In vitro inhibition of *Erwinia amylovora* Romanian isolates by new antagonistic bacterial strains

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Abstract

Biological control of fire blights has proven to be promising and challenging. In order to select antagonistic agents to Erwinia amylovora, natural virulent isolates of pathogenic bacteria are necessary. In a screening experiment, several bacterial species were isolated from pears and quince leaves and flowers and subjected to identification using semiselective media, API20E system and RAPD analysis. Among them, two strains exhibit the characteristics of Erwinia amylovora, other belong to Pantoea, Serratia or Enterobacter genus. In order to select potential biological control agents efficient against fire blight, 25 bacterial strains belonging to Bacillus and Pseudomonas genus were tested. Several strains seem to be inhibitory for the new isolates of Erwinia amylovora after in vitro tests and they were examined for preliminary characterization, in order to establish the species they belong. API system, Biolog identification as well as molecular analysis (ITS-PCR and RAPD) allow the identification of selected inhibitory strains as P.aeruginosa (P14 and P10), P.fluorescens (P20), P.putida (P5) and Bacillus amyloliquefaciens.

Keywords: *Erwinia amylovora*, antagonistic bacteria, *Bacillus amyloliquefaciens,* RAPD analysis, ITS-PCR

Introduction

Erwinia amylovora is a Gram negative rod-shaped bacterium belonging to the family *Enterobacteriaceae* and causes fire blight, a devastating disease of pears, apples and several rosaceous ornamentals and infects most members of the subfamily *Pomoideae* [6] Rapid identification of the pathogen before extensive symptoms are installed is necessary. Several assays have been developed for detection of this pathogen. Classical methods include plating on semi selective media [3], serological assays [**7**] and molecular methods using PCR technology.

Fire blight is a complex disease where the infective agent is continually associated with plant tissue, after the infection occurred. It is well known that the primary route of infection is through the blossom and the infection could be spread by insects or rainfall [6]. The studies performed during the past decade proposed a number of epiphytic bacteria (belonging to several species like *Pseudomonas fluorescens, Erwinia herbicola* or *Bacillus subtilis*) as biocontrol agents. Several bacteria are proposed to be used as biocontrol agents, the best characterized being *Pseudomonas fluorescens* strain A-50 (BlightBan), *Bacillus subtilis QWT713* (Serenade) and *Pantoea agglomerans* (Blossom Bless) [11]. These biological agents are proposed to block colonization sites on the stigma, stimulate host defense, and in some instances produce antibiotic compounds that inhibit *E.amylovora* growth.

The application of such products remains a challenge for most products. An interesting approach for spread the products is the use of honeybees but in this case it is necessary to identify the species the antagonist bacterial isolates belong [5].

The aim of this work was to isolate and identify bacterial isolates from affected fruit trees, as well as to select the appropriate inhibitory bacteria, potential useful as *Erwinia amylovora* biocontrol agents.

Material and methods

1. Bacterial pathogens isolation. The plant samples for isolation of *Erwinia amylovora* strains were obtained from pears and quince leaves and flowers with small necrosis spots.

The samples were processed in sterile antioxidant maceration buffer as indicated by Gorris et al. [7]. The plant material (0,1 g aprox.) was cut in small pieces into plastic bags and 4,5 ml of the antioxidant maceration buffer were added and the mixture was incubated on ice for few minutes. 3 x 1ml aliquots of each macerate were transferred to sterile Eppendorf tubes by decantation. 50µl of diluted (1:10 and 1:100) and undiluted macerate were spread at the surface of separate plates containing different media: King`s B, Nutrient Sucrose Agar (NSA) and Crosse-Goodman agar (CG). The colonies appeared on these media were plated on MM1-Cu, MM2-Cu (Bereswill et al., 1998) or Luria Bertani agar and the aspect was examined. The strains isolated were subjected to API 20E tests (bioMerieux) which

allowed the identification as *Erwinia* species. The results of a GN2 Microlog test (Biolog, Inc., Hayward,Calif) identified them with highest probability as belonging to *Pantoea spp* or *Erwinia amylovora*. The confirmation of *E.amylovora* was performed by testing the virulence on immature pear fruits as well as by genetic analysis [1].

2. Other bacterial strains. In order to select inhibitory bacterial strains, various bacteria were isolated from ICDPP or USAMV Bucharest orchards. Among the new isolated, in our experiments were used 22, both pseudomonads and bacilli (table 1).

Table 1. Bacteria used in experiments

Nr.	Species	Strain	Source
1	*Pseudomonas putida*	P5	ICDPP
2	*Pseudomonas spp.*	P10	Biotehnol
3	*Pseudomonas spp.*	P11	
4	*Pseudomonas spp.*	P14	
5	*P.aeruginosa* ATCC 27853	P1	
6	*Pseudomonas spp.*	P20	
7	*Pseudomonas fluorescens ATCC13525*	P22	
8	*Pseudomonas putida ATCC49128*	P23	
9	*Bacillus spp.*	Bw	
10	*Bacilus spp.*	Bcpc	
11	*B.licheniformis*	B40	
12	*B.subtilis*	B2R	
13	*B.subtilis*	B005	ICDPP
14	*B.subtilis*	45B	
15	*B.subtilis*	Lob1b	
16	*B.subtilis*	Sal1	
17	*Bacillus spp.*	R3P5M	
18	*Bacillus spp.*	R6P4G	
19	*Bacillus spp.*	SV66a	
20	*Bacillus spp.*	SV84	
21	*Bacillus spp.*	SV100	
22	*Bacillus spp.*	SV165	
23	*Bacillus spp.*	SV170	
24	*Bacillus spp.*	SV171	
25	*Bacillus spp.*	SV166	

3. Antagonism detection. Two methods were used for anti-erwinia bacteria: streak assay and the observation of growth inhibition zones caused by test bacteria on a lawn of pathogen on King B medium.

4. DNA isolation and manipulation. Genomic bacterial DNA was isolated by Wizard R Genomic DNA Purification Kit. Restriction endonucleases were purchased from Promega Corp. (Madison, Wis.) and digestion of DNA was carried out as recommended by the manufacturer.

5. PCR amplification. Primers used are listed in table 2.

Table 2. Primers used in experiments

Primer	Nucleotide sequence	Target and aim of use	References
Primer A	CGG TTT TTA ACG CTG GG	Identification and characterization of *Erwinia amylovora*	2
Primer B	GGG CAA ATA CTC GGA TT		
ITS1F	AAG TCG GTA ACA AGG TAG	Characterization of *Bacillus* and *Pseudomonas* species	9
ITS2R	GAC CAT ATA TAA CCC CAA G		

For PCR amplification, reactions were carried out in 25 µl containing 40 ng DNA and 1 µM/4 µM primers, in 40 cycles (94^{o}C - 1 min., 58/60^{o}C - 1 min., 72^{o}C-2 min.) [4]. The primers were synthesized and obtained from Biosearch Technologies Inc., USA. The amplicons obtained with ITS1/ITS2 primers were subjected to several restriction enzymes and the electrophoretic pattern was examined [9].

For RAPD analysis of *B.amyloliquefaciens* reactions were carried out in 25 µl containing 40ng DNA, 1x-buffer; $MgCl_2$-2,5mM, Taq polymerase 1U, 1,3µM primer and 0,2mM dNTPs, in 36 cycles (94^{o}C - 1 min., 36^{o}C - 1 min., 72^{o}C-2 min and, finally, 72 ^{o}C - 10 min) [12].

PCR products were separated by gel electrophoresis using a 1,5% agarose gel in 0,5x Tris-borate-EDTA buffer at 5,0 V/cm and visualized in UV light.

Results and discussion

1. Isolation and characterization of *Erwinia amylovora*

The isolation of *E.amylovora* from symptomatic samples is relatively easy because the number of culturable bacteria in them is usually high. When symptoms are reduced the number of *E.amylovora* culturable cells can be very low and enrichment is necessary.

In our experiments, the plants presented reduced symptoms (leaves and flowers with small necrosis spots). Plating the dilutions on six types of culture media allowed the observation of different bacterial colonies. Based on literature the morphology of *E.amylovora* colonies depends on culture medium. For example, colonies of *E.amylovora* on CCT appear at about 48 h and are pale-violet, circular, smooth and mucoid after 72 h (this medium inhibits most pseudomonads but not *Pantoea agglomerans*). Colonies of *E.amylovora* on King's B appear at 24 h and are creamy white, circular, intending to spread and non-fluorescent under UV light after 48 h, property that allows the distinction from fluorescent pseudomonads. On Levan medium, colonies of *E. amylovora* are whitish, circular, and highly mucoid after 48h of cultivation [3].

According Bereswill et al. [3], a good discrimination among *Erwinia* species and other Gram negative bacteria is provided by the colony morphology on LB agar and on two minimal media: MM1-Cu and MM2-Cu: *E.amylovora* presented white colonies on LB agar, yellow, mostly mucoid non-fluorescent colonies on MM2-Cu medium (fig.1) instead of a strong growth inhibition on MM1-Cu agar.

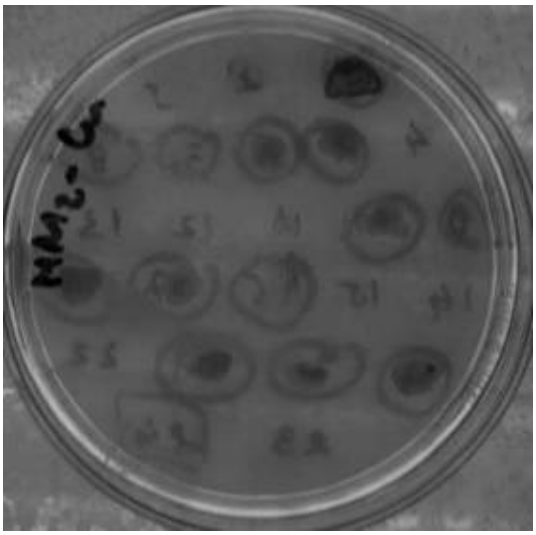

Figure 1. Aspect of the colonies selected from plant material grown on MM2-Cu medium: yellow, mostly mucoid non-fluorescent colonies which could be *E.amylovora*

The results obtained with the bacteria isolated from plant material are presented in table 3.

Table 3. Growth pattern of plant-associated bacteria on different culture media

Strain	LB	MM2-Cu	MM1-Cu	CCT
1	Yellow	Yellow, flat	+	+, pale-violet
2	White	White, mucoid	-	-
3	White	White, mucoid	-	-

4	White	White, mucoid	-	-
5	Yellow	Yellow, flat	+	+, pale-violet
6	Yellowish	Yellow, flat	-	+, pale-violet
7	Yellowish	Yellowish, mucoid	-	+, pale-violet
8	White	Yellowish, mucoid	-	+, pale-violet
9	White	Yellowish, mucoid	-	+, pale-violet
10	Yelowish	Yellow, flat	+	+, pale-violet
11	White	White, mucoid	-	-
12	White	White, mucoid	-	-
13	white	Absence of growth	-	-
14	White	White, mucoid	-	-
15	White	White, mucoid	-	-
16	White	Yellowish, mucoid	-	+, pale-violet
17	White	White, mucoid	+	+, pale-violet
18	White	White, mucoid	+	+, pale-violet
19	Yellow	Yellow, flat	+	+, pale-violet
20	Yellow	Yellow, flat	+	+, pale-violet
21	Yellow	Yellow, flat	+	+, pale-violet
22	White	White, mucoid	-	-
23	White	White, mucoid	-	-
24	White	Yellowish, mucoid	-	+, pale-violet
25	Yellow	Yellowish, flat	+	+, pale-violet
26	Yellow	Yellowish, flat	+	+, pale-violet
27	Yellow	Absence of growth	-	+, pale-violet
28	White	White, mucoid	-	-
29	White	White, mucoid	-	-
30	white	Yellow, flat	+	+, pale-violet
31	White	Yellowish, mucoid	-	+, pale-violet
32	White	White, mucoid	-	-
33	White	White, mucoid	-	-
34	White	White, mucoid	-	-

For the identification of these strains were used two commercial tests: API 20E and BIOLOG Gram-negative kit. Despite the information from literature related to the efficient identification through the mentioned systems, in our experiments the results were surprising: with BIOLOG tests no results were obtained; with API 20E the codes revealed for the selected strains were characteristic for *Pantoea* genus. These strains were tested for pathogenicity against immature pear fruits belonging to Cascade variety (sensitive to fire blight) at different incubation period (5-7 days), in wet chamber, at 20-24^{0}C. The strains proved to produce the largest damages to fruits (brown spots and mucoid exudates) were considered as *E.amylovora*.

The confirmation of *E.amylovora* was performed by the use of two kind of PCR methods: standard PCR with a single pair of primers (primer A and primer B) that produce an amplicon of 900 bp length [2, 3] and nested PCR, when two pairs of primers were applied simultaneous (AJ75/AJ76 and PEANT1/PEANT2) and allow the amplification of a product of 391 bp [10]. In our experiments, the pair of primers A/B, corresponding to a specific region from plasmid pEA29 from *Erwinia amylovora*, allowed the amplification of a 900 bp product, that was absent in other *Erwinia* species. Our results were similar to those presented by Kim et al. [8] in a study related to pathogenic bacteria in Japan. The expected amplicons were detected only in the case of two strains, designated as Ew1 (formerly colony no.7 from table 3) and SVEa, these strains exhibiting the most severe symptoms on plant material (fig.2).

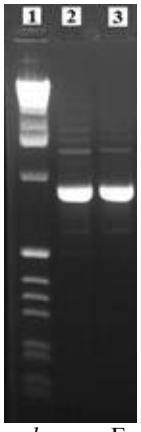

Figure 2. PCR of DNA isolated from two strains of *Erwinia amylovora*: Ew1 and SVEa. 1 – marker DNA (1kb); 2 – Ew1; 3 – SVEa. The arrow indicates the position of 900 bp amplicon obtained with primer A/primer B pair.

The results obtained with the other set of primers (AJ75/AJ76 and PEANT1/ PEANT2), were not so concluding: with nested PCR according to Llop et al. (2001) an amplicon of 350 bp was observed. However, the previous results with primerA/primer B allowed the

These strains were used for studies related to their inhibition by various bacteria (*Pseudomonas spp.* and *Bacillus spp.*). The selection of antagonistic bacteria was performed by direct action of various bacterial strains on a lawn of pathogenic bacteria.

2. Screening of antagonistic strains of bacteria

A number of 204 antagonistic and non-antagonistic bacterial strains were isolated from soil (rhizosphere), leaves, shoots and blossoms of apple and pear trees from the orchard of the Institute for Plant Protection Bucharest.

In order to select new bacterial strains able to inhibit the growth of *E.amylovora* isolates, 8 strains of *Pseudomonas spp.* and 17 *Bacillus spp.* strains were tested. The interactions were examined on two media: King B and nutrient agar. The best results, appreciated as the dimension of inhibition zones, were observed on King B, similar observation being communicated by other authors [11]. For example, good inhibitory potential have some strains of *Bacillus subtilis* designated as B2R, B005, Sal 1, 45B şi R6P4G (fig.3)

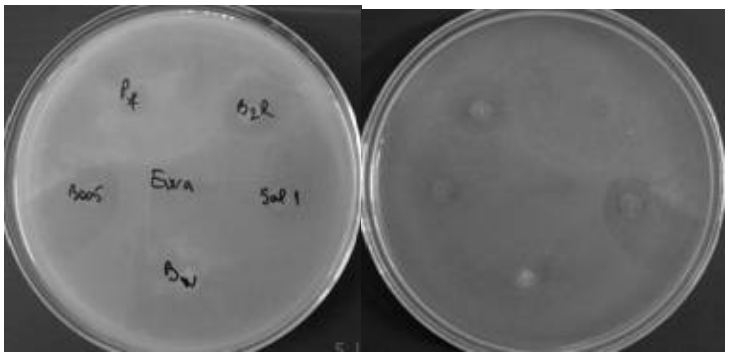

Figure 3. Inhibitory action of some bacteria against *Erwinia amylovora* SVEa

Clear inhibition areas were also detected in *Pseudomonas spp* P10, P14, P5 and P20 (fig.4).

Figure 4. Inhibition of *E.amylovora* SVEa by *Pseudomonas* strains after 48 h of incubation on King B medium

Similar results were also obtained with some new *Bacillus spp.* isolates, at least five strains producing the *in vitro* inhibition of *E.amylovora* (fig.5).

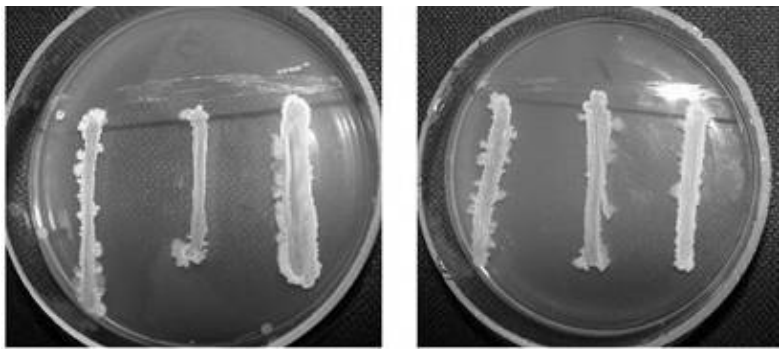

Figure 5. Antagonism between several *Bacillus spp.* and *E.amylovora* Ew1 (streak assay). From left to right (vertical lines): SV 100; SV 84; SV 170; SV 166, SV 165 and SV171.

In order to identify the antagonists, both microbiological and molecular techniques were applied. The use of API 20E and Biolog system allowed the identification of *Pseudomonas* strains as following: the strains P10 and P14 belonging to *P.aeruginosa,* P20 was identified as *P.fluorescens* and P5 as *P.putida.* Similar results were obtained when ITS-PCR was applied. The amplicons, obtained with ITS1/ITS2 primer pair, were digested with Alu I and the patterns were compared with those of reference strains (fig.6).

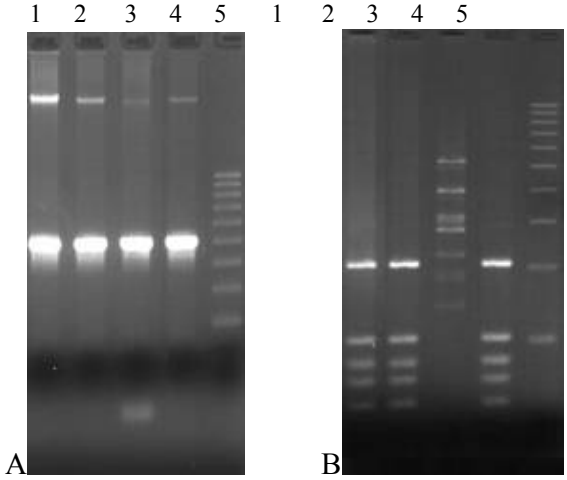

Figure 6. Electrophoretic pattern of amplicons obtained after ITS-PCR (A) and restriction products resulted after Alu I digestion (B). 1 = *P.aeruginosa* ATCC 27853; 2 = P10; 3 = P11; 4 = P14; 5 = 100 bp ladder

The same primer pair (ITS1/ITS2) was used for characterization of new *Bacillus* strains. No significant differences were observed related to amplicon dimension or in electrophoretic pattern of restriction fragments generated by Alu I or HhaI enzymes (fig.7).

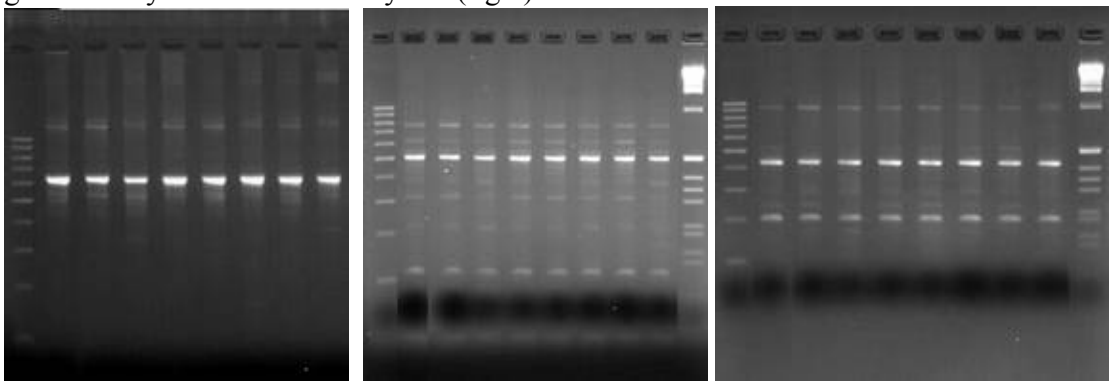

Figure 7. Electrophoretic profile of amplicons generated by ITS1/ITS2 primers (A) and cleaved with AluI (B) or HhaI (C) restriction enzymes. Samples order (from left to right): 100 bp ladder, SV166, SV165, SV84, SV100, SV170, SV66a, SV171, BCPC, 1 kbp ladder

BIOLOG system as well API 20E and API CHB tests identified the isolates SV100, SV165, SV170, SV171, SV166 and Bcpc as *Bacillus amyloliquefaciens*. For the others these systems gave contradictory results but all belong to *Bacillus* genus. Further molecular studies were realized with two out of the antagonists that shown the best

inhibitory action: SV100 and SV165, comparing with Bw, in order to detect the differences between them. RADP analysis using 10 arbitrary primers (UBC and OP types): UBC1, UBC 2, UBC 51, UBC 52, pgs2 pgs3, OPG5, OPG6, OPG14 and OPA17 (fig.8).

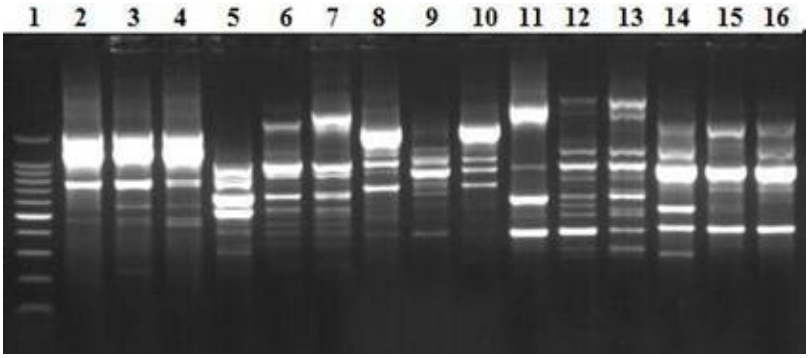

Figure 8. Eletrophoretic pattern amplification products obtained with the decameric primers produced by UBC: UBC1, UBC 2, UBC 51, UBC 52 şi pgs2. The order of samples: 1- 100 bp ladder Promega, 2, 5, 8, 11, 14 – Bw; 3, 6, 9, 12, 15 – SV100; 4, 7, 10, 13, 16 – SV165

It is obvious that the strains designated as SV100 and SV165 belong to the same species, instead Bw could belong to another *Bacillus* species. Molecular polymorphism among the *B.amyloliquefaciens* strains tested in our experiments was observed when the primer UBC51 was used. It this aspect will be proved also with other strains belonging to the same species and the amplicon of 1100 bp remains specific for SV165 strain, it will be useful as molecular marker for monitoring the strain in natural ecosystems. Moreover, the application of various molecular techniques could be very useful in order to validate markers that allow the development of a method for monitoring and assessment of the environmental fate of antagonist strains [1].

Conclusions

- Comparative studies on various culture media for selection and preliminary identification of *Erwinia amylovora* allowed the selection of a pair of minimal media: MM1-Cu and MM2-Cu, which proved a good discrimination among bacterial isolates;
- Two new *Erwinia amylovora* strains were isolated from infected material, the identification being performed by microbiological and genetic methods;
- In vitro experiments permits the selection of several bacterial strains, belonging to *Pseudomonas* and *Bacillus* genus, that exhibited and increases inhibitory action against bacterial pathogen *E.amylovora;*
- Preliminary tests performed for the identification of bacterial antagonists allowed the identification of two *P.aeruginosa* strains, one *P.fluorescens,* one *P.putida* and 5 *B.amyloliquefaciens* strains.
- Slight differences were observed among two of *B.amyloliquefaciens* strains that exhibited the best inhibitory activities: SV100 and SV165 when RAPD analysis with 10 primers was performed.
- The application of various molecular techniques could be very useful in order to validate markers that allow the development of a method for monitoring and assessment of the environmental fate of antagonist strains

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