The influence of spring barley extracts on *Pseudomonas putida* PCL1760

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Abstract. The aim of the present study was to evaluate the influence of different extracts (water, ethanol, enzymatic) from germinated seeds of spring barley on the development of *Pseudomonas putida* bacteria. The tests on optical density were performed to study the influence of different extracts on the development of model extracts. The analysis of the influence of different extracts from germinated seeds of spring barley on biological agent - *Pseudomonas putida* PCL1760, contained in biopreparations for crop protection showed that ethanol extract from Raushan variety of spring barley increased the resistance of *Pseudomonas putida* PCL1760 to the heat and osmotic stresses. All the types of extracts did not contribute to the increase of bacteria resistance to low temperatures. There was no significant difference established between the activity of spring barley extracts with vernalization and without vernalization. The materials of the study showed that ethanol extracts from spring barley can be used for the development of *Pseudomonas* spp. based on biopreparations for the enhancement of biocontrol microorganisms (BCMs) activity.

1 Introduction

Nowadays, biofungicides are frequently used for crop protection. Activity of biofungicides determined by their biological agent [1-4]. When the seeds are treated with biofungicides, the biocontrol microorganism is exposed to different chemical compounds. Seed germination is characterized by a number of compounds (exudates), many of them exert increased biological activity, including antimicrobial activity as well [5-13]. The influence of such exudates on the activity of biological agents is poorly understood. To reveal influence of seedling exudates, active compounds were extracted using different protocols and tested on biofungicide bioagents.

Currently, plant-derived physiologically active substances obtained by different extraction methods attract researchers' interest [10, 13-19]. In particular, amino acids and proteins obtained during plant extraction show significant growth stimulation effect during application for different agricultural crops [1]. The extracts demonstrate significant positive effect on the nitrogen consumption, optimization of C:N ratio as well as plant hormonal

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status [3]. Protein hydrolysates stimulate the growth and development of different microorganisms, including soil borne fungi and bacteria [4, 21].

Seed extracts are easy to prepare from the germinating seeds and roots are quite similar [18], and (iii) effective interaction between a number of biocontrol microorganisms and plants is observed at the stage of seed germination [7]

Different methods are used for extraction of plant-derived physiologically active substances. The most widespread methods are water, ethanol and enzymatic extraction [14]. The composition of an extract changes depending on the method of extraction. In particular, hot water is used primarily for extraction of water-soluble proteins (albumins, etc.), ethanol – for alcohol-soluble proteins (prolamins, etc.), and enzymes – for different amino acids. Plants can be used as a source of material for extracts that enhance BCMs resistance to stress. However, particular interest is drawn to spring barley.

In present research, we obtained compounds from barley seedlings using water, ethanol, and enzymatic extractions. These compounds were tested to determine their influence on growth of biocontrol agent *Pseudomonas putida* strain PCL1760.

2 Materials and Methods

The study was conducted at the Department of General farming, crop protection and breeding at the Kazan State Agricultural University.

Seed preparation.

Spring barley (*Hordeum distichon* L.) of variety Raushan was used for preparation of extracts. Before germination, the seeds were sterilized in 70% ethanol for 5 minutes. After drying out in sterile filter paper, the seeds (100 pcs x 3 replications) were placed in moist chambers (plant culturing units with sterile filter paper and sterile water). Germination was performed by two methods. The first method: germination was performed at +24°C (without vernalization) for 7 days. The second method: germination was performed at +24°C for 3 days and then the seeds were placed in a cooling chamber at + 8°C for 3 days (with vernalization). Seven days after, the seeds were dried out to 14% of moisture and milled in a lab mill. As a result, milled crop for extraction was obtained. Fined air-dried dark grey forest soil (humus content 5.2%) was used as a standard sample. One gram of ground materials was used for further extraction.

Water extraction: 85°C 10 ml of sterile distilled water was added to 1 g of the material. Time of extraction was 12 hours. After the extraction, supernatant fluid was filtered through a paper filter. 5 ml of extract was added sterile water to the volume of 10 ml. All the tests were replicated three times.

Ethanol extract: 25°C 10 ml of 95% ethanol was added to 1 g of the material. The time of extraction was 12 hours. After the extraction, supernatant fluid was filtered through paper filter. Ethanol was evaporated, and the residue was diluted in 10 ml of sterile water.

Enzymatic extract: 1 g of the material was added 1 mg of pancreatin (digestive enzyme, 10 000 UA) and 35°C 10 ml of distilled water. The time of extraction was 12 hours. After the extraction, supernatant fluid was filtered through a paper filter. 5 ml of extract was added distilled water to 10 ml. All the tests were replicated three times.

Tests variants: 1. water extract without vernalization - WEWV, 2. ethanol extract without vernalization - EEWV, 3. enzyme extract without vernalization - EnEWV, 4. water extract with vernalization - WEV, 5. ethanol extract with vernalization - EEV, 6. enzyme extract with vernalization - EnEV, 7. soil water extract - SWE, 8. soil ethanol extract - SEE, 9. soil Enzyme extract - SEnE.

Evaluation of the extracts activity towards *Pseudomonas putida*

The seeds were germinated in the growing medium BM containing 7.6 g/L $K_2HPO_4 \cdot 3H_2O$, 3 g/L KH_2PO_4 , 1 g/L $(NH_4)_2SO_4$ and 0.2 g/L $MgSO_4 \cdot 7H_2O$ with or without 2% glucose solution. This medium is a modified succinate Meyer medium [12].

Pseudomonas putida PCL1760 strain was used for the tests on the evaluation of influence of plant extracts on microorganisms. This strain was isolated from rhizosphere of avocado and have been proved to be an active colonizer of plant rhizosphere [16]. Moreover, PCL1760 is a biological control agent of tomato root rot caused by *Fusarium oxysporum*. It protects the plants using mechanism of competition for ecological niches [17]. The strain *P. putida* PCL1760 is a prototroph, i.e. it grows in mineral media and its optimal temperature for growth is 30°C. To evaluate its influence on PCL1760 strain, 50 µL of each extract was added to 150 µL of BM medium already mixed with a suspension of PCL1760 strain cells to the concentration of ($OD_{600} = 0.01$), which corresponds to 10^7 cells/ml. Each extract was tested in three replicates of for statistical processing of the data. The plates were incubated at 30 °C for evaluation of the extracts influence on the growth of PCL1760, and for evaluation of the extracts influence under salt, osmotic and cold stress. For evaluation of extracts influence on the strain PCL1760 under the heat stress, the plates were incubated under 37°C. Optical density of the culture was measured with iMark (Bio-Rad Laboratories, USA) at wavelength 60 nm.

3 Results

To define the test parameters, the authors obtained the growth curve for *P. putida* PCL1760 strain in the growing medium BM containing glucose (Fig. 1). After inoculation of 0.01, the culture achieved the density of 0.1 (OD_{600}), which corresponded to the density of 10^8 cells/ml of culture.

Fig. 1. Growth curve of the *P. putida* PCL1760 strain in the essential medium BM, containing 2% glucose at 30°C.

The results of the extracts influence in the growth of *P. putida* bacteria in minimum essential medium BM are presented in Table 1.

Table 1. Influence of extracts on the development of *P. putida* PCL1760 in the minimum essential medium BM without glucose (optical density at 600 nm), 2017.

Variant	1 st series of tests	2 nd series of tests	Mean	Significance of difference to control
Water control -				
WEWV	0.056	0.066	0.061	–
EEWV	0.294*	0.163*	0.229*	+
EnEWV	0.073	0.067	0.070	–
WEV	0.080	0.082	0.081	–
EEV	0.098*	0.094*	0.096*	+
EnEV	0.074	0.071	0.073	–
SWE	0.051	0.052	0.052	–
SEE	0.075	0.076	0.076	–
SEnE	0.052	0.056	0.054	–

Note: * – the difference as compared to the control is significant at P=0.05.

Strong stimulating effect on the development of *P. putida* in the growing medium without glucose was exerted by ethanol extract without vernalization and with vernalization. Water and enzymatic extracts did not influence on optical density, as well as all the other soil extracts.

Table 2. Influence of extracts on the growth of *P. putida* PCL1760 in the minimum essential medium BM without glucose (optical density at 600 nm, T=30° C), 2017.

Variant	1 st series of tests	2 nd series of tests	Mean	Significance of difference to control
Water control -	0.104	0.103	0.103	
WEWV	0.104	0.063*	0.083	–
EEWV	0.136*	0.126*	0.131*	+
EnEWV	0.071*	0.057*	0.064	–
WEV	0.102	0.074*	0.088	–
EEV	0.118	0.149*	0.134*	+
EnEV	0.091	0.094	0.093	–
SWE	0.064*	0.070*	0.067*	+
SEE	0.202*	0.134*	0.168*	+
SEnE	0.092	0.100	0.096	–

Note: * – the difference as compared to the control is significant at P=0.05.

Soil and spring barley seeds ethanol extract positively influenced on the development of *P. putida* in the medium with glucose. Water and enzymatic extracts did not influence on this parameter.

Cold stress test results brought some interesting results (Table 3).

Table 3. Influence of the extracts on the development of *P. putida* PCL1760 in the minimum essential medium BM with glucose (optical density at 600 nm, T=–20° C), 2017.

Variant	1 st series of tests	2 nd series of tests	Mean	Significance of difference to control
Water control -	0.076	0.087	0.082	
WEWV	0.064	0.066	0.065	–
EEWV	0.094	0.110	0.102	–
EnEWV	0.081	0.088	0.085	–

WEV	0.075	0.073	0.074	–
EEV	0.087	0.092	0.089	–
EnEV	0.087	0.092	0.090	–
SWE	0.066	0.072	0.069	–
SEE	0.101*	0.130*	0.115*	+
SEnE	0.065	0.099	0.082	–

Note: * – the difference as compared to the control is significant at $P=0.05$.

Positive changes in parameters of optical density were observed only when soil ethanol extracts were used, while in other test variants no significant difference was observed as compared to the control.

To model the influence of the heat stress, *P. putida* was cultivated at + 37° C (Table 4).

Table 4. Influence of the extracts on the development of *P. putida* PCL1760 in the minimum essential medium BM with glucose (optical density at 600 nm, $T=+37^{\circ}\text{C}$), 2017.

Variant	1 st series of tests	2 nd series of tests	Mean	Significance of difference to control
Water - control	0.072	0.078	0.075	
WEWV	0.084	0.063	0.073	–
EEWV	0.096	0.180*	0.138*	+
EnEWV	0.071	0.063	0.067	–
WEV	0.072	0.074	0.073	–
EEV	0.078	0.167*	0.122*	+
EnEV	0.081	0.088	0.085	–
SWE	0.064	0.064	0.064	–
SEE	0.103	0.134*	0.119*	+
SEnE	0.066	0.075	0.071	–

Note: * – the difference as compared to the control is significant at $P=0.05$.

Under the conditions of the heat stress, spring barley seeds ethanol extracts had significant positive effect on the growth of *P. putida*. There was no significant difference between barley seeds extracts without vernalization and with vernalization.

The results of the tests on the extracts influence on the development of the bacteria under osmotic stress are shown in Table 5.

Table 5. Influence of the extracts on the development of *P. putida* PCL1760 in the minimum essential medium BM in the presence of glucose (optical density at 600 nm, $T=+30^{\circ}\text{C}$, 3% solution of NaCl), 2017.

Variant	1 st series of tests	2 nd series of tests	Mean	Significance of difference to control
Water - control	0.081	0.081	0.081	
WEWV	0.083	0.119*	0.101	–
EEWV	0.133*	0.206*	0.170*	+
EnEWV	0.100	0.118*	0.109	–
WEV	0.102	0.164*	0.133*	+
EEV	0.090	0.138*	0.114*	+
EnEV	0.122*	0.140*	0.131*	+
SWE	0.080	0.108	0.094	–
SEE	0.125*	0.154*	0.139*	+
SEnE	0.064	0.104	0.084	–

Maximum values of the optical density parameter were observed in the variant with spring barley seeds extract without vernalization. Positive results were obtained in the tests of all the seeds extracts with vernalization and soil ethanol extracts.

4 Conclusions

The present study showed that the influence of different groups of extracts on biological agent *P. putida* PCL1760 is diverse. The most significant positive effect had ethanol extracts, which is explained by the presence of high concentrations of amino acid proline that is a well-known protectors of microorganisms from the stress impact under stress conditions [20]. It can be suggested that the expression of this amino acid by the germinating seeds allows for biological agent activity maintenance under conditions of abiotic stresses. The observed effect can be used as a basis for the development of specialized adaptogene drugs for the maintenance of biofungicide activity under unfavorable conditions. The development of this direction will allow the producers to increase the effectiveness of crops bioprotection from the diseases.

Raushan spring barley seeds ethanol extracts have positive effect on the increase of *P. putida* PCL1760 resistance to heat and osmotic stresses, but do not influence on the resistance to low temperatures. No differences were observed between the anti-stress activity of ethanol seed extracts without vernalization and with vernalization.

5 Recommendations

Application of ethanol extracts from germinated spring barley seeds can significantly increase the resistance of biocontrol microorganisms (BCMs) contained in biopesticides to the influence of unfavorable abiotic factors.

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