Biological Control 121 (2018) 151–158

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Enhancing the efficacy of yeast biocontrol agents against postharvest pathogens through nutrient profiling and the use of other additives

Betina R. Gramisci[a], M. Cecilia Lutz[b], Christian A. Lopes[a,b], Marcela P. Sangorrín[a,*]

[a] Grupo de Biodiversidad y Biotecnología de Levaduras, Instituto de Investigación y Desarrollo en Ingeniería de Procesos, Biotecnología y Energías Alternativas (PROBIEN, Consejo Nacional de Investigaciones Científicas y Tecnológicas – Universidad Nacional del Comahue), Buenos Aires, 1400 (8300) Neuquén, Argentina
[b] Facultad de Ciencias Agrarias, Universidad Nacional del Comahue, Argentina

ARTICLE INFO

Keywords:
Postharvest diseases
Antagonist yeast
Biocontrol
Nutritional profile

ABSTRACT

Vishniacozyma victoriae and *Pichia membranifaciens* were selected in a previous work for their biocontrol effectiveness against *Penicillium expansum* and *Botrytis cinerea*, the causal agents of blue and grey mold of pear fruits. The objective of this study was to determine the effects of different nutrient or additives on the growth of the two antagonist yeasts and of the two fungal pathogens in order to develop a rational selection based on nutritional profiles to be used in biocontrol enhancement of decay in pear fruits. Twenty-six different nutrient sources including 18 amino acids, five sugars, three inorganic nitrogen and one iron source were tested *in vitro* for their effect on the growth of the two yeasts and the two pathogens. Nutrients that promoted the growth of the yeasts and inhibited the growth of pathogens were applied with the antagonist to wounded fruits to evaluate their effect on enhancing biocontrol in cold storage. In general, the effect of each additive was specific to the yeasts and pathogens used in the *in situ* assays. The combination of the yeast and some additives resulted in a significantly higher activity with respect to the single treatments applied separately, producing synergistic effects. The effect of the exogenous application of $CaCl_2$ and chitosan together with the antagonist yeasts was also evaluated against the two pathogens. The most effective mixtures were the $CaCl_2$ with the two antagonist yeasts. Our research demonstrates that manipulating the chemical environment by adding several amino acid and/or Cl_2Ca results in improved antagonistic activity of *Vishniacozyma victoriae* and *Pichia membranifaciens* against two postharvest diseases of pear fruits.

1. Introduction

Postharvest diseases are responsible for consistent losses of fresh fruits and vegetables (up to 50–60% of fresh products) (Nunes, 2012; Usall et al., 2016). Today, the control of postharvest pathogens still relies mainly on the use of synthetic fungicides. However, the public demands to reduce the use of fungicides and the development of fungicide resistant pathogens bring about an urgent need for searching alternative control strategies. The biological control agents (BCAs) have been the focus of considerable research over the last 30 years by many scientists and several commercial companies worldwide (Droby et al., 2016; Wisniewski et al., 2016).

As a generalization, BCA must be better adapted than the pathogen to the environmental and nutritional conditions of the fruit or vegetable wound, growing rapidly and using nutrients effectively at low concentrations. In this sense, different chemical compounds (additives) have been proposed as an alternative to enhance biocontrol activity (Bencheqroun et al., 2007; Liu et al., 2013; Nunes et al., 2001). Improvement of biocontrol with additives may result from direct inhibition of the pathogen, from elicitation of systemic acquired resistance in the host tissue and/or from stimulation of antagonistic activity. The combinations of yeast with other compounds have also been demonstrated to be an effective method for improving biocontrol performance (Liu et al., 2013). Moreover, additives can make it possible to use a lower antagonist concentration without affecting the protection level (Bastiaanse et al., 2010; Liu et al., 2013), the presence of specific additives in the formulation of BCAs appears as an essential prerequisite to the commercial success of BCAs. As an example, chitosan (poly-β-(1 → 4) N-acetyl-D-glucosamine), together with its derivatives, has been reported to control postharvest diseases through its antifungal properties and ability to elicit host defence responses (El Ghaouth et al., 2004; Palou et al., 2015). Another group of additives used alone or in combination with biocontrol agents is formed by inorganic salts and minerals, such as calcium chloride, ammonium molybdate and sodium bicarbonate (Droby et al., 2016; Wisniewski et al., 2016).

Blue mold and grey mold decays caused by *Penicillium expansum* and

* Corresponding author.
E-mail address: sangorrinmarcela@conicet.gov.ar (M.P. Sangorrín).

https://doi.org/10.1016/j.biocontrol.2018.03.001
Received 30 September 2017; Received in revised form 28 February 2018; Accepted 1 March 2018
Available online 02 March 2018

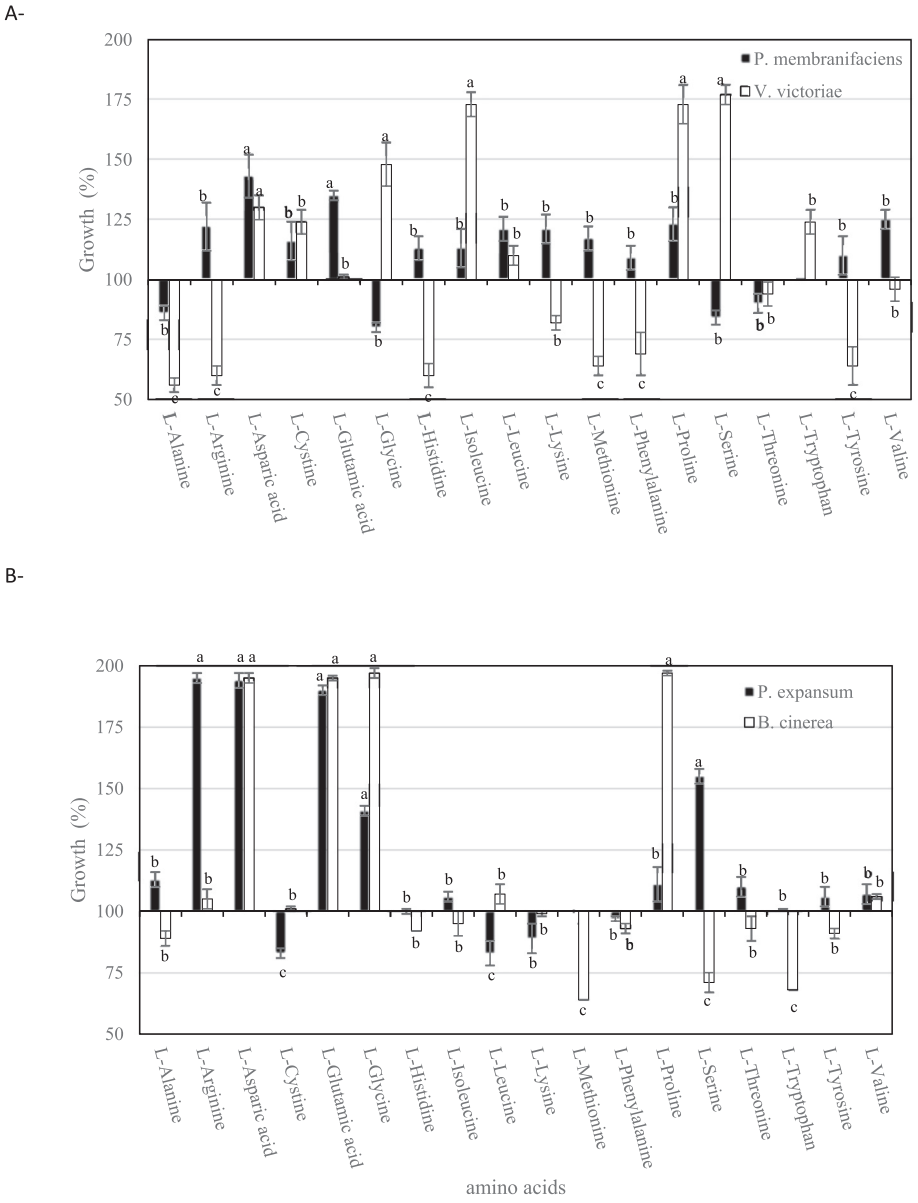

Fig. 1. Effect of the addition of amino acids on yeasts (A) and molds (B) growth. The growth percentage (%) for each treatment was calculated by comparison with the control (100%) without additives. Bars represent standard deviations. Letter at the top bars indicate significantly difference according to Tukey's test at $p \leq .05$.

Botrytis cinerea respectively, are the most important postharvest pear diseases (Yu et al., 2007). Recently, we isolated and identified the epiphytic yeasts *Pichia membranifaciens* NPCC 1250 and *Vishiniacozyma victoriae* NPCC 1263, ex *Cryptoccocus victoriae* (Lui et al., 2015), during cold postharvest storage of pear fruits in Argentinian North Patagonia and we tested their efficacy for controlling the postharvest diseases of pears caused by these two pathogens (Lutz et al., 2013, 2012). Previously reported works have shown that epiphytic antagonists are influenced by a number of biological and environmental factors and storage practices (Droby et al., 2016; Fiori et al., 2012). In this sense, pear fruits are a rich source of carbohydrates but are poor in nitrogen (Yim and Nam, 2016). Thus, nitrogen sources could be a growth limiting factor for both antagonist and pathogen and could be manipulated to enhance antagonist activity.

The objective of this study was to determine the effects of different additives on the growth of *P. membranifaciens* and *V. victoriae* and of the fungal pathogens *P. expansum* and *B. cinerea* in order to develop a rational selection based on nutritional profiles to be used in biocontrol enhancement of decay in pear fruits.

2. Materials and methods

2.1. Microorganisms and culture conditions

Both spoilage fungi (*Botrytis cinerea* NPCC 2049 and *P. expansum* NPCC 2023) and antagonist yeasts (*Pichia membranifaciens* NPCC 1250 and *Vishiniacozyma victoriae* NPCC 1263) were isolated from pear fruits Packhaḿs Triumph cultivars after six months of storage at -1 ± 0 °C (Lutz et al., 2012). All microorganisms were preserved in glycerol 20% (v/v) and stored at -20 °C in the North Patagonian Culture Collection (NPCC), Neuquén, Argentina.

B. cinerea NPCC 2046 and *P. expansum* NPCC 2023 cultures were grown at 26 °C on potato–dextrose agar (PDA) and kept at 4 °C until use. The conidia suspension was obtained from 7-days-old cultures in darkness at 20 °C, and adjusted to a Minimal Conidia Concentration (MCC) of 5×10^3 or 1×10^4 conidia/mL, respectively, adjusted by direct courting using a Neubauer chamber.

V. victoriae NPCC 1263 and *P. membranifaciens* NPCC 1250 cells suspensions were prepared by growing cultures on Glucose Peptone

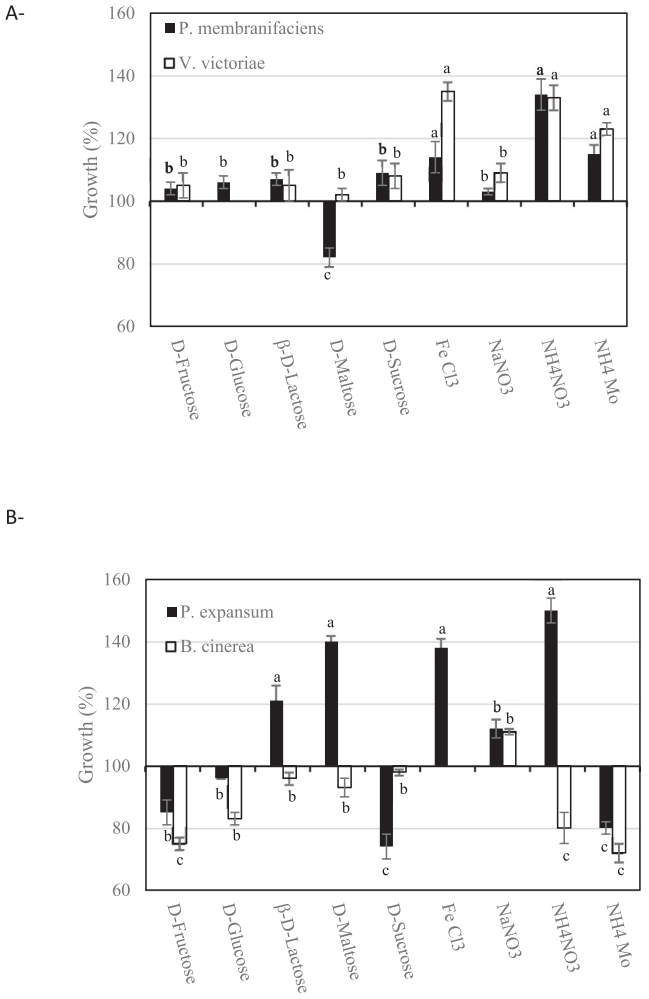

Fig. 2. Effect of the addition of sugars and salts on yeasts (A) and molds (B) growth. The growth percentage (%) for each treatment was calculated by comparison with the control (100%) without additives. Bars represent standard deviations. Letter at the top bars indicate significantly difference according to Tukey's test at $p \leq .05$.

Table 1
Selected compounds from nutritional profile of microorganisms.

Pathogen	Antagonist Yeast	Compounds that stimulate yeasts growth and inhibit molds growth (Fig. 3 and Table 2)	Compounds that stimulate yeasts and molds growth (Fig. 4 and Table 3)
B. cinerea	*P. membranifaciens*	L-Methionine NH_4Mo NO_3NH_4	L-Aspartic acid L-Glutamic acid L-Prolina
	V. victoriae	L- Serine L-Tryptophan NH_4Mo NO_3NH_4	L-Aspartic acid L-Prolina L-Glycine
P. expansum	*P. membranifaciens*	L-Cysteine L-Leucine NH_4Mo	L-Arginine L-Glutamic acid $FeCl_3$ NO_3NH_4
	V. victoriae	L-Cysteine L-Leucine NH_4 Mo	L-Serine L-Aspartic acid $FeCl_3$ L-Glycine NO_3NH_4

Yeast-agar (GPY-agar: glucose, 40 g/L; peptone 5 g/L; yeast extract, 5 g/L; agar, 20 g/L) for 48 h at 20 °C. A loop of the respective yeast culture was removed from the agar surface and suspended in sterile water at concentration of 10^6 cell/mL adjusted by direct courting using a Neubauer chamber.

2.2. Nutritional profile of microorganisms

Nutritional profile was evaluated *in vitro* as the ability of both the BCAs and the pathogens to grow in pear juice medium supplemented or not with a nutrient. Pear juice was prepared by homogenization with distilled water (15% v/v), and the homogenate was filtrated through a Whatman No 1 filter. Twenty-six different nutrient sources including five sugars, 18 amino acids, three inorganic nitrogen sources and one inorganic iron sources were used. Amino acids known to be present in apple tissues were used at concentration 10 times the amount reported for pear tissues (USDA, 2016). The criterion for selecting these concentrations was supported by previous reference (Bencheqroun et al., 2007)

These amino acids and sugar were tested containing per litre (g) : L-Alanine (1.12), L-Arginine (0.8), L-Asparic acid (8.4), L-Cystine (0.16), L-Glutamic acid (2.4), L-Glycine (1.04), L-Histidine (0.16), L-Isoleucine (0.88), L-Leucine (1.52), L-Lysine (1.36), L-Methionine (0.16), L-Phenylalanine (0.88), L-Proline (1.68), L-Serine (1.2), L-Threonine (0.88), L-Tryptophan (0.16), L-Tyrosine (0.16), L-Valine (1.36), D-Fructose (1092), D-Glucose (4 8 4). β-D-Lactose (2), D-Maltose (2) and Sucrose (1 3 7). The iron source ($FeCl_2$) and the nitrogenous compounds (NH_4NO_3, $NaNO_3$ and $NH_4Mo_7O_{24}$) were used at a concentration of 0.1%, supported by some preliminary studies (Chanchaichaovivat et al., 2008; Lutz et al., 2013). All solutions were filter sterilized.

Tests for utilization of nitrogenous compounds were conducted on a Raytor microplates reader (BibbySterlin Ltd, Stone, Staffs, UK), by employing the technique validated by (Langvad et al., 1999). The experiments were done in quadruplicate: 10 µL aliquots of each yeast or fungal suspension at 10^6 cells/ml were independently transferred into each well of a 96-well polystyrene plate containing pear juice supplemented or not with each nutrient. The plates were incubated at 20 °C under agitation, 150o.p.m (orbits per minute), for up to three days for *P. membranifaciens* and *V. victoriae,* four days for *B. cinerea* and five days for *P. expansum*. Yeast and fungal growth was assessed by measuring the optical density of the culture media at 550 nm. The relationships between Absorbance and dry weight in our tests were linear for the four cases.

2.3. Evaluation of nutrients or additives as biocontrol enhancers.

The effect of the exogenous application of nutrient on the biocontrol activity of *P. membranifaciens* and *V. victoriae* against *P. expansum* and *B. cinerea* was evaluated on wounded pears. Calcium chloride (2% v/v) and Chitosan (molecular weight between 500 and 800 kDa) (1% v/v) were also tested.

Packham's Triumph pear fruits stored for two months at −1 ± 0 °C were surface sterilised with 70% (v/v) ethanol, and air dried prior to wounding. One wound −3 mm deep and 3 mm wide - in the equatorial region of each fruit, was performed using a sterile cork borer. Each wound was inoculated with 20 uL of a 24 h yeast culture suspension (10^6 cells/mL). An aliquot (10 uL) of the corresponding nutrient solution was also pipetted into the wound 30 min after inoculation of the antagonist yeast. Pear wounds inoculated with 20 uL of distilled water, instead of yeasts, were used as controls in every case. After 60 min, the treated wounds were inoculated with 10 µL of a conidial suspension of $5x10^3$ conidia/mL (*B. cinerea*) or 1×10^4 conidia/mL (*P. expansum*). Fruits were placed on trays packs in boxes with polyethylene bags and stored for 120 days at 0 ± 1 °C and 95% relative humidity. The fruits were examined for decay every 15 days and disease incidence (DI) was calculated as the number of decay wounds over the total number of

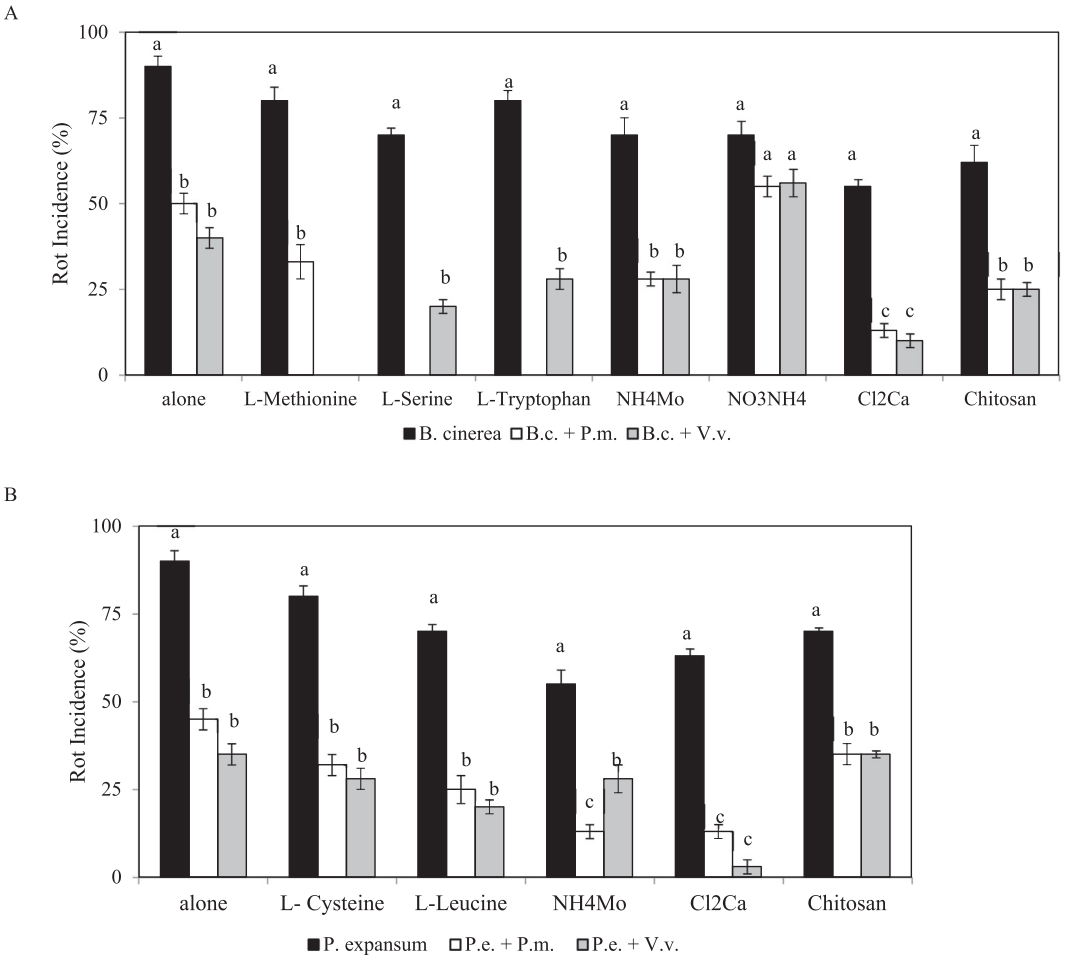

Fig. 3. Percentage of rot incidence caused by *Botrytis cinerea* (A) and *Penicillium expansum* (B) on pears inoculated with both antagonists with or without nutrients that stimulate yeasts growth and inhibit molds growth (Table 1) and additives (Cl_2Ca and Chitosan). **B.c.**: *Botrytis cinerea*; **P.e.**: *Penicillium expansum*; **P.m.**: *Pichia membranifaciens*; **V.v.**: *Vishniacozyma victoriae.* Bars represent standard deviations. Letter at the top bars indicate significantly difference according to Tukey's test at $p \leq .05$.

wounds. Ten fruits were used per treatment and each treatment was repeated three times. The biocontrol percentage (C%) for each treatment was calculated by comparison with the control (pathogen alone). The whole experiment described above was conducted twice.

2.4. Data analysis

To assess the advantage of combined postharvest treatments (BCA + additives) with respect to the same treatments applied alone (BCA or additives), the type of interaction (additive, synergistic or antagonistic) was evaluated. The synergy factor (SF) was calculated according to the Abbott's formula (Levy et al., 1986): SF = E.obs./E.exp., where Eobs. and Eexp. are, respectively, the observed and expected biocontrol percentage (C%) of the combination. Eexp. was calculated as follows: (Ea + Eb)–(Ea * Eb/100), where Ea = C% of postharvest treatment a (BCA); Eb = C% of postharvest treatment b (additives). If SF = 1, the interaction between the combination treatments was identified as additive; if SF < 1, the interaction was antagonistic and if SF > 1, the interaction was synergistic.

Data were subjected to a two-way analysis of variance (ANOVA) and means were separated according to the Tukey test (P = .05) using STATISTICA data analysis software system, version 8 (Stat-Soft, 2007, France).

3. Results

3.1. Nutritional profile of microorganisms

The growth of both yeasts and pathogens was affected differentially by the addition of nutrients in *in vitro* assays. Eleven out of 18 amino acids tested were utilized by *P. membranifaciens* and only seven of them were utilized by *V. victoriae* (Fig. 1a). In particular, L-Aspartic acid, L-Cysteine, L-Isoleucine, L-Leucine and L-Proline amino acids significantly improved the growth of both *V. victoriae* and *P. membranifaciens* (Fig. 1). Regarding the effect of all amino acids on pathogens growth, four and six amino acids were strongly utilized by *B. cinerea* and *P. expansum* respectively (Fig. 1b), three of them including L-Aspartic, L-Glutamic and L-Glycine caused significant increase in the growth of the two pathogens. Contrarily, nine amino acids caused an inhibition in the growth of *B. cinerea* and two amino acids (L-Cysteine and L-Leucine) inhibited the growth of *P. expansum* (growth percentages < 100%, Fig. 1b).

From a total of five carbon compounds tested, none of them stimulated significantly the growth of both *V. victoriae* and *P. membranifaciens* (Fig. 2a) and only lactose and maltose significantly stimulated the growth of *P. expansum* (Fig. 2b). On the other hand, the growth of the two pathogens was negatively affected by fructose and glucose.

The addition of the nitrogen salts NH_4Mo and NH_4NO_3 stimulated the growth of both antagonist yeasts (Fig. 2a) and only NH_4NO_3 increased the growth of *P. expansum*. $NH_4Mo_7O_{24}$ (NH_4Mo) inhibited the growth of both *B. cinerea* and *P. expansum.* Finally, the iron salt ($FeCl_3$)

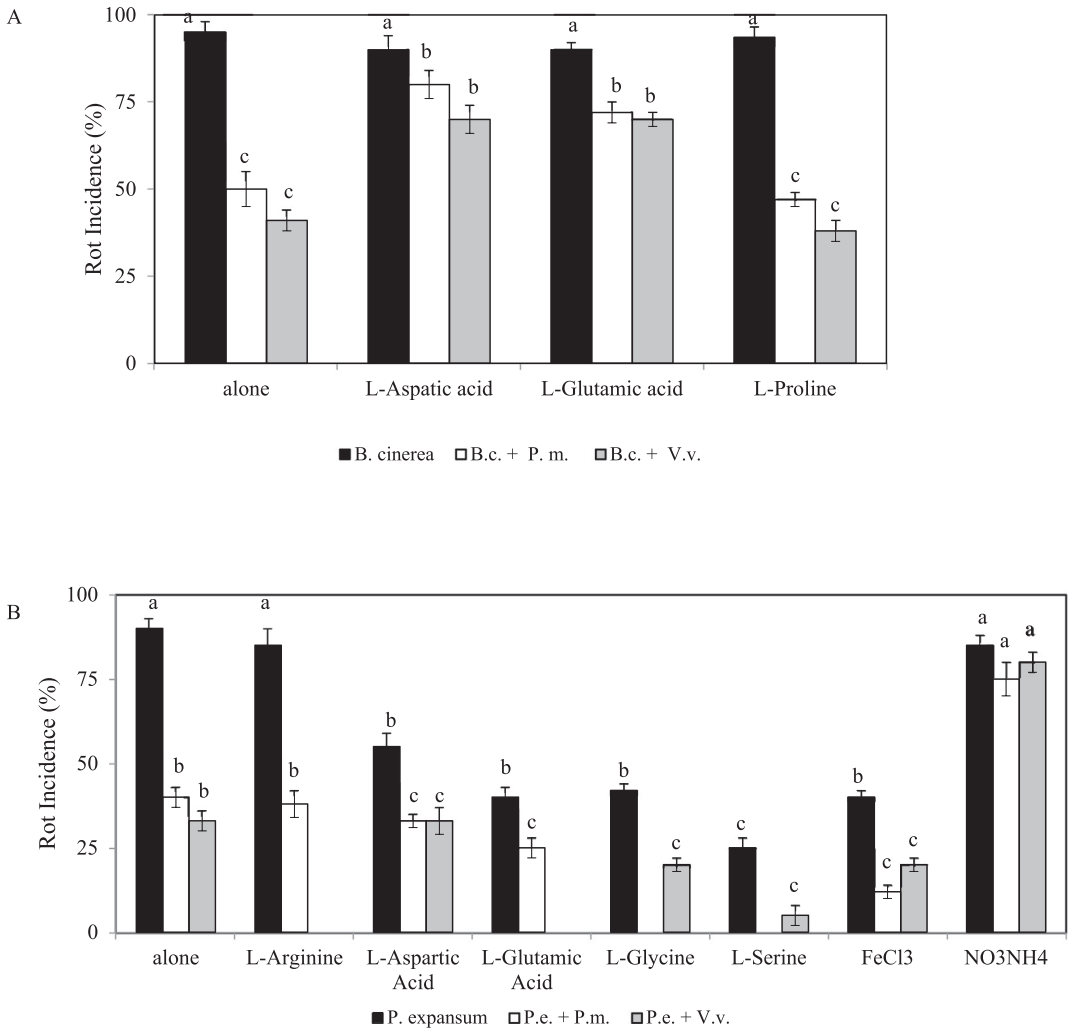

Fig. 4. Percentage of rot incidence caused by *Botrytis cinerea* (A) and *Penicillium expansum* (B) on pears inoculated with both antagonists with or without nutrients that stimulate yeasts and molds growth (Table 1). **B.c.**: *Botrytis cinerea*; **P.e.**: *Penicillium expansum*; **P.m.**: *Pichia membranifaciens*; **V.v.**: *Vishniacozyma victoriae.* Bars represent standard deviations. Letter at the top bars indicate significantly difference according to Tukey's test at $p \leq .05$.

was strongly utilized by both yeasts and increased the growth of *P. expansum* (Fig. 2b).

3.2. Enhancement of biocontrol efficacy

In situ tests using the same nutrient concentrations employed in the *in vitro* assays were carried out. Nutrients that promoted BCAs growth and, at the same time, inhibited pathogens growth, as well as, nutrients showing stimulatory effect on both microorganisms growth were examined (Table 1). These tests were evaluated for each yeast-pathogen combination and all the additives were tested alone (Figs. 3 and 4). The synergistic factor (SF) of each additive-BCA combination was further calculated (Tables 2 and 3) to ascertain the type of interaction (antagonistic, additive, or synergistic).

Three amino acids and the two nitrogen salts were tested as nutrients that promoted the growth of the BCAs and, at the same time, inhibited the growth of *B. cinerea* (Fig. 3). L-Methionine, L-Tryptophan and L-Serine amino acids significantly improved the biocontrol activity of the two antagonist yeasts, evidencing a synergistic effect (SF > 1) (Table 2). NH_4Mo only improved the biocontrol activity of *P. membranifaciens* (SF = 1.07) and NH_4NO_3 showed an antagonistic interaction (SF < 1) with the two yeasts. Regarding *P. expansum,* both L-Leucine and L-Cysteine amino acids were tested evidencing an enhanced biocontrol (synergy factor greater than 1, Table 2). Again, NH_4Mo improved the biocontrol activity of *P. membranifaciens* (SF = 1.07). On the other hand, all amino acids applied individually slightly inhibited the pathogens development in pear wounds (10–30%) (Fig. 3). Application of NH_4Mo alone, greatly reduced the incidence of *P. expansum* (40% of control), but the two ammonium salts had a lesser effect against *B. cinerea* (20% control in two cases). The effect of the exogenous application of the additives, $CaCl_2$ and chitosan, together with the antagonist yeasts was also evaluated against the two pathogens. The most effective mixtures, i.e., those with higher values of synergistic factor, were the $CaCl_2$ with the two BCAs (Table 2).

Finally, nutrients showing stimulatory effect on both antagonists and pathogens growth were tested in their capacity to improve the antagonist activity in *in situ* assays (Fig. 4). The seven compounds were tested individually against *P. expansum*, five of them showed a high inhibition of mold development in pear wounds (40–60% control), however, the four amino acids had a lesser effect against *B. cinerea* (5–10% control). Out of 16 evaluations, only the two that contained L-Proline showed SF = 1, evidencing an additive interaction, and none of the 16 showed a synergistic effect (Table 3).

4. Discussion

Despite its great potential for improving biocontrol, manipulation of the chemical environment has not been widely exploited. This may be

Table 2
Effect of compounds that stimulate yeast growth and inhibit molds growth in pears disease control.

Pathogen	Compounds(*)	Antagonist Yeast	Disease Control (%)	Ee	Synergy factor (SF)
B. cinerea		*P. membranifaciens*	45		
		V. victoriae	56		
	L-Methionine	———	10		
	L-Methionine	*P. membranifaciens*	60	50.5	1.19
	L-Serine	———	20		
	L-Serine	*V. victoriae*	70	60.8	1.08
	L-Tryptophan	———	10		
	L-Tryptophan	*V. victoriae*	65	60.4	1.08
	NH_4Mo	———	20		
	NH_4Mo	*P. membranifaciens*	60	56	1.07
	NH_4Mo	*V. victoriae*	60	64.8	0.93
	NO_3NH_4	———	20		
	NO_3NH_4	*P. membranifaciens*	20	56	0.36
	NO_3NH_4	*V. victoriae*	20	64.8	0.31
	Cl_2Ca	———	40		
	Cl_2Ca	*P. membranifaciens*	75	67	1.12
	Cl_2Ca	*V. victoriae*	80	73.6	1.09
	Chitosan	———	30		
	Chitosan	*P. membranifaciens*	60	61.5	0.98
	Chitosan	*V. victoriae*	60	68.5	0.88
P. expansum		*P. membranifaciens*	50		
		V. victoriae	60		
	L-Cysteine	———	10		
	L-Cysteine	*P. membranifaciens*	60	46	1.09
	L-Cysteine	*V. victoriae*	65	55	1.09
	L-Leucine	———	20		
	L-Leucine	*P. membranifaciens*	65	60	1.08
	L-Leucine	*V. victoriae*	70	60	1.17
	NH_4Mo	———	40		
	NH_4Mo	*P. membranifaciens*	75	70	1.07
	NH_4Mo	*V. victoriae*	50	76	0.66
	Cl_2Ca	———	30		
	Cl_2Ca	*P. membranifaciens*	80	65	1.26
	Cl_2Ca	*V. victoriae*	95	72	1.38
	Chitosan	———	20		
	Chitosan	*P. membranifaciens*	50	60	0.83
	Chitosan	*V. victoriae*	50	68	0.74

(*) Selected nutrients (from Table 1) and additives (bold type). **Disease control:** biocontrol percentage for each treatment was calculated by comparison with the control (pathogen + water). **Ee:** Expected effect or expected biocontrol percentage, **SF**: synergy factor, see Materials and Methods.

attributable to limited knowledge of the mechanisms of biocontrol. If nutrients limiting pathogens or antagonists growth were known, they could be manipulated to stimulate antagonist populations and/or biocontrol mechanisms (Bastiaanse et al., 2010; Spadaro and Droby, 2016). The need for suitable alternatives to fungicides for the control of postharvest decay has prompted research aiming to devise an integrated control strategy combining various alternatives and having the same efficacy as fungicide treatments. The probability of successfully finding stimulatory compounds can be increased by a rational selection of the nutrient in *in vitro* tests before testing on fruit. The objective of the present study was to evaluate the feasibility of the combined application of BCAs with nutritional compounds or additives for the rot biocontrol on pear. This objective was successfully achieved, because different compounds improved the biocontrol activity of the tested antagonists against both *P. expansum* and *B. cinerea*.

Our previous results showed that the biocontrol capability of the two selected yeasts identified as *P. membranifaciens* and *V. victoriae* was significantly affected in the presence of the same carbon and nitrogenous sources used in this work (Lutz et al., 2013), showing competition for nutrients and germination inhibition. However, the effect of individual amino acids usually present in the pear have not been evaluated (USDA, 2016). Improved biocontrol with nutritional amendments can result from stimulation of antagonist growth and inhibition of the pathogen. For this reason, in this work we first tested *in vitro* the ability of the two yeasts and the two pathogens to grow in the presence of 26 different nutrient sources including 18 amino acids, five sugars, three inorganic nitrogen and one iron sources. The broad nitrogen-utilizing capacity of the two yeasts observed in our work suggests that BCAs are better competitors in the limiting nitrogen conditions typical of pear wounds.

Nearly 60% of the total nitrogen in pear fruits is present in the form of amino acids, being L-Aspartic acid, L-Glutamic acid and L-Proline the most abundant amino acids in pear fruits (Yim and Nam, 2016). In our work, both, yeasts and pathogens, showed to be stimulated by the majority amino acids of pear indicate that they would have to compete to colonize fruits wounds. Although sugar is required for conidial germination and initiation of the pathogenic process, due to the high concentration of sugars naturally present in pears, it is unlikely that sugars could play a significant role in this biocontrol system. In our assays, fructose and glucose *per se* inhibited the growth *in vitro* of *P. expansum* and *B. cinerea* at the evaluated concentrations. Previous studies have also shown the inhibitory effect of fructose and glucose on *P. expansum* by reduction of spore germination (Lutz et al., 2013; Scherm et al., 2003); and similar effects were reported for the same two sugars as well as sucrose on *Botrytis* conidial germination (Card et al., 2009; Lutz et al., 2013)

In order to achieve a nutritional manipulation that enhances the biocontrol activity, we proceeded to the search of the nutrients that specifically enhance the growth of the antagonist yeasts but inhibit the pathogens growth. The amino acids in our system were utilized readily by the antagonists but poorly by the pathogens, however the sugar did not stimulate significantly the growth of both yeasts and molds. *V. victoriae* and *P. membranifaciens* were stimulated by eleven and seven amino acids respectively, while only four and five were strongly utilized

Table 3
Effect of compounds that stimulate growth yeast and molds on disease control on pears.

Pathogen	Compounds(*)	Antagonist Yeast	Disease Control (%)	Ee	Synergy factor (SF)
B. cinerea		*P. membranifaciens*	47		
		V. victoriae	57		
	L-Aspatic acid	———————	10		
	L-Aspatic acid	*P. membranifaciens*	10	52.3	0.19
	L-Aspatic acid	*V. victoriae*	20	61.3	0.33
	L-Glutamic acid	———————	10		
	L-Glutamic acid	*P. membranifaciens*	20	52.3	0.38
	L-Glycine	———————	10		
	L-Glycine	*V. victoriae*	20	61.3	0.33
	L-Prolina	———————	5		
	L-Prolina	*P. membranifaciens*	50	49.65	1.00
	L-Prolina	*V. victoriae*	60	59.15	1.00
P. expansum		*P. membranifaciens*	55		
		V. victoriae	63		
	L- Arginine	————————	6		
	L- Arginine	*P. membranifaciens*	57	57.25	0.99
	L-Aspatic acid	————————	40		
	L-Aspatic acid	*P. membranifaciens*	40	73	0.55
	L-Aspatic acid	*V. victoriae*	40	77.8	0.51
	L-Glutamic acid	———————	60		
	L-Glutamic acid	*P. membranifaciens*	40	82	0.49
	L-Glycine	———————	50		
	L-Glycine	*V. victoriae*	50	81.5	0.61
	L-Serina	———————	70		
	L-Serina	*V. victoriae*	80	88.9	0.90
	$FeCl_3$	———————	70		
	$FeCl_3$	*P. membranifaciens*	70	86.5	0.81
	$FeCl_3$	*V. victoriae*	50	88.9	0.56
	NO_3NH_4	———————	5		
	NO_3NH_4	*P. membranifaciens*	10	57.25	0.17
	NO_3NH_4	*V. victoriae*	5	64.85	0.08

(*) Selected nutrients (from Table 1). **Disease control:** biocontrol percentage for each treatment was calculated by comparison with the control (pathogen + water). **Ee**: Expected effect or expected biocontrol percentage, **SF**: synergy factor, see Materials and Methods.

by *B. cinerea* and *P. expansum,* respectively. This discrepancy resulted in an increased growth of the antagonist yeasts, shifting the balance of the antagonist-pathogen system to favour the antagonist. As a result, an enhanced biocontrol was observed in the high values of synergy obtained in this work after adding selected nutrients in pear wounds (from a total of thirteen evaluations, nine were synergistic, SF > 1). The interaction between BCA and nutrients was also highly significant, and the activity of each BCA was improved at different levels by the same additive.

These results are consistent with observations in various biocontrol systems on fruit; however, it has been previously observed that, for each particular pathogen-antagonist-fruit system, a particular relationship must be found. Janisiewicz et al., (1992) have already demonstrated that L-Asparagine and L-Proline enhanced the biocontrol of blue mold on apples by the *Peudomonas syringae*. They have also shown that the two amino acids were utilized readily by the antagonist but had little effect on pathogen radial growth in *in vitro* assays. In another work, the bioefficacy of *Candida sake* against *P. expansum* on apples was enhanced significantly with the addition of L-Serine and L-Aspartic (Nunes et al., 2001). Instead, Vero et al., (2002) have already demonstrated that the addition of yeasts suspended in the amino acid solution did not result in an enhancement of the biocontrol. These authors only demonstrated that the amino acids increased the *in situ* development of the yeast, but did not demonstrate the effect of these amino acids on the pathogen growth. Our work is the first report that has evaluated the nutritional profile of both the two antagonist yeasts and the two pathogen molds considering the fruit composition, pears in our case, and testing the biocontrol activity of these compounds on fruits under storage conditions. In this work, we show that L-Cysteine and L-Leucine enhanced biocontrol of *P. expansum* and *B. cinerea* on pears, whereas L-Methionine, L-Serine and L-Tryptophan enhanced biocontrol of *B. cinerea*. The application *in situ* of these amino acids individually did not have a significant reduction of blue and grey decay.

On the other hand, we combined the nutrients that stimulated the growth of both BCAs and pathogens. In this case, the nutrients significantly weakened the biocontrol effect of the two antagonists against the two pathogens on pears (synergy factor < 1). Only in the case of L-Proline, which stimulates the growth of *B. cinerea* and the two yeast antagonists, an additive effect (SF = 1) was observed with no improvement in biocontrol. We can conclude from our data that the addition of these nutrients does not increase the synergistic effect, since only two cases out of sixteen evaluated in Table 3 presented a SF > 1. Instead, the addition of the selected nutrients for their ability to stimulate yeasts growth and to inhibit molds growth (Table 2), does increase the synergistic effect, eleven of thirteen evaluations were synergistic (SF > 1). Therefore, the probability to find successfully stimulatory compounds can be increased by a rational pre-selection of a large list of compounds in *in vitro* tests before testing them on fruits.

Despite the positive results obtained with the amino acids, the application of these nutrients as additives in commercial scale is economically difficult. In the search for lower additives costs, natural compounds included in the GRAS (Generally Recognized as Safe) list could be evaluated (Usall et al., 2016; Wisniewski et al., 2016). Mineral compounds are more extensively used in combined treatment studies and have generally been proposed to enhance the antagonists biocontrol efficacy (Liu et al., 2013; Yu et al., 2012). Our results demonstrated that combining NH_4Mo with the two BCAs stimulated *in vitro* growth and inhibited the development of the two pathogens. In our case, different effects were observed with each BCA on pear wounds: synergic effect with *P. membranifaciens* (SF = 1.07) and antagonist effect with *V. victoriae* (SF < 1) against the two pathogens evaluated. The ability of $CaCl_2$, to improve the biocontrol activity of antagonist yeasts against several postharvest diseases of fruits has been widely demonstrated (Lima et al., 2011; Yu et al., 2012; Wisniewski et al., 2016). In our

results, $CaCl_2$ alone showed a reduction of the incidence of rot caused by *B. cinerea* (40% control) and *P. expansum* (30% control), the combination of both BCAs with $CaCl_2$ resulted in synergistically enhanced biocontrol activity (SF > 1) against the two pathogens. On the other hand, positive results were reported adding chitosan to improve the performance of biocontrol agents in controlling postharvest pathogens (Romanazzi et al., 2016; Yu et al., 2012). In our work, the application of chitosan alone had a lesser effect against both pathogens, 20–30% control in two cases and antagonistic effects (SF < 1) were observed on pear wounds.

The addition of particular compounds to antagonist yeasts suspensions could be employed to enhance their antagonistic activity, but the influence of each additive or nutrient will be different, depending on the BCAs, fruits and pathogens should be evaluated. Our research demonstrates that manipulating the chemical environment by adding several amino acids and Cl_2Ca results in improved antagonistic activity of *P. membranifaciens* and *V. victoriae* against two postharvest diseases of pear fruits.

Acknowledgments

This work was supported by Universidad Nacional del Comahue, ANPCyT PICT 2780 and CONICET PIP 470. Lutz, M.C and Gramsci, B. are the recipient of a fellowship from CONICET.

References

Bastiaanse, H., de Lapeyre de Bellaire, L., Lassois, L., Misson, C., Jijakli, M.H., 2010. Integrated control of crown rot of banana with *Candida oleophila* strain O, calcium chloride and modified atmosphere packaging. Biol. Control 53, 100–107. http://dx.doi.org/10.1016/j.biocontrol.2009.10.012.

Bencheqroun, S.K., Bajji, M., Massart, S., Labhilili, M., Jaafari, S. El, Jijakli, M.H., 2007. In vitro and in situ study of postharvest apple blue mold biocontrol by *Aureobasidium pullulans:* evidence for the involvement of competition for nutrients. Postharvest Biol. Technol. 46, 128–135. http://dx.doi.org/10.1016/j.postharvbio.2007.05.005.

Card, S.D., Walter, M., Jaspers, M.V., Sztejnberg, A., Stewart, D., 2009. Targeted selection of antagonistic microorganisms for control of Botrytis cinerea of strawberry in New Zealand. Australas. Plant Path. 38, 183–192.

Chanchaichaovivat, A., Panijpan, B., Ruenwongsa, P., 2008. Putative modes of action of *Pichia guilliermondii* strain R13 in controlling chilli anthracnose after harvest. Biol. Control 47, 207–215. http://dx.doi.org/10.1016/j.biocontrol.2008.07.018.

Droby, S., Wisniewski, M., Teixidó, N., Spadaro, D., Jijakli, M.H., 2016. The science, development, and commercialization of postharvest biocontrol products. Postharvest Biol. Technol. 122, 22–29. http://dx.doi.org/10.1016/j.postharvbio.2016.04.006.

El Ghaouth, A., Wilson, C., Wisniewski, M., 2004. Biologically-based alternatives to synthetic fungicides for the control of postharvest diseases of fruit and vegetables. In: Naqvi, S.A.M.H. (Ed.), Diseases of Fruits and Vegetables: Volume II: Diagnosis and Management. Springer, Netherlands, Dordrecht, pp. 511–535. http://dx.doi.org/10.1007/1-4020-2607-2_14.

Fiori, S., Scherm, B., Liu, J., Farrell, R., Mannazzu, I., Budroni, M., Maserti, B.E., Wisniewski, M.E., Migheli, Q., 2012. Identification of differentially expressed genes associated with changes in the morphology of Pichia fermentans on apple and peach fruit. FEMS Yeast Res. 12, 785–795. http://dx.doi.org/10.1111/j.1567-1364.2012.00829.x.

Janisiewicz, W.J., Usall, J., Bors, B., 1992. Nutritional enhancement of biocontrol of blue mold on apples. Phytopathology 82, 1364–1370.

Langvad, F., 1999. A rapid and efficient method for growth measurement of filamentous fungi. J. Microbiol. Methods 37, 97–100. http://dx.doi.org/10.1016/S0167-7012(99)00053-6.

Levy, Y., Benderly, M., Cohen, Y., Gisi, U., Bassand, D., 1986. The joint action of fungicides in mixtures: comparison of two methods for synergy calculation. EPPO Bull. 16, 651–657. http://dx.doi.org/10.1111/j.1365-2338.1986.tb00338.x.

Lima, G., Castoria, R., De Curtis, F., Raiola, A., Ritieni, A., De Cicco, V., 2011. Integrated control of blue mould using new fungicides and biocontrol yeasts lowers levels of fungicide residues and patulin contamination in apples. Postharvest Biol. Technol. 60, 164–172. http://dx.doi.org/10.1016/j.postharvbio.2010.12.010.

Liu, J., Sui, Y., Wisniewski, M., Droby, S., Liu, Y., 2013. Review: utilization of antagonistic yeasts to manage postharvest fungal diseases of fruit. Int. J. Food Microbiol. 167, 153–160. http://dx.doi.org/10.1016/j.ijfoodmicro.2013.09.004.

Liu, X.-Z., Wang, Q.-M., Göker, M., Groenewald, M., Kachalkin, A.V., Lumbsch, H.T., Millanes, A.M., Wedin, M., Yurkov, A.M., Boekhout, T., Bai, F.-Y., 2015. Towards an integrated phylogenetic classification of the *Tremellomycetes*. Stud. Mycol. 81, 85–147. http://dx.doi.org/10.1016/j.simyco.2015.12.001.

Lutz, M.C., Lopes, C.A., Rodriguez, M.E., Sosa, M.C., Sangorrin, M.P., 2013. Efficacy and putative mode of action of native and commercial antagonistic yeasts against postharvest pathogens of pear. Int. J. Food Microbiol. 164, 166–172. http://dx.doi.org/10.1016/j.ijfoodmicro.2013.04.005.

Lutz, M.C., Lopes, C.A., Sosa, M.C., Sangorrín, M.P., 2012. A new improved strategy for the selection of cold-adapted antagonist yeasts to control postharvest pear diseases. Biocontrol Sci. Technol. 22, 1465–1483. http://dx.doi.org/10.1080/09583157.2012.735223.

Nunes, C.A., 2012. Biological control of postharvest diseases of fruit. Eur. J. Plant Pathol. 133, 181–196. http://dx.doi.org/10.1007/s10658-011-9919-7.

Nunes, C., Usall, J., Teixidó, N., Miró, M., Viñas, I., 2001. Nutritional enhancement of biocontrol activity of *Candida sake* (CPA-1) against *Penicillium expansum* on apples and pears. Eur. J. Plant Pathol. 107, 543–551. http://dx.doi.org/10.1023/A:1011288710875.

Palou, L., Valencia-Chamorro, S., Pérez-Gago, M., 2015. Antifungal edible coatings for fresh citrus fruit: a review. Coatings 5, 962–986. http://dx.doi.org/10.3390/coatings5040962.

Romanazzi, G., Smilanick, J.L., Feliziani, E., Droby, S., 2016. Postharvest biology and technology integrated management of postharvest gray mold on fruit crops. Postharvest Biol. Technol. 113, 69–76. http://dx.doi.org/10.1016/j.postharvbio.2015.11.003.

Scherm, B., Ortu, G., Muzzu, A., Budroni, M., Arras, G., Migheli, Q., 2003. Biocontrol activity of antagonistic yeasts against *Penicillium expansum* on apple. J. Plant Pathol. 85, 205–213. http://dx.doi.org/10.1007/s11274-007-9557-6.

Spadaro, D., Droby, S., 2016. Development of biocontrol products for postharvest diseases of fruit: the importance of elucidating the mechanisms of action of yeast antagonists. Trends Food Sci. Technol. 47, 39–49. http://dx.doi.org/10.1016/j.tifs.2015.11.003.

Usall, J., Torres, R., Teixidó, N., 2016. Biological control of postharvest diseases on fruit: a suitable alternative? Curr. Opin. Food Sci. 11, 51–55. http://dx.doi.org/10.1016/j.cofs.2016.09.002.

USDA, 2016. National Nutrient Database for Standard Reference Release 28, https://ndb.nal.usda.gov/ndb/foods/show/2418.

Vero, S., Mondino, P., Burgueño, J., Soubes, M., Wisniewski, M., 2002. Characterization of biocontrol activity of two yeast strains from Uruguay against blue mold of apple. Postharvest Biol. Technol. 26, 91–98. http://dx.doi.org/10.1016/S0925-5214(01)00199-5.

Wisniewski, M., Droby, S., Norelli, J., Liu, J., Schena, L., 2016. Alternative management technologies for postharvest disease control: The journey from simplicity to complexity. Postharvest Biol. Technol. 122, 3–10. http://dx.doi.org/10.1016/j.postharvbio.2016.05.012.

Yim, S.-H., Nam, S.-H., 2016. Physiochemical, nutritional and functional characterization of 10 different pear cultivars (Pyrus spp.). J. Appl. Bot. Food Qual. 89, 73–81. http://dx.doi.org/10.5073/JABFQ.2016.089.009.

Yu, T., Chen, J., Chen, R., Huang, B., Liu, D., Zheng, X., 2007. Biocontrol of blue and gray mold diseases of pear fruit by integration of antagonistic yeast with salicylic acid. Int. J. Food Microbiol. 116, 339–345. http://dx.doi.org/10.1016/j.ijfoodmicro.2007.02.005.

Yu, T., Yu, C., Chen, F., Sheng, K., Zhou, T., Zunun, M., Abudu, O., Yang, S., Zheng, X., 2012. Integrated control of blue mold in pear fruit by combined application of chitosan, a biocontrol yeast and calcium chloride. Postharvest Biol. Technol. 69, 49–53. http://dx.doi.org/10.1016/j.postharvbio.2012.02.007.