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Microbial inoculation to improve plant performance in mine-waste substrates: A test using pigeon pea (*Cajanus cajan*)

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Abstract

Mining activities alter soil physicochemical and biological properties that are critical for plant establishment. Revitalisation of soil biological properties via microbial inoculations can potentially be adopted to improve vegetation restoration. Here, we evaluate the feasibility of using beneficial microorganisms in the form of commercially available inoculants to enhance plant performance in a non-toxic and infertile mine-waste substrate, using pigeon pea [*Cajanus cajan* (L) Millsp.] as a test plant. Six treatments were established to investigate the effects of inoculants (*Bradyrhizobium* spp., microbial mix and uninoculated controls) and water availability (low and moderate) in a factorial design over 6 months. Plant performance was determined by physiological parameters (leaf gas exchange, leaf carbon, nitrogen and stable isotopes) and growth (height and biomass). Plant xylem sap phytohormones were measured to determine the plants' physiological status and effects of inoculation treatments. Results revealed that water had a greater effect on plant growth than inoculation treatments. Inoculation treatments, however, improved some physiological parameters. This study suggests that physical conditions such as soil moisture and nutrient availability may occlude more subtle (direct or interactive) effects of beneficial soil microbes on plant growth and plant condition. Prior knowledge on the biological and physicochemical properties of the soil to be amended, and on plant species-specific responses, would be needed to customise microbial inoculants for maximum benefits to ecological restoration, to support future adoption of this practice.

KEYWORDS
gas exchange, microbial inoculation, mine site restoration, phytohormones, soil amendments, xylem sap

1 | INTRODUCTION

Globally, mine site restoration faces great challenges due to legacy effects of mining operations such as disturbed soil structure (Sheoran et al., 2010), and soil and groundwater pollution due to heavy metals and chemical leaching (Duruibe et al., 2007; Jung, 2001; Wong, 2003). One of the greatest challenges in mine site restoration is the re-establishment of self-sustaining vegetation (Thavamani et al., 2017) in substrates that have been biologically degraded (Harris et al., 1993). Mine site restoration in arid and semiarid zone systems such as Western Australia (WA) is further challenged by climatic factors including seasonal aridity and high temperatures (Groom & Lamont, 2015), soils of low organic matter (Murphy et al., 1998) and low phosphorus content (Soil Quality Pty Ltd., 2017). To date, mine site vegetation restoration success rates have been low (Lamb et al., 2015; Suding, 2011), so restoration practices need to be improved to increase the success rates.

Current common practice in large and long-term WA mine sites involves the stripping and stockpiling of topsoils, before spreading the topsoils onto engineered landforms for vegetation restoration. The depth of topsoils stripping ranges between 5 and 100 cm, depending on soil types, and varies between locations and company practices (Evolution Mining, 2015; Sustainable Soils Management Pty Ltd., 2013). Guidelines on topsoil handling have been established to ensure that the soil retains its full functionality for restoration use (LPSDP, 2016; Main Roads Western Australia, 2016; MHFD, 2020). However, the physicochemical and biological properties that determine 'soil quality' and functionality of these topsoils are often altered in the process (Delgado & Gomez, 2016; Golos & Dixon, 2014; Vincent et al., 2018). The stripping, stockpiling and spreading of the topsoils lead to drastic changes in soil structures (Wick et al., 2009). Rearrangement of mineral particles, organic matter and pore space among these particles may cause compaction, ground fissures and alter soil hydraulics and water retention properties, which are important for plant water access and uptake (Bünemann et al., 2018; Delgado & Gomez, 2016) and impact the soil biogeochemical cycles and distribution of soil organisms (Bi et al., 2019; Buscot, 2005; Wong & Bradshaw, 2003). Topsoil compaction may also impose penetration resistance to root growth and various physiological dysfunctions resulting in poor plant growth (Bünemann et al., 2018; Kozlowski, 1999).

Long-term stockpiling without vegetation cover also alters soil physicochemical and biological properties. Wind erosion and leaching may cause the loss of organic matter and mineral nutrients, reducing the fertility of the topsoils. More importantly, prolonged absence of plants in the topsoils ceases rhizodeposition, the input of plant organic carbon to the soil system via root turn-over and root exudation (Delgado & Gomez, 2016; Golos & Dixon, 2014; Gougoulias et al., 2014). This in turn affects soil microorganisms, which are dependent upon rhizodeposition as energy source (Raaijmakers et al., 2009). Lack of plant presence also reduces niche areas (i.e., rhizosphere) for soil microbial activities and colonization sites for beneficial microorganisms such as *Rhizobium* and mycorrhizal fungi, which are dependent on plant roots for physical support. The reduction in soil microorganisms in turn leads to decreased soil biological properties, which are important for supporting plant growth.

Revitalisation of soil biological properties by applying microbial inoculants directly or indirectly through organic amendments are potential methods to help increase mine site restoration success (Abbott et al., 2018; Hueso-González et al., 2017; Rivera et al., 2014; Vincent et al., 2018). Multiple studies in agricultural and forestry systems have revealed that soil microorganisms, including mycorrhizal fungi and bacteria, can enhance plant nutrient uptake and promote growth (Grover et al., 2011; Hayat et al., 2010; Pii et al., 2015; Trabelsi & Mhamdi, 2013; Yong et al., 2014). Soil microorganisms also help increase plant resistance against drought stress via various mechanisms (de Vries et al., 2020; Ngumbi & Kloepper, 2016; Tobar et al., 1994; Zhao et al., 2015). Use of microbial inoculants and organic amendments to achieve sustainable agriculture is also being advocated (Abbott et al., 2018; Backer et al., 2018; de Vries et al., 2020; Finkel et al., 2017; Wong et al., 2020) as the understanding of beneficial plant–microbe interactions is increasing with research and technological advances. Likewise, these beneficial interactions could be exploited to improve mine site vegetation restoration through increasing rhizospheric nutrient and bioactive metabolite availability, improved plant nutrient and water uptake and increased stress tolerance. For example, enhancement of mineral nutrient and water uptake via root architecture modification due to mycorrhizal symbiosis, or root growth stimulation from microbial metabolites like phytohormones, for example, auxins and cytokinins (Bi et al., 2019; Boivin et al., 2016; Cox et al., 2018), could benefit plants in nutrient-poor and arid environments such as WA mine sites. Past studies have also shown increased survival in plants inoculated with beneficial microorganisms (Ngumbi & Kloepper, 2016).

As facilitators of plant–microbe interactions, phytohormones are involved in many belowground interactions between roots, soil and the microbiome, mediating microbial symbiosis, root morphology, nutrient acquisition, plant growth, resilience and immunity to diseases (de Vries et al., 2020; Naseem et al., 2014; Ngumbi & Kloepper, 2016; Pérez-Montaño et al., 2014; Wong et al., 2020). Our understanding of the communication pathway for phytohormones along this soil–microbe–root–shoot continuum is improving. Current evidence indicated that the phytohormonal signals were transferred from the soil and microbes (rhizosphere) to the roots, entering the xylem channel and finally reaching the shoots to optimise physiological responses to match the prevailing growth conditions (de Vries et al., 2020; Dodd et al., 2010; Gupta et al., 2020; Kiba et al., 2019; Yong et al., 2000, 2014). Thus, assessing the xylem phytohormonal profiles of test plants might offer valuable insights to assess the status of any plant–microbe interactions.

Despite the great potential for the use of microorganisms to increase mine site restoration success, there is a knowledge gap in the growth benefits microorganisms can confer to plants under mine site conditions. The available literature on microorganisms in a mining context is mostly focused on the microbial community structure shifts, diversity, functionality (Banning et al., 2011; Degrood et al., 2005;

Harris et al., 1989; Kumaresan et al., 2017; Li et al., 2014) and phytoremediation of pollutants (Fashola et al., 2016; Thavamani et al., 2017; Wong, 2003). However, works investigating growth benefits that microorganisms can confer on plants under mine site conditions are limited. In one recent study by Moreira-Grez et al. (2019), the effects of a commercial inoculant and mineral fertilization on seedling emergence of *Acacia ancistrocarpa*, a native legume commonly used in restoration, was investigated. The study concluded that the commercial inoculant reduced seedling emergence and did not enhance plant fitness determined via shoot: root ratio measurements on plants subjected to 12 weeks of growth. In contrast, Aggangan & Anarna (2019) found that microbial inoculated seedlings of *Acacia mangium*, *Eucalyptus urophylla* and *Pterocarpus indicus* performed better in terms of survival, biomass and microbial population after 27 months of growth in substrates subjected to additional amendments (lime, vermicompost and inorganic fertilisers). Contrasting findings between both studies highlighted that much work is still required to determine the effects of commercial inoculants on the growth and physiological condition of plant species over longer growth periods.

Thus, the aim of the present work was to further evaluate the feasibility of using beneficial microorganisms in the form of commercially available inoculants to enhance plant performance in a non-toxic but infertile mine-waste substrate, using pigeon pea [*Cajanus cajan* (L) Millsp.] as a test plant. Six treatments were established to investigate the effects of inoculants (*Bradyrhizobium* spp., microbial mix and uninoculated controls) and water availability (low and moderate) in a factorial design over 6 months. Plant performance was determined by physiological parameters and xylem sap phytohormone concentrations were measured to determine the plants' physiological status and effects of inoculation treatments. We hypothesised that pigeon pea subjected to microbial inoculation would exhibit better growth performance and drought tolerance with corresponding changes in xylem sap phytohormones.

2 | METHODS

2.1 | Plant species selection

Pigeon pea [*Cajanus cajan* (L.) Millsp.], a fast-growing legume able to withstand arid conditions, was selected as the test plant in this experiment. Pigeon pea has been widely used in plant-growth-promoting rhizobacteria interaction studies (Gopalakrishnan et al., 2016; Sonawane et al., 2019) and drought stress tolerance experiments (Qiao et al., 2011). Information on phytohormone profile changes in pigeon pea with microbial inoculation is also available (Upadhyaya et al., 1991; Yong et al., 2014) to help determine the efficacy of the inoculation treatments in regulating plant physiology.

2.2 | Plant growth conditions

Seeds of pigeon pea, sourced from seed company Perth Hills Veggie Co., Perth, WA, were sown in plastic tapered square pots (60 mm × 60 mm × 200 mm; Garden City Plastics, Forrestfield, WA) containing 640 ml sieved (12.5 mm) and homogenised substrate (25% topsoil and 75% overburden) collected from a Pilbara mine site. The Pilbara region, situated in the north of Western Australia, is a biodiverse semiarid ecosystem but also one of the most heavily mined regions in the State (Department of Primary Industries and Regional Development, 2017; Muñoz-Rojas et al., 2016). In the local restoration operations, overburden consisting of rocks and soil that originates from the layer surrounding the ore body being mined (Oggeri et al., 2019) is commonly used in landform reconstruction and as vegetation growth media in mixture with topsoil (Muñoz-Rojas et al., 2016). Topsoil and overburden originated from an iron ore mine near Newman, WA. Both substrates were stockpiled on-site for 5+ years before being stored dry in steel drums for an additional 5+ years. Hence, the substrates were considered infertile. The homogenised substrate had a water holding capacity of approximately 22%. The chemical properties of the substrate were determined by CSBP Soil and Plant Analysis Laboratory (Bibra Lake, WA) and are presented in Table 1. The plants were grown in a

TABLE 1 Chemical properties of the substrate used in this experiment

Chemical properties	
Ammonium Nitrogen (mg kg^{-1})	6.67 ± 0.67
Nitrate Nitrogen (mg kg^{-1})	7.00 ± <0.01
Colwell Phosphorus (mg kg^{-1})	<2
Colwell Potassium (mg kg^{-1})	82.7 ± 1.67
Sulphate Sulphur (mg kg^{-1})	130 ± 3.35
Organic Carbon (mg 100 g^{-1})	270 ± 20.0
Conductivity (dS m^{-1})	0.19 ± <0.01
pH ($CaCl_2$)	7.53 ± 0.03
pH (H_2O)	8.27 ± 0.07
DTPA Copper (mg kg^{-1})	0.21 ± 0.01
DTPA Iron (mg kg^{-1})	3.30 ± 0.24
DTPA Manganese (mg kg^{-1})	1.92 ± 0.04
DTPA Zinc (mg kg^{-1})	0.63 ± 0.05
Exc. Aluminium (meq 100 g^{-1})	0.06 ± 0.01
Exc. Calcium (meq 100 g^{-1})	5.41 ± 0.03
Exc. Magnesium (meq 100 g^{-1})	1.01 ± <0.01
Exc. Potassium (meq 100 g^{-1})	0.16 ± 0
Exc. Sodium (meq 100 g^{-1})	0.22 ± 0
Aluminium $CaCl_2$ (mg kg^{-1})	<0.01
Boron hot $CaCl_2$ (mg kg^{-1})	<0.01
Total Nitrogen (mg 100 g^{-1})	<10
Total Phosphorus (mg kg^{-1})	253 ± 8.27
Total Carbon (mg 100 g^{-1})	710 ± 20.0
Exc. Acidity (meq 100 g^{-1})	<0.01
KCl exc. Aluminium (meq 100 g^{-1})	<0.01
KCl exc. Hydrogen (meq 100 g^{-1})	<0.01

Abbreviations: DTPA, diethylene-triamine-penta-acetic acid; Exc., exchangeable

glasshouse at the University of Western Australia (UWA) under daytime average photosynthetically active radiation (PAR) of 600 μmol m^{-2} s^{-1} between September 2017 and March 2018, in simulated Pilbara climatic conditions of 34 ± 1°C day, 25 ± 1°C night and average relative humidity of 55%.

2.3 | Experimental procedure

Six treatments were established to investigate the effects of microbial inoculants (*Bradyrhizobium* spp., microbial mix and uninoculated controls) and water availability (low and moderate) on the growth and physiology of pigeon pea. During sowing, rhizobial inoculated treatments were treated with a commercially available *Bradyrhizobium* spp. (obtained from Perth Hills Veggie Co., Perth, WA) at 0.25 g inoculant per 100 g of seeds (Drew et al., 2012). *Bradyrhizobium* spp. is widely used for pigeon pea culture in Australia (Drew et al., 2012). Microbial mix inoculated treatments were treated with a freeze-dried commercial microbial mix (Langley Fertilizers Troforte® Microbe Blend – Cropping, Sunpalm Australia Pty Ltd, Wangara, WA), comprised of beneficial bacteria and fungi (Appendix 1 of Supporting information), reconstituted in deionised water, in addition to the commercial *Bradyrhizobium* spp. inoculant. Five hundred microliter inoculant (equivalent to 0.1 g microbial mix) was applied around the seeds, covered loosely with fine substrate, and kept moist until seedlings emerged. These inoculation treatments are hereby referred to as 'Rhizobia' and 'Microbes' inoculated treatments, respectively. Controls of uninoculated plants were also included. Each treatment group had five replicates. All treatments received 0.47 g commercial controlled-release fertiliser (10: 1.5: 4.5 NPK plus trace elements, release pattern 3 months, Sunpalm Australia Pty. Ltd., Wangara, WA) 10 days after the seeds were sown. Fertilization was delayed to avoid down-regulation of plant–microbial symbiosis observed in fertilised plants (Porter & Sachs, 2020; Upadhyaya et al., 1991; Yong et al., 2014). Uniform seedlings were subsequently selected to achieve final density of one plant per pot. Initially, the seedlings were given 36 ml of water daily for 2 weeks before being subjected to low and moderate watering regimes adapted from Muñoz-Rojas et al. (2016). In brief, low water treatments received 2 × 54 ml and moderate water treatments received 3 × 54 ml water per week via manual administration of deionised water using a 50 ml syringe. The moisture content of the substrates ranged between 10.1%–15.7% and 10.6%–16.3% (HydroSense II, Campbell Scientific Australia Pty. Ltd.) for the low and moderate treatment groups, respectively, at harvest.

Plant performance was determined by physiological parameters (leaf gas exchange, leaf carbon and nitrogen content) and growth (height and biomass). Plant physiological performances were further evaluated by measuring foliar stable carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$) and oxygen ($\delta^{18}O$) isotopes, which function as surrogate variables that integrate various physiological processes (Robinson et al., 2000). Briefly, plant $\delta^{15}N$ signatures correlate with levels of N fixation through symbiosis with N-fixing microorganisms (Yoneyama, 2017). Plant $\delta^{13}C$ signatures provide a surrogate measurement of the plants' water-use-efficiency (WUE), and combination with $\delta^{18}O$ allows an assessment of variation in stomatal regulation and photosynthetic capacity (Cernusak et al., 2013; Dawson et al., 2002; Flanagan & Farquhar, 2014). Xylem sap phytohormone concentrations were measured to determine the plants' physiological status and effects of inoculation treatments (Yong et al., 2014).

2.4 | Leaf physiology measurements

Leaf gas exchange was measured 2 weeks prior to the plants' harvest, using a portable open system (LI-6400XT, Licor, Lincoln, NE) equipped with the standard leaf chamber LED light source and CO_2 injector system. All measurements were made between the hours of 8 am and 12 pm, at PAR of 1200 μmol m^{-2} s^{-1}, sample CO_2 at 382–399 μmol CO_2 mol^{-1} air, and air temperature 27.8–29.6°C, on surviving plants (n = 4–5) one day after watering. Intrinsic water-use efficiency (WUE_i) was determined as photosynthetic rate divided by stomatal conductance (Hatfield & Dold, 2019).

2.5 | Biomass and foliar carbon, nitrogen and stable isotope measurements

Leaves fallen off the plants were collected throughout the experiment and presented as ratio to the harvested shoot mass (referred to as 'shed leaves'). Roots were removed from the soil, brushed and gently washed to remove attached soil particles. The ratio of root mass to total biomass (root mass fraction) was explored to determine differences in biomass partitioning. Shoot and root dry mass were determined after drying the plant material to a constant weight at 70°C for approximately 72 h.

Single, newly mature whole-leaf samples were oven-dried and ground for $\delta^{15}N$ and $\delta^{13}C$ analysis using a continuous flow system consisting of a Delta V Plus mass spectrometer connected with a Thermo Flash 1112 via Conflo IV (Thermo-Finnigan, Bremen, Germany). The samples were also analysed for $\delta^{18}O$ using a high-temperature conversion elemental analyser (TC/EA) coupled with Delta XL mass spectrometer in continuous flow mode (Thermo-Fisher Scientific, Bremen, Germany). All isotopic analyses were carried out by the West Australian Biogeochemistry Centre (WABC, UWA, Perth).

2.6 | Xylem sap collection and analysis

Phytohormone analyses were conducted on xylem sap collected pre-dawn prior to harvesting plants. Plants were watered 1 day prior to harvest. During pre-dawn xylem sap collection, plants were cut at about 2 cm above soil level and placed into a pressure chamber (PMS-600, PMS Instrument Company, Albany, OR). The cut surfaces were blotted with methanol:formic acid:water (14:1:2, vol vol^{-1}) to inhibit enzymatic reactions from breaking down

phytohormones and to remove contaminating cell debris (Yong et al., 2000). Plant cuttings were placed in a pressure chamber and subjected to increasing pressure until bleeding occurred and then maintained at that constant pressure for sap collection for approximately 5 min to prevent collection of exudates apart from xylem sap. The first drops of xylem sap were discarded to avoid contamination. Xylem sap was collected using a micropipette and transferred into microcentrifuge tubes containing 25 μl concentrated formic acid and placed on ice. On average, xylem sap collected from individual plants ranged between 50 and 200 μl. Collected sap samples were stored in darkness at −80°C until analysis.

Due to the low volumes of xylem sap collected, samples were pooled within treatments and split into two sets for analysis using ultra-performance liquid chromatography-electrospray ionization-tandem mass (UPLC-ESI-MS/MS) in ESI positive (auxins and cytokinins) and ESI negative (abscisic acid and salicylic acid) mode. The samples were spiked with deuterated standards (Table 2) (OlChemIm Ltd., Olomouc, Czech Republic) close to endogenous concentrations (Gosetti et al., 2010) and dried down in a rotary evaporator (Eppendorf Vacufuge plus) at room temperature. The concentrated samples were reconstituted with starting mobile phase 5% acetonitrile (ACN) and 10% ACN for ESI positive and ESI negative modes, respectively, both with 0.01% formic acid (FA) for analysis. Samples were analysed at ×10 concentration and endogenous concentration in ESI positive and ESI negative modes, respectively. Reconstituted samples were analysed in duplicates using an Acquity UPLC® I-Class System equipped with a Binary Solvent Manager, a Sample Manager with 10 μl loop needle, and an Acquity UPLC® CSH™ C18 column (2.1 × 100 mm, particle size of 1.7 μm) coupled to a triple quadrupole mass spectrometer Xevo® TQ-S micro (Waters, Singapore). The UPLC mobile phase consisted of ACN with 0.01% (vol vol^{-1}) FA (A) and water with 0.01% (vol vol^{-1}) FA (B), flowing at 0.5 ml min^{-1}. Specific gradients were used for each mode of analysis (Appendix 2 of Supporting information). Column temperature was held at 50°C for both ESI modes. System control, data acquisition and data analysis were performed with the MassLynx™- version 4.1 software (Waters, Milford, MA). Phytohormone concentrations were quantified according to Equation (1). The results reported are the mean value of duplicate samples that met the criteria of signal-to-noise (S/N) ratio >10 and relative standard deviation percentage (RSD%) <20. The results with S/N ratio <10 were deemed below limits of quantification (<LOQ). Phytohormones analysed with their respective LOQ and analytical parameters are presented in Table 2.

$$\text{Phytohormone concentration} = \text{Peak area of endogenous phytohormone} \times \frac{\text{Concentration of deuterated standard}}{\text{Peak area of deuterated standard}} \quad (1)$$

2.7 | Statistical analysis

Two-way analysis of variance (ANOVA) with Tukey's HSD post hoc tests were performed to determine if growth and physiological variables differed significantly among the treatments, including water and inoculation treatments and the interactions between both. Effects of water and inoculation treatments on plant photosynthetic rates were determined by analysis of covariance (ANCOVA) with stomatal conductance as a covariate. All parameters investigated were tested for normality and variance homogeneity using Shapiro–Wilk and Levene's tests, respectively, and the data were square root or log-transformed when required. All the ANOVA, ANCOVA and post hoc tests were performed using JMP® 14.1.0 (SAS Institute Inc.). Correlations between measured variables presented in the form of a correlogram were generated by R (R Core Team, 2020) package corrplot (Wei & Simko, 2017).

3 | RESULTS

3.1 | Growth

Plants subjected to low water treatments were shorter and had lower total biomass than plants given moderate water in both controls and inoculated plants (Table 3). Despite improved biomass growth, plants subjected to moderate water treatment shed more leaves, with the highest rate observed in the control treatment group (Table 3). Phenotypically, the control plants in both water treatments had leaves that were smaller and less green compared with the inoculated plants (Figure 1a).

Overall, watering treatments contributed significantly ($p < 0.0001$) to the differences observed in height, total biomass and biomass allocations (Table 3). Differences existed among water treatments in shoot mass and root mass, as indicated by the post hoc Tukey test, with the general patterns being similar to that observed in the total biomass. There were no significant differences in root mass fraction (Table 3).

Inoculation had no direct nor interactive effects on the plant growth parameters measured but increased the number of nodules (Table 3). Nodule counts in all the plants were generally low, with an overall mean value of 3.68 and standard error of 0.65. Inoculated plants had higher number of nodules (4.94 ± 0.78) than control plants (1.4 ± 0.79) (Figure 3b).

3.2 | Gas exchange

Gas exchange measurements revealed large variations in photosynthetic rates among the treatments, which strongly correlated with stomatal conductance (Figure 2a and Table 4). In general, inoculated plants of the low water treatment tended to have higher photosynthetic rates than non-inoculated plants in that treatment, and than most plants in the moderate water treatment (Figure 2a). ANCOVA analysis with stomatal conductance as covariate revealed that inoculation treatments had a significant effect on photosynthetic rates (Table 4). This appears to correspond with generally lower WUE_i at a given stomatal conductance for control compared with inoculated treatments (Figure 2b: most control plants are below the fitted line).

TABLE 2 Analytical parameters for the phytohormones and respective deuterated standards examined

Phytohormone class	Analyte	Transition	Cone voltage (V)	Collision voltage (V)	Retention time (min)	LOQ (ng ml^{-1})	Deuterated standard	Transition	Cone voltage (V)	Collision voltage (V)	Retention time (min)	Spiked concentration (ng ml^{-1})
Auxin	Indole-3-Acetic Acid (IAA)	176 > 130	15	20	5.95	0.25	[$^2H_5$]-IAA	181 > 134	20	25	5.94	0.10
	Indole-3-Butyric Acid (IBA)	204 > 186	22	15	6.74	0.50	[$^2H_5$]-IAA	181 > 134	20	25	5.94	0.10
Cytokinin	N^6-Benzyladenine (BAP)	226 > 91	23	22	5.50	0.05	[$^2H_7$]-BAP	233 > 98	25	24	5.41	0.10
	N^6-Benzyladenosine (BAPR)	358 > 226	10	20	6.07	0.10	[$^2H_7$]-BAP	233 > 98	25	24	5.41	0.10
	cis-Zeatin (*cZ*)	220 > 136	17	25	1.81	0.10	[$^2H_5$]-*tZ*	225 > 46	18	21	1.43	0.10
	Dihydrozeatin (DHZ)	222 > 136	20	23	1.56	0.50	[$^2H_3$]-DHZ	225 > 136	20	26	1.55	0.10
	Dihydrozeatin-O-Glucoside (DHZOG)	384 > 222	24	19	1.58	0.50	[$^2H_3$]-DHZ	225 > 136	20	26	1.55	0.10
	Dihydrozeatin Riboside (DHZR)	354 > 136	14	40	3.80	0.05	[$^2H_3$]-DHZ	225 > 136	20	26	1.55	0.10
	N^6-Isopentenyladenine (iP)	204 > 136	17	17	5.05	0.25	[$^2H_6$]-iP	210 > 137	20	23	5.01	0.10
	N^6-Isopentenyladenosine (iPR)	336 > 136	22	30	5.99	0.05	[$^2H_6$]-iP	210 > 137	20	23	5.01	0.10
	Kinetin (K)	216 > 81	20	28	3.39	0.25	[$^2H_5$]-*tZ*	225 > 46	18	21	1.43	0.10
	trans-Zeatin (*tZ*)	220 > 136	21	19	1.47	0.05	[$^2H_5$]-*tZ*	225 > 46	18	21	1.43	0.10
	trans-Zeatin-O-Glucoside (*tZOG*)	382 > 220	20	23	1.48	0.50	[$^2H_5$]-*tZOG*	387 > 225	17	17	1.41	0.10
	trans-Zeatin Riboside (*tZR*)	352 > 220	14	21	3.60	0.50	[$^2H_5$]-*tZ*	225 > 46	18	21	1.43	0.10
Abscisic acid	Abscisic acid (ABA)	263 > 153	20	15	4.94	5.00	[$^2H_6$]-ABA	269 > 159	25	16	4.91	10.00
Salicylic acid	Salicylic acid (SA)	137 > 93	25	17	3.92	5.00	[$^2H_4$]-SA	141 > 97	25	27	3.88	10.00

TABLE 3 Height, biomass and biomass distribution of plants subjected to different inoculation (control, Rhizobia, microbes) and water (L: Low; M: Moderate) treatments and effects of water, inoculation and their interactions on the respective measured parameters

Treatments	Height (mm)	Total biomass (g)	Shoot dry mass (g)	Root dry mass (g)	Shed leaves (%)	Root mass fraction	Nodules
Control – L	227 ± 30^{b}	2.04 ± 0.08^{c}	0.92 ± 0.10^{c}	1.13 ± 0.04^{b}	29.8 ± 2.7^{b}	0.56 ± 0.03^{a}	0.2 ± 0.2^{b}
Control – M	391 ± 11^{a}	3.69 ± 0.47^{ab}	1.43 ± 0.16^{abc}	2.26 ± 0.32^{a}	41.9 ± 2.2^{a}	0.61 ± 0.01^{a}	2.6 ± 1.44^{ab}
Rhizobia – L	267 ± 13^{b}	2.25 ± 0.14^{bc}	1.07 ± 0.10^{bc}	1.18 ± 0.06^{b}	30.2 ± 1.2^{b}	0.53 ± 0.02^{a}	7.5 ± 2.72^{a}
Rhizobia – M	393 ± 36^{a}	4.17 ± 0.37^{a}	1.43 ± 0.16^{a}	2.28 ± 0.14^{a}	38.0 ± 2.1^{a}	0.56 ± 0.03^{a}	3.8 ± 1.07^{ab}
Microbes – L	247 ± 19^{b}	2.42 ± 0.22^{bc}	1.04 ± 0.17^{c}	1.39 ± 0.09^{b}	25.6 ± 2.0^{a}	0.58 ± 0.04^{a}	3.6 ± 1.12^{ab}
Microbes – M	404 ± 28^{a}	4.48 ± 0.59^{a}	1.87 ± 0.17^{ab}	2.60 ± 0.46^{a}	38.9 ± 3.3^{b}	0.57 ± 0.03^{a}	5.5 ± 0.65^{ab}
Water	51.86***	48.06***	25.07***	61.11***	33.16***	1.00^{NS}	0.68^{NS}
Inoculation	0.40^{NS}	1.57^{NS}	1.85^{NS}	1.62^{NS}	1.20^{NS}	1.08^{NS}	4.82*
Water*Inoculation	0.31^{NS}	0.10^{NS}	0.54^{NS}	0.06^{NS}	0.74^{NS}	0.53^{NS}	0.84^{NS}

Note: Values shown for measured parameters are mean ± standard error ($n = 4–5$). Different letters indicate significant differences between treatments at $p < 0.05$ (ANOVA with post hoc Tukey HSD test). Values shown for the effect test are *F* ratio; statistical significance: NS: not significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

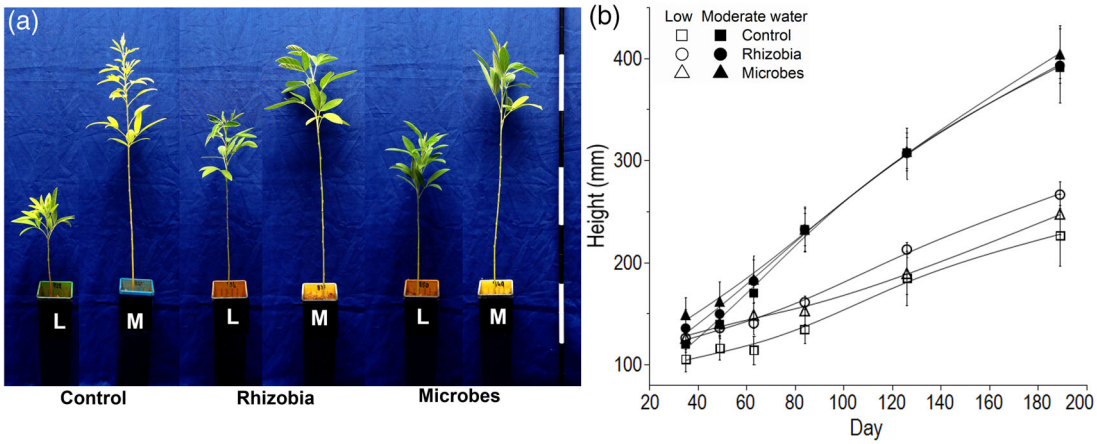

FIGURE 1 Representative plants (a) and growth in height (b) of pigeon pea plants subjected to different inoculation treatments and two watering regimes (i.e., low and moderate indicated by L and M and open and closed symbols respectively). Scale bar denotes 10 cm intervals. Square symbols represent control plants, circles for Rhizobia treatment and triangles for microbes inoculated plants [Colour figure can be viewed at wileyonlinelibrary.com]

3.3 | Foliar carbon, nitrogen and isotopes

Plants subjected to moderate water treatment had higher foliar nitrogen content than plants in the low water treatment (Table 5). Within each water treatment, inoculated plants had 1.2–2.3-fold higher foliar nitrogen content compared with respective controls (Figure 3a). Foliar nitrogen content was significantly influenced by both water and inoculation but not their interaction (Table 5). Foliar carbon content was not significantly different in the two water treatments but was increased from 44 to 47 g g^{-1} by inoculation (Table 5). There were no statistical differences in foliar $\delta^{18}O$ and $\delta^{13}C$ content among the treatments. There was however a slight positive correlation ($R^2 = 0.203$) between foliar $\delta^{18}O$ and $\delta^{13}C$ (Figure 3c). Foliar $\delta^{15}N$ was affected by water and inoculation treatment, and interactions between both factors (Table 5). The low water control group, which had less nodules, had significantly higher foliar $\delta^{15}N$ than inoculated treatments and the moderate water treatment groups regardless of inoculation treatment (Figure 3b).

3.4 | Phytohormones

Plant growth associated phytohormones including cytokinins, in the ribosylated form, namely N^6-isopentenyladenosine (iPR), dihydrozeatin riboside (DHZR) and *trans*-zeatin riboside (*t*ZR) were detected in the plant xylem sap pooled within treatment groups. Most treatments had a similar iPR concentration, around 0.04 nmol L^{-1}, except for a modest increase in low water treated Rhizobia and Microbes inoculated plants (Table 6). A marked increase of *t*ZR was detected in Rhizobia inoculated plants subjected to low water availability. There was also a general trend that plants subjected to low water availability had higher *t*ZR concentration

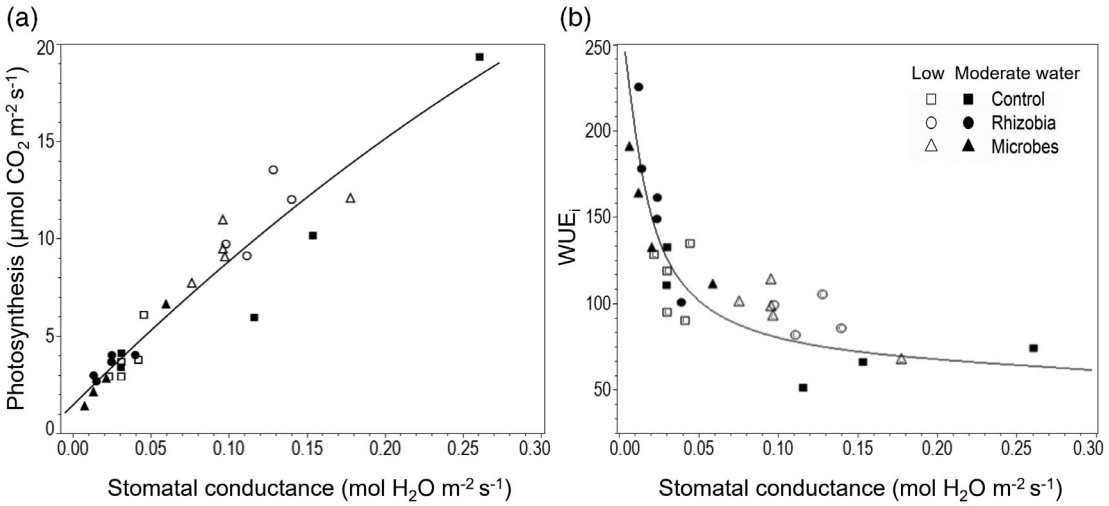

FIGURE 2 Photosynthetic rates (a) and intrinsic water-use-efficiency (WUE_i) (b) plotted against stomatal conductance of pigeon pea plants subjected to different inoculation treatments and two watering regimes (low and moderate indicated by open and closed symbols, respectively). Square symbols represent control plants, circles for rhizobia treatment and triangles for microbes inoculated plants

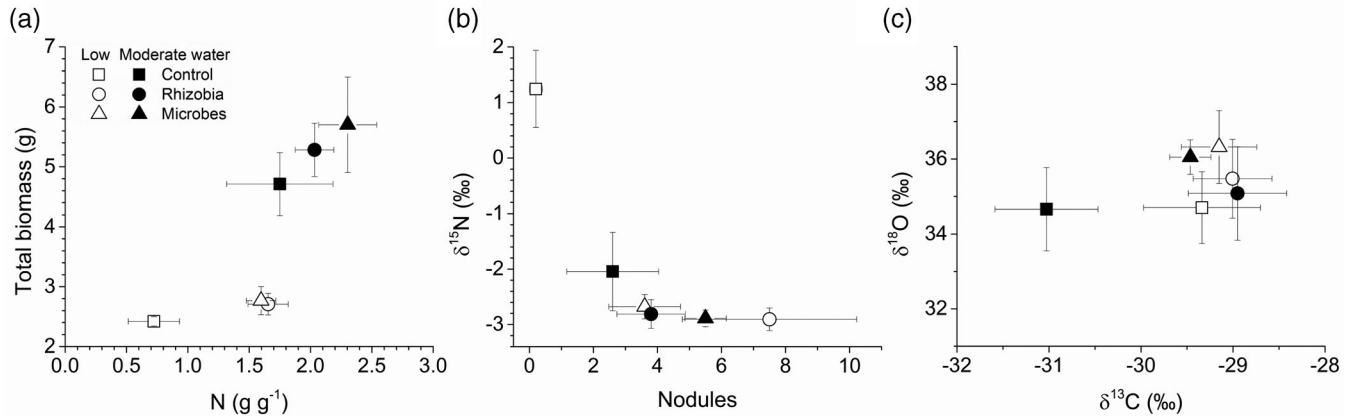

FIGURE 3 Relationships between (a) total plant biomass and foliar N content, (b) foliar $\delta^{15}N$ and nodule count, and (c) foliar $\delta^{18}O$ and foliar $\delta^{13}C$ of pigeon pea plants subjected to different inoculation treatments and two watering regimes (low and moderate indicated by open and closed symbols respectively). Square symbols represent control plants, circles for rhizobia treatment and triangles for microbes inoculated plants

TABLE 4 Analysis of covariance (ANCOVA) for photosynthetic rates by water and inoculation treatments with stomatal conductance as covariate

Model	DF	Sum of squares	*F*	*p*
Water	1	0.104	2.44	0.132
Inoculation	2	0.312	3.65	0.042
Stomatal conductance	1	13.717	320.89	< 0.0001

compared with moderate water treated plants. A similar trend was observed for DHZR, with exceptions in the Microbes inoculated group.

Plant stress-related phytohormones, including abscisic acid (ABA) and salicylic acid (SA), were also detected. Inoculated plants had higher ABA concentrations under a moderate water regime than under a low water regime, but the opposite was found for control plants. Water regimes did not have consistent effects on SA, but inoculated plants generally had lower concentrations than controls.

3.5 | Relationships among plant traits and phytohormones

Relationships between all measured parameters evaluated within this experiment were explored through correlation and presented in the form of a correlogram (Figure 4). Significant positive correlations between growth parameters such as height with root mass and height with total or shoot biomass were observed, as expected. Both $\delta^{13}C$

TABLE 5 Foliar nutrient and stable isotopes content and respective effects of water, inoculation and their interactions

Treatments	C (g g^{-1})	N (g g^{-1})	C/N	$\delta^{18}O$ (‰)	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Control – L	44.46 ± 0.65^{b}	0.72 ± 0.21^{b}	79.88 ± 16.78^{a}	34.70 ± 0.96^{a}	−29.34 ± 0.64^{a}	1.24 ± 0.69^{a}
Control – M	44.84 ± 0.84^{ab}	1.75 ± 0.43^{b}	45.21 ± 22.38^{ab}	34.66 ± 1.11^{a}	−31.03 ± 0.56^{a}	−2.04 ± 0.71^{b}
Rhizobia – L	46.23 ± 0.31^{ab}	1.65 ± 0.16^{ab}	42.94 ± 15.72^{ab}	35.48 ± 1.05^{a}	−29.01 ± 0.43^{a}	−2.91 ± 0.20^{b}
Rhizobia – M	47.55 ± 0.22^{a}	2.03 ± 0.16^{a}	23.99 ± 1.93^{b}	35.08 ± 1.25^{a}	−28.95 ± 0.53^{a}	−2.81 ± 0.26^{b}
Microbes – L	47.40 ± 0.15^{ab}	1.60 ± 0.12^{a}	30.33 ± 2.18^{ab}	36.32 ± 0.98^{a}	−29.15 ± 0.41^{a}	−2.68 ± 0.22^{b}
Microbes – M	47.51 ± 0.32^{a}	2.30 ± 0.24^{a}	21.36 ± 1.93^{b}	36.05 ± 0.46^{a}	−29.46 ± 0.22^{a}	−2.89 ± 0.13^{b}
Water	1.73^{NS}	11.47*	2.51^{NS}	0.077^{NS}	2.44^{NS}	8.62*
Inoculation	17.78***	4.75*	5.82*	1.08^{NS}	3.09^{NS}	18.18***
Water*Inoculation	0.56^{NS}	0.83^{NS}	0.85^{NS}	0.012^{NS}	1.69^{NS}	8.12*

Note: Values presented for foliar nutrient and stable isotopes are mean ± standard error ($n = 4–5$) . Different letters indicate significant differences between treatments at $p < 0.05$ (from ANOVA with post hoc Tukey HSD test). Values shown for the effect test are *F* ratio; statistical significance: NS: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

TABLE 6 Phytohormone concentrations (nmol L^{-1}) detected in xylem sap samples

Treatments	iPR	DHZR	*t*ZR	Total cytokinin	ABA	SA
Control – L	0.0474	4.24	3.16	7.45	94.2	2433
Control – M	0.0447	2.43	<LOQ	2.47	61.7	4424
Rhizobia – L	0.0641	15.06	5.29	20.41	72.6	1723
Rhizobia – M	0.0471	4.67	0.74	5.46	130.9	854
Microbes – L	0.0531	3.96	1.45	5.46	65.8	1115
Microbes – M	0.0447	6.96	0.94	7.94	87.8	1426

Note: Xylem sap of replicate plants was pooled into one sample per treatment, which was analysed twice.
<LOQ indicates concentration below limits of quantification
Abbreviations: ABA, abscisic acid; DHZR, dihydrozeatin riboside; iPR, N^{6}-isopentenyladenosine; SA, salicylic acid; *t*ZR, *trans*-zeatin riboside

and ABA were also observed to have a significant positive correlation with plant WUE_i.

Through the correlation analysis, some interesting relationships were found between phytohormone concentrations and physiological measurements. For example, cytokinins (iPR and DHZR) were positively correlated with each other and with gas exchange parameters (photosynthetic rates and stomatal conductance) and number of nodules. Representative relationships with stronger correlations, such as DHZR with number of nodules, and iPR with photosynthetic rates are presented in Figure 5a,b, respectively. ABA was found to be negatively correlated with stomatal conductance (Figure 5c), SA (Figure 5d) and photosynthetic rates (Figure 4).

4 | DISCUSSION

Effects of two different inoculants, namely *Bradyrhizobium* spp. (Rhizobia) and a mixture of soil microorganisms (Microbes), on plant physiological performance under two different watering regimes, low and moderate, were studied using a test plant, pigeon pea. Overall, inoculation treatments impacted plant physiological parameters (e.g. endogenous phytohormones) but not biomass or height growth, which was mainly influenced by water availability. These observations indicated that the use of microbial inoculants could potentially be beneficial for improving certain plant health functions in mine site restoration environments. For example, enhanced photosynthetic WUE may not increase growth rates but could contribute to increased stress resilience against the harsh environment of restored mine sites.

In terms of plant growth, water availability had a greater influence on the differences observed in the plants' height and biomass (Table 3) compared with inoculation treatments. It was expected that inoculation treatments might influence biomass partitioning, but root mass fraction (Table 3) revealed otherwise. Inoculation did, however, have a significant effect on nodule count, with consistently higher nodule counts for both the Rhizobia and Microbes treatments than uninoculated control treatments (Table 3; Figure 3b). This indicated that the symbiotic plant–rhizobia relationships were successfully established, but may not necessarily lead to growth benefits under the prevailing conditions. Evidence of plant–microbial symbiosis via nitrogen fixation in the inoculated plants was confirmed by the more negative foliar $\delta^{15}N$ values (Yoneyama, 2017), which were close to

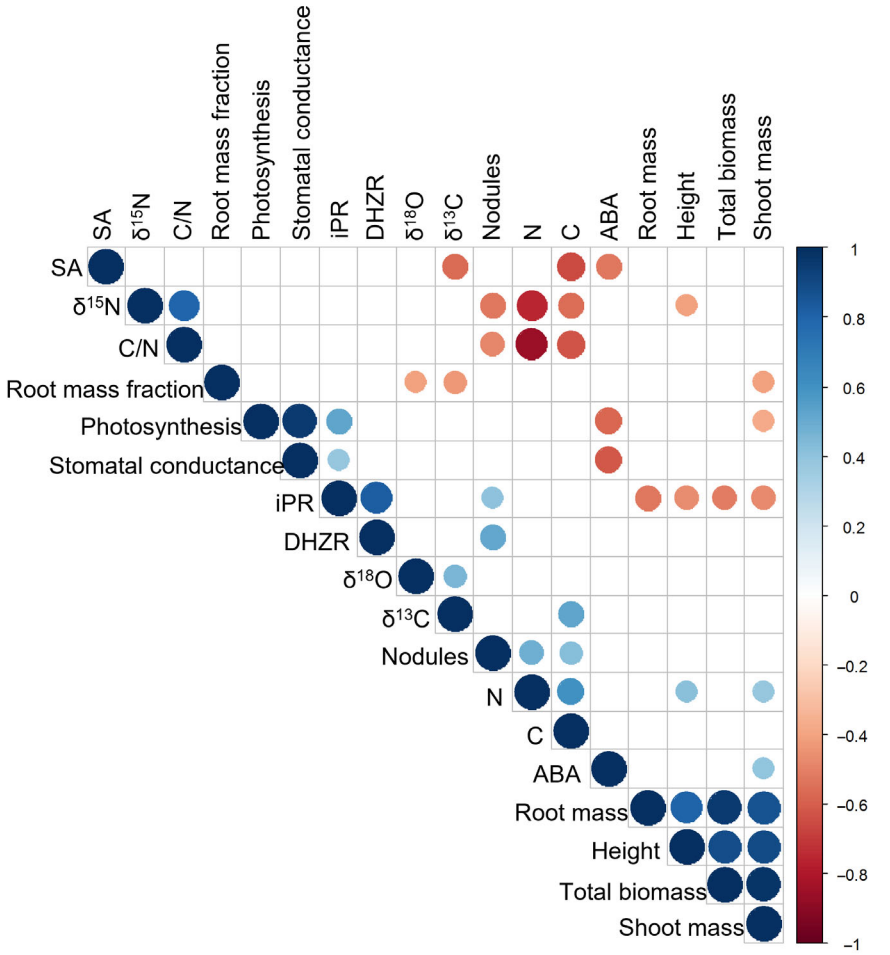

FIGURE 4 Correlogram for the measured plant growth parameters (total, shoot and root biomass, root mass fraction, height and nodules), foliar chemistry (C, N and C/N), foliar isotope composition ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{18}O$), gas-exchange measurements (photosynthesis, stomatal conductance and intrinsic water-use efficiency (WUE_i)), and xylem sap phytohormones (ABA, SA, iPR and DHZR). Circle size is proportional to the correlation coefficient. Positive correlation is indicated by blue, while negative correlation is indicated by red. Blank squares indicate that the correlation was not significant ($\alpha = 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com]

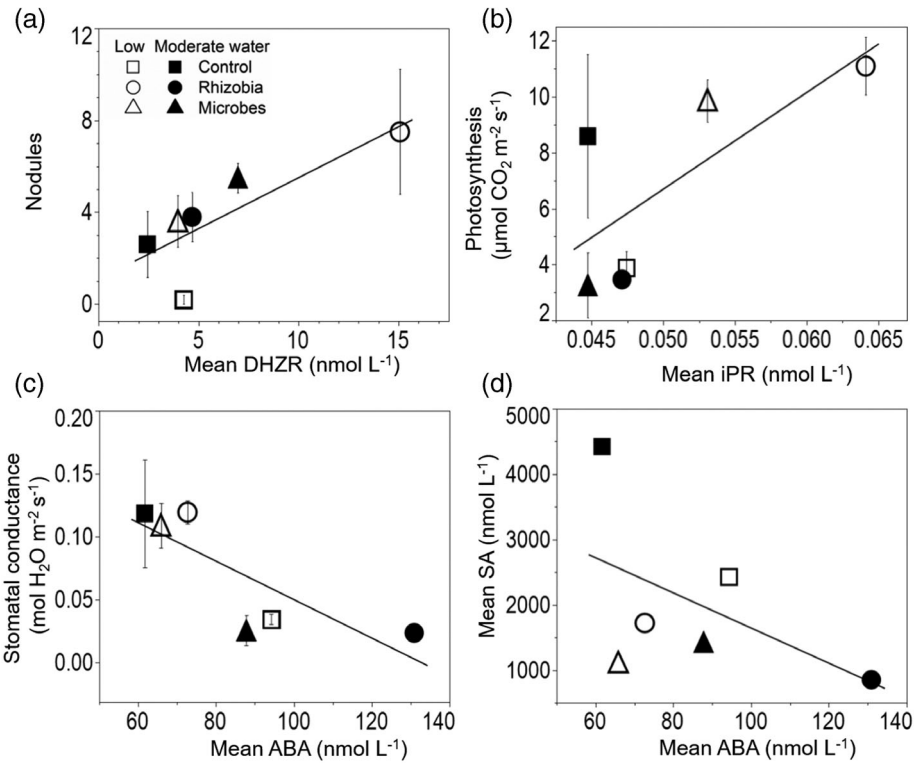

FIGURE 5 Relationship between (a) nodules and xylem sap DHZR, (b) plant photosynthetic rate and xylem sap iPR, (c) plant stomatal conductance and xylem sap ABA, and (d) xylem sap SA and ABA

the reported value of −1.6 ± 0.44‰ by Kumar Rao et al. (1996). Results of foliar nitrogen content suggested that the nitrogen fixation resulting from a rather modest increase in nodule counts in inoculated treatments could have supplied the plants with additional nitrogen, resulting in higher foliar nitrogen content (Table 5; Figure 3a). Foliar nitrogen content detected across treatments was, however, lower than the average value of 5% in similar pigeon pea leaves grown in Alfisol soil (Sanetra et al., 1998) and closer to that observed by Nichols (1965) in nutrient-deficient pigeon pea. This could be due to the low basal nutrient content in the growth substrate. For example, nitrogen content of the growth substrate with total nitrogen <100 mg kg^{-1} was considered extremely low (Rayment & Lyons, 2010) despite fertilization, especially so for a legume crop species like the pigeon pea. To overcome nutrient limitation in these substrates, pigeon pea plants abscised older leaves (Figure 1a) to remobilise nitrogen for newer growth, as evident in the relatively rapid leaf shedding rate of 26%–42% across treatments (Table 3). An overall low number of nodules was observed in all the plants (3.68 ± 0.65), especially the inoculated treatments (4.94 ± 0.78) in which a higher number of nodules, ranging between 7 and 10 (Rajendran et al., 2008), was expected. The low number of nodules might have resulted from phosphorus deficiency. In a mineral nutrition study conducted by Nichols (1965), the omission of phosphorus significantly reduced nodule counts in pigeon pea compared with most other elements. The observation of increasing nodule count with increasing phosphorus supply in soybean (*Glycine max*) further illustrated the importance of phosphorus in nodule formation (Qiao et al., 2007). Further investigations, such as tissue nutrient analysis, are required to confirm that the pigeon pea plants in this study were not phosphorus deficient.

In this experiment, water was the main factor accounting for the differences observed in growth, except for nodule counts attributed to the inoculation treatments. A strong negative impact of water deficiency on plant biomass was also previously reported in cowpea (*Vigna unguiculata*) by Rocha et al. (2019). Rocha et al. (2019) also found that inoculation treatments mitigated the growth inhibition of water deficiency on cowpea. In contrast with their findings, the overriding effect of water might have masked the beneficial effects of inoculation treatments on the growth of pigeon pea, despite significant effects on their physiology (e.g., biological nitrogen fixation, gas exchange). It is likely that the absence of improved growth in inoculated pigeon pea might have resulted from insufficient soil nutrients in our mine site substrates. As discussed earlier, deficiency in nitrogen and phosphorus, and possibly other nutrients, would have affected the efficacy of the inoculation treatments on pigeon pea. The effect of substrate nutrients on plant growth and how that may have changed with inoculation were, however, not investigated in this experiment.

Unexpectedly, plants in the low water treatments exhibited higher conductance and photosynthesis compared with plants in the moderate water treatment (Figure 2a). It is reasonable to assume that, on average, the plants that were given more water had higher rates of plant-level photosynthesis and transpiration, and that the greater carbon fixation resulted in their higher biomass and greater height (Table 3). Being larger plants, they also had greater leaf area (Figure 1). Thus, the rates of photosynthesis and transpiration per unit leaf area were not necessarily higher for the plants in the moderate water treatment. Also, the photosynthesis rates and stomatal conductance may have dropped faster with time after the last watering event. This is however only detectable with continuous monitoring of the plants' gas exchange, which was not conducted. The point measurements of gas exchange may therefore not represent longer-term physiology. It is useful, however, to explore possible differences in WUE. Inoculated plants tended to have higher WUE_i at a given stomatal conductance, with more data points above the fitted line than control plants in Figure 2b. To further determine if the gas exchange differences were mainly due to stomatal conductance or photosynthetic capacity, which could have been enhanced by inoculation treatment, foliar $\delta^{13}C$ and $\delta^{18}O$ were analysed (Flanagan & Farquhar, 2014). The slight positive correlation between foliar $\delta^{13}C$ and $\delta^{18}O$ (Figure 3c) indicated that stomatal conductance was the main factor (Flanagan & Farquhar, 2014). The multi-measurements approach of using short-term gas-exchange measurements and stable isotopes revealed that the inoculated plants had higher photosynthesis and WUE_i, which were due to stomatal conductance, and not enhanced photosynthetic capacity.

Microorganisms (*Azotobacter*, *Azospirillum*, *Bacilli*, *Pseudomonas*, *Streptomyces*, *Saccharomyces*, *Trichoderma* and various fungi) formulated into the commercial microbial inoculant (Appendix 1 of Supporting information) and *Bradyrhizobium* spp. used as rhizobial inoculant have been reported to produce phytohormones (Chanclud & Morel, 2016; Dodd et al., 2010; Sathya et al., 2017; Sumbul et al., 2020). The ribosylated forms of cytokinins that are produced by microorganisms (García de Salamone et al., 2001; Madhaiyan et al., 2006; Upadhyaya et al., 1991) could be easily transported to the shoots (Kudoyarova et al., 2019) from the roots via the xylem (Park et al., 2017; Yong et al., 2014). Hence, an increase in xylem phytohormone concentration of the inoculated plants would indicate higher levels of phytohormones *in planta* that are available for physiological functions (Kiba et al., 2019) and these are produced by the plant (mainly root tips) and its associated microorganisms (Lu et al., 2021).

Phytohormones are typically present in the plant xylem sap at very low concentrations, and therefore xylem sap samples collected had to be concentrated for analysis. Due to the low yield, samples within the same treatment group were pooled, making statistical analysis impossible. While this prevented us from assessing the statistical differences between treatments, the analyses revealed useful biological relationships between the phytohormones and plant performances (Figures 4 and 5). The phytohormone analytical results are of high standard, meeting the criteria of strong S/N ratio >10 and RSD <20% for duplicated analysis of each pooled sample. Cytokinins of the ribosylated forms, *t*ZR and DHZR, were detected in similar concentrations as those quantified in pigeon pea by Upadhyaya et al. (1991).

Positive correlations between nodule counts and xylem concentrations of the cytokinins DHZR and iPR (Figure 4) could be due to two different processes. Firstly, the higher concentrations of

cytokinins in inoculated plants (Table 6) could have resulted from root production upon rhizobial infection. This could be a mechanism to prevent the formation of excessive numbers of nodules (autoregulation of nodulation), which could otherwise inhibit the growth of host plants (Sasaki et al., 2014). Secondly, increased cytokinin could also have resulted from plant uptake of cytokinins or precursors produced by the microbes (Dodd et al., 2010). This is potentially beneficial for the plants, as ribosylated cytokinins can be easily transported to the shoots to stimulate plant growth (Kiba et al., 2019; Kudoyarova et al., 2019). The low SA but high ABA observed in the inoculated plants suggest that the inoculation treatments could have helped resist pathogens and increase WUE to enhance drought tolerance (Jorge et al., 2019; Naseem et al., 2014): both traits could be highly beneficial for plants under field conditions with low water availability and encountering possible biotic stress. The negative correlations of SA with ABA (Figure 5d) and WUE were also previously reported by Mosher et al. (2010).

Overall, phytohormone analysis results indicated that inoculation treatments impacted the plants' xylem sap phytohormone concentration with strong correlations with physical traits, specifically nodule counts, and plant photosynthetic rates and stomatal conductance. The benefits of this can only be speculated until greater understanding is gained into the roles of each phytohormone and the implications of increasing or decreasing its concentration *in planta*. It is also important to note that only representative phytohormones in plant xylem sap were investigated due to the limited sap volume. There is a possibility that phytohormones or other bioactive substances not investigated here may show greater changes to inoculation treatments. Investigation of phytohormone differences in other plant organs, such as roots and leaves, may also provide more insights on the impacts of inoculation treatments under low, moderate and high water availability.

In conclusion, soil microbial inoculation improved the physiology of pigeon pea growing in a challenging substrate under water limitation. The inoculation treatments helped the plants to optimise water use via phytohormone regulation and provided the plants with additional nitrogen. Water availability, however, had a greater effect on the plants' growth than microbial inoculations. Plant survival and resilience, rather than rapid growth, is often a priority for ecological restoration projects in challenging environments, and therefore the potential use of soil amendments that improve plant resilience can be very beneficial in restoration projects (Hueso-González et al., 2017; Rivera et al., 2014; Valliere et al., 2020; Wang, 2017). To validate the feasibility of applying soil microbial inoculation for mine site restoration, the effects of inoculation treatments under field conditions and on target species for restoration, which are often native species, must also be investigated, following the earlier controlled environmental experiments (e.g., pots, greenhouse). Validation on the efficacy of the inoculation treatments should not only include growth parameters, which could be masked by other factors such as water availability, but to also include other physiological measures to assess stress tolerance under a range of abiotic conditions. This study has highlighted that resource conditions such as soil moisture and nutrient availability could have strong effects on the potential of soil microbes to positively influence plant growth during restoration. Further prior knowledge on the properties of the soil to be amended, including soil type and indigenous microorganisms, seed banks, and plant species-specific responses, are needed to customise the inoculant for maximum benefits to ecological restoration and to support future adoption of this practice.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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