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RESEARCH ARTICLE

Nitrogen fertiliser replacement by single and multi-strain rhizobial inoculants for black gram, green gram and soybean cultivation in Sri Lanka

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Abstract: Various environmental, economic and health problems have arisen in the world due to the continuous application of N-fertilisers for crop production, especially in the third world countries. The current study was undertaken to develop effective rhizobial inoculants for three major legume crops in Sri Lanka, namely *Vigna mungo, Vigna radiata* and *Glycine max* to replace the application of nitrogen fertilisers. Rhizobial isolates were obtained from the root nodules of different cultivars of edible legumes, non-edible wild legumes and wild relatives of *Vigna* spp. Authentication and screening for effectiveness of the isolates were carried out, and five strains were selected as effective isolates and cross inoculated with the three legumes along with a stress tolerant strain, which was previously screened. A pot experiment was followed by a field trial in the dry zone of Sri Lanka under farmers' conditions as single and multi-strain inoculations. The results of the pot experiment indicated that the addition of rhizobial inoculants increased the growth performance in all treatments. In the field trial, both single and multi-strain inoculants gave significant increases in yield, compared to N-fertiliser application in all three crops, *viz*; an increase of the yield from 3 % to 39 % in *V. mungo*, 5 % to 14 % in *V. radiata*, and 4 % to 13 % in *G. max*. In conclusion, the current study has shown that single and multi-strain rhizobial inoculants are capable of completely replacing urea application to *V. mungo, V. radiata* and *G. max,* in Sri Lanka without any yield reduction.

Keywords: *Glycine max*, multi strain, N-fertiliser rhizobial inoculants, single-strain rhizobial inoculants, *Vigna mungo, Vigna radiata.*

INTRODUCTION

Rudimentary inoculation practices, such as moving soil from the previously cultivated fields, with well nodulated legumes, were recommended soon after Hellriegel's 1886 report on 'legumes could fix N_2'. The commercial use of pure cultures of rhizobia as inoculants was then patented in 1896 by Nobbe and Hiltner (Fred *et al*., 1932). Innovations in inoculant product formulations has led the establishment of inoculant manufacturing industries in Europe, North America and Australia. Despite the long history of legume inoculant use and development, only a few farmers in the developing countries of Asia have adopted inoculation into their legume cultivation practices. With time legume inoculation with rhizobia has gained importance in agricultural biotechnology (Aurora *et al*., 2017; Santos *et al*., 2019).

Examination of the legume inoculation technology identifies a potential for rapid penetration into the Asian agricultural input markets. The use of legume inoculation is inexpensive and requires little technical knowledge. The economic and environmental risk associated with inoculant use is minimal (Hettiarachchi *et al*., 2014). Biological nitrogen fixation by legumes is an essential important component of small-holder and low-input cropping systems commonly practiced in Asia. The

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limited use of inoculant in Asia is not surprising because rhizobial inoculant production and marketing in many parts of Asia have been hampered by problems common to many developing countries. However, evidence is available to ascertain that many farmers could derive economic benefit by using inoculants (Ojiem *et al*., 2014; Chekanai *et al*., 2018; Rurangwa *et al*., 2018).

The overuse of chemical nitrogen fertilisers has caused an imbalance in the nitrogen cycle and aggravated the pollution in surface and groundwater. Increased loads of nitrogen fertiliser to freshwater and marine ecosystems have caused eutrophication (Khan *et al*., 2017). There is no doubt that the excessive use of chemical fertiliser and other agrochemicals is not healthy for the ecosystem, environment and human beings. Despite these problems, many farmers continue to use excessive amounts of fertilisers and toxic agrochemicals (pesticides and herbicides), with the false expectation of higher crop yield. Overuse or abuse of artificial fertilisers and other agrochemicals has increased during the last two decades and is widely used by farmers in Sri Lanka. Both the agrochemicals and petrochemicals act as environmental pollutants as well as a contributor of certain diseases, such as chronic kidney disease of unknown etiology (CKDu); a disease mainly affecting the North Central Province and spreading to other areas in Sri Lanka (Rajapakse *et al*., 2016), and cancer which is prevailing in several parts of Sri Lanka. Approximately 3.5 % of the government budget is currently being spent on the agro-subsidies.

In Sri Lanka, the Rhizobial Inoculant Production Facility, initiated at the Department of Botany, University of Peradeniya which has later moved to the National Institute of Fundamental Studies (NIFS), Kandy has reported that the rhizobial inoculants prepared with ground coir dust as the carrier material, could produce high yields of soybean compared to 50 kg per hectare of urea (maximum level recommended by the Department of Agriculture) (Kulasooriya *et al.,*2017). The selling price of a 250 g inoculum packet recommended for use for one acre is Rs. 400 (≈ $ 2). The usual rate of application of urea among soybean farmers is 40 kg/acre and is applied in two doses; 20 kg as basal and 20 kg as a top dressing. As of current market price, this costs Rs. 4250 (≈ $ 22) for a farmer, in addition to the cost of Rs. 3000 (≈ $15) for 5 labourers required for the application of fertiliser. In addition, the farmer has to bear transport cost of urea from the purchasing point to his field. It has also been observed that weed growth is reduced at least by 50 % in crop cultivations inoculated with rhizobial inoculants (Kulasooriya *et al*., 2017) compared to those fertilised with urea. A farmer could save another Rs. 4000 (≈ $ 20) per acre on the cost of agrochemicals needed for weed and pest control. Therefore, the use of the inoculants could make a total saving of Rs. 11,000 (≈ $ 55) per acre to a farmer compared to a field receiving the complete package of recommended agro-chemicals. The use of rhizobial inoculants produced by the NIFS is gaining popularity in Sri Lanka (Kulasooriya *et al*., 2017).

It is a challenge to develop a novel rhizobial inoculant that can promote higher levels of nitrogen fixation under practical field conditions. When the factors such as moisture, temperature, soil pH and salinity become extreme, improving yield becomes complicated (Giddens *et al*., 1982). Natural rhizobia of wild legumes growing under adverse conditions, such as salt stress, elevated temperatures and drought, generally exhibit higher tolerance to such conditions. The rhizobia of wild legumes in arid zones exhibit higher tolerance to prevailing adverse conditions, such as salt stress, elevated temperatures and desiccation (Zahran, 2001). Studies are needed to test the possibility of using effective strains isolated from black gram, green gram and soybean along with stress-tolerant rhizobia as inoculants to increase N-content of these edible legumes under stress conditions and the yield. Arora *et al.* (2017) reported that stress tolerant rhizobial species can be incorporated in developing bioformulations that can withstand salinity, drought and high temperatures. Although research has been undertaken on these lines, only a few contributed in developing inoculants. Ahmad *et al.* (2013) stated that halo-tolerant, auxin producing *Rhizobium* strains improve osmotic stress tolerance in mung bean. Further, Tewari and Arora (2014) recorded the use of exopolysaccharides (EPS) in bioformulation, as EPS protects inoculated rhizobial cells from stress factors such as salinity, desiccation and pH.

Multi-strain rhizobial inoculation of African Acacias under nursery conditions showed a significant increase in total nitrogen than that of the control plants in six out of the seven species (Sutherland *et al.*, 2000). Significant increases in dry weight and total nitrogen over controls ranged from 19 % to 75 % and 11 % to 89 %, respectively. On the other hand, studies conducted on common bean (*Phaseolus vulgaris* L.) showed that three rhizobial strains evaluated were equally effective in the accumulation of total shoot N and that the multi-strain inoculant offered no consistent advantage over the single-strain inoculants. In the United States multi-strain inoculants are produced commercially (Burton *et al*., 1980) to provide a compensatory mechanism to theoretically meet the constraints imposed by the host-

strain-environment interactions, which is impossible or limited with single-strain inoculants. A very few systematic studies have been carried out to evaluate the performances of single-strain and multi-strain inoculants and limited information is available demonstrating the effects of different rhizobial strains for efficient nitrogen fixation.

There is evidence indicating that differential competition for nodule occupancy between strains of *Bradyrhizobium japonicum* in the presence of nitrate in sand cultures (McNeil *et al*., 1982). According to Somasegaran and Bohlool (1990) the nitrogen-fixing effectiveness of multistrain inoculants was found to be determined by both the effectiveness of the component strains and the percentage of the nodules occupied by them. Multistrain formulations were equally effective as most effective single-strain inoculants (Kyei-Boahen *et al.*, 2005) or intermediate between the most and the least effective. The percentage of nodules occupied and the amount of nitrogen fixed by the component strains of a multi-strain inoculant showed highly significant linear correlation.

Black gram (*Vigna mungo* L. Hepper), a member of the Asian *Vigna* crop group is an annual pulse crop native to Central Asia. It is the staple crop in Central and south East Asia. This crop plays an important role in daily diets because of its high protein content (20–25 %), which is double of wheat and three times of rice (FAO, 1994). Green gram (*Vigna radiata*) seed is more palatable, nutritive, digestible and non-flatulent than other pulses grown. Its seeds contain 24.2 % protein, 1.3 % fat and 60.4 % carbohydrates and 118 mg and 340 mg of calcium and phosphorous, respectively per 100 g of seeds. It is rich in vitamin A and considered as a substitute for animal protein and forms a balanced diet when used with cereals (Consideine, 1992). Soybean (*Glycine max* L.) is the most important grain legume crop in the world in terms of total production and international trade. Soybean seeds contain 18 % to 23 % oil and about 38 % to 44 % protein (Hymowizt *et al*., 1998). These three crops are the most important grain legumes cultivated in the rain fed farming systems in dry and intermediate zones of Sri Lanka.

Comparative studies between single-strain and multi-strain inoculants have not been reported in Sri Lanka. A combination of effective high nitrogen fixing rhizobial strains with stress tolerant strains, if shown to be superior and applicable to a wider range of habitats, could be advantageous to any commercial inoculant producer. Therefore, the main objective of the current study was to evaluate the efficiency of single and multistrain rhizobial inoculants on black gram, green gram and soybean in Sri Lanka.

METHODOLOGY

Pot experiment under semi aseptic conditions

A pot experiment was carried out in a plant house under semi aseptic conditions, in the Department of Botany, Faculty of Science, University of Peradeniya, Sri Lanka (7°15'60.00" N 80°35'59.99" E). Three crop legumes, namely, *Vigna mungo* (black gram), *Vigna radiata* (mung bean) and *Glycine max* (soybean), were used in the study.

Preparation of pots

All the pots (black plastic: 43 cm diameter) were surface sterilised using bleaching powder (4 g) dissolved in water (100 mL), followed by rinsing with hot water. A rigifoam (polystyrene foam) disc of 1 cm thickness was placed at the bottom of a pot and filled with washed autoclaved river sand till the level of sand reached 1 inch from the top of the pot. Pots were filled with boiling water and drained 2 to 3 times to remove any soluble nitrogen present.

Seed preparation and germination

Vigna mungo, *V. radiata* and *G. max* seeds were surface sterilised separately by immersing in 70 % alcohol for 1–2 min followed by rinsing 5 times with sterilised water. Seeds were planted after sterilisation. A sterilised plastic tube (diameter ½ inch) was inserted in the middle to supply nutrients and water during the experiment. After planting of seeds the pots were covered using cling wrap prior to inoculation to prevent air borne contamination.

Strain selection

Five rhizobial strains (C8, C10, M5, M6 and VD1) isolated from different host plants (Table 1), which were previously screened (Hettiarachchi *et al*., 2014) as effective nitrogen fixing, and high nodulating strains along with K7 (isolated from *Vigna trilobata*), which was previously screened (Hettiarachchi *et al*., 2013) as a stress tolerant strain were used for inoculation as single and multi-strain inoculant combinations as described in

Table 1: Rhizobial strain isolated host plant, Gene Bank accession number of rhizobial strains, rhizobia cross inoculated crop plant and different combinations of rhizobia used for treatment

Host Plant	Rhizobial strains	Gene Bank Accession No	Crop species	Rhizobial combinations used
Mimosa pudica	M5 (ef)	KF008230	*V. mungo*	M5VD1K7, M5VD1, M5K7, VD1K7, M5, VD1, K7
Vigna dalzelliana	VD1 (ef)	KF008232		
Crotalaria brownie	C10 (ef)	KF008228	*V. radiata*	C10M6K7, C10M6, C10K7, M6K7, C10, M6, K7
Mimosa pudica	M6 (ef)	KF008231		
Crotalaria brownie	C8 (ef)	KF008227	*G. max*	C8VD1K7, C8VD1, C8K7, VD1K7, C8, VD1, K7
Vigna trilobata	K7 (st)			

* ef - effective st - stress tolerant

Table 1. Sequencing of 16S rRNA region was carried out for all effective isolates and submitted to the Gene Bank and Accession numbers were obtained. For K7, DNA fingerprinting was carried out using ERIC primer.

Inoculant preparation

Single-strain rhizobial inocula were cultured separately on ½ Lupin agar (LA) medium. The inocula were separately obtained using 1 % sucrose solution and the resulting rhizobial broths were separately transferred into autoclaved beakers. Multi-strain inoculants were prepared by adding equal volumes from each single-strain, resulting in equal volumes for each treatment.

Inoculation

After 3 days of seeding of the host plant, using a micropipette, 1 mL of the rhizobial inoculum was inoculated directly on the seedlings. As controls, the uninoculated and nitrogen controls were injected with 1 mL of distilled water. Pots were covered with sterilised gravel to prevent air borne contamination.

Experimental design

Four replicate pots were used with three plants per pot (12 plants) for each *Rhizobium* inoculum with nitrogen positive and negative controls. These were arranged in a complete randomised design (CRD).

Plant house conditions, nutrients and watering

Autoclaved water and nutrient solutions (60 mL, devoid of nitrogen) (N^-) were added to all the plants on designated days of the week. In addition, 5 mL of 1 % KNO_3 was added weekly to the nitrogen positive control (N^+) plants (Master class in Rhizobial Technology, 2012).

Plant and nodule assessment

After 8 weeks, the plants were visually rated (scale of 0–10, based on plant growth performance) and then harvested. The roots were carefully washed, and the nodules were detached, and wrapped with absorbent tissue paper and allowed to dry at room temperature. The nodules, shoots and roots were oven-dried at 70 °C for 48 h and weighed using an analytical balance (KeRn ABS - 220-4 No. WB 1210059).

Strain effectiveness

The effectiveness of the strains was calculated using the equation below (Fernando *et al.*, 2005). Dry mass (DM) of the strain-inoculated plant was compared with the N^- and N^+ controls.

$$\% \text{ Strain effectiveness} = \frac{[\text{DM of the inoculated plant} - \text{DM of the N- control}] \times 100}{[\text{DM of the N+ control} - \text{DM of the N- control}]}$$

Field trials in the dry zone

Field description

Field trials were carried out in the dry zone with the same combinations of rhizobial inoculants, as used for the pot experiments. *Vigna mungo* and *V. radiata* fields were in Bulagala dry-zone (7°54'0''N80°37'60''E) and *G. max* fields were located at in Galnawa dry zone (8°02' 02 " N80 °28' 45" E).

Experimental design

A randomised complete block design (RCBD) was used with three replicate blocks per treatment. The plot sizes were 2.74 × 1.52 m for *V. mungo*, 3.66 × 1.52 m for V. *radiata* and 4.27 × 1.83 m for *G. max.* The three rows of plants adjacent to the edge of each plot on all four sides were not considered when taking readings, in order to minimise the edge effect from all sides of each plot. Nitrogen positives and negatives were used as controls.

Single-strain and multi-strain rhizobial inoculum preparation

Single and multi-strain inoculants were prepared similar to the pot experiment. Rhizobial broth cultures were injected into autoclaved, powdered and packeted coir dust (Kulasooriya *et al*., 2007).

Seeding and agronomic practices

Seeds were mixed with the coir dust-based inoculum and sown (Kulasooriya *et al*., 2008). The recommended seed requirement was used. Plots were irrigated once a week and recommended agronomic practices were applied (Table 2). Before the addition of the basal dressing the plants were thinned according to the recommendations. A basal dressing of fertiliser urea (only for the N^+ control), Triple Super Phosphate (TSP) and Muriate of Potash (MOP) (to all the treatments) was added 12 days after sowing. A top dressing of urea was added at flowering, only to the nitrogen positive control.

Nodule and dry weight assessment

Before harvesting (after 8 weeks), 30 plants were visually rated (scale of 0–10, based on plant growth performance). The roots were carefully washed and the nodules were detached and wrapped with absorbent tissue paper and dried at room temperature. The nodules, shoots and roots were oven-dried at 70 °C for 48 h and weighed.

Yield data

Yield and yield component data of remaining plants (*V. mungo* ~ 80 plants, *V. radiata* ~ 100 plants and *G. max* ~ 200 plants) were recorded after 80–90 days of planting.

Statistical analysis

Data on nodule number (NN), nodule dry weight (ND), shoot dry weight (SD), root dry weight (RD), total dry weight (TD), yield and yield component data were subjected to analysis of variance (ANOVA) followed by Duncan's Multiple Range Tests (DMRT) to separate the treatment means.

RESULTS AND DISCUSSION

Pot experiment

Vigna mungo

Morphological differences were observed within treatments showing variations in the effectiveness of the rhizobial treatment. The highest average visual rate (AVR) of nine was observed with multi-strain M5K7 treatment, whereas the lowest (seven) was with the N minus control (N^- control) and rest of the treatments showed a value of eight (Supplementary Table 1). A significant increase in nodulation (ANN) and nodule dry weight (AND) was observed with the multi-strain M5K7 compared to the rest of the treatments. All the rhizobial inoculated treatments, including the N+ control, showed no significant difference in average shoot dry weight (ASD), Average Root Dry weight (ARD) and total dry matter production. These values were significantly higher than the N^- control.

Vigna radiata

Single-strain C10 showed the highest value for AVR (nine), ANN, AND, ASD and ARD (Supplementary Table 2). When considering the total dry matter production, single-strain C10 gave the highest value, which was not significantly different from the value obtained by K7 treatment.

Glycine max

Multi-strain VD1K7 showed the highest value (nine) and the N- showed the lowest value (six) with respect to AVR. With respect to ANN, AND, ASD, ARD and total dry weight, multi-strain VD1K7 gave significantly higher values than all other treatments (Supplementary Table 3).

Table 2: Seedling and agronomic practices: requirements, recommendations

Host Plant	Seed requirement	Spacing between plants	Basal fertiliser (kg/h)	Top dressing (only to N+ control)
V. mungo	30 kg/ha	40 cm × 10 cm	TSP 100, MOP 75, Urea 30 (only to N+ control)	Urea 35 kg/h
V. radiata	30 kg/ha	40 cm × 10 cm	TSP 100, MOP 75, Urea 30 (only to N+ control)	Urea 35 kg/h
G. max	55 kg / ha	40 cm × 5 cm	TSP 150, MOP 75, Urea 50 (only to N+ control)	Urea 50 kg/h

The Strain Effectiveness (SE) values indicated that all the treatments resulted in a high level of effectiveness in *Vigna mungo* (Table 3), according to Beck *et al*. (1993). SE values of the isolates were rated as highly effective (> 80 %), effective (50 – 80 %) and ineffective (< 35%). *Vigna radiata* SE values showed that other than three treatments, all other treatments had high effectiveness. With regards to *G. max*, except C8VD1K7 treatment, all other treatments showed high effectiveness.

The pot experiment was mainly done in order to evaluate the infective ability of the single-strain (SS) and multi strain (MS) inoculants. All the tested inoculants produced nodules in the three crops under investigation. There was no nodulation in the uninoculated controls in the pot experiment, demonstrating that aseptic conditions were met in the experimental set up and maintenance of the plants in the greenhouse was adequate (Bala *et al.*, 2003). Overall, the multi-strain inoculants produced more nodules than the single-strain inoculants in the pot experiments

Similar to previous studies, host biomass production in the pot experiment was used as the criterion for strain effectiveness in N_2 fixation. Compared to N^- application, in all three crops, dry weights increased in inoculated treatments. Hoben (1994) and Peoples *et al.* (2002) explained that shoot dry matter is a good indicator of relative isolate effectiveness. The current results show that the same or higher dry matter could be obtained by rhizobial inoculation without adding N fertilisers.

Field trial

The pot experiments were conducted under aseptic conditions in a greenhouse whereas in the field trials, the inoculant strains had to compete with the indigenous rhizobia and other indigenous microbes present in the soil. Although some strains are effective in N_2 fixation, they may not be able to compete with the indigenous rhizobia and other soil microorganisms for substrates and space in most locations (Santos *et al.*, 2019). Also, the naturally occurring rhizobium populations often occur in high numbers in soil and can compete strongly with the introduced rhizobium inoculants. Better N_2 fixation can be achieved by selecting superior rhizobia. However, the selection of these rhizobia would need to take into consideration not only their N_2 fixing capacity but also the competitive ability against native rhizobia, which are frequently ineffective in N_2 fixation (Hettiarachchi *et al*., 2014). Superior N_2-fixing strains have to outcompete native rhizobia and occupy a significant proportion in nodules. For this to be achieved, rhizobia have to be selected under natural conditions in competition with the native rhizobia (Rengel, 2002) emphasizing the necessity of conducting a field trial.

Plant growth parameters

Vigna mungo

With respect to AVR values, single-strain VD1 and multi-strain M5K7 gave the highest value (nine), whereas the rest of the treatments gave a similar value (eight). With respect to ANN, multi-strain M5VD1K7 and K7 gave significantly higher values than both N controls. When considering the AND values, M5VD1K7 and M5VD1 gave significantly higher values than the N controls (Supplementary Table 4). Multi-strain M5K7 gave a significantly higher ASD value than the N controls. When looking at the ARD values, no significant difference was observed within the treatments, whereas multi-strain M5K7 and single-strain VD1 gave the highest values. Although the multi-strain M5K7 and the single-strain

Table 3: Percentage strain effectiveness (SE) based on total dry matter production of the targeted legume crops in comparison with $^{N+}$ and $^{N-}$ controls (pot experiment).

V. mungo		*V. radiata*		*G. max*	
Strain	%SE	Strain	%SE	Strain	%SE
M5VD1K7	95.71	C10M6K7	91.37	C8VD1K7	39.53
M5VD1	114.87	C10M6	19.32	C8K7	198.97
M5K7	148.58	C10K7	116.87	C8VD1	126.27
VD1K7	95.22	M6K7	26.67	VD1K7	462.18
M5	89.29	C10	245.49	C8	93.84
VD1	103.36	M6	72.83	VD1	219.08
K7	106.28	K7	172.16	K7	198.91
N+	100	N+	100	N+	100
N -	0	N-	0	N-	0

VD1 gave comparatively higher values with respect to total dry matter production, no significant difference was observed within the treatments.

Vigna radiata

Except the multi-strain C10M6, the rest of the rhizobial strain inoculated treatments gave higher AVR values than both N controls, in which multi-strain C10M6K7, C10K7 and M6K7 and single-strain M6 gave the highest value of nine (Supplementary Table 5). Multi-strain C10M6K7 gave the highest values for both ANN and AND, which were significantly higher than both N controls. Multi-strain C10K7 gave the highest value for ASD and ARD and is again significantly higher than both N controls. No significant difference was observed in the total dry matter production, in which multi-strains C10M6K7 and C10K7 gave the highest values.

Glycine max

In *G. max*, multi-strain C8K7 and single-strain VD1 gave the highest values for AVR (9 and 8.5 respectively). Multi-strain VD1K7 gave the highest value for ANN, which was significantly higher than the rest of the treatments (Supplementary Table 6). Some of the rhizobial strain inoculated treatments gave significantly higher values than both controls with respect to AND values. Only the multi-strain C8K7 gave significantly higher values for ASD, ARD and total dry matter production than the rest of the treatments. Others showed significantly similar values.

In the current study, differences in Average Number of Nodules (ANN) were observed for the same treatment in the pot and field experiments. ANN results showed that only two rhizobial inoculants gave lower values than the control values, indicating that these were not able to compete with the native soil rhizobial population. Similar to the previous field trials, with N^+ treatment, only a few number of nodules were observed indicating the negative effect of nitrogen fertiliser application on nodulation of the legume plants (Crews *et al.*, 2004; Xuan *et al*., 2017). Nodule dry weight is essential in strain evaluation, as it serves as an indicator for symbiotic efficiency (Graham *et al.*, 2004).

With reference to AVR values, most of the time, multi- strain inoculants were superior to the single-strain inoculants in both pot and field experiments. This could be attributed to the multi-strain's ability to effectively nodulate and enhance solubilisation of other essential soil minerals, such as phosphorus (Koskey *et al*., 2017). The rhizobial strain inoculated treatments always showed higher AVR values compared to the N^- control. In some instances, even higher than the N^+ control. Since AVR values correlate with other growth and yield parameters investigated, it was found that the growth performance has increased with the addition of both single-strain and multi-strain rhizobial inoculants. Zablotowicz *et al*. (1991) found that increasing rhizobial diversity enhances the shoot dry weight in bean plants. In some trials, there were no significant differences among single-strain and multi strain treatments with respect to the total dry matter production. When considering the infectivity and effectivity in these inoculants with respect to the pot experiment, in *V. mungo* although the infectivity varied within the inoculants the effectivity was fairly high in all the inoculants. In *V. radiata* one single-strain inoculant and in *G. max* one multi strain inoculant showed high infectivity as well as high effectivity. In the field trial, in *V. mungo* both single-strain and multi strain inoculants seems to be effective. In *V. radiata* although the single-strain inoculant performed better in the pot experiment, the multi strain inoculants performed better in the field trial. With respect to *G. max* the multi strain inoculants seem to be better than the single-strain inoculants. A proper combination of different infective and effective Rhizobium strains could enhance nodule, occupancy hence biological fixation of nitrogen. Hungria *et al*. (2000) noted that a combination of specific rhizobia strains, performs better in promoting N^-fixation and growth of different bean cultivars as compared to the use of individual rhizobia strains. These results support the claims made by Kawaka *et al*. (2014) Korir *et al*., (2017) and Koskey *et al*. (2017), that there is a direct association among nodulation, plant growth and nitrogen accumulation in legume plants.

Yield data

Vigna mungo

Multi-strain M5K7 and the single-strain VD1 gave significantly higher NP values compared to the N^+ treatment (Table 4). Most of the rhizobial strain inoculated treatments other than M5 gave significantly higher PL values than the N^- control. Although the values were not significantly higher, most of the rhizobial strain inoculated treatments gave higher number of seeds per pod than both controls. All the rhizobial strain inoculated treatments gave a higher yield than both controls (N^+ and N^-). Significantly higher yield values were obtained from multi-strains M5VD1 and M5K7, and single-strains VD1 and K7.

Vigna radiata

Other than C10, all other rhizobial strain inoculated treatments gave significantly higher values than both controls with respect to NP values (Table 4). When considering PL values, only multi-strain M6K7 and the single-strain K7 showed significantly higher values than the N^- control. When considering the seed yield, all except single-strain C10 gave numerically higher values than the N^+ control. However, only the multi-strain C10K7 and single-strain K7 gave statistically significant higher values.

Glycine max

All the treatments gave significantly higher values than the N- control with respect to NP values, among which multi-strains C8K7 and VD1K7 gave significantly higher values (Table 4). With respect to PL all the treatments gave similar values, which were significantly higher than the N^- control. The multi-strain C8K7 showed a significantly higher number of seeds/pods than the rest of the treatments. All the treatments showed significantly higher value than the N- control, with respect to 100 seed weight.

Table 4: Yield and yield component data

Strain	NP	PL(cm)	No. seeds per Pod	100 seed weight (g)	Seed yield /plant (g)	Estimated seed yield (kg/ha)
A) *V. mungo*						
M5VD1K7	32.71^{bc}	4.74^{a}	7.10^{a}	4.78^{ab}	9.25^{c}	2398.25^{c}
M5VD1	39.29^{ab}	4.85^{a}	7.20^{a}	5.41^{a}	11.96^{a}	3101.44^{a}
M5K7	41.43^{a}	5.17^{a}	7.21^{a}	4.99^{ab}	11.05^{a}	2864.92^{a}
VD1K7	36.86^{ab}	5.04^{a}	7.12^{a}	4.55^{ab}	9.43^{b}	2444.50^{b}
VD1	41.14^{a}	5.13^{a}	6.34^{ab}	5.61^{a}	11.48^{a}	2976.22^{a}
M5	32.29^{bc}	4.40^{ab}	7.50^{a}	4.42^{ab}	8.82^{c}	2285.81^{c}
K7	36.00^{b}	5.089^{a}	7.45^{a}	4.92^{ab}	10.72^{a}	2779.86^{a}
N+	31.56^{bc}	4.74^{a}	6.58^{ab}	4.13^{b}	8.56^{c}	2218.82^{c}
N-	28.11^{c}	4.23^{b}	6.17^{ab}	3.93^{b}	7.48^{d}	1938.53^{d}
B) *V. radiata*						
C10M6K7	21.86^{ab}	7.06^{ab}	7.25^{ab}	5.57^{a}	8.73^{ab}	2183.00^{ab}
C10M6	21.29^{ab}	7.05^{ab}	6.44^{b}	5.00^{ab}	8.43^{ab}	2108.82^{ab}
C10K7	22.63^{ab}	6.89^{ab}	6.22^{b}	5.41^{a}	9.01^{a}	2252.00^{a}
M6K7	21.37^{ab}	7.87^{a}	8.45^{a}	5.03^{ab}	8.58^{ab}	2144.60^{ab}
C10	20.14^{bc}	6.03^{b}	6.57^{b}	4.51^{b}	7.74^{bc}	1935.00^{bc}
M6	22.71^{a}	6.66^{ab}	6.68^{b}	5.00^{ab}	8.44^{ab}	2109.18^{ab}
K7	22.29^{ab}	7.68^{a}	7.60^{a}	5.87^{a}	9.21^{a}	2303.73^{a}
N+	19.67^{c}	7.12^{ab}	8.01^{a}	4.40^{b}	8.02^{bc}	2006.00^{bc}
N-	17.44^{d}	6.57^{b}	6.52^{b}	3.51^{c}	6.80^{d}	1699.50^{d}
C) *G.max*						
C8VD1K7	49.33^{c}	3.25^{a}	2.10^{c}	14.45^{ab}	16.64^{e}	4402.80^{e}
C8VD1	47.22^{d}	3.24^{a}	2.19^{a}	14.95^{ab}	16.86^{e}	4459.55^{e}
C8K7	62.66^{a}	3.03^{a}	2.18^{a}	16.92^{a}	20.42^{a}	5401.56^{a}
VD1K7	60.22^{a}	3.24^{a}	2.10^{c}	16.37^{a}	20.31^{a}	5373.60^{a}
C8	57.77^{b}	3.10^{a}	2.10^{c}	16.04^{a}	18.94^{b}	5011.32^{b}
VD1	52.77^{bc}	3.13^{a}	2.05^{d}	15.91^{a}	17.63^{d}	4663.47^{d}
K7	50.75^{c}	3.11^{a}	2.15^{b}	15.98^{a}	18.84^{b}	4985.03^{b}
N+	55.00^{b}	3.03^{a}	2.11^{c}	15.03^{ab}	18.07^{cd}	4779.52^{cd}
N-	44.29^{e}	2.10^{b}	2.03^{d}	13.07^{C}	14.60^{f}	3859.89^{f}

NP = Average number of pods; PL = average pod length values in the same column [separately for sections A), B) and C)] followed by the same letter are not significantly different at 5 % probability level.

Multi-strain C8K7 and VD1K7, and single-strain C8 and K7 gave significantly higher yield values compared to the N^+ control.

Comparison of yield performance considering all three crops investigated under inoculation with fertiliser applications ascertains that certain strains (both single and multi) had given higher responses for the three crops tested. For *V. mungo*, all the inoculants gave higher values than the N^+ fertiliser application in which two multi strain inoculants (M5VD1 and M5K7) and two SS inoculants (VD1 and K7) gave comparatively higher values than the N^+ fertiliser application. In *V. radiata*, only one single-strain inoculant (VD1) gave a lower value than N^+ fertiliser application. In *G. max*, two multi-strain inoculants (M5VD1K7 and M5VD1) and one single-strain inoculant gave a lower value than N^+ fertiliser application (Figure 1).

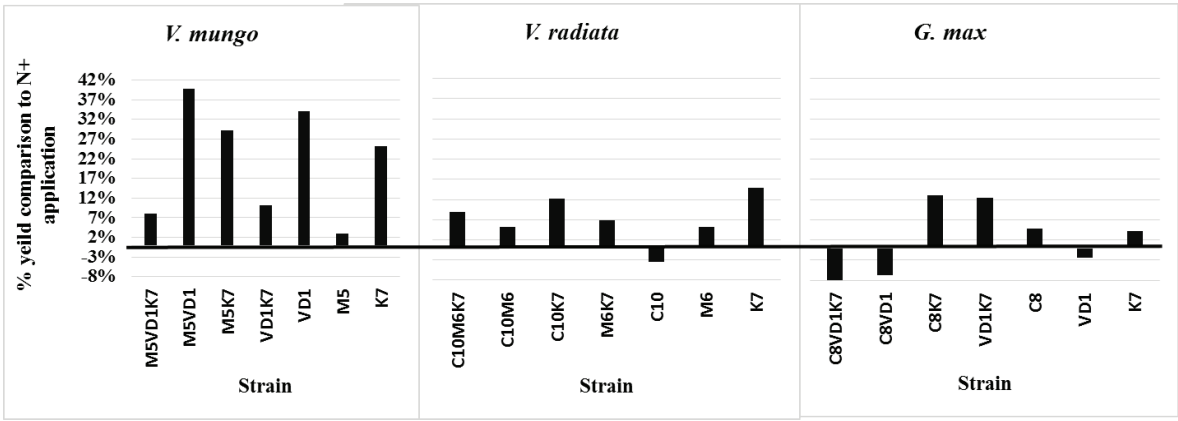

Figure 1: Percentage yield increase/decrease in comparison to N^+ fertiliser application (zero line indicates N^+ fertilizer)

According to the results obtained, both the single-strain and multi-strain inoculants are capable of completely replacing N^- fertiliser application. However, multi-strain inoculants seem to be superior to single-strain inoculants (Figure 2).

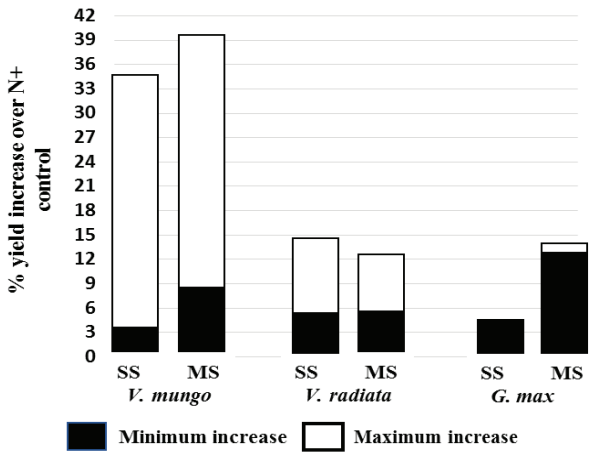

Figure 2: Yield comparison between single-strain and multi strain rhizobial inoculants

In Australia, in early studies with rhizobial inoculation, only single-strain inoculants were used as inoculants to prevent possible dominance and antagonistic effects of a particular strain in the mixture (Schwinghamer *et al*., 1979) to diagnose loss of effectiveness, and to facilitate quality control (Thompson *et al*., 1980). However, in the United States, multi strain inoculants were produced commercially (Burton *et al*., 1980) to provide a compensatory mechanism to theoretically meet the constraints imposed by the host-strain-environment interactions, which is impossible with single-strain inoculants.

Unlike many free-living diazotrophs, rhizobia are able to exhibit highly efficient nitrogen fixation, only when they are in the host nodule cells in endosymbiotic form as bacteroid (Hakoyama *et al*., 2009). Rhizobial nitrogen-fixing activity is restricted to symbiotic bacteroids, and free-living rhizobia do not fix atmospheric nitrogen, a feature unique to legume/rhizobium symbiosis (Kneip *et al*., 2007).

It appears that the effectiveness of mixed inoculants is dependent on the effectiveness and competitiveness of the strains in the mixture. Although much work has been documented in rhizobial ecology in evaluating the success of inoculant strains (measured as percent nodule occupancy) in numerous competition experiments, meaningful interpretations of nodule occupancies in relation to the nitrogen fixed by the nodule occupants have apparently not been quantified. Furthermore, it is noted that little information has been reported on rhizobial inoculants in the past decade (Aurora *et al*., 2017) and this limits a thorough discussion on this area of study.

Alhough the increase of nodulation through rhizobial inoculation resulted in elevated growth performances and enhanced yield, multi locational field testing over two or more seasons is needed before these inoculants are recommended for farmer use. Santos *et al*. (2019) states that farmers are more receptive to use of inoculants due to high-quality products and the availability of multi-strains, which cost less than chemical fertilisers. In the context of sustainable agriculture, microbial inoculants play a major role to alleviate the negative environmental impact caused by chemical fertilisers (Santos *et al*., 2019).

CONCLUSIONS

In conclusion, the current study has shown that the addition of rhizobial inoculants, both single-strain and multi-strain has completely replaced urea applications to *V. mungo*, *V. radiata* and *G. max* crop cultivation in Sri Lanka while increasing the yields of all three crops investigated, significantly.

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Supplementary data

Supplementary Table 1: Plants visual rate, nodule number, nodule dry weight, shoot dry weight and root dry weight of *V. mungo*

STRAIN	AVR	ANN	AND	ASD	ARD
M5VD1K7	8.00	5.9701^{c}	0.0097^{bc}	0.6560^{a}	0.0937^{a}
M5VD1	8.00	11.6667^{b}	0.0133^{b}	0.6939^{a}	0.0991^{a}
M5K7	9.00	18.3333^{a}	0.0198^{a}	0.7605^{a}	0.1087^{a}
VD1K7	8.00	8.0000^{bc}	0.0122^{b}	0.6550^{a}	0.0936^{a}
M5	8.00	4.2812^{c}	0.0095^{bc}	0.6433^{a}	0.0919^{a}
VD1	8.00	11.7252^{b}	0.0130^{b}	0.6711^{a}	0.0958^{a}
K7	8.00	9.3521b^{c}	0.1200^{b}	0.6769^{a}	0.0967^{a}
N+	8.00	0.0000^{d}	0.0000^{d}	0.6645^{a}	0.0949^{a}
N-	7.00	0.0000^{d}	0.0000^{d}	0.4668^{b}	0.0667^{b}

AVR = average visual rate, ANN = average no. nodules/plant, AND = average nodule dry weight (g/plant), ASD = average shoot dry weight (g/plant), ARD = average Root dry weight (g/plant). Values in the same column followed by the same letter are not significantly different at 5 % probability level.

Supplementary Table 1: Plants visual rate, nodule number, nodule dry weight, shoot dry weight and root dry weight of *V. radiata*

STRAIN	AVR	ANN	AND	ASD	ARD
C10M6K7	8	12.8333^{c}	0.0133^{b}	0.6571^{b}	0.0939^{b}
C10M6	8	6.4721^{d}	0.0073^{c}	0.5439^{bc}	0.0777^{bc}
C10K7	8.5	17.3562^{b}	0.0153^{b}	0.6972^{b}	0.0996^{b}
M6K7	8	13.2542^{c}	0.0134^{b}	0.5545^{bc}	0.0794^{bc}
C10	9	21.0000^{a}	0.0196^{a}	0.8993^{a}	0.1285^{a}
M6	8	6.7522^{d}	0.0075^{c}	0.6280^{b}	0.0897^{b}
K7	8.5	16.6452^{b}	0.0169^{ab}	0.7841^{ab}	0.1120^{ab}
N+	8	0.0000^{e}	0.0000^{d}	0.6707^{b}	0.0958^{b}
N-	7	0.0000^{e}	0.0000^{d}	0.5135^{bc}	0.0734^{bc}

AVR = average visual rate, ANN = average number of nodules, AND = average nodule dry weight (g/plant), ASH = average shoot height (cm), ARL= average root length (cm), ASD = average shoot dry weight (g/plant), ARD = average root dry weight (g/plant). Values in the same column followed by the same letter are not significantly different at 5 % probability level.

Supplementary Table 3: Plants visual rate, nodule number, nodule dry weight, shoot dry weight and root dry weight of *G. max*

STRAIN	AVR	ANN	AND	ASD	ARD
C8VD1K7	8	5.7210^{d}	0.0062^{d}	0.8856^{b}	0.1265^{b}
C8K7	7.5	18.4572^{b}	0.0162^{b}	1.0896^{b}	0.1557^{b}
C8VD1	7.5	6.0000^{d}	0.0078^{d}	0.9965^{b}	0.1425^{b}
VD1K7	9	27.4251^{a}	0.0211^{a}	1.4262^{a}	0.2038^{a}
C8	8	16.8521^{bc}	0.0129^{bc}	0.9551^{b}	0.1364^{b}
VD1	8	19.7542^{b}	0.0139^{bc}	1.1152^{b}	0.1593^{b}
K7	8	13.3342^{c}	0.0132^{bc}	1.0895^{b}	0.1556^{b}
N+	8	0.0000^{e}	0.0000^{e}	0.9630^{b}	0.1376^{b}
N-	6	0.0000^{e}	0.0000^{e}	0.8350^{bc}	0.1193^{b}

AVR = average visual rate, ANN = average number of nodules, AND = average nodule dry weight (g/plant), ASH = average shoot height (cm), ARL= average root length (cm), ASD = average shoot dry weight (g/plant), ARD = average root dry weight (g/plant). Values in the same column followed by the same letter are not significantly different at 5 % probability level.

Field experiment

Supplementary Table 4: Plants visual rate, nodule number, nodule dry weight, shoot dry weight and root dry weight of *V. mungo*

STRAIN	AVR	ANN	AND	ASD	ARD
M5VD1K7	8	51.00^{a}	0.1386^{a}	11.6545^{bc}	1.6649^{ab}
M5VD1	8	47.33^{ab}	0.1111^{b}	13.0484^{b}	1.8641^{ab}
M5K7	9	29.29^{c}	0.0878^{c}	15.7730^{a}	2.2533^{a}
VD1K7	8	38.88^{b}	0.0857^{c}	12.7199^{b}	1.8171^{ab}
M5	8	33.50^{c}	0.0806^{c}	12.5093^{b}	1.7871^{ab}
VD1	9	46.22^{ab}	0.0904^{c}	15.2458^{ab}	2.1779^{a}
K7	8	48.67^{a}	0.0901^{c}	12.7244^{b}	1.8177^{ab}
N+	8	42.86^{b}	0.0803^{c}	11.5312^{bc}	1.6473^{ab}
N-	8	39.67^{b}	0.0700^{c}	11.6473^{bc}	1.6341^{ab}

AVR = average visual rate, ANN = average number of nodules, AND = average nodule dry weight (g/plant), ASH = average shoot height (cm), ARL= average root length (cm), ASD = average shoot dry weight (g/plant), ARD = average root dry weight (g/plant) Values in the same column followed by the same letter are not significantly different at 5% probability level.

Supplementary Table 5: Plants visual rate, nodule number, nodule dry weight, shoot dry weight and Root dry weight of *V. radiata*

STRAIN	AVR	ANN	AND	ASD	ARD
C10M6K7	9	47.5^{a}	0.0580^{a}	4.2858^{ab}	0.6123^{ab}
C10M6	7	44.33^{ab}	0.0378^{bc}	3.2323^{b}	0.4618^{b}
C10K7	9	39.21^{b}	0.0465^{b}	4.4582^{a}	0.6369^{a}
M6K7	9	43.16^{ab}	0.0443^{b}	3.8511^{b}	0.5502^{b}
C10	8	40.67^{b}	0.0331^{c}	3.4288^{b}	0.4898^{b}
M6	9	40.89^{b}	0.0413^{b}	3.7989^{b}	0.5427^{b}
K7	8.5	39.56^{b}	0.0489^{b}	4.1168^{ab}	0.5881^{b}
N+	8	34.57^{bc}	0.0283^{c}	3.6981^{b}	0.5283^{b}
N-	7.5	29.75^{c}	0.0277^{c}	3.2204^{b}	0.4601^{b}

AVR = average visual rate, ANN = average number of nodules, AND = average nodule dry weight (g/plant), ASH = average shoot height (cm), ARL= average root length (cm), ASD = average shoot dry weight (g/plant), ARD = average root dry weight (g/plant). Values in the same column followed by the same letter are not significantly different at 5 % probability level.

Supplementary Table 6: Plants visual rate, nodule number, nodule dry weight, shoot dry weight and root dry weight of *G. max*

STRAIN	AVR	ANN	AND	ASD	ARD
C8VD1K7	7.5	50.71^{b}	0.2462^{c}	6.8668^{b}	0.9810^{b}
C8K7	9	53.71^{b}	0.3927^{ab}	8.9506^{a}	1.2787^{a}
C8VD1	7	53.63^{b}	0.2254^{c}	6.6441^{b}	0.9492^{b}
VD1K7	8	73.00^{a}	0.4262^{a}	7.2959^{b}	1.0423^{b}
C8	8	55.38^{b}	0.3117^{b}	6.9658^{b}	0.9951^{b}
VD1	8.5	59.57^{b}	0.2865^{b}	7.2616^{b}	1.0374^{b}
K7	8	56.33^{b}	0.3152^{b}	7.2634^{b}	1.0376^{b}
N+	8	43.43^{c}	0.2205^{c}	6.8877^{b}	0.9840^{b}
N-	7	30.00^{cd}	0.2065^{c}	6.4960^{b}	0.9280^{b}

AVR = average visual rate, ANN = average number of nodules, AND = average nodule dry weight (g/plant), ASH = average shoot height (cm), ARL= average root length (cm), ASD = average shoot dry weight (g/plant), ARD = average root dry weight (g/plant). Values in the same column followed by the same letter are not significantly different at 5 % probability level.