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Growth enhancement of maize (*Zea mays* L) through *Azospirillum lipoferum* inoculation : effect of plant genotype and bacterial concentration

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Summary — Germination tests and pot experiments were carried out to study the specificity between an *Azospirillum lipoferum* strain and *Zea mays* hybrids. In both types of trial differences between hybrids were exhibited, and the influence of the plant genotype was clearly demonstrated. The optimal level for bacterial concentration was found to be 10^7 bacteria per plant and the importance of an early inoculation was shown. Pot experiments were less precise than germination tests. Such germination tests for screening plant varieties using the criterion of plant-growth promotion appeared to be a very useful tool.

***Zea mays* / *Azospirillum lipoferum* / germination tests / plant genotype / plant-growth promotion**

Résumé — Promotion de croissance du maïs *(Zea mays)* par inoculation avec *Azospirillum lipoferum* : influence du génotype de la plante hôte et de la dose d'inoculum. *Des essais de germination et des essais en pots ont été utilisés pour étudier la spécificité de l'association entre une souche* d'Azospirillum lipoferum *et diverses variétés hybrides de maïs ainsi que la dose d'inoculum. L'influence du génotype de la plante hôte est clairement montrée dans les 2 types d'essais comme le montrent la figure 1 et le tableau V. Le niveau optimal de l'inoculation a été estimé à 10^7 bactéries par plante et l'importance d'une inoculation précoce est démontrée (tableaux I, II, III). Les essais en pots sont moins sélectifs que les tests de germination (tableau IV). Ceux-ci s'avèrent très performants pour le criblage des variétés sur le critère de la croissance végétale.*

Zea mays / Azospirillum lipoferum / *test de germination / génotype de la plante hôte / promotion de croissance végétale*

INTRODUCTION

Rhizospheric bacteria of the genus *Azospirillum* are known for their ability to improve grain and dry matter yields of crop plants (Yahalom *et al,* 1984; Wani *et al,* 1985; Fayez and Daw, 1987; Tilak and Subba Rao, 1987; Sarig *et al,* 1988).

The mechanism by which *Azospirillum* contributes to plant growth has remained a matter of controversy for years. Nowadays it is know that associative N_2 fixation is often negligible in temperate regions and that additional processes should be considered in the beneficial effects observed (Michiels *et al,* 1989). An early effect of inoculation on root morphology and physiology has been shown: localization on and within wheat roots (Levanony *et al,* 1989), wheat root-hair branching (Jain and Patriquin, 1984), enhancement of mineral absorption (Baldani *et al,* 1983; Lin *et al,* 1983; Morgenstern and Okon, 1987; Sarig *et al,* 1988), root length, respiration rate and ATP-saving on tomato (Hadas and Okon, 1987).

The specificity of the plant–bacterium association is still controversial. According to Jain and Patriquin (1984) and Baldani and Döbereiner (1980), the results depend on the strain–host plant specificity, whereas for Okon and Hadar (1987) the effect is not strain-dependent within a species of *Azospirillum* or within different plant species.

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In *Zea mays,* enhancement of root and shoot length were observed under controlled conditions (Fallik *et al,* 1988); in the field, Hegazi *et al* (1983) obtained yield increases in the Nile valley.

This paper describes the comparative use of 2 methods for measuring the inoculation effects of an *Azospirillum lipoferum* strain on *Zea mays* seedlings. The influence of bacterial concentration and plant genotype are investigated.

MATERIALS AND METHODS

Cultivars

In the germination tests and pot experiments, hybrids INRA 258, Dea (Pioneer Hi-bred Int) were used, and hybrids Pioneer Hi-bred Int, Marina, Licea, Dea, Eva, Fulvia, Volga and Sirena in the comparison between cultivars.

Micro-organism

The strain used was *Azospirillum lipoferum* CRT1 isolated from maize rhizosphere (Fages and Mulard, 1988).

Medium

AFM 10 medium was used (Fages, 1990); the composition was as follows (in g/l): $MgSO_4$ H_2O, 0.2; NaCl, 0.1; $CaCl_2$, 0.02; $FeSO_4$ $7H_2O$, 0.01; Na_2MoO_4 $2H_2O$, 0.002; KH_2PO_4, 0.4; K_2HPO_4, 0.6; yeast extract, 3.0; glucose, 5.0; pH adjusted to 7.2 with NaOH 10 N. Sterilization was by autoclaving for 20 min at 121°C. Glucose was separately sterilized under the same conditions.

Inoculum preparation

Inoculum was prepared in a 250-ml Erlenmeyer flask containing 150 ml medium. These flasks were incubated at 30°C on a rotative shaker for 24 h. These cultures in late log-phase were centrifuged for 15 min at 4 000 g. Cells were resuspended in sterile distilled water, homogenized with a turbine (Ultraturrax, Ika) and diluted until the desired cell concentration was reached for plantlet inoculation. Microbial population was determined by dilution plate counts on AFM 10 medium complemented with agar. Results were expressed as colony forming units (cfu).

Germination tests

One hundred *Zea mays* seeds were placed in a polystyrene box (18 x 15 x 3 cm) containing sterile Loire sand (from the Loire river, France) humidified with bacterial suspension (sand: suspension ratio 5:1 v/v, equivalent to 2.5 ml inoculum per seed). The previously autoclaved inoculum was used as control; its concentration varied from one experiment to another. Seeds were covered with a 1-cm thick sand layer then placed in a germination chamber at 18°C with a relative humidity of 70% for 5 days in darkness. Percentage of germination as well as coleoptile and primary root lengths were measured. Ten replicates of each treatment were made. Germination percentages were determined by deduction of dead or abnormal plantlets from total plant count. Lengths were measured on 10 plants sampled randomly.

Pot experiments

Twelve or 20 *Zea mays* seeds were placed in a pot over a water tank containing 2.5 l of sterile Loire sand. The sand had been previously humidified with demineralized water (sand: water ratio, 5:1 v/v). Inoculation was achieved by placing 1 ml bacterial suspension on each seed. The previously autoclaved inoculum was used as control. When several inoculum concentrations were tested, the intermediate level (10^7 cfu/ml) was chosen for autoclaving. Seeds were then covered with a 1-cm thick layer of sand. Ten replicates of each treatment were made. Pots were placed in a germination chamber for 14 days with a photoperiod of 14 h light and 10 h dark and a synchronous thermoperiod of 25/20°C. Plant nutritive requirements were covered by the following presterilized solution (in mg/l): KNO_3, 384; K_2HPO_4, 54; KH_2PO_4, 109; $NaNO_3$, 17; NaCl, 12; NH_4NO_3, 16; $Ca(NO_3)_2$ $4H_2O$, 732; $MgSO_4$ $7H_2O$, 185; $FeSO_4$ $7H_2O$, 1; $ZnSO_4$ H_2O, 1; H_3BO_3, 1; $CuSO_4$ $5H_2O$, 0.03; $MnSO_4$ $4H_2O$, 0.1; $(NH_4)_2MoO_4$, 0.03. At emergence (after 4 days), thinning out of seedlings was carried out leaving 9 out of 12 or 16 out of 20 plantlets per pot. At harvest after 14 days, the following measurements were performed: root surface area, root and shoot dry matter.

Titrimetric measurement of root surface area (Carley and Watson, 1966)

The root system was washed with demineralized water to eliminate all the sand particles remaining on the root system. After excision, roots were drained for 3 h. Each root system was then soaked for 15 s in HCl 3 N. Excess acid elimination was performed in a manual centrifugal dryer. Roots were then left in 300 ml of demineralized water with gentle shaking. 100 ml of this solution was then titrated with NaOH 0.25 N and phenolphtalein as dye indicator. Results were ex-

pressed in ml of poured NaOH. Roots and shoots were then placed in a ventilated drying oven for 48 h at 85°C for dry matter determination.

Statistical analysis

Variance analyses were followed by Newman–Keuls test when Fisher test probability was < 5%.

RESULTS

Germination tests

No modification in the percentages of germination was observed (data not shown).

Effect of bacterial concentration

A preliminary experiment carried out with 6 x 10^9 cfu/ml (table I) showed an inhibition of root growth. A non-significant decrease in shoot-length was observed with both cultivars.

Another experiment was set up with 3 different bacterial concentrations of inoculum: 0.9 x 10^9, 0.9 x 10^7 and 0.9 x 10^5 cfu/ml. At the highest concentration, no difference in length from control was observed (table II). A significant increase in primary root elongation was obtained with the 2 other bacterial concentrations. However, a decrease in shoot length with the lowest bacterial density was obtained.

Table III shows results from trials using the same protocol but carried out at different dates. Four inoculum levels were tested in these trials.

Table I. Germination tests. Effect of inoculation with *A lipoferum* CRT1 at 6.0 x 10^9 cfu/ml on root and shoot lengths of 2 hybrids of *Zea mays*.

Hybrid	*INRA 258*		*DEA*	
	Control	*Inoculated*	*Control*	*Inoculated*
Shoot length (mm)	41.8 a	40.6 a	46.7 a	43.4 a
Primary root length (mm)	93.3 b	80.3 c	121.9 b	86.1 c

Mean values having common letter within each cultivar are not significantly different at 0.05 level (Newman–Keuls test).

Table II. Germination tests. Effect of bacterial concentration of *A lipoferum* CRT1 on root and shoot lengths of *Zea mays* (cv Dea).

Bacterial concentration (cfu/ml)	*Shoot length (mm)*	*Primary root length (mm)*
0.9 x 10^9	37.0 a	94.3 ab
0.9 x 10^7	37.6 a	95.5 a
0.9 x 10^5	33.8 b	95.8 a
Autoclaved control	36.3 a	91.2 b

Mean values having common letter within a column are not significantly different at 0.05 level (Newman–Keuls test). Control is the intermediate concentration culture autoclaved.

Table III. Germination tests. Effect of bacterial concentration of *A lipoferum* CRT1 in 4 trials on root and shoot lengths of *Zea mays* (cv Dea).

Trial No	*Bacterial concentration (cfu/ml)*	*Shoot length (mm)*		*Primary root length (mm)*	
		Control	*Inoculated*	*Control*	*Inoculated*
1	1.0 x 10^6	42.9 a	45.3 b	106.2 c	108.1 c
2	5.0 x 10^6	47.1 a	48.6 b	105.2 c	110.3 d
3	1.0 x 10^7	34.7 a	37.6 b	80.0 c	92.4 d
4	1.0 x 10^8	40.4 a	42.5 b	87.6 c	90.7 d

Mean values having common letter within a trial are not significantly different at 0.05 level (Neuwman–Keuls test). Length variations between trials are caused by test duration which may vary by a few hours after 5 days. For each trial, control is the bacterial culture autoclaved.

Apart from the lowest concentration which exhibited no difference, all the other inoculations led to a significant increase in root elongation. The highest increases were obtained with the 2 intermediate concentrations. The bacterial density effect on shoot length was less pronounced, since a significant increase was observed with all the inoculum concentrations.

Effect of the genotypes of 7 maize hybrids

To test host-plant genotype, the level of 1.0 x 10^7 cfu/ml was chosen. Besides the Dea hybrid already tested with this concentration, 6 new cultivars were tested (fig 1). Four hybrids, Eva, Fulvia, Dea and Marina, exhibited a positive response to both criteria. Shoot elongation was respectively increased by 13.1%, 10.2%, 8.4% and 7.8%. Root elongation was respectively increased by 4.3%, 3.0%, 5.0% and 3.1%. In Volga, a significant increase in root elongation of 3.8% was observed. In Licea, in spite of a 5% increase in shoot length, no significant difference was observed. Finally an inhibition of root growth was obtained with Sirena.

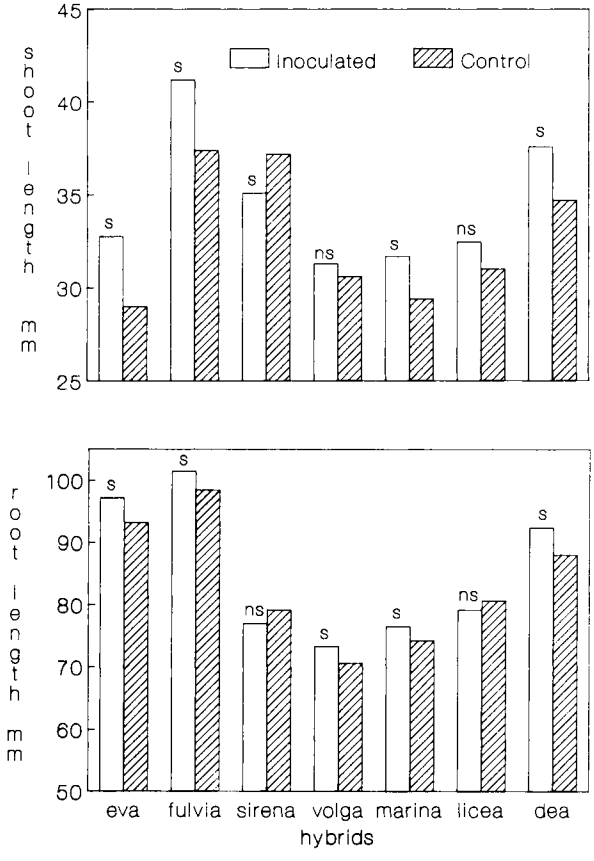

Fig 1. Germination tests. Primary root and shoot lengths of *Zea mays* hybrids through inoculation with *A lipoferum* CRT1 at 1.0 x 107 cfu/ml s, significantly different at 0.05 level (Newman–Keuls tests); ns, not significantly different at 0.05 level (Newman–Keuls test).

Pot experiments

Effect of bacterial concentration

In the first experiment, 3 bacterial concentrations of 0.9 x 10^9, 0.9 x 10^7 and 0.9 x 10^5 cfu/ml were tested for 14 days with 16 plants per pot. As shown in table IV, a significant increase in root surface area (13.5%) was obtained for the intermediate level. No significant difference with the other parameters was observed, due to too wide a biological variation.

Nevertheless, in all cases measurements on inoculated plants gave higher values than those obtained with control plants. In a second trial carried out with 9 plants, 3 other concentrations, 1.0 x 10^8, 1.0 x 10^7 and 1.0 x 10^6 cfu/ml, were tested.

There was too great a dispersion in the measurements for the statistical threshold to be reached on root dry matter and root surface area. However, the intermediate inoculum concentration gave the highest values. On aerial parts, a significant increase in dry matter was obtained whatever the inoculum concentration.

Effect of the genotypes of 5 maize hybrids

Two hybrids, Volga and Licea, tested in the germination tests, were not assayed here. The lar-

Table IV. Pot experiment. Effect of bacterial concentration of *A lipoferum* CRT1 on root surface area and dry weight of *Zea mays* (cv Dea).

Bacterial concentration (cfu/ml)	*Root surface area (ml NaOH/pot)*	*Shoot dry weight (g/pot)*	*Root dry weight (g/pot)*
Trial 1			
0.9 = 10^9	14.8 a	2.45 a	2.07 a
0.9 x 10^7	15.1 b	2.40 a	2.18 a
0.9 x 10^5	13.5 a	2.36 a	2.01 a
Control	13.3 a	2.33 a	1.99 a
Trial 2			
1.0 x 10^8	10.3 a	1.99 a	0.56 a
1.0 x 10^7	11.1 a	2.02 a	0.61 a
1.0 x 10^6	9.6 a	2.08 a	0.54 a
Control	9.4 a	1.75 b	0.57 a

Mean value having common letter within trial and within a column are not significantly different at 0.05 level (Newman–Keuls test). Control is the intermediate concentration culture autoclaved.

gest significant effects were obtained on Dea root surface area (+ 21% over control) and on Eva shoot dry matter (+ 7%). No other differences were obtained with these 2 cultivars (table V). Responses of the 3 other plant varieties to inoculation were non-significant.

Table V. Pot experiments. Effect of inoculation with *A lipoferum* CRT1 at 1.0 x 10^7 cfu/ml on root surface area and dry weight of different *Zea mays* hybrids.

Hybrid	*Root surface area (ml NaOH/pot)*		*Root dry weight (g/pot)*		*Shoot dry weight (g/pot)*	
	Control	*Inoc*	*Control*	*Inoc*	*Control*	*Inoc*
Marina	9.6a	9.7a	0.64c	0.65c	1.97d	2.03d
Dea	13.4a	17.0b	0.65c	0.68c	2.34d	2.37d
Eva	8.3a	8.0a	0.80c	0.77c	1.49d	1.60e
Fulvia	17.8a	17.1a	0.88c	0.91c	2.38d	2.42d
Sirena	10.3a	10.6a	0.59c	0.60c	2.21d	2.26d

Mean values having common letter within a hybrid are not significantly different at 0.05 level (Newman–Keuls test).

DISCUSSION

The different parameters checked in germination tests did not show the same sensitivity to bacterial inoculation. The percentage of germination was not affected. This qualitative criterion may not be suitable for demonstrating growth promotion. Moreover, it is related to seed physiology rather than to external effects.

Root elongation was the best parameter for displaying differences between inoculum concentrations. This is evidence that it is the root development of plantlets which is directly affected by bacterial inoculation. Under our experimental conditions, the optimal inoculum level was 1.0 x 10^7 cfu/ml or 2.5 x 10^7 cfu/plant. Lower bacterial densities led to lower responses. These results are in agreement with the findings of Fallik *et al* (1988) on *Zea mays,* while Kapulnik *et al* (1985) found an optimal inoculum level for wheat of between 10^5 cfu/plant and 10^6 cfu/plant. Beyond 10^9 cfu/ml, a clear inhibition was observed.

Shoot elongation was also increased through inoculation. However, this parameter was less sensitive to the number of bacteria applied.

These results show a very early effect of *Azospirillum* inoculation on *Zea mays.* With another crop *(Panicum miliaceum),* similar effects were obtained by Harari *et al* (1989) on root elongation and root-hair development.

The effect of *Zea mays* inoculation by *Azospirillum lipoferum* CRT1 was host-plant genotype dependent since coleoptile elongation varied from –5.6% to +13.1% and primary root length varied from –2.8% to +5.8% over control. Figure 1 illustrates these results, showing that germination tests are a powerful tool for the screening of plant–bacteria associations on the plant-growth promotion criterion. This is a decisive step forward compared with classical screening methods performed with indirect criteria such as biological nitrogen fixation (Ela *et al,* 1982; Thomas-Bauzon *et al,* 1982) or phytohormone production (Zimmer and Böthe, 1989).

Pot experiment results showed that inoculation effects were still observable after 2 weeks' growth. The increases obtained had the same order of magnitude as those observed after 5 days. However, the biological variation was at this development stage more pronounced, and consequently, results were less often statistically significant.

For root surface area improvement, the optimal inoculum level appeared to be 1.0 x 10^7 cfu/plant. No correlation between root dry matter and root surface area was found. The better development of the root system which can be easily observed *de visu* was not converted into root dry matter variations measurables after 14 days of growth. Titrimetric measurement of root surface area is the best method for evidencing differences among inoculum concentrations. As shown by the trial with several plant varieties, this method was not suitable for hybrid screening. Compared with the germination tests method, it has 3 major disadvantages. It is longer, involves more work and is less discriminative.

Azospirillum lipoferum CRT1 growth-promotion effect on *Zea mays* began from the very early plant development stages and remained observable after 2 weeks. It could be measured on several parameters, and depended on both the inoculum concentration and the host-plant genotype. These findings suggest that the yield enhancements in the field came, at least partly, from such early root-growth promotion effects.

The methods described here, particularly the germination tests, indicated which bacterial strain-cultivar pair should be preferably chosen and which inoculum level was optimal. Moreover, the precocity of the observed effects showed that the period immediately following planting is crucial. Hence, one wonders, if the fate of the inoculated strain in the rhizosphere is really important beyond the few days during which the growth-promotion effect acts. The inoculum formulation (Fages, 1990) and the methodology of field application is therefore very important and must allow a good root colonization at the beginning of plant growth. These methods are under current investigation in our laboratory.

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