RESEARCH

Effects of the bacterial-fungal interaction between Tsukamurella paurometabola *C 924 and* Glomus fasciculatum *and* Glomus clarum *fungi on lettuce microrrizal colonization and foliar weight*

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ABSTRACT

The study of fungal-bacterial interactions in soils is not only interesting from a basic point of view but has also yielded findings of societal and economical relevance, such as in the application of biological controls of plant diseases. This study evaluated the effect of *Tsukamurella paurometabola* C 924, a bacterium with nematocidal action isolated from banana rhizosphere, as single inoculant or combined with arbuscular mycorrhizal fungi (AMF) *Glomus fasciculatum* and *Glomus clarum* in lettuce (*Lactuca sativa* L.). Controls included non-bacteria non-AMF, and each AMF species alone. Five replicates were used. AMF did not show any influence, neither in *T. paurometabola* C 924 c.f.u. counts in soil, nor over its phenotypic nematocidal characters. On the other hand, the bacterium stimulated AM colonization for both fungi species as well as an early infection. Combined inoculation improved significantly fresh weight of plants as compared with the microorganisms separately or the non-inoculated control.

Keywords: *Tsukamurella*, *Glomus*, arbuscular mycorrhiza, fungal-bacterial interaction, growth promotion, lettuce

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RESUMEN

Effecto de la interacción entre la bacteria *Tsukamurella paurometabola* C 924 y los hongos *Glomus fasciculatum* y *Glomus clarum* en la colonización micorrízica y el peso foliar en lechuga. El estudio de las interacciones entre bacterias y hongos en suelos no solo es interesante desde el punto de vista científico, también tiene relevancia económica y social, como en la aplicación de controles biológicos a enfermedades de plantas. Este trabajo evalúa el efecto de *Tsukamurella paurometabola* C 924, bacteria con actividad nematicida aislada de la rizosfera de banano, como inoculante único o combinado con los hongos micorrízicos *Glomus fasciculatum* y *G clarum* en lechuga (*Lactuca sativa* L.). Se incluyeron controles sin inoculantes microbianos, así como cada especie de hongo micorrízico por separado. Se emplearon cinco réplicas por tratamiento. Los hongos micorrízicos no mostraron influencia sobre las concentraciones de *T. paurometabola* C 924 en suelos, ni sobre sus caracteres fenotípicos nematicidas. Por otra parte, la bacteria estimuló la colonización fúngica del sistema radicular de las plantas con ambas especies de hongos, así como la colonización temprana de las raíces. La inoculación conjunta de la bacteria y los hongos incrementó de forma significativa el peso fresco de las plantas al compararlos con las plantas inoculadas con cada microorganismo de forma separada o con el control sin inocular.

Palabras clave: *Tsukamurella*, *Glomus*, micorrizas arbusculares, interacción bacteriana, promoción del crecimiento, lechuga

*I*ntroduction

Interest in biological control and microbial pesticides has increased in the last years worldwide because of the need to find alternatives to chemical products and to protect the environment. However, the success in using this kind of products depends largely of the knowledge of their mechanism of action and its interaction with other microorganisms on the plant rhizosphere.

Nematocidal action of the bacterium *Tsukamurella paurometabola* (formerly *Corynebacterium paurometabolum*) C 924 has been reported [1]. This strain isolated from banana rhizosphere in Cuban soil, whose mechanism of action is based on the activity of chitinases and hydrogen sulfide production, is a good candidate for the biological control of plant parasitic nematodes.

The arbuscular mycorrhizal fungi (AMF) constitute an important group of symbionts associated to agricultural crops. This symbiosis may enhance the root systems ability to absorb and carry soil elements of low mobility, by means of a network of mycelia, thus promoting plant growth [2]. However, AMF differ in their ability to enhance nutrients uptake and the different physiology of AMF-colonized roots may alter conditions for rhizosphere microbial groups [3].

The effect of inoculants on bacterial and fungal populations in the rhizosphere is decisive for maximizing plant nutrient availability, since the soil microbial community in the rhizosphere plays a key role in plant nutrition. Many authors have observed different effects of inoculation of soil bacteria and AMF, on different species of plants. Results have shown that the interaction among the two groups of microorganisms may improve [4, 5] or not [6] plant growth.

Considering that a biotechnological goal is to use a combined inoculation of selected rhizosphere microorganisms to minimize fertilizer application and to maximize plant growth and nutrition, the purpose of this study was to examine the influence of AMF on *T. paurometabola* C 924 population in soil, the effect of

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T. paurometabola C 924 on colonies of lettuce (*Lactuca sativa* L.) roots by AMF and the influence of both AMF and bacterial inoculation as functional inoculum in lettuce (*Lactuca sativa* L.) growth.

Materials and methods

Microorganisms

T. paurometabola C 924 inoculum was produced by growing the strain 18 hours in a culture shaken at 28 ºC in 200 mL of LB medium (NaCl 10 g/L, Triptone 10 g/L and Yeast Extract 5 g/L). At the log phase of growth, bacterial suspension was centrifuged (10 000 rpm for 20 min at 4 ºC) and washed twice with saline solution (NaCl 0.85%). Bacterial concentration was adjusted to 10^8 c.f.u./mL, according to the previously established correlation between optical density and c.f.u. number [7]. It was applied at a rate of 1 mL per pot (10^8 c.f.u./mL) seven days before planting the seeds.

AMF *G. fasciculatum* and *G. clarum*, were obtained from the collection of the National Institute of Agricultural Research [8]. Inocula consisted of soil containing fungal spores and hyphae. They were applied on a concentration of 200 g m^{-3} at the sowing time.

Plant growth conditions

Lettuce (*Lactuca sativa* L.) plants were grown for 30 days in a greenhouse under controlled conditions at 28 ºC during the day, and 23 ºC during the night , with a 14 h photoperiod on pots of 1 dm^3. Pots were daily weighed throughout the experiment, and water loss replaced daily by top watering to maintain soil moisture close to 100% field capacity during the period of plant growth.

Experimental design

The experiment consisted in three treatments for *T. paurometabola* C 924. It was inoculated separately or in co-inoculation with *G. fasciculatum* or *G. clarum*. Non bacterial controls inoculated with each AM fungus were also used, as well as non-treated plants. All treatments were replicated five times with a total of 30 pots and placed in a completely randomized block design.

Plant growth was evaluated at 7, 14, 21, and 30 days after being sowed. Results were statistically evaluated by factorial analysis of variance and mean values were compared by the Duncan test ($p \leq 0.05$).

Analytical procedures

In order to determine the *T. paurometabola* C 924 concentration in rhizosphere, 1 g of soil was taken at 0, 7, 14, 21, and 30 days after sowing the seeds. It was suspended for one hour in 99 mL of saline solution plus Tween 80 (0.1%). Bacterial concentration was determined by plate counts of 100 µL aliquots from serial ten fold dilutions from the suspension, using a selective medium designed for this strain (LB medium plus Ampicilline 50 µg/mL, Kanamycin 50 µg/mL and Potassium telluride 0.0025%) [9].

T. paurometabola C 924 phenotypic characters enhancing nematocidal activity (production of hydrogen sulfide and chitinases) were also tested to the isolated strains from lettuce (*Lactuca sativa* L.) rhizosphere.

Production of chitinases was tested by streaking the bacterium on Minimal Medium (M9) amended with colloidal chitin 0.5%, bacterial growth was considered as positive. Hydrogen sulfide production was determined inoculating bacterium in tubes with 8 mL of Nutrient Broth and placing on the top a filter paper embedded in lead acetate. A black precipitate on it was considered a positive result.

Mycorrizal densities were determined by visual determination on root system according to Grand and Harvey, 1982 [10].

Plant growth was evaluated by measuring the foliar area of the plant in a technical balance.

Results

AMF did not affect *T. paurometabola* C 924 c.f.u. counts in soil. No significant differences were found among the treatments throughout the experiment, ranging between 10^5 and 10^4 c.f.u/g rhizosphere soil. Only at 14 days after sowed, counts were higher for the single inoculation. Nevertheless, *T. paurometa-bola* C 924 counts decreased throughout the experiment for each treatment (Figure 1). No bacterial counts were found in the negative control.

T. paurometabola C 924 nematocidal characters were not affected by AMF. They remain present in all the isolates during the experiment (Figure 2).

Root colonization by AMF was successful for all treatments, even for those that did not receive fungi. Association increase, in terms of visual density, was progressive. Higher densities were found in *T. paurometabola* C 924 coinoculation with *G. clarum*, followed by *G. clarum* separately and bacterial co-inoculation with *G. fasciculatum* (Figure 3).

Early mycorrhization was found in both species of AMF (7 days) as well as statistically higher colonization densities throughout the experiment in treatments where they were inoculated with *T. paurometabola* C 924 as compared to AMF separately. We also observed higher mycorrhization rates in plants treated with *T. paurometabola* C 924 separately, than in non-inoculated.

These results are proportionally related to plant growth. Plants co-inoculated with the bacterium and

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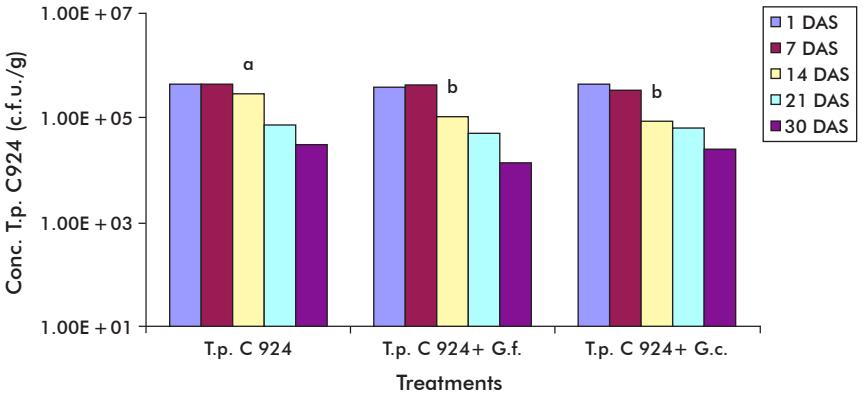

Figure 1. *T. paurometabola* C 924 c.f.u. per gram of soil counts in the inoculated soils during the interaction experiment with *G. fasciculatum* or *G. clarum* in lettuce (*Lactuca sativa* L.). Each value is the mean of triplicates. Means with different letters are significantly different according to Duncan test ($p \leq 0.05$). DAS: days after sowed; T.p. C 924: *T. paurometabola* C 924; G.c.: *G. clarum*; G.f.: *G. fasciculatum*.

AMF, showed significant higher fresh weight than those inoculated separately or non-inoculated (Table 1).

Discussion

Understanding the mechanisms through rhizosphere microbial populations (bacteria and/or fungi) interact is very important for the management of sustainable systems. However, it is difficult to predict the outcome of these interactions in relation to microbial activity, as well as the meaning of this activity regarding the interaction of specific microorganisms groups on plant growth [11].

It is known that inoculation with AMF has different effect on free living rhizobacteria [12]. In this study, the presence of AMF did not show any influence neither in *T. paurometabola* C 924 concentration in soil, nor over its phenotypic nematocidal characters. Similar effects have been observed by Raimam *et al*., 2007 [13] who found that AMF did not affect free living N fixing bacteria population in rice.

Attempts to introduce foreign microorganisms in ecosystems have often failed due to the strains inability to succeed in the space and nutrient competition with native microorganisms better adapted to the environment [14]. The finding of the decreasing of *T. paurometabola* C 924 population during the experiment is in accordance with previous studies with this strain. In field trials, Mena *et al*., 2002 [9], observed that population of strain C 924 was not definitively established in terrestrial ecosystems where it was introduced. It showed a trend to decrease until disappearing on detectable limits in an approximately two years' period. Other researchers have found similar effects with Azospirillum spp strains in shorter time periods [15].

This behaviour can also be related to soil washing produced by irrigation. The decreasing of *T. paurometabola* C 924 population is a positive fact, because it can minimize any negative effect upon the native biota in agriculture environment such as the displacement of wild population causing imbalance in natural ecosystems.

Several authors have described different effects of bacteria on AMF colonization [12, 16,]. In our study the presence of *T. paurometabola* C 924 had a positive influence on AMF colonization as well as stimulation of early infection. These results are in accordance with previous works of Barea *et al*., 1997 [17], who found that inoculation of free-living diazotrophs improve fungal infectivity, and that from Von Alten *et al*. (1993) [18], who obtained similar results with a Bacillus mycoides strain. This result correlates with the extent of growth enhancement [19]. A greater ability of dual inoculated roots to form external hyphae could also explain bacterial effectiveness [20], since it affects mycorrhizal nutrient uptake.

In the same way, Vonderwell *et al*., 2000 [6], studied the effect of different plant growth-promoting rhizobacteria on AMF colonization in Pine seedlings and observed positive as well as negative results on mycorrhization according to the bacterial dose applied.

Root colonization by AMF cause physiological changes in root system. Inside the root, the fungi grows both inter- and intra-cellularly, and the intracellular arbuscules have an important function in nutrient exchange with the plant. These changes are favourable for plant growth and allow a better response to plant environmental stress [21] and at the same time improve the quality of soil [22, 23].

The soilborne or extramatrical hyphae take up nutrients from the soil solution and transport them to the root. By means of this mechanism, mycorrhizae increase the effective absorptive surface area of the plant. In nutrient-poor or moisture-deficient soils, nu-

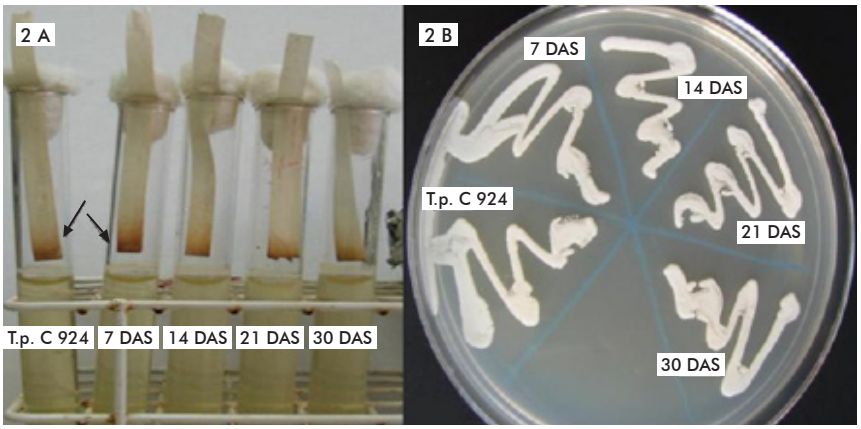

Figure 2. Hydrogen sulfide production (2A) and chitinases (2B) in different isolates of *T. paurometabola* C 924. Black arrows indicate lead sulfide. DAS: days after sowed.

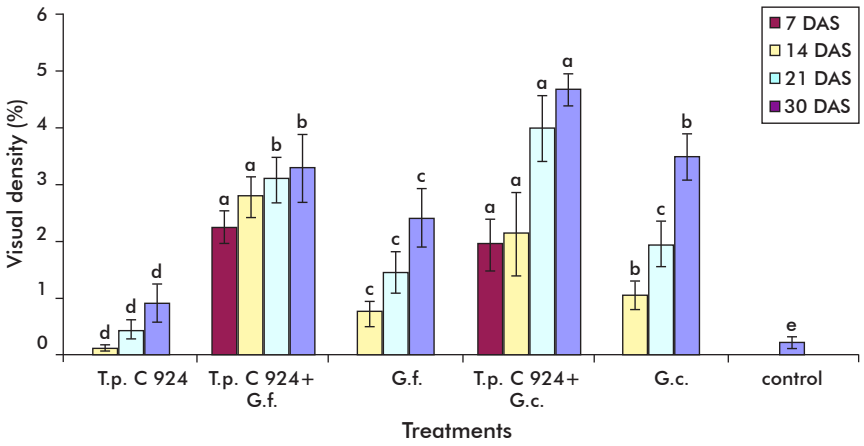

Figure 3. Mycorrhizal colonization of root lettuce (*Lactuca sativa* L.). Vertical bars represent standard deviations. Means with different letters are significantly different according to Duncan test ($p \leq 0.05$). DAS: Days after sowed; Tp: *T. paurometabola* C 924; Gc: *G. clarum*; Gf: *G. fasciculatum*.

Table 1 Fresh weights (g per plant) of lettuce (*Lactuca sativa* L.) plants 30 days after being sowed*

Treatments	Foliar fresh weight (g)
control	0.98 ± 0.13^{e}
G. fasciculatum	17.22 ± 2.2^{c}
T. paurometabola C 924 + *G. fasciculatum*	23.05 ± 1.6^{b}
T. paurometabola C 924 + *G. clarum*	29.50 ± 2.3^{a}
G. clarum	25.30 ± 1.7^{b}
T. paurometabola C 924	3.56 ± 1.3^{d}
ANOVA (*p* value)	< 0.0001

*Values are the means of five replicates + Standard deviation.
[a-e]Means with different letter are significantly different according to Duncan test ($p \leq 0.05$)

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trients taken up by the extramatrical hyphae can lead to an improved plant growth and reproduction. As a result, mycorrhizal plants are often more competitive and better able to tolerate environmental stresses than are non-mycorrhizal plants [24].

Plant growth benefits have been described in bacteria and AMF coinoculated plant cultures. Requena *et al*., 1997 [25] found dry weight increases in *Anthyllis cyti* when inoculated with rhizobacteria and AMF. Recent studies of co-application of *Bacillus sp* and *G. manihotis* in banana vitroplants have shown better growth and adaptation than non- inoculated controls [26]. Other plant growth promoting bacteria as *Rhizobium* and *Azotobacter* have also shown better results in plant growth when co-inoculated with AMF [27, 28]. *T. paurometabola* C 924 combined with AMF also significantly improve the fresh weight of plants as compared to the microorganisms applied separately or the non- inoculated plants.

These results show, as previously reported, the existence of "functional compatibilities" between saprotrophic and symbiotic microorganisms [16]. On the other hand, the plant response may be diagnostic for the success of the association which was dependent on the AMF involved. We observed significant better and separate plant growth with *G. clarum* co-inoculated with *T. paurometabola* C 924, than with plants inoculated with *G. fasciculatum*.

It is a fact that AMF play a relevant role in NH_4^+ plant uptake [29]. *T. paurometabola* C 924 produces a high amount of extracellular NH_4^+ during organic matter decomposition [30] and it could partially explain the results here shown.

This strain, besides its nematocidal characters, has shown the production of plant growth promoting compounds [31] that can stimulate plant susceptibility to mycorrhizal colonization, spore germination or the growth of mycelium, thereby increasing the chance of contacts between fungal hyphae and plant roots and, consequently, increasing mycorrhizal establishment and plant growth [32]. This fact can also explain the significant differences found in the fresh weight on plants treated separately with *T. paurometabola* C 924 as compared with the non-inoculated control.

Conclusions

We conclude that AMF did not affect *T. paurometabola* C 924 population in soil. The nematocidal bacterium improved the colonization of lettuce roots by the AMF tested. Coinoculation of *T. paurometabola* C 924 and *G. clarum* or *G. fasciculatum* promotes lettuce growth.

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