MOLECULAR EVIDENCE OF *TAPHRINA WIESNERI* IN LEAVES AND BUDS OF HEALTHY SWEET CHERRY: A POSSIBLE ENDOPHYTISM?

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SUMMARY

Analysis of DNAs from leaves and buds of several sweet cherry trees (*Prunus avium*) in two different Italian regions with different climates, has revealed the presence of *Taphrina wiesneri*, in all samples analyzed. The presence of other fungi was only occasional. The presence of *T. wiesneri* was shown by sequencing segments of rDNA genes amplified by PCR. Analysis of genes coding for the elongation factor-1α (EF1-α) and RNA-polymerase II subunit 1 (RPB1), confirmed the result. The *T. wiesneri* sequences were variants of those reported in data bases. *T. wiesneri* was not detected in buds of walnut, apricot and sour cherry growing close to the analysed sweet cherry trees. Determination of the relative numbers of *T. wiesneri* and sweet cherry genomes in samples prepared from the same buds of 4 different trees all gave similar values indicating massive presence of the fungus. However, *in situ* hybridization experiments on bud sections using a *T. wiesneri*-specific 18S rDNA probe and stains for fungi, labelled structures not clearly representing hyphae.

Key words: stone fruits, PCR, EF1-α, rRNA genes, RPB1.

INTRODUCTION

A molecular study performed to characterize the fungal population related to cherry chlorotic rusty spot (CCRS), a disease whose aetiological agent was supposed to be a fungus because of its consistent association with mycelium-like structures (Alioto *et al.*, 2003) and mycoviruses (Di Serio *et al.*, 1996, 1997; Covelli *et al.*, 2004; Coutts *et al.*, 2004; Kozlakidis *et al.*, 2006), unexpectedly revealed the presence of *Taphrina wiesneri* in symptomless cherry trees and those with CCRS.

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T. wiesneri (synonym *T. cerasi*) is the causal agent of witches' broom and leaf curl on cherry and apricot trees and is found in practically all regions where these trees are grown. The fungus is transmitted by blastosporic conidia or ascospores from infected tissues, and overwinters on bud scales and in infected wood (Mix, 1949). As other fungi belonging to order Taphrinales this pathogen is dimorphic, forming mycelium and asci in its parasitic phase and budding yeast cells in its saprotrophic phase with no ascomata (ascomycetous fruiting bodies) (Kramer 1973, 1987).

Here, we report the presence of *T. wiesneri* in leaves and in the inner parts of buds of all the sweet cherry trees analysed, irrespective of disease symptoms, geographical area and altitude.

MATERIALS AND METHODS

Origin of samples and DNA extraction. DNA from leaves and buds of sweet cherry trees located in several areas of the Campania and Tuscany regions of Italy was extracted as reported (Caputo *et al.*, 1991). In Campania, four CCRS-affected trees came from Ariano Irpino (AV) (altitude of 700 m); four apparently healthy trees growing at sea level, were from Portici (NA), Sessa Aurunca (CE), Procida (NA) and Naples. In Tuscany, four apparently healthy trees were from Pratovecchio (400 m altitude), two trees from Lonnano (700 m altitude) and one tree from Prato alle Cogne (1054 m altitude), all in the province of Arezzo (AR); one tree was from Scandicci (FI), at sea level. The trees from Pratovecchio, Ariano Irpino and Portici were analysed systematically over a period of 3 years. The other trees were analysed occasionally with the main aim of finding a sweet cherry tree free of *Taphrina*. DNA samples from buds of a walnut, apricot and sour cherry tree (*Prunus cerasus*), located in the areas of analyzed *Prunus avium* trees, were used as negative controls. The walnut was from Pratovecchio, the apricot and sour cherry from Ariano Irpino.

Polymerase chain reaction and sequence analysis. Primers EF4/EF3 (Smit *et al.*, 1999), were used to amplify the region of 1600 bp typical of all fungal rDNA

18S genes. Primers P3 (5'-GGCTACCACATCCAAGGAAGGCAGC-3') and M7 (5'- GGTCAACCAAGGCCAAGGTTCAGC-3'), designed on the *T. wiesneri* 18S rDNA sequence (accession No. AY548293), were used to amplify a 252 bp 18S rDNA segment to detect the *Taphrina* 18S rDNA gene in sweet cherry trees and to be used as a specific *in situ* hybridization probe on bud sections. Primer P2 (5'-GATTGTCGTTGGGCTGCTGG-3') was designed on a *T. wiesneri* rDNA sequence (accession No. AY548293) and primer M49 (5'-CGCGTATTCCTACCTGATCTGAG-3') on a further *T. wiesneri* rDNA sequence (accession No. AB435051) (Yamamoto *et al*., 2008), to amplify a region encompassing both ITS1 and ITS2 spacers to distinguish *Taphrina* species and variants (Tavares *et al*., 2004; Marongiu *et al*., 2003). Primers for the gene coding for RNA polymerase II subunit 1 (RPB1), RPB-F (5'-AGGTAGATGCAAATGACCC-3') and RPB-R (5'-CCGGCTCATGTTATCAAGG-3'), and for the elongation factor-1α (EF1-α), EF1-F (5'-CGGAAAGTCAACCACTACCG-3') and EF1-R (5'-CAGTCCGACCATCAGACGAGC-3'), of *T. wiesneri* were designed on GenBank sequences (accession Nos DQ471134 and DQ479936). PCR incubation mixtures contained 50 ng of DNA, 1.5 mM $MgCl_2$, 200 µM each dNTP, 400 nM forward and reverse primers and 2.5 U of *Taq* DNA polymerase (Sigma-Aldrich, USA). All reagents were combined and maintained at 95°C for 3 min. Thirty-five cycles were performed using primers EF4 and EF3, by heating at 95°C for 30 sec, 48°C for 30 sec, and 72°C for 1 min, followed by a final period at 72°C for 10 min. Other primers were used in the same conditions except that annealing temperatures were 66°C for primers P3/M7, 65°C for P2/M49, 62°C for primers EF1-F/EF1-R, and 57°C for RPB1-F/RPB1-R.

PCR products, of the expected lengths, were isolated by electrophoresis on agarose gels and purified from the excised bands using NucleoSpin Extract kit (Macherey-Nagel, Germany). The purified PCR products were ligated in pDRIVE vectors (Qiagen, USA), used to transform competent TG1 cells and plated on agar LB in the presence of 100 µg/ml Ampicillin. Transformed bacterial colonies were identified by Blue/White selection. The inserts of at least 5 positive clones for each experiment were sequenced (BMR Genomics, Padova, Italy or MWG Biotech, Bochum, Germany) and analyzed using BLASTn program with default parameters (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analysis was performed using the program MEGA v. 3.1 (Kumar *et al.*, 2004), Neighbour-Joining method, and the validity of the branches was estimated by performing bootstrap analysis (1,000 replicas). The relative numbers of genome copies of *Taphrina* and of sweet cherry in the same DNA samples isolated from buds of 4 different cherry trees were evaluated comparing the relative fluorescence intensities of rDNA fragments produced by PCR amplifications. Primers for fungal DNA were P3/M7 that amplify a 252 bp 18S rDNA segment. Primers for cherry tree DNA were ITSa/ITSb (Adachi *et al*., 1994) that amplify a 350 bp segment spanning from the 3' region of 18S to 3' region of 5.8S rDNA of sweet cherry.

***In situ* hybridization.** To study fungal localization, cherry bud sections were prepared using a standard procedure. In brief, buds were split into halves, fixed in 10% formaldehyde for 3 days at room temperature, washed in 0.1 M phosphate buffer pH 7.0, dehydrated in an ethanol series from 30 to 100%, incubated in xylene, embedded in paraffin at 57-59°C and sectioned (section thickness 7 µm). Bud sections for *in situ* hybridization experiments were treated with the probe of 252 bp, specific for the 18S rDNA gene of *Taphrina* genus, prepared by PCR amplification with primers P3/M7 in the presence of digoxigenin-11-dUTP (Roche, Switzerland) as a label, in a ratio of 5% DIG-11-dUTP and 95% dTTP as reported (De Rienzo *et al.*, 2001). Sections hybridized with the digoxigenin labelled probe were treated with anti-digoxigenin antibodies conjugated to alkaline phosphatase (Roche, Switzerland) and revealed with BM-purple substrate (Roche, Switzerland). Sections were stained with Trypan-blue and Sudan III to reveal fungi, as reported (Barrow, 2003) and Lactophenol-blue was used according to the manufacturer's instructions (Sigma-Aldrich, USA).

RESULTS

Identification of *T. wiesneri*. All samples of DNA extracted from leaves and buds of cherry trees located in different areas at different altitudes and climatic conditions showed the presence of rDNA genes of *Taphrina* genus. These findings were the results of several experiments in which the DNA fragments obtained by PCR amplification using primers EF4/EF3, generic for 18S rDNA of fungi, were cloned and sequenced. In each experiment 40 to 60% of the clones isolated consistently showed sequences corresponding to *Taphrina* as revealed by BLASTn analyses (data not shown). Among the sequences producing significant alignments, *T. wiesneri* was among those with the best score (data not shown). The sequences of the other clones produced alignments to different fungal genera not detected in the DNAs of all analyzed cherry trees (data not shown). These results were obtained for all cherry trees analysed, independently of CCRS symptoms, geographical area and altitude. As a negative control, bud DNAs were used of a sour cherry and an apricot tree from Ariano Irpino and of a walnut tree from Pratovecchio growing in close proximity to the analyzed sweet cherry trees. *Taphrina* rDNA was never detected in 10 different

clones amplified from the bud DNA of each of these trees. This indicates that the finding of *Taphrina* in *P. avium* trees did not depend on the presence of this fungus in a particular environment.

To identify the *Taphrina* species, DNA was amplified with primers P2/M49 encompassing ITS regions of rDNA genes in 100 clones derived from 10 different trees. BLASTn sequence analyses of individual clones revealed the presence of *T. wiesneri* in all these samples. Table 1 shows the accession numbers of two sequences deposited in GenBank, representing the two major variant sequences showing an identity to each other of 99% isolated from the DNAs of the 9 cherry trees studied for a period of 3 years. The other analyzed *T. wiesneri* sequences differed in few nucleotide positions in the ITS regions (data not shown). This is in line with the findings of Rodrigues and Fonseca (2003), who demonstrated intraspecific ITS sequence polymorphisms that correlated with host species. The two deposited sequences (Table 1), were used to construct an unrooted phylogenetic tree with bootstrap values (Fig. 1). The two sequences cluster with *T. wiesneri* with a bootstrap value of 98, in line with BLASTn results.

To analyze tissues less exposed to external contamination, the presence of *Taphrina* was also investigated in dissected embryonic gamete structures of buds collect-

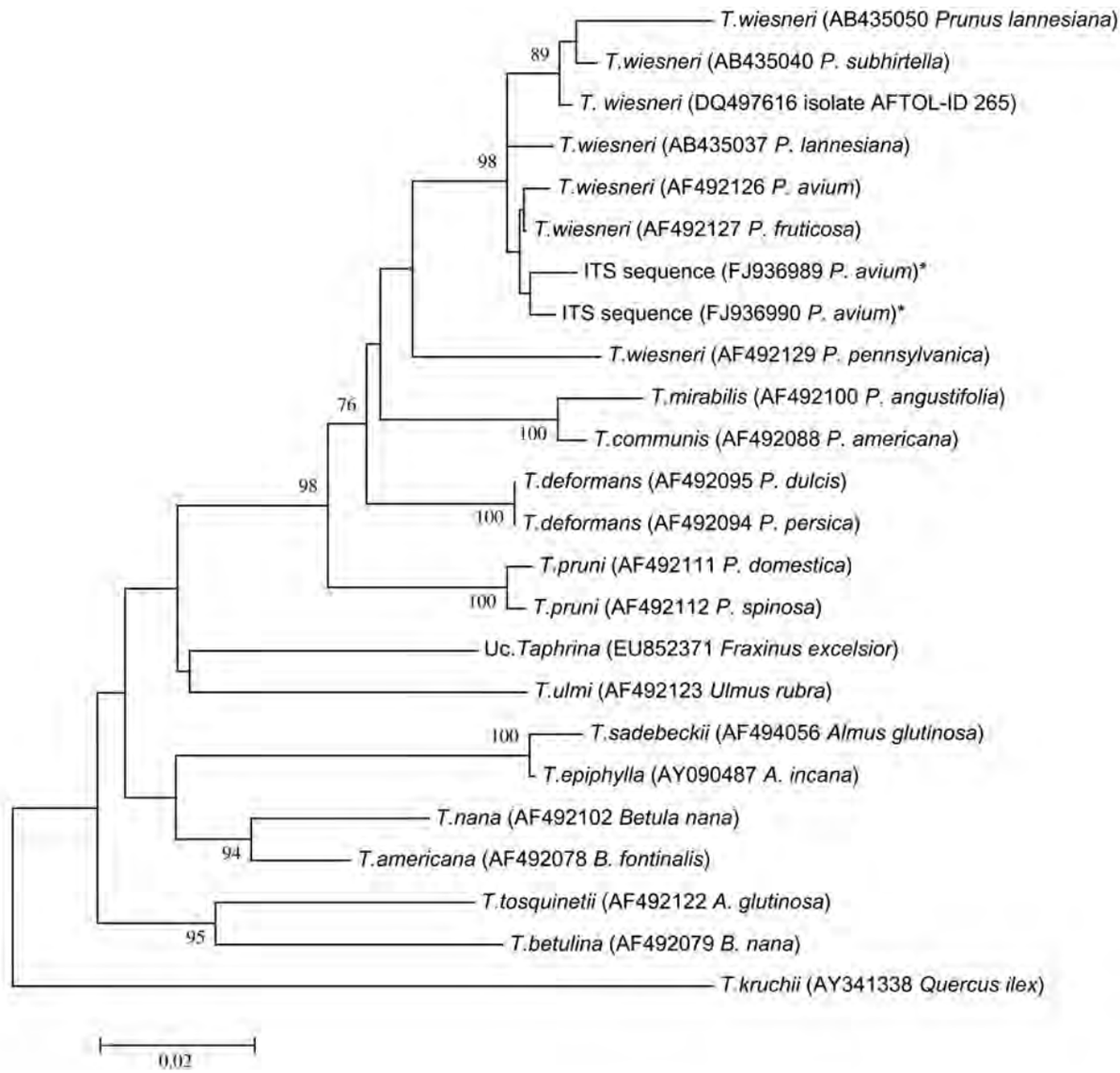

Fig. 1. Unrooted phylogenetic tree calculated from the ITS region sequences of fungi belonging to the *Taphrina* genus present in databases and from the 2 ITS region sequences deposited by the authors. The tree was calculated using the program MEGA v. 3.1 (Kumar *et al.*, 2004), Neighbour-Joining method, and the validity of the branches was measured by bootstrap analysis (1,000 replicas). Bootstrap values above 70% are shown. The scale bar represents 2% divergence. Accession numbers and hosts of reference sequences are indicated in brackets. * ITS sequences obtained in this work, deposited in GenBank.

Table 1. PCR identification of *Taphrina* species from bud DNA of Italian *P. avium.*

Used primers (expected sequence length)	Accession numbers		BLASTn results		
	This paper	Reference sequence from GenBank	Identity (%)	Score (bits)	e-value
EF4-EF3 (1600bp, sequenced 921bp, 18S rDNA)	FJ936988	*Taphrina wiesneri* (AY495827)	98 (902/919)	1594	0.0
P3-M7 (252bp, 18S rDNA)	Identical to deposited sequence	*Taphrina wiesneri* (AY548293)	100 (252/252)	466	$2e^{-128}$
P2-M49* (690bp, spacers rDNA)	FJ936989 FJ936990	*Taphrina wiesneri* (DQ497616)	98 (601/610)	1080	0.0
EF1-forw/EF1-rev (727 bp, EF1_ protein)	FJ975773	*Taphrina wiesneri* (DQ479936)	98 (716/727)	1282	0.0
RPB1-forw/RPB1-rev (550bp, RPB1 protein)	FJ972197	*Taphrina wiesneri* (DQ471134)	98 (538/546)	949	0.0

*two variants deposited.

ed in December, May and October from 4 different trees: 1 tree from Portici, 2 from Pratovecchio and 1 from Ariano Irpino. PCR amplifications were performed using primers P3/M7 designed by us to specifically identify the *Taphrina* genus: BLASTn analyses of the sequences of the amplified products confirmed specificity of the primers because only 18S rRNA genes of *Taphrina* genus were found in the analyzed clones of each cherry tree.

The presence of *T. wiesneri* genome was also searched by analysing the sequences of genes coding for RNA polymerase II subunit 1 (RPB1) and for the elongation factor-1α (EF1-α) proteins, used for fungal phylogeny (Spatafora *et al.,* 2006). Genes were amplified on bud DNAs of two sweet cherry trees, one from Portici and one from Prato alle Cogne. Sequence determinations on the 5 clones of PCR products showed the presence of a single variant of the two genes. BLASTn analysis on EF1-α gene sequence showed 98% identity with the only *T. wiesneri* sequence and 97% with the only *T. deformans* sequence present in data bases. Similar analysis on RPB1 gene sequence showed 98% identity with the only *T. wiesneri* sequence and 81% identity with the two *T. deformans* sequences present in data bases. This adds evidence to the identification of *T. wiesneri* in DNA of *Prunus avium*.

Determination of *Taphrina* gene copy number and immunodetection. The relative numbers of *Taphrina* and sweet cherry genomes in the DNA extracted from cherry tree buds were calculated using a value of $2\text{-}4\times10^7$ for *Taphrina* genome size and of 3.38×10^8 for the genome of cherry tree (Arumuganathan and Earle, 1991). Values calculated from fluorescence intensities corresponding to different loading quantities of amplified DNA fragments (Fig. 2) resulted in a minimal value of about 0.3 fungal genomes per cherry tree genome, assuming a similar number of rDNA repetitions in both organisms. In fact, if the cherry tree should contain a larger number of rDNA transcriptional unit repetitions the estimate of the number of fungal genomes would increase.

In situ hybridization experiments were carried out on transversal sections of cherry tree buds, using a digoxigenin-labelled probe specific for *Taphrina* 18S rDNA. This probe, revealed by enzymatic immunoassay, identified series of dots that, although not clearly typical of fungi, might correspond to cross sections of hyphae (Fig. 3b). The unstained bud sections of Fig. 3a, and the Safranin and Light-green stained section of 3c, show that bud structures were well preserved. Adjacent serial sections stained, respectively, with Lactophenol-blue and with Trypan-blue (Fig. 3d and 3e) provided similar results. Detail of a structure stained with Trypan-blue is shown in panel f.

DISCUSSION

Our results indicate the presence of *T. wiesneri* in leaves and micro and mega-sporophylls inside closed buds of sweet cherry trees either free of leaf-curl or witches' broom or affected by CCRS. Repeated washings and sterilization aiming at removing externally contaminating organisms (Cardinali *et al*., 1994) did not substantially change the results. *T. wiesneri* was invari-

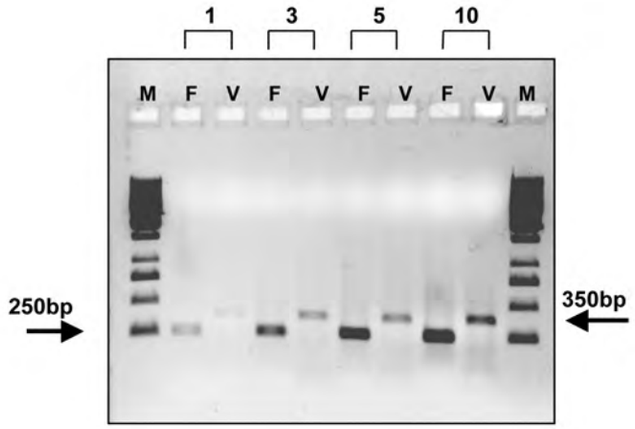

Fig. 2. Electrophoretic analyses of sweet cherry (V) and *Taphrina* (F) rDNA fragments amplified by PCR from the same DNA samples isolated from sweet cherry buds. The relative numbers of genomes were estimated from the relative fluorescence intensities of the respective amplified DNA bands. From left to right: 1, 3, 5, 10 µl loaded solution of PCR product. M = 1Kb marker DNA, bp = basepairs.

ably found in all tissues, including the inner reproductive structures of buds, as shown by amplification of fragments both of rDNA and of two genes coding for protein. Our search for a cherry tree in Campania and Tuscany not carrying *T. wiesneri* failed. This is different from the molecular detection of *T. deformans* in washings of some symptomless peach tree buds, before the appearance of leaf curl symptoms (Tavares *et al.*, 2004).

Analysis of the number of *T. wiesneri* and *P. avium* genomes in the same DNA samples extracted from buds of different trees (Fig. 2), indicates that the fungus is not only present occasionally, since a minimum number of about 0.3 *T. wiesneri* genomes per *P. avium* genome was found in different experiments. This ratio, calculated assuming a similar number of repetitions of rDNA genes in the fungus and in the plant, is increased if the plant genome has a higher number of repetitions of rDNA genes.

Surprisingly, a series of *in situ* hybridization experiments, using an rDNA probe specific for *Taphrina*, la-

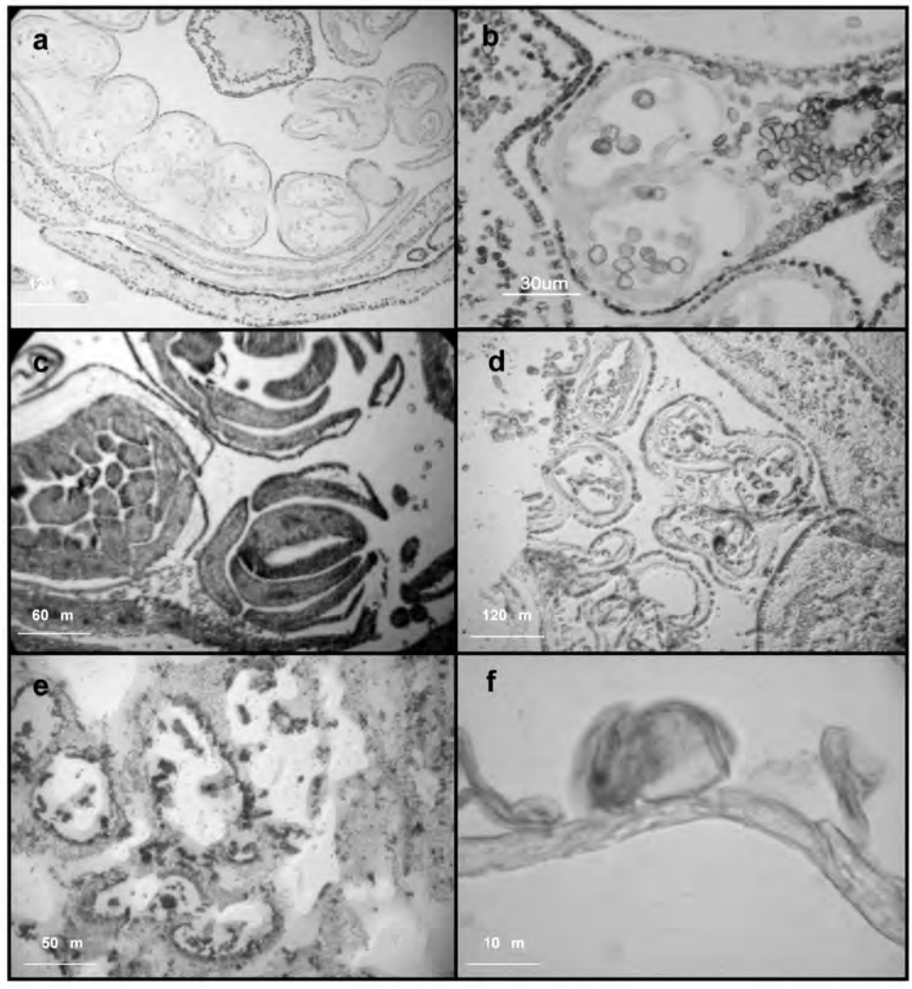

Fig. 3. Micrographs of cherry tree bud sections analyzed to detect *Taphrina wiesneri.* Panel (a) unstained; (b) hybridized *in situ* with 18S rRNA probe; (c) dual-stained with Safranin and Light-green; (d) stained with Lactophenol blue; (e) dual-stained with Trypan blue and Sudan III; (f) detail of the Trypan blue-stained section.

belled structures not clearly typical of fungi on symptomless sweet cherry tree bud sections. Similar results were observed using stains generally used to detect fungi (Fig. 3). On the other hand, details of the morphology of Trypan-blue stained structures shown in Fig. 3f, are intriguing for their clear interaction with a stain specific for chitin (Barrow, 2003). This poses a question concerning possible adaptive modification of the fungal structures mainly due to the fact that all analysed trees, in all habitats, showed no particular sign of *Taphrina* infection (leaf curl or witches' broom) (Schmitz, 1916; Mix, 1949). Moreover, some of the cherry trees analysed in Florence and Arezzo provinces, were in areas where no fungicide was applied during the investigation period.

Symptoms caused by *T. wiesneri* are described in all plant pathology textbooks not so much for their economic importance as for the peculiarity. In Campania, the disease is rarely observed and only on old and declining trees, often relicts of previously existing orchards. While the disease is now of no economic importance, the data reported here show that *T. wiesneri* is normally present not only in cherry leaves, but also inside buds suggesting that although normally innocuous, the fungus may turn pathogenic in particular conditions (Ogawa and English, 1991).

The finding that the same analytical procedure, based on PCR amplification, cloning of DNA fragments and sequence analysis of 10 individual clones, revealed no *T. wiesneri* in the buds of control trees (walnut, apricot and sour cherry), suggests that *T. wiesneri* is not an occasional contaminant. The results indicate the possibility that *T. wiesneri* may have established an endophyte-type of relation with the sweet cherry tree. This would be interesting because several plant species are reported to harbour endophytes (Redman *et al*., 2002; Arnold *et al*., 2003; Márquez *et al*., 2007) but none are fruit trees. It is interesting that *T. wiesneri* produces cytokinin (Johnston and Trione, 1974; Kern and Naef-Roth, 1975; Yamada *et al*., 1990; Matsuyama and Misawa, 1966) that might play a role in a mutual endophytic relationship.

As a final consideration, it should be remembered that many plant pathology text books on fruit trees suggest that *T. wiesneri* may be present in cherry tree tissues (Goidanich, 1964; Mac Swan and Koepsell, 1974; Atkinson, 1971; Ogawa and English, 1991). Our data support this idea and indicate that the invariable presence of *T. wiesneri* in healthy cherry trees may be due to endophytism.

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