Journal of Soil Science and Plant Nutrition (2026) 26:8193–8206
https://doi.org/10.1007/s42729-026-03393-4

ORIGINAL PAPER

Enhancing Rhizobial Inoculum Effectiveness Through Novel Formulation of Biochar-Nutrients-Biopolymers and Superabsorbent Polymers

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Received: 15 January 2026 / Accepted: 7 June 2026 / Published online: 15 June 2026
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Abstract

Peat is the most widely used commercial carrier for rhizobial inoculants, but it is non-renewable and increasingly scarce. We aimed to formulate pinewood biochar (PWBC) carrier blended at defined ratios with a biopolymer (xanthan gum), a superabsorbent polymer (polyacrylamide), and waste-derived organics (eggshell, mulch hay) to improve moisture buffering and rhizobial persistence, nodulation and nitrogen (N) fixation. Shelf-life and survival rate were first determined across six carriers {peat; PWBC; PWBC + xanthan (PWBC-X); PWBC + xanthan + polyacrylamide (PWBC-XP); PWBC + xanthan + polyacrylamide + eggshell (PWBC-XPE); PWBC + xanthan + polyacrylamide + eggshell + mulch hay (PWBC-XPEM)} for 120 days, stored at 28 °C. The foremost carrier PWBC-XPEM was then selected for glasshouse evaluation against uninoculated control, peat, and PWBC. Soybean (*Glycine max* L.) was grown under three watering regimes (D0: 55% water holding capacity (WHC), well-watered; D1 30%WHC, moderate drought; D2: 15%WHC, severe drought). Results from this study revealed that PWBC-XPEM showed the highest storage performance, maintaining a rhizobial population of 11.06 log10 CFU g^{-1} and 95% survival after 120 days ($p < 0.05$) as compared to peat and other formulations. Under D2, soybean plants inoculated with PWBC-XPEM increased shoot dry weight (90%), root length (189%), and nodule number (163%) relative to the uninoculated control, and enhanced biological nitrogen fixation (BNF) (87%), nitrogenaseactivity (1.10 μmol C_2H_4 h^{-1} $plant^{-1}$), chlorophyll and membrane stability. Overall, PWBC-XPEM emerges as a suitable peat alternative, combining better shelf life and improved soybean growth performance particularly under severe drought conditions, supporting dryland-oriented inoculant applications and offering clear prospects for the commercial application.

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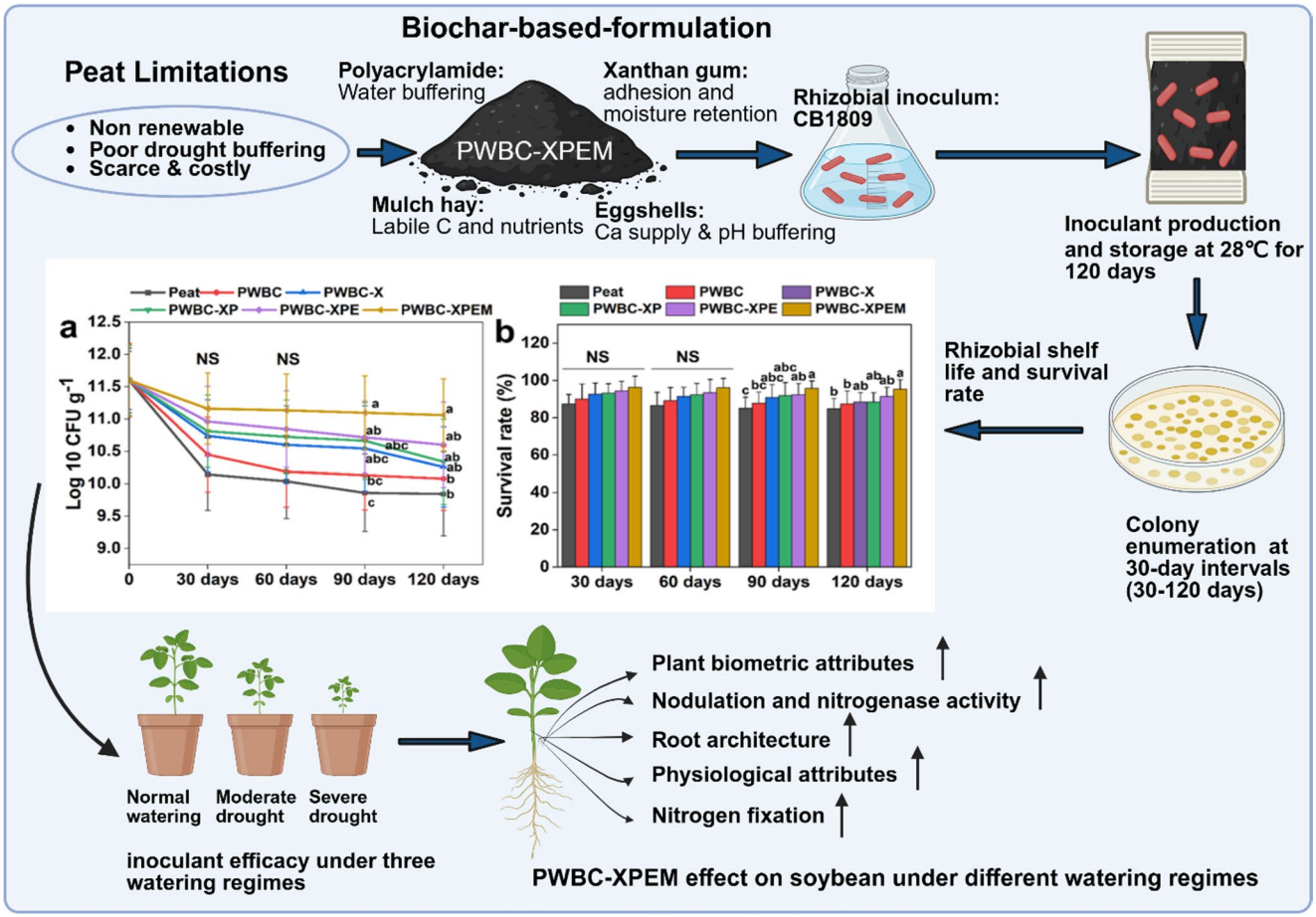
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Graphical Abstract



Keywords Alternative carriers · Shelf-life · Survival rate · Soybean · Drought

1 Introduction

Nitrogen (N), as an essential constituent of plants, is a significant part of amino acids for protein synthesis and the formation of DNA and RNA necessary for plant growth. Biological N fixation (BNF) through the symbiosis between rhizobia and the legume plant provides essential N sources for plant growth. In Australia, rhizobia facilitate BNF of approximately 110 kg N/ha/year through symbiotic relationships with pasture and pulse legumes, which provide a $4 billion annual national benefit (Drew et al. 2012). Rhizobia are introduced to the soil through inoculants, typically consisting of carrier materials (inorganic, organic, and salt) and rhizobia. Inoculants are commonly created by blending effective rhizobia with powdered or granulated carrier materials, which crucially offer a protective habitat for the strain's survival and growth. An effective carrier should possess certain features such as practical structures (e.g., porous or layer structures), an abundance of labile carbon (C) and essential elements such as N, calcium (Ca), oxygen (O), potassium (K), and phosphorus (P), a high water holding capacity (WHC), a neutral pH, cost-effectiveness and environmental friendliness (Hale et al. 2015; Shabir et al. 2023b). Despite considerable attempts to explore innovative carriers for the manufacturing of high-quality inoculants, many potential options remain underdeveloped for commercial applications (Ajeng et al. 2020; Allaga et al. 2020; Siyuan Tao 2018). In the current scenario, the primary commercial carrier materials are peat or lignite-based; however, these materials face limitations that impede further product quality enhancement (Poppe et al. 2021). Consequently, the selection of appropriate carrier materials, specifically for commercial purposes, remains critical.

Among different kinds of materials, biochar and biopolymer materials have received notable attention owing to their excellent contributions to agriculture. Specifically, biochar has vast applications in agricultural soils, such as soil health improvement through enhanced physical, chemical, and biological properties, alongside reductions in greenhouse gas emissions and C sequestration (Fang et al. 2014; Zhou et al. 2025). Novel applications of biopolymers in agriculture include their use as fungicides, soil modifiers and plant activators (Rashti et al. 2019; Ren et al. 2011). Similarly, in the realm of agricultural farming, both materials (biochar and biopolymers) are regarded as emerging microbial carriers (Igalavithana et al. 2017; Shabir et al. 2023b; Tittabutr et al. 2007). For instance, Shabir et al. (2023b) demonstrated that pine wood biochar (600 °C) was the most effective carrier for *Bradyrhizobium japonicum* (CB1809), maintaining high viability (approximately 10.11 and 9.76 log 10 CFU g^{-1}) after 90 days at 28 °C and 38 °C, respectively, due to favourable physicochemical properties such as high surface area and functional group abundance. Similarly, an increase in the population of *Pseudomonas putida* UW4 in biochar carrier material was also reported by Sun et al. (2015). Different biopolymers, including cellulose, xanthan gum, alginate, starch, agar, chitin/chitosan, have been reported as microbial carriers with substantial nutrient values (Lobo et al. 2019; Vassilev et al. 2020). Superabsorbent biopolymers (hydrogels) have also been recognised for immobilising and protecting rhizobia and other N-fixing microbes (Bashan 1986). Cell immobilisation entails physically containing living microbial cells within a defined carrier material region to restrict their movement while preserving catalytic capacity and reusability (Żur et al. 2016). Hydrogels are polymeric materials containing hydrophilic functional groups exhibiting high water-absorbing and swelling properties (Lapponi et al. 2022). Several other organic-based alternatives, such as plant by-products, bagasse, rice bran, and manure, have been studied for suitability to host specific rhizobial strains (Albareda et al. 2008; Kumar et al. 2015).

Biochar was pre-treated with a range of biopolymers to evaluate shelf life and rhizobial survival rate (Shabir et al. 2023a). While biopolymer amendments demonstrated some potential to enhance rhizobial shelf life and survival, their effects did not significantly differ from those observed in the peat control. Consequently, in the present study, we further improved biochar by incorporating superabsorbent biopolymer (polyacrylamide: excellent WHC) and organic waste materials (eggshells and mulch hay: which have excellent nutrient availability) in varying ratios to enhance rhizobial growth and survival within biochar. This unique combination of biochar, biopolymers, superabsorbent biopolymers, and organic materials has not been previously reported. We hypothesised that integrating biochar with biopolymers, superabsorbent polymers, and nutrient-rich organic materials would enhance rhizobial survival and shelf life and improve symbiotic performance and plant growth under varying moisture conditions compared with conventional peat-based carriers. Based on this hypothesis, the present study aimed to evaluate and compare the effectiveness of these carrier formulations on rhizobial growth, survival, and shelf life, as well as their impact on soybean nodulation, BNF, plant growth, and physiological responses under different soil moisture regimes.

2 Materials and Methods

2.1 Biochar Preparation, Modification, and Inoculant Production

As the primary carrier material, pinewood biochar (PWBC) was pyrolysed (slow pyrolysis) at 400 °C. The procedure was accomplished in an anoxic environment. Nitrogen gas (N_2) at 0.5 LPM was used as the intake gas. A 10 °C min^{-1} heating rate was used to produce biochar for one hour, gradually increasing the temperature by 50 °C to reach the 400 °C targeted peak temperature (Shabir et al. 2023b; Zhang et al. 2017). After thoroughly processing, the biochar was allowed to cool at ambient temperature, milled into a fine powder, sieved (250 µm) and packed into plastic bags. The physicochemical characterisation of biochar is previously described (Shabir et al. 2023b). Additional materials, such as xanthan gum and polyacrylamide, were purchased from Sigma-Aldrich Pty Ltd. and MIBA Rescaype AB Pty Ltd., respectively. Chicken eggshells and mulch hay (obtained from Rocky Point sugar mills, Australia) were air dried for 24 h before oven drying at 70 °C for two days, milled and powder was sieved (250 µm). These materials were mixed with biochar to create formulations. Detailed proportions are commercially confidential; only the carrier substrates and additives are described here. The formulations and their abbreviations are mentioned in Table S1. Peat was generously provided by Green Microbes Pty. Ltd., Australia and used as the control.

Bradyrhizobium japonicum (CB 1809) rhizobial strain was used and cultured in yeast mannitol broth (YMB) (Vincent 1970). The previously outlined procedure (Shabir et al. 2023b) was used to prepare the inoculants after the materials had been sterilised at 121 °C. The inoculants' moisture was adjusted to 60% moisture content (dry basis) and stored at 28 °C.

3 Assessment of Shelf-life

The viability of the rhizobial strain CB 1809 in the carriers was assessed by counting rhizobial colony-forming units (CFU) at 30, 60, 90, and 120 days. These measurements were used to evaluate the shelf life of rhizobia within the carrier formulations. To calculate the CFU, 1 g of inoculant was diluted with 9 mL of YMB solution. The dilutions were agitated for 30 min at 150 rpm on a gyratory shaker to liberate the rhizobial strains. After dilutions up to 10^{-6}, and 10^{-7}, 100 µL of the final dilution was then dispersed in triplicate on nutrient agar plates.

All plates were then incubated for 6–8 days at 28 °C. The colonies on the yeast mannitol agar (YMA) plates were counted to determine the number of viable bacteria (CFU) per gram of carrier material, expressed as Log 10 CFU g^{-1}.

4 Pot Trials

A glasshouse pot experiment was conducted using a completely randomised design to evaluate the effects of carrier materials and moisture regimes on soybean (*Glycine max* L.) growth and nodulation. Glasshouse conditions were maintained at 22 °C (day/night), 50–60% relative humidity, and a 16 h photoperiod. The experiment followed a factorial arrangement with two factors: (i) carrier material (uninoculated control, peat, PWBC, and PWBC-XPEM), and (ii) soil moisture: three moisture levels were applied relative to the soil's WHC of 28.7%, including 55% WHC (well-watered, represented as D0), 30% WHC (mild drought, represented as D1), and 15% WHC (severe drought, represented as D2). This resulted in 12 treatment combinations, each replicated four times, giving a total of 48 pots.

Each plastic pot (14 cm × 11.5 cm) was filled with 1 kg of oven-dried sandy loam soil. The primary properties of the soil were pH 5.30, EC 69 µS cm^{-1}, total C 0.90%, total N 0.60% hot water extractable organic C 0.35 g kg^{-1}, and hot water extractable organic N 0.07 g kg^{-1} (Shabir et al. 2023a).

Soybean seeds were surface sterilised with ethanol and rinsed thoroughly with sterile Milli Q water before air-drying. Seeds were inoculated using a slurry containing inoculant (5 g kg^{-1} seed) and 10% sucrose solution as an adhesive (Vincent 1970), then air-dried under shade prior to sowing. Five seeds were sown per pot, and seedlings were thinned to three plants per pot 10–15 days after emergence. Soil moisture levels were maintained gravimetrically throughout the experiment according to the designated WHC treatments.

5 Harvesting and Plant Growth Attributes

Soybean seeds were sown on December 11, 2021, and harvested on February 20, 2021. At the harvest, we recorded shoot and root lengths, and the number of root nodules. After separating the roots from the shoots, they were thoroughly cleaned and then dried on paper towels. To determine the dry weight, plant root and shoot samples were oven-dried for 48 h at 60 °C.

6 Determination of Biological N-Fixation

BNF in soybean plants in the pot trial was estimated by the ^{15}N-natural abundance method using a Sercon Hydra 20–22 Europa EA-GSL isotope-ratio mass spectrometer. The ratios for stable ^{15}N isotopes were calculated using the methods explained previously (Shabir et al. 2024).

N derived from the atmosphere (% Ndfa) was estimated using Eq. 1:

$$\%\ \text{Ndfa} = \frac{\delta\ ^{15}\text{N reference plant} - \delta\ ^{15}\text{N legume}}{\delta\ ^{15}\text{N reference plat} - \text{B value}} \times 100 \quad (1)$$

where B is the ^{15}N natural abundance of N in soybeans that solely depends on BNF, and δ^{15}Nref and δ^{15}Nsoy are the natural ^{15}N abundance of the reference (wheat) and soybean plants, respectively.

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Plant N and N fixed were calculated using Eqs. 2 and 3.

$$\text{Plant N}\ (\text{g plant}^{-1}) = \frac{\text{legume shoot dry biomass}\ (\text{g}) \times\ \text{Shoot N}\ (\%)}{100} \quad (2)$$

$$\text{N fixed per plant}\ (\text{g}) = \text{Legume N uptake}\ (\text{g}) \times\ \text{Ndfa}\ (\%) \times 1.5 \quad (3)$$

*For the soybean plants, factor 1.5 is used to include an estimate for the contribution of belowground N (Howieson and Dilworth 2016).

6.1 Nitrogenase Activity

Nitrogenase enzyme activity in plant roots was determined using the acetylene reduction assay (ARA) (Hardy et al. 1973). For each treatment group, fresh roots were washed, patted dry with paper towels, and placed in incubation bottles (250 mL). 10% of the air inside the bottles was exchanged with acetylene gas, and the bottles were kept in a dark place at room temperature for 1 day (Bahulikar et al. 2021). A 500 µL sample from each incubated bottle was injected into the gas chromatograph (GC-2010, Shimadzu

Plus) to estimate ethylene production. N_2 gas with a steady flow rate of 10 ml min^{-1} was used as make-up gas. To quantify the ethylene produced, standard curves were created using known amounts of ethylene (standards). Finally, the quantity of ethylene produced per gram of fresh root weight per hour was used to measure the nitrogenase activity (Bahulikar et al. 2021).

7 Root Architecture

After cleaning, the root systems from each treatment were scanned using WinRhizo software. The scans, obtained at 400 dpi with 8-bit grayscale settings, allowed measurement of root characteristics such as surface area, average diameter, volume, and the counts of tips, forks, and crossings (Himmelbauer et al. 2004).

8 Physiological Attributes

The amounts of chlorophyll a, chlorophyll b, and total chlorophyll (a+b) in the leaves were determined following the method established by Lichtenthaler (1987). To extract pigments, 0.5 g of leaf samples were first frozen in liquid N_2 and then ground up with a solution of 80% acetone. The mixture was spun in a centrifuge at 3,000 times gravity for 10 min. The clear liquid (supernatant) was collected, and its light absorption at specific wavelengths of 663.2 nm, 646.8 nm, and 470 nm was measured using a UV/Vis spectrophotometer (model UV1800, Shimadzu, China). Lichtenthaler (1987) method was applied to calculate the amount of each pigment based on the measured light absorption (extinction coefficients) and a specific equation.

Leaf membrane stability index (MSI) was calculated using the method proposed by Sairam et al. (2002). Briefly, two distinct samples of 0.5 g of fresh leaves were submerged in 10 mL of milli-Q water. Samples were kept for 30 min at 40 °C (EC1) and then 10 min at 100 °C (EC2), during which their electrical conductivities were monitored. The MSI was computed using Eq. 4.

$$MSI = 1 - \left(\frac{\mathrm{EC1}}{EC2}\right) \times 100 \quad (4)$$

Using the newest, fresh apical leaves of soybean plants, the relative water content (RWC) was calculated using the procedure described by Sairam et al. (2002).

8.1 Statistical Analysis

Rhizobial viable counts (Log 10 CFU g^{-1}) within carriers were calculated after logarithmic adjustment. Testing for normality and homogeneity of variance proceeds statistical analysis. The average difference between the carrier's shelf life and survival rate was calculated using Fisher's test when the analysis of variance (one-way ANOVA) was significant ($p<0.05$). For the pot trial experiment, data were analysed using two-way ANOVA to evaluate the effects of inoculant treatments, watering regimes, and their interaction. Where significant effects were detected ($p<0.05$), mean comparisons were performed using Fisher's least significant difference (LSD) test, and differences among treatments were indicated by different letters (Steel 1997). Principal component analysis (PCA) was conducted on the correlation matrix between the variables to separate the impact of watering regimes and treatments. Minitab 18 was utilised for all analyses.

9 Results

9.1 Shelf-Life Study

The CFU counts varied between the carrier materials and sampling dates throughout the 120-day storage at 28 °C (Fig. 1a, b). On days 30 and 60, no significant differences ($p>0.05$) in CFU values were observed across the carrier materials, although all carrier materials performed slightly better than peat (Fig. 1a). Amid all the inoculants, PWBC-XPEM showed superior shelf life (11.16 Log 10 CFU g^{-1}) and survival rate (96%) at day 60. The CFU counts for the PWBC-XPE and PWBC-XPEM materials significantly exceeded ($p<0.05$) those of peat on day 90, corresponding to shelf-life values of 10.72 and 11.10 Log 10 CFU $g^{-1,}$ respectively. Moreover, at the end of day 120, only PWBC-XPEM showed a significantly higher ($p<0.05$) CFU value than peat, accompanied by the highest survival rate (95%) (Fig. 1b).

9.2 Plant Growth Characteristics

Soybean plants inoculated with PWBC and PWBC-XPEM exhibited substantial growth improvements ($p<0.05$) compared to the control and peat, with PWBC-XPEM consistently providing the strongest response (Table 1). Under D0, the PWBC treatment significantly increased ($p<0.05$) shoot and root dry weights by 104% and 56% over the

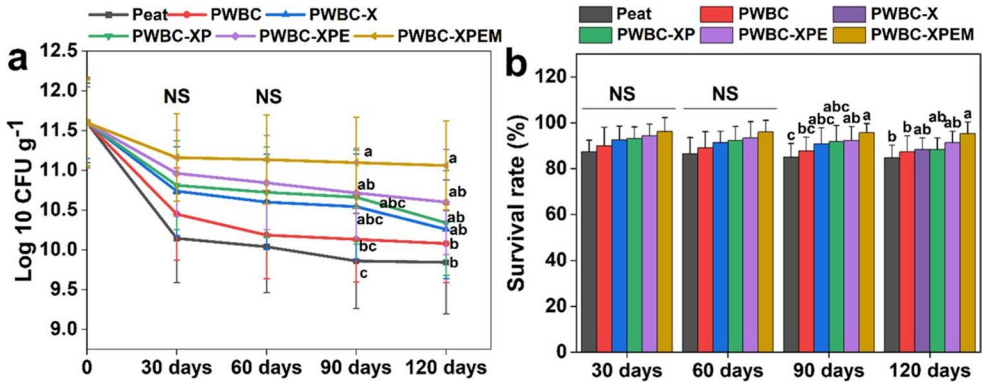


Fig. 1 Rhizobial shelf-life and survival rates across carrier materials during the 120-day storage period at 28 °C (**a**) Log 10 CFU g^{-1} value and (**b**) survival rate. The values (mean ± SD, $n = 3$) that do not share the same letter for different storage times are significantly different (LSD test, $p < 0.05$). PWBC: Pinewood biochar; PWBC-X: PWBC + xanthan gum; PWBC-XP: PWBC + xanthan gum + polyacrylamide; PWBC-XPE: PWBC + xanthan gum + polyacrylamide + eggshells; PWBC-XPEM: PWBC + xanthan gum + polyacrylamide + eggshells + mulch hay

Table 1 Effect of carrier materials on soybean growth parameters (plant^{-1}) under different watering regimes

Watering regimes	Treatments	Shoot dry weight (g)	Root dry weight (g)	Plant height (cm)	Root length (cm)	No. of nodules (*n*)
D0						
	Control	0.71 ± 0.09 f	0.63 ± 0.04 f	40 ± 3.3 de	29.8 ± 2.4 de	0.00 ± 0.0 f
	Peat	0.96 ± 0.10 cd	0.87 ± 0.10 c	47.3 ± 4.5 c	37.0 ± 3.2 cd	18.3 ± 2.5 c
	PWBC	1.45 ± 0.11 b	0.98 ± 0.12 b	52.5 ± 4.8 ab	41.0 ± 4.2 bc	24.8 ± 2.5 b
	PWBC-XPEM	1.86 ± 0.12 a	1.41 ± 0.13 a	57.6 ± 5.36 a	47.0 ± 4.3 ab	30.0 ± 3.6 a
D1						
	Control	0.55 ± 0.09 g	0.48 ± 0.07 g	30.6 ± 3.7 f	22.8 ± 2.3 ef	0.0 ± 0.0 f
	Peat	0.72 ± 0.07 f	0.64 ± 0.05 ef	40.1 ± 4.0 de	30.8 ± 3.4 de	14.0 ± 1.4 d
	PWBC	0.86 ± 0.10 de	0.80 ± 0.10 cd	50.7 ± 4.3 bc	39.0 ± 4.4 c	18.5 ± 2.7 c
	PWBC-XPEM	1.04 ± 0.07 c	0.87 ± 0.11 c	57.1 ± 6.0 a	49.0 ± 3.3 a	25.0 ± 3.4 b
D2						
	Control	0.40 ± 0.05 h	0.34 ± 0.07 h	24.2 ± 2.1 g	15.5 ± 2.6 f	0.00 ± 0.0 f
	Peat	0.58 ± 0.07 g	0.50 ± 0.06 g	34.1 ± 4.1 ef	28.3 ± 3.6 e	7.50 ± 1.2 e
	PWBC	0.66 ± 0.08 fg	0.62 ± 0.08 f	45.8 ± 4.3 cd	38.4 ± 3.5 c	11.3 ± 1.3 d
	PWBC-XPEM	0.76 ± 0.10 ef	0.73 ± 0.10 de	56.2 ± 4.1 ab	44.6 ± 4.7 abc	19.8 ± 2.3 c

For each parameter, the values (mean + SD, n = 4) do not share the same letter are statistically significant (LSD, $p < 0.05$). D0: 55%WHC (water holding capacity); D1: 30%WHC; D2:15%WHC. PWBC: Pinewood biochar; PWBC-XPEM: PWBC + xanthan gum + polyacrylamide + eggshells + mulch hay

uninoculated control, while PWBC-XPEM achieved increases ($p < 0.05$) of 162% and 124% for the shoot and root dry weights, respectively. Relative to peat, PWBC-XPEM enhanced shoot and root dry weights by 94% and 62% and shoot and root lengths also improved compared with the peat inoculant. Nodulation was absent in the control; PWBC produced 24.8 nodules per plant (36% higher than peat), while PWBC-XPEM supported 30 nodules (64% higher than peat) (Table 1).

Similarly, under D1, PWBC-XPEM maintained its advantage, significantly ($p < 0.05$) increasing shoot and root biomass by 89% and 81% compared with the control, and by 44% and 36% over peat. Shoot and root lengths rose by 87% and 115% relative to the control, while nodulation reached 25 nodules per plant, a 79% improvement over peat (Table 1). Even D2, PWBC-XPEM sustained significant benefits for plant biometric attributes.

9.3 Biological Nitrogen Fixation

The PWBC-XPEM and PWBC revealed consistent and substantial improvements in N assimilation and fixation across all watering regimes as compared to peat and control (Table 2). Under D0, peat improved plant N uptake and N fixed relative to the uninoculated control, but both PWBC and PWBC-XPEM showed further statistically significant ($p < 0.05$) gains for some N fixation parameters. PWBC increased plant N uptake by 54.7% and N fixed by 61.2% compared with peat. The enhanced formulation PWBC-XPEM provided even greater improvements, significantly

Table 2 Effect of carrier materials on nitrogen fixation (plant^{-1}) of the soybean plant under different watering regimes

Watering regimes	Treatments	Shoot *N* (%)	Plant *N* uptake (mg)	Ndfa (%)	*N* fixed (mg)
D0					
	Control	2.25 ± 0.01e	15.94 ± 2.8f	41.79 ± 0.7 h	9.98 ± 1.6 fg
	Peat	2.31 ± 0.01c	22.26 ± 0.4 cd	69.64 ± 1.8c	23.26 ± 1.0c
	PWBC	2.37 ± 0.02b	34.43 ± 4.8b	72.56 ± 0.7b	37.49 ± 5.4b
	PWBC-XPEM	2.47 ± 0.02a	45.88 ± 1.2a	77.74 ± 3.3a	53.55 ± 3.6a
D1					
	Control	2.15 ± 0.01 g	11.89 ± 2.1 g	39.70 ± 0.7i	7.08 ± 1.2gh
	Peat	2.22 ± 0.02f	15.95 ± 2.5f	62.26 ± 0.3de	14.90 ± 2.3e
	PWBC	2.28 ± 0.01d	19.52 ± 2.0de	63.45 ± 0.5d	18.58 ± 2.0d
	PWBC-XPEM	2.36 ± 0.01b	24.51 ± 1.9c	70.36 ± 0.4c	25.87 ± 1.9c
D2					
	Control	2.11 ± 0.01 h	8.44 ± 0.8 h	37.98 ± 0.3j	4.81 ± 0.4 h
	Peat	2.16 ± 0.01 g	12.44 ± 1.5 g	45.18 ± 0.3 g	8.43 ± 1.0 g
	PWBC	2.22 ± 0.01f	14.54 ± 0.9 fg	58.87 ± 0.6f	12.84 ± 0.8ef
	PWBC-XPEM	2.29 ± 0.01d	17.31 ± 1.0ef	60.89 ± 0.4e	15.81 ± 0.8de

For each parameter, the values (mean + SD, $n = 4$) do not share the same letter are statistically significant (LSD, $p < 0.05$). D0: 55%WHC (water holding capacity); D1: 30%WHC; D2:15%WHC. PWBC: Pinewood biochar; PWBC-XPEM: PWBC + xanthan gum + polyacrylamide + eggshells + mulch hay

increasing ($p < 0.05$) shoot N by 6.9%, plant N by 106%, Ndfa by 11.6%, and N fixed by 130% relative to peat, confirming its superior efficiency under optimal conditions (Table 2).

Moreover, under D1, biochar-based carriers maintained their advantage. For example, PWBC-XPEM further boosted performance, with shoot N increasing by 6.3%, plant N uptake by 53.7%, Ndfa by 13.0%, and N fixed by 73.6% compared with peat. These findings demonstrate the resilience of PWBC-XPEM in sustaining BNF under water-limited conditions (Table 2).

A similar trend was observed under D2. PWBC-XPEM again delivered significantly the highest values ($p < 0.05$) compared with peat (Table 2). While peat improved plant N traits compared with the control, PWBC and especially PWBC-XPEM provided significantly greater increases across all regimes. These results highlight the promise of biochar-based inoculant carriers, particularly PWBC-XPEM, as more effective and resilient alternatives to peat under both optimal and drought-stressed environments.

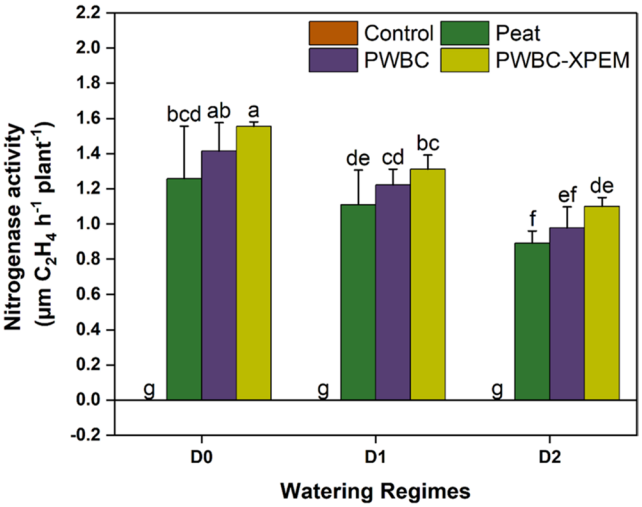


Fig. 2 Effect of different carrier materials on nitrogenase activity using the acetylene reduction assay. D0: 55%WHC (water holding capacity); D1: 30%WHC; D2:15%WHC. PWBC: Pinewood biochar; PWBC-XPEM: PWBC + xanthan gum + polyacrylamide + eggshells + mulch hay

9.4 Nitrogenase Activity Through the Acetylene Reduction Assay

Nitrogenase activity, measured by the acetylene reduction assay (ARA), was strongly influenced by carrier treatments and watering regimes (Fig. 2). No activity was detected in uninoculated controls under any condition, confirming the absence of BNF without inoculation. Under D0, peat supported moderate activity (1.26 µm C_2H_4 h^{-1} $plant^{-1}$), while PWBC recorded slightly higher activity (1.42 µm C_2H_4 h^{-1} $plant^{-1}$). PWBC-XPEM achieved the highest activity (1.56 µm C_2H_4 h^{-1} $plant^{-1}$), representing a 24% improvement ($p < 0.05$) over peat and 10% over PWBC, indicating enhanced rhizobial performance and nodule function (Fig. 2).

At D1, nitrogenase activity declined across all treatments but remained significantly higher ($p < 0.05$) in inoculated plants than in controls. Peat recorded 1.11 µm C_2H_4 h^{-1} $plant^{-1}$, while PWBC increased activity by 10%, and PWBC-XPEM further improved activity by 18% ($p < 0.05$) compared with peat (Fig. 2). Under D2, overall nitrogenase activity was markedly reduced, yet the inoculated treatments continued to sustain measurable activity. PWBC-XPEM reached 1.10 µm C_2H_4 h^{-1} $plant^{-1}$, a 24% improvement over peat (Fig. 2).

9.5 Root Morphology

Root architectural traits were significantly enhanced by inoculation, with PWBC-XPEM consistently outperforming peat and the uninoculated control across all watering regimes (Table 3). Under D0, peat increased root growth compared with the control, but PWBC-XPEM achieved the strongest improvements, raising surface area by 27.2% and root volume by 18.0%, while tips and forks more than doubled (by 130.6% and by 106.2%), and crossings increased by 70.3% relative to peat. These results highlight the extensive root proliferation and branching capacity conferred by the biochar-enriched formulation (Table 3).

Under D1, the overall decline in root growth was evident, yet the inoculated treatments demonstrated resilience compared with the control. While peat sustained only modest improvements, PWBC added incremental gains in surface area (by 12.7%) and root volume (by 14.0%), but little advantage in branching. In contrast, PWBC-XPEM markedly altered root system architecture, nearly offsetting the negative effects of water limitation. Relative to peat, it increased root volume by almost half (by 46.5%), generated 70% more tips, and boosted forks (by 30.9%) and crossings (by 23.8%) (Table 3).

The impact became even more striking under D2, where controls displayed highly stunted roots with minimal branching. However, PWBC-XPEM stood out as a robust enhancer of root morphology under stress. Compared with peat, it expanded root surface area by 32.2% and volume by 57.1%, while doubling tip numbers (by 112.5%) and significantly increasing forks (by 25.9%) and crossings (by 46.7%) (Table 3).

9.6 Membrane Stability Index and Relative Water Content

The influence of carrier formulations on soybean physiological responses was evident in both the membrane MSI and RWC under all watering regimes (Fig. 3a, b). Under D0, PWBC-XPEM showed the significantly highest ($p<0.05$) MSI value, representing a 27% increase compared with the control and 14% higher than peat (Fig. 3a). Similarly, RWC in PWBC-XPEM plants showed a 30% increase over the control and 17% increase over peat (Fig. 3b). PWBC also improved MSI and RWC by 19% and 16% over the control, respectively.

At D1, MSI values declined across treatments, but PWBC-XPEM maintained significantly higher ($p<0.05$) stability, representing a 25% increase over the control and 14% over peat. RWC followed a comparable trend, with PWBC-XPEM recording a 23% increase over the control. Under D2, the differences became more pronounced. PWBC-XPEM sustained the highest MSI, representing 34% and 36% increases over the control, respectively, and 17% and 14% improvements over peat.

9.7 Chlorophyll Content

Chlorophyll content was significantly influenced by inoculation, with both biochar-based carriers surpassing peat across all watering regimes (Fig. 4a, b, c). Under D0, peat enhanced chlorophyll levels compared with the control, but PWBC and PWBC-XPEM provided additional gains. The enriched formulation, PWBC-XPEM, showed even greater

Table 3 Effect of carrier materials on root morphology of the soybean (plant^{-1}) under different watering regimes

Watering regimes	Treatments	Surface Area (cm^2)	Average Diameter (mm)	Root volume (cm^3)	Tips (number)	Forks (number)	Crossings (number)
D0							
	Control	37.76 ± 4.3de	0.58 ± 0.04bcde	0.53 ± 0.07bcd	1558 ± 20.6e	1111 ± 34.7e	129.0 ± 9.1e
	Peat	46.69 ± 5.9bc	0.66 ± 0.09abc	0.61 ± 0.09abc	2225 ± 54.5c	1349 ± 10.9c	175.5 ± 12.6c
	PWBC	51.52 ± 5.8ab	0.69 ± 0.10ab	0.66 ± 0.13a	2618 ± 52.7b	1730 ± 31.8b	270.8 ± 10.2b
	PWBC-XPEM	59.39 ± 10.6a	0.75 ± 0.15a	0.72 ± 0.09a	5132 ± 56.7a	2782 ± 98.3a	298.8 ± 32.1a
D1							
	Control	27.55 ± 3.4 fg	0.55 ± 0.09cde	0.39 ± 0.06ef	811 ± 15.0 h	796 ± 24.6 h	94.25 ± 6.9f
	Peat	33.46 ± 3.4ef	0.56 ± 0.11cde	0.43 ± 0.09de	1107 ± 55.9f	929 ± 11.7f	124.0 ± 11.0e
	PWBC	37.72 ± 6.4de	0.60 ± 0.06bcd	0.49 ± 0.13cde	1153 ± 22.7f	934 ± 19.6f	133.8 ± 6.3e
	PWBC-XPEM	43.14 ± 6.6 cd	0.69 ± 0.10ab	0.63 ± 0.05ab	1883 ± 57.0d	1216 ± 31.2d	153.5 ± 12.6d
D2							
	Control	19.53 ± 2.7 h	0.41 ± 0.03f	0.20 ± 0.06 g	429 ± 11.7j	652 ± 22.7j	66.25 ± 10.5 g
	Peat	24.23 ± 3.3gh	0.47 ± 0.06ef	0.28 ± 0.08 fg	463 ± 17.8j	693 ± 13.7ij	82.50 ± 8.7 fg
	PWBC	27.78 ± 3.9 fg	0.51 ± 0.06def	0.37 ± 0.13ef	613 ± 7.4i	726 ± 19.4i	84.25 ± 6.9 fg
	PWBC-XPEM	32.03 ± 4.6efg	0.56 ± 0.07cde	0.44 ± 0.07de	984 ± 21.8 g	873 ± 19.4 g	121.0 ± 7.7e

For each parameter, the values (mean + SD, $n=4$) do not share the same letter are statistically significant (LSD, $p<0.05$). D0: 55%WHC (water holding capacity); D1: 30%WHC; D2:15%WHC. PWBC: Pinewood biochar; PWBC-XPEM: PWBC + xanthan gum + polyacrylamide + eggshells + mulch hay

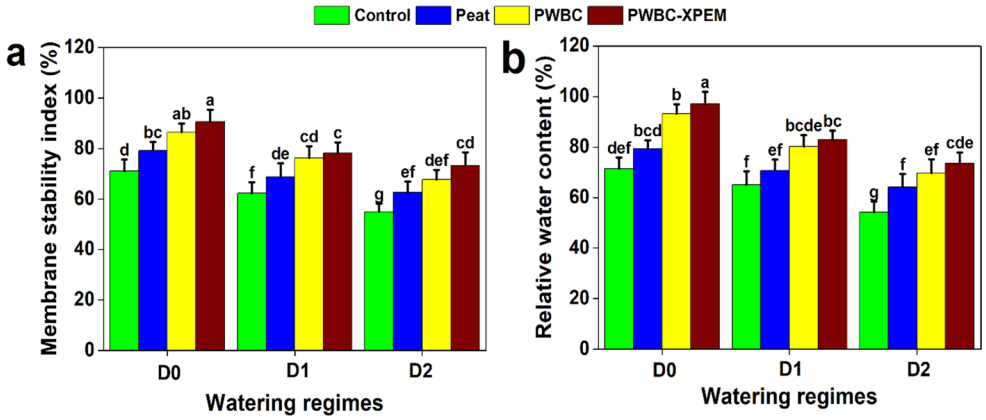


Fig. 3 Effect of different carrier materials on (**a**) membrane stability index (MSI) and (**b**) relative water content (RWC) under different watering regimes. For the parameters, the values (mean ± SD, $n=4$) that do not share the same letter are significantly different (LSD test, $p<0.05$). D0: 55%WHC (water holding capacity); D1: 30%WHC; D2:15%WHC. PWBC: Pinewood biochar; PWBC-XPEM: PWBC + xanthan gum + polyacrylamide + eggshells + mulch hay

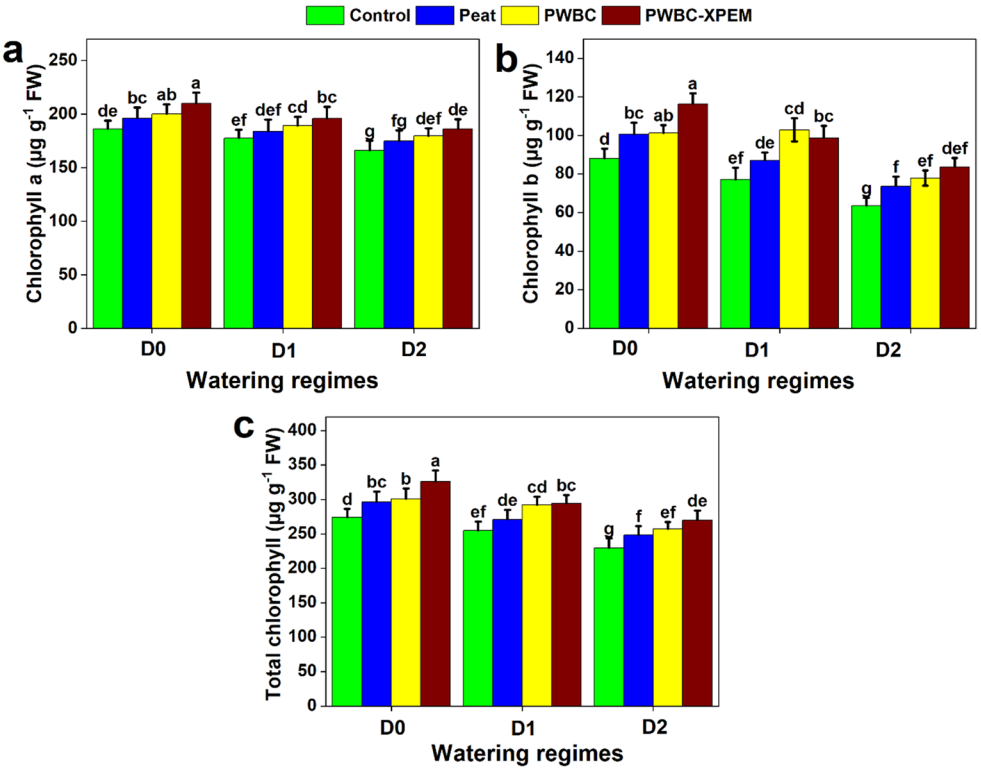


Fig. 4 Effect of carrier materials on (**a**) chlorophyll a, (**b**) chlorophyll b and (**c**) total chlorophyll in soybean plants under different watering regimes. For the parameters, the values (mean ± SD, $n=4$) that do not share the same letter are significantly different (LSD test, $p<0.05$). D0: 55%WHC (water holding capacity); D1: 30%WHC; D2:15%WHC. PWBC: Pinewood biochar; PWBC-XPEM: PWBC + xanthan gum + polyacrylamide + eggshells + mulch hay

improvements, with chlorophyll a increasing by 6.5%, chlorophyll b by 13.4%, and total chlorophyll by 8.6%.

Under D1, overall chlorophyll levels declined slightly, but inoculation mitigated the impact. Compared with peat, PWBC-XPEM again showed the most consistent benefits, enhancing chlorophyll a, chlorophyll b, and total chlorophyll. The stronger stimulation of chlorophyll b by biochar carriers is notable, as this pigment complements chlorophyll a in light harvesting and contributes to maintaining photosynthetic efficiency under stress.

Likewise, under D2, PWBC-XPEM once again exhibited the greatest resilience, raising chlorophyll b by 15.6% and total chlorophyll by 10.0% relative to peat.

9.8 Multivariate Analysis

Principal component analysis (PCA) was used to analyse the correlation between different treatments and response variables (Fig. 5a, b). F1 and F2 were shown to be the primary contributors, explaining 81% and 7.5% of the variability in

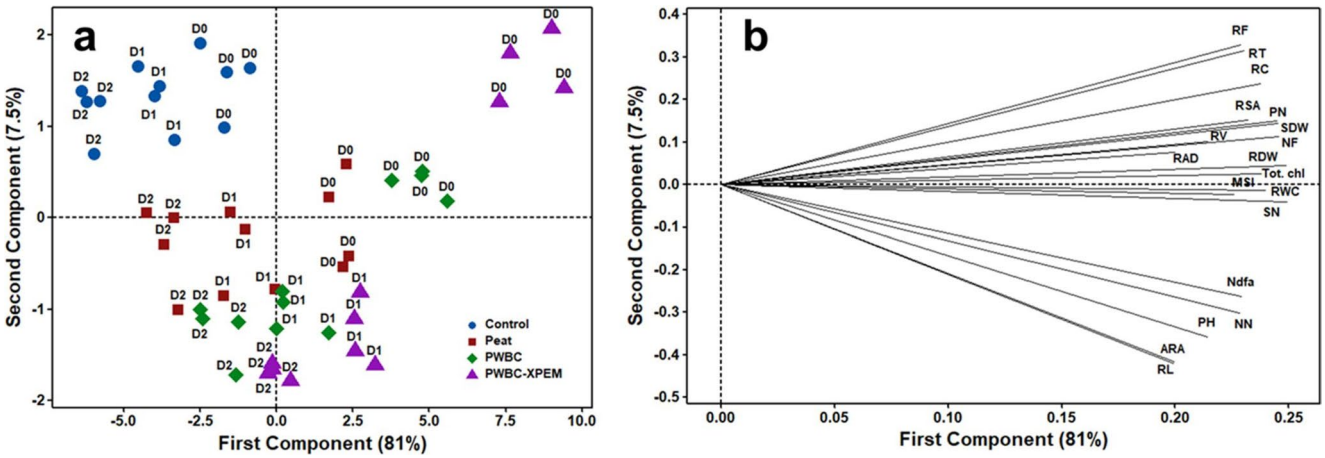


Fig. 5 Comparison of different treatments (**a**) and response variables of soybean (**b**) using principal component analysis (PCA). SDW: shoot dry weight; RDW: root dry weight; NN: number of nodules; PH: plant height; RL: root length; NA: nitrogenase activity; RSA: root surface area; RAD: root average diameter; RV: root volume; RT: root tips; RF: root forks; RC: root crossings; Tot. chl: total chlorophyll; MSI: Membrane stability index; RWC: relative water content; SN: shoot nitrogen; PNU: plant nitrogen uptake; NF: nitrogen fixed. PWBC: Pinewood biochar; PWBC-XPEM: PWBC + xanthan gum + polyacrylamide + eggshells + mulch hay

the analysed data, respectively, even though thirteen factors (F1 to F13) contributed to the variability in the data overall. The PCA clearly differentiated treatments and watering regimes based on plant growth and N fixation traits.

In (Fig. 5a), control samples (blue circles) clustered strongly on the negative axis of PC1, particularly under drought (D1, D2), reflecting poor performance across most parameters. Peat treatments (red squares) were separated from controls along PC1, indicating moderate improvements but still overlapping with drought-stressed conditions. PWBC treatments (green diamonds) shifted further towards the positive PC1 axis, especially under D0, showing improved nutrient uptake and biomass accumulation. Notably, PWBC-XPEM (purple triangles) was distinctly grouped on the far positive side of PC1, especially under D0 and D1, highlighting its consistent superiority across environments.

In (Fig. 5b), trait loadings show that variables contributing most strongly to PC1 include shoot N (SN), Ndfa, nodulation (NN), root length (RL), acetylene reduction assay (ARA), and photosynthetic parameters (PH, PN, total chlorophyll). These vectors aligned positively with PWBC-XPEM and, to a lesser degree, PWBC, explaining their strong clustering along the positive PC1 axis. In contrast, control and peat treatments aligned negatively with these variables, reinforcing their relatively lower contributions to N fixation and growth performance.

Overall, the PCA demonstrates that PWBC-XPEM consistently improved plant performance across both optimal and drought conditions, with clear separation from peat and control, confirming its effectiveness as a superior inoculant carrier.

10 Discussion

Across all storage intervals, the PWBC-XPEM formulation sustained rhizobial viability for up to 120 days, outperforming the other carriers. This outcome is consistent with the notion that combining biochar with biopolymers, superabsorbent polymers, and organic amendments can create a more hospitable microenvironment, a higher WHC, additional nutrients, and stabilising physicochemical properties than biochar alone. Given the sensitivity of rhizobial survival to pH, the capacity of these additives to buffer the carrier toward neutrality is likely significant, as most rhizobia grow best at a pH of 7.0–7.5 (Thomas et al. 1994). The specific ratio of biochar to biopolymers and organics appears to matter as it modulates WHC, biochar chemistry (C–C/C–H, C–OH, C = O, O–C = O, and porosity) under low pyrolysis 400 °C retain more O/N-functional groups (greater hydrophilicity), features linked with improved shelf stability (Bolan et al. 2023; Sashidhar et al. 2020). In parallel, biopolymer extends shelf-life while biochar provides micro-habitats that can shelter cells and increase survival rate (Shabir et al. 2023a). Another factor influencing the inoculants' microbial survival rate is moisture (Bashan et al. 2014). The ideal moisture content for bacterial development and activity is known to be between 40 and 80% of WHC (Głodowska et al. 2016). Due to the existence of sponge-like matrices, biochar was found to be effective at retaining moisture (Shabir et al. 2023b; Zhang et al. 2017). It also demonstrated an improved shelf life when used as an alternative carrier for microbial inoculum in comparison to other carrier materials, such as peat (Saranya et al. 2011). According to Ali et al. (2019), biochar is also thought to offer microorganisms appropriate protection against desiccation.

Rhizobia support legume development by influencing BNF processes, facilitating more efficient uptake of water and essential nutrients (Ojuederie et al. 2019). In the present study, the plant responses mirror these carrier advantages. Relative to the uninoculated control and conventional peat, PWBC-XPEM consistently improved growth traits across watering regimes. Plants receiving PWBC-XPEM showed higher shoot and root biomass, longer shoots and roots, and more nodules. Moreover, under D0, PWBC-XPEM outperformed peat for N acquisition and fixation, with plant N uptake and N fixed more than doubling (by 106% and by 130%). Similarly, under D1 and D2, the formulation sustained clear margins over peat, where plant N uptake rose by 39% and N fixed by 88% relative to peat under D2. These trends suggest a formulation that maintains N inputs during water limitation while safeguarding the rhizobia host symbiosis. Mechanistically, biochar enhanced nutrient mobilisation, increased porosity and moisture retention, improved rhizosphere conditions surrounding the seed or roots, and increased surface area (Ajeng et al. 2020; Lehmann and Joseph 2015). Similar biochar-enabled gains under drought stress have been reported for lupin and cucumber (Egamberdieva et al. 2017; Nadeem et al. 2017), and pine-derived biochar has been highlighted as an effective carrier for maize and cucumber (Głodowska et al. 2016; Hale et al. 2014). Nitrogenase activity followed this hierarchy. In contrast to peat, PWBC-XPEM enhanced activity by around 26% under D0, supporting the notion that appropriately enriched biochar can establish a physical-chemical habitat that facilitates BNF (Xiu et al. 2021). The present findings reinforce earlier reports by Azeem et al. (2021), indicating that biochar formulations stimulated soybean nodulation, increasing both nodule number and mass. By enhancing the soil's physical structure and chemical properties, biochar appears to reorient root foraging, encouraging greater root proliferation and infection events, which in turn strengthen BNF. The biochar also seemed to slow the degradative trajectory often seen under continuous soybean cultivation, helping maintain a fertile, biologically active rhizosphere. Taken together, these changes provide a sound basis for vigorous plant growth and more resilient production systems (Wu et al. 2022).

Root system architecture helps connect these physiological and N outcomes. Under D0, PWBC-XPEM substantially increased ($p<0.05$) root surface area and volume relative to peat and stimulated lateral proliferation (tips, forks, crossings). Under D1, the formulation continued to reshape the root network despite drought stress. Even at D2, when growth is typically most constrained, the root morphology was significantly higher as compared to peat, showing an increase in surface area (32.2%), diameter (19.1%) and volume (57.1%). A finer, highly branched root system would increase soil exploration and resource interception, helping to maintain RWC, protect membranes (MSI), and feed the symbiosis the C it needs to keep fixing N under stress. Biochar's advantageous control of the rhizosphere soil's physical and chemical characteristics produced a more conducive soil microecological environment for root development. It was reported that using biochar can significantly enhance soybean root biomass, encourage the overall root length and surface area of soybean seedlings, and also increase root activity. Using biochar is regarded as a crucial management technique to increase root surface area and root length density (Reyes-Cabrera et al. 2017; Zhu et al. 2018).

The physiological indicators of stress tolerance also showed a similar trend. Inoculation elevated RWC, MSI, and chlorophylls (a, b, total) compared with the control under D1 and D2, with PWBC-XPEM repeatedly the top performer. Chlorophyll responses were robust and consistent: relative to peat, BC-XPEM increased chlorophyll a, b, and total chlorophyll by 6.5%, by 13.3%, and by 8.6% at D1; and by 7.1%, by 15.6%, and by 10.0% at D2. The stronger effect on chlorophyll b across regimes is noteworthy because it complements chlorophyll a in light capture, which could help explain the maintenance of photosynthesis under stress. In line with this, prior work links improved soil structure and water retention under biochar to higher pigment pools and stress mitigation (Abideen et al. 2020; Kammann et al. 2011; Kim et al. 2021).

The coherence of the root, pigment, and N-fixation suggests that the formulation did not just act on a single trait; it shifted the whole plant-microbe-soil system toward resilience. While glasshouse data are informative, multi-site, multi-season trials will help quantify durability across soils, climates, and management, as others have observed species- and context-specific responses to biochar carriers. Despite these considerations, the convergence of shelf-life, univariate traits, and BNF makes a strong case that PWBC-XPEM acts as an appropriate carrier with benefits that persist from storage to field-relevant stress.

11 Conclusions

The findings from this study have demonstrated that integrating biochar with biopolymers, a superabsorbent polymer, and nutrient-rich organic materials creates a carrier environment that enhances rhizobial survival by improving moisture retention, nutrient availability, and protective microhabitats. These conditions supported sustained bacterial viability and translated into improved nodulation, biological nitrogen fixation, and plant growth under both optimal and water-limited conditions. The results have supported the hypothesis that combining

biochar with functional polymeric and organic components can outperform conventional peat-based carriers by simultaneously improving microbial persistence and symbiotic efficiency. This highlights a clear mechanistic link between carrier composition, microbial survival, and plant performance. The study introduces a novel multi-component carrier formulation that offers a promising alternative to peat, with potential applications in sustainable legume production, particularly in environments prone to water stress. Further field-based validation across diverse soil and climatic conditions will be essential to confirm its broader applicability.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42729-026-03393-4.

Acknowledgements We would like to thank Griffith University and Soil CRC for providing financial assistance to Rahat Shabir through the Griffith University Post Graduate Scholarship and Soil CRC top-up scholarship, respectively. The *Bradyrhizobium japonicum* strain CB1809 was graciously provided by the Australian Inoculants Research Group (AIRG), for which the authors are very thankful.

Author Contributions RS, YL, and CC contributed to the idea and experimental design of the work. RS and YL performed material formulations, pot trial setup, and data collection and curation. RS performed the data analysis and interpretation. RS wrote the first draft of the manuscript, and all authors (YL, CC, MM, and YF) commented on previous versions of the manuscript. CC supervised the project and acquired funding. All authors read and approved the final manuscript.

Funding Open Access funding enabled and organized by CAUL and its Member Institutions. This project has been supported by the Cooperative Research Centre for High Performance Soils, whose activities are funded by the Australian Government's Cooperative Research Centre Program (PJA3.4.001).

Data Availability The manuscript files include all data analysed for this study. Data with replicates will be available upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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