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Seed Quality, Chlorophyll and Carotene Content in *Brassica juncea* L. Leaves at Two Growth Stages in Response to Rhizospheric Bacteria

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Abstract

Leaf pigments play a crucial role in photosynthesis and protection, which drives plant growth. Rhizospheric bacteria playing a pivotal role in promoting plant development, also affects leaf pigmentation. The present study was therefore aimed to assess the influence of plant growth-promoting rhizobacterial (PGPR) treatments on leaf pigments and plant growth at early stages in *Brassica juncea* (L.). Pot experiments were conducted with selected rhizobacteria for 2 months. Although plant responses varied among the different PGPR inoculants, *Pseudomonas azotoformans* (JRBHU5) and *Pseudomonas gessardii* RRBHU-1 (P21) exhibited, notable improvements in germination percentage, seedling vigor index, biomass and leaf variables viz. relative water content (RWC), live fine fuel moisture (LFFM), leaf pigment ratio and leaf dry matter content (LDMC). The pigments found in leaves (β and α carotene, and chlorophyll a and b) of mustard got remarkably increased in JRBHU5 and P21 treatments, analyzed through absorption spectrum analysis. The absorption spectrum of *Brassica* leaf extracts revealed red-shifts in absorption peak influenced by solvent polarity and growth stages. Fluorescence studies indicated enhanced leaf pigment fluorescence under UV light in JRBHU5, JRBHU6, P21, and JRBHU1 treated plants suggesting an efficient chlorophyll synthesis in the treated plants. PGPR inoculation maintained photosynthetic efficiency, promoting growth and delaying senescence.

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Introduction

The contemporary vegetable production systems, heavily reliant on soil fertilizers, have led to an escalation in soil health degradation. The World Food Program (WFP) aiming to improve food production efficiency is equally concerned with minimizing the adverse impacts on ecosystems and human health. In pursuit of this goal, the utilization

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of plant biostimulants, natural substances distinct from fertilizers and pesticides, has emerged as a promising alternative to address the challenges stemming from the industrialization of agriculture as advocated by the World Bank.

Brassica juncea L., a member of Brassicaceae family, stands out as a commercially important major oilseed crop grown widely in North America, Asia, Europe, and Northern Africa. *Brassica juncea* is a high-fiber, mineral-rich, and phytochemical-rich food crop grown annually with major contributors in global annual production being the European Union (30.87%), Canada (26.36%), China (20.41%), India (8.54%), Australia (4.93%), and Others (8.89%) (Source: Agricultural statistics at a Glance 2022). Plant growth and developments are significantly influenced by PGPR which modifies the chemistry of the rhizosphere providing disease resistance/and higher nutrient absorption, resulting in high biomass production. Microbial-inoculated *Brassica* sp. has been reported to improve photosynthesis under stress by reducing stomatal resistance, thereby enhancing biomass.[2,3] This increase in yield can be attributed to higher photosynthetic activities and an efficient uptake of nutrients and water.

Microbial inoculants are largely being studied for their remedial effects on stress generated oxidative bursts affecting the photosynthetic apparatus, proteins, membrane lipids and other cellular components.[4] The composition of leaf chlorophyll and carotenoid undergoes changes in trees across different phenological stages[5] and along light canopy gradients,[6] as well as in response to various biotic[7,8] and abiotic stressors[9] like heat-waves, drought,[10,11] UV-B[12], and elevated CO_2 and O_3.[13,14] Leaf pigments are vital for seedling growth and protection. As seedling mature, the balance and amount of leaf pigments adjust to optimize photosynthesis and protection. While PGPRs are well studied for their effects on promoting plant growth, their influence on content of various leaf pigments and their variations has not been thoroughly investigated. Therefore, the study aimed to explore the impact of various PGPRs on the growth, photosynthetic efficiency, and leaf pigments of *Brassica juncea* seedlings.

Materials and Methods

The study was performed at Banaras Hindu University (25.2677° N, 82.9913° E) situated in district Varanasi of India. It has a lenient, hot, and temperate climate with an average annual temperature of 26.1°C and rainfall of 1110 mm.

Seed Bio-priming and Growth Conditions

The seeds of Indian mustard (*Brassica juncea* L.) procured from the Indian Agriculture Research Institute (IARI), New Delhi, India, were surface sterilized by 1% sodium hypochlorite (NaOCl) for 30 seconds, then allowed to air dry after two rounds of rinsing in sterile distilled water under laminar air-flow. Bacterial strains isolated from fields of northern India with plant growth-promoting traits tested on wheat were selected for bio-priming mustard seeds. The selected PGPRs were screened for biochemical and growth-promoting traits in our lab as already reported.[15] Molecular identification (16S rRNA) of bacterial strains was done earlier and Gene Bank accession number obtained were viz JRBHU1 (*Burkholderia paludism*-MK439528), JRBHU4 (*Pseudomonas lactis*-MK500865), JRBHU5 (*Pseudomonas azotoformans*-MK500938), JRBHU6 (*Burkholderia seminalis*-MK500868), JRBHU9 (*Enterobacter hormaechei*-MK500940), JRBHU10 (*Enterobacter cloacae*-MK501756), JRBHU11 (*Bacillus subtilis*-MN759630), and P21 (Pseudomonas gessardii RRBHU-1-OK427346). Each of the eight bacterial strains was grown in nutrient broth medium on a rotary shaker at 27°C for 24 hours. Subsequently, the mustard seeds were immersed in 25 millilitres of nutrient media having pre-screened bacterial suspensions (109 CFU/ml) and were maintained at 28 ± 2°C at 90 rpm for 10-12 hours and seeds were sown without any treatment in the control group. Seeds were further dried overnight under laminar air flow and used for greenhouse experiment. An amalgamation of soil, sand, and vermiculite (4:1:1 v/v) was autoclaved for three consecutive days. Bio-primed seeds were then sown in a greenhouse at 25/20°C, 16/8 h day/night photoperiod, 500-700 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), and 70% relative humidity. The treatments were randomly distributed with three replications each.

Seed Quality And Seedling Parameter

The germination percentage was calculated after five days and paper towel method was used to determine the Vigour index (VI). After 15 days 3 plants from each treatment were carefully removed from pots and properly washed for analyzing the

different plant growth parameters. Measurements of shoot and root length, leaf number and area, live fine fuel moisture (LFFM) fresh and dry biomasses and relative water content (RWC) were taken for all treatments along-with controls. The shoots and roots were individually oven-dried at 105°C for 24 hours to determine their dry weight. Fresh samples were used for leaf pigment analyses.

Leaf Chlorophyll and Carotene Extraction

Chlorophylls and carotenoids are fat-soluble compounds, and acetone provides sharp absorption peaks, making it the preferred solvent for chlorophyll assays.[16] Therefore, approximately equal amounts of leaf tissue were collected from all three uprooted plants and bulked for each treatment. Leaf tissue (20mg) was macerated in a pestle and mortar in 5ml of 80% acetone. These extracts were adjusted up-to 10 ml of final volume. To avoid pigments photo-bleaching, extraction and concentration procedures were performed in subdued light.

UV Induced Chlorophyll Fluorescence

Preliminary chlorophyll estimations in samples were done based on fluorescence emission on UV exposure.[17] The fluorescence intensities emitted were dependent on the amount of light absorbed by the chloroplast present in the mesophyll and were graded as +++ (very high), ++ (high), and + (moderate) based on visual observation. To compare the fluorescence intensities emitted by the chloroplast equal amounts of *Brassica* leaf extract were taken in culture tubes and UV radiations were passed through Benchmark UV lamp (UV intensity = +500 µW/cm2, and wavelength = 254 nm) in a dark room maintained at 20° C.

Spectrophotometry

The absorbance of leaf extracts was measured using *Jasco* V-670 UV-VIS-NIR spectrophotometer model in a range of 400–700 nm. The absorption peaks of chlorophylls can shift to longer wavelengths when the solvent's polarity and/or water concentration increase, with the shift being more pronounced in the blue region than the red region. Chlorophyll a and chlorophyll b absorb light in narrow bands in the blue and red spectrum ranges, whereas carotenoids have broader absorption bands in the blue range.[18] For the identification and quantification of carotenoids, spectral absorption was used and α-carotene was measured at 444nm, and β-carotene at 452nm.[19] Peaks of carotenoids' absorption can differ depending on the solvent type and water content, with shifts occurring at higher water content. This shift can be one nm or more, depending on the solvent and carotenoid type.[20]

Data Analysis

Chlorophylls, total chlorophylls, and carotenoids concentration were calculated by the below mentioned formulae. The pigments maximum absorption wavelength was determined as it shifted with the growth stages, as mentioned in equations 1 to 6.

Seedlings stage,

Chl a=12.21 A_{663}–2.81 A_{646}, Chl b=20.13 A_{646}–5.03 A_{663} ...(1)

Chlorophyll (a + b) = (8.05A_{663} + 20.29A_{646}) ...(2)

For β carotene (1000A_{452}) or for α carotene (1000A_{444})-1.82chl a_c–85.02chl b_c/198 ...(3)

One-month plant stage,

Chl a=12.21 A_{676}–2.81 A_{646}, Chl b=20.13 A_{646}–5.03 A_{676} ...(4)

Chlorophyll (a + b) = (8.05A676 + 20.29A646) ...(5)

For β carotene (1000A_{537}) or for α carotene (1000A_{524}) -1.82chl ac–85.02chl bc/198 ...(6)

Abbreviations: A = absorbance, chl = chlorophyll, c = pigment content in µg/ml of extract.

The experiments were conducted in triplicates in a randomized design, and the data were analyzed using software SPSS-version 20. The LSD test was used to compare means, and a 0.05 probability level was set for analyzing the critical difference among isolates in each treatment.

Results

Rhizobacterial Effects on Plant Growth

It was over-all observed that rhizospheric bacteria improved plant growth and nearly 100% seed germination was recorded in treatments viz. JRBHU1, JRBHU5, JRBHU6, and P21 (figure 1). Treatment of JRBHU5 resulted in the longest

root with 84% increase, followed by P21 (79%). JRBHU5 also showed the highest length of shoot with an increase of 127%, followed by P21 with 120% increase. Other strains exhibited substantially increases. The VI showed significant differences in JRBHU1, JRBHU5, JRBHU6, and P21 treatments. Additionally, the root:shoot length ratios, first and second internode distances were also obtained, as mentioned in Table 1.

Table 1: Seed germination and seedling parameters measured after different bacterial treatments.

Bacterial isolates	Germination (%)	Root length (cm)	Shoot length (cm)	Seedling vigour index	Root: Shoot ratio	First internode distance (cm)	Second internode distance (cm)	Weight of Root (g)		Weight of Shoot (g)	
								Fresh	Dry	Fresh	Dry
JRBHU1	80.940 ± 4.770^{ab}	7.300 ± 0.430^{abcd}	11.767 ± 0.140^{a}	1634.210 ± 49.810^{b}	0.621 ± 0.030^{a}	5.770± 0.140^{abc}	1.933 ± 0.060^{a}	0.018 ± 3.340^{cd}	0.007 ± 5.770^{b}	0.667 ± 0.012^{a}	0.040 ± 0.002^{cd}
JRBHU4	52.380 ± 4.780^{c}	6.167± 0.140^{de}	9.167 ± 0.270^{b}	806.130± 89.350^{c}	0.674 ± 0.010^{a}	5.200 ± 0.050^{c}	1.333 ± 0.1200^{b}	0.008 ± 5.780^{e}	0.004 ± 3.330^{b}	0.285 ± 0.003^{de}	0.034 ± 0.004^{b}
JRBHU5	95.230 ± 4.770^{a}	8.400 ± 0.200^{a}	12.700 ± 0.110^{a}	2110.000 ± 32.1^{a}	0.663± 0.020^{a}	6.200 ± 0.050^{a}	2.133 ± 0.060^{a}	0.026 ± 5.780^{a}	0.013 ± 0.023^{a}	0.57 0± 0.022^{b}	0.056 ± 0.003^{d}
JRBHU6	90.480 ± 4.730^{a}	7.500 ± 0.320^{abc}	11.934 ± 0.140^{a}	1762.330 ± 134.7^{ab}	0.629 ± 0.020^{a}	5.900 ± 0.110^{ab}	1.966 ± 0.030^{a}	0.021 ± 0.001^{bc}	0.007 ± 3.330^{b}	0.618 ± 0.016^{ab}	0.018 ± 0.004^{d}
JRBHU9	52.380 ± 4.770^{c}	6.234 ± 0.23^{cde}	9.334 ± 0.230^{b}	889.480 ± 13.330^{c}	0.671 ± 0.040^{a}	5.230 ± 0.030^{bc}	1.366 ± 0.120^{b}	0.008 ± 6.670^{e}	0.004 ± 3.330^{b}	0.306 ± 0.009^{de}	0.035 ± 0.004^{ab}
JRBHU10	61.900 ± 4.760^{bc}	6.967± 0.080^{bcde}	9.834 ± 0.210^{b}	1040.880 ± 88.190^{c}	0.713 ± 0.080^{a}	5.300 ± 0.150^{bc}	1.733 ± 0.120^{ab}	0.017 ± 3.340^{d}	0.005 ± 3.330^{b}	0.244 ± 0.025^{e}	0.035 ± 0.004^{bc}
JRBHU11	61.900 ± 4.760^{bc}	6.334 ± 0.320^{cde}	9.434 ± 0.340^{b}	973.270 ± 58.090^{c}	0.854 ± 0.220^{a}	5.270 ± 0.030^{bc}	1.400 ± 0.050^{b}	0.009 ± 3.340^{e}	0.004 ± 3.330^{b}	0.330 ± 0.016^{c}	0.037 ± 0.004^{ab}
P21	95.240 ± 4.770^{a}	7.934 ± 0.180^{ab}	12.0 34± 0.310^{a}	1905.2 20± 132.360^{ab}	0.660 ± 0.090^{a}	6.000 ± 0.110^{a}	2.100 ± 0.050^{a}	0.022 ± 8.820^{ab}	0.009 ± 5.770^{b}	0.625 ± 0.016^{ab}	0.048 ± 0.00^{3d}
Control	47.620 ± 4.700^{c}	5.834 ± 0.170^{e}	7.434 ± 0.520^{c}	633.270 ± 74.610^{c}	0.796 ± 0.070^{a}	4.430± 0.290^{d}	1.266 ± 0.170^{b}	0.006 ± 8.82^{e}	0.003 ± 5.77^{b}	0.396 ± 0.009^{c}	0.015 ± 0.004^{a}

The data represents the means of three replicates ± SE, with significant differences observed within the same column represented by different letters at p-value of ≤ 0.05.

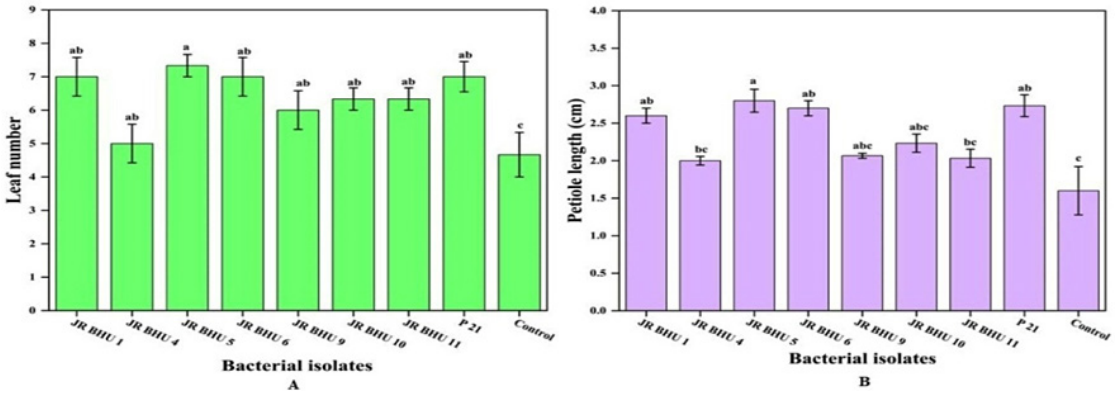
Fig. 1: Plant growth and vigour index of *Brassica juncea* L. after various PGPR treatments at two different growth stages (A) Seedlings (B) One-month plants

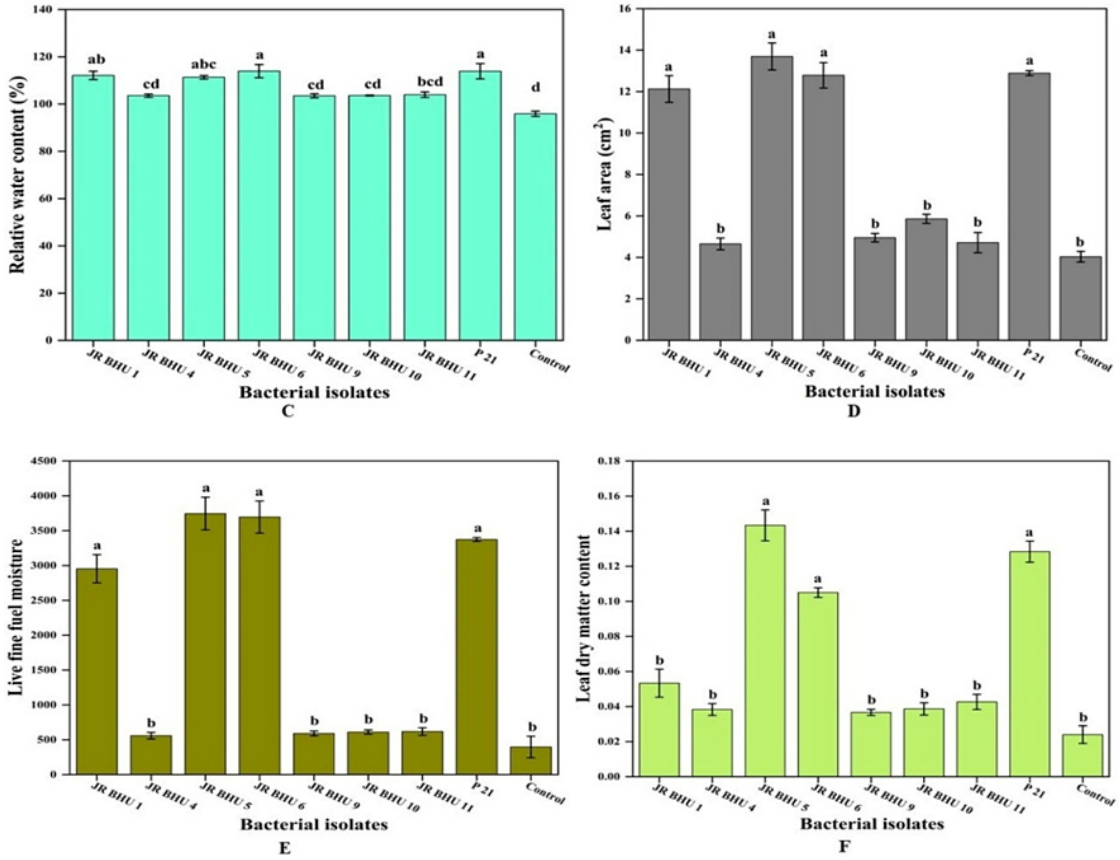
Impact of PGPR on Plant Biomass

The effects of rhizospheric bacteria on plant biomass was mostly insignificant for most of the isolates except for JRBHU5 which showed an increase of 1.3% in root dry biomass followed by P21 with 0.9% increase. Similarly, an increase of 5.6% in shoot dry weight was observed in JRBHU5 followed by P21 (4.8%) (Table1). Plants inoculated with PGPR isolates showed a significant increase in live fine fuel moisture content in shoots, with treatment JRBHU5 showing the highest LFFM value followed by JRBHU6 and P21 as depicted in figure 2E.

Impact of PGPR on Leaf Attributes

Evaluation of leaf variables viz. leaf number, petiole length, leaf area, RWC, and LDMC showed considerable effects of different bacterial treatments. The leaf counts and petiole length showed variations among which JRBHU5 followed by P21 treated plants had the highest leaf count as well as petiole length among all the treatments (Figures 2A and B). Inoculation of JRBHU5 also increased the leaf area maximally among all (Figure 2D). A slight rise in RWC was observed after different treatments for most of the isolates. However significant increase in RWC was observed in JRBHU6 (117.934%), and P21 (117.9%) inoculated plants (Figure 2C). Highest impact on LDMC with 14.5% increment was observed after JRBHU5 treatment followed by P21 (12.84%) and JRBHU6 (10.5%) (Figure 2F). The effect of bacterial isolates JRBHU1, JRBHU5, JRBHU6, and P21 on LFFM was highly significant.





Turkey's multiple range test at p ≤ 0.05 was used to compare treatments, with results shown as means of three replicates and slanting bars suggest standard error of mean.

Fig. 2: Effects of bacterial seed bio-priming on different leaf parameters (A) Leaf number (B) Petiole length (C) RWC (D) Leaf area (E) LFFM (F) LDMC

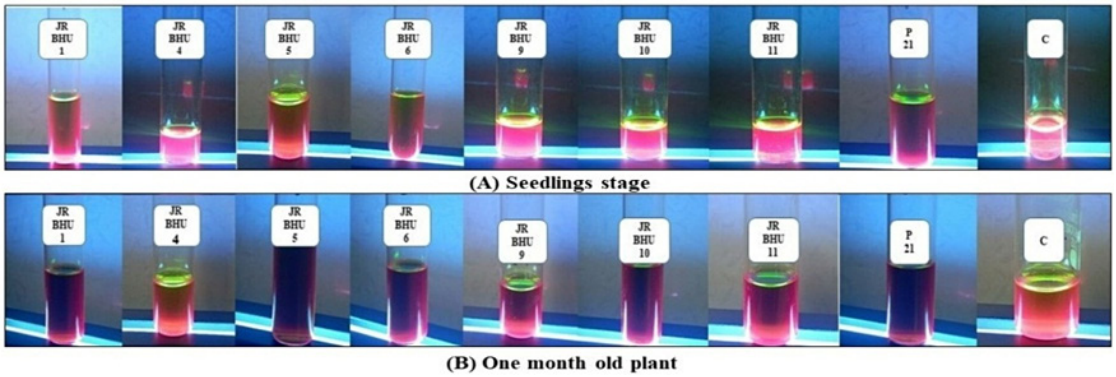


Fig. 3: Leaf pigment fluorescence under UV light exposure at different growth stages (A) Seedlings (B) One-month plants

Bacterial Effects on Leaf Pigments

To get a preliminary idea on effects of bacterial treatments on *B. juncea* leaf pigments, a fluorescence study was done. Chlorophyll molecules extracted from *Brassica* plant leaves re-emit red light as fluorescence in varying amounts under UV light exposure depending upon the chlorophyll content which was graded in the scale (+, ++, +++) based on visual observation (Table 2). Bacterial treated young seedlings and one-month-old treated plants showed a dark ruby color as compared to the control. An immense rise in red intimacy was visually observed in treatments JRBHU1, JRBHU5, JRBHU6, and P21 at both seedlings and one-month stage as depicted in Figures 3 A and B.

Table 2: Pursuance of PGPRs on UV-induced chlorophyll fluorescence

Bacterial isolates	Seedlings	One-month plants
JRBHU1	+ + +	+ + +
JRBHU4	+ +	+ +
JRBHU5	+ + +	+ + +
JRBHU6	+ + +	+ + +
JRBHU9	+ +	+ +
JRBHU10	+ +	+ +
JRBHU11	+ +	+ +
P21	+ + +	+ + +
Control	+	+

+: Visual intensity of red fluorescence

Absorption Maxima and Spectra

The absorption maxima of leaf pigments being very narrow are often affected by the spectrophotometer instrument.[21] To measure the accurate effect of bacterial treatments on leaf pigment content at different growth stages the absorption maxima were first calculated for each pigment (chlorophylls, carotenoids) in the range 400–700 nm with the laboratory spectrophotometer (model *Jasco* V-670). The literature clearly advocates that at wavelength deviation of more than 1nm, it is advisable to measure absorbance maxima. Similarly, the wavelength regions should not exceed 2nm for the same equations to be applied to any extraction solvent. The differences in absorption maxima of different pigments in acetone extract as mentioned in literature are given in Supplementary Table 1. The absorption spectrum of *Brassica* leaf extract containing mixtures of chl a and b, β and α carotenes obtained over acetone solvent at two different growth stages were measured to find the absorption peak. Accordingly, the spectrophotometer readings were made as to 663nm for chl a and 646nm for chl b in seedlings, 676nm for chl a and 646nm for chl b in one-month plant. Similarly, the wavelengths for β and α carotenes were set at 452 and 444nm (seedlings) and 537 and 524nm (one-month plant) as depicted in Figure 4A and B. The solvent-specific calculation of the pigment content at different growth stages was assessed by using the relevant equations (Equations 1 to 6).

Supplementary Table 1: Wavelength maxima (Amax) of leaf chlorophyll (a and b), and carotenes (α and β) in acetone solvent

A_{max} for Chl a [nm]	A_{max} for Chl b [nm]	A_{max} for (x + c) [nm]	A_{max} for β car [nm]	A_{max} for α car [nm]	Reference
428.5_{blue}	452.5_{blue}				22
			452_{blue}	444_{blue}	19
428_{blue}	453_{blue}	470_{blue}			21
663_{red}	646_{red}				23
663_{red}	645_{red}				24
663_{red}	645_{red}	470_{blue}			25
663_{red}	645_{red}	480_{blue} and 510_{blue}			26
663_{red}	645_{red}	470_{blue}			27

Abbreviations: Chl- Chlorophyll, Car- Carotenes, (x + c) = Total carotenoids

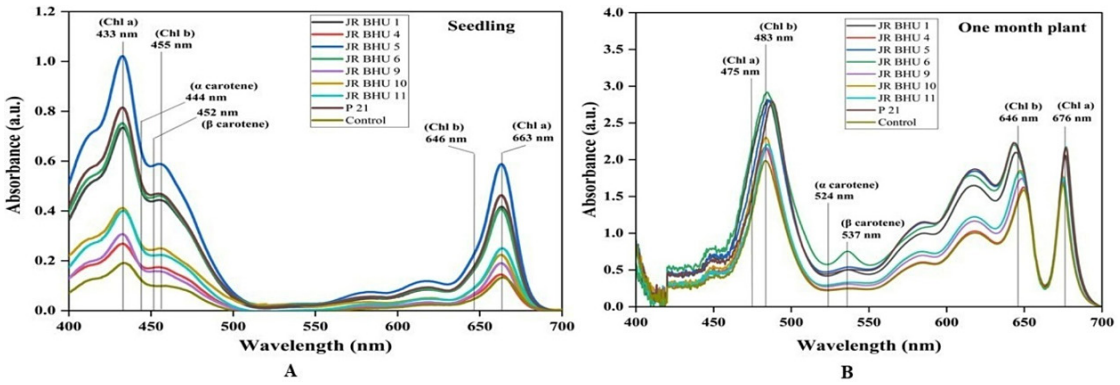


Fig. 4: Absorption spectra of different pigments of B. juncea L. leaf after different bacterial treatments at different growth stages (A) Seedlings (B) One-month.

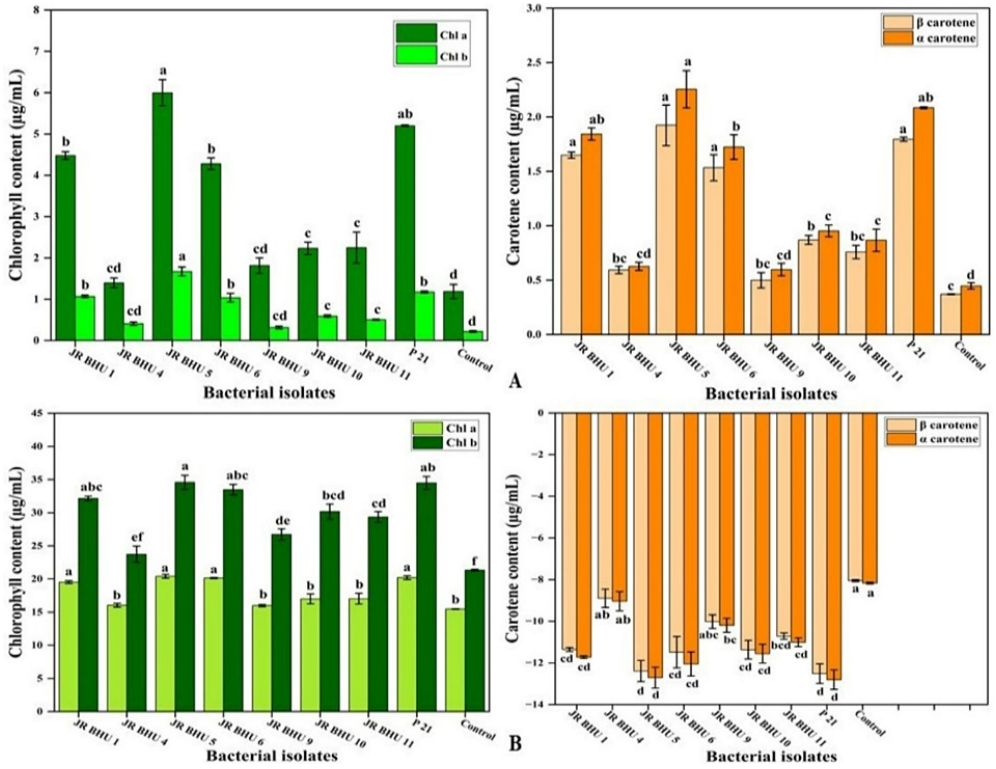


Fig. 5: Effect of bacterial seed biopriming on leaf chlorophyll and carotenoid contents at different growth stages (A) Seedlings (B) One-month

Quantification of Pigments

Leaf pigments are critical indicators of plant's photosynthetic and photo-protection status. Their concentrations vary across different growth stages and are affected by multiple factors. It was found that the chlorophyll content in one-month plants was significantly higher than carotenoids whereas the reverse was observed at seedlings stage. Chl a content was much higher than that of Chl b at the seedlings stage (Figure 5A) but was found to degrade rapidly with plant development as at one-month stage, the content of Chl b increased (Figure 5B). The content of α carotene was slightly higher than that of β carotene at seedlings (Figure 5A),

while after one-month the β carotene got increased. (Figure 5B). With plant growth, the chlorophyll contents improved while the carotenoid contents decreased. Similarly, among the chlorophyll pigments, the chlorophyll b content increased from 0.317 to 34.577 mg/mL, ranging from the seedling stage to the one-month stage (Figures 5 A and B). The trend of variation in leaf pigments with growth stages was observed to be similar in both control and bacterial treated plants. However, the different bacterial treatments showed significant effects on amount of chlorophyll and carotenoid contents. At seedlings stage, the bacterial influence was more on chl a, which was high compared to chl b. Similarly, at one month stage, the influence was more on chl b which was higher in amount than chl a. This overall reflected that the rhizospheric bacteria had strong influence on plant physiological and biochemical pathways involved in synthesis and degradation. Among all the tested bacteria higher chl b content at one month stage was observed in JRBHU5 and P21 inoculated plants that was 23 and 29 folds higher than the seedlings plants respectively. Most of the bacterial strains also influenced the carotenoid contents of *Brassica*. PGPR strains JRBHU5 and P21 were most influential in affecting both synthesis and degradation of carotenoid compounds.

Table 3: Percent increment in leaf pigments of *B. juncea* L. after different PGPR treatments

(A) Seedlings				
Bacterial isolates	**Chl a%**	**Chl b%**	**β car %**	**α car %**
JRBHU1	292.2 ± 51.120^{a}	397.2± 40.925^{bc}	343.6 ± 3.815^{ab}	313.4 ± 14.205^{bc}
JRBHU4	20.0 ± 7.944^{c}	88.0 ± 2.168^{d}	62.0 ± 8.191^{c}	40.4± 0.905^{e}
JRBHU5	420.2 ± 50.658^{a}	695.1 ± 109.480^{a}	416.9 ± 45.929^{a}	402.6 ± 5.875^{a}
JRBHU6	273.5 ± 44.151^{ab}	375.8 ± 5.414^{bc}	312.2 ± 28.580^{b}	284.8 ± 0.409^{c}
JRBHU9	54.9 ± 6.974^{c}	45.1± 0.990^{d}	43.4 ± 10.754^{c}	33.0 ± 4.311^{e}
JRBHU10	92.9 ± 16.312^{bc}	174.1 ± 17.382^{bcd}	134.3 ± 9.153^{c}	112.9 ± 1.531^{d}
JRBHU11	88.4 ± 4.258^{bc}	135.1 ± 19.672^{cd}	104.5 ± 14.869^{c}	92.2± 10.446^{de}
P21	359.1 ± 70.920^{a}	452.8 ± 73.480^{ab}	383.2± 0.994a^{b}	369.6 ± 32.401^{ab}
(B) One-month plants				
Bacterial isolates	**Chl a%**	**Chl b%**	**β car%**	**α car%**
JRBHU1	26.2 ± 0.982^{a}	50.6 ± 0.569^{abc}	41.1 ± 0.307^{ab}	43.6± 0.021^{abc}
JRBHU4	3.6 ± 1.522^{b}	11.1 ± 5.049^{e}	10.5 ± 4.842^{c}	10.6 ± 5.015^{d}
JRBHU5	31.9 ± 1.531^{a}	61.9± 3.909^{a}	53.8 ± 5.410^{a}	55.5 ± 5.309^{ab}
JRBHU6	30.1 ± 0.204^{a}	56.8 ± 2.668^{ab}	42.5 ± 8.554^{ab}	47.5 ± 6.259^{ab}
JRBHU9	3.3 ± 1.357^{b}	25.2 ± 3.141^{de}	24.4 ± 3.372^{bc}	24.8 ± 3.447^{cd}
JRBHU10	9.8 ± 4.521^{b}	41.3 ± 4.401^{bcd}	41.0 ± 4.758^{ab}	41.4 ± 4.738^{abc}
JRBHU11	10.0 ± 4.951^{b}	37.5 ± 2.936^{cd}	33.1 ± 1.074^{abc}	34.8 ± 1.865^{bc}
P21	30.6 ± 1.481^{a}	61.5 ± 3.531^{a}	55.4 ± 4.983^{a}	56.7 ± 4.876^{a}

Abbreviation: Chl- chlorophyll, Car- carotene
The data represents the means of three replicates ± SE, with significant differences observed within the same column with different letters at p-value ≤ 0.05.

different growth stages (A) Seedlings (B) One-month The pigment ratio in *Brassica* varied with different rhizobacterial treatments and growth stages. Chl a:Chl b showed 53.83% and 47.27% increases in JRBHU9 and control plants at the seedling stage, whereas 72.6% and 67.9% increases in control and JRBHU4 plants was observed after one month stage (Figure 6A). Total chlorophyll (a+b) content

was higher in treatments, with JRBHU5 showing an increase of 76.71% followed by P21 with 63.75% increase at seedlings stage whereas at one-month plants this was 110% and 109% for the same treatments (Figure 6B). Total carotenoids (X+C) increased in treatments JRBHU5 (41.79%), and P21 (38.81%) at seedlings stage while control (-32.45%), and JRBHU4 (-35.88%) decreased in one-month plants (Figure 6C). Ratio of total chlorophyll to total carotenoids was higher in JRBHU5 (18.55%), and P21 (16.44%) in seedlings while P21 (-21.61%) and JRBHU1 (-21.72%) decreased in one-month plants (Figure 6D). Photosynthetic pigment concentration increased by a certain percentage compared to the control in *Brassica juncea* (Table 3).

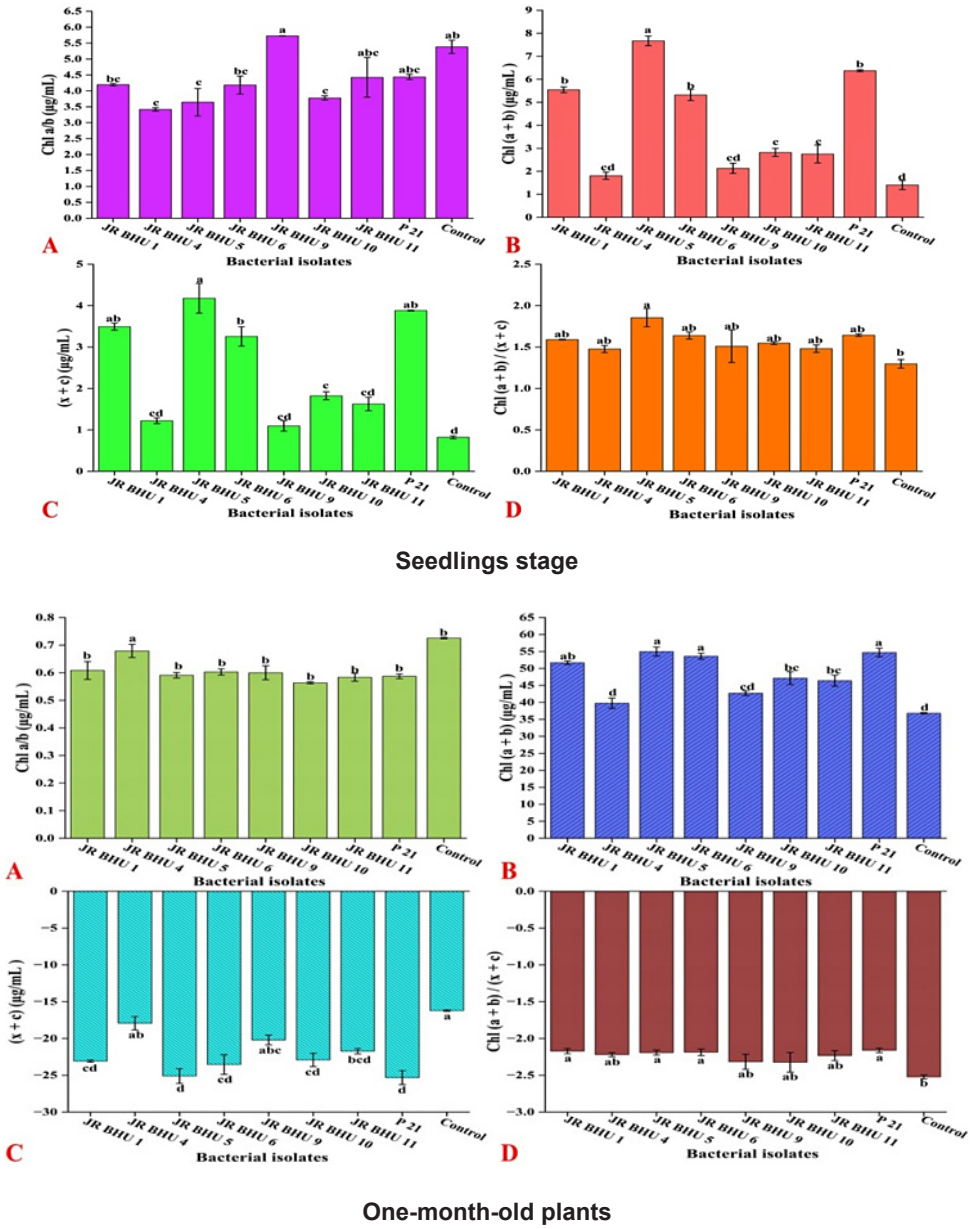


Fig. 6: Impact of PGPRs on the concentration of photosynthetic pigments of *B. juncea* L. at seedlings and one month stage (A) Chl a/b (B) Chl (a+b) (C) (X+C) (D) Chl (a+b) / (X+C)

Pearson correlation coefficients computed among the photosynthetic pigments of *Brassica* to identify the linearity of the correlation among the pigments with respect to growth stages was found to be significant at $p \leq 0.05$ at both growth stages. The correlation results showed that carotenoid content was strongly related to chlorophyll content expressed at different growth conditions. The data analysis showed that chl b was strongly and highly impacted by chl a, and carotenes at seedlings stage, while at one month stage chlorophyll a was found to be positively and highly correlated with chlorophyll b, α carotene, and β carotene. While a positive correlation was found among the different chlorophyll and carotenoid pigments at seedlings stage, after one month a negative correlation was observed among the different chlorophyll and carotenoid pigments due to steady decrease in chlorophyll a and α carotene with plant growth. Thus, increase in carotenoids at seedling stage promoted the chl a synthesis while after one month it promoted the chl b synthesis.

Discussion

The study investigated the influence of eight rhizobacterial isolates on the plant growth, development and pigment concentrations of *B. juncea* L. (Indian mustard). PGPRs in eukaryotic hosts can produce reactive oxygen species (ROS) and cause oxidation bursts within the endophytic cell due to severe conditions like reduced space, nutrition, and host-mediated regulation.[28] Screening an efficient PGPR by analyzing its effect on various physiological and morphological plant parameters in a good practice.[3] The vigour index was chosen as the preliminary screening factor, as the growth of a plant is directly influenced by three crucial growth attributes: germination percentage, root length, and shoot length.[29,30] Vigour index of plants after being inoculated by JRBHU5 and P21 increased by 105.5% and 95.25% compared to the control, indicating that JRBHU5 and P21 were the finest strains. PGPR treatments resulted in increased chlorophyll content, root-shoot diameter, root-shoot length and weight showing delayed leaf senescence compared to non-inoculated controls in cauliflower, muskmelon, and watermelon transplants.[31,32] *Cicer arietinum* seedlings,[33] maize seedlings inoculated with *Burkholderia phytofirmans* and *Enterobacter* sp.[34] and cabbage seedlings inoculated with *Bacillus megaterium* and *Pseudomonas agglomerans*.[35,36] Inoculation of JRBHU5 exceeded the control's shoot dry biomass, possibly due to enhanced photosynthate assimilation and biomass aggregation. Plants inoculated with JRBHU5 showed comparable shoot and roots elongation, suggesting both below and above ground portions growing in response to PGPR. Root water intake and transpiration loss significantly impact the moisture content in shoots and leaves. LFFM, or shoot water content, is an indicator of combustibility. Inoculation of bacterial isolates showed increased shoot moisture, rehydrating the plant and contributing to biomass formation. JRBHU6 (92.4%) and P21 (84.35%) displayed significant increases in LFFM, with JRBHU5 (93.65%) showing the highest value. Plants inoculated with JRBHU5 showed a significant increase in leaf area, which improved the photosynthetic and energy harvesting capabilities, thereby improving plant yield.[37] Improvements were observed in the RWC of plants treated with JRBHU6 and P21 rhizobacterial strains, indicating lateral roots' role in nutrient and water transport, as they increase root surface area for absorption38. *Bacillus*52 and *Pseudomonas putida*51 treatment has been reported to induce soil micro-aggregation, leading to increased soil water content and water availability in the leaves of *Eclipta prostrate*[39]. Plants in drought-prone areas respond better to steady tissue hydration and as rhizospheric bacterial strains accelerate imbibition it also influenced the RWC.[40] PGPRs were crucial in affecting leaf mass and water retention processes along with a significant impact of LDMC.[41,42]

Rhizobacterial treatments also significantly affected the *Brassica*'s leaf pigments content at different growth stages. PGPR inoculation responses are higher at early growth stages, possibly due to root nutrient exchanges becoming more intense during vegetative growth.[43] Exposure to UV light to study the chlorophyll fluorescence revealed a significant increase in red intensity in treatments JRBHU1, JRBHU5, JRBHU6, and P21 at both seedling and one-month stages. UV light promotes electrons from the S0 state to the S2 state in chlorophyll, allowing pigments to observe only blue-violet wavelengths. When excited with ultraviolet light, in the absence of an electron transport chain, chlorophyll molecules fluoresce in red zone, indicating their return to their

ground state.[44] The *Brassica* leaf extract exhibited variations in absorption maxima at different growth stages due to redshift. The study also confirms the influence of refractive index and dielectric constant of solvents on the wavelength shifts in chlorophyll absorption.[45] It is well documented that the interaction between chlorophyll molecules can cause a red shift in absorption spectra.[18,46] The rhizobacterial treatments prominently increased the pigment concentrations over the control. Compared among the treatments JRBHU5, JRBHU6, JRBHU1, and P21 showed a higher pigment amount. In seedlings, Chl a was abundant compared to Chl b, but ratio got reversed in one-month old plants. The concentration of α carotene was higher than β carotene at seedlings stage whereas in one-month plants carotene ratio were found negative due to higher concentration of chl b than chl a. Carotene is an accessory light-harvesting pigment transferring its excitation energy to chlorophyll.[47] Rhizospheric bacterial treatments significantly affected chlorophyll and carotenoid ratios, with JRBHU5 and P21 being most effective. Seedlings had higher chl a: chl b, (x+c), and (chl a+b: x+c), while after one-month plants had higher (chl a+b) and the chl a:chl b in leaves was approximately 3:1. The study clearly indicated that growth conditions and environmental factors have significant impact on pigments ratio. Low light and shade increases LHCPs proportion, resulting in lower chlorophyll a/b values, higher chlorophyll a/ carotene values and total chlorophyll/carotenoids, indicating functional pigment equipment towards light adaptation.[48] Lichtenthaler and Buschmann (1984) suggested that a decrease in Chl a: Chl b could be attributed to the expansion of PS II's antenna system, as the pigment antenna system is the sole reservoir for Chl b.[49] Bacterial strains inoculated in *B. juncea* L. influenced the PS II antenna system size, as an increased chl b concentration was observed after treatments. Leaf greenness which is an indication of the higher ratio of total chlorophyll to carotenoids was increased in response to PGPR, while senescence/stress/damage triggers faster chlorophyll breakdown.[21] The content of chlorophylls to carotenes in leaves after treatments was in the following order JRBHU5>P21>JRBHU6>JRBHU1 indicating a delayed senescence. Thus, the bacteria showed significant effects on maintaining the photosynthetic efficiency of plants, delaying senescence, and regulating the aging process of plants probably by gene duplication, telomerase activity or by increasing meristematic cells.[50] The acdS gene, which codes for ACC deaminase activity, has been identified in *Pseudomonas sp* which helps in stress alleviation, subsequently increasing the total chlorophyll content.[51] Studies have reported the variation in chlorophyll pigments according to season. Variations in the ratio of chl a: total chlorophyll in trees are reported to be highest in early season and minimal in mid-season, with no variability observed in chl b: total chlorophyll across seasons. Pigment derivatives and degradation products influence chlorophyll renewal especially in the mid-growing season.[52] Similarly in the present study variations in the concentrations of chlorophyll a and b was observed during various growth phases. It is crucial to note that chl a and b are reported to be inter-convertible in the chlorophyll cycle,[53] as chl b is synthesized from chl a through chlorophyllide anoxygenase (CAO) enzyme.[54] In the present study, the concentration of photosynthetic pigment got enhanced relative to the control, indicating a considerable effect of PGPRs treatments. Total chlorophyll content was also higher in *Bacillus licheniformis* inoculated plants compared to *Pseudomonas plecoglossicida* and control plants. Among all the bacterial treatments, JRBHU5 and P21 were found to be most influential in affecting the leaf pigments. Both these bacteria promoted the synthesis of chlorophyll a during the seedling stage, and after one-month, their effects were more pronounced on chlorophyll b favoring the normally observed inter-conversions in plant pigments at different growth stages. As plants grow from seedling to juvenile stage, the amounts of chlorophyll increase which affects the chlorophyll to carotenoid ratio, and the bacteria were found to promote the natural process quantitatively as well.

Conclusion

Rhizobacteria Burkholderia spp. (JRBHU1, JRBHU6), *Enterobacter* spp. (JRBHU9, JRBHU10), *Bacillus* spp. (JRBHU11), and *Pseudomonas* spp. (JRBHU4, JRBHU5, P21) enhanced plant growth and development, particularly by influencing photosynthetic pigments in Indian mustard (*Brassica juncea* L.) at various growth stages. Chlorophyll content in one-month plants was higher than carotenoids, compared to the seedling stage. Studies on chlorophyll regeneration can be facilitated by examining pigment derivatives, precursors, and degradation products of key

pigments. JRBHU5 as well as P21 exhibited the most significant influence on leaf pigments ratio, promoting the synthesis of chl a during the seedling stage also facilitating inter-conversions in plant pigments at different growth stages.

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Conflict of Interest
The authors declare that there is no conflict of interest.

Data Availability
This article has all the data that was generated or evaluated during this investigation, and the data can be reproduced and is provided by the authors upon request.

Author's Contribution
Surya Prakash Dube: Data curation; Formal analysis; Investigation; Methodology; Visualization; Roles/Writing – original draft. Riddha Dey: Validation; Visualization; Writing. Seema Devi: Validation; Visualization; Writing. Richa Raghuwanshi: Conceptualization; Project administration; Resources; Supervision; Validation; Visualization; Review & editing.

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