Available online at www.sciencedirect.com
ScienceDirect
International Journal of Food Microbiology 113 (2007) 251–257
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Biological control of postharvest spoilage caused by *Penicillium expansum* and *Botrytis cinerea* in apple by using the bacterium *Rahnella aquatilis*

Juan Calvo, Viviana Calvente, María Edith de Orellano, Delia Benuzzi, Maria Isabel Sanz de Tosetti *

Área de Tecnología Química y Biotecnología, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Chacabuco y Pedernera, 5700-San Luis, Argentina

Received 23 November 2005; received in revised form 24 May 2006; accepted 26 July 2006

Abstract

The epiphytic bacterium *Rahnella aquatilis*, isolated from fruit and leaves of apples, was tested for antagonistic properties against *Penicillium expansum* and *Botrytis cinerea* on Red Delicious apple fruit. In "in vitro" assays, this bacterium inhibited completely the germination of *P. expansum* and *B. cinerea* spores, but it needed direct contact with the spores to do it. However the putative mechanism seemed be different for the two pathogens. The bacterium did not produce extracellular antibiotic substances and when the acute toxicity test was performed no mortality, toxicity symptoms or organ alterations of the test animals (Wistar rats) were observed.

Assays of biological control of *P. expansum* and *B. cinerea* on apple fruit were carried out at different temperatures. At 15 °C and 90% RH, the incidence of disease caused by *P. expansum* on apples stored for 20 days, was reduced by nearly 100% by *R. aquatilis* (10^6 cells/ml), while in the case of *B. cinerea*, the reduction of decay severity was nearly 64% but there was no reduction in the incidence of disease. At 4 °C and 90% RH the treatment with the bacterium significantly inhibited the development of *B. cinerea* on apples stored for 40 days and the incidence of disease was reduced by nearly 100%, while the incidence of disease caused by *P. expansum* at 4 °C was 60%. The results obtained show that *R. aquatilis* would be an interesting microorganism to be used as a biocontrol agent.

Keywords: *Rahnella aquatilis*; Biocontrol; Postharvest diseases; Blue mould; *Botrytis cinerea*; Gray mould; *Penicillium expansum*

1. Introduction

Some fruits, apples among them, are usually stored after harvest. During cold storage losses of economic importance are produced by several decays due to fungal rot. *Penicillium expansum* and *Botrytis cinerea* are well-known postharvest pathogens. They produce blue and gray rots, respectively (Goepfert, 1980). The use of synthetic chemicals as fungicides is a primary method of control of postharvest fungal decay of apple fruit. However, several fungicides are not used for postharvest treatment or have been removed from the market due to possible toxicological risks. Alternative methods of control are needed because of the negative public perceptions about the use of pesticides, development of resistance to fungicides among fungal pathogens, and high development costs of new chemicals. In recent years, biological control of postharvest diseases of fruits has become an important field for research. A number of yeasts and bacteria have been report to inhibit postharvest decay of fruit effectively (Janisiewicz and Korsten, 2002).

Among bacteria, a number of Gram positive and Gram negative bacteria have been evaluated as Biological Control Agents (BCAs). Several strains of the genus *Bacillus* have received much attention as BCAs. *Bacillus subtilis* isolated from citrus fruit surface was successfully evaluated for control of citrus green and blue moulds caused by *Penicillium digitatum* and *P. italicum* respectively (Obagwu and Korsten, 2003), and *Bacillus licheniformis* was reported as an effective BCA against tomato gray mould caused by *B. cinerea* (Jae Pil Lee et al., in press). Among Gram negative bacteria, *Pseudomonas cepacia* (Janisiewicz and Roitman, 1988), *Pseudomonas syringae* (Janisiewicz and Jeffers, 1997) *Pantoea agglomerans* (Nunes et al., 2001, 2002) and

* Corresponding author. Fax: +54 652 30224.
E-mail address: msanz@unsl.edu.ar (M.I. Sanz de Tosetti).

0168-1605/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2006.07.003

Serratia plymuthica (Meziane et al., 2006) were reported as effective BCAs against diseases of different fruits.

Searching for BCAs, in the permanent screening program of our laboratory, a coliform bacterium, identified as *Rahnella aquatilis*, was the bacterium mostly isolated from different sources. The natural habitat of *R. aquatilis* is water, but the organism has also been isolated from plant leaves (Hashidoko et al., 2002), soil (Tallgren et al., 1999), foods (Jensen et al., 2001) and non-environmental samples as blood, surgical wounds, urine, sputum, and bronchial washings. Most clinical cases have occurred in compromised hosts and young children and there have been no reports of *Rahnella*-associated mortality (Tasch, 2005).

This microorganism considered to be a little public health significance, has not yet been searched as a BCA against postharvest diseases, however it was reported as an antagonistic agent of *Xanthomonas campestri* which cause bacterial spot of cucumber and tomato (El-Hendawy et al., 2003, 2005). Then, we evaluated the bacterium *R. aquatilis* as an antagonist against the two major postharvest pathogens of apple, *P. expansum* and *B. cinerea*, because there are not any reports in this matter.

The present study was conducted to determine: (1) the potential of *R. aquatilis* for control of postharvest decays caused by *P. expansum* and *B. cinerea* on apple, (2) the influence of antagonist concentration on biocontrol efficacy and its behavior in wound apples, and (3) the putative mechanism of action.

2. Materials and methods

2.1. Bacterial antagonist and fungal pathogen strains

R. aquatilis (bSL1strain) was isolated in our laboratory from microbial consortiums obtained by picking the surface of apple fruit throughout the growing season. It was identified by using the API 20 E and API 50 CHE systems (bioMérieux, France). Additional test for motility, Voges–Proskauer (VP), Simmons citrate and methyl red were conducted according methods prescribed by Krieg and Gerhardt (1994), and Smibert and Krieg (1994). The acute oral toxicity of the bacterium was determined by the "Centro de Servicio Farmacológico" of the Universidad Nacional de San Luis. Assays were carried out by using Wistar rats. Six male and six female (between 200 g and 250 g) were starved during 4 h before the administration of a *R. aquatilis* suspension (4×10^{11} CFU kg^{-1} live weight) or the same volume of distilled water (control). Rats were observed during 14 days to evaluate mortality or any other symptom. After this time, the rats were killed and macroscopic observation of their organs was carried out.

P. expansum came from CEREMIC (Mycological Reference Center), Rosario, Argentina and *B. cinerea* was a kind gift to us from INTA (National Institute of Agricultural Technology). Both moulds were maintained on potato dextrose agar medium (PDA medium).

2.2. Preparation of bacteria and pathogen spore suspensions

R. aquatilis strain was grown on YGA (yeast extract 5 g/l, glucose 10 g/l, agar–agar 20 g/l) in Roux's bottles, suspended in sterile distilled water and concentrations adjusted according to standard curve with a Metrolab1500 UV–VIS Spectrophotometer by measuring the optical density at 635 nm.

For conidial production, *B. cinerea* were grown on PDA (Potato Dextrose Agar) at 20–25 °C. When the mycelium appeared, cultures were kept at 15 °C for inducing sporulation. After a week, spores were harvested and suspended in 10 ml of sterile distilled water containing 0.05% (v/v) Tween 80. The concentration of spore suspension was determined with a Neubauer chamber and adjusted with sterile distilled water to 1×10^6 spores/ml. Conidia of *P. expansum* were obtained from 7 day old PDA cultures grown at 20–25 °C. The concentration was also adjusted to 1×10^6 spores/ml.

2.3. Inhibition of germination of P. expansum and B. cinerea spores

In order to evaluate antagonistic activity of *R. aquatilis* against *P. expansum* and *B. cinerea*, in vitro assays on special slides for micro culture were performed. In these assays was also determined the minimum *Rahnella* concentration necessary for inhibiting the conidial germination of the phytopathogens. The composition of GP medium used in these assays was as follows: Glucose 5 g/l, $(NH_4)_2SO_4$ 0.5 g/l, Peptone 0.05 g/l, PO_4H_2K 0.2 g/l, $Cl_2Mg{\cdot}6H_2O$ 0.2 g/l, $FeSO_4$ 0.005 g/l. pH 4.5.

Aliquots of 20 μl of *R. aquatilis* suspensions in GP medium (10^2, 10^3, 10^4, 10^5, and 10^6 cells/ml) were put on the slide, immediately 20 μl of conidial suspension (10^6 spores/ml) were added. Controls were made with 20 μl of GP medium without antagonist and 20 μl of conidial suspension (10^6 spores/ml). Slides were incubated in a wet chamber at 28 °C during 48 h. After this time, these preparations were observed with a light microscope (Olympia) at a magnification of ×500. Conidial germination was assessed counting at least 100 conidia per slide. Preparations were also observed by SEM using a scanning electron microscope LEO 1450 VP in the Laboratory of Electronic Microscopy and Micro analysis. (LABMEM-UNSL).

2.4. Source of fruit material

Red Delicious apples used in the biological control assays were obtained from a commercial orchard. Fruits selected were free of wounds and rots and as much as possible homogeneous in maturity and size. The fruit were used after a short time of storage at 1 °C (no longer than 3 months).

2.5. Assays of control of P. expansum and B. cinerea on apple fruit

In order to evaluate the antagonistic activity against *P. expansum* and *B. cinerea* on apple fruit, surface apples were disinfected by immersion for 1 min in a dilute solution of sodium hypochlorite (1% active chlorine), washed two times by immersion in distilled water, and left in a dry place to remove excess water off the surface. Then, fruit were wounded ($3\times3\times3$ mm^3) in two places (midway between the calyx and the stem end) with a punch and 20 μl of *R. aquatilis* suspension (10^6 cells/ml) in sterile distilled water was put in each wound.