

Effect of Plant Growth Regulators on Protease Activity in Forest Floor of Norway Spruce Stand

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Abstract: Soil proteases are involved in organic matter transformation processes and, thus, influence ecosystem nutrient turnovers. Phytohormones, similarly to proteases, are synthesized and secreted into soil by fungi and microorganisms, and regulate plant rhizosphere activity. The aim of this study was to determine the effect of auxins, cytokinins, ethephon, and chlorocholine chloride on spruce forest floor protease activity. It was concluded that the presence of auxins stimulated native proteolytic activity, specifically synthetic auxin 2-naphthoxyacetic acid (16% increase at added quantity of 5 µg) and naturally occurring indole-3-acetic acid (18%, 5 µg). On the contrary, cytokinins, ethephon and chlorocholine chloride inhibited native soil protease activity, where ethephon (36% decrease at 50 µg) and chlorocholine chloride (34%, 100 µg) showed the highest inhibitory effects. It was concluded that negative phytohormonal effects on native proteolytic activity may slow down organic matter decomposition rates and hence complicate plant nutrition. The study enhances the understanding of rhizosphere exudate effects on soil microbial activity and soil nitrogen cycle.

Keywords: auxins; cytokinins; soil protease; spruce forest



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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 [1]. Rhizosphere bacteria can play a key role in the transformation and mobilization of macro- and micronutrients in the soil, improving the nutritional state of plants [2,3]. Phytohormones synthesized and secreted into their surroundings by plant growth-promoting rhizobacteria (PGPR), located in the rhizosphere (close to the plant roots) [4] act as plant growth regulators (PGR). Fungi, much like bacteria, can interact with plants in a beneficial way, for example, in the form of mycorrhizal symbiosis [5] or can pose a threat, i.e., in the form of fungal diseases and pathogens [6]. Either way, in both instances enter plant tissues or plant cells in order to obtain nutrients. Fungi have, therefore, developed various tools allowing them to invade plant cells, including the production of plant hormone-like compounds (auxins, cytokinins, ethylene, abscisic acid, jasmonic acid, salicylic acid) [7–9]. PGRs are organic substances that regulate the growth and development of plants (including their physiological processes) in extremely low concentrations [10]. PGRs primarily include auxins, cytokinins, gibberellins, ethylene, and abscisic acid [11]. Microbial phytohormonal synthesis of auxin, especially indole-3-acetic acid (IAA), has been long acknowledged [12] and so it has been recognized that up to 80% of rhizosphere microorganisms are able to produce or release auxins as secondary metabolites [13]. Cytokinins, similarly to auxins, are produced by bacteria, plants, and algae [1] and affect lateral root system development [14,15].

This study was performed to determine the effects of auxins, cytokinins and plant growth regulators (PGR) on native protease activity in organic horizon of forest soils at a Norway spruce stand. The working hypothesis expected that auxins increase native soil protease activity, while cytokinins and plant growth regulators inhibit the activity. In this

short communication, two naturally occurring auxins (indole-3-acetic acid and indole-3-butyric acid), two synthetic auxins (1-naphthaleneacetic acid, 2-naphthoxyacetic acid), and two cytokinins (6-benzylaminopurine, adenine hemisulphate) were tested. Additionally, ethephon (ethylene-releasing compound) [16] and chlorocholin chloride [17] were used as PGRs. At the present time, phytohormones are applied to improve the vitality and increase the rooting success of young plants grown in substrates. The ability to regulate native protease activity by the presence of phytohormones may help to better understand the role of proteases in the decomposition of soil organic matter.

2. Materials and Methods

2.1. Study Site

Soil samples were collected at the Bílý Kříž ecological experimental site, part of the European infrastructure integrated carbon observation system (ICOS) project incorporated in the European Strategy Forum on Research Infrastructures (ESFRI). It is located in the Moravian-Silesian Beskids, eastern part of the Czech Republic (N 49°30'17", E 18°32'28"), and it is characterized by an annual rainfall of 1239 mm, an average annual temperature of 6.7 °C and an altitude of 825–860 m above the sea level. The experimental area constitutes of a 35-year-old spruce tree stand (*Picea abies* /L./Karst.) on haplic Podzsol soil type [18].

2.2. Experiment Description

Sampling was performed halfway through the vegetation season (end of June) within the highest expected soil biological activity. Topsoil samples were collected from the organic layer (Oe horizon). Three composite samples of about 500 g in weight, each assembled from three randomly selected points, were collected from the Oe horizons. The samples were sieved through a 2 mm mesh and stored in PET bags at a temperature of 5 °C.

Following chemical compounds were selected for the experiment: indole-3-acetic acid (IAA) ($C_{10}H_8NNaO_2$, ≥98% sodium salt, Sigma–Aldrich), indole-3-butyric acid (IBA) ($C_{12}H_{12}KNO_2$, ≥98% potassium salt, Sigma–Aldrich) (naturally occurring auxins) and 1-naphthaleneacetic acid (NAA) ($C_{12}H_9O_2K$, ≥95% potassium salt, Sigma–Aldrich), 2-naphthoxyacetic acid (NOA) ($C_{12}H_9O_3Na$, ≥95% sodium salt, Sigma–Aldrich) (synthetic auxins). Selected cytokinins: 6-benzylaminopurine (BAP) ($C_{12}H_{11}N_5 \cdot HCl$, ≥98%, hydrochloride, Sigma–Aldrich), adenine hemisulfate (AH) ($C_5H_5N_5 \cdot 1/2H_2SO_4$, ≥99%, salt, Sigma–Aldrich) and two PGRs ethephon (ET) ($ClCH_2CH_2P(O)(OH)_2$, ≥96%, powder, Sigma–Aldrich) and chlorocholine chloride (CCC) (≥99%, powder, Sigma–Aldrich). Auxins were selected according to the IAA effect similarity on microorganisms and plants. IBA is an IAA precursor (or a separate auxin—according to some authors). NAA and NOA are synthetic auxins and their influence on cells is similar to IAAs. ET and CCC were selected for their indirect effect on cells (e.g., initiation of ethylene production).

2.3. Laboratory Analysis and Data Evaluation

Native proteolytic enzyme activity was determined by casein hydrolysis. The amount of produced l-tyrosine was measured according to the methodology by Rejsek et al. [19]: Two grams of soil were incubated at 50 °C with 2 mL of casein solution, 2 mL of distilled water and 100 µL of distilled water with dissolved PGR. Resulting supernatants (1 mL) were mixed with 3.7% disodium carbonate, 1 mL of 0.06% copper sulphate and 1 mL of Folin–Ciocalteu reagent (1:3 water ratio) and measured at 578 nm. Protease activity measurements were carried out in triplicates, where each soil sample replicate weighed 2 g. Control samples were incubated without the addition of PGR (0 µg PGR). Added chemicals were dissolved in distilled water. Prior to incubation, PGRs were dissolved in 100 µL of distilled water in amounts of 0, 5, 50, and 100 µg per 2 g of dry soil and added to 2 g of fresh soil sample. Sample incubation time was 2 h. The soil pH was measured in 1:2.5 soil:water and soil:KCl (1M KCl) suspensions. LECO TruSpec CN analyzer (MI, USA) was used for total carbon (Ct) and total nitrogen (Nt) determination; calibrated

by the Tobacco 1016 standard (LECO standards). Selected soil chemical properties shown in Table 1 were tested.

Table 1. Selected chemical properties of the tested soils.

Plot	C _t (%)	N _t (%)	C/N	pH H ₂ O	pH 1M KCl
Spruce, Oe horizon (haplic Podzsol)	19.53	0.89	21.9	4.35	3.12

C_t—total carbon, N_t—total nitrogen.

Statistical analyses were performed using Statistica 13.3 (Tibco.com, accessed on 18 April 2021). The results were evaluated using the Shapiro-Wilk test to test the normality of the data and using parametric single factor ANOVA and by multiple comparison of HSD with the means of the Tukey test. Statistical data evaluation was according to [20]. Table 2 presents resulting regression analysis Pearson coefficient data.

Table 2. Resulting soil protease activity after the addition of phytohormones and PGRs to samples in the quantities of 0, 5, 50, and 100 µg g^{−1} dry soil.

Treatment	0	5	50	100
Auxin				
NAA	78.43 ± 0.01	80.70 ± 2.48	84.11 ± 0.57	84.97 ± 1.02 *
NOA	78.43 ± 0.01	91.22 ± 2.26 *	88.66 ± 1.77 *	88.38 ± 0.57 *
IBA	78.43 ± 0.01	82.41 ± 2.71	85.25 ± 0.85	84.68 ± 2.05
IAA	78.43 ± 0.01	92.36 ± 0.75 *	82.98 ± 1.50	76.73 ± 1.30
Cytokinin				
BAP	78.43 ± 0.01	71.04 ± 1.02 *	64.22 ± 2.05 *	62.80 ± 2.22 *
AH	78.43 ± 0.01	73.60 ± 3.76	78.72 ± 2.33	73.88 ± 2.71
ET	78.43 ± 0.01	69.34 ± 2.27 *	50.30 ± 0.49 *	50.87 ± 2.71 *
CCC	78.43 ± 0.01	71.33 ± 2.88	59.96 ± 1.24 *	51.44 ± 2.84 *
Correlation coefficients (p)				
Auxin				
NAA	1.0000			
NOA	0.6290 **	1.0000		
IBA	0.8177 **	0.7663 **	1.0000	
IAA	−0.2721	0.4634	−0.0451	1.0000
Cytokinin				
BAP	1.0000			
AH	0.1620	1.0000		
ET	0.8573 **	0.1027	1.0000	
CCC	0.8875 **	0.1990	0.9062 **	1.0000

The results in the table show the amount l-tyrosine produced in µg/h/g^{−1} dry soil with standard error ± SE for the Tukey HSD test ($p < 0.05$; $n = 3$). * are statistically significant ($p < 0.05$; $n = 3$) and statistically significant correlations ($p < 0.05$; $n = 12$) are designated **.

3. Results and Discussion

The NOA synthetic auxin stimulated native protease activity the most in all studied quantities, with the highest stimulation achieved at 5 µg (16%, 91.22 µg l-tyrosine produced). The second synthetic auxin used (NAA) stimulated the activity of native proteases to a smaller degree by 84.97 µg l-tyrosine produced at 100 µg (8%) (Table 2). Gómez and Carpena [21] identified that the same auxins stimulated root exudation in addition to the inhibition of root growth. Similarly, such negative effect of synthetic auxins on root growth was detected by Márquez et al. [22]. Naturally occurring auxins showed generally lower stimulating effect on native soil protease activity with the exception of IAA at the 5 µg quantity (92.36 µg), therefore, a stimulating response to the addition of 5 µg IAA was detected in the Oe horizon of the spruce stand. Tsavkelova et al. [23] investigated the influence on bacterial biomass size change in several bacterial strains. They concluded that

adding 100 µg/mL of IAA produced maximum stimulation in the strains of *Mycobacterium* sp. and *Sphingomonas* spp., while *Rhizobium* sp. was stimulated the most at 10 µg/mL of IAA, which corresponds with our findings of bacterial reactions being dependent on the amount of IAA added.

The IBA auxin did not exhibit an apparent effect ($p < 0.05$) on native soil proteolytic activity (Table 2). When comparing our research to similar studies focusing on the growth and development of plant roots in both IBA-added environments (IBA of synthetic origin) and naturally created IBA environments (IBA occurring after PGPR inoculation) our spruce stand results are in contradiction to those by Márquez et al. [22] who determined that root growth inhibition occurs with increasing IBA auxin concentration. Similar findings have also been documented by Wang et al. [24] who established that increasing IBA concentration led to a decrease in prolonging root growth.

ET, CCC, and cytokinin BAP inhibited native proteolytic activity (Table 2). The AH cytokinin had no statistically significant ($p < 0.05$) effect on 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 (−20%, 100 µg PGR). An identical inhibitory effect of cytokinins was identified in our earlier study by Holik et al. [5] conducted in a spruce forest on haplic Cambisol soil type and where native soil proteases were inhibited in organic and organomineral horizons. Cytokinins can also negatively influence the growth and development of roots [25]. Similarly, cytokinins can stimulate the activity of certain enzymes in plant roots as indicated by Veselov et al. [26] as for catalase activity subjected to 6-benzylaminopurine application or else by Chang et al. [27] as for nitrate reductases. ET inhibited native proteases in all applied quantities similarly to BAP cytokinin (Table 2). ET reduced native protease activity in terms of l-tyrosine production from 78.43 µg (0 µg PGR) to 50.30 µg (−36%, 50 µg PGR). We have come to the same conclusion as in our former study by Holik et al. [5], where spruce forest native soil proteolytic activity was equivalently inhibited. ET inhibitory effect on plant growth, photosynthesis, biomass N accumulation ability inducing overall low N levels was documented by Khan et al. [16]. ET can also inhibit root mycorrhiza, as demonstrated by Rupp et al. [28] in the study of mycorrhiza in *Pinus mugo*. CCC inhibited native proteolytic activity of soils only in the quantities of 50 and 100 µg, reducing l-tyrosine production to 51.44 µg (−34%, 100 µg PGR). CCC, as well as ET, showed an inhibitory effect on the activity of native soil proteases. CCC also exhibited a negative effect on rhizospheric microflora when inhibiting the mycelium growth of *Fusarium* and *Penicillium* [29]. Nevertheless, CCC can also have a stimulating effect on selected plant enzymes as demonstrated by Anosheh et al. [30] on catalase and peroxidase activity while superoxide dismutase was unaffected.

Positive result correlations were found between NAA and NOA (Table 2), as well as 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 found between ET and CCC (Table 2).

It appears to be very difficult to compare our results, i.e., the effect of auxins, cytokinins, and PGRs on soil enzyme activity, with research works of other authors, since results comparable to our research study are absent in literature worldwide. However, remotely related studies can be found, just as the effect of phytohormones on root mycorrhiza, e.g., [9,31], the resistance of plants to soil salinity [26,32], or phosphorus solubility [33,34].

4. Conclusions

The effect of phytohormones and PGRs on native protease activity in spruce forest stands was examined in our research study. The results show that auxins have a positive effect on forest soil protease activity while cytokinins and PGRs have a negative effect. Due to the absence of similar studies, the contribution to better understanding of phytohormone and PGR effects on the enzymatic activity of soil microorganisms and the availability of nitrogen in the soil nitrogen cycle is perceived as the most beneficial part of the study.

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