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# Microbial synergy in fertilizer composites increases the uptake of nutrients by plants

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## Abstract

Intensive fertilization has been required in order to provide the nutrients needed for plant growth under the current agricultural practices being applied to meet the global food demands. Micronutrients such as zinc, manganese, and copper are required in small quantities when compared to macronutrients (such as nitrogen, phosphorus and potassium), but they are essential for the plant growth cycle and consequently for increasing productivity. Mineral oxides such as ZnO, MnO, and CuO are used in agriculture as micronutrient sources, but their low solubility limits practical applications in plant nutrition. Similarly, elemental sulfur ($S^0$) can provide a high concentration source of sulfate, but its availability is limited by the ability of the soil to promote $S^0$ oxidation. We propose here the integration of these nutrients in a composite based on a biodegradable starch matrix containing mineral oxides and $S^0$ in a dispersion that allowed encapsulation of the acidifying agent *Aspergillus niger*, a native soil fungus. This strategy effectively improved the final nutrient solubility, with the composite starch/$S^0$/oxide$_{\text{mixture}}$ multinutrient fertilizer showing remarkable results for solubilization of the oxides, hence confirming a synergic effect of $S^0$ oxidation and microbial solubilization. This composite exhibited an extended shelf life and soil-plant experiments with Italian ryegrass (*Lolium multiflorum* Lam.) confirmed high efficiencies for dry matter production, nutrient uptake and recovery. These findings can contribute to the development of environmentally friendly fertilizers towards a more sustainable agriculture and could open up new applications for formulations containing poorly soluble oxide sources.

**Keywords:** Oxides solubilization, sulfur oxidation, starch composite, *Aspergillus niger*, eco-friendly fertilization.

## Introduction

The intensive use of soils for crop production and livestock pasture, together with inappropriate soil agricultural practices, has decreased their natural fertility. Consequently, intensive fertilization has been required in order to provide the nutrients needed for plant growth. Micronutrients such as zinc, manganese, and copper are required in small quantities when compared to macronutrients (such as nitrogen, phosphorus and potassium), but they are essential for the plant growth cycle and consequently for increasing productivity [1, 2]. Generally, these micronutrients are provided to plants by applying highly soluble sulfate and chlorate salts [1, 2]. However, these soluble nutrient sources are produced from their corresponding oxide minerals by chemical processing using strong inorganic acids and high-energy consumption, which results in generation of industrial waste that cause harmful environmental impacts. Hence, the direct use of oxide mineral sources as plant micronutrients would be desirable in order to reduce both environmental impacts and costs, with the additional advantage of providing higher nutrient contents than the corresponding salts [3, 4]. However, the high chemical stability of the oxides limits their solubility to a level far lower than that required for practical applications [5].

Recent studies have shown that the solubility of oxides increases when they are dispersed in elemental sulfur ($S^0$) matrices, due to the local acidification promoted by $S^0$ oxidation [6, 7]. However, $S^0$ oxidation is limited by the microbiological soil activity and in practice tends to be very low, making this strategy unfeasible [8-10]. An ideal fertilizer product should provide the means to transport microorganisms together with the nutrients, as well as provide a good environment for microorganism activation and proliferation. For this purpose, the native soil filamentous fungus *Aspergillus niger* has been used to promote the solubilization of phosphorus from phosphate minerals, due to its excellent ability to produce organic acids capable of complexing (chelating) metals from oxides [11, 12]. In addition, previous studies have found that *A. niger* can promote the biological oxidation of $S^0$ [6, 7, 13, 14]. Therefore, the challenge is to develop a material that would favor the interaction of mineral oxide particles with particles of $S^0$, as well as enable the simultaneous encapsulation of microorganisms in a single granule. A possible option would be to apply the concept of "bioreactor granule", which has been successfully demonstrated to improve the solubilization of phosphate minerals [7, 15]. This strategy is based on the preparation of a composite using gelatinized starch as a matrix for the encapsulation of microrganisms and the simultaneous dispersal of mineral particles to form a granule.

The present work proposes the development of a multi-nutrient fertilizer based on starch composites, which is biologically activated by *A. niger* in order to supply micronutrients and S to plants. Three commercial mineral oxides of low reactivity ($ZnO_{Mineral}$, $MnO_{Mineral}$, and $CuO_{Mineral}$) and $S^0$ were used to produce different composite formulations. The results indicated a synergic effect among elemental sulfur, the mineral oxide dispersion and microorganism activity, resulting in improved efficiency of nutrient uptake by plants.

## Materials and Methods

The filamentous fungus *Aspergillus niger C* (BRMCTAA 82), obtained from the Embrapa Food Technology collection (Rio de Janeiro, RJ, Brazil), was used here as a model microorganism to promote oxide solubilization. Among eight filamentous fungi, this *A. niger* strain was selected by Klaic et al. [11] as the best for the solubilization of phosphate minerals. Spore suspensions of the fungus strain were kept at -18 °C in a solution of water with glycerol (10 wt.%) and NaCl (0.9 wt.%). Spores were germinated at 30 °C on Petri dishes containing potato dextrose agar. After 96 h, a suspension of grown spores was harvested by adding distilled water. The spore concentration was determined using a Neubauer chamber.

## Materials

Corn starch (St) (Amidex 3001, 30% amylose and 70% amylopectin) was supplied by Ingredion (Brazil), urea was from Synth (Brazil), and elemental sulfur (86 wt.%) was from Sigma-Aldrich (St. Louis, USA). The mineral oxide sources of zinc oxide ($ZnO_{Mineral}$), manganese oxide ($MnO_{Mineral}$), and copper oxide ($CuO_{Mineral}$) were supplied by Heringer Fertilizers (Brazil). Sulfurgran®, a commercial fertilizer source of S, was supplied by Produquímica Fertilizers (Brazil). All the materials were used as received. The chemical compositions of the three mineral oxides and the elemental sulfur were characterized by X-ray fluorescence and X-ray diffractometry, while the morphologies were analyzed by scanning electron microscopy. In addition, the particle size and surface area were measured by dynamic light scattering and isothermal nitrogen adsorption, respectively. Details concerning the characterization methods used are provided in the Supplementary Material.

## Biological solubilization of oxides

Experiments were carried out to evaluate the potential of *A. niger* to solubilize mineral oxides under submerged culture. Firstly, the pre-culture was initiated in 250 mL Erlenmeyer flasks containing 50 mL of the nutrient medium (Czaped Dox) and the density of spores adjusted was 1.2x$10^7$ spores per mL of the nutrient medium. Czaped Dox nutrient medium was used, as described by Grayston et al. [13], containing (w/v): sucrose, 3%; $NaNO_3$, 0.3%; NaCl, 0.2%; $MgCl_2.7H_2O$, 0.05%; KCl, 0.05%; $KH_2PO_4$, 0.1%. The incubation for the pre-culture step was carried out for 48 h in an orbital shaker incubator, at 30 °C and 220 rpm. After, solubilization of the oxides was initiated by transferring a volume of pre-culture suspension corresponding to 10% (v/v) to 50 mL of a new culture medium with the same composition. In this cultivation step, the oxides were added and the suspension consisting of cells and oxides was kept for 96 h in an orbital shaker incubator at 30 °C and 220 rpm. The mass of element (Zn, Mn and Cu) added was standardized to obtain a concentration of 50 mg/L. Hence, the mineral oxides presents different impurities, the masses of $ZnO_{Mineral}$, $MnO_{Mineral}$, and $CuO_{Mineral}$ added were 4.22, 5.00, and 11.34 mg, respectively. After the bioactivation period, the resulting suspension was vacuum filtered using Whatman No. 1 filter paper, followed by centrifugation for 20 min at 11000 rpm and 20 °C. The clear supernatant was analyzed for the determination of solubilized Zn, Mn, and Cu, using inductively coupled plasma (ICP). Details concerning the ICP method are provided in the Supplementary Material. All experiments were carried out in triplicate and the data were calculated as means ± standard deviations.

## Preparation of the composites

The first step in preparation of the composites was the gelatinization of starch (St) by dispersing 8 wt.% starch and 1 wt.% urea in distilled water (250 mL) and keeping the dispersion at about 90 ºC for 30 min, under stirring, until a sticky starch paste gel was formed, following the protocol proposed by Giroto et al. [16]. The oxide particles were then transferred to the medium and were dispersed in the starch gel by vigorous agitation for 15 min. The oxides were added to the starch paste gel using a proportion of 1:2 between starch (33.6 wt.%) and the oxide/mixed oxides (66.4 wt.%). For the composites with elemental sulfur, the oxides were mixed with the elemental sulfur using a beaker and spatula, prior to dispersion in the starch gel. The different proportions of starch, oxides, and elemental sulfur used to produce the composites are shown in Table S1, together with their nomenclatures. After dispersion of the oxides, the temperature was reduced to 30 ºC for incorporation of the *Aspergillus niger* spores (spore suspensions were prepared as previously described, in order to achieve a concentration of 1x$10^8$ spores per gram of the material). The mixed gel was kept at

40 ºC for at least 12 h, under air circulation, in order to obtain a dry solid gel. The dried composite was ground in a laboratory knife mill (Model MA048, Marconi), with particles sized 1-4 mm being collected using a sieve and stored in dry boxes. The composites were characterized by scanning electron microscopy, energy dispersive X-ray spectroscopy, and X-ray tomography. Details concerning these characterization methods are provided in the Supplementary Material.

## Bioactivation of the composites

Incubation of the composites in a liquid medium was conducted to evaluate the potential of *A. niger* encapsulated in the matrix to germinate and solubilize the mineral oxides during fungal growth. The nutrient medium used was Czapek Dox, as described in Section 2.3. Cultivations were performed in 250 mL Erlenmeyer flasks by adding a volume of 50 mL of the nutrient medium. The incubation was carried out in an orbital shaker incubator, at 30 °C and 220 rpm. The mass of element added was standardized to obtain the same concentration in solution (50 mg $L^{-1}$). Hence, the masses of composites St/Zn, St/Mn, St/CuO, St/Mix, and St/Mix+S added were 6.33, 7.50, 17.00, 30.8, and 65.96 mg, respectively. All the experiments were carried out using triplicates. After this period, the resulting material was vacuum filtered using Whatman No. 1 filter paper and was then centrifuged for 15 min at 11000 rpm and 20 °C. The clear supernatant was analyzed by ICP to determine the concentrations of solubilized Zn, Mn, and Cu. The sulfate ($SO_4^{2-}$) produced from the oxidation of $S^0$ was determined by the turbidimetric method proposed by Camargo et al. [17]. Details concerning these methods are provided in the Supplementary Material.

## Soil-plant experiments

The efficacies of the St/Mix+S composites as fertilizer sources of Zn, Mn, Cu, and S for plants were evaluated in a greenhouse experiment conducted at Embrapa Pecuaria Sudeste, in São Carlos (São Paulo State, Brazil). The composites were applied to two Oxisols, soil series of São Carlos (a sandy loam and a clay soil) that were poor in organic matter, Zn, Mn, Cu, and S. The two soils were collected at pasture sites, at a depth of 0-20 cm. Before use, the soil was air-dried, crushed to pass through a 2 mm screen in order to obtain a homogeneous soil granulometry, and chemically and physically characterized [18, 19]. The chemical and physical properties of the soils are shown in Table S3 (Supplementary Material).

The trial followed a fully randomized block design with three treatments and four replicates, together with four additional pots to serve as unlabeled controls (without S, Cu, Zn, and Mn fertilization), resulting in 16 pots that were each filled with 3 kg of soil. The forage was grown in 4 L ceramic pots. At the start of the experiment, the base saturation "V" of the soils was increased to 60% by liming, as described by Cantarella and Furlani [20]. Before planting the seeds, the soil samples were homogenized and incubated for 30 days, with the moisture content of the soil maintained close to 70% of its water retention capacity. About 20-30 seeds of Italian ryegrass (*Lolium multiflorum* Lam.) were sown at a depth of 1 cm from the soil surface. An initial fertilization with macro and micronutrients (except Zn, Mn, Cu, and S) was performed immediately after sowing, with the application of 82 mg kg$^{-1}$ of P and 70 mg kg$^{-1}$ of N as diammonium phosphate (DAP) onto the soil surface. Each pot also received a solution containing 130 mg kg$^{-1}$ of N (as urea), 118 mg kg$^{-1}$ of P and 150 mg kg$^{-1}$ of K (as $KH_2PO_5$), 0.8 mg kg$^{-1}$ of B (as $H_3BO_3$), and 0.15 mg kg$^{-1}$ of Mo (as $(NH_4)_6Mo_7O_{24}$), which was prepared as described by Novais et al. [21]. The St/Mix+S composite fertilizer was transferred to the surface of the soil at a suitable rate to supply 100 mg kg$^{-1}$ of S and 12 mg kg$^{-1}$ of Cu, Zn, and Mn. The S and micronutrients were also supplied, in the same ratio, as a mixture of oxides + Sulfurgran® or as soluble sources of micronutrients ($CuCl_2$, $ZnCl_2$, and $MnCl_2$) + Sulfurgran®. In each treatment, the fertilizers were applied to the surface of the soil. After fertilization and sowing of Italian ryegrass, the soil moisture was adjusted by irrigation whenever necessary (determined visually), by gently applying water. After emergence of the Italian ryegrass, thinning was performed in order to obtain a final stand of five plants per pot.

The dry matter production by the Italian ryegrass was evaluated in 5 consecutive cuts, made at a height of 5 cm to allow for subsequent regrowth, at 40, 55, 70, 90, and 130 days after fertilization. The plant material collected in each cut was dried in a forced-air oven at 70 °C for 72 h, followed by weighing the material and grinding to <1 mm with a Wiley mill. Samples of the dry matter were digested in nitric-perchloric acid solution, as described by Miyazawa et al. [22]. The solution from the digestion was analyzed by ICP to determine the concentrations of solubilized S, Zn, Mn, and Cu.

Uptake of the nutrients (S, Zn, Mn, and Cu, mg of micronutrient per pot$^{-1}$ units) was calculated from the dry matter mass of each cut (DM, in kg pot$^{-1}$) and the nutrient content (NC, in mg kg$^{-1}$) obtained by ICP, using Equation 1:

$$\text{Nutrient uptake} = DM \times NC \qquad \text{Equation 1}$$

The crop recovery efficiency (RE, mg increase of nutrient uptake per mg of nutrient applied to the soil) was calculated using Equation 2:

$$RE = (U1 - U0) / F \qquad \text{Equation 2}$$

where F is the amount of fertilizer nutrient applied, U1 is the total S, Zn, Mn, or Cu uptake in the aboveground biomass in an experimental pot that received S, Zn, Mn, and Cu, and U0 is the total S, Zn, Mn, or Cu uptake in the aboveground biomass in a pot that was not fertilized with these nutrients (control). The dry matter, nutrient uptake, and RE data obtained during the experimental period of 130 days were subjected to statistical analysis.

## Results

### Characterization of the mineral oxides and composites

Table S2 shows the characterization data for all the mineral oxide sources. The chemical compositions showed that $ZnO_{Mineral}$ (74 wt.% of ZnO) and $MnO_{Mineral}$ (65 wt.% of MnO) presented relatively high oxide contents, compared to $CuO_{Mineral}$ (28 wt.% of CuO). The low Cu content in $CuO_{Mineral}$ was due to the significant amounts of clay or quartz (based on the Al and Si contents). The amounts of oxides of Fe (such as hematite) and Ca (such as calcite) were also significant in the composition of $CuO_{Mineral}$, as well as in the composition of $MnO_{Mineral}$. It is important to emphasize that these materials were naturally occurring mineral oxide sources, so these were typical compositions as well as the expected phases (Fig. S1, Supplementary Material). The particle sizes of the three mineral oxides were similar, although the smaller $ZnO_{Mineral}$ surface area (1 $m^2$ $g^{-1}$) indicated differences in particle aggregation. Such differences in particle aggregation were confirmed by morphological analysis (Fig. 1), with $ZnO_{Mineral}$ (Fig. 1A) presenting well-formed crystals, while $MnO_{Mineral}$ and $CuO_{Mineral}$ (Fig. 1B and 1C) showed morphologies that were more irregular, suggesting that the higher surface areas of $MnO_{Mineral}$ and $CuO_{Mineral}$ were related to their greater roughness. Comparison with the elemental analysis data indicated that this agglomeration was probably related to the clay fractions, which were not significant in the case of $ZnO_{Mineral}$.

The dispersion of the mineral oxide particles in the starch composites was analyzed using X-ray microtomography, as shown in Fig. 1D-H, where the bright dots represent oxide particles, while the dark dots are the starch matrix. This analysis revealed that the $ZnO_{Mineral}$

particles were homogeneously distributed in the starch matrix, while greater tendency for particle agglomeration was observed for the St/Mn, St/Cu, and St/Mix composites. This was probably related to the higher particle agglomeration and roughness observed for $MnO_{Mineral}$ and $CuO_{Mineral}$ (Fig. 1B-C). The flat $ZnO_{Mineral}$ surfaces contributed to a better distribution of the starch over the particles, despite the lower surface area. The St/Mix+S composite was also very homogeneous (Fig. 1H), although some particle agglomerates around 100 µm in size were present. These results showed that the elemental sulfur ($S^0$) added in this composite had a good interaction with the starch and increased the dispersion of the oxides. The elemental distribution in the St/Mix+S composite was also analyzed by energy dispersive X-ray (EDX) spectroscopy (Fig.1I), which corroborated the X-ray microtomography results and showed that S, Zn, Mn, and Cu were homogeneously distributed in all the composites.

## Biological solubilization of the mineral oxides

This set of experiments was carried out to evaluate the potential of *A. niger* to solubilize the mineral oxides sources under submerged cultivation, as well as to understand the mechanisms involved in the solubilization process. Fig. 2A shows the oxide solubilization during submerged cultivation of *A. niger* (free cells), together with the control experiments (without *A. niger* inoculation). In the first 24 h of *A. niger* cultivation, all three oxides ($ZnO_{Mineral}$, $MnO_{Mineral}$, and $CuO_{Mineral}$) showed much higher solubility, compared to the control treatments. The increased solubilization could be attributed to the acidification of the medium, since the pH decreased during the course of the cultivation period, due to the production of organic acids by *A. niger* (Fig. 2B). The solubilization rates for the three oxides increased gradually up to 96 h, reaching 80% for $ZnO_{Mineral}$, 74% for $MnO_{Mineral}$, and 45% for $CuO_{Mineral}$. The temporal solubilization profiles for $ZnO_{Mineral}$ and $MnO_{Mineral}$ were very similar, while less intense solubilization was observed for $CuO_{Mineral}$, probably due to the antimicrobial activity associated to the copper oxide [23, 24].

## Effect of bioactivation of the composites on oxides solubilization

Three starch-based composites were initially designed using the mineral oxides (St/Zn, St/Mn, and St/Cu, Table S1) and a set of experiments was carried out to evaluate the synergy among mineral oxide particles dispersed in a matrix together with encapsulated *A. niger* to promote solubilization of the micronutrients. In these experiments, the composites were incubated in a liquid nutrient medium that provided the minimum conditions required for spore germination and fungal growth.

Fig. 2 (C-D) shows the solubilization achieved from bioactivation of the starch-based composites St/Zn, St/Mn, and St/Cu, together with the corresponding controls (without *A. niger* spores). The temporal profiles of solubilization (Fig. 2C) revealed that the composites were bio-activated at different times, with some delay that was probably due to the slow diffusion of water through the starch matrix. The St/Zn and St/Mn composites were rapidly bio-activated by *A. niger*, resulting in reduction of the pH (Fig. 2D) and increased solubilization, while the St/Cu composite was only bio-activated after incubation for 48 h, probably due to the antimicrobial effect of CuO [23, 24].

Fig. 2 (E-F) shows the effect of bioactivation of the St/Mix composite on oxide solubilization and pH during the incubation. Solubilization of the three oxides only increased after 48 h, due to the acidity of the culture medium. The presence of the mixture of mineral oxides in a single composite did not affect activation of the *A. niger* spores, which occurred at the same time observed for the St/Zn, St/Mn, and St/Cu composites that contained each oxide separately (Fig. 2C-D). The solubilization of Zn and Mn from the St/Mix composite remained high, with values above 90% after 288 h of incubation. On the other hand, the solubilization of Cu showed a small decrease from 75 to 65%, compared to the St/Cu composite.

In order to optimize the effect of this multi-nutrient composite, elemental sulfur ($S^0$) was incorporated into the starch (Table S1, Fig. 1H). Fig. 3 shows the effect of bioactivation of the resulting St/Mix+S composite on oxide solubilization, pH, and $S^0$ oxidation. The St/Mix+S composite showed faster bioactivation, compared to the other composites analyzed previously. In the first 24 h of incubation, there was intense acidification of the medium, with decrease of the pH to around 4.5, while the pH of the St/Mix composite (Fig. 2F) and the other oxide composites (Fig. 2D) showed no acidification in the same period. After 24 h, acidification of the medium continued to increase, as evidenced by the decreasing pH. This acidification led to substantial increases of the solubilization of ZnO and MnO, with values near 100% reached in only 48 h. However, the solubilization of CuO was similar to the values obtained for the St/Mix and St/Cu composites, not exceeding 60% even after 288 h. It was also observed that after the solubilization of ZnO and MnO reached 100%, there were small reductions in the amounts of Zn and Mn in solution, which could be attributed to the effects of complexation and precipitation of these elements with organic acids and other compounds present in the solution [25-27].

Shelf life experiments using St/Mix+S indicated that bioactivation of the composite could be easily achieved after storage in plastic bags at room temperature. The results (Fig. S4, Supplementary Material) showed that similar levels of composite bioactivation were obtained for the initial material (10 days) and after two and six months of storage. This showed that the gelatinized starch matrix was able to protect the *A. niger* spores, with encapsulation of the spores ensuring a long shelf life of the product.

**Soil-plant experiments**

Greenhouse experiments were carried out to evaluate the performance of the St/Mix+S composite in a soil-plant system. The aim was to determine the effectiveness of the bio-activated St/Mix+S composite in supplying micronutrients and sulfur, compared to commercial fertilizers and a control treatment (without fertilization). After 7 days, visual analysis of the composite added to the soil revealed bioactivation by *A. niger* and initiation of the solubilization process in the granules (Fig. S5, Supplementary Material). Fig. 4 shows the results for dry matter production by the Italian ryegrass during 130 days of growth on two soils with different characteristics (a clay soil and a sandy loam soil). As expected, the two selected soils responded differently to fertilization, as can be seen from the images of the ryegrass acquired prior to the five cuts, up to 130 days after planting (Fig. 4 A-B).

After 130 days of plant growth, the average dry matter production (considering all the treatments) was slightly higher for the clay soil (24 g pot$^{-1}$), compared to the sandy loam soil (20 g pot$^{-1}$). An increase of up to 16 g pot$^{-1}$ was provided by fertilization with the St/Mix+S composite, compared to the control treatment. The lower value for the sandy loam soil could be attributed to the lower concentration of S in this soil (Supplementary Material, Table S3), which limited growth after the 3rd cut (70 days after planting/fertilization), as shown in Fig. 4D. In the case of the clay soil, the nutrients provided by the St/Mix+S composite sustained DM production throughout the entire period of growth, with a production profile superior to that obtained for soil fertilized with the oxides + Sulfurgran® (Fig. 4C). In contrast, fertilization of the sandy loam soil with the two different nutrient sources resulted in similar DM production profiles (Fig. 4D).

Fig. 5 shows the cumulative uptakes of S, Zn, Mn, and Cu by the Italian ryegrass for the five cuts performed during cultivation for 130 days on the two soils, without fertilization or with fertilization using the different sources. Analysis of S showed that for both soils, there was a response to the presence of S during the growth period. The forage cultivated on the soil without fertilization (control) absorbed almost all the S available in the soil of each pot

(45 and 15 mg for the clay soil and the sandy loam soil, respectively) (Table S3, Supplementary Material). The forage fertilized with St/Mix+S showed higher S uptake during the entire growth period, compared to use of the Sulfurgran® fertilizer (Fig. 5A-B). During most of the growth period, the uptakes of Zn, Mn, and Cu from the St/Mix+S composite were higher for both types of soil, relative to uptake of the micronutrients from the oxides or the soluble sources (Fig. 5C-H).

Table 1 summarizes the cumulative dry matter production of the Italian ryegrass, the total uptakes of Zn, Mn, Cu, and S, and the crop recovery efficiency (RE) for all five cuts during the experimental period of 130 days. The cumulative dry matter production using the clay soil fertilized with St/Mix+S was around 30 g pot$^{-1}$, which was similar to the value achieved using the commercial high solubility sources ($CuCl_2$ + $ZnCl_2$ + $MnCl_2$ + Sulfurgran®) and higher than for the treatment with the insoluble sources (oxides + Sulfurgran®) or for the control. In contrast, no statistically significant difference in the cumulative DM production was observed using the fertilized sandy loam soil. The results demonstrated the potential of the St/Mix+S composite to increase biomass production of the Italian ryegrass on both soils, showing results similar to those obtained with commercial fertilizers. A significant increase of S uptake was observed for the sandy loam soil fertilized with the St/Mix+S composite. This evidenced that $S^0$ oxidation was higher in the case of the St/Mix+S composite, compared to Sulfurgran®, due to the dispersion of $S^0$ in the starch matrix and the oxidation promoted by *A. niger* [14]. In contrast, the $S^0$ from Sulfurgran® was only oxidized by the soil microbial population.

## Discussion

The direct use of oxide mineral sources as plant micronutrients would be desirable in order to reduce both environmental impacts and costs in agriculture. However, their low solubility limits practical applications in plant nutrition. Therefore, the aim of this work was to develop a multi-nutrient fertilizer with mineral oxides of low reactivity ($ZnO_{Mineral}$, $MnO_{Mineral}$, and $CuO_{Mineral}$) and $S^0$ in starch composites formulations biologically activated by *A. niger*. Different proportions of starch, oxides, and elemental sulfur were used to produce the composites (Table S1) in which *A. niger* espores were encapsulated. The characterization of the composites containing the individual oxides (St/Zn, St/Mn, St/Cu), the mixture of oxides (St/Mix) and the mixture of oxides plus $S^0$ (St/Mix+S) revealed that Zn, Mn, Cu and S were homogeneously distributed in all the composites. Interestingly, the elemental sulfur ($S^0$)

added in the composite had a good interaction with the starch and increased the dispersion of the oxides.

In order to understand the mechanisms involved in the biological solubilization process, a preliminary set of experiments was carried out to evaluate the potential of *A. niger* free cells to solubilize the isolated mineral oxides sources under submerged cultivation. The results indicated that the mechanisms of solubilization of $ZnO_{Mineral}$, $MnO_{Mineral}$, and $CuO_{Mineral}$ were similar to those reported for bioleaching, where the solubilization of oxides is a consequence of acidification of the medium by the microbial production of organic acids [28-31]. In order to investigate whether this behavior was directly related to organic acid production, solubilization experiments were performed to determine the solubility of the oxides in three synthetic organic acids (citric, oxalic, and gluconic acids), as well as in water as a control (Fig. S2, Supplementary Material). As expected, the solubility of the three oxides in water was less than 0.1%, while highly significant solubility was observed in most of the organic acids, with the exception of ZnO and MnO in oxalic acid.

The organic acids can dissolve heavy metals by direct displacement of metal ions from the ore matrix by hydrogen ions, as well as by the formation of soluble metal complexes and chelates [32]. In addition, there are others compounds possessing at least two hydrophilic reactive groups (such as phenol derivatives), which may also be excreted into the culture medium during microbial cultivation and have a solubilizing action on mineral oxides. Specifically for manganese, solubilization of the metal could also occur due to the enzymatic reduction of highly oxidized metal compounds, although the enzymatic effect is low in comparison to acidification by organic acids [32, 33].

Given the potential of *A. niger* free cells to solubilize the mineral oxides, a second set of experiments was carried out to evaluate the synergy among mineral oxide particles dispersed in the starch matrix with encapsulated *A. niger* to promote solubilization of the micronutrients. Although the solubilization was delayed, compared to the original oxides, the composites formed by the starch-based matrix with the oxides were effective in maintaining the viability of the *A. niger* spores, even after the drying/milling processing steps. For example, the St/Mn composite showed high solubilization of 80% after 96 h, followed by a slower increase to 100% solubilization after 288 h. For the St/Zn and St/Cu composites, solubilization was less intense during the first 96 h, while final values around 90% and 70% were obtained after 288 h, respectively (Fig. 2C-D). The solubilization rates obtained for the St/Mn and St/Cu composites during 96 h were similar to those obtained for submerged culture with *A. niger* free cells (when the oxides were solubilized in the culture medium without formation of composites; Fig. 2A). In the case of the St/Zn composite, the solubilization was slightly lower, although the difference was not statistically significant.

An analysis of effect of bioactivation of the St/Mix composite on oxide solubilization revealed that the presence of the mixture of mineral oxides in a single composite did not affect activation of the *A. niger* spores, which occurred at the same time observed for the St/Zn, St/Mn, and St/Cu composites that contained each oxide separately. Solubilization of the oxides from the control samples without *A. niger* spores remained below 1% up to 288 h, as expected due to the small pH change during the incubation period. These results corroborated the hypothesis that the acidification of the culture medium caused by the microbial production of organic acids was the main factor influencing solubilization of the St/Mix composite.

A visual comparison of the composite (solid granules) and the structure resulting after 288 h of bioactivation revealed that the polymer matrix was progressively replaced by microorganism biomass (Fig. S3, Supplementary Material). The microbial biomass contributed to maintaining the composite structure, which could intensify the effect on the dispersed oxide particles. These results suggested the suitability of the starch-based oxide composites for use as granulated fertilizers, which would enable an application method appropriate for field conditions, since for agronomic purposes, it is generally impractical to apply mineral oxides in the form of powder.

Aiming to further optimize the effect of this multi-nutrient composite, elemental sulfur ($S^0$) was incorporated into the starch. The St/Mix+S composite showed faster bioactivation, compared to the other composites analyzed previously. An intense acidification of the medium was observed already in the first 24 h of incubation, with decrease of the pH to around 4.5. Evaluation of $S^0$ oxidation revealed that approximately 50% of the $S^0$ was oxidized in 96 h (Fig. 3C), which contributed to increased acidity of the medium (since the $S^0$ oxidation reaction produces acidity) and further assisted solubilization of the oxides, especially ZnO and MnO [6, 13, 34]. After 96 h of incubation, *A. niger* continued to promote $S^0$ oxidation, but at lower intensity, although oxidation did not exceed 80%, even after 288 h (12 days). In addition, there was excellent dispersion of the $S^0$ within the starch matrix (Fig. 1I), which could have played a key role in achieving high interaction between the $S^0$ and the microorganism, resulting in a high degree of oxidation. It is worth to mention that *A. niger* unlike some bacteria (such as *Acidithiobacillus thioxidaans* and *Acidithiobacillus ferrooxidans*) promotes the $S^0$ oxidation by indirect mechanisms, such as enzyme secretion and reduction of pH by the production of organic acids [6, 13, 35]. In overall, the results showed that the $S^0$ incorporation in starch matrix was efficient to improve the micronutrients solubilization, since the total acidity increased by synergisms between production of organic acids by *A. niger* and the acidity produced by $S^0$ oxidation.

Promising results were also found during shelf life experiments using St/Mix+S composites stored in plastic bags at room temperature, in which similar levels of bioactivation were obtained for the initial material (10 days) and after two and six months of storage. Nevertheless, greenhouse experiments are necessary to evaluate the performance of the St/Mix+S composite in a soil-plant system. Therefore, the effectiveness of the bio-activated St/Mix+S composite in supplying micronutrients and sulfur to Italian ryegrass was compared to commercial fertilizers on two soils with different characteristics (a clay soil and a sandy loam soil).

In terms of dry matter production, an increase of up to 16 g $pot^{-1}$ was provided by fertilization with the St/Mix+S composite, compared to the control treatment. For the clay type of soil, the nutrients provided by the St/Mix+S composite sustained DM production throughout the entire period of growth, with a production profile superior to that obtained for soil fertilized with the oxides + Sulfurgran® (Fig. 4C). Besides the DM production, the cumulative uptakes of Zn, Mn, and Cu from the St/Mix+S composite were higher for both types of soil, relative to uptake of the micronutrients from the oxides or the soluble sources (Fig. 5).

The uptake of micronutrients from the control soil was lower than from the fertilized soil, due to the low micronutrient contents of both soils (except for Cu in the clay soil) (Table S3), with this difference increasing in the final two forage cuts. On the other hand, the progressive solubilization of micronutrients from the St/Mix+S composite was as effective as the soluble sources in supplying Zn, Mn, and Cu to the forage. Despite the lower application frequency, the oxides applied to the soil also provided micronutrient contents similar to those obtained with the soluble sources. However, the profile of the micronutrients supply to the forage from the St/Mix+S composite was higher than the profile obtained with the oxides (Fig. 5C-H). These results showed that the dispersion of the oxide particles in the starch+$S^0$ composite matrix, together with the acidification provided by the production of organic acids and the oxidation of $S^0$ by *A. niger*, led to increased solubilization of Zn, Mn, and Cu, confirming the results of the incubation experiment (Fig. 3).

The efficiency of the St/Mix+S composite in supplying S to the forage was most evident for the sandy loam soil, where the S uptake was 1.8 times higher than obtained with Sulfurgran® (Table 1). The forage grown on the clay soil showed higher total uptakes of Zn, Mn, and Cu from the St/Mix+S composite than from the oxides. In addition, for this soil, the supply of Zn from the composite was also more abundant, compared to the soluble sources. In the case of the sandy loam soil, only the total Zn uptake from the composite was higher than obtained using the oxides. The low concentrations of Mn and Cu in the sandy loam soil (Table S3) could have contributed to the better performance of the oxides in supplying the micronutrients, with a smaller difference between the sources.

The RE represents the increase in nutrient uptake (in mg) per mg of nutrient applied to the soil. For the clay soil, the RE for sulfur from the St/Mix+S composite was similar to that for Sulfurgran®, with values between 11.6 and 15.9%. On the other hand, for the sandy loam soil, the RE value for sulfur from the composite was two times higher than for Sulfurgran®. This higher efficiency observed for the sandy loam soil could be attributed to the low S oxidation capacities of these soils. According to Germida and Jazen [36], sandy soils with low OM contents have lower capacities for the oxidation of sulfur, due to their low microbial activities. Hence, the dispersion of $S^0$ in the starch matrix and the activity of *A. niger* inoculated in the composite potentiated the oxidation of $S^0$ [14], even in the sandy loam soil. Similarly, Mattiello et al. [6] also observed increased oxidation of S from elemental sulfur granulated with ZnO, with increased Zn solubilization attributed to the acidification provided by the oxidation of elemental sulfur by *A. niger* or *Acidithiobacillus thiooxidans*.

The total RE for Zn from St/Mix+S was 1.5-fold higher than for Zn from the oxides and soluble sources in the clay soil, and 2-fold higher than for Zn from the oxides in the sandy loam soil. For both soils, the St/Mix+S composite showed Mn and Cu RE values similar to those for the soluble sources. No differences were observed for the oxides RE values, with the exception of the clay soil, for which the Mn RE for the composite was 2.4-fold higher than for the oxides. The low Zn, Mn, and Cu RE values obtained with the Italian ryegrass could be attributed to the high application rate of the nutrients to the soil (12 mg $kg^{-1}$) and the short growing period (130 days). The results demonstrated that the St/Mix+S composite fertilizer can be effective for soil application, given that the uptakes of S, Zn, Mn, and Cu from the fertilizer were similar to or even higher than from a soluble source of micronutrients combined with Sulfurgran®.

## Conclusions

The findings of this work showed that organic acids production and $S^0$ oxidation promoted by encapsulated *A. niger* favored the uptake of micronutrients from mineral sources, using a synergic strategy in which the composite structure based on a starch matrix was an essential feature. This material represents a proof of concept, characterizing a reactor-in-a-granule system that is activated following application to soil, locally promoting a sequence of reactions that increase fertilizer efficiency. Furthermore, the strategy is environmentally friendly, since it reduces the need for pre-processing of nutrients, while also minimizing application procedures. In greenhouse soil-plant experiments, the system showed efficiency similar to that achieved using optimized commercial fertilizer application, in terms of dry matter production, nutrient uptake, and recovery. Therefore, these findings demonstrate the potential of the St/Mix+S composite as a granulated fertilizer that is a source of multi-nutrients (S, Zn, Mn, and Cu), opening up new routes for the development of smart fertilizers capable of making it feasible to use low solubility sources of nutrients required for efficient plant development.

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## Figure Captions

**Fig. 1** FEG-SEM images of **A)** $ZnO_{Mineral}$, **B)** $MnO_{Mineral}$, and **C)** $CuO_{Mineral}$. The X-ray tomography cross-sectional images of the composites are shown for **D)** St/Zn, **E)** St/Mn, **F)** St/Cu, **G)** St/Mix, and **H)** St/Mix+S. **I)** Images of distributions of the elements (S, Zn, Mn, and Cu) in the St/Mix+S composite by EDX analysis.

**Fig. 2** Biological solubilization of $ZnO_{Mineral}$, $MnO_{Mineral}$, $CuO_{Mineral}$, and controls (without *A. niger* spores) **A)** Temporal profile of solubilization. **B)** Temporal profile of pH. · **--** · Temporal profile of **C)** solubilization. **D)** pH for the bioactivated St/Zn, St/Mn, and St/Cu composite and the control (without *A. niger* spores). · **--** · Temporal profiles of **E)** solubilization and **F)** pH for the bioactivated St/Mix composite and the control (without *A. niger* spores). All experiments were carried out using Czapek Dox medium, 30 °C, 220 rpm, and $pH_{initial}$ 7,

**Fig. 3** Temporal profiles of **A)** solubilization, **B)** pH, and **C)** sulfur oxidation for the bioactivated St/Mix+S composite and the control (without *A. niger* spores), using Czapek Dox medium, 30 °C, 220 rpm, and $pH_{initial}$ 7.

**Fig. 4** Cumulative dry matter production by Italian ryegrass during 130 days on two soils without fertilization (control) or fertilized with oxides + Sulfurgran®, the St/Mix+S composite, and $CuCl_2$ + $ZnCl_2$ + $MnCl_2$ + Sulfurgran®. Photographs of the Italian ryegrass cultivated using the clay soil **A**) and the sandy loam soil **B**), before the 5th cut. Cumulative masses of dry matter obtained using the clay soil **C**) and the sandy loam soil **D**).

**Fig. 5** Cumulative uptakes of S, Zn, Mn, and Cu during 130 days by the Italian ryegrass on the two soils without fertilization (control) or fertilized with oxides + Sulfurgran®, the St/Mix+S composite, and $CuCl_2$ + $ZnCl_2$ + $MnCl_2$ + Sulfurgran®. S uptake using clay soil **A**) or sandy loam soil **B**); Zn uptake using clay soil **C**) or sandy loam soil **D**); Mn uptake using clay soil **E**) or sandy loam soil **F**); Cu uptake using clay soil **G**) or sandy loam soil **H**).

**Table 1** Cumulative dry matter production (DM), total uptakes of Zn, Mn, Cu, and S, and total recovery efficiency (RE) for the Italian ryegrass during the trial period of 130 days.

| **Soil** | **Treatment** | **DM** | **S** | **Zn** | **Mn** | **Cu** | **S** | **Zn** | **Mn** | **Cu** |
|---|---|---|---|---|---|---|---|---|---|---|
| | | (g pot$^{-1}$) | Uptake (mg pot$^{-1}$) | | | | RE (%) | | | |
| **Clay** | **Control** | 16.77 c | 39.62 b | 0.15 c | 1.60 c | 0.24 c | - | - | - | - |
| | **Oxides + Sulfurgran®** | 22.70 cb | 74.37 a | 1.02 b | 2.46 b | 0.34 cb | 11.58 a | 2.40 b | 2.38 b | 0.27 b |
| | **St/Mix+S** | 29.74 a | 98.19 a | 1.44 a | 3.69 a | 0.46 a | 15.92 a | 3.58 a | 5.80 a | 0.60 a |
| | **ZnCuMn + Sulfurgran®** | 28.21 ba | 80.08 a | 1.01 b | 3.52 a | 0.39 ba | 13.48 a | 2.38 b | 5.30 a | 0.42 ab |
| **Sandy loam** | **Control** | 8.96 b | 15.56 c | 0.18 c | 1.07 b | 0.07 c | - | - | - | - |
| | **Oxides + Sulfurgran®** | 23.45 a | 47.00 b | 1.18 b | 3.09 a | 0.24 ba | 10.48 b | 2.78 b | 5.60 a | 0.46 ab |
| | **St/Mix+S** | 24.80 a | 86.97 a | 2.18 a | 3.69 a | 0.32 a | 23.80 a | 5.55 a | 7.26 a | 0.68 a |
| | **ZnCuMn + Sulfurgran®** | 22.26 a | 45.47 b | 2.03 a | 2.93 a | 0.22 b | 9.97 b | 5.14 a | 5.15 a | 0.42 b |

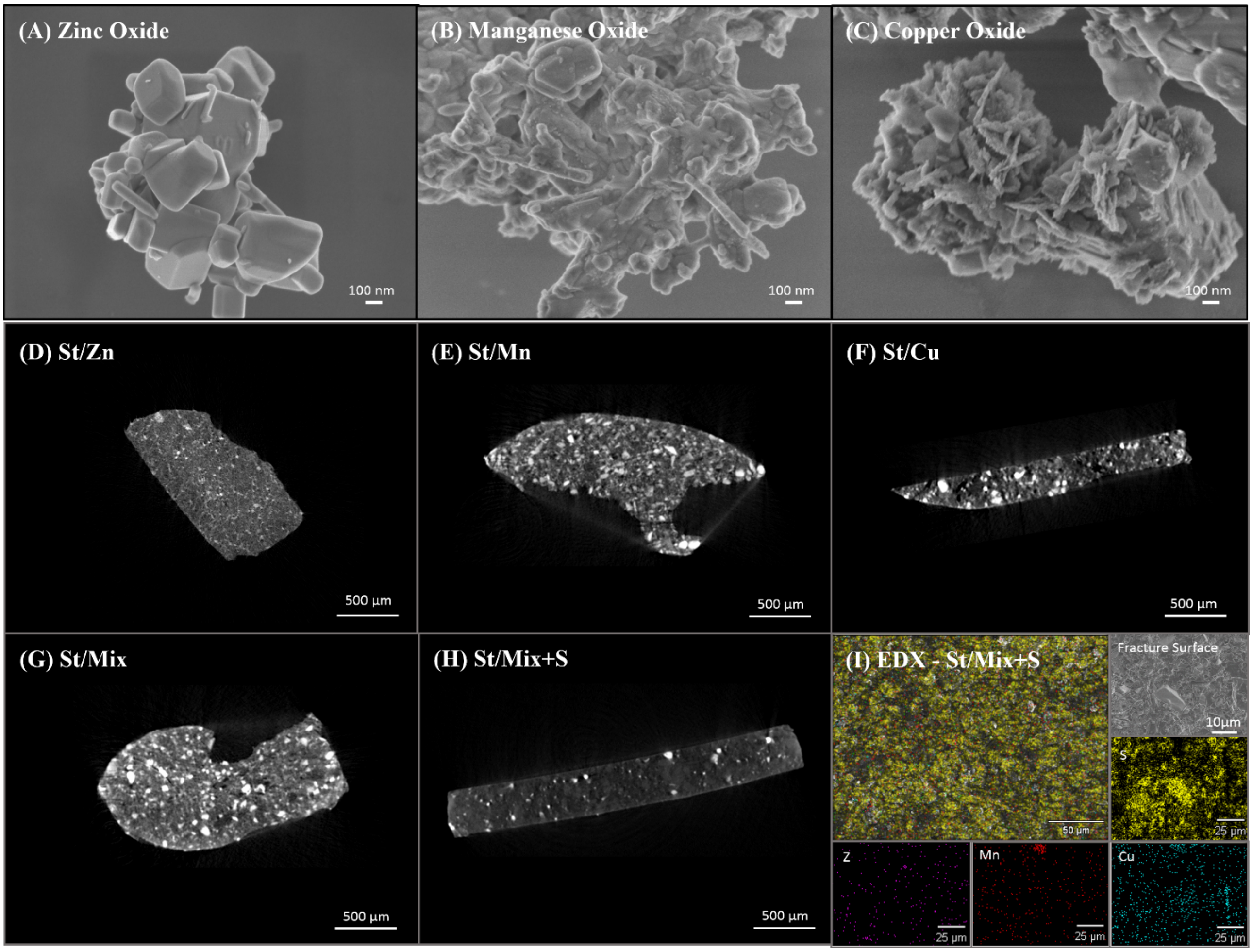
(A) Zinc Oxide
100 nm
(B) Manganese Oxide
100 nm
(C) Copper Oxide
100 nm
(D) St/Zn
500 µm
(E) St/Mn
500 µm
(F) St/Cu
500 µm
(G) St/Mix
500 µm
(H) St/Mix+S
500 µm
(I) EDX - St/Mix+S
50 µm
Fracture Surface
10µm
S
25 µm
Z
25 µm
Mn
25 µm
Cu
25 µm

**Fig. 1**

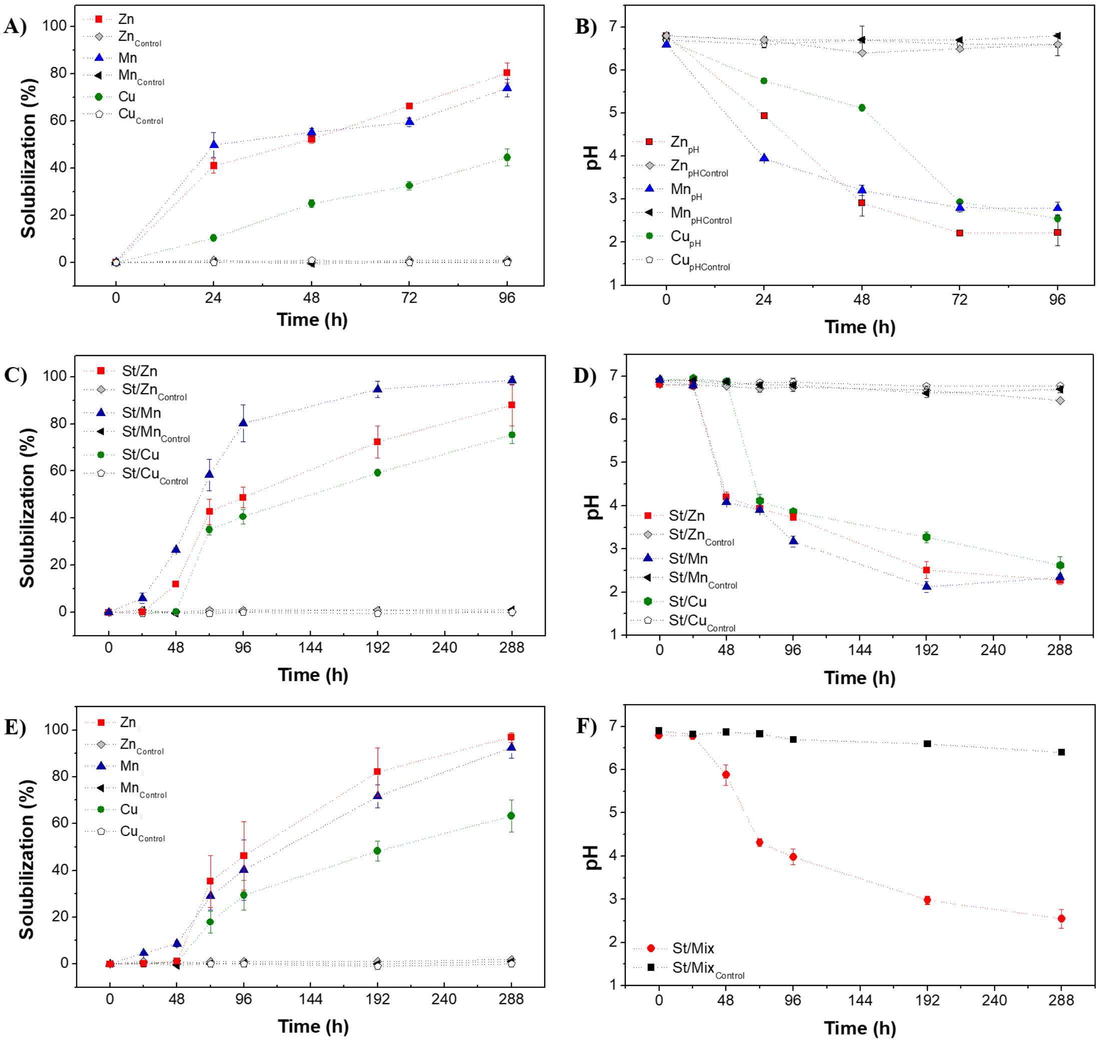
A)
Solubilization (%)
Time (h)
Zn
Zn$_{Control}$
Mn
Mn$_{Control}$
Cu
Cu$_{Control}$
B)
pH
Time (h)
Zn$_{pH}$
Zn$_{pHControl}$
Mn$_{pH}$
Mn$_{pHControl}$
Cu$_{pH}$
Cu$_{pHControl}$
C)
Solubilization (%)
Time (h)
St/Zn
St/Zn$_{Control}$
St/Mn
St/Mn$_{Control}$
St/Cu
St/Cu$_{Control}$
D)
pH
Time (h)
St/Zn
St/Zn$_{Control}$
St/Mn
St/Mn$_{Control}$
St/Cu
St/Cu$_{Control}$
E)
Solubilization (%)
Time (h)
Zn
Zn$_{Control}$
Mn
Mn$_{Control}$
Cu
Cu$_{Control}$
F)
pH
Time (h)
St/Mix
St/Mix$_{Control}$
0
24
48
72
96
144
192
240
288
0
20
40
60
80
100
1
2
3
4
5
6
7

**Fig. 2**

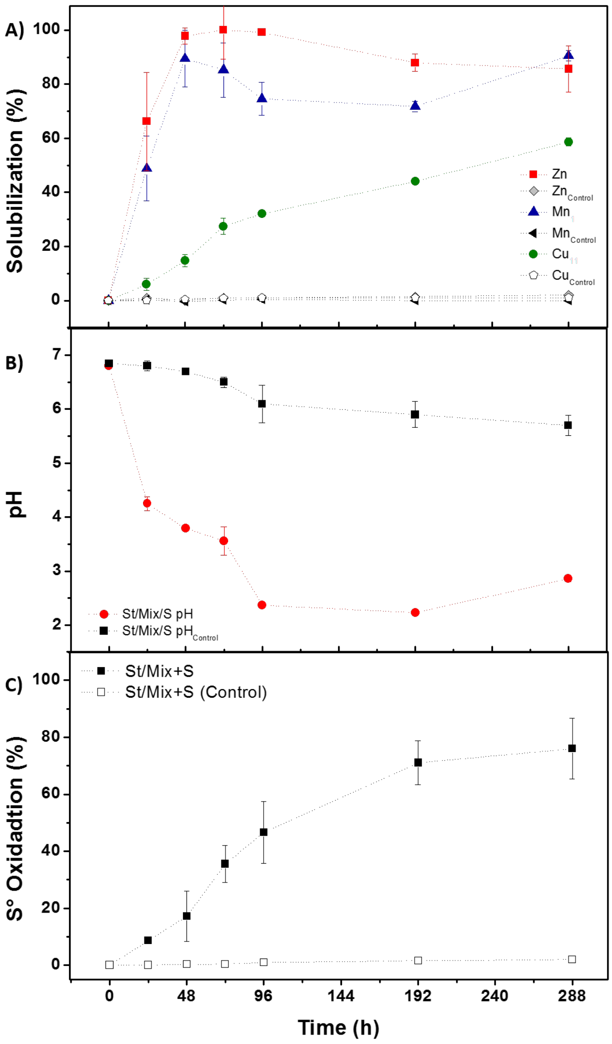
A)
Solubilization (%)
100
80
60
40
20
0
Zn
Zn$_{Control}$
Mn
Mn$_{Control}$
Cu
Cu$_{Control}$
B)
pH
7
6
5
4
3
2
St/Mix/S pH
St/Mix/S pH$_{Control}$
C)
S° Oxidadtion (%)
100
80
60
40
20
0
St/Mix+S
St/Mix+S (Control)
0
48
96
144
192
240
288
Time (h)

**Fig. 3**

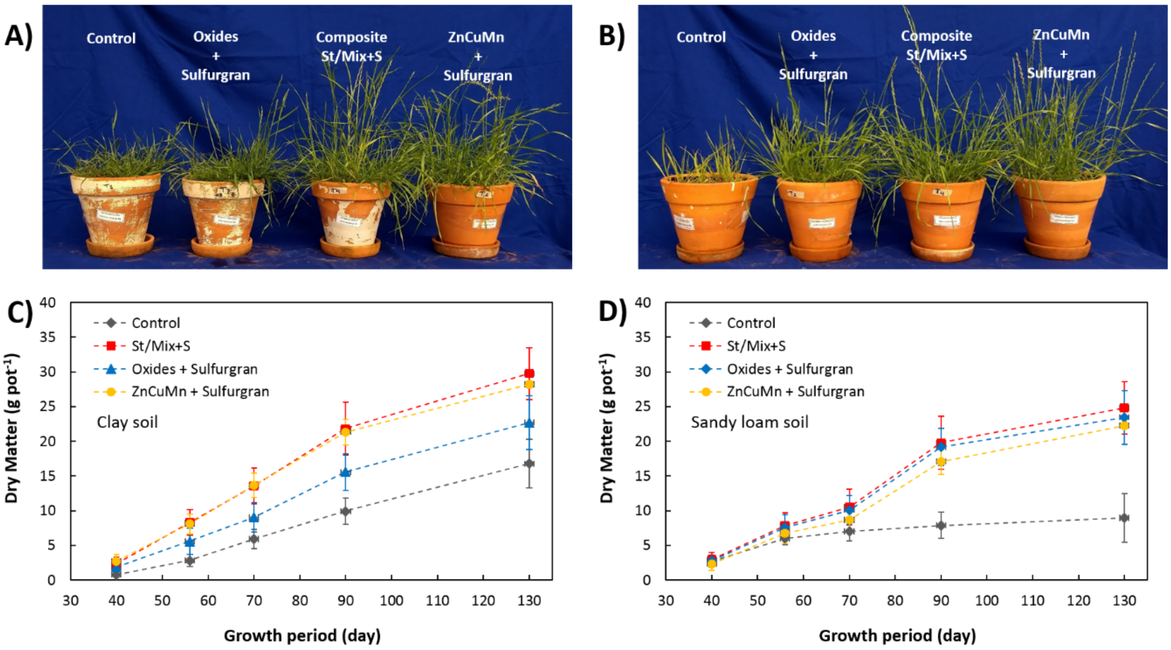
A)
Control
Oxides + Sulfurgran
Composite St/Mix+S
ZnCuMn + Sulfurgran
B)
Control
Oxides + Sulfurgran
Composite St/Mix+S
ZnCuMn + Sulfurgran
C)
Control
St/Mix+S
Oxides + Sulfurgran
ZnCuMn + Sulfurgran
Clay soil
Dry Matter (g pot-1)
0
5
10
15
20
25
30
35
40
30
40
50
60
70
80
90
100
110
120
130
Growth period (day)
D)
Control
St/Mix+S
Oxides + Sulfurgran
ZnCuMn + Sulfurgran
Sandy loam soil
Dry Matter (g pot-1)
Growth period (day)

Fig. 4

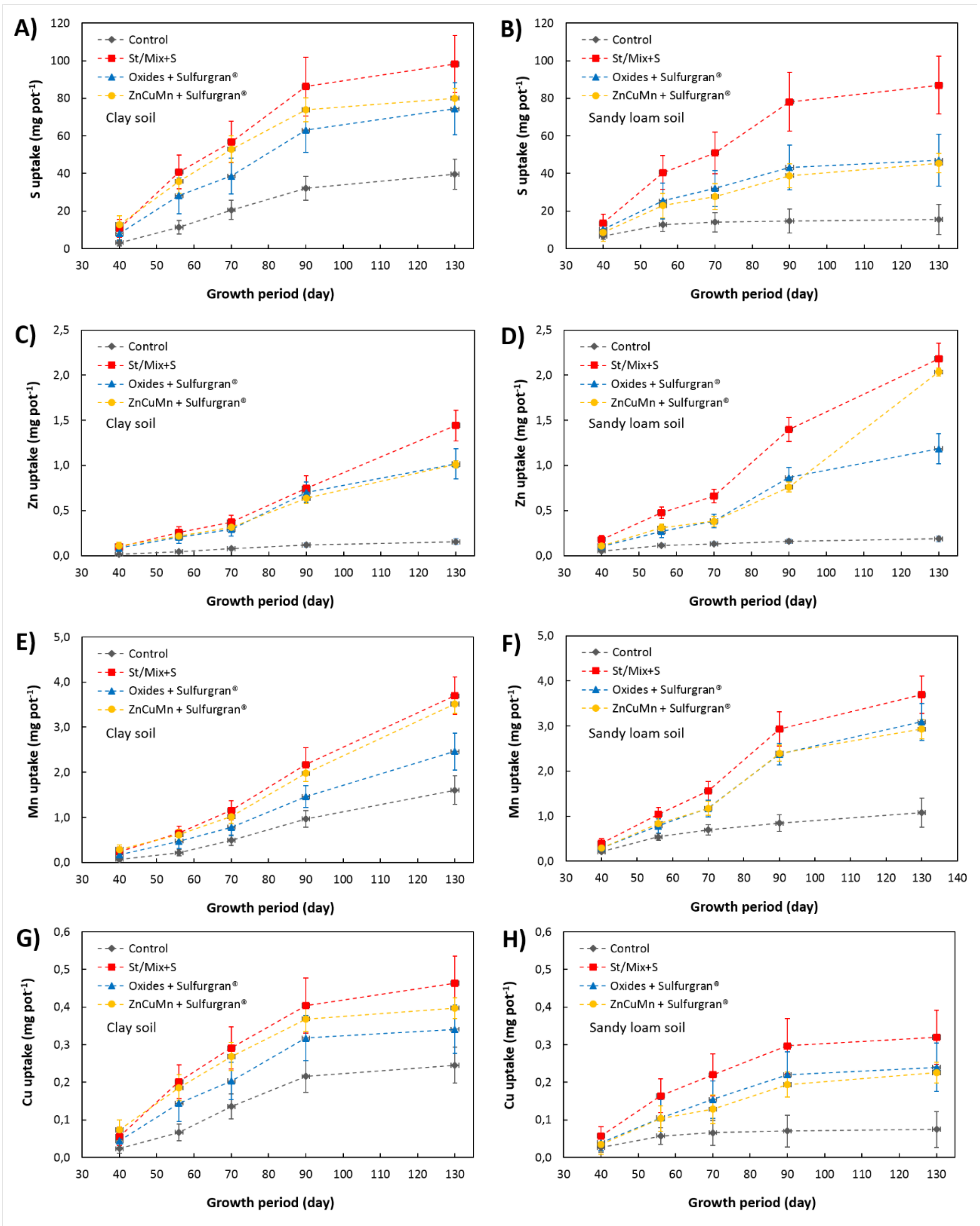
A)
Control
St/Mix+S
Oxides + Sulfurgran®
ZnCuMn + Sulfurgran®
Clay soil
S uptake (mg pot$^{-1}$)
Growth period (day)
B)
Sandy loam soil
S uptake (mg pot$^{-1}$)
C)
Clay soil
Zn uptake (mg pot$^{-1}$)
D)
Sandy loam soil
Zn uptake (mg pot$^{-1}$)
E)
Clay soil
Mn uptake (mg pot$^{-1}$)
F)
Sandy loam soil
Mn uptake (mg pot$^{-1}$)
G)
Clay soil
Cu uptake (mg pot$^{-1}$)
H)
Sandy loam soil
Cu uptake (mg pot$^{-1}$)
0
20
40
60
80
100
120
0,0
0,5
1,0
1,5
2,0
2,5
0,0
1,0
2,0
3,0
4,0
5,0
0,0
0,1
0,2
0,3
0,4
0,5
0,6
30
40
50
60
70
80
90
100
110
120
130
140

Fig. 5

# Supplementary Material

## Summary

### Material and Methods

### Figures

### Tables

## Supplementary Material

### Materials and Methods

#### Determination of surface area

The specific surface area of the three oxides were determined by isothermal nitrogen adsorption using a Micromeritics ASAP-2020 instrument, according to the 5-point B.E.T. (Brunauer–Emmett–Teller) method.

#### Determination of particle size

The particle size was determined by Dynamic Light Scattering (DLS). The analyses were carried out on a High Performance Particle ZetaSizer (Malvern Instruments) to obtain the average particle sizes of phosphates rock before and after miling. 1 mg of each sample was transferred to a 25 ml glass bottle, which contained 10 ml of distilled water. Before the DLS measurements, the solution was sonicated (Bransom 450 W) for 10 min in order to disperse the particles within the solution.

#### X-ray fluorescence (XRF)

X-ray fluorescence (XRF) for the three oxides were performed to identify the most common chemical components present in oxide structure using the lithium tetraborate fusion technique.

#### X-ray Diffraction (XRD)

X-ray diffraction (XRD) analyses of the mineral oxides ($ZnO_{Mineral}$, $MnO_{Mineral}$ and $CuO_{Mineral}$) samples were performed using a LabX XRD-6000 diffractometer (Shimadzu, Japan) operated with Cu-Kα radiation ($\lambda = 1.54056$ Å), voltage of 30 kV, and current of 30 mA. The diffractograms were recorded for $2\theta$ from 5° to 55°, using a continuous scanning speed of 2° $min^{-1}$.

#### Scanning electron microscopy (SEM)

The morphologies of the three oxides and of the nanocomposites produced were observed by field emission gun scanning electron microscopy (FEG-SEM), using a JSM-6701F microscope (JEOL, Japan) operated at an acceleration voltage of 15 kV, with a working distance of 10 mm and a secondary electron detector. Then, the disc was coated with carbon in a Leica EMSCD050 chamber and imaged using the secondary electron mode. Semi-quantitative atomic compositions were evaluated by energy-dispersive X-ray (EDX) spectroscopy using an EDX Link Analytical device (Isis System Series 200) coupled with a LEO 440 scanning electron microscope.

#### X-ray Microtomography (MicroCt)

In addition, the nanocomposites were also analyzed using X-ray microtomography (MicroCt) (model 1172 SkyScan) to observe the particle morphologies and degree of particle dispersion of oxides in the starch matrix. The composite samples were placed in a rotating steel support and the images were acquired using the following parameters: unaltered, spatial resolution (voxel size) of 2 mm, 0.3º step rotation, 180º rotation, and averaging of 6 frames. The images were reconstructed from the tomographic sections using NRecon software (SkyScan), applying the following settings: smoothing 5, ring artifact correction 5, and beam hardening correction of 60%.

## Determination of Zn, Mn and Cu

An ICP-OES system with a dichroic spectral combiner for simultaneous collection of data in radial and axial viewing mode was used to perform all measurements (VistaRL CCD-Simultaneous ICP-AES, Varian). The instrument arrangement involves a vertical argon plasma with a flow rate of 15.0 L $min^{-1}$, and a limit of detection (L.O.D.) for Zn, Mn and Cu of 0.01 mg $L^{-1.}$

## Determination of sulfur-oxidation in liquid medium

The sulfate ($SO_4^{2-}$) from the oxidation of S° were analyzed for turbid metric as proposed by Camargo et al. (2009). The method is based on the $BaSO_4$ precipitation after addition of $BaCl_2$ into the solution and the $SO_4^{2-}$ concentration was estimated by the solution turbidity. Then concentration of $SO_4^{2-}$ were determined by UV-Vis spectrometry at a wavelength of 420 nm in a Lambda 25 UV-Vis Spectrophotometer (Perkin Elmer).

## Supplementary Material

## Figures

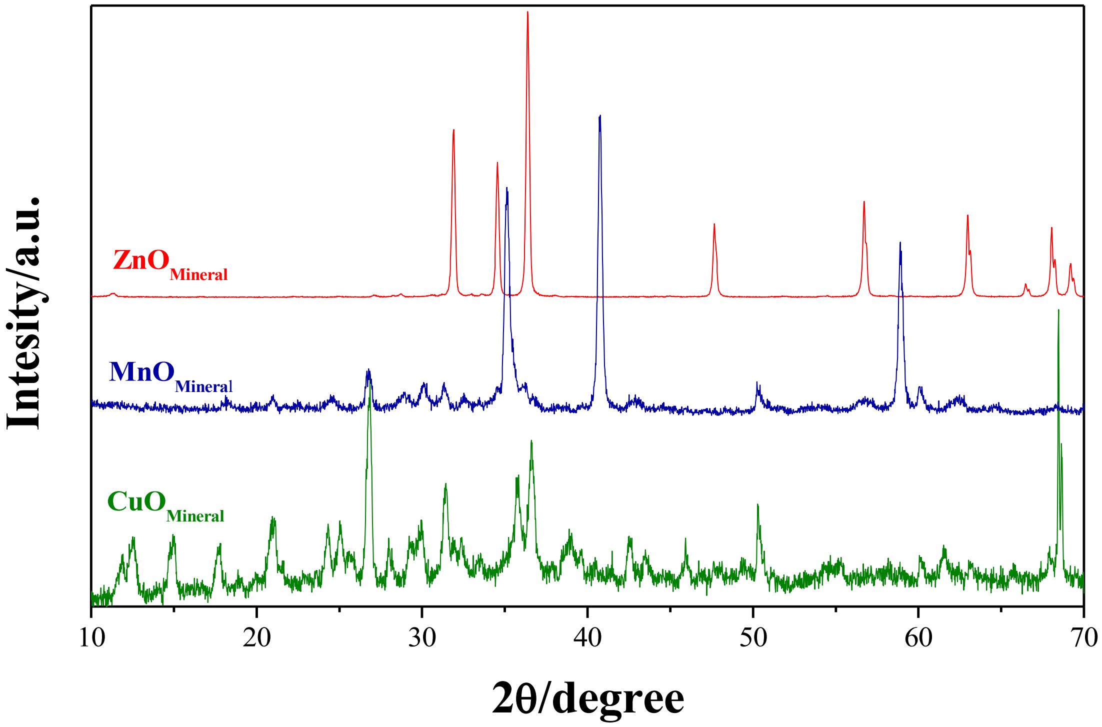

**Figure S1.** X-ray diffraction (XRD) for mineral oxides samples of ZnO$_{\text{Mineral}}$, MnO$_{\text{Mineral}}$ and CuO$_{\text{Mineral}}$.

## Supplementary Material

## Figures

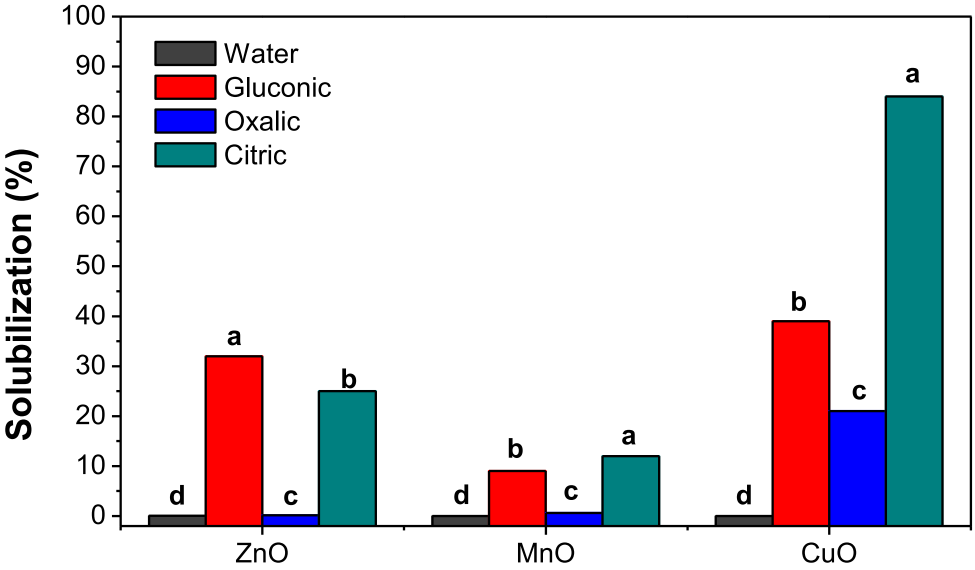

**Figure S2.** Solubility of oxides in a solution of organic acids 2 wt.%.

Prepare 100 ml of an organic acid solution (2 wt%) in a 250 ml beaker. Heat until slightly boiled and add 1 g of the sample (oxide). Cover the beaker with watch glass and keep in slight boiling for 10 min. Then, turn off and allow to cool to room temperature, transfer to a 200 ml volumetric flask and complete the volume with distilled water and homogenise. Keep at rest for 10 min and filter using filter paper (medium to fine porosity). From the filtrate obtained the concentration of the micronutrient was determined by ICP.

## Supplementary Material

### Figures

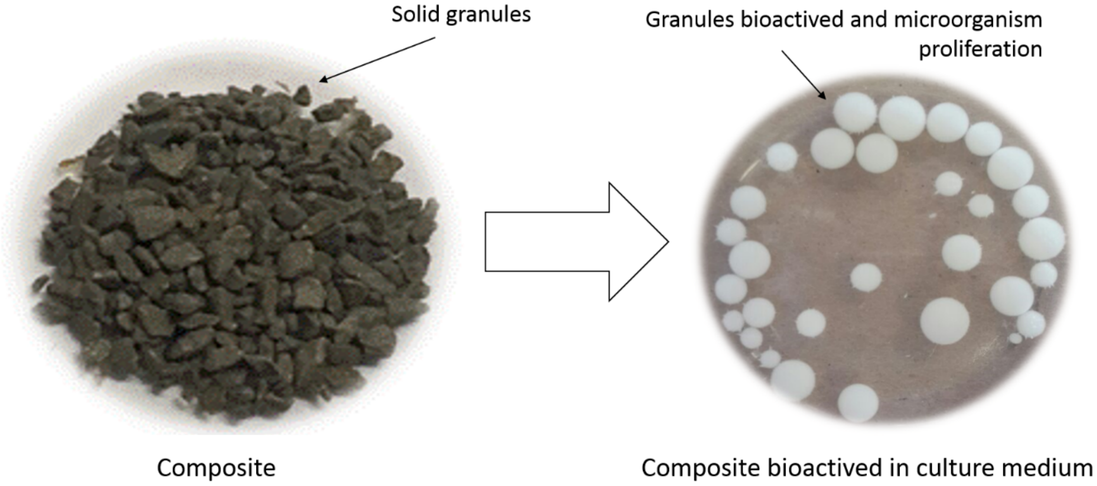

**Figure S3**. Visual comparison between the initial composite (solid granules) and after 288 h of bio-activation at culture medium.

## Supplementary Material

### Figures

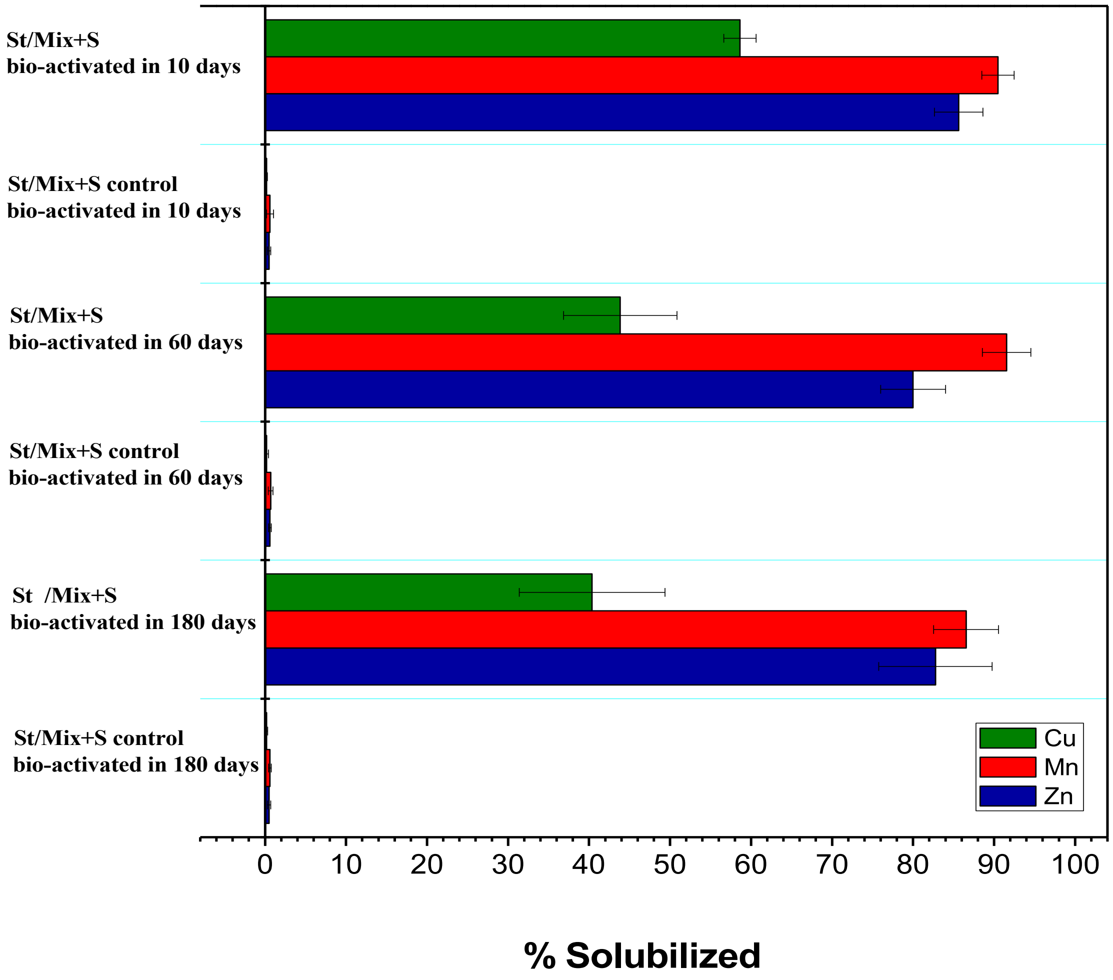

**Figure S4.** Effect of the shelf time on bio-activation of St/Mix+S and control (without *A. niger* spores) using Czapek Dox medium, 30 °C, 220 rpm, $pH_{initial}$ 7 after 288 h of incubation.

**Supplementary Material**

**Figures**

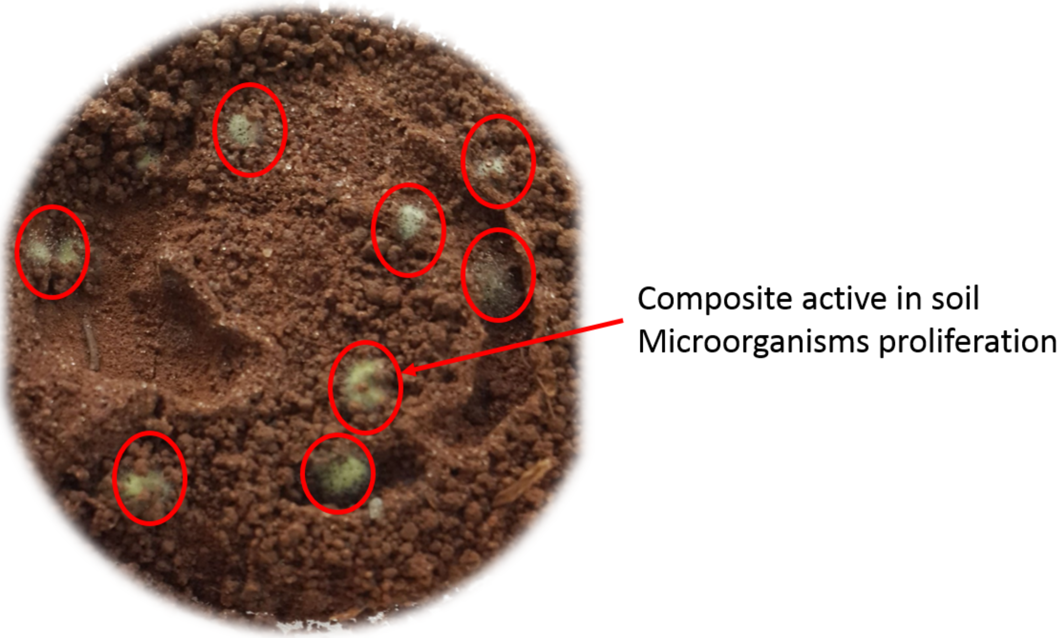

**Figure S5**. Visual analysis of the bio-activation of composite (solid granules) and after seven days of the granule placed in soil.

## Supplementary Material

### Table

**Table S1.** Compositions of the composites in terms of starch, oxides, and elemental sulfur.

| Composite | % (by mass) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | % of mineral oxide added in the formulation | | | | | % of the element in the final composite | | | |
| | Starch (St) | Zinc oxide ($ZnO_{Mineral}$) | Manganese oxide ($MnO_{Mineral}$) | Copper oxide ($CuO_{Mineral}$) | Elemental sulfur ($S^0$) | Zinc (Zn) | Manganese (Mn) | Copper (Cu) | Sulfur (S) |
| St/ZnO | 33.44 | 66.66 | - | - | - | 39.48 | - | - | - |
| St/MnO | 33.44 | - | 66.66 | - | - | - | 33.31 | - | - |
| St/CuO | 33.44 | - | - | 66.66 | - | - | - | 14.70 | - |
| St/Mix | 33.44 | 13.70 | 16.23 | 36.73 | - | 8.11 | 8.11 | 8.11 | - |
| St/Mix+S | 33.44 | 6.40 | 7.58 | 17.17 | 35.52 | 3.79 | 3.79 | 3.79 | 30.55 |

## Supplementary Material

## Table

**Table S2.** Chemical analysis by X-ray fluorescence of the mineral sources of zinc oxide, manganese oxide, and copper oxide, together with the corresponding surface areas and particle sizes.

| **Composition** | **Mineral oxide (% by mass)** | | |
|---|---|---|---|
| | **$ZnO_{Mineral}$** | **$MnO_{Mineral}$** | **$CuO_{Mineral}$** |
| **ZnO** | **73.726** | 0.105 | 2.462 |
| **MnO** | 0.069 | **64.572** | 0.724 |
| **CuO** | 0.060 | 0.028 | **27.618** |
| $Na_2O$ | 22.967 | 0.07 | 0.786 |
| MgO | 0.330 | 0.038 | 0.799 |
| $Al_2O_3$ | 0.766 | 4.271 | 10.749 |
| $SiO_2$ | 0.557 | 6.185 | 18.923 |
| $P_2O_5$ | 0.004 | 0.258 | 0.690 |
| $SO_3$ | 0.873 | 0.079 | 8.365 |
| $K_2O$ | 0.044 | 1.264 | 0.827 |
| CaO | 0.209 | 0.504 | 11.125 |
| $TiO_2$ | Nd* | 1.619 | 0.503 |
| $Fe_2O_3$ | 0.285 | 17.705 | 14.71 |
| Others | ≤ 1 | ≤ 1 | ≤ 1 |
| **Total (%)** | **≈ 100** | **≈ 100** | **≈ 100** |
| **Surface area ($m^2$ $g^{-1}$)** | **1.063** | **9.259** | **5.359** |
| **Particle size (nm)** | **446±46** | **325±56** | **302±51** |

*Nd: Not detected.

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**Supplementary Material**

**Table**

**Table S3**: Chemical and physical properties of soil studied

| Soil | pH[1] | K | Ca | Mg | H+Al | CTC[2] | V[3] | P | S | Zn | Cu | Fe | Mn | OC[4] | Sand | Silt | Clay |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | ........................... mmol$_c$ kg$^{-1}$ ......................... | | | % | | | | ........................... mg kg$^{-1}$ .............................. | | | | | ........... g kg$^{-1}$ ............ | |
| **Sandy loam** | 5.1 | 1.3 | 9.0 | 2 | 35 | 47 | 26 | 4 | 5 | 0.6 | 0.9 | 14 | 2 | 8 | 663 | 34 | 303 |
| **Clay** | 5.8 | 0.7 | 19 | 9 | 25 | 54 | 53 | 4 | 15 | 0.3 | 9.6 | 21 | 2.5 | 13 | 319 | 218 | 463 |

[1] pH = determined in $H_2O$

[2] CEC = cation-exchange capacity.

[3] V = percentage of soil base saturation.

[4] OC = total organic C.

## Supplementary Material

## References

O. A. Camargo, A. C. Moniz, J. A. Jorge, J. M. A. S. Valadares, *Métodos de Análise Química, Mineralógica e Física de Solos do Instituto Agronômico de Campinas*, Instituto Agronômico, Campinas, SP, Brazil, **2009**.

A. S. Oliveira, A. R. A. Nogueira, C. A. F. S. Carmo, D. G. Almeida, F. D. Fernandes, G. V. E. Pitta, G. M. Carlos, H. Oliveira, J. B. Mamão, M. J. A. Armelin, M. F. C. Saldanha, M. Miyazawa, S. Scramin, W. O. Barreto, Y. A. Ruffini, *Tecidos vegetais*. In: Nogueira, A. R. A., Souza, G. B. (Ed.). *Manual de laboratórios: solo, água, nutrição vegetal, nutrição animal e alimentos*. São Carlos, SP: Embrapa Pecuária Sudeste, p. 25-33, **2005**.