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Influence of diesel and biodiesel fuel-contaminated soil on microorganisms, growth and development of plants

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ABSTRACT

The paper presents the results of studies concerning the phytotoxicity of biodiesel and its diesel oil blends with a germination and root elongation test. The paper also analyses the effect of fuel on the number and activity of soil microorganisms and the reaction of plants used in the research. Fuel was introduced into the soil at a concentration of 10 and 50 g/kg dry mass soil. Based on the test results, it was found that from among 19 plants species representing 5 families taxonomically, only 4 species showed resistance to the presence of the fuel in soil, regardless of their type and dose (*Glycine max* (L.) Merill, *Helianthus annuus* L., *Lupinus luteus* L., cv. Lord and *Pisum sativum* L., cv. Eureka). Fuel generally reduced the number of heterotrophic microorganisms, and stimulated the growth of decomposing microorganisms and content of biomass. Significant differences in the number and activity of microorganisms were associated with the presence of biodiesel in the soil. The fuel had a negative influence on the biometric and physiological parameters of plants. A shorter length of shoots and roots was noted, especially in objects with biodiesel, reduced water content, and general content of assimilation pigments.

Keywords: contamination; petroleum; remediation; bioindicator; microflora; microbial biomass

Soil contamination with petroleum compounds reduces the growth of plants, among others, by the inhibition of germination and growth, photosynthesis, and respiration processes. The anatomical changes of roots, deformation of cells, reduction of the amount of root hair, vascular obstruction, and oil accumulation in tissues and their dehydration were observed (Siddiqui and Adams 2002, Ziółkowska and Wyszkowski 2010). The presence of petroleum hydrocarbons in the soil also influences the number and activity of the microorganisms colonising it, wherein the reaction of microorganisms depends on the type of contamination, although it mostly depends on its concentration in the environment (Hawrot-Paw 2011a). Petroleum hydrocarbon contamination changes the carbon/nitrogen ratio. The presence of carbon promotes the growth and development of many microorganisms, although the lack of C:N balance may lead to the immobilisation of nitrogen by microbial biomass, making it unavailable to plants (Adam and Duncan 2003). Reduction of the negative impact of petroleum derivatives on the environment should also be favoured by the use of ecological fuels such as biodiesel, which has better properties compared to conventional fuel; it is non-toxic and almost free of sulphur and aromatic compounds (Demirbas 2009).

Populations of microorganisms are an integral part of the soil and their activity, among others, in the transformation processes of many chemical substances, which is essential for its proper functioning (Watanabe et al. 2002, Winding et al. 2005), while phytotoxicity tests are not only the method of plant selection with the desired remediation properties. They are also a way of finding potential bioindicators of the presence of fuel in the environment and an assessment method of the effectiveness of the conducted remediation treatments (Hawrot and Nowak 2005).

The aim of this study was to determine the phytotoxic influence of biodiesel and its mixtures with diesel fuel on the growth of plants and the relationship between soil pollution and the reaction of soil microflora and plants.

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MATERIAL AND METHODS

Within the study, experiments evaluated the phytotoxicity of fuel (experiment No. 1) with untreated seeds of 19 plants species (several of them in different cultivars), belonging to 5 families (Asteraceae, Fabaceae, Brassicaceae, Polygonaceae, Poaceae), and the influence of the contamination on soil microflora and selected biometric and physiological parameters of plants (experiment No. 2). The influence of diesel oil and biodiesel or their mixture was assessed with the germination and root elongation test (Włodkowic and Tomaszewska 2003). Clean fuel was introduced to the soil (B0 – diesel fuel, B100 – biodiesel) and their mixture in suitable proportions – B5, B20, B50 (the number determines the percentage share of biodiesel in the mixture) – in two doses: 10 and 50 g/kg dry mass (DM) soil. The experiment involved three repetitions for each variant. The obtained values were substituted into the formula given below, determining the germination index (%, GI) for each tested plant species. A germination index value over 100 proves the stimulating impact of fuel:

$$GI = \frac{100 \times (G_S \times L_S)}{(G_C \times L_C)}$$

G_S and G_C – number of seeds that germinated in the research sample and in the control sample, L_S and L_C – length of roots in the research sample and in the control sample.

In experiment No. 2, soil was divided into samples weighing 900 g, polluted with fuel in the dose of 50 g/kg DM soil, and then placed in pots with the capacity of 1000 g. In the pots, at a depth of 1.5 cm, seeds of pea (*Pisum sativum* L., cv. Eureka), the plant selected in experiment No. 1, were sown. For each treatment of the experiment, 8 pots were prepared. Seeds were planted in half of the pots (P) and half was used without plants. In the study, AGRO 400 sodium lamps were used. The daily photoperiod was determined at 12 h day/12 h night. The pots were incubated at temperature ± 20°C. After 43 days, soil samples were taken from the plant rhizosphere zone. Heterotrophs of the nutrient agar medium (Biocorp) after 48-h incubation at 28°C and the number of diesel and biodiesel decomposing microorganisms in the Bushnell-Haas medium (Bushnell and Haas 1941) with diesel or biodiesel fuel at a dose of 1% (v/v), after 7 days of incubation in 28°C, were determined using the plate method. The activity of microorganisms was determined based on the content of biomass by the physiological method (Anderson and Domsch 1978). The number and activity of microorganisms were measured in three repetitions.

Selected biometric and physiological parameters of the tested plant were also determined in the experiment: the length of the ground parts and root elongation (mm), and water balance according to Bandurska (1991) using two indicators: RWC – relative water content, and WSD – deficit of water saturation, content of assimilation pigments (chlorophyll *a*, chlorophyll *b*, total chlorophyll *a* + *b*, carotenoids) based on the method of Lichtenthaler and Wellburn (1983). Biometric measurements were taken from all plants in each pot, for all variants of the experiment. All analyses of physiological parameters were done in three repetitions.

The analysis of variance (ANOVA) and the Tukey's test at the $P < 0.05$ level were used to analyze the experimental results. Statistical calculations were carried out using the Statistica 10.0 program (StatSoft, Krakow, Poland).

RESULTS AND DISCUSSION

The ability of the studied plants to germinate in soil contaminated with fuel was varied and largely depended on the plant species. Most of the plants were not resistant to fuel presence in the soil. Only 4 plants showed the tendency for growth in the contaminated environment, regardless of the type of fuel present in the soil (Table 1). Similar results, in which the species from the family of Fabaceae (lucerne and beans) showed the greatest germination index, were obtained by Hawrot-Paw and Hreczuk (2009). According to Adam and Duncan (2002), the degree of germination inhibition and plant growth depends not only on the plant species, its cultivar, time of exposure, and contamination concentration, but also on the volatile content of the fuel fraction. The stimulating effect of biodiesel in the mixture with diesel fuel was visible mainly with its 20% and 50% addition, both in lower and higher dose of contamination. This is likely to be the result of a lower toxicity of biodiesel in relation to diesel oil (Lapinskiene et al. 2006). Conventional fuel has an adverse effect on the water-air relations in the soil, creating an impermeable oily film layer around the seeds or

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Table 1. Germination and elongation index

Plant	Cultivar	Dose of fuels (g/kg)									
		10					50				
		B0	B5	B20	B50	B100	B0	B5	B20	B50	B100
Helianthus annuus L.		171	217	199	227	118	161	261	174	252	222
Pisum sativum L.	Eureka	256	277	331	244	266	239	304	241	215	270
Pisum sativum L.	Tarchalska	93	84	101	79	109	64	80	74	71	99
Trifolium repens L.	Haifa	82	81	87	88	79	59	74	69	70	96
Trifolium hybridum L.	Aurora	63	69	64	67	55	43	60	54	57	102
Trifolium pratense L.	Raba	66	84	96	107	124	70	101	90	111	76
Trifolium resupinatum L.		67	62	66	66	92	63	74	88	85	59
Medicago sativa L.	Beda	38	44	37	67	99	49	72	87	117	75
Medicago sativa L.		59	95	99	113	50	61	94	362	91	83
Lupinus angustifolius L.	Bajor	77	69	85	100	85	44	51	51	28	87
Lupinus luteus L.	Lord	138	130	149	155	123	117	147	157	167	145
Glycine max (L.) Merill		149	214	186	203	188	175	195	154	206	195
Vicia sativa L.	Hanka	114	132	105	120	91	89	106	114	130	95
Sinapis alba L.	Maryna	124	80	105	91	95	80	65	71	66	93
Brassica juncea (L.) Czern.		111	82	110	109	103	93	78	82	95	101
Brassica napus L. (partim)	Markiz	109	78	138	129	86	140	118	153	141	61
Fagopyrum esculentum Moench	Hruszowska	80	70	56	64	76	41	40	58	71	73
Fagopyrum esculentum Moench	Kora	102	70	107	78	100	72	91	79	66	51
Hordeum vulgare L.	Antek	74	64	70	79	64	45	80	51	75	49
Festuca rubra L.		89	69	36	92	73	47	40	57	32	61
Phleum pratense L.		57	63	78	89	66	23	32	64	64	50
Secale cereale L.	Caroass	82	70	63	67	63	69	60	77	85	49
Secale cereale L.		32	47	32	58	66	83	70	70	43	33

B0 – pure petroleum diesel; B5 – 5% biodiesel and 95% petroleum diesel; B20 – 20% biodiesel and 80% petroleum diesel; B50 – 50% biodiesel and 50% petroleum diesel; B100 – pure biodiesel

roots, and interfering with proper germination and growth of plants (Adam and Duncan 2002, Ziółkowska and Wyszkowski 2010).

For experiment No. 2, pea (cv. Eureka) was selected, which is characterised by the highest resistance to contamination, and the germination index reached its highest values. After 43 days of incubation in the objects, in which diesel and its mixture with biodiesel was introduced to the soil, a reduction in the number of heterotrophic microorganisms (Figure 1a) was noted. A different effect of conventional fuel and biofuel on soil microflora was observed by Hawrot and Nowak (2004) and Hawrot-Paw (2011a). According to Baran (2000), contamination with petroleum compounds distorts the ratio of carbon to nitrogen and phosphorus in the soil, and their scarcity makes some microorganisms fully use the energy contained in hydrocarbons, hence the inhibition of their growth and development is possible. Above the control values, the number of cells increased only in object B50, although this was a non-significant change. The beneficial effect of the presence of plants was observed in object B0 + plants (P). The presence of diesel and biodiesel in the soil stimulated the development of microorganisms, which use the components of fuel as the source of carbon and energy (Figures 1b,c). Values below the control ones were only observed in object B0 for biodiesel decomposing microorganisms. With the increasing share of the biocomponent, there was a reduction in the number of bacteria. In the experiment, the highest number was determined in object B5 for diesel degrading microorganisms and B20 in the case of biodiesel degrading microorganisms. The plants had a beneficial influence on the growth and develop-

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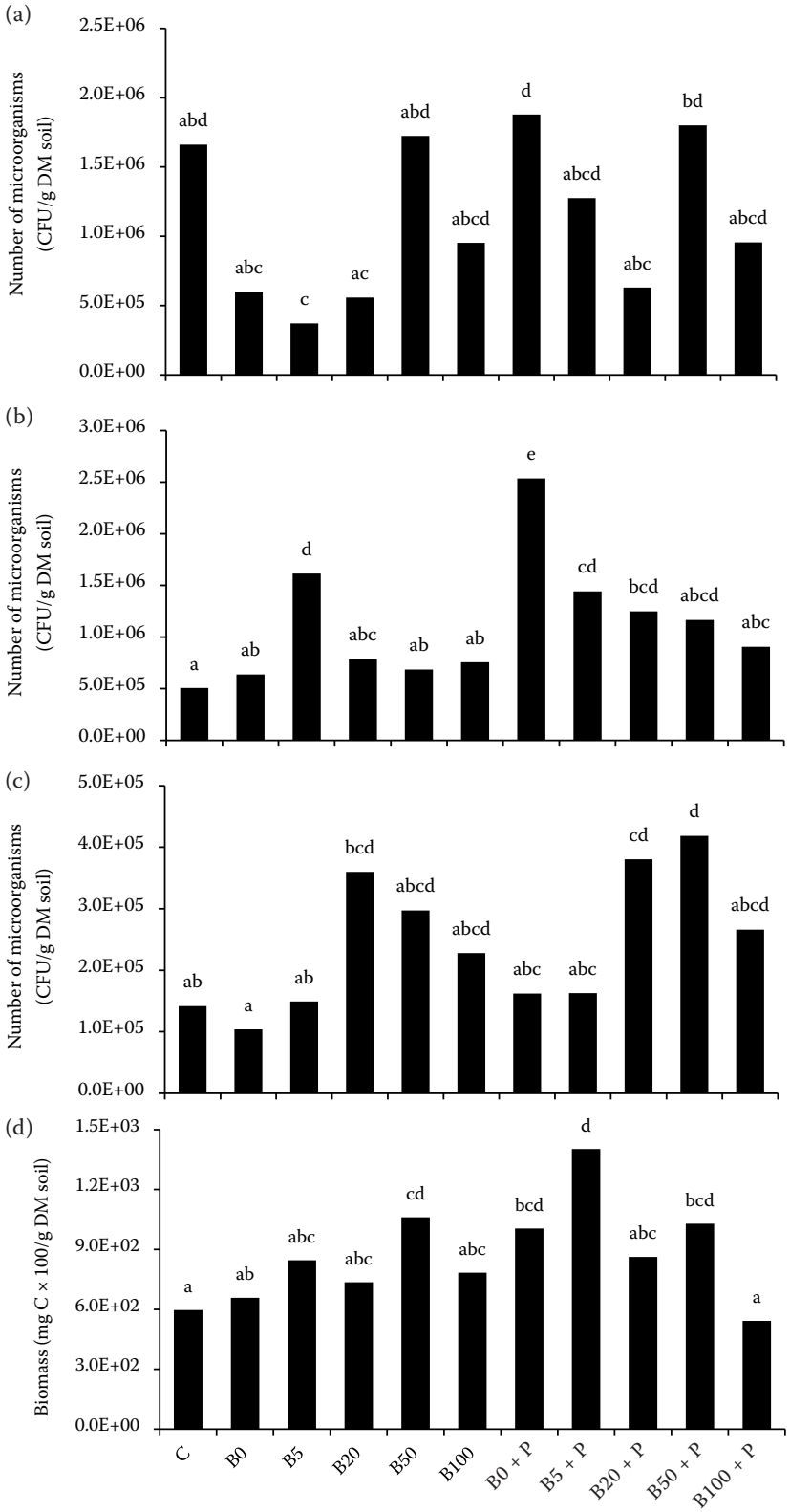

Figure 1. The number of (a) heterotrophic microorganisms; (b) diesel-oil degrading microorganisms; (c) biodiesel degrading microorganisms in 1 g dry mass soil, and (d) the content of the living organisms biomass. Mean over each column not marked with the same letter is significantly different at $P < 0.05$. C – control; B0 – pure petroleum diesel; B5 – 5% biodiesel and 95% petroleum diesel; B20 – 20% biodiesel and 80% petroleum diesel; B50 – 50% biodiesel and 50% petroleum diesel; B100 – pure biodiesel; B0 + plants – pure petroleum diesel; B5 + plants – 5% biodiesel and 95% petroleum diesel; B20 + plants – 20% biodiesel and 80% petroleum diesel; B50 + plants – 50% biodiesel and 50% petroleum diesel; B100 + plants – pure biodiesel; CFU – colony forming units

ment of microorganisms. Statistically significant changes, in comparison to the contaminated soils without the plants, were only noted in object B0 + P, for diesel-degrading microorganisms.

The content of the biomass of living organisms, like enzymatic activity, can serve as an indicator of soil contamination and its biological activity (Hawrot-Paw et al. 2010). The presence of diesel oil may stimu-

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late (Hawrot-Paw 2011b) and reduce the content of biomass (Hawrot-Paw and Martynus 2010). After 43 days from the introduction of fuel into the soil, the biomass content in all objects apart from B100 + P was higher compared to the control. Biodiesel in the mixture with diesel increased the biomass content of microorganisms in the soil covered with vegetation, although over a biodiesel content of 50%, the biomass of microorganisms decreases. Pure biodiesel, both in soil with plants and without them, caused 4% and 7% inhibition, respectively.

The plants can stimulate the microbiological activity of soils (Reilley et al. 1996). The multiplication of microorganism in the root zone of the plants may be the result of the increased availability of nutrients produced by the plants, which also improves the oxygen conditions of the soil (Schwab et al. 2006). In the conducted microbiological studies, in the rhizosphere area of the pea plants, stimulation of the development of heterotrophic microorganisms (over 30% increase), degrading diesel fuel (over 85% increase), and the content of biomass in the soil contaminated with pure diesel oil by over 20% in relation to the control and soil not covered with vegetation were noted. Similar effects were noted by Merkl et al. (2005) studying the effect of diesel fuel and growing legumes on the change of the number of microorganisms in the soil.

The presence of fuel had a negative effect on the studied parameters of plants (Table 2). Their growth on soil contaminated by pure diesel fuel was characterised by the smaller length of shoots and roots, and in general increasing the share of biodiesel caused deterioration of these values. The performed statistical analysis confirmed the presence of statistically significant differences between the average values of the length of the shoots and roots in the individual objects of experience. In addition, other authors observed that the contamination of soil with petroleum derivatives caused a reduction in the mass of yellow lupine plants by almost 50% Wyszkowska and Kucharski (2005), and complete inhibition of barley germination Ziółkowska and Wyszkowski (2010). Fuel had an adverse effect on the water content in the plant tissues, and the highest water deficit was found in plants growing on soil contaminated by pure diesel oil. Fuel also had an adverse effect on the content of assimilation pigments, the amount of which was smaller in general than the control values, although the observed changes were not statistically significant. The reduction of chlorophyll content is connected with the reduction in energy sources available for the plant, which results in the reduction in biomass growth and even death, depending on the applied dose.

The varied response of microorganisms and plants to the presence of diesel fuel and biodiesel may have been caused e.g. by a different chemical composition of the two types of fuel. While petroleum hydrocarbons present in conventional fuel may constitute a source of carbon and energy required to grow and develop for some microorganisms, they are toxic for many other microorganisms. The negative response of plants may have resulted from changes to the water-air conditions in the roots or from an accumulation of petroleum products

Table 2. Results of biometric measurements, water balance and the content of assimilation pigments for *Pisum sativum* L., cv. Eureka

Treatment	Biometric measurement length (mm)		Water balance (%)		Assimilation pigments (mg/g fresh mass)			
	shoot	root	RWC	WSD	chlorophyll *a*	chlorophyll *b*	total chlorophyll	carotenoids
C + plants	45^{a}	207^{a}	84.21^{e}	15.78^{b}	0.78^{a}	0.48^{a}	1.26^{a}	1.77^{a}
B0 + plants	37^{c}	105^{b}	54.54^{a}	45.45^{f}	0.69^{a}	0.41^{a}	1.11^{a}	1.59^{a}
B5 + plants	41^{b}	121^{c}	80.70^{c}	19.29^{d}	0.89^{a}	0.36^{a}	1.26^{a}	2.12^{a}
B20 + plants	22^{d}	38^{e}	86.36^{f}	13.63^{a}	1.01^{a}	0.49^{a}	1.5^{a}	2.58^{a}
B50 + plants	19^{e}	43^{d}	82.60^{d}	17.39^{c}	0.28^{a}	0.45^{a}	0.74^{a}	1.72^{a}
B100 + plants	4^{f}	29^{f}	68.96^{b}	31.03^{e}	0.36^{a}	0.37^{a}	0.73^{a}	1.47^{a}

Mean over each column not marked with the same letter is significantly different at $P < 0.05$. RWC – relative water content; WSD – deficit of water saturation; C – control, B0 – pure petroleum diesel; B5 – 5% biodiesel and 95% petroleum diesel; B20 – 20% biodiesel and 80% petroleum diesel; B50 – 50% biodiesel and 50% petroleum diesel; B100 – pure biodiesel

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in plant tissue. Biodiesel is primarily composed of long chain fatty acid esters. However, it also contains antioxidants (which have a confirmed negative effect on the growth and activity of some organisms), stabilisers, and biocides. The negative effect of biodiesel may stem either directly from its harmful impact on microorganism cells and plant tissue, or, as in the case of diesel fuel, indirectly from changes caused to the environment. The impact of biodiesel on the environment may involve changes to the structure and pH of soil. It is worth noting that while biodiesel is considered easily biodegradable, it still constitutes an alien substance in the environment. This indicates the need to determine any metabolites appearing throughout the process.

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