# *IN VIVO* GIBBERELLIN $A_9$ METABOLISM BY *AZOSPIRILLUM* SP. IN *dy* DWARF RICE MUTANT SEEDLINGS

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## ABSTRACT

*Azospirillum* sp. are bacteria beneficial to inoculated plants, and the effect is partially attributed to gibberellin production. Deutero-labelled $GA_9$ was fed to *dy* mutant rice-dwarf seedlings inoculated with *A. lipoferum* or *A. brasilense* and/or treated with Prohexadione-Ca, an inhibitor of late steps in gibberellin biosynthesis. According to the shoot growth response, the mutant showed the ability to perform 3?-hydroxylation of the d2$GA_9$ fed. This ability was partially blocked by Prohexadione-Ca, suggesting that $GA_9$ may have some activity *per se*. After the feeding was done along with *Azospirillum* sp. inoculation, the bacteria performed 3?-hydroxylation as shown by shoot growth promotion and corroborated by GC-MS detection of d2$GA_3$. The growth promotion was blocked by Prohexadione-Ca but in a lower extent as compared with non-inoculated controls, suggesting that the inhibitor worked on the 3?-hydroxylation performed on the substrate fed but on the microorganism capacity to produce "active" gibberellins.


***Keywods:*** *Azospirillum, gibberellins, rice*

## INTRODUCTION

Although there are more than 130 gibberellins (GAs) known, works with mutants and inhibitors of the GA biosynthesis led to the conclusion that few are active *per se*, being the others precursors or byproducts (Nakayama et al., 1991). In the fungus *Gibberella fujikuroi*, the GA activation takes place through $GA_1$ and $GA_3$ formation from $GA_9$ via $GA_4$ by 3?-hydroxylation (Crozier 1982). In corn, however, $GA_{20}$ is the immediate precursor for $GA_1$ (Spray et al. 1984) and of $GA_3$ via $GA_5$ (Smith et al. 1991). In rice it also occurs the $GA_{20}$ conversion (and "activation") to $GA_1$ through 3?-hydroxylation (Kobayashi et al. 1989). However, fungal 3?-hydroxylation is catalyzed by membrane-linked cytochrome P450 monoxigenases while plant GA 3?-hydroxylases are 2-oxoglutarate dependent soluble dioxygenases (Hedden et al. 1999).

Among the different substances known to block the GA pathway Prohexadione-Ca is an acylciclohexatrione which "mimics" the 2-oxoglutarato structure, thus inhibiting the 3?-hydroxylative activating step (Nakayama et al. 1991). In fact, the enzymatic kinetics demonstrates that the acylciclohexatrione is highly competitive with regard to 2-oxoglutarate, which is co-substrate of the dioxygenases. The GA biosynthesis in the fungi *G. fujikuroi* and *Sphaceloma manihoticola*, contrary to the higher plants, is not affected by the acylciclohexatrione. This is explained as Prohexadione-Ca undergoes rapid disintegration in the fungi liquid cultures but also by the fact that cyclases and momooxigenases, but not dioxygenases, have been identified in *G. fujikuroi* (Hedden et al. 1999).

*Azospirillum* spp. are rhizospheric and/or endophytic bacteria that improve growth and yield of several cereals (Okon and Labandera-Gonzáles 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. 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* sp. produce $GA_1$ and $GA_3$ *in vitro* in chemically defined

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media (Bottini et al. 1989, Jansen et al. 1992). 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, being $GA_1$ the result of the 3?-hydroxylation of $GA_{20}$ (Piccoli and Bottini, 1994) while $GA_3$ could come from $GA_9$ in a early non-hydroxylative pathway (Piccoli et al. 1996). This is also sustained because the production of $GA_1$ and $GA_3$ in cultures of *A. lipoferum* is differentially stimulated by blue light (Piccoli and Bottini, 1996). However, actual evidence *in vivo* occurrence processes has to be demonstrated. Recently it has been found that *Azospirillum* sp. metabolize $d2GA_{20}$ to $d2GA_1$ *in vivo* in *dy* rice mutant seedlings (Cassán et al., 2001a and b).

The *dy* dwarf mutant of rice (*Oryza sativa* L.) possesses a genetic blockage in the *dy* gene that controls the 3?-hydroxylation of $GA_{20}$ to $GA_1$ (Kobayashi et al. 1994). The present work provides evidence related to the 3?-hydroxylation of $GA_9$ by *Azospirillum* sp. in seedlings of the *dy* mutant inoculated with these bacteria, and confirm previous results that such mechanism is present in the response of the plant to bacterial inoculation.

## MATERIAL AND METHODS

The bacterial strains used were: *Azospirillum lipoferum* strain USA 5b (a gift from Dr. V. Baldani, EMBRAPA, Brasil) and *A. 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 Resarch Institute of Vegetables, Ornamental Plants and Tea, Tsukuba, Japan. Both *Azospirillum* sp. strains were grown in nitrogen free biotin-based (NFb) medium with malic acid (5 g $l^{-1}$) and $NH_4Cl$ (1.25 g $l^{-1}$) as described by Piccoli et al. (1996), in a water bath with orbital shaking (80 rpm) at 30 °C, until an $OD_{540}$ of 1.0 corresponding to a concentration of ca. $10^8$ CFU (colony forming units) $ml^{-1}$. Bacteria were harvested by centrifugation at 8000 rpm 15 min and 4 °C. Cell pellet was washed twice with 0.85 % NaCl and re-suspended in the same volume of buffer phosphate 0.05 M for further inoculation. The ?17,17-$^2H_2$?$GA_9$ was provided by Prof. L. Mander, University of Adelaide, Australia, and ?$^3$H?$GA_9$ was a gift of Prof. R. P. Pharis, University of Calgary, Canada. Seeds of rice were disinfected, pre-germinated and sowed as previously reported (Cassán et al. 2001b) in (20 x 200 mm) glass tubes (one seed per tube, 5 tubes per treatment) containing Fahraëus (1957) solution. Some of the treatments included the addition of 20 mg $l^{-1}$ of Prohexadione-Ca (BX-112, a gift of W. Rademacher, BASF, Linburgerof, Germany) in the medium. Seedlings were then incubated 48 h at 30 °C under continuous cold-fluorescent light (2000-3000 lux) and 100 % RH. After 72 h of growing under the conditions of above, the seedlings were inoculated via the roots with *A. lipoferum* strain USA 5b or *A. brasilense* strain Cd, in 100 ?l of phosphate buffer 0.05 M containing a bacterial titer of 3 $10^6$ CFU $plant^{-1}$. After another 72 h 1 ?g of ?17,17-$^2H_2$?$GA_9$ plus 6000 dpm of ?17-$^3$H?$GA_9$ were added dissolved in 1 ?l 95 % ethanol to the first leaf of each seedling with the aid of a microsyringe. The treatments were done with and without Prohexadione-Ca in the medium. Controls were carried out with only buffer phosphate and ethanol (absolute control, treatment 1 in Figure 1), Prohexadione-Ca alone (treatment 2), ?17,17-$^2H_2$?$GA_9$ alone (treatment 3), ?17,17-$^2H_2$?$GA_9$ plus Prohexadione-Ca (treatment 4), inoculated with *A. brasilense* Cd (treatment 9), inoculated with *A. lipoferum* USA 5b (treatment 10). All the treatments were done by triplicate and under aseptic conditions. At 72 h after application of the growth regulators second leaf length and root length of seedlings were measured. Values were analyzed by *a posteriori* Tuckey Test with p ? 0.05. For GA purification five seedlings from each treatment were taken and freeze dried. The frozen material was homogenized, extracted and purified as informed (Cassán et al. 2001b). To quantify radioactivity, aliquots of 100 ?l from each HPLC fraction were dissolved in 4 ml of scintillation cocktail and radioactivity was measured with a Beckman Instruments, Mod. LS 6000 IC, radio counter. Radioactive fractions of the purified GA extracts were derivatized and analyzed by GC-MS as previously reported (Cassán et al. 2001b). In each case characteristic ions were monitored for ?17,17-$^2H_2$?$GA_1$, ?17,17-$^2H_2$?$GA_3$, ?17,17-$^2H_2$?$GA_4$, ?17,17-$^2H_2$?$GA_8$ and ?17,17-$^2H_2$?$GA_9$ at the proper retention times. Tentative quantification was done by calculation of the area for the respective $[M]^+$.

## RESULTS AND DISCUSSION

Growth response of rice *dy* mutant seedlings treated with ?17,17-$^{2}H_{2}$?$GA_{9}$ and/or inoculated with *A. brasilense* Cd and *A. lipoferum* USA 5b, and/or aditionof Prohexadione-Ca is observed in Figure 1.

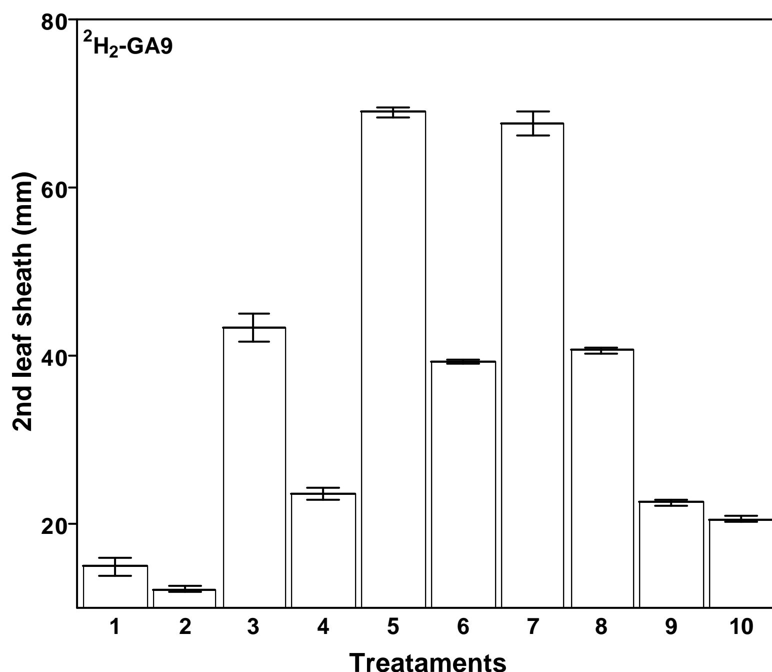


**Figure 1.** Second leaf growth (in mm) of *dy* mutant seedlings. **1,** Control (buffer phosphate 0.05 M + 1 ?l ethanol 95 %); **2,** Prohexadione-Ca 20 mg $l^{-1}$; **3,** 1 ?g ?17,17-$^{2}H_{2}$?$GA_{9}$ $plant^{-1}$; **4,** 1 ?g ?17,17-$^{2}H_{2}$?$GA_{9}$ $plant^{-1}$ + Pprohexadione-Ca 20 mg $l^{-1}$; **5,** 1 ?g ?17,17-$^{2}H_{2}$?$GA_{9}$ $plant^{-1}$ + *A. lipoferum* USA 5b; **6,** 1 ?g ?17,17-$^{2}H_{2}$?$GA_{9}$ $plant^{-1}$ + *A. lipoferum* USA 5b + Prohexadione-Ca 20 mg $l^{-1}$; **7,** 1 ?g ?17,17-$^{2}H_{2}$?$GA_{9}$ $plant^{-1}$ + *A. brasilense* Cd; **8,** 1 ?g ?17,17-$^{2}H_{2}$?$GA_{9}$ $plant^{-1}$ + *A. brasilense* Cd + Prohexadione-Ca 20 mg $l^{-1}$; **9,** *A. brasilense* Cd; **10,** *A. lipoferum* USA 5b.

Growth of second leaf sheath was improved respect to the control seedlings (treatment 1) by the inoculation with *Azospirillum* sp. (treatments 9 and 10). This promotion may be due to GA 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 and 1997). Also root length of seedlings was higher (data not shown).

When ?17,17-$^{2}H_{2}$?$GA_{9}$ was applied alone (treatment 3) it estimulated shoot internode elongation significantly. Even though the *dy* mutant possess a blockage in the 3?-hydroxylative step (Kobayashi et al. 1989), this is not absolute as $GA_{9}$ is relatively active as compared to $GA_{1/3}$ in promoting shoot elongation in rice (Murakami 1972). Since the seedlings were fed with an excess of $GA_{9}$ (1?g $plant^{-1}$), this amount may be sufficient to promote the measured elongation. Alternatively, the results also suggests that $GA_{9}$ may be active *per se*. Notwithstanding, those seedlings treated with ?17,17-$^{2}H_{2}$?$GA_{9}$ and inoculated with *A. brasilense* Cd or *A. lipoferum* USA 5b showed the highest significant increases (treatments 5 and 7). However, when the inoculated and hormone-fed seedlings were cultured in the presence of the late step of GA biosynthesis inhibitor, Prohexadione-Ca, they showed less growth (treatments 6 and 8 ) similar to the [17,17-$^{2}H_{2}$]GA9 treatment (Figure 1, treatment 3). Again, this fact suggests tha $GA_{9}$ may have the intrinsic capacity of being active *per se*.

When the rice seedlings treated with ?17,17-$^{2}H_{2}$?$GA_{9}$ and inoculated with *A. brasilense* Cd and *A. lipoferum* USA 5b were analyzed for GA content, characteristic ions of ?17,17-$^{2}H_{2}$?$GA_{3}$ (m/z m/z 506, 491, 447) were found at the retention times and matching the abundance of those of authentic standard. No

ions for ?17,17-$^2H_2$?$GA_1$ (e.g., m/z 508, 493, 450) or ?17,17-$^2H_2$?$GA_4$ (i.e., m/z 420, 286, 228) were found at the proper retention times in treatments fed with ?17,17-$^2H_2$?$GA_9$. An estimation of the presence for ?$^2H_2$?$GA_9$ and ?$^2H_2$?$GA_3$ is shown in Table 1.

**Table 1.** Estimation of deutero gibberellins in *dy* mutant seedlings fed with ?17,17-$^2H_2$?$GA_9$, control and inoculated with *A. lipoferum* USA 5b or *A. brasilense* Cd.

| Treatments | M+ 332 area x $10^3$ [$^2H_2$?$GA_9$ | M+ 506 area x $10^3$ [$^2H_2$?$GA_3$ |
|---|---|---|
| ?17,17-$^2H_2$?$GA_9$ control | 1915 | 70 |
| ?17,17-$^2H_2$?$GA_9$ + *A. lipoferum* USA5b | 1267 | 485 |
| ?17,17-$^2H_2$?$GA_9$ +*A. brasilense* Cd | 915 | 720 |

Based on the calculation of areas for the respective parent ions from injection of sample aliquots, in those plants inoculated with *Azospirillum* sp., a relatively abundant amount of ?17,17-$^2H_2$?$GA_3$ was found. However, the presence of Prohexadione-Ca in the medium completely abolished the $GA_9$ ? $GA_3$ conversion, since the latter was not detected (data not shown).

In inoculated seedlings the presence of endophytic *Azospirillum* sp. was found. Root tissues contained more bacteria than stem and leaves (data not shown). 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 3?-hydroxylation performed by the bacteria. Characterization of ?17,17-$^2H_2$?$GA_3$ in inoculated seedlings correlated with reversion of genetic dwarfism observed 72 h after ?17,17-$^2H_2$?$GA_9$ application. These results are confirmatory of those obtained *in vitro* by Piccoli and Bottini (1994) and *in vivo* Cassán et al. (2001b) regarding the 3?-hydroxylating capacity of *Azospirillum* spp. grown in chemically-defined medium and inoculated to *dy* seedlings. This 3? hydroxylating step seems to be performed by a dioxygenase oxoglutarate dependent, like those found in plants (Hedden 1999), since the process was abolished by the presence of Prohexadione-Ca (results presented in this paper and those of Cassán et al. 2001b). The fact that ?17,17-$^2H_2$?$GA_1$ had not been found in treatments fed with ?17,17-$^2H_2$?$GA_9$ also confirms that $GA_3$ and $GA_1$ come from different pathways in the bacterium metabolism, as it has been previously postulated (Piccoli and Bottini 1996, Piccoli et al. 1996, Cassán et al., 2001b). The results presented in this work and other previous (Bottini et al. 1989, , Janzen et al. 1992, Lucangeli and Bottini 1997, Piccoli and Bottini 1994 and 1996, Piccoli et al. 1996, Cassán et al. 2001a and b) sustain the idea of a direct involvement of increased levels of GAs physiologically active in plants inoculated with the microorganism in the promotion of the plant growth and yield.

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