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Mycorrhizal and bacterial inoculants enhance photosynthesis, water relations, and leaf thermal regulation in *Vigna Radiata* L. under soilless substrate culture

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Plant growth-promoting microorganisms (PGPMs) and biochar are increasingly recognized as sustainable strategies to enhance crop performance; however, their comparative and integrative effects on carbon–water coordination and leaf thermal regulation under soilless substrate conditions remain poorly resolved. We investigated the physiological responses of *Vigna radiata* L. to bacterial biostimulant (BB), arbuscular mycorrhizal (AM) fungi, their co-inoculation (BA), and BA combined with biochar (BiB). All treatments enhanced biomass relative to the uninoculated control, but through distinct physiological mechanisms. BB reduced leaf temperature (LT) primarily via increased stomatal conductance and transpiration, supporting higher net photosynthesis. In contrast, AM enhanced plant water status, as reflected by higher relative water content, and maintained intrinsic water-use efficiency comparable to the control but greater than in the other inoculation treatments. The reduction in LT under AM, despite unchanged transpiration, may reflect differences in plant water relations rather than purely evaporative cooling. BA integrated these complementary functions, resulting in the lowest LT, and maximum biomass. BiB further enhanced photosynthetic rate and maintained intrinsic water-use efficiency, although biomass was slightly lower than in BA. Collectively, the results demonstrate that the distinct physiological roles of bacterial and mycorrhizal inoculants, stomatal-driven carbon acquisition versus hydraulic stabilization, become functionally complementary under co-inoculation, enabling coordinated regulation of carbon assimilation, water balance, and leaf thermal dynamics to maximize biomass production. By reducing leaf temperature, PGPMs could contribute to maintaining photosynthetic efficiency under potential heat stress.

Keywords PGPB, Mycorrhiza, PGPM, Biochar, Photosynthesis

Global agricultural productivity is increasingly challenged by climatic extremes, resource limitations, and environmental stressors, including heat, and drought. In response, sustainable strategies that enhance plant performance while minimizing chemical inputs are urgently needed. Among these, plant growth-promoting microorganisms (PGPMs), including beneficial bacteria and arbuscular mycorrhizal (AM) fungi, have emerged as key biotic tools to improve crop growth and stress resilience through multiple physiological and biochemical pathways.

Vigna radiata L. was selected due to its agronomic, nutritional, and socio-economic importance. As a protein-rich legume widely cultivated in traditional farming systems, it plays a significant role in food security and rural livelihoods. Its rapid growth and high biomass production make it well-suited for controlled

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cultivation systems. Although primarily grown in soil, soilless culture systems are increasingly used to study plant–microbe interactions and improve nutrient use efficiency. To our knowledge, this study represents one of the first evaluations of *V. radiata* L. under soilless conditions combined with microbial consortia and biochar, providing a controlled framework to assess physiological responses[1–3].

Plant growth-promoting bacteria (PGPB) can stimulate plant growth by enhancing nutrient availability, producing phytohormones, and promoting stomatal conductance, thereby improving carbon assimilation and water-use dynamics[4,5]. AM fungi, in contrast, enhance root hydraulic conductivity, water uptake, and nutrient foraging, contributing to improved tissue hydration and intrinsic water-use efficiency[6,7]. In parallel, biochar has gained attention for its ability to improve substrate aeration, nutrient retention, and water-holding capacity, potentially enhancing the effectiveness of microbial inoculants[8,9].

Photosynthesis is the central process driving plant growth, and its regulation depends on both diffusional and biochemical factors. Net photosynthetic rate (Pn) reflects the overall capacity for carbon assimilation, integrating stomatal and metabolic limitations. Stomatal conductance (gs) and transpiration rate (E) primarily represent the diffusional control of CO_2 entry and water vapor loss, thereby linking carbon gain with plant water status and evaporative cooling. Intercellular CO_2 concentration (Ci) provides mechanistic insight into whether reductions in Pn arise from stomatal limitation (low Ci) or from non-stomatal/biochemical constraints such as impaired Rubisco activity or electron transport (normal or elevated Ci). Together, these parameters allow discrimination between hydraulic, stomatal, and metabolic controls over photosynthesis and enable evaluation of water-use efficiency. Assessing how PGPB and AM fungi influence these interconnected processes under controlled conditions therefore provides functional insight into their roles in regulating carbon assimilation, water relations, and thermal balance.

Soilless culture offers a powerful experimental platform, as its precise control of nutrients and elimination of soil heterogeneity enhance the resolution of physiological measurements. This enables clearer attribution of changes in gas exchange, water-use efficiency, and leaf temperature to specific microbial inoculants. Because root–microbe interactions occur in a defined nutrient solution environment, this system also permits clearer attribution of plant responses to specific microbial inoculants by minimizing confounding soil effects[10–12]

An underexplored dimension of microbial influence is thermal regulation at the leaf level. Leaf temperature is a critical determinant of photosynthetic performance, water conservation, and stress resilience, as it reflects the balance between absorbed radiation, transpiration, and tissue hydration[13,14]. While microbial inoculants are known to improve water relations and stomatal function, their potential role in modulating leaf temperature dynamics has not been systematically investigated, particularly in soilless substrate conditions.

In this study, we investigated the physiological and growth responses of *V. radiata* L. grown in soilless system (sand:perlite) to bacterial inoculants (BB), arbuscular mycorrhizal (AM) fungi, their combination (BA), and a triple treatment including biochar (BiB).

The specific objectives were to:

1. Assess the effects of PGPM separately and in addition to biochar on photosynthetic performance, carbon assimilation, and water-use efficiency.
2. Examine the potential role of PGPBs and AM fungi in thermal regulation by evaluating leaf-level physiological traits.
3. Determine how these coordinated physiological adjustments translate into biomass accumulation.

By integrating microbial inoculation and biochar application in the controlled sand:perlite substrate culture, this study investigates how microbial consortia regulate carbon assimilation, water balance, and biomass accumulation. Stomatal conductance is central to coordinating CO_2 uptake with transpirational water loss, thereby linking photosynthesis to plant hydraulic status, while leaf temperature serves as an integrative indicator of energy balance and transpirational cooling. Comparing bacterial and fungal inoculants enables mechanistic differentiation between stomatal-driven carbon acquisition and hydraulic stabilization. Assessing these effects individually and in combination provides a framework to determine whether functional complementarity among microbial partners can harmonize carbon–water coordination and optimize crop performance.

Materials and methods

Seeds of *V. radiata* L., representing a local germplasm traditionally cultivated in the region, were collected from Ashkan Village, Alan Rural District, Sardasht County, West Azerbaijan Province, Iran. A seed sample was deposited at Zist Asa Clinic, Urmia, where seed identification and collection were confirmed (Fig. 1).

Seed sterilization and preparation of microbial inoculants

Seeds of *V. radiata* L. were surface-sterilized with 5% (v/v) sodium hypochlorite for 10 min and rinsed three times with sterile distilled water. Sterilized seeds were air-dried under sterile conditions prior to inoculation. The seeds were divided into five groups: control (no inoculation), bacterial biofertilizers (BB), arbuscular mycorrhizal (AM) fungi, a combination of BB and AM (BA), and a combination of BB, AM, and biochar (BiB).

Bacterial inoculants consisted of nitrogen-fixing bacteria (*Pantoea agglomerans* O4), phosphorus-solubilizing bacteria (*Pseudomonas putida* P13 and *Pantoea agglomerans* P5), potassium-solubilizing bacteria (*Pseudomonas vancouverensis* S19 and *Pseudomonas koreensis* S14), and zinc/iron-solubilizing bacteria (*Pseudomonas japonica* FZ.21-1 and FZ.29-1).

These strains were selected based on previously documented plant growth-promoting traits, including nitrogen fixation[15], phosphate solubilization[16,17], potassium solubilization[18], and zinc/iron solubilization[19]. All inoculants were obtained from Green Biotech Incorporation, Tehran, Iran.



Fig. 1. Seeds of *Vigna radiata* L. used in this study.

For bacterial inoculation, 0.1 g of solid biological NPK formulation (10^7–10^8 CFU g^{-1}) was dissolved in 200 mL sterile distilled water. Separately, 3 mL of FeZn biofertilizer (10^7–10^8 CFU g^{-1}) was diluted in 300 mL sterile distilled water. This formulation is intended to improve the availability of iron and zinc to the plants. Equal volumes (100 mL each) of the prepared suspensions were mixed thoroughly and uniformly sprayed onto the seeds. Approximately 20 seeds were inoculated per treatment.

AM fungal inoculum contained *Glomus etunicatum*, *Glomus mosseae*, and *Glomus intraradices*. For inoculation, 5 mg of solid AM inoculum (10^7–10^8 propagules g^{-1}) was suspended in 200 mL sterile distilled water and applied uniformly to the seeds. For the combined BA treatment, equal volumes (100 mL) of bacterial and AM suspensions were mixed prior to application. The inoculated seeds were air-dried for 1 h prior to sowing. Seeds in the BiB treatment were inoculated with the same microbial consortium used in the BA treatment, with the addition of biochar to the growth substrate. Biochar (< 2 mm particle size) was incorporated into the substrate at 5% (w/w) prior to planting.

Seeds were sown in plastic pots (17 × 18 cm) containing a 1:1 (v/v) mixture of river sand and perlite. River sand was washed and sterilized by autoclaving at 120 °C for 20 min before use. Ten seeds were sown per pot, and seedlings were thinned to seven uniform plants per pot at the two-leaf stage.

Biochar preparation and analysis

Dried apple wood was pyrolyzed at 550 °C for 5 h under anaerobic conditions to produce biochar[20]. The biochar was then ground and sieved to obtain particles smaller than 2 mm for use in BiB treatment. A 1:20 (w/v) biochar:distilled water suspension was prepared and shaken for 2 h at room temperature to measure pH and electrical conductivity (EC) using a Hanna conductivity meter (HI 8819, Portugal) and a Corning pH meter 7 (UK). Ash and volatile matter were determined in a muffle furnace following standard methods, and organic matter content was calculated using the loss-on-ignition method[21]. Total nutrients (N, P, K) were measured after acid digestion with $HNO_3/HClO_4$ mixture (3:1) in a perchloric-acid-rated fume hood. The resulting biochar had a pH of 8.35, EC of 0.79 dS/m, organic matter content of 53%, total N of 0.9%, P of 0.23%, K of 0.41%, and a C/N ratio of 58.8.

Growth conditions

Plants were grown under controlled environmental conditions (Fig. 2) with a light intensity of approximately 150 µmol photons m^{-2} s^{-1} (equivalent to 7,000 lx) provided by a combination of red and blue TD lamps. Temperature ranged from 17 °C (minimum) to 33 °C (maximum). The photoperiod was 16 h light / 8 h dark, and relative humidity was maintained at approximately 60%.

A modified Hoagland nutrient solution[22] was prepared in 20 L batches. Macronutrient stock solutions (1 M) were made in separate 1 L containers at the following concentrations: KNO_3 101.10 g L^{-1}, $Ca(NO_3)_2{\cdot}4H_2O$ 236.16 g L^{-1}, $NH_4H_2PO_4$ 115.08 g L^{-1}, and $MgSO_4{\cdot}7H_2O$ 246.49 g L^{-1}. For each 20 L batch, 120 mL of KNO_3 stock, 80 mL of $Ca(NO_3)_2{\cdot}4H_2O$, 40 mL of $NH_4H_2PO_4$, and 20 mL of $MgSO_4{\cdot}7H_2O$ were added to 18 L of water. A combined micronutrient stock (KCl 1.864 g L^{-1}, H_3BO_3 0.773 g L^{-1}, $MnSO_4{\cdot}H_2O$ 0.169 g L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.288 g L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.062 g L^{-1}, H_2MoO_4 0.040 g L^{-1}) was prepared, and 40 mL of this stock (2 mL L^{-1}) was added to the batch. Iron was supplied from a separate NaFeDTPA (10% Fe) stock at 30.0 g L^{-1}, adding 10 mL per batch (0.5 mL L^{-1}). The pH was adjusted to 6.5 ± 0.1 using 1 M KOH or HCl, and the volume was brought to 20 L with water. This nutrient solution was used daily to irrigate the pots.

Seedlings were initially acclimated with ¼-strength solution for the first two days, followed by ½-strength solution for an additional two days, before switching to full-strength solution for the remainder of the experiment.



Fig. 2. Left: *Vigna radiata* L. at the two-leaf stage. Right: plant at the time of physiological and growth analysis.

To prevent salt accumulation in the substrate, pots were leached once per week with approximately 600 mL of distilled water.

Physiological and growth measurements

Three weeks after sowing, gas exchange parameters were measured on the terminal leaflet of the third fully expanded trifoliate leaf of one of the seven cultured plant in the pot using a photosynthesis system (Walz HCM-1000, Heinz Walz GmbH, Germany) while plants remained in pots. The measured parameters included net photosynthetic rate (Pn, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}), transpiration rate (E, mmol H_2O m^{-2} s^{-1}), intercellular CO_2 concentration (Ci, µmol mol^{-1}), and leaf temperature (T_leaf, °C).

Instantaneous water-use efficiency (WUEins) and intrinsic water-use efficiency (WUEint) were calculated as Pn/E and Pn/gs, respectively. Measurements were conducted under ambient CO_2 concentration (~ 400 µmol mol^{-1}), chamber temperature of 25–26 °C, relative humidity of 50–55%, and photosynthetically active radiation (PAR) of 1700–1800 µmol m^{-2} s^{-1}.

Leaf area (LA) of the measured leaflet was determined using a leaf area meter. Relative water content (RWC) was measured according to Barrs & Weatherley[23], as described by Piršelová et al.[24]. For biomass determination, after harvesting, three plants per pot were chosen. Shoot dry weight (SDW) and root dry weight (RDW) were recorded after oven-drying at 70 °C for 48 h to constant weight.

Experimental design and statistical analysis

The experiment was arranged in a completely randomized design (CRD) consisting of four inoculation treatments (BB, AM, BA, and BiB) and an uninoculated control (C), each replicated three times. Each pot was considered a biological replicate and contained seven plants after thinning. Data were analyzed using SAS software. Analysis of variance (ANOVA) was performed to determine treatment effects. When significant differences were detected, means were compared using Tukey's honestly significant difference (HSD) test at $P \leq 0.05$. Data normality and homogeneity of variance were verified prior to ANOVA.

In this experiment the absence of a biochar-only control represents a limitation of the experimental design.

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Results

Photosynthetic gas exchange

Microbial treatments significantly influenced net photosynthesis (Pn) and related gas-exchange parameters (Fig. 3 and Table 1). For Pn, the BiB treatment (13.44) and BB (12.51) recorded the highest values and were statistically similar to each other (both sharing letter a and ba respectively), while BA (11.14, bc) and AM (9.63, dc) showed intermediate values that did not differ significantly from the control (C, 8.97, d). Intercellular CO_2 concentration (Ci) was highest in BiB (18.6) and BB (18.25), both sharing letter a, followed by BA (17.52, ba), whereas AM (15.66) and the control (15.58) had significantly lower values (letter b). Transpiration rate (E) was highest in BA (4.03) and BB (3.82), both with letter a, while the control (2.51, b) and AM (2.17, b) showed significantly lower rates. Stomatal conductance (g_s) was highest in BB and BA (both 0.14, letter a), followed by BiB (0.10, b) and then AM and control (both 0.07, letter b). These results indicate that bacterial-containing treatments (BB, BA, BiB) generally enhanced gas exchange, whereas AM alone did not significantly improve Pn or g_s relative to the control.

Water relations and water-use efficiency

Relative water content (RWC) differed significantly among treatments (Fig. 3 and Table 1). AM-treated plants had the highest RWC (0.89, letter a), followed by BA (0.79, ba) and the control (0.75, bc), while BB (0.65) and BiB (0.66) showed the lowest values (both letter c). Instantaneous water-use efficiency (WUE_{ins}) was highest in AM (4.43, letter a), intermediate in the control (3.59, ba) and BiB (3.59, ba), and lowest in BB (3.29, b) and BA (2.76, b). Intrinsic water-use efficiency (WUE_{int}) was also highest in AM (127.68, a) and BiB (121.45, a), followed by the control (114.47, a), whereas BB (86.5, b) and BA (76.17, b) had significantly lower values. These patterns highlight contrasting water-use strategies: AM supported high hydration and high water-use efficiency, while bacterial treatments (BB, BA) favored higher transpiration.

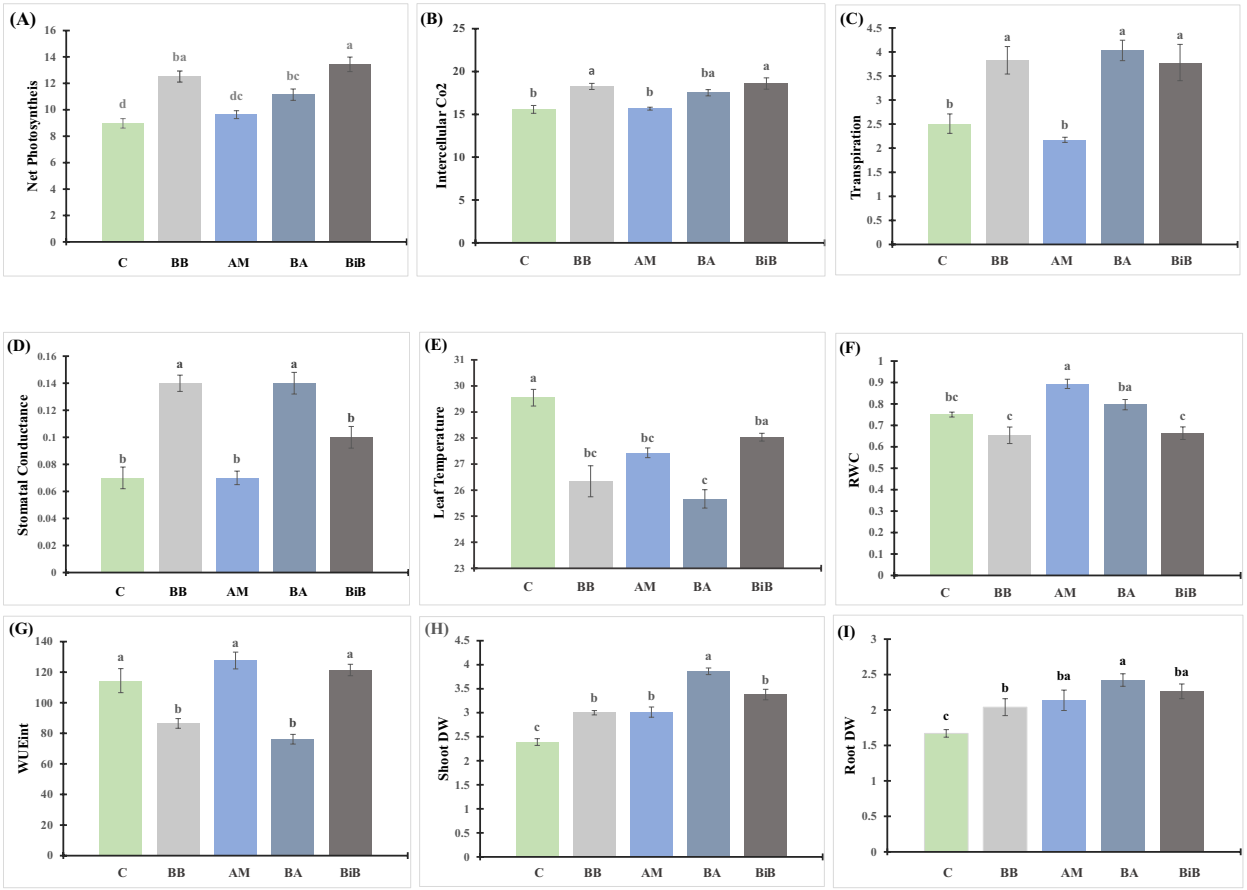


Fig. 3. Photosynthesis and growth responses of *Vigna radiata* L under different treatments. Parameters: (**A**) net photosynthesis rate (Pn, µmol CO_2 m^{-2} s^{-1}); (**B**) intercellular CO_2 concentration (Ci, µmol mol^{-1}); (**C**) transpiration rate (E, mmol H_2O m^{-2} s^{-1}); (**D**) stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}); (**E**) leaf temperature (LT, °C); (**F**) relative water content (RWC); (**G**) intrinsic water use efficiency (WUEint, µmol CO_2 mol^{-1} H_2O); (**H**); shoot dry weight (SDW, g $plant^{-1}$); (**K**) root dry weight (RDW, g $plant^{-1}$). Treatments: BB = bacterial biostimulators; AM = arbuscular mycorrhiza; BA = BB + AM; BiB = BB + AM + biochar; and C = Control. Different letters show a significant difference at the $P < 0.05$ level based on Tukey's test.

Leaf temperature (LT)

Leaf temperature was significantly affected by treatments (Fig. 3 and Table 1). The control had the highest leaf temperature (29.55 °C, letter a). Among the inoculated treatments, BA showed the lowest temperature (25.66, c), which was significantly lower than the control and also lower than AM (27.43, bc) and BiB (28.03, ba). BB (26.34) and AM (27.43) were statistically similar (both sharing bc), whereas BiB (ba) was intermediate and not significantly different from the control. Therefore, not all microbial treatments significantly reduced leaf temperature relative to the control; BA produced the greatest reduction.

Leaf area and biomass

Microbial inoculation significantly enhanced leaf expansion and biomass accumulation (Fig. 3 and Table 1). Leaf area (LA) was highest in BA (16.66 cm^2, letter a), followed by BiB (16.16, ba), AM (15.80, ba), BB (15.20, ba), and the control (14.16, b). BA was significantly higher than the control, but the other treatments did not differ statistically from each other (sharing ba). Shoot dry weight (SDW) was highest in BA (3.86, a), followed by BiB (3.37, b), BB (3.00, b), and AM (3.01, b), all significantly higher than the control (2.39, c). Root dry weight (RDW) followed a similar pattern: BA produced the highest (2.42, a), BiB (2.26, ba) and AM (2.13, ba) were intermediate, BB (2.04, b) was lower, and the control had the lowest (1.66, c).

Correlation analysis

Pearson's correlation analysis, performed on treatment means (n = 5), showed coordination among gas exchange, water-use efficiency, and biomass traits (Table 2). Pn was positively correlated with Ci ($r = 0.979$, $P < 0.01$), while Ci was positively associated with E ($r = 0.914$, $P < 0.05$) and E with gs ($r = 0.903$, $P < 0.05$). WUEint showed a negative correlation with gs ($r = -0.902$, $P < 0.05$), and WUEins was positively correlated with WUEint ($r = 0.887$, $P < 0.05$), reflecting coordinated adjustments in water-use efficiency. Biomass traits formed a highly

Treatments	Pn	Ci	E	gs	LT	WUEins	WUEint	RWC	LA	SDW	RDW
C	8.97 ± 0.36d	15.58 ± 0.45b	2.51 ± 0.2b	0.07 ± 0.008b	29.55 ± 0.31a	3.59 ± 0.15ba	114.47 ± 7.86a	0.75 ± 0.01bc	14.16 ± 0.16b	2.39 ± 0.07c	1.66 ± 0.05c
BB	12.51 ± 0.42ba	18.25 ± 0.35a	3.82 ± 0.28a	0.14 ± 0.006a	26.34 ± 0.59bc	3.29 ± 0.2b	86.5 ± 3.18b	0.65 ± 0.03c	15.2 ± 0.49ba	3 ± 0.04b	2.04 ± 0.11b
AM	9.63 ± 0.3dc	15.66 ± 0.17b	2.17 ± 0.05b	0.07 ± 0.005b	27.43 ± 0.18bc	4.43 ± 0.16a	127.68 ± 5.48a	0.89 ± 0.02a	15.8 ± 0.28ba	3.01 ± 0.1b	2.13 ± 0.14ba
BA	11.14 ± 0.43bc	17.52 ± 0.37ba	4.03 ± 0.21a	0.14 ± 0.008a	25.66 ± 0.35c	2.76 ± 0.05b	76.17 ± 3.18b	0.79 ± 0.02ba	16.66 ± 0.13a	3.86 ± 0.06a	2.42 ± 0.08a
BiB	13.44 ± 0.55a	18.6 ± 0.66a	3.78 ± 0.37a	0.1 ± 0.008b	28.03 ± 0.15ba	3.59 ± 0.19ba	121.45 ± 3.7a	0.66 ± 0.02c	16.16 ± 0.44ba	3.37 ± 0.1b	2.26 ± 0.1ba

Table 1. Mean comparison of photosynthetic, water relation, thermal, and biomass traits of *Vigna radiata* L. under microbial treatments. Values are means ± SE (n = 3). Different letters within a column indicate significant differences among treatments according to Tukey's HSD test ($p < 0.05$). Parameters: Pn, net photosynthesis rate (μmol CO_2 m^{-2} s^{-1}); Ci, intercellular CO_2 concentration (μmol CO_2 mol^{-1}); E, transpiration rate (mmol H_2O m^{-2} s^{-1}); g_s, stomatal conductance (mol H_2O m^{-2} s^{-1}); LT, leaf temperature (°C); RWC, relative water content (%); WUEint, intrinsic water-use efficiency (μmol CO_2 mol^{-1} H_2O); WUEins, instantaneous water-use efficiency (μmol CO_2 $mmol^{-1}$ H_2O); LA, leaf area (cm^2); SDW, shoot dry weight (g); RDW, root dry weight (g). Treatments: BB, bacterial biostimulant; AM, arbuscular mycorrhizal fungi; BA, BB + AM; BiB, BB + AM + biochar; C, control.

Traits	Pn	Ci	E	gs	LT	WUEins	WUEint	RWC	LA	SDW	RDW
Pn	1	0.98 **	0.81	0.62	-0.41	-0.37	-0.25	-0.74	0.49	0.53	0.55
Ci	0.98 **	1	0.91 *	0.75	-0.5	-0.55	-0.43	-0.77	0.49	0.59	0.57
E	0.81	0.91 *	1	0.90 *	-0.63	-0.83	-0.72	-0.66	0.53	0.70	0.61
gs	0.626	0.75	0.90 *	1	-0.83	-0.81	-0.90 *	-0.47	0.47	0.66	0.56
LT	-0.41	-0.5	-0.62	-0.83	1	0.48	0.74	-0.02	-0.71	-0.79	-0.77
WUEins	-0.37	-0.55	-0.83	-0.81	0.48	1	0.89 *	0.47	-0.25	-0.51	-0.32
WUEint	-0.25	-0.43	-0.72	-0.90 *	0.74	0.88 *	1	0.26	-0.26	-0.5	-0.35
RWC	-0.74	-0.77	-0.66	-0.47	-0.03	0.47	0.26	1	0.16	0.02	0.08
LA	0.49	0.49	0.53	0.47	-0.71	-0.25	-0.26	0.16	1	0.96 **	0.99 **
SDW	0.53	0.59	0.70	0.66	-0.78	-0.51	-0.49	0.02	0.96 **	1	0.97 **
RDW	0.55	0.57	0.61	0.56	-0.77	-0.32	-0.34	0.08	0.99 **	0.97 **	1

Table 2. Pearson correlation coefficients among photosynthetic, water relation, thermal, and growth traits of *Vigna radiata* L. under microbial and biochar treatments (n = 5, treatment means). Significant correlations at $P < 0.05$ (*) and $P < 0.01$ (**) are indicated. The table highlights coordinated responses among gas exchange, water-use efficiency, and biomass traits. Parameters: Pn, net photosynthetic rate; Ci, intercellular CO_2 concentration; E, transpiration rate; g_s, stomatal conductance; LT, leaf temperature; WUEins, instantaneous water-use efficiency; WUEint, intrinsic water-use efficiency; RWC, relative water content; LA, leaf area; SDW, shoot dry weight; RDW, root dry weight.

integrated cluster, with LA correlated with SDW ($r = 0.961$, $P < 0.01$) and RDW ($r = 0.993$, $P < 0.01$). In contrast, RWC showed no significant relationships with most physiological traits.

Discussion

This study reveals that distinct microbial configurations differentially shaped carbon assimilation, water-use dynamics, thermal regulation, and biomass accumulation in *V. radiata* L. under a soilless substrate culture (sand:perlite). Bacterial biostimulate (BB) and biochar plus microbial inoculation (BiB) maximized net photosynthesis, whereas co-inoculation of AM fungi and bacteria (BA) translated physiological gains into the greatest increases in leaf area and total biomass. Intrinsic water-use efficiency (WUEint) remained comparable to control levels in AM and BiB treatments; however, the control accumulated the least biomass. In contrast, BB reduced WUEint due to elevated stomatal conductance and transpiration accompanying enhanced CO_2 assimilation, suggesting that bacterial-mediated biomass gains were achieved at the cost of greater water expenditure. These patterns highlight a functional trade-off between carbon gain and water conservation across treatments. Collectively, the findings suggest complementary physiological roles: BB primarily enhanced stomatal-driven gas exchange, BA optimized the translation of assimilated carbon into structural biomass, and the BiB and AM supported high biomass performance while maintaining WUEint. Moreover, the consistent reduction in leaf temperature across microbial treatments indicates coordinated modulation of transpiration and energy balance.

Physiological measurements were conducted at the early vegetative stage (three weeks post-germination) to capture the initial effects of microbial inoculation on carbon–water relations and leaf thermal regulation. This stage represents the period when seedlings establish root–microbe interactions and initiate active photosynthesis, enabling the detection of early functional differences between bacterial and mycorrhizal inoculants. While the results reveal distinct physiological patterns at this stage, plant responses may shift during later developmental phases due to changes in sink–source dynamics and biomass allocation. Future studies should determine whether the functional complementarity observed here persists or evolves throughout subsequent vegetative and reproductive stages.

Linking microbial traits to physiological outcomes

The bacterial and fungal strains used in this study possess specific plant-growth-promoting (PGP) traits documented in the literature, which help explain the observed treatment effects. The nitrogen-fixing *Pantoea agglomerans* O4[15] and the phosphate-solubilizing *Pseudomonas putida* P13 and *Pantoea agglomerans* P5[16,17] likely enhanced nitrogen and phosphorus availability, supporting the higher photosynthetic rates (Pn) and biomass accumulation in BB and BiB treatments. The potassium-solubilizing *Pseudomonas vancouverensis* S19 and *P. koreensis* S14[18] may have improved K^+ uptake, which is known to regulate stomatal function, consistent with the increased stomatal conductance (g_s) and transpiration (E) observed in bacterial treatments. The Zn/Fe-solubilizing *Pseudomonas japonica* strains FZ.21-1 and FZ.29-1[19] could have alleviated micronutrient limitations, aiding chlorophyll synthesis and overall photosynthetic efficiency. The AM fungi (*G. etunicatum*, *G. mosseae*, and *G. intraradices*) are known to enhance root hydraulic conductivity and osmotic adjustment[25,26], which aligns with their higher relative water content (RWC) and WUE_{int} without elevated stomatal conductance. Explicitly linking these strain-specific traits to our measured variables provides a stronger biological basis for interpreting the distinct physiological and growth responses among treatments.

Photosynthetic performance and carbon assimilation

The physiological responses of *V. radiata* L. to microbial inoculation revealed two complementary routes to improved carbon gain. BiB and BB exhibited the highest photosynthetic rates, whereas AM alone maintained lower Pn while preserving relatively high WUEint, comparable to the control and BiB. The application of PGPMs can enhance photosynthetic performance in plants[27,28]. This improvement is often attributed to the stimulation of stomatal opening, increasing the uptake of CO_2 from the atmosphere. For example, inoculating sweet pepper with *Azospirillum brasilense* and *Pantoea dispersa* maintained higher stomatal conductance under salt stress[29], while *Sphingobacterium changzhouense* enhanced photosynthesis and stomatal conductance in maize under drought conditions[30].

Elevated Pn in BB and BiB aligns with bacterial stimulation of stomatal opening and nutrient-driven enhancement of photosynthetic biochemistry, including improved nitrogen and phosphorus acquisition[4,5]. Similarly, microbial inoculants have been reported to enhance the biochemical aspects of photosynthesis by improving the plant's nutritional status[31,32]. A consortium of AM fungi, yeasts, and bacteria significantly improved N, P, and K concentrations in strawberry plants, correlating with higher photosynthetic rates under drought[31].

In our previous study, PGPM inoculation, with the exception of AM alone, significantly increased chlorophyll a and b contents, suggesting that elevated pigment levels contributed to the higher photosynthetic capacity observed in these treatments[2]. Similar findings have been reported in other studies, where increased chlorophyll content enhanced light-harvesting capacity, supporting greater photosynthetic efficiency and carbon assimilation[28,30].

The addition of biochar, particularly in combination with microbial inoculants, has been reported to enhance photosynthetic performance in soil-based systems[27]. In such systems, biochar can improve substrate physicochemical properties, including porosity, water retention, and nutrient availability, thereby indirectly supporting root development and microbial activity[9,33,34]. However, the present study was conducted under soilless conditions, where soil structural improvements and nutrient retention mechanisms are not directly applicable. Moreover, parameters such as nutrient dynamics, and root–rhizosphere interactions were not directly assessed. Therefore, the responses observed under the BiB treatment may reflect indirect or interactive effects between biochar and microbial inoculants rather than confirmed changes in substrate properties.

Stomatal vs. non-stomatal mechanisms

Different microbial treatments can enhance photosynthesis through distinct mechanisms, which can be broadly categorized as stomatal and non-stomatal (or biochemical) factors. Stomatal-driven enhancement: Some microbial inoculants primarily increase CO_2 uptake by promoting higher stomatal conductance[29]. While effective, this mechanism makes the plant's carbon gain more reliant on environmental factors such as water status and vapor pressure deficit. Non-stomatal enhancement: Other treatments improve photosynthesis by targeting biochemical limitations. For instance, a study on *Aronia melanocarpa* found that a microbial inoculant helped the plant avoid a "noon break" in photosynthesis caused by non-stomatal factors, boosting the intrinsic efficiency of the photosynthetic apparatus[35]. Enhancements in chlorophyll content, nutrient uptake, and photosynthetic enzyme activity also contribute to this category[28,31].

In the present study, the BiB treatment achieved the highest Pn despite only moderate stomatal conductance, whereas BB and BA exhibited higher gs but lower Pn. This partial decoupling of Pn from gs indicates that photosynthetic enhancement under BiB was not solely governed by stomatal limitation and suggests a contribution of non-stomatal processes. Although biochemical determinants such as mesophyll conductance, carboxylation capacity, or chlorophyll content were not measured, previous studies have shown that microbial–biochar interactions can influence these components of the photosynthetic machinery[27,36].

By contrast, BB responses appeared more directly linked to stomatal-driven CO_2 diffusion, as increases in gs were closely aligned with changes in Pn. Notably, BA produced greater biomass than BB despite comparable stomatal conductance, implying differences in carbon-use efficiency or post-assimilatory allocation. Collectively, these findings suggest that the relative contributions of stomatal and biochemical factors varied among treatments, warranting targeted investigation of photosynthetic limitations to resolve the underlying mechanisms.

Thermal regulation and water relations

Microbial inoculations exerted clear effects on leaf temperature (LT), transpiration (E), stomatal conductance (g_s), and relative water content (RWC), revealing distinct, and sometimes complementary, strategies of thermal regulation. RWC, a reliable indicator of tissue hydration and cellular water balance, reflects the leaf's capacity to maintain turgor and sustain metabolic activity[37]. In our experiment, BB and BA treatments exhibited high g_s and E, which translated into lower LT, especially in BA. These results support the role of PGPB in promoting stomatal opening, enhancing evaporative cooling and short-term carbon assimilation[5,29]. At the same time, the high transpiration observed in BB coincided with reduced tissue hydration, illustrating the trade-off between evaporative cooling and maintenance of leaf water status.

By contrast, AM-inoculated plants sustained high RWC despite low g_s and E and a moderate LT. This pattern is consistent with the idea that AM-mediated thermal regulation may rely less on stomatal evaporative cooling and more on improved whole-plant water relations, such as enhanced root hydraulic conductivity or osmotic adjustment, as proposed in previous studies[25]. AM symbioses have been shown to upregulate aquaporin expression and alter root hydraulic architecture, thereby sustaining leaf water potential and allowing more conservative stomatal behavior without a catastrophic loss of photosynthetic capacity[26,38,39].

Co-inoculation (BA) integrated these complementary strategies: BA achieved the lowest LT while maintaining relatively high RWC and elevated transpiration, indicating a synergistic balance between stomatal cooling and hydraulic support. This synergy likely reflects coordinated improvements in nutrient status, hormonal signalling,

and root–microbe interactions that allow stomata to operate more effectively without excessively draining tissue water reserves[13,40].

Mechanistically, PGPB and AM fungi can regulate g_s through multiple, partly overlapping pathways. PGPBs modulate phytohormones (ABA, cytokinins, auxins), produce ACC-deaminase, solubilize minerals, and stimulate osmolyte synthesis, all of which can shift stomatal sensitivity and plant water use toward a more favourable carbon–water balance under stress[34,41–45]. Some reports further suggest that certain bacteria alter ABA signaling or its sensitivity in leaves, giving plants a finer control of stomatal aperture under fluctuating water availability[44,46]. At the guard-cell level, microbial partners promote osmolyte accumulation and antioxidant activity that maintain guard-cell turgor and responsiveness. Microbial inoculation has also been associated with changes in stomatal anatomy (density and pore traits)[47].

The regulation of leaf temperature by microbial inoculants is an emerging and rapidly developing field. While transpiration is the primary driver of leaf cooling[48], recent studies using thermal imaging and physiological assays have directly shown that PGPB consortia can lower leaf temperature under water stress by sustaining stomatal opening and improving plant water status[39,49]. Conversely, AM fungi may buffer thermal extremes, potentially through improved osmotic adjustment and increased water uptake capacity, as suggested by previous studies[50,51]. The complementary functions of PGPB (evaporative cooling) and AM fungi (hydration-based buffering) explain why co-inoculation frequently produces additive or synergistic benefits for leaf energy balance, photosynthesis, and growth across taxa[42,52].

The magnitude and direction of microbial effects depend on strain identity, host genotype, environmental context, and the timing of inoculation[40,53]. Some studies report no synergy under certain conditions, underscoring the importance of matching microbial consortia to crop and environment. Nevertheless, our soilless experiment demonstrates that a combined AMF + PGPB treatment (BA) lowers leaf temperature more effectively than single inoculants, while also protecting tissue hydration and supporting transpiration-driven cooling, represents a novel contribution to the literature[7]. These results suggest that microbial consortia could re-balance the leaf energy budget through integrated hydraulic and stomatal mechanisms, a possibility consistent with the literature.

Growth responses: leaf area and biomass

Growth responses of *V. radiata* L. paralleled the physiological trends observed among treatments. BA produced the greatest leaf area and biomass accumulation, resulting in the highest shoot and root dry weight. The BiB treatment also enhanced growth relative to single inoculants and the control; however, shoot dry weight remained significantly lower than BA. Overall, biomass responses followed the hierarchy: BA > BiB > single inoculants > control.

Interestingly, this pattern differs from our previous soil-based study, in which the combined application of PGPMs and biochar resulted in the highest biomass (PGPM + biochar > PGPMs > single inoculants > single biochar > control)[2]. The contrasting outcomes highlight the complexity of plant–microbe–substrate interactions and suggest that responses to biochar plus PGPM may differ substantially between soil systems and soilless sand:perlite media supplied with nutrient solution.

The superior performance of BA in the present system suggests complementary functional interactions between AM fungi and bacteria, potentially enhancing nutrient utilization and carbon assimilation efficiency. It has been proposed that AM fungal hyphae may facilitate bacterial dispersal along the hyphosphere, thereby promoting closer spatial association between microorganisms and roots[54]. While such mechanisms were not directly assessed here, they may partly explain the enhanced growth observed under co-inoculation. Hormonal signaling has also been implicated in microbial synergy in other systems; however, hormone-mediated effects were not evaluated in this study and therefore remain speculative.

Correlation analysis revealed coordinated relationships among gas exchange, water-use efficiency, and biomass traits, highlighting the interconnected nature of plant functional responses under microbial treatments. Pn was correlated with Ci, which in turn was positively associated with E and gs, indicating that carbon assimilation was closely linked to stomatal regulation. WUEint was negatively correlated with gs, while WUEins aligned positively with WUEint, reflecting coordinated adjustments in water-use efficiency among treatments. Biomass traits formed a highly integrated cluster, with leaf area associated with both shoot and root dry weight. These results support the view that microbial inoculation influenced multiple functional traits simultaneously, resulting in associative patterns that contributed to overall plant productivity. While correlations cannot establish causation, they highlight key functional modules, gas exchange that underlie treatment-specific responses and provide a framework for targeted mechanistic studies in the future.

Limitations of the study

Several limitations should be considered when interpreting the present findings. First, the experimental design did not include a biochar-only control; therefore, the specific contribution of biochar cannot be disentangled from that of the microbial inoculants. Second, although we discuss possible mechanisms such as hydraulic conductivity, osmotic adjustment, and stomatal regulation, these processes were not directly measured in this study. Our interpretations are therefore based on literature-supported plausibility rather than direct evidence. Third, the correlation analysis was performed on treatment means (n = 5), which limits the statistical inference and should be viewed as exploratory. Acknowledging these limitations does not diminish the value of the results but provides a transparent context for their interpretation and for designing future mechanistic studies.

Conclusion

This study investigated the individual and combined effects of plant growth–promoting bacteria (PGPB) and arbuscular mycorrhizal (AM) fungi on *Vigna radiata* L. cultivated under soilless conditions. The findings

clearly demonstrate that co-inoculation (BA) produces complementary and synergistic effects that enhance physiological performance and biomass accumulation more effectively than single inoculations.

The BA treatment resulted in the greatest leaf area and shoot and root dry weights, reflecting the integration of enhanced stomatal CO_2 assimilation with improved plant hydraulic status. While PGPB (and BiB) maximized net photosynthetic rates, AM fungi independently improved tissue hydration and intrinsic water-use efficiency. Notably, co-inoculation significantly reduced leaf temperature, suggesting coordinated microbial-mediated thermal regulation through the combined effects of stomatal cooling and hydration buffering.

Overall, microbial consortia optimized carbon assimilation, water-use efficiency, and leaf energy balance, leading to consistent increases in biomass. These results provide physiological evidence supporting the potential use of beneficial microbial combinations to improve early vegetative performance, carbon–water coordination, and thermal status in *V. radiata* L. under soilless substrate conditions.

Data availability

Data Availability Statement The manuscript includes all experimental data and analyses necessary to support the conclusions. Additional datasets generated and analyzed during the current study are available from the corresponding author upon request.

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Author contributions

Author contributions Shirwan Malaie[1*] designed and conducted the experiments, collected and analyzed the data, and wrote the original draft of the manuscript. Latifeh Pourakbar[1] and Sina Siavash Moghaddam[2,3] supervised the study, provided guidance on experimental design, and critically reviewed the manuscript. Nabi Khezrinejad[4] contributed to experimental analyses. All authors reviewed, edited, and approved the final version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
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