Assessment of the antifungal activity of the violacein-forming strain *Janthinobacterium* sp. B-3515 against the mould fungus *Alternaria brassicicola* F-1864

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Assessment of the antifungal activity of the violacein-forming strain *Janthinobacterium* sp. B-3515 against the mould fungus *Alternaria brassicicola* F-1864

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Abstract. A study of antifungal properties of violacein-forming strain *Janthinobacterium* sp. B-3515 as well as its secondary metabolite, violacein, against *Alternaria brassicicola* F-1864 is presented. Regardless of the presence of bacteria, mycelium growth in the first two days proceeded at the same rate. The effect of the bacterial strain was manifested after the third day of incubation. In general, during co-culture, the bacterial strain statistically significantly reduced the average growth of the mycelium of the mould fungus by 10%. The average growth of *A. brassicicola* F-1864 was decreased in the presence of an aqueous solution of violacein in the nutrient medium (1%, 3%, and 5%). The pigment in 5% concentration had the greatest effect, as the difference between the average growth of the control group and the experimental group was 18%. The mycostatic activity of bacteria of genus *Janthinobacterium* and violacein against mould fungus *Alternaria brassicicola* F-1864 was shown for the first time.

1. Introduction

The mould fungus *Alternaria brassicicola* has been shown to be the causative agent of seed pod disease in *Brassica oleracea* crops [1]. Many varieties of cabbage (*Brassica* sp.) are widely cultivated in Europe, Asia, New Zealand, and North America as a staple food for humans and cattle. However, no known cultivar is completely resistant to *A. brassicicola* to date [2]. One of the ways how the fungus penetrates plant cells of the host is through the action of several serine esterases that are bound to the spore surface on the cuticle [3]. On this basis, some fungicides may be aimed at inhibiting the enzymatic activity of *A. brassicicola* spores, but if aggressive agents are used, the plant itself may be affected.

Current trends in the development of plant protection products aim at biologization, namely the replacement of active chemical pesticides with biological ones (e.g. antagonist microorganisms and their secondary metabolites). This direction requires the search for new effective producers of antibiotic substances and the development of a set of optimal parameters for obtaining biologically active substances.

One of the most promising compounds for the development of plant protection agents could be the blue-violet pigment violacein (figure 1).

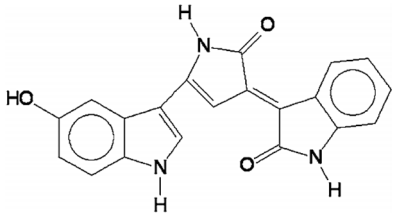

Figure 1. The structural formula of violacein [5].

There is evidence that violacein produced by many bacteria (*Chromobacterium violaceum, Janthinobacterium lividium*, etc.) has antibacterial, antifungal, antiviral (against herpes simplex virus), and antitumor properties [5]. In addition, the pigment had a stimulating effect on the formation of immunoglobulin G by the ovary cells of the Chinese hamster [6].

Violacein showed antifungal activity against the phytopathogenic mould fungus *Rosselinia necatrix* [5]. The bacteria *Janthinobacterium lividium* were found to be active against the mould *Batrachochytrium dendrobatidis*, the causative agent of frog disease (chytridiomycosis), which is a serious problem for the ecological condition of water bodies [7]. The study aims to evaluate the antifungal activity of the violacein-forming bacterium *Janthinobacterium* sp. B-3515 and pigment against the mould fungus *Alternaria brassicicola* F-1864.

2. Materials and methods

The antagonistic potential of indigenous violacein-forming strain *Janthinobacterium* sp. B-3515 against mould fungus *Alternaria brassicicola* F-1864 was evaluated by co-culture method [8] on agarized Sabouraud's medium composition (g/l): glucose, 40.0; peptone, 10.0; yeast extract, 5; agar, 18.0. A suspension of cells of B-3515 strain equal to 0.1 optical density (OD), prepared using a Microscan Turbidity Meter (manufactured by Siemens, USA), was introduced into agar wells (100 µl per well), made in a solid nutrient medium with a sterile 11 mm diameter drill bit. The volume of nutrient medium in each Petri dish was 20 ml. As a control, the mould fungus *A. brassicicola* F-1864 was cultured without *Janthinobacterium* sp. B-3515.

Mold fungus F-1864 was replaced in the centre of a Petri dish using the imprinting method [9]. The cultures were incubated at 25ºC for 5 days. Every 24 hours, the colony diameter of the fungus was measured and averaged according to the formula (1) [10]:

$$S = \sqrt{\frac{\sum V^2}{n}} \quad (1)$$

where S is root mean square, V is a date, n – is the number of measurements.

To obtain violacein, the culture of *Janthinobacterium* sp. B-3515 was grown in a liquid nutrient medium (3% peptone) for 5 days without stirring so that a biofilm was formed on the interface. After incubation, the biomass of the pigmented culture was separated from the culture liquid by centrifugation at 4,400 g for 10 min (using a Neofuge 1600R centrifuge, Heal Force, China). The supernatant was discarded, the precipitate was mixt with 50 ml of 95% ethanol and stirred for 1 hour at 35ºC (ThermoStable IS-30 shaker incubator, Daihan Scientific, Korea). The mixture was centrifuged again, the supernatant pigmented solution was collected and another 50 ml of ethanol was added. The mixture was resuspended and centrifuged as descript above. The supernatant was collected so as the total volume of the crude ethanol extract was 100 mL. The obtained solution was evaporated using a vacuum rotary evaporator (N-1000R). The evaporated violacein was dried in a desiccator (ThermoStable OF-50, Daihan Scientific, Korea) at 60ºC until the moisture evaporated completely. The resulting crystalline pigment powder was collected and the aqueous violacein solution was analysed using a UV-1900i spectrophotometer, Shimadzu, Japan at λ = 200-700 nm.

Antifungal activity of aqueous solutions of crude dry extract of violacein (concentrations were 1%, 3%, and 5%) against *A. brassicicola* F-1864 was determined by disc-diffusion method [8].

For this purpose, a suspension of mould spores equal to 0.1 optical density at λ = 600 nm (Microscan Turbidity Meter, Siemens, USA) was "lawn-passed" into Petri dishes each containing 20 ml of agarized Sabouraud nutrient medium. Sterile paper disks ("white tape"), 9 mm in diameter, were soaked in 40 ml of violacein solution and placed in cups in 6 replicates. The cultures were incubated for 72 hours at 25ºC. The average radii of the growth inhibition zones of the mould fungus were used to judge the antifungal activity of violacein.

The effect of the pigment on the growth of the fungus was investigated by linear growth of mycelium, in which aqueous solutions of violacein were added to the agar (1,000 µl per cup). The volume of nutrient medium in each Petri dish was 20 ml. *A. brassicicola* F-1864 was used as a control without adding the studied solutions. The antifungal properties of the pigment were judged by the mean square diameters of the colony of mould fungus F-1864.

Confidence intervals (Δ) were calculated according to the formula (2) [11]:

$$\Delta = t_{st} \cdot m \,, \tag{2}$$

where t_{st} is the standard value of the Student test, m is the representativeness error of the mean square and is calculated according to the formula (3) [11]:

$$m = \frac{\sigma}{\sqrt{n}} \,, \tag{3}$$

where σ is the standard deviation and is calculated as (4) [11]:

$$\sigma = \sqrt{\frac{\sum(V-\mathrm{X})^2}{n-1}} \,, \tag{4}$$

where V is the date, X is the arithmetic mean (or S is the root mean square of the colony diameter), and n is the number of measurements.

Average mycelial growth was calculated using the formula (5,6) [11]:

$$\mathrm{X} = (\lg \mathrm{G}_{v+1} - 1) \cdot 100\% \,, \tag{5}$$

if

$$\lg G_{v+1} = \frac{\sum \lg(V+1)}{n} \tag{6}$$

The significance of the difference between the averages was calculated statistically using the difference-in-differences method [12].

3. Results and discussion

The study of antagonistic activity of strain *Janthinobacterium* sp. B-3515 revealed that the colony growth of *A. brassicicola* F-1864 in control and experimental groups was statistically insignificantly different within 3 days (figure 2). However, a statistically significant difference in the mean square diameters of the mould fungus colonies in both the control and the experimental groups at days 1 and 3 was observed, as the calculated Student's *t*-test was higher than the table value at the error level of $p < 0.001$. The colony diameters of the control and experimental groups increased statistically significantly from 3 to 4. The calculated Student's t-test was higher than the table values with an error level of $p < 0.001$ (control group) and $p > 0.01$ (experimental group). On day 5, the mean squared colony diameter of the mould fungus in the control group significantly increased, as the calculated Student's *t*-test was higher than the standard test at $p < 0.05$). In contrast, in the experimental group, there was no mycelial growth on day 5, relative to day 4 (table 1).

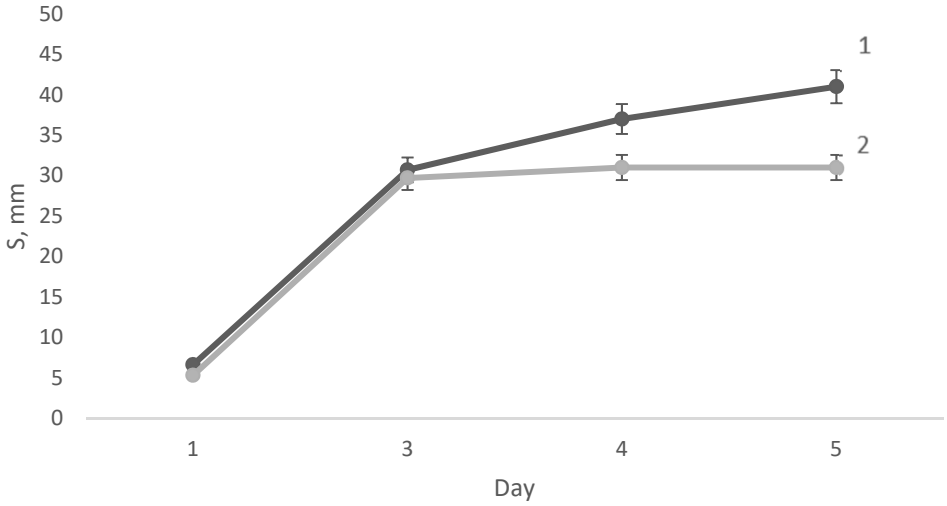

Figure 2. Growth of *Alternaria brassicicola* F-1864 mould fungus colonies without *Janthinobacterium* sp. B-3515 (1) and in the presence of the bacterial strain (2).

The mean quadratic diameter of the mould fungus colonies in the experimental group was statistically insignificant compared to the control group up to 3 days of incubation. On the 4th and 5th days, mycelial growth in the presence of bacterial strain B-3515 significantly slowed down in comparison with the control, as the calculated Student's *t*-test was higher than the table test at $p < 0.001$ (table 1).

Table 1. Mean square colony diameter of the mould fungus *Alternaria brassicicola F*-1864 as an indicator of the antifungal properties of strain *Janthinobacterium* sp. B-3515.

Variants Incubation	Incubation time, days	Mean square, $S \pm \Delta$	Error of variance, *Sd*	Student's *t*-test, *t*	Mean colony growth of *A. brassicicola* F-1864, *X*, %
Control	$1_{(1)}$	6.6 ± 1.7	$Sd_{(1-2)} = 0.75$ $Sd_{(1-1.1)} = 0.5$	$t_{(1-2)} = 32.1^{c}$ $t_{(1-1.1)} = 2.7^{a}$	
	$3_{(2)}$	30.7 ± 0.08	$Sd_{(2-3)} = 0.75$	$t_{(2-3)} = 10.2^{c}$	60
	$4_{(3)}$	37.7 ± 2.4	$Sd_{(3-4)} = 1.4$	$t_{(3-4)} = 2$	
	$5_{(4)}$	41.0 ± 3.5	$Sd_{(2-1.2)} = 0.3$	$t_{(2-1.2)} = 2.4$	
Experiment	$1_{(1.1)}$	5.3 ± 1.4	$Sd_{(1.1-1.2)} = 0.4$ $Sd_{(3-1.3)} = 0.9$	$t_{(1.1-1.2)} = 59.8^{c}$ $t_{(3-1.3)} = 6.6^{c}$	
	$3_{(1.2)}$	29.7 ± 0.08	$Sd_{(1.2-1.3)} = 0.3$	$t_{(1.2-1.3)} = 4.5^{b}$	50
	$4_{(1.3)}$	31.0 ± 1.6	$Sd_{(1.3-1.4)} = 0.4$	$t_{(1.3-1.4)} = 0$	
	$5_{(1.4)}$	31.0 ± 1.6	$Sd_{(4-1.4)} = 1.1$	$t_{(4-1.4)} = 8.7^{c}$	

[a] – the difference is statistically significant at the level of error $p < 0.05$;
[b] – the difference is statistically significant at the level of error $p < 0.01$;
[c] – the difference is statistically significant at the level of error $p < 0.001$.

Thus, based on the data obtained, when *A. brassicicola* F-1864 and *Janthinobacterium* sp. B-3515 wear co-cultured, the bacterium has a mycostatic effect. This statement is confirmed by the fact that the average mycelial growth (figure 3) in the control group is 10% higher than in the experimental group (table 1).

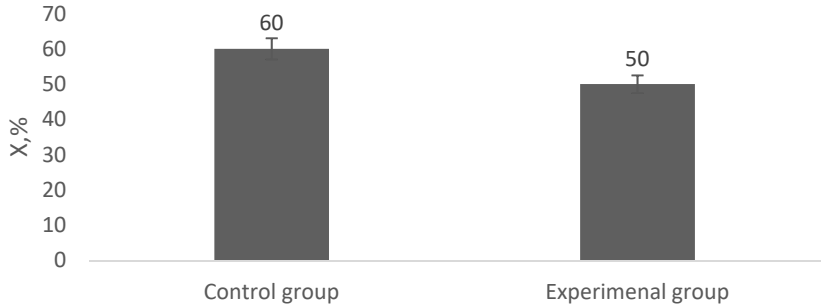

Figure 3. Average mycelial growth of *Alternaria brassicicola* F-1864 mould fungus without *Janthinobacterium* sp. B-3515 (control group) and in the presence of the strain (experimental group).

During the cultivation of *Janthinobacterium* sp. B-3515 as a producer, 0.4 g of dried violacein was obtained from 100 ml of the crude ethanol extract. Spectrophotometric analysis of the aqueous solution showed that the absorption maximum was at 576 nm, which approximated the property of violacein (based on literature data, the absorption maximum of methanol extract of violacein is λ = 575 nm).

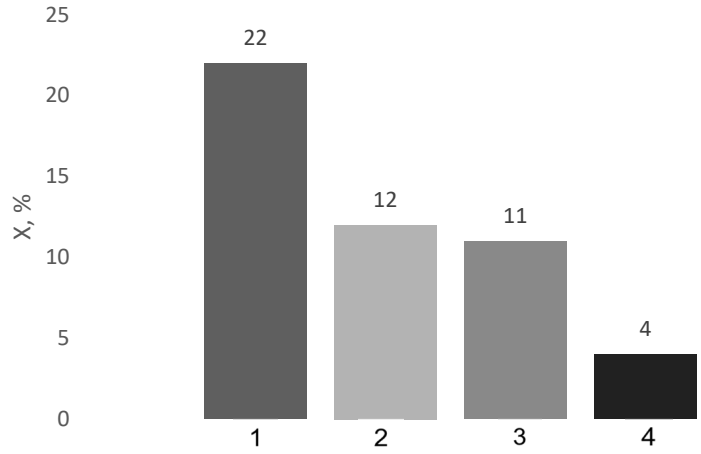

Figure 4. Average growth of mycelium of fungus *Alternaria brassicicola* F-1864 in the control sample (1), in the presence of 1% (2), 3% (3), and 5% (4) pigment in growth medium.

A study of the antagonistic activity of an aqueous solution of violacein revealed that the pigment mixed in Saburo nutrient medium (1,000 µl of the aqueous solution with pigment concentration of 1%, 3%, and 5%) had a weak mycostatic effect. Average growth of the mycelium of *A. brassicicola* F-1864 on the 5th day of incubation (figure 4) was statistically significantly reduced by 10% (for 1%), 11% (for 3%), and 18% (for 5%), compared to the control group.

Thus, the investigation revealed a moderate mycostatic activity of the strain *Janthinobacterium* sp. B-3515 and its secondary metabolite, violacein, against mould fungus *Alternaria brassicicola* F-1864.

4. Conclusion

In nature, microorganisms exist in permanent competition. This has led to the development of a multitude of ways to implement mechanisms of antagonistic interactions, including those which lead to the development of diseases in cultivated plants. However, compounds with proven antagonistic activity and the strains synthesizing them can be used as plant protection agents. In this work, the mycostatic efficacy of violacein-produced strain *Janthinobacterium* sp. B-3515 and its secondary metabolite against the mould fungus *Alternaria brassicicola* F-1864 were investigated. It was shown that during co-cultivation on agarized Sabouraud nutrient medium, the bacterium statistically significantly reduced the average mycelial growth by 10%, while the aqueous solution of violacein in the medium, in concentrations of 1%, 3%, and 5%, had a similar impact by 10%, 11%, and 18%, respectively. On this basis, it can be assumed that the mycostatic efficacy of an aqueous solution of violacein increased with increasing of its concentration. The search for the optimal pigment concentration will continue.

Acknowledgments

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References

[1] Humpherson-Jones F M and Maude R B 1982 *Annals of App.* **100** 61–71
[2] Macioszek V, Gapinska M, Zmienko A, Sobczak M, Skoczowski A, Oliwa J and Kononowicz A 2020 *Cells* **9** 2329
[3] Berto P, Commenil P, Belingheri L and Dehorter B 1999 *FEMS Microbiol.* **180**(2) 183–9
[4] Marinelli F, Genilloud O, Fedorenko V and Ron E Z 2015 *BioMed. Res*. 276964
[5] Duran N, Justo G Z, Ferreira CV, Melo P S, Cordi L and Martins D 2010 *Biotechnology and Applied Biochemistry* 127–33
[6] Kido M, Idogaki H, Nishikawa K and Omasa T 2020 *Cytotechnology* 1–14
[7] Ackleh A S, Carter J, Chellamuthu V K and Ma B 2016 *Ecological Modelling* **320** 158–69
[8] Egorov N S 1965 *Microbial Antagonists and Biological Methods for Determining Antibiotic Activity* (Moscow: Higher School) p 211
[9] Dudka I A 1982 *Methods in Experimental Mycology* Reference book (Kyiv: Naukova Dumka) p 535
[10] Lyakhovchenko N S et al. 2021 *Microorganisms* **9** p 102
[11] Snegin E A 2016 *Workshop on Biometrics: Tutorial* (Belgorod: Publishing house “Belgorod”, NRU “BelSU”) p 56
[12] Moiseychenko V F, Trifonova M F, Zaveryukha A X and Eshchenko V E 1996 *Fundamentals of Scientific Research in Agronomy* (Moscow: Kolos) p 336