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Wild bradyrhizobia that occur in the Burdekin region of Queensland are as effective as commercial inoculum for mungbean (*Vigna radiata* (L.)) and black gram (*Vigna mungo* (L.)) in fixing nitrogen and dry matter production

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ABSTRACT

Anecdotal reports have suggested wild bradyrhizobia (*Bradyrhizobium* spp.) were as effective as commercial inoculum for mungbean (*Vigna radiata* (L.)) grown in the Burdekin region of far north Queensland, Australia. To test this hypothesis, we sampled the diversity of bradyrhizobium strains at two field sites in the Burdekin region of north Queensland, and from plants grown in inoculated or uninoculated soil collected from one field site in Millmerran, southern Queensland. We then compared two wild bradyrhizobium with the commercial strain for their ability to fix nitrogen and promote biomass production on plants in a glasshouse experiment. Ten mungbean nodules were collected from each of four plants, from each of two sites in the Burdekin region. One site had been treated with commercial bradyrhizobium inoculum strain CB1015, the other had never been treated with commercial inoculum. Protein analysis of the nodules using matrix assisted desorption ionization time of flight mass spectrometry (MALDI-TOF MS) showed a total of eight different strains of bradyrhizobia, distinctly different to CB1015, were present in the nodules of these plants. Of these eight strains, two strains dominated, one at each of the two collection sites. On the other hand, mungbean plants grown in soils from Millmerran, on the Darling Downs, hosted only CB1015 where inoculated, and did not nodulate when not inoculated. The two dominant wild bradyrhizobia were cultured from nodules from these field collected plants, and used in a controlled glasshouse experiment. In the glasshouse experiment, two mungbean cultivars and one black gram (*Vigna mungo* (L.)) cultivar were: (i) not inoculated and not supplied with nitrogen, (ii) not inoculated and supplied with nitrogen, (iii) inoculated with the commercial bradyrhizobia strain CB1015, (iv) inoculated with the dominant wild bradyrhizobia strain from Site 1, or (v) inoculated with the dominant wild bradyrhizobia strain from Site 2. Cultivars inoculated with either of the two dominant wild bradyrhizobia strains were similar in biomass and fixed a similar amount of nitrogen, to those inoculated with CB1015. There was no significant difference between the three inoculated treatments for shoot biomass nor nodule biomass per plant. Nodule number did not differ significantly from CB1015 for either of the wild bradyrizobia treatments. For root biomass per plant, the two mungbean cultivars did not differ between the three inoculated treatments, but the black gram had significantly less root biomass compared to the CB1015 treatment for one of the two wild bradyrhizobia treatments. For nitrogen fixed per plant, none of the cultivars showed significant difference between inoculated treatments. Our findings are consistent with the hypothesis that in some parts of Queensland some wild bradyrhozobia may be as effective as commercial inoculum for mungbean and black gram, potentially abrogating the need for artificial inoculation, and perhaps offering new options for the future development of better adapted commercial inoculum.

Abbreviations: eBLUEs, empirical best linear unbiased estimates; LSD, least significant difference; MALDI-TOF MS, matrix assisted laser desorption ionization time of flight mass spectrometry; PCA, principal component analysis; REML, residual maximum likelihood; SARAMIS™, spectral archive and microbial identification system; %Ndfa, percentage of plant N derived from atmospheric N_2

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1. Introduction

Mungbean (*Vigna radiata* (L.) R. Wilczek) and black gram (*Vigna mungo* (L.) Hepper) are profitable leguminous crops grown in tropical and sub-tropical agricultural regions of Australia (Drew et al., 2014). Legumes can fix nitrogen from the atmosphere in association with bacteria known as rhizobia. The bacteria form a symbiotic association with the plant in nodules on their roots. Nitrogen is fixed in the form of ammonia that is easily taken up by plants and incorporated into amino acids and proteins. This nitrogen is used by the legume plant to maximise grain yield, and some residual nitrogen may be left in the soil to the potential benefit of crops that follow (McInnes and Haq, 2003). To maximise nitrogen fixation, strains (genetic variants or subtypes) of rhizobia need to be matched with the legume species and cultivar, and with the soil type and environmental conditions (Bushby, 1988; Bushby and Lawn, 1992). For mungbean, the currently recommended inoculum in Australia is the *Bradyrhizobium* species strain CB1015, originally from India (Bullard et al., 2005). Commercially produced bradyrhizobia matched to leguminous crops are applied as inoculum at planting. Under some conditions bradyrhizobia can persist in the soil, so crops are not always inoculated if being grown on land where mungbeans have been grown in recent seasons (Bullard et al., 2005). Even where commercial inoculum has not been applied, some wild, potentially nitrogen-fixing, bacteria often live in the soil. These can be native or naturalised from deliberate or accidental introductions with crops, pastures or weeds. Generally these wild bradyrhizobia have been thought to be detrimental to leguminous crops, because they sometimes outcompete commercial inoculum, while they may not be as effective in fixing nitrogen (McInnes and Haq, 2003; Drew et al., 2014). However, anecdotal reports have suggested wild bradyrhizobia (*Bradyrhizobium* spp.) are as effective as commercial inoculum for mungbean grown in the Burdekin region of far north Queensland.

If some native bradyrhizobia strains are as effective as commercial strains with regard to nodulation and nitrogen fixation, there may be no need to artificially inoculate in some regions. Simplifying the operations involved in growing mungbeans could encourage more farmers to incorporate this crop into their farming system, potentially bringing both economic and environmental benefits through reducing the need for artificial nitrogen fertilizers. Furthermore, wild bradyrhizobia strains could be investigated as commercial replacements for CB1015.

To distinguish between strains of bradyrhizobia, a relatively new methodology, matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS), has been used in this study. This method involves ionizing intracellular proteins from cell micro-colonies. These proteins are separated according to their mass to charge ratio, which together form a fingerprint characteristic of a bacterial sample. These spectra can be compared with each other or matched against reference strains in databases (Ziegler et al., 2012; Zeigler et al., 2015). Zeigler et al. (2015) demonstrated MALDI-TOF MS could accurately characterize root nodule bacteria and, through comparison with a spectral database, could identify the major rhizobial genera and some species. MALDI-TOF MS provides a rapid, inexpensive method for fingerprinting bradyrhizobia directly from nodules, without the need to produce cultures or have prior knowledge about strains being tested.

The aim of this study was to test the hypothesis that some wild bradyrhizobia are as effective as commercial inoculum for mungbean in the Burdekin region of far north Queensland. Based on the findings, preliminary information can be provided to growers on the effectiveness of commercial inoculation compared with not inoculating and relying on wild bradyrhizobia for nodulation and nitrogen fixation.

2. Materials and methods

2.1. Plant and nodule collection from field sites and field collected soil

Whole mungbean plants, including roots, were collected from two sites in the Burdekin catchment area of north Queensland in November 2015. At each site, plants were at the mid- to late-flowering growth stage at the time of collection. Mungbean plants were collected from Site 1: At 19°46′44″S and 147°11′40″E, a site never inoculated with bradyrhizobia, with a black cracking clay vertisol soil type, and a cropping history that did not include legumes. The mungbean cultivar at this site was Jade-AU. Plants were also collected from Site 2: At 19°31′54.53″S and 147°26′36.52″E, a site inoculated with bradyrhizobia, with a sandy loam soil overlaying delta sand. Mungbean had been grown at this site six years previously, followed by sugarcane. At this site, mungbeans were inoculated with the recommended CB1015 inoculum. The mungbean cultivar at this site was Crystal.

Ten nodules were collected from each of four plants from each Burdekin site for testing protein profiles by MALDI-TOF MS (Mabritec AG). This represented between about 15 and 25% of total nodules from each plant. In general, plants from Site 1 had fewer, larger nodules, compared with those from Site 2. Reference culture plates of CB1015, CB756 (the previously recommended commercial inoculum) and CB1809 (used to inoculate soybean) were included in the analysis. An additional ten nodules from each of the four plants were used to establish cultures on yeast mannitol agar (Hungria et al., 2016).

Soil was collected from a third site near Millmerran on the Darling Downs in southern Queensland, Site 3: At approximately 27°46′29″S and 151°14′49″E, with sandy loam soil that had never grown legumes and had never been inoculated. Soil was collected to a depth of 15 cm. Jade-AU mungbean plants were grown in this soil in pots in a glasshouse. Three plants were collected from pots to which CB1015 had been added and three from pots left uninoculated. Five nodules were collected from each of the three plants grown in pots inoculated with CB1015. Plants were at the early- to mid-flowering stage when harvested.

2.2. Glasshouse experiment

A glasshouse experiment was conducted to compare the effect of two dominant wild strains identified from mungbean plants collected from the Burdekin region of north Queensland and the commercial strain of bradyrhizobia, CB1015, on the growth and nitrogen fixation of two mungbean cultivars and a black gram cultivar. The experiment was conducted in a glasshouse between 17th of February and 28th of March 2016, under natural light and humidity conditions, with cooling restricting maximum temperature to 30 °C.

Five treatments were applied to three cultivars, each replicated in four pots laid out in a randomised block design. Each pot was planted with four plants. Treatments were: (1) uninoculated; (2) uninoculated with added nitrogen (KNO_3 was applied as a 10 g L^{-1} solution, with 5 mL added four times and 10 mL added three times over the experimental period); (3) CB1015 inoculum added to pots; (4) Brady 5 inoculum added to the pots (bradyrhizobia Cluster 5; the most common bradyrhizobium identified from nodules collected from Site 1); and (5) Brady 9 added to pots (bradyrhizobium Cluster 9; the most common bradyrhizobium identified from nodules collected from Site 2 inoculum). The cultivars were Crystal and Jade-AU mungbean and Regur black gram. Sorghum (*Sorghum bicolor* (L.) Moench) was included as a non-nitrogen fixing standard.

Seeds were surface sterilised (70% ethanol for 1 min then 5% sodium hypochlorite for 3 min then rinsed in 6 changes of deionised, sterile water), pre-germinated and planted four per 15 cm pot of sterile sand and vermiculite. Inoculum (cultured rhizobia suspended in sterile 1% sucrose solution) or sterile 1% sucrose solution (2 mL) was added to each seedling at planting. Inoculum concentration was 8.25×10^9 colony forming units per mL (CFU mL^{-1}) for CB1015 and 1.70×10^{10} CFU mL^{-1} for Brady 9, calculated using the Miles and Misra drop plate count method (Vincent, 1970). Concentration for Brady 5 was not determined due to technical issues, but was likely similar to Brady 9. During the six weeks of the experiment, 30 mL per pot