Effect of bacterium *Pseudomonas fluorescens* and low fungicide dose seed treatments on parasite fungus *Aphanomyces cochlioides* and sugar beet yield and quality

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ABSTRACT

Effect of sugar beet seed inoculation with a bacterium *Pseudomonas fluorescens* and treatment by fungicides Thiram 42-S and Dithane S-60 with and without seed inoculation aiming to control *Aphanomyces cochlioides* – root decay agent was studied. The trial lasted for three years on two soil types (Mollic Gleysols and Eutric Cambisols). The following parameters of sugar beet yield and quality were investigated: root yield, sugar content, sugar in molasses, sugar yield as well as percentage of the infected and decayed plants as a consequence of parasite fungus infestation. The highest average sugar beet root yield was obtained in the variant of the seed treated with fungicide Thiram 42-S and inoculated with bacterium *P. fluorescens* (85.15 t/ha). However, there were no statistically significant differences ($P < 0.05$) between the above-mentioned variant and the one with seed inoculated only with bacterium *P. fluorescens* (84.63 t/ha). The highest mean sugar content of 16.39% was also accomplished during the three-year investigation in the variant of the inoculated seed treated by fungicide Thiram 42-S. All other variants accomplished statistically very significantly lower values of this parameter. The same variant was characterized by the highest mean sugar yield value (12.79 t/ha) on both soil types. Namely, an average sugar yield of the variants inoculated with bacteria was 11.22 t/ha and by 44.22% higher compared to an average yield of non-inoculated variants. The highest percent of the infected and decayed plants (average value on both soil types in the three year investigation) was reported in the control variant (28.92% infected and 25.00% decayed plants) whereas the lowest one was detected in the variant of the seed inoculated with bacterium *P. fluorescens* in combination with low dose of fungicide Thiram 42-S (4.70% infected plants and 2.88% decayed plants). An average percent of the infected plants inoculated with bacterium *P. fluorescens* was 9.13% whereas the aforesaid value of the plants infected with parasitic fungus *A. cochlioides* in non-inoculated variants was by 146.00% higher being 22.42%.

Keywords: *Pseudomonas fluorescens*; *Aphanomyces cochlioides*; fungicides; sugar beet; seed inoculation; yield; quality

Pseudomonas fluorescens belongs to rod-like, asporogenous, gram negative bacteria being as saprophyte forms widespread in soils and waters. These bacteria belong to soil microorganisms that develop one of the very important soil process – denitrification.

However, owing to antimicrobial agents production, these bacteria show a distinguished antibiosis against pathogenic microorganisms – disease agents on arable crops, by inactivating their growth and reproduction (Whipps 2001).

Due to cyclic lipopeptides production (Thrane et al. 2000, Koch et al. 2002, Andersen et al. 2003) these bacteria are more and more used as biological agents (Nielsen et al. 1999, Sørensen et al. 2001). The investigation results of numerous authors prove that purified lipopeptids show antagonistic activity against fungi responsible for sugar beet root decay such as *Rhizoctonia solani* (Nielsen et al. 2000, 2002, Andersen et al. 2003), *Aphanomyces cochlioides (*Nielsen et al. 1999, Sørensen et al. 2001), *Pythium ultimum* and *Pythium debarianum* (Lee et al. 2000, Nielsen et al. 2000, Thrane et al. 2000, Andersen et al. 2003). The above-mentioned researches indicate potential role of bacteria producing lipopeptides in fungi diseases bio-control (Thrane et al. 2000, 2001). Lipopeptides may also function as biosurfactants (Desai and Banat 1997), which can facilitate bacterial growth on water – insoluble carbon sources (Koch et al. 1991, Ron and Rosenberg 2001) or their interaction with hydrophobic surfaces (Neu 1996), e.g., surface motility (Lindum et al. 1998).

Young sugar beet plants are subjected to various soil pathogens comprising basidiomycetes *Rhizoctonia solani* and oomycetes *Pythium ultimum*, *Pythium debarianum* and *Aphanomyces cochlioides*. These pathogenic fungi cause sugar beet plants to decay even prior to soil surface emergence, although the highest decay turned out to be from emergence phase to 2–4 true leaves phase. However, most parts of the diseased plants survive. Their further growth is slowed down thus they never reach normal size. The aforesaid finally is reflected in yield and quality elements.

Fungicide treated seed mainly prevents these fungi development. However, fungicides largely affect human health and environment whereas pathogenic fungi became very fast resistant to them. For that reason, seed inoculation with bacteria showing antagonism against pathogenic fungi is an acceptable alternative to chemical pesticides application (Andersen et al. 2003).

Since *Pseudomonas* spp. are usually not especially fungicides sensitive (Pedersen et al. 2002) seed is possible to be treated in combination with low doses of these chemical agents aiming at effective control of pathogenic fungi growth and reproduction resulting in a positive effect on all sugar beet yield and quality indicators.

MATERIAL AND METHODS

The experiment was set up on two soil types – Mollic Gleysols (FAO 1998) and Eutric Cambisols (Table 1) being, in the period 2001–2002, known for detected pathogenic fungi – sugar beet root decay agents – *Aphanomyces cochlioides*. In 2002, 2003 and 2004 the experiment was set up by completely randomised block design in 4 repetitions and 8 various seed treating variants: 1. untreated seed; 2. Thiram 42-S fungicide treated seed (48% Tiram, 600 ml/100 kg seed); 3. Dithane S-60 fungicide treated seed (60% Mankozeb, 800 g/100 kg seed); 4. seed treated with Thiram 42-S (300 ml/100 kg seed) + Dithane S-60 (400 g/100 kg seed) fungicides; 5. seed inoculated with *Pseudomonas fluorescens* bacterium; 6. seed treated with fungicide Thiram 42-S (300 ml/100 kg seed) + inoculated with bacterium *Pseudomonas fluorescens*; 7. seed treated with fungicide Dithane S-60 (400 g/100 kg seed) + inoculated with bacterium *Pseudomonas fluorescens*; 8. seed treated with fungicides Thiram 42-S (300 ml/100 kg seed) + Dithane S-60 (400 g/100 kg seed) + inoculated with bacterium *Pseudomonas fluorescens*.

Soil samples were taken in autumn after harvest of the preceding crop. Soil analyses were carried out by standard methods (Table 1): organic matter content was determined by bichromate method, pH in H_2O and KCl, phosphorus and potassium content by ammonium-lactate method according to Egner-Riehm-Domingo (Page 1982).

Pseudomonas fluorescens was isolated from sugar beet seedling rhizosphere (Thrane et al. 2000) and cultivated on King's B medium (Pseudomonas F; Difco catalog No. 0448-17-1). Fluorescent *Pseudomonas* spp. could be detected by illuminating agar plates with UV light (254 nm) and randomly picking of fluorescent colonies. The inoculant was applied on the seed directly prior sowing in amount of approximately 8×10^4 bacteria/seed, i.e. $0.8–1.4 \times 10^{10}$ bacteria/hectare.

Hybrid of OS Sana sugar beet (Ploidity – $2n = 3x = 27$) was used in the sowing. Both investigation years were characterized by the sowing conducted in the second March decade. The row spacing was 50 cm and within row 20 cm.

Percent of the plants infected with pathogenic fungus *Aphanomyces cochlioides* as well as percent of decayed plants was stipulated in 4–6 true leaves phase. The sugar beet digging, conducted in the mid October, was followed by determination of root yield (t/ha), sugar content (%), sugar in molasses (%) and sugar yield (t/ha).

Weather conditions in the investigation years (Table 2) differed and affected sugar beet growth, root yield and quality. The 2002 growing season was

Table 1. Soil characteristics

Investigated properties in a field	Soil type	
Layer (0–0.3 m)	Mollic Gleysols	Eutric Cambisols
pH (H_2O)	7.42	6.46
pH (KCl)	6.44	5.98
Decomposed organic matter (%)	3.22	1.87
P (mg/100 g soil)	24.44	22.50
K (mg/100 g soil)	29.57	23.11

Table 2. The precipitation and mean monthly air temperatures for sugar beet in Osijek (2002– 2004)

Month	Quantity of rainfalls (mm)				Mean monthly temperature (°C)			
	1901–1991	2002	2003	2004	1901–1991	2002	2003	2004
April	56	58	9	122	11.2	11.5	11.5	11.4
May	63	156	43	63	16.8	19.1	20.5	15.4
June	88	48	25	88	19.4	22.1	24.7	19.8
July	66	85	70	58	21.2	23.0	22.9	22.1
August	61	55	23	105	20.4	21.3	24.6	21.4
September	46	75	49	45	16.8	16.0	16.7	15.8
Sum	380	477	219	481				
Average					17.6	18.8	20.2	17.7

characterized by increased month air temperature compared to long term average by 1.2°C. Especially warm temperatures in June and July were 22.1°C i.e. 23.0°C, respectively. Precipitations amount of the growing season was 477 mm, being higher by 97 mm compared to long-term average for this area. Unlike the first one, the second investigation year (2003) was markedly dry. Only 219 mm precipitated in the growing season. Sowing time (March) was characterized by low precipitation, only 2.9 mm, as well as emergence period (April) with only 9.1 mm of rain. Such drought period brought about difficult emergence and slight sugar beet growth. High temperature, also, contributed to poor growth. Mean monthly air temperature was in the growing season even 20.2°C i.e. by 2.6°C higher compared to long term average. Especially June, July and August were characterized by high temperatures (24.7, 22.9, 24.6°C) affecting sugar increase and its concentration. The third research year (2004) was characterized by the lowest average growing season monthly air temperatures (17.7°C) in the investigated period on long term average level. Temperatures provided more suitable conditions for sugar beet growth and development compared to the previous two years although this year July and August temperatures were too high for maximum photosynthesis. Year 2004 was known for 10 mm of precipitations being higher compared to this area average. However, precipitations, primarily in July and August favorably affected root growth and obtained sugar beet yield.

Results were processed by modern statistical methods (ANOVA) using the computer program StatSoft Inc. (2001) STATISTICA (data analysis software system), version 6.

RESULTS AND DISCUSSION

The three year investigation conducted on soil types Eutric Cambisols and Mollic Gleysols was known for the highest average sugar beet root yield (Table 3) accomplished in the variant of the seed treated with fungicide Thiram 42-S and bacterium *Pseudomonas fluorescens* inoculation (variant 6). However, there was no statistically significant difference ($P < 0.05$) between variant 6 and the one with seed inoculated with bacterium *Pseudomonas fluorescens* (variant 5).

Soil type Eutric Cambisols was characterized by the highest average sugar beet yield obtained in the variant 6 (seed treated with Thiram 42-S and inoculated with bacterium *Pseudomonas fluorescens*) in all three-investigation years. All other variants obtained statistically very significant ($P < 0.01$) lower average sugar beet root yields.

The investigation results of Pytlarz-Kozicka (2005) prove that sugar beet yielding depend, among others, of anti-fungal plant protection.

In the first investigation year soil type Mollic Gleysols was known for the highest average root yield accomplished in the variant with seed treated by fungicide Thiram 42-S and inoculated with bacteria *Pseudomonas fluorescens* (variant 6). All other variants obtained statistically very significant ($P < 0.01$) lower root yields. In the second investigation year (2003) there was no statistically significant difference between variant 6 and variant 5 (seed inoculated with bacterium *Pseudomonas fluorescens*). All other variants accomplished statistically very significant ($P < 0.01$) lower average sugar beet root yields.

The second investigation year (2003) is characterized by an outstanding high temperatures

Table 3. Investigated parameters by the localities (soil types) and years of investigation

Investigated parameter	Variants	Mollic Gleysols			Eutric Cambisols			Average
		2002	2003	2004	2002	2003	2004	
Root yield (t/ha)	1	58.62	45.27	75.15	55.61	35.52	73.87	57.34
	2	71.32	55.31	90.03	66.39	51.24	83.51	69.63
	3	58.61	43.45	78.00	58.40	43.10	72.95	59.08
	4	66.49	50.03	84.15	52.73	38.66	77.82	61.65
	5	81.93	67.20	103.27	77.85	62.91	96.61	84.63
	6	88.14	68.00	97.41	85.03	66.43	105.91	85.15
	7	77.75	53.78	88.94	77.10	52.11	92.00	73.61
	8	71.48	56.42	90.52	70.19	54.98	85.48	71.51
$LSD_{0.05}$		2.05	1.12	2.19	2.08	1.82	2.14	1.49
$LSD_{0.01}$		2.65	1.88	2.84	3.16	2.56	3.08	2.32
Sugar content (%)	1	12.05	13.01	13.39	12.47	12.74	13.95	12.94
	2	14.63	15.49	16.51	14.75	15.56	16.89	15.64
	3	11.89	14.00	13.91	12.11	12.88	13.90	13.12
	4	11.88	13.63	13.58	11.97	13.41	14.15	13.10
	5	14.63	15.41	16.81	14.90	16.21	16.44	15.73
	6	15.07	15.89	17.32	15.31	16.78	17.95	16.39
	7	14.05	15.11	17.00	13.79	16.01	16.58	15.42
	8	14.01	15.03	15.98	13.98	16.09	16.51	15.27
$LSD_{0.05}$		0.49	0.27	0.24	0.32	0.30	0.42	0.34
$LSD_{0.01}$		0.66	0.39	0.37	0.48	0.44	0.58	0.52
Sugar in molasses (%)	1	2.11	2.30	2.00	2.07	2.24	1.95	2.11
	2	1.50	2.61	1.48	1.41	1.58	1.29	1.65
	3	2.07	2.12	1.95	2.00	2.17	1.87	2.03
	4	1.95	2.03	1.53	2.01	1.79	1.56	1.81
	5	1.12	1.28	1.26	1.47	1.39	1.14	1.28
	6	1.51	1.69	1.35	1.48	1.51	1.21	1.46
	7	1.63	1.75	1.48	1.43	1.39	1.26	1.49
	8	1.49	1.60	1.39	1.45	1.51	1.22	1.44
$LSD_{0.05}$		0.16	0.19	0.06	0.03	0.08	0.06	0.11
$LSD_{0.01}$		0.24	0.28	0.11	0.05	0.12	0.09	0.15
Sugar yield (t/ha)	1	5.83	4.85	8.56	5.78	3.73	8.86	6.27
	2	9.36	7.12	13.53	8.86	7.16	13.03	9.79
	3	5.75	5.16	9.33	5.90	4.62	8.78	6.59
	4	6.60	5.80	10.14	5.25	4.49	9.80	6.96
	5	11.07	9.49	16.06	10.63	9.32	14.78	11.83
	6	11.95	9.66	15.55	11.76	10.14	17.73	12.79
	7	9.66	7.18	13.80	9.53	7.62	14.09	10.31
	8	8.95	7.58	13.21	8.79	8.02	13.07	9.94
$LSD_{0.05}$		0.52	0.56	0.40	0.51	0.39	0.72	0.48
$LSD_{0.01}$		0.70	0.78	0.58	0.84	0.60	0.98	0.69

1. Untreated seed. 2. Thiram 42-S fungicide treated seed (600 ml/100 kg seed). 3. Dithane S-60 fungicide treated (800 g/100 kg seed). 4. Seed treated with Thiram 42-S (300 ml/100 kg seed) + Dithane S-60 fungicides (400 g/100 kg seed). 5. Seed inoculated with *Pseudomonas fluorescens* bacterium. 6. Seed treated with fungicide Thiram 42-S (300 ml/100 kg seed) + inoculated with bacterium *P. fluorescens.* 7. Seed treated with fungicide Dithane S-60 (400 g/100 kg seed) + inoculated with bacterium *P. fluorescens.* 8. Seed treated with fungicides Thiram 42-S (300 ml/100 kg seed) + Dithane S-60 (400 g/100 kg seed) + inoculated with bacterium *P. fluorescens*

drought. The growing season was known for 161 mm rainfall less and temperatures were by 2.6° C higher compared to the long-term average. This was the reason why average sugar beet root yield was by (23.50%; 37.89%) lower on the soil type Mollic Gleysols, i.e. by (25.46%; 41.14%) on the soil type Eutric Cambisols compared to the first and the third investigation year. Due to the pronounced water shortage occurred from sugar beet sowing to the end of the growing season, bacteria *Aphanomyces cochlioides* soil prevalence was lower. However, due to the long-term emergence and slowed growth of young sugar beet plants, these evident damages were caused by the aforementioned pathogenic fungi. Since Mollic Gleysols soil was known for its favorable physical chemical properties *Pseudomonas fluorescens* was more abundant compared to the soil Eutric Cambisols. Also, due to the soil water lack in 2003 year this benefit bacterium was reported to be less soil dominant.

The third investigation year (2004) was known for the highest average sugar beet root yield accomplished by the variant inoculated with bacterium *Pseudomonas fluorescens* (variant 5) on Mollic Gleysols. Statistically very significant lower yields ($P < 0.01$) were obtained by all other variants.

An average sugar beet root yield in the variant inoculated with bacterium *Pseudomonas fluorescens* (variants 5, 6, 7, 8) was 78.73 t/ha and by 24.08% higher compared to an average yield in the only fungicides treated seed variants (variants 2, 3, 4).

Obtained results are in the harmony with investigations conducted by Whipps (2001) who stated that plants inoculated with bacterium *Pseudomonas fluorescens* are characterized by fast initial growth allowing faster passage of the most sensitive phase known for the most pronounced pathogenic attack consequences. Owing to an outstanding antagonistic bacterium effect against sugar beet root agent – fungus *Aphanomyces cochlioides*, high percent of survived inoculated plants was accomplished compared to non-inoculated ones as well as reduction of infected plant consequences. The aforementioned was reflected in sugar beet root yield.

The highest sugar content was achieved in all three-investigation years, on both soil types in the variant of the seed treated with fungicide Thiram 42-S and inoculated by the bacterium *Pseudomonas fluorescens* (variant 6). However, in the first investigation year soil type Mollic Gleysols did not show statistically very significant differences ($P < 0.01$) between the aforementioned variant, variant 2 (Thiram 42-S fungicide treated seed) and 5 (*Pseudomonas fluorescens* bacterium inoculated seed), in the second year variant 2 and the third year variant 7 (seed inoculated with *Pseudomonas fluorescens* bacterium and Dithane S-60 fungicide treated). In the first investigation year Eutric Cambisols showed no statistically very significant differences ($P < 0.01$) between the aforementioned variant (variant 6) and variant 5 (seed treated only with *Pseudomonas fluorescens* bacterium). However, differences between variant 6 in the second and the third investigation year and all other investigation variants were very significant ($P < 0.01$).

An average sugar content of the variants non-inoculated with *Pseudomonas fluorescens* bacterium was 13.70% and by 12.74% lower compared to an average sugar content of inoculated variants being 15.70%.

The lowest average values of the investigated parameter – sugar in molasses appeared to be in the variants of the seed inoculated only with *Pseudomonas fluorescens* bacterium. Only the first investigation year was an exception where the lowest average value of this parameter was obtained on the soil type Eutric Cambisols in the variant with only Thiram 42-S fungicide treated seed (variant 2). There were no statistically significant differences ($P < 0.05$) with the variant of the seed inoculated with *Pseudomonas fluorescens* bacterium and treated with Dithane S-60 fungicide (variant 7) i.e. statistically very significant difference ($P < 0.01$) in the variant of the seed inoculated with bacterium *Pseudomonas fluorescens* and treated with fungicides Thiram 42-S and Dithane S-60 (variant 8).

An average molasses sugar value in the *Pseudomonas fluorescens* bacterium inoculated variants (5, 6, 7 and 8) was 1.42% and by 22.41% lower compared to the average value of this parameter where seed was treated only with fungicides (variants 2, 3 and 4). Namely, bacteria *Pseudomonas fluorescens* ssp. mobilize soil phosphorus making it available for plants affecting reduction of adverse nitrogen effect. This results in a balanced plant nutrition and reduced production of alpha amino nitrogen, potassium and sodium. Namely, it leads to molasses share reduction and increased sugar yield.

Sugar yield is defined by the root yield, sugar content and level of potassium, sodium and alpha-amino nitrogen (molasses sugar). This investigated parameter was in a very significant positive cor-

relation with root yield (r = 0.967**) and sugar content (r = 0.917**).

The highest average sugar yield value was accomplished in the variant where seed was treated with Thiram 42-S fungicide and inoculated with *Pseudomonas fluorescens* bacterium (12.79 t/ha). The exception is the third investigation year on the soil type Mollic Gleysols where the highest value of this parameter was accomplished in the variant of the seed inoculated with *Pseudomonas fluorescens* bacterium (variant 5). However, there were no statistically very significant differences (P < 0.01) between the variant inoculated with *Pseudomonas fluorescens* bacterium and treated with Thiram 42-S fungicide (variant 6). Differences between bacterium treated variants (5, 6, 7 and 8) and those where seed was treated only with fungicides (variants 2, 3 and 4) could be seen here. Namely, an average sugar yield of the variants bacteria inoculated was 11.22 t/ha and by 44.22% higher compared to an average yield of non-inoculated variants.

Percent of sugar beet plants infected with *Aphanomyces cochlioides* fungus (Table 4) and decayed plants percent was in significant positive correlation with root yield (r = 0.896**) and sugar content (r = 0.905**). The highest percent of infected and decayed plants (average value on both soil types during the three year investigation) was detected in the control variant (28.92% infected, 25.00% decayed plants). The lowest percent was found out in the variant of the seed inoculated with *Pseudomonas fluorescens* in combination with low dose of Thiram 42-S fungicide (4.70% infected plants, 2.88% decayed plants). An average percent of infected plants inoculated with *Pseudomonas fluorescens* bacterium was 9.13% whereas reported value of the plants infected by parasitic fungus *Aphanomyces cochlioides* with non-inoculated variants was by 146.00% higher being 22.42%. An average percent of decayed plants inoculated with this benefit bacterium was 5.92% whereas an average percent of decayed plants caused by this pathogenic fungus infection with non-inoculated variants was by 212.67% higher being 18.51%.

All investigated parameters of sugar beet root yield and quality in all three investigative years were known for best results obtained by the seed inoculated with *Pseudomonas fluorescens* bacterium and treated with low doses of Thiram 42-S fungicide. The aforesaid approves pronounced antagonistic activity of *Pseudomonas fluorescens* bacterium against parasitic fungus *Aphanomyces cochlioides* and insensibility of this benefit bacterium against fungicide low doses (Pedersen et al. 2002, Andersen et al. 2003). Combined seed treatment with bacterium *Pseudomonas fluorescens* and low doses of these chemical agents resulted in

Table 4. Percentage of the infected and decayed plants as a consequence of parasite fungus (*Aphanomyces cochlioides*) infestation in 4–6 true leaves phase

Investigated parameter	Variants	Mollic Gleysols			Eutric Cambisols			Average
		2002	2003	2004	2002	2003	2004	
Infected plants (%)	1	30.9	28.3	29.1	26.4	31.7	27.1	28.92
	2	18.8	17.9	14.9	17.7	20.4	6.6	16.05
	3	28.1	24.2	19.4	27.0	27.8	16.0	23.75
	4	24.3	19.8	17.2	29.1	21.5	13.8	20.95
	5	6.2	10.8	1.6	8.6	13.6	4.9	7.62
	6	1.8	9.2	2.3	1.6	10.8	2.5	4.70
	7	12.2	13.6	11.7	11.8	13.9	5.7	11.48
	8	17.6	11.4	8.9	19.2	12.5	6.6	12.70
Decayed plants (%)	1	26.4	23.1	24.0	23.8	27.8	24.9	25.00
	2	15.0	14.6	9.8	13.9	18.0	4.8	12.68
	3	22.4	21.0	12.6	24.4	24.5	13.2	19.68
	4	19.6	13.9	11.0	25.8	18.3	9.7	16.38
	5	4.9	6.2	0.4	5.2	8.2	2.1	4.50
	6	0.7	5.7	1.5	1.1	6.5	1.8	2.88
	7	9.8	8.4	8.3	8.9	8.9	3.0	7.88
	8	14.4	6.1	5.2	11.8	9.0	3.9	8.40

Variants see Table 3

efficient control of the pathogenic fungi growth and reproduction which in turns reflected on all sugar beet yield and quality indicators.

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