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Effects of rhizobia and arbuscular mycorrhizal fungi on yield, size distribution and fatty acid of soybean seeds grown under drought stress

Nicholas O. Igiehon [a], Olubukola O. Babalola [a,*], Xavier Cheseto [b], Baldwyn Torto [b]

[a] Food Security and Safety Niche, Faculty of Natural and Agricultural Sciences, Private Mail Bag X2046, North-West University, Mmabatho 2735, South Africa
[b] International Centre of Insect Physiology and Ecology, P.O. Box 30772-00100, Nairobi, Kenya

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ABSTRACT

Soybean (*Glycine max* L.) is among the most economically important legumes that provide more than 1/4 of food (for man) and animal feed. However, its yield is comparatively low, most especially under drought stress. The aim of this study therefore was to assess the ability of *Rhizobium* spp. and mycorrhizal fungi to enhance the yield, seed size and fatty acid content of soybean grown under semi-arid environment. *Rhizobium* sp. strain R1 was found to possess nitrogen-fixing gene coniferyl aldehyde dehydrogenase function while *Rhizobium cellulosilyticum* strain R3 was found to have nitrogen-fixing genes cysteine desulfurase SufS and cysteine desulfurase IscS activity. Soybean (*Glycine max* L) seeds inoculated with *Rhizobium* spp. and mycorrhizal fungi were cultivated in soil exposed to drought stress. *Rhizobium* spp. inoculation and mycorrhization alleviate drought stress and increase yield, size and fat content of soybean seeds. This increase in the aboveground parameters was accompanied with an increase in belowground mycorrhizal spore number, percentage root mycorrhization and aboveground shoot relative water content (RWC) in the dually inoculated (R1 + R3MY) soybean plants. In particular, the dually inoculated (R1 + R3MY) soybean plants revealed 34.3 g fresh weight, 15.1 g dry weight and soybean plants singly inoculated with *Rhizobium* sp. strain R1 (R1) produced more large seeds with 12.03 g dry weight. The non-inoculated (control) seeds contained a higher percentage of moisture content compared to the microbially amended seeds while seeds co-inoculated with *Rhizobium cellulosilyticum* strain R3 and mycorrhizal consortium revealed the highest percent (8.4 %) of fat. Several fatty acids that are of significant health benefits to humans were observed in the soybean seeds. In order to gain insights into the bacterial communities of rhizospheric soil collected at different stages of soybean growth, class-based Heat-map analysis was performed on the Miseq sequenced data. The core bacteria that were found in the rhizospheric soil were *Verrumicrobia*, *Proteobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Cyanobacteria*, *Chloroflexi*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Deinococcus thermus* and *Nitrospira* suggesting that the rhizobia and fungi used in this study can also improve soil microbial diversity.

1. Introduction

Soybean (*Glycine max* L.) is among the most economically important legumes that provide more than 1/4 of food (for man) and animal feed (Graham and Vance, 2003), however, its yield is comparatively low (Egli, 2008) most especially under drought stress. Drought is one of the most crucial abiotic factors affecting soybean yield (Pathan et al., 2014; Purcell and Specht, 2004). It has been acknowledged that drought tolerance is an important target area for crop development and enhancement (Pennisi, 2008). Soybean cultivation mainly relies on natural rainfall, but irregular delivery of rainfall can cause yield difference in the same field, and soybean is exceedingly sensitive to drought, especially in the reproductive growth phase (Oya et al., 2004). Irrigation is not a possible alternative for the cultivation of soybean in most parts of the world because of its high cost. Therefore there is an urgent need for the development of cost-effective means to alleviate drought stress and improve seed yield particularly in the semi-arid environment.

"Seed yield per unit area is the product of the number of plants per unit area, number of seeds per plant and 100-seed weight. The 100-seed weight is affected by seed size, measured by length, width and thickness (Niu et al., 2013)". According to Boyer and Wisniewski-Dyé (2009), seed

* Corresponding author.
E-mail address: olubukola.babalola@nwu.ac.za (O.O. Babalola).

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yield reduced maximally during blooming and early pod formation phase. The decrease in yield is mainly due to a reduction in pod number per plant (Kokubun et al., 2001). Previous studies have indicated that the decrease in the number of pod per plant under water stress is a result of the increased rate of flower loss (Momen et al., 1979).

Additionally, variations in weather conditions, especially rainfall and temperature, can affect plant yield (Egli, 2008). Crop models have been used by previously Anderson et al. (2001) to assess the effects of weather on soybean and corn yields. These researchers found that improved weather was responsible for some of the yield increase. Lobell and Asner (2003); Egli (2008) reported that the yield of soybean and corn cultivated within a certain period at some locations were improved by decreasing temperatures, 'but the effects were the same for both crops.' However, their outcomes were queried on technical grounds (Gu, 2003). Low temperature and/or drought are also known to affect soybean yield components such as seed weight and size.

Drought stress may decrease seed weight and size (during seed fill) depending on the time, duration and severity of the stress. Soybean plants subjected to severe drought at a particular stage of their life cycle diminished individual seed weight from 0.21 to 0.18 g in one greenhouse trial and from 0.20 to 0.17 g in another trial experiment (Dornbos et al., 1989). Vieira et al. (1998) reported that soybean plants that were exposed to drought at the beginning of seed fill reduced individual seed weight from 0.14 to 0.09 g per seed. However, Smiciklas et al. (1992) reported that soybean plants exposed to water stress during the reproductive phase marginally reduced seed weight from 0.164 to 0.155 g, which was not significantly different from seeds harvested from well-watered plants. Soybean plants exposed to drought stress prior to the linear phase of seed fill reduced seed number and also "seed number was maintained by remobilization of reserves" when plants were subjected to drought stress after linear filling stage (New et al., 1994; Samarah et al., 2004). But when reserves were depleted under prolonged stress, seed size reduced due to the fact that the seed filling period was abridged (New et al., 1994).

Moreover, the fatty acid content is a quality indicator use to classify soybean cultivars and soy-based products, and such classification can influence consumers' choice for this crop (Lee et al., 2013). Soybean contains 40–42 % protein and 18–22 % oil in which 85 % is unsaturated fatty acid (Anwar et al., 2016) and so to produce and sustain soybean with high quality, particularly in semi-arid environments, the use of *Rhizobium* spp. and mycorrhizal fungi is encouraged. The genus *Rhizobium* associated with plant growth promoting rhizobacteria (PGPR) is well-known for its plant growth-promoting traits (Igiehon and Babalola, 2018). "*Rhizobium*, *Pseudomonas*, *Azospirillum* and *Bacillus*, have been found to have positive impacts on crops by enhancing both above and belowground biomass and could, therefore, play positive roles in achieving sustainable agriculture (Igiehon and Babalola, 2018)". Although, an increase in nutrient uptake and yield in soybean inoculated with mycorrhizal fungi and *Rhizobium* spp. has previously been reported (Igiehon and Babalola, 2017; Liu et al., 2012; Tian et al., 2013; Igiehon and Babalola, 2018), limited information is available on rhizobial and mycorrhizal fungal enhancement of soybean aboveground components particularly its nutritional contents under drought or semi-arid environments. However, it has been reported that the contribution of mycorrhizal fungal symbiosis to plant drought tolerance is connected to nutrient accumulation (Aliasgharzad et al., 2006; Augé, 2001). Microbial improvement of yield and organic nutrient(s) in seeds from soybean plants exposed to drought stress can be a vital response in water stress tolerance. Also, enhancement of soybean yield by microbial inoculation can be correlated with improvement in soil microbial diversity in the rhizosphere since it was previously reported by Liu et al. (2019) that microbial communities could be affected by fertilizer amendment as well as *Rhizobium* inoculation. The aim of this study, therefore, was to investigate the effects of microbial inoculants on soybean plants' yield, seed size, fat content, fatty acid and soil microbial diversity under drought stress in semi-arid environment.

2. Materials and ethods

2.1. Soybean seeds

In this study, we used soybean seeds (PAN 1532 R cultivar) obtained from Agricultural Research Council (ARC) located at Potchefstroom, South Africa and classified as "moderately drought-sensitive cultivar" in our previous experiment. The viability of the seeds was confirmed by the ARC representative.

2.2. Sources of bacterial species and mycorrhizal consortium

The rhizobial species used in this study were isolated from Bambara groundnut (*Vigna subterranea*) rhizosphere and identified by molecular method in our previous study. The rhizobial species which possess plant growth promoting traits were found to be drought-tolerant in our previous drought-tolerant experiment using polyethylene glycol (PEG) as a drought factor (Igiehon et al., 2019). On the other hand, the mycorrhizal consortium was acquired from the Department of Biochemistry and Microbiology, Rhodes University, Grahamstown, South Africa. The mycorrhizal consortium was confirmed to be drought tolerant. A combination of arbuscular mycorrhizal fungi (AMF), which include *Rhizophagus clarus* (previously *Glomus clarum*), *Gigaspora gigantea*, *Funneliformis mosseae* (previously *Glomus mosseae*), *Claroideoglomus etunicatum* (previously *Glomus etunicatum*) and *Paraglomus occulum* (molecular determination) were used for soybean inoculation in this study. The infectivity potential of the fungi was evaluated by culturing them in pot cultures of sorghum plants for three (3) months. The AMF spore number was 8–10 per gram with the most probable number (MPN) of propagules between 50 -100 per gram.

2.3. Screening for nitrogen-fixing genes in rhizobia species

Nitrogenase activity was ascertained by screening for nitrogen-fixing genes in the rhizobia species used in this study. This feat was accomplished by performing whole genome sequencing (HiSeq system-illumina) sequencing of the bacterial species as underpinned below:

2.4. DNA extraction

DNA was extracted from fresh culture of *Rhizobium* sp. strain R1 and *Rhizobium cellulosilyticum* strain R3 using Zymo DNA extraction kit according to manufacturer's instructions. The success of the extraction process and DNA relative band thickness were confirmed in 1 % agarose. Also, NanoDrop spectrophotometer (Thermo Scientific, NANODROP LITE Spectrophotometer) analysis showed that *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 had DNA concentrations of 40 ng/μL and 45 ng/μL, respectively. Then, 40 μL of DNA of each rhizobial species was sent to Molecular Research Laboratory (Mr. DNA), Texas, USA, for HiSeq system (illumina) sequencing.

2.5. Sequencing and de novo assembly of DNA reads

According to manufacturer's instructions, KAPA HyperPlus kits (Roche) were used to prepare DNA libraries from 25−50 ng of DNA extract. Thereafter, Qubit® dsDNA HS Assay Kit (Life Technologies) was used to determine DNA concentration, and average library size was estimated using Agilent 2100 Bio-analyzer (Agilent Technologies). Enzymatic degradation of DNA samples was performed to obtain ds DNA fragments followed by end repair and A-tailing to achieve 'end-repaired, 5'-phosphorylated, 3'-dA-tailed ds DNA fragments.' Then, ds DNA adapters with 3′-dTMP overhangs were ligated to 3′-dA-tailed DNA molecules and DNA libraries were amplified by using high fidelity and low-bias PCR. Amplified DNA libraries were diluted and 'sequenced paired-end for 500 cycles using the HiSeq system (Illumina).

Illumina sequenced data were uploaded into Kbase, quality checked

was performed using FastQC (v1.0.4), and trimmomatic was used to trim off low-quality sequence and adapter (Bolger et al., 2014). Sequenced reads were thereafter *de novo* assembled to create contigs using ARAST.

2.6. Annotation

Upon assembly, the genome *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 were annotated using Kbase Prokka (V1.12) annotation pipeline as well as rapid annotation using subsystem technology (Aziz et al., 2008). These systems allow the identification of functional annotations, introns and 'manual curation of gene annotations'. The data for both rhizobial species were given Bioproject number PRJNA496421, while R1 strain was solely assigned Biosample number SAMN10240937 and SRA Accession number SRR8060784 but R3 strain was exclusively allocated Biosample number SAMN10245972 and SRA Accession number SRR8061690 upon submission to GenBank database.

2.7. Greenhouse experiment

Greenhouse experiment was performed according to the methods described by Zarik et al. (2016) with little modifications. In brevity, topsoil (10−25 cm) used for the greenhouse experiment was obtained from North-West University. Upon physicochemical analyses, the soil was found to have a pH of 7.390 and contain 22 % clay, 8 % silt, 70 % sand, 0.095 % total N, 639.0 mg/kg Mg, 406.0 mg/kg K, 3.190 % organic matter, 1.260 % organic C, 45.400 mg/kg Mn and 1.950 mg/kg Fe. Then, the soil was sieve and sterilized at 121 °C for 1800 s, and 8000 g of the sterile soil was weighed into plastic pots with a diameter of 30 cm and a height of 29 cm. A pot experiment was set-up in the greenhouse using a completely randomized design (CRD), and drought stress was induced by using three regimes of water, namely: 100, 70 and 40 % field capacity (FC). Twenty four (24) treatments with 8 replicates resulting in 192 replicates were used.

Rhizobium sp. strain R1 and *R. cellulosilyticum* strain R3 used for pot inoculation were cultured in broth and harvested by spinning in sterile 0.85 % saline water. The optical density (OD) of both rhizobial strains was set at 1.3, and strain R1 and R3 had 30×10^9 CFU/mL, and 31×10^9 CFU/mL colony counts, respectively. Two hundred ml (200 mL) of each rhizobial strain was used to inoculate 240 soybean seeds that were surface sterilized in 75 % ethanol and 1 % sodium hypochlorite and rinsed in sterile distilled water. For co-inoculation, 100 mL of strain R1 suspension mixed with 100 mL of strain R3 suspension was used to inoculate 240 surfaced sterilized soybean seeds. By contrast, 480 soybean seeds used for control and mycorrhizal treatments were suspended in 0.85 % saline water. Afterward, soybean seeds were subjected to shaking in a shaker incubator at room temperature for 24 h and aseptically air-dried in a Laminar flow cabinet. Prior to sowing, sterile soil in pots was watered to 100 % FC with 360 mL of H_2O and 5 seeds were sowed per pot. For treatments involving AMF inoculation, 5 g of mycorrhizal consortium was placed in the soil (approximately 6−8 cm deep) before seed sowing. Two weeks after emergence, soybean seedlings were thinned to 2 per pot, and all the pots were watered every 3 days to 100 % FC for about 3 weeks after seed emergence, after which drought or water stress was introduced in the 70 and 40 % FC treatments. Consequently, 252 and 144 mL of water were used to water 70 and 40 % FC treatments, respectively, while 360 mL of water was continuously supplied to the 100 % FC treatments. After the initiation of drought stress, water was supplied to seedlings every two days for 3 weeks, and water stress was increased by watering every 3 days for extra 30 days prior to counting branch and leaf number of one of the soybean plants in the pots.

2.8. Physicochemical parameters of soil for the field experiment

Prior to planting, soil samples were collected 10 cm below the topsoil from five (05) different spots in the field, homogenized and kept at 4 °C before analyses. The homogenized soil sample was sieved using 2 mm sieve. Iron, manganese, organic carbon, organic matter, potassium, magnesium content, pH, total nitrogen, percentage sand, silt and clay of soil sample were determined according to the methods of Rascovan et al. (2016); Tajini et al. (2012) with little modifications.

2.9. Weather time series of experimental field area

The amount of rainfall was the drought index used in this study. The amount of rainfall for the period of soybean growth in the experimental area was monitored on a daily basis by South Africa Weather Service and compared with previous rainfall data for the past 10 years.

2.10. Bacterial growth and preparation for field experiment

Rhizobium spp. growth and processing was carried out as described by Prakamhang et al. (2015) with little modifications. Briefly, *R. cellulosilyticum* strain R3 was grown in two bottles containing 500 mL Luria Bertani (LB) broth and *Rhizobium* sp. strain R1 was grown in 800 mL LB broth, and both were incubated in a shaker incubator at ambient temperature (28 ± 2 °C). Bacteria were grown to early exponential phase within 5 days, and the bacterial broth was harvested by centrifuging at 10,000 *g* for 15 min. Bacterial pellet was suspended in 0.85 % (w/v) sterile NaCl. Bacterial inocula were quantified by spectrophotometric and plate count methods. The suspended bacterial pellet optical density (OD) was obtained and adjusted at 600 nm to 1.301. One ml (1 mL) of bacterial suspension was serially diluted, and samples from 10^{-6} dilution were cultured by pour plate method and incubated at 37 °C for 48 h. *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 colonies were counted and recorded as 520×10^7 and 524×10^7 CFU (colony forming unit) $ml^{-1,}$ respectively.

2.11. Soybean preparation and inoculation for field experiment

Soybean seeds (PAN 1532 R) were prepared for inoculation as described by Ndeddy Aka and Babalola (2016) with little modifications. For the single bacterial inoculation, 100 mL of *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 suspension was used to inoculate approximately 2000 soybean seeds. For the co-inoculation, 50 mL each of *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 were used to inoculate approximately 2000 soybean seeds in sterile flasks. The flasks were top-up by adding 600 mL of 0.85 % (w/v) NaCl. Seven hundred ml (700 mL) of 0.85 % (w/v) NaCl was added to approximately 2000 seeds (for the arbuscular mycorrhizal treatment) and approximately 2000 seeds (for the control). Seeds were subjected to shaking at 180 rpm in a shaker incubator at room temperature for 24 h and air-dried in a sterilized laminar flow before sowing.

2.12. Field experiment

The field experiment was conducted as described by Prakamhang et al. (2015) with little modifications. Eight (8) different treatments were used as follow: (1) Non-inoculated (control) soybean seeds, (2) R1; single inoculation of soybean seeds with *Rhizobium* sp. strain R1, (3) R3; single inoculation of soybean seeds with *R. cellulosilyticum* strain R3 (4) R1 + R3; co-inoculation with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 (5) MY; inoculation of soybean seeds with arbuscular mycorrhizal fungal (AMF) consortium, (6) R1MY; co-inoculation of soybean seeds with *Rhizobium* sp. strain R1 and AMF consortium, (7) R3MY; co-inoculation of soybean seeds with *R. cellulosilyticum* strain R3 and AMF consortium and (8) R1 + R3MY; co-inoculation of soybean seeds with *Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and AMF consortium. The field site was located in North-West University school farm, Mafikeng campus (GPS coordinates 25.82202S:025.61430E), North-West Province, South Africa, which has a history of maize cultivations but no history of fertilizer

application. The experiment was conducted within December 2017 – April 2018. The experiment was set-up using 2 factorial randomized complete block design (RCBD) by dividing into eight (8) treatments (as described above), and each treatment was replicated eight (8) times, making a total of sixty-four (64) plots. Five-row plots were used with 0.5 m inter-row spacing, 0.13 m intra-row spacing, 1 m inter-plot spacing and 1 m inter-block spacing. Each plot size was 2 × 0.52 m (1.04 m^2). Bacterial inoculated seeds and control seeds were placed 10 cm below top-soil, and for treatments involving AMF consortium inoculation, approximately 5 g of AMF consortium was poured 12 cm into the soil and covered sparingly with soil before seeds were added. Four (4) seeds were sowed per hole. Two weeks after emergence, soybean seedlings were thinned to one. "The regular agricultural practices were done except chemical fertilizer application and pesticide spraying (Prakamhang et al., 2015)". For bacterial community study, rhizospheric soils were collected at the beginning of blooming (BB), beginning of pod development (BP), beginning of seed development (BS), full seed (FS) and at full maturity (FM) from soybean plants treated with R1 + R3MY. Rhizospheric soils were collected from the eight replicates, mixed together, immediately transferred in an ice box to the laboratory and stored at −20 °C before DNA extraction. Furthermore, plants were harvested at maturity and data were collected from the soybean plants at the middle of the plots and not from the edge to avoid border effects. The number of the pod, seed number per pod, seed area were determined according to previous methods (Tiquia et al., 1996; Samarah et al., 2004). Seed sizes were determined according to the method of Samarah et al. (2004) with little modifications. Briefly, Soybean seeds were classified into small and large sizes by passing the seeds through a 5.6 mm diameter sieve. Large seeds represented seeds retained above a 5.6 mm diameter round sieve, and small seeds represented seeds that passed through the 5.6 mm diameter round sieve. Pods were separated by hand, and seed yield (fresh and dry weight) and weight per seed (fresh and dry) were determined by weighing on a weighing machine (Radwag weighing machine by Lasec, made in Poland). Similarly, nodules were separated from soybean plant roots, counted and fresh and dry weights were determined as described above.

2.13. Percentage (%) moisture

Percentage (%) moisture content of soybean samples was calculated using the formula:

$$\%\ \text{moisture} = \frac{100\ \text{x}[(\text{filter bag weight} + \text{sample weight}) - \text{Weight after drying}]}{\text{Sample weight}}$$

2.14. Total lipids

The fat analysis was carried out on 1 g of each of the flour samples according to a protocol adapted from Folch et al. (1957). The samples were separately extracted in 10 mL solution of Chloroform: Methanol (2:1v/v), vortexed for 2 min and allowed to stand for 30 min. Thereafter, the samples were filtered through a Whatman paper No 1 into a weighed 50 mL Falcon tubes and the filtrate allowed to evaporate to dryness in the hood. % Total extracted fats were calculated using the following equation:

/ initial weight of sample) * 100 = % Total Extracted Fat. The fat content and the weights were recorded.(weight of the falcon tube plus fat – the weight of the falcon tube)

2.15. Gas chromatography coupled mass spectrometry analysis (GC–MS) of fatty acids

Methyl esterification was carried out on 100 mg of each of the flour samples according to a protocol adapted from a recent (Cheseto et al., 2020). A solution of sodium methoxide (15 mg/mL) was prepared in dry methanol and added (500 μL) to each sample. The samples were then vortexed for 1 min, sonicated for 5 min and incubated at 60 °C for 1 h, thereafter quenched by adding 100 μL deionized water followed by vortexing for another 1 min. The resulting methyl esters were extracted using GC-grade hexane (Sigma–Aldrich, St. Louis, MO, USA) and centrifuged at 14, 000 rpm for 5 min. The supernatant was dried over anhydrous Na_2SO_4 and analyzed (1.0 μL) on a 7890A gas chromatograph linked to a 5975 C mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA). The GC was fitted with a HP5 MS low bleed capillary column (30 m ×0.25 mm i.d., 0.25 μm) (J&W, Folsom, CA, USA). Helium at a flow rate of 1.25 mL min^{-1} served as the carrier gas. The oven temperature was programmed from 35 to 285 °C with the initial temperature maintained for 5 min then 10 °C min^{-1} to 280 °C, held at this temperature for 20.4 min. The mass selective detector was maintained at an ion source temperature of 230 °C and a quadrupole temperature of 180 °C. Electron impact (EI) mass spectra were obtained at the acceleration energy of 70 eV. Fragment ions were analyzed over 40–550 *m/z* mass range in the full scan mode. The filament delay time was set at 3.3 min. The fatty acids were identified as their methyl esters by comparison of gas chromatographic retention times and fragmentation patterns with those of authentic standards and reference spectra published by library–MS databases: National Institute of Standards and Technology (NIST) 05, 08, and 11. Serial dilution of methyl hexadecanoate (1−100 ng/μL) prepared from n-hexadecanoic acid, (≥ 98 % purity), (Sigma-Aldrich, St. Louis, MO) was also analyzed by GC–MS to generate a linear calibration curve (peak area *vs* concentration) with the following equation; [y = 5E+07x + 2E + 07 (R2 = 0.9972)], which was used for external quantification.

2.16. Percentage mycorrhizal colonization

The percentage of AMF root colonization was calculated by the methods of Ortiz et al. (2015); Phillips and Hayman (1970), with little modifications. Briefly, soybean root samples (4 replicates per treatment) were treated with 10 % KOH and stained with trypan blue solution prepared by dissolving 0.82 g of trypan blue powder in a mixture of 640 mL of distilled water, 480 mL glycerol and 520 mL lactic acid. Stained root samples were examined under a stereo-microscope at X4 and X3 magnification.

2.17. AMF spore count

AMF spore counts were performed by wet sieving and decanting techniques according to the methods of Pacioni (1992) with little modifications. One hundred g (100 g) of soil samples were collected from soybean rhizosphere and mixed with 1000 mL of sterile distilled water and poured through 212, 106, 63 and 53 μm sieves stacked together from the top to the base in descending order. The content of the 53 μm was backwashed into vials and centrifuged at 1800 rpm for 5 min. One ml (1 mL) of the supernatant was mixed with 0.5 mL of 60 % sucrose in a fresh vial and centrifuged at 1800 rpm for 5 min. With a syringe, AMF spore suspension was removed from the supernatant and poured in a sterile Petri-dish and examined under a stereomicroscope at X4 and X3 magnification. Four (4) replicates of each treatment were used for the spore counts.

2.18. Relative water content

Fifty mm (50 mm) of shoot relative water content from 4 replicates of each treatment was estimated in soybean plants as follows: (FW-DW)/ TW-DW) x 100 (Ortiz et al., 2015; Aroca et al., 2003).

2.19. DNA extraction

Microbial DNA was extracted from 0.25 g of the soybean rhizospheric soil using DNeasy Power Soil Kit (QIAGEN GmbH, Hilden, GERMANY) according to the manufacturer's instructions. The presence of microbial DNA was confirmed in 1 % agarose while; the DNA concentrations were checked using NanoDrop spectrophotometer (Thermo Scientific, NANODROP LITE Spectrophotometer). Then, 40 μL of extracted DNA from BB, BP, BS, FS and FM soil samples were sent to Molecular Research Laboratory (Mr. DNA), Texas, the USA for Miseq sequencing (Next Generation Sequencing-NGS).

Miseq Sequencing of DNA samplesPolymerase chain reaction primers 515/806 which target V4 variable region of 16S rRNA with a forward primer attached to a barcode were deployed to amplify the DNA extract using HotStarTaq Plus Master Mix Kit (Qiagen, USA) according to the following conditions: 94 °C for 180 s, 94 °C for 30 s, 72 °C for 60 s and 72 °C for 300 s (elongation step). PCR products were checked in 2 % agarose. Thereafter, in the same proportion, multiple samples were homogenized based on their DNA concentrations and molecular weight. Mixed samples were purified using calibrated ampure XP beads, followed by Illumina DNA library preparation. According to the manufacturer's instructions, Purified samples were sequenced on Miseq at MR DNA (www.mrdnalab.com, Shallowater, TX, USA), and sequenced data were processed using MR DNA analysis pipeline (MR DNA, Shallowater, TX, USA). Briefly, sequences were merged, 'depleted of barcode' and those less than 150bp and 'ambiguous base call' were expunged. Sequences were further denoised, followed by the generation of operational taxonomic units (OTU) and removal of chimeras. 'OTUs were defined by clustering at 3 % divergence (97 % similarity)' and taxonomically classified using 'BLASTn against curated database'' acquired from RDPII and NCBI (www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu).

2.20. Bioinformatics analysis

Sequenced data of soil microbial community were analyzed using MicrobiomeAnalyst pipeline (Dhariwal et al., 2017). Heatmap of fatty acid methyl esters was generated using Perseus version 1.6.2.3.

2.21. Statistical analysis

Data were submitted to normality and homogeneity of variances. Data that were not normally distributed were transformed to satisfy the assumptions of analysis of variance (ANOVA). Differences between means were determined using Duncan test and differences at $P < 0.05$ were considered significant (Igiehon, 2015; Dytham, 2011).

3. Results

Analysis of the whole genome sequence of *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 for the presence of genes that could be responsible for nitrogenase activity showed that both rhizobial strains possess a few nitrogen-fixing genes but two of the genes are reported for each strain in this study (Fig. 1a, b). The two nitrogenase genes reported for strain R1 has the coniferyl aldehyde dehydrogenase function with the same aliases and gene length (Table 1). On the contrary, the two nitrogen-fixing genes described for *R. cellulosilyticum* strain R3 have cysteine desulfurase SufS and cysteine desulfurase IscS activity (Table 1) with different aliases and gene locations (Fig. 1c, d).

Under drought conditions in the greenhouse, R1 + R3MY had the greatest significant impact on soybean leaf numbers under severe drought conditions (Fig. 2). Similarly, the dual inocula R1MY and R3MY proved effective in enhancing leaf numbers at 40 % FC, but sole inoculation with the mycorrhizal consortium (MY) showed a better effect than its combination with either of the rhizobial strains. Under moderate drought conditions, R1 + R3 inoculum increased leaf number more than the non-inoculated soybean counterpart. As a matter of fact, at 70 % FC, leaf number was enhanced by all the microbial treatments, including single inoculation with R1, R3 and MY, but the control treatment showed the lowest leaf number (Fig. 2). Under well-watered conditions (100 % FC), soybean plants amended with microbial species also produced more leaves than the non-inoculated plants (Fig. 2).

As regards branch number, under severe drought stress condition (40 % FC), co-inoculation of rhizobial strain R1 and mycorrhizal consortium (R1MY) had the greatest effect on soybean branch number with a mean branch number of 3.88 followed by co-inoculation of strain R1, R3 and mycorrhizal consortium (R1 + R3MY). Treatments involving dual inoculation of strain R3 and mycorrhizal consortium (R3MY) and co-inoculation of both rhizobial strains (R1 + R3) had the same mean branch number of 3.50 (Fig. 2). By contrast, mycorrhizal consortium (MY) amendment had a better effect on branch number under moderate drought stress condition (70 % FC) followed by R1 + R3 and R1 + R3MY treatments. But, under moderate drought stress conditions, a significant decrease in branch number was observed in the non-inoculated (control)

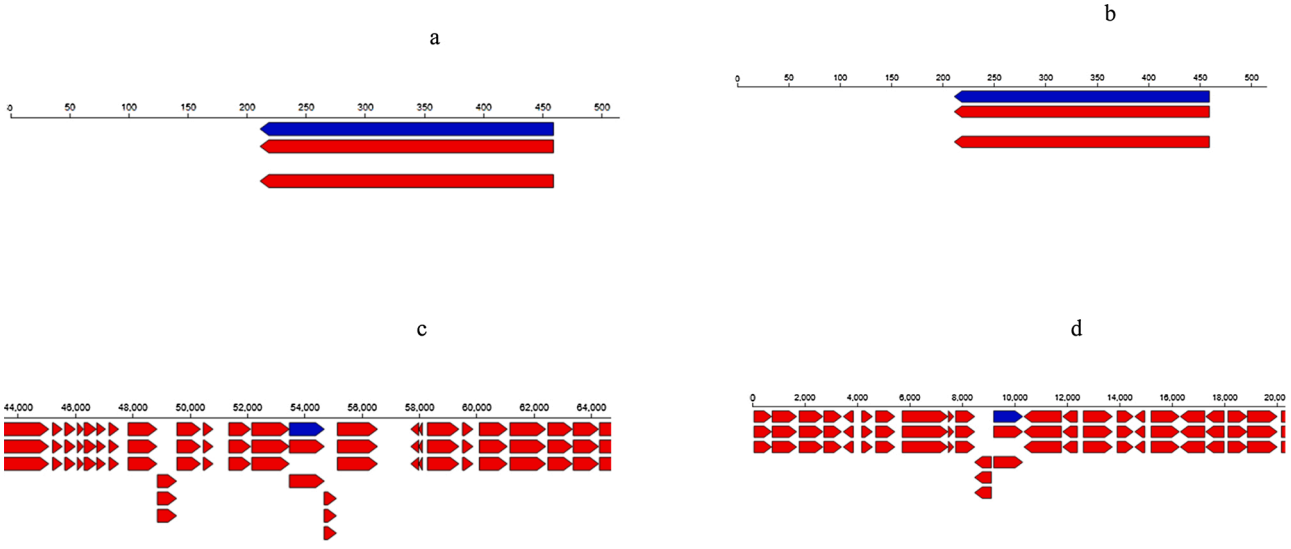

Fig. 1. Contig locations of nitrogen fixing genes of (a) R1 strain Coniferyl aldehyde dehydrogenase, (b) R1 strain Coniferyl aldehyde dehydrogenase, (c) R3 strain Cysteine desulfurase SufS and (d) R3 strain Cysteine desulfurase IscS. The blue bars represent the gene locations.

Table 1
Selected nitrogen-fixing genes found in the genome of *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3.

Feature ID	Type	Function	Ontology	Aliases	Start	Strand	length	Location
R1 strain								
JKFNCFJO_07150	Gene	Coniferyl aldehyde dehydrogenase	–	calB, 1.2.1.68	459	–	249	NODE_15016_length_487_cov_3.476386
JKFNCFJO_07150_CDS	CDS	Coniferyl aldehyde dehydrogenase	–	calB, 1.2.1.68	459	–	249	NODE_15016_length_487_cov_3.476386
R3 strain								
LKJIOFBO_01056	Gene	Cysteine desulfurase SufS	SSO:000001294- CBSS-345074.3.peg.1627: Cysteine desulfurase (EC 2.8.1.7) SSO:000001856- Cysteine desulfurase (EC 2.8.1.7) SSO:000001857- Cysteine desulfurase, IscS subfamily (EC 2.8.1.7) SSO:000001858- Cysteine desulfurase, NifS subfamily (EC 2.8.1.7) SSO:000001859- Cysteine desulfurase, SufS subfamily (EC 2.8.1.7) SSO:000001860- Cysteine desulfurase CsdA-CsdE, main protein CsdA (EC 2.8.1.7)	sufS, 2.8.1.7	53,465	+	1221	NODE_18_length_99415_cov_30.341991
LKJIOFBO_01588	Gene	Cysteine desulfurase IscS	SSO:000001294- CBSS-345074.3.peg.1627: Cysteine desulfurase (EC 2.8.1.7) SSO:000001856- Cysteine desulfurase (EC 2.8.1.7) SSO:000001857- Cysteine desulfurase, IscS subfamily (EC 2.8.1.7) SSO:000001858- Cysteine desulfurase, NifS subfamily (EC 2.8.1.7) SSO:000001859- Cysteine desulfurase, SufS subfamily (EC 2.8.1.7) SSO:000001860- Cysteine desulfurase CsdA-CsdE, main protein CsdA (EC 2.8.1.7)	iscS_3, 2.8.1.7	9200	+	1113	NODE_32_length_40656_cov_31.105864

soybean plants. A similar result was observed under 100 % FC in which the non-inoculated soybean plants showed the lowest branch number with a mean value of 5 (Fig. 2). However, under 100 % FC, R1 + R3MY treatment showed the highest branch number. Furthermore, the physical properties of the experimental field soil that were analyzed are percentage sand, silt and clay. The soil had the highest percentage of sand (68 %) followed by clay (26 %) and silt (6 %). Chemical analyses of the soil showed that the field soil had 858, 344, 43.5 and 3.9 mg/kg of magnesium (mg), potassium (K), manganese (Mn) and iron (Fe) respectively. The pH of the soil was closed to neutral (pH 7.62), with 0.07 % of total nitrogen. However, 3.23 % of the soil represents organic matter and 1.06 % organic carbon (Table 2).

The location of the experimental field was North-West University, Mafikeng campus in North-West Province, which is semi-arid due to the low amount of rainfall the Province experiences annually. Between December and April, this region had recorded less than 5 mm of rainfall for the past 10 years except February 2017 in which approximately 9 mm of rainfall was recorded (Fig. 3). Soybean is a summer crop since it grows well during this season and the summer season is usually between October and April in South Africa. During the period of this current study, the highest amount of rainfall was 3.88 mm recorded in February, and the least was 0.95 mm in April (Fig. 3). These data indicate that this region does experience a low amount of rainfall, and therefore, crops grown therein are exposed to drought stress.

In this study, seed yield was evaluated by weighing the fresh and dry weights of soybean seeds. The highest fresh weight (34.3 g) was recorded for soybean plants co-inoculated with *Rhizobium* sp. strain R1/*R. cellulosilyticum* strain R3 and mycorrhizal fungi (R1 + R3MY) and the least fresh weight (15.1 g) was recorded for the non-inoculated control plants. *Rhizobium* sp. strain R1 (R1), *R. cellulosilyticum* strain R3 (R3), *Rhizobium* sp. strain R1/*R. cellulosilyticum* strain R3 (R1 + R3), mycorrhizal consortium (MY), *Rhizobium* sp. strain R1 and mycorrhizal consortium (R1MY) and *R. cellulosilyticum* strain R3 and mycorrhizal consortium (R3MY) had fresh weights of 19.9, 21.2, 31.9, 27.3, 20.6 and 32.6 g respectively (Fig. 4). Seed dry weight was also significantly increased by fungal and rhizobial inoculation compared to the non-inoculated plants (Fig. 4). Soybean seeds amended with R1 + R3MY had the highest dry weight, followed by seeds treated with R3MY (Fig. 4).

Similarly, the weight of individual soybean seeds similarly showed that R1 + R3MY effectively increased the fresh and dry weight of soybean seed (Fig. 5). This indicates that the combined interaction among the microbial inocula used in this study had a positive effect on soybean seeds. However, there was no significant difference between the individual seed fresh weight of non-inoculated soybean plant and soybean plants inoculated with the different microbial inocula. Similarly, the dry weight per seed for all the treatments was approximately 0.1 g.

Additionally, the highest (mean of 202. 2) seed area, which is a product of seed length, width and 2.3, was observed in soybean plants amended with MY. On the contrary, the least seed area was recorded for the control treatment (Fig. 6). Soybean plants co-inoculated with R1 and R3 had the highest number of seed per pod with an average of 18.3 followed by the non-inoculated plants with a mean of 17.3. MY, R1MY, R3MY and R1 + R3MY treatments had similar seed number per pod of approximately 16.2 while the least number was observed in soybean inoculated with R1 (Fig. 7).

Soybean seeds classified into small and large sizes showed that soybean plants inoculated with R1 produced more seeds with large sizes seconded by plants treated with R1 + R2MY. The non-inoculated soybean plants produced large seeds with a mean dry weight of approximately 2.40 g, which was significantly different from that of R1 treatment (Fig. 8). The outcome of this fieldwork showed that R1 + R3, MY, R1MY, R3MY and non-inoculated treatments produced more of large seeds than small seeds.

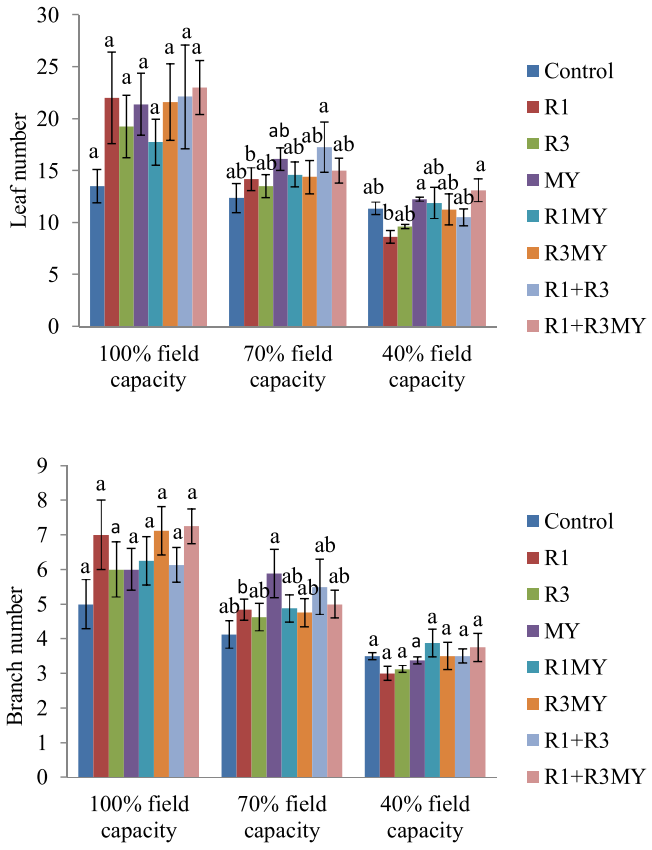

Fig. 2. Leaf and branch number of inoculated and non-inoculated soybean plants exposed to a 52 day period of drought stress in the greenhouse. Control – non-inoculated treatment, R1–*Rhizobium* sp. strain R1, R3–*R. cellulosilyticum* strain R3, MY – mycorrhizal consortium, R1MY–*Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY–*R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1 + R3–*Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1 + R3MY–*Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium. 100 %, 70 % and 40 % represent the different water regimes. Data represent mean ± SE. According to Duncan's multiple range test (n = 8), values that have common letters are not different significantly (P > 0.05).

Table 2
Physicochemical parameters of the field soil sample.

Parameter	Result
pH (H_2O)	7.62
Fe (mg/kg)	3.87
Mn (mg/kg)	43.50
Organic carbon (%)	1.06
Organic matter (%)	3.23
Phosphorus	N/D
Potassium (mg/kg)	344.00
Magnesium (mg/kg)	858.00
Total nitrogen (%)	0.07
Sand (%)	68.00
Silt (%)	6.00
Clay (%)	26.00

In addition, soybean plants treated with R3MY produced the highest number of root nodules (Fig. 9). It was found that root nodule numbers correlated positively with nodule fresh and dry weights since R3MY treatment also produced nodules with greater fresh and dry weight (Fig. 9). Also, soybean plants amended with other microbial treatments produced nodules but at different levels, while the non-inoculated soybean plants did not produce nodules at all.

The % moisture content of the homogenized seed samples used to determine % fat content ranged from 7.20 to 3.20 % (Fig. 10). The least (3.20 %) percentage moisture content was observed in soybean amended with R1 and the highest (7.20) was observed in the control treatment. There were no significant differences ($P > 0.05$) in the % moisture content of soybean seeds treated with the different microbial inocula. The % moisture content of soybean samples inoculated with R3, R1 + R3, MY, R1MY, R3MY and R1 + R3MY were 4.0, 4.4, 3.6, 3.8, 3.4 and 3.5 respectively.

Moreover, crude fatty analysis of the soybean seeds revealed the highest amounts of fat in soybean seeds co-inoculated with R1 + R3 and R3MY, which were significantly different ($p < 0.05$) from seeds amended with R3 and R1MY (Table 3). On the contrary, seeds inoculated with mycorrhizal fungi had the lowest amount of fatty, which was significantly different ($p < 0.05$) from every other treatment.

The results of the fatty acid analysis of soybean seeds determined as methyl esters using GC–MS are presented in Fig. 11 including the essential fatty acids polyunsaturated (PUFA) and monounsaturated fatty acids (MUFA), as well saturated fatty acids (SFA). Treatment effects alterered both the qualitative and quantitative profiles of these fatty acids. Notably, of the PUFA, except in the control, methyl (9*Z*, 12*Z*) octadecadienoate was downregulated in all the treatments at varying levels. On the other hand, methyl (9*Z*, 12*Z*, 15*Z*) octadecatrienoate was upregulated in in the combination R1 + R3 and R1MY. Likewise, methyl (9*Z*, 11*E*, 13*E*) octadecatrienoate was upregulated in the combination R1MY. Strikingly, most MUFA were downregulated in the combination treatments. For instance, except for MY, methyl 6-octadecenoate was downregulated in all treatments. Likewise, except for R1 and C, methyl (9*Z*)-hexadecenoate was downregulated in all treatments. The high presence of methyl (13*E*) octadecenoate in the control C was downregulated in all other treatments. Of the SFA, combinations of MY led to downregulation of methyl octadecenoate, whereas combining RI and MY upregulated methyl heptadecanoate.

Non-inoculated (control) treatments showed the least colonization with AMF in soybean roots. Colonization rate increased significantly notably in plants inoculated with R1 + R3MY. The colonization rate of R1 + R3MY was 97.8 %, and control was 19.3 % (Fig. 12). However, compared to the control, increased colonization rate was observed in soybean plants inoculated with R1, R3, R1 + R3, MY, R1MY and R3MY. Similar results were also obtained for some fungal spores in soybean rhizosphere with soybean plants amended with R1 + R3MY showing the highest number of spore and non-inoculated plants had the least spore number (Fig. 12).

Percentage (%) relative water content (RWC) is one of the parameters used to determine the response of plants to environmental stress. As depicted in Fig. 13, RWC increased in the inoculated soybean plants under this semi-arid condition. How RWC increase was particularly glaring in soybean plants co-inoculated with R1 + R3MY. The synergistic interaction of the two rhizobial species and mycorrhizal consortium increased RWC at 63.30 %, was higher than that of the non-inoculated control at 43.60 %. Co-inoculation of R3 and mycorrhizal consortium also resulted in a significant increase of RWC by 58.80 % compared to the control.

In order to gain insights into the bacterial communities of rhizospheric soil collected at different stages of soybean growth, class-based Heat-map analysis was performed on the Miseq sequenced data (Fig. 14). The core bacteria that were found in the rhizospheric soil were *Verrumicrobia*, *Proteobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Cyanobacteria*, *Chloroflexi*, *Bacteroidetes*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Deinococcus_thermus* and *Nitrospirae* (Fig. 14). Bacteria in the class *Actinobacteria* were more abundant at BS, FS and FM stages, while bacteria in the class *Proteobacteria* were more abundant at BB and BP stages. Bacteria in the class *Candidatus_tectomicrobia*, *Fibrobacteres*, *Fusobacteria*, *Spirochaetes* and *Tenericutes* were minimally present the soil (Fig. 14). Core microbiome analysis showed the highest prevalence of one (1) for *Verrumicrobia*, *Proteobacteria*, *Gemmatimonadetes*, *Firmicutes*, *Cyanobacteria*, *Chloroflexi*, *Bacteroidetes*, *Actinobacteria* and

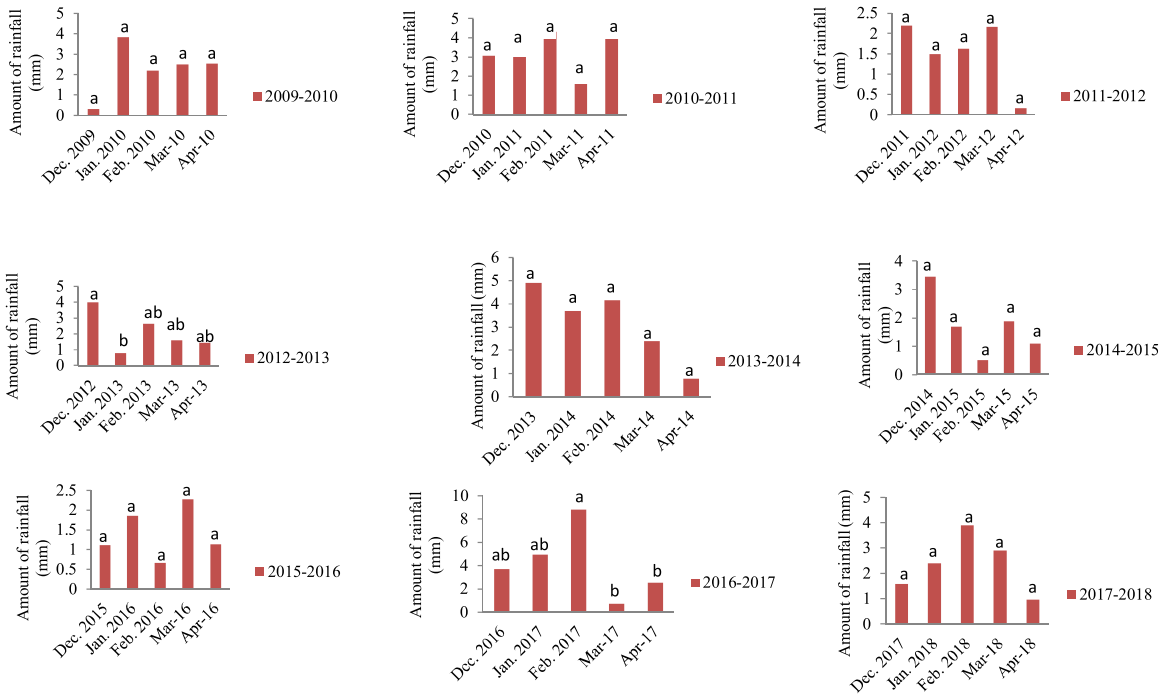

Fig. 3. Amount of rainfall recorded in the study area between 2009 and 2018 for the months of December to April.

Acidobacteria. By contrast, *Planctomycetes* and *Deinococcus_thermus* showed a prevalence of 0.8 and *Nitrospirae* had a prevalence of 0.2 (Fig. 15).

3.1. Online data availability

BB with the online name B_L001: Bioproject number PRJNA496421, Biosample number SAMN10341378, SRA Accession number SRR8127646
https://www.ncbi.nlm.nih.gov/sra/SRX4948854[accn]

BP with the online name P_L001: Bioproject number PRJNA496421, Biosample number SAMN10341908, SRA Accession number SRR8127966
https://www.ncbi.nlm.nih.gov/sra/SRX4949174[accn]

BS with the online name AMPLICON: Bioproject number PRJNA496421, Biosample number SAMN10341954, SRA Accession number SRR8128850
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FM with the online name H_L001: Bioproject number PRJNA496421, Biosample number SAMN10342630, SRA Accession number SRR8129599
https://www.ncbi.nlm.nih.gov/sra/SRX4950721[accn]

4. Discussion

The two rhizobial species *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 isolated from Bambara groundnut (*Vigna subterranea*) rhizosphere in our previous study possess nitrogen-fixing (*nif*) genes and thus could have the potential to fix nitrogen in the soil. In particular, strain R1 possesses *nif* genes that have coniferyl aldehyde dehydrogenase function while; strain R3 was found to have cysteine desulfurase SufS and cysteine desulfurase IscS function. Also, strain R1 *nif* genes possess calB, 1.2.1.68 and calB, 1.2.1.68 aliases whereas strain R3 *nif* genes were found to have sufS, 2.8.1.7 and iscS_3, 2.8.1.7 aliases. In addition, *nif* genes of R1 strain were negatively stranded, which contradict the positive-stranded *nif* genes found in strain R3 (Table 1). The presence of the *nif* genes in these strains could be an indication that these bacteria possess nitrogenase activity and may have the potential to supply plants with the needed nitrogen for growth in the greenhouse and under natural soil conditions.

Furthermore, it is commonly known that research targeting to achieve agricultural sustainability start in a controlled environment (e.g., greenhouse). Thus in the current study, the impacts of rhizobial species on soybean above-ground parameters such as branch and leaf number under drought conditions using different water regimes as a drought factor were considered.

An increase in leaf number was observed in soybean plant amended with R1 + R3MY under severe drought conditions. But, under 70 % FC, all the microbial treatments enhanced soybean leaf number compared to the control experiments. A similar result was observed for soybean leaves grown under well-watered condition. The improvement in leaf number in soybean plants co-inoculated with rhizobial and mycorrhizal fungi especially under severe drought condition could be due to the contributive effects of the mycorrhizal symbiont since it is presently acknowledged that mycorrhizal symbiosis shield plant from the devastating effects of drought and that such contributive effects are traceable to a combination of physical, cellular and nutritional effects (Ruiz-Lozano, 2003). Generally, mycorrhizal and rhizobial symbioses improve host plant development due to enhanced plant nutrition (Igiehon and Babalola, 2018; Ruiz-Lozano, 2003; Safir et al., 1972; Davies et al., 1992; Roldán et al., 2008) and direct H_2O adsorption and transport through mycorrhizal hyphae (Augé et al., 2003; Ruiz-Lozano and Azcón, 1995).

In addition, under greenhouse condition, a significant increase in branch number was observed in most of the co-inoculated soybean plants irrespective of the water regime (Fig. 2). The positive effects noticed in co-inoculated soybean plants could be due to the synergistic plant growth-promoting contributions from both species. However, single inoculations with either rhizobial species strain R1 or strain R3 also resulted in enhanced branch numbers, particularly under 100 % and 70 % field capacity (Fig. 2). The branch number is a critical component of plants as its increase could result in an increase in leaf number.

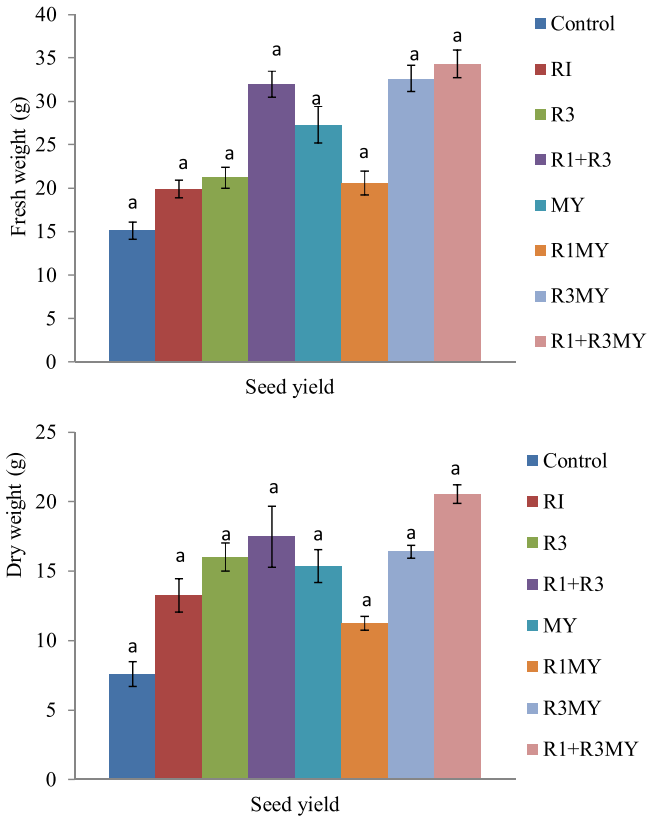

Fig. 4. Fresh and dry weight of entire seed of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1 + R3), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1 + R3MY) under semi-arid environment. Data represent mean ± SE. According to Duncan's multiple range test (n = 8), values that have common letters are not different significantly (P > 0.05).

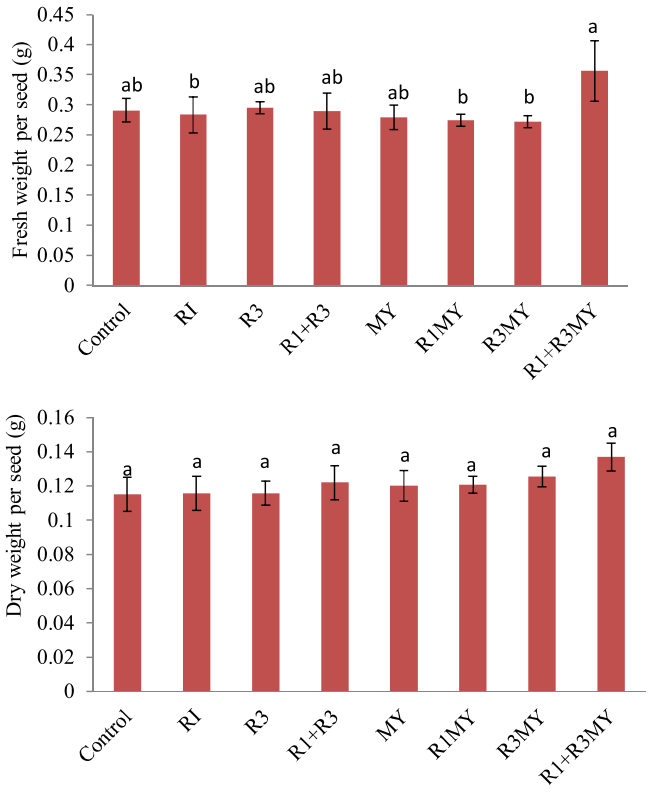

Fig. 5. Fresh and dry weight of individual seed of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1 + R), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1 + R3MY) under semi-arid environment. Data represent mean ± SE. According to Duncan's multiple range test (n = 8), values that have common letters are not different significantly (P > 0.05).

Considering all factors acting equally, the increase in leaf number may lead to an increase in carbon substrate produce through the process of photosynthesis and consequently resulting in plant growth and yield enhancement.

Table 2 shows that the pH of the soil in the study site was 7.62 suggesting that the soil is slightly above the optimum pH range of 5.5–7 (Gurmu, 2007; Zerihun et al., 2015) needed for soybean growth. However, this pH will support the growth of bacteria but may not favor the growth of fungi since the latter grows best in acidic environments.

From the rainfall data (Fig. 3), low rainfall was recorded during the experimental period (December-April), which also corresponds with the amount of rainfall recorded for the same period for the past ten years in the study area. In addition, in Thailand, Prakamhang et al. (2015) reported a mean rainfall of 31.1 mm in a well-watered field study involving *Bradyrhizobium* spp. and soybean, which is above the highest mean rainfall of 3.88 mm observed in February in this present study (Fig. 3). These data suggest that the study area for the present study (which may not be able to support the growth of most agricultural crops such as soybean) is not well-watered and therefore is semi-arid since it was also reported that soybean grows best with an annual rainfall of 900-1300 mm (Gurmu, 2007) which is far above the amount of rainfall record in the study area; as results shown in this study represent significant data for the yearly rainy period in this region.

Therefore, in the current study, the effects of rhizobia and mycorrhizal fungi on the yield, size and fatty acid content of soybean grown under the semi-arid environment of South Africa were evaluated. It was

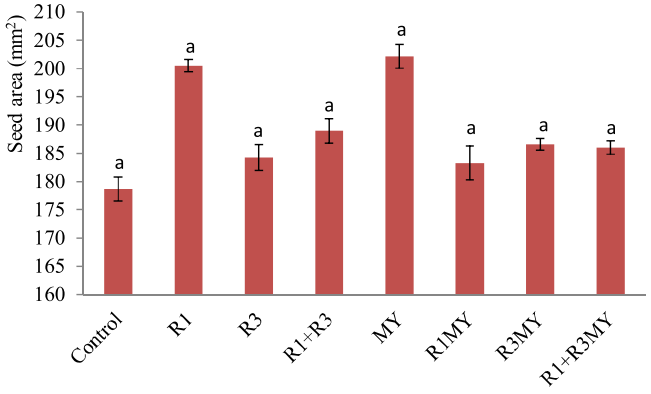

Fig. 6. Seed area of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1 + R), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1 + R3MY) under semi-arid environment. Data represent mean ± SE. According to Duncan's multiple range test (n = 8), values that have common letters are not different significantly (P > 0.05).

observed that the co-inoculation of soybean plants with *Rhizobium* sp. strain R1/*Rhizobium cellulosilyticum* strain R3 and mycorrhizal consortium (R1 + R3MY) resulted in a significant increase in the fresh and

Fig. 7. Number of seed per pod of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1 + R), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1 + R3MY) under semi-arid environment. Data represent mean ± SE. According to Duncan's multiple range test (n = 8), values that have common letters are not different significantly (P > 0.05).

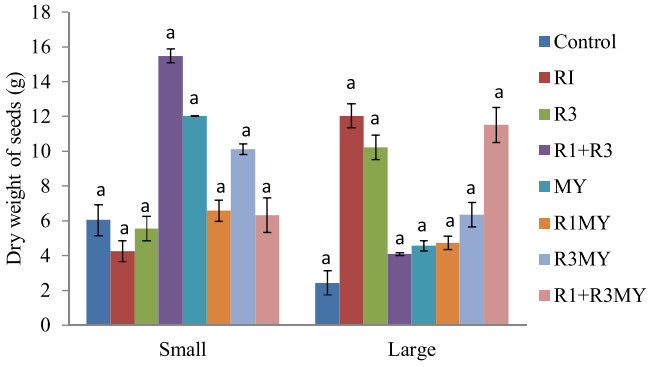

Fig. 8. Dry weight of small and large soybean seeds harvested from non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1 + R), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1 + R3MY) under semi-arid environment. Large seeds represented seeds retained above a 5.6 mm diameter round sieve; small seeds represented seeds that passed through the 5.6 mm diameter round sieve. Data represent mean ± SE. According to Duncan's multiple range test (n = 8), values that have common letters are not different significantly (P > 0.05).

dry weights of soybean seeds compared to the non-inoculated control plants. Similarly, other microbial treatments were effective in enhancing the fresh and dry weight (parameters that were used to determine seed yield in this study), but the least fresh (19.9 g) and dry (13.2 g) weights were obtained for soybean treated with *Rhizobium* sp. strain R1 (R1) unlike *R. cellulosilyticum* strain R3 treatment (R3) which was more effective in improving soybean yield (Fig. 4). These results agree with our previous findings focused on *in vitro* soybean seed germination experiment (data are not shown) in which soybean seedlings singly inoculated with R3 showed a better performance than those amended with R1 or *Sinorhizobium meliloti* strain R5. Amazingly, R3 treatment produced seeds with dry weight (16 g) greater than soybean inoculated with mycorrhizal consortium even though this consortium contains five (5) different drought-tolerant AMF: namely *Rhizophagus clarus*,

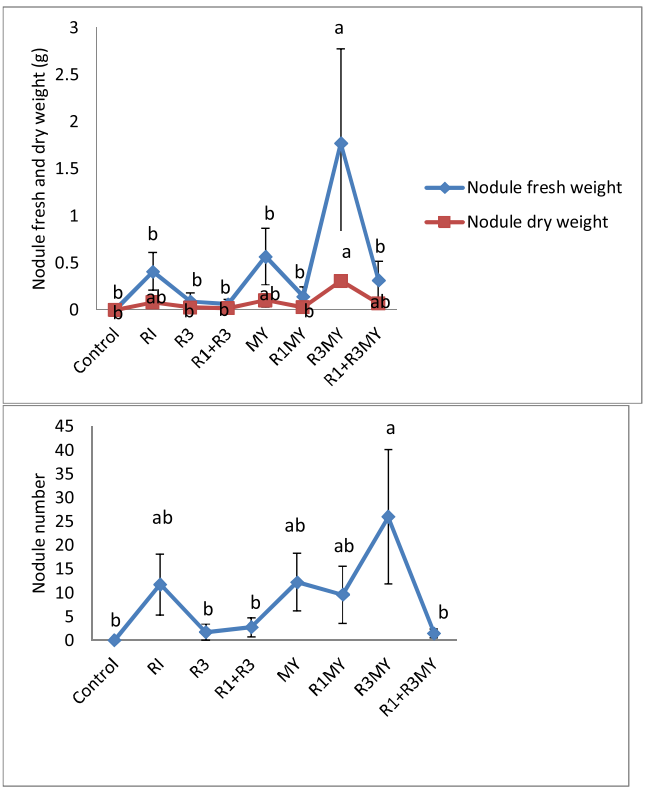

Fig. 9. Nodule fresh and dry weight and nodule number of inoculated and non-inoculated soybean plants harvest at full maturity. Control – non-inoculated treatment, R1–*Rhizobium* sp. strain R1, R3–*R. cellulosilyticum* strain R3, MY – mycorrhizal consortium, R1MY–*Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY–*R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1 + R3–*Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1 + R3MY–*Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium. Data represent mean ± SE. According to Duncan's multiple range test (n = 8), values that have common letters are not different significantly (P > 0.05).

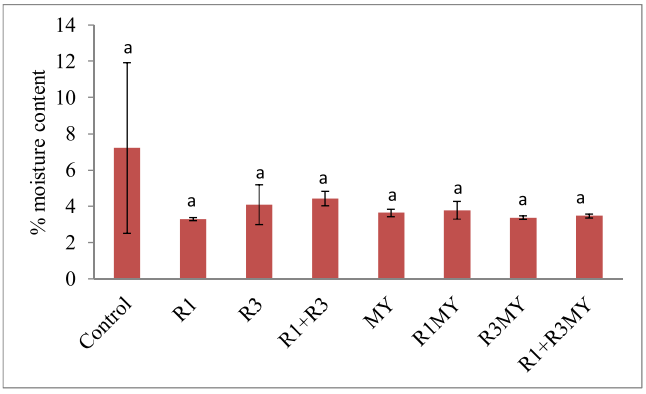

Fig. 10. Percentage moisture content in soybean seeds of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1 + R), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1 + R3MY) under semi-arid environment. Data represent mean ± SE. According to Duncan's multiple range test (n = 8), values that have common letters are not different significantly (P > 0.05).

Table 3
Crude fatty analysis in inoculated and non-inoculated (control) soybean seeds harvested from the field.

Treatment	Crude fat (g)	Crude fat (%)
C	0.08 ± 0.01^{a}	7.82 ± 0.71^{a}
R1	0.08 ± 0.01^{a}	7.80 ± 0.68^{a}
R3	0.05 ± 0.02^{ab}	4.81 ± 1.76^{ab}
R1 + R3	0.08 ± 0.005^{a}	8.45 ± 0.45^{a}
MY	0.04 ± 0.02^{b}	3.73 ± 2.50^{b}
R1MY	0.07 ± 0.001^{ab}	7.35 ± 0.49^{ab}
R3MY	0.08 ± 0.01^{a}	8.40 ± 1.05^{a}
R1 + R3MY	0.08 ± 0.002^{a}	7.70 ± 0.21^{a}

Legend: Control – non-inoculated treatment, R1–*Rhizobium* sp. strain R1, R3–*R. cellulosilyticum* strain R3, MY – mycorrhizal consortium, R1MY–*Rhizobium* sp. strain R1 and mycorrhizal fungal consortium, R3MY–*R. cellulosilyticum* strain R3 and mycorrhizal consortium, R1 + R3–*Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3, R1 + R3MY–*Rhizobium* sp. strain R1, *R. cellulosilyticum* strain R3 and mycorrhizal consortium. Data represent mean ± SE. According to Duncan's multiple range test (n = 3), values that have common letters are not different significantly ($P > 0.05$).

Gigaspora gigantea, *Funneliformis mosseae*, *Claroideoglomus etunicatum* and *Paraglomus occulum.* A significant increase ($P < 0.05$) was, however observed in the yield of soybean dually inoculated with *R. cellulosilyticum* strain R3 and mycorrhizal consortium (R3MY).

There were no significant differences ($P > 0.05$) among the treatments as regards dry weight per seed (Fig. 5). However, the highest fresh (0.35 g) and dry (0.13 g) weights were recorded in soybean seed co-inoculated with R1 + R3MY. This increase may be due to synergistic interaction between the rhizobia and mycorrhizal fungi since many researchers have reported that yield of legumes is affected by AMF and rhizobia interactions (Ahmad, 1995; Redecker et al., 1997; Xavier and Germida, 2003). In comparing the impacts of associations between *Glomus aggregatum, Sclerocystis microcarp* and *Glomus pallidum,* and four *Rhizobium phaseoli* on the yield of different kidney bean genotypes, Ahmad (1995) observed that the symbiotic efficacy was reliant on the actual combination of the mycorrhizal species, bacterial strain and the host genotype. However, contrary to other studies, both effective and ineffective *Rhizobium* species were included in the study so as to determine the role of *Rhizobium* spp. in the tripartite association. It was found that co-inoculation of an effective *Rhizobium* strain LX43 with an AM fungus enhanced the yield, nitrogen and phosphorus content of the plant. Similarly, the co-inoculation of less effective *Rhizobium* isolate with another AM fungus increased the yield of the legume. However, this significantly enhanced productivity than that of an effective Rhizobium-AMF combination.

The area of seed randomly picked from each treatment revealed that soybean treated with the mycorrhizal consortium (MY) had the greatest area of 202.18 mm^2. In addition, R1 treatment seed area (200. 48 mm^2) was relatively higher than other treatments. This result indicates that MY and R1 relatively affect the length and width of soybean seeds, although the effect was not significant compared to the non-inoculated (control) soybean plants (Fig. 6). The effect of seed area was very pronounced in the seed size distribution of R1 since this treatment produced large seeds with the greatest weight (Fig. 8). However, seed area effect did not significantly affect yield (fresh and dry weight of entire seed produced) since much yield increase was observed in soybean dually inoculated with *Rhizobium* sp. strain R1 and *R. cellulosilyticum* strain R3 (R1 + R3), R1MY and R1 + R3MY.

The number of seeds per pod is also a component that can affect soybean seed yield. Rhizobia and fungi did not significantly ($P > 0.05$) affect the number of seed per pod; soybean dually inoculated with R1 + R3 had the highest number of seed per pod with an average number of 18.27 however (Fig. 7). Single inoculation with either R1 or R3 resulted in either 14.4 or 15.69 seed numbers per pod, respectively. But co-inoculation with only the rhizobia strains slightly affected pod seed number. This effect slightly decreased when each of the rhizobial strains was co-inoculated with mycorrhizal consortium. Interestingly, such a slight decrease was also observed in soybean dually inoculated with R1 + R3MY. Perhaps, the significant increase in the yield of soybean (Fig. 4) dually inoculated with R1 + R3MY can be related to the production of more pods per plant than other treatments which confirm the conclusions drawn by Liu et al. (2003); Westgate and Peterson (1993) that yield increase under drought stress is caused by the increase in the number of the pod.

R1, R3 and R1 + R3MY were more effective in producing large soybean seeds since these treatments produced large seeds with greater weight (Fig. 8). Comparatively, R1 + R3 and MY treatments did not favor the production of large seeds suggesting that the effects seen in soybean seeds amended with R1 + R3MY treatment could largely be attributed to the synergistic interaction between the rhizobial species and mycorrhizal consortium. Naturally, leguminous crops such as soybean are able to form symbiotic interactions with both AMF and rhizobia (Xavier and Germida, 2003; Wang et al., 2011). Tripartite mutualistic symbiotic interactions with AMF and rhizobia should be very crucial for soybean productivity (Igiehon and Babalola, 2018) and the impacts of soybean inoculation with rhizobia and AMF need further research.

However, R1 + R3MY treatment was not very effective with respect to nodule formation as an average number of 1.5 nodules were produced by the treatment. But, the positive effects of synergistic interaction were observed in soybean plants treated with R1MY and R3MY (Fig. 9). Single inoculation with R1 strain produced a mean nodule number of 11.8, and such effect declined drastically in soybean plants treated with R3 strain, which produced a mean number of 1.8 nodules. Overall, R1, MY, R1MY and R3MY had relatively better impacts on nodule number, as well as nodule fresh and dry weights.

However, the % moisture content of non-inoculated (control) soybean seeds was relatively higher than the inoculated soybean seeds. The seeds of the non-inoculated plants showed 7.2 % of moisture, unlike the seeds of soybean plants amended with rhizobial species and mycorrhizal fungi, which showed relatively low moisture content. This indicates that the microbial candidates used in this study may not only improve the nutrient status of soybean seeds but could also be harnessed to produce soybean with a long shelf-life.

Furthermore, the interactions between rhizobial species and mycorrhizal fungi enhanced the fat content of soybean seeds. The highest percentage (8.4 %) was observed in soybean seeds treated with R3MY and the least (3.7 %) % fat was recorded for soybean plants treated with mycorrhizal consortium (Table 3). These results showed that drought stress enhanced the fatty acid contents in seeds of some inoculated soybean plants, either through upregulation or down-regualtion of specific fatty acids, which is in agreement with the work of Hou et al. (2006) who reported that drought stress is among the environmental factors that affect the content and/or composition of fat in soybean seeds. However, there was no significant difference in the % fat of soybean seeds inoculated with R3 and R1MY.

Also, in this present study, fatty acid analysis revealed the presence of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in soybean seeds. The upregulation of specific unsaturated fatty acids is noteworthy because in humans some of these fatty acids help to alleviate cholesterol levels, which is frequently linked to heart diseases (Grundy, 1989). For instance, the presence of the essential omega-3 fatty acid, methyl (9*Z*, 12*Z*, 15*Z*) octadecatrienoate (α-linolenic acid, ALA), is associated with several health and nutritional benefits including, antitumor effects on human colorectal cancer cells (González-Fernández et al., 2020), protects against hypertension (Li et al., 2020) and reduction of blood lipid profiles in obesity patients (Yue et al., 2020). On the other hand, methyl (9*Z*, 11*E*, 13*E*) octadecatrienoate (α-eleostearic acid), induces necroptosis in tumor cells and reduces human colon cancer growth in mice (Van Aken and Pogson, 2017), while the odd-chain saturated fatty acids: methyl heptadecanoate is associated with lower risks of adiposity, chronic inflammation, cardiovascular disease, metabolic syndrome, type 2 diabetes, nonalcoholic

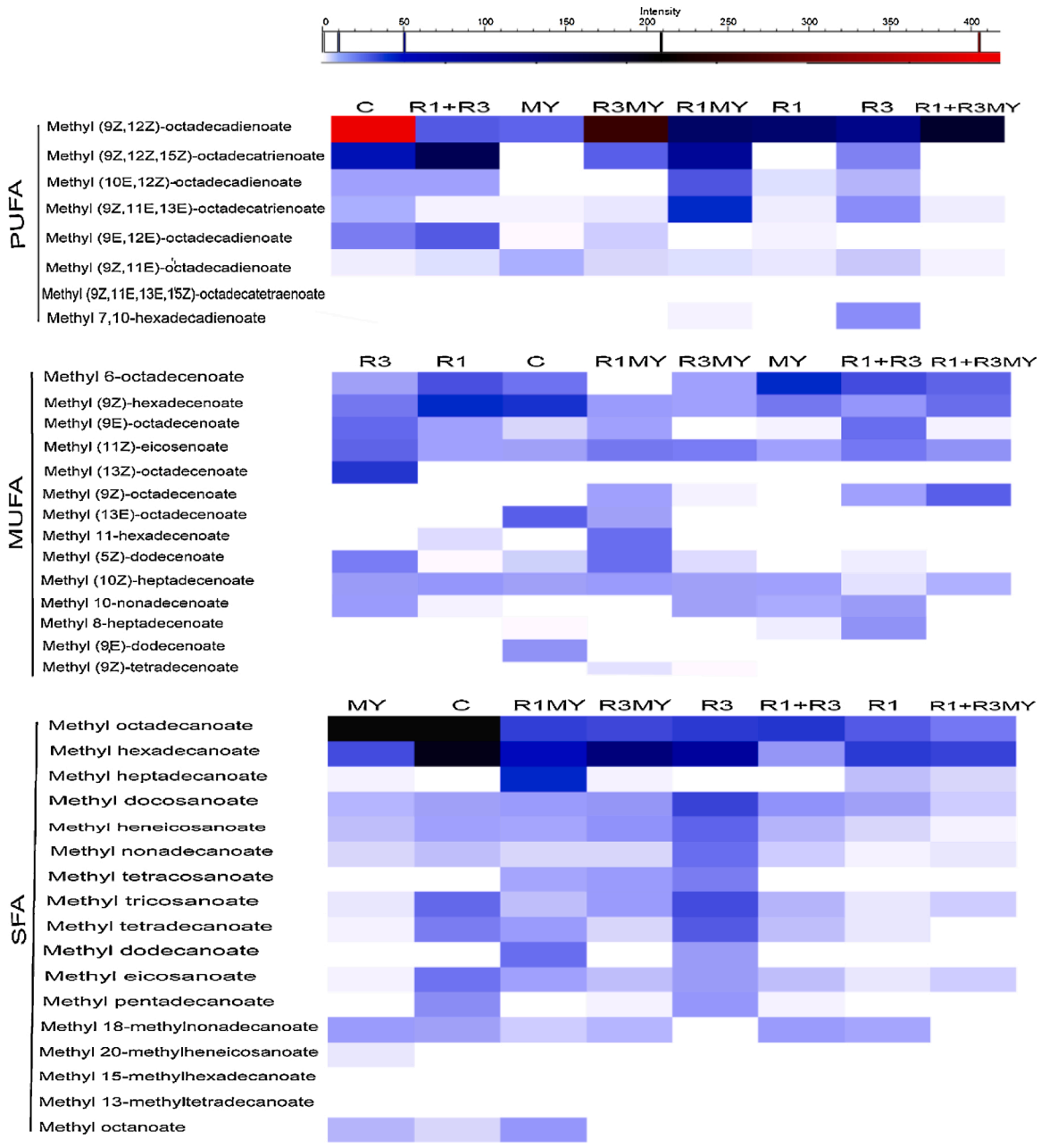

Fig. 11. Heatmap of Upregulated and downregulated fatty acid composition in soybean seeds of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1 + R), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1 + R3MY) under semi-arid environment.

steatohepatitis, chronic obstructive pulmonary disease and pancreatic cancer (Venn-Watson et al., 2020).

Also, the quality of soybean is evaluated based on the concentration and composition of fat, oil and protein. Soybean seeds have been reported to contain 24 % oleic acid, 8 % linolenic acid and 54 % linoleic acid (Schnebly and Fehr, 1993). A higher concentration of oleic acid is a desirable quality for long shelf-life and stability of oil for industrial purposes, but higher concentrations of polyunsaturated fatty acids such as linolenic acids are needed for human nutrition (Bellaloui, 2011). However, fatty acids (such as oleic acid) are less vulnerable to oxidative modifications during processing, storage cum frying. Therefore, the food industry is currently concerned in producing soybean seed that contains a high concentration of oleic acid (Rahman et al., 2001). According to Opperman and Varia (2011), in 2010, South Africans demand for soybean oil was at 1.3 million tons, followed by other countries such as Mozambique and Zambia, with 0.2 million tons demand each for soybean oil in the same year. Thus, the demand for soybean seeds can greatly be influenced by its fatty acid content and production of soybean rich in fatty acid will, therefore, contribute positively to human nutrition and economy growth since it has also been reported that availability of high-quality seed plays a significant role in the growth and development of any crop-producing enterprise (Dlamini et al., 2014).

In addition, soybean root dually inoculated with R1 + R3MY was most heavily colonized by AMF (Fig. 12b). The % mycorrhization of roots of soybean inoculated with MY was 82, which was slightly less than plants dually inoculated with *Rhizobium* sp. strain R1/mycorrhizal consortium (R1MY), R3MY and R1 + R3MY. The increase in mycorrhizal colonization level observed in the R1MY, R3MY and R1 + R3MY

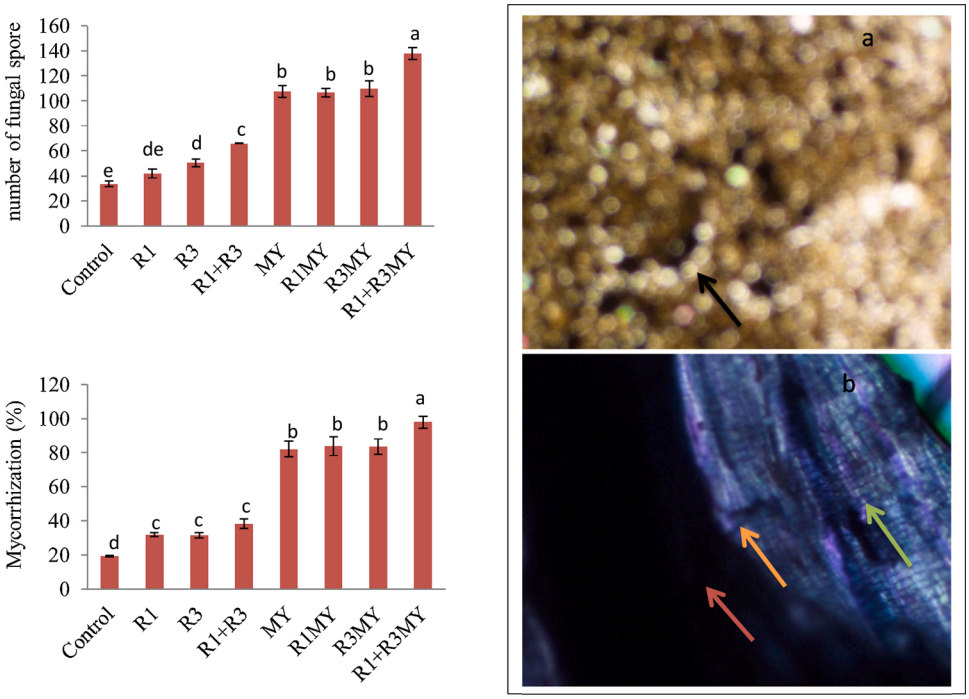

Fig. 12. Number of fungal spore and percentage mycorrhizal colonization in non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1 + R), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1 + R3MY) under semi-arid environment. Data represent mean ± SE. (a) represents AMF spores (b) represent represents root colonized with AMF. Black, green, orange and red arrows point towards fungal spores, arbuscule, hyphal and soybean plant root respectively.

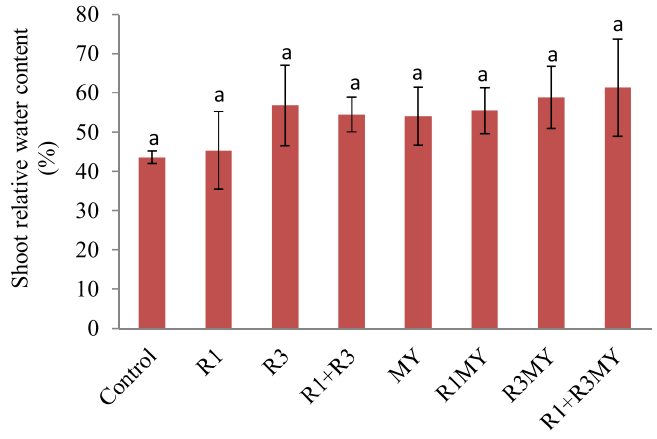

Fig. 13. Relative water content in the shoot of non-inoculated control soybean plants (control), soybean singly inoculated with *Rhizobium* sp. (R1), *Rhizobium cellulosilyticum* (R3), dually inoculated with *Rhizobium* sp. and *Rhizobium cellulosilyticum* (R1 + R), mycorrhizal consortium (MY), *Rhizobium* sp. and mycorrhizal consortium (R1MY), *Rhizobium cellulosilyticum* and mycorrhizal consortium (R3MY), *Rhizobium* sp./*Rhizobium cellulosilyticum* and mycorrhizal consortium (R1 + R3MY) under semi-arid environment. Data represent mean ± SE. (a) represents AMF spores (b) represent represents root colonized with AMF.

treatments might be traced to the fact that the rhizobial species aided the mycorrhizal fungi to penetrate the soybean roots. Also, the efficacy of dual inocula, particularly R1 + R3MY, could be linked to % mycorrhizal colonization, as previously reported (Marulanda et al., 2009). In contrast, Ortiz et al. (2015) highlighted that the efficiency of dual inoculation in a study carried out under drought conditions in the field was not related to the colonization level. However, in this study, mycorrhizal vesicles, arbuscules and hyphae were also seen in soybean plant roots inoculated singly with R1, R3 and the non-inoculated (control) plant roots but at a relatively low colonization level. These soybean roots may have been colonized by indigenous mycorrhizal fungi present in the soil (Igiehon and Babalola, 2017). Similar results were also obtained for spore number (Fig. 12a) in which soybean plant rhizosphere co-inoculated with R1 + R3MY showed the highest number of fungal spore (average of 137.75) and the non-inoculated plant rhizosphere showed the lowest fungal spore number with an average number of 33.5.

One new and remarkable result obtained in the current research under semi-arid environmental conditions is the inherent ability of the microorganisms to cope and tolerate drought conditions. Additionally, not only rhizobial, but also mycorrhizal inoculants proved competent in assisting inoculated plants to reduce the harmful drought effects on yield and fatty acid content of soybean seeds.

In this present study, most of the inocula applied were able to increase water content that reduced yield and nutrient content caused by drought. Rhizobial and fungal inocula applied induced different natural processes that helped soybean plants to grow and develop under drought.

Relative water content (RWC) is 'involved in plant response to drought' (Aliasgharzad et al., 2006; Ortiz et al., 2015). The lowest RWC observed in control soybean plants showed that they were relatively less effective in coping with drought (Fig. 13). In this study, plants colonized by rhizobial species and mycorrhizal consortium increased plants RWC content by 45.3 % (R1), 56.8 % (R3), 54.5 % (R1 + R3), 54.1 % (MY), 55.5 % (R1MY), 58.9 % (R3MY), 61.3 % (R1 + R3MY). However, the increase in RWC was not significantly different ($p > 0.05$).

Class-based Heatmap analysis grouped bacterial found in soybean rhizosphere at different growth stages into seventy (17) classes (Fig. 14). Nevertheless, core microbiome analysis showed that the soybean rhizosphere harbored twelve (12) core bacterial classes (Fig. 15). Bacterial in most of these classes can enhance plant growth since they are known to possess plant growth-promoting traits.

In conclusion, although, most of the soybean plants singly and dually inoculated with *Rhizobium* spp. and mycorrhizal consortium enhanced yield, seed fresh weight and dry weight, seed size, seed fatty acid content and shoot water content, the results of this present study largely suggest that synergistic interactions are involved in the enhancement of yield, seed size and fatty acid content of soybean plants while influencing soil microbial diversity. This interaction between rhizobia and mycorrhizal fungi is an effective biotechnological strategy to improve plant yield and nutrition in semi-arid environments. However, insights into the adaptation cum functioning of microorganisms and plants to extreme

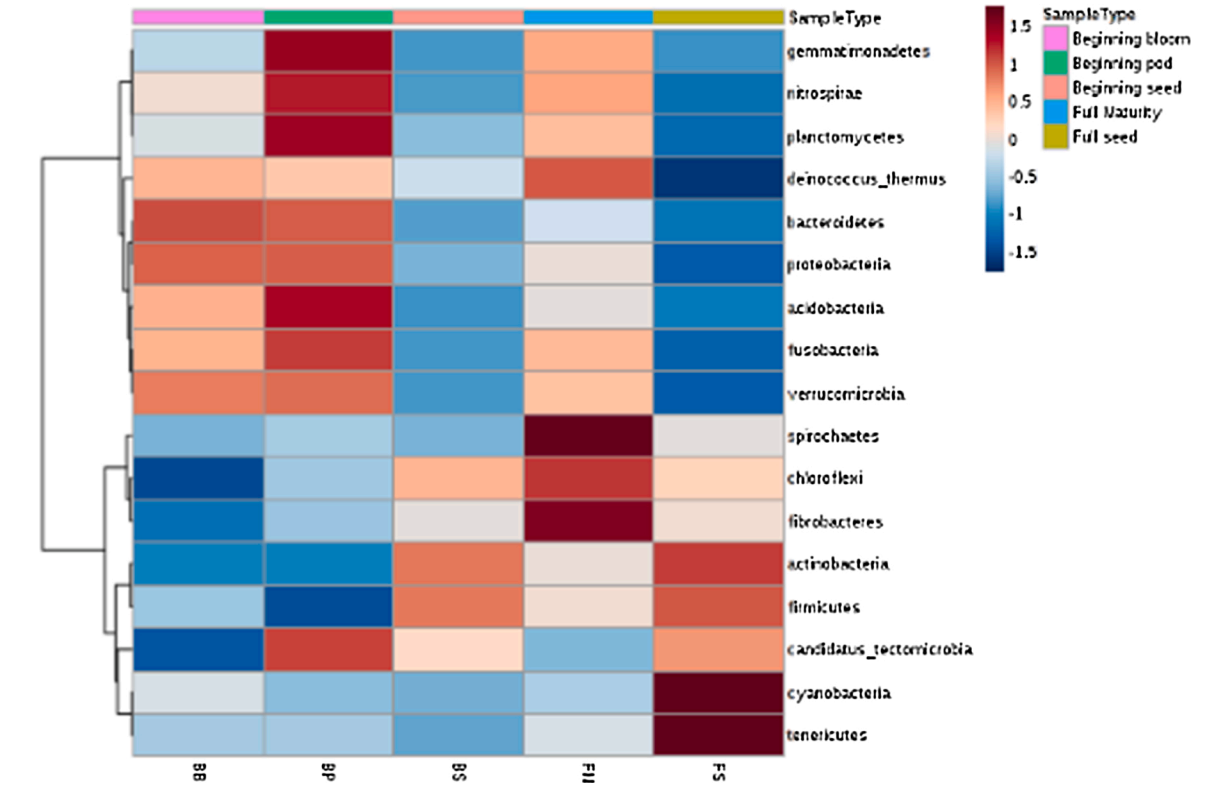

Fig. 14. Class based Heat-map visualization of bacterial communities at different stages of soybean growth. Colour intensity of each box is directly proportional to the taxon abundance. BB – beginning of blooming, BP – beginning of pod development, BS – beginning of seed development, FM – full seed, FM – full maturity.

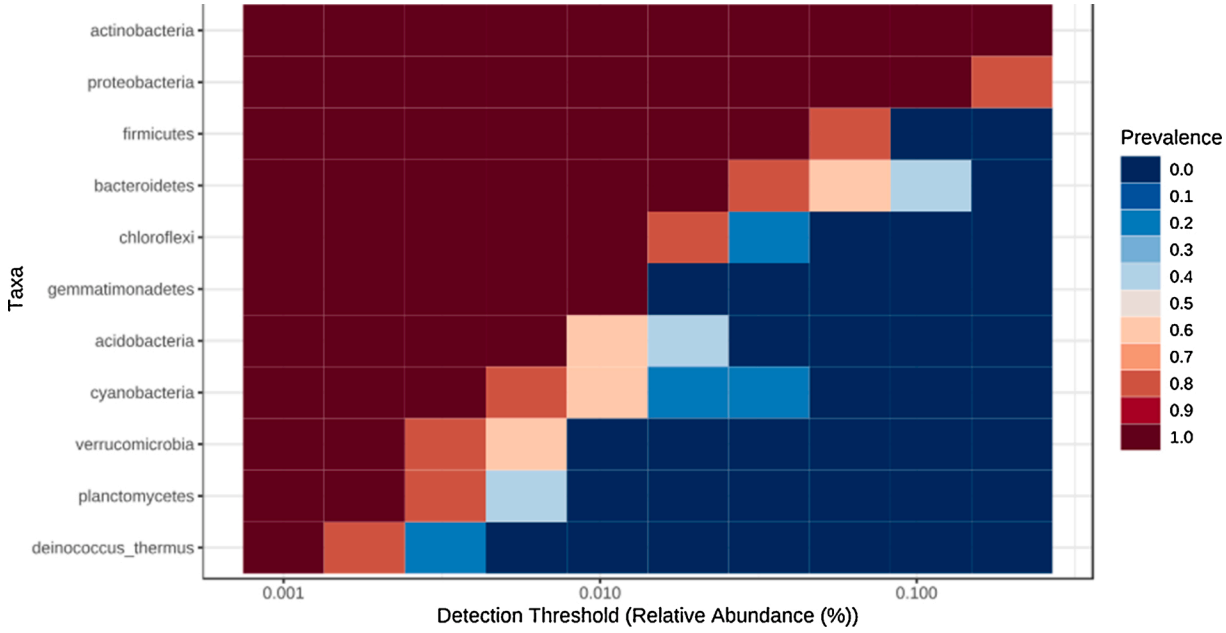

Fig. 15. Class based core bacteria present in soybean rhizosphere throughout the growth stages.

environmental conditions need further studies.

Authors' contribution

NOI designed the study, performed the experiments, did statistical and bioinformatics analyses of all the data and wrote the article; OOB conceptualizes the work, secured funding, led the sample collection, provided academic input and expertise in writing the manuscript, commented on the manuscript at all stages and thoroughly critiqued the article; while XC and BT did fatty acid analyses of soybean seeds. All authors approved the article for publication.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors

Declaration of Competing Interest

All authors declare no conflict of interest

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