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SIGNIFICANCE OF TIMING AND LEVEL OF INOCULATION WITH RHIZOSPHERE BACTERIA ON WHEAT PLANTS

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Summary—The importance of time of inoculation and bacterial concentration in the inoculum on the response of wheat plants was evaluated, using eight strains of rhizosphere bacteria. The optimal bacterial concentration, for all strains, was 10^5–10^6 colony forming units ml^{-1}. Plant response was highest when seeds had been inoculated but was less when seedlings were inoculated. Successive inoculations somewhat increased plant response. Early inoculations resulted in an increased colonization of plant roots at later stages of growth. It was concluded that time of inoculation and the concentration of bacteria in the inoculum were of significant importance in plant response to inoculation and they may govern the inconsistency found in inoculation experiments using beneficial bacteria.

INTRODUCTION

The time of inoculation of plants with beneficial rhizosphere bacteria is of significant importance. Most of the mechanisns involved in the association are either unknown or highly speculative (Okon, 1985; Patriquin *et al.*, 1983). It is important to supply the bacteria and ensure their survival in high numbers for relatively long periods. Crops, such as legumes and summer cereals, which are sown under wet conditions or irrigated immediately after sowing, are usually inoculated at sowing or immediately afterwards (Burton, 1976; Kloepper, 1983). The situation becomes even more problematic in crops, sown in dry soil long before the rain, in which application of bacteria at soaking stage of the carrier is practically impossible because too few of the inoculated bacteria survive.

The aims of this study were to determine the best time to inoculate wheat under fully controlled conditions, to evaluate the number of bacteria required and to explain the inconsistent results obtained when plants were inoculated with beneficial bacteria.

MATERIALS AND METHODS

Organisms

The following bacterial strains were used: *Azospirillum brasilense* Cd, (ATCC 29710) *Azospirillum* sp. T_1 (82008) and T_2 (82012) isolated from *Cynodon dactylon* in Israel and five other unidentified rhizosphere bacteria (82006, 82011, 82021, 84780, 84680) isolated from the rhizosphere of wild cereals in Israel. Wheat (*Triticum aestivum*) cv. "Deganit", served as the host plant.

Plant growth conditions

Wheat seeds, surface disinfested with 1% NaOCl for 3 min and thoroughly washed with tapwater to avoid traces of hypochlorite, were imbibed for 3 h and then either sown in Rehovot brown-red degrading sand soil (5 seeds per 5 l pot, thinned to two seedlings after emergence) or transferred to a hydroponic system (100 seeds per system) consisting of the following parts: a 5 l plastic container filled with 3 l Hoagland's solution (replaced twice a week), a plastic stand with cheese-cloth on its upper surface and, inside the solution, an air ventilation system (a 25 cm glass tube with 10 air exits) supplied with filtered air (to avoid contamination). The experiment was conducted in fully controlled growth chambers (Conviron, model EF7H, Controlled Environments, Canada) at 20 ± 2°C, 10 h light and 14 h darkness or, alternatively, in a controlled greenhouse at 22 ± 3°C (natural illumination).

Inoculation methods

Bacteria, grown in nutrient broth (Difco) for 24–48 h in a rotary shaker, 200 rpm at 15 ± 2°C (*A. brasilense* was grown at 30 ± 2°C), were centrifuged (12,000 *g* min^{-1}) and the harvested cells were washed twice in a saline solution (0.85% NaCl). Then, every culture was diluted to 10^6 colony forming units (cfu)ml^{-1}. Various bacterial concentrations were tested in a single experiment (Fig. 1). Pots were inoculated by irrigating the soil with a bacterial suspension. Each pot was placed on a separate bench to avoid cross-contamination by excess drainage water and a 3 cm layer of sterile vermiculite was spread on the soil surface to prevent airborne dispersal of bacteria within the controlled greenhouse. The hydroponic system was inoculated by pumping out the nutrient solution, washing twice in 4.5 l of tapwater, and then filling the container with 3 l of bacterial suspension. After 12–14 h this suspension was pumped out, the container washed and a new Hoagland's solution applied. Seeds were inoculated by dipping (only slightly covering) disinfected seeds into bacterial suspensions for 12 h. After washing,

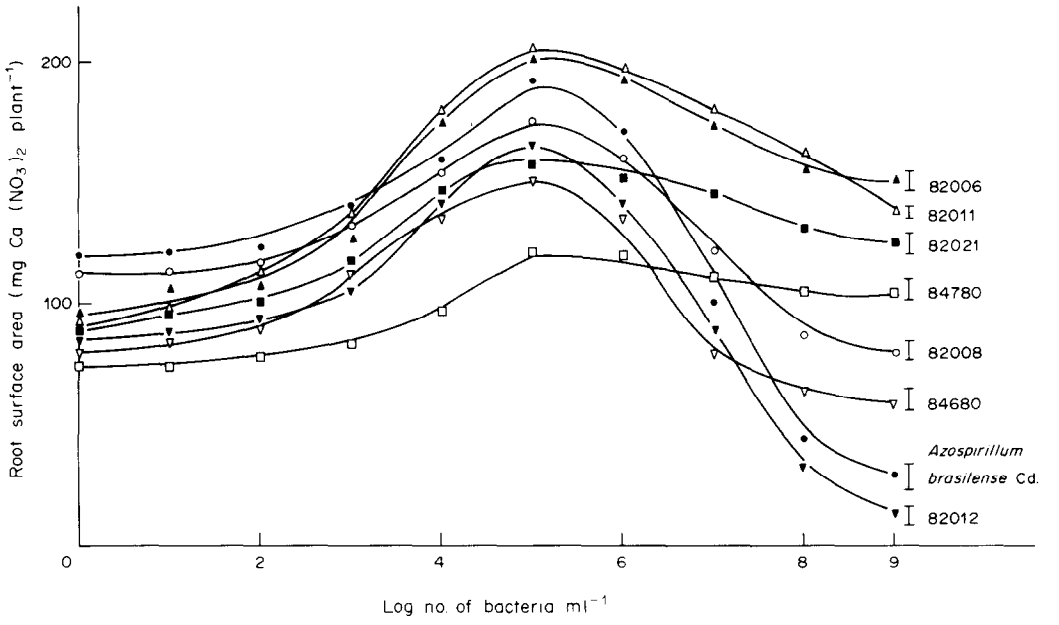

Fig. 1. Response of wheat plant to inoculations with rhizosphere bacteria at various concentrations. Bar near each line represents the standard error of the line.

these seeds were either sown or transferred to the hydroponic system.

Bacterial counts in roots

A. brasilense Cd. was counted in roots by an MPN determination and grown on selective media (Bashan and Levanony, 1985). Other rhizosphere bacteria were counted by homogenizing roots in a fine glass homogenizer (Kontes, U.S.A.) and counting the bacteria on nutrient agar (Difco) plates by the plate count method.

Measurements of plant growth

Root surface area was determined by Carley and Watson's (1966) gravimetric method using $Ca(NO_3)_2$, after gently washing off all soil particles, unless taken directly from hydroponic systems, and air drying them.

Dry weight was determined immediately after drying the foliage in a forced-air oven at 50°C for 72 h.

Experimental design and statistical analysis

All experiments were carried out in a random design, with five replicates per treatment and repeated 2–3 times. Data presented are from representative experiments. A replicate consisted of 10 pots or five hydroponic systems. Significance is given by either $P \leqslant 0.05$ or standard error.

RESULTS

Optimal bacterial level

Eight different rhizosphere bacterial strains were tested, at concentrations ranging from 10^2 to 10^8 cfu ml^{-1}, to determine the optimal level for bacterial inoculation of wheat plants. Plant reaction was measured as the increase in root surface area.

All bacterial strains tested showed the same trend in influencing plant roots. The optimal level, for all the isolates, was 10^5–10^6 cfu ml^{-1}. Decreasing bacterial concentration to low levels such as 10^2–10^4 cfu ml^{-1} had a smaller positive effect, whereas increasing bacterial concentration to 10^8 cfu ml^{-1}, decreased root surface area (Fig. 1).

Effect of time of inoculation

The effect of time of inoculation, using three isolates (*A. brasilense* Cd. 82006 and 84780), was tested in hydroponic systems (for short periods, up to 24 h after imbibition) and in the soil (up to 20 days after seedling emergence). Inoculating seeds, shortly after imbibition, yielded the best results, significantly increasing both root surface area and foliage dry weight as compared with the non-inoculated controls (Fig. 2a and c). Though the effect of the isolates did not differ significantly at any time of inoculation. Late seedling inoculations (up to 20 days after emergence) resulted in a constant decrease in plant response. Soil inoculations (several days after seedling emergence) were even less effective (Fig. 2b and d). Inoculating plants 20 days after seedling emergence was totally ineffective (Fig. 2b and d).

Bacterial counts in the rhizosphere of inoculated wheat plants were constant, unaffected by all but one inoculation timing 20 days post-emergence—which yielded smaller colonization levels (Fig. 2e and f).

Effect of successive inoculations on plants and bacterial colonization of roots

Successive bacterial inoculations of wheat plants grown on soil had only a small effect on root surface area and foliage dry weight. However, after four such

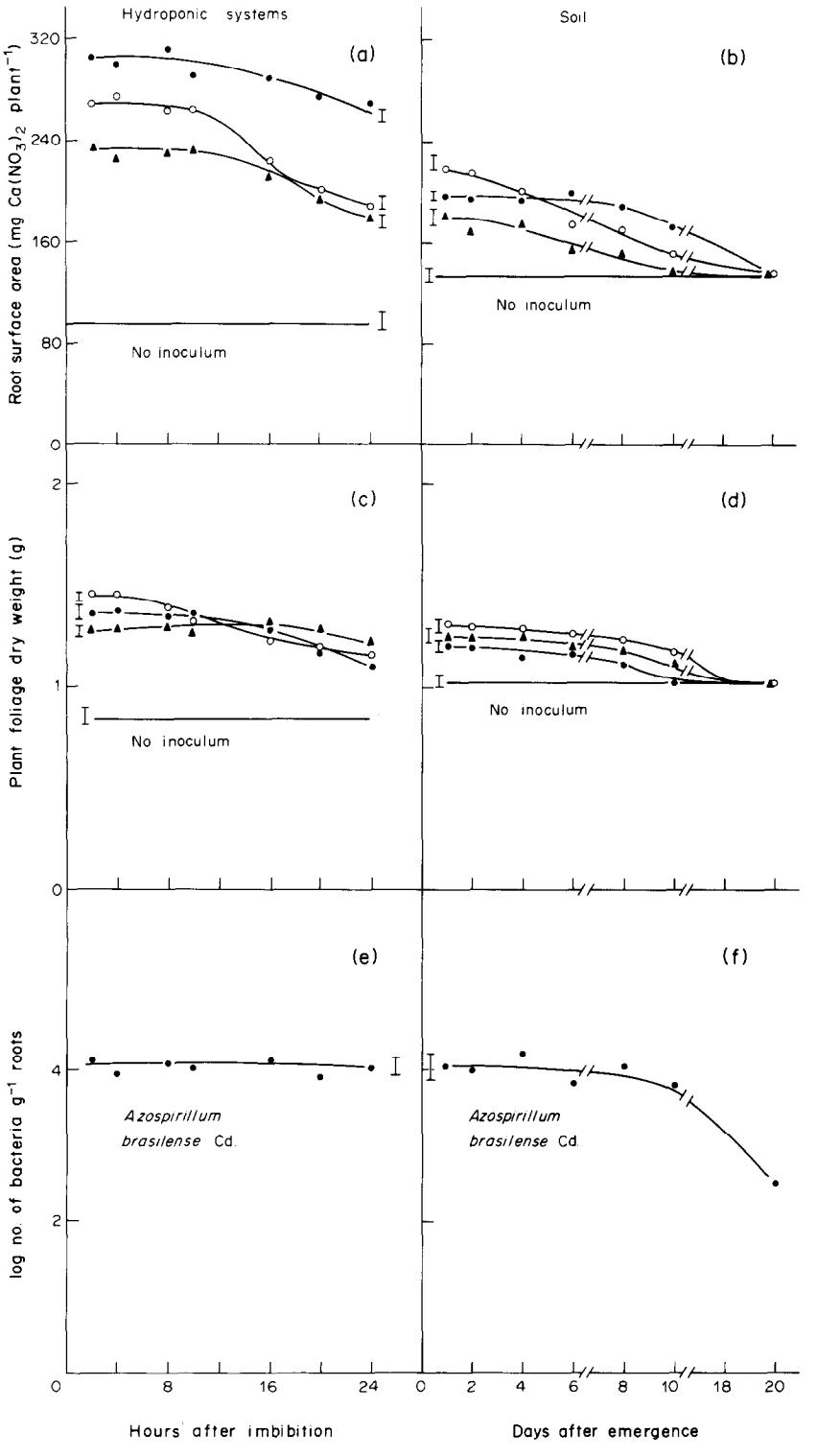

Fig. 2. Response of wheat plants and root colonization to inoculation with rhizosphere bacteria at different inoculation timings, in hydroponic systems (ace) and in soil (bdf). ●—*A. brasilense*. Cd., ○—82006, ▲—84780. Bars near each line represent the standard error of the line.

inoculations, both root surface area and foliage dry weight increased significantly (Fig. 3a and b).

Successive inoculations had a slight effect on bacterial colonization of roots. The slightly increased bacterial counts in the rhizosphere, as a result of successive inoculations, soon returned to its normal level, similar to that following a single inoculation (Fig. 3c).

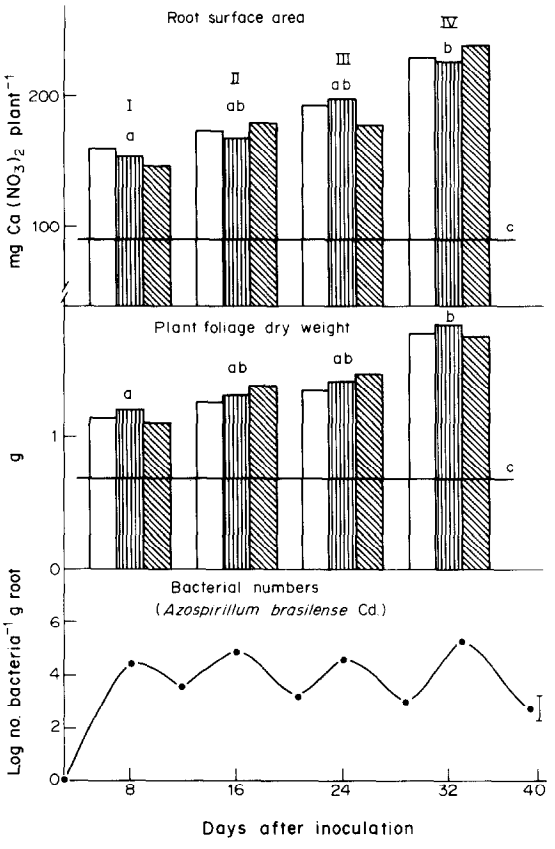

Fig. 3. Effect of successive bacterial inoculation on (a) wheat root surface area; (b) foliage dry weight. ● or □—*A. brasilense* Cd, ▥—82006, ▨—84780, ——— —non-inoculated plants. Columns followed by different letters at each sub-figure, differ significantly at $P \leqslant 0.05$; (c) bacterial count in the wheat rhizosphere. Bar represents standard error of the line.

Root colonization at various time of inoculation

The effectiveness of time of inoculation (at sowing or seedling emergence, the first true leaf, the second leaf and 4–5 leaves) on wheat root colonization by the three rhizosphere bacteria was tested. Root colonization was measured, in each plant separately, 3 weeks after inoculation. *A. brasilense* Cd. successfully colonized the roots during the early stage of growth; $83 \pm 4\%$ of plants were colonized when inoculation was carried out at sowing; $80 \pm 3\%$ of plant roots were colonized when inoculated immediately after emergence; $48 \pm 5\%$ at the first leaf stage and $28 \pm 4\%$ when inoculated at two-leaf stage. When plants were inoculated at the age of 4–5 leaves, most of the root systems ($92 \pm 2\%$) were not colonized. Similar trends, though at lower levels, were obtained by the other two bacteria tested.

DISCUSSION

The effect of different times of inoculation of beneficial bacteria has not been accurately elucidated. There is no substantial documentation of any similarity of overlapping in inoculation timing between experiments. The time span of bacterial application is from sowing until flowering or even later. When sowing under wet conditions, most inoculations are performed at sowing or immediately before or after it (Kloepper, 1983; Bowen and Rovira, 1961; Kloepper *et al.*, 1980; Smith *et al.*, 1984; Sarig *et al.*, 1984; Albrecht *et al.*, 1981; Kapulnik *et al.*, 1981; Baldani *et al.*, 1983; Burton, 1976; Millet *et al.*, 1984), sometime on sowing day or a day or two after seedling emergence (Rovira, 1963; Kapulnik *et al.*, 1985; Tien *et al.*, 1979; Kapulnik *et al.*, 1981; Okon *et al.*, 1983; Thomas-Bauzon *et al.*, 1982), from several days after emergence until the appearance of one to three leaves (Albrecht *et al.*, 1981; Millet and Feldman, 1984; Darbyshire and Greaves, 1970; Kapulnik *et al.*, 1985), or on relatively old plants (Marocco *et al.*, 1983; Nur *et al.*, 1980; Reynders and Vlassak, 1982). However, to the best of my knowledge, a study of one model of association between plant and bacteria, over various periods of time, has not been published.

Kapulnik *et al.* (1985) measured the responses of wheat plants to beneficial *Azospirillum* by analyzing root surface area and foliage dry weight for several weeks following inoculation. These two variables indicated that the optimal timing for inoculation was the first 24 h after seed imbibition. The efficiency of later inoculations decreased, so that inoculating plants 20 days after emergence was totally ineffective. Since root colonization levels were similar, regardlesss of inoculation time and the effects on plants can be measured, it may be concluded that the bacteria affects the seed mainly at germination, and less at the root colonization stage. Hence, inoculation timing is of vast importance. Additionally, early inoculation enhanced root colonization by the beneficial bacteria. Inoculation at sowing or immediately after emergence resulted in 80% root colonization, whereas inoculation at the three to four leaf stage yielded less than 30% colonization.

In experiments in various parts of the world, plants have been inoculated with varying amounts of bacteria, to induce plant response. Although the initial bacterial level was relatively high (10^8–10^{11} cells m^{-2}) no generalization can be drawn as to the optimal bacterial level for best inoculation. Kapulnik *et al.* (1985) found that plant response to high levels of *Azospirillum* was negative and that the optimal bacterial level of inoculation in hydroponic systems was 10^5–10^6 cells ml^{-1}. My study confirms these findings, extending them to include many beneficial rhizosphere bacteria. High bacterial levels decreased plant response, whereas lower levels were apparently ineffective. Over 70 different rhizosphere bacteria isolates showed similar trends (Bashan, Levanony and Avivi, unpublished).

The number of inoculation treatments is also an unknown factor. For practical reasons, most studies were conducted after a single application of bacteria (Kapulnik *et al.*, 1981; Kloepper, 1983; Smith *et al.*, 1984; Burton, 1976). Some experiments were conducted with two (Albrecht *et al.*, 1981) or even three inoculations (Millet *et al.*, 1984). My study shows that multiple inoculations have a marginal effect on plant growth. Only four successive inoculations increased plant response, but their effect on bacterial level in the roots was minimal, perhaps because most

of the inoculation sites on the roots were already occupied with bacteria. Hence, successive inoculations are of minor importance. Furthermore, inoculating relatively large plants is most difficult.

Inoculation timing seems to be crucial in determining successful colonization and influencing plant growth. Under practical field conditions, at least part of the inoculations are not carried out at the optimal time. Thus, part of the applied bacterial population may die, resulting in partial root colonization by these beneficial bacteria and finally, variability in plant response. However, this particular point should be established in controlled field experiments as well. It is, therefore, concluded that inoculating at the wrong time and with inconsistent bacterial levels may result in poor and non-uniform colonization of the root system, thus explaining the inconsistent results obtained from field experiments.

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