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Arbuscular mycorrhizal fungi and *Bradyrhizobium* co-inoculation enhances nitrogen fixation and growth of green grams (*Vigna radiata* L.) under water stress

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ABSTRACT

Green gram (*Vigna radiata* L. Wildzek) is a neglected crop with great potential to boost food security among small scale farmers. Inoculation of this crop with beneficial soil microbiota can sustainably improve its production especially under water stress conditions. Here, we aimed at determining the effect of bradyrhizobia and arbuscular mycorrhizal fungi (AMF) co-inoculation on growth and nitrogen fixation of green grams under water stress conditions. Potted green grams variety KS20 were planted in sterilized soil and inoculated with *Bradyrhizobium, Funneliformis mosseae, Rhizophagus irregularis*, a commercial consortium of four AMF isolates (*Rhizophagus irregularis, Funneliformis mosseae*, *Glomus etunicatum* and *Glomus aggregatum*) and a consortium of the four aforementioned AMF isolates and *Bradyrhizobium*. The plants were subjected to three water stress levels, irrigation after interval of 4 days, 8 days and 12 days and maintained for 50 days. After harvesting, the plants were measured for nodulation, percentage root mycorrhizal colonization, growth parameters and shoot nitrogen (N), phosphorous (P) and potassium (K). Remarkably, all AMF inoculations significantly $P \leq 0.05$ increased shoot dry weight (SDW), root dry weight (RDW) and P compared to *Bradyrhizobium* and control plants under all the watering regimes. Inoculation with *Rhizophagus irregularis* resulted to highest shoot dry weight, root dry weight and P under water regime 3, which provided the most intense water stress condition. Moreover, strong positive correlation ($R^2 = 0.8765$, $P = 0.006$) was observed between shoot dry weight, P and AMF root colonization under all watering regimes.

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Bradyrhizobium; arbuscular mycorrhizal fungi; green grams; agroecosystem resilience

Introduction

Green grams (*Vigna radiata* L. Wildzek), a native of India, is currently an important annual leguminous crop in Kenya. This crop is a source of food, animal feeds and income (Mathu et al. 2012), especially in arid and semi-arid tropics (ASAL). Green grams also play a significant role in nutrient cycling, especially biological nitrogen fixation (Swaminathan, Singh, and Nepalia 2012) in ASAL areas. However, maximum production of green grams has not been realized due to soil infertility and inadequate rainfall.

Beneficial rhizospheric microorganisms such as arbuscular mycorrhizal fungi (AMF) and rhizobia provide essential agroecosystem services which include enhanced crop nutritional

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absorption and tolerance to both abiotic and biotic stresses (Oruru and Njeru 2016; Nyamwange et al. 2018). Inoculation of green grams with bradyrhizobia enriches the soil with nitrogen which is expensive to smallholder farmers if done by procuring inorganic fertilizers (Ouma et al. 2016). The bradyrhizobia increases plant nitrogen content which in turn enhances plant growth and grain production (Koskey et al. 2017). Co-inoculation of green grams with AMF and bradyrhizobia benefits the plant by promoting a tripartite symbiosis where AMF absorb nutrients especially phosphates that are used to generate energy required for biological nitrogen fixation. In return, the leguminous plant provides AMF and bradyrhizobia with photosynthetic carbohydrates (Habibzadeh et al. 2013).

Inoculation of green grams with bradyrhizobia and AMF could enhance productivity under drought stress since AMF makes the plant to have adequate hydration in all tissues evidenced by higher water content of the plant (Miransari 2014). Colonization of green grams by AMF increases drought resistance by enhancing root hydraulic conductivity and increased root growth which makes the plant to have large surface area for absorption of nutrients and water (Bhuvaneswari et al. 2014). Moreover, AMF enhances resistance to drought by increasing uptake of essential plant nutrients such as N and P, which in turn promotes faster growth of host plant enhancing drought resistance (Abdullahi and Sheriff 2013; Nyamwange et al. 2018). Symbiotic association of legumes with both AMF and rhizobia bacteria enhanced symbiotic nitrogen fixation, increased nodule number and nodule dry weight and enhanced shoot nitrogen content (Barea, Azcon-Aguilar, and Azcon-Aguilar 1992).

In this work, we tested the hypothesis that co-inoculation of green grams with bradyrhizobia and AMF will increase delivery of agroecosystem services promoting plant growth under water stress conditions. The specific objectives were to; (1) determine the effect of irrigation regimes and microbial inoculation on nodulation and AMF root colonization, (2) examine the effect of irrigation regimes and microbial inoculation on root dry weight and shoot dry weight, and (3) assess the effect of irrigation regimes and microbial inoculation on shoot P, N and K.

Material and methods

Experimental soil

Soil samples for greenhouse experiment were obtained from five farms in Kitui Central Sub-County, Kitui County which lies in 1°22′S 38°01′E, about 160 km east of Nairobi County. Kitui County is hot and dry with temperatures ranging between 14°C and 34°C. The county receives between 500 mm and 1050 mm of rainfall annually, with an average of 900 mm of rainfall per annum. This represents one of the main ASAL areas predominated by smallholder farmers where agriculture is the main economic activity. Green grams are among the key crops that are grown for food and income generation. The soil samples were mixed to obtain a homogenous soil sample for determination of soil physicochemical properties which were as follows; slightly acidic soil pH of 5.94, 0.25% N, 0.014% P, 0.00028% K, 2.90% C, 9.20% Ca and 3.65% Mg.

Fungal inoculums

The AMF inoculums were made up of Myco apply custom super concentrate powder containing 220,200 mycorrhizal propagules gram^{-1}. The consortium of AMF consisting of 4 mycorrhizal species, *(Glomus aggregatum, Funneliformis mosseae, Rhizophagus irregularis* and *Glomus etunicatum)* was donated by Symyco, a joint venture between Symbiotic Sciences based in New Delhi, India and Mycorrhizal Application Inc., based in Grants Pass, Oregon. The single isolates of *Rhizophagus irregularis* and *Funneliformis mosseae* inoculums were obtained from International Bank of the Glomeromycota INRA, France. One hundred and sixty grams of each inoculum was

multiplied in roots of Bermuda grass (*Cynodon dactylon*) in Kenyatta University greenhouse for four months. Thereafter, fragments of infected roots of *Cynodon dactylon*, spores and soil medium in which the inoculum was cultured and fragments of AM fungi hyphae were used as crude AMF inoculums.

Bradyrhizobia field trap cultures

The trapping of native bradyrhizobia was carried out between December 2015 and January 2016 in five agricultural farms in Kitui Central Sub-County. Green gram seeds obtained from Kenya Seed Company Limited, were planted in the five farms that were prepared by plowing on the onset of rains. Ten plants from each of the farms were randomly sampled and harvested forty days after germination by carefully digging out the root system with the nodules intact. The root nodules with red or pink coloration were carefully plucked from the plant and put in sterile vials with cotton wool and silica gel for desiccation. The vials were screwed with caps and transported to Kenyatta University Microbiology laboratories for *Bradyrhizobium* isolation and storage.

Isolation of bradyrhizobium in broth cultures

The air dried intact round nodules were placed into a sample bottle containing cool sterile water and allowed to imbibe water for two hours. They were then, immersed in 70% ethanol for 2 minutes and 3% sodium hypochlorite for 3 minutes (Somasegaran and Hoben 1994). The sterilized nodules were rinsed with 7 changes of sterilized water. They were put together in a petri-dish with a drop of sterile water and crushed with a sterile glass rod into a nodule suspension. A loopful of the suspension was streaked on yeast extract manitol agar (YEMA) plate and incubated at 28°C in the dark for 6 days. Well isolated colonies were streaked in YEMA with Congo Red and YEMA with Bromothymol Blue (BTB), incubated and observations made daily for 12 days. The blue colonies formed in YEMA with BTB were sub-cultured to get pure bradyrhizobia cultures. To obtain a broth culture, a loopful of pure bradyrhizobia colonies was transferred into McCartney bottles with yeast extract mannitol broth (YEMB) and incubated for 5 days at 28 °C. The growth of *Bradyrhizobium* in the broth was revealed by observing the turbidity of the medium.

Experimental design

The experimental design used in the greenhouse was randomized complete block design (RCBD) with three irrigation regimes as the main treatment and six microbial inoculations as the sub-treatments. The three irrigation regimes included; Regime I - irrigated once after every 4 days, regime II - irrigated once after every 8 days and regime III - irrigated once after every 12 days with 250 ml of distilled water. Microbial inoculant treatments were, *Bradyrhizobium* (B), *Funneliformis mosseae* (M1), *Rhizophagus irregularis* (M2), a commercial consortium (M3) of four isolates (*Rhizophagus irregularis, Funneliformis mosseae*, *Glomus etunicatum*, *Glomus aggregatum*), co-inoculation with bradyrhizobia and the commercial consortium (B + M3) of the four isolates and control (C) which was left uninoculated. The experiment was replicated six times and a total of 108 pots were used each measuring 30 cm height and 22 cm diameter.

Planting and maintenance of greenhouse bioassays

Mycorrhizal inoculation was done by mixing sterilized soil with inoculum at a depth of 3 to 6 cm before sowing. The AM fungi inoculum was mixed thoroughly with planting soil to ensure even

Table 1. Effect of water stress and microbial inoculation on growth parameters.

Treatment	% MC	NN	NDW (mg)	SDW ($g^{plant-1}$)	RDW ($g^{plant-1}$)
Microbial inoculation					
C	0.0	0.0	0.0	0.48 ± 0.03d	0.07 ± 0.01d
B	0.0	4.50 ± 0.81b	8.00 ± 2.00b	1.03 ± 0.08c	0.12 ± 0.02c
B + M3	48.61 ± 1.75c	11.83 ± 1.18a	30.30 ± 4.00a	1.71 ± 0.01ab	0.19 ± 0.01b
M1	56.23 ± 2.13b	0.0	0.0	1.86 ± 0.14a	0.22 ± 0.01a
M2	77.01 ± 2.54a	0.0	0.0	1.98 ± 0.13a	0.25 ± 0.02a
M3	32.24 ± 1.04d	0.0	0.0	1.46 ± 0.09b	0.16 ± 0.07b
Water stress					
R1	31.73 ± 3.50b	7.67 ± 0.83a	18.50 ± 3.00a	1.76 ± 0.11a	0.21 ± 0.02a
R2	34.89 ± 4.89ab	11.50 ± 0.92a	27.50 ± 2.00a	1.31 ± 0.09b	0.18 ± 0.01b
R3	36.93 ± 5.06a	5.33 ± 0.58b	11.50 ± 2.00b	1.19 ± 0.08b	0.12 ± 0.01c
P values for main factors and their interactions					
Microbial inoculation	<0.001	<0.001	<0.001	<0.001	<0.001
Water stress	0.006	<0.001	<0.001	<0.001	<0.001
Microbial inoculation × water stress	0.242	<0.001	<0.001	0.011	0.001

MC, myorrhizal colonization; SDW, shoot dry weight; NN, Nodule number; NDW, Nodule dry weight; RDW, Root dry weight; C, control; B, *Bradyrhizobium*; M1, *Funneliformis mosseae*; M2, *Rhizophagus irregularis;* M3, Consortium of (*Funneliformis mosseae, Rhizophagus irregularis, Glomus aggregatum* and *Glomus etunicatum*); B + M3, co-inoculation of *Bradyrhizobium* and the consortium. The means followed by same letter within the same column are not significantly different at $P \leq 0.05$ (Tukey's HSD).

distribution of inoculum. Two surface sterilized green gram seeds were planted per pot, watered immediately and thereafter watered daily with distilled water. A total of 108 pots were used in this experiment. Three days after germination, the seedlings were thinned to one per pot, and inoculated with *Bradyrhizobium* by placing 1 ml (10^9 cfu/ml) of bradyrhizobia broth into the pot just close to the base of the seedling. After 8 days, the plants were put in different irrigation regimes up to 50^{th} day.

Plant sampling and analyses

The plants were sampled 50 days after sowing and carefully washed to preserve the roots. Nodules were plucked from the roots and their number recorded after counting. The shoots and roots were carefully separated. About hundred pieces of root segments from each treatment were washed thoroughly with tap water and put in falcon tubes where they were cleared using 10% potassium hydroxide in water bath at 80°C for 15 min. The roots were neutralized in 2% hydrochloric acid solution and stained in 0.05% trypan blue in lactic acid. The roots were observed for AMF colonization by use of dissecting microscope at ×40 magnification using gridline intersect method (Giovannetti and Mosse 1980). Dry weight of roots, shoots and nodules was determined after drying the samples in the oven at 65°C to a constant weight. Shoot samples from each treatment were dried followed by ashing.

Determination of shoot nutrients

About 1 gram of the ground samples from each treatment was digested in 1 ml of concentrated sulfuric acid at 300°C prior to nutrient analysis. Total nitrogen was determined using Kjedahl method by measuring the amount of ammonium ions transformed from organic N (Bremner 1982). The shoot P was determined by colorimetric method by determining the concentration in parts per million (Kissel and Sonon 2008). The shoot K content was determined in neutral ammonium acetate extract after sulfuric acid digestion (Page, Miller, and Keeney 1982).

Table 2. Effect of inoculation of AMF and *Bradyrhizobium* on shoot nutrients.

Treatment	Nitrogen (%)	Phosphorous (ppm)	Potassium (ppm)
Microbial inoculation			
C	1.14 ± 0.05b	762.50 ± 35.74f	8500.00 ± 116.93c
B	1.63 ± 0.17ab	952.78 ± 42.97e	8800.00 ± 186.30c
M1	1.98 ± 0.16a	2254.00 ± 103.34b	10083.30 ± 122.42ab
M2	2.13 ± 0.14a	2475.00 ± 140.92a	10488.30 ± 278.82a
M3	1.41 ± 0.06b	1258.33 ± 105.84d	8833.30 ± 113.80c
B + M3	1.64 ± 0.09ab	1647.89 ± 112.92c	9533.30 ± 171.33b
Water stress			
R1	1.66 ± 0.12a	1120.06 ± 74.59c	9683.30 ± 211.60a
R2	1.56 ± 0.09a	1852.28 ± 113.54a	9083.30 ± 153.27b
R3	1.72 ± 0.09a	1697.92 ± 146.99b	9352.50 ± 127.28ab
P values for main factors and their interactions			
Microbial inoculation	<0.001	<0.001	<0.001
Water stress	0.551	<0.001	0.004
Microbial inoculation × water stress	0.822	<0.001	0.830

C, control; B, Bradyrhizobium, M1, Funneliformis mosseae; M2, Rhizophagus irregularis; M3, Consortium of (Funneliformis mosseae, Rhizophagus irregularis, Glomus aggregatum and Glomus etunicatum); B + M3, co-inoculation of Bradyrhizobium and the consortium; R1, regime 1; R2, regime 2; R3, regime 3. The means followed by same letter within the same column are not significantly different at $P \leq 0.05$ (Tukey's HSD).

Data analyses

The data on plant biomass, nodule dry weight, nodule number and shoot N, P and K were checked for homogeneity of variance using Bartlett test followed by two-way analysis of variance (ANOVA). Data on percentage AMF colonization were arcsine ($\sqrt{x}$) transformed to fulfill the assumptions of ANOVA. The relationship between changes in plant biomass, shoot nutrients and root AMF colonization was determined by Pearson correlation coefficient. Wherever feasible, post hoc test was performed using Tukey's HSD test ($P < 0.05$). All statistical analyses were performed with SAS software (version 9.1).

Results

Effect of irrigation regimes and microbial inoculants on green grams nodulation and percentage mycorrhizal colonization

Mycorrhizal root colonization only occurred in AMF inoculated treatments showing that all the AMF inocula were active. Accordingly, percentage AMF colonization was different ($P < 0.001$) across all AMF treatments with M2 recording the highest percentage colonization of 77.01% (Table 1). Interestingly, co-inoculation of bradyrhizobia with AMF consortium (B + M3) had higher effect on AMF colonization than AMF consortium (M3) alone. Watering regime R3 recorded the highest percentage of mycorrhizal colonization with an average of 36.93% while watering regime R1 recorded the lowest percentage of mycorrhizal colonization of 31.73%. Nodule dry weight and nodule number were affected ($P < 0.001$) by bradyrhizobia and AMF inoculation whereby plants inoculated with bradyrhizobia alone had lower number of nodules than plants with AMF co-inoculated with bradyrhizobia (B + M3). Water stress affected ($P < 0.001$) the nodule number. Plants subjected to watering regime R2 had the highest nodule number while watering regime R3 had the least number of nodules. Shoot dry weight and root dry weight were affected ($P < 0.001$) by microbial inoculation and water stress. Inoculation of *Funneliformis mosseae* (M1) and *Rhizophagus irregularis* (M2) produced the highest shoot dry weight and root dry weight whereas the control treatment recorded the lowest shoot dry weight and root dry weight. Water stress x microbial inoculation interaction had an effect ($P < 0.001$) on the number of nodules in the plants, nodule dry weight and root dry weight (Table 1).

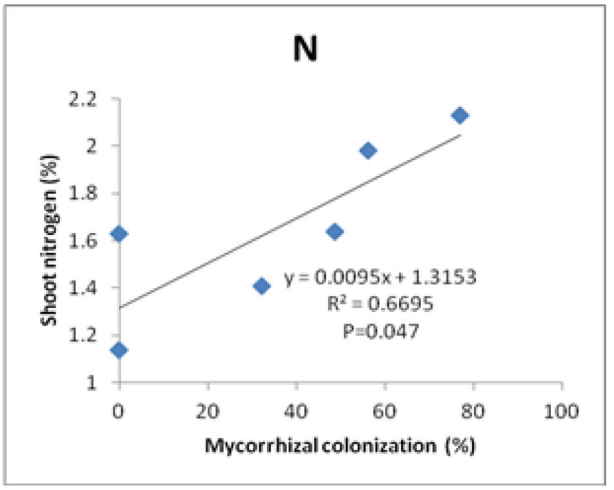

Figure 1. Correlation between root mycorrhizal colonization and shoot nitrogen.

Effect of irrigation regimes and microbial inoculants on N, P and K uptake

The shoot N, P and K were influenced ($P < 0.001$) by bradyrhizobia and AMF inoculation whereby M2 recorded the highest amount of shoot N, P and K compared to control treatments (Table 2). The amount of N in plants inoculated with B + M3 and M3 was not different. Moreover, there was positive correlation between percentage mycorrhizal root colonization and shoot nitrogen content ($R^2 = 0.6695$, $P = 0.047$) as shown in Figure 1.

Water stress affected ($P < 0.001$) shoot P and K with irrigation regime R2 recording the highest amount of P with an average of 1852.28 ppm while regime R1 recorded the lowest amount of P with an average of 1120.06 ppm. Regime R1 recorded the highest amount of shoot K with an average of 9683.30 ppm whereas regime R2 recorded the lowest amount of shoot K with an average of 9083.30 ppm. Moreover, correlation analysis showed a strong positive correlation ($R^2 = 0.8814$, $P = 0.006$) between percentage root AMF colonization and shoot potassium content (Figure 2). Microbial inoculation x water stress interaction affected ($P < 0.001$) the shoot P (Table 2).

Effect of irrigation regimes and microbial inoculants on green gram growth parameters

Interaction of watering regimes with AMF co-inoculated with bradyrhizobia (B + M3) produced higher SDW than each of the two inoculants alone. Interactions of watering regime 3 with *R. irregularis* (M2) had the highest root dry weight while interaction of regime R3 with AMF consortium (M3) had least RDW among the AMF inoculants. Co-inoculation of bradyrhizobia and AMF consortium (B + M3) had higher RDW on interaction with regime 3 compared to interaction of bradyrhizobia and M3 independently (Table 3). Bradyrhizobia (B) recorded the highest P on interaction with watering regime R2 while the lowest value was obtained on interaction with regime R1. M1 had the highest value of P on interaction with R3 while the lowest amount of P was absorbed on interaction with regime R1. AMF consortium (M3) had the highest amount of phosphorus on interaction with watering regime R2 while the lowest absorption of phosphorus on interaction with M3 occurred in regime R1. Co-inoculation of AMF consortium with bradyrhizobia had the highest absorption of phosphorus on interaction with watering regime R3 while the lowest phosphorus absorption was observed on interaction with regime R1 (Table 3).

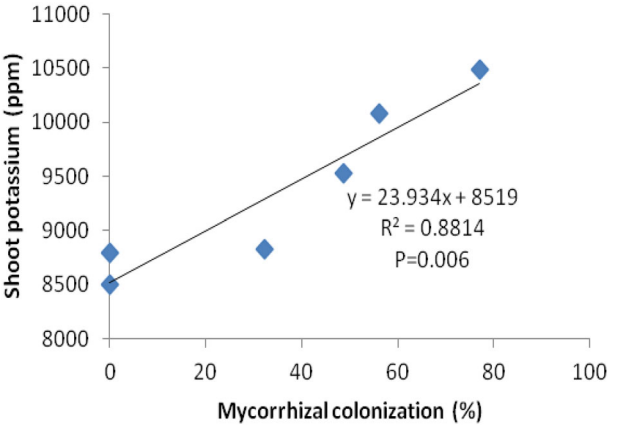

Figure 2. Correlation between percentage root mycorrhizal colonization and shoot potassium (ppm).

Table 3. Effect of interaction between watering regime and microbial inoculation on growth parameters and shoot phosphorus.

MI	Water stress	SDW (g)	RDW (g)	NN	NDW (mg)	Shoot P (ppm)
C	R1	0.42 ± 0.09i	0.08 ± 0.01g	0.0	0.0	612.50 ± 11.58j
	R2	0.50 ± 0.03i	0.09 ± 0.01g	0.0	0.0	952.50 ± 16.52h
	R3	0.52 ± 0.03i	0.06 ± 0.01h	0.0	0.0	712.00 ± 11.79i
B	R1	1.19 ± 0.17f	0.16 ± 0.02d	3.33 ± 0.84d	8.00 ± 1.25d	745.83 ± 17.06i
	R2	1.10 ± 0.15g	0.13 ± 0.01e	8.33 ± 0.95c	15.00 ± 1.00c	1175.00 ± 27.03g
	R3	0.80 ± 0.03h	0.09 ± 0.01g	1.83 ± 0.60e	5.00 ± 0.95e	937.50 ± 13.75h
B + M3	R1	2.30 ± 0.04ab	0.23 ± 0.02c	12.00 ± 2.11b	29.00 ± 2.34b	987.50 ± 14.76h
	R2	1.44 ± 0.12e	0.20 ± 0.01c	14.67 ± 2.19a	40.00 ± 4.02a	1901.17 ± 40.28de
	R3	1.39 ± 0.07e	0.13 ± 0.01e	8.83 ± 1.22c	18.00 ± 1.87c	2025.00 ± 51.08d
M1	R1	2.34 ± 0.32ab	0.29 ± 0.03a	0.0	0.0	1687.00 ± 21.25f
	R2	1.67 ± 0.15c	0.23 ± 0.01c	0.0	0.0	2387.50 ± 50.79c
	R3	1.56 ± 0.12d	0.14 ± 0.01e	0.0	0.0	2687.65 ± 67.45b
M2	R1	2.40 ± 0.30a	0.31 ± 0.02a	0.0	0.0	1750.00 ± 24.56ef
	R2	1.87 ± 0.06bc	0.27 ± 0.01b	0.0	0.0	2812.46 ± 80.45a
	R3	1.67 ± 0.08c	0.17 ± 0.01d	0.0	0.0	2862.95 ± 85.34a
M3	R1	1.91 ± 0.08b	0.21 ± 0.03c	0.0	0.0	937.34 ± 14.56h
	R2	1.25 ± 0.06f	0.15 ± 0.01de	0.0	0.0	1875.23 ± 32.50e
	R3	1.21 ± 0.05f	0.11 ± 0.02f	0.0	0.0	962.50 ± 15.45h

MI, microbial inoculation; SDW, shoot dry weight; NN, Nodule number; NDW, Nodule dry weight; RDW, Root dry weight; C, control; B, *Bradyrhizobium*; M1, *Funneliformis mosseae*; M2, *Rhizophagus irregularis;* M3, Consortium of (*Funneliformis mosseae, Rhizophagus irregularis, Glomus aggregatum* and *Glomus etunicatum*); B + M3, co-inoculation of *Bradyrhizobium* and the consortium; R1- regime 1; R2, regime 2; R3, regime 3. The means followed by same letter within the same column are not significantly different at $P \leq 0.05$ (Tukey's HSD).

Discussion

Effect of watering regime and microbial inoculation on nodulation and mycorrhizal colonization

Inoculation of green grams with AMF improved the plant growth parameters such as shoot dry weight and root dry weight. AMF improves soil structure (Miller and Jastrow 2000) and increases plant growth (Bhuvaneswari et al. 2014). Moreover, AMF increases CO_2 assimilation rate which results in improved plant growth (Amerian, Stewart, and Griffiths 2001). These results are similar to those reported by Vanitha, Srikar, and Eranna (2005) who recorded significant increase in number of leaves, plant height, dry weight and fresh weight in *Osmium kilimandscharicum* on treatment with *Glomus fasciculatum* compared to plants un-inoculated with AMF.

The higher percentage colonization of green grams by AMF caused higher absorption of nutrients in M2 which were assimilated into the plant forming new structures (Parkash 2004;

Lekberg and Koide 2005). Single AMF inoculants M1 and M2 were better than the AMF consortium, M3. This could be due to competition among the isolates for infection sites on the host plants (Thonar et al. 2014). The isolates in the consortium could also be incompatible with one another or had competition for fixed carbon. Similar results were observed when *Gigaspora species* and *Rhizophagus species* were co-inoculated in *Medicago truncatula* the plants had lower growth and phosphorus content than the same plants co-inoculated with *Claroideoglomus species* and *Rhizophagus species* which had synergetic effect on nutrient absorption and enhanced growth parameters (Thonar et al. 2014). *Rhizophagus irregularis,* M2 was better colonizer of green grams among the AMF isolates used and this was in agreement with Singh et al. (2012) who found that some varieties of crops are compatible to certain AMF species while being incompatible to other AMF species. The incompatible AMF inoculants are denied access to root mycorrhizal infection sites hence colonization fails to occur (Sanders, Koch, and Kuhn 2003).

Effect of irrigation regimes and microbial inoculants on shoot nutrients

In this study, mycorrhizal plants had higher amount of shoot N, P and K than non-mycorrhizal control plants. The AMF hyphae absorbs nutrients especially P (Habibzadeh et al. 2013) together with other nutrients such as K, Zn, Cu, S, Fe, Ca, Mg and Mn which are essential in promoting growth of plants (Smith et al. 1994; Malik et al. 2006). This is in agreement with the study done on soybeans, where AMF increased shoot nutrients in the infected plants leading to higher shoot and root dry weights (Aliasgharzad, Neyshabouri, and Salimi 2006).

Green gram plants infected with bradyrhizobia alone had higher amount of shoot N and P than the non-inoculated plants because of improved nodulation following bradyrhizobia inoculation (Smith and Read 2008), increasing concentration of N and P in plant leaves. These findings are in agreement with those reported by Singh, Samajpati, and Paul (2011) where green gram plants inoculated with viable strains of *Bradyrhizobium* enhanced plant height, dry biomass and increased grain yield.

The AMF and bradyrhizobia increase uptake of P and N, which are crucial for increasing plant production (Johansen 1999). Co-infection of plants with mycorrhiza and bradyrhizobia increased concentration of shoot N and K compared to crops that were not co-inoculated with the two microorganisms. The increased concentration of nitrogen and potassium played an important role in osmotic alteration and stomatal behavior which is essential in increasing plant production (Aliasgharzad, Neyshabouri, and Salimi 2006).

The current study shows that co-infection of green gram plants with AMF and bradyrhizobia increased nodulation and enhanced plant biomass in AMF inoculated plants. Nodulation and Nitrogen fixation ability in legumes are enhanced by mycorrhizal inoculation because AMF improves P uptake by the host plant, providing higher amount of energy for nitrogen fixation by *Bradyrhizobium* (Oruru and Njeru 2016). Therefore, co-inoculation of plants with bradyrhizobia and AMF would show synergistic effects on nodulation and nitrogen fixation. Cluett and Boucher (1983), after analyzing published data on relationship between AMF colonization and nodulation concluded that, there was a positive functional correlation between Bradyrhizobia and mycorrhiza, hence they can coexist and be active in one host enhancing nutrition and plant growth (Tobar et al. 1996).

The beneficial effect of AM fungi to N fixation is by amassing higher amount of P used in nodulation (Antunes and Goss 2005). The extensive and rapid mycorrhizal colonization to a plant leads into earlier nodule formation leading to higher Nitrogen fixation in plants co-inoculated with rhizobia and AMF (Goss et al. 2002). The mycorrhizal interaction with rhizobia is due to flavanoids produced by the host plant (Antunes and Goss 2005). Both rhizobia and AM fungi have evolved functionally similar ability to recognize signal compounds produced by the host plant (Xie et al. 1995). Several flavanoids produced by host plant have the potential of stimulating

hyphal growth and branching, which in turn stimulates nodulation with increase in N_2 fixation (Antunes and Goss 2005).

Effect of tripartite symbiosis on growth and nitrogen fixation of green grams under water stress

The tripartite symbiosis in this study was investigated by co-inoculation of AMF consortium with bradyrhizobia (B + M3). The results on plant growth provided in this study reveal that, green gram plants co-inoculated with mycorrhizal consortium and bradyrhizobia (B + M3) had higher enhancement of SDW than those infected with each microorganism alone (either M3 or B alone). This showed synergy between the *Bradyrhizobium* and AMF consortium microbes in enhancing absorption of nutrients and general plant growth. Both rhizobia and mycorrhiza can co-infect leguminous crops increasing nitrogen fixation and soil nutrient absorption through the fungal mycelia hence the host plants get double benefit from the microbes in exchange of fixed carbon (Antunes and Goss 2005).

The AMF and bradyrhizobia increase uptake of P and N, which are crucial for increasing plant production (Johansen 1999). Soybeans co-inoculated with mycorrhiza and bradyrhizobia were found to have up to 150% increase in dry weight compared to un-inoculated plants (Aliasgharzad, Neyshabouri, and Salimi 2006). Co-infection of plants with mycorrhiza and bradyrhizobia increased plant nutrients as was observed in soybeans when plants were co-inoculated with both mycorrhiza and *Bradyrhizobium* were reported to have higher concentration of shoot N and K compared to crops that were not co-inoculated with the two microorganisms (Aliasgharzad, Neyshabouri, and Salimi 2006).

Tripartite symbiosis interaction was observed to have significant effect on nitrogen fixation and growth of plants (Antunes 2004). In dual inoculation, rhizobia and AMF increased the number of nodules, number of spores, AMF % colonization of roots and P uptake above the plants inoculated with each microbe alone (Mirdhe and Lakshman 2014). Bagyaraj, Manjunath, and Patil (1979) observed higher N content in soybean, co-inoculated with AMF and rhizobia, in their experiment, higher soybean grain production and dry biomass was observed. After review of much research work, on co-inoculation of AM fungi and rhizobia, Michael, Shalaby, and Hanna (2000) reported significant increase on the activity of nitrate reductase, shoot dry weight, tissue P and N, nodulation and mycorrhizal colonization rates. Hazarika et al. (2000) observed that co-inoculation of AMF and rhizobia can enhance N_2 fixation, nodulation and general production of green grams. The plants co-inoculated with rhizobia and AMF had higher uptake of soil nutrients due to increased root surface area enhanced by extra-radical mycelia of the fungi especially when rhizobia and *Glomus mosseae* were co-inoculated (Abbott et al. 1992).

G. mosseae was reported to be a good inoculant for green grams in association with rhizobia (Ray and Valsalakumar 2010). Co-inoculation of *Bradyrhizobium* and *Glomus mosseae* enhanced yield of green gram plants and enhanced all the growth parameters of the infected plants as compared to single inoculation of either bradyrhizobia or *Glomus mosseae* (Singh, Samajpati, and Paul 2011). Rhizobia and *Glomus* sp. co-inoculation significantly increased the RDW, nodule number and SDW of faba bean plants (Ahmed and Elsheikh 1998). AMF and bradyrhizobia co-inoculation was reported to enhance production and growth characteristics in *Medicago sativa*, tomatoes and soybeans (Singh, Samajpati, and Paul 2011).

Conclusion

Although water stress reduced growth in green grams, inoculation of plants with bradyrhizobia and AM fungi improved growth and drought stress tolerance. The single AMF inoculants (M1 and M2) did better than the consortium (M3). Growth and shoot nutrients content were higher

in plants inoculated with single isolates compared to AMF consortium. Among the AMF isolates, *Rhizophagus irregularis* (M2) was the best inoculant for green grams. Co-inoculation of green grams with bradyrhizobia and AMF consortium (B + M3) increased growth and nutrients acquisition above the value attained when each microbe was inoculated to green grams alone. This reveals some synergy in the activity of bradyrhizobia and AMF. There was strong correlation between AMF colonization and plant biomass and shoot nutrients. The plant growth parameters were increased in correlation to percentage AMF root colonization.

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