Theses for doctoral dissertation (PhD)

EXAMINATION OF THE EFFECTS OF NITROGEN STABILIZER AND FOLIAR FERTILIZER TREATMENTS IN MAIZE (*ZEA MAYS* L.)

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1. INTRODUCTION AND OBJECTIVES OF THE DOCTORAL THESIS

Among the studies aimed at improving the general condition of field crops due to their increased exposure to extreme weather conditions, research on nutrient supply and increasing the utilization of applied nutrients is of great importance, as the proper condition of plants can be ensured and improved primarily by optimal and satisfactory nutrient supply (Kundu et al. 2022). The role of N fertilizers is now fundamental and their use in crop production has long been a regular agrotechnical practice in crop production around the world, but numerous studies have pointed out that the utilization of N fertilizers is not satisfactory due to various losses of N (Omara et al, 2019, Dimpka et al, 2020). A significant part of N losses is a consequence of leaching of nitrate ions (Zhang et al., 2017), which, in addition to occasional sudden large N losses in soil, leads to environmental and human health problems through groundwater contamination (Dimpka et al., 2020). Efforts to reduce N losses are therefore an increasingly urgent task for agriculture. Nitrification inhibitors promise to address the problem of N loss; they delay the conversion of ammonium ions to nitrite and then to nitrate ions by slowing down the nitrification of ammonia-oxidizing bacteria, thus reducing the leaching of nitrate ions to groundwater and increasing the efficiency of N fertilization, (Zhong-Qing et al, 2020). Among the nitrification inhibitors, nitrapyrin has been the most widely used active ingredient in America and China over the past 40 years (Keerthisinghe et al., 1993), but has only recently become available in Hungary, therefore the amount of experience, literature and research on its use is limited.

In addition to increasing the efficiency of N fertilisation, foliar fertilisation is gaining ground as a complementary nutrient supply technology to improve plant condition, initially applied in horticultural crops to treat deficiency diseases (Fernández and Eichert, 2009), However, due to the increasing demand for improved yields, the extremes of hydro-meteorological factors, heat stress, unfavourable soil conditions and low micronutrient content, its use is nowadays also widespread in arable crops to maintain and improve general condition (Brankov et al., 2020).

Based on the expected climatic trends, the greatest difficulty in crop production due to global climate change is caused by the poor nutrient supply capacity of soils and the significant leaching of mobile nutrients, resulting in deficiency diseases and reduced plant condition (Nagy et al, 2011). Thus, the study of the effects of foliar fertilizer treatments that enhance N utilization and other nutrients is a major basis of agricultural research. Due to its versatility, maize cultivation is of major economic importance not only in Hungary but also worldwide (Ranum et al., 2014), and the results of maize studies are therefore highly exploitable. For my research, I aimed to study the effects of nitrapyrin and foliar fertilizer treatments on maize physiology and quantitative and qualitative parameters of the yield. Since no comprehensive study has been carried out to present the results of the domestic application of nitrapyrin and since there is a growing justification for the inclusion of foliar fertilizer treatments in general agricultural practice in the future, my research will provide a better understanding of the potential of these future strategies.

My research objectives are to investigate the use and plant health effects of the nitrification inhibitor nitrapyrin in domestic fields, to determine the optimal timing of nitrapyrin treatment, and to study the effects of nitrapyrin and foliar fertilizer treatments in combination through physiological characterization of maize specific pathogen resistance, abiotic stress responses in maize, and general maize health-related parameters.

2. MATERIAL AND METHOD

Our field experiments were set up in parallel at multiple locations. The exact details of the experimental sites are shown in *Table 1.* The classification of the field experiments according to the objective of the related studies is shown in *Table 2.*

Table 1: Field trial sites

Experiment		
AG-19	Year:	2019
	Location:	Agrárgazdaság Ltd. (Debrecen-Józsa)
HD-19	Year:	2019
	Location:	Hajdúhadházi Bocskai Mg. Ltd. (Bocskaikert)
BK-20	Year:	2020
	Location:	University of Debrecen MÉK Demonstration Garden (Debrecen)
KF-20	Year:	2020
	Location:	Kurucz Farm Ltd (Ebes)
AG-21	Year:	2021
	Location:	Agrárgazdaság Ltd. (Debrecen)
BK-21	Year:	2021
	Location:	University of Debrecen MÉK Demonstration Garden (Debrecen)
KF-21	Year:	2021
	Location:	Kurucz Farm Ltd (Debrecen)

The codes, consisting of letters and numbers, represent the abbreviation of the name of the company that owns the site (letters) and the abbreviation of the year of the experiment setup (numbers).

Table 2: Classification of field trials according to the objective of the trials

Objectives	Experiments
1. Study of the combined effect of nitrapyrin and foliar fertilizer treatments	AG-19, BK-20, KF-20, AG-21, BK-21, KF-21
- Testing the resistance to specific pathogens	BK-20
- Physiological characterisation of abiotic stress responses	BK-21
2. Determination of the optimal timing of nitrapyrin treatment	BK-20, BK-21
3. Testing the effectiveness of nitrapyrin on sandy soils	HD-19

2.1. The used nitrapyrin and its application

The nitrogen stabiliser formulation used in our experiments (nitrapyrin, N Lock, Corteva Agriscience, Wilmington, USA) was applied at the manufacturer's recommended dose (1.7 ha $^{-1}$; 300 ha^{-1} water volume). The material sprayed before sowing was also applied immediately into the 6-8 cm layer of soil, while the post-sowing treatment was timed based on weather forecasts, i.e. rainfall was expected after spraying. In the BEMK-20 and BEMK-21 experiments, nitrapyrin treatments were also applied post-sowing, with 45 mm of rainfall within two weeks of application in 2020 (OMSZ, 2020), while in 2021, 4 mm of rainfall within a few hours and an additional 12 mm within two weeks helped the active ingredient to penetrate the soil (OMSZ, 2021).

2.2. The used foliar fertilizer and its application

The nutrient composition of the foliar fertilizer used in the experiments (Zsendítő, Hed-Land Hungária Ltd.) *is* shown in *Table 3.* The treatments were applied in the morning and midday, in cloudy weather, free from strong sunlight, and only in maize with sufficient foliage (minimum 8 leaves at maturity) to ensure efficient absorption of the nutrients applied. The treatments were applied at the doses and amounts recommended by the manufacturer.

Table 3: Foliar fertiliser preparations used in the experiments

Nutrients	**Foliar fertilizer (g ha^{-1})**
Total nitrogen (N) [a]	800
Phosphorus pentoxide (P_2O_5)	700
Potassium oxide (K_2O)	700
Sulphur trioxide (SO_3)	700
Boron (B) [b]	1.27
Copper (Cu) [b]	1.62
Iron (Fe) [b]	2.84
Manganese (Mn) [b]	2.65
Zinc (Zn) [b]	0.38

[a] -urea nitrogen; [b] - EDTA metal ion-chelate complex

2.3. Determination of soil nitrate content

The effectiveness of the nitrapyrin treatment was monitored by determining the nitrate content of treated and untreated soil at 0-30 cm depth, which can be compared to provide indirect information on nitrification activity. Soil samples were taken randomly, following a "W" shape, taking into account the heterogeneity of the site. Soil sampling was carried out every 2-3 weeks to adapt to weather conditions. Soil samples were taken from the areas not treated with nitrapyrin (Kt: control, Lt: treated with foliar fertilizer) and from the areas treated with nitrapyrin (Np: treated with nitrapyrin, Np + Lt: treated with nitrapyrin and foliar fertilizer). This can be explained by the fact that foliar fertiliser treatment alone does not significantly influence the change in soil nitrate content, as the amount of liquid that drips off the leaf surface and that reaches the soil surface directly and leaches into the soil is negligible. The nitrate content was determined using a selective nitrate electrode (Nitrat 2000, Stelzner, Germany) following the steps recommended by the manufacturer.

2.4. Determination of relative chlorophyll content

In all cases, the relative chlorophyll content of maize leaves was determined at least one week after foliar fertilization to ensure that the applied nutrients were utilized by the time of measurement. The measurements were made on the mature leaves of maize near the ear and were based on an average of 5 measurements per leaf (20 plants per treatment). A SPAD-502 Plus device (Konica Minolta, Japan) was used for the measurements.

2.5. Determination of root mass and stem thickness

Since the foliar fertilizer treatment does not affect the root growth of the plants, root samples were taken from the untreated (Kt, Lt) and nitrapyrin-treated (Np, Np+Lt) stocks. The bias due to different root sizes was based on the root weight of randomly selected 10 plants per treatment, 30 cm^3 from the soil surface per unit soil volume. After sampling, roots were cleaned of soil residues and dried to constant weight in a drying oven at 60 °C and the weight of the resulting clean and dry root samples was measured. Stem thickness was also determined as an indicator of biomass gain using a caliper. Stem diameter of plants was measured in the same way as for root samples and nitrate content determination in the untreated (Kt, Lt) and treated (Np, Np + Lt) stands. Measurements were taken uniformly from the diameter of the lower three stems above the soil surface and averaged over the three stems (20 plants per treatment, randomly selected).

2.7. Determination of quantitative and qualitative parameters of the yield

Quantitative analyses of the yield included the determination of the kernel weight per thousand kernels, the length and diameter of the maize ear after removal of the ear leaves (20 plants per treatment, randomly selected). The diameter of the maize ear was determined at half the length of the maize ear measured with a tape measure using a caliper.

For the determination of the nutrient parameters of the yield, a homogeneous mixture of 500 g of grain from 20 ears of maize per treatment was tested in one measurement. The grain protein, oil and starch contents were measured using the Infratech 1241 Grain Analyzer (FOSS, Hilleroed, Denmark).

2.8 Artificial infection of maize

To test the resistance of maize to pathogens, artificial infection of maize ears with pure cultures of *Aspergillus flavus* NVK 20 (NCAIM F.00048) and *Fusarium verticillioides* MRC 826 (NCAIM F.00935) was performed. To prepare inocula, strains were inoculated on potato dextrose agar (PDA) medium (Beever and Bollard, 1970; Merck Millipore, Germany) and after inoculation, cultures were incubated for 7 days at 25 °C (*F. verticillioides*) or 37 °C (*A. flavus*) (CLN 240 laboratory incubator, Pol-Eko-Apparatura, Poland). Conidia from freshly cultured colonies were resuspended in sterile water and filtered through sterile Miracloth tissue (Merck Millipore, Germany). Cell counts of spore suspensions were determined using a haematocytometer (Bürker chamber). *F. verticillioides*, the pathogen causing ear blight, was used for infection of the ears on 31 July 2020, at the time of maize tasselling, while *A. flavus was* used on 18 August 2020, during the period of intensive insect damage. Infestations were carried out only on healthy, symptom-free ears. For both pathogens, 1 ml of spore suspension (5×10^6 spore count) was injected through the tip of the ear using a sterile syringe (Chirana T. Injecta, Slovakia). Since both pathogens pose a potential risk to human health and the environment, artificial infection was performed with extreme care and caution, wearing a well-fitting N95 face mask, goggles, protective clothing and gloves. Sterile needles were used for both infections. The infected maize ears were then covered with a waterproof plastic bag for 7 days, creating a humid microclimate around the ear that is favourable for fungal infection. After the 7th day, the plastic bags were replaced with paper bags, which were removed only at the time of maize harvest on 15 September 2020 (Zuber et al., 1978). The extent of infection was assessed as a percentage (%) of each treatment, determined by the number of grains on the maize ear showing visible signs of infection.

2.9 Physiological examination of leaf tissue

For physiological studies of leaf tissues, young fully developed maize leaves were used. For the determination of the nutrient content of leaf tissues, leaf samples were taken after foliar fertilizer treatment, while for the determination of the other parameters, leaf samples were taken at the R1 (flowering initiation) phenophase of maize. During sampling, fresh tissue samples were immediately frozen using liquid nitrogen and stored at -70 °C until further use.

Leaf sampling for ***element content*** determinations was always carried out 1-1.5 weeks after foliar fertilizer treatments to ensure that the applied nutrients were utilised by the time of sampling. Sampling was done from the whole leaf blade. Determination of the element content of leaf samples was performed by the HL-LAB Environmental and Soil Testing Laboratory (Debrecen).

The chlorophyll a, chlorophyll b and carotenoid contents of the leaf samples were quantified according to the procedure of Moran and Porath (1980). For the measurements, fresh tissue samples from fully developed young maize leaves were stored in liquid nitrogen until processing, and then 50 mg of the samples were dissolved in 5 ml of N,N-dimethylformamide (4°C, 72 h). The absorbance of the resulting extracts was determined spectrophotometrically at 470 nm, 647 nm and 664 nm (Nicolet Evolution 300 UV-vis spectrometer; Thermo Fisher Scientific, Waltham, MA, USA) The results were analysed following the methodology of Wellburn (1990).

Superoxide dismutase (SOD) activity was determined by adapting the methods of Giannopolitis and Ries (1977) and Beyer and Fridovich (1987). 400 mg sections of tissue samples were homogenized in 4 ml $NaPO_4$ buffer (50 mM, pH 7.8), which also contained 1 mM phenylmethanesulphonyl fluoride (PMSF), 0.1 mM EDTA and 1 *w/v%* polyvinylpyrrolidone (PVP). After centrifugation (10,000 *g*, 4 °C, 15 min), the SOD activity of the supernatant extracts was monitored spectrophotometrically by oxidation of 70 µM nitroblue tetrazolium (NBT) at a wavelength of 560 nm (Nicolet Evolution 300 UV-Vis Spectrometer).

For the determination of ***proline,*** tissue samples were crumbled in liquid nitrogen and their 300 mg weight fractions were homogenized in 600 µl ethanol (70 *v/v%*) (Hummel et al., 2010). After centrifugation (10000 *g*, 4 °C, 15 min), 500 µl of the supernatant was mixed with 500 µl of ninhydrin solution (1 *w/v%*; 60 *w/v%* in acetic acid) and 500 µl of 20 *v/v%* ethanol. The reaction mixtures were incubated at 95 °C (20 min) and the absorbance values were detected after centrifugation (12000 *g*, 4 °C, 1 min) at 520 nm (Nicolet Evolution 300 UV-Vis spectrometer). The proline contents were determined using a standard curve (Carillo and Gibon 2011).

For the determination of ***malondialdehyde,*** tissue samples were crumbled in liquid nitrogen and homogenised in 100 mg of 100 mg by weight in a 1 ml volume of 0.25 *w/v %* thiobarbituric acid (TBA) and 10 *w/v* % trichloroacetic acid (TCA). After centrifugation (10 000 *g*, 4 °C, 15 min), 800 µl of reagent was added to 200 µl of supernatant, composed of 0.5 *w/v* % TBA and 20 *w/v* % TCA. The reaction mixtures were transferred to a shaker incubator (Thermo-Shaker, Bioshan TS-100) (95 °C, 30 min) and subsequently cooled on an ice bed. For the determination of MDA content, the absorbance of the samples was detected at 532 nm and 600 nm (Heath and Packer 1968).

2.10. Description of the soil incubation experiment

The effectiveness of nitrapyrin treatment was also monitored under laboratory conditions through changes in nitrate content in soil samples at different temperatures. To eliminate bias due to differences in soil properties, soil samples were taken from the same location in the DE MoE Demonstration Garden, 200 g of soil were placed in 300 ml plastic containers, to which 12 mg of ammonium sulphate was added to activate nitrification activity, followed by 1 ml of nitrapyrin solution (300 mg ml^{-1}) to a portion of the soil samples (Powell and Prosser, 1986). The samples were then incubated at 10, 15, 20 and 25 °C for 3 weeks (CLN 240 laboratory incubator, Pol-Eko-Apparatura, Poland), trying to maintain the moisture content at 55-65%. Soil nitrate content was determined once a week (3 pots per treatment) using a nitrate ion-selective electrode (Nitrat 2000, Stelzner, Germany) according to the steps recommended by the manufacturer.

2.11. Statistical studies

Statistical analyses were carried out using the R programming language (*v4.1.1*; R Core Team 2021) and the R package "agricolae" (*v1.3-5*; De Mendiburu 2021). Student's *t-test* and Duncan's multiple range test with one-factor analysis of variance (ANOVA) at 5% significance level ($p < 0.05$) were used to compare sample means.

3. RESULTS

3.1 Results of soil nitrate measurements in the experimental years

To investigate the effectiveness of nitrapyrin, we also monitored changes in soil nitrate content. In all experimental years, the treatment resulted in significant differences in soil nitrate content compared to untreated soil, which is important information because the magnitude and duration of the difference in nitrate content between nitrapyrin-treated and untreated soils may indicate the mechanism of action of nitrapyrin. The lower or higher nitrate levels measured as a result of nitrapyrin treatment may also support the efficacy of nitrapyrin, since the quantitative evolution of nitrate levels as a result of treatment may be influenced by several factors such as nitrifying bacterial activity, rainfall, soil organic matter content, soil physical properties and soil temperature. It is important to stress, however, that soil nitrate levels at the time of sampling are only an indication of the current state of the soil and do not allow a clear conclusion to be drawn about the effectiveness of nitrapyrin, nor do they confirm the beneficial effects of nitrapyrin on plant nitrogen supply, so it is essential to take into account the evolution of other parameters that assess the health of the plants in order to ascertain the beneficial effects of nitrapyrin.

3.4. Results of relative chlorophyll content measurements in the experimental years

The effectiveness of nitrapyrin and foliar fertilizer treatments was also monitored by measuring the relative chlorophyll content of maize leaves, the results of which are shown in Table 9.

Table 7: Changes in relative chlorophyll content as a function of treatments

Year	Experiments	Kt	Np	Lt	Np+Lt	Np^+
2019	AG-19	55.93 ± 5.07^a	61.35 ± 3.52^b	62.62 ± 3.07^{bc}	64.52 ± 3.34^c	-
	HD-19	47.14 ± 11.29^a	72.74 ± 16.51^b	-	-	-
2020	BK-20	42.19 ± 3.35^a	48.19 ± 3.78^b	41.79 ± 4.31^a	52.63 ± 5.40^b	53.72 ± 3.28^c
	KF-20	61.91 ± 3.66^a	63.41 ± 4.17^a	62.71 ± 2.63^a	66.97 ± 4.98^b	-
2021	AG-21	47.21 ± 4.35^a	51.03 ± 3.96^b	46.91 ± 4.94^a	48.75 ± 2.13^{ab}	-
	BK-21	41.38 ± 3.71^a	49.59 ± 3.81^b	39.81 ± 3.85^a	48.69 ± 3.98^b	45.99 ± 5.05^{ab}
	KF-21	47.97 ± 3.63^a	51.07 ± 4.29^b	49.89 ± 4.12^{ab}	55.22 ± 3.49^c	-

The table shows the mean and standard deviation of the SPAD values of 20 independent leaf samples per treatment. Different lower case letters (a, b, c) indicate significant difference between treatments in a given experiment (one-factor ANOVA and Duncan's post hoc test; $p < 0.05$). Kt: untreated; Np: nitrapyrin (pre-seeding); Lt: foliar fertilizer; Np + Lt: nitrapyrin (pre-seeding) + foliar fertilizer; Np^+ : nitrapyrin (post-seeding).

Summarizing the results, the relative chlorophyll content of the plants increased in all experiments as a result of the nitrapyrin treatment, suggesting that plants in the treated areas had access to more nitrogen, which is closely correlated with the amount of chlorophyll. In contrast to nitrapyrin treatment, treatment with foliar fertiliser alone did not increase relative chlorophyll content to any significant extent, but the efficacy of nitrapyrin with the addition of foliar fertiliser has already resulted in further significant increases in several cases. This is presumably due to the improved nitrogen supply resulting from the combination of nitrapyrin and the significantly improved nitrogen supply to the plants from the combined treatment with nitrogen-containing foliar fertiliser. In conclusion, the higher relative chlorophyll content in leaf tissue is mainly due to the nitrapyrin treatments, which, when supplemented with foliar fertilizer, resulted in a further significant increase in some cases. This is probably due to the even more favourable N supply conditions for the plant resulting from the combined treatment with N-containing foliar fertiliser.

3.5 Results of root mass and stem thickness measurements in the experimental years

In order to examine the effect of nitrapyrin on maize biomass production, the root mass and stalk thickness of untreated (Kt) and nitrapyrin-treated (Np) stands were also determined, the results of which are shown in Table 10.

Table 8: Changes in root mass and stem thickness due to nitrapyrin treatment

		Root mass (g)			**Stem thickness (mm)**		
Year	**Experiment**	Kt	Np	Np^+	Kt	Np	Np^+
2019	AG-19	20.14 ± 7.64^a	27.91 ± 8.61^b	-	23.71 ± 2.41^a	27.19 ± 2.51^b	-
	HD-19	23.58 ± 6.08^a	31.02 ± 7.18^b	-	19.56 ± 1.40^a	23.18 ± 1.38^b	-
2020	KF-20	33.80 ± 8.53^a	39.10 ± 6.98^a	-	25.99 ± 2.22^a	27.76 ± 1.18^b	-
	BK-20	14.72 ± 5.63^a	28.06 ± 6.68^b	45.30 ± 8.13^c	17.65 ± 1.39^a	20.67 ± 1.18^b	23.27 ± 1.22^c
2021	AG-21	25.67 ± 3.06^a	32.39 ± 4.53^b	-	19.91 ± 1.30^a	22.47 ± 1.57^b	-
	BK-21	11.85 ± 2.26^a	21.67 ± 2.10^b	18.94 ± 1.83^b	17.87 ± 1.54^a	21.43 ± 1.20^b	21.16 ± 0.63^b
	KF-21	31.80 ± 3.70^a	39.60 ± 2.70^b	-	17.47 ± 2.28^a	23.60 ± 1.24^b	-

The table shows the mean and standard deviation of 20 independent measurements per treatment. Different lower case letters (a, b, c) indicate significant difference between treatments in a given experiment (one-factor ANOVA and Duncan's post hoc test; $p < 0.05$). Kt: untreated; Np: nitrapyrin (pre-seeding); Np^+ : nitrapyrin (post-seeding).

Given that the primary objective of the foliar fertilizer treatment is not to increase plant biomass, the parameters indicating this were only tested to verify the efficacy of nitrapyrin. Our results show that nitrapyrin treatment resulted in increased root mass and stem thickness of plants compared to untreated plants, i.e., nitrapyrin contributed significantly to the long-term provision of available nitrogen forms for uptake by plants.

3.6. Results of measurements of quantitative and qualitative parameters of the crop in the experimental years

When examining ***ear length***, ***ear thickness*** and ***weight per thousand grains,*** significant improvements were observed compared to untreated stands for both nitrapyrin alone and for plants treated with foliar fertilizer alone and a combination of both, but there was no clear difference in the efficacy of these treatments in all cases. It can be said that the positive change in these parameters was mainly due to the nitrapyrin treatment, but the addition of foliar fertilizer to the treatment resulted in further significant increases in certain cases. This can also be explained by the more favourable nitrogen supply conditions in the soil as a result of the nitrapyrin treatment and, through this, the improved nitrogen uptake by the plants. For ***protein***, ***oil*** and ***starch content of the crop,*** only small changes were observed as a result of the treatments, but it is noticeable that the increase in protein content was mainly due to the nitrapyrin-treated stocks, which can similarly be explained by the improved nitrogen supply, an essential mineral for protein formation.

3.7. Results of artificial infection

Changes in tolerance to artificial infestation with specific maize pathogens (*Aspergillus flavus: Af; Fusarium verticillioides*: *Fv*) were also monitored in relation to nitrapyrin (Np), foliar fertilizer (Lt) and combined treatments (Np + Lt). The extent of infection in response to each treatment is illustrated in *Figure* 7.

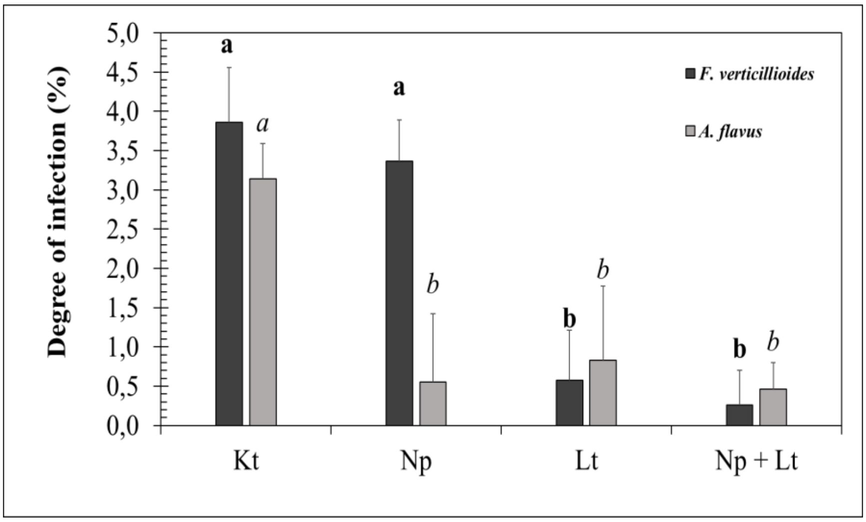

Figure 7: Degree of ear infestation by *A. flavus* and *F. verticillioides* pathogens as a function of treatments.

The figure shows the mean and standard deviation of 10 independent measurements per treatment. Different lower case letters (a, b) indicate significant difference between treatments (one-way ANOVA and Duncan's post hoc test; $p < 0.05$). Kt: untreated; Np: nitrapyrin (pre-sowing); Lt: foliar fertilizer; Np + Lt: nitrapyrin (pre-sowing) + foliar fertilizer.

It was found that following artificial beak infection with the two pathogens, the appearance of visible symptoms was most pronounced in untreated stocks and least in maize treated with the combined treatment. Thus, the highest tolerance to fungal infection of maize was obtained in the most nutrient-rich stands due to nitrapyrin and foliar fertilizer treatments.

3.8. Results on the elemental content of maize leaves in the experimental years

The results of the leaf analysis show that in most experiments, treatments did not significantly increase the amount of nutrients below the critical level, but in the HD-19 experiment, a significant improvement in the nitrogen supply of the stand was achieved with the nitrapyrin treatment. This is also important because the area was sandy soil, which suggests that in loose, organic matter-poor soils, treatments to supplement nutrient supply can be more effective. Our results also show that deficiency diseases were most frequent in 2021, with deficiencies of nitrogen, phosphorus, potassium and sulphur being found at all experimental sites. These results can be explained by the unfavourable hydro-meteorological data of the year, as the intensive period of maize development and nutrient uptake was not accompanied by sufficient rainfall, so that the plants could not use the nutrients in sufficient quantities due to the lack of solvents. The results of the leaf analysis also highlight the importance of latent deficiency diseases, as early detection of deficiency diseases is difficult in the absence of symptoms indicating low levels of most nutrients. In addition to nitrogen, maize is physiologically more sensitive to magnesium and zinc. Our results also show that magnesium and zinc deficiencies were frequent and did not increase significantly as a result of foliar fertilisation. A possible explanation for this is that the composition of the applied foliar fertilizer was general, containing only low amounts of zinc and no magnesium. Consequently, it is not known to what extent zinc and magnesium supply would have increased if a foliar fertiliser with a nutrient composition adapted to the nutrient requirements of maize had been applied. However, our results also support previous research calling for greater attention to magnesium and zinc supply in maize.

3.9 Results on photosynthetic pigment content, superoxide dismutase activity, proline and malondialdehyde content

Leaf physiological studies were performed to determine the chlorophyll-a, chlorophyll-b, carotenoid pigment content, MDA concentration, SOD activity and proline content of leaf tissues in the BK-21 experiment. The results of the tests are summarized in *Table 15.*

Table 15: Results of leaf physiological tests

Parameter	Kt	Np	Lt	Np + Lt
Chlorophyll *a* (mg kg^{-1} raw weight)	18.5 ± 1.0^{a}	19.9 ± 0.7^{b}	19.7 ± 0.3^{b}	21.2 ± 0.2^{c}
Chlorophyll *b* (mg g^{-1} raw weight)	4.8 ± 0.2^{a}	5.4 ± 0.7^{a}	5.2 ± 0.6^{a}	6.7 ± 0.7^{b}
Carotenoid (mg g^{-1} raw weight)	13.2 ± 0.7^{a}	12.8 ± 0.4^{a}	13.9 ± 0.5^{ab}	14.8 ± 0.8^{b}
MDA concentration (nmol g^{-1} raw weight)	154.8 ± 11.2^{b}	121.4 ± 16.6^{a}	113.5 ± 14.6^{a}	105.1 ± 12.2^{a}
SOD activity (U g^{-1} raw weight)	0.2 ± 0.0^{d}	0.2 ± 0.0^{c}	0.1 ± 0.0^{b}	0.1 ± 0.0^{a}
Proline content (μ ml^{-1})	291.0 ± 32.7^{c}	219.8 ± 11.9^{ab}	240.0 ± 14.4^{b}	195.0 ± 20.1^{a}

The table shows the mean and standard deviation of 5 independent measurements per treatment from the BK-21 experiment area. The different lower case letters (a, b, c) indicate significant difference between treatments (one-factor ANOVA and Duncan's post hoc test; $p < 0.05$). Kt: untreated; Np: nitrapyrin (preplanting); Lt: foliar fertilizer; Np + Lt: nitrapyrin (preplanting) + foliar fertilizer.

The highest values of chlorophyll a and chlorophyll b pigments, which are also good indicators of maize health, were found in the stocks treated with nitrapyrin and foliar fertilizer, while the lowest values of SOD, proline and MDA content activated by oxidative stress were also found in the combined treatment plants. These results illustrate the extreme importance of nutrient supply for plant health and stress tolerance.

3.10. Laboratory study of the temperature dependence of nitrapyrin

The efficacy of nitrapyrin was tested both in laboratory and field conditions. The results of the soil incubation experiment are illustrated in *Figure 5,* where the efficacy of nitrapyrin was tested by the change in nitrate content of soil samples at different temperatures. As the experiment was set up without plant cover, the nitrate content of the soil samples was not affected by the plant nutrient cycling due to nitrapyrin treatment, so the change in nitrate content provides information on the dynamics of nitrification activity in the soil.

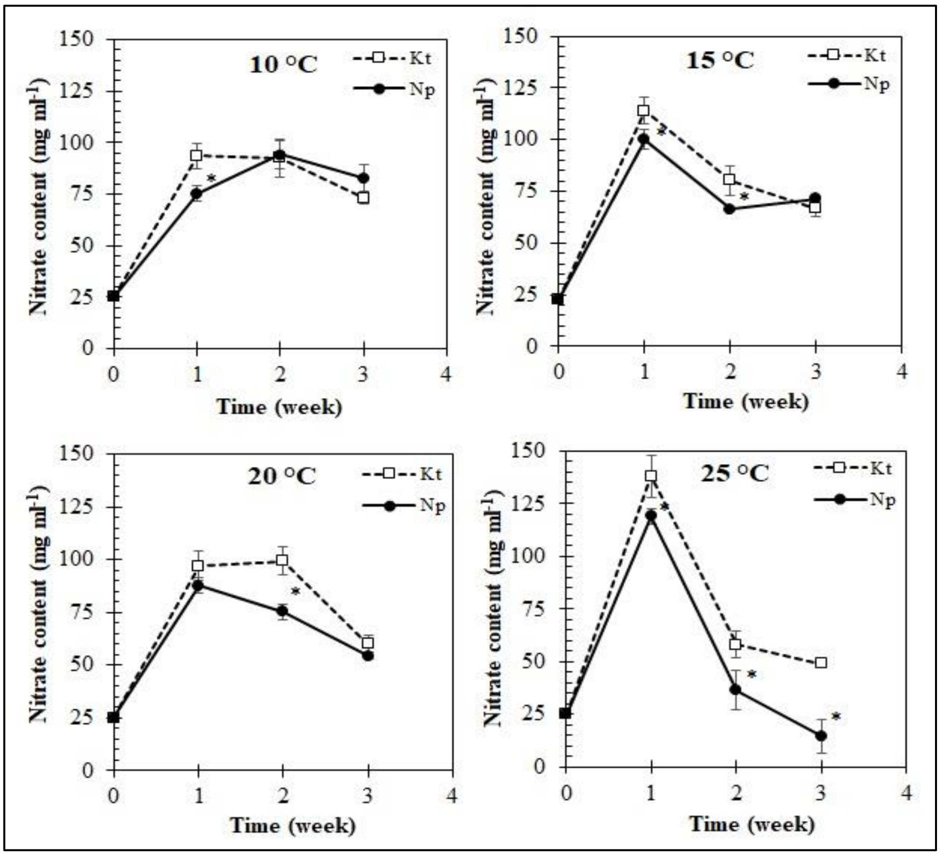

Figure 8: Changes in nitrate content of soils at different temperatures after nitrapyrin treatment

The figure shows the mean and standard deviation of 3 independent measurements per treatment. * - Significant difference compared to untreated soils for nitrate (Student's t-test; $p < 0.05$).

Of the experimental years for optimal timing of nitrapyrin treatment, 2020 was the most favourable, with significantly more rainfall during the critical development period for maize, compared to 2021. After evaluating our results, it can be concluded that a delayed application of nitrapyrin is only justified if higher rainfall is expected in the post-treatment period, which will help to make more efficient use of the higher amounts of nitrogen available from the treatment. Considering that the application of nitrapyrin alone is an additional labour input, in practice it is recommended to apply it in parallel with postemergent weed control of maize.

3.11. Field trial on the timing of nitrapyrin application

In order to determine the optimal timing of nitrapyrin treatment, nitrapyrin was delayed in 2020 in plots separated within the BK-20 and 2021 in the BK-21 experiments (labeled Np^+), so that we could compare the efficacy of the pre-seeding and post-seeding delayed treatments.

In 2020, monitoring the change in ***soil nitrate content in the*** BK-20 experiment showed that the delayed application of nitrapyrin was more beneficial, as it resulted in more N available in the soil during the intensive nutrient uptake period of maize. However, it is important to underline that the post-treatment period (13 June 2020) was very wet, which contributed to a more efficient N uptake through the soil during this critical period. Singh and Nelson (2019) also emphasized that during a rainy period, inhibitors can be applied more efficiently to mitigate N loss. Our results are also in agreement with the research of Pittelkow et al. (2017), in which nitrapyrin treatment in autumn (early) did not significantly reduce N loss and thus did not increase yield. Overall, in 2020, late application of nitrapyrin proved to be more effective considering the nutrient uptake dynamics of maize, however, the higher efficiency of late application is presumably mainly due to the rainfall period following treatment. In contrast, in 2021, in the BK-21 experiment, significantly lower nitrate levels were measured as a result of the late treatment compared to the early treatment *(*Since in 2021, the late application of nitrapyrin (10 June 2021) was followed by a period of very low rainfall compared to 2020, so essentially the nitrate leaching loss could also be lower. The significantly lower nitrate content as a result of the late treatment can be assumed to be due to the effective slowing down of nitrification activity, so that the conversion of ammonium ions to nitrate ions may have been slower.

By observing the change in ***relative chlorophyll content,*** significantly higher SPAD values were measured in 2020 in the BK-20 experiment compared to the early nitrapyrin treatment, which can be explained by more efficient N utilization by the plants. In contrast, in 2021, in the BK-21 experiment, the efficiency of the late nitrapyrin treatment was no longer superior to the early treatment. The difference in the results between the two years is most likely due to the climatic conditions in the two years. While in the period of intensive maize development 2020 was more abundant in precipitation, 2021 was significantly less favourable, which factors determined to a large extent the N uptake efficiency of the plants from the soil and, through this, the relative chlorophyll content.

Observing changes in ***root mass*** and ***stem thickness in*** 2020, both root mass and stem thickness parameters increased significantly in the BK-20 experiment with late nitrapyrin treatment compared to early treatment. In contrast, in 2021, in the BK-21 experiment, there was no significant difference between the efficacy of nitrapyrin applied at the two time points, which could also be due to the conditions of the year. In 2020, the abundant rainfall following the late application of nitrapyrin presumably created more favourable N uptake conditions for the plants, whereas in 2021, drought could have significantly impaired the amount of N taken up through the soil, thus reducing biomass gain.

Although in the BK-20 experiment in 2020, the late nitrapyrin treatment resulted in nearly the same ***thousand grain weight***, while ***ear length*** and ***ear thickness*** increased significantly, so the later nitrapyrin treatment was more beneficial. In contrast, in 2021, in the BK-21 experiment, no favourable changes were observed for any of the parameters after the late treatment, and statistically verifiable lower values were found for the parameters of thousand grain weight and ear length. The reason for this is probably the different rainfall in each year, which in 2020 was much more favourable for maize development.

The effects of applying nitrapyrin at two dates were also examined through changes in the ***nutritive characteristics of the crop.*** Overall, slight differences in protein, oil and starch content were observed in both years. In 2020, in the BK-20 experiment, the increase in protein content was most pronounced with late nitrapyrin treatment, while no significant differences in oil and starch content were observed. In comparison, our data show that in 2021, in the BK-21 experiment, the late application of nitrapyrin even slightly decreased the protein content.

Considering the effects of nitrapyrin treatments ***on leaf tissue nutrient content, in the*** 2020 BK-20 trial, late nitrapyrin treatment significantly increased leaf tissue N content, preventing severe N deficiency disease in pre-sowing nitrapyrin-treated stocks. The significant improvement in N supply resulting from the late nitrapyrin treatment may have been due to the improved N uptake conditions in the soil as a result of the delayed treatment, which may have been greatly influenced by the abundant rainfall following application. In addition to N, the late treatment with nitrapyrin improved, although not significantly, the Mg and S availability of the plants. In other words, delayed application of nitrapyrin in 2020 proved to be more effective than early application. In contrast, in 2021, in the BK-21 trial, these beneficial effects of late application of nitrapyrin were no longer observed, as no increase in either nutrient was observed as a result of the treatment. This is most likely due to the unfavourable precipitation conditions of the year. In 2020, the precipitation yield was significantly higher than in 2021 during the intensive nutrient uptake period of maize, so the effectiveness of late nitrapyrin treatment was significantly higher in 2020 than in 2020 compared to early treatment.

4. NEW SCIENTIFIC FINDINGS

1. **Based on our research results, nitrapyrin treatment can increase the nitrogen utilisation of maize** and improve the nitrogen supply parameters of the plant.

2. **Based on a soil incubation study, we have demonstrated that the efficacy of nitrapyrin increases with increasing soil temperature.** We have demonstrated that nitrapyrin inhibition is enhanced by accelerated nitrification at warmer soil temperatures.

3. **We have demonstrated that the beneficial effects of delayed post-emergence application of nitrapyrin can be expected in a rainy season.**

4. **The nitrapyrin treatment is highly effective when applied to sandy soils.** Spectacular physiological improvement of maize stands has been demonstrated in sandy soils.

5. **We have demonstrated that the addition of foliar fertilizer treatment to nitrapyrin results in further favourable plant physiological changes in maize.** We have found that the combination of nitrapyrin and foliar fertilizer containing nitrogen to reduce nitrogen loss and thus improve nitrogen uptake can improve plant physiology. We have demonstrated that the combination of nitrapyrin and foliar fertilizer treatment reduces the physiological stress levels in maize leaves.

5. PRACTICAL APPLICABILITY OF THE FINDINGS

1. A nitrification inhibitor containing the active ingredient nitrapyrin, which aims to reduce nitrogen losses, can be successfully used to supply nitrogen more efficiently to crops with high nitrogen demand. My results show that the application of nitrapyrin can increase parameters that are closely correlated with the nitrogen supply of plants, such as chlorophyll content, biomass gain, thousand grain weight and protein content of the crop. The results obtained are of great practical importance, as the judicious application of nitrapyrin can increase the yield of rapeseed and oilseed rape crops in addition to maize.

2. Based on studies on the optimal timing of nitrapyrin application, it was found that if more rainy days can be expected based on forecasts after the planned date of delayed application of nitrapyrin after sowing, or if treatment is planned in an area with irrigation, a higher degree of effectiveness of nitrapyrin can be expected.

3. The application of nitrapyrin on loose sandy soils significantly helps to improve the nitrogen use efficiency of crops, and therefore the treatment is most appropriate in areas where nitrogen losses are more significant due to the physical properties of the soil.

4. Foliar fertilizer treatment alone is not sufficient to improve the health of the crop. Our studies have shown that it is advisable to use preparations with high Zn and Mg content adapted to the nutrient requirements of maize, as the results of laboratory leaf analysis have highlighted the increased sensitivity of maize to Zn and Mg deficiency.

5. Laboratory tests of nutrient content in leaf tissue at most of the sites of our experiments draw attention to common hidden deficiencies in plants, the timely detection and treatment of which is a fundamental task for all farmers.

6. Nowadays, research into improving stress tolerance in plants is at the heart of much research. The combination of nitrapyrin and foliar fertilizer treatments can increase the tolerance of plants to biotic (pathogen infection) and abiotic stresses, so the practical use of both nitrification inhibitors to promote nutrient utilisation and foliar fertilizer treatments to supplement nutrient supply is justified to achieve better plant health.

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7. LIST OF PUBLICATIONS

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List of publications related to the dissertation

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