# BIOCONTROL OF BLUE MOLD ON APPLE FRUITS BY *AUREOBASIDIUM PULLULANS* (STRAIN ACH1-1): *IN VITRO* AND *IN SITU* EVIDENCE FOR THE POSSIBLE INVOLVEMENT OF COMPETITION FOR NUTRIENTS

**S. KRIMI BENCHEQROUN[1,4], M. BAJJI[1], S. MASSART[1], F. BENTATA[2], M. LABHILILI[2], H. ACHBANI[3], S. EL JAAFARI[4] & M.H. JIJAKLI[1,*]**

[1] Unité de Phytopathologie, Faculté Universitaire des Sciences Agronomiques de Gembloux, Passage des Déportés 2, BE-5030 Gembloux, Belgium

[2] Institut National de la Recherche Agronomique Guich, BP 415, Rabat, Morocco

[3] Institut National de la Recherche Agronomique, BP 578, Meknés, Morocco

[4] Université de Moulay Ismaïl, BP 4010, Meknés, Morocco

[*] E-mail: jijakli.h@fsagx.ac.be

## SUMMARY

*Aureobasidium pullulans* strain Ach1-1 was recently isolated for its biocontrol effectiveness against *Penicillium expansum*, the causal agent of blue mold on harvested apples. In the present study, strain Ach1-1 was found to be very effective in controlling *P. expansum* on apple wounds. For *in vitro* tests, strain Ach1-1 and *P. expansum* were cocultured in the presence of apple juice (0 - 5%) using a system preventing direct contact between both agents. The presence of the antagonist greatly reduced germination of conidia at low (0.1, 0.5 and 1%) but not at high (5%) juice concentrations. Germination of previously inhibited conidia at 0.5% apple juice was partially restored in the presence of the antagonist when fresh juice was added at a final concentration of 5%, and completely recovered at both 0.5 and 5% juice concentrations in the absence of the antagonist. These data show that *P. expansum* conidia are able to germinate when cocultered with strain Ach1-1 in conditions of sufficient rather than limited nutrient availability and that the antagonist does not affect the viability of these conidia, indicating that the inhibitory effect of strain Ach1-1 on conidia germination may be due to a competition for nutrients. Such observation was confirmed *in situ* since the application of high amounts of exogenous amino acids, vitamins or sugars on apple wounds significantly reduced the protective level of strain Ach1-1 against *P. expansum*, the most important effect was obtained with amino acids followed by vitamins and then by sugars. The present work provides both *in vitro* and *in situ* evidence that the biocontrol activity of strain Ach1-1 against *P. expansum* essentially relies on competition for apple fruit nutrients, especially amino acids.

**Key words:** Apple fruit, *Aureobasidium pullulans*, biocontrol, *Penicillium expansum*, postharvest

## INTRODUCTION

The yeast-like fungus *Aureobasidium pullulans* (de Bary) Arnaud is one of the most widespread and well adapted saprophytes (Blakeman and Fokkema, 1982). Different strains were isolated and have shown to be effective in controlling the main postharvest pathogens (e.g. *Botrytis cinerea* and *Penicillium expansum*) on several important crops (e.g. apple, Table grape and strawberry) (Lima *et al.*, 1999; Ippolito *et al.*, 2000; Castoria *et al.*, 2001; Adikaram *et al.*, 2002; Lima *et al.*, 2003; Ippolito *et al.*, 2005). As a cosmopolitan microorganism and due to its ability to protect a wide range of hosts from major postharvest pathogens, *A. pullulans* may be considered as a po-

tential biocontrol agent for plant diseases. However, a good understanding of the mechanisms underlying its biocontrol activity is a prerequisite to identify pivotal biocontrol features and to develop appropriate formulations allowing their expression (Massart *et al.*, 2006). Moreover, knowledge of such mechanisms is very important to establish specific criteria for a better screening of new potential biocontrol agents as well as to facilitate registration procedures. The modes of action of *A. pullulans* appear to rely essentially on competition for nutrients but also on induction of defence responses and on production of lytic enzymes (Lima *et al.*, 1997; Ippolito *et al.*, 2000; Castoria *et al.*, 2001).

In a previous study, *A. pullulans* strain Ach1-1 was selected for its high biocontrol activity against *P. expansum* and *B. cinerea* on wounded *Golden delicious* apples (Achbani *et al.*, 2005). In order to maximize its potential use as a biocontrol agent, an in-depth study has been undertaken and focused on competition for nutrients, the mechanism repeatedly reported to be responsible for antagonism of most *A. pullulans* strains. Unlike previous studies (*e.g.* Lima *et al.*, 1997; Janisiwicz *et al.*, 2000; Castoria *et al.*, 2001), both *in vitro* and *in situ* tests have been considered with an emphasis on nutrients present in apple fruit tissues.

## MATERIAL AND METHODS

### Biological material

The pathogen *P. expansum* strain 880 was isolated from infected apple fruits (INRA Meknès, Morocco). It was maintained on potato dextrose agar (PDA) medium at 4°C. A conidia suspension was obtained by flooding a 10-day-old PDA culture with sterile water containing 0.05% (v/v) Tween 20 and its concentration determined with a hemacytometer.

*A. pullulans* strain Ach1-1 was isolated from the surface of *Golden Delicious* healthy apples (Belgium) and was maintained on PDA at 4°C. Before its application, it was grown on PDA at 25°C with three subcultures of 24 h. The cell suspension was obtained by flooding cultures with isotonic water (0.85% NaCl) and its concentration was estimated using a hemacytometer.

Apple fruits (cultivar *Golden Delicious*) used for *in situ* tests were bought from a local market and maintained in the dark at 1°C.

### Biocontrol assay on apple

The efficacy of *A. pullulans* strain Ach1-1 against *P. expansum* was evaluated on apple wounds as previously described (Jijakli and Lepoivre, 1993) with some modifications. Fruits were wounded with a cork-borer (3 wounds of 4-mm diameter and 4-mm depth per apple) and treated with 40 µl of the antagonist suspension (or isotonic water for controls) at either $10^7$ or $10^8$ cfu/ml. One hour later, the wound sites were inoculated with 40 µl of *P. expansum* preparation ($10^5$ conidia/ml). Apples were incubated on wet filter paper in closed plastic boxes at 25°C in the dark for 5 d before measuring diameters of decay lesions. The protective level was estimated according to the formula: (Dc-Dt)/Dc×100, where Dc and Dt are respectively the diameter lesion of the control and treated apples.

### *In vitro* competition for nutrients

*In vitro* competition for nutrients was studied according to the method developed by Janisiewicz *et al.* (2000). Tissue culture plates and cylinder inserts equipped with a membrane filter (0.4 µm pore size) attached to their bottom part were used. Two different tests were conducted. In the first one, the plate wells were filled with 600 µl apple juice at various concentrations (0, 0.1, 0.5, 1 and 5%) in the absence (controls) or in the presence of the antagonist (strain Ach1-1 at $10^7$ cfu/ml). The cylinder inserts were placed in the plate wells and filled with 400 µl of a conidia suspension (*P. expansum* at $2x10^5$ conidia/ml). The plates containing the inserts were incubated in the dark at 25°C for 24 h, and then the number of germinating conidia on the membrane was scored. The second test initially consisted in testing 0.5% apple juice concentration as in the first test, and then *P. expansum* conidia previously incubated for 24 h in the presence of the antagonist were submitted to new nutritional conditions. For a set of samples, cylinders were kept in the same wells (with the antagonist) in which fresh apple juice was added to obtain a final concentration of 0.5 or 5%. For the remaining samples, cylinders were moved to new wells containing apple juice at 0, 0.5 or 5% without the antagonist. After an additional 24 h of incubation in the same conditions as above, the number of germinating conidia on the membrane was recorded.

### *In situ* competition for nutrients

The effect of an addition of exogenous nutrients (amino acids, vitamins and sugars) to apple wounds on the biocontrol efficacy of strain Ach1-1 ($10^7$ cfu/ml) against *P. expansum* ($10^5$ conidia/ml) was assessed. To this end, biocontrol assays were performed on apple wounds as described above with an additional step consisting in the application of 40 µl per wound of either water (none) of one of the nutrient solutions (amino acids, vitamins or sugars) 1 h after the inoculation of the pathogen. Concentrations of amino acids, vitamins and sugars correspond respectively to 20, 20 and 5 times those reported for apple tissues (USDA nutrient database for standard reference, release 14, 2001). Diameter lesions were measured after 5 d of incubation in the dark at 25°C. The protective level was evaluated as mentioned above.

### Statistical analysis

For biocontrol assays, 15 apple fruits were used per treatment. For *in vitro* tests (germination), 100 conidia per treatment were considered. Each test was conducted twice and data were subjected to analysis of variance. Means were separated using the Student-Newman-keul's test at $P \leq 0.05$. All analyses were performed using the Statistical Analysis System (SAS/STAT) software.

## RESULTS

### Biocontrol assay on apple

The mean diameter of the lesions caused by *P. expansum* in the absence of strain Ach1-1 was about 2.8 cm (Table 1). The effect of the pathogen has been significantly reduced (88%) when the antagonist was applied at $10^7$ cfu/ml and quasi totally prevented at $10^8$ cfu/ml. The corresponding protective levels were 83.8 and 99.0%, respectively (Table 1).

**Table 1.** Lesion diameters (cm) developed by *P. expansum* strain 880 on apple wounds after 5 days of incubation in the absence (Control) or in the presence of *A. pullulans* strain Ach1-1 (Strain Ach1-1) at two different concentrations ($10^7$ and $10^8$cfu/ml) and the corresponding levels of protection (%). Values with the same letter are not significantly different (P≤0.05)

| Treatment | Lesion diameter (cm) | Protective level (%) |
|---|---|---|
| Control | 2.81 ± 0.03a | - |
| Strain Ach1-1 at $10^7$ cfu/ml | 0.35 ± 0.05b | 83.8 |
| Strain Ach1-1 at $10^8$ cfu/ml | 0.02 ± 0.01c | 99.0 |

### *In vitro* competition for nutrients

The germination percentages of *P. expansum* conidia after 24 h of incubation at different apple juice concentrations without (control) or with (strain Ach1-1) the antagonist are shown in Table 2. The smallest percentages were obtained at 0% apple juice in the absence as well as in the presence of strain Ach1-1. In controls, apple juice at all tested concentrations allowed germination percentages ranging from 82.0 to 98.5%. However, the presence of the antagonist greatly reduced germination of conidia in apple juice except at the highest concentration (Table 2).

**Table 2.** Germination percentages of *P. expansum* strain 880 conidia after 24 h of incubation without (Control) or with *A. pullulans* strain Ach1-1 (Strain Ach1-1) at various juice concentrations. Values with the same letter are not significantly different (P≤0.05)

| Apple juice (%) | Conidial germination (%) | |
|---|---|---|
| | Control | Strain Ach1-1 |
| 0.0 | 19.0 ± 0.0d | 20.0 ± 1.0d |
| 0.1 | 82.0 ± 4.0b | 30.5 ± 2.5d |
| 0.5 | 98.5 ± 1.5a | 28.5 ± 5.5d |
| 1.0 | 91.0 ± 4.0ab | 51.5 ± 4.5c |
| 5.0 | 93.5 ± 3.5ab | 96.5 ± 3.5ab |

In the case of conidia previously incubated with the antagonist for 24 h in 0.5% apple juice, the addition of fresh juice in the same wells to obtain either 0.5 or 5% resulted, after an additional 24 h of incubation, in a germination percentage of 34.0 or 67.8%, respectively (Table 3). When cylinders containing inhibited conidia were moved to new wells filled with apple juice at 0, 0.5 or 5% without strain Ach1-1, germination after 24 h of incubation was

still inhibited at 0% apple juice but restored at 0.5 and especially at 5% (Table 3).

**Table 3.** Germination percentages of *P. expansum* strain 880 conidia previously exposed to *A. pullulans* strain Ach1-1 in 0.5% apple juice for 24 h and then submitted to new nutritional conditions in the presence (+) or the absence (-) of strain Ach 1-1 for an additional 24 h. Values with the same letter are not significantly different (P≤0.05)

| Treatment | | Conidial germination |
|---|---|---|
| Strain Ach1-1 | Apple juice (%) | (%) |
| + | 0.5 | 34.0 ± 4.5c |
| | 5.0 | 67.8 ± 4.5b |
| - | 0.0 | 29.3 ± 7.5c |
| | 0.5 | 94.3 ± 4.5a |
| | 5.0 | 100.0 ± 0.0a |

### *In situ* competition for nutrients

Once again, biocontrol assay revealed the great effectiveness of strain Ach1-1 in controlling *P. expansum* on apple, the protective level being 97.1% (Table 4). The addition of exogenous amino acids, vitamins or sugars in wound apples significantly increased lesion diameters developed by the pathogen in the absence of the antagonist (control). The greatest effect was obtained with amino acids and vitamins compared to sugars. In the presence of the antagonist, lesion diameters were significantly higher in response to than in the absence of nutrient supply (Table 4). The protective level provided by strain Ach1-1 was strongly reduced in wounds supplemented with exogenous nutrients.

**Table 4.** Effect of exogenous nutrient (amino acids, vitamins or sugars) application on lesion diameters (cm) developed by *P. expansum* strain 880 on apple wounds after 5 days of incubation in the absence (Control) or in the presence of *A. pullulans* strain Ach1-1 (Strain Ach1-1) and the corresponding levels of protection (%). Values with the same letter are not significantly different (P≤0.05)

| Treatment | Lesion diameter (cm) | | Protective |
|---|---|---|---|
| | Control | Strain Ach1-1 | level (%) |
| None | 1.98 ± 0.04c | 0.06 ± 0.03f | 97.1 |
| Amino acids | 2.32 ± 0.02a | 1.66 ± 0.06d | 28.4 |
| Vitamins | 2.14 ± 0.04b | 1.51 ± 0.06ed | 29.4 |
| Sugars | 2.33 ± 0.04a | 1.42 ± 0.10e | 39.2 |

## DISCUSSION

In the present work, *A. pullulans* strain Ach1-1 was very effective in suppressing *P. expansum* on apple wounds (Table 1). This is consistent with data of a previous study (Achbani *et al.* 2005) in which strain Ach1-1, among different potential antagonistic microorganisms isolated from apple surfaces, has shown a protective level of more than 90% against *P. expansum*. Other strains of *A. pullulans* reported in other works also displayed high antagonistic activities not only against *P. expansum* on apples but also against major postharvest pathogens on several important crops (Lima *et al.*, 1999; Ippolito

*et al.*, 2000; Janisiwicz *et al.*, 2000; Castoria *et al.*, 2001; Adikaram *et al.*, 2002; Schena *et al.*, 2003).

In the absence of the antagonist, higher *in vitro* germination percentages of *P. expansum* conidia were recorded in the presence than in the absence of apple juice (Table 2), and the largest lesion diameters were obtained in apple wounds supplemented with exogenous nutrients (Table 4). This confirms that *P. expansum* is nutrient-dependent and, as a necrotrophic pathogen, requires sufficient nutrients for its conidial germination and hyphal development. In the presence of the antagonist, however, *in vitro* germination of the pathogen conidia was inhibited except at the highest apple juice concentration (Table 2). Moreover, inhibited conidia were still able to germinate when submitted to high apple juice concentrations with as well as without the antagonist (Table 3). These data suggest that *A. pullulans* strain Ach1-1 outcompetes *P. expansum* conidia for apple juice nutrients without affecting their viability. Such observation has been strengthened by *in situ* tests in which the supply of additional nutrients significantly reduced the biocontrol activity of the antagonist (Table 4), the extent of the reduction being dependent on the nature of the added nutrients. In previous works dealing with nutrient competition in the antagonistic activity of *A. pullulans* (Lima *et al.*, 1997; Castoria *et al.*, 2001), *in situ* tests were performed using the Nutrient Yeast Dextrose Broth medium whose composition is not in relation with that of apple fruit tissues. In the present study, however, such tests were conducted using concentrations of amino acids, vitamins and sugars corresponding respectively to 20, 20 and 5 times those reported for apple fruit tissues (USDA nutrient database for standard reference, release 14, 2001). Depending on the assays, the most important reduction of the antagonistic activity of strain Ach1-1 against *P. expansum* was obtained with amino acids (71 - 78%) followed by vitamins (51 - 70%) and finally by sugars (38 - 60%). Amino acids seem thus to be more involved in competition between the antagonist and the pathogen conidia than vitamins and most particularly than sugars, at least considering our experimental conditions.

As a conclusion, our data provide strong evidence from both *in vitro* and *in situ* tests that competition for apple nutrients would be one of the main mechanisms underlying the biocontrol activity of *A. pullulans* strain Ach1-1 against *P. expansum* on stored apple fruits, amino acids being the most limited nutrients. The investigation will continue in this direction in order to find out among amino acids the one(s) that are the most limited as well as the gene(s) involved in their uptake and metabolisation by the antagonist cells.

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