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# Potential for biocontrol of *Pleurotus ostreatus* green mould disease by *Aureobasidium pullulans* De Bary (Arnaud)

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## ABSTRACT

*Pleurotus ostreatus*, commonly known as “oyster mushroom”, is an edible fungus economically important worldwide. The green mould, caused by *Trichoderma pleuroti* and *T. pleuroticola*, is a very important fungal disease, and it is commonly controlled by the use of fungicides. The yeast-like fungus *Aureobasidium pullulans* is a biocontrol agent naturally found throughout a wide range of habitats. The effect of *A. pullulans* L1 and L8 strains on *P. ostreatus*, *T. pleuroti* and *T. pleuroticola* was studied in *in vitro* assays. Both yeast strains resulted compatible with *P. ostreatus* growth, and effective in reducing the *T. pleuroticola* and *T. pleuroti* colony growth. The inhibitory effect of L1 and L8 was similar in the majority of the *Trichoderma*-*A. pullulans* combinations on agar plates. Both strains were more efficient than *Trichoderma* in substrate colonization, and produced volatile and nonvolatile metabolites which reduced *Trichoderma* growth. When the activity of L1 and L8 was tested against the green mould disease of *P. ostreatus* under controlled conditions similar to those of a mushroom farm, only L8 was effective in controlling the disease. It showed an effect similar to that of the fungicide prochloraz against *T. pleuroticola*, the less aggressive pathogen, and lower than that of the fungicide against *T. pleuroti*, the most aggressive. The antagonism was the result of mechanisms like antibiosis and competition for space and nutrients, whereas the direct attachment of *A. pullulans* with hyphae of the pathogens did not play a role.

## 1. Introduction

*Pleurotus ostreatus* (Jacq.) Kumm., commonly known as “oyster mushroom”, is an edible basidiomycete fungus economically important worldwide. It is susceptible to a wide range of biotic diseases, and green mould is one of the most important fungal diseases (Sharma and Vijay, 1996; Woo et al., 2004, 2009; Kredics et al., 2006; Park et al., 2006; Hatvani et al., 2007; 2012; Alfonso et al., 2008; Sobieralski et al., 2012; Innocenti et al., 2019) which is caused by *Trichoderma pleuroti* S. H. Yu and M.S. Park (2006; previously *T. pleurotum*) and *T. pleuroticola* S. H. Yu and M. S. Park (2006) (Hatvani et al., 2007; Komon-Zelazowska et al., 2007; Innocenti et al., 2019). Typical symptoms of the disease are green sporulation areas on the surface of cultivation substrate that is exposed to the green mould infection mostly during the spawn run phase.

The disease control is commonly based on fungicide application, and currently only prochloraz is allowed for use in mushroom farms in Italy (Sponix Flow, Adama Makhteshim Chemical Works Ltd, Beer-Sheva, Israel, Reg. n. 13671 16/03/2007, modified 19/06/2017, art.

7(1), D.P.R. n. 55/2012). However, in accordance with the aims of the European Regulation EC 1107/2009, concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC, priority should be given to non-chemical and natural alternatives wherever possible. Considering also the consumer demand of healthy foods obtained with cultivation system with low environmental impact, products based on antagonistic microorganisms could be useful tools. *Bacillus amyloliquefaciens*, *B. subtilis* and *B. licheniformis* isolates seemed to be appropriate to be developed as biocontrol products to overcome the *Trichoderma* green mould problem of *P. ostreatus* (Nagy et al., 2012; Mwangi et al., 2017). A product based on *B. amyloliquefaciens* D747 strain (Amylo-X<sup>®</sup>, Biogard, CBC (Europe) S.r.l., Nova Milanese, MB, Italy) is commercialized in Italy only against *T. aggressivum* which is responsible of *Agaricus bisporus* green mould.

*Aureobasidium pullulans* (De Bary) Arnaud is a yeast-like fungus, naturally found throughout a wide range of terrestrial and aquatic habitats under temperate, tropical and arctic environment (Samson et al., 2004; Gunde-Cimerman et al., 2000; Zalar et al., 2008), and able to live under controlled atmosphere gases (Martínez-Romero et al., 2012). *Aureobasidium pullulans* is well known for its biotechnological significance

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as a producer of the biodegradable extracellular polysaccharide pullulan, and of a variety of the hydrolytic enzymes (Zalar et al., 2008). It naturally occurs as endophyte (Pugh and Buckley, 1971; Martini et al., 2009; Parsa et al., 2016) or epiphyte (Andrews, 2006) of a wide range of plant species and tissues. *Aureobasidium pullulans* showed biocontrol activity against postharvest diseases such as nectarine brown rot (Janisiewicz et al., 2010; Mari et al., 2012), kiwifruit grey mould (Di Francesco et al., 2018b), and strawberry rot (Lima et al., 1997). It was effective also against field diseases such as vineyard sour rot (Dimakopoulou et al., 2008), tomato late blight (Di Francesco et al., 2017), and winter wheat stem base diseases (Wachowska and Borowska, 2014). Mechanisms involved in its biocontrol activity are competition for nutrients, antibiosis, parasitism, and induction of plant resistance (Ippolito et al., 2000; Bencheqroun et al., 2007; Zhang et al., 2010; Di Francesco et al., 2015b, 2017). To our knowledge, no studies have been carried out on *A. pullulans* potential for the biological control of *P. ostreatus* green mould disease.

The aim of this study was to evaluate: (i) the compatibility of *A. pullulans* with *P. ostreatus* growth in *in vitro* assays; (ii) the effect of *A. pullulans* against *T. pleuroti* and *T. pleuroticola* growth in *in vitro* assays; (iii) the potential biocontrol effect against green mould disease caused by *Trichoderma* under controlled conditions similar to those of a mushroom farm.

## 2. Materials and methods

### 2.1. *Aureobasidium pullulans*, *Trichoderma* spp. and *Pleurotus ostreatus*

In all experiments *A. pullulans* L1 and L8 strains were used. They were isolated from “Red haven” peach carposphere harvested in the experimental orchard of Bologna University located in Cadriano, Bologna, Italy (Mari et al., 2012). They were molecularly identified (Di Francesco et al., 2018a) and maintained on nutrient yeast dextrose agar (NYDA, 8 g L<sup>-1</sup> nutrient broth, 5 g L<sup>-1</sup> yeast extract, 10 g L<sup>-1</sup> dextrose, 25 g L<sup>-1</sup> technical agar (Oxoid, UK), at 4 °C in the dark, in the culture collection of CRIOF – Department of Agricultural and Food Sciences (Distal), University of Bologna. For the experiments, they were transferred on NYDA plates and incubated at 25 °C for two days in the dark. Yeast cell suspensions were then obtained by adding distilled sterile water containing 0.05% (v/v) Tween 80, and by scraping the colony surface with a sterile loop. The final concentration used in all experiments was 10<sup>8</sup> cells mL<sup>-1</sup>.

*Trichoderma pleuroticola* AFS 432 (Tpa 432) and AFS 488 (Tpa 488), and *T. pleuroti* AFS 492 (Tpi 492) and AFS 497 (Tpi 497) strains were used. They were isolated from green mould-affected samples of wheat straw substrate of *P. ostreatus* from a mushroom farm located in Emilia Romagna, Italy. They were molecularly identified by amplification of the *tefl* gene fragment (Innocenti et al., 2019), and maintained in potato dextrose broth (PDB, Difco) added with 15% glycerol at -80 °C, in the culture collection of Distal, University of Bologna, under AFS codes.

The commercial *P. ostreatus* strains ‘Soppo’ (PoSp; Sylvan, Somycel, Langeais, France) and K12 (Micelio Fungisem S.A., La Rioja, Spain), widely cultivated in Italian mushroom farms, were utilised. The spawn consisted in sterile millet seeds colonised by the mycelium.

### 2.2. Effect of *Aureobasidium pullulans* on *Pleurotus ostreatus* and *Trichoderma* colony growth

The effect of *A. pullulans* L1 and L8 strains on the mycelial growth of PoSp and K12, Tpa 432 and 488, and Tpi 492 and 497 was investigated by the following assays: (i) dual culture; (ii) volatile organic compounds (VOCs); (iii) nonvolatile organic compounds (NVOCs). The mycelium of *P. ostreatus* PoSp and K12 was obtained by plating millet

kernels of commercial spawns on Potato Dextrose Agar (PDA, 39 g L<sup>-1</sup>, Oxoid, UK) for 7 days at 20 °C in the dark.

In the dual culture assay, each *P. ostreatus* or *Trichoderma* strain was separately co-cultured with each yeast strain on PDA plates. In each plate, a mycelial plug (6 mm in diam.) from 7 day-old colony of *P. ostreatus* or *Trichoderma* was inoculated at 30-mm distance from the plate edge. *Aureobasidium pullulans* inoculum (100 µL, 10<sup>8</sup> cells mL<sup>-1</sup>) from 48 h-old colony on NYDA was streaked by a sterile loop 30 mm from the plug (Di Francesco et al., 2017). In control plates, only *P. ostreatus* or *Trichoderma* plugs were inoculated. Plates were incubated at 25 °C, in the dark for 3 days, then the radius of fungal colony was measured on a line from the centre of the plug to yeast colony.

For the VOC assay, the technique of Dennis and Webster (1971a) was used. *Aureobasidium pullulans* was inoculated by spreading 100 µL of suspension, 10<sup>8</sup> cells mL<sup>-1</sup> (Di Francesco et al., 2015b) in each NYDA plate. After 48-h incubation at 25 °C, the lid of the plate was replaced by a bottom PDA plate inoculated with 6 mm *P. ostreatus* or *Trichoderma* mycelial plug, then the two bottoms were tapped together with a double layer of Parafilm. In the control plates, PDA bottoms not inoculated with yeast strains were tapped together with *P. ostreatus* or *Trichoderma* bottoms. The diameter of *Trichoderma* or *P. ostreatus* colony was measured after 25 °C incubation in the dark respectively for 3 or 7 days.

For the NVOC assay, the technique of Dennis and Webster (1971b) was used. A cell suspension (100 µL) of each *A. pullulans* strain obtained from 48 h-old colonies on NYDA, was spread on a sterile cellophane layer PTT (Safta, PC, Italy) positioned on a 20 mL NYDA plate. In control plates, 100 µL of sterile water was spread on the cellophane. After 48-h incubation at 25 °C, the cellophane was removed, and a mycelial plug (6 mm diam.) of each *P. ostreatus* or *Trichoderma* was inoculated in the centrum. The diameter of *Trichoderma* or *P. ostreatus* colony was measured after 25 °C incubation in the dark respectively for 3 or 7 days.

In all experiments, three plates (replicates) were used for each combination and for the controls.

### 2.3. *In vivo* assay

The *P. ostreatus* growing substrate was prepared by using the following technique similar to that used in mushroom farm. Chopped wheat straw (2–5 cm) was steam pasteurised at 90 °C for 1 h (Sobieralski et al., 2012) and added to PoSp spawn (2% v/w). An amount of 100 g of substrate + PoSp was then distributed in each perforated transparent plastic food container (15 × 8 × 10 cm). The substrate was treated with 20 mL of *A. pullulans* L1 or L8 (2.4 × 10<sup>7</sup> cells/g substrate) suspension separately, or with prochloraz (Sponix Flow, 450 g L<sup>-1</sup>, Adama Makhteshim LTD, Beer-Sheva, Israel) at 1.25 µL L<sup>-1</sup> dose (field dose) as chemical control. *Trichoderma* inoculum was obtained from abundantly sporulating colonies on PDA plates maintained at 25 °C for 4 days in the dark, then under natural light. A 3 mL Tpa 432 or Tpi 492 water spore suspension was separately inoculated in the substrate (5 × 10<sup>3</sup> spore/g substrate) immediately after yeast or prochloraz treatment.

Each container was then wrapped in a plastic bag closed by a cotton plug. Controls consisted in containers with PoSp + water (23 mL), PoSp + *Trichoderma* (3 mL) + water (20 mL), PoSp + L1 (20 mL) + water (3 mL) and PoSp + L8 (20 mL) + water (3 mL). The treatment prochloraz + L8 was not considered, because in a preliminary *in vitro* assay, prochloraz completely inhibited the yeast colony growth. The time and the dose of prochloraz treatment were comparable to those used in mushroom farms, where the fungicide is applied to substrate once at spawn. Six replicates were performed for each treatment for a total of 54 containers that were located in a growth chamber following a complete randomized design at 23–25 °C and at 80%

relative humidity. Four weeks later, during the spawn run phase, the colonisation of substrate by *Trichoderma* was visually assessed for each container using a six-point scale where: 0, no colonisation; 1, sporadic growth few small green areas; 2, growth (colonization) less than 20% of substrate colonized by green mould; 3, 20–50% of colonized substrate; 4, 51–70% of colonized substrate and 5, more than 70% colonisation by green mould (Innocenti et al., 2019, modified). The colonization index for each treatment and control was then calculated as the mean of the values of the replicates. The efficacy of green mould disease control was calculated with the following formula:  $((Ic - It)/Ic) \times 100$ , where  $Ic$  is the colonization index in the control and  $It$  is the colonization index in the treatment. At the end of the experiment, *A. pullulans* was randomly re-isolated from the substrate and identified as described by Di Francesco et al. (2018a).

All *in vitro* and *in vivo* experiments were conducted twice with similar results. The data of both experiments are reported.

## 2.4. Statistical analysis

Data were subjected to two-way ANOVA. Separation of means, when possible, was performed using the Student Newman Keuls test. Data were reported as mean values  $\pm$  standard deviation (SD). Analyses were performed with the software Statgraphic Plus Version 2.1 (Statistical Graphics Corp., USA 1996).

## 3. Results

### 3.1. Effect of *Aureobasidium pullulans* on *Pleurotus ostreatus* colony growth

The co-culturing of *A. pullulans* L1 and L8 and PoSp and K12 showed that the colony growth of both strains of *P. ostreatus* were not influenced by yeast strains. Indeed two-way ANOVA analysis showed the following values for what concerns the experiment 1, *A. pullulans* strain factor,  $F = 0.09$  and  $P = 0.92$ , for *P. ostreatus* strain factor,  $F = 1.25$  and  $P = 0.29$ , and for interaction between the two factors,  $F = 0.87$  and  $P = 0.44$ . For what concerns the experiment 2, values are the following: *A. pullulans* strain factor,  $F = 1.53$  and  $P = 0.26$ , for *P. ostreatus* strain factor,  $F = 0.25$  and  $P = 0.62$ , and for interaction between the two factors,  $F = 0.07$  and  $P = 0.93$ .

The effect of L1 and L8 VOCs on PoSp and K12 colony growth is reported in Table 1. In both experiments, *A. pullulans* L8 significantly increased the mean value of both *P. ostreatus* strains colony diameter in comparison to the mean of untreated controls. The effect of L1 treat-

**Table 1**

Effect of volatile organic compounds produced by *Aureobasidium pullulans* L1 and L8 strains on colony diameter of *Pleurotus ostreatus* ‘Spoppo’ and ‘K12’ strains, 7 days after inoculation.

Treatment	<i>P. ostreatus</i> diameter (mm)		Mean
	K12	PoS <span>P</span>	
<i>Experiment 1</i>			
<i>A. pullulans</i> L1	68.7 ± 1.2	57.0 ± 4.6	62.8 ± 7.1 a
<i>A. pullulans</i> L8	84.0 ± 5.3	65.7 ± 4.5	74.8 ± 11.0 b
Untreated	64.3 ± 5.1	58.7 ± 4.9	61.5 ± 5.6 a
Mean	70.3 ± 9.7 B	60.4 ± 5.7 A	
<i>Experiment 2</i>			
<i>A. pullulans</i> L1	67.0 ± 1.7	56.0 ± 4.0	61.5 ± 6.6 a
<i>A. pullulans</i> L8	86.0 ± 5.2	66.3 ± 4.2	76.2 ± 11.6 b
Untreated	64.7 ± 4.5	56.7 ± 6.1	60.7 ± 6.5 a
Mean	72.6 ± 10.7 B	59.7 ± 6.5 A	

In both experiments, treatment and *P. ostreatus* strain factors are significant and their interaction is not significant, according to two-way ANOVA ( $P < 0.05$ ). Mean values  $\pm$  SD followed by the same upper case letter in a line, and by the same lower case letter in a column are not significantly different according to SNK test ( $P < 0.01$ ).

ment was not different from that of untreated control. The mean diameter of *P. ostreatus* K12 colonies was significantly higher than that of PoSp independently from treatment.

The effect of L1 and L8 NVOs on PoSp and K12 colony growth is reported in Table 2. In both experiments, the mean colony diameter of *P. ostreatus* strains was significantly increased by *A. pullulans* L8 in comparison to control means. *Pleurotus ostreatus* colony treated with the strain L1 showed a similar growth of untreated control. The mean diameter of *P. ostreatus* K12 colony was significantly higher than that of PoSp.

### 3.2. Effect of *Aureobasidium pullulans* on *Trichoderma* colony growth

The co-culturing of *A. pullulans* and *Trichoderma* strains showed that the colony growth of all *Trichoderma* strains were influenced by yeast strains (Table 3). In both experiments, the colony growth of Tpi 492 and Tpi 497 was reduced by L1 and L8, and no significant differences were observed between the two *A. pullulans* strains. Both yeast strains reduced more Tpi 492 colony growth than that of Tpi 497. The untreated control Tpi 492 showed a lower growth than that of Tpi 497.

**Table 2**

Effect of nonvolatile organic compounds produced by *Aureobasidium pullulans* L1 and L8 strains on colony diameter of *Pleurotus ostreatus* ‘Spoppo’ and ‘K12’ strains 7 days after inoculation.

Treatment	<i>P. ostreatus</i> diameter (mm)		Mean
	K12	PoS <span>P</span>	
<i>Experiment 1</i>			
<i>A. pullulans</i> L1	64.0 ± 4.6	54.3 ± 5.5	59.2 ± 7.0 a
<i>A. pullulans</i> L8	71.0 ± 1.0	60.0 ± 1.0	65.5 ± 6.1 b
Untreated	62.0 ± 3.0	54.1 ± 3.6	59.0 ± 6.2 a
Mean	66.3 ± 4.5 B	56.1 ± 4.4 A	
<i>Experiment 2</i>			
<i>A. pullulans</i> L1	65.3 ± 4.5	54.0 ± 7.0	59.7 ± 8.1 a
<i>A. pullulans</i> L8	72.7 ± 2.5	61.0 ± 2.0	66.8 ± 6.7 b
Untreated	64.0 ± 3.0	56.3 ± 2.5	60.2 ± 4.9 a
Mean	67.3 ± 5.0 B	57.1 ± 4.9 A	

In both experiments, treatment and *P. ostreatus* strain factors are significant and their interaction is not significant, according to two-way ANOVA ( $P < 0.05$ ). Mean values  $\pm$  SD followed by the same upper case letter in a line, and by the same lower case letter in a column are not significantly different according to SNK test ( $P < 0.01$ ).

**Table 3**

Effect of *Aureobasidium pullulans* L1 and L8 strains on colony growth (mm) of *Trichoderma pleuroti* AFS 492 and AFS 497, and *T. pleuroticola* AFS 432 and AFS 488 after 3 days of incubation on PDA plates in co-culturing conditions.

Treatment	<i>T. pleuroti</i>		<i>T. pleuroticola</i>	
	AFS 492	AFS 497	AFS 432	AFS 488
<i>Experiment 1</i>				
<i>A. pullulans</i> L1	16.7 $\pm$ 2.1 aA	20.0 $\pm$ 0.0 aB	20.7 $\pm$ 1.2 bA	19.7 $\pm$ 1.5 aA
<i>A. pullulans</i> L8	15.8 $\pm$ 4.0 aA	20.8 $\pm$ 1.3 aB	15.7 $\pm$ 0.8 aA	20.0 $\pm$ 1.0 aB
Untreated	20.3 $\pm$ 2.1 bA	33.0 $\pm$ 1.0 bB	30.0 $\pm$ 0.0 cA	34.0 $\pm$ 1.0 bB
<i>Experiment 2</i>				
<i>A. pullulans</i> L1	15.7 $\pm$ 0.6 aA	21.0 $\pm$ 1.0 aB	21.3 $\pm$ 1.5 aA	20.3 $\pm$ 2.1 aA
<i>A. pullulans</i> L8	16.4 $\pm$ 2.0 aA	21.4 $\pm$ 1.9 aB	19.7 $\pm$ 0.6 aA	22.7 $\pm$ 1.0 aB
Untreated	21.3 $\pm$ 2.1 bA	30.3 $\pm$ 1.5 bB	31.0 $\pm$ 1.0 bA	35.3 $\pm$ 1.5 bB

In both experiments, *Trichoderma* species, *Trichoderma* strain, and treatment (L1 and L8) factors and their interaction are significant according to two-way ANOVA ( $P < 0.05$ ). Mean values  $\pm$  SD followed by the same upper case letter in a line and by the same lower case letter in a column are not significantly different according to SNK test ( $P < 0.01$ ).

In both experiments, L1 and L8 strains significantly reduced the colony growth of Tpa 432 and Tpa 488 compared to untreated controls. In experiment 1, *A. pullulans* L8 reduced Tpa 432 colony growth more than L1, and in experiment 2, no difference was found between the two yeast strains. In both experiments, L1 and L8 strains showed a similar effect in reducing Tpa 488 colony growth. The L8 strain reduced Tpa 432 colony growth more than Tpa 488 in both experiments. The untreated control Tpa 432 showed a lower growth than that of Tpa 488.

For what concerns the effect of L1 and L8 VOCs on Tpi 492 and Tpi 497 colony growth (Table 4), two-way ANOVA indicated that, in the experiment 1, there was no significant interaction between treatment and *Trichoderma* strain factors, then a comparison was made among the mean values of each factor. Both L1 and L8 yeast strains similarly reduced *Trichoderma* colony growth with respect to untreated control. Mean value of Tpi 492 growth was significantly lower than that of Tpi 497. In experiment 2, there was significant interaction between treatment and *Trichoderma* strain factors. L1 and L8 VOCs significantly and similarly reduced Tpi 492 and Tpi 497 colony growth with respect to untreated control. Both yeast strains reduced Tpi 492 colony growth more than that of Tpi 497. For what concerns *T. pleuroticola*, in both experiments L1 and L8 strains significantly reduced Tpa 432 and Tpa 488 colony growth compared to untreated controls, and similarly reduced the colony growth of Tpa 432 strain. In the experiment 2, L8 strain reduced Tpa 488 colony growth more than L1. Both yeast strains reduced the Tpa 432 colony growth more than that of Tpa 488.

The effect of NVOCs produced by L1 and L8 on *Trichoderma* colony growth is reported in Table 5. For what concerns *T. pleuroti*, in both experiments, L1 and L8 strains significantly reduced the colony growth of Tpi 492 and Tpi 497 compared to the untreated control. In the first experiment, L8 strain reduced Tpi 492 colony growth more than L1. In both experiments, the two *A. pullulans* strains similarly reduced Tpi 497 colony growth. For what concerns *T. pleuroticola*, two-way ANOVA indicated no significant interaction between treatment and *Trichoderma* strains factors, then a comparison was made among the mean values of each factor for both experiments. *Aureobasidium pullulans* L1 and L8 similarly reduced *T. pleuroticola* colony growth independently of *Trichoderma* strain. The mean growth of Tpa 488 was similar to that of Tpa 432.

### 3.3. In vivo assay

In this assay, Tpa 432 and Tpi 492 were used, due to the fact that in *in vitro* experiments they were the most sensitive strains to *A. pullulans*. No symptoms of green mould disease were observed in uninfected control. The effect of L1 and L8 yeast strains against the green mould disease of *P. ostreatus* 'Spoppo' strain is reported in Table 6. The L8 treatment was significantly effective against Tpi 492 by reducing the substrate colonization index by 18.8% and by 30% compared with that of the infected control in experiment 1 and 2, respectively. In both experiments, L1 treatment was not effective against the disease caused by Tpi 492. Prochloraz was the most effective treatment against Tpi 492 disease, it reduced the colonization index of substrate by 62.5% and by

**Table 4**

Effect of volatile organic compounds produced by *Aureobasidium pullulans* L1 and L8 strains on colony growth (mm) of *Trichoderma pleuroti* AFS 492 and AFS 497 and *T. pleuroticola* AFS 432 and AFS 488 after 3 days of incubation on PDA plates.

Treatment	<i>T. pleuroti</i>			<i>T. pleuroticola</i>	
	AFS 492	AFS 497	Mean	AFS 432	AFS 488
<i>Experiment 1</i>					
<i>A. pullulans</i> L1	51.3 ± 4.2	73.7 ± 6.5	62.5 ± 13.2 a	60.3 ± 6.8 aA	72.3 ± 2.5 aB
<i>A. pullulans</i> L8	49.3 ± 4.5	66.3 ± 3.2	57.8 ± 9.9 a	56.3 ± 5.1 aA	74.3 ± 4.0 aB
Untreated	73.3 ± 2.0	90.0 ± 0.0	81.7 ± 9.2 b	90.0 ± 0.0 bA	90.0 ± 0.0 bA
Mean	58.0 ± 11.9 A	76.7 ± 11.1 B			
<i>Experiment 2</i>					
<i>A. pullulans</i> L1	55.6 ± 2.1 aA	69.0 ± 1.0 aB		49.7 ± 3.8 aA	74.7 ± 5.5 bB
<i>A. pullulans</i> L8	56.3 ± 3.2 aA	64.0 ± 3.6 aB		52.3 ± 2.1 aA	65.3 ± 3.2 aB
Untreated	88.7 ± 1.0 bA	87.0 ± 2.6 bA		83.0 ± 2.6 bA	88.3 ± 1.5 cA

In experiment 1, *T. pleuroti*, treatment (L1 and L8) and strain (AFS 492 and 497) factors are significant, and their interaction is not significant. For *T. pleuroticola*, treatment (L1 and L8) and strain (AFS 432 and 488) factors, and their interaction are significant, according to two-way ANOVA ( $P < 0.05$ ). In experiment 2, for both *T. pleuroti* and *T. pleuroticola*, treatment and strain factors, and their interaction are significant, according to two-way ANOVA ( $P < 0.05$ ). Mean values ± SD followed by the same upper case letter in a line and by the same lower case letter in a column are not significantly different according to SNK test ( $P < 0.01$ ).

**Table 5**

Effect of nonvolatile organic compounds produced by *Aureobasidium pullulans* L1 and L8 strains on colony growth (mm) of *Trichoderma pleuroti* AFS 492 and AFS 497, and *T. pleuroticola* AFS 432 and AFS 488 after 3 days of incubation on PDA plates.

Treatment	<i>T. pleuroti</i>		<i>T. pleuroticola</i>		
	AFS 492	AFS 497	AFS 432	AFS 488	Mean
<i>Experiment 1</i>					
<i>A. pullulans</i> L1	35.7 ± 1.5 bA	38.6 ± 1.6 aA	46.3 ± 1.5	42.3 ± 4.0	44.3 ± 3.5 a
<i>A. pullulans</i> L8	22.0 ± 1.0 aA	37.0 ± 1.7 aA	47.7 ± 2.5	40.7 ± 1.5	44.2 ± 4.3 a
Untreated	59.0 ± 2.6 cA	66.7 ± 0.6 bB	77.7 ± 0.6	76.3 ± 0.6	77.0 ± 0.9 b
Mean			57.2 ± 15.4 A	53.1 ± 17.6 A	
<i>Experiment 2</i>					
<i>A. pullulans</i> L1	36.3 ± 2.3 aA	38.1 ± 2.6 aA	45.3 ± 1.5	41.0 ± 3.6	43.2 ± 3.4 a
<i>A. pullulans</i> L8	32.3 ± 2.5 aA	31.7 ± 3.5 aA	49.0 ± 2.6	44.3 ± 2.1	46.7 ± 3.3 a
Untreated	62.7 ± 2.1 bA	71.7 ± 1.5 bB	82.3 ± 2.5	81.7 ± 2.5	82.0 ± 2.3 b
Mean			58.9 ± 17.8 A	55.7 ± 19.7 A	

In both experiments, for *T. pleuroti*, treatment (L1 and L8) and *Trichoderma* strain (AFS 492 and 497) factors, and their interaction are significant. For *T. pleuroticola*, treatment (L1 and L8) and strain (AFS 432 and 488) factors are significant, and their interaction is not significant, according to two-way ANOVA ( $P < 0.05$ ). Mean values ± SD followed by the same upper case letter in a line and by the same lower case letter in a column are not significantly different according to SNK test ( $P < 0.01$ ).

**Table 6**

*In vivo* assay: effect of *Aureobasidium pullulans* L1 and L8 strains and prochloraz on substrate colonisation rate by *Trichoderma pleuroti* AFS 492 and *T. pleuroticola* AFS 432, 28 days after *Pleurotus ostreatus* 'Spoppo' strain spawning under growth chamber conditions.

Treatment <sup>a</sup>	Experiment 1 – Colonization index (0–5)		Experiment 2 – Colonization index (0–5)	
	<i>T. pleuroti</i> AFS 492	<i>T. pleuroticola</i> AFS 432	<i>T. pleuroti</i> AFS 492	<i>T. pleuroticola</i> AFS 432
<i>A. pullulans</i> L1	4.58 ± 0.66 cB	1.75 ± 1.25 bA	4.67 ± 0.52 cB	2.25 ± 0.76 bcA
<i>A. pullulans</i> L8	3.83 ± 0.41 bB	0.92 ± 0.80 aA	3.50 ± 0.55 bB	1.25 ± 0.76 abA
Prochloraz	1.75 ± 0.42 aB	0.50 ± 0.55 aA	2.17 ± 0.41 aB	0.67 ± 0.52 aA
Infected control	4.67 ± 0.52 cB	2.67 ± 0.82 bA	5.0 ± 0.0 cB	2.33 ± 0.52 cA

In both experiments, treatment and *Trichoderma* species factors, and their interaction are significant according to two-way ANOVA ( $P < 0.05$ ). Mean values ± SD followed by the same upper case letter in a line and by the same lower case letter in a column are not significantly different according to SNK test ( $P < 0.05$ ).

<sup>a</sup> *A. pullulans* L1 and L8 concentration,  $5 \times 10^3$  spore/g substrate; prochloraz dose,  $1.25 \mu\text{L L}^{-1}$ .

56.6% in experiment 1 and 2, respectively. The L8 treatment was effective against Tpa 432 by reducing the colonization index by 65.5% and by 46.3%, compared with that of infected control in experiment 1 and 2, respectively. It showed a colonization index value statistically similar to that of prochloraz. The treatment with L1 was not effective. In infected controls, the colonization index value of Tpi 492 was higher than that of Tpa 432.

#### 4. Discussion

The green mould caused by *Trichoderma pleuroticola* and *T. pleuroti* is one of the most serious diseases of *Pleurotus ostreatus* that frequently causes dramatic production losses (Hatvani et al., 2007, 2012; Bellettini et al., 2018; Innocenti et al., 2019). Currently, the only tool available to control the disease in *P. ostreatus* Italian farms is the prochloraz application at spawn phase. The cultivation substrate can be treated only at this phase, because it is bagged immediately after. Innocenti et al. (2019) showed that prochloraz was effective against both *Trichoderma* species responsible for the disease, however they observed that the treatment at the spawn phase was not sufficient to ensure protection during the whole oyster cultivation cycle, probably because of the fungicide activity reduction under mushroom farm conditions, and of the absence of natural antagonists in the growing substrate. Therefore, new alternative methods more durable and sustainable for both human and environmental health, have to be investigated. Biological control methods of *P. ostreatus* green mould have been scarcely studied. Nagy et al. (2012) and Mwangi et al. (2017) showed the efficacy of *B. amyloliquefaciens*, *B. subtilis* and *B. licheniformis* isolates against the disease. Zhang et al. (2010) observed that *A. pullulans* PL5 strain was active against postharvest pathogens of peach, apple and plum. Moreover, investigating the modes of action of PL5 strain, they suggested that the production of antibiotics was not involved, then they concluded that *A. pullulans* PL5 could be potentially registered as a biocontrol agent. At present, two strains of *A. pullulans*, DSM 14940 and DSM 14941, are commercialized as biopesticides for the control of strawberry, grape and tomato grey mould (Botector<sup>®</sup>), of fruit tree fire blight (Blossom Protect<sup>™</sup>), and of apple fruit post-harvest diseases (Boni Protect). In previous works, Mari et al. (2012) and Di Francesco et al. (2017, 2018b) demonstrated that *A. pullulans* L1 and L8 strains were effective against stone fruit brown rot, kiwifruit grey

mould and tomato blight. Our study showed that the same *A. pullulans* strains were fully compatible with *P. ostreatus* growth. Indeed, in the co-culturing assay, they did not hampered *P. ostreatus* colony growth, which was promoted by volatile and nonvolatile compounds produced by L8 strain as showed in specific assays. On the basis of these results, the effect of L1 and L8 against *Trichoderma* species responsible of green mold disease was studied under laboratory and growth chamber conditions. In co-culturing and volatile and nonvolatile assays, both *A. pullulans* strains reduced the colony growth of *T. pleuroticola* and *T. pleuroti* strains. In *in vitro* assays, the inhibitory effect of L1 and L8 was similar in the majority of the *Trichoderma*-*A. pullulans* combinations. The competition for space and nutrients and the production of volatile and nonvolatile compounds seemed to be responsible for the biocontrol activity, while hyphal interaction seemed not to be involved in the antagonistic activity of the yeast as observed also by Zhang et al. (2010) for *A. pullulans* PL5 strain against post-harvest pathogens. Similarly, Di Francesco et al. (2015a) showed that the same mechanisms reported above played a significant role in the antagonism of L1 and L8 strains against *Monilinia laxa*, causal agent of peach brown rot. The competition for space and nutrients and the absence of hyphal interaction were also showed by the *A. pullulans* LS-30 strain against *Botrytis cinerea*, *Penicillium expansum*, *Rhizopus stolonifera* and *Aspergillus niger*, and no antibiosis was involved in its antagonistic activity (Castoria et al. 2001). Di Francesco et al. (2015b) identified four volatile organic compounds produced by L1 and L8 strains as phenethyl alcohol, 1-butanol-3-methyl, 1-butanol-2-methyl and 1-propanol-2-methyl belonging to the group of alcohols which were active against several postharvest pathogens. *Aureobasidium pullulans* is well known also for the production of non-volatile metabolites, such as the pullulan which has numerous applications in medicine, pharmacy, food industry and other fields (Leathers, 2003; Cheng et al., 2011), and a  $\beta$ -glucan consumed as health-promoting food with beneficial immunomodulatory effects (Tada et al., 2009; Muramatsu et al., 2012). To our knowledge, no data on the identification of yeast nonvolatile metabolites effective against plant pathogens are available. Considering that these compounds produced by L1 and L8 strains were effective not only against *Trichoderma* species pathogenic of *P. ostreatus*, but also against *Phytophthora infestans* responsible of tomato late blight (Di Francesco et al., 2017), their identification is ongoing, in order to verify the absence of metabolites with risk for environment and human health. *Aureobasidium pullulans* is a saprophytic fungus that is widely distributed in the environment, and like many saprophytic fungi, in the right host *A. pullulans* can be an opportunistic human pathogen (Mehta et al., 2017). Indeed, this fungus caused infections in immunocompromised or chronically ill patients (Chan et al., 2011; Mershon-Shier et al., 2011; Najafzadeh et al., 2014; Chowdhary et al., 2015; Mehta et al., 2017). It is supposed that extracellular enzymatic activities could also be involved in *A. pullulans* antagonistic activity towards *T. pleuroti* and *T. pleuroticola*. Castoria et al. (2001) showed that exochitinase (N-acetyl-b-D-glucosaminidase) and  $\beta$ -1-3-glucanase activities were involved in the antagonism of *A. pullulans* LS-30 strain against several plant pathogens.

When the biocontrol activity of L1 and L8 was tested against the green mould disease caused by *T. pleuroti* 492 and *T. pleuroticola* 432, only L8 strain was effective. We highlight that the effectiveness of L8 against *T. pleuroticola* 432 disease was similar to that of prochloraz used at field dose, whereas the biocontrol effect of L8 against *T. pleuroti* 492 disease was lower than that showed by the fungicide. *Trichoderma pleuroti* 492 was more aggressive than *T. pleuroticola* 432 against *P. ostreatus*, consistently to Innocenti et al. (2019). The different aggressiveness of *T. pleuroti* 492 and *T. pleuroticola* 432 could justify the different biocontrol efficacy showed by L8.

Another important outcome of *A. pullulans* application in mushroom crop could be the improvement of *P. ostreatus* fruit-body nutraceutical properties, which are deeply influenced by the substrate cultivation

(Carrasco-Gonzalez et al., 2017). In a previous work, the amino acid analysis of kiwifruit juice amended with L1 and L8 showed that both aspartic and glutamic acid content was increased, and that new amino acids such as serine, glycine, threonine, arginine, alanine and valine were produced (Di Francesco et al., 2018b). This aspect is now under investigation in *P. ostreatus* fruit bodies produced in a substrate amended with *A. pullulans* strains.

In conclusion, we demonstrated, for the first time, the effectiveness of *A. pullulans* in reducing *Trichoderma* colony growth in *in vitro* assays, and the control of green mould disease under conditions comparable to those of a mushroom farm, where only L8 strain was effective. In the case of the less aggressive *Trichoderma* species, the strain was even effective as the chemical. Based on the above, the patent n. 102018000007085 was developed by Bologna University for the application of L8 strain in *P. ostreatus* farm against the green mould disease. CRediT authorship contribution statement

**Roberta Roberti:** Formal analysis, Methodology, Data curation, Writing - review & editing. **Alessandra Di Francesco:** Methodology. **Gloria Innocenti:** Conceptualization, Resources. **Marta Mari:** Supervision.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biocontrol.2019.04.016>.

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