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**Citation style:** Rusin Milena, Gospodarek Janina, Nadgórska-Socha Aleksandra. (2020). Soil Pollution by Petroleum-Derived Substances and its Bioremediation: The Effect on *Aphis fabae* Scop. Infestation and Antioxidant Response in *Vicia faba* L. "Agronomy" (Vol. 10 (2020), Art. No. 147), doi 10.3390/agronomy10010147



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Article

# Soil Pollution by Petroleum-Derived Substances and its Bioremediation: The Effect on *Aphis fabae* Scop. Infestation and Antioxidant Response in *Vicia faba* L.

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Received: 23 December 2019; Accepted: 16 January 2020; Published: 19 January 2020



**Abstract:** In this study, the effects of soil contamination with petroleum-derived substances (PDSs) (petrol, diesel fuel and used engine oil) and its bioremediation using biopreparation ZB-01 on broad bean infestation by black bean aphid *Aphis fabae* Scop., as well as on the antioxidant enzymes activity (superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (POD)) and antioxidant (non-protein thiols and proline) content in plant leaves, were determined. Results showed that after three years from the moment of soil contamination PDSs limited infestation of broad bean by *A. fabae*. However, the adverse effects on aphids' life cycles were not proven. The lowered infestation may result from the lower attractiveness of contaminated plants to pests. PDSs significantly affected the activities of enzymes and the antioxidants content, with that effect being diversified. The increased activity of SOD was found in plants exposed to diesel fuel, together with the lowest numbers of aphids accompanying it, which can suggest a certain role of the enzyme in pest response to the stress caused by this PDS. The ZB-01 biopreparation limited the adverse effect of PDSs on the degree of broad bean plant infestation by *A. fabae*. Its influence on the antioxidant response was diversified. In the plants exposed to EO, changes in antioxidant response were reduced under the influence of ZB-01.

**Keywords:** agricultural soil contamination; bacterial degradation; antioxidants; antioxidant enzymes; host plant; herbivores

## 1. Introduction

Soil contamination with petroleum derived substances (PDSs) has become a global problem because of the effects that these compounds exert on the natural environment. The chief sources of contamination are production processes, transportation pipeline and refinery malfunctions, tank leakages, or accidents [1]. It is commonly known that the soil contamination with PDSs constitutes a potential danger to human health and life; therefore, in recent years, efforts have been undertaken in order to find effective, inexpensive, and environmentally friendly ways to amend contaminated soils [2–5]. The most commonly recommended method is biological remediation [6–8]. The microorganisms naturally occurring in the environment decompose the contaminants, which removes them from the environment or transforms them into less toxic, or non-toxic and less durable forms in the environment (alcohols, acids, carbon dioxide, and water). The microbiological degradation of petroleum-derived carbohydrates makes use of the enzyme catalytic activities of microorganisms in order to speed up the rate of the pollutants' decomposition [9,10].

Even low concentrations of PDSs in soil adversely affect the growth and development of cultivated plants [11,12] and provoke the manifestation of stress reactions in them, for example, in the changes in the antioxidant enzymes and in the content of antioxidants [13,14]. Among the most prominent enzymes superoxide dismutase (SOD), catalase (CAT) and H<sub>2</sub>O<sub>2</sub>—reducing peroxidases like guaiacol type peroxidases (POD), as well as compounds like thiols and proline (important non-enzymatic antioxidants) should be mentioned. These enzymes and antioxidants take part in detoxification of reactive oxygen species (ROS); in particular SOD is an enzyme that alternatively catalyzes the dismutation of superoxide (O<sub>2</sub><sup>-</sup>) into either ordinary molecular oxygen or H<sub>2</sub>O<sub>2</sub>. The last compound is eliminated by catalase, by several classes of peroxidases and by the action of ascorbate-glutathione cycle. Non proteins thiols and proline are active in some antioxidative and regulatory properties [15–17].

The changes triggered by the PDSs in plants can indirectly affect the feeding by phytophages [18,19]; however, studies in this field are still rare and conducted mostly after the contamination has occurred, while any data on the follow-up (distant in time) effects of pollutants is lacking. Aphids are one of the more dangerous pests of plants that are particularly vulnerable to changes in the quality of food consumed, which is rapidly reflected in their developmental cycle [18,20]. Additionally, it was found that the increase in the activity of enzymes responsible for protective reaction in plants, such as CAT, POD, and SOD can lead to the increased resistance of plants against aphids [21,22].

Broad bean (*Vicia faba* L.), which is one of the principal host plants of black bean aphid *Aphis fabae* Scop., is a suitable test plant both as a bioindicator of soil contamination with PDSs [23] and also in detecting mutagenic substances [24–26].

We attempted to find the answers to the following questions: (1) Does the stress caused by distant-in-time (after 3 years) soil contamination with PDSs such as petrol, diesel fuel, and used engine oil affect the infestation of broad bean plants by black bean aphid? (2) Do the possible differences in the infestation of plants under the effect of PDSs result from the changes in their developmental parameters? (3) Could the antioxidative response of plants induced by soil contamination be associated with the infestation of plants by *A. fabae*? (4) How are the aforementioned parameters (enzyme activity, antioxidants content, and the infestation of plants by aphids) affected by the bioremediation of soil contaminated with PDSs using biopreparation ZB-01?

## 2. Materials and Methods

### 2.1. Experimental Setup

The field experiment was conducted at the Experimental Station of the University of Agriculture in Krakow, located in Mydlniki near Krakow (Poland; 50.0815° N, 19.84730° E). In November 2009, indigenous soil (loamy-sand) (detailed characteristics of the soil was given in a previous papers [14,27]) was placed in special containers of 1 m<sup>3</sup> volume, retaining the natural arrangement of layers. The containers were sunk in the ground so that their upper edge was at the same level as the surface of the soil. The description of the containers and its arrangement in the experimental field were presented earlier [27]. In June 2010, the soil surface was artificially contaminated with petrol (BP Unleaded 95) (P), used engine oil (PLATINUM Classic Semisynthetic 10W-40, used for one year in a petrol engine) (EO), and diesel oil (BP Diesel Fuel) (DF) in a quantity of 6000 mg of petroleum product per 1 kg of soil dry mass in the container (i.e., typical for medium-contaminated soils), by pouring it on the soil. The non-contaminated soil in identical containers served as a control (C). After one week, biopreparation ZB-01 was added to the soil in the series with bioremediation (R). The biopreparation was specially prepared for this experiment in the Biochemistry Department of University of Agriculture in Krakow and contained selected prokaryotic organisms, isolated from sites heavily polluted with organic compounds. It consisted mainly bacteria: *Stenotrophomonas*, *Pseudomonas*, *Moraxella*, *Acinetobacter*, *Alcaligenes*, *Ochrobactrum*, *Comamonas*, *Burkholderia*, *Corynebacterium*, *Oligella*. The ZB-01 treatment was proceeded by fertilization with the use of “Azofoska” compound fertilizer (Inco Group, Susz, Poland; 13.6% N, 6.4% P<sub>2</sub>O<sub>5</sub>, 19.1% K<sub>2</sub>O, 4.5% MgO, 23.0% SO<sub>3</sub>, 0.045% B, 0.18%

Cu, 0.17% Fe, 0.27% Mn, 0.04% Mo, 0.045% Zn) at a dose of 100 g per container. The procedure of adding ZB-01 and fertilizer was repeated one year later (2011). The experiment was established in four replications, in line with the randomised blocks method.

The detailed monitoring of total petroleum hydrocarbon (TPH) content in the experimental soil was conducted every month over two years from the moment of soil pollution [27]. The analysis showed that after 24 months from the moment of soil contamination, TPH content in the soil contaminated with EO was more than 11 times higher than in the control soil, while in the soil contaminated with DF it was more than twice as high (16,530 mg kg<sup>-1</sup> in EO, 3852 mg kg<sup>-1</sup> in DF and 1458 mg kg<sup>-1</sup> in control). The content of TPH in the soil contaminated with petrol (2661 mg kg<sup>-1</sup>) was similar to the control. The applied ZB-01 biopreparation in the case of soils contaminated with EO and DF caused a significant decrease in the content of TPH, however in the case of EO it was still almost five times higher than in the control (9205 mg kg<sup>-1</sup> in EO R, 1980 mg kg<sup>-1</sup> in DF R and 2006 mg kg<sup>-1</sup> in C R).

In order to monitor changes in PDSs content in the experimental soil after completion of the experiment (i.e., four years after contamination), an analysis of the soil regarding the hydrocarbon content divided into C<sub>6</sub>-C<sub>12</sub> hydrocarbons (in acc. with the standard PN-ISO 22155: 2013) and C<sub>12</sub>-C<sub>36</sub> hydrocarbons (in acc. with the standard PN-EN ISO 16703: 2011) was made [14]. The amount of C<sub>12</sub>-C<sub>36</sub> hydrocarbons was still much greater in the soil polluted with EO and DF than in the control soil (1000 mg kg<sup>-1</sup> in EO, 750 mg kg<sup>-1</sup> in DF and <6 mg kg<sup>-1</sup> in control). In the case of P it was 12 mg kg<sup>-1</sup>. The biopreparation used caused a significant decrease in C<sub>12</sub>-C<sub>36</sub> hydrocarbons content in all contaminated soils (about two times in the case of EO and P and over three times in the case of DF).

## 2.2. Plants

The seeds of the Windsor White variety of broad bean were sown in the containers at the beginning of April 2013, after earlier preparation of the soil (i.e., loosening and fertilizing) in the amounts provided in the standard seeding rate for that plant, that is, 30 seeds per container. Pre-sowing soil fertilization with NPK fertilizer ('polifoska') was applied, providing 2.88 g N, 3.77 g P, 7.16 g K and 1.30 g S per container. After sprouting, 25 plants were left in each container.

## 2.3. *Aphis fabae* Scop

The observations pertaining to the occurrence of black bean aphids were carried out from the time of noticing the first winged female migrants till the end of their foraging, in intervals of 4–5 days, on 15 randomly chosen and marked plants in each container. The numbers of particular morphotic forms (wingless females, winged females, larvae) and the position of the colonies on plants were recorded. When a colony numbered less than 100 individuals, all of the individuals were precisely counted. With a higher number of aphids, their total number was determined approximately. Additionally, the percentage of plants infested by aphids in each container was determined during each observation.

Moreover, observations of the effect of PDSs on the developmental parameters in the *A. fabae* aphid lifecycle over two generations were carried out, that is, the average lifespan, fecundity, and the population intrinsic growth rate (the effect of bioremediation was not analysed because first of all we wanted to check if the PDSs affect the parameters in any way). For this part of the study four broad bean plants in each container not covered by observation of the occurrence of black bean aphids were used. The observations were conducted with the use of cylindrical cages (12 cm diameter × 20 cm height) made of closely woven airy fabric placed on broad bean leaves (at the same level on each plant to avoid leaf age influence on the pest biology). One cage covered one whole single broad bean leaf used for investigating the life history traits of a single aphid female. The cages were attached to the leaves and carefully closed on both sides in order to avoid the escape of aphids. The investigations were conducted on *A. fabae* individuals from the culture of the Microbiology and Biomonitoring Department maintained on the same host plant, that is, broad bean, Windsor White c.v. Three wingless aphid females were placed in each cage and removed completely once they gave birth to their first larvae. One larva was left in each cage. After it reached sexual maturity, its fecundity was determined every

day, while new larvae were removed each time, except the first one, which was transferred immediately to a new cage in order to determine the demographic indicators of the second generation. Cages used to monitor the second generation were placed on different branches of the broad bean plants, adopting the rule that it should always be on leaves from the same foliage level of the plant in each treatment and generation analysed. Aphid lifespan and fecundity were assessed for 16 females of each generation and treatment. The population intrinsic growth rate was calculated using the formula developed by Wyatt and White [28]:

$$r_m = (0.738 \cdot \ln M_d)/d \quad (1)$$

where:

$r_m$  is the population intrinsic growth rate,

$d$  is the duration of pre-reproductive period (from birth to producing the first offspring),

$M_d$  is the mean number of larvae born in the period from  $d$  to  $2d$  days from birth.

The constant value of 0.738 is an approximation of the proportion of the total fecundity produced by a female in the period from 1 day to 2 days from birth.

#### 2.4. Analysis of the Biochemical Parameters of the Plants

In order to determine antioxidant enzymes activity and antioxidant content, randomly selected plants from each container were taken during the flowering stage, the highest metabolic point during the plant life cycle [29], and the physiological performance of *V. faba* was determined by using the fully expanded, mature and undamaged leaves [17]. Previously, protocols of enzyme activity analyses, as well as proline, non-protein thiols and protein content, were described in detail by Nadgórska-Socha et al. [26,30]. Separate homogenates of leaves were ground in mortar with quartz sand and on ice cold extraction medium containing 100 mM phosphate buffer (pH 6.8) for POD and 50 mM (K/Na) phosphate buffer for CAT and centrifuged at  $12,000 \times g$ , at  $4^\circ\text{C}$  for 20 min. The supernatant was used to determine the enzyme activity levels. The activity of POD was measured at 470 nm according to Fang and Kao [31] using guaiacol as the substrate, and it was expressed in  $\mu\text{mol}$  of tetra-guaiacol  $\text{mg}$  of protein $^{-1}$   $\text{min}^{-1}$ . The catalase activity was assayed using method of Aebi [32] and expressed in  $\mu\text{mol}$   $\text{H}_2\text{O}_2$   $\text{mg}$  protein $^{-1}$   $\text{min}^{-1}$ . The analysis of superoxide dismutase (SOD) activity was established following the procedure described by Beauchamp and Fridovich [33], and it was expressed in U. The total protein was estimated using the Bradford [34] method. To measure the contents of non-protein thiols it was used method of Mass et al. [35]. The proline content was measured using acidic ninhydrin method and it was calculated as described by Bates et al. [36], expressed in  $\mu\text{mol}$  proline  $\text{g}^{-1}$  fresh weight.

#### 2.5. Statistical Analysis

The results obtained were analysed, checked for normality (Shapiro–Wilk test with Lilliefors correction) and equality of variance (Levene’s test). The significance of differences between the means were tested by two-factor variance analysis (STATISTICA 13.1 software), and the means were differentiated by Fisher’s LSD test at  $p < 0.05$ . Principal Component Analysis (STATISTICA 13.1 software) assessed the similarities and relations between *A. fabae* occurrence, biochemical parameters and soil contamination with PDSs.

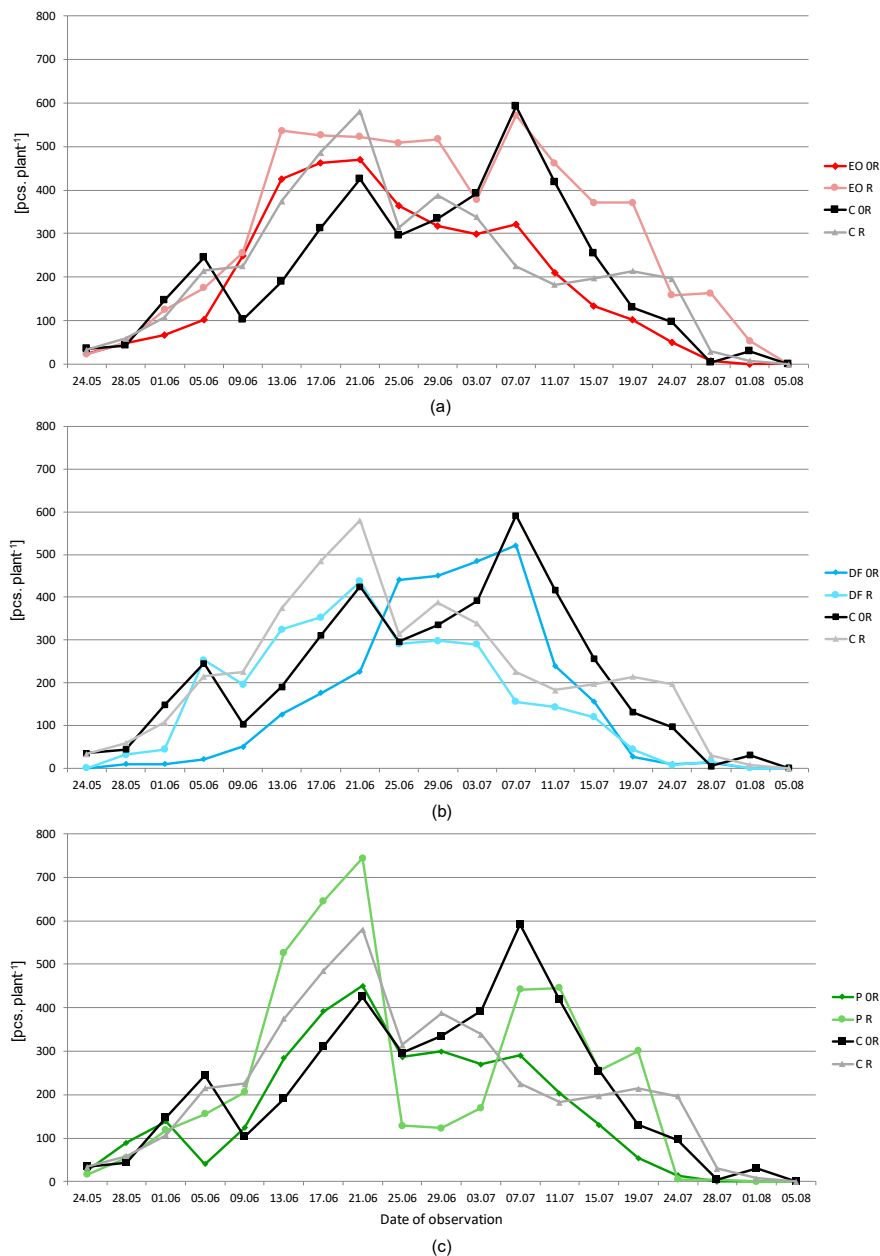
### 3. Results

#### 3.1. Aphids

The analysis of the course of dynamics in the occurrence of aphids on broad bean plants cultivated three years past the time of soil contamination with PDSs demonstrated their appearance on plants in the end of May, with the appearance on plants cultivated on the DF-contaminated soil, it was delayed by approximately 4 days, compared with the appearance of aphids on control plants and on plants



growing on the soil contaminated with EO and P (Figure 1). In the control treatment, the number of aphids increased gradually with certain fluctuations, reaching the maximum on 7 July, and later their number decreased systematically almost to the end of the period of conducted observations. The biopreparation ZB-01 applied to the control soil initially did not caused significant differences in the numbers of pests recorded; however, from around the 10th of June until the end of that month, the mean number of aphids per plant was higher than that on the plants growing on soil without added biopreparation. However, in the subsequent period, the trend was reversed, and in the control treatment with biopreparation application, fewer aphids were noted than on plants without it.



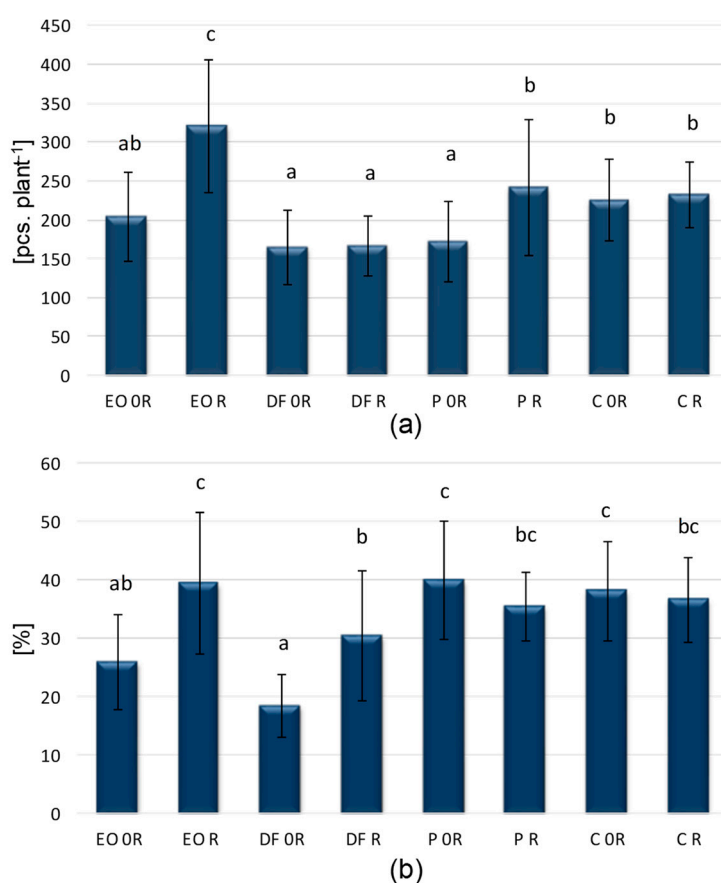
**Figure 1.** The dynamics of *Aphis fabae* Scop occurrence on broad bean plants after three years from the soil contamination with petroleum-derived substances and the use of ZB-01 biopreparation (pcs. plant<sup>-1</sup>); plants exposed to engine oil (a), diesel fuel (b) and petrol (c). EO—soil contaminated with engine oil, DF—soil contaminated with diesel fuel, P—soil contaminated with petrol, C—control soil, OR—without bioremediation, R—with bioremediation.

In the section contaminated with EO, the number of aphids on plants increased with time, reaching their maximum numbers faster than in the control treatment, that is, on the 21st of June (Figure 1a); however, in the later period of foraging, the number of pests was lower than on non-contaminated plants. On plants cultivated on the soil contaminated with EO, when biopreparation was applied, more aphids were noted at almost all dates of observations than on plants growing without the use of ZB-01.

The course of the dynamics of aphid numbers on plants exposed to DF was similar to that in the control treatment (Figure 1b). On the plants growing in the soil undergoing bioremediation, the number of aphids was initially (till the end of June) higher than that on the plants in the soils without biopreparation, but later stayed at a lower level than on plants in the section without the use of ZB-01. Therefore, the trend of changes was very similar to that observed in control plants.

Almost until the end of June, the number of aphids in the P-contaminated section was similar to that on control plants; however, as early as from the beginning of July, a remarkable decrease in their numbers was noted, compared with the control treatment (Figure 1c). The applied biopreparation evidently favoured the occurrence of aphids in the second ten days of June.

In analysing the mean number of aphids on broad bean plants in the season, it was found that all PDSs applied contributed to the decrease in the value of this parameter, and the statistically significant differences compared with the control were found in the case of soils contaminated with DF and P (by 60 and 53 individuals less, respectively) (Figure 2a). The bioremediation resulted in a significant increase in the numbers of aphids on plants in the sections contaminated with EO and P by more than 30%. In the remaining cases, it had no significant effect on the trait analysed.



**Figure 2.** The effect of petroleum-derived substances and ZB-01 biopreparation on the number of *Aphis fabae* Scop. on broad bean plants (pcs. plant<sup>-1</sup>) (average in the research season)—(a) and the percentage of plants infested by *Aphis fabae* Scop. [%] (average in the research season)—(b). Values marked with the same letters do not differ significantly according to LSD test at  $p < 0.05$ . Vertical bars mean SE (standard error). Symbols as in Figure 1.

The soil contamination with DF and EO resulted in a significant decrease in the percentage of broad bean plants infested by black bean aphids, respectively, by 54% and 33% (Figure 2b). Petrol contamination had no significant effect on the trait analysed, and the percentage of plants colonised by pests in this treatment was similar to that in the control (approximately 40%). The biopreparation used caused an increase in the percentage of plants infested by aphids in the sections contaminated with oils (EO and DF). In the cases of the control section and the section contaminated with P, no significant effect of bioremediation on the analysed parameter was found.

There was no significant effect of PDSs upon the proportions of particular morphotic forms of black bean aphids (Table 1). Wingless females constituted an average of approximately 30%, the proportion of winged females did not exceed 1.85%, and the proportion of larvae was generally slightly below 70%. Only in the soil contaminated with DF did the use of biopreparation led to a significant (more than threefold) decrease in the proportion of winged females.

**Table 1.** The effect of petroleum-derived substances and ZB-01 biopreparation on the proportions of particular morphotic forms of *Aphis fabae* Scop. (% of the total number of aphids) (average in the research season).

Treatment	Wingless Females	Winged Females	Larvae
EO OR	29.21 ( $\pm 3.50$ ) <sup>a,*</sup>	1.19 ( $\pm 0.75$ ) <sup>abc</sup>	69.60 ( $\pm 12.58$ ) <sup>a</sup>
EO R	29.57 ( $\pm 7.76$ ) <sup>a</sup>	0.89 ( $\pm 0.33$ ) <sup>abc</sup>	69.54 ( $\pm 10.80$ ) <sup>a</sup>
DF OR	29.14 ( $\pm 5.34$ ) <sup>a</sup>	1.85 ( $\pm 0.50$ ) <sup>c</sup>	69.01 ( $\pm 11.66$ ) <sup>a</sup>
DF R	30.00 ( $\pm 3.88$ ) <sup>a</sup>	0.57 ( $\pm 0.22$ ) <sup>ab</sup>	69.43 ( $\pm 7.42$ ) <sup>a</sup>
P OR	29.52 ( $\pm 6.36$ ) <sup>a</sup>	0.83 ( $\pm 0.57$ ) <sup>ab</sup>	69.65 ( $\pm 9.34$ ) <sup>a</sup>
P R	29.17 ( $\pm 4.14$ ) <sup>a</sup>	0.53 ( $\pm 0.22$ ) <sup>a</sup>	70.30 ( $\pm 3.74$ ) <sup>a</sup>
C OR	29.25 ( $\pm 2.33$ ) <sup>a</sup>	1.58 ( $\pm 0.76$ ) <sup>bc</sup>	69.17 ( $\pm 5.60$ ) <sup>a</sup>
C R	27.94 ( $\pm 1.92$ ) <sup>a</sup>	1.24 ( $\pm 0.58$ ) <sup>abc</sup>	70.82 ( $\pm 4.18$ ) <sup>a</sup>

\* Means  $\pm$  SE (standard error) in columns marked with the same letters do not differ significantly according to LSD test at  $p < 0.05$ . Symbols as in Figure 1.

No significant effect of PDSs on the position of the black bean aphid colonies on broad bean plants was found (Table 2). In all treatments, approximately half of the pests occurred on shoots. The second most frequently occupied position was on the apexes of plants (22–35% of the total number of aphids) and their leaves (3–20%). Particular treatments did not differ either in the numbers of aphids foraging on flowers or pods. In the section contaminated with DF and subjected to bioremediation, a higher percentage of aphids foraged on the apexes of plants, at the expense of those foraging on leaves, compared with plants of the same treatment but without the use of ZB-01. The ZB-01 biopreparation also contributed to a significant increase in the proportion of aphids foraging on shoots at the expense of those foraging on leaves, flowers, and pods in the section contaminated with P.

**Table 2.** The effect of petroleum-derived substances and ZB-01 biopreparation on the position of *Aphis fabae* Scop. colonies (% of the total number of aphids) (average in the research season).

Treatment	Apexes of Plants	Shoots	Leaves	Flowers	Pods
EO OR	25.94 ( $\pm 6.32$ ) <sup>a,*</sup>	52.37 ( $\pm 12.57$ ) <sup>ab</sup>	12.78 ( $\pm 3.22$ ) <sup>ab</sup>	4.99 ( $\pm 1.38$ ) <sup>a</sup>	3.92 ( $\pm 2.38$ ) <sup>a</sup>
EO R	25.39 ( $\pm 4.49$ ) <sup>a</sup>	47.45 ( $\pm 10.77$ ) <sup>a</sup>	20.57 ( $\pm 6.14$ ) <sup>b</sup>	3.28 ( $\pm 0.94$ ) <sup>a</sup>	3.31 ( $\pm 1.76$ ) <sup>a</sup>
DF OR	22.69 ( $\pm 7.13$ ) <sup>a</sup>	44.87 ( $\pm 9.54$ ) <sup>a</sup>	19.84 ( $\pm 5.76$ ) <sup>b</sup>	3.04 ( $\pm 1.20$ ) <sup>a</sup>	9.57 ( $\pm 3.20$ ) <sup>a</sup>
DF R	34.04 ( $\pm 4.64$ ) <sup>b</sup>	54.20 ( $\pm 7.81$ ) <sup>ab</sup>	5.66 ( $\pm 1.27$ ) <sup>a</sup>	0.87 ( $\pm 0.62$ ) <sup>a</sup>	5.22 ( $\pm 2.62$ ) <sup>a</sup>
P OR	33.86 ( $\pm 5.67$ ) <sup>ab</sup>	47.05 ( $\pm 11.26$ ) <sup>a</sup>	9.56 ( $\pm 2.77$ ) <sup>a</sup>	7.00 ( $\pm 2.27$ ) <sup>a</sup>	2.52 ( $\pm 1.68$ ) <sup>a</sup>
P R	35.92 ( $\pm 8.79$ ) <sup>b</sup>	56.44 ( $\pm 5.39$ ) <sup>b</sup>	3.74 ( $\pm 1.89$ ) <sup>a</sup>	3.37 ( $\pm 1.50$ ) <sup>a</sup>	0.52 ( $\pm 0.50$ ) <sup>a</sup>
C OR	30.32 ( $\pm 6.43$ ) <sup>ab</sup>	47.62 ( $\pm 8.26$ ) <sup>a</sup>	14.92 ( $\pm 6.83$ ) <sup>ab</sup>	0.62 ( $\pm 0.39$ ) <sup>a</sup>	6.51 ( $\pm 3.50$ ) <sup>a</sup>
C R	35.87 ( $\pm 7.53$ ) <sup>b</sup>	45.60 ( $\pm 5.65$ ) <sup>a</sup>	11.61 ( $\pm 4.50$ ) <sup>ab</sup>	4.53 ( $\pm 1.71$ ) <sup>a</sup>	2.38 ( $\pm 1.57$ ) <sup>a</sup>

\* Means  $\pm$  SE (standard error) in columns marked with the same letters do not differ significantly according to LSD test at  $p < 0.05$ . Symbols as in Figure 1.



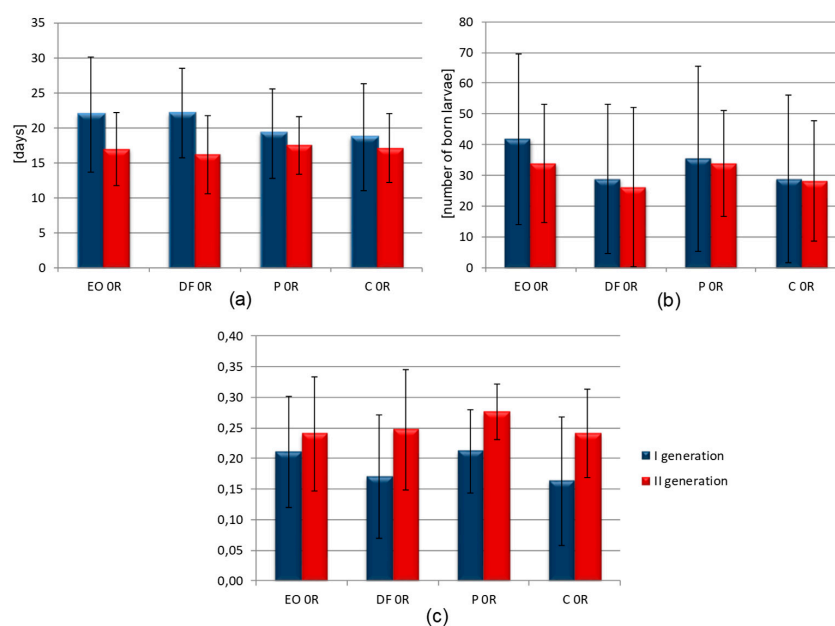
The length of the pre-reproductive period (d) in *A. fabae* ranged from 11.13 to 11.75 days for the first generation, and from 8.69 to 9.25 days for the second generation (Table 3). None of the PDSs applied had any significant effect on the value of that parameter. The mean number of larvae born by females during the reproduction period equal to d (parameter  $M_d$ ) in the first generation was the highest in the section contaminated with EO (41.78), and for the second generation—in the section contaminated with DF (34.00). Similarly to the previously analysed case, no significant effect of PDSs on the value of parameter  $M_d$  was found.

**Table 3.** The effect of petroleum-derived substances on some biological parameters (d—duration of pre-reproductive period,  $M_d$ —mean number of larvae born in time = d) of *Aphis fabae* Scop.

Details	d		$M_d$	
	I	II	I	II
EO	11.44 (±0.81)	8.69 (±0.59)	41.78 (±27.68)	28.31 (±19.34)
DF	11.75 (±4.42)	8.88 (±0.74)	28.80 (±24.30)	34.00 (±25.77)
P	11.19 (±0.98)	9.06 (±0.25)	35.40 (±30.22)	33.93 (±17.25)
C	11.13 (±1.41)	9.25 (±0.68)	26.66 (±27.17)	26.19 (±19.57)

Symbols as in Figure 1. There were no statistically significant differences between treatments.

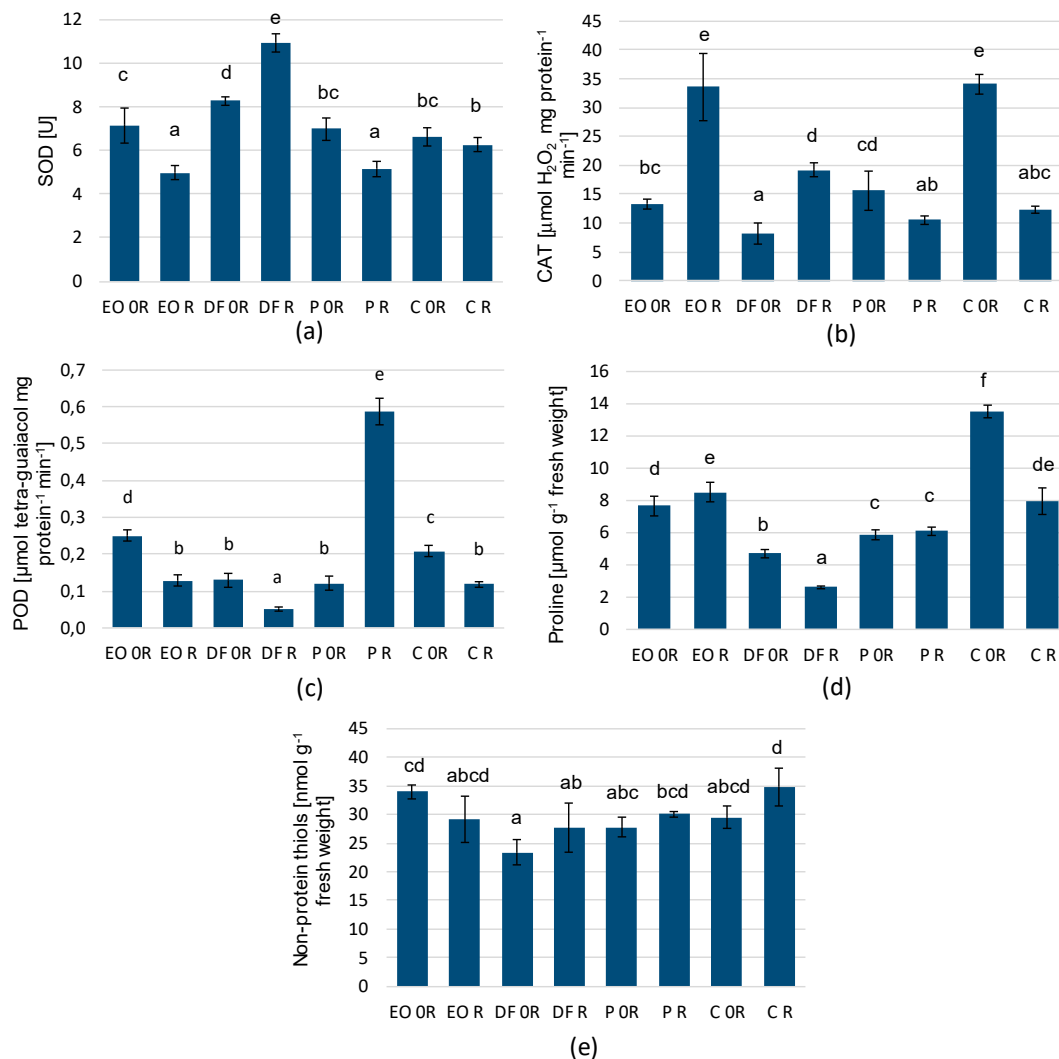
There was no significant effect of any of PDSs upon the mean lifespan in black bean aphids, their fecundity, and on the population intrinsic growth rate in both the first and second generation of the analysed pest (Figure 3). The mean lifespan of females in the first generation amounted to approximately 20 days. In the second generation, it was shorter by more than three days (Figure 3a). The fecundity of the first-generation females ranged from 28.84 larvae born by one female in the section contaminated by DF to 41.78 larvae in the section contaminated with EO (Figure 3b). The fecundity of the second-generation aphids was slightly lower than that of the first-generation aphids. Furthermore, it was noted that the population intrinsic growth rate in black bean aphids was higher in the second generation than that in the first generation in all treatments analysed, but it did not exceed 0.3 (Figure 3c).



**Figure 3.** The effect of petroleum-derived substances on: mean lifespan [days]—(a); mean fecundity—(b); population intrinsic growth rate ( $r_m$ )—(c) of *Aphis fabae* Scop. Vertical bars mean SE (standard error). Symbols as in Figure 1. There were no statistically significant differences between the treatments.

### 3.2. Analysis of the Biochemical Parameters of the Plants

All PDSs caused a significant decrease in the activity of CAT (Figure 4b), as well as a decrease in the content of proline (Figure 4d) in the broad bean plants; however, they had no effect on the content of non-protein thiol groups (Figure 4e). DF contributed to the increase in the activity of SOD (Figure 4a), but such a relationship was not noted in the case of the remaining PDSs. Both DF and P led to an almost twofold decrease in the activity of POD (Figure 4c).

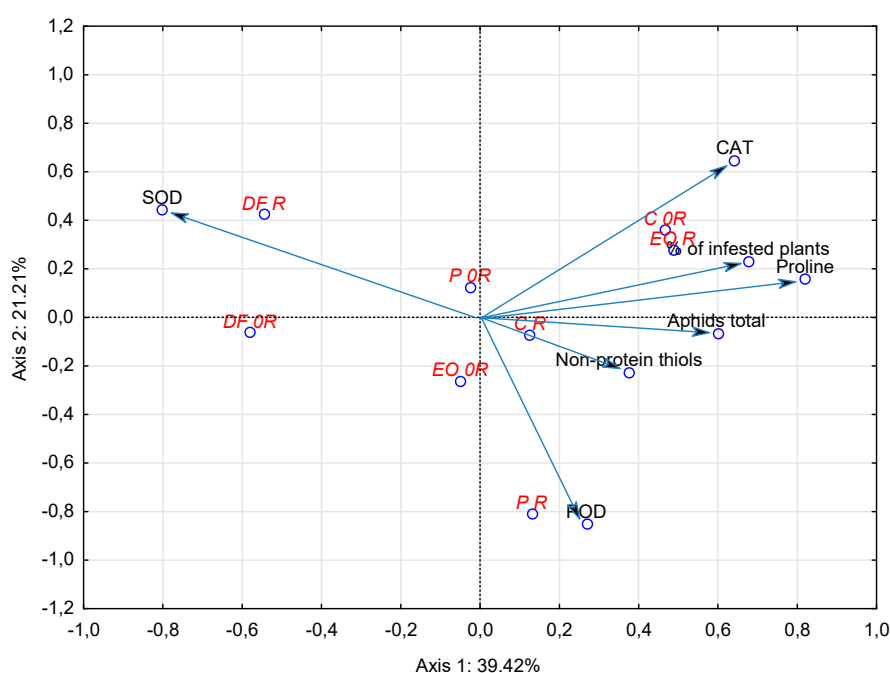


**Figure 4.** The effect of petroleum products and ZB-01 biopreparation on the activity of antioxidant enzymes: (a)—superoxide dismutase, (b)—catalase, (c)—guaiacol peroxidase, and antioxidants: (d)—proline, (e)—non-protein thiols in broad bean leaves. Values marked with the same letters do not differ significantly according to LSD test at  $p < 0.05$ . Vertical bars mean SE (standard error). Symbols as in Figure 1.

On the one hand, ZB-01 biopreparation applied to the soil contaminated with EO caused an increase in the activity of CAT and an increase in the content of proline in plant leaves. On the other hand, it led to a decrease in the activities of SOD and POD. In the section contaminated with DF, the biopreparation caused a decrease in the activity of POD and in proline content, but it also contributed to an increase in the activities of SOD and CAT. In the section contaminated with P, the biopreparation led to a more than a fivefold increase in POD, but the activities of SOD and CAT were significantly decreased. In the case of control treatment, the biopreparation caused a general decrease in the activities of anti-oxidant enzymes and in the proline content in the broad bean plants.

### 3.3. Relationships between *Aphis fabae* Occurrence, Biochemical Parameters and Soil Contamination with PDSs

Principal component analysis (PCA) of *A. fabae* occurrence, the biochemical parameters of the plant and soil contamination with PDSs showed that the 1st and 2nd ordination axes together explain 60.63 percentage of variation, dividing the samples into two sets (set one: DF R, DF 0R; set two: EO R, C 0R, P R) (Figure 5). Percentage of plants infested by *A. fabae* and the total number of aphids per plant, as well as CAT activity and proline content, were positively correlated with the 1st axis, while the activity of SOD was negatively correlated with this axis. POD activity was negatively correlated with 2nd ordination axis. The PCA analysis confirmed negative influence of DF on aphids as well as POD and CAT activity and proline and non-protein thiols contents, while SOD activity increased in DF treatment.



**Figure 5.** Principal component analysis of *Aphis fabae* occurrence, biochemical parameters and soil contamination with PDSs. Symbols as in Figure 1.

## 4. Discussion

The course of changes in dynamics of the occurrence of black bean aphids on the broad bean plants was not principally different from that described in the literature, although the period of foraging can be considered to be fairly long [37,38]. The percentage of broad bean plants infested by aphids at the time of the highest intensity of the pest occurrence can reach to 80–100% according to published data, with the mean for the season at approximately 40%, depending on the variety [37,39,40]. In the experiment conducted, the value of the analysed parameter in the control treatment showed similar levels. Initially, the aphids colonise the apexes of plants, and then they move onto shoots and flowers, to appear on leaves and pods in the final stage of foraging. The highest numbers of aphids appear usually on shoots and the apex, fewer appear on leaves, and the least appear on the generative parts [39]. All of these observations were confirmed in the experiment conducted.

Davies et al. [41] found that, under laboratory conditions, the larvae of black bean aphids reach sexual maturity as early as after  $5.85 \pm 0.35$  days; however, under field conditions, this period is usually extended and is generally between 7.65 and 11.3 days [18,40]. In our studies, the period amounted to 11.13 days for the first generation, and 9.25 days for the second generation. The fecundity of the wingless females of *A. fabae* foraging on broad bean plants ranges between 15.3 to 59.2 larvae born [37,42], and their lifespan amounts to 16–25 days [18,40,42,43]. In our experiment, the females

foraging on control plants gave birth to an average of approximately 29 larvae, and their lifespan was approximately 16–18 days. According to various authors [18,40,43], the intrinsic growth rate in the population of black bean aphids foraging on broad bean plants ranges from 0.159 to 0.345, and it is different in different generations of the insect. In our experiment, the rate in the first generation amounted 0.16 to 0.21 and from 0.24 to 0.28 in the second.

The soil contamination with DF and P contributed to a significant reduction in the mean number of aphids on broad bean plants (by an average of approximately 30%), while the percentage of plants infested by aphids in the treatments with EO and DF was lower by approximately 30% and 50%, respectively, compared with the control treatment. The reason behind the different degrees of the infestation of broad bean plants by aphids can be their different attractiveness at the time of in-flight of winged individuals, and, by this token, the number of plants colonised can be different, as well as the number of migrating females that decide to stay on a given plant. Yet another cause can appear as early as during the period of permanent foraging by the pest and can be a consequence of the effect of forage quality upon the rate of the pest population growth. The third possibility can pertain to discouraging aphids to forage on contaminated plants and their earlier migration to other host plants. In this latter case, it could appear as an increased number of winged individuals on contaminated plants, but this was not found in the experiment conducted.

During our experiment we also checked the second possibility, that is, the influence of quality of the host plant on developmental parameters. As indicated by the data available in earlier publications, the effect of PDSs upon the developmental parameters of aphids depends on the type of contaminating substance, its dose, as well as on the species and generation of pest. Rusin et al. [18] demonstrated that, with soil contamination with PDSs, the duration of the pre-reproduction period in *A. fabae* aphids can extend up to 12.2 days. Soil contamination with EO or DF, as well as with P in the dose of  $9 \text{ g kg}^{-1}$ , results in the reduction of fecundity and of the lifespan of *A. fabae* by up to 70% and 30%, respectively, as well as in almost complete inhibition of the population intrinsic growth rate [18]. The lower doses of PDSs ( $6 \text{ g kg}^{-1}$ ) result in the reduction of fecundity by nearly 25%, but they do not affect the lifespan of wingless *A. fabae* females [40]. The adverse effects of PDSs upon the length of life, fecundity, and the population intrinsic growth rate were also confirmed with respect to *Rhopalosiphum padi* L. aphid [19]. In the experiment presented here, there were no significant effects of any of the PDSs applied upon the duration of the pre-reproduction period, or upon the remaining developmental parameters of two subsequent generations of black bean aphids. The differences can stem from the time passed from the instants of soil contamination and of course a dose. Despite the similar dose ( $6 \text{ g kg}^{-1}$ ) our experiment was conducted three years after contamination (which means that the contaminants could be partially decomposed), whereas the experiments referenced to above were conducted immediately after the soil contamination event. In available publications, there is no information about a follow-up (remote in time) effect of the soil contamination with PDSs upon developmental parameters of herbivores.

The PDSs often contribute to the weakening of growth and development of cultivated plants, and they modify the contents of heavy metals, and macro- and micro-components in these plants, which can result in the worsening of the pest forage quality (decreased content of phosphorus, nitrogen, protein, and chlorophyll) [18,19,44,45], and this can also be associated with the modification of the process of colonising the cultivars. The adverse effects of PDSs can also be associated with the migration of harmful substances to the aboveground parts of plants, which are principal places of foraging by aphids [39]. In the initial stage of observations, the numbers of aphids on the broad bean plants was generally higher on the control plants, which could testify to the fact that aphids were more willing to colonise healthy plants, with greater vigour. This confirms that the possible reason for reduction in aphid infestations under the influence of PDSs could be the different attractiveness of host plant at the time of in-flight of winged individuals.

In available publications, there is no information pertaining to the effect of the bioremediation upon the foraging by herbivores on plants growing on contaminated soil. The available studies in this field pertain principally to soil organisms. These studies indicate, however, that the use of

biopreparations can level the adverse effect of PDSs [27,46,47], which was also confirmed in the present experiment, particularly with respect to the soil contaminated with EO and P. The numbers of aphids on the plants growing on soil in those treatments subjected to bioremediation was similar to the numbers of aphids on control plants, or was even higher. Moreover, in the case of the plants exposed to DF, after applying ZB-01 biopreparation, the percentage of plants colonised by aphids increased significantly, as well as the numbers of aphids foraging on the apexes of plants. The proportion of winged females in the aphid population decreased in this case. All this indirectly indicates the improvement of feeding conditions due to the use of biopreparation.

The experiment conducted demonstrated that the PDSs had significantly affected the activities of antioxidative enzymes and the content of antioxidants in the leaves of broad bean plants. Both the oils (EO and DF) and P led to a more than a twofold decrease of the activity of CAT. Similar regularities in the application of oils were noted in the study by Rusin et al. [14], where the test plant was winter wheat and the measurements were obtained four years after the instance of soil contamination. Tabassum et al. [48] showed an almost threefold decrease in the activity of CAT in the aboveground parts of *Mirabilis jalapa* under the effect of used engine oil at a 2% concentration, which also corresponds to our results. It is worth mentioning, however, that the cited authors conducted their studies 48 days after the instance of soil contamination; and, in the present experiment, this effect still continues after three years. Achuba's [49] investigation indicated that contaminations by petroleum products (kerosene, diesel, engine oil, and petrol) at 2% concentrations caused a decrease by approximately 10% of CAT activity in the leaves of cowpea and maize seedlings, with these studies continuing only up to 12 days after the soil contamination.

Soil pollutants such as heavy metals (whose source can also be PDSs) often result in the increased activity of POD in plants [26,50,51]. A similar effect was noted in our experiment, only with regard to the soil contamination with EO. The remaining PDSs caused a decrease in the activity of this enzyme in the leaves of the broad bean plants. Marti et al. [15] noted the lower activity of POD in the leaves of alfalfa growing on the soil contaminated by sediments from a refinery containing major quantities of hydrocarbons. However, Rusin et al. [14], in their experiments on winter wheat, noted reversed relationships, i.e., the increase of the POD activity by nearly 20% under the effect of DF and P, and the decrease by 20% in the case of EO. The obtained discrepancies are indicative of the fact that the activities of antioxidative enzymes in plants depend on many factors, such as plant species, tissues analysed, and the conditions of the experiments, for example, pollutant concentration, and the type of pollutant [15,52–54].

In the present experiment, DF caused a significant increase in the activity of SOD in broad bean leaves. Dazy et al. [29] also found that the activity of this enzyme in *Oenothera biennis* plants increases under the effect of the presence of PAHs and heavy metals. At the same time, the above authors demonstrated that the phenomenon was accompanied by the limiting of plant growth and a decrease in the biomass quantity. Similar regularities were also noted in the leaves of alfalfa growing on the soil contaminated by hydrocarbons and heavy metals [15] and in the leaves of *Mirabilis jalapa* growing on EO-contaminated soil [48]. Shen et al. [55] found that SOD next to carotenoids are the main effective antioxidants when H<sub>2</sub>O<sub>2</sub> and ROS increase in leaf tissues and cells at the condition of PAH (polycyclic aromatic hydrocarbons) accumulation.

Soil contamination by heavy metals can cause an increase in the proline content in plants [26,51,52,56]. In our experiment, we noted a reverse relationship, that is, all PDSs applied caused significant decreases in the content of proline in the leaves of broad bean plants. In the studies performed by Rusin et al. [14] pertaining to the effects of PDSs upon the antioxidant responses in winter wheat, it was found that EO causes a significant decrease in the proline content in the leaves of plants, while DF and P cause its increase. Nadgórska-Socha et al. [30] emphasised that the content of that antioxidant depends on the species of plant. The authors demonstrated that, within the area contaminated with heavy metals, the proline content in the leaves of *Cardaminopsis arenosa* increased,

but, in the leaves of *Plantago lanceolata*, it decreased. Next, the soil contamination with cadmium and lead cause the decrease in the content of proline in the leaves of *Brassica juncea* [57].

No significant effect of PDSs upon the content of non-protein thiol groups was found. The data concerning the effect of PDSs and heavy metal soil contamination upon the content of -SH groups is discrepant depending on the species of plant and the kind of contaminant [14,26,51,58]. Sulphur-containing molecules occur in many plant cells where they perform a multitude of various functions, owing to which they can be independently regulated.

The effect of ZB-01 biopreparation upon the antioxidative enzymes and on the content of antioxidants in the leaves of broad bean plants was diverse and depended on the type of contaminant and on the type of enzyme or antioxidant. On the one hand, ZB-01 caused a decrease in the activities of SOD and POD in the plants exposed to EO, which can testify to the decreased level of stress in plants; however, on the other hand, it caused an increase in the activity of CAT and an increase in proline content in this treatment. In these cases, it just showed the action somewhat levelling the changes caused by that PDS. In the case of DF, however, the biopreparation caused a more intense decrease in the activity of POD and the content of proline, as well as a further elevation of SOD activity. Its effect had the nature of levelling the effect of DF only in the case of CAT. In the section contaminated with petrol, ZB-01 led to a decrease in the activities of SOD and CAT, but to increase in the case of POD. In the control treatment, it generally caused a drop in the activities of antioxidative enzymes, and in the content of proline. The diverse effects of biopreparation upon these parameters was also demonstrated in the study by Rusin et al. [14] with respect to winter wheat, which suggests that the research in this area should be continued.

The effect of aphid presence on the induction of antioxidant response in host plants has been demonstrated in many published studies [51,59–61]. Additionally, Kerchev et al. [59] showed that the perturbations in the leaf antioxidant system are intrinsic to the potato leaf response to peach-potato aphid (*Myzus persicae* Sulzer). In the available literature on the topic of the relationships between the activity of antioxidating enzymes and antioxidant content in plants, which were previously changed as a result of the stress factors, information on the infestation of plants by aphids is very scarce, and with respect to contaminants, there is no data at all. In their analysis of the relationship between antioxidants induced by nymphs of *B. tabaci* feeding on tobacco and aphid resistance, Zhao et al. [22] point at CAT as the main enzyme responsible for inducing tobacco resistance to aphids. Next, He et al. [21] indicated SOD and POD as the enzymes responsible for the resistance of two varieties of chrysanthemum to aphids. The activities of these two enzymes increased rapidly immediately after aphid infestation and remained at a high level to as late as after 168 h after inoculation. The studies conducted by us found that the lowest number of aphids was noted on plants exposed to DF. At the same time, in those plants, an increased activity was noted, but only in the case of SOD, while remaining enzymes showed decreased activities. The proline content was also evidently lower. The levelling effect of ZB-01 biopreparation towards the changes in enzyme activities under the conditions prevailing in the soil contaminated with EO brought about a significant increase in the number of aphids, which was not noted in the object contaminated with DF, where ZB-01 led to an even further increase in SOD. Thus, it could confirm the role of this enzyme in the process of plant infestation, as suggested by He et al. [21].

## 5. Conclusions

In the three years after the instance of soil contamination, the PDSs studied had a limiting effect on the degree of infestation of broad bean plants by *A. fabae*, which was demonstrated by the lower percentage of plants infested by aphids (EO and DF contaminated plants) and the lower numbers of aphids on plants (all PDSs).

It was not demonstrated if any observed differences in the aphid numbers had been the results of adverse effects of applied substances on the developmental parameters in aphids, such as lifespan, fecundity, or the population intrinsic growth rate. The lower degree of infestation by aphids of broad bean plants exposed to PDSs was probably the effect of a lower number of aphids starting the



development of a colony, which, in its turn, could result from the lower attractiveness of contaminated plants to *A. fabae*.

The PDSs significantly affected the activities of antioxidating enzymes as well as the content of antioxidants in the leaves of broad bean plants, with that effect being diversified, depending on the kind of PDS and the enzyme or antioxidant analysed. Increased activity of SOD was found in plants exposed to DF, together with the lowest numbers of aphids accompanying it, which could suggest a certain role of the enzyme in pest response to the stress caused by this PDS.

The ZB-01 bioreparation partly levelled the adverse effect of PDSs on the degree of broad bean plant infestation by *A. fabae*. Its influence on the activities of antioxidative enzymes and the content of antioxidants was diversified and depended on the kind of contaminating substance, and on the kind of enzyme or antioxidant. In the plants exposed to EO the changes in antioxidant response in *V. faba* caused by this PDS were reduced under the influence of ZB-01.

**Author Contributions:** Conceptualization, J.G. and A.N.-S.; Investigation, M.R., J.G. and A.N.-S.; Methodology, J.G. and A.N.-S.; Writing—original draft, M.R., J.G. and A.N.-S. All authors have read and agreed to the published version of the manuscript.

**Funding:** Scientific publication financed from the funds for science in 2009–2013 as a research project (N N305 151537) and by the Ministry of Science and Higher Education of the Republic of Poland.

**Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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