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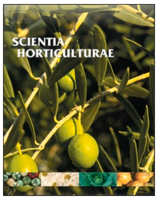

Apple leaf macro- and micronutrient content as affected by soil treatments with fertilizers and microorganisms

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ABSTRACT

The effects of bacterial and fungal inocula on the nutritional status of ‘Sampion’ apple trees was evaluated in three-years study (2018–2020). The experiment was set up in a two-factor system: (i) fertilization (without fertilization or mineral fertilization with nitrogen, phosphorus, potassium); (ii) application of beneficial microorganisms (without application, application of fungi or bacteria). The mixture of beneficial soil fungi contained two species: *Aspergillus niger* and *Purpureocillium lilacinum*. The mixture of beneficial bacteria contained three strains of *Bacillus* (*Bacillus* sp., *Bacillus amyloliquefaciens* and *Paenibacillus polymyxa*). The analysis of mineral content and leaf greenness (SPAD index) was performed on the samples collected from the trees in August. The lack of mineral fertilization caused a significant decrease in the nitrogen content of apple leaves, which resulted in their lighter color (lower SPAD index). The application of filamentous fungi or bacterial strains had a modifying effect on the mineral composition of apple leaves, what became evident especially in the third year of the study. An increase in the concentration of nitrogen, calcium, magnesium, sulphur and most microelements (except for boron) was found as a result of the influence of tree mineral fertilization and microorganisms applied to the soil. In the absence of mineral fertilization of the trees, the applied microbial inocula resulted in the significant increase of concentration of most macro- and microelements in the leaf tissue (except for magnesium and boron).

1. Introduction

Apple (*Malus domestica*) is one of the most popular and favorite fruits in the world. Due to its economic importance, apple cultivation has a significant impact on the development of Polish agriculture. Poland ranks as the number one producer of apples in Europe and as the third in the world (GUS, 2017). In 2019, apple orchards accounted for 72.3% of the fruit-tree cultivation area, and apples accounted for 78.8% of the fruit harvest in orchards in Poland. The world apple production has increased from 17.05 to 89.33 million tonnes during the period 1961–2016, showing a rapid growth of global apple consumption (FAO, 2018). The reason for the global increase in apple production is the delicious flavor and abundant health benefits of the apple fruit (Boyer and Liu, 2004).

In the past years, very intensive fruit-growing systems have been developed in all fruit-growing centers. This method of farming requires application of excessive amounts of chemical fertilizers, pesticides and herbicides, which could be harmful to soil microorganisms, human beings, animals and the entire natural environment (Boye and Arcand, 2013; Mosa et al., 2016; Wang et al., 2017; Garima, 2019).

Because of concerns for food and environmental safety, we must limit the use of chemicals and mineral fertilizers. In the case of fertilization, the proposed solution is to use natural organic fertilizers; however, their amounts might be limited. Another possible solution is to reduce mineral fertilization by increasing its efficiency by enriching the soil with beneficial microorganisms – both fungi and bacteria (Boye and Arcand, 2013; Derkowska et al., 2015; Kumar et al., 2018; Sas-Paszt et al., 2019a). Mineral fertilizers and beneficial microorganisms can be added to the soil separately or in combination as biofertilizers.

Results of numerous experiments have revealed that biofertilizers enriched with mycorrhizal fungi and filamentous fungi produce stimulating effects on the growth and reproduction of several plant species (Sas-Paszt et al., 2015, 2019a; Kumar et al., 2018). Beneficial microorganisms assist the roots in absorbing minerals from the soil and

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strengthen plant physiology (Mahdi et al., 2010; Garima, 2019). Microorganisms play a vital role in maintaining long-term soil fertility and sustainability by fixing atmospheric di-nitrogen, mobilizing fixed macro- and micronutrients, or converting insoluble phosphorus (P) in the soil into forms available to plants (Mahdi et al., 2010).

The effect of inoculation with beneficial bacteria and fungi on the changes in the content of minerals in soil and nutrients in leaf tissue was analyzed in the cultivation of apple trees. In the study performed by Kurek et al. (2013), a phosphate solubilizing bacterium *Pseudomonas luteola* BN0834 was introduced into the soil in a young apple tree orchard (cv 'Ligol'). When the ten times higher than the number of native phosphate solubilizing microorganisms (PSM) in soil (*P. luteola* BN0834 × 10) was introduced without a mineral fertilizer added near the surface of the roots, positive correlations were found between the number of PSM in the tree rhizosphere and the content of available P in non-rhizosphere soil and also between the number of PSM in the apple tree rhizosphere and the amount of P, potassium (K) and calcium (Ca) in plant leaves (Kurek et al., 2013).

Karlidag et al. (2007) showed that root inoculation of strains *Bacillus* M3, *Bacillus* OSU-142 and *Microbacterium* FS01 increased leaf nutrient contents (nitrogen, phosphorus, potassium, calcium, iron, manganese, zinc) significantly in apples (cv. Granny Smith). The results of this study suggest that these microorganisms (applied alone or in combinations) have the great potential for use as plant-growth-promoting rhizobacteria to increase the yield, growth and nutrition of apple trees (Karlidag et al., 2007).

The application of commercial Bokashi biostimulant (containing more than 60 selected strains of effective microorganisms) to soil of 15-year-old apple trees (cv. Anna) increased some macro and micro elements (i.e. N, P, K, Fe, Zn, and Mn) content in soil as compared to the control one (Sahain et al., 2007). Przybyłko et al. (2021) inoculated several cultivars of apple trees with commercially available microbial inoculum Micosat F (containing spores and mycelium of arbuscular mycorrhizal fungi: *Glomus mosseae* GP11, *G. viscosum* GC41, *G. intraradices* GB67 and plant-growth-promoting rhizobacteria *Bacillus subtilis* BA41 and *Streptomyces* spp. SB19). As a result, the authors observed better nitrogen nutrition status that promoted vigorous growth of apple trees and more efficient uptake of magnesium from the soil. On the other hand, lower phosphorus and potassium content in the tree leaves was observed. According to the authors, this phenomenon may have been a result of fungus - plant competition in conditions of this element deficiency in soil (Przybyłko et al., 2021).

Knowledge of mineral nutrition, due to its expressiveness, is of great importance when recommending a certain amount of fertilizers. Leaf analysis is a good way to diagnose potential insufficiency or excess, and offers the possibility of determining the nutritional status of crops and correcting deficiencies, if necessary (Montañés et al., 1991). Assessment of the mineral nutrition status of plants is based on comparing the concentrations of nutrients in plant tissues with reference concentrations obtained from a population growing under optimal nutrition (Lucena, 1997; Mourao Filho, 2004).

The nutritional status of plants can also be assessed on the basis of plant appearance, by assessing their growth vigor and the color of their leaves. Dark green leaves and vigorous vegetative growth are characteristics of plants with adequate N supply. Leaf color gives a good indication of the chlorophyll content of leaves, which has a close correlation with plant N status (Treder and Cieśliński, 2003; Uddling et al., 2007). Minolta Co. (Japan) has developed a chlorophyll metre, SPAD-502, which can be used to assess foliar N content (Treder et al., 2016).

The aim of the study was to evaluate the effects of using bacterial and fungal inocula on the nutritional status of apple trees as expressed by the concentrations of minerals in their leaves.

2. Material and methods

2.1. Experimental site and material

The experiment was established in the spring of 2018 in the Experimental Orchard of the National Institute of Horticultural Research in Dąbrowice (Central Poland, 51°54′51.1″N 20°06′41.0″E (51.914188, 20.111389), 145 m a.s.l.) and was run for three consecutive years. The subjects of the research were dwarf apple trees of the cultivar 'Sampion' grafted on M.9 rootstock. They were planted in early April on a podzolic soil underlaid by sandy loam, rated as soil quality class 3b. At planting time, the soil pH was slightly acidic at pH 6.2 (in KCl), and the average humus content of the soil was 1.2%. The levels of minerals in the soil, including macroelements, was as follows: phosphorus (P) - 75, potassium (K) - 124, magnesium (Mg) - 58 mg kg^{-1}, and microelements: boron (B) - 2.4, copper (Cu) - 4.8, iron (Fe) - 862, manganese (Mn) - 75.5, sodium (Na) - 4.35, zinc (Zn) - 3.7 mg kg^{-1}. Available P and K in the soil were determined using a solution of Ca-lactate (at pH 3.6), and available magnesium (Mg) was determined using a solution of 0.0125 M Ca-chloride (Ostrowska et al., 1991). The contents of micronutrients were determined using 1 M HCl extraction reagent (Mercik, 2004). The details of used soil analysis methods are presented in the paper by Wójcik and Filipczak (2015).

The trees were spaced 4 m between rows and 2 m in the row. The experiment was established in a random block design in four replications. Each experimental combination was represented by 12 trees.

2.2. Experimental design

The experiment was set up in a two-factor system. fertilization: 1.1 without fertilization, or 1.2 mineral fertilization with NPK beneficial microorganisms : 2.1 control, 2.2 fungi, or 2.3 bacteria

The experiment included the following experimental treatments:

1 Control (no fertilization).
2 Standard NPK – soil fertilization before planting in doses of 20 g of Super Fos Dar 40 granulated fertilizer, 160 g of potassium salt per plot (12 m^2) and 55 g of urea were applied under each individual tree (6 m^2).
3 Control + beneficial fungi – beneficial soil fungi on their own in the amount of 5.25 g per plot were applied. The mixture of beneficial soil fungi contained two species: *Aspergillus niger* and *Purpureocillium lilacinum*. The fungal spores concentration was approx. 10^8 cfu g^{-1}.
4 Control + beneficial bacteria – beneficial soil bacteria on their own in the amount of 3.83 g was applied. The mixture of beneficial bacteria contained three strains of *Bacillus* (*Bacillus* sp., *Bacillus amyloliquefaciens* and *Paenibacillus polymyxa*).
5 Standard NPK + beneficial fungi – soil fertilization as in point 2 combined with the beneficial soil fungi listed in point 3.
6 Standard NPK + beneficial bacteria – soil fertilization as in point 2 and the beneficial bacteria applied to the soil as in point 4.

All the treatments were repeated in the second and third year after planting (2019, 2020). In the first year of the experiment, mineral fertilizers were applied on the soil surface around the trees at planting time. The microorganisms were mixed with the soil in the planting hole. In the second and third year, the fertilizers were applied onto the soil surface, whereas the microorganisms were mixed with the topsoil. The experimental field was fitted with concrete poles reinforced in tree rows, with wires stretched between the poles and bamboo canes tied to the wires and the trees. Drip irrigation was established after planting. The trees were irrigated in a dry season from May to September (on the basis of soil moisture monitoring with 5TE dielectric probes (METER Group, Inc., USA)). In the first year, from the spring to August, the soil was kept in clean cultivation with rotating mechanical implements, the alleyways were grassed down and frequently moved. From the second year, 'Basta' herbicide was applied within tree rows to control weeds. To keep the trees in healthy condition, six sprayings with fungicides and four with insecticides were essential. In the first year after planting, the flower buds that appeared on some trees were removed so that the fruit would not inhibit the growth of the young plants. No flower or fruit thinning was done in the subsequent years.

2.3. Meteorological data

During the experiment, meteorological data was monitored by the weather station installed in the orchard (iMETOS, Pessl Instruments, Austria; Table 1). The average air temperature in the growing season (April–October) during the measurement period was at the level of the 30-year average. The highest average temperatures were recorded in 2018, with the values 1.97 °C higher than the multi-year average, while the lowest values were recorded in 2020 (0.52 °C lower than the multi-year average). The highest total rainfall was recorded in 2020, 176.8 mm higher than the 30-year average. The lowest rainfall occurred in 2019, 74.2 mm lower than the multi-year average.

2.4. Measurements

2.4.1. Assessment of mineral nutrition

Samples of twenty leaves from the middle part of shoots were selected at random from each replicate in early August to measure leaf mineral content. Prior to the chemical analyses of the leaves, indirect measurements of relative chlorophyll content were carried out using a SPAD-502 metre (Konica Minolta Co., Ltd, Japan).

The concentrations of macro- and microelements in leaf tissue were analyzed by the Chemical Laboratory of the National Institute of Horticultural Research in Skierniewice, Poland. The collected samples of the leaves from each treatment were placed for 48 h in a forced-air dryer at 70 °C. After grinding and wet mineralization in acids, the concentrations of macronutrients (P, K, Ca, Mg, S) and micronutrients (Fe, Mn, Cu, Zn, B) were determined using a sequential emission spectrometer with inductively coupled plasma (ICP Perkin-Elmer model Optima 2000 DV, Boston, Massachusetts, USA). The selected elements were determined at their characteristic wavelengths (Boss and Fredeen, 2004). The N content in plant samples was analyzed using the Kjeldahl method (Latimer, 2012) (the Kjeldahl apparatus Vapodest, Königswinter, Germany). All the nutrients were determined in three repetitions.

2.4.2. Deviation from optimum percentage (DOP index)

The DOP index is an alternative method to the traditional diagnosis, which is capable of accurately defining the quantity and quality of each nutrient in plants: optimal (DOP = 0), deficiency (DOP < 0), or excess (DOP > 0) (Montañés et al., 1991). The DOP index was obtained from leaf chemical analyses by the following formula:

$$DOP = \left(\frac{C_n}{C_o} - 1\right) x\ 100$$

where:

C_n – foliar concentration of the analyzed nutrient

C_o – optimum nutrient content for apple trees proposed by Sadowski et al. (1990).

2.5. Data analysis

The results were statistically analyzed using two-way analyses of variance with the Duncan test, α = 0.05, using the statistical program Statistica 13.1. Due to the significant influence of the years, the analyses were statistically conducted separately for each study year. Data not significantly different from each other were marked with the same letters. The error bars on the graphs indicate the standard error.

3. Results

3.1. SPAD index

In the first year of the study (2018), the analysis of variance did not show a significant effect of the applied experimental variants on the value of the SPAD index of apple leaves (Fig. 1). In the following year, such an effect was found only after the use of fungal and bacterial inocula, and in the third year of the study (2020) both for the applied microorganisms and the applied fertilization. In the second year of cultivation, the highest values of the SPAD index were those of the leaves of NPK-fertilized trees growing on the plots where the soil had been inoculated with the fungi or bacteria. These indices were not only significantly higher than those measured for the control non-fertilized trees and the control trees with the soil inoculated with fungi, but also those fertilized with NPK without the additional soil treatment with microorganisms (Fig. 1). In the third year of cultivation, the effect of the lack of fertilization on the color of apple leaves (SPAD index) became apparent. The leaves of the non-fertilized trees were much lighter than the leaves of the apple trees for which mineral fertilization had been applied. Significantly the highest SPAD index was found for the leaves of the trees growing on the fertilized plots and inoculated with fungi.

3.2. Nitrogen content of leaves

Chemical analyses of the leaves were carried out in 2018 and 2020. In the first year of the study, no significant influence of the tested factors on the nitrogen content of apple leaves was found. Such an influence was proved in the third year of orchard cultivation (Fig. 2). In 2020, a significant reduction in the nitrogen content of leaves could be seen on non-fertilized plots. Significantly the lowest nitrogen content was found in the leaves of the control non-fertilized trees where no microorganisms were used. The very addition of the fungal or bacterial inocula had a significant effect on increasing the nitrogen concentration in apple leaves. The highest nitrogen concentration was found in apple trees under mineral fertilization with the simultaneous application of the

Table 1

Average air temperature and total precipitation in Dąbrowice in the 2018–2020 growing seasons in comparison with multi-year averages.

Average air temperature (°C)								
Year/month	Apr	May	Jun	Jul	Aug	Sep	Oct	Average Apr-Oct
2018	13.2	16.5	18.5	20.3	20.1	15.1	9.6	16.18
2019	9.6	12.5	21.7	18.3	19.8	13.9	10.0	15.11
2020	8.0	11.1	17.7	16.8	18.9	14.0	9.3	13.68
1991–2020*	8.7	13.7	17.2	19.1	18.5	13.9	8.4	14.21
Total precipitation (mm)								
Year/month	Apr	May	Jun	Jul	Aug	Sep	Oct	Total Apr-Oct
2018	28.6	51.6	30.0	155.0	55.6	70.6	64.8	456.2
2019	15.6	52.2	36.4	50.8	59.2	82.2	26.4	322.8
2020	9.6	81.6	135.0	63.2	110.4	74.8	99.2	573.8
1991–2020*	40.7	60.3	71.7	75.3	55.6	50.1	43.3	397.0

* 30-year average

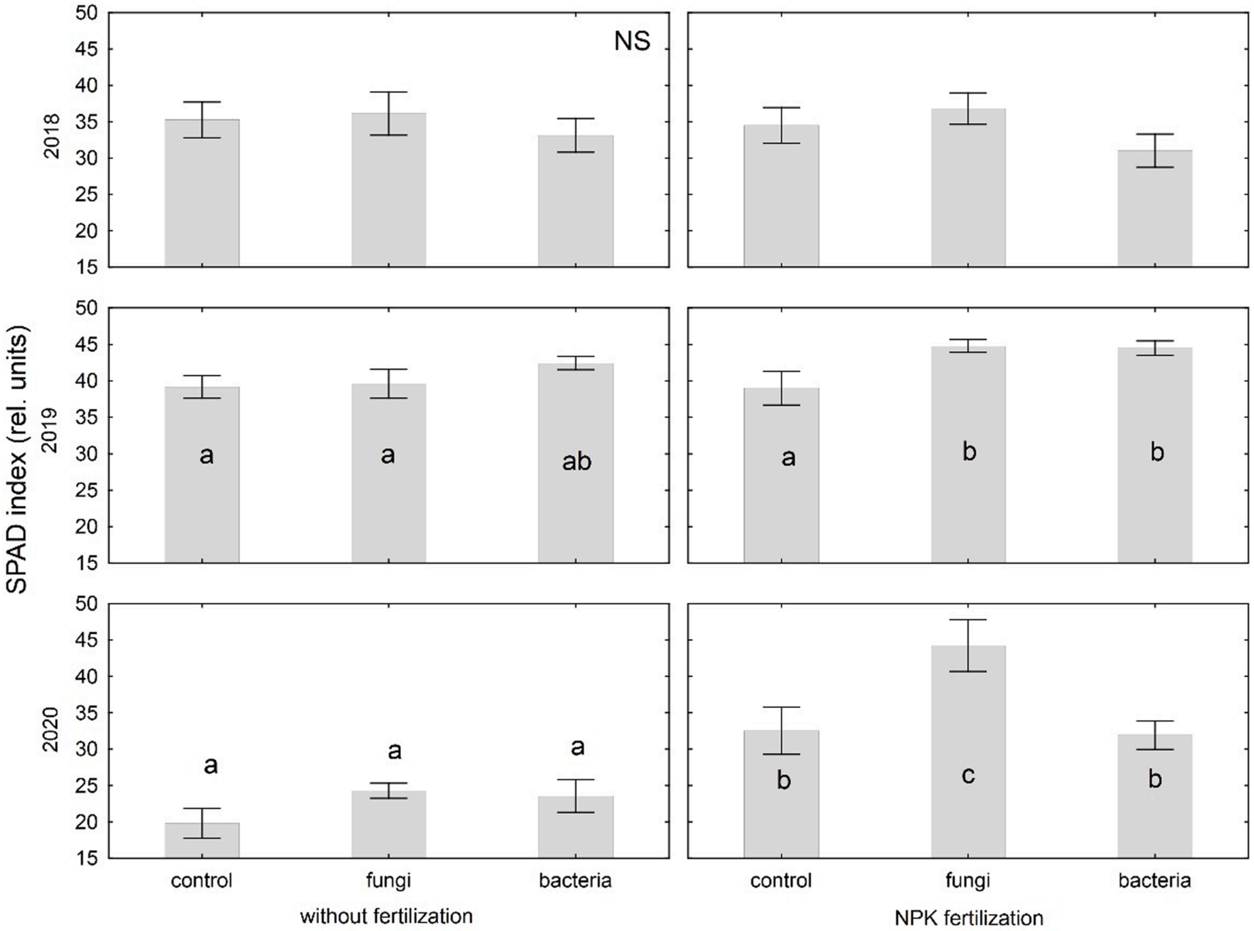

Fig. 1. Effect of the use of beneficial microorganisms and mineral fertilization on the value of the SPAD index of apple trees. Statistical analyses are presented separately for each year. Means marked with the same letters do not differ significantly at α = 0.05, ns – differences not significant.

fungal or bacterial inoculum.

A high level of correlation was found between the nitrogen content of the leaves and the SPAD index values (Fig. 3). This indicates the possibility of using this parameter (the measurement is non-destructive) as an indicator of the nitrogen nutrition status of apple trees (although it is necessary to establish the relationship between these parameters for a specific cultivar, tree development stage, and cultivation technology).

3.3. *Phosphorus content of leaves*

As in the case of nitrogen, no significant influence of the applied factors on the phosphorus content of apple leaves was found in the first year of the study. It was in the third year of the study that the chemical analyses of leaves showed such an effect. Significantly the lowest phosphorus content was found in the leaves of NPK-fertilized trees whose soil had been treated with the inoculum containing fungi or the inoculum containing bacteria (Fig. 4). Much higher phosphorus concentrations were found in the leaves of non-fertilized trees. Here, too, significant differences were found between the plots treated with the inocula containing microorganisms and the control plots. Statistically, the highest concentration of phosphorus was found in the leaves of non-fertilized trees growing on the plots where the soil was enriched with beneficial fungi.

3.4. *Potassium content of leaves*

Already in the first year of the study, the effects of fertilization and of the use of microorganisms on the potassium content of apple leaves were evident (Fig. 5). Application of the inoculum containing microorganisms significantly increased the concentration of potassium in the leaves of both non-fertilized and fertilized trees. In the third year of cultivation, the potassium content of the leaves was significantly lower compared with the results obtained in the first year. In the case of the plots where no mineral fertilization was applied, the application of fungi or bacterial inoculum increased the potassium content of apple leaves.

3.5. *Calcium content*

In the first year of the study, there was no significant influence of the applied factors on the concentration of calcium in apple leaves (Table 2). However, such an effect was shown by the chemical analyses of the leaves in the third year of the study. In 2020, the leaves of the control trees (without inocula) had significantly the lowest calcium content. Inoculation of the soil with microorganisms resulted in a significant increase in the concentration of calcium in apple tree leaves. Such differences were found both for the plots without mineral fertilization and for those where fertilization was applied. The highest calcium content was found in the leaves of fertilized trees growing in the soil enriched with inocula containing microorganisms. Statistically, the highest concentration of calcium was found in the leaves of NPK-fertilized trees growing on the plots where the soil was enriched with the inoculum containing bacteria.

3.6. *Magnesium content*

As in the case of potassium, the effects of fertilization and of the use

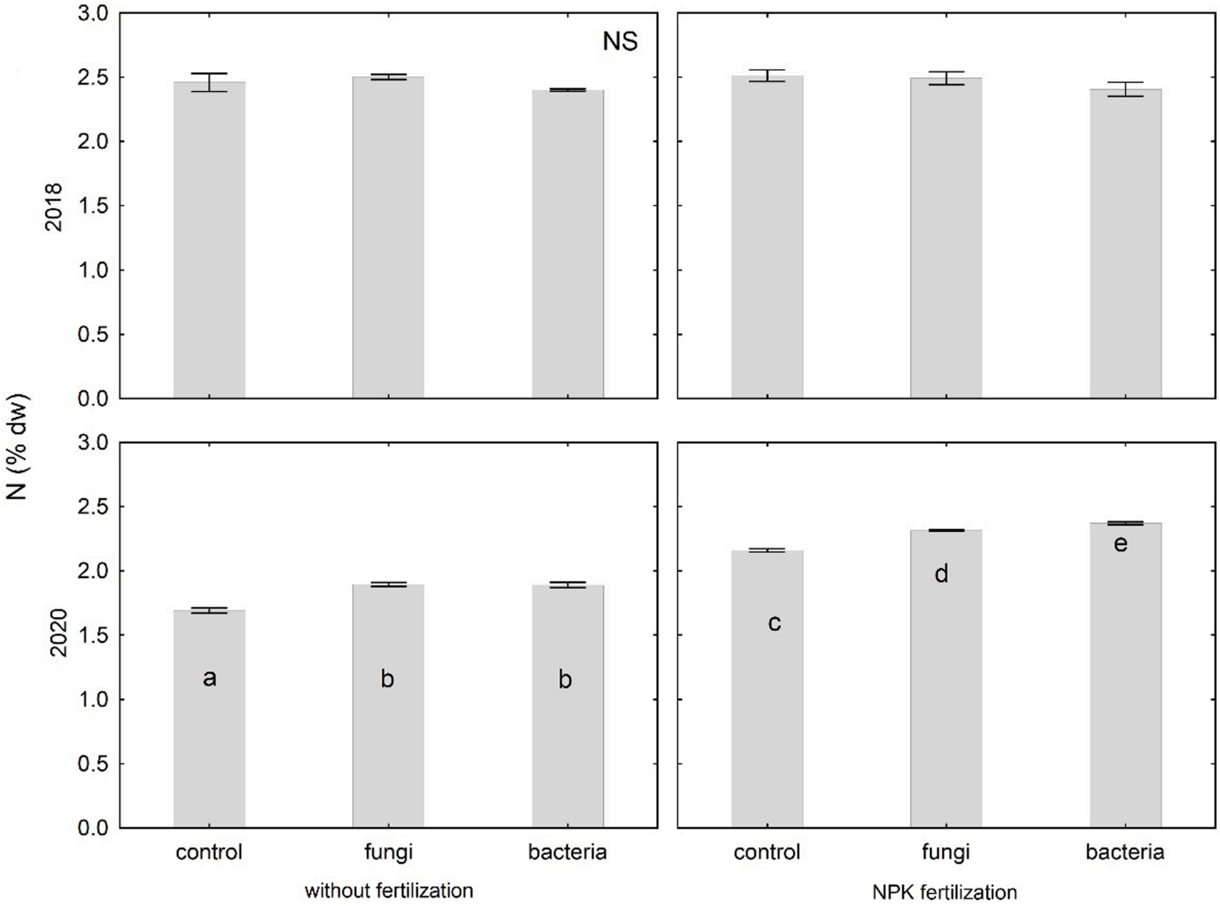

Fig. 2. Effect of the use of beneficial microorganisms and mineral fertilization on the nitrogen (N) leaf content. Statistical analyses are presented separately for each year. Means marked with the same letters do not differ significantly at α = 0.05, ns – differences not significant.

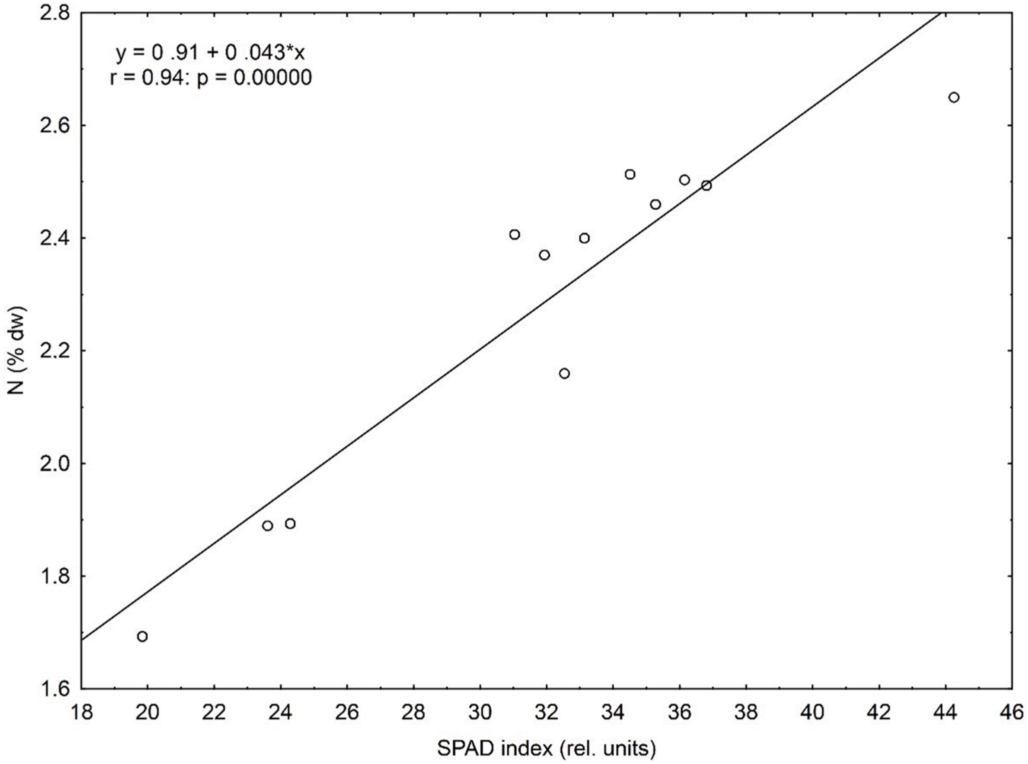

Fig. 3. Correlation between the values of the SPAD index and the concentration of nitrogen in apple leaves.

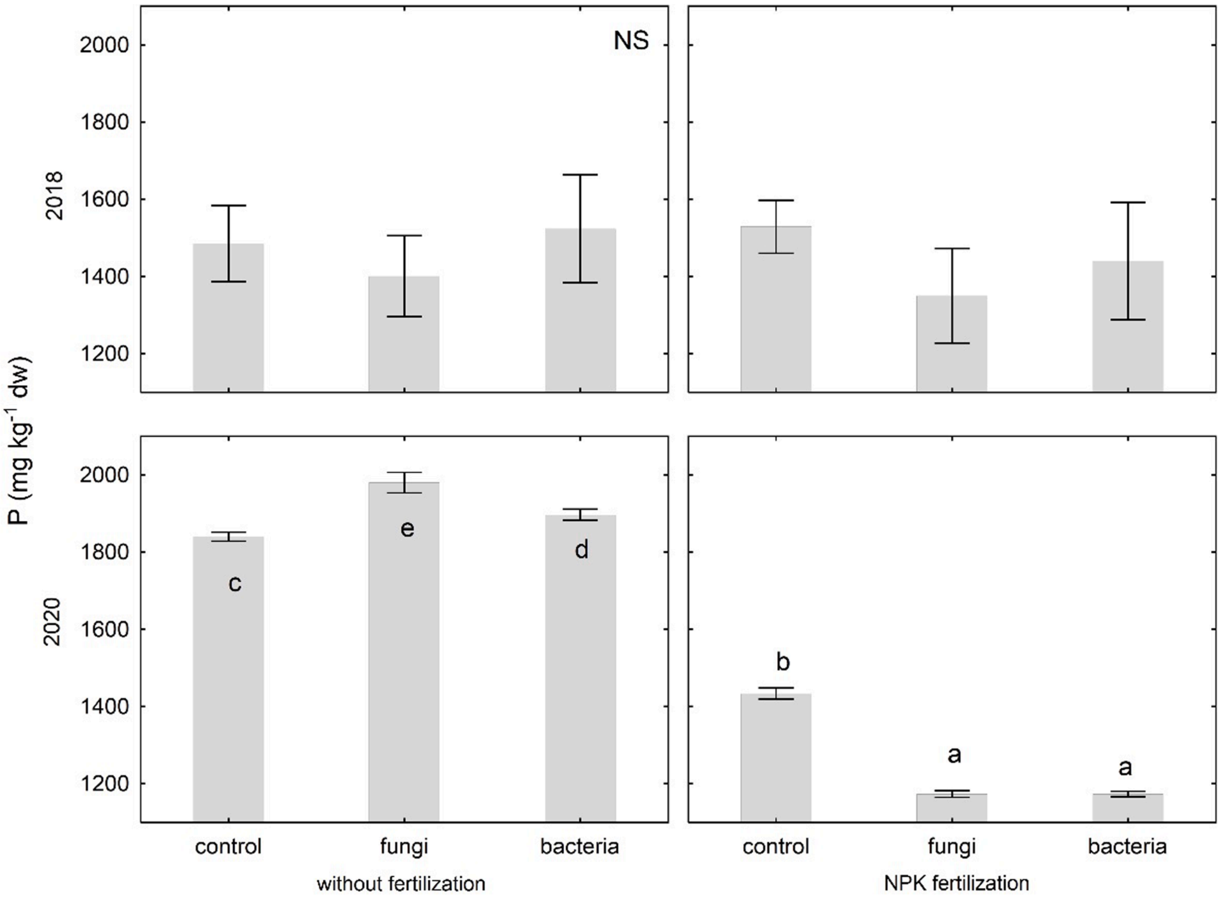

Fig. 4. Effect of the use of beneficial microorganisms and mineral fertilization on the phosphorus (P) leaf content. Statistical analyses are presented separately for each year. Means marked with the same letters do not differ significantly at α = 0.05, ns – differences not significant.

of microorganisms on the concentration of magnesium in apple leaves were demonstrated already in the first year of the study (Table 2). The analyses performed in the first growing season showed significantly the highest levels of magnesium in the leaves of the non-fertilized control trees growing on the plots where the soil had been inoculated with bacteria. In the case of fertilized plots, the opposite situation was evident. The trees fertilized and inoculated with the bacterial medium had significantly the lowest magnesium content in the leaves. A similar level of magnesium in apple leaves was found in the third year of cultivation, but in that growing season the highest magnesium concentrations were found in the leaves of NPK-fertilized trees growing on the plots inoculated with microorganisms.

3.7. *Sulphur content*

In the case of leaf sulphur content, the significance of differences between the experimental variants was demonstrated in the third year of cultivation (Table 2). We can see here a marked reduction in the sulphur content of the leaves on the non-fertilized plots. Significantly the lowest sulphur content was found in the leaves of the non-fertilized control trees where no microorganisms were used. The very addition of the fungal or bacterial inocula had a significant impact on increasing the sulphur concentration in apple leaves. The highest sulphur concentration was found in the apple trees under mineral fertilization with the simultaneous application of fungal or bacterial inocula.

3.8. *Microelements*

Apart from macronutrients in leaves, the study also assessed the concentrations of micronutrients such as iron (Fe), boron (B), manganese (Mn), zinc (Zn), and copper (Cu). For all the estimated microelements, significant differences between the analyzed results were found in the third year of the study (Table 3).

Fe

The level of average iron concentrations in leaves in the third growing season was considerably lower than the results obtained in the first season. In 2020, significantly the highest iron content was found in the leaves of the trees under mineral fertilization and growing on the plots inoculated with bacteria. In the absence of fertilization, the applied fungal inoculum significantly increased the concentration of this component in the leaves.

B

In general, the level of boron in apple leaves did not change with the age of the trees. In the third year of cultivation, significantly the highest boron concentration was found in the leaves of the control trees. In the case of both non-fertilized and NPK-fertilized trees, significantly lower boron levels were found in the leaves of trees growing on the plots where the soil was inoculated with microorganisms.

Mn

In the third year of cultivation, significantly the lowest manganese content was found in the leaves of the control trees. Both in the absence of fertilization and under mineral fertilization, the addition of the fungal or bacterial inocula had a significant effect on increasing the manganese concentration in apple leaves. The leaves of the trees under mineral fertilization with simultaneous application of fungal inoculum had the highest manganese content.

Zn

The concentrations of zinc in apple leaves in the third growing season were higher than the results obtained in the first season. In 2020, the leaves of non-fertilized trees growing on the plots inoculated with fungi

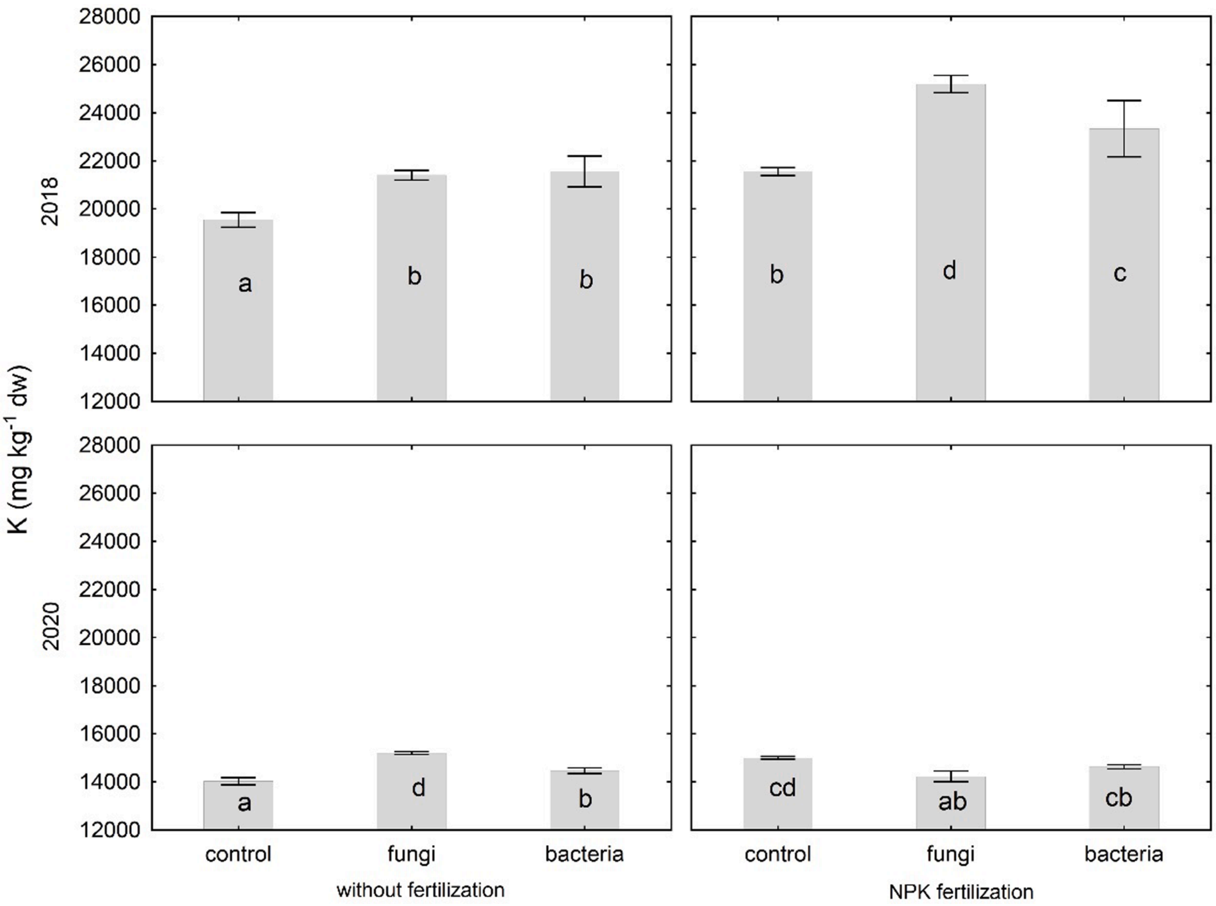

Fig. 5. Effect of the use of beneficial microorganisms and mineral fertilization on the potassium (K) leaf content. Statistical analyses are presented separately for each year. Means marked with the same letters do not differ significantly at α = 0.05.

Table 2
Effect of the use of beneficial microorganisms and mineral fertilization on the calcium (Ca), magnesium (Mg), and sulphur (S-SO_4) content (mg kg^{-1} dw) of apple leaves.

Macroelement	Fertilization	Year	Microorganisms		
			None	Fungi	Bacteria
Ca	Control	2018	9319 a	8858 a	7790 a
	NPK		9704 a	8858 a	9138 a
	Control	2020	15,000 a	17,500 c	16,556 b
	NPK		17,767 c	18,867 d	19,767 d
Mg	Control	2018	2398 bc	2641 cd	2757 d
	NPK		2297 b	2360 b	2004 a
	Control	2020	2500 c	2520 c	2410 b
	NPK		2337 a	2590 d	2563 cd
S-SO_4	Control	2018	1607 a	1621a	1603 a
	NPK		1624 a	1561 a	1554 a
	Control	2020	1182 a	1327 b	1340 b
	NPK		1446 c	1576 d	1619 d

Statistical analyses are presented separately for each year. Means marked with the same letters do not differ significantly at α = 0.05.

Table 3
Effect of the use of beneficial microorganisms and mineral fertilization on the iron, boron, manganese, zinc, and copper content (mg kg^{-1} dw) of apple leaves.

Microelement	Fertilization	Year	Microorganisms		
			None	Fungi	Bacteria
Fe	Control	2018	78.9 a	79.4 a	79.3 a
	NPK		82.8 a	75.8 a	75.8 a
	Control	2020	52.5 a	61.0 b	54.1 a
	NPK		60.9 b	59.8 b	66.8 c
B	Control	2018	33.9 a	27.7 a	24.9 a
	NPK		31.7 a	22.7 a	24.7 a
	Control	2020	41.7 d	33.8 c	30.2 b
	NPK		33.5 c	28.2 ab	26.4 a
Mn	Control	2018	75.8 a	81.1 a	66.8 a
	NPK		87.1 a	70.0 a	81.5 a
	Control	2020	16.5 a	18.3 b	23.1 d
	NPK		20.6 c	57.0 f	33.7 e
Zn	Control	2018	24.0 a	22.5 a	26.6 a
	NPK		26.8 a	21.9 a	22.6 a
	Control	2020	29.1 b	37.6 f	34.5 e
	NPK		27.0 a	32.4 d	30.0 c
Cu	Control	2018	7.5 a	7.2 a	7.7 a
	NPK		7.3 a	7.3 a	6.7 a
	Control	2020	5.9 a	6.6 c	6.3 b
	NPK		6.3 b	6.9 d	6.8 d

Statistical analyses are presented separately for each year. Means marked with the same letters do not differ significantly at α = 0.05.

had significantly the highest zinc content. Both in the absence of fertilization and under mineral fertilization, the addition of the fungal or bacterial inocula had a significant effect on increasing the concentration of zinc in apple leaves.

Cu

In 2020, significantly the highest copper content was found in the leaves of NPK-fertilized trees growing on the plots where the soil had been inoculated with fungi or bacteria. Both in the absence of fertilization and under mineral fertilization, the addition of the fungal or bacterial inocula had a significant effect on increasing the concentration of copper in apple leaves.

3.9. Correlations

The statistical analysis showed a number of highly significant correlations between the concentrations of minerals in apple leaves (Table 4). The nitrogen content of apple leaves was highly positively correlated ($r \geq 0.7$) with the concentrations of potassium, sulphur, iron, and manganese. Phosphorus showed a highly negative correlation with nitrogen ($r \leq -0.7$). A high negative correlation ($r = -0.91$) was demonstrated between the concentrations of potassium and calcium, as well as calcium and iron. The leaf magnesium content did not correlate significantly with any of the other analyzed macro- or microelements. No high correlation coefficients between the analyzed components were found for copper, zinc, and boron.

3.10. DOP index

The data in Table 5 shows a relative deviation from the optimum leaf macronutrient content in the treatments. In both years of leaf analyses, the DOP$_K$ (i.e. for potassium) was positive for all the treatments. In the case of nitrogen, a positive DOP$_N$ for all the variants of the experiment was obtained only in the first year of the study. In the third year, positive (optimal) DOP$_N$ values were obtained only for the fertilized trees growing on the plots inoculated with the fungal or bacterial inocula. The DOP$_{P;Mg}$ were negative for all the combinations in both test years.

Table 5
DOP index determined from apple leaf macronutrient content after different treatments with fertilizers and microorganisms.

Element	Fertilization		Microorganisms		
		Year	None	Fungi	Bacteria
N	Control	2018	6.96	8.70	4.35
	NPK		9.13	8.26	4.78
	Control	2020	-26.52	-17.83	-17.83
	NPK		-6.09	0.43	3.04
P	Control	2018	-29.29	-33.29	-27.48
	NPK		-27.19	-35.71	-31.43
	Control	2020	-12.38	-5.71	-9.67
	NPK		-31.76	-44.14	-44.14
K	Control	2018	56.34	71.22	72.48
	NPK		72.42	101.54	86.66
	Control	2020	12.26	21.60	15.74
	NPK		20.00	13.86	17.06
Mg	Control	2018	-11.19	-2.19	2.11
	NPK		-14.93	-12.59	-25.78
	Control	2020	-7.41	-6.67	-10.74
	NPK		-13.44	-4.07	-5.07

4. Discussion

Significant differences in most of the analyzed parameters became apparent only in the third year of the study. This is perfectly normal when research is done with woody plants grown in a nutrient-rich soil. During the first stage of growth, a tree can use the reserve compounds stored in the wood (reutilization) (Cheng et al., 2004). The length of the apple tree's response to lack of fertilization depends on the fertility of the soil, the weather pattern, the strength of tree growth and the yield (Treder, 2003). The soil analysis carried out prior to establishing the experiment showed high concentrations of N, P, K, Mg, Ca, and optimal pH. However, the humus content was very low, only 1.2%. The very low organic matter content of the soil was not favorable for natural multiplication of microorganisms.

Evidence of the influence of mineral fertilization and the microorganisms applied to the soil on the increase in the concentration of most of the analyzed macro- and microelements was observed in the third year of cultivation. The lack of mineral fertilization caused a significant decrease in nitrogen levels in apple leaves, which directly resulted in their lighter color (lower SPAD index). Similar relationships had been previously demonstrated by Treder and Cieśliński (2003). The authors recommend the use of the SPAD-502 m for quick and non-invasive assessment of plant nutrition with nitrogen (although it is necessary to establish the relationship between these parameters). In the third year of cultivation, the estimated DOP$_N$ values for non-fertilized trees were negative.

The complete lack of fertilization caused the nitrogen content to drop to a deficit level (below 1.8% according to the reference values recommended for apple by Sadowski et al. 1990). The application of microorganisms to the soil resulted in a significant increase in the nitrogen content of leaves of the non-fertilized trees (the N content was above 1.8% - a threshold value of the low range proposed by Sadowski et al. (1990). This beneficial effect might be of big importance for the management and optimization of plant nutrition with this element. What is very important is the fact that the microorganisms also had a significant impact on the increase in nitrogen content in relation not only to the control trees but also those fertilized with NPK. Significantly the highest nitrogen content was found in the leaves of NPK-fertilized trees growing in the soil inoculated with beneficial bacteria. Sas-Paszt et al. (2019b), too, while conducting research on strawberry, had found that an NPK fertilizer with bacteria significantly increased the nitrogen (N) level in the leaves. In 2018, the DOP$_N$ values for the NPK-fertilized trees growing in the soil inoculated with microorganisms were positive, close to zero, which proves the optimal nitrogen content.

Our research has also shown a positive effect of microorganisms (when using mineral fertilization) on the nutrition of apple trees with calcium, magnesium, and sulphur. These results are especially important when we relate them to the growth and yielding of trees, which were also the highest for these variants of the experiment (article in preparation). The influence of microorganisms on the nitrogen nutrition of apple trees was so strong that even with relatively vigorous tree growth there was no "dilution" effect, described by Tagliavini et al. (1992) as a tendency to lower the concentrations of N, Ca, and Mg in leaves due to the more vigorous tree growth. According to Olszewski (2001), the potassium content decreases with the age of trees, which was also confirmed by our study. In the first year of cultivation, the leaf

Table 4
Correlations between the concentrations of individual components in apple leaves ($n = 36$).

	P	K	Ca	Mg	S	Fe	Mn	Cu	Zn	B
N	-0.72	0.70	-0.55	-0.09	0.86	0.82	0.80	0.57	-0.65	-0.46
	P	-0.21	-0.01	-0.06	-0.60	-0.34	-0.49	-0.07	0.52	0.18
		K	-0.91	-0.26	0.51	0.77	0.74	0.53	-0.68	-0.49
			Ca	0.18	-0.40	-0.75	-0.68	-0.57	0.63	0.33
				Mg	-0.05	-0.11	-0.18	0.16	0.16	0.13
					S	0.75	0.75	0.65	-0.28	-0.64
						Fe	0.78	0.67	-0.56	-0.36
							Mn	0.47	-0.53	-0.37
								Cu	-0.14	-0.59
									Zn	-0.01

potassium concentration reached a very high level (according to the reference values for apple trees described by Sadowski et al. 1990). In the case of both non-fertilized and fertilized trees, the highest potassium content was found in the trees growing on the plots where the inocula with fungi or bacteria were applied. In the third year of cultivation, the potassium concentration in the leaves was much lower, but it was still within an optimal range (1–1.5%) recommended for apple by Sadowski et al. (1990). No beneficial effect of microorganism application on the potassium nutrition of the trees (grown under mineral fertilization) was found here.

The phosphorus content in the leaves was inversely correlated with the nitrogen content. The lowest phosphorus content was found in the leaves of the trees well-nourished with nitrogen, whose soil had been treated with the microbial inocula. For both the trees (fertilized) growing on control and microbiologically enriched plots, the leaf phosphorus concentration was within the low range (< 0.15%, according to Sadowski et al. 1990). The decrease of P content observed after the application of microbial inocula might negatively affect tree vigor and this effect should be taken into consideration during optimization of fertilization strategy for high yields and high nutrient use efficiencies.

Treder (2003) had demonstrated the phenomenon of a marked increase in phosphorus content with a decrease in nitrogen content in non-fertilized apple trees. Bojic et al. (1985), too, had shown a decrease in the phosphorus content of apple leaves with an increase in nitrogen fertilization rates. In the case of the non-fertilized trees, a significant increase in the phosphorus content in a leaf tissue, after application of microbial inocula was observed. However, for both control and inoculated plants the recorded concentration of this element was within the optimal range (0.15–0.26%) described by Sadowski et al. (1990).

The influence of the applied factors on the concentration of microelements in apple leaves was demonstrated only in the third year of the study. The concentration of boron, which is a trace element essential for normal growth and development of apple trees was within the optimal level (Wójcik et al., 2008; Wójcik, 2009). The highest concentrations of this element were found in the leaves of the trees growing on the control plots. The boron content of apple leaves was inversely correlated with the nitrogen content.

Enriching the soil with the microbial inocula significantly increased the concentrations of manganese (Mn), zinc (Zn), and copper (Cu) in apple leaves. Addition of the fungal substrate (fertilized plots) resulted in an increase of content of Mn to the optimal level (within the range of 41–100 mg kg^{-1} proposed by Sadowski et al. 1990). In a study with strawberry, Sas-Paszt al. (2019b) had shown similar relationships only for zinc (Zn). For the other microelements (Fe, Zn, Cu), the recorded concentrations were within the optimal or even high range (according to Wójcik 2009). In the case of iron, an increase in the content of this element was confirmed only for the fungal inocula used on the control plots without fertilization and the bacterial inocula where mineral fertilization was applied.

5. Conclusions

The presence of filamentous fungi and bacterial strains had a modifying effect on the mineral composition of apple leaves.

Significant variation in most of the analyzed parameters became evident in the third year after tree planting. At that time, an increase in the concentration of most macro- and microelements was observed as a result of the influence of mineral fertilization and the microorganisms applied to the soil.

The intensity of the green color of the leaf is a sensitive indicator of changes in nitrogen content. The lack of mineral fertilization caused a significant decrease in the nitrogen content of apple leaves, which directly resulted in their lighter color (lower SPAD index).

CRediT authorship contribution statement

Waldemar Treder: Conceptualization, Methodology, Writing – original draft, Supervision. **Krzysztof Klamkowski:** Methodology, Investigation, Writing – original draft, Writing – review & editing. **Katarzyna Wójcik:** Investigation, Formal analysis, Writing – original draft. **Anna Tryngiel-Gać:** Investigation, Formal analysis, Writing – review & editing. **Lidia Sas-Paszt:** Conceptualization, Methodology. **Augustyn Mika:** Investigation. **Waldemar Kowalczyk:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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