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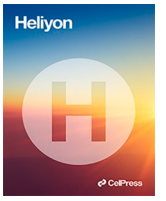

Research article

The impact of single and combined amendment of elemental sulphur and graphene oxide on soil microbiome and nutrient transformation activities

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ABSTRACT

Background: Sulphur (S) deficiency has emerged in recent years in European soils due to the decreased occurrence of acid rains. Elemental sulphur (S^0) is highly beneficial as a source of S in agriculture, but it must be oxidized to a plant-accessible form. Micro- or nano-formulated S^0 may undergo accelerated transformation, as the oxidation rate of S^0 indirectly depends on particle size. Graphene oxide (GO) is a 2D-carbon-based nanomaterial with benefits as soil amendment, which could modulate the processes of S^0 oxidation. Micro-and nano-sized composites, comprised of S^0 and GO, were tested as soil amendments in a pot experiment with unplanted soil to assess their effects on soil microbial biomass, activity, and transformation to sulphates. Fourteen different variants were tested, based on solely added GO, solely added micro- or nano-sized S^0 (each in three different doses) and on a combination of all S^0 doses with GO.
Results: Compared to unamended soil, nano-S^0 and nano-S^0+GO increased soil pH($CaCl_2$). Micro-S^0 (at a dose 4 g kg^{-1}) increased soil pH($CaCl_2$), whereas micro-S^0+GO (at a dose 4 g kg^{-1}) decreased soil pH($CaCl_2$). The total bacterial and ammonium oxidizer microbial abundance decreased due to micro-S^0 and nano-S^0 amendment, with an indirect dependence on the amended

Abbreviations: AOB, Ammonium oxidizing bacteria; ARS, Arylsulfatase; dsr, Sulphur reducing bacteria; NP, Nanoparticle; GO, Graphene oxide; GP, Graphene; p, P-value; PCA, Principal component analysis; Phos, Phosphatase; r, Correlation coefficient; rGO, Reduced graphene oxide; S^0, Elemental sulphur; SW, Water-soluble sulphur; Ure, Urease; 16S, 16S rDNA; 18S, 18 rDNA.

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dose. This trend was alleviated by the co-application of GO. Urease activity showed a distinct response to micro-S^0+GO (decreased value) and nano-S^0+GO amendment (increased value). Arylsulfatase was enhanced by micro-S^0+GO, while sulphur reducing bacteria (dsr) increased proliferation due to high micro-S^0 and nano-S^0, and co-amendment of both with GO. In comparison to nano-S^0, the amendment of micro-S^0+GO more increased soluble sulphur content more significantly.

Conclusions: Under the conditions of this soil experiment, graphene oxide exhibited a significant effect on the process of sulphur oxidation.

1. Introduction

Sulphur is an important nutrient for all living organisms, utilized in the formation of amino acids, proteins, and sulpholipids in plants. It also participates in chlorophyll formation, promotes nodulation in legumes, and aids in the development and activation of certain enzymes and vitamins [1]. Plants primarily acquire sulphur in the form of sulphates [2] and sulphites, which are soluble and prone to leaching from the soil [3], potentially leading to sulphur deficiency in soils [4] with several negative impacts on plant health and crop yield [5]. Sulphur deficiency has become more emerging in recent years due to reduced atmospheric inputs [6] to the soil in the form of acid rains, resulting from a decline in SO_2 emissions by approximately 20 % compared to levels from 30 years ago [7,8]. Systematic soil analyses conducted in the Czech Republic revealed low sulphur content in 85 % of all soils [9].

One environmentally sound solution to this problem could involve more frequent application of elemental sulphur S^0, yielded e.g. through the desulphurization of biogas production processes [10–12]. Elemental sulphur is highly beneficial in agriculture [13–15]. Apart from serving as a source of S nutrient for microorganisms and plants [13,16], in particular for canola [17,18], S^0 offers various advantages to the physico-chemical properties of soil [16,19]. It regulates the pH of alkalic soil or composted matter [20] as well as sewage sludges used for fertilization [21], incerases the availability of other nutrients [13,22], particularly in calcareous soils [23,24], enhances crop yields [22,25] and promotes tolerance to abiotic and biotic stresses in plants [26].

Elemental sulphur is insoluble in water, it does not leach from the soil [27,28]. To be solubilized, it requires bacteria capable of oxidizing it into plant-available sulphates (SO_4^{2-}) [29]. Several specific soil taxa, such as the genus *Thiobacillus* [30] and *Betaproteobacteria* [31] facilitates S^0 oxidation, and their relative abundance respond positively to S^0 content [32,33]. On the contrary, S^0 adversely affects other soil microorganisms, mainly fungi [34,35]. The oxidation of S^0 depends on factors such as soil water potential, temperature, aeration [30], hydrophobicity of particles and indirectly on their size [36]. Thus, fine formulated (micro-sized, nano-sized) S^0 may be transformed more rapidly into sulphates [25,37], potentially enhancing plant nutrition efficiency [25,38]. Furthermore, micro-/nano-sized S^0 alleviates metalloid toxicity [39], and enhances soil pH modulation [40,41], as well as aiding in plant pest control [42]. Various methods exist for the preparation of micro- or nano-sized S^0 such as crushing, ball milling [43,44], chemical precipitation [45,46] or sonication [47].

Other nanomaterials with increasing applicability in soil include 2D carbon-based amendments such as graphene (GP), graphene oxide (GO), and reduced graphene oxide (rGO) [48–50]. GO typically contains 42–62 % wt carbon and 24–36 % wt oxygen. GO exhibits high mobility in soil [51,52], but aggregation mediated by Ca^{2+} at concentrations $\geq$0.5 mM [53] can efficiently reduce the risk of leaching. Soil amendment with GO alters its hygroscopic and adsorptive capabilities, affecting water content. GO in soil mitigates drought stress impacts on plants [50,54] and enhances the uptake of mineral micronutrients by acting as a carrier with controlled release [55–58]. These positive effects on soil nutritional properties benefit plant growth and physiology [59,60], while also protecting against other plant-harming factors [61]. The adsorption of metalloids by GO enables the environmental detoxication of soil or water [62,63]. GO exhibits variable impacts on bacteria, fungi, algae: while high susceptible gram-positive bacteria [64] and rhizobacteria [65] show inhibition of growth or enzymatic activity [66], other soil microbes exhibit neutral to positive responses to GO [67,68] or GO altered bacterial community richness [67,69,70] in a concentration-dependent manner [71].

Combining GO with other nanomaterials may further improve its impact on soil [72,73] and provide new specific materials [74,75], which could, for example, ameliorate salinity stress in crops [76]. Therefore, a combination of finely formulated S^0 and carbon-based materials hold promise for environmental and forestry applications [77–79]. However, the combined use of nano- or microsized S^0 + GO and the evaluation of their impact on soil microbiota, their activities (respiration, enzymes activity) are rarely addressed [72,80]. The impact of finely formulated S^0 on soil microbial properties, especially soil enzymes transforming nutrients, is more significant for nano-S^0 than micro-S^0 [81]. Therefore, the aim of this work was to contribute to the understanding the impacts of the mutual interaction of soil amendments GO and elemental sulphur in a novel type of a composite product designed for gradual oxidation and transformation into a plant-available form of sulphur. The composite, as a presumably more easily colonizable matrix compared to the sole S^0, should provide transformable finely formulated elemental sulphur to microbial oxidizers while concurrently protecting against undesirable losses due to excessive solubilization and leaching. Thus, in this work we aimed to verify the following hypotheses.

1. Nano-sized S^0 exerts a larger effect than micro-sized S^0 on various soil properties.
2. GO interferes with sulphur oxidation.
3. The co-effect of GO and S^0 depends on the sulphur particle size.

2. Material and methods

2.1. Preparation and characterization of materials

The synthesis procedures for micro-/nano-sized composites, consisting of elemental sulphur and GO, were carried out as previously described [72] in the liquid phase, using ultrapure water. Prior to the further dilution of the produced stock suspensions, all materials were sonicated twice for 5 min with a 2 min break, employing an ultrasound needle Bandelin Sonopuls HD 2070 homogenizer (Berlin, Germany), operating at a frequency of 20 Hz. Subsequently, they were agitated for 24 h at 140 rpm.

The composite product of nano-/micro-S^0 + GO underwent identical characterization as described in the preceding study [72]. This involved employing the same method for surface characterization (SEM conducted on a Tescan MAIA 3 equipped with a FEG; Tescan Ltd., Brno, Czech Republic), dynamic light scattering (DLS, using the Zetasizer Nano ZS instrument; Malvern Instrument Ltd., Worcestershire, UK), atomic force microscopy (AFM, utilizing a Bruker Dimension FastScan microscope; Bruker Nano Surfaces, Santa Barbara, CA, USA), and C/H/N/S/O elemental analysis (conducted using FLASH 2000 organic elemental analyser; ThermoFisher Scientific Inc., Waltham, MA, USA).

2.2. Pot experiment design

The experimental soil was collected from a rural area near the town of Troubsko, Czech Republic (49°10′28"N 16°29′32"E). The soil was taken from a depth of 0–15 cm and characterized as a silty clay loam according to the USDA Textural Triangle and classified as a Haplic Luvisol according to the WRB soil classification system. To eliminate coarse particles, the soil was sieved through a mesh with mesh with a 2 mm aperture.

The following soil properties were determined: total C 14.0 g kg^{-1}, total N 1.60 g kg^{-1}, available other nutrients - P 0.097 g kg^{-1}, S 0.145 g kg^{-1}, Ca 3.26 g kg^{-1}, Mg 0.236 g kg^{-1}, K 0.231 g kg^{-1} and pH ($CaCl_2$) 7.3. Total C and N were measured using the Vario Macro Cube (Elementar Analysensysteme GmbH, Langenselbold, Germany) according to Refs. [82,83], P, S, and mineral was measured by inductively coupled plasma mass spectrometry (ICP-MS) in the soil extract prepared according to Mehlich 3 standard method [84], and pH($CaCl_2$) was measured according to ISO 10390:2005 [85].

A quantity of 250 g of soil was mixed with 50 mL of a suspension containing either graphene oxide (GO), or nano- (S^{0-NP}) or micro-elemental sulphur (S^0), or a composite of both GO and S^0 (or S^{0-NP}) at various concentrations, as detailed in Table 1. The used concentrations were chosen to obtain modified (higher) doses of S^0 (S^{0-NP}) and the equal doses of GO as used in authors' previous work [80]. Each treatment was replicated three times. The soil mixtures were then transferred to pots of size 9 × 9 × 10 cm, randomly placed in a growth chamber (CLF Plant Climatics GmbH, Germany) and incubated under controlled conditions for 32 weeks: full-spectrum LED lighting with an intensity 370 μmol m^{-2} s^{-1}, a 12-h long photoperiod, temperature (day/night) 20 °C/12 °C, relative air humidity (day/night) 45 %/70 %, soil moisture maintained at 65 % of the water holding capacity.

2.3. Soil sampling and analyses

After 32 weeks, a mixed soil sample was collected from each pot. These soil samples were sieved through a 2 mm mesh sieve and subsampled for further analyses. The air-dried portion of each sample was used to determine pH($CaCl_2$) according to ISO 10390:2005 [85] and for soil sulphur (SW) measurement using an inductively coupled plasma optical emission spectrometer (ICP-OES spectrometer Spectro, Arcos) at a soil:water ratio of 1:5 (w/v) [86].

Freeze-dried subsamples were analyzed for enzymatic activities - arylsulfatase (ARS), phosphatase (Phos), urease (Ure) – according to ISO 20130:2018 [87], and for real-time qPCR analysis of microbial biomass in soil. DNA for real-time qPCR was extracted from soil samples using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, USA). Isolated DNA was quantified using Nanodrop One (Thermo Scientific, USA). The SYBR-Green platform was used on a CFX96 Real-Time PCR detection system (Bio-Rad Laboratories, USA). Real-time qPCR

Table 1
Experimental variants determined by type and dose of amended materials.

Abbr.	Variant	Type of S^0	S^0 conc. [g·L^{-1}]	S^0 dose [g·kg^{-1}]	GO conc. [mg·L^{-1}]	GO dose [mg·kg^{-1}]
I.	Control	–	0	0	0	0
II.	GO	–	0	0	50	10
III.	$_1S^0$	micro-sized	5	1	0	0
IV.	$_1S^0$+GO	micro-sized	5	1	50	10
V.	$_2S^0$	micro-sized	10	2	0	0
VI.	$_2S^0$+GO	micro-sized	10	2	50	10
VII.	$_4S^0$	micro-sized	20	4	0	0
VIII.	$_4S^0$+GO	micro-sized	20	4	50	10
IX.	$_{0.05}S^{0-NP}$	nano-sized	0.25	0.05	0	0
X.	$_{0.05}S^{0-NP}$ + GO	nano-sized	0.25	0.05	50	10
XI.	$_{0.5}S^{0-NP}$	nano-sized	2.5	0.5	0	0
XII.	$_{0.5}S^{0-NP}$ + GO	nano-sized	2.5	0.5	50	10
XIII.	$_1S^{0-NP}$	nano-sized	5	1	0	0
XIV.	$_1S^{0-NP}$ + GO	nano-sized	5	1	50	10

was performed to quantify partial bacterial and fungal genes coding for ribosomal RNA in soil DNA extracts. The primers used were 1108F (5′ ATGGYTGTCGTCAGCTCGTG 3′) and 1132R (5′ GGGTTGCGCTCGTTGC 3′) for bacterial 16S [88], FF390 (5′ AICCATTCAATCGGTAIT 3′) and FR1 (5′ CGATAACGAACGAGACCT 3′) for fungal 18S rDNA [89], AMOA1F (5′ GGGGTTTCTACTGGTGGT 3′) and AMOA2R (5′ CCCCTCKGSAAAGCCTTCTTC 3′) for ammonium-oxidizing bacteria (AOB) [90], RH1-DSR-F (5′ GCCGTTACTGTGACCAGCC 3′) and RH3-DSR-R (5′ GGTGGAGCCGTGCATGTT 3’) for S-reducing microorganisms (dsr) [91].

2.4. Data processing and statistical analyses

Data processing was conducted using Microsoft Excel 365 online version, and statistical analyses were performed using freely available software R, version 4.2.2 [92]. along with the RStudio script editor [93]. Principal component analysis (PCA) and one-way analysis of variance (ANOVA) type I (sequential) sum of squares at a 5 % significance level were used to characterize the relationship between the treatments and selected soil properties [94]. Effect size was measured by using of Eta-squared. Tukey's honestly significant difference (HSD) test was employed for the determination of statistically significant differences among treatments following ANOVA. The factor level means for each treatment were calculated using "treatment contrast" which was also utilized to detect statistically significant difference among factor level means, with the control group serving as the "intercept". The assumption of selected models was verified after statistical analysis, also at a significance level of 0.05, by testing for normality and homoscedasticity using diagnostic plots and appropriate statistical tests [95].

3. Results

3.1. Effects on pH and soil microbial biomass

Based on the results of principal component analysis (PCA, Fig. 1A), approximately 54.7 % of the variation is explained by the first two eigenvalues together (the first eigenvalue 30.8 % and the second is 23.9 %) in this presented experiment. The total contribution of soil properties on PC1 and PC2 together is the following (in descending order): SW, AOB, dsr, Ure and pH($CaCl_2$).

Soil pH($CaCl_2$) was evaluated as a key property, which affects the soil microbiome in terms of abundance, composition, and activity. A sole elemental sulphur (without GO) amendment did not promote soil acidification, as only the $_4S^0$+GO variant showed significantly lower pH ($CaCl_2$) than the Control (Fig. 2A). Contrary to expectations, micro- and nano-S^0 promoted an increase in pH ($CaCl_2$), when applied solely (without GO, except for the $_2S^0$ variant) or in combination with GO, which interaction was distinct for micro-S^0+GO (tendency to an even higher drop in pH ($CaCl_2$)) and for nano-S^0+GO (tendency to a slightly increased pH($CaCl_2$), Table S1). PCA showed that pH ($CaCl_2$) contributed to the variability of soil properties by 16.31 % (in the 1st dimension), and it was positively related to 18S rDNA and negatively to SW (Fig. 1A). Pearson's correlation was the most significant (and positive) between pH($CaCl_2$) and Ure (Pearson's $r(42) = 0.53$ (95 % CI[0.27, 0.72]), $p < 0.001$), wherease it was negative between pH($CaCl_2$) and SW (Pearson's $r(42) = -0.71$ (95 % CI[-0.83, −0.51]), $p < 0.001$) (Fig. 1B).

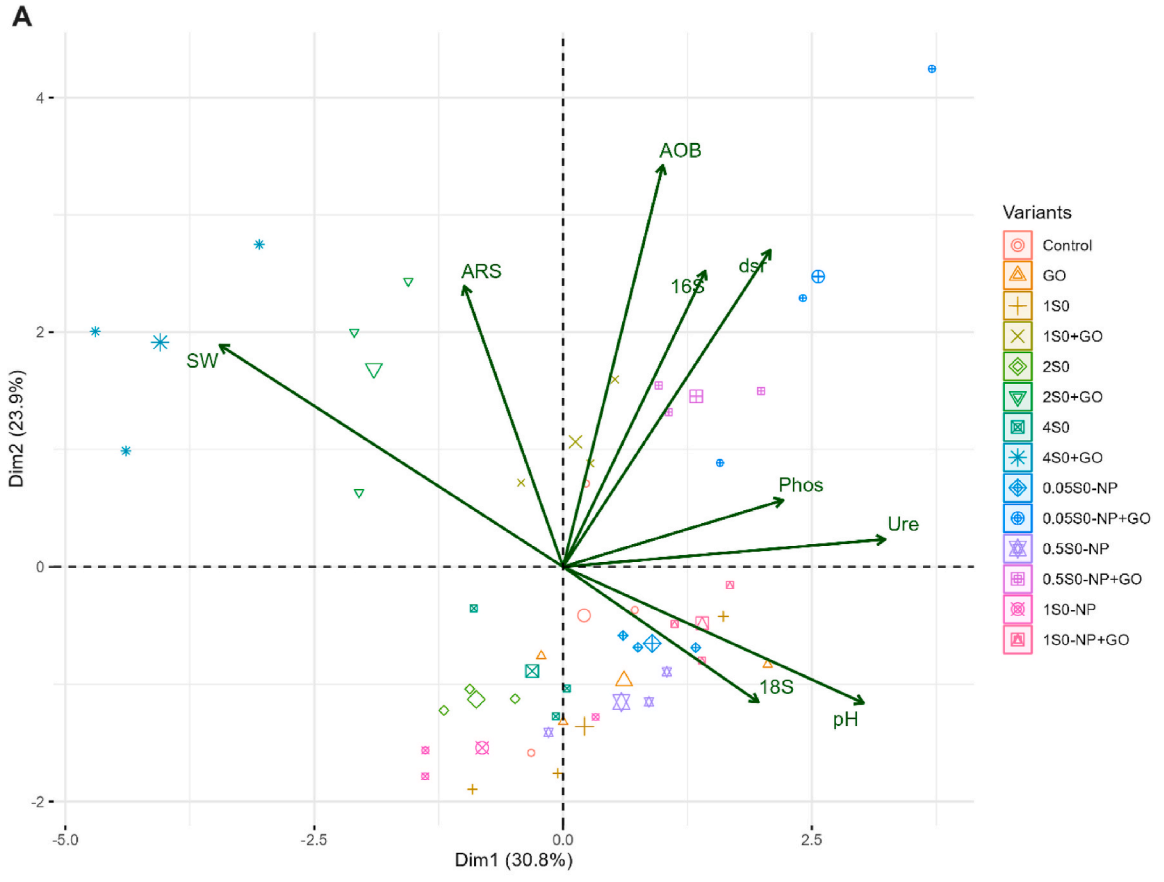
The abundance of gene copies of 16S rDNA was determined as an indicator of total bacterial biomass in soil. Neither sole nor combined (with GO) amendment of elemental sulphur (regardless to the size of particles) changed significantly total bacterial biomass in comparison to the control (and between respective other variants each other; Fig. 2B). The only significant decrease in 16S rDNA was revealed for $_1S^{0-NP}$, which value was lower than in $_{0.05}S^{0-NP}$ + GO. This finding suggested that nano-S^0 has a stronger negative effect (on soil bacterial population) than micro-S^0 and combination of nano-S^0 with GO mitigated the decrease in 16S more than micro-S^0+GO (Table S1). PCA showed that 16S rDNA contributed to the variability of soil properties by 21.41 % (in 2nd dimension), it showed a positive relation with AOB and dsr, corroborated by positive correlation between 16S and AOB (Pearson's $r(42) = 0.64$ (95 % CI[0.41, 0.79]), $p < 0.001$), 16S and dsr (Pearson's $r(42) = 0.44$ (95 % CI[0.16, 0.66]), $p = 0.003$; Fig. 1A and B).

In contrast to the total bacterial biomass in soil, biomass of ammonium-oxidizing bacteria (AOB, determined as the copy number of gene *amoA*) was significantly decreased by medium nano-S^0 dose in the variant $_{0.5}S^{0-NP}$ as compared to the control (Fig. 2C). Despite the statistical insignificance of differences in AOB values, solely amended micro- and nano-S^0 derived lower soil abundance of ammonium oxidizers. On the contrary, co-application of GO with nano-S^0 led to significantly increased AOB in variants $_{0.05}S^{0-NP}$ + GO and $_{0.5}S^{0-NP}$ + GO in comparison to the control and the tendence to higher AOB in all GO + S^0-treated variants was significantly higher under combination nano-S^0 + GO (Fig. 2C–Table S1). AOB contributed to the variability of soil properties by 32.26 % (in 2nd dimension) on PCA biplot, overall it was the second highest contribution to variability (Fig. 1A).

The fungi comprise a fraction of soil microbiome, which are responsive to the elemental sulphur. Therefore, fungi were also determined at the end of experiment as 18S rDNA as the copy number per dry soil weight. However, 18S rDNA did not vary significantly between any of experimental variant and the control, there was revealed on significant effect of GO on 18S values (Fig. 2D). PCA biplot showed negative relation of 18S rDNA to SW and ARS (Fig. 1A) and corroborated by negative relation of 18S and ARS (Pearson's $r(42) = -0.54$ (95 % CI[-0.72, −0.28]), $p < 0.001$).

3.2. Effects on enzymatic activities and sulphur mineralization

Urease (Ure) is an indicator of nitrogen mineralization and deamination, therefore, it is related to the values of AOB in soil. However, only the Ure of the variant $_4S^0$ + GO significantly decreased compared to the control (Fig. 3A), whereas no significant increase in urease activity due to the amendment of micro-/nano-S^0 or GO was observed. However, the combination of nano-S^0 and GO showed a tendency to increase Ure compared to each variant with the respective equal dose of sole nano-S^0 (Fig. 3A–Table S1). Urease



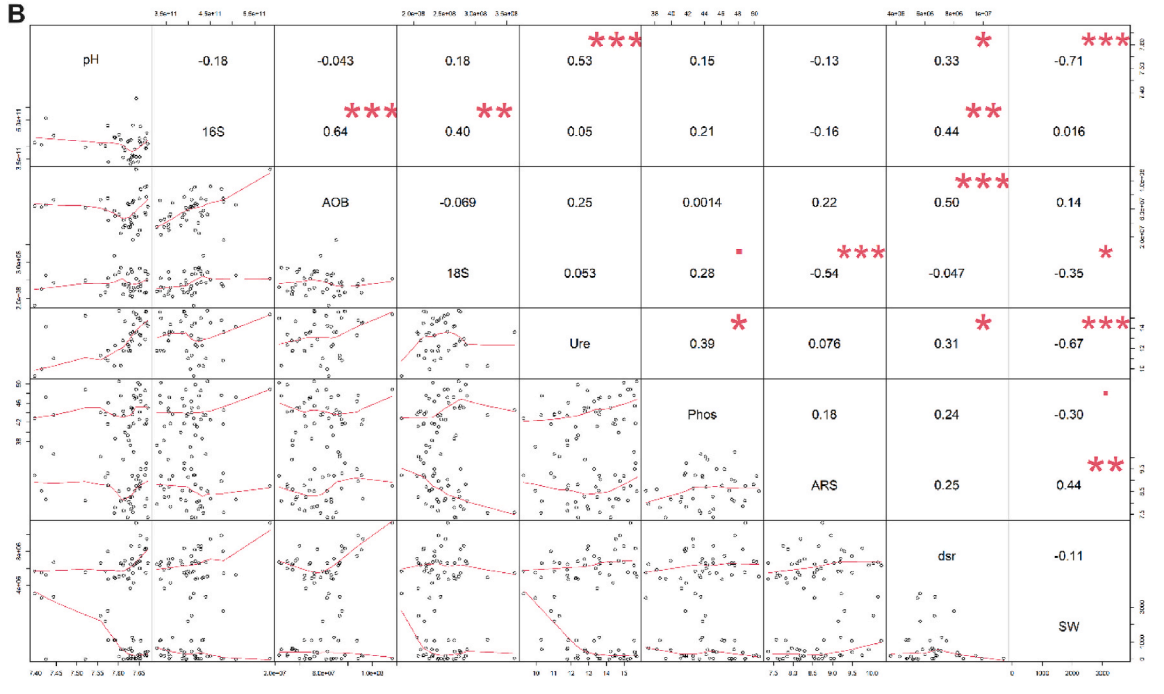


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Fig. 1. Principal component analysis (PCA biplot, A) and Pearson's correlation analysis (B) of soil properties
The length of vector on Rohlf's PCA biplot indicates a strength of contribution of the respective property to the variability among variants in the first two principal components; the values on the Pearson's correlation chart indicate correlation coefficients, calculated on the statistical level of significance indicated by red marks: ˙ $p \leq 0.1$, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

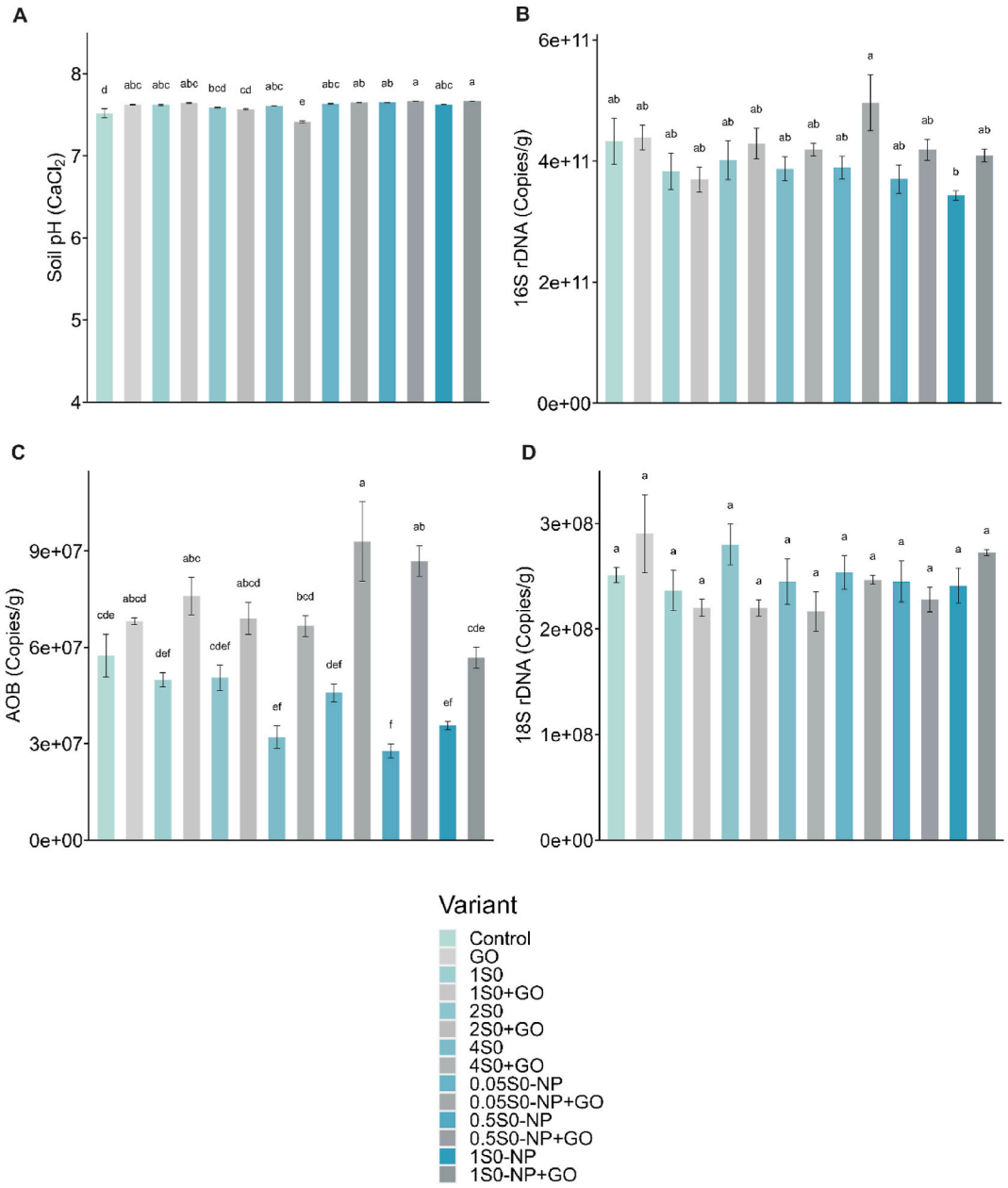

Fig. 2. Soil pH($CaCl_2$) and microbial biomass of soil amended with micro- and nano-sized elemental sulphur, graphene oxide, and combination
Mean values (n = 3) ± standard error of mean of (A) pH ($CaCl_2$), (B) bacterial abundance (16S rDNA), (C) abundance of ammonium oxidizing bacteria (AOB), (D) fungal abundance (18S rDNA). Letters indicate the differences between variants (calculated by Tukey's HSD posthoc test) at the statistical level of significance $p \leq 0.05$.

showed a noticeable contribution to the variability of soil properties on the PCA biplot - by 15.62 % (in the 1st dimension) – with a positive relation to Phos and pH (and a negative with SW, Fig. 1A). Pearson's correlation plot (Fig. 1B) revealed a relation between Ure and SW (Pearson's $r(42) = -0.67$ (95 % CI[-0.81, −0.45]), $p < 0.001$).

Phosphatase activity (Phos) is presumably coupled with S-derived changes in pH($CaCl_2$). However, no significant difference in Phos

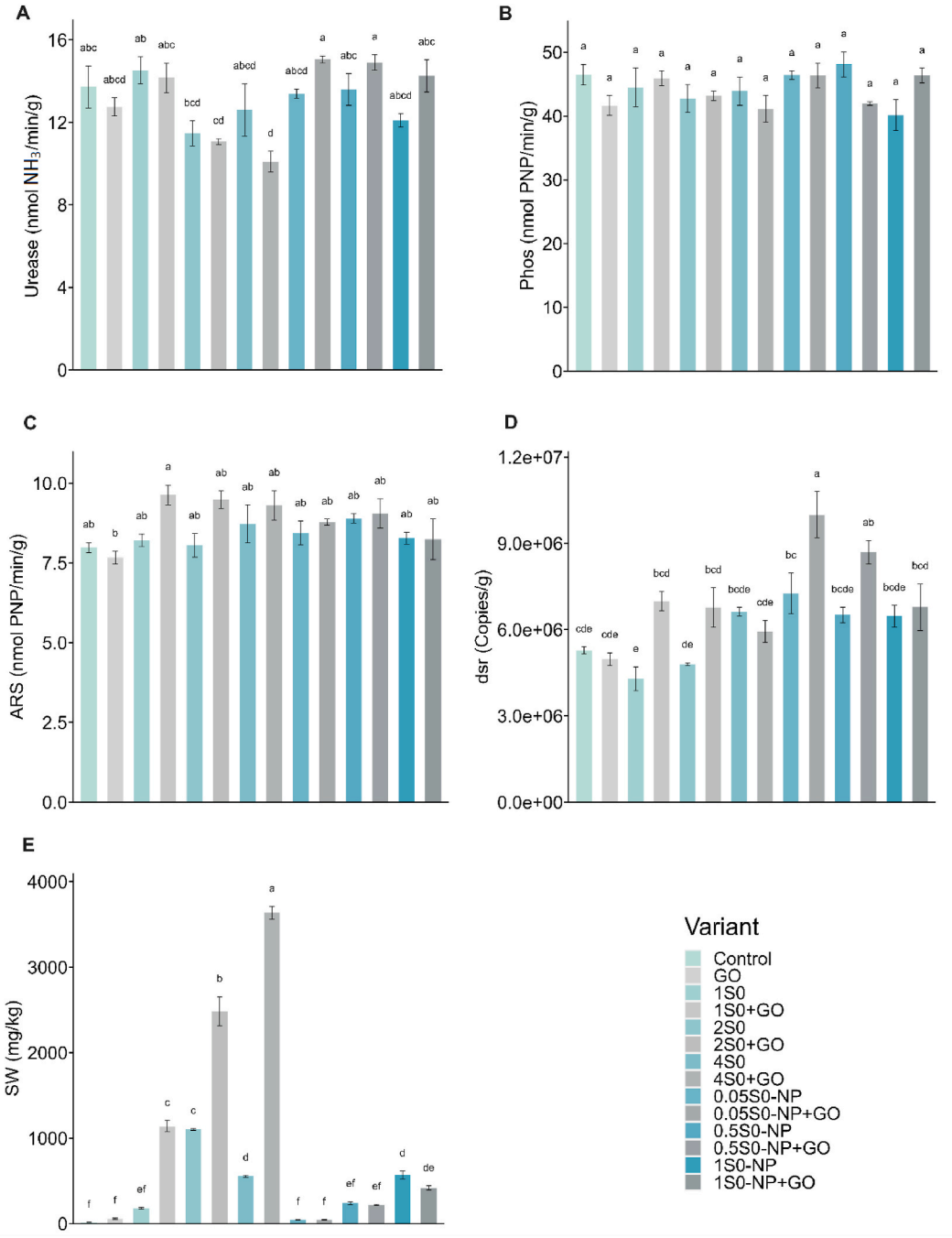

Fig. 3. Enzymatic activities and indicators of sulphur mineralization in soil amended with micro- and nano-sized elemental sulphur, GO, and combination

Mean values (n = 3) ± standard error of mean or (A) urease (Ure), (B) phosphatase (Phos), (C) arylsulfatase (ARS), (D) abundance of sulphur-reducing microorganisms (dsr), (E) soluble sulphur content (SW). Letters indicate the differences between variants (calculated by Tukey's HSD posthoc test) at the statistical level of significance $p \leq 0.05$.

between any of the experimental variant was detected (Fig. 3B). Phos had an overall weak contribution to the variability in the soil properties without significant relation to any of them (Fig. 3B).

Arylsulfatase (ARS), which catalyzes a step in organic sulphate mineralization, differed between most of the variants (and the control) insignificantly, the lowest value was found in the variants GO, significantly decreased only compared to ARS in $_1$ S^0 + GO (Fig. 3C). Nevertheless, GO co-applied with micro-S^0 tended to increase average ARS values compared to the sole micro-S^0-amended variants (Table S1). ARS showed on the PCA biplot a negative relation with pH($CaCl_2$) and 18S rDNA, Pearson's correlation showed the

relation of ARS and 18S (Pearson's r(42) = - 0.54, $p < 0.001$), but revealed also a positive relation between ARS and SW (Pearson's r (42) = 0.44 (95 % CI[0.15, 0.65]), $p < 0.001$, Fig. 3C).

The biomass of soil sulphur-reducing bacteria (measured as the copy number of genes coding for sulphur reductase, dsr) was comparable between variants, except for those with GO and nano-S^0 (where dsr significantly increased in $_{0.05}S^{0-NP}$ + GO and $_{0.5}S^{0-NP}$ + GO) when compared to the control (Fig. 3D). GO co-amended with micro-/nano-S^0 (namely 1 and 2 g micro-S^0 ·kg^{-1} and 0.05, 0.5 g nano-S^0 ·kg^{-1}) tended to increase the abundance of dsr in comparison to the variants solely amended with micro-/nano-S^0 (Table S1). PCA showed that dsr contributed to the variability of soil properties by 25.75 % (in 2nd dimension), its relations to the other properties were already mentioned.

Water-soluble sulphur (SW), represented in soil mostly by sulphates (SO_4^{2-}), was found (in comparison to the Control and GO variants) significantly increased in all variants amended with ≥1 g kg^{-1}. GO combined with micro-S^0 significantly increased SW values compared to the sole micro-S^0-amended variants (Table S1), but no similar effect was observed for GO + nano-S^0 (Fig. 3E). PCA showed that SW contributed to the variability of soil properties by 25 % (in the 2nd dimension), its relations to the other properties were already mentioned.

4. Discussion

4.1. Discussion to effects on soil microbial biomass

The use of nano-sized materials, including elemental sulphur (S^0), in agriculture has been explored for their potential to enhance nutrient delivery to plants due to their high surface area-to-volume ratio, which can increase the reactivity and solubility of nutrients. The transformation of elemental sulphur (S^0) into a soluble form is a redox and pH-modifying process that can lead to a decrease in soil pH though the production of sulphurous or even sulphuric acids, depending on pH, moisture content, microbial activity, and the presence of other substances in the soil [96]. However, it may also stabilize pH depending on the soil's mineral composition though the formation of sulphates with alkaline ions like Ca^{2+}. While most studies have reported significant soil acidification following the addition of elemental sulphur [28,40,97], our experiments revealed a contrasting increase in pH in the tested soil due to S^0 addition. This effect was particularly pronounced when GO was combined with higher doses (0.5; 1.0 g kg^{-1}) of nano-sized S^0. A similar non-acidifying (insignificant) change in soil pH under increased ionic strength and a dose of 0.2 g kg^{-1} of soil was reported in the study of Zhao et al. [98]. Although GO, especially when combined with micro-sized elemental sulphur, enhanced S^0 oxidation (as detailed in section 3.2, Fig. 3E), and increased bacterial abundance in the soil (section 3.1, Fig. 2B), it likely mitigated acidification through its deprotonation, leading to the formation of reduced GO (rGO). Similar enhanced formation of rGO via proton effects and microbial activity has already been reported [99,100]. This notion supports our hypothesis No. 2 about GO interference with sulphur oxidation.

Sulphur-oxidizing bacteria serve as the primary mediators of elemental sulphur oxidation [28,33,101] and their abundance changes in response to S^0 amendments in soil. Moreover, S^0 oxidation is typically accompanied by changes in the abundances of other bacterial groups [30,31,33,102], reflecting overall alterations in soil conditions, including nutrient transformation processes. While the reduction of total bacterial biomass (16S rDNA) due to S^0 amendment was statistically insignificant, it aligns with the referred negative impacts of sulphur oxidation intermediates (e.g. SO_2) on certain soil bacteria [72,103,104]. Sole application of GO insignificantly affected bacterial abundance, consistent with negligible or no effect on absolute microbial biomass [66]. However, other studies have reported positive effects of GO on relative bacterial abundance [69,105,106]. This may explain the positive effect of GO on the bacterial biomass in the double-amended (S^0 + GO) variants, attributed to specific amplification of sulphur oxidizers, as referred in several studies [13,31,107]. This GO-mediated mitigation of S^0-induced reduction in bacterial abundance, particularly significant in nano S^0-amended soil, supports hypotheses 2 and 3 (about GO interference with sulphur oxidation and dependence of GO and S^0 co-effect on the sulphur particle size).

PCA and Pearson's correlation analyses revealed a mutual relation between 16S rDNA and the indicator of nitrification AOB (as well as dsr microbial indicators, Fig. 2C; 1A,B; 3D), supporting the hypothesis that changes in absolute bacterial abundance depend largely on shifts in the relative richness of distinct bacterial groups. The observed tendency towards S^0-specific reduction in ammonia-oxidizing bacteria abundance (though significant only in the $_{0.5}S^{0-NP}$ + GO variant) and the indirect dependence of AOB on S^0 amendments to soil have been observed by other authors under conditions of very low pH [108]. While, in our work we observed that GO insignificantly affected AOB values (Fig. 2C), this contrasts with the reported adverse effect of GO on the abundance of ammonia oxidizers and nitrifiers [105,109]. On the contrary, significantly higher AOB in variants $_{0.05}S^{0-NP}$ + GO, $_{0.5}S^{0-NP}$ + GO suggest a beneficial effect of combined GO + S^0 on the nitrification process, possibly due to GO's ability to adsorb ammonium, as reported by Wu et al. [110]. Furthermore, GO likely contributed to alleviating the negative impact of sulphur oxidation intermediates on the soil bacterial biomass (as mentioned earlier), as sulphites exhibit high reactivity with GO (leading to its reduction) [111]. Considering the elemental sulphur effect again, variants S^0+GO (both micro- and nano-sized) exhibited an indirect dependence between AOB and the dose of elemental sulphur (Fig. 2C). A significant relation of AOB, ARS, and dsr supports the presumption of broad-effective, nano-S^0+ GO-promoted enhancement in the abundance of nitrifying and sulphur reducing bacteria, which modifies of sulphur mineralization and availability in soil, as evidenced by the sulphur content in soil. These findings regarding the specific effect of nano-S^0+ GO support hypotheses 2 and 3 (about GO interference with sulphur oxidation and dependence of GO and S^0 co-effect on the sulphur particle size).

Soil fungal abundance has been reported to be significantly affected by elemental sulphur [112], as fungi belong to the important group of S^0-oxidizers [30,33]. However, this study revealed only insignificant differences between the tested variants in fungal abundance (determined as 18S rDNA copy number, Fig. 2D). Nevertheless, there was a detectable tendency towards the most negative effect of combined micro-S^0+GO on 18S values of the respective treated variants. This observation suggests that GO tended to reduce

fungal biomass in combination with elemental sulphur, which contrasts with the reported positive impact of GO on fungal growth in soil [113] due to its partial ability to metabolize GO. Contrary results regarding fungal biomass change (determined by of N-acetyl-β-D-glucosaminidase) in soil with nano-S^0 + GO, planted with lettuce, were reported by Hammerschmiedt et al. [72] too. The insignificance of the differences found for 18S values in this study, in contrast to the results reported by Hammerschmiedt et al. [72], could be attributed to distinct factors impacting the soil microbiome, including the absence of a plant rhizosphere effect.

4.2. Discussion to effects on enzymatic activities and sulphur mineralization

Enzyme activities such as Ure (an indicator of nitrification), Phos (with a phosphate solubilizing function), and ARS (involved in sulphur mineralization) were consistently unaffected by solely amended GO (Fig. 3A–C). In contrast, the amendment of the tested soil with higher doses of sole elemental sulphur (specifically micro-S^0) tended to negatively impact urease activity (an indicator of nitrification), conversely to the reported positive impact of elemental sulphur on Ure [72,114–116]. Thus, the hypothesis 1 was disproven (i.e. nano-sized S^0 did not exert a larger effect than micro-sized S^0 on various soil properties). This relation between S^0 and both Ure and AOB suggests an adverse effect of sulphur oxidation processes on soil nitrification. GO in combination with nano-S^0 tended to positively effect Ure (Fig. 3A, Tab, S1), which was in contrast to other authors' findings [105,117]. Although inconsistent with previous studies, this finding verifies the hypotheses 2 and 3 (about GO interference with sulphur oxidation and the dependence of GO and S^0 co-effect on the sulphur particle size). The positive impact of GO combined with nano-sized elemental sulphur has been observed previously [72].

A less pronounced positive relation of pH and Phos (PCA Fig. 1A) suggests that the increased pH with S^0 amendment could correspond to uninhibited phosphatase activity, partially in line with increased Phos activity in response to elemental sulphur addition to soil [72,114–116,118]. Nevertheless, Phos values in S^0-treated variants remained unchanged, with insignificant changes compared to the Control values (Fig. 3B). Similarly, the combined impact of GO and S^0 was insignificant, contrary to overall findings in other studies [66,117,119]. Again, these results were mainly attributed to minimally changed soil pH (without acidification).

Nano-sized S^0, as well as other types of elemental sulphur [13,114,120], added to soil, have been previously shown to enhance ARS activity due to increased S-transformation rates and cycling in soil. However, ARS tended to be enhanced only by the amendment of sole nano-S^0 only in one variant ($_{0.5}S^{0-NP}$) in this study (Fig. 3C); neither sole micro-S^0 treatment exhibited an increase in average ARS (except for $_4S^0$). GO did not positively affect ARS when applied alone, in line with other studies [72,121]. In contrast, the co-application of GO and elemental sulphur showed a tendency to enhanced ARS in both nano-S^0+GO and micro- S^0+GO combinations, particularly promoting a significant increase in enzyme activity in $_1S^0$+GO, compared to the GO variant (Fig. 3C). This is another piece of evidence of GO-coupled beneficial effect on sulphur mineralization in soil, corroborating hypothesis 2 (about GO interference with sulphur oxidation), although hypotheses 1 and 3 (about the larger effect of nano-sized S^0 and about the dependence of GO and S^0 co-effect on the sulphur particle size) were disproven.

The weaker positive impact of nano-S^0 combined with GO on ARS was attributed to the generally smaller yield of sulphur oxidation, indicated by lower sulphur content (Fig. 3E), as well as higher sulphur-reducing bacteria biomass, dsr (Fig. 3D). ARS might have also been slightly negatively affected by the reductive transformation of sulphur, as abundancies of dsr were increased on average in all nano-S^0-treated variants compared to the Control (Fig. 3D), but only in one micro-S^0-treated variant ($_4S^0$), which exhibited a much higher content of sulphur (Fig. 3E). Therefore, it was presumed that the processes of sulphur oxidation and reduction occurred simultaneously, interdependently, and were differently promoted by micro- and nano-sized elemental sulphur, with the latter likely promoting higher overall solubility and reactivity, affecting both processes more significantly. The amplification of sulphur-reducing bacteria in soil due to S^0 addition has been previously reported [32]. Although solely added GO did not increase the abundance of dsr (compared to the Control), GO co-amended with S^0 (both micro- and nano-sized) tended to multiply the biomass of sulphur reducers, which was coupled with significant increase in dsr of nano-S^0+GO variants and brought verification of the hypothesis 2 (about GO interference with sulphur oxidation). However, Table S1 showed that at low and moderate micro- S^0 dose (+GO), the increase in dsr biomass was proportionally higher compared to sole elemental sulphur treatment than was revealed from nano-S^0+GO, partially disproving hypothesis 3 (dependence of GO and S^0 co-effect on the sulphur particle size).

The content of soluble sulphur (Fig. 3E) was more positively affected by lower to moderate doses of solely amended micro-S^0 (1, 2 g kg^{-1}) than nano-S^0 (at doses 0.05, 0.5 g kg^{-1}). Moreover, a much stronger positive impact of co-amended micro-S^0 and GO on SW content was observed, which was not found for nano-sized elemental sulphur. While the benefit and direct dependence of elemental sulphur on the availability of solubilizes sulphur in soil is well known [26,101,122], the role of GO in the process of S^0 complete oxidation to sulphates and their solubilization has been largely unknown and unreported until know. As hypothesized (point 2), GO, as a potential electron acceptor, likely interferes more with molecular nanosulphur in the process of oxidation and electron reception [123], as well as though higher absorption of S^0 nanoparticles on its surface, hindering further solubilization, while providing S^0 for bacterially-mediated reduction (indicated by dsr).

The distinct responses of soil enzymes such as urease and arylsulfatase to amendments with micro-S^0+GO and nano-S^0+GO suggest that the size and combination of these materials modulate enzyme activities in soil. This is in line with the understanding that soil enzymes, which are crucial for nutrient cycling and organic matter decomposition, can be sensitive to changes in soil chemistry and microbial community structure induced by amendments. Urease activity, being central to nitrogen cycling, and arylsulfatase, important for sulphur cycling, showing differential responses indicate that nano-materials could be engineered to target specific nutrient cycling pathways. This aligns with existing knowledge that suggests nano-materials can influence soil enzyme activities, either by directly affecting the enzymes or by altering the soil environment and microbial communities that produce these enzymes.

In summary, the findings, how nano-S^0 and GO affect soil properties, microbial biomass, and sulphur transformation processes,

align with current knowledge in the fields of nanotechnology and soil science. They contribute to an evolving understanding of how these materials can be used to address specific agricultural challenges like sulphur deficiency in soils. However, it is needed also to highlight the importance of careful consideration of their broader environmental impacts, because the growing interest in using nanomaterials in agriculture includes a parallel concern for their environmental and safety implications. There is still a need for comprehensive assessments of their long-term impacts on soil health, crop safety, and potential accumulation in the food chain.

5. Conclusions

The results of the pot experiment support the conclusion that the particle size of elemental sulphur is crucial for its oxidation in soil, which is coupled with distinct effects of nano-S^0 and micro-S^0 mainly on the content of soil-soluble sulphur, the abundance of sulphur-reducing bacteria (dsr), ammonia-oxidizers (AOB), activities of enzymes such as urease and arylsulfatase, and soil pH($CaCl_2$). While micro-S^0 tended to enhance the transformation to sulphates more, nano-S^0 likely contributed to sulphur reduction by dsr microbes, presumably due to a different rate in sulphur mineralization. The combination of both S^0 types with graphene oxide (GO) in the composite amendments emphasized these differences even further, which were formulation- and dose-dependent. The lowest dose of nano-S^0 in composite with GO was the most stimulating for soil nitrogen mineralization, but surprisingly, it also increased the abundance of dsr (sulphur reducers). On the contrary, the most enhanced S^0 oxidation likely resulted from the highest dose of micro-S^0 in composite with GO, which was coupled with significant soil acidification and concurrent mitigation of sulphur reduction and nitrogen mineralization. Finally, the amendment of micro-S^0+GO increased the content of soluble sulphur more (in comparison to nano-S^0) both without or with GO. Under the conditions of this soil experiment, GO with both types of S^0 exhibited a noticeable effect on the oxidation processes of both sulphur and nitrogen. In conclusion, while the results regarding the effects of GO and nano-S^0 on soil properties, microbial communities, and enzyme activities are consistent with the broad trends observed in the application of nanotechnology in agriculture, they also provide new insights into the potential for these materials to be used in combination or synergistically to address specific soil and crop management challenges. Further research, especially field-based studies, is essential to fully understand the long-term implications of these findings and to optimize the use of nanotechnology in sustainable agriculture practices.

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

CRediT authorship contribution statement

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Declaration of competing interest

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

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Appendix A. Supplementary data

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