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INFLUENCE OF CULTURAL FACTORS ON ANTIMICROBIAL ACTIVITY OF BIOLOGICAL CONTROL AGENTS FOR LEGUME CROPS PROTECTION

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Abstract

Active bacteria antagonistic towards most deleterious pathogens of soybean and lupine were selected. Experiments on antagonistic and plant-protective properties of isolated strains were performed *in vitro* and *in vivo*. Fermentation medium was optimized for highly promising strain *Bacillus subtilis* M-1 showing increased complex antimicrobial activity against fungal and bacterial pathogens of plants.

Key words: biopreparation, microbial antagonists, lupine and soybean pathogens

Introduction

Cultivars of fodder lupine and soybean included into State register of breeding achievements in Belarus do not fully meet production criteria mainly due to unstable annual crop yields caused by diseases – anthracnose (*Colletotrichum lupini*) (Thomas and Sweetingham 2000, Frencel et al. 2000, Yakusheva 1997), brown spot (*Pleiochaeta setosa*) (Kuptsov 1999), grey mould (*Botrytis cinerea*) (Penaloza et al. 1996, Dorozhkin et al. 1978), bacterial blight (*Pseudomonas syringae*) (Leshhenko et al. 1987), etc. Effective suppression of phytopathogens and reduction of negative effects of chemicals widely applied in agricultural practice requires introduction of ecologically safe biological control agents. Application of antagonistic microorganisms capable of inhibiting phytopathogens development by secreted metabolites seems an attractive approach. There are no registered biopreparations in Belarus against pathogens in legume crops so that selection and characterization of microbial species antagonistic to pathological agents of lupine and soybean appears extremely relevant task. Screening for active microbial antagonists as a vital

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stage in biopreparations design should be complemented by optimization of their growing conditions for maximal expression of microbial potential.

The investigation was aimed at selection of microbial variants with high antagonistic activity towards fungal and bacterial pathogens and choice of optimum cultivation parameters favouring manifestation of increased biological efficiency considering pathogens of grain-legume crops.

Materials and methods

Antagonistic bacteria *Bacillus subtilis* M-1, *B. subtilis* M-22 and *Pseudomonas aurantiaca* S-1 isolated from soil samples from various regions of Belarus and strains *B. subtilis* BIM B-377, *B. pumilus* BIM B-263, *Streptomyces griseoviridis* BIM B-264 and *P. fluorescens* BIM B-152 deposited at collection of non-pathogenic cultures, Institute of Microbiology, National Academy of Science, Belarus, were taken for investigation. The antagonists were grown in submersed culture in Erlenmeyer flasks on the shaker (200 rpm) at 28°C for four days on Meynell media: molasses – 20.0, K_2HPO_4 – 7.0, KH_2PO_4 – 3.0, $MgSO_4$ – 0.1, sodium citrate – 0.5, $(NH_4)_2SO_4$ – 1.0, H_2O – adjusted to 1 l, pH 7.0 (Meynell and Meynell 1967). To optimize nutrient medium different concentrations of carbon (glucose, sucrose, molasses and peas, rye, oat, corn and soybean flour) and nitrogen ($(NH_4)_2SO_4$, NH_4NO_3, KNO_3, urea, yeast extract, corn extract, peptone) sources were used.

Preliminary screening of antagonistic bacteria was carried out by spot testing on agar medium with meat peptone broth (MPB) and meat worth broth (MWB) (Segy 1983). Originally isolated lupine pathogens (*Colletotrichum lupini, Botrytis cinerea, Pleiochaeta setosa*) and soybean pathogens (*Pseudomonas syringae, Xanthomonas phaseoli*) from the collection of Institute of Microbiology were chosen for tests. *Colletotrichum lupine* and *P. setosa* were cultured on medium with tomato worth (tomato worth – 200 ml, $CaCO_3$ – 5.4 g/l, H_2O – 1 l, pH 6.0), *B. cinerea* – on potato-glucose broth (potatoes – 200 g, glucose – 10 g, H_2O – 1 l, pH 6.0), while phytopathogenic bacteria *P. syringae, X. phaseoli* – were grown on Hottinger broth medium (Hottinger broth – 50 ml/l, peptone – 5 g/l, NaCl – 5 g/l, glucose – 10 g/l, H_2O – 1 l, pH 7.0) in Erlenmeyer flasks on the shaker (200 rpm) at 25–28°C for two days.

Comparative evaluation of antagonistic activity of metabolites contained in cell-free filtrate of isolated cultures was conducted using wells technique (Segy 1983), in four replications with two wells per plate. Cell-free filtrate was obtained by sieving cultural liquid of antagonistic species through membrane filter (pore diameter – 22 μm). Suppressive effect of antagonists on phytopathogens development was evaluated as zone diameter of its absence or poor growth around the wells containing bacterial metabolites.

Phytoprotective effect of antagonists was estimated by sequential treatment of lupine and soybean seeds with pathogens and cell-free filtrate of antagonistic bacteria (Frencel et al. 2000, Leshhenko et al. 1987). Lupine and soybean seeds were exposed to fungal spore suspension (1×10^6 conidia per 1 ml) or bacterial culture

(1×10^8 cfu per 1 ml) during 3 min and then treated with antagonist cell-free filtrate diluted 20 times in tap water. Seeds (10 seeds per plate in four replications) were incubated in Petri plates containing wet filters at 24°C. Disease development (%) on plant seedlings was calculated after seven days as follows: (no. of plants × score disease severity) / (total no. of plants × highest score) × 100. Disease severity was measured using scale 0–4, where 0 – no symptoms and 4 – dead seedlings.

Statistical processing of experimental data (Rokitsky 1973) was carried out by calculating arithmetical means and least significant difference at probability level 95%.

Results

After screening with wells technique the tested cultures of antagonists differed in activity with respect to specific phytopathogens (Table 1).

Microscopic examination showed that bacteria strains *B. subtilis* BIM B-377, *P. aurantiaca* S-1 and *S. griseoviridis* BIM B-264 inhibited spore germination, disrupted normal growth of germ tubes and mycelium formation in pathogens *B. cinerea, P. setosa* and *C. lupini*. In addition, strains *S. griseoviridis* BIM B-264, *P. fluorescens* BIM B-152 and *B. subtilis* M-1 possessed high bactericidal activity causing cell death of *P. syringae*.

Table 1

Comparative evaluation of antagonistic activity of examined cultures towards phytopathogens of grain legume crops in *in vitro* experiments

Strain	Width of antagonistic zones (mm)									
	Colletotrichum lupini		*Botrytis cinerea*		*Pleiochaeta setosa*		*Pseudomonas syringae*		*Xanthomonas phaseoli*	
	AG	PG	AG	PG	AG	PG	AG	PG	AG	PG
B. subtilis M-1	19±1.2	0	16±1.3	22±1.1	27±0.9	0	19±0.7	0	17±1.0	0
B. subtilis M-22	18±1.0	0	14±1.0	34±0.9	20±1.0	0	17±1.0	0	13±1.1	0
B. subtilis BIM B-377	29±1.4	0	27±0.7	8±1.2	24±1.4	0	0	0	11±1.0	0
B. pumilus BIM B-263	17±0.9	0	0	0	0	0	0	0	0	0
P. aurantiaca S-1	29±1.1	0	27±0.9	0	26±1.2	0	32±1.2	0	19±0.6	0
P. fluorescens BIM B-152	21±0.9	0	19±1.1	0	0	15.0±1.3	30±0.5	0	20±1.0	0
S. griseoviridis BIM B-264	25±1.0	0	30±1.2	0	36±1.1	0	33±1.2	0	25±1.2	0

AG – zone showing absence of phytopathogen growth, PG – zone of poor phytopathogen growth.

Revealed characteristics of antagonistic cultures were confirmed in experiments estimating plant protective potential on soybean and lupine seedlings (Tables 2 and 3).

Table 2

Efficiency of phytoprotective action of bacterial free-cell filtrate against soybean bacterial blight in *in vivo* experiment

Type of seed treatment	Disease development (%)
P. syringae **(control)**	**53**
P. syringae + *B. subtilis* BIM B-377	53
P. syringae + *B. subtilis* M-1	21
P. syringae + *P. fluorescens* BIM B-152	25
P. syringae + *S. griseoviridis* BIM B-264	25
$LSD_{0.05}$	1.4

Table 3

Efficiency of phytoprotective action of bacterial free-cell filtrate against grey mould and anthracnose of lupine in *in vivo* experiment

Type of seed treatment	Disease development (%)
Water	0
B. cinerea **(control)**	**100**
B. cinerea + *B. subtilis* M-22	9.0
B. cinerea + *B. subtilis* M-1	8.0
B. cinerea + *B. pumilus* BIM B-263	70.0
B. cinerea + *P. aurantiaca* S-1	64.0
B. cinerea + *P. fluorescens* BIM B-152	100.0
B. cinerea + *B. subtilis* BIM B-377	10.0
B. cinerea + *S. griseoviridis* BIM B-264	75.0
$LSD_{0.05}$	1.3
C. lupini **(control)**	**100**
C. lupini + *P. aurantiaca* S-1	48
C. lupini + *B. subtilis* BIM B-377	71
C. lupini + *P. fluorescens* BIM B-152	100
C. lupini + *S. griseoviridis* BIM B-264	40
$LSD_{0.05}$	1.9

Strain *B. subtilis* M-1 displaying high antimicrobial activity to fungal and bacterial pathogens of lupine and soybean was chosen for further studies.

To increase antagonistic activity of the selected strain, a series of experiments was performed to optimize composition of nutrient medium (including tests on carbon and nitrogen sources, and examination of their ratios in the Meynell medium). Antimicrobial activity of *B. subtilis* M-1 was correlated with diameter of growth suppression zones for phytopathogens *B. cinerea* and *P. syringae.* Effects of various carbon and nitrogen sources on activity of *B. subtilis* M-1 are presented in Tables 4 and 5.

Table 4

Effect of diverse carbon sources on antimicrobial activity of *Bacillus subtilis* M-1

Carbon source	Concentration (% vol.)	Titer		Width of antagonistic zones (mm)			
		total per 1 ml × 10^9	spores per 1 ml × 10^9	*B. cinerea*		*P. syringae*	
				AG	PG	AG	PG
Glucose	1.0	1.8±0.1	1.5±0.2	16±0.4	31±1.1	17±0.5	0
Sucrose	1.0	1.6±0.2	1.6±0.1	16±0.6	33±0.7	19±0.4	0
Molasses	2.0	2.3±0.3	2.1±0.1	17±0.3	33±0.8	20±0.4	0
Molasses	3.0	1.8±0.3	1.8±0.4	16±0.9	33±0.3	21±0.1	0
Molasses	4.0	1.6±0.2	0.16±0.01	13±1.1	36±0.4	21±0.2	0
Peas meal	2.0	2.5±0.1	2.2±0.2	15±0.4	28±0.7	20±0.7	0
Rye flour	2.0	2.5±0.1	2.3±0.2	15±0.5	32±0.7	20±1.1	0
Oat meal	2.0	2.0±0.2	1.9±0.1	17±1.2	30±1.1	20±0.8	0
Corn meal	2.0	1.9±0.2	1.9±0.4	14±0.4	34±0.9	19±0.4	0
Soya meal	2.0	2.9±0.4	2.0±0.3	15±0.3	32±0.6	16±0.5	0
Barley meal	2.0	1.9±0.1	1.6±0.1	15±0.4	36±0.4	19±0.4	0

AG – zone showing absence of phytopathogen growth, PG – zone of poor phytopathogen growth.

Table 5

Effect of diverse nitrogen sources on antimicrobial activity of *Bacillus subtilis* M-1 grown on Meynell medium containing molasses (2%)

Nitrogen source	Content in the medium (g/l)	Titer		Width of antagonistic zones (mm)			
		total per 1 ml × 10^9	spores per 1 ml × 10^9	*B. cinerea*		*P. syringae*	
				AG	PG	AG	PG
$(NH_4)_2SO_4$ (control)	**1.0**	**1.9±0.1**	**1.8±0.4**	**17±0.4**	**28±0.5**	**21±0.6**	**0**
NH_4NO_3	0.6	1.6±0.1	1.6±0.1	15±0.8	25±0.6	20±0.7	0
KNO_3	1.5	1.4±0.3	1.6±0.1	13±0.7	22±0.5	19±0.4	0
Urea	0.45	2.2±0.2	2.0±0.3	14±0.5	23±0.7	21±0.5	0
Yeast extract	5.0	1.9±0.1	1.8±0.2	18±1.0	40±0.1	23±0.4	0
$(NH_4)_2SO_4$ + yeast extract	1.0+0.025	1.9±0.2	1.8±0.2	18±0.6	39±0.3	23±0.7	0
Corn extract	5.0	2.0±0.2	1.9±0.1	14±0.3	26±0.7	21±0.6	0
Peptone	5.0	1.9±0.1	1.9±0.3	16±0.8	24±0.9	22±0.7	0
$LSD_{0.05}$		0.1	0.1	0.5	0.8	0.5	

AG – zone showing absence of phytopathogen growth, PG – zone of poor phytopathogen growth.

Both qualitative make-up and quantitative ratios of medium components proved key factors for expression of antagonistic activity. Changes in antagonistic activity of *B. subtilis* M-1 as a function of different C/N ratios were traced (Table 6). The most favourable medium for bacterial cell growth and expressing antimicrobial activity was Meynell medium containing 2% molasses as carbon

Table 6

Effect of C/N ratio on antimicrobial activity of *Bacillus subtilis* M-1 grown on Meynell medium containing yeast extract (25 mg/l)

Content of $(NH_4)_2SO_4$ (g/l)	C/N	Titer (spores per 1 ml × 10^9)	Width of antagonistic zones (mm)			
			B. cinerea		*P. syringae*	
			AG	PG	AG	PG
0.75*	27	1.7±0.2	15±0.5	25±0.7	23±0.6	0
1.0* (control)	**20**	**2.5±0.1**	**18±1.1**	**38±0.8**	**24±0.5**	**0**
1.5*	13	1.9±0.3	17±0.4	28±0.5	23±0.9	0
3*	7	2.2±0.2	16±0.7	32±1.0	21±1.0	0
0.5**	60	1.6±0.1	18±0.6	28±0.8	22±0.7	0
1**	30	1.3±0.3	17±0.6	24±0.5	21±0.5	0
2**	15	1.8±0.1	18±0.5	32±0.6	21±1.1	0
$LSD_{0.05}$		0.1	0.3	0.8	0.4	

AG – zone showing absence of phytopathogen growth, PG – zone of poor phytopathogen growth.
*Molasses at 2% concentration was used as carbon source for bacterial culture.
**Molasses at 3% concentration was used as carbon source for bacterial culture.

source enriched with 0.25% yeast extract. Antagonist culture grown on optimized medium resulted in enlarged zones showing lack of phytopathogen growth (width increase by 13% and 26% for *P. syringae* and *B. cinerea*, respectively) as compared to control without yeast extract.

Seed treatment with cell-free filtrate of *B. subtilis* M-1 culture grown on optimized medium increased biological efficiency towards bacterial blight and grey mould by 4% as compared to results recorded on control medium (Table 7).

Table 7

Efficiency of phytoprotective action of bacterial free-cell filtrate against soybean bacterial blight and lupine grey mould in *in vivo* experiment

Type of seed treatment	Disease development (%)	
	control medium	optimized medium
***P. syringae* (control)**	**53**	**53**
P. syringae + *B. subtilis* M-1	21	17
$LSD_{0.05}$	1.4	1.3
***B. cinerea* (control)**	**100**	**100**
B. cinerea + *B. subtilis* M-1	8.0	4.0
$LSD_{0.05}$	1.3	1.6

Discussion

The majority of antagonists considered were characterized by causing lack or poor growth of pathogens in zones, ranging from 11 to 36 mm width. Mechanism of antagonistic action was based on antibiosis displayed as fungicidal and bactericidal effects of agar diffused metabolites. Antifungal activity of *B. subtilis* and actinomycetes (*Streptomyces* sp.) was also demonstrated by Belgian and Brazilian researchers in laboratory tests against *B. cinerea* and *C. gloeosporioides* (Toure et al. 2004, Gomes et al. 2000).

Development of anthracnose on lupine seedlings may be controlled by strains *P. aurantiaca* S-1 and *S. griseoviridis* BIM B-264 (biological efficiency 52–60%). The microbial species slow down germination of pathogen spores and reduce infection rate on plant seedlings. Biological efficiency of lupine anthracnose control was higher than it was demonstrated by Chinese researchers on mango fruits (Chuang and Ann 1997), where bacterial antagonists (*B. subtilis* and *P. fluorescens*), yeast isolates *Pichia ohmeri* and *Sporobolomyces* sp. decreased anthracnose necrotic spots on average by 20–45%.

Strains *B. subtilis* BIM B-377, *B. subtilis* M-1 and *Bacillus* sp. M-22 exhibited high phytoprotective effect against lupine grey mould (90–92%) caused by their ability of inhibiting penetration of pathogen germ tubes into plant tissue. Our results are very close to data obtained in biological control of grey mould on apple fruits where 80% protection level was maintained over 10 days after antagonist application (Toure et al. 2004). *Bacillus subtilis* M-1, *P. fluorescens* BIM B-152 and *S. griseoviridis* BIM B-264 showed high phytoprotective effect against soybean bacterial blight (75–79%). It seems that *B. subtilis* M-1 strain may by highly attractive as a basis of biopreparation for control of lupine grey mould and soybean bacterial blight.

The production of antimicrobial metabolites by *B. subtilis* M-1 was increased by optimization of nutrient medium by with most favourable carbon and nitrogen sources. The favourable carbon sources were molasses, sucrose, glucose and starch rich flour which were already described in literature (Egorov 1986). Due to low cost and good progress in cells growth and antimicrobial activity molasses (2%) was selected as a main carbon source for further work.

Initial concentration of N-containing salts was calculated based on their content in Meynell control medium (1 g of nitrogen per litre), with carbon sources – 2% molasses. Supply of yeast extract into the medium stimulated antimicrobial activity of *B. subtilis* M-1. Since increase in activity was caused even by micro doses of yeast extract, a variant combining yeast extract with $(NH_4)_2SO_4$ was suggested for large scale cultivation to reduce production costs.

No only quality of carbon and nitrogen sources but also their quantitative ratio may contribute to high antagonistic activity of cell-free filtrate of *B. subtilis* M-1. Optimum C/N ratio for expression of antimicrobial activity was 20:1.

Summarizing, the following composition of nutrient medium for growth of antagonistic bacteria was defined (g/l): molasses – 20.0, K_2HPO_4 – 7.0, KH_2PO_4 – 3.0, $MgSO_4$ – 0.1, sodium citrate – 0.5, $(NH_4)_2SO_4$ – 1.0, yeast extract – 25 mg, H_2O – to 1 l.

Cell-free filtrate of *B. subtilis* M-1 grown on optimized medium inhibited bacterial blight and grey mould pathogens on seedlings of lupine and soybean by 83% and 96%, respectively.

Good prospects of biological method for control of lupine and soybean diseases were proved. Yet, antagonists differing in range of antimicrobial action and efficiency towards specific pathogens. That is why microorganisms showing a broad spectrum of antagonistic action and increased plant protective potential are required for comprehensive biological control of legume diseases.

Streszczenie

WPŁYW CZYNNIKÓW HODOWLANYCH W KULTURACH NA AKTYWNOŚĆ ANTYBIOTYCZNĄ CZYNNIKÓW OCHRONY BIOLOGICZNEJ W OCHRONIE ROŚLIN MOTYLKOWATYCH

Antybiotyczny potencjał bakterii wyizolowanych z gleby oraz pochodzących z kolekcji Instytutu Mikrobiologii Białoruskiej Państwowej Akademii Nauk badano *in vitro* oraz *in vivo* wobec patogenów łubinu i soi. Izolaty *Pseudomonas aurantiaca* S-1 i *Streptomyces griseoviridis* BIM B-264 okazały się aktywne wobec patogenu powodującego antraknozę łubinu (*Colletotrichum lupini*). Bakteria *Bacillus subtilis* M-1 wykazała kompleksowe właściwości ochronne na łubinie przeciwko *Botrytis cinerea,* sprawcy szarej pleśni, i na soi przeciwko *Pseudomonas syringae,* sprawcy bakteryjnej plamistości soi. Optymalizacja warunków wzrostu bakterii *B. subtilis* M-1 wpłynęła na lepszą biologiczną ochronę roślin motylkowatych przed czynnikami chorobotwórczymi.

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