown. In inoculated seedlings the presence of endophytic *Azospirillum* spp. was found. Root tissues contained more bacteria than stem and leaves. Also there were more bacteria in seedlings infected with *A. brasilense* Cd than in those inoculated with *A. lipoferum* USA 5b, in correlation with the growth response of the seedlings to which [17,17-$^2H_2$]GA_{20} had been added (treatments 5 and 7, Fig. 1).

Thus, the results of this work prove that the reversion of genetic dwarfism in seedlings of rice (*Oryza sativa* L.) *dy* mutant inoculated with *A. brasilense* Cd and *A. lipoferum* USA 5b is due to 3β-hydroxylation performed by the bacteria. The finding of [17,17-$^2H_2$]GA_8 as a minor catabolite may be due to the conversion of GA_1 to GA_8 by the plant, since in the previous in vitro work GA_8 has not been obtained as a product of GA_{20} by bacteria (Piccoli and Bottini 1994, Piccoli et al. 1996). The characterization of [17,17-$^2H_2$]GA_1 (the main GA active in regulating stem elongation in rice; Kobayashi et al. 1989) in inoculated seedlings correlated with reversion of genetic dwarfism observed 72 h after [17,17-$^2H_2$]GA_{20} application. These results are confirmatory of those obtained in vitro by Piccoli and Bottini (1994) regarding the 3β-hydroxylating capacity of *Azospirillum* spp. grown in chemically-defined medium. This 3β hydroxylating step seems to be performed by a 2-oxoglutarate-dependent dioxygenase, like those found in plants (Hedden 1999), since Prohexadione-Ca inhibits the conversion of GA_{20} oxidase and GA 3β-hydroxylase (Nakayama et al. 1990a, Nakayama et al. 1990b).

The fact that [17,17-$^2H_2$]GA_3 had not been found also suggests that GA_3 and GA_1 come from different pathways in the bacterium metabolism, as has been previously postulated (Piccoli and Bottini 1996, Piccoli et al. 1996).

The results presented in this work and other previous studies (Bottini et al. 1989, Jansen et al. 1992, Fulchieri et al. 1993, Piccoli and Bottini 1994, Lucangeli and Bottini 1996, Piccoli and Bottini 1996, Piccoli et al. 1996, Lucangeli and Bottini 1997) sustain the idea of a direct involvement of increased levels of physiologically active gibberellins in plants inoculated with the microorganism in the promotion of the plant growth and yield.

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