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In vitro and *in situ* study of postharvest apple blue mold biocontrol by *Aureobasidium pullulans*: Evidence for the involvement of competition for nutrients

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Abstract

Aureobasidium pullulans strain Ach1-1 was selected for its effectiveness against blue mold caused by *Penicillium expansum* on stored apple fruit. The possible involvement of competition for nutrients in the biocontrol activity of this antagonistic strain was investigated both *in vitro* and *in situ*. For *in vitro* assays, the effect of strain Ach1-1 on germination percentages of *P. expansum* conidia was evaluated after a 24 h incubation period in the presence of increasing apple juice concentrations (0–5%) using a system allowing the physical separation of both agents. In the absence of strain Ach1-1, conidial germination was strongly promoted by apple juice whatever the concentration. However, germination was significantly reduced by the presence of strain Ach1-1 except at the highest juice concentration. For conidia previously inhibited at 0.5% juice, germination after 24 h of incubation was partially recovered in the presence of strain Ach1-1 when fresh juice was added to a final concentration of 5%, and completely restored at both 0.5 and 5% juice concentrations in the absence of strain Ach1-1. For *in situ* assays, strain Ach1-1 was very protective against *P. expansum* on postharvest wounded apples. However, the application of high concentrations of exogenous sugars, vitamins and most particularly amino acids, significantly reduced such protection. Time-course analysis of apple amino acids at the wound site revealed that these compounds were more depleted in wounds treated with strain Ach1-1 alone and especially in those treated with both agents (strain Ach1-1 and *P. expansum*) compared to wounds treated with *P. expansum* alone or to untreated ones. Exogenous amino acids, applied at high concentrations on apple wounds as a mixture of specific amino acid groups or as individuals, significantly decreased strain Ach1-1 efficacy against *P. expansum*. The present study provides *in vitro* and *in situ* evidence that competition for apple nutrients, most particularly amino acids, may be a main mechanism of the biocontrol activity of *A. pullulans* strain Ach1-1 against blue mold caused by *P. expansum* on harvested apple fruit.

Keywords: Amino acids; *Aureobasidium pullulans*; Biocontrol; Nutrient competition; *Penicillium expansum*; Postharvest apples

1. Introduction

Apple production is a complex process involving orchard, storage and marketing phases. Long-term storage of this important fruit has become possible due to modern postharvest technologies. However, postharvest diseases, especially those originating from wound infection at harvest or during postharvest handling and packing, can be a serious limiting factor for such storage (Bondoux, 1992). Blue mold caused by *Penicillium expansum* Link is an important postharvest disease on apples worldwide and is an economic concern not only for the fresh fruit industry but also for the fruit processing industry because *P. expansum* is generally regarded as the major producer of the mycotoxin patulin (McKinley and Carlton, 1991). Until now, this disease has essentially been controlled by pre- and postharvest handling practices and the application of synthetic fungicides (Jijakli and Lepoivre, 2004). However, several reasons including development of fungicide-resistant pathogens, fungicide toxicity, and deregistration of some effective products, have motivated the search for alternative approaches. Biological control has emerged as one of the most promising disease

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management alternatives with different mechanisms of action compared to chemical fungicides (Punja and Utkhede, 2003; Spadaro and Gullino, 2004; Fravel, 2005; Massart and Jijakli, 2007). A number of yeasts and bacteria have been reported to control postharvest fruit decay effectively (Janisiewicz and Korsten, 2002). Despite progress in understanding their modes of action, only a few biocontrol products are currently registered for such control in Europe (Alabouvette et al., 2006), in comparison with other countries, especially the USA (e.g. Fravel, 2005). In this context, several strains of the yeast-like fungus *Aureobasidium pullulans* (de Bary) Arnaud, one of the most widespread and well adapted saprophytes (Blakeman and Fokkema, 1982), have been isolated and appear to be potential biocontrol agents based on their great effectiveness in controlling the main postharvest pathogens (e.g. *Botrytis cinerea* and *P. expansum*) on several important crops including apple, table grape and strawberry (Lima et al., 1999, 2003; Ippolito et al., 2000, 2005; Castoria et al., 2001; Adikaram et al., 2002).

Recently, a new potential strain named Ach1-1 was isolated from the surface of 'Golden Delicious' apples and selected for its strong antagonistic properties against both *P. expansum* and *B. cinerea* pathogens (Achbani et al., 2005), although the mechanism(s) underlying its biocontrol activity are still unknown. Nevertheless, a good understanding of such mechanisms is a prerequisite for identifying the main biocontrol features for a better screening of new potential biocontrol agents, to develop appropriate formulations allowing their expression, and to facilitate registration procedures. The present work was thus undertaken to investigate the possible involvement of competition for nutrients in the biocontrol activity of strain Ach1-1 against *P. expansum*. Although several possible mechanisms have been reported (Droby and Chalutz, 1994; Janisiewicz and Korsten, 2002; Massart and Jijakli, 2007), the main mode of action of yeast(-like) biocontrol agents is believed to be competition for nutrients and space (Droby and Chalutz, 1994; Lima et al., 1997; Janisiewicz et al., 2000; Castoria et al., 2001). According to the literature dealing with competition for nutrients and space using different antagonist/pathogen(/host) systems (Castoria et al., 1997, 2001; Lima et al., 1997; Janisiewicz et al., 2000; Guetsky et al., 2002; Vero et al., 2002): (i) most studies have considered either the *in vitro* or the *in situ* approach but not both, (ii) the contribution of the competition for nutrients and competition for space has rarely been considered separately, and (iii) *in situ* assays have been often performed using the Nutrient Yeast Dextrose Broth medium whose composition is not comparable with that of host tissues. In the present study, competition for nutrients as a mode of action of *A. pullulans* strain Ach1-1 against *P. expansum* was first examined *in vitro* using a system that allows discrimination between competition for nutrients and that for space (Janisiewicz et al., 2000) using increasing concentrations of apple juice. Then, the study was complemented by *in situ* biocontrol assays using high concentrations of major apple components (amino acids, vitamins and sugars) as well as by a time-course analysis of specific nutrients at the wound site in different contexts.

2. Materials and methods

2.1. Microorganisms and fruit material

The pathogen *P. expansum* strain 880 was isolated from infected apple fruit (INRA, Meknès, Morocco) and has been one of the most aggressive isolates in the collection (unpublished data). It was maintained on potato dextrose agar (PDA) at 4 °C. A conidial suspension was obtained by flooding 10-day-old PDA cultures of *P. expansum* with sterile water containing 0.05% (v/v) Tween 20. Conidia concentration of the pathogen was determined with a hemacytometer.

The biocontrol agent used in this study was the yeast-like fungus *A. pullulans* (De Bary) Arnaud strain Ach1-1 isolated from the surface of healthy 'Golden Delicious' apples (Belgium). It was selected for its high antagonistic properties against *P. expansum* and *B. cinerea* (Achbani et al., 2005). Before each application, strain Ach1-1 was grown on PDA at 25 °C for three subcultures of 24 h. A cell suspension was obtained by flooding cultures with isotonic water (0.85% NaCl). The cell concentration was determined and adjusted to obtain 10^7 colony-forming units (CFU) mL^{-1} using a hemacytometer.

Apple fruits ('Golden Delicious') were bought from a local market and maintained for 1–2 weeks in the dark at 1 °C until used. These fruits were produced by Limdor Bourdelas group (St.-Yrieix-La Perche, France). After harvest at their optimal maturity, they were stored in a controlled cold atmosphere until marketing without any chemical treatment. The same product was used in the present work for the period December 2005–April 2006.

2.2. In vitro competition for nutrients

For all *in vitro* tests, competition for nutrients was studied using the non-destructive method developed by Janisiewicz et al. (2000). Tissue culture plates with 24 wells (TC-test plates) and culture plate inserts provided with a diffusing membrane of 0.4 μm pore size (Millicell-CM, Millipore, Belford, MA) attached to the bottom part of the cylinder were used. Such a system allows media nutrients and metabolite interchange with physical separation of the antagonist and the pathogen. Suspensions containing the biocontrol agent Ach1-1 (10^7 cfu mL^{-1}) in apple juice at various concentrations (0, 0.1, 0.5, 1 and 5%) were dispensed into the wells of culture plates (600 μL per well). Cylinder inserts were placed in the wells and *P. expansum* suspensions (2×10^5 conidia mL^{-1}) were dispensed inside the inserts (400 μL per cylinder). The plates were then incubated in the dark at 25 °C for 24 h. Controls were represented by cultures of *P. expansum* alone in the same apple juice concentrations as above. After incubation, inserts were removed from the wells and blotted from the bottom with a tissue paper. For each insert, the membrane was cut with a sharp scalpel, transferred to a glass slide, stained with lactophenol-cotton blue and mounted for optical microscope observation. The effect of strain Ach1-1 on *P. expansum* on the different media was assessed by conidial germination percentage compared to the corresponding controls. Germinated conidia were counted

within a sample of 100 conidia and using two membranes per treatment.

When an inhibitory effect on *P. expansum* conidia was observed during the first assay, a parallel set of culture plates with the inserts was prepared as above to evaluate the viability of the conidia and the possible suppression of the observed inhibition. Inhibited conidia in the antagonist suspension at 0.5% apple juice concentration were submitted to new nutritional conditions in the presence or absence of the antagonist. These new nutritional conditions were obtained in two ways. In one, an amount of apple juice was added to the same media of the first assay containing the antagonist to obtain concentrations of 0.5 or 5%. In the other, inserts containing inhibited conidia were removed from culture plates and inserted into new ones containing apple juice at concentrations of 0, 0.5 or 5%. After an additional 24 h of incubation in the dark at 25 °C, the percentage of germinated conidia was evaluated using two membranes per treatment and a total of 100 conidia per membrane.

2.3. Biocontrol assays on apple wounds

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 × 4 mm × 2 mm per apple) and treated with 40 μL of the antagonist suspension (or isotonic water for controls) at 10^7 CFU mL^{-1}. One hour later, the wounded sites were inoculated with 40 μL of *P. expansum* preparation (10^5 conidia mL^{-1}). Apples were then incubated on wet filter paper in closed plastic boxes at 25 °C in the dark for 5 days before measuring diameters of decay lesions. The percentage of lesion reduction was estimated according to the formula $(D_c - D_t)/D_c \times 100$, where D_c and D_t are, respectively, the lesion diameters of the control and treated apples.

2.4. In situ competition for nutrients

To study a possible nutrient competition mechanism *in situ*, the effect of exogenous application of major apple components (amino acids, vitamins and sugars) on the biocontrol activity of strain Ach1-1 against *P. expansum* was evaluated on wounded apples. Biocontrol assays were performed as described above with an additional step consisting of the application of nutrient solutions in apple wounds. Three solutions were prepared by mixing most of the amino acids, vitamins and sugars known to be present in apple tissues to obtain concentrations higher than those reported for apple tissues (20, 20 and 5 times, respectively) (Table 1) (USDA nutrient database for standard reference, release 14, 2001). One hour after inoculation of *P. expansum*, 40 μL of either water (none) or one of the nutrient solutions were added per wound. Controls were treated with the same solutions in the absence of strain Ach1-1. Lesion diameters were measured after 5 days of incubation in the dark at 25 °C. The percentage of lesion reduction was evaluated as described above using 15 apples per treatment.

Table 1
Identity and concentration (g L^{-1}) of amino acids, vitamins and sugars used for *in situ* competition for nutrients of *A. pullulans* (strain Ach1-1) against *P. expansum* on postharvest apples

Nutrient group	Specific nutrient	Concentration (g L^{-1})[a]
Amino acids		
Group 1	Serine	1.9
	Glycine	1.9
	Glutamic acid	0.3
Group 2	Threonine	1.7
	Arginine	1.4
	Histidine	0.7
	Alanine	1.7
	Aspartic acid	0.2
	Proline	1.7
Group 3	Tryptophan	0.5
	Leucine	1.0
	Lysine	2.9
	Methionine	0.5
	Phenylalanine	1.2
	Tyrosine	0.01
	Valine	2.1
Vitamins	Ascorbic acid	1357.1
	Thiamin	4.0
	Riboflavin	3.3
	Niacin	18.3
	Pantothenic acid	145.2
	Pyrodixin	11.4
	Tocopherol	76.2
Sugars	Sucrose	88.6
	Glucose	104.0
	Fructose	252.5

[a] 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).

2.5. In situ competition for amino acids

The effect of exogenous application of increasing concentrations of amino acids on the protective level of strain Ach1-1 against *P. expansum* was assessed using the same procedure as above. Amino acids known to be present in apple tissues were used at concentrations 2, 10 or 20 times the amount reported for apple tissues (Table 1) (USDA nutrient database for standard reference, release 14, 2001).

The effect of exogenous supply of amino acids on strain Ach1-1 growth was also evaluated. After 24 h of incubation, apple wound tissues were removed with a scalpel, placed in a sterile mortar with 5 mL of KPT buffer (0.05 M of potassium phosphate at pH 6.5 and 0.005% Tween 80) and ground with a pestle. Five apples with three wounds each were used. For each apple, recovered cell suspensions from the three wounds were pooled, diluted 10^3 and 10^4 times, and plated on agar plates (50 μL per plate) using one plate per dilution (i.e. 5 plates per dilution and per treatment). Plates were then incubated at 25 °C for 2 days and the number of strain Ach1-1 colonies was recorded.

2.6. Assimilation of apple amino acids by the antagonist and/or the pathogen

Wounded apples were distributed into four sets (10 apples/set): (1) non-treated apples (control), (2) apples treated with strain Ach1-1 alone (antagonist), (3) apples inoculated with *P. expansum* alone (pathogen), and (4) apples treated with strain Ach1-1 and then inoculated with *P. expansum* (antagonist + pathogen). The two microorganisms were used following the same concentrations and timing as for biocontrol assays. For each set, 50 μL of KPT buffer were added to each wound and then recovered by pipeting 0, 4, 6, 14 and 24 h after the application of the antagonist. Samples of each set (30 samples) were pooled and their amino acid concentrations were determined by high-pressure liquid chromatography (HPLC) and detected by fluorescence. Samples were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ.Fluor) and separated on an amino acid column (Waters AccQ.Tag column) thermostatized at 37 °C. Amino acids were detected by a fluorescence detector (Waters 2475) with an excitation wavelength at 250 nm and an emission wavelength at 395 nm. The identity of amino acids was confirmed by retention times using standards (Sigma Chemical Co.).

A parallel biocontrol assay was performed as previously described to evaluate the efficacy of strain Ach1-1 against *P. expansum* in the same conditions as for the test above.

2.7. In situ competition for specific amino acids

The effect of exogenous application of specific amino acids on the protective level of strain Ach1-1 against *P. expansum* was assessed as above. These amino acids were selected based on HPLC analysis (see Section 3) and were applied either as a mixture of amino acids or individually at a concentration 20 times that reported for apple tissues (Table 1).

2.8. Statistical analysis

For each experiment, a completely randomized design was used. Each experiment was repeated at least once with similar results. Data from one experiment were subjected to a two-way analysis of variance (ANOVA) using the Statistical Analysis System (SAS/STAT) software. The two independent factors were juice concentration and strain Ach1-1 (Figs. 1 and 2), 'nutrient' concentration and strain Ach1-1 (Figs. 3, 4, 7 and 8), and incubation time and treatment (Fig. 6). Data of Fig. 5 were subjected to a one-way analysis of variance with amino acid concentration as the independent factor. When significant differences were found, means were compared using the Tukey's HSD test at $P \leq 0.05$.

3. Results

3.1. In vitro competition for nutrients

Fig. 1 shows germination percentages of *P. expansum* conidia after 24 h of incubation at increasing apple juice concentrations

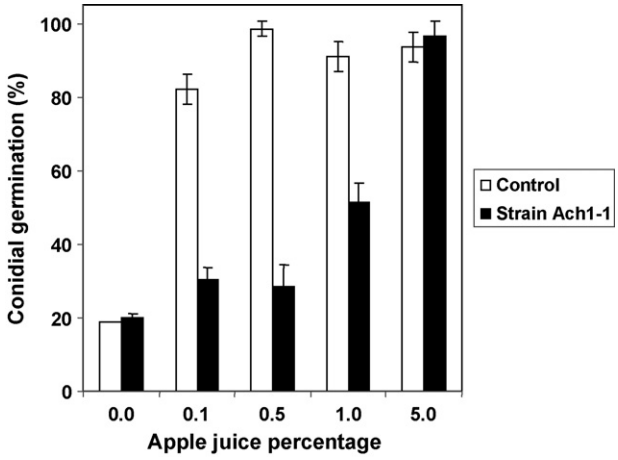

Fig. 1. Germination percentages of *P. expansum* conidia after 24 h of incubation without (control) or with *A. pullulans* (strain Ach1-1) as a function of apple juice concentration. Vertical bars represent S.E. ($n = 2$).

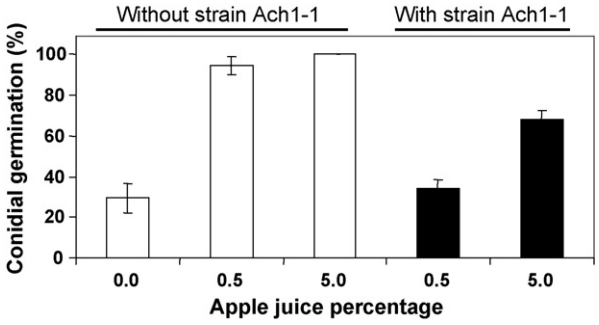

Fig. 2. Germination percentages of *P. expansum* conidia after 24 h of incubation without or with *A. pullulans* strain Ach1-1 as a function of apple juice concentration. These conidia were previously exposed to strain Ach1-1 in 0.5% apple juice for 24 h. Vertical bars represent S.E. ($n = 2$).

without (control) or with the antagonist (strain Ach1-1). Analysis of variance showed a significant effect of juice concentration, presence of strain Ach1-1 and their interaction (data not shown). The lowest percentages were recorded at 0% juice concentration whatever the treatment (control or strain Ach1-1). In the absence of strain Ach1-1 (control), conidia of *P. expansum* ger-

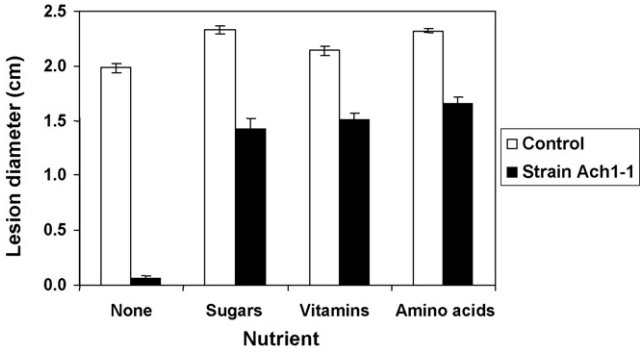

Fig. 3. Effect of exogenous application of sugars, vitamins or amino acids in apple wounds on lesion diameters (cm) induced by *P. expansum* in the absence (control) or in the presence of *A. pullulans* (strain Ach1-1) after 5 days of incubation. Vertical bars represent S.E. ($n = 45$).

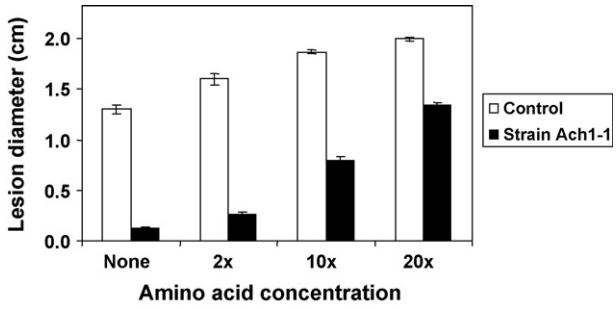

Fig. 4. Effect of exogenous amino acid application in apple wounds on lesion diameters (cm) induced by *P. expansum* in the absence (control) or in the presence of *A. pullulans* (strain Ach1-1) after 5 days of incubation. Vertical bars represent S.E. ($n = 45$).

minated (82.5–98.5%) within the first 24 h of incubation at all apple juice concentrations tested (Fig. 1). In the presence of strain Ach1-1, however, conidial germination was significantly reduced in apple juice except at the highest concentration. The highest inhibitory effect (71%) of the biocontrol agent strain Ach1-1 was obtained at 0.5% juice concentration.

When cylinders containing inhibited conidia (after 24 h of incubation) at 0.5% juice with strain Ach1-1 were transferred to new wells containing increasing concentrations (0, 0.5 or 5%) of fresh apple juice without strain Ach1-1, conidial germination after an additional incubation of 24 h was still inhibited in the absence of apple juice (0%) but completely restored in the presence of both 0.5 and 5% apple juice concentrations (Fig. 2). However, the addition of fresh juice into wells containing inhibited conidia (after 24 h of incubation with the antagonist) to obtain concentrations of either 0.5 or 5% did not totally recover conidial germination 24 h later; germination percentages were 34.0 and 67.8%, respectively (Fig. 2).

3.2. In situ competition for nutrients

In the absence of exogenous nutrients, strain Ach1-1 seemed very effective in reducing the lesion diameters provoked by *P. expansum* on wounded apples, the corresponding reduction percentage being 97.1% (Fig. 3). Analysis of variance showed a significant effect of the presence of strain Ach1-1 on the extent of infection (detailed data not presented). In controls, nutrient supply in apple wounds significantly enhanced the diameter of lesions. In the presence of strain Ach1-1, nutrient application strongly increased lesions caused by the pathogen (Fig. 3); percentages of lesion reduction afforded by strain Ach1-1 in response to sugars, vitamins and amino acids were 39.8, 29.4 and 28.4%, respectively. 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%) (detailed data not shown).

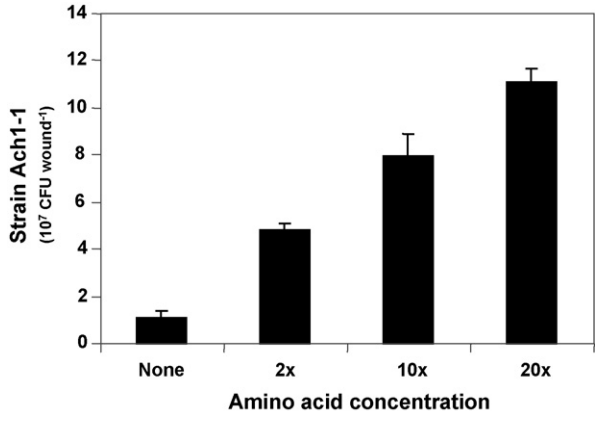

Fig. 5. Effect of exogenous amino acid application in apple wounds on population size (10^7 CFU wound^{-1}) of *A. pullulans* strain Ach1-1 after 24 h of incubation. Vertical bars represent S.E. ($n = 5$).

3.3. In situ competition for amino acids

In both controls and strain Ach1-1-treated wounds, lesion diameters developed by *P. expansum* significantly increased with increasing concentrations of amino acids supplied (Fig. 4). The application of strain Ach1-1 significantly reduced infection lesions whatever the amino acid concentration; the extent of the reduction decreased with increasing amino acid concentrations. The most important loss of strain Ach1-1 protection against *P. expansum* was obtained with the supply of the highest (20×) amino acid concentration (Fig. 4).

Fig. 5 shows the effect of exogenous supply of amino acids on strain Ach1-1 growth in apple wounds after 24 h of incubation. Population size of the strain was significantly increased in response to the application of amino acids; the higher the amino acid concentration, the larger was the population size of the antagonist.

3.4. Assimilation of apple amino acids by the antagonist and/or the pathogen

HPLC analysis of the time-course pattern of (endogenous) amino acids in apple wounds indicates that the total amount of these compounds decreased during apple incubation, especially during the first 14 h following strain Ach1-1 application (Fig. 6). The most important reduction was observed in apple wounds treated with strain Ach1-1 and especially in those con-

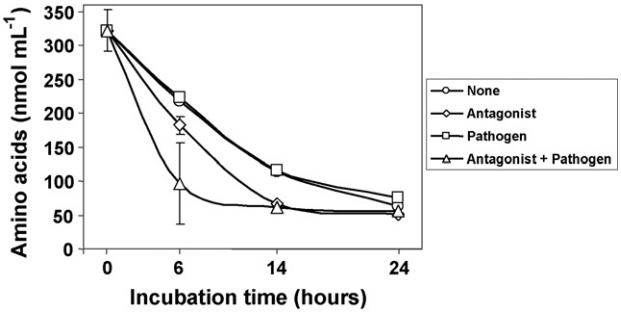

Fig. 6. Concentration (nmol mL^{-1}) of total amino acids in apple wounds during the first 24 h of incubation. Wounds were either intact (none), treated with *A. pullulans* strain Ach1-1 alone (antagonist), inoculated with *P. expansum* alone (pathogen) or treated with strain Ach1-1 and then inoculated with *P. expansum* (antagonist + pathogen). Vertical bars represent S.E. ($n = 2$).

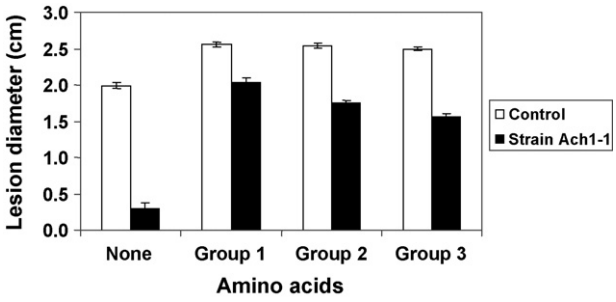

Fig. 7. Effect of exogenous application of different groups of specific amino acid in apple wounds on lesion diameters (cm) induced by *P. expansum* in the absence (control) or in the presence of *A. pullulans* (strain Ach1-1) after 5 days of incubation. Vertical bars represent S.E. ($n = 45$).

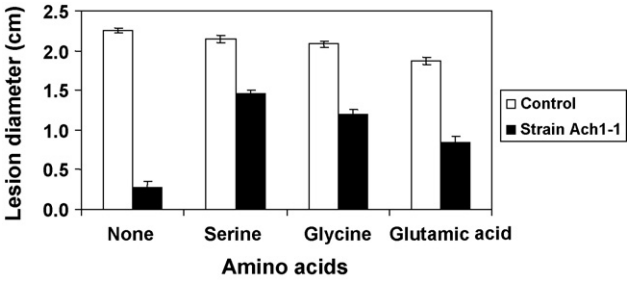

Fig. 8. Effect of exogenous application of serine, glycine or glutamic acid in apple wounds on lesion diameters (cm) induced by *P. expansum* in the absence (control) or in the presence of *A. pullulans* (strain Ach1-1) after 5 days of incubation. Vertical bars represent S.E. ($n = 45$).

taining strain Ach1-1 and *P. expansum*. The detailed analysis of HPLC data allowed us to classify the different amino acids analyzed into three groups based on their individual concentration and changes during incubation (Table 1). The first group includes serine, glycine and glutamic acid whose concentrations were largely reduced with incubation time. The second group contains amino acids (aspartic acid, histidine, arginine, threonine, alanine and proline) whose concentrations remained roughly unchanged during incubation. The remaining amino acids (tyrosine, valine, methionine, lysine, leucine, tryptophan and phenylalanine), which were present at very low concentrations, constitute the third group (detailed data not shown).

3.5. *In situ* competition for specific amino acids

Analysis of variance showed that lesion diameters developed by *P. expansum* were significantly affected by the amino acid group, by the presence of strain Ach1-1 and by their interaction (data not shown). In controls, lesion diameters developed by *P. expansum* were significantly higher in the presence than in the absence of exogenous amino acids independently of the group used (Fig. 7). In the presence of strain Ach1-1, amino acid supply significantly extended infection lesions; the most important effect was obtained with the first group (serine, glycine and glutamic acid). For these amino acids, the protection level was 20% only compared to 83.1% without any additional amino acids (Fig. 7).

Fig. 8 shows that the extent of lesions caused by *P. expansum* in the absence of strain Ach1-1 was not affected by individual application of serine or glycine but was reduced by the application of glutamic acid. Strain Ach1-1 significantly reduced the development of the pathogen regardless of the presence or absence of amino acids. The lowest level of protection was obtained with serine (32.7%).

4. Discussion

A. pullulans strain Ach1-1 is a new potential biocontrol agent against postharvest apple blue mold. It was selected based on its high level of protection against *P. expansum* on wounded apples (Achbani et al., 2005). In the present work, the ability of this strain Ach1-1 to suppress *P. expansum* development was repeatedly observed both *in vitro* and *in situ*. It prevented conidia germination in diluted apple juice concentrations (Fig. 1) and significantly reduced infection on wounded apples (Figs. 3, 4, 7 and 8) with a percentage of lesion reduction ranging from 83 to 97% depending on the assays. In order to maximize the potential use of strain Ach1-1 for the control of apple postharvest decay, an understanding of its mode(s) of action was necessary.

In the absence of strain Ach1-1, higher *in vitro* germination percentages of *P. expansum* conidia were recorded in the presence than in the absence of apple juice (Fig. 1), and the largest lesion diameters were obtained in apple wounds supplemented with exogenous nutrients (Figs. 3, 4 and 7). 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 strain Ach1-1, however, *in vitro* germination of the pathogen conidia was inhibited except at the highest apple juice concentration (Fig. 1) and the diameter of wound lesions was enhanced by supply of exogenous apple nutrients compared to non-treated wounds (Fig. 3). This suggests that strain Ach1-1 was effective against *P. expansum* in nutrient-limited environments only. Moreover, inhibited conidia were viable as they were still able to germinate when incubated at high apple juice concentrations without and even with the antagonist (Fig. 2). These data suggest therefore, that *A. pullulans* strain Ach1-1 out-competes *P. expansum* conidia for apple nutrients without affecting their viability. Its biocontrol activity was concentration-dependent (data not shown) and can be reversed either *in vitro* at high apple juice concentrations (Figs. 1 and 2) or *in situ* by the addition of exogenous apple nutrients (Figs. 3, 4, 7 and 8), which constitute supporting evidence for a possible involvement of competition for nutrients in the biocontrol activity of strain Ach1-1 against blue mold caused by *P. expansum* on stored apple fruit (Droby et al., 1989; Lima et al., 1997; Janisiewicz et al., 2000; Castoria et al., 2001; Meziane et al., 2006). If this is the case, strain Ach1-1 applied on apple surfaces will exert a fungistatic rather than fungicidal activity on *P. expansum* as it will deplete (limiting) nutrients available at the infection site (wounds) and inhibit conidia germination that depends on exogenous nutrients without affecting their viability. This will delay or prevent apple decay after harvest depending on the biocontrol agent concentration. In this way, competition for nutrients may be particularly effective as a sole mode of

action or it may weaken the pathogen and predispose it to other mechanisms. In fact, other modes of action, including elicitation of host defence responses or production of lytic enzymes, might contribute simultaneously or sequentially with competition for nutrients to the biocontrol activity of strain Ach1-1 as was reported for other strains of the same species (Ippolito et al., 2000; Castoria et al., 2001).

It is important to mention that our *in situ* assays were conducted using high concentrations of amino acids, vitamins and sugars considering those reported for apple fruit tissues (USDA nutrient database for standard reference, release 14, 2001). Among these apple nutrients, amino acids seem to be more involved in competition between strain Ach1-1 and *P. expansum* than vitamins and more particularly than sugars, at least under our experimental conditions (Fig. 3). Moreover, an exogenous application of increasing concentrations of amino acids in apple wounds significantly lowered (by 64% at the highest concentration) the biocontrol activity of strain Ach1-1 against *P. expansum* (Fig. 4) without altering the development of these microorganisms (Figs. 4 and 5), suggesting that competition for apple amino acids by strain Ach1-1 plays an important role in suppressing *P. expansum*. This finding was strengthened by a time-course analysis of wound amino acids during apple incubation in which these nutrients were more depleted in the presence of strain Ach1-1 than in the presence of *P. expansum* and that the most rapid depletion was obtained in the presence of both agents (Fig. 6). This suggests that strain Ach1-1 assimilates apple amino acids better than *P. expansum*, most particularly serine, glycine and glutamic acid. Serine and glutamic acid were found to be two of three major amino acids in diluted apple juice and were completely depleted during the 24 h of incubation with another *A. pullulans* strain (ST1-A24) *in vitro* (Janisiewicz et al., 2000). Therefore, these amino acids may be more involved than the other amino acids in the mechanism of competition. Indeed, their application on apple wounds at high concentrations either as a mixture (Fig. 7) or individually (Fig. 8) strongly lowered the biocontrol activity of strain Ach1-1, which indicates that these amino acids, and especially serine, appear to be the most limited nutrients in this competition.

Overall, our data provide strong evidence from both *in vitro* and *in situ* assays 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. Among these amino acids, glycine, glutamic acid and especially serine appear to be the most limited nutrients in this mechanism. The investigation will continue to find out the gene(s) involved in the uptake and the metabolism by the antagonist cells of these limited amino acids.

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