

# Fresh-weight measurements of roots provide inaccurate estimates of the effects of plant growth-promoting bacteria on root growth: a critical examination

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## Abstract

Four strains of plant growth-promoting bacteria (PGPB), including three strains of *Azospirillum* and *Pseudomonas fluorescens* 313 were used to inoculate seeds of wheat, tomato, pepper, and cotton. Inoculated seedlings were grown to the two or three-leaf stage. After harvest, seven different environmental and technical conditions were evaluated to determine the effect of these conditions on the reproducibility of fresh and dry root weight measurements. Dry root weight of each sample showed no significant variations (smaller than 1%), despite the variations in measurement conditions. Root fresh weights varied greatly (usually in the range of 4–10%, but up to 18%), and were significantly affected ( $P \leq 0.05$ ) by air temperature and relative humidity, air currents, different light intensities during extraction of plants from the substrate, duration of the extraction from soil (depending on the size of the experiment and type of plant growth substrate), and the type of absorbing paper used to blot excess water from harvested roots. Measurements by different technicians did not influence fresh or dry weight values. We conclude that fresh weight determinations are altered by factors independent of the intended experimental variables and should not be used to evaluate the effect of PGPB on plants.

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**Keywords:** Erroneous methodology; Plant dry weight; Plant growth-promoting bacteria; PGPR; Rhizobacteria

## 1. Introduction

With the use of plant growth-promoting bacteria (PGPB) gaining acceptance, numerous bacterial species have been isolated and evaluated for their capacity to promote plant growth (Glick, 1995; Bashan and Holguin, 1997; Hallmann et al., 1997; Bashan et al., 2004; Bashan and de-Bashan, 2005).

When plant species are inoculated with PGPB, increases in yield are expected, comparable to those obtained in previous studies that used the same bacterium-host pair. Some studies have used final measurements of yields of the inoculated plants as their principal characteristic (Berge et al., 1990; Gagné et al., 1993; Purcino et al., 1996;

Pandey et al., 1998). However, most studies, especially those concentrating on screening of new isolates, are short-term, and usually measure the effect of a PGPB on plant growth by determining plant root and foliage weight. Two types of determination are common in the literature, root fresh weight (Heijnen et al., 1993; Kurek and Jaroszk, 1994; Shishido et al., 1995; Creus et al., 1996; Lucangeli and Bottini, 1996; Olivares et al., 1996; Bouillant et al., 1997) and root dry weight (Iswandi et al., 1987; de Freitas and Germida, 1990; Milus and Rothrock, 1993; Chanway and Holl, 1994; Sturz and Christie, 1995; Ratti and Janardhanan, 1996; Bashan et al., 1998).

Despite efforts to duplicate experimental procedures, final weight values may vary between experiments because of variations in environmental and technical factors during preparation of tissues for weighing. Variable environmental factors may include laboratory temperature, relative humidity (RH), air currents during exposure of roots to laboratory air, while other sources of variation may include the size of the experiment, type of absorbent paper used to blot excess

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moisture from tissues, and the time required to extract plants from the substrate.

One may argue that dry weight determinations, by definition, are more reliable. Yet, there is a continuous use of the two procedures or methodologies when evaluating PGPB performance. Sometimes authors used the two methodologies interchangeably in different scientific reports published at about the same time (Nairn and Chanway, 1999; Shishido and Chanway, 1999; Burd et al., 2000). As recently as the two latest conferences on Plant Growth-Promoting Bacteria (5th International PGPR workshop, Villa Carlos Paz, Argentina, 29 October–3 November 2000, Auburn University Web Sites: <http://www.ag.auburn.edu/argentina/> and 6th International PGPR workshop, Calicut, India, 5–10 October 2003, <http://www.ag.auburn.edu/india/>), authors are continuing to use one of two methodologies: fresh weight (4 papers) and fresh and dry terms in the same paper (8 papers). Additionally, referees of submitted manuscripts frequently encounter fresh weight measurements. For example, 14 of 75 manuscripts evaluated in 2004, 12 of 86 manuscripts in 2003 and 15 of 76 manuscripts in 2002 used fresh weight measurements (Y. Bashan, unpublished data). Other referees and editors find this is a general situation, one that is discouraged by editors (P. Nannipieri, personal communication). This perspective motivated our data analysis on the causes of fluctuation of dry and fresh weight of inoculated plant material.

The specific objective of this study was to evaluate experimentally a common mistake in the literature and experimentation, namely, that fresh weight measurements provide accurate determinations. This critical examination demonstrated that root fresh weight determinations used to evaluate effects of PGPB-inoculation are inherently faulty and recommend eliminating this common practice in scientific experimentation.

## 2. Materials and methods

### 2.1. Organisms and growth conditions

Bacterial strains used to inoculate plants: *Azospirillum brasilense* strains Cd (DSM 7030, Braunschweig, Germany) and Sp-245 (donated by the late J. Döbereiner, CNPBS, Rio de Janeiro, Brazil), *A. lipoferum* 1842 (DSM 1842), and *Pseudomonas fluorescens* 313 (Bashan and Gonzalez, 1999).

Plant strains used: wheat (*Triticum aestivum* cv. Deganit, Zeraim Gedera Co., Israel), tomato (*Lycopersicon esculentum* Mill. cv. M-82-1-8-VF), pepper (*Capsicum annuum* cv. Ma'or), and cotton (*Gossypium barbadense* cv. Pima S-5), all seeds (obtained from Hazera Co., Haifa, Israel), were inoculated with bacteria after standard disinfection of the seeds.

*Azospirillum* strains were grown by standard procedures for this genus (Bashan et al., 1993) and *P. fluorescens* in

Nutrient Broth (Difco, MI) for 16 h at  $30 \pm 1$  °C with agitation at  $200 \text{ rev min}^{-1}$ . All bacteria were prepared for inoculation as described by Bashan (1986).

Plants were grown for 4 weeks in oven-sterilized quartz sand (100% coarse sand > 200  $\mu\text{m}$  grain size; no N, P, K, clay, or silt present; no measurable amounts of organic matter,  $\text{CaCO}_3$  4.3%; pH 7.3; and conductivity  $0.77 \text{ mS cm}^{-1}$ ) supplemented with acid-treated finely ground vermiculite in a ratio of 40:1 (sand:vermiculite, w/w) to create a water-holding capacity of 24%. Before sowing, substrate was fertilized once with 1% N:P:K (30:30:30) commercial garden fertilizer containing microelements. Plants were also grown in agricultural sandy soil (Haplic Xerosol plus Eutric Regosol with coarse texture, containing 0.14% N; 1.68% P; 0.3% K; 10.53% clay; 53.3% silt; 36.2% sand; 13.5% water-holding capacity; 0.86%  $\text{CaCO}_3$ ; 2.49% organic matter at pH 7.5, and conductivity of  $0.5 \text{ mS cm}^{-1}$ ) in 500-ml black plastic pots (see Bashan et al., 1989).

All growth substrate materials were sterilized by a standard Tyndelization-autoclave procedure. Because the pots were not steam resistant, they were first washed with commercial dish detergent, thoroughly rinsed with reverse-osmosis drinking water, and then surface disinfected by spraying with 70% technical-grade ethanol. The sterile growth substrate was placed in the pots in a room disinfected by spraying all surfaces with 70% alcohol (Carrillo et al., 1996). Pots with substrate added were immediately sown with inoculated seeds. Because the pots were open to the air, they were later contaminated by airborne microorganisms with a maximum of  $10^5 \text{ cfu g}^{-1}$  dry substrate. However, these contaminating organisms colonized the roots of the inoculated plants during the 30-d experiments in negligible numbers (described later).

Pots containing wheat were kept in a growth chamber at  $22 \pm 2$  °C, 14 h illumination of  $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$  (Conviron TC 16, Controlled Environments, Winnipeg, Canada). All other plants were grown likewise but at  $28 \pm 2$  °C.

### 2.2. Bacterial inoculation

Seeds were inoculated with the bacterial strains at a final concentration of  $10^6 \text{ cfu ml}^{-1}$  by standard vacuum seed inoculation (Puente and Bashan, 1993). Immediately after cotyledon emergence, plants were inoculated a second time by directly irrigating the substrate (Bashan, 1986) with double-washed, 50 ml, bacteria suspended in 60 mM phosphate buffer saline pH 7.0 (PBS) at a final concentration of  $10^6 \text{ cfu ml}^{-1}$ .

### 2.3. Measurements of fresh and dry weight

To avoid root damage or loss during harvest, each pot was first submerged in a water bath for 5–30 min at ambient temperature and then plants were lightly shaken to release them from the substrate. Shorter submersion

periods were used for sand and longer periods for soil. The roots were then rinsed with tap water until all visible sand and soil particles were removed. Particles that adhered strongly to the roots were manually removed with tweezers. Dry weight was measured by placing large root systems into small, tared brown paper bags or small root systems into tared aluminum foil pouches. Samples were dried in a forced-draft oven at  $75 \pm 2$  °C for 16 h. Extended dry periods did not change the results. The hot aluminum foil pouches were allowed to cool at room ambient condition for 5 min. Only a few pouches (10–15) were removed for each batch measurement to avoid absorption of moisture from the air. Dried roots were weighed on a laboratory scale (Mettler AE 163, Switzerland) located in the same laboratory as the oven.

After blotting excess moisture from the roots with absorbent paper (see details below), root fresh weight was measured under the following conditions. (i) Laboratory environment: ambient temperature, 25 or 32 °C, air current created by the air conditioning system, 0 and  $4.3 \text{ m s}^{-1}$ , 30 or 74% relative humidity, and light intensities of 60 or  $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$ . Roots were subjected to these conditions for 1 h, which is the average time required to extract 50 plants from a sand substrate. (ii) Time factors: time elapsed between extraction of plants from the substrate and weight measurement, (0.5–2 h for a 100-plant experiment); and extraction time determined by the size of the experiment, which was 1 h (50–60 plants) to 5 h (250–300 plants), and the two types of plant growth substrate (reflecting the difficulty of extracting the plants). (iii) Technical factors: type of absorbent paper used, Whatman No. 1 filter paper (Whatman, Brentford, Middlesex, UK), coffee filter paper (Melitta, Clearwater, FL), fine adsorbing tissue (Kleenex 100, Mexico), and common white paper ( $78 \text{ g m}^{-2}$ , Leeds bond, Kimberly-Clark, Mexico), and two technicians doing extraction and measurement procedures in the same experiment. Standard conditions, when a single variable was changed, were air temperature 25 °C, RH 30%, light intensity  $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , 100 plant samples, and water absorption from the extracted roots with fine absorbing tissue. Fresh weight measurements were made on the same material which was reported by dry weight.

#### 2.4. Bacterial counts from roots

After harvesting the plants, root colonization by *Azospirillum* strains was determined with a standard ELISA method (Levanony et al., 1987) and by *P. fluorescens*, using the plate count method on Nutrient Broth supplemented with 200 mg tetracycline  $\text{l}^{-1}$  (Bashan and Gonzalez, 1999). Total root colonization by the plate count method on nutrient agar (Difco) routinely detected less than  $10^2 \text{ cfu g}^{-1} \text{ dw}$  of unidentified contaminating bacteria.

#### 2.5. Experimental design and statistical analysis

For each condition, five replicates of five plants (a total of 25 plants) were used. All means were based on 25 measurements. Each plant was grown in a separate pot. Experiments were performed twice. Results of single experiments showed similar statistical differences among treatments. Therefore, the results were pooled for one-way ANOVA or Student's *t*-test. Results in percentages were transformed to arcsin before analysis and were analyzed with Statistica software (StatSoft, Inc., Tulsa, OK). Actual *P* values are given for all analyses and all graphical data accompanied by standard error bars.

### 3. Results

While environmental variables caused negligible variability to dry weight values taken immediately after retrieving the samples from the drying oven (standard errors in Fig. 1), environmental variables affected fresh weight determinations. In all four inoculated wheat, tomato, pepper, and cotton cultivars grown in sand and sandy soil (data only shown for wheat and tomato in sand and sandy soil; Tables 1–4) changes in air temperature, air currents created by the air conditioning system, and RH in the lab, caused a variation of 4–14% in root fresh weight. Light intensity in the lab (60 and  $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ) resulted in fresh weight variation of 2–8%, but usually had a marginal effect (Tables 1–4). Variables related to the time between extractions of plants from the substrate and weighing the samples, the size of the experiment, and ease of extraction from the substrate were responsible for differences up to 18% (Tables 1–4). Technical factors, such as type of absorbent paper for blotting excess water, gave results that varied up to 15% (Tables 1–4). Fresh weight variations obtained by different technicians were not statistically different (Tables 1–4). Because measurements for pepper and cotton plants provided similar results, within the same range of values and variability as wheat and tomato plants, these analyses are not presented.

Most bacterial inoculations increased root dry weight within a range of 16–21%, consistent with the general response of these plant species (Fig. 1) (Bashan et al., 1989; Bashan and Gonzalez, 1999). All PGPB colonized host roots at numbers of  $10^4$ – $10^6 \text{ cfu g dw}^{-1} \text{ root}$  (Fig. 2), numbers that had previously been documented (Bashan et al., 1989; Bashan and Gonzalez, 1999).

### 4. Discussion

Obtaining reproducible results is one of the primary goals of scientific studies and is the cornerstone of the peer-review process. It appears that environmental and technical variables, not related to bacterial inoculation, are an

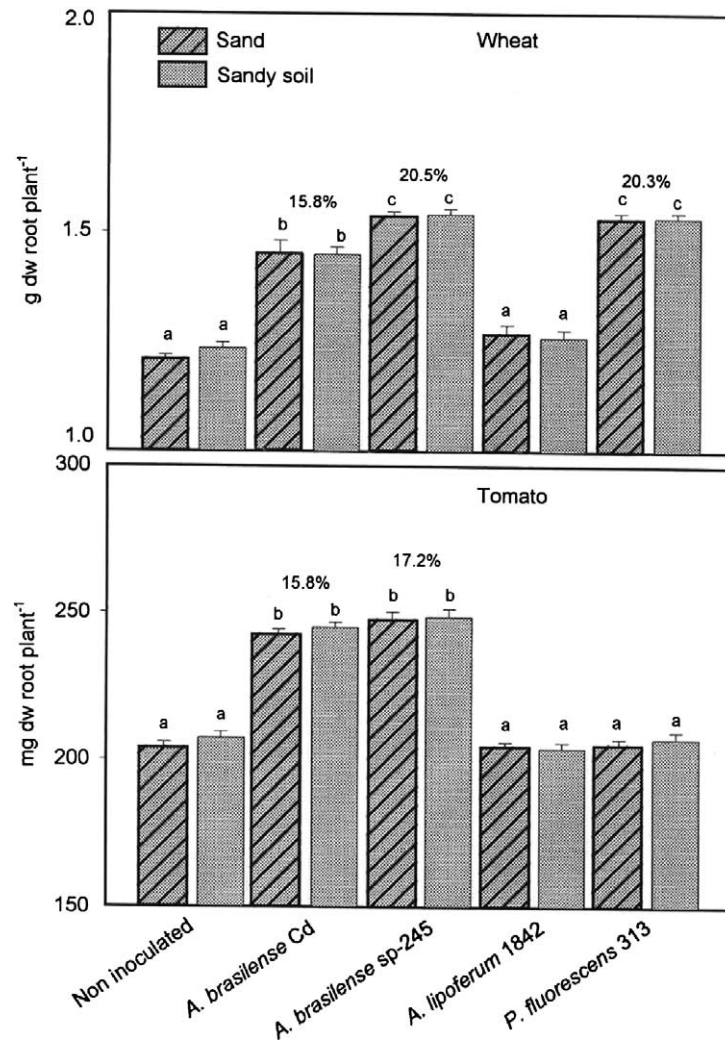


Fig. 1. Effect of inoculation with four plant-growth-promoting bacteria on wheat and tomato plants grown in sand and sandy soil. In each subfigure, columns denoted by a different letter indicate significantly different values at  $P \leq 0.05$  in one-way ANOVA. Number above inoculated columns indicates the average increase in plant dry weight as a result of inoculation. Values of two soils in each treatment were combined because the differences between growth in sand or soil for each treatment are not statistically different. Bars represent standard error.

important issue and can significantly influence fresh weight measurements used to assess the plant growth promoting effect of bacteria. The variances between different determinations can be attributed to the degree that roots become desiccated by prevailing environmental conditions during the course of each experiment.

Almost no variation was detected in dry weight determinations (less than 1% between different persons performing the analysis), whereas the variations in fresh weight were from 0 to 18%, many of these were highly significant statistically. An additional advantage for dry weight measurements was that, in case of accidentally absorbed humidity when working in high RH environments, samples could be dehydrated again and produce identical results. Less than 0.1% variability was detected when samples are dehydrated again (data not shown).

The causes for variability in measurements of fresh weight samples from temperature, air currents, and RH

correlated well with water loss during the sampling process. However, in some cases, the extent of the variation depends on the presence or absence of inoculated PGPB and the inoculated plant (see Tables). Variations in wheat were larger than in tomato, pepper, or cotton plants.

We assume these are real effects based on properties of inoculated plants (Bashan and Holguin, 1997; Bashan et al., 2004), and not a matter of statistical differences of the values without any relation to reality. Furthermore, the data for dry weight and fresh weight suggest that, although there is a natural variation in samples with dry weight measurements, the range of variation in fresh weight samples are far greater (compare Fig. 1 and the Tables). In theory, a large number of samples in each set of fresh weight measurements might decrease the standard deviation of their values enough to make those values statistically comparable to dry weight values, in day-to-day laboratory

Table 1  
Fresh weight determinations (g fresh plant<sup>-1</sup>) of wheat roots growing in sand and each inoculated with one of four PGPB or not inoculated when evaluated under varying laboratory conditions

Variable	Not inoculated	AD (%) <i>P</i>	<i>A. brasilense</i> Cd	AD (%) <i>P</i>	<i>A. brasilense</i> Sp-245	AD (%) <i>P</i>	<i>A. lipoferum</i> 1842	AD (%) <i>P</i>	<i>P. fluorescens</i>	AD (%) <i>P</i>
<i>Laboratory environmental variables</i>										
Temp 25 °C	14.12 a		14.4 a		15.2 a		12.8 a		15.3 a	
Temp 32 °C	12.26 b	13.2***	13.28 b	1.7***	14.46 b	4.8*	12.02 a	NS	15.18 a	NS
Air current 0 m s <sup>-1</sup>	13.98 a		15.12 a		14.98 a		13.58 a		15.12 a	
Air current 4.3 m s <sup>-1</sup>	12.02 b	14.0***	14.36 b	5.0*	14.14 b	5.6*	12.36 b	9.0***	14.6 a	NS
30% R H	12.44 a		14.22 a		14.88 a		12.38 a		14.6 a	
74% R H	13.8 b	9.8***	14.96 a	NS	15.58 b	4.5*	13.74 b	9.9***	15.6 b	6.4*
Light intensity 60 μmol m <sup>-2</sup> s <sup>-1</sup>	12.16 a		13.92 a		15.14 a		13.64 a		15.26 a	
Light intensity 200 μmol m <sup>-2</sup> s <sup>-1</sup>	13.36 b	9.1***	14.64 b	5.0*	15.6 a	NS	13.9 a	NS	15.26 a	NS
<i>Extraction times</i>										
0.5 h	14.22 a		14.6 a		15.6 a		13.46 a		15.3 a	
1 h	11.88 b	18.2***	14.06 ab	7.2*	14.18 b	NS	12.58 b	11.5***	15.12 a	NS
2 h	11.86 b		14.34 a		14.24 b		12.7 b		14.9 a	
5 h	11.64 b		13.56 b		13.54 b		11.92 c		14.84 a	
<i>Technical variables</i>										
Filter paper	12.26 a		14.34 a		15.08 a		12.88 a		15.52 a	
Kleenex paper	12.2 a		14.10 a		15.36 a		13.16 ab		15.36 a	
Coffee filter	13.1 b	15.1***	14.22 a	8.7**	15.24 a	7.0**	13.54 b	9.9**	15.30 a	5.8*
White paper	14.36 c		15.44 b		16.20 b		14.28 c		16.24 b	
Technician 1	11.9 a		14.44 a		15.0 a		13.24 a		15.76 a	
Technician 2	12.12 a	NS	14.12 a	NS	15.12 a	NS	13.18 a	NS	15.66 a	NS

Treatment variables and the technician variable were compared by Student's *t*-test. Time variables and types of blotting paper were compared by one-way ANOVA: \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ . AD = average difference between values expressed as a percentage of the highest value. NS = not significantly different.

Table 2

Fresh weight determinations (g fresh plant<sup>-1</sup>) of wheat roots growing in sandy soil and each inoculated with one of four PGPB or not inoculated, as affected by laboratory variables during the determination of the variables

Variable	Not inoculated	AD (%) <i>P</i>	<i>A. brasilense</i> Cd	AD (%) <i>P</i>	<i>A. brasilense</i> Sp-245	AD (%) <i>P</i>	<i>A. lipoferum</i> 1842	AD (%) <i>P</i>	<i>P. fluorescens</i>	AD (%) <i>P</i>
<i>Laboratory environmental variables</i>										
Temp 25 °C	12.96 a		14.72 a		15.76 a		13.12 a		15.52 a	
Temp 32 °C	12.42 b	4.16*	14.14 a	NS	15.12 b	4.0*	12.84 a	NS	15.08 a	NS
Air current 0 m s <sup>-1</sup>	13.02 a		15.1 a		15.92 a		13.2 a		15.48 a	
Air current 4.3 m s <sup>-1</sup>	12.34 b	5.2**	14.36 b	5.0*	15.18 b	4.65**	12.8 a	NS	15.32 a	NS
30% R H	12.64 a		14.16 a		15.26 a		12.74 a		15.24 a	
74% R H	13.76 b	8.14**	15.26 b	7.2***	16.4 b	6.95*	13.32 a	NS	16.14 b	5.6*
Light intensity 60 μmol m <sup>-2</sup> s <sup>-1</sup>	12.62 a		15.08 a		15.28 a		13.38 a		15.56 a	
Light intensity 200 μmol m <sup>-2</sup> s <sup>-1</sup>	13.02 b	3.0*	15.32 a	NS	15.56 a	1.8**	13.52 a	NS	15.92 a	NS
<i>Extraction times</i>										
0.5 h	13.52 a		15.42 a		15.32 a		13.22 a		15.72 a	
1 h	12.5 b		14.48 b		15.26 a		13.02 ab		15.42 a	
2 h	12.24 b	10.7***	14.38 b	6.8***	15.08 a	NS	12.84 bc	4.6**	15.12 a	NS
5 h	12.08 b		14.26 b		15.16 a		12.62 c		15.04 a	
<i>Technical variables</i>										
Filter paper	13.08 a		15.36 a		15.3 a		12.86 a		15.52 a	
Kleenex paper	13.2 a		15.46 a		15.52 a		12.82 a		15.58 a	
Coffee filter	13.26 a	7.9***	15.32 a	4.0**	15.38 a	8.2***	13.14 a	10.5***	15.54 a	7.2**
White paper	14.2 b		15.98 b		16.66 b		14.32 b		16.72 b	
Technician 1	13.46 a		14.78 a		15.32 a		13.28 a		15.64 a	
Technician 2	13.38 a	NS	14.82 a	NS	15.52 a	NS	13.22 a	NS	15.4 a	NS

Treatment variables and the technician variable were compared by Student's *t*-test. Time variables and types of blotting paper were compared by one-way ANOVA: \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ . AD = average difference between values expressed as a percentage of the highest value. NS = not significantly different.



Table 3

Fresh weight determinations (mg fresh plant<sup>-1</sup>) of tomato roots growing in sand and each inoculated with one of four PGPB or not inoculated affected by laboratory variables persisting during the determinations

Variable	Not inoculated	AD (%) <i>P</i>	<i>A. brasilense</i> Cd	AD (%) <i>P</i>	<i>A. brasilense</i> Sp-245	AD (%) <i>P</i>	<i>A. lipoferum</i> 1842	AD (%) <i>P</i>	<i>P. fluorescens</i>	AD (%) <i>P</i>
<i>Laboratory environmental variables</i>										
Temperature 25 °C	2064 a		2436 a		2566 a		2084 a		2050 a	
Temperature 32 °C	2004 a	NS	2314 b	5.0*	2442 b	4.8***	2006 b	3.7*	1938 b	5.5***
Air current 0 m s <sup>-1</sup>	2074 a		2430 a		2400 a		2056 a		2046 a	
Air current 4.3 m s <sup>-1</sup>	1974 a	NS	2302 b	5.3***	2318 b	3.4*	2018 a	NS	1870 b	8.6***
30% R H	2010 a		2272 a		2344 a		1994 a		1992 a	
74% R H	2068 a	NS	2436 b	6.8***	2524 b	7.1***	2186 b	8.8**	2102 b	5.2***
Light intensity 60 μmol m <sup>-2</sup> s <sup>-1</sup>	2056 a		2438 a		2472 a		2228 a		2058 a	
Light intensity 200 μmol m <sup>-2</sup> s <sup>-1</sup>	2086 a	NS	2466 a	NS	2516 b	1.75*	2236 a	NS	2058 a	NS
<i>Extraction times</i>										
0.5 h	2052 a		2448 a		2480 a		2108 a		2064 a	
1 h	1974 b		2372 b		2350 b		2058 a		1950 b	
2 h	1984 b	5.2**	2378 ab	7.1**	2332 b	7.4***	2042 a	NS	1926 b	10.9***
5 h	1946 b		2276 c		2298 c		1970 a		1840 c	
<i>Technical variables</i>										
Filter paper	2050 a		2440 a		2430 a		2160 a		2048 a	
Kleenex paper	2048 a		2442 a		2424 a		2144 a		2058 a	
Coffee filter	2042 a	6.4***	2426 a	3.1*	2426 a	6.4***	2118 a	NS	2116 ab	7.0*
White paper	2184 b		2516 b		2588 b		2260 a		2200 b	
Technician 1	2038 a		2448 a		2538 a		2150 a		2130 a	
Technician 2	2040 a	NS	2454 a	NS	2524 a	NS	2192 a	NS	2138 a	NS

Treatment variables and the technician variable were compared by Student's *t*-test. Time variable and the type of blotting paper were compared by one-way ANOVA: \* =  $P < 0.05$ , \*\* =  $P < 0.01$ , \*\*\* =  $P < 0.001$ . AD = average difference between values expressed as a percentage of the highest value. NS = not significantly different.

Table 4

Fresh weight determinations (mg fresh plant<sup>-1</sup>) of tomato roots growing in sandy soil and each inoculated with one of four PGPB or not inoculated affected by variables persisting during the determinations

Variable	Not inoculated	AD (%) <i>P</i>	<i>A. brasilense</i> Cd	AD (%) <i>P</i>	<i>A. brasilense</i> Sp-245	AD (%) <i>P</i>	<i>A. lipoferum</i> 1842	AD (%) <i>P</i>	<i>P. fluorescens</i>	AD (%) <i>P</i>
<i>Laboratory environmental variables</i>										
Temp 25 °C	2072 a		2486 a		2500 a		2066 a		2060 a	
Temp 32 °C	2014 a	NS	2358 b	5.1*	2350 b	6.0***	1952 b	5.5***	1950 b	5.3**
Air current 0 m s <sup>-1</sup>	2040 a		2460 a		2510 a		2076 a		2072 a	
Air current 4.3 m s <sup>-1</sup>	2008 a	NS	2306 b	6.3***	2348 b	6.5***	1894	8.8***	1894 b	8.6***
30% R H	2002 a		2370 a		2346 a		1968 a		1974 a	
74% R H	2074 b	3.5*	2562 b	7.5***	2488 b	5.7***	2070 b	4.9**	2082 b	5.2*
Light intensity 60 μmol m <sup>-2</sup> s <sup>-1</sup>	2074 a		2494 a		2442 a		2086 a		2082 a	
Light intensity 200 μmol m <sup>-2</sup> s <sup>-1</sup>	2094 a	NS	2570 b	3*	2456 a	NS	2114 a	NS	2076 a	NS
<i>Extraction times</i>										
0.5 h	2060 a		2476 a		2426 a		2026 a		2046 a	
1 h	2008 b		2304 bc		2360 b		1974 ab		1966 d	
2 h	1974 bc	5.4***	2330 b	8.5***	2336 bc	5.9**	1964 b	7.8***	1892 b	9.1***
5 h	1950 c		2268 c		2284 c		1868 c		1860 bc	
<i>Technical variables</i>										
Filter paper	2068 a		2456 a		2512 a		2066 a		2062 a	
Kleenex paper	2102 a		2456 a		2504 a		2072 a		2080 a	
Coffee filter	2088 a	4.8*	2450 a	4.9**	2520 a	NS	2074 a	NS	2074 a	NS
White paper	2172 b		2576 b		2562 a		2152 a		2116 a	
Technician 1	2074 a		2442 a		2518 a		2134 a		2112 a	
Technician 2	2054 a	NS	2456 a	NS	2508 a	NS	2154 a	NS	2118 a	NS

Treatment variables and the technician variable were each compared by Student's *t*-test. Time variables and type of blotting paper were compared by one-way ANOVA: \*=*P*<0.05, \*\*=*P*<0.01, \*\*\*=*P*<0.001. AD=average difference between values expressed as a percentage of the highest value. NS=not significantly different.



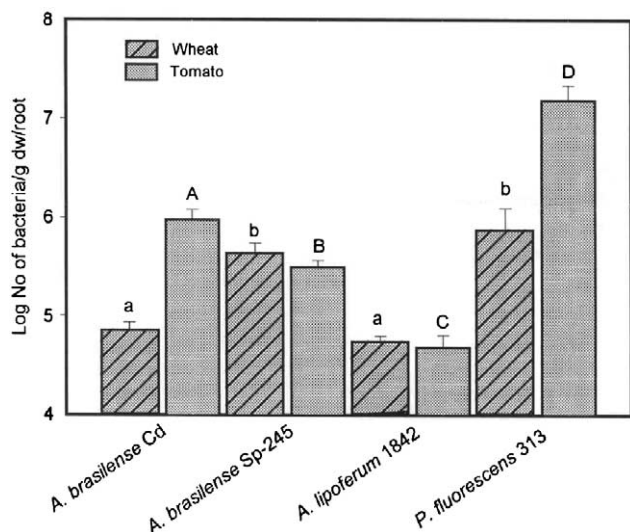


Fig. 2. Root colonization of wheat and tomato roots by *A. brasilense* strains Cd and Sp-245, *A. lipoferum* 1842, and *P. fluorescens* 313. Columns denoted by a different letter indicate significantly different values at  $P \leq 0.05$  in one-way ANOVA. Bars represent standard error.

work, the time and cost of intensive labor make this impractical.

Our results indicate that attention must be paid to conditions under which root fresh weight is measured and that details of these conditions be provided for peer review. Because dry weight does not seem to be affected by the laboratory environment, it should be considered as the standard approach to determine effects of PGPB on plants, and the fresh weight approach to measuring root biomass should be abandoned.

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## References

- Bashan, Y., 1986. Significance of timing and level of inoculation with rhizosphere bacteria on wheat plants. *Soil Biology & Biochemistry* 18, 297–301.
- Bashan, Y., de-Bashan, L.E., 2005. In: Hillel, D. (Ed.), *Bacteria/plant growth-promotion Encyclopedia of Soils in the Environment*, vol. 1. Elsevier, Oxford, pp. 103–115.
- Bashan, Y., Gonzalez, L.E., 1999. Long-term survival of the plant-growth-promoting bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* in dry alginate inoculant. *Applied Microbiology and Biotechnology* 51, 262–266.

- Bashan, Y., Holguin, G., 1997. *Azospirillum*-plant relationships: environmental and physiological advances (1990–1996). *Canadian Journal of Microbiology* 43, 103–121.
- Bashan, Y., Ream, Y., Levanony, H., Sade, A., 1989. Nonspecific responses in plant growth, yield, and root colonization of noncereal crop plants to inoculation with *Azospirillum brasilense* Cd. *Canadian Journal of Botany* 67, 1317–1324.
- Bashan, Y., Holguin, G., Lifshitz, R., 1993. In: Glick, B.R., Thompson, J.E. (Eds.), *Isolation and characterization of plant growth-promoting rhizobacteria Methods in Plant Molecular Biology and Biotechnology*. CRC Press, Boca Raton, FL, pp. 331–345.
- Bashan, Y., Puente, M.E., Myrold, D.D., Toledo, G., 1998. In vitro transfer of fixed nitrogen from diazotrophic filamentous cyanobacteria to black mangrove seedlings. *FEMS Microbiology Ecology* 26, 165–170.
- Bashan, Y., Holguin, G., de-Bashan, L.E., 2004. *Azospirillum*-plant relationships: physiological, molecular, agricultural, and environmental advances (1997–2003). *Canadian Journal of Microbiology* 50, 521–577.
- Berge, O., Fages, J., Mulard, D., Balandreau, J., 1990. Effects of inoculation with *Bacillus circulans* and *Azospirillum lipoferum* on crop-yield in field grown maize. *Symbiosis* 9, 259–266.
- Bouillant, M.L., Miché, L., Ouedraogo, O., Alexandre, G., Jacoud, C., Sallé, G., Bally, R., 1997. Inhibition of *Striga* seed germination associated with sorghum growth promotion by soil bacteria. *CR Academy of Sciences of Paris* 320, 159–162.
- Burd, G.I., Dixon, D.D., Glick, B.R., 2000. Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. *Canadian Journal of Microbiology* 46, 237–245.
- Carrillo, A., Puente, M.E., Bashan, Y., 1996. Application of diluted chlorine dioxide to radish and lettuce nurseries insignificantly reduced plant development. *Ecotoxicology and Environmental Safety* 35, 57–66.
- Chanway, C.P., Holl, F.B., 1994. Growth of outplanted lodgepole pine seedlings one year after inoculation with plant growth promoting rhizobacteria. *Forest Science* 40, 238–246.
- Creus, C.M., Sueldo, R.J., Barassi, C.A., 1996. *Azospirillum* inoculation in pregerminating wheat seeds. *Canadian Journal of Microbiology* 42, 83–86.
- de Freitas, J.R., Germida, J.J., 1990. Plant growth promoting rhizobacteria for winter wheat. *Canadian Journal of Microbiology* 36, 265–272.
- Gagné, S., Dehbi, L., Le Quére, D., Cayer, F., Morin, J.-L., Lemay, R., Fournier, N., 1993. Increase of greenhouse tomato fruit yields by plant growth-promoting rhizobacteria (PGPR) inoculated into the peat-based growing media. *Soil Biology & Biochemistry* 25, 269–272.
- Glick, B.R., 1995. The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology* 41, 109–117.
- Hallmann, J., Quadt-Hallmann, A., Mahaffee, W.F., Kloepper, J.W., 1997. Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology* 43, 895–914.
- Heijnen, C.E., Hok-A-Hin, C.H., Van Elsas, J.D., 1993. Root colonization by *Pseudomonas fluorescens* introduced into soil amended with bentonite. *Soil Biology & Biochemistry* 25, 239–246.
- Iswandi, A., Bossier, P., Vandenberghe, J., Verstraete, W., 1987. Effect of seed inoculation with the rhizopseudomonad strain 7NSK2 on the root microbiota of maize (*Zea mays*) and barley (*Hordeum vulgare*). *Biology and Fertility of Soils* 3, 153–158.
- Kurek, E., Jaroszek, J., 1994. Treatment of rye (*Secale cereale* L.) seedlings with growth promoting *Pseudomonas* strains: plant growth responses and survival of bacteria in the rhizosphere. *Acta Microbiologica Polonica* 43, 189–198.
- Levanony, H., Bashan, Y., Kahana, Z.E., 1987. Enzyme-linked immunosorbent assay for specific identification and enumeration of *Azospirillum brasilense* Cd. in cereal roots. *Applied and Environmental Microbiology* 53, 358–364.

- Lucangeli, C.D., Bottini, R., 1996. Reversion of dwarfism in *dwarf-1* maize (*Zea mays* L.) and *dwarf-x* rice (*Oryza sativa* L.) mutants by endophytic *Azospirillum* spp. *Biocell* 20, 221–226.
- Milus, E.A., Rothrock, C.S., 1993. Rhizosphere colonization of wheat by selected soil bacteria over diverse environments. *Canadian Journal of Microbiology* 39, 335–341.
- Nairn, J.D., Chanway, C.P., 1999. Recovery of a rhizosphere-colonizing GEM from inside wheat roots. *Canadian Journal of Microbiology* 45, 612–615.
- Olivares, F.L., Baldani, V.L.D., Reis, V.M., Baldani, J.I., Döbereiner, J., 1996. Occurrence of the endophytic diazotrophs *Herbaspirillum* spp. in roots, stems, and leaves, predominantly of Gramineae. *Biology and Fertility of Soils* 21, 197–200.
- Pandey, A., Sharma, E., Palni, L.M.S., 1998. Influence of bacterial inoculation on maize in upland farming systems of the Sikkim Himalaya. *Soil Biology & Biochemistry* 30, 379–384.
- Puente, M.E., Bashan, Y., 1993. Effect of inoculation with *Azospirillum brasilense* strains on the germination and seedlings growth of the giant columnar cactus (*Pachycereus pringlei*). *Symbiosis* 15, 49–60.
- Purcino, A.A.C., Paiva, E., Silva, M.R.C., de Andrade, S.R.M., 1996. Influence of *Azospirillum* inoculation and nitrogen supply on grain yield, and carbon- and nitrogen-assimilating enzymes in maize. *Journal of Plant Nutrition* 19, 1045–1060.
- Ratti, N., Janardhanan, K.K., 1996. Response of dual inoculation with VAM and *Azospirillum* on the yield and oil content of *Palmarosa* (*Cymbopogon martinii* var. *motia*). *Microbiological Research* 151, 325–328.
- Shishido, M., Chanway, C.P., 1999. Spruce growth response specificity after treatment with plant growth-promoting *Pseudomonas*. *Canadian Journal of Botany* 77, 22–31.
- Shishido, M., Loeb, B.M., Chanway, C.P., 1995. External and internal root colonization of lodgepole pine seedlings by two growth-promoting *Bacillus* strains originated from different root microsites. *Canadian Journal of Microbiology* 41, 707–713.
- Sturz, A.V., Christie, B.R., 1995. Endophytic bacterial systems governing red clover growth and development. *Annals of Applied Biology* 126, 285–290.