Phytopathol. Mediterr. (2008) 47, 122–131

Interaction between *Acremonium byssoides* and *Plasmopara viticola* in *Vitis vinifera*

Santella BURRUANO[1], Antonio ALFONZO[1], Sandra LO PICCOLO[1], Gaetano CONIGLIARO[1], Vincenzo MONDELLO[1] , Livio TORTA[1], Maddalena MORETTI[2] and Gemma ASSANTE[2]

[1]Dipartimento S.En.Fi.Mi.Zo. Sezione di Patologia Vegetale e Microbiologia Agraria, Università degli Studi di Palermo, Viale delle Scienze 2, 90128 Palermo, Italy
[2] Istituto di Patologia Vegetale, Facoltà di Agraria, Via Celoria 2, 20133 Milano, Italy

Summary. The endophytic fungus *Acremonium byssoides* was isolated from the leaves of grapevine cv. Regina Bianca to determine whether it could act as an antagonistic endophyte of the downy mildew agent *Plasmopara viticola*. The occurrence of this fungus was ascertained in 34 grapevines (cultivars Regina Bianca, Catarratto and Insolia) representing all the grapevine plants in an experimental vineyard located in Sicily and which had never been treated with any fungicides. The isolation frequency of the endophytic *Acremonium* strains was assessed in several plant organs by means of monthly samplings over a 2-year period. The endophyte was found in all the grapevines, with higher frequencies in the cv. Regina Bianca in the buds, and in the cooler months of the year. All the strains collected from the leaves, and most of the strains from the buds of cv. Insolia were identified as *A. byssoides* by conventional means. The presence and spatial distribution of *A. byssoides* was determined in asymptomatic or *P. viticola*-infected leaves of the grapevine cv. Insolia using an optimised staining technique and differential interferential contrast light microscopy, as well as laser scanning microscopy. *A. byssoides* was a natural colonizer of grapevines and actively parasitized *P. viticola*. Culture filtrates and the crude extract of *A. byssoides* obtained from a cv. Insolia foliar strain, completely inhibited *P. viticola* sporangia.

Key words: acremines, antagonism, confocal microscopy, downy mildew, endophytes.

Introduction

The biology and epidemiology of grapevine downy mildew caused by the oomycete *Plasmopara viticola* are directly affected by climate and weather. In the southern and insular areas of Italy, which are hot and dry, it is humidity rather than the temperature that represents the main factor limiting the establishment and development of downy mildew. Epidemics break out only during rainy summers. Disease control based on forecasting is successful elsewhere, but it is seldom accurate in Sicilian vineyards, so that control measures here are often unsuccessful and the outcome of their implementation difficult to predict. The life cycle of *P. viticola* has been studied for three decades (Burruano *et al.*, 2006), in order to elucidated the local epidemiology of the disease. Earlier investigations found several leaf samples that contained collapsed *P. viticola* sporangiophores and sporangia, and also showed that a fungus that grew and sporulated in those leaves had heavily parasitized the pathogen structures. The hyperparasitic fungus was isolated, and identified as *Acremonium byssoides* W. Gams & G. Lim (Burruano *et al.*, 1998). Preliminary observations using differential interferential contrast light microscopy (DIC) and laser scanning microscopy (LSM) revealed that *A. byssoides* was an endophyte in *Vitis vinifera*, cv. Regina Bianca. The structures of the fungus (hyphae, phialides, and conidia) were

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

detected in the mid-ribs of leaves of asymptomatic vines and also in the intercellular spaces of leaf tissues naturally damaged by insects (*Altica* sp.) or by pathogens such as *Erysiphe necator* (Burruano *et al.*, 2003). These studies also showed that the endophytic growth and differentiation of the reproductive structures of the fungus was triggered by the damaged host cells themselves. Furthermore, investigations into the secondary metabolites of *A. byssoides* strain A20 showed that it produced the acremines A-F, a class of compounds with a cyclohexene or aromatic component replaced or cyclised by a prenyl moiety, which inhibited sporangial germination more or less effectively, depending on the acremine type (Assante *et al.*, 2005).

That fungal endophytes occur in many perennial herbaceous plants is well-known, and they have been advocated as a possible protection strategy to cope with insect and pathogen attacks, high heavy metal levels, and the depredations of mammalian herbivores, which are often associated with toxic phenomena in livestock raised on contaminated fodder (Anselmi *et al.*, 2000; Saikkonen *et al.*, 2004). In woody plants, endophyte-host associations display a diverse range of durable symbioses and may affect the phyllosphere habitat of the host, enhancing its vegetative performance and its resistance to drought and infection (Bills, 1996; Ragazzi *et al.*, 2004). Many species in the polyphyletic genus *Acremonium* (Glenn *et al.*, 1996; Rossman *et al.*, 2001) are described either as having antagonistic properties against plant pathogens, or as being endophytes living in asymptomatic hosts (Schardl, 1996, 2001; Schardl *et al.*, 2004; Wicklow *et al.*, 2005). So far there have been only a few reports of microbial endophytism in grapevine (Bell *et al.*, 1995; Mostert *et al.*, 2000, Musetti *et al.*, 2006).

We therefore conducted a survey to evaluate *Acremonium* strains that were endophytes in the grapevine organs of three native Italian cultivars, Regina Bianca, Catarratto, and Insolia in a Sicilian vineyard that had never been treated with any fungicides, since it served as our experimental field. Using DIC and confocal LSM, we also analysed the type of interaction that occurred between *A. byssoides* and *P. viticola* in asymptomatic and *P. viticola*-infected leaf tissues of the grapevine cv. Insolia that had been naturally colonized by the *Acremonium* endophyte. We further investigated the influence of the culture filtrates (CFs) and crude extracts (CEs) of a foliar *A. byssoides* strain on pathogen sporangial germination. The aim of the investigation was to serve as a basis for evaluating whether this antagonistic symbiosis can be effectively exploited in the biocontrol of *P. viticola*.

Materials and methods

Sampling of grapevine organs and isolation of the endophyte

The experimental vineyard was 50 m^2 wide, on a level surface and was located in the garden of the S.En.Fi.Mi.Zo., Department of Palermo University (38° 0,6' 27" N; 13° 21' E; 42 m above sea level) and comprised 34 10-year-old grapevines: 16 cv. Regina Bianca (a table grape variety), 16 cv. Catarratto, and two cv. Insolia (wine-grape varieties). Organ samples were taken at monthly intervals from each vine for two consecutive years, depending on the seasonal presence or absence of organs. Each months 18 buds, nine leaves, nine leaf petioles, and nine shoots were sampled from each vine. Ninety seeds per plant from the cv. Insolia were also included at harvest time. The endophytes were isolated as follows: shortly after plant sample collection, the samples were surface-sterilized by shaking them in 70% ethanol for 1 min, dipping them in a water sodium hypochlorite solution (5% v:v) for 2 min, and shaking them in 70% ethanol again for 1 min. The samples were then rinsed in sterile distilled water, sectioned, and placed in Petri dishes containing 2% malt extract agar (MA) amended with 50 mg l^{-1} oxytetracycline hydrochloride (Sigma Chemicals Co., Milan, Italy). Bud samples were sectioned into five fragments each, while 10 fragments were cut from each leaf (0.6-mm diam. disks), leaf petiole (0.5-cm segments), and shoot sample (0.5-cm segments), and kept at 22–24°C. After 3 days, fragments were inspected daily by stereomicroscopy for 3 weeks, and the number of fungal colonies that developed was recorded. All colonies thought to belong to the genus *Acremonium* were transferred to MA Petri dishes in pure culture. Identification of the genus was on the basis of morphological characteristics observed in agar cultures and from slide cultures examined under the light microscope, using the keys of Barnett (1965), Von Arx (1974), and Domsch *et al.* (1980). The species epithet for *A. byssoides* was based on the key of Gams (1975). Two indices for the isolation frequency (IF) of *Acremonium* strains were calculated: $IF_o = (Ni_o/Nt_o)\times100$,

where Ni_o is the number of organs from which the fungus was isolated, and Nt_o the total number of organs examined; and $IF_f = (Ni_f/Nt_f)\times100$, where Ni_f is the number of fragments colonized by the fungus, and Nt_f the total number of fragments examined (Ragazzi *et al.*, 2001).

Preparing samples for microscopy

Four asymptomatic leaves were collected from each cv. Insolia grapevine every 15 days, from January through June. To ensure that the plant material examined was of the same age, we sampled the fourth leaf below each of the apical vine-shoots. The leaves were surface-sterilized, placed at 22°C and 100% relative humidity in sterile 150-mm Petri dishes lined with paper disk filters that had been soaked in distilled water, and divided into two groups of four leaves each. One group was immediately inoculated with 10 drops (100 μl) per leaf of a water suspension of *P. viticola* sporangia (8×10^5 ml^{-1}, obtained by shaking heavily infected leaf pieces in water), and maintained at 20±1°C. The other group was used to determine and study the natural occurrence and activity of *A. byssoides* as an endophyte.

Tissue disks (0.9-mm diam., five disks per leaf) were cut from non-inoculated and inoculated leaves after 2, 4, and 8 days of leaf incubation, soaked for 24 h in 80% ethanol in water, boiled in 10% potassium hydroxide (w:v) for 15 min, rinsed in the same alkaline solution, and transferred to a 10% hydrogen peroxide solution at room temperature until completely discoloured (20–40 min). To visualize *A. byssoides* hyphae, the disks, after rinsing in distilled water were acidified by rapid immersion in 10% hydrogen chloride, and then boiled for 1–2 min in a lactophenol solution containing 1 g l^{-1} acid fuchsin, a stain specific for chitin in fungal walls. The disks were then rinsed twice in distilled water, and mounted in a cold lactophenol solution (25 g phenol crystal, 25 ml lactic acid, 25 ml glycerol, 25 ml water). In order to visualize the *P. viticola* structures, which were not stained by the acid fuchsin because they lack chitin, mechanically inoculated samples were contrast-stained with aniline blue diammonium salt (Sigma Chemicals Co., Milan, Italy). The entire disk of all the samples was observed, first with normal DIC (Axiophot, Carl Zeiss equipped with Normarsky Contrast DIC, Axiophot Zeiss, Oberkochen, Germany), then with LSM (Mod. 510, Carl Zeiss, Inc.) equipped with argon and helium-neon lasers (at 458–488 and 543 nm, respectively), which enabled the specimens to be examined through serial z-axis optical sections. The three-dimensional (3-D) reconstructions of the specimens were generated using an IBM RS/6000 Unix workstation where the image files were transferred for further processing using LSM3D projection software (Carl Zeiss), in panoramic transparent mode.

Bioassay with CFs and CE of *A. byssoides* on germination of *P. viticola* sporangia

Acremonium byssoides strain A21, isolated from the leaves of cv. Insolia, was grown at 24°C for two weeks in Erlenmeyer flasks containing one of two liquid media: a malt extract-peptone-glucose-broth (MPGB: 30 g l^{-1} malt extract, 3 g l^{-1} peptone, 30 g l^{-1} glucose), or a corn steep broth (CSB: 10 g l^{-1} corn steep liquor, 90 g l^{-1} glucose, 100 g l^{-1} sucrose, 5 g l^{-1} yeast extract, 2 g l^{-1} potassium phosphate dibasic). The medium ingredients were obtained from Merck KGaA (Frankfurt, Germany), except for corn steep liquor, which was obtained from Sigma Chemicals Co. The CFs were obtained after two weeks of growth by sterile-filtering the cultures in a vacuum on a 500-ml Stericup (0.45 μm HV Durapore membrane, Millipore Co., Bedford, MA, USA). The CE was obtained from cultures on MPGB plus agar (MPGA), by extraction with Ethyl-acetate/MeOH (100:1). The CFs and the CE were chromatographed on TLC aluminium sheets (Silica gel 60 F_{254}, Merck KGaA) in two solvent mixtures (dichloromethane/methanol 15:1; or hexane/ethyl-acetate 1:1) and the R_f values of the fluorescent spots were compared under UV lighting (256 nm) with acremines, obtained as previously described, added as pure standards (Assante *et al.*, 2005).

The inhibition of *P. viticola* sporangia germination was tested in 96-well flat-bottom plates (Sigma Chemicals). The CE previously dissolved in DMSO (not exceeding 0.3% final volume) and diluted in water was assayed at a 100 μg ml,$^{-1}$ final concentration. The CFs (100 μl per well) were similarly assayed. The controls were water, DMSO, and culture broths (MPGB and CSB not inoculated with *A. byssoides*), correctly diluted and adjusted to the same pH as that measured in the test solutions. A suspension of *P. viticola* sporangia was freshly prepared in deionised sterile water (10^4 ml^{-1}) and immediately loaded into the multi-well plates, 100 μl per well,

diluted 1:1 with CE, CF, or a control solution, and maintained at 22°C. After 2 h, sporangia germination was halted by staining the suspension with 0.05% trypan blue in Amman's lactophenol (20 g phenol crystal, 16 ml lactic acid, 31 ml glycerol per 100 ml water). Germination of the sporangia was determined by light microscopy, scoring 100 propagules in each well, 6 wells per test suspension. Empty (germinated) sporangia were easily distinguished from degenerated or non-germinated sporangia. The germination percentages were compared with those of the controls. We performed two independent experiments, comprising readings on 1200 sporangia for each treatment.

Statistical analyses

Data on the activity of the CFs and the CE of *A. byssoides* against the germination of *P. viticola* sporangia were transformed by raising them to the power of 0.225, as suggested by the statistical software, and then subjected to ANOVA. Because the variances of the examined groups were not homogenous ($P<0.05$ Levene's test), we used Tamhane's test (Tamhane, 1977), which is a *post hoc* test that does not assume the homogeneity of the variances. Statistical analysis was performed using SPSS version 8.0 (SPSS Science, Chicago, IL, USA).

Results

Isolation of *Acremonium* strains from grapevine organs

All of the grapevines of the vineyard were colonized by *Acremonium*. *Acremonium* strains represented 90.4% of the endophytes isolated from the 24,012 organs (211,320 fragments) collected over 2 years. Other endophytic strains belonging to the genera *Alternaria, Cladosporium, Gliocladium, Hyalodendron,* and *Trichoderma* were also occasionally found, but they were never associated with *P. viticola*.

The IF of the endophytic *Acremonium* strains was calculated in relation to the number of organs examined (Table 1). The average *Acremonium* incidence over the 2 years of sampling ranged from 3% in the cv. Catarratto, to 4.1% in cv. Insolia and 5.8% in cv. Regina Bianca. The endophyte was isolated more frequently during the cooler months; in particular, cv. Catarratto grapevines were positive for the endophyte from February through March and in May, while in the cv. Regina Bianca and Insolia, *Acremonium* was isolated, though at low frequencies, during 10 and 8 months respectively. The month of April for cv. Regina Bianca, May, November, and December for cv. Insolia, and August for the three cultivars were exceptions to this trend; the IF_o in these months was zero.

The highest IF_o values occurred in the buds, followed by the shoots of the cv. Regina Bianca and Catarratto, and the leaves of cv. Insolia. The lowest values were always in the leaf petioles.

As regards the isolation frequency of the fragments (IF_f) the total incidence of *Acremonium* in the fragments was very low, ranging from 1% in the cv. Catarratto to 1.2% in the cv. Insolia and 1.4% in the cv. Regina Bianca.

We collected 198 *Acremonium* isolates, 73 from the cv. Regina Bianca, 31 from the cv. Catarratto, and 94 from the cv. Insolia. Of the 94 *Acremonium* isolates from cv. Insolia, all the 58 isolates from the leaves and 10 of the 20 isolates from the buds were identified as *A. byssoides* on the basis of the morphological characteristics, using the taxonomic keys and culture conditions described earlier. The remaining isolates had to await species assignment by more accurate molecular identification, which is in progress.

Interaction between *A. byssoides* and *P. viticola* in the foliar tissues of the grapevine cv. Insolia

Differences in the size and morphology of mycelium, the occurrence of haustoria, and the reproductive structures made it possible to distinguish *A. byssoides* mycelium from *P. viticola* mycelium using LSM combined with optimised discolouration and the double-staining contrast technique. We could thus observe the growth of the endophyte in asymptomatic, naturally colonized leaves, as well as the interaction between *A. byssoides* and *P. viticola* in mechanically inoculated leaves.

Non-inoculated leaves.

DIC examination of non-inoculated leaves of cv. Insolia, collected from the end of January to the middle of March, revealed thin hyphae in the veins, clearly visible, as red strings because of the fuchsin staining (Fig. 1a). Other hyphae ran through the intercellular spaces from the veins to the mesophyll cells (Fig. 1b), and some host cells of the lower epidermis had red-stained walls (Fig. 1c) indicating typical *A. byssoides* phialides and conidia (Fig. 1d, red colour).

Table 1. Isolation frequencies (IF_o and, in brackets, IF_f) of *Acremonium* strains in 34 grapevines belonging to three cultivars (cv. Regina Bianca, Catarratto and Insolia) of a vineyard located in Palermo, Sicily, Italy[a].

1a. cv. Regina Bianca[b]

Organ	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Shoot	20.1[c] (3.3[d])	20.5 (1.6)	20.5 (3.3)	0 (0)	13.5 (2.2)	9.4 (1.1)	14.6 (1.9)	0 (0)	2.1 (0.2)	0 (0)	0 (0)	0 (0)
Leaf	-	-	-	0 (0)	5.6 (0.6)	4.5 (0.5)	2.8 (0.3)	0 (0)	7.3 (1.1)	0 (0)	0 (0)	0 (0)
Bud	11.1 (3.3)	0 (0)	35.2 (27)	0 (0)	-	-	-	-	-	-	-	-
Petiole	-	-	-	0 (0)	0 (0)	5.2 (0.5)	0 (0)	0 (0)	0 (0)	4.2 (0.6)	4.5 (0.5)	2.1 (0.2)

Total IF_o value of the cultivar 5.8 (IF_f 1.4).

1b. cv. Catarratto[b]

Organ	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Shoot	0 (0)	27.8 (10)	19.4 (5)	0 (0)	25.0 (6.6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Leaf	-	-	-	0 (0)	5.6 (1.1)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Bud	0 (0)	0 (0)	18.8 (12)	0 (0)	-	-	-	-	-	-	-	-
Petiole	-	-	-	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Total IF_o value of the cultivar 3.0 (IF_f 1.0).

1c. cv. Insoliae[e]

Organ	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Shoot	5.6 (1.1)	13.9 (1.7)	13.9 (2.2)	2.8 (0.3)	0 (0)	5.6 (0.6)	2.8 (0.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Leaf	66.7	(19.4)	0 (0)	0 (0)	0 (0)	8.3 (1.4)	2.8 (0.3)	0 (0)	2.8 (0.3)	2.8 (0.3)	0 (0)	0 (0)
Bud	0 (0)	16.7 (7.5)	0 (0)	11.1 (3.6)	0 (0)	18.1 (9.2)	-	-	-	-	0 (0)	0 (0)
Petiole	0 (0)	-	0 (0)	0 (0)	0 (0)	13.9 (2.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Seed	-	-	-	-	-	-	-	-	-	0.3 (0.3)	-	-

Total IF_o value of the cultivar 4.1 (IF_f 1.2).

[a] For a period of two years, 90 fragments were obtained every month from each grapevine and each type of organ, as described in Materials and methods. Seasonal absence of organs led to periods of non-sampling, which is indicated by bar (-).

[b] Samples from 16 grapevines.

[c] $IF_0 = (Ni_0/Nt_0)\times100$, where Ni_0 is the number of positive organs and Nt_o is the total number of examined organs.

[d] $IF_f = (Ni_f/Nt_f)\times100$, where Ni_f is the number of positive fragments and Nt_f is the total number of examined fragments. Values are in brackets.

[e] Samples from two grapevines.

DIC examination of non-inoculated leaves collected from March through June showed that the intercellular spaces of leaf mesophyll tissue were colonized by the endophytic hyphae of *A. byssoides*, and also showed that sporulation was occurring between the stomata and the cells of the lower epidermis.

Some host cells were also stained by fuchsin (Fig. 1e). Moreover, sporadic red-stained granular masses were noted along the veins (Fig. 1f). LSM optical sectioning of the foliar disks in transmitted light showed that the granular masses consisted of hyphal ribbons, phialides, and conidia of the endophyte. LSM also revealed that the red-stained cells were surrounded by hyphae of *A. byssoides*, which passed from the veins to the lower epidermis and granular masses in a continuum through the focal planes (Fig. 1g, h, i).

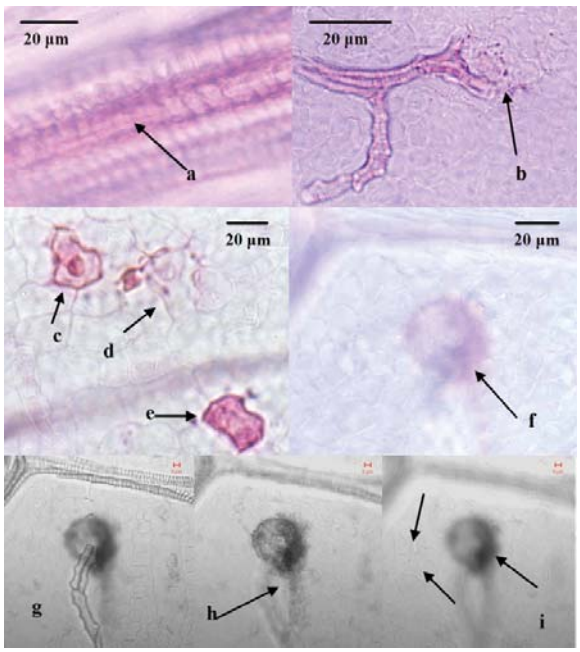

Fig. 1. Occurrence of *Acremonium byssoides* in non-inoculated grapevine (cv. Insolia) leaf mesophyll observed by differential interferential contrast light microscopy (a–f) and optical scanning confocal microscopy (55 μm; g–i). (a) Hyphae in the leaf veins. (b) Intercellular endophytic hyphae in the mesophyll. (c) Fuchsin-stained (red) walls of host epidermal cells indicate *A. byssoides* evasion. (d) *A. byssoides* fructifications emerging from among the epidermal cells. (e) A group of cells totally stained by fuchsin. (f) Fuchsin-stained granular mass of sub-stomatal tissue, showing the hyphal ribbons of *A. byssoides*. (g–i) *A. byssoides* hyphae developing from the vein and extending through the mesophyll to the epidermal cells.

Inoculated leaves.

In disks of cv. Insolia leaves in which no endophyte was detected the pathogen usually emerged and sporulated 4–5 days post inoculation (dpi) under controlled conditions. Sporulation was evenly distributed over the entire inoculation area, and well-formed sporangia and sporangiophores were visible by stereomicroscopy. In leaves naturally colonized by the endophyte, on the other hand, the emergence and sporulation of the oomycete was delayed and did not occur until 7–8 dpi. Sporangiophore branches were rarely detected in the leaves collected from January through March, and became even more sporadic later in the year (from March to June); when they occurred they were limited to the edge of the inoculation zone (Fig. 3a). Leaf samples removed at the end of January after discolouration and staining with fuchsin, were examined by DIC microscopy. Endophyte hyphae passed from the veins to the intercellular spaces at 2 dpi (Fig. 2a), and reached and partially invaded the hyaline oomycete mycelium in a further 4–6 days (Fig. 2b). The pathogen hyphae gave rise to anomalous and rare sporangiophores within red-stained granular masses emerging from the stomata at 8 dpi (Fig. 2c). We examined these masses at 100× magnification and found them to consist of hyphal ribbons, phialides, and conidia of *A. byssoides*.

In leaves removed from the middle of March through June, the hyphae of the endophyte had already colonized the pathogen mycelium by 2 dpi, and had invaded and deformed its asexual structures (sporangiophores and sporangia) by 5 dpi. Sexual structures (gametangia and oospores), if any, were produced by 7–8 dpi (Fig. 2d). Surprisingly, the sexual structures of *P. viticola* were produced in endophyte-colonized leaves earlier than in endophyte-free leaves. Numerous thin fuchsin-stained hyphae were visible around deformed host cells and pathogen structures in conjunction with typical conidia of *A. byssoides*. Moreover, the endophyte produced phialides and conidia emerging from the stomata and intercellular spaces, and at the same time some plant host cells were deformed and stained red (Fig. 2e).

The foliar disks were inspected and optically sectioned from the veins to the lower epidermis by

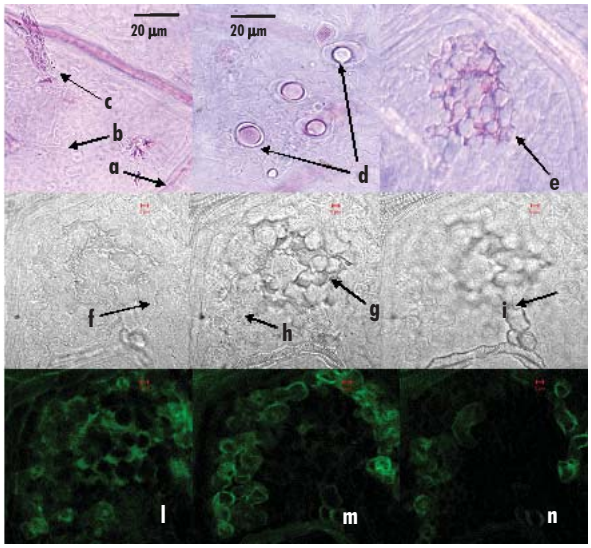

Fig. 2. *Acremonium byssoides* growth in leaf tissues inoculated with *Plasmopara viticola* (late January). (a–e) Differential interferential contrast light microscopy images. (a) Hyphae of *A. byssoides* growing out of the veins (2 dpi), passing between parenchyma cells, (b) reaching the hyaline oomycete mycelium, partly invading it, and (c) anomalous sporangiophores within red-stained granular masses emerging from the stomata. (d) Mycelium and sexual structures (gametangia and oospores) of *P. viticola*, invaded and deformed by *A. byssoides*. (e) Phialides and conidia of *A. byssoides* emerging from the stomata and the intercellular spaces of the abaxial epidermis. The associated deformed host cells are also stained red. (f–n) A series of confocal images through the focal planes of discoloured and aniline-stained *P. viticola*-infected fragments using transmitted and fluorescent light. (f) Deformed host cells with endophyte tracks. (g) Host cells surrounded by *A. byssoides* hyphae. (h) *A. byssoides* phialides with conidia inside and/or outside the host cells. (i) *A. byssoides* hyphae passing from the secondary veins into the host cells. (l–n) Hyphae of *P. viticola* between host cells, extending into the mesophylls.

LSM in transmitted (argon) and fluorescent (helium-neon) light. Scan images of the specimen area by transmitted light revealed that the mycelium was continuous composed of thin hyphae, and that it colonized the mesophyll in the intercellular spaces; the mycelium surrounded and passed through and into the plant host cells (Fig. 2f, g, h, i). The same series of scan images in fluorescent light showed *P. viticola* hyphae around the plant host cells, with decreasing fluorescence from the sub-epidermal layers toward the inner tissues in proximity of the veins. By comparing the data from these two types of microscopic examination, we determined that the degeneration of *P. viticola* mycelium induced by *A. byssoides* was associated with minor fluorescence (Fig. 2l, m, n).

Effect of CFs and CE of an *A. byssoides* strain on *P. viticola* sporangia germination

Germination of sporangia in the control solutions (water and DMSO, Fig. 3b) exceeded 80%, but it was significantly lower in the culture broth controls (32–37%), in which sporangial degeneration was also seen (Table 2). Germination was strongly inhibited in the CFs and CE, where it was significantly lower than all the controls. Moreover, in the CFs and the CE the sporangia were generally degenerated, with collapsed cellular membranes (Fig. 3c, d). That the CFs and CE of *A. byssoides* cultures contained acremines was confirmed by TLC and the pure reference standards.

Table 2. Percent germination of *Plasmopara viticola* sporangia after 2 h contact with culture filtrates (CF_s), or crude extract (CE) of *Acremonium byssoides* strain A21 or with culture media.

Sporangia germination medium	Germination (%)
Distilled water	89.7 a[f]
DMSO[a]	83.7 b
MPGB[b]	32.4 c
CSB[c]	36.6 c
CF MPGB[d]	0.9 e
CF CSB[d]	3.6 d
CE MPGA[e]	0.0 f

[a] DMSO, Dimethylsulfoxide in water (0.3% v:v).
[b] MPGB, malt extract - peptone - glucose broth.
[c] CSB, Corn steep broth.
[d] CF, Culture filtrates were diluted 1:1 with a sporangial suspension.
[e] CE MPGA, Crude extract from malt extract – peptone - glucose agar was tested at 100 μg ml^{-1}.
[f] Means followed by the same letter are not significantly different at P=0.05 (Tamhane's test).

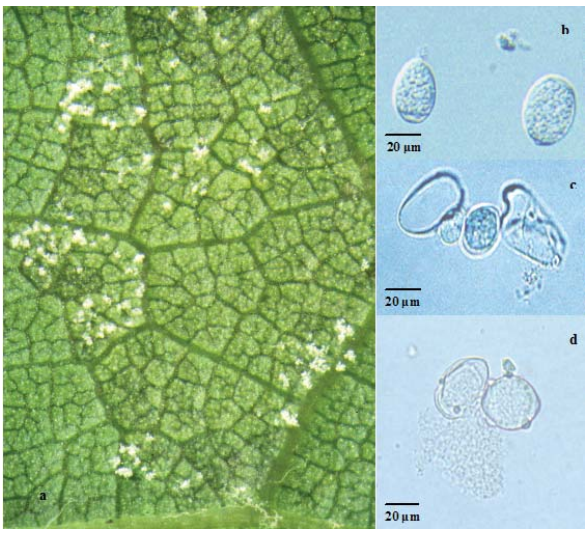

Fig. 3. (a) Rare and sporadic sporulation of *P. viticola* emerging 8 dpi from the edge of the inoculation point in a leaf inoculated in April. (b) *P. viticola* sporangia germinating in control solutions (water and DMSO), and (c) in the culture filtrate of *A. byssoides* grown in MPGB medium. (d) Degenerated sporangia treated with crude extract of *A. byssoides* grown in MPGA.

Discussion

Endophytic fungi and bacteria can greatly affect the health of the host (Chanway, 1998; Schulz and Boyle, 2005; Tanaka *et al.*, 2006; Tintier & Rudgers, 2006), as has been demonstrated for the *Acremonium/Neotyphodium* genera associated with grasses (Christensen *et al.*, 2002; Faeth *et al.*, 2006; Zhang *et al.*, 2006). There is also evidence that in organic agriculture, in the absence of agrochemical treatments, plants synthesize higher amounts of defence metabolites. This has been reported to occur with flavones and ascorbic acid in tomatoes, with polyphenols and glycoalkaloids in potatoes, with flavonols in apples, and with resveratrol in grapevines (Haislova *et al.*, 2005; Niggly and Leifert, 2007).

The no-tillage management of the test vineyard and the lack of agrochemicals provided us with a rare and valuable opportunity to investigate the ecological relationship between the endophytic *Acremonium* and *V. vinifera*. The interaction of these two is normally impaired by human interference, i.e. programs of disease control that are normally quite intense. This is the first report of a continuous presence of *Acremonium* as the only residential endophyte (Mocali *et al.*, 2003) in *V. vinifera* , which must thus be included as one of the symptomless hosts of this fungus.

The incidence of *Acremonium* increased during moist, cool periods, and in the buds and shoots, which are probably the source of the endophyte during re-colonization when the season grows warm again. After winter dormancy and with increasing temperatures, vine plants begin to grow rapidly, expanding their preformed shoots, internodes, and leaves. This vigorous growth may explain why attempts to isolate the endophyte in April (cv. Regina Bianca and Catarratto) and in May (cv. Insolia) were unsuccessful: *A. byssoides* needs time to colonize the newly formed vine tissues. The endophyte was still isolated at a time when the main shoots grew at a slower rate, while microscope inspection of the cv. Insolia leaves from January through June always revealed quiescent endophyte hyphae in the veins, even though in culture the leaf fragments did not test positive for the viable endophyte at that time.

In summer, endophyte growth in the plant organs seemed to come to a halt. The endophyte was regularly found in the autumn samples, however it seems that the adverse environmental conditions of the hottest months negatively affected the physiological water status parameters of the vines. As a result, the apoplastic water content and the concentration of nutrients in the leaves decreased and this had a direct negative impact on the microenvironment in which *A. byssoides* developed. Drought and high temperatures lowered the IF of *A. byssoides* and caused wide variations in the level of its colonization, as has also been found in other endophyte-host combinations (Collado *et al.*, 1999; Bahnweg *et al.*, 2005).

The majority of the other organisms isolated from the cv. Insolia were identified as *A. byssoides*. This fungus remained latent inside the vine organs without any visible effect on the plants. Our results suggested that there was a mutualistic association between *A. byssoides* and *V. vinifera*, because the fungus grew actively within the plant without causing symptoms, and at the same time made the environment within the plant unfit for *P. viticola*. Burruano *et al.* (2003) described how *A. byssoides* changed rapidly from a quiescent to an active state as a result of powdery mildew infection, *Altica* sp. punctures, or other wounds caused by climatic or physiological conditions to which the vine cells are subjected, and the present study (LSM, DIC and IF seasonal variation) confirmed the great plasticity of this endophyte. In non-inoculated

leaf samples, the leaf cells occasionally became damaged, and whenever this occurred, the endophyte always grew rapidly, and LSM revealed some red-stained epidermal cell walls near the *A. byssoides* phialides and conidia, even when there was no sign of the pathogen itself. Similarly, in grapevine leaves inoculated with *P. viticola* in January, the endophyte reached and hyperparasitized the pathogen in 4-6 days; formation of sporangiophores and sporangia was rare. During the growing season (March to June) when vegetation became abundant, *A. byssoides* was in an activated state in the parenchyma of the plant, and the endophyte already hyperparisitized the pathogen by 2 dpi. The asexual reproduction of the pathogen was partly to completely inhibited, and thin and deformed sporangiophores were found only at the points of inoculation. It is possible that the stress experienced by *P. viticola* induced early formation of the sexual structures in an attempt to ensure its survival.

The optimised staining technique and the use of DIC as well as LSM gave the best results for checking and evaluating the interaction between host, endophyte and pathogen. The phenological and sanitary stage of the host can influence the morphology of *A. byssoides,* and above all its antagonistic capacity to *P. viticola* in relation to the date of the assay. For this reason, careful monitoring of fungal endophytes in a plant could give useful information on the susceptibility of that plant to various pathogens and could also make it possible to rationalize the protection afforded by the endophyte.

Culture filtrates and CE of a strain of *A. byssoides* isolated from the leaves of cv. Insolia completely inhibited sporangial germination by *P. viticola*; and, in preliminary observations, the same CFs and CE also effectively delayed the growth *in vitro* of *Botrytis cinerea*, *Colletotrichum* sp., *Fusarium* sp. and *Phytophthora capsici* (Alfonzo, 2004). The CFs and CE contained acremines previously isolated from *A. byssoides* in the cv. Regina Bianca. The greater activity of the CFs and CE as compared to the pure compounds (Assante *et al.*, 2005) suggested however that the acremines alone were not responsible for inhibiting pathogen germination. Whether the reduction in disease development was due to the direct antagonism of the endophyte acting as a hyperparasite of the pathogenic organism or was an indirect effect of host activation (defence compounds and induced resistance; Faeth & Sullivan, 2003; Schulz & Boyle, 2005) remained unclear. Furthermore, because secondary metabolite production is strongly affected by environmental and nutritional factors, and varies from strain to strain within the same species (Yong *et al.*, 1998; Tan & Zou, 2001; Schulz *et al*. 2002; Strobel, 2003;), the involvement of such factors in the relationship of *A. byssoides* with both *V. vinifera* and *P. viticola* needs to be elucidated. Further study of functional factors involved in the *V. vinifera/A. byssoides* symbiosis and of the possible application of *A. byssoides* as a biocontrol agent of *P. viticola* is under way.

Literature cited

Alfonzo A., 2004. *Indagini Sui Metaboliti Secondari Prodotti da Funghi Antagonisti e Micotossinogeni*. PhD Thesis, University of Palermo, Italy, 41–43.

Anselmi N., A. Tannini and A. Mazzaglia, 2000. The role of endophytes in oak decline. In: *Decline of Oak Species in Italy. Problems and Perspectives*. (A. Ragazzi, I. Dellavalle, ed.). Accademia Italiana di Scienze Forestali, Firenze, Italy, 129–144.

Assante G., S. Dallavalle, L. Malpezzi, G. Nasini, S. Burruano and L. Torta, 2005. Acremines A-F, novel secondary metabolites produced by a strain of an endophytic *Acremonium*, isolated from sporangiophores of *Plasmopara viticola* in grapevine leaves. *Tetrahedron* 61, 7686–7692.

Bahnweg G., W. Heller, S. Stich, C. Knappe, G. Betz, C. Heerdt, R.D. Kehr, D. Ernst, C. Langebartels, A.J. Nunn, J. Rothenburger, R. Schubert, P. Wallis, G. Muller-Starck, H. Werner, R.Matyssek and H.Jr. Sandermann, 2005. Beech leaf colonization by the endophyte *Apiognomonia errabunda* dramatically depends on light exposure and climatic conditions. *Plant Biology* 7, 659–669.

Barnett H.L., 1965. *Illustrated Genera of Imperfect Fungi*. 2nd. edition, Burges Publishing Company, Houston, TX, USA.

Bell C.R., C.A. Dickie, W.L.G. Harvey and J.W.F. Chan, 1995. Endophytic bacteria in grapevine. *Canadian Journal of Microbiology* 41, 46–53.

Bills G.F., 1996. Isolation and analysis of endophytic fungal communities from woody plants. In: *Endophytic Fungi in Grasses and Woody Plants: Systematics, Ecology and Evolution*. S.C. Redlin and L.M. Carris, APS Press ed., St Paul, MN, USA, 31–65.

Burruano S., G. Conigliaro and L. Torta, 1998. Associazione tra *Acremonium byssoides* e *Plasmopora viticola*. *Micologia Italiana* 1, 19–22.

Burruano S., G. Conigliaro, S. Lo Piccolo and L. Torta, 2003. Detection of endophytic *Acremonium* sp. in healthy or naturally damaged leaves of *Vitis vinifera*. *Journal of Plant Pathology* 85, 318 (abstract).

Burruano S., G. Conigliaro, S. Lo Piccolo, A. Alfonzo and L. Torta, 2006. *Plasmopara viticola*: three decades of observation in Sicily. *Proceedings of the 5th International Workshop on Grapevine Downy and Powdery Mildew*. 18-23 June 2008. San Michele all'Adige, Italy, 57–60.

Chanway C.P., 1998. Bacterial endophytes: ecological and practical implications. *Sydowia* 50, 149–170.

Christensen M.J., R.J. Bennett and J. Schmid, 2002. Growth of *Epichloe/Neotyphodium* and p-endophytes in leaves of *Lolium* and *Festuca* grasses. *Mycological Research* 106, 93–106.

Collado J., G. Platas, I. Gonzales and F. Pelaez, 1999. Geographical and seasonal influences on the distribution of fungal endophytes in *Quercus ilex* L. *New Phytologist* 144, 525–532.

Domsch K.H., W. Gams and T.H. Anderson, 1980. *Compendium of Soil Fungi*. Academic Press, Oxford, England, 16–29.

Faeth S.H. and T.J. Sullivan, 2003. Mutualistic asexual endophytes in a native grass are usually parasitic. *American Naturalist* 161, 310–325.

Faeth S.H., D.R. Gardner, C.J. Hayes, A. Jani, S.K. Wittlinger and T.A. Jones, 2006. Temporal and spatial variation in alkaloid levels in *Achnatherum robustum*, a native grass infected with the endophyte *Neotyphodium*. *Journal of Chemical Ecology* 32, 307–324.

Gams W., 1975. *Cephalosporium*-like *Hyphomycetes*: some tropical species. *Transaction British Mycological Society* 64, 389–404.

Glenn A.E., C.W. Bacon, R. Price and R.T. Hanlin, 1996. Molecular phylogeny of *Acremonium* and its taxonomic implications. *Mycologia* 88, 369–383.

Haislovà J., V. Schulzovà, P. Slanina, K. Jannè, K.E. Hellenas and C.H. Andersson, 2005. Quality of organically and conventionally grown potatoes: four-year study of micronutrients, metals, secondary metabolites, enzymic browning and organoleptic properties. *Food Additives and Contaminants* 22, 514–534.

Mocali S., E. Bertelli, F. di Cello, A. Mengoni, A. Sfalanga, F. Viliani, A. Caciotti, S. Tegli, G. Surico and R. Fani, 2003. Fluctuation of bacteria isolated from elm tissues during different seasons and from different plant organs. *Research in Microbiology* 154, 105–114.

Mostert L., P.W. Crous and O. Petrini, 2000. Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. *Sydowia* 52, 46–58.

Musetti R., A. Vecchione, L. Stringher, S. Borselli, L. Zulini, C. Marzani, M. D'Ambrosio, L.S. Di Toppi and I. Pertot, 2006. Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew by the endophytic fungus *Alternaria alternata*. *Phytopathology* 96, 689–698.

Niggli U. and C. Leifert, 2007. Improving sustainability in organic and low input food production systems. *Proceedings of the 3rd International Congress of the European Integrated Project Quality Low Input Food (QLIF),* 2007. *Hohenheim, Germany*, 15–22.

Ragazzi A., S. Moricca and I. Dellavalle, 2004. *Endophytism in Forest Trees*. Accademia Italiana di Scienze Forestali, Firenze, Italy, 15-32.

Ragazzi A., S. Moricca, P. Capretti, I. Dellavalle and E. Turco, 2001. Endophytic fungi in *Quercus cerris*: isolation frequency in relation to phenological phase, tree health and the organ affected. *Phytopathologia Mediterranea* 40,165–171.

Rossman A.Y., J.M. McKemy, R.A. Pardo-Schultheiss and H.J. Schroers, 2001. Molecular studies of the Bionectriaceae using large subunit rDNA sequences. *Mycologia* 93, 100–110.

Saikkonen K., P. Wäli, M. Helander and S.H. Faeth, 2004. Evolution of endophyte-plant symbioses. *Trends in Plant Science* 9, 275–280.

Schardl C.L., 1996. *Epichloë* species: fungal symbionts of grasses. *Annual Review of Phytopathology* 34, 109–130.

Schardl C.L., 2001. *Epichloë festucae* and related mutualistic symbionts of grasses. *Fungal Genetics and Biology* 33, 69–82.

Schardl C.L., A. Leuchtmann and M.J. Spiering, 2004. Symbioses of grasses with seedborne fungal endophytes. *Annual Review of Plant Biology* 55, 315–340.

Schulz B. and C. Boyle, 2005. The endophytic continuum. *Mycological Research* 109, 661–686.

Schulz B., C. Boyle, S. Draeger, A.K. Rommert and K. Krohn, 2002. Endophytic fungi: a source of novel biologically active secondary metabolites. *Mycological Research* 106, 996–1004.

Strobel G.A., 2003. Endophytes as source of bioactive products. *Microbes and Infection* 5, 535–544.

Tan R.X. and W.X Zou, 2001. Endophytes: a rich source of functional metabolites. *Natural Products Reports* 18, 448–459.

Tamhane A.C., 1977. Multiple comparisons in model I one-way ANOVA with unequal variances. Communications in Statistics. *Theory and Methods* 6, 15–32.

Tanaka A., M.J. Christensen, D. Takemoto, P. Park and B. Scott, 2006. Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. *Plant Cell* 18, 1052–1066.

Tintier T. and A. Rudgers, 2006. Grass-herbivore interactions altered by strains of a native endophyte. *New Phytologist* 170, 513–521.

Von-Arx J.A., 1974. *The Genera of Fungi Sporulating in Pure Culture*. J. Cramer Vaduz, Liechtenstein, 100–194.

Wicklow D.T., S. Roth, S.T. Deyrup and J.B. Gloer, 2005. A protective endophyte of maize: *Acremonium zeae* antibiotics inhibitory to *Aspergillus flavus* and *Fusarium verticillioides*. *Mycological Research* 109, 610–618.

Yong J., J.N. Sacalis and C.C. Still, 1998. Bioactive flavonoids from endophyte-infected blue grass (*Poa ampla*). *Journal of Agricultural and Food Chemistry* 46, 3785–3788.

Zhang N.X., V. Scott, T.H. Al-Samarrai, Y.Y. Tan, M.J. Spiering, L.K. McMillan, G.A. Lane, D.B. Scott, M.J. Christensen and J. Schmid, 2006. Transformation of the ryegrass endophyte *Neotyphodium lolii* can alter its *in planta* mycelial morphology. *Mycological Research* 110, 601–601.

Accepted for publication: July 13 , 2008