*Mycopath* (2017) 15(1): 13-21 **Research Article**

# Halophilic bacteria induce resistance and promote growth of stem canker-infected tomato plants grown under greenhouse conditions

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## Abstract

The present study was carried out to investigate potential of halophilic bacteria to induce plant resistance as well as to promote growth of tomato. The results indicated that halophilic bacteria were able to induce PR-proteins and total proteins in treating tomato. Among four halophilic bacteria tested, strain M3-23 of *Virgibacillus marismortui* was strongly able to induce PR-proteins, especially chitinase and glucanase produced by tomato plants infected by *Botrytis cinerea*. The tomato growth was also promoted by the halophilic bacteria. Furthermore, *in vitro* antifungal activities proved the most efficiency of strain J9 of *Bacillus subtilis* as well as the strain J31of *Terribacillus halophilus* on mycelial biomass reduction, hyphae destruction and inhibition of spore germination of the fungal pathogen. This study demonstrates the efficacy of the halophilic bacteria to improve tomato defense and protection against stem canker.

**Keywords**: *Botrytis cinerea*, halophilic bacteria, induced resistance, PR-proteins , tomato.

## Introduction

Stem canker of tomato caused by *Botrytis cinerea* is a severe disease that causes serious damage to tomato crops particularly prominent in Tunisia The current control method in North Africa is the application of fungicides at flowering stage but the rapid emergence of resistant strains of *B. cinerea* has reduced fungicide effectiveness and consequently their constant use (Hmouni *et al*., 2006). Moreover, fungicides potentially cause soil pollution and may have detrimental effects on humans (Martínez-Romero *et al*., 2008). Biological control offers an alternative to the use of synthetic fungicides and has become a well established principle over the last few decades. In recent studies, we describe the ability of halophilic bacteria, to reduce grey mold infection on tomato and strawberry caused by *B. cinerea* (Sadfi- Zouaoui, *et al*., 2007; 2008a; Essghaier *et al*., 2009)

Higher plants have a broad range of mechanisms to protect themselves against various threats, including, physical, chemical and biological stresses as well as pathogen attacks (Ryals *et al*., 1996). Plant reactions to these factors are very complex and involve the activation of a set of genes, encoding different proteins. Among these, production and accumulation of pathogenesis related proteins (PR proteins) in plants in response to invading pathogen and/or stress situation is very important. PR proteins were accumulated locally in the infected and surrounding tissues, and also in remote uninfected tissues. Production of PR proteins in the uninfected parts of plants can prevent the affected plants from further infection (Agrios, 2005). Currently PR-proteins were categorized into 17 families, according to their properties and functions, including β-1,3-glucanases, chitinases, thaumatin-like proteins, peroxidises (Van Loon and Van Strien, 1999). Among these PR proteins chitinases (PR-3 and PR-4) and β-1,3-glucanases (PR-2) are important hydrolytic enzymes that are abundant in many plant species after infection by different types of pathogens. They play the main role of defense reaction against fungal pathogen since chitin and β-1,3-glucan are major structural component of the cell walls of many pathogenic fungi. β-1,3-glucanases appears to be co-ordinately expressed along with chitinases after fungal infection. This co-induction of the two hydrolytic enzymes has been described in many plant species, including pea, bean, tomato, tobacco, maize, potato and others (Li *et al*., 2001).

A group of important antagonistic bacteria exhibit diverse modes of actions, including parasitizing, competing for nutrient uptake, inducing systemic resistance of plants, promoting plant health, producing toxins, antibiotics or enzymes. To our knowledge, there are few reports mentioning the ability of halophilic bacteria to control the activity of phytopathogenic fungi and to produce bioactive compounds (Essghaier *et al*., 2009). Moreover, it should be noted that the ability of such halophilic bacteria to induce resistance in the plant has not been

reported. Based on data explained above, present study was undertaken to evaluate the ability of halophilic bacteria isolated from Tunisian sebkha to induce the production of PR proteins in tomato plants, since they play an important role in plant defense responses to *Botrytis cinerea* infection.

# Material and Methods

**Microorganisms**

Four moderately halophilic bacteria previously isolated from a Tunisian Sebkha and selected for their ability to reduce *B. cinerea*, were chosen in this work: *Bacillus subtilis* (strain J9), *Virgibacillus marismortui* (strain M3-23), *Planococcus rifietoensis* (strain M2-26) and *Terribacillus halophilus* (strain J31). *B. cinerea* isolate BCt was obtained from the collection of the Laboratory of Biotechnology applied to the agriculture of the National Institute for Agronomic Research of Tunisia (INRAT). The fungi were grown on potato dextrose agar (PDA) at 28 °C.

**Treatments**

Tomato plants (hybrid F1 Maria) used in this study were grown in 25-cm diameter pots in a plastic house and *B. cinerea* isolate BCt from tomato canker was used for inoculation. Eight weeks after transplanting, two lesions were made per plant by pruning petioles (0.5 cm long) and placing a 6-mm square agar plug from actively growing *B. cinerea* on PDA medium onto the resulting wound.

The bacterial antagonists were grown on TSA (Tryptic Soy Agar, Difco) supplemented with 5% NaCl (w/v). After 48 h incubation at 30 °C, the cells were then scraped from the Petri dishes, diluted in sterilized 1% NaCl saline solution and adjusted to $10^8$ CFU $mL^{-1}$. Bacterial treatments were applied as spray at different time 10, 20, 30 and 40 days after first inoculation with *B. cinerea*. Each treatment had 10 replicate plants. All plants were grown in the greenhouse at about 22 °C. Two types of treatments were applied in this study. Preventive treatment involves spraying the tomato stem with a bacterial suspension. After that a fungal disc was placed as detailed above. Curative treatment involves spraying the bacterial antagonist after the development of stem canker on a tomato plant. Thus bacterial treatments begin after a period of 10 days from the first day of the artificial infection made with the rod by *B. cinerea.*

Ten days after bacterial sprays for each treatment, the tomato leaves were harvested for enzymatic assay. The negative control plants were untreated plants (C-) which were spraying only with 0.1% NaCl solution and positive control plant were only infected by the pathogen and don't treat by biological solution (C+). Lesion length of the stem was measured for each infected site three times during the course of the trial. Stem length of plant was measured to indicate tomato growth.

**Protein extraction from harvested leaves**

Tomato leaves are ground in the cold mortar in the presence of a small amount of Fontainebleau sand in the presence of the buffer extraction (25 mM Tris HCl pH7, 100 mM KCl, 1.5% PVP (w/v) which were added at the rate of 2:1. After homogenization in the cold, the plant extract is then centrifuged at 3500 rpm for 15 min at 4 °C. The obtained supernatant was then centrifuged at 12000 rpm for 20 min at 4 °C, and finally the supernatant containing the proteins extracted from the harvested tomato leaves obtained from each treatment were stored at 4 °C for further use. Total protein present in the proteins extracted from harvested tomato leaves sample were determined by the method of Bradford (1976).

**Chitinase assay**

Chitinase was determined according to the method of Gomez-Ramirez *et al*. (2004). The sample and 1% colloidal chitin suspension was incubated for 1 h at 37 °C. The product was determined by DNS assay and the absorbance was measured at 540 nm. The activity was defined as the amount of enzyme required to produce 1 µmol $h^{-1}$ $mg^{-1}$ of N-acetylglucosamine (NAG, Sigma) (Roja-Avelizapa *et al*., 1999).

**Glucanase assay**

Enzyme was performed according to the method of Leelasuphakul *et al*. (2006). The standard assay contained 100 µL of the sample and 500 µL of 1% laminarin in 0.1 M sodium acetate buffer pH 5 and 200 µL of 50 mM sodium acetate buffer pH 5. After incubation at 5 °C for 30 min, the reaction was stopped by boiling for 10 min then 0.2 mL of 1% DNS and boiled for 5 min. The optical absorbance was measured at 540 nm. The glucanase activity was expressed in units (µmol glucose equivalent $min^{-1}$).

**Protease assay**

Five hundred microliters of 0.5% azocasein (sigma) in 0.1 M Tris-HCl buffer pH 8 and 100 µL of the sample were incubated for 30 min at 37 °C. The reaction was stopped by adding 500 µLof 10% TCA. This was left for 15 min at room temperature and centrifuged at 8000 rpm at 4 °C for 10 min. One milliliter of supernatant was added to 1 mL of 1 M NaOH and absorption was measured at 440 nm. One unit (U) of protease activity was defined as the amount of enzyme that liberated 1µmol of tyrosine per min at 37 °C (Olajuyigbe and Ajele, 2005).

***In vitro* antifungal activity of halophilic bacteria**

Cell free supernatant from each bacterium were obtained by centrifugation of broth culture of each bacterium and filtration throughout Millipore filter 0.22 µm diameter stored at -20 °C until use. The investigation of the bacterial effect on biomass fungal growth was tested on PDA plates after five

days incubation at 25 °C. Mycelium suspension in Tris-HCl buffer (0.01 M, pH 8), was incubated with each bacterial cell free supernatant. The mixture was incubated at 37 °C for 14 h. After that, optical density was measured at 540 nm. Increase of OD compared to control tube (containing only mycelial suspension), indicates destruction of fungal hyphae by cell free supernatant. A sample of 100 µL of the mixture was then directly observed by optical microscopy.

**Effect of halophilic bacteria on pathogen sporulation**

Spore germination assay was done by harvesting spores aseptically from 9 day old culture maintained on PDA, using sterilized distilled water. Antifungal effect of each sample was tested by using cavity slides. A 50 µL spore suspension adjusted to $10^6$ conidia $mL^{-1}$ and 50 µL of cell free supernatant were taken on separate cavity slides .These slides were incubated at 21±2 °C in moist chambers for 24 h. Each slide was then observed under the microscope for spore germination. The spores were enumerated and percentage of spore germination was calculated. One cavity slide was inoculated only with fungal spores. The percentage of spore germination inhibition I (%) was determined by microscopic examination of spores in the presence of the bacterial supernatant from each bacterium (E), compared to control tube containing only the spore suspensions. The percentage of spores germination was determined as; I (%): (C-E)/C × 100. Three replicates were used for each treatment (Essghaier *et al.*, 2009).

**Statistical analysis**

Mean separations were carried out based on LSD test made with test of multiple comparisons (PPDS test). Analysis were performed using logicial XLSTAT, test ANOVA. Data are reported in the text and figures as mean values for all replicated experiments ± standard error of the mean.

# Results and Discussion

**Effect of halophilic bacteria on the induction resistance of tomato plant**

During curative treatment, all biological treatment induced the production of total protein after ten days with maximum of induction was obtained with strain M2-26 (4547.5 (U $mg^{-1}$). However only *T. halophilus* strain J31 was able to maintain the same amount of protein throughout the curative treatment under different time (10, 20, 30 and 40 days after application of first treatment) with 3625 U $mg^{-1}$ of the harvested leaves. During the preventive treatment, only strain M3-23 was able to induce the production of total proteins during treatments with an optimum of 4994 U $mg^{-1}$ of the harvested leaves at 10 days.

**Effect of halophilic bacteria on the induction of the production of PR proteins in tomato**

Chitinase production measured ten days after biological treatment revealed a highly significant increase ($P<0.001$) of this enzyme in tomato leaves attacked only with the pathogen (C+) compared with untreated plant (Fig. 1). During curative treatment, the maximum chitinase production following treatments (from 10 to 40 days) was obtained with the biological treatment with strain J31, and value varying from 20,493 to 54,9 U $mg^{-1}$. In contrast, strain M2-26 did not induce chitinase (Fig. 1A). In preventive treatment, chitinase induction in tomato leaves was induced by all biological treatment tested and the highest value of chitinase was obtained at 40 days with the application of strain J31 with71,5 U $mg^{-1}$. However, at 20 days, the maximum chitinase production was recorded by following sprays with *V. marismortui* strain M3-23 (Fig. 1B).

For glucanase induction the results showed that during the curative treatment, the application of biological treatment was unable to induce glucanase production compared to untreated tomato. Unlike the preventive treatment, all bacteria were able to induce glucanase production in tomato (Fig. 2). Moreover, the maximum glucanase production was given by the application of strain M3-23 with value varying from 82,3 to 460.8 U $mg^{-1}$. The strain M2-26, strain J9 and strain J31 were able to induce glucanase production by tomato according to time application from 10 to 40 days. Also during preventive treatment, a significant production of glucanase was obtained in the tomato plants only infected by the pathogen *B. cinerea* and compared to healthy plants (C-) ( Fig. 2A). However, during the curative treatment, such induction was not detected according to the PPDS test.

The results showed a highly significant increase of protease production in tomato only infected by the pathogen compared with untreated plant. On the other hand, none of the biological treatment was able to induce protease production in tomato neither under curative or preventive treatment according to PPDS test.

**Comparison of the effectiveness of curative or preventive treatment**

Under preventive treatment, total protein and glucanase were induced more in tomato by the application of biological treatment compared to curative treatment as shown in Fig. 3. Chitinase production in tomato was induced both by preventive and curative treatments by the application of all biological solution tested (strains M3-23, M2-26, J9 and J31). However, there was no protease induction by halophilic bacteria. So the preventive treatment was more effective in the induction of resistance to the tomato plant compared with curative treatment. In the light of results obtained it would be interesting to conduct a preventive treatment in order to ensure

induction of resistance in tomato attacked by *B. cinerea*. The results obtained in this work, have also shown a difference between halophilic bacteria tested here. Strain M3-23 of *V. marismortui* was the best to induce PR-proteins in infected tomato since this strain was able to give the highest production of chitinase, glucanase and the total protein followed by strain J31. The result of halophilic bacteria effect on tomato growth was expressed as stem length in the preventive treatment were given in Table 1. The result has shown that the best tomato growth was obtained by the strain M3-23 of *V. marismortui* (Table 1). Data reported the effect of halophilic bacteria on reduction of disease on tomato plant is presented in Table 2, markedly showed the efficiency of the strain M3-23 to reduce disease both in curative and preventive treatment by comparison with other halophilic bacteria tested in this study.

The *in vitro* antifungal activities of halophilic bacteria tested *in vivo* on tomato plant were investigated to compare their efficiency against fungi. The effect of cell free supernatant of each halophilic bacteria used in the present work given in Table 3, shows the marked efficiency of all bacteria, but the strains *B. subtilis* J9 and *T. halophilus* J31were the most efficient. The morphological effect of such bacteria on mycelial destruction is demonstrated in the Fig. 4

Halophilic bacteria have been much studied for their ability to produce a variety of compounds of industrial interest (Prakasha *et al.,* 2012). However, few works investigated the ability of halophilic bacteria in biological control (Sadfi *et al*., 2002) and these have shown the effectiveness of halophilic bacteria to control gray mold of tomato (Sadfi-Zouaoui *et al.,* 2008a) and strawberries in storage conditions and in the field (Essghaier *et al.,* 2009; Essghaier *et al*., 2012). Various modes of action were mentioned by the antagonist such as antibiosis (Ramarathnam *et al*., 2007), antifungal enzymes (Dunlap *et al*., 1998), nutrient competition (Lepoivre, 2003) and the induction of resistance of plant (Essalmani and Lahlou, 2003). Our previous work has focused on the ability of halophilic bacteria to produce antibiotic and volatile compounds, antifungal enzymes as well as their ability to inhibit spore germination of the pathogen *in vitro* (Essghaier *et al.,* 2009, 2012). In the present work, we have confirmed the ability of these halophilic bacteria to induce plant resistance in tomato artificially infected by *B. cinerea* and to promote growth of tomato plant. Since the results have shown the efficiency of the tested halophilic bacteria to increase a total protein expressed in units per mg of leaves harvested. During the curative test, the most effective strain was strain J31 of *T. halophilus*. However, at a preventive treatment only strain M3-23 was able to induce the production of total proteins. In addition, during the curative treatment, all bacteria tested induced chitinase production and the highest production was shown by strain J31. Other studies have shown the ability of biological antagonists to induce systemic resistance in the plant through induction of chitinase activity, such as *Bacillus subtilis* by inducing chitinase activity in grapevine infected by *B. cinerea* (Trotel-Aziz *et al.,* 2008), Pseudomonas syringae can induce resistance of Chinese cabbage infected by *B. cinerea* by induction of chitinase (Daulagala and Allan, 2003). Other investigators also showed the ability of the pathogen *B. cinerea* to induce plant resistance leading to an increase in chitinase activity in the vine (Robert *et al*., 2002), which is in agreement with our results showing a net increase in chitinase in tomato leaves infected with *B. cinerea* and untreated compared with leaves unscathed. During the curative treatment, any strain was able to record a significant increase in glucanase activity compared with healthy plants. However, at preventive treatment, all the bacteria had managed to induce glucanase activity in the plant and the greatest activity was obtained from the leaves treated with strain M3-23. In addition, other studies have shown the ability of chemical treatments with S-methyl benzoic acid (BTH) to induce resistance by increasing the activity of glucanase and chitinase in rose leaves infected by *Diplocarpon rosae* (Suo *et al.,* 2001). In addition, exposure to UV-C radiation could induce a significant increase in glucanase activity in tomato fruits infested by *B. cinerea* (Charles *et al*., 2009). Previous researchers investigated the ability of halophilic bacteria to reduce or inhibit *Botrytis* growth but here we firstly determinate the effect of cell free supernatants from these bacteria on mycelial biomass reduction and destruction under *in vitro conditions.* The present work shows the efficiency of halophilic bacteria and their adverse affects on pathogen hyphal morphology and mycelia growth. On one hand, we can conclude that the strain M3-23 seems to be the most effective in inducing the high important activity threshold chitinase, glucanase and total protein. On the second hand, in light of the results obtained it would make sense to conduct a preventive treatment in order to ensure induction of resistance in tomato attacked by *B. cinerea* viewed as biological antagonists were able to increase much glucanase, chitinase and the total protein sensed in the leaves during the preventive treatment.

# Conclusion

Halophilic bacteria were able to induce resistance in tomato artificially infected by *B. cinerea* especially by the application of a preventive treatment. The enzyme activity induced the resistance in tomato and the promotion of growth has been attributed to *V. marismortui* strain M3-23. This work clearly demonstrated that these halophilic bacteria are good biocontrol candidates and further work is needed to more elucidate their modes of action

**Table 1:** Effect of halophilic bacteria after preventive treatment on tomato growth, expressed by stem length measured in mm.

| | Tomato stem length in mm | | | |
|---|---|---|---|---|
| | **T1** | **T2** | **T3** | **T4** |
| **C-** | 16,66 ± 1,5 | 20,33 ± 3,2 | 23,33 ± 3 | 29 ±2,6 |
| **C+** | 16 ± 0 | 15,75 ± 1,7 | 20 ± 0 | 22,5 ± 0,7 |
| **M3-23** | 15,33 ± 1,5 | 22,33 ± 0,5 | 29,33 ± 3,2 | 32 ± 3,6 |
| **M2-26** | 15,66 ± 2 | 16 ± 2.4 | 20,5 ± 3,5 | 29 ± 0.7 |
| **J9** | 16,5 ± 0,7 | 19 ± 0 | 23 ± 0 | 30.5 ± 0,7 |
| **J31** | 14,66 ± 2 | 17,66 ± 1,5 | 24,33 ± 3.2 | 28,33 ± 5 |

**C-:** Negative control (untreated plant), **C+:** positive control (plant artificially infected by *B. cinerea*), M3-23, M2-26, J31 and J9: infected plant by *B. cinerea* and treated by halophilic bacteria, respectively. M3-23, M2-26, J31 and J9. T1, T2, T3 and T4 means 10 d, 20 d, 30 d and 40 d: time of application after first treatment, respectively.

**Table 2:** Effect of halophilic bacteria on disease reduction expressed by lesion length after treatment of 40 days

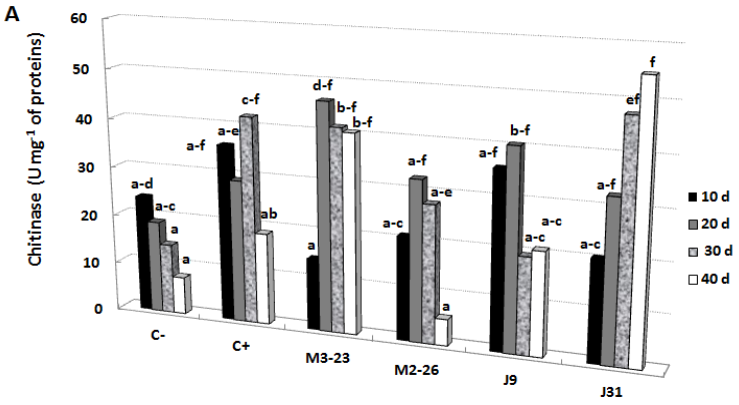
| | Length of the lesion (mm) | |
|---|---|---|
| **Treatment** | **Curative** | **Preventive** |
| **C+** | 60±1.3 | 28±0.7 |
| **M3-23** | 2±0.7 | 1.7±0 |
| **M2-26** | 13±0.5 | 9±1.5 |
| **J9** | 14±1.3 | 9±1.5 |
| **J31** | 6±0.9 | 5.4±0 |

C+: positive control (plant artificially infected by B. cinerea), M3-23, M2-26, J31 and J9: infected plant by *B. cinerea* and treated by halophilic bacteria respectively M3-23, M2-26, J31 and J9.

**Table 3:** Comparison of halophilic bacterial effect on *Botrytis cinerea* biomass and germination.

| | **Mycelia biomass inhibition ( %)** | **Mycelial destruction (UA mL$^{-1}$)** | **Spores inhibition ( %)** |
|---|---|---|---|
| **Control** | 0 ± 0 | 0 ± 0 | 0 ± 0 |
| **J9** | 83 ± 0 | 1.3 ± 0.15 | 95.8 ± 0.17 |
| **M3-23** | 66 ± 0 | 0.6 ± 0 | 0 ± 0 |
| **M2-26** | 65.5 ± 0 | 0.6 ± 0.13 | 57.4 ± 0.35 |
| **J31** | 63 ± 0 | 1.4 ± 0.22 | 98.5 ± 0.17 |

Control: Fungal growth without bacteria



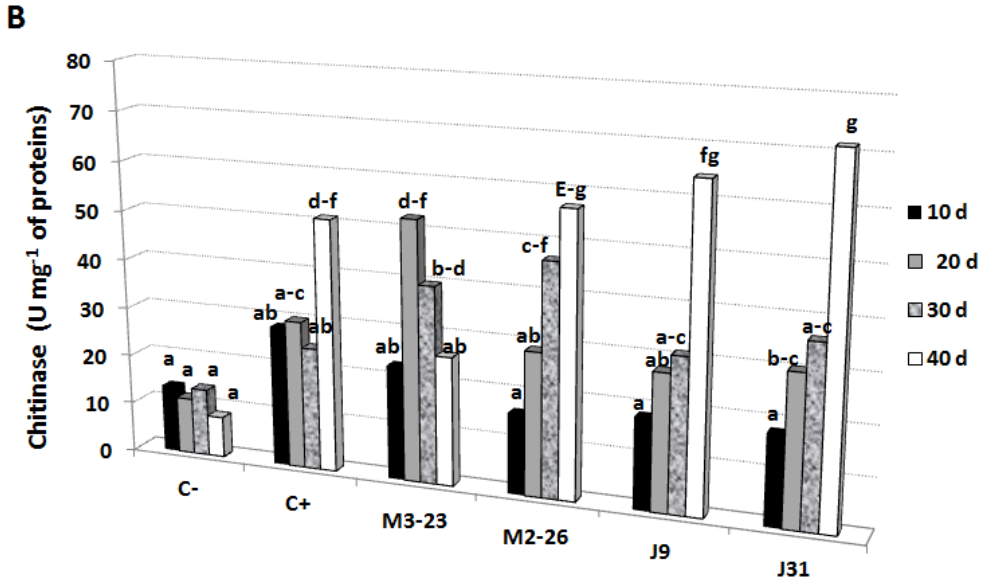



**Fig. 1:** Quantification of chitinase expressed in U $mg^{-1}$ in revolted leaves after curative (A) and preventive (B) treatment with the different halophilic bacteria as compared to controls
C-: Negative control (untreated plant); C+: positive control (plant artificially infected by *Botrytis cinerea*); M3-23, M2-26, J31 and J9: plant infected by *B. cinerea* and treated by *Virgibacillus marismortui* strain M3-23, *Planococcus rifitoensis* strain M2-26, *Terribacillus halophilus* strain J31 and *Bacillus subtilis* strain J9,respectively.; 10 d, 20 d, 30 d and 40 d: 10, 20, 30 and 40 days after application of the first treatment, respectively. For each timing, bars with the same letter did not differ significantly at P = 0.05 by PPDS test.

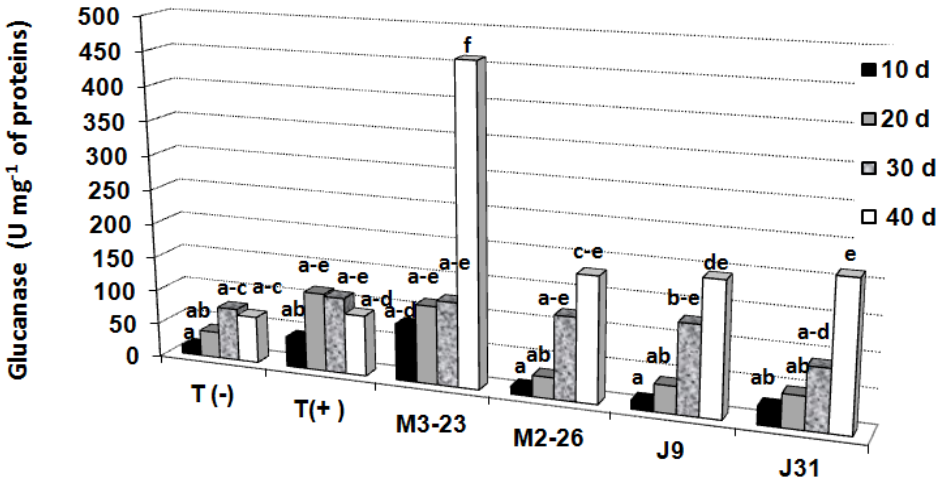



**Fig. 2**: Glucanase activities in revolted leaves after preventive treatment, expressed in U $mg^{-1}$.
C-: Negative control (untreated plant); C+: positive control (plant artificially infected by *Botrytis cinerea*); M3-23, M2-26, J31 and J9: plant infected by *B. cinerea* and treated by *Virgibacillus marismortui* strain M3-23, *Planococcus rifitoensis* strain M2-26, *Terribacillus halophilus* strain J31 and *Bacillus subtilis* strain J9, respectively; 10d, 20d 30d and 40d: 10, 20, 30 and 40 days after application of the first treatment. Bars show the standard errors of the means. For each timing, bars with the same letter are not significantly different according to LSD test at *P* = 0.05.

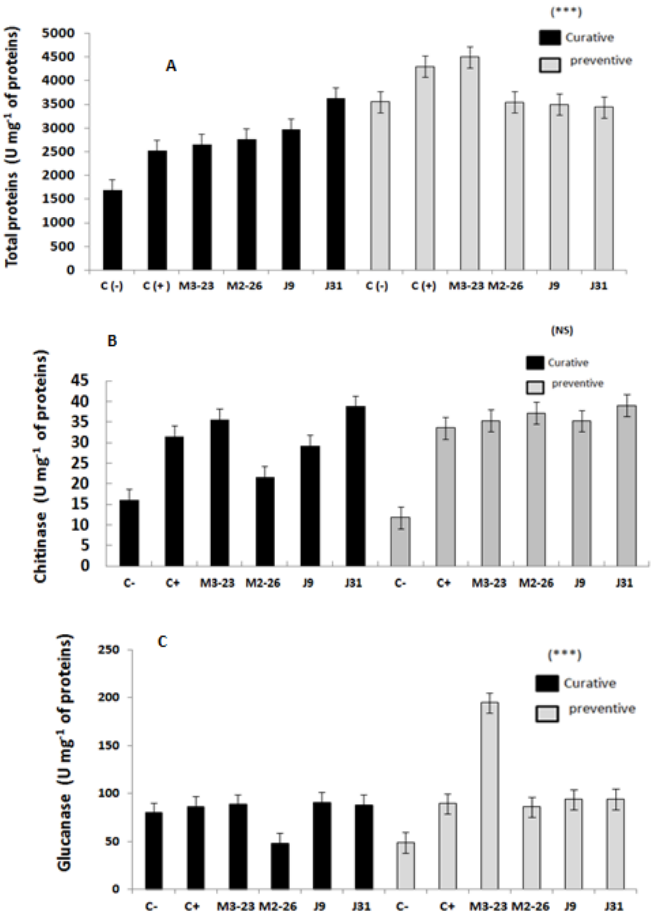



**Fig. 3:** Comparison of the efficiency of the curative and preventive treatments on tomato proteins induction, as measured by total proteins, chitinase and glucanase contents.
C-: Negative control (untreated plant); C+: positive control (plant artificially infected by *Botrytis cinerea*); M3-23, M2-26, J31 and J9: plant infected by *B. cinerea* and treated by *Virgibacillus marismortui* strain M3-23, *Planococcus rifitoensis* strain M2-26, *Terribacillus halophilus* strain J31 and *Bacillus subtilis* strain J9, respectively; 10d, 20d 30d and 40d: 10, 20, 30 and 40 days after application of the first treatment. Pr, cur: respectively the preventive and curative biological treatments. ***: Highly significant difference with $0.001<P<0.01$ and NS: not significant difference $P>0.05$ according to the test PPDS. Histograms presenting the same letter do not differ significantly as determined by the PPDS test.

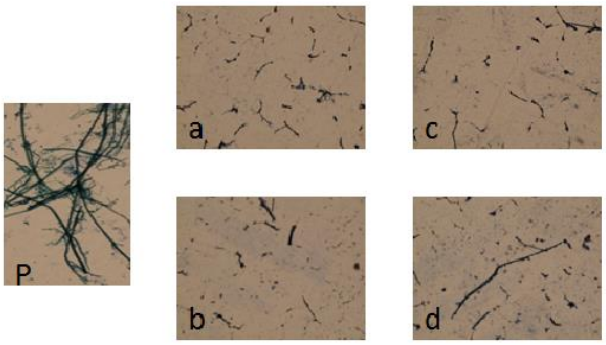



**Fig. 4:** Microscopic observations showing the effect of the cell free supernatant of tested halophilic bacteria on mycelia destruction of *Botrytis cinerea*.
P: Fungal mycelial growth without cell free bacterial supernatants a ,b, c and d: Fungal mycelial growth in presence of cell free supernatants of *Virgibacillus marismortui* strain M3-23, *Bacillus subtilis* strain J9,, *Terribacillus halophilus* strain J31, and *Planococcus rifitoensis* strain M2-26, respectively.

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