

Secondary screening of strains of antagonists to a *Phoma* pathogen on sunflower

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Abstract. There are presented data of the first stage of the secondary screening of antagonist strains from a collection of the biological methods laboratory of V.S. Pustovoit All-Russian Research Institute of Oil Crops to the aggressive isolate of *Phoma macdonaldii* Boerema infecting sunflower. We used methods of ‘agar blocks’ and ‘perforated Petri dishes’. As a result of a screening of 55 antagonist strains to *Phoma macdonaldii* pathogen we selected 17 promising strains-producers of microbiological preparations having biological efficiency exceeding 40.0 %. In control variant without seed treatment sunflower seedlings infection was up to 76.2 %, and maximal biological efficiency of laboratory samples from fungi-producers were determined for a strain M-24 *Penicillium* sp. (73.8 %); from bacteria of a genus *Bacillus* – for a strain D-10 *Bacillus* sp. (74.2%); from bacteria of a genus *Pseudomonas* – for a strain Sgc-1 *Pseudomonas* sp. (73.8 %). Maximal colonizing activity were noted for fungi-producers M-24 *Penicillium* sp. (85.7 %) and Xk-1 *Ch. olivaceum* (71.4 %), as well as for bacteria of a genus *Bacillus* – 5B-1 *B. subtilis* (71.4 %) and a genus *Pseudomonas* – Oif 2-1 *Pseudomonas* sp. and 14-3 *P. chlororaphis* (71.4 %) against *Phoma* infection in control of 70.0 %.

1 Introduction

Last years the world agricultural production aimed to wider usage of elements of organic farming. Thus, development of biotechnologies of creation and application of the modern competitive microbiological preparations for agriculture became a high-priority task [1-6]. The biological methods laboratory of the V.S. Pustovoit All-Russian Research Institute of Oil Crops is one of the few ones in Russia which develops microbiological protection preparations for oil crops. As a base of a worked out by us conception of task-oriented development of microbiological preparations for protection of sunflower and other agricultural crops against diseases we use a search of antagonist strains which are safety for people, non-phytotoxic, possessing high activity in widely varying environments against a complex of pathogens, being of polyfunctional action. As a result of long-term researches we have created a collection of promising strains of fungi and bacteria-antagonists to a

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wide range of pathogens infecting oil and other agricultural crops. We studied protection actions of microbiological preparations for sunflower, soybean and rapeseed against pathogens of white rot, phomopsis, fusariose and downy mildew. We are continuing researches on microbiological protection of oil flax against fusariose pathogen [7, 8].

The subject of the present research was selected as harmfulness of Phoma rot (*Phoma macdonaldii* Boerema) on sunflower in the world [9-14] and last years in Russia [15, 16] has become more and more serious.

There are no released and registered microbiological preparations against the Phoma rot on sunflower in Russia.

Two bacterial bio-preparations were registered against the Phome rot on root crops: on sugar beet – BFTIM KS-2, L based on the bacterium *Bacillus amyloliquefaciens* and on carrots – Fitosporin-M, L based on *Bacillus subtilis*. We did not find in the literature information on the development of biological protection measures against the Phoma rot on sunflower, except for testing biological preparations against the Phoma rot on tea [17].

We isolated an aggressive isolate of the pathogen and conducted a primary screening of 65 antagonist strains from the laboratory collection. At the first stage of screening, we tested fungal strains represented by genera *Trichoderma*, *Penicillium*, *Chaetomium*, *Trichothecium*, *Sordaria*, *Talaromyces*, *Aspergillus*, *Metarhizium* and a class *Basidiomycetes*, and bacteria from genera *Bacillus* and *Pseudomonas*.

As a result of the primary screening to *Phoma macdonaldii* on sunflower, we selected 55 strains having one or more types of mechanism of action from the collection of antagonists.

In connection with the search among fungi and bacteria for promising strains-producers of microbiological preparations to protect sunflower from *Phoma macdonaldii*, an important aspect is not only their manifestation of antifungal activity *in vitro*, but also a protective and colonizing effect for seeds and seedlings in laboratory and fields. Therefore, the next step in our work was to identify, among the selected antagonists, the most active strains-producers of microbiological preparations having a protective and colonizing effect against *Phoma macdonaldii* in conditions of artificial infection in the laboratory in a moist chamber (the first stage of the secondary screening).

2 Materials and methods

To conduct the first stage of the secondary screening based on the promising strains, we accumulated laboratory samples of microbiological preparations in a preparative form 'liquid culture' (LC). Antagonists were cultivated on liquid nutrient mediums: fungi – on Rudakov's, bacteria of a genus *Bacillus* – Tylon's-3, of a genus *Pseudomonas* – King's B. Deep cultivation were done in nutator with a spin speed 200 rpm, in Erlenmeyer flasks, of a volume 750 ml, at medium volume 150 ml. Amount of colony-forming units (CFU) in a liquid culture (LC) of microbiological preparations was determined by a Koch's method.

To determine the protective effect of laboratory samples of microbiological preparations in a moist chamber, we used the method of "agar blocks" developed by Zaichuk (1983). For this, sunflower seeds of the variety R-453 we treated manually with the laboratory samples of the microbiological preparations in Erlenmeyer flasks, in circular rotational movements, with a preparation application rate of 3.0 liter per a ton and a working fluid rate of 10 liter per a ton. After a day, the treated seeds were laid out in a germinator on moistened filter paper with a layer of cotton, an agar block with pathogen mycelium, 3.0 × 4.0 mm in size, was applied on top and a moist chamber was created. Empirically, we have established the optimal nutrient medium is potato-sucrose agar (KSA) and the pathogen cultivation period is 10 days. As a control we used untreated seeds infected with a pathogen. Accounting for

the infection level on sunflower seedlings was carried out in seven days after infecting with a pathogen.

The colonizing activity of the strains that showed the best protective effect by the method of “agar blocks” we determined by the method of “perforated Petri dishes” using the modified Antonova and Saukova’s method (2005). For this, two-day sunflower seedlings obtained by germinating seeds treated with the laboratory samples of the microbiological preparations were placed in the holes of a perforated Petri dish so that the tips of the roots touched the colony of pathogenic isolate of *Phoma macdonaldii*, the exposure time was 3 hours at a temperature of +25.0 °C. In the control variant, the tips of the sunflower roots were immersed into filter paper moistened with sterile water. Then the infected and control seedlings were kept in a moist chamber for four days.

The infection degree of sunflower seedlings by the pathogen of *Phoma macdonaldii* was assessed according to the scale we developed:

0 points – healthy seedlings

1 point – darkening of the root tip, intensive formation of lateral roots;

2 points – darkening of the root by a third or to the middle, but the intensive formation of lateral roots;

3 points – overwinding of rot in the middle of the root or between the hypocotyl and the root, but the intensive formation of lateral roots;

4 points – root rotting to the middle or overwinding of rot between the hypocotyl and the root, lateral roots are poorly developed;

5 points – complete root rotting, lateral roots are poorly developed or absent;

1–3 – viable seedlings;

4–5 – non-viable seedlings.

Accounting of the biological effectiveness of the laboratory samples when determining the colonizing activity was conducted according to the spread of the disease, taking into account the infection degree of sunflower seedlings by the *Phoma macdonaldii* pathogen in a day after infection.

An experiment included three repetitions. The biological effectiveness of the laboratory samples of the microbiological preparations was determined by the formula:

$$C = 100 (a - b)/a, \quad (1)$$

where: C – biological activity, %;

a – amount of diseased plants in a control;

b – amount of diseased plants in a variant.

3 Results and discussing

As a result of the evaluation of the laboratory samples of the microbiological preparations in the preparative form of LC based on 55 strains of fungi and bacteria-antagonists isolated in the initial screening, we selected 17 promising strains-producer that showed biological efficacy exceeding 40.0% in conditions of high artificial infection of sunflower with *Phoma macdonaldii* in the laboratory using the “agar block” method. Ten strains-producers of the microbiological preparations demonstrated the maximum efficiency against the pathogen in seven days after infection (Table. 1).

Among 23 fungal antagonist strains, we found out the high biological efficiency of the laboratory samples for seven strains, while the maximum was found in M-24 *Penicillium* sp. (73.8 %) and Xk-1 *Chaetomium olivaceum* (67.2 %) under artificial infection of sunflower seedlings with *Phoma macdonaldii* in the control without treatment an average of 76.2 %.

Table 1. Efficiency of seed treatment of sunflower variety R-453 with the laboratory samples of the microbiological preparations containing fungi or bacteria-producers against *Phoma macdonaldii* in lab conditions under artificial infection using a method of 'agar blocks'

| Variant | Titer, CFU per g | Biological efficiency, % |
|--|----------------------|--------------------------|
| Control, without treatment | - | 76.2* |
| Fungi-producers | | |
| M-24 <i>Penicillium</i> sp. | 7.8x10 ⁹ | 73.8 |
| Xk-1 <i>Chaetomium olivaceum</i> | 4.0x10 ⁷ | 67.2 |
| T-2 <i>Trichoderma</i> sp. | 2.8x10 ⁹ | 60.6 |
| Pbc-1 <i>P. brevicompactum</i> | 9.0x10 ⁹ | 60.6 |
| Tt-1 <i>Talaromyces trachispermus</i> | 6.8x10 ⁷ | 60.6 |
| Pr-1 <i>Penicillium rugulosum</i> | 6.0x10 ⁹ | 47.5 |
| A-1 <i>Basidiomycetes</i> | 5.4x10 ⁷ | 47.5 |
| Bacteria-producers | | |
| D-10 <i>Bacillus</i> sp. | 3.8x10 ¹⁰ | 74.2 |
| 5-3 <i>Bacillus</i> sp. | 3.0x10 ¹⁰ | 60.6 |
| B-5 <i>B. licheniformis</i> | 4.8x10 ¹⁰ | 60.6 |
| 1a <i>B. polymyxa</i> | 5.4x10 ¹⁰ | 54.0 |
| 11-1 <i>Bacillus</i> sp. | 2.0x10 ¹⁰ | 47.5 |
| 5B-1 <i>B. subtilis</i> | 5.0x10 ¹⁰ | 41.2 |
| K 1-2 <i>Bacillus</i> sp. | 4.4x10 ¹⁰ | 41.2 |
| Sgc-1 <i>Pseudomonas</i> sp. | 2.8x10 ¹¹ | 73.8 |
| 14-3 <i>P. chlororaphis</i> | 3.0x10 ¹¹ | 47.5 |
| Oif 2-1 <i>Pseudomonas</i> sp. | 1.8x10 ¹¹ | 40.9 |
| *Infection of sunflower seedlings with <i>Phoma</i> pathogen in control without treatment, distribution, % | | |

Among 24 bacterial strains-producers of the genus *Bacillus*, the high efficiency of laboratory samples against the *Phoma macdonaldii* pathogen was also found for seven strains, with the maximum in D-10 *Bacillus* sp. (74.2 %), 5-3 *Bacillus* sp. and B-5 *B. licheniformis* (60.6 %).

Three strains from eight bacterial antagonist strains of the genus *Pseudomonas* had the high efficiency of the laboratory samples against *Phoma macdonaldii*, while the maximum was established in Sgc-1 *Pseudomonas* sp. (73.8 %).

We determined colonizing activity of strains-producers that showed the best protective effect by the method of "agar blocks" in a day after infection. It should be emphasized we infected only the tips of the roots of the two-day-old sunflower seedlings which touched the pathogen colony for three hours. Thus, infecting the roots that have grown from the treated seeds, we can view the colonizing activity of the strains-producers that is their ability to move beyond the growing root.

It is important to say, that already in a day after infection with *Phoma macdonaldii* we saw more intense root damage in 70.0 % of the seedlings of the control variant (2-3 points). Whereas in the best variants of strains-producers, the roots were 80.0-90.0 % healthy and only 10.0-20.0 % showed only darkening of the tip or third of the root (0-2 points) (Table 2).

Among the fungal strains, the fungi-producers M-24 *Penicillium* sp. and Xk-1 *Ch. olivaceum* demonstrated maximal colonizing activity and protection effect. When seedlings of sunflower variety R-453 were infected with *Phoma macdonaldii* up to 70.0 %, biological effectiveness of the best fungal strains-producers was 71.4–85.7 %. Among bacterial strains from a genus *Bacillus*, the strain 5B-1 *B. subtilis* had the highest biological effectiveness

(71.4 %). The strains Oif 2-1 *Pseudomonas* sp. and 14-3 *P. chlororaphis* appeared to be the most effective (71.4 %) among all of a genus *Pseudomonas*.

Table 2. Influence of treatment of sunflower seeds of variety R-453 with the laboratory samples of the microbiological preparations on a level of seedlings infection with *Phoma macdonaldii* in lab conditions under artificial infection using a method of ‘agar blocks’

| Variant | Titer, CRU per g | Infected seedlings, % | | | | | Biological efficiency, % |
|--|----------------------|--|------|------|------|---------|--------------------------|
| | | Infection level of the main root, points | | | | Totally | |
| | | 0 | 1 | 2 | 3 | | |
| Control, without treatment | - | 0 | 0 | 30.0 | 40.0 | 70.0* | - |
| Fungi-producers | | | | | | | |
| M-24 <i>Penicillium</i> sp. | 7.8x10 ⁹ | 90.0 | 0 | 10.0 | 0 | 10.0 | 85.7 |
| Xk-1 <i>Chaetomium olivaceum</i> | 4.0x10 ⁷ | 80.0 | 0 | 20.0 | 0 | 20.0 | 71.4 |
| Pr-1 <i>Penicillium rugulosum</i> | 6.0x10 ⁹ | 70.0 | 0 | 0 | 30.0 | 30.0 | 57.1 |
| T-2 <i>Trichoderma</i> sp. | 2.8x10 ⁹ | 60.0 | 30.0 | 0 | 10.0 | 40.0 | 42.9 |
| Tt-1 <i>Talaromyces trachispermus</i> | 6.8x10 ⁷ | 60.0 | 10.0 | 10.0 | 20.0 | 40.0 | 42.9 |
| Bacteria-producers | | | | | | | |
| 5B-1 <i>B. subtilis</i> | 5,0x10 ¹⁰ | 80.0 | 0 | 20.0 | 0 | 20.0 | 71.4 |
| 5-3 <i>Bacillus</i> sp. | 3,0x10 ¹⁰ | 70.0 | 0 | 0 | 30.0 | 30.0 | 57.1 |
| B-5 <i>B. licheniformis</i> | 4,8x10 ¹⁰ | 70.0 | 10.0 | 10.0 | 10.0 | 30.0 | 57.1 |
| 11-1 <i>Bacillus</i> sp. | 2,0x10 ¹⁰ | 70.0 | 20.0 | 10,0 | 0 | 30.0 | 57.1 |
| Oif 2-1 <i>Pseudomonas</i> sp. | 1,8x10 ¹¹ | 80.0 | 0 | 10.0 | 10.0 | 20.0 | 71.4 |
| 14-3 <i>P. chlororaphis</i> | 3,0x10 ¹¹ | 80.0 | 0 | 10.0 | 10.0 | 20.0 | 71.4 |
| Sgc-1 <i>Pseudomonas</i> sp. | 2,8x10 ¹¹ | 70.0 | 20.0 | 10.0 | 0 | 30.0 | 57.1 |
| *Note: a degree of sunflower seedlings infection, points: 0 – healthy root; 1 – darkening of root tips, formation of lateral roots; 2 – darkening of root by one third or to the middle, formation of lateral roots; 3 – overwinding of rot in the middle part of a root or between hypocotyl and root, formation of lateral roots | | | | | | | |

All the selected promising antagonist strains will be evaluated in the following screening stages. We will determine the growth-stimulating activity for a sunflower crop and the protective effect of laboratory samples of the microbiological preparations under artificial infection with a pathogen in the soil in laboratory and field. We plan to develop the microbiological preparations in the form of “wetable powder” with a prolonged shelf life on a base of the strains-producers that showed the maximum protective and colonizing effects, and having a growth-stimulating activity.

4 Conclusion

As a result of the first stage of the secondary screening of 55 antagonist strains to *Phoma macdonaldii* on sunflower we selected 17 promising strains-producers of

microbiological preparations having shown biological activity to infected seedlings more than 40.0 %.

Maximal efficiency of the laboratory samples were stated for the strains: from fungi-producers – M-24 *Penicillium* sp. (73.8 %); from bacteria of a genus *Bacillus* – D-10 *Bacillus* sp. (74.2 %); from bacteria of a genus *Pseudomonas* – Sgc-1 *Pseudomonas* sp. (73.8 %) comparing to a control variant without seed treatment with sunflower seedlings infection of 76.2 %.

Fungi-producers M-24 *Penicillium* sp. (85.7 %) and Xk-1 *Ch. olivaceum* (71.4 %), as well as bacteria from a genus *Bacillus* – 5B-1 *B. subtilis* (71.4 %) and a genus *Pseudomonas* – Oif 2-1 *Pseudomonas* sp. and 14-3 *P. chlororaphis* (71.4 %) demonstrated maximal colonizing activity and protection effect compared to a control variant without seed treatment with sunflower seedlings infection of 70.0 %.

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