Growth, nutritional quality and antioxidant capacity of lettuce grown on two different soils with Sulphur-based fertilizer, organic and chemical fertilizers

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Abstract

Organic and industrial wastes represent a great opportunity to produce organic-mineral fertilizers for land restoration and crop quality improvement. The principal interest of this work was to use sulphur recovered from the residues of hydrocarbon refining processes, and orange wastes from food industry processing stabilized with bentonite (SBO) to cultivate lettuce (*Lactuca sativa* L.), one of the most produced green leafy vegetable worldwide. The aim of the present study was to cultivate lettuce in open field, in two different soils, using the mineral-organic fertilizer (SBO) at different concentrations, in comparison to chemical fertilizer (NPK), horse manure (HM) and to a control (unfertilized soil). The impact of the new fertilizer on lettuce growth and quality (nutrients and antioxidants) was investigated. Results evidenced that sulphur-based-fertilizer increased mainly the quality rather than the growth of lettuce independently from the soil characteristics. The fertilization with SBO increased the amount of potassium and sulphate in lettuce grown in both soils in respect to fertilizer concentration. In both locations, a stimulatory effect of SBO fertilizer, more on secondary metabolites than primary metabolites, in particular flavonoids, phenols, vitamins C and E, was observed. The antioxidant activities expressed as DPPH and TAC, also increased in lettuce grown with SBO at all concentrations compared to CTR and the other treatments, the increase was dependent on the SBO concentration. In short, lettuce was found enriched of anti-inflammatory compounds and vitamins when cultivated with SBO. The highest antioxidant activities, in SBO grown lettuce, were correlated to the high amount of phenols for DPPH, and flavonoids, vitamin C and E for TAC. These results can be useful for both nutraceutical and agronomic purposes.

Keywords: bioactive compounds, crop yield, chlorophylls, flavonoids, phenols, vitamins.

1. Introduction

There is, nowadays, between people, a major awareness regards the health benefits of vegetable daily consumption and their contribution to the prevention of numerous diseases such as cancer and cardiovascular disorders. All this is reflected in an increasing consumer demand for plant-based food, that rises also from a major attention to particular themes as environment, animal welfare, rejection of meat and at last but not the least religious beliefs (de Boer and Aiking, 2021). The increasing vegetable food demand leaded to an immoderate use of chemical fertilizers that degraded as much as one-third of the world's potential farmland with a detrimental impact on soil and human health. Because of the increasing sensibleness in this field, and the European guidelines (Green deal) aiming to accelerate the transition to a sustainable food system production to generate fairer economic returns with a neutral or positive environmental impact, it is becoming of great interest for farmers to shift from chemical to organic farming systems. There are controversial opinions on organic agriculture, some claim that organic farming systems are more profitable and environmentally friendly others question the organic agriculture for its lower productivity which in turn generates higher consumer prices (Herencia et al., 2011). Controversies aside, numerous authors evidenced great advantages of using organic fertilizers, more related to a nutraceutical improvement of organic farming vegetables rather than yields. Crop grown with organic fertilizers contained more phytochemicals (polyphenols in organic potatoes or tomatoes) (Montgomery and Biklè, 2021; Golijan and Sečanski, 2021) with antioxidant properties and beneficial effects on human health. Therefore, for many authors (Liu et al. 2014; Pane et al. 2015; Bergstrand, 2022), using organic matter and minerals readily utilizable from crop species can represent the most suitable method to fertilize agricultural soils for improving crop quality. Organic and industrial wastes can represent a great opportunity to produce organic-mineral fertilizers by using the three key principles (recycling, reusing, and reducing) of circular economy with numerous advantages for agriculture and environment. On the basis of the above considerations and of our previous works (Muscolo et al., 2017; 2019; 2021; Panuccio et al., 2016; 2019) focused on the recycle of different kinds of biomasses for land restoration and crop quality improvement, the principal interest of this work was to use sulphur recovered from the residues of hydrocarbon refining processes, and orange wastes from food industry processing stabilized with bentonite (Muscolo et al., 2021), to cultivate lettuce (*Lactuca sativa* L.), one of the most produced agri-food products worldwide. Lettuce, belonging to the Asteraceae family, is the leafy vegetable consumed as raw in salad, rich in minerals (Kapoulas et al., 2017) that occupies the largest cultivation area in Italy and can be successfully grown all year round (Ronga et al., 2019) in a variety of soil types with pH ranging from 6.0 to 6.8 and temperatures between 15 and 18.5 °C. even if it can withstand temperatures up to 5 ° C. Lettuce production is destined to increase for the continuous market demand due to the rapidly world population increase. Lettuce yield improvement has been achieved up to now by using chemical fertilizers or poultry manure whose main problem is the risk of microbiologically contamination of vegetables that are generally consumed fresh (Atidégla et al., 2016). Recently, Abdalla et al. (2021) showed that sulphur nutrition improved plant growth, quality, secondary metabolites, and antibacterial and radical scavenging activities of hydroponically grown lettuce cultivars. Additionally, Zenda et al. (2021), in light of the emerging problems of soil fertility exhaustion and climate change-exacerbated environmental stresses, evidenced as sulphur assumes special importance in crop production, particularly under intensively cropped areas. Considering that little crop research on Sulphur nutrient has been conducted and very scarce information has been reported (Zenda et al., 2021), the objective of the proposed study was to verify the efficacy of sulphur-based fertilizer for lettuce cultivation with the aim to mitigate problems associated with the use of other chemical or unbeneficial organic fertilizers. The experiment was carried on in open field, in two different soils, using the new mineral-organic fertilizer (SBO) at different concentrations, in comparison to chemical fertilizer (NPK), horse manure (HM) and to a control (unfertilized soil). The nutritional quality of lettuce was investigated as bio-active compounds (phenols, flavonoids, vitamins). The specific aims were to: 1) assess the impact of the new fertilizer on lettuce growth; 2) verify if the new fertilizer affected lettuce quality (nutrients and antioxidants) and the extent of its impact 3) verify if the fertilizer capacity was affected by soil characteristics.

2. Materials and Methods

2.1. Fertilizer manufacturing

Steel Belt System s.r.l. was in charge of the manufacturing process to prepare sulphur tablets with a diameter of 3/4 mm as reported in a previous paper by Muscolo et al. (2017; 2019). Sulphur was linked to 10%of bentonite clay as inert support. Sulphur-bentonite was mixed with an organic waste, residues of orange industry (pastazzo) (O). Elemental S was in percentage the main constituent of fertilizer (Muscolo et al., 2021). The new fertilizer, in the form of pads, was analysed for pathogens (total coliforms, faecal coliforms, salmonella spp and Escherichia coli) and heavy metals to avoid any toxic and harmful effects on soil and crops. The samples have been also analyzed by atomic absorption spectroscopy (GBC mod. 908) as reported in Muscolo et al. (2021). The same samples were also analyzed for Salmonella spp., as reported in Ben Said et al. (2017). Results evidenced absence of pathogens and confirmed that SBO was heavy metal free (Muscolo et al., 2021).

2.2. Pad analysis

The experiment was performed in open field in two different location on soils that differed for chemical and biological properties (Table 1). Locations: Motta San Giovanni, Loc. Liso, Italy (37.9991° N, 15.6999° E), with a sandy-loam soil (11.85% clay, 23.21% silt, and 64.94% sand) and location Lazzaro 37.9724° N, 15.6657° with a sandy clay loam (23% Clay, 27% silt, 50% sand) soil. Textural class were detected according to the Food and Agriculture Organization of the United Nations (FAO) soil classification system (FAO, 2007). The pH of both soils ranged from 8.43 for Motta San Giovanni and 8.47 for Lazzaro, close to be strongly alkaline. Soil organic matter was 3.42% in Motta San Giovanni and 2.4% in Lazzaro respectively. The soils were fertilized with sulphur bentonite orange pads (SBO), distributed at a depth of 10/15 centimetres. Four different percentage of SBO have been identified for fertilizing purposes. The different doses were 476 kg S ha^{-1} (SBO, 1.4), 952 kg S ha^{-1} (SBO, 2.8), 1428 kg S ha^{-1} (SBO, 4.2) and 1904 kg S ha^{-1} (SBO, 5.6) (Severson and Shacklette, 1988; Muscolo et al., 2017). SBO pads, analysed for their chemical composition, contained low amounts of the main nutrients as reported in Muscolo et al. (2022). Unfertilized soil was used as control (CTR), nitrogen: phosphorous: potassium, NPK (20/10/10) at 170 kg ha^{-1} was used as chemical fertilizer, and horse manure (HM) at 430 Kg ha^{-1} as organic fertilizer. Soil amendment was performed in triplicates in field. Fertilizer application: for each typology of fertilizer three parcel of 1 m square each was used. In each parcel, 3–4 plants/m^2 of lettuce have been planted. In total 36 plants for each treatment. The distance from one plant to another was 40 cm and from row to row 50 cm. During the experiment the parcel were irrigated to ensure that the water content was kept at 70% of the field capacity and soil humidity was monitored with direct read soil pH/moisture meter - R181 to maintain the 70% of the field capacity constant in both soils.

2.4. Plant analysis

The experiment was terminated at lettuce maturity, three months after sowing. Leaf and root length were measured with a meter. Plants were harvested and separated into shoots, and roots. Fresh weights were measured by weighing, and the individual plant parts were then dried at 70 ˚C in an oven. Dry weights (Biomass) were determined and plant materials were ground to pass a 20-mesh screen. Growth parameters detected: plant height (PH, cm), measured from the soil level to the highest point of lettuce plant; leaf number (LF): number of leaves from the basal leaves to the last open leaf; lettuce wide as circumference (HC, cm): with a meter around the lettuce head; leaf area (LA), using areas known as blades as reported by da Silva Ribeiro (2018).

2.5 Determination of antioxidant compounds in plants

Antioxidants and antioxidants activity, are considered markers of crop quality because are involved in the neutralization of free radicals that cause tissue damages with detrimental effects on human health. Antioxidant compounds and antioxidant activities were detected in lettuce leaves, the edible part of this vegetable (Adesso et al., 2016; Rasouli et al., 2022).

Lettuce leaves were blended and homogenized with 15 mL of EthOH: H_2O (80:20), centrifuged at 3000 rpm for 15 min, and filtered through a 0.45 μm filter (Millipore Corporation, Bedford, TX, USA). The extract was frozen at -80 °C until analysis.

Total phenols (TPHE), were measured with Folin–Ciocalteu assay (Muscolo et al., 2020). The sample absorbance was read at 760 nm on a calibration curve with gallic acid. Results were expressed as g gallic acid equivalent kg^{-1} DW.

Total flavonoids (FLA) were analyzed according to Muscolo et al. (2020). The absorbance was measured at 430 nm on a calibration curve of rutin and the results expressed as g rutin equivalent kg^{-1} DW. Carotenoids, were detected as reported by Muscolo et al. (2019)

Vitamin C was assayed according to Muscolo et al. (2020). The measurement was done in a UV–Vis Agilent 8453 spectrophotometer (Agilent Technologies, CA, USA) using ascorbic acid calibration curve, the results were reported as μg of ascorbic acid /g fresh weight of lettuce.

Vitamin E was detected as described by Prieto et al. (1999), Absorbance was measured at 695 nm against blank. Vitamin E was quantified based on the molar absorption coefficient of the phosphomolybdenum complex.

Chlorophylls a (Chl a) and b (Chl b) were determined as described in Muscolo et al. (2019). Fifty mg of leaves in 25 mL of absolute ethanol. After overnight incubation in the dark at room temperature; supernatant absorbances were read at 663 nm and 645 nm (Arnon, 1949). Chlorophyll a and b amount was expressed as mg g^{-1} dry weight (DW).

Carotenoids (CAR) were extracted by leaves grinding 50 mg in 25 mL cold acetone (Lichtenthaler, 1987). After overnight incubation at room temperature and centrifugation for 5 min at 14,000 rpm, the absorbance of samples was measured at 537, 647, and 663 nm. The amount of carotenoid content was expressed as mg g^{-1} dry weight (DW).

Vitamin A was detected as reported in Aremu and Nweze (2017). Absorbance was read at 436 nm and Vitamin A was expressed as Retinol Equivalent (RE).

2.6. Determination of antioxidant activities in plants

The antioxidant activity against DPPH radical (2,2-diphenyl-1-picryl-hydrazyl-hydrate) was determined with the method reported in Muscolo et al. (2020). The DPPH concentration in the cuvette was chosen to give absorbance values of ∼1.0. Absorbance changes of the violet solution were recorded at 517 nm after 30min of incubation at 37 ∘C. The inhibition I (%) of radical-scavenging activity was calculated as:

$$I\ (\%) = [(A0 - AS)/A0] \times 100,$$

where A0 is the absorbance of the control and AS is the absorbance of the sample after 30 min of incubation. Results were expressed as Trolox equivalent (TE).

The total antioxidant capacity (TAC) was performed according to Muscolo et al. (2020). Sample absorbance was measured at 695 nm using UV–visible spectrophotometer. Methanol (0.3 ml) in place of extract was used as blank. The antioxidant activity was expressed as μg of α-tocopherol g^{-1} DW on a calibration curve.

2.7. Statistical analysis

Analysis of variance was carried out for all the data sets. One-way ANOVA with Tukey's Honestly. Significant Difference test were carried out to analyse the effects of fertilizers on each of the various parameters measured. ANOVA and T-test were carried out using SPSS software (IBM Corp.2012). Effects were significant at $p \leq 0.01$. To explore relationships among different fertilizers and lettuce parameters in the two different locations, datasets were analysed using Principal Component Analysis (PCA).

3. Results and discussion

Lettuce growth was influenced by the diverse typology of fertilizations and at minor extent by the type of soil used for cultivation. Lettuces cultivated in Motta were significantly smaller (see fresh weight, leaf number and plant height in Fig. 1) than those grown in Lazzaro, both in control soil and in fertilized soils. In both locations, even if at different concentrations, lettuces cultivated with SBO, had the better growth in terms of less water content and more dry mass compared to the control and the other treatments. The best growth was observed in Lazzaro than Motta location (Fig. 1). No significant differences were observed in leaf number and in leaf area among the treatments in both the experimental locations (Motta and Lazzaro), while an increase in head circumference was observed in presence of SBO at concentrations ranging from 2.8 to 5.6 in both soils in respect to the other treatments (Fig. 1). The effect of fertilizer typologies was mostly evident on lettuce metabolism than growth, confirming previous finding of Yang et al. (2018). demonstrating that various factors, among who's fertilization, can cause variations in the quality of vegetation. Panuccio et al. (2021a) evidenced that the fertilizing power of digestate was more dependent on the concentration used than the type of soil, and that the effects of digestate on the different soils were different in extent but in any case, positive. Results obtained showed differences in essential mineral elements (cations) in lettuce grown with the different fertilizers and in the diverse locations (Fig. 2). The macronutrients (calcium, magnesium and phosphorous) in lettuce leaves grown in Motta didn't evidence significant differences in amount, except for potassium that increased remarkably with SBO 2.8 and 4.2 and ammonium that increased, increasing SBO concentration in respect to CTR. In Lazzaro, the greatest potassium amount was observed in lettuce grown with SBO 4.2 and NPK, while ammonium was the highest in HM grown lettuce. No remarkable differences were detected for the other macronutrients. Regarding anions (Fig. 3), lettuce grown in Motta contained more phosphate and sulphate with SBO 2.8, while lettuce grown in Lazzaro showed the greatest amount of phosphate with NPK, and sulphate with SBO 2.8, 4.2 and 5.6 (Fig. 3). The fertilizers increased also Ca/Mg ratio and the greatest increase was detected in presence of SBO 2.8 in Motta and 4.2 in Lazzaro with a value of 1.8 and 2.0 respectively. The optimal Ca/Mg ratio in the human diet varies between 1.70 and 2.60 (weight to weight), and excessive or less consumption or the imbalanced consumption is related to an increased risk of different types of cancer and metabolic syndrome (Costello et al., 2021; Moore-Schiltz et al., 2015). Results highlighted the beneficial effects of SBO in increasing the essential nutrients amount in lettuce. Muscolo et al. (2020) discriminated the effect of organic fertilizers one from each other on the quality of red Topepo, highlighting as fertilizer composition resulted largely responsible for the synthesis of bioactive compounds, flavour, and aroma of this fruit. Panuccio et al. (2021b) found, on tomato fertilized with different digestates, a strict relationship between fertilizer characteristics and plant metabolic profile, highlighting as the intrinsic characteristics of a fertilizer can impact plant metabolism, shifting the metabolic pathway from primary to secondary. Secondary metabolites (SMs) are natural products coming from shikimic acid through the shikimate pathway (Bruce and Onyegbule, 2021) and are involved in plant defences and in stress tolerance. Plant SMs are also utilized for medical, pharmaceutical and culinary purposes (Chiocchio et al., 2021). Data obtained evidenced, in both locations, a stimulatory effect of fertilizers more on secondary metabolites than primary metabolites. Total proteins increased only in lettuce with SBO 2.8 in Motta and lettuce with NPK, HM, SBO 1.4 and 2.8 in Lazzaro. Total carbohydrates did not change significantly in quantity in respect to the fertilizer used (Table 2), while changes in quantities were observed for total phenols, flavonoids and vitamins (Table 2). TPHE was more prominent in lettuce from Motta soil fertilized with SBO 2.8, than in the other treated lettuces. Conversely in Lazzaro the greatest increase was observed in lettuce cultivated with NPK followed by HM and SBO at all concentrations in respect to control (Table 3). In Lazzaro flavonoids progressively and significantly increased in lettuce, with increasing the concentrations of SBO compared to control, NPK and HM. A similar trend was observed for vitamin E and C (Tables, 2-3). In Motta, flavonoids and vitamin E were the highest with SBO. No changes among the treatments were observed for vitamin A and C. Flavonoids, vitamin E and C are considered bioactive compounds with protective effects against cardiovascular disease, some cancer typologies and/or photosensitivity diseases (Pietta, 2000). Vitamin C is used as an antioxidant supplement, and claimed to increase resistance to diseases and oxidative stress (Yimcharoen et al., 2019). Some recent studies demonstrated a significant reduction in the risk of lung cancer by increased dietary vitamin C uptake (Villagran et al., 2021). Vitamin E rich food consuming demonstrated a decrease in the incidence of cardiovascular diseases, providing protection against LDL cholesterol oxidation and reducing risk of heart disease (Ziegler et al., 2020). Flavonoids, in addition to their mode of action as antioxidant, can reduce carcinogenesis modulating the metabolism of food-born carcinogens by inhibiting or inducing the phase I and II biotransformation enzymes, and by suppressing the abnormal proliferation of early, preneoplastic lesions (Kopustinskiene, et al., 2020; Dharmawansa et al. 2020). A part the bioactive compounds, also the antioxidant activities expressed as DPPH and TAC, increased in lettuce grown with SBO at all concentrations compared to CTR and the other treatments, in Motta location (Fig. 4). In Lazzaro increased DPPH with NPK, followed by HM and the highest concentrations of SBO fertilizer. TAC increased remarkably only with SBO 1.4 and 2.8 (Fig. 5). DPPH and TAC are two recognized mechanisms for the radical scavenging reactions (Csepregi et al., 2016). Antioxidant activities are strictly dependent on the richness and type of phytochemicals and helping to prevent the development of diverse oxidative stresses are considered responsible for the therapeutic properties of plants (Zhang et al., 2012). Bioactive compounds mainly include flavonoids, phenolics and vitamins. In this study a strict relationship between the quantity of TPHE and DPPH activity was observed in lettuces grown in both locations (Tables 2-3). In presence of SBO 2.8 and 4.2 an increase in flavonoids, vitamin E and C concomitantly to DPPH and TAC was detected (Tables 2-3; Figs.4-5). NPK increased mainly total phenol content that was not related to the TAC increase but explicitly correlated to an enhancement of DPPH. Chlorophylls and carotenoids, recognized as antioxidant compounds are natural pigments that can be intake daily with the regular ingestion of fruits and vegetables. No significant differences have been detected among the treatments and between the location (Table 4) in the carotenoids and chlorophyll quantities in lettuce, suggesting that carotenoids and chlorophyll were not overproduced to be used as scavengers but rather they were mainly produced to be used in the light-harvesting process of photosynthetic process and in the protection against photo-oxidative damage as demonstrated by data on growth parameters and antioxidant capacity of the lettuces. PCA analysis confirmed this assertion, evidencing that CAR and Chlorophyll a and b were not correlated to any antioxidant's activity both in Motta and in Lazzaro (Fig. 7A, B). The Pearson correlation between LA with PH and HC (Table 5) evidenced how leaf area has a key role in the light interception capacity which in turn affects growth and metabolism of a crop, representing a linear relationship between accumulated biomass and cumulative intercepted photosynthetically active radiation (Bai et al., 2016). The correlation between FLA, VIT C, and E with TAC in lettuce grown in Motta and between DPPH, and TPHE in lettuce grown in Lazzaro evidenced a different activation of the antioxidant power of lettuce in respect to soil and fertilizer combination (Fig. 7A, B). For growth parameters, in Motta, PC1 accounted for 46.15% of the variance, and PC2 accounted for 26.49%, and their sum explained 72.64% of total variance (Fig. 6A). Data evidenced, important growth parameters as LA, HC, PH and FW were mainly influenced by SBO 4.2. In Lazzaro, PC1 accounted for 59.23% of the variance, and PC2 accounted for 31.95%, and their sum explained 91.18% of total variance (Fig. 6B). Data evidenced a correlation between SBO 2.8 and 4.2 with Biomass, HC and LA, and between SBO 5.6 with LN, PH and FW (Fig. 6B). Regarding bioactive compounds, in Motta, PC1 accounted for 37.83% of the variance, and PC2 accounted for 26.70%, and their sum explained

64.53% of total variance (Fig. 7A). Data showed that SBO 2.8 was positively associated with TPRO, Vit C, CAR and Chlorophyll a. SBO 4.2 was correlated to FLA, TAC, VITA, Vit E and CARB. CTR and HM were in the left top quadrant associated with Chl b, TPHE and DPPH. No correlation between primary and secondary metabolites with NPK and SBO 1.4 located in the negative left quadrant (Fig. 7A) was observed. In Lazzaro PC1 accounted for 44.99% and PC2 for 24.91% of the variance, and their sum explained 69.9% of total variance (Fig. 7B) showed a different scenario, on the right top quadrant HM, and NPK were associated with TPHE, TCARB and Chlb, on the left top quadrant SBO 2.8 and 4.2 were correlated with TPRO, VIT E, DPPH, and FLA. Conversely in the negative quadrant on the right were located VIT A, Chl a, CAR and CTR. In the left down-negative side SBO 1.4 and 5.6 were associated with VIT C and TAC. Data evidenced a strict correlation with SBO (2.8, 4.2 and 5.6) with TPRO, antioxidant and antioxidant activities. Regarding the anions PC1 accounted for 51.36% of the variance, and PC2 accounted for 27%, and their sum explained 78.36% of total variance (Fig. 8). Anions were mainly correlated to SBO 1.4 (Malate, Sulphate and Chloride), and SBO 4.2 with Sulphate and Bromide in both Motta and Lazzaro grown lettuces (Fig. 8A, B). As reported in Fig. 8, PC1 accounted for 66.31% of the variance, and PC2 accounted for 21.75%, and their sum explained 88.06% of the total variance. Ammonium correlated with SBO 4.2 in Motta, and with HM in Lazzaro. Magnesium and Calcium only in Lazzaro-grown lettuce correlated with SBO 1.4 and NPK (Fig. 9A, B). In short, we observed that the mineral organic-fertilizer had a positive effect on lettuce metabolism, stimulating the synthesis of bioactive compounds and antioxidative activity mainly when used at a concentration (SBO, 2.8) corresponding to 952 kg S ha^{-1} in both soils.

In conclusion, this study evidenced that sulphur can have a key role for improving crop productivity while enhancing crop nutritive-value, satisfying the increasing healthy food request from the growing human population. Sulphur based fertilizer manufacturing is a low environmental impact process, that recycles wastes, minimally processed, with suitable organic matter and sulphur in a unique industrial production process. The low environmental impact comes from the significant reductions of greenhouse gas emissions in the atmosphere for the elimination of a large amount of hazardous materials from the environment. The effects observed in the current study suggest that we need to redirect our attention to sulphur as a nutrient also for its synergistic relationship with other nutrients. Further efforts should involve exploring the demand for S from crops to optimize its use. Matching the S demand of crops with specific soil properties, is essential to facilitate the right dose of sulphur in agricultural production, improving food production while maintaining nutrient balance in a sustainable and environmentally friendly way.

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.

Funding: this research was financial supported by Steel Belt Systems s.r.l., Venegono (Va), Italy and it was conducted as part of the project LIFE20 ENV/IT/000229 –LIFE RecOrgFert PLUS, funded by the LIFE Programme of the European Union.

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Table 1 Main physical and chemical properties of soils located in Motta San Giovanni and Lazzaro. Electric conductivity (EC), organic carbon (OC), organic matter (OM), total nitrogen (N), microbial biomass (MBC), Cation exchange capacity (CEC).

	MOTTA SG	Lazzaro
pH (H_2O)	8.43	8.47
pH (KCl)	6.94	6.99
EC (µS/cm)	302	301
OC (%)	1.981	1.4
OM	3.42	2.91
N (%)	0.198	0.2344
C/N	10	6.08
MBC µg C g^{-1} dry soil	835	1132
CEC cmol($^{+}$) Kg^{-1}).	19	14

Table 2. Total proteins (TPRO, µg g^{-1} dw), total carbohydrates (TCARB, mg glucose g^{-1} dw) total phenols (TPHE, µg tannic acid g^{-1} dw), total flavonoids (TFLA, µg quercetin g^{-1} dw), total carotenoids (CAR, mg g^{-1} dw), 2,2′-diphenyl-1-picrylhydrazyl radical activity assay (DPPH•, % inhibition) vitamin A (VIT A, µg retinol g^{-1} dw), vitamin C (VIT C, µg ascorbate g^{-1} dw) vitamin E (VIT E, µg alpha-tocopherol g^{-1} dw) in lettuce leaves cultivated on Motta soils without fertilizer (control, CTR), with nitrogen:phosporous:potassium (NPK), horse manure (HM) and different concentrations of sulfur-bentonite based fertilizers (SBO 1.4; 2.8; 4.2; 5.6), Data are the means ±standard errors of three replicates of three independent experiments (n=36). *Different letters indicate significant differences per $p \leq 0.01$.

	CTR	**NPK**	**HM**	**SBO 1.4**	**SBO 2.8**	**SBO 4.2**	**SBO 5.6**
TPRO	2243±20[b]	2279±28[b]	2294±23[b]	2207±26[c]	2369±25[a]	2255±21[b]	2253±22[b]
TCARB	22±2[a]	23±1[a]	23±2[a]	24±2[a]	23±2[a]	23±3[a]	24±25[a]
TPHE	4943±28[d]	5535±25[b]	4785±35[c]	4404±25[d]	5828±35[a]	5295±36[c]	4106±41[e]
FLA	24±2[a]	19±2[b]	15±2[b]	34±4[a]	29±3[a]	27±3[a]	24±2[a]
DPPH %	582±15[c]	783±11[a]	565±9[d]	613±10[c]	590±13[c]	650±15[b]	601±15[c]
VIT A	0.27±0.02[a]	0.28±0.02[a]	0.23±0.03[a]	0.27±0.02[a]	0.30±0.01[a]	0.28±0.03[a]	0.28±0.02[a]
VIT C	46±1[a]	48±1[a]	49±2[a]	49±3[a]	53±3[a]	52±2[a]	52±2[a]
VIT E	34±3[c]	34±2[c]	43±2[b]	47±3[b]	57±2[a]	59±3[a]	64±3[a]

Table 3. Total proteins (TPRO, µg g^{-1} dw), total carbohydrates (TCARB, mg glucose g^{-1} dw) total phenols (TPHE, µg tannic acid g^{-1} dw), total flavonoids (TFLA, µg quercetin g^{-1} dw), total carotenoids (CAR, mg g^{-1} dw), 2,2′-diphenyl-1-picrylhydrazyl radical activity assay (DPPH•, % inhibition) vitamin A (VIT A, µg retinol g^{-1} dw), vitamin C (VIT C, µg ascorbate g^{-1} dw) vitamin E (VIT E, µg alpha-tocopherol g^{-1} dw) in lettuce leaves cultivated on Lazzaro soils without fertilizer (control, CTR), with nitrogen:phosporous:potassium (NPK), horse manure (HM) and different concentrations of sulfur-bentonite based fertilizers (SBO 1.4; 2.8; 4.2; 5.6), Data are the means ±standard errors of three replicates of three independent experiments (n=36). *Different letters indicate significant differences per $p \leq 0.01$

	CTR	NPK	HM	SBO 1.4	SBO 2.8	SBO 4.2	SBO 5.6
TPRO	2128±25^{c}	2260±35^{a}	2262±25^{a}	2270±25^{a}	2320 ^{a} ±28	2227±18^{b}	2176±32^{c}
TCARB	22.4±1.5^{a}	23.6±2^{a}	22.2±2.5^{a}	20.5±2^{a}	22.3±0.5^{a}	22.8±1.5^{a}	21.6±1.8^{a}
TPHE	7245±35^{d}	12683±45^{a}	9008±20^{b}	8700±29c	8738±30^{c}	9015±35^{b}	8860±20^{c}
FLA	29±2^{bc}	31±2^{b}	35±2.5^{b}	26±1.5^{c}	35±2^{b}	46±2.5^{a}	47±3^{a}
DPPH	623±21^{b}	683±18^{a}	579±25^{b}	446±20^{d}	546±18^{c}	593±28^{b}	378±20^{e}
VIT A	0.35±0.02^{a}	0.30±0.02^{a}	0.30±0.03^{a}	0.29±0.03^{a}	0.29±0.01^{a}	0.29±0.02^{a}	0.28±0.03^{a}
VIT C	49±2^{b}	48±1^{b}	47±1^{b}	55±2^{a}	57±3^{a}	53±3^{a}	51±2^{ab}
VIT E	34±2^{b}	35±3^{b}	24±2^{c}	30±4^{bc}	57±4^{a}	57±5^{a}	63±5^{a}

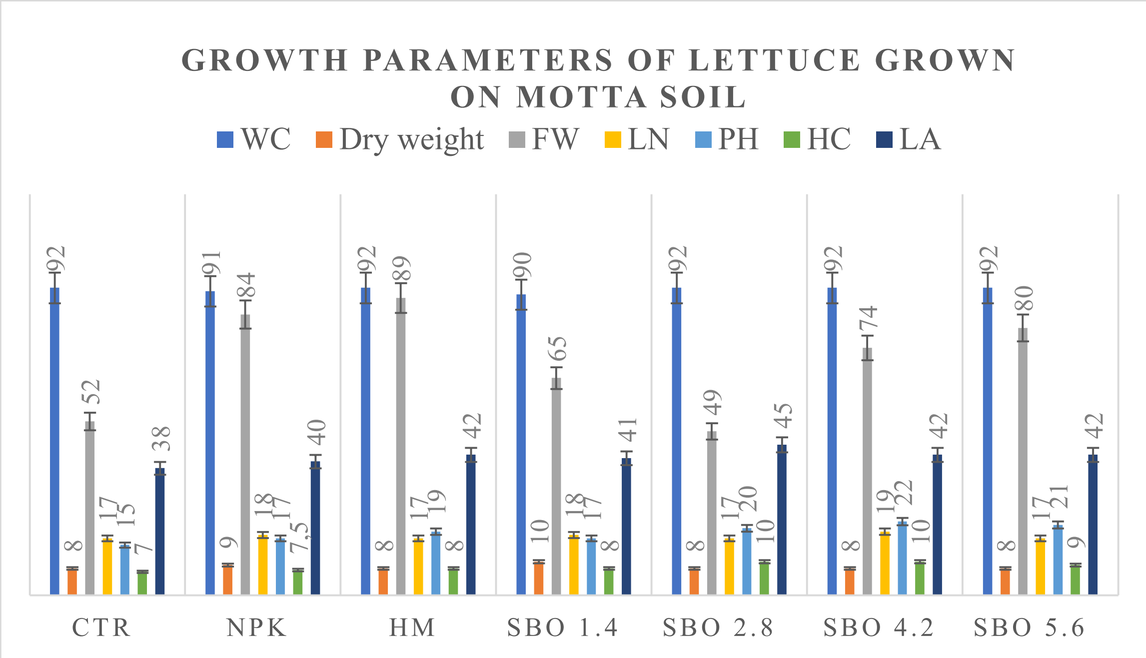
Table 4. Chlorophyll a (Chl a, mg/100g fw), chlorophyll b (Chl b, mg/100g fw) total chlorophyll (Chl a+Chl b), Chlorophyll a/ Chlorophyll b ratio (Chl a/Chl b), total carotenoids (CAR, mg/100g fw), in lettuce leaves cultivated on Motta and Lazzaro soils without fertilizer (control, CTR), with nitrogen:phosporous:potassium (NPK), horse manure (HM) and different concentrations of sulfur-bentonite based fertilizers (SBO 1.4; 2.8; 4.2; 5.6), Data are the means of three replicates of three independent experiments (n=36). *Different lowercase superscript letters in the same row indicate significant differences among the treatments per $p \leq 0.01$. § Different capital letters in the same column indicate significant differences into the same treatment for each parameter per $p \leq 0.01$

Motta	**CTR**	**NPK**	**HM**	**SBO 1.4**	**SBO 2.8**	**SBO 4.2**	**SBO 5.6**
Chl a	3.45^{a*}A§	3.38^{b}A	3.54^{a}B	3.28^{b}A	3.64^{a}A	3.44^{a}A	3.57^{a}A
Chl b	0.44^{a}A	0.31^{c}A	0.38^{b}B	0.31^{c}A	0.38^{b}A	0.31^{c}A	0.37^{b}A
Chl a+Chl b	3.99^{a}A	3.69^{b}A	3.92^{a}B	3.59^{b}A	4.02^{a}A	3.75^{b}A	3.94^{a}A
Chl a/Chl b	7.84^{c}B	10.9^{a}A	9.31^{b}A	10.58^{a}B	9.57^{b}B	11.1^{a}B	9.64^{b}B
CAR	1.8^{a}A	1.7^{a}A	1.8^{a}B	1.7^{a}A	1.9^{a}A	1.8^{a}A	$1.8^{\pm}$A
Lazzaro	**CTR**	**NPK**	**HM**	**SBO 1.4**	**SBO 2.8**	**SBO 4.2**	**SBO 5.6**
Chl a	3.57^{b}A	3.13^{c}A	4.19^{a}A	2.74^{d}B	2.66^{d}B	2.27^{e}B	2.79^{d}B
Chl b	0.37^{b}A	0.27^{c}A	0.58^{a}A	0.11^{d}B	0.10^{d}B	0.17^{d}B	0.16^{d}B
Chl a+Chl b	3.94^{b}A	3.40^{c}A	4.77^{a}A	2.85^{d}B	2.76^{d}B	2.44^{e}B	2.95^{d}B
Chl a/Chl b	9.64^{d}A	11.59^{c}A	7.22^{e}B	24.9^{a}A	26.6^{a}A	13.4^{c}A	17.4^{b}A
CAR	1.8^{b}A	1.6^{c}A	2.2^{a}A	1.3^{d}B	1.3^{d}B	1.1^{e}B	1.4^{e}B

Table5 Pearson's correlations (r) between different typologies of fertilizer, and growth parameters of lettuces grown in Motta (A) and in Lazzaro (B). *Values in bold are different from 0 with a significance level alpha=0,05*

Correlation matrix (Pearson (n):							
A							
	WC	Biomass	FW	LN	PH	HC	LA
WC	**1**	**-1**	-0,04	-0,35	0,436	0,356	0,225
Biomass	**-1**	**1**	0,037	0,346	-0,44	-0,36	-0,23
FW	-0,04	0,037	**1**	0,207	0,282	-0,13	-0,06
LN	-0,35	0,346	0,207	**1**	0,267	0,267	-0,07
PH	0,436	-0,44	0,282	0,267	**1**	**0,897**	0,741
HC	0,356	-0,36	-0,13	0,267	**0,897**	**1**	**0,847**
LA	0,225	-0,23	-0,06	-0,07	0,741	**0,847**	**1**

Correlation matrix (Pearson (n):							
B							
Variables	WC	Biomass	FW	LN	PH	HC	LA
WC	**1**	**-1**	0,153	-0,18	-0,12	**-0,81**	-0,54
Biomass	**-1**	**1**	-0,15	0,183	0,122	**0,808**	0,54
FW	0,153	-0,15	**1**	**0,794**	0,751	0,347	0,495
LN	-0,18	0,183	**0,794**	**1**	**0,932**	0,692	0,577
PH	-0,12	0,122	0,751	**0,932**	**1**	0,631	0,672
HC	**-0,81**	**0,808**	0,347	0,692	0,631	**1**	0,747
LA	-0,54	0,54	0,495	0,577	0,672	0,747	**1**



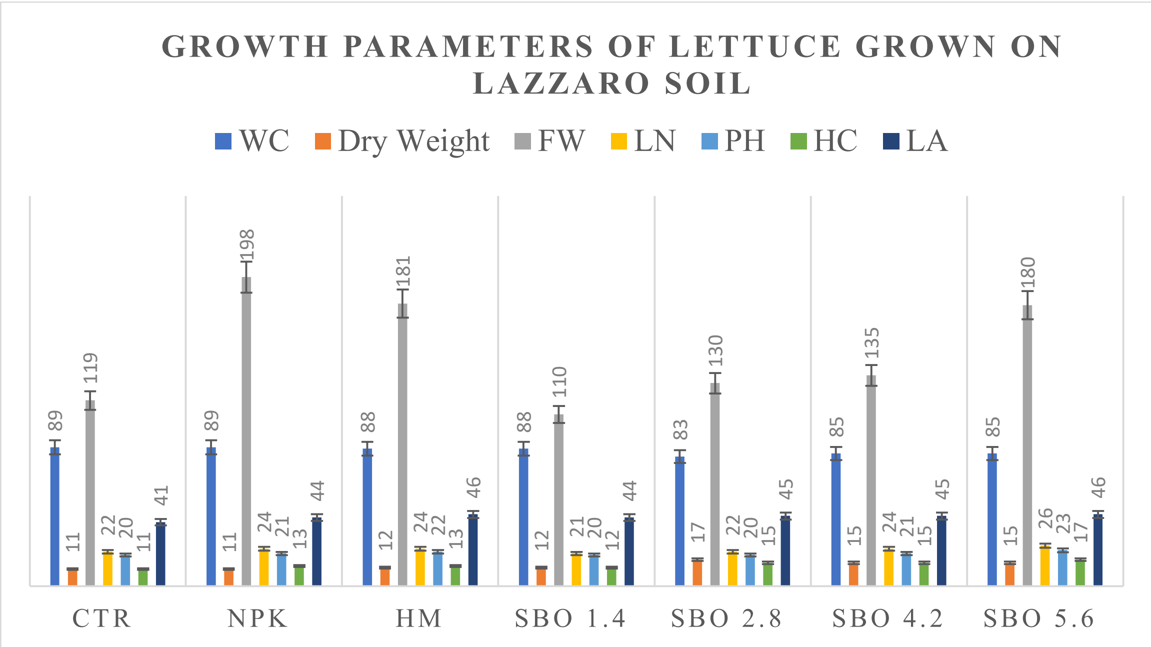
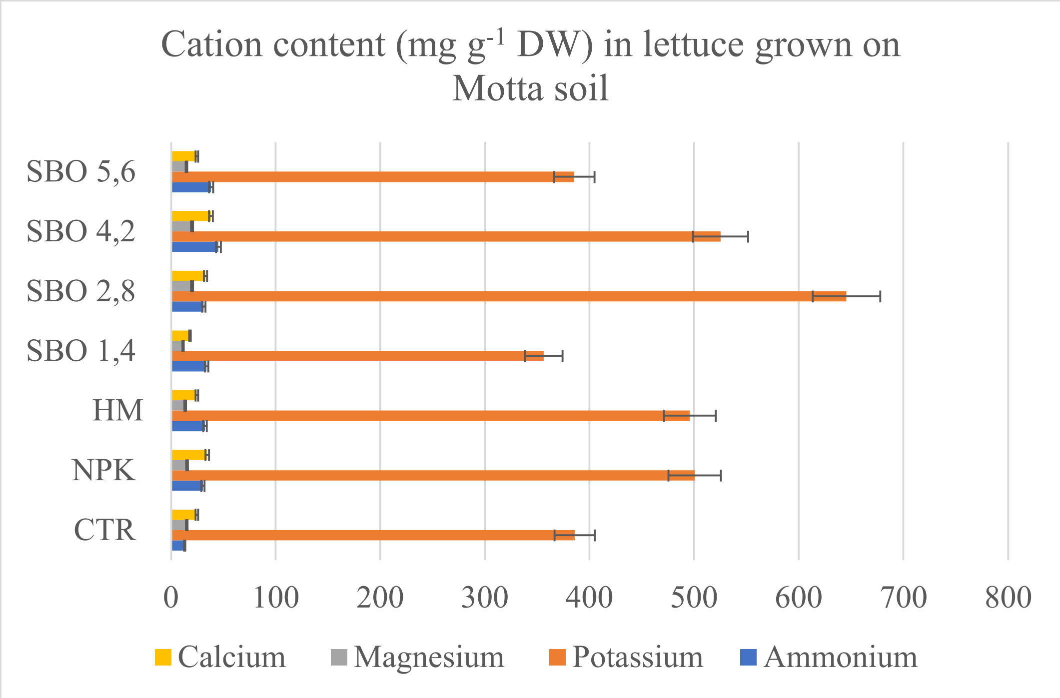


Figure 1 lettuce growth parameters: biomass as dry weight (% dw) and fresh weight (g/plant), leaf number (LN), plant height (PH, cm), head circumference (HC, cm), leaf area (LA, cm^2) and water content (WC, %) detected at the end of growth period (3 months) on Motta and Lazzaro soils unfertilized (CTR) and fertilized with nitrogen phosphorous and potassium (NPK, 20/10/10), horse manure (HM), and sulphur-bentonite-orange wastes (SBO) at different concentrations. Data are the mean ±standard error of three replicates of three independent experiments (n=36).



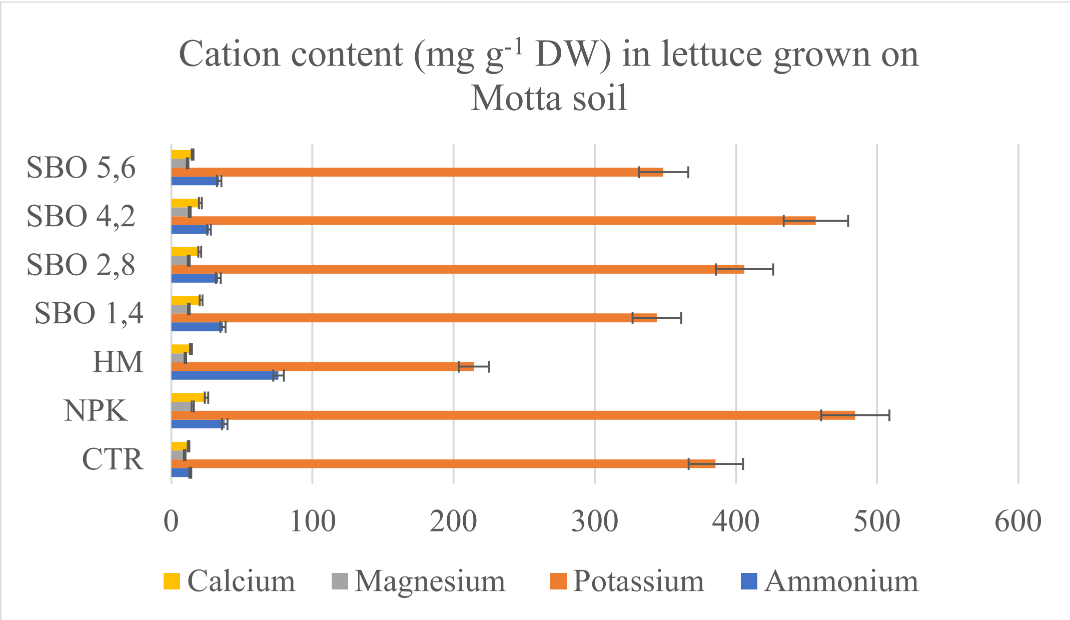
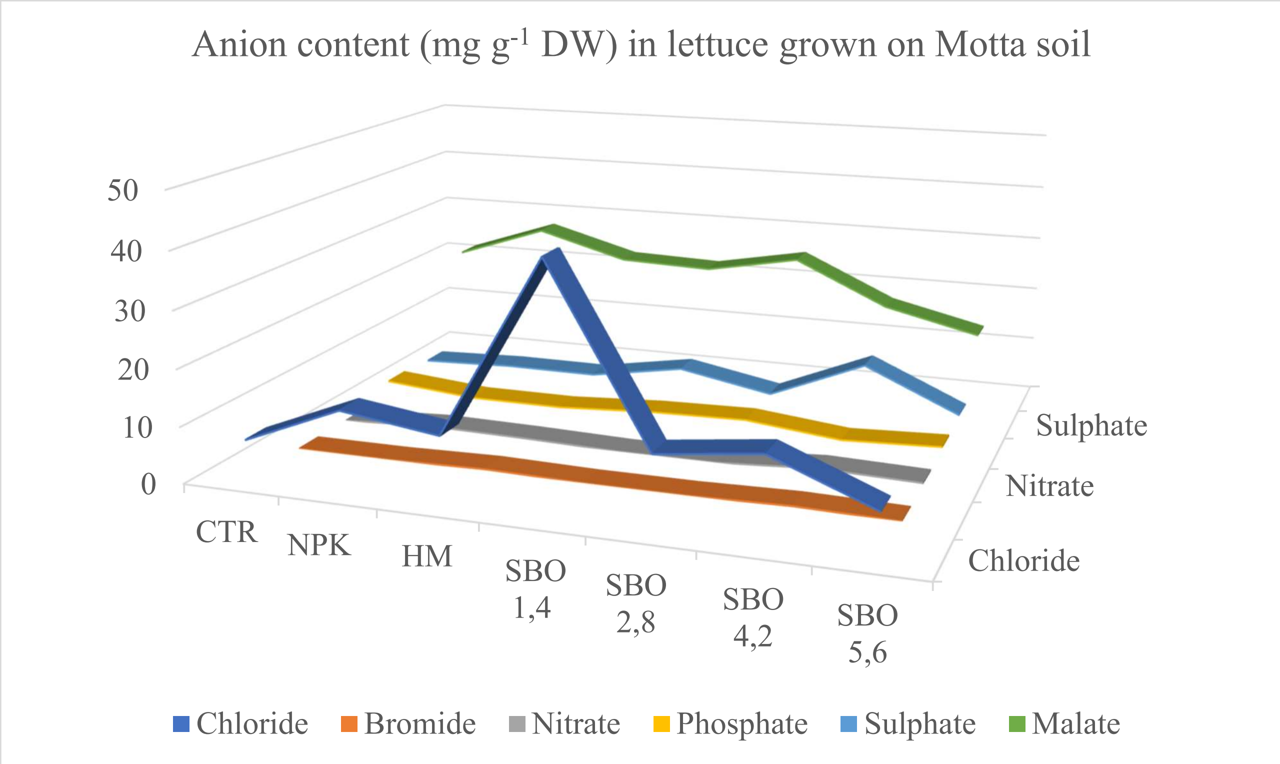


Figure 2 Cation content in leaves of lettuce grown on Motta and Lazzaro soils cultivated without fertilizers (Control, CTR) and with nitrogen:phosporous:potassium (NPK), horse manure (HM). And different concentrations of sulphur-bentonite based fertilizers (SBO 1.4; 2.8; 4.2; 5.6), Data are the means of three replicates of three independent experiments (n=36).



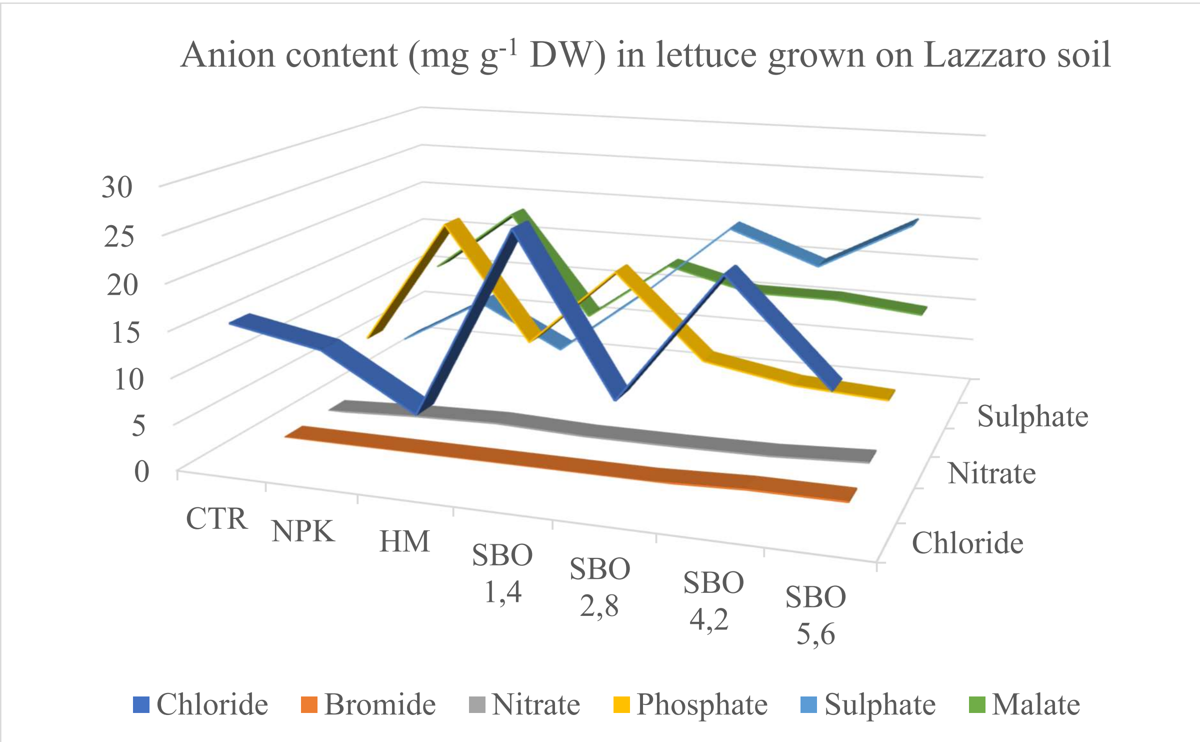
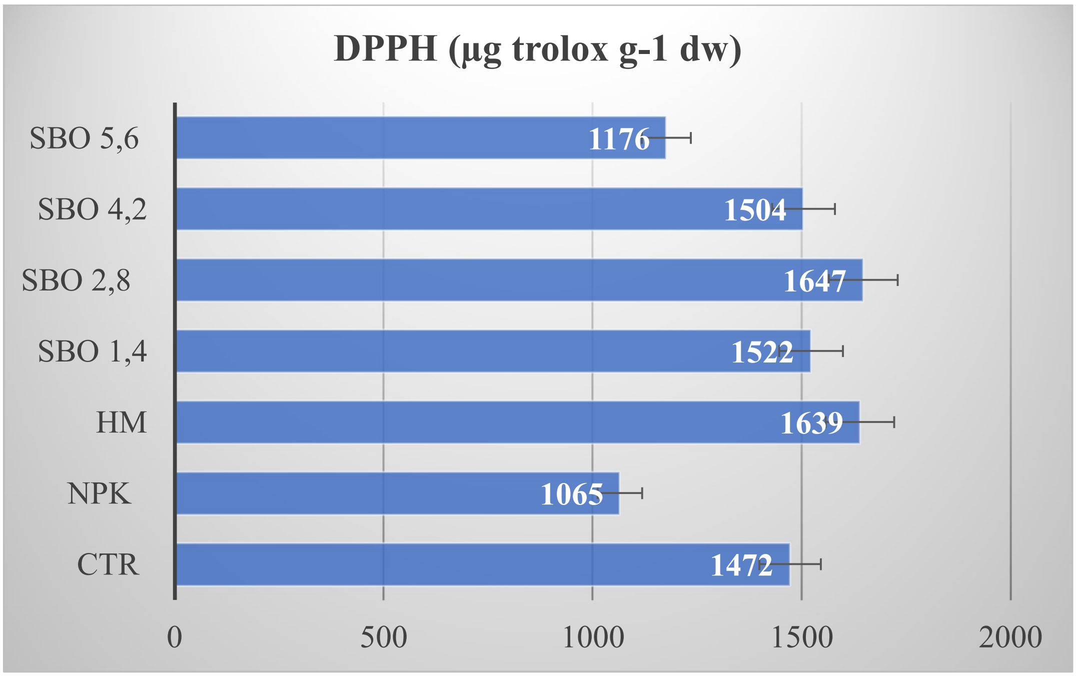


Figure 3 Anion content in leaves of lettuce grown on Motta and Lazzaro soils cultivated without fertilizers (Control, CTR) and with nitrogen:phosporous:potassium (NPK), horse manure (HM). And different concentrations of sulphur-bentonite based fertilizers (SBO 1.4; 2.8; 4.2; 5.6), Data are the means of three replicates of three independent experiments (n=36).



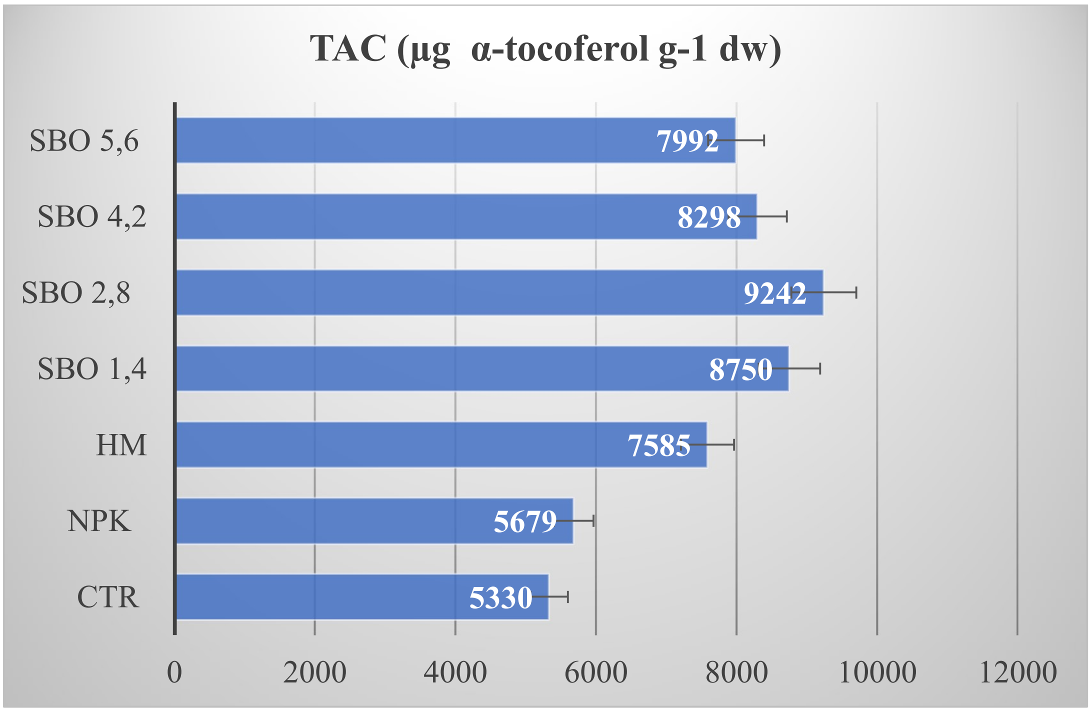
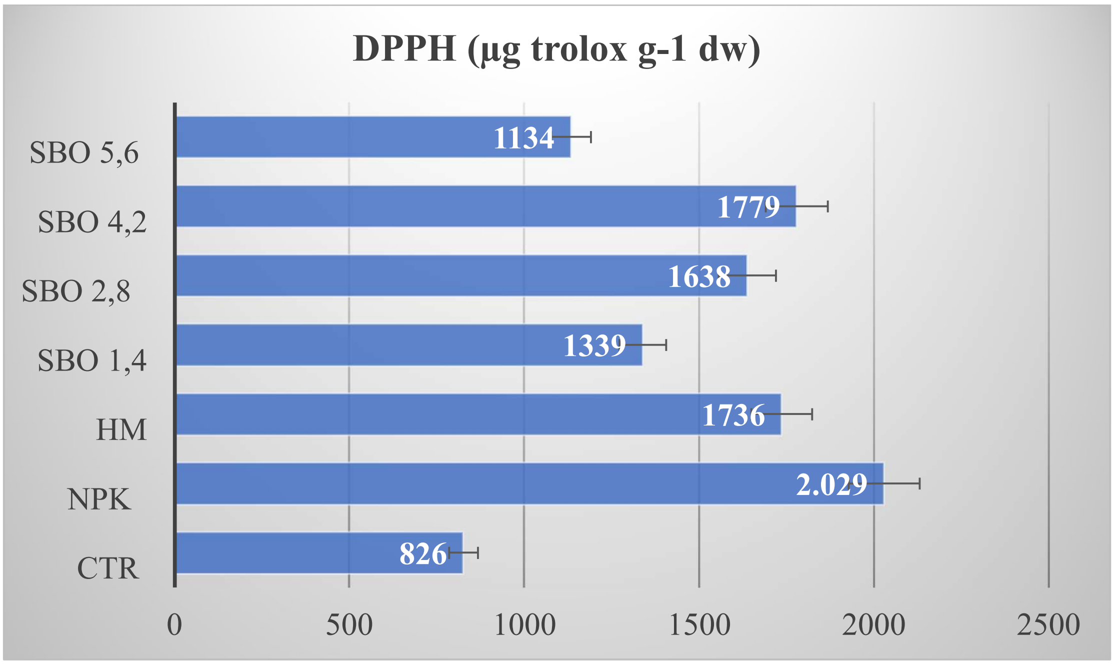


Figure 4 Antioxidant capacity expressed as DPPH and total antioxidant capacity (TAC) detected in lettuce leaves at the end of cultivation period **on Motta soil** unfertilized (CTR) and fertilized with nitrogen phosphorous and potassium (NPK, 20/10/10), horse manure (HM), and sulphur-bentonite-orange by-product (SBO) at different concentrations.



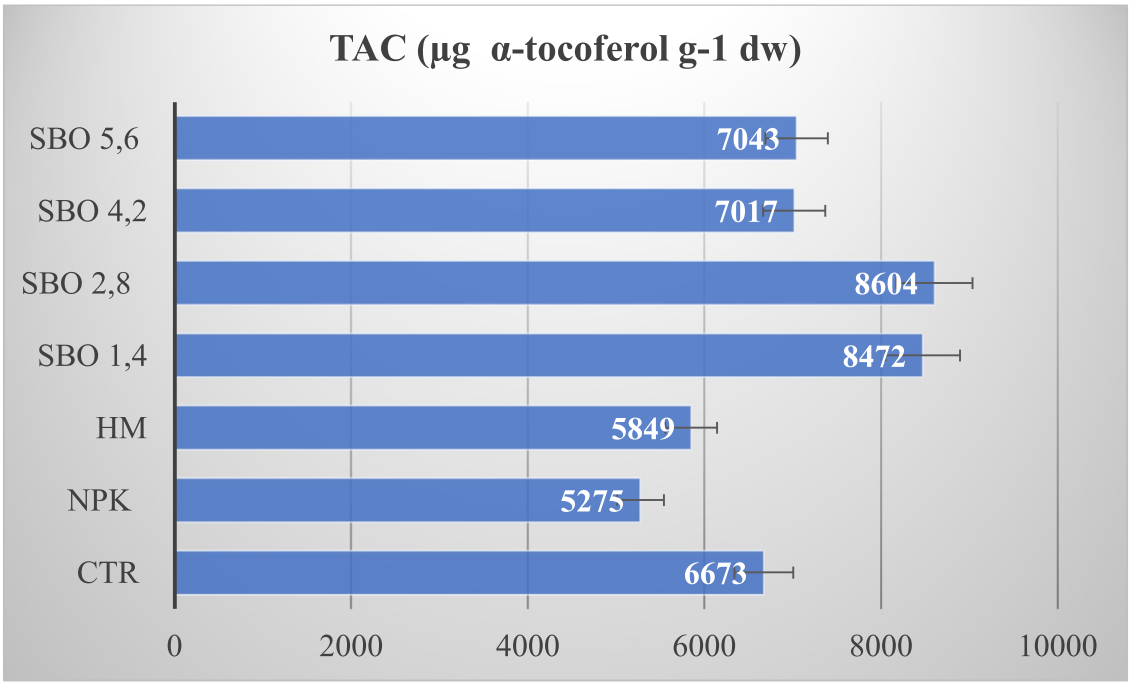
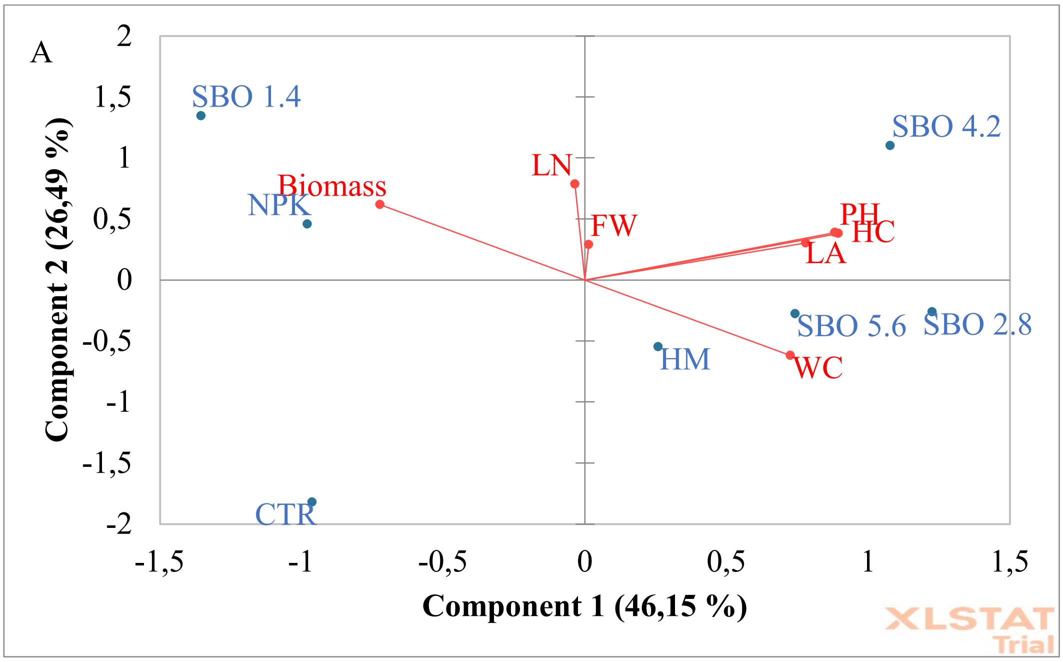


Figure 5 Antioxidant capacity expressed as DPPH and total antioxidant capacity (TAC) detected in lettuce leaves at the end of cultivation period **on Lazzaro soil** unfertilized (CTR) and fertilized with nitrogen phosphorous and potassium (NPK, 20/10/10), horse manure (HM), and sulphur-bentonite-orange by-product (SBO) at different concentrations



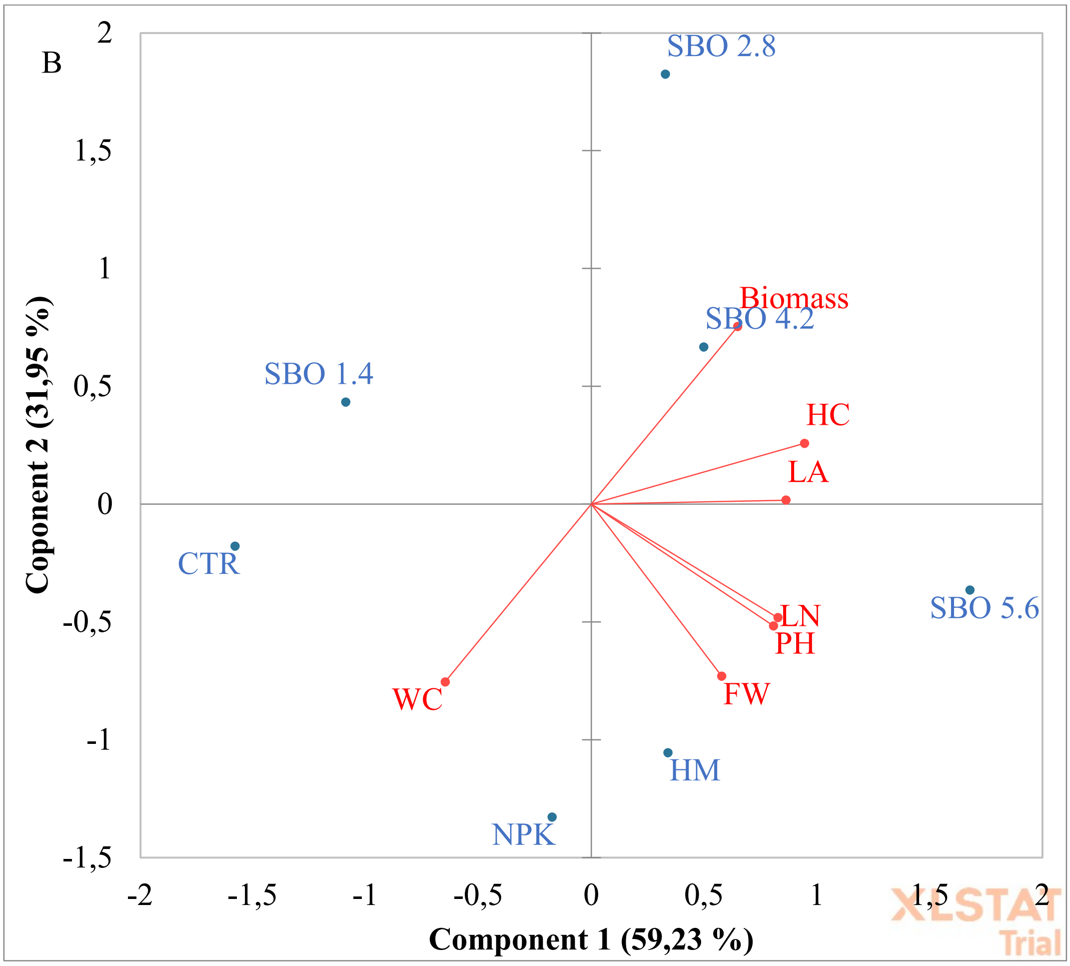


Figure 6 PCA (principal component analysis) diagram of growth parameters of lettuce cultivated in Motta (A) and Lazzaro (B) in soils without fertilizer (CTR) and with different fertilizers nitrogen:phosporous:potassium (NPK), horse manure (HM) and different concentrations of sulphur-bentonite based fertilizers (SBO 1.4; 2.8; 4.2; 5.6).

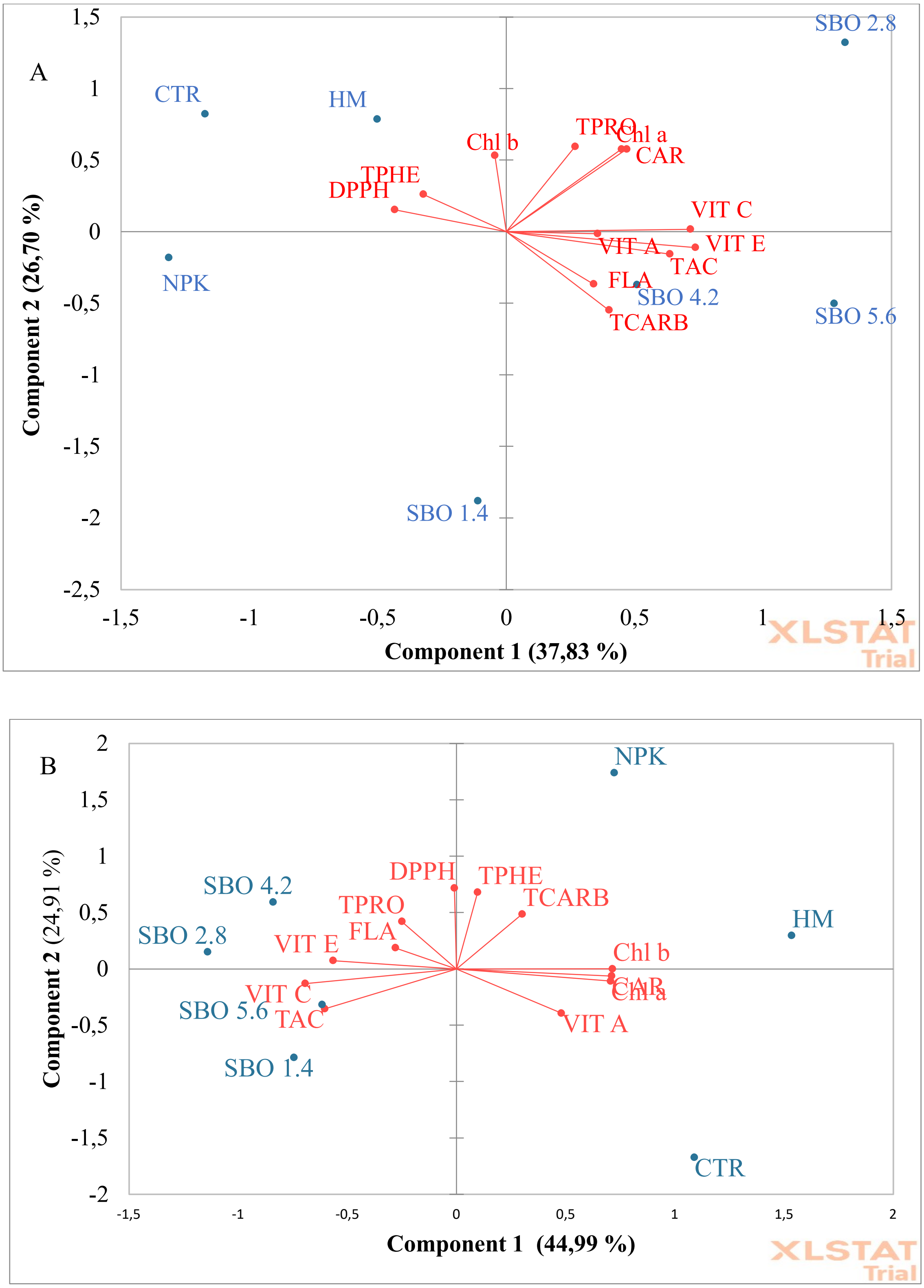
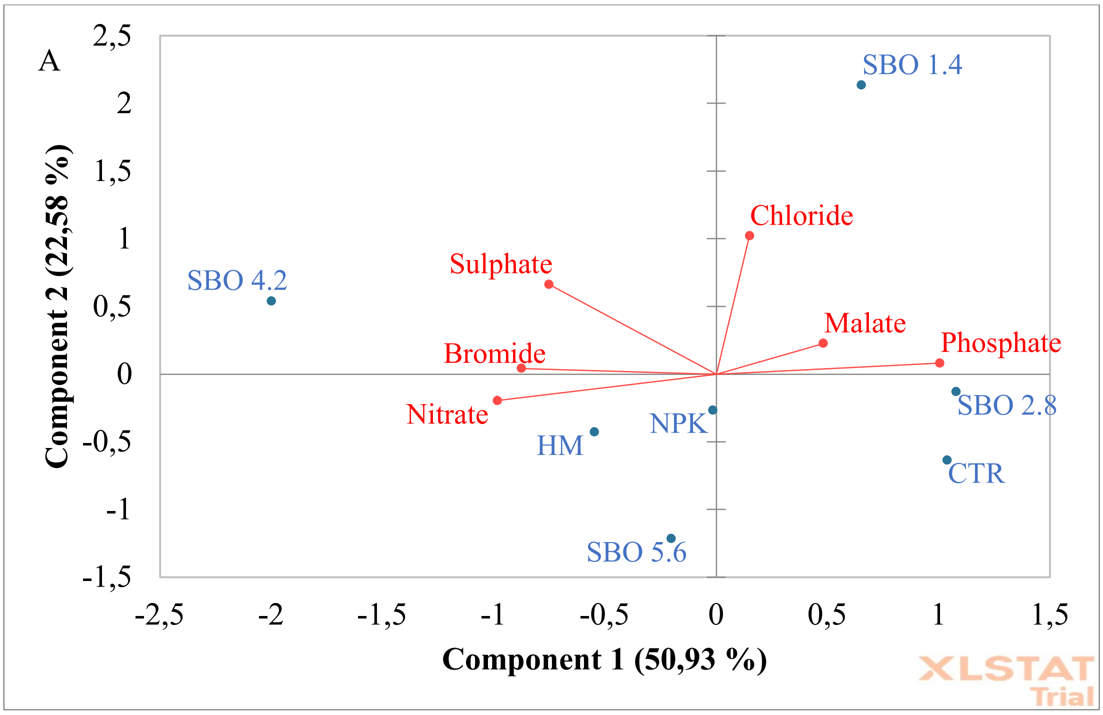


Figure 7 PCA (principal component analysis) diagram of primary and secondary metabolites in lettuce cultivated in Motta (A) and Lazzaro (B) in soils without fertilizer (CTR) and with different fertilizers nitrogen:phosporous:potassium (NPK), horse manure (HM) and different concentrations of sulphur-bentonite based fertilizers (SBO 1.4; 2.8; 4.2; 5.6).



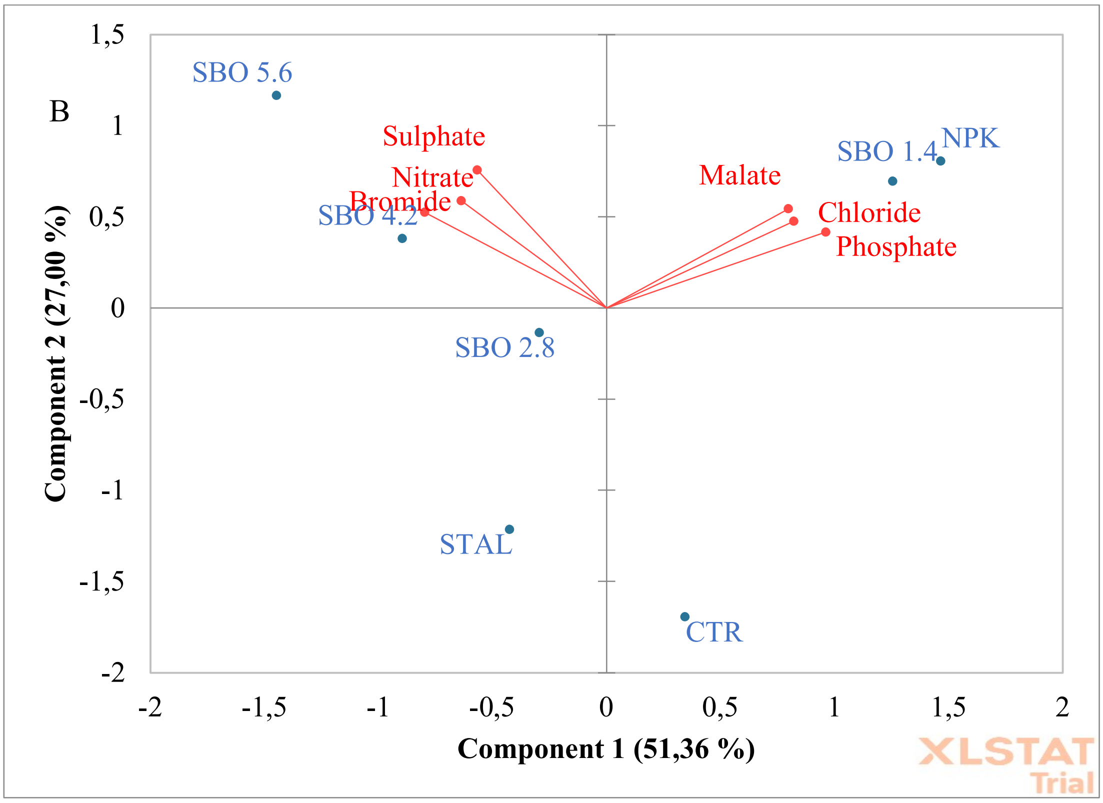


Figure 8 PCA (principal component analysis) diagram of anions in lettuce cultivated in Motta (A) and Lazzaro (B) in soils without fertilizer (CTR) and with different fertilizers nitrogen:phosporous:potassium (NPK), horse manure (HM) and different concentrations of sulphur-bentonite based fertilizers (SBO 1.4; 2.8; 4.2; 5.6).

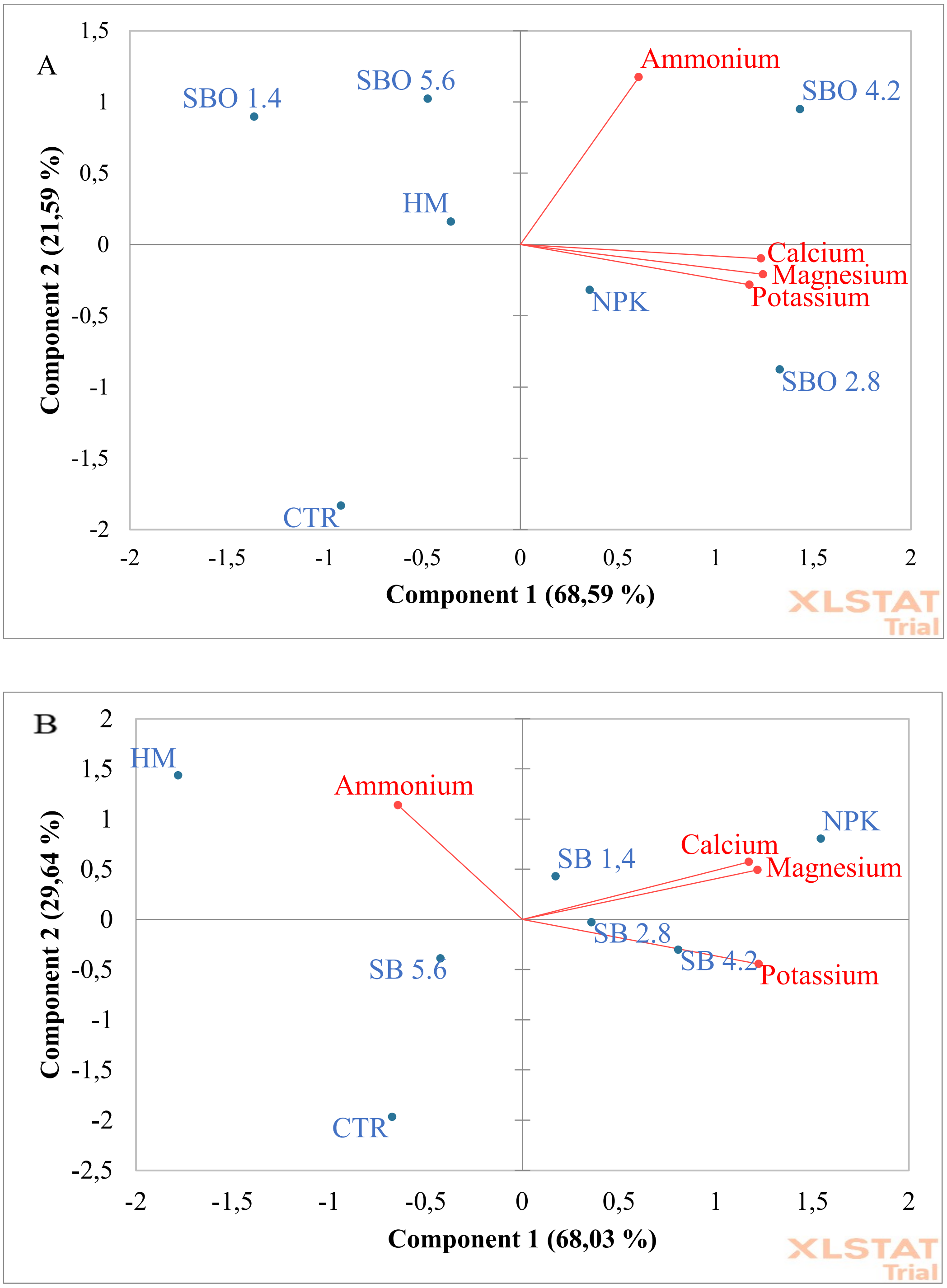


Figure 9 PCA (principal component analysis) diagram of canions in lettuce cultivated in Motta (A) and Lazzaro (B) in soils without fertilizer (CTR) and with different fertilizers nitrogen:phosporous:potassium (NPK), horse manure (HM) and different concentrations of sulphur-bentonite based fertilizers (SB 1.4; 2.8; 4.2; 5.6).