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Using Microgranular-Based Biostimulant in Vegetable Transplant Production to Enhance Growth and Nitrogen Uptake

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Abstract: Vegetable growers need high-quality transplants to ensure the success of their crops. Treating seedlings with protein hydrolysates and beneficial fungus *Trichoderma atroviride* has the potential to improve the health and quality of vegetable transplants via various biostimulant activities, but the best rates and application methods to achieve these benefits are still unclear. Therefore, the aim of the studies described in this manuscript were to: (i) identify the optimal rate of a microgranular-based biostimulant containing vegetal-derived protein hydrolysate (PH) and the beneficial fungus *T. atroviride* MUCL45632 on lettuce and tomato transplant production (Experiment 1); and (ii) determine whether combining the *T. atroviride* inoculant with the PH in microgranular or liquid form would best support the synergistic effects of these products using greenhouse and laboratory experiments (Experiments 2, 3 and 4). Mixing the microgranular-based PH directly into the substrate prior to sowing resulted in a significant dose-dependent increase in shoot fresh and dry biomass, root dry weight, root to shoot ratio, leaf N content and chlorophyll content (Soil-Plant Analysis Development index) in both lettuce and tomato transplants up to a biostimulant rate of 2 g L^{-1}. The positive effect of the microgranular-based PH on plant growth, leaf N and chlorophyll content in both the lettuce and tomato transplants was also observed in the second experiment. However, the PH-mediated enhancement of shoot fresh biomass was more pronounced when *Trichoderma* was combined with the liquid instead of the microgranule PH. In contrast, the microgranule containing PH and *Trichoderma* was more effective in increasing the plant root to shoot ratios than the combined application of liquid PH and *Trichoderma*. In the laboratory experiments, the application of PH to sandy soil enhanced the number of *Trichoderma* colonies and stimulated *Trichoderma*-induced respiration for up to two and six days for the liquid and microgranular PHs, respectively. These results demonstrate that mixing microgranules containing PH and *Trichoderma* in the substrate prior to sowing at a rate of 2 g L^{-1} is the best approach to enhance shoot and especially root growth of both tomato and lettuce plantlets, while also ensuring high N uptake and leaf chlorophyll content.

Keywords: *Lactuca sativa* L.; plant biostimulants; protein hydrolysate; *Solanum lycopersicum* L.; *Trichoderma* spp.; vegetable seedlings; soil respiration test

1. Introduction

High-quality vegetable transplant production is important to guarantee a good establishment of plantlets after transplanting, and for most of the vegetable crops, it is common to use transplants as starting vegetal material in field and greenhouse production. Transplants are considered to be of high-quality if they have the following characteristics: (i) disease- and insect-free; (ii) ability to overcome transplanting shock; and (iii) well-developed aerial (i.e., leaf area and no visual defects) and root system: [1]. In addition, other seedling characteristics that are useful indicators of transplant quality and especially post-transplant performance include: root to shoot ratio, stem diameter [2], plant dry weight [3], ability to generate new roots after transplantation and [4] nutritional status. A promising strategy to obtain high-quality vegetable transplant production in nurseries is the use of microbial and non-microbial plant biostimulants (PBs), since their application may have positive effects on several quality traits of vegetable transplants. Moreover, the application of PBs is useful also for promoting the rapid establishment of plantlets after transplanting [4] and better crop performance in field and greenhouse conditions [5,6].

Among non-microbial PBs, protein hydrolysates (PHs) have gained prominence due to their ability to exert both a biostimulant and nutritional action on crops [7]. Several studies have reported beneficial effects of PHs application on quality traits of vegetable transplants and crop performance after transplanting. Vinković et al. [8] reported that using PHs before and after transplanting resulted in improved growth of root, stem and leaves, and the ability of tomato plants to cope with transplant stress. Moreover, the hormone-like activity of a legume-derived PH, such as auxin- and gibberellin-like activity, resulted in better crop performance driven by an enhanced nitrogen uptake associated with better development of roots and stimulation of N metabolism [5]. Furthermore, PHs can also enhance plant growth indirectly by stimulating the growth of beneficial microorganisms like *Trichoderma* spp. This promising plant growth-promoting fungus improves plant growth and productivity mainly by increasing root growth and enhancing soil nutrient availability, thus increasing plant nutrient uptake (mainly N, P, Fe, Mn and Zn) [9].

The establishment of *Trichoderma* in plant roots and benefits to plant health and productivity may be promoted by PHs application. Indeed, PHs can act on the root microbiome by modifying the root architecture and enhancing the biological activity. This is likely to contribute to the beneficial effects on plant growth and development observed after the application of PHs [10]. For example, by combining endophytic fungi such as *Trichoderma atroviride* with a vegetal-derived PH, lettuce plants were better able to tolerate stress conditions like salinity and alkalinity. The beneficial effects of this combination of products were correlated with a greater root surface area, leaf chlorophyll content, and proline accumulation, and an increase in catalase and glutathione peroxidase enzyme activities [11]. Thus, the combined application of a vegetal-derived PH and *Trichoderma atroviride* induced synergistic effects in terms of crop growth stimulation and nutrient uptake. Thus, combining these products can both address global food security and promote sustainability (by improving nitrogen use efficiency) [12], and ensure that growers can obtain high-quality transplants. PHs are mainly applied as a foliar spray or soil/substrate drench. However, both non-microbial and microbial PBs can also be applied on seeds (i.e., seed coating) [13] to guarantee the rapid germination of high-quality seedlings. When these products are in solid form as a microgranule, PBs can be mixed into the substrate before sowing without any additional biostimulant application. This simplifies the application procedures for transplant production in nurseries, and ensures the production of high-quality plantlets.

As the synergistic effects derived from the combined application of PH and *Trichoderma* on plant growth stimulation and nutrient uptake seem to be promising especially when applied at an early stage of crop growth, the following studies were conducted to further optimize these benefits during transplant production. Specifically, we carried out four experiments under greenhouse or laboratory conditions to: (i) identify the rate that would best optimize the biostimulant action of a microgranular-based protein hydrolysate containing the beneficial fungus *Trichoderma atroviride* MUCL45632 (microgranular-based PH) on lettuce and tomato transplant production (Experiment 1);

(ii) compare the combined effect of PH and the beneficial fungus *Trichoderma atroviride* MUCL45632 [14] on lettuce and tomato transplant growth performance when the PH was in microgranular versus liquid form (Experiment 2); and (iii) quantify the effect of adding different rates or forms (microgranule or liquid) of PH on the growth and respiration of *Trichoderma atroviride* under laboratory conditions (Experiments 3 and 4).

2. Materials and Methods

2.1. Experiment 1

The first experiment was conducted in a glass-glazed greenhouse in the Department of Horticulture and Landscape Architecture at Purdue University, West Lafayette, IN (lat. 40 N, 129 long. 86 W; altitude 188 m above sea level). The average day/night air temperatures were 23.8 ± 0.8/20.3 ± 0.9 °C. The experiment was set up in a randomized complete block design with five replicates to evaluate the biostimulant action of a microgranular-based PH containing *Trichoderma atroviride* MUCL45632 mixed into the substrate prior to sowing at the following rates: 0, 1, 2 and 4 g L^{-1}. The microgranular-based PH had a particle size range of 1.7–2.5 mm, a specific weight of 1.0 kg L^{-1} and contained 40 g kg^{-1} of N as amino acids and signaling peptides (lateral root promoting peptide) obtained from a legume-derived protein source, and 5×10^3 CFU g^{-1} of *Trichoderma atroviride* MUCL45632. The mineral carrier was a phyllosilicate mineral. The microgranular-based PH was manufactured by Italpollina USA Inc. (Anderson, IN, US). Tomato (*Solanum lycopersicum* L. cultivar Corborino-Reimer Seeds, Saint Leonard, MD, USA) and lettuce (*Lactuca sativa* L. cultivar Black seeded Simpson-American Seed Company, Spring Grove, PA, US) were sowed on 27 September 2016 in 36-hole plastic trays (264.2 cm^3 per cell) filled with a commercial potting mix (Fafard 2P Mix; Conrad Fafard, Agawam, MA, US). Each experimental unit included 36 plants for both tomato and lettuce. Irrigation was provided every day by subirrigation using an ebb-and-flow system; briefly, trays were flooded with water to a depth of 2 cm for about 20 min and then water was drained back to the reservoir tank. Hoagland's nutrient solution was applied weekly through the ebb-and-flow system [15]. The plants were grown without any application of pesticides. At the end of the trial (24 October; 27 days after sowing; DAS), the Soil-Plant Analysis Development (SPAD) index was measured using a portable chlorophyll meter SPAD-502 (Konica Minolta, Tokyo, Japan). Twenty readings per experimental unit were taken on fully expanded leaves and averaged to a single SPAD value for each biostimulant application treatment. Plants were harvested to determine the shoot fresh weight and dry weight of the plant parts (shoot and roots). The dry weight of the above and below ground biomass was determined in a forced-air oven at 80 °C for 3 days. Root to shoot ratio was calculated on a dry weight basis. Dry leaf dry biomass was ground to quantify the nitrogen content. The nitrogen concentration was determined by the Dumas method using the elemental analyzer LECO FP 528.

2.2. Experiment 2

The second experiment on tomato and lettuce was carried out from 9 January to 14 February 2017 using the same crop varieties and growing conditions reported for Experiment 1. Three treatments were tested with five replicates as follows: untreated control, microgranular-based PH (as in the Experiment 1; 40 g kg^{-1} of N and 5×10^3 CFU g^{-1} of *Trichoderma atroviride* MUCL45632) and liquid-based PH (50 g kg^{-1} of N; Trainer®, Italpollina USA, Inc., Anderson, IN, USA) plus *Trichoderma atroviride* MUCL45632 (Condor®, Italpollina USA, Inc., Anderson, IN, USA). Both microgranule and liquid products contained a PH obtained from enzymatic hydrolysis of a legume-derived protein source. The application rates of both microgranule and liquid PHs were normalized (2.0 and 1.6 g L^{-1}, respectively) in order to provide the same amount of nitrogen (80 mg N L^{-1} of substrate), while *Trichoderma atroviride* MUCL45632 was normalized to the *Trichoderma* level in the microgranular-treated substrate (1×10^4 CFU L^{-1} of substrate) by adding *Trichoderma atroviride* MUCL45632 inoculum together with the liquid-based PH. The biostimulant products were applied just prior to sowing in the substrate. Cultural practices were the same as those reported for Experiment 1. At the end of the trial (36 DAS), the SPAD index, growth parameters and leaf N content were assessed as in Experiment 1.

2.3. Experiment 3

An incubation test was performed in Petri dishes filled with sandy soil to evaluate the influence of different rates of PH on the growth of *Trichoderma atroviride* MUCL45632 under sterile conditions at the Biotechnology Lab of Tuscia University, Italy. Twenty polystyrene Petri dishes (size 100 mm × 15 mm) were filled with 65 g of dry sterile sandy soil each. A water suspension of *Trichoderma atroviride* MUCL45632 was prepared with a *Trichoderma* concentration of 1×10^3 CFU mL^{-1}, and added to the sandy soil of five Petri dishes at a rate of 0.15 g water per g sandy soil. A water suspension of *Trichoderma atroviride* MUCL45632 (1×10^3 CFU mL^{-1}) that also contained the liquid PH tested in Experiment 2 was added to the remaining 20 Petri dishes at rates of 1, 2 or 4 g L^{-1} (5 Petri dishes for each PH rate). After *Trichoderma* inoculation, the petri dishes were kept for 4 days in a growth chamber at 24 °C under dark conditions. After the incubation period, the number of *Trichoderma* colonies on the soil surface of each plate was counted using a stereo-microscope (32 x).

2.4. Experiment 4

An incubation test was carried out to quantify the CO_2 flux from the soil enriched with *Trichoderma atroviride* MUCL45632 alone or in combination with microgranular- or liquid-based PH at the Soil Microbial Ecology Lab of Purdue University, IN, US. A sandy soil from a nearby field that had grown vegetables for at least ten years was collected and air-dried; soil was first screened (through a 5-mm sieve), then sterilized in an autoclave (at 121 °C for 30 min) and brought to 70% of water holding capacity. The soil had 0.6% organic matter and a pH of 6.5. Three treatments were tested as follows: microgranular-based *Trichoderma atroviride* MUCL45632, microgranular-based PH containing *Trichoderma atroviride* MUCL45632 and liquid-based PH containing *Trichoderma atroviride* MUCL45632. The application rates of microgranular-based *Trichoderma*, microgranular-based PH containing *Trichoderma* and liquid-based PH plus *Trichoderma* were normalized (2.0, 2.0 and 1.6 g L^{-1}, respectively) in order to provide the same amount of *Trichoderma atroviride* MUCL45632 (1×10^4 CFU L^{-1} of substrate) and nitrogen (80 mg N L^{-1} of substrate only for microgranular-based PH and liquid-based PH treatments). For the respiration rate determination, three replicates of each product/soil blend were used. The products/soil blends were placed into centrifuge tubes. Each tube was placed in a 500 mL flask. In each flask, a tube containing NaOH (1 N) solution was added. After 48 h of incubation at 25 °C, absorbed CO_2 in the NaOH solution was precipitated with $BaCl_2$ (0.75 N) as insoluble $BaCO_3$ and titrated with HCl (1 M) to determine the amount of unreacted NaOH [16].

2.5. Statistical Analysis

All experimental data were subjected to an analysis of variance (ANOVA) and Duncan's test ($p \leq 0.05$) to determine significant differences between treatments. All statistical analyses were performed using the SPSS software package (SPSS 10 for Windows 2001).

3. Results and Discussion

3.1. Experiment 1: Effects of Microgranular-Based Biostimulant Rates on Lettuce and Tomato Transplants

Positive effects in terms of biomass production following the application of PHs have been reported in several studies [5,7,10,17–20]. In our experiment, the results obtained by using a microgranular-based biostimulant directly mixed into the substrate were consistent with these previous observations, and in particular, a significant increase in shoot fresh biomass for both crops, lettuce (Table 1) and tomato (Table 2), was evident. For both crops, the maximum shoot growth was reached with 2 g L^{-1}, whereas no significant differences were found between the control treatment and the microgranule application rate of 1 g L^{-1}.

Table 1. Effect of biostimulant treatments on dry weight of shoots and roots, root to shoot ratio and nitrogen content, and Soil-Plant Analysis Development (SPAD) index of leaves in lettuce transplants (Experiment 1).

Microgranular Biostimulant (g L^{-1})	Shoot Fresh Weight (g $plant^{-1}$)	Shoot Dry Weight (g $plant^{-1}$)	Root Dry Weight (g $plant^{-1}$)	Root to Shoot	Leaf	
					N (g kg^{-1} d.w.)	SPAD
0	1.83 b	0.15 b	0.04 b	0.30 b	34.8 b	10.9 b
1	2.03 b	0.16 ab	0.05 a	0.35 a	33.6 b	12.8 a
2	3.10 a	0.21 a	0.05 a	0.34 a	42.2 ab	13.0 a
4	2.98 a	0.20 ab	0.05 a	0.35 a	54.4 a	14.2 a
Significance	***	*	*	***	**	**

*, **, *** Significant at $p \leq 0.05$, 0.01 and 0.001. Different letters within each column indicate significant differences according to Duncan's test ($p \leq 0.05$).

Table 2. Effect of biostimulant treatments on dry weight of shoots and roots, root to shoot ratio and nitrogen content, and Soil-Plant Analysis Development (SPAD) index of leaves in tomato transplants (Experiment 1).

Microgranular Biostimulant (g L^{-1})	Shoot Fresh Weight (g $plant^{-1}$)	Shoot Dry Weight (g $plant^{-1}$)	Root Dry Weight (g $plant^{-1}$)	Root to Shoot	Leaf	
					N (g kg^{-1} d.w.)	SPAD
0	0.81 b	0.10 b	0.03 b	0.26 b	20.3 c	31.7 bc
1	0.90 b	0.11 b	0.04 ab	0.33 a	19.9 c	30.2 c
2	1.49 a	0.17 a	0.05 a	0.32 a	26.6 b	32.1 b
4	1.71 a	0.17 a	0.05 a	0.30 a	37.5 a	34.3 a
Significance	***	***	***	***	***	**

, * Significant at $p \leq 0.01$ and 0.001. Different letters within each column indicate significant differences according to Duncan's test ($p \leq 0.05$).

In lettuce, shoot dry weight significantly increased only with application of the micrthe microgranular-based PH at 2 g L^{-1} (+40%), while in tomato, the increase was significant and of the same magnitude at 2 and 4 g L^{-1} (+70%) in comparison with the untreated control (Tables 1 and 2). For shoot dry weight, no differences were found between control plants of lettuce or tomato, and those grown with the lower concentration of the microgranular-based PH (1 g L^{-1}).

A previous study demonstrated that by increasing the dose of a plant-derived PH, shoot, root and total plant dry biomass also increased concurrently [5]. This was also the case in the current experiment, since a dose-dependent increase in shoot biomass in both lettuce and tomato transplants was evident. However, the effect was more pronounced for tomato, indicating that tomato is more responsive to this product than lettuce. Root dry weight was positively affected in lettuce with 1 and 2 g L^{-1} (+12%) of the product, but not with 4 g L^{-1}, while in tomato, root dry weight increased up to 2 g L^{-1} (+66%) with similar values in the 2 and 4 g L^{-1} treatments (Tables 1 and 2). Finally, the root to shoot ratio was significantly enhanced in both crops by the microgranule application with no significant differences among the microgranule rates.

These findings demonstrate that mixing a microgranular-based biostimulant directly into the growth substrate prior sowing can improve transplant quality in term of overall growth, and alter the root to shoot ratio, which could promote the health and productivity of transplants. The stimulation of root growth by this product could be ascribed to the presence of signaling compounds, which are easily perceived by roots, and may stimulate endogenous phytohormone biosynthesis such as auxins and brassinosteroids [12,21]. In particular, the presence of the bioactive peptide LRPP (lateral root promoting peptide) may elicit hormone-like activity, thus boosting root growth. Moreover, it has been reported that *Trichoderma atroviride* MUCL 45,632 can produce auxin-like compounds, increasing plant rooting via stimulation of primary root elongation, lateral root development and root hair formation [4]. Moreover, better development of roots could improve the nutrient uptake and utilization efficiency, resulting in greater biomass production.

Previous experiments evaluating positive crop responses following microgranular-based biostimulant application on plant biomass production have been associated with greater leaf N [5] and chlorophyll contents [22]. This was also the case in the current experiment, as both parameters were positively correlated with increases in lettuce (Table 1) and tomato (Table 2). In lettuce, the positive effect on N content was significantly different compared with the control treatment with 4 g L^{-1} of the microgranule (+56%), while in tomato, the highest level was reached at 4 g L^{-1} (+84%), followed by 2 g L^{-1} (+31%). The chlorophyll content in lettuce was significantly increased by all rates of microgranule application, ranging from 17 to 30%, with no significant differences among the microgranule application rates. In tomato transplants, the chlorophyll content was positively influenced by the microgranule application only with 4 g L^{-1} (+8%) in comparison with the control treatment.

The positive effects of PH and *Trichoderma* application on plant growth, chlorophyll and leaf nitrogen content are likely due to greater nitrogen uptake and utilization through root growth stimulation, nutrient uptake and assimilation processes [23]. In addition, high rates of PH can also act as a nitrogen source (N-fertilizer effect), leading to an increase in N content in plants. Indeed, the greater N availability in turn will lead to a greater chlorophyll content and biomass production, and consequently better crop performance.

3.2. Experiment 2: Effect of Biostimulant in Microgranular or Liquid Form on Lettuce and Tomato Transplants

The effects of the microgranular-based PH containing *Trichoderma atroviride* MUCL45632 and liquid PH plus *Trichoderma atroviride* MUCL45632 applications on lettuce and tomato are reported respectively in Tables 3 and 4. Lettuce plants had significantly greater shoot fresh (+38%) and dry weight (+28%) compared with control plants when treated with the liquid PH plus *Trichoderma*. In contrast, shoot fresh weight of lettuce treated with the combination of *Trichoderma* with the microgranular-based PH was significantly higher than the control (+16%), but there were no differences between these two treatments in terms of shoot dry weight (Table 3). The same responses occurred for tomato transplants, with significant increases in shoot fresh and dry weight compared with the control in plants grown treated with the liquid PH plus *Trichoderma* in comparison with the control treatment. Moreover, shoot fresh weight was 66% higher in tomato transplants treated with the liquid PH plus *Trichoderma*, than those treated with the microgranular-based PH + *Trichoderma* combination. In addition, there was no difference in tomato shoot dry weight between the microgranular PH + *Trichoderma* treatment that the liquid PH + *Trichoderma* and control treatments (Table 4). Thus, in terms of shoot growth, the combined application of *Trichoderma* with liquid PHs seems to be more efficient than the combination with the microgranular-based PHs. However, the lack of significant differences in shoot dry weight between liquid and microgranular-treated plants of lettuce and tomato indicates that the liquid-based PH did not enhance the carbon assimilation process in comparison with the microgranular-based PH; therefore, the highest shoot fresh weight in plants treated with liquid-based PH resulted from an increase in water content in the shoot tissues. The lower water content in plants treated with the microgranule-based PH in comparison with those treated with the liquid-based PH can be considered a positive quality trait for minimizing transplant shock [24].

Table 3. Effect of biostimulant treatments on dry weight of shoots and roots, root to shoot ratio and nitrogen content, and Soil-Plant Analysis Development (SPAD) index of leaves in lettuce transplants (Experiment 2).

Treatment	Shoot Fresh Weight (g plant^{-1})	Shoot Dry Weight (g plant^{-1})	Root Dry Weight (g plant^{-1})	Root to Shoot	Leaf	
					N (g kg^{-1} d.w.)	SPAD
Control	3.00 c	0.21 b	0.07 b	0.31 b	23.9 b	10.7 b
Liquid PH + *Trichoderma*	4.17 a	0.27 a	0.07 b	0.26 c	37.0 a	12.7 a
Microgranular PH + *Trichoderma*	3.58 b	0.23 ab	0.08 a	0.35 a	37.8 a	11.3 ab
Significance	***	*	*	**	***	*

*, **, *** Significant at $p \leq 0.05$, 0.01 and 0.001. Different letters within each column indicate significant differences according to Duncan's test ($p \leq 0.05$).

Table 4. Effect of biostimulant treatments on dry weight of shoots and roots, root to shoot ratio and nitrogen content, and Soil-Plant Analysis Development (SPAD) index of leaves in tomato transplants (Experiment 2).

Treatment	Shoot Fresh Weight (g plant^{-1})	Shoot Dry Weight (g plant^{-1})	Root Dry Weight (g plant^{-1})	Root to Shoot	Leaf	
					N (g kg^{-1} d.w.)	SPAD
Control	1.39 c	0.17 b	0.05 b	0.29 b	11.0 b	30.4 b
Liquid PH + *Trichoderma*	4.00 a	0.34 a	0.08 a	0.24 c	20.4 a	34.4 a
Microgranular PH + *Trichoderma*	2.40 b	0.20 ab	0.07 a	0.34 a	20.1 a	32.5 ab
Significance	***	***	***	***	***	*

*, *** Significant at $p \leq 0.05$, and 0.001. Different letters within each column indicate significant differences according to Duncan's test ($p \leq 0.05$).

In contrast to the results for shoot fresh weight, root dry weight was positively affected by the application of the microgranular-based PH containing *Trichoderma* in both lettuce (+14%) and tomato (+40%) transplants (Tables 3 and 4). However, there were no differences between this treatment and the liquid PH plus *Trichoderma* application in tomato. Interestingly the positive effect of the PH treatments on the root to shoot ratio was observed for both crops with the application of the microgranular-based PH containing *Trichoderma*. In particular, the root to shoot ratio increased by 12% and 37% in the lettuce and tomato plants, respectively, in comparison with the control plants. On the contrary, combining *Trichoderma* with the liquid PH led to a significant decrease in the root to shoot ratio of both plants in comparison with the untreated control, since this treatment strongly stimulated the growth of the aerial parts of the plants. Good development of plant roots is critical for successful transplants. Consequently, the positive effect of the microgranular-based PH combined with *Trichoderma* on root dry weights and root to shoot ratios observed in this study indicates that this approach would be better for transplant production than the liquid formulation. As described above, the stimulation of root growth in response to the microgranular-based PH + *Trichoderma* formulation may be ascribed to the presence of an LRPP bioactive peptide in this PH and the auxin-like compounds released by *Trichoderma atroviride* MUCL45632 [4].

Increases in leaf N content were significant and similar for both treatments. In particular, N content was 55% and 85% greater in lettuce and tomato leaves, respectively, when compared with the control. However, chlorophyll content was significantly improved only by the liquid PH plus *Trichoderma* application (+18% in lettuce and +13% in tomato) in comparison with the control treatment. Nevertheless, the leaf chlorophyll content of both crops treated with the microgranular PHs coupled with *Trichoderma* was not significantly different from the liquid PH plus *Trichoderma* treatment. Consequently, both treatment formulations have potential to increase the greenness (SPAD index) and N concentrations of leaves in lettuce and tomato transplants.

When the SPAD readings and nitrogen values of leaves from both Experiments 1 and 2 were pooled, a significant linear relationship between the SPAD readings and N contents was recorded for lettuce leaves, whereas this relationship was not significant for tomato leaves (Figure 1). The SPAD readings of tomato leaves were always higher, of about 22 units, than the leaf lettuce values for a similar nitrogen content. Similarly, the relationship of the SPAD readings to leaf N content was significantly different between crop species [25]; this variability of the SPAD readings across crop species is due to the differences in leaf features that cause different light reflection and/or scattering effects [25].

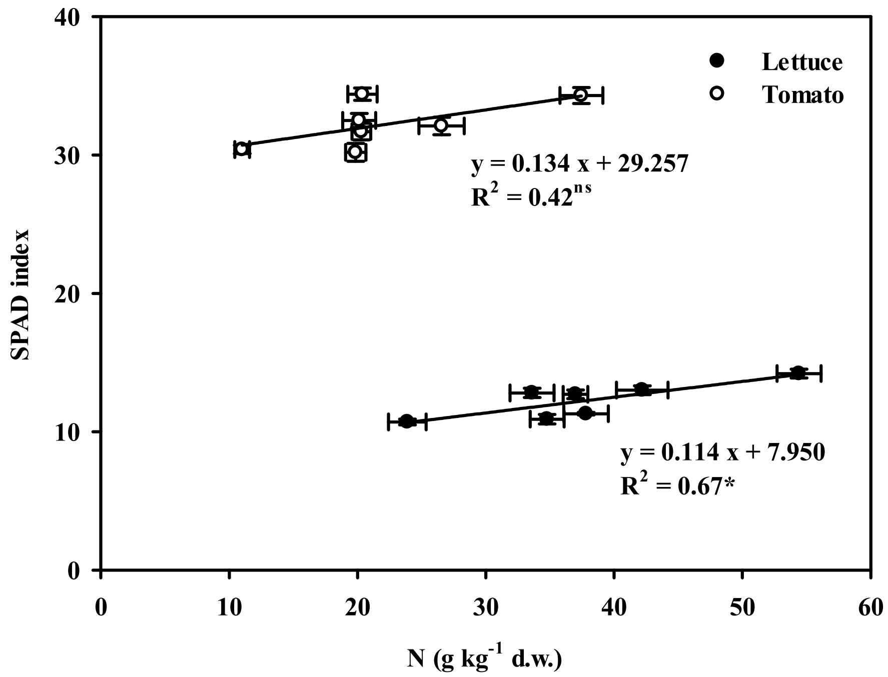

Figure 1. Relationship between the Soil-Plant Analysis Development (SPAD) index and nitrogen (N) content of leaves in tomato and lettuce. Bars represent standard errors of the means (Experiments 1 and 2).

The different crop responses induced by the liquid and microgranular-based biostimulants could be related to differences in the availability of LRPP bioactive peptides for plant uptake caused by the slow release action of PH–phyllosilicate complexes over time. Indeed, silicate minerals such as zeolites that have a high cation exchange capacity have been reported to adsorb amino [26] and humic acids [27]. In particular, it has been reported that the interaction between Ala and zeolite occurs via the NH_3^+ group, while the one between Met and zeolite occurs via the COO-, NH_3^+ and CH_3 groups [26]. In the case of humic acids, the adsorption seems to depend on the cations' capacity to create stable bridges with organic matter, and the selectivity for their adsorption [27]. Moreover, zeolite's strong affinity for NH_4^+ is useful for maximizing N use efficiency [28].

The results of this experiment indicate that combining *Trichoderma* with the microgranular-based PH may be a more efficient approach for generating quality transplants than when combining *Trichoderma* with a liquid PH, since the microgranular combination leads to better root development, a more balanced root to shoot ratio and good levels of N and chlorophyll in leaves.

3.3. Experiments 3 and 4: Effect of PH on Growth and Respiration of Trichoderma atroviride

It has been reported that PHs can be a food source for *Trichoderma*, thereby stimulating *Trichoderma* growth and increasing its establishment in the soil/root system [7]. This was the case in the current experiment, since a stimulation of *Trichoderma* growth with the PH application was observed in the Petri dish trial where the number of *Trichoderma* colonies per plate increased significantly by increasing the PH rate up to 2 g L^{-1} (Figure 2). To investigate the effect of PH form (microgranule or liquid) on *Trichoderma* activity, the rate of CO_2 release over time was quantified in *Trichoderma*-inoculated soil treated or untreated with the two PH forms. After two days from the PH application, soil respiration for both PH forms strongly increased with the same magnitude in comparison with the untreated control (Figure 3). In contrast, after six days of incubation, soil respiration was significantly higher than the control treatment only when the PH was added to the *Trichoderma*-inoculated soil as a microgranule. This result may be ascribed to the fact that organic nitrogen-containing compounds are adsorbed in the microgranular pores, thus ensuring a longer release time, while the organic nitrogen-containing compounds in the liquid PH are subjected to more rapid use by the *Trichoderma atroviride* MUCL45632 isolate [29]. Finally, at 10 and especially 14 days of incubation, the soil respiration rate decreased, reaching the same level for all treatments, indicating that substrates in the PHs were completely mineralized.

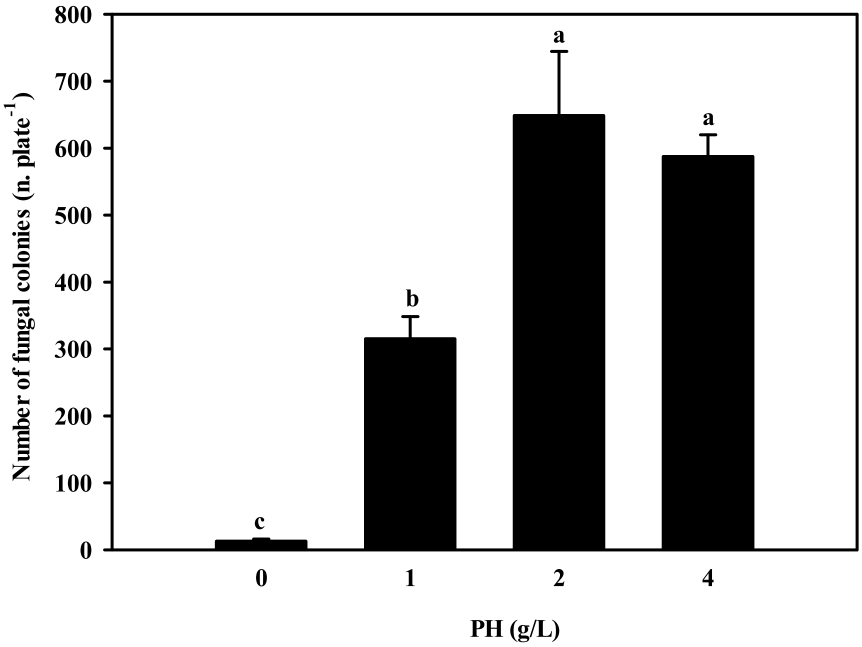

Figure 2. Effect of protein hydrolysate (PH) rate on number of *Trichoderma atroviride* colonies per plate after 4 days of incubation. Bars represent standard errors of the means. Means with different letters are significantly different according to Duncan's test ($p \leq 0.05$) (Experiment 3).

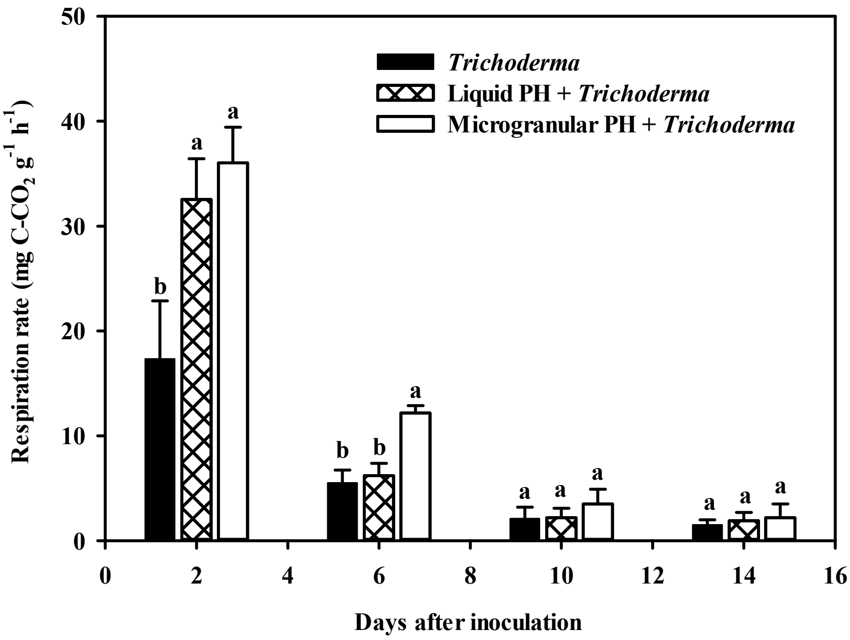

Figure 3. Soil respiration rate after application of *Trichoderma atroviride* alone or in combination with microgranular or liquid protein hydrolysate (PH). Bars represent standard errors of the means. Within each set of bars, means with different letters are significantly different according to Duncan's test ($p \leq 0.05$) (Experiment 4).

4. Conclusions

The production of high-quality vegetable transplants is critical for good crop establishment after transplanting [30], especially for vegetable crops like tomato and lettuce. Treating seeds and seedlings with vegetal-derived PHs, a category of plant biostimulants, has received a lot of interest due to the positive effects these products have been shown to have on many crops. In this study, we demonstrated that mixing 2 g of a microgranular-based PH directly into one liter of substrate prior to sowing can enhance the morpho-physiological quality traits of both tomato and lettuce transplants. The microgranular-PH increased the stimulation of shoot and especially root growth, clearly demonstrated by the increased root to shoot ratio, which could be ascribed to the presence of an LRPP in the product. We also demonstrated that combining PHs with *Trichoderma* can provide further benefits to tomato and lettuce transplants, however, the formulation of the PH is important. Combining *Trichoderma* with PH in liquid form reduced the root to shoot ratio of both crops, whereas combining this beneficial fungus with the microgranular-based PH promoted several transplant traits. Based on results of the plate assays and respiration tests, the synergistic effects derived from the combined application of the microgranular-based PH and *Trichoderma* appear to be due, at least in part, to the provision of nutrients needed to establish the beneficial fungus. The above findings open new research opportunities for developing slow release microgranular biostimulants able to maximize the synergistic action of PHs containing signaling peptides (e.g., LRPP) and beneficial fungi like *Trichoderma atroviride*. This approach is currently applied by R&D of Italpollina Company for developing microgranular-based biostimulants such as Tandem® (Italpollina USA, Inc., Anderson, IN, USA). Finally, the PH microgranular with a *Trichoderma* formulation would simplify the application of these products to seedling substrates in the nursery or to field crops at planting (side dress applications), providing further economic benefits to growers.

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