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Pyrolysis temperature affects biochar suitability as an alternative rhizobial carrier

Rahat Shabir[1,2] · Yantao Li[1,2,3] · Mallavarapu Megharaj[2,4] · Chengrong Chen[1,2]

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Abstract

Biochars produced from different feedstocks and at different pyrolysis temperatures may have various chemical and physical properties, affecting their potential use as alternative microbial carrier materials. In this study, biochars were produced from pine wood and oak feedstocks at various temperatures (400°C, 500°C, 600°C, 700°C and 800°C), characterized, and assessed for their potential as carriers for *Bradyrhizobium japonicum* (CB1809) strain. The biochars were then stored at two different storage temperatures (28°C and 38°C) for up to 90 days. Furthermore, the study also explored the role of potentially ideal carriers as inoculants in the growth of *Glycine max L.* (soybean) under different moisture levels i.e., 55% water holding capacity (WHC) (D0), 30% WHC (D1) and, 15% WHC (D2) using a mixture of 50% garden soil and 50% sand. The results were compared to a control group (without inoculants) and a peat inoculant. Among all the materials derived from pine wood and oak, pine wood biochar pyrolyzed at 400°C (P-BC400) exhibited the highest CFU count, with values of 10.34 and 9.74 Log 10 CFU g^{-1} after 90 days of storage at 28°C and 38°C, respectively. This was notably higher compared to other biochars and peat carriers. Significant ($p < 0.05$) increases in plant properties: shoot and root dry biomass (174% and 367%), shoot and root length (89% and 85%), number of leaves (71%), membrane stability index (27%), relative water content (26%), and total chlorophyll (140%) were observed in plants treated with P-BC400 carrier inoculant compared to the control at D2; however, lower enrichment of $\delta^{13}C$ (37%) and $\delta^{15}N$ (108%) with highest number of root nodules (8.3 ± 1.26) and nitrogenase activity (0.869 ± 0.04) were observed under D2, as evident through PCA analysis, showing more nitrogen (N) fixation and photosynthetic activity. Overall, this experiment concluded that biochar pyrolyzed at lower temperatures, especially P-BC400, was the most suitable candidate for rhizobial inoculum and promoted soybean growth.

Keywords Biochar · Pyrolysis temperature · Rhizobial inoculant · Shelf life · Soybean · Drought

Introduction

Peat is the most commonly used carrier for rhizobia inoculum (Atieno et al. 2018). However, its availability has declined in recent years since it is a non-renewable resource (Hardy and Knight 2020). Also, the extraction (mining), processing, and sterilization of peat incur high costs, besides its availability on a large scale is limited (Beck 1991; Bilney 1997; Buntić et al. 2019). Therefore, it is critical to develop new rhizobial carriers as a substitute for peat in order to keep the inoculant industry and agricultural production afloat (Araujo et al. 2020; Hale et al. 2015; Kumar et al. 2017). Considerable efforts have been made to explore alternative materials for rhizobial carriers in the recent time (Albareda et al. 2008; Araujo et al. 2020). A suitable carrier should have some distinctive properties, such as practicable

✉ Chengrong Chen
c.chen@griffith.edu.au

1 Australian Rivers Institute, School of Environment and Science, Griffith University, Nathan Campus, Nathan, QLD 4111, Australia

2 Cooperative Research Centre for High Performance Soils, Callaghan, NSW, Australia

3 College of Chemical Engineering, Qingdao University of Science and Technology, Qingdao 266042, China

4 Global Centre for Environmental Remediation, College of Engineering, Science and Environment, The University of Newcastle, University Drive, Callaghan, NSW 2308, Australia

architectures (e.g., porous structures, channel, or layer structures), abundant nutrients [(e.g., carbon (C), N, oxygen (O), phosphorous (P), potassium (K), and calcium (Ca)], appropriate pH (near neutral), high water holding capacity (WHC), and cost-effectiveness (Bolan et al. 2023; Li et al. 2023; Pacheco-Aguirre et al. 2017; Shabir et al. 2023b). Despite significant efforts to exploit novel carrier materials such as sawdust, charcoal, sugarcane bagasse, cow manure, neem leaves, Buffel grass, alginate/starch blends and rice bran for high-quality bioinoculant production, many potential materials have only been published in scientific journals and rarely developed for commercial use (Arora et al. 2008; Hassan et al. 2018; Kumar et al. 2015; Rohman et al. 2021). The recommended viable cell count for each carrier material is considered to be 10^7 CFU g^{-1} (Sohaib et al. 2020).

Biochar can be made from a variety of feedstocks such as organic waste from agriculture/forestry, animal waste/manure, food waste and green waste, and the physical and chemical properties of the material vary depending on the feedstock type and the pyrolysis process (Ippolito et al. 2020; Tomczyk et al. 2020; Zhang et al. 2017). Pyrolysis temperature plays a vital part in determining the properties and function of biochar materials. Typically, the pyrolysis temperature ranges from 200°C to 1000°C (Sohi et al. 2010) to produce sterilized biochar products. Zhang et al. (2017) reported that an increase in pyrolysis temperature (350°C to 550°C) can increase the pH of biochar. Similarly, Hale et al. (2015) noted that biochars pyrolyzed at 600°C showed higher pH (alkaline) as compared to those pyrolyzed at 300°C, which showed lower pH (acidic) values. Moreover, biochars pyrolyzed at lower temperatures contain a higher amount of amorphous C (Keiluweit et al. 2010; Tomczyk et al. 2020), which could facilitate microbial growth and protect them from toxins (Allen et al. 2021). Additionally, biochar pyrolyzed at higher temperatures has been found to exhibit a high surface area and environmental stability (Ahmad et al. 2012; Mašek et al. 2013; Shabir et al. 2023b). However, elevated pyrolysis temperature results in less N content leading to lower N availability for plant uptake (Uchimiya and Hiradate 2014). On the other hand, biochars pyrolyzed at lower temperatures from various feedstocks (pinewood, sugarcane, peanut shell, and oak) have shown a significant amount of nutrients (Mn, K, Fe, N, P Ca, Na, and Mg) (Zhang et al. 2017). Biochar has vast applications in agricultural soil due to its exceptional physicochemical properties. It has been extensively studied and documented as a nutrient (C, P) sequestrating agent, soil rehabilitating modifier, rhizosphere soil protector, and soil conditioner (Alipour et al. 2021; Amini et al. 2016; Rashti et al. 2019a, b; Reverchon et al. 2015). However, the sole application of biochar in the soil does not make it a comprehensive fertilizer capable of supplying a significant amount of essential nutrients for plant growth. Combining beneficial microbes such as rhizobia and/or plant growth-promoting rhizobacteria with biochar enhances its effectiveness as a soil amendment, making it a valuable and promising tool for sustainable agriculture, especially in the legume sector (Ajeng et al. 2020; Hariz et al. 2015; Shabir et al. 2023a). Recent reports have highlighted the role of biochar as a microbial carrier, and studies on this topic have been published (Azeem et al. 2021; Głodowska et al. 2016, 2017; Hale et al. 2015; Tittabutr et al. 2007). For Instance, Głodowska et al. (2017) reported that biochar produced from soft wood feedstock (Pyrovac-PR) showed the highest viable cell count (6.3 $\log_{10}$ CFU/mL) of *Bradyrhizobium japonicum* strain (532 C) after 37 weeks of storage at 21°C as compared to peat and other biochars. Moreover, significant number of root nodules (29.58 ± 3.91 plant $^{-1}$) were observed in Uncoated + PR + *B. japonicum* treated soybean plants as compared to uncoated seeds (0.83 ± 0.28 plant $^{-1}$) under N free Hoagland's solution.

Biochar-based bacterial inoculants have the potential to significantly contribute to sustainable agriculture by promoting plant growth (Hale et al. 2014), enhancing soil fertility, and reducing the dependence on mineral fertilizers (Azeem et al. 2021). However, less attention has been paid to investigating the influence of pyrolysis temperature on biochar as an alternative microbial carrier and such studies are seldom reported. Furthermore, the carrier potential of pinewood and oak-derived biochars pyrolyzed at a wide range of temperatures (400°C, 500°C, 600°C, 700°C and 800°C) remains unexplored.

In this study, we aimed to investigate the physicochemical characteristics of low-cost biochar pyrolyzed at various temperatures using advanced analytical methods. Furthermore, we assessed its suitability as an alternative rhizobial carrier. The survival rate and shelf life of rhizobia were monitored and compared with peat, which served as the control. Additionally, we examined the impact of rhizobial inoculation on the growth, physiology, and isotopic signatures of soybean plants at the end of the plant growth experiment. We hypothesized that different pyrolysis temperatures (400°C, 500°C, 600°C, 700°C, and 800°C) could have different physicochemical properties and some of these biochar could be more suitable for shelf life and survival of rhizobia as an alternative rhizobial carrier material due to their better surface and chemical properties.

Methodology

Feedstocks and slow pyrolysis operation for biochar manufacturing

Australian peat was purchased from Green Microbes Aust Pty Ltd. Oak (*Allocasuarina torulosa*) and pinewood (*Pinus radiata*) feedstocks were selected from Narrongin region, Western Australia (32.936°S 117.178°E). The feedstocks were air-dried and converted into small pieces (3–4 cm) prior to oven dried at 60°C for 3–4 days. Slow pyrolysis method was used to produce biochar at laboratory scale. Biochar preparation was carried out using horizontal furnace (HTF 80/12e3/1, Laboratory Equip. Pty. Ltd., Australia) with 300 mm long tube under anoxic conditions, maintaining temperature uniformity within 5°C. N_2 gas was used as inlet gas with flow rate 0.5 LPM. The residence time of biochar manufacturing was 1 h at 10°C min^{-1} heating rate with gradual increase of 50°C to target the peak temperatures 400°C, 500°C, 600°C, 700°C and 800°C, respectively. The biochar materials produced were given the names P-BC400, P-BC500, P-BC600 P-BC700, P-BC800, and O-BC400, O-BC500, O-BC600 O-BC700, O-BC800 where the prefix letters P and O stand for pinewood, and oak, and the suffix numbers stand for pyrolysis temperatures. Biochar materials were finely ground to powder form and sieved (250 µm) prior to use.

Physicochemical analysis of biochar

Biochar total C (TC) and N (TN) were analysed using LECO (CNS-2000, LECO Corporation, MI, USA) analyzer (Zhang et al. 2017). Hot water extractable organic C (HWEOC) in biochar samples was measured following the method recommended by Chen et al. (2000). Briefly, 1.5 g of biochar samples (oven dried) were mixed with 30 mL distilled water in capped Falcon tubes and incubated for 18 h at 70°C. Afterward, the Falcon tubes were placed in an end-over-end shaker for five minutes to ensure uniform mixing. The mixtures were then filtered using a Whatman 42 filter paper (Whatman Ltd., Maidstone, UK). The filtrate was used to determine HWEOC value using a TOCN analyzer (SHIMADZU TOC-VCPH, Kyoto, Japan) (Liu et al. 2021; Yao et al. 2021). To analyze major and trace elements, wet digestion of biochar samples was performed using HNO_3 and $HCLO_4$ in a 4:1 ratio. Inductively coupled plasma optical emission spectroscopy (ICP-OES, Perlin Elmer, USA) was used to measure the concentrations (Bahadori et al. 2019). The results were reported on an oven-dry basis. pH and electrical conductivity (EC) were determined using a 1:20 volumetric ratio of biochar samples to distilled water. The mixtures were placed in the end-to-end shaker at 70 rpm for one h to ensure uniform shaking. Measurements were taken using an Accumet® basic AB15 pH/cond. meter (Rajkovich et al. 2012). The biochar water holding capacity (WHC) was determined by saturating the biochar materials in water for 24 h and then allowing them to drain in the air for 1–3 h (properly covered to reduce the aerial loss). The mass of water retained in the material per gram of dry material was used to calculate the percentage WHC values (Hale et al. 2015; Shabir et al. 2023b). The functional groups of the biochars were determined using FTIR (PerkinElmer Spectrum Two IR spectrometer, PerkinElmer, USA). FTIR measurements were recorded using the Spotlight 400 series in the spectrum range of 650–4000 cm^{-1}. Physical properties such as porous and layered structures, pore size, and specific surface area (SSA) were characterised using a scanning electron microscope (SEM) (JSM-7001 F) and a Brunauer-Emmett-Teller (BET) analyzer.

Strain culture condition, materials sterilization, and inoculation

Australian Inoculants Research Group, Department of Primary Industries, New South Wales (NSW), Australia. provided the *Bradyrhizobium japonicum* (CB 1809) strain. CB 1809 was cultured in YMB (yeast mannitol broth) in a 250 mL Erlenmeyer flask and shaken for five days at 28 °C at 150–180 rev min^{-1} on a gyratory shaker (Vincent 1970). For sterilization, all sieved biochar and peat materials were placed in autoclave bags, with each bag containing 10 g of each material. All the bags were then sterilized in an autoclave for 20 min at 121 °C. A sufficient volume of five-day-old, incubated CB 1809 strains (2.14×10^9 ml^{-1}) was aseptically injected into the autoclave bags to create the inoculant while maintaining a moisture content (MC) of 40% (oven dried basis) in biochar and peat materials. The biochar materials inside the bags were thoroughly mixed by hand, and 60–70% space was left in the bags to allow for proper aeration. Bags were kept at 28°C and 38°C for up to 90 days.

Determination of shelf life

The population density of strain CB 1809 in each inoculant was examined by measuring the Colony-forming unit (CFU) value at 20 days, 40 days, 60 days, and 90 days to determine the shelf life of prepared inoculants at 28°C and 38°C. To calculate the CFU value, 1 g of inoculant was suspended in 9 mL of YMB broth. To ensure the complete release of the rhizobial strains from carriers, the suspensions were shaken for 30 min at 150 rpm on a gyratory shaker. Serial dilutions of the formulations were made up to $10^{-6} - 10^{-7}$, and 100 µl of the final dilution was spread onto nutrient agar

petri plates. Each dilution was repeated 3 times. All plates were then incubated at 28 ± 2 °C for 2–3 days. The number of microbial colonies that appeared on each plate was counted, and the inoculum log CFU g^{-1} of the carrier material was calculated.

Greenhouse pot trial

Experimental setup

The suitability of biochars produced at different pyrolysis temperatures as the inoculants of CB1809 for soybean (*Glycine max L.*) growth and nodulation against the carrier control (without any carrier and/or with peat) was determined through pot trial that was performed in the greenhouse (day/night temperature 22°C; humidity 50–60%, day length 16 h). For the pot trial, P-BC400, which showed the highest shelf life (10.34 Log 10 CFU g^{-1}), and O-BC400, which had the second highest shelf-life (9.76 Log 10 CFU g^{-1}) after 90 days of storage, were selected. Furthermore, the inoculants P-BC800 and O-BC800, which had the worst Log 10 CFU g^{-1} Fig. 3 were also used for comparison. Prior to the pot trial, the seeds were dipped into ethanol (95% C_2H_6O) for 5 min for surface sterilization and rinsed thoroughly with sterile double distilled water (Kumar et al. 2017). Afterward, the seeds were air-dried for 30 min. The seeds were gently mixed with 4–5 days old inoculants. To coat the seeds, 4 g of each inoculant and a 10% sucrose solution (as a sticking agent) were utilized. The coated seeds were then placed in the shade for dry aeration. Plastic pots (14 cm × 11.5 cm) were filled with a mixture of 1 kg of garden soil (50%) and sand (50%). We used loamy garden soil and other important properties of soil mixture includes HWEOC (0.45 g kg^{-1}), TN (0.12%), and pH (5.25) (Table S1).

Moisture treatments

Three different moisture levels of soil were adjusted based on WHC: 55%, 30%, and 15% of WHC (65.7%), to achieve water (drought) stress at varying rates. Normal irrigation, mild drought, and severe drought were represented by D0 (55%WHC), D1 (30%WHC), and D2 (15%WHC), respectively. In total, eighteen treatments (Table S2) with four replicates were used in this study, resulting in a total of 72 treatment units (pots). Before sowing, oven-dried soil in each pot (72) was watered to normal watering level (D0) with 361 ml of water. Five inoculated seeds were sown in each pot, and thinning was done to retain three seedlings in each pot about 10–15 days following emergence. Water (drought) stress was applied to soybean seedlings after thinning in both mild (D1) and severe drought (D2) treatments. For the mild treatment, 197 ml of water was used, and for the severe drought treatment, 98 ml of water was used. Every day, pots were weighed and watered daily to maintain the proper moisture level. Each pot was given another six weeks to allow for the establishment of healthy seedlings.

Harvesting and plant biometric attributes

On May 10, 2020, soybean seeds were planted, and on July 20, 2020, they were harvested. After harvest, the length of the shoots and roots, the number of leaves, and the number of root nodules were all measured and recorded. The roots were separated from the shoots and rinsed well to remove any debris before being dried on paper towels. The root and shoot samples were oven dried for two days at 70°C to calculate the dry weight. Following the assessment of nitrogenase activity, root dry weights were calculated.

Acetylene reduction assay

There are few methods to determine the N fixation such as N-balance (determine N fixation on area basis: kg/ha), N-difference (calculate N-fixation per plant or area basis), ^{15}N /ureide method (provides percentage of N fixed through BNF) and acetylene reduction assay (ARA: nitrogenase activity to catalyse N-fixation) (Unkovich et al. 2008). In this study ARA was used to evaluate the activity of nitrogenase enzymes in the root system of plants (Hardy et al. 1973). Briefly, the entire root system of each treatment (washed and paper towel-dried) was placed in 250 ml incubation bottles and carefully sealed with a cap. To prevent the roots from drying out during the assay, damp paper towels were placed in each bottle. 10% of the air was replaced with acetylene gas, and the system was left in the dark at room temperature for 24 h (Bahulikar et al. 2021). To measure ethylene production, a 500 µl sample was obtained from each incubation bottle and fed into a gas chromatograph (GC-2010, Shimadzu Plus). The GC was set up with a 30 M gas pro column, a 50°C oven, and 50°C and 270°C front inlet and FID detector temperatures, respectively. N_2 gas was used as a make-up gas at a constant flow rate of 10 ml min^{-1}. Standard curves of GC peak area were established using repeated dilutions of a known quantity (standards) of ethylene to record the concentration of ethylene produced in ARA. The amount of ethylene collected per unit fresh weight of roots per hour was used to calculate the nitrogenase activity.

Leaf pigmentation, membrane stability index (MSI) and relative water content (RWC) analysis

The leaf pigments chlorophyll (chl.) a, b, and total chl. were determined using the Lichtenthaler (1987) method. In

summary, 1.0 g of fresh leaf samples was taken and immediately immersed in liquid nitrogen to stop metabolic activity in the leaf tissue. Then, 80% acetone was mixed with the leaf tissues and pulverized until homogeneity was reached. The homogenized mixture was then centrifuged at a speed of 3000×g for 10 min. The resultant supernatant was used in a UV–spectrophotometer (UV1800, Shimadzu, Japan) to measure absorbance at wavelengths of 663.2 and 646.8 nm. The chlorophyll contents were determined using Lichtenthaler (1987) formulas. The results were reported in μg g^{-1} of fresh leaf weight.

The leaf membrane stability index (MSI) was calculated. For this purpose, 0.5 g fresh leaves were immersed in 10 ml of distilled water, and samples were divided into two groups. The electrical conductivity (EC1) of the first group was measured after heating the samples in an oven at 40°C for half an hour, while the electrical conductivity (EC2) of the second group was measured after heating in an oven at 100 °C for half-hour. The following equation was used to calculate MSI.

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

The relative water content (RWC) of soybean leaves was determined using the youngest fresh apical leaves, following the methods explained by Sairam et al. (2002).

Determination of stable C ($\delta^{13}C$) and N ($\delta^{15}N$) isotopes

Fresh apical soybean leaves were obtained and air-dried before being oven dried at 70 °C for 48 h to determine $\delta^{13}C$ and $\delta^{15}N$. The dried leaf samples were ground into powder, and 4–5 mg of each sample was pelletized into tin capsules. Isotope-ratio mass spectrometer (Sercon Hydra 20–22 Europa EA-GSL) was used to analyse the pelletized materials for $\delta\ ^{13}C$ and $\delta\ ^{15}N$. The following equation is used to explain ratios for stable C and N isotopes in δ (standard delta) and ‰ (notation per mil): $y = [(R_{sample} / R_{standard})\ 1] \times 1000$, where Y is either $\delta^{13}C$ or $\delta^{15}N$ and R is the heavier to lighter isotope ratio of the sample and standard, which is $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively. As a standard values for C and N, Pee Dee Belemnite (PDB) limestone and ambient N were used (Bahadori et al. 2019; Garzon-Garcia et al. 2017).

Statistical analysis

Rhizobial counts (Log 10 CFU g^{-1}) in carriers were estimated after logarithmic transformation. The average difference in shelf life and survival rate of carriers was tested using Fisher's test when ANOVA was significant at $p < 0.05$. For the plant analysis, the data were analysed by two-way analysis of variance (ANOVA) with watering regimes and inoculant treatments as factors. The interaction data were estimated and presented between soil watering regimes and treatments. Mean values were compared by the least significant difference (LSD) test at a 5% ($p < 0.05$) probability level (Steel 1997). To evaluate the effects of watering regimes and treatments, principal component analysis (PCA) was used to analyze the correlation matrix between the variables. Minitab 18 software was used to carry out the analysis.

Results

Characteristics of carrier materials

Results indicated that TC% of biochars produced from both pine wood and oak feedstocks increased with pyrolysis temperature, however, TN% and HWEOC% showed inverse relationship to TC% with increase of pyrolysis temperature (Table 1). At lower pyrolysis temperatures (400°C), the pH values of all pine wood and oak-derived biochars were close to neutral. For instance, P-BC400 and O-BC400 had pH values of 7.24 and 7.34 pH, respectively. Similarly, biochars pyrolyzed at lower temperatures (400°C and 500°C) exhibited lower EC, SSA, and pore size compared to those pyrolyzed at higher temperatures. Pine wood-derived biochars did not show a significant difference in WHC, with values ranging from 102% to 107%. However, a relatively wide range of WHC (40% – 101%) was observed in oak-derived biochars. In terms of element analysis, all the derived biochars showed abundant elements such as K, P, S, Ca, Mg, and Al. Notably, the highest concentration was observed for Ca, which is a critical nutrient for strain survival and growth. The concentration of toxic metals (e.g., Ni, Cd, Pb, Hg, As) in all biochars was below the detection limit, indicating their low toxicity to rhizobia and the environment.

Figure 1 shows the SEM characterization of the morphologies of all biochars. The surfaces of all biochars appeared coarse, with channels and pores indicated by white arrows and yellow circles, respectively.

Abundant functional groups and characteristic bands in the biochars, as identified by FTIR spectra, are presented in Fig. 2. There were no significant differences in C structures of the biochars than the feedstock types. However, the pyrolysis temperature had a greater impact on the C structure of the biochars than the feedstock type (Fig. 2a, b). This could be attributed to dehydration and decarboxylation during pyrolysis. As the pyrolysis temperature increased from 400°C to 800°C, the OH and C=O stretching vibrations (3617–3671 cm^{-1} and 1693 cm^{-1}, respectively) decreased.

Table 1 Physico-chemical properties of pinewood and oak derived biochars as a carrier material (Mean ± SD; $n = 4$)

Properties	P-BC400	P-BC500	P-BC600	P-BC700	P-BC800	O-BC 400	O-BC500	O-BC600	O-BC700	O-BC800
Physico-chemical characteristics										
TC (%)	74.2	80.1	81.2	81.4	81.7	72.7	78.3	79.7	80.5	82.4
TN (%)	0.41	0.44	0.40	0.31	0.29	0.39	0.46	0.38	0.37	0.34
C: N ratio	181	184	200	263	282	186	170	210	218	242
HWEOC (g kg^{-1})	2.1	1.03	0.92	0.81	1.50	1.72	1.14	1.12	0.93	0.89
pH (1:20; w/v)	7.24	7.43	7.84	7.90	8.11	7.34	7.92	8.55	8.75	8.79
EC (µS)	63.4	76	94.3	109	109	81	102	158.4	174	169
WHC (%)	102.2	104.3	106.8	104.5	106.8	39.7	101.1	100.4	78.8	49.8
Specific surface area (m^2 g^{-1})	14.2	25.5	142.7	167.4	154.4	11.5	28.4	138.4	154.2	152.4
Pore size (µm)	3.64	4.52	6.35	6.87	6.24	3.24	5.32	6.04	6.49	6.05
Elemental concentration (g kg^{-1})										
K	2.44	2.66	3.35	3.64	2.82	2.93	3.57	3.54	3.68	3.96
P	0.57	0.61	0.79	0.83	0.63	1.46	1.57	1.59	1.74	1.88
Ca	6.04	6.98	8.26	8.58	6.43	3.62	9.83	9.30	9.93	9.06
S	0.31	0.26	0.34	0.31	0.27	0.42	0.36	0.41	0.40	0.39
Mg	1.20	1.37	1.65	1.83	1.42	1.19	1.58	1.52	1.74	1.62
Al	0.90	0.77	0.99	0.97	0.75	0.04	0.10	0.13	0.36	0.07
Na	0.38	0.39	0.50	0.49	0.38	1.30	1.59	1.52	1.60	1.68
Zn	0.30	0.09	0.14	0.05	0.02	0.06	0.04	0.17	0.04	0.02
Fe	0.24	0.16	0.23	0.21	0.15	0.02	0.04	0.02	0.29	0.04
Mn	0.38	0.44	0.54	0.53	0.45	0.23	0.30	0.32	0.35	0.32
Ni, Cd, As, Hg, Pb	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl	<dl

TC: total C; TN: total N, HWEOC: hot water extractable organic C; EC: electrical conductivity; WHC: water holding capacity; <dl: below detection limit

Similarly, within this temperature range (400°C to 800°C), the aliphatic CH_2 and CH_3 stretching and deformation vibrations (2916–2985 cm^{-1} and 1388–1395 cm^{-1}, respectively) decreased with increasing pyrolysis temperature. Strong aromatic C = C stretching vibrations (1584–1594 cm^{-1}) were noted in the biochars produced at 400°C, and these vibrations remained present in all biochars until the temperature surpassed 600°C. Moreover, C-O stretching vibrations (1062–1195 cm^{-1}) were observed in all biochars. However, these C-O stretching vibrations were more pronounced in oak-derived biochars compared to pine wood-derived biochars (Fig. 2a, b), where these peaks were only observed at lower temperatures (400°C − 600°C). In oak-derived biochars, an additional C-O stretching vibration (e.g., acetyl esters) and OH plane deformation appeared at 1233 cm^{-1}. The CO_3^{2-} ion deformation vibration (872–885 cm^{-1}) was present in all biochars, regardless of the feedstock type and pyrolysis temperature.

Rhizobial shelf life and survival rate

As shown in Fig. 3 and Figure S1, among the evaluated pine wood and oak-derived biochar carriers, P-BC400 exhibited the most favourable results for the shelf life and survival rate of rhizobia. At 28°C (Figure S1), the strain population (survival rate) of all biochar (pine wood and oak) derived products ranged between 79% − 95% at the end of 90 days storage period, which was not significantly different ($p > 0.05$) from peat (86% − 91%). Among the pine wood-derived biochars, the biochar pyrolyzed at a lower temperature (P-BC400) showed the highest shelf life (10.34 log10 CFU g^{-1} value) (Fig. 3a) with a corresponding survival rate of 95% (Figure S1a). The lowest shelf life (8.53 log10 CFU g^{-1}) and survival rate (78%) were observed in O-BC800 (Fig. 3b, Figure S1b). At 38°C, all tested carrier materials experienced different levels of colony count reduction, but the survival rate of strains remained above 72%, with the highest value recorded in P-BC400 (90%) compared to peat (86%) and other biochar carriers (Figure S1 c, d). From 0 to 20 days, under both storage temperatures (28°C and 38°C), a rapid decrease in shelf life and survival rate was observed in biochars that were pyrolyzed at relatively high temperatures (O-BC800 > O-BC700 > O-BC600) (Fig. 3, Figure S1). However, an overall gradual decrease was observed from 20 to 90 days among all carriers, and viable cell count (survival rate) did not deteriorate significantly, maintaining 78% (under 28°C) and 72% (under 38°C).

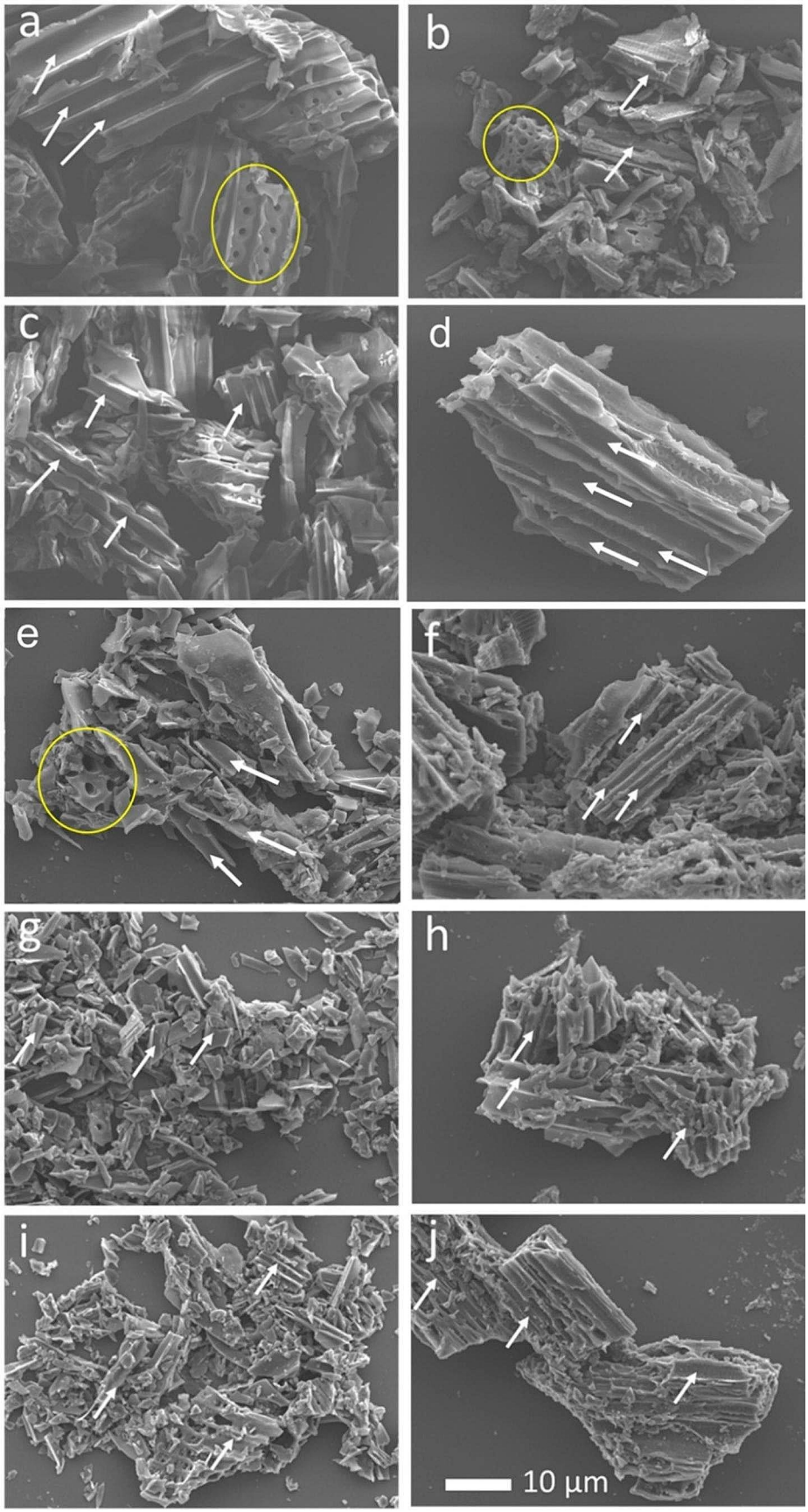

Fig. 1 Representative SEM images of P-BC400 (**a**), O-BC400 (**b**), P-BC500 (**c**), O-BC500 (**d**), P-BC600 (**e**), O-BC600 (**f**), P-BC700 (**g**), O-BC700 (**h**), P-BC800 (*i*) and O-BC800 (**j**). White arrows and yellow circles represent the channel and pores, respectively. P-BC400: pine wood biochar pyrolyzed at 400°C; O-BC400: oak O-BC500 biochar pyrolyzed at 400°C; P-BC500: pine wood biochar pyrolyzed at 500°C; O-BC500: oak O-BC500 biochar pyrolyzed at 500°C; P-BC600: pine wood biochar pyrolyzed at 600°C; O-BC600: oak wood biochar pyrolyzed at 600°C; P-BC700: pine wood biochar pyrolyzed at 700°C; O-BC700: oak wood biochar pyrolyzed at 700°C; P-BC800: pine wood biochar pyrolyzed at 800°C; O-BC800: oak wood biochar pyrolyzed at 800°C

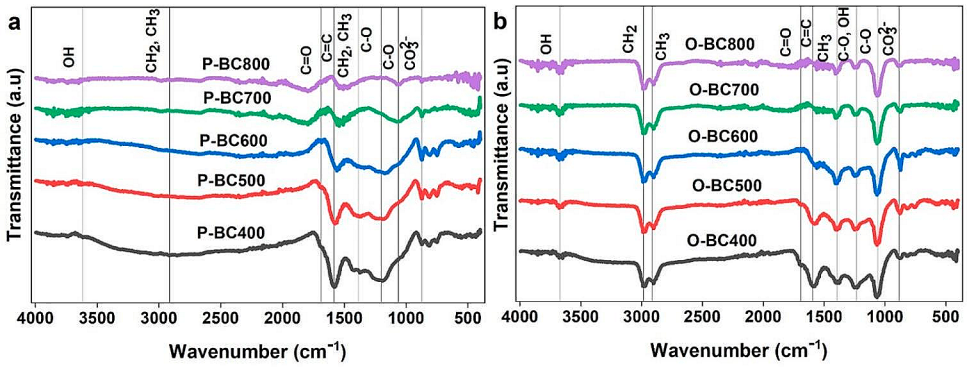

Fig. 2 (**a**) FTIR spectra of P-BC400, P-BC500, P-BC600, P-BC700, and P-BC800. (**b**) O-BC400, O-BC500, O-BC600, O-BC700, O-BC800. For the abbreviations, please see the caption of Fig. 1

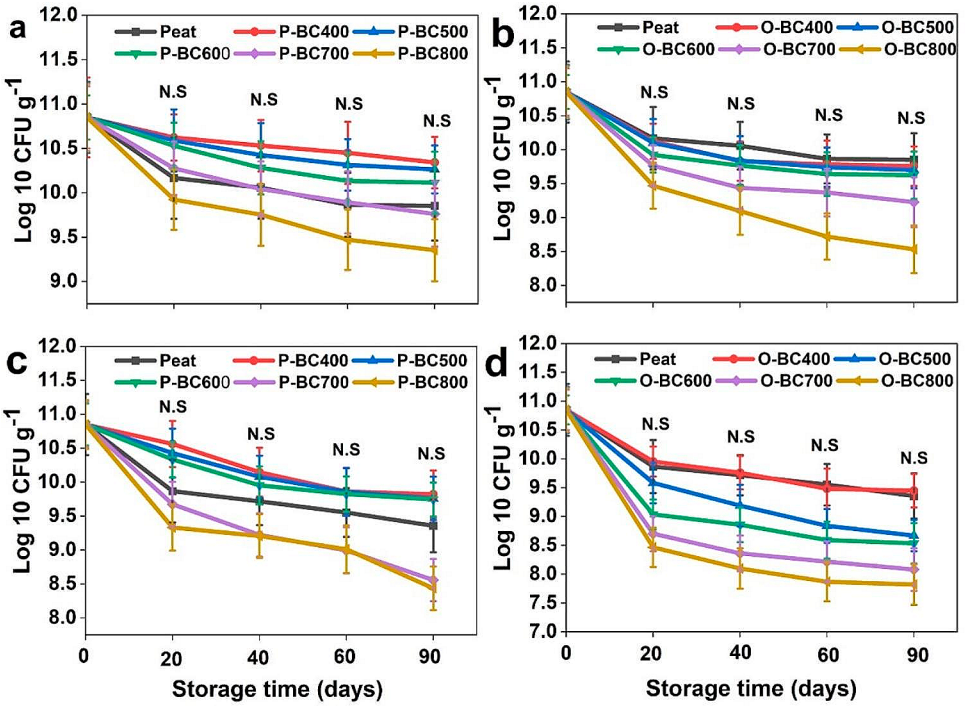

Fig. 3 Log 10 CFU g^{-1} value of peat and pine wood-based inoculants under 28°C (**a**) and peat and oak based inoculants under 28°C (**b**). Log 10 CFU g^{-} value of peat and pine wood-based inoculants under 38°C (**c**) and peat and oak based inoculants under 38°C (**d**). Storage time was 0–90 days (Mean ± SD, $n = 3$). N.S, non-significant (p. Storage time was 0–90 days (Mean ± SD, $n = 3$). N.S, non-signif

Plant growth attributes

The results (Table 2) revealed a significant difference ($p < 0.05$) in the growth attributes (shoot/root dry biomass, plant height, root length, number of leaves, and root nodules) of soybean plants grown with different treatments (control, peat, P-BC400, P-BC800, O-BC400, O-BC800) under different watering regimes (D0, D2, and D3). An obvious decrease in plant growth was observed with increased drought stress in each treatment. However, inoculant applications significantly reduced the negative impact of stress.

Plants showed better growth with P-BC400 inoculants followed by O-BC400 as compared to peat and control. For example, P-BC400 treated plants grown under severe drought stress (D2) showed greater shoot dry weight (by 174%) and root dry weight (by 367%) as compared to control. Notable increases in shoot length (by 86%) and root length (by 85%) were observed in plants inoculated with P-BC400 under severe drought stress (Table 2, Figure S2) as compared to those treated with the peat. On the other hand, at D2 (severe drought stress), plants inoculated with O-BC400 showed 116% and 200% increases in shoot dry weight and root dry weight, respectively, as compared to control (uninoculated) (Table 2). A 68% increase in shoot length and 62% increase in root length were recorded in soybean plants inoculated with O-BC400 as compared to the peat at D2 (Table 2). Moreover, P-BC400 and O-BC400 treatments caused a significant increase in the numbers of leaves and root nodules of soybean plants (Table 2). Maximum increase in number of leaves (by 25%) and root nodules (by 135%) were observed in P-BC400 treated plants followed by O-BC400 treated plants (that showed 15% increase in number of leaves and 85% increase in root nodules) at D2, as compared to the peat (Table 2). Consequently, relatively

Table 2 Effect of carrier materials on growth parameters of soybean plant under different watering regimes

Watering regimes	Treatments	Shoot dry weight (g plant^{-1})	Root dry weight (g plant^{-1})	Shoot length (cm)	Root length (cm)	No. of leaves (plant^{-1})	No. of root nodules (plant^{-1})
D0							
	Control	0.37 ± 0.02 hi	0.07 ± 0.00 j	44.0 ± 1.94 jk	11.9 ± 0.96 ij	10.5 ± 0.58 efg	0.0 ± 0.00 k
	Peat	0.44 ± 0.02 f	0.12 ± 0.01 fg	61.9 ± 1.50 fg	14.6 ± 0.76 gh	11.5 ± 0.58 de	8.5 ± 0.58 cdef
	O-BC400	0.69 ± 0.02 c	0.15 ± 0.00 d	71.4 ± 1.61 c	20.7 ± 1.71 bc	13.3 ± 0.50 b	11.5 ± 0.58 b
	O-BC800	0.51 ± 0.02 e	0.13 ± 0.00 ef	62.4 ± 2.02 efg	17.0 ± 0.82 ef	11.75 ± 0.50 d	9.5 ± 0.58 cd
	P-BC400	0.95 ± 0.03 a	0.26 ± 0.01 a	84.7 ± 2.25 a	29.6 ± 0.78 a	15.75 ± 0.96 a	16.0 ± 0.82 a
	P-BC800	0.61 ± 0.02 d	0.16 ± 0.00 cd	64.6 ± 1.58 e	19.3 ± 1.41 cd	13 ± 0.82 bc	12.5 ± 0.58 b
D1							
	Control	0.31 ± 0.02 j	0.05 ± 0.01 k	31.1 ± 1.56 m	8.3 ± 0.51 k	8.8 ± 0.96 hi	0.0 ± 0.00 k
	Peat	0.34 ± 0.02 ij	0.08 ± 0.00 ij	51.6 ± 1.79 kl	10.6 ± 0.91 j	11.0 ± 1.41 def	7.5 ± 1.29 fgh
	O-BC400	0.52 ± 0.05 e	0.11 ± 0.00 g	68.0 ± 2.56 d	16.7 ± 1.90 ef	13.3 ± 0.96 b	9.8 ± 1.71 c
	O-BC800	0.44 ± 0.02 f	0.09 ± 0.00 hi	60.0 ± 1.18 g	13.9 ± 1.48 h	11.5 ± 0.58 de	8.0 ± 0.82 efg
	P-BC400	0.72 ± 0.02 b	0.21 ± 0.00 b	75.4 ± 2.35 b	21.0 ± 1.58 b	15.0 ± 0.82 a	12.0 ± 1.63 b
	P-BC800	0.51 ± 0.03 e	0.14 ± 0.01 e	63.3 ± 2.43 ef	17.9 ± 1.00 de	12.0 ± 0.82 cd	9.3 ± 0.96 cde
D2							
	Control	0.19 ± 0.02 l	0.03 ± 0.00 l	25.7 ± 1.50 n	6.2 ± 0.43 l	7.3 ± 0.96 j	0.0 ± 0.00 k
	Peat	0.26 ± 0.02 k	0.06 ± 0.01 k	28.9 ± 1.83 m	8.6 ± 0.76 k	10.0 ± 0.82 fg	3.5 ± 1.29 j
	O-BC400	0.41 ± 0.02 fg	0.09 ± 0.00 hi	48.5 ± 2.08 i	13.9 ± 1.03 h	11.5 ± 1.29 de	6.5 ± 1.29 h
	O-BC800	0.34 ± 0.02 i	0.07 ± 0.00 j	40.1 ± 1.71 l	11.4 ± 0.92 ij	8.3 ± 0.95 ij	5.0 ± 0.82 i
	P-BC400	0.52 ± 0.03 e	0.14 ± 0.02 c	53.8 ± 2.17 h	15.9 ± 0.68 fg	12.5 ± 0.58 de	8.3 ± 1.26 def
	P-BC800	0.39 ± 0.02 gh	0.09 ± 0.00 h	50 ± 0.99 ij	12.2 ± 0.69 i	9.5 ± 0.58 gh	6.8 ± 0.96 gh

For each parameter, the values (mean + SD, $n = 4$) do not share a same letter are significantly different (LSD test, $p < 0.05$). O-BC400: oak biochar pyrolyzed at 400°C; O-BC800: oak biochar pyrolyzed at 800°C; P-BC400: pine wood biochar pyrolyzed at 400°C; P-BC800: pine wood biochar pyrolyzed at 800°C. D0: 55%WHC (water holding capacity); D1: 30%WHC; D2:15%WHC

similar trend regarding plant growth attributes (shoot/root dry weights, shoot/root length, number of leaves and root nodules) was observed under mild stress (D1) and normal irrigated (D0) plants using the same treatments. However, maximum plant growth was recorded under normal irrigation (Table 2).

Acetylene reduction assay

The nitrogenase activity value differed significantly ($p < 0.05$) in all biochar-based carrier inoculants compared to peat and control (Table 3). A significantly greater nitrogenase activity was observed in plants inoculated with P-BC400 followed by O-BC400 at all watering regimes (D0, D1 and D2). For instance, the nitrogenase activity in P-BC400 treated plants increased by 16% (D0), 10% (D1), and 4% (D2), respectively compared to peat-treated plants (significant at $p < 0.05$). The nitrogenase activity in O-BC400 and O-BC800 treated plants showed a significant difference ($p < 0.05$) to each other at D0 and at D1, but the values did not show a significant ($p > 0.05$) difference at D2. However, the nitrogenase activity of O-BC400 and O-BC800 treated plants were significantly ($p < 0.05$) higher to peat treated plants at each watering regime (Table 3). Similarly, a significant ($p < 0.05$) difference in nitrogenase activity was also observed among P-BC400 and P-BC800 treated plants at each watering regime.

$\delta^{13}C$ and $\delta^{15}N$ signatures

Soybean plants treated with the P-BC400 had significantly lower bulk leaf $\delta^{13}C$ enrichment (-30.0‰ at D0; -29.0‰ at D1; -28.8‰ at D2) than the peat and control (Table 3). While, for the $\delta^{13}C$ signature, there were no significant ($p > 0.05$) differences among different biochar-based treatments (P-BC400, P-BC800, O-BC400 and O-BC800) at D0 and D1, but P-BC400 at D2 showed a significant ($p < 0.05$) reduction in $\delta^{13}C$ enrichment as compared to other biochar-based carriers (Table 3).

For the $\delta^{15}N$ signature, P-BC400 treated plants had significantly lower $\delta^{15}N$ enrichment ($p < 0.05$) (-5.91‰ at D0 and −4.39‰ at D2) as compared to peat and control (Table 3). Apart from O-BC800, insignificant differences ($p > 0.05$) were observed among all biochar-based inoculants at the same watering regimes (D0 and D2) (Table 3). At D1 only P-BC400 showed significantly ($p < 0.05$) lower $\delta^{15}N$ enrichment (-4.39‰) as compared to the control.

Table 3 Effect of carrier materials on nitrogenase activity (µm C_2H_4 h^{-1} $plant^{-1}$), $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) of soybean plant under different watering regimes

Watering regimes	Treatments	Nitrogenase activity (µm C_2H_4 h^{-1} $plant^{-1}$)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
D0				
	Control	0.00 ± 0.00 j	-24.5 ± 0.47 cde	-4.13 ± 0.08 c-g
	Peat	0.866 ± 0.04 f	-25.7 ± 0.47 def	-4.40 ± 0.08 d-g
	O-BC400	0.942 ± 0.01 b	-27.0 ± 0.47 f	-5.13 ± 0.08 gh
	O-BC800	0.892 ± 0.04 d	-26.0 ± 0.47 ef	-4.86 ± 0.08 fgh
	P-BC400	1.002 ± 0.08 a	-30.0 ± 0.47 g	-5.91 ± 0.08 h
	P-BC800	0.924 ± 0.02 c	-26.5 ± 0.47 ef	-5.19 ± 0.08 gh
D1				
	Control	0.00 ± 0.00 j	-23.0 ± 0.47 bc	-3.25 ± 0.08 a-d
	Peat	0.864 ± 0.01 fg	-24.4 ± 0.47 de	-3.61 ± 0.08 b-e
	O-BC400	0.899 ± 0.06 d	-25.4 ± 0.47 cde	-3.76 ± 0.08 b-f
	O-BC800	0.876 ± 0.06 e	-24.9 ± 0.47 de	-3.67 ± 0.08 b-f
	P-BC400	0.947 ± 0.03 b	-29.0 ± 0.47 g	-4.53 ± 0.08 efg
	P-BC800	0.892 ± 0.01 d	-25.2 ± 0.47 de	-4.41 ± 0.08 d-g
D2				
	Control	0.00 ± 0.00 j	-21.0 ± 0.47 a	-2.11 ± 0.08 a
	Peat	0.832 ± 0.00 i	-22.7 ± 0.47 ab	-2.89 ± 0.08 ab
	O-BC400	0.849 ± 0.03 h	-24.9 ± 0.47 de	-3.34 ± 0.08 b-e
	O-BC800	0.843 ± 0.03 h	-23.1 ± 0.47 bc	-3.15 ± 0.08 abc
	P-BC400	0.869 ± 0.04 ef	-28.8 ± 0.47 g	-4.39 ± 0.08 d-g
	P-BC800	0.858 ± 0.08 g	-24.2 ± 0.47 bcd	-3.29 ± 0.08 a-d

The values (mean + SD, $n = 4$) do not share a same letter are significantly different (LSD test, $p < 0.05$). For the abbreviations, please see Table 2

Leaf MSI (%) and RWC (%)

In terms of MSI, the combined effect of inoculants and watering regimes was significant ($p < 0.05$), as shown in Fig. 4a. Among all carrier inoculants, the P-BC400 inoculant significantly increased the MSI of soybean plants compared to other inoculants and the control under all watering regimes (Fig. 4a). For instance, the MSI value increase ($p < 0.05$) of P-BC400 over the control was 51.30% at D0, 33.59% at D1, and 26.85% at D2, respectively. However, as compared to peat a significant increase ($p < 0.05$) of 13.10%, 13.28%, and 18.23% was also observed at D0, D1, and D2, respectively. The O-BC400 also showed significantly ($p < 0.05$) higher MSI in soybean plants as compared to peat and control at each watering regime, except for an insignificant ($p > 0.05$) effect on peat at D1. The observed values of MSI in soybean plants for other biochar carriers, such as O-BC800 and P-BC800 were not significantly ($p > 0.05$) higher from peat but were observed significantly ($p < 0.05$) higher from the control at each watering regime.

RWC elevated in the inoculated soybean plants under each watering regime compared to the control, as elucidated in Fig. 4b. In soybean plants treated with P-BC400, the rise in RWC was particularly noticeable at each watering regime (D0, D1 and D2) as compared to control and peat. For instance, compared to control and peat (Fig. 4b), the RWC of P-BC400 treated plants significantly ($p < 0.05$) increased by 31.6% and 17.8%, respectively, at D0, followed by 26.3% and 15.6% increase (significant at $p < 0.05$), respectively, at D2. Moreover, the O-BC400 treated plants also showed significantly ($p < 0.05$) higher RWC than peat and control at three watering regimes, but all the values were less than P-BC400.

Leaf pigmentation

A significant difference was observed regarding the chlorophyl contents (chl. a, chl. b and total chl.) among different carrier inoculants and control at every watering regime (Fig. 5). Among all carrier inoculants P-BC400 inoculant showed significant increases in chl. a, chl. b, and total chl. content as compared to control at each watering regime (D0, D1 and D2) (Fig. 5). Moreover, P-BC400 inoculant impacted more on chl. b than chl. a, and total chl. (Fig. 5b).

Multivariate analysis

The coalition between various treatments (control, peat, P-BC400, P-BC800, O-BC400 and O-BC800) and response variables (Shoot/root dry weight, shoot/root length, number of leaves, number of root nodules, ARA, MSI, chlorophyll contents, RWC, $\delta^{13}C$ and $\delta^{15}N$) was determined using principal component analysis (PCA) (Fig. 6a, b). Twelve factors (F1 to F12) were identified as the sources of overall variability in the data, but only two components were the main contributors, accounting for 80.1%, and 13.7%, of the variability. Different treatments with different watering regimes were clustered along different axes based on their contributions (Fig. 6a). The control treatment with D0, D1 and D2 was scattered away from the other peat and biochar-based treatments, positioned along the y-axis. This treatment

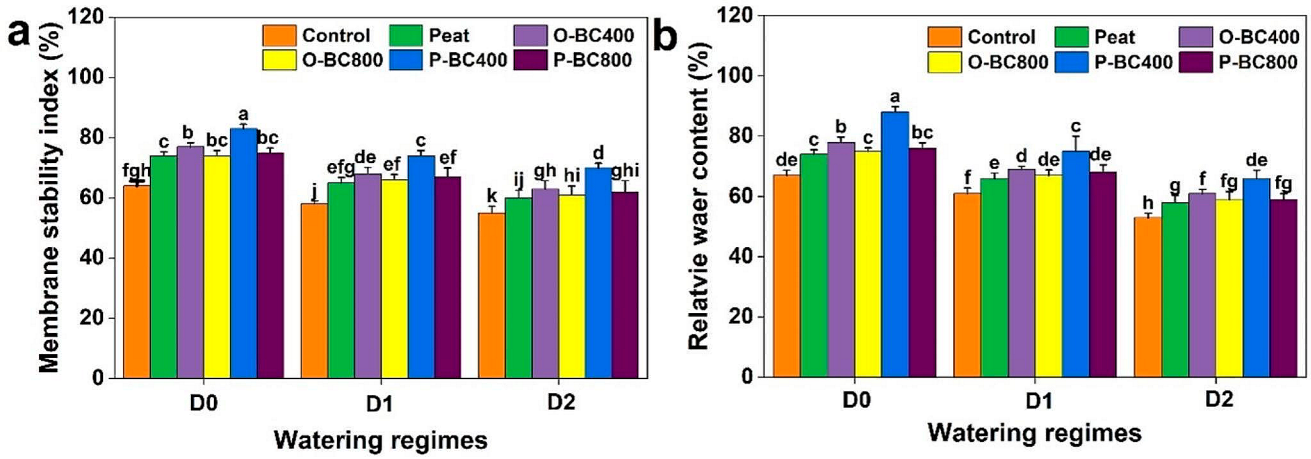

Fig. 4 Effect of peat and biochar-based inoculants on membrane stability index (MSI) (**a**) and relative water content (RWC) (**b**) For the parameters, the values (mean ± SD, $n = 4$) do not share a same letter are significantly different (LSD test, p For the D0: 55%WHC (water holding capacity); D1: 30%WHC; D2:15%WHC. For the other abbreviations please see the caption of Fig. 1

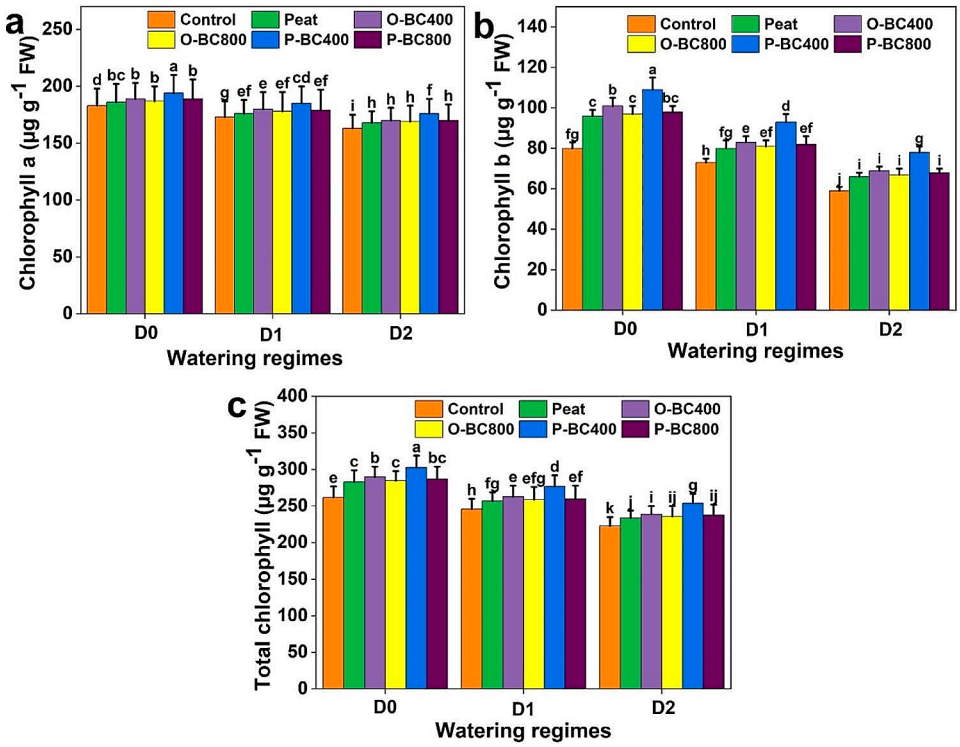

Fig. 5 Effect of peat and biochar-based inoculants on chlorophyl a (**a**) chlorophyll b (**b**) and total chlorophyll (**c**) in soybean plants. For the parameters, the values (meanoculants onD1: 30%WHC; D2:15%WHC. For the other abbreviations please see the cap$p$ or the paD0: 55%WHC (water holding capacity); D1: 30%WHC; D2:15%WHC. For the other abbreviations please see the caption of Fig.D2

exhibited lower growth, physiological attributes, and higher isotopic signatures enrichment for each watering regime. The P-BC400 treatment with D0 was positioned at the far-right side along the x-axis, indicating higher growth and physiological attributes, as well as lower $\delta^{15}N$ (indicative of higher nodulation) and $\delta^{13}C$ enrichment. The other treatments (O-BC400, O-BC800, peat, and P-BC800) with D0 were clustered near the centre of the x-axis. P-BC400 with D1 and D2 was placed at the top right side of the y-axis, characterized by better plant growth and chlorophyll values compared to other treatments scattered on the left side along the y-axis at the same watering regimes (D1 and D2). All the variables except $\delta^{13}C$ content, exhibited negative correlations with $\delta^{15}N$ enrichment (clustered on the same direction) resulting in their clustering closer to each other (Fig. 6b). For factor F1, the major contributors were shoot dry weight, root dry weight, number of nodules, MSI, RWC, total chlorophyll, shoot length, root length, and number of leaves. Therefore, these variables were grouped closer to each other near the x-axis (Fig. 6b). The main contributors

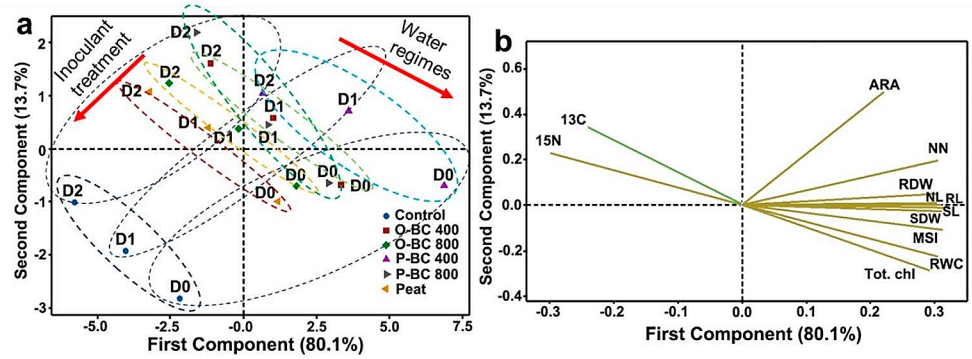

Fig. 6 Comparison of different response variables of soybean grown under different watering regimes [D0: 55%WHC (water holding capacity); D1: 30%WHC; D2:15%WHC)] with different treatments (control, peat, P-BC400, P-BC800, O-BC400, and O-BC800) using principal component analysis (PCA). ARA: acetylene reduction assay value; NN: number of nodules; SDW: shoot dry weight; RDW: root dry weight; NL: number of leaves; SL: shoot length; RL: root length; Tot. chl: total chlorophyll; MSI: Membrane stability index; RWC: relative water content. For the other abbreviations please see the caption of Fig. 1

of F2 included $\delta^{13}C$ and $\delta^{15}N$, which were scattered away from the x-axis. ARA content made the major contribution to F3 (Table S3).

Discussion

Biochars characterization and shelf life

Various carrier materials that offer protected habitats and protection from predators have been widely exploited to enhance the longevity and survival rate of rhizobia. Several researchers claimed that biochar is a viable carrier for creating formulations, either on its own or in combination with other materials (Ajeng et al. 2020; Kumar et al. 2017; Sun et al. 2016). The present study examined different biochar materials (P-BC400-PBC800 and O-BC400-OB-800), all of which exhibited significant physical (WHC), chemical (pH, plant-available nutrients), and structural (SSA, pore size, and functional groups) characteristics, indicating their suitability for developing bioformulation/biofertilizer.

In our research, we hypothesized that the pyrolysis temperature not only affects the suitability of biochar as a carrier for microorganisms but also influences plant growth. The findings of this study showed that biochar pyrolyzed at lower temperatures (especially P-BC400) was the appropriate carrier. For instance, after 90 days of storage, the population of the strain in the P-BC400 carrier remained as high as 10.34 log10 CFU g^{-1} at 28°C and 9.74 logs 10 CFU g^{-1} at 38°C, respectively, surpassing industrial standards of 10^7 g^{-1} (Balume et al. 2015). The pH of the carrier material significantly influenced the shelf life of the rhizobia (Hale et al. 2015). Depending on the feedstock, pyrolysis temperature, and oxidation level, biochar pH can range from below 4 to above 12 (Lehmann 2007) and increases with the increase of pyrolysis temperature (Zhang et al. 2017). Microorganisms generally thrive in a pH range of 7.0–7.5 (Thomas et al. 1994). Our study observed the highest CFU values in biochars (pyrolyzed at lower temperature: P-BC400, O-BC400) that had a pH value close to 7, while the lowest CFU values were found in biochars pyrolyzed at higher temperature (P-BC800 and O-BC800), likely due to their highly alkaline pH values (Table 1). Similarly, Głodowska et al. (2016) demonstrated that *Pseudomonas libanensis* population was more abundant and viable in biochars (dynamotive, pyrovac, and basque) with pH values close to neutral compared to those with higher pH values.

SSA and pore size were other crucial factors associated with the increased shelf life of rhizobial inoculants (Głodowska et al. 2016). In our current study, it was noted that SSA and pore size increased with higher pyrolysis temperature (up to 600°C), reaching the maximum in P-BC800 (152.4 m^{-2} g^{-1}) and O-BC800 (154.4 m^{-2} g^{-1}) (Table 1). However, these biochars exhibited a shorter shelf life than P-BC400 and O-BC400. Hale et al. (2015) also affirmed that Pit600 and Shell600 biochars had the largest SSA, but they did not affect the survival rates of the inoculum. Although these materials might have numerous nano-/micropores, bacteria cannot access them, thus not accurately reflecting the material's ability to serve as an inoculum carrier. In reality, the macro porosity on biochar surfaces constitutes a small portion (Hardie et al. 2014). FTIR analysis revealed the presence of several functional groups, such as C-C/C-H, C-OH, C = O, and O-C = O, in all types of biochars (Fig. 2). These functional groups have a positive impact in extending the shelf life of rhizobia. The surface chemistry of biochar is very diverse and contains organic C aromatic structures (C-C/C-H) with heteroatoms, which create microenvironments within the biochar that contain both acidic and basic moieties. Because of this unique feature, biochar can be used as a carrier material for various inoculum ingredients with different pH requirements (Sashidhar et al. 2020).

Furthermore, the existence of these natural surface functional groups makes biochars particularly valuable compared to other carrier materials such as peat, clay minerals, and polymers, as they have the ability to attract surrounding nutrients that are useful for bacterial growth (Sashidhar et al. 2020).

Inoculant effects on plant growth attributes and N fixation

In our findings, we observed significant improvements in various plant growth attributes, including shoot and root dry weight, shoot and root length, number of leaves, and number of nodules, when using biochar-based inoculants compared to peat and the control group. Interestingly, soybean plants inoculated with P-BC400 showed better growth compared to other treatments under each watering regime. This could be due to the positive effects of biochar-based inoculants on soil porosity, moisture retention (WHC), nutrients mobilization, and increased surface around the rhizosphere (Ajeng et al. 2020; Lehmann and Joseph 2015; Lehmann et al. 2011). Biochar absorb water in its pores that dissolves soluble minerals and organic compounds on outer and inner surface of biochar when applied into the soil. These solutes raise the pH, cations/anions in the soil solution, increasing dissolved organic C, which lowers Eh and raises electrical conductivity. By altering the physical characteristics of the soil, biochar also affects how efficiently plants absorb and use water under desiccation. For example, biochar lowers the bulk density of soil, which may help soils retain more water. Furthermore, changes in soil aggregate formation may result from interactions between soil particles and biochar surfaces, which may in turn impact the linkages between soil, water, and plants (Ajayi et al. 2016; Fu et al. 2022; Mannan et al. 2021). Our findings are consistent with the studies done by Egamberdieva et al. (2017) and Nadeem et al. (2017), which reported significant growth improvements in lupin and cucumber, respectively, under drought stress conditions with the application of biochar-based inoculants. Furthermore, other studies have shown that pine wood serves as an excellent carrier material for promoting the growth of cucumber and corn crops (Głodowska et al. 2016; Hale et al. 2014).

To assess the role of nitrogenase in nitrogen fixation, we conducted ARA. We found that nitrogenase activity in soybean plants inoculated by P-BC400 showed a significant increase of 15.75% compared to peat inoculants under normal watering conditions (D0). The increased ARA values in P-BC400 inoculated plants corresponded to the higher number of nodules. For instance, P-BC400 showed an 88.23% increase in the number of root nodules compared to peat under D0. Biochar has the ability to promote nodule formation by facilitating chemical signalling between the host plant and symbiotic organisms, thereby facilitating the trapping nod factors and flavonoids (Mia et al. 2018; Sashidhar et al. 2020). Our results align with the findings of Xiu et al. (2021), who examined the role of corn straw biochar at different concentrations and found that a medium concentration (30 g kg^{-1}) of biochar was optimal for the maximizing nitrogen fixation through ARA in soybean plants. Although, determination of nitrogenase activity by ARA is questionable because nitrogenase is not the only enzyme (hydrogenase also reduces ethylene to acetylene: may interfere results) that mediates the conversion of acetylene to ethylene, as measured by ARA (Hamilton et al. 1964). The acetylene reduction assay's simplicity and capacity to yield semi-quantitative data make it a popular technique for determining nitrogenase activity despite these limitations (Soper et al. 2021).

Influence on physiological parameters and isotopes ($\delta^{15}N$ and $\delta^{13}C$)

In comparison to control group, all inoculant treatments resulted in a significant increase ($p < 0.05$) in the levels of chl. a, b, total chl, RWC, and MSI in soybean plants experiencing water deficit conditions (D1 and D2). However, the plants inoculated with P-BC400 exhibited prominent chlorophyll values. Numerous mechanisms explain how the addition of biochar affects plant physiological attributes. Adding biochar to soil improves its pH and cation exchange capacity, increases soil availability and retention of N, promotes the growth of beneficial microorganisms, and restricts the bioavailability of heavy metals, all of which are linked to the increased plant physiological parameters (chlorophyll, RWC and MSI) (Bornø et al. 2022; He et al. 2020; Ni et al. 2023; Wang et al. 2023). Previous research by Abideen et al. (2020) has established that a higher concentration of leaf physicochemical attributes indicates plant sensitivity to stress tolerance. This observation is consistent with the findings of Kammann et al. (2011), who observed an increase in chlorophyll content in *Chenopodium quinoa* cultivated in soil treated with biochar. The enhanced leaf chlorophyll content could boost the photosynthetic capability and ameliorate plant tolerance and growth against drought stress. This is further supported by the increased RWC and MSI values, which reveal that P-BC400 inoculated plants showed superior RWC and MSI values compared to both the control group (uninoculated) and plants inoculated with other carriers. Biochar has the ability to enhance the mesoporous structure of the soil, thus improving its capacity to retain water (Kim et al. 2021). This improved water retention capacity of the soil can contribute to increased RWC and MSI in plants under drought stress. Nadeem et al. (2017)

also reported similar findings, demonstrating a significant increase in RWC and MSI in cucumber leaves when biochar was applied under drought-stress conditions (D1 and D2).

Analysing $\delta^{13}C$ and $\delta^{15}N$ isotopes can reveal information on the metabolic and physicochemical processes involved in C and N transformations (Gouveia et al. 2019). These isotopes ($\delta^{13}C$ and $\delta^{15}N$) have been widely used as indicators of various environmental stresses, including drought, to assess the tolerance of plants in soil-plant systems (Gouveia et al. 2019; Lauteri et al. 1993; Robinson et al. 2000). Previous work revealed a higher $\delta^{13}C$ concentration in *Medicago truncatula* inoculated with *Sinorhizobium medicae* exposed to drought stress compared to normal watering conditions (Staudinger et al. 2016). Additionally, Robinson et al. (2000) and Gouveia et al. (2019) reported enrichment of $\delta^{13}C$ in the wild barley and taro plants, respectively, under drought conditions, considering it a favourable response to environmental stress (drought). Similarly, in our study, we observed a significant retention of $\delta^{13}C$ at a D2 across almost all treatments compared to D0, and D1. However, under severe drought stress (D2) soybean plants inoculated with P-BC400 exhibited a remarkably lower $\delta^{13}C$ enrichment compared to control and peat. To conserve water, plants under drought stress often close their stomata, which lessens their capacity to absorb carbon dioxide (CO_2). N availability in this scenario might operate as a limiting factor for photosynthesis because it is a necessary component of chlorophyll and other photosynthetic enzymes. Inoculating soybeans with N-fixing rhizobia allows them to acquire an additional supply of atmospheric N through a process known as biological nitrogen fixation (BNF) (Bazzer et al. 2020). There are two ways that biochar can enhance BNF. Firstly, biochar directly affects the soil by raising its pH and enhancing its molybdenum (Mo), sulphur (S), and bioavailable phosphorus (P) content. These soil alterations increase BNF and soybean nodulation. Secondly, by adsorbing flavonoids and Nod factors, it can also enhance chemical signalling between the symbiont and host that could enhance the process of BNF (Gul and Whalen 2016; Van Zwieten et al. 2015; Xiu et al. 2021).

Increased photosynthetic activity can result from this additional N supplementation, which can also help to mitigate some of the constraints brought on by drought stress. Therefore, compared to plants without inoculants, soybean plants may have less $\delta^{13}C$ enrichment (Wong et al. 2022).

The difference in $\delta^{15}N$ values in soybean leaves under different treatments provides insights into how carrier inoculants affect plant N contents under conditions of water scarcity (drought). $\delta^{15}N$ can serve as a potential benchmark for determining plant growth and N metabolism (Serret et al. 2018). Moreover, $\delta^{15}N$ has a negative correlation with BNF (Bazzer et al. 2020), as evident in our results where soybean plants inoculated with P-BC400 showed a lower $\delta^{15}N$ value (-6.03‰) compared to the control (-1.92‰) at D0 as evidenced by ARA (Table 3). Compared to plants that rely on mineral N as a source of N, BNF leads to the dilution of $\delta^{15}N$ in plants actively fixing N. A lower $\delta^{15}N$ value indicates a greater dilution of $\delta^{15}N$ due to BNF (Bazzer et al. 2020; Doughton et al. 1995). However, contrary to the $\delta^{13}C$ value, we observed a decrease in $\delta^{15}N$ in soybean plant leaves under drought stress (D1 and D2). The reduction in $\delta^{15}N$ in plants due to drought stress has been previously reported (Araus et al. 2013; Bort et al. 2014; Gouveia et al. 2019; Serret et al. 2018).

Conclusion

P-BC400 has demonstrated a significant improvement ($p < 0.05$) in the shelf life of CB1809 compared to other biochars and peat. The inoculum with P-BC400 has effectively enhanced soybean plant growth and nodulation, increased plant physiological attributes such as chlorophyll contents, RWC and MSI, and reduced $\delta^{13}C$ enrichment under drought stress conditions. These findings highlight the great potential of the developed inoculant to substantially enhance soybean production and serve as a sustainable alternative to peat-based inoculants. It is important to note that while this research was conducted in a greenhouse and yielded promising results with biochar-based inoculants at lower pyrolysis temperatures, further investigation at the field level is required to validate the efficiency of these novel inoculants under diverse agroecological conditions. Furthermore, to support agronomic advancements, more research is needed on the formulation of inoculants using different waste materials that can be developed into commercial products.

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Credit authorship contribution statement.

Rahat Shabir: Conceptualization, Methodology, Experimenting, Software, Original draft Writing– review & editing, Investigation, Formal analysis. **Yantao Li**: Conceptualization, Review & editing, Software, Investigation, Formal analysis. **Mallavarapu Megharaj**: Writing – review & editing, Resources. **Chengrong Chen**: Supervision, Conceptualization, Methodology, Writing – review & editing, Funding acquisition, Data curation, Validation, Correspondence.

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Declarations

Competing of interests 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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