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Phytotoxicity Assessment of Biofertilizer Produced from Bioreactor Composting Technology Using Lettuce (*Lactuca sativa* L.) Seeds

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Abstract

Establishing reliable technological information on the safety of biofertilizers produced from a bioreactor composting technique is a must prior to its commercialization. A phytotoxicity study of biofertilizer made from the bioreactor composting technology at Aklan State University, Banga, Aklan, Philippines was conducted for fourteen (14) days using commercially available lettuce seeds (*Lactuca sativa L.*). Standard phytotoxicity attributes such as hypocotyl length, radicle length, relative germination percentage, and relative radicle growth observed during the germination stage were evaluated. Results revealed no significant difference in the radicle lengths of the germinated lettuce seeds as affected by the varying levels of biofertilizer dilution at $H(3) = 10.567$, $p = 0.061 > 0.05$. *On the other hand*, *the* hypocotyl length of the lettuce showed significant differences in response to varying levels of biofertilizer dilution with Welch's $F(5, 5.163) = 8.175$, $p = 0.017 < 0.05$. Also, the different levels of biofertilizer affected significantly the germination percentage of lettuce seeds $F(5, 12) = 5.822$, $p = 0.006 < 0.05$. All levels of biofertilizer treatments indicated a decrease in relative germination percentage. However, those seeds applied with 10% biofertilizer have the highest reduction of germination percentage, equivalent to 86.9% (RGP = 13.10%). All levels of biofertilizer showed an increase in radicle growth in contrast to the negative control plant except for the one given a 10% level of biofertilizer. Seeds that received 10% biofertilizer showed an extremely high reduction in radicle growth, equivalent to 72.22% (RRG = 27.78%). The study shows that applying low levels of the bioreactor-produced biofertilizer will observably reduce the measure of the germination characteristics of lettuce seeds, but not necessari-

ly low enough to be considered phytotoxic. However, the application of at least 10% bioreactor-produced biofertilizer can presumptively lead to phytotoxicity.

Keywords

Biofertilizer, Bioreactor, Germination, Lettuce, And Phytotoxicity

1. Introduction

Fertilizer is considered one of the primary means to improve agricultural productivity, supporting the aim of sustainable development goal (SGD) in doubling the agricultural production and income of food producers. With the continued global unrest, the cost of major fertilizer supplies is expected to increase. Providing farmers with locally sourced market-available fertilizer materials is the best strategy to improve their ability to increase their crop yields, their income, and their financial security. Initiatives have been introduced to cater to this concern through the Bioreactor Composting Technology that produces locally available farm-based biofertilizers. Biofertilizer is recognized as a promising, cost-effective, eco-friendly, renewable source of plant nutrients for supplementing chemical fertilizers, as well as being helpful for the remediation of polluted soils [1]. Studies conducted in the past suggest the crucial role of biofertilizer in improving the growth and yield of plants and that biofertilizers can be used for improving soil health, even on highly contaminated sites. In recent years, biofertilizers have emerged as an important component for biological nitrogen fixation. They offer an economically attractive and ecologically sound route for providing nutrients to plants [2].

Biofertilizers are substances that contain living microorganisms that colonize the rhizosphere of plants and increase the supply or availability of primary nutrients and/or growth stimulus to any crop [3] [4]. The absence of phytotoxicity is one of the most important criteria for the use of biofertilizers and their carriers must be able to maintain their activity efficiently until used [5]. Phytotoxicity is the delay of seed germination, inhibition of plant growth, or any adverse effects on plants caused by specific substances (known as phytotoxins) or growing conditions [6] [7]. The term for plant damage is "phytotoxicity" and it can also be caused by pesticides, nutrients, or physical and environmental damage (wind, sun, hail, etc.) [8]. Phytotoxicity appears in several ways on plants but five types of damage most commonly occur which are burn, necrosis, chlorosis, leaf distortion, and stunting [9]. Phytotoxicity of compost is often best evaluated by conducting germination or growth tests [10] [11].

The bases for conducting phytotoxicity trials include availability, ease of germination, and sensitivity to compost toxicity [12]. This creates a perfect opportunity of using lettuce seeds (*Lactuca sativa L.*) since lettuce seeds work well in running bioassays for toxicity tests and exhibits high sensitivity. The purpose of

this study is to assess the phytotoxicity of bioreactor-produced biofertilizer from Aklan State University-Banga, Aklan, Philippines, using lettuce. Specifically, it aims to determine the nutrient composition of biofertilizers, determine the growth of lettuce (*Lactuca sativa L.*) seeds, and identify the significant differences in radicle length, hypocotyl length, germination percentage as affected by the varying levels of biofertilizer dilution. As there is no established study yet on the phytotoxicity of biofertilizers from the bioreactor composting facility, the data generated from this study is essential in biofertilizer formulations that are safe for the environment, effective, and cheap source of nutrients that are essential for the sustainability of agricultural crop production.

2. Materials and Methods

2.1. Materials

The materials and reagents used in the experiment are biofertilizers, lettuce seeds, distilled water, zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), 90 × 14 mm diameter sterile petri dishes, filter paper, 10 mL syringe, graduated cylinder, beakers, graduated ruler, tweezers, paper towels, aluminum foil, plastic cling wrap, record book, and laptop for encoding purposes.

2.2. Methods

2.2.1. Experimental Design and Treatments

The experiment was conducted at Aklan State University, Banga, Aklan. A laboratory set-up was established that permits limited exposure to hazards that could affect the experiment and that ensures that the experimental units will not be exposed to direct sunlight. The experimental units were laid out in a completely randomized design (CRD). The study has a single factor with three replicates of five variating dilutions of biofertilizers, excluding two control groups to determine its phytotoxicity. The experimental units were left without disturbance for ten (10) days. After such period, data gathering on the experimental units was conducted.

2.2.2. Production of Biofertilizer

The study followed the methodology described in the paper of Tansengco *et al.*, (2016) [13]. The biofertilizer was generated from various biodegradable wastes, mainly vegetable scraps and leftover foods. A ratio of 40 percent Nitrogen source and 60 percent carbon source was observed in the study. These wastes have a high moisture content (81.4% to 83.6%), pH of 5.0 to 5.5, and total organic matter of 91.0% to 93.2%. Dried yard waste as bulking materials composed of fallen leaves had low moisture content (9.1% to 10.0%), pH of 5.4 to 5.6, and total organic matter of 84.6% to 90.0%. The moisture content of the mixture was adjusted by adding water to achieve 50% - 60% moisture.

2.2.3. Preparations of Biofertilizer Samples

Biofertilizer samples used in the study were air-dried. Dried samples were pulve-

rized using a mortar and pestle and sieved using a standard < 2 mm soil siever. One (1) kilogram of representative samples was placed in an airtight bag for analysis.

2.2.4. Biofertilizer Sampling for Laboratory Analysis

Representative samples of the biofertilizer were submitted for physico-chemical (nutrient) analysis. The composite sample was brought to the Department of Agriculture – Region VI laboratory in Iloilo City, Philippines, for routine biofertilizer chemical (nutrient) analysis.

2.2.5. Preparing the Control Solutions

Biofertilizer toxicity was assessed using the standard phytotoxicity test. Negative control solution C(-) was prepared using distilled water, while zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) was utilized as the positive control solution [14].

2.2.6. Preparations of Biofertilizer Solutions

The bio-fertilizer samples were air-dried, crushed, and sieved. One hundred (100) grams of fine biofertilizers were dissolved in 900 mL of distilled water. The mixture was then filtered using filter paper. The aqueous extract was collected, and dilutions of 0.1, 1, 10, 100, and 1000 ppm biofertilizer solutions were prepared by adding distilled water.

The treatments were formulated as follows: Treatment 1 (0.1 ppm)—0.001% aqueous biofertilizer extract and 99.999% distilled water; Treatment 2 (1 ppm)—0.01% aqueous biofertilizer extract and 99.990% distilled water; Treatment 3 (10 ppm)—0.1% aqueous biofertilizer extract and 99.900% distilled water; Treatment 4 (100 ppm)—1% aqueous biofertilizer extract and 99% distilled water; and Treatment 5—10% aqueous biofertilizer extract and 90% distilled water.

2.2.7. Testing Procedures

Before the testing, all glassware was sterilized using an autoclave. The glassware was wrapped in clean paper, secured with paper tapes, placed inside the autoclave, and sterilized for 30 minutes. After such, the glassware was left inside for another 30 minutes to cool down. The lettuce seeds that were used in the study were produced by East-West Seeds Philippines with a germination rate of 96% and a purity rate of 99%. Before the experiment, a trial on the germination rate of the seeds was conducted to test the viability of the seeds.

The methodologies of Sobrero and Ronco (2004) [15] were adapted in the experimental testing. One (1) milliliter of the previously prepared dilution C(-), and biofertilizer solutions (Treatments 1, 2, 3, 4, and 5) were poured into each Petri dish containing a filter paper cut to fit the dish. The dilution covered the whole filter paper area. The filter paper absorbed most of the dilution to prevent the seeds from getting too soaked. In each petri dish, ten seeds of lettuce were placed in an orderly and spacious manner using a tweezer to allow the root growth of the test crop. The seed plates were sealed with cling wrap to avoid moisture loss and were kept in a designated area for ten days (240 hours) at

room temperature.

2.2.8. Data Gathering

The following data were gathered for the study:

1) Radicle Length

After ten days, the radicle length of each seedling in each sample replicate was carefully measured using a graduated ruler. Radicle elongation measured is the length from the knot (the thicker transition region between the radicle and hypocotyl) to the root apex.

2) Hypocotyl Length

Hypocotyl elongation considered the length from the knot (the thicker transition region between the radicle and hypocotyl) to the beginning of the insertion of the two cotyledons.

3) Relative Germination Percentage

Determined from the ten (10) lettuce seeds per treatment using the formula below, where *Gs* is the number of germinated seeds in the sample, and *Gc* is the number of germinated seeds in control:

$$\text{RGP } (\%) = Gs/Gc \times 100\%$$

4) Relative Radicle Growth

Determined using the formula below, where *Ls* is the radicle length of the germinated seeds in the sample, and *Lc* is the radicle length of the germinated seeds in control:

$$\text{RRG } (\%) = Ls/Lc \times 100\%$$

2.2.9. Statistical Analysis

Descriptive statistics were generated for the parameters displayed by the experimental units. Statistical significance test among treatment groups was performed using Kruskal-Wallis Test to address violations of normality for One-way Analysis of Variance (ANOVA). Welch's ANOVA was selected to evaluate differences in group means for hypocotyl length due to heterogeneous populations of treatment groups. The traditional One-way ANOVA was then performed to test differences in germination rate in response to varying levels of biofertilizers. Post hoc analysis was executed using Games-Howell for multiple pairwise comparisons of treatment groups concerning hypocotyl length and Least Significant Differences for germination rate at 5% significance level. All statistical tests were performed using SPSS (Statistical Package for Social Sciences) Version 21.

3. Results and Discussion

3.1. Nutrient Composition of Biofertilizer

A sample of the biofertilizer was taken to the Regional Soils Laboratory of the Department of Agriculture located in Parola, Iloilo City, Philippines. Soil test data (Table 1) from the laboratory analysis reflected that the biofertilizer produced from the bioreactor composting technology has pH level of 8.23, 1.51

Table 1. Soil test data of the biofertilizers.

Measure/Component	Level/Content
pH Level	8.23
Total Nitrogen (N)	1.51%
Total Organic Matter	30.2%
Phosphorus (P)	117 ppm
Potassium (K)	2,311 ppm

percent nitrogen content, 30.2 percent total organic matter, 117 ppm phosphorus content, and 2311 ppm potassium content. The pH level of the biofertilizer (8.23) is notably higher than the optimum soil pH range recommended for lettuce growth. This conforms with the previous results observed by Hanapi *et al.* (2013) [16] which obtained a biofertilizer pH range of 8.20 - 8.50. Though higher than the neutral pH, the slightly alkaline pH is beneficial because it will contribute to neutralizing acidic agricultural soil [17] that is dominant in the locality. Chang and Yang (2009) concluded in their study that inoculated biofertilizers with tested microbes had a significantly higher ash, pH, total nitrogen, and soluble phosphorus content. Adding microbes can shorten the period of maturity, improve the quality, increase the soluble phosphorus content, and enhance the populations of phosphate-solubilizing and proteolytic bacteria in the biofertilizers [18].

Notably, the NPK composition of the biofertilizer is very high compared to the NPK requirement for lettuce growth. Lettuce requires medium levels of fertilization, growing best with 150 to 200, which significantly increases plant height, number of leaves per plant, dry weight, and yield [19] [20]. Healthy P levels in soil range from 25 to 50 ppm [21]. When soil test P levels are above 40 ppm, only small amounts of P are required which are best applied as starter. With soil K test values above 150 ppm, K fertilization is not required as lettuce will likely not respond to K fertilization. A positive yield response is possible with an intermediate K availability (100 - 150 ppm) [22] [23].

3.2. Radicle Length

After ten days of observation, the radicle length of lettuce seedlings in each sample replicate was carefully measured using a graduated ruler. As observed (**Table 2**), the negative control plant produced the longest radicle length of lettuce with a mean of 44.30 (SE = 12.57). Treatment 5, which has the highest level of biofertilizer dilution (10%) yielded the lowest radicle length with a mean of 2.22 (SE = 1.47). The application of varying levels of biofertilizer dilution does not significantly affect the growth of lettuce seeds in terms of their radicle length $H(3) = 10.567$, $p = 0.061 > 0.05$.

The variations in radicle development may be attributed to the other phytotoxic component of the biofertilizer. Aside from the excessive amount of nutrient present in the materials, other components such as heavy metals and other

Table 2. Statistical significance test results (One-way ANOVA, Kruskal-Wallis, and Welch's ANOVA) of Radicle Length (mm), Hypocotyl Length (mm), and Germination Percentage (%) of lettuce seeds after subjected to varying levels of biofertilizer dilution.

Treatments	Radicle length	Result	Hypocotyl length	Result	Germination Percentage	Result
C-	44.30 ± 12.57	H(3) = 10.567, p = 0.061	14.39 ± 6.10 abc	Welch's F(5, 5.163) = 8.175, p = 0.017	76.67 ± 3.33 c	F(5.12) = 5.822, p = 0.006
T1 (0.001%)	22.31 ± 9.72		19.89 ± 1.03 cd		73.33 ± 3.33 bc	
T2 (0.01%)	22.54 ± 7.11		8.93 ± 4.56 abc		43.33 ± 12.02 b	
T3 (0.1%)	24.30 ± 9.79		13.10 ± 0.57 ab		60.00 ± 11.55 bc	
T4 (1%)	22.78 ± 9.56		15.79 ± 2.18 abc		53.33 ± 16.67 bc	
T5 (10%)	2.22 ± 1.47		2.33 ± 2.33 a		10.00 ± 5.77 a	

Values are mean ± standard error S.E. Values with same letters in the same column do not differ significantly at $p < 0.05$ using Dunn-Bonferroni test for hypocotyl length and Games-Howell for germination percentage.

organic pollutants are present as well. In the study of Zhou, Wieslander, & Wu (2016), heavy metals harm lettuce seeds which impede their germination and radicle growth. According to Zhou *et al.* (2016), leafy vegetables are highly able to uptake and accumulate heavy metals [24]. Heavy metals can be readily taken up by vegetable roots, and can be accumulated at high levels in the edible parts of vegetables, even heavy metal in the medium is at low levels [25] [26].

3.3. Hypocotyl Length

The hypocotyl length of the lettuce showed significant differences in response to varying levels of biofertilizer dilution with Welch's $F(5, 5.163) = 8.175$, $p = 0.017 < 0.05$. Lettuce seeds applied with 0.001% biofertilizer were the tallest with mean of 19.89 mm (SE = 1.03) which significantly differ to those applied with 10% biofertilizer which produced the lowest hypocotyl length with mean of 2.33 (SE = 2.33). Among the levels of biofertilizers, there are no significant mean differences compared to the negative control plant.

In most cases, lettuce prefers fertile soil with a pH ranging from 6 to 6.8. In a study conducted by Roosta (2011) magnesium (Mg) concentration in the leaves increased with elevating pH up to 7.0 and then decreased at 8.0, but iron (Fe), manganese (Mn), and zinc (Zn) concentrations decreased at higher solution pH levels [27].

The level of pH in the growth media determines the availability and functions of nutrients in them. Excessive amounts of nutrients in the medium can cause toxicity in the growth of the plants. As noted in the nutrient analysis of the biofertilizer, high levels of P and K are present and are higher that the ideal amount required. Alkaline pH increases and decreases micronutrients affecting the early growth development of plants.

Poor hypocotyl development may also be attributed to the presence of other toxic substances like heavy metals. Studies suggest that heavy metals might cause an inhibition in the initial growth that alters water balance and nutrient absorp-

tion, thereby affecting their transportation to the aboveground plant parts and thus negatively affecting shoot growth and ultimately decreasing biomass accumulation [28]. Hence, it is non-germination of the lettuce seeds. This result, however, contradicts the observation of Spiassi *et al.* (2015) in their study for they found a relative relationship between the elongation of hypocotyl and increased levels of biofertilizer [29].

3.4. Germination Percentage

Different levels of biofertilizer affected significantly the germination percentage of lettuce seeds $F(5, 12) = 5.822$, $p = 0.006 < 0.05$. Seeds subjected to negative control solution have the highest germination percentage with a mean of 76.67% (SE = 3.33) while those seeds subjected to 10% biofertilizer dilution have the lowest germination percentage with a mean of 10.00% (SE = 5.77). In contrast to the negative control plant, those group of seeds applied with 0.01% and 10% biofertilizer dilution differed significantly in terms of germination. Notably, all levels of biofertilizer dilution compared to 10% level of biofertilizer dilution have a significant mean difference in germination percentage.

Biofertilizers are substances containing variety of microbes having the capacity to enhance plant nutrient uptake by colonizing the rhizosphere and make the nutrients easily accessible to plant root hairs. In the study conducted by Bákonyi *et al.* (2013), they concluded that the usage of biofertilizers make germination more effective, thus significantly increasing the number of germinated seeds [30].

3.5. Relative Germination Percentage

The relative germination percentage is the determination of the viable seeds sown. It measures the total seeds germinated at the right conditions from the seed sown. Relative germination percentage (RGP) reflects germination behavior of seeds of a single batch subjected to different levels of treatment in contrast to the negative control plant. All levels of biofertilizer treatments indicated a decrease in relative germination percentage (**Figure 1**). Seeds subjected to 0.001% biofertilizer have the lowest decline of germination compared to the negative control plant, equivalent to only 4.17% (RGP = 95.83%). Those seeds applied with 10% biofertilizer have the highest reduction of germination percentage, equal to 86.9% (RGP = 13.10%).

As noted, biofertilizers can be good sources of nutrients such as Nitrogen, Phosphorous, Potassium, and total organic matter (TOM). During the early development of seedlings, a minimum amount of nutrients is required considering that seeds can initially utilize the nutrients within their system. Exposure of the seedlings to a higher concentration of nutrients at this stage can be detrimental to the development of the plant. Some studies showed that the increase in the dose of N in both the laboratory and field conditions decreased the germination rate of seedlings [31]. Statistical analysis proved that increasing the N dose

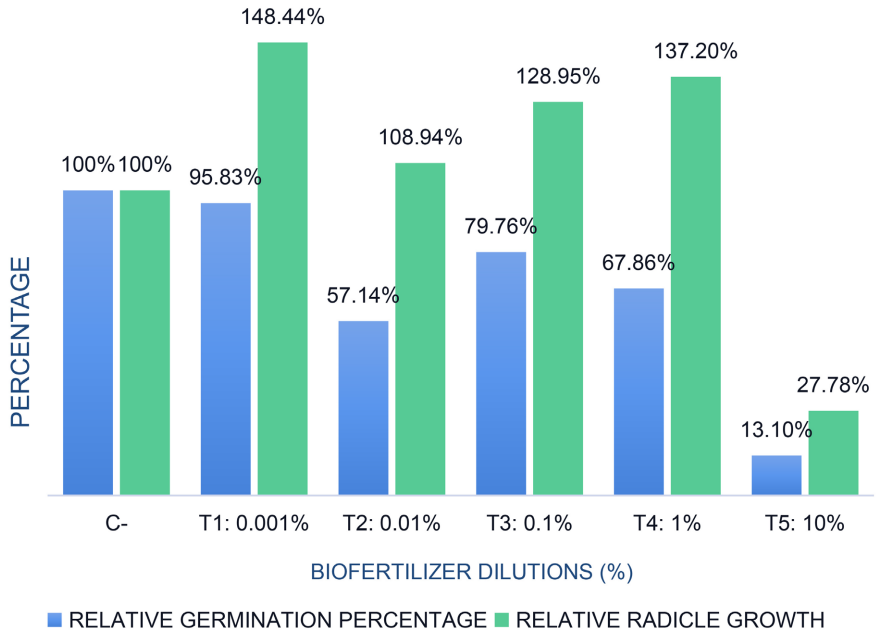

Figure 1. Relative germination percentage (RGP) and relative radicle length (RRL).

decreased the vigor value of wheat seeds, however, other authors found the opposite [32], claiming that the vigor of the seeds improved by increasing the N dose. According to the study of Aydinalp & Marinova (2009), there was a reduction in seed germination as metal concentrations in the growing media increased in general [33]. The major effects of heavy metals on seeds are manifested by overall abnormalities and decrease in germination, reduced root and shoot elongation, dry weight, total soluble protein level [34] [35], oxidative damage, membrane alteration, altered sugar and protein metabolisms, nutrient loss [24]-[36] all contributing to seed toxicity and productivity loss [34].

3.6. Relative Radicle Growth

Radicle growth is the primary root that emerges from the seeds upon germination. It grows downward in the soil and is used to seeds anchorage for a better crop stand. The relative radicle growth of seeds from different levels of biofertilizer versus the negative control plant reflects the viability of a population of seeds. All levels of biofertilizer dilutions showed increased radicle growth compared to the negative control solution except for the 10% biofertilizer dilution. Notably, the application of 0.1% biofertilizer dilution increased the efficiency and viability of the seeds by 48.44%, compared to the negative control which was the highest increase among the levels of biofertilizer. This was followed by seeds with 1% biofertilizer dilution with an increase of 37.20%. Seeds with 0.1% biofertilizer had a 28.95% increase and seeds applied with 0.01% dilution had an increase of 8.94%. Seeds that received the 10% biofertilizer showed an extremely high reduction in radicle growth, equivalent to 72.22% (RRG = 27.78%).

Though lettuce applied with 0.001% biofertilizer showed a slight decrease in

germination percentage (4.17%), it produced substantially longer radicle length in contrast to the negative control plant by 48.44%. On the other hand, the highest decline in germination percentage (RRG = 13.10%) and radicle length (RRL = 27.78%) were both observed from the lettuce applied with 10% biofertilizer. These findings suggest that the low number of lettuce seeds that were able to germinate correspondingly produced significantly shorter radicle length when applied with a high concentration of biofertilizer in contrast to the negative control plant. One observed properties of biofertilizers that may contribute to the significantly lower relative radicle length is their high level of pH.

During seed germination, alkalinity/salinity results in many disorders and metabolic changes, such as solute leakage, K^+ efflux, and α-amylase activity [37]. Firstly, it reduces moisture availability by inducing osmotic stress, creating nutrient imbalance and ionic toxicity [38]. Cell membranes are the hotspots for controlling solute's active and passive transfer and regulating plant nutrient uptake [39]. Alkali salts, such as $NaHCO_3$ and Na_2CO_3, are the primary ion sources found in saline soils; Na^+, K^+, Ca^{2+}, and Mg^{2+} are the main cations and $C1^-$, NO_3^-, HCO_3^-, CO_3^{2-}, and SO_4^{2-} are the main anions. Indeed, studies have confirmed that alkaline salts damage plants more than neutral salts [40] [41] [42] [43] as cited by Wang *et al.* (2022).

4. Conclusions

The biofertilizer produced from the bioreactor composting technology of Aklan State University is alkaline and has a nitrogen content of 30.2 percent OM, 1.51 percent nitrogen, 117 ppm phosphorus; and 2311 ppm potassium. Application of low levels of the bioreactor-produced biofertilizer affected the hypocotyl length and germination percentage of the lettuce seeds but not its radicle length. Application of at least 10% bioreactor-produced biofertilizer can presumptively lead to phytotoxicity.

Based on the study conducted, it is recommended to have a thorough chemical analysis of the nutrient content, heavy metal levels, and even substance of emerging concerns of the bioreactor-produced biofertilizer to assess its growth effects and the safety of high-value crops. Studies may also be conducted on the effects of the bioreactor-produced biofertilizer on the yield and nutritional quality of other high-value crops. Better and more effective formulations of biofertilizers as a nutrient start-up may be considered in future research.

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Conflicts of Interest

All authors declare no conflicts of interest in this paper.

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