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Biological control of leaf disease of barley with *Bacillus* strain

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A new *Bacillus amyloliquefaciens* subsp. *plantarum* strain IMV B-7404 was selected by an *in-vitro* agar plate assay as a potential biological control agent. The strain strongly inhibited the growth of 12 important plant pathogenic fungi.

In growth chamber assays, the severity of spot blotch of barley decreased when culture filtrate was applied on leaves or was introduced into the plant growth medium before fungal inoculation. Apparently, the metabolites of strain IMV B-7404 play a role in disease prevention, which indicates that the strain can stimulate induced systemic resistance (ISR). When the cell suspension was sprayed onto leaves, the disease severity depended on environmental and, probably, some other conditions.

We suggest that effective biological control by *Bacillus*-based products depends on spraying leaves with the cell suspension to co-ordinate with the process of plant infection by the pathogen. The ecological behaviour and biology of both the antagonist and the target pathogen are the key elements in enhancing the efficacy of biocontrol products. It is necessary to know the stage of infection that is most vulnerable. This information needs to be identified individually for each pathosystem and in relation to environmental conditions.

Keywords: *Bacillus*, antagonism, biological control, barley, *Bipolaris sorokiniana*

INTRODUCTION

In recent years much attention has been paid to the use of biological agents for plant disease control. Important among these diseases are those caused by fungi in the genus *Bipolaris* (syn. *Helminthosporium, Drechslera*). *Bipolaris sorokiniana* (Sacc.) Shoemaker (teleomorph *Cochliobolus sativus* Ito & Kuribayashi) in particular causes spot blotch, common root rot and "black point" of barley, affecting, respectively, leaves, roots, and seed. The distribution of *Bipolaris* disease of barley in Ukraine has reached catastrophic proportions that sometimes affect 75.2% of crops (Mykhailenko, 2005). Chemical fungicides are used for protection of barley. However, since barley is a dietary product, the potential of biological control of barley diseases with antagonistic bacteria is of essential interest.

Bacillus spp. are aerobic endospore-forming bacteria ubiquitous in agricultural systems. These bacteria have a number of valuable traits and are a potential major source of microbial biopesticides

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for several reasons. First, *Bacillus* spp., such as *B. subtilis*, are well-studied organisms. Second, this species is recognized as non-pathogenic and safe to humans. Third, *Bacillus* spp. have the capacity to produce spores which are extremely resistant to unfavourable environmental conditions (Cawoy et al., 2011). These bacteria are known to produce a variety of antimicrobial compounds. *Bacillus* spp. are also of great interest because of the diversity of their modes of action. *Bacillus* strains have been successfully used for crop protection, and numerous products are currently commercialized throughout the world.

A new strain of *B. amyloliquefaciens* subsp. *plantarum* was selected among strains of Ukrainian collection of microorganisms that showed a suppressive activity on *Fusarium* root rot and eyespot of wheat (Kriuchkova et al., 2015). Here we describe the effects of *B. amyloliquefaciens* subsp. *plantarum* strain IMV B-7404 on spot blotch of barley when applied to leaves as a cell suspension or metabolites. Additional objectives of the study were to determine the antifungal spectrum and to indicate the incubation conditions suitable for disease inhibition.

MATERIALS AND METHODS

Microbial strains and culture media

The microbial strain *B. amyloliquefaciens* subsp. *plantarum* IMV B-7404, used in this study, was selected from among the Ukrainian collection of microorganisms of the Zabolotny Institute Microbiology and Virology National Academy of Science of Ukraine (IMV). The well documented strain *B. amyloliquefaciens* IMV B-7100, the active ingredient of the commercial biofungicide "Phytosporin", was used for comparison.

The *Bacillus* strains were grown in flasks (750 ml) with a liquid nutrient medium containing dextrose – 2%, $(NH_4)_2HPO_4$ – 4.75%, sodium citrate – 1.29%, KH_2PO_4 – 9.6%, NaOH – 0.18% (pH 6.5–7.0) on a shaker at 200 rpm for 18–24 h at 37°C. The culture filtrate was obtained by centrifugation at 8000–9000 g. The precipitate was washed with sterile tap water and cell suspensions were prepared with a titer of 10^9 CFU ml^{-1} (colony forming units per mL).

Cultures of fungal phytopathogens were obtained from the working collection of the Plant Pathology Department of the National University of Life and Environmental Science of Ukraine. Potato dextrose agar (PDA) was used for subculture and experiments.

Fungal inhibition assay

The inhibition of hyphal growth of 12 species of plant pathogenic fungi by strain IMV B-7404 was assayed on PDA in Petri dishes as described by Keel et al. (Keel et al., 1992). The bacterial strain was spotted in the centre of each dish and these cultures were incubated at 25°C. One day later an 8-mm agar disk of each fungus was placed at the edge of the plate. After 4–14 days the growth of fungi on each test plate was compared with growth on a control plate. Inhibition was expressed as the percentage of inhibition obtained with that on a control plate. The fungi that were inhibited by more than 30% were identified as sensitive to the strain (Milner et al., 1996).

Bioassay for biocontrol activity against spot blotch on barley leaves

Barley seedlings used in the experiment were grown in plastic pots (2.5 cm diameter × 6 cm high) until 1–2 leaves had developed. The culture filtrate or the cell suspension was individually spread over the leaves with a hand-held sprayer. The culture filtrate was also inoculated into the substrate (sterile sand) in which the barley was grown and any suppressive effect was compared with that where the leaves were treated. The applications were made three days before or three days after pathogen inoculation.

For pathogen inoculation, barley seedlings were misted with conidial suspension (10^4 ml^{-1}) of *B. sorokiniana* using a hand-held sprayer. Immediately following inoculation, the plants were moved into a dew chamber to facilitate fungal penetration, and 18 h later transferred to the greenhouse conditions at 25°C for disease development. Disease symptoms were scored at seven days after inoculation, and disease ratings were expressed on the basis of the diseased leaf area and the lesion type using a 1 to 5 disease severity scale: 1 – no infection or less than 2% of

the leaf area infected with small brown specks less than 1 mm in diameter; 2 – less than 10% of the leaf area infected with brown spot lesions with grey to white centres, about 1 to 3 mm in diameter; 3 – average, of about 25% of the leaf area infected with brown spot lesions with grey to white centres, about 1 to 3 mm in diameter; 4 – average, of about 50% of the leaf area infected with typical spindle-shaped lesions, 3 mm or longer with necrotic grey centres and water-soaked or reddish brown margins, with little or no coalescence of lesions; 5 – more than 75% of the leaf area infected with coalescing spindle-shaped lesions (De Vleesschauwer et al., 2010).

For experiments to test different temperature conditions for their influence on the performance of the biocontrol agent, after fungal inoculation the plants were transferred into greenhouse conditions at 17°C. After seven days the pots were transferred to a growth chamber at 25°C to stimulate disease development. Disease symptoms were scored at 14 days after inoculation, and disease ratings were expressed on the basis of the diseased leaf area and the lesion type using a 1 to 5 disease severity scale as described above.

Data analysis

The experiments with strain IMV B-7404 were conducted at least twice, with similar results, and the data from single experiments are presented here. In all experiments, treatments were arranged in a randomized complete block with six replications. All means of the data were tested by the least significant difference (LSD) at $P = 0.05$.

RESULTS

Antifungal spectrum of strain IMV B-7404

B. amyloliquefaciens subsp. *plantarum* strain IMV B-7404 had a significant inhibitory effect on the growth of all fungal pathogens in comparison with their respective controls (Table 1).

The presence of an inhibition zone without a direct contact indicated the production of some diffusible non-volatile metabolites by strain IMV B-7404, particularly antibiotics and cell wall degrading enzymes such as chitinases and glucanases that break down polysaccharides, chitins and β-glucanase, thereby destroying the cell wall integrity (Tapwal et al., 2011). Similar findings on the interaction of *Bacillus* species and their derivatives with fungal pathogens were recorded by McKeen et al. (McKeen et al., 1986), Milner et al (Milner et al., 1996) and others. Among the fungi tested, the most sensitive were the slow-growing species which, however, are important causal agents of cereal diseases: common root rot, "black point" and spot blotch (*B. sorokiniana*), eyespot (*O. yallundae*), take-all (*G. graminis*), and sharp eyespot (*Rh. cerealis*).

Fusarium species were less sensitive but, nevertheless, the use of the *Bacillus*-based biofungicides against barley diseases is very promising.

Spot blotch inhibition by strain B-7404

Figure 1 shows the effects of the culture filtrate and the cell suspension of strain IMV B-7404 on the severity of spot blotch on barley

Table 1. ***In-vitro*** **growth inhibition of fungal phytopathogens by** ***B. amyloliquefaciens*** **subsp.** ***plantarum*** **strain IMV B-7404**

Fungi	Inhibition, %
Cochliobolus sativus (*Bipolaris sorokiniana*)	55.7
Oculimacula yallundae	56.7
Gaeumannomyces graminis	52.0
Rhizoctonia cerealis (*Ceratobasidium cereale*)	47.3
Gibberella zeae (*Fusarium graminearum*)	33.3
Fusarium poae	38.3
Gibberella fujikuroi (*Fusarium moniliforme*)	37.3
Nectria haematococca (*Fusarium solani*)	21.7
Fusarium oxyporum	31.7
Bipolaris maydis	55.6
Botrytis cinerea (*Botryotinia cinerea*)	60.0
Rhizoctoniasolani (*Thanatephorus* sp.)	45.7

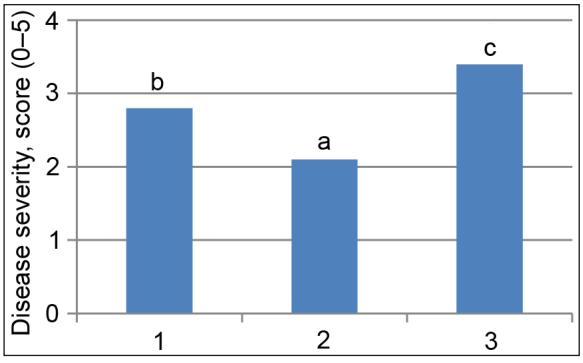

Fig. 1. Spot blotch on barley leaves treated with components of shake-cultures of *B. amyloliquefaciens* subsp. *plantarum* strain IMV B-7404. 1 – no treatment (control), 2 – culture filtrate, 3 – cell suspension. Means with the same letter are not significantly different at $P = 0.05$

leaves. Development of spot blotch lesions on the leaves was inhibited only by treatment with the culture filtrate. Washed bacterial cells not only failed to inhibit the disease but also promoted symptom development.

The culture filtrate of IMV B-7404 inhibited spot blotch when applied either by spraying onto leaves or by introduction into the plant growth medium (Fig. 2). Applying the culture filtrate to the growth medium, so that active substances were in contact with the root, tended to suppress the disease even more (non-significantly) than when the culture filtrate was applied to leaves.

This suggests that the metabolites of strain IMV B-7404 can stimulate induced systemic resistance in barley seedlings. Disease suppression was evidence of induced resistance rather than of direct inhibition of the pathogen.

Whilst spot blotch symptoms were significantly suppressed on the leaves sprayed with the culture filtrate before inoculation with the pathogen, its application after pathogen inoculation, however, did not suppress the severity of the disease but tended to increase it (Fig. 3).

Disease was not inhibited when the cell suspension was sprayed onto the leaves before and after fungal inoculation (results not shown).

Symptom development was hampered when the barley seedlings were grown at 17°C rather than at 25°C. The disease was stimulated seven days after inoculation by transferring the pots to a growth chamber at 25°C. As a result, the first symptoms were detected 14 days after inoculation. The inhibition of disease severity was detected when the treatments were applied after fungal inoculation (Fig. 4). There was sig-

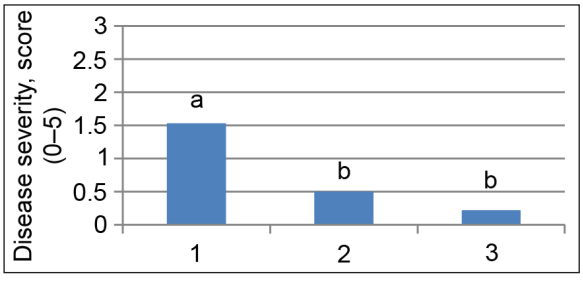

Fig. 2. Spot blotch on barley leaves treated with the culture filtrate of *B. amyloliquefaciens* subsp. *plantarum* strain IMV B-7404. Treatments: 1 – no treatment (control), 2 – sprayed onto leaves; 3 – introduction into growth medium (sterile sand)

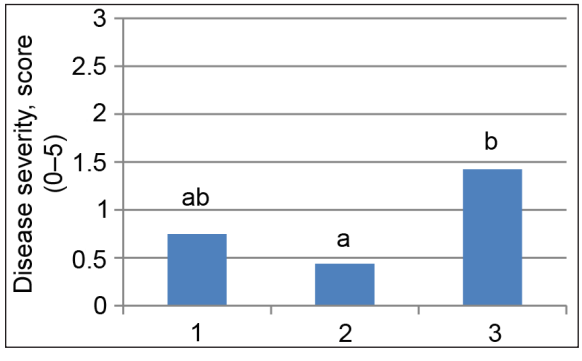

Fig. 3. Spot blotch on barley leaves treated with the culture filtrate of *B. amyloliquefaciens* subsp. *plantarum* strain IMV B-7404 before (2) and after (3) fungal inoculation. 1 – no treatment (control)

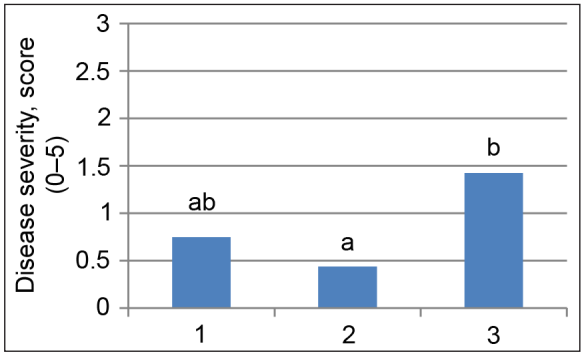

Fig. 4. Spot blotch on barley leaves treated with components of shake-cultures of *B. amyloliquefaciens* subsp. *plantarum* strain IMV B-7404 after fungal inoculation at 17°C. Treatments: 1 – no treatment (control); 2 – cell suspension; 3 – culture filtrate

nificant inhibition only by the culture filtrate, although the cell suspension also tended to suppress the disease. There was no inhibition when treatment was applied before fungal inoculation (results not shown).

This result suggests differences in effects depending on the stages of pathogen infection. Stronger effects may become manifest later when disease development is slow (at 17°C), coinciding with the active stage of pathogen development.

DISCUSSION

Not all strains of *Bacillus* spp. are capable of becoming active ingredients in biocontrol products for commercial use. The main constraint to large-scale application of *Bacillus*-based biofungicides is their inconsistent performance in the field, from site to site and from year to year (Notz et al., 2001). The strains with most prospects are those which act through several mechanisms (Cawoy et al., 2011).

Strain IMV B-7404 was identified as *B. amyloliquefaciens* subsp. *plantarum*, which is rather similar to *B. subtilis*, from which it can be distinguished by a slightly higher molecular percentage of G+C content in its DNA (Yoshida et al., 2001). This study of strain IMV B-7404 demonstrated the inhibition of barley leaf disease by its metabolites when the seedlings were sprayed at the 1–2 leaf stage. Moreover, introducing the culture filtrate into the plant growth medium also resulted in inhibition of spot blotch on the leaves. This suggests that the strain can stimulate induced systemic resistance (ISR) in the plants.

Strain IMV B-7404 reduced the severity of spot blotch on barley leaves caused by *B. sorokiniana* when the filtrate was applied before, but not after fungal inoculation, suggesting that the filtrate does not have a therapeutic effect but exerts a preventive effect on the leaf disease.

However, at 17°C there was significant inhibition of the leaf disease when treatment was applied after fungal inoculation. This may be explained by a slower progress of disease development, and later sensitivity of the pathogen to *Bacillus* metabolites. The infection process of *B. sorokiniana*, as well as other fungal pathogens, consists of several stages: attachment to the host, spore germination, reception of signals from the plant that is required for appressorium formation, and secretion of extracellular enzymes for further penetration of the plant surface. Infection by *B. sorokiniana* is known to be highly variable and very sensitive to environmental conditions (Duveiller, Garcia Altamirano, 2000). Conidia of *Bipolaris* may therefore be unable to germinate at 17°C and may not be subject to antagonistic bacteria before germination.

We propose that effective biological control by *Bacillus*-based products depends on spraying leaves with a cell suspension to co-ordinate with the process of plant infection by the pathogen. It is necessary to know the most sensitive stage of infection. This information needs to be identified individually for each pathosystem and in relation to environmental conditions. The ecological behaviour and biology of both the antagonist and the target pathogen are the key elements in enhancing the efficacy of biocontrol products. Most *Bacillus*-based products include dry inactive and active ingredients (biomass) intended to be applied as a suspension in liquid, generally to seed or foliage (Schisler et al., 2004).

The results of our research show that the survival of bacterial biomass on leaves may depend on abiotic factors (temperature). Although *B. amyloliquefaciens* has been frequently isolated from leaves (Yoshida et al., 2001), the cell suspension (10^9 CFU ml^{-1}) of IMV B-7404 did not inhibit symptoms on barley leaves where disease development was rapid at 25°C, but tended to be effective at 17°C, where the disease process was slower.

Further studies are needed to assess the ecological behaviour of strain IMV B-7404 on leaves in relation to other abiotic (e.g., temperature, humidity, light) and biotic (e.g., host genotype, host age) factors, which can affect the survival and biocontrol activity of both fungal and bacterial inoculants and development of leaf diseases.

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MIEŽIŲ LAPŲ LIGŲ BIOLOGINĖ KONTROLĖ SU *BACILLUS* PADERME

Santrauka

Nauja *Bacillus amyloliguefaciens* subsp. *plantarum* IMV B-7404 padermė buvo pasirinkta kaip potencialus biologinės kontrolės agentas tiriant *in vitro* agaro plokšteles. Ši padermė stipriai slopino 12 svarbių augalų patogeninių grybų augimą. IMV B-7404 padermės metabolitai yra svarbūs ligų prevencijai, todėl ši padermė gali stimuliuoti sukeltą sisteminį atsparumą. Mūsų nuomone, efektyvią biologinę kontrolę *Bacillus* pagrindu pagamintais produktais lemia augalo patogeninės infekcijos eiga. Tiek antagonisto, tiek tikslinio patogeno ekologinė elgsena ir biologija yra pagrindinis biokontrolės produktų veiksmingumą didinantis elementas. Būtina išsiaiškinti kiekvienos patogeninės sistemos pažeidžiamiausią etapą bei atsižvelgti į aplinkos sąlygas.

Raktažodžiai: *Bacillus*, antagonizmas, biologinė kontrolė, miežiai, *Bipolaris sorokiniana*