Antagonism of *Bacillus subtilis* strain AG1 against vine wood fungal pathogens

Antonio ALFONZO, Gaetano CONIGLIARO, Livio TORTA,
Santella BURRUANO and Giancarlo MOSCHETTI

Dipartimento S.En.Fi.Mi.Zo., Sez. Patologia vegetale e Microbiologia agraria, Viale delle Scienze, 90128,
Università degli Studi, Palermo, Italy

Summary. Antagonistic substances produced by a *Bacillus subtilis* strain (AG1), which were previously found to slow down the growth of esca fungi *in vitro*, were produced in an artificial medium, isolated from the cell-free medium by precipitation and acidification (to less than pH 2.5) and extracted from the precipitate with 96% ethanol. The crude extract employed in antibiotic assays confirmed, *in vitro*, the antagonism of *B. subtilis* against *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*, and also showed antifungal activity toward *Verticillium dahliae* and *Botryosphaeria rhodina*.

Key words: grapevine, tracheomycosis, biological control, antibiosis.

Grapevine trunk diseases cause considerable losses in the wine industry worldwide. Symptoms of wood diseases are dead spurs, arms, cordons and sometimes entire vines, due to the wood colonization by various wood pathogens (Larignon *et al.*, 1997; Mugnai *et al.*, 1999; Fischer *et al.*, 2003; Wood *et al.*, 2005; Surico *et al.*, 2006; Van Niekerk *et al.*, 2006; Bonfiglioli *et al.*, 2007; Zanzotto *et al.*, 2007; Burruano *et al.*, 2008).

Since there is no direct control of these diseases, new ways must be found to control them.

Recent investigations on esca in Sicilian vineyards found that the genus *Bacillus* occurred in grapevine wood tissue. A spore-producing Gram-positive bacterium isolated from black dots on grapevine wood, named AG1, was identified as *Bacillus subtilis* subsp. *subtilis,* on the basis of a partial 16S rRNA sequence comparison. This species has a well know activity as an antagonist of many plant pathogens, including the agents of grapevine trunk diseases (Ferreira *et al.*, 1991; Romero *et al.*, 2007; Leelasuphakul *et al.*, 2008). Moreover, in direct and indirect assays, this strain was antagonistic against the well-known tracheomycotic pathogens of grapevine such as the main agent of esca of grapevine, *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora* (Alfonzo *et al.*, 2008).

As part of a program aimed at confirming and elucidating the mechanism of the antagonism, the AG1 crude metabolite extract was also tested against some other pathogenic fungi of grapevine.

The bacterial strain used was originally isolated on malt agar (MA), and maintained on the same medium at 4°C. The antibiotic substance(s) produced by *B. subtilis* were extracted according to the method of McKeen *et al.* (1986), with some modification.

Bacterial cultures were plated out on MA and incubated at 28°C for 1 day. Loopfuls of the bacterial colony were streaked in tubes containing po-

Corresponding author: S. Burruano
Fax +39 091 7028855
E-mail: santella@unipa.it

tato-dextrose agar (PDA) and incubated for 24 h at 28°C. After 1 day a loopful of the colonies obtained was suspended in a peptone water solution (0.1% w:v), vortexed and inoculated in flasks containing an antibiotic production medium composed of 20 g l^{-1} malt extract broth (MEB), with pH adjusted to 6.0–6.2 with 5 N NaOH. The medium was dispensed to 300 ml Erlenmeyer flasks (100 ml per flask) and autoclaved at 120°C for 20 min.

The inoculated flasks were incubated on a shaker at 170 rpm and 28°C for 3 days and then the medium was centrifuged for 20 min at 16,500 g to remove the cells. To inactivate the *B. subtilis* spores, the supernatant was sonicated at 20 KhZ for 300 seconds and the antibiotics were precipitated, adjusting the pH to 2.5 with concentrated HCl. This material was centrifuged for 10 min at 16,500 g and the pellet containing the active fraction was extracted three times with 96% ethanol. The ethanol extract was dried under vacuum at 45°C in a rotary evaporator. Inactive substances were removed by sequential extraction with ethyl acetate and acetone according to the method of McKeen. The resulting residue containing the active substances was dissolved in 96% ethanol, designated as crude metabolite extract (CMe), and stored in capped bottles at 4°C.

The antagonistic activity of *B. subtilis* CMe was assayed against the following grapevine pathogens: *P. aleophilum*, *P. chlamydospora*, *V. dahliae* and *B. rhodina*. All isolates were previously grown on MA at 28°C.

The agar diffusion technique of Villani *et al*. (1995), with slight modifications, was employed to quantify the bio-activity of CMe against the phytopathogenic strains.

After sporulation, the spores of each pathogen were harvested in a peptone water solution (0.1% w: v) to give a concentration of approximately 10^6–10^7 spores ml^{-1}. Aliquots (0.1 ml) of each suspension were spread in 60 mm Petri dishes (one aliquot per dish) and overlaid with 10 ml of MA. After solidification, wells (Ø 6 mm) were bored in the MA plates.

To detect the inhibition zone, 50 μl of twofold serial dilutions of CMe were pipetted into the wells and incubated at the optimal growth temperatures of each pathogen (28°C for *P. aleophilum*, *P. chlamydospora*, and *B. rhodina*; 25°C for *V. dahliae*) for seven days. The titre of the CMe in Arbitrary Units (AU) ml^{-1} was expressed as the reciprocal of the highest dilution indicating a definite zone of inhibition (>1mm).

In order to evaluate the stability of CMe, enzymatic and thermal tests were carried out. CMe was treated with the following enzymes (all from Sigma Chemical Co, Brooklyn, NY, USA), dissolved in distilled water at a concentration of 1 mg ml^{-1}: trypsin (pH 8.1), pepsin (pH 1.5), pronase (pH 7.5), α-chymotrypsin (pH 7.5), papain (pH 6.5), α-amylase (pH 6.1) and proteinase K (pH 8.5). The pH of distilled water was adjusted (by NaOH or HCl) to the pH indicated for the optimum activity of each enzyme. Antifungal metabolites (1 mg ml^{-1}) were added to the enzyme solutions and incubated for 2 h at various temperatures, depending on the type of enzyme (37°C for trypsin, pepsin, α-chymotrypsin, α-amylase and proteinase K; 40°C for pronase; 65°C for papain; Munimbazi *et al*., 1998). The controls consisted of CMe without enzymes, and only enzyme solutions, without CMe.

The residual activity of CMe was evaluated by the agar diffusion technique mentioned above, using colonies of *P. chlamydospora*.

The effect of temperature on metabolite activity was tested in a range of 4–120°C (4, 20, 40, 60, 80, 100, 120°C) for 20 min and the remaining activity was assayed using the technique on *P. chlamydospora* as described above.

The techniques of production and extraction of the antibiotic substance(s), used in the study yielded about 150 mg l^{-1} of crude metabolite extract of *B. subtilis*.

The antagonistic assays indicated that metabolites of AG1 inhibited mycelial growth (AU ml^{-1}) of all the pathogens tested (Fig. 1). *B. rhodina* was the most sensitive to CMe, followed in order by *V. dahliae, P. chlamydospora* and *P. aleophilum* (Table 1).

The enzymatic and thermal tests carried out to elucidate the chemical nature of the active compounds of CMe showed that it was stable at high temperature (60–120°C; Fig. 2) and resistant to enzymatic degradation, which are well known features of polypeptide antibiotics produced by *Bacillus* spp. (Romero *et al*., 2007; Leelasuphakul *et al*., 2008).

Studies to characterise the crude extract chemically and to identify the molecules involved in bioactivity are in progress.

The determination of the antagonistic mechanism and of the toxicity of the secondary metabolites of AG1 will enable this strain to be employed in low-impact defensive strategies.
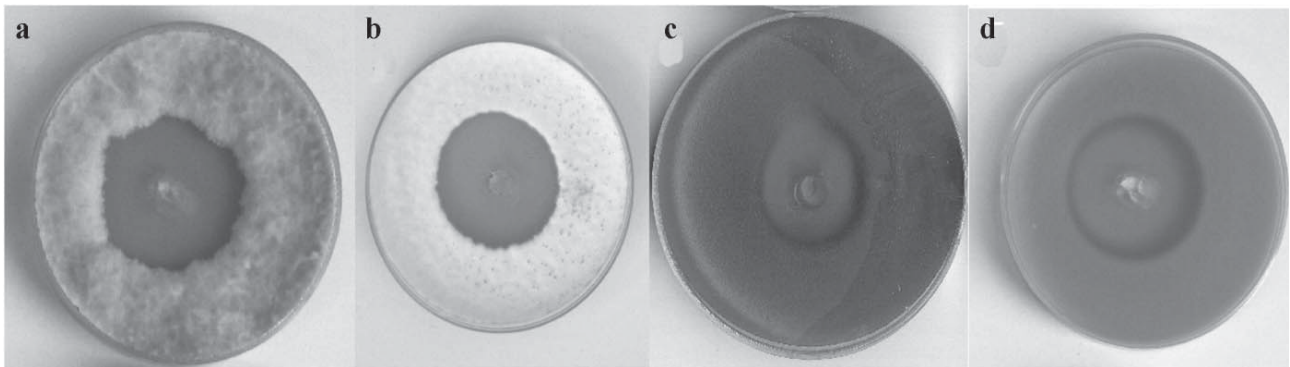
Fig. 1. Antifungal effect of crude metabolite extract (CMe) from *Bacillus subtilis* AG1 on: a, *Botryosphaeria rhodina*; b, *Verticillium dahliae*; c, *Phaeomoniella chlamydospora,* and d, *Phaeoacremonium aleophilum*.

Table 1. Indicator strains and their sensitivity to crude metabolite extract as determined by the critical dilution assay.

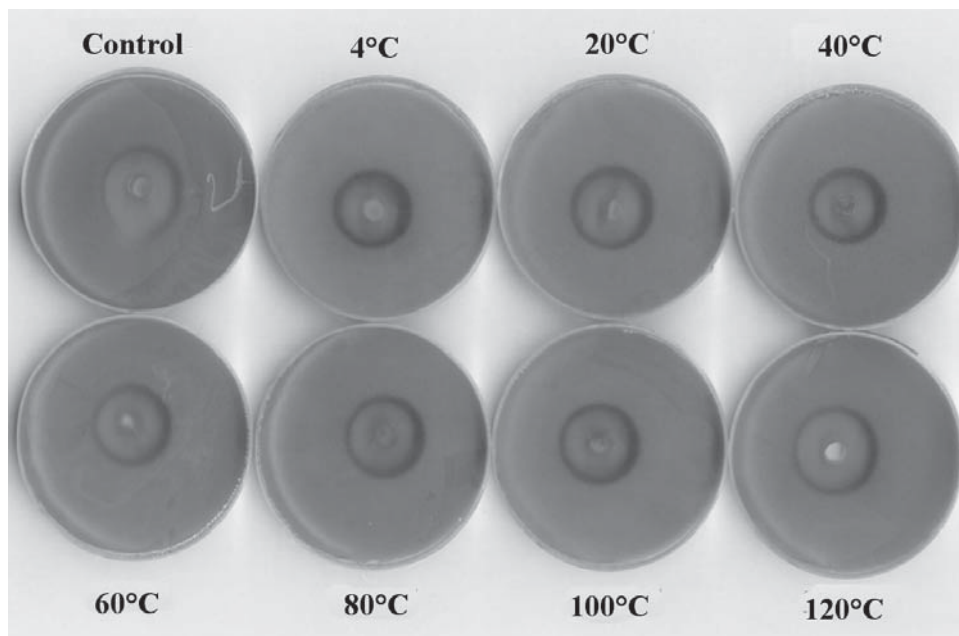| Indicator fungal strain	Mycelial growth inhibition (AU ml^{-1})
Phaeoacremonium aleophilum	440
Phaeomoniella chlamydospora	480
Botryosphaeria rhodina	4480
Verticillium dahliae	960

Fig. 2. Stable activity of crude metabolite extract (CMe) against *Phaeomoniella chlamydospora* at various temperatures. Note the resistance to high temperatures (60–120°C).

Literature cited

Alfonzo A., V. Ventorino, L. Torta, S. Burruano and G. Moschetti, 2008. *In vitro* antagonism of a grapevine endophytic *Bacillus subtilis* strain towards "esca" fungi. *Integrated Protection in Viticulture IOBC/wprs Bullettin* 36, 19–24.

Bonfiglioli R. and S. McGregor, 2007. *Verticillium* wilt in New Zealand vines. *The Australian & New Zealand Grapegrower & Winemaker*, 30–31.

Burruano S., V. Mondello, G. Conigliaro, A. Alfonzo, A. Spagnolo and L. Mugnai, 2008. Grapevine decline in Italy caused by *Lasiodiplodia theobromae. Phytopathologia Mediterranea 2, 132–136.*

Ferreira, J. H. S., F. N. Matthee, and A. C. Thomas, 1991. Biological control of *Eutypa lata* on grapevine by an antagonistic strain of *Bacillus subtilis. Phytopathology* 81, 283–287.

Fischer M. and H.H. Kassemeyer, 2003. Fungi associated with Esca disease of grapevine in Germany. *Vitis* 42(3), 109–116.

Larignon P. and B. Dubos, 1997. Fungi associated with esca disease in grapevine. *European Journal of Plant Pathology* (103), 147–157.

Leelasuphakul, W., P. Hemmanee and S. Chuenchitt, 2008. Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. *Postharvest Biology and Technology* 48(1), 113–121.

McKeen C.D., C.C. Reilly and P.L. Pusey, 1986. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis. Phytopathology* 76, 136–139.

Mugnai L., G. Graniti and G. Surico, 1999. Esca (black measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant Disease* 83(5), 404–418.

Munimbazi C. and L.B. Bullerman, 1998. Isolation and partial characterization of antifungal metabolites of *Bacillus pumilus. Journal of Applied Microbiology* 84(6), 959–968.

Romero D., A. De Vincente, R.H. Rakotoaly, S.E. Dufour, J.W. Veening, E. Arrebola, F.M. Cazorla, O.P. Kuipers, M. Paquot and A. Pérez-García, 2007. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* towards *Podosphaera fusca. Molecular Plant-Microbe Interaction* 20(4), 430–440.

Surico G., L. Mugnai and G. Marchi, 2006. Older and more recent observations on esca, a critical overview. *Phytopathologia Mediterranea* 45 Supplement, S68–S86.

Van Niekerk J.M., P. Fourie, F. Halleen and P.W. Crous, 2006. *Botryosphaeria* spp. as grapevine trunk disease pathogens. *Phytopathologia Mediterranea* 45, supplement S43–S54.

Villani F., O. Pepe, G. Mauriello, G. Salzano, G. Moschetti and S. Coppola, 1995. Antilisterial activity of thermophilin 347, a bacteriocin produced by *Streptococcus thermophilus. International Journal of Food Microbiology* 25(2), 179–190.

Wood P.M. and C.E. Wood, 2005. Cane dieback of dawn seedless table grapevines (*Vitis vinifera*) in Western Australia caused by *Botryosphaeria rhodina. Australasian Plant Pathology* 34(3), 393–395.

Zanzotto A., F. Autiero, D. Bellotto, G. Dal Cortivo, G. Lucchetta and M. Borgo, 2007. Occurrence of *Phaeoacremonium* spp. and *Phaeomoniella chlamydospora* in grape propagation material and young grapevines. *European Journal of Plant Pathology* 119, 183–192.