



Synergistic effects of nitrogen and plant growth-promoting rhizobacteria inoculation on the growth, physiological traits and nutrient absorption of intercropped *Eucalyptus urophylla* × *Eucalyptus grandis* and *Dalbergia odorifera*

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Abstract

Key message Legume/nonlegume intercropping systems equipped with moderate nitrogen (N) application and PGPR inoculation could be exploited in eucalyptus growing areas and degraded land as an ecologically sustainable system to avoid excessive fertilization and enhance nitrogen use efficiency.

Abstract Hazardous nitrogenous fertilizers in eucalyptus monocultures are challenging for the balance between eucalyptus wood production and ecological service functions. To assess whether plant growth-promoting rhizobacteria (PGPR) inoculation coupled with N application may improve plant growth by increasing biomass, photosynthesis, soil nutrient supply, and nutrient uptake capacity in the intercropping system. A pot experiment was performed to evaluate the synergistic effects of N application and N-fixing PGPR on growth, physiological parameters, N accumulation and nitrogen use efficiency (NUE) in intercropped *Eucalyptus urophylla* × *Eucalyptus grandis* (*E. urophylla* × *E. grandis*) and *Dalbergia odorifera* (*D. odorifera*). N fertilization positively influenced the plant height, dry matter yield, photosynthetic characteristics, N accumulation and nitrate reductase activity of *E. urophylla* × *E. grandis* under both inoculations. The growth and physiological traits of *D. odorifera* improved under the N₂ (6 g N pot⁻¹) application level. *E. urophylla* × *E. grandis* inoculated with *Rhizobium japonicum* IOC 113-2 showed higher values for plant height, biomass accumulation, N accumulation and photosynthesis with N fertilization, but the growth and physiological parameters of *D. odorifera* responded differentially to different inoculation treatments. The results demonstrated that an appropriate N supply in combination with N-fixing PGPR inoculation of legumes could increase nutrient absorption, NUE and yield advantages in intercropped *E. urophylla* × *E. grandis* and *D. odorifera*; thus, this method could be recommended as an alternative planting system under N-limited conditions in agroecosystems.

Keywords Intercropping · Nitrogen application · PGPR inoculation · Resource utilization · Yield advantages

Introduction

Intercropping, the simultaneous growth of multiple crop or tree species in a single field, has been widely practiced due to its economic, ecological and environmental benefits (Martin-Guay et al. 2018; Kanagendran et al. 2019; Dowl- ing et al. 2021). Indeed, legume/nonlegume intercropping, which is considered a fertility-enhancing and beneficial mixed-planting system, may achieve the optimal exploitation

of soil and other nutrients (Tian et al. 2020; Xu et al. 2021; Cabreira et al. 2022), reduce reliance on external nitrogen (N) inputs through N₂ fixation from the atmosphere and N transfer effects (Yong et al. 2018; Xianyu et al. 2019; Zhao et al. 2020), reduce pests and weeds and increase yield per unit area (Cong et al. 2018; Lei et al. 2021). Legume/non-legume intercropping is a widely practiced tree planting method in China, especially in plantings of high N-demanding woody perennials such as *Eucalyptus*. Approximately 5.46 Mha of *Eucalyptus* plantations have been established to meet the growing demands of the timber industry in China (Hu et al. 2017; Chu et al. 2018; Zhang 2019). In this context, *Eucalyptus* intercropped with *Dalbergia odorifera*, a semideciduous leguminous plant, has become a common

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cultivation system for its economic benefits and sustainability in Guangxi, China (Zhang et al. 2021).

Compared with *Eucalyptus* monocultures, intercropping is able to achieve the optimal exploitation of light, temperature, space and atmospheric N sources through plant-plant and plant-microbe interactions and offers beneficial growth conditions to both plants (Zheng et al. 2016; Santos et al. 2017; Zhang et al. 2019; Ye et al. 2020). Previous studies have revealed that the introduction of *D. odorifera* into *Eucalyptus* plantations increased plant biomass, N use efficiency (NUE) and N₂ fixation through root interactions, in which beneficial microbes play a key role in driving nutrient cycling and absorption (Xianyu et al. 2019, 2021; Yao et al. 2021). Moreover, it has been reported that the productivity of intercropped legumes, as the less competitive plants in intercropping systems, was inhibited by a companion plant (*Eucalyptus*) due to the low availability of soil N (Verret et al. 2017). As a result, limitations of soil N stimulated the biological nitrogen fixation (BNF) process of legumes to fix N from the atmosphere (Vanlauwe et al. 2019). In fact, N fixation and utilization can be encouraged by promoting symbiotic N fixation in legumes and elevating the association between diazotrophs and their nonleguminous host plants (Santi et al. 2013; Schweiger 2016). However, the highly specific processes of diazotrophic bacteria-plant associations do not automatically occur in plant rhizospheres since they are energetically expensive processes (Rosenblueth et al. 2018). Under such circumstances, applying appropriate microbial practices to improve plant productivity, nutrient absorption and soil health is considered to be a potential and promising strategy for sustainable agroforestry (Etesami and Maheshwari 2018).

The application of plant growth-promoting rhizobacteria (PGPR) has been extensively studied in agroforestry due to their positive effects on yield and ecosystem functioning (Gupta et al. 2021; Barbosa et al. 2022), which result in improvements in plant growth (Agarwal et al. 2019; Rosa et al. 2022), nutrient uptake (Wang et al. 2019; Wu et al. 2019; Xie et al. 2022), control of plant diseases (Chu et al. 2019; Kong et al. 2020) and maintenance of systemic resistance in plants (Ferus et al. 2019; Renoud et al. 2022). Such inoculation could enhance photosynthetic efficiency and physiological parameters related to abiotic stress tolerance (Gui et al. 2020; Sharma et al. 2021). Moreover, this method has been used with considerable success to improve the growth, physiology and productivity of eucalyptus (Kanagendran, et al. 2019; Ren et al. 2020), olive trees (Bizos et al. 2020), *Pinus pseudostrobus* and *Eysenhardtia polystachya* (Gómez-Romero et al. 2019); additionally, the reduced application of N fertilizer in combination with PGPR inoculation increased plant growth, physical parameters and nutrient uptake in comparison to that of full fertilizer applications (Huang et al. 2015; Salto et al. 2020).

The use of PGPR in combination with fertilization also significantly increased plant productivity, total chlorophyll, N uptake and soil chemical and biological fertility (Ozturk et al. 2003; Sun et al. 2020). Although some studies have employed PGPR inoculation coupled with N fertilization to investigate the growth promotion effects on plants, they have focused on commercial crops in agriculture (Swarinalakshmi et al. 2020; Tshewang et al. 2020; Kumar et al. 2021). Regardless of the yield advantages with intercropping, only a few studies have considered how the variation and combination of inputs such as PGPR inoculants and N fertilizers may influence the yield advantages and nutrient uptake of timber intercropping systems (Wang et al. 2021; Chalk et al. 2022).

With the purpose of exploring the full potential of yield advantages and resource utilization in agroforestry systems, the combined effects of N fertilizer application and PGPR inoculation in intercropping systems need to receive more attention for the development of sustainable agroforestry practices. The comparison of plant growth and physiological responses in the *E. urophylla* × *E. grandis* and *D. odorifera* intercropping systems induced by different N rates and inoculation was performed in a controlled greenhouse system, which was also a continuation of our previous research work (Xianyu, et al. 2019). Our objectives were to (1) investigate the necessity of N fertilizer at the beginning of plant development in nonlegume/legume intercropping systems; (2) compare the effect of different N levels and PGPR inoculation on plant growth, physiological variables and NUE; and (3) provide a preferable fertilization and inoculation strategy for *Eucalyptus* plantations to avoid the problems of N deficiency and lessen the ecological impacts of agroforestry.

Materials and methods

Experimental site and design

The experiment was carried out in a greenhouse located at Guangxi University, Nanning, China (108°17'30.3"E, 22°51'4.79"N). The mean annual temperature of the study area is 21.6 °C, and the annual average precipitation is 1304 mm. The selected soil physical and chemical characteristics were as follows: pH 4.65 and organic matter, total N, phosphorus (P) and potassium (K) contents of 26.52, 1.22, 0.57 and 11.85 g kg⁻¹, respectively. The greenhouse experiment was carried out to check the intercropped *E. urophylla* × *E. grandis* and *D. odorifera* growth performance in response to the varying N levels and PGPR inoculation, which included 4 levels of N fertilization (CK, no N addition; N₁, 3 g N pot⁻¹; N₂, 6 g N pot⁻¹; N₃, 12 g N pot⁻¹) and inoculation treatments: *Bacillus megaterium* (*B. megaterium*) strain DU 07 (an effective PGPR isolated from the

rhizosphere soil of *E. urophylla* × *E. grandis* provided by Huang Baoling Researcher at Guangxi University) and *Rhizobium japonicum* (*R. japonicum*) IOC 113-2 (strain provided by Shanghai Bioresource Collection Center), including an uninoculated control, with 4 replications of each treatment beginning on 16 March 2016. The intercropping systems comprised 3-month-old *E. urophylla* × *E. grandis* and 1-year-old *D. odorifera* trees at a proportion of 1:1, and N was applied as urea ($\text{CO}(\text{NH}_2)_2$). Each pot was 50 cm in diameter and 45 cm in depth and contained 25 kg of soil and 1 kg of perlite. Plants were continuously watered as needed, and pesticide and herbicide applications were performed according to local practices.

Inoculation of PGPR

Before planting, trees were inoculated with *B. megaterium* or *R. japonicum*. The strains were inoculated into liquid beef extract peptone medium in a conical flask, incubated under agitation at 28 °C for 5 d, and then diluted with sterile distilled water at a proportion of 1:2. For treatments, the washed *E. urophylla* × *E. grandis* and *D. odorifera* roots were treated by drenching with a bacterial cell suspension of *B. megaterium* DU 07 and *R. japonicum* IOC 113-2 for 30 min, respectively.

Sample collection and index measurements

To analyze the response to N treatments and PGPR inoculation in terms of growth, developmental characteristics and nutrient uptake in plants, samples were collected 6 months after planting. The plant height was measured as the distance from the soil surface to the tip of a main stem. Soil samples were collected from an area in the middle of each pot between two trees. Then, the soil samples were separated into two sections: the first section was sieved through a 2 mm net for the measurements of ammonium N (NH_4^+ -N) and nitrate N (NO_3^- -N) contents; the second section was dried and sieved with a 0.2 mm mesh for chemical analyses. The whole plants were harvested and divided into leaves, stems and roots and then oven dried at 65 °C for 48 h to a constant weight, and the dry matter (DM) yield was calculated.

N content analysis

The dried material was pulverized in a ball mill (< 0.1 mm) and mixed thoroughly; the leaves (100 mg), stems (200 mg), roots (200 mg) and soil samples (500 mg) were used for N analysis with an automatic discontinuous chemical analyzer (Smartchem200, AMS, Italy) after digestion in a mixture of concentrated H_2SO_4 and H_2O_2 .

Physiological measurements

Eight fresh and healthy leaves were randomly selected from each tree of *E. urophylla* × *E. grandis* and *D. odorifera* for physiological measurements in the morning at 180 days after fertilization. Leaf veins were removed and mixed with the remaining material and then cut into pieces. Leaf tissues (0.5 g) were incubated in a mixture of 5 ml of 95% (v/v) acetic acid and 5 ml of 80% (v/v) acetone in the dark overnight, and chlorophyll content was determined by spectrophotometry (Ultrospec 2100 pro, Biochrom, US). Nitrate reductase (NR) and catalase (CAT) activities were assayed by Coomassie brilliant blue, anthrone colorimetry, sulfonamide colorimetry and ultraviolet spectrophotometry (Ultrospec 2100 pro, Biochrom, US) (Ping and Mingjun 2007). The concentration of malondialdehyde (MDA) was determined according to the method described by Mahmoud et al. (2017).

Three healthy leaves from four sides of each plant were used for measuring photosynthesis with an LI-6400XT (LI-COR, USA) portable photosynthesis measurement system. The instantaneous net photosynthetic rate (P_n), transpiration rate (T_r), stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) of plants were determined three times at a light intensity of $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ or $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ for *E. urophylla* × *E. grandis* and $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ for *D. odorifera*; the CO_2 concentration of the incoming air was $380 \mu\text{mol m}^{-1}$ with a 500 mL min^{-1} flow rate.

Calculations

The N accumulation in the plant and NUE were calculated as follows:

$$\begin{aligned} \text{N accumulation (g plant}^{-1}) \\ = \text{dry matter (g)} \times \text{N concentration (g kg}^{-1}) \end{aligned} \quad (1)$$

$$\text{NUE\%} = \frac{\text{NA}_T - \text{NA}_C}{\text{N}_a} \times 100\% \quad (2)$$

where NA_T is the N accumulation per pot under different N treatments (g), NA_C is the N accumulation in CK and N_a is the N application rate.

Statistical analysis

The significance of N application and inoculation treatments was tested by two-factor design analysis. Differences in plant growth, physiological variables and NUE under four N levels and three inoculation treatments were assessed by one-way analysis of variance (ANOVA). Interactions between

independent variables were analyzed by two-way ANOVA. One-way and two-way ANOVA were conducted with SPSS software (SPSS Inc., Chicago, IL, USA). Pairwise comparisons between least square means were based on the adjusted Tukey test at the 0.05 probability level ($P < 0.05$) and a confidence level of 0.95. The figures were generated using Sigmaplot 10.0 software.

Results

Growth

Compared with the uninoculated control, the plant height of *E. urophylla* × *E. grandis* with the *R. japonicum* IOC 113-2 treatments was significantly increased by 28.75%, 11.40%, 24.56% and 10.33% in the CK, N₁, N₂ and N₃ treatments, respectively, while *D. odorifera* inoculated with *B. megaterium* DU 07 had significantly higher plant height than the uninoculated control by 28.15%, 5.77%, 8.20% and 11.90% (Fig. S1). The analysis of variance shows a significant effect on the interaction of N level and inoculation treatment. However, it is also worth noting that the high N treatment (N₃) decreased the plant height (Fig. S1) and DM (Fig. 1) of *D. odorifera*. The use of different concentrations of N and different inoculations resulted in significant variation in the DM allocation of both species (Fig. 1). The DM distribution of *E. urophylla* × *E. grandis* under different treatments followed this trend: stems > leaves > roots, while *D. odorifera* followed this trend: stems > roots > leaves. The comparison of all inoculation treatments showed that the allocation ratio of leaves under the *R. japonicum* IOC 113-2 treatments was

significantly higher than that under the other treatments, while the *B. megaterium* DU 07 treatments produced higher root biomass than the other treatments.

Chlorophyll pigments

The results showed that the chlorophyll a + b content (chl content) of *E. urophylla* × *E. grandis* was improved by the N₂ level with PGPR inoculation (Fig. S2). The highest chl a content (2.61 mg g⁻¹), chl b content (0.54 mg g⁻¹) and chl a + b content (3.03 mg g⁻¹) of *E. urophylla* × *E. grandis* were achieved with *R. japonicum* IOC 113-2 inoculation with N₂ application (R-N₂) and *R. japonicum* IOC 113-2 inoculation with N₃ application (R-N₃). The highest chlorophyll content of *D. odorifera* (4.01 mg g⁻¹) was achieved with the *B. megaterium* DU 07 inoculation with N₂ application (B-N₂), and the lowest (1.91 mg g⁻¹) was achieved with the uninoculated control with no N application (U-CK). Analysis of variance indicated that all attributes were influenced by N level and inoculation treatment; only the interaction of N × inoculation (I) showed very significant effects on the chl content of *E. urophylla* × *E. grandis*.

MDA contents and NR and CAT activity

With increasing N levels, the MDA content of *E. urophylla* × *E. grandis* and *D. odorifera* under all inoculation treatments varied in the order N₃ < N₂ < N₁ < CK (Table 1). The lowest MDA contents of *E. urophylla* × *E. grandis* and *D. odorifera* were achieved with the B-N₃ and R-N₃ treatments, respectively. N application caused a significant increase in the NR activity of *E. urophylla* × *E. grandis* to

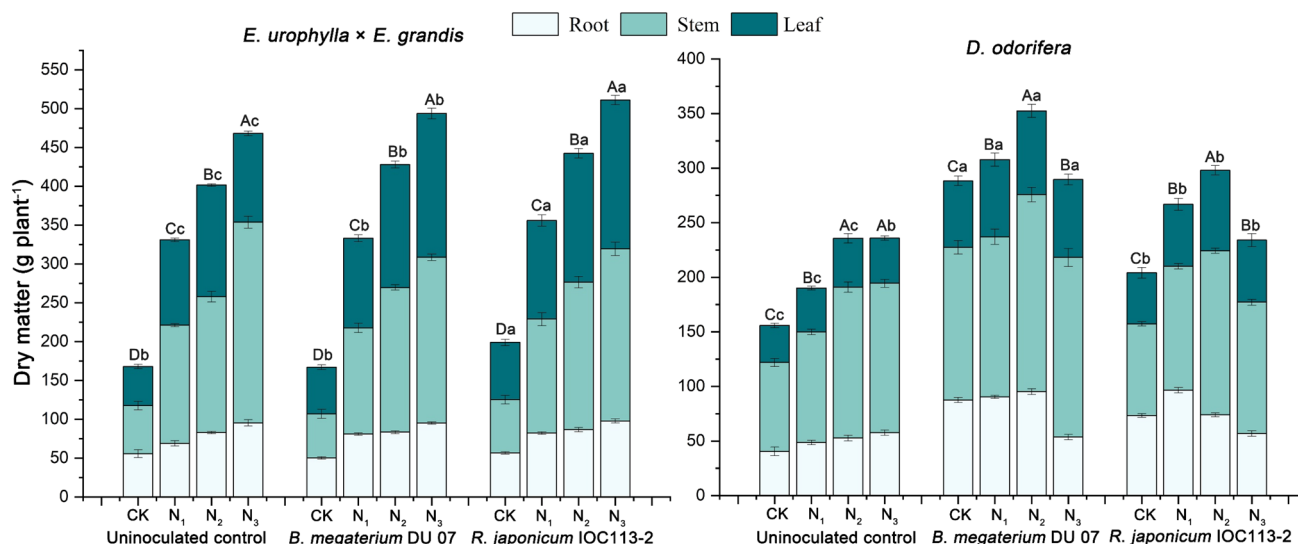


Fig. 1 The effects of N application and inoculation treatments on dry matter yield in intercropped *E. urophylla* × *E. grandis* and *D. odorifera* plants

Table 1 MDA content, and NR and CAT activities in the leaves of *E. urophylla* × *E. grandis* and *D. odorifera* under different N levels and inoculation treatments

Inoculation treatment	N level	MDA content ($\mu\text{mol}\cdot\text{g}^{-1}$)		NR activity ($\mu\text{g}\cdot\text{g}^{-1}\cdot\text{h}^{-1}$)		CAT content ($\mu\text{g}\cdot\text{g}^{-1}\cdot\text{min}^{-1}\cdot\text{FW}$)	
		<i>E. urophylla</i> × <i>E. grandis</i>	<i>D. odorifera</i>	<i>E. urophylla</i> × <i>E. grandis</i>	<i>D. odorifera</i>	<i>E. urophylla</i> × <i>E. grandis</i>	<i>D. odorifera</i>
Uninoculated control	CK	102.95 ± 2.45 Aa	71.07 ± 1.09 Aa	45.48 ± 1.81 Dc	173.45 ± 14.18 Da	46.44 ± 1.17 Da	70.68 ± 4.06 Ca
	N ₁	84.72 ± 4.02 Ba	65.66 ± 3.69 Ba	61.32 ± 1.38 Cc	205.35 ± 9.71 Ca	77.32 ± 0.33 Ca	108.80 ± 8.29 Ba
	N ₂	68.43 ± 3.76 Ca	59.45 ± 1.25 Ca	66.26 ± 2.36 Bc	233.83 ± 8.57 Ba	86.74 ± 1.32 Ba	114.64 ± 2.05 Ba
	N ₃	55.99 ± 2.76 Db	53.76 ± 1.64 Da	78.13 ± 2.65 Ac	271.66 ± 19.41 Aa	94.12 ± 0.20 Aa	143.20 ± 3.85 Aa
<i>B. megaterium</i> DU 07	CK	102.71 ± 2.47 Aa	71.33 ± 1.92 Aa	85.78 ± 2.94 Cb	114.47 ± 7.54 Ab	48.26 ± 2.90 BCa	40.44 ± 1.35 Ab
	N ₁	85.36 ± 3.57 Ba	66.07 ± 2.89 Ba	88.14 ± 3.36 Cb	104.67 ± 8.65 Bc	50.60 ± 2.38 Bb	40.60 ± 1.36 Ab
	N ₂	68.21 ± 3.04 Ca	59.40 ± 2.96 Ca	110.09 ± 3.87 Bb	111.43 ± 6.76 Abc	56.09 ± 3.30 Ab	40.39 ± 1.37 Ab
	N ₃	55.73 ± 2.25 Db	54.41 ± 2.50 Da	204.20 ± 4.28 Ab	119.80 ± 7.41 Ab	43.87 ± 2.88 Cb	37.46 ± 1.38 Ab
<i>R. japonicum</i> IOC 113-2	CK	105.89 ± 1.99 Aa	69.35 ± 2.50 Aa	93.32 ± 2.99 Da	65.14 ± 3.95 Cc	40.38 ± 2.46 Cb	40.08 ± 2.78 Ab
	N ₁	86.88 ± 2.44 Ba	63.47 ± 2.67 Ba	104.45 ± 3.59 Ca	116.28 ± 4.76 Bb	42.78 ± 2.70 BCc	41.21 ± 2.18 Ab
	N ₂	71.96 ± 2.38 Ca	56.82 ± 3.25 Ca	117.48 ± 3.51 Ba	122.13 ± 7.59 Bb	49.78 ± 2.72 Ac	42.70 ± 3.53 Ab
	N ₃	60.07 ± 1.69 Da	42.30 ± 2.14 Db	227.35 ± 9.16 Aa	134.65 ± 5.16 Ab	47.04 ± 3.59 ABb	36.96 ± 2.44 Bb
Sources of variation		F value from two-way ANOVA test					
		MDA content		NR activity		CAT	
		<i>E. urophylla</i> × <i>E. grandis</i>	<i>D. odorifera</i>	<i>E. urophylla</i> × <i>E. grandis</i>	<i>D. odorifera</i>	<i>E. urophylla</i> × <i>E. grandis</i>	<i>D. odorifera</i>
N levels (N)		614.944***	149.084***	990.394***	58.473***	148.560***	77.980***
Inoculation treatments (I)		6.806*	18.673***	1061.834***	578.263***	771.816***	1934.653***
N × I		0.196 ^{NS}	4.873*	138.195***	18.003***	91.065***	96.467***

The data in the table are the mean ± standard deviation (n=4). The different capital letters within the same columns indicate significant differences ($p < 0.05$) among the N treatments within each inoculation treatments and the different lowercase letters within the same columns indicate significant differences ($p < 0.05$) among the inoculation treatments within each N treatment. *, ** and *** represent significant effects at $P < 0.05$, $P < 0.001$ and $P < 0.0001$, respectively, NS represents nonsignificant effects. (the same applies below)

138.05% and 143.62% of the control (CK) with *B. megaterium* DU 07 and *R. japonicum* IOC 113-2 inoculation, respectively (Table 1). N fertilizer application significantly increased the NR activity of both crop species, except that the NR activity of *D. odorifera* was restrained by PGPR inoculation. Similar to that of the MDA contents, PGPR inoculation had no significant effect on increasing the CAT activity of *E. urophylla* × *E. grandis* and *D. odorifera*, and only N application improved the CAT activity under the same inoculation (Table 1).

Photosynthetic characteristics

Different inoculation treatments had different impacts on the *Pn* of *E. urophylla* × *E. grandis* and *D. odorifera*, and N application improved the *Pn* of both species (Fig. S3a). Compared to that of *B. megaterium* DU 07, the *Pn* of *E. urophylla* × *E. grandis* treated with *R. japonicum* IOC 113-2 increased significantly by 10.85%, 9.11%, 7.90% and 7.86% under CK, N₁, N₂ and N₃, respectively. The *Pn* of *D. odorifera* first increased and then decreased

with increasing N application rates and reached the highest values of $12.06 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $0.71 \mu\text{mol m}^{-2} \text{s}^{-1}$ at the N₂ level under the *B. megaterium* DU 07 and *R. japonicum* IOC 113-2 treatments, respectively. N application had significant effects on the *Tr* of both species, and only the high N level (N₃) had a certain inhibitory effect on the *Tr* of *D. odorifera* under both inoculation treatments (Fig. S3b). Thus, the highest *Tr* values of *E. urophylla* × *E. grandis* ($15.78 \mu\text{mol m}^{-2} \text{s}^{-1}$) and *D. odorifera* ($4.66 \mu\text{mol m}^{-2} \text{s}^{-1}$) were achieved with the R-N₃ and B-N₂ treatments, respectively. Compared to *B. megaterium* DU 07, *R. japonicum* IOC 113-2 increased the *Tr* of *E. urophylla* × *E. grandis* by 16.12%, 14.25%, 18.82% and 19.73% under the CK, N₁, N₂ and N₃ levels, respectively. In contrast, *B. megaterium* DU 07 increased the *Tr* of *D. odorifera* by 0.67%, 0.56%, 1.69% and 2.50% when compared with those under the *R. japonicum* IOC 113-2 treatment under CK, N₁, N₂ and N₃, respectively. The *gs* of *E. urophylla* × *E. grandis* and *D. odorifera* were not statistically different for the interaction of N level and inoculation treatment but increased with increasing N fertilizer level

and PGPR inoculation (Fig. S3c). The effect of N application and inoculation treatments on *Ci* of *E. urophylla* × *E. grandis* and *D. odorifera* was contrary to that observed for *gs*, N application and PGPR inoculation both decreased the *Ci*, except the effect of N_3 on *Ci* of *D. odorifera* was unsatisfactory (Fig. S3d).

Plant N accumulation and nitrogen use efficiency (NUE)

The results showed that a higher N accumulation of *E. urophylla* × *E. grandis* was observed under *R. japonicum* IOC 113-2 inoculation, and the response of N accumulation to N application and inoculation treatments in *D. odorifera* varied in different organs (Fig. S4). The application of R-CK, U-CK, R-CK and R-CK resulted in 13.86 g plant⁻¹, 7.16 g plant⁻¹, 16.86 g plant⁻¹ and 37.76 g plant⁻¹ N accumulation in the roots, stems, leaves and whole plants of *D. odorifera*, respectively, which were the maximum values. The interaction between N level and inoculation treatment showed very significant effects on the N accumulation of *E. urophylla* × *E. grandis* and *D. odorifera*.

The interaction between the N level and inoculation treatments significantly affected the mixed nutrient absorption rates of *E. urophylla* × *E. grandis* and *D. odorifera* (Fig. 2). The NUE under different inoculation treatments was increased by 15.2% compared to that of the uninoculated control, and the highest NUE (59.66%) was achieved at the R- N_2 level. When the same N level was applied, a higher NUE of the *E. urophylla* × *E. grandis* and *D. odorifera* intercropping system was observed under the

R. japonicum IOC 113-2 inoculation, with NUE ranging between 35.86 and 59.66%.

Principal component analysis (PCA)

The results of the pot experiment were subjected to PCA, which showed that the first factor accounted for 65.9% and 61.5% of the variation for *E. urophylla* × *E. grandis* and *D. odorifera*, respectively. The treatments with higher plant height, yield and photosynthesis are distributed throughout the first and fourth quadrants, and they correspond to the *Bacillus megaterium* and *Rhizobium japonicum* inoculation of both species (Fig. 3), with the uninoculated control clearly separated from the two groups. PCA showed that N application and inoculation modified the growth and physiological profiles of *E. urophylla* × *E. grandis* and *D. odorifera*, and *E. urophylla* × *E. grandis* exhibited more significant spatial heterogeneity based on N levels than *D. odorifera*.

Discussion

Effects of N application on plant growth, physiological characteristics and NUE

The results of this study are highly consistent with those of previous studies (Xianyu et al. 2019). The plant growth, biomass, photosynthesis and N accumulation of *D. odorifera* peaked at the N_2 level (6 g N pot⁻¹). High N addition caused significant inhibition of chl, photosynthetic NUE (Sun et al. 2018) and BNF (Bahulikar et al. 2021), as well as root development (Li et al. 2021), and decreased root absorptivity, resulting in lower leaf N acquisition (Chen et al. 2018) and consequently influencing plant growth and the photosynthetic rate (Tilba and Sinegovskaya 2012; Zhang et al. 2013). The inhibition of root growth could lead to changes in phytohormones via root–shoot communication, which in turn alters the photosynthetic apparatus and DM accumulation (Noga et al. 2019). In addition, such variation comes with the dysfunction of nutrient-recycling microbial ecosystems in soil. In contrast, higher concentrations of N promoted plant growth, NR and photosynthesis of *E. urophylla* × *E. grandis*, indicating that the rapid growth of *E. urophylla* × *E. grandis* requires a high demand for N fertilizer (Fonseca et al. 2018) through the rapid consumption of soil N. NR activity has an adverse effect on the senescence of leaves and thus is able to prolong the photosynthetic period, resulting in yield advantages (Kichey et al. 2007). The CAT activity also increased consistently with the chlorophyll content; the strengthened antioxidant activity was mostly due to the enhancement of leaf physiological characteristics. A similar argument was mentioned by Waraich et al. (2011), who noted that N input promotes antioxidant ability, protects

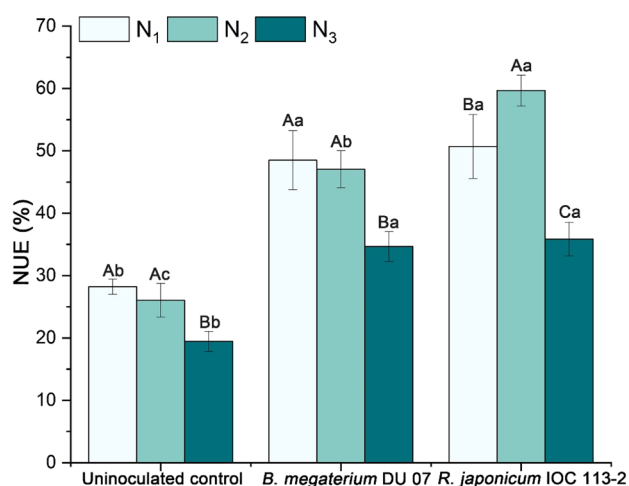


Fig. 2 Nitrogen uptake efficiency (NUE) of intercropped *E. urophylla* × *E. grandis* and *D. odorifera* under different N application and inoculation treatments

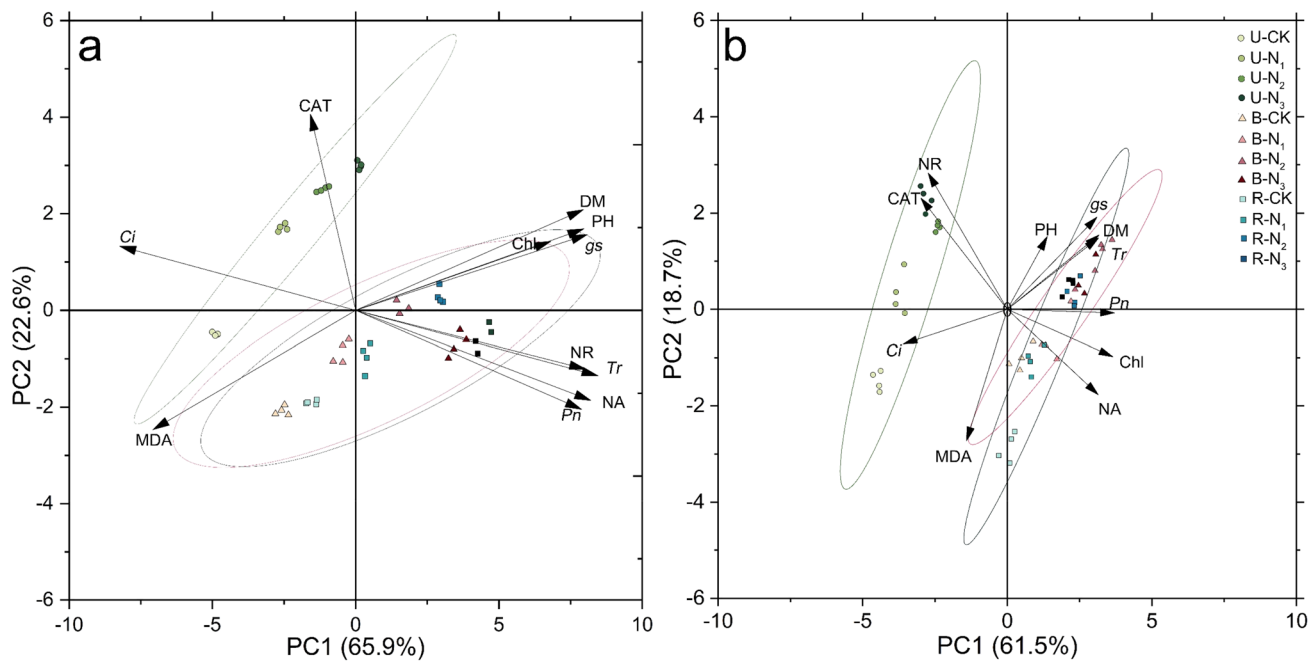


Fig. 3 Principal component analyses (PCA) of *E. urophylla* × *E. grandis* (a) and *D. odorifera* (b) under uninoculated control (U), *Bacillus megaterium* (B) and *Rhizobium japonicum* (R) inoculation treatment with CK, N₁, N₂, and N₃ application. PH plant height, DM dry

matter, Chl chlorophyll pigments, MDA malondialdehyde, NR nitrate reductase, CAT catalase, Pn photosynthetic rate, Tr transpiration rate, gs stomatal conductance, Ci intercellular CO₂ concentration, NA N accumulation

plants from photooxidative damage and delays leaf senescence. The CK (no N addition) groups in this study showed weak performance in terms of growth and physiological parameters, indicating that appropriate N levels in *E. urophylla* × *E. grandis*/*D. odorifera* intercropping are necessary for a productive system, consistent with previous studies in other intercropping systems (Du et al. 2020; Gao et al. 2020). N inputs help relieve the N depletion caused by eucalyptus (Santos et al. 2020) and intense interspecific N competition; appropriate N application benefits the production and N assimilation of legumes under relatively adverse interspecific interactions (Chen et al. 2019; Zeng et al. 2021).

Comparison of plant growth, physiological characteristics and NUE under inoculation with different PGPR

One of the alternative solutions to controlled release fertilizer is employing functional microorganisms involved in N₂ fixation abilities and NUE in intercropping systems (Muthukumar and Udaiyan 2018). Beneficial soil microbes, such as rhizobium, are able to establish mutualistic relationships between legumes and rhizobia and stimulate BNF (Sun et al. 2020) and increase the yield advantage of cereal–legume intercropping systems (Vanlauwe et al. 2019); the combined inoculation of rhizobia and PGPR also significantly increased the nodule number, biomass, nutrient uptake and

seedling quality index (Korir et al. 2017; Karthikeyan and Arunprasad 2021; Leite et al. 2022). These results have also been confirmed by Mei et al. (2021), who reported that rhizobial inoculation enhances productivity and symbiotic N₂ fixation and reduces apparent N losses in maize/faba bean intercropping. *B. megaterium* is known to play a leading role in mineral phosphate solubility, while phosphate solubility is mediated by organic acid and proton release and ultimately regulates NH₄ assimilation and, consequently, N intake increases (Bulut 2013). The *B. megaterium* used in this study was isolated from the rhizosphere soil of *E. urophylla* × *E. grandis* with strong N₂ fixation ability. In the present study, all *B. megaterium* DU 07 treatments had significant effects on the N content of *E. urophylla* × *E. grandis*, indicating that combined N application and P intake by microorganisms may affect the intake of both elements. Likewise, efficient PGPR selected from fenugreek nodules improve the growth and P content of barley–fenugreek intercrops (Toukabri et al. 2021). *D. odorifera* inoculated with *R. japonicum* IOC 113-2 showed higher plant height, biomass accumulation, photosynthesis, and NUE associated with N fertilization; however, variations in the growth and physiological parameters of *D. odorifera* in response to inoculations differed between the two inoculation treatments. In this study, inoculation with PGPR increased the nonsymbiotic N fixation of *E. urophylla* × *E. grandis* and the symbiotic N fixation of *D. odorifera*. These results may be attributed to

the high N consumption of *E. urophylla* × *E. grandis*, reducing the concentration of N in the root environment, which resulted in a better nodulation and N₂ fixation capacity in *D. odorifera*.

PGPR inoculation coupled with N application is preferable for yield and nutrient absorption

PGPR inoculation increased the plant height of both plants regardless of N application, and a higher increment was observed in CK (Fig. S1). In accordance with the high N addition causing the growth inhibition of *D. odorifera*, the potential of N fixation by PGPR can be limited or even inhibited in the presence of high N concentrations, which would prevent it from reaching its full potential (Carvalho et al. 2014). However, data from our study also indicated that PGPR inoculation along with 6 g N pot⁻¹ (N₂ level) led to the highest plant height, biomass and N content of *E. urophylla* × *E. grandis* and *D. odorifera*, reflecting the beneficial behavior of bacterial inoculation along with N fertilization. This may be because plants usually prefer the available N source in soil to maintain their own growth due to the high energy cost of N₂ fixation (plants supply bacteria with photosynthesis-derived carbon metabolites for ammonium) (Jeudy et al. 2010). A higher photosynthetic rate and NR and CAT activity were recorded in plants with *R. japonicum* IOC 113-2 inoculation than with *B. megaterium* DU 07 inoculation, illustrating that *R. japonicum* IOC 113-2 exerted stronger effects on plant physiological metabolic activity in the present study. The positive interaction of N application with *R. japonicum* IOC 113-2 inoculation contributed to the increased biomass and stronger photosynthetic capacity of *E. urophylla* × *E. grandis* and had a counteractive effect on the senescence and transpiration rate. Both inoculations inhibited the chl b content compared to the uninoculated control but enhanced the chl a/b value, which helped plants fit into different light availability across different environments (Li et al. 2018). Singh and Prasad (2012) and Chen et al. (2021) also documented that the beneficial effects of inoculation bring an increase in organic substances to soil and improve soil fertility as well as increase nutrient availability and absorptivity for plants.

Interestingly, *D. odorifera* inoculated with PGPR increased the growth and biomass of the neighboring plant more than that of itself, and a similar situation also occurred in *E. urophylla* × *E. grandis*. A possible explanation for this finding is that when *E. urophylla* × *E. grandis* is inoculated with *B. megaterium* DU 07, the phosphorus-solubilizing bacteria in the intercropping system may support the growth of *D. odorifera* by stimulating N₂ fixation, synthesizing phytohormones and enhancing the bioavailability of elements such as zinc and iron (Domínguez-Castillo et al. 2020). In another case, N₂ fixation of *D. odorifera* reduced its reliance

on soil N, and N transfer from *D. odorifera* was beneficial for *E. urophylla* × *E. grandis* when *D. odorifera* was inoculated with *R. japonicum* IOC 113-2. The parameters regarding the biomass of the aboveground and underground parts of both plants were significantly positively correlated overall (Fig. 3). This is in accordance with previous studies, which have documented that intercropping accelerated the growth and N accumulation of *E. urophylla* × *E. grandis* but restricted those of legumes (Voigtlaender et al. 2019). When *E. urophylla* × *E. grandis* was grown in the presence of legume plants, *E. urophylla* × *E. grandis* had advantages over *D. odorifera* in terms of competition for soil N, thus stimulating the BNF of *D. odorifera*. In addition, the biomass and N content of *E. urophylla* × *E. grandis* were obviously higher when grown with *D. odorifera* due to the N transfer between *D. odorifera* and *E. urophylla* × *E. grandis*. The specific allocation and efficient synthetic utilization of nutrients determine the interspecific facilitation of intercropping (Li et al. 2016). In intercropping systems, N accumulation in *E. urophylla* × *E. grandis* was derived primarily from the soil N content, while *D. odorifera* benefited from atmospheric N. As a result of the N contribution from BNF and N transfer, NUE was greatly promoted by the intercropping system but decreased with increasing N rates. Thus, the outcomes from this study support that the combined application of N fertilizer and inoculation effectively enhances plant yield and improves nutrient uptake in intercropping systems, and this approach could be considered an example of sustainable management for nutrient-limited conditions (Varinderpal et al. 2021).

Conclusions

In summary, we grew intercropped *E. urophylla* × *E. grandis* and *D. odorifera* with various N levels and PGPR inoculation. Nitrogen application effectively met the N requirements at the beginning of plant development in the intercropping system, while PGPR inoculation stimulated N uptake in soil, which could be supplemented by N application, thus jointly promoting the growth and photosynthesis of aboveground parts. *R. japonicum* IOC 113-2 inoculation combined with moderate N application resulted in greater productivity, N accumulation and nutrient absorption than those of the other combined treatments. We suggest that this combination represents an economic approach that can be used to provide optimum productivity and counteract the negative effects of excessive fertilization. It is suggested that intercropping with proper management can offer a solution to the conundrum of maximizing both land sparing and sharing; in other words, a combination of moderate N application rates and N-fixing PGPR inoculation applied in the eucalyptus and

D. odorifera intercropping system is recommended under N-limited conditions.

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Declarations

Conflicts of interest The authors declare that they have no conflicts of interest.

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