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Bacterial Isolates as Potential Inoculants for Organic Kale (*Brassica oleracea* L.) Production in Nueva Ecija

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Abstract

Organic agriculture greatly depends on soil microorganisms to produce the optimum amount of nutrients available for plant use. This study was conducted to evaluate the yield and yield components of organic Kale (*Brassica oleracea* L.) in response to bacterial inoculants. The experiment was conducted in a ten-year-old organic farm at Ramon Magsaysay-Center for Agricultural Resources and Environment Studies, Central Luzon State University (RM-CARES-CLSU), Nueva Ecija. All treatments used 5t/ha recommended rate of organic fertilizer (RROF), varying only with inoculants *Enterobacter aerogenes*, *Enterobacter cloacea*, and *Bacillus subtilis*. Plants applied with organic fertilizer inoculated with *E. cloacea* increased yield by 27% compared to plants applied with solely organic fertilizer. *E. cloacea* was also the best inoculant for organic fertilizer based on yield weight per plot and per hectare and the number of survived plants. It is also the most efficient bacterial inoculant for organic fertilizer to enhance the availability of nutrients for optimum organic Kale production. *E. aerogenes*, on the other hand, stimulated the nitrogen uptake significantly, and although comparable with other treatments, it can induce high dry matter yield and high nitrogen and potassium content of the plant.

Introduction

Organic farming is expanding worldwide and is supported by regional and national government. However, this system is facing many problems mostly related to nutrient supply and disease control limitations. According to the Research Institute of Organic Agriculture FiBL latest survey, the organic farmland, the number of organic producers, and organic retail sales continue to grow. Currently, 1.4 % of the world's agricultural land is organic (Willer et al., 2019). Plants are always in a symbiotic relationship with soil microbes (bacteria and fungi) during their growth and development. The free-living soil microorganisms inhabiting the rhizosphere of many plant species have diverse beneficial effects on the host plants (Raza et al., 2016a,b) through different mechanisms such as nitrogen fixation, nodulation, and phosphorus solubilization. Microorganisms are responsible for releasing nutrients from organic matter and can positively influence plant growth. Progress in developing successful technologies through research and development on plant growth-promoting bacteria (PGPB) is gaining influence. PGPB is instrumental to crucial processes that ensure the stability and productivity of agroecosystems towards an ideal agricultural system.

Variability in the performance of PGPB may be due to various environmental factors that affect their growth which exerts their effects on plants. These factors include climate, weather conditions, and soil characteristics or the composition or activity of the indigenous microbial flora of the soil (Gupta et al., 2015). Moreover, the impact of PGPB positively lies in its influence on crop productivity and the functional ecosystem. The use of PGPB as inoculants offer an attractive way to replace chemical fertilizers and pesticides, especially in organic farming, where chemicals are not recommended. Indiscriminate use of synthetic fertilizers, particularly nitrogen and phosphorus, has led to substantial soil pollution by reducing pH and exchangeable bases; thus, making these nutrients unavailable to crops, resulting in loss of productivity (Gupta et al., 2015).

Plant growth-promoting bacteria facilitate plant growth directly by either assisting in resource acquisition (nitrogen, phosphorus, and essential minerals) or modulating plant hormone levels and indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the form of biocontrol agents. Furthermore, plant growth-promoting bacteria can be used to reduce chemical fertilizer application, which is economically and environmentally beneficial for plant production. They are also recognized as the best soil and crop management practice to achieve better soil fertility for more sustainable agriculture (Maheshwari et al., 2012).

In this study, kale, a member of the Brassica family, was used as a test plant. It is rich in vitamins A and C and super-loaded with vitamin K. It grows best in full sun and cool, moist soil enriched with organic compost. Kale is used as salads or simply as a garnish. It is not popular, but accordingly, it has components that can reduce cancer risk and is the most nutrient-dense food in existence (Gunnars, 2018). Plant growth in the organic system greatly depends on organic matter inputs and the functional activities of soil microorganisms (Tu et al., 2006). This study evaluated the growth-promoting effects of three bacterial inoculants, namely: *Enterobacter aerogenes, Enterobacter cloaceae*, and *Bacillus subtilis*, on Kale production. A field trial using mixed formulations of bacterial inoculants has been reported to increase yield and decrease plant disease incidence. The general objective of this study is to evaluate the growth and yield performance of organically grown Kale plants as influenced by plant growth-promoting bacteria (PGPB) under field conditions Specifically, the study was conducted to assess the plant growth promoting effects of bacterial inoculants; evaluate the yield and yield components of kale in response to applied inoculants; determine the inoculants' effect on kale plant nutrient uptake.

Methodology

The growth promotion of the kale experiment was conducted in the experimental area of RM-CARES. Var. Toscana was used with characteristics of dwarf green and curled dark green leaves and a slightly crinkled appearance. The agronomic characteristics and nutrient composition of the plant were evaluated 45 days after transplanting. Kale prefers well-drained sandy loam soils with good organic matter and soil pH of 5.5 to 6.5 (slightly acidic). The following treatments were used:

T1 - Control, recommended rate of organic fertilizer (RROF) at 5t/ha, no PGPB application)

T2 - RROF at 5t/ha, inoculated with A (*Enterobacter aerogenes*)

T3 - RROF at 5t/ha, inoculated with B (*Enterobacter cloacea*)

T4 - RROF at 5t/ha, inoculated with C (*Bacillus subtilis*)

Sources of PGPB Isolates

The pure culture isolates of PGPB were provided by RM-CARES, CLSU. Accordingly, these PGPB were isolated from different animal manure such as carabao (*E. aerogenes*), rabbit (*E. cloacea*), and goat (*B. subtilis*). Isolates were sub-cultured and mass-produced in rice bran broth.

Bacterial Inoculants

These three PGPB isolates were used as inoculants in this study: A (*Enterobacter aerogenes*), B (*Enterobacter cloaceae*), and C (*Bacillus *subtilis*) for the evaluation of plant growth promotion. Each bacterium was streaked on plated nutrient agar (NA) and incubated at room temperature for five days. PGPB cultures were harvested in sterile distilled water (SDW) and set aside to be added to the PSB-culture rice bran broth.

Preparation of Mother Inoculant

Twenty-four hours old pure culture of phosphorus-solubilizing bacteria (PSB) was scraped using sterilized distilled water. Scraped culture served as a mother culture. Meanwhile, rice bran broth, dispensed in 250ml flasks were sterilized and then inoculated with 1 ml each of the prepared mother cultures. The mother inoculant was shaken for 5 days using a rotary shaker at 120 rpm for mass production.

Viable Cell Count of Inoculants

One (1) ml from 10-5 dilution was plated in National Botanical Research Institute's Phosphate (NBRIP) agar with three replicates. The plates were incubated at room temperature for 48-72 hrs. Colonies grown in media were counted for colony-forming units (cfu). Viable cell count was determined using hemocytometer.

Application of Prepared Inoculant in the Experimental Field

Bacterial inoculants were incorporated into the organic fertilizer at 200ml of bacterial broth per 2kg of organic fertilizer per plot as per recommendation and incubated for 7 days.

Land Preparation and Soil Sample Collection

An initial soil sample was collected in the experimental area after cultivation. The area was divided into four blocks where each block was subdivided into four plots measuring 2m x 2m.

Experimental Layout

The different treatments were randomly assigned in each block following the Randomized Complete Block Design (RCBD) (Figure 1).

Cultural Management Practices

Seedling Production

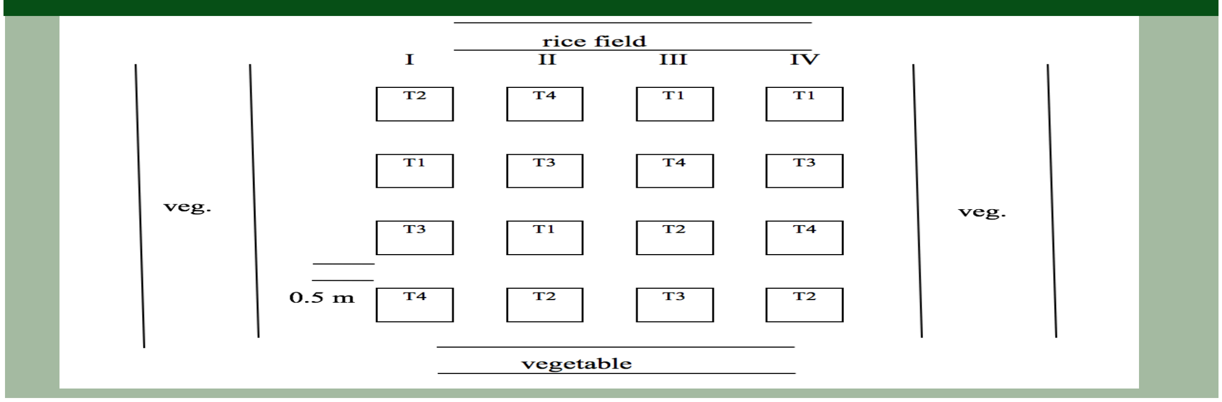
The seeds were sown 1 inch deep in seedling trays using 1:1:1 ratio (organic fertilizer, carbonized rice hull, and soil) of the potting media following the protocol of RM-CARES for seedling production. Thinning was done after a week, and the soil was kept evenly moistened or at field capacity throughout its growth period. Seedlings were kept away fromdirect sunlight by covering them with a nylon net to avoid the burning of leaves.

Transplanting

Seedlings were grown in July under a typical greenhouse and were transplanted when they already had 3–4 well-developed leaves. Seedlings were transplanted at 25cm between rows and 25cm between hills at one seedling per hill. Proper cultural management practices, such as watering, weeding, etc. were strictly

Figure 1

Field Layout



implemented to maintain a good plant stand.

Fertilization and Water Management

Organic fertilizer was applied as basal during transplanting at 5 tons/hectare recommendation. Inoculant was applied as drench: 10% of the RROF is the equivalent volume (L) of inoculant applied. A total of 200 ml inoculant, inoculated with 2kgs. organic fertilizer per plot was applied. The plants were irrigated immediately after transplanting in the field using the manual sprinkler and as needed.

Weeding and Pest and Disease Management

Weeds were removed manually near the base of the plants to minimize nutrient competition, but some along the rows were not completely removed to protect the soil and encourage beneficial insects as part of organic farming (PCAARRD, 2015). For insect pests and diseases management, oriental herbal nutrient (OHN) was used as an organic pesticide. OHN composition includes turmeric, chili pepper, and molasses at 1:1:1 ratio of 1kg mixture. The ingredients were finely chopped, mixed, and fermented for one week.

Harvesting

Kale plants were harvested 30 days after transplanting by cutting the aboveground parts. Tagged plant samples were harvested separately for the needed data.

Data Gathered

Climatic Data

Mean data on weekly rainfall and temperature during the growing period from planting to harvesting were collected from the PAG-ASA station of CLSU.

Agronomic Plant Characteristics

Ten tagged sample plants were used for gathering the following data:

Weekly growth rate. The height of sample plants in each plot was measured from the base of the plant to the tip of the longest leaf at weekly intervals starting from 7 days after transplanting (DAT). The formula used was:

WGR = FH-IH/number of weeks-1

where:

WGR- weekly growth rate

FH- final height; number of plants at weekly interval/number of weeks

IH- initial height

Number and weight of the marketable leaves per plot. The total number of marketable leaves was taken from the sample plants. The leaves are considered marketable when it reaches the proper maturity at 60 days from seed emergence.

Computed marketable yield per hectare. The total weight of all the marketable plants per plot was converted on a per hectare basis using the formula:

$$\text{Computed yield per hectare} = \frac{(\text{Yield per plot} * 10000)}{\text{net area}}$$

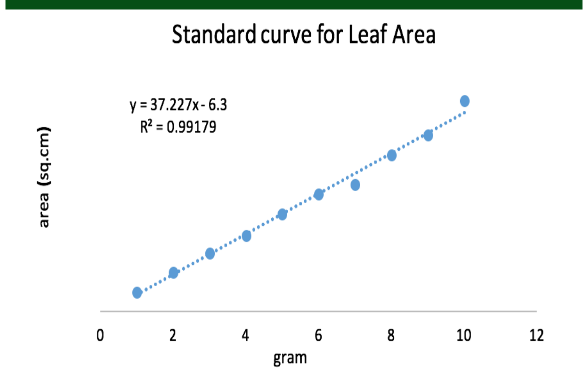
Leaf area. The leaf area was regressed on leaf mass using a linear model (Figure 2) following relationship: y = mx + b or Leaf area = slope (leaf mass) + y-intercept. The procedure used in determining leaf area was modified from the procedure of Awal et al. (2004).

Plant height at harvest. Plant height was measured from the base of the stem to the tip of the highest leaf. Height at harvest is an important parameter used to compare the growth of the plants according to treatments.

Stand count at harvest. Stand count at

Figure 2

Linear Model on Leaf Area



harvest is the total number of plants harvested per plot. The number of healthy live plants were counted in the measured area. These were done multiple times to obtain the average count. Average number of plants were multiplied by 4 to obtain the per plot analysis.

Nutrient Composition of the Plant

Plant tissues of tagged samples/treatment were submitted to the Regional Soils Laboratory, San Fernando City, Pampanga for N, P, K analyses. All samples were collected and prepared for the following analysis:

Total N:	Kjeldahl method
Phosphorus (P):	Vanadomolybdate method
Potassium (K):	Flame photometer
Nutrient Uptake:	This was computed using the following formula:

Nutrient uptake = dry matter yield x (% nutrient content of the plant/100)

Soil Chemical Analysis

Soil analysis was done before planting and after harvesting. The data gathered and methods used were:

Soil pH:	Potentiometric method using 1:1 soil-water ratio
% Organic Matter:	Walkey-black method
Total N:	Based on OM analysis
Available P:	Olsen's method
Exchangeable K:	Cold sulfuric extraction method

Data Analysis

Collected data were organized, tabulated, and subjected to Analysis of Variance (ANOVA) for single factor Randomized Complete Block Design (RCBD) using IRRI Statistical Tool for Agricultural Research or IRRI STAR (version 2.0.1) software. The analysis considered 5% level of significance. Means were compared using Tukey's HSD (honestly significant difference) test.

Results and Discussion

Physical and Chemical Properties of the Soil

Table 1 shows the physical and chemical properties of soil collected prior to the experiment. Initially, the soil had a pH value of 7.19, which is considered neutral ranging from 6.6-7.3 (State University of New York, 2015). The content of primary macronutrients nitrogen, phosphorus, and potassium was noted to be low (0.07%), high (42.62ppm), and low (110ppm), respectively. The soil had percentage sand of 25%, 30.52% clay, and 43.95% silt, which is under the texture classification of sandy clay loam. The organicmatter of the soil increased from 1.4% to 3.99% at harvest. These chemical properties of soil favors the process in the production of crops indicating good soil fertility status.

Cell Count of Inoculants

Table 2 shows the initial count of the inoculants applied in the organic fertilizer. *E. cloacea* has the highest count with 4905cfu/250ml of the inoculants, followed by

Table 1

Initial and Final Physical and Chemical Properties of Soil in the Experimental Area

Soil Properties	Initial	Final
1. Total Nitrogen, %	0.07	0.19
2. Organic Matter (%)	1.4	3.99
3. Available Phosphorus, ppm	42.62	-
4. Exchangeable Potassiu, ppm	110.00	-
5. pH	7.9	6.72
6. Texture	Sandy Clay Loam	Sandy Clay Loam

Table 2

Initial Count of Bacterial Isolates as Inoculants

Microorganism	cfu/250ml
1. *E. aerogenes*	138
2. *E. cloacea*	4905
3. *B. subtilis*	2646

B. subtilis with 2646cfu/250ml. *E. aerogenes* has the least colony with 138cfu/250ml among the three isolates.

Climatic Data

Heavy rainfall was observed in the third week of July, while only minimal rainfall was noticed in the last week of August (Table 3). Total rainfall (1362mm) contributed to the good growth of kale plants. According to Charwizira, E. et al., 2014, the optimum rainfall for kale is 1,500mm.

Temperature ranges from 23-24°C in the months of July-September during the conduct of the experiment with an average of 24°C (Table 4). Greenlife Crop Protection Africa [GCPA] (2014) reported that the temperature requirement of kale ranges from 16-21°C.

Agronomic Characteristics of the Plant

An increasing trend of plant height (Figure 3) was observed during the 6-month growing period of kale, however, the height of the plants among the treatments did not differ significantly (Table 5 and Figure 3). Organically grown kale had an average height of 14.89cm.

Table 5 presents the average yield per plot. Kale applied with organic fertilizer inoculated with *E. cloacea* significantly showed the highest weight of 124.75g, followed by *E. aerogenes* (120.6) and *B. subtilis* with 101.78g but comparable to the control plants. A similar trend of data was obtained from the yield at harvest per hectare basis. As to leaf area and plant height at harvest, all plants, regardless of treatment, are comparable (Table 5). In summary, *E. cloacea*, significantly outranked all treatments and stands out among inoculants used in terms of weight of harvest per plot and hectare and the number of plants that survived in the field (stand count).

Table 3

The Rainfall (mm) Amount During the Conduct of the Study

Week	July	August	September
1	57.9	174.8	198
2	62.7	152.0	114
3	395.2	78.5	111
4	213.9	35.4	20
Total	729.7	440.7	443

Table 4

Minimum Temperature (°C) During the Conduct of the Study

Week	July	August	September
1	24.5	24.1	24.0
2	24.3	24.1	22.4
3	23.8	23.9	23.2
4	23.6	23.0	23.6
Mean	24.1	23.8	23.3

The application of organic fertilizer inoculated with bacterial isolates improved the yield of Kale compared to the control plants. Plants applied with organic fertilizer inoculated with *E. cloacea* obtained the highest yield with 311.88kg/ha. This yield is 27% higher compared to plants applied with organic fertilizer alone (control). The next highest yield was observed from plants inoculated with *E. aerogenes* with a 23% increase. However, plants treated with *Bacillus subtilis* only had a 4% higher yield compared to the control. In California, Kale produced 4.17tons/ha by conventional farming (Monterey County Agricultural Commissioner's Office, 2017). However, the researchers did not find any literature investigating organic kale production. Kale is typically a cool weather plant (Thomas, 2018); hence, the tropical condition in the study site may have adversely affected its mean stand and yield.

Table 5 further shows that the survival performance of kale plants applied with inoculant B (*E. cloacea*) was significantly higher compared with the other two inoculants and

Figure 3

Weekly Height of Kale as Influenced by Different Bacterial Inoculants

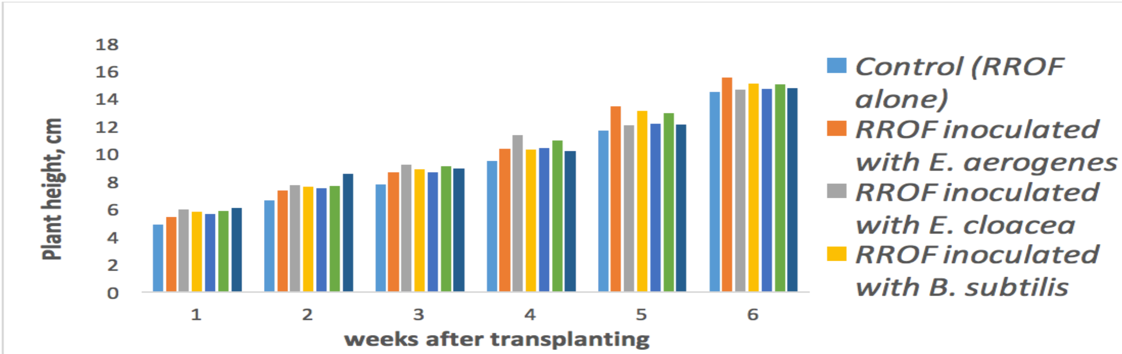


Table 5

Agronomic Characteristics of Kale as Influenced by Different Bacterial Isolates as Potential Inoculant

Treatment	Height at Harvest (cm)	Average Growth Rate (cm/wk)	Leaf area (cm^2)	Weight per plot (g)	Yield at harvest (kg/ha)	Stand Count
1. Control (RROF alone)	14.49	1.65^{ab}	10.43	97.95^{c}	244.88^{c}	32^{b}
2. RROF inoculated with *E. aerogenes*	15.53	1.82^{a}	12.25	120.6^{ab}	301.5^{ab}	40^{ab}
3. RROF inoculated with *E. cloacea*	14.64	1.66^{ab}	12.48	124.75^{a}	311.88^{a}	47^{a}
4. RROF inoculated with *B. subtilis*	15.07	1.68^{ab}	10.85	101.78^{bc}	254.44^{bc}	41^{ab}

(Means with the same letter indicates significantly the same)

without inoculant. The increase in kale yield with inoculation of *E. cloacea* is probably due to the higher count of its colony-forming unit, as plants are always associated with soil microbes (bacteria and fungi) during their growth and development. This association through symbiosis could contribute to the favorable nutrient chemical reaction within the soil ecosystem. Plants absorb nitrogen from the soil in the form of nitrate (NO_3^-) and ammonium (NH_4^+), which are essential nutrients for growth and development. Biological N_2-fixing PGPB in crops revitalizes growth-promoting activity, disease management and maintains the nitrogen level in agricultural soil (Damam et al., 2016).

Findings from this study validate the fact that *E. cloacea* is an ammonia producer. The bacterium is also a phosphate solubilizer and makes nitrogen more readily available for plant use. As reported, *E. cloacae* recovered from the soybean rhizosphere significantly enhanced the growth of soybean and wheat (Ramesh et al., 2014). Also, this bacterium is a starch degrader and phosphorus-solubilizer and thus, can augment energy production in kale (Ramesh et al., 2014).

Furthermore, Deepta et al. (2010) commented that *Enterobacter* functions in three ways: synthesizing particular compounds for the plants, facilitating the uptake of certain nutrients from the soil, and lessening or preventing the plants from diseases.

Dry Matter Yield, Nutrient Concentration, and Nutrient Uptake of the Plant

Results showed that the treatments applied with inoculants had no effect on the dry matter yield of kale as well as on the nutrient (N, P, and K) contents of the crop (Table 6). Plant uptake of potassium and phosphorus were also comparable. From among the macro elements, only nitrogen uptake was shown significantly affected by the inoculant used, where *E. aerogenes* having 3.97g/plant outranked the other two inoculants withcomparable efficiency in increasing the N uptake of kale. Numerically, *E. aerogenes* gave high P and K with 0.679 and 2.13g/plant, respectively. The decreasing order of the macronutrient contents in kale leaves was: N>K>Ca>S>P>Mg, in which nitrogen is the most extracted nutrient, followed by potassium and calcium. This result differs from previous studies conducted with brassicas, in which greater potassium extractions were observed in kale leaves hence nutrient uptake of the plant (Aquino et al., 2009; Correa et al., 2013).

Analysis of Soil After Harvest

Soil pH, percent organic matter, and percent nitrogen at harvest are comparable among the treatments. As shown in Table 7, the pH of the soil ranged from 6.68 to 6.76, which falls under slightly acidic conditions. It was observed that soil pH slightly reduced at harvest from pH 7.19 to 6.72. However, the total average nitrogen increased from 0.07 to 0.2 percent. *E. cloacea* obtained the highest final N concentration in the soil numerically. According to Khalifa et al., 2016, it exhibited traits for plant-growth-promoting and could be developed as an eco-friendly biofertilizer for *P. sativum* and probably for other

Table 6

Analysis of the Dry Matter Yield, Nutrient Content and Nutrient Uptake of Kale Harvest as Influenced by Different Bacterial Isolates as Potential Inoculant

	Dry Matter Yield (g)	Total Nutrient of the Plant (%)			Nutrient Uptake of the Plant (g/plant)		
		N	P	K	N	P	K
1. Control	66.24	4.56	0.89	2.39	3.07[b]	0.609	1.65
2. RROF inoculated with *E. aerogenes*	72.66	5.21	0.89	2.78	3.97[a]	0.679	2.13
3. RROF inoculated with *E. cloacea*	65.39	4.30	0.96	2.72	2.90[b]	0.652	1.82
4. RROF inoculated with *B. subtilis*	66.35	4.48	0.89	2.55	3.04[b]	0.609	1.74

(Means with the same letter indicates significantly the same)

Table 7

Chemical Analysis of the Soil After Harvest as Influenced by Different Bacterial Isolate as Potential Inoculant

Treatments	Ph	OM (%)	N (%)
Intial	7.19	1.4	0.07
Final	6.73	3.98	0.20
Control	6.72	3.84	0.19
RROF inoculated with *E. aerogenes*	6.7	4.08	0.2
RROF inoculated with *E. cloacea*	6.73	4.20	0.21
RROF inoculated with *B. subtilis*	6.76	3.81	0.19

(RROF-Recommended Rate of Organic Fertilizer)

important plant species in the future. Thus, plants inoculated with this isolate increased the yield of organic kale. Added plant growth-promoting bacteria could have enhanced N availability (Khalifa et al., 2016).

Conclusions

The research proved the effectiveness of *E. aerogenes*, *E. cloacea* and *B. subtilis* as potential bacterial inoculants of organic fertilizer for organic farming. These isolates increased the growth and yield performance of organic kale. *E. cloacea* significantly better than *E. aerogenes* and *B. subtilis* in terms of yield weight per plot and per hectare, and plant survival in the field. Further, plants applied with organic fertilizer inoculated with *E. cloacea* had bigger leaves and higher phosphorus content numerically, but comparable to other inoculants used and the uninoculated. On the other hand, *E. aerogenes*, as inoculant of organic fertilizer, significantly increased the plant growth rate and high nitrogen uptake of the plants. Although comparable with the other two inoculants, *E. aerogenes* stimulates better dry matter yield, higher nitrogen in plant tissues, increased phosphorus, and potassium uptake, and induces plants to grow taller. This research also revealed that increased productivity of kale could be attained using either organic fertilizer alone or inoculated with *B. subtilis*. Still, better yield and enhanced morphological traits of plants can be achieved when organic fertilizer is inoculated with *E. cloacea* and *E. aerogenes* bacteria for added product value.

Recommendations

Based on the study results, the application of organic fertilizer inoculated with *Enterobacter spp.* is recommended for improving the growth and yield performance of kale in organic farming system.

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