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Journal of Plant Nutrition

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/lpla20

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To cite this article: Juan Carlos Quezada & Luca Bragazza (22 Nov 2023): Foliar applications of a zeolite-based biostimulant affect soil enzyme activity and N uptake in maize and wheat under different levels of nitrogen fertilization, Journal of Plant Nutrition, DOI: 10.1080/01904167.2023.2280124

To link to this article: https://doi.org/10.1080/01904167.2023.2280124

Published online: 22 Nov 2023.

JOURNAL OF PLANT NUTRITION
https://doi.org/10.1080/01904167.2023.2280124

Foliar applications of a zeolite-based biostimulant affect soil enzyme activity and N uptake in maize and wheat under different levels of nitrogen fertilization

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ABSTRACT

There is a growing interest in developing agricultural practices that can improve crop performance while preserving natural resources. Plant biostimulants are thought to play a role in reaching this goal, in particular by increasing the nitrogen use efficiency. However, a notable research gap exists concerning the effects of foliar applications of natural zeolites as plant biostimulants on crop performance. To address this knowledge gap, a greenhouse experiment was set up in order to study the response of maize and wheat traits, specifically the biomass productivity and nitrogen uptake, as well as the response of soil extracellular enzymes to the foliar applications of a natural zeolite in combination with different levels of nitrogen fertilization, that is, 100%, 75%, and 50% of the optimal dose. Foliar application of zeolite in wheat and maize plants led to an increase in root nitrogen concentration of about 10%, particularly at the lowest nitrogen fertilization rate. This response was accompanied by an increase in aboveground to belowground uptake nitrogen ratio. Furthermore, there was a significant reduction of about 20% in root biomass in both crops with zeolite application across the entire nitrogen fertilization gradient. These plant-level responses were associated with a significant increase in the activity of carbon-degrading and nitrogen-degrading enzymes at the soil level in response to zeolite applications. Our findings provide a compelling proof-of-concept for the beneficial effects of foliar-applied zeolite as a biostimulant for crops, emphasizing the critical need for additional field research to validate our greenhouse results.

ARTICLE HISTORY
Received 13 March 2023
Accepted 30 October 2023

KEYWORDS
Beta-glucosidase; leucine-aminopeptidase; root biomass; *Triticum aestivum* L.; *Zea mays* L

Introduction

Meeting the demands of a rapidly expanding world population and minimizing the environmental consequences of agronomic practices pose significant challenges for modern agriculture (Clark and Tilman 2017; Lynch et al. 2021). Plant biostimulants (PBs) have been identified as important components in promoting sustainable agriculture (Cataldo et al. 2021). Indeed, PBs are defined as substances, mixtures or microorganisms that are capable of enhancing nutrient use efficiency, abiotic stress tolerance and/or crop traits (Rouphael and Colla 2020; Del Buono 2021; Du Jardin 2015) and can be classified into six categories, that is, humic and fulvic acids, protein

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hydrolysates, seaweed and other plant extracts, chitosan, beneficial fungi and bacteria, as well as inorganic compounds (Du Jardin 2015; Yakhin et al. 2016).

While the mode of actions of different PBs are yet to be fully understood (Yakhin et al. 2017), recent application of plant phenomics combined with metabolomics seems promising to elucidate how different PBs can promote plant growth (Nephali et al. 2020; Li et al. 2022; Zhang et al. 2023). PBs action should translate into economic and environmental advantages such as higher crop yield, increased crop quality, reduced fertilization rate, and/or higher stress tolerance. Recent meta-analyses have shown that PBs can enhance crop yield (Li, van Gerrewey, and Geelen 2022) and nutrient use efficiency (Herrmann et al. 2022) depending on type of biostimulant, application mode, crop species and environmental conditions.

The PBs category of inorganic compounds includes essential elements or inorganic salts such as, for example, silicates, phosphites, and carbonates. Siliceous natural nanomaterials such as zeolites can potentially be included in this category of inorganic PBs (Constantinescu-Aruxandei et al. 2020; Mondal et al. 2021). Natural zeolites are nanoporous, crystalline, hydrated aluminosilicates of alkali and alkaline earth cations that are characterized by reversible dehydration, large volume of free space, and high cation exchange capacity (Mumpton 1999). When applied as soil amendment, recent studies have shown that natural zeolites can affect soil microbial structure (Ferretti et al. 2018), reduce nitrogen losses (Ruser and Schulz 2015; Faccini et al. 2018; Akbari et al. 2021; Roumani and Olfs 2021) and increase plant nutrient use efficiency (de Campos Bernardi et al. 2016; Mehrab et al. 2016; Medoro et al. 2022). Interestingly, zeolite addition to agricultural soils has been linked to changes in extracellular enzyme activities so suggesting that zeolite can also affect microbial activity and, ultimately, nutrient cycling (Garau et al. 2007; Gondek et al. 2023; Khati et al. 2017; Tzanakakis et al. 2021). However, studies that test natural zeolites as PBs for plant nutrition *via* foliar application are limited (e.g. El-Gabiery and Ata Allah 2017; Moale et al. 2021; Petoumenou 2023; Sirbu et al. 2023) and, to our knowledge, no study has yet examined the effects of zeolite on crop nutrition (but see Sedaghat et al. 2022 as a strategy against drought in wheat plants).

In order to understand the potentials of zeolites as biostimulant for crops, a greenhouse experiment has been performed where maize and spring wheat plants were treated with a natural zeolite *via* foliar application in combination with three levels of nitrogen (N) fertilization, that is, 100%, 75% and 50% of the recommended optimal dose. The tested zeolite as biostimulant (Fertiroc®) is a combination of natural zeolite (chabazite) and natural soft calcium carbonate. Our study aims to show the potential of zeolites as biostimulants in crop production by improving nutrient use and soil enzyme activity. Specifically, we want to answer the following questions: (1) how does foliar-applied zeolite affect aboveground and belowground biomass as well as the corresponding N uptake in maize and wheat plants? (2) Does soil activity of carbon, phosphorus, and N-degrading enzymes respond to foliar application of zeolite? The overarching hypothesis of this study is that, under reduced levels of N fertilization, the application of zeolite can not only improve the N nutrition of the two crops, but also stimulate the activity of soil N-degrading enzymes.

Material and methods

Experimental setup

A greenhouse experiment was conducted at the research station of the Swiss Institute for Agricultural Research (Agroscope) in Changins, Switzerland (46°24′ N, 06°14′ E), from April to June 2021. Spring wheat (*Triticum aestivum* L. cv. Diavel) and maize (*Zea mays* L. cv. LG31211) plants were grown in two distinct greenhouse modules under the same conditions, that is, a light/dark regime of 14/10 h with supplemental lighting of 400 W m^{-2} at 22/15 °C and relative humidity of 50%. Wheat and maize plants were grown in plastic pots of, respectively, 20 cm (diameter) × 15 cm (height) and 20 cm (diameter) × 25 cm (height) containing 3 kg of soil for the wheat and 6 kg of soil for the maize plants. All pots within each greenhouse module were placed on tables and randomly reshuffled every week to avoid any potential effects of microclimatic gradients.

Five and three seeds of, respectively, wheat and maize were sown in each pot. After seed emergence, maize plants were thinned to one plant per pot whereas five plants of wheat were left in each pot. All the pots contained a loamy (338 g kg^{-1} sand, 425 g kg^{-1} silt and 237 g kg^{-1} clay) soil that was manually collected from the surface layer (0–15 cm) of an agricultural soil classified as Calcaric Cambisol and situated close to the greenhouse. Before potting, the soil was air-dried, ground, sieved through a 1-cm mesh and mixed thoroughly. The initial soil physico-chemical properties were as follow: pH (1:2.5, H_2O) of 7.9, total N content of 1.21 g kg^{-1}, total organic C content of 13.3 g kg^{-1}, available P content of 0.14 g kg^{-1}, and available K content 0.29 g kg^{-1}. All plants received deionized water two or three times per week so to keep soil moisture content at 60-80% of field capacity throughout the experimental period. Both crop species were allowed to grow for nine weeks. All pots received a basal fertilization treatment consisting of P (as KH_2PO_4) and K (as KCl).

Nitrogen and zeolite treatments

Pots were arranged in a replicated and completely randomized design. Nitrogen fertilization was applied at three different rates (= treatments), that is, the amount of 110 and 120 kg N ha^{-1} for maize and wheat, respectively, in accordance with the official recommendations for optimal N fertilization (Sinaj and Richner 2017), and two lower amounts of 60 kg N ha^{-1} and 90 kg N ha^{-1} for both crops. Nitrogen was applied as ammonium nitrate in two dates for maize, that is, at V4 (4 leaf collars present) and V6 (six leaf collars present) growth stage, and in three dates for wheat, that is, at Zadok's growth scale of 16 (six unfolded leaves), 18 (eight unfolded leaves) and 19 (nine unfolded leaves) (Zadoks et al. 1974). In the case of maize, each of the two dates of N fertilization provided half of the total optimal N dose. Similarly, in the case of wheat fertilization, each of the three dates of N addition provided one-third of the total optimal N dose. The three N fertilization treatments (hereafter N120/N110, N60, and N90 kg ha^{-1}) were coupled with (+Z) or without (0Z) foliar application of zeolite. Thereby, the overall experiment design resulted in six treatments, that is, 3 N levels × 2 zeolite levels. More specifically, the maize experiment consisted of 24 experimental units (each pot containing one plant was one experimental unit). For each N treatment (i.e. 110, 90 and 60 kg ha^{-1}), maize plants received two levels of foliar zeolite (addition and no-addition, see below for the specific amount) with four replicates for each treatment. For the wheat experiment, each experimental unit was represented by a pot containing five plants. The N levels and the zeolite levels followed the same protocol as for the maize so yielding to 24 experimental units. All the treatments with the foliar zeolite were sprayed on the same days when the N fertilization was applied (30, 36, and 42 days after sowing for the wheat, and 36 and 51 days after sowing for the maize). The commercial product Fertiroc® was used as natural zeolite and was sprayed over the plants with a pressurized portable sprayer of 1 L. The Fertiroc® product is a composition of natural zeolite (chabazite) mixed with a proportion of natural soft calcium carbonate. To be effective, the composition is micronized and works extremely fine according to the know-how of the producer company Power the Nature SA (Lausanne, Switzerland and Paris, France). For wheat, the proper amount of Fertiroc was prepared based on the sur-face that was covered by 12 pots (i.e. 1.2 m^2) following the manufacturer's recommendations of 10 L of Fertiroc© ha^{-1} in 600 l of water. This resulted in the application of a solution of 72 mL of water containing 1.2 mL of Fertiroc©. For the maize, the proper amount of zeolite for 24 pots was prepared with the equivalent of 15 L of Fertiroc© ha^{-1} in 600 L resulting in a solution of

72 ml of water containing 1.8 mL of Fertiroc©. The controls of the zeolite treatment (0Z) were sprayed with the same amount of deionized water on the same dates of foliar applications of Fertiroc®.

Sampling and laboratory analyses of plant and soil samples

Wheat and maize plants were sampled after 9 weeks from the sowing in order to recover the aboveground and belowground biomass (roots). The wheat plants were sampled at the dough development stages (growth stage 85), that is, when spikes were already emerged and the shoots, including the flag leaves, were yellow (Zadoks et al. 1974). On the other hand, maize plants were sampled at the tasseling stage. For wheat, all the five plants in each pot were sampled together and then separated into roots, aboveground biomass and spike biomass. Maize plants, one per pot, were separated into aboveground (tassels were included) biomass and roots. Aboveground biomass was cut at the soil level and roots were gently washed by hand over a sieve using tap water to remove soil particles. All the separated parts were oven dried at 60 °C for 72 h.

After drying, the roots, the aboveground biomass and the spike biomass samples were ground with a rotor mill to a fine powder (<2 mm). Total N content (%) was determined by Dumas dry combustion. The *N* uptake in the aboveground as well as in the belowground biomass was calculated as: [*N* concentration (%) × dry weight (g plant^{-1})]. Upon harvest, composite soil samples made of three soil cores (2.5 cm diameter × 10 cm depth) per pot were collected and thoroughly mixed. Fresh subsamples were immediately frozen at −20 °C for soil enzyme analysis.

Soil extracellular enzymatic activity

The activities of five selected soil enzymes related to C-cycling, i.e. β-glucosidase (BG), β-xylosidase (BX), to C and N-cycling, that is, β-N-acetylglucosaminidase (NAG), to N-cycling, that is, leucine aminopeptidase (LAP), and to P-cycling, that is, acid phosphatase (AP), were measured using synthetic fluorogenic substrates according to a modified procedure by (Marx et al. 2001; German et al. 2011). Fluorogenic 4-methylumbelliferone (MUF)-based substrate was used to determine the activities of BG, BX, NAG and AP (Sigma-Aldrich, St. Louis, MO). Fluorogenic 7-amino-4-methylcoumarin (MUC)-based substrate was used to determine the activity of LAP (Sigma–Aldrich, St. Louis, MO). The fluorescence was measured by a microplate reader (BioTek, Instruments, US) with excitation wavelength of 355 nm and emission wavelength of 460 nm. For calibration and quenching effects, a set of standards were prepared with 200 μL of soil slurry solution (for each individual sample) with a range of increasing concentrations of MUF or MUC standards. Enzyme activities were calculated from the regression slopes of the standard measurements along with the fluorescence average values of the triplicates for each sample and they were reported as µmol substrate (MUF or MUC) g^{-1} dry soil h^{-1}.

Statistical analyses

Univariate and multivariate statistical analyses were performed using the software Statistica v. 13.5.1.17 (TIBCO Software). The experimental design had two factors, that is, N fertilization (three levels) and zeolite biostimulant (two levels). For all studied variables, a two-way analysis of variance (ANOVA) was used to test the effects of N fertilization, zeolite and their interaction. A significant interaction indicates that the effect of zeolite depends on the level of N fertilization. Within each level of N fertilization, the effect of zeolite application was tested by means of a *t*-Student test. Finally, a principal component analysis (PCA) was applied to determine the correlations and explore the variability between plant and soil parameters.

Results

Effect of N fertilization and FertiRoc addition on aboveground biomass

In maize plants, on the basis of two-way ANOVA, a positive effect of N fertilization on aboveground biomass and the correspondent N concentration was observed (Figure 1A, B). Instead, the zeolite addition did not have any effect either on aboveground biomass or correspondent N concentration, with the only exception of the lowest N fertilization treatment (N60) where the application of zeolite significantly increased the N concentration in aboveground biomass (c. +13%) compared to the no-zeolite treatment (Figure 1B).

In wheat plants, neither N fertilization nor zeolite application had a significant impact on aboveground biomass (Figure 2A). Differently, N fertilization positively affected the N

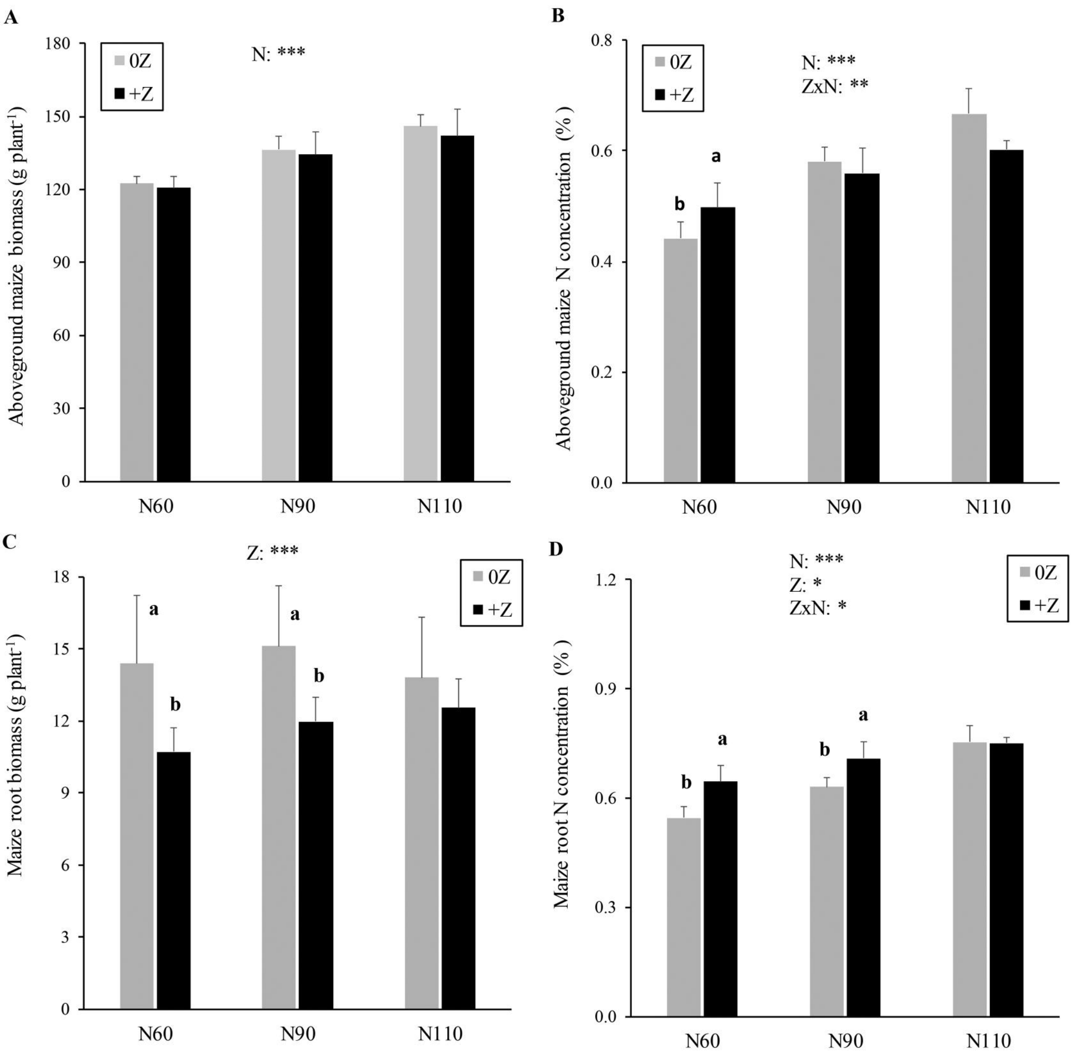

Figure 1. Mean ($n = 4$) abovegroundbiomass (a), abovegroundbiomass N concentration(B), belowgroundbiomass (C), and belowgroundbiomass N concentration(D) in maize plants under different N (N60, N90, and N110kg ha^{-1}) and zeolite (0Z = control, +Z = addition) treatments. Error bars representstandard deviation. The significantfactors (N and Z) and their interaction(ZxN) from the two-wayANOVAare indicated(*p <.10; **p <.05; ***p <.01) in relationto N and zeolitetreatment. Superscriptletters indicate significantdifferences($p < 0.085$) betweenzeolite treatmentsfor the same rate of N fertilization basedon *t*-Studenttest.

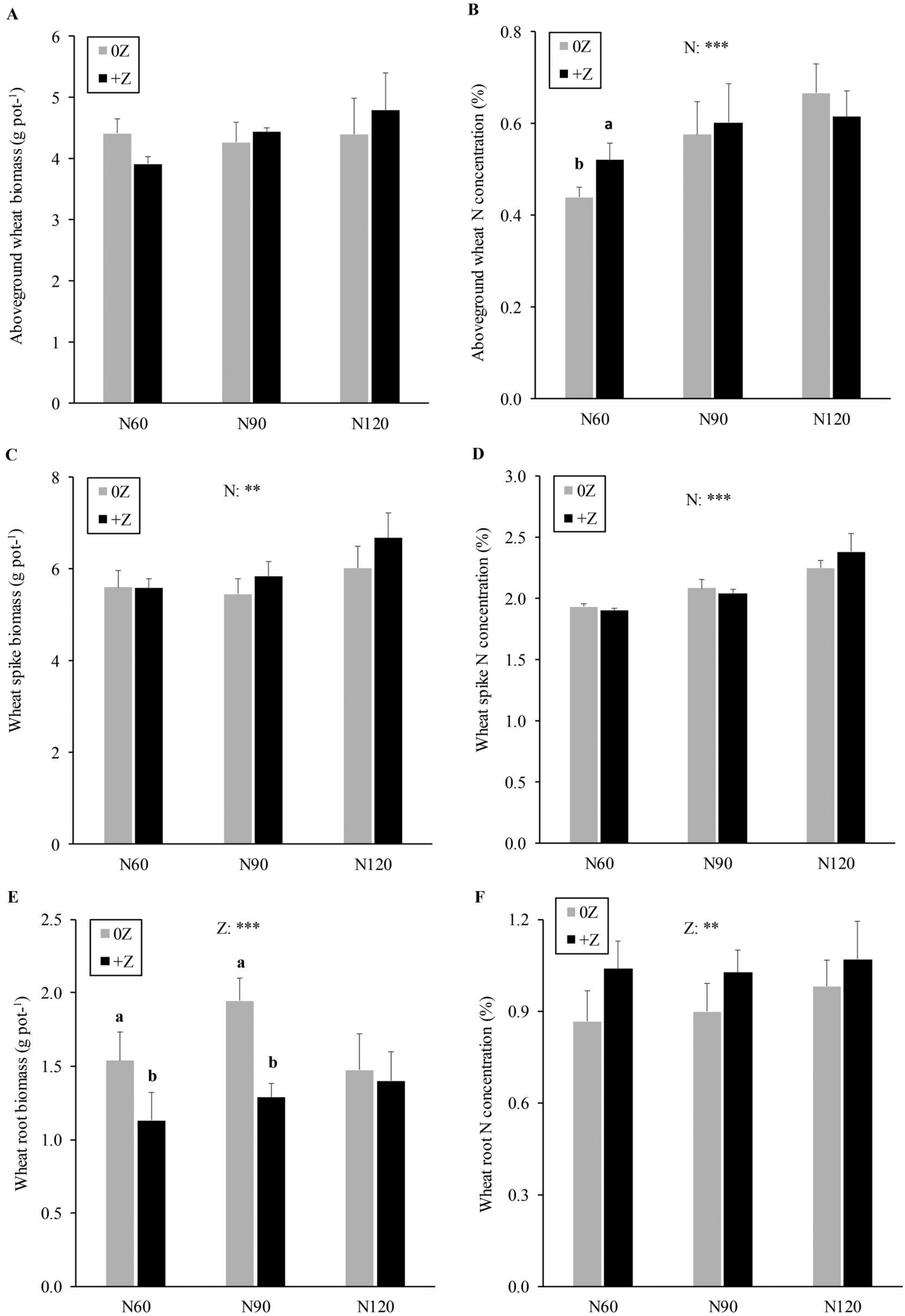

Figure 2. Mean ($n = 4$) aboveground biomass (a), aboveground biomass N concentration (B), spike biomass (C), N concentration in spike biomass (D), root biomass (E), and belowground biomass N concentration (F) in wheat plants under different nitrogen (N60, N90, and N120 kg ha^{-1}) and zeolite (0Z = control, +Z = addition) treatments. Error bars represent standard deviation. The significant factors (N and Z) and their interaction (ZxN) of the two-way ANOVA are indicated ($*p < 0.10$; $**p < 0.05$; $***p < 0.01$) in relation to nitrogen and zeolite treatment. Superscript letters indicate significant differences ($p < 0.040$) between zeolite treatments for the same rate of N fertilization based on *t*-Student test.

concentration in aboveground biomass (Figure 2B). Zeolite application did not affect the N concentration in aboveground biomass of wheat, but it did lead to a significant increase (c. +19%) in N concentration at the lowest N fertilization level compared to the no-zeolite treatment (Figure 2B). Regarding the spike biomass and N concentration in spikes (Figure 2C, D), N fertilization had a significant effect, while zeolite application did not. Nevertheless, there was a trend toward higher mean values of spike biomass with zeolite addition along the N gradient (Figure 2C).

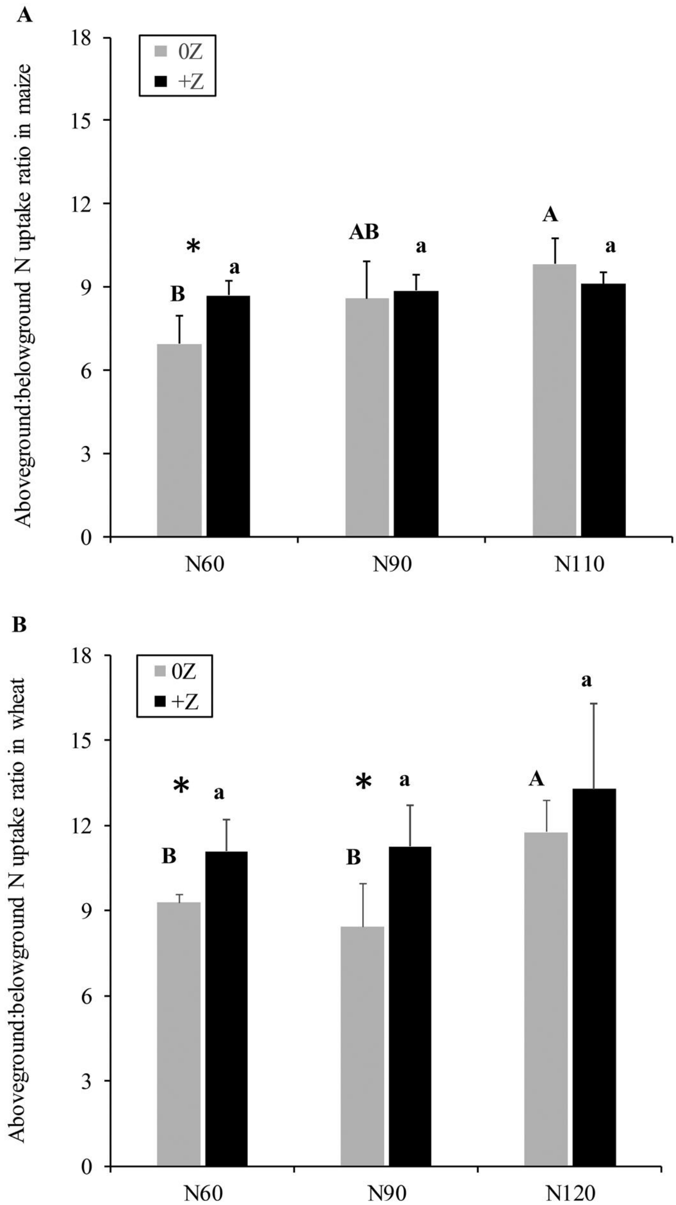

Figure 3. Mean ($n = 4$) ratio of aboveground to belowground N uptake in maize plants (A) and in wheat plants (B) under different N (N60, N90, and N110/120 kg ha^{-1}) and zeolite (0Z = control, +Z = addition) treatments. In the case of wheat, the aboveground biomass includes the aerial biomass and the spike biomass. Error bars represent the standard deviation. Superscript letters indicate significant differences ($p < 0.05$) between N fertilization treatments for the same zeolite treatment (uppercase = 0Z, lowercase = +Z) based on one-way ANOVA and post-hoc Fisher LSD test. The asterisks indicate a significant difference ($p < 0.05$) between zeolite treatments for the same N fertilization level based on *t*-Student test.

Table 1. Effect of nitrogen (N) fertilization and zeolite (Z) addition on mean activity of C, N, and P-targeting enzymes in maize and wheat soil.

Soil	Enzyme activity (µmol MUF or MUC g^{-1} soil h^{-1})	Zeolite treatment		Statistical analysis		
		0Z	+Z	Z effect	N effect	ZxN interaction
Maize	Acid phosphatase (AP)	10.7[a]	6.6[b]	***	NS	NS
	β-glucosidase (BG)	39.4[b]	73.3[a]	***	NS	NS
	β-xylosidase (BX)	10.2[a]	4.4[b]	***	NS	NS
	N-acetylglucosaminidase (NAG)	9.3[a]	3.9[b]	***	NS	NS
	Leucine-aminopeptidase (LAP)	19.2[b]	34.3[a]	***	NS	NS
Wheat	Acid phosphatase (AP)	18.9[a]	9.9[b]	***	**	NS
	β-glucosidase (BG)	30.2[b]	93.7[a]	***	***	***
	β-xylosidase (BX)	6.9[b]	21.3[a]	***	NS	NS
	N-acetylglucosaminidase (NAG)	8.7[a]	5.5[b]	**	***	NS
	Leucine aminopeptidase (LAP)	17.1[b]	36.8[a]	***	NS	NS

Note: Values are the average ($n = 12$) across three N fertilization rates for the two zeolite treatments. The 0Z treatment indicates no-zeolite addition (control), whereas + Z indicates zeolite addition according to manufacturing indications. The statistical analysis, based on a two-way NOVA, shows the significance of zeolite and nitrogen effect as well as their interaction: NS = no-significant ($p > 0.10$), * = $p \leq 0.10$, ** = $p \leq 0.05$, *** = $p < 0.01$. For each enzyme, the significance of the Fisher-LSD post-doc comparison between zeolite treatments is indicated by different small case letters ($p \leq 0.05$).

Effect of N fertilization and FertiRoc addition on belowground biomass

For what concerns the belowground biomass of both crops, N fertilization did not have a significant impact on root productivity. However, there was a tendency for root biomass to decrease at higher N fertilization rates, particularly in maize (Figures 1C and 2E). Conversely, the addition of Fertiroc resulted in a significant decrease of root biomass for both crop species (Figures 1C and 2E) and an increase in root N concentration (Figures 1D and 2F) in all the N fertilization treatments.

Effect of FertiRoc addition on N uptake

The aboveground to belowground N uptake ratio was positively affected by Fertiroc addition for both crop species, particularly at lower N fertilization levels (Figure 3). Indeed, without zeolite application the plants receiving a higher N fertilization were characterized by a significantly higher aboveground to belowground N uptake ratio. However, with zeolite application no signifi-cative differences were observed in the N uptake ratio among the three N fertilization levels (Figure 3). Notably at the lowest N fertilization levels (N60 for maize and N60 and N90 for wheat), zeolite application resulted in a significant increase in the aboveground to belowground N uptake ratio compared to the corresponding control (Figure 3).

Effect of FertiRoc addition on soil enzyme activity

The foliar application of Fertiroc was associated with a significant change in the activity of soil enzymes in both maize and wheat soil. Specifically, the activity of β-glucosidase (BG) and leu-cine-aminopeptidase (LAP) increased more than double, whereas the activity of acid phosphatase (AP) and N-acetylglucosaminidase (NAG) decreased with the application of zeolite (Table 1). Furthermore, the activity of β-xylosidase (BX) increased in wheat soil, but decreased in maize soil upon zeolite application (Table 1).

Discussion

The observed higher aboveground biomass in response to N fertilization is in agreement with previous studies reporting a positive effect of increasing N availability for maize and wheat productivity (Miao et al. 2006; Morris et al. 2018; Struck et al. 2019; Ordonez et al. 2020).

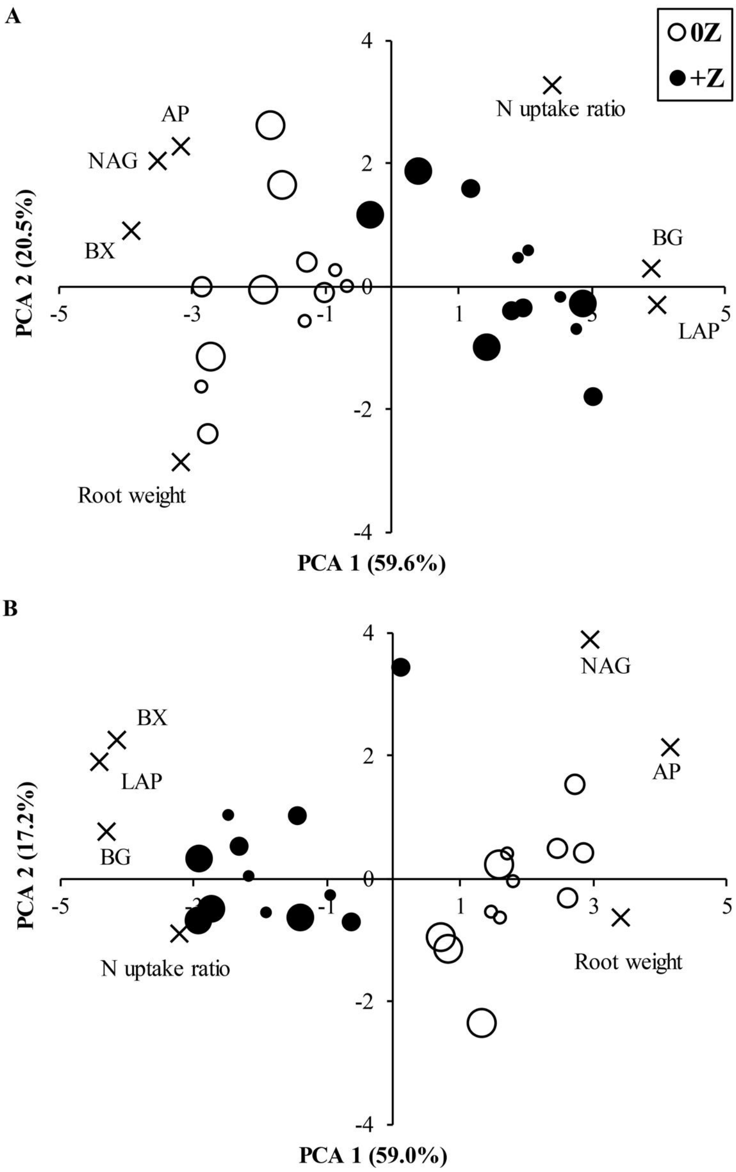

Figure 4. PCA ordination of maize (A) and wheat (B) samples on the basis of soil enzymatic activity of acid phosphatase (AP), β-glucosidase (BG), β-xylosidase (BX), N-acetylglucosaminidase (NAG), and leucine aminopeptidase (LAP) as well as on the basis of aboveground to belowground N uptake ratio and root weight under zeolite addition (+Z) and no-zeolite addition (0Z). The three N fertilization rates are represented by the increasing size of the circles (120/110 kg N ha^{-1} = biggest size, 60 kg N ha^{-1} = lowest size).

Interestingly, the application of Fertiroc in the lowest N fertilization treatment resulted in higher N concentration in aboveground biomass for both crop species, suggesting that Fertiroc may, directly or indirectly, enhance the N uptake under lower N availability.

Regarding the belowground productivity, previous studies have demonstrated that plants tend to allocate more dry matter to belowground biomass in order to acquire more nutrients when faced with lower N availability (Chen et al. 2015; Liu et al. 2017; Duncan et al. 2018; Ordonez et al. 2020; Kubar et al. 2022). This explains the observed increase in root biomass in response to decreasing N fertilization, a response particularly pronounced in maize. At the same time, the observed decrease of root biomass with Fertiroc application may, indeed, reflect an increase of N availability, so resulting in less investment in root growth (Figures 1D and 2F). Despite the decrease in root biomass, the data indicate that Fertiroc application was associated with an improvement of the efficiency of N uptake, particularly under lower N fertilization where a higher N concentration in aboveground biomass was detected with Fertiroc application (Figures 1B and 2B).

The foliar application of Fertiroc led to a stabilization of the N uptake ratio across all the levels of N fertilization, particularly at low N fertilization rates (Figure 3). This effect was observed in both studied crops and was not evident in the absence of Fertiroc application. The direct and indirect mechanisms of zeolite for promoting a better N uptake at lower N fertilization level are still unclear and further studies are necessary. We can speculate that zeolite can either directly affect N metabolism of the plant (Li et al. 2022; Savarese et al. 2022) or indirectly through plant-soil microbial interactions (Pantigoso et al. 2022; Costa et al. 2023). For our study, we hypothesize that a role in the improvement of N uptake is also played by a change in soil enzyme activity with Fertiroc application (Table 1). Specifically, higher activities of both β-glucosidase (BG) and leucine-aminopeptidase (LAP) were associated to higher aboveground to belowground N ratio and lower root biomass in both crops (Figure 4). The increased LAP activity indicates an increase in amino acid degradation and, consequently, more N available to the crops (Siwik-Ziomek and Szczepanek 2019; Greenfield et al. 2021). In parallel, the increase in BG activity may reflect a change in root exudate quality and quantity so to promote soil microbial activity (Sanaullah et al. 2016) and accelerate soil N cycling (Henneron et al. 2020). The observed decrease in NAG activity is consistent with previous findings on the response of this enzyme to increased N availability (Olander and Vitousek 2000) and to changes in root exudate composition (Hao et al. 2022). The decrease of AP activity may be attributed to an increased mobilization of inorganic phosphorus resulting from the release of organic acids by plant roots (Zhang et al. 2019). Taken together, we hypothesize that the observed changes in soil enzyme activities induced by the Fertiroc application may reflect a functional response of the soil microbiome to changes in crop root exudates (Sieradzki et al. 2023). This link between soil enzyme activity and soil microbiome composition has been already reported in response to the addition of other types of PBs (Khati et al. 2017; Barone et al. 2019; Mattarozzi et al. 2020; Trivedi et al. 2021).

In conclusion, this study demonstrates that foliar application of the Fertiroc® has significant effects at both plant and soil level. For both maize and wheat, Fertiroc addition reduced root bio-mass but increased root N concentration, especially at lower N fertilization rates. Remarkably, Fertiroc addition maintained a consistent aboveground to belowground N uptake ratio, even under reduced N input. At soil level, Fertiroc addition increased the activity of β-glucosidase and leucine- aminopeptidase enzymes potentially leading to increased N availability. This research provides a proof-of-concept for the potential benefits of foliar application of zeolite as biostimulant for crops. However, the complexity of the effects merits further in-depth investigation to fully eluci-date the underlying mechanisms. This is particularly important if foliar application of zeolite can be combined with strategies aiming at reducing N fertilization levels while maintaining crop yield quality and quantity.

Acknowledgments

For technical support during the greenhouse experiment, thanks are due to Yves Grosjean and Said Elfouki.

Disclosure statement

The authors declare no conflict of interest. The funder “Power the Nature” had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Funding

This research was partly funded by "Power the Nature." In addition, as part of the ArbeitProgram22-25, this study was also partly funded by Agroscope.

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Data availability statement

The data presented in this study are available on request from the corresponding author.

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