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Assessing The Efficacy Of Microbial Bioformulations In Enhancing Biomass And Defence Related Compounds Of Spinach (*Spinacia Oleracea L.*)

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ABSTRACT

Large amount of chemical fertilizers are commonly used in agricultural sector for enhancing the crop productivity to meet the global food demand which also plays a major role to deteriorate environment as well as human health. The use of microbial bioformulations for crop cultivation particularly raw vegetables is a good practice considering human health and environmental sustainability. The present study was carried out to analyze the effect of microbial bioformulations on the plant growth, biochemical profile (chlorophyll, carotenoids and protein content) and defence related compounds (flavonoids and phenolic content) of spinach. In this study, a bioformulation consisting consortium of four bacterial strains (*Bacillus filamentosus* RS3B, *Bacillus pseudomycoides* RS6B, *Bacillus paramycoides* RPB3, *Alcaligenes faecalis* RS10B) in two carrier material viz. Bentonite and Talc+Gluten named as T1 (Bentonite+microbial consortium (MC)) and T2 (talc+gluten +MC) along with inoculated broth medium(T3) uninoculated carriers (T4 and T5), chemical fertilizer (T6) and non-inoculated soil (T7) were applied for the cultivation of spinach crop. The results depicted that inoculation of bioformulation containing MC considerably increased the concentration of total phenolics in both bentonite based (T1) and talc+gluten based bioformulation(T2) by 48.7% and 100.43%, the flavonoids were also increased by 235.02 and 310.46%, total chlorophyll 71.8-162%, carotenoids 156-209.5%, protein 35.2-87% and dry biomass by 275-703% over untreated control. Therefore, our findings indicate, that carrier-based bioformulations (T1 and T2) are excellent plant growth regulators, which increase the yield and overall quality of spinach, an iron rich common vegetable.

Keywords: *biochemical, bioformulation, defence related, flavonoids, phenolics*.

Introduction

Human innate immunity against chronic diseases is increased by the consumption of leafy vegetables and fruits (Bagchi *et al*. 2003). Agricultural practices have a major impact on their nutritive value and yield. Overuse of synthetic chemicals to enhance the yield and quality of plant species could become an imminence to ecosystem and degrade environment (Wang *et al*., 2018). Long term use of synthetic fertilizers for crop improvement causes water resource degradation and deteriorate human health (Riahi *et al*., 2020). Biofertilization is a good alternative to chemical fertilizer towards improving the yield with quality of the leafy vegetables (Fekry and Nawar 2017). Spinach (*Spinacia oleracea* L.) is one among the extensively consumed leafy vegetable which is cheap, readily available, and a rich source of vitamins, amino acids, and minerals (Fe and Zn) as well as phytochemicals like fibres and polyphenols (Yoon *et al*., 2017; Parwada *et al*., 2020). The crop is a rich source of defence related compounds like flavonoids and and phenolic (Metha and Belemkar 2014; Khalid *et al*., 2017). Phenolic compounds attract insects for seed dispersion, pollination and act as a

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natural resistance against harmful microorganisms and even controlling plant hormones (Khanam *et al*., 2012). Flavonoids play many important roles in biological functions like the prevention of oxidation of low-density lipoprotein by Quercetin, (Cartea, *et al*., 2011). Spinach also contains high biological value compounds especially Beta-carotene and lutein which both have antioxidant and anticancer properties (Toledo *et al*., 2003). Traditionally spinach was consumed cooked, but now it is also eaten in raw form as salads, including that sold ready-to-eat, which contain smaller and more tender leaves than those used for cooking (Gómez *et al*., 2018).

The type of fertilizer used, growing condition and nutritional value determine the quality of fresh vegetables (Gómez *et al*., 2018). Due to its theraputive properties and increased raw consumption, it could be considered an applicant for cultivation with microbe-based bioformulation (Cakmakci *et al.,* 2007). Microbial inoculation for improved crop productivity of leafy vegetables is not a new method (Bashan *et al*., (2014). Chamangasht *et al*., (2012) also reported increased lettuce growth (plant height, number of leaves, leaf area) and yield upon inoculation of bioformulation as compared to control. However single strain inoculation did not show promising results under field conditions, due to the poor flexibility of inoculants to changing environmental conditions (Mishra *et al*., 2021). Hu *et al*., 2017 also reported that multistrain inoculants improved plant growth more effectively compared to a single strain. The main issue with the microbial application is to maintain a suitable number of inoculants in the field for a longer period. Hence, inoculation of microbes in the form of carrier-based formulation could be a promising approach to achieve the benefits of microbial inoculants (Parveen *et al*., 2022; Maheshwari *et al*., 2015). Carriers used for bioformulation must have good moisture retaining capacity, be well-buffered, environmentally safe, convenient to use, and economical (Zayed, 2016; Bhattacharyya *et al*., 2020). The carrier must also possess the ability to support the target organism for growth to sustain a desirable population over an acceptable period (Macik *et al*., 2020). For the commercialization of microbial inoculants, they must be developed into a carrier-based bioformulation (Aamir *et al*., 2020). Liquid bioformulation is commonly used for plant growth and provides a microenvironment to microbes, but it can reduce viability and multiplication rates as compared to solid carriers (Berninger *et al*., 2018). Maggio *et al*. (2013) reported that fertilization dose and farming system (organic or conventional) are the important factors in determining the content of antioxidant compounds in different crops. Cultivation of tomato and black pepper under organic conditions with the application of plant growth promoting bacteria (*B. subtilis* and *B. cereus*) have been recorded with more flavonoid contents than those cultivated by traditional farming (Hallmann, 2012; Babu *et al*., 2015). Khalid *et al*., 2017 also reported enhanced content of flavonoids and phenolics in spinach treated with microbial inoculants as compared to control plants. Hence, in this study, Bentonite and talc+gluten-based bioformulation consisting microbial consortium were assessed to show their effect on the growth, biochemical profile, phenolics and flavonoid content of spinach as compared to controls (inoculated broth, uninoculated carriers, chemical fertilizer, uninoculated soil, etc.).

2. Methodology

2.1. Microbial strains and carrier material

In this study, four pre isolated and characterized as good plant growth promoting bacterial (PGPB) stains (*Bacillus filamentosus* RS3B, *Bacillus pseudomycoides* RS6B, *Bacillus paramycoides* RPB3, *Alcaligenes faecalis* RS10B, (Maddhesiya *et al*., 2020) were procured

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from the departmental laboratory (research group of Prof Rana Pratap Singh) of school for earth and environmental sciences, BBA University, Lucknow, India. The carrier materials i.e., bentonite, Talc and gluten for the preparation of bioformulation were purchased from G-1082 basement Sushant Lok-2 sector 57 Gurugram Haryana, 122011 IN and HiMedia Lab Pvt. Ltd, Mumbai, India.

The compatibility of bacterial strains was tested based on the formation of overlapping or inhibition zone between the paired culture by following the method of James and Mathew, (2017). A consortium (MC) of compatible microbial strain were prepared by following the method described by Mishra *et. al.,* (2021).

In order to evaluate the bioformulation's effect on spinach growth, a pot study was conducted in which two carrier-based bioformulation T1 (bentonite+MC) and T2 (talc+gluten (3:1 ratio) +MC) along with control treatments T3 (MC in Nutrient Broth), T4 (bentonite), T5 (talc+gluten), T6 (chemical fertilizer) and T7 (soil only) were used.

2.2. Bioformulation preparation and its application in the Pot experiment

Firstly, the carrier materials (Bentonite and Talc+Gluten (3:1 ratio) were dried, ground, sieved and sterilized by following the method described by Tripathi *et al.,* (2014). The carrier-based granular bio formulations were prepared by using freshly prepared microbial consortium ($1x10^8$CFU) in 2:1 ratio (100g carrier and 50 ml bioformulation) following the methods of Kumar *et al*., (2014). The granular bioformulations were spread in trays and then sealed in low-density polythene bags (Maheshwari *et. al*., 2015).

The experiment was conducted in triplicates in earthen pots, in the month of November -December for two consecutive years (2019 and 2020). The pots were arranged randomly in three blocks under open greenhouse conditions (Inostroza *et. al*. 2016) at Environmental Science Research Station, Babasaheb Bhimrao Ambedkar University, Lucknow, India (26° 72 E, 80° 85 N). Sandy loam soil, (pH 6.5, EC 4.6 ds/m, organic matter 0.65%, available nitrogen 15.00mg/kg, available potassium 92.88mg/kg, available phosphorus 1.48mg/kg, Alkaline phosphatase 1.17µg/g, MBC 0.64µg/g) was collected from the non-agricultural land of BBAU campus. The soil was mixed thoroughly before the experiment and 7 kg of non-autoclaved soil was filled in each earthen pot (45x15x15cm). The bioformulation (0.5%/100g of soil) was added once into the soil at the time of seed sowing. Seeds of spinach were sterilized with 3% hydrogen peroxide solution before sowing (Bakhsh *et al*., 2016). In this study 10 seeds per pot were sowed and 5 saplings per pot were maintained by thinning after germination for homogeneity. The pots were irrigated on alternate days, and weed removal was done when required. The plants were harvested 60 days after sowing (DAS), rinsed with tap water and were assessed for plant above and below ground length and biomass following the method of Seymen (2021). The uniform leaf area index was collected from each treatment by using Image J software (Wayne Rasband NIH, http://imagej.nih.gov/ij/index.html) Vitale *et al.*, (2020).

2.3. Biochemical analysis

Estimation of photosynthetic pigments (chlorophyll and carotenoids) and protein content was done by following the methods described by Mastan, *et al*, (2019). Photosynthetic Pigments extraction from the sample was done by crushing the sample with 100% acetone (ice-cold) using mortar and pestle and centrifugation at 5,000 rpm for 5 min. The absorbance of supernatants was quantified by a spectrophotometer at, 645 and 662nm (chlorophyll) 470nm(carotenoids). Protein content estimation was done by following the method of Lowry *et al.,* (1951).

2.4. Phytochemical analysis

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The leaves of spinach were collected and dried in a hot air oven at 70°C for 48 hours. Dried leaves were powdered with the help of a grinder. Methanolic extracts of the dried leaf powder were prepared by following the method by Khan *et al*. (2012). For determination of total phenolic content, in a 10 ml of volumetric flask 1 mL of plant sample extract (methanolic extract) was added and incubated for 2-3minutes then 5 mL Folin–Ciocalteau reagent (1:10 H_2O) and4 ml of Na_2Co_3(7.5% w/v) was added to it. Gallic acid (Hi-Media) was used as standard (0.10, 0.15, 0.20, 0.25, 0.30, 0.35,0.40 and 0.45 mg/L) to determine total phenolic. After keeping in dark for 30 min, absorbance was recorded at 765 nm using a reagent blank on spectrophotometer (Khan *et al*., 2012). Content of total phenolics was expressed as gallic acid equivalent (GAE) in milligram per gram plant dry weight basis.

Total flavonoid content (TFC) was assessed by following aluminium trichloride colorimetric assay described by Khan *et al.,* (2012). For flavonoid determination, in a 10ml of volumetric flask, 1ml of sample or catechin standard solution (20, 40, 60, 80 and 100 mg/L), 4ml of distilled water and 0.3 ml of 5% $NaNO_2$ was added and kept undisturbed for 5 minutes. After 5 min, 0.3 ml of $AlCl_3$ (10%) was added and after 1minute, 2ml of 1 M NaOH was added, finally the total volume was made up to 10 ml using deionized distilled water (DDW). The reaction mixture was mixed properly and the absorbance was recorded at 510 nm using a reagent blank. Amount of total flavonoids content was expressed milligram per gram dry plant materials as catechin equivalent.

2.5. Soil sampling and analysis

The soil was collected from each earthen pot at the depth of 10 cm pre- and post-experiment. Collected samples were sieved (2 mm sieve) to remove concrete and other debris and homogenized manually. For physicochemical analysis, the soil was oven dried at 65ºC temperature (for 24 h), while fresh and moist soil was used to analyze microbial and enzymatic activities. The physicochemical properties including pH, electrical conductivity, and available nitrogen (AN), phosphorus (AP) potassium (AK), organic carbon, alkaline phosphatse (AlkP) microbial biomass carbon (MBC) of the soil were estimated by following the standard methods of Jackson (1973). Rhizospheric colonization (CFU/g of root) was checked at 30 and 60 days after sowing (DAS)by following Cavaglieri *et al*. (2009). Plants were harvested from the pots. The soil adhering with the roots was removed by washing, and then blended, weighed, and re-suspended in distilled water. The soil suspension was then serially diluted and plated on respective agar media (Nutrient agar for bacteria and Potato dextrose agar for fungi) (Hi-media) and CFU per gram of root were estimated (Fatima and Arora 2021).

2.6. Statistical analysis: Statistical analysis of the data was done using MS Excel and IBM SPSS statistics 20. Data were analyzed by one-way analysis of variance, and mean values were compared using Duncan's multiple range test ($P<0.05$).

3. Results and discussion

Based on microbial compatibility and good PGP traits four bacterial strains were selected to design a consortium using various carrier material. Bioformulations application to spinach helped to enhance the quality and growth of plants and also improved soil fertility.

3.1. Effect of experimental treatments on plant growth and photosynthetic activity

After two months of spinach cultivation, the obtained results showed that the applied bio formulations (T1 and T2) have highest efficacy to enhance spinach growth and productivity. The results revealed that, transplants subjected to different carrier-based bioformulation exhibited a remarkable visual increase in their shoot and root length as compared to the non-

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inoculated plants (fig. 1 and fig. 2). Inoculation of carrier-based T2 bioformulation to spinach after 60 DAS stimulated maximum plant growth in terms of shoot length (76-142%), root length (28-200%), shoot fresh and dry biomass (9.5-109%) and (21-1300%), root biomass fresh (21-207%) and dry (3300-4376%), Leaf area (106-340%) respectively over non- PGP carrier material, chemical fertilizer and uninoculated plants at 60 DAS. Bioformulation (T1) inoculation to spinach also increased shoot length (73-109%), root length (10- 156%), fresh and dry weight of shoot (74-334% and 238-1366%), fresh and dry weight of root (75-300% and 3300-5566%), leaf area (103-333%) over all control treatments (T3-T7). Overall T1 and T2 bioformulation were proved to be the best treatments for highest plant growth as compared to other treatments. Maximum growth in bioformulation treated spinach plants might be due longer shelf life of microbes in carrier which in turn enhanced their PGP activity through various mechanisms like solubilization of insoluble nutrients, production of growth hormone and induction of biochemical. Another possible explanation for this result is colonization capacities of the bioformulations. These results are in agreement with the findings of Tripti *et al*., (2017), highlighting, carrier based bioformulation increased tomato plant growth as compared to uninoculated control. The study is also in lineation with the results of Ahemad and Kibret, (2014) and Shahzad *et al*., (2017) that reported enhanced plant growth parameters like plant height, biomass and number of branches upon application of carrier based bioformulation over liquid inoculum and other control treatments. The results are in agreement with Subba (2001), who stated that microbes in the rhizosphere are capable of secreting growth substances, antibiotics and secondary metabolites which aids better plant growth and productivity.

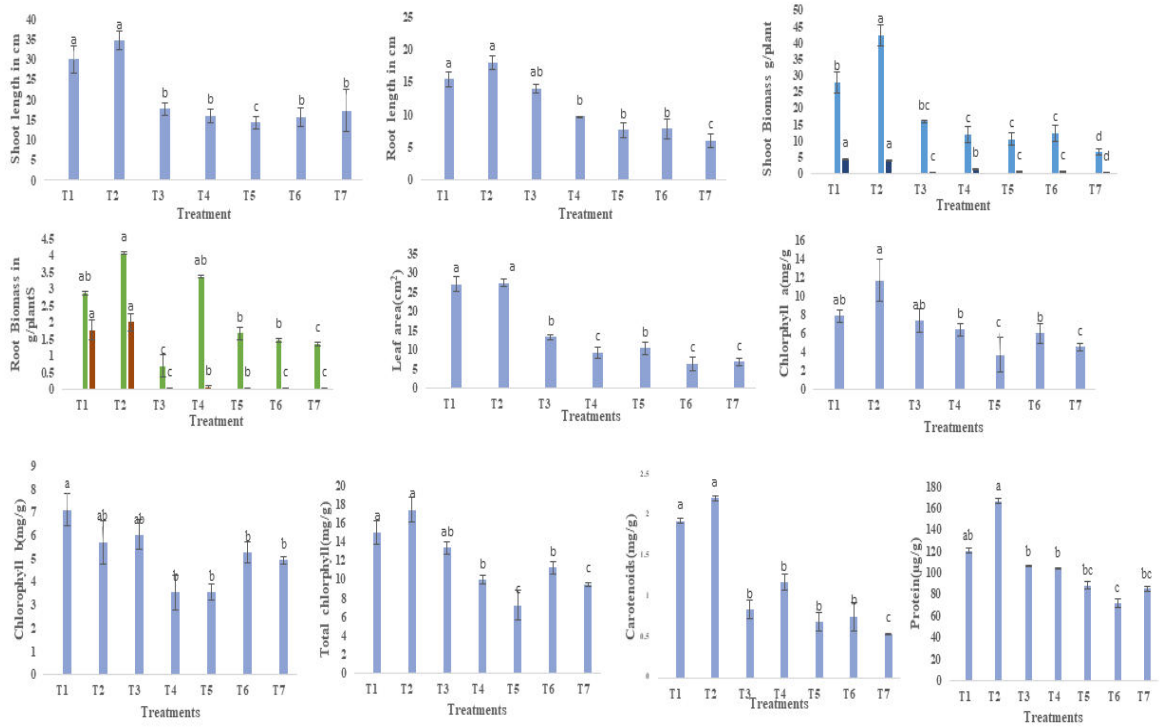

Fig. 1: Effect of bioformulation and other treatments on Plant growth and photosynthetic pigments of spinach

T1= Bentonite +MC, T2=talc+gluten +MC, T3 =nutrient broth+MC, T4= bentonite, T5=talc+gluten, T6=chemical fertilizer and T7= soil only, *mg=milligram, cm=centimetres,*

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g=grams, µg=microgram, Data are mean of three replicas, in duplicate determination (n=6) ± standard error of means. Means, followed by the same letter in a column are not significantly different by Duncan's multivariate test (DMRT)

also show promising results for enhanced chlorophyll, carotenoids and protein content of the plant. In the current study, the bioformulation T1 enhanced chlorophyll a (6.8-116%), chlorophyll b (18-102%), total chlorophyll (12- 106%), carotenoids (72-220%) and protein content (14-69%) over non-PGP carrier material, chemical fertilizer and uninoculated control. Similarly, T2 bioformulation showed significantly increased chlorophyll a (58-222%), chlorophyll b (6.8-31%),

total chlorophyll (21-125%), carotenoids (100-340%) and protein content (58-133%) as compared to chemical treatment as well as other controls. The increased pigmentation and protein content could be attributed to increased water availability and minerals due to inoculation of carrier based bioformulation. These results are in lineation with the results of Prabhu and Thomas (2002); Barman *et al*., (2016). Findings are also supported by the results of Khalid *et al*., (2017) who found that the inoculation of *A. chroococcum, B. megaterium and B. mucilaginous* bacterial strains for cultivation of spinach showed significant increase in chlorophyll a content as compared to untreated control plant sets.

Fig. 2: Pot experiment showing visual effect of bioformulation on plant growth

T1= Bentonite +MC, T2=talc+gluten +MC, T3 =nutrient broth+MC, T4= bentonite, T5=talc+gluten, T6=chemical fertilizer and T7= soil only

3.2. Influence of treatments on defence related compounds of spinach

Considering the inherent medicinal accountability of defence related compounds in chronic diseases such as cancer, cardiovascular and neurodegenerative disorders (Riedel *et al*., 2012), the secondary metabolites of spinach like total phenolics and total flavonoids were analysed. The results of the current study showed that the contents of total phenolic and flavonoids under different experimental treatments were found to vary significantly. In bioformulation treated plants, the amount of total phenolic contents in T1(47-60%) and in T2 (84-118%)

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were higher as compared to chemical fertilizer and un-inoculated control plants (Fig. 3 A). Possible reason for increased phenolic content in the plant treated with bioformulation might be production of Indole acetic acid by the microbial strains which is also supported by the findings of Cisternas-Jamet *et al*., (2020). It was observed that experimental plants inoculated with T1 also showed (68-640%) and T2 (113-840%) increased content of flavonoids than that of chemical fertilizer, un-inoculated carrier control and non-treated control plants. While, treatment T3 i.e liquid inoculum, showed 72.3% and 272.5% higher phenolics and flavonoids content than those of other non-treated plants, which was still less than that of T1 and T2. Insignificant difference was observed between application of other treatments for flavonoids and total phenolic content. The most possible reason for enhanced phenolics and flavonoids in bioformulation treated plants could be longer shelf life of microbes in bioformulation, which will help in secretion of growth hormone and metabolites. Few other studies also revealed the higher microbial performance for enhanced metabolites production when incorporated into carrier material as compared to liquid inoculum application (Bona *et al*., 2018).

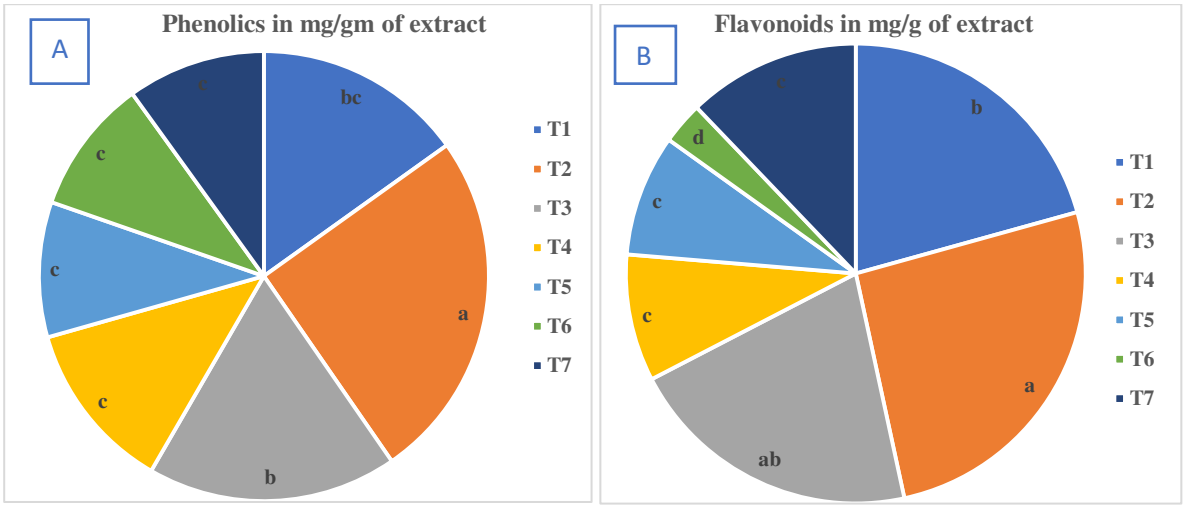

Fig 3: Effect of experimental treatment on health-promoting compounds in the spinach leaf extract. A =total phenolic, B= Flavonoids

T1= Bentonite +MC, T2=talc+gluten +MC, T3 =nutrient broth+MC, T4= bentonite, T5=talc+gluten, T6=chemical fertilizer and T7= soil only, *mg=milligram, Data are mean of three replicas, in duplicate determination (n=6) ± standard error of means. Means, followed by the same letter in a column are not significantly different by Duncan's multivariate test (DMRT)*

The Results are also in lineation with the findings of Riahi *et al.,* (2020), illustrating enhanced content of flavonoids and phenolics content in *Pelargonium graveolens* upon inoculation of *Pseudomonas rhizophila*, *Halomonas desertis* and *Oceanobacillus iheyensis* as compared to uninoculated plants. Barman *et al*., (2016), also reported phenolic compound accumulation in seedlings inoculated with coco-peat. The reason might be due to its better growing condition like the water maintenance ability of fermented cocopeat (Saikia and Upadhyaya 2011). Similar to our findings, Khalid *et al*., (2017), also reported an enhanced concentration of total phenolic, flavonoids and phenolic acid in Spinach leaves upon inoculation of bacterial strains (*Glomus fasciculatum, Glomus mosseae* and PGP *Azotobacter*

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chroococcum, Bacillus mucilaginous and *B. megaterium)* as uninoculated plants. Further results were supported by the findinds of Helaly *et al*., (2020), who reported enhanced content of flavonoids and phenolics in collard plant upon inoculation of *Serratia marcescens*, *Pseudomonas poae*, *Plantibacter flavus* and *Bacillus amyloliquefaciens* as compared to uninoculated plant sets. Overall, in the present study, the improvement of pigments, proteins, phenolics and flavonoids in spinach leaves with the application of bioformulation have potential to increase its nutritional and commercial value.

3.2. Changes in soil physicochemical, enzymatic properties and microbial population with application of bioformulation

The effect of bioformulations on fertility of pot soil was assessed and results are presented in table 1. All the studied parameters showed a remarkable difference between carrier-based bioformulation and other treatments. The maximum percentage increase in soil organic matter was observed by T1 (44-331%) and T2 (61.8-382%) as compared to other treatments. Similarly, Kushwaha (2011), also reported an increased percentage of organic carbon in soil upon the addition of bioformulation. Other soil nutrients like AN, AP and AK were increased by (2.28-123.5%), (26.5-309%) and 7.53-85.5%) respectively, with application of T1 while T2 showed (167-475%), (343-12656%), (30-487%) increased available NPK over uninoculated carrier material (T4 and T5), chemical fertilizer (T6) and non-treated plants(T7). Analysis of pot soil after crop harvesting showed increased concentration of nutrients like AN, AP and AK upon inoculation of pot soil with bioformulations, which might be facilitated by the ability of microbes that solubilize insoluble nutrients. The results are in agreement with the results of Kumar *et al*., (2013) and Tripti *et al*., (2017). Enzymes like MBC and Alkaline phosphatase also increased (22-321%) and (49-385.1%) with T1 and (8.3-273%)

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Table 1: Effect of different treatments on soil physio- chemical and enzymatic properties.

	Treatment	pH	EC (ds/m)	OM (%)	AN (mg/kg)	AP (mg/kg)	AK (mg/kg)	ALkP(µg/g)	SMBC(µg/g)
Pre-sowing	0 day	6.50±0.10^{c}	4.60±0.20^{a}	0.65±0.03^{e}	15.00±2.50^{e}	1.48±0.89^{d}	92.88±14.58^{d}	1.17±0.81^{e}	64.13±9.07^{e}
Post experiment	T1	6.20±0.10^{c}	1.55±0.22^{c}	2.28±0.01^{b}	62.50±2.50^{b}	10.15±0.05^{b}	184.68±5.51^{b}	18.41±4.50^{b}	455.32±12.43^{b}
	T2	8.23±0.12^{a}	1.19±0.06^{d}	2.47±0.01^{a}	136.67±10.41^{a}	31.74±0.50^{a}	204.20±9.54^{a}	66.43±1.40^{a}	403.23±13.22^{a}
	T3	8.30±0.10^{a}	1.93±0.05^{c}	2.32±0.01^{a}	54.17±7.22^{c}	8.38±5.85^{b}	159.67±14.58^{c}	10.49±2.42^{c}	372.20±5.24^{b}
	T4	7.57±0.29^{b}	1.21±0.03^{d}	1.78±0.03^{c}	36.67±2.89^{d}	0.61±0.18^{e}	178.75±14.58^{bc}	18.41±3.52^{b}	108.41±7.62^{d}
	T5	7.93±0.38^{b}	3.55±0.19^{b}	1.49±0.03^{c}	61.67±1.44^{b}	0.27±0.05^{e}	166.03±25.25^{c}	9.09±2.42^{c}	188.210±8.37^{c}
	T6	7.90±0.44^{b}	1.89±0.09^{c}	1.03±0.03^{d}	54.17±7.22^{c}	1.72±0.05^{d}	111.96±5.51^{cd}	8.62±1.61^{d}	182.30±10.39^{c}
	T7	7.10±0.35^{c}	3.60±0.16^{b}	1.52±0.04^{c}	57.50±6.61^{c}	7.40±2.44^{c}	111.96±5.51^{cd}	11.42±0.81^{c}	112.20±12.30^{c}

T1= Bentonite +MC, T2=talc+gluten +MC, T3 =nutrient broth+MC, T4= bentonite, T5=talc+gluten, T6=chemical fertilizer and T7= soil only, ***AN****=Available nitrogen;* ***AP****= available phosphorus;* ***AK*** *=available potassium;* ***OM****= organic matter;* ***WHC*** *= water holding capacity;* ***mg/kg****= milligram per kilograms;* ***%*** *=percentage;* ***EC*** *= Electrical conductivity;* ***ds/m****= desicimen per meter, Data are mean of three replicas, in duplicate determination (n=6) ± standard error of means. Means, followed by the same letter in a column are not significantly different by Duncan's multivariate test (DMRT)*

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Table 2: Effect of different treatments on rhizospheric colonization of microbial population.

	Treatments ⟹	T1	T2	T3	T4	T5	T6	T7
30 days	**Bacteria CFU/g root**	$8.00x10^{7}$±1.00	$12.00x10^{7}$±2.00	$6.00x10^{6}$±1.00	$7.67x10^{5}$±1.53	$4.00x10^{5}$±1.00	$4.00x10^{4}$±1.00	$5.00x10^{4}$±1.00
	Fungal CFU/g of root	$11.00x10^{6}$±1.00	$14.33x10^{6}$±1.53	$6.67x10^{5}$±0.58	$6.00x10^{4}$±1.00	$6.33x10^{4}$±0.58	$3.00x10^{3}$±1.00	$5.00x10^{3}$±1.00
60 days	**Bacteria CFU/g of root**	$14.00x10^{7}$±1.00	$22.67x10^{7}$±1.53	$8.50x10^{6}$±1.53	$10.50x10^{5}$±1.53	$8.50x10^{5}$±0.58	$6.00x10^{4}$±1.15	$7.00x10^{4}$±1.15
	Fungal CFU/g of soil root	$15.33x10^{7}$±1.53	$29.67x10^{7}$±1.15	$11.00x10^{6}$±1.00	$9.33x10^{5}$±1.53	$9.00x10^{4}$±1.00	$7.33x10^{3}$±2.08	$6.67x10^{3}$±1.15

T1= Bentonite +MC, T2=talc+gluten +MC, T3 =nutrient broth+MC, T4= bentonite, T5=talc+gluten, T6=chemical fertilizer and T7= soil only; CFU= colony forming unit, Data are mean of three replicas, in duplicate determination (n=6) ± standard error of means. Means, followed by the same letter in a column are not significantly different by Duncan's multivariate test (DMRT

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and (285-728%) with T2 respectively, over control treatments(T3-T7). After inoculation of bioformulation in the pot soil, significant increase in enzymes like MBC and Alkaline phosphatase was observed, which might be due to increased population of PGPM in the soil resulting in improved soil fertility. Previous study by Mishra *et al*., (2021) and Mishra and Singh, (2022) also reported increased percentage of soil MBC upon inoculation of bioformulation, which in turn have a positive effect on interaction of microbes with native microbes and on colonization.

The results further showed that inoculation of bioformulation helps in the microbial colonization in the rhizosphere. T1 and T2 showed maximum enhanced population density of $14x10^7$ and $22.6x10^7$ cfu/g of root for bacteria, while $15.33x10^6$ and $29.67x10^7$ cfu/g of root for fungi respectively at 60 DAS (table 2). Comparatively, lesser CFU was reported for other treatments T3-T7 with maximum colonization of to $8.5x10^6$ and $11.6x10^6$ cfu at 60 DAS for bacteria and fungi respectively and a minimum of $6x10^4$ for bacteria and $6.6x10^3$ for fungi at 60 DAS. The results indicated that carrier-based bioformulation helped in maintaining the population of microbes in the rhizosphere. The competency of microbial strain in the rhizosphere indicates about their capacity to be a plant growth promotor (Compant *et al*., 2019; Rilling *et al*., 2019). Similar to the current study, Novinscak and Filion 2020, reported that talc and peat based bioformulation of *Pseudomonas fluorescens* and *P. synxantha* could enhance the rhizospheric colonization. Aeron *et al*., 2011 also reported that bioformulation increased root colonization as compared to sole bacteria application and control.

4.0. Conclusions

The present study concluded that carrier material stimulates the activity of microbial consortium by enhancing root length, shoot length, biomass, leaf area, pigments, protein and secondary metabolites in spinach leaves. Apart from plant growth the microbial bioformulation also improve soil health by enhancing the availability of essential nutrients for plant growth. Overall, the outcome of the study reflected that the formulation of microbes in carrier material is an alternative to conventional unsustainable approaches for plant growth. Thus, it can be concluded that spinach inoculated with microbial bioformulation can easily reduce the dependence on chemical-based fertilizer for sustaining soil fertility and plant growth.

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