Article

Effect of *Methylobacterium extorquens* on tomato development in the presence or absence of *Fusarium oxysporum*

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Abstract

The wilting of tomato (*Fusarium oxysporum*) promotes the excessive use of synthetic fungicides, which are not economically or environmentally viable. This work proposes the use of *Methylobacterium extorquens* to determine its effect on plant development and the signaling pathway, for the defense of tomato plants, against phytopathogens. From tomato seeds var. SUN 7705 embedded and plants sprinkled with the bacteria at a concentration of 10^9 CFU mL^{-1}, were challenged to the plant with the fungus in two tests. Variables of root length in seedlings, plant height, leaf amplitude, yield, total dry weight of the plant and dry root weight were measured. A completely randomized design with six treatments and four repetitions was used, two plants formed the experimental unit. Likewise, an essay was carried out on the expression of defense genes, in tomato seedlings of the same variety. Significant statistical differences ($p\leq 0.05$) were found between treatments embedded and sprinkled with *M. extorquens* and the treatments inoculated with *F. oxysporum* and the controls, reflecting in the seedling root length, plant height, leaf amplitude, dry weight of the plant and the root, as well as in the yield. However, the genes encoding the phenylalanine ammonium lyase (PAL), protein (PR-6), superoxide dismutase (SOD) and lipoxygenase (LOX) enzyme group, were not significantly expressed in seedlings treated with *M. extorquens*.

Keywords: *Fusarium*, *Methylobacterium extorquens*, *Solanum lycopersicum*, *Oxysporum*, biological control.

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Introduction

In Mexico, the tomato is one of the main vegetables produced in irrigated and temporary areas, due to the considerable number of farmers dedicated to this activity, its prices that can become attractive, its acceptance by consumers and the collection of foreign exchange for this country (Vargas and Martínez, 2004). The tomato production in the greenhouse has also shown a considerable increase; however, the wilting caused particularly by *Fusarium oxysporum* f. sp. *lycopersici*, has caused significant losses (Carrillo-Fasio *et al*., 2003; Ascencio-Álvarez *et al*., 2008).

In the search for environmentally friendly alternatives for the control of phytopathogens, different beneficial bacteria for plants and antagonistic to phytopathogenic fungi have been found, within which the members are the genus *Methylobacterium* themselves that by their ubiquitous character, colonize different habitats including soil, water, leaf surfaces, nodules, seeds and air (Corpe, 1985; Corpe and Rheem, 1989; Nobrega-Dourado *et al*., 2015), as well as in the host's internal tissues; that is, they can act as endosymbionts (Sy *et al*., 2005) and inhibit and antagonize fungi among them some of the genus *Fusarium* (Poorniammal, 2009; Nobrega-Dourado *et al*., 2015).

Some bacteria that contribute to nitrogen fixation, increased nutrient intake, synthesis and fixation of phytohormones may be linked to plant development and are considered in the group of *BPCV* growth promoting bacteria (Loredo-Osti, 2004). In the last decade, some researchers have called aerobic methylotrophic bacteria for their phytosymbiotic properties, related to the production of auxins (indole-3-acetic acid, indole-3-pyruvic and indole-3-butyric acid) and cytokinins (zeatin, trans zeatin and trans-zeatin riboside), responsible for plant growth; through the cell division and the increase in elongation of the cells, both of the roots and of the aerial part; also, for its ability to induce effects of defensive responses in plants against phytopathogens.

It has also been shown that the use of *Methylobacterium* spp. has had a positive effect on the development of rice, sugarcane and peanut plants; as well as in the elongation of the root in seedlings of chili and tomato (Madhaiyan *et al*., 2004, 2005, 2006; Ryu *et al*., 2006). Based on the foregoing, the objective of this work was to determine the effect of *Methylobacterium extorquens* on plant development and the signaling pathway, for the defense of tomato plants, against phytopathogens.

Materials and methods

The research was carried out at the Interdisciplinary Research Center for Regional Integral Development (CIIDIR-IPN, Michoacán), located in the city of Jiquilpan, Michoacán, Mexico, between the coordinates 19º 59' north latitude and 102º 43' west longitude, at a height of 1 550 meters above sea level.

Isolation and identification of *Methylobacterium extorquens*

Starting from tomato leaves, the isolation of *Methylobacterium* sp. in a solid selective medium of ammonium and mineral salts (AMS), which contained per liter: 0.8 g of agar, 0.5 g of ammonium chloride, (NH_4Cl), 0.54 g of potassium diacid phosphate, (KH_2PO_4), 0.7 g of dipotassium

phosphate, (KH_2PO_4), 1 g of magnesium sulfate heptahydrate, ($MgSO_4\ 7H_2O$), 0.2 g of calcium chloride dihydrate, ($CaCl_2.2H_2O$), 4 mg of iron sulfate heptahydrate ($FeSO_4\ 7H_2O$), 0.1 mg zinc sulfate heptahydrate ($ZnSO_4\ 7H_2O$), 0.03 mg manganese chloride tetrahydrate ($MnCl_2\ 4H_2O$), 0.3 mg boric acid, (H_3BO_3), 0.2 mg cobalt chloride hexahydrate ($CoCl_2\ 6H_2O$), 0.01 mg of copper chloride dihydrate ($CuCl_2\ 2H_2O$), 0.02 mg of nickel chloride hexahydrate ($NiCl_2\ 6H_2O$), 0.06 mg of sodium molybdate dihydrate ($Na_2Mo\ O_4\ 2H_2O$).

The pH was adjusted to 6.8 ±0.2 at 25 °C and 0.5 mL of methanol with 0.05 gL^{-1} of PCNB was added. Petri dishes were placed in the incubator at 26 °C for a period of 72 hours for the development of the bacteria (Ryu *et al*., 2006). The inoculum was prepared by developing the bacteria in liquid culture medium in its exponential mid-phase with an optical density of 600 nm (OD_{600}= 1.2), followed by centrifugation for 15 min at 3 000 x g, washing and resuspending in a sulfate solution of magnesium ($MgSO_4$) 30 mM until obtaining a cell density of 10^9 CFU mL^{-1} (OD_{600}= 1.2). Followed by an orbital agitation for 24 °C for 48 h.

The 16S gene was amplified by PCR from the extracted DNA, using the first according to the methodology described by Weisburg *et al*. (1991). The sequencing process was carried out in the Genomic Services Laboratory of the National Laboratory of Genomics for Biodiversity (LANGEBIO). The nucleotide sequence obtained was compared with 16S rDNA gene sequences in the Silva databases (http://www.arb-silva.de) to identify the bacterial species to which the sequence belonged, in this case *M. extorquens*.

Isolation and identification of *Fusarium oxysporum*

The fungus was isolated from the root of the diseased tomato plant with wilting symptoms. Necrotic roots were cut into sections of 1 to 2 cm in length and placed in a solution with 3% sodium hypochlorite for 2 min, rinsed 3 times with sterile water and dried in aseptic sanitas, then placed in the middle of potato dextrose agar (PDA) culture at a temperature of 25 °C ±1 C for a period of 5 days for fungus development.

Which was purified by the hyphae technique and identified based on its morphological characteristics, cited by Nelson *et al*. (1983). The pathogenicity of the strain was tested according to Koch's postulates. For which four healthy plants were inoculated at the base of the stem and tomato roots with small wounds, with a concentration of 1 x 10^6 CFU mL^{-1}; from pure cultures of the fungus. The four control plants were also injured, but without inoculum.

Effect of *M. extorquens* on seed germination and root length

Tomato seeds variety SUN 7705 were aseptized in 95% ethanol, they were also treated with 50 v/v sodium hypochlorite and 10 µl of 20% tween, in both cases they were repeatedly rinsed with sterile water. 20 seeds were placed in a flask containing 100 mL of a 0.03 M $MgSO_4$ solution, to which the inoculum of *M. extorquens* was added, from pure culture with a bacteriological handle, until a concentration of 10^9 CFU mL^{-1} was obtained (treatment M). 20 seeds considered as control (C), were equally aseptized and embedded in the same solution, but without the bacteria.

The 40 seeds of both treatments were placed individually in cavities of a germination tray with peat moss and vermiculite (2:1). At seven days after sowing (dds) the germination percentage was determined and at 21 dds the root length was measured. The test was repeated twice and a Student's t-test was done to determine statistical differences.

Effect of *M. extorquens* on tomato plant

The tomato cultivation variety SUN 7705 was carried out in pots of 5 L capacity with substrate based on peat moss and vermiculite (2:1) sterilized by solarization for 30 days. The following treatments were formed from seeds embedded with 0.03 M $MgSO_4$ for 12 h under stirring, with and without inoculum of *M. extorquens*: 1) plants from seeds embedded without bacteria (C), control 1; 2) embedded seeds and plants sprinkled with 0.03 M $MgSO_4$ without bacteria (CA), control 2; 3) plants from seeds embedded without bacteria and inoculated with *F. oxysporum* (F); 4) plants. From seeds embedded with *M. extorquens* (M); 5) plants from seeds embedded with bacteria and inoculated with the fungus (MF); and 6) embedded seeds and plants sprinkled with the bacteria and inoculated with the fungus (MFA).

Inoculations in the plants were made 41 days after the emergency; in the case of the fungus, they were made to the neck of the tomato plant with 12 mL of a suspension of *F. oxysporum* at a concentration of 10^6 CFU mL^{-1}, the bacterial suspension was sprinkled in the leaf area, with 50 ml of a solution of 0.03 M $MgSO_4$ at a concentration of 10^9 CFU mL^{-1} for 3 consecutive days.

Variables evaluated

Variables of height and amplitude of the plant were carried out at 30, 60 and 90 days after sowing (dds), fruit production was quantified during the cultivation cycle and at the end of this cycle the total dry weight of the plant and root was recorded on an electronic scale Ohaus CS 2000 brand, after drying in an oven brand FELISA at 70 °C to obtain constant weight. A completely randomized design with six treatments and four repetitions was used, the experimental unit was constituted by two plants. The analysis of variance (Anova) and the comparison of averages by the Tukey test ($p \leq 0.05$) was done using the statistical package SAS (Statistical Analysis System) for Windows v. 9.

Gene expression assays

Tomato seeds variety SUN 7705 were placed in a solution of 0.03 M $MgSO_4$ inoculated by the handle technique, with a pure culture of *M. extorquens* at a concentration of 10^9 CFU mL^{-1} for 12 h under stirring (treatment M); other seeds were put in the same solution and under the same conditions, without inoculating the bacteria (treatment C). Seeds of both treatments were sown in germination trays with a substrate based on sterile pedestrian moss and 2:1 vermiculite; thus, they remained in a growth chamber at 28 °C, with 18 h light and 8 h dark.

At 22 seedling growth, leaves of each treatment were collected, frozen and ground with liquid nitrogen. To determine the possible signaling pathway that *Methylobacterium* could induce, for the defense of the tomato plant against phytopathogens, RNA extraction was performed by means of the Column Extraction Kit and an RT-PCR was performed using two probes: P6 coding for the

marker gene of the salicylic acid route and Pin2 related to the jasmonic acid route, ribosomal 18S was used as a control. Standard protocols according to taq. Used (Invitrogen) were considered; for the RT the commercial of ferments was used, where the expected products are of the size P6= 450 bp, Pin2= 302 and 18S= 506 bp.

28 cycles were run for the markers of both signaling pathways (P6 and Pin2), while for 18S only 22 cycles were used. On the other hand, the seedlings of treatment M were sprayed with 20 ml of the bacterial suspension at a concentration of 10^9 CFU mL^{-1} and were collected at 24 and 72 h to make the corresponding test in duplicate (M1 and M2), seedlings of treatment C were sprinkled with the same solution without the bacteria and collected at time 0 (C1 and C2). Once the samples were obtained, the RNA was extracted using the Plant RNA Reagent technique and the cDNA Promise, a first PCR was performed where the control was the ribosomal 18S and a second with the Pal and P6 probes for the salicylic acid pathway, with a size expected of 687 bp and 450 bp respectively.

The SOD and LOX probes were also used for the jasmonic acid pathway. In both cases the standard protocol was used according to the taq used (Invitrogen). The RT-PCR conditions were the same as those of the first test, unlike the number of cycles which were for PAL= 25 cycles, P6= 25, SOD= 22 and LOX= 33 cycles. To determine the statistical difference in the intensity of gene expression, the Image 1.44 program and the Statgraphics 4.0 statistical package were used with an Anova at 95% reliability. The intensity at which resistance genes were expressed in the tomato plant were plotted for each of the treatments in which response was sought.

Results and discussion

Effect of *M. extorquens* on seed germination and root length

The average germination percentage in both tests was higher in tomato seeds inoculated with *M. extorquens* (97%) compared to seeds embedded without bacteria (92%) (Table 1). Similar responses with the application of this bacterium have been reported in the germination of wheat, barley and corn (Abanda-Nkpwatt, 2006).

This may be related to the increase in the production of vitamin B12 and hormones such as cytokinins (zeatin, trans-zeatin and trans-zeatin riboside) and auxins (indole-3-acetic acid, indole-3-pyruvic and indole acid -3-butyric) which stimulated the germination of seeds and the growth of rice plants (Maghaiyan *et al*., 2004).

The root length in tomato seedlings of three weeks of age was statistically greater ($p \leq 0.05$) in the treatment with *Methylobacterium* compared to the control (Table 1). These results agree with those reported in tomato and red pepper (Ryu *et al*., 2006), in rice (Maghaiyan *et al*., 2004), chili and tomato, where an increase in the production of auxins (indol-3-acetic acid) and cytokines in plants treated with said bacteria was also verified (Ryu *et al*., 2006), which may explain the increase in the elongation of the tomato root, even though these phytohormones were not quantified in this study.

Table 1. Root length of tomato seedlings, with and without *M. extorquens*.

Treatments	Seed germination	Root length (mm)	
		Test 1	Test 2
C♦	92%	39.5 b*	50 b
M	97%	75.9 a	90.8 a

*= values with different letters within each column are statistically different, Tukey ($p \leq 0.05$); ♦C= seeds embedded in 0.03 M $MgSO_4$ (control), M= seeds embedded in 0.03 M $MgSO_4$ inoculated with *Methylobacterium extorquens*.

Effect of *M. extorquens* on tomato plant

Results regarding the height and breadth of tomato plants, were similar in both crop cycles, where treatment M (seeds embedded with *M. extorquens*), exerted statistical differences in plant height from 27.2 cm to 90 dds in the first test (Table 2) and 16.7 cm with respect to the control 1 (C) in the second test (Table 3).

Table 2. Height and breadth of tomato plants in the greenhouse, with and without *M. extorquens* (first test).

Treatments	Plant height (cm)			Foliage diameter (cm)		
	30 dds	60 dds	90 dds	30 dds	60 dds	90 dds
C♦	16.5 c*	78.5 bcd	87 bc	25.5 c	42.7 b	45 c
CA	15.7 c	75.7 cd	84 bc	25.2 c	41.5 b	44 c
F	14 c	67.2 d	77.7 c	21.7 d	36.7 c	38.7 d
M	24.2 a	93 a	114.2 a	37.2 a	56 a	59.5 a
MF	19.7 b	81.7 abc	98.5 ab	31.7 b	52.2 a	55.5 b
MFA	19.7 b	89.2 ab	107.7 a	33.2 b	53.5 a	56.7 ab

*= values with different letters within each column are statistically different, Tukey ($p \leq 0.05$); ♦C= seeds embedded in 0.03 M $MgSO_4$ (control 1); CA= embedded seeds and plants sprinkled with 0.03 M $MgSO_4$ (control 2); F= plants inoculated with *F. oxysporum*; M= seeds embedded with *Methylobacterium*; MF= plants from seeds embedded with the bacteria and inoculated with the fungus; MFA= plants from seeds embedded with the bacteria and inoculated with the fungus.

Table 3. Height and breadth of tomato plants in the greenhouse, with and without *M. extorquens* (second test).

Treatments	Plant height (cm)			Foliage diameter (cm)		
	30 dds	60 dds	90 dds	30 dds	60 dds	90 dds
C♦	16.7 cd*	61.5 bcd	93.5 c	26.7 c	43.5 cd	45.2 c
CA	16.7 cd	59.5 cd	93 c	25.2 cd	42 cd	44.5 c
F	14.5 d	56.5 d	76 d	23.2 d	40 d	39.7 d
M	22.2 a	76.2 a	110.2 a	36.7 a	53.5 a	57.5 a
MF	18.7 bc	67.7 abc	97.5 bc	28.5 bc	46.2 bc	51.5 b
MFA	20 ab	69 ab	102.2 ab	30.2 b	49.2 ab	53.5 ab

*= values with different letters within each column are statistically different, Tukey ($p \leq 0.05$); ♦C= seeds embedded in 0.03 M $MgSO_4$ (control 1); CA= embedded seeds and plants sprinkled with 0.03 M $MgSO_4$ (control 2); F= plants inoculated with *F. oxysporum*; M= seeds embedded with *Methylobacterium*; MF= plants from seeds embedded with the bacteria and inoculated with the fungus; MFA= plants from seeds embedded with the bacteria and inoculated with the fungus.

Likewise, there were significant differences in the diameter of the plant (foliage amplitude), measured at 90 days in the treatments inoculated with the bacteria, with respect to treatment C and inoculated with *F. oxysporum* (Table 2 and 3). These results were similar to those reported in sugarcane, where *Methylobacterium* sp. embedded in seed and sprinkled on the plant, it registered significant statistical differences in plant height and leaf area with respect to C, effects that could be due to the production or synthesis of phytohormones (Madhaiyan *et al*., 2005; Ryu *et al*., 2006).

The average tomato production in both tests, statistically contrasted ($p \leq 0.05$) among those who involved *M. extorquens* (M, MF and MFA) with the controls (C and CA) and the F treatment, inoculated with *F. oxysporum* (Table 4), which could indicate, that the bacteria presented a symbiotic relationship, because it can be associated with leafy plants, stem, root and meristems (Abanda-Nkpwatt *et al*., 2006).

Table 4. Production, average fruit weight and dry weight of tomato plants in the greenhouse, with and without *M. extorquens* (first test).

Treatments	Production (g)	Average fruit weight (g)	Dry weight of the plant (g)
C♦	434 bc*	75.7 a	48.5 b
CA	333.7 cd	72 a	51 b
F	182 d	47.5 b	47.2 b
M	647 a	71.7 a	63.5 a
MF	550.5 ab	72.5 a	52.2 b
MFA	594.2 a	70.7 a	52.5 b

*= values with different letters within each column are statistically different, Tukey ($p \leq 0.05$); ♦C= seeds embedded in 0.03 M $MgSO_4$ (control 1); CA= embedded seeds and plants sprinkled with 0.03 M $MgSO_4$ (control 2); F= plants inoculated with *F. oxysporum*; M= seeds embedded with *Methylobacterium*; MF= plants from seeds embedded with the bacteria and inoculated with the fungus; MFA= embedded seeds and plants sprinkled with the bacteria and inoculated with the fungus.

Allowing the development of the tomato plant even under conditions of stress due to the presence of the pathogen (Trotsenko *et al*., 2001). It should be noted that, under '*in vitro*' conditions, several species of *Methylobacterium* have restricted the development of *F. oxysporum* in papa dextrose agar culture medium (Savitha *et al*., 2015), which has also occurred with *M. extorquens* (Poorniammal, 2009).

With respect to the dry weight of the plant and the root, there were only significant differences between the plants treated with *M. extorquens* by imbibition of the seeds (M) with the rest of the treatments (Table 5), where it seems that the presence of the fungus, which is attributed to root necrosis and vascular wilting (Tello and Lacasa, 1988; Fernández *et al*., 2007), which would imply less development and consequently a statistical decrease in the dry weight of the treatment F and in this case also in the average weight of the fruit (Table 4 and 5).

Table 5. Production, dry weight of the plant and tomato root in the greenhouse, with and without *M. extorquens* (second test).

Treatments	Production (g)	Dry weight of the plant (g)	Dry root weight (g)
C♦	931.2 c*	53 cb	16 cb
CA	953.5 c	60.2 b	14.5 cb
F	807.5 d	40 c	12.2 c
M	1 248 a	75.5 a	31 a
MF	1 067.5 b	60.5 b	20.7 b
MFA	1 068.7 b	59.7 b	19 cb

*= values with different letters within each column are statistically different, Tukey ($p \leq 0.05$); ♦C= seeds embedded in 0.03 M $MgSO_4$ (control 1); CA= embedded seeds and plants sprinkled with 0.03 M $MgSO_4$ (control 2); F= plants inoculated with *F. oxysporum*; M= seeds embedded with *Methylobacterium*; MF= plants from seeds embedded with the bacteria and inoculated with the fungus; MFA= embedded seeds and plants sprinkled with the bacteria and inoculated with the fungus.

Expression of genes related to the defense of tomato plants

The genes encoding the phenylalanine ammonium lyase (PAL), protein (PR-6), superoxide dismutase (SOD) and lipoxygenase (LOX) enzyme group, were not significantly expressed in seedlings treated 22 dds, with *M. extorquens*. Although PR-6 and PAL are referred to as markers related to the salicylic acid-mediated defense signal, in the case of PAL (Figure 1) it is not expressed as much as PR-6 does just after 24 h (Figure 2) what could result from the route by which it is expressed, because the PAL derives from a longer sequence than that of PR-6.

Regarding SOD, it is partially repressed during the recognition phase and then fired at 72 h (Figure 3) with a tendency to repress again after that time, which makes it clear that these are not the routes by which induction occurs of resistance in plants inoculated with *M. extorquens*. LOX was not expressed in any of the tests, so the gel intensity of the corresponding image could not be obtained.

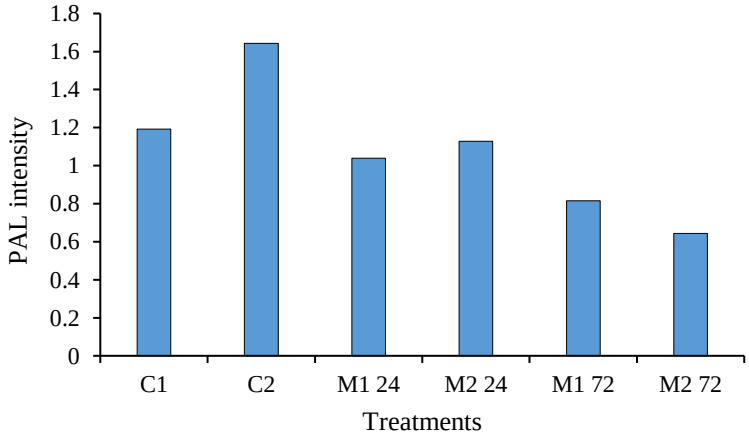

Figure 1. Intensity of the PAL gene in treatments with *M. extorquens* (M1 and M2 at 24 and 72 h) and without the bacteria (C1 and C2 at 0 h), with a n= 3 and an α 0.05.

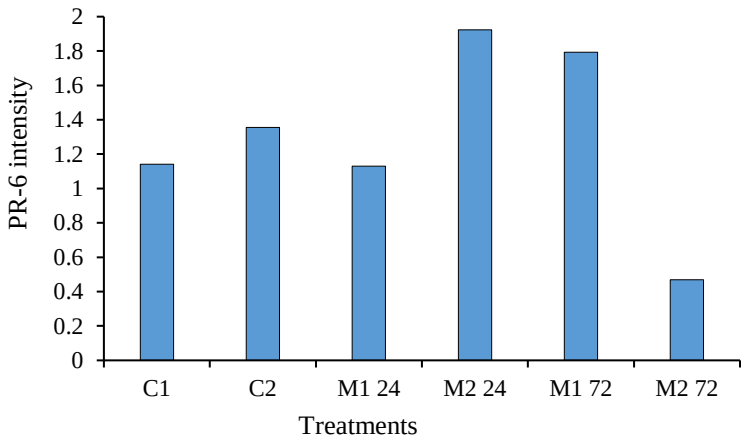

Figure 2. Intensity of the PR-6 gene in treatments with *M. extorquens* (M1 and M2 at 24 and 72 h) and without the bacteria (C1 and C2 at 0 h), with a n= 3 and an α 0.05.

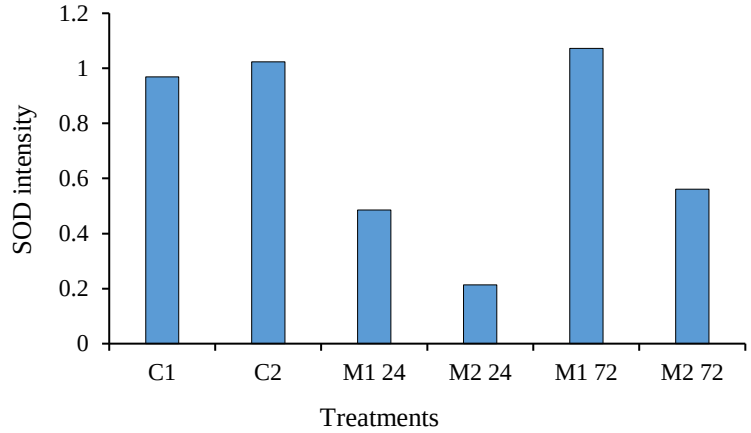

Figure 3. Intensity of the SOD gene in treatments with *M. extorquens* (M1 and M2 at 24 and 72 h) and without the bacteria (C1 and C2 at 0 h), with a n= 3 and an α 0.05.

It is likely that the enzymatic activity related to defense mechanisms has not increased, because, in the evaluations carried out, the plants with the phytopathogen under study were not challenged, as happened in the case of wheat genotypes, where observed an increase in PAL activity, which was higher in resistant genotypes (HD 29 and DWL 5023) during infection of the pathogen *Neovossia indica* (Gogoi *et al*., 2000) or the importance of the LOX enzyme.

In the tomato-*Alternaria solani* interaction, where the intensity of the bands obtained with LOX were higher in resistant varieties than in susceptible varieties, a protein that may be involved with the defense mechanisms in that interaction (Solorzano *et al*., 2006). However, it has also been found that the inoculation of *Metylobacterium* sp., has reduced the effect of *Rhizoctonia solani* on rice plants, increased the production of pathogenesis-related proteins (PR) and the production of phenolic compounds in the plant.

A large number of enzymes, including peroxidase (PO), PAL, LOX, β-1, 3-glucanase and chitinase have been associated with systemic resistance, although the increase in activity and accumulation of these enzymes depends mainly on the inducing agent, as well as the genotype of the plant, the physiological state and the pathogen (Madhaiyan *et al*., 2004). Another similar case was reported in peanuts inoculated with *Methylobacterium* sp., where protection was induced against *Aspergillus niger* and also significantly improved germination and vigor of seedlings, also increasing PR proteins and phenols compared to controls, and therefore the induction of physical and chemical barriers caused by the increase in associated enzymatic activities (Madhaiyan *et al*., 2006).

Conclusions

The imbibition of tomato seeds variety SUN 7705, in $MgSO_4$ solution, inoculated with *Methylobacterium extorquens* at a concentration of 10^9 CFU mL^{-1}, promoted the root length of tomato seedlings and at the same time, the growth and development of the plant, reflected in greater dry weight of plants and roots, as well as in their yield. The enzymatic activity related to defense mechanisms was not increased, probably because, in the resistance gene expression tests, the plant was not challenged with the pathogen, so it is recommended to involve it so that it can cause some kind of stress and also analyze a greater number of enzymes.

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