313
OK

SELECTION OF ANTAGONISTS OF POSTHARVEST APPLE PARASITES: *PENICILLIUM EXPANSUM* AND *BOTRYTIS CINEREA*

E.H. ACHBANI[1], R. MOUNIR[1], S. JAAFARI[2], A. DOUIRA[3], BENBOUAZZA[1] & M.H. JIJAKLI[4]

[1] Laboratoire de Phytobactériologie, INRA Méknès, BP 579 Méknès VN, Maroc
[2] Laboratoire de Biotechnologie et Amélioration des Plantes (UMI-BAP), Université Moulay Ismail, BP 4010, Meknès, Maroc
[3] Laboratoire de Biologie et de Protection des cultures, Universiré Ibn Tofail Faculté des Scioences, BP 133, 14000 Kénitra, Maroc
[4] Unité de Phytopathologie, Faculté universitaire des Sciences Agronomiques Passage des Déportés 2, BE-5030 Gembloux, Belgium

ABSTRACT

The objectives of this study were to constitute a collection of pathogenic agents of economic importance which cause losses of apple fruits after harvest namely *Botrytis cinerea* and *Penicillium expansum* and to select *in vivo* efficient antagonistic strains able to protect fruits against both pathogens at 5°C (*P. expansum*) and 25°C (*P. expansum* & *B. cinerea*). Twenty strains of *P. expansum* and ten strains of *B. cinerea* have been isolated from infected apple fruits. Potential antagonistic micro-organisms (thirty three isolates) belonging to yeast, bacteria and fungi have been isolated from apple surface. Six of them (strains Ach1-1, Ach2-1, Ach2-2 belonging to *Aureobasidium pullulans* (De Bary) Arnaud, and strains 1112-3, 1113-10 and 1113-5 belonging to *Aureobasidium pullulans* (de Bary) Arn. v. *pullulans*) showed a high level of protection (more than 80%) at 25°C, once inoculated with *P. expansum* or *B. cinerea* for 5 days. The highest level of protection against *P. expansum* (96%) was observed with the application of Ach 2-1. Six days after inoculation of *B. cinerea*, strains Ach 2-2 and Ach 2-1 insured 100% and 96 % of protection, respectively. At lower temperature (5°C), first symptoms of *P. expansum* appeared 13 days after its inoculation. Percentages of protection observed after apple treatment with one of the six antagonistic strains were ranged from 78% to 94% 20 days after *P. expansum* inoculation. Strains labelled Ach showed a protective level higher than 90% against this pathogen, followed by strain 1113-10 (90%), strain 1113-5 (89%) and strain 1112-3 (82%). At 26 days post-inoculation, levels of protection decreased but remained higher than 60% (more than 80% with strain Ach2-2 and strain 1113-5, 75% with strain Ach2-1 and 1113-10, 72% with ach1-1, 61% for the other strains). Strain Ach2-2 and 1113-10 were retained as the best antagonists for the subsequent studies.

Key-words: antagonists, postharvest, apple, *Penicillium expansum*, *Botrytis cinerea*.

INTRODUCTION

Morocco contributes for 30% of apples production in Africa, which places it in second position after South Africa. Thirty two of this operated area (approximately 9000 ha) are located in Meknès area and its surroundings, with refrigerating capacity of 45 000 tons. Postharvest pathogens cause substantial losses in this Moroccan apple production. The losses can reach 60% according to farmers. *Botrytis cinerea* and *Penicillium expansum* are two important wound fungal pathogens partially responsible of these losses during storage. The incidence of each pathogen remains to be determined, but it

seems that *B. cinerea* and *P. expansum*, two wound pathogens, are responsible of many infections observed on fruits (Bondoux, 1992).
Control of postharvest pathogens still relies mainly on the use of synthetic fungicides like benzidimazoles family, but the development of fungicide-resistant and a very strict legislation to reduce pesticides use have increased the search for alternative control strategies (Janisiewicz *et al.*, 1994). Among the alternative control methods, a considerable attention was attributed to biological control methods (Roberts, 1990; Wilson and Wisniewski, 1992; Bull *et al.*, 1997; Chand-Goyal and Spotts, 1997; El Ghaouth *et al.*, 1998).
The results obtained during the ten last years showed that several antagonists were used against postharvest diseases of fruit and vegetables such as *Bacillus subtilis*, *Pseudomonas syringae*, *Pichia anomala*, *P. guillermondii*, *Candida oleophila*, *Cryptococcus laurentii*. Two biofungicides already have been commercialized as postharvest biofungicides on apples and pears: Aspires (Ecogen, Inc., Langhorne, Pa) and Bio-Save 110 (Ecoscience, Worcester, MA).
The current study was conducted to constitute a collection of *B. cinerea* and *P. expansum* and select *in vivo* efficient antagonistic strains able to protect fruits against *P. expansum* at 5°C and *P. expansum* and *B. cinerea* 25°C.

MATERIAL AND METHODS

Constitution of a collection of postharvest pathogens on apple: *P. expansum and B cinerea*

Isolates of *P. expansum and B. cinerea* were obtained from infected apples tissue with various symptoms of decay collected during 2003 and 2004 either in the markets or in the private storage stations [Meknes (N33°53 734, W5° 32 227), Azrou (N33° 26 683, W5° 13 845) and Midelt (N32° 41 570, W4 43 721)]

Symptoms reproduction

For long-term storage, fungi isolates were suspended in 25% glycerol at -86°C. For short-term storage isolates were kept at 4°C on Potato Dextriose Agar (PDA) plates.
The spore suspension of *P. exapansum* and *B. cinerea* were prepared from 10 to 15 day-old cultures cultivated on PDA plates medium incubated at 25°C by scarping the surface the surface of the colonies recovered with Tween 20 (0.05%). Pathogens cell were counted with Bürker's cell and adjusted to a concentration of 10^6 spores/ml.
"Golden Delicious" apples were surface-sterilized with Na-hypochlorite (2 min in a 10% solution), rinsed three times with sterile distilled water *et allowed* to dry before wounding. Wounds were created by removing plugs of 5mm in diameter and 3 mm in depth from the surface (Two wounds per apple). These wounds were inoculated with 70 µl of 10^6 CFU/ml (in isotonic water 8.5% NaCl) for each fungal strain. The apples were then placed in boxes on filter paper soaked with 3 ml of sterile distilled water to maintain high humidity. Diameters of lesions were determined after 4 or 5 days at 25°C.

Isolation and cultures of potential antagonistic micro-organisms

From the various batches of apples (Golden Delicious) bought in market, 4 apples of each batch are introduced into a plastic bag (3000 ml) with 1000 ml of washing water KPBT (Potassium phosphate 0.05M with 0.005% of Tween 80). After agitation on a regulated electric shaker (120 rpm), 5 ml of washing water were serially diluted (5x, 10x, 50x) and x100 in KPBT. Hundred µl of each dilution were spread on PDA plate. Three repetitions for each dilution were carried out. Plates were then incubated for 2 or 3 days at 25°C. The various microorganisms colonies observed on the medium were described (size, color, opacity, etc.) and cultured at 25°C for 3 successive generations on PDA plates with an interval of 48 hours. Two optical densities (OD 0.5 and OD 0.75) at 595 nm were used per isolate at first and then, concentrations of microorganisms (yeasts or bacteria) were determined with Bürker's cell and adjusted to 10^7 cfu/ml in isotonic water 8.5% NaCl.

In vivo selection of the antagonists of apple postharvest disease

A technique of selection of antagonists of apples postharvest diseases (*P. expansum and B. cinerea*) was applied using a protocol of Jijakli (personal communication) with slight modifications: i) "Golden Delicious" apples were surface-sterilized with Na-hypochlorite (2 min in a 10% solution), rinsed twice successively with sterile distilled water *et a*llowed to dry before wounding. Fruits were wounded in the equatorial zone by removing plugs 6 or 8 mm in diameter and 3 mm in depth from the surface and placed on plastic boxes containing a humid filter paper to maintain high humidity. Fifty or seventy µl of 10^7 CFU/ml of micro-organisms cell suspension were pipetted into each wound (5 fruits per isolate) and 24h later, these wounds were inoculated with 50 or 70 µl of 10^6 spores/ml of *P. expansum* and *Botrytis cinerea* (0.05% Tween 20). Fruits were replaced at 25°C. Diameters of lesions (blue and gray mold) compared to control were determined between 5 and 11 days at 25°C or 20 and 26 days at 5°C. Biocontrol activity of antagonist agents was evaluated according to the following formula: [(Dm of control-0.6 (or 0.8)) – (Dm of X -0.6 (or 0.8)) X 100]/ (Dm of control-0.6 (or 0.8)) where Dm = Decay average diameter (of 10 wounds); 0.6 (or 0.8) = Diameter of wounds on cm ; Control = Wound inoculated only by the pathogen agent; X = "antagonist".

Characterization of efficient isolates

Identification of microorganisms was made by Braunschweing company in Germany DSMZ 0(Deutsche Sammlung von Mikroorganismen und Zellkulturen GMBH (for Ach1.*) and by BCCM/MUCL Louvain-la-Neuve in Belgium for the rest of microorganisms.

RESULTS

Constitution of a collection of postharvest pathogens, *P. expansum* and *B cinerea* on apple

The typical symptoms of *P. expansum* (blue mold) and *B. cinerea* (gray mold) were induced by the various strains isolated from decayed apples fruits during 2003 and 2004 when inoculated on wounded apples (Golden delicious). Twenty isolates of *P. expansum* and 10 ioslates of *B. cinerea* were obtained. According to this study, *P. expansum* strain 880 and strain 881 were the most aggressive of all isolates (data no shown).

In vivo selection of the antagonists of apple postharvest

Two screening assays of antagonists were conducted on apples at first in Belgium (at "Unité de Phytopathologie de Gembloux") against *P. expansum* and *B. cinerea*. Among 9 epiphytic micro-organisms (yeast's and bacteria), three strains of yeast (strain Ach1-1, Ach2-1 and Ach2-2) were selected. These strains were effective in inhibiting *P. expansum* (>90% of protection level at 25°C after 5 days of incubation) (Table 1). The highest level of protection (96%) was observed with Ach2-1 strain.
After 7 days of incubation, the level of protection decreased but there remained higher than 72%, the maximum rate being expressed by Ach1-1 isolate with 85% of protection. Protection level after 11 days fluctuated between 71 (Ach2-1) and 85% (Ach1-1) (Table 1). Against *B. cinerea* (Table 2), protection level offered by strain Ach2-2 was 100% until 11 days of incubation while Ach2-1 strain offered 95% and 91% respectively 7 and 11 days after pathogen inoculation.

Table 1. Biocontrol activity of some strains against *P. expansum* on wounded Golden Delicious apples at 25°C (NC: no counted).

Strain	DO 0.5			DO 0.75		
	After 5 days of incubation	After 7 days of incubation	After 11 days of incubation	After 5 days of incubation	After 7 days of incubation	After 11 days of incubation
	% Protection/control					
C.oleophila	42.45	42.93	40.11	67.63	45.55	39.55
Rinsing water	49.64	40.31	33.15	49.64	40.31	33.15
Ach 1.1	84.89	71.20	62.12	91.37	85.34	85.52
Ach 1.2	46.04	35.08	NC	28.06	29.84	NC
Ach 1.3	74.82	60.21	65.46	69.07	53.93	43.18
Ach 1.4	40.29	33.51	NC	33.09	25.65	NC
Ach 1.5	33.81	25.65	NC	28.78	26.70	NC
Ach 1.6	42.45	36.65	NC	33.09	35.08	NC
Ach 1.7	33.09	29.32	NC	29.50	20.42	NC
Ach 2.1	88.49	80.11	72.42	95.68	75.39	71.03
Ach 2.2	87.05	80.11	74.65	92.09	72.25	75.49

A third screening of antagonists from rinsing water of G.D. apple was undertaken in Morocco (INRA Phytobacteriology laboratory in Meknes). Among 25 isolates belonging to bacteria, yeasts and fungi, 3 strains were effective in

inhibiting *P. expansum*, exhibiting a level of protection exceeding 84% at 25°C after 5 days of incubation (Table 3).

Table 2. Biocontrol activity of some strains (at a concentration of DO 0.75 at 495 nm) against *B. cinerea* on wounded Golden Delicious apples at 25°C

Strain	After 5 days of incubation	After 6 days of incubation	After 8 days of incubation
	% Protection/Control		
C.o 10^5	87.94	82.81	83.39
C.o 10^7	100	100	100
Ach 1.3 DO 0.75	87.94	79.37	70
Ach 2.1 DO 0.75	100	95.70	91.86
Ach 2.2 DO 0.75	100	100	97.29

Table 3. Biocontrol activity other strains (at a concentration of 10^7 cfu/ml) against *P. expansum* on wounded Golden Delicious apples 5 days after incubation at 25°C

Strain	% protection/Control
Control *Pe*	0
Ach2-2	68.34
1113-4	77.89
1113-9	68.84
1113-10	85.43
1112-3	84.42
1112-2	67.84
1113-5	85.93
1113-6	67.34
1112-1	68.34

At 5°C (Table 4) for 20 days, Ach's strains expressed a high antagonism against *P. expansum* exceeding 91% of protection, followed by strains 1113-10 (90%), 1113-5 (89%) and 1112-3 (82%). After 26 days of incubation, the efficacy decreased, but remained higher than 60%. Strain Ach2-2 and 1113-10 were retained as the best antagonists for the subsequent studies.

Table 4. Biocontrol activity of some isolates (at a concentration of 10^7 cfu/ml) against *P. expansum* on wounded Golden Delicious apples at 5°C

Strain	after 20 days	after 26 days
	% protection/Control	
Control *P.e*	0	0
Ach1-1	90.91	71.75
ACH2-1	91.56	75.34
ACH2-2	93.51	88.79
1113-10	90.26	75.34
1113-5	88.96	86.55
1112-3	82.47	60.54

Identification of isolates

Isolates 1113-5, 1113-9 and 1113-10 were identified as *Aureobasidium pullulans* (of Bary) Arn.v. *pullulans* whereas strain Ach1-1, Ach2-1 and Ach2-2 were identified as *Aureobasidium pullulans* (of Bary) Arn.

DISCUSSION

Microorganisms exhibiting antagonistic properties against *P. expansum* and *B. cinerea* have been isolated from soil, leaves of apple tree or from fruits or leaves of other plant. In this respect, the current study was conducted to select *in vivo* efficient antagonistic strains able to protect fruits against both pathogens at 5°C (*P. expansum*) and 25°C (*P. expansum* and *B. cinerea*) by using a method of antagonist isolation adopted by Jijakli and Lepoivre (1993). Surface of the apple fruit seems to be a favorable site to select efficient antagonists against postharvest diseases of apples. Janisiewicz and Korsten (2002) reported that the selection of the antagonists must be carried out on healthy fruits in the orchard or in storage, preferably from the fruits of biological orchards where the natural populations are not disturbed by the use of chemicals.

In our study, few micro-organisms isolated from surface fruits expressed a high antagonism against both parasites, and confirmed as well as that the apple surface is a favorable site to select efficient antagonists against apple postharvest diseases.

Study carried out on certain antagonists showed that the concentrations giving a high protection against postharvest pathogens varied between 10^9 cfu/ml for bacteria and 10^7 cfu/ml for yeasts (Janisiewicz, 1998). The application of 10^7 cfu/ml of *Pichia anomala* (strain K) or *C. oelophila* (strain O) was necessary to completely inhibit lesions caused by *B. cinerea* upon inoculation with 50µl of 10^6 spores/ml (Jijakli *et al.*, 1999). Our results are similar with those reported by these authors. The concentrations used for antagonistic and pathogen strains were respectively 10^7 (10^8) and 10^6 (10^7) cfu/ml.

The antagonist strains belong to *Aureobasidium pullulans* (De Bary) Arnaud (Ach's strains), or to *Aureobasidium pullulans* (de Bary) Arn. v. *pullulans*) (1112-3, 1113-10 and 1113-5) and were already reported by others studies as a potential antagonist species against postharvest diseases of some fruits and vegetables (Lima *et al.*, 1997; Leibinger *et al.*, 1997). These species are not pathogenic for human, nor recognized as producer of mycotoxins (www.mold-survivor).

In this study, two strains of *Aurebasidium pullulans* (strain Ach1-1 and 1113-5) were selected for their high biocontrol activity against *P. expansum* and *B. cinerea* on wounded Golden Delicious apples. In order to increase their efficacy, it is important to evaluate their ecology. In prospect, three objectives will be carried out successively: a) the in vitro influence of the physical factors (temperature, water activity, pH and UV) on the growth of antagonists, b) their in vivo influence, and finally, c) the identification of additives allowing to improve survival and antagonist control in spite of the unfavorable parameters.

ACKNOWLEDGEMENTS

The authors thank CUD Belgium for funding this study that constitute a part of the "PIC" project "Biological control of apple postharvest diseases".

LITERATURE

BONDOUX P., 1992. Maladies de conservation des fruits à pépins, pommes et poires. Institut National de la Recherche Agronomique Paris :173p.

BULL. C. T., STRACK J.P. & SMILANICK J.L., 1997. *Pseudomonas syringae* strains ESC-10 and ESC-11 survive in wounds on citrus and control green and blue moulds of citrus. Biol. Control. **8**:81-88.

CHAND-GOYAL T. & SPOTTS R.A., 1997. Biological control of postharvest diseases of apple and pear under semi-commercial and commercial conditions using three saprophytic yeasts. Biol. Control **10**:199-206.

EL GHAOUTH A., WILSON C.L. & WISNIEWSKI M., 1998. Ultrastructural and cytochemical aspect of the biocotrol activity of *Candida saitoana* in apple fruit. Phytopathology **88**:282-291.

JANISIEWICZ W.J. 1998. Biocontrol of postharvest disease of temperature fruits. Challenges and opportunities. Marcel Dekker, Inc. New York, United States of America. 171-197.

JANISIEWICZ W.J. & KORSTEN L., 2002. Biological control of postharvest diseases of fruits. Annu. Rev. Phytopathol. **40**:411-441

JANISIEWICZ W.J., PETERSON D.L. & BORS R., 1994. Control of storage decay of apples with *Sporobolomyces roseus*. Plant Dis **78**:466-470.

JIJAKLI M.H. & LEPOIVRE P., 1993. Biological control of postharvest *Botrytis cinerea* and *Penicillium* on apples, IOBC/WPRS Bulletin: Biological Control of Foliar and Postharvest Disease **16**:106-110.

JIJAKLI M.H., LEPOIVRE P. GREVESSE C., 1999. Yeast species for biocontrol of apple postharvest diseases: An encouraging case of study for practical use. Biotechnological Approche in Biocontrol of plant pathogens. Edit. by Mukerji *et al.*, Kluwer Academic/Plenum Publishes, New York. 31-49.

LEIBINGER W., BREUKER B., HAHN M. & MENDGEN K., 1997. Control of postharvest pathogens and colonization of the apple surface by antagonistic microorganisms in the field. Phytopathology **87**:1103-1110.

LIMA G., IPPOLITO A., NIGRO F. & SALERNO M., 1997. Effectiveness of *Aureobasidium pullulans* and *Candida oleophila* against postharvest strawberry rots. Posharvest Biol. Technol. **10**:169-178.

ROBERTS R.G., 1990. Postharvest biological control of gray mold of apple by *Cryptococcus laurentii*. Phytopathology **80**:526-530.

WILSON C.L. & WISNIEWSKI M.E., 1989. Biological control of postharvest diseases of fruits and vegetables: an emerging technology, Rev. Phytopathol. **27**:425-441.

WILSON C.L. & WISNIEWSKI M.E., 1992. Future alternative to synthetic fungicides for the control of postharvest diseases. *in* Tjamos E.S. *et al.* (Eds.), Biological Control of Plant Disease. Plenum Press, New York :133-148.