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Effect of *Aureobasidium pullulans* strains against *Botrytis cinerea* on kiwifruit during storage and on fruit nutritional composition

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ABSTRACT

Kiwifruit, wounded at the equator or by pedicel removal, to simulate the stem end wound, were treated with *Aureobasidium pullulans* (L1 and L8 strains) and subsequently inoculated with conidia of *Botrytis cinerea*. Fruits were stored at -1 °C in normal refrigeration (NR) or in controlled atmosphere (CA) (2% O_2; 4.5% CO_2). After 4 months, both antagonists significantly reduced the disease in all experiments, L1 better than L8. In NR, their efficacy was higher than 80%. In CA, the disease reduction was lower: between 30% (L1) and 60% (L8). The ability of both strains to compete with the pathogen for nutrients was tested in kiwifruit juice (0.5%) by *in vitro* experiments. Antagonists significantly reduced pathogen conidia germination in water and in juice. An HPLC analysis was performed to define the amino acid composition of kiwifruit juice upon L1 and L8 treatment. L1 and L8 greatly increased the concentration of both glutamic and aspartic acids and stimulated the production of new amino acids, although at low concentrations. Each amino acid displayed an antifungal effect against mycelium growth of *B. cinerea*. Finally, L1 and L8, cold tolerant and active strains in CA, can be effectively applied to control the stem end rot of kiwifruit in long storage.

1. Introduction

"Hayward" kiwifruit was planted worldwide in temperate-zone countries (Michaelidis and Elmer, 2000) and, following New Zealand's success at marketing, other countries were encouraged to follow suit, by developing commercial plantings of this cultivar. *Botrytis cinerea* (Pers: Fr) has always been considered one of the most important pathogens for stored kiwifruit (Costa et al., 1991), becoming a serious problem over the last 15 years in kiwifruit production (Michaelidis and Elmer, 2000). Pathogens can colonize kiwifruit during fruit harvest (Elmer et al., 1997) through vasculature tissue (Sharrock and Hallett, 1991), and after pedicel removal (Pennycook, 1985). Sure enough, fruit surface wounds caused by poor handling practices can also act as an entry point of *B. cinerea*. Gray mold symptoms are usually visible at the stem end after 3-4 weeks of cold storage and after 3-4 months of storage in controlled atmosphere (CA). In Italian integrated pest management guidelines, fungicide postharvest treatments are allowed for kiwifruits, although the issues related to chemical residues on fruit make these treatments difficult to justify. Curing, a delay between harvest and cool storage, is able to reduce the *B. cinerea* incidence by 4- and 2-fold, compared to uncured fruit, depending on kiwifruit varieties (Wurms, 2005). Furthermore, among alternative means for disease control, the application of biocontrol agents (BCAs), in particular bacteria such as *Bacillus subtilis*, *Pseudomonas* spp (Duncan, 1991; Franicevic, 1993). and yeasts as *Cryptococcus laurentii* (Duncan, 1991), *Candida sake*, *C. pulcherrima* (Cook et al., 1999), have been proposed. Moreover, an additive positive effect against gray mold of kiwifruit was reported when a BCA was combined with curing (Cook et al., 1999).

The application of the yeast *Aureobasidium pullulans* (De Bary) against several postharvest pathogens including *B. cinerea*, may represent an effective alternative to fungicidal treatments (Ippolito et al., 2000, Di Francesco et al., 2015a). Its efficacy can be attributed to different mechanisms of action such as: the production of antifungal compounds and cell wall degrading enzymes (Di Francesco et al., 2015a, b, Parafati et al., 2015, Zhang et al., 2010), the competition for nutrients and space (Di Francesco et al., 2017) and

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also its ability to colonize and adapt to different kinds of surfaces (Mari et al., 2012b; Di Francesco et al., 2017) and environmental conditions.

Considering this, the main objective of this study was to evaluate the effect of two *A. pullulans* strains (L1 and L8) against *B. cinerea* of kiwifruit under the standard protocols for fruit storage (normal refrigeration (NR) and CA), depleted of the curing procedure. Moreover, the ability of both strains to modify the nutritional component of kiwifruit was analysed through *in vitro* experiments based on HPLC analysis of the amino acid composition of kiwifruit juice treated or not with BCA. The effect of these modifications was then evaluated on the pathogen mycelium growth.

2. Materials and methods

2.1. Antagonists

A. pullulans L1 and L8strains, previously identified by Mari et al. (2012a), were maintained on nutrient yeast dextrose agar (NYDA: 8 g of nutrient broth, 5 g of yeast extract, 10 g of dextrose and 15 g of technical agar (Oxoid, Basingstoke, UK) in 1 L of distilled water) and stored at 4 °C until use. Two days before the experiments, each antagonist was grown on NYDA at 25 °C, and yeast cells were collected in sterile distilled water containing 0.05% (v/v) Tween80 (Sigma-Aldrich, St. Louis, MO, USA) then adjusted to a final concentration of 10^8 CFU mL^{-1}.

2.2. Pathogen

Botrytis cinerea (CRIOF-DipSA collection) derived from a rotted kiwifruit was grown on oat meal agar (60 g of oat meal, 10 g of sodium nitrate, 30 g of sucrose, 12 g of agar per 1 L of distilled water) and incubated at 25 °C for 10 days. Pathogen conidia were collected and suspended in sterile distilled water containing 0.05% (v/v) Tween 80 and the suspension was adjusted to 10^4 CFU mL^{-1}, with a haemocytometer.

2.3. Fruit

"Hayward" kiwifruit (*Actinidia deliciosa* (A. Chev.) C.F. Liang & A.R. Ferguson) obtained from an orchard located in Cesena (Italy) were used. Fruits harvested at commercial maturity (7.2° Brix) were picked from trees with a small (3 cm) portion of pedicel. Immediately before the experiment, fruits were divided in two lots: fruits of the first lot were wounded with a sterile nail (3x3x3 mm) on the equator (EQW) (one wound per fruit); in fruits of the second lot, the pedicel was removed to simulate the stem end wound (SEW).

2.4. Effect of BCA application and storage conditions on Botrytis cinerea infections: in vivo experiments

L1 and L8 suspensions (10^8 CFU mL^{-1}) were utilized for *in vivo* assays. Aliquots of 20 μL of each yeast strain were pipetted either into EQWs or into SEWs of kiwifruit. After air drying (1 h), 20 μL of pathogen conidia suspension (10^4 conidia mL^{-1}) were pipetted into the two different types of wounds. The kiwifruits were then divided in two groups containing equal numbers of fruits with EQWs and SEWs and stored at -1 °C in CA (2% O_2; 4.5% CO_2) or in NR with 21% of O_2. The treatment and storage condition effects were recorded after 3 and 4 months. The control samples were represented by kiwifruits treated with distilled water and inoculated with pathogen conidia suspension. Each treatment (BCA and type of wound) was represented by 80 fruits (four replicates of 20 fruits each). The experiment was performed twice.

2.5. Population dynamic

In order to study the population dynamics of both yeast strains in kiwifruit, fruits were wounded on the equator as described above and treated with 20 μL of cell suspension of L1 or L8 (10^8 CFU mL^{-1}); fruits were then kept at -1 °C in CA. Antagonist cell were collected 0, 3, and 4 month after the treatment. The concentration of yeast cells in wounds was determined as described by Mari et al. (1996) with some modifications. The wounded tissue was removed with a sterile 6 mm diameter cork-borer (about 10 mm deep) and transferred into a sterile stomacher bag containing 10 mL of sterile distilled water and Tween 80 (0.05%). The bag was stomached for 10 min (Bag Mixer 400; Interscience, St Nom, France). The resulting slurry, with prior dilution in sterile distilled water, was surface-plated on NYDA. Petri dishes were incubated at 25 °C for 2 days. The sample unit was represented by three fruits replicated three times per treatment and the experiment was performed twice.

2.6. Competition for nutrients

The nutrient depletion by L1 and L8 was assayed on the germination and the germ tube elongation of *B. cinerea* conidia in kiwifruit juice. The juice was obtained by homogenizing 100 g of peeled kiwifruit and centrifuging the homogenate for 15 min at 4800 rpm. The supernatant was then diluted with distilled water at the required concentrations and sterilized by a sterile microfilter (pore size 0.45 μm), as described by Di Francesco et al. (2017). Tissue culture plates with 24 wells per plate (Costar, Corning Inc., Corning, NY) and culture plate inserts Millicell-CM (Millipore Corp., Bedford, MA) were used as reported by Janisiewicz et al. (2000) with some modifications. The inserts consisted of a polystyrene cylinder and a hydrophilic polytetrafluoroethylene (PTFE) membrane (pore size 0.45 μm). Aliquots (120 μL) of fruit juice at 5%, 0.5% and water were dispensed in the wells of culture plates with 40 μL of the pathogen conidia suspension (10^5 CFU mL^{-1}), while the same aliquots of L1 or L8 cell suspensions (10^8 CFU mL^{-1}) were dispensed inside the cylinder inserts. The system allows for the interchange of nutrients and metabolites in the kiwifruit juice without physical contact between antagonist cells and pathogen conidia. The plates with the cylinders were placed at 25 °C on a rotary shaker at 50 rpm; after 6 h of incubation, cylinders were removed from the wells and 20 μL of juice were transferred to a glass slide for microscope (Nikon Eclipse TE2000–E) observations. The percentage of conidia germination and the germ tube elongation (μm) was determined on 90 conidia per treatment (3 microscopic fields, replicates, with 30 conidia each). The growth rate of the germ tube was evaluated from the slope of the straight line. Cylinder inserts, without L1 and L8 strains, containing sterile distilled water were considered as a control. The experiment was performed twice.

2.7. Amino acid analysis by HPLC

Amino acids were determined by reversed phase HPLC analysis and automated pre-column derivatization with o-phtalaldehyde-3-mercaptopropionic acid (OPA) for primary and 9-fluorenylmethylchloroformate (FMOC) for secondary amino acids, according to Agilent procedures (Application note 5990-4547 EN, 2010), with some modifications. A Hewlett-Packard Model series 1100 system, coupled with a diode array detector (UV wave length set at 338 and 262 nm), a GeminiC18 column (110 Å–4.6 × 250 mm, 5 μm Phenomenex) and the corresponding guard column, were used. The column was conditioned with a mobile phase consisting of 98% of 20 mM sodium phosphate buffer, pH 7.8 (phase A) and 2% of acetonitrile/methanol/water, 45:45:10 (phase

B). A mobile phased gradient was used, starting from 2% B, increasing B from 2 to 44.5 in 44.5 min, and then to 61% by the 45-46th min, to 73% by the 58th min and to 100% by the 59th min, remaining at 100% for 3 min, before finally returning to 2%. Quantitative analysis was performed by using norvaline and sarcosine as internal standards for primary and secondary amino acids respectively. Single peak response factors for twenty amino acids relative to internal standard were calculated as the ratio between calibration curve slopes of each amino acid standard and the internal standard. Calibration curves were constructed from amino acid standard solutions in 0.1 NHCl, prepared following Agilent procedures. The analytical limit of quantification was determined for each amino acid (0.8–2 mg L^{-1}). Samples of kiwifruit juice (10%) amended with L1 and L8 suspension (10^8 CFU mL^{-1}) or distilled water (control) (3:1 v/v), were filtered (0.22 μm) prior to injection, after 12 h of incubation at 25 °C.

2.8. Effect of amino acids on Botrytis cinerea growth

The influence of aspartic acid, glutamic acid, serine, glycine, threonine, arginine, alanine, valine on pathogen growth was evaluated through plates containing malt extract agar (MEA, Oxoid, Cambridge, UK) amended with different concentrations of each amino acid (Sigma Aldrich) (Table 3). Glutamine and leucine amino acids were not tested because the first was detected only in kiwifruit juice amended with L1, the second with L8.

The concentrations used were similar to those found in kiwifruit juice (10%) by HPLC analysis. Plates were inoculated with an agar plug (6 mm in diameter) obtained from a 6-day *B. cinerea* colony. The control was represented by MEA plates without amendments. The colony growth was assessed after 4 days of incubation at 25 °C. The sample unit was represented by 10 plates (replicates) for each amino acid. The experiment was performed twice.

2.9. Statistical analysis

All data were analysed using univariate analysis of variance (ANOVA) and the significant differences were subjected to least significant difference (LSD) to separate differences among the means; statistical significance was considered as $P < 0.05$. All analyses were performed with Statgraphics software (version centurion 15.0). The experiments were carried out in a completely randomized block design.

3. Results

3.1. Effect of BCA application and storage conditions on Botrytis cinerea infections

The efficacy of two BCAs (L1 and L8) was tested on differently wounded kiwifruits (EQW and SEW), artificially inoculated with *B. cinerea* (10^4 CFU mL^{-1}) and stored in NR or CA. Low oxygen concentrations stimulated the pathogen (data not shown), as all control fruits were totally infected, while in untreated fruits, stored in NR, the incidence of disease was lower, although related to the type of wound: EQW (75%) and SEW (15%) (Fig. 1). Both L1 and L8 strains significantly reduced the gray mold (Fig. 2) in all experiments, although L1 showed greater effectiveness than L8. In NR their efficacy was highest; L1 strain completely inhibited the pathogen in both types of wound, while L8 showed a reduction of 100% and 80% for SEW and EQW, respectively. In fruits treated with BCAs and stored in CA, the reduction of disease was lower, ranging between 30% (L1) and 60% (L8), for both EQW and SEW inocula.

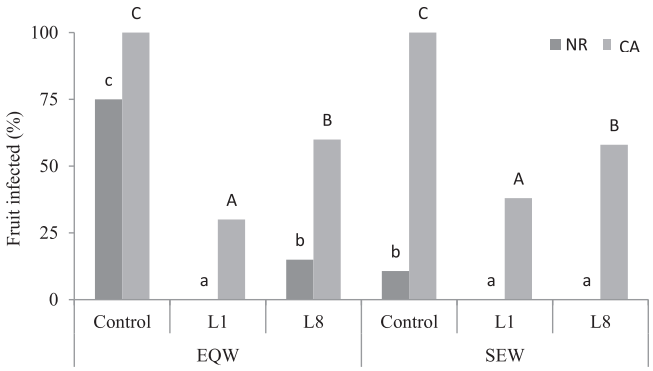

Fig. 1. Incidence of *Botrytis cinerea* in kiwifruit stored in normal refrigeration (NR) or in controlled atmosphere (CA) at -1 °C for 4 months. Fruits were wounded with a sterile nail at the equator (EQW). Fruits were harvested with a small portion of pedicel which was immediately removed before treatment to simulate a stem end wound at the stem end (SEW). Fruits were treated with BCA cell suspension (10^8 cell mL^{-1}) and then inoculated with pathogen conidia suspension (10^4 conidia mL^{-1}). The data are the mean of four replicates of 20 fruits each. Same letters (lower case – NR; capital letter – CA) are not statistically significant according to LSD test; $P < 0.05$.

3.2. Population dynamic

The growth dynamics of both *A. pullulans* L1 and L8 strains was similar in kiwifruit stored in CA at -1 °C for 4 months (Fig. 3). The population of BCAs decreased during the first three months of storage, more quickly for L8 (2×10^6 CFU $wound^{-1}$) than L1 strain (2.5×10^6 CFU $wound^{-1}$). At the end of storage after 4 months, the population of the two antagonists was almost the same (2.1×10^6 CFU $wound^{-1}$ and 2×10^6 CFU $wound^{-1}$ for L1 and L8 respectively). Similar results were found while analysing the population dynamics of both yeasts in NR (data not shown).

3.3. Competition for nutrients

After 8 h of incubation, *B. cinerea* conidia dipped in water showed a low percentage of germination (31%) compared to conidia dipped in kiwifruit juice at 0.5% (66%) and 5% (80%). Both antagonists significantly reduced the conidia germination of *B. cinerea* both in water (42% on average) and in kiwifruit juice. The highest inhibition of conidia germination produced by BCAs strains was shown in 0.5% kiwifruit juice: 75.8% for L1 and 78.7% for L8.

In contrast with the results obtained from conidia germination, the reduction of germ tube length shown by L1 and L8 was higher in water than in kiwifruit juice at both tested concentrations; however, in all cases the antagonists achieved a significant inhibition of germ tube length (Table 1).

3.4. Amino acids HPLC analysis

Diluted kiwifruit juice was analyzed for amino acid content in the presence of antagonist strains L1 or L8, in order to verify the BCA amino acid consumption after 12 h of incubation at 25 °C and their possible role in the competition for nutrients. Twelve hours were chosen as incubation time since no differences in amino acid composition was detectable before that time (data not shown). In this experiment a kiwifruit juice concentrated at 10% was used in order to increase the sensitivity of the HPLC analysis.

After 12 h of incubation, aspartic and glutamic acid were the major amino acids of the diluted kiwifruit juice (6.4 mg L^{-1} and 10.2 mg L^{-1} respectively). The addition of yeast modified the amino acid composition by an increase of both detected amino acids, more consistent for L8 (11.6 and 18.3 mg L^{-1} for aspartic acid and

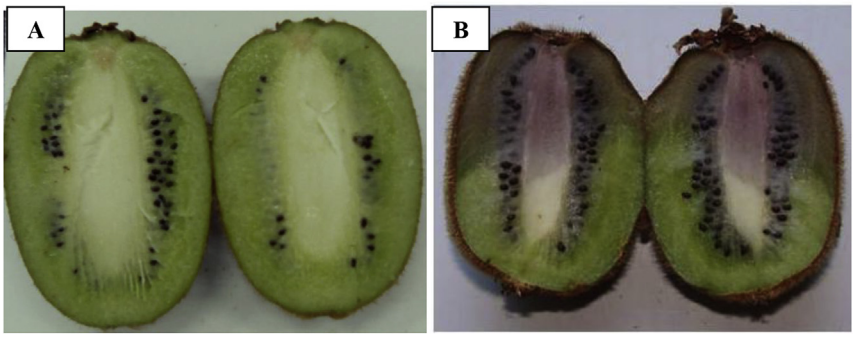

Fig. 2. Symptoms related to the stem end wound (SEW) inoculation with *Botrytis cinerea* (10^4 conidia mL^{-1}) after 4 months of storage in controlled atmosphere (CA). (A) Kiwifruit treated with *Aureobasidium pullulans* (10^8 CFU mL^{-1}); (B) control kiwifruit.

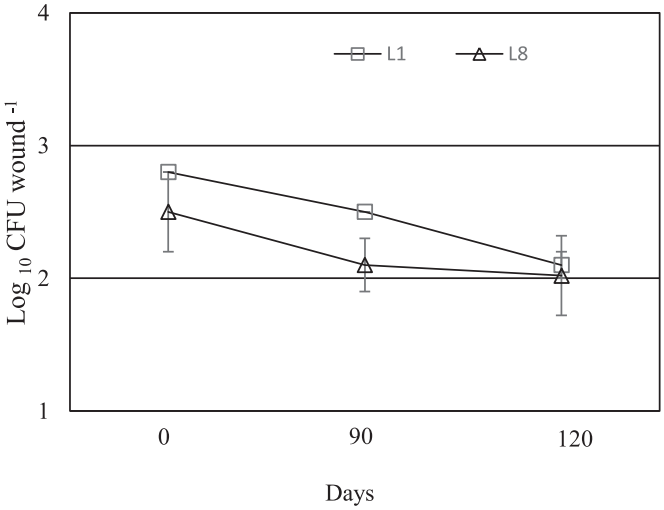

Fig. 3. Population dynamics of *Aureobasidium pullulans* (L1 and L8 strains) in kiwifruit wound at 0 °C in controlled atmosphere (CA) conditions for 120 days. Each point represents the mean of the number of colony forming units (CFUs) from three replicates (kiwifruit), each plated in triplicate at each sampling time.

glutamic acid respectively) than L1 strain (8.6 and 13.5 mg L^{-1}). At the same time, in fruit juice supplemented with yeast, other amino acids appeared, such as serine, glycine, threonine, arginine, alanine, valine, leucine, glutamine (for L1) and isoleucine (for L8), even if in lower amounts, indicating the ongoing metabolism of the antagonists. After 24 h of incubation, the amino acid distribution did not change (data not shown). In general, mainly L8 seemed to increase kiwifruit juice amino acids content respect to L1 (Table 2).

Table 2
Amino acid content (mg L^{-1}) in kiwifruit juice (10%) in the presence of water (control)/L1/L8 solutions (3:1 v/v) detected by HPLC-DAD after 12 h of incubation at 25 °C.

Amino acids	Control	L1	L8
Aspartic acid	6.4	8.6	11.6
Glutamic acid	10.2	13.5	18.3
Serine	nd[a]	3.4	4.9
Glutamine	nd	2.6	nd
Glycine	nd	1.1	3.5
Threonine	nd	1.8	4.8
Arginine	nd	4.8	4.7
Alanine	nd	4.3	7.7
Valine	nd	4.4	6.9
Isoleucine	nd	nd	5.6
Leucine	nd	6.6	7.2

[a] nd: not detectable.

3.5. Amino acid effect on Botrytis cinerea mycelium growth

The effect of the main detected amino acids, present in kiwifruit juice before and after yeast addiction, was assayed on *B. cinerea* mycelium growth using pure chemical products at different concentrations, chosen on the basis of (and encompassing) the previously determined amounts by HPLC analysis. All tested concentrations significantly reduced the pathogen growth, the best combinations being 5 mg L^{-1} for aspartic acid, 8 mg L^{-1} for glutamic acid, 6 mg L^{-1} for serine, 5 mg L^{-1} for glycine, 3 mg L^{-1} for threonine, 5 mg L^{-1} for arginine, 10 mg L^{-1} for alanine and 5 mg L^{-1} for valine, showing inhibition of 40%, 37.1%, 34.2%, 28.5%, 28.5%, 22.8%, 34.2% and 28.5% respectively (Table 3).

Table 1
Percentage of germinated conidia and germ tube length (μm) of *Botrytis cinerea* after 8 h of incubation with and without *Aureobasidium pullulans* (L1 and L8 strains) in water (0) or kiwifruit juice (0.5 and 5%).

	% Germinated *B. cinerea* conidia			Germ tube length (μm) of *B. cinerea* conidia		
	Kiwifruit juice %			Kiwifruit juice %		
	0	0.5	5	0	0.5	5
B. cinerea	31 ± 0.7b*	66 ± 1.7b	80 ± 0.58c	21.7 ± 1.87b**	21.0 ± 1.85b	23.0 ± 1.62b
B. cinerea + L1	19 ± 1.2a	16 ± 1.8a	44 ± 0.33b	9.15 ± 1.42a	15.3 ± 1.95a	18.0 ± 1.61a
B. cinerea + L8	17 ± 1.0a	14 ± 1.2a	28 ± 2.41a	6.42 ± 1.82a	14.3 ± 0.82a	16.5 ± 1.39a

*Values are the means of three repetitions (30 conidia each) per treatment. Means within the same column followed by different letters are significantly different ($P < 0.05$) according to LSD test.
**Values are the means of germ tube length of 90 germinated conidia. Means within the same column followed by different letters are significantly different ($P < 0.05$) according to LSD test.

Table 3
Effect of pure amino acids (mg L^{-1}) on *Botrytis cinerea* mycelial growth.

Aspartic acid (mg L^{-1})	Colony diameter (mm)	Glutamic acid (mg L^{-1})	Colony diameter (mm)	Serine (mg L^{-1})	Colony diameter (mm)	Glycine (mg L^{-1})	Colony diameter (mm)
0	35 ± 0.42c	0	35 ± 0.42c	0	35 + 0.42c	0	35 + 0.42c
2.5	26 ± 0.39b	4	24 ± 0.36b	2.5	25 + 0.32b	2.5	32 + 0.23b
5	21 ± 0.47a	8	22 ± 0.48a	4	25 + 0.30b	5	25 + 0.41a
10	22 ± 0.62a	16	23 ± 0.48 ab	6	23 + 0.45a	10	25 + 0.38a
Threonine (mg L^{-1})	Colony diameter (mm)	Arginine (mg L^{-1})	Colony diameter (mm)	Alanine (mg L^{-1})	Colony diameter (mm)	Valine (mg L^{-1})	Colony diameter (mm)
0	35 ± 0.42c	0	35 ± 0.42c	0	35 + 0.42d	0	35 + 0.42c
1.5	30 ± 0.29b	2.5	32 ± 0.26b	2.5	28 + 0.22c	2.5	30 + 0.33b
3	25 ± 0.30a	5	27 ± 0.30a	5	25 + 0.30b	5	25 + 0.31a
6	25 ± 0.42a	10	28 ± 0.38a	10	23 + 0.21a	10	25 + 0.27a

A mycelial plug (6 mm) from an actively growing pathogen culture was placed in the center of malt extract agar plates, incubated at 25 °C for 4 days. The control was represented by plates without amendments.
Data represent the mean of ten Petri dishes. Within a column, values followed by the same letters are not statistically significant at the LSD test ($P < 0.05$). Amino acids values were treated separately.

4. Discussion

In the present study, the application of *A. pullulans* strains significantly reduced the gray mold incidence of kiwifruit, stored in NR and CA. Postharvest application of BCAs for the control of fruit diseases was reported previously in a variety of plant host/pathogen systems (Cook et al. 1999, Wilson and Wisniewski, 1989) including *B. cinerea* of kiwifruit, controlled by yeasts and bacteria (Cheah et al., 1996, Cook et al. 1999). CA and temperature at -1 °C are the normal conditions for kiwifruit storage for a long time (3-5 months); however, these conditions increased the incidence of stem end rot (Brigati et al., 2003). Our results confirmed that the storage in CA favours the appearance of gray mold in previously inoculated fruit, achieving an incidence of 100% in control fruit; nevertheless, both BCAs, L1 and L8 strains, were able to reduce the disease. Moreover, a lower efficacy of antagonists was observed in kiwifruit stored in CA than fruit stored in RN, confirming the results previously obtained against *B. cinerea* of apple with *Trichosporon* sp. and *C. albidus* (Tian et al., 2002). In the present work, fruits were artificially infected either by removing the pedicel, simulating a stem end wound, the preferred entry point for *B. cinerea,* or by making a wound, at the equator of the fruit, with a sterile nail. Both types of wound were colonized by pathogen, although when kiwifruit were stored in NR only 15% of the stem end wound of untreated fruits showed symptoms of gray mold. Although it is known that kiwifruit are highly susceptible to *B. cinerea* infection through wounds in the pericarp, not all fruits develop rots when inoculated through the picking wound (Poole and McLeod, 1994). A high level of resistance to infection through this entry path was observed in stored fruit, suggesting that some components of these tissues were able to inhibit the germination of *B. cinerea* conidia (Poole and McLeod, 1991).

Both strains highlighted the ability to be cold tolerant (Mari et al., 2012a) and able to survive in the fruit wound also at low concentrations of O_2 (2%) and relatively high concentrations of CO_2 (4.5%). Many yeasts require CO_2 as a carbon source for growth (Rockwell and Highberger, 1927) and when CO_2 dissolves in water, carbonic acid is produced and pH is lowered. The yeast-like form of *Aureobasidium* spp. is predominant at pH 6.0–8.0, while the mycelial form prefers lower pH (2.0–2.5) (Lee et al., 2002); this could explain the population decline of both BCAs throughout the storage in CA and their lower efficacy against *B. cinerea* in fruit stored at 4.5% CO_2.

Better results were detected in NR, where L1 strain totally reduced disease development and L8 strain controlled the pathogen by over 80%, both after 4 months of storage. Nevertheless, storage at low oxygen concentrations could alter fruit primary metabolism (Tonutti, 2015) and indirectly reduce the antagonistic effect of BCAs.

Pennycook and Manning (1992) found that in kiwifruit pedicel removal triggered a significance increase in host resistance against *B. cinerea*, with maximum values when fruit was kept at 10 °C for 3 days and minimum values when fruit was constantly incubated at 0 °C (Bautista Banos et al., 1997). Nevertheless, the disease control obtained with curing is not always satisfactory, depending on the year and kind of antagonist. The application of BCAs in addition to curing improved the biocontrol activity but only when BCAs were supplemented after 96 h of fruit curing. The host resistance mechanisms in the first 24-48 h of curing are probably not specific to *B. cinerea* but also affect other epiphytic microbes (Cook et al., 1999).

L1 and L8 strains were previously investigated for their mechanisms of actions (Di Francesco et al., 2016); in particular the competition for nutrients and space represents their fundamental mode of action (Di Francesco et al., 2017). The protocol set up by Janisiewicz et al. (2000), that separates the pathogen from the antagonist cells while keeping both in the same medium, was used in several antagonist/pathogen interactions such as *A. pullulans*/*Monilinia laxa* (Di Francesco et al., 2017), *A. pullulans/Penicillium expansum* (Bencheqroun et al., 2007) and *Kloeckera apiculata/P. italicum* (Liu et al., 2013). In the present study, the germination rate of *B. cinerea* conidia was low when the pathogen was dipped only in water, in agreement with previous observations on germination of necrotrophic pathogens (Di Francesco et al., 2017; Byrde and Willetts, 1977), which need sufficient nutrients for conidia germination and hyphal development (Bencheqroun et al., 2007). In water, L1 and L8 strains reduced *B. cinerea* germination probably involving other mechanisms of action such as antibiosis (Di Francesco et al., 2015b). In kiwifruit juice, the germination of *B. cinerea* conidia was reduced more at a concentration of 0.5% than at a concentration of 5%. Previous results obtained in peach juice, with the same antagonists and *M. laxa* conidia, showed a similar behaviour (Di Francesco et al., 2017). A greater availability of nutrients (5%) stimulated the pathogen conidia germination and reduced the inhibitory effect of BCAs as formerly observed by Edwards (1970) on microbial kinetics.

The amino acid HPLC analysis of kiwifruit juice detected aspartic and glutamic acid as the main amino acids present. These data are quite different from those obtained in peach juice, where the main amino acid was asparagine, followed by aspartic acid. However, after 12 h of incubation in both kiwifruit and peach juice, the antagonists L1 and L8 increased the concentration of aspartic and

glutamic acid. In addition, new amino acids such as serine, glycine, threonine, arginine, alanine and valine were produced in both juices, although at low concentrations but probably with an additive effects against *B. cinerea* growth. Not all organisms are able to synthesize amino acids despite this feature being highly prominent and specific and exploited in different industrial requirements, such as glutamic acid widely used in recent years for its extensive application in the food industry (Mahmood, 2015).

In our trials, the detected amino acids were assayed at similar detected concentrations in juice, after 12 h of antagonist incubation, revealed an antifungal effect against mycelium growth of *B. cinerea*, confirming the results obtained against *M. laxa* by aspartic acid (Di Francesco et al., 2017). Although the two amino acids aspartic and glutamic acids displayed a partial reduction of *B. cinerea* growth *in vitro*, actually similar to the one shown by the two amino acids concentration present in kiwifruit juice without the yeasts, it is possible that the BCAs activity could result from the combined action of the other different amino acids de novo produced by L1 and L8. For example glycine, serine, alanine and leucine all appeared to slightly inhibit *B. cinerea* growth and they were all previously used like antiseptic agents due to their low level of toxicity (Minami et al., 2004; Baisa et al., 2013; Barreteau et al., 2008; Fox et al., 1944).

An effective antagonist has to be compatible with postharvest conditions; in this study, *A. pullulans* L1 and L8, cold tolerant and active strains in CA, showed how it can be effectively applied to control the stem end rot of kiwifruit during long storage. Many aspects on the mechanisms of action of these BCAs still have to be clarified; previous results highlighted that the emission of volatile organic compounds was involved in the growth reduction of *B. cinerea* (Di Francesco et al., 2015b) or that the production of hydrolytic enzymes played an important role in the inhibition of *M. laxa* conidia germination (Di Francesco et al., 2015b). In the present work, only BCA application was tested, and further studies are required for a multifactor approach where kiwifruits are cured, keeping them at 10 °C for 4 days, treated with L1 or L8 strains and then placed in NR or CA at - 1 °C for storage. With this approach, curing and BCA application are safe, making adequate decay control possible without the use of fungicides.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2017.11.010.

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