Pseudomonas graminis as biocontrol agent of fire blight

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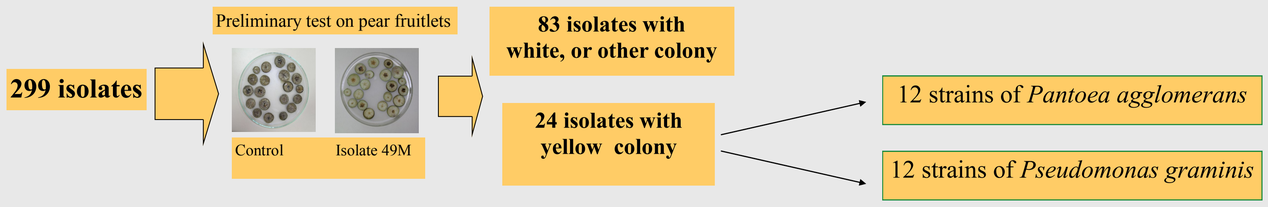
Aim

Selection of bacterial isolates effective for control of fire blight and trails to elucidate mechanism of action.

Screening on pear fruitlets and identification

Out of 299 isolates originating from leaves and soil 107 showed high efficacy in protection of pear fruitlets cv. 'Conference' against fire blight. Yellow colony isolates (24) were characterized using physiological and biochemical tests according to keys of Bradbury (1988) and Schad et al. (2001), and by 16S r RNA sequence analyses (Weisburg et al.1991; Drancourt et al. 1997).



Strain 49M of *P. gramins* was used for detailed studies

Protection of apple blossoms

Blossoms on Idared/M.26 trees growing in pot in the greenhouse were sprayed with water suspension of strain 49M at concentrations of 10^7 and 10^8 cfu/ml. After 24 h blossoms were spray inoculated with water suspension of *Erwinia amylovora* strain Ea659 at 10^7 cfu/ml. The presence of blight symptoms was recorded after 5 to 9 days. The preparations: Miedzian 50WP (copper oxichloride), Aliette 80WG (fosetyl-Al), isolate A506 (*Pseudomonas fluorescens*) and Blight Ban A506 (USA) were used for comparison.

0-4: degree scale of disease severity

Experiment 1

Treatment	Disease severity Days after inoculation: 5	7
Control	1.2 b* (0)**	1.8 b (0)
49M 10^8 cfu/ml	0.1 a (89.0)	0.3 a (82.8)
49M 10^7 cfu/ml	0.3 a (71.2)	0.7 a (62.3)
Miedzian 50WP 0.15%	0.4 a (63.5)	1.1 a (36.0)
Miedzian 50WP 0.3%	0.3 a (72.0)	0.8 a (55.9)
Aliette 80WG 0.25%	0.5 a (57.6)	1.0 ab (42.4)

Experiment 2

Treatment	6	9
Control	1.5 b* (0)**	2.6 b (0)
49M 10^8 cfu/ml	0.3 a (77.2)	0.9 a (62.6)
A506 10^8 cfu/ml	0.4 a (72.7)	0.8 a (67.3)
Blight Ban A506	0.2 a (81.8)	1.0 a (60.3)

*rating scale of severity: 0 = no necrosis; 4 = total necrosis of ovary and peduncle, analyses were made separately for each day;
**numbers in brakets show efficacy (%)

Protection of M.26 rootstock shoots

Tips of terminal shoots on one-year-old apple M.26 growing in pots in the greenhouse were cut off with sterile scissors and afterwards sprayed with water suspension of 49M at 10^8cfu/ml. BlightBan A 506 was included for comparison. Immediately after spraying the shoots were covered with plastic bags. After 6 hours they were spray inoculated with water suspension of *E .amylovora* strain Ea 659 at 10^7 cfu/ml and again covered with plastic bags for 24 hours. After 7, 10 and 17 days the measurement of total length of shoots and the length of necrotized part of shoots was made.

Treatment	Percentage of M.26 shoots infestation by fire blight Days after inoculations: 7	10	17
Control	6.5 b* (0)**	21.0 b (0)	37.6 c (0)
49M	0.05 a (99.2)	1.6 a (92.5)	5.2 a (86.3)
Blight BanA506	6.0 b (6.6)	9.0 a (57.0)	16.7 b (55.5)

*lenght of shoot lesion/total lenght of shoot x 100; analyses were made separately for each day
** numbers in the brakets show efficacy (%)

Survival of bacteria on apple blossoms in orchard

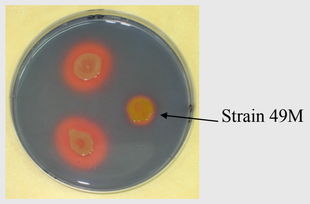
1. Blossoms on apple trees cv. Jonagored, were sprayed with water suspensions of isolate 49M and strain C9-1 of *Pantoea agglomerans;* control blossoms were sprayed with sterile distilled water
2. After 2, 5 and 10 days 10 randomly selected blossoms were cut off at the base of calyx. Each blossom was placed into10 ml of sterile distilled water in test tube and after 15 min. of shaking aliquot was poured on NAS medium
3. Bacterial colonies with characteristic morphology were counted after 48 h of incubation at 24°C

Combinations	Number of bacteria after days 2	6	10
Control*	$0 - 2x10^3$	$700 - 4x10^5$	$2.0x10^3 - 1.0x10^5$
C9-1	$3.5x10^5 - 3.7x10^6$	$9x10^4 - 4.3x10^6$	$2.3x10^5 - 2.5x10^6$
49M	$7.4x10^5 - 1.4x10^7$	$1.9x10^5 - 1.4x10^7$	$2.0x10^4 - 6.7x10^6$

* only native bacteria

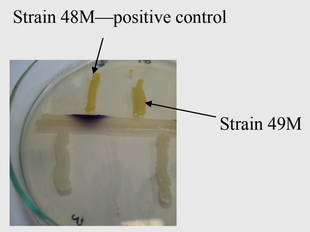
Mechanisms of action

Siderophores production



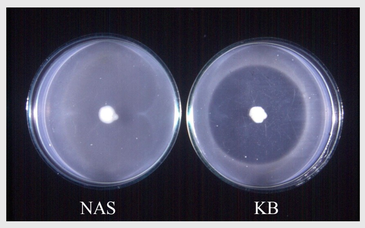
Isolate 49M produced siderofores on medium containing complex: CAS - Fe $^{3+}$ - HDTMA prepared according to Schwyn and Neilands (1987).

Signaling molecules N - acyl homoserine lactones (AHL)



Strain 49M did not produced N - acyl homoserine lactones in test with AHL - indicator strain *Chromobacterium violaceum* CV026 according to the method of McClean et al. (1997). Tested bacteria were cultivated in LB medium, the discoloration of bacterial growth was recorded after 2-3 days of co-cultivation.

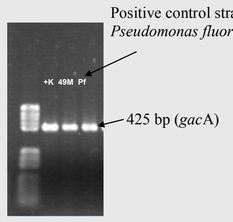
Biotic relationships on artificial media



Strains	NAS 24	NAS 48 h	KB 24	KB 48 h	LB 24	LB 48 h
49M	0	0	11.0	0	0	0
A506	0	0	11.5	0	0	0
C9-1	0	0	0	0	0	0

Bacteria were seeded on each medium in the center of Petri dishes and after 3 days of incubation at 26°C they were killed by vapors of chloroform and then flooded with soft agar containing *E. amylovora*, strain Ea 659. The radius of inhibition zones around tested strains was measured after 24 and 48 hours.

Analysis of genes coding antibiotics



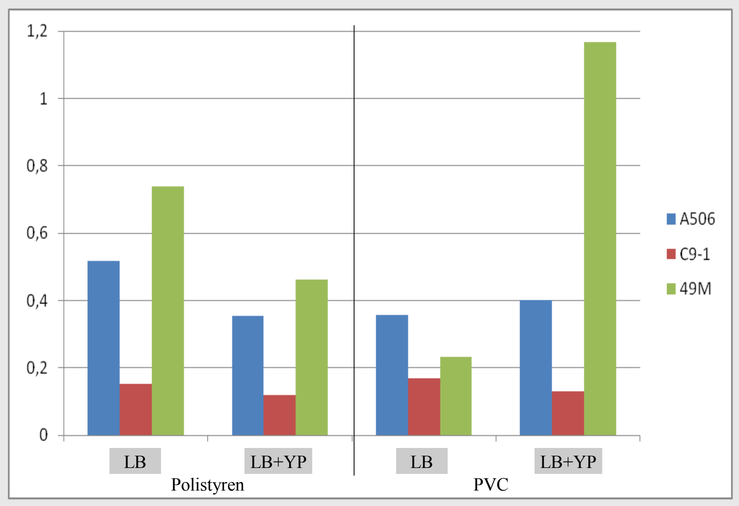
Isolates	Presence of genes involved in antibiotic biosynthesis: 2,4 - diacethylphloroglucinol *phl*D	Phenazine *phz*A , *phz*F, *phzC, phzD*	Pyoluteorine *plt*B, *plt*C	Pyrrolnitrin *prn*D	Regulatory gen *gac*A
49M	-	- - - -	- -	-	+
A506	-	- - - -	- -	-	-
59M*	+	- - - -	+ +	-	+

* strain *Pseudomonas* spp. isolated from soil used for comparison

For all PCR tests, as a template 1 µl of boiled bacteria suspension (1 colony in 0.5 ml H_2O) was used. Bacterial DNA was amplified with primers: *phlD*, *phzA, phzF, phzC, phzD*, *plt*B, *prn*D, *gac*A1 and *gac*A2.

Biofilm formation

Biofilm formation was assayed by the ability of cells to adhere to the wells of 96-well microtiter plates made of polyvinyl chloride (PVC) and polistyren. Bacterial strains were grown overnight in LB or YP and LB media, diluted in indicated medium and added to each well of the microtiter plates and incubated for 12h at 27°C according the method Hossain and Tsuyumu 2006. Strains A506 and C9-1 were used for comparison.



Summary

- Strain 49M significantly protected apple blossoms and shoots against fire blight. High efficacy was obtained when 49M was applied at concentration 10^8 cfu/ml (89.0-82.8%)
- Survival of 49M bacteria on apple blossoms cv. Jonagored in orchard was high; 10 days from their introduction by spraying with water suspension at 10^8 cfu/ml $2.0x10^4$—6.7×10^6 cfu/ blossom were detected
- Strain 49M produced siderophores and biofilm but not N - acyl homoserine lactones (AHL).
- The presence of regulatory gen *gac*A influencing the production of several secondary metabolites including antibiotics was found in 49M; however, no *phl*D, *phz*D, *plt*B, *plt*C and *prn*D genes were not detected
- Study on biotic relationship between tested isolate and *E. amylovora* on 3 microbiological media (Nutrient Agar with sucrose, King B and LB) showed that 49M inhibited growth of pathogen only on King B medium.

Acknowledgement:
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