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Effect of Nitrogen Fertilization in the Sour Cherry Orchard on Soil Enzymatic Activities, Microbial Population, and Fruit Quality

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Abstract: Sour cherry (*Prunus cerasus* L.) is one of the most important fruit crops in Poland and 'Łutówka' is the dominant cultivar in commercial orchards. The experiment was carried out in 2007–2013 in three orchards; in each of them, three levels of fertilization were applied: 0 N kg ha^{-1}, 60 kg N ha^{-1}, and 120 kg N ha^{-1}. The activity of dehydrogenase and protease in the soil was studied depending on nitrogen fertilization. The abundance of soil microorganisms was assessed: bacteria, fungi, actinomycetes, and nitrogenous bacteria (*Azospirillum* and *Azotobacter*) in the years during the experiments carried out with fertilization of 60 kg N ha^{-1} in all orchards. The enzyme activity of dehydrogenases increased after the use of 60 kg N ha^{-1} from 3.8 to 6.7 (cm^3 H_2 24 h^{-1} kg^{-1} DW soil), but a further increase in the dose to 120 kg N ha^{-1} caused a decrease in activity to 5.1 (cm^3 H_2 24 h^{-1} kg^{-1} DW soil). The activity of proteases was dependent on nitrogen fertilization, but to a large extent it was related to the course of climatic conditions. There is no relationship between the growth and the activity of proteases. The yield and selected quality parameters of the cherry fruits were associated with both dehydrogenases and proteases. The use of lower doses of nitrogen fertilizers allows for maintaining biological balance in the soil and a more efficient use of nutrients, contributing to less environmental pollution.

Keywords: soil; biodiversity; sour cherry; dehydrogenase; protease; bacteria; fungi; enzymatic activity

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1. Introduction

Fruit production is an important branch of agriculture in Poland. Sour cherry production, depending on the year, amounts to approximately 180–200 thousand tonnes [1], except in the years when spring frosts damage flowers and fruit buds [2–4], which is quite common in Poland [5,6]. The date of flowering is very variable [7], and, therefore, flower production is late in this cultivar. Such cultivar is self-pollinated 'Łutówka', which accounts for about 80% of cherry plantings [8]. Cherry fruits are used primarily as industrial fruits with high nutritional value, which is due to the high content of various antioxidant compounds, such as flavonoids and phenolic compounds [9,10]. Anthocyanins are the main component of the antioxidant capacity of cherries, commonly referred to as ORAC (Oxygen Radical Absorption Capacity) [11]. They primarily act as antioxidants, have anti-inflammatory, anti-cancerous, and anti-aging properties [12–14].

Soil quality assessment is important for fruit crops due to the perennial nature of crops. This leads to unilateral depletion of nutrients in the soil, deterioration of the soil structure, and microbiological imbalance. It consists of increasing the number of parasitic fungi, bacteria, and actinomycetes, which can weaken growth [15,16]. The accumulation of parasitic microorganisms cannot be avoided in field cultivation of fruit trees, as in the cultivation of strawberries, where it is possible to grow in an artificial medium such as coconut fiber to eliminate the problems associated with soil diseases [17]. Among the fungi that can negatively affect tree growth, the following are noted: *Pythium*,

Thielaviopsis, *Rosellinia*, *Phytophthora*, *Cylindrocarpon*, *Fusarium*, *Rhizoctonia*, *Penicillium*, and *Alternaria* [18–20]. Among the bacteria that are responsible for a growth disorder are *Bacillus subtilis and Pseudomonas putida* [21–23]. Microorganisms of the genus *Actinomyces* penetrate the cuticle of the roots or root hairs into the epidermis, causing destruction and death [24].

Performing a microbiological analysis of the soil is very time consuming and does not give a complete answer to the fertility of the soil. Therefore, another sensitive indicator of soil quality is sought. The metabolism of organic matter in the soil is related to the activity of microorganisms and the enzymes they produce [25]. Enzymes are prone to adverse environmental effects. Therefore, changes in the activity of soil enzymes quickly reflect environmental disturbances affecting soil and plants that are the result of agrotechnical treatments or monoculture cultivation [26]. They participate in biochemical transformations and play an important role in catalysing reactions such as the decomposition of organic residues and the circulation of nutrients in the soil. The most important in the decomposition processes of plant residues are those that are directly involved in the degradation of lignin and cellulose and in the mineralization of organic compounds of nitrogen, phosphorus, and sulphur [27]. Enzymes belonging to oxidoreductases and hydrolases play a more important role [28].

Enzymatic activity is dependent on many factors. The most important are soil type, cultivation method, use of chemicals, soil condition, and climate [29–32]. Therefore, to characterize soil quality, data related to soil enzyme activity, soil mineral content, microbial abundance, and taxonomic diversity [33–35] should be combined. The use of organic matter in the rows increases the enzymatic activity of the soil under the trees, compared to the herbicide strip in the interrow [22].

The activity of soil microorganisms is stimulated by photosynthetic products secreted by the root system into the soil [36,37]. Limiting the availability of carbon substrates such as cellulose and lignin is a factor that limits the activity of soil dehydrogenase [38]. Soil inhabited by plants contains a higher concentration of soluble carbon compared to non-inhabited soil [39]. The species of fruit trees has an impact on the formation of enzymatic activity. The soil from the cherry and plum orchards was characterized by much greater enzymatic activity than the soil from the apple and pear orchards [40]. It is caused by the specific species composition of bacteria inhabiting the roots of trees [41] as well as the secretion by apple and pear trees of phytoncides that inhibit the activity of enzymes [42,43].

Lowering soil moisture with increasing temperature affects the decrease in the activity of dehydrogenases [44], as does too high of a humidity in freshly flooded soils [45,46]. On the other hand, a change in temperature, without a change in soil moisture, does not cause changes in the activity of dehydrogenase [46].

Mineral fertilization plays an important role in enzymatic activity [47,48]. However, regular use of too high doses of nitrogen fertilizers can lead not only to too intensive growth of trees, deterioration of fruit quality, but also to disruption of the functioning of soil ecosystems [47–49]. High nitrogen fertilization can alter the balance of minerals and, as a result, reduce the enzymatic activity of the soil, as well as reduce the biomass of microorganisms [50,51].

Bacteria are the most studied potential microbiological indicators of soil quality [52,53]. The degree of development of microorganisms in the soil is a function of agroecological factors, both their chemical and physical properties, especially in the abundance of organic matter [54]. The abundance of representatives of Proteobacteria was positively associated with the overall improvement in the availability of N and the content of organic matter in the soil (SOM) of orchards [55,56]. It is believed that the ratio of bacterial colony-forming units of *Azospirillum* (CFU) to phytotoxic micromycetes may serve as an indicator of the severity of soil fatigue in an apple orchard [57].

Increasing doses of nitrogen fertilization in the replanted apple orchard limited the abundance to a greater extent than *Azospirillum*. The pH of the soil was of greater importance for the abundance of *Azospirillum*. This is confirmed by the fact that soil

application with the same nitrogen fertilization caused an increase in the abundance of *Azospirillum*. The abundance of nitrogenous bacteria was mostly dependent on soil moisture levels, sampling dates and soil reactions [58]. Bacteria of the genus *Azospirillum* are characterized by poor survival in the soil for a longer period, and the decisive influence here is the joint interaction of many chemical factors (nitrogen, organic matter, salinity) and physical (consistency, moisture) [59]. An important factor differentiating the state of microflora (endophytic) are weather conditions, crop species, fertilization, and the development phase of the plant [54]. A better indicator of changes in the soil environment is *Azotobacter*, which reacts strongly to chemical and physical changes in the soil [54] and is found in various soils around the world. However, they are present primarily in neutral or alkaline soils, while in acidic soils with a pH below 6.0, these bacteria are generally absent or present in very small quantities [60].

Biofertilizers produced based on microorganisms can be described as microbial inoculants that are supposed to improve plant growth. *Azospirillum* and *Azotobacter* have been intensively used in economically important crops, mainly in monocotyledonous species, but recently on other species, such as strawberry [61]. When comparing the effects of *Azotobacter* and *Azospirillum* on the growth of olive trees, only *Azotobacter* affected growth and yield [62].

Sour cherries have relatively high nitrogen requirements [63], however, the recommendations vary depending on the country, organic matter content, and the method of cultivation. Although the use of nitrogen fertilization at a dose above 100 kg N ha^{-1} increased the yield of trees, there was no significant effect of 120 kg N ha^{-1} of nitrogen on vegetative growth and yield of sour cherries [64–67]. In France, 30–40 kg N ha^{-1} is recommended for young orchards, and 80–100 kg N ha^{-1} for fully fruiting. On soils with a high content of organic matter, doses should be reduced [68,69]. According to the recommendations for the integrated production of sweet cherries in Poland, it is recommended to fertilize older trees with nitrogen throughout the area with a dose of 50–80 kg N ha^{-1} or a reduced dose of 30–50 kg N ha^{-1} in strips with a width of approximately 2 m [69]. Comparable doses of nitrogen fertilization are recommended in integrated jab production, where 20–80 kg N ha^{-1} is recommended depending on the content of organic matter in the soil. Above 2.6% of organic matter, 20–40 kg is recommended N ha^{-1} [70]. Yield and average fruit size at a dose of 60 kg N ha^{-1} were the most optimal for growing cherries of the 'Lapins' cultivar on Gisela 5 rootstock. Doses of approximately 120 or 250 kg N ha^{-1} increased the N content of the leaves but did not have a positive effect on the growth, yield, and quality [71–73].

The aim of this study was to evaluate: (1) assessment of changes in the enzymatic activity of the soil according to nitrogen fertilization, orchard age, and sampling date, (2) determination of the abundance of microorganisms depending on the age of the orchard, and the date of sampling, (3) assessment of the effect in climatic conditions on the enzymatic activity of the soil and the microbiological activity of the soil.

2. Materials and Methods

2.1. Location

The experiment was carried out at the experimental farm of the Poznań University of Life Sciences (52°31′22.9″ N 16°39′14.4″ E) in fawn soil, which was made of clay sands that are deposited in clay of light order. The content of floating parts was 21% and humus was 1.25%.

The research was carried out in cherry orchards (*Prunus cerasus* L.), which were planted in spring in 3 different years: 1999 (OR 1), 2001 (OR 2), and 2002 (OR 3). The cherry trees of the 'Łutówka' cultivar grafted on the rootstock of mahaleb cherry (*Prunus mahaleb* L.) were planted in a spacing of 4 m × 1.3 m.

All agrotechnical treatments in the orchard were carried out in accordance with the recommendations for production orchards, based on the current cherry production program. In the inter-rows, grass was maintained, which, depending on the course of

climatic conditions, was mowed several times. The herbicide strip was kept under the trees using glyphosate twice, during spring and once in autumn. During vegetative growth periods, if there were weeds, a mechanical scythe was used.

2.2. Experiment Layout

The experiment was carried out in 2007–2013 in the system of random blocks.

In each of the orchards, three levels of nitrogen fertilizers were applied:

- level 0 (N0)—no fertilization,
- level 1 (N60)—fertilization 60 kg N ha^{-1},
- level 2 (N120)—fertilization 120 kg N ha^{-1}

The trees covered by the experiment grew in one row. Each level of fertilization was repeated 4 times. There were 8 trees in each repetition. The insulation gap between the individual plots consisted of 2 trees.

Nitrogen fertilization was carried out in the spring before the start of the growing season with ammonium nitrate (34% N).

2.3. Sampling

Samples for biochemical analyses were collected three times each year:

- (Date I) in the spring, flowering (BBCH 60–69) in end of April, and early May;
- (Date II) in the summer after fruit harvesting, (BBCH 93–97) in late July, and early August;
- (Date III) in the fall, at the end of the vegetation, beginning of dormancy (BBCH 87–89) in October.

Soil samples were collected for microbiological analysis during two terms corresponding to successive phases of development. The terms were as follows: Date I—two weeks after flowering to before second fruit fall (BBCH 71–72) in the second half of May and early June, Date II—after harvest, when shoot growth was complete but leaves were still green (BBCH 91–92) in the second half of September and early October.

The sampling was carried out during weak sunlight, in the morning, in the herbicide strip, at a distance of 50–70 cm from the tree trunk of the layer covering the accumulation level of Ap (0–20 cm). A kilogram soil sample was taken from each repetition. After taking the samples, they were immediately transported to the laboratory of the Department of Fruit Growing at the Poznań University of Life Sciences and stored at a temperature of 5 °C in the refrigerator until the analysis was performed.

2.4. Chemical Analysis

Dehydrogenase (ADh) by the colorimetric method according to Thalmann [74] using as a substrate at 1% solution of TTC (triphenyl tetrazole chloride), after 24 h of incubation at 30 °C at a wavelength of 485 nm (TTC test), expressed its activity in cubic centimetres $H_2 \cdot 24\ h^{-1} \cdot kg^{-1}$ DW of the soil.

Protease (AP) by spectrophotometric method according to Ladd and Butler [75] using a substrate of a 1% sodium caseinate solution, after 1 h of incubation at 50 °C at a wavelength of 578 nm, the enzyme activity was expressed in milligrams of tyrosine $h^{-1} \cdot kg^{-1}$ DW of soil.

2.5. Microbiological Analyses

Soil samples were taken on two dates, spring and fall. Microbiological analysis was performed on the basis of the serial dilution method and involved determining (using selective substrate in three replications) the numbers of colony forming units (CFU g^{-1} DW of soil) of the total number of bacteria, actinobacteria, and fungi. Estimation of CFU number of the above-mentioned microorganisms is a measure of the intensity of their current metabolic activity.

- total bacterial count was determined on Merck standard agar after 5 days of incubation at 28 °C,
- actinobacteria on Pochon agar after seven days of incubation at 24 °C [76],
- fungi in Martin medium after five days of incubation at 24 °C [77],
- *Azotobacter* abundance, assessed according to the method developed by Fenglerowa after four days of incubation at 28 °C [78],
- *Azospirillum* on liquid NFB medium as a titre according to Döbereiner [59].

Cultures were carried out in five repetitions; the number of microorganisms was converted to 1 g of fresh soil mass and the abundance of *Azospirillum* was calculated using the NPL method.

The CFU method used offers only a limited amount of information compared to genetic methods; however, despite its limitations, this method is still used in the study of soil microbiological ecology, because it provides a reliable picture of its response to changes in the soil environment, which is indicated by the significant correlation between the types of bacteria detected by genetic methods and those identified by culture [79].

2.6. Analysis of Weather Conditions

Meteorological data were taken from a meteorological station (type of station, manufacturer), located in the orchard. The measured temperature was the air temperature at a height of 2 m, the temperature on the ground, the temperature of the soil, and precipitation.

The rainfall in 2007–2013 during the growing season was the highest in 2009 and was 498.0 mm, while the least precipitation was recorded in 2008 (343.0 mm). The average temperature of the growing season was the lowest in 2008. In 2011, a much higher temperature was recorded, where the average was 16.5 °C (Table 1).

Table 1. Weather Conditions in the Vegetation Period (April-October).

Months	Year						
	2007	**2008**	**2009**	**2010**	**2011**	**2012**	**2013**
	Precipitation (mm)						
April	5.4	56.2	19.6	19.0	9.2	9.8	85.2
May	98.8	9.0	85.4	110.1	32.8	57.0	99.4
Jun	85.6	15.2	160.0	13.0	56.2	127.8	46.0
July	95.8	62.0	79.4	111.4	182.4	121.8	37.6
August	34.8	116.4	32.8	124.1	32.4	39.0	38.2
September	29.4	27.0	52.4	72.4	27.8	24.6	81.0
October	20.2	57.2	68.4	5.3	27.4	64.4	23.8
Precipitation	370.0	343.0	498.0	455.3	368.2	444.4	411.2
	Temperature (°C)						
April	9.6	7.9	11.3	8.7	12.6	9.8	9.5
May	14.2	13.6	12.5	11.6	16.5	15.4	14.4
Jun	18.1	17.4	14.6	17.0	21.3	16.0	17.4
July	17.7	19.0	18.5	22.0	18.6	19.1	19.7
August	18.2	17.6	18.8	18.8	20.6	18.3	18.7
September	12.8	12.7	14.8	12.7	16.2	14.2	12.6
October	7.4	8.6	6.8	6.4	9.4	8.2	10.2
Mean	14.0	13.8	13.9	13.9	16.5	14.4	14.6

2.7. Statistical Analysis

The results were subjected to a multivariate analysis of variance and the significance of differences between the averages was assessed on the basis of the Duncan Test at the significance level $\alpha = 0.05$.

The Pearson correlation coefficient between biochemical, microbiological, and meteorological properties was also calculated. The principal component analysis (PCA) was used to determine the relationship between enzymatic activity, number of microorganisms, cross-sectional area of the tree, and yield.

Statistical calculations were performed using Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA).

3. Results and Discussion

3.1. Dehydrogenase Enzyme Activity

The use of nitrogen fertilization causes an increase in enzymatic activity dehydrogenases (Tables 2 and 3; Figures 1A–D and 2A–C). It is most visible in the first sampling date (Figure 1D). In the next two days, the activity of dehydrogenase is less. The application of fertilization of 60 kg N ha^{-1} caused the highest level of activity. Increasing the dose to 120 kg N ha^{-1} resulted in a decrease in activity, and so in each orchard and at any time it was higher than without nitrogen fertilization (Figure 1C). This is confirmed by other studies in which the activity of dehydrogenase under the influence of nitrogen fertilization was curvilinear, initially showing a strong increase and later a clear decrease. In annual crops fertilized more abundantly than in ornamental crops, the activity of dehydrogenase was shown to be high for 50 and 150 kg N ha^{-1}, while the dose of 100 and 200 kg N ha^{-1} reduced the activity of this enzyme [49].

The effect of mineral fertilization on the activity of other enzymes is also described in the literature [80]. Therefore, the activity of phosphatases increased after increasing the absorbable phosphorus in the soil. On the other hand, an increase in the amount of ammonium compounds reduced protease activity in the soil [81]. However, the effect of mineral fertilization can be modified by cultivation factors, because the change in water–air conditions caused by the use of mechanical cultivation under trees was found to affect the reduction of enzymatic activity [80].

The activity of dehydrogenase, like other enzymes, changes during the growing season, which was found to be in the three orchards studied. It was the highest at the beginning of the growing season, after the flowering of trees (Figure 1D). Evaluation of the enzymatic activity carried out in the cultivation of an annual plant (soybean—*Glycine max* L.) also showed that enzymatic activity was highest in the flowering phase in the 15–20 cm layer, where the main mass of the root system was located [82,83]. In summer and autumn, the activity of dehydrogenase decreases. The high activity of dehydrogenase in the spring period is probably related to the higher activity of the root system and the accompanying root secretions during this period, which are a very good source of nutrients for microorganisms in the rhizosphere [25,29,49]. Perhaps the age of the orchard may also be a factor influencing the activity of dehydrogenase, since in this comparison the activity of dehydrogenase was significantly higher in the oldest orchard, which was founded in 1999 (OR 1). In the 2001 established autumn period, only the orchard (OR 2) was characterized by significantly lower activity than the others (Figure 2B).

Nitrogen fertilization had a significant impact on the activity of dehydrogenase in the spring period. In other words, in general, fertilizing the orchard at a dose of 60 kg N ha^{-1} increased the enzyme activity of dehydrogenase (Figure 1C). Sawicka et al. (2020) obtained similar results, but also in the cultivation of an annual plant (*Zea mays* L. maize), proving that nitrogen fertilization at the level of 100 and 150 kg N ha^{-1} did not affect the increase in dehydrogenase activity in the second half of the year. Furthermore, in maize cultivation, very high nitrogen fertilization resulted in lower dehydrogenase activity in the soil [49,84]. Furthermore, in perennial plants, high nitrogen fertilization limited

dehydrogenase activity [85]. Slightly different relationships were found in the soil in which perennial crops are grown in the replanted apple orchard because the use of mineral fertilization with nitrogen (130 kg ha^{-1}) and potassium (190 kg ha^{-1}) significantly increased the enzyme activity of dehydrogenase [27]. Variability is observed in perennial crops, such as the walnut orchard. Organic fertilization, applied on the surface or as mulch, significantly increases the activity of dehydrogenase, which is the largest in the upper layer of the soil and much higher than the use of mineral fertilization [86].

Our research, conducted on a 10-year-old cherry orchard, indicated that the applied nitrogen fertilization increases the activity of dehydrogenase.

Table 2. Influence of nitrogen fertilization on the activity of dehydrogenases in 2008–2010 (ADh cm^3 H_2 24 h^{-1} kg^{-1} DW soil).

Orchard	Fertilization	Date I	Date II	Date III
Orchard 1	0 N	4.3 $^{a\,1}$	2.9 ^{ab}	2.6 ^{a}
	60 N	11.3 ^{e}	5.9 ^{c}	6.7 ^{c}
	120 N	8.4 ^{c}	3.4 ^{ab}	4.0 ^{ab}
Orchard 2	0 N	6.9 ^{bc}	2.1 ^{a}	2.5 ^{a}
	60 N	8.8 ^{cd}	3.5 ^{ab}	3.9 ^{ab}
	120 N	8.2 ^{c}	3.1 ^{ab}	2.8 ^{a}
Orchard 3	0 N	5.7 ^{ab}	3.0 ^{ab}	3.8 ^{ab}
	60 N	10.7 ^{de}	4.2 ^{b}	5.6 ^{bc}
	120 N	7.9 ^{bc}	3.2 ^{ab}	5.2 ^{bc}
Mean of orchard	OR 1	8.0 $^{a\,2}$	4.1 ^{b}	4.5 ^{b}
	OR 2	8.0 ^{a}	2.9 ^{a}	3.1 ^{a}
	OR 3	8.1 ^{a}	3.5 ^{ab}	4.9 ^{b}
Mean of fertilization	0 N	5.6 $^{a\,3}$	2.7 ^{a}	3.0 ^{a}
	60 N	10.3 ^{c}	4.5 ^{b}	5.4 ^{b}
	120 N	8.2 ^{b}	3.2 ^{a}	4.0 ^{a}
Main effects ^{4}				
Orchard (A)		ns	*	**
Fertilization (B)		***	***	***
Interaction				
A × B		*	**	**

^{1} year × fertilization; the same letters in the column are not significantly different at = 0.05 (Duncan's test). ^{2,3} the orchard and fertilization; the same letters in the column are not significantly different at = 0.05 (Duncan's test). The ^{4} *p*–value of the F ratio: ns, not significantly different; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 3. Effect of nitrogen fertilization on dehydrogenase activity in 2011–2013 (ADh cm^3 H_2 24 h^{-1} kg^{-1} DW soil).

Fertilization	Date I	Date II	Date III
0 N	3.2 $^{a\,1}$	1.6 ^{a}	1.8 ^{a}
60 N	6.1 ^{b}	2.5 ^{b}	3.4 ^{b}
120 N	5.0 ^{ab}	1.9 ^{a}	2.8 ^{ab}
Main effects ^{2}			
Fertilization	*	**	*

^{1} one-way analysis of variance; data in the same column marked with the same letter are not significantly different at $\alpha = 0.05$ (Duncan's test). ^{2} *p*–value of the F ratio: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

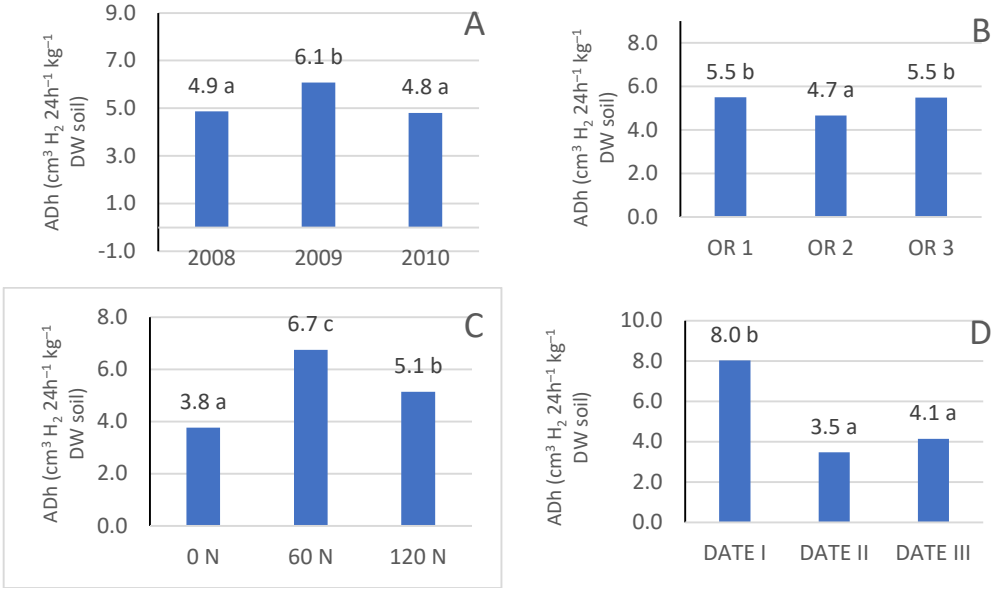


Figure 1. Dehydrogenase activity in 2008–2010. (**A**)—depending on the year of conducting the research; (**B**)—depending on the orchard; (**C**)—depending on the level of fertilization; (**D**)—depending on the timing of sampling.

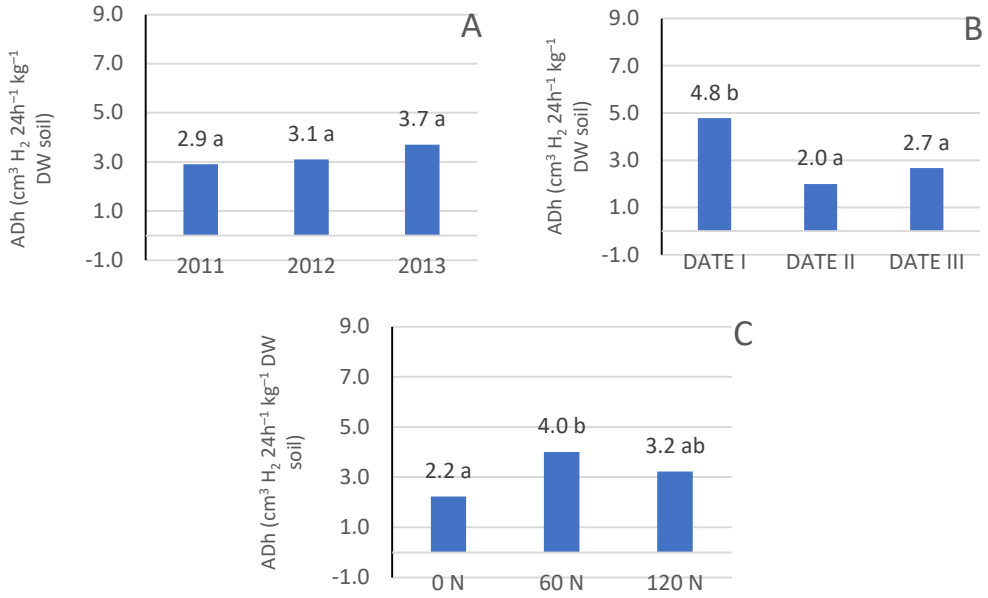


Figure 2. Activity of dehydrogenases in 2011–2013 (**A**)—depending on the year; (**B**)—depending on the timing of sampling; (**C**)—depending on the level of fertilization.

3.2. Protease Enzyme Activity

Studies of protease activity conducted in cherry orchards in 2008–2010 showed a high variability under the influence of each of the factors (Tables 4 and 5). The highest activity of the protease was recorded in 2009, which coincided with the highest rainfall (Table 1). However, in 2008, when the rainfall was below 400 mm, protease activity was the lowest (Figure 3A). Therefore, it can be assumed that the use of irrigation in the orchard will increase the activity of this enzyme. This effect was obtained in an apple orchard, where irrigation caused a significant increase in protease activity [27] and, as expected, the opposite effect was achieved by drying the soil leading to the denaturation of the enzyme [87,88].

In all the orchards studied, the activity of proteolytic enzymes increased during the growing season. The lowest was in the spring period and the highest in autumn at the end of the growing season (Table 4. Figures 3D and 4B).

Nitrogen fertilization had no significant effect on proteolytic enzyme activity between 2008 and 2010 (Figure 3C). No other result was expected, as numerous studies showed that differentiated short-term and long-term nitrogen fertilization does not cause a change in protease activity [49,89]. We found that the application of nitrogen fertilization increased protease activity compared to the control (no nitrogen fertilization); however, the differences between fertilization combinations no longer affected the significance of the changes in activity (Figure 3C). This is probably related to the N mobility in soil [90].

In 2011–2013, in the orchard established in 2001 (OR2), proteolytic enzyme activity was the lowest in 2012 (Figure 4A), when at the time of sampling there was a positive water balance, and it was the year with the highest rainfall during the growing season in 2011–2013 and one of the highest in the last 30 years. A high linear correlation coefficient was found between protease activity and water balance (Table 6). Maintaining soil moisture at 90% of the field water capacity reduced the activity of protease relative to humidity at the level of 60% of the field water capacity [27]. As in previous years, proteolytic enzyme activity increased during the growing season and was highest during the autumn period. This is confirmed by the results obtained in agricultural crops, where the activity of proteases increased throughout the growing season and had the high activity in September and October [91]. The use of nitrogen fertilization increased the protease level, but only at a dose of 60 kg N ha^{-1}. An additional increase in nitrogen fertilization to 120 kg N ha^{-1} reduced proteolytic bacteria (Tables 4 and 5, Figures 3C and 4C). Similar results were obtained in an apple orchard, where increasing nitrogen fertilization from 65 kg N ha^{-1} to 130 kg N ha^{-1} resulted in a decrease in protease activity [27].

Table 4. Influence of nitrogen fertilization on protease activity in 2008–2010 (mg tyrosine 24 h^{-1} kg^{-1} DW soil).

Orchard	Fertilization	Date I	Date II	Date III
OR 1	0 N	7.6 $^{b\ 1}$	3.1 ^{a}	5.5 ^{a}
	60 N	7.6 ^{b}	6.1 ^{ab}	9.8 $^{b–d}$
	120 N	3.5 ^{a}	9.5 ^{b}	12.4 ^{cd}
OR 2	0 N	4.8 ^{ab}	5.0 ^{a}	9.5 $^{b–d}$
	60 N	2.9 ^{a}	5.0 ^{a}	9.0 $^{a–c}$
	120 N	3.8 ^{a}	3.6 ^{a}	8.1 ^{ab}
OR 3	0 N	3.9 ^{a}	6.5 ^{ab}	12.9 ^{d}
	60 N	4.9 ^{ab}	6.2 ^{ab}	11.7 $^{b–d}$
	120 N	5.2 ^{ab}	3.6 ^{a}	9.9 $^{b–d}$
Mean of orchard	OR 1	6.2 $^{B\ 2}$	6.2 ^{a}	9.3 ^{a}
	OR 2	3.8 ^{a}	4.5 ^{a}	8.9 ^{a}
	OR 3	4.7 ^{ab}	5.5 ^{a}	11.5 ^{b}
Mean of fertilization	0 N	5.5 $^{B\ 3}$	4.9 ^{a}	9.3 ^{a}
	60 N	5.1 ^{b}	5.8 ^{b}	10.2 ^{ab}
	120 N	4.1 ^{a}	5.6 ^{b}	12.5 ^{b}
Main effects ^{4}				
Orchard (A)		*	ns	*
Fertilization (B)		**	*	*
Interaction A × B		*	**	**

^{1} year × fertilization; the same letters in the column are not significantly different at =0.05 (Duncan's test). ^{2,3} the orchard and fertilization; the same letters in the column are not significantly different at = 0.05 (Duncan's test). The ^{4} *p*–value of the F ratio: ns, not significantly different; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 5. Influence of nitrogen fertilization on protease activity in 2011–2013 (mg tyrosine 24 h^{-1} kg^{-1} DW soil).

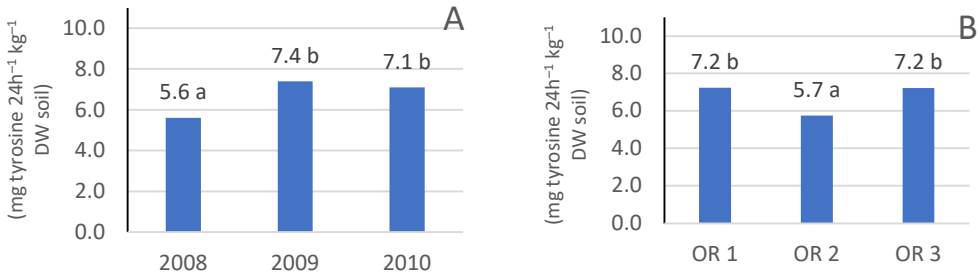
Fertilization	Date I	Date II	Date III
0 N	6.5 $^{a\ 1}$	5.8 ^{a}	7.7 ^{a}
60 N	9.3 ^{b}	6.4 ^{b}	9.8 ^{b}
120 N	2.8 ^{ab}	4.9 ^{a}	8.0 ^{ab}
Main effects ^{2}			
	*	**	*

^{1} one-way analysis of variance; data in the same column marked with the same letter are not significantly different at $\alpha = 0.05$ (Duncan's test). The ^{2} p–value of the F ratio: * $p < 0.05$; ** $p < 0.01$.

Table 6. Value of linear correlation coefficient.

	Term I		Term II		Term III	
	Dehydrogenase Activity	Protease Activity	Dehydrogenase Activity	Protease Activity	Dehydrogenase Activity	Protease Activity
			2008–2010			
pH	0.23	0.18	0.45 ***	0.44 ***	0.81 **	0.20
Air temperature	0.34	0.11	–0.13	–0.39 **	–0.61	–0.36
Evapotranspiration	–0.07	–0.64 ***	–0.02	–0.54 **	–0.46	–0.27
Water balance	–0.05	–0.49	0.09	–0.59 *	–0.56	–0.32
Soil temperature	0.13	–0.07	–0.07	–0.55 **	–0.49	–0.30
Yield	–0.19	–0.03	0.42 ***	0.20	0.47	0.58
Total soluble solids	0.04	0.27	0.03	–0.61 *	–0.68 ***	–0.35
L*	0.53	0.68 ***	0.04	–0.07	–0.43	–0.31
			2011–2013			
Air temperature	–0.51	0.24	–0.84	0.93 *	0.45	0.53
Evapotranspiration	–0.62	0.39	0.06	0.91 *	0.11	0.36
Water balance	0.88 **	–0.80	–0.78 *	0.12	–0.82 **	–0.72 *
Soil temperature	–0.60	0.34	0.13	0.97 *	0.50	0.55
Yield	0.35	–0.75 ***	–0.56	–0.02	–0.57	–0.73 **
Total soluble solids	0.10	0.40	0.32	0.43	0.04	0.34
Firmness	0.25	–0.75 ***	–0.76 *	–0.06	–0.48	–0.88 **
L*	0.89 **	–0.80 ***	–0.34	0.65	–0.82 **	–0.61
C^*_{ab}	–0.83 **	0.30	0.50	0.77 *	0.90 *	0.49
H_{ab}	0.74 *	–0.31	–0.21	0.71 *	–0.56	–0.30

* Significant levels $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$. Explanations: L*—indicator of darkening of fruit at the time of harvest; C^*_{ab}—Chroma = $((a^*)^2 + (b^*)^2)^{0.5}$; H_{ab}—($H° = \tan^{-1} b^*/a^*$).



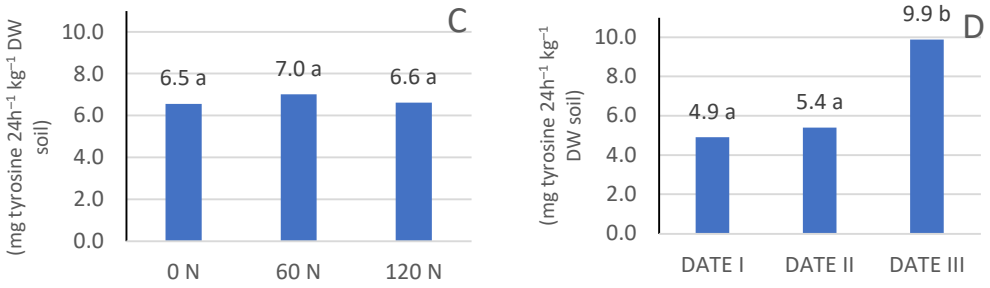


Figure 3. Protease activity in 2008–2010. (**A**)—depending on the year; (**B**)—depending on the orchard; (**C**)—depending on the level of fertilization; (**D**)—depending on the timing of sampling.

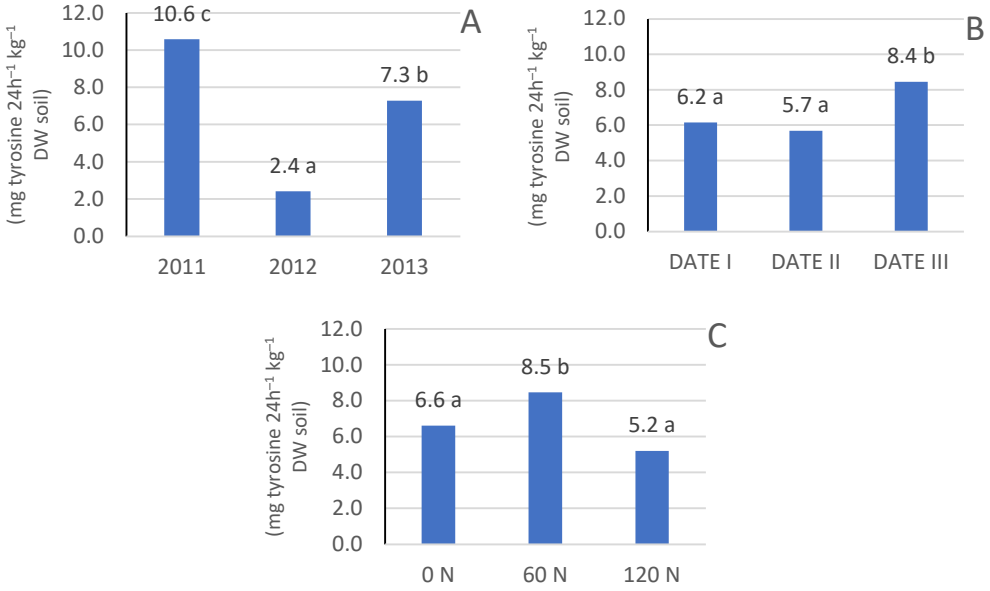


Figure 4. Protease activity in 2011–2013 (**A**)–depending on the year; (**B**)–depending on the timing of sampling; (**C**)–activity of proteases depending on the level of fertilization.

3.3. Microbiological Activity

3.3.1. Total Number of Bacteria

The timing of the sampling had a significant impact on the abundance of bacteria (Table 7). A similar relationship was found earlier in the apple orchard, where the abundance of soil microorganisms depended on the date of sampling and the year of research [92]. Seasonal changes in the abundance of microbes in the soil are associated with periodic changes in soil reaction, which is higher in spring and autumn [93]. The abundance of microorganisms present in the soil is also influenced by the conditions of its cultivation, and irrigation [92,94]. A very large source of seasonal variability can also be other agrotechnical treatments, mainly mineral fertilization and organic fertilization. Both have an impact on the abundance of bacteria. Organic fertilization as a source of numerous bacterial taxa usually causes their multiplication in the soil environment, and mineral torches most often limits the number of bacteria [95].

The highest number of bacteria was recorded in autumn of 2009 (Table 7, Figure 5A). This growing season saw the highest amount of precipitation. In addition, the last two months preceding sampling exceeded 120 mm. Bacteria and actinobacteria take up more water than fungi [96]. The number of bacteria was higher in the fall period and only in 2008 was there a higher activity in the spring period. In the remaining years of the study, the total number of bacteria in the initial period was lower in spring (Figure 5A). The age

of the orchard had no significant effect on the total number of bacteria population (Figure 5B). However, the results of studies conducted in citrus orchards of different ages indicate that the abundance of bacteria was higher in orchards of 7 and 11 years than in 3-year orchards. It should be emphasized that in this experiment the difference between the orchards was 3 years and the trees have already completed intensive growth and reached the assumed size. After a juvenile period of intensive fruit trees, the demand for nutrients from the soil is stable, as is the abundance of bacteria [97].

Table 7. Influence of the date of sampling and sour cherry orchards on the number of microorganisms in the soil during the vegetation season 2007–2010.

Year	Date	Bacteria CFU ×10^5 g^{-1} DW Soil)	Actinobacteria (CFU ×10^5 g^{-1} DW Soil)	Fungi (CFU ×10^4 g^{-1} DW Soil)	*Azotobacter* (CFU g^{-1} DW Soil)	*Azospirillum* (CFU ×10^3 g^{-1} DW Soil)
2007	Date I	26.8 $^{a\ 1}$	37.2 ^{bc}	4.0 ^{a}	17.4 ^{a}	55.1 ^{a}
	Date II	135.2 ^{c}	70.0 ^{d}	9.7 ^{b}	20.5 ^{ab}	62.5 ^{a}
2008	Date I	168.5 ^{d}	151.0 ^{f}	9.1 ^{b}	31.0 $^{b–d}$	407.0 ^{b}
	Date II	128.8 ^{c}	95.3 ^{e}	15.9 ^{c}	34.7 $^{c–e}$	382.8 ^{b}
2009	Date I	65.5 ^{b}	42.3 ^{c}	7.9 ^{b}	44.2 ^{e}	36.2 ^{a}
	Date II	226.5 ^{e}	155.1 ^{f}	17.6 ^{c}	38.4 ^{de}	138.7 ^{a}
2010	Date I	21.3 ^{a}	4.7 ^{a}	2.6 ^{a}	24.5 $^{a–c}$	37.2 ^{a}
	Date II	27.8 ^{a}	20.3 ^{ab}	3.9 ^{a}	20.6 ^{ab}	59.4 ^{a}
	OR 1	95.3 ^{a}	64.6 ^{a}	7.9 ^{a}	25.4 ^{a}	237.2 ^{b}
	OR 2	97.0 ^{a}	74.6 ^{a}	7.9 ^{a}	34.6 ^{b}	75.8 ^{a}
	OR 3	107.9 ^{a}	76.8 ^{a}	10.7 ^{b}	26.8 ^{a}	129.0 ^{a}

[1] one–way analysis of variance; data in the same column marked with the same letter are not significantly different at $\alpha = 0.05$ (Duncan's test). CFU—colony forming unit.

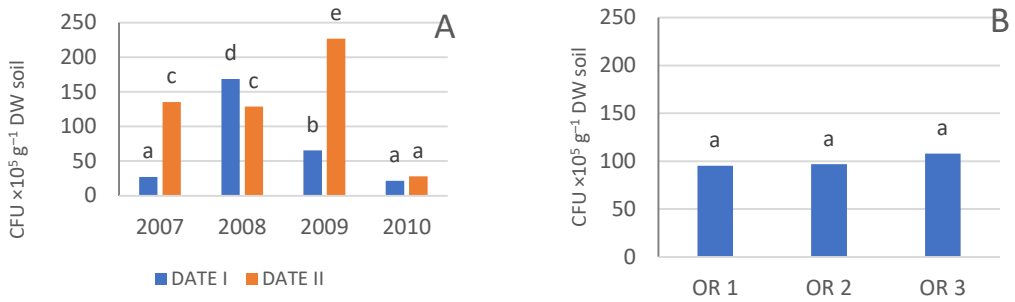


Figure 5. Total number of bacteria in 2007–2010: (**A**)—depending on the year and date of sampling; (**B**)—depending on the orchard.

3.3.2. Actinobacteria

The timing of the sampling had a significant impact on the abundance of actinobacteria (Table 7). The highest number of actinobacteria was found in the spring period in the year 2008 (Figure 6A).

The number of actinobacteria in the orchards studied was variable and a significantly higher number of actinobacteria was found in the cherry orchards established later, that is, in 2001 and 2002 (Table 5, Figure 6B). With the age of the orchard, the number of actinobacteria decreased. Presumably, the roots of *Prunus mahaleb* L. are not affected by actinobacteria and over time their numbers decrease. This is confirmed by German studies, where only the roots of apple or pear trees were colonized by actinobacteria and were damaged by them, while such pathogenicity was not found on the roots of *Prunus mahaleb* L. [98]

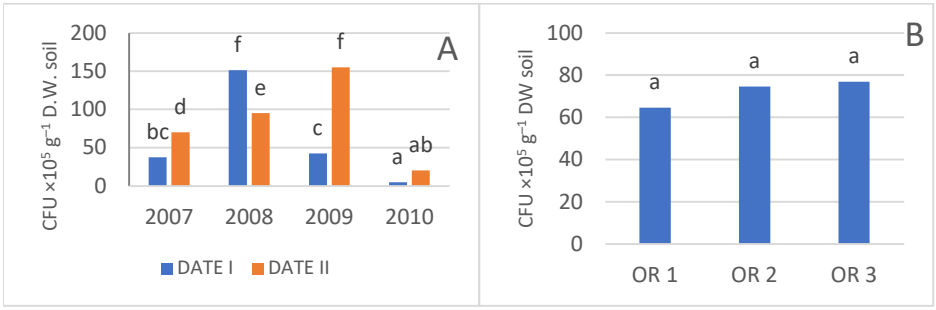


Figure 6. Number of actinomycetes in 2007–2010: (**A**)—depending on the year and date of sampling; (**B**)—depending on the orchard.

3.3.3. Fungi

The site from which the samples were taken was important for the abundance of fungi and nitrogenous bacteria (Table 7). The total abundance of fungi was highest in OR 3 (Figure 7B). During the growing season, the number of fungi increased. Significant increases were found in 2008 and 2009 (Figure 7A). In the reference studies conducted in the apple orchard, it was the highest in spring or fall depending on the year of the research, and the irrigation used limited the number of fungi. In the autumn period, the number of fungi was on average 35% higher than in spring [92,99].

A significantly higher number of fungi was found in the youngest cherry orchard, founded in 2002 (OR 3) (Figure 7B). In the soil, the abundance of bacteria and fungi is variable, and it is often difficult to find clear regularities. In the research conducted in China, the largest and the population of fungi was in the orchard 7 years and older. However, the abundance of fungi may vary depending on soil conditions [79,95]. It is a complex ecosystem in which different types of fungi can dominate. The variability of the results can be caused by organic fertilization, which can cause an increase in the number of fungi [95]. However, the results obtained in older citrus orchards show a different relationship, because in them, the number of fungi increased with age [97]. A relationship was found between fungus increase in the abundance and the yield per tree cross-sectional area of cherry trees (Figure 8).

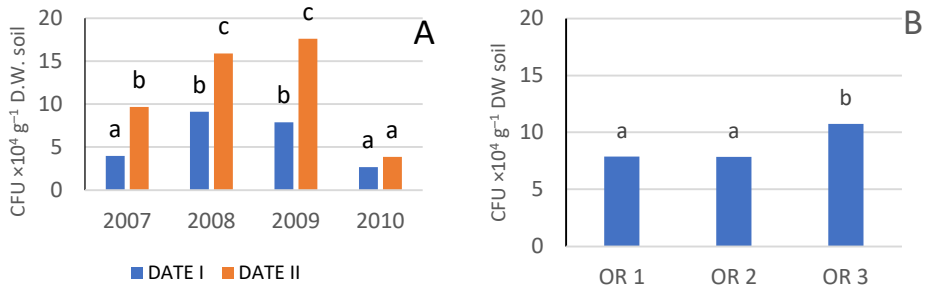


Figure 7. Number of fungi in 2007–2010: (**A**)—depending on the year and date of sampling; (**B**)—depending on the orchard.

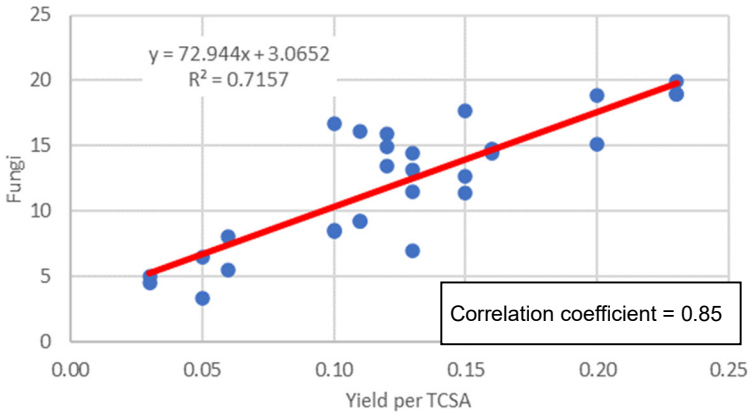


Figure 8. Correlation between yield per tree cross-sectional area and fungi in 2008–2010.

3.3.4. *Azotobacter*

A high variability in the abundance of nitrogenous *Azotobacter* bacteria was found (Tables 7). The influence of the season and the position was observed. In the first two years, the abundance of this bacterium was higher in spring than in the fall period, while in the next two years it was the opposite of the highest value recorded in 2009. This variability is confirmed by the results of the authors who showed that the abundance of *Azotobacter* is the highest during fruit harvest [92]. The yield of trees depended on the abundance of *Azotobacter*, especially if this activity was studied in the fall period (Table 8).

Significant differences in the abundance of nitrogenous bacteria were found depending on the orchard. In the cherry orchard founded in 2001 (OR 2), the number *of Azotobacter* was significantly higher than in other orchards (Figure 9B). The abundance of *Azotobacter* in studies in the cherry orchard was higher when irrigation was carried out [92].

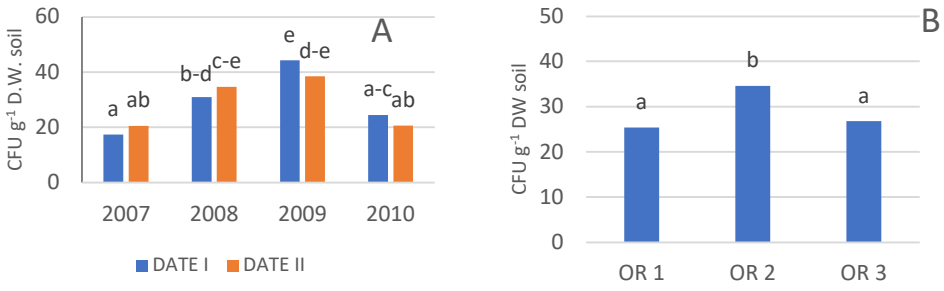


Figure 9. Number *Azotobacter* in 2007–2010: (**A**)—depending on the year and date of sampling; (**B**)—depending on the orchard.

3.3.5. Azospirillum

During the research period, the abundance of nitrogenous *Azospirillum* bacteria was also very diverse. The highest values were recorded in 2008 in the spring term, and it was significantly higher than in other dates. In most years, there were more bacteria than at the beginning of vegetation (Figure 10A). Their occurrence in the soil is influenced by physical and chemical factors. The importance of nitrogen content, organic matter, salinity, moisture, and soil compactness was found [100]. The greatest effect of *Azospirillum* on plant growth is on moderately fertilized soils. On soils with very low fertilisation and over-fertilisation, the impact will be less pronounced [101]. The use of high doses of mineral fertilizers reduces the number of bacteria from the genera *Azospirillum* and *Azotobacter*. The course of weather conditions also affects the abundance of *Azospirillum*. With dry soil, the low osmotic potential limits the viability and activity of bacteria [102]. *Azospirillum* abundance had a greater impact on the activity of proteolytic enzymes than the activity of dehydrogenases (Figure 11A, Table 8). The PCA showed that a relationship was

found between the activity of nitrogenous bacteria and the yield of trees. The increase in sour cherry was associated with an abundance of diazotrophic bacteria (Figure 11B).

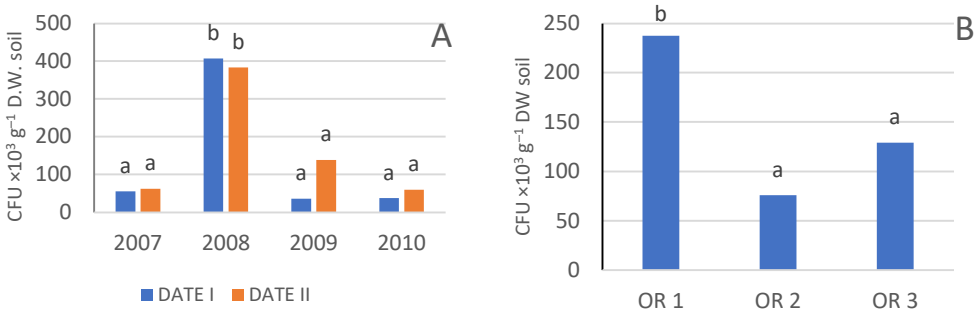
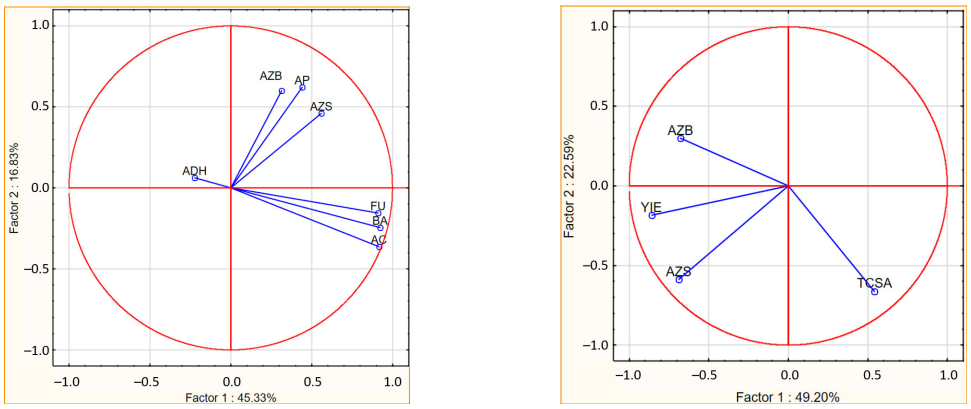


Figure 10. Number of *Azospirillum* 2007–2010: (**A**)—depending on the year and date of sampling; (**B**)—depending on the orchard.

Table 8. Value of the linear correlation coefficient depending on the abundance of soil microorganisms.

	Bacteria		**Actinobacteria**		**Fungi**		***Azotobacter***		***Azospirillum***	
	S	**A**	**S**	**A**	**S**	**A**	**S**	**A**	**S**	**A**
Dehydrogenase activity	0.21	0.51	–0.34	0.42	–0.19	0.54	–0.19	0.43	0.02	–0.15
Protease activity	0.11	0.23	0.05	0.13	–0.04	0.31	–0.25	0.82 **	0.72 *	0.23
Air temperature	–0.87 **	–0.30	–0.78 *	–0.23	–0.81 **	0.04	–0.48	–0.07	–0.45	0.57 *
Evapotranspiration	–0.48	–0.11	–0.42	–0.05	–0.45	0.27	0.27	0.12	–0.59	0.64 *
Water balance	–0.51	–0.25	–0.45	–0.18	–0.48	0.12	0.29	0.02	–0.61	0.59
Soil temperature	–0.83 **	–0.14	–0.70 *	–0.08	–0.79 *	0.39	–0.55	0.05	–0.71 *	0.67 *
Yield	0.61 *	0.48	0.52 *	0.34	0.50 *	0.70 *	0.13	0.91 ***	0.48 *	0.59 *
Firmness	0.65 *	–0.32	0.55	–0.29	0.67 *	0.07	–0.33	0.04	0.44	0.33
Mass fruit	–0.78 *	–0.29	–0.82 *	–0.28	–0.82 **	–0.41	–0.31	–0.41	–0.56	–0.54
Total soluble solids	0.67 *	–0.26	0.68 *	–0.15	0.70 **	0.08	–0.12	–0.04	0.61 *	0.47
Titratable acidity	–0.09	0.63	–0.05	0.50	–0.08	0.36	0.51 *	0.46	–0.36	–0.24
Tree cross sectional area	–0.62 *	–0.22	–0.73 *	–0.25	–0.69 *	–0.47	–0.16	–0.39	–0.17	–0.12
L*	–0.62 *	–0.73 *	–0.62 *	–0.69 *	–0.62	–0.88 **	–0.34	–0.58 *	0.06	–0.02
a*	0.87 **	–0.01	0.87 **	0.05	0.85 **	0.43	–0.12	0.30	0.84 **	0.80 **
b*	–0.14	–0.52	–0.04	–0.46	–0.15	–0.21	–0.69 *	–0.15	0.31	0.32
C^*_{ab}	0.87 **	–0.02	0.87 **	0.05	0.84 ***	0.43	–0.13	0.29	0.84 **	0.81 **
H_{ab}	–0.57 *	0.42	–0.61 *	0.39	–0.63 *	0.01	–0.36	–0.22	–0.56	–0.46

* Significant levels $p < 0.001$, ** $p < 0.01$, *** $p < 0.05$. Explanations: L*—indicator of darkening of fruit at the time of harvest; C^*_{ab}—Chroma = $((a^*)^2 + (b^*)^2)^{0.5}$; H_{ab}—($H° = \tan^{-1} b^*/a^*$).



(**A**) (**B**)

Figure 11. (**A**)—enzymatic activity dehydrogenases and proteases on the abundance of soil microorganisms, (**B**)—the effect of diazotrophic bacteria on yield and growth. ADH—dehydrogenases, AP—proteases, AZB—*Azotobacter*, AZS—*Azospirillum*, FU—fungi, BA—bacteria, AC—actinobacteria, YIE—yield kg/tree, TCSA—cross-sectional area of the trunk.

3.4. Yield, Growth, and Quality of Fruit

Nitrogen fertilization by affecting enzymes and soil microorganisms had to affect the availability of nutrients as well as the condition of the root system. The aim of this experiment was not to analyse the yield and quality of the fruit, but, in order to assess the impact of the soil ecosystem that has these characteristics, the average values obtained in all orchards and in all years of research were analysed. The largest trunk diameter had trees fertilized with a dose of 60 kg N ha^{-1} and the increase in the dose caused a slight decrease in tree growth (Figure 11A). Similar relationships were found in tree yield, where the optimal level of fertilization was 60 kg N ha^{-1} (Figure 12B).

The weight of the fruit increased with the applied nitrogen fertilization and the largest fruits were in combination with the highest dose of fertilization (Figure 2C). On the other hand, nitrogen fertilization had no significant effect on fruit firmness (Figure 13).

The lowest values were found with the highest nitrogen fertilization (Figure 13). As a result of nitrogen fertilization, the brightness of the color (L*) did not change significantly. On the other hand, the share of red in fruits (a*) increased. Similarly, the parameter b*, which indicates a higher proportion of yellow also increased (Figure 14).

The yield of trees still depended on the activity of soil enzymes, namely it was inversely proportional to the activity of protease, especially if this activity was studied in the second period of analysis (Table 6).

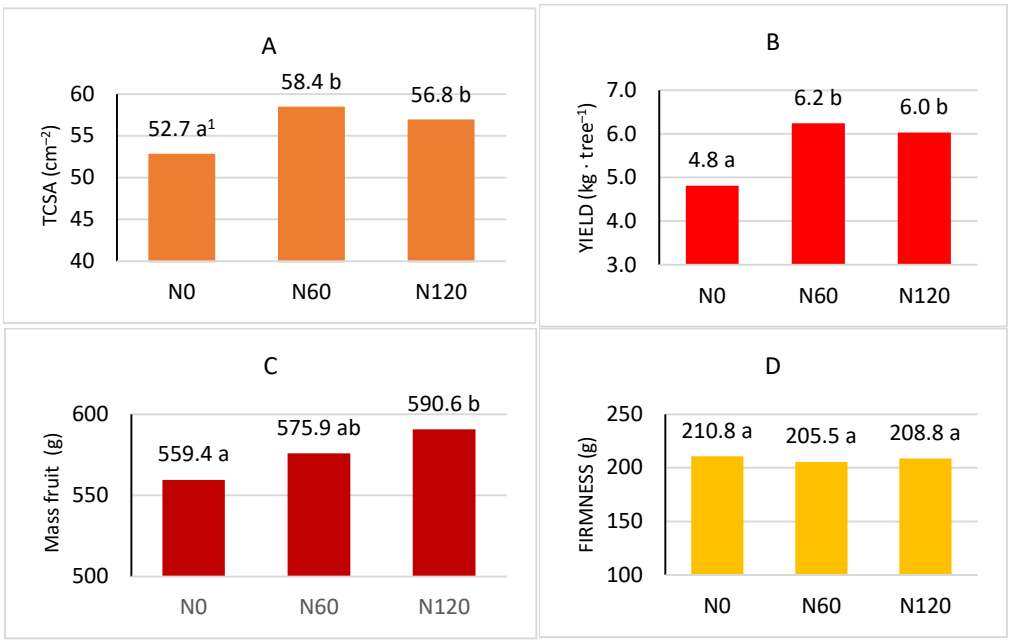


Figure 12. The effect of nitrogen fertilization on (**A**)—tree cross sectional area (TCSA) (cm^{-2}), (**B**)—yield (kg $tree^{-1}$), (**C**)—mass of fruit (g), (**D**)—firmness of fruit (g). Average for all orchards (OR 1, OR 2, OR 2) in years 2007–2013. [1] means that the same letters are not significantly different at α = 0.05 (Duncan's test).

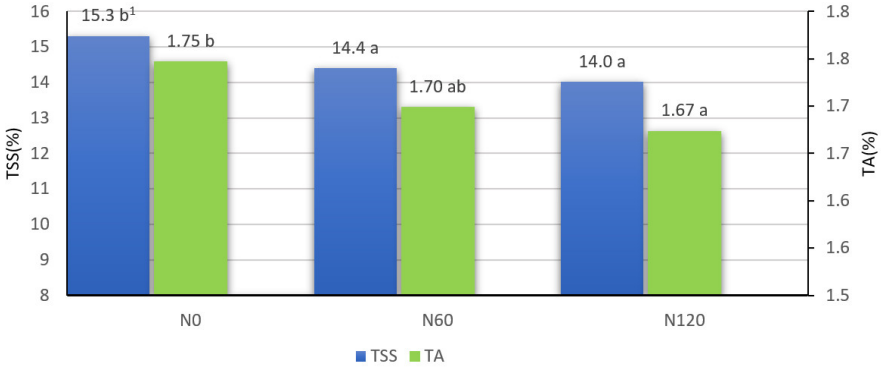


Figure 13. The effect of nitrogen fertilization on total soluble solids (TSS), titratable acidity (TA). Average for all orchards (OR 1, OR 2, OR 2) in years 2007–2013. [1] means that the same letters are not significantly different at $\alpha = 0.05$ (Duncan's test).

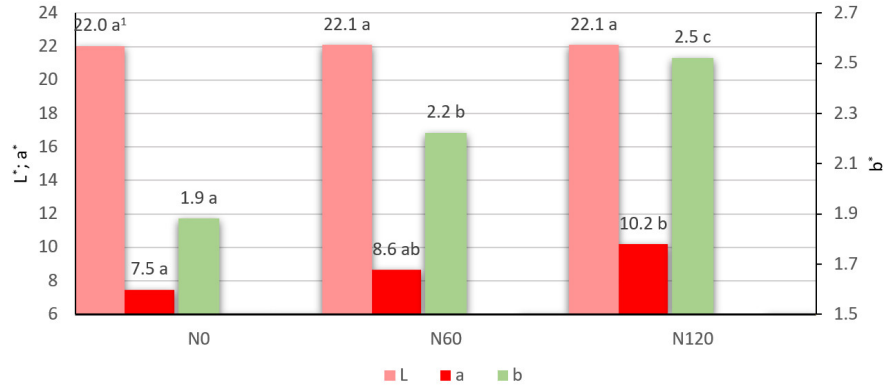


Figure 14. The effect of nitrogen fertilization on the colour. Average for all orchards (OR 1, OR 2, OR 2) in years 2007–2013. L* indicates darkening of fruit at the time of harvest; a* indicates chromaticity on a green (–) to red (+) axis; b* chromaticity on a blue (–) to yellow (+) axis. [1] means that the same letters are not significantly different at $\alpha = 0.05$ (Duncan's test).

4. Conclusions

The richness or fertility reflects enzymatic activity. Our research proves that it is from nitrogen fertilization. Enzymatic activity grew after the application of 60 kg N ha^{-1}, but a more recent increase in nitrogen dose to 120 kg N ha^{-1} caused a decrease in activity. This may indicate that too much of nitrogen fertilization causes a decrease in the number of soil microorganisms. The use of high doses of mineral nitrogen fertilizers inhibits the activity of nitrogenase but also reduces the number of diazotrophic bacteria, which respond to better use of nutrients and, consequently, reduce mineral fertilization. This allows us to conclude that too high doses of fertilization do not have a positive effect on soil quality. Furthermore, the activity of proteases, depending on the site and years of research, was dependent on nitrogen fertilization. However, protease activity is largely related to the course of climatic conditions. Soil moisture and temperature are crucial.

A relationship was found between protease activity and cherry yield, as well as between dehydrogenase and protease activity and the quality of cherry fruits.

Our research shows that both enzymes studied are a good indicator of soil microbial activity. Of the soil microorganisms studied, fungi and *Azotobacter* or *Azospirillum* bacteria are the most correlated with cherry yields.

The use of lower doses of nitrogen fertilizers will allow for maintaining biological balance in the soil and a more effective use of nitrogen fertilizers, the effectiveness of which is very low. This reduces the cost of fruit production while reducing denitrification and increasing nitrogen leaching, which pollutes the natural environment.

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