

Effect of N and P nutrition on extracellular secretion of lumichrome, riboflavin and indole acetic acid by N₂-fixing bacteria and endophytes isolated from *Psoralea* nodules

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Abstract Soils of the Cape Fynbos in South Africa are very low in nutrients, especially N and P, which affect bacterial growth and metabolism. In this study, the effect of supplying nitrate (14.8 and 59.3 mM NO₃⁻), ammonium (28.1 and 112.0 mM NH₄⁺) and phosphorus (1.4 and 5.7 mM P) to five N₂-fixing and 11 non-nodulating bacterial strains isolated from root nodules of *Psoralea* species in the Cape Fynbos was assessed. The data revealed marked variation in the secretion of lumichrome, riboflavin and IAA into culture filtrate. There was generally greater production of lumichrome, riboflavin and IAA by the N₂-fixing bacteria than those unable to nodulate *P. pinnata* and *siratro*, with much greater concentrations of lumichrome and riboflavin in culture filtrate at high P than low P. At low and high P, symbiotic strain TUT57pp produced 2.2-fold and 3.2-fold more IAA than TUT65prp and TUT33pap respectively, (two non-nodulating strains also with greater IAA production). Although ammonium nutrition has no effect on riboflavin production, it altered lumichrome concentrations in culture filtrate. While ammonium application had no effect, supplying bacterial cells with high nitrate concentration

significantly decreased cellular production of lumichrome and riboflavin, two important symbiotic signal molecules. The observed nitrate inhibition of lumichrome and riboflavin biosynthesis and release is in addition to its depressive effect on nodulation and N₂ fixation in symbiotic legumes.

Keywords Cape Fynbos · Culture filtrate · Psoraleae · Rhizobia · Nitrate inhibition of lumichrome · Ammonium · Metabolites

1 Introduction

The Cape Fynbos biome in the Western Cape Province of South Africa is unique in terms of its high proportion of indigenous and endemic plant species. The dominant families include the Ericaceae, Proteaceae, Restionaceae and Leguminosae (Fabaceae). The Fynbos is dominated by many evergreen shrubs with tiny, spiny, needle-like leaves, occurring mostly on acidic (pH 2.9–7.0), coarse-grained soils derived from heavily leached sandstones or limestones of the Cape Mountains. As a result, the Cape Fynbos soils are poor in nutrients, especially nitrogen and phosphorus (0.4–3.7 μg P g⁻¹, Cramer 2010). Members of the tribe Psoraleae belong to the family Leguminosae, and the majority are indigenous to the Cape Fynbos. The *Psoralea* species and their associated microsymbionts generally occur in a wide range of habitats with varying soil ecologies and environmental factors.

In addition to their effect on plant growth, soil factors such as pH, temperature and salinity are reported to alter the synthesis and extracellular release of metabolites such as lumichrome, riboflavin and indole acetic acid in bacteria isolated from *Psoralea* nodules (Kanu and Dakora 2009). Soil pH, salinity and temperature can also influence the

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availability of mineral nutrients in soil (Marschner 1995) as low pH tends to increase aluminium and trace element (Fe, Zn, Cu, B, Mo) toxicity in soils (Brady 1990).

The secretion of plant root exudates into low-nutrient soils can solubilize mineral nutrients such as Ca, P, Fe, Mn, Mo, Cu and Zn (Gardner et al. 1983; Marschner 1995; Dakora and Phillips 2002) for uptake by both plants and microbes. In such nutrient-poor soils, bacterial species such as rhizobia can also benefit from the solubilization of nutrient elements via the use of siderophores and components of bacterial exudates to chelate minerals (Jurkevitch et al. 1986; Chabot et al. 1996; Antoun et al. 1998). Although P is generally limiting in agricultural soils (<1.0 μM , Reisenauer 1966) and Cape Fynbos soils (0.4–3.7 $\mu\text{g Pg}^{-1}$, Cramer 2010), its nutrition is important for rhizobial growth (Keyser and Munns 1979; Chakraharti et al. 1981; Smart et al. 1984). Low P depresses rhizobial secretion of lipo-chitooligosaccharide Nod factors (McKay and Djordjevic 1993), which are important signals for nodule formation. Rhizobial cell viability is also reduced within 48 h (Leung and Bottomley 1987) under conditions of P deficiency. As a result, root-nodule bacteria tend to increase alkaline phosphatase activity in order to increase P concentrations in soils (Smart et al. 1984; Al-Niemi et al. 1997), thus leading to enhanced P availability and uptake.

As with P, N is also low in Fynbos soils. Yet it is required as a major component of the genetic material (e.g. DNA, RNA, nucleic acids, pyrimidines, etc.) in microbes, and is needed for enzyme formation. However, combined N (in the form of ammonium) can limit nodulation in legumes (Streeter 1988) through its effects on the expression of nodABC genes in symbiotic rhizobia (Dusha et al. 1989). High nitrate concentrations can also depress the levels and profile of bacterial Nod factors (McKay and Djordjevic 1993), and thus limit nodule formation in symbiotic legumes.

Three other biologically-active molecules identified from rhizobial exudates that affect plant growth include lumichrome, riboflavin and indole acetic acid (Phillips et al. 1999; Matiru and Dakora 2005a, b; Kanu et al. 2007; Kanu and Dakora 2009). Although lumichrome is commonly synthesized by microbes and plants, it is also a degradation product of riboflavin (Phillips et al. 1999; Cooper 2007). Lumichrome and riboflavin are both reported to induce root respiration, often leading to increased rhizosphere concentration of CO_2 , needed for rhizobial growth and mycorrhizal symbiosis (Becard and Piche 1989). Additionally, lumichrome is not only reported to elicit early initiation of trifoliolate leaf development and expansion of trifoliolate and unifoliolate leaves in symbiotic legumes (Matiru and Dakora 2005a), it also alters stomatal conductance when applied to the rhizosphere of monocots and dicots, an event that leads to water-saving when stomatal conductance is reduced (Matiru and Dakora 2005b). Foliar application of lumichrome has also

been shown to enhance photosynthetic rates and increase plant growth in soybean and corn (Khan et al. 2008).

Furthermore, both lumichrome and riboflavin have been implicated as quorum-sensing molecules in bacteria (Rajamani et al. 2008). The role of riboflavin as a signal molecule was also underscored by the finding that *Sinorhizobium meliloti* strains carrying extra copies of the riboflavin biosynthesis gene *rib* BA were found to release 15 % more riboflavin than wild-type, and were 55 % more efficient in alfalfa root colonization for nodule formation (Yang et al. 2002).

Although we know the effect of N and P nutrition on Nod factor production in symbiotic rhizobia (McKay and Djordjevic 1993), little information currently exists on the effects of these nutrients on the biosynthesis of other symbiotically-important metabolites. The aim of this study was to determine the effects of P and N as nitrate (NO_3^-) or ammonium (NH_4^+), on the synthesis and release of lumichrome, riboflavin and indole acetic acid by bacteria isolated from *Psoralea* root nodules.

2 Materials and methods

2.1 Bacterial strain origin and history

Sixteen bacterial strains isolated from root nodules of eight *Psoralea* species were used in this study. The origin and history of the test strains are indicated in Table 1. Due to unavailability of seed material for the eight *Psoralea* species (except *P. pinnata*), bacterial isolates were authenticated and proven to be nodule-forming bacteria using *P. pinnata* and *Macropitium atropurpureum*, a known promiscuous host (Kanu and Dakora 2012).

2.2 Preparation of bacterial growth media, extraction and quantification of lumichrome and riboflavin

Bacterial broth cultures were prepared as described by Phillips et al. (1999) for quantifying lumichrome and riboflavin released by *Sinorhizobium meliloti*. Broth culture solutions were prepared with either two phosphate (1.4 and 5.7 mM K_2HPO_4), two nitrate (14.8 and 59.3 mM KNO_3), or two ammonium concentrations (28.1 and 112.0 mM NH_4Cl). These broth solutions were autoclaved at 121 °C for 15 min, and cooled to room temperature. Each broth medium was then inoculated with the different bacterial strains isolated from *Psoralea* species and incubated at 25 °C with continuous shaking in the dark for 7 d to reach stationary phase. In all experiments, 4 replicate broth cultures were used for each strain. The extraction and quantification of lumichrome and riboflavin from culture filtrate was done using thin-layer chromatography (see Phillips

Table 1 Origin and nodulation history of strains used in this study

	Isolates	Host	Site	Nodulation ability	
				<i>P. pinnata</i>	<i>Macropodium atropurpureum</i>
	TUT71pas	<i>P. asarina</i>	KNR	+	–
	TUT57pp	<i>P. pinnata</i>	K	+	–
	TUT1pm	<i>P. monophylla</i>	KNR	–	+
	TUT18pac	<i>P. aculeata</i>	BB	±	–
	TUT23prt	<i>P. restioides</i>	K	±	+
	TUT67pl	<i>P. laxa</i>	BB	–	–
Key	TUT66pl	<i>P. laxa</i>	BB	–	–
<i>BB</i> Betty's Bay; <i>K</i> Kleinmond;	TUT65prp	<i>P. repens</i>	RNR	–	–
<i>KNR</i> Koggelberg Nature Reserve;	TUT61pp	<i>P. pinnata</i>	BB	–	–
<i>RNR</i> Rooiles Nature Reserve	TUT33pap	<i>P. aphylla</i>	BB	–	–
Due to unavailability of seed material for all <i>Psoralea</i> species (except <i>P. pinnata</i>), bacterial isolates were authenticated using <i>P. pinnata</i> and <i>Macropodium atropurpureum</i> (a known promiscuous host). +=effective nodulation; ±=ineffective nodulation, -=not nodulated; nd=not determined	TUT34pap	<i>P. aphylla</i>	BB	–	–
	TUT72pas	<i>P. asarina</i>	KNR	–	–
	TUT64prp	<i>P. repens</i>	RNR	–	–
	TUT10pm	<i>P. monophylla</i>	KNR	–	–
	TUT13pac	<i>P. aculeata</i>	KNR	–	–
	TUT22prt	<i>P. restioides</i>	K	–	–

et al. 1999; Kanu et al. 2007; Kanu and Dakora 2009). The cultures were centrifuged at 10 000×g for 10 min to pellet the bacterial cells; 5 mL of the supernatant was then passed through a C18 cartridge, rinsed three times with deionised water to remove salts, and the lumichrome and riboflavin eluted with methanol. The eluates were dried down, and then resolubilized in 30 µL methanol for thin-layer chromatography (TLC) as described by Phillips et al. (1999). All experimental procedures were conducted under low light conditions to avoid degradation of riboflavin.

The separation of lumichrome and riboflavin in culture filtrates was carried out as described by Phillips et al. (1999). The resolubilized lumichrome and riboflavin were spotted on silica-gel-coated glass plates (Alltech 0.2×100×100 mm HPTLC silica gel plates). The compounds in the lipophilic fraction were separated using chloroform or methanol or water (17.5:12.5:1.5) mixture. The TLC plates were viewed on a UV-light box (Ultra-Violet Products Ltd, Science Part, Milton Road, Cambridge, UK) and photographed with Polaroid film (Thermal paper, High density type, Kyoto, Japan).

Standards of known concentrations of riboflavin and lumichrome were also spotted, ran on the TLC plates, and photographed as described above. Once more, all operations were done under low light conditions to avoid degradation of riboflavin into lumichrome (Kanu et al. 2007). The spots indicating the positions of lumichrome and riboflavin were located under a UV lamp for both the standards and compounds extracted from the bacterial culture filtrates and marked on the TLC plates. The spots were then scraped off the plates, eluted and their absorbances measured at 444 nm for riboflavin and 249 nm for lumichrome using a

spectrophotometer (DU-64 Beckman Instruments Inc., Fullerton, Canada). For each strain, three independently grown bacterial broth cultures were used, with each assay being a replicate. For visual comparison, the three replicates of each strain were run side by side on the TLC plates. Because this method proved to be tedious, a modified version for the extraction and quantification of lumichrome and riboflavin from culture filtrate (D A Phillips, personal communication) was adopted, tested, and used for quantifying the bacterial metabolites (Kanu and Dakora 2009). The broth culture for each bacterial strain or isolate was centrifuged at 10,000×g for 10 min to pellet the bacterial cells, and the supernatant collected. Samples (5 mL) of the supernatant were passed through C18 cartridges, to trap the two metabolites out of water. The 3 mL C18 cartridges were each rinsed three times with deionised water (2 mL each time) to remove all salts and the riboflavin and lumichrome eluted with 300 µL methanol as described by Phillips et al. (1999). The absorbances of eluates were measured at 444 nm for riboflavin and 249 nm for lumichrome using a standard spectrophotometer (DU-64 Beckman Instruments Inc., Fullerton, Canada). Incubation of broth culture for riboflavin production was done in the dark, and all other experimental procedures conducted under very low light conditions in order to avoid degradation of this metabolite into lumichrome.

2.3 Bacterial count in broth cultures

In order to be able to express metabolite release on a per-cell basis, cell counts were done for only phosphorus application. The number of viable bacterial cells (CFUs) in each

broth culture was estimated using the Plate Count method (Vincent 1970) with duplicate plates per each dilution series, as described by Kanu and Dakora (2009).

2.4 Bioassay for indole acetic acid (IAA) in bacterial culture filtrates

The culture filtrates of 16 bacterial strains were assayed for IAA production in response to phosphorus (P) only. Broth culture solutions were prepared with two P concentrations (1.4, and 5.7 mM K_2HPO_4). Cell counts were however not done for the release of this metabolite. The colorimetric method of Gordon and Weber (1951) was used in this study, as described by Kanu and Dakora (2009). The cultures were grown in low light for 7 days, centrifuged at $15000 \times g$ for 10 min, and following the addition of two parts of 0.01 M $FeCl_3$ in 35 % $HClO_4$ to one part supernatant, IAA was measured colorimetrically at 530 nm after 25 min. The recorded absorbances were read off a standard curve prepared from pure indole acetic acid. Three separate assays were performed, and their average used for estimating IAA formation.

2.5 Statistical analysis

A comparison of the amounts of lumichrome and riboflavin released per cell by bacteria in response to phosphorus were statistically analyzed using one-Way ANOVA (Statistica 2007 software). One-Way analyses were also done for IAA, lumichrome and riboflavin concentrations expressed on a per-mL basis. The levels of metabolite secretion at the two concentrations of each nutrient (i.e. P and N) were also compared using 2-Way ANOVA. In all analyses, the means were separated using the Duncan Multiple Range test at different $p \leq 0.05$.

3 Results

3.1 Effects of phosphorus nutrition on bacterial secretion of lumichrome and riboflavin

A 2-Way ANOVA analysis showed that, with the five N_2 -fixing strains, greater amounts of lumichrome and riboflavin were released at high P concentration compared to low P (Table 2). A 1-way ANOVA analysis with Duncan test also revealed significant ($p \leq 0.05$) differences in the release of lumichrome and riboflavin by bacterial cells in response to P nutrition. In general, more lumichrome and riboflavin were released by the nodule-forming bacteria than those unable to nodulate *P. pinnata* and *siratro* (Table 2). The five N_2 -fixing strains also differed in their levels of extracellular lumichrome secretion, with TUT23prt releasing the most

lumichrome at low P, and TUT18pac the least. At high P, TUT23prt again secreted more lumichrome than the other four strains (Table 2). With P nutrition, a similar pattern was obtained for riboflavin concentrations in bacterial culture filtrate. Strain TUT23prt again released much more riboflavin per cell at low P than the others, while at high P TUT71pas from *P. asarina* produced the most riboflavin (Table 2).

Of the 11 non-nodulating isolates on *P. pinnata* and *siratro*, TUT67pl, TUT61pp, and TUT13pac produced the same amounts of lumichrome and riboflavin irrespective of P levels (Table 2), while strains TUT22prt, TUT66pl and TUT72pas secreted the most lumichrome and riboflavin at both low and high P concentrations (Table 2). In contrast, TUT67pl and TUT61pp produced the least amount of metabolites at the two P levels (Table 2). Except for three strains, the rest released more metabolites at high P than low P (Table 2).

3.2 Effect of phosphorus nutrition on bacterial production of indole acetic acid (IAA)

Of the five symbiotic isolates, strain TUT57pp produced the most IAA at both low and high P (Table 3). There were however no significant differences in culture filtrate concentrations of IAA between low and high P (Table 3). After TUT57pp, isolate TUT65prp (a non-nodulating strain from *P. repens*) was the second highest in IAA production at 1.4 mM P, but not 5.7 mM P (Table 3). Except for TUT33-pap and TUT10pm, which showed an increase in IAA production at high P, the remaining strains were unaffected by high P (Table 3).

3.3 Effects of mineral nitrogen (NO_3^- and NH_4^+) on bacterial production of lumichrome and riboflavin

Supplying 14.8 mM and 59.3 mM nitrate to bacterial isolates from *Psoralea* species revealed marked differences in the concentrations of lumichrome and riboflavin in culture filtrate. As shown in Table 4, lumichrome levels were decreased by high nitrate concentration for all bacterial isolates. At low nitrate (14.8 mM), the concentration of lumichrome was highest in the culture filtrate of TUT22prt (which is non-nodulating on *P. pinnata* and *siratro*) followed by symbiotic strains TUT1pm and TUT57pp, and least in strain TUT72pas (Table 4).

Relative to low nitrate, riboflavin concentration in culture filtrate was decreased by high nitrate, and quite significantly for nine strains (Table 4). At low nitrate, symbiotic strain TUT23prt and two non-nodulating isolates on *P. pinnata* and *siratro* (i.e. TUT66pl and TUT22prt) showed much greater release of riboflavin into culture filtrate when compared with the other bacterial strains (Table 4). Although the

Table 2 Effect of phosphorus nutrition on the release of lumichrome and riboflavin by N_2 -fixing rhizobial strains and bacterial endophytes isolated from *Psoralea* nodules

Isolates	Lumichrome released (ng CFU ⁻¹ × 10 ⁸)		Riboflavin released (ng CFU ⁻¹ × 10 ⁸)	
	1.4 mM P	5.7 mM P	1.4 mM P	5.7 mM P
Nodule formers				
TUT71pas	17.9±4.9cB	44.6±9.7bA	11.7±1.17cdB	100.5±63.6aA
TUT57pp	29.7±3.2bA	35.8±9.7bcA	34.2±2.94bA	34.8±11.9bcA
TUT1pm	2.7±0.7efB	26.7±4.4bcdA	2.6±0.2eA	36.5±13.1bcA
TUT18pac	2.4±0.7efA	2.7±1.5eA	3.2±0.2deA	6.78±2.6bcA
TUT23prt	57.9±1.0aB	121.2±24.9aA	50.4±6.6aA	61.7±10.7abA
Non-nodule formers				
TUT67pl	0.1±0.0fA	0.1±0.0eA	0.1±0.0eA	0.1±0.0cA
TUT66pl	11.0±2.8dA	14.0±0.9cdeA	12.6±0.2cA	24.6±3.1bcA
TUT33pap	2.6±0.7efA	12.2±1.3cdeA	3.0±0.6deA	3.0±0.4cA
TUT34pap	0.2±0.0fA	0.2±0.0eA	0.2±0.0eA	0.4±0.1cA
TUT72pas	7.0±3.4deB	28.8±0.5bcA	8.4±3.3cdeA	16.7±2.7bcA
TUT65prp	2.4±0.7efA	4.0±2.6deA	2.1±0.2eA	3.1±0.3cA
TUT64prp	0.3±0.0fA	0.5±0.0eA	0.5±0.1eA	0.9±0.0cA
TUT61pp	0.1±0.0fA	0.1±0.0eA	0.1±0.0eA	0.1±0.01cA
TUT10pm	2.8±0.35efA	4.0±0.1AdeA	2.9±0.5deA	10.4±1.6bcA
TUT13pac	0.3±0.1fA	0.3±0.0eA	0.3±0.0eA	0.3±0.0cA
TUT22prt	26.7±0.7bA	18.0±3.8cdeA	12.9±7.4cA	27.9±1.4bcA

Values (Mean±SE) followed by dissimilar letters (in lower case) within a column for each concentration are significantly different at $p \leq 0.05$. Values (Mean±SE) followed by dissimilar letters (in upper case) within a row for each metabolite are significantly different at $p \leq 0.05$

Table 3 Effect of phosphorus nutrition on the release of indole acetic acid (IAA) by N_2 -fixing rhizobial strains and bacterial endophytes isolated from *Psoralea* nodules

Isolates	IAA released (ng mL ⁻¹)	
	1.4 mM P	5.7 mM P
Nodule formers		
TUT71pas	0.2±0.0efA	1.0±0.2efA
TUT57pp	27.6±1.2aA	25.4±1.6aA
TUT1pm	1.0±0.1defA	1.4±0.2efA
TUT18pac	0.8±0.1defA	0.6±0.0efA
TUT23prt	0.6±0.0defA	2.1±0.2cdeA
Non-nodule formers		
TUT66pl	2.2±0.3cdA	3.3±0.6cdA
TUT67pl	0.6±0.0defA	0.3±0.0eA
TUT33pap	0.4±0.0efB	7.9±0.4bA
TUT34pap	1.9±0.3cdeA	1.7±0.5defA
TUT72pas	0.4±0.2efA	0.5±0.0efA
TUT65prp	12.4±1.1bA	1.0±0.1efB
TUT64prp	0.5±0.0defA	0.4±0.0eA
TUT61pp	2.9±0.2cA	3.2±0.1cdA
TUT10pm	0.2±0.0efB	3.7±0.3cA
TUT13pac	0.7±0.0defA	1.1±0.1efA
TUT22prt	0.2±0.0fA	1.9±0.4defA

Values (Mean±SE) followed by dissimilar letters (in lower case) within a column for each concentration are significantly different at $p \leq 0.05$. Values (Mean±SE) followed by dissimilar letters (in upper case) within a row for IAA metabolite are significantly different at $p \leq 0.05$

riboflavin produced by TUT23prt dropped from 14.4 ng mL⁻¹ at low nitrate to 7.2 ng mL⁻¹ at high nitrate, this strain together with TUT67pl and TUT34pap were still leading producers of riboflavin at high nitrate concentration (Table 4). Isolates TUT10pm and TUT13pac showed the least production of riboflavin at high nitrate, just as they were among the least lumichrome producers at high nitrate (Table 4).

Supplying 28.1 mM and 112.0 mM ammonium had no effect on the synthesis and release of riboflavin. However, lumichrome production responded to ammonium nutrition. At 28.1 mM ammonium, the concentration of lumichrome was high in the culture filtrates of symbiotic strains TUT23prt and TUT57pp as well as in non-nodulating strains TUT67pl, TUT72pas and TUT33pap, but low in strains TUT64prp and TUT1pm (Table 5). At high ammonium supply, isolate TUT23prt together with TUT33pap again produced more lumichrome than the other strains (Table 5).

4 Discussion

In this study, N and P were supplied to five N_2 -fixing and 11 non-nodulating bacterial strains isolated from root nodules of *Psoralea* species in order to assess their effect on the synthesis and release of three metabolites, lumichrome, riboflavin and IAA. The data showed marked variation in the secretion of the three metabolites in response to mineral N and P nutrition irrespective of whether they were

Table 4 Effect of nitrate nutrition on the release of lumichrome and riboflavin by N_2 -fixing rhizobial strains and bacterial endophytes isolated from *Psoralea* nodules

Isolates	Lumichrome released (ng CFU ⁻¹ × 10 ⁸)		Riboflavin released (ng CFU ⁻¹ × 10 ⁸)	
	14.8 mM NO ₃ ⁻	59.3 mM NO ₃ ⁻	14.8 mM NO ₃ ⁻	59.3 mM NO ₃ ⁻
Nodule formers				
TUT71pas	3.4±0.7bcdeA	1.1±0.1abcdB	7.0±0.7eA	5.6±0.8abcA
TUT57pp	4.3±0.3abcA	1.1±0.3abcdB	7.8±0.3cdeA	6.3±1.2abA
TUT1pm	4.7±0.2abA	1.3±0.1abB	8.2±1.1cdeA	5.8±0.09abcA
TUT18pac	3.7±0.2bcdA	0.6±0.1deB	11.3±0.7bA	6.6±0.7abB
TUT23prt	2.6±0.2defA	1.2±0.2abcB	14.4±1.1aA	7.2±0.92aB
Non-nodule formers				
TUT67pl	2.0±0.1efA	0.6±0.1deB	7.3±0.1deA	7.3±0.5aA
TUT66pl	2.1±0.1efA	0.3±0.0eB	14.7±2.1aA	3.8±0.9cdeB
TUT33pap	2.2±0.9efA	1.1±0.3abcdB	7.2±0.5deA	4.7±0.2bcdB
TUT34pap	3.9±0.5bcdA	0.9±0.1bcdE	7.7±0.2cdeA	6.9±0.2aA
TUT72pas	0.6±0.1gA	0.58±0.1deA	7.3±0.2deA	5.7±0.1abcA
TUT65prp	2.6±0.6defA	0.8±0.0bcdeB	8.0±0.6cdeA	3.0±0.5deB
TUT64prp	4.0±0.4bcdA	1.5±0.3aB	7.3±0.2deA	5.7±0.4abcA
TUT61pp	3.0±0.2cdefA	0.6±0.0deB	8.3±0.4cdeA	5.7±0.6abcB
TUT10pm	1.6±0.1fgA	0.4±0.2eB	9.8±0.8bcdA	2.7±0.1eB
TUT13pac	2.0±0.5fgA	0.4±0.0eB	10.4±0.7bcA	2.9±0.3eB
TUT22prt	5.3±0.5aA	0.7±0.3cdeB	14.1±0.7aA	6.3±0.6abB

Values (Mean±SE) followed by dissimilar letters (in lower case) within a column for each concentration are significantly different at $p \leq 0.05$. Values (M±SE) followed by dissimilar letters (in upper case) within a row for each metabolite are significantly different at $p \leq 0.05$

expressed on a per-cell or per-mL basis (Tables 2–5). Whether at low or high P, there was generally greater production of lumichrome and riboflavin by the N_2 -fixing bacteria than those unable to nodulate *P. pinnata* and *siratro* (Table 2). Furthermore, with the five N_2 -fixing strains, greater amounts

of lumichrome and riboflavin were produced at high P compared to low P concentration (Table 2), a finding consistent with the reportedly greater secretion of lipo-chito-oligosaccharide Nod factors at high P than low P in *Rhizobium leguminosarum* bv. *trifolii* (McKay and Djordjevic 1993).

Table 5 Effect of ammonium nutrition on the release of lumichrome and riboflavin by N_2 -fixing rhizobial strains and bacterial endophytes isolated from *Psoralea* nodules

Isolates	Lumichrome released (ng CFU ⁻¹ × 10 ⁸)		Riboflavin released (ng CFU ⁻¹ × 10 ⁸)	
	28.1 mM NH ₄ ⁺	112.0 mM NH ₄ ⁺	28.1 mM NH ₄ ⁺	112.0 mM NH ₄ ⁺
Nodule formers				
TUT71pas	2.6±0.4abA	3.1±0.3bA	8.5±4.1 aA	5.5±1.4 aA
TUT57pp	3.7±0.3aA	2.4±0.5bA	4.6±1.4 aA	5.9±1.1 aA
TUT1pm	1.9±0.4bA	0.7±0.1cA	5.8±1.3 aA	5.0±2.4 aA
TUT18pac	3.2±0.4abA	2.2±0.3bA	4.9±0.7 aA	6.3±1.5 aA
TUT23prt	3.9±0.3aA	4.6±1.3aA	6.7±0.9 aA	8.6±3.0 aA
Non-nodule formers				
TUT67pl	3.8±0.6aA	2.9±0.3bA	7.6±1.1aA	6.0±0.4 aA
TUT66pl	3.3±0.6abA	3.1±0.3bA	6.0±1.9 aA	5.8±0.6 aA
TUT33pap	3.5±0.6aA	3.4±0.5aA	7.8±1.9 aA	3.9±0.7 aA
TUT34pap	2.7±0.5abA	2.5±0.6bA	7.0±2.1 aA	6.2±0.7 aA
TUT72pas	3.8±0.4aA	2.3±0.4bA	6.2±0.2 aA	5.3±0.6 aA
TUT65prp	3.2±0.1abA	2.2±0.3bA	8.9±0.8 aA	5.2±0.5 aA
TUT64prp	1.8±0.2bA	2.4±0.4bA	4.3±0.5 aA	7.3±0.9 aA
TUT61pp	2.8±0.6abA	2.3±0.3bA	4.0±0.8 aA	4.2±0.5 aA
TUT10pm	3.3±0.4abA	1.8±0.8bcA	5.7±0.9 aA	4.7±0.8 aA
TUT13pac	2.9±0.4abA	2.9±0.6bA	4.4±0.6 aA	6.8±0.6 aA
TUT22prt	3.1±0.2abA	2.04±0.2bcA	5.9±0.4 aA	6.1±1.4 aA

Values (Mean±SE) followed by dissimilar letters (in lower case) within a column for each concentration are significantly different at $p \leq 0.05$. Values (Mean±SE) followed by dissimilar letters (in upper case) within a row for each metabolite are significantly different at $p \leq 0.05$

Although root-nodule bacteria and other rhizosphere microbes such as *Pseudomonas fluorescens* and *Azospirillum brazelense* are known to produce riboflavin and other vitamins for their own growth (West and Wilson 1938; Yang et al. 2002; Dakora et al. 2005), the greater secretion of lumichrome and riboflavin induced by P nutrition in N₂-fixing strains but not in non-nodulating bacteria could be symbiosis related. This argument is supported by the finding that *Sinorhizobium meliloti* strains carrying extra copies of riboflavin biosynthesis gene *rib BA* released 15 % more riboflavin than the wild-type, and were 55 % more efficient in alfalfa root colonization (Yang et al. 2002), a first step towards enhanced nodulation. Their role in symbiotic establishment and promotion of plant growth (Matiru and Dakora 2005a, b) is further supported by the fact that the production and release of lumichrome and riboflavin was found to be greater in symbiotic microbes than other bacterial types (Phillips et al. 1999). Furthermore, the extracellular concentrations of riboflavin in exudates of some bacteria can be 8 times greater than that of internal cellular concentration (Phillips et al. 1999), suggesting that the two molecules have evolved directly or indirectly as rhizosphere signals influencing the outcomes of plant/bacterial interactions (Dakora et al. 2005).

The synthesis and release of IAA, another important bacterial morphogen involved in early symbiotic establishment (Badenoch-Jones et al. 1983; Hirsch 1992; Mathesius et al. 1998; Lambrecht et al. 2000), was affected by P nutrition. Although the differences in IAA exudation between symbiotic strains and non-nodulating isolates were not as marked as lumichrome and riboflavin production when P was supplied, it is important to note that, at 1.4 mM P, N₂-fixing strain TUT57pp produced 27.6 ng mL⁻¹ IAA relative to 12.4 ng mL⁻¹ IAA by the non-nodulating strain TUT65prp (the second highest producer). Similarly, at 5.7 mM P, symbiotic TUT57pp produced 25.4 ng mL⁻¹ IAA relative to 7.9 ng mL⁻¹ IAA by the non-nodulating strain TUT33pap (the second highest producer at that concentration). The greater production of IAA under both low and high P conditions by this N₂-fixing strain TUT57pp indicates its adaptation to environments with differing P status. Shokri and Emtiazi (2010) however found no differences ($p \leq 0.05$) in IAA production between symbiotic and non-symbiotic bacteria and concluded that there was no relationship between IAA production and nodulating ability of the microsymbionts.

Supplying 59.3 mM nitrate to five N₂-fixing and 11 non-nodulating bacterial isolates generally resulted in significantly decreased levels of lumichrome and riboflavin in culture filtrate, indicating an inhibitory effect of nitrate on the synthesis and extracellular release of the two molecules. McKay and Djordjevic (1993) also found a substantial decrease in Nod factor production, following nitrate supply to *Rhizobium leguminosarum* bv. *trifolii*. Because lumichrome

and riboflavin are symbiotic signals, their decreased concentrations in the rhizosphere with nitrate supply is likely to reduce lumichrome-induced root production of CO₂ (Phillips et al. 1999), needed for rhizobial growth (Lowe and Evans 1962). The ultimate result would be impaired nodulation and N₂ fixation in legumes from poor growth of bacterial populations. Although in this study ammonium nutrition altered bacterial production of lumichrome, it had no effect on riboflavin (Table 5), a finding consistent with the lack of response of Nod factor secretion to ammonium supply (McKay and Djordjevic 1993). However, both nitrate and ammonium are known for their inhibition of legume nodule formation and nitrogenase activity in N₂-fixing diazotrophs (Streeter 1988).

In conclusion, P application significantly increased lumichrome, riboflavin and IAA production in symbiotic strains over their non-nodulating counterparts, with much greater concentrations of lumichrome and riboflavin being produced and released into culture filtrate at high P than low P. While ammonium application had no effect, supplying bacterial cells with high nitrate concentration decreased the synthesis and release of lumichrome and riboflavin, two important symbiotic signal molecules. This is in addition to the already known negative effects of nitrate and ammonium nutrition on N₂-fixing diazotrophs and the legume symbiosis, which include ammonium inhibition of common *nodABC* gene expression (Wang and Stacey 1990; Patriarca et al. 2002), nitrate and ammonium suppression of legume nodule formation and nitrogenase activity in root nodules (Streeter 1988), and nitrate inhibition of *nod*-gene-inducing isoflavone biosynthesis in symbiotic legumes (Cho and Harper 1991).

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