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Legume inoculant application methods: effects on nodulation patterns, nitrogen fixation, crop growth and yield in narrow-leaf lupin and faba bean

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Abstract

Aims

Liquid and granular rhizobial inoculants have some practical advantages for delivering rhizobial inoculants to legume crops in terms of ease-of-use and in separating rhizobia from potentially harmful seed-applied pesticides. The aim of this research was to determine whether inoculant application methodologies altered the patterns of nodulation on roots, inputs of symbiotic nitrogen (N_2) fixation, the accumulation of legume shoot dry matter (DM), grain yield, and grain nitrogen (N).

Methods

Eight field experiments were established at four different locations in south-eastern Australia to quantify the response of lupin (*Lupinus angustifolius* L.) and faba bean (*Vicia faba* L.) to three inoculant application methods (on-seed application as a peat slurry, in-furrow peat inoculant delivered as a liquid suspension at seeding, in-furrow peat granules delivered at seeding) compared with uninoculated treatments. N_2 fixation was assessed using the ^{15}N natural abundance method and canola was included as a non-legume reference.

Results

Inoculation significantly improved crown nodulation, from 0.05 to 13 nodules plant^{-1} in lupin at two sites and from 0.17 to 21.3 nodules plant^{-1} in faba bean at three sites. Nodulation responses were decreased for faba bean treatments at sites with low pH, and for both lupin and faba bean at sites where soils contained large populations of naturally-occurring rhizobia.

Inoculation increased grain yield from 0.48 to 1.94 t ha^{-1} faba bean relative to uninoculated treatments; N_2 fixation increased by 175 kg N ha^{-1} in lupin at one site and by 46 to 280 kg N ha^{-1} in faba bean at two sites. The different inoculant application methods led to minor differences in crown and lateral root nodulation patterns but only impacted N_2 fixation and grain yield at one site with faba bean, where peat slurry treatments had 186 to 195 kg N ha^{-1} more N_2 fixation than other treatments and peat slurry and granules provided 0.8 to 1.0 t ha^{-1} more grain yield than liquid inoculants.

Conclusion

On-seed application of peat slurry always provided the best nodulation, grain yield and N_2 fixation. Small changes in nodulation patterns using in-furrow inoculants only resulted in reduced N_2 fixation in faba bean at one site. At that site faba bean grain yield was reduced by 1.0 t ha^{-1} in liquid inoculant treatments, compared with on-seed peat slurry treatments.

Keywords: ^{15}N natural abundance, faba bean, inoculation, lupin, nodulation, rhizobia

Introduction

Symbiotic nitrogen (N_2) fixation is a key biological process in legumes that supports plant growth and the production of high-protein seed and forage (Peoples et al. 2009). The establishment of legume root nodules to provide a functioning N_2-fixing symbiosis requires that an adequate population of appropriate root nodule bacteria (rhizobia) be already established in the soil or that rhizobia be supplied at sowing by inoculation (Roughley et al. 1993; Peoples et al. 2009; Denton et al. 2013; Thilakarathna and Raizada 2017). Inoculants traditionally used are peat-based and are applied to seed in slurry form, often with adhesive to enhance attachment. This procedure ensures that inoculant rhizobia are delivered into the soil at a point in the immediate vicinity of the emerging root (Brockwell et al. 1995, Deaker et al. 2004). However, the procedure is somewhat time-consuming, especially with larger-seeded legumes, and it may represent a bottleneck at time of sowing. Recently, Australian pulse legume producers have been using liquid and solid inoculant formulations to deliver rhizobial inoculants directly into the furrow. This is known as soil-applied (in-furrow) inoculation, to distinguish it from the seed-applied (on-seed) procedure. It has the advantage of providing more flexibility in delivery than the traditional method of inoculation (Denton et al. 2009; Drew et al. 2014) and it has the potential to mitigate against certain drawbacks of seed-applied inoculation. In-furrow inoculation separates the inoculant from seed-applied fungicides and insecticides that may be hazardous to rhizobial survival (Brockwell et al. 1980). The use of soil-applied inoculants is preferred for use in crops such as peanut (*Arachis hypogaea* L.), where seed-applied inoculant may damage the seed (Drew et al. 2014). Furthermore, soil-applied inoculants provide advantages to legumes that have epigeal germination, such as soybean (*Glycine max* L.) and subterranean clover (*Trifolium subterraneum* L.), whose germinating habit often lifts the seed coat – and much of the on-seed inoculant – away from the point of emergence of the seedling root (Brockwell et al. 1980). Thus, despite a long history of effective use of peat inoculants, there are some potential advantages in the use of granular or liquid inoculants for the promotion of nodulation and N_2 fixation of legumes.

The type of inoculant formulation has the potential to alter the pattern of root nodulation. Rhizobia are poorly motile in soils (Wadisirisuk et al. 1989) so the point of delivery of rhizobia into the soil is a determinant of nodulation pattern. In soils with low background populations of rhizobia, on-seed inoculation typically results in nodules clustered around the crown region of the root system (Valverde and Wall 2002, Remmler et al. 2014) with fewer nodules at depth. In contrast, plants inoculated with in-furrow granular inoculants are more likely to have greater lateral root nodulation (Kyei-Boahen et al. 2002). Compared with on-seed peat slurry inoculation, alternative in-furrow inoculant application methods have been shown in field studies to provide greater crop growth and grain yield (Brockwell et al. 1980; Nleya et al. 2001, Kyei-Boahen et al. 2002; Gan et al. 2005). However, responses appear to depend on the soil conditions into which the seed is sown, such as soil moisture status, as well the prevailing environmental conditions (Brockwell et al. 1980; Brockwell et al. 1988a). Nodulation deeper in the soil profile on lateral roots resulting from granular inoculation has been associated with increased grain yield, particularly due to increased N_2 fixation later in the season (Kyei-Boahen et al. 2002). The authors attributed the result to greater availability of soil moisture at depth. Although there is the potential that altering nodulation patterns through the use of different inoculant application methods may improve N_2 fixation and grain yield, this has not been documented in rainfed environments in Australia. There has been a recent increase in the use of different inoculant application methods, but many questions remain as to the benefits of these methods in different environments and cropping systems.

The aim of this study was to assess whether different inoculant application methods (peat slurry, peat applied as a liquid into the furrow, peat granules) altered nodulation patterns and, if so, whether this influenced N_2 fixation, crop growth or grain yield of narrow-leaf lupin

(*Lupinus angustifolius* L.) and faba bean (*Vicia faba* L.). Eight field experiments were conducted at four locations in south-eastern Australia to test the hypothesis that granular and liquid inoculants will increase lateral nodulation, with subsequent positive impacts on legume productivity.

Materials and methods

Field sites

The questions were addressed in field experiments that included two pulse legumes - narrow-leaf lupin and faba bean - established at four different locations in Victoria and New South Wales, Australia. Details of location, date of experimentation, soil type, plant treatments, plot dimensions and replication are shown in Table 1. At each site, lupin and faba bean experiments were adjacent to each other. Each experiment included a non-legume reference plant (canola, *Brassica napus* L. cv. Thunder TT). Prior to sowing, soil cores were taken to depths of 0.6 m to 1.2 m, soil samples were oven-dried at 40 ^{o}C for 24 hours, and soil chemistry was determined by CSBP Soil and Plant Analysis Service (Bibra Lake, Western Australia). Chemical characteristics of the soils are summarized in Supplementary Table 1.

Counting rhizobia

The numbers of rhizobia in soils at sowing was estimated using the most probable number (MPN) method of Brockwell (1963) following sampling of 25 soil cores (0-10 cm) across each site. Vetch (*Vicia sativa* L.) cv. Morava was used for counting *Rhizobium leguminosarum* bv. *viciae* (rhizobia for faba bean) and lupin (cv. Mandelup) was used for counting *Bradyrhizobium lupini*. The lower limit of detection using the technique is 4 rhizobia g^{-1} soil, equivalent to 6.0 x 10^9 rhizobia per hectare, to a depth of 10 cm, assuming a bulk density of 1.0 g cm^{-3}. At locations where rhizobia were undetectable, populations were regarded as zero. With the technique, a difference of 1.16 $\log_{10}$ units is required for differences between samples to be significant (P <0.05).

Sowing and experimental design

Seed was sown into moist soil and all plots received single superphosphate (120 kg ha^{-1}) at sowing. Legume treatments were inoculated with commercial inoculants using three different inoculant delivery systems - one on-seed and two in-furrow treatments: 1) peat inoculant slurry on-seed, 2) granular inoculant delivered in-furrow, 3) peat inoculant suspended in water and delivered in-furrow. Each experiment included uninoculated controls. Inoculation was performed according to the manufacturer's recommendations. For treatment 1, peat (BASF, Southbank, Australia) was applied to seed prior to sowing at a rate of 250 g peat to 100 kg seed. For treatment 2, peat granules (BASF, Southbank, Australia) were delivered in-furrow with the seed at a rate of 6 kg ha^{-1}. For treatment 3, peat inoculant suspension was delivered in-furrow at a rate of 50 l ha – equivalent to 250 g of peat per 100 kg of seed. Dilution plate counts were used to estimate the number of viable rhizobia delivered by each inoculation method (Table 2). The experiments were sown one treatment at a time using a cone seeder (eight rows with 17.5 cm spacing), and all sowing equipment was decontaminated with ethanol after each treatment to prevent inter-treatment contamination. Rainfall data collected at each site is presented in Figure 1. Experiments were monitored throughout the growing season and weeds, pests, and fungal pathogens were controlled using the appropriate chemical sprays, at rates according to manufacturers' recommendations.

Sampling and sample processing

The experiment at the Boorhaman North was repeatedly grazed by sulphur-crested cockatoos (*Cacatua galerita*). Above-ground sampling at this site therefore was not feasible and only below-ground data were collected.

Nodulation, peak biomass production, seed yield, N content, and N_2 fixation were measured for all treatments. For nodulation measurements, 10 individual plants were removed from each plot at approximately 18 weeks after sowing, using a spade to excavate the entire root system. Crown and lateral nodule number and weight were determined as outlined in detail in Denton et al. (2013). Peak biomass measurements were taken approximately 21 weeks after sowing, by manual sampling 0.788 m^2 of each plot (removing all plants from five separate 1 m sections of a drill row per plot). These samples were dried for 4 days at 70 °C to determine shoot dry matter (DM). Grain yields were collected using a mechanical plot harvester to capture the entire plot, approximately 30 weeks after sowing, in November or December (indicated in Fig. 1), depending on year.

Calculations of N_2 fixation

Estimates of N_2 fixation were obtained using the ^{15}N natural abundance technique (Unkovich et al. 2008). Shoot %N content and ^{15}N natural abundance composition (δ^{15}N; ‰) were analysed and N_2 fixation estimated using unfertilised canola as the non-N_2 fixing reference species. Subsamples of shoot DM were ground and analysed for total N concentration (mg N g^{-1}), and ^{15}N composition using an automatic nitrogen and carbon analyser (ANCA-SL) interfaced to a 20–20 stable isotope mass spectrometer (Europa Scientific, Crewe, UK). The proportion of legume N derived from atmospheric N_2 (%N_{dfa}) was calculated by comparing the ^{15}N natural abundance (expressed as $\delta^{15}N$ or parts per thousand (‰) relative to the ^{15}N composition of atmospheric N_2) of legume shoot N ($\delta^{15}N$ legume) to the $\delta^{15}N$ of the non-legume reference species canola that was assumed to reflect the $\delta^{15}N$ of the plant-available soil N ($\delta^{15}N$ soil) using the following equation [1]:

$$\%N_{dfa} = 100 \text{ x } (\delta^{15}N \text{ soil} - \delta^{15}N \text{ legume}) / (\delta^{15}N \text{ soil} - B) \qquad [1]$$

where B represents the $\delta^{15}N$ of faba bean shoots (-0.50‰) or lupin shoots (-0.57‰) (Unkovich et al. 2008).

The mean $\delta^{15}N$ of the reference canola shoot $\delta^{15}N$ varied from between 3.4 and 4.4‰ at Mininera, 3.1 and 3.8‰ at Rutherglen, and 4.7 and 6.0‰ at Culcairn. These values were substantially greater than the +2‰ generally considered the lowest reference $\delta^{15}N$ required to provide reliable measures of N_2 fixation (Unkovich et al. 1994).

The amounts of N_2 fixed were calculated from estimates of legume %Ndfa, shoot DM and N content (%N) as follows using equations [2] and [3]:

$$\text{Legume shoot N} = \%N/100 \text{ x (legume shoot DM)} \qquad [2]$$

$$\text{Amount shoot N fixed} = \%Ndfa/100 \text{ x (legume shoot N)} \qquad [3]$$

However, shoot-based estimates of N_2 fixation underestimate total inputs of fixed N since substantial amounts of legume N can also be associated with, or derived and released from, the nodulated roots (McNeill and Fillery 2008; Wichern et al. 2008; Peoples et al. 2009). In the case of field-grown faba bean, below-ground pools of N have been reported to represent between 24-40% of the total plant N (Rochester et al. 1998; Khan et al. 2003). Consequently, the total amounts of N_2 fixed were determined by multiplying the shoot values calculated with equation [3] by a factor of 1.52 for faba bean and 1.33 for lupin, to include below-ground contributions of fixed N_2, as described by Unkovich et al. (2010).

Net N balances were calculated for Culcairn by comparing N removed in grain with the estimates of the total amounts of fixed N_2 accumulated in both the shoot and below-ground components over the growing season.

Statistical analysis

Differences between parameters of plant productivity, nodulation, and N_2 fixation were examined by analysis of variance (ANOVA), followed by a Tukey *post hoc* test to determine where significant differences occurred, using GenStat (14th edition, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK). Normality was assessed using the Shapiro-Wilk's W statistic. Relationships between measures were assessed by correlation analysis, using Pearson's r if data were normal and Spearman's rho if data were not normal (normality assessed by Shapiro-Wilk's W), in the statistical software package PAST (v2.17; Hammer et al., 2001). All significant correlations were assessed visually to ensure outliers did not influence results.

Results

Soil rhizobia

MPN estimates did not detect the presence of lupin rhizobia at Culcairn and Mininera, although lupin rhizobia were estimated to be present at the Boorhaman North at 810 rhizobia g^{-1} soil, and at 2180 rhizobia g^{-1} soil at the Rutherglen site (Table 2); faba bean rhizobia were undetected using MPNs at Boorhaman North and Culcairn while Rutherglen contained 2020 rhizobia g^{-1} soil and there were 21 rhizobia g^{-1} soil at Mininera.

Lupin nodulation

In the Mininera experiment, inoculation resulted in small but significant increases in nodule numbers compared with uninoculated treatments; on-seed inoculation increased nodule mass on the crown or lateral roots and increased average nodule mass (Table 3). In the Culcairn experiment, inoculation of lupin with peat slurry on-seed significantly increased crown and lateral nodule numbers and crown nodule mass compared with in-furrow inoculation. Nodulation in the in-furrow (liquid) and in-furrow (granules) treatments was similar. All forms of inoculation were better in terms of nodulation than the uninoculated control. In the Rutherglen and Boorahman North experiments however, both of which had soil populations of *B. lupini* exceeded $\log_{10}$ 12.0 rhizobia ha^{-1} prior to the studies, there were few treatment differences in nodulation numbers, mass or average mass of nodules (Table 3).

Faba bean nodulation

In the Mininera experiment, on-seed peat slurry inoculation increased crown and lateral nodule numbers and crown nodule mass, in-furrow liquid inoculant increased crown and lateral nodule number and mass, and granular inoculant increased crown nodule mass, relative to uninoculated treatments (Table 4). At the Rutherglen site, which had measurable background soil rhizobia prior to the study, peat slurry inoculation increased crown nodule mass compared with no inoculation and granular inoculation increased lateral nodule mass compared with peat slurry (Table 4, Figure 2). At Culcairn and Boorhaman North, peat slurry inoculation increased crown nodulation number and mass compared with all other treatments, while granular inoculants increased lateral root nodulation number and mass, compared with most other treatments (Table 4). The size of naturally-occurring populations of *R. leguminosarum* bv. *viciae*, the rhizobia for faba bean, appeared pertinent. At Rutherglen, where nodulation did not respond to inoculation, there were $\log_{10}$ 12.5 faba bean rhizobia ha^{-1}. At Boorhaman North, where there was a small response to inoculation, the naturally-occurring population was $\log_{10}$ 10.5 rhizobia ha^{-1}. At Mininera and Culcairn, where responses were greater, naturally-occurring rhizobia were undetectable.

Estimates of N_2 fixation

Lupin N_2 fixation did not increase with inoculation at the Rutherglen and Mininera field sites (Table 5). At Culcairn however, all inoculant treatments increased both shoot N concentration and the proportion of the lupin N derived from atmospheric N_2 (Ndfa %), and peat slurry and liquid inoculation treatments resulted in significant increases in the amounts shoot N and total N_2 fixed, relative to uninoculated plots (Table 5).

Shoot N in faba bean, including total and fixed N, was increased through inoculation at Mininera and Culcairn sites, but no responses were observed at Rutherglen (Table 6). At Mininera, shoot N concentration increased with peat slurry inoculation and all inoculants enhanced shoot N accumulation, %Ndfa and total N_2 fixed, compared with uninoculated treatments (Table 6). At Culcairn, inoculation with peat slurry on-seed increased the shoot N and the total amount of N_2 fixed above that achieved by in-furrow granules, liquid inoculation or no inoculation. Inoculation of all formulations increased shoot N concentration and %Ndfa compared with uninoculated treatments (Table 6).

Legume shoot dry matter and grain yield

Inoculation had no effect on either lupin grain yield or shoot DM production at any of the experimental sites (Table 7). There was also no effect of inoculation on faba bean at Rutherglen, but both grain yield and shoot DM production of faba bean significantly increased compared with uninoculated treatments with peat slurry inoculation at Culcairn and Mininera, and with liquid inoculation at Mininera. At Culcairn, faba bean yields were lower with liquid inoculation compared with peat slurry or granular inoculation (Table 7). For both legumes at the Culcairn site, peat slurry inoculation significantly increased total grain N and net N balance compared with uninoculated treatments (Table 8). Uninoculated lupins removed 95 kg of soil N, while inoculated lupins contributed 48 kg N ha^{-1}, after accounting for N removal in grain. Uninoculated faba bean removed 17 kg N ha^{-1} from the soil, while inoculated faba bean contributed 153 kg N ha^{-1} after accounting for N removed in grain harvest (Table 8).

Nodulation patterns were associated with different productivity outcomes in both lupin and faba bean treatments at different sites. For lupin there were significant but weak correlations between lateral root nodules and grain yield ($r = 0.4$ $p < 0.05$) at Mininera and between average nodule size and grain yield ($r = 0.420$ $p < 0.05$) at Mininera and Culcairn (Table 9). For faba bean, crown and lateral nodule numbers and nodule mass were significantly correlated with grain yield at Culcairn and Mininera (Table 10; $r > 0.533$ $p < 0.01$). In addition, crown and lateral nodulation was related to peak shoot DM ($r > 0.715$ $p < 0.001$) at Culcairn, while lateral nodule mass was related to peak shoot DM ($r = 0.632$ $p < 0.001$) at Mininera (Table 10). At Rutherglen, peak shoot DM was correlated with crown nodule number ($r = 0.432$ $p < 0.05$; Table 10).

The relative impact of on-seed or in-furrow inoculation treatments was significant relative to uninoculated treatments. The productivity impacts at sites without background rhizobia (Culcairn for lupin and faba bean and Mininera for faba bean) are indicated in Supplementary Table 2.

Discussion

In this study, all three methods of inoculation (peat slurry, granules or liquid) generally increased nodulation, N_2 fixation and grain yield, relative to uninoculated treatments. At the most responsive sites, nodulation due to inoculation increased by up to 125% in faba bean and up to 260% in lupin, N_2 fixation due to inoculation increased by up to 280 kg N ha^{-1} in faba bean and up to 175 kg N ha^{-1} in lupin, and grain yield due to inoculation in faba bean increased from 0.48 to 1.94 tha^{-1}. There were, however, fewer differences among inoculant methods. Across all experiments, on-seed peat slurry inoculant produced greater or equal grain yield and N_2 fixation, relative to in-furrow granular and liquid inoculants. Although the application of different inoculant methods led to differences in nodulation patterns in lupin and faba bean, significant differences in grain yield and fixed N were only observed for one crop, faba bean, at the Culcairn site. At that site, peat slurry inoculation of faba bean provided greater total N_2 fixation than either granular or liquid inoculants, and greater grain yield than liquid inoculant application. These differences were associated with increases in crown nodule number and mass in the peat slurry treatments, as indicated by correlations among the data for faba bean at Culcairn. At Culcairn, strong spring rainfall allowed the crop to fix more N than elsewhere, through greater biomass accumulation, which led to differences in grain yield. Despite differences in nodule numbers and nodule mass among inoculant treatments, lupin N_2 fixation and grain yield at Culcairn were both unaffected by inoculant application treatments. Thus, while inoculation application methods may alter nodulation patterns, they are unlikely to improve N_2 fixation or grain yields, relative to on-seed peat slurry inoculation.

On-seed inoculation is the conventional form of legume inoculation and has served legume growers well for more than 100 years. The point of delivery of rhizobia into the soil is proximal to where the crown of the root system will develop. In-furrow inoculation, using liquid or granular inoculants is more recent; it often delivers rhizobia at a point in the soil profile where lateral roots will form. In Saskatchewan, Canada, where granular inoculants were placed at 25 to 80 mm below chickpea (*Cicer arietinum* L.), these treatments had greater lateral root nodulation, and enhanced yield (Kyei-Boahen et al. 2002). In our experiments, nodulation patterns in both lupin and faba bean were not as markedly influenced by inoculant application methods. Lateral root nodules potentially form later than crown root nodules, as particular loci on legume roots are only transiently susceptible to nodulation (Bhuvaneswari et al. 1981). Although lateral root nodules have less potential for total N_2 fixation, due to a shorter active period when formed later, they can increase late-season, post pod-fill N_2 fixation (Hardarson et al. 1989), during a period of high crop demand for N (Zapata et al. 1987, Bergersen *et al.* 1992). In our, on-seed peat slurry inoculation, which increased crown nodulation, had generally higher grain yields than other treatments. In contrast, in-furrow granular and liquid inoculants had more varied yield patterns. These data suggest that the benefits of deeper nodulation may be dependent on other factors. It is likely that soil water availability, potential protection of the inoculant from adverse conditions (low pH, desiccation, high temperatures), and seasonal environmental conditions will all influence N_2 fixation and grain yield outcomes (Peoples et al. 2009). These considerations aside, the evidence from our experiments was conclusive: in terms of most parameters of nodulation and crop productivity, on-seed inoculation was consistently superior to in-furrow inoculation. However, there were few differences between the two forms of in-furrow inoculation – liquid and granules.

Populations of rhizobia for lupin (*B. lupini*) and/or faba bean (*R. leguminosarum* bv. *viciae*) occurred naturally at Rutherglen, Mininera and Boorhaman North, but not at Culcairn. The populations at Rutherglen exceeded 2000 rhizobia g^{-1} of soil (0-10 cm) – equivalent to more than one million per seed. The number of naturally-occurring lupin rhizobia in Boorhaman North soil was 810 g^{-1} soil. There was no response to any inoculation method by either lupins or faba beans at Rutherglen and lupins did not respond to inoculation at Boorhaman North. At these sites, plants in all treatments developed abundant crown and lateral nodules, in numbers generally exceeding those found in inoculated plants at sites with few soil rhizobia. Previous research has indicated the unlikeliness of an inoculation response where soil rhizobial backgrounds such as these exist (Brockwell et al. 1995, Herridge 2008, Denton et al. 2011). In the Mininera soil a small population (21 per gram) of faba bean rhizobia occurred, which is equivalent to around 100,000 per seed and was greatly outnumbered by inoculant rhizobia – more the 10 million per seed, as previously observed (Denton et al. 2013). Faba beans responded to inoculation at Mininera, a finding that is consistent with the concepts of Brockwell et al. (1995) and Herridge (2008) above.

The superiority of on-seed inoculation compared with in-furrow inoculation may be inflated in an experimental system. Commercial growers of pulse legumes such as lupins and faba beans that require high seeding rates find that even relatively small areas of crop require several tonnes of seed. For the grower, the logistics of on-seed inoculation on this scale are formidable. In addition, there is a considerable risk of high rates of inoculant mortality when on-seed inoculant is applied to seed as much as 24 hours (or more) in advance of seeding (Brockwell et al. 1995). As a rule, this problem is not encountered in experiments as seed is usually sown within an hour of inoculation. On the other hand, the commercial grower may choose the option of in-furrow inoculation, either liquid of granular, because of (i) its convenience and (ii) a lower risk of inoculant mortality. Our work has demonstrated that nodulation, N_2 fixation and crop production of lupins and faba beans respond satisfactorily to in-furrow delivery of legume inoculant.

Total faba bean nodule numbers were higher following peat slurry inoculation at Rutherglen (78 plant^{-1}) and Mininera (64 plant^{-1}) than at Culcairn (14 plant^{-1}) and Boorhaman North (9 plant^{-1}), which was likely to be due to low soil pH at the latter two sites. Low soil pH is well known to limit the survival and persistence of inoculant rhizobia (Hungria and Vargas 2000). There is potential that low pH in microsites of the soil might have limited the survival of inoculant rhizobia, as our measures were made from bulk soil. There were differences among the two species; lupin had less nodules than faba bean overall, as is commonly observed (Drew et al. 2014), but this did not appear to be influenced by soil pH or the resident soil population of rhizobia. Total lupin nodules were lowest at Mininera, but the reasons for this were not clear.

Legume dependence on N_2 fixation (%N_{dfa}) was low for inoculated legumes at Mininera (24 to 38% for lupin and 36 to 44% for faba bean), potential due to lower yields reducing the demand for N_2 fixation. Dependence on N_2 fixation was greater at Rutherglen and Culcairn, with ranges for inoculated lupin (50 to 69%) and inoculated faba bean (56 to 72%) that were closer to values observed in multiple studies in southern Australia (75% mean value for lupin, 65% mean value for faba bean; Unkovich et al. 2010). Fixed N in shoots and roots of faba bean and lupin at Culcairn was estimated to be over 300 kg N ha^{-1}. These values are in the upper range of measures of fixed N in shoots and roots of faba bean of 17 to 456 kg N ha^{-1} (mean 185 kg N ha^{-1}) and those of lupin of 39 to 441 kg N ha^{-1} (mean 103 kg N ha^{-1}), estimated from field grown crops reported in Unkovich et al. (2010). Inoculation also improved grain N, as observed in faba bean by Youseif et al. (2017). Inoculation had a significant contribution to the residual net N balance after accounting for the N removed from the system in grain at harvest. In uninoculated crops at Culcairn, soil N use differed markedly between crops, with lupin accessing significantly more soil N compared with faba bean (calculated as: [Total crop N] – [N fixed]). Inoculation resulted in 50 to 150 kg more residual N ha^{-1}, representing a difference of 140 to 170 kg N ha^{-1} between treatments for the two crops. The data from Culcairn illustrated the economic benefits derived from using a quality inoculant at an approximate cost of AUD$10 ha^{-1} when soil rhizobia numbers are either low or absent (AUD$ = USD$0.75). Under these conditions, the contribution of the additional N_2 fixed and net N returned to soil in legume residues was equivalent to an input of AUD$95 to AUD$115 ha^{-1} of urea fertiliser, and the increase observed in faba bean grain yield of 1.94 t ha^{-1} was worth >AUD$775 ha^{-1}, based on the prevailing grain prices at the time. Similar benefits have recently been reported for soybean in Nigeria, due, principally, to the low cost of inoculant, relative to its potential benefits (Ronner et al. 2016).

Legume seeds were sown into moist soils, following favourable pre-season rainfall in our experiments (Figure 1). However, in southern Australia, lupin and faba bean crops are frequently sown into dry soil early in the season, prior to breaking rain (Thomson et al. 1997). At these times, soil moisture deficits are likely to be variable, and potentially high, with the resulting survival of rhizobia inoculated onto seed affected by dry soil (Brockwell et al. 1988b). The survival of seed-applied rhizobia when sown into dry soils is poorly understood, with little practical information to guide legume growers. It has been suggested that soil inoculants, such as granular and liquid inoculants delivered deeper in the soil, may be exposed to greater moisture, which could improve rhizobial survival and nodulation (Brockwell et al. 1980, 1988a; Hardarson et al. 1989, Kyei-Boahen et al. 2002), but the detailed comparative studies of the value of different inoculant formulations under conditions of water deficit has yet to be fully evaluated in southern Australian systems. The conditions in Australia, which rely on season-breaking rainfall, contrast with the north American experience of initial moisture from snow melt, so northern hemisphere examples may not necessarily provide a useful guide to the relative performance of different approaches to inoculation in Australia. Therefore, any potential advantage of sowing in-furrow inoculants in dry environments remains to be tested.

Nodulation patterns are hypothesised to have an impact on later season N_2 fixation, when substantial N_2 fixation can occur (Zapata et al. 1987). The variable climates of southern Australia and other Mediterranean-type environments often lead to end-of-season water deficits or ‘terminal drought’ (Turner and Asseng 2005) that leads to drying of the surface soil. The impact that surface soil drying has upon nodule function is not well understood, but it can potentially reduce nodule number, nodule biomass and N_2 fixation, since nodules in dry soil do not appear to be hydrated from water deeper in the soil profile (Abdelhamid et al. 2011), even though shoot mass may not decrease. If inoculant types can be supplied that alter nodulation patterns and provide nodulation deeper in the soil profile, then there is potential for maintenance of N_2 fixation following surface soil drying, relative to crown-only nodulated plants. Whether this impacts on N_2 fixation under Australian field conditions would require detailed assessment in variable seasons that differ in their late-season rainfall. In the current experiments, a lack of surface moisture was not observed late in 2008 and 2010, so we did not directly evaluate this scenario. In our experiments, nodulation from liquid and granular inoculants typically provided less nodule mass than peat inoculants, potentially due to inefficiencies in inoculant delivery to deeper roots, or to differences in the ability for the carrier material to provide for rhizobia survival, which may have limited the effects. In circumstances where surface soil drying occurs later in the season, when N_2 fixation is required to contribute significant N for grain-filling, then in-furrow inoculants delivered effectually may provide an advantage to legume crops.

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Table 1. Locations and dates of experiments to examine the benefits of alternative forms of inoculation, showing soil type, plant treatments, plot dimensions and replication.

Location	Coordinates	Date	Soil type (Isbell 1996)	Plant treatments	Seeding rate, sowing depth	Plot dimensions	Replicates
Mininera, Victoria	37° 35' 54.95" S 142° 57' 3.89" E	2008	Sodosol	Faba bean cv. Farah Lupin cv. Mandelup Canola cv. Thunder TT	210 kg ha^{-1}, 8 cm 120 kg ha^{-1}, 3 cm 7 kg ha^{-1}, 1 cm	10m x 1.42m	6
Rutherglen, Victoria	36° 6' 4.91" S 146° 30' 46.34" E	2008	Brown sodosol	Faba bean cv. Farah Lupin cv. Mandelup Canola cv. Thunder TT	210 kg ha^{-1}, 8 cm 120 kg ha^{-1}, 3 cm 7 kg ha^{-1}, 1 cm	15m x 1.42m	8
Culcairn, New South Wales	35° 39’ 2.5914” S 147° 0' 53.3196" E	2010	Sodosol	Faba bean cv. Farah Lupin cv. Jindalee Canola cv. Thunder TT	210 kg ha^{-1}, 8 cm 120 kg ha^{-1}, 3 cm 5 kg ha^{-1}, 1 cm	15m x 1.42m	6
Boorhaman North, Victoria	36° 6' 27.4926" S 146° 14' 28.125" E	2010	Red chromosol	Faba bean cv. Farah Lupin cv. Jindalee Canola cv. Thunder TT	210 kg ha^{-1}, 8 cm 120 kg ha^{-1}, 3 cm 5 kg ha^{-1}, 1 cm	15m x 1.42m	6

Table 2. Soil rhizobial populations and rhizobial numbers delivered from inoculant treatments for lupin and faba beans at the four field sites. Note that rhizobial values are expressed on a per area or per seed basis.

Location	Soil rhizobia ($\log_{10}$ ha^{-1})		Soil rhizobia ($\log_{10}$ seed^{-1})		Inoculant rhizobia ($\log_{10}$ seed^{-1})					
					Peat slurry on-seed		***In-furrow inoculation***			
							Liquid		*Granules*	
	for lupin	faba bean	lupin	faba bean	lupin	faba bean	lupin	faba bean	lupin	faba bean
Mininera	0.0	10.5	0.0	5.2	7.3	7.9	6.3	7.0	6.7	7.2
Rutherglen	12.5	12.5	6.7	7.2	7.3	7.9	6.3	7.0	6.7	7.2
Culcairn	0.0	0.0	0.0	0.0	7.3	7.9	6.3	7.0	6.7	7.2
Boorhaman North	12.1	0.0	6.3	0.0	7.3	7.9	6.3	7.0	6.7	7.2

Table 3. The impact of inoculant application methods including uninoculated, peat slurry on-seed (peat), peat granules (granules) in-furrow or application of liquid inoculant in-furrow (liquid) on lupin nodule number, weight and size.

Inoculation treatment	Nodule number per plant		Nodule weight per plant (mg)		Average nodule weight (mg) *
	crown	lateral	crown	lateral	
			Mininera		
Uninoculated	0.1 c	0.1 b	0.4 b	0.5 b	2.7 b
Peat	4.8 a	1.9 a	20.6 a	8.5 a	3.9 a
Granules	1.1 bc	1.0 ab	13.5 a	9.5 a	8.6 a
Liquid	3.1 ab	0.9 ab	11.0 a	5.6 a	6.9 a
	$P< 0.001$	$P <0.05$	$P <0.01$	$P <0.05$	$P < 0.01$
			Rutherglen		
Uninoculated	33.9 a	46.5 a	19.4 a	4.0 ab	9.9 a
Peat	14.8 a	22.2 a	27.7 a	5.9 a	14.2 a
Granules	32.1 a	36.9 a	18.8 a	3.4 b	9.9 a
Liquid	20.3 a	34.4 a	24.4 a	6.0 a	14.8 a
	ns	*ns*	*ns*	$P< 0.05$	*ns*
			Culcairn		
Uninoculated	0.0 d	0.0 c	0.1 c	0.0	0.3 c
Peat	20.7 a	3.0 a	89.7 a	11.2	4.3 a
Granules	4.4 c	1.1 bc	39.4 b	3.5	8.5 b
Liquid	9.0 b	2.0 ab	61.4 b	18.7	7.5 b
	$P <0.001$	$P <0.01$	$P <0.001$	*ns*	$P <0.001$
			Boorhaman North		
Uninoculated	13.2 b	6.6 a	47.2 a	16.7 a	3.2 a
Peat	14.1 ab	5.7 a	53.1 a	15.6 a	3.4 a
Granules	10.4 b	5.1 a	38.5 a	16.7 a	3.5 a
Liquid	17.3 a	9.5 a	48.6 a	25.5 a	2.6 a
	$P< 0.001$	*ns*	*ns*	*ns*	*ns*

Data are provided as means (Rutherglen n= 8; all other sites n=6). Means in a single sub-column followed by a different letter are significantly different at the given P values, *ns* indicates non-significance. *Average nodule weight per plant divided by total number of nodules per plant.

Table 4. The impact of inoculant application methods including uninoculated, peat slurry on-seed (peat), peat granules (granules) in-furrow or application of liquid inoculant in-furrow (liquid) on faba bean nodule number, weight and size.

Inoculation treatment	Nodule number per plant		Nodule weight per plant (mg)		Average nodule weight (mg)*
	crown	lateral	crown	lateral	
			Mininera		
Uninoculated	0.4 c	0.3 c	1.2 c	0.4 b	12.0 a
Peat	42.8 a	20.9 a	93.7 a	19.7 a	11.2 a
Granules	7.6 bc	5.0 bc	41.3 b	10.5 ab	32.2 a
Liquid	17.6 b	12.8 ab	46.4 b	20.4 a	14.3 a
	P <0.001	*P <0.001*	*P <0.001*	*P <0.01*	*ns*
			Rutherglen		
Uninoculated	50.2 a	33.6 a	148.3 b	70.2 ab	22.4 a
Peat	52.0 a	26.3 a	208.3 a	43.3 b	25.2 a
Granules	40.7 a	31.4 a	159.7 ab	83.5 a	22.1 a
Liquid	54.8 a	38.6 a	186.9 ab	77.0 ab	18.9 a
	ns	*ns*	*P <0.05*	*P <0.05*	*ns*
			Culcairn		
Uninoculated	0.1 b	0.0 b	0.4 d	0.0 b	4.0 c
Peat	12.9 a	1.2 a	172.5 a	4.9 ab	16.0 bc
Granules	4.0 b	1.0 a	106.6 b	20.3 a	33.6 a
Liquid	1.7 b	0.7 ab	40.9 c	9.7 ab	21.9 ab
	P <0.001	*P <0.01*	*P <0.001*	*P <0.05*	*P <0.001*
			Boorhaman North		
Uninoculated	0.0 b	0.0 b	0.0 b	0.0 b	0.0 b
Peat	7.7 a	0.6 b	63.3 a	1.7 b	7.9 ab
Granules	1.0 b	1.7 a	31.3 b	11.6 a	18.1 ab
Liquid	0.3 b	0.2 b	2.4 b	5.4 ab	19.0 a
	P <0.001	*P <0.001*	*P <0.001*	*P <0.001*	*P<0.05*

Data are provided as means (Rutherglen n= 8; all other sites n=6). Means in a single sub-column followed by a different letter are significantly different at the given P values, *ns* indicates non-significance. *Average nodule weight per plant divided by total number of nodules per plant.

Table 5. Impact of inoculant application method including uninoculated, peat slurry on-seed (peat), peat granules (granules) in-furrow or application of liquid inoculant in-furrow (liquid) on lupin N and the proportion (%Ndfa) and amount of N_2 fixed.

Site	Treatment	Shoot %N	Shoot N (kg N ha^{-1})	Ndfa (%)	Total N fixed (kg N ha^{-1})
Mininera	Uninoculated	2.3 a	74 a	15 a	16 a
	Peat	2.4 a	65 a	24 a	21 a
	Granules	2.3 a	65 a	38 a	35 a
	Liquid	2.2 a	54 a	24 a	17 a
		ns	*ns*	*ns*	*ns*
Rutherglen	Uninoculated	2.9 a	157 a	63 a	130 a
	Peat	2.9 a	154 a	69 a	142 a
	Granules	3.0 a	159 a	67 a	144 a
	Liquid	2.9 a	147 a	60 a	118 a
		ns	*ns*	*ns*	*ns*
Culcairn	Uninoculated	1.9 b	144 b	26 b	50 b
	Peat	3.1 a	287 a	58 a	225 a
	Granules	3.0 a	217 ab	50 a	148 ab
	Liquid	2.9 a	231 a	56 a	177 a
		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Data are provided as means (n=4). Means in a single sub-column followed by a different letter are significantly different at the given P values, *ns* indicates non-significance. %N is the percentage of shoot N; Ndfa is the percentage of legume N derived from atmospheric N_2. Total N_2 fixed at the paddock level incorporates estimates of fixed N in both the shoot and roots (kgN ha^{-1}).

Table 6. Impact of inoculation method including uninoculated, peat slurry on-seed (peat), peat granules (granules) in-furrow or application of liquid inoculant in-furrow (liquid) on faba bean N, and the proportion (%Ndfa) and amount of N_2 fixed.

Site	Treatment	Shoot %N	Shoot N (kg N ha^{-1})	Ndfa (%)	Total N fixed (kg N ha^{-1})*
Mininera	Uninoculated	1.8 b	68 b	18 b	17 b
	Peat	2.3 a	98 a	44 a	63 a
	Granules	2.1 ab	81 a	36 a	44 a
	Liquid	2.2 ab	106 a	41 a	66 a
		$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.001$
Rutherglen	Uninoculated	2.5 a	153 a	65 a	152 a
	Peat	2.6 a	152 a	70 a	163 a
	Granules	2.6 a	152 a	67 a	156 a
	Liquid	2.7 a	114 a	72 a	125 a
		ns	*ns*	*ns*	*ns*
Culcairn	Uninoculated	1.8 c	106 b	23 b	36 c
	Peat	2.9 a	325 a	64 a	316 a
	Granules	2.5 ab	139 b	62 a	130 b
	Liquid	2.5 b	145 b	56 a	121 b
		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Data are provided as means (n=4). Means in a single sub-column followed by a different letter are significantly different at the given P values, *ns* indicates non-significance. %N is the percentage of shoot N; Ndfa is the percentage of legume N derived from atmospheric N_2. Total N_2 fixed at the paddock level incorporates estimates of fixed N in both the shoot and roots (kgN ha^{-1}). Note that rounding of shoot %N in granules (2.54) and liquid inoculants (2.48) for Culcairn data obscures the differences in these treatments and their significance relative to peat slurry.

Table 7. Impact of inoculation method including uninoculated, peat slurry on-seed (peat), peat granules (granules) in-furrow or application of liquid inoculant in-furrow (liquid) on legume grain yield and peak shoot dry matter (DM) production.

		Lupin		Faba bean	
Site	Treatment	Grain yield (t ha^{-1})	Peak DM (t ha^{-1})	Grain yield (t ha^{-1})	Peak DM (t ha^{-1})
Mininera	Uninoculated	1.16	3.2	0.94 b	3.7 b
	Peat	1.17	2.9	1.42 a	4.8 a
	Granules	1.26	2.8	1.13 ab	4.0 ab
	Liquid	1.10	2.6	1.37 a	4.8 a
		ns	*ns*	$P < 0.01$	$P < 0.05$
Rutherglen	Uninoculated	0.75	5.5	2.06	5.8
	Peat	0.75	5.4	2.09	5.6
	Granules	0.77	5.5	2.05	5.4
	Liquid	0.73	5.5	2.12	4.7
		ns	*ns*	*ns*	*ns*
Culcairn	Uninoculated	3.45	7.8	1.75 c	6.0 b
	Peat	3.69	9.2	3.69 a	11.6 a
	Granules	3.67	7.4	3.50 a	9.0 ab
	Liquid	3.92	8.1	2.70 b	8.4 ab
		ns	*ns*	$P < 0.001$	$P < 0.001$

Data are provided as means (Rutherglen n= 8; all other sites n=6). Means in a single sub-column followed by a different letter are significantly different at the given P values, *ns* indicates non-significance. Treatments across sites values indicate the effect of inoculation treatment, with treatment nested within site.

Table 8. Total grain nitrogen and net nitrogen balances (residual fixed N in below-ground and above-ground material after the removal of grain N) at the Culcairn site, following the comparison of inoculation with peat slurry inoculated (on-seed) and uninoculated lupin and faba bean.

Crop	Inoculation treatment	Total grain N (kg ha^{-1})	Net N balance (kg ha^{-1})
Lupins	On-seed	+177 a	+48 a
	Uninoculated	+145 b	-95 b
		P<0.001	*P<0.001*
Faba beans	On-seed	+163 a	+153 a
	Uninoculated	+53 b	-17 b
		P<0.001	*P<0.001*

Table 9. Relationship between lupin nodulation and productivity at the Culcairn, Rutherglen, and Mininera field sites.

Site	Crop parameters	Nodule number per plant		Nodule weight per plant (mg)		Average nodule weight (mg)
		crown	lateral	crown	lateral	
Mininera *n=24*	Grain yield	-0.080	-0.006	0.118	**0.398***	**0.374***
	Peak shoot DM	-0.114	-0.112	-0.154	-0.044	-0.191
Rutherglen *n=32*	Grain yield	0.136	0.293	-0.049	-0.231	-0.272
	Peak shoot DM	-0.021	0.107	0.125	0.102	0.129
Culcairn *n=24*	Grain yield	0.204	0.186	0.175	0.300	**0.420***
	Peak shoot DM	0.209	0.180	0.240	0.277	0.012

Relationship assessed by correlation analysis in PAST, using Pearson's r if data normal and Spearmans rho if data were not normal. Normality was assessed by Shapiro-Wilk's W. All significant correlations assessed visually to ensure outliers not influencing results. Bolded correlations are significant at P ≤ *0.05 .

Table 10. Relationship between faba bean nodulation and productivity at the Culcairn, Rutherglen, and Mininera field sites

Site	Crop parameters	Nodule number per plant		Nodule weight per plant (mg)		Average nodule weight (mg)
		crown	lateral	crown	lateral	
Mininera *n=24*	Grain yield	**0.609****	**0.533****	**0.531****	**0.547****	-0.032
	Peak shoot DM	0.395	**0.491***	0.367	**0.632*****	0.052
Rutherglen *n=32*	Grain yield	0.079	-0.051	0.092	0.019	-0.051
	Peak shoot DM	**0.413***	0.115	0.025	0.003	-0.325
Culcairn *n=24*	Grain yield	**0.870*****	**0.768*****	**0.863*****	**0.514***	0.220
	Peak shoot DM	**0.715*****	**0.736*****	**0.737*****	0.393	0.100

Relationship assessed by correlation analysis in PAST, using Pearson's r if data normal and Spearman's rho if data were not normal. Normality was assessed by Shapiro-Wilk's W. All significant correlations assessed visually to ensure outliers not influencing results. Bolded correlations are significant at P ≤ *0.05, **0.01, ***0.001

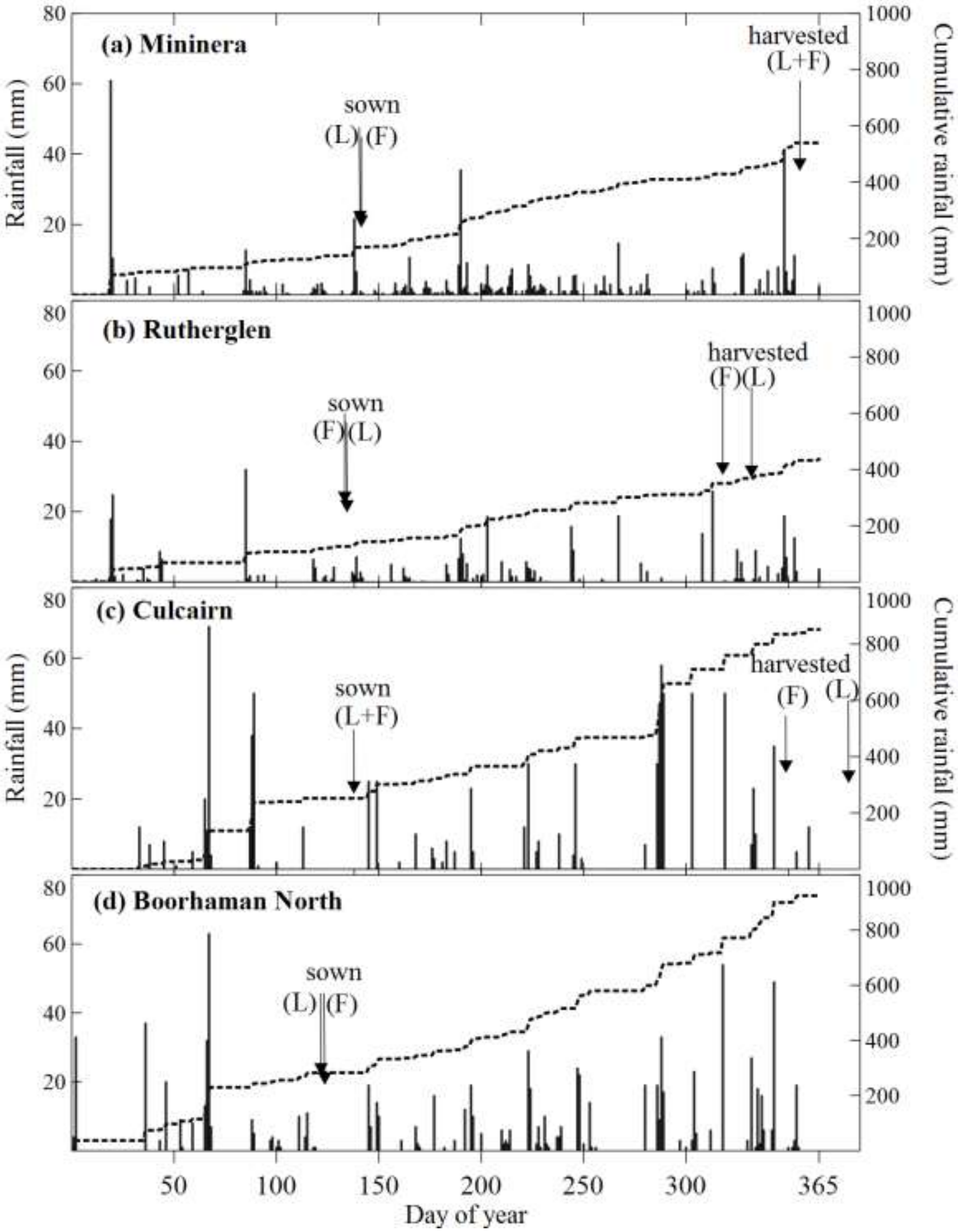

Figure 1. Seasonal rainfall patterns at (a) Mininera, and (b) Rutherglen in 2008 and at (c) Boorhaman North and (d) Culcairn in 2010. Arrows in each panel represent the time of sowing and harvest of faba bean (F) and lupin (L). Note that legumes were not harvested at Boorhaman North due to crop damage.

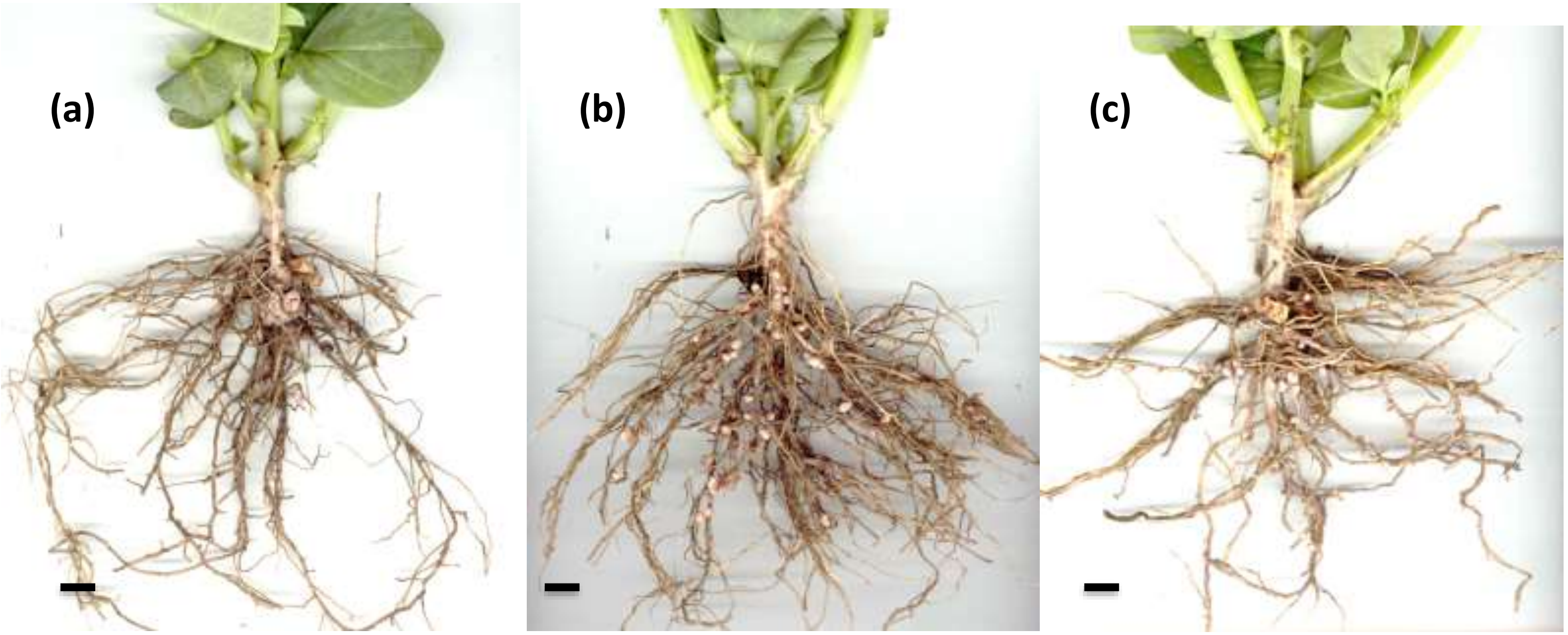

Figure 2. Nodulation patterns on selected faba bean roots following inoculation with different application methods, including (a) crown nodulation from on-seed peat slurry inoculant application, (b) granular inoculant sown in-furrow, and (c) liquid inoculant delivered in-furrow. Plants were sampled from the Rutherglen field site 106 days after sowing. Examples indicate differences in nodule distribution between the upper crown region and lateral roots. Scale indicates 1 cm.

Supplementary Table 1. Soil characteristics at sowing for the four experimental field sites.

Site and depth	NH_4^+	NO_3^-	P	K	S	Organic-C (%)	EC (dS m^{-1})	pH (H_20)
	mg kg^{-1}							
Mininera								
0-20 cm	2.8	29.1	31.0	274.7	12.6	1.89	0.10	5.78
20-40 cm	1.4	11.4						
40-100 cm*	1.0	4.0						
Rutherglen								
0-20 cm	1.4	13.1	29.7	145.5	7.4	0.44	0.04	6.10
20-40 cm	1.0	2.8						
40-100 cm*	1.0	1.7						
Culcairn								
0-10 cm	6.3	53.0	39.0	314.0	20.1	1.93	0.17	4.80
10-30 cm	2.3	6.5	9.5	214.5	9.4	0.40	0.05	5.05
30-120 cm**	2.3	5.4						
Boorhaman North								
0-10 cm	5.5	28.5	27.0	472.0	4.6	1.17	0.10	4.95
10-30 cm	2.3	13.3	6.5	283.0	2.5	0.39	0.04	5.60
30-120 cm**	2.7	8.4						

*average of data from 3 x 20 cm increments; ** average of data from 3 x 30 cm increments.

Supplementary Table 2. Productivity increases (expressed as a percentage of the production of uninoculated controls) in lupins and faba beans due to on-seed (peat slurry) and in-furrow (liquid and granules pooled) inoculation.

Parameters of productivity	Lupins		Faba beans			
	Culcairn		Culcairn		Mininera	
	On-seed	In-furrow	On-seed	In-furrow	On-seed	In-furrow
Shoot DM (t ha^{-1})	18	0	93	45	30	11
Shoot N (%)	63	55	58	47	28	18
Shoot N (kg ha^{-1})	99	56	207	34	44	38
Ndfa (%)	123	104	178	157	144	114
Total N fixed (kg ha^{-1})	300	225	778	249	271	165
Seed yield (t ha^{-1})	7	10	129	77	51	33