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Microbial consortia inoculation of woody legume *Erythrina brucei* increases nodulation and shoot nitrogen and phosphorus under greenhouse conditions

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ABSTRACT

The legume-rhizobium symbiosis provides Nitrogen (N), while Legume-AMF symbiosis improves Phosphorus (P) supply to plants. This research was conducted to evaluate the symbiotic effectiveness of the Bradyrhizobium spp. and consortial inoculation of plant growth promoting bacteria -*Bradyrhizobium shewense* (AU27) and *Acinetobacter soli* (AU4), and arbuscular mycorrhizhal fungi *Glomus* sp.1 (AMF1) and *Acaulospora* sp.1 (AMF2), on growth, production and shoot N and P content of *Erythrina brucei*.The bacterial and mycorrhizal species were evaluated for phyto-beneficial properties in the greenhouse as individual as well as consortial inoculation.. All Bradyrhizobium species were effective for symbiotic nitrogen fixation. Consortial inoculations comprising of *B. shewense* (AU27) + *A. soli* (AU4) + *Glomus* sp.1 (AMF1) + *Acaulospora* sp.1 (AMF2) (T7) increased shoot length and shoot dry weight by 140% and 268%, respectively compared to un-inoculated control. Inoculations that involved *B. shewense* (AU27) + *A. soli* (AU4) increased shoot nitrogen by 260%, and 1200% increment of shoot P was recorded with inoculations of *B. shewense* (AU27) + *Glomus* sp.1 (AMF1) compared to un-inoculated control. These microbial inputs could be candidates for growth enhancement and shoot nitrogen and phosphorus improvement in *Erythrina brucei* and also as sustainable and eco-friendly agriculture input.

1. Introduction

Nitrogen (N), phosphorous (P) and Potassium (K) are the three most important nutrients that determine soil fertility and limit plant growth. However, it is established that plant growth and health is not only determined by the availability of these nutrients, but also by the presence of consortium of microorganisms in the vicinity of the root surface known as the rhizosphere. About 2-5% of the rhizosphere competitive microbes exert phyto-beneficial effects. The use of plant growth-promoting microbes (PGPM) is a potentially advantageous technique for improving crop productivity, food quality and security in more sustainable and eco-friendly agricultural systems [2, 14, 24]. These microorganisms are engaged in symbiotic relationships with a multitude of above- and belowground plant parts that constitute phyto-beneficial microbes, including rhizobia, mycorrhizal fungi, and endophytes [34].

Rhizosphere associated bacteria referred to as rhizobacteria and the Mycorrhizha majorly contribute to plant growth promoting functions. Somers et al. [38] have classified these rhizosphere associated microorganisms based on their roles as (i) biofertilizers (increasing the availability of nutrients to plant), (ii) phytostimulators (plant growth promotion, generally through phytohormones production), (iii) rhizoremediators (degrading organic pollutants) and (iv) biopesticides (controlling diseases, mainly by the production of antibiotics, antifungal metabolites, synthesis of fungal cell wall and its component degrading enzymes). Plant growth promoting rhizobacteria have the potential to produce different types of metabolites that help the host plants to improve minerals such as phosphorus and iron, promote growth and protect them from phytopathogens, and enhance their tolerance to abiotic stresses [3, 32].

The symbiotic association between leguminous plants and rhizobium

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that leads to biological nitrogen fixation (BNF) [44], is very important in nitrogen nutrition in any ecosystem. Arbuscular mycorrhizal fungi (AMF) are present in nearly all soils, forming a symbiotic association with roots of approximately more than 80% of terrestrial plant species [36]. Arbuscular mycorrhizal fungi mediates up to 70% of total P uptake by plants and other immobile nutrients by producing extensive hyphae that grow out from roots and effectively increase exploratory areas of roots and help in harnessing fixed/immobile P [10].

The symbiotic association involving rhizobium, AMF and the legume plant is referred to as tripartite symbiosis, and is a mutually beneficial interactions and plays a pivotal role in natural ecosystems by influencing plant productivity, nutrition, and community structure [3]. Dual inoculation of leguminous plants with rhizobium and AMF is being recommended to improve legume plant growth, nodulation and to increase shoot N and P content [3, 45]. However, the effectiveness of the rhizobium-AMF-plant interactions varies with host plant species, Rhizobium strains, arbuscular mycorrhizal fungal species and soil conditions [30, 39].

Erythrina brucei is a woody legume tree characterized by very important agro forestry attributes such as rapid establishment, tolerance to light, possession of spreading canopy, high rate of litter production, rapid litter decomposition and very soft woody nature [16, 28]. It is extensively planted in the farmlands in southern Ethiopia in the agro forestry systems. Its biomass has been used as green manure for crop growth enhancement. The local farmers in southern and southwestern Ethiopia commonly plant E. brucei inside their farmlands, while cultivating crops such as barley, wheat and maize. The farmers prune the branches and leaves of E. Brucei and mulch it under soil before sowing the grains. They also collect the branches and leaves of E. brucei from forests, shades, home gardens and land boundary fences and transport to farmlands as low-cost agricultural inputs. The legume is well integrated in Sidama agro-forestry system [12] in southern Ethiopia. The plant is reported to improve soil fertility as it fixes atmospheric nitrogen in association with root nodulating bacteria [1, 43]. The symbiotic association of this host plant with arbuscular mycorrhizal fungi has also been reported [12, 25]. Megersa and Assefa [25] have also shown that the dual inoculation of root nodulating bacteria and AMF (Gigaspora and/or Glomus) improved its biomass production threefold compared to the control plants under greenhouse conditions.

Despite the agro-forestry importance of the tree species E. brucei, there is still a dearth of information regarding enrichment of E. brucei biomass with N and P through single inoculation and consortial inoculations with selected and effective endosymbionts to improve its growth, nodulation and N and P status in the biomass.

This work was a part of a long term plan that targets on understanding the rhizosphere microbes of E .brucei for use in its growth promotion. The rhizosphere associated indigeneous (Rhizobia, AMF and rhizobacteria) can be used as microbial inputs for the enhancement of the symbiotic association among the host legume, Rhizobium and AMF which could lead to improved nitrogen fixation, and utilization of phosphorus and other immobile plant nutrients. The use of effective and compatible microbial inoculants could be sustainable and ecofriendly biotechnological inputs to improve the traditional agro-forestry practices in the southern and southwestern Ethiopia.

Therefore, this study was conducted to investigate the effects of combined inoculation of *B. shewense* (AU27) and *Glomus* sp.1 (AMF1) and/or *Acaulospora* sp.1 (AMF2) and/or *Acinetobacter soli* (AU4) on growth, nodulation and shoot nitrogen and phosphorus contents of *E. brucei* under greenhouse condition.

2. Materials and methods

2.1. Sources of the bacterial species

*Bradyrhizobium shewense (*AU27), *Bradyrhizobium cytisi* (HU3) and *Bradyrhizobium cajani* (HO2) were previously isolated from the root nodules of E. brucei ([5]a). These bacterial species were studied for their eco-physiological stress tolerance, carbon and nitrogen substrate utilization versatility and identified using 16S rRNA gene partial sequence (Berza et al., [5]a). *Acinetobacter soli* (AU4), accession number (MK370560) was obtained from the culture collection of the Department of Microbial, Cellular and Molecular Biology, Addis Ababa University.

2.2. Phytobeneficial properties of the bacterial species

2.2.1. Evaluation of the bacterial species for inorganic phosphates solubilization potential

The *E. brucei* root nodule bacteria were evaluated for their potential for solubilizing insoluble inorganic phosphate using Pikovskaya's agar medium (PA) (Pikovskaya, 1948). In iron phosphate ($FePO_4$) or aluminum phosphate ($AlPO_4$) solubilization studies, the quantity of tricalcium phosphate ($Ca_3(PO_4)_2$ in the PA medium was substituted with $FePO_4$ or $AlPO_4$. Ten (10 μL) 48-120 h old culture (10^8 CFU mL^{-1}) of each isolate was spot inoculated on PA medium amended with $Ca_3(PO_4)_2$ or $AlPO_4$ or $FePO_4$ and incubated at 28 °C for 7-10 days. A clear halo zone around the colonies was considered as an indication of phosphate solubilization. The phosphate solubilization (PS) index was calculated according the methods described by Premono et al. (1996).

$$PS = \frac{colonydiameter + Halozonediameter}{Colonydiameter}$$

2.2.2. Quantification of solubilized inorganic phosphate

The inorganic phosphate solubilizing potential of the strains was determined using National Botanical Research Institute's phosphate growth medium (NBRIP) (Nautiyal, 1999). The individual isolates were grown in YEM broth for 72 h from which 10 μl (10^8CFU mL^{-1}) was inoculated into 20 mL NBRIP liquid medium. The quantity of $Ca_3(PO_4)_2$ in NBRIP medium was substituted with $FePO_4$ and $AlPO_4$ in these phosphates solubilization studies. The pH of each medium was adjusted to neutral before the experiment. The un-inoculated medium served as control and both the control and experimental tubes were incubated at 28 °C with gentle shaking at 120 rpm for three consecutive days. Ten (10) mL culture was removed and centrifuged at 12,500 rpm for 10 minutes and the supernatant was used for determining the pH and the amount of phosphate released in the medium. The released phosphate was determined using colorimetric methods as described by Murphy &Riley, (1962).The intensity of color was read using spectrophotometer (Jenway, 6405, UV–VIS Spectrophotometer, England) at 430 nm. The amount of P-solubilized was extrapolated from the standard curve constructed using analytical grade KH_2PO_4. The amount of solubilized phosphate was quantified by subtracting the phosphate released in un-inoculated control from the inoculated cultures. The experiments were conducted in triplicates and values were expressed as mean.

2.2.3. Quantification ofIndole acetic acid (IAA) production

IAA production was estimated according to Gordon and Weber, (1951), indicated by the appearance of pink color. The un-inoculated broth medium served as a control. The amount of IAA produced was quantified by measuring absorbance at 535 nm using a spectrophotometer (Jenway, 6405 Uv/vis spectrophotometer, England) and compared against a standard curve prepared using a known concentrations of analytical grade IAA. The intensity of the pink color was rated as ++ for deep pink; + for pale red and – for no color change.

2.2.4. Determination of Hydrogen cyanide (HCN) and Ammonia (NH_3) production

Production of HCN was qualitatively measured according to the method described by Dinesh et al. (2015), as indicated by the change in color of the filter paper strips from yellow to brown to red. The intensity of color changes was recorded as + for production, and - for no production. The production of NH_3 was determined qualitatively according

to the methods described in Cappuccino and Sherman (1992) indicated by precipitate color change; +, for brown, - for yellow.

2.2.5. Determination of Hydrolytic enzymes

Chitinase production was tested according to the method described by Saima et al.(2013). Similarly, the production of protease was carried out following the procedures described by Dinesh et al. (2015) and Lipase production was detected according to the methods described in Smibert et al. (1994).

2.2.6. Evaluation of symbiotic effectiveness of Bradyrhizobium spp.in sand culture

Greenhouse pot experiments were conducted to evaluate nodulation and symbiotic effectiveness of Bradyrhizobium species in sand culture at College of Natural Sciences Addis Ababa University.

Erythrina brucei seeds were surface sterilized by soaking in 70% ethanol for 1min followed by 3% (v/v) sodium hypochlorite for 8 min and repeatedly washed with sterile water for about four to six times. The surface sterilized seeds were germinated on 1% water agar (w/v) [37]. Four to five healthy seedlings were transplanted in to surface sterilized 3kg capacity plastic pot filled with acid washed coarse river sand. The seedlings were thinned down to three per pot after successful establishment. *B. shewense* (AU27), *B. cajani* (HO2), and *B. cytisi* (HU3) were grown to exponential phase in YEM broth for 6 days at 28°C [42] from which 2 ml (10^6 CFU mL^{-1}) of the suspension, was inoculated to each seedling a week after transplanting. Pots without bacterial inoculation were included as negative control and nitrogen fertilized (0.05% KNO_3) pots as positive control.

The experiments were carried out in triplicates and in a completely randomized design in a greenhouse with 12 h photo period and 22 °C and 15 °C day and night temperatures, respectively. Each pot was fertilized with 50 ml of N-free nutrient solution [8] at 15-day interval. The N-free nutrient solution consisted of (g/l); KH_2PO_4, 0.136.1; $CaCl_2.2H_2O$, 294.1; $MgSO_4.7H_2O$, 123.3; $FeC_6H_5O_7.3H_2O$, 6.7; K_2SO_4, 87.0; $MnSO_4.H_2O$, 0.338; H_3BO_3, 0.247; $ZnSO_4.7H_2O$, 0.288; $CuSO_4.5H_2O$, 0.1; $CoSO_4.7H_2O$, 0.056; $Na_2MoO_2.2H_2O$, 0.048. The solution pH was adjusted to 7.0 with 1 N NaOH. The positive control pots were fertilized with KNO_3 (0.05%) (w/v) every week.

All the pots were watered regularly at two days' interval with sterile water. Plants were uprooted 90 days after planting (DAP) to collect root nodules and plant growth parameters such as nodule number, nodule dry weight, shoot length and shoot dry weights were measured. The relative symbiotic effectiveness (SE) of the Bradyrhizobium species was computed using the formula:

$$SE(\%) = \frac{shoot\ dry\ weight\ of\ the\ inoculated\ plants}{shoot\ dry\ weight\ of\ N\ supplied\ plants} x100$$

Where the relative symbiotic effectiveness (SE) values were rated as highly effective (HE) (if % SE value > 80), effective (E) (% SE value between 50-80), moderately effective (ME) (% SE value between 35-49) and ineffective (I) (% SE value < 35) [4].

2.3. Multiple inoculation Experiment

2.3.1. Compatibility test

The compatibility between *B. shewense* (AU27) and *A. soli* (AU4) was determined by following cross streaking method [33]. In brief, each of the co-inoculated strains were grown in the yeast extract mannitol (YEM) broth medium at 28°C for 72-120 h in shaking incubator at 120 rpm. A loopful (10^6 CFU mL^{-1}) of a bacterial isolate was streaked perpendicularly on freshly prepared YEMA medium; i.e., after the first isolate was allowed to grow at 28°C for 72-144 h, the second isolate was streaked at an angle of approximately 90° going outward from the emerged colonies of the first isolate. Then the second isolate was incubated for 72-144 h at 28°C,if there was growth inhibition it was indicated by the appearance of clear zones at the crossing points,which indicated that the isolates were not compatible.

2.3.2. Microbial inocula preparation for green house experiments

The arbuscular mycorrhizal fungi species *Glomus* sp.1 (AMF1) and *Acaulospora* sp.1 (AMF2) were selected based on their most common occurrence in the rhizosphere of E. brucei ([6]b). During AMF inocula multiplication, 72 spores of *Glomus* sp.1 (AMF1) or 43 *Acualospora* sp.1 (AMF2) spores were inoculated. The mycorrhizal inocula were multiplied using maize (*Zea mays*) host plant. The maize seeds were surface sterilized using 70% ethanol for 1 min followed by 3% sodium hypochlorite for 3 min and rinsed several times using sterile distilled water. The seeds were pre-germinated on 1% water agar and transplanted to pots. About 4 kg capacity surface sterilized plastic pots filled with sterile soil-sand (2:1) mixture were planted with maize seedlings and grown for 90 days. The plants were watered with tap water 2 to 3 times a week for the first 60 days and then the duration of watering was reduced to once a week for the next 15 days. Watering was completely terminated for the final 15 days and the plants were pruned. Plants were uprooted after 90 days after planting and the AMF spore density 100 g^{-1} of dry soil was enumerated using sucrose density gradient centrifugation technique according to the methods described in Brundrett et al. [9]. The AMF root colonization of maize plant was also checked and determined according to the methods described in McGonigle et al. [26]. The AMF colonized maize plant roots and soils were air dried, and roots were chopped in to 1cm length and mixed well with the soil-sand mixture (2:1)and this preparation served as crude inocula.

The *B. shewense* (AU27) was selected for multiple inoculation experiments based on its symbiotic effectiveness, whereas the endophyte *A. soli* (AU4) was selected for the same experiments based on its multiple plant growth promoting properties, eco-physiological stress tolerance and versatility in C and N substrate utilization as previously studied ([5] a). The two bacterial inoculants were grown in YEM broth and nutrient broth for 144 h and 72 h at 28°C, respectively and adjusted to 10^8 CFU mL^{-1}.

2.3.3. The greenhouse experiment

The seeds of *E. brucei* were surface sterilized using 70% ethanol for 1min followed by 3% sodium hypochlorite for 8 min and rinsed several times with sterile distilled water and were incubated to germinate on 1% water agar for seven days. Five seedlings were transplanted into ten (10) kg capacity plastic pots filled with soil: sand (2:1) mixture. The soil used in this experiment was obtained from Menagesha; Gallica Flowers P.L.C farm, 20 kms from Addis Ababa. The physicochemical characteristics of the soil were; pH (1:2.5;H_2O) 7.02; EC 0.13 (us/cm); available P 36.04 mg/kg; K+ 865.76 mg/kg; Ca++ 4170.80 mg/kg; Mg++ 765.93 mg/kg; TN (%) 0.3; OM (%) 5.48.The total nitrogen (TN) was determined using the Kjeldahl method [20], and the soil available phosphorus was determined using the method described by Olsen et al. [29]. The nutrient analysis of the soil samples was outsourced to CROPNUTS Laboratory services, Nairobi, Kenya. The sand used in this experiment was river sand. AMF spore density 100 g^{-1} of dry soil used in this experiment was enumerated using sucrose density gradient centrifugation technique according to the methods described in Brundrett et al. [9].

The seedlings were thinned down to three after establishment to which 150 g crude *Glomus* sp.1 (AMF1) inocula that consisted -120 spores 100 g^{-1} soil: sand mixture (2:1) and 68% AMF colonized maize root segments) or *Acaulospora* sp.1 (AMF2) 85 spores 100 g^{-1} soil: sand mixture (2:1) and 52% AMF colonized maize root segments were inoculated into each pot depending upon the experimental treatment requirements. In combined inoculation treatments involving both AMF species, 75 g of each AMF species crude inocula mixture was used. Similarly, 2 ml of *B. shewense* and/or *A. soli* were inoculated into the respective treatments.

In the greenhouse, pots were arranged in a completely randomized design (CRD) with the following treatments: (T1) -Un-inoculated

control; (T2)- *B. shewense* (AU27) + *Glomus* sp.1 (AMF1); (T3) -*B. shewense* (AU27) + *Acaulospora* sp.1 (AMF2); (T4) -*B. shewense* (AU27) + *Acinetobacter soli* (AU4); (T5) -*B. shewense* (AU27) + *Glomus* sp.1 (AMF1) + *A. soli* (AU4); (T6) -*B. shewense* (AU27) + *Acaulospora* sp.1 (AMF2) + *A. soli* (AU4) and (T7) -*B. shewense* (AU27) + *Glomus* sp.1 (AMF1) + *Acaulospora* sp.1 (AMF2) + *A. soli* (AU4). The greenhouse experiment was carried by maintaining triplicates.

The pots were watered 2-3 times a week for 90 days and also fertilized with N-free nutrient solutions [8] once in two weeks from which the phosphate source (KH_2PO_4) was excluded. This N-free solution contained the nutrients in the following final concentration (gL^{-1}): $CaCl_2.2H_2O$, 294.1; $FeC_6H_5O_7.3H_2O$, 6.7; $MgSO_4.7H_2O$, 123.3; K_2SO_4, 87.0; $MnSO_4.H_2O$, 0.338; H_3BO_3, 0.247; $ZnSO_4.7H_2O$, 0.288; $CuSO_4.5H_2O$, 0.100; $CoSO_4.7H_2O$, 0.056 and $Na_2MoO_2.2H_2O$, 0.048.

After 90 days, plants were harvested, and the shoot and root length, shoot dry weight, nodule number and AMF root length colonization were measured. The total shoot nitrogen content was determined according the Kjeldahl method as described by Hinds & Lowe [20] and shoot phosphorous was determined according to the method described in Olsen et al. [29]. AMF spore density 100 g^{-1} of dry soil-sand mixture was enumerated using sucrose density gradient centrifugation technique according to the methods described in Brundrett et al. [9]. The number of nodules per plant was determined for each treatment with *B. shewense* (AU27) inoculation and AMF root colonization was also assessed for both native and inoculated AMF species treatments according to McGonigle et al. [26]. Shoot and root dry weights were determined by drying the shoots and roots at 70 °C for 72 h until constant weight is recorded.

2.4. Statistical analysis

One-way ANOVA was employed to test significant differences in different parameters within and between inoculation treatments using SAS version 9.2. Tukey's HSD multiple range test was conducted to test for mean separation ($p<0.05$).

3. Results

3.1. Determination of phyto-beneficial properties of the bacterial species

The bacterial species involved in this multiple inoculation experiment were evaluated for different plant growth promoting properties. Among the bacterial species evaluated, *A. soli* (AU4) exhibited multiple phyto-beneficial properties (Table 1). It produced 171.65 μg L^{-1} IAA and solubilized 108.96 mg L^{-1}, 87.33 mg L^{-1} and 84.08 mg L^{-1} of calcium triphosphate, aluminum phosphate and ferric phosphate, respectively (Table 1). In addition to IAA production and inorganic phosphate solubilization, *A. soli* (AU4) also exhibited synthesis of different volatile secondary metabolites (HCN and NH_3) and hydrolytic enzymes lipase and protease (Table 1). Among Bradyrhizobium species evaluated, only *B. shewense* (AU27) exhibited phyto-beneficial trait (phosphate solubilization). Bradyrhizobium species did not exhibit the production of volatile secondary metabolites and hydrolytic enzymes that were evaluated. As result of its possession of multiple phyto-beneficial properties, *A. soli* (AU4) was selected for further multiple inoculation studies in the greenhouse conditions using the host plant species. Moreover, the Bradyrhizobium species were evaluated for nitrogen fixing potentials in sand culture studies.

3.2. Nodulation and symbiotic effectiveness of Bradyrhizobium species in sand culture

The relative symbiotic effectiveness of the Bradyrhizobium species under greenhouse conditions is presented in Table 2.

The Bradyrhizobium species exhibited variations in the induction of number of root nodules. Root nodules were not recorded in the negative control treatment revealing sufficient care has been taken to avoid contamination. The highest number of 30±1 nodules per plant was recorded from *E. brucei* inoculated with *B. cytisi* (HU3) followed by 26±1.5 nodules per plant induced by *B. shewense* (AU27) (Table 2). The size of the root nodules was large, round shaped and more concentrated on tap roots and also evenly distributed on lateral roots. The inoculated *E. brucei* plants also showed variation in the dry weight of root nodule which ranged between 0.28±0.01 g $plant^{-1}$ and 0.65±0.01 g $plant^{-1}$. *B. shewense* (AU27) exhibited the highest nodule dry weight per plant of 0.65±0.01 g followed by *B. cytisi* (HU3) which resulted in 0.43±0.01 g per plant. Bradyrhizobium spp. *B. shewense* (AU27) & *B. cytisi* (HU3) performed better compared to *B. cajani* (HO2) with regard to nodule dry weight.

The shoot length of plants inoculated with Bradyrhizobium species also exhibited variations among treatments and these ranged between 6.5±0.1 cm and 7.83±0.1cm $plant^{-1}$ which was much lower than the N-fertilized control plants with (8.90±0.1cm). All of the inoculated plants exhibited 2-3-fold increase in shoot length compared to the un-inoculated control plants (Table 2).

The shoot dry weight due to Bradyrhizobium species inoculation also exhibited differences among the treatments. All the inoculants produced significantly ($p<0.05$) higher shoot dry weight which ranged between 3.16±0.1 g and 5.45±0.1 g $plant^{-1}$. Bradyrhizobium species inoculated treatments produced much higher shoot dry weight compared to the un-inoculated and unfertilized control plants (1.36±0.1 g $plant^{-1}$) (Table 2). Particularly, inoculation with *B. shewense* (AU27) produced significantly ($p<0.05$) higher shoot dry weigh (5.45±0.1 g $plant^{-1}$) followed by *B. cytisi* (HU3) (4.05±0.01 g $plant^{-1}$) and *B. cajani* (HO2) (3.16±0.1 g $plant^{1}$). Based on its better performance in plant growth parameters and symbiotic effectiveness in the sand culture, *B. shewense* (AU27) was selected for multiple inoculations studies in the greenhouse conditions using the host plants.

3.3. Multiple inoculations Experiment

3.3.1. Plant growth improvement by inoculations with phyto-beneficial microbial inputs

The bacterial species evaluated in this particular study were compatible to each other. Bacterial growth was recorded all over cross streaks without exhibiting any inhibition zones.

The shoot length of *E. brucei* plants inoculated with *B. shewense* (AU27), *A. soli* (AU4) and AMF species *Glomus* sp. (AMF1) & *Acaulospora*

Table 1
Phytobeneficial properties of bacterial species obtained from root nodules of *E. brucei*

Isolates	Strains	IAA/μg/mL	PSI	$Ca_3(PO4)_2$(mg/L)	$AlPO_4$(mg/L)	$FePO_4$(mg/L)	HCN	NH_3	Chitinase	Lipase	Protease
AU4	Acinetobacter soli	171.65	6.0	108.96	87.33	84.08	+	+	-	+	+
AU27	Bradyrhizobium shewense	-	1.0	ND	ND	ND	-	-	-	-	-
HO2	Bradyrhizobium cajan	-	0.0	ND	ND	ND	-	-	-	-	-
HU3	Bradyrhizobium cytisi	-	0.0	ND	ND	ND	-	-	-	-	-

ND-not determined; PSI-phosphate solubilization index;
+ Producer
- non-producer

Table 2
Nodule number (NN), nodule dry weight (Ndwt), shoot length (SL), and shoot dry weight (Sdwt) and relative symbiotic effectiveness (SE) of Bradyrhizobium species isolates from E. brucei root nodule after 90days of growth in a greenhouse.

S. No	Strains	NN	Ndwt (g/ plant^{-1})	SL(cm)	Shdwt (g/plant^{-1})	SE (%)	SE Status
1	HU3	30.0±1a	0.43±0.01b	7.56±0.011b	4.05±0.01c	94	HE
2	AU27	26.33±1.5a	0.65±0.01a	7.83±0.1ba	5.45±0.1a	126	HE
3	HO2	6.33±1.5b	0.28±0.01c	6.50±0.1ba	3.16±0.1d	73	E
4	+ control	-	-	8.90±0.1a	4.32±0.011b	-	-
5	- control	-	-	2.53±0.01c	1.36±0.1e	-	-

HE-Highly effective, E-Effective. Values are mean±SD and are expressed as means of triplicate experiments. Means with the same letter in the same column are not significantly different at p <0.05 by Tukey's HSD test.

sp.1 (AMF2) were recorded across growth periods at 30, 60 and 90 days after planting (DAP). Fig.1 presents the shoot length of *E. brucei* plants inoculated with *B. shewense* (AU27) and/or different combinations of AMF species *Glomus* sp. (AMF1) & *Acaulospora* sp.1 (AMF2) and/or *A. soli* (AU4). The shoot lengths of the inoculated plants varied between 12.0±0.1 cm and 17.33±0.1 cm during the first 30 DAP, with range of 5.33±0.1 cm. The highest shoot length (17.33±0.1 cm) was recorded by plants inoculated with all microbial inputs *B. shewense* (AU27) + *A. soli* (AU4) + *Glomus* sp. (AMF1) + Acaulospora sp.1 (AMF2) (T7) followed by (15.33±0.1 cm) due to *B. shewense* (AU27) + *A. soli* (AU4) + *Glomus* sp. (AMF1) inoculation during first 30 days after planting.

The shoot length also varied between 26.50±0.1 cm in un-inoculated control and inoculations contained *B. shewense* (AU27) + *A. soli* (AU4) to 37.66±1 cm in inoculations that involved all the inoculants (T7) with a range of 11.16±0.1 cm at 60 DAP. The shoot length of the host plants at least doubled during 60 DAP compared to shoot length produced at the first 30 days (Fig.1). Similarly, at 90 DAP; the shoot length also varied between 32±0.1 cm in un-inoculated plants and 76.67±1 cm in inoculation treatment that involved all the inoculants (T7) with a range of 44.67±1 cm plant length (Fig 1).Moreover, inoculations with multiple phyto-beneficial microbial inputs exhibited increased shoot length compared to the dual inoculations. However, among dual inoculation treatments, those that involved *B. shewense* (AU27) + *Glomus* sp. (AMF1) + *Acaulospora* sp.1 (AMF2) improved plant performance in terms of plant height compared to B. shewense (AU27) + A. soli (AU4) dual inoculation at the 90 DAP (Fig.1).

3.3.2. *Effects of inoculations of phyto-beneficial bacterial and AMF species on nodulation*

We did not record root nodules on un-inoculated control plants revealing the absence of compatible E. brucei nodulating native rhizobia in the soil used in this study. A significant ($p<0.05$) variation in the number of root nodules was observed among inoculation treatments that contained *B. shewense* (AU4). The highest number of 18±0.10 nodules per plant was recorded in treatments with consortial inoculation that contained all inoculants (T7) followed by (17±0.11.00) nodules per plant produced by treatment involved *B. shewense* (AU27) + *Glomus* sp.1 (AMF1) + *A. soli* (AU4) (T5) (Fig.2). The representative dissected root nodules exhibited pink colorations as indication of effectiveness.

3.3.3. *Effects of inoculations of phyto-beneficial bacterial and AMF species on shoot dry weight, shoot length and root length*

The effect of dual and consortial inoculations of the selected phyto-beneficial bacterial and arbuscular mycorrhizal fungi species on growth and production of the host plant were evaluated in a soil-sand culture under greenhouse conditions (Table 3). The consortial microbial inoculations involving dual, triple or multiple isolates exhibited statistically significant ($p<0.05$) differences in shoot dry weight within the treatments. The highest shoot dry weight of 57.67±1.01 g per plant was recorded from the treatment that involved consortia inoculation of all the test microbes (T7) followed by (51.80±0.5 g) per plant from triple inoculation treatment (T6) that was inoculated with all inoculants, except the *Glomus* sp.1 (AMF1) (Table 3).

Dual inoculations that involved *B. shewense* (AU4) and *Glomus* sp.1 (AMF1) (T2) or *Acaulospora* sp.1 (AMF2) (T3) exhibited significantly ($p<0.05$) higher shoot dry weight of 38±1.1 g and 35±1 g, respectively compared to dual inoculations with *B. shewense* (AU27) and *A. soli* (AU4) (T4) which exhibited 26±0.3 g shoot dry weight (Table 3).

Similarly, the shoot length recorded among treatments also significantly ($p<0.05$) varied between 32±1 cm in un-inoculated control and 76.67±1.5 cm in inoculations that involved all inoculants. The highest shoot length of 76.67±1.5 cm was recorded from the consortia inoculation treatment that contained all the inoculants (T7) followed by 73.67±1.1 cm that involved triple inoculation of treatment of

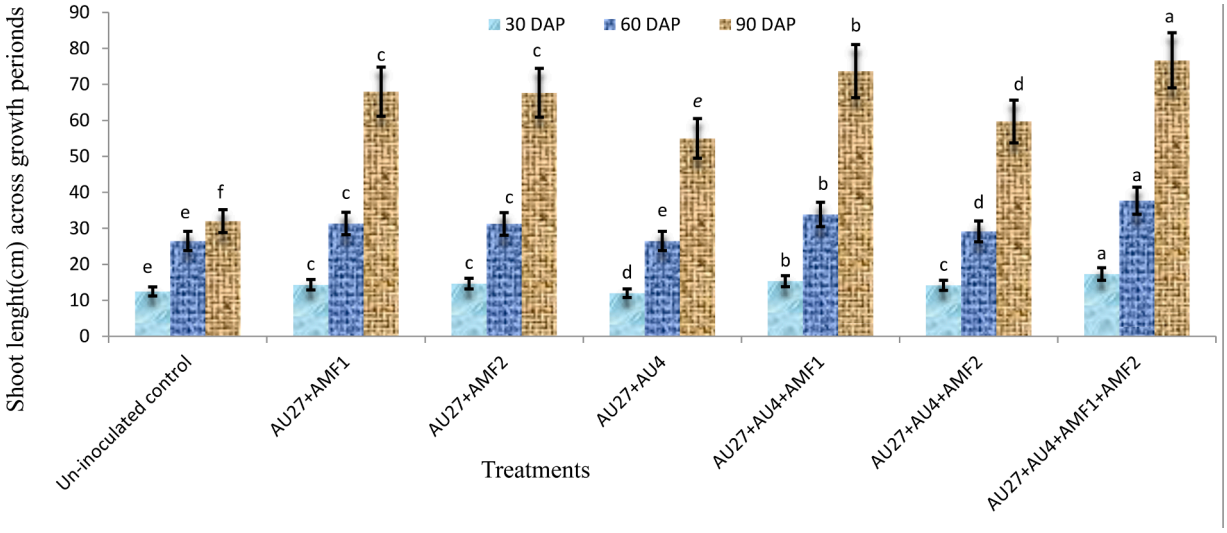

Figure 1. The effects of the inoculation of microbial consortia on the shoot length of E. brucei across growth periods under greenhouse conditions.

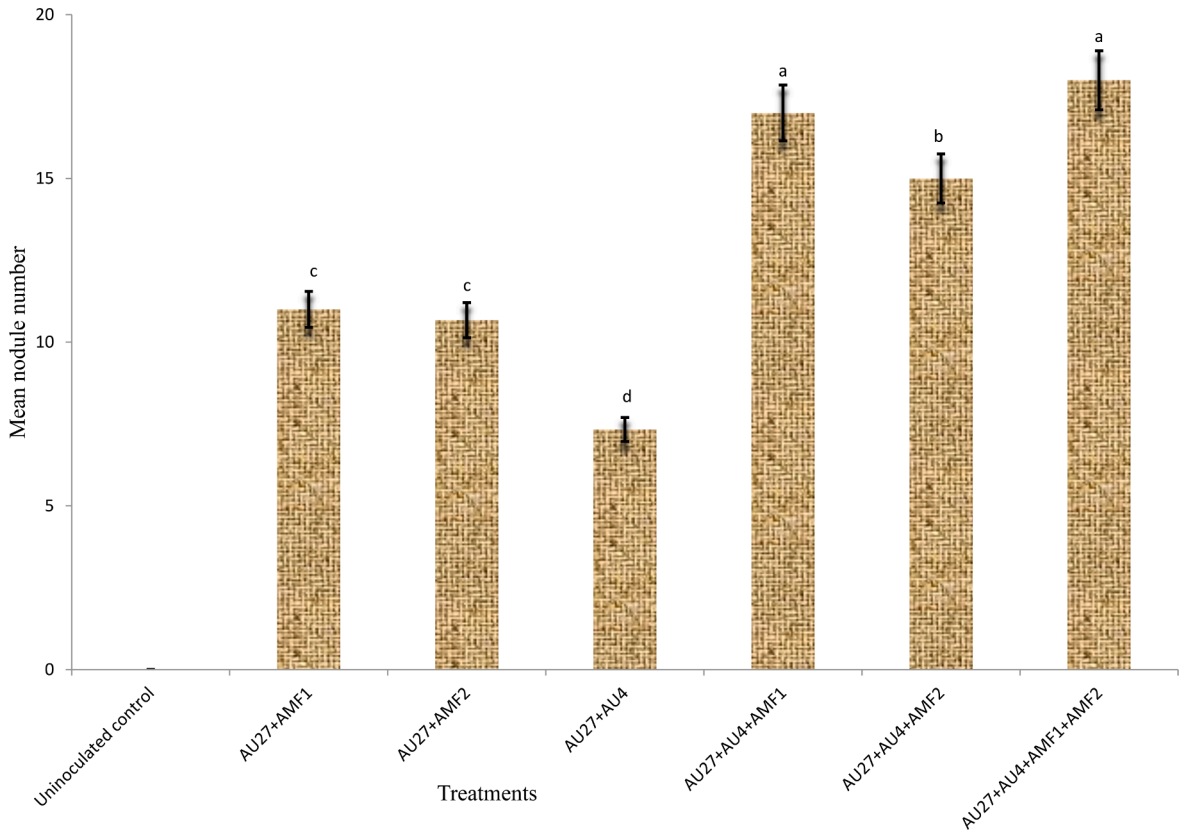

Figure 2. The effects of inoculation of microbial consortia on the root nodule number of E. brucei 90 days after planting under greenhouse conditions

Table 3
The mean shoot and root length, shoot dry weight, nodule number and AMF root length colonization of *E. brucei* inoculated with *B. shewense* (AU27) and *Glomus* sp.1 (AMF1) and/or *Acaulospora* sp.1(AMF2) and/or *A. soli* (AU4) grown for 90 days in greenhouse condition.

Treatments	Treatment code	Shoot length (cm)	Root length (cm)	Shoot dry weight (g)
Un-inoculated control	T1	32.00±1.5d	21.00±1.8e	15.68±1.2f
AU27+AMF1	T2	68.00±3.2ba	58.53±1.9cb	38.17±2.4d
AU27+AMF2	T3	67.67±2.3ba	55.73±1.1cd	35.70±3.3d
AU27+AU4	T4	55.00±1.8c	63.00±4.3b	26.04±2.5e
AU27+AU4+AMF1	T5	73.67±4.23a	76.00±2.1a	51.80±3.2b
AU27+AU4+AMF2	T6	59.67±1.6bc	51.67±2.9d	44.33±1.7c
AU27+AMF1+AMF2+AU4	T7	76.67±5.2a	73.67±3.4a	57.67±2.7a

Values are mean±SD and are expressed as means of triplicate experiments. Means with the same letter in the same column are not significantly different at $p < 0.05$ by Tukey's HSD test.

B. shewense (AU27) + *A. soli* (AU4) +*Glomus* sp.1 (AMF1). Inoculation with these microbial inputs increased the host plant shoot length between 1.7 and 2.4 fold compared to the negative control (Table 3).

Likewise, the inoculation of *E. brucei* with different microbial inoculants also exhibited variations in root length. The longest root (76.0±1.5 cm) was recorded from the inoculation treatment involved *B. shewense* (AU27) + *A .soli* (AU4) + *Glomus* sp.1 (AMF1) (T5) followed by 73.67±1.1 cm root length as a result of multiple inoculation with all the inoculants (T7) (Table 3). The dual inoculations of the host plant with *B. shewense* (AU27) and A. soli (AU4) (T4) produced longer roots compared to dual inoculations that consisted of *B. shewense* (AU27) and *Glomus* sp.1 (AMF1) (T2) or *Acaulospora* sp.1 (AMF2) (T3).

3.3.4. Effect of phyto-beneficial bacteria and AMF inoculation on Root length colonization

Mean percentage root length colonization recorded from dual, triple and multiple inoculation treatments in a greenhouse soil-sand pot culture is presented in Fig.3. We recorded root colonization in un-inoculated control and AU4 inoculated treatments. The root colonization with AMF species inoculation could be due to native AMF species in the soil used in this greenhouse study. The mean percentage of AMF root length colonization varied significantly ($p<0.05$) among the inoculated treatments. The average AMF root colonization ranged between 37.4% in *B. shewense* (AU27) + *Glomus* sp.1 (AMF1) (T2) plants and 54.93% in consortia inoculation treatment that involved *B. shewense* (AU27) + *A. soli* (AU4) + *Glomus* sp.1 (AMF1) + *Acaulospora* sp.1 (AMF2) (T7) (Fig. 3). We did not observe significant difference in root length colonization for dual inoculations that involved *B. shewense* (AU27) and AMF treatments (T2 & T3). Statistically significant ($p<0.05$) differences were recorded in root length colonization between triple inoculated treatments (T5 & T6). In general, multiple inoculation treatments (T5, T6 & T7) exhibited a higher mean percentage root length colonization compared to the dual inoculation treatments regardless of the inoculation types (Fig.3).

3.3.5. Effects of phyto-beneficial bacteria and AMF inoculations on shoot nitrogen and phosphorous content

The dual and multiple inoculations resulted in statistically significant

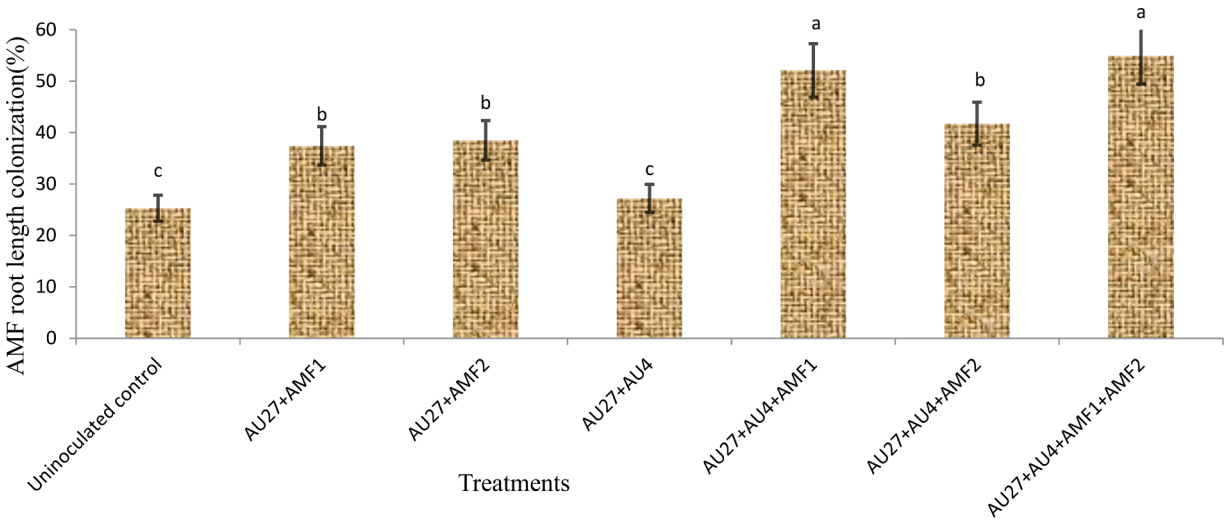

Figure 3. The effects of inoculation of microbial consortia on the AMF root length colonization of E. brucei 90 days after planting under greenhouse conditions

($p<0.05$) difference in shoot total nitrogen and phosphorous contents (Table 4**)** among various inoculation treatments. The shoot total nitrogen (TN) content varied between 2.72% in *B. shewense* (AU27) and *Acaulospora* sp.1 (AMF2) inoculated treatments (T3) and 4.50% in *B. shewense* (AU27) and A. soli (AU4) involved treatments (T4). In general, inoculation of *E. brucei* with microbial inputs increased shoot total nitrogen content at least by 2.2 to 3.6-fold compared to un-inoculated control plants. Similarly, the dual and consortia inoculations showed variation in accumulated ($p<0.05$) shoot P that varied between 1.04% in treatments involved *B. shewense* (AU27) + *A. soli* (AU4) + *Glomus* sp.1 (AMF1) (T5) and *B. shewense* (AU27) + *A. soli* (AU4) + *Acaulospora* sp.1 (AMF2) (T6), and 1.56% in treatment which contained *B.shewense* (AU27) and Glomus sp.1 (AMF1) (T2). The highest shoot P content was recorded from inoculation treatments which consisted of dual inoculations of *B. shewense* (AU27) and *Glomus* sp.1 (T2) followed by *B. shewense* (AU27) and *Acaulospora* sp.1 (AMF2) (T3). In general, inoculation of *E. brucei* with microbial inputs exhibited shoot phosphorous between at least 8.6 and 13-fold increment compared to un-inoculated control.

4. Discussion

Rhizosphere associated plant growth promoting bacteria and AMF are reported to enhance growth, development and improve biomass nitrogen and phosphorus contents of woody legume E. brucei. This host woody legume is used in agro-forestry as a low cost agricultural input for improving soil fertility by smallholder farmers in southern Ethiopia. It is being planted in the home gardens, farmlands, land boundaries and forests. Smallholder farmers use the biomass of *E. brucei* in farmland for mulching, as green manure and cover plant material and as a substitute to chemical fertilizers to improve crop productivity.

Several symbiotic and non-symbiotic bacteria have been previously reported from the root nodules of E. brucei (Amsalu et al., 2012, 2013; [5]a). These authors also reported various phyto-beneficial properties like IAA production, inorganic phosphate solubilization, synthesis of volatile secondary metabolites and hydrolytic enzymes exhibited by E. brucei root nodule bacteria. Similarly, Dobo et al. [12] and Berza et al. [5] have reported other phyto-beneficial microorganisms, arbuscular mycorrhizal fungi (AMF) from the rhizosphere of the same host plant implying that the rhizosphere and root nodules of this particular plant are rich in phyto-beneficial microbes which can be inoculated to E. brucei to enhance its growth and improve its biomass nitrogen and phosphorous and to be used as low cost agricultural input by smallholder farmers in Ethiopia.

As revealed by the shoot dry matter accumulation, *B. shewense* (AU27), and *B. cytisi* (HU3) are highly effective since they accumulated shoot dry matter more than 80% of the N-fertilized plants [27]). However, *B. cajani* (HO2) enabled the host plant to accumulate 73% of the shoot dry matter of the positive control plants, and is rated as effective. As indicated in the results, a significant differences in plant height and shoot biomass between the inoculated treatments and the control under greenhouse conditions. This can be explained by an increase in the supply of nitrogen by symbiotic association with the inoculated nitrogen fixing bacterial strains. This increment in the supply of nitrogen was reflected in better plant growth. Similar results were reported by [18] who have showed that rhizobial inoculation improves nitrogen fixation, photosynthetic capacity and total biomass of cowpea plants. Other researchers have also emphasized that symbiotic nitrogen fixation is directly related to shoot dry matter produced as a result of rhizobium inoculation [27, 37]. It is interesting to note that inoculation with B. shewense (AU27) accumulated shoot dry matter more than the N-fertilized control plants (129%) (Table 1) indicating that the inoculant not only fixed atmospheric nitrogen through the symbiotic interaction with the host plant, but also enhanced the host plant growth with a possible production of different types of phyto-beneficial traits by the Rhizobium species. The accumulation of 3.16±0.1 to 5.45±0.1 g shoot dry weight per plant by the Bradyrhizobia species in this study was much higher than those reported by Megersa and Assefa [25], which was 2.49 g shoot dry weight per plant. Apart from the inherent effectiveness of the inoculants, the big difference could be attributed to the long growing period (90 days) of the plant growth in this experiment compared to the

Table 4
Shoot total nitrogen (TN) (%) and phosphorus (P) (%) of *E. brucei* dual, triple and multiple inoculated with *B. shewense* (AU27) and *Glomus* sp.1 (AMF1) and/or *Acaulospora* sp.1(AMF2) and /or *A. soli* (AU4) and grown in greenhouse for 90 days.

Treatments	Treatment code	Shoot nitrogen	Shoot phosphorus
Un-inoculated control	T1	1.25±0.5g	0.12±0.1f
AU27+AU4	T4	4.50±0.1a	1.25±0.8c
AU27+AU4+AMF1	T5	3.35±0.2d	1.04±0.3e
AU27+AU4+AMF2	T6	3.93±0.6b	1.04±0.7e
AU27+AMF1	T2	2.82±0.9e	1.56±0.5a
AU27+AMF2	T3	2.72±0.3f	1.49±0.2b
AU27+AMF1+AMF2+AU4	T7	3.72±0.4c	1.18±0.1d

Values are mean±SD and are expressed as means of triplicate experiments. Means with the same letter in the same column are not significantly different at $p<0.05$ by Tukey's HSD test.

60 days by the other study. Although all the Bradyrhizobium species were effective and highly effective, B. shewense (AU27) and B. cytisi (HU3) could be used as potential candidates for field trial to enhance the growth, development and biomass nitrogen content of the host plant.

In the sand culture experiment, we recorded nodule number between 6±1.5 and 30±1 per plant. However, between 25±1 and 50±1.1 nodules per plant were recorded during isolation of root nodule bacteria using plant infection method from E. brucei rhizosphere soil samples. On the other hand, in the multiple inoculation experiment, we recorded between 7±0.1 and 18±0.1 nodules per plant. These small numbers of root nodules could be attributed to the absence of competing ineffective native E. brucei nodulating bacteria as revealed by the lack of nodules in negative controls.

Megersa and Assefa [25] have reported the presence of root nodules between 125 and 143 per plant in greenhouse experiment that involved E. brucei single, dual and consortia inoculations in soil. The number of root nodules produced by a legume plant may be determined by the variety of the host plant and the type of the Bradyrhizobium strain [30]. Hence, the number of nodules produced could depend on the growth stage of the host plant , the demand of fixed nitrogen by the host plant and its rhizosphere micro flora and symbiotic nitrogen fixing efficiency of nodulating rhizobial species.

Interestingly, the inoculated plants in this study exhibited fast growth as revealed by shoot length improvement records across the growth periods. The highest shoot length, 39% and 23% increment compared to the un-inoculated control was recorded by treatments that involved the consortia of all inoculants (T7) and consortia of B. shewense (AU27) + A. soli (AU4) + AMF1 (T5), respectively during the first 30 DAP. The same treatments increased plant height by 19.5% and 5.7%, respectively compared to the dual inoculations comprised of B. shewense (AU27) + AMF during the same growth period. Similarly, inoculation treatments T7 and T5 increased shoot length by 42% and 27.6%, respectively compared to the un-inoculated control plants during the first 60 DAP. Likewise, consortia inoculation treatments, T7 & T5 increased plant height by 20% and 8%, respectively compared to dual inoculation treatments that comprised of B. shewense (AU27) and AMF during 60 days' growth period. The role of AMF inoculation is well expressed among dual inoculation treatments (T2, T3 & T4). AMF + phyto-beneficial bacteria dual inoculations exhibited better plant performance with regard to shoot length compared to phyto-beneficial bacteria +phyto-beneficial bacteria co-inoculation (Fig.1) across the growth periods.

Consortia inoculation with B. shewense (AU27) + A. soli (AU4) + Glomus sp.1 (AMF1) (T5) increased shoot length by 130% and the other treatment that comprised of all inoculants (T7) exhibited the highest increase in shoot length (140%) compared to un-inoculated control at 90 DAP. These treatments (T7&T5) increased shoot length by 13% and 9%, respectively compared to dual inoculation treatments that comprised of B. shewense (AU27) and AMF during the same growth period.

Dual inoculation of *E. brucei* with *B. shewense* (AU27) + *Glomus* sp.1 (AMF1) (T2) and *B. shewense* (AU27) + *Acaulospora* sp.1 (AMF2) (T3) increased plant shoot length by 113% and 111%, respectively compared to un-inoculated control 90 DAP. However, dual inoculation with *B. shewense* (AU27) + *A. soli* (AU4) (T4) increased the shoot length by 72% compared to un-inoculated control 90 DAP. This indicates that AMF species (*Glomus* sp.1 or *Acaulospora* sp.1) co-inoculations enhanced plant shoot length by 39% compared to *A. soli* (AU4)co-inoculation.

Inoculated plants showed better development (shoot length and shoot dry weight) compared to un-inoculated plants. These results can be explained by the improved mineral nutrition availability to the inoculated plants,which was reflected in improved vertical growth and biomass. The variations between AMF and/or A. soli (AU4) inoculations could be attributed to the differences in the rhizosphere function between AMF and phyto-beneficial bacteria. Arbuscular mycorrhizal fungi are well known for their mobilization of available P, macro and/or micronutrients and water beyond the root depletion zone and translocation to the associated host plants. AMF also play vital role in the rhizosphere by solubilizing organic phosphate by producing phosphatase enzyme [3, 31], while phyto-beneficial bacteria have crucial role in inorganic phosphate solubilization. Inoculations with all inoculants highly enhanced plant growth with reference to plant height which might be attributed to the synergistic interactions among *B. shewense* (AU27), AMF species and the *A. soli*(AU4). Vafadar et al. [41] have reported that legumes benefit very much from dual symbiosis (rhizobia and AMF) to improve their growth, biomass and nutrient assimilation. In this experiment, the increased shoot length could be attributed to AMF species (Glomus sp.1 and Acaulospora sp.1)and A. soli (AU4) (which is producer of multiple plant growth promoting traits such IAA, phosphate solubilizing, production of volatile secondary metabolites and synthesis of hydrolytic enzymes (Table 1) which improved plant growth parameters and nutrient uptake. In general, inoculation of this woody legume tree with microbial consortia enhanced growth which is characterized by shoot length increment and production of high amount of litter compared to un-inoculated control plants. The consortia inoculations which comprised of all inoculants (T7) and triple inoculation treatment which contained all inoculants except AMF2 (T5) increased the shoot dry weight by 268% and 230%, respectively compared to the un-inoculated control plants. Similarly, co-inoculations involved AU27 + AMF1 (T2) and AU27 + AMF2 (T3) increased shoot dry weight by 143% and 127%, respectively compared to the un-inoculated control. In addition, co-inoculation of AU27 + AU4 (T4) increased shoot dry weight by 66% compared to the un-inoculated control.

Co-inoculations that comprised of AMF (T2 & T3) increased plant biomass accumulation at least 61% compared to inoculations that involved AU27 + AU4 (T4). Moreover, consortia inoculation treatments, T7, T5 & T6 increased shoot dry weight by 56%, 40% and 20%, respectively compared to the dual inoculation treatments that involved *B. shewense* and AMF. Previously, Megersa and Assefa [25] have reported shoot dry weight increment between17% and 45.3% using the same plant as result of single, dual and consortia inoculations compared to un-inoculated control in the greenhouse experiment.

Phyto-beneficial interactions are observed when a microbial consortium is inoculated to enhance plant growth which could be expressed as an additive or synergistic interaction, in part, are due to the fact that multiple microbial species can perform a variety of tasks in an ecosystem like in the rhizosphere [34]. Therefore, the phyto-beneficial mechanisms of plant growth stimulation like enhanced nutrient availability, phytohormone modulation, biocontrol, biotic and abiotic stress tolerance are exerted by different microbial players within the rhizosphere, such as phyto-beneficial bacteria and arbuscular mycorrhizal fungi [17, 35].

The disparities in plant biomass accumulation as indicated by shoot dry weight in the present study and Megersa and Assefa [25] could be attributed to several different factors. The soils used in this greenhouse experiment were characterized by a higher organic matter content (5.48%) compared to soil used by the other authors, (with 1.53%) organic content. Higher soil organic matter content has a multifaceted strategy to improve soil quality such as increment in bioavailability of soil nutrients like P [11]. The soil organic matter also provides substrates and energy and therefore, increases bioavailability of macro and micro nutrients, allowing the maintenance of soil quality and ecosystem functionality [15, 22]. These could enhance symbiotic nitrogen fixation and photosynthetic rates that could be expressed in terms of biomass accumulation. In addition, relatively higher AMF root colonization in this study might have contributed to the enhanced nitrogen fixation compared to Megersa and Assefa [25]. As demonstrated by results, AMF inoculated treatments performed higher compared to A. soli (AU4) inoculated treatments. This difference could be attributed to the fact that AMF infection often results in increased allocation of C to the root system, implying increased root biomass, respiration and mycelial biomass. Thus, the root and mycelia mass could explore larger soil volume, beyond the depletion zone for nutrients, resulting in a higher nutrient uptake rates [7].

The mean root length colonization as the result of inoculation with microbial inputs varied between 27% and 55%. Similarly, Megersa and Assefa [25] have recorded between 25% and 28% AMF root length colonization in inoculation studies carried out in greenhouse using the same host plant. The higher AMF root length colonization in the present study could be attributed partly to the soil physicochemical properties such as available P and soil organic carbon. The soils used in this experiment were characterized by an available P concentration of 27.05 mg kg^{-1}which falls in medium soil available P range [19, 23]. However, Megersa and Assefa [25] have used soils with 37mg kg^{-1} of available P which falls in higher soil available P range [19, 23]. It is well established that higher soil P content inhibits AMF root length colonization. In addition, the higher soil organic carbon content (3.18 %) in this experiment might have also enhanced AMF root infection compared to soil organic carbon content (0.88 %) in the other authors.

The root nodule bacterial inocula in the presentexperiment might have assisted the germination of AMF spores, thus leading to higher infection percentage [44]. In this context, the plant growth promoting bacteria might have acted as mycorrhiza helper bacteria (MHB). The MHB could promote mycelial growth, improve host recognition and change root system architecture and improve receptivity of roots [40]. The root nodule bacteria, *A.soli* (AU4) applied in the present study was IAA producer (Table 1**)**. Hence, this strain might have enhanced *E. brucei* root growth and development, which in turn stimulated the AMF root length colonization [40].

The E. brucei root growth and development as indicated by root length was also varied among different inoculation treatments. The consortia inoculations in treatments (T5) and (T7) increased the root length by 261.9% and 250.8% respectively compared to un-inoculated control plants.

The increased root length in the treatments (T5 and T7) which received consortial inoculation could be associated to the phytohormone production (IAA) by A. soli (AU4) that probably enhanced root extension, growth and development. The stimulation of root hairs growth and lateral roots elongation by IAA might provide more active sites and access for dual symbiotic association with rhizobia and AMF to improve root architecture, length and dry weight [13].

The inoculation of different microbial inputs enhanced E. brucei biomass, nitrogen and phosphorus contents as exhibited remarkable improvements compared to un-inoculated control. The dual and triple inoculations with B. shewense (AU27) + A. soli (AU4) (T4) and B. shewense (AU27) + Acaulospora sp.1 (AMF2) + A. soli (AU4) (T6) increased the shoot total nitrogen content by 260%, and 214.4%, respectively compared to un-inoculated control. The higher shoot total nitrogen content in the dual inoculated treatments might be due to a higher nitrogenase activity as a result of better P nutrition corroborating to the enhanced nitrogen fixation reported by Bai et al. [3]. The higher shoot total nitrogen and phosphorus contents could allow for increased shoot growth and photosynthetic rates and lead to increased total N and P levels that resulted from the improved biological nitrogen fixation [41].

Likewise, shoot P content significantly ($p<0.05$) differed among inoculated treatments. The inoculation treatments consisted of *B. shewense* (AU27) + *Glomus* sp.1 (AMF1) (T2) and *B. shewense* (AU27) + *Acaulospora* sp.1 (AMF2) (T3) increased the shoot P content by 1200% and 1141.6%, respectively compared to un-inoculated control. Though, A. soli (AU4) was previously confirmed as good inorganic phosphate solubilizer (Table 1), inoculations of B. shewense (AU27) with *Glomus* sp.1 (AMF1) or *Acaulospora* sp.1 (AMF2) enhanced better shoot P accumulation compared to inoculation with *B. shewense* (AU27) and *A. soli* (AU4) (Table 4). The results clearly indicated that inoculation of AMF contributed to higher shoot P accumulation compared to bacterial inoculations. This difference could be attributed to increased available P exploration beyond the depletion zone and translocation to the plant roots by AMF compared to bacterial species which play role in inorganic P solubilization [3, 31].

5. Conclusion

In this experiment root nodule bacteria *A. soli* (AU4) exhibited multiple phyto-beneficial properties and the three Bradyrhizobium species were effective nitrogen fixers, but *B. shewense* (AU27) was the best performer. Multiple inoculation of *E. brucei* with microbial inoculants comprised of *B. shewense* (AU27) + *Glomus* sp.1 (AMF1) + *Acaulospora* sp.1 (AMF2) + *A. soli* (AU4) highly increased plant growth with reference to the shoot length and dry weight. Similarly, total shoot nitrogen content was highly improved as result of inoculation with *B. shewense* (AU27) and *A. soli* (AU4) and the plant biomass was enriched with fixed nitrogen as result of dual and/ormultiple inoculations. Likewise, shoot phosphorus content was highly improved due to dual inoculations comprised of *B. shewense* (AU27) and AMF. In general, shoot P content was improved as a result of dual and/or multiple inoculations. Therefore, inoculation of the host plant with phyto-beneficial microbial inputs can enhance its growth and improve the shoot nitrogen and phosphorous and the enriched plant biomass can be applied as low cost agricultural inputs by small holder farmers in order to achieve sustainable and eco-friendly agriculture.

Declaration of Competing Interest

None

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00707.

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