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ORIGINAL ARTICLE

Evaluation of the utility of surfactin produced by the native strain of *Bacillus subtilis natto* BS19 in reducing the feeding and development of *Oulema melanopus* and *Oulema gallaeciana*

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Abstract

The study objective was to investigate the influence of microbiologically obtained surfactin on the feeding and development of *Oulema melanopus* and *Oulema gallaeciana* on spring wheat (*Triticum aestivum*) and spring barley (*Hordeum vulgare*). The purified bioproduct was applied to the leaves of cereal plants at a concentration of 660.5 mg · l^{-1}. The tests were conducted as a no-choice test and a choice test. Pest feeding and egg-laying were analyzed. The addition of surfactin to the food reduced the feeding of female and male tested insects as compared to controls. Male pests caused less damage to plants than females. Insect feeding on surfactin-treated plants was low in the first days of the experiment. The tested insects laid fewer eggs on plants treated with the biosurfactant. In terms of food selection, both female and male *Oulema* spp. were much more likely to choose food to which surfactin had not been applied. It can thus be concluded that surfactin can contribute positively to the biological control of beetles of the genus *Oulema* under natural conditions. However, further research is needed to better understand the mechanisms by which analogues of this compound limit the development of this cereal pest in its natural environment.

Keywords: *Bacillus subtilis* natto, *Oulema melanopus*, *Oulema gallaeciana*, pest control methods, surfactin

Introduction

There is an ongoing global search for pest control methods that are more environmentally friendly than extensively applied chemical treatments. Biological methods are therefore of interest to many researchers (Lamparski *et al.* 2021; Denoirjean *et al.* 2022). Microorganisms of the genus *Bacillus* and their bioproducts play a special role since they can be used as microbiological agents for biological control (Mnif *et al.* 2013; Salazar *et al.* 2022). Thus, they play a key role in Integrated Pest Management (IPM) (Sosnowska 2018). Among the bacteria of the genus *Bacillus*, *B. thuringiensis* receives special attention as a source of biopesticides (Chakrabarty *et al.* 2020), as well as other species, such as *B. amyloliquefaciens* (Geetha *et al.* 2011) or *B. subtilis* (Mnif *et al.* 2013).

One of the biosynthesic products of bacteria of the genus *Bacillus*, and also a secondary metabolite, is surfactin. This is a biosurfactant that, in addition to its strong surfactant properties, is also characterized by low toxicity, high biodegradability and stability under various environmental conditions (Liu *et al.* 2015). The compound's ability to reduce the surface tension of water from 72 to 27 mN · m^{-1} and interfacial tension (water-n-hexadecane) from 43 to 1 mN · m^{-1} results from its amphiphilic structure (Chen *et al.* 2015). Surfactin consists of seven ring-forming amino acids and a β-hydroxy fatty acid chain of 13–16 carbon atoms. Surfactin is produced as a mixture of isoforms, and structural differences (e.g., length of carbon chain) which affect the properties of these compounds

(Liu *et al.* 2012a). Previous studies confirm the role of surfactin as a compound with broad biological activity against fungi, bacteria and viruses (Liu *et al.* 2012b; Liu *et al.* 2019; Kiesewalter 2021). Researchers are also interested in the effect surfactin has on insects. Geetha *et al.* (2011) provide evidence that this compound causes hemolysis with potent larvicidal activity. They showed that bioproducts synthesized by the VCRC B483 strain of *B. amyloliquefaciens* caused death of both larval and pupal stages of mosquitoes. In turn, research by Rodríguez *et al.* (2018) confirmed 59.8% mortality of aphids within 24 hours of the application of a mixture of biosurfactants produced by *B. atrophaeus* L193.

Under field conditions, cereal plants are exposed to attack by various phytophages, including cereal leaf beetles (*Oulema melanopus* L. and *Oulema gallaeciana* Heyden). The cereal leaf beetle is considered to be a major pest of wheat, barley and several other cereal plants. Both of these species are common pests in European and North American cereal fields. In Poland, there are five species of *Oulema*, among which *O. melanopus* and *O. gallaeciana* predominate. In central and northern Poland, the dominant species is cereal leaf beetle, whereas *O. gallaeciana* dominates in the country's southeast (Mazurkiewicz *et al.* 2019). The feeding of cereal leaf beetle, and especially of its larvae when they occur *en masse*, has a significant economic impact (Philips *et al.* 2011). This insect is responsible for damaging the assimilation apparatus of leaves, which has a decisive impact on yield sizes and the formation of the dry mass of grain. Foraging larvae damage the upper husk and parenchymal tissue, while the lower husk dries up and turns white. Meanwhile, adults cause damage in the form of small, shallow fissures spread randomly across the upper leaf surface (Császár *et al.* 2021). Several chemical, agrotechnical and biological methods are used to combat these plant pests (Philips *et al.* 2011). However, as previously mentioned, the primary goal is to use biological methods that exploit their natural enemies, while also taking into consideration the developmental stage of the cereal leaf beetle. Hence, it is indicated that, *inter alia*, predatory insects of the family Carabidae (Cleoptera), *Tetrastichus julis* Yang, *Trichogramma* sp., species of the order *Hymenoptera*, are important for the biological control of *Oulema* sp. *Alternaria alternata* (Fr.) Keissl., *B. thuringiensis* bacteria and nematodes are also seen as entomopathogens that can reduce the abundance of the pest (Mazurkiewicz *et al.* 2019). However, there is still no information on the possible effects of lipopeptide biosurfactants, including surfactins, on this pest species.

Hence, the primary aim of this study was to quantify the feeding and evaluate the development of *O. melanopus* and *O. gallaeciana* on spring wheat (*Triticum aestivum*) and spring barley (*Hordeum vulgare*) treated with surfactin produced by the native *B. subtilis* BS19 strain. In addition, in this study, the obtained bioproduct was characterized, including, in particular, an assessment of its surface-active properties under various environmental conditions (pH, temperature, salinity) and thus its potential suitability for use in plant protection.

Materials and Methods

The *Bacillus subtilis* natto BS19 strain

The *B. subtilis* natto BS19 strain was isolated from natto (which is a food product containing fermented soybean), according to the procedure of Koim-Puchowska *et al.* (2019). The bacterium was identified according to a sequencing of the 16S rRNA gene. The isolate was placed in cryobanks (Grasso Biotech, Poland) and stored at –20°C until analysis.

Bacillus subtilis natto BS19 culturing

To obtain surfactin, submerged culturing (SmF) was carried out in flasks (v = 250 ml) on a substrate whose composition was modified according to Koim-Puchowska *et al.* (2021) and consisted of 40 g · l^{-1} starch; yeast extract 4 g · l^{-1}; KH_2PO_4 4.08 g · l^{-1}; $Na_2HPO_4 \times 2H_2O$ 7.12 g · l^{-1}; $MgSO_4 \times 7H_2O$ 0.2 g · l^{-1}; $CaCl_2$ 0.0008 g · l^{-1}; $FeSO_4 \times 7H_2O$ 0.0011 g · l^{-1}; EDTA 0.0012 g · l^{-1}; pH 7.0. Cultivation was performed at 37°C for 120 h in a Thermo Scientific, MaxQ 4450 shaking incubator (Thermo Fisher Scientific, Waltham, MA, USA). The medium was inoculated with the inoculum obtained from the 24 h culturing of *B. subtilis* natto BS19 on nutrient broth (bacteriological tryptone peptone 5 g · l^{-1}; yeast extract 2.5 g · l^{-1}; glucose 1 g · l^{-1}; pH 7.2–7.4) at 37°C at rpm = 180.

Surfactin isolation

Surfactin was isolated according to the modified method of Hsieh *et al.* (2004). After culturing, the biomass was centrifuged (2400 g, t = 15 min, T = 4°C), and the pH of the obtained supernatant was successively lowered to 2.0 using concentrated hydrochloric acid. The solution was left for 24 h at +4°C for acidic precipitation. The obtained residue was centrifuged (2400 g, t = 15 min, T = 4°C) and dissolved in 50 ml of distilled water at pH 8.0. Surfactin was extracted from the obtained solution successively on two occasions using methylene chloride (50 ml). After evaporation of the solvent under reduced pressure on each occasion, the residue was dissolved in distilled water at pH 8.0, recrystallized with 1M HCl and finally dissolved in

5 ml of distilled water at pH 8.0. An aqueous solution of the mixture of surfactin isoforms thus obtained was used in the experiment.

Qualitative and quantitative determination of surfactin

Qualitative and quantitative determinations of the mixture of surfactin isoforms synthesized using individual waste products were preceded by the extraction of an aqueous solution of this compound (pH = 8.0) by affinity chromatography using an SPE (solid-phase extraction) system. This was done using columns of Agilent Technologies Bond Elut C18 specific for the isolation of hydrophobic compounds as presented in the work of Koim-Puchowska *et al.* (2019). The concentration of the tested surface compound was determined by high-performance liquid chromatography (HPLC) using an Agilent Technologies device (1220 model) equipped with a diode detector. The chromatographic separation was carried out under the following conditions: Poroshell 120 EC-C18 column (4.6 × 150 mm), mobile phase 80 : 20 [acetonitrile: 3.8 mM (trifluoroacetic acid), temperature 40°C, detection 205 nm). The concentration was determined from the sum of the surfactin peaks using the ESTD external standards method.

Analysis of surfactin structure by Fourier transform infrared spectroscopy (FTIR)

The aqueous surfactin solution was freeze-dried in an Alpha model 1–2 LDplus freeze drier (Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for 24 h. The obtained lyophilized product was subjected to qualitative analysis using a Thermo Scientific Nicolet iS5 FTIR spectrometer (Thermo Fisher Scientific, USA) at room temperature (25°C). The presence of the main functional groups of the obtained product was analyzed between 400 and 4000 wavenumbers (cm^{-1}) at a resolution of 2 cm^{-1}.

Evaluation of the surface-active properties of surfactin depending on various environmental conditions

The stability of the surface-active properties of the obtained bioproduct was verified at t = 1 h in different ranges of pH (2–10), salinity (1–7%) and temperature (25, 50, 70, 90, 100°C). The surfactin solution was also autoclaved at 121°C, t = 15 min. The surface tension of the surfactin solution with a concentration of 35 mg · l^{-1} was measured using a tensiometer (PI-MT1M model, Donserv) according Koim-Puchowska *et al.* (2019).

Insect experiment procedure

The study was performed in 2021 in the laboratory of the Department of Biology and Plant Protection, Bydgoszcz University of Science and Technology, Bydgoszcz, Poland (53°07'N and 18°00'E).

Laboratory tests began with the import of soil, which was bought in a gardening shop (universal soil by "Biovita" in 50-liter bags) and spring wheat and spring barley seeds of varieties treated with Omnix 025 FS seed treatment, provided by a friendly farmer from the Kujawsko-Pomorskie province.

The following experimental factors were adopted: I – plant cultivar (respectively, spring wheat (*T. aestivum* cultivar Goplana) and spring barley (*H. vulgare* cultivar Radek), II – insect species (respectively, *O. melanopus* and *O. gallaeciana*) and III – application of bioproduct (respectively, provided with or without surfactin).

In the laboratory, in 2021, from the third week of March until mid-April, spring wheat and spring barley were sown in pots (Ø = 13 cm, 10 cm tall). In each pot, 10 seeds were sown 2.5–3.0 cm deep.

During plant growth, the plants were irrigated once a week with "Florovit uniwersalny" liquid fertilizer (also intended for agricultural plants) in the amount of 0.2 ml per 100 ml of water per pot. The plants were grown in an air-conditioned room (21 ± 1°C, L16 : D8 photoperiod and 70% Relative Humidity).

Laboratory tests

Seven weeks after sowing, the plants reached the BBCH-32 stage (beginning of stem elongation). *O. melanopus* and *O. gallaeciana* had been imported from the surrounding fields a few days earlier. They were immediately put into isolators with *T. aestivum* or *H. vulgare*. Then, *O. melanopus* or *O. gallaeciana* (sex differentiation – *in copula*) were transferred to a Petri dish (Ø = 10 cm). The end segment of the top leaf of each plant was placed on each dish, and then surfactin was provided (with a small brush) or not provided (as a control). All plants were used for the following laboratory tests:

1. No-choice test – determine *O. melanopus* and *O. gallaeciana* feeding on plants provided or not provided with surfactin

Three days after *O. melanopus* and *O. gallaeciana* feeding, on a 5-cm-long leaf, injuries were measured (total scar length). The results (Tables 1–2) were presented as the mean total scar length (in mm) caused by one female or one male of pest insects within a period of 1 day (Clement *et al.* 2011). Data are presented as mean ± SD. In Fig. 3A–D, we showed the total feeding scar length on the 1st, 2nd and 3rd days of the experiment. The experiments were performed in five replications.

2. No-choice test – determination of *O. melanopus* and *O. gallaeciana* oviposition on plants provided or not provided with surfactin

To evaluate the effect of the application of surfactin on egg-laying, one pair of beetles was placed on Petri dishes for 3 days, the eggs laid were counted and the results (Table 3) were given as the number of eggs per one pair. Data are presented as mean ± SD. The experiments were done in five replications.

3. Choice test – a comparison of *O. melanopus* and *O. gallaeciana* feeding on *T. aestivum* and *H. vulgare* provided with or without surfactin

The pots with plants were put on racks with plastic Petri dishes (10 cm in diameter). On the bottom of the dish, in the middle, two end sections of top plant leaves were affixed 2 cm apart with a short piece of transparent tape. Then, one insect was placed inside. After 3 days, the insect was removed and injuries on leaves were counted. The results (Table 4) were presented as the total feeding scar length (in mm) on 5-cm-long leaves caused by one female or one male *O. melanopus* or *O. gallaeciana* within a period of 1 day. Data are presented as mean ± SD. The experiments were done in five replications.

Statistical analysis

The results were subjected to an analysis of variance (ANOVA) in a completely random system using the program Statistica 2013. The results had previously been log transformed. Normal distribution of data in individual groups was confirmed by the Shapiro–Wilk test. The significance of the differences between the object averages were estimated based on Tukey's test at the significance level $p < 0.05$. The results of all no-choice and choice tests are expressed as arithmetic mean plus or minus standard deviation (± SD).

Results

Surfactin

The concentration of surfactin obtained from microbiological synthesis by the native strain *Bacillus subtilis* natto BS19 was 660.5 mg · l^{-1}. On the basis of the spectrum of the tested compound (Fig. 1), the presence of N–H stretch vibrations in the wavelength range 3300–3500 cm^{-1} was recorded. Progressively increased absorbance was observed in the ranges 1710–1750 cm^{-1} and 1600–1700 cm^{-1}, which is related to the appearance of C = O stretch vibrations. The increase in absorbance at wavelengths of 1500–1600 and 1300–1400 may be related to the occurrence of N = O stretch and bend vibrations. The appearance of the absorption bands mentioned above clearly indicated the presence of amide and nitro groups in the tested compound. In addition to the appearance of the aforementioned absorption band in the 1710–1750 cm^{-1} and 1600–1700 cm^{-1} ranges, C–O stretch vibrations were observed at wavelengths in the 1000–1300 range, which may suggest the presence of ester groups. Moreover, increased absorbance was found in the range 2850–2950 cm^{-1}, which suggested the presence of an aliphatic chain (C–H stretch vibrations). It can thus be concluded that the tested product was a structurally diverse organic compound.

The practical applications of surfactin are limited by its surfactant activity stability at various temperatures, salinities and environmental pH ranges. Analysis of the results confirmed that the salinity of the solution did not affect the strength of surface activity of the surfactin. With increasing concentration of sodium chloride (NaCl) from 1 to 7% in the solution, no significant fluctuations in surface tension of the tested solution were found (Fig. 2A). Likewise, increasing the temperature of the solution had no effect on the surface activity of the surfactin.

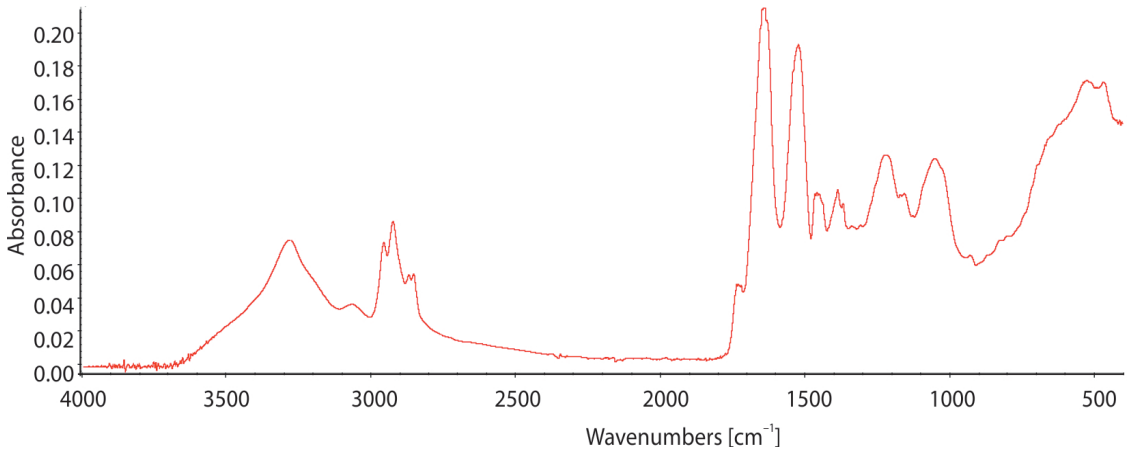

Fig. 1. FTIR spectra of freez-dried analogs of surfactin produced by *Bacillus subtilis* BS19

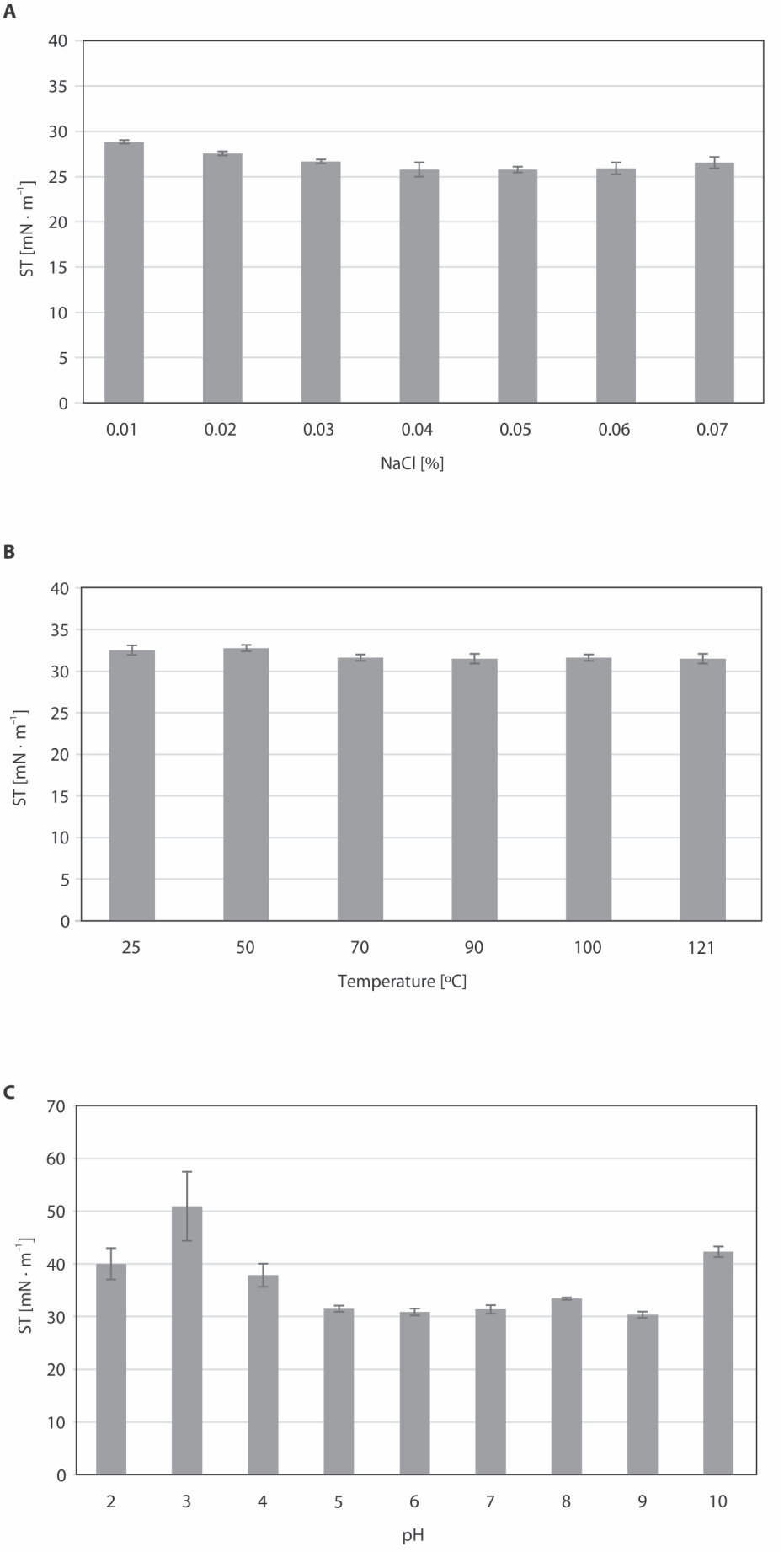

Fig. 2A–C. Stability of the surface-active properties of biosurfactants produced by *Bacillus subtilis* BS19 under different salinities – A, temperature – B and pH – C. The results represent the mean ± standard deviation. ST – surface tension

The surface tension of the solution after 1 hour's exposure to a temperature from 25 to 100°C was <35 mN · m^{-1} (Fig. 2B). Sterilizing the surfactin-containing solution in an autoclave (temperature 121°C, t = 15 min) did not reduce the surfactant activity of the tested compounds. On the other hand, among the examined factors, environmental reaction seemed to have a significant effect on the surface activity of the tested compound. At pH < 5, surfactin precipitates from the solution, and, hence, its surface activity is low. However, activity was found to be clearly stable in the range pH 5–9 (ST <35 mN · m^{-1}). At pH 10, surface tension was slightly higher than in this range of pH values, i.e., around 40 mN · m^{-1} (Fig. 2C).

Insect experiments

Oulema melanopus and Oulema gallaeciana feeding on plants provided with or not provided with surfactin (no-choice test)

It was found that females of insects fed with varying intensity on the investigated cereal plants (Tab. 1). Insects (regardless of species) were more likely to damage wheat plants than barley (34.9 and 32.8 mm, respectively). Regardless of plant species, *O. melanopus* damaged them more than *O. gallaeciana* (35.2 and 32.4 mm, respectively). The addition of *surfactin* to the food resulted in a significant difference in the feeding of the tested insects – 32.8 mm compared to the control plants (34.8 mm).

We found that the males of the pest insects fed with varying intensity on the investigated cereal plants (Table 2). Both insect species more eagerly damaged spring wheat than spring barley plants (31.1 and 29.8 mm, respectively). Regardless of the plant species, *O. melanopus* damaged them more than *O. gallaeciana* (31.4 and 29.6 mm, respectively). The addition of *surfactin* to the food resulted in a significant difference in the feeding of the tested insects – 29.2 mm compared to the control plants (31.7 mm).

It was noted that males and females fed on wheat and barley plants with different intensities, with 30.5 and 33.8 mm total scar length, respectively (Tables 1–2).

It was observed that the females fed on plants at intensities that differed between days of the experiment (Fig. 3A–B). They fed far less intensely on *surfactin*-treated plants on days 1 and 2 than on day 3. By contrast, in the control plants, the differences in the increment of damage between days 1, 2 and 3 were smaller. As with the females, the males also fed on plants with different intensities on different days of the experiment (Fig. 3C–D). Thus, the pests fed far less intensely on *surfactin*-treated plants on days 1 and 2 than on day 3. By contrast, in the control plants, the differences in the increment of damage on days 1, 2 and 3 were smaller.

Oulema melanopus and Oulema gallaeciana oviposition on plants provided with or without surfactin (no-choice test)

We found that female insects laid eggs with different intensities on the investigated cereal plants (Table 3). Insects were more likely (regardless of species) to lay eggs on wheat plants than on barley (24.6 and 22.5 ind., respectively). Regardless of plant species, female *O. melanopus* laid more eggs than *O. gallaeciana*

Table 1. Effect of the application of surfactin in *Triticum aestivum* and *Hordeum vulgare* on *Oulema melanopus* and *Oulema gallaeciana* female feeding (no-choice test) (mm). Data (total scar length) are presented as mean ± SD

I – Plant	II – Insect	III – Application of bioproduct		Mean
		control	surfactin	
Triticum aestivum	*Oulema melanopus*	37.0 ± 1.22	34.8 ± 1.64	35.9 ± 1.79
	Oulema gallaeciana	34.2 ± 1.30	33.4 ± 1.67	33.8 ± 1.48
Mean		35.6 ± 1.90	34.1 ± 1.73	34.9 ± 1.93
Hordeum vulgare	*Oulema melanopus*	35.8 ± 1.48	33.2 ± 1.30	34.5 ± 1.90
	Oulema gallaeciana	32.2 ± 0.84	29.8 ± 1.30	31.0 ± 1.63
Mean		34.0 ± 2.21	31.5 ± 2.17	32.8 ± 2.49
Oulema melanopus		36.4 ± 1.43	34.0 ± 1.63	35.2 ± 1.94
Oulema gallaeciana		33.2 ± 1.48	31.6 ± 2.37	32.4 ± 2.09
Mean		34.8 ± 2.17	32.8 ± 2.33	33.8 ± 2.44

$HSD_{0.05}$ – I = 0.876*** (F = 23.52, α = 0.001); II = 0.876*** (F = 41.81, α = 0.001); II/I = 1.238; I/II = 1.212; III = 0.876*** (F = 21.33, α = 0.001); III/I = 1.238; I/III = 1.212; III/II = 1.238; II/III = 1.212

Data (total scar length) are presented as mean ± SD. The differences were tested using Tukey's test. Level of significance: *p < 0.05, **p < 0.01, ***p < 0.001

Table 2. Effect of the application of surfactin in *Triticum aestivum* and *Hordeum vulgare* on *Oulema melanopus* and *Oulema gallaeciana* male feeding (no-choice test) (mm). Data (total scar length) are presented as mean ± SD

I – Plant	II – Insect	III – Application of bioproduct		Mean
		control	surfactin	
Triticum aestivum	*Oulema melanopus*	32.8 ± 1.30	30.8 ± 1.30	31.8 ± 1.62
	Oulema gallaeciana	32.0 ± 1.41	28.8 ± 2.05	30.4 ± 2.37
Mean		32.4 ± 1.35	29.8 ± 1.93	31.1 ± 2.10
Hordeum vulgare	*Oulema melanopus*	31.8 ± 1.79	30.0 ± 1.58	30.9 ± 1.85
	Oulema gallaeciana	30.2 ± 1.30	27.2 ± 1.64	28.7 ± 2.11
Mean		31.0 ± 1.70	28.6 ± 2.12	29.8 ± 2.24
Oulema melanopus		32.3 ± 1.57	30.4 ± 1.43	31.4 ± 1.76
Oulema gallaeciana		31.1 ± 1.60	28.0 ± 1.94	29.6 ± 2.35
Mean		31.7 ±1.66	29.2 ± 2.07	30.5 ± 2.24
$HSD_{0.05}$ – I = 1.004* (F = 6.86, α = 0.05); II = 1.004*** (F = 13.16, α = 0.001); II/I = 1.419; I/II = 1.389; III = 1.004*** (F = 25.38, α = 0.001); III/I = 1.419; I/III = 1.389; III/II = 1.419; II/III = 1.389				

Data (total scar length) are presented as mean ± SD. The differences were tested using Tukey's test.
Level of significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 3. Effect of the application of surfactin in *Triticum aestivum* and *Hordeum vulgare* on *Oulema melanopus* and *Oulema gallaeciana* oviposition (no-choice test) [ind.]. Data (ind. per 1 pair of pests by 3 days) are presented as mean ± SD

I – Plant	II – Insect	III – Application of bioproduct		Mean
		control	surfactin	
Triticum aestivum	*Oulema melanopus*	26.2 ± 1.48	24.2 ± 1.30	25.2 ± 1.69
	Oulema gallaeciana	25.0 ± 1.00	22.8 ± 2.05	23.9 ± 1.91
Mean		25.6 ± 1.35	23.5 ± 1.78	24.6 ± 1.88
Hordeum vulgare	*Oulema melanopus*	24.8 ± 0.84	22.4 ± 0.89	23.6 ± 1.51
	Oulema gallaeciana	22.8 ± 1.64	20.0 ± 1.41	21.4 ± 2.07
Mean		23.8 ± 1.62	21.2 ± 1.69	22.5 ± 2.09
Oulema melanopus		25.5 ± 1.35	23.3 ± 1.42	24.4 ± 1.76
Oulema gallaeciana		23.9 ± 1.73	21.4 ± 2.22	22.7 ± 2.32
Mean		24.7 ± 1.72	22.4 ± 2.06	23.5 ± 2.22
$HSD_{0.05}$ – I = 0.884*** (F = 21.97, α = 0.001); II = 0.884*** (F = 16.01, α = 0.001); II/I = 1.251; I/II = 1.224; III = 0.884*** (F = 28.88, α = 0.001); III/I = 1.251; I/III = 1.224; III/II = 1.251; II/III = 1.224				

Data (total scar length) are presented as mean ± SD. The differences were tested using Tukey's test. Level of significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

(24.4 and 22.7 ind., respectively). The addition of *surfactin* to the food resulted in a significant difference in oviposition of insects – 22.4 ind. compared to 24.7 ind. in control plants. On average, the insects laid 23.5 eggs.

Comparison in choice test, Oulema melanopus and Oulema gallaeciana feeding on Triticum aestivum and Hordeum vulgare provided with or without surfactin

We found that the tested insects, when they had the option of choosing their food, foraged with varying intensity on cereal plants (Table 4). Insects (irrespective of species) damaged the cereal plants treated with *surfactin* much less than the control plants. It was noted that the difference between feeding on plants treated with *lipopeptid* and feeding on the control plants was smaller for male pests (13.3 and 17.0 mm, respectively) than it was for female; for females, this difference was much more pronounced (11.8 and 22.4 mm, respectively).

As in the case of tests performed under conditions in which pests could choose, the most damage was recorded in the case of *O. melanopus* on *T. aestivum*, for both male and female pests (for plants treated with *surfactin* and for control plants: 13.8 and 17.8 mm and 13.2 and 23.2 mm, respectively).

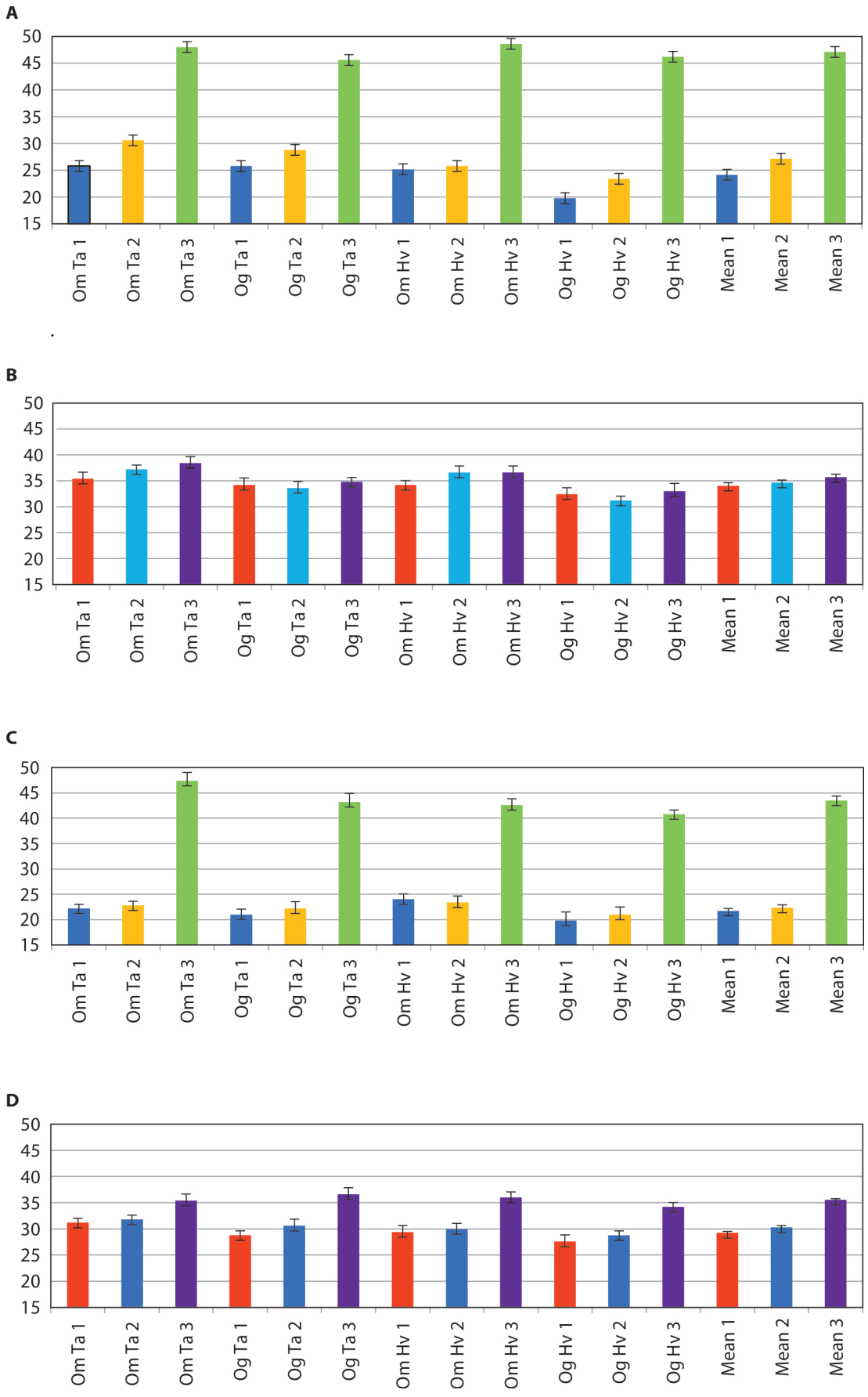

Fig. 3A–D. *Oulema melanopus* (Om) and *Oulema gallaeciana* (Og) female feeding in 1st, 2nd and 3rd day of the experiment on *Triticum aestivum* (Ta) and *Hordeum vulgare* (Hv) provided with surfactin (no-choice test) (mm) – A and without surfactin (no-choice test) (mm) – B, *Oulema melanopus* (Om) and *Oulema gallaeciana* (Og) male feeding in 1st, 2nd and 3rd day of the experiment on *Triticum aestivum* (Ta) and *Hordeum vulgare* (Hv) provided with surfactin (no-choice test) (mm) – C and without surfactin (no-choice test) (mm) – D

Table 4. Comparison in choice test, *Oulema melanopus* and *Oulema gallaeciana* male and female feeding on *Triticum aestivum* and *Hordeum vulgare* provided with surfactin or not (control) (mm)

Surfactin		Control		HSD
		Male		
O. melanopus *T. aestivum*	13.8 ± 1.48	*O. melanopus* *T. aestivum*	17.8 ± 1.92	2.504**
O. gallaeciana *T. aestivum*	13.2 ± 1.30	*O. gallaeciana* *T. aestivum*	16.8 ± 1.92	2.396**
O. melanopus *H. vulgare*	14.0 ± 2.12	*O. melanopus* *H. vulgare*	17.2 ± 0.84	2.351*
O. gallaeciana *H. vulgare*	12.2 ± 1.79	*O. gallaeciana* *H. vulgare*	16.0 ± 1.00	2.113**
Mean	13.3 ± 0.60	Mean	17.0 ± 0.54	0.837***
		Female		
O. melanopus *T. aestivum*	13.2 ± 1.79	*O. melanopus* *T. aestivum*	23.2 ± 2.39	3.075***
O. gallaeciana *T. aestivum*	11.8 ± 2.05	*O. gallaeciana* *T. aestivum*	22.2 ± 1.64	2.708***
O. melanopus *H. vulgare*	11.8 ± 2.49	*O. melanopus* *H. vulgare*	23.0 ± 1.41	2.952***
O. gallaeciana *H. vulgare*	10.2 ± 1.30	*O. gallaeciana* *H. vulgare*	21.2 ± 2.59	2.988***
Mean	11.8 ± 1.57	Mean	22.4 ± 0.55	1.734***

Data (total scar length) are presented as mean ± SD. The differences were tested using Tukey's test.
Level of significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Discussion

Cereal leaf beetles and many other insect pests are managed using conventional methods. For this reason, other methods of reducing their numbers should be sought. It would be best if these new methods were in harmony with nature. These can be non-chemical methods for supporting plants by reducing the number of pests (Lemanowicz *et al.* 2020; Lamparski *et al.* 2021).

It has been shown that surfactin produced by bacterial strains of the genus *Bacillus* is a biodegradable compound that is non-toxic to the environment (Chen *et al.* 2015). It may therefore have potential for the biological control of plant pests. Moreover, our research confirmed that surfactin produced by *B. subtilis* natto BS19 is stable in the face of differences in environmental salinity or temperature. Similar to our results, Liu *et al.* (2015) showed that temperature changes (in the range 30–120°C) did not significantly affect the surface tension of the surfactin-containing solution (which remained nearly constant at around 28–30 $mN \cdot m^{-1}$). The surfactin produced by strain BS-37 was also quite stable under a strongly alkaline environment (as high as 50 $g \cdot l^{-1}$ $NaCl^{+}$, 110 $g \cdot l^{-1}$ $CaCl_2$). Our research, too, confirmed the stability of the surface activity of surfactin in the pH range of 5–9. Meanwhile, Janek *et al.* (2021) indicated that surfactin produced by *B. subtilis* #309 was stable at a pH in the range 6–12, with maximum surface activity at pH 6.0–8.0. Similar to the results presented here, other researchers confirm a decrease in surfactin activity in acidic environments (Liu *et al.* 2015; Janek *et al.* 2021). This is related to the precipitation of biosurfactants at pH 2 and 4 and thus a decrease in the surface activity of this compound (Janek *et al.* 2021).

We found that adding surfactin to the food resulted in a significant difference in the feeding of female insects (32.8 mm compared to 34.8 mm for control plants) and of male pests (29.2 mm compared to 31.7 mm for control plants). It was noted that females and males of both *Oulema* species each feed on plants with an intensity that differed between successive days of the experiment – plants treated with surfactin fed significantly less on days 1 and 2 than on day 3. On the other hand, in the control plants, the differences in the increment of damage on days 1, 2 and 3 were smaller. The addition of surfactin to the food also resulted in a significant difference in the oviposition of insects (22.4 ind. compared to 24.7 ind. in control plants). It was found that when the tested insects could choose their food, they foraged with varying intensity on cereal crops. Insects (irrespective of species) damaged cereal plants treated with surfactin much less than control plants. The FTIR spectra indicated that

the biosurfactant contained both aliphatic and peptide moieties which was similar to the results of others (Gurjar and Sengupta 2015).

So far, there have been no studies on the effect of surfactin from *B. subtilis* natto BS19 on the feeding and development of cereal leaf beetles. Therefore, in this article, we compared our results to studies on other insects. Other researchers report testing various *Bacillus*-based products and surfactin obtained from it on insects belonging to different orders – butterflies, flies and true bugs. Denoirjean *et al.* (2022) found that lipopeptides produced by the genus *Bacillus* may be new biopesticides. Research confirms the possibility of using these compounds in combating black bean aphid *Aphis fabae*, i.e., both adults and nymphs in the larval stage. Surfactin concentration at the level of 1 g · l^{-1} resulted in a decrease in the locomotor activity of aphids as well as reductions in feeding activity. Ghribi *et al.* (2011) tested biosurfactants produced by *B. subtilis* SPB1 against third instar larvae of midgut *Prays olea*. Biosurfactants caused *P. olea* mortality with an LC50 and LC90 of 142 and 369 μg · ml^{-1}, respectively. Additionally, the surviving larvae exposed to SPB1 biosurfactant remained at the third instar stage for more than 9 days in contrast to untreated larvae. In futher research, Ghribi *et al.* (2012a) found that *B. subtilis* SPB1 biosurfactant showed activity against the Egyptian cotton leaf worm *Spodoptera littoralis* and it displayed toxicity with an LC50 of 251 ng · cm^{-2}. A number of histopathological changes have been shown in both *P. olea* and *S. littoralis* larvae after biosurfactant treatment. Among other things, cellular vacuolisation or destruction of epithelial cells and their boundaries have been demonstrated. Ghribi *et al.* (2012b) also reported that lepidopteran larvicidal potency of the biosurfactant secreted by *B. subtilis* SPB1 strain was determined. The LC50 of the biosurfactant against third instar larvae of *Ephestia kuehniella* was 57.0 μg · g^{-1} at 6 days post treatment. Additionally, this bioproduct was stable in a high range of temperature, pH and UV/salinity. Mnif *et al.* (2013) also demonstrated high insecticidal activity of the biosurfactant synthesized by *B. subtilis* SPB1 against the carob moth *Ectomyelois ceratoniae* Zeller. The LC50 and LC90 values after 6 days of contact were 152 mg · g^{-1} and 641 mg · g^{-1}, respectively. In other experiments, Abd El-Salam *et al.* (2011) investigated the biological activity of two bacterial strains *B. subtilis* NRC313 (BS NRC313) and *B. thuringiensis* NRC335 (BT NRC335) against third larval instars of the cotton leafworm *Spodoptera littoralis* (Boisd.). They found that bioproducts synthesized by bacteria caused a reduction of adult emergance and an extension of the generation period. The application of *B. thuringiensis and B subtilis* resulted in a reduction of *Spodoptera littoralis* larvae by 55.6 and 67.4, respectively, in clover plants under field conditions (Abd El-Salam *et al.* 2011).

Geetha *et al.* (2012) showed that surfactin is a potential agent used as an ultra-low volume spray against adult mosquitoes, which are vectors of malaria. Knockdown dosage (KD50) and KD (90) were, respectively, 10.73 and 26.39 mg · m^{-3}, while the lethal doses of LD (50) and LD (90) were 16.13 and 39.21 mg · m^{-3}.

Conclusions

Both *O. melanopus* L. and *O. gallaeciana* Heyden are major pests of cereal plants in Europe and North America. These widespread species generate significant yield losses in grain crops, especially wheat or barley. Scientists are still looking for pest control methods that are less invasive to the environment than chemical plant protection products. Surfactin analogs are easily biodegradable compounds and therefore environmentally friendly. An additional advantage is that the surface ativity of a mixture of isoforms produced by *Bacillus subtilis* natto BS19 is stable in a wide temperature range (25–100°C and even 121°C), environmental reaction (5–9) and salinity (1–7% NaCl). Moreover, it is characterized by a low level of the critical concentration of micellization (12 mg · l^{-1}). Our research confirmed the possibility of using surfactin isoforms as a means of limiting the feeding of adult grain beetles. The application of this compound at a concentration of about 660.5 mg · l^{-1} on the leaves of cereal plants caused reductions in the feeding of female and male insects as compared to controls. We found that male pests caused less damage to plants than females and insect feeding on surfactin-treated plants was limited, especially at the beginning of the experiment. We noticed that the insects laid fewer eggs on plants treated with the biosurfactant. In further research we plan to focus on elucidating the mechanisms of action of surfactin analogs on pest insects in their natural environment.

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