Enhancement of cell division in wheat root tips and growth of root elongation zone induced by *Azospirillum brasilense* Cd

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Inoculation by bacterial infiltration of germinating wheat seeds with 10^6-10^8 colony-forming units of the beneficial rhizosphere bacteria, *Azospirillum brasilense* Cd, significantly increased cell division in root tips during germination. The phenomenon occurred mainly in the second wave, i.e., 24 h after inoculation, of cell division in the meristem. Seed inoculation significantly enlarged the elongation zone of their roots. These inoculation effects suggest that the larger root system, which is usually observed in inoculated plants, may originate in part from the enhancement of cell division and the intensive growth of the elongation zone of seminal roots.

Key words: *Azospirillium*, beneficial bacteria, bacteria–root interaction, cell division, rhizosphere bacteria, root growth.

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L'inoculation par infiltration des graines de blé en germination avec 10^6-10^8 unités formatrices de colonies d'une bactérie bénéfique de la rhizosphère, *Azospirillum brasilense* Cd, accroît significativement la division cellulaire dans les apex racinaires durant la germination. Le phénomène se produit principalement dans la seconde vague (24 h après l'inoculation) de la division cellulaire dans la zone méristématique. L'inoculation des graines agrandit significativement la zone d'élongation de leurs racines. Ces effets de l'inoculation suggèrent que le système racinaire plus développé que l'on observe ordinairement chez les plantes inoculées peut en partie originer de la stimulation de la division cellulaire et de la croissance intensive de la zone d'élongation des racines séminales.

Mots clés : *Azospirillum*, bactérie bénéfique, interaction racine–bactéries, division cellulaire, bactérie de la rhizosphère, croissance racinaire.

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Introduction

Inoculating plants with various species of the beneficial rhizosphere bacterium *Azospirillum* resulted in an increased plant foliage and yield. However, these effects were inconsistent (for review, see 20, 21). The mechanism(s) by which bacterial cells affect plant cells are not clearly understood. Several effects on root morphology and functions were proposed, e.g., production of growth substances by the bacteria that increase the number of the reproductive parts and plant growth (3, 9, 12, 23), increase in root surface area (4, 16), promotion of root branching and root-hair development (11, 13, 15, 16, 21, 24), increase in mineral uptake (2, 15, 18), increased root exudation (8, 25), and biological N_2 fixation at different magnitudes of importance (1, 14, 19, 22).

However, none of the above mechanisms is fully accepted as the major contribution of the bacteria. Currently accumulating data indicate that several mechanisms, each small in magnitude, may simultaneously contribute to the overall effect observed in plants.

The purpose of the present study was to define the origin of one of the most marked effects of inoculation on plant growth, the development of a larger root system.

Material and methods

Organisms, growth conditions, and inoculation

The plant-beneficial bacterium, *Azospirillum brasilense* Cd (ATCC 29710), and wheat seedlings of *Triticum aestivum* cv. Deganit were used as models. *Azospirillum brasilense* Cd was cultured in a nutrient broth (Difco) medium and prepared for inoculation as previously described (5). Wheat seeds were disinfected in 1% NaOCl for 5 min, thoroughly rinsed in tap water (free of chlorine, fluor, or dirt), and imbibed at an ambient temperature under vacuum to facilitate penetration of the following liquid mixtures: (*i*) tap water as control, (*ii*) a double-washed *A. brasilense* Cd suspension containing 10^6 colony-forming units (cfu)/mL in tap water, and (*iii*) a similar suspension containing 10^8 cfu/mL. The seeds were germinated on wet cheesecloth and maintained in Parafilm-sealed Petri dishes at 25 ± 1°C for 24 h.

Measurement of cell division

After 24 h, emerged roots were excised and immediately immersed for 2.5 h at ambient temperature in a saturated solution of α-bromo naphthalene dissolved in tap water to arrest mitosis. Roots were then fixed and stained in a solution of 2% acetocarmine in 45% glacial acetic acid for 4–6 d. To determine the rate of cell division, the root tip (approximately 3 mm from the tip) was excised and hydrolyzed in a boiling solution of acetocarmine and 1 M HCl (3:1 v/v). The meristem was then carefully squashed in acetocarmine (7). These preparations were examined under a Zeiss photomicroscope. Interphase and mitotic nuclei were counted. The mitotic index was determined by dividing the number of cells in mitosis by the total number of cells present in the microscopic area under a magnification of 500.

Measurement of elongation zone of roots

Roots of 48-hour-old seedlings were cut and immersed in a 2-mL boiling solution of concentrated lactic acid (Analar, Poole, UK) containing 5–7 drops of 1% lacmoid (ICN, Plainview, NY, U.S.A.) dissolved in 45% glacial acetic acid for 3–4 min. In the treated roots, the meristem and the elongation zone were clearly identified under a light microscope. Termination of the elongation zone and beginning of the differentiation zone was marked by the first root hairs and the appearance of protoxylem elements. The length of the root from the

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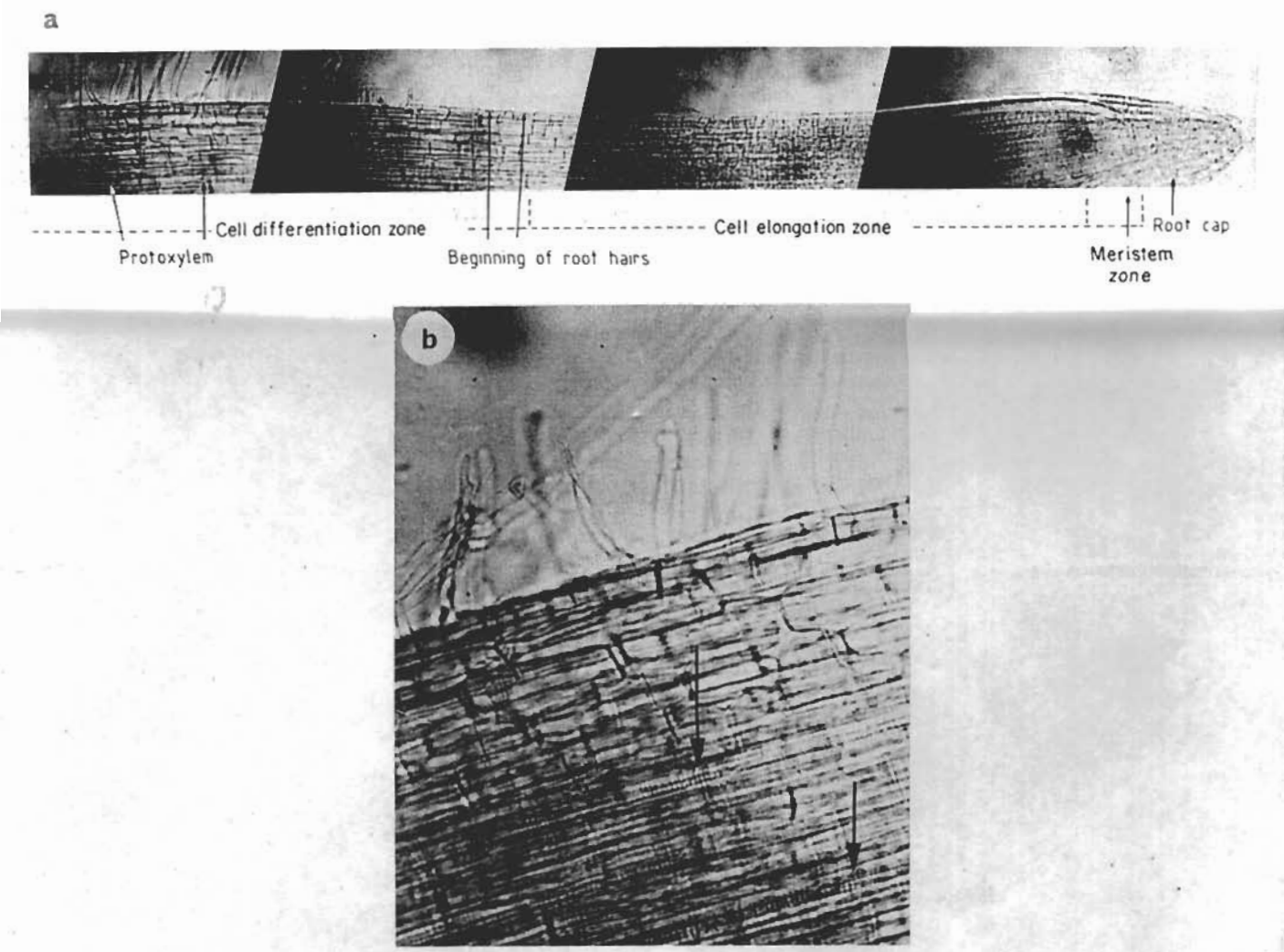

FIG. 1. (*a*) Photograph of intact root of wheat under experimental conditions showing the different root zones. ×125. (*b*) Inset of Fig. 1*a* showing protoxylem elements that were the border of length measurements. Arrows show the beginning of protoxylem elements. ×187.

meristem through the elongation zone was measured at a microscopic magnification of 125 (Fig. 1).

Azospirillium brasilense *Cd counts in wheat roots*

Identification of *A. brasilense* Cd and bacterial counts were determined by competition enzyme-linked immunosorbent assay (ELISA) and by indirect ELISA developed especially for detection and enumeration of this bacterial strain (17).

Experimental design and statistical analysis

Cell division experiments were repeated twice. Six roots originating from six different plantlets were analyzed for each treatment. Twenty randomly chosen areas were analyzed in each root. More than 9000 cells were examined in each experiment. Results presented for the mitotic index are from a representative experiment. Measurements of the elongation zone, carried out on 450 roots in seven experiments, were statistically analyzed together. Significance is given at $P \leq 0.05$ using Duncan's multiple range test.

Results and discussion

The most common morphological change in grasses inoculated with beneficial bacteria of the genus *Azospirillum* is a significant increase in the volume of the root system. The phenomenon was observed in hydroponic cultures and has been observed in field-grown plants (20, 21). Larger root systems may originate from branching of seminal and adventitious roots, enlargement of single roots, and the expansion of cells in the elongation zone. Only the branching effect is, as yet, sufficiently proven by several studies (11, 15, 24). Kapulnik et al. (15) proposed that inoculation has a positive effect on root length.

In the present study, the mitotic index and the size of the elongation zone were determined in young roots of inoculated and noninoculated wheat plants, since these are known to be very sensitive to external treatments (10). A significant enhancement of cell division in root tips (Table 1) 24 h after inoculation at the optimal inoculation level (10^6 cfu/mL (4)) and an increase of 17−29% in the size of the elongation zone (Table 2) 48 h after inoculation were demonstrated in inoculated plants. The two effects occurred immediately after germination. The magnitude of these effects was lower when measured at later stages of the inoculation process.

Kapulnik et al. (16), working with the same *Azospirillum* strain (Cd) inoculated on wheat seedlings, showed that inoculation increased root length but had no significant effect on the size of the elongation zone at the regular inoculum concentration (10^5 cfu/mL). At a high nonoptimal inoculum level (10^8 cfu/mL), a significant decrease in the size of the elonga-

TABLE 1. Rate of cell division in meristems of wheat seminal roots noninoculated and inoculated by *Azospirillum brasilence* Cd, 24 h after inoculation

Inoculum level (cfu/mL)	Total no. of cells evaluated	Interphase cells in mitosis	Mitotic index (%)
0 (water control)	3378	283	7.46*a*
10^6	2141	348	13.72*b*
10^8	4513	564	11.05*ab*

NOTE: Numbers followed by different letters differ significantly at $P \leq 0.05$ using Duncan's multiple range test.

TABLE 2. Size of the elongation zone in seminal roots of noninoculated and inoculated wheat seedlings by *Azospirillum brasilense* Cd, 48 h after inoculation

Inoculum level	Total no. of roots measured	Size of the elongation zone (μm)
0 (water control)	148	20.59*c*
10^6	141	24.07*b*
10^8	161	26.61*a*

NOTE: Numbers followed by different letters differ significantly at $P \leq 0.05$ in Duncan's multiple range test.

tion zone was observed. Our results, which show an increase in the size of the elongation zone *per se* but not a significant enhancement of cell division in this high inoculum level, differ from these previous results, although we share the same opinion that inoculation increases the root size of wheat. One possible explanation for this difference may be the different inoculation and sampling protocols, i.e., Kapulnik et al. (16) measured the effect on the elongation zone approximately 100 h after initial seed wetting, whereas we measured it only 48 h after imbibing. Thus, it may be concluded that the significant effect of *Azospirillum* on the elongation zone of the root diminished quickly. However, this crucial point needs further detailed study. These findings corroborate a previously suggested hypothesis that the marked effect of *Azospirillum* on wheat plant development occurs in the initial stages of seed germination (4).

Estimation of the *A. brasilense* Cd population in the roots revealed a surface population ranging from 5.0×10^4 to 2.0×10^5 cfu/g fresh weight of roots, whereas the internal root population ranged from 1.0×10^6 to 5.0×10^6 cfu/g fresh weight of roots. It was established earlier that the level of inoculation, whether 10^6 or 10^8 cfu/mL, has no effect on the number of wheat roots actually colonized by *A. brasilense* Cd (6). Under the experimental conditions described in this study, the roots were always colonized by *A. brasilense* Cd after inoculation.

It is therefore suggested that the larger root volume of inoculated wheat plants may be caused by the growth of single roots in addition to the known root branching effect (11, 12). Each root of the inoculated plants contained more cells and its elongation zone was larger. Hence, a larger root system supporting the foliage may have formed.

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