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ORIGINAL PAPER

Solid-state Fermentation of Municipal Green Waste with *Trichoderma viride*: IAA Production in Tray Reactors and its Biostimulant Effect on Early Growth of Lettuce Seedlings (*Lactuca sativa* L.)

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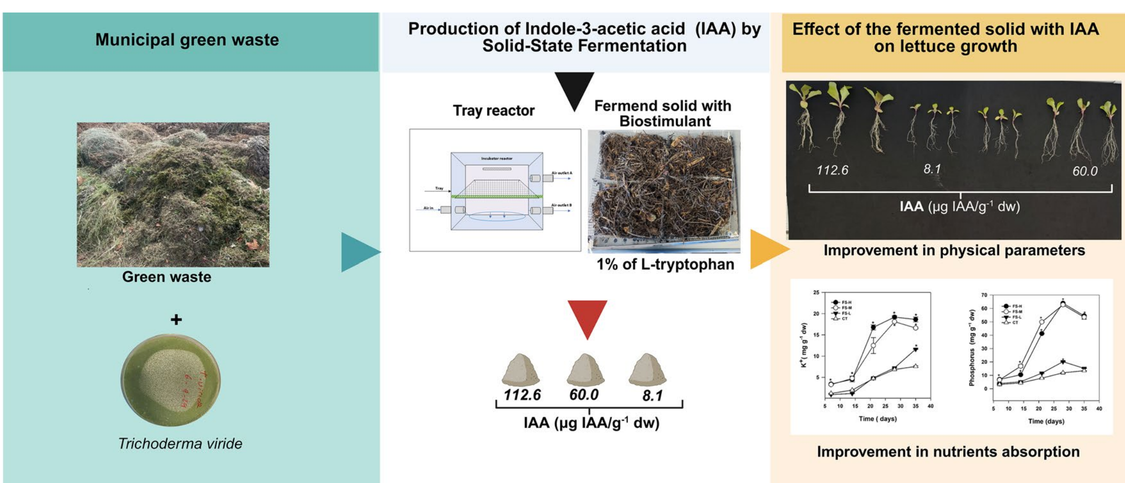
Abstract

The world-wide expansion of urban areas has led to an increase of municipal green waste (MGW) generation. In this study, indole-3-acetic acid (IAA), a known biostimulant, was produced using *Trichoderma viride* through solid-state fermentation (SSF) of MGW in a tray bioreactor. The production of IAA was optimized under SSF conditions, achieving a maximum yield of 112.6 μg IAA g^{-1} dry weight at 28 °C, 65% humidity, and 1% of L-tryptophan supplementation based on total weight. The biostimulant effectiveness was further evaluated in lettuce (*Lactuca sativa* L.) germination pot tests, showing significant improvements in growth parameters, photosynthetic pigments and nutrient uptake. Fermented solids with two different IAA concentrations (112.6 and 60.0 μg IAA g^{-1} dw) were tested on lettuce plants, resulting in higher biomass, root and shoot length, and overall vigour plant compared to a control without IAA at 7, 14, 21, 28, and 35 days after planting. Additionally, chlorophyll content, carotenoids, and the absorption of key nutrients (K^+, Ca^{2+}, Mg^{2+}, P, S) were significantly enhanced. These findings highlight the potential of *Trichoderma viride* as a biostimulant producer, aligning this strategy to circular economy principles by valorising MGW into agricultural useful bioproducts, thus reducing the dependency on chemical fertilizers, while promoting sustainable agriculture.

Graphical Abstract



Keywords Indole-3-acetic acid · *Trichoderma viride* · Municipal green waste · Circular bioeconomy · Biostimulant · Lettuce germination

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Introduction

Municipal green waste (MGW), which includes grass clippings, leaves, pruned branches, and other plant residues collected from parks and gardens, represents a significant proportion of urban waste [1, 2]. Improper management of these residues can lead to negative environmental impacts. Therefore, the valorisation of MGW emerges as a promising alternative for the sustainable utilization of this resource [3].

Sustainable agriculture faces challenges due to limiting factors such as climate change, depletion of natural resources, and the increasing global demand for food. Agricultural systems have been forced to enhance their productivity to meet these demands [4]. In this context, it is crucial to develop innovative and sustainable solutions based on marketable bioproducts that contribute to a thriving bioeconomy [5]. Among them, biostimulants derived from green waste have gained attention as a novel approach [6].

Biostimulants are substances or microorganisms applied to plants to enhance nutrient efficiency, improve tolerance to abiotic stress, and increase crop quality [7]. *Trichoderma viride* is widely recognized for its biocontrol potential against various phytopathogenic fungi, although its biostimulant effect is less known [8, 9]. Specifically, *Trichoderma viride* produces auxins, such as indole-3-acetic acid (IAA), which work as biostimulants by promoting plant growth and development [10]. IAA is one of the most influential phytohormones, playing a crucial role in plant growth and in the plant responses to both biotic and abiotic stress factors [11, 12]. Additionally, Tryptophan (Trp) is an aromatic amino acid synthesized through the shikimate/chorysmate pathway, being a precursor in secondary metabolism, including IAA biosynthesis [13]. While some references can be found using *Trichoderma harzianum* for this purpose.

Solid-state fermentation (SSF) is a process carried out in the absence or near absence of free water and has been widely used to produce various marketable products from several organic waste, including green waste [14]. In this context, utilizing SSF to produce biostimulants from green waste has emerged as a strategy to scale up the production of these compounds, with direct use in crop growth [15, 16]. *Trichoderma harzianum* has been reported to produce spore and indole-3-acetic acid in SSF both with biostimulant effects in agriculture applications. Porras et al. [17] reported a final concentration of 119.02 μg IAA g^{-1} dw using green waste as substrate for *Trichoderma harzianum* in a bench scale SSF reactor demonstrating the effects of the fermented solid on lettuce seeds germination.

Lettuce (*Lactuca sativa* L.) is a widely known crop consumed globally, primarily in salads and valued for its excellent health properties. It is one of the most consumed leafy vegetables due to its taste and high nutritional value [18].

In the context of climate change and resource scarcity, circular bioeconomy emerges as a key strategy to recycle green waste, adding value to this waste. This study aims to maximize and evaluate the production of biostimulants from MGW using *Trichoderma viride* through SSF in laboratory-scale tray bioreactors to ascertain its performance as compared to *Trichoderma harzianum*, more commonly used in this application. Specifically, it seeks to determine the maximum IAA production and assess its biostimulant effect on growth parameters, photosynthetic pigments, and nutrient uptake in lettuce plants. The influence of initial L-tryptophan concentration in IAA production was investigated. The novelty of the work lies first, in the use of *Trichoderma viride* as producer of biostimulants as this *Trichoderma* species is not commonly applied with this purpose and, second, in its comprehensiveness facing SSF production of a fermented solid with biostimulant properties from an abundant residue in an easily scalable bioreactor configuration and the determination of its effects on lettuce seeds germination, in a completely circular and zero-waste strategy.

Materials and Methods

Municipal Green Waste and Inoculum

Green Waste and Wood Chips

The MGW used in this study consisted of grass clippings and wood chips collected from parks and gardens within the campus of the Universitat Autònoma de Barcelona (Spain).

MGW was cut into approximately 1.5 × 1.5 cm pieces, washed with tap water, and then air-dried to remove excess moisture [6]. Afterwards it was transferred to a 50 L cylindrical vessel with a perforated stainless-steel plate in the bottom through which air was supplied at a constant flow rate of 5 L min^{-1} at room temperature for 10 days, to achieve uniform drying of the substrate. The MGW was manually stirred once per day. The airflow rate was regulated using a flow meter (Tecfluid, Spain, model: 2150).

After the drying process, the MGW was stored at room temperature. Prior to SSF, the MGW was autoclaved twice at 121 °C for 30 min to minimize contamination.

Inoculum

The fungal strain used in this study was *Trichoderma viride*, obtained from the National Agricultural Health Service (SENASA) in Peru.

Trichoderma viride was preserved at −80 °C in sterilized cryovials containing 10% glycerol. To prepare the inoculum, *Trichoderma viride* was cultured on potato dextrose agar (PDA) plates at room temperature for seven days. The spores were then harvested and suspended in a Tween 80 solution at 0.1% to prepare a stock solution. This spore suspension was subsequently used to inoculate the SSF matrix, ensuring a final concentration of approximately 10^7 spores g^{-1} dw.

SSF Tray Bioreactor Configuration

Tray-based SSF

SSF experiments were conducted at laboratory scale. The tray-based bioreactor comprised an incubator (Memmert GmbH + Co. KG, Schwabach, Germany) equipped with two metal-mesh trays. Each tray was fabricated from mesh sheets and measured 39.5 cm in length and 27.5 cm in width, providing a working bed height of roughly 3 cm. The incubator was supplied with a continuous airflow of 500 mL min^{-1}. A metal tray was placed in the bioreactor and divided into two parts where two treatments were performed, each containing 400 g of MGW. Figure 1 provides a scheme of the tray bioreactor. In the first treatment, 1% L-tryptophan (concentration at which best results were obtained following the procedure explained in next section) was added, while in the second treatment, no L-tryptophan was used. Temperature and relative humidity inside the bioreactor were monitored throughout the SSF process using temperature and humidity sensors (Standard Thermochron iButton device, Maxim Integrated, USA). Once the SSF process was completed, pH, electrical conductivity (EC) and organic matter of the fermented material were analyzed. Subsequently, the material was stored in a − 20 °C freezer until its application.

Influence of L-tryptophan Concentration and Process Time in IAA Production

A time-course experiment was conducted to determine IAA production by *Trichoderma viride* over a defined period. The experiment was set up for 10 days.

To determine the influence of L-tryptophan concentration in IAA production, L-tryptophan was supplemented at concentrations of 0%, 0.25%, 0.5%, and 1% relative to the moist weight of MGW into four different tray sections. The SSF process was conducted at 28 ºC with a moisture content of 65%. IAA production and *Trichoderma viride* spore count were assessed on days 1, 2, 3, 5, 7, and 10.

Characterization of the Biostimulant Produced by SSF

IAA Evaluation

For the extraction of IAA produced through SSF with *Trichoderma viride*, 25 g of fermented solid were mixed with 125 mL of ultrapure water in a 500 mL Erlenmeyer flask. The mixture was then agitated in an incubator at 180 rpm for 30 min at 15 °C. After agitation, the liquid phase was separated and centrifuged at 10,000 rpm for 15 min at 4 °C. The supernatant was collected and filtered through a 0.22 µm nylon filter. The final extract was stored at −20 °C for further analysis.

IAA quantification was performed using an HPLC system (Dionex Ultimate 3000, Dionex, Idstein, Germany). This system was equipped with an UltiMate 3000 autosampler, a column compartment, and a photodiode array detector. The chromatography data system (CDS) was controlled by Chromeleon software version 6.8 (Thermo Scientific). The chromatographic column used was an LC Kinetex® 5 µm EVO C18 100 Å (250 × 4.6 mm). The mobile phase

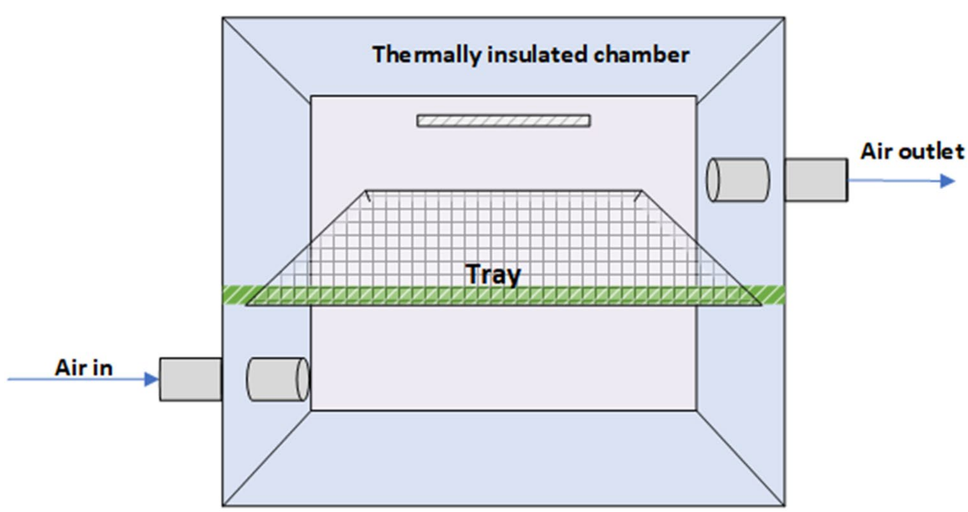

Fig. 1 General setup of the lab-scale tray bioreactor with forced aeration used for solid-state fermentation

consisted of: Phase A: 2.5% acetonitrile in ultrapure water, Phase B: 80% acetonitrile in ultrapure water. The detection wavelength was set at 280 nm. The chromatographic method followed the protocol described by [6], with slight modifications. For the preparation of the standard curve, IAA (98%) from Merck Sigma-Aldrich (CAS 87–51-4) was used at different concentrations. To improve IAA evaluation and minimize matrix effects, 5 ppm of IAA were added to the samples. The IAA analysis was performed in triplicate for each treatment.

Trichoderma viride Spore Count

To determine the spore count of *Trichoderma viride*, 20 g of fermented solid were added to 100 mL of 0.1% (v/v) Tween 80 solution. The mixture was then agitated at 180 rpm for 30 min at 15 °C, followed by a 1:1000 dilution with Tween solution.

Spore quantification was performed using a Neubauer counting chamber and observed under an optical microscope (Olympus BH2). The evaluation was conducted in triplicate. The number of *Trichoderma viride* spores per gram of dry matter is presented in Eq. 1:

$$\text{Spore concentration} = \frac{S}{DF \times CV} \times \frac{EV}{SWW} \times \frac{SWW}{DM} \tag{1}$$

where: Spore concentration is the number of spores per gram of dry green waste and wood chips (spores g^{-1} dw); S is the number of spores counted in the Neubauer chamber; DF is the dilution factor; CV is the sample volume in the Neubauer chamber (mL); EV is the extraction volume (mL); SWW is the wet weight of the solid sample (g ww); and DM is the dry matter weight of the solid sample (g dw).

Biostimulant Application and Effect on Lettuce Seeds Germination

Plant Material and Growth Conditions

The experiments were conducted in a greenhouse at the Experimental Station of the Universitat Autònoma de Barcelona, located in Cerdanyola del Vallès. Lettuce (*Lactuca sativa*) seeds of the "Maravilla" variety were used. The experiment was carried out from October 8 to November 11, 2024. The climatic conditions during the growth period are presented in Fig. S1 (Supplementary Material). The maximum and minimum air temperatures recorded during lettuce growth were 39.0 °C and 7.5 °C, respectively.

Polystyrene germination trays (47.4 cm × 69.2 cm, 150 cells each) were used, with each cell measuring 3.5 cm in length, 3.5 cm in width, and 7 cm in depth.

Biostimulant Application and Irrigation

Four treatments were established:

(1) FS-H: Addition of fermented solid containing 112.6 µg IAA g^{-1} dw (high IAA concentration, obtained from fresh fermented solid).
(2) FS-M: Addition of fermented solid containing 60.1 µg IAA g^{-1} dw (medium IAA concentration, obtained from a fermented solid that was stored for 6 months).
(3) FS-L: Application of fermented solid containing 8.1 µg IAA g^{-1} dw (low IAA concentration, obtained from a fermentation where tryptophan was not used as precursor for IAA biosynthesis).
(4) CT (Control Treatment): without fermented solid application.

For the germination tests, the fermented solid was mixed with peat at a 1:10 (w/w) ratio [17]. Initially, 10 g of peat were added to each cell, followed by 1 g of fermented solid (FS-H, FS-M, or FS-L). Based on the IAA content of the fermented solids, the 1:10 (w/w) application ratio resulted in initial concentrations of 11.26, 6.00, and 0.81 µg IAA g^{-1} of substrate mixture for FS-H, FS-M, and FS-L, respectively. One lettuce seed was placed in each cell of the germination trays. Each cell received 10 mL of distilled water per plant at the beginning of the experiment.

Each treatment was replicated three times, with each replicate consisting of 50 germination tray cells (one seed per cell). A completely randomized design (CRD) was implemented, consisting of four treatments and three replicates. The treatments were numbered as follows: Treatment 1: (FS-H); Treatment 2 (FS-M); Treatment 3 (FS-L); Treatment 4 (CT).

The irrigation system was an automated sprinkler system. Three soil moisture sensors (TEROS 10 model, Meter Group, USA) were installed, configured with a ZL6 datalogger, and monitored in real-time via the Zentra Cloud platform to track soil water content percentage throughout the vegetative period [19]. The moisture sensors were placed in pots filled with peat at a depth of 7 cm.

Three standard curves were constructed to determine the water content in the peat (%), using three sensors, and the most representative curve was selected based on its field capacity. Soil moisture readings were expressed as percentages, with 100% representing fully irrigated peat at field capacity and 0% indicating completely dry peat. The irrigation levels in the experiment were adjusted between 90 and 100% of field capacity. Crop management followed until 35 days after planting (DAP).

Measurement of Biomass Weight, Length, and Plant Vigor

Three randomly selected seedlings per replicate were evaluated at 7, 14, 21, 28, and 35 DAP, consisting of 9 seedlings per treatment. The following parameters were measured: Total fresh weight, shoot fresh weight, and root fresh weight using an analytical balance (PB3002-S, Mettler Toledo). Dry weight was determined after drying the samples in an oven at 40 °C for three days until a constant weight was achieved. Total plant length (from root tip to shoot tip), root length, and shoot length were measured through image digitization. Seedlings were photographed using a digital camera, and the images were processed with ImageJ 1.54 g software, (National Institute of Health (NIH), USA) to determine root, shoot, and total seedling measurements. All measurements were expressed in centimetres (cm) and recorded at 7, 14, 21, 28, and 35 DAP.

The Plant Vigour Index (PVI) was calculated using Eq. 2 [20]:

$$PVI=PG \times BL \quad (2)$$

where: PG is the germination percentage at 7 days and BL is the plant length (cm). The PVI was evaluated at 7, 14, 24, 28, and 31 DAP.

Measurement of Chlorophyll Content, Carotenoids, Cations, and Anions in Plants

For the extraction of chlorophyll a, chlorophyll b, total pigments, and carotenoids, 0.20 g of leaf and root samples were weighed, ground in a mortar with liquid nitrogen, and then mixed with 96% ethanol. The sample was then vortexed at 120 rpm for 5 min, followed by centrifugation at 6000 rpm at 15 °C, and the supernatant was collected.

Chlorophyll a, chlorophyll b, total pigments, and carotenoids were quantified using spectrophotometry (Hach Lange DR 3900) by measuring the absorbance of the supernatant at 665 nm, 649 nm, and 470 nm, respectively. The concentrations were calculated using the equations proposed by [21]. The results were expressed in mg per gram of fresh weight (mg g^{-1} fw). The evaluation of chlorophylls, total pigments, and carotenoids was performed in triplicate for each treatment at 7, 14, 21, 28, and 35 DAP.

The concentrations of cations, potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}) and anions, phosphate (PO_4^{3-}) and sulphate (SO_4^{2-}) were analysed. A 0.20 g sample of fresh leaves and roots was ground in a mortar with liquid nitrogen and then mixed with ultrapure water. The sample was agitated at 120 rpm for 5 min, followed by centrifugation at 7000 rpm at 15 °C. The supernatant was separated and filtered using a 0.22 µm nylon membrane filter.

Cation quantification was performed using an ICS 2000 ion chromatography system (Dionex Corporation, USA), a preconfigured ion chromatography system designed for isocratic and gradient separations with conductivity detection. The system was controlled using Chromeleon software version 6.8.

The chromatographic column used was a Dionex IonPac CS16 with a cation-exchange capacity of 5 × 250 mm: 8.4 milliequivalents. The mobile phase consisted of 38 mM methanesulfonic acid (Merck Sigma Aldrich, CAS 75–75-2) in ultrapure water. A standard calibration curve was prepared for each anion. Cation evaluation was performed in triplicate for each treatment at 7, 14, 21, 28, and 35 DAP.

For anion quantification, a Dionex DRS 600 (Thermo Fisher Scientific, USA) system was used. This dynamically regenerated suppressor is specifically designed for ion chromatography, enabling effective suppression of eluents during anion analysis. The system was controlled using Chromeleon software version 7. The chromatographic column used was a Dionex™ IonPac™ AS11-HC-4 µm, with dimensions of 2 × 250 mm. The mobile phase consisted of 100 mM potassium hydroxide (KOH) in ultrapure water. A standard calibration curve was prepared for each anion. Anion evaluation was performed in triplicate for each treatment at 7, 14, 21, 28, and 35 DAP.

To express the results in terms of elemental sulphur (S) and phosphorus (P), a stoichiometric conversion was applied to sulphate (SO_4^{2-}) and phosphate (PO_4^{3-}) concentrations obtained through ion chromatography.

Statistical Analysis

All data were analysed using analysis of variance (ANOVA) with the statistical software packages SigmaPlot 12.5, and Infostat 2020. When the data did not meet the normality assumption, the Kruskal–Wallis test was applied for non-parametric data. For parametric data, mean differences were evaluated using Tukey's post hoc test ($p \leq 0.05$). Additionally, principal component analysis (PCA) was performed to assess the relationships between the parameters related to IAA production.

Results

Biostimulant Production by SSF Using a Tray Bioreactor

Determination of the Influence of L-tryptophan Concentration in *Trichoderma viride* IAA and Spore Production

The fermentation process was conducted in a tray bioreactor for 10 days, using the optimal temperature range of 25–28 °C for *Trichoderma viride*, as reported by Kumar et al. [10]. The substrate consisted of 56% wood chips and 44% grass waste, following the optimized conditions established by Ghoreishi et al. [6] with an initial moisture content of 65%. L-tryptophan was supplemented at 0%, 0.25%, 0.5%, and 1% (w/w).

The highest IAA production curve reached its peak on day 2, with a concentration of 122.42 ± 1.06 μg IAA g^{-1} dw at 1% (w/w) L-tryptophan (Fig. 2A).The spore count of *Trichoderma viride* peaked on day 7, reaching $8.24\times10^9 \pm 5.85\times10^9$ spores g^{-1} dw at 0.25% (w/w) L-tryptophan, whereas the lowest values were $2.96\times10^8 \pm 1.47\times10^8$ spores g^{-1} dw, recorded on day 1 at 0% (w/w) L-tryptophan (Fig. 2B).

Based on these findings, the conditions for maximizing IAA production under the experimental conditions, are by supplementing 1% (w/w) L-tryptophan in the initial mixture and at an SSF time of 2 days. These conditions favour the highest IAA yield while achieving half the spore production measured as maximum (obtained with 0.25% L-tryptofan) (Fig. 2).

Biostimulant Production Through SSF from MGW with *Trichoderma viride*

The production of the material with biostimulant properties to be applied in the lettuce seeds germination tests was performed in a tray bioreactor under the above reported conditions, to maintain maximum IAA production. Another fermentation was performed in the same conditions but without the addition of precursor (L-tryptophan). This experiment consisted of three fermentation cycles in trays (two using L-tryptophan as precursor). Three samples were taken from each treatment, and the mean values ± standard deviation were calculated.

Higher IAA production through SSF was clearly observed in the two cases where 1% (w/w) L-tryptophan was initially added yielding 112.86 ± 1.46 μg IAA g^{-1} dw with a spore count of $5.93\times10^8 \pm 1.70\times10^8$ spores g^{-1} dw and 112.6 ± 5.47 μg IAA g^{-1} dw with a spore count of $6.30\times10^8 \pm 1.28\times10^8$ spores g^{-1} dw respectively. In contrast, the treatment without L-tryptophan resulted in a lower IAA production of 8.1 ± 0.12 μg IAA g^{-1} dw, with spores count of $3.79\times10^8 \pm 5.68\times10^7$ spores g^{-1} dw.

The final product of the first fermentation was stored for 60 days at room temperature before application reaching 60.1 ± 1.52 μg IAA g^{-1} dw. This material was used as the FS-M treatment (medium IAA concentration). Across all treatments, the bioreactor temperature remained stable at 27–28 °C throughout the run (Table 1).

Effect of Fermented Solid with Biostimulant Application on Lettuce Seedlings

Time Course-evaluation of Physical Growth Parameters

To assess the biostimulant effect of the fermented solid containing IAA, a time course evaluation of the physical parameters of lettuce seedlings was conducted from germination until 7, 14, 21, 28, and 35 DAP. These results are presented in Table S1 (Supplementary material), including fresh biomass weight, root weight, shoot weight, plant length, root length, shoot length, and plant vigour index.

Significant physical differences were observed in lettuce seedlings, with FS-H and FS-M showing a notable increase in comparison to CT throughout the growth period (Fig. 3).

Specifically, fresh weight (Fig. 3A), shoot weight (Fig. 3B), and root weight (Fig. 3C) in the FS-H and FS-M treatments were significantly higher compared to CT and FS-L at 7, 14, 21, 28, and 35 DAP. At 35 DAP, fresh weight was statistically higher in FS-H and FS-M, showing increments of 360.3% and 154.1%, respectively, compared to CT. Shoot weight increased significantly by 370% and 137%, respectively, while root weight showed a significant increase of 331% and 209%, respectively, compared to CT.

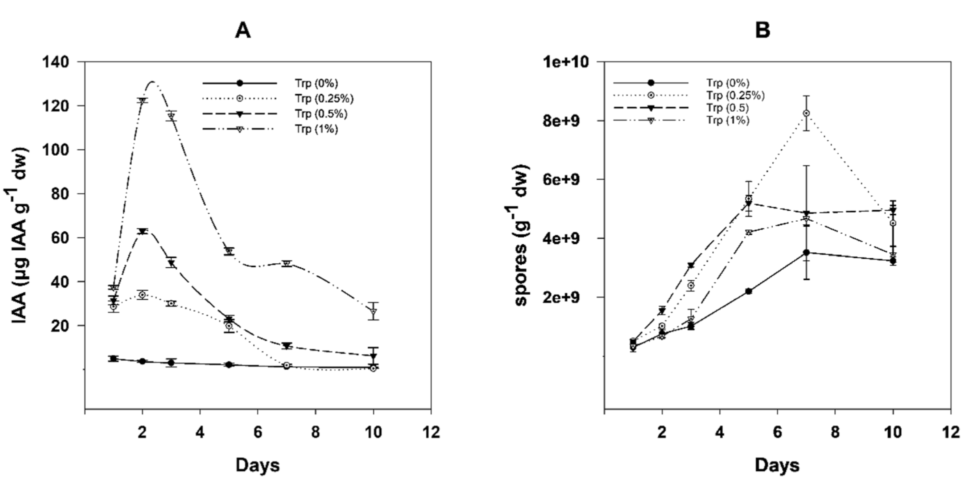

Fig. 2 Evolution of tray reactor SSF over 10 days under different L-tryptophan initial concentrations on the substrate: 0 g (0%), 0.5 g (0.25%), 1 g (0.5%), and 2 g (1%). **A** Evolution of IAA production (μg IAA g^{-1} dw) **B** evolution of *Trichoderma viride* spore production (spores $g.^{-1}$ dw)

Table 1 Characterization of the fermented solid with different concentrations of IAA produced by SSF in a tray bioreactor with *Trichoderma viride* using MGW (IAA: indole-3-acetic acid, dw: dry weight, EC: electrical conductivity)

Substrate	Fermented solid	L-tryptophan (%)	Final IAA concentration (μg IAA g^{-1} dw)	SSF conditions		Final properties			
				Initial moisture (%)	Temperature range (°C)|	pH	EC (mS/cm)	Final moisture (%)	Organic matter (%)
Grass (44%)	FS-H	1	112.60	65	27–28	6.62	1.38	57.84	8.42
Wood chips (56%)	FS-M	1	112.86			6.71	1.47	54.37	8.59
	FS-L	0	8.1			6.55	1.30	55.24	8.60

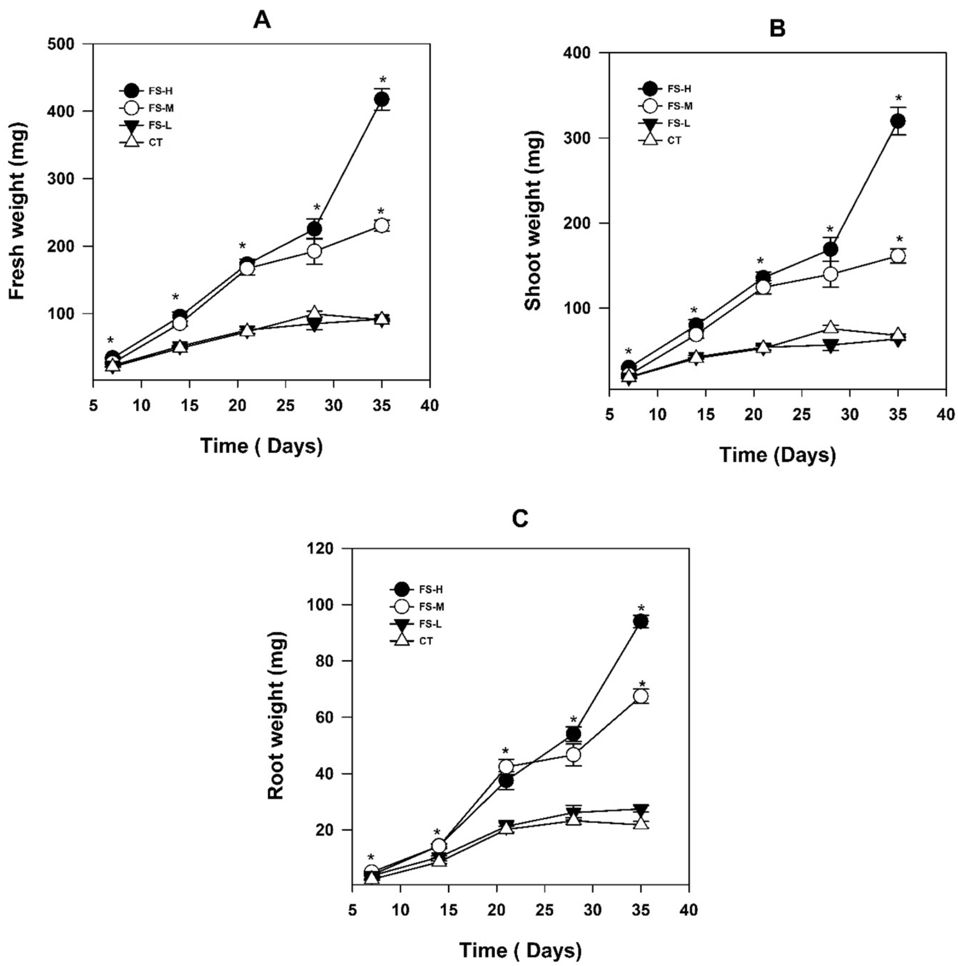

Fig. 3 Effect of FS-H, FS-M, FS-L, and CT treatments on lettuce growth parameters. Fresh weight (A), shoot weight (B), and root weight (C) are shown as median ± experimental error. Asterisks (*) indicate statistically significant differences ($p \leq 0.05$) between treatments in the time-course evaluation

Additionally, also for FS-H and FS-M treatments compared to CT, total plant length increased significantly by 65.4% and 48.1%, while shoot length increased by 72.19% and 58.94%, and root length showed significant growth of 61.8% and 47.3%, respectively. Finally, the plant vigor index presented a significant increase of 68.7% and 50.1%, respectively for FS-H and FS-M.

According to our results, the application of fermented solid with higher IAA doses (FS-H, FS-M) significantly improved weight, growth, and vigor in lettuce seedlings. However, the FS-L treatment did not show statistically significant differences compared to CT.

Time-course Evaluation of Photosynthetic Pigments, Carotenoids, and Nutrients Content

The photosynthetic pigments, carotenoids, and nutrient content (K^+, Ca^{2+}, Mg^{2+}, P, S) in lettuce seedlings, including both leaves and roots, were measured at 7, 14, 21, 28, and 35 DAP, as presented in Tables S2 and S3 (Supplementary material).The results showed that all these parameters significantly increased in FS-H and FS-M compared to CT in lettuce seedlings.

Figure 4 presents time-course evaluation of photosynthetic pigments and carotenoids over time (DAP) for the four different treatments. The chlorophyll a content in the FS-H and FS-M treatments was statistically higher than in CT throughout the evaluation period (7, 14, 21, 28, and 35 DAP). At 35 DAP, chlorophyll increased by 306% and 304% respectively, while in FS-L, it increased only by 20% (Fig. 4A). Similarly, chlorophyll b increased significantly by 383.3% and 291.7%, while FS-L showed a smaller increase of 16.7% (Fig. 4B) and the increment in total pigments was of 405.6% and 311.1%, for FS-H and FS-M respectively and 22.2% for FS-L (Fig. 4C). Likewise, carotenoid content increased significantly by 371.4% and 314.3% respectively with FS-L showing an increase of 28.6% (Fig. 4D).

Calcium (Ca^{2+}), magnesium (Mg^{2+}), potassium (K^{+}), phosphorus (P), and sulphur (S) content in lettuce is presented in Fig. 5. The content of these elements in FS-H and FS-M treatments was statistically higher than in CT throughout the evaluation period (7, 14, 21, 28, and 35 DAP). At 35 DAP, the contents of K^{+}, Ca^{2+}, Mg^{2+}, P, and S were significantly higher in FS-H and FS-M compared to the control. The greatest increases were observed in Mg^{2+} (302% and 252.9%), Ca^{2+} (214.5% and 169.3%), and P (304% and 300%) in FS-H and FS-M, respectively. K^{+} and S also rose markedly, by 146.2% and 101% in FS-H, and by 119.6% and 40.7% in FS-M. In contrast, FS-L showed no significant differences for Ca^{2+}, P, and S, and only modest increases for K^{+} (53.8%) and Mg^{2+} (45.9%) (Fig. 5A–E).

Discussion

Evaluation of L-tryptophan Levels for IAA Production in Tray Bioreactor

As previously reported, the production of biostimulants from MGW through SSF represents an innovative alternative for

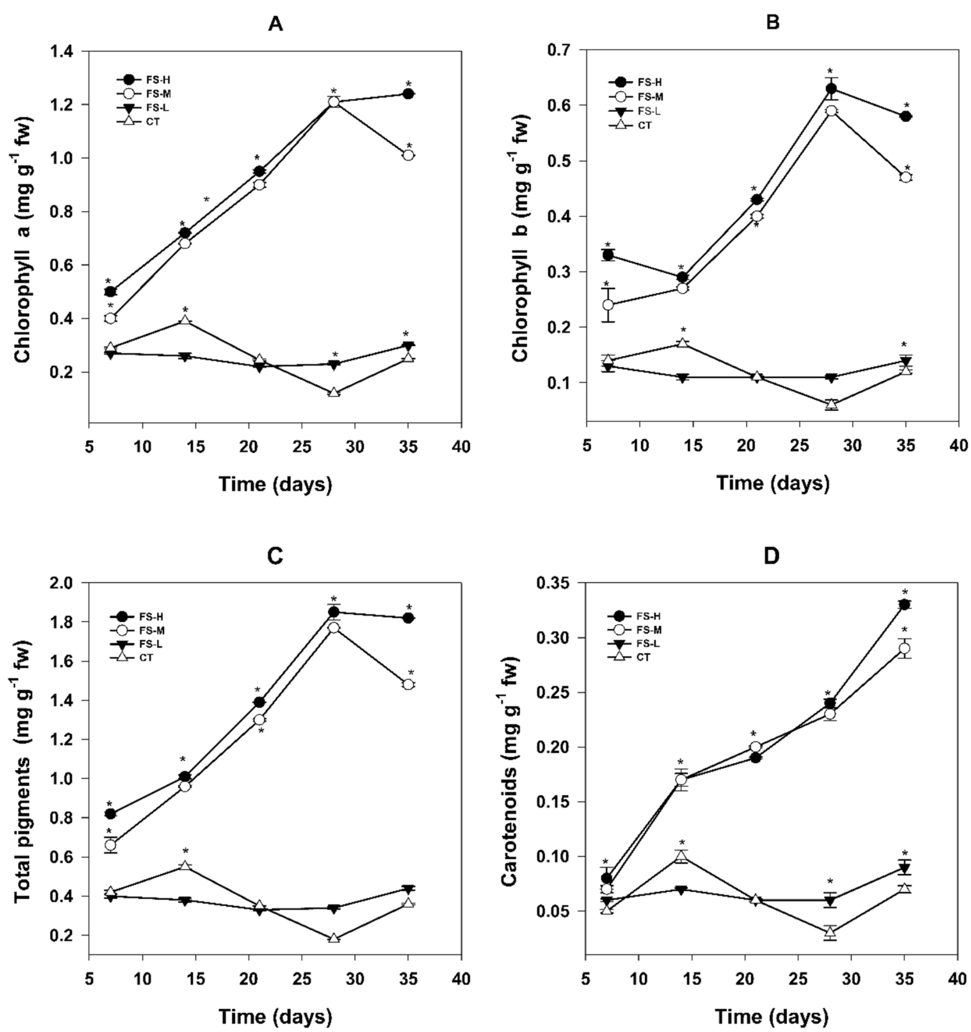

Fig. 4 Effect of FS-H, FS-M, FS-L, and CT treatments on photosynthetic pigments and carotenoid content in lettuce cultivation. Chlorophyll a (A) is shown as median ± experimental error; chlorophyll b (B) and carotenoids (D) are shown as median ± experimental error. Asterisks (*) indicate statistically significant differences ($p \leq 0.05$) between treatments in the time-course evaluation

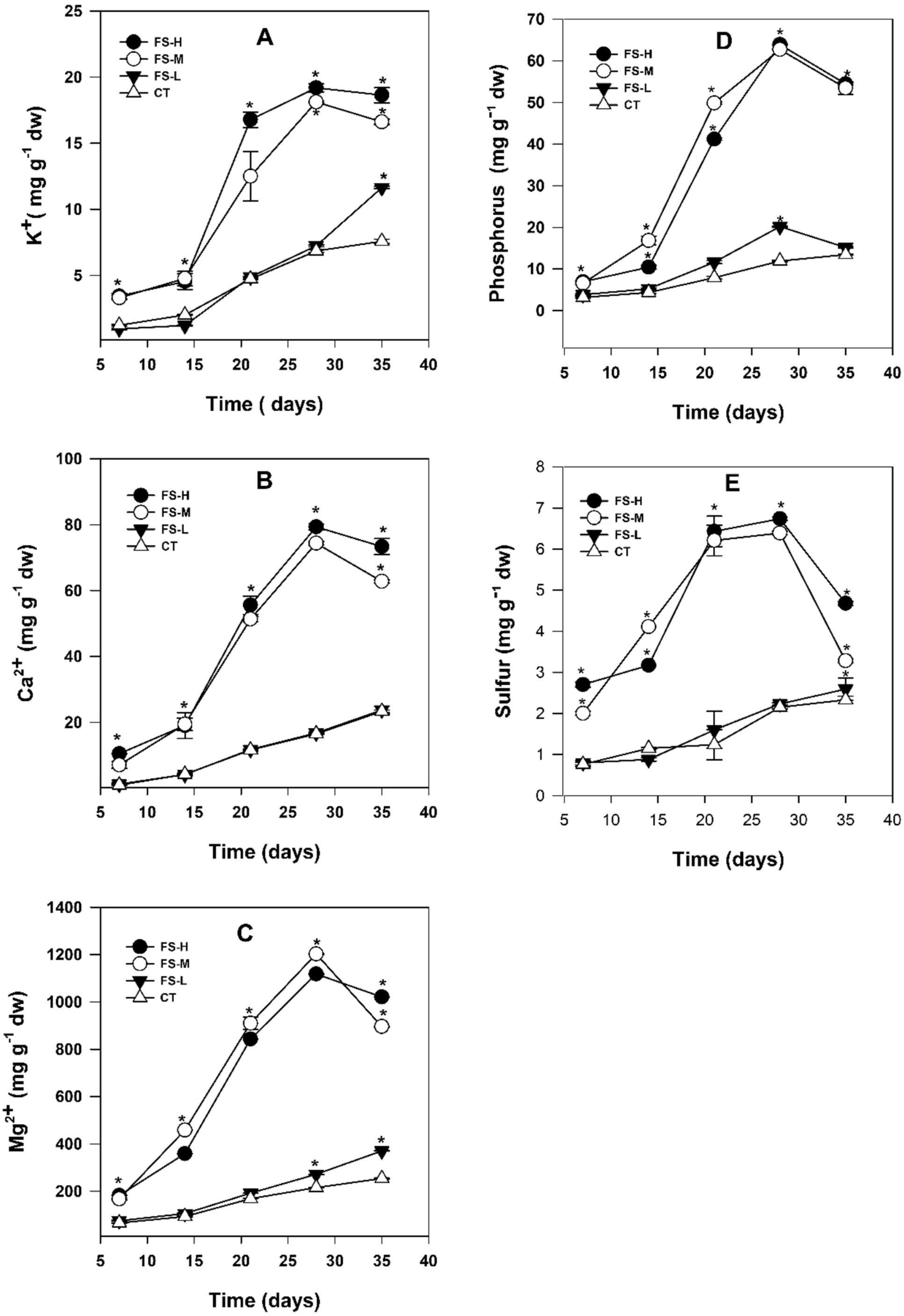

Fig. 5 Effect of FS-H, FS-M, FS-L, and CT treatments on nutrient content in lettuce seedlings at 35 DAP. Nutrient concentrations are shown for **A** potassium (K^+), **B** calcium (Ca^{2+}), **C** magnesium (Mg^{2+}), **D** phosphorus (P), and **E** sulphur (S), expressed as median ± experimental error. Asterisks (*) indicate statistically significant differences ($p \leq 0.05$) between treatments in the time-course evaluation

valorising this waste [6]. In this study, we aimed to maximize biostimulant production, specifically IAA yield. The optimization of tray bioreactors for SSF has been previously explored [22], highlighting that temperature and relative humidity are crucial factors for microbial growth and the quality of fermentation of protein content. Other authors also point substrate moisture and air temperature as key variables also mentioning fermentation time to play a fundamental role in determining the final yield of the fermented solid in the production of protease enzymes by *Aspergillus niger* and in single-cell protein production from sugarcane bagasse using *Saccharomyces cerevisiae* [23, 24]. Also, Ghoreishi et al. [15] reported that *Trichoderma harzianum* produced 120 µg IAA g^{-1} dry matter and 1.3×10^9 spores g^{-1} dry matter when using green waste and wood chips as substrate.

The use of L-tryptophan has been reported essential for IAA production in *Pseudomonas aeruginosa* isolates, with supplementation at 1 mg mL^{-1} of broth at 32 ± 2 °C for 96 h according to Goswami et al. [25]. Similarly, [6] reported that SSF with *Trichoderma viride* yielded 4.0 μg IAA g^{-1} dw on day 10. Our results confirm this finding, as without L-tryptophan supplementation during SSF, IAA production remained below 10 μg IAA g^{-1} dw throughout the 10-day time-course experiment (Fig. S2, Supplementary Material).

Other studies have reported the use of L-tryptophan as an IAA precursor at 0.45% (w/w) with *Trichoderma harzianum* produced 101.46 μg g^{-1} dw [6] and 1% (w/w) using *Aspergillus flavipes*, achieving 183 μg IAA mL^{-1} [26]. Variations in L-tryptophan needs could be influenced by factors such as microbial strain, substrate type, and particle size, leading to variability in IAA production. For instance, Kumar et al. [10] reported that *Trichoderma viride* produced 115 μg IAA/mL with 0.5% L-tryptophan at 28 °C ± 2 °C under submerged fermentation conditions.

Beyond its biocontrol function, *Trichoderma viride* has limited research on its biostimulant activity compared to other strains such as *Trichoderma harzianum*. This study represents the first attempt to produce IAA using *Trichoderma viride* and demonstrates its potential for IAA and spore production from MGW using a tray bioreactor. Furthermore, the tray bioreactor represents a scalable strategy, optimizing energy and material use compared to other types of bioreactors. Additionally, the tray bioreactor design facilitates operational handling.

Biostimulant Effect on Growth Parameters in Lettuce Cultivation

As previously reported, some strains of *Trichoderma* exhibit predominant biostimulant activity, making them unique for widespread use in horticulture. These strains engage in multi-level communication within root and shoot systems, releasing auxins at the root level, which promotes root branching, enhances nutrient absorption, and ultimately boosts crop growth and yield [27, 28].

Several studies have confirmed that *Trichoderma viride* and other strains can synthesize IAA and effectively promote plant and crop growth [10, 29], acting as a cell elongation promoter that stimulates root elongation and development in soybean [30]. The effect of IAA on plants depends on its concentration and the species. Some studies have shown that treating maize grains with *Solenostemma argel*, an IAA-producing bacterial strain, supplemented with 2% (w/v) L-tryptophan, led to increased root elongation, seedling vigour, fresh and dry biomass, as well as higher chlorophyll, phenolic, and anthocyanin content [31]. Additionally, the application of a fermented solid with biostimulant properties, produced by *Trichoderma harzianum* containing 119.02 μg IAA g^{-1} dw to lettuce cultivation significantly improved physical parameters such as fresh weight, height, number of leaves, and leaf area, as well as chemical parameters like photosynthetic pigments and DPPH [16]. However, with 11.8 μg IAA g^{-1} dw and without L-tryptophan supplementation under normal irrigation (100–70% field capacity), no significant differences in fresh weight were observed compared to the control.

Furthermore, the application of IAA at concentrations of 40, 80, and 120 mg IAA kg^{-1}, produced by *Aspergillus flavipes* through SSF and applied to eucalyptus, resulted in a significant increase in root length, shoot length, and root fresh weight compared to the control (0 mg IAA kg^{-1}) [26]. Other authors reported that the exogenous application of IAA (20 mg L^{-1}) in cotton cultivation significantly enhanced root length, plant height, shoot and root fresh weight, and photosynthetic efficiency in leaves compared to the control without IAA [32].

The results of the present study showed that the application of FS-H and FS-M, produced by *Trichoderma viride* in lettuce seeds germination, lead to results statistically superior to CT at 7, 14, 21, and 35 DAP in physical parameters, and plant vigour index. Meanwhile, FS-L did not show statistically significant differences with CT. These findings are in accordance with Contreras-Cornejo et al. [33] who reported that the inoculation of *Trichoderma virens* in Arabidopsis induced growth regulators, including auxins, leading to increased biomass production and stimulated lateral root development. Moreover, a strain of *Trichoderma hamatum* GD12 was found to promote root and shoot growth in lettuce through bran extract treatments [34].

The application of a biostimulant produced with *Trichoderma viride* through SSF using MGW may be considered as an alternative to reducing synthetic fertilizer use, thereby lowering environmental impact. Furthermore, within the framework of a circular economy, this approach closes the organic waste cycle, integrating these residues into agricultural systems and promoting a sustainable waste management model.

Biostimulant Effect on Photosynthetic Pigments and Nutrient Uptake in Lettuce Cultivation

In addition to its role in plant growth and development, IAA enhances the structural and functional attributes of the photosynthetic system, including chlorophyll content, total pigments, and photosynthetic activity in eggplant seedlings [35].

In this study, the application of biostimulant produced by *Trichoderma viride* through SSF had a significant effect on chlorophyll a, chlorophyll b, carotenoids, and total pigments in the FS-H and FS-M treatments at 7, 14, 21, 28, and 35 DAP, compared to the CT. The FS-L treatment showed statistical differences only at 28 and 35 DAP. These findings align with other studies evaluating the comparative effect of two IAA-producing *Trichoderma* strains, which resulted in improved root length and chlorophyll content in maize when supplemented with 1 g L^{-1} of L-tryptophan [36]. Similarly, maize plants inoculated with *Trichoderma asperelloides* exhibited significant improvement in chlorophyll content [37]. Photosynthetic pigments are essential indicators of plant stress tolerance. Rice cultivation, supplementation with *Trichoderma reesei* improved photosynthetic pigments, IAA production, and nutrient uptake [38]. Additionally, chlorophyll has been used to predict bioavailable phosphorus in barley cultivation [39]. These results are consistent with our study, where photosynthetic pigments showed significantly higher accumulation in FS-H and FS-M, along with increased nutrient absorption (phosphorus, potassium, sulphur, calcium, and magnesium) in lettuce plants, demonstrating the biostimulant effect of *Trichoderma viride*. This effect suggests a potential reduction in the need for chemical fertilizers.

A recent study reported that inoculation with *Trichoderma harzianum* increased fresh weight by 18.62%, phosphorus uptake by 170%, and potassium uptake by 73.17% in Lollo Rosso lettuce compared to uninoculated plants [40]. Additionally, *Arabidopsis thaliana* plants cultivated in the presence of *Trichoderma viride* exhibited a 45% increase in biomass and 58% higher chlorophyll content, confirming its growth-promoting effects [41].

Root modifications induced by the qid74 gene from *Trichoderma harzianum* enhanced total root absorption surface, improving nutrient uptake and translocation to shoots in tomato and cucumber, resulting in higher biomass production and more efficient NPK and micronutrient use [42]. Moreover, a strain of *Trichoderma asperellum* CHF 78 functioned as a growth promoter, producing 8.59 µg IAA mL^{-1}, which significantly increased dry weight and plant height in tomato plants, even when infected with Fusarium, reducing disease severity and enhancing nutrient absorption (P, K, Mg, and Zn) [43].

This study demonstrated the biostimulant effect of *Trichoderma viride* in improving biomass production, photosynthetic pigment content, and nutrient uptake in lettuce cultivation.

Conclusions

This study demonstrated the feasibility of producing a fermented solid with IAA using *Trichoderma viride* in a tray bioreactor with MGW through SSF. Additionally, L-tryptophan supplementation was identified as an essential factor for enhancing IAA production.

These results highlight the potential of *Trichoderma viride* as a biostimulant producer, offering a sustainable alternative to chemical products. Furthermore, this approach promotes the recycling and reduction of municipal green waste from parks and gardens, repurposing it as a value-added agricultural input within a circular economy model. These differences highlight the need for further research to determine optimal L-tryptophan concentrations and fermentation conditions for maximizing IAA production.

These findings lay the groundwork for future research on process scalability and field applications. However, further research on large-scale production is required to achieve similar yields and evaluate its efficacy under real agricultural conditions.

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Data Availability The data presented in this study are available upon request to the corresponding author.

Declarations

Conflict of interest The authors have no financial or non-financial interests to disclose.

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