Azospirillum brasilense and *Azospirillum lipoferum* Hydrolyze Conjugates of GA_{20} and Metabolize the Resultant Aglycones to GA_1 in Seedlings of Rice Dwarf Mutants[1]

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Azospirillum species are plant growth-promotive bacteria whose beneficial effects have been postulated to be partially due to production of phytohormones, including gibberellins (GAs). In this work, *Azospirillum brasilense* strain Cd and *Azospirillum lipoferum* strain USA 5b promoted sheath elongation growth of two single gene GA-deficient dwarf rice (*Oryza sativa*) mutants, *dy* and *dx*, when the inoculated seedlings were supplied with [17,17-$^2H_2$]GA_{20}-glucosyl ester or [17,17-$^2H_2$]GA_{20}-glucosyl ether. Results of capillary gas chromatography-mass spectrometry analysis show that this growth was due primarily to release of the aglycone [17,17-$^2H_2$]GA_{20} and its subsequent 3β-hydroxylation to [17,17-$^2H_2$]GA_1 by the microorganism for the *dy* mutant, and by both the rice plant and microorganism for the *dx* mutant.

Azospirillum spp. are considered to be important plant growth promotive rhizobacteria that can improve the growth and yield of at least several plant species (Okon and Labandera-González, 1994). However, the mechanism by which *Azospirillum* spp. and other promotive rhizobacteria promote plant growth has yet to be elucidated (Glick et al., 1999, and literature cited therein). Phytohormone production (Tien et al., 1979; Okon and Kapulnik, 1986), including gibberellins (GAs; Bottini et al., 1989; Fulchieri et al., 1993; Lucangeli and Bottini, 1997), is one mechanism that has been proposed.

GAs are a class of phytohormones with many demonstrated effects on a number of physiological processes (Davies, 1995). Among the 130 GAs identified up to now from plants, fungi, and bacteria are GA_1, GA_3, and GA_4, the three most common directly effective GA shoot elongation promoters. Their levels in plant tissues appear to be regulated by three processes: (a) biosynthesis, (b) reversible conjugation, and (c) catabolism. The activation step in the biosynthesis of growth-promotive GAs is 3β-hydroxylation, i.e. conversion of the 3-deoxy GA_{20} into the bioactive GA_1 (Kobayashi et al., 1994). Conjugation of GAs is almost exclusively with Glc, either via the carboxyl group forming glucosyl esters or via the hydroxyl group generating glucosyl ethers in a range of isomeric forms. These glucosyl conjugates do not appear to be biologically active per se; rather, their role is proposed to be as reserve, transport, or entry to catabolism forms (Schneider and Schliemann, 1994).

GA production in vitro by *Azospirillum* spp. and the effects of *Azospirillum* spp. on infected plants have been studied earlier. Thus, GA_1 and GA_3 were characterized by capillary gas chromatography-mass spectrometry (GC-MS) from gnotobiotic cultures of *Azospirillum lipoferum* (Bottini et al., 1989) and *Azospirillum brasilense* (Janzen et al., 1992). In addition, the bacteria have also been shown to metabolize exogenous GAs (Piccoli and Bottini, 1994; Piccoli et al., 1996). External factors such as light quality (Piccoli and Bottini, 1996) or oxygen availability and osmotic strength (Piccoli et al., 1999) may also influence the amount and type of GA produced. It was also found that application of GA_3 in concentrations similar to those produced by the microorganism, or by inoculation with different *Azospirillum* spp. strains, can promote growth of roots in corn (*Zea mays*) seedlings (Fulchieri et al., 1990). It is interesting that inoculation with *A. lipoferum* promoted the appearance of free forms of GA_3 in inoculated plant roots, whereas putative GA conjugates predominated in controls (Fulchieri et al., 1993). The latter results, however, did not establish whether the higher content of GA_3 found in the inoculated roots was due to de novo production, hydrolysis by the microorganism per se, or by the plant under bacterial influence. Regarding this issue, Piccoli et al. (1997) demonstrated that *A.*

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Table I. *Length of second leaf sheath (in mm, $\pm p < 0.05$ confidence limits) of rice dy seedlings treated with GA A_{20} glucosyl conjugates and inoculated with Azospirillum spp.*

Treatments	Noninoculated	*A. lipoferum* Strain USA 5b	*A. brasilense* Strain Cd
[17,17-$^2H_2$] GA_{20}-G (μg plant^{-1})			
10^{-1}	12.02 ± 0.63	18.20 ± 1.46	15.60 ± 0.48
10^{-2}	11.10 ± 0.89	13.30 ± 0.47	10.80 ± 0.40
10^{-3}	10.40 ± 0.48	12.00 ± 1.09	10.60 ± 0.80
[17,17-$^2H_2$] GA_{20}-GE (μg plant^{-1})			
10^{-1}	11.80 ± 0.40	17.20 ± 1.46	17.40 ± 0.80
10^{-2}	11.60 ± 0.80	13.40 ± 0.48	13.40 ± 1.01
10^{-3}	11.00 ± 0.63	11.75 ± 0.82	11.00 ± 0.89
Ethanol 95% (v/v) + buffer	9.33 ± 0.74	10.60 ± 0.80	10.40 ± 0.48

lipoferum grown in chemically defined medium has the capacity to hydrolyze in vitro [17,17-$^2H_2$] GA_{20} glucosyl ester (GA_{20}-GE) and [17,17-$^2H_2$] GA_{20} glucosyl ether (GA_{20}-G).

In a number of rice (*Oryza sativa*) cultivars the dwarf phenotype is expressed in several single-gene mutants. One mutant, *dx*, blocks at the level of kaurene oxidation; a second dwarf, *dy*, blocks the 3β-hydroxylation step (Takahashi and Kobayashi, 1991; Kobayashi et al., 1994).

We report herein the bacterial hydrolysis and metabolism of GA conjugates in these two dwarf mutants of rice by endophytic *Azospirillum* spp.

RESULTS

Reversal of the Dwarf (*dy*) Phenotype in Rice

The responses of the *dy* rice mutant seedlings treated with [17,17-$^2H_2$]GA_{20}-G and [17,17-$^2H_2$]GA_{20}-GE at several concentrations and/or inoculated with *A. brasilense* strain Cd and *A. lipoferum* strain USA 5b are shown in Table I. Both strains were effective in promoting growth of seedlings and reversing dwarfism when the two types of GA_{20}-glucosyl conjugates were added. The promotive effects were similar for each of the two forms of GA_{20}-glucosyl conjugates. Inoculated control plants (but without GA_{20} conjugate application) also showed dwarfism reversal, having a significantly greater growth than the ethanol-treated controls. Treatments with deuterated GA glucosyl conjugates alone (no *Azospirillum* spp.) could also slightly reverse dwarfism, relative to ethanol control treatments (Table I). Thus, it seems likely that the plant has some intrinsic capacity to hydrolyze GA-glucosyl conjugates. The difference in growth for inoculated versus noninoculated seedlings can best be explained as hydrolytic plus metabolic activity of the bacteria on the conjugate. Although dwarfism reversal was observed in treatments with each addition of GA-glucosyl conjugates, or with independent inoculation, the greatest growth increases were observed for those seedlings that received the conjugated hormones as well as the microorganism. There were no significant differences between seedlings treated with [17,17-$^2H_2$]GA_{20}-G or [17,17-$^2H_2$]GA_{20}-GE. Thus, no particular *Azospirillum* spp. specificity for substrate was found.

Reversal of the Dwarf (*dx*) Phenotype in Rice

The responses of the rice *dx* mutant treated with decreasing concentrations of [17,17-$^2H_2$]GA_{20}-G and [17,17-$^2H_2$]GA_{20}-GE and inoculated with both *A. brasilense* strain Cd and *A. lipoferum* strain USA 5b are shown in Table II. Internode length of inoculated seedlings was significantly longer relative to noninoculated treatments for the higher concentration of [17,17-$^2H_2$]GA_{20}-G and [17,17-$^2H_2$]GA_{20}-GE. *A. brasilense* strain Cd and *A. lipoferum* strain USA 5b were effective in promoting growth of seedlings and reversing dwarfism when deuterated glucosyl conjugates were added, relative to noninoculated treat-

Table II. *Length of second leaf sheath (in mm, $\pm p < 0.05$ confidence limits) of rice dx seedlings treated with GA A_{20} glucosyl conjugates and inoculated with Azospirillum spp.*

Treatments	Noninoculated	*A. lipoferum* Strain USA 5b	*A. brasilense* Strain Cd
[17,17-$^2H_2$] GA_{20}-G (μg plant^{-1})			
10^{-1}	17.60 ± 1.85	21.80 ± 1.32	23.60 ± 1.35
10^{-2}	12.60 ± 0.48	14.20 ± 0.97	13.40 ± 0.80
10^{-3}	10.00 ± 0.63	10.40 ± 0.48	12.00 ± 0.63
[17,17-$^2H_2$] GA_{20}-GE (μg plant^{-1})			
10^{-1}	21.50 ± 0.50	29.80 ± 1.32	30.80 ± 1.46
10^{-2}	15.60 ± 0.48	17.40 ± 0.48	17.00 ± 0.89
10^{-3}	10.40 ± 1.01	11.20 ± 0.74	11.40 ± 1.35
Ethanol 95% (v/v) + buffer	12.30 ± 0.50	13.60 ± 1.01	13.40 ± 0.74

ments. *A. brasilense* strain Cd and *A. lipoferum* strain USA 5b (no GA_{20} conjugates) showed similar growth-promoting effects. Again, as for the *dy* mutant, although reversal of dwarfism was observed in treatments with addition of hormones or with independent inoculation, the greatest growth differences were observed in those seedlings that received hormones and microorganism together. Table II also shows that for the *dx* mutant there was an apparent preference for the glucosyl ester as substrate.

The *dx* mutant was much more efficient than the *dy* mutant in utilizing the glucosyl conjugates of $[17,17^2H_2]GA_{20}$ for sheath elongation growth. It is likely due to a higher inherent capacity of the *dx* mutant to 3β-hydroxylate the aglycone, $[17,17^2H_2]GA_{20}$, to the bioactive $[17,17^2H_2]GA_1$, i.e. the genetic lesion in the *dy* mutant results in a very much reduced ability to 3β-hydroxylate 3-deoxy GAs (Takahashi and Kobayashi, 1991; Kobayashi et al., 1994).

Endophytic Bacteria

Endophytic presence of the bacteria in roots and stems of inoculated plants was shown for both cultivars of all experiments (summarized in Table III).

Identification of GAs and Their Quantitation by GC-MS

Identification of $[17,17\text{-}^2H_2]GA_{20}$ (the aglycone of the conjugates applied) and $[17,17\text{-}^2H_2]GA_1$ (the 3β-hydroxylated metabolite of $[17,17\text{-}^2H_2]GA_{20}$) was based on the peak area counts of eight diagnostic ions (Table IV). Both $[17,17\text{-}^2H_2]GA_{20}$ and its metabolite $[17,17\text{-}^2H_2]GA_1$ were identified for the two rice mutants.

Quantification of $[17,17\text{-}^2H_2]GA_{20}$ (the aglycone) and $[17,17\text{-}^2H_2]GA_1$ (its 3β-hydroxylated metabolite) were made using the absolute areas of the molecular ions (M^+) at *m/z* 420 and 508 by GC-MS for the *dy* seedlings, with or without *Azospirillum* spp. inoculation (Table V). There is a high correlation between the application of deuterated GA conjugates and the growth response of the rice mutant seedlings in the presence of *Azospirillum* spp. It was a coincidence that $[17,17\text{-}^2H_2]GA_{20}$ and $[17,17\text{-}^2H_2]GA_1$ M^+ peak areas were higher for those treatments where inoculation with *Azospirillum* spp. was a cotreatment (Tables V and VI). Thus, it is apparent that inoculation with the microorganism allows for deconjugation (release of the aglycone $[17,17\text{-}^2H_2]GA_{20}$) and its metabolism to $[17,17\text{-}^2H_2]GA_1$ in both *dx* and *dy* mutants of rice seedlings. These results also prove that the bacteria can 3β-hydroxylate GA_{20} because in noninoculated $[17,17\text{-}^2H_2]GA_{20}$ glucosyl conjugate treatments of *dy*, $[17,17\text{-}^2H_2]GA_1$ was not identified (Table V) and the *dy* mutant seedlings showed very little growth (Table I). However, *dx* seedlings without *Azospirillum* spp. inoculation showed fair uptake and good conversion to $[17,17\text{-}^2H_2]GA_1$ (Table VI), as well as good growth (Table II). It is noteworthy that GA_1 levels are higher in the *Azospirillum*-inoculated *dx* mutant after treating with GA_{20}-G than after treating with GA_{20}-GE (Table VI). This seems to be inconsistent with the internode length results obtained (Table II); i.e. GA_{20}-GE was more effective than GA_{20}-G to induce the second leaf sheath growth in the *Azospirillum*-inoculated *dx* mutant. However, this could be because the GA measurements were done using whole seedlings.

DISCUSSION

Sheath elongation growth of two single gene dwarf rice mutants, *dy* and *dx*, can be promoted when seedlings inoculated with *A. brasilense* strain Cd and A. *lipoferum* strain USA 5b are also supplied with $[17,17\text{-}^2H_2]GA_{20}$-GE or $[17,17\text{-}^2H_2]GA_{20}$-G. This growth is due primarily to release of the aglycone, $[17,17\text{-}^2H_2]GA_{20}$, and its subsequent 3$\beta$-hydroxylation to $[17,17\text{-}^2H_2]GA_1$ by the microorganism for the *dy* mutant, and by both the plant and microorganism for the *dx* mutant. This mutant retained the ability to 3β-hydroxylate GA_{20}. Hence, *Azospirillum* spp. can effectively hydrolyze GA-glucosyl conjugates in vivo. This confirms earlier work by Piccoli et al. (1997) where in vitro cultures of *A. lipoferum* effectively released the GA aglycone in chemically defined medium.

The identification of $[17,17\text{-}^2H_2]GA_1$ only in inoculated treatments of *dy* seedlings (Table II) further demonstrates that *Azospirillum* spp. has the in vivo capacity to 3β-hydroxylate $[17,17\text{-}^2H_2]GA_{20}$ to $[17,17\text{-}^2H_2]GA_1$ because the *dy* mutant is blocked in this biosynthetic step. As with the *dx* mutant, the current results confirm, in vivo, the 3β-hydroxylation abilities of *A. lipoferum* previously shown in vitro (Piccoli and Bottini, 1994; Piccoli et al., 1996). The finding that

Table III. *Bacterial counts 72 h after inoculation*

Strain Inoculated	Fraction	*dx*, Colony Forming Units (CFU) g plant^{-1}	*dy*, CFU g plant^{-1}
A. lipoferum strain USA 5b	Root	6.10^6	3.10^6
A. lipoferum strain USA 5b	Stem + leaves	3.10^4	5.10^4
A. brasilense strain CD	Root	4.10^7	2.10^7
A. brasilense strain CD	Stem + leaves	5.10^5	7.10^5
Buffer phosphate	Root + stem + leaves	Nd[a]	Nd

[a] Nd, Not detected.

Table IV. *Gibberellin identification by GC-MS based on relative abundance (in %) of characteristic ions*

Rice seedlings had been treated with GA-A_{20}-glucosyl conjugates and inoculated with Azospirillum spp.

Compound	Abundance in % of Characteristic Ions
[17,17-$^2H_2$]GA_{20} standard	420 (100), 405 (13), 377 (66), 361 (18), 347 (5), 303 (20), 237 (9), and 209 (43)
[17,17-$^2H_2$]GA_{20} putative	420 (100), 405 (14), 377 (62), 361 (17), 347 (7), 303 (21), 237 (12), and 209 (57)
[17,17-$^2H_2$]GA_1 standard	508 (100), 493 (9), 450 (21), 378 (20), 315 (11), 239 (12), 225 (11), and 209 (44)
[17,17-$^2H_2$]GA_1 putative	508 (100), 493 (10), 450 (21), 378 (17), 315 (14), 239 (13), 225 (10), and 209 (58)

only [17,17-$^2H_2$]GA_1 was a metabolic product of [17,17-$^2H_2$]GA_{20} (the deconjugated aglycone) strongly implies that GA_1 and GA_3 have different precursors in the route of synthesis in *Azospirillum* spp. (Piccoli and Bottini, 1996; Piccoli et al., 1996).

As expected, *dx* seedlings, with or without inoculation, that were treated with [17,17-$^2H_2$]GA_{20}-GE and [17,17-$^2H_2$]GA_{20}-G showed greater growth when compared with similarly treated *dy* seedlings (Tables I and II). Seedlings of *dx* also produced [17,17-$^2H_2$]GA_1 from [17,17-$^2H_2$]GA_{20} in noninoculated treatments.

These responses by the *dx* seedlings are consistent with earlier studies (Takahashi and Kobayashi, 1991; Kobayashi et al., 1994) that place the dwarfing lesion of the *dx* mutation early in the GA biosynthesis pathway. In a similar manner, the inability of the *dy* seedlings to grow or to produce [17,17-$^2H_2$]GA_1 without inoculation again confirm that this dwarf has its genetic lesion at the later, 3β-hydroxylation step of GA biosynthesis.

In conclusion, the beneficial effect of *Azospirillum* spp. on growth and yield (Okon and Labandera-González, 1994) or water stress alleviation (Creus et al., 1997) of graminaceous plants can likely be explained, at least in part, by: (a) GA production by the bacteria (Kucey, 1988; Bottini et al., 1989; Fulchieri et al., 1990; Janzen et al., 1992; Fulchieri et al., 1993; Lucangeli and Bottini 1997; Piccoli et al., 1999), (b) deconjugation of GA-glucosyl conjugates (Piccoli et al., 1997), and (c) 3β-hydroxylation of inactive 3-deoxy GAs present in roots to active forms (Kobayashi et al., 1994; Piccoli and Bottini, 1994; Piccoli et al., 1996).

MATERIALS AND METHODS

Biological Material

The following bacteria were used: *Azospirillum lipoferum* strain USA5b (kindly provided by Dr. Vera Baldani, EMBRAPA, Itajai, Brazil) and *Azospirillum brasilense* strain Cd (ATCC accession no. 29710); and the dwarf rice (*Oryza sativa*) mutants *dy* (rice cv Waito C) and *dx* (rice cv Tanginbozu; mutants were gifts from Dr. Masaji Koshioka, National Research Institute of Vegetables, Ornamental Plants and Tea, Mie, Japan).

Bacterial Growth

Bacterial strains were grown in liquid nitrogen-free biotin-based (NFb) medium with malic acid (5 g L^{-1}) and NH_4Cl (1.25 g L^{-1}; Piccoli et al., 1997) on an orbital shaker at 30°C and 80 rpm, until an optical density at 540 nm of 1.0 was reached. This corresponds with a concentration of approximately 10^8 CFU mL^{-1}. Bacteria were harvested by centrifugation at 8,000 rpm and 4°C for 15 min. The cellular pellet was washed twice with 0.85% (w/v) NaCl solution and resuspended in 0.05 M phosphate buffer to obtain a titer of 3×10^6 CFU mL^{-1} for later inoculation.

Deuterated GA Conjugates

The deuterated GA conjugates [17,17-$^2H_2$] GA_{20}-glucosyl ester ([17,17-$^2H_2$] GA_{20}-GE) and [17,17-$^2H_2$] GA_{20}-glucosyl ether ([17,17-$^2H_2$] GA_{20}-G) were prepared from [17,17-$^2H_2$] GA_{20} (96% [v/v] $^2H_2$) as previously described (Schneider et al., 1989, 1990).

Table V. *Absolute areas of peak abundance for the M^+ of [17,17-$^2H_2$]GA_{20} (m/z 420) and [17,17-$^2H_2$]GA_1 (m/z 508) obtained by GC-MS for dy seedlings (equal sample aliquots were injected; see "Materials and Methods" for details)*

Results represent a typical subsample from one replicate, injected several times without noticeable differences.

Treatment (10^{-1} μg $plant^{-1}$)	M^+ 420 Area $\times 10^3$ ([$^2H_2$]GA_{20})	M^+ 508 Area $\times 10^3$ ([$^2H_2$]GA_1)	Total Peak Areas $\times 10^3$ of ([$^2H_2$]GA_{20}) + ([$^2H_2$]GA_1)
[$^2H_2$]GA_{20}-G without *Azospirillum* spp.	73	Nd[a]	73
[$^2H_2$]GA_{20}-GE without *Azospirillum* spp.	67	Nd	67
[$^2H_2$]GA_{20}-G + *A. lipoferum* strain USA5b	570	140	710
[$^2H_2$]GA_{20}-GE + *A. lipoferum* strain USA5b	480	360	840
[$^2H_2$]GA_{20}-G + *A. brasilense* strain Cd	1,100	160	1,260
[$^2H_2$]GA_{20}-GE + *A. brasilense* strain Cd	400	130	530

[a] Nd, Not detected.

Table VI. *Absolute areas of peak abundance for the M^+ of [17,17-$^2H_2$]GA_{20} (m/z 420) and [17,17-$^2H_2$]GA_1 (m/z 508) obtained by GC-MS for dx seedlings (equal sample aliquots were injected; see "Materials and Methods" for details)*

Results represent a typical subsample from one replicate, injected several times without noticeable differences.

Treatment (10^{-1} μg plant^{-1})	M^+ 420 Area × 10^3 ([$^2H_2$]GA_{20})	M^+ 508 Area × 10^3 ([$^2H_2$]GA_1)	Total Peak Areas × 10^3 of ([$^2H_2$]GA_{20}) + ([$^2H_2$]GA_1)
[$^2H_2$]GA_{20}-G without *Azospirillum* spp.	7	45	52
[$^2H_2$]GA_{20}-GE without *Azospirillum* spp.	12	260	272
[$^2H_2$]GA_{20}-G + *A. lipoferum* strain USA5b	2,100	410	2,510
[$^2H_2$]GA_{20}-GE + *A. lipoferum* strain USA5b	310	78	388
[$^2H_2$]GA_{20}-G + *A. brasilense* strain Cd	530	110	640
[$^2H_2$]GA_{20}-GE + *A. brasilense* strain Cd	240	38	278

Seedling Growth

Seeds of both rice mutants were surface sterilized with ethanol (70% [v/v]) for 20 s, then with sodium hypochlorite (2% [v/v]) for 20 min, finally being washed with sterile distilled water. They were pregerminated in 80 μM Uniconazole (S-3307D, Sumimoto Chem. Co., Nagoya, Japan), an early-stage GA biosynthesis inhibitor, at 30°C for 48 h to deplete their endogenous GA levels. Then after again washing with sterile distilled water, five seeds were sown in glass beakers containing 2 mL of agar (0.8% [w/v]) and incubated for 48 to 72 h at 30°C under continuous fluorescent light (20 μmol m^{-2} s^{-1}) in a saturated humidity.

Treatments

After 72 h roots, of seedlings of both rice mutants were inoculated with the *Azospirillum* spp. strains using a titer of 3×10^6 CFU plant^{-1}. Control plants received an equivalent amount of buffer. After another 72 h, [17,17-$^2H_2$]GA_{20}-GE or [17,17-$^2H_2$]GA_{20}-G, dissolved in 1 μL of ethanol (95% [v/v]), were carefully placed on the surface of the seedling roots with the aid of a microsyringe. Treatments are summarized as follows: treatment 1, 1 μL ethanol (95% [v/v]) + 0.05 M buffer phosphate; treatments 2 through 6, 10^{-1} to 10^{-5} μg plant^{-1} of [17,17-$^2H_2$] GA_{20}-GE; treatments 7 through 11, 10^{-1} to 10^{-5} μg plant^{-1} of [17,17-$^2H_2$] GA_{20}-GE + *A. lipoferum* strain USA 5b; treatments 12 through 16, 10^{-1} to 10^{-5} μg plant^{-1} of [17,17-$^2H_2$] GA_{20}-GE + *A. brasilense* strain Cd; treatments 17 through 21, 10^{-1} to 10^{-5} μg plant^{-1} of [17,17-$^2H_2$] GA_{20}-G; treatments 22 through 26, 10^{-1} to 10^{-5} μg plant^{-1} of [17,17-$^2H_2$] GA_{20}-G + *A. lipoferum* strain USA 5b; treatments 27 through 31, 10^{-1} to 10^{-5} μg plant^{-1} of [17,17-$^2H_2$] GA_{20}-G + *A. brasilense* strain Cd; treatment 32, *A. lipoferum* strain USA 5b; and treatment 33, *A. brasilense* strain Cd.

Measurements and Statistical Analysis

After 72 h in the presence of the deuterated GA conjugates, the length of the first internode was measured. Seedlings (roots + shoots) were frozen in liquid N_2 and stored at −30°C until processed. Each experiment was carried out in a random design, with five repetitions per treatment. Data were analyzed by analysis of variance (ANOVA) followed by Tukey's *t* test at $P \leq 0.05$.

Bacterial Counts in Stems and Roots

Bacterial counts were made for both shoots and roots of control and treated rice seedlings using plates of NFb agar. Plant tissue was macerated using a mortar and pestle with 0.05 M phosphate buffer. Serially diluted inoculations were applied to plates containing agar NFb. After incubating for 72 h at 30°C, the number of CFU was assessed.

Evaluation of [17,17-$^2H_2$] GAs

Seedlings (including roots) treated with 10^{-1} μg plant^{-1} of GA_{20}-G and GA_{20}-GE were homogenized 72 h after treatment in 500 mL of methanol:water (4:1) and kept at 4°C overnight. After filtration the solid residue was extracted again and filtered. Filtrates were combined and methanol evaporated under reduced atmospheric pressure. The aqueous phase was adjusted to a pH of 2.5 with acetic acid (1% [v/v]) and partitioned four times with the same volume of water-saturated (1% [v/v] acetic acid) ethyl acetate. Acidic ethyl acetate was evaporated and diluted with acetic acid:methanol:water (1:10:89), then filtered through 0.45-μm membranes and injected into an HPLC system (KNK-500, Konic Inc., Barcelona) with a C_{18} reverse phase column (μBondapack, 300 × 3.9 mm, Waters Associates, Milford, MA). Elution was at 2 mL min^{-1} using a gradient 10% to 73% (v/v) methanol in 1% (v/v) acetic acid over 30 min until the 73% (v/v) methanol concentration was reached. The fractions corresponding with the retention times of [17,17-$^2H_2$]GA_{20} and [17,17-$^2H_2$]GA_1 were subjected to a second HPLC using a Nucleosil 5[$N(CH_3)_2$] column (15 cm × 4.6 mm, Waters Associates). Elution was carried out with acetic acid:methanol (0.1:99.9) at 1 mL min^{-1}. The fractions that would contain [17,17-$^2H_2$]GA_{20} and [17,17-$^2H_2$]GA_1 were again collected, evaporated, and processed as described by Volmaro et al. (1998) prior to assessment by capillary GC-MS. The only change was that a Hewlett-Packard-1 cross-linked methyl silicone capillary column (25-m length × 0.25-mm i.d. × 0.22-μm film thickness) with a GC temperature program of 100°C to 195°C at 15°C min^{-1}, then to 260°C at 4°C min^{-1}, was used. For each analysis, full-scan spectra of [17,17-$^2H_2$]GA_{20} and [17,17-$^2H_2$]GA_1 were monitored at the retention times of authentic [17,17-$^2H_2$]GA_{20} and [17,17-$^2H_2$]GA_1 standards.

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LITERATURE CITED

Bottini R, Fulchieri M, Pearce D, Pharis R (1989) Identification of gibberellins A_1, A_3, and iso-A_3 in cultures of *Azospirillum lipoferum*. Plant Physiol **90:** 45–47

Creus C, Sueldo R, Barassi C (1997) Shoot growth and water status in *Azospirillum*-inoculated wheat seedlings grown under osmotic and salt stresses. Plant Physiol Biochem **35:** 939–944

Davies PJ (1995) The plant hormones: their nature, occurrence and functions. *In* PJ Davies, ed, Plant Hormones: Physiology, Biochemistry and Molecular Biology. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 1–12

Fulchieri M, Lucangeli C, Bottini R (1990) Effects of *A. lipoferum* and GA_3 on root growth in corn. *In* M Polsinelli, R Materassi, M Vicenzini, eds, Nitrogen Fixation: Proceedings of the Fifth International Symposium on Nitrogen Fixation by Non-Legumes. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 297–298

Fulchieri M, Lucangeli C, Bottini R (1993) Inoculation with *A. lipoferum* affects growth and gibberellin status of corn seedlings roots. Plant Cell Physiol **l34:** 1305–1309

Glick BR, Patten CL, Holguin G, Penrose DM (1999) Biochemical and Genetic Mechanisms Used by Plant Growth Promoting Bacteria. Imperial College Press, London, pp 1–13

Janzen R, Rood S, Dormar J, McGill W (1992) *Azospirillum brasilense* produces gibberellins in pure culture and chemically-medium and in co-culture on straw. Soil Biol Biochem **24:** 1061–1064

Kobayashi M, Gaskin P, Spray C, Phinney B, Mac Millan J (1994) The metabolism of gibberellin A_{20} to gibberellin A_1 by tall and dwarf mutants of *Oryza sativa* and *Arabidopsis thaliana*. Plant Physiol **106:** 1367–1372

Kucey RMN (1988) Plant growth-altering effects of *Azospirillum brasilense* and *Bacillus* C-11–25 on two wheat cultivars. J Appl Bacteriol **64:** 187–196

Lucangeli C, Bottini R (1997) Effects of *Azospirillum* spp. on endogenous gibberellins content and growth of maize (*Zea mays* L.) treated with uniconazole. Symbiosis **23:** 63–72

Okon Y, Kapulnik Y (1986) Development and function of *Azospirillum*-inoculated roots. Plant Soil **90:** 3–16

Okon Y, Labandera-González C (1994) Agronomic applications of *Azospirillum*: an evaluation of 20 years of worldwide field inoculation. Soil Biol Biochem **26:** 1591–1601

Piccoli P, Bottini R (1994) Metabolism of 17,17-[$^2H_2$]-gibberellin A_{20} to 17,17-[$^2H_2$]gibberellin A_1 by *Azospirillum lipoferum* cultures. AgriScientia **11:** 13–15

Piccoli P, Bottini R (1996) Gibberellin production in *Azospirillum lipoferum* cultures is enhanced by light. Biocell **20:** 185–190

Piccoli P, Lucangeli C, Schneider G, Bottini R (1997) Hydrolisis of 17,17-[$^2H_2$]-gibberellin A_{20}-glucoside and 17,17-[$^2H_2$]-gibberellin A_{20}-glucosyl esther by *Azospirillum lipoferum* cultured in nitrogen-free biotin-based chemically defined medium. Plant Growth Regul **23:** 179–182

Piccoli P, Masciarelli O, Bottini R (1996) Metabolism of 17,17-[$^2H_2$]-gibberellin A_4, A_9 and A_{20} by *Azospirillum lipoferum* in chemically defined culture medium. Symbiosis **21:** 263–274

Piccoli P, Masciarelli O, Bottini R (1999) Gibberellin production by *Azospirillum lipoferum* cultured in chemically defined medium as affected by water status and oxygen availability. Symbiosis **27:** 135–146

Schneider G, Schliemann W (1994) Gibberellin conjugates: an overview. Plant Growth Regul **15:** 247–260

Schneider G, Schreiber K, Jensen E, Phinney BO (1990) Synthesis of gibberellin A_{29}-β-D-glucosides as well as -β-D-glucosyl derivatives of [17-^{13}C,$^3H_2$] gibberellin A_5, A_{20} and A_{29}. Liebigs Ann Chem **1990:** 491–494

Schneider G, Sembdner G, Schreiber K (1989) Partial synthesis of some physiologically relevant gibberellin glucosyl conjugates. Tethraedron **45:** 1355–1364

Takahashi N, Kobayashi M (1991) Organ-specific gibberellins in rice: roles and biosynthesis. *In* N Takahashi, BO Phinney, J MacMillan, eds, Gibberellins. Springer-Verlag, New York, pp 9–21

Tien T, Gaskin M, Hubbel D (1979) Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl Environ Microbiol **37:** 1016–1024

Volmaro C, Pontin M, Luna V, Baraldi R, Bottini R (1998) Blue light control of hypocotyl elongation in etiolated seedlings of *Lactuca sativa* (L.) cv. Grand Rapids related to exogenous growth regulators and endogenous IAA, GA_3 and abscisic acid. Plant Growth Regul **26:** 165–173