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REGULAR ARTICLE

Effects of endophytic *Streptomyces* and mineral nitrogen on Lucerne (*Medicago sativa* L.) growth and its symbiosis with rhizobia

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Abstract
Background and Aims The effects of three endophytic *Streptomyces* on plant growth and the symbiosis of Lucerne and its rhizobial partner were examined in the presence of three levels of soil nitrogen.
Methods Three *Streptomyces* strains, LuP30 and LuP47B isolated from the roots of Lucerne (*Medicago sativa* L.) and EN23 isolated from roots of wheat (*Triticum aestivum* L.) were added as spores to Lucerne seeds (with and without *Sinorhizobium meliloti* RRI 128) at three levels of applied NH_4NO_3: 3, 25 and 50 mg/kg of soil.
Results Plant growth increased with the addition of the actinobacteria strains alone from 19 % to 33 %. Co-inoculation of LuP30 with rhizobia strain RRI 128 produced the largest increase in shoot weight (46 %) of Lucerne plants growing in soil with 25 mg/kg NH_4NO_3. Co-inoculation with each of the actinobacteria with the rhizobia increased the number of nodules by more than 100 % compared with RRI128 alone, 4 weeks after rhizobial inoculation. A labelled ^{15}N experiment showed co-inoculation with rhizobia and LuP30 or LuP47B enhanced N_2-fixation 47 % and 72 %, respectively.
Conclusions The actinobacteria significantly improved plant growth and N_2-fixation when applied with the rhizobia strain RRI 128 to Lucerne plants growing in soil supplied with 25 mg/kg NH_4NO_3.

Keywords Alfalfa · Streptomycete actinomycetes · Nitrogen · Endophytes · Nodulation

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Introduction

The establishment of nodulation and nitrogen fixation in legumes is affected by different soil factors such as pH, availability of nitrogen, calcium, and interactions with rhizosphere microorganisms (Solans et al. 2009). Although N is required in significant amounts (~30 kg/t DM) to optimise plant growth (Pattison et al. 2010), larger amounts (>50 kg/ha) of available N in soil generally have a negative effect on legume nodulation and nitrogen fixation (Streeter 1985, 1988; Puiatti and Sodek 1999; Lucínski et al. 2002; Herridge et al. 2005), with many stages of the symbiotic process inhibited (Dogra and Dudeja 1993; Dusha 2002; Mortier et al. 2012). However, a low, static concentration of ammonium (0.5 mM NH_4^+ or 7 mg/kg N) can result in stimulation of nodulation in pea (*Pisum sativum*) (Gudden and Vessey 1997; Fei and Vessey 2003).

Plant mutation and breeding approaches have been used to produce nitrate tolerant soybean (Carroll et al. 1985a, 1985b; Reid et al. 2011), but the results of that work have not been widely adopted due to other agronomic shortcomings. Manipulation of the soil microbiology and in particular the application of bacteria,

including actinobacteria, is another strategy that is currently being investigated (Li and Alexander 1988, 1990; Le et al. 2014). The potential for actinobacteria to modify legume symbioses was raised by (Sharma et al. 2005) who found a high abundance of actinobacteria in the rhizosphere of three different legumes, faba beans (*Vicia faba* L., cv. Scirocco), peas (*Pisum sativum* L., cv. Duel) and white lupin (*Lupinus albus* L., cv. Amiga). Recent studies on the endophytic actinobacteria show that they also colonise and live within the root nodules of *Lupinus angustifolius* (Trujillo et al. 2006; Trujillo et al. 2007; Trujillo et al. 2010) and nodules of pea (*Pisum sativum* L.) (Carro et al. 2007; García et al. 2010). A number of actinobacteria have been shown to improve the growth, nodulation and N_2-fixation of soybean (Gregor et al. 2003; Soe et al. 2012; Nimmnoi et al. 2014), pea (*Pisum sativum* L.) (Tokala et al. 2002), bean (*Phaseolus vulgaris* L.) (El-Tarabily et al. 2008), chickpea (Gopalakrishnan et al. 2015b) and Lucerne (*Medicago sativa* L.) (Samac et al. 2003; Martínez-Hidalgo et al. 2014). With regard to overcoming N inhibition of the symbiosis, Solans et al. (2009) and Solans et al. (2015) found that some actinobacteria isolated from the root nodule surface of *Discaria trinervis* promoted the nodulation (42 % increase in nodule mass) of Lucerne and enhanced the plant biomass and nodulation of *Lotus tenuis* inoculated with *Mesorhizobium loti* when grown in the presence of high levels of soil N (7 mM NH_4NO_3). This study examines the application of three endophytic actinobacteria (*Streptomyces* spp.) isolated from wheat and Lucerne on the nodulation and growth of Lucerne plants growing in potting media supplied with three levels of NH_4NO_3.

Materials and methods

Actinobacteria, rhizobia and Lucerne seeds

Seeds of the Lucerne cultivar 'SARDI Ten' and *Sinorhizobium meliloti* strain RRI 128 (the strain used to produce commercial rhizobia inoculants for Lucerne in Australia) were used in all experiments. The three actinobacteria used were *Streptomyces* sp. EN23, isolated from healthy wheat roots, and *Str.* spp. LuP30 and LuP47B isolated from healthy Lucerne roots. All three actinobacteria had been shown to increase the growth of Lucerne nodulated with the *S. meliloti* strain RRI 128 in previous studies, under low soil N conditions (3 mg NH_4NO_3/kg soil) (Le et al. 2014).

Growth of actinobacteria and rhizobia

Actinobacteria were grown on Mannitol Soya Flour agar (MS) plates and incubated at 27 °C for 7–14 days to obtain spores. The colony-forming units (CFU) of the actinobacterial spores were enumerated as described by the Miles and Misra (1938) technique. *Sinorhizobium meliloti* strain RRI 128 was streaked onto Yeast Mannitol Agar (YMA) and a single pure colony selected, transferred and maintained on YMA plates or slants for use in the experiments. A standard curve of the relationship between *S. meliloti* CFU (Miles and Misra 1938) and OD_{600nm} was determined and used to standardise the titre of rhizobial cells in each experiment to approximately 10^8 cells/ml.

Plant establishment

Seeds of Lucerne cultivar 'SARDI Ten' were surface sterilised following the protocol of Coombs and Franco (2003). Seeds were removed from the final water rinse after 10 min, and placed in a laminar flow cabinet overnight to dry. Actinobacteria were applied to the surface of the sterilised Lucerne seeds as a suspension in 0.3 % xanthan gum to provide 10^8 CFU/g seed and the seeds sown (10 seeds/pot) into a pasteurised (by autoclaving) potting mix (1 kg/pot of 50:50 by volume of sand:vermiculite) contained in 1.25 l self-watering pots (Décor Watermatic™). Seedlings were thinned to 4/pot after 6 days, before inoculation with rhizobia strain RRI 128.

Experimental treatments and design

The factorial experiment comprised (i) 3 strains of actinobacteria, (ii) ± inoculation with *S. meliloti* RRI 128 and (iii) 3 levels of soil NH_4NO_3 (3, 25 and 50 mg/kg) which were added with 200 mL of McKnight's solution on planting day (McKnight 1949). Milli-Q (MQ) water was added to the pots as required in the following weeks. Plants designated to rhizobia treatments were inoculated with an aqueous suspension (1 ml per plant containing 10^8 CFU) of rhizobia 6 days after sowing. Each treatment was replicated eight times. Pots were arranged in a completely randomised design in a greenhouse and moved weekly

to reduce spatial effects. Four pots (replicates) of each treatment were harvested at 4 and 7 weeks after inoculation. The experiment was carried out in the winter of 2013 (approx. Mean 10 h/14 h day/night, temp. range 5-20 °C).

Experiment 2

The ^{15}N experiment to determine the relative contribution of soil and fixed N sources at different stages of plant growth was carried out as described in the above experiment except that only two strains, LuP30 or LuP47B, were applied separately to the seeds prior to inoculation with rhizobia strain RRI 128. A control treatment (rhizobia strain RRI 128 only) was also included. Each treatment was replicated 12 times. Plants were supplied with 25 mg $^{15}NH_4{}^{15}NO_3$ (98 %, Cambridge Isotope Laboratories, Inc.), per kg of soil mix with 200 mL of McKnight's solution (McKnight 1949) on planting day. The experiment was carried out in the winter of 2014 (approx. Mean 10 h/14 h day/night temp. Range 5-20 °C). Four pots (replicates) of each treatment were harvested at 1, 3 and 5 weeks after inoculation with rhizobia strain RRI 128.

Parameters measured

After harvest the height of shoots, dry weight of shoots and roots, and the number and dry weight of nodules per plant were measured. The height of the shoots was measured as the distance from the base of the dominant shoot at sand level to the youngest leaf, and an average calculated for the four plants in each pot. Shoots and roots were dried separately in an oven at 60 °C for 48 h to constant weight and average dry weights calculated for the four plants in each pot. The number and total mass of nodules per plant were determined from sampling 2 plants per pot. Nodules in the top 5 cm of roots and large nodules (diameter ≥ 1 mm) were counted. There were four pots for each treatment.

Elemental and isotopic composition analyses

Four shoots per pot and four pots for each treatment were sampled. The whole shoots were dried at 60 °C for 48 h and ground using a clean mortar and pestle to about 1 mm in size and sent to the Waite Analytical Services, University of Adelaide, Australia to determine N content (%), P, K and other macro and trace elements.

In experiment 2: the N content of the shoots and roots was analysed by mass spectrometry to determine the proportions of plant N derived from the atmosphere and soil. All four shoots and roots from each pot were ground separately to about 1 mm in size. About 3 mg of each sample were analysed for total N and ^{15} N on an isotope ratio mass spectrometer (Sercon, Crewe, Cheshire, UK).

The At% of ^{15}N has been calculated taking account of the ^{15}N in air with the formula below

$$\mathrm{At\%} = [100 \times (0.3663/(100-0.3663)) \times ((\mathrm{deltaN}/1000) + 1)]/1 + [(0.3663/(100-0.3663)) \times ((\mathrm{deltaN}/100) + 1)].$$

Statistical analysis

All data were assessed for normality and log transformed prior to ANOVA where necessary. Data was analysed using ANOVA and significant differences between means determined using Duncan's Multiple Range test. All references to significance in the text imply statistical significance at $P < 0.05$, unless otherwise stated.

Results

Effect of actinobacteria on Lucerne growth in the absence of rhizobia

In the absence of the rhizobial partner, the three actinobacteria EN23, LuP30 and LuP47B significantly increased the shoot dry weights by between 19 % to 33 % in the 25 mg/kg NH_4NO_3 treatment; and 23 % to 24 % in the 50 mg/kg NH_4NO_3 treatment (Table 1). In contrast, root dry weight was reduced significantly by LuP30 (−21 %) and LuP47B (−29 %) at 25 mg and EN23 (−18 %) at 50 mg NH_4NO_3 after 7 weeks. The actinobacteria significantly increased the shoot:root ratio at 25 mg/kg and 50 mg/kg NH_4NO_3 while there were no effects at 3 mg/kg NH_4NO_3. Treatment with all 3 actinobacteria significantly reduced the iron (~50 %), copper (~40 %) and manganese (~30 %) concentrations in the plant shoots (Table 2). None of the actinobacteria increased shoot N concentration. However, total shoot N was increased (+28 %) by LuP47B at 50 mg NH_4NO_3 compared with the control treatment (Table 2).

Effect of actinobacteria on Lucerne growth when applied with rhizobia

The application of actinobacteria with rhizobia significantly increased the shoot weight of Lucerne (inoculated with rhizobia) at the 4 (data not shown) and 7 week harvests at 25 mg/kg NH_4NO_3 by 19 % to 36 % and 27 % to 46 %, respectively. The effect of LuP30 was consistent across N levels (7 week harvest). LuP47B had no effect on shoot weight at 50 mg/kg NH_4NO_3, while EN23 had no effect at 3 mg/kg NH_4NO_3 (Table 1). Effects of the actinobacteria on root weight were variable. Whilst EN23 and LuP30 increased the root dry weight in the 25 mg/kg NH_4NO_3 treatment at 7 weeks, LuP30 reduced the root dry weight at 3 and 50 mg/kg NH_4NO_3 (Table 1). LuP30 and LuP47B both significantly increased the shoot: root ratio at 3 mg/kg NH_4NO_3 but only LuP47B showed an increase at 25 mg/kg NH_4NO_3 while LuP30 increased the ratio at 50 mg/kg NH_4NO_3 at 7 weeks.

Application of actinobacteria affected the nutrient status of the Lucerne shoots (Table 2). In general, the application of actinobacteria with rhizobia increased or had no effect, on the accumulated amount of several nutrients (N, P, Fe, Bo Cu and Mn). Other nutrients (e.g. K) were never affected (data not shown). At 4 weeks after inoculation with rhizobia the *Streptomyces* spp. EN23, LuP30 and LuP47B significantly increased the total N (μg), P (μg), Bo (μg) and Mn (μg) at 25 mg NH_4NO_3. For example, co-inoculation with either EN23, LuP30 or LuP47B and rhizobia RRI 128 increased total N in shoot by 26, 50 and 27 % respectively though the *Streptomyces* spp. did not increase significantly the N concentration of shoots (Table 2). Moreover, where actinobacteria were applied, the numbers of positive/neutral/negative responses (across all NH_4NO_3 treatments at the 7 week harvest) were for N (4/4/1), for P (6/3/0), for Fe (4/4/1), for Bo (2/7/0), for Cu (2/6/1) and for Mn (0/9/0). Of the 18 positive responses, 7 were attributable to inoculation with LuP47B, 6 to LuP30 and 5 to EN23.

Nutrient accumulation is the product of shoot weight and nutrient concentration. In general, nutrient concentration in the Lucerne shoots was not affected or decreased, with the application of actinobacteria. The numbers of positive/neutral/negative responses (across all NH_4NO_3 treatments at the 7 week harvest) were for N (0/6/3), for P (1/7/1), for Fe (1/6/2), for Bo (0/5/4), for Cu (1/5/3) and for Mn (1/6/2). Of the 15 negative responses, 5 were attributable to each of the actinobacteria. Three of the 4 positive responses (not P) were attributable to EN23 in the 3 mg/kg NH_4NO_3 treatment.

Impacts on nodulation

The number of nodules per plant increased with the increasing N supply. This was associated with improved plant growth at the higher N levels. However, number of nodules per mg of root decreased significantly from 2.6 to 1.2 to 0.9 nodules in the 3, 25 and 50 mg/kg NH_4NO_3treatments at 4 weeks, respectively, but

Table 1 Effect of endophytic actinobacteria (*Streptomyces* spp. EN23, LuP30 and LuP47B individually or in combination with *S.meliloti* strain RRI 128) and soil N on Lucerne shoot and root weight at 7 weeks after inoculation

Treatment	Shoot weight DM (mg/plant)			Root weight DM (mg/plant)			Shoot:root		
	3 mg NH_4NO_3	25 mg NH_4NO_3	50 mg NH_4NO_3	3 mg NH_4NO_3	25 mg NH_4NO_3	50 mg NH_4NO_3	3 mg NH_4NO_3	25 mg NH_4NO_3	50 mg NH_4NO_3
Inoculation with *Streptomyces*									
Not inoculated	20^{a}	79^{a}	173^{a}	17.8^{a}	56^{b}	91^{b}	1.1^{a}	1.4^{a}	1.9^{a}
+ EN23	20^{a}	105^{b}	216^{b}	17.3^{a}	49^{ab}	75^{a}	1.2^{a}	2.1^{b}	2.9^{b}
+ LuP30	20^{a}	94^{b}	216^{b}	19.6^{a}	44^{a}	84^{ab}	1.0^{a}	2.2^{b}	2.6^{b}
+ LuP47B	20^{a}	103^{b}	212^{b}	17.1^{a}	40^{a}	79^{ab}	1.2^{a}	2.6^{b}	2.7^{b}
Inoculation with *Sinorhizobium* and *Streptomyces*									
RRI 128 only	229^{a}	268^{a}	348^{a}	129^{c}	100^{a}	169^{b}	1.8^{a}	2.7^{a}	2.1^{a}
RRI 128 + EN23	238^{a}	340^{bc}	394^{c}	114^{bc}	151^{b}	172^{b}	2.1^{ab}	2.3^{a}	2.5^{a}
RRI 128 + LuP30	283^{b}	392^{d}	379^{bc}	76^{a}	141^{b}	142^{a}	3.7^{bc}	2.8^{a}	2.7^{b}
RRI 128 + LuP47B	284^{b}	365^{c}	331^{a}	101^{ab}	109^{a}	163^{b}	2.8^{b}	3.5^{b}	2.0^{a}

Different letters in the same column indicate means are significantly different ($P < 0.05$)

Table 2 Effect of inoculation with *Streptomyces* spp. (individually or in combination with RRI 128) on the concentration (mg/kg) and total accumulation of nutrients (mg or μg) in Lucerne shoots at 4 and 7 weeks after inoculation

Treatment	NH_4NO_3 level (mg/kg)	[N] (mg/kg)	Total N (μg)	[P] (mg/kg)	Total P (μg)	[Fe] (mg/kg)	Total Fe (μg)	[Bo] (mg/kg)	Total Bo (μg)	[Cu] (mg/kg)	Total Cu (μg)	[Mn] (mg/kg)	Total Mn (μg)
Inoculation with *Streptomyces* (7 week harvest)													
Not inoculated	25	9220	793	1845	148	102	8.2	70	5.6	18.0	1.4	260	20.8
EN23	25	8000	834	1807	189	59*	6.2*	62*	6.5	10.8*	1.1*	170*	17.7
LuP30	25	8620	823	1883	180	66*	6.3	68	6.5	11.5*	1.1*	182*	17.4
LuP47B	25	8200	845	1810	187	62*	6.5	67	6.9	10.6*	1.1*	175*	18.2
Not inoculated	50	9530	1647	1077	186	123	21.2	63	10.9	14.3	2.5	134	23.1
EN23	50	9110	1984	1027	224	63*	13.6*	48*	10.4	9.09*	2.0*	97**	21.0
LuP30	50	9250	2028	1050	229	64*	14.1*	52*	11.5	9.04*	2.0*	108*	23.7
LuP47B	50	9910	2110*	1040	222	39**	8.3**	53*	11.3	8.68*	1.8*	99*	21.2
Inoculation with *Sinorhizobium* and *Streptomyces* (4 week harvest)													
RRI 128 only	25	30,900	2037	2000	132	135	8.8	60	3.9	12	0.8	97	6.4
RRI 128 + EN23	25	30,600	2568*	2133	180*	127	10.6	59	5.0*	11	0.9	116	9.7*
RRI 128 + LuP30	25	33,640	3049**	1877	170*	202*	18.2**	57	5.2*	13	1.2**	119*	10.8**
RRI 128 + LuP47B	25	32,540	2595*	2250*	180*	145	11.6*	59	4.7*	12	1.0*	108	8.6*
RRI 128 only	50	24,950	2178	1523	134	149	13.0	59	5.1	12	1.1	103	9.0
RRI 128 + EN23	50	28,230	2494	1730**	152*	137	12.1	58	5.1	12	1.1	95	8.4
RRI 128 + LuP30	50	26,910	2612*	1630*	158*	198*	19.3*	59	5.7	11	1.1	109	10.6
RRI 128 + LuP47B	50	27,560	2477	1733**	156*	184	16.5	63	5.7	13	1.2	105	9.4
Inoculation with *Sinorhizobium* and *Streptomyces* (7 week harvest)													
RRI 128 only	3	22,640	5198	823	189	130	29.8	45	10.4	8.7	2.0	98	22.4
RRI 128 + EN23	3	24,270	5799	883	211	267*	63.8**	45	10.9	12.8*	3.0*	113*	26.9
RRI 128 + LuP30	3	21,990	6530*	767	220*	167	48.1*	40*	11.5	7.7	2.2	86	24.7
RRI 128 + LuP47B	3	20,590	7295**	743	223*	202	60.4**	41*	12.2	7.7	2.3	90	27.3
RRI 128 only	25	19,410	5194	690	185	147	39.2	49	13.0	8.0	2.1	88	23.6
RRI 128 + EN23	25	16,270**	5516	637*	216	101	34.3	40*	13.4	6.4*	2.2	69*	23.4
RRI 128 + LuP30	25	17,740	6952*	687	269*	119	46.8	41*	15.9	6.9	2.7	71*	27.9
RRI 128 + LuP47B	25	16,550**	6059	723	264*	182	67.2*	48	17.4*	8.6	3.2*	90	32.9
RRI 128 only	50	25,120	8440	887	298	182	61.2	44	14.9	9.6	3.2	103	34.7
RRI 128 + EN23	50	25,400	9934*	977	384*	156	60.9	46	18.0*	9.0	3.4	97	38.1
RRI 128 + LuP30	50	21,490	8191	983*	375*	113*	43.3	39	14.8	7.4*	2.8	93	35.3
RRI 128 + LuP47B	50	21,350*	7016*	913	298	117*	38.5*	40	13.1	7.8*	2.5*	102	33.4

Asterisks indicate significant differences from control treatment at the same soil N level at $P < 0.05$ (*) or $P < 0.01$(**)

they were similar at the 7 week harvest (Table 3). The promptness of nodulation (proportion of nodules on the top 5 cm of the root) was also decreased from 14.5/22, 12.3/24 and 2.5/40 in the 3, 25 and 50 mg/kg NH_4NO_3 treatments (Table 4).

The addition of actinobacteria increased nodule number per plant at each level of N compared to the rhizobia treatment alone, after 4 weeks plant growth. For example, at 25 mg/kg NH_4NO_3 all three actinobacteria increased the number of nodules per plant by more than 75 %. EN23 and LuP30 also increased the number of nodules at 50 mg/kg NH_4NO_3 (Table 3). By 7 weeks, the increased nodulation associated with the actinobacteria only persisted in the 3 mg/kg NH_4NO_3 treatment for LuP30 and LuP47B. Inoculation with actinobacteria and N level also affected the position of nodules on the root system (Table 4). The number of nodules in the top 5 cm of the root was reduced by

Table 3 Effect of actinobacteria and soil N on nodule number per plant and number of nodules per mg of root at 4 and 7 weeks after inoculation with RRI 128

Treatment	Number of nodules						Number of nodules per mg of root					
	4 weeks			7 weeks			4 weeks			7 weeks		
	3 mg NH_4NO_3	25 mg NH_4NO_3	50 mg NH_4NO_3	3 mg NH_4NO_3	25 mg NH_4NO_3	50 mg NH_4NO_3	3 mg NH_4NO_3	25 mg NH_4NO_3	50 mg NH_4NO_3	3 mg NH_4NO_3	25 mg NH_4NO_3	50 mg NH_4NO_3
RRI 128 only	22^{a}	24^{a}	40^{a}	27^{a}	47^{a}	69^{b}	2.6^{a}	1.2^{a}	0.9^{a}	0.2^{a}	0.5^{b}	0.4^{ab}
RRI 128 + EN23	21^{a}	53^{b}	52^{b}	30^{ab}	51^{a}	56^{a}	2.5^{a}	1.6^{b}	1.2^{b}	0.3^{a}	0.3^{a}	0.3^{a}
RRI 128 + LuP30	31^{b}	51^{b}	48^{b}	41^{c}	57^{a}	66^{ab}	2.6^{a}	1.4^{ab}	1.1^{b}	0.5^{b}	0.4^{ab}	0.5^{b}
RRI 128 + LuP47B	24^{a}	42^{b}	36^{a}	37^{bc}	49^{a}	71^{b}	2.5^{a}	1.6^{b}	0.9^{a}	0.4^{b}	0.4^{ab}	0.4^{ab}

Different letters in the same column indicate means are significantly different ($P < 0.05$)

increasing the amount of N, but the effect was moderated by the application of EN23 or LuP30 especially in the 3 mg/kg NH_4NO_3 treatment after 4 weeks. Nodule size was also increased with actinobacterial treatment (Table 4). There were several examples where total nodule mass/plant increased with the addition of actinobacteria (Fig. 1). At the 4 week harvest with 25 mg/kg NH_4NO_3, strains EN23, LuP30 and LuP4B increased nodule mass per plant by 125 %, 140 % and 78 % respectively. At 50 mg/kg NH_4NO_3, strains EN23 and LuP30 increased nodule mass/plant by 63 % and 42 % respectively. At the 7 week harvest, EN23 at 50 mg/kg NH_4NO_3 was the only treatment that increased nodule mass.

^{15}N experiment

The addition of LuP30 and LuP47B generally increased shoot weight and number of nodules per plant in the 3 and 5 week harvests, confirming previous measures of their efficacy (Table 5). None of the actinobacteria increased total plant N (roots and shoots) at 3 weeks, although there was a small increase in the amount of ^{15}N for the LuP47B treatment (Fig. 2). At the 5 week harvest, the addition of LuP30 or LuP47B increased total plant N by 40 % and 60 %, respectively. This was mostly due to greater accumulation of ^{14}N (derived from N_2-fixation) which was increased by LuP30 or LuP47B by 47 % and 72 %, respectively. The total N and ^{14}N were distributed more in the shoots (about 70 %) and roots (~30 %) at the 5 weeks after inoculation with rhizobia (Table 6). LuP47B significantly increased the total amount of ^{15}N in the shoots at 3 weeks and roots at the 3 and 5 weeks harvest (Table 6).

Discussion

In the absence of rhizobia, the three strains of *Streptomyces* increased Lucerne shoot weight by 19 % to 33 % compared to untreated plants after 7 weeks growth at the two higher N levels, indicating some of the benefits were independent of the rhizobial symbiosis. Non-symbiotic benefits of actinobacteria have been reported for non-legumes such as cucumber (El-Tarabily et al. 2010) and been attributed to the solubilisation of phosphate, and in wheat to the control of soil borne disease (Franco et al. 2007; Hamdali et al. 2008). LuP30 and LuP47B have been shown to produce siderophores and

Table 4 Distribution of total nodules in the top 5 cm of roots and large nodules (diameter ≥ 1 mm) due to treatment with actinobacteria at different rates of NH_4NO_3. Data from harvests at 4 and 7 weeks after inoculation with RRI 128 ($n = 4$)

Treatment	Nodules in the top 5 cm of roots						Large nodules (diameter ≥ 1 mm)					
	4 weeks			7 weeks			4 weeks			7 weeks		
	3 mg	25 mg	50 mg	3 mg	25 mg	50 mg	3 mg	25 mg	50 mg	3 mg	25 mg	50 mg
RRI 128 only	14.5^{b}	12.3^{ab}	2.5^{a}	14.3^{bc}	11.8^{a}	9.8^{b}	16.3^{a}	17.4^{a}	13.5^{a}	15.5^{a}	18.9^{a}	16.6^{a}
RRI 128 + EN23	10.0^{a}	15.5^{b}	4.3^{a}	16.0^{c}	13.1^{a}	5.5^{a}	14.1^{a}	24.8^{b}	16.4^{ab}	15.0^{a}	21.8^{b}	21.5^{b}
RRI 128 + LuP30	12.0^{a}	15.5^{b}	5.0^{a}	11.3^{a}	13.5^{a}	6.0^{a}	16.0^{a}	23.8^{b}	19.6^{b}	15.1^{a}	19.6^{ab}	14.8^{a}
RRI 128 + LuP47B	14.8^{b}	9.5^{a}	7.0^{a}	13.3^{ab}	15.3^{a}	9.6^{b}	17.1^{a}	21.1^{ab}	12.0^{a}	17.8^{a}	21.1^{ab}	22.0^{b}

Different letters in the same column indicate means are significantly different ($P < 0.05$)

IAA, and LuP30 has the ability to solubilise phosphate (Le et al. 2015). Nimmnoi et al. (2014) found that the siderophore producing actinobacteria increased the iron content of plants. Martínez-Hidalgo et al. (2014) conducted a similar experiment with Lucerne plants and concluded that *Micromonospora* spp. enhanced nitrogen uptake efficiency and/or improved nitrogen availability in soil. EN23, LuP30 and LuP47B have characteristics that are consistent with those reported in other actinobacteria that improved the growth of non-legumes, and so some non-symbiotic benefits are likely to contribute to their efficacy.

Plant growth and nodule number/plant were increased by the level of applied N and microbial treatment. Increases associated with N application were attributable to increased plant vigour. Although the larger plants had more nodules overall, they were observed to have delayed nodulation and fewer nodules/mg of root. N levels in the experiment were not high enough to reduce nodulation to the extent reported by others (Heichel and Vance 1979), but were still sufficient to delay nodulation and provide the opportunity for improvement with actinobacterial inoculation.

When LuP30 and LuP47B were co-inoculated with rhizobia strain RRI 128, Lucerne growth and nodulation were increased. Most improvements in nodulation were measured in the first 4 weeks of plant growth, indicating early involvement of the actinobacteria in the regulation of nodulation. The isotope experiment provides evidence that actinobacteria strain LuP47B increased nitrogen (^{15}N) uptake from the soil, but this benefit was relatively small. By comparison, increases in early nodulation (number and density) were large. Martínez-Hidalgo et al. (2014) proposed that *Micromonospora* play a role as rhizobia helper bacteria (RHB) and since the actinobacteria were coated onto Lucerne seeds prior

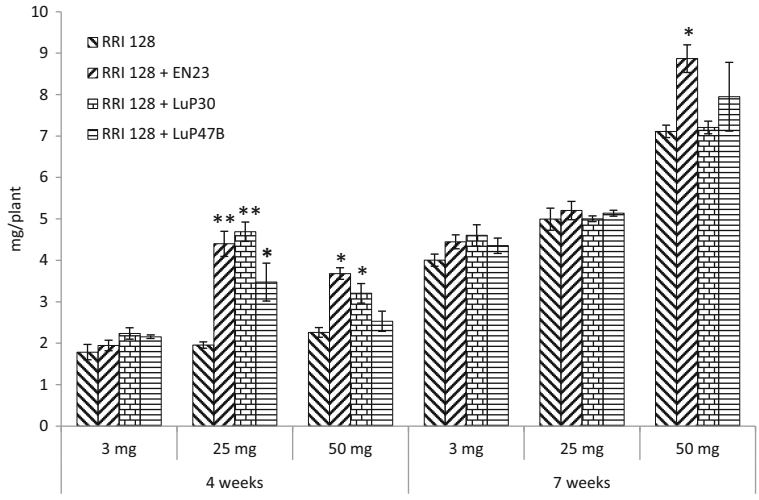

Fig. 1 Effect of actinobacteria and soil N (NH_4NO_3) on Lucerne nodule weight (mg DM/plant) at 4 and 7 weeks after inoculation ($n = 4$). Asterisks indicate significant differences from RRI 128 treatment at $P < 0.05$ (*) or $P < 0.01$(**)

Table 5 Effect of endophytic actinobacteria (*Streptomyces* spp. EN23, LuP30 and LuP47B in combination with *S.meliloti* strain RRI 128) on Lucerne shoot and root weight at 1, 3 and 5 weeks at 25 mg $^{15}NH_4{}^{15}NO_3$ per kg sand and vermiculite after inoculation ($n = 4$)

Treatment	Shoot:root			Shoot weight (mg DM/plant)			Root weight (mg DM/plant)			Number of nodules (#/plant)		
	1w	3w	5w	1w	3w	5w	1w	3w	5w	1w	3w	5w
Inoculation with *Sinorhizobium* and *Streptomyces*												
RRI 128 only	2.5	1.9	1.9	5.8	25.9	87.6	2.3	13.7	45.9	0	18.6	30.0
RRI 128 + LuP30	2.3	2.2	2.1	5.8	33.7*	110.2*	2.5	15.3	52.8	0	24.6*	48.6*
RRI 128 + LuP47B	2.3	2.1	1.9	5.4	34.3*	110.7*	2.3	16.1	59.6*	0	22.8*	37.2

Asterisks indicate significant differences from control treatment at the same soil N level at $P < 0.05$ (*) or $P < 0.01$(**)

to inoculation with rhizobia, they could have primed the plant root for rhizobial infection and nodulation (Tokala et al. 2002). Measures of earlier root hair curling and reduced time to nodule appearance (Le, unpublished data) suggest that the effects of the actinobacteria occur very early and therefore may be associated with rhizobial colonisation, plant/rhizobia signalling or *Nod* factor synthesis. More generalised PGPR effects associated with the production of phyto-hormones such as auxins, cytokinins, and gibberellins could also be important (Glloudemans and Bisseling 1989) because of their effects on root growth and plant development, even if they are not specifically involved in the complex signalling that regulates nodulation. Changed hormone levels resulting from the application of actinobacteria have been postulated to be significant to increased nodulation of both Lucerne (Solans et al. 2015) and chickpea (Gopalakrishnan et al. 2015a; Gopalakrishnan et al. 2015b).The large body of work on hyper- and super-nodulating soybeans that are tolerant of soil nitrate has shown nodulation in the presence of nitrate is strongly controlled by a feed-back signal from the plant shoot (Li et al. 2009; Ferguson et al. 2010; Reid et al. 2011). Changes to the concentration of nutrients in the Lucerne shoots when inoculated with actinobacteria suggests they had some effect on the broader plant physiology and so modification of the feedback loop for control of nodulation needs to be considered. Nimmnoi et al. (2014) proposed that co-inoculation with both actinobacteria and rhizobia increased plant growth by improving the uptake of nutrients and El-Tarabily et al. (2008) also found similar results with actinobacteria in mungbean (*Phaseolus vulgaris* L.). Although changes to the nutritional status of the Lucerne plants were measured, it is unlikely that nutrient levels per se were critical to the improvements in plant growth, because with the exception of N, they should not have been limiting to growth.

Equalisation of nodule number by the latter harvest at 25 and 50 mg NH_4NO_3 was probably the result of

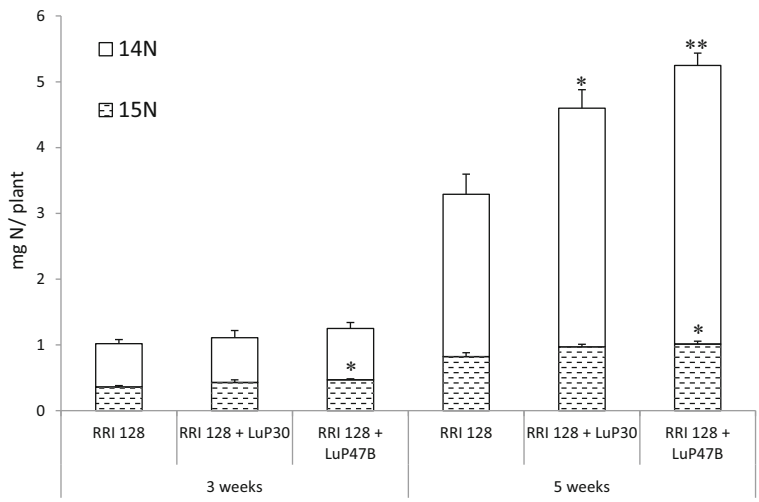

Fig. 2 Accumulation of N (^{14}N and ^{15}N) in each Lucerne plant (shoot and root) inoculated with rhizobia and actinobacteria. Asterisks indicate significant differences at $P < 0.05$ (*) or $P < 0.01$(**)

Table 6 Effect of inoculation treatment on the distribution of accumulated N (^{14} N and ^{15}N) between plant roots and shoots

Treatment	Shoot (mg)						Root (mg)					
	3 weeks			5 weeks			3 weeks			5 weeks		
	^{15}N	^{14}N	Total N	^{15}N	^{14}N	Total N	^{15}N	^{14}N	Total N	^{15}N	^{14}N	Total N
RRI 128 only	0.26^{a}	0.49^{a}	0.75^{a}	0.54^{a}	1.73^{a}	2.28^{a}	0.10^{a}	0.18^{a}	0.28^{a}	0.28^{a}	0.74^{a}	1.01^{a}
RRI 128 + LuP30	0.31^{ab}	0.50^{a}	0.82^{a}	0.66^{a}	2.56^{b}	3.22^{b}	0.12^{ab}	0.18^{a}	0.30^{a}	0.31^{ab}	1.06^{ab}	1.38^{ab}
RRI 128 + LuP47B	0.34^{b}	0.59^{a}	0.93^{a}	0.65^{a}	2.96^{b}	3.61^{b}	0.13^{b}	0.20^{a}	0.32^{a}	0.36^{b}	1.28^{b}	1.64^{b}

Different letters in the same column indicate means are significantly different ($P < 0.05$)

compensatory nodulation in the control treatment (RRI 128) as soil N declined through the course of the experiment. Whilst it shows that the effects of the actinobacteria may be quite transient, their potential value is not diminished, because early plant vigour is critically important to successful Lucerne establishment and its subsequent persistence and production.

Conclusion

This study confirms that the selected actinobacteria can improve the growth, nodulation and nitrogen fixation of Lucerne plants inoculated with rhizobia strain RRI 128. Both non-symbiotic and symbiotic effects are likely to be contributing to the improvement. The actinobacteria were most effective at 25 mg NH_4NO_3/kg of soil and in the first four weeks of growth, indicating their use would be best targeted to encourage early plant vigour and aid pasture establishment in soils with low/moderate N levels. The efficacy and durability of the actinobacteria in field soils containing complex micro-flora including different strains of rhizobia is still to be determined, as are the mechanisms of action that promote early nodulation and the enhancement of the plant rhizobial symbiosis. Overall, actinobacteria strain LuP30 showed most promise for use as a co-inoculant with rhizobia based on its benefits to nodulation and plant growth. Strain LuP30 provided the most consistent benefit to nodulation and Lucerne growth across the three N levels in this study.

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References

Carro L, Pukall R, Spoker C, Kroppenstedt RM, Trujillo ME (2007) *Micromonospora cremea* sp. nov. and *Micromonospora zamorensis* sp. nov., isolated from the rhizosphere of *Pisum sativum*. Int J Syst Evol Microbiol 62: 2971–2977

Carroll BJ, McNeil DL, Gresshoff PM (1985a) Isolation and properties of soybean [*Glycine max* (L.) merr.] mutants that nodulate in the presence of high nitrate concentrations. Proc Natl Acad Sci U S A 82:4162–4166

Carroll BJ, McNeil DL, Gresshoff PM (1985b) A supernodulation and nitrate-tolerant symbiotic (nst) soybean mutant. Plant Physiol 78:34–40

Coombs TJ, Franco CMM (2003) Isolation and identification of actinobacteria from surface-sterilized wheat roots. Appl Environ Microbiol 69:5603–5608

Dogra RC, Dudeja SS (1993) Fertiliser N and nitrogen fixation in legume-*Rhizobium* symbiosis. Ann Biol 9:149–164

Dusha I (2002) Nitrogen control of bacterial signal production in *Rhizobium meliloti*- alfalfa symbiosis. Indian J Exp Biol 40: 981–988

El-Tarabily KA, Hardy GESJ, Sivasithamparam K (2010) Performance of three endophytic actinomycetes in relation to plant growth promotion and biological control of *Pythium aphanidermatum*, a pathogen of cucumber under commercial field production conditions in the United Arab Emirates. Eur J Plant Pathol 128:527–539

El-Tarabily KA, Nassar HA, Sivasithamparam K (2008) Promotion of growth of bean (*Phaseolus vulgaris* L.) in a calcareous soil by a phosphate-solubilizing, rhizosphere-competent isolate of *Micromonospora endolithica*. Appl Soil Ecol 39:161–171

Fei H, Vessey JK (2003) Involvement of cytokinin in the stimulation of nodulation by low concentrations of ammonium in *Pisum sativum* L. Physiol Plant 118:447–455

Ferguson BJ, Indrasumunar A, Hayashi S, Lin M, Lin Y, Reid DE, Gresshoff PM (2010) Molecular analysis of legume nodule development and autoregulation. J Integr Plant Biol 52:61–76

Franco CMM, Michelsen P, Percy N, Conn V, Listiana E, Moll S, Loria R, Coombs TJ (2007) Actinobacterial endophytes for improved crop performance. Australas Plant Pathol 36:524–531

García LC, Martínez-Molina E, Trujillo ME (2010) *Micromonospora pisi* sp. nov., isolated from root nodules of *Pisum sativum*. Int J Syst Evol Microbiol 60:331–337

Glloudemans T, Bisseling T (1989) Plant gene expression in early stages of rhizobium legume symbiosis. Plant Sci 65:1–13

Gopalakrishnan S, Srinivas V, Alekhya G, Prakash B, Kudapa H, Rathore A, Varshney RK (2015a) The extent of grain yield and plant growth enhancement by plant growth-promoting broad-spectrum *Streptomyces* sp. in chickpea. Springerplus 4:1–10

Gopalakrishnan S, Srinivas V, Alekhya G, Prakash B, Kudapa H, Varshney RK (2015b) Evaluation of *Streptomyces* sp. obtained from herbal vermicompost for broad spectrum of plant growth-promoting activities in chickpea. Org Agric 5:123–133

Gregor AK, Klubek B, Varsa EC (2003) Identification and use of actinomycetes for enhanced nodulation of soybean co-inoculated with *Bradyrhizobium japonicum*. Can J Microbiol 49:483–491

Gudden RH, Vessey JK (1997) The stimulating effect of ammonium on nodulation in *Pisum sativum* L. is not long lived once ammonium supply is discontinued. Plant Soil 195:195–205

Hamdali H, Hafidi M, Virolle MJ, Ouhdouch Y (2008) Rock phosphate-solubilizing actinomycetes: screening for plant growth-promoting activities. World J Microbiol Biotechnol 24:2565–2575

Heichel RWF, Vance CP (1979) Nitrate-N and *Rhizobium* strain roles in alfalfa seedling nodulation and growth. Crop Sci 19: 512–518

Herridge DF, Robertson MJ, Cocks B, Peoples MB, Holland JF, Heuke L (2005) Low nodulation and nitrogen fixation of mungbean reduce biomass and grain yields. Aust J Exp Agric 45:269–277

Le HX, Franco CMM, Ballard RA (2014) Isolation and characterisation of endophytic actinobacteria and their effect on the early growth and nodulation of Lucerne (*Medicago sativa* L.). In: VVSR Gupta, M Unkovich, BN Kaiser (eds) The 17th Australian nitrogen fixation conference, Adelaide: Australia.

Le HX, Franco CMM, Ballard RA, Drew EA (2015) Isolation and characterisation of endophytic actinobacteria and their effect on the early growth and nodulation of Lucerne (*Medicago sativa* L.). Plant Soil. doi:10.1007/s11104-015-2652-9

Li D-M, Alexander M (1988) Co-inoculation with antibiotic-producing bacteria to increase colonization and nodulation by rhizobia. Plant Soil 108:211–219

Li D-M, Alexander M (1990) Factors affecting co-inoculation with antibiotic-producing bacteria to enhance rhizobial colonization and nodulation. Plant Soil 129:195–201

Li D, Kinkema M, Gresshoff PM (2009) Autoregulation of nodulation (AON) in *Pisum sativum* (pea) involves signalling events associated with both nodule primordia development and nitrogen fixation. J Plant Physiol 166:955–967

Lucínski R, Polcyn W, Ratajczak L (2002) Nitrate reduction and nitrogen fixation in symbiotic association *Rhizobium*-legumes. Acta Biochim Pol 49:537–546

Martínez-Hidalgo P, Galindo-Villardón P, Trujillo ME, Igual JM, Martínez-Molina E (2014) Micromonospora from nitrogen fixing nodules of alfalfa (*Medicago sativa* L.). a new promising plant probiotic bacteria. Sci Rep 4:1–9

McKnight T (1949) Efficiency of isolates of *Rhizobium* in the cowpea (*Vigna unguiculata*) group, with proposed additions to this group. Qd J Agric Sci 6:61–76

Miles AA, Misra SS (1938) The estimation of the bactericidal power of the blood. J Hyg 38:732–749

Mortier V, Holsters M, Goormatchtig S (2012) Never too many? How legumes control nodule numbers. Plant Cell Environ 35:245–258

Nimmnoi P, Pongsilp N, Lumyong S (2014) Co-inoculation of soybean (*Glycine max*) with actinomycetes and *Bradyrhizobium japonicum* enhances plant growth, nitrogenase activity and plant growth. J Plant Nutr 37:432–446

Pattison T, Moody P, Bagshaw J (2010) Soil health for vegetable production in Australia. The State of Queensland, Department of Employment, Economic Developement and Innovation

Puiatti M, Sodek L (1999) Ethylene and the inhibition of nodulation and nodule activity by nitrate in soybean. Rev Bras Fisiol Veg 11:169–174

Reid DE, Ferguson BJ, Hayashi S, Lin Y, Gresshoff PM (2011) Molecular mechanisms controlling legume autoregulation of nodulation. Ann Bot 108:789–795

Samac DA, Willert AM, Mcbride MJ, Kinkel LL (2003) Effect of antibiotic-proucing *Streptomyces* on nodulation and leaf spot in alfalfa. Appl Soil Ecol 22:55–66

Sharma S, Aneja MK, Mayer J, Munch JC, Schloter M (2005) Characterization of bacterial community structure in rhizosphere soil of grain legumes. Microb Ecol 49:407–415

Soe KM, Bhromsiri A, Karladee D, Yamakawa T (2012) Effects of endophytic actinomycetes and *Bradyrhizobium japonicum* strains on growth, nodulation, nitrogen fixation and seed weight of different soybean varieties. Soil Sci Plant Nutr 58:319–325

Solans M, Ruiz OA, Wall LG (2015) Effect of actinobacteria on *Lotus tenuis-Mesorhizobium loti* symbiosis: preliminary study. Symbiosis 65:33–37

Solans M, Vobis G, Wall LG (2009) Saprophytic actinomycetes promote nodulation in *Medicago sativa-Sinorhizobium meliloti* symbiosis in the presence of high nitrogen. J Plant Growth Regul 28:106–114

Streeter JG (1985) Nitrate inhibition of legume nodule growth and activity. Plant Physiol 77:325–328

Streeter JG (1988) Inhibition of legume nodule formation and N_2 fixation by nitrate. Crit Rev Plant Sci 7:1–23

Tokala RK, Strap JL, Jung CM, Crawford DL, Salove MH, Deobald LA, Bailey JF, Morra MJ (2002) Novel plant-microbe rhizosphere interaction involving *Streptomyces lydicus* WYEC108 and the pea plant (*Pisum sativum*). Appl Environ Microbiol 68:2161–2171

Trujillo ME, Alonso-Vega P, Rodríguez R, Carro L, Cerda E, Alonso P, Martínez-Molina E (2010) The genus *Micromonospora* is widespread in legume root nodules: the example of *Lupinus angustifolius*. ISME J 4:1265–1281

Trujillo ME, Kroppenstedt RM, Fernández-Molonero C, Schumann P, Martínez-Molina E (2007) *Micromonospora lupini* sp. nov. and *Micromonospora saelicesensis* sp. nov., isolated from root nodules of lupinus angustifolius. Int J Syst Evol Microbiol 57:2799–2804

Trujillo ME, Kroppenstedt RM, Schumann P, Martínez-Molina E (2006) *Kribbella lupini* sp. nov., isolated from the roots of *Lupinus angustifolius*. Int J Syst Evol Microbiol 56:407–411