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Physiological characteristics (SDH and ALP activities) of arbuscular mycorrhizal colonization as affected by *Bacillus thuringiensis* inoculation under two phosphorus levels

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Abstract

The effect of *Bacillus thuringiensis* (*B.t.*) inoculation on plant growth and on the intra- and extraradical mycorrhizal development of lettuce roots colonized by *Glomus mosseae* or *Glomus intraradices* was examined in an inert, soil-less substrate. Histochemical determination of succinate dehydrogenase (SDH) and alkaline phosphatase (ALP) activities which indicate active fungal metabolism was carried out at two phosphorus (P) levels. The presence of *B.t.* increased extra- and intraradical colonization [measured as frequency (*%F*), intensity (*%I*) and percentage of arbuscules (%A)] for both arbuscular mycorrhizal fungi (AMF) rather than plant growth or nutrition regardless P level. Under the lowest level of P fertilization, *B.t.* enhanced to a similar extent the extra- and intraradical development of both endophytes, but the proportion of fungal tissue showing SDH or ALP was increased in *G. intraradices*-colonized plants. [SDH: 458% (M) and 512% (A); ALP: 358% (M) and 300% (A)]. P supply decreased *G. intraradices* colonization to a higher extent than *G. mosseae*. Nevertheless, the totality of *G. intraradices* structures developed in P-amended medium showed intraradical o extraradical activity, while in *G. mosseae*-colonized roots, SDH and ALP activities highly decreased relative to fungal tissue determined by TB staining as affected by P. Our results show that bacterial inoculation compensates the negative effect of P on the intraradical fungal growth and vitality. P amendment reduced in a higher extent *G. intraradices* infection intensity (non-vital and vital staining) and *G. mosseae* activity (ALP staining). Thus, big differences in the proportion of SDH-active infection showing ALP activity in mycelium developed by each endophyte were noted at the highest P level. Physiological plant parameters such as photosynthetic activity did not explain specific changes on each arbuscular-mycorrhizal fungus as affected by P or *B.t.* inoculation. The increased extraradical mycelium development and metabolic fungal activity as a result of *B.t.* inoculation positively affected N and P plant content and photosynthetic rate in *G. intraradices*-colonized plants under the lowest P conditions. In general, the increased metabolically active fungal biomass in co-inoculated plants was irrespective of P level and was not related to the P plant uptake from the inert soil-less substrate. These results show the bacterial effect increasing the physiological and metabolic status of AM endophytes, which not only confirms but also extends previous findings on arbuscular mycorrhizae-bacteria interactions. The present study emphasizes the ecological and practical importance of rhizosphere free-living bacteria as mycorrhizae-helper microorganisms.

Keywords: *Bacillus thuringiensis*; Mycorrhiza–bacteria interaction; SDH and ALP fungal activities; Phosphorus

1. Introduction

Arbuscular mycorrhizal (AM) fungi and other beneficial rhizosphere microorganisms are important in sustainable agriculture (Bethlenfalvay, 1992) since nutrient cycling and soil productivity are influenced by the activity of rhizosphere microbial populations (Barea et al., 1997). Of particular interest are the interrelationships between AM fungi and saprophyte, ubiquitous soil-inhabiting bacteria involved in nutrient transformations (Toro et al., 1990a,b). Studies on dual AM fungi–bacterial interaction have focused on obtaining synergim

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on plant growth (Bowen and Rovira, 1999). Nevertheless, a better understanding of interactions between soil microorganisms is needed for optimal utilization of microbial inoculants in order to improve growth and nutrition of plants (Barea et al., 1997). Rational management of selected microorganisms has become relevant for sustainable agriculture purposes (Elliott and Lynch, 1995).

AM effectiveness has been related to the extent of fungal colonization and the metabolic characteristics of the intra or extraradical fungal structures (Tisserant et al., 1993; Guillemin et al., 1995). In this context, AM fungi and rhizosphere microorganisms can influence their mutual development resulting in a synergistic interaction affecting plant responses (Azcón, 1989). This effect can be exerted indirectly through the host plant or directly by the fungus (Schwab et al., 1983). The use of vital staining has been proposed as a good method for making evaluations concerning the effect of biotic or abiotic factors on the less explored metabolic fungal aspects (Tisserant et al., 1993). Alkaline phosphatase (ALP) activity appears to be indicative of the efficiency of AM fungus on plant nutrition (Guillemin et al., 1995). Physiological basis of phosphorus (P) absorption and translocation by AM fungi are processes metabolically dependent (Thomson et al., 1990a,b).

Rhizosphere bacteria are able to improve a symbiotic growth of AM fungi (Azcón, 1987). Germination, hyphal growth and vegetative spore's production of *Glomus mosseae* under axenic conditions were increased in the presence of a bacterial inoculum (Azcón-Aguilar and Barea, 1985; Azcón-Aguilar et al., 1986; Azcón, 1987).

A logical next step is to test if such mycorrhizal growth enhancement by bacterial inoculation is followed by metabolic and physiological stimulation of AM fungus and on the AM functioning. Thus, the aim of this study was to ascertain whether a selected soil bacterium, *B.t.*, which normally enhance plant growth under drought stress conditions, is able to stimulate mycorrhizal metabolism. This was carried out by using fungal succinate dehydrogenase (SDH) and ALP activities as index of vitality and activity (Tisserant et al., 1993; Guillemin et al., 1995). These two enzymes, are considered to play key metabolic roles in the mycorrhizal functioning.

We selected therefore, an inert soil-less substrate in which ion concentrations and water were controlled. It permits the growth of AM plants with a constant supply of nutrients. For testing microbial interaction on AM fungal physiological status, the use of a non-adsorbing medium having readily available nutrients would allow us to distinguish direct from host mediated nutritional effects. Intra- and extraradical fungal development, as well as metabolic characteristics of fungal colonization determining the vitality and activity of AM symbiosis as effected by AM fungus-bacteria interaction are evaluated at two levels of P in the medium. Also, changes in the host plant photosynthetic capacity and related effects were determined.

2. Materials and methods

The experiment consisted of a two-factor randomised complete block design of: (i) single or dual inoculation of a bacterial isolated and two AM fungi, and (ii) two levels of P added to the soil. For each treatment, one-half of the plants were maintained without extra P application and the other half were P fertilized. Six replicates per treatments were made given a total of 60 pots.

2.1. Experimental set up

Lettuce (*Lactuca sativa* L. cv. Romana) plants were grown during ten weeks in sterilized sand (< 1 mm) vermiculite clay type (0.6–3.3 mm) sepiolite (0.5–3.3 mm) (1 : 1 : 1, *v/v/v*) medium. Surface sterilized seeds were sown in pots of 300 ml capacity. Two AM fungi were used as inocula, *G. mosseae* (Nicol. And Gerd) Gerd. and Trappe and *G. intraradices* (Thaxter sensu Gerd). The AM inoculum consisted of spores, hyphae and mycorrhizal root fragments (70% of colonization) from an stock culture of each fungus, with the same host plant, which belong to the collection of the Estación Experimental del Zaidín (Granada, Spain). The AM inoculum was placed (5 g per pot) directly below the seeds in each pot. Control nonmycorrhizal treatments, received the same amount of autoclaved inoculum together with a 2-ml aliquot of a filtrate (< 20 μm) of the AM inoculum in order to equal microbial population other than AMF.

The bacterial inoculum (*B.t.*) used was isolated from a local desertified soil from the Alicante province (Easten Spain). It was isolated following a standard procedure, identified using molecular techniques and cultivated in our laboratory. Database searches for 16S rDNA sequences similarity using FASTA and BLAST algorithms unambiguously identified the isolate as member of the genus *Bacillus*. The 16S rDNA sequence from this bacterial isolated showed its highest similarity (>98%) with *Bacillus thuringiensis*. The culture was grown in a rotatory shaker at 150 ppm at 28 °C for 48 h in 250 ml flasks containing 50 ml of a nutrient broth (8 g l^{-1}) medium. One-ml $plant^{-1}$ having 10^6 CFU ml^{-1} was added per pot when appropriated. Two seeds were sown and thinned to one seedling per pot after shoot emergence.

Plants were grown in a controlled environmental chamber (70–75% relative humidity, day/night temperatures of 26 and 17 °C respectively, 14 h photoperiod). The photosynthetic photon flux density was 503 μmol m^{-2} s^{-1} as measured with a light meter (LICOR, model LI-188B).

Plants were treated twice a week (50 ml/pot/week) with a Hewitt half P strength nutrient solution (Hewitt, 1952) which gives the plant its normal requirements. The pH was

adjusted to 6.8–7. In the last three weeks, half of the plants were fertilized with the same nutrient solution having complete P content. The amendment applied was 30 mg P kg^{-1} medium (10.0 mg P per pot).

Before harvest, CO_2 exchange rate, transpiration rate and instantaneous water use efficiency (WUE) were measured on the fourth leaf of each plant. Atmospheric CO_2 was measured 5 m above ground level. Photosynthetic photon flux density was 1180 μmol m^{-2} s^{-1}, which ensured that no limitation in photon irradiance occurred (Long and Hällgren, 1987). Light was provided by a portable halogen lamp a model LCA-3. (General Electric 300 PAR 56/WFL). An integrated infrared CO_2 analyser (Analytical Development Co., Hoddesdon, UK, Model ADC-3) was used for these determinations. Measurements were made 2 h after the light was turned on.

2.2. Biochemical determinations

Proline content in leaves and roots were evaluated by colorimetry (Bates et al., 1973).

The relative water content (RWC) was determined (Baars and Wheatherley, 1962) in five leaf discs (1 cm diameter).

At harvest (10 weeks after planting), the root system was separated from the shoot and dry weight were determined of the shoots and root tissues.

Plant leaves were weighed and dried in a forced-draught oven at 70 °C for 1 day and ground in a Wiley Mill to pass a 0.5 mm mesh. The material was digested with H_2SO_4. Concentrations of N (Baethgen and Alley, 1989), P and K (Lachica et al., 1973) in plant tissue were determined.

Roots were carefully washed and then divided into three batches: one was stained by the classical non-vital Trypan blue (TB) staining (Phillips and Hayman, 1970) and the others were used for histochemical vital staining (SDH or ALP activities) in order to measure total (TB), living (SDH) or active (ALP) AM fungal development.

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–HCl pH 7.0, 2.5 M sodium-succinate hexahydrate, 4 mg ml^{-1} 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.

ALP was determined according to the procedure described by Tisserant et al. (1993), which confirmed the specify of staining methods for ALP. Roots were immersed in a freshly made solution containing 50 mM Tris–citric acid pH 9.2, 1 mg ml^{-1} alfa-naphthyl acid phosphate (monosodium salt), 0.05% $MgCl_2$ anhydrous, 0.05% $MnCl_2$ tetrahydrate and 1 mg ml^{-1} fast blue RR salt. Root fragments were stained overnight at room temperature and cleared for 15–20 min in 1% active chlorine solution in sodium hypochlorite.

Mycorrhizal development, was evaluated by the method of Trouvelot et al. (1986) and expressed as frequency of AM colonization (*F%*, percentage of root fragments showing fungal colonization), intensity of AM colonization (*M%* which gives an estimation of the amount of root cortex that became mycorrhiza and is referred to the whole root system while *m%* refers only to the mycorrhizal root fraction). *A%* is the arbuscule abundance and gives a estimation of the arbuscule richness in the whole root system, while a% referres to the mycorrhizal root fraction only. Extraradical mycelium was determined by adaptation of methods described by Jones et al. (1998); Newman (1966). Data were subjected to ANOVA with microbial treatment, phosphorus level and microbial treatment-phosphorus level interaction as sources of variations. When the main effect was significant ($P < 0.05$), differences among means were evaluated for significance ($P < 0.05$) by Duncan's test. For the percentage values an Arc Sin transformation was made before the statistical analysis.

3. Results

Results reported in this study confirmed the effectivity of *G. mosseae* and *G. intraradices* for plant growth in soil-less rooting medium. The two inoculated AM fungi showed similar effectiveness on shoot and root biomass production when they were singly or co-inoculated with *B.t.* irrespective of P level (Table 1). Mycorrhizal-colonization effectivity on root growth was only evidenced, at the lowest phosphorus level in the growing medium, in dual *G. intraradices* plus *B.t.* treated plants. In spite of non-significant differences in growth between the plants colonized by each endophyte, *G. mosseae* seemed to be more effective alone and *G. intraradices* increased shoot growth by 25% when associated with the bacterial strain in the lowest P fertilized medium (Table 1).

Table 1
Effect of inoculation with *B. thuringiensis* (*B.t.*) or AM fungi *G. mosseae* and *G. intraradices* with or without *B.t.* on the fresh weight (mg $plant^{-1}$) of the shoots and roots of 10 weeks old *Lactuca sativa* plants cultivated in an inert soil-less substrate in the presence of two levels of P

AM	30 mg *P* pot^{-1}		40 mg *P* pot^{-1}	
	Shoots	Roots	Shoots	Roots
Without B.t.				
Control	11.0a	7.4ab	17.2a	13.0a
G. mosseae	17.5c	9.3bc	21.3b	14.1a
G. intraradices	14.9bc	8.3ab	22.5b	11.1a
With B.t.				
Control	9.5a	6.0a	17.4a	11.8a
G. mosseae	15.8c	10.3c	20.1ab	17.2a
G. intraradices	18.5c	10.4c	22.2b	9.6a

In each column means follwed by the same letter are not significantly different ($P < 0.05$) according to the Duncan's test.

Table 2
Effect of inoculation with *B. thuringiensis* (*B.t.*) or AM fungi *G. mosseae* (M) and *G. intraradices* (I) with or without *B.t.* on transpiration rate (mM $H_2O\ m^{-2}\ s^{-1}$), photosynthetic rate (mM $CO_2\ m^{-2}\ s^{-1}$); Water used efficiency (WUE) (mM $CO_2\ m^{-2}\ s^{-1}$/mM $H_2O\ m^{-2}\ s^{-1}$), stomatal conductance (M $m^{-2}\ s^{-1}$) and requirements (1/quantum efficiency) of 10 weeks old *Lactuca sativa* plants cultivated in an inert soil-less substrate in the presence of two levels of *P*

Microbial treatments	Transpiration	Photo-synthetic activity	WUE	Stomatal conductance	Requirements
30 mg *P* pot^{-1}					
B.t.	2.25a	0.063c	0.03b	13.2d	9.129bc
M	1.34c	0.110b	0.08b	32.2a	7.535c
I	1.59b	0.120b	0.08b	24.4c	4.811cd
M + *B.t.*	1.63b	0.098b	0.06b	23.7c	28.970a
I + *B.t.*	1.50b	0.250a	0.17a	27.8b	2.384d
40 *mg P pot*$^{-1}$					
B.t.	1.12c	0.47c	0.42c	40.4a	1224a
M	1.84a	1.75a	0.95a	19.1c	322c
I	1.89a	0.73b	0.39c	17.6c	784b
M + *B.t.*	1.48b	0.70b	0.47c	27.2b	808b
I + *B.t.*	1.47b	0.81b	0.55c	27.9b	706b

In each column means follwed by the same letter are not significantly different ($P < 0.05$) according to the Duncan's test.

Table 3
Effect of inoculation with *B. thuringiensis* (*B.t.*) or AM fungi *G. mosseae* and *G. intraradices* with or without *B.t.* on N, P and K content (mg $plant^{-1}$) of 10 weeks old *Lactuca sativa* plants cultivated in an inert soil-less substrate in the presence of two levels of *P*

AM	30 mg *P* pot^{-1}			40 mg P pot^{-1}		
	N	P	K	N	P	K
Without B.t.						
Control	30.9c	2.5c	52.9c	49.4b	3.2b	79.2c
G. mosseae	39.1c	4.7b	90.1a	54.3ab	4.5a	111.0b
G. intraradices	34.1c	4.0b	78.8b	68.7a	4.8a	123.6ab
With B.t.						
Control	33.0c	2.1c	48.9c	63.4ab	3.1b	85.7c
G. mosseae	51.6a	4.6b	85.7a	64.7ab	4.5a	111.6b
G. intraradices	50.0a	6.1a	72.4b	58.6ab	5.1a	127.5a

In each column means follwed by the same letter are not significantly different ($P < 0.05$) according to the Duncan's test.

Some physiological parameters of the plant were changed by the microbial-inoculation (Table 2). AM colonization as well as a higher P concentration increased photosynthetic activity and water use-efficiency (WUE). Stomatal conductance was increased by AM infection only at the lowest P levels. CO_2 assimilation and WUE were highest in *G. intraradices* plus *B.t.* inoculated plants when compared to the rest of the treatments at the lowest P fertilization level. Nevertheless, in the richest P medium, *G. mosseae* colonization reached highest levels for most of the physiological plant parameters evaluated (Table 2).

Under the lowest P content in the medium, P and K uptake by the plant was enhanced by mycorrhizal colonization. *G. intraradices* increased phosphorus plant acquisition in a highest extent when co-inoculated with *B.t.* and *G. mosseae* resulted in the most effective fungus in lettuce K uptake. Plant uptake of N was maximized in dually inoculated plants. P amendment maintained the enhancing mycorrhizal effect particularly on P and K plant acquisition (Table 3).

B.t. was effective in enhancing N and P uptake in *G. intraradices*-colonizal plants and only N in *G. mosseae*-colonized plants in the poorest P medium. Such bacterial effects were not found under the highest P ammendments (Table 3).

In general *B.t.* inoculation affected the values of mycorrhizal colonization in a higher extent than plant growth (Fig. 1). The effect of *B.t.* on AM infective values followed a particular pattern depending on the AM fungal species, P level in the medium, and staining procedure used (Fig. 1).

Under the lowest P conditions, intraradical mycorrhizal development increased following *B.t.* inoculation in *G. mosseae* and in *G. intraradices* colonized roots to similar extent. Nevertheless, regarding living and active fungal tissue (SDH and ALP staining) *B.t.* increased *G. intraradices* more than *G. mosseae*. In general, the different stimulating effect of *B.t.* on intraradical development of both endophytes was also evidenced at the highest level of P in the medium (Fig. 1).

At the highest P level, *G. intraradices* showed reduced fungal development compared to *G. mosseae* but nearly the totality of *G. intraradices* intraradical biomass resulted to be alive (SDH staining) and active (ALP staining). In contrast, the AM colonization developed by *G. mosseae*, estimated by TB and SDH staining, was higher but ALP was lower than that found in *G. intraradices*. Different behaviour between the two endophytes after P application was found. In *G. mosseae* colonized roots, SDH and ALP activities tended to decrease relative to fungal colonization reaching values about a half (SDH) and less than 10% (ALP) compared to TB. The depressing effect of P on fungal colonizing values (TB, SDH and ALP) was compensated by bacterial inoculation particularly in *G. intraradices* treatment (Fig. 1).

The most important result found is the general stimulating role of *B.t.* on, vitality and activity of AM fungal mycelium colonizing roots tissue at whatever P concentration in the medium. In fact, the detrimental effect of an increasing P supply on mycorrhizal colonizing parameters was not observed in co-inoculated AM-*B.t.* treatments (Fig. 1).

RWC was enhanced by P application and the effect of bacterial treatment was not so relevant enhancing this value (Fig. 2).

Proline accumulation in roots was improved by single mycorrhizal treatments. The proportions of proline

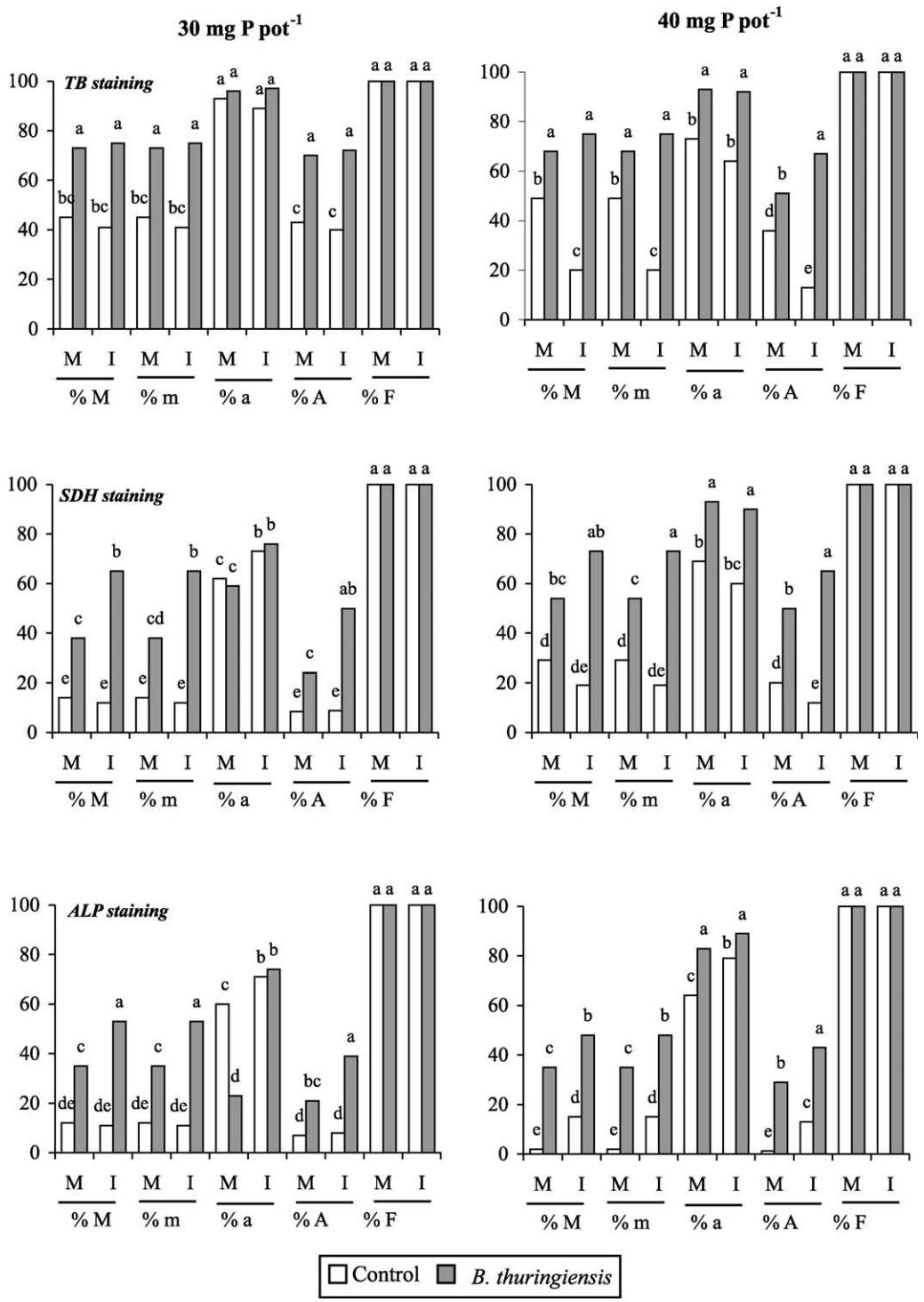

Fig. 1. Root colonization by *G. mosseae* (M) or *G. intraradices* (I) observed after TB, succinate dehydrogenase (SDH) or alkaline phosphatase (ALP) staining. Plants (*L. sativa*) were either inoculated with *B. thuringiensis* or remained un-inoculated (control) and were cultivated for 10 weeks in an inert soil-less substrate amended with two P levels. Mycorrhizal parameters are as follow: *M%* is the colonization intensity and indicates the amount of root cortex that became mycorrhiza, referred to the whole root system, while m% is referred only to the mycorrhizal root fraction. *A%* is the arbuscule abundance in the whole root system, while *a%* represents the arbuscule abundance in the mycorrhizal root fraction only. *F%* is the colonization frequency and gives the ratio between colonized root fragments and the total root fragments observed. Into each P level means followed by the same letter are not significantly different ($P < 0.05$) according to the Duncan's text.

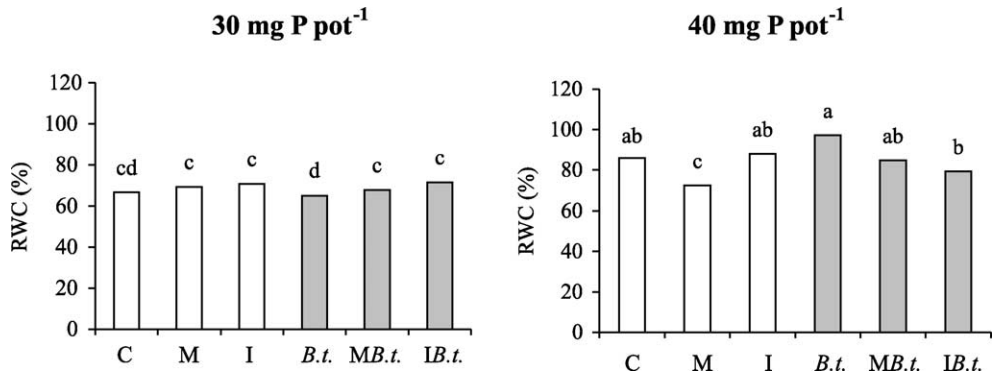

Fig. 2. Relative water content (RWC) of *Lactuca sativa* plants cultivated for 10 weeks in an inert soil-less substrate amended with two P levels. Plants were uninoculated control (C) or inoculated with *B. thuringiensis* (*B.t.*). or the AM fungi G. *mosseae* (M) or *G. intraradices* (I) with or without *B.t.* Into each P level means followed by the same letter are not significantly different ($P < 0.05$) according to the Duncan's text.

allocated in root and shoot changed according to P application. In the lowest P amendment the proportion of proline in roots was the greatest (Fig. 3).

Differences between the two AM endophytes in extraradical mycelium production were found. The stimulating effect of *B.t.* increasing such AM fungal growth in the soil was only evident in the poorest P medium (Fig. 4). The P effect reducing external hyphae was strongest on *G. intraradices*-colonized roots (Fig. 4).

4. Discussion

Our results confirm the positive effect of *B.t.* in the development of extra- and intraradical AM colonization in the symbiosis formed by *G. mosseae* or *G. intraradices* with lettuce roots. An important effect of *B.t.* is the enhancement in the proportion of the AM mycelium that was alive and active in AM colonized root tissue under whatever P levels assayed.

The results presented are relevant under the particular experimental conditions used, since bacterial treatments did not improve shoot growth of mycorrhizal plants. The lack of nutritional limitation in the growing medium may be the cause of this. In the case of dual *G. intraradices-B.t.* inoculation having a greater root development, the bacterial effect on percentage of AM colonization alive and active (as expressed in Fig. 1) was the greatest in terms of total length of infected roots, colonization density and fungal structures of the infection.

The increased SDH and ALP activities in *B.t.* treatments indicate the existence of an enhancement of the functional arbuscular endomycorrhization in terms of P nutrition, but this effect was only expressed in *G. intraradices*-colonized plants under the lowest P level. At the highest P level, ALP did not provide useful physiological index of an effective symbiotic association in terms of P nutrition as Smith et al. (1990) reported. ALP is involved in phosphate uptake by AM roots (Dexheimer et al., 1982). Thus, when its activity was reduced by P application, particularly in *G. mosseae*-colonized plants, this probably decreased the mycorrhizal effect on P uptake and growth stimulation as also reported by Gianinazzi-Pearson and Gianinazzi (1983).

The main objective of this study was to determine the role and influence of the selected bacterial strain on the extent but also on the metabolic abilities of the intraradical fungal structures formed within the colonized roots. SDH and ALP activities related to active metabolism, were greatly increased by *B.t.* presence. Moreover, the phosphorus application (as detrimental factor on such values,

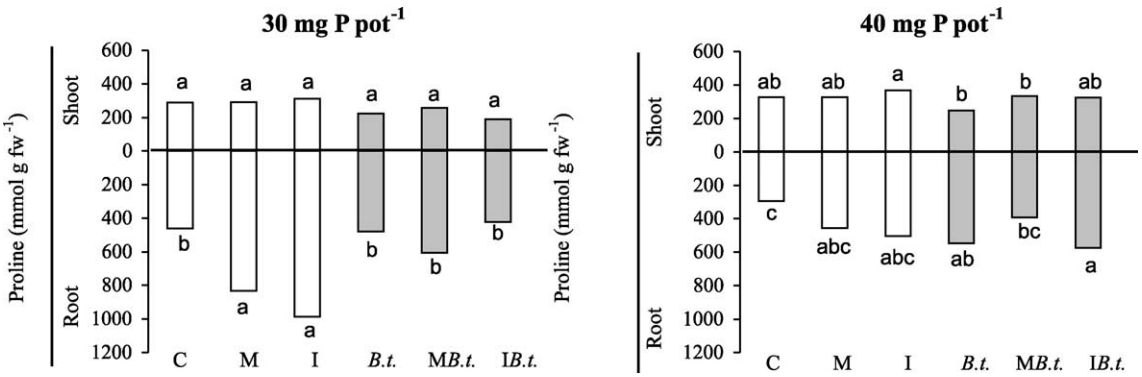

Fig. 3. Proline accumulation (mmol g fw^{-1}) by *Lactuca sativa* plants cultivated for 10 weeks in an inert soil-less substrate amended with two P levels. Plants were un-inoculated control (C) or inoculated with *B. thuringiensis* (*B.t.*) or the AM fungi *G. mosseae* (M) or *G. intraradices* (I) with or without *B.t.* Into each P level means followed by the same letter are not significantly different ($P < 0.05$) according to the Duncan's text.

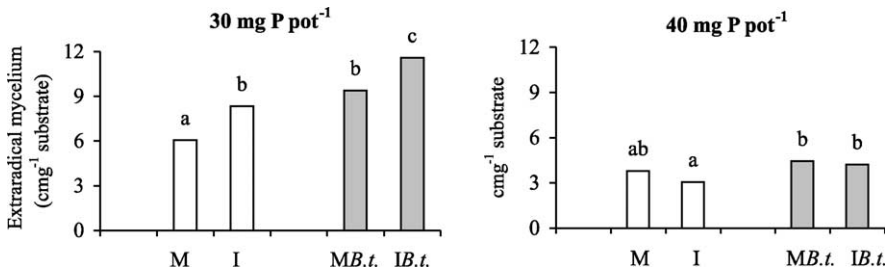

Fig. 4. Extraradical mycelium produced by AM fungi in pots containing *Lactuca sativa* plants cultivated for 10 weeks in an inert soil-less substrate amended with two P levels. Plants were uninoculated control (C) or inoculated with *B. thuringiensis* (*B.t.*) or the AM fungi G. *mosseae* (M) or *G. intraradices* (I) with or without *B.t.* Into each P level means followed by the same letter are not significantly different ($P < 0.05$) according to the Duncan's text.

Guillemin et al., 1995) did not decrease the fungal vitality and activity in the co-inoculated plants. In *G. intraradices*-colonized roots the beneficial *B.t.* effect reached the highest values.

An outstanding result is the ability of *B.t.* to maintain a great proportion of the intraradical mycelium alive (SDH) and active (ALP) even in adverse conditions, which suggests to be an indication on a direct bacterial effect on AM fungal metabolic status. Moreover this did not result, under the experimental conditions tested, in an increased P acquisition.

In previous work we found that some soil bacteria increased plant growth and AM colonization (Azcón et al., 1978). More specific studies also demonstrated the effect of selected soil microorganisms on spore germination and hyphal growth (Azcón-Aguilar et al., 1985, 1986; Azcón, 1987). Our results confirm and extend these findings. No information existed previously on the role of *B.t.* in increasing the physiological and metabolic state of *G. mosseae* and *G. intraradices* during colonization. The ALP activity has been associated with an active phosphate metabolism (Tisserant et al., 1993). But, in a soilless medium, the increase in the proportion of active ALP intraradical mycelium did not always precede the AM growth stimulation. In fact, the experimental conditions used were chosen to avoid the expression of the fungal metabolic properties on nutrients acquisition, since non-mycorrhizal plants were under non-limiting nutritional conditions. Nevertheless, nitrogen uptake was improved by *B.t.* in mycorrhizal plants.

The *B.t.* effect did not seem to be due to mechanisms operating via physiological status in the host (Table 2). It appears that changes in root exudation patterns (Graham, 1982) and in hormonal balance (Azcón et al., 1978) in the host plant are involved in the development of the AM symbiosis (Azcón and Ocampo, 1981). The *B.t.* strain is able to produce phytohormones (IAA) which could change root cell permeability and the rates of root exudation. But, according to the results reported here, a direct bacterial effect on each fungus inducing physiological changes seems to be involved in the observed mycorrhizal stimulation. Mycorrhiza helper bacteria (MHB) were described as being able to stimulate mycelial growth or enhance the colonization of AM fungi (Garbaye, 1994; Azcón-Aguilar and Barea, 1995; Barea, 1997). Our results show the role of *B.t.* as a MHB acting on the metabolic status of AM fungi.

In general, the more active fungal metabolism in bacterial-AM treatments found in this study did not significantly contribute to increase plant growth. Root growth, N and P nutrition as well as photosynthetic values were enhanced in dual *B.t.* and *G. intraradices*-colonized plants at the lowest P level. In fact, the highest frequency of SDH and ALP active arbuscules in *G. intraradices-B.t.* co-inoculated plants could reflect an increased surface area for nutrient exchange between living symbionts (Smith and Dickson, 1991). Guillemin et al. (1995) observed a positive correlation between arbuscular activity (frequency [% A] after ALP staining) and the mycorrhizal responses. Such correlation was only tested on N uptake in *G. mosseae*-colonized plants. In this study, at the highest P level applied, increases in active (SDH and ALP) infection did not represent growth stimulation neither increased P uptake.

Nevertheless, the amendment of P decreased *G. intraradices* infection intensity (TB staining) and *G. mosseae* mycorrhizal activity (ALP staining). The P effect was selective on each endophytes despite on increased SDH activity in plants colonized by any of these two fungi. This observation, agrees with that reported by Guillemin et al. (1995), and indicates the highest vitality of AM fungi in the root of P fertilized plants. Under these experimental conditions, the bacterial effect was evident in increasing live and active AM fungal biomass even when having no effect on P status in the host plant.

Different responses in the metabolic activity of *G. mosseae* and *G. intraradices*-colonized roots were noted as affected by environmental changes such as P application or *B.t.* inoculation. In fact, the level of AM colonization by *G. mosseae* did not vary with P application while increased SDH-active infection and reduced ALP values. Comparatively, the active *G. intraradices* colonization shows a different response to P-fertilization, since P amendment decreased the colonization level and did not change ALP activity.

In contrast to negative effect of P, the presence of *B.t.* enhanced particularly the active (SDH and ALP) mycelial

phase of both fungus. Nevertheless, the physiological and/or nutritional plant parameters determined did not explain such particular and specific mycorrhizal metabolic activities.

It has been suggested that the delay on AM formation by P fertilization may be due to a direct effect on hyphal growth (Schwab et al., 1983). Such reduction in growth of extraradical mycelium was here also observed (Fig. 2).

In this study, both endophytes showed different ability to produce extraradical mycelium in the poorest P medium as Abbott and Robson (1985) also noted. Extraradical mycelium developed was not indicative of AM efficiency in plant growth and nutrition as Sylvia (1988); Graham et al. (1982) suggested. But the *B.t.* effect was more relevant on *G. intraradices* colonized plants, where also increased growth and N, P content in the poorest P substrate. This could be correlated with *B.t.* stimulating effect on the extraradical mycelium. Under the highest P conditions, the enhancing promoted by *B.t.* was only observed in *G. intraradices* colonized roots compensating to some extent the negative P effect, particularly for *G. intraradices* extraradical development.

The physiological bases of the effect of phosphorus and/or bacterial on mycorrhizal characteristics is not known. In the case of *G. intraradices,* the highest AM fungal development and activity in the poorest P medium when co-inoculated with *B.t.* may be the result of an increased carbohydrate supply from the plant to the fungus (Hetrick, 1989). But in the case of *G. mosseae*-colonized roots, the *B.t.* stimulating effect cannot be explained by such mechanism. The ability of *B.t.* for producing indole acetic acid (2.95 mg l^{-1} as determined in axenic liquid culture medium) may cause such mycorrhizal-stimulating effect (Azcón et al., 1978; Azcón, 1993). Regarding to P content in plants dually inoculated, the observed effects can not be interpreted as a direct physiological control through internal P status of plant tissues, as it has been suggested by Amijee et al. (1989a); Braunberger et al. (1991).

The reduced extraradical fungal growth following P application seems not to be the result of a limited carbohydrate supply by the plant to the fungus as Amijee et al. (1989b); Hetrick (1989) suggested. Photosynthetic rate was consistently higher in *G. mosseae*-colonized roots at the highest P level and *B.t.* restricted assimilation of CO_2 (limiting supply of carbohydrate to the root) but increased physiological fungal activities. Thus, a direct *B.t.* helping effect on AM fungi seem to be at work.

Our results highlight the diversity in the way, function and reaction of AM symbiosis formed by different species of AM fungi. Our data reflect the complexity of P transfer and interactive mechanisms operating in the AM symbiosis as it was also reported by Boddington and Dodd (1998). While carbon demand by AM fungi is considered as a cost of the symbiotic system (Wang et al., 1989; Wright et al., 1998a,b) this is more or less compensated by AM colonization according to bacterial interaction since it affected the process of C assimilation as observed here. Nevertheless, the mycorrhizal effect increasing photosynthetic activity was independent of the nutritional status, as also Wright et al. (1998a,b); Fay et al. (1996); Ruiz-Lozano et al. (1995a,b) found. Ezawa et al. (1999) suggested links between ALP and C/P transfer in AM fungi. In fact, plants respond differently to AM colonization if the symbionts involved have different carbon requirements.

Glomus spp and *B.t.* are adapted to, and tend to prevail in stressed, poor and dry environments and to cope with stress conditions. Under such limiting conditions, the microbial interactions, like the ones studied here, play a crucial role in mycorrhizal vitality and thus, in plant survival. In fact, our results emphasize the link between rhizosphere bacterial populations and AM symbiosis in agricultural and natural systems. This interesting research field deserve further study.

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