Azospirillum Inoculation of Maize Seed During Imbibition

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Summary

Maize seeds (*Zea mays*, L.) hybrid DK 636 (FAO 530) were inoculated either with live (L) or control (autoclaved) *Azospirillum brasilense* BR 11005 cells during imbibition. Seeds were then dried up to 14% humidity, and stored at 15 - 20°C in the dark. After 0, 5, 15, and 25 days of storage, seeds were germinated in the dark in a humid chamber at 20°C, and seedlings were grown for 5 days. Afterwards, length, fresh weight (FW), dry weight (DW), and water content (WC) were determined in coleoptiles, while roots were used to determine *Azospirillum* most probable number (MPN), DW and root surface. The growth-promoting effect of *Azospirillum* on root DW and radical surface was significantly evident in all periods of seed storage. However, the effects on coleoptile length, DW and WC were significant only after 15 days of storage. On the other hand, *Azospirillum* MPN in roots of seedlings from L seeds was 10^8 cells g^{-1} at 0 and after 5 days of seed storage, while decreasing to 10^7 and 10^5 at 15 and 25 days, respectively. These results indicate an optimal bacterial concentration of 10^8 - 10^7 cells g^{-1} of roots, for a general growth promotion effect on maize seedlings, and 10^5 to have an effect in roots only. The inoculation system adopted here was able to maintain this last MPN even after drying and storing seeds for up to 25 days.

Introduction

Biotechnologies based on the use of rhizobacteria to enhance plant growth and crop yield are highly dependent on a successful colonization either of the rhizosphere, the surface and/or the interior of the root (Harris et al., 1989; Summer, 1990; Okon and Itzigsohn, 1995). The definitive bacterial establishment is affected by various edaphic, abiotic and biotic factors, which also have direct influence on the plant growth and the functioning of the root. Humidity and temperature regimes, pH, texture, availability of O_2 and nutrients, are the principal abiotic factors affecting the survival of the bacteria introduced in the soil (Davies and Whitbread, 1989; Tsai et al., 1992). The most important biologic factors are predation by protozoa (Acea and Alexander, 1988), antagonism and rivalry with other microorganisms (Acea et al., 1988; Fallik et al., 1988 a), and physiological state of the bacterium introduced (Vandenhove et al., 1993; Vande Broek and Vanderleyden, 1995).

In general, the techniques previously developed for the inoculants with *Rhizobium* have been adapted to inoculate *Azospirillum*. The supports commonly used in association with this bacteria are peat, vermiculite or alginate, as well as liquid formulations. Hence, present inoculation systems are applied to sowing, rather than to germination or else in post-emergence. But all of them are based on the bacterial contribution made externally to the seed or to the seedling. Therefore, in these systems the final bacterial settlement into the plant is limited by most of the abiotic and biotic factors cited previously.

The inoculation method must also ensure the liberation of the inoculum in the field in a timely and precise way, allowing the bacterial cells to remain viable and to keep their ability to colonize the roots in an adequate number (Harris et al., 1989; Fages, 1994; Okon and Labandera-González, 1994). This number varies according to plant species, the recommended one being 10^5-10^6 bacteria per plant in the case of wheat (Kapulnik et al., 1985), and 10^7 in the case of maize (Arsac et al., 1990), 2-3 weeks after sowing.

Experiments performed by Creus et al. (1996) demonstrated the feasibility of inoculating wheat seeds with *Azospirillum* prior to germination by means of imbibition of the seeds with the inoculum. In the seeds dried and stored, 3.7 x 10^6 viable cells g^{-1} were detected after 27 days of storage. In the case of maize, the adaptation of this method would be easier due to the greater size of the seeds, since their greater area favor the contact with the inoculum.

The objectives of this work were to introduce *A. brasilense* into maize seeds during imbibition, and after drying the seeds, to study the effect of storage on the bacterial ability to colonize the root of the emerging seedling, and in addition, to evaluate several growth parameters in coleoptiles and roots of seedlings obtained from the inoculated, stored seeds.

Materials and Methods

Bacterial culture and inoculation of seeds

Azospirillum brasilense BR 11005 (ex Sp. 245) was multiplied in congo red agar medium (Rodríguez Cáceres, 1982) for 72 h at 30°C, transferred to OAB liquid medium containing 0.1% NH_4Cl (Okon et al., 1977), and incubated for 24 h at 32°C with orbital agitation (100 rpm). Cells were harvested by 10 min centrifugation at 8,142 *x g* in a SS43 Sorvall rotor and resuspended in 66 mM phosphate buffer (pH 7), to 10^9 bacteria ml^{-1}.

Seeds of the DK 636 (FAO 530) hybrid maize (*Zea mays* L.) were surface-sterilized in 1% NaOCl for 5 min and washed thrice with sterile distilled water (SDW). Preimbibition in water was accomplished during 4 h. Later, the seeds were inoculated either with live (L) or control (C) autoclaved *Azospirillum* cells by immersion for 4 h in the inoculum containing 10^7 bacterial cells $seed^{-1}$ (Creus et al., 1996, Casanovas, 1997).

Once inoculation was finished, seeds were air-dried in a laminar flow cabinet to 14% humidity, and stored at 15 - 20°C in the dark up to 25 days.

Colonization assessment and growth parameters in seedlings

After 0, 5, 15, and 25 days of storage, groups of 30 L and C seeds were transferred to 20 cm diam. x 8 cm high polycarbonate containers having in the bottom a 0.5 cm layer of cotton and filter paper soaked in SDW. These containers were placed in a growth chamber at 20°C, where seedlings were left to grow in the dark for up to 5 days (Casanovas, 1997). Seedlings were left to grow under these conditions for up to 5 days. Afterwards, length, fresh weight (FW), and dry weight (DW) of the coleoptiles were measured, while roots were used to determine *Azospirillum* colonization, DW and root surface.

One g of roots obtained from pools of 15-20 five-day-old L and C seedlings developed from seeds stored for different periods of time, were immersed in 3% chloramine-T for 10 min and washed twice for 10 min with SDW. After homogenization in a mortar with 4.5 mL 66 mM phosphate buffer (pH 7), nine 1:10 serial dilutions up to 10^{-9} were obtained. Three 0.1 mL replicates from each dilution were cultured in semisolid NFb medium containing 0.1% NH_4Cl (Döbereiner and Day, 1976), and bacterial most probable numbers (MPN) per gram of FW were estimated according to Postgate (1969). Bacterial colonies were then transferred to congo red agar medium to detect typical *A. brasilense* growth (Rodríguez-Cáceres, 1982).

Growth parameters were determined in tissues dissected from the remaining 10-15 five-day-old seedlings developed from seeds stored at different times.

DW was determined by drying coleoptiles and roots in an oven at 65°C, until constant weight. Root surface was determined by titration (Wilde and Voigt, 1949; Carley and Watson, 1966), and expressed as mL of 0.03N NaOH. Water content (WC) of coleoptiles was calculated as follows: $WC = (FW - DW) \times 100 \times DW^{-1}$.

Experimental design and statistical analysis

The experimental design was in completely randomized blocks, with three repetitions. The results were analyzed through PROC GLM procedure, using the SAS statistical package (SAS Institute, 1994). Differences between means were determined using the Tukey's test ($P < 0.05$).

Results and Discussion

Effect of the inoculated seed storage time on the ability of *A. brasilense* BR 11005 to colonize the seedlings

MPN of *Azospirillum* in roots of seedlings from C seeds remained fairly constant during the whole period of study, at levels no lower than 10^4 cells g^{-1} (Table 1). In the roots of seedlings from L seeds, at zero time and after 5 days of storage 10^8 bacteria g^{-1} were detected. This concentration diminished in one order of magnitude at 15 days, and in three at 25 days of storage (Table 1). These results are similar to those reported for wheat by Creus et al. (1996), who used the same inoculation method.

The presence of *Azospirillum* in the controls treated with autoclaved bacterium indicate the existence of an 'endogenous pool' of the bacterium, as already reported by various authors in different graminaceous plants (Rao and Venkateswarlu, 1988; Volkogon et al., 1995). Nevertheless, the existence of an airborne phase of the *Azospirillum* sp. could explain the presence of the bacterium in the control, despite of all the precautions taken (Bashan, 1991).

Table 1. Viable *Azospirillum* cells, in roots of 5-day-old seedlings grown from maize seeds inoculated during imbibition, and stored dry

Storage time (Days)	Treatment	MPN (*Azospirillum* cells g^{-1} FW)
0	L	1.1 (±0,80) x 10^8
	C	5.7 (±3,10) x 10^4
5	L	1.8 (±0,23) x 10^8
	C	1.1 (±0,13) x 10^5
15	L	1.1 (±0,80) x 10^7
	C	1.2 (±0,31) x 10^5
25	L	4.5 (±0,00) x 10^5
	C	1.4 (±0,16) x 10^4

Means of most probable number (MPN) of *Azospirillum* viable cells and their standard deviations (in parentheses) were obtained from triplicates. FW: fresh weight. L and C: seeds inoculated with live and control, autoclaved *A. brasilense* cells, respectively.

Storage of *Azospirillum*-inoculated maize seeds versus growth promotion effects on seedlings

DW of L roots significantly exceeded that of C roots during all the experimental period (Fig. 1). The per cent increase ranged from 63 % at 0 time, to 24 % at day 25 of seed storage.

In addition, *Azospirillum* inoculation induced a significant increase of the root surface during the storage period under study. In this parameter, the per cent of increase due to inoculation varied between 50 and 80 % (Fig. 1).

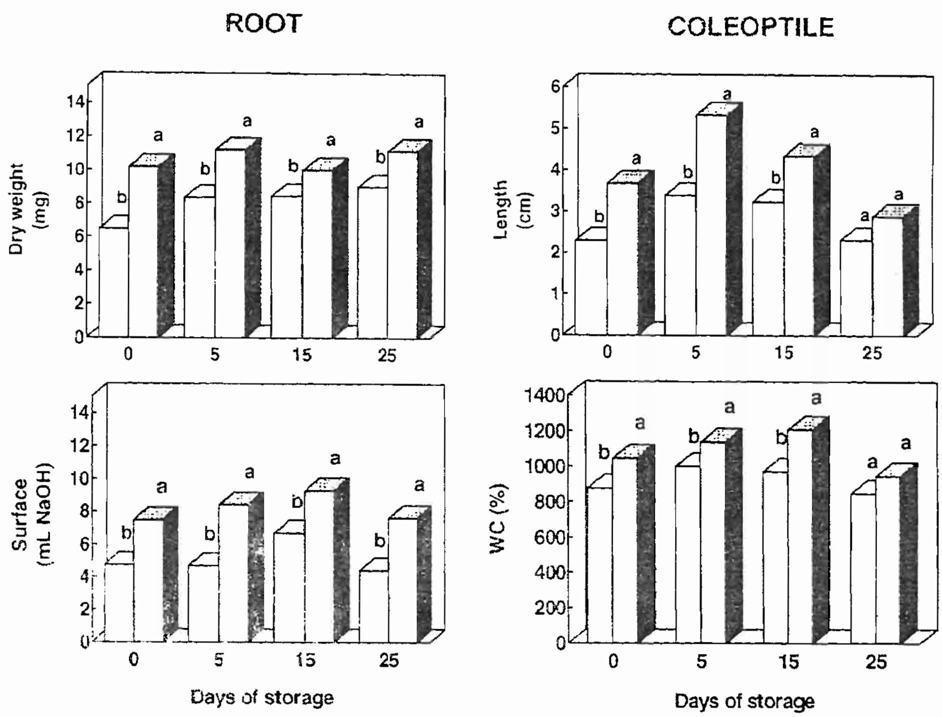
These results extend those obtained by Okon and Kapulnik (1986), who reported an increase in both the number and ramifications of radical hair and lateral roots, not accompanied by significant changes in root biomass during the first three weeks after inoculation. In contrast, the inoculation system adopted in the present work could probably allow a better expression of the bacterial effects during the first days of growth. This, in turn, could result in an increased root DW (Fig. 1).

Figure 1 shows that at 0, 5, and 15 days of seed storage, the coleoptile length from L seeds was significantly greater than those from C seeds, with increases of 61

%, 56 %, and 34 %, respectively. Even though this difference between L and C coleoptiles was 22 % after 25 days of seed storage, it was not significant ($P < 0.05$).

The DW of coleoptiles from L seeds exceeded that of C ones in 54 %, 47 %, 37 %, and 18 % during the period under study. These differences were significant in the first

Fig. 1. *Azospirillum* effects on growth parameters and water content in 5-day-old seedlings grown from maize seeds inoculated during imbibition, and stored dry



White and shadowed bars correspond to seeds inoculated with autoclaved and living *A. brasilense* BR11005 cells, respectively. WC: water content. Different letters on top of bars mean significant differences (P<0.05). Data are means of at least 10 seedlings.

three storage periods (data not shown).

The WC of L coleoptiles significantly differed from that of C ones during 15 days of seed storage, being 19 %, 14 %, and 24 % higher at 0, 5, and 15 days, respectively (Fig. 1). At 25 days the difference was not significant (Fig. 1). These increases in WC are compatible with the effects of inoculation on promoting a better water status and hence, a better radical growth as reported by other authors (Fallik et al., 1988 b).

It is important to emphasize that in all the parameters evaluated, the growth-promotion effect due to *Azospirillum* inoculation (Fig. 1) decreases together with the MPN of live cells found in the root (Table 1).

Several authors have reported that the expression and magnitude of the beneficial effects of the plant-bacterium interaction would depend on the number of cells that colonize the root, and this number differs according to the plant species (Kapulnik et al., 1985; Fallik et al., 1988a; Zaady et al., 1993). In maize, an optimal number of *Azospirillum* cells of 10^7 $plant^{-1}$, 2-3 weeks after sowing, has been recommended (Arsac et al., 1990). These results cannot be compared directly with ours. However, considering that we used a maximum of 20 seedlings to collect 1 g of roots, the figures obtained from seeds at 0 time and after 5 days of storage could be roughly calculated, as 5.5×10^6 and 9.0×10^6 cells $plant^{-1}$, respectively. These figures are very close to the optimal ones reported by Arsac et al. (1990), but considerably lower after 15 and 25 days of seed storage.

However, the effect of inoculated seed aging on MPN is not the same as on seedling growth. Indeed, while the MPN drops from roughly 10^6 to 10^5 cells $plant^{-1}$ in 15 days storage, there are no significant differences on the growth promotion effect exerted by *Azospirillum* on both root and coleoptile growth during that period (Fig. 1). Moreover, even though after 25 days of storage MPN dropped from 1.1×10^8, to 4.5 $\times 10^5$ bacteria g^{-1} of root FW (Table 1), the effect of *Azospirillum* in promoting root growth is still significant (Fig. 1). These latter results could indicate that the number of *Azospirillum* cells reported as optimal by Arsac et al. (1990) could be lower, at least in maize seedlings.

Conclusions

The method proposed for inoculating maize seeds with *A. brasilense* BR 11005 does not require adhesives or supports to obtain seeds carrying viable cells that later colonize the radical system in an adequate number. It is also valid to express the beneficial effects of the bacteria after germination, which would favor the establishment of the crop by speeding root proliferation, emergence, and seedling development.

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