Biostimulants as a Tool for Improving Environmental Sustainability of Greenhouse Vegetable Crops

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Abstract: Plant biostimulants have gained great interest from the agrochemical industry and farmers because of their ability to enhance nutrient use efficiency and increase abiotic stress tolerance in crop production. However, despite the considerable potential of biostimulants for the sustainable development of the agricultural sector, the environmental evaluation of the application of biostimulants is still missing. Hence, this is the first study that focuses on the environmental assessment of the biostimulant action of arbuscular mycorrhizal fungus *Glomus intraradices* and vegetal-derived protein hydrolysate on two greenhouse vegetable crops, spinach and zucchini squash, under different fertilization regimes. The life cycle assessment from a cradle to gate perspective, which covers all processes related to crop cultivation up to harvest, was carried out to calculate the carbon footprint of the production chain for these two crops. The results of the comparative analysis revealed that the CO_2 equivalent emissions of both crops were reduced due to the biostimulant applications. In particular, the effect of the mycorrhization on the reduction of carbon emissions compared to the un-mycorrhized control was higher in zucchini plants under organic fertilization (12%) than under mineral fertilization (7%). In addition, organic fertilization increased the total carbon footprint of zucchini (52%) compared with mineral fertilization. The results also showed that an increase of nitrogen fertilization from 15 to 45 kg N ha^{-1} in spinach production enhanced the total CO_2 emissions per ton of harvested leaves in comparison with treatments that involved the foliar applications of protein hydrolysate together with a lower nitrogen input; this increase was 4% compared to the unfertilized treatment with application of biostimulant. This study can support decision-making in terms of agronomic technique choices in line with sustainable development of vegetable crop production.

Keywords: life cycle assessment; carbon footprint; mycorrhizal fungi; protein hydrolysate; spinach; zucchini squash

1. Introduction

Growing population and global demand for food have led traditional agriculture to excessive use of agrochemicals for increasing crop productivity. This attitude has eventually resulted in a seriously negative impact on soil quality as well as the deterioration of nonagricultural terrestrial and aquatic ecosystems [1]. Hence, alternatives that can substitute for chemical fertilizers without affecting productivity and economic output are encouraging [2]. In this context, the use of substances

and/or microorganisms known as plant biostimulants, or products able to enhance a crop's use of nutritive elements, mitigate the adverse effects of abiotic stress on crops and boost edible product quality, is of particular interest [3,4].

According to the European Regulation on fertilizers [5] recently approved by the European Parliament, "plant biostimulants" are defined as any "product that stimulates the nutritional processes of plants regardless of nutrient content, to improve one or more of the following plant characteristics or plant rhizosphere: a) nutrient use efficiency; b) tolerance to abiotic stress; c) qualitative characteristics; d) availability of nutrients confined to the soil or in the rhizosphere ". The market for these products also recorded exponential increases. In 2015, the turnover of biostimulants in Europe was around half a billion euros, with 29.9% of the global market [6]. The latest analyses conducted by [7] show that in 2025 the global market will experience a turnover of more than four billion dollars. Apart from the availability of products with more effective biostimulant action, this outstanding rise can be justified by the growing need to reduce the damage imposed on crops subject to abiotic stress, and the need to increase the use efficiency of agrochemical inputs and to reduce the environmental impact of production systems. Root inoculation with microbial biostimulants such as arbuscular mycorrhizal fungi has proven to be effective in enhancing yield and quality in many crops through the increase in nutrient and water uptake and the stimulation of primary and secondary plant metabolism, especially under low soil fertile conditions [8]. Similarly, several studies demonstrated that foliar sprays and/or root applications of protein hydrolysates can increase yield and produce quality in many crops by stimulating C and N metabolism, nutrient uptake and crop resistance against abiotic stresses [4].

While many studies addressed the importance of biostimulants as effective products on crop productivity [9–14], an investigation of biostimulant role from the environmental point of view has remained untouched. Therefore, the current study intends to make a comparative assertion via a life cycle assessment approach of two case studies: mycorrhized or unmycorrhized zucchini squash crops grown under organic or mineral fertilization and spinach crops foliarly treated or untreated with a vegetal-derived protein hydrolysate—both under different nitrogen rates. The main reason for conducting the research is to comprehend whether, and to what extent, environmental benefits can be achieved through different farming strategies for growing these important vegetable crops, zucchini squash and spinach. The main audiences of this study are farmers, extension specialists and scientists following confident approaches to boost the quality and quantity of their products and policymakers who promote biostimulants for sustainable development.

2. Materials and Methods

2.1. Life Cycle Assessment and Carbon Footprint

Life cycle assessment (LCA) is a comprehensive approach that assesses environmental concerns correlated with a product's complete life cycle (i.e., any good or service) [15]. In compliance with LCA guidelines [15,16], carbon footprint estimates greenhouse gas (GHG) emissions associated to products. This indicator is controversial not only for companies along the production chain but also for policymakers. The term ''product carbon footprint'' denotes the GHG emissions of a product across its life cycle (raw materials, manufacturing, distribution, end use and disposal and recycling) [17]. It encompasses the greenhouse gases (carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O)), together with families of gases including hydrofluorocarbons (HFCs) and perfluorocarbons (PFCs).

This LCA study achieves the following objectives:

- an assessment of the overall environmental impact of the production chain for crops following a cradle to gate perspective (plant cultivation phase up to harvest) considering both the direct emissions of the different phases of the process and the indirect emissions associated with the production of raw materials as inputs in the production chain;
- an environmental comparison of different ways of managing the production chain that considers 1 ton of cultivated spinach and zucchini squash as a functional unit to identify the most sustainable way.

The spinach and zucchini squash production chain under assessment is illustrated in Figure 1.
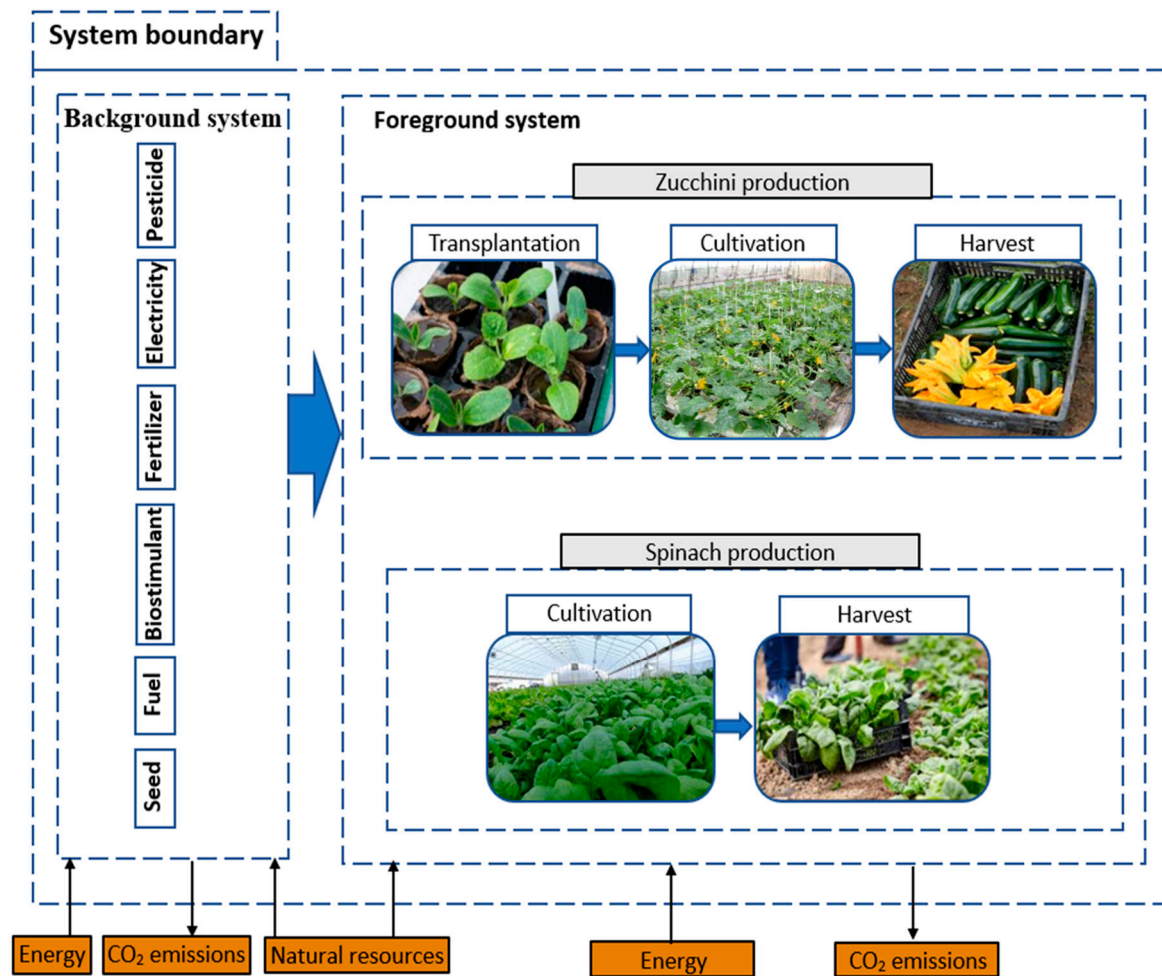

Figure 1. The diagram of the system boundary for selected crops.

2.1.1. Inventory Data Collection

The foreground data requirements for greenhouse zucchini and spinach production, namely, the specific data for modelling these product systems (e.g., quantity of fertilizer or pesticide consumption) were obtained from two scientific publications [14,18]; missing data (e.g., diesel consumption, lubricant, electricity) were extracted from the agriculture handbooks [19] and [20–22], which refer to the cultivation of spinach and zucchini. However, background data reflecting the data for the production of input materials (e.g., energy, seed, mineral fertilizer, protein hydrolysate, arbuscular mycorrhizal fungi) were extracted from [23–25]. CO_2 eq emission resulting from the production of 1 kg of vegetal-protein hydrolysate was taken from [26]. Inputs used for calculation of CO_2 emissions from the production of 100 kg of mycorrhizal inoculum were estimated considering a greenhouse mycorrhizal multiplication process on host plants of corn as follow: 70 corn seeds, 0.83 m^3 of vermiculite substrate, 70 L of irrigation water and 0.1 kg of mineral fertilizer.

In the case of the fertilizer pellet production process, all energy and material flows were estimated based on [27–30]: electricity consumption for feeding screw, hammermills, volume pumps and conditioners was assumed to be 0.07 kWh per kg of pellet while heat for drying was assumed to be 3732 MJ heat per ton of moisture [29,30]. In this study, CO_2 emissions due to soil respiration were considered negligible for both crops due to the lack of soil in zucchini production and the short cycle of winter spinach.

The experimental trial on the zucchini crop was carried out starting from the transplant of seedlings on 5 March 2009 in a greenhouse located at the Experimental Farm of Tuscia University, Italy [18].

Zucchini (*Cucurbita pepo* L.—cultivar "Tempra") seeds were previously sown in 84 cell-plug trays (cell diameter 9 mm) containing 26.5 cm^3 of peat-based commercial substrate. Prior to sowing, half of the trays were inoculated with a commercial mycorrhizal inoculum carrying *Glomus intraradices* and bacteria of the rhizosphere (Aegis Argilla, Italpollina S.p.A., Rivoli Veronese, Italy). After 14 days, the seedlings, which were at the two true-leaf stage, were transplanted in pots filled with fluvial sand (9 L pot^{-1}) arranged in double rows at a distance of 1.4 m apart, and the space between plants within a row was 0.5 m (plant density 20,000 seedlings per ha). Irrigation was carried out using a drip system with a flow rate of 2 L h^{-1} for each dripper. Two fertilization regimes were tested as follow: (1) mineral fertilization and (2) fertilization according to EU organic farming legislation EC 834/2007 (organic fertilization). In both fertilization regimes, the total fertilizer requirements were accommodated, partly in pre-plant fertilization and partly in the fertigation stage. Granular mineral-based slow release fertilizer (20-5-10-3), iron sulfate, triple superphosphate and pelletized organic-based fertilizer were applied in pre-plant fertilization, in mineral fertilization and organic fertilization regimes, respectively. Pre-plant fertilizers were accurately mixed into the substrate before filling the pots. On the one hand, calcium nitrate, monopotassium phosphate, potassium sulphate, potassium nitrate, magnesium nitrate and micronutrient fertilizer were supplied through fertigation to plants in mineral fertilization treatment, whereas organically-treated plants were fertigated with an organic liquid fertilizer containing fluid distillation-residue, so called 'borlanda', and magnesium sulphate enriched with micronutrients [18].

The experimental trial resulted in the comparison of four treatments: (M) standard fertilization with mineral fertilizers; (M + AM) standard fertilization with mineral fertilizers and mycorrhizal inoculation; (O) organic fertilization; (O + AM) organic fertilization and mycorrhizal inoculation. The harvest started on 19 May. Table 1 shows the inventory of data related to producing zucchini under the four experimental treatments.

The agronomic trial on greenhouse baby spinach (*Spinacia oleracea* L.—cultivar "Platypus") was conducted during three months from 19 January to 16 March 2018 in a greenhouse located at the Department of Agricultural Sciences, University of Naples Federico II, Italy [14]. The treatments involved various combinations of N fertilization regimes and weekly foliar applications of a protein-hydrolysate based biostimulant (Trainer®, Italpollina S.p.A., Rivoli Veronese, Italy). Trainer® is an enzymatically derived protein hydrolysate of vegetal origin that has 50 g kg^{-1} of N as free amino acids and soluble peptides; the aminogram of the product in g kg^{-1} was Ala (12), Arg (18), Asp (34), Cys (3), Glu (54), Gly (12), His (8), Ile (13), Leu (22), Lys (18), Met (4), Phe (15), Pro (15), Thr (11), Trp (3), Tyr (11) and Val (14). The following fertilization treatments were compared: unfertilized control ($N0$), unfertilized control plus foliar applications of biostimulant ($N0 + B$), soil mineral fertilization with 15 kg of N per ha (N_{15}), mineral fertilization with 15 kg of N per ha plus foliar applications of biostimulant ($N_{15} + B$), mineral fertilization with 30 kg of N per ha (N_{30}), mineral fertilization with 30 kg of N per ha plus foliar applications of biostimulant ($N_{30} + B$), mineral fertilization with 45 kg of N per ha (N_{45}), mineral fertilization with 45 kg of N per ha plus foliar applications of biostimulant ($N_{45} + B$). The nitrogen was provided as ammonium nitrate and applied through an overhead irrigation system in three weekly applications starting 7 days after sowing. The biostimulant-treated plants were sprayed uniformly five times (at 25, 32, 39, 46 and 53 days after sowing) during the growing cycle at 7 days interval with a solution containing 4 mL L^{-1} of Trainer®. Further details of the test are reported in [14]. Table 2 indicates the inventory data of spinach production for the six experimental treatments.

2.1.2. Calculation of the Carbon Footprint

Upon collection of relevant specific data, definitive product-level GHG emissions were calculated, which means that the carbon footprint for zucchini and spinach produced in the various experimental treatments was calculated. The values of global warming potentials (GWP100) were

quantified in accordance with the assessment of Intergovernmental Panel on Climate Change (IPCC 2013) method, which transforms GHG emissions into kg of CO_2 eq. SimaPro 9 (PRé Sustainability, LE Amersfoort, The Netherlands) was applied for the computation of the carbon footprint.

Table 1. Inventory data for zucchini production in relation to experimental treatments[1].

Items	Unit	Quantity (Unit ha^{-1})			
		M	**M+AM**	**O**	**O+M**
Output to technosphere					
Fruit yield	kg	88,634	96,024	56,696	64,838
Input from the environment					
Water	m^3	2440	2440	2440	2440
Inputs from technosphere					
Seedling production					
Seeds	n	20,000	20,000	20,000	20,000
Mycorrhizal inoculum	kg	0	7.94	0	7.94
Peat based substrate	m^3	0.53	0.53	0.53	0.53
Calcium carbonate	kg	1	1	1	1
Calcium nitrate	kg	0.50	0.50	0.50	0.50
Triple superphosphate	kg	0.15	0.15	0.15	0.15
Potassium sulphate	kg	0.25	0.25	0.25	0.25
Gasoline	kg	264.60	264.60	264.60	264.60
Fruit production					
Pre-plant fertilization					
Slow-release mineral fertilizer	kg	360	360	0	0
Triple superphosphate	kg	353	353	0	0
Iron sulfate	kg	0.55	0.55	0	0
Pelletized organic-based organic fertilizer	kg	0	0	1206	1206
Fertigation					
Calcium nitrate	kg	991.70	991.70	0	0
Monopotassium phosphate	kg	66.60	66.60	0	0
Potassium sulfate	kg	423	423	0	0
Potassium nitrate	kg	247.70	247.70	0	0
Magnesium nitrate	kg	468.50	468.50	0	0
Micronutrient mix	kg	38.80	38.80	0	0
Organic liquid fertilizer	kg	0	0	8840.09	8840.09
Magnesium sulphate enriched with micronutrients	kg	0	0	454.80	454.80
Pesticides					
Flonicamid	kg	0.40	0.40	0.40	0.40
Deltametrin	L	0.50	0.50	0.50	0.50
Abamectin	L	1.20	1.20	1.20	1.20
Penconazole	L	1.50	1.50	1.50	1.50
Sulfur	kg	10	10	10	10
Electricity	kWh	11.10	11.10	11.10	11.10
Diesel	kg	46,512	46,513	46,524	46,527
Lubricant	kg	3.50	3.50	3.50	3.50
Output to environment					
Emissions to air [2]					
CO_2	kg	193,491.70	193,495.82	193,541.58	193,554.06
CH_4	kg	23.25	23.25	23.26	23.26
N_2O	kg	1.39	1.39	1.39	1.39

[1] M = mineral fertilization; O = organic fertilization; AM = inoculation with the arbuscular mycorrhizal fungus *Glomus intraradices*; [2] Only those emissions related to global warming.

Table 2. Inventory data for spinach production in relation to experimental treatments[1].

Items	Unit	Quantity (Unit ha^{-1})							
		N_0	N_0+B	N_{15}	N_{15}+B	N_{30}	N_{30}+B	N_{45}	N_{45}+B
Output to technosphere									
Leaf yield	kg	4780	6370	8420	10,529	10,290	11,970	11,950	12,900
Input from the environment									
Water	m^3	620	620	620	620	620	620	620	620
Inputs from technosphere									
Seeds	kg	167	167	167	167	167	167	167	167
Mineral fertilizer									
Ammonium nitrate	kg	0	0	44	44	88	88	132	132
Biostimulant									
Protein hydrolysate	kg	0	8.5	0	8.5	0	8.5	0	8.5
Pesticides									
Pyrethrin	kg	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96
Copper oxychloride	kg	7	7	7	7	7	7	7	7
Electricity	kWh	210	210	210	210	210	210	210	210
Diesel	kg	164	166	169	171	171	173	173	174
Lubricant	kg	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2
Output to environment									
Emissions to air [2]									
CO_2	kg	682.24	690.56	703.04	711.36	711.36	719.68	719.68	719.68
CH_4	kg	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.08
N_2O	kg	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005

[1] N_0 = unfertilized plants; N15 = plants fertilized with 15 kg N ha^{-1}; N30 = plants fertilized with 30 kg N ha^{-1}; N45 = plants fertilized with 45 kg N ha^{-1}; B= biostimulant applications. [2] Only those emissions related to global warming.

3. Results and Discussion

The marketable production of zucchini was significantly different among treatments with the highest values found in mycorrhized plants subjected to standard mineral fertilization (M + AM = 96.02 t ha^{-1}), followed by non-mycorrhized plants treated with standard mineral fertilization (M = 88.63 t ha^{-1}) and then mycorrhized plants with organic fertilization (O + AM = 64.83 t ha^{-1}), while non-mycorrhized plants with organic fertilization provided the lowest fruit yield (O = 56.69 t ha^{-1}). Since the functional unit selected is 1 ton of harvested crop, an increase or a decrease in fruit yield has a reverse impact on total CO_2 emissions. The results reported in Table 3 showed that the mycorrhization reduced the total emissions of carbon dioxide equivalent per ton of marketable zucchini, in correspondence with both mineral and organic fertilization treatments. The effect of the mycorrhization on the reduction of CO_2 emissions concerning control treatments (M and O) was more evident in plants subjected to fertilization with organic alternatives (−12%) compared to those grown under mineral fertilization (−7%). The lowest absolute values of carbon dioxide equivalent emissions were obtained in the treatment that involved the mycorrhization of plants with mineral fertilization (M + AM). The mycorrhization reduced the emissions of all inputs used per ton of marketable zucchini due to an effect that increased the production of marketable zucchini with the same input consumption. In fact, mycorrhization improved the efficiency of resource use (e.g., fertilizers), which has a direct connection with controlling carbon emissions.

On the other hand, organic fertilization intensified emissions of carbon per ton of marketable zucchini due to a reduction in fruit yield compared to mineral fertilization treatments.

In case of process contribution, diesel, consumed mainly for greenhouse heating, was the main contributor to the total carbon footprint. In addition, calcium nitrate applied in post-plant fertilization induced a relatively significant impact on GWP because of the high energy cost in the fertilizer production (Figure 2).
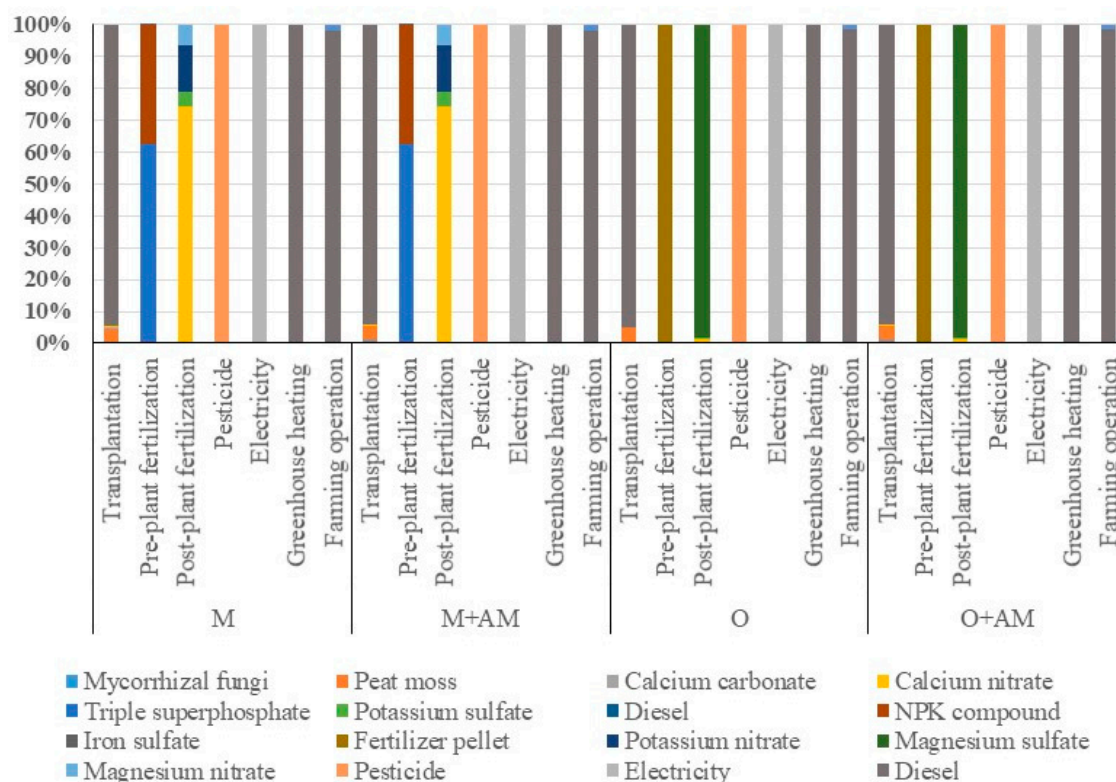
Figure 2. Process contribution to global warming potential in different experimental treatments (M = mineral fertilization; O = organic fertilization; AM = inoculation with the arbuscular mycorrhizal fungus *Glomus intraradices*).

The production of spinach leaves was increased by increasing the nitrogen fertilization rate, while biostimulant applications enhanced spinach yield, especially under low nitrogen supply. Therefore, the highest spinach yield was obtained in N_{45} + B treatment (12.90 t ha^{-1}) and the lowest one in N_0 treatment. The nitrogen use efficiency was higher in the treatment that included the leaf supply of biostimulant compared to those with only mineral fertilization of the soil.

Greenhouse gas emissions of spinach production under the various experimental treatments were estimated based on the data reported in Table 3.

Table 3. Calculation of the carbon footprint for each ton of harvested zucchini fruits under different treatments[1] (IPCC 2013, FU: 1 t).

Input	kg CO$_2$ eq t^{-1}			
	M	**M+AM**	**O**	**O+AM**
Nursery transplant production	16.30	15.11	25.39	22.38
Pre-plant fertilization	9.91	9.14	2.63	2.30
Post-plant fertilization	45.56	42.05	1.96	1.71
Pesticides	1.38	1.28	2.16	1.89
Electricity	0.05	0.04	0.08	0.07
Diesel	2465.80	2277.18	3848.1	3366.78
Total	2539.00	2344.80	3880.32	3395.13

[1] M = mineral fertilization; O = organic fertilization; AM = inoculation with the arbuscular mycorrhizal fungus *Glomus intraradices*.

Carbon dioxide emissions related to biostimulant production were obtained using the values reported in [26]. The emissions of carbon dioxide relevant to the lubricant inputs were negligible, and therefore they were not reported in Table 4. The key results in Table 4 revealed that the foliar applications of biostimulant (N_0 + B, N_{15} + B, N_{30} + B, N_{45} + B) lead to a reduction of the total carbon

emissions per ton of spinach harvested compared to the treatments with the same amount of fertilizer but without foliar applications of biostimulant (N0, N15, N30, N45). The highest carbon emission reduction (24%) was related to the unfertilized treatment together with application of biostimulant (N0 + B), whereas this reduction was limited to only 7% with the application of biostimulant (N45 + B).

The above findings demonstrated for the first time in the scientific literature that plant biostimulants such as mycorrhizal fungi and protein hydrolysates have the potential to reduce the greenhouse gas emissions of vegetable production under greenhouse conditions.

Table 4. Calculation of the carbon footprint for each ton of harvested spinach production under different treatments[1] (IPCC 2013, FU: 1 t).

Input	kg CO_2 eq t^{-1}							
	N0	N0+B	N15	N15+B	N30	N30+B	N45	N45+B
Seeds	6.30	4.72	3.57	2.86	2.97	2.51	2.52	2.33
Biostimulant	0	0.18	0	0.11	0	0.09	0	0.09
Ammonium nitrate	0	0	41.32	33.04	67.63	58.14	87.33	80.92
Pesticides	9.64	7.14	5.40	4.32	4.42	3.80	3.80	3.52
Electricity	18.78	14.09	10.66	8.53	8.72	7.50	7.51	6.96
Diesel	160.30	121.94	93.77	75.99	77.64	67.62	67.63	63.11
Total	195.02	148.07	154.72	124.85	161.38	139.66	168.79	156.93

[1] N0 = unfertilized plants; N15 = plants fertilized with 15 kg N ha^{-1}; N30 = plants fertilized with 30 kg N ha^{-1}; N45 = plants fertilized with 45 kg N ha^{-1}; B= biostimulant applications.

Moreover, the biostimulant-mediated reduction of carbon footprint was more pronounced under low input farming system conditions such as low rates of N fertilizer inputs or organic-based fertilization regimes. The results of LCA analysis showed a major contribution of diesel to total carbon emissions for both greenhouse crops. Application of alternative heat sources such as heat recovery from the existing power plants and geothermal heat or innovative design of greenhouse structure for harnessing solar energy and energy-saving approach (e.g., thermal screens, double-layer plastic cover) can diminish the carbon emission to a large extent [31–33]. Environmental parameters such as air temperature, relative humidity, physical and chemical soil characteristics, soil water content and soil temperature can also influence CO_2 emissions by respiration processes [34,35]. However, these parameters are quite stable under protected cultivations, especially in high tech greenhouses where it is possible to accurately control the microclimate parameters and to reduce CO_2 emissions from soil respiration processes using mineral-based substrate production systems like the one used in the zucchini trial [18].

4. Conclusions

The results obtained from the carbon footprint estimation demonstrated that both mycorrhization and foliar applications of vegetal-derived protein hydrolysate can lead to a 7–12% and 7–24% reduction in the global warming potential of greenhouse-grown zucchini and spinach, respectively. These reductions were the result of an increase in crop productivity linked with root mycorrhization and foliar applications of vegetal-derived protein hydrolysate.

Carbon footprinting is a useful tool to support the decision-making process since it is a practice for navigating impacts of agronomic techniques on global warming potential. In this way, the agricultural sector can identify and prioritize efficient management according to technique choices that lead to mitigation of greenhouse gas emissions in a specific production system. Therefore, the findings of this study provide useful insights into the sustainable management of vegetable crops and, in particular, into biostimulant use.

In the cultivation of zucchini and spinach, biostimulants such as mycorrhizal fungus *Glomus intraradices* and vegetal-derived protein hydrolysate complemented by the use of other sustainable agricultural practices represent a valid tool to increase the efficiency of fertilizer application, to enhance crop yield and to reduce greenhouse gas emissions per marketable production unit.

However, future studies should evaluate the benefits of mycorrhizal fungi and protein hydrolysates (alone or in combination) on carbon footprint across different growing seasons and soil conditions in order to fully understand the potential of biostimulants in reducing CO_2 emission. It should be emphasized that the quantification of the carbon footprint associated with a product constitutes an interesting commercial opportunity since it allows the development of marketing strategies aimed at the mitigation of the carbon footprint of products. The label of the carbon footprint is certainly a distinctive element and an indication of the quality improvement of a product. Marketing strategies focused on environment protection would promote consumption of green products. In fact, these strategies make purchasing choices into responsible choices based on considerations of the environmental impact associated with the product.

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