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Reducing N Fertilization without Yield Penalties in Maize with a Commercially Available Seed Dressing

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Abstract: Introducing smart and sustainable tools for climate change adaptation and mitigation is a major need to support agriculture's productivity potential. We assessed the effects of the processed gypsum seed dressing SOP® COCUS MAIZE+ (SCM), combined with a gradient of N fertilization rates (i.e. 0 %, 70 % equal to 160 kg N ha^{-1}, and 100 % equal to 230 kg N ha^{-1}) in maize (*Zea mays* L.), on: (i) grain yield, (ii) root length density (RLD) and diameter class length (DCL), (iii) biodiversity of soil bacteria and fungi, and (iv) Greenhouse Gases (GHGs, i.e. N_2O, CO_2, and CH_4) emission. Grain yield increased with SCM by 1 Mg ha^{-1} (+8 %). The same occurred for overall RLD (+12 %) and DCL of very fine, fine, and medium root classes. At anthesis, soil microbial biodiversity was not affected by treatments, suggesting earlier plant-rhizosphere interactions. Soil GHGs showed that (i) the main driver of N losses as N_2O is the N-fertilization level, and (ii) decreasing N-fertilization in maize from 100 % to 70 % decreased N_2O emissions by 509 mg N-N_2O m^{-2} y^{-1}. Since maize grain yield under SCM with 70 % N-fertilization was similar to that under Control with 100 % N-fertilization, we concluded that under our experimental conditions SCM may be used for reducing N input (-30 %) and N_2O emissions (-23 %), while contemporarily maintaining maize yield. Hence, SCM can be considered an available tool to improve agriculture's alignment to the United Nation Sustainable Development Goals (UN SDGs) and to comply with Europe's Farm to Fork strategy for reducing N-fertilizer inputs.

Keywords: maize; fertilization reduction; climate change mitigation; SDG; Farm to Fork; food security; sustainability; GHGs

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1. Introduction

Conventional management of agroecosystems has often depleted soil quality and altered soil processes involved in the provision of multiple ecosystem services [1]. The combination of intensive tillage and high nitrogen (N) fertilization has increased soil organic carbon (SOC) mineralization [2], thus mining yield potential and exacerbating the contribution of agricultural soils to the increase of Greenhouse Gases (GHGs) concentration in the atmosphere [3]. To further complicate matters is the high pressure

mounted on agriculture in recent years to support world population growth with an appropriate food supply [4]. Therefore, introducing smart and sustainable tools for building resilience of agro-ecosystems is a major need to ensure an efficient use of agricultural inputs and support productivity, while facing the challenge of climate change at the same time [5].

Gypsum is a relatively cheap [6] and common mineral amendment [7], with a range of favorable effects on both soil physical and chemical properties [8]. Gypsum also provides calcium (Ca) and sulfur (S) to plants [9]. The improvement of soil conditions and relative plant responses have the potential to increase crop yields [9], by increasing root system development and establishment, and enhancing water and nutrients uptake by plants [10]. This can be explained by the fact that calcium is a main component of the root cell wall, and acts on cell elongation and proliferation [11]. In addition, Kost et al. [9] previously reported that gypsum can also promote crop growth and yield by enhancing positive plant-soil-microbes interactions. Nevertheless, the degree of plant response to gypsum application is far from being fully understood [8].

Soil microbial communities and their biodiversity play essential roles in the biogeochemical cycles of soil nutrients [12]. The use of mineral fertilizers in agriculture has an impact on soil microbial communities [13]. Increasing N fertilization, for instance, had consistent impact on the richness, diversity and composition of soil bacterial communities [14], while fungal communities remained relatively unaffected [13]. Gypsum can stimulate the denitrification process mediated by soil microbes and contribute to plant growth by mitigating N leaching and runoff from soils [15]. In combination with different soil fertilization approaches, gypsum may also cause significant alterations in soil microbial communities' biodiversity due to changes in soil physicochemical properties [16,17] Consequently, soil functions and nutrient cycling are impacted. The interaction of all changes in soil promoted by gypsum with soil microbiota is an important open question, especially in intensive agricultural systems [18].

The crucial link between agricultural growth and the Sustainable Development Goals (SDGs) set by the United Nations Development Program is established through the efficient use of nitrogen in cereal production systems [19]. At the same time, the European Commission (EU) recently set ambitious goals for reducing fertilizer use significantly (-20 %) at the field level by 2030 [20]. The main leading reasons are the massive and still inefficient fertilizer use in the agricultural system and the consequent unacceptable risks for environmental and human health [2]. Above all, excess of N fertilization (especially if mismatched with plant needs) not always results in increased crop yield, but often leads to high N losses in surface- and ground-water bodies via N leaching and runoff, as well as in the atmosphere via GHGs emission [21].

Greenhouse gases emission to the atmosphere and the impact on global climate are among the greatest environmental concerns of current times [22]. Agriculture activities contribute to around 10–14 % of GHGs emission globally [22]. Nitrous oxide (N_2O) emission is strictly related to N fertilization [23], being a main driver of the overall GHGs emissions. Over the last 150 years, atmospheric N_2O levels have risen by 18 %. Measurements of N_2O and its isotopic composition in air suggest that the increase, at least since the early 1950s, is dominated by emissions from soil treated with synthetic and organic N-fertilizer [24–26]**.** Conversely, the role of fertilization in CO_2 emissions is not fully understood: suppression [27], increase [28], or no effect [29] were shown. The same is true for methane (CH_4), since increasing N fertilization was reported to increase [30], inhibits [31], or to have no effects on CH_4 oxidation in soil [32].

Although research on the effect of gypsum is reported in the scientific literature, no comprehensive study connecting the concomitant responses of crop yield, root development, microbial diversity, and GHGs emission has been previously performed. In this context, the objective of the present study was to evaluate the effect of the processed gypsum seed dressing SQG377, commercial name SOP® COCUS MAIZE+ (SCM) combined with different N-fertilization rates, on maize yield, root density and root classes

distribution, biodiversity of soil bacteria and fungi, and GHGs (i.e., N_2O, CO_2, and CH_4) emission. Our hypothesis was that SCM would allow for a reduction of N-fertilization (and GHGs) without impact on maize yield by supporting functional soil biodiversity and root development.

2. Materials and Methods

2.1. Site and Soil Characteristics

The field experiment was conducted between April 2019 and April 2020, at the CERZOO experimental station in Piacenza (45°00′21.6″N, 9°42′27.1″E; altitude 68 m a.s.l.), Po Valley, Northern Italy. The location is characterized by a temperate climate (Cfa following Köppen classification), with an average annual temperature of 13.2 °C and cumulative annual precipitation of 837 mm. Weather data during the experiment were acquired with an automatic meteorological station (Figure 1).

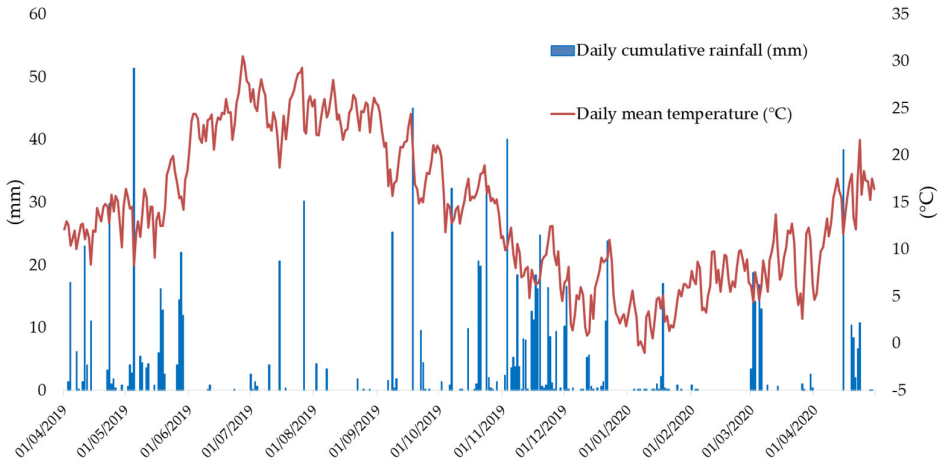


Figure 1. Daily rainfall (columns) and air temperature (line) during the field experiment (April 2019 – April 2020).

The soil is a fine, mixed, mesic Udertic Haplustalf, based on the Keys to Soil Taxonomy [33]. The physicochemical properties measured before the beginning of the experiment in the top 0 – 30 cm soil layer were: organic matter content 30 g kg^{-1}; pH H_2O 7.8; bulk density 1.36 g cm^{-3}; sand 127 g kg^{-1}; silt 445 g kg^{-1}; clay 428 g kg^{-1}; soil total N 1.7 g kg^{-1}; available P (Olsen) 32 mg kg^{-1}; exchangeable K (NH_4^+ Ac) 294 mg kg^{-1}; and cation exchange capacity 30 $cmol^+$ kg^{-1}.

2.2. Experimental Design, Treatments and Crop Management

A split-plot (SP) experimental design was set i[to assess the effects of a commercially available product named SQG377 - SOP® COCUS MAIZE+ (SOP Srl, Italy), hereafter SCM, during the cultivation of maize (*Zea mays* L.). SCM is a seed dressing product made of 100 % natural calcium sulfate (gypsum), processed with SOP proprietary technology. The seed dressing was obtained by mixing the maize seeds (around 82,000 seeds ha^{-1}) with SCM at a dosage of 200 g ha^{-1}, according to the manufacturer's specifications provided on the Technical Data Sheet of the product. An automated mixer was used to homogenize the seed dressing with the seeds immediately before planting. The main factor in the SP experimental design was the presence/absence of SCM, with two levels: (i) SCM treatment; and (ii) no seed treatment as the Control. Then, the secondary factor was the chemical N-fertilization rate (Urea 46 % N), with three levels: (i) 230 kg N ha^{-1} as the 100 % N-fertilization; (ii) 160 kg N ha^{-1} as the 70 %

N-fertilization; and (iii) 0 kg N ha^{-1} as the no chemical fertilization control (0 % N-fertilization). The 100 % N-fertilization rate was estimated according to the N balance, considering crop and soil-climate variables [34]. The N-fertilizer was applied once at the V5 – V6 phenological stage (6 June 2019), and was incorporated into the soil during distribution. The number of replicates was four (4 blocks), giving a total of 24 plots. The single plot size was 200 m^3 (each 25 m long and 8 m wide).

All plots were cultivated with continuous maize prior to starting the experiment. Two weeks before starting tillage operations (i.e. 30 cm subsoiling plus 15-cm rotary harrowing), cattle slurry at a rate of 28 m^3 ha^{-1} (equal to 50 kg of efficient N, as computed according to the European Nitrates Directive; [35]) were homogeneously distributed in all plots, according to the common practices of the area. The main cattle slurry characteristics were total solids 3.83 %; volatile solids 2.34 %; total N 2.37 g kg^{-1}, of which 50.5 % as ammonia N, P 0.37 g kg^{-1}; K 2.02 g kg^{-1}; pH 7.6; electrical conductivity 17.1 mS cm^{-1}).

Maize was planted on April 19th, 2019, with 75 cm spacing between rows. Maize was sprinkler-irrigated five times at doses of 40, 40, 45, 20 and 45 mm in order to prevent water stress. A detailed description of irrigation water doses estimation from the maize crop evapotranspiration, crop coefficients (Kc) calculation, and crop irrigation requirements (CIR) is reported in Fiorini et al. [36]. The field was treated with 3.3 L ha^{-1} of the preemergence herbicide Trophy (Acetochlor 40 % + Dichlormid 6 %) and 1 L ha^{-1} of post-emergence herbicide Fluoxypyr 20 % (to control *Abutilon theophrasti* M.), plus 1.5 L ha^{-1} of Nicosulfuron to control *Sorghum halepense* L. Maize was harvested on 4 October 2019, with a plot-scale combine and the maize residues were partially incorporated into the soil (c.a. 40 %) by chiselling.

2.3. Measurements of Maize Grain Yield and Root Density

Yield of maize was determined as follows: for each plot, plant ears from three randomly selected areas of 6 m^2 were manually harvested at the BBCH 89 and weighed. After the separation from bracts and cob, grains were pooled and mixed. A total number of 24 samples were obtained. About 100 g subsamples of each grain sample was oven-dried at 65 °C until constant weight to determine dry matter content.

Maize root sampling was carried out at anthesis, BBCH 69 [37], on 21 July 2019, with a self-constructed "Shelby" tube sampler of 7 cm diameter. The tube was pushed into the soil through the hydraulic arm of a digger to collect an intact 0 – 30 cm soil core. In each plot, two positions on the perpendicular of the crop row were identified: 0 cm (on the row, i.e. close to the base of the sampled plant but not including the maize stalk) and at 37.5 cm (mid-row). Root biomass was isolated from the surrounding soil following procedures reported in Fiorini et al. [38]. After extraction, roots were scanned at 600 dpi with the Epson Expression 10000xl scanner (Epson America, Los Alamitos, CA, USA), equipped with a double light source to avoid roots overlapping. The determination of Root Length Density (RLD, cm cm^{-3}) and root diameter was performed by using the winRHIZO Reg 2012 software (Regent Instruments, Québec, QC, Canada). The Diameter Class Length (DCL, mm cm^{-3}) was then calculated for very fine (0.0 – 0.075 mm), fine (0.075 – 0.2 mm), medium (0.2 – 1.0 mm), and coarse (> 1 mm) diameters for the whole soil profile, as adapted from Reinhardt and Miller [39].

2.4. Soil DNA Extraction, Amplification and Bioinformatics Analyses

Soil sampling for DNA extraction and amplification also occurred on 21 July 2019, following the same procedures reported above for maize root sampling. Soil samples adhered to maize plants' roots were manually separated from the surrounding bulk soil, collected into separate sterile containers, and kept at −20 °C until analysis. According to the manufacturer's protocol, the whole soil DNA was extracted using the DNeasy PowerSoil Kit (Ref 12888-100, QIAGEN GmbH, Hilden, Germany). Samples were amplified by primer pairs 343F/802R for bacterial 16S rRNA and ITS-1 /ITS-2 for

ITS region of fungi. Two-step PCR amplification protocols were adopted for both, as detailed in [40,41]. Sequence data preparation and concomitant statistical analyses were carried out as previously detailed [42]. Briefly, paired-reads were assembled with the "pandaseq" script [43] and demultiplexed using the Fastx-toolkit. Mothur v.1.32.1 [44] was applied in order to remove sequences with large homopolymers (≥ 10), non-aligning, and chimeric sequences [45]. The resulting high-quality sequences were analysed with Mothur and R [46] following two main approaches: the operational taxonomic unit (OTU) and the taxonomy-based approach. Sequences were first aligned against the SILVA reference aligned database for bacteria [47] using the NAST algorithm and a kmer approach [48,49], and then clustered at 3 % distance using the average linkage algorithm for the former approach. For the latter, sequences were classified into taxa using an amended version of the Greengenes database [50].

2.5. Gas Sampling and Quantification

Nitrous oxide, CO_2 and CH_4 fluxes were measured using the static closed-chamber method [51] with sampling performed manually throughout a 1-year period, from April 2019 to April 2020. Each cylindrical static chamber (40 cm diameter and 25 cm high) was made of polyvinyl chloride (PVC), of white colour to reduce the impact of directly radiating heat during gas sampling. During the experimental period, the chambers were fitted inside stainless-steel rings (39 cm diameter and 15 cm height) that were inserted 10 cm into the soil (one steel ring per plot). Each ring was installed into the soil at the beginning of the field experiment and kept in the original place throughout the whole experiment. Rings were temporarily removed (for few hours) from the soil only when tillage, planting, fertilizer distribution, and harvesting occurred. In each chamber, an internal battery-operated fan was installed to provide air mixing.

Fluxes were measured on 25 sampling events during the experimental period. In detail: after slurry distribution and soil tillage, GHGs were measured five times during a three-week period; then twice between planting and top-dress application of N-fertilizer (one week after planting maize and two days before N application). After N-fertilizer application, gas fluxes were evaluated three times per week for the following week, and twice per week in the second and third weeks after fertilization. Subsequently, the frequency decreased to once per week until the harvest and then one sample every three weeks in winter. Sampling was always performed between 9:00 and 12:00 a.m., following the recommendation of Maris et al. [52]) to minimize diurnal variation in flux patterns. At the time of gas sampling, the air temperatures outside and inside the chambers were measured simultaneously.

On each sampling date, the standard procedure was to take six samples of ambient air at chamber closure (i.e. 0 min after chamber closure), and then two samples of the chamber headspace were withdrawn at 15 and 30 min after closure. A volume of 60 mL of the air accumulated in the headspace of each chamber was sampled with 100 mL syringes. Before transferring the gas into a proper vial, a volume of 30 mL was discarded for purging the sampling syringe. The remaining gas was then transferred in 20 mL pre-evacuated LabcoExetainer® glass vials (Labco, Lampeter, Wales, UK) fitted with butyl rubber stoppers and analysed in the laboratory by gas chromatography (Agilent 7890A with a Gerstel Maestro MPS2 autosampler, Agilent Technologies Inc., Wilmington, DE, USA), equipped with electron capture and flame ionization detectors for the quantification of N_2O, CO_2 and CH_4, respectively. Details of the procedures used for gas analysis and fluxes calculation are described in Peyron et al. [53].

The GHG emission fluxes were determined from the linear increase of the gas concentration at each sampling time during the time of chamber closure. The applied equation is:

$$\boldsymbol{F = \frac{\delta C}{\delta t}\frac{p \cdot V}{R \cdot T \cdot A}} \quad (1)$$

where F is the flux (μg m^{-2} s^{-1}) from top atmosphere, C is the gas concentration (μmol mol^{-1}), t is the time (s), p is the atmospheric pressure (Pa, constant), V is the headspace volume (m^3), R is the universal gas constant (8.3145 m^3 Pa K^{-1} mol^{-1}), T is the ambient air temperature (°K), and A is the surface area enclosed by the chamber (m^2). The linear regression approach uses the slope obtained from the least-squares linear regression of C versus t to estimate δC and δt to be used in the Equation.

The average GHGs flux for each treatment presented is the arithmetic mean of three replications per treatment. The cumulative GHGs emission throughout the entire one-year measurement was calculated by integrating the emission flux curves over time.

2.6. Soil Properties Affecting GHGs

Soil samples (0 – 30 cm depth) were taken from each plot to evaluate the mineral N content: once before the application of the N fertilizer treatments, and then every 2 weeks for the first two months after N fertilizer addition and subsequently less frequently (Figure S1a in the Supplementary Material). The soil samples were transported immediately to the laboratory for nitrate (NO_3^-) and water content analyses. The soil NO_3^- concentrations were analysed using 5 g of homogeneously mixed soil extracted with 50 mL of HCl (1 M), and pipetted into 96-well quartz microplates. Nitrate-N was then analysed with dual-wavelength UV spectroscopy (275, 220 nm).

On each gas sampling, the gravimetric water content was determined (at 0 – 10 cm) by drying soil samples at 105 °C until constant weight. Soil bulk density (at 0 – 30 cm) was determined with the cylinder method. Soil porosity was estimated assuming a soil particle density of 2.65 g cm^{-3}. The value of water-filled pore space (WFPS) was then calculated by using values of soil water content, bulk density, and particle densities, as described in Maris et al. [54] (Figure S1b in the Supplementary Material).

2.7. Statistical Analyses

Data on (i) maize grain yield, (ii) root density and distribution (i.e. RLD and DCL), and (iii) cumulated GHGs emission were analysed through an analysis of variance (ANOVA) with a split plot design following procedures outlined by Gomez and Gomez [55] and using the "agricolae" package of RStudio 3.3.3. The main factor was the presence/absence of SCM, while the secondary factor was the N-fertilization rate. The assumptions of ANOVA were verified through the Shapiro-Wilk and the Levene's tests. Tukey's honestly significant difference (HSD) as a post hoc was used to explore significant differences (*p*-value 0.05; 0.01 and 0.001) between treatments.

Statistical analyses of OTU and taxonomy matrixes were performed in Mothur and R and included hierarchical clustering with the average linkage algorithm at different taxonomic levels, Principal Component Analysis (PCA) to assess the grouping of the unconstrained samples, and Canonical Correspondence Analyses (CCA) to assess the significance of different treatments on the analysed diversity.

3. Results

3.1. Grain Yield of Maize

Maize grain yield was significantly affected by the presence of SCM and N-fertilization rate (Table 1). Overall, maize grain yield was higher with the SCM treatment than with the Control (+ 8 %). On average, maize grain yield increased with increasing N fertilization rate. In detail, the 100 % fertilization treatment had higher maize grain yield than with 70 % fertilization, which in turn had a higher yield than the 0 % fertilization treatment. The interaction P/A × N did not show any statistical difference.

Table 1. Grain yield (Mg ha^{-1}) of maize as affected by the presence/absence of SCM (SCM vs control) and N-fertilization rate (0 %, 70 %, and 100 % N-fertilization). Different letters within the same source of variation indicate statistical significant differences between means.

Source of Variation	Main Factor	Secondary Factor	Grain Yield (Mg ha^{-1})
presence/absence of SOP® COCUS MAIZE (SCM) (P/A)	SCM	-	13.36 ± 2.14 a
	Control	-	12.35 ± 1.85 b
	p (F)		< 0.01
N-fertilization rate (N)	-	0 % N-fertilization	10.34 ± 0.59 c
	-	70 % N-fertilization	13.49 ± 0.79 b
	-	100 % N-fertilization	14.74 ± 0.83 a
	p (F)		< 0.001
P/A × N	SCM	0 % N-fertilization	10.63 ± 0.40
		70 % N-fertilization	14.14 ± 0.32
		100 % N-fertilization	15.32 ± 0.83
	Control	0 % N-fertilization	10.05 ± 0.65
		70 % N-fertilization	12.85 ± 0.50
		100 % N-fertilization	14.17 ± 0.22
	p (F)		n.s.

Mean values ± raw standard deviations are reported.

3.2. Root Length Density (RLD) and Diameter Class Length (DCL)

As for grain yield, SCM and the N-fertilization rate had a significant effect on maize RLD (Table 2). In detail, (i) the SCM treatment increased on average maize RLD (+ 12 %) compared with the Control treatment, and (ii) maize RLD increased with increasing N fertilization rate in the order 0 % < 70 % < 100 % N-fertilization. The interaction P/A × N did not show any difference in this case.

Table 2. Root Length Density (RLD; cm cm^{-3}) as affected by presence/absence of SCM (SCM vs control) and N-fertilization rate (0 %, 70 %, and 100 % N-fertilization). Different letters within the same source of variation indicate statistical significant differences between means.

Source of Variation	Main Factor	Secondary Factor	RLD (cm cm^{-3})
presence/absence of SCM (P/A)	SCM	-	3.71 ± 1.45 a
	Control	-	3.32 ± 1.17 b
	p (F)		< 0.05
N-fertilization rate (N)	-	0 % N-fertilization	2.70 ± 0.80 c
	-	70 % N-fertilization	3.54 ± 1.19 b
	-	100 % N-fertilization	4.30 ± 1.42 a
	p (F)		< 0.001
P/A × N	SCM	0 % N-fertilization	2.80 ± 0.96
		70 % N-fertilization	3.85 ± 1.45
		100 % N-fertilization	4.47 ± 1.56
	Control	0 % N-fertilization	2.59 ± 0.67
		70 % N-fertilization	3.23 ± 0.88
		100 % N-fertilization	4.13 ± 1.40
	p (F)		n.s.

Mean values ± raw standard deviations are reported.

Overall, the SCM treatment significantly affected DCL for very fine, fine, medium, and coarse root diameters at various extents in 70 % and 100 % N-fertilization, while not in 0 % N-fertilization (Table 3). In the 70 % N-fertilization sub-treatment, DCL for very fine, fine, and medium root diameters increased under SCM compared with under Control. Conversely, DCL for coarse root diameter was higher under Control than under the SCM treatment. A similar pattern was observed in the 100 % N-fertilization sub-treatment, although a significant effect due to SMC treatment was found only for very fine (increase) and coarse (decrease) root diameters.

Table 3. Diameter class length (DCL) for very fine (ø = 0 – 0.075 mm), fine (ø = 0.075 – 0.2 mm), medium (ø = 0.2–1 mm) and coarse (ø > 1 mm) root diameters as affected by the interaction between presence/absence of SCM (SCM vs Control) and N-fertilization rate (0 %, 70 %, and 100 % N-fertilization). Letters indicate differences SCM vs Control as obtained by the Tukey's test performed for each level of N-fertilization rate (0 %, 70 %, and 100 % N-fertilization) and distance from the row (0 cm, 35 cm); blank is not significant.

		0 % N-fertilization		**70 % N-fertilization**			**100 % N-fertilization**		
		SCM	**Control**	**SCM**		**Control**	**SCM**		**Control**
Root Diameter Class	**Distance from the row**	**DCL (cm cm^{-3})**		**DCL (cm cm^{-3})**			**DCL (cm cm^{-3})**		
ø = 0 – 0.075 mm	0 cm	0.54 ± 0.16	0.49 ± 0.05	1.20 ± 0.08	a	0.83 ± 0.10 b	1.24 ± 0.17	a	0.89 ± 0.19 b
	37.5 cm	0.30 ± 0.16	0.30 ± 0.10	0.43 ± 0.07		0.50 ± 0.16	0.62 ± 0.10		0.56 ± 0.10
ø = 0.075 – 0.2 mm	0 cm	1.39 ± 0.24	1.31 ± 0.21	2.25 ± 0.14	a	1.48 ± 0.29 b	2.20 ± 0.11		2.05 ± 0.37
	37.5 cm	0.68 ± 0.08	0.82 ± 0.06	1.02 ± 0.22		1.02 ± 0.10	1.30 ± 0.26		1.07 ± 0.12
ø = 0.2 – 1 mm	0 cm	1.55 ± 0.30	1.29 ± 0.26	1.93 ± 0.10	a	1.46 ± 0.16 b	2.23 ± 0.57		2.00 ± 0.46
	37.5 cm	1.02 ± 0.14	0.88 ± 0.09	1.06 ± 0.28		0.93 ± 0.32	1.17 ± 0.24		1.41 ± 0.29
ø > 1 mm	0 cm	0.10 ± 0.05	0.06 ± 0.01	0.08 ± 0.01	b	0.20 ± 0.01 a	0.15 ± 0.03	b	0.27 ± 0.12 a
	37.5 cm	0.01 ± 0.01	0.03 ± 0.02	0.02 ± 0.01		0.03 ± 0.03	0.03 ± 0.02		0.02 ± 0.01

Mean values ± raw standard deviations are reported.

3.3. Biodiversity of Soil Bacteria and Fungi

There were no significant differences between presence or absence of SCM and N-fertilization levels in species richness and diversity of soil bacteria and fungi according to Sobs (number of observed species), Chao and Simpson's indexes (Figure 2).

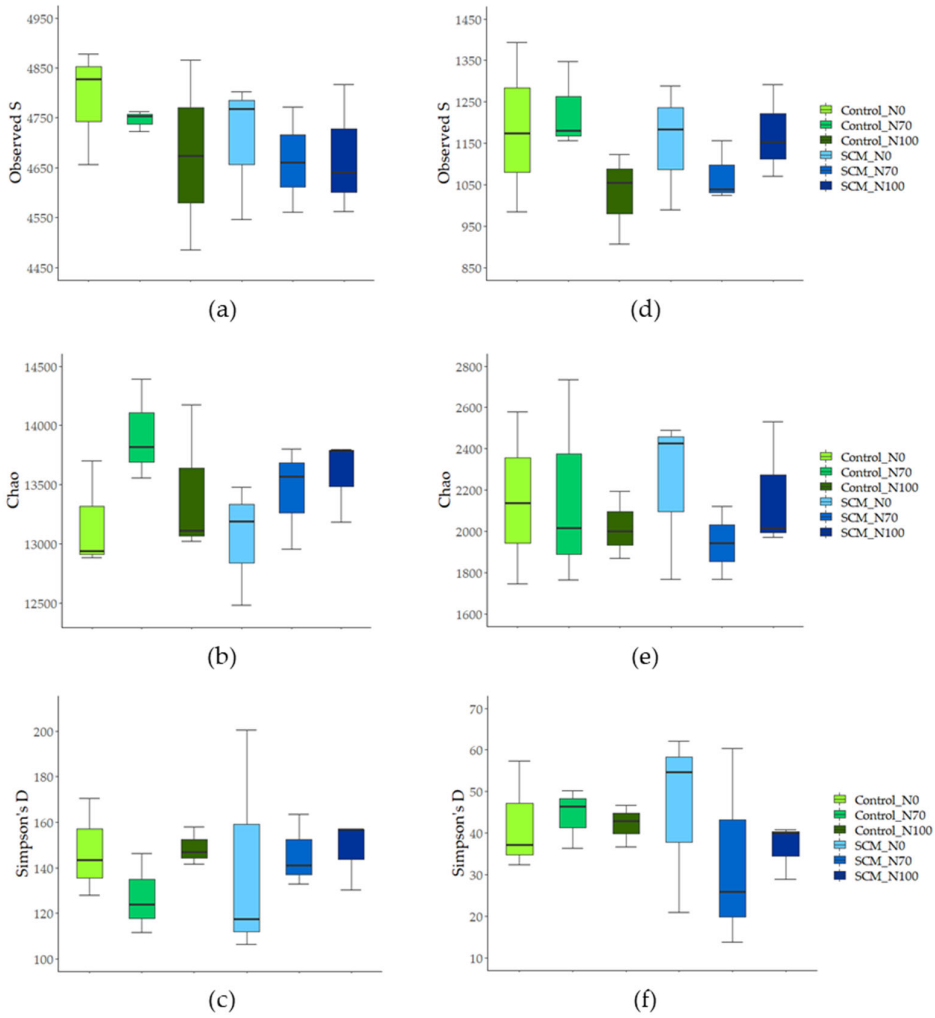


Figure 2. Estimation of α-diversity indexes and richness of each field separately for bacteria (**a–c**) and fungi (**d–f**). Control_N0 = Control with 0 % N-fertilization; Control_N70 = Control with 70 % N-fertilization; Control_N100 = Control with 100 % N-fertilization; SCM_N0 = SCM with 0 % N-fertilization; SCM_N70 = SCM with 70 % N-fertilization; SCM_ N100 = SCM with 100 % N-fertilization.

Taxonomically, samples had a quite heterogeneous distribution that did not significantly differ between relative abundances of any particular group of treatments and controls. In soil bacterial community classes of Acidobacteria-6, Chloracidobacteria, Acidimicrobiia, Actinobacteria, Thermoleophilia, Anaerolineae, Bacilli, Nitrospira and, in particular at order level, iii1-15, RB41, envOPS12, PK29 were of high relative abundance. In the soil fungal community, Dothideomycetes, Sordariomycetes, Blastocladiomycetes and Tremellomycete classes and their Pleosporales, Hypocreales, Sordariales, Tremellales, Blastocladiales, Mortierellales orders were found in high relative abundances across all samples (Figure 3).

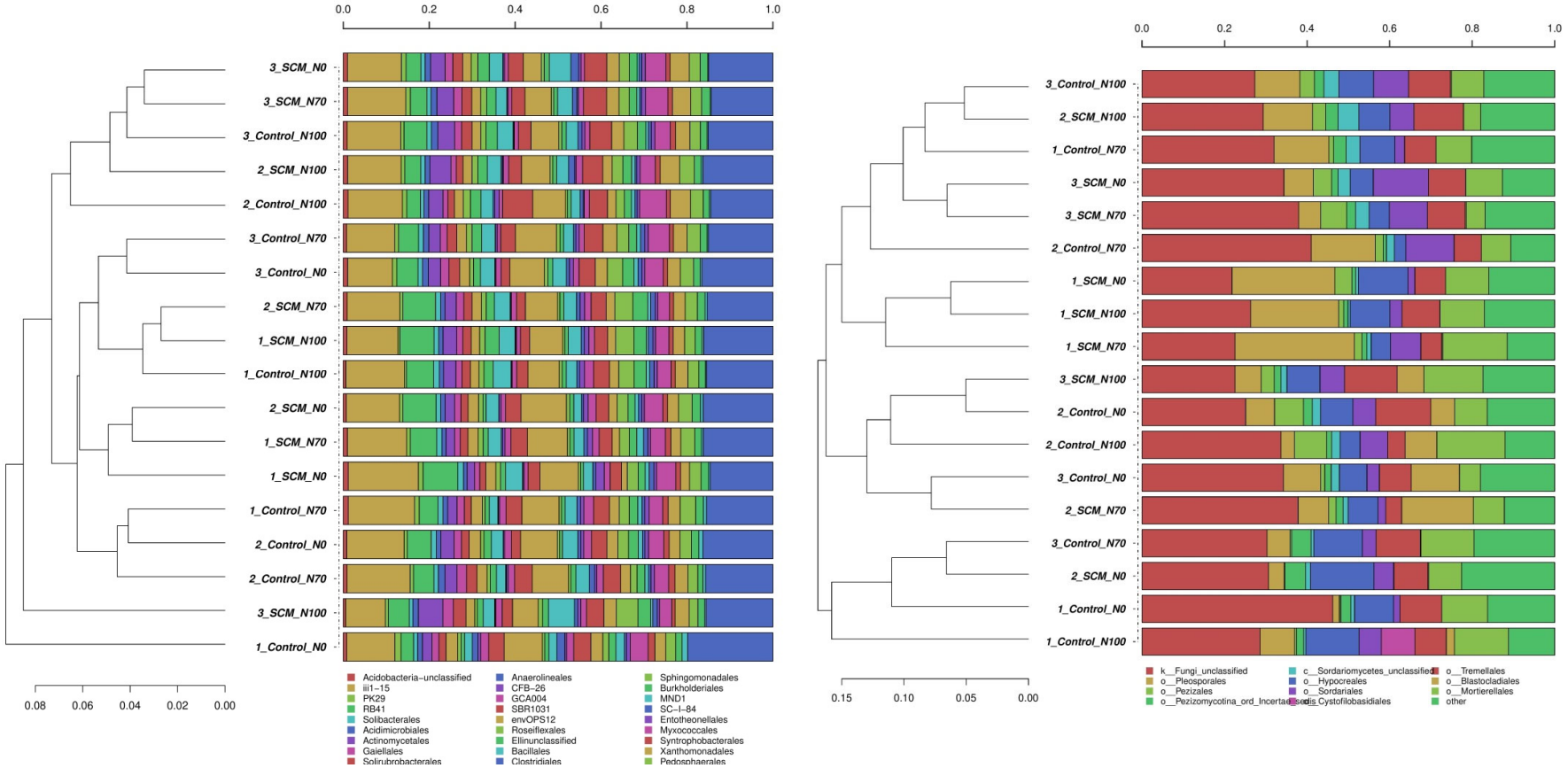
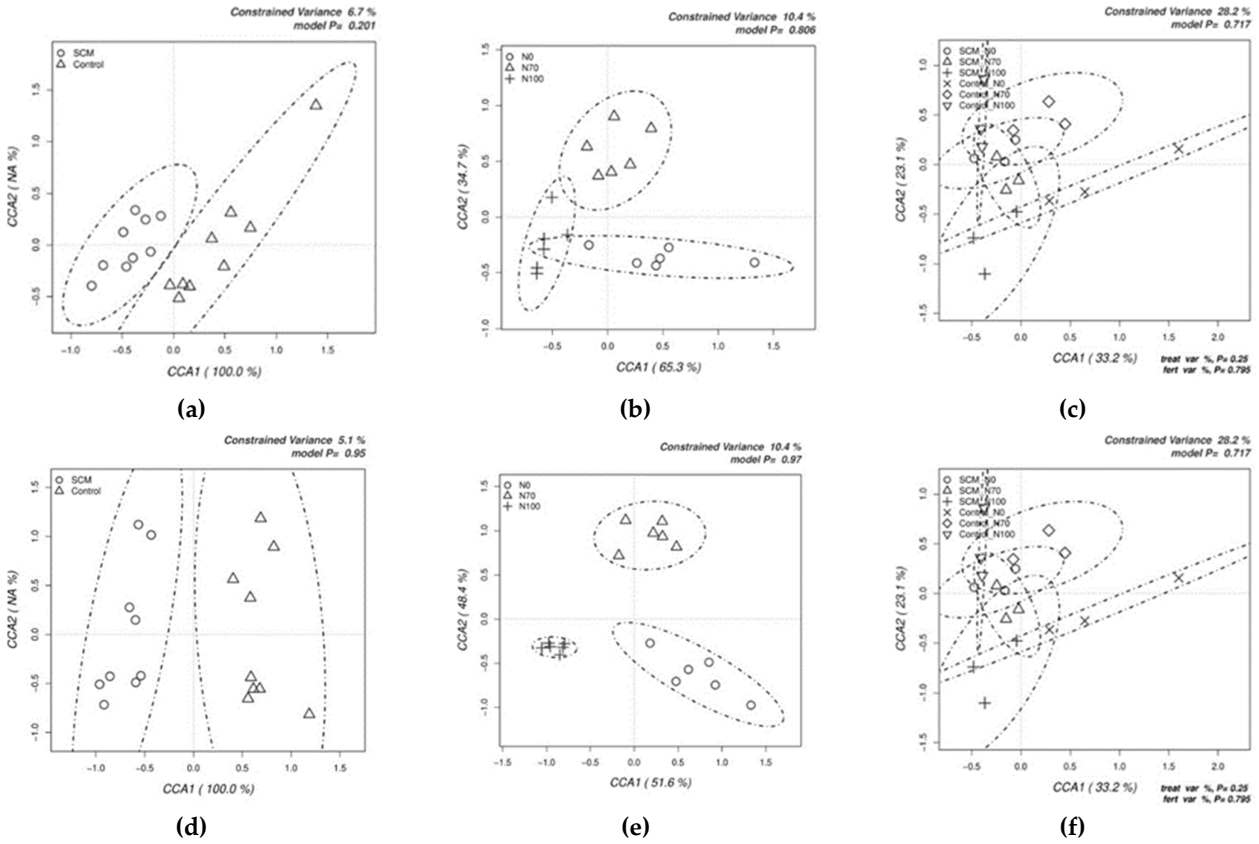


Figure 3. Taxonomic comparison of all samples (a, bacteria; b, fungi) through hierarchical clustering of microbial communities at the order level across all samples used in this study. Clusters were identified with the average linkage algorithm for taxa that contributed at least 5 % to a single sample. Taxa that contributed less than this threshold were added to the sequence group denoted "other". Control_N0 = Control with 0 % N-fertilization; Control_N70 = Control with 70 % N-fertilization; Control_N100 = Control with 100 % N-fertilization; SCM_N0 = SCM with 0 % N-fertilization; SCM_N70 = SCM with 70 % N-fertilization; SCM_ N100 = SCM with 100 % N-fertilization.

Canonical correspondence analysis (CCA) plots, in which bacterial and fungal community structures in samples were investigated, indicated trends in differences between N fertilization levels and treatments in respect to control. However, these differences were not significant in either bacterial or fungal communities (Figure 4).



igure 4. Canonical correspondence analyses (CCAs) on the impact of the treatments on the structure of bacterial (**a**, **b**, **c**) and fungal (**d**, **e**, **f**) communities. Determined by the relative abundances of all the operational taxonomic units OTUs obtained by Illumina sequencing of bacterial 16S and fungal ITS amplicons. Control_N0 = Control with 0 % N-fertilization; Control_N70 = Control with 70 % N-fertilization; Control_N100 = Control with 100 % N-fertilization; SCM_N0 = SCM with 0 % N-fertilization; SCM_N70 = SCM with 70 % N-fertilization; SCM_ N100 = SCM with 100 % N-fertilization.

3.4. Greenhouse Gas Emissions: Nitrous Oxide, Carbon Dioxide and Methane

Nitrous oxide emissions were not significantly affected by the presence/absence of SCM, but they were affected by the N-fertilization rate (Table 4). In detail, the highest values were recorded for 100 % N-fertilization (1701 mg N_2O-N m^{-2} y^{-1}), followed by 70 % N-fertilization (1192 mg N_2O-N m^{-2} y^{-1}) and control (464 mg N_2O-N m^{-2} y^{-1}). No significant interaction between P/A × N was observed (Table 4).

Average CO_2 emissions did not show statistically significant differences with the presence/absence of SCM (Table 4). Conversely, they were significantly affected by the N-fertilization rate with a higher value for 0 % N-fertilization (4135 g CO_2-C m^{-2} y^{-1}) than for 70 % N-fertilization (2689 g CO_2-C m^{-2} y^{-1}) and 100 % N-fertilization (2850 g CO_2-C m^{-2} y^{-1}). The interaction P/A × N did not show any difference in this case also (Table 4).

Average CH_4 emissions were neither affected by the presence/absence of SCM nor by the N-fertilization rate (Table 4). All treatments acted as a sink, and the highest daily average CH_4 consumption was registered for SCM (-110 mg CH_4-C m^{-2} y^{-1}) and 100 % N-fertilization (-120 mg CH_4-C m^{-2} y^{-1}). No significant interaction P/A × N was found (Table 4).

Table 4. Cumulated greenhouse gas emissions as affected by presence/absence of SCM (SCM vs control) and N-fertilization rate (0 %, 70 %, and 100 % N-fertilization) during the entire experimental year (April 2019-April 2020). Different letters within the same source of variation indicate statistical significant differences between means.

Source of variation	Main factor	Secondary factor	N_2O (mg N-N_2O m^{-2} y^{-1})		CO_2 (g C-CO_2 m^{-2} y^{-1})		CH_4 (mg C-CH_4 m^{-2} y^{-1})	
presence/absence of SCM (P/A)	SCM	-	1171 ± 627		3230 ± 925		-110 ± 47	
	Control	-	1067 ± 574		3220 ± 1032		-75 ± 56	
	p (F)		n.s.		n.s.		n.s.	
N-fertilization rate (N)	-	0 % N-fertilization	464 ± 131	a	4135 ± 652	a	-78 ± 57	
	-	70 % N-fertilization	1192 ± 234	b	2689 ± 855	b	-80 ± 53	
	-	100 % N-fertilization	1701 ± 408	c	2850 ± 641	b	-120 ± 47	
	p (F)		< 0.001		< 0.01		n.s.	
P/A × N	SCM	0 % N-fertilization	494 ± 118		4219 ± 584		-93 ± 40	
		70 % N-fertilization	1253 ± 318		2415 ± 408		-115 ± 39	
		100 % N-fertilization	1766 ± 479		3057 ± 639		-122 ± 70	
	Control	0 % N-fertilization	434 ± 162		4051 ± 838		-62 ± 76	
		70 % N-fertilization	1132 ± 158		2964 ± 1197		-45 ± 44	
		100 % N-fertilization	1635 ± 417		2643 ± 700		-117 ± 25	
	p (F)		n.s.		n.s.		n.s.	

Mean values ± raw standard deviations are reported.

4. Discussion

4.1. Responses of maize grain yield, RLD, and DCL to SCM and N-fertilization

Our results showed that grain yield and RLD of maize were both significantly affected by the application of SCM. On average, maize seeds treated with SCM developed 12 % higher RLD than those under Control condition. It is widely accepted that a well-established and developed root system is essential for efficient absorption of water [56]. In addition, maize plants with high root density tend to accumulate more macro- and micro-nutrients than plants with low RLD [57]. It follows that increasing root development of maize plants often results in increased yield performance [58], as supported by our findings.

Maize grain yield in our study ranged between 10.05 and 15.32 Mg ha^{-1}, which is in line with typical values of the area [59]. Regardless of N-fertilization, here we corroborated the hypothesis that applying SCM to maize seed during planting could be considered as a mean to increase root development and grain yield (c.a. 1 Mg ha^{-1}), thus probably promoting a more efficient use of inputs (i.e., water and N). Previous studies suggested that crop yield and RLD might be increased (+ 2– 5 %) with gypsum application (e.g. da Costa and Crusciol [60]; Crusciol et al. [61]). However, these authors referred to different crops and to an application rate of > 2 Mg ha^{-1} of gypsum. Besides, the yield gap in their results was lower than in ours (+8 %). Earlier findings reporting that further increasing gypsum rates (up to 6 Mg ha^{-1}) reduces crop yield [62] complete the picture of this matter in the literature.

Beyond the effect of SCM, our study also confirmed that the N-fertilization rate significantly affected RLD and in turn maize yield. The highest grain yield (14.74 Mg ha^{-1}) was obtained in the 100 % N-fertilization treatment, which also had the highest RLD (4.30 cm cm^{-3}). These results are mainly due to the fact that 100 % N-fertilization treatment received the N rate estimated according to the N balance and computed on maize needs (see Materials and Methods). However, the increase in N absorption by plants may likely have also increased the absorption and metabolism of other nutrients [63] and water [64] in 100 % N-fertilized plants.

In the present study, the application of SCM had a significant impact on the diameter class distribution of roots. In detail, SCM increased (or tended to increase) DCL of very fine, fine, and medium diameter classes, while decreased DCL of coarse roots. These effects were evident in 70 % N-fertilized and in 100 % N-fertilized plots, and at 0 cm distance from the maize row. Beyond the enhancing effects of SCM on RLD, results on DCL indicate that SCM, increasing the root hair surface can enhance the absorption of nutrients and water by maize plants, which ultimately results in crop yield increase.

On the contrary, DCL of coarse diameter root class was higher under the Control treatment. In this regard, it was reported that plants develop thick roots during mid-late phenological phases to support carbohydrate storage in grains and nutrients absorption from the soil profile [65]. In this context, our results suggest that maize plants in Control plots developed roots with higher diameter to ensure the optimal level of nutrients uptake from the soil. Conversely, the presence of SCM may have enhanced early root development of plants, thus resulting in greater uptake since the beginning and better matching to nutrients needs than under Control.

Maize yield performance under SCM in our study, which showed values of grain yield with 70 % N-fertilization similar to those under Control with 100 % N-fertilization, highlights the potential of this seed dressing product, especially in light of the recent claims from the EU about the reduction of N-fertilizer use [20], without reducing crop yield.

4.2. Relationships between treatments and biodiversity of soil bacteria and fungi

At anthesis, results indicated that seed with SCM at a rate of 200 g ha^{-1} had no significant impact on the diversity of soil bacteria and fungi in the proximity of the roots. These findings can be explained by the minimal rate of material used in our study when compared to the previously reported positive impact at 2 Mg·ha^{-1} and negative impact at the rates exceeding 4 Mg ha^{-1} [66] of gypsum. Observed trends, in which little changes were occurring without causing significant alterations, could be attributed to the ecological niches occupied by some bacteria in the soil without having a major impact on the general phylogenetic composition of the bacterial community [67]. Nevertheless, an overall positive impact of SCM in the agronomic part of our study was recorded. This was probably due to the increased rhizosphere volume, which was the consequence of the increased density of maize roots in plots with SCM, compared with the Control. However, such an increased root development, which was a win-win situation for our experimental conditions, could not be attributed to a direct impact on the soil bacterial and fungal community at anthesis. It can be attributed to the fact that, together with microbial diversity, the plant-rhizosphere interactions change at the different growth levels [68] and specific microbial recruitment by the plants at the juvenile stages are crucial for the overall success of the crops in terms of yield and growth [69]. Therefore, it is possible that SCM had an impact at the earlier stages of the plant growth reflected at the later stages as the density of maize roots in plots with SCM.

It should also be kept in mind that the 100 % N-fertilization treatment (both SCM and Control) received a carefully computed N rate estimation according to the N need of the maize. Therefore, the indifference of the microbial community to the N treatment rate can be attributed to the fact that there was no excessive N for the microbial community as the highest rate was the right amount needed for the plant itself. It is known that changes in soil nutrients may influence microbial biodiversity. However, the lack of significance of our experimental treatments on soil microbial diversity could be attributed to the minimal/very limited fluctuation in the availability of N that might affect plant nutrient uptake [70] as evidenced by similar values of grain yield in SCM with 70 % N-fertilization and in Control with 100 % N-fertilization in our study. Significant changes in soil microbial communities structure can be either positive or negative depending on the microbial guilds involved; in this study, the lack of changes indicated that the tested product at the given dose certainly has no ecotoxicological relevance at the microbial level.

4.3. Nitrous oxide, carbon dioxide, and methane emissions as affected by SCM and N-fertilization

Nitrous oxide emissions in this study (ranging from 434 to 1766 mg N_2O-N m^{-2} y^{-1}) were higher than those measured in maize under similar environmental conditions [71]. This was mainly a consequence of: (i) the fine soil texture, which is known to increase N_2O emissions [72]; the relatively high soil organic matter concentration, stimulating N_2O producing processes [73]; the seasonal pattern of rainfall which often show WFPS exceeding 60 % (Figure S1b in the Supplementary Material) and steers soil denitrification activity [54,74].

Our results confirmed that the main driver for GHG emissions is the N-fertilizer rate: the application of increasing rate of N fertilizer led to increased N_2O emissions due to enhanced availability of N in soil [75], thus stimulating nitrification and/or denitrification microbial activity [76]. In detail, decreasing N-fertilization from 100 % to 70 % decreased N_2O emissions by 509 mg N-N_2O m^{-2} y^{-1}. The results of this study are in substantial agreement with those of Hansen et al. [77] and Shcherbak et al. [78], who showed an exponential increase in N_2O emissions in maize with increasing rates of N fertilizer applied. This is mainly because applying high mineral fertilizer rates (i) increases the N_2O produced as a by product during the microbial oxidation of ammonium

(NH_4^+) to nitrate (NO_3^-), and (ii) stimulates N_2O production by other processes such as dissimilatory NO_3^- reduction to NH_4^+ and co-denitrification [79,80].

The N_2O emissions during the postharvest period were very low in our study (ranging from 13 to 30 mg N_2O-N m^{-2} y^{-1}; data not shown), representing less than 1 % of the total cumulative N_2O-N emissions during the entire experimental year. Main reasons were that the NO_3^- in the soil after harvest was very low for all treatments (Figure S1a) and the WFPS was contemporarily relatively high (above 70 % ; Figure S1b), which favored the reduction of N_2O to N_2 [81]. This demonstrated that whether N-fertilization rate is estimated according to the actual N needs (as N balance), when considering crop and soil-climate variables, N_2O emissions during the off-season may be kept under control without reducing yield.

The yield performance of SCM with 70 % N-fertilization, which was similar to that of Control with 100 % N-fertilization (as reported earlier), suggests an indirect potential of this product for reducing N_2O emissions (-23 %) from intensively managed maize by reducing N-fertilization without losing yield.

Annual CO_2 emissions, ranging from 2415 to 4135 g CO_2-C m^{-2} y^{-1}, fall in the ranges obtained by Abalos et al. [74], Plaza-Bonilla et al. [82] (2014), and Maris et al. [52], in intensive maize systems, under similar environmental conditions. Here we found that SCM had no effects on CO_2, while increasing N-fertilization significantly reduced CO_2 emissions (between -30 to -38 % in N-fertilized plots). This could be related to the fact that N-fertilization was shown to inhibit microbial growth and soil respiration, hence leading to reduced CO_2 emissions [27]. Our findings are in agreement with those of DeForest et al. [83], Burton et al. [84], and Maris et al. [54], who observed that N-fertilized plots had fewer CO_2 emissions (between 15 to 41 %) than unfertilized ones.

Annual CH_4 emissions obtained in our study, which ranged between -45 to -122 mg CH_4-C m^{-2} y^{-1}, are in line with the values previously observed in intensive maize systems (e.g. Abalos et al. [85]; Plaza-Bonilla et al. [82]). Although no significant difference was found, we observed that SCM and the highest N-fertilization rate tended to reduce emissions and/or increase CH_4 oxidation rate. It was indeed reported that gypsum-based fertilizers or high N-fertilization rates could increase the potential behavior of soil as a sink for atmospheric CH_4, by modifying the structure of microbial populations responsible for CH_4 oxidation [86].

5. Conclusions

The present field study demonstrated that SOP® COCUS MAIZE+ (SCM) is an effective tool to reduce N-fertilization input by 30 % without yield penalties. This was possible in our condition because SCM enhanced maize root development, especially that of very fine, fine, and medium roots classes.

At anthesis, no significant effect was found on biodiversity of soil bacteria and fungi in the rhizosphere, which suggests that SCM has no ecotoxicological relevance at the microbial level.

Our results highlight that decreasing N-fertilization in maize from 230 to 160 kg N ha^{-1} results into the reduction of considerable N losses as N_2O. Since maize grain yield under SCM with 160 kg N ha^{-1} was similar to that under Control with 230 kg N ha^{-1}, this suggests an indirect potential of this product for reducing N_2O emissions by stimulating a more efficient N use and yield.

Hence, our results indicate that SCM seed dressing may be considered an available tool for improving the alignment of intensive agro-ecosystems to the SDG framework and satisfying the requests from the European strategy Farm to Fork.

However, this study was conducted only in one year. Although weather conditions during this period could be considered as typical, further studies are needed to verify that results remain consistent in wetter and/or drier years and in the middle- and long-term.

Supplementary Materials: The following are available online at www.mdpi.com/2073-4395/11/3/407/s1, Figure S1. Annual pattern (April 2019 – April 2020) of soil NO_3- concentrations (a) and WFPS (b) under all treatments. Control_0% = Control with 0 % N-fertilization; Control_70% = Control with 70 % N-fertilization; Control_100% = Control with 100 % N-fertilization; SCM_0% = SCM with 0 % N-fertilization; SCM_70% = SCM with 70 % N-fertilization; SCM_100% = SCM with 100 % N-fertilization.

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