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RESEARCH ARTICLE

Seed biopriming at different concentrations to assess the effects of Cyanobacteria on germination and seedling performance of keystone arid species

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Abstract

Introduction: Biocrust cyanobacteria have a large potential as biofertilizers for restoring degraded ecosystems because of their ability to improve soil nutrition and stabilisation, and to produce metabolites such as phytohormones to enhance plant growth. However, important aspects regarding the effects of cyanobacteria on native plants, such as metabolite production or concentration of inoculants, remain unknown. Here, we investigated the effects of different concentrations of cyanobacteria, on the germination and seedling growth of keystone plant species used in dryland restoration. We hypothesised that the studied inoculant would improve germination and seedling growth rates, with specific effects associated with the inoculant's concentration and metabolomic profiles.

Methods: We bioprimed seeds of four native plant species, using a cyanobacterial inoculant with different proportions of *Nostoc* and *Leptolyngbya* at two different concentrations. We recorded germination, measured seedling growth, and determined the corrected vigour for each treatment and species. Metabolites produced by the cyanobacterial inoculant were assessed to identify plant growth hormones potentially driving any effects.

Results: There was a clear positive effect on the total germination of *Triodia epactia* and *Triodia wiseana*, but negative impacts for *Senna notabilis* and *Grevillea wickhamii*. There were also positive effects on root growth, but only for *T. epactia*, with negative or neutral impacts on the root and shoot growth of other species tested. We detected phytohormones, salicylic acid and indole-3-acetic acid, that were produced by our cyanobacteria inoculant, which are strongly linked to positive effects in early plant growth stages, but also known to inhibit growth when in higher concentrations.

Conclusion: The positive effects of the biopriming protocol used are not uniform and highlight the need to improve our understanding of the effects provided both from different consortia and the concentrations applied when inoculating. There is a very high

value in improving restoration outcomes for native vegetation communities in arid and semi-arid regions.

KEYWORDS
auxins, biopriming, cyanobacteria, hormones, salicylic acid

1 | INTRODUCTION

Cyanobacteria are primary components of biological soil crusts or 'biocrusts' that have essential roles in soils and ecosystems, including nitrogen and carbon fixation, promotion of phosphorous bioavailability, protection against erosion and improvement of soil hydrology (Chamizo et al., 2018; Mager & Thomas, 2011; Singh et al., 2016). Because of these abilities, biocrust cyanobacteria have been harnessed for improving soil stabilisation and chemical nutrition within the context of ecological restoration (Giraldo-Silva et al., 2019; Román et al., 2020; Su et al., 2009; Wang et al., 2009). Cyanobacteria can also influence seed germination and plant growth through exudated phytohormones such as auxins (Shariatmadari et al., 2015; Zarezadeh et al., 2020) gibberellic acid, (Rodríguez et al., 2006), and cytokinins (Bayona-Morcillo et al., 2020). Thus, cyanobacterial-based inoculants are commonly used in agriculture to stimulate root and shoot growth and increase seedling weight, grain yield, levels of carbohydrates, proteins and oils (Gavilanes et al., 2020; Osman et al., 2020; Righini et al., 2021; Zarezadeh et al., 2020). Despite their extended use as a plant growth-promoting bacteria for agricultural applications, these organisms have only recently emerged as a potentially beneficial tool for promoting plant growth in the context of natural ecosystem restoration (Chua et al., 2020; Muñoz-Rojas et al., 2018).

Cyanobacteria is a broad phylum with multiple functions, and variation in morphology, physiology and ecology (Whitton & Potts, 2012). The family Nostocaceae, which belongs to the order Nostocales, are characterised by their unbranched filaments of cells and the development of heterocysts amongst the cells of the filaments. Cyanobacteria from this family, for example, those from the genera *Nostoc*, have been targeted for soil restoration because of their ability to fix nitrogen and potentially contribute to the total nitrogen input in ecosystems with poor soils, such as in semi-arid and arid lands where nitrogen may be limited (Yeager et al., 2007). In addition, species of *Nostocaceae* can form symbiotic relationships with particular plants (Meeks, 2007). Other types of cyanobacteria inhabiting arid ecosystems and forming the so-called 'light biocrusts' are the filamentous and nonheterocytous cyanobacteria, such as *Microcoleus* and *Leptolyngbya* (Pietrasiak et al., 2013). *Leptolyngbya* is less cited in studies involving land restoration, (Pietrasiak et al., 2013) however, species within this genus have the ability to arrange filaments entangled with soil grains, produce exopolysaccharides and bind soil particles (Mager & Thomas, 2011; Mugnai et al., 2018). Moreover, *Leptolyngbya* has shown a substantial potential for ex situ cultivation, unlike *Microcoleus*, (Prufert-Bebout & Garcia-Pichel, 1994) which could help overcome the current barriers to the production of cyanobacterial biomass for large-scale application in soils (Roncero-Ramos et al., 2019). Therefore, the genus can be considered an effective inoculant able to enhance soil cohesion and stabilisation.

Seed biopriming is a seed enhancement technology that refers to the inoculation of seeds with beneficial organisms, such as microorganisms, to increase the number of seeds germinating and germination rates (speed) in suboptimal environments, suppress disease, control plant pathogens, promote growth, restrain side effects caused by drought, and increase tolerance to high temperatures, salinity and heavy metals (Bisen et al., 2015; Pedrini et al., 2020). Seed biopriming with cyanobacterial consortia has shown positive or neutral effects on the germination and growth of several dryland native plants, (Chua et al., 2020; Muñoz-Rojas et al., 2018) but the responses have been species-specific and there are still several knowledge gaps related to these plant–microbial relationships. Furthermore, we still do not know whether different concentrations of cyanobacteria, and therefore potentially larger amounts of metabolites, would result in distinct effects on seed germination and seedling growth. Previous studies have shown that variants of the same bacteria species (wild vs. mutant) can produce different concentrations of auxins (Xie et al., 1996). Also, different concentrations of inoculant, a producer of indole-3-acetic acid (IAA—a primary auxin in plants), can generate a range of beneficial and deleterious effects in plant roots (Persello-Cartieaux et al., 2001). However, previous studies investigating the use of cyanobacteria as bioprimers for seed restoration have not tested the production of plant hormones by the inoculants, missing the link of cause and effect.

Here, we bioprimed seeds of four keystone native plant species used in dryland restoration, *Senna notabilis*, *Grevillea wickhamii*, *Triodia epactia*, *Triodia wiseana*, using a cyanobacterial inoculant with different proportions of Nostocaceae and *Leptolyngbya* cyanobacteria, to assess their effects on seed germination and seedling growth. In a novel approach, we also analysed the metabolites produced by this inoculant to identify plant growth hormones potentially driving these effects, and we tested two different concentrations, that is, 1 and 5 g L^{-1} of our inoculant. To evaluate whether phytohormone concentration was the driver of the effects, we also bioprimed seeds of two of our study species, *T. wiseana* and *T. epactia*, with IAA alone. This allowed an evaluation of the response caused by a single chemical component rather than a combination of chemicals potentially found in our tested inoculant. We hypothesised that our inoculant would improve germination and seedling growth rates, with specific effects associated with the metabolomic profiles and concentration of the cyanobacteria inoculants. Higher concentrations of inoculant were expected to induce stronger responses in the

studied plants whereas similar responses were expected in closely related plant species.

2 | MATERIALS AND METHODS

2.1 | Study region

Seeds and cyanobacteria used in this study were native to the Pilbara region in the northwest of Western Australia (22°03′S, 118°07′E to 23°19′S, 119°43′E). This region is semi-arid, with low annual rainfall (250–400 mm $year^{-1}$) and temperatures regularly exceeding 40°C in summer (Bureau of Meteorology, Australian Government Website, 2022). The region covers 179,000 km^2 and contains a high diversity of plant species, many of them endemic to the Pilbara (Doughty, 2013). Dominant genera include *Acacia* and *Senna* from the Fabaceae family, *Grevillea* from the Proteaceae family and *Triodia* from the Poaceae family, (Erickson et al., 2016) in hummock grasslands, tussock grasslands and sclerophyll shrubland communities. Due to its large iron ore deposits, the region experiences high-intensity iron ore extraction which disturbs more than 2300 km^2 of the surrounding habitat, (Environmental Protection Authority, 2014) placing stress on the native vegetation communities that inhabit the region.

2.2 | Cyanobacteria culturing and identification

A mixed-cyanobacterial culture composed predominantly of *Leptolyngbya* and other Nostocaceae (hereby referred as 'LeptoNos'), available at the Ecology Laboratory at the School of Biological, Earth and Environmental Sciences laboratories (UNSW, Sydney) was selected for the biopriming experiment (Supporting Information: Figure S1a).

Cyanobacteria had been previously isolated from soil biocrusts collected from Gallery Hill (Pilbara region), and cultured in BG11 medium (Cyanobacteria BG11 Freshwater Solution; Merck) (Muñoz-Rojas et al., 2018). Trichomes and colonies (lately mats) of different cyanobacteria were repeatedly inoculated in new tubes for the establishment of fresh cultures. The mixed culture, composed of *Leptolyngbya* and Nostocaceae, showed a higher growth rate and they were therefore cultivated together. The culture was maintained in nonaxenic conditions, as many studies have found that the association of cyanobacteria with other bacteria develop symbiotic or mutually beneficial relationships, resulting in nutrient availability and growth promotion for the cyanobacteria (Salomon et al., 2003; Wang et al., 2022). Rossi et al. (2022) support the idea that the cyanobacteria and the bacteria in the cyanosphere create a microcosm that involves crucial nutrient exchange processes. These authors also caution against working with axenic cultures, which is counterproductive (Rossi et al., 2022). We maintained the proportion of bacteria in the cultures low (Supporting Information: Figure S1) through careful manipulation of the initial colonies and trichomes (being clean while dragged over BG11 solid media), and during the following constant subculturing. Since cyanobacteria is the dominant bacteria in our consortium, it is likely that it is primarily responsible for the majority of metabolic production. Nevertheless, we stress the significance of considering our consortium as a unified entity that comprises other bacteria as well. Populations were cultivated and maintained under a regime of 24°C ± 1°C and a 16:8 h light–dark cycle (80 µmol photons m^{-2} s^{-1} of irradiance) in plant growth chambers (Thermoline Scientific Climatron 2400-TH-CO_2).

To confirm the composition of the culture, we used molecular methods. The total biomass was extracted using the DNeasy PowerSoil Kit (Qiagen) following the manufacturer's instructions. The 16S V1–V3 region from 16S ribosomal RNA gene was then amplified by polymerase chain reaction (PCR) using the barcoded primers 27F (AGAGTTTGATCMTGGCTCAG) and 519R (GWATTACCGCGGCKGCTG) including an overhand Illumina adapter. Subsequently, a second PCR was performed to incorporate index barcodes using the Nextera XT Index Kit (Illumina). A paired-end sequencing was performed on an Illumina Miseq sequence platform (Illumina) using the Miseq Reagent kit v3. 2 × 300 cycle. Amplifications and sequencing were performed by the Ramaciotti Centre for Genomics (UNSW, Australia). Raw sequence data processing was carried as in Machado de Lima et al. (2021) Briefly, the OTUreporter v1.0.0-beta (9b72c8e) pipeline (https://bitbucket.org/xvazquezc/otureporter) based on Mothur v1.39.5 (Schloss et al., 2009) was used to ensure quality filtering, assignments of respective samples, and trimmings of reads. Chimeras were detected through the script Chimera. vsearch, (Rognes et al., 2016) and taxonomy was assigned by comparison with the SILVA database v 132 (Quast et al., 2013). The unprocessed sequence information has been shared with NCBI and is accessible to the public through the BioProject identifier PRJNA944785.

2.3 | Seed preparation and Cyanobacteria biopriming of seeds

We used seeds of *T. wiseana* C. A. Gardner, *T. epactia* S. W. L. Jacobs, *S. notabilis* (F. Muell.) Randell and *G. wickhamii* Meisn. previously collected from the Pilbara region and stored in a controlled environment room at 15°C and 15% relative humidity at Kings Park Science (Department of Biodiversity, Conservation and Attractions, Western Australia). These native species, representative of Australian semi-arid environments, have been prioritised in restoration programs in the Pilbara region (Bateman et al., 2018; Erickson et al., 2016). Seeds were checked for viability by microscopy and surface sterilised in 1% (wt/vol) calcium hypochlorite solution for 30 min. Then, the seeds were washed with autoclaved milli-Q water. *S. notabilis* seeds were immersed in hot water at 90°C for 2 min and *T. wiseana* seeds had their covering floret structure removed to overcome dormancy (Erickson et al., 2016).

Cyanobacterial biomass from the selected culture was filtered and resuspended in milli-Q water, to maintain the biopriming inoculant at concentrations of 1 and 5 g L^{-1}. Biomass concentration was determined based on cyanobacteria dry weight, by oven-drying filtered subsamples at 60°C for 24 h. Seeds were grouped in separate plastic tubes by species and treatment. The cyanobacteria biopriming

inoculant was added to the tubes at both concentrations (1 and 5 g L^{-1}), except for the control treatment which contained the same volume of milli-Q water only (Supporting Information: Figure S1b). Then, plastic tubes were agitated on an orbital shaker (Ratek EOM5) for 20 h at low speed and 25°C. After agitation, seeds were transferred to Petri dishes for the germination step.

2.4 | Seed preparation and biopriming different concentrations of auxin, germination and early seedling growth

Batches of *T. wiseana* and *T. epactia* seeds were used in a second experiment, where they were bioprimed with IAA. As per the original approach, seeds were checked for viability by microscopy and surface sterilised in 1% (wt/vol) calcium hypochlorite solution for 30 min, and then washed with autoclaved milli-Q water. A stock solution of 10 mg/mL of IAA was diluted to produce solutions of 2 and 50 µg/mL. Seeds of each species were immersed in tubes containing 2 and 50 µg/mL of IAA, and also in autoclaved milli-Q water as a control. Plastic tubes were agitated on an orbital shaker (Ratek EOM5) for 25 h at low speed and 25°C. After agitation, seeds were transferred to Petri dishes for germination assessment.

2.5 | Seed germination and early seedling growth measurement

Seeds bioprimed with cyanobacteria or hydro-primed were plated on 90 mm Petri dishes with agar 5 g L^{-1} (n = 4 dishes per treatment; 25 seeds per dish = 100 seeds per treatment). For the IAA and control (hydro-primed) experiment, *T. wiseana* and *T. epactia* seeds were also plated on 90 mm Petri dishes with agar 5 g L^{-1} but at slightly different levels of replication (n = 3 dishes per treatment; 33 seeds per dish = 99 seeds per treatment). All seeds were incubated under 16/8 h alternating temperature (20°C/29°C) and light:dark cycles in a plant growth chamber (Thermoline Scientific Climatron 2400-TH-CO_2). Germination was scored on the emergence of the radicle and was recorded daily until no new germination was observed for 3 days. After that, seedlings bioprimed with cyanobacteria or hydro-primed were randomly selected (around 30–40 seedlings per species) and measured root and shoot length. While seedlings bioprimed with IAA and hydro-primed were also randomly selected, but 16–24 seedlings were selected per species, and measured root and shoot length.

2.6 | Metabolomics analyses

For the metabolomic analyses, 250 mL of cyanobacterial culture were grown in BG-11 growth medium under a 16:8 day–night cycle at 25°C until sufficiently dense, following established protocols (Hussain et al., 2010). The cyanobacterial culture was then filtered using 11 µm pore size filter paper (Whatman Ltd.) and the resulting pellets freeze-dried to remove any excess water. Metabolites were extracted via the addition of 50 mL methanol to the lyophilised pellets followed by 24 h incubation at 4°C, and the methanol was then filtered using 11 µm pore size filter paper (this step was repeated twice). Methanol was collected and evaporated under a fume hood before derivatization. To derivatize the samples for gas chromatography–mass spectrometry (GC–MS) analysis, 40 µL *N,O*-is (trimethylsilyl)trifluoroacetamide (BSTFA) and 20 µL of molecular sieved acetonitrile were added to the extracts before incubation at 55°C for 1 h. The derivatized extracts were analysed using a Focus DSQII GC-MS (GER; Thermo Fisher Scientific). A Thermo TriPlus II autosampler set-up was used to inject liquid samples, with a sample volume of 10 µL injected splitless, and five pre- and postwashes carried out for each injection using acetonitrile. The run was repeated three times. The carrier gas was helium with a flow rate of 1.7 mL/min. The initial oven temperature was set at 70°C, ramping to 75°C over 5 min before increasing to 325°C at 5.6 C min^{-1} with a 10 min holding time, following previously published metabolite analysis methods (Wong et al., 2015). The instrument was run using positive electron ionisation full scan mode from 40 to 650 Da.

2.7 | Data analysis

All the analyses were performed using the R statistical platform Version 4.0.2 (R Core Team, 2021). Boxplots and line plots were constructed using the package 'ggplot2' (Wickham, 2011). Germination was checked from the day after biopriming until the third day without any new observed germination. Seeds germinated during this period were used to calculate the total proportion of seeds germinated. Additionally, 'T50' (time to reach 50% germination), 'slope' (indication of how long seed germination reaches completion), and 'Max' (maximum germination rate) (Kniss & Streibig, 2018) were calculated using the dose–response models in the RStudio drc package, (Ritz et al., 2015) and the germination rate was plotted using cumulative germination over time. We used the 'compParm' function to evaluate the differences in the 'T50', 'Slope' and 'Max'. The package 'SeedCalc' (Silva et al., 2019) was used to calculate the corrected vigour index (Medeiros & Pereira, 2018). Using a linear model framework, we investigated how root and shoot lengths and corrected vigor were influenced by inoculants, while the 'emtrends' function ('emmeans' package) (Lenth et al., 2019) was applied to make comparisons across treatments. A similar approach was applied for total germination data, but instead using a generalised linear model with binomial error distribution. For metabolomic data processing and identification, we used the AMDIS software package, identifying known plant metabolites. The identities of peaks were ascertained using the NIST library version 11.

3 | RESULTS

Overall, our results showed that the effects of the different concentrations of cyanobacterial inoculant on seed germination and seedling growth were specific to each plant species. *For T. epactia*,

LeptoNos consortia increased the maximum germination ($p < 0.05$) 10.2% (Table 1 and Supporting Information: Figure S2) when used at high concentrations (5 g L^{-1}). Roots inoculated with the LeptoNos consortium at 5 g L^{-1} were 1.5 times longer compared to those in the control ($p < 0.05$) (Figure 1 and Supporting Information: Table S1). The corrected vigour index was 78% higher ($p < 0.001$) than the control at the highest dose (5 g L^{-1}; Figure 2 and Supporting Information: Table S2).

Germination of *T. wiseana* inoculated with LeptoNos consortium was 9.2% higher ($p < 0.005$) than that found in the control (Table 1 and Supporting Information: Figure S2). However, the consortium reduced root lengths by 22.2% compared to the control ($p < 0.05$), and shoots by 17.8% and 16% at 1 and 5 g L^{-1} concentrations, respectively (Figure 1 and Supporting Information: Table S1). The corrected vigour index was significantly lower (18%; $p = 0.01$) in the seeds treated with the lower concentration (1 g L^{-1}), while the higher concentration of this consortium did not show any effect. Germination rates of *S. notabilis* decreased (7.1% on average; $p < 0.005$) in response to the consortia and their different concentrations. Inoculation with LeptoNos (both concentrations) increased the germination time T50 (Table 1 and Supporting Information: Figure S2), and reduced root length (28%; $p < 0.0001$) and shoot length (over 22%; $p < 0.005$) (Figure 1 and Supporting Information: Table S1). Lower vigor index values (18.0%–29.7%; $p < 0.001$) were observed in seeds treated with either concentration of the LeptoNos consortium (Figure 2). The LeptoNos 1 g L^{-1} consortium reduced maximum germination of *G. wickhamii* by 7.4% ($p = 0.01$; Table 1 and Supporting Information: Figure S2), while both concentrations resulted in none of the seeds reaching the seedling developmental stage.

Metabolomic analysis through GC–MS identified the common plant hormone IAA in the consortia using the NIST database (Supporting Information: Figure S3). Another potential phytohormone, that is, salicylic acid was also identified using the NIST database in the consortia (Supporting Information: Figure S4).

T. epactia seeds treated with 2 and 50 µg/mL of IAA did not show significant changes in their maximum germination (Table 1), however, presented significantly longer roots in comparison with the control when treated with 2 µg/mL ($p < 0.005$; Figure 3). Conversely, *T. wiseana* bioprimed with IAA, displayed no significant differences in their germination rate (Table 1) and root length when treated with the lower dose of 2 µg/mL, but showed shorter root length when treated with the higher dose ($p < 0.0001$; Figure 3).

4 | DISCUSSION

Overall, our results suggest that the LeptoNos consortium can be beneficial, but the contrasting responses across our native study species and at different life-cycle stages uncovered a more complex relationship. There was a clear positive effect on the total germination of *T. epactia* and *T. wiseana*, but negative impacts for *S. notabilis* and *G. wickhamii*. There were also positive effects on root growth, but only for *T. epactia*, with negative or neutral impacts on the root and shoot growth of other species tested. We detected two phytohormones, salicylic acid and IAA, that were produced by our cyanobacteria inoculant, both of which are strongly linked to positive effects in early plant growth stages, but also known to inhibit growth when applied at higher concentrations (Toribio et al., 2020; Zarezadeh et al., 2020). This suggests a potential mechanism for the mixed growth rate response, however, other metabolites may also play a role, for example, toxic metabolites at a critical concentration and further work is required to confirm the biological mechanism behind the observed responses. While this research analysed recognised plant growth-promoting metabolites, it is conceivable that there are additional unknown metabolites being produced that are not documented in metabolite libraries or detectable by GC–MS. The contrasting results, when compared to previous studies that have included the same native species, highlight the need to understand how different cyanobacterial compositions,

TABLE 1 Maximum germination (%) and T50 (days) for each plant species and treatment (mean ± SE).

	Triodia epactia		*Triodia wiseana*		*Senna notabilis*		*Grevillea wickhamii*	
Treatment	**Max**	**T50**	**Max**	**T50**	**Max**	**T50**	**Max**	**T50**
Control	36.3 ± 3.0	2.89 ± 0.2	74.6 ± 1.6	1.90 ± 0.1	98.2 ± 0.0	0.69 ± 0.1	13.4 ± 1.6	6.54 ± 0.5
LeptoNos 1 g L^{-1}	40.5 ± 2.9	2.74 ± 0.2	74.3 ± 1.4	1.85 ± 0.3	**89.0 ± 1.6***	**1.15 ± 0.1***	**6.0 ± 1.0***	6.71 ± 0.7
LeptoNos 5 g L^{-1}	**46.5 ± 3.8***	2.775 ± 0.2	**83.8 ± 1.2***	1.811 ± 0.3	**93.1 ± 1.3***	**1.37 ± 0.7***	8.6 ± 1.3	6.26 ± 0.6
Control	66.9 ± 2.1[a]	1.9	21.8 ± 0.02[b]	2.33 ± 0.3	–	–	–	–
Auxin 2 µg/mL	70.0 ± 2.0	1.9	19.4 ± 0.02	2.20 ± 0.4	–	–	–	–
Auxin 50 µg/mL	66.8 ± 2.3	2.00 ± 0.06	18.8 ± 0.02	3.35 ± 0.4	–	–	–	–

Note: Asterisks (*) and bold indicates significance levels at $p < 0.05$.

Assessing treatment efficacy: utilising a generalised linear model approach with binomial error distribution with the 'emtrends' function to uncover significant differences between control and treatment groups.

[a]Value generated using around 16 seedlings per treatment.

[b]Value generated using around 24 seedlings per treatment.

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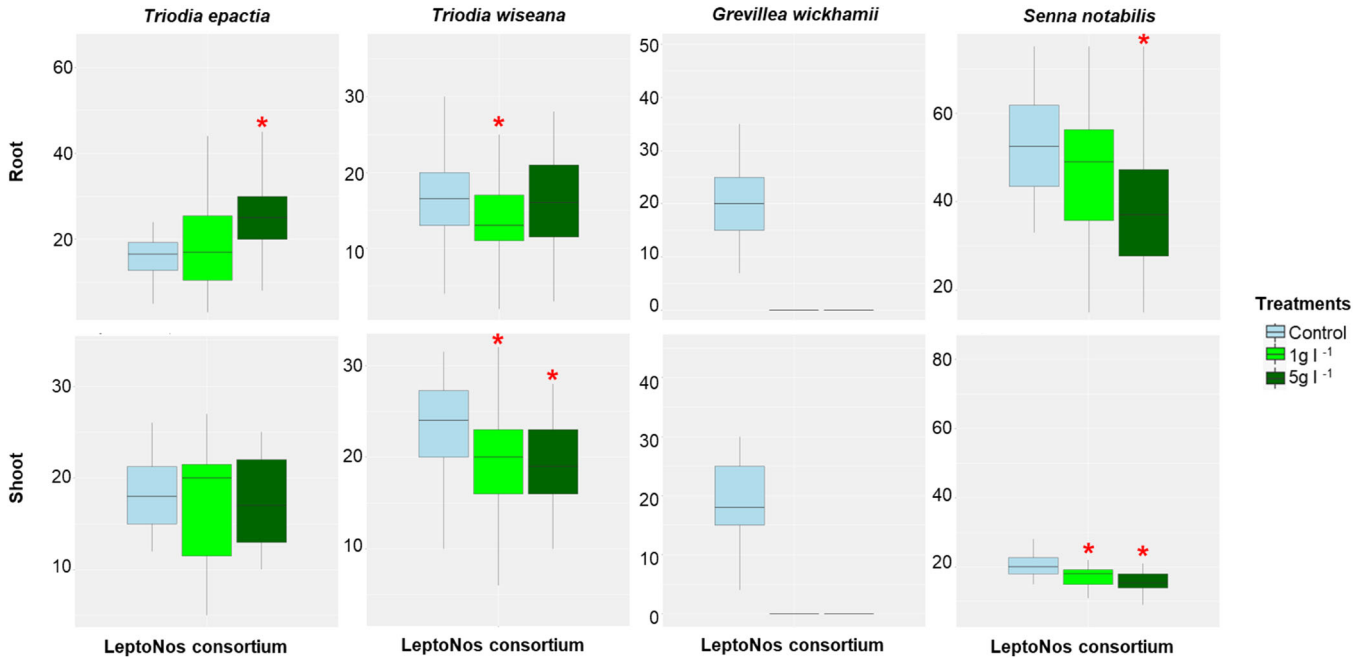

FIGURE 1 Seedling length (mm) for *Triodia epactia*, *Triodia wiseana*, *Grevillea wickhamii* and *Senna notabilis*. Levels of significance represented by red asterisks $*p < 0.05$. Assessing treatment efficacy: utilising a linear model approach with the 'emtrends' function to uncover significant differences between control and treatment groups.

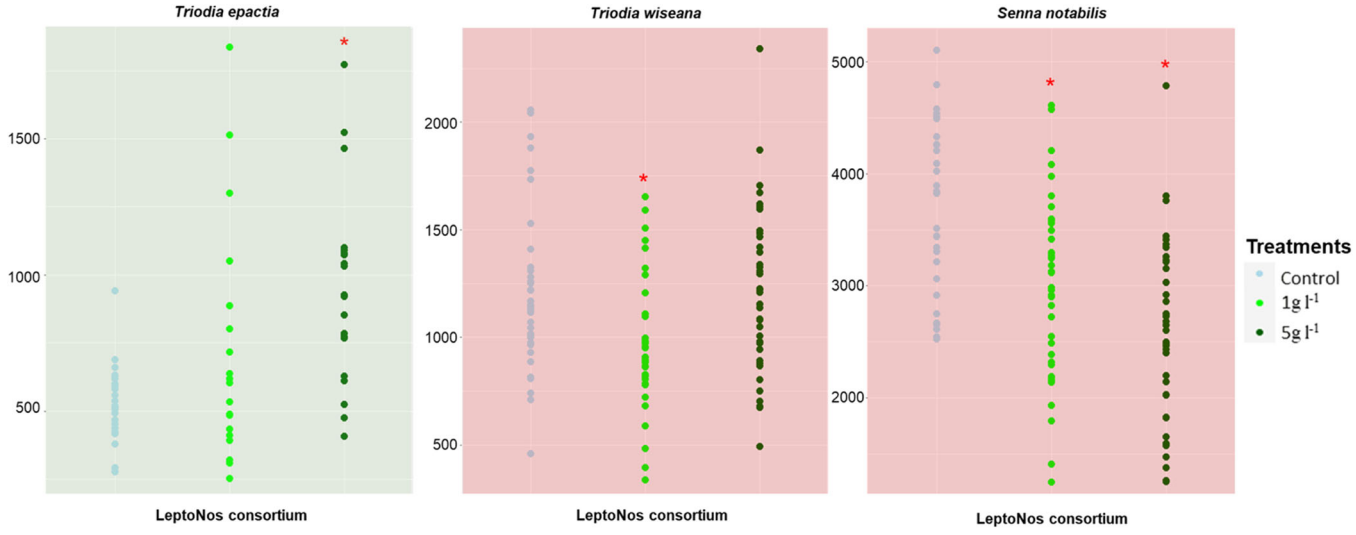

FIGURE 2 Corrected vigor index for the four studied species (*Triodia epactia*, *Triodia wiseana* and *Senna notabilis*) and its respective treatments (LeptNos consortia, both concentrations, 1 and 5 g L^{-1}). Levels of significance represented by red asterisks $*p < 0.05$. Assessing treatment efficacy: utilising a linear model approach with the 'emtrends' function to uncover significant differences between control and treatment groups. Graphs highlighted in green represent the treatments that positively impacted the tested seed, while the ones highlighted in red represented the negatively impacted.

and the concentrations of their different metabolites, interact with native species. This is particularly important for clarifying the potential benefits of using cyanobacteria in native vegetation restoration.

IAA has been previously identified as a product of cyanobacteria organisms, with several benefits provided to plants, including growth promotion (Zarezadeh et al., 2020). However, the concentration of IAA determines whether effects are positive or negative, with many studies showing that excessive amounts of IAA can inhibit growth, have negative effects on plant physiology, and potentially promote seed dormancy (Gamalero & Glick, 2011; Liu et al., 2013; Noel et al., 1996; Tsavkelova et al., 2007). Positive plant responses are therefore concentration-dependent, as well as controlled by the spatial-temporal distribution of IAA, (Cao et al., 2020) meaning that

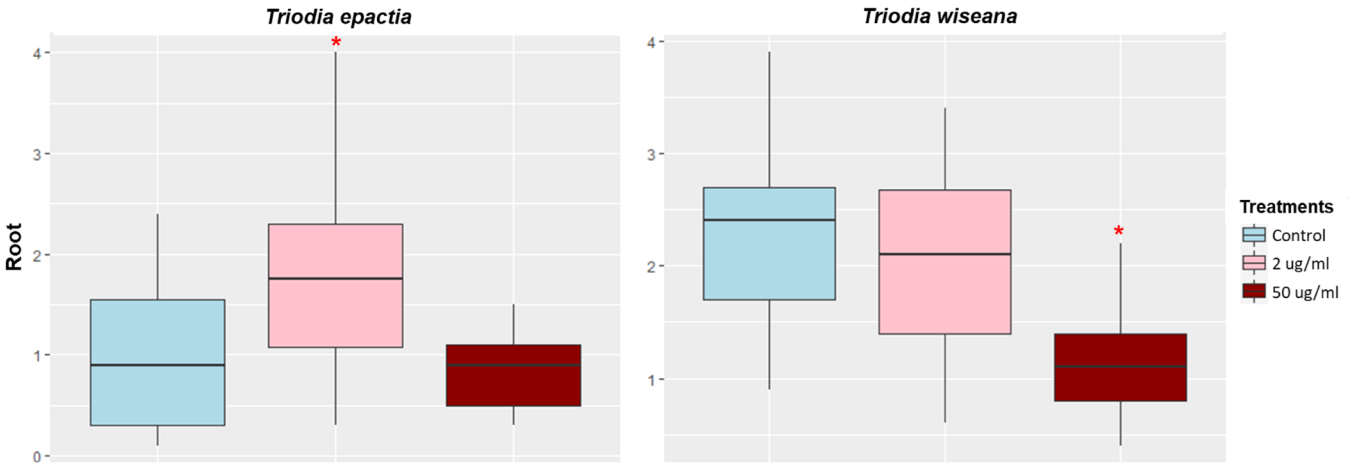

FIGURE 3 Seedling length (mm) for *Triodia epactia* and *Triodia wiseana* bioprimed with different concentrations of Auxin. Levels of significance represented by red asterisks $*p < 0.05$. Assessing treatment efficacy: utilising a linear model approach with the 'emtrends' function to uncover significant differences between control and treatment groups.

optimal levels can vary between plant species and as a result of plant tissue sensitivity to auxins (Gamalero & Glick, 2011). One of our study species, *S. notabilis*, was very sensitive to the LeptoNos inoculant, suffering negative effects regardless of the applied inoculant concentration. This followed different findings for the same species in other studies where only low concentration of inoculant and different inoculant composition were used, with positive effects on germination (Chua et al., 2020) and seedling growth (Muñoz-Rojas et al., 2018). This suggests that much lower concentrations of our inoculant need to be tested to identify whether our inoculant composition can provide promotive effects for *Senna*.

Perhaps the clearest example of the importance of inoculant concentration and composition is found in the response of *Triodia*. Previous studies found no effects of cynobacteria biopriming on either of our *Triodia* study species' germination, however, both had used inoculant concentrations of 1 g/L and different cyanobacterial composition (Chua et al., 2020; Muñoz-Rojas et al., 2018). In our study, both *Triodia* species produced significantly greater germination but only at the high 5 g/L concentration. Previously undetected effects of biopriming on these species could therefore be a result of the concentration of inoculant applied. Impacts on *Triodia* root and shoot growth was more complex, with *T. wiseana* growth reduced and *T. epactia* growth increased by cyanobacteria biopriming. These different response patterns in growth were mirrored in the experiment using isolated IAA, where *T. epactia* was positively influenced at moderate but not high concentrations of IAA, while *T. wiseana* showed a clear negative response. The different responses of these two closely related species suggest that they have different sensitivities to the growth hormones tested, and potentially others present in the consortium, at the seedling growth stage.

Species-specific testing and the determination of the concentration of hormones produced by the inoculant may be beneficial in the restoration context when aiming to use cyanobacteria as a plant promoter. This approach is already considered in agricultural settings and has helped to develop a much finer-scale understanding of biopriming with inoculants. For example, Dubeikovsky et al. (1993) experimentally tested the bacteria *Pseudomonas fluorescens*, an IAA overproducing mutant, and observed positive effects in the root development of blackcurrant cuttings. However, the same bacterium caused an inhibitory effect in cherry cuttings. Glick interpreted these results as being driven by prior suboptimal levels of IAA in the blackcurrant cuttings, which had become optimal after the bacterial effect (Glick, 2012). The auxin levels of cherry cuttings, however, were already optimal before bacterium addition, and the generated supraopitimal level produced an inhibitory response. These findings are aligned with the fact that plants have their own ontogeny and react differently to the organisms on their surrounding, (Havrilla et al., 2019) being very specific in relation to their microbial recruitment (Ramakrishna et al., 2019). For species with high restoration value, like *Triodia* which is a keystone genus across much of arid and semi-arid Australia, investment in research at a similar resolution to that conducted in the agricultural sector would help to identify the full potential of biopriming, particularly in large-scale restoration.

Differences in response of our study species to the inoculant and IAA applied highlights the importance that concentration can play in determining outcomes for improved germination and growth in restoration. However, factors beyond microbial hormonal production should also be considered when interpreting our, and future, results. Cyanobacteria are able to produce a multitude of chemical compounds, and some might be deleterious or have impacts on plant establishment and growth while affecting their symbiotic bacteria. For example, cyanobacteria can produce secondary metabolites possessing antibacterial activity, (Gupta & Vyas, 2021) able to impact plant resilience. Also, they can affect plants directly through the production of toxins that deplete processes in plant tissues (Romanowska-Duda et al., 2002). While our study has demonstrated the complexity surrounding biopriming, it highlights the need for

further studies to understand associations between bacterial inoculation and plant growth. This work also reaffirms the importance of plant–soil microbial interactions at early growth stages (seed germination and seedling growth), particularly in the design of bioinoculants for plant growth and health promotion.

The positive relationships we found between our inoculant and the Triodia species studied, in addition to previous studies investigating arid-zone species, (Chua et al., 2020; Muñoz-Rojas et al., 2018) support the fact that biopriming native plant species with cyanobacteria provides a pathway forward for improving restoration. At the same time, the mixed results for *G. wickhamii* and *S. notabilis* (when comparing our study with previous positive results [Chua et al., 2020]) highlights the need to improve our understanding of the effects provided both from different consortia and the concentrations applied when inoculating. There is a very high value in improving restoration outcomes for native vegetation communities in arid and semi-arid regions, and particularly their keystone species, which are subject to multiple pressures from large-scale clearing, for example, *Triodia* has a wide distribution, extending from arid inland to coastal areas, and has multiple important uses, including in restoration practices and traditional indigenous culture (Erickson et al., 2016; Gamage et al., 2012). The hummock grasslands where *Triodia* dominates covers over 18% of the Australian continent (Department of the Environment and Water Resources, 2007). Subsequently, *Triodia* is the primary species in the Pilbara, and other regions, that practitioners have long focused on to restore areas that have been disrupted by mining activities (Erickson et al., 2016). However, seed-based restoration in arid zones has had limited success, (Bateman et al., 2019) highlighting the urgent need for new seed enhancement technologies, especially for Triodia, given its tightly regulated recruitment patterns (Lewandrowski et al., 2018). The benefits of fine-tuning the application of a bioinoculant like cyanobacteria are not only the positive outcomes for germination and growth of keystone species like *Triodia*, but also its dual role as a bio-tool, which can enhance soil fertility and initiate the recovery of soil functions in dryland infertile substrates. The development of artificial cyanobacteria-dominated biocrust could lead to other benefits, including a reduced need for external fertiliser and amendment inputs (Muñoz-Rojas et al., 2018).

AUTHOR CONTRIBUTIONS

All authors: Study conception and design. Nathali M. Machado-de-Lima, Jana Stewart, Miriam Muñoz-Rojas: Material preparation, data collection and analysis. James Charlesworth: Chemical analyses. Nathali M. Machado-de-Lima: Writing of the first draft of the manuscript. All authors: Reading, commenting on previous versions of the manuscript, approval of the final manuscript. Nathali M. Machado-de-Lima: Review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The authors confirm that the unprocessed sequence information relevant to the findings presented in this manuscript has been deposited in the National Centre for Biotechnology Information (NCBI) and is publicly available through the BioProject identifier PRJNA944785. The data can be accessed by interested parties and is freely available for download and use, ensuring transparency and reproducibility of our study.

ETHICS STATEMENT

The research on 'Seed biopriming at different concentrations to assess the effects of cyanobacteria on germination and seedling performance of keystone arid species' and the resulting manuscript have been carried out in accordance with scientific ethics principles and relevant regulations and guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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