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Characterization of Natural Biostimulants and Their Effect on Soil Properties

Charakterizace přírodních biostimulantů a jejich vliv na vlastnosti půdy

BAKALÁŘSKÁ PRÁCE

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Abstract

Biostimulants represent an environmentally friendly way of protecting plants and supporting their growth. Here, fermented nettle was characterized in terms of antioxidant properties, protein content, and activities of selected enzymes. Furthermore, this study focused on the effects of biostimulants (fermented nettle and vermicompost) on soil properties.

Fermented nettle showed high content of phenolic compounds and high antioxidant capacity. Activity of proteases, as well as acid and alkaline phosphatases was successfully determined in fermented nettle. Alkaline phosphatase activity was significantly higher than that of acid phosphatase.

Protease, endo-β-1,3-glucanase, and total peroxidase activities were determined in soils of plants treated with biostimulants – fermented nettle (N), vermicompost (V) – and compared with control (C). All groups of plants were also exposed to drought stress (D). Application of biostimulants did not affect protease activity in watered groups but D groups showed a significant increase in proteolysis. Endo-β-1,3-glucanase activity was higher in the groups treated with biostimulants and drought did not affect the activity. The group VD showed significantly higher total peroxidase activity than other groups. The protein content in groups treated with biostimulants was higher than in the control group; however, ND showed significant decrease in protein concentration. Based on the measured parameters, these biostimulants appear to alleviate drought stress.

Chemical plant protection products, while effective, have some severe disadvantages. Heavy utilization of pesticides leads to environmental pollution. Triazole fungicides are widely used to combat fungal pathogens and it is their impact on soil properties that was analysed. Triazole fungicides (penconazole (P) and tebuconazole (T)) were applied either as soil drench (s) or foliar spray (f). Application of triazoles did not affect soil protein content. Proteolytic activity was decreased in sPT, fP, and fPT groups compared with the control group. Groups sPT, fP, and sT showed decrease in endo-β-1,3-glucanase activity. Total peroxidase activity was significantly higher in every group (except fT) than in the control group. The soil drench of both triazoles (sPT) and foliar spray of penconazole (fP) decreased most of the determined enzyme activities, thus representing the most detrimental applications for soil parameters.

Key words: antioxidant properties, biostimulant, drought stress, phenolic compounds, soil enzymes, triazole fungicides

Abstrakt

Biostimulanty představují ekologický způsob ochrany rostlin a podpory jejich růstu. V této práci byla charakterizována kopřivová jícha z hlediska antioxidačních vlastností, obsahu proteinů a aktivit vybraných enzymů. Dále byly sledovány účinky biostimulantů (kopřivové jíchy a vermikompostu) na vlastnosti půdy.

Kopřivová jícha vykazovala vysoký obsah fenolických látek a vysokou antioxidační kapacitu. V kopřivové jíše byla úspěšně stanovena aktivita proteas a také kyselých a alkalických fosfatas. Aktivita alkalických fosfatas byla výrazně vyšší než aktivita kyselých fosfatas.

V půdách rostlin ošetřených biostimulanty (kopřivová jícha (N), vermikompost (V)) byla stanovena aktivita proteas, endo-β-1,3-glukanasy a peroxidas. Tato stanovení byla porovnána s kontrolní skupinou. Všechny skupiny rostlin byly navíc vystaveny suchu (D). Aplikace biostimulantů neovlivnila aktivitu proteas v zavlažovaných skupinách, ale v D skupinách došlo k výraznému zvýšení proteolýzy. Aktivita endo-β-1,3-glukanasy byla vyšší ve skupinách ošetřených biostimulanty a nebyla ovlivněna suchem. Skupina VD vykazovala značně vyšší celkovou aktivitu peroxidas než ostatní skupiny. Obsah bílkovin ve skupinách ošetřených biostimulanty byl vyšší než v kontrole, avšak skupina ND vykazovala výrazný pokles koncentrace proteinů. Na základě naměřených parametrů se zdá, že tyto biostimulanty zmírňují stres suchem.

Chemické přípravky na ochranu rostlin jsou sice účinné, mají však i závažné nevýhody. Intenzivní používání pesticidů vede ke znečištění životního prostředí. Triazolové fungicidy jsou široce používány v boji proti houbovým patogenům, a právě jejich vliv na půdní vlastnosti byl analyzován. Triazolové fungicidy (penkonazol (P) a tebukonazol (T)) byly aplikovány buď jako zálivka do půdy (s), nebo postřik na listy (f). Aplikace triazolů neovlivnila obsah bílkovin v půdě. Proteolytická aktivita byla snížena ve skupinách sPT, fP a fPT ve srovnání s kontrolní skupinou. Skupiny sPT, fP a sT vykazovaly pokles aktivity endo-β-1,3-glukanasy. Celková aktivita peroxidas byla u všech skupin (kromě fT) výrazně vyšší oproti kontrolní skupině. Půdní zálivka obsahující oba triazoly (sPT) a postřik na listy penkonazolem (fP) snížily většinu stanovených enzymových aktivit, představují tedy nejškodlivější aplikace pro půdní parametry.

Klíčová slova: antioxidační vlastnosti, biostimulant, fenolické látky, půdní enzymy, stres suchem, triazolové fungicidy

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List of abbreviations

% (v/v)	Volume per volume percentage
% (w/v)	Weight per volume percentage
AMP·HCl	2-Amino-2-methyl-1-propanol hydrochloride
ANOVA	Analysis of variance
APS	Ammonium persulfate
APX	Ascorbate peroxidase
BCA	Bicinchoninic acid (disodium salt)
BSA	Bovine serum albumin
C	Control group of plants
CAT	Catalase
CD	Control group of plants that underwent drought stress
DAB	3,3′-Diaminobenzidine
DPPH	2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazine-1-yl
DTT	1,4-Dithiothreitol
F-C	Folin-Ciocalteu
FN	Fermented nettle
fP	Group of plants treated with penconazole applied as foliar spray
fPT	Group of plants treated with a mixture of penconazole and tebuconazole applied as foliar spray
FRAP	Ferric Ion Reducing Antioxidant Power
FRET	Fluorescence Resonance Energy Transfer
fT	Group of plants treated with tebuconazole applied as foliar spray
FTC-Casein	Native casein labelled with fluorescein isothiocyanate
Glc	Glucose
GPX	Glutathione peroxidase
GR	Glutathione reductase

MBTH	3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate
MOPS	3-(Morpholin-4-yl)propane-1-sulfonic acid
N	Group of plants treated with fermented nettle
ND	Group of plants treated with fermented nettle that underwent drought stress
pNP	4-Nitrophenol
pNPP	4-Nitrophenyl phosphate (disodium salt hexahydrate)
PVP	Polyvinylpyrrolidone
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
sP	Group of plants treated with penconazole applied into the soil
sPT	Group of plants treated with a mixture of penconazole and tebuconazole applied into the soil
sT	Group of plants treated with tebuconazole applied into the soil
TCA	Trichloroacetic acid
TEMED	*N*,*N*,*N*′,*N*′-Tetramethylethane-1,2-diamine
TPTZ	2,4,6-Tris(2-pyridyl)-*s*-triazine
Tris	Tris(hydroxymethyl)aminomethane
V	Group of plants treated with vermicompost
VD	Group of plants treated with vermicompost that underwent drought stress

1. Introduction

The research of plant biostimulants is getting more and more attractive, since there is a prevailing need to increase crop yield to feed the growing human population, while also minimising the detrimental impact of fertilizers and pesticides (e.g., fungicides) on the environment [1]. Biostimulants hold promise in terms of improving crop production and mitigating abiotic stresses, such as drought, salinity, or heat [2]. This thesis focuses mainly on two plant biostimulants – fermented nettle and vermicompost leachate, as well as their effect on soil properties.

Fermented nettle is prepared by soaking stinging nettle (*Urtica dioica* L.) plants in water for four weeks. Vermicompost leachate is a by-product of vermicomposting – waste conversion utilizing microorganisms and earthworms.

The chemical composition of both biostimulants is well documented. Fermented nettle is rich in potassium, sulphur, calcium, magnesium, phosphorus, and nitrogen [3]. Vermicompost contains a high percentage of potassium, calcium, magnesium, phosphorus, and nitrogen [4].

In this work, rather than focusing on the above-mentioned chemical composition of fermented nettle and vermicompost, the aim was to test antioxidant properties, determine the protein concentration and the activity of selected enzymes – phosphatases and proteases.

The other aim was to analyse the effect of fermented nettle and vermicompost on soil properties. Both biostimulants were applied into the soil to stimulate the growth of maize (*Zea mays* L.) in a plant growth chamber under standardized conditions. The results of aforementioned treatments were compared with a control group. Every group also underwent drought stress. Altogether, six groups were analysed – control (C), control + drought (CD), fermented nettle treatment (N), fermented nettle treatment + drought (ND), vermicompost treatment (V) and vermicompost treatment + drought (VD).

Finally, the last objective was to analyse the soil in which tomato plants (*Solanum lycopersicum*, cv. Cherrola) treated with two triazole fungicides – penconazole (P), tebuconazole (T) and their combination (PT) were grown. The fungicides were applied in two ways: as a foliar spray (f) or as a soil drench (s). In total, including untreated control plants (C), samples of soil were collected from seven groups of plants – C, sP, sT, sPT, fP, fT, and fPT.

The effects of these treatments on the soil were determined by the activity of various enzymes, such as proteases, endoglycosidases, and peroxidases.

2. Literature Review

2.1 Plant Growth and Protection

Chemical products which promote plant growth and facilitate plant protection, while still widely used, are losing their popularity. The high efficacy of chemical fertilizers and pesticides does not outweigh the numerous detrimental effects, such as pollution of environment, disruption of the biological equilibrium, their accumulation in soil and ground water, and their residue in produce [5]. Even the effectiveness of chemical pesticides can be questioned. Those aimed at weed control have insufficient selectivity in some cases and may harm crops [6]. Crops affected by pesticides have shown reduction of biomass production, chlorophyll concentration, and nutrient uptake [7],[8]. The fate of pesticides in the environment is summarized in Figure 1 [9].

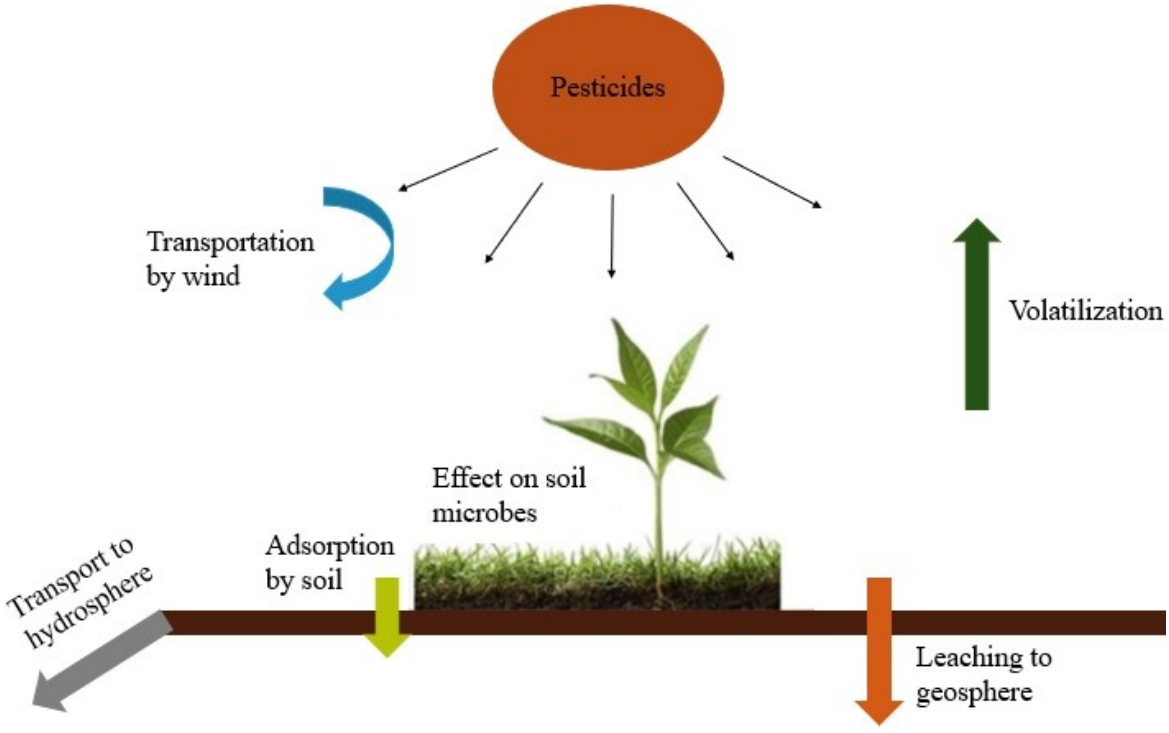

Figure 1: The fate of pesticides in environment. Pesticides affect soil microorganisms, leach into soil and eventually into groundwater. They can also be transported by the wind, evaporated from the soil and reach other plants in the form of rainfall. Taken and edited from [9].

The search for environmentally friendly substitution of chemical fertilizers and pesticides has led to an increasingly greater focus on biostimulants [10]. Plant biostimulants

are not considered fertilizers nor pesticides. The function of these substances or microorganisms is to enhance nutrient availability and uptake and to mitigate abiotic stresses [11]. The complex composition and mechanism of biostimulants prevent the introduction of a single definition and categorization, and therefore they vary from author to author [12]. Multitude of studies have been conducted that illustrate the beneficial effects of biostimulants, for example on plant growth [13],[14],[15], yield [16],[17],[18], and drought stress tolerance [19],[20],[21]. The effects of biostimulants on plants are summarized in Figure 2 [22].

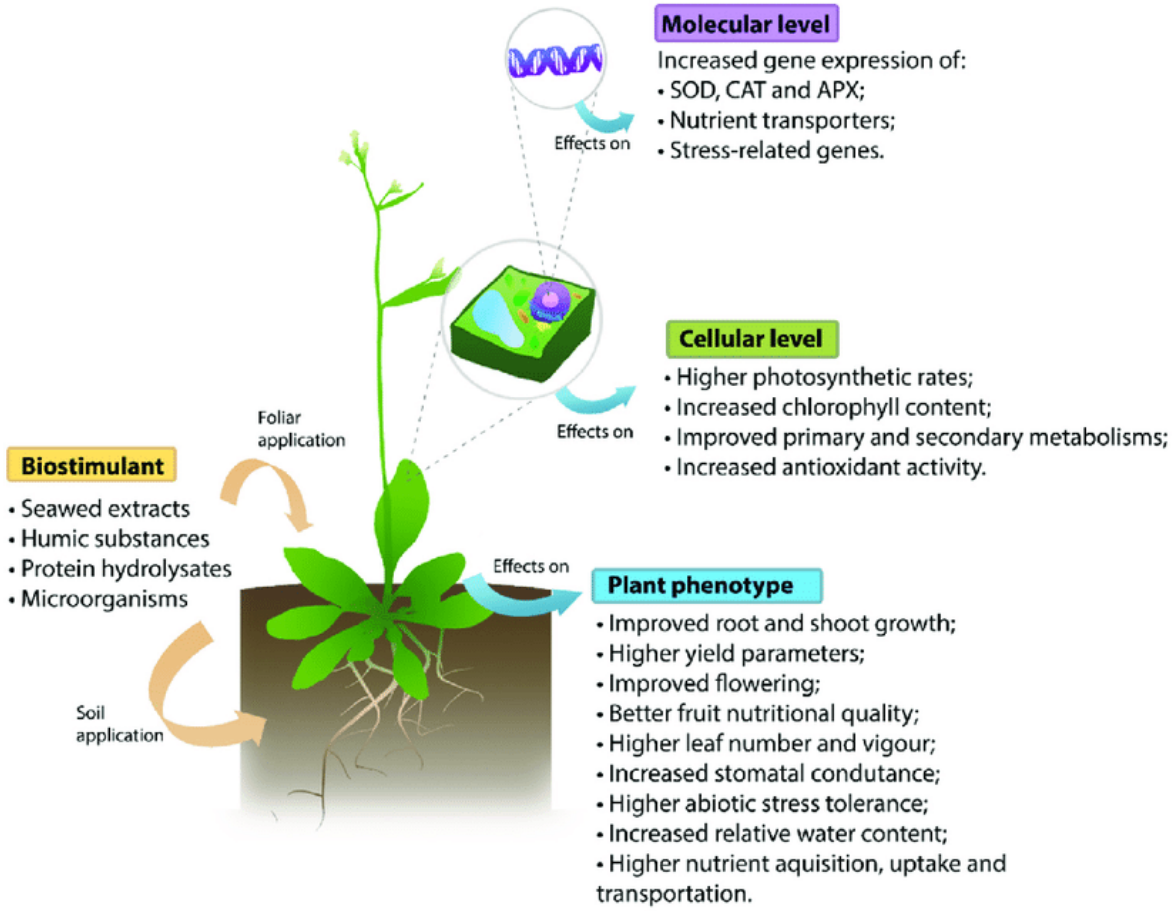

Figure 2: The summarization of the effects of biostimulants on plants. Affected is not only the plant phenotype but the plant is also affected on cellular and molecular level. Abbreviations: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX). Taken from [22].

In the following chapters mainly the impact of chemical protection, specifically triazole fungicides, on soil is discussed.

2.2 Effects of Pesticides on Soil Properties

The soil is a diverse material that changes over time consisting of inorganics (air, water, minerals) and organic matter. It covers most of the land and is a vital part of Earth's ecosystem. Soil offers structural support for plants, stores water, and holds biota [23]. Each soil type differs in biological, chemical, and physical properties. These properties can be negatively affected by long-term agricultural practices [24]. For instance, pesticides reduce microbial populations and enzyme activity in soil [25].

Soil enzymes promote nutrient cycling and energy flow by decomposing organic matter in soil, and thus are vital in agriculture. The primary source of soil enzymes are microorganisms and to a lesser extent plants and animals. Extracellular enzymes accumulate in the soil, forming complexes with clay or humic colloids. Conversely, intracellular enzymes can be found in cytoplasm or bound to cell walls [26].

Soil enzymes respond to changes in environment faster than other properties, therefore their activity can be used to assess soil quality. Enzymes analysed in soil are most commonly: dehydrogenases, exoglycosidases, endoglycosidases, phosphatases, and proteases [26].

Dehydrogenases are intracellular enzymes whose activity correlates with microbial respiration [27]. Many studies were conducted with the aim to analyse the effect of pesticides on dehydrogenase activity. Overall, pesticides either have no effect or inhibit dehydrogenase activity [28],[29],[30].

β-Glucosidase activity follows the same trend as dehydrogenase activity, meaning the application of pesticides has either no effect or an inhibitory one [31],[32]. Fungicides and herbicides have slight inhibitory impact on cellulase activity [33],[34]. Surprisingly, insecticides (at low concentrations) stimulated the activity of cellulase [35].

Phosphatase activity showed a considerable decrease after fungicide application [36],[37],[38]. Some fungicides increased the activity of acid phosphatase and in contrast inhibited the activity of alkaline phosphatase [39]. Herbicides have overall negative effect on phosphatase activity [40]. Lastly, insecticides inhibited acid phosphatase activity [41],[42].

In one study, protease activity increased after herbicide application [43], yet in another study protease activity first decreased, but then increased after one week of incubation [44].

2.2.1 Triazole Fungicides

Triazole fungicides are all derived from 1,2,4-triazole [45]. They are used worldwide to prevent the growth and spread of various fungi on crops, vegetables, etc. [46]. The mechanism of triazole fungicide protection is based on the inhibition of sterol 14-α-demethylase (EC 1.14.14.154), thus preventing biosynthesis of ergosterol, an important part of fungal plasma membrane [47],[48]. Examples of commonly used triazole fungicides include triticonazole, epoxiconazole, myclobutanil, propiconazole, hexaconazole, difenoconazole, paclobutrazole, metconazole, tebuconazole, and penconazole.

Tebuconazole's broad-spectrum of application has some downsides. First, some fungal pathogens have gained resistance, *Sphaerotheca fuliginea*, *Puccinia triticina*, *Zymoseptoria tritici*, and *Penicillium digitatum* [49]. Second, fungicides can be toxic to non-target organisms [50]. Third, the frequent use has led to contamination of soil, underground water and air [45] (Figure 1).

The half-life of tebuconazole in soil is 30-50 days [51] and the residues of triazoles in soil affect microorganisms and enzymes therein [45]. A study conducted with a triazole fungicide myclobutanil showed that in high doses this fungicide inhibited the activity of dehydrogenases and urease [48]. Different study used a mixture of fungicides (spiroxamine, tebuconazole and triadimenol) and concluded that the activity of dehydrogenases, catalase, urease, alkaline phosphatase, and especially acid phosphatase has dramatically decreased. The fungicides also pose a serious threat to soil microorganisms [52].

The effects of triazole fungicides on soil health depend on time and dose. To avoid the decrease in soil microorganisms and enzyme activities, it is advised to follow recommended doses of fungicides for each type of soil or crop [45].

2.3 Biostimulants and Abiotic Stress

Nature of biostimulants is, as previously stated, very diverse. Nonetheless, they share the ability to enhance abiotic stress tolerance [11]. Abiotic stress (e.g., drought, heat, cold, salinity stress, and heavy metal pollution) in plants causes the generation of reactive oxygen species (ROS), photosynthesis impairment, and decreases plant growth and yield [53] (Figure 3).

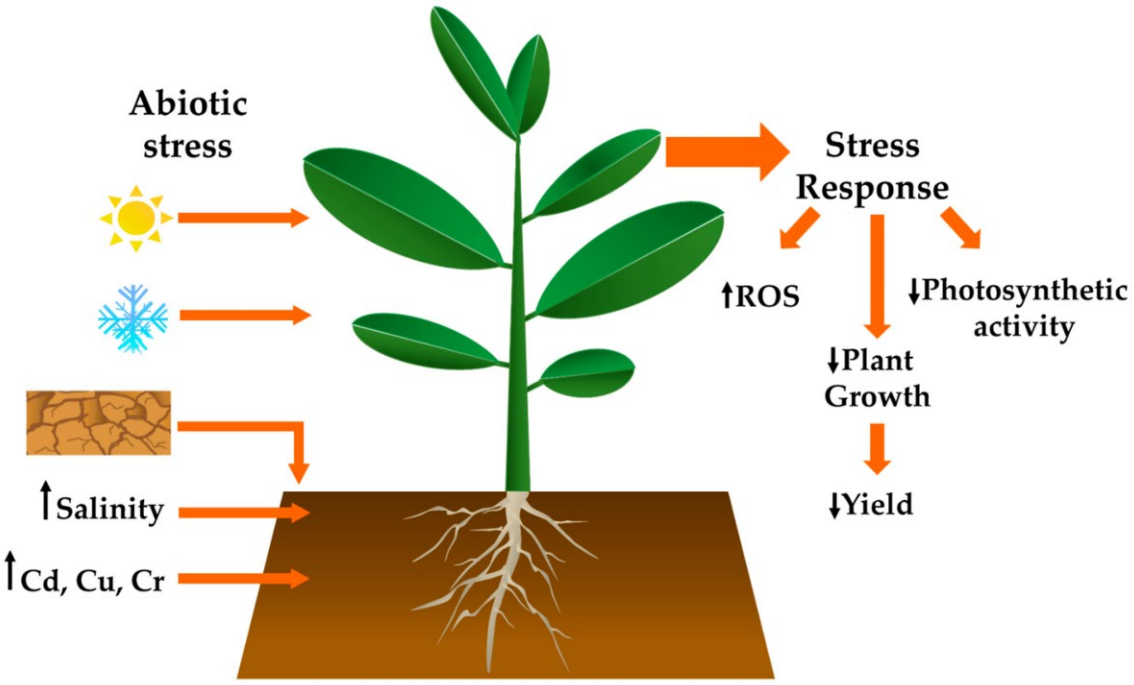

Figure 3: The detrimental effects of abiotic stress on plants. There is an increase in the generation of ROS and decrease in plant growth, yield, and photosynthetic activity. Abbreviation: reactive oxygen species (ROS). Taken from [53].

Plants have developed a defence system regulated by phytohormones against abiotic stress. Such phytohormones are, for example, abscisic, jasmonic, and salicylic acid. Plants under abiotic stress generate ROS and their accumulation induces oxidative stress [54]. Antioxidants (nonenzymatic or enzymatic) suppress oxidative stress by scavenging the ROS. Enzymatic antioxidants include superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), ascorbate peroxidase (APX, EC 1.11.1.1), and glutathione reductase (GR, EC 1.6.4.2), on the other hand, nonenzymatic antioxidants are, for example, ascorbic acid, glutathione, carotenoids, and phenolic compounds [55]. Tolerance to abiotic stresses can also be elevated by microorganisms in the rhizosphere. Rhizobacteria and mycorrhizae organisms are able

to, for instance, stimulate the production of phytohormones and increase the expression of antioxidant genes [56]. These microorganisms also reduce oxidative stress by enhancing the production of secondary metabolites (e.g., terpenes, alkaloids, phenolic compounds) [57].

Biostimulants ameliorate abiotic stress by increasing phenolic, flavonoid, and jasmonic acid concentration, upregulating genes related to metabolism of ascorbate and glutathione, improving antioxidant activity, increasing activity of antioxidant enzymes (SOD and CAT), and upregulating ROS scavengers [22]. In soil, biostimulants improved water retention and stimulated the activity of alkaline phosphatase, glycosidase, urease, and dehydrogenase [58],[59],[60]. There are still pieces missing from the complex puzzle that is the action mechanism of biostimulants in plants. Their effect on soil remains even more of an underexplored area.

The categorization of biostimulants is complex, still, experts generally recognize the following categories. Humic and fulvic acids, protein hydrolysates and other N-containing compounds, seaweed extracts and botanicals, chitosan and other biopolymers, inorganic compounds, beneficial fungi, and beneficial bacteria [11]. The following chapters focus on humic and fulvic acids, and then seaweed extracts and botanicals.

2.3.1 Humic and fulvic acids

Humic and fulvic acids belong to a group called humates [61]. Source of the substances is microbial activity and decomposition of biomass in soil. Humates are a major component in organic soil matter [62]. These acids differ in molecular weight and solubility. Humic acids have higher molecular weight and are soluble in alkaline solutions. In contrast, fulvic acids have predominantly lower molecular weight and are soluble in both alkaline and basic solvents [2]. The stability of the structure of the humic substances depends on pH. Acidic environment destabilizes the conformation of these substances. They typically form supramolecular associations containing a large variety of functional groups, the most represented being carboxylic and phenolic hydroxyl groups [63]. See Figure 4 for the example structures of humic and fulvic acids.

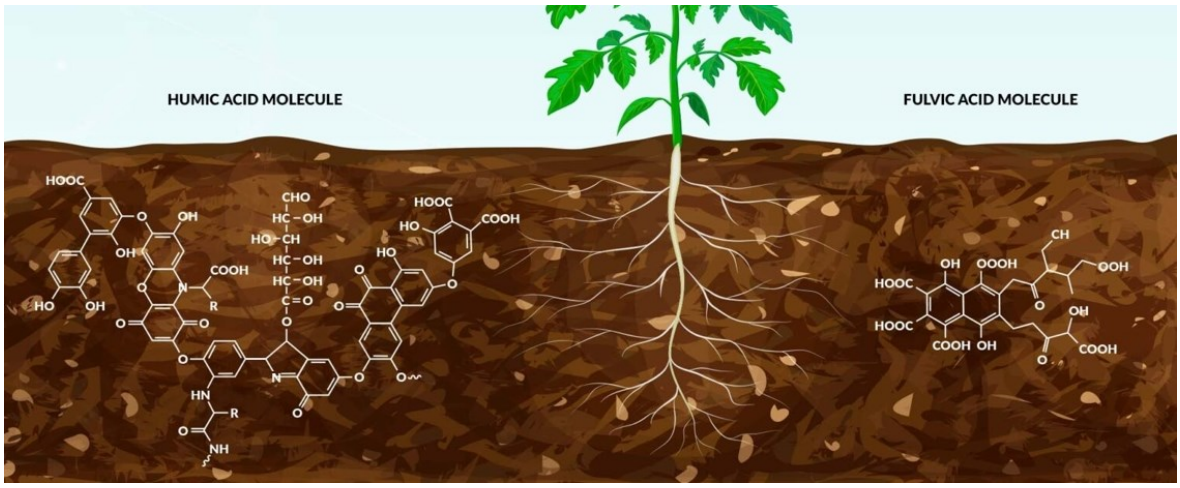

Figure 4: The example of the structures of humic and fulvic acid molecules. Taken and edited from [61].

Humates improve soil airing, root penetration, nutrient availability, water retention, and overall soil fertility [64]. Humic substances have been proven to induce various morphological changes in plants, leading to increased plant growth and yield [2]. Under saline stress, humic substances are able to activate antioxidant enzymes. It has also been shown that soil drench application is more effective than foliar [65].

Application of humic acids significantly increased plant growth, yield, and nutrient uptake [2]. Adani et al. (1998) recorded enhanced nutrient uptake (N, P, Fe, Cu) as well as root and shoot growth [66]. Another study showed increased yield of *Solanum tuberosum* L. and higher protein content in tubers [67]. Humic acids mitigated oxidative stress in plants under water stress and promoted plant growth [68].

In soil, humic acids increased the concentration of labile and moderately labile organic P, as opposed to the concentration of highly and moderately resistant organic P, which was reduced. The concentration of total organic P was not affected [69]. Piccolo et al. (1996) showed that humic acids improve the structural properties and the ability of soil to hold water [70]. Furthermore, Huang (2022) used humic acids to decrease soil salinity and promote water retention in soil [71].

Fulvic acids have the ability to complex metal ions, e.g., Fe, Al, thus releasing P from aluminium phosphate and iron phosphate [72]. Another study stated that these acids not only increased the crop yield, but also stimulated the activity of urease and alkaline phosphatase in soil [73]. Under water stress, fulvic acids promoted yield production, enhanced chlorophyl content and stimulated the activity of SOD and CAT [74].

It should be noted, however, that some studies do not report any observed changes associated with the application of humates. In some cases, overly high concentration of humic substances can even limit plant growth [2],[75].

2.3.2 Seaweed extracts and botanicals

Using seaweed as a fertilizer is an ancient practise, however its utilization as a biostimulant is fairly new [11]. There are a number of commercial seaweed extracts on the market, e.g., AgroKelp, Kelpro, Synergy and others [76]. Most of them are made from brown seaweeds, especially *Ascophyllum nodosum* [2]. Seaweed extracts are a mixture of diverse and unique substances. Polysaccharides like laminarin, fucoidan and alginates cannot be found in land plants. They also contain macro- and micronutrients, phytohormones, phenolic compounds, and vitamins [64],[77].

Seaweed extracts help mitigate abiotic stress and promote production of biomass. Moreover they enhance root system and improve the soil structure, the uptake of mineral nutrients, and the quality of produce [78]. Seaweed treatment stimulates seedling growth in both unstressed and salt-stressed environments. Treated stressed plants also produced more nonenzymatic as well as enzymatic antioxidants [79]. Cucumber plants treated with *Macrocystis pyrifera* extract showed high antioxidant capacity and contained significantly higher concentration of total phenols and vitamin C in the fruits compared to the control group [80]. Algae extracts ameliorated drought stress by increasing relative water content, osmotic potential, antioxidant enzyme activity (SOD, CAT, APX) and by decreasing H_2O_2 content [81].

Effects of seaweed extracts on soil include improved structure and consequently water retention, increased microbial activity, and boosted root system. In soils under water stress treated with algae products, the activities of sucrase, glucosidase, urease, and phosphatase were stimulated [59].

The effectiveness of algae extracts application depends on several factors – the method (soil drench or foliar spray), the type of crop, and the source of seaweed [82].

As for the other botanicals, examples include *Borago officinalis* L. (borage) or *Urtica dioica* L. (nettle). Borage extract treatment increased the production of phenolics and flavonoids and the antioxidant activity [83]. The effects of nettle are discussed in the following chapter.

2.4 Fermented Nettle

Stinging nettle (*Urtica dioica* L.) is an herbaceous plant with a long history of use in traditional medicine mainly for its antioxidant and anti-inflammatory properties [84],[85],[86]. Nettle contains plethora of bioactive molecules such as flavonoids, carotenoids, phenolic compounds, polysaccharides, fatty acids, vitamins, and minerals [86],[87],[88],[89]. Of the minerals, iron, boron, zinc, potassium, magnesium, and phosphor are all abundant in the plant, though it depends on harvesting time [89],[90]. Water-soluble vitamins C, B_1, B_2, B_3, B_6, B_9, as well as fat-soluble vitamins A, D, E, K are represented in nettle plants [86],[88],[89],[91].

Thanks to its extraordinary properties, stinging nettle makes for a suitable biostimulant. Fermented nettle is prepared by immersing nettle plants into tap water and then allowing them to ferment in a plastic container without a lid at ambient temperature (ca 15–25 °C). The fermentative process indicated by small bubbles lasts for about 14 days. The mixture is occasionally stirred during that period. Before use, fermented nettle is filtered and diluted with distilled water. Fermented nettle can be applied either as a foliar spray or, more often, as a soil drench [3],[58],[92],[93]. The ratio of nettles to water given in [3],[58] is 183 g of nettles in 10 l of water, in [92],[93] the ratio is 200 g of nettles in 1 l of water. This ratio then affects the final dilution of fermented nettle before use. The less concentrated mixture is diluted 1:3 with distilled water and the other is diluted at a 1:10 ratio. Analyses of the chemical composition of fermented nettle showed high concentration of ammonium ions, iron, potassium, boron, and calcium [58].

As other biostimulants, fermented nettle can stimulate microbial activity, plant growth, and nutrient uptake [94]. A study by Domenico (2019) reported stimulation of plant growth and flowering, as well as increase in chlorophyll content [94]. Fermented nettle increased root growth [58], shoot length and weight (both fresh and dry) [95], and iron content in leaves [3]. In contrast, foliar treatment of fermented nettle had no significant effect on potato (*Solanum tuberosum* L.) yield or growth [96].

There is one study that deals with the effect of fermented nettle on soil properties. The study reported improvement in soil respiration as well as increase in dehydrogenase and alkaline phosphatase activity [58].

No study yet has been conducted on the ability of fermented nettle to alleviate abiotic stress. Nevertheless, it has a great potential. Mitigation of abiotic stresses is generally related to increased soil nutrient availability and microbial activity [2], which, as have confirmed

several studies [58],[94], fermented nettle is capable of. Stinging nettles also contain antioxidant substances (flavonoids, phenolic compounds, vitamin C) [86],[89], thus it may help decrease oxidative stress.

2.5 Vermicompost

Vermicomposting is a process in which earthworms and microorganisms convert solid organic waste into nutritional vermicompost. It is an eco-friendly and low-cost way of managing solid waste. Earthworms break down the waste by ingesting it and their movement aerates the matter. Thus, earthworms fragment and modify the substrate so that microorganisms can more efficiently decompose the waste [97],[98],[99].

Depending on the amount of vermicompost produced in a year and the structure used, vermicomposting can be divided into a small-scale and a large-scale one. Large-scale vermicomposting produces up to 100 tonnes of vermicompost and serves for commercial purposes. The small-scale one is for personal use and up to 10 tonnes of vermicompost can be obtained. Small-scale vermicomposter is shown in Figure 5 [100].

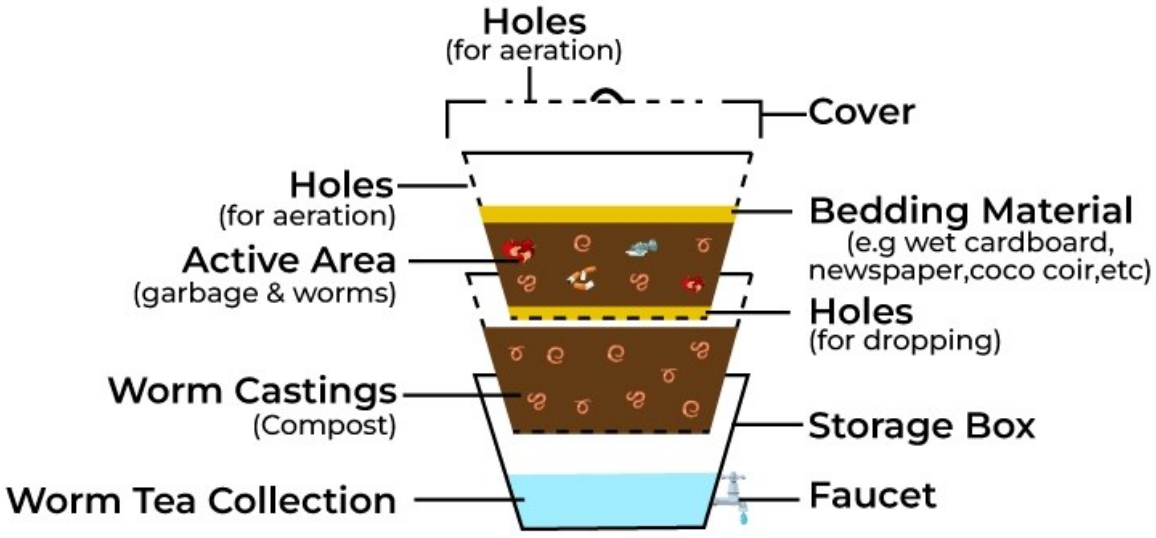

Figure 5: Diagram of a small-scale vermicomposter. Worm tea is a colloquial name for liquid by-product of vermicomposting (vermicompost leachate). Taken and edited from [100].

Vermicompost leachate (i.e. worm tea) contains the previously discussed humic acids (chapter 2.3.1, p. 16), macro- and micronutrients, and phytohormones. In one study, the application of vermicompost leachate improved plant growth, increased Cu concentration in leaves, and stimulated antioxidant activity in bulbs [101]. It can also increase grain yield of

sunflower [102], concentration of phenols, flavonoids and ascorbic acid in leaves [103], and mineral nutrient availability [104].

As other biostimulants, vermicompost is able to mitigate abiotic stress, especially osmotic stress. Under salt stress, the vermicompost leachate treatment improved plant growth and reduced accumulation of sodium cations in young leaves [105]. Reyes-Perez et al. (2021) reported similar results, the treatment also increased fresh and dry weight of shoot [106]. Another study showed that after vermicompost treatment, stressed plants have higher total phenolic, potassium, and chlorophyll a and b content compared to the control group. In addition, the activity of antioxidant enzymes, SOD and CAT, was increased [107].

In soil, vermicompost increased soil mycorrhizal inoculum potential activity [102], P, Ca, K, and Mg content [108], the activity of urease, acid phosphatase, dehydrogenase, and glucosidase, as well as the amount of beneficial bacteria [109].

3. Aims of the Thesis

This bachelor thesis has two main objectives – characterization of fermented nettle and analysis of selected properties in soils treated with either plant biostimulants (fermented nettle, vermicompost) or triazole fungicides (penconazole, tebuconazole).

1. Characterization of fermented nettle in terms of phenolic content, antioxidant capacity and selected enzyme activities.
2. Optimalization of the determination of selected enzyme activities (proteases, peroxidases, endoglycosidases) in soil.
3. Analysis of soil treated with biostimulants for selected enzyme activities.
4. Determination of selected enzyme activities in soil where plants treated with triazole fungicides were grown.

4. Materials and Methods

4.1 List of Chemicals

1,4-Dithiothreitol	Sigma-Aldrich, USA
2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazine-1-yl	Sigma-Aldrich, USA
2,4,6-Tris(2-pyridyl)-*s*-triazine	Sigma-Aldrich, USA
2-Amino-2-methyl-1-propanol hydrochloride	Merck, Germany
2-Mercaptoethanol	Sigma-Aldrich, USA
3-(Morpholin-4-yl)propane-1-sulfonic acid	Sigma-Aldrich, USA
3,3′-Diaminobenzidine	Sigma-Aldrich, USA
3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate	Sigma-Aldrich, USA
4-Nitrophenol	Sigma-Aldrich, USA
4-Nitrophenyl phosphate disodium salt hexahydrate	ITW Reagents, Italy
6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)	Sigma-Aldrich, USA
Acetic acid	Lachema, CZ
Acetone	Lach-Ner, CZ
Acrylamide	Sigma-Aldrich, USA
Ammonium iron(III) sulfate dodecahydrate	Sigma-Aldrich, USA
Ammonium persulfate	Sigma-Aldrich, USA
Ascorbic acid	Sigma-Aldrich, USA
Azocasein	Sigma-Aldrich, USA
Bicinchoninic acid disodium salt	ChemCruz, USA
Bisacrylamide	Serva, Germany
Bovine serum albumin	Sigma-Aldrich, USA
Brilliant Blue R 250	Sigma-Aldrich, USA
Bromophenol Blue	Sigma-Aldrich, USA
Calcium chloride anhydrous	Ubichem, UK
Citric acid anhydrous	Penta, CZ

Copper(II) sulfate pentahydrate	Lachema, CZ
D-Glucose	Sigma-Aldrich, USA
Disodium hydrogen phosphate dodecahydrate	Lachema, CZ
Ethanol 96%	VWR Chemicals, USA
Folin-Ciocalteu's reagent	VWR Chemicals, USA
Formaldehyde 36–38%	Lach-Ner, CZ
FTC-Casein	Thermo Scientific, USA
Gelatine from bovine skin, Type B	Sigma-Aldrich, USA
Glycerol anhydrous	Lach-Ner, CZ
Glycine	VWR Life Science, USA
Hydrochloric acid 35%	Lach-Ner, CZ
Hydrogen peroxide 30%	VWR Chemicals, USA
Iron(III) chloride anhydrous	VWR Chemicals, USA
Laminarin from Laminaria digitata	Sigma-Aldrich, USA
Methanol	Lach-Ner, CZ
N,*N*,*N*′,*N*′-Tetramethylethane-1,2-diamine	Thermo Scientific, USA
Phenol	Sigma-Aldrich, USA
Polyvinylpyrrolidone	Sigma-Aldrich, USA
Protein Assay Dye Reagent Concentrate	Bio-Rad, USA
Silver nitrate	Sigma-Aldrich, USA
Sodium acetate trihydrate	Lachema, CZ
Sodium carbonate	VWR Life Science, USA
Sodium dihydrogen phosphate dihydrate	Lachema, CZ
Sodium dodecyl sulfate	Serva, Germany
Sodium hydrogencarbonate	Lachema, CZ
Sodium hydroxide	Penta, CZ
Sodium tartrate dihydrate	Lachema, CZ
Sodium thiosulfate pentahydrate	Lachema, CZ
Sulfamic acid	Sigma-Aldrich, USA

Trichloroacetic acid	Sigma-Aldrich, USA
Tris(hydroxymethyl)aminomethane	VWR Life Science, USA
Triton X-100	Sigma-Aldrich, USA

4.2 List of Instruments

Analytical Balance AE163	Mettler Toledo, Switzerland
Analytical Balance Entris	Sartorius, Germany
Benchtop Incubator Shaker	BenchTop Lab Systems, USA
Centrifuge MiniSpin	Eppendorf, Germany
Centrifuge Universal Z 300	Hermle Labortechnik, Germany
Electrophoresis Mini-PROTEAN System	Bio-Rad, USA
Electrophoresis Multigel System	Analytik Jena (Biometra), Germany
Elite Dry Bath Incubator	Major Science, Taiwan
Incubator with natural circulation IB-01E	Schoeller Instruments, CZ
Laboratory balance Kern 440-45	Kern, Germany
Light Plate Slimlite Plano	Kaiser Fototechnik, Germany
Lyovac GT 2 E	STERIS Finn-Aqua, Finland
Mini Incubator	Labnet International, USA
pH Meter UltraBASIC UB-10	Denver Instrument, USA
Spectrophotometer Infinite M200 PRO	Tecan Group, Switzerland
Spectrophotometer Multiskan GO	Thermo Scientific, USA
Thermal Shaker *lite*	VWR International, USA
Vortex 4 basic	IKA, Germany

4.3 Methods

Unless otherwise specified in the text, “solution” means an “aqueous solution”.

4.3.1 Preparation of Fermented Nettle

The stinging nettles (*Urtica dioica* L.) used in this experiment were grown in Hradištko (Czech Republic) GPS N 49°52.24997', E 14°24.61278' (altitude 321 m a. s. l., average annual temperature 7–8 °C, average growing season temperature 13–14 °C, average annual precipitation 600 mm of water column) and collected in September 2022. In a plastic container, 100 g of nettles were left to ferment in 3 l of tap water. The fermentation lasted for four weeks in the dark at 22 °C during which the mixture was occasionally stirred. The next step was to filter the mixture through two layers of gauze. The fermented nettle thus prepared was then lyophilized and stored at ambient temperature (20–24 °C).

4.3.2 Preparation of Fermented Nettle Extracts

Accurately about 20 mg (50 mg) of fermented nettle was weighed out into a micro test tube. In the case of determination of phosphatase and protease activity, approximately 3 mg of polyvinylpyrrolidone (PVP) was added to counteract the undesirable (e.g., inhibitory) effects of secondary metabolites on enzyme activity. Thereafter, 1.0 ml of extracting solvent was added. The micro test tube containing the mixture was incubated for 30 min at 25 °C (30 °C) under orbital agitation (900 min^{-1}). The extracting solvent used, and the incubation temperature are shown in Table *1*.

Table 1. The amount of fermented nettle (FN), the extracting solvents used, and the temperature at which individual extracts were prepared.

Assay	*m*(FN) [mg]	Extraction reagent	*T* [°C]
Phenolic compounds	20	50% (v/v) Ethanol	25
Antioxidant capacity			
Proteins		Distilled water	
Proteases	50	McIlvaine buffer, pH 7	30
Acid phosphatases	20	McIlvaine buffer, pH 6	
Alkaline phosphatases		250mM AMP·NaOH buffer, pH 9	

The mixture was then centrifuged at 12,100× g for 5 min at ambient temperature. The supernatant was further used for the subsequent analyses.

The McIlvaine buffers were prepared as a mixture of 0.1M citric acid and 0.2M disodium hydrogen phosphate. The ratio of the two solutions was chosen according to the desired pH.

4.3.3 Determination of Total Phenolic Content in Fermented Nettle

Total phenolic content was determined using the Folin-Ciocalteu (F-C) assay. This assay is based on the reaction between molybdates/tungstates present in the F-C reagent and phenolic compounds. Under alkaline conditions, phenolate ions are formed which can then transfer electrons to the F-C reagent. This reaction leads to blue colour formation [110].

First, 20 µl of suitably diluted fermented nettle extract was pipetted in triplicate into the wells of a microtiter plate. Subsequently, 100 µl of 10% (v/v) F-C reagent was added into the microtiter plate. After incubation (4 min) at ambient temperature, 80 µl of 75 g/l (w/v) sodium carbonate solution was added to achieve alkaline environment. After 30 min incubation period at ambient temperature, the absorbance was measured at 760 nm. The absorbance of a blank solution, where the fermented nettle extract was substituted for 50% (v/v) ethanol, was also measured. Standard phenol solutions in the range 100–700 µM were used for the calibration (Figure 6).

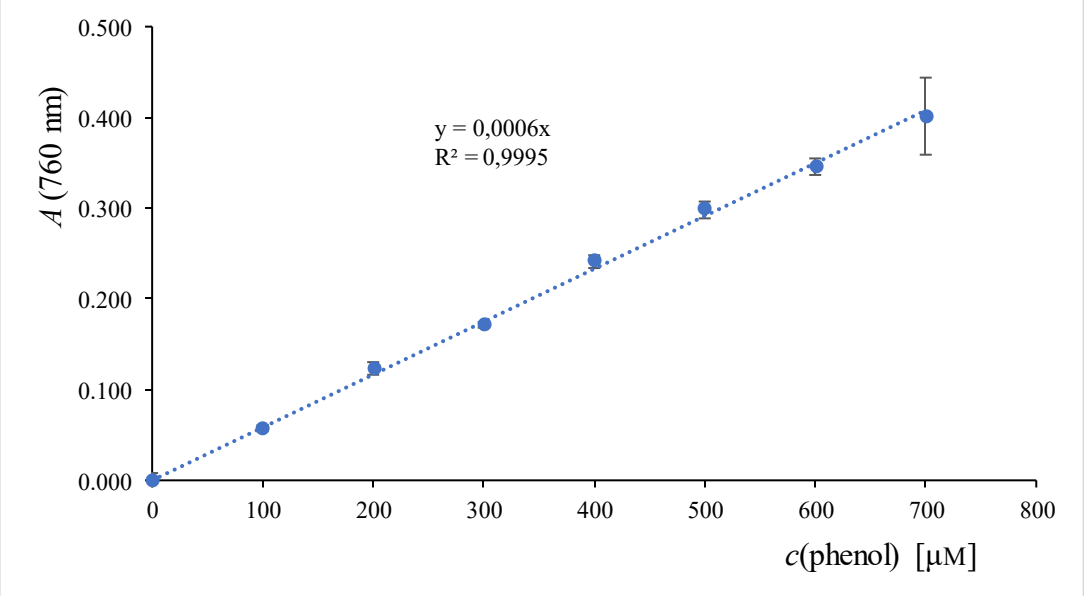

Figure 6: The calibration curve where the absorbance of standard phenol solutions at 760 nm is plotted against their concentration (µM). This calibration was used to determine the total phenolic content of fermented nettle by the F-C assay.

4.3.4 Determination of Antioxidant Capacity in Fermented Nettle

Two methods, FRAP (Ferric Ion Reducing Antioxidant Power) and DPPH (2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazine-1-yl), were used to determine the antioxidant capacity in fermented nettle. These measurements determine the efficiency at which the antioxidant compounds in fermented nettle are able to mitigate the impacts of oxidants and free radicals [111].

4.3.4.1 Determination of Antioxidant Capacity in Fermented Nettle by FRAP Assay

The determination of antioxidant capacity by the FRAP assay is based on the ability of antioxidants to reduce a ferric complex $[Fe(TPTZ)_2]^{3+}$ to a violet ferrous complex $[Fe(TPTZ)_2]^{2+}$ under acidic conditions [111],[112].

The FRAP reagent used for this assay consisted of three solutions. (1) 700μM TPTZ solution in 40mM hydrochloric acid, (2) 20mM ferric chloride solution, and (3) 300mM acetate buffer, pH 3.6. These solutions (1), (2), (3) were mixed in a volume ratio 5:2:13. The FRAP reagent was always prepared fresh and protected from light. A total of 200 μl of FRAP reagent was added in triplicate to 40 μl of appropriately diluted fermented nettle extract. After a 10 min incubation period at ambient temperature, the absorbance of the solution was measured at 593 nm. Fermented nettle extract was replaced by 50% (v/v) ethanol in a blank solution.

The calibration curve was constructed using standard ascorbic acid solutions in the range of 10–120 μM (Figure 7).

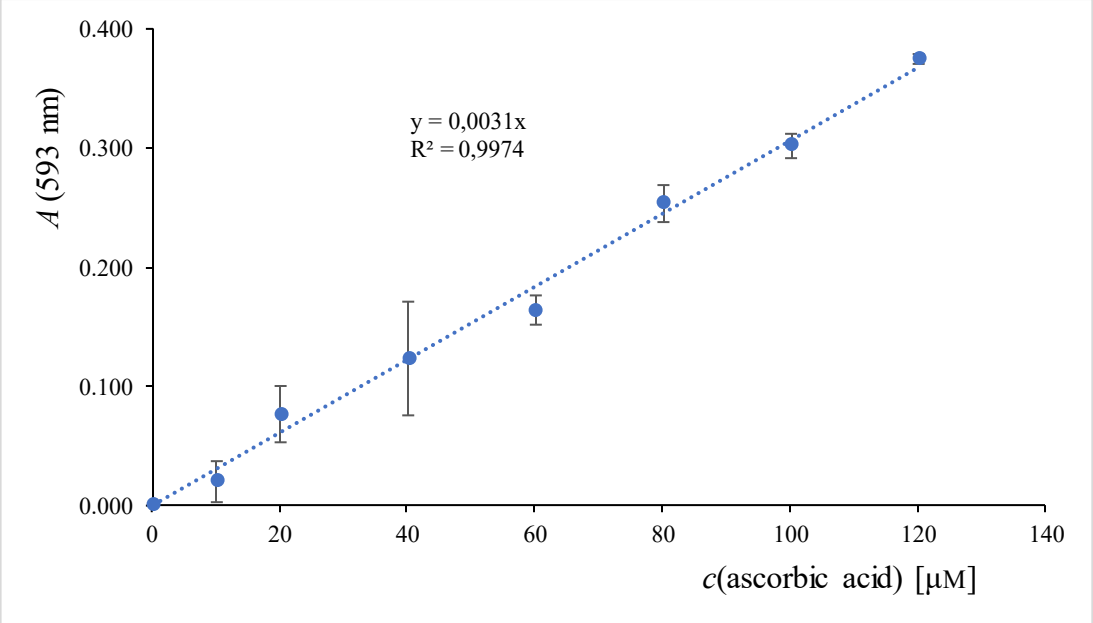

Figure 7: The calibration curve where the absorbance of standard ascorbic acid solutions at 593 nm is plotted against their concentration (µM). This calibration was used to determine the antioxidant capacity of fermented nettle by the FRAP assay.

4.3.4.2 Determination of Antioxidant Capacity in Fermented Nettle by DPPH Assay

This method evaluates the free radical scavenging ability of fermented nettle. A stable free radical with deep violet colour, DPPH, was used for this assay. Antioxidants can donate a hydrogen atom to the DPPH radical, thus causing the formation of its reduced form (DPPH-H). This radical reduction leads to a colour change from violet to light yellow. The resulting antioxidant capacity is determined as a decrease in violet colour, i.e. decrease of DPPH radicals in comparison with a reference sample [113].

First, 50 µl of suitably diluted fermented nettle extract was pipetted in triplicate into a microtiter plate. Then 100 µl of 96% (v/v) ethanol and 50 µl of 400µM DPPH solution in 96% (v/v) ethanol were added. The DPPH solution was always prepared fresh and shielded from light. A blank and a reference were also prepared. In the blank solution, fermented nettle extract was substituted for distilled water and the DPPH solution for 96% (v/v) ethanol. In the reference solution, fermented nettle extract was swapped for distilled water. The samples were incubated for 10 min at ambient temperature. The absorbance of the solutions was measured at 517 nm.

The calibration was obtained using trolox solutions in the range of 20–150 µM as standards (Figure 8).

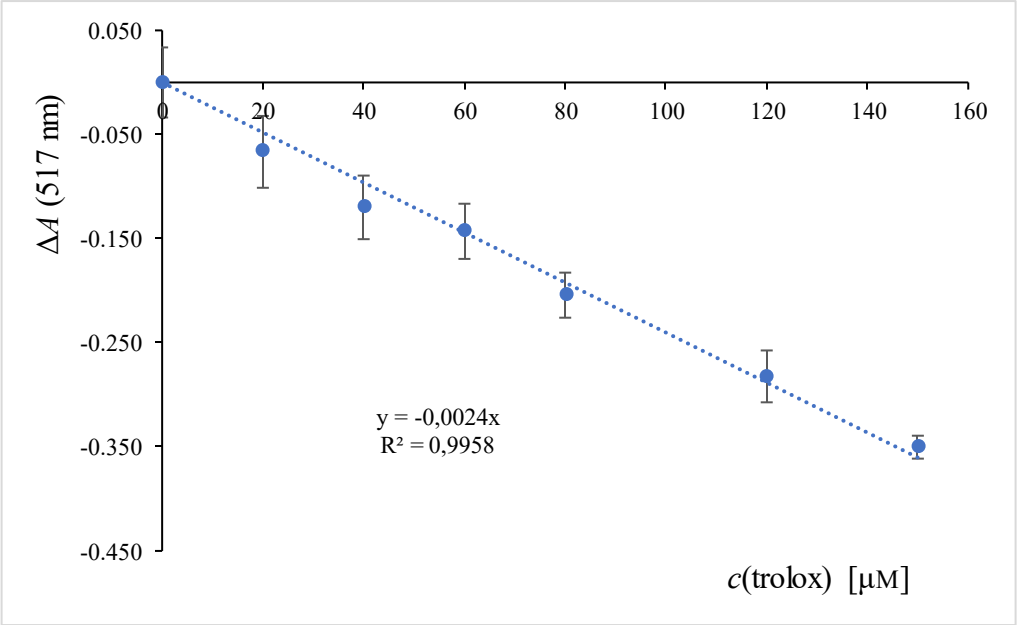

Figure 8: The calibration curve where the absorbance of standard trolox solutions at 517 nm is plotted against their concentration (μM). This calibration was used to determine the antioxidant capacity of fermented nettle by the DPPH assay. The Δ*A* (517 nm) is determined as a difference of absorbances at 517 nm between the sample and the reference containing no antioxidants.

4.3.5 Determination of Protein Concentration in Fermented Nettle

The protein concentration was determined using three methods – the Bradford protein assay, the Lowry protein assay, and the Bicinchoninic acid (BCA) protein assay.

4.3.5.1 Determination of Protein Concentration in Fermented Nettle by Bradford Assay

This method of quantifying the protein content of fermented nettle is based on binding of Coomassie Brilliant Blue G-250 dye to protein. The bond between the dye and protein shifts the peak absorbance of the dye from 465 to 595 nm. The concurring colour change is from brown to blue [114].

20 μl of appropriately diluted fermented nettle extract was pipetted in triplicate into the wells of a microtiter plate. After the addition of 200 μl of the Bradford reagent, the samples were incubated for 10 min at ambient temperature. The absorbance of the solution was measured at 595 nm and 450 nm. A blank solution was prepared by replacing fermented nettle extract with distilled water.

Bovine serum albumin (BSA) solutions in the range 0.1–0.5 mg/ml were used as standards for the construction of the calibration curve (Figure 9).

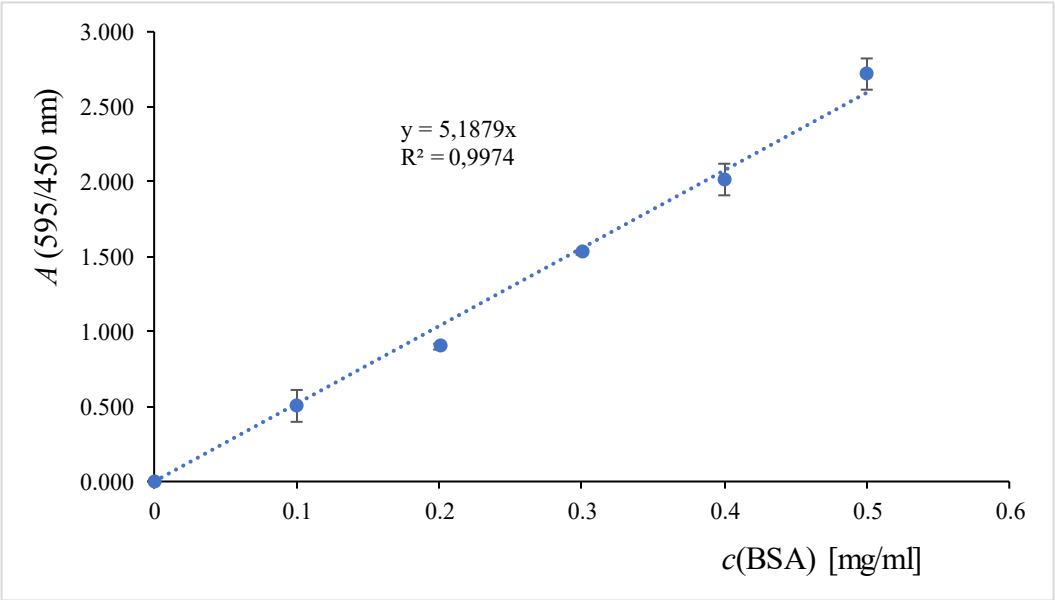

Figure 9: The calibration curve where the absorbance of standard BSA solutions at 595/450 nm is plotted against their concentration (mg/ml). This calibration was used to determine the protein content of fermented nettle by the Bradford assay.

4.3.5.2 Determination of Protein Concentration in Fermented Nettle by Lowry Assay

This method is based on the reduction of cupric ions to cuprous ions and the subsequent reaction of Cu^{+} ions with F-C reagent. The cupric ions are reduced in a reaction with a peptide bond in an alkaline environment. The F-C reagent is then reduced by the cuprous ions, resulting in blue colour production. The reaction of the F-C reagent with tryptophan and tyrosine also contributes to the blue colour [115].

First, the solution (1) of 2% (w/v) sodium carbonate in 0.1M sodium hydroxide and the solution (2) of 0.5% (w/v) copper(II) sulfate pentahydrate in 1% (w/v) sodium tartrate were mixed in a volume ratio 50:1. The solution (3) obtained was always prepared fresh. Thereafter, 15 µl of suitably diluted fermented nettle extract was pipetted in triplicate into a microtiter plate. The addition of 165 µl of the solution (3) to the samples was followed by a 10 min incubation period at ambient temperature. Then, 20 µl of 50% (v/v) F-C reagent was added into the microtiter plate and the samples were incubated for 30 min at ambient

temperature. Finally, the absorbance of the samples (and of the blank solution where fermented nettle extract was swapped for distilled water) was measured at 700 nm.

The calibration was obtained using standard BSA solutions in the range 0.05–0.7 mg/ml (Figure 10).

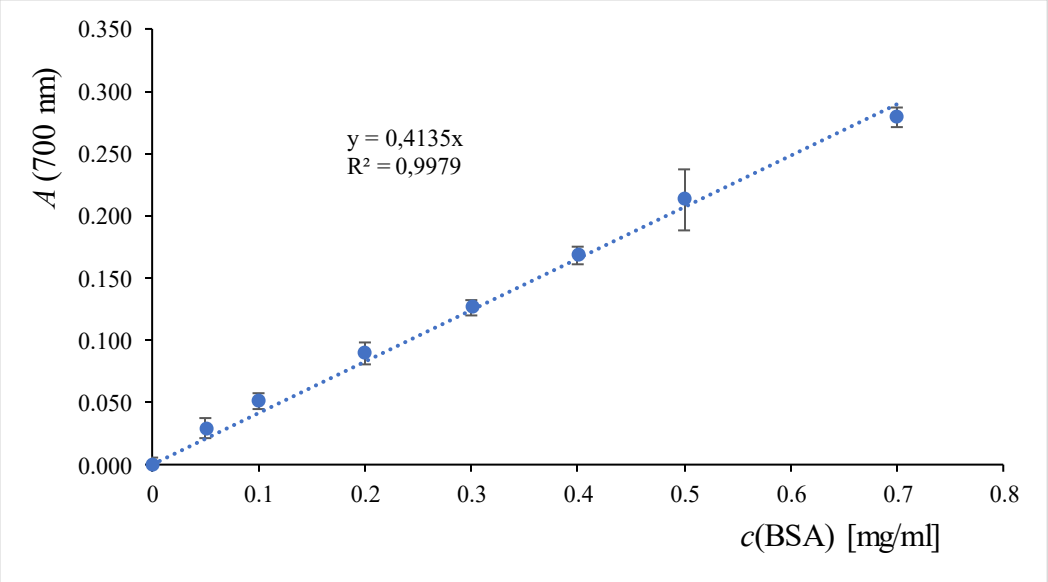

Figure 10: The calibration curve where the absorbance of standard BSA solutions at 700 nm is plotted against their concentration (mg/ml). This calibration was used to determine the protein content of fermented nettle by the Lowry assay.

4.3.5.3 Determination of Protein Concentration in Fermented Nettle by BCA Assay

The principle of this method is the same as in the Lowry assay, with the exception of the F-C reagent being replaced by bicinchoninic acid (BCA). Under alkaline conditions, the BCA is capable of binding cuprous ions to form an intense purple complex [116].

To start with, solution (1) of 2% (w/v) sodium carbonate, 0.95% (w/v) sodium bicarbonate, 0.16% (w/v) sodium tartrate, 0.4% (w/v) sodium hydroxide, 1% (w/v) BCA (sodium salt), pH 11.25 and solution (2) of 4% (w/v) copper(II) sulfate pentahydrate were mixed in a volume ratio 50:1. Thus obtained reagent is always prepared fresh. 200 µl of the reagent was added to 10 µl of the suitably diluted fermented nettle extract, which had already been pipetted in triplicate into the microtiter plate. After a 1 h incubation period at 37 °C, the absorbance of the samples was measured at 562 nm. A blank sample was prepared by substituting fermented nettle extract for distilled water.

Standard BSA solutions in the range 0.05–0.7 mg/ml were used for the calibration (Figure 11).

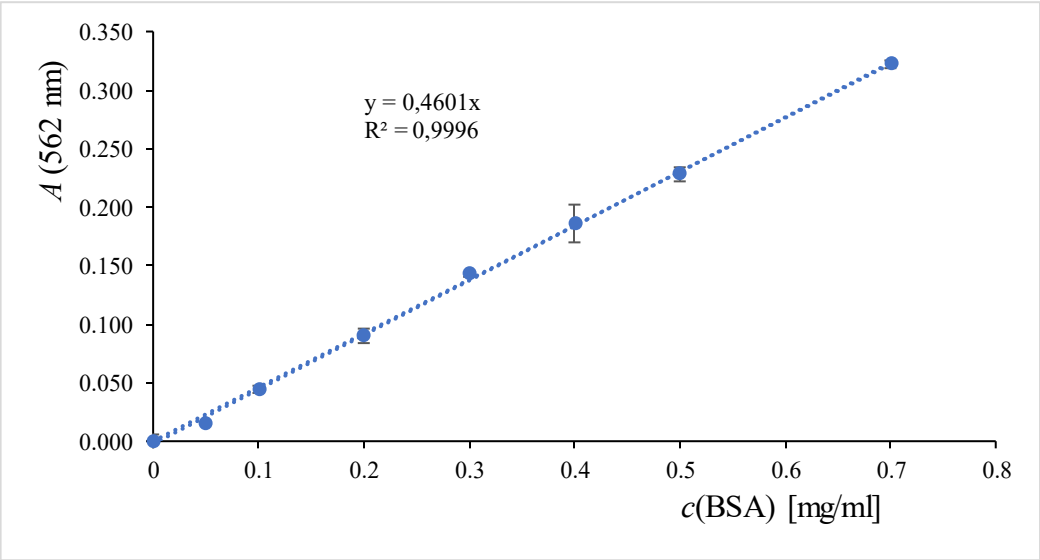

Figure 11: The calibration curve where the absorbance of standard BSA solutions at 562 nm is plotted against their concentration (mg/ml). This calibration was used to determine the protein content of fermented nettle by the BCA assay.

4.3.6 SDS Polyacrylamide Gel Electrophoresis of Proteins in Fermented Nettle

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a method for separating proteins based on their size. This is achieved by the ability of SDS to coat the proteins and to provide a net negative electrical charge to the protein molecules [117]. The proteins were detected using acid form of silver staining. This method is based on the reduction of silver ions to silver metal by proteins. Sites with proteins are revealed as dark bands on light background [118].

Gel Preparation

The gel was prepared using Bio-Rad's vertical electrophoresis kit. First, a mixture for the preparation of 12% (w/v) resolving gel was pipetted between two glass plates placed in a casting frame and clipped on a casting stand. The mixture consisted of 4.9 ml of distilled water, 6.0 ml of 30% (w/v) acrylamide/bisacrylamide solution, 3.8 ml of 1.5M Tris·HCl (pH 8.8), 150 µl of 10% (w/v) sodium dodecyl sulfate (SDS), 6 µl of tetramethylethylenediamine (TEMED), and 150 µl of 10% (w/v) ammonium persulfate

(APS). TEMED and APS was added last to initiate polymerization. The resolving gel mixture was overlaid with distilled water and allowed to solidify for about 30 min.

After the distilled water was removed, 5% (w/v) stacking gel mixture was poured onto the resolving gel. The stacking gel mixture contained 2.7 ml of distilled water, 0.67 ml of 30% (w/v) acrylamide/bisacrylamide solution, 0.5 ml of 0.5M Tris·HCl (pH 6.8), 40 µl of 10% (w/v) SDS, 4 µl of TEMED, and 40 µl of 10% (w/v) APS. Immediately after the mixture was pipetted, a 10-well gel comb was placed between the glass plates and the stacking gel was allowed to polymerize for about 20 min. The comb was removed, and the glass plates containing the gel were placed into a buffer tank. The buffer tank was filled with an electrode buffer consisting of 25mM Tris, 192mM glycine, and 0,1% (w/v) SDS; pH 8.3.

Sample Application and Separation Process

The samples of fermented nettle were prepared as follows. Aqueous fermented nettle extract obtained as described in chapter 3.3.2 was mixed with chilled acetone in a 1:3 ration to precipitate proteins. This mixture was left in a freezer at −20 °C for 24 h. The samples were centrifuged for 5 min at 12,100× g and ambient temperature. Then the supernatant was removed, and the sediment was allowed to air-dry. The precipitated proteins were dissolved in 200 µl of distilled water.

20 µl of thus prepared samples was added to 20 µl of reducing sample buffer (2× concentrated). This buffer consisted of 2.4 ml of distilled water, 2.6 ml of 0.5M Tris·HCl (pH 6.8), 2 ml of glycerol, 2 ml of 10% (w/v) SDS, 0.5 ml of 2-mercaptoethanol and 0.5 ml of 0.1% (w/v) bromophenol blue. All the samples as well as Bio-Rad's Precision Plus Protein Kaleidoscope Prestained Protein Standard were boiled for 5 min in an aluminium heat block and subsequently allowed to cool to ambient temperature.

Afterwards, 25 µl of the samples and 5 µl of the protein standard were pipetted into the wells of the stacking gel. To start with, the voltage was set at 70 V, until bromophenol blue reached the resolving gel. Then the voltage was increased to 140 V. Electrophoresis was terminated when the dye front reached the lower edge of the resolving gel.

Silver Staining

The first step was to soak the gel in a fixation solution consisting of 50% (v/v) ethanol, 12% (v/v) acetic acid, and 0.05% (v/v) formaldehyde. The gel was left in this solution overnight. Subsequently, the gel was twice thoroughly rinsed in 20% (v/v) ethanol solution and twice in distilled water within 20 minutes. This was followed by sensitization

in 0.02% (w/v) sodium thiosulfate solution for 2 min. The gel was twice briefly rinsed in distilled water. The next step was impregnation with a solution containing 0.2% (w/v) silver nitrate and 0.076% (v/v) formaldehyde for 20 min. Then the gel was quickly washed in distilled water. The gel was briefly rinsed in a development solution which consisted of 6% (w/v) sodium carbonate, 0.0004% (w/v) sodium thiosulfate, and 0.05% (v/v) formaldehyde. After that the gel was immersed in the development solution until the desired staining intensity occurred. The development was terminated by the addition of 12% (v/v) acetic acid solution.

4.3.7 Determination of Protease Activity in Fermented Nettle

4.3.7.1 Determination of Protease Activity in Fermented Nettle by Azocasein Assay

The activity of proteases (EC 3.4) was determined using azocasein as a substrate. The enzymes digest the substrate, resulting in the release of chromophoric groups from azocasein. These chromophoric groups are then soluble in trichloroacetic acid (TCA) [119].

Two sample procedures were used to determine protease activity in fermented nettle. One used fermented nettle extract and the other used fermented nettle directly. The first reaction was initiated by adding 50 µl of fermented nettle extract to 50 µl of 1.25% (w/v) azocasein solution and 40 µl of McIlvaine buffer (pH 7). This mixture was incubated for 1 h at 30 °C, under orbital agitation (900 min^{-1}). To terminate the reaction, 17 µl of 25% (w/v) TCA was added. This was followed by 5 min of centrifugation at 12,100× g and ambient temperature. Subsequently, 100 µl of the supernatant was pipetted into a microtiter plate. After adding 100 µl of 2M sodium hydroxide to the sample, the absorbance was measured at 450 nm. A blank solution was prepared by mixing 50 µl of 1.25% (w/v) azocasein solution, 40 µl of McIlvaine buffer (pH 7), 17 µl of 25% (w/v) TCA, and 50 µl of fermented nettle extract, in this order. Then the blank solution was incubated and processed in the same way as the other samples.

The second procedure differs only in that PVP, 200 µl of McIlvaine buffer (pH 7), and 250 µl of 1.25% (w/v) azocasein solution were added to 5 mg of fermented nettle. The incubation was caried out the same way as in the first procedure and the reaction was terminated by adding 85 µl of 25% (w/v) TCA. A blank solution was prepared by mixing 5 mg of fermented nettle, 200 µl of McIlvaine buffer (pH 7), 85 µl of 25% (w/v) TCA, and

250 µl of 1.25% (w/v) azocasein solution, in this order. The further steps followed the first procedure.

The calibration curve was constructed using a two-fold serial dilution of an azocasein solution in the range 0.05–3.1 mg (Figure 12).

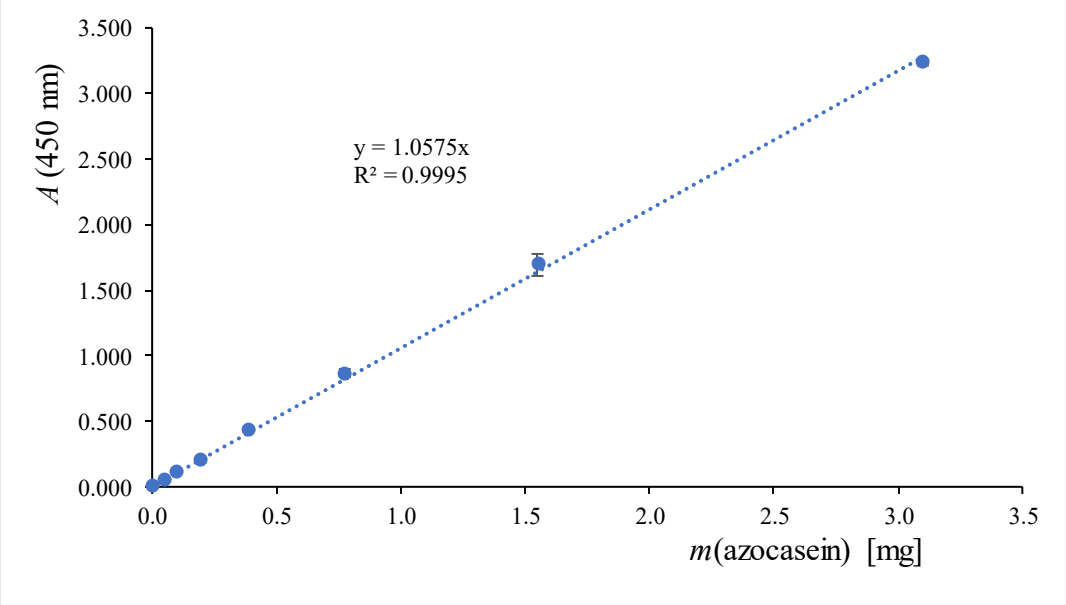

Figure 12: The calibration curve where the absorbance of azocasein solutions at 450 nm is plotted against the amount of azocasein in the solutions (mg). This calibration was used to determine protease activity in fermented nettle.

4.3.7.2 Determination of Protease Activity in Fermented Nettle by Zymography

This method in based on SDS-PAGE with gels containing gelatine. After the gels were run and the SDS removed, proteases recover their activity, thus being able to digest the gelatine. The Coomassie Brilliant Blue staining then indicates the sites of lysis as white bands on dark background [120].

Gel Preparation

The gels ware prepared using Biometra's vertical electrophoresis set. First, 12% (w/v) resolving gel mixture was prepared. The mixture contained 3.3 ml of distilled water, 4.0 ml of 30% (w/v) acrylamide/bisacrylamide solution, 2.5 ml of 1.5M Tris·HCl (pH 8.8), 100 µl of 10% (w/v) SDS, 0.12% (w/v) gelatine, 4.5 µl of TEMED, and 100 µl of 10% (w/v) APS. The resolving gel mixture was covered with distilled water and allowed to polymerize for about 30 min.

The distilled water was removed and then 4% (w/v) stacking gel mixture was poured onto the resolving gel. The stacking gel mixture consisted of 1.52 ml of distilled water, 3.25 ml of 30% (w/v) acrylamide/bisacrylamide solution, 625 µl of 0.5M Tris·HCl (pH 6.8), 25 µl of 10% (w/v) SDS, 3.8 µl of TEMED, and 35 µl of 10% (w/v) APS. A 12-well gel comb was placed between the glass plates right after the mixture was pipetted and the stacking gel was allowed to solidify for about 20 min. The comb was carefully removed and the glass plates containing the gel were placed into a buffer tank. The buffer tank was filled with an electrode buffer consisting of 25mM Tris, 192mM glycine, and 0,1% (w/v) SDS; pH 8.3.

Sample Preparation, Application and Separation Process

The samples of fermented nettle were prepared as follows. Into micro test tubes, 5 mg of fermented nettle was added to 1 ml of non-reducing sample buffer. This buffer contained 63mM Tris·HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, and 0.0013% (w/v) bromophenol blue. The samples were incubated for 1 h at 37 °C, under orbital agitation (900 min^{-1}). After the incubation period, the samples were centrifuged for 5 min at 12,100× g and ambient temperature. The supernatant was pipetted into new micro test tubes. GE Healthcare's Amersham Low Molecular Weight Kit for SDS Electrophoresis was boiled for 5 min in an aluminium heat block and subsequently allowed to cool to ambient temperature.

Then, 5 µl of the samples as well as 7 µl of the protein standard were pipetted into the wells of the stacking gel. First, the voltage was set to 70 V till the dye front reached the resolving gel. Afterwards the voltage was increased to 140 V and the electrophoresis was terminated when bromophenol blue reached the lower edge of the resolving gel.

Staining

Right after the gels were removed from glass plates, they were briefly washed in distilled water. Then the gels were immersed in 2.5% (v/v) Triton X-100 for 1 h. The gels were twice quickly rinsed in an incubation solution in which they were afterwards incubated for 6 h at 37 °C. The composition of this solution was: 50mM Tris·HCl (pH 8.4) and 5mM calcium chloride. Thereafter the incubation period and two brief washes in distilled water, the gels were stained in Coomassie Brilliant Blue solution (0.1% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid) for 2 h. The gels were soaked

in a destaining solution (10% (v/v) acetic acid in 25% (v/v) ethanol) until white bands were detected.

4.3.8 Determination of Phosphatase Activity in Fermented Nettle

4.3.8.1 Determination of Acid Phosphatase Activity in Fermented Nettle

Acid phosphatase (EC 3.1.3.2) activity was measured using *p*-nitrophenylphosphate (pNPP) as substrate, which is hydrolysed by the enzymes. The concentration of the yellow reaction product, *p*-nitrophenol (pNP), was determined [121].

Two sample procedures were used. One using fermented nettle extract and the other fermented nettle directly.

The first reaction was initiated by adding 20 µl of fermented nettle extract to 10 µl of 20mM *p*-nitrophenylphosphate (pNPP) solution and 10 µl of McIlvaine buffer (pH 6). After a 1 h incubation period at 30 °C, under orbital agitation (900 min^{-1}), the reaction was terminated by adding 50 µl of 250mM AMP·NaOH buffer (pH 9). This mixture was pipetted into a microtitration plate and the absorbance was measured at 405 nm. A blank solution was prepared by mixing 10 µl of 20mM pNPP solution, 10 µl of McIlvaine buffer (pH 6), 80 µl of 250mM AMP·NaOH buffer (pH 9), and 20 µl of fermented nettle extract, in this order. Then the blank solution was incubated and processed with the other samples.

In the second procedure, PVP, 100 µl of McIlvaine buffer (pH 6), and 100 µl of 20mM pNPP were added to 5 mg of fermented nettle. The incubation proceeded as in the first procedure and the reaction was terminated by adding 800 µl of 250mM AMP·NaOH buffer (pH 9). The mixture was centrifuged for 5 min at 12,100× g and ambient temperature. After that 120 µl of the supernatant was pipetted into a microtiter plate and the absorbance was measured at 405 nm. A blank solution was prepared by mixing 5 mg of fermented nettle, 100 µl of McIlvaine buffer (pH 6), 800 µl of 250mM AMP·NaOH buffer (pH 9), and 100 µl of 20mM pNPP solution, in this order. After incubation and centrifugation, the absorbance was measured at 405 nm.

Standard pNP solutions in the range 0.05–0.5 µmol were used to obtain the calibration curve (Figure 13).

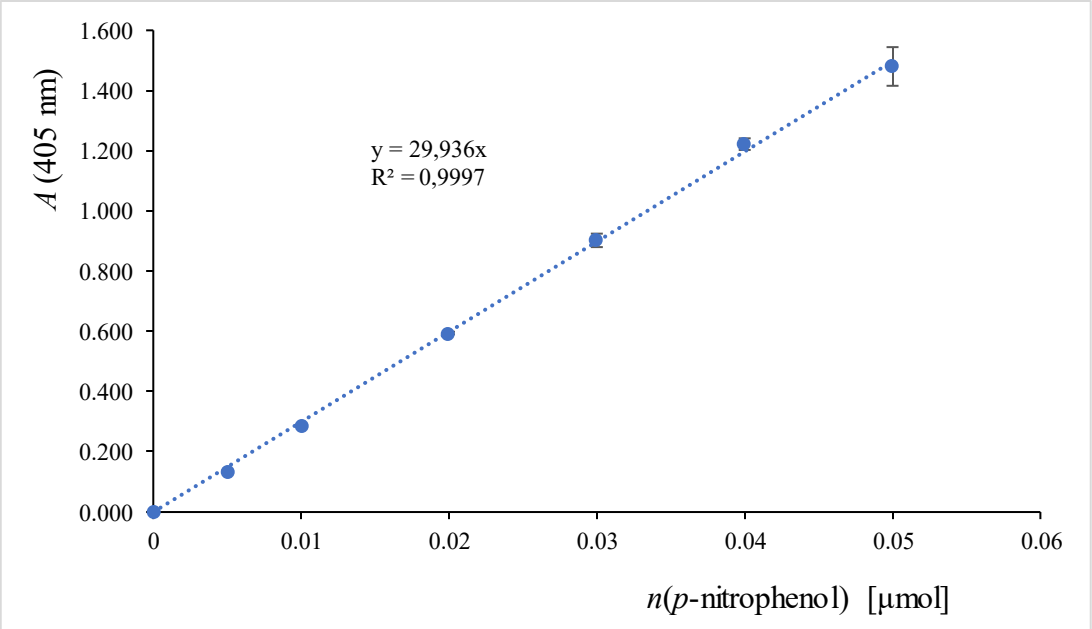

Figure 13: The calibration curve where the absorbance of standard pNP solutions at 405 nm is plotted against their amount of substance (µmol). This calibration was used to determine acid phosphatase activity in fermented nettle.

4.3.8.2 Determination of Alkaline Phosphatase Activity in Fermented Nettle

The determination of alkaline phosphatase (EC 3.1.3.1) activity follows the same principle as that of acid phosphatase. Thus, the enzymes dephosphorylate pNPP resulting in yellow pNP [122].

First, 20 µl of 20mM pNPP solution and 20 µl of 250mM AMP·NaOH buffer (pH 9) were pipetted in triplicate into the wells of a microtiter plate. The reaction was initiated by adding 20 µl of nettle water extract into the mixture. The absorbance of the samples was measured every 2 min for 1 h at 405 nm.

p-nitrophenol solutions in the range 0.05–0.5 µmol were used as standards for the construction of the calibration curve (Figure 14).

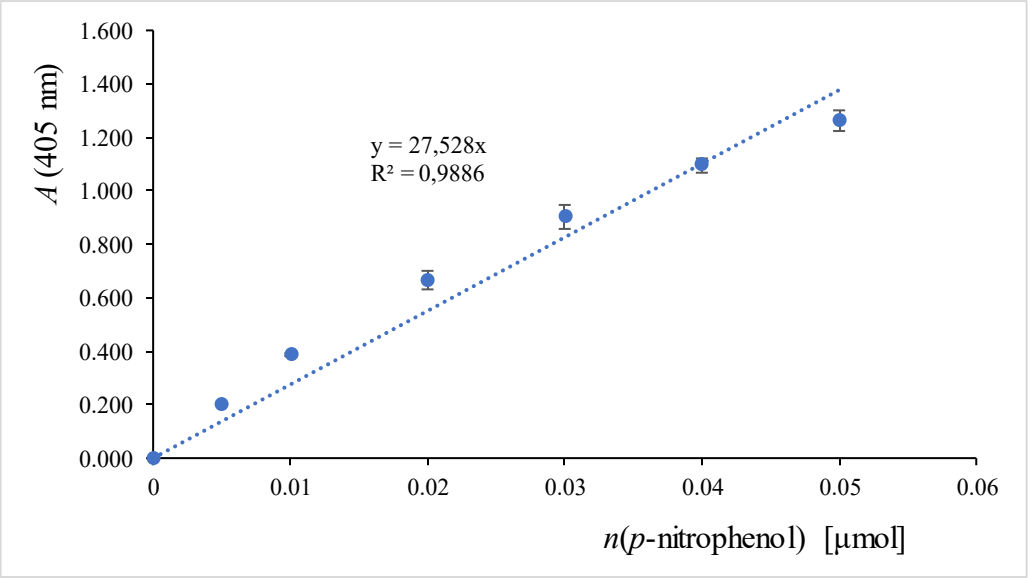

Figure 14: The calibration curve where the absorbance of standard pNP solutions at 405 nm is plotted against their amount of substance (µmol). This calibration was used to determine alkaline phosphatase activity in fermented nettle.

4.3.9 Cultivation of Plants under Drought Stress

The maize plants (*Zea mays* L., cultivar DKC 3969, Monsanto, CZ) used in this experiment were grown in a plant growth chamber under standardized conditions (MDZ3; Malapa). The temperature ranged between 23–27 °C and the lighting (Zeus 600W; Lumatek), available to the plants for 8 hours per day, was set at 50 %.

The maize seeds were planted 1 cm deep in a substrate with active humus by Agro. The substrate was watered and the pots were covered with a lid to retain moisture. After about one week, the plants germinated and were treated with 20 ml of biostimulants. The two biostimulants used for this treatment were fermented nettle (N) and vermicompost leachate (V).

The preparation of fermented nettle is described in chapter 4.3.1 (p. 27). The vermicompost leachate was, like fermented nettle, lyophilized. Both biostimulants were applied in the form of a 20 g/l suspension of given lyophilizates.

The plants were treated with the biostimulants twice a week for one week. Half of the plants in this experiment were exposed to drought (D) for 8 days before harvest. All the plants were harvested two weeks after the first treatment. Together with untreated control group (C), this experiment contained 6 groups of plants (C, CD, N, ND, V, and VD) with 7 plants per group.

After the harvest, soil samples were collected as follows. In each group the soil was mixed to form a composite sample, which was then lyophilized. The samples were sifted through a 500µm sieve and stored at −20 °C in a freezer.

4.3.10 Cultivation of Plants Treated with Triazole Fungicides

The soil used for the analyses described below was obtained from an experiment conducted in 2021 [123]. Briefly, tomato plants (*Solanum lycopersicum*, cv. Cherrola) grown in substrate Agro with active humus and fertilized with Kristalon fertilizer were treated with triazole fungicides. Two fungicides were chosen for treatment: penconazole (P), tebuconazole (T) and their combination (PT) in a 1:1 ratio. The fungicides (3.52 µmol per plant) were applied as a foliar spray (f) or injected directly into the soil (s). Together with untreated control plants (C), there were seven groups of plants (C, sP, sT, sPT, fP, fT, and fPT). Each group consisted of six tomato plants. During the experiment, the plants were treated 5 times with triazoles and 5 times with fertilizer. After six weeks of growth, soil samples were collected, lyophilized and stored in a freezer at −20 °C. Prior to the analyses, the soil was sifted through a 500µm sieve.

4.3.11 Determination of Protein Concentration in Soil by Lowry Assay

First, 3.5 ml of 50mM phosphate buffer (pH 7) was added to 0.5 g of soil. The mixture was incubated for 1 h at 30 °C, under orbital agitation (200 min^{-1}). Thereafter, the samples were centrifuged for 10 min at 4,000× g and ambient temperature. Approximately 1 ml of the supernatant was pipetted into a micro test tube. The supernatant was once again centrifuged for 10 min, now at 12,100× g and ambient temperature. The protein concentration of the suitably diluted extract was measured using the method described in chapter 4.3.5.2 (p. 32).

4.3.12 Determination of Protease Activity in Soil

4.3.12.1 Determination of Protease Activity in Soil by FRET Assay

Measurement of protease activity (EC 3.4) based on fluorescence resonance energy transfer (FRET) detects increase in total fluorescence caused by the digestion of fluorescein-labelled casein [124].

FTC-Casein stock solution of concentration 5 mg/ml was diluted 1:500 in 100mM Tris·HCl buffer, pH 7. 200 µl of thus prepared FTC-Casein working reagent was added to 5 mg of soil and 200 µl of Tris·HCl buffer, pH 7. This mixture was incubated for 1 h at 30 °C,

under orbital agitation (900 min^{-1}), and shielded from light. The samples were centrifuged for 5 min at 12,100× g and ambient temperature. Finally, 200 µl of supernatant was pipetted into a microtiter plate and 485/538 nm excitation/emission maxima was measured. A blank solution was prepared by substituting FTC-Casein working reagent for 100mM Tris·HCl buffer, pH 7.

4.3.12.2 Determination of Protease Activity in Soil by Zymography

This method followed the procedure described in chapter 4.3.7.2 (p. 37) with some minor changes. First, 5 mg of fermented nettle was replaced by 5 mg of soil and only 300 µl of non-reducing sample buffer was added. Second, 25 µl of samples were loaded on the gel and no protein standard was run with the samples. Third, the gels were incubated in the incubation solution for 18 h at 37 °C.

4.3.13 Determination of Endoglycosidase Activity in Soil

The activity of endo-β-1,3-glucanase (EC 3.2.1.39) was measured by a method using laminarin as substrate and 3-methyl-2-benzothiazolinone hydrozone (MBTH) as reagent. This reagent is used to quantify the amount of reducing sugars released throughout this method. First, the sugar reacts with only one molecule of MBTH but in acid and oxidizing environment the MBTH adduct of sugar can bind another MBTH molecule. This second binding produces more intensely coloured blue product [125].

7.0 ml of 100mM MOPS·NaOH buffer, pH 5 was added to 1.0 g of soil. After a 1 h incubation period at 30 °C, under orbital agitation (200 min^{-1}), 40 mg of wet soil was weighted out into a micro test tube. The former mixture was centrifuged for 10 min at 4,000× g and ambient temperature, then approximately 1 ml of the supernatant was pipetted into a micro test tube. The supernatant was once again centrifuged for 10 min at 12,100× g and ambient temperature.

The MBTH reagent used for this assay was prepared by mixing 3 mg/ml MBTH solution and 1 mg/ml DTT (dithiothreitol) solution in a volume ratio 1:1. This reagent was always prepared fresh. The acidic Fe solution used contains 0.5% (w/v) ferric ammonium sulfate dodecahydrate and 0.5% (w/v) sulfamic acid solution in 0.25M hydrochloric acid.

The first reaction was initiated by adding 8 µl of soil extract into a micro test tube containing 20 µl of 100mM MOPS·NaOH buffer (pH 5) and 12 µl of 1.6 mg/ml laminarin solution. The second reaction was started by adding 100 µl of 100mM MOPS·NaOH buffer (pH 5) and 60 µl of 1.6 mg/ml laminarin solution to the micro test tube with 40 mg of wet

soil. All the samples were incubated for 1 h at 37 °C, under orbital agitation (900 min^{-1}). After the incubation, the samples containing wet soil were centrifuged for 5 min at 12,100× g and ambient temperature. Then 40 µl of the supernatant was pipetted into a new micro test tube. The following procedure was the same for the samples prepared with soil extract and the ones prepared with wet soil. 40 µl of 0.5M NaOH and 40 µl of the MBTH reagent were added to all micro test tubes. After a 15 min incubation period at 80 °C in an aluminium heat block, 80 µl of the acidic Fe solution was added. The contents of the micro test tubes were mixed and 100 µl of the samples was pipetted into a microtiter plate. The absorbance of the samples was measured at 630 nm. A blank solution was prepared by mixing 8 µl of soil extract with 40 µl of 0.5M NaOH and then heating this mixture at 80 °C for 30 min in an aluminium heat block. Subsequently, 12 µl of distilled water, 20 µl of 100mM MOPS·NaOH buffer (pH 5), 40 µl of the MBTH reagent, 40 µl of 0.5M NaOH, and 80 µl of the acidic Fe solution were added.

The calibration curve was obtained using standard glucose solutions in the range 5–80 nmol (Figure 15).

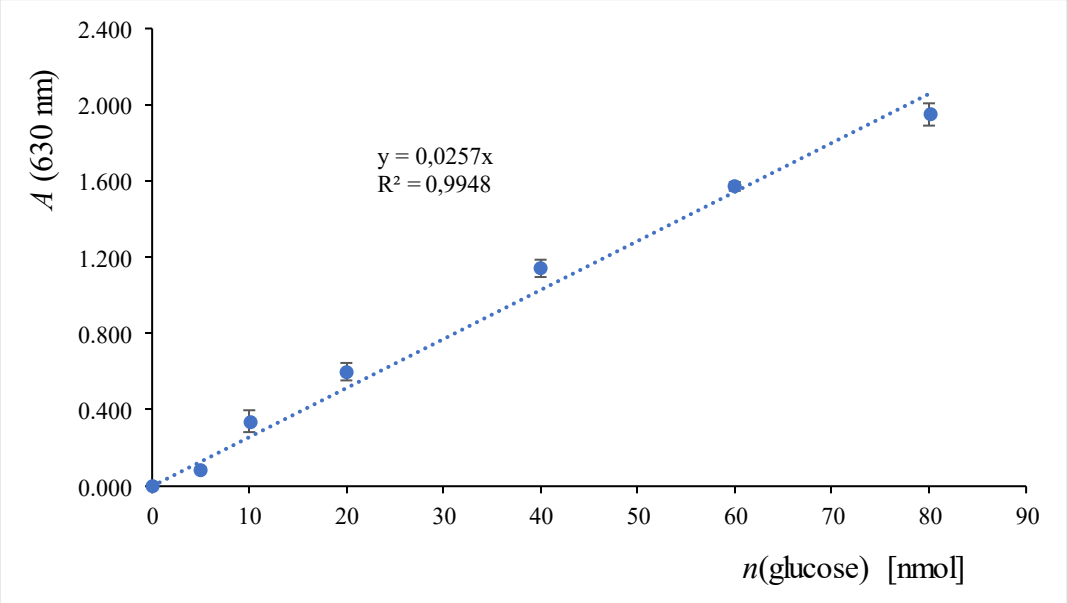

Figure 15: The calibration curve where the absorbance of standard glucose solutions at 630 nm is plotted against their amount of substance (nmol). This calibration was used to determine the endoglycosidase activity in soil.

4.3.14 Determination of Peroxidase Activity in Soil

The activity of total soluble and total bound peroxidases (EC 1.11.1.7) was detected by an assay using 3,3'-diaminobenzidine (DAB) as a substrate. The enzymes oxidize the substrate, resulting in the formation of a red complex [126].

3.5 ml of 50mM phosphate buffer (pH 7) was added to 0.5 g of soil. After a 1 h extraction period at 30 °C, under orbital agitation (200 min^{-1}), the samples were centrifuged for 10 min at 4,000× g and ambient temperature. Approximately 1 ml of the supernatant was pipetted into a micro test tube. The supernatant was once again centrifuged for 10 min at 12,100× g and ambient temperature. 20 µl of soil extract was pipetted in triplicate into a microtiter plate. Subsequently, 155 µl of 50mM phosphate buffer (pH 7), 5 µl of 49mM hydrogen peroxide, and 20 µl of 4 mg/ml DAB solution in 96% (v/v) ethanol was added. The absorbance was measured at 430 nm every 5 min for 1 h at 25 °C.

4.3.15 Statistical Analysis

The data from measurements in fermented nettle were analysed in MS Excel version 2403. The statistical analysis of data from soil measurements was performed by one-way ANOVA (protein concentration, protease activity, peroxidase activity) or two-way ANOVA (comparison of two procedures measuring endoglycosidase activity) in R program 4.1.2.

In the case of total peroxidase and protease activity, one-way ANOVA was chosen. The analysis of the endo-β-1,3-glucanase activity results was slightly different since this enzyme activity was determined using two methods. The first step was to conduct one-way ANOVA comparing the two methods. Then, two one-way ANOVAs were conducted to find differences between groups measured by the two methods separately. Finally, two-way ANOVA was used to compare all the results of measuring endo-β-1,3-glucanase activity between each other.

The post-hoc test used to determine significant difference between groups at $p < 0.05$ was Tukey test. Statistically significant differences are indicated by different letters above bars in the graphs. All graphs were created in MS Excel 2403.

5. Results

In this thesis, fermented nettle was characterized in terms of antioxidants and selected enzyme activities. The results are expressed per gram of dry weight of fermented nettle. The influence of biostimulants (fermented nettle and vermicompost) as well as chemical treatment (triazole fungicides) on soil properties was analysed. The results are expressed per gram of dry weight of soil.

5.1 Analyses of Fermented Nettle

5.1.1 Total Phenolic Content and Antioxidant Capacity in Fermented Nettle

Total phenolic content in fermented nettle was determined by the method described in chapter 4.3.3 (p. 28). The results are expressed as µmol of phenol equivalent per g of dry weight of fermented nettle (FN) (Figure 16). Antioxidant capacity in fermented nettle was measured using two methods – FRAP (p. 29) and DPPH (p. 30). The results of FRAP assay are expressed as µmol of ascorbic acid equivalent per g of dry weight of fermented nettle (FN) (Figure 16). The results of DPPH assay are expressed as µmol of trolox equivalent per g of dry weight of fermented nettle (FN) (Figure 16).

Total phenolic content in fermented nettle was (56 ± 13) µmol(phenol equivalent)/g(FN). Antioxidant capacity in fermented nettle is slightly higher when measured by FRAP assay ((19 ± 1) µmol(ascorbic acid equivalent)/g(FN)) than by DPPH assay (14 ± 4) µmol(trolox equivalent)/g(FN)).

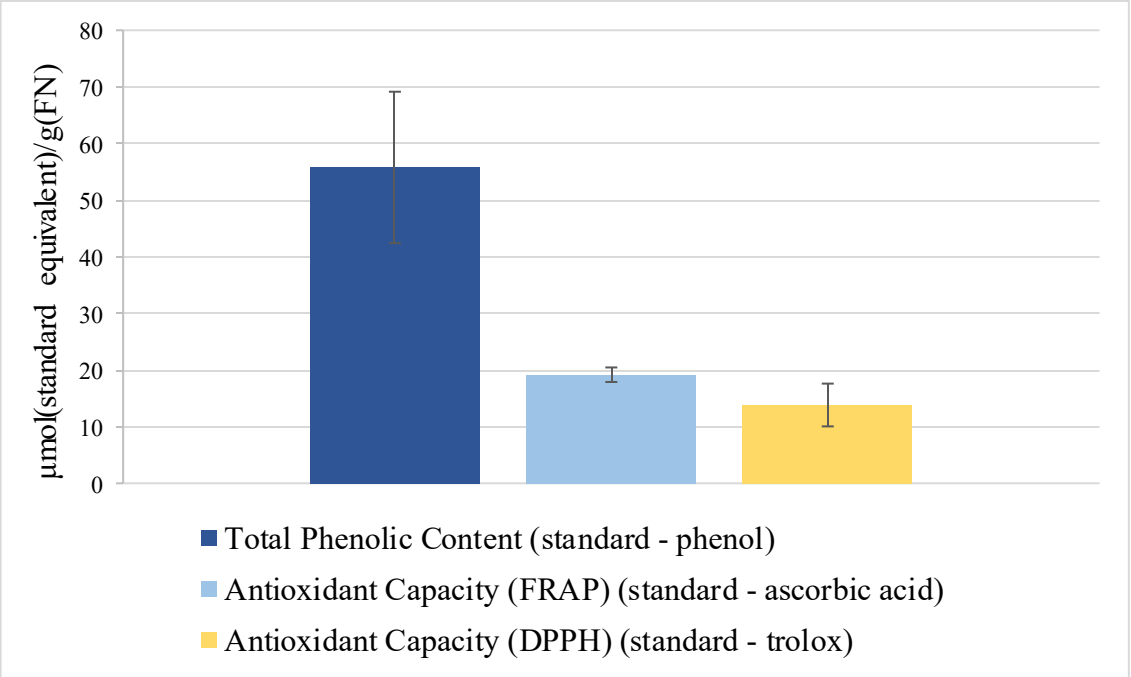

Figure 16: Total phenolic content in fermented nettle expressed as µmol of phenol equivalent per g of dry weight of fermented nettle. Antioxidant capacity in fermented nettle measured by FRAP assay expressed as µmol of ascorbic acid equivalents per g of dry weight of fermented nettle. Antioxidant capacity in fermented nettle measured by DPPH assay expressed as µmol of trolox equivalent per g of dry weight of fermented nettle. Abbreviation: fermented nettle (FN).

5.1.2 Protein Concentration in Fermented Nettle

Three methods were used to determine protein concentration in fermented nettle. The Bradford assay is described in chapter 4.3.5.1 (p. 31), the Lowry assay in chapter 4.3.5.2 (p. 32), and the BCA assay in chapter 4.3.5.3 (p. 33). All results are expressed as mg of protein per g of dry weight of fermented nettle (FN) (Figure 17).

The highest concentration of protein was determined by BCA assay ((220 ± 39) mg(protein)/g(FN)). On the other hand, the lowest protein concentration was measured using Bradford assay ((20 ± 1) mg(protein)/g(FN)). The protein concentration determined by Lowry assay was (134 ± 15) mg(protein)/g(FN).

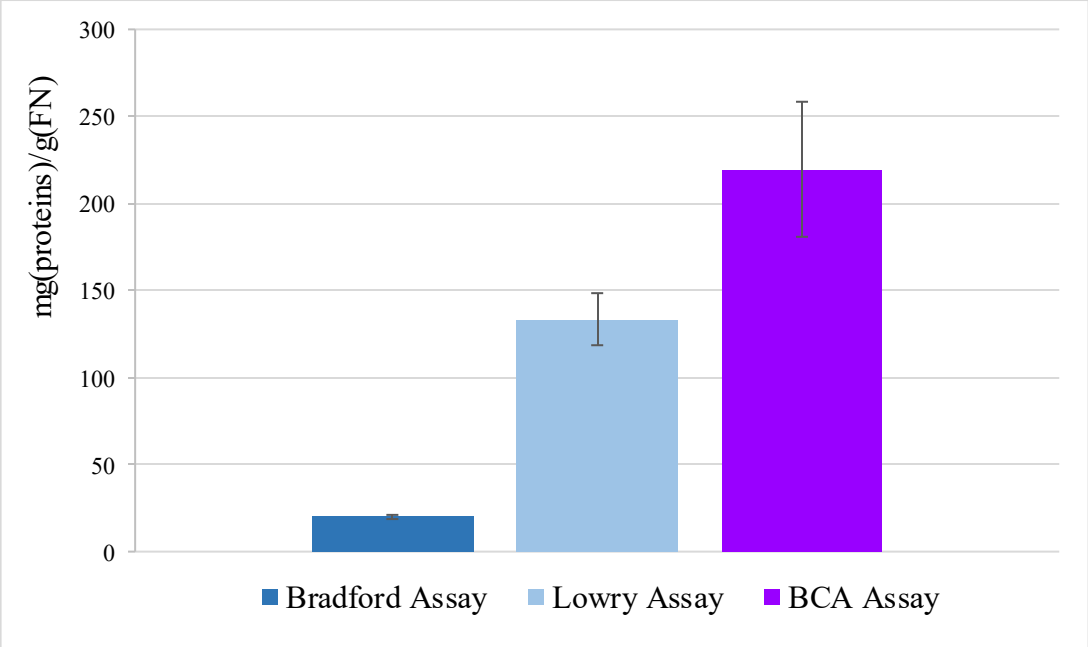

Figure 17: Protein concentration in fermented nettle expressed as mg of protein per g of dry weight of fermented nettle. The three methods used: Bradford assay, Lowry assay, and BCA assay. Abbreviations: fermented nettle (FN), bicinchoninic acid (BCA).

5.1.3 Electrophoretic Separation of Proteins in Fermented Nettle

This method is described in chapter 4.3.6 (p. 34). The protein standard contained proteins with molecular weight 10–250 kDa. The results of SDS-PAGE of proteins in fermented nettle are shown in Figure 18. A total of nine bands of proteins were detected.

Two of the three most intense bands (1, 2) contain proteins with molecular weight greater than 250 kDa. The most intense band (3) contains proteins with molecular weight around 200 kDa.

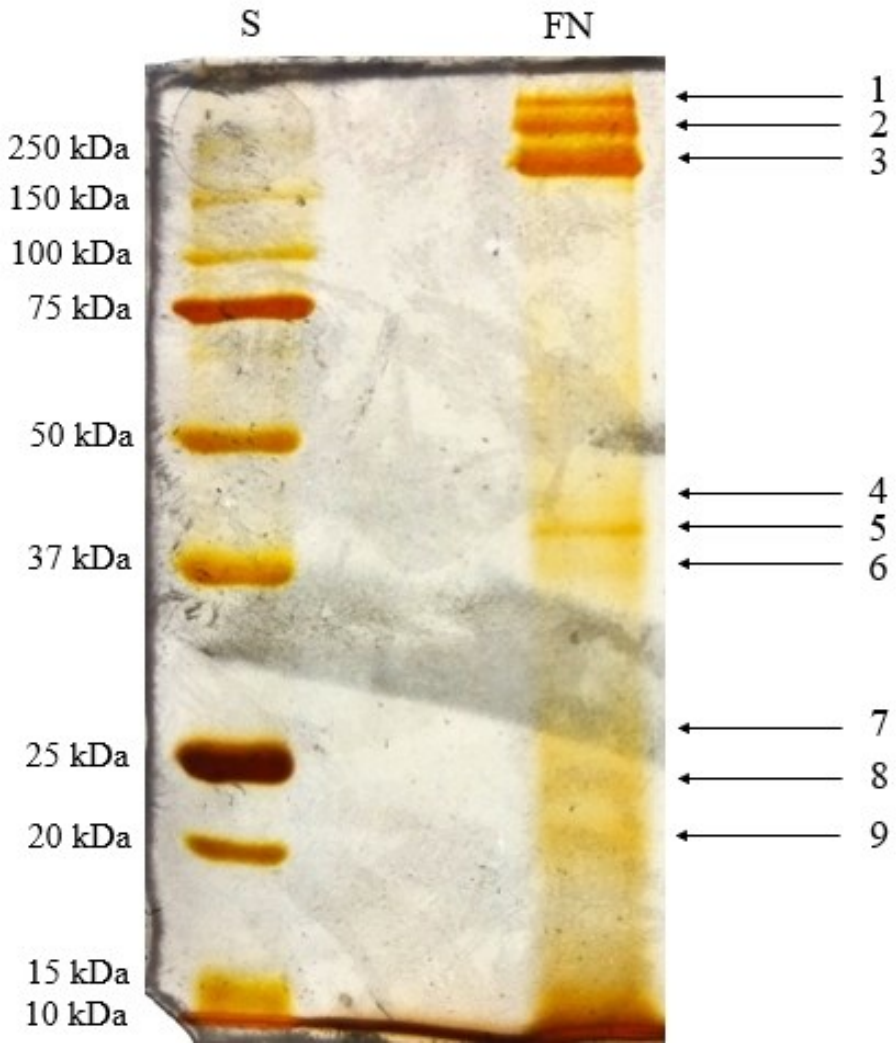

Figure 18: SDS-PAGE (12% polyacrylamide gel) of proteins in fermented nettle after silver staining. Legend: S marks the protein standard, FN marks the lane where fermented nettle was applied. Numbered black arrows indicate the bands of protein contained in fermented nettle.

5.1.4 Proteolytic Activity in Fermented Nettle

Protease activity in fermented nettle was determined by azocasein assay. This method is described in chapter 4.3.7.1 (p. 36). Two procedures were used: one using fermented nettle extract and the other using solid lyophilized fermented nettle directly. The results are expressed as mg of hydrolysed substrate (azocasein) per min per g of dry weight of fermented nettle (Figure 19).

Protease activity was higher when fermented nettle extract was used ((2.4 ± 0.2) mg(azocasein)/min/g(FN)) rather than fermented nettle directly ((0.79 ± 0.03) mg(azocasein)/min/g(FN)).

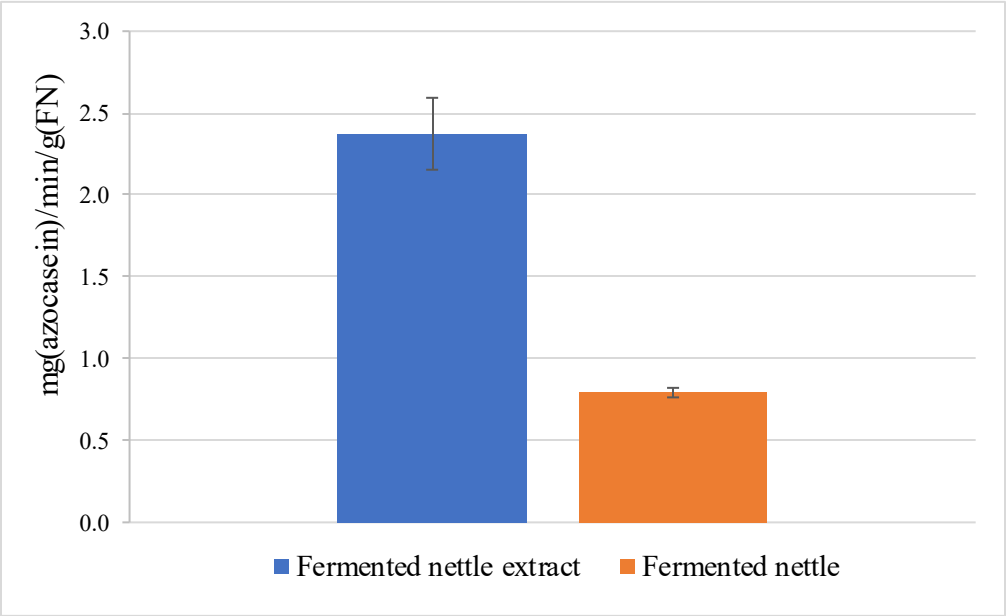

Figure 19: Protease activity of fermented nettle expressed as mg of substrate hydrolysed (azocasein) per min per g of dry weight of fermented nettle. Abbreviation: fermented nettle (FN).

The second method used to detect protease activity was zymography. This method is described in chapter 4.3.7.2 (p. 37). The results of zymography of fermented nettle are shown in Figure 20. The white bands indicate the most represented proteases. Two bands (1, 2) contain proteases with molecular weight greater than 97.0 kDa. The most intense band (3) contains proteases with molecular weight around 97 kDa. The lowest molecular weight of protease in fermented nettle is around 30 kDa (band 7).

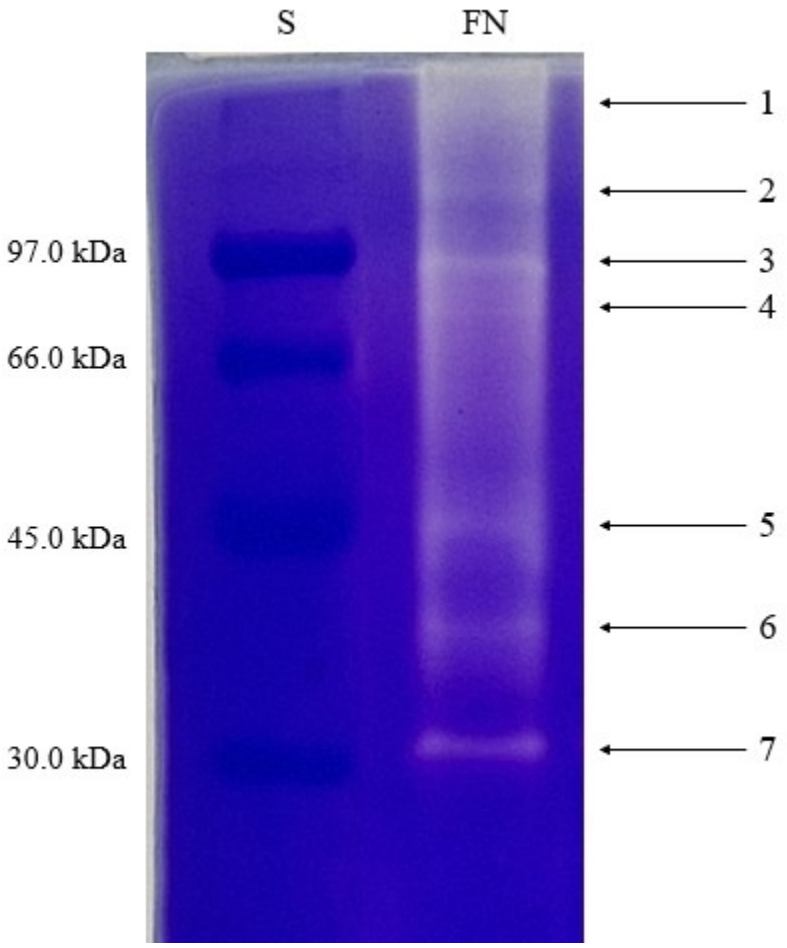

Figure 20: Zymogram of proteases in fermented nettle (12% polyacrylamide gel containing 0.12% (w/v) gelatine). Legend: S marks the protein standard and FN marks the lane where fermented nettle was applied. Numbered black arrows indicated the most represented proteases.

5.1.5 Phosphatase Activity in Fermented Nettle

In fermented nettle, both acid phosphatase activity and alkaline phosphatase activity were determined. Acid phosphatase activity was determined using two procedures, one using fermented nettle extract and the other solid lyophilized fermented nettle directly. Both procedures are described in chapter 4.3.8.1 (p. 39). Alkaline phosphatase was determined using only FN extract and this method is described in chapter 4.3.8.2 (p. 40). The results are expressed as nmol of product formed (*p*-nitrophenol) per min per g of dry weight of fermented nettle (FN) (Figure 21).

Lower acid phosphatase activity was determined when using the procedure with fermented nettle ((43.0 ± 5.9) mg(pNP)/min/g(FN)), rather than the procedure with fermented nettle extract ((91.8 ± 14.1) mg(pNP)/min/g(FN)). Alkaline phosphatase activity

was more than four times higher ((409.0 ± 76.7) mg(pNP)/min/g(FN)) than acid phosphatase activity.

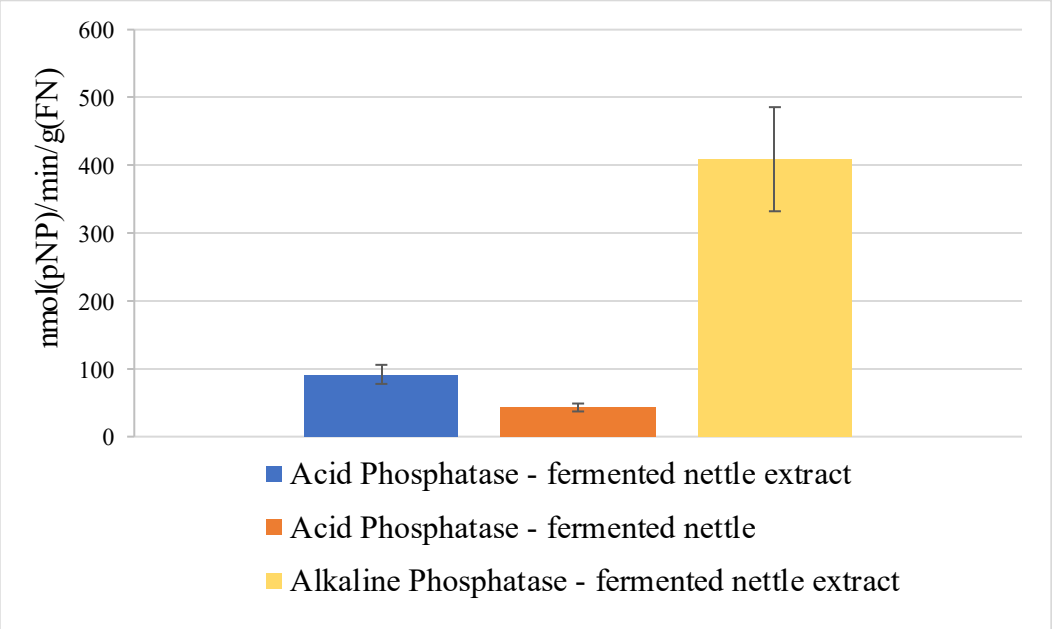

Figure 21: Acid and alkaline phosphatase activity of fermented nettle expressed as nmol of substrate formed (pNP) per min per g of dry weight of fermented nettle. Abbreviations: p-nitrophenol (pNP), fermented nettle (FN).

5.2 Analyses of Soil Treated with Biostimulants

5.2.1 Protein Concentration in Soil Treated with Biostimulants

Protein concentration in soil was determined using Lowry assay. This method is described in chapter 4.3.11 (p. 42). The results are expressed as mg of protein per g of dry weight of soil (Figure 22).

The protein concentration in soil is the lowest in the group of plants treated with fermented nettle that underwent drought stress (ND) and highest in the group of plants treated with vermicompost (V). Every group that underwent drought stress has lower protein concentration than the corresponding group which was watered regularly. Protein content was significantly lower in the group treated with fermented nettle that underwent drought stress (ND) than in the corresponding, regularly watered group.

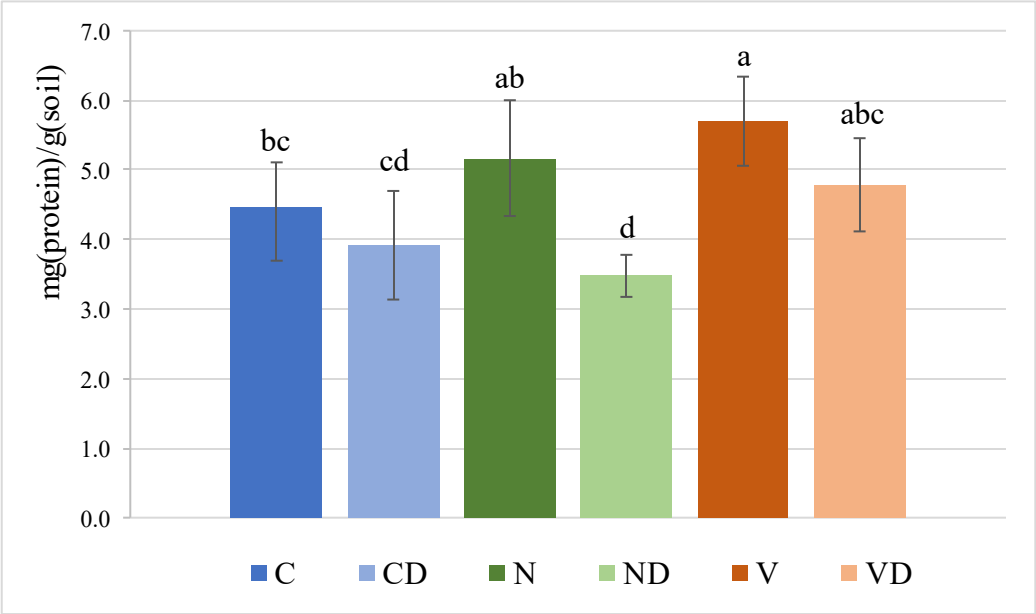

Figure 22: Protein concentration determined by Lowry assay in soil treated with biostimulants expressed as mg of protein per g of dry weight of soil. The different letters above the bars indicate statistically significant differences between groups. Abbreviations: control group (C), group treated with fermented nettle (N), group treated with vermicompost (V), groups exposed to drought (CD, ND, VD).

5.2.2 Proteolytic Activity in Soil Treated with Biostimulants

Protease activity in soil was determined by FRET assay described in chapter 4.3.12.1 (p. 42). The results are expressed as mega relative fluorescence units (MRFU) per h per g of dry weight of soil (Figure 23).

Protease activity in soil of the control group and the groups treated with biostimulants shows no significant difference. The groups that underwent drought have higher protease activity than the groups that were regularly watered. The group treated with fermented nettle that underwent drought (ND) shows the highest protease activity.

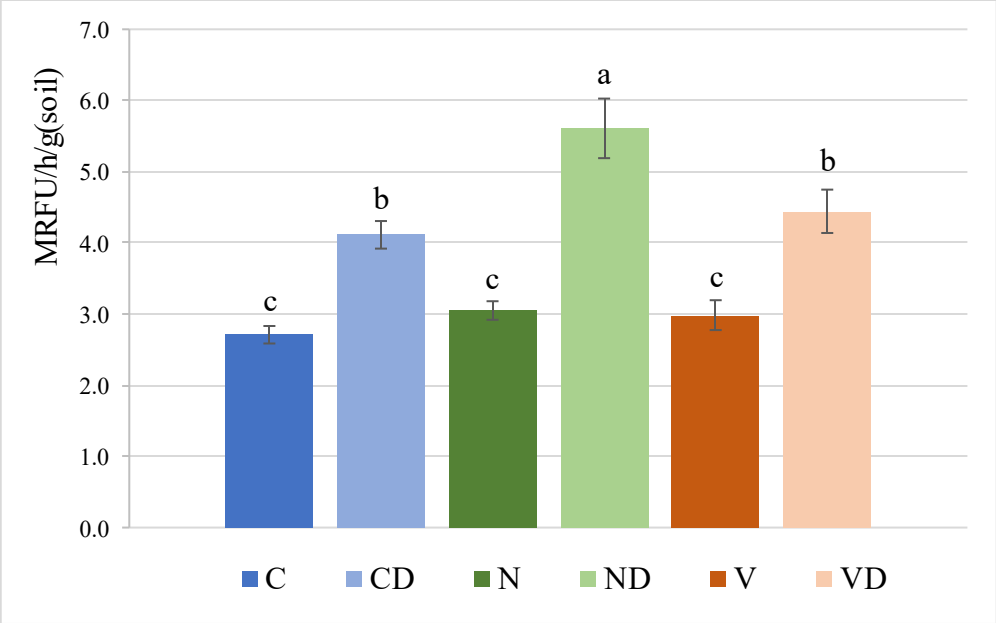

Figure 23: Protease activity of soil treated with biostimulants expressed as MRFU per h per g of dry weight of soil. The different letters above the bars indicate statistically significant differences between groups. Abbreviations: mega relative fluorescence units (MRFU), control group (C), group treated with fermented nettle (N), group treated with vermicompost (V), groups exposed to drought (CD, ND, VD).

The second method used to determine protease activity and distribution in soil was zymography (Figure 24). This method is described in chapter 4.3.12.2 (p. 43). Three zymograms from independent biological repeats were performed.

In two repetitions, the highest protease activity was detected in the control group (C). In one repetition, the highest protease activity showed the group treated with vermicompost (V). According to the FRET assay, the highest protease activity should be detected in group treated with fermented nettle that underwent drought stress (ND). Indeed, this group showed high protease activity.

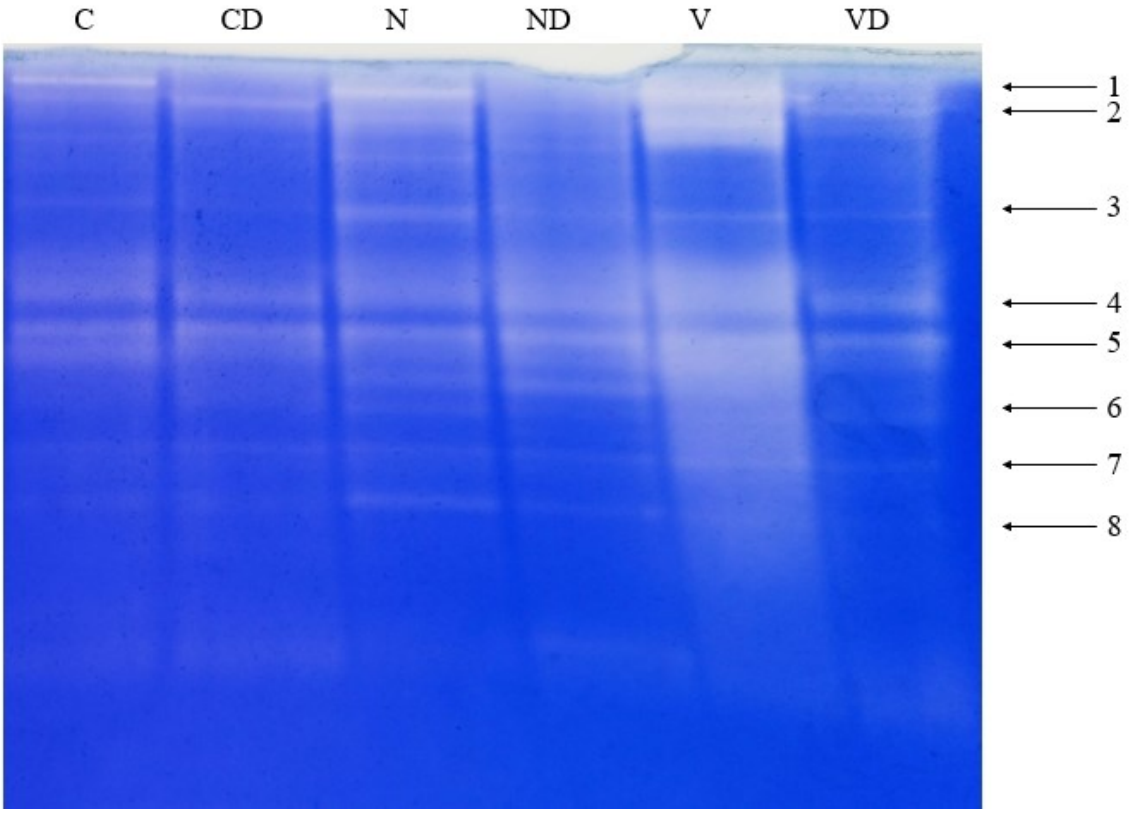

Figure 24: Zymogram of proteases in soil treated with biostimulants. Legend: control group (C), group treated with fermented nettle (N), group treated with vermicompost (V), groups exposed to drought (CD, ND, VD). Numbered black arrows indicate proteases.

5.2.3 Endoglycosidase Activity in Soil Treated with Biostimulants

Endoglycosidase activity (endo-β-1,3-glucanase) was determined by the method described in chapter 4.3.13 (p. 43). The results are expressed as μmol of the product formed (glucose) per h per g of dry weight of soil (Figure *25*).

Endo-β-1,3-glucanase activity measured in soil extract of a control group was lower than in the groups treated with biostimulants. The drought stressed groups have higher endo-β-1,3-glucanase activity than the group that were regularly watered. The procedure where wet soil was used shows overall lower endo-β-1,3-glucanase activity than the procedure with soil extract. The activity measured in wet soil indicates no significant difference between the groups.

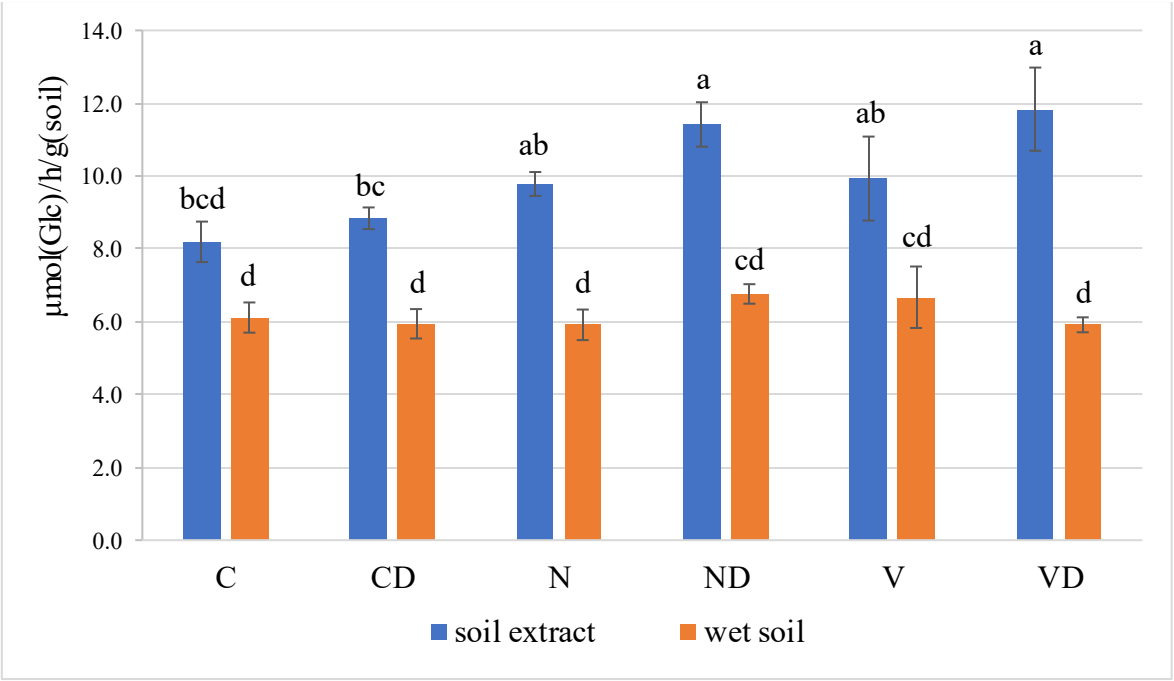

Figure 25: Endo-β-1,3-glucanase activity of soil treated with biostimulants expressed as µmol of product formed (Glc) per h per g of dry weight of soil. The different letters above the bars indicate statistically significant differences between groups. Abbreviations: glucose (Glc), control group (C), group treated with fermented nettle (N), group treated with vermicompost (V), groups exposed to drought (CD, ND, VD).

5.2.4 Peroxidase Activity in Soil Treated with Biostimulants

Peroxidase activity was determined by the method described in chapter 4.3.14 (p. 45). The results are expressed as absorbance change per h per g of dry weight of soil (Figure 26).

The lowest peroxidase activity in soil was detected in the control group (C). Groups treated with biostimulants have slightly higher peroxidase activity. The groups that underwent drought show higher peroxidase activity than the groups that were regularly watered. The group treated with vermicompost that underwent drought (VD) shows the highest peroxidase activity, over two times higher than other experimental groups.

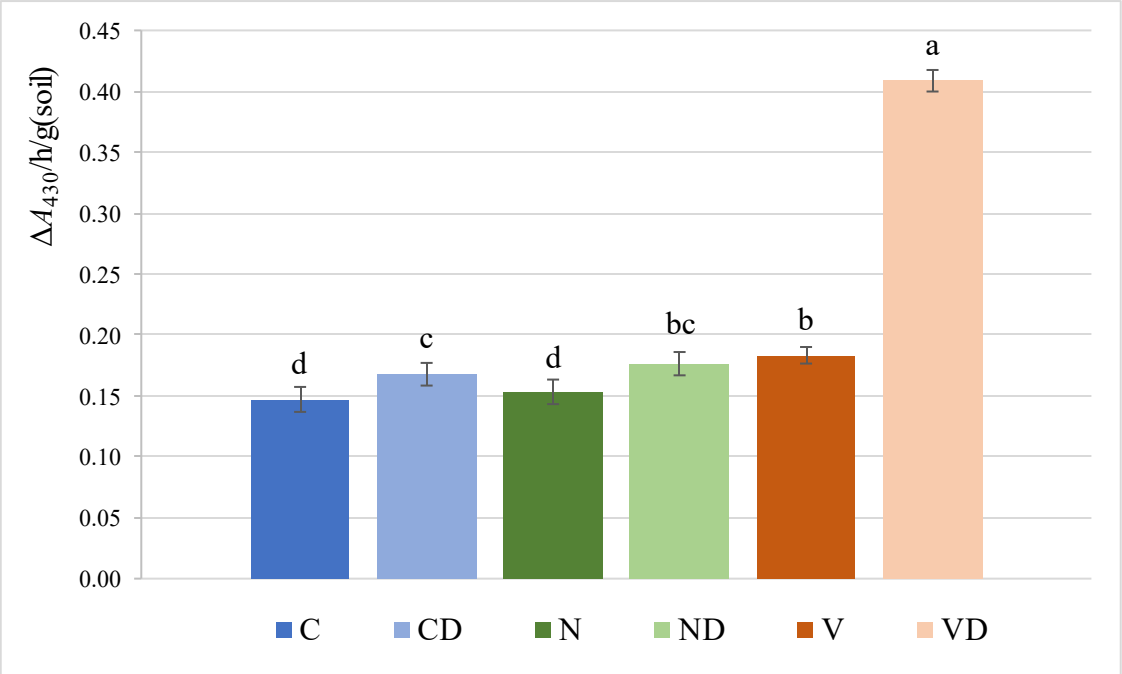

Figure 26: Peroxidase activity of soil treated with biostimulants expressed as absorbance change (at 430 nm) per h per g of dry weight of soil. The different letters above the bars indicate statistically significant differences between groups. Abbreviations: absorbance change (ΔA_{430}), control group (C), group treated with fermented nettle (N), group treated with vermicompost (V), groups exposed to drought (CD, ND, VD).

5.3 Analyses of Soil Treated with Triazole Fungicides

5.3.1 Protein Concentration in Soil Treated with Triazole Fungicides

Protein concentration in soil was determined by Lowry assay. Description of this method can be found in chapter 4.3.11 (p. 42). The results are expressed as mg of protein per g of dry weight of soil (Figure 27).

The protein concentration in soil is the highest in the group of plants treated with a soil drench mixture of penconazole and tebuconazole (sPT). The lowest protein concentration was detected in the control group (C) and the group treated with a foliar spray of a mixture of penconazole and tebuconazole (fPT). In general, the protein concentration in each group does not significantly differ.

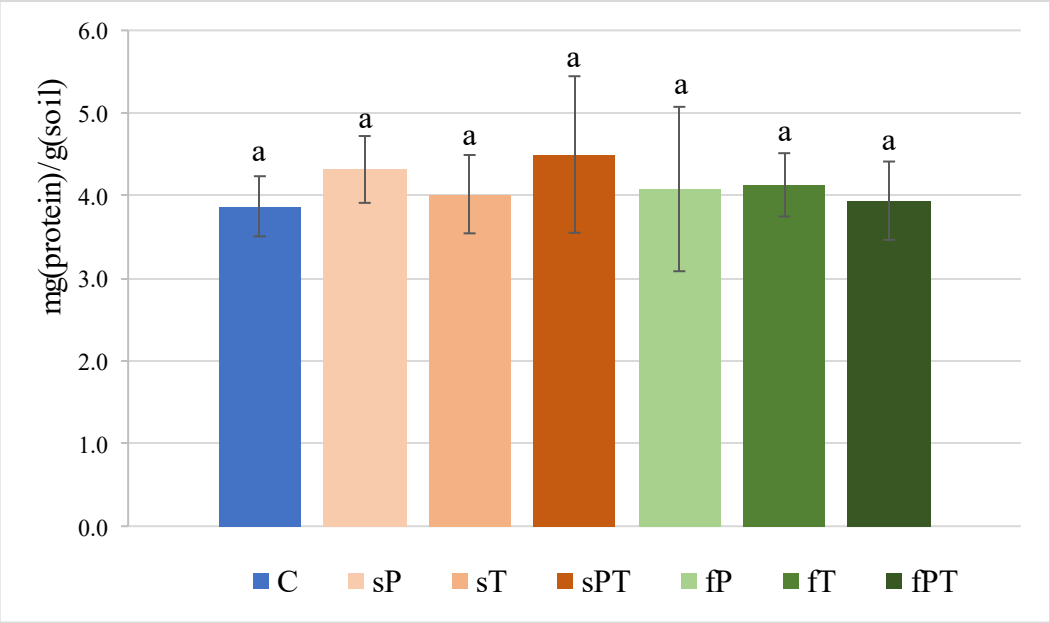

Figure 27: Protein concentration determined by Lowry assay in soil treated with triazole fungicides expressed as mg of protein per g of dry weight of soil. The same letters above the bars indicate no statistically significant difference between groups. Abbreviations: control group (C), group treated with penconazole (P), group treated with tebuconazole (T), group treated with both triazoles (PT), soil drench (s), foliar spray (f).

5.3.2 Proteolytic Activity in Soil Treated with Triazole Fungicides

Protease activity in soil was determined by FRET assay described in chapter 4.3.12.1 (p. 42). The results are expressed as mega relative fluorescence units (MRFU) per h per g of dry weight of soil (Figure 28).

Protease activity is the highest in soil treated with a soil drench of penconazole (sP). The lowest protease activity was determined in the group treated with a soil drench mixture of penconazole and tebuconazole (sPT). Compared to the control group (C), groups sPT, fP, and fPT show statistically significant lower protease activity.

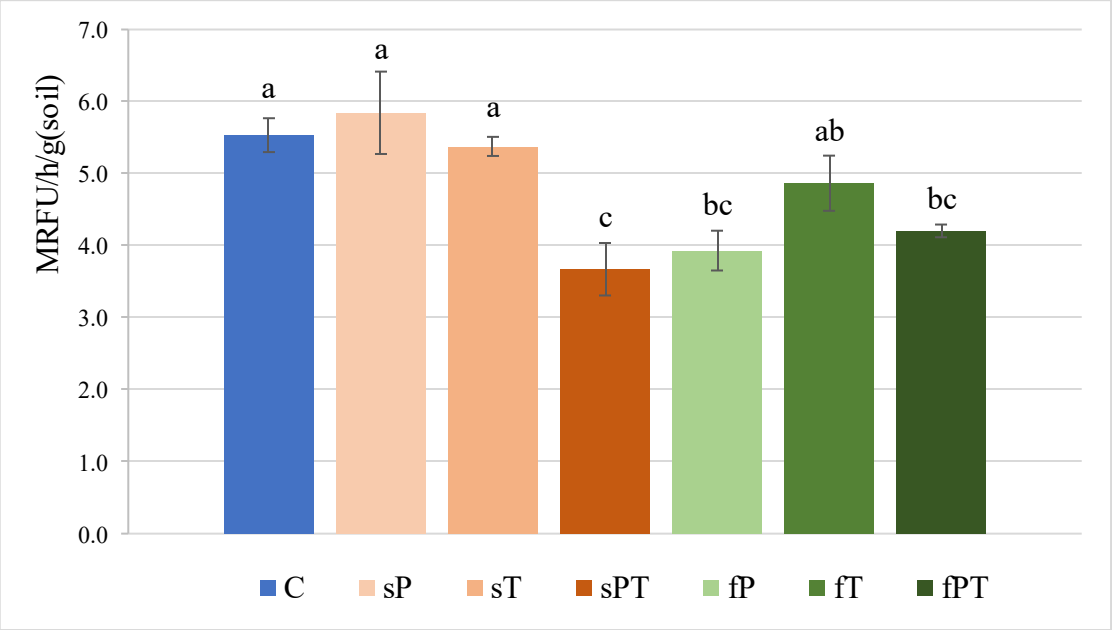

Figure 28: Protease activity of soil treated with triazole fungicides expressed as MRFU per h per g of dry weight of soil. The different letters above the bars indicate statistically significant differences between groups. Abbreviations: mega relative fluorescence units (MRFU), control group (C), group treated with penconazole (P), group treated with tebuconazole (T), group treated with both triazoles (PT), soil drench (s), foliar spray (f).

Protease activity in soil was also determined by zymography. This method is described in chapter 4.3.12.2 (p. 43). The results of zymography are shown in a zymogram (Figure 29).

Protease activity determined by zymography is approximately the same for every group. The groups treated with a soil drench of penconazole and tebuconazole had slightly higher intensity of bands than the other groups. According to the FRET assay, the same groups showed high protease activity.

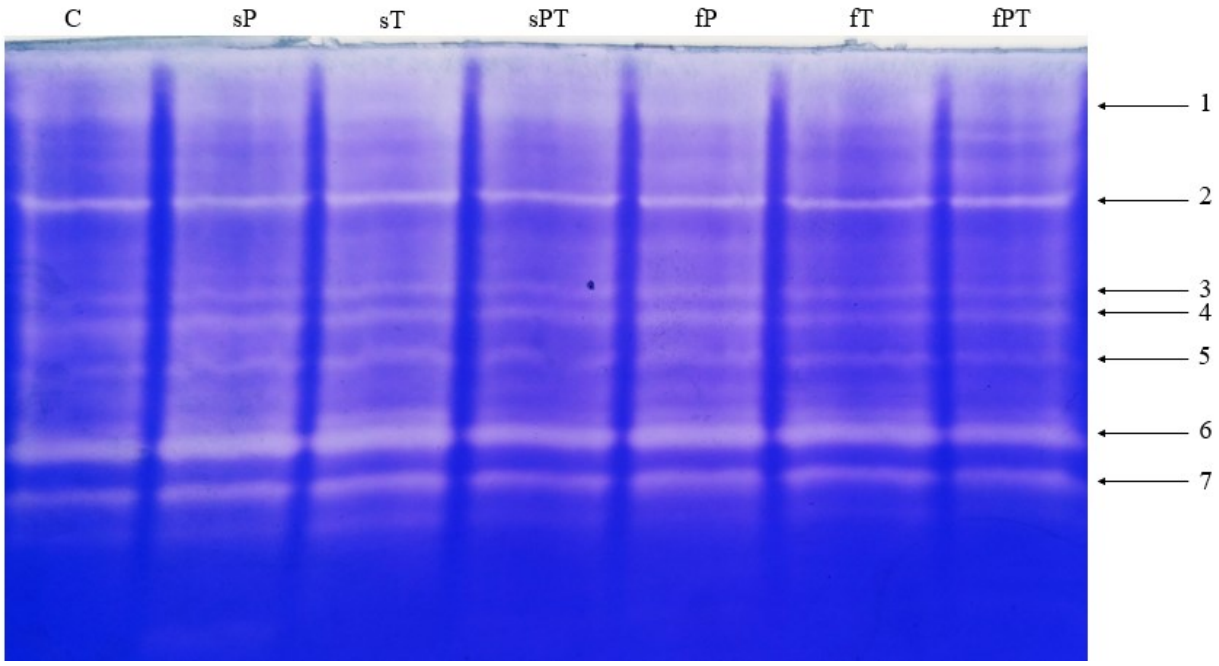

Figure 29: Zymogram of proteases in soil treated with triazole fungicides. Legend: control group (C), group treated with penconazole (P), group treated with tebuconazole (T), group treated with both triazoles (PT), soil drench (s), foliar spray (f). Numbered black arrows indicate proteases.

5.3.4 Endoglycosidase Activity in Soil Treated with Triazole Fungicides

The method used to determine endoglycosidase activity (endo-β-1,3-glucanase) is described in chapter 4.3.13 (p. 43). The results are expressed as μmol of the product formed (glucose) per h per g of dry weight of soil (Figure *30*).

The highest endo-β-1,3-glucanase activity in soil extract was determined in group treated with foliar spray mixture of penconazole and tebuconazole (fPT). Endo-β-1,3-glucanase activity in soil extract was the lowest in the group treated with soil drench of tebuconazole (sT). The procedure using wet soil shows overall lower endo-β-1,3-glucanase activity and no significant difference between groups.

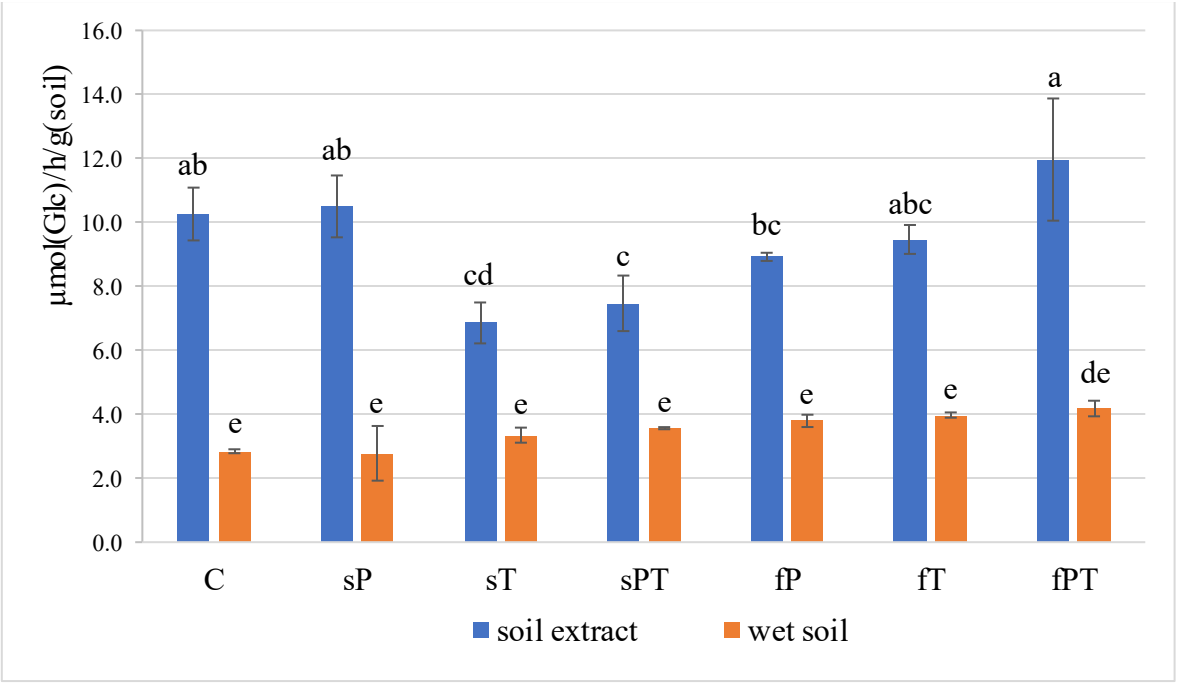

Figure 30: Endo-β-1,3-glucanase activity of soil treated with triazole fungicides expressed as µmol of product formed (Glc) per h per g of dry weight of soil. The different letters above the bars indicate statistically significant differences between groups. Abbreviations: glucose (Glc), control group (C), group treated with penconazole (P), group treated with tebuconazole (T), group treated with both triazoles (PT), soil drench (s), foliar spray (f).

5.3.5 Peroxidase Activity in Soil Treated with Triazole Fungicides

Peroxidase activity was determined by the method described in chapter 4.3.14 (p. 45). The results are expressed as absorbance change per h per g of dry weight of soil (Figure 31).

The highest peroxidase activity was detected in the group treated with soil drench of penconazole (sP). On the other hand, the control group (C) and the group treated with penconazole foliar spray (fP) showed the lowest peroxidase activity. The groups treated with soil drench have higher peroxidase activity than the groups treated with foliar spray.

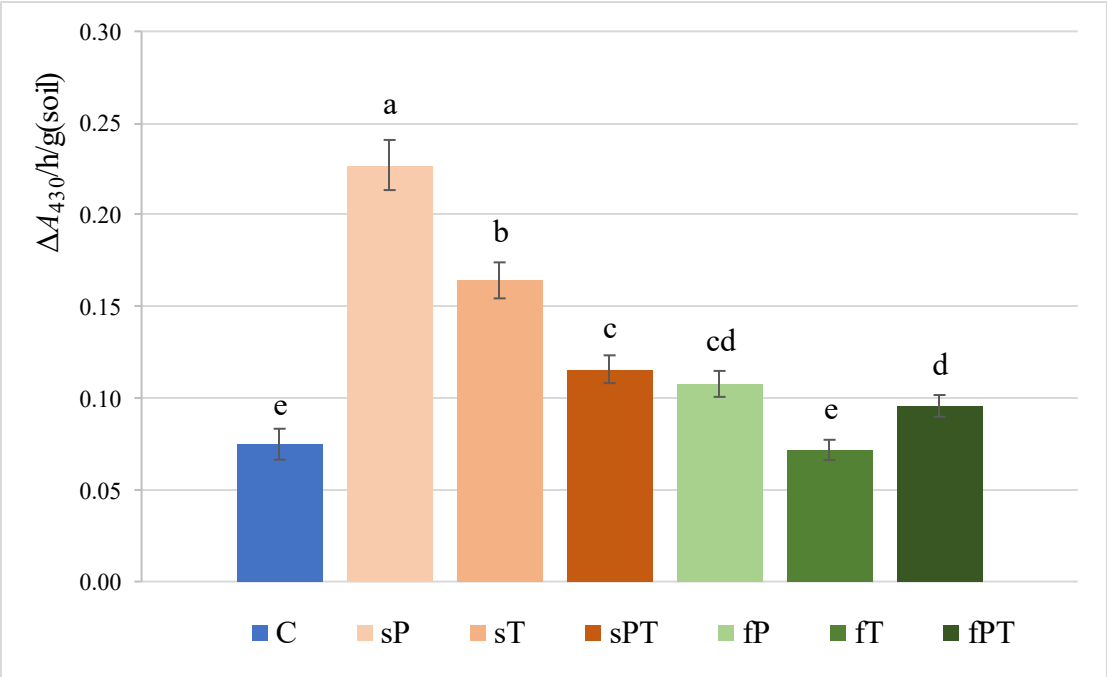

Figure 31: Peroxidase activity of soil treated with triazole fungicides expressed as absorbance change (at 430 nm) per h per g of dry weight of soil. The different letters above the bars indicate statistically significant differences between groups. Abbreviations: absorbance change (ΔA_{430}), control group (C), group treated with penconazole (P), group treated with tebuconazole (T), group treated with both triazoles (PT), soil drench (s), foliar spray (f).

6. Discussion

There is a need to find ways to improve crop yields in efficient and sustainable ways to provide enough food for the ever-increasing population [1]. One option is to start using more biostimulants in agriculture. They have proven very useful in mitigating various abiotic stresses, such as drought [127]. Due to climate change, drought is nowadays one of the more pressing abiotic stresses to address [128]. As far as plant protection products are concerned, it should be considered how they affect the environment before application [5]. For example, frequently used triazole fungicides can negatively affect the activity of soil enzymes [50].

In this thesis, fermented nettle was characterized, the effects of fermented nettle and vermicompost on soil properties were assessed, and the soil treated with penconazole and tebuconazole was analysed. The experiment with biostimulants – fermented nettle, vermicompost – conducted on maize plants (*Zea mays* L.) consists of six groups. The group treated with fermented nettle (N), the group treated with vermicompost (V), the control group (C), and groups that underwent drought stress (ND, VD, CD). Tomato plants (*Solanum lycopersicum*, cv. Cherrola) were treated with triazole fungicides – penconazole (P), tebuconazole (T), and mixture of both (PT) – either in the form of a soil drench (s) or a foliar spray (f). The seven resulting groups are labelled: sP, sT, sPT, fP, fT, fPT, and the control group (C).

The phenolic content, antioxidant capacity, protein concentration, activity of acid phosphatases (EC 3.1.3.2), alkaline phosphatases (EC 3.1.3.1), and proteases (EC 3.4) were determined in fermented nettle. The activities of endo-β-1,3-glucanase (EC 3.2.1.39), total peroxidases (EC 1.11.1.7), and proteases (EC 3.4) were measured in the soil. Soil enzymes come from, among other things, microorganisms and root exudates [129].

Fermented nettle is a product of stinging nettle (*Urtica dioica* L.). Hence, it contains a high amount of total phenolic compounds (Figure 16) [130]. Antioxidant capacity in fermented nettle was measured using two methods – FRAP and DPPH (Figure 16). The results of these two methods are comparable. Nettle is a natural antioxidant with the ability to donate hydrogen and high free radical scavenging activity. It is rich in phenolics, flavonoids and vitamins (e.g., vitamin C), giving it the formerly mentioned antioxidant properties [87].

Protein concentration in fermented nettle was determined by three methods – the Bradford, Lowry, and BCA assay (Figure 17). The Bradford assay detects the lowest protein content in FN. In contrast the BCA assay shows the highest protein concentration in FN.

Studies comparing these three methods of protein content determination (in different substances) agree that BCA assay is the most precise and the Bradford assay the least accurate [131],[132],[133]. However, fermented nettle is an intricate material and some substances in it (e.g., reducing saccharides, phenolic compounds) may interfere with the determinations. To choose the best method for the determination of protein content in FN, it would be necessary to compare the results with the Kjeldahl method or subject the protein samples to amino acid analysis after acid hydrolysis. SDS-PAGE of proteins in fermented nettle shows that most proteins have a molecular weight around 250 kDa (Figure 18). Proteins can be a source of nitrogen for plants [134].

Proteolytic activity measured in fermented nettle extract was higher than when measured in fermented nettle directly (Figure 19). This could be due to a presence of inhibitors that reduce protease activity. PVP was used to repress the inhibitory effect of secondary metabolites, nonetheless different inhibitors (that are not extracted) might be present. Another reason may be adsorption of proteolytic products (released azo-peptides from azocasein) to the solid fermented nettle and thus their contribution to the activity is not detected in the spectrophotometric measurement. Zymography of FN shows seven different proteases (Figure 20). Molecular weight of the most represented protease (3) is around 97 kDa. Proteases are degradative enzymes that digest proteins. The low-molecular-mass products of digestion are then an accessible source of organic nitrogen to plants [134].

Acid phosphatase activity in FN evinces similar trend as proteolytic activity. Higher acid phosphatase activity was measured in fermented nettle extract than in solid lyophilized FN (Figure 21). The reasons for inhibition may be the same as for proteolytic activity. Alkaline phosphatase activity was only determined in FN extract and is about four times higher than acid phosphatase activity (Figure 21). Both acid and alkaline phosphatases hydrolyse organic phosphorous compounds and release phosphate groups [135].

Soil treated with biostimulants was analysed in terms of protein content and enzyme activity (endo-β-1,3-glucanase, total peroxidases, and proteases). The total protein content was lower in the groups that underwent water deficit (Figure 22). This decrease is statistically significant only in the groups treated with fermented nettle (ND). Concentration of proteins in soil under water stress is not much researched. On the other hand, decrease in protein content in plants under drought stress is confirmed [136],[137].

Endo-β-1,3-glucanase activity in soil extracts of groups C, CD, N, and V shows no statistically significant differences (Figure *25*). The groups treated with biostimulants that underwent water deficit exhibit significantly higher endo-β-1,3-glucanase activity than the

control group. This enzyme activity is mainly of microbial origin. It has been confirmed that drought reduces the microbial community and thus depresses activity of endo-β-1,3-glucanase. However, the abundance of microorganisms in the soil can be improved by treating the soil with various organic compounds [128]. In fact, the groups ND and VD show the highest endo-β-1,3-glucanase activity.

The activity of this enzyme was also determined in wet soil (Figure *25*). This procedure shows overall decrease in endo-β-1,3-glucanase activity compared to the procedure with soil extract. Furthermore, no significant differences between groups were detected. The adsorbed enzymes on soil particles vary in their catalytic properties and may be partially (or fully) active. Enzyme adsorption depends on available surfaces, temperature, pH, moisture, and ionic strength [138]. Here, the remaining activity in wet soil may represent "a pool of enzymes" that are released in the extraction procedure, but if adsorbed they are either inhibited by interfering substances in the soil, or not fully active. Also, the incubation of substrates with wet soil may lead to their adsorption to the soil particles and thus not being available to the enzymes. Furthermore, the products may interact with the soil surface as well and become unavailable to the detection spectrophotometric procedure.

The groups that were exposed to water deficit show higher peroxidase activity compared to the corresponding groups that were watered (Figure 26). VD shows the highest peroxidase activity. Peroxidases help mitigate consequences of drought stress. Therefore, their activity increases in arid conditions. Stefanovits-Bányai et al. (1998) found that the increase of peroxidase activity in plants corresponds with increasing water deficit [139]. Furthermore, humic acid (vermicompost component) substantially stimulates the activity of antioxidant enzymes in dry conditions [140].

Proteolytic activity of regularly watered groups shows no significant difference between the groups (Figure 23). Soils that underwent drought stress show significant increase of protease activity compared to the C, N and V groups. ND group evinces the highest proteolytic activity. Water deficit can decrease nitrogen uptake of a plant and thereby increase the need for proteolytic activity. Proteases can eliminate proteins damaged by the osmotic stress [141]. Song et al. (2012) exposed untreated rhizosphere soil to water stress and observed gradual decrease in protease activity [142]. In contrast, group CD shows increased proteolytic compared to C group. Zymogram of these soils shows eight proteases (Figure 24). Higher intensities are indicated in groups that were regularly watered, which is the exact opposite result compared to the former procedure. Protease spectrophotometric assay uses fluorescently tagged casein and was performed at pH 7, while zymography is

based on the cleavage of gelatine in gel at pH 8.4. The difference in results of both methods may lie in the involvement of different proteases at these pH values as well as in increased activity of gelatinases in zymography.

The effects of triazole fungicides on soil were observed by activities of defence enzymes (endo-β-1,3-glucanase, total peroxidases) and degradative enzymes (proteases). Furthermore, total concentration of proteins in soil was determined. Increased activity of defence enzymes in plants is a well-known reaction to invading pathogens. Endo-β-1,3-glucanase belongs to the group of pathogenesis-related proteins and cleaves β-1,3-glucans found in fungal cell walls. The defensive nature of peroxidases (antioxidant enzymes) stems from their involvement in synthesis of phenolic compounds [143]. Proteases play a key role in the cycle of nitrogen, which is vital for plant nutrition [144]. One of the major sources of proteases in soil are microorganisms [145]. The use of pesticides can lower microbial activity [25] and thus also depress proteolytic activity.

The protein concentration in soil is not affected by the triazole fungicides treatment (Figure 27). Endo-β-1,3-glucanase activity (Figure *30*) measured in soil extract is the lowest in the sT group while the highest in the fPT group. The same determination performed by Račko (2022) shows similar trend [123]. Endo-β-1,3-glucanase may directly destroy the integrity of the fungal pathogen membrane/cell wall by hydrolysing β-1,3-glucans, or triggering the plant defence by releasing cleaved elicitors [146].

The decrease in endo-β-1,3-glucanase activity determined in aforementioned group can be attributed to the efficiency of given treatments. The fungicides rid the plants of the fungi infection and consequently alleviate the need for a stress response. The activity of this enzyme was measured not only in soil extract but also directly in wet soil (Figure *30*). Following this procedure, the endo-β-1,3-glucanase activity, apart from being significantly lower than when determined in soil extract, indicates no differences between groups. Again, the aforementioned "adsorption effect" of enzymes, substrates or products on soil particles might have influenced these results.

The group sP shows the highest total peroxidase activity, followed by the sT, sPT, sPT, fP, and fPT groups, in decreasing order (Figure 31). The lowest peroxidase activity was determined in fT group and this result is comparable to the activity in the C group. Multiple studies note that triazoles applied by soil drenching increase activities of antioxidant enzymes (SOD and APX) in plants [147],[148],[149], therefore activity in soil is likely affected, too. Indeed, groups treated with soil drench of triazoles show higher peroxidase activity than the control group and the groups treated with foliar spray.

Proteolytic activity in groups sP, sT, and fT is comparable to the activity of the control group (Figure 28). Statistically significant decrease in protease activity was determined in sPT, fP, and fPT groups. Enzyme activity is linked to the number of microorganisms in soil [150], thus, a decrease in activity in the sP, sT, and sPT groups was expected because the triazoles were applied directly into the soil. In a study by Roman et al. (2023), triazole fungicide applied into the soil slightly decreased proteolytic activity [48]. However, lower protease activity was detected in groups with foliar application. The foliar application of triazoles may represent a greater burden on plant metabolism than soil drench and therefore root enzyme secretion may be reduced. In this triazole experiment, it is expected that the soil enzymes were rather of plant origin, since microbial activity by total dehydrogenase assay was measured (data not shown) but it was under limit of detection. Proteolytic activity was also detected by zymography (Figure 29). The zymogram shows seven proteases. The most represented proteases are under numbers two and six. While the protease activity is approximately the same in each group, sP and sT show slightly higher activity. This result corresponds with the former assay.

7. Conclusion

Fermented nettle contained a significant amount of phenolic compounds and had high antioxidant capacity. According to SDS-PAGE, fermented nettle also contained predominantly proteins with molecular weight around 250 kDa. Enzyme activity, more precisely acid and alkaline phosphatase, as well as protease activity was successfully measured in fermented nettle. The activity of alkaline phosphatases was over four times higher than that of acid phosphatases.

The experiment where plants were treated with biostimulants (fermented nettle, vermicompost) also involved drought stress. The effects of the biostimulants and drought on soil were analysed. The protein content was lower in the arid groups (CD, ND, VD) but only ND showed statistically significant decrease. Activity of protease, endo-β-1,3-glucanase, and total peroxidase was measured and the groups that underwent drought stress showed an increase in enzyme activity when compared to their corresponding group. In the case of endo-β-1,3-glucanase, the stimulation of the activity was not statistically significant.

Triazole treatment (penconazole, tebuconazole and their mixture) did not affect the protein concentration in the soil. Proteolytic activity decreased compared to the control group in the following groups: sPT, fP, and fPT. Groups sT, sPT, and fP indicated lower endo-β-1,3-glucanase activity than control group when measured in soil extract. Total peroxidase activity increased in every group, except for fT, which had activity comparable to the control group.

8. References

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