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Bacillus Induces Phenolic Compounds and Enhances Resistance to *Uncinula necator* Infection in Grapevine Leaves

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ABSTRACT

Thirty bacteria, identified as *Bacillus* sp., were tested for their biocontrol effects against *Uncinula necator in vivo* by using a leaf disc bioassay. Six bacteria were selected and tested in the greenhouse. Among these bacterial isolates B27 and B29 reduced disease development significantly compared to the untreated control. Isolates B27 and B29 revealed a mass disease index (MDI) of 50 and 60%, respectively, while the infected plants showed an MDI of 115%. From our study it also appears that isolate B27 has the advantage of influencing the host's response to pathogen attack. In fact, HPLC analysis demonstrated that B27 isolate induces the accumulation of phenolic compounds in grapevine leaves, particularly flavonoids, that are toxic to the pathogen.

Keywords: *Bacillus* sp., biological control, HPLC analysis, powdery mildew

INTRODUCTION

Grapevine (*Vitis vinifera*) is economically the most important fruit species globally because of the numerous uses of its fruit in the production of wine, juice, table grapes, dried fruit and organic compounds. Fungal pathogens are a major problem affecting grapevine yield either by a direct infection of berries or by a reduction of plant vigor (Pool *et al.* 1984; Gadoury *et al.* 2001). Gray mold caused by *Botrytis cinerea*, downy mildew caused by *Perenospora viticola* and powdery mildew caused by *Uncinula necator* are probably the most common and widely distributed diseases of grape vine throughout the world (Viret 1996). Powdery mildew is of economic importance in all grape-producing areas in the world (Gadoury *et al.* 1995; Ypema and Gubler 1997). Infected vines show generally low pruning weight, reduced winter hardiness of canes (Pool *et al.* 1984), low yield and reduced fruit quality, because of higher acidity, resulting often in the production of wines of lower quality. Control of *U. necator* through chemical sprays has been only partially successful (Ypema *et al.* 1997). The economic costs and negative environmental impact associated with these applications has led to a recent search for alternative strategies involving biological stimulation of host defense mechanisms (Ait Barka *et al.* 2002).

In fact, most biocontrol studies have focused on the use of the antagonistic fungus *Ampelomyces quisqualis* as a naturally occurring mycoparasite on both the anamorph and the teleomorph of many species of powdery mildew (Falk *et al.* 1995). Norton *et al.* (2000) showed that a mycophagous mite in the family *Tydeidae*, *Orthotydeus lambi* (Baker), can substantially reduce the development of a biotrophic fungal pathogen, *U. necator* (Schwein.) Burrill, the causal agent of grapevine powdery mildew. Considerable work has been conducted on the interaction of plant pathogenic fungi with antagonistic bacteria (Jones and Roane 1982; Walker *et al.* 1996). Bacteria have been shown to reduce fungal development *in vitro* and *in vivo* on *B. cinerea* in grape vine plants (Ait Barka *et al.* 2000, 2002). *Bacillus* is a bacterium with a strong antagonistic activity against many phytopathologic fungi although little is known about the effect of these bacteria on *U. necator*. Biocontrol bacteria may protect plants against pathogens by direct antagonistic interactions between the biocontrol agent and the pathogen, as well as by induction of the host resistance. The biocontrol depends on a wide variety of traits, such as the production by the biocontrol strain of various antibiotic compounds, iron chelators and exoenzymes such as proteases, lipases, chitinases, and glucanases (Krechel *et al.* 2002; Sessitsch *et al.* 2004); as well as competitive root colonization (Chin-A-Woeng *et al.* 2000; Lugtenberg *et al.* 2001), and induced resistance in the host plant (Trotel Aziz *et al.* 2008).

Resistance induction has been observed and suggested as an alternative control method in different pathosystems, such as wheat-*Bipolaris sorokiniana* and *Drechslera teres* (Bach 1997); coffee-*Hemileia vastatrix* (Guzzo *et al.* 1993) and pepper-*Phytophthora capsici* (Hwang *et al.* 1997). There are often large increases in phenolic synthesis in plants after infection with plant pathogens (Matern *et al.* 1995). Several studies have been published on the production of *trans*-resveratrol in vine plants and on its inevitable accumulation either in leaves or in grapes during several injuries (Paul *et al.* 1998; Lopez *et al.* 2001; Ferreira *et al.* 2004). Phenolic compounds are important constituents of plant cells and are associated with physiological defense against infection by bacteria, viruses and fungi (El Hadrami *et al.* 1997; Daayf *et al.* 2003).

In the current study 30 *Bacillus* isolates were studied for their antagonistic activity against *U. necator* on leaf discs. Among these isolates, six were selected and studied further for their biocontrol effects against powdery mildew development under greenhouse conditions. In order to elucidate at least in part, the mode of action of these bacteria *in vivo*, the effects of two of the most effective *Bacillus* iso-

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lates on the accumulation of phenolic compounds in grape leaves were investigated by using spectrophotometry and HPLC analysis. In this work we report that induced resistance, exemplified by the accumulation of phenolic compounds in grapevine leaves in the presence of some *Bacillus* isolates, may play a significant role in the biocontrol of powdery mildew.

MATERIALS AND METHODS

Fungi

A single spore isolate of *U. necator*, collected in 2002 from a vineyard in Takelsa (Nabeul, Tunisia) and maintained on seedlings of grape cv. 'Carignan' grown in a greenhouse, was used for all inoculations. This cultivar, which was used for all performed assays, was chosen for its availability.

Antagonists

Thirty bacteria were previously isolated from salty soils in the south of Tunisia and identified as *Bacillus* sp. (Sadfi *et al.* 2001). These bacteria were maintained on nutrient agar and stored at 4°C for further treatments. The bacterial inoculum was grown on nutrient growth medium by transferring two loops of each bacterium to 100 ml liquid medium in a 250 ml Erlenmeyer flask and incubating them at 28°C at 120 rpm for 24 h. Bacteria were collected by centrifugation (7000 × *g*, 5 min) and washed twice with sterile distilled water (SDW). The bacterial concentration was adjusted to 10^8 CFU/ml by using the serial dilution technique.

Leaf disc bioassay

A leaf disc bioassay was performed according to Ypema and Gubler (1997); leaves were collected from *Vitis vinifera* L. cv. 'Carignan' cuttings instead of seedlings. Fully expanded, 6-day old leaves were used to produce leaf discs. Detached grapevine leaves were surface sterilized by 30 s immersion in a 5% sodium hypochlorite solution and rinsed several times in SDW. 20 mm diameter discs were punched out of them. Leaf discs were immersed for 30 min in an aqueous bacterial solution and drained for excess liquid before being placed in Petri plates (9 cm diameter) lined with moistened paper towels. The remaining film of solution was allowed to dry overnight. Leaf discs were inoculated with conidia from one inoculum source of *U. necator* by means of a vacuum operated setting tower. This inoculation method provided a uniform inoculum density among the leaf discs. For each treatment three Petri dishes, each containing 7 leaf discs, were used. Inoculum density was determined with a hemacytometer and ranged from 600 to 700 conidia/cm^2. Leaf discs were incubated at 25°C and a 16 h/day photoperiod and a light intensity of 150 µE m^{-2} s^{-1}. Ten days after inoculation, leaf discs were scored for percent surface area colonized by *U. necator*. The percentage of inhibition at each bacterium was determined by comparison to water-treated, inoculated leaf discs. Isolates that did not show any potential for powdery mildew control were discarded. To confirm the results, each bacterium was evaluated at least twice.

Greenhouse assay

In order to confirm the results obtained in the leaf discs bioassay, six bacteria from the first group (B29, B27, B3, B8, B15 and B7) and three other bacteria that were shown to be effective in other plant pathogen interactions against *Fusarium oxysporum* f.sp. *albedinis* on date palm (BTX, X16 and 55T) (El Hassni *et al.* 2004) were tested in the greenhouse. Besides these bacteria, an isolate of *Trichoderma viridae* was also used in this assay to be tested for its ability to inhibit *U. necator* leaf colonization. Two controls were considered: inoculated and non-treated plants and plants inoculated and treated with the fungicide trifloxystrobin (Flint 50 WG, Bayer, Germany). The antifungal activities of these treatments against powdery mildew development were qualitatively and quantitatively examined in the present assay. Cuttings were obtained from grapevine cv. 'Carignan'. Rooted plants were placed in a greenhouse until they developed 2-4 leaves. These plants were placed in 10-L pots (containing sand and soil mixture, 2: 1, v/v) and four vines were placed in each pot. Plants were fertilized twice weekly with a 0.1%, 20/20/20 (N/P/K) fertilizer (Engrais Fruitier, Espace vert, Tunisia) solution.

The foliage of potted 'Carignan' vines was treated with a suspension of each bacterium. The concentration of inoculum for each isolate tested consisted of a propagule suspension prepared from pure cultures. The suspension was adjusted to a concentration of 10^8 propagules/ml in SDW. All plants were sprayed to run-off using an atomizer to deliver the inocula uniformly. A second treatment was applied 15 days later (8-10 leaf stage) with the same bacteria. Plants used as non-treated control were sprayed with SDW. Treated plants were allowed to air dry for approximately 1 hr before being moved to an isolate compartment in the greenhouse for 24 hr to allow for the establishment of the test microorganisms.

The vines were inoculated by dusting conidia from mildew colonies on previously infected leaves. After inoculation, the pots were placed in a greenhouse at 25°C to allow for the establishment of *U. necator.*

The incidence and severity of fungal infection were evaluated on foliage. Severity was estimated as the mass disease index (MDI): $MDI = \sum Xi*I/5$ (Ben Maachia 2001).

A scale was performed and modified in this work (adapted to the powdery mildew on grape) with 6 classes (i=0: 0%; i=1: 0-5%; i=2: 5-25%; i=3: 25-50%; i=4: 50-75%; i=5: 75-100%) where Xi was the number of leaves that have the index I and *U. necator* incidence was reported as the percentage of leaves infected.

Effect of bacteria on the accumulation of phenolic compounds

In this experiment, we tried to elucidate, at least in part, the mode of action of the two bacteria B27 and B29 *in vivo*. So, the experiment in the greenhouse was repeated with only the two selected bacteria, B27 and B29. The effect of bacterial treatment on the accumulation of total phenolic compounds was estimated using four treatments:

Treatment A: vines were not inoculated by *U. necator*, and not treated by bacteria.
Treatment B: vines were only inoculated by *U. necator* and treated by SDW.
Treatment C: vines were inoculated and treated by *Bacillus* B27.
Treatment D: vines were inoculated and treated by *Bacillus* B29.

Leaves were collected from the vines in each pot four times throughout the incubation period. At each sampling time, leaves were cut at random from the treated vines. Each collection of leaves was extracted for phenolic compounds. The first sampling was performed one day after the first treatment. The second sampling was performed three days after inoculation. The third sampling was performed two days after symptom appearance on the control vines. The fourth sampling was performed one day after the second treatment.

Extraction and analysis of soluble phenolics

Samples (1 g) were ground at 4°C in a mortar with 5 ml of methanol, and spun for 10 min at 10,000 rpm. These operations were repeated three times. The extracts were de-pigmented with petroleum ether and phenolics were extracted with ethyl acetate, evaporated to dryness and finally dissolved in methanol HPLC grade according to a previously described method (El Hadrami *et al.* 1997). Total phenolic level was determined using the Folin-Ciocalteu method as described by Macheix *et al.* (1990). Samples (200 µL, two replicates) were introduced into test tubes; 1.0 mL of Folin-Ciocalteu's reagent and 0.8 mL of sodium carbonate (7.5%) were added. The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured using a Perkin-Elmer ì15 UV-vis spectrophotometer (Norwalk, CT). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram fresh material.

Phenolic compound analyses were carried out using Waters HPLC equipment. 600E supplemented with a photodiode array

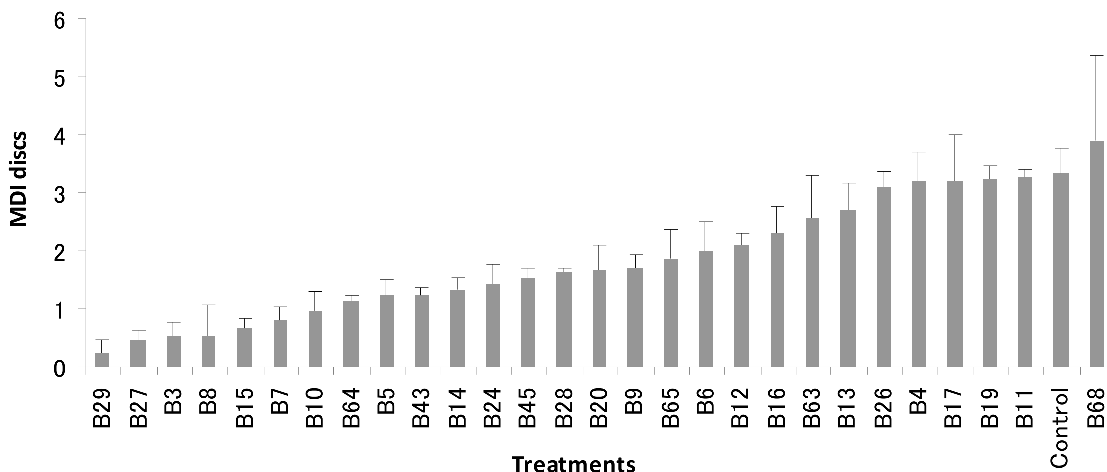

Fig. 1 Effect of biological treatment on the mass disease index (MDI) on discs of vine 10 days after the inoculation by *U. necator*. The control was treated with distilled water and inoculated with the fungus. Error bars are standard errors of means from three replicates.

detector Waters 990 and a Millipore software for data analysis. An efficient gradient of acetonitrile-*o*-phosphoric acidified bidistilled water (pH 2.6) was used on an Interchrom C18 reversed phase analytical column (4.6 mm × 250 mm, 5 m). Multiple channel wavelengths, including 280, 320 and 350 nm were used during the elution. Phenolics were identified on the basis of their retention times and their characteristic spectra in comparison with standards supplied by Sigma-Aldrich. When necessary, co-injection and elution with standards were used to ensure the identity of the compounds. Quantities of hydroxycinnamic acid derivatives and flavonoids were estimated respectively by measuring the area under the peaks and comparing it to chlorogenic acid and rutin used as external standards.

Many reference standards were used in this study such as *p*-coumaric, caffeic and ferulic acids and quercitin.

Statistical analysis

All the experiments schemes were randomized complete blocks. At least five replicates per treatment were performed. Data from all experiments conducted in this work were analyzed using analysis of variance (ANOVA) and the Statistica system (version 5, 97 Edn). The treatments were compared using the LSD analysis with a critical probability of $p = 0.05$.

RESULTS

Leaf discs bioassay

Based on the percentage of inhibition of fungal growth, 10 days after inoculation, leaf discs were scored for the MDI. Twenty-two of the 30 bacteria applied delayed disease progress significantly compared with disease progress in the inoculated control treatments (**Fig. 1**). Symptoms obtained from *U. necator* cultures treated with the first group of bacteria (B29, B27, B3, B8, B15, B7, B10) resulted in significantly ($P<0.001$) reduced disease development. Leaf discs are susceptible to fungal attack, and when inoculated with *U. necator*, the control produced characteristic symptoms within 7 to 10 days. In contrast, under the same inoculation and growth conditions, discs treated with this group of bacteria appeared healthy and exhibited only small surface necroses. The first group of bacteria (B29, B27, B3, B8, B15, B7, B10) shown a higher percentage of necroses reduction (from 71 to 92%). The last group of bacteria (B68, B11, B19, B17, B4, B26, B13) failed to provide protection. They had the same behaviour as the control (treated with SDW).

Greenhouse assay

In order to confirm the result obtained in the leaf discs bioassay, six bacteria from the first group were tested in the greenhouse (B29, B27, B3, B8, B15 and B7) with other bacteria that showed to be functional in other interactions, namely plant-pathogens BTX, X16 and 55T. An isolate of *Trichoderma viridae* was used in this assay to test its ability to inhibit *U. necator* leaf colonization. Two controls were used: plants inoculated and non-treated and plants inoculated and treated with fungicides (trifloxystrobin). The antifungal activities of these bacteria against powdery mildew examined in the present assay and their potency were qualitatively and quantitatively assessed by the pre-

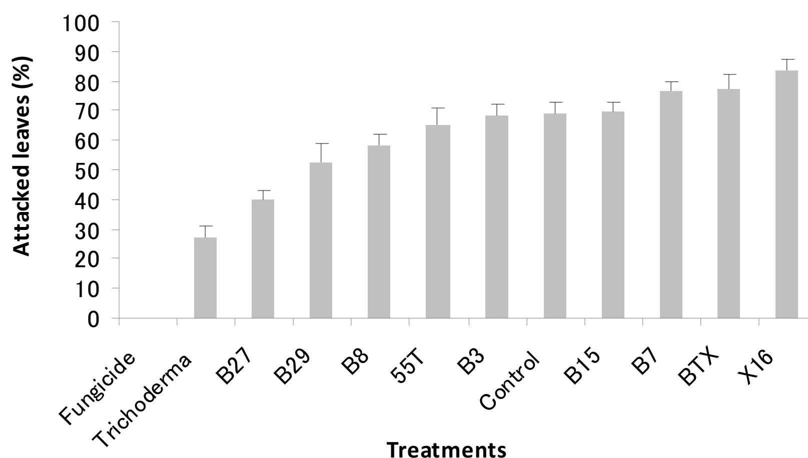

Fig. 2 Effect of biological treatments on the % of attacked leaves of grapevine inoculated by *Uncinula necator*. The control was treated with distilled water and inoculated with the fungus. Fungicide was the control treated with trifloxystrobin and inoculated with the pathogen. Error bars are standard errors of means from five replicates.

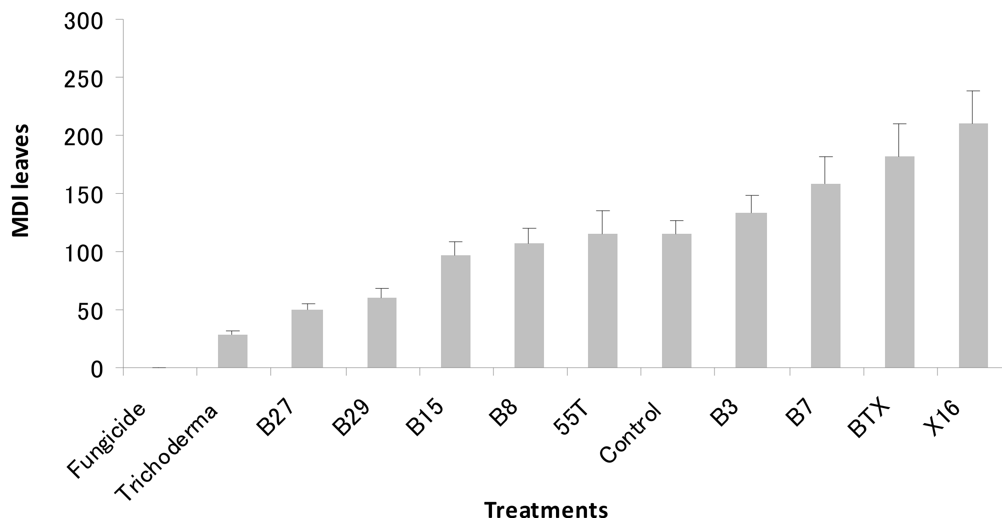

Fig. 3 Effect of biological treatment on the MDI on leaves of vine inoculated by *U. necator*. The control was treated with distilled water and inoculated with the fungus. Fungicide was the control treated with trifloxystrobin and inoculated with the pathogen. Error bars are standard errors of means from five replicates.

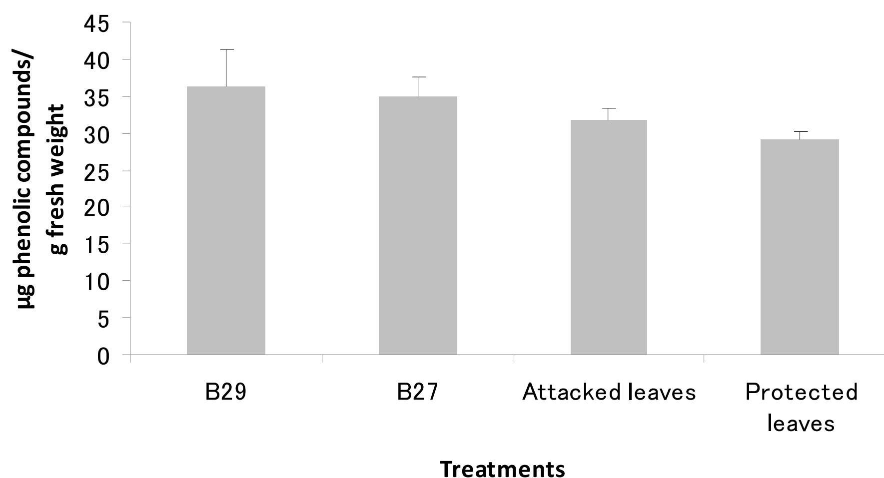

Fig. 4 Effect of biological treatments on the accumulation of total phenolic compounds in vine leaf tissue in control non inoculated (protected leaves), in control inoculated (attacked leaves), in leaves treated by the bacterium B29 and in leaves treated by the bacterium B27. Bars represent the standard error (mean ± S.E., n=3).

sence or absence of inhibition of the development of the fungus on leaves. The importance of this development is also discussed. The results appear in **Figs. 2** and **3**. The results show that the two bacteria (B27 and B29) and the *Trichoderma viridae* isolate have an antifungal activity against *U. necator*. The difference was mainly expressed as greater reduction in disease incidence (**Fig. 2**) and severity (**Fig. 3**) on leaves than the other bacteria, although both bacteria (B27 and B29) reduced disease development significantly, compared with untreated controls. The percentage of protection was over 60, 42 and 23% in plants treated with *Trichoderma*, B27 and B29, respectively compared with the infected plants (**Fig. 2**). The treatment by the two bacteria (B27 and B29) provided far better disease control on leaves than those treated by the other bacteria (B8, 55T, B3, B15, B7, BTX and X16; **Figs. 2**, **3**).

The bacterium B15 reduced disease severity on leaves (**Fig. 3**), but failed to provide satisfactory protection (**Fig. 2**). In fact, B15 failed to protect leaves from pathogen attack, and > 70% of leaves were infected by the fungus. The severity of disease development on vine leaves was pronounced when *U. necator* was inoculated alone (control treatment). However, *T. viridae* limited disease severity substantially (**Fig. 2**). The bacteria B7, BTX and X16 seemed to increase the infection and development of the pathogen. Indeed, an increase of severity and the impact of the disease were noted after treatment with these bacteria.

The efficacy of the fungicide trifloxystrobin in controlling powdery mildew on leaves of grapevine was significantly superior to all provided by the other biological treatments. No development of the fungus was noted on grapevines treated with this fungicide.

Effect of bacterial treatment on the accumulation of phenolic compounds

1. Total amount of phenolic compounds

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives. In many cases, these substances serve as plants defense mechanisms against predation by microorganisms.

Based on the absorbance value of the methanol extract solution, reacted with the Folin-Ciocalteu reagent, the amount of total phenolics was not significantly different. All the treatments showed a similar pattern of phenols. The amount of total phenolics varied widely in grapevine leaves and ranged from 0.2 to 0.45 mg GAE/g dry material. At the end of the period studied, the plants treated with the two bacteria (B27 and B29) showed a change in the pattern of accumulation, accompanied by a small increase in response to inoculation, in both cases (**Fig. 4**).

2. Identification of phenolic compounds by HPLC

Total phenolic compounds, measured by the Folin-Ciocalteu method, only gives an estimate of the phenolic content. It does not separate nor does it give a quantitative measurement of compounds. That is why these extracts were analyzed by HPLC at three different wavelengths with the diode array detector. The hydroxybenzoic acid derivatives, flavan-3-ols and dihydrochalcones were quantified at 280 nm, hydroxycinnamic acid derivatives at 320 nm and the flavonols at 360 nm.

A preliminary study was devoted to the optimization of chromatographic conditions to obtain good separation of these compounds within a short analysis time. Using acetonitrile as solvent, the major phenolics were identified by their retention times and characteristic spectra. HPLC studies point to the presence of caffeic, *p*-coumaric and ferulic acids and quercetin derivatives (**Fig. 5A**). No hydroxybenzoic acid derivatives, flavan-3-ols and dihydrochalcones were found. Thus, hydroxycinnamic acids were the major phenolic compounds in the samples tested. The inoculation of grapevine leaves with *U. necator* resulted in a quantitative change of the phenolic pool of these plants, enhancing the accumulation of hydroxycinnamic acids (**Fig. 5B**). The significant accumulation of hydroxycinnamic acids is observed especially for caffeic and ferulic acids. The effect of the inoculation of plants by powdery mildew and the development of symptoms was the induced accumulation of coumaric acid derivatives. However, phytoalexin production by vine leaves such us resveratrol has been studied, and it was noted that diseased tissue did not shown any induced compounds (**Fig. 5**).

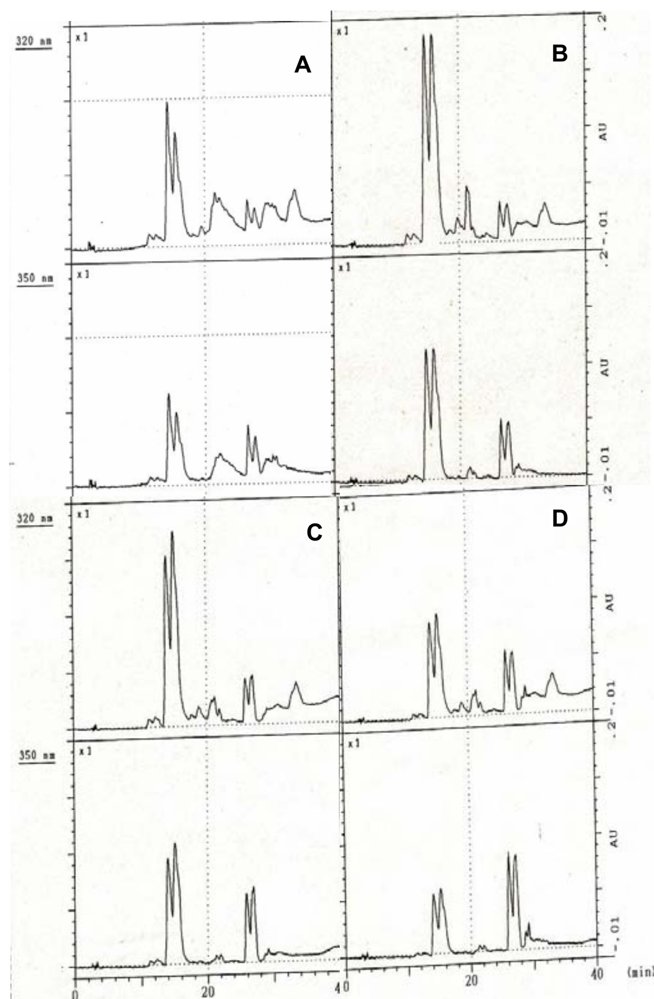

Fig. 5 HPLC profiles of grapevine leaves phenolic compounds. Non inoculated control (A), *U. necator* inoculated control (B), Inoculted and treated with B29 (C), Inoculated and treated with B27 (D).

The accumulation of ferulic and caffeic acids is observed with the same amplitude in the leaves treated with the B29 isolate and inoculated by the fungus, but they do not shown any disease symptoms. Further, the level detected of coumaric acid derivatives is less than in the control showing powdery mildew development (**Fig. 5C**).

A large increase of quercetine derivatives was recorded after treatment with the B27 isolate (**Fig. 5D**) compared to the other treatments and to the controls.

Figs. 6 and **7** show the major phenolics identified and quantified in the leaves. The phenolic content in the leaves was calculated on the basis of the results of the addition of total hydroxycinnamic acid and total flavonoids. There was considerable variability in flavonoid concentration in leaves treated with B27, B29 and the controls (**Fig. 6**). Remarkable differences were also observed for hydroxycinnamic acid content in the same samples (**Fig. 7**). However, it has to be noted that leaf samples treated with B27 accumulated more flavonoids whereas control leaves accumulated more hydroxycinnamic acids.

DISCUSSION

Gram-positive bacteria, especially *Bacillus* species, have received much attention as effective biological control agents and have some formulation advantages over Gram-negative bacteria (Emmert and Handelsman 1999; Schisler *et al.* 2004). In this study, 29 bacterial strains have been tested, 6 isolates with high biocontrol activity against *U. necator* were selected by using leaf discs assay from grapevine cv. 'Carignan'.

It is generally recognized that expression of antagonism by a microorganism towards a pathogen *in vitro* cannot be regarded as evidence that the microorganism will control the pathogen in the field (Reddy and Hynes 1994). In contrast, results obtained in the present study indicated a correlation between antagonisms recorded *in situ* and the effectiveness of these *Bacillus* at the development of powdery mildew on grape leaves in greenhouse trials.

On the *in planta* grapevine test, two bacteria, B27 and B29, seemed to be most effective against this pathogen fungus. The bacteria used in our study are all isolated from soil. Many *Bacillus* spp., such as *B. subtilis*, are considered ubiquitous in soil and can protect against fungal pathogens (Asaka and Shoda 1996; Emmert and Handelsman 1999; Trotel-Aziz *et al.* 2008). *Bacillus* spp. have been reported to be effective in the biocontrol of multiple plant diseases owing to their production of several broad-spectrum antibiotics and their longer shelf lives as a result of their ability to form endospores (Emmert and Handelsman 1999). Mechanisms contributing to disease control by microbial agents include direct antagonistic mechanisms and/or induced resistance in the host plant (Trotel-Aziz *et al.* 2008). The antifungal activity of these strains is probably due to the presence of extracellular compounds, perhaps polymers having functions of cellular aggregation, inhibition of germination, and lengthening of the germinative hyphae (Mari *et al.* 1996). In fact, an *in vitro* agar plate pairing assay was used to determine if the *Bacillus* were directly antagonistic to *B. cinerea*; these isolates showed high inhibition ability toward this fungus (data not shown). It is also possible that these bacteria induced plant defense products. Melnick *et al.* (2008) demonstrated that different *Bacillus* strains had the ability to colonize cacao leaves. They were also able to establish long-term colonization and to induce the ISR (induced systemic resistance) phenomena in plants.

The *Trichoderma* isolate seems to be effective against powdery mildew in controlled conditions. Similar results have been obtained *in vitro* with *T. harzianum* on *B. cinerea* by Kapat *et al.* (1998). Further, biocontrol agents are usually considered to control only a narrow range of pathogens, sometimes only a single strain, and even then they may be effective only under localized conditions. *Trichoderma* is considered to be an effective antagonistic fungus against many plant pathogenic fungi including *B. cinerea*, *Crini-*

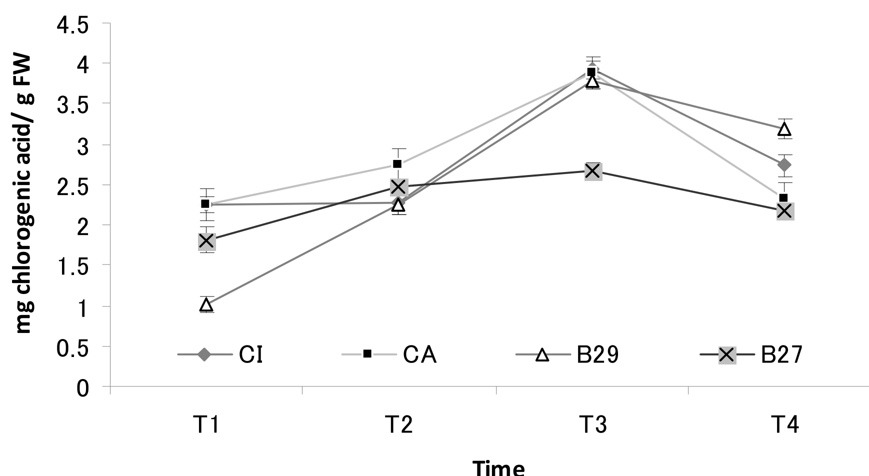

Fig. 6 Effect of biological treatments on the accumulation of hydroxycinnamic acids in vine leaf tissue in control non inoculated (CI), in control inoculated (CA), in leaves treated by the bacterium B29 and in leaves treated by the bacterium B27. Bars represent the standard error (mean ± S.E., n=3).

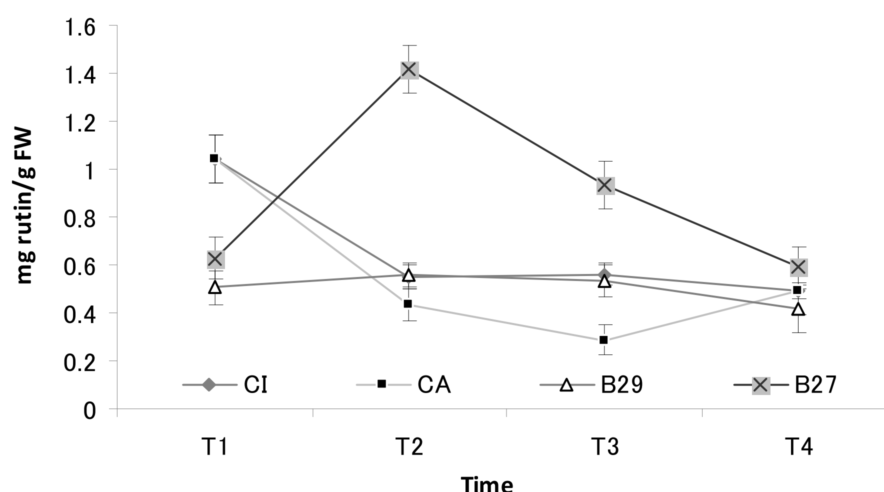

Fig. 7 Effect of biological treatments on the accumulation of flavonoids in vine leaf tissue, in control non inoculated (CI), in control inoculated (CA), in leaves treated by the bacterium B29 and in leaves treated by the bacterium B27. Bars represent the standard error (mean ± S.E., n=3).

pellis perniciosa, and soil-borne fungi such as *Rhizoctonia*, *Sclerotinia*, *Pythium* and *Fusarium* (Bastos 1996; Conney and Lauren 1998). Furthermore, *Trichoderma* spp. was used against Botrytis bunch rot on grape (Harman *et al.* 1996) and against powdery mildew [*Erysiphe* (Sect. *Microsphaera*) *pulchra*] in *Cornus florida* (Mmbaga *et al.* 2008).

Phenolic compounds are among the most widely distributed secondary products in the plant kingdom. Since they are known to accumulate in response to infection in some species, it has also been suggested that they play a potential role in disease resistance (Gayoso *et al.* 2004).

Hydroxycinnamic acids are the precursors of the cellular support material lignin in plants, but they also provide defense against plant pathogens (Dixon and Paiva 1995) and other stress factors such as wounding (Housti *et al.* 2002) and intense solar radiation (Jaakola *et al.* 2004). Hydroxycinnamic acids such as caffeic acids are common representatives of a wide group of phenyl-derived compounds, which are in the highest oxidation state. Hydroxycinnamic acids are effective against viruses, bacteria and fungi (Brantner *et al.* 1996) and have also been shown to increase in response to injury and infection (Strange *et al.* 2001). Researchers studying other fungi have recently reported that caffeic acid reduced the production of cutinase by *Monilinia fructicola in vitro*, but did not affect fungal growth (Bostock *et al.* 1999). This compound as well as the other hydroxycinnamic acids is produced following the appearance of symptoms and therefore following the propagation of the fungus inside the host plant. Flavonoid compounds are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. Since, they are known to be synthesized by plants in response to microbial infection (Kortekamp 2006) it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins. In fact, in addition to providing a source of stable free radicals, flavonoids are known to complex irreversibly with nucleophilic amino acids in proteins, often leading to inactivation of the protein and loss of function. For that reason, the potential range of flavonoid antimicrobial effects is great (Cowan 1999). Moreover, Kolb *et al.* (2001) observed a high concentration of hydroxycinnamic acids in grape leaves exposed to visible light. However, biosynthesis of kaempferol and quercetin was specifically increased by UV-B radiation.

The results obtained in this investigation are in agreement with previous research for flavonoids and hydroxycinnamic acids in leaves (Kortekamp 2006). On the other hand, findings of this study provide deeper knowledge regarding the content of these antioxidants in grape products.

Phytoalexin production by vine leaves has been studied, and it was noted that diseased tissue did not show any induced compounds. Phenolic-based defense responses are characterized by an early accumulation of phenolic compounds at the infection site. It is thought that rapid accumu-

lation of toxic phenols may result in the effective isolation of the pathogen at the original site of entrance (Fernandez and Heath 1998). These defense mechanisms appear to be important determinants affecting the host response, and may be possible contributing factors to the resistance mechanism in vine. These results were obtained with the bacterium B27, which accumulates a significant amount of flavonoids from the second sampling time (three days after inoculation). On the other hand, the other treatments (controls and leaves treated by the bacterium B29; **Fig. 6**) show an accumulation of hydroxycinnamic acids at the third sampling time (two days after symptoms appearance on the control vines).

These studies demonstrate a great potential for the utilization of bacteria as an alternative to fungicides in plant disease management. In fact, plants are endowed with diverse mechanisms that protect them from pathogenic microorganisms. The bacterium B27 has the advantage of influencing the host's response to pathogen attack. The two bacteria (B27 and B29) stopped the development of the fungus. They have the ability to protect grapevine plants against *U. necator*. B27 was more efficient than B29, and seemed to inhibit the growth of the fungus by secreting toxic substances. All these results tend to show that the plant reacts to treatment with the bacterium B27 and produces a high amount of flavonoids, which seems to be toxic to the fungus.

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