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The Effect of a Genetically Modified *Rhizobium meliloti* Inoculant on Fungal Alkaline Phosphatase and Succinate Dehydrogenase Activities in Mycorrhizal Alfalfa Plants as Affected by the Water Status in Soil

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Abstract

A time-course pot experiment was designed to compare the effect of two *Rhizobium meliloti* strains, the wild type (WT) and its genetically modified (GM) derivate, on the physiological activity of the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* during the colonization of alfalfa (*Medicago sativa* L.) roots as affected by the water status in the growing medium using histochemical staining methods as succinate dehydrogenase (SDH) and alkaline phosphatase (ALP) enzyme markers. At each harvest time, and for each water level, there were no significant differences in plant growth response between the effect of the two rhizobial WT and GM treatments on AM-plants. This is an unexpected result because the GM strain usually behaves as an improved rhizobial inoculant. Nodulation followed already described patterns, i.e. the GM strain produced less, but bigger, nodules than the WT strain. In spite of the lack of an improved growth response to the GM strain, this did not adversely affect the development of the AM symbiosis (*Glomus mosseae-Medicago sativa*). It was also found that, under well-watered conditions, about 80% of the AM mycelium in plants inoculated with the GM *Rhizobium* was alive (SDH activity) throughout the experiment, while only 10–20% of the intraradical

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mycelium remained alive in plants inoculated with the WT strain. Both rhizobial strains behaved similarly under water-limiting conditions in regard to AM development.

Keywords: Arbuscular mycorrhizas, *Rhizobium*, genetically modified micro-organisms, fungal succinate dehydrogenase and alkaline phosphatase

1. Introduction

Soil productivity and nutrient cycling may be influenced by the interactions occurring between soil microbial populations, particularly those living in the rhizosphere (Bethlenfalvay and Schüepp, 1994; Barea et al., 1997). Because rational management of soil microorganisms has become an issue of substantial import, the use of microbial inoculant is being explored for sustainable agriculture purposes (Elliot and Lynch, 1995). However, current interest in environmental problems do establish that risk assessment must be considered first, when microbial inoculants are being applied, particularly when they are genetically-modified (GM) microbial inoculants (O'Gara et al., 1994). Previous results (Barea et al., 1996) in relation to biosafety purposes for an inoculant release demonstrated that a genetically modified (GM) *Rhizobium meliloti* strain did not disturb arbuscular mycorrhiza (AM) formation and function in comparison to its parent wild type (WT). Moreover, the GM *Rhizobium* strain, which was developed to improve the nodulation competitiveness of the WT strain (Sanjuan and Olivares, 1991), also increased the number of mycorrhizal entry points, as well as mycorrhizal plant response.

However, it was necessary to test such GM behaviour in other soil and conditions. When this was done, it was corroborated that the GM rhizobial strain: (i) always produced less but more effective nodules than the WT strain; (ii) did not adversely affect the performance of the AM symbiosis; (iii) in most cases, although with some exceptions, the GM strain benefited AM functioning more than the WT strain (Toro, 1996). Where the effects produced by the GM and WT strains were similar, it was hypothesized that the GM strain was benefiting some AM symbiotic related processes, while interfering with others. For this reason, a study was proposed to examine whether or not such GM *Rhizobium* would affect any of the key metabolic activities related to AM fungal functioning.

In fact, AM symbiosis effectiveness has been related to the metabolic characteristics of fungal colonization (Guillemin et al., 1995). In this context, fungal succinate dehydrogenase and alkaline phosphatase activities have been proposed as enzyme markers (Guillemin et al., 1995) because of their key position in primary fungal metabolism. Histochemical staining methods have

been developed for these enzymatic activities (Tisserant et al., 1993). Succinate dehydrogenase (SDH), a mitochondrial enzyme, may be a marker of a metabolically active fungus (Smith and Gianinazzi-Pearson, 1990). Alkaline phosphatase (ALP), which is specific to AM colonization, and localized within the phosphate-accumulating vacuoles of the fungal mycelium (Gianinazzi-Pearson and Gianinazzi, 1978) appears to be involved in polyphosphate breakdown. This enzyme has been associated to the processes of phosphorus acquisition in mycorrhizal plants, and may therefore be a potential marker of efficiency for the symbiosis (Tisserant et al., 1993).

In the present study the effect on the physiological activity of AM colonization of a *Rhizobium meliloti* wild type strain (WT), were compared with these produced by the genetically modified derivate strain (GM) which was developed to improve nodulation competitiveness in the WT strain (Sanjuan and Olivares, 1991). In particular, the impact of these two *Rhizobium* strains on metabolic activity (SDH and ALP) in mycorrhizal alfalfa plants was examined using a time-course experiment with harvest at 20, 30, 40 and 50 days after inoculation, and following histochemical staining methods.

Because of the interest in predicting the behaviour of these mycorrhizal-*Rhizobium* strains interactions over a range of environmental conditions, this assay included changes in the water status of the test soil.

2. Material and Methods

Alfalfa (*Medicago sativa* L., cultivar Aragón) was the test plant. Surface sterilized seeds were sown in pots of 200 ml capacity. The mycorrhizal inoculum consisted of spores, hyphae and AM colonized root fragments (70% of fractional colonization) from the stock culture of a fungus with *Lactuca sativa*, which belong to the collection of the Estación Experimental del Zaidín. The AM fungus was *Glomus mosseae* (Nicol. and Gerd.) Gerd and Trappe. Inoculum (5 g/pot) was placed directly below the seeds in each pot. All plants were inoculated with the AM inoculum. There were two rhizobial treatments: the WT *Rhizobium meliloti* strain (GR4) and its genetically modified derivate GR4(pCK3) (Sanjuan and Olivares, 1991). The rhizobial inoculum consisted of 1 ml per pot of the corresponding *Rhizobium* strain culture. The rhizobial cultures were prepared following standard procedures (Azcón et al., 1991) and contained 10^9 cells ml^{-1}. These two rhizobial treatments were replicated 24 times giving a total of 48 microcosm units so as to allow plants to grow under two water regimes: 80% and 100% of the soil water holding capacity, respectively.

Soil was collected from the experimental field of the Estación Experimental del Zaidín, Granada, sieved (2 mm pore size), diluted with quartz (1/1, v/v) and autoclaved (100°C, 1 h 3 consecutive days). The characteristic of this

agricultural soil used were: pH 7.8; 2.07% organic matter; 0.1% total N; 32 µg P/g ($NaHCO_3^-$ extractable P); 311.2 µg K/g (exchangeable); with 35.86% sand; 43.6% silt and 20.54% clay. Pots were filled with 200 g of sterilized soil/sand mixture.

Plants were grown in a controlled environmental chamber under conditions of 50% relative humidity, day and night temperatures of 27°C and 18°C, respectively, and a photoperiod of 14 h. Photosynthetic photon flux density (PPFD) was 503 µmol/m^2/s as measured with a lightmeter (LICOR, model LI-188B). Distilled water was supplied by daily weighing to maintain the required water level (80% and 100% of water holding capacity) of the test soil/sand mixture throughout the experiment.

Three pots from each treatment were harvested after 20, 30, 40 and 50 days of plant growth. At each harvest, plant biomass accumulation and nodulation were first estimated (Barea et al., 1996). The roots were then carefully washed and divided into three batches: one stained by the classical non-vital trypan blue (TB), and staining of fungal tissue (Phyllips and Hayman, 1970), and the others were used for histochemical vital staining (SDH or ALP activities) of the mycorrhizal roots in order to measure total (TB), living (SDH) or functional (ALP) mycorrhizal fungal development.

Succinate dehydrogenase (SDH) activity was revealed according to the procedure described by Smith and Gianinazzi-Pearson (1990). The roots were immersed in a freshly made solution containing 0.2 M Tris-ClH pH 7.0, 2.5 M sodium-succinate 6-hydrate, 4 mg/ml nitro blue tetrazolium, 5 mM $MgCl_2$. Root fragments were stained overnight at room temperature and then cleared for 15–20 min in a 3% active chlorine solution of sodium hypochlorite.

Alkaline phosphatase (ALP) was determined according to the procedure described by Tisserant et al. (1993), which confirmed the specificity of staining methods for ALP. The roots were immersed in a freshly made solution containing 50 mM Tris-citric acid pH 9.2, 1 mg/ml alfa-naphthyl acid phosphate (monosodium salt), 0.05% $MgCl_2$ anhidro, 0.05% $MnCl_2$ tetrahydrate and 1 mg/ml Fast Blue RR. Root fragments were stained overnight at room temperature and after that were cleared for 15–20 min in a 1% active chlorine solution of sodium hypochlorite.

Mycorrhizal development, after either non-vital or vital procedures, was evaluated by the method of Trouvelot et al. (1986) and expressed as frequency of AM colonization (F%, percentage of root fragments with fungal colonization), intensity of AM colonization (M%, the fractional colonization extent), arbuscule frequency (A%, percentage of root cortex with arbuscules). Differences among means were evaluated for significance ($P<0.05$) by Duncan's test. For the percentages values an Arc Sin transformation was made before statistical analysis.

3. Results

At each harvest time, and for each water level, there were no significant differences in growth response between the effect of the two rhizobial WT and GM treatments tested in AM-plants. Nodulation (data not shown) followed already described patterns (Barea et al., 1996) i.e. the GM strain produced less, but bigger, nodules than the WT strain. All the three parameters of mycorrhizal development evaluated, i.e. frequency of mycorrhization (F%, Fig. 1), colonization intensity (M%, Fig. 2) and arbuscule frequency (A%, Fig. 3) followed a similar pattern for each staining procedure, *Rhizobium* strain and water level applied.

After 30 days of inoculation under well-watered conditions the total amount of fungal tissue (TB staining) corresponded to both a living (SDH staining) and an active (ALP staining) fungal development, for all the three mycorrhization parameters (F%, Fig. 1), (M%, Fig. 2) and (A%, Fig. 3). This was also true with regard to the A% parameter (Fig. 3) at 80% of the water holding capacity of soil. However, the fungus starts to function (ALP activity) later at water-limiting conditions (Figs. 1–3), probably because arbuscule developments were delayed (Fig. 3) under such stress situation.

The fractional AM colonization estimated by TB staining increased at 40 days and then remained constant to the end of the assay, whereas the AM colonization curves with vital staining (SDH and ALP) tended to decrease earlier (between 30 to 40 days). However, the ALP activity actually disappeared under water-limiting conditions. Another important point is that staining for SDH activity in GM-*Rhizobium* inoculated plants, revealed that about 80% of mycelium colonizing root tissues was alive throughout the experiment under well-watered conditions. In contrast, only 10–20% of mycelium remained alive in plants inoculated with the WT strain, under the same water regime (100% of the water holding capacity), at the end of the assay. Both rhizobial strains behaved similarly under water-limiting conditions.

4. Discussion

The results reported in this study show that the GM strain of *Rhizobium meliloti* developed by Sanjuan and Olivares (1991), and confirmed to be an improved inoculant (Barea et al., 1996), did not adversely affect the development of the AM symbiosis (*Glomus mosseae-Medicago sativa*) even in the case studied here where under the particular experimental conditions, the GM strain did not improve the effects on plant growth produced by its parent WT strain.

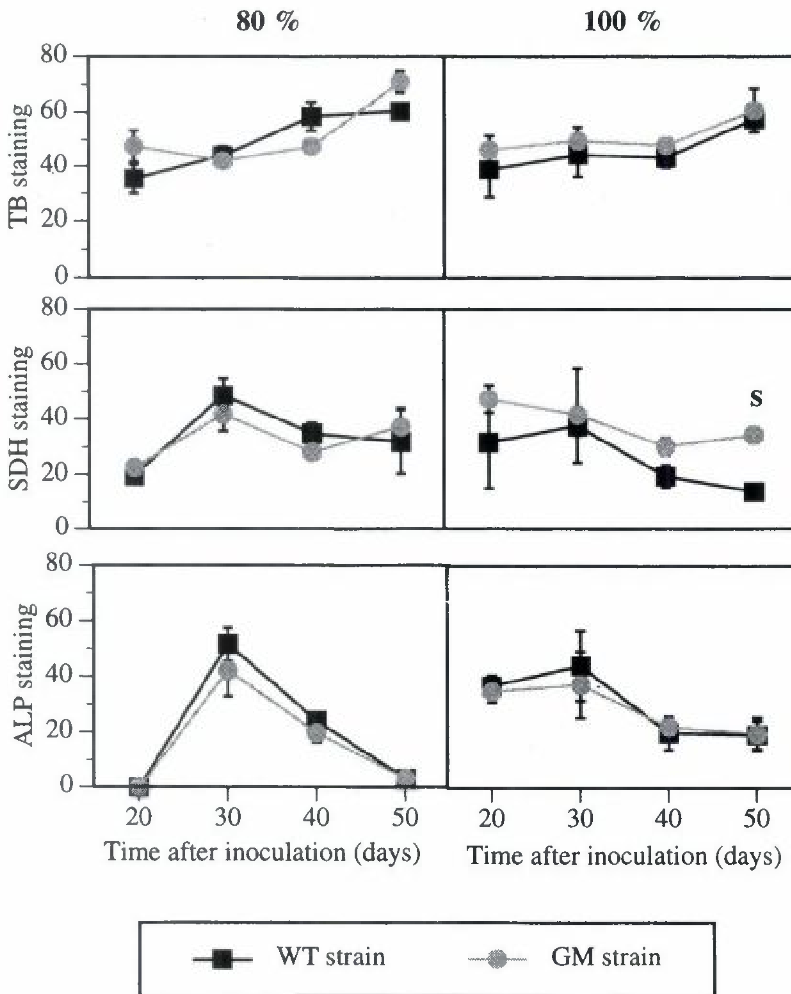

Figure 1. Effect of two *Rhizobium meliloti* strains, the wild type (GR4) and its genetically modified derivative (GR4(pCK3)) at two water levels in soil (80% and 100% of the water holding capacity), on the frequency of AM colonization (F%) observed with trypan blue (TB) staining, succinate dehydrogenase activity (SDH) and alkaline phosphatase activity (ALP) during the development of colonization of *Medicago sativa* roots with *Glomus mosseae*. Bars represent S.E. The S symbols indicates significant differences between GM and WT *Rhizobium* strains according to Duncan's test ($P \leq 0.05$).

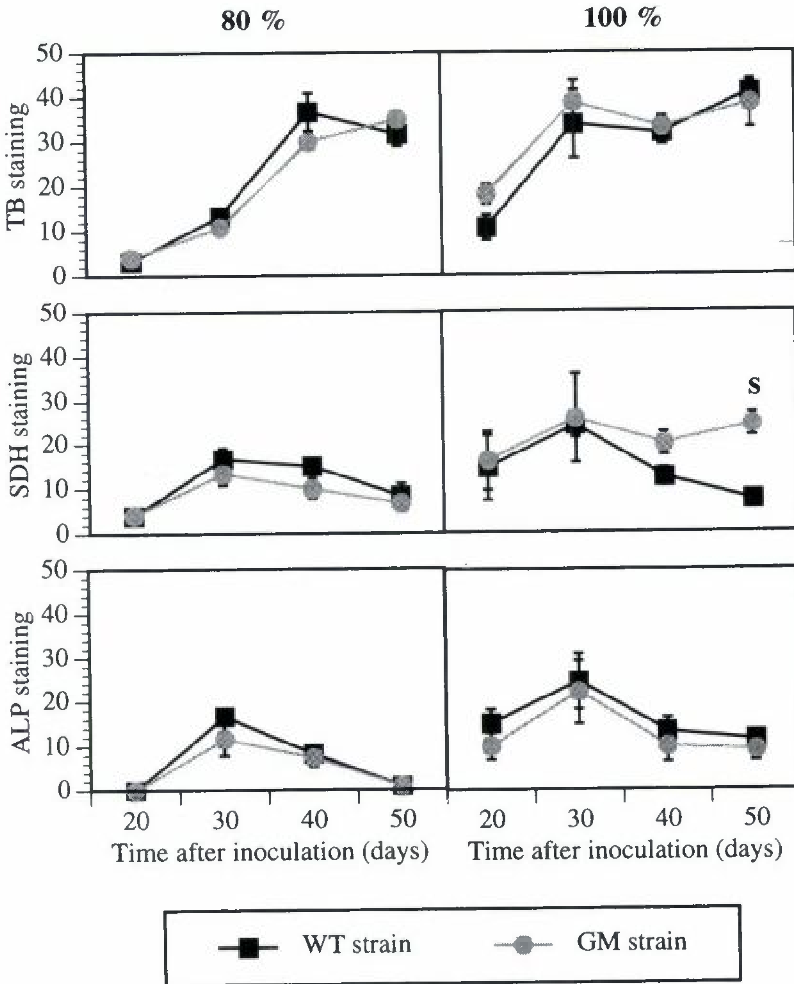

Figure 2. Effect of two *Rhizobium meliloti* strains, the wild type (GR4) and its genetically modified derivative (GR4(pCK3)) at two water levels in soil (80% and 100% of the water holding capacity), on intensity of AM colonization (M%) observed with trypan blue (TB) staining, succinate dehydrogenase activity (SDH) and alkaline phosphatase activity (ALP) during the development of colonization of *Medicago sativa* roots with *Glomus mosseae*. Bars represent S.E. The S symbols indicates significant differences between GM and WT *Rhizobium* strains according to Duncan's test ($P \leq 0.05$).

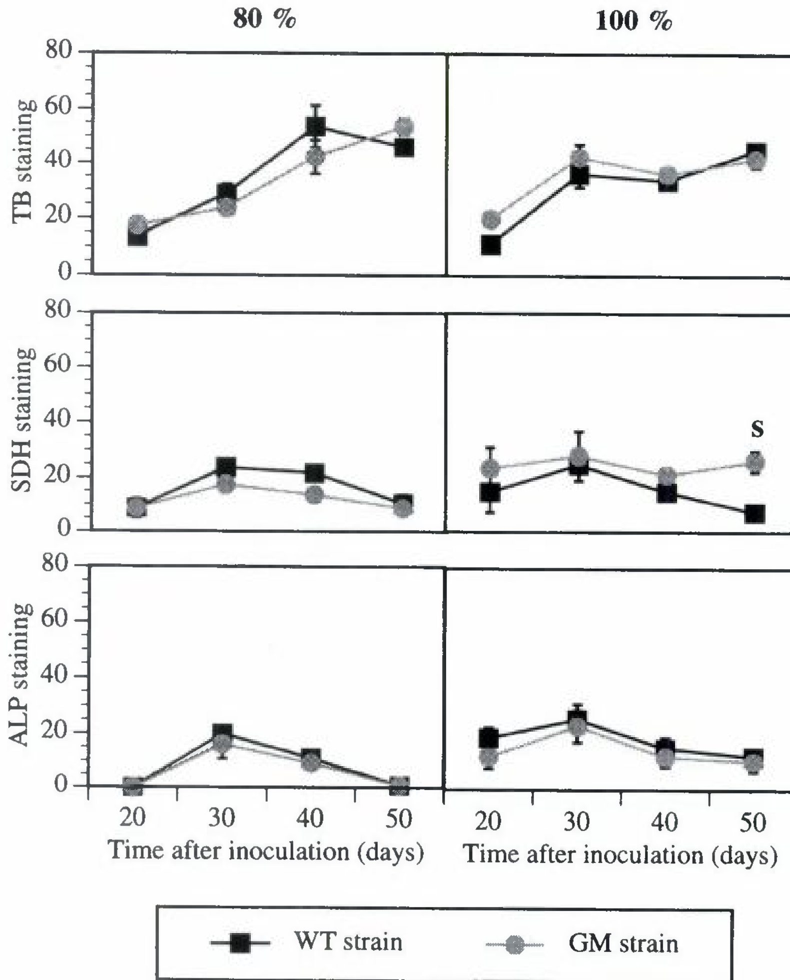

Figure 3. Effect of two *Rhizobium meliloti* strains, the wild type (GR4) and its genetically modified derivative (GR4(pCK3)) at two water levels in soil (80% and 100% of the water holding capacity), on arbuscule frequency (A%) observed with trypan blue (TB) staining, succinate dehydrogenase activity (SDH) and alkaline phosphatase activity (ALP) during the development of colonization of *Medicago sativa* roots with *Glomus mosseae*. Bars represent S.E. The S symbols indicates significant differences between GM and WT *Rhizobium* strains according to Duncan's test ($P \leq 0.05$).

The application of staining techniques, such as those using trypan blue (Phillips and Hayman, 1970), which do not distinguish living from dead mycelium, gave a time-course development pattern similar to the one frequently described in the literature (Smith and Read, 1997). However, the use of vital stains allowed us to make observations, which concern new and less explored aspects of the differential behaviour of each *Rhizobium* strain.

One outstanding result is the demonstrated ability of the GM *Rhizobium* strain to maintain a high proportion of the intraradical AM mycelium alive (SDH stain) throughout the experiment. However, this mycelium show low levels of ALP activity at the last stages of plant growth. Probably the experimental conditions used, as discussed below, could limit such activity.

At 80% of the water holding capacity of the soil a lag phase took place in the expression of the ALP activity, and only a small proportion of the intraradical mycelium showed activity of this enzyme during the earliest phases of AM colonization. However, under well watered conditions, all the fungal mycelium (TB) was living (SDH) and active (ALP) up to 30 days after inoculation. This indicates that the experimental conditions can affect the time-course expression of these fungal metabolic properties, as has been found in other situations (Tisserant et al., 1993).

Because of the aim of our experiment was to determine metabolic variations during the first few weeks in the developmental stages of AM colonization, as affected by rhizobial and water stress treatments, small pots were used. Therefore, the results can only account for the target aims but not for other effects that cannot be expressed under such limited plant growth conditions. For example, the SDH and ALP activities linked to active metabolic fungal performances tend to decrease, and even disappear under water stress conditions at only seven weeks of plant growth. In spite of the ALP activity that decreases as AM colonization ages (Tisserant et al., 1993), its total disappearance under water-limiting situations reported here is a new result which deserves some further research. It can be argued that the relative small soil volume explored by the AM fungus, as well as the difficulties with which P diffusion meets, those imposed by water limitation may lower P supply and this could make the fungal ALP activity unnecessary. From an operational point of view, it is not logical that this occurs only seven weeks after inoculation in an agricultural-like situation.

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