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Alteration in the Native Proteolytic Activity in the Forest Soil after the Application of Phytohormones

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Abstract: Soil proteases are involved in the transformation of organic matter and thus influence the nutrient turnover in the ecosystem. Phytohormones, similarly to proteases, are synthesized and secreted into the soil by fungi and microorganisms and regulating their activity in the rhizosphere. The aim of our work was to find out how the presence of auxins, cytokinins, ethephone and chlorocholine chloride affects the activity of native soil proteases at the spruce tree stand. Auxins stimulated the native proteolytic activity in the spruce tree stand. Synthetic auxins most stimulated the activity of 2-naphthoxyacetic acid and the naturally occurring auxins of indole-3-acetic acid in the organic horizon of the spruce forest. Cytokinins, ethephone and chlorocholine chloride inhibited the activity of native soil proteases in the spruce tree stand. The highest inhibitory effect was found in ethephone and chlorocholine chloride. Overall, the negative effect of phytohormones on the activity of the native proteolytic activity may slow down the decomposition of organic matter and thus make plant nutrition more difficult. The outcomes of our work assist with understanding of the effect of substances produced by the rhizosphere on the activity of soil microorganisms and the soil nitrogen cycle.

Keywords: Forest soils, Soil enzyme aktivity, Soil microorganisms

1. Introduction

Bacteria are ubiquitous organisms distributed unevenly in the soil environment. The highest concentrations of bacteria are found around the roots of plants in the so-called rhizosphere a [1]. Rhizosphere bacteria can play a key role in the transformation and mobilisation of macro- and micronutrients in the soil, improving the nutritional state of the plants [2,3]. These bacteria are referred to as plant growth-promoting rhizobacteria (PGPR). PGPRs synthesize and secrete phytohormones [4], which are plant growth regulators (PGRs), into their surroundings. These PGRs are organic substances that regulate the growth and development of plants, including their physiological processes, in extremely low concentrations [5]. Among the PGR to include, for example, auxins, cytokinins, gibberellins, ethylene, abscisic acid [6]. Microbial synthesis of the phytohormone auxin, especially indole-3-acetic acid (IAA), has been known for a long time and it is stated that that up to 80% of microorganisms in the rhizosphere are able to produce or release auxins as secondary metabolites [7]. Cytokinins, like auxins, are produced by bacteria, plants and algae [1] and affect the lateral root system development [8,9].

This research study has been performed to identify what effect auxins and cytokinins or more precisely plant growth regulators (PGRs) have on the native protease activity of the forest soil (Oe horizon). We tested naturally occurring indole-3-acetic acid and indole-3-butyric acid auxins, two synthetic auxins 1-naphthaleneacetic acid and 2-naphthoxyacetic acid, two cytokinins 6-benzylaminopurine and adenine hemisulfate in the research study. Ethephone, which is an ethylene-

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46 releasing compound [10] and chlorocholine chloride used as a plant growth regulator [11] were the
47 other studied substances.

48 **2. Materials and Methods**

49 The soil samples for the experiments were taken from the Bílý Kříž experimental ecological site
50 (the part of the European infrastructure of the Project ICOS (Integrated Carbon Observation System)
51 incorporated in the international research infrastructure ESFRI (European Strategy Forum on
52 Research Infrastructures), located in the Moravian-Silesian Beskids in the eastern part of the Czech
53 Republic (N 49°30'17'', E 18°32'28'') with an altitude of 825-860 m above the sea level, an average
54 annual temperature of 6.7°C and an annual rainfall of 1 239 mm. The experimental area was the 35-
55 year-old spruce tree stand (*Picea abies* / L./ Karst.) with the soil type haplic Podzosol [12] and the soil
56 samples were taken from the organic horizon (Oe horizon). Sampling was performed in the middle
57 of the growing season (end of June) when the highest biological activity is to be expected. Three
58 mixed samples were collected (each sample collected from three sample sites) from the organic
59 horizon in weight of cca 500 g. The samples were sieved through 2 mm sieve and stored in PET bags
60 at a temperature of 5°C. The selected chemical properties of the tested soil are given in Table 1.
61
62 **Table 1.** Selected chemical properties of the tested soils.

Plot	C_t (%)	N_t (%)	C/N	pH H_2O	pH 1M KCl
Spruce, Oe horizon (haplic Podzosol)	19.53	0.89	21.9	4.35	3.12

63 C_t – total carbon, N_t – total nitrogen
64
65 The activity of native soil proteases was determined spectrophotometrically by hydrolysis of
66 casein as a substrate and the amount of μg l-tyrosine produced was measured according to the
67 methodology by Rejsek et al. [13]. A total of 1 g of soil was incubated with 2 ml of distilled water and
68 2 ml of casein solution at 50 °C. The sample incubation time was 2 hours. After mixing 1 ml of
69 supernatant with 3.7% disodium carbonate, 1 ml of 0.06% copper sulfate and 1 ml of Folin-Ciocalteu
70 reagent (1:3 water ratio) it was measured at 578 nm. 100 μl of distilled water with dissolved PGR at
71 0, 5, 50 and 100 μg per gram of dry soil were added to 1 gram of the soil sample. Naturally occurring
72 auxins indole-3-acetic acid (IAA) (≥98% sodium salt, Sigma Aldrich), indole-3-butyric acid (IBA)
73 (≥98% potassium salt, Sigma Aldrich) and two synthetic auxins 1-naphthaleneacetic acid (NAA)
74 (≥95% potassium salt, Sigma Aldrich), 2-naphthoxyacetic acid (NOA) (sodium salt, Sigma Aldrich)
75 and cytokinins 6-benzylaminopurine (BAP) (≥98%, hydrochloride, Sigma Aldrich), adenine
76 hemisulfate (AH) (≥99%, salt, Sigma Aldrich) and two PGRs ethephon (ET) (≥96%, powder, Sigma
77 Aldrich) and chlorocholine chloride (CCC) (≥99%, powder, Sigma Aldrich) were selected with in the
78 experiment. Auxines were selected based on similarity to the effect of IAA on microorganisms and
79 plants. IBA is an IAA precursor (or a separate auxin - according to some authors). NAA and NOA
80 are synthetic auxins and their influence on cells is similar to IAAs. ET and CCC were selected for
81 their indirect effect on cells (eg initiation of ethylene production). The soil pH was measured in a
82 soil:water suspension with a ratio of 1:2.5 in 1M KCl. Total carbon (Ct) and total nitrogen (Nt) was
83 detrmined on LECO TruSpec Analyzer (MI, USA). Calibrated to LECO standards: Tobacco 1016.
84 Statistical evaluation of the results of the native protease activity of the individual samples was
85 performed using single factor ANOVA and multiple comparison of HSD by means of Tukey test in
86 Statistica 13.3 (Tibco.com). Table 2 presents the Pearson coefficients from the regression analysis of
87 the data.

88 **3. Results and Discussion**

89 The synthetic auxin NOA stimulated the native protease activity the most in all the studied
90 quantities, with the highest stimulation being achieved at 5 μg NOA. The second synthetic auxin
91 NAA stimulated the activity of native proteases only slightly, by 84.97 μg l-tyrosine at 100 μg NAA

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(Table 2). The positive influence of NAA and NOA on the native protease activity was found out in the forest tree stand. Gómez and Carpena [14] identified that these auxins stimulated root exudation in addition to the inhibition of the root growth. Similarly, the negative effect of synthetic auxins on root growth was detected by Márquez et al. [15]. Naturally occurring auxins had little stimulating effect on the activity of native soil proteases. Only IAA was stimulated with 5 µg IAA (92.36 µg l-tyrosine). A stimulating response to the addition of IAA was detected at 5 µg IAA in the spruce tree stand. Tsavkelova et al. [16] investigated the influence on bacterial biomass size change in several bacterial strains. Bacterial reactions, as mentioned in our work, were dependent on the amount of IAA added. For instance, the amount of 100 µg/ml IAA produced maximum stimulation in the strains of *Mycobacterium* sp. and *Sphingomonas* spp., while *Rhizobium* sp. was stimulated the most at 10 µg/ml IAA [16].

Table 2. The native proteolytic activity after addition of auxins, cytokinins, ethephon and chlorocholine chloride in amounts from 0, 5, 50 a 100 µg g^{-1} dry soil in spruce stand with soil type haplic Podzol.

Plot	**0**	**5**	**50**	**100**
Auxine				
1-naphthaleneacetic acid	78.43±0.01	80.70±2.48	84.11±0.57	84.97±1.02*
2-naphthoxyacetic acid	78.43±0.01	91.22±2.26*	88.66±1.77*	88.38±0.57*
Indole-3-butyric acid	78.43±0.01	82.41±2.71	85.25±0.85	84.68±2.05
Indole-3-acetic acid	78.43±0.01	92.36±0.75*	82.98±1.50	76.73±1.30
Cytokinine				
6-benzylaminopurine	78.43±0.01	71.04±1.02*	64.22±2.05*	62.80±2.22*
Adenine hemisulfate	78.43±0.01	73.60±3.76	78.72±2.33	73.88±2.71
Ethephon	78.43±0.01	69.34±2.27*	50.30±0.49*	50.87±2.71*
Chlorocholine chloride	78.43±0.01	71.33±2.88	59.96±1.24*	51.44±2.84*
Correlation coefficients (p)				
Auxine				
1-naphthaleneacetic acid	1.0000			
2-naphthoxyacetic acid	0.6290**	1.0000		
Indole-3-butyric acid	0.8177**	0.7663**	1.0000	
Indole-3-acetic acid	-0.2721	0.4634	-0.0451	1.0000
Cytokinine				
6-benzylaminopurine	1.0000			
Adenine hemisulfate	0.1620	1.0000		
Ethephon	0.8573**	0.1027	1.0000	
Chlorocholine chloride	0.8875**	0.1990	0.9062**	1.0000

Results represent the amount of produced L-tyrosine in µg /h /g^{-1} dry soil, * are statistically significant ($P < 0.05$; $n = 3$). Standard error ± SE for Tukey HSD test ($P < 0.05$; $n = 3$). A statistically significant results are designated *. A statistically significant correlation ($P < 0.05$; $n = 12$) are designated **

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Auxin IBA did not have a statistically significant effect ($P<0.05$) on the native soil proteolytic activity (Table 2). Our results can be compared to the studies focusing on the growth and development of plant roots in the IBA-added environment that is of the synthetic origin, or is created in the environment after inoculation of PGPR. Our results from the forest stand are in contradiction to those ones by Márquez et al. [15], where the root growth inhibition occurs with the increasing IBA auxin concentration. Similar findings have also been found out by Wang et al. [17] when the decrease of the prolonging root growth occurred with the increasing concentration of IBA.

ET, CCC and cytokinine BAP inhibited the native proteolytic activity of the forest soil (Table 2). The cytokinine AH had no statistically significant ($P< 0.05$) effect on the native protease activity. BAP inhibited native soil proteases in all the observed quantities, with a production decrease from 78.43 μg l-tyrosine (0 μg PGR) to 62.80 μg l-tyrosine (100 μg PGR). The same inhibitory effect of cytokinins was also identified in our study by Holik et al. [5] in the spruce forest with the soil type of haplic Cambisol, where native soil proteases were inhibited in the organic and organomineral horizon. Cytokinins can also negatively act on the growth and the development of roots [18]. Cytokinins can also stimulate the activity of certain enzymes in plant roots as found out by Veselov et al. [19] for catalase activity after the application of 6-benzylaminopurine or Chang et al. [20] for nitrate reductases. ET inhibited native proteases in all the measured amounts similarly to the cytokinine BAP (Table 2). ET reduced the activity of native proteases from 78.43 μg l-tyrosine (0 μg PGR) to 50.30 μg l-tyrosine (50 μg PGR). We have come to the same conclusion in our former study by Holik et al. [5], there was the inhibition of the native soil proteolytic activity in the spruce forest. The inhibitory effect of ET was also found out by Khan et al. [10], where the plant growth, photosynthesis and accumulation of nitrogen in their biomass decreased with the low level of nitrogen. ET can also inhibit root mycorrhiza, as demonstrated by Rupp et al. [21] in the study of mycorrhiza in *Pinus mugo*. CCC inhibited the native proteolytic activity of soils only at 50 μg CCC and 100 μg CCC, reducing the amount of l-tyrosine to 51.44 μg (100 μg PGR). CCC as well as ET had an inhibitory effect on the activity of native soil proteases. CCC also has a negative effect on rhizospheric mycoflora, where CCC inhibited the growth of the mycelium of *Fusarium* and *Penicilium* [22]. CCC can also have a stimulating effect on plant enzymes. Anosheh et al. [23] demonstrated the CCC stimulating effect on catalase and peroxidase activity in the study, but it did not affect the activity of superoxide dismutase.

The positive correlations of the results between NAA and NOA were found out at the forest site (Table 2), and the results also correlated positively between IBA and both synthetic auxins (NAA and NOA). The outcomes of IBA strongly correlated with PGR ET and CCC and a strong positive correlation was also revealed between ET and CCC (Table 2).

The comparison of our results, i.e. the effect of auxins, cytokinins and PGRs on the soil enzyme activity, with the research works of other authors is very difficult, because similar results to our research study are absent in the literature worldwide. We can find studies that deal with related topics, such as the effect of phytohormones on root mycorrhiza e.g. [24,25], the resistance of plants to soil salinity [19,26], or phosphorus solubility [27,28].

4. Conclusions

The effect of auxins, cytokinins and PGRs on the native protease activity in the forest soil was studied in our research work. The results show that auxins have a positive effect on the activity of soil proteases in the forest soil. Cytokinins and PGRs also had a negative effect on the activity of native soil proteases both at the forest soil. Due to the absence of similar studies, this work is beneficial to the better understanding of the effects of auxins, cytokinins and PGRs on the activity of soil microorganisms and the availability of organic nitrogen to plants.

Author Contributions: LH conceived the idea and conducted the experiments, VV analyzed the data and created the tables. LH wrote the paper.

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