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Characterisation of the yeast *Pichia membranifaciens* and its possible use in the biological control of *Botrytis cinerea*, causing the grey mould disease of grapevine

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Abstract

Pichia membranifaciens strain FY-101, isolated from grape skins, was found to be antagonistic to *Botrytis cinerea*, the causal organism of the grey mould disease of the grapevine. When grown together on solid as well as liquid media, the yeast brings about the inhibition of this parasitic fungus, coagulation and leakage of its cytoplasm, and suppression of its ability to produce the characteristic grey mould symptoms on the grapevine plantlets. In vitro experiments confirm that this yeast can be used as a biological control organism against *B. cinerea*. An account of the molecular characterisation of *P. membranifaciens* (complete sequence of the ITS region of its ribosomal DNA, GenBank accession No. AF 270935), as well as the interaction between *B. cinerea* and the yeast, are given here.

Keywords: Antagonism; Internal transcribed spacer; Nuclear ribosomal DNA; Grapevine; *Botrytis cinerea*; *Pichia membranifaciens*; *Vitis vinifera*

1. Introduction

Grey mould of the grapevine caused by *Botrytis cinerea* is a well known disease, and causes heavy losses of yield in table and wine grapes in many places around the world. The control of this and other fungal diseases of grapevine is mainly by use of chemical fungicides. Widespread use of chemical fungicides have certainly decreased the incidence of fungal diseases, but at the same time have contributed to the appearance of fungicide-resistant strains of the pathogens. Due to consumer resistance to chemical residues in food and public concern for environmental safety, there is an increasing demand to develop alternative methods for disease control [1].

One of the potential non-hazardous alternatives to the chemical fungicides is biological control, which consists of the use of biological processes to lower inoculum density of the pathogen in order to reduce crop loss [2]. Many microorganisms have been reported in the literature to suppress the pathogenic activity of *B. cinerea*. Bacteria on the surface of chrysanthemum leaves were found to inhibit the spores of this fungus [3]; *Bacillus circulans* has been reported to suppress *B. cinerea* and its pectinolytic activities, while *Bacillus* sp. inhibits the fungal pathogen while increasing host resistance by triggering the formation of stilbene-type phytoalexins [4,5]. The use of mycoparasites to control the incidence of *B. cinerea* is also well known. Fungi such as *Trichoderma* and *Gliocladium* have been extensively studied [6]. Species of *Pythium* have also been found to antagonise and suppress the grey mould pathogen [7].

Several species of yeast are also known to inhibit fungi. *Candida oleophila* is active against *B. cinerea* and has been used to protect apples after harvest [8]. Other yeasts are reported to be antagonists of a diverse group of phytopathogens: *Debaryomyces hansenii* against *Penicillium di-*

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gitatum on grapefruit, *Pichia guillermondii* against *Botrytis*, *Rhizopus*, and *Alternaria* rots of tomato fruits, *Cryptococcus laurentii*, *Cryptococcus flavus*, and *Cryptococcus albidus* against *Mucor* rot of pear, *Candida sake* against major postharvest pathogens of apple including *B. cinerea* and *Rhizopus nigricans* [9]. Some biocontrol products such as 'Aspire' using yeasts like *C. oleophila* have already been commercialised by Ecoscience Corporation (Worcester, MA, USA).

Yeasts are taxonomically diverse and include ascomycetes and basidiomycetes. A third group, the imperfect yeasts, have both ascomycetous and basidiomycetous affinities. The ascomycetous species are a heterogeneous group with the perfect states belonging to various genera. The morphological taxonomy of these organisms is usually difficult and time-consuming to assess. Increasingly the taxonomic description of fungi combines morphological descriptions and molecular data. Molecular taxonomy through DNA probes, restriction fragment length polymorphism (RFLP) of polymerase chain reaction (PCR)-amplified rDNA, RFLP of mitochondrial DNA, RFLP of total DNA, species-specific primers, karyotype analysis using pulsed field gel electrophoresis, and RAPDs are more and more used to finger print a wide range of microorganisms [10]. The PCR coupled to RFLP analysis (PCR-RFLP) has become a useful tool in fungal taxonomy and is currently used to identify different fungi [11]. Amplification of the ribosomal gene is used for the genetic identification of many organisms since it comprises both highly conserved sequences during evolution and highly variable sequences which resolve on various taxonomic scales. The ribosomal nuclear DNA consists of transcribed and non-transcribed regions. The ITS1 and ITS2 (internal transcribed spacers) are non-conserved regions and have been amplified with the PCR method using universal primers ITS1 and ITS4. Complete sequences of the ITS region of many yeast are currently available on the GenBank.

An account of the possible biological control of the grey mould disease of grapevine caused by *B. cinerea* by the application of *Pichia membranifaciens* is being described here for the first time. Morphological and molecular characteristics of the yeast, such as the sequences of the PCR amplified ITS region of its ribosomal nuclear DNA, are also given in this article.

2. Materials and methods

B. cinerea strain BCO3 used in this study was taken from the corresponding author's personal collection of the fungi and were grown on solid medium such as potato dextrose agar (PDA). Strain FY-101 of *P. membranifaciens* was isolated from the grape berries taken in the Burgundian region of France, and was grown on PDA, malt extract agar (MEA) and potato dextrose broth (PDB: same composition as PDA but devoid of agar) and also on YNB (yeast nitrogen broth). The agar plates were incubated at 25°C while the broths were placed on a rotary shaker at 25°C. Grapevine plantlets in glass tubes (vitro-plants) of *Vitis vinifera* cultivar 'Chardonnay' and 'Pinot noir' were taken from the 'Laboratoire des Sciences de la Vigne' of our institute, where they are grown on a regular basis.

2.1. Assay of antifungal activity

Antagonism between the *Botrytis* and the yeast was observed by placing both these organisms on the same PDA plate and incubating them at 25°C for 7 days. This was also done in liquid culture by introducing spores and mycelium of *B. cinerea* (BCO3) in a 3 day old PDB broth containing *P. membranifaciens* (FY-101) and incubating them at 25°C. Mycelium and spores of *B. cinerea* were taken out periodically for microscopic examination.

Infection on the two cultivars (Chardonnay and Pinot noir) of *V. vinifera* was done on 2 month old vitro-plants grown on MS medium (Murashig and Skoog). Three sets of six vitro-plants were used in inoculation experiments. Fifty microlitres of a fungal spore suspension of *B. cinerea* (3×10^5 spores ml^{-1}) was placed on the undersurface of the leaves of the first set of the vitro-plants, 50 μl of the mixture of BCO3+ *P. membranifaciens* was inoculated on the leaves of the second set of the vitro-plants, while the third set was inoculated with 50 μl of the broth containing only *P. membranifaciens*.

2.2. DNA isolation and PCR

The yeast *P. membranifaciens* was grown in PDB. The culture condition, DNA isolation and the PCR amplification of the complete ITS region of its nuclear ribosomal DNA were done as described earlier [12] using universal primers ITS-1 (TCC GTA GGT GAA CCT GCG G) and ITS-4 (TCC TCC GCT TAT TGA TAT GC). The primers were synthesised and the DNA sequence was realised by Oligo Express (Paris, France). The ITS sequences of *P. membranifaciens* were compared with those of related species and were submitted to the GenBank.

3. Results

The yeast *P. membranifaciens*, when grown on 5% MEA (malt extract agar) for 3 days at 25°C, showed cells which were oval to elongate, occurred singly, in pairs, in chains or clusters (Fig. 1). Growth was yellowish-tan, dull and smooth. When viewed under the microscope, *P. membranifaciens* had moderately branched pseudohyphae. True hyphae were not observed.

When *B. cinerea* (BCO3) was grown together with the antagonist yeast *P. membranifaciens* on the same agar plate, a small zone of inhibition appeared around the yeast

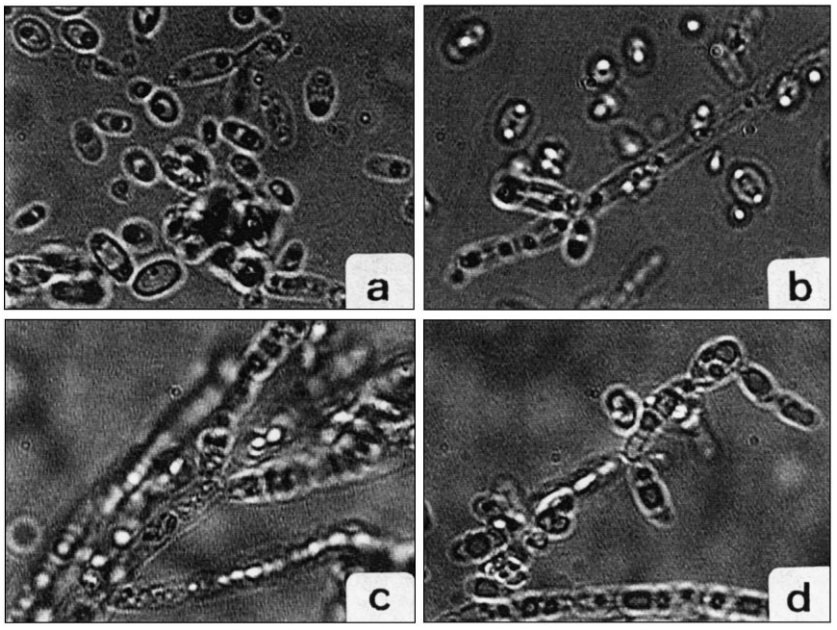

Fig. 1. *P. membranifaciens* strain FY-101. a: Oval to cylindrical isolated cells; b: budding; c,d: pseudo-hyphae. All panels, bar = 25 μm.

inoculum. Hyphae developing in the vicinity of the inhibition failed to sporulate. When the mycelium of BCO3 was grown together with *P. membranifaciens* strain FY-101 in PDB broth, it failed to germinate and produce the greyish colonies on fresh PDA plates. Microscopic examination of the BCO3 mycelium in contact with *P. membranifaciens* showed extensive coagulation of its protoplasm, and many of the hyphal cells were observed to be completely empty (Fig. 2).

Experiments with the grapevine vitro-plants showed that, when inoculated by *B. cinerea*, the plants developed the characteristic grey mould symptoms and eventually died, while the second set of vitro-plants inoculated with a mixture of fungal conidia and *P. membranifaciens* were fully developed, vigorous and viable. The third set of vitro-plants inoculated with *P. membranifaciens* (FY-101) were perfectly healthy (Fig. 3).

The ITS region and the flanking 18S, 5.8S, and 28S

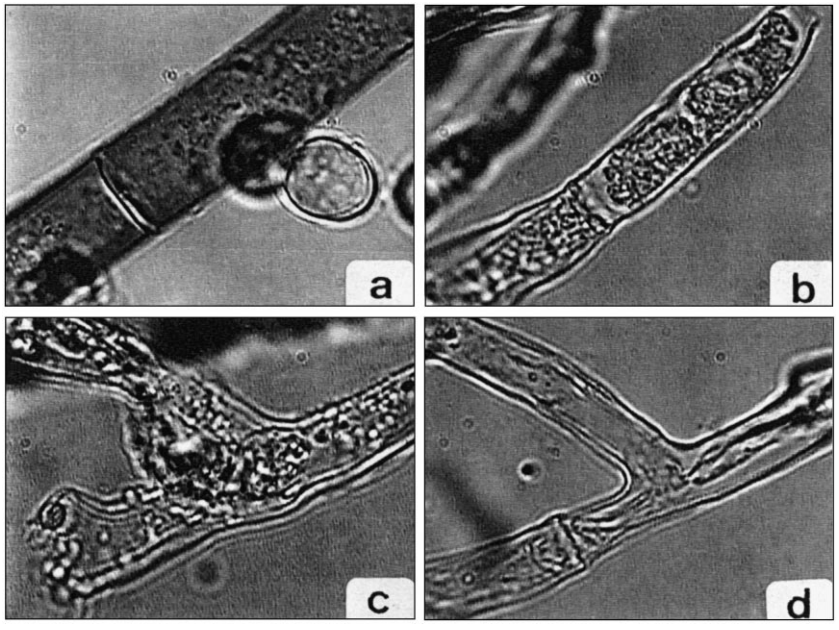

Fig. 2. *B. cinerea* strain BCO3. a: Normal hypha; b: coagulation of hyphal contents; c,d: emptied hyphal cells in contact with the yeast. All panels, bar = 25 μm.

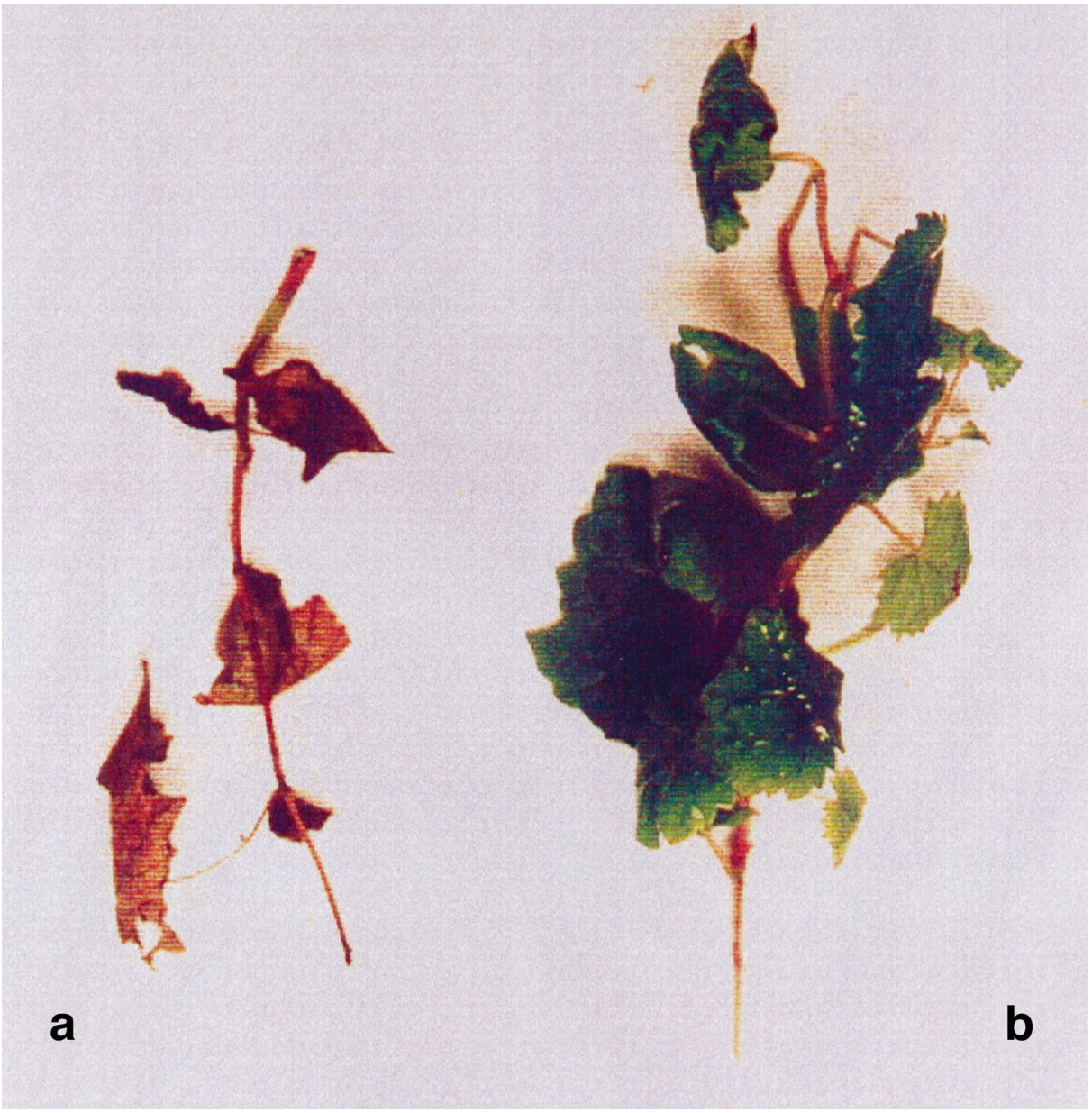

Fig. 3. *V. vinifera*. a: Plant infected with *B. cinerea* showing grey mould symptoms; b: vigorous grapevine plant inoculated with a mixture of *B. cinerea* and *P. membranifaciens*.

genes of the nuclear ribosomal DNA of *P. membranifaciens* are comprised of the following bases: 1–7 = **18S** gene (partial sequence), 8–95 = **ITS1**, 96–252 = **5.8S** gene, 253–397 = **ITS2**, 398–451 = **28S** gene (GenBank accession No. AF 270935):

1 **atcatta**ctg tgattatacc aacaccacac tgtgtgggcg cacaaaacac ctaaacctgg
61 agtatacaca cgtcaacaaa agatctaaaa gaa-ta**aaact ttcaacaacg gatctcttgg**
121 ttctcgcatc gatgaagagc gcagcgaaat gcgataccta gtgtgaattg cagccatcgt
181 gaatcatcga gttcttgaac gcacattgcg cccgtcggta ttccggcggg catgcctgtc
241 tgagcgtcgt ttccttcttg tgcaccgcgg ggtctttgca gatcctctct gcgcagagct
301 ggccgtgcca ctggcccggc cgaaaagaaa cgttgcggac gaagcgaact acatcgggac
361 gctttggccg ccgagcgaaa aaaaaacacc att-gagc**tcg acctcagatc aggtaggagt**
421 acccgctgaa cttaagcata tcaataagcg g

The multiple sequence alignment of the ITS regions of *P. membranifaciens* and those of related yeast species are given in Fig. 4.

4. Discussion

Many yeast strains are known to exhibit antagonism

Fig. 4. CLUSTAL W. (1.8) multiple alignment of *P. guillermondii*, *C. sake*, *B. custersianus*, *D. anomala*, *P. membranifaciens*, *I. orientalis* and *P. fermentans*.

against *B. cinerea* causing postharvest diseases. The high frequency of yeasts among the antagonistic agents reported could be related to the fact that yeasts are tolerant to extreme environmental conditions of storage (temperature close to 0°C, high relative humidity etc.) and also because they are adapted to high sugar concentrations, high osmotic pressure and tolerant of low pH [8].

Our study shows that *P. membranifaciens* is a good antagonistic agent towards *B. cinerea* causing the grey mould disease of the grapevine. The yeast may bring about the destruction of *B. cinerea* by secreting exo- and endo-β-1,3-glucanases (unpublished) like those observed in the case of *Trichoderma harzianum* [13], *P. guillermondii* [14] and *Serratia marescens* [15], but other enzymes may be involved as well [15].

The ITS region of the ribosomal nuclear DNA of *P. membranifaciens* is comprised of 390 (ITS1+5.8S+ITS2) bases, and has a very small ITS1 region of only 88 bases. As far as our knowledge is concerned this constitutes the smallest ITS1 region amongst all the yeasts, and hence it shows few similarities when compared with others. The closest yeast is *Issatchenkia orientalis* (GenBank No. AF 246989) with a 73.0% similarity. Other yeasts have fewer degrees of resemblance: *Pichia fermentans* (AF 218998) 68.6%; *Brettanomyces custersianus* (AF 043511) 64.1%; *C. sake* (AF 013529) 62%; *Dekkera anomala* (AF 043510) 58.7%; and *P. guillermondii* (AB 032176) 53.9%.

The mycelium of *B. cinerea* infected with *P. membranifaciens* failed to develop the characteristic grey mould symptoms when re-inoculated onto grapevine, and since *P. membranifaciens* is not at all pathogenic to *V. vinifera*, it should be an effective biocontrol agent against *B. cinerea*. However, field trials and formulations have to be studied before its acceptance as a biofungicide.

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