IMPROVING BENEFICIAL PLANT-MICROBE INTERACTIONS IN ACIDIC AND SALINE SOIL

A dissertation

by

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ABSTRACT

Soil acidity and salinity are major constraints for food productivity around the world. Beneficial plant-microbe interactions (BPMI) could improve plant tolerance to acidity and salinity and increase crop yields. However, soil acidity and salinity can adversely impact many BPMI including the symbiosis between plants and arbuscular mycorrhizal fungi (AMF) and N_2-fixing bacteria. Soil amendments or foliar applied signaling compounds enhance microbial diversity and functions. However, it was not clear how native soil microbial community of an acidic and a saline soil respond to these amendments and impact BPMI of a legume crop. Two greenhouse studies were conducted using a legume crop (cowpeas, *Vigna unguiculata* (L.) Walp.). Goal for the first study was to evaluate the impacts of biochar (BC) as a soil amendment and salicylic acid (SA) as a foliar stimulant on plant nutrient concentrations, rhizosphere and endophytic microbiome, AMF colonization, nodulation and pod yield of cowpea plants grown in an acidic soil. Goal for the second study was to evaluate the impacts of compost (CMP) as soil amendment and foliar application of strigolactones (SL), SA and coumarins (COU) on plant nutrient concentrations, rhizosphere and endophytic microbiome, AMF colonization, nodulation and pod yield of cowpea plants grown in a saline soil. Results from the first study showed that soil acidity reduced nodulation, nutrient uptake, rhizosphere microbiome diversity and pod yield. Biochar (BC) was more effective in increasing soil pH, nodulation, plant nutrient concentrations and pod yields than SA treatment. Biochar (BC) treatment also increased AMF colonization and abundance of several plant beneficial

taxa compared to control. It was concluded that BC application to soil was effective in improving BPMI and cowpea pod yield in acidic soils. Results from the second study showed that soil salinity adversely impacted plant nutrient uptake, AMF colonization and pod yields. Among the treatments, SL+SA produced highest nodulation, AMF colonization and pod yields. Relative abundance of several AMF and plant beneficial microbial taxa were higher in SL+SA treatment. It was concluded that foliar application of SL+ SA was most effective in improving BPMI and cowpea pod yield in saline soils.

DEDICATION

I dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving parents, Shiva Nath Jha and Priti Jha whose words of encouragement has always motivated me to do better. My sister Mahima and my brother Sumit who have never left my side and are very special. I also dedicate this dissertation to my friends Cara, Javid, Jaimin, Sunny and Archana who have supported me throughout my PhD.

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NOMENCLATURE

Al	Aluminum
H^+	Proton ion
N	Nitrogen
P	Phosphorus
K	Potassium
Ca	Calcium
Mg	Magnesium
Mo	Molybdenum
Fe	Iron
Mn	Manganese
Na	Sodium
Cl	Chlorine
S	Sulfur
NO_3	Nitrate
PO_4	Phosphate
N	Nitrogen
EC	Electrical conductivity
KH_2PO_4	Potassium phosphate, monobasic
$MgSO_4.7H_2O$	Magnesium sulfate, heptahydrate
K_2SO_4	Potassium sulfate

$CaCl_2.H_2O$	Calcium chloride, monohydrate
H_3BO_3	Boric acid
$MnSO_4.H_2O$	Manganese sulfate, monohydrate
$CuSO_4.5H_2O$	Copper sulfate, pentahydrate
$ZnCl_2$	Zinc chloride
EDTA	Ethylenediaminetetraacetic acid
DTPA	Diethylenetriamine pentaacetate
RLD	Root Length Density
ROS	Reactive oxygen species
SOD	Superoxide dismutase
CAT	Catalase
POD	Peroxidase
APX	Ascorbate peroxidase
PGP	Plant-growth-promoting
PGPB	Plant-growth-promoting bacteria
PGPF	Plant-growth-promoting fungi
NFB	Nitrogen-fixing bacteria
AMF	Arbuscular mycorrhizal fungi
BPMI	Beneficial plant-microbe interactions
PSB	Phosphate-solubilizing bacteria
PSM	Phosphate-solubilizing microorganisms
BC	Biochar

SA	Salicylic acid
AC	Acidic control
NC	Neutral control
CMP	Compost
SL	Strigolactones
COU	Coumarins
GYP	Gypsum
MYCO	Mycorrhizal inoculant
CS	Control Saline
WAG	Weeks after seed germination
DNA	Deoxyribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
qPCR	Quantitative Polymerase Chain Reaction
NTC	No template control
ITS	Internal transcribed spacer
OTU	Operational Taxonomic Units
CSS	Cumulative Sum Scaling
QIIME	Quantitative Insights Into Microbial Ecology
PCoA	Principal Coordinates Analysis
LEfSe	Linear discriminant analysis effect size
mg	Milligram
Kg	Kilogram

mL	Milliliter
cm	Centimeter
°C	Centigrade
TX	Texas
OR	Oregon
MO	Missouri
WI	Wisconsin
CA	California
FL	Florida
USA	United States of America

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Impacts of soil acidity and salinity on soil quality and plant growth

1.1.1 Soil acidity impacts on plant growth

Soil acidity is a major abiotic stress for plant growth and agricultural productivity around the world. About 30-40% of the world's arable land and about 40.9% in USA are impacted by soil acidity (Von Uexküll and Mutert, 1995). Soil pH below 6.5 is considered acidic and severe impacts on plant growth are noted below pH 5.5. Thus, soil pH between the neutral range of 6.5-7.5 is required for optimum crop growth of most crop plants including legumes (Soares et al., 2014).

Natural soil acidification can occur due to acidic soil minerals such as granites or high rainfall induced leaching of soil base cations from the rooting zone and increasing aluminum (Al) speciation and concentration (Aguilera et al., 2015; Fageria and Baligar, 2008). Soil acidification can also be accelerated by agricultural practices including use of nitrate-forming fertilizers (Goulding, 2016). Acidic soils are generally deficient in phosphorus (P), calcium (Ca), potassium (K), magnesium (Mg) and molybdenum (Mo) (Fageria and Baligar, 2008). Acidic pH range increases the solubility of toxic metal ions such as aluminum (Al), iron (Fe) and manganese (Mn), which are detrimental to crop growth at higher concentrations. Among these, Al is the most abundant and thus, its toxicity is a most common problem in acidic soils (Bose et al., 2015; Gupta et al., 2013; Kochian et al., 2004; Marschner, 1991; Robson, 2012). Excessive Al and H^+ ions in acidic

soils interfere with plant adsorption of P, Mg, Ca, K, and Mo causing plant growth reduction and yield loss (Bhuyan et al., 2019). Furthermore, increased concentrations of Al species [Al^{3+}, $Al(OH)^{3+}$, $Al(OH)^{3+}$, and $Al(OH)^{4+}$] can inhibit root cell division by damaging the cell structure of the root apex, resulting in poor root growth and development (Seguel et al., 2013), consequently decreasing root biomass (Kolawole et al., 2000) and altering the root morphology (Wang et al., 2020a). Phosphorous (P) deficiency is also common in acid soils (Iqbal, 2012) due to high P fixing capacity of Al and Fe-minerals under acidic conditions and precipitation as poorly soluble Al–Pi complexes in the rhizosphere, which limits phosphorus availability (Cumming and Ning, 2003).

1.1.2 Soil salinity impacts on plant growth

A saline soil generally has higher pH range above 8.5, with higher range of electrical conductivity (EC) of the saturation extract in the root zone exceeding 4 dS m^{-1} at 25 °C and exchangeable sodium concentration of more than 15% (Qadir et al., 2007). According to FAO (2012), the area under perpetual salinization had almost reached 34 million irrigated hectares by the year 2012 (Session, 2020). In USA, about 146 million ha (15.8%) of total arable land and 4.2 million ha (23%) of irrigated land was salt-affected in the year 1988 (Shahid et al., 2018). Soil salinization has been rapidly increasing due to poor agricultural practices and it is expected to impact more than 50% of the world arable land by the year 2050 (Jamil et al., 2011; Shrivastava and Kumar, 2015; Wang et al., 2003). Saline soils generally occur in dry regions where a combination of climatic factors such as poor water transmission properties of subsoil horizons, low rainfall, high transpiration and evaporation result in salts accumulation in the surface rooting zone

(Rengasamy, 2002). Salinity levels are also increased by soluble salts addition through saline irrigation water and increased usage of salt forming fertilizers (Al-Karaki, 2000).

In saline soil conditions, crop productivity is lower and yields are reduced (Munns, 2005). Higher salt concentrations in the rooting zone decreases water absorption capacity and adversely affects plant metabolic processes and osmotic balance, nutrient absorbance, hydraulic conductivity and intercellular CO_2 concentrations (Al-Karaki, 2001). Higher concentration of Na^+ and Cl^- in plant tissues alters osmotic balance and reduce plant's ability to absorb other essential nutrient ions such as K^+, $Ca^{2+,}$ and Mn^{2+} (Hasegawa et al., 2000). Toxicity of Na^+ can also disrupt several enzyme structures and damage cell organelles and plasma membrane (Feng et al., 2002). Moreover, optimum K^+: Na^+ ratio is vital to activate enzymes in plant cell cytoplasm necessary for maintenance of plant growth. Although K^+ is generally at adequate amounts in saline soils, it is poorly adsorbed due to interference of Na^+ which competes with K^+ in plant uptake, and thus reducing K^+: Na^+ ratio in plant tissues and severely affecting plant growth (Wakeel, 2013). Higher Na^+ accumulation in root tissues disrupts the root cell membrane causing decreased root growth, root biomass (Zhang et al., 2013a) and root length density (RLD) (Snapp and Shennan, 1992) leading to reduced nutrient uptake in plants.

In addition, both acidic and salinity stress in plants trigger the generation of reactive oxygen species (ROS) in the internal tissues causing severe oxidative damages to the cells such as peroxidation of membrane lipids, oxidation of proteins and DNA strand breakage (Esfandiari et al., 2007; Ma, 2005; Shi et al., 2006). Plants could prevent such oxidative stress by increasing the production of enzymatic antioxidants such as superoxide

dismutase (SOD), catalase (CAT), peroxidases (POD) and ascorbate peroxidases (APX) and non-enzymatic antioxidants such as ascorbate and glutathione (Mittler, 2002; Srivastava and Dubey, 2011a). However, plants that are sensitive to acidic and salt stress conditions have shown reduced capability to produce these enzymes and fail to mitigate ROS in cells causing stunted growth and sometimes death of the plant (Sharma and Dubey, 2007; Yasar et al., 2008).

1.2 Importance of plant-microbe interactions in acidic and saline soil

It is well known that most plants establish interactions with a large variety of microorganisms (Brundrett, 2009). Some microbes are commensals or pathogenic for their hosts. Whereas some microbes are beneficial (symbiotic and mutualistic) and are known to support plant growth and increase plant tolerance to biotic and abiotic stresses (Bent, 2006) and enhance yields (Dimkpa et al., 2009).

Several plant-growth-promoting bacteria (PGPB) such as *Bacillus* (Din et al., 2019), *Pseudomonas* (Zerrouk et al., 2019) and *Streptomyces* (Sadeghi et al., 2012) have shown to promote plant growth under acidic and saline conditions. These beneficial microorganisms help plants to overcome soil fertility constraints through diverse mechanisms such as enhanced nutrient assimilation by biological nitrogen fixation (Kuan et al., 2016), P solubilization (Sharma et al., 2013) and Fe solubilization and acquisition (Jin et al., 2014) and control pathogens by antagonism and competition (Chowdhury et al., 2015). Many plant-growth-promoting fungi (PGPF) such as *Penicillium*, *Aspergillus* and *Trichoderma* promote plant tolerance to acidic and saline conditions by various mechanisms such as buffering pH by releasing organic compounds (Liao et al., 2018), bio

sequestration of toxic ions such as Al or Na (Ghorbani et al., 2008) or enhancing production of plant-growth-promoting (PGP) metabolites and antioxidant enzymes to mitigate oxidative damages under salt and acidic stress conditions (Anam et al., 2019).

Rhizobia-legume interaction is a major symbiosis, in which rhizobia live in root nodules of leguminous plants and convert N_2 to NH_4 for plants in exchange for other nutrients (Oldroyd et al., 2011). Nodulation under acidic and saline soil conditions have also shown to be positively correlated with N_2-fixation, N uptake and plant total N concentrations in several studies (Allito et al., 2020; Aydi et al., 2008; Franco and Munns, 1982) suggesting that improving rhizobia-legume symbiotic interactions play a major role in enhancing plant nutrient content under stressed conditions.

Endophytes are microorganisms residing inside plant tissues, and are mostly mutualistic in nature. The diversity and composition of rhizosphere and endophytic microbial community can vary in response to soil conditions such as soil pH (Liu et al., 2017). Root endophytic microbiome are generally less diverse than the rhizosphere microbiome and more diverse than leaf/shoot endophytes (Bodenhausen et al., 2013). It was also noted that bacterial phyla *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were more abundant in the roots than in the rhizosphere while *Gemmatimonadetes* and *Acidobacteria* were depleted in the roots. It is hypothesized that composition of shoot and root endophytic microbiomes are overwhelmingly similar due to translocation via apoplast in xylem vessels (Chi et al., 2005). Another study noted that *Proteobacteria*, *Actinobacteria* and *Firmicutes* were dominant in shoots of legume plants (Costa et al., 2012). Many of these endophytes promote plants growth under stressed conditions of

acidity and salinity by providing N through N_2-fixation (Moyes et al., 2016), solubilization of P (Ghosh and Mandal, 2020; Hariprasad and Niranjana, 2009), producing various plant hormones (Kusari et al., 2013; Lata et al., 2018) and siderophores (Rungin et al., 2012). One report noted that several endophytes promoted antioxidant enzyme activity and ROS detoxification under stressed conditions (Sheibani-Tezerji et al., 2015). Several endophytic PGPB such as *Burkholderia* (Sheibani-Tezerji et al., 2015; Stopnisek et al., 2014), *Pseudomonas* (Labanca et al., 2020) and PGPF such as *Chaetomium* (Haruma et al., 2019) and *Pantoea* (Chen et al., 2014) were predominant in acidic conditions. Whereas, bacteria *Bacillus* (Abd_Allah et al., 2018), *Pseudomonas* (Ali et al., 2014a; Win et al., 2018), *Streptomyces* (Singh and Gaur, 2017), *Klebsiella*, *Serratia*, *Arthrobacter* and *Microbacterium* (Qin et al., 2014) were abundant in saline soils. Some endophytic fungi such as *Piriformospora indica*, *Penicillium* spp. and *Aspergillus* spp. were noted to promote plant growth in acidic (Khan et al., 2015a) and saline soils (Baltruschat et al., 2008; Khan et al., 2011).

Another example of symbiotic association between fungi and plants is arbuscular mycorrhizal fungi (AMF). AMF are obligate biotrophs (can only grow in the presence of host) that form symbiotic association with roots of about 80% of plant species and establish a bidirectional interchange of nutrients. Elaborate AMF hyphal networks in the soil improves soil exploration and supply of nutrients and water to host plants in exchange for carbon compounds (Aguilera et al., 2015). AMF forms arbuscules inside the root cortical cells which serve as the nutrient exchange sites (Dodd, 2000). Additionally, AMF can also form vesicles between the cortical cells and store nutrients. AMF are able to

establish extensive network of extraradical hyphae, sometime extending beyond the rhizosphere and into intra-mineral and soil aggregates where roots cannot reach (Selvakumar et al., 2014). AMF have shown to increase uptake of P, K and Ca in acidic (Alloush and Clark, 2001) and saline soils (Hajiboland et al., 2010; Turkmen et al., 2008).

1.2.1 Plant tolerance to soil acidity mediated by beneficial plant-microbes

1.2.1.1 Tolerance to Al toxicity

Plant resistance to Al is often attributed to organic acid exudation from plant roots and chelation of Al^{3+} in the rhizosphere (Gaume et al., 2001). Symbiotic associations with AMF can alleviate Al toxicity by minimizing Al availability through their influence on exudation from roots (Aguilera et al., 2015). Secretion of organic acids like citrate and malate have shown to increase in plants associated symbiotically with AMF (Cumming and Ning, 2003) or other rhizosphere and endophytic microbes (Barra et al., 2018; de la Luz Mora et al., 2017) and thus enhancing the chelation of toxic Al^{3+} in acidic soils. In addition to organic acids, glomalin-related proteins produced by AMF can sequester Al (Aguilera et al., 2015), which are recalcitrant complex with high residence time (Rillig et al., 2001). Moreover, some rhizosphere and endophytic bacteria produce siderophores that can bind with Al and reduce Al-toxicity (de la Luz Mora et al., 2017; Haruma et al., 2018).

1.2.1.2 Increase nutrient availability

Poor availability of several essential nutrients under acidic conditions, such as P, Ca, Mg and Mo) contribute to their deficiency and adversely impact plant growth in acid soils (Marschner, 1991). Availability and plant uptake of nutrients can be increased by beneficial microbial interactions (Borie et al., 2010). For example, several phosphate-

solubilizing microorganisms (PSM) can increase P availability to plants grown in acidic soil (Collavino et al., 2010). Several microbes such as *Bacillus*, *Pseudomonas* and *Burkholderia* can increase solubility and mineralization of insoluble P minerals or P-organic complexes (Mehmood et al., 2018). Mycorrhizal associations can also increase P availability by extending hyphal networks (Dutta and Bora, 2019; Seguel et al., 2013). Moreover, bioavailability of occluded or insoluble P-minerals was facilitated by AMF through production of citric acid, malic acid and gluconic acid (Klugh-Stewart and Cumming, 2009). These organic acids are also be produced by rhizosphere and endophytic microbes, which can increase P availability to plants (Ribeiro et al., 2018). Microbes also produce extracellular phosphatase, which can solubilize phytate complexes and increase P availability in the rhizosphere (Rubio et al., 2002).

1.2.1.3 Plant tolerance to oxidative stress mediated by plant beneficial microbes in acid soils

Acidic soils can lead to increased production of ROS in plants and oxidative damage of plant biomolecules (Boscolo et al., 2003). Many studies have shown that AMF and other beneficial microbes can improve plant tolerance to oxidative stress by increasing the activity of antioxidant enzymes such as SOD, POD, CAT and APX and also, by augmenting the concentrations of non-enzymatic antioxidants such as glutathione and ascorbic acid when exposed to soil acidity and Al-toxicity (Bilal et al., 2018a; Dudhane et al., 2012; Khan et al., 2015a). Superoxide anions, one of the ROS generated in plants, are dismutated to hydrogen peroxide (H_2O_2) by the action of SOD. Hydrogen peroxide is further scavenged by CAT, POD and APX enzymes by reducing H_2O_2 to water molecules

(Sharma and Dubey, 2007). Many studies have shown that the expression levels of these antioxidant enzymes are increased in the presence of beneficial rhizosphere and endophytic microbes under abiotic stressed conditions (Afridi et al., 2019; Bharti et al., 2016).

1.2.2 Plant tolerance to soil salinity stress mediated by plant beneficial microbes

1.2.2.1 Increase nutrient uptake and ion homeostasis in plant tissues

Soil salinity significantly reduces the absorption of several essential nutrients, particularly P, as PO_4 binds strongly to Ca^{2+} and Mg^{2+} at higher pH and becomes unavailable to plants (de Aguilar et al., 1979). Mycorrhizal symbiosis can further increase P availability by the extensive hyphal network (Ruiz-Lozano and Azcón, 2000). In addition, several phosphate-solubilizing bacteria (PSB) such as *Pseudomonas* spp., *Bacillus* spp. and *Enterobacter* can also interact with AMF and increase P availability to plant (Osorio, 2011). These bacteria produce organic acids which can reduce soil pH and increase solubilization of P from Ca bound phosphate (or rock phosphate) (Wahid et al., 2016). Released P is taken up by AMF hyphae and thereby maintaining a low soluble P concentration in soil for a continuous and sustained release of P by the associated bacteria (Osorio, 2011).

Plants exposed to salt stress also suffer from Na^+ toxicity and K^+ deficiency, since the acquisition of K^+ is disrupted by excess Na^+ concentration in soil (Porcel et al., 2016). Rhizosphere and endophytic microbes including AMF can facilitate K^+ uptake while preventing Na^+ absorption and translocation to the shoots (Abdelaziz et al., 2017; Evelin

et al., 2009; Ilangumaran and Smith, 2017). Some endophytic microbes such as *Piriformospora indica* has shown to enhance the transcript levels of the genes encoding different K channels e.g., high affinity potassium transporter 1 (HKT1) and the inward-rectifying K^+ channels KAT1 and KAT2, under salt stress (Abdelaziz et al., 2017) and therefore improve K uptake.

1.2.2.2 Plant tolerance to soil salinity induced oxidative stress mediated by plant beneficial microbes in saline soils

Symbiotic association of plants with AMF and endophytic microbes can reduce the production of ROS, and protect cellular membrane structures from different oxidative damages due to salinity (Baltruschat et al., 2008; Han et al., 2014). Increased activity of enzymatic antioxidants (SOD, CAT, POD and APX) and non-enzyme antioxidants (ascorbate and glutathione) has been observed in plants colonized with beneficial microbes under saline conditions (Asaf et al., 2018; Hajiboland et al., 2010; Hashem et al., 2016; Li et al., 2017) and thus preventing plants from the oxidative stress induced by salinity.

1.2.2.3 Osmotic adjustment and photosynthesis

Balancing plant osmotic status and turgor pressure of leaves in saline soils is critical for plant growth and to maintain the balance between photosynthesis and transpiration, water use efficiency and stomatal conductance in the symbiont plants (Augé et al., 2008; Cho et al., 2006). These processes are facilitated by the improved hydraulic conductivity of the root at low water potential (Kapoor et al., 2008). The root conductance is improved

by longer root length and by altering root system morphology, which are induced by AMF associations (Evelin et al., 2009).

Increasing salinity causes a reduction in chlorophyll content (Sheng et al., 2008) due to suppression of specific enzymes that are responsible for the synthesis of photosynthetic pigments (Murkute et al., 2006). A reduction in the uptake of nutrient elements (e.g. Mg) needed for chlorophyll biosynthesis also reduces the chlorophyll concentration in the leaf (El-Desouky, 1998). A higher chlorophyll content in leaves of plants associated with beneficial microbes and AMF under saline conditions was reported in several studies (Giri and Mukerji, 2004; Rojas-Tapias et al., 2012; Sannazzaro et al., 2006; Sheng et al., 2008).

1.3 Impact of soil acidity and salinity on beneficial microbe interactions

Soil pH is a major driver of microbial diversity and composition in the plant rhizosphere (Zeng et al., 2019) and endosphere (Papik et al., 2020). Higher proton (H^+) concentration in acidic soil can impact microbial community by disrupting cell membranes, cell division and altering enzyme activity (Sullivan et al., 2017). Inhibition of microbial growth and activity in acidic conditions can reduce abundance and diversity of rhizosphere and endophytic microbiome (Wan et al., 2020). A recent study showed that rhizosphere and endospheric microbiome of plants grown in acidic soil were more abundant in bacterial phyla *Acidobacteria*, *Firmicutes* and *Chloroflexi* and depleted in *Actinobacteria* and *Bacteroidetes* (Wan et al., 2020). Among fungi, *Ascomycota* and *Basidiomycota* were dominant phyla in acidic soil conditions (Zhang et al., 2016b).

Soil acidity significantly reduces nodulation and N_2-fixation in legumes (Lin et al., 2012). High H^+ and Al^{3+} ions in the root zone and plant tissues reduce the flavonoid secretion from the roots, which further decreases rhizobia *nod* gene induction and Nod-driven metabolite secretion (McKAY and Djordjevic, 1993). This inhibits initiation of rhizobia interactions and colonization resulting in reduced nodule formation (Ferguson et al., 2013). However, some strains of *Rhizobium* spp. appeared to be tolerant to soil acidity, such as *R. tropici* and *R. loti* (Cunningham and Munns, 1984; Wood et al., 1988). Some *Bradyrhizobium* spp. were also tolerant to soil acidity compared to *Rhizobium* spp. (Spaink et al., 2012). Several species of *Burkholderia* are also tolerant to soil acidity and successfully form nodules and fix N under acidic soil conditions (Angus et al., 2013; Garau et al., 2009)

Decrease in AMF root colonization, spore germination and germ tube growth was observed at low pH with high Al levels (Klugh-Stewart and Cumming, 2009). However, negative impacts of soil acidity on AMF was varied as different species were able tolerate a range of pH and Al toxicity (Clark et al., 1999b). Among the AMF species found in acidic soils, species of *Rhizophagus, Glomus, Acaulospora, Gigaspora,* and *Scutellospora* were predominant (Aguilera et al., 2015; Maki et al., 2008). Several AMF species within *Gigaspora* and *Scutellospora* were adapted to low pH conditions and promoted plant growth in acidic soil, but were unable to produce similar results in neutral soil (Bartolome-Esteban and Schenck, 1994). Thus, it is evident that different AMF species are predominant in acidic versus neutral soil, and specific AMF species may establish efficient symbiosis in acid soil.

Accumulation of soluble salts significantly impacts the soil microbial community structure in saline soils (Andronov et al., 2012). Beneficial plant-microbe interactions (BPMI) such as those with AMF (Jahromi et al., 2008) and endophytes (Pirhadi et al., 2018) were also impacted. The osmotic stress due to higher salt concentrations can minimize cell growth and even cause death of sensitive microbes, and generally lead to decreased microbial abundance (Yuan et al., 2007). However, some microbes are adapted to saline soils by evolving different salt tolerant mechanisms and were noted to form beneficial interactions with plants (more details are in section 1.3.3).

Diversity and composition of rhizosphere and endophytic microbiome in saline soil could be different from acid or neutral soil. Some of species of bacteria such as *Bacillus*, *Enterobacter*, and *Streptomyces* have been observed to promote plant growth under highly saline soil conditions (Jiang et al., 2019). The most dominant fungi in saline soils include members of phylum *Ascomycota* such as *Penicillium*, *Fusarium*, *Paecilomyces* and *Trichoderma* (Bronicka et al., 2007) Furthermore, salinity hampers the growth and multiplication of rhizobia and thus inhibiting its symbiosis with legumes leading to decreased nodulation (Tu, 1981). Salinity can also impact spore germination and growth of hyphae of AMF (Giri et al., 2007; Jahromi et al., 2008) as excess salt concentrations increases osmotic stress and lysis of fungal hyphae (Evelin et al., 2009; Juniper and Abbott, 2006). Whereas, several species within *Rhizophagus*, *Funneliformis*, *Claroidoglomus* and *Septoglomus* were able to tolerate higher salt concentrations and promoted plant growth in saline soil conditions (Lumini et al., 2020; Zhang et al., 2020). Moreover, AMF community in legumes differ in composition than non-legumes due to

high plant N content and higher requirement of P for the nodulation in legumes (Xiao et al., 2019). For instance, legumes were observed to be more highly abundant in a strain of *Glomus* (*Rhizophagus*) *intraradices* than in non-legumes in one study (Scheublin et al., 2004). Thus, it is evident that AMF diversity and interactions are impacted by soil conditions and plant selection, which must be deciphered to identify key stone species for a specific soil condition. This could also be beneficial to identify effective soil management practices to improve their abundance and interactions in acid and saline soils.

1.4 Mitigation avenues to improve beneficial plant-microbe interactions in acid and saline soils

1.4.1 Biochar soil amendment to improve plant-beneficial interactions in acidic soils

Traditionally, lime has been widely used for pH correction in acidic soils (Dent, 1992). Lime induced pH increase and nutrient availability are short term and require continuous applications (Goulding, 2016). Lime application contributes to higher CO_2 emissions (West and McBride, 2005), hardening and reacidification of the soil (Wang and Xian-Jun, 2017) and also increased leaching loss of some minerals like Mg^{2+} and NO_3^- have been noted (Lundell et al., 2001). On the other hand, addition of biochar in acidic soil increases the soil pH buffering capacity (Xu et al., 2012), soil fertility and also has other soil health benefits such as carbon sequestration (Biederman and Harpole, 2013). These benefits makes it a promising substitute to lime application in acidic soil (Wu et al., 2020). Biochar is produced by pyrolysis of biomass in a low oxygen environment (Kookana et al., 2011). Biochar has been successfully used as soil amendment with many

beneficial effects on soil health such as increasing in soil pH in acid soils (Murray et al., 2015; Pietri and Brookes, 2009), increasing CEC and availability of nutrients (Chintala et al., 2014; Major et al., 2010; Yuan and Xu, 2012) and consequently stimulating plant growth and yields (Kolton et al., 2017; Kolton et al., 2011). Biochar increases the soil pH in acidic soil due to its inherent alkalinity resulting from the pyrolysis and high base cation content (Shetty and Prakash, 2020). Increasing pH towards the neutral range can increase solubility of Al/Fe- PO_4 complexes and release Al bound-PO_4 (Devau et al., 2009) resulting into increased availability of P (Cui et al., 2011; Xiang et al., 2017; Yao et al., 2019). Biochar also increases the availability of K in soil due to high content of K in biochar ash and also reduced K leaching of biochar (Laird et al., 2010). Biochar addition in acidic soil was noted to increase nodulation (Wang et al., 2018a; Xiang et al., 2017). The main reason proposed was adsorption of flavonoids and nod factors on the surface of biochar can increase the longevity of these signaling molecules in soil and thus increased chances of these signals being received by rhizobia in soil (Thies and Rillig, 2009). In addition, rhizobia also tends to live in the pores on the biochar surface as a strategy to protect itself from pathogens (Sun et al., 2020). These effects could therefore facilitate the exchange of nodulation signals between plant roots and rhizobia (Thies and Rillig, 2009).

Biochar can reduce Al toxicity in acidic soils by increasing soil pH and promote oxidation of highly toxic Al^{3+} ions to $Al(OH)_2^+$ and $Al(OH)^{2+}$ and adsorbing Al species on surfaces by complexation with carboxyl groups (Qian et al., 2013). These Al mitigation properties of biochar also facilitates root growth and development in acid soils (Dai et al., 2017). Some studies indicated that root architecture parameters such as root length, root

volume (Xiang et al., 2017) and RLD (Xiao et al., 2016) were increased by biochar. Impact of biochar on root traits has also been attributed to improved soil aeration, water retention and soil structure (Xiao et al., 2016). However, biochar impacts on root biomass are variable, as some studies noted an increase (Prendergast-Miller et al., 2014; Xiang et al., 2017; Xiao et al., 2016), while others reported a decrease (Varela Milla et al., 2013) or no effect (Keith et al., 2015).

Studies have shown that the application of biochar to soil significantly influences microbial diversity and composition mainly due to the increased soil pH and nutrient availability (Kolton et al., 2017; Meng et al., 2019). Increased pH and nutrient availability generally leads to higher abundance of *Chloroflexi*, *Gemmatimonadetes* and *Bacteroidetes* while decreasing the abundance of *Acidobacteria,* when biochar was applied to a acidic soil (Sheng and Zhu, 2018). Similarly, biochar impacts soil fungal community composition (Hu et al., 2014; Yao et al., 2017). For instance, increased abundance of *Trichoderma* and *Paecilomyces,* and decreased abundance of plant pathogenic *Fusarium* was observed in biochar applied soils. The impact of biochar on abundance and colonization of AMF is dependent on the bioavailability of P in the soil (Madiba et al., 2016). Under low P conditions, biochar increased the AMF colonization in plant roots (Ezawa et al., 2002; Matsubara et al., 2002) whereas decreased AMF colonization was noted under sufficient P conditions in biochar added soil (Madiba et al., 2016; Warnock et al., 2010).

1.4.2 Compost application to saline soils

Compost amendments are used to provide essential nutrients (N, P, K, and micronutrients) and organic matter (Lakhdar et al., 2008). Compost is also used to improve soil physico-chemical properties (Hanay et al., 2004). It has been demonstrated that the application of compost to saline soils can accelerate Na^+ leaching, decrease the exchangeable sodium percentage and EC (Qadir et al., 2001; Walker and Bernal, 2008) and increase water infiltration (El-Shakweer et al., 1998), porosity, water-holding capacity and aggregate stability (Shiralipour et al., 1992). Improved root growth, root biomass and modification of root architecture including increased RLD were noted in compost applied soils (Leogrande and Vitti, 2019). Mineralization of compost increases P-availability (Meena et al., 2018) and plant available potassium in saline soils (Walker and Bernal, 2008), and thus increase K^+: Na^+ ratio in plant tissue (Liang et al., 2003).

Favorable impacts of compost application on soil microbial community and plant-microbe interactions are also well established. Addition of compost to a saline soil improved nodulation in legumes, mainly by increasing concentration Ca^{2+} in the soil and exchanging with Na^+ ions in the nodules, which negatively impacts nodulation (Lawson et al., 2004; Lawson et al., 1995). Compost application to saline soils was also noted to impact the diversity and composition of plant-associated microorganisms (Manasa et al., 2020; Shi et al., 2019). For example, compost applied saline soil was noted to have increased bacterial abundance and diversity and altered microbial composition in few studies (Lu et al., 2015; Yang et al., 2020b). Moreover, compost application has also shown to improve interaction of plants with symbiotic microbes such as N_2-fixing bacteria

NFB (Lawson et al., 2004) and AMF (Yang et al., 2018) thus improving plant N and P uptake. However, when added to a saline soil, it can result in accumulation of more salts and heavy metals and reduced oxygen in the soil-root zone that could adversely affect plant-microbe interactions, plant growth and development (Carvajal-Muñoz and Carmona-Garcia, 2012; Lakhdar et al., 2009). Thus, despite of several benefits of compost on improving physico-chemical properties of soil and plant-microbe interactions, its addition to saline soils is not highly recommended yet and needs further investigation (Lakhdar et al., 2009).

1.4.3 Exogenous application of stimulants and signaling compounds

Soil acidity and salinity can adversely impact the exchange of signaling between plants and beneficial microbes and establishment of symbiosis with native NFB (Morón et al., 2005) and AMF (Liu et al., 2020) could be impaired. Therefore, increasing the signaling between plants and such stress-tolerant symbiotic microbes could be an effective approach to improve nutrient availability and plant growth under acidic or saline soil conditions. For this purpose, exogenous application of signaling compounds has been increasingly explored in the recent years (Andreo-Jimenez et al., 2015; Lebeis et al., 2015; Pandey et al., 2013a). Some of these signaling compounds include strigolactones (SL) (Aroca et al., 2013), salicylic acid (SA) (Lebeis et al., 2015) and coumarins (COU) (Stringlis et al., 2019).

1.4.3.1 Salicylic acid

Salicylic Acid (SA) is a small phenolic compound produced by plants and some microorganisms for inducing plant immune responses, particularly under abiotic stress

conditions including soil acidity and salinity (Reyes-Díaz et al., 2016). One study noted that SA enhanced antioxidant enzyme activity and protected tissue from oxidative stress (Pandey et al., 2013a). There are reports in support of SA's role in modulating microbiome structure and interactions. One study noted that SA influenced colonization by endophytes (Chen et al., 2020b) including AMF (Medina et al., 2003). Other studies reported that SA influenced diversity (Kniskern et al., 2007) and composition (Lebeis et al., 2015) of plant microbiome in stressed plants. It was reported that SA regulated plant immune system and improved ion homeostasis. The endophytic microbiome of SA-treated Arabidospis plants was enriched in beneficial, stress-tolerant and non-pathogenic microbes (Lebeis et al., 2015). Application of SA induced the accumulation of a wide range of secondary metabolites in plants including indole glucosinolates, phytoalexins and alkamides, which play a role in communication of plants with microbial populations (Ortíz-Castro et al., 2009). However, it is not clear whether foliar application of SA on a legume crop like cowpea exposed to acidity and salinity would also impact rhizopshere and endophytic microbiome structure. It also needs to be explored whether microbiome modualtion would improve beneficial microbial interaction and plant growth.

1.4.3.2 Strigolactones

Strigolactones (SL) are secondary plant metabolites and signaling molecules that regulate or stimulate plant interactions (Siddiqi and Husen, 2017). Their role in promoting plant interactions with AMF and colonization is well established (Abdelhalim et al., 2019; Rochange et al., 2019), including for plants exposed to saline soils (Aroca et al., 2013; Van Ha et al., 2014). Strigolactones are exuded from roots to induce colonization (Khosla

and Nelson, 2016; Siddiqi and Husen, 2017), to stimulate AMF hyphal branching, growth and spore germination (Aroca et al., 2013). Recently several studies reported their role in promoting rhizobia-legume symbiosis as well (McAdam et al., 2017; Peláez-Vico et al., 2016). Strigolactones (SLs) influenced nodulation by promoting infection thread formation by rhizobia in legume roots and increased the number of nodules (McAdam et al., 2017; Peláez-Vico et al., 2016). Thus, SL could potentially be utilized to promote two important beneficial plant-microbial interactions. However, it is not clear whether SL could also be effective under saline and acid soils. It is also not clear whether SLs when applied exogenously would produce quantifiable impacts in a legume plant exposed to acidity and salinity. Understanding abundance and diversity of beneficial rhizosphere and endophytic microbes will be valuable to develop SL application as a potential tool for modulating beneficial microbial interactions. For instance, in a recent study by Carvalhais et al. (2019), it was shown that SLs producing plants had more pronounced effect on the fungal diversity than bacterial diversity. However, this study only involved the rhizosphere microbial community but no other plant-associated microbiome. Therefore, a comprehensive analysis of SLs impacts on both rhizosphere and endophytic microbiome needs to be undertaken to gain insight of practical application of SL analogues in soils under abiotic stress.

1.4.3.3 Coumarins

Coumarins (COU) are another group of plant secondary metabolite and signaling molecules that are excreted by roots. It is believed that they are exuded primarily to increase iron availability and uptake under iron deficient conditions such as in saline or

alkaline soils (Clemens and Weber, 2016; Stringlis et al., 2019; Tsai and Schmidt, 2017). Recently, it was noted that COU modulates plant responses to Fe and P deficiency under saline conditions, and interactions between plant roots and beneficial microbes (Niro et al., 2016; Stringlis et al., 2018). Studies showed that COU contributed to Fe uptake by chelation and/or reduction of Fe^{3+} to Fe^{2+} which was then transported by roots (Rajniak et al., 2018; Schmidt et al., 2014). It was also indicated that COU play a role in modulating the composition of root and rhizosphere microbiome (Stringlis et al., 2019; Stringlis et al., 2018; Voges et al., 2019). For instance, it was shown in a recent study by Stringlis et al. (2018) that COU caused differential abundance of specific microbes in roots. It was proposed that COU stimulated antimicrobial action in the rhizosphere inhibiting plant pathogens while selecting for the beneficial microbiome. Coumarins were also noted to increase AMF colonization, by acting as a signaling molecule under P starvation conditions (Wang et al., 2018c). They were also shown to induce antioxidant enzymes or directly act as antioxidants and thus reduce the oxidative stress in plants under abiotic and biotic stressed conditions (Qin et al., 2019; Saleh and Madany, 2015). Thus, COU could potentially improve Fe and P fertilization in saline soils. However, there is no evidence whether COU influence other beneficial microbial interactions such as BNF and AMF in saline soils.

1.5 Research gaps and study objectives

Soil acidity and salinity are major constraints for agricultural productivity around the world (Pessarakli and Szabolcs, 1999; Rorison, 1972). To sustainably mitigate acidity and salinity stress, soil management approaches must focus on improving beneficial

microbial interactions with plants (Shrivastava and Kumar, 2015; Sorty et al., 2018). One of the recent approaches used by researchers to mitigate abiotic stressed in plants is to apply various amendments and stimulants that potentially impact the diversity and composition of microbial communities in rhizosphere and plant endosphere. However, major knowledge gaps exist for a clear understanding of whether the shift in native microbial community composition under acidic and saline conditions also influence plant physiological functions, yield and productivity. Moreover, it is not clear how exogenous application of combination of signaling compounds compare to soil amendments for their impacts on rhizosphere and endosphere microbiome structure, particularly the plant beneficial microbes that can influence plant tolerance to acidity and salinity stress. A comprehensive assessment of plant physiological attributes, plant growth and development, and microbiome interactions was also lacking for a clear understanding of these interactions in saline and acidic soils and functioning of BPMI such as legume-rhizobia and plant root-AMF symbiosis (Kafle et al., 2018). Studies on endophytic microbial responses to soil acidity and salinity stress and their role in promoting plant tolerance are lacking in an agriculturally important legume such as cowpea (Suryanarayanan, 2020; Tosi et al., 2020). Current research findings on using biochar as soil amendment or plant stimulants such as SA and COU to influence stress tolerance are promising, but studies mostly focused on their direct impacts on plant physiological responses and crop yield (Sedaghat et al., 2017). Studies on plant beneficial microbial community were limited to few model plant systems such as *Arabidopsis* (Lebeis et al., 2015; Voges et al., 2019). To address these major knowledge gaps, we conducted two

experiments with following objectives. Objectives for the first experiment were 1) to study the impacts of soil acidity on cowpea rhizosphere and endophyte microbiome composition, AMF and rhizobia interactions, 2) to evaluate biochar application to soil and SA foliar application for their impacts on BPMI and cowpea growth and yield grown in acidic soil. Objectives for the second experiment were 1) to study the impacts of soil salinity on cowpea rhizosphere and endophytic microbiome composition, AMF and rhizobia interactions, 2) to evaluate soil amendments and foliar application of signaling compounds for their impact on BPMI and cowpea growth and yield grown in saline soil.

CHAPTER II

IMPROVING BENEFICIAL PLANT-MICROBE INTERACTIONS IN ACIDIC SOIL USING BIOCHAR AND SALICYLIC ACID

2.1 Synopsis

Soil acidity is a major constraint for soil fertility and crop productivity in many regions globally. Major impacts of soil acidity include Al toxicity effects on root growth and causing deficiency of several plant nutrients. Low pH and Al toxicity adversely impacts the beneficial plant microbe interactions (BPMI) such as those with arbuscular mycorrhizal fungi (AMF), N_2-fixing bacteria (NFB) and endophytes. However, certain PGP microbes are acid tolerant and therefore have potential to improve crop yields and productivity. Identifying suitable amendments to modulate the rhizosphere and endophytic microbiome to improve plant beneficial interactions could be an effective approach to improve plant nutrient uptake and yields in acid soils. Biochar (BC) is a potential alternative to liming, for sustainably managing soil acidity, as it was noted to improve soil conditions and soil health attributes including plant-beneficial interactions in many soils. Alternatively, plant foliar sprays of signaling compounds such as salicylic acid (SA) were noted to modulate plant-microbiome interactions and improve plant stress tolerance. This study was conducted to evaluate BC as a soil amendment and SA as a foliar applied stimulant for their impacts on nodulation, AMF colonization, diversity and composition of rhizosphere and endophytic microbiome of cowpea plants grown in acidic

soils. Treatment effects on soil pH, soil and plant nutrient concentrations, root biomass and plant yield were also determined.

Results showed that soil acidity reduced nodulation, plant nutrient (N, P, K, Ca and Mg) concentrations, diversity of rhizosphere microbes and pod yield. Biochar (BC) amendment was more effective in improving plant nutrient uptake and pod yields than SA treatment. Soil pH was increased to around 5.8 ± 0.2 in the BC treatment compared to control (5.0 ± 0.2). Similarly, nodulation numbers were higher in BC treatment, which resulted in higher N concentrations in the leaves compared to SA treatment. Percent AMF colonization was also increased significantly in BC treatment, which recorded higher leaf P concentrations. Treatment of SA significantly improved AMF colonization and abundance of AMF taxa in the rhizosphere, however, plant nutrient concentrations and pod yield did not significantly change compared to control. Both BC and SA significantly altered the microbial composition in the rhizosphere and plant endosphere. However, only BC treatment significantly increased the relative abundance of several plant beneficial taxa such as *Bacillus*, *Pseudomonas, Penicillium*, *Rhizobium* and *Bradyrhizobium.* Based on the results of this study it was concluded that BC application to an acidic soil was effective in improving BPMI and pod yields of cowpea plants grown in an acidic soil.

2.2 Introduction

About one-third of the global arable land is affected by soil acidity and more than 67% of these acidic soils have low agricultural productivity (Von Uexküll and Mutert, 1995). Soil acidity restricts plant growth and productivity mainly due to toxicity of H^+, Al and Mn, and deficiency of nutrients such as Ca, Mg, P or Mo (Dinkecha and Tsegaye,

2017; Fageria and Baligar, 2003; Opala et al., 2018). Soil acidity also restricts root growth due to higher concentration of Al^{3+} ions, which can disrupt root cells, Ca homeostasis and signal transduction pathways in the plants (Ma, 2007). Plants exposed to soil acidity stress generate higher levels of reactive oxygen species (ROS) in the internal tissues causing severe oxidative damages to the cells such as peroxidation of membrane lipids, oxidation of proteins and DNA strand breakage (Ma, 2005; Shi et al., 2006). In addition, severe subsoil acidity is commonly noticed in many Ultisols that have a clay-rich B horizon with accumulation of Al, where crop yield reductions appear more frequently and are difficult to restore (Langdale and Shrader, 1982; Sumner and Yamada, 2002).

Plant beneficial microbes could help plants to tolerate acidic stress by reducing Al toxicity (Aguilera et al., 2015), increasing root length and root length density (RLD) (Dal Cortivo et al., 2017), increasing nutrient uptake (Collavino et al., 2010) as well as increasing the production of antioxidant enzymes and synthesis of anti-stress secondary metabolites helping plants to reduce oxidative damages (Bilal et al., 2018b; Lata et al., 2018; Malinowski and Belesky, 1999).

Adverse impacts of acidity on beneficial plant-microbe interactions (BPMI) are evident and can limit plant -growth-promoting (PGP) microbes in the rhizosphere (Clark et al., 1999a; Ferguson et al., 2013; Klugh-Stewart and Cumming, 2009). For instance, Al-toxicity can inhibit signaling between host plant and rhizobia and thus causes reduced nodulation (Ferguson et al., 2013). Moreover, growth of rhizosphere microbes are restricted in acidic conditions due to high H^+ ions that inhibit cell division and cause disruption of cell membranes (Sullivan et al., 2017). Inhibition of AMF spore germination

and germ tube formation under acidic conditions can delay or hinder the establishment of root-AMF symbiosis (Klugh-Stewart and Cumming, 2009).

Correcting soil acidity using lime is a common practice but is not sustainable and has negative environmental impacts such as emitting CO_2 from soil (West and McBride, 2005). Moreover, long term lime application results in reacidification and hardening of the soil (Wang and Xian-Jun, 2017). Biochar is a viable and sustainable option due to its carbon sequestration benefits (Biederman and Harpole, 2013) and many other beneficial effects on soil health properties (Krishnakumar et al., 2014). Beneficial impacts of biochar on soil physicochemical properties include soil pH buffering (Chintala et al., 2014), increasing CEC (Xu et al., 2014) and nutrient availability (Nelson et al., 2011). Biochar applied soil was noted to have significantly higher microbial diversity and different composition (Chen et al., 2017a; Huang et al., 2019). For instance, biochar application increased the abundance of bacterial phyla *Protobacteria* and *Bacteroidetes* in acidic soil (Xu et al., 2014). Some recent studies are available on the positive impact of biochar on plant-beneficial microbes (Chen et al., 2020a; Wang et al., 2020c), and impacts on microbial community diversity and composition (Huang et al., 2019; Li et al., 2020; Zhang et al., 2017).

Soil acidity weakens the signaling between plants and beneficial microbes due to low pH and high concentration of Al^{3+} ions consequently impairing the establishment of symbiosis with NFB (Morón et al., 2005) and AMF (Liu et al., 2020). Biochar is known to improve signaling between plants and microbes by adsorbing the signaling factors on its surface and therefore facilitates the exchange of signals between plants and symbiotic

microbes (Thies and Rillig, 2009). It is well established that rhizosphere microbiome structure and assembly is driven by several soil conditions including soil pH and plant responses to those conditions (Chaparro et al., 2014; Pérez-Jaramillo et al., 2019). Thus, it can be anticipated that rhizosphere microbiome of an acidic soil, compared to a neutral soil, would be different and comprise more acid-tolerant microbes (Wan et al., 2020). Several PGP microbes are acid tolerant and impact crop growth and development in acid soils (de la Luz Mora et al., 2017; Silambarasan et al., 2019). For instance, *Acidobacteria* and *Chloroflexi* (Wan et al., 2020) as well as some NFB such as *Burkholderia* (Aizawa et al., 2010), some strains of *Bradyrhizobium* (Appunu and Dhar, 2006) and *Rhizobium* (Appunu and Dhar, 2006) were found to be dominant under acidic conditions. Stimulating their interactions using exogeneous signaling compounds could be a viable option to increase plant-microbe interactions in acidic soil. Salicylic acid (SA) is one such signaling phytohormone, which was noted to induce defense responses, particularly under soil acidity (Reyes-Díaz et al., 2016). Exogenous application of SA has shown to promote tolerance in plants to acidity mainly by enhancing the activity of antioxidant enzymes such as SOD, POD and APX (Pandey et al., 2013b). SA was noted to influence production of secondary metabolites in plants that are known to minimize toxicity effects of Al-ions (Yang et al., 2003). In *Arabidopsis*, application of SA significantly modified the diversity and composition of plant microbiome (Chen et al., 2020b; Lebeis et al., 2015) and triggered plant immune responses (Lebeis et al., 2015). However, impact of SA application on BPMI are varied and not clearly understood. For instance, influence of SA on legume-rhizobia symbiosis has been shown to be both positive (Hegazi and El-Shraiy,

2007) and negative (Mabood and Smith, 2006). Similarly, root AMF colonization was increased in some plants, but no effects were noted on stressed plants (Medina et al., 2003). Moreover, its effect on rhizosphere microbes and endophytes is not explored in agriculturally relevant crops like cowpea legume.

It was hypothesized that application of biochar to acidic soil would significantly increase microbial diversity in the rhizosphere and enhance beneficial interactions of AMF and NFB. Similarly, exogenous application of SA would enhance endophytic, AMF and NFB. As a result, BPMI (nodulation and AMF colonization), nutrient concentrations and crop yield will be higher in biochar and SA treatments. The objectives were 1) to study the impacts of soil acidity on cowpea rhizosphere and endophyte microbiome composition, AMF and rhizobia interactions and 2) to evaluate biochar application to soil and SA foliar application for their impacts on plant-microbe interactions and cowpea growth and yield grown in acidic soil.

2.3 Materials and Methods

2.3.1 Soil, plant materials and chemicals

Both acidic subsoil and neutral soil used in this study were collected near Texas A&M AgriLife Research and Extension Center, Overton in Rusk county, Texas (32.2746° N, 94.9786° W). The acidic soil was a Kirvin soil series and classified as fine, mixed, semiactive and thermic Typic Hapludults (NRCS, USDA web soil survey). The subsoil horizons (28 cm – 58 cm) of these soils are red clay and very highly acidic. The neutral soil used in this study was a Lilbert soil series and classified as loamy, siliceous, semiactive, thermic Arenic Plinthic Paleudults (NRCS, USDA web soil survey). Soil

texture, pH, NO_3 and available P of these soils were estimated by the Soil, Water and Forage Testing Laboratory**,** Department of Soil and Crop Sciences, Texas A&M University (Table 2.1). A greenhouse study was conducted using Texas Cream 40 variety of cowpea (*Vigna unguiculata* (L.) Walp.) as the plant host. Plant containers (Tree seedling nursery Containers, Stuewe & Sons, Inc., Tangent, OR, USA;15.2 -cm diameter, 30.5-cm length, 4.26 L volume) were used to grow the plants. Biochar (Wakefield Biochar, WI, USA) 5% wt/wt was used as soil amendment and salicylic acid (Sigma Aldrich, St. Louis, MO, USA) 0.5mM was used as a plant amendment applied as foliar spray in every 3 days after seed emergence. Properties of biochar used in the study are detailed as reported by manufacturer in Table 2.2

Table 2.1. Characteristics of the native acidic and neutral soil used in the experiment.

Parameter	**Acidic soil**	**Neutral soil**
pH	4.9	6.5
Texture	Clay	Loamy fine sand
Conductivity	172 µmho/cm	80 µmho/cm
Nitrate-N	5 mg/kg	18 mg/kg
Phosphorus	2 mg/kg	62 mg/kg
Potassium	111 mg/kg	252 mg/kg
Calcium	1211 mg/kg	1302 mg/kg
Magnesium	368 mg/kg	71 mg/kg
Sulfur	45 mg/kg	15 mg/kg

Table 2.2. Physical and chemical properties of biochar (as reported by manufacturer).

Property	**Specification/Value**
Pyrolysis temperature	500 °C
Feedstock material	Soft wood (Pine)
Bulk density	0.48 g/cm^3
Total organic matter	95.12 % total mass
Total carbon	88.01 % total mass
Total organic carbon	87.67 % total mass
Total inorganic carbon	0.34 % total mass
Total ash	4.88 % total mass
pH	7.4
Nitrogen (N)	0.59 % wt.
Total phosphate	4.53 mg/kg
Potassium (K)	614 mg/kg
Calcium (Ca)	4128 mg/kg
Iron (Fe)	595 mg/kg
Magnesium (Mg)	1225 mg/kg
Manganese (Mn)	234 mg/kg
Zinc (Zn)	4.59 mg/kg
Surface area	365.69^2/dry g

2.3.2 Experimental design and growth conditions

The experiment had a completely randomized design consisting of 6 treatments and 3 controls with 3 replicates. The names and details of treatments are provided in Table 2.3. Seeds were inoculated with *Bradyrhizobium* sp. (Vigna) (Exceed superior legume inoculant, Visjon Biologics, Wichita Falls, TX) a day before sowing. The plants were grown for 3 weeks for sampling in first time point, 6 weeks for sampling in second time point and 9 weeks for sampling in third time point in a greenhouse at Texas A&M AgriLife Research and Extension Center, Overton, Texas and watered daily to 70% water holding capacity (determined based on maximum water holding capacity using saturating method).

The plants were irrigated two times during the entire growing season with half strength modified Hoagland nutrient solution, the composition of which is detailed in Table 2.4.

Table 2.3. Name and details of each treatment used in the experiment.

Treatment number	**Treatment name**	**Treatment details**
T1	BC	Biochar amended acidic soil
T2	SA	Salicylic acid treatment for plant grown in acidic soil
T3	BC+SA	Biochar amendment + salicylic acid treatment in acidic soil
T4	AC	Acidic soil control, unamended (pH 4.9 ± 0.1)
T5	NC	Neutral soil control, unamended (pH 6.5 ± 0.2)

Table 2.4. Composition of modified Hoagland nutrient solution used in the experiment.

Compounds	Concentration of stock solution (mM)	Volume of stock solution (ml) per liter of final solution	Volume of final solution (ml) added to the pot
KH_2PO_4	1000	2.0	1.4
$MgSO_4.7H_2O$	2000	1.0	0.7
K_2SO_4	2000	1.25	0.875
$CaCl_2.H_2O$	1000	1.25	0.875
H_3BO_3	6.25	2.0	1.4
$MnSO_4.H_2O$	2.5		
$CuSO_4.5H_2O$	0.2		
$ZnCl_2$	0.1		
Ammonium Molybdate	0.05		
FeNaEDTA	64	1.0	0.7

2.3.3 Sampling, root scanning, nodulation and root AMF colonization

Sampling of rhizosphere, roots, shoots and leaves were done at three distinct plant developmental stages (time points). First sampling time corresponded with vegetative stage or 3 weeks after seed germination (3 WAG), second sampling time corresponded with flowering stage or 6 weeks after seed germination (6 WAG) and third sampling time corresponded with pod maturity stage or 9 weeks after seed germination (9 WAG) of the plant.

At each sampling time, the pots were destructively sampled for rhizosphere soil, roots, shoots and leaves, processed accordingly for different analysis and stored at -80°C until analysis. The shoots were harvested, weighed and then some leaf samples were kept separately for DNA and nutrient analysis (stored at -80°C until analysis). In the remaining soil, rhizosphere and some roots were stored separately for DNA and some rhizosphere soil was kept separately in tubes for pH and nutrient analysis. Remaining soil in the pots were washed to retain only the roots. These roots were then blotted, weighed, counted for number of nodules and then stored at -20°C for estimation of AMF colonization percentage, root biomass and root length density. For estimation of biomass, roots and shoots (obtained from harvest) were dried at 65°C in a forced-air oven for 48 h, and weighed.

2.3.4 Root scanning

Roots stored at -20 °C were first scanned for root length and root density quantification using Epson WinRHIZO scanner (Regent Instruments Inc., Quebec, Canada). The whole root system was spread into a plastic transparent tray filled with 3

mm of water so that individual roots and neighbor lateral roots did not overlap and stick. The roots were imaged by scanning (STD 4800, Regent Instruments Inc., Quebec, Canada) and their length measured by Epson WinRHIZO software version 2017a (Instruments Regent Inc., Quebec, Canada). Root length density was measured by dividing root length (cm) obtained by the volume of soil used in the experiment (cm^3).

2.3.5 Estimation of percentage of root AMF colonization

One gram of root stored at -80 °C was used to measure percentage of root colonization by AMF. Roots were gently removed from soil and washed under tap water, and then stained with trypan blue following a modified procedure of Phillips and Hayman (1970). Roots were placed in tissue cassettes (Fischer Scientific Inc., Hampton, NH, USA) and submerged in pre-boiled 10 % KOH for 10 min to remove host cytoplasm and nuclei. Cassettes were then washed 5X with tap water and submerged in 2 % HCl for 30 min, followed by 5X washing with tap water. The cassettes were then submerged in 0.05 % trypan blue solution (water, glycerin, lactic acid in 1:1:1 (v/v/v)) at 90ºC for 5 min. The cassettes were then washed 5X with tap water and stored at 4 °C for 7 days immersed in distilled water to remove excess stain. The percentage of AMF colonization was then determined using the gridline intersection method (Giovannetti and Mosse, 1980).

2.3.6 Estimation of pH and nutrient concentration of rhizosphere soil

Change in soil pH was determined using the method by Schofield and Taylor (1955). The pH was determined in a 1:2 ratio of soil to water extract of the soil using deionized water. Samples were stirred and allowed to equilibrate for a minimum of 30

minutes after adding the water. The actual determination was made using a hydrogen selective electrode and pH values are reported on a dry soil basis only.

For nutrient analysis of soil, a slightly modified method of Haney et al. (2006) was used. Soil extractant H3A was used to extract NO_3, P, K, Ca and Mg from soil. The extractant was prepared by dissolving the following chemicals in one liter of water: Lithium citrate (5.0 g); citric acid (0.5 g); malic acid (0.5 g); oxalic acid (0.5 g); EDTA (0.25 g) and DTPA (0.25 g). Soils obtained from each treatment were weighed (4.0 g) separately in 50 mL centrifuge tubes and extracted with 40 mL of H3A. Soil samples were shaken for 30 minutes and centrifuged at 3000 rpm for 8 minutes and then filtered through Whatman 2V pleated filter paper in 2 mL vials. Nutrients were then quantified by Ion Chromatography (Thermo Electron North America LLC, Madison, WI, USA) method.

2.3.7 Estimation of leaf tissue elemental concentrations

Dried leaf samples (at 65^0C in a forced-air oven for 48 h) were crushed and weighed (0.5-1.0 g) into a 50 mL Taylor tube and extracted with conc. nitric acid overnight and then analyzed for nutrient ions (P, K, Ca, Mg, Fe and Na) using Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) method (Havlin and Soltanpour, 1980). Total N in leaves was measured separately using dry combustion C/N analyzer (Elementar Inc.).

2.3.8 Extraction of DNA from rhizosphere and plant tissues

Soil DNA was extracted from 0.5 g of soils (-80 °C) from the rhizosphere using DNeasy Power Soil Pro DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) and DNA from plant tissues (root and leaf) were extracted using Power plant kit (Qiagen

Inc.) following the manufacturer's instructions. After extraction, all DNA samples were quantified to detect DNA quality using a spectrophotometer (SimpliNano, GE Healthcare LifeSciences, Inc.).

2.3.9 Estimation of abundance of bacteria, fungi, AMF and NFB in rhizosphere and plant tissues

Quantitative real-time PCR (qPCR) was used to quantify the abundances of total bacterial 16S rRNA, total AMF 18S rRNA, total fungal internal transcribed spacer (ITS) and total *nifH* gene targets in both rhizosphere and plant tissues. For quality control, all qPCR runs included 5 different concentrations of DNA standards (gBlock standards, Integrated DNA Technologies Inc.) for each target gene (for standard curve), details on these standards are provided in Table 2.5, no-template control (NTC), positive control, negative control, and 2 spiked random samples from the study's DNA samples with one of the standards to test for possible qPCR inhibitors. Standards and NTC were run in triplicate, and the rest of controls and experimental samples were run in duplicate. Positive and negative controls for each target gene, R^2 values and reaction efficiency of standard curves obtained in each run are listed in Table 2.6. Primers were obtained from Integrated DNA Technologies Inc. and are outlined in Table 2.7. Amplifications of DNA was performed using RotorGene SYBR® Green qPCR kit, with gene abundance measured using RotorGene Q Software version 2.3.1.49 (QIAGEN, Hilden, Germany).

Table 2.5. Details of qPCR standards.

Target gene	Microbial source for sequence included in the gBlock standards	Dilution range of the standards having the targeted gene (copies/2 µl)
16S rRNA	*Pseudomonas denitrificans*	$10^7 – 10^3$
ITS	*Rhizopus microsporus*	$10^7 – 10^3$
AMF-18S rRNA	*Glomus intraradices*	$10^6 – 10^2$
nifH	*Rhizobium leguminosarum*	$10^6 – 10^2$

2.3.10 Estimation of rhizosphere and endophytic microbial diversity and composition

Microbial DNA from soil, roots and leaves was sequenced in the V4 region of 16S rRNA gene marker amplified by primers 515F- 5'-GTGYCAGCMGCCGCGGTAA-3' (Parada et al., 2016) and 806R- 5'-GGACTACNVGGGTWTCTAAT-3' (Apprill et al., 2015) and the ITS marker with primers ITS1F- 5'-CTTGGTCATTTAGAGGAAGTAA-3' (Gardes and Bruns, 1993) and ITS2R- 5'-GCTGCGTTCTTCATCGATGC-3' (White et al., 1990). DNA libraries were prepared as described in the Illumina 16S rRNA Metagenomic Sequencing Library Preparation protocol, except that dual 6 bp instead of 8 bp index sequences were attached to each amplicon during indexing PCR and were loaded on Illumina Miseq instrument for paired-end sequencing following manufacturer's protocol (Illumina, San Diego, CA). Qiime1.9.1 (Caporaso et al., 2010) and USEARCH 8.0.1 (Edgar, 2010) software packages were used to process the raw sequencing reads

Table 2.6. Quality control details of the qPCR runs in the experiment.

Target microbial gene	Positive control	Negative control	R^2 value of standard curve for rhizosphere	Reaction efficiency for rhizosphere	R^2 value of standard curve for plant tissue	Reaction Efficiency for plant tissues
16S rRNA	*Escherichia coli* K-12	*Methanospirillum hungatei*	0.98	1.00	0.99	1.01
ITS	*Rhizopus microsporus*	*Escherichia coli* K-12	0.99	0.94	0.98	0.99
AMF 18S rRNA	*Glomus intraradices*	*Escherichia coli* K-12	0.97	1.00	0.98	0.95
nifH	*Rhizobium leguminosarum*	*Rhizopus microsporus*	0.98	0.99	0.97	0.94

Table 2.7. Details of primers and PCR conditions used for the qPCR assays in the experiment.

Target microbial group	Primers and sequences	qPCR reaction mixture	Thermal profile	Reference
Total bacteria (16S rRNA)	341f-(5'-CCTACGGGAGGCAGCAG-3')/ 797r-(5'-GGACTACCAGGGTATCTAATCCTGTT-3')	7.5 µl SYBR Green (2x) Master Mix, 0.225 µl F primer (0.3 µM), 0.675 µl R primer (0.9 µM), 2 µl DNA template, 4.6 nuclease free H_2O.	3 min at 98°C for initial denaturation; 40 cycles of 30 s at 98°C, 30 s at 61.5°C, extension for 20 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 50-99°C (1° and 5 s/cycle melt) after a pre-melt conditioning for 90 s at 50°C.	Modified after (Harter et al., 2014)
Total AMF (18S rRNA)	GC-AMV4.5NF- (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G [GC clamp] AAG CTC GTA GTT GAA TTT CG-3′)/ AMDGR-(5′-CCC AAC TAT CCC TAT TAA TCA T-3′)	7.5 µl SYBR Green (2x) Master Mix, 1.5 µl each primer (5 µM), 2 µl DNA template, 2.5 nuclease free H_2O	10 min at 98°C for initial denaturation; 35 cycles of 30 s at 98°C, 30 s at 55°C, extension for 45 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 50-98°C (1° and 5 s/cycle melt).	Modified after (Sato et al., 2005)

Table 2.7. Continued.

Total fungi (ITS)	ITS1f-(5'-TCC GTA GGT GAA CCT GCG G3')/5.8s-(5'-CGC TGC GTT CTT CAT CG-3')	7.5 µl SYBR Green (2x) Master Mix, 1.5 µl each primer (5 µM), 2 µl DNA template, 2.5 nuclease free H_2O.	10 min at 98°C for initial denaturation; 35 cycles of 60 s at 98°C, 30 s at 53°C, extension for 45 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 48-98°C (1° and 5 s/cycle melt)	Modified after (Fierer et al., 2005)
Total *nifH*- harboring bacteria	PolF-(5'-TGC GAY CCS AAR GCB GAC TC3')/PolR- (5'-ATS GCC ATC ATY TCR CCG GA3') where Y = C/T; S = G/C; R = A/G; B = C/G/T	7.5 µl SYBR Green (2x) Master Mix, 0.225 µl F primer (0.3 µM), 0.675 µl R primer (0.9 µM), 2 µl DNA template, 4.6 nuclease free H_2O.	10 min at 98°C for initial denaturation; 35 cycles of 1 min at 98°C, 1 min at 55°C, extension for 1 min at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 50-98°C (1° and 5 s/cycle melt).	Modified after (Poly et al., 2001)

obtained from Illumina Miseq. Each ITS sequence tags were compared to the UNITE ITS sequence database (Abarenkov et al., 2010) and 16S rRNA sequences were compared to the Greengenes database (Release 13.5) (DeSantis et al., 2006) using UCLUST (Edgar, 2010) in order to pick referenced-based (prokaryotes) or open-reference (fungi) operational taxonomic units (OTUs) at 97% similarity, and then were recorded assignments for each OTU. The OTU abundance dataset was further normalized using cumulative sum scaling (CSS) transformation (Paulson et al., 2013) available on the QIIME platform. Samples with less than 1000 sequences were discarded.

2.3.11 Data analysis

Differences among treatments for change in soil pH, shoot biomass, root biomass, nutrient concentrations (NO_3, PO_4, K, Ca and Mg), N in leaves, nodulation and % AMF colonization were statistically analyzed using ANOVA in SAS software (SAS Inc.), using PROC GLM procedure. Differences between treatments were obtained using Fisher's least-significant-difference (LSD) test at a *p*-value of <0.05. Pearson's correlation coefficient was determined for pairwise comparison between leaf nutrient concentration, nodulation, AMF colonization and pod yield and correlation plot was created using "corrplot" package(Wei et al., 2017) in R. Calculations of alpha-diversity (Shannon) and observed species richness and estimated richness (Chao1) were done using QIIME. Principal Coordinate Analysis (PCoA) was performed to visualize the effect of different treatments on microbial community composition and a two-way non-parametric multivariate analysis of variance (PERMANOVA) was used to test the significant differences in rhizosphere and endophytic microbial community composition between the

experimental treatments using the Phyloseq package (McMurdie and Holmes, 2013) on R version 3.6.1 based on a Bray-Curtis distance measure between the groups. Linear discriminant analysis effect size (LEfSe) was performed to identify significant differences in bacterial and fungal taxa between treatments and controls. The Kruskal-Wallis (KW) sum-rank test is used in LEfSe analysis to detect the features with significantly different abundances between assigned classes, and then linear discriminant analysis (LDA) is performed to estimate the effect size of each differentially abundant taxon (Segata et al., 2011). Significant taxa were used to generate taxonomic cladograms illustrating differences between sample classes on the website http://huttenhower.sph.harvard.edu/galaxy.

Phylogenetic investigation of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict the Kyoto Encyclopedia of Genes and Genomes (KEGG). PICRUSt predicted metagenomes from 16S rRNA data by using evolutionary modeling and comparing with a reference genome database. Generally, OTUs of 16S rRNA sequences were normalized by PICRUSt, and then the metagenomes predicted by PICRUSt algorithm were collapsed into clusters of orthologous groups of proteins (COGs) and KEGG. Predictive COGs and KEGGs were screened out and visualized by Statistical Analysis of Metagenomic Profiles (STAMP) software package v 2.1.3 (Parks et al., 2014). Pairwise comparison of the KEGG pathways was performed applying a Welch's (two-tailed) *t* test with 95 % confidence intervals, and values were considered significant at $P < 0.05$.

Mantel tests were used to calculate the correlations between variations in microbial composition (based on Bray-Curtis distances) and different soil and plant growth parameters using vegan package in R (Dixon, 2003). Pearson correlation coefficients were used to test for the correlations between dissimilarity matrices using 9999 permutations. Bray-Curtis dissimilarities were used for microbial community while Euclidean distance dissimilarities were used for soil and plant growth parameters.

2.4 Results

2.4.1 Impact of experimental treatments on pH and nutrient concentrations in the rhizosphere soil

No significant change in rhizosphere soil pH was observed in AC treatment (natural acidic soil) during the entire plant growing season and remained at around pH 5.0 at both depths (0-5" and 5-10") (Table 2.8). Among the experimental treatments, rhizosphere soil pH increased significantly in biochar treatments (BC and BC+SA) at 6 WAG and 9 WAG. However, BC treatment had a higher increase in pH than BC+SA at both time points. Whereas foliar treatment of SA did not significantly alter the rhizosphere pH. Similar results were observed in the rhizosphere pH at subsurface (5-10") soil layer, with highest increase in soil pH observed in the BC treatment followed by the BC+SA treatment.

Concentration of NO_3, PO_4 and K were observed to be significantly lower ($p < 0.05$) in the rhizosphere soil of AC treatment than NC (neutral control) indicating a negative impact of soil acidity on plant nutrient availability (Table 2.9). Among all the experimental treatments, rhizosphere nutrient concentrations were highest and significantly different in the BC treatment at 6 WAG and 9 WAG and in both surface (0-

Table 2.8. Rhizosphere pH in the experimental treatments measured at 3, 6 and 9 weeks after germination (WAG).

Treatment	pH at depth 0-5"			pH at depth 5-10"		
	3 WAG	**6 WAG**	**9 WAG**	**3 WAG**	**6 WAG**	**9 WAG**
BC	5.15 ± 0.0.29b	5.85 ± 0.43a	5.84 ± 0.10a	5.14 ± 0.02b	5.71 ± 0.07a	5.65 ± 0.16a
SA	5.08 ± 0.28b	5.19 ± 0.16ab	5.17 ± 0.10bc	5.02 ± 0.03b	4.97 ± 0.05b	4.91 ± 0.03b
BC+SA	5.17 ± 0.24b	5.82 ± 0.26a	5.64 ± 0.24b	5.08 ± 0.04b	5.69 ± 0.07ab	5.67 ± 0.02a
NC	6.41 ± 0.12a	6.09 ± 0.19a	5.98 ± 0.12a	6.10 ± 0.02a	5.95 ± 0.14a	5.83 ± 0.52a
AC	5.01 ± 0.26b	5.02 ± 0.10b	5.08 ± 0.19c	5.03 ± 0.33b	4.97 ± 0.03b	4.89 ± 0.03b

Note: data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

Table 2.9. Rhizosphere nutrient concentration in experimental treatments measured at 6 WAG and 9 WAG.

Depth	Treatment	Rhizosphere at 6 WAG (mg/kg)			Rhizosphere at 9 WAG (mg/kg)		
		NO_3	P	K	NO_3	P	K
0-5”	BC	6.33 ± 0.29a	14.43 ± 4.34b	226.71 ± 12.59b	5.18 ± 0.82a	16.87 ± 6.47b	203.97 ± 9.96b
	SA	5.20 ± 0.43b	7.11 ± 1.95bc	80.09 ± 5.77d	2.30 ± 0.68b	6.96 ± 2.13c	72.10 ± 7.82d
	BC+SA	2.46 ± 0.36c	15.07 ± 1.82b	149.60 ± 12.00c	2.57 ± 1.19b	10.66 ± 0.61bc	123.05 ± 12.62c
	NC	5.58 ± 0.55ab	68.34 ± 3.36a	269.15 ± 14.96a	5.14 ± 0.54a	60.91 ± 6.30a	231.61 ± 15.15a
	AC	2.42 ± 0.72c	3.97 ± 0.27c	84.90 ± 2.90d	1.65 ± 0.67b	3.37 ± 0.68c	74.44 ± 4.17d
5-10”	BC	7.81 ± 2.01a	6.64 ± 0.14b	176.71 ± 19.18b	7.07 ± 2.28a	7.12 ± 1.12b	136.40 ± 37.43ab
	SA	5.75 ± 1.88ab	4.26 ± 0.13b	66.76 ± 5.23d	3.74 ± 2.42b	3.21 ± 0.08bc	59.98 ± 9.50c
	BC+SA	1.70 ± 0.95c	8.07 ± 0.42b	116.27 ± 7.14c	1.23 ± 0.36b	6.80 ± 0.67b	92.33 ± 5.32bc
	NC	3.49 ± 1.67bc	54.72 ± 3.20a	235.81 ± 5.74a	3.02 ± 1.74b	45.17 ± 4.59a	163.08 ± 38.44a
	AC	1.55 ± 0.97c	3.49 ± 0.20b	73.90 ± 6.71d	1.23 ± 0.76b	2.04 ± 0.73c	63.56 ± 5.36c

Note: data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan’s multiple range test; n=3 for all time points.

Table 2.10. Root dry matter (g) in experimental treatments measured at 3, 6 and 9 WAG.

Treatment	Root dry matter (g) at depth 0-5”			Root dry matter (g) at depth 5-10”		
	3 WAG	**6 WAG**	**9 WAG**	**3 WAG**	**6 WAG**	**9 WAG**
BC	0.433 ± 0.13a	0.826 ± 0.12a	2.49 ± 0.05a	0.275 ± 0.26a	0.500 ± 0.13ab	1.03 ± 0.21ab
SA	0.382 ± 0.12a	0.662 ± 0.13a	0.88 ± 0.24b	0.298 ± 0.03a	0.398 ± 0.13b	0.66 ± 0.09c
BC+SA	0.388 ± 0.07a	0.739 ± 0.24a	2.38 ± 0.88a	0.239 ± 0.11a	0.421 ± 1.86b	0.96 ± 0.02b
NC	0.553 ± 0.09a	1.734 ± 0.39a	2.82 ± 0.88a	0.264 ± 0.09a	0.972 ± 2.86a	1.27 ± 0.19a
AC	0.372 ± 0.02a	0.731 ± 0.07a	1.10 ± 0.52b	0.337 ± 0.05a	0.407 ± 0.14b	0.95 ± 0.12b

Note: data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan’s multiple range test; n=3 for all time points.

5”) and subsurface (5-10”) soil layers. Treatment of SA significantly increased the concentration of NO_3 in the rhizosphere soil compared to AC treatment at 6 WAG but not at 9 WAG. Whereas, treatment BC+SA significantly increased the concentration of P and K in the rhizosphere of surface layer (0-5”) compared to the AC treatment.

2.4.2 Impact of experimental treatments on root biomass and root length density

Total root biomass (root dry matter) was quantified for surface and subsurface soil layers in individual pots at three time points (Table 2.10). No significant change was noted in root biomass between the two initial stages (3 & 6 WAG) of plant growth. However, at pod maturity stage (9 WAG), root biomass was significantly lower ($p < 0.05$) in AC (1.1 g/pot at 9 WAG) than NC (2.8 g/pot at 9 WAG). Moreover, treatments of biochar (BC and BC+SA) significantly increased root biomass at 9 WAG (2.4 g/pot), whereas SA treatment did not significantly impact root biomass and remained around 0.9 g/pot at all time points.

Root length density (RLD), measured as cm of root/cm^3 of soil, was not significantly different between AC and NC treatments (Table 2.11). However, BC and BC+SA treatments significantly increased RLD (3.5 cm/cm^3 at 9 WAG) while SA treatment significantly decreased RLD at pod maturity phase of the plant (1.7 cm/cm^3 at 9 WAG) compared to AC treatment (2.5 cm/cm^3 at 9 WAG).

2.4.3 Impact of experimental treatments on nutrient concentrations in the plant leaf tissue

Concentration of N, P, K, Ca and Mg were significantly reduced ($p < 0.05$) in the AC treatment compared to NC treatment (Table 2.12, 2.13). Experimental treatments of BC, SA and BC+SA significantly increased N and P concentrations compared to AC. At 6 WAG, highest N (129 mg/kg) and P (7.4 mg/kg) were observed in BC treatment. BC treatment also increased N at 9 WAG compared to AC treatment but more than SA treatment. Additionally, BC treatment increased the concentration of K, Ca and Mg, whereas SA treatment did not show significant differences compared to AC treatment.

Concentration of Al was significantly higher ($p < 0.05$) in AC treatment (305 mg/kg) than all other experimental treatments (Figure 2.1). Experimental treatments of BC, SA and BC+SA significantly reduced Al concentration (22 mg/kg, 30 mg/kg and 108 mg/kg respectively) compared to AC treatment.

Table 2.11. Root length density (cm of root per cm^3 of soil) measured in experimental treatments at 3, 6 and 9 WAG.

Treatment	Root length density (RLD) (cm/cm^3) at depth 0-5"			Root length density (RLD) (cm/cm^3) at depth 5-10"		
	3 WAG	6 WAG	9 WAG	3 WAG	6 WAG	9 WAG
BC	0.813 ± 0.64a	2.92 ± 0.79a	3.51 ± 0.31 a	0.445 ± 0.17bc	2.60 ± 0.36a	3.08 ± 0.07a
SA	0.787 ± 0.19a	2.11 ± 0.76a	1.70 ± 0.17d	0.736 ± 0.19a	2.14 ± 0.40a	1.69 ± 0.24c
BC+SA	0.682 ± 0.07a	2.52 ± 1.09a	3.31 ± 0.57ab	0.555 ± 0.21ab	2.43 ± 0.74a	2.90 ± 0.14a
NC	0.618 ± 0.21a	2.98 ± 0.19a	2.77 ± 0.35bc	0.228 ± 1.75c	2.12 ± 0.70a	2.28 ± 0.14b
AC	0.741 ± 0.03a	2.49 ± 0.66a	2.49 ± 0.22c	0.610 ± 0.07ab	1.73 ± 0.36a	2.26 ± 0.40b

Note: data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

Table 2.12. Leaf N concentration in experimental treatments measured at 6 and 9 WAG.

Treatment	N (mg/plant)	
	6 WAG	**9 WAG**
BC	129.02 ± 7.53b	234.73 ± 17.41b
SA	124.38 ± 1.01b	89.72 ± 14.80c
BC+SA	133.07 ± 17.15b	164.74 ± 74.55bc
NC	316.21 ± 18.61a	397.47 ± 38.83a
AC	72.62 ± 2.75c	53.44 ± 7.34c

Note: data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

Table 2.13. Leaf nutrient concentration in experimental treatments measured at 6 WAG.

Treatment	P (mg/plant)	K (mg/plant)	Ca (mg/plant)	Mg (mg/plant)
BC	7.37 ± 1.28b	137.14 ± 18.44a	71.77 ± 5.02b	21.00 ± 1.06b
SA	6.15 ± 0.29b	84.69 ± 12.62b	26.04 ± 1.96c	13.00 ± 0.64c
BC+SA	6.61 ± 0.79b	120.65 ± 6.40ab	64.35 ± 9.57bc	18.57 ± 1.52bc
AC	3.49 ± 0.33c	87.87 ± 9.51b	26.80 ± 1.54c	15.43 ± 2.20bc
NC	48.38 ± 5.70a	137.10 ± 5.38a	324.43 ± 52.68a	78.55 ± 14.57a

Note: data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

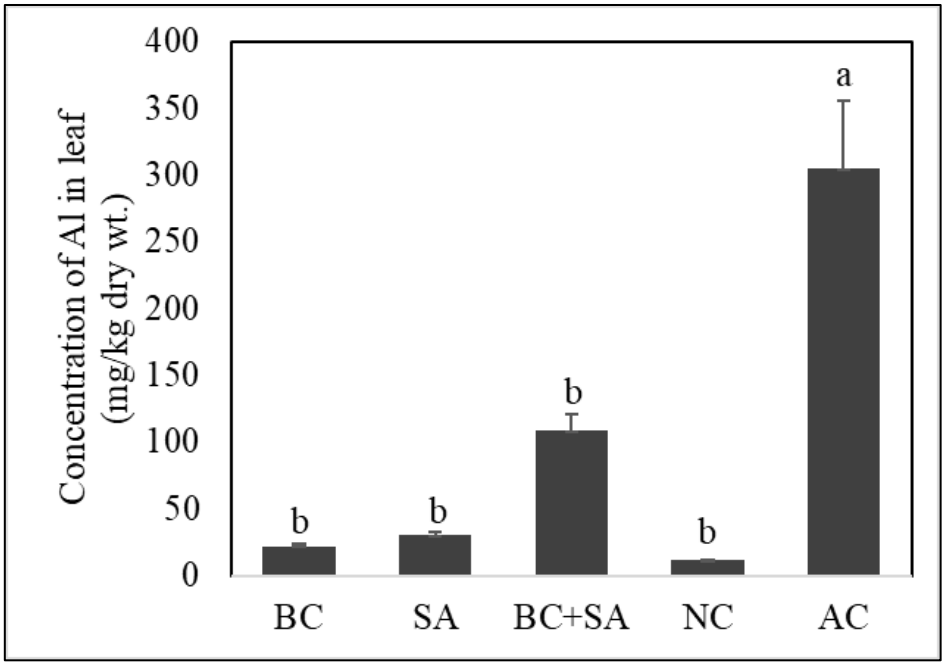

Figure 2.1. Concentration of Al in leaves (mg per kg dry wt. of leaf) of experimental treatments at 6 WAG.
Note: data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

2.4.4 Impact of acidity and experimental treatments on nodulation and percentage of root AMF colonization

Total number of nodules per pot were significantly lower ($p < 0.05$) in AC treatment than NC treatment at all three growth stages of cowpea (Figure 2.2). Experimental treatments of BC, SA and BC+SA significantly improved ($p < 0.05$) nodulation, but only up to 6 WAG. Highest number of nodules were recorded in BC treated plants at 27 nodules/plant. However, the difference between treatments were smaller after 6 WAG and no significant differences were observed between the experimental treatments at 9 WAG.

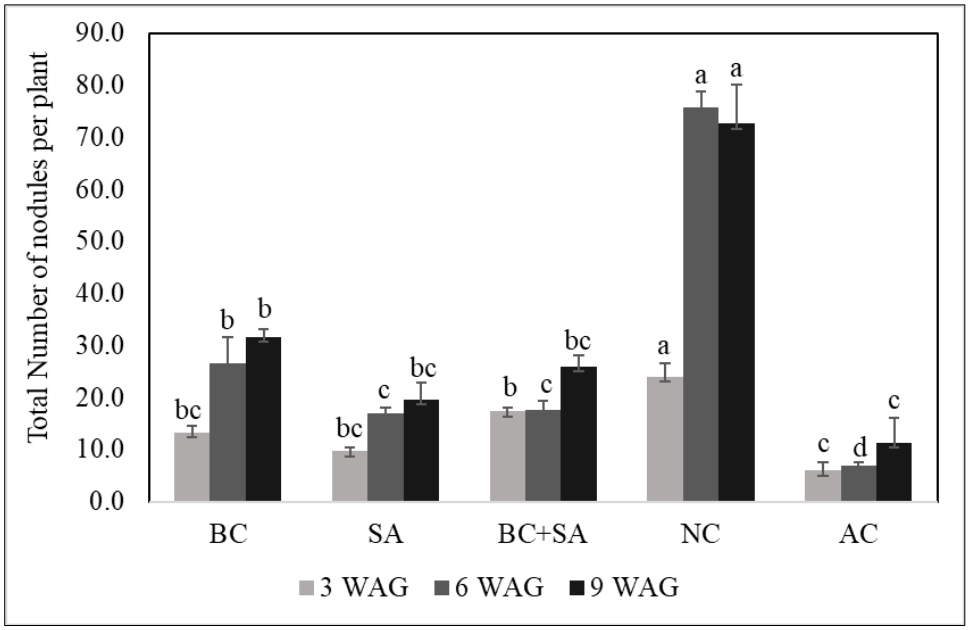

Figure 2.2. Total number of nodules in experimental treatments measured at 3, 6 and 9 WAG.
Note: Data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

Negative impact of soil acidity on root colonization by AMF was observed between the treatments at all time points, but trends were inconsistent (Figure 2.3). Percent colonization was significantly higher in most treatments and highest in NC treatment (38 %) compared to AC treatment (12 %) at 3 WAG. However, at 6 WAG, lowest colonization was recorded in NC treatment at 40 % and there were no significant differences between the remaining treatments. At 9 WAG, highest colonization was noted in SA (75 %), and

were significantly higher in both BC and SA treatments compared to AC (55 %). There was no significant difference between other treatments.

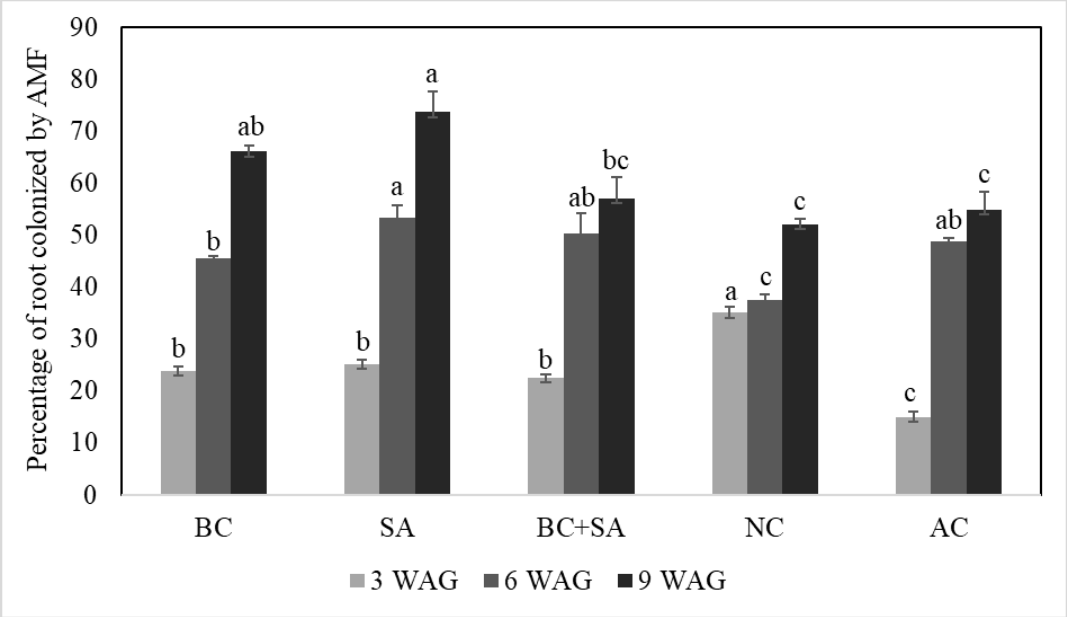

Figure 2.3. Percentage of root colonized by AMF in the experimental treatments measured at 3, 6 and 9 WAG**.**

Note: data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

2.4.5 Impact of acidity and experimental treatments on pod yield

Pod yield (g) was significantly reduced ($p < 0.05$) in AC treatment (3.8 g/pot) compared to NC treatment (9 g/pot) indicating the negative impact of acidic stress on plant productivity (Figure 2.4). Highest pod yield after NC treatment was observed in BC treatment (7 g/pot) and showed significant increase ($p < 0.05$) than AC treatment. Treatment of biochar with SA (BC+SA) also significantly increased the pod yield of cowpea beans. Foliar application of SA also increased the pod yield but was not

significantly higher than AC treatment. Correlation network analysis between different plant growth parameters and pod yield revealed that pod yield was highly (positively) correlated to nodulation (r = 0.93), N (r = 0.91) and rhizosphere pH (r = 0.92) and negatively correlated to leaf Al concentration (r = -0.73) (Figure 2.5). Moreover, leaf N was positively correlated to the number of nodules.

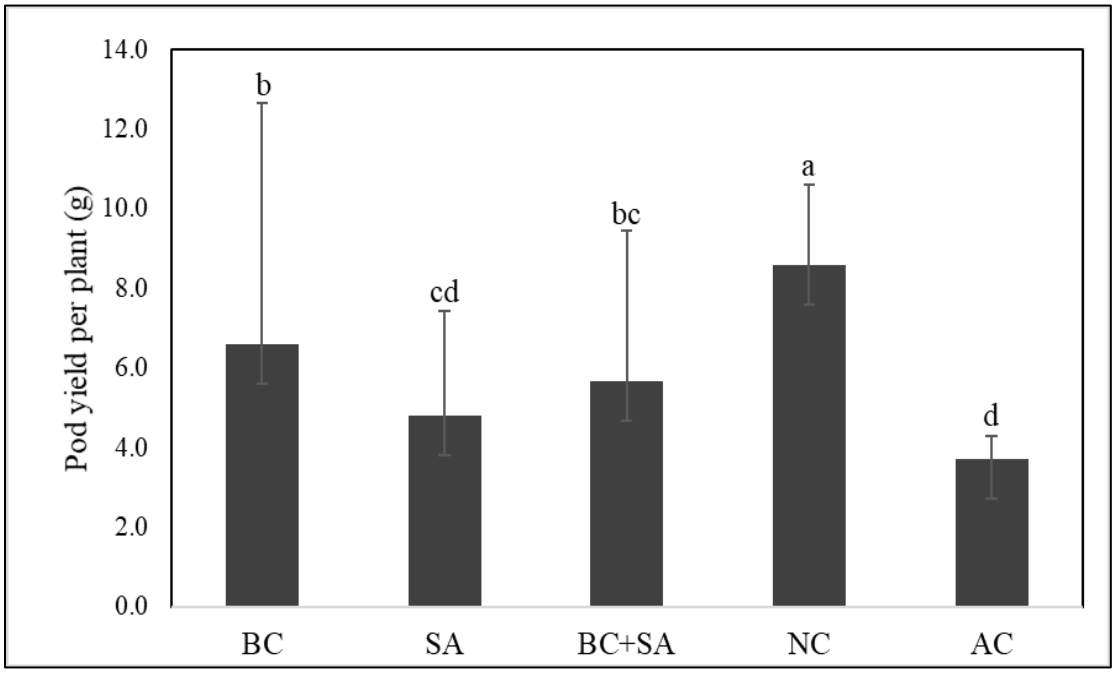

Figure 2.4. Pod yield per plant measured in all the experimental treatments after 9 WAG.

Note: data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

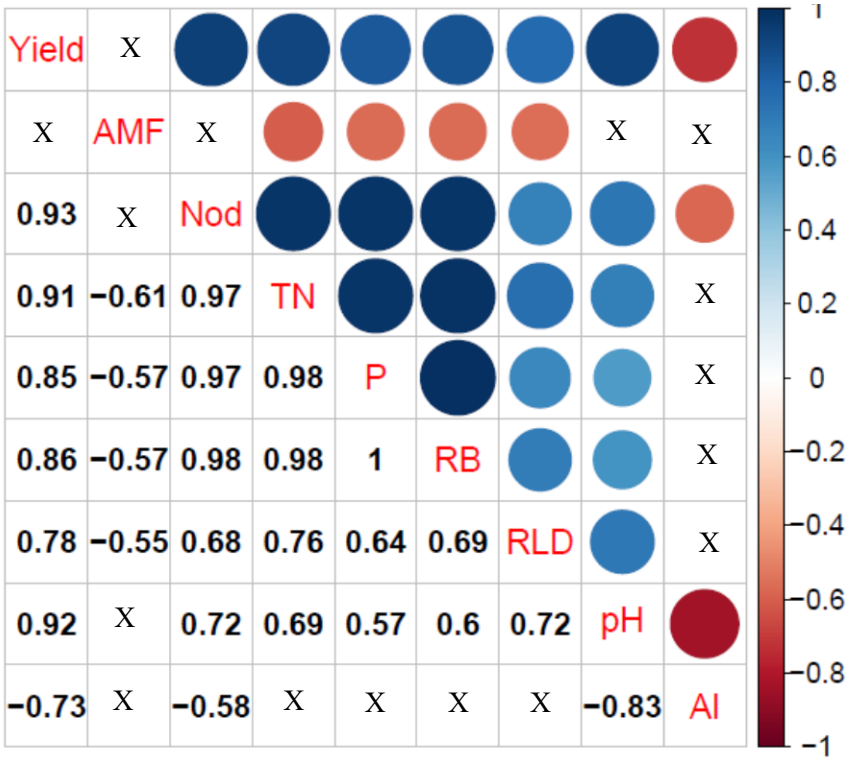

Figure 2.5. Pairwise comparisons between different plant growth parameters, pH and pod yield using Pearson's correlation coefficient.

Note: the color bar is representing range of Pearson's correlation coefficient. Blue color represents positive correlation range and red for negative. Circle size corresponds to coefficient value range from smaller (zero) to larger (1). the insignifcant ($p > 0.05$) correlations are marked 'X' in plot. AMF (percentage of root AMF colonization); K.Na; Nod (Number of nodules); TN (total N leaf concentration); P (total P leaf concentration); RB (Root biomass), RLD (Root length density), Yield (pod yield).

2.4.6 Impact of experimental treatments on relative abundance of bacteria, fungi, AMF and NFB

Abundance of prokaryotes (16S rRNA), fungi (ITS), AMF (AMF specific 18S) and NFB (*nifH*) were quantified in the rhizosphere, root and leaf samples by qPCR assays and results are presented in Table 2.14-2.16. In the rhizosphere, abundance of prokaryotes, fungi and AMF were significantly lower ($p < 0.05$) in AC treatment than NC treatment. Biochar treatment (BC) increased the abundance of prokaryotes in the rhizosphere and roots, although not at significant level. Whereas SA treatment significantly increased prokaryotic gene abundance in roots but not in the rhizosphere and leaves. Abundance of AMF was significantly increased in the rhizosphere of BC and SA treated plants than AC treatment. No significant differences were observed in abundances of fungi and NFB in the rhizosphere and plant tissues of experimental treatments and AC treatment.

2.4.7 Impact of soil acidity and experimental treatments on diversity and composition of rhizosphere and endophytic microbial community

Shannon and Simpson diversity indices represent species richness (measurement of OTU abundances) and evenness (measure of relative abundance of rare and abundant species) of microbial community with more weightage of species richness in Shannon index and that of species evenness on Simpson index (Kim et al., 2017a). Chao1 estimates projected richness based on rarefaction curves (measurement of OTUs expected in a given sample) and sensitive to changes in the rare species (Wang et al., 2018b).

Table 2.14. Impact of experimental treatments on gene copy number abundance of 16S rRNA, ITS, AMF and *nifH* in the rhizosphere at 6 WAG.

Treatment	log (16S rRNA gene copies g^{-1} soil)	log (ITS gene copies g^{-1} soil)	log (AMF gene copies g^{-1} soil)	log (*nifH* gene copies g^{-1} soil)
BC	8.41 ± 0.85ab	7.87 ± 0.48b	6.85 ± 0.38b	6.87 ± 0.76a
SA	7.66 ± 0.33b	7.38 ± 0.34b	6.69 ± 0.40b	6.09 ± 0.31a
NC	9.21 ± 0.10a	9.20 ± 0.15a	7.56 ± 0.45a	6.52 ± 0.57a
AC	7.67 ± 0.10b	7.28 ± 0.09b	6.08 ± 0.17c	6.11 ± 0.03a

Note: Data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

Table 2.15. Impact of experimental treatments on gene copy number abundance of 16S rRNA, ITS, AMF and *nifH* in the root endosphere at 6 WAG.

Treatment	log (16S rRNA gene copies g^{-1} root)	log (ITS gene copies g^{-1} root)	log (AMF gene copies g^{-1} root)	log (*nifH* gene copies g^{-1} root)
BC	10.46 ± 1.18ab	9.67 ± 0.79a	7.40 ± 0.36a	9.85 ± 2.04a
SA	10.79 ± 0.54a	10.00 ± 0.61a	7.57 ± 0.48a	10.08 ± 0.33a
NC	10.31 ± 0.42ab	10.00 ± 0.21a	7.83 ± 0.42a	9.43 ± 0.95a
AC	9.16 ± 0.73b	9.02 ± 0.73a	7.24 ± 0.14a	9.01 ± 1.09a

Note: data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

Table 2.16. Impact of experimental treatments on gene copy number abundance of 16S rRNA, ITS in the leaf endosphere at 6 WAG.

Treatment	Log (16S rRNA gene copies g^{-1} leaf)	Log (ITS gene copies g^{-1} leaf)
BC	8.00 ± 0.42b	6.81 ± 0.17b
SA	7.76 ± 0.34b	6.80 ± 0.02b
NC	8.96 ± 0.19a	7.36 ± 0.13a
AC	8.26 ± 0.57ab	6.80 ± 0.24b

Note: data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

The impact of soil acidity and treatments on bacterial and fungal diversity was larger in the rhizosphere than in the endosphere. Soil acidity significantly reduced the bacterial and fungal diversity (Shannon index) and abundance in the rhizosphere and richness in the roots of AC treatments as compared to NC treatment (Figure 2.6A, 2.7A; Table 2.17,2.18). Treatments BC and SA significantly improved the fungal diversity in the rhizosphere as compared to AC treatment (Figure 2.7A). Microbial diversity and species evenness were observed to be significantly increased in the leaves of AC treatment than NC treatment (Figure 2.6C, 2.7C). However, no significant differences were observed between the treatments for microbial diversity of roots and leaves.

A PCoA plot of Bray-Curtis distances for bacterial and fungal OTUs in the rhizosphere and endosphere are shown in Figure 2.8 and 2.9, respectively. Permanova test results are shown in Table 2.19 and 2.20. PCoA plots for bacterial and fungal community in the rhizosphere showed a clear separation by experimental treatments, with larger separation observed between AC and NC treatments. Permanova test confirmed that

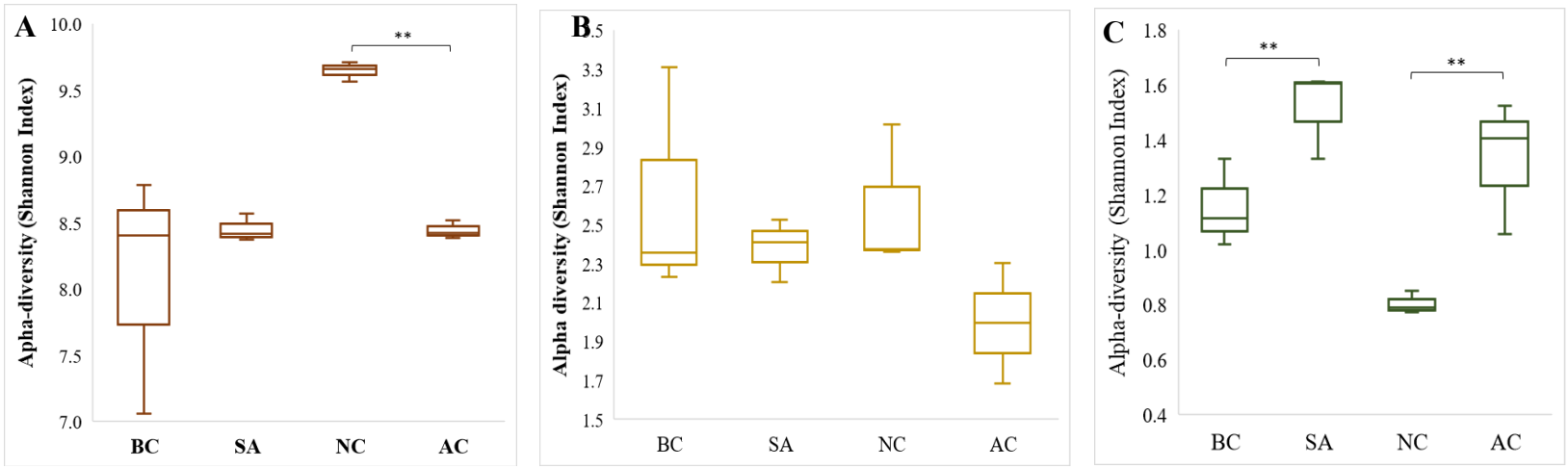

Figure 2.6. Shannon indices (Alpha-diversity) of bacterial community in the rhizosphere (A), roots (B) and leaves (C) of all the experimental treatments. Statistical analyses were performed by ANOVA and significance is denoted by asterisks where $**p < 0.05$.

Table 2.17. OTU numbers, Simpson and Chao1 for bacterial community in rhizosphere, root and leaf of the experimental treatments.

Treatment	Rhizosphere			Root			Leaf		
	Observed OTUs	Simpson	Chao1	Observed OTUs	Simpson	Chao1	Observed OTUs	Simpson	Chao1
BC	815b	0.9871b	1745b	176a	0.6715a	339ab	110ab	0.2757b	194a
SA	753b	0.9925ab	1574b	130a	0.6734a	256b	127a	0.3856a	259a
NC	1348a	0.9974a	3209a	177a	0.6632a	386a	85b	0.1827c	192a
AC	821b	0.9932ab	1108c	121a	0.5370a	237b	110ab	0.3284ab	200a

Note: Data presented are the means for 3 replicates. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

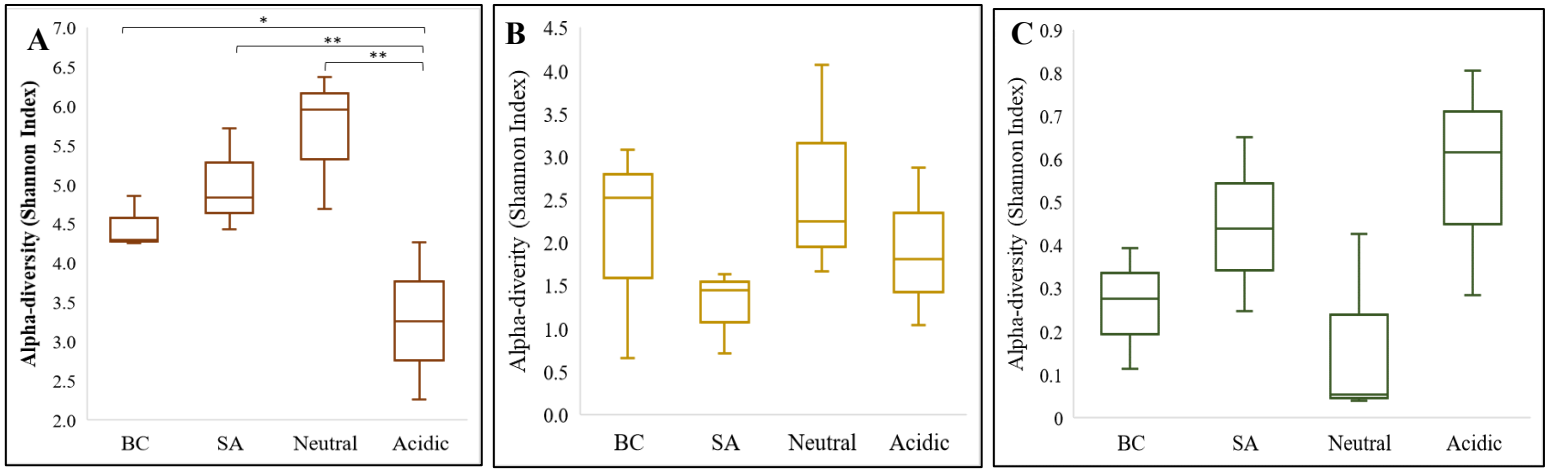

Figure 2.7. Shannon indices (Alpha-diversity) of fungal communities in the rhizosphere (A), roots (B) and shoots (C) of all the experimental treatments. Statistical analyses were performed by ANOVA and significance is denoted by asterisks where **P < 0.05.

Table 2.18. OTU numbers, Simpson and Chao1 for fungal community in rhizosphere, root and leaf of the experimental treatments.

Treatment	Rhizosphere			Root			Leaf		
	Observed OTUs	Simpson	Chao1	Observed OTUs	Simpson	Chao1	Observed OTUs	Simpson	Chao1
BC	103ab	0.8895a	134ab	31a	0.5460a	41a	17a	0.0544a	29a
SA	155a	0.8940a	187ab	29a	0.3138a	40a	14a	0.1206a	23a
NC	197a	0.9488a	244a	53a	0.6469a	65a	11a	0.0438a	20a
AC	34b	0.8570a	69b	33a	0.5162a	52a	18a	0.1518a	22a

Note: data presented are the means for 3 replicates. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

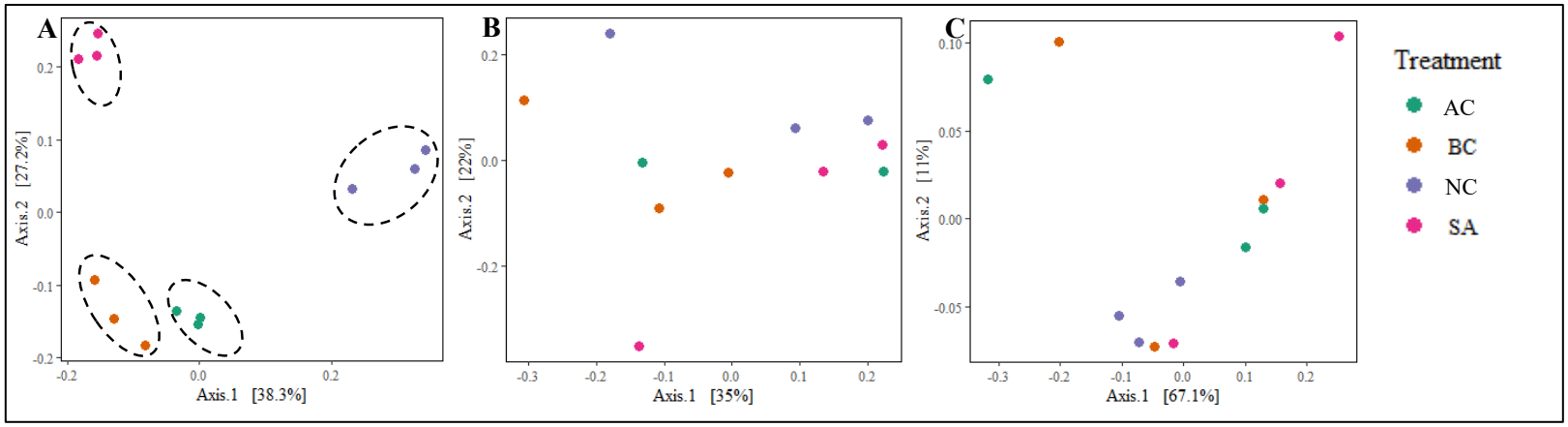

Figure 2.8. Principal Coordinate Analysis (PCOA) of bacterial community in rhizosphere (A), roots (B) and leaves (C) for individual samples from all the treatments using Bray-Curtis dissimilarity distance matrix. Replicates of each treatment are surrounded by dashed ovals to indicate the differences in bacterial community composition between different treatments.

Table 2.19. PERMANOVA p-values from pairwise comparisons of the treatments for bacterial OTUs based on Bray-Curtis dissimilarity index.

Compartment	Treatment	BC	SA	NC	AC
Rhizosphere	BC		0.0997	0.1032	0.1937
	SA	0.0997		0.1035	0.1002
	NC	0.1032	0.1035		0.0974
	AC	0.1937	0.1002	0.0974	
Root	BC		0.1004	0.397	0.3971
	SA	0.1004		0.1034	0.3989
	NC	0.397	0.1034		0.3934
	AC	0.3971	0.3989	0.3934	
Leaf	BC		0.2998	0.6044	0.0995
	SA	0.2998		0.1021	0.0997
	NC	0.6044	0.1021		0.1995
	AC	0.0995	0.0997	0.1995	

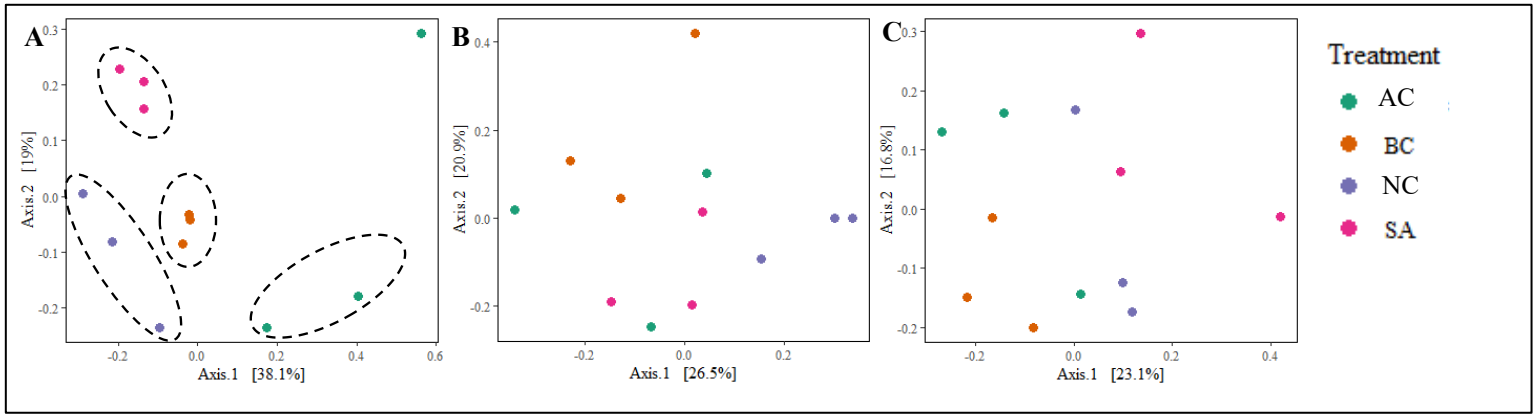

Figure 2.9. Principal Coordinate Analysis (PCOA) of fungal community in rhizosphere (A), roots (B) and leaves (C) for individual samples from all the treatments using Bray-Curtis dissimilarity distance matrix. Replicates of each treatment are surrounded by dashed ovals to indicate the differences in bacterial community composition between different treatments.

Table 2.20. PERMANOVA p-values from pairwise comparisons of the treatments for fungal OTUs based on Bray-Curtis dissimilarity index.

Compartment	Treatment	BC	SA	NC	AC
Rhizosphere	BC		0.1003	0.1050	0.0957
	SA	0.1003		0.0965	0.0983
	NC	0.1050	0.0965		0.0972
	AC	0.0957	0.0983	0.0972	
Root	BC		0.0976	0.1008	0.2937
	SA	0.0976		0.1009	0.5024
	NC	0.1008	0.1009		0.0981
	AC	0.2937	0.5024	0.0981	
Leaf	BC		0.1042	0.0983	0.0999
	SA	0.1042		0.1006	0.0969
	NC	0.0983	0.1006		0.6011
	AC	0.0999	0.0969	0.6011	

rhizosphere microbial community were significantly different ($p < 0.1$) between BC and SA, and AC treatment. Bacterial and fungal community in the endosphere of neutral (NC) and acidic (AC) control treatments were separated, but not between BC, SA and AC treatments.

Relative abundance of bacterial and fungal phyla is presented in Figure 2.10 and 2.11 respectively. The predominant bacterial phyla were *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes, Proteobacteria*, *Planctomycetes* and *Verrucomicrobia* (Figure 2.10). In the rhizosphere, relative abundance of *Acidobacteria* and *Chloroflexi* were significantly higher ($p < 0.05$), while *Bacteroidetes* and *Firmicutes* were significantly lower in AC treatment than NC treatments.

No significant difference was observed in the relative abundance of *Proteobacteria* in the rhizosphere of AC and NC treatments. In the rhizosphere of BC treatment, relative abundance of *Acidobacteria* was significantly decreased ($p < 0.05$) while that of *Firmicutes* was significantly increased than AC treatment. Also, relative abundance of *Bacteroidetes*, *Planctomycetes* and *Verrucomicrobia* was significantly higher ($p < 0.05$), in the rhizosphere of SA treatment than AC treatment. Additionally, linear discriminant analysis effect size (LEfSe) was performed on OTU abundance data to identify significantly different microbial taxa between pairwise comparison of treatments (Figure 2.12-2.15). LEfSe analysis revealed that *Alphaproteobacteria*, *Gammaproteobacteria*, *Bacillus*, *Paenibacillus*, *Rhizobiales*, *Hyphomicrobium* and *Rhodoplanes* were significantly more abundant in the rhizosphere of NC treatment than AC treatment.

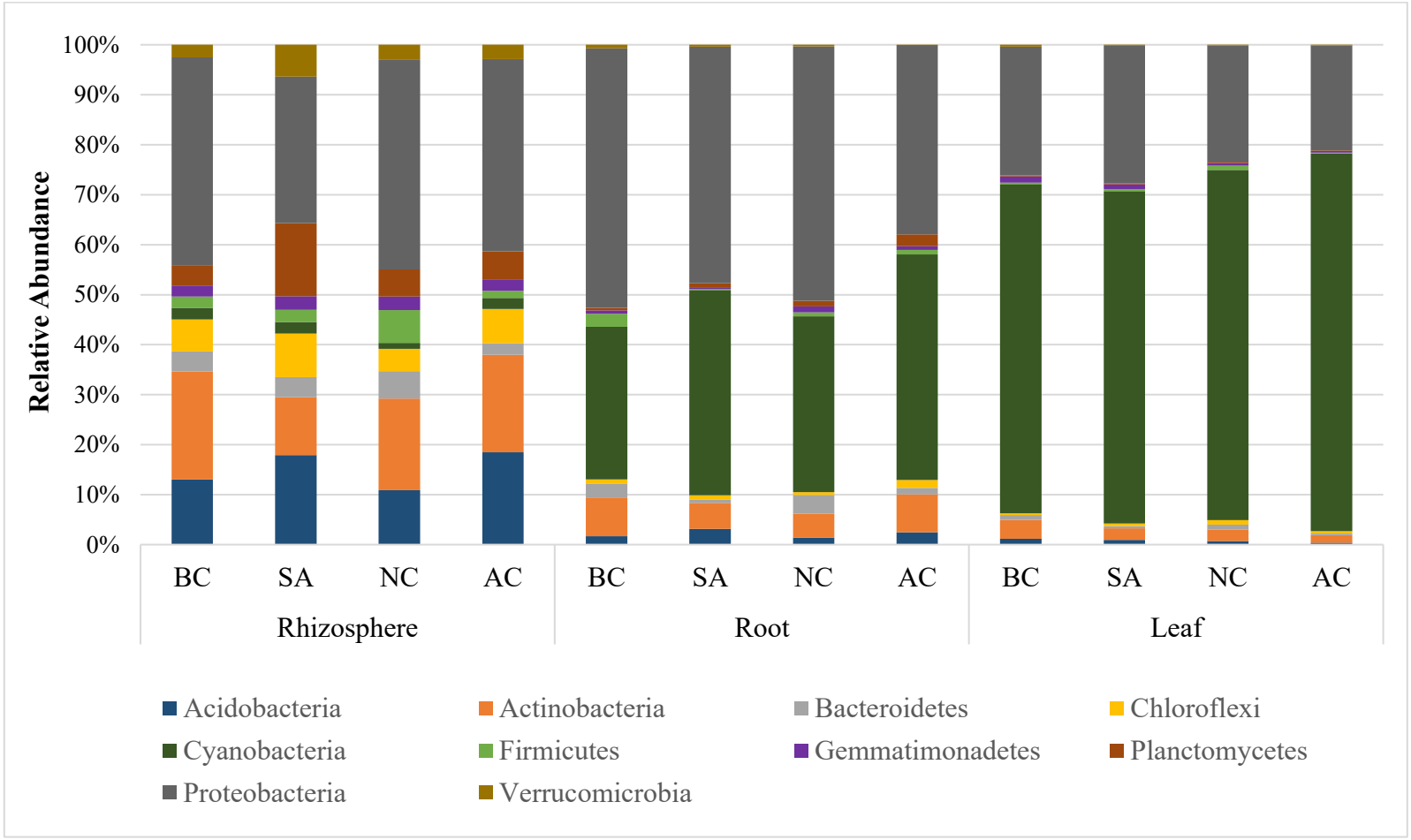

Figure 2.10. The relative abundance of bacterial phyla in the rhizosphere and endosphere of the treatments.

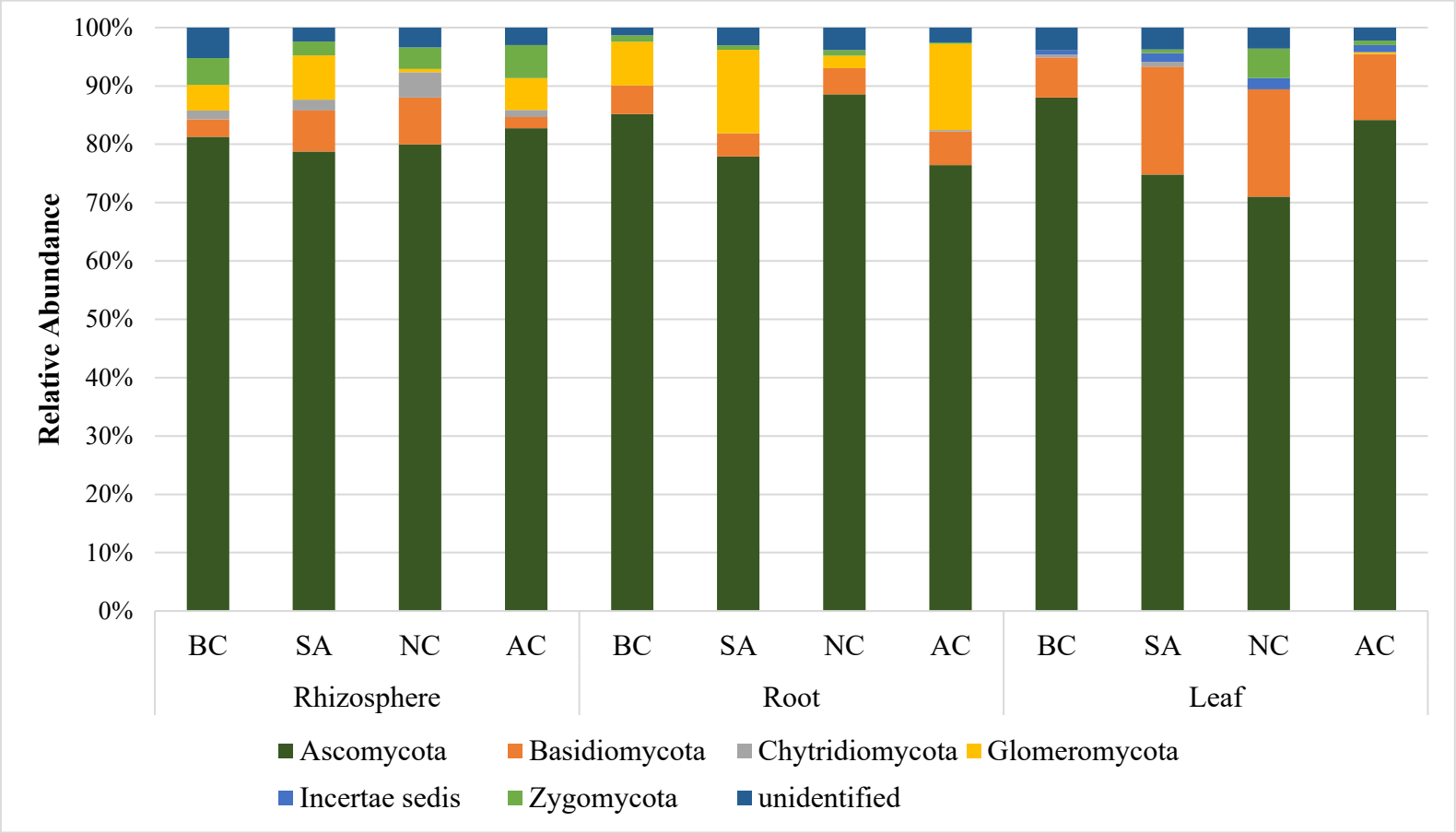

Figure 2.11. The relative abundance of fungal phyla in the rhizosphere and endosphere of the treatments.

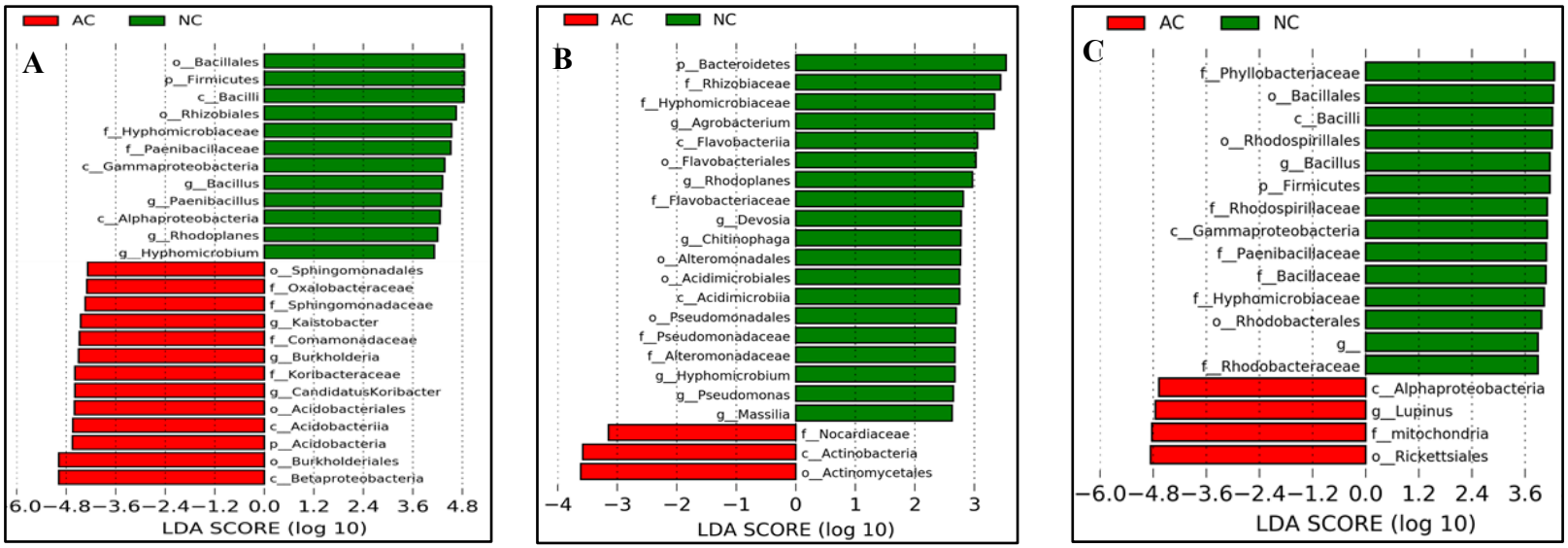

Figure 2.12. Significantly different bacterial taxa in rhizosphere (A), roots (B) and leaves (C) between neutral control (NC) and acidic control (AC) comparisons based on linear discriminant analysis effect size (LEfSe) method. Only taxa meeting a linear discriminant analysis (LDA) significance threshold of > 2.5 are presented.

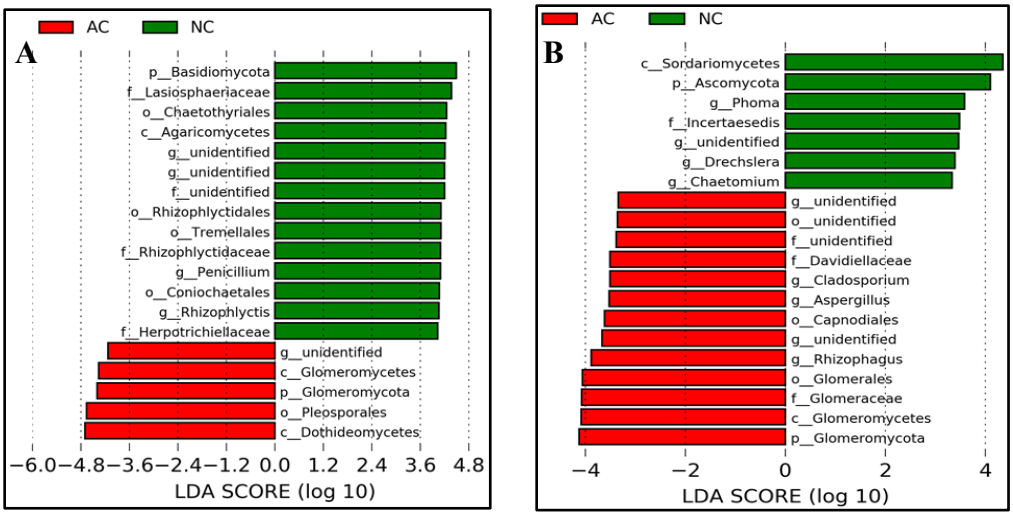

Figure 2.13. Significantly different fungal in rhizosphere (A) and roots (B) between neutral control (NC) and acidic control (AC) comparisons based on linear discriminant analysis effect size (LEfSe) method. Only taxa meeting a linear discriminant analysis (LDA) significance threshold of > 2.5 are presented.

Whereas relative abundance of *Betaproteobacteria*, *Acidobacteria* and *Burkholderia* were significantly higher in the rhizosphere of AC treatment (Figure 2.12A). Moreover, *Bacillus*, *Rhizobium*, *Bradyrhizobium* and *Flavisolibacter* were significantly more abundant in the rhizosphere of BC treatment than AC treatment (Figure 2.14A). Relative abundance of *Bacteroidetes* and *Firmicutes* was significantly lower in AC treatment than NC treatment in roots and leaf endosphere, respectively (Figure 2.10). Also, several bacterial taxa including *Rhizobiaceae, Flavobacterium* spp., *Pseudomonas* spp., *Chitinophaga* spp. and *Hyphomicrobium* spp. were significantly abundant in the roots. Genera *Bacillus* and *Paenibacillus* were significantly abundant in the leaf of NC treatment than AC treatment (Figure 2.12B). Furthermore, relative abundance of *Firmicutes* was significantly increased in BC treatment roots while abundance of *Cyanobacteria* and *Chloroflexi* were significantly reduced in the shoots of BC treated plants compared to AC

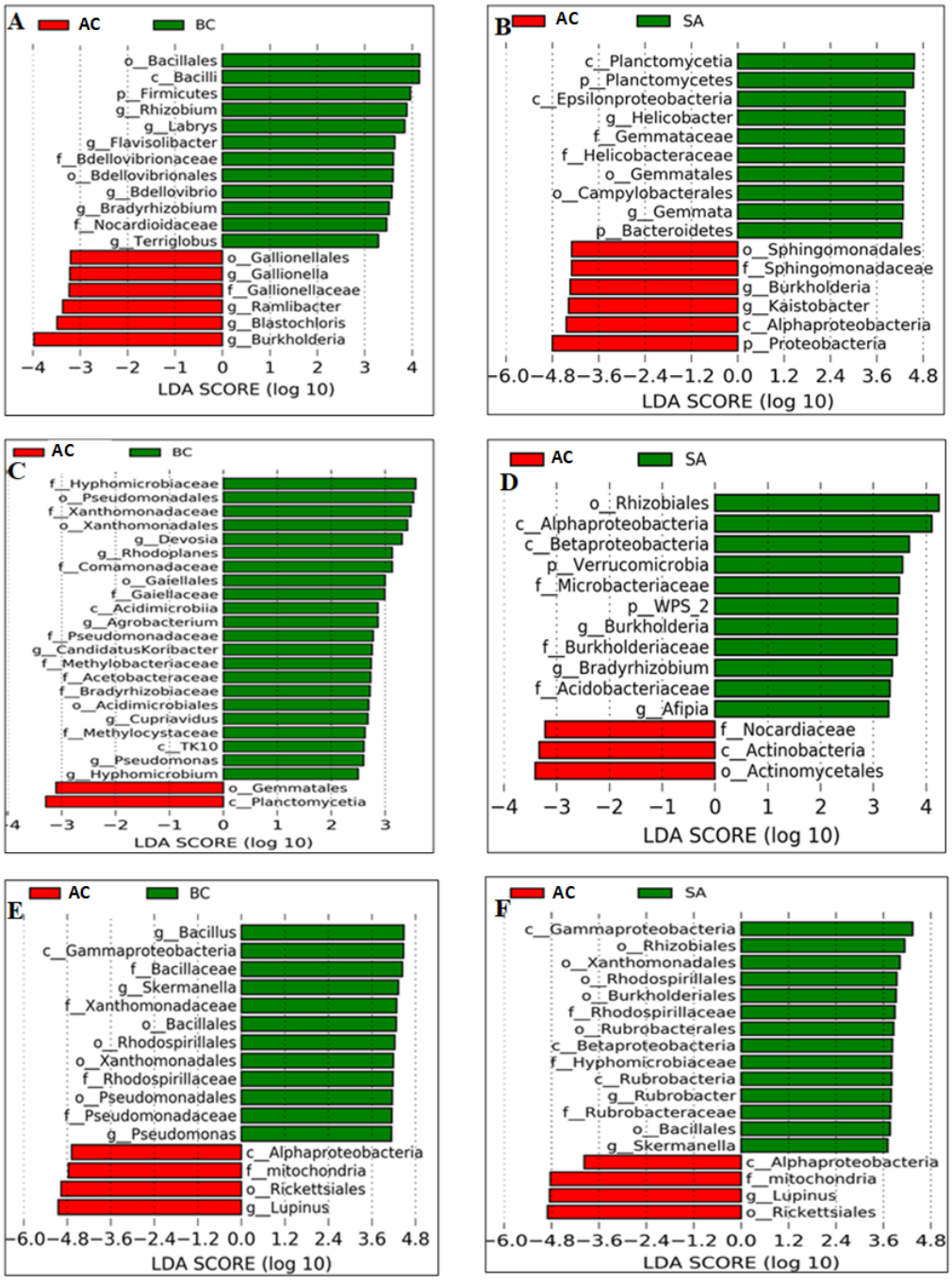

Figure 2.14. Significantly different bacterial taxa in rhizosphere (A, B), roots (C, D) and leaves (E, F) between each treatment and control (AC) comparisons based on linear discriminant analysis effect size (LEfSe) method. Only taxa meeting a linear discriminant analysis (LDA) significance threshold of > 2.5 are presented.

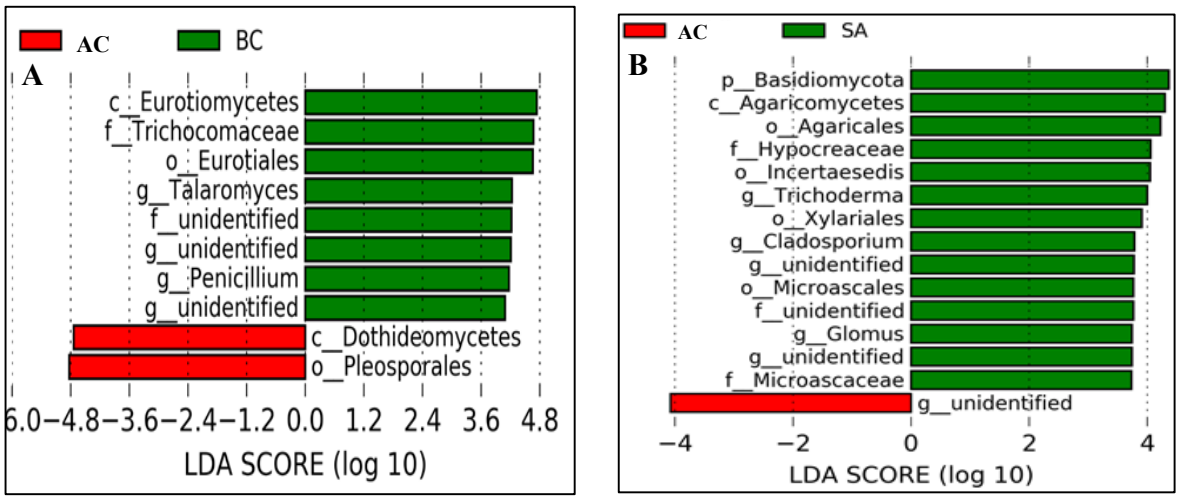

Figure 2.15. Significantly different fungal taxa in rhizosphere between each treatment and control (AC) comparisons based on linear discriminant analysis effect size (LEfSe) method. Only taxa meeting a linear discriminant analysis (LDA) significance threshold of > 2.5 are presented.

treatment plants. Increased relative abundance of phylum *Proteobacteria* was observed in the leaf endosphere of both BC and SA treatments compared to AC treatment. LEfSe analysis further revealed that several bacterial taxa including genera *Pseudomonas*, *Hyphomicrobium*, *Rhodoplanes* and families *Xanthomaondaceae* and *Bradyrhizobiaceae* were significantly more abundant in roots and leaves of BC treated plants (Figure 2.14C, 2.14E). In addition, significant increase in the abundance of *Burkholderia* spp., *Bradyrhizobium* spp. and order *Rhizobiales* was observed in the roots and leaves of SA treatment compared to AC treatment (Figure 2.14D, 2.14F).

The predominant fungal phyla were *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Glomeromycota, Incertae sedis* and *Zygomycota* (Figure 2.11). Rhizosphere of AC treatment was found significantly lower in relative abundance of fungal phylum *Basidiomycota* than NC treatment. LEfSe analysis further revealed that AC treatment had

significantly higher relative abundance of AMF phylum *Glomeromycota* in rhizosphere and roots and AMF genus *Rhizophagus* was more abundant in the roots of AC treatment than NC treatment (Figure 2.13 Relative abundance of phylum *Glomeromycota* was significantly higher in the rhizosphere of SA treated plants. LEfSe analysis further revealed increased abundance of fungal genus *Penicillium* spp. and family *Trichocomaceae* in rhizosphere of BC treatment while abundance of *Trichoderma* spp. and AMF genus *Glomus* in rhizosphere of SA treated plants compared to AC treatment (Figure 3.15). No significant impact of experimental treatments (BC and SA) was observed on the abundance of fungal endophytes in roots and leaves.

2.4.8 Influence of soil and plant growth parameters on microbial community composition

Mantel tests were performed to measure the Pearson's correlations between soil and plant growth parameters (soil pH, nutrient concentration, leaf Al content and RLD) and microbial community composition (based on Bray-Curtis distances) in rhizosphere and plant tissues (Table 2.21,2.22). Results showed that bacterial community in rhizosphere of acidic stressed plants were significantly influenced by several parameters and positively correlated to rhizosphere soil pH and leaf nutrient concentration (N, P, Ca and Mg) with strongest impact of P content. Leaf bacterial community was significantly correlated to only K content of leaves. Fungal community composition in rhizosphere was strongly correlated with pH of rhizosphere soil while those in roots were significantly influenced by nutrient concentration (N, P, Ca and Mg) in the plant tissues.

Table 2.21. Mantel tests between soil and plant growth parameters and abundance of bacterial community in rhizosphere and endosphere of different treatments using Pearson's correlation coefficient.

Parameters	Rhizosphere		Root		Leaf	
	r	p-value	r	p-value	r	p-value
pH	0.2325	**0.0488**	0.1313	0.1584	-0.1433	0.8738
Leaf N	0.4807	**0.0015**	-0.0829	0.5823	-0.0077	0.4331
Leaf P	0.5874	**0.0011**	-0.1359	0.6881	-0.0120	0.4575
Leaf K	0.0988	0.2093	0.0981	0.2502	0.3993	**0.0062**
Leaf Ca	0.5639	**0.0017**	0.0440	0.3731	0.0533	0.3665
Leaf Mg	0.5476	**0.0011**	-0.0223	0.4740	0.0555	0.3549
Al	-0.0369	0.5735	0.1080	0.2369	-0.1240	0.7735
RLD	-0.087	0.7438	0.0880	0.2739	-0.1059	0.7169

Note: Pearson correlation coefficients were used to test for the correlations between dissimilarity matrices using 9999 permutations. Bray-Curtis dissimilarities were used for bacterial community while Euclidean distance dissimilarities were used for soil and plant growth parameters; r: Pearson's correlation coefficient; pH: rhizosphere soil pH; N, P, K, Ca, Mg, Al denotes to total concentration of these nutrients in leaf tissues and RLD is root length density.

Table 2.22. Mantel tests between soil and plant growth parameters and abundance of fungal community in rhizosphere and endosphere of different treatments using Pearson's correlation coefficient.

Parameters	Rhizosphere		Root		Leaf	
	r	p-value	r	p-value	r	p-value
pH	0.3573	**0.0043**	0.0873	0.2314	-0.0216	0.5517
Leaf N	0.1419	0.2167	0.5154	**0.0034**	0.2176	0.1235
Leaf P	0.2827	0.4150	0.5178	**0.0052**	0.1986	0.1445
Leaf K	0.0014	0.4565	0.0763	0.2882	-0.1026	0.7668
Leaf Ca	0.1116	0.2632	0.4673	**0.0103**	0.2193	0.1337
Leaf Mg	0.1128	0.2663	0.4682	**0.0096**	-0.0203	0.5311
Al	0.1580	0.1547	0.0664	0.3179	0.2376	0.1014
RLD	0.0606	0.3160	-0.0120	0.5133	0.3936	**0.0327**

Note: Pearson correlation coefficients were used to test for the correlations between dissimilarity matrices using 9999 permutations. Bray-Curtis dissimilarities were used for fungal community while Euclidean distance dissimilarities were used for soil and plant growth parameters; r: Pearson's correlation coefficient; pH: rhizosphere soil pH; N, P, K, Ca, Mg, Al: total concentration of these nutrients in leaf tissues; RLD: root length density.

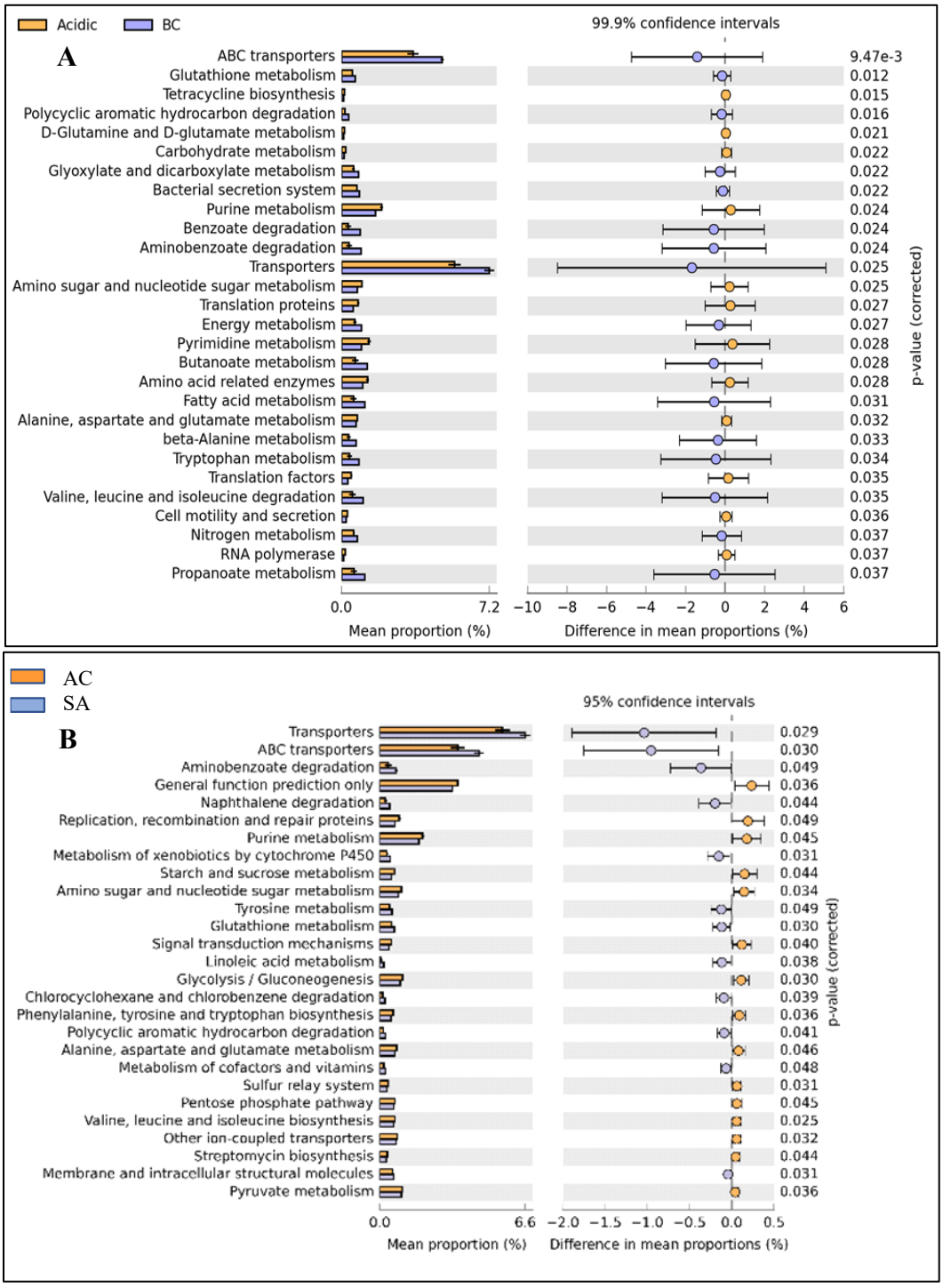

Figure 2.16. STAMP analysis on the KEGG pathways that differed between BC treatment and AC treatment in roots. The figure shows an extended error bar plot for the comparison of KEGG pathways in AC vs BC treatment (A) and in AC vs SA treatment (B). Only functions with $P < 0.05$ are shown.

2.4.9 Impact of experimental treatments on functional profile of microbial community

Potential KEGG based functional pathways associated with the bacterial OTUs of the experimental treatments were predicted using PICRUST. Further, STAMP analysis was used to identify significant differences in the abundances of these predicted functional pathways (Figure 2.16). Both BC and SA treatments had significant influences on the predicted functional pathways in roots. Abundance of genes related to "transporters" and "ABC transporters" were significantly

higher in BC and SA treatments than AC treatment. Also, roots of BC and SA treatment had higher abundance of several genes associated with metabolic pathways such as those for metabolism of glutathione and several amino acids than AC treatment. BC-treated roots were also abundant in genes for "nitrogen metabolism". Also, a gene associated with "membrane and intracellular structural molecules" was significantly abundant in SA-treated roots.

2.5 Discussion

2.5.1 Impacts of soil acidity on soil and plant growth parameters and microbial diversity and composition

Cowpea plants grown in acidic soil (pH ~4.9) and a neutral pH soil (pH ~6.5) were compared to assess the impacts of acidity on plant growth, diversity and composition of rhizosphere and endophytic microbial community and BPMI.

Results showed that concentration of nutrients (N, P, K, Ca and Mg) in soils and leaves were significantly lower in AC treatment compared to NC treatment. Total N

concentration in leaf tissue was positively correlated to the number of nodules similar to other studies (Allito et al., 2020; Aydi et al., 2008). Therefore, the decreased N in leaves of AC treatment plants was likely due to the decreased N_2-fixation and reduced nodulation. Moreover, leaf Al concentration was significantly higher than the threshold concentration of 30 mg/kg for legumes (Wallace and Romney, 1977), suggesting hyper accumulation of Al by cowpea plants grown in the acidic soil used in the study. Impact of Al toxicity and soil acidity was also evident on root growth and development, as root biomass and RLD decreased significantly in AC compared to NC treatment. It is well established that high Al concentration in soil can inhibit root growth and root cell division by damaging the root apex cells (Seguel et al., 2013) and consequently decreasing root biomass (Kolawole et al., 2000). High Al toxicity can also alter root morphology and decrease RLD under acidic conditions (Caires et al., 2008; Wang et al., 2020a). Furthermore, reduced root biomass and RLD impairs the ability of plant roots to uptake nutrients (Wendling et al., 2016).

Soil acidity significantly reduced the bacterial and fungal diversity and richness in the rhizosphere and root endosphere compared to neutral soil conditions. A larger decline was noted in the rhizosphere than in the root and leaf endophytes. Also, results of PCoA plots showed a clear separation in the composition of rhizosphere microbial community of AC and NC treatment which was not noted for endosphere microbial community. This was similar to the results obtained by Han et al. (2020) who noted that rhizosphere community were influenced by fluctuations in soil pH but not endophytes. These results support the prevailing hypothesis that soil pH is a major driver of microbial community

composition (Lauber et al., 2009; Wan et al., 2020; Zeng et al., 2019). Furthermore, significantly higher relative abundance of *Acidobacteria* was noted in the rhizosphere of AC treatment than NC treatment. *Acidobacteria* are well known to survive under low pH and nutrient-deficient conditions prevalent in an acidic soil (Fierer et al., 2007). It was revealed by LEfSe analysis that various taxa under phylum *Firmicutes* such as *Bacillus* and *Paenibacillus* were significantly depleted in the rhizosphere of AC treatment compared to NC treatment. Moreover, abundance of *Pseudomonas* and *Flavobacterium* were also significantly lower in the roots of AC treatment than NC treatment. These bacteria are well known for their PGP attributes and are also capable of solubilizing P in acid soils and improve plant P-uptake (Achkouk et al., 2020; Qureshi et al., 2012; Soltani et al., 2010). Therefore, significantly lower concentration of P noted in the rhizosphere and leaves of AC treatment compared to NC treatment could be due to the lower abundance of these phosphate-solubilizing bacteria (PSB) in the AC treatment rhizosphere. Abundance of several NFBs such as *Rhizobiales* and *Rhizobiaceae* were also significantly depleted in the rhizosphere and roots of AC treatment than NC. This suggests that common NFBs might be sensitive to acidic pH and may not successfully establish nodulation as evidenced by lower nodulation in AC treatment. One study noted that increased H^+ ion concentration causes cellular pH instability and growth inhibition of inoculated *Bradyrhizobium* in acidic soil (Graham et al., 1994). It was also noted that *Burkholderia,* which are acid tolerant NFB than Rhizobia (Garau et al., 2009), had significantly higher relative abundance in the rhizosphere of AC treatment. However, their higher relative abundance did not produce higher nodulation in AC treatment suggesting

that poor nodulation was probably not due to lack of competent NFB in the rhizosphere. It could be due to disruption of signal exchange between the plant and NFB by low pH conditions (Ferguson et al., 2013). It was noted that high H^+ in the root zone and plant tissues reduces the flavonoid secretion from the roots, which decreased *Nod* gene induction in NFB and Nod driven metabolite secretion (McKAY and Djordjevic, 1993). High H^+ disrupted exchange of signals between the plant and bacterial partners causing root hair deformation and root hair curling (Miransari et al., 2006). Also, the attachment of NFB to legume root hairs requires Ca^{2+}-dependent adhesions (Smit et al., 1992) and therefore limited availability of Ca^{2+} in acidic soils (Ramirez et al., 2001) can impair the process of bacterial attachment to root hairs and infection thread formation (Gage, 2004). Collectively, low pH conditions in acidic soils disrupts the signaling exchange between plant roots and NFB reducing nodulation consequently reducing N-uptake. Moreover, many NFB and PSB were noted to be sensitive to acidic soil, and may have resulted in reduced nutrient uptake and pod yields in AC treatment compared to NC treatment.

Alpha-diversity of fungal community in the rhizosphere of AC treatment was significantly lower than neutral soil, suggesting that fungal community was also sensitive to acidic conditions. Previous studies noted a similar decrease in fungal diversity as soil pH decreased (Wang et al., 2015; Zhou et al., 2016). Among fungal phyla, *Basidiomycota* decreased in the rhizosphere of acidic soil, which was in contrast to a previous report that it was one of the dominant phyla in acidic conditions (Zhang et al., 2016b). Interestingly, AMF phylum *Glomeromycota* was at higher relative abundance in cowpea roots of acidic soil conditions than neutral soil. This was further confirmed by LEfSe analysis which

showed that several AMF taxa including genus *Rhizophagus* and order *Glomerales* were significantly abundant in the rhizosphere and roots of unamended acidic soil compared to neutral soil conditions. Many AMF species within *Rhizophagus* were reported to be widely distributed in acid soils and demonstrated tolerance to Al^{3+} (Aguilera et al., 2015; Maki et al., 2008). Thus, abundance and diversity of AMF was not significantly impacted by high soil acidity and Al toxicity in the present study. This also confirms the comparable percentage of root colonization by AMF observed in AC treatment with NC treatment. However, the P concentration in the rhizosphere and leaves was significantly lower in the AC treatment indicating that AMF could not solubilize enough P in the acidic soil. This could be possibly due to limited interaction of AMF with other phosphate-solubilizing microbes (PSM) in the soil due to their low abundance in AC treatment as discussed earlier. This has been recently reported in several studies that the interaction of AMF with other PSM increase P-uptake by plants under acidic conditions (Sharma et al., 2020; Souchie et al., 2006; Zhang et al., 2018). Several PSM can solubilize Al-bound phosphates by releasing phosphatases and also mineralize the organic P by releasing phytases and organic acids, subsequently translocated by AMF hyphae to the host plant (Masrahi et al., 2020; Wahid et al., 2020).

In conclusion, soil acidity and Al-toxicity negatively impacted nodulation, leaf N concentration, microbial diversity of rhizosphere and pod yield. Although, AMF colonization was not impacted by soil acidity. However, lower abundance of PSM and their limited interaction with AMF possibly caused reduction in P-availability in the

rhizosphere and total P in the leaves of the plants. Therefore, reduced nutrient availability and uptake significantly reduced pod yield of AC treatment compared to NC treatment.

2.5.2 Impacts of biochar on rhizosphere chemistry, microbial diversity and composition, and plant growth parameters

Biochar amendment to acid soil significantly increased soil pH and NO_3 and P concentrations in the rhizosphere and leaf tissues. The soil pH buffering potential of biochar can be attributed to its inherent alkalinity due to enrichment of carboxyl groups during pyrolysis and high cation exchange capacity (Chintala et al., 2014). Biochar used in this study had a pH of 7.4. BC treatment also significantly increased root biomass and RLD as compared to AC treatment which could be due to improvement of soil physical properties changes brought by biochar in the soil (Devereux et al., 2012). Biochar increases the soil aeration and porosity which facilitate the root proliferation and thus improve overall root growth (Bruun et al., 2014). Increased RLD also contributed to nutrient availability by increasing the soil volume explored by the root system (Faye et al., 2019). Biochar increased nutrient concentrations (N, P, K, Ca and Mg) and decreased Al concentration in leaves. Similar results have been shown in other studies where biochar amended soil led to increased leaf elemental concentration while decreasing uptake of toxic Al ions (Lauricella et al., 2020; Xia et al., 2020). Additionally, increased microbial diversity and shifted community composition may have also contributed to nutrient mobilization and availability (DeLuca et al., 2015).

Results obtained from alpha diversity indices and observed OTU richness indicated that despite a significant increase in soil pH and nutrient availability by biochar, there was

no significant impact on bacterial diversity and richness in the rhizosphere and plant endosphere. Biochar impacts on soil microbial community are variable, as some studies noted increased bacterial diversity (Chen et al., 2013; Mitchell et al., 2015), whereas others noted a decrease in the bacterial diversity after biochar application (Gómez-Luna et al., 2012; Khodadad et al., 2011). However, fungal diversity in the rhizosphere of biochar amended plants increased significantly than acidic control (AC). Furthermore, biochar led to a significant change in the composition of bacterial community in both rhizosphere and plant endosphere and fungal community in the rhizosphere. Several N-transforming and NFB such as *Rhizobium*, *Bradyrhizobium*, *Hyphomicrobium* and *Rhodoplanes* had relatively higher abundance in the rhizosphere and roots of BC-treated plants compared to AC treatment. Increased abundance of NFB in BC treatment consequently increased nodulation as noted in other studies (Wang et al., 2018a; Xiang et al., 2017). The increased soil pH in BC treatment decreased H^+ ion concentration in the rhizosphere and its inhibitory effect on flavonoids production and bacterial *nod* gene induction (McKAY and Djordjevic (1993). Moreover, adsorption of flavonoids and nod factors on the surface of biochar promoted the residence time of these signaling molecules in soil and initiation of rhizobia interactions (Thies and Rillig, 2009). Biochar may also provide protection to rhizobia as it tends to survive well in pores of biochar (Sun et al., 2020). All these effects could therefore facilitate the exchange of nodulation signals between plant roots and N_2-fixing bacterial partner (Thies and Rillig, 2009) leading to higher N-fixation and N in BC treatment than AC and SA treatment.

Bacterial phylum *Firmicutes* was noted to be significantly higher in abundance in the rhizosphere and roots of BC treatment than AC treatment. Among *Firmicutes,* the major genus significantly higher in relative abundance in BC treatment was *Bacillus.* Several *Bacillus* species occur in the rhizosphere and root endophere and have shown plant growth promotion under abiotic stress conditions (Lopes et al., 2018). Many *Bacillus* were noted to influence plant growth by producing phytohormones, solubilizing nutrients and some are capable of fixing nitrogen (Lopes et al., 2018). Moreover, several species of *Bacillus* can solubilize phytate (organic P) and increase P availability to plants (Ahmad et al., 2018). Additionally, LEfSe analysis revealed significant increase in relative abundance of *Pseudomonas* in roots and leaves of biochar amended plants. Several *Pseudomonas* spp. are beneficial and are known for their role in plant growth promotion under abiotic stress (Mercado-Blanco and Bakker, 2007; O'sullivan and O'Gara, 1992). Several species of *Pseudomonas* can produce siderophores that can bind with Al^{3+} ions and minimize Al-toxicity (Zerrouk et al., 2016). Reduced Al concentrations and increased root activity in the BC treatment was probably influenced by abundance of these beneficial microbes. Furthermore, some endophytic *Pseudomonas* secrete organic acids to solubilize mineral phosphates and increase P uptake by plants (Kuklinsky-Sobral et al., 2004). Biochar also led to increased abundance of PGP fungi *Penicillium* in the rhizosphere, which was noted to mobilize inorganic-P complexes increase and increase P-uptake by plants (Wakelin et al., 2007). It was also interesting to note that BC treatment significantly increased AMF colonization compared to AC treatment only around 9 WAG, which was probably to meet the higher P demand by cowpea at later growth stages (Kahiluoto et al., 2001). Therefore,

it is evident that the positive influence of biochar on AMF colonization and abundance of native P solubilizing microorganisms (*Pseudomonas*, *Bacillus* and *Penicillium* spp.) led to increased P availability in soil and therefore increased P concentration in leaves, similar to results noted in other studies (Chabot et al., 1996; Wahid et al., 2020).

BC-treated roots showed a significant increase in abundance of many functional genes predicted by PICRUST analysis. Several enriched KEGG pathways related to transporters and ABC transporter pathways, which are involved in nutrient uptake in the rhizosphere and exchange of carbohydrates and amino acids (Ali et al., 2014b), were significantly more abundant in BC-treated roots. Similarly, Glutathione metabolism was signficnatly more abduanct in BC treatment. Glutathione is a non-enzymatic antioxidant molecule, which plays a role in protecting plants against oxidative damage caused by ROS produced under abiotic stressed conditions such as soil acidity (Szalai et al., 2009). These results indicate that microbiome structure under BC treatment improved nutrient availability and uptake functions, and decreased oxidative stress caused by Al toxicity and low pH conditions (Kamran et al., 2019).

Addition of BC in acidic soil significantly increased pod yield compared to AC and SA treatments. It can be concluded that BC treatment shifted microbial community in acid soil rhizosphere and stimulated enhanced nodulation, N-fixation, P availability and nutrient uptake and higher pod yield. It is also clear that BC treatment improved plant tolerance to acidic by increasing soil pH and reduced Al toxicity.

2.5.3 Impacts of SA on soil and plant growth parameters and microbial diversity and composition

Salicylic acid (SA) treatment significantly reduced the concentration of Al in the leaves of cowpea plants indicating reduction in acidic stress conditions. This is in accordance with a study by Pandey et al. (2013b) who reported that SA reduced the adverse effects of Al toxicity in *Oryza sativa* seedlings by suppressing the uptake of Al by root tips and by inducing the production of antioxidant enzymes inhibiting the accumulation of ROS in plants. However, SA treatment did not significantly change the pH of rhizosphere compared to AC treatment.

Alpha-diversity indices for microbial community in SA treated plants showed increased diversity of fungal community in the rhizosphere and beta-diversity was also significantly different for bacterial and fungal community in the rhizosphere. No significant changes were observed in the diversity of endophytic community of roots and leaves which is similar to a study by Liu et al. (2018), who noted that SA treatment did not alter diversity of root associated microbiome of *Triticum aestivum* plants. However, SA treatment modified the composition of microbial community at specific taxa level as evidenced by LEfSe analysis. Similarly, Lebeis et al. (2015) reported that SA altered root microbiome at specific bacterial taxa level in *Arabidopsis*. Several NFB such as *Rhizobium*, *Bradyrhizobium* and *Burkholderia* were significantly abundant in roots and leaves of SA treated plants. It is well established that *Burkholderia* are predominant NFB in acidic soils (Garau et al., 2009) and forms nodules and contribute significantly to N_2-fixation in legumes (Estrada-De Los Santos et al., 2001). This probably contributed to

increased nodulation and leaf N in SA treated plants up to 6 WAG compared to AC treatment. However, both nodulation and concentration of leaf N decreased at 9 WAG in SA- treated plants and was not significantly different to AC treatment. Significantly lower nodulation, leaf N cocnentrations and pod yields were noted in SA treatment than BC. Thus, it is possible that the applied *Rhizobium* inoculum or native *Burkholderia* could not establish nodulation in SA-treated plants due to poor exchange of nodulation signals under lower soil pH conditions as compared to BC treatment. Moreover, limited rhizosphere soil P availability reduced growth and activity of rhizobia further limiting the nitrogen fixation efficiency of SA-treated plants (Binkley et al., 2003; O'Hara, 2001) and thus decreasing the leaf N and pod yield.

Among fungi, AMF taxa *Glomus* and *Glomeromycota* were observed to be significantly abundant in the rhizosphere of SA treated plants. Moreover, SA treatment significantly increased the root AMF colonization at 9 WAG compared to AC treatment. This is in contrast to a report that application of SA either decreases or does not influence AMF colonization (Hause et al., 2007). Similarly, another study noted that foliar application of SA decreased the root AMF colonization in *Cucumis sativas* plants (Ludwig-Müller et al., 2002). Interestingly, AMF colonization in SA treated plants was higher than BC treatment, which was probably to meet high P demand and improve P uptake (Lin et al., 2020). It was also noted that SA treatment increases the signaling between AMF and plants by increasing the export of sugars from leaves to roots providing more carbon to AMF and thus facilitating the symbiosis (Garg and Bharti, 2018). However, it is interesting that SA treatment only improved AMF interactions and

colonization, but not nodulation. These contradicting efffects of SA on AMF and NFB needs further attention to identify suitable combination of signaling compounds to elicit comprehensive benefits on BPMI.

Trichoderma was another fungus that was significantly abundant in the rhizosphere of SA treated plants. *Trichoderma* are biocontrol agents that are also known to promote plant growth under acidic soil conditions (Mercl et al., 2020) through several mechanisms such as production of phytohormones, solubilization of sparingly soluble minerals for P (Li et al., 2015) and the regulation of abundance of other rhizosphere microbiome (Vinale et al., 2006). Thus, increase in AMF colonization and PSB *Burkholderia* and fungi *Trichoderma* significantly improved the P content in leaf tissues of SA-treated plants as compared to AC treatment.

SA-treated roots also showed a significant increase in abundance of several functional genes predicted by PICRUST analysis. Glutathione metabolism was more abudannt in the SA treated roots, which indicate potential role of SA in protecting plants from oxidative stress caused by soil acidity as previously shown in other studies (Pandey et al., 2013b). Moreover, abundance of predicted KEGG pathway "membrane and intracellular structural molecules" indicates a possible mechanism by SA to protect from membrane injury and lipid peroxidation caused by oxidative damages by ROS molecules under acidic stressed conditions (Srivastava and Dubey, 2011b).

Despite the significantly improved P in leaves, reduced oxidative stress and decreased Al-toxicity, the pod yield of SA-treated plants was not significantly different as compared to AC treatment and was significantly lower than BC treatment. Cowpea pod

yield was significantly correlated with pH, nodulation and leaf N in the present study. Therefore, lower pH and nodulation at 9 WAG under SA treatment diminished plant productivity. It was also noted that the SA-treated plants at 6 WAG had lower shoot height (Appendix- A Figure1,3) and demonstrated early signs of nutrient deficiency, primarily N deficiency, as leaves turned yellow prematurely (Appendix Figure 4,5). These evidences suggest later stage N-deficiency in SA treatment. No significant impact of SA on root biomass and significantly reduced RLD at 9 WAG also further confirms the lower uptake of N in SA-treated plants (Wendling et al., 2016). Moreover, SA induced early flowering and pod formation than other treatments (Appendix-A Figure 6). It has been reported in few studies that SA induces flowering and pod formation in plants as a protective strategy from various abiotic stressed conditions including nutrient-deficiency (Afshari et al., 2013; Hayat et al., 2010). Besides, the effects of SA treatment in plants under stressed conditions can also be influenced by the duration of treatment, plant species, age and treated plant organ (Khan et al., 2015b; Miura and Tada, 2014; Shi et al., 2009). It was mentioned in a study by Kováčik et al. (2009), that SA could either produce plant growth promotion or inhibition depending on the concentration of SA used exogenously. Thus, future studies must focus on determining appropriate dosage of SA for a specific crop to elicit favorable effects.

2.5.4 Implications on soil fertility management and plant production in acidic soils

As expected, soil acidity reduced plant nutrient concentrations and pod yields due to low pH and high Al concentrations in the leaf. It is well established that Al toxicity and

deficiency of N, P and Ca in acidic soil are major constraints for plant yield and productivity (Rahman et al., 2018; Rao et al., 2016). Lime application to acid soils is the common approach to improve soil fertility and productivity (Fageria and Baligar, 2008). However, lime has several disadvantages including reacidification over time, CO_2 emissions (West and McBride, 2005) and hardening of soils after continuous applications (Wang and Xian-Jun, 2017). In this study, use of BC increased soil pH of a highly acidic soil (~4.9) to around 6.0, and reduced the concentration of Al in leaf tissue. Additionally, biochar improved nodulation, AMF colonization, RLD, plant nutrient concentrations (N, P, K and Ca) and pod yields. Biochar improved microbial diversity and altered composition in the rhizosphere by increasing relative abundance of several beneficial bacteria (*Bacillus* and *Pseudomonas*) and fungi (*Penicillium*). Therefore, it can be concluded that biochar is a sustainable alternative to improve soil health in acid soils, as it produced comprehensive benefits on soil properties and improved BPMI. Long-term impacts with respect to soil pH buffering capacity and long-term carbon sequestration are added benefits of biochar (Chintala et al., 2014). Use of biochar is therefore recommended for correcting soil acidity and improving plant yields and productivity in acidic soils.

2.6 Conclusions

Cowpea plants grown under acidic soil accumulated higher Al concentrations in the leaves and showed adverse impacts on nutrient availability, plant growth and pod yield. Rhizosphere microbiome structure was significantly different from a neutral soil rhizosphere microbiome. Biochar amendment improved soil pH and decreased Al accumulation, and increased nutrient availability and concentration in leaf tissues, and

increased pod yield. Biochar amendment to acid soils significantly increased nodulation, uptake of nutrients and the abundance of beneficial PGP microbes such as *Bacillus*, *Pseudomonas, Penicillium* and NFB such as *Rhizobium* and *Bradyrhizobium* in the rhizosphere and endosphere. Foliar application of SA decreased Al concentrations and increased nutrient concentrations in leaf tissue compared to acidic control, but beneficial effects were lower than BC treatment. SA increased the AMF colonization and abundance of PGP microbes such as *Burkholderia* spp., *Trichoderma* spp. and AMF *Glomus* spp. in the rhizosphere and roots of the plant. However, nodulation, leaf N and pod yields were lower than BC treatment. Soil pH did not change significantly in SA treatment. Based on this study results, it can be concluded that symbiotic interactions of legumes with NFB are more sensitive to the adverse impacts of soil acidity. Whereas AMF interactions appeared to be not sensitive to soil pH, but rather were influenced by both SA and BC treatments.

CHAPTER III

IMPROVING BENEFICIAL PLANT-MICROBE INTERACTIONS IN SALINE SOIL USING AMENDMENTS AND STIMULANTS

3.1 Synopsis

Soil salinity is a major problem impacting the agricultural productivity all around the world. Improving interactions of plants with beneficial microbes in the rhizosphere and endosphere can be an effective and sustainable approach to increase improve salinity tolerance, crop yield and productivity in saline soils. This study was conducted to evaluate compost (CMP) as a soil amendment and foliar application of several signaling compounds such as strigolactones (SL), salicylic acid (SA) and coumarins (COU) for their impacts on diversity and composition of rhizosphere and endophytic microbiome, arbuscular mycorrhizal fungi (AMF) colonization, nodulation, plant nutrient concentrations and pod yield. Results showed that soil salinity adversely impacted plant nutrient uptake, AMF colonization and pod yields. Among the amendment treatments, SL+SA treatment produced the highest cowpea pod yield followed by CMP amendment. The highest nodulation and root colonization were noted in SL+SA treated plants. Significant higher relative abundance of *Streptomyces* and several AMF (*Rhizophagus* and *Diversispora*) were noted in the rhizosphere and roots of SL+SA treated plants. There were no significant changes in plant growth, yield and microbiome composition in COU treatment compared to control (CS). It can be concluded that foliar application of SL and

SA together can be a very effective strategy to alleviate the adverse impact of soil salinity on plants.

3.2 Introduction

Soil salinization is a major agricultural problem particularly in arid and semi-arid areas in the world (Chen et al., 2017b; Porcel et al., 2015). It has been estimated that worldwide 20% of total cultivated land and 33% of irrigated agricultural land is affected by salinity (Gupta et al., 2020). Saline soils are characterized by an electrical conductivity of the saturation extract (ECe) in the root zone exceeding 4 dSm^{-1} at 25 ºC and more than 15% (w/v) of exchangeable sodium (Quirk, 1971). Higher Na^+ concentration in the root zone leads to higher uptake of Na^+ and lower cellular K^+: Na^+ ratios in plant tissues (Wakeel, 2013). This leads to ionic imbalance in plant cells reducing their water absorption capacity, photosynthesis efficiency and plant growth (Ashraf, 2004). Lower K^+: Na^+ ratios in the cytosol will also disrupt many enzyme activity, protein synthesis, turgor maintenance and stomatal movement (Evelin et al., 2019). Soil salinity causes a significant reduction in P adsorption due to fixation of PO_4^- ions with Ca^{2+}, Mg^{2+} and Zn^{2+} ions in the soil (de Aguilar et al., 1979). Higher Na^+ and Cl^- concentrations in the root zone of saline soil compete and reduce the uptake of NH_4^+ and NO_3^- respectively (Fageria et al., 2011). Moreover, salinity also disrupts nitrogen fixation by reducing nodulation and inhibiting the growth of rhizobia or impairing their ability to infect root hairs (Tu, 1981). Thus, N supply to legumes grown in saline soils is reduced drastically (Fageria et al., 2011). Higher pH in saline soil reduces the solubility of Fe in the soil due to the formation

of insoluble Fe hydroxides and oxides, limiting the bioavailability of Fe for the plants (Kakei et al., 2012) resulting in leaf chlorosis and reduced plant growth (Li et al., 2016). Moreover, Fe deficiency in legumes also decreases nodulation and N_2-fixation because it is an essential component of nitrogenase and leghemoglobin (Evans and Russell, 1971). High Na^+ ions in saline soils reduces the root length, root density and root hair development further decreasing the uptake of essential nutrients (Shabala et al., 2003). Moreover, soil salinity also leads to increased accumulation of reactive oxygen species (ROS) inducing oxidative stress in plants (Zhang et al., 2013b).

Studies suggest that plants under salinity stress can benefit from microbial interactions in the rhizosphere and endosphere, and show enhanced tolerance (Ali et al., 2014a; Hajiboland et al., 2010; Khan et al., 2017). Beneficial plant-microbial interactions (BPMI) were noted to improve ion homeostasis (K^+: Na^+ ratio) (Evelin et al., 2019), induce production of antioxidant enzymes (Zhang et al., 2016a) and modulate root architecture and increase nutrient uptake (Yun et al., 2018). Microbial interactions can increase K^+: Na^+ ratio in plants by increasing K^+ uptake and restrict the transport of Na^+ to leaves by modulating the expression level of Na^+ and K^+ ion channels such as high affinity potassium transporter 1 (HKT1) and the inward rectifying K^+ channels KAT1 and KAT2, which play key roles in regulating Na^+ and K^+ homeostasis (Abdelaziz et al., 2017). Modification of root architecture, root length and root density are also presumed to be influenced by beneficial microbiome interactions, which may be involved in regulating salt acquisition and translocation and also increasing nutrient uptake by plants (Gupta et al., 2020; Jung and McCouch, 2013). These microbial associations were also noted to

reduce oxidative damage in plant cellular components due to the production of ROS during salt stress by producing various antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD), glutathione reductase (GR) and dehydroascorbate reductases (DHAR) (Ruiz-Lozano et al., 2012).

Use of gypsum has been suggested in many studies to reclaim saline soils due to its ability to exchange Na^+ ions with Ca^{2+} ions reducing the toxic concentration of Na^+ ions in the soil (Amezketa et al., 2005; Mahmoodabadi et al., 2013). However, additions of gypsum to the soil may interfere with nutrient (K and Mg) availability for plants, and consequently, nutrient absorption and plant growth (Favaretto et al., 2008). Manipulation of soil conditions by application of suitable soil amendments such as compost is an alternative to gypsum and also has potential to improve BPMI. Application of compost amendments in saline soils was shown to impact microbial diversity and composition by increasing the availability of nutrients through mineralization, increasing K^+: Na^+ ratio through leaching of Na^+ ions, and influencing enzymatic activities (Lakhdar et al., 2009; Shen et al., 1997).

Higher concentrations of soluble salts in saline soils also adversely impact BPMI (Jahromi et al., 2008) and it appears that tolerance to such conditions is influenced by taxonomic variations in the associated microbes. For instance, certain bacterial and fungal genera were dominant in saline conditions and promoted growth and salt resistance of the host plant (Arora et al., 2012; Estrada et al., 2013). Several *Bacillus*, *Enterobacter*, and *Streptomyces* have been observed to promote plant growth under highly saline soil conditions (Jiang et al., 2019). The most dominant plant growth promoting fungi in saline

soils include *Penicillium*, *Paecilomyces* and *Trichoderma* (Bronicka et al., 2007). Use of stimulants that can increase interactions with saline tolerant plant beneficial microbes could be an effective approach to improve plant growth and productivity in salt-affected soils (Colla et al., 2017; Quiza et al., 2015). Some of these stimulants include strigolactones (SL) (Aroca et al., 2013), salicylic acid (SA) (Lebeis et al., 2015) and coumarins (COU) (Stringlis et al., 2019).

Strigolactones (SLs) are signaling compounds that are known to promote and establish symbiosis between plant and beneficial microbes such as AMF (Aroca et al., 2013) and rhizobia (McAdam et al., 2017) under nutrient deficient conditions. . It was noted that SLs induced interactions between plants and AMF and stimulated AMF hyphal branching and spore germination (Aroca et al., 2013). Recently several studies also reported that SLs promoted *Rhizobium*-legume symbiosis (McAdam et al., 2017; Peláez-Vico et al., 2016), by promoting infection thread formation by rhizobia in legume roots and thus increasing the number of nodules (McAdam et al., 2017; Peláez-Vico et al., 2016). However, impact of exogenously applied SLs on microbiome interactions with a legume plants exposed to saline conditions is not clearly understood. In a recent study by Carvalhais et al. (2019), it was shown that SL-producing plants had more pronounced effect on the fungal diversity than bacterial diversity. However, this study only looked at the rhizosphere microbial community but not the other plant-associated microbiome. Therefore, a comprehensive analysis of SLs impacts on both rhizosphere and endophytic microbiome will provide more insights and potential for practical application of SL analogues in salt affected soils.

Salicylic acid (SA) is a phytohormone well known to protect plants from soil salinity mainly by inducing the production of antioxidant enzymes (Pandey et al., 2013a). It was noted that SA modulated plant microbiome under stressed conditions by serving as a key regulator of plant immune system (Lebeis et al., 2015). In this study, it was reported that the endophytic microbiome of SA-treated *Arabidopsis* plants was enriched in beneficial stress-tolerant and non-pathogenic community (Lebeis et al., 2015). The impact of SA on other BPMI such as those of plant roots with AMF and/or rhizobia are not clearly understood. Treatment of plants with SA have shown to either increase (Ansari et al., 2016) or decrease (Medina et al., 2003) or have no effect (Ludwig-Müller et al., 2002) on AMF colonization. Impact of SA on nodulation and plant-rhizobia symbiosis affected by salinity stress is also not clearly understood (Akhtar et al., 2013). Ione study noted increased number of nodules in SA treated plants, which was attributed to the protection of root nodules by antioxidant enzymes induced by SA application under saline conditions (Palma et al. (2013).

Coumarins (COU) are secondary metabolites produced by both plants and some microbes, and act as a signaling molecule to increase nutrient uptake under nutrient (mainly Fe and P) deficiency conditions (Clemens and Weber, 2016). One study noted that COU exudates inhibited plant pathogens by their selective antimicrobial action in soil (Stringlis et al., 2018). It was also noted that COU modulates plant responses to Fe and P deficiency prevalent under saline conditions and the interaction between plant roots and beneficial microbes in the rhizosphere and roots (Niro et al., 2016; Stringlis et al., 2018). Another study noted that COU increased AMF colonization by acting as a signaling

molecule under P starvation conditions (Wang et al., 2018c). They were also shown to induce antioxidant enzymes and thus reduce the oxidative stress in plants under abiotic stressed conditions (Qin et al., 2019; Saleh and Madany, 2015). However, it is not clear whether these signaling compounds influence beneficial microflora in saline soils.

It was hypothesized that use of soil amendments and stimulants as foliar sprays on a legume crop grown in saline soil would positively impact nodulation, AMF colonization, diversity and composition of beneficial rhizosphere and endophytic microbiome. Improved beneficial interactions were anticipated to improve plant nutrient uptake, improved salinity tolerance (K^+: Na^+ ratio) and yield. Objectives of the study were 1) to study the impact of salt stress on cowpea rhizosphere and endophyte microbiome composition, AMF and rhizobia interactions and 2) to evaluate compost application to soil and foliar application of SA, SL and COU for their impacts on BPMI and cowpea growth and yield grown in saline soil.

3.3 Materials and methods

3.3.1 Soil, plant materials and chemicals

Surface soil was collected near Texas A&M AgriLife Research and Extension Center at Pecos in Reeves County, Texas (31.4229° N, 103.4932° W). Majority of soils found in this region are saline and moderately alkaline. The soil sample used for this study was a Dalby clay soil series and classified as Fine, smectic, frigid Oxyaquic Vertic Hapludalfs. Soil texture, pH, ECe, NO_3 and available P (Mehlich-3) were reported by the Soil, Water and Forage Testing Laboratory Department of Soil and Crop Sciences, Texas A&M University (Table 3.1).

Table 3.1 Characteristics of the native saline soil used in the experiment.

Parameter	Value
pH	8.5
ECe	6.33
Texture	Clay
P(Mehlich-3)	39 mg/kg
NO_3	24 mg/kg
K	506 mg/kg

Texas Cream 40 variety of cowpea (*Vigna unguiculata* (L.) Walp.) was used as the plant host and seeds were obtained from Texas A&M AgriLife Research and Extension Center at Overton, Texas. Small plant containers (KBW Supply, Tyler, TX; 22.5 cm diameter, 22 cm length, 7.5 L volume) were used to grow the plants.

3.3.2 Experimental design and growth conditions

The experiment had a completely randomized design consisting of 6 treatments and 3 controls with 3 replicates to a total of 27 samples. The treatments details are provided in Table 3.2. Compost from cow manure was used as an organic amendment and was collected from Texas A&M AgriLife research and extension center at Overton (0.5% N, 0.5% P, 0.5% K, pH ~ 7.0) and was applied in soil @5% wt./wt. Three stimulants/signaling compounds used in the experiment were 5 µM synthetic SL analog GR24 (ChemPep, Inc. Wellington, FL) (stock solution of 3.3 mM SL was prepared by mixing 2 mg of SL GR24

in 2 mL of acetone and further diluted to 5 µM by adding 1.5 mL of the stock solution to 1 L of Millipore water), 0.1mM SA (Sigma Aldrich, St. Luis, MO, USA) (0.07g of SA was dissolved in 1 L of Millipore water) and 50 ppm coumarin (2H-chromen-2-one; COU) dissolved in 0.1% ethanol in Millipore water. Stimulants were applied as foliar spray at every 3 days after seed emergence. Endophytic mycorrhizal inoculum MycoApply® Soluble Maxx (Mycorrhizal Applications, Grants Pass, OR) was incorporated in soil at 3g/pot (200 propagules/g of soil).

Seeds were inoculated with *Bradyrhizobium* sp. (Vigna) (Exceed superior legume inoculant, Visjon Biologics, Wichita Falls, TX) a day before sowing. The plants were grown for 6 weeks for sampling in first time point and 9 weeks for sampling in second time point in a greenhouse at Texas A&M AgriLife Research and Extension Center, Overton, Texas and watered daily to 70% water holding capacity (determined based on maximum water holding capacity using saturating method). The plants were irrigated two times during the entire growing season with a half strength modified Hoagland nutrient solution, the composition of which is detailed in Table 3.3.

3.3.3 Sampling, root scanning, nodulation and root AMF colonization

Sampling of rhizosphere, roots, shoots and leaves were done at two distinct plant developmental stages (time points). First sampling time corresponded with flowering stage or 6 weeks after seed germination (6 WAG) and second sampling time corresponded with pod maturity stage or 9 weeks after seed germination (9 WAG) of the plant. At each sampling time, the pots were destructively sampled for roots, rhizosphere soil and leaves, processed accordingly for different analysis and stored at -80 °C until analysis.

Table 3.2 Name and details of each treatment used in the experiment.

Treatment number	Name	Details
T1	CMP	Matured compost mixed with saline soil
T2	SL	Strigolactones treatment for plants grown in saline soil
T3	SA	Salicylic acid treatment for plants grown in saline soil
T4	SL+SA	Strigolactone + Salicylic acid treatment for plants grown in saline soil
T5	COU	Coumarin treatment for plants grown in saline soil
T6	COU+SL	Coumarin + Strigolactone treatment for plants grown in saline soil
T7	CS	Saline soil, control, unamended
T8	GYP	Gypsum amended saline soil
T9	GYP+MYCO	Gypsum amended saline soil inoculated with endophytic mycorrhiza

Table 3.3 Composition of modified Hoagland nutrient solution used in the experiment.

Compounds	Concentration of stock solution (mM)	Volume of stock solution (ml) per liter of final solution	Volume of final solution added per pot (ml)
KH_2PO_4	1000	2.0	1.0
$MgSO_4.7H_2O$	2000	1.0	0.5
K_2SO_4	2000	1.25	0.625
$CaCl_2.H_2O$	1000	1.25	0.62
H_3BO_3	6.25	2.0	1.0
$MnSO_4.H_2O$	2.5		
$CuSO_4.5H_2O$	0.2		
$ZnCl_2$	0.1		
Ammonium Molybdate	0.05		
FeNaEDTA	64	1.0	0.5

The shoots were harvested, weighed and then a representative set of leaves were separated for DNA extaction and nutrient analysis, and stored at -80 °C until analysis. Entire soil media (after removal of soil and roots attached on the edges of pot) wihtin the pots was composited in a ziplock bags before sampling. Aproximately 5g rhizosphere soil (soil in contact with the roots and collected by gently shaking the roots) and a set of root fragments of approximately 500 mg were collected for DNA extraction and stored at -80 °C after washing with tap water followed by first rinsing with 0.6% bleach (to remove the epiphytic microflora) and second rinsing with molecular-grade water. Rhizosphere soil was stored separately for pH and nutrient analysis. Remaining soil in the ziplock bags was washed to retain only the roots. These roots were then blotted, weighed, counted for number of nodules and then stored at -20°C for estimation of AMF colonization percentage, root biomass and root length density. For estimation of dry biomass, roots and shoots (obtained from harvest) were dried at 65 °C in a forced-air oven for 48 h, and weighed.

3.3.4 Root scanning

Roots frozen and stored at -20 °C for 5-7 days were first scanned for root length and root density quantification. The whole root system was spread into a plastic transparent tray filled with 3 mm of water so that individual roots and neighbor lateral roots did not overlap and stick. The roots were imaged on a scanner and their length estimated. Root length density (RLD) was estimated as a ratio of root length (cm) to the volume of soil used in the experiment (cm^3). After scanning, these roots were stored at -20 °C again to determine the percentage of AMF colonization.

3.3.5 Estimation of percentage of root AMF colonization

Approximately a gram of root stored at -20 °C (after scanning) was used to estimate percentage of root colonization by AMF. Roots were gently removed from soil and washed under tap water, and then stained with trypan blue following a modified procedure (Phillips and Hayman, 1970). Roots were placed in tissue cassettes and submerged in pre-boiled 10 % KOH for 10 min to remove host cytoplasm and nuclei. Cassettes were then washed 5X with tap water and submerged in 2 % HCl for 30 min, followed by 5X washing with tap water. The cassettes were then submerged in 0.05 % trypan blue solution (water, glycerin, lactic acid in 1:1:1 (v/v/v)) at 90ºC for 5 min. The cassettes were then washed 5X with tap water and stored at 4 °C for 7 days immersed in distilled water to remove excess stain. The percentage of AMF colonization was then determined using the gridline intersection method (Giovannetti and Mosse, 1980).

3.3.6 Estimation of pH and nutrient concentration of rhizosphere soil

Change in soil pH was determined using the method by Schofield and Taylor (1955). The pH was determined in a 1:2 ratio of soil to water extract of the soil using deionized water. Samples were stirred and allowed to equilibrate for a minimum of 30 minutes after adding the water. The actual determination was made using a hydrogen selective electrode and pH values were reported on a dry soil basis only.

For nutrient analysis of soil, a slightly modified method of Haney et al. (2006) was used. Soil extractant H3A was used to extract nitrate, ammonium, phosphate, potassium, calcium and magnesium from soil. The extractant was prepared by dissolving in one liter

of water: Lithium citrate (5.0 g); citric acid (0.5 g); malic acid (0.5 g); oxalic acid (0.5 g); EDTA (0.25 g) and DTPA (0.25 g). Soils obtained from each treatment were weighed (4.0 g) separately in 50 mL centrifuge tubes and extracted with 40 mL of H3A. Soil samples were shaken for 30 minutes and centrifuged at 3000 rpm for 8 minutes and then filtered through Whatman 2V pleated filter paper in 2 mL vials. Nutrients were then quantified by Ion Chromatography (Thermo Electron North America LLC, Madison, WI, USA).

3.3.7 Estimation of leaf tissue elemental concentrations

Dried leaf samples (at 65^0C in a forced-air oven for 48 h) were crushed and weighed (0.5-1.0 g) into a 50 mL Taylor tube and extracted with conc. nitric acid overnight and then analyzed for nutrient ions (P, K, Ca, Mg, Fe and Na) using Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) method (Havlin and Soltanpour, 1980). Total N in leaves were measured separately using dry combustion C/N analyzer (Elementar Inc.).

3.3.8 Extraction of DNA from rhizosphere and plant tissues

Soil DNA was extracted from 0.5 g of rhizosphere soil (was preciously stored at-80 °C) using DNeasy Power Soil Pro DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) and DNA from plant tissues (root and leaf) were extracted using Power plant kit (Qiagen Inc.) following the manufacturer's instructions. Quality and quantity of DNA was determined using a spectrophotometer (SimpliNano, GE Healthcare LifeSciences, Inc.).

3.3.9 Estimation of abundance of bacteria, fungi, AMF and NFB in rhizosphere and plant tissues

Quantitative real-time PCR (qPCR) was used to quantify the abundance of total bacteria by targeting the16S rRNA gene, total AMF by targeting the AMF specific 18S rRNA gene, total fungi by targeting the internal transcribed spacer (ITS) and N_2-fixing bacteria (NFB) by targeting the *nifH* gene targets in both rhizosphere and plant tissues. For quality control, all qPCR runs included 5 different concentrations of DNA standards (gBlock standards, Integrated DNA Technologies Inc.) for each target gene to develop standard curve. Details on these standards are provided previously in Table 2.5. No-template control (NTC), positive control, negative control, and 2 spiked random samples from the study's DNA samples with one of the standards to test for possible qPCR inhibitors were included in each qPCR run. Standards and NTC were run in triplicate, and rest of samples were run in duplicates. Details on controls, standard curve R^2 value and reaction efficiency are listed in Table 3.4. Primers were obtained from Integrated DNA Technologies Inc. and are outlined in Table 3.5. Amplifications of DNA was performed using RotorGene SYBR® Green qPCR kit, with gene abundance measured using RotorGene Q Software version 2.3.1.49 (QIAGEN, Hilden, Germany).

3.3.10 Estimation of rhizosphere and endophytic microbial diversity and composition

Microbial DNA from soil, roots and leaves was sequenced in the V4 region of 16S rRNA gene marker amplified by primers 515F- 5' GTGYCAGCMGCCGCGGTAA-3'

(Parada et al., 2016) and 806R- 5'-GGACTACNVGGGTWTCTAAT-3' (Apprill et al., 2015) and the ITS marker with primers ITS1F- 5'CTTGGTCATTTAGAGGAAGTAA-3' (Gardes and Bruns, 1993) and ITS2R- 5'-GCTGCGTTCTTCATCGATGC-3' (White et al., 1990). DNA libraries were prepared as described in the Swift amplicon 16S+ITS panel library preparation protocol and were on Illumina Miseq instrument for paired-end sequencing following manufacturer's protocol (Illumina, San Diego, CA) by Experimental Genomics Core facility at Texas A&M university, College Station, TX. The raw sequence reads obtained from Illumina Miseq were processed to remove adapters, primer sequences and short (< 100bp) and low quality reads (< Phred-33 of 20) using Trimmomatic software (Bolger et al., 2014). These paired ends were assembled using Qiime1.9.1 (Caporaso et al., 2010) scripts and USEARCH 8.0.1 (Edgar, 2010) software was then used to remove chimeric sequences. Each ITS sequence tags were compared to the UNITE ITS sequence database (Abarenkov et al., 2010) and 16S rRNA sequences were compared to the Greengenes database (Release 13.5) (DeSantis et al., 2006) using UCLUST (Edgar, 2010) in order to pick referenced-based (prokaryotes) or open-reference (fungi) operational taxonomic units (OTUs) at 97% similarity, and then were recorded assignments for each OTU. The OTU abundance dataset was further normalized using cumulative sum scaling (CSS) transformation (Paulson et al., 2013) available on the QIIME platform. Samples with less than 1000 sequences were discarded.

Table 3.4. Quality control details of the qPCR runs in the experiment.

Target microbial gene	**Positive control**	**Negative control**	**R^2 value of standard curve for rhizosphere**	**Reaction efficiency for rhizosphere**	**R^2 value of standard curve for plant endosphere**	**Reaction Efficiency for plant endosphere**
16S rRNA	*Escherichia coli* K-12	*Methanospirillum hungatei*	0.99	0.91	0.99	1.01
AMF 18S rRNA	*Glomus intraradices*	*Escherichia coli* K-12	0.99	1.01	0.98	0.95
ITS	*Rhizopus microsporus*	*Escherichia coli* K-12	0.98	1.00	0.99	0.98
nifH	*Rhizobium leguminosarum*	*Rhizopus microsporus*	0.99	0.94	0.98	1.04

Table 3.5. Details of primers and PCR conditions used for the qPCR assays in the experiment.

Target microbial group	Primers and sequences	qPCR reaction mixture	Thermal profile	Reference
Total bacteria (16S rRNA)	341f-(5'-CCTACGGGAGGCAG CAG-3')/ 797r-(5'-GGACTACCAGGGTA TCTAATCCTGTT-3')	7.5 µl SYBR Green (2x) Master Mix, 0.225 µl F primer (0.3 µM), 0.675 µl R primer (0.9 µM), 2 µl DNA template, 4.6 nuclease free H_2O.	3 min at 98°C for initial denaturation; 40 cycles of 30 s at 98°C, 30 s at 61.5°C, extension for 20 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 50-99°C (1° and 5 s/cycle melt) after a pre-melt conditioning for 90 s at 50°C.	Modified after (Harter et al., 2014)
Total AMF (18S rRNA)	GC-AMV4.5NF- (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G [GC clamp] AAG CTC GTA GTT GAA TTT CG-3′)/ AMDGR-(5′-CCC AAC TAT CCC TAT TAA TCA T-3′)	7.5 µl SYBR Green (2x) Master Mix, 1.5 µl each primer (5 µM), 2 µl DNA template, 2.5 nuclease free H_2O	10 min at 98°C for initial denaturation; 35 cycles of 30 s at 98°C, 30 s at 55°C, extension for 45 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 50- 98°C (1° and 5 s/cycle melt).	Modified after (Sato et al., 2005)

Table 3.5. Continued.

Total fungi (ITS)	ITS1f-(5'-TCC GTA GGT GAA CCT GCG G3')/5.8s-(5'-CGC TGC GTT CTT CAT CG-3')	7.5 µl SYBR Green (2x) Master Mix, 1.5 µl each primer (5 µM), 2 µl DNA template, 2.5 nuclease free H_2O.	10 min at 98°C for initial denaturation; 35 cycles of 60 s at 98°C, 30 s at 53°C, extension for 45 s at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 48- 98°C (1° and 5 s/cycle melt)	Modified after (Fierer et al., 2005)
Total *nifH*- harboring bacteria	PolF-(5'-TGC GAY CCS AAR GCB GAC TC3')/PolR-(5'-ATS GCC ATC ATY TCR CCG GA3') where Y = C/T; S = G/C; R = A/G; B = C/G/T	7.5 µl SYBR Green (2x) Master Mix, 0.225 µl F primer (0.3 µM), 0.675 µl R primer (0.9 µM), 2 µl DNA template, 4.6 nuclease free H_2O.	10 min at 98°C for initial denaturation; 35 cycles of 1 min at 98°C, 1 min at 55°C, extension for 1 min at 72°C, and acquisition for 10 s at 82°C. Melt curve produced at 50- 98°C (1° and 5 s/cycle melt).	Modified after (Poly et al., 2001)

3.3.11 Data analysis

Differences among treatments for change in soil pH, shoot biomass, root biomass, nutrient concentrations (NO_3, PO_4, K, Ca and Mg), N in leaves, nodulation and % AMF colonization were statistically analyzed using ANOVA in SAS software (SAS Inc.), using PROC GLM procedure. Statistical mean differences between the treatments were based on using Fisher's least-significant-difference (LSD) test at a *p*-value of <0.05. Pearson's correlation coefficient was determined for pairwise comparison between leaf nutrient concentration, nodulation, AMF colonization and pod yield and correlation plot was created using "corrplot" package(Wei et al., 2017) in R. Calculations of alpha-diversity (Shannon) and observed species richness and estimated richness (Chao1) were done using QIIME. Principal Coordinate Analysis (PCoA) was performed to visualize the effect of treatments on microbial community composition. Two-way non-parametric multivariate analysis of variance (PERMANOVA) was used to test the significant differences in rhizosphere and endophytic microbial community composition between the experimental treatments using the Phyloseq package (McMurdie and Holmes, 2013) on R version 3.6.1 based on a Bray-Curtis distance measure between the groups. Linear discriminant analysis effect size (LEfSe) was performed to identify significant differences in bacterial and fungal taxa between treatments and controls. The Kruskal-Wallis (KW) sum-rank test is used in LEfSe analysis to detect the features with significantly different abundances between assigned classes, and then linear discriminant analysis (LDA) is performed to estimate the effect size of each differentially abundant taxon (Segata et al., 2011). Significant taxa were used to generate taxonomic cladograms illustrating differences

between sample classes on the website http://huttenhower.sph.harvard.edu/galaxy. Mantel tests were used to calculate the correlations between variations in microbial composition (based on Bray-Curtis distances) and different soil and plant growth parameters using vegan package in R (Dixon, 2003). Pearson correlation coefficients were used to test for the correlations between dissimilarity matrices using 9999 permutations. Bray-Curtis dissimilarities were used for microbial community while Euclidean distance dissimilarities were used for soil and plant growth parameters.

3.4 Results

3.4.1 Impacts of experimental treatments on pH and nutrient ion concentrations in the rhizosphere soil.

No significant change in rhizosphere soil pH was observed in the CS treatment (native saline soil only) during the plant growing season with the pH remaining around 8.5 (Figure 3.1). Among all the experimental treatments, soil pH decreased significantly ($p < 0.05$) to <7.5 in GYP and GYP+MYCO treatments, compared to CS treatment. Use of 5% CMP did not change the pH significantly at both time points compared to CS treatment. Foliar spray treatments of SL when applied alone (SL) or in combination with COU (COU+SL) or SA (SL+SA) decreased soil pH significantly at 9 WAG. Whereas, COU and SA treatments did not significantly alter the pH.

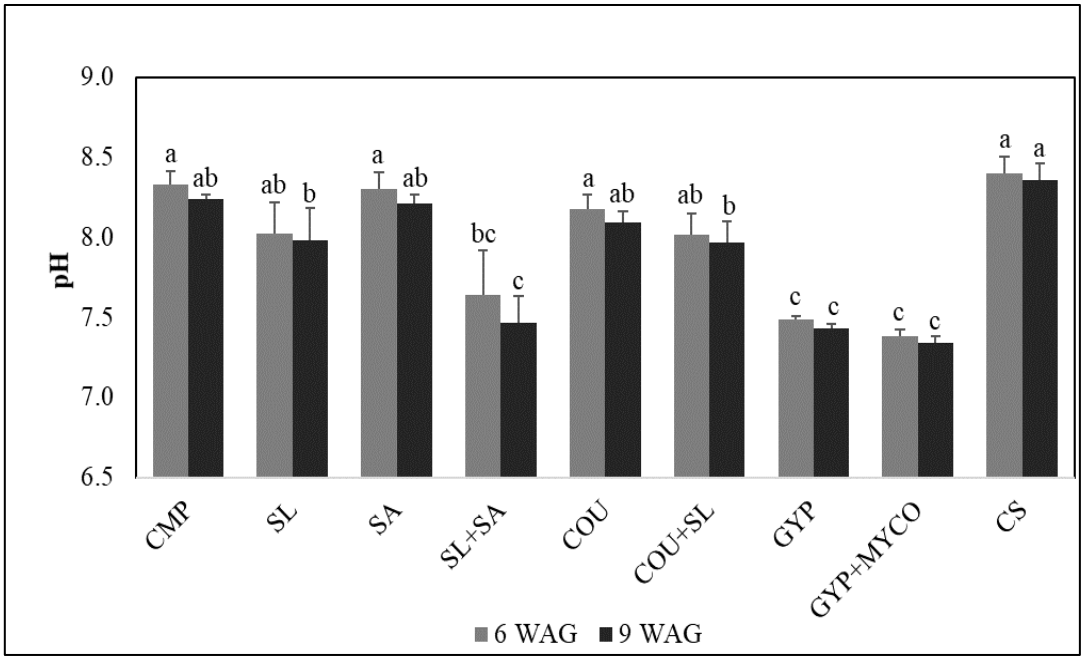

Figure 3.1 Rhizosphere pH in the experimental treatments measured at 6 and 9 WAG.

Note: data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

Addition of gypsum (GYP and GYP+MYCO treatments) significantly increased ($p < 0.05$) Ca compared to CS (Table 3.6). Compost (CMP) treatment had a significant increase in P compared to the CS treatment but did not change the concentration of any other nutrient significantly. Among stimulants, foliar application of COU, SL alone and SL in combination with SA (SL+SA) significantly increased the concentration of NO_3 and P in the soil as compared to CS treatment. SA treatment significantly increased the NO_3 concentration while also significantly decreased the concentration of K in the soil. No impact of combined COU and SL (COU+SL) was observed on the concentration of any of the nutrient ions in the soil.

Table 3.6. Rhizosphere nutrient concentration in the experimental treatments measured at 6 and 9 WAG.

Time	Treatment	NO_3 (mg/kg)	P (mg/kg)	K (mg/kg)	Ca (mg/kg)
6 WAG	CMP	24.55 ± 2.98bc	55.21 ± 3.46a	681.25 ± 33.87a	19.94 ± 5.63c
	SL	44 .24±2.25ab	57.79 ± 0.53a	705.54 ± 51.53a	88.07 ± 7.60c
	SA	43.58 ± 6.15ab	50.37 ± 6.91abc	423.25 ± 63.97b	32.22 ± 5.90c
	SL+SA	48.54 ± 1.54a	53.51 ± 2.83ab	751.54 ± 14.17a	48.97 ± 7.08c
	COU	46.98 ± 1.99ab	54.12 ± 3.47ab	737.89 ± 41.87a	51.45 ± 11.70c
	COU+SL	27.54 ± 2.43abc	51.43 ± 5.05abc	755.45 ± 60.10a	111.97 ± 27.29c
	GYP	26.89 ± 3.62abc	50.73 ± 1.73abc	732.78 ± 30.10a	449.96 ± 4.64b
	GYP+MYCO	28.54 ± 4.35abc	47.36 ± 4.83bc	737.05± 42.07a	1001.92 ± 90.06a
	CS	19.97 ± 4.63c	45.61 ± 1.31c	722.07 ± 13.98a	34.05 ± 5.16c
9 WAG	CMP	29.12 ± 3.12bc	63.29 ± 1.83ab	716.47 ± 54.41a	29.82 ± 4.91e
	SL	47.51 ± 1.71a	66.11 ± 2.56a	749.69 ± 17.52a	93.70 ± 7.42c
	SA	46.79 ± 5.51a	57.20 ± 5.48bcd	493.99 ± 254.08b	30.93 ± 8.10e
	SL+SA	52.34 ± 3.02a	67.50 ± 0.79a	774.56 ± 10.90a	78.59 ± 1.83cd
	COU	46.42 ± 1.22ab	58.82 ± 3.91bc	762.47 ± 15.92a	48.36 ± 20.81de
	COU+SL	28.52 ± 1.85bc	55.46 ± 5.05cd	748.95 ± 22.62a	106.84 ± 39.91c
	GYP	29.05 ± 15.96bc	54.23 ± 3.30cd	751.06 ± 12.57a	432.36 ± 37.16b
	GYP+MYCO	30.82 ± 4.06bc	52.60 ± 5.44cd	760.02 ± 24.70a	959.89 ± 38.41a
	CS	21.92 ± 5.64c	51.28 ± 1.28d	737.69 ± 6.94a	29.48 ± 3.51e

Note: data presented are the means for 3 replicates with standard error. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points

3.4.2 Impact of experimental treatments on root biomass and root length density

Total root biomass (dry matter) was quanitfied per pot (5 kg of soil) and ranged between 2.39 g in CMP treatment to a low of 0.42 g in the CS treatment (Table 3.7). Root biomass was highest in the CMP treatment at both time points. Treatments of SL and SA and their combination (SL+SA) also significantly increased the ($p < 0.05$) root biomass compared to CS, but was lower than CMP. Other treatments slightly increased the root biomass compared to CS but changes were not statistically significant.

Table 3.7. Root dry matter of the experimental treatments measured at 6 and 9 WAG.

Treatment	Root dry matter per pot (g)	
	6 WAG	9 WAG
CMP	2.01 ± 0.41a	2.39 ± 0.52a
SL	1.28 ± 0.51ab	1.82 ± 0.34abc
SA	1.17 ± 0.34bc	1.73 ± 0.38abc
SL+SA	1.47 ± 0.28ab	2.07 ± 0.40ab
COU	0.68 ± 0.45bc	1.15 ± 0.40cd
COU+SL	1.11 ± 0.21bc	1.52 ± 0.17bcd
GYP	1.03 ± 0.61bc	1.56 ± 0.51bcd
GYP+MYCO	1.09 ± 0.59bc	1.50 ± 0.48bcd
CS	0.42 ± 0.10c	0.95 ± 0.17d

Note: data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

Root length density (RLD) measured as cm of root per cm^3 of soil, was found to be significantly increased ($p < 0.05$) in GYP treatment than CS indicating adverse effect of salt stress on root system architechture (Table 3.8). Treatment CMP significantly improved the RLD in plants at 6 weeks. Foliar application of SL and SA alone or in

combination (SL+SA) also increased the RLD significantly while application of COU or COU+SL did not have any significant effect on plants of either of the time points.

Table 3.8. Root length density (RLD) of the experimental treatments at 6 and 9 WAG.

Treatment	Root length density (RLD) (cm of root per cm^3 of soil)	
	6 WAG	9 WAG
CMP	1.19 ± 0.14a	1.11 ± 0.17ab
SL	1.00 ± 0.21ab	1.16 ± 0.38ab
SA	0.94 ± 0.09ab	1.41 ± 0.12a
SL+SA	1.20 ± 0.09a	1.41 ± 0.19a
COU	0.55 ± 0.24bc	1.18 ± 0.19b
COU+SL	1.14 ± 0.26bc	1.20 ± 0.27ab
GYP	0.91 ± 0.39ab	1.47 ± 0.05a
GYP+MYCO	0.85 ± 0.39ab	1.16 ± 0.21b
CS	0.30 ± 0.05c	0.89 ± 0.34b

Note: data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

3.4.3 Impact of experimental treatments on nutrient concentrations in the plant leaf tissue

Results obtained from dry combustion of dried leaf samples for N showed that CMP treatment and foliar spray of combined SL and SA (SL+SA) significantly increased ($p < 0.05$) the N content in leaf tissues as compared to CS while other treatments did not have a sigfnicant impact (Table 3.9). Total concentrations for other nutrient elements were obtained from IC-P analysis of acid-digested leaf tissues. Results showed that CMP, SL and SL+SA treatments significantly increased ($p < 0.05$) total P, total K, total Ca, total Mg

Table 3.9. Leaf nutrient concentration in the experimental treatments measured at 6 WAG.

Treatment	N (mg/plant)	P (mg/plant)	K (mg/plant)	Ca (mg/plant)	Mg (mg/plant)	Fe (mg/plant)
CMP	113.49 ± 19.51ab	17.00 ± 3.14ab	391.95 ± 50.96a	349.79 ± 46.85ab	63.45 ± 13.37a	0.69 ± 0.07a
SL	108.73 ± 11.61abc	17.81 ± 3.16a	367.34 ± 54.19a	348.07 ± 15.38ab	59.63 ± 6.86ab	0.67 ± 0.19a
SA	66.73 ± 13.18c	10.51 ± 5.35abc	339.61 ± 33.55ab	150.85 ± 37.04bc	38.66 ± 6.68abc	0.38 ± 0.13c
SL+SA	138.65 ± 34.73a	16.88 ± 5.64ab	371.41 ± 54.70a	355.60 ± 48.19a	56.54 ± 15.50ab	0.64 ± 0.31ab
COU	78.64 ± 30.53bc	10.94 ± 4.54abc	356.93 ± 60.07ab	175.09 ± 44.44abc	41.49 ± 9.53abc	0.41 ± 0.17bc
COU+SL	83.37 ± 18.75bc	9.71 ± 3.43bc	369.58 ± 58.30ab	158.10 ± 38.37abc	40.66 ± 10.79abc	0.31 ± 0.07c
GYP	94.83 ± 16.49abc	11.30 ± 3.56abc	367.35 ± 39.93ab	309.33 ± 40.04ab	48.38 ± 11.03abc	0.30 ± 0.07c
GYP+MYCO	77.95 ± 16.30bc	10.37 ± 5.53abc	333.77 ± 38.83ab	147.69 ± 18.80bc	34.73 ± 6.71bc	0.31 ± 0.03c
CS	65.41 ± 15.15c	7.13 ± 0.64c	135.05 ± 31.36b	105.57 ± 30.93c	33.54 ± 5.48c	0.36 ± 0.33c

Note: data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

and total Fe concentrations in leaf tissues. GYP treatment significantly increased total Ca in the leaf tissues.

3.4.4 Impact of experimental treatments on K^+: Na^+ ratio in leaf tissues

Leaf K^+:Na^+ ratio was obtained to determine the effect of soil salinity in leaf tissues and was evaluated based on total elemental concentration obtained from ICP analysis (Figure 3.2). ratio. Among treatments, only CMP and SL+SA had a significant effect on K^+:Na^+ and increased the ratio by more than 90% in the leaf tissues compared to CS treatment.

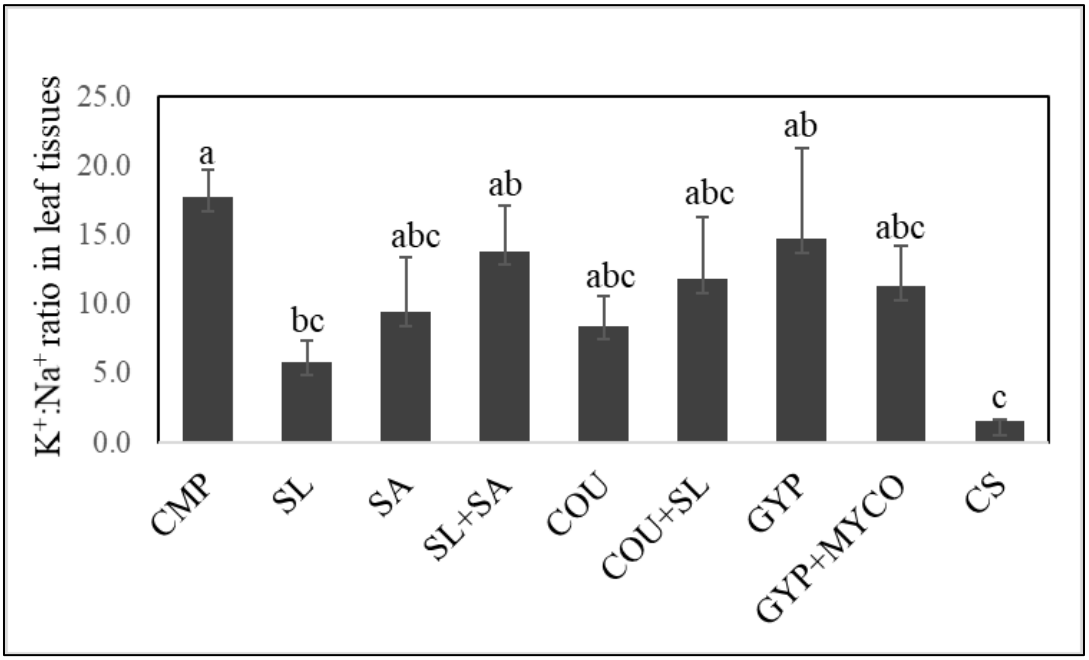

Figure 3.2. Leaf K^+/Na^+ in the experimental treatments.
Note: data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

3.4.5 Impact of experimental treatments on nodulation and percentage of root AMF colonization

Total number of nodules per pot were lowest in CS treatment at around <10 at both time points (Table 3.10). Treatments of CMP, SL, SA and SL+SA significantly increased the ($p < 0.05$) the nodule numbers. Highest nodules were recorded in CMP treatment at 6 weeks at 32/plant and in SL+SA treatment at 36 nodules/plant. No significant changes were noted in other treatments compared to CS treatment.

Percentage of root colonization by AMF significantly increased ($p < 0.05$) in GYP treatment compared to CS treatment (Figure 3.3). Treatments of GYP+MYCO and COU showed no significacnt difference in percentage of root colonized by AMF. Remaining experimental treatments significantly increased ($P < 0.05$) AMF colonization. Highest root AMF colonization was observed in SL+SA (up to 82%) followed by SL (up to 78%).

Table 3.10. Total number of nodules in the experimental treatments measured at 6 and 9 WAG.

Treatment	Total number of nodules per plant	
	6 WAG	9WAG
CMP	32 ± 1.15a	34 ± 1.15a
SL	29 ± 2.08a	35 ± 1.00a
SA	23 ± 3.61b	26 ± 1.53b
SL+SA	29 ± 2.52a	36 ± 1.53a
COU	13 ± 3.00c	17 ± 1.53c
COU+SL	14 ± 5.13c	18 ± 5.86c
GYP	19 ± 2.65c	23 ± 1.53c
GYP+MYCO	2 ± 0.58e	14 ± 2.08e
CS	8 ± 1.73d	9 ± 1.15d

Note: data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

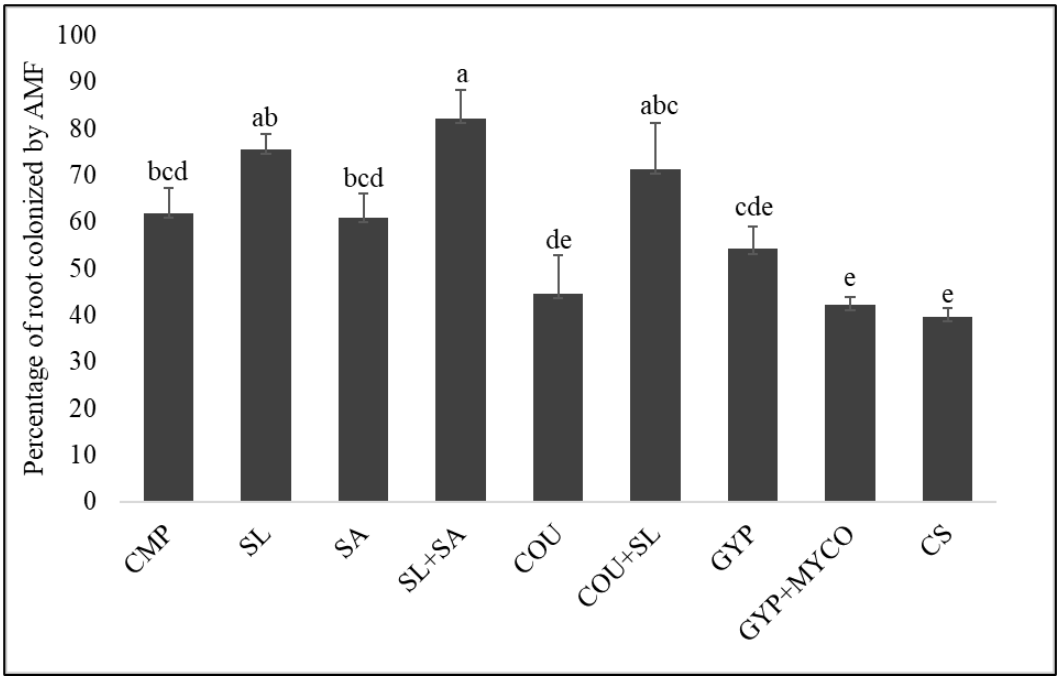

Figure 3.3. Percentage of root colonized by AMF in the experimental treatments. Note: data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

3.4.6 Impact of experimental treatments on pod yield

Pod yield (g) of cowpea was lowest (0.3 g) in plants grown under natural saline conditions (CS) indicating a negative impact of salt stress on plant productivity (Figure 3.4). Gypsum treatment (GYP) did not increase the yield significantly. Treatments of CMP, SL, SA and SL+SA significantly increased ($p < 0.05$) pod yield than CS treatment and highest pod yield per plant (4.3 g) was noted in SL+SA treatment.

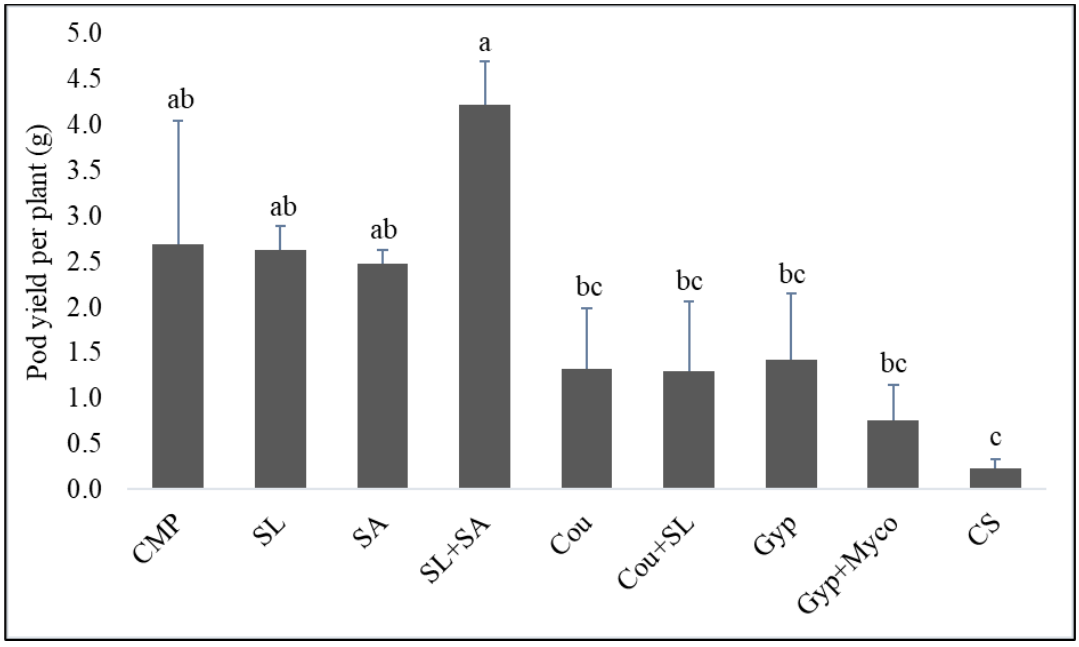

Figure 3.4. Pod yield (g) per plant in the experimental treatments.
Note: data presented are the means of 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

The correlation between different plant parameters and yield showed that yield was most significantly ($p < 0.05$) correlated (positively) to nodulation ($r = 0.91$) followed by P content in shoot tissues ($r = 0.83$) and AMF colonization percentage ($r = 0.82$) (Figure 3.5).

3.4.7 Impact of experimental treatments on relative abundance of bacteria, fungi, AMF and NFB in rhizosphere, roots and leaves

Abundance of prokaryotes (16S rRNA), fungi (ITS), AMF (AMF specific 18S) and NFB were quantified in the rhizosphere, root and leaf samples by qPCR assays and results are presented in Table 3.11-3.13.

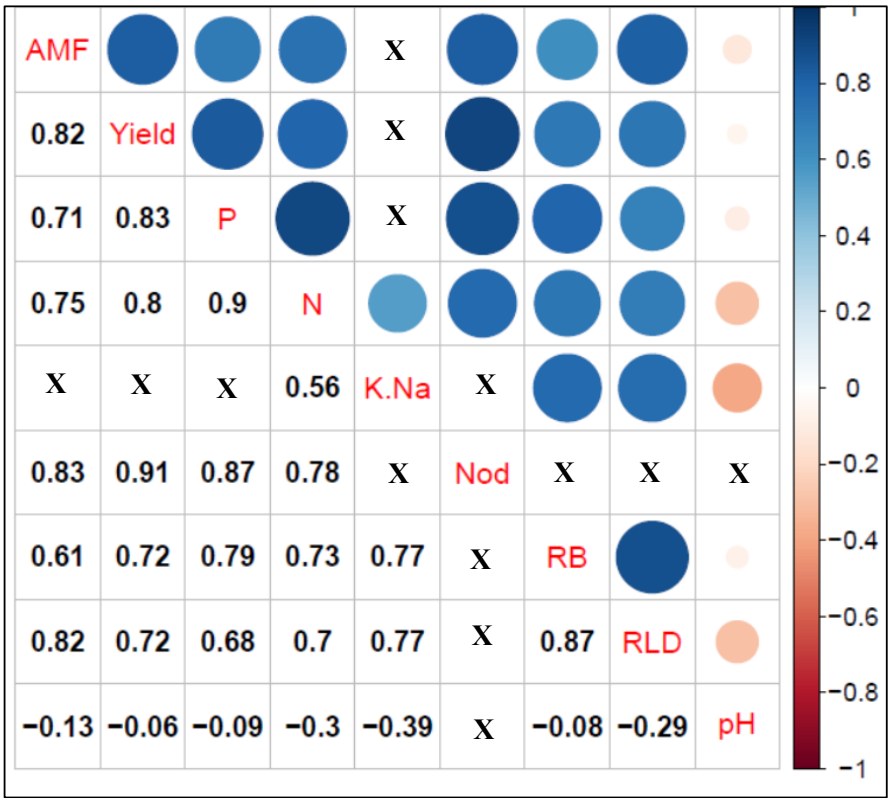

Figure 3.5. Pairwise comparisons between different plant growth parameters, AMF colonization, nodulation, pH and pod yield using Pearson's correlation coefficient.
The color bar is represnting range of Pearson's correlation coefficient. Blue color represents positive correlation range and orange for negative. Circle size corresponds to coefficient value range from smaller (zero) to larger (1). the insignifcant ($p > 0.05$) correlations are marked 'X' in plot. AMF (percentage of root AMF colonization); K.Na (K+: Na+ ratio in shoots); Nod (Number of nodules); RB (Root biomass) and RLD (Root length density).

In the rhizosphere, abundance of prokaryotes was significantly higher ($p < 0.05$) in SL+SA treatment compared to the CS treatment (Table 3.11). No significant differences were observed in any other treatments. There were no signifcant difference between the treatments for abundances of fungi, AMF and NFB. In roots, abundance of prokaryotes was significantly higher ($p < 0.05$) in plants treated with foliar application of SL, SA and combination of SL and SA (SL+SA) (Table 3.12). No significant differences were

observed in fungal abundances in roots between the treatments and CS. Abundances for AMF in roots significantly increased ($p < 0.05$) in all the SL based treatments (SL, SL+SA, COU+SL) and in CMP treated plants. No effect of COU and GYP was observed in the AMF gene abundances in treated plant roots. Abundances of *nifH* gene were found to be significantly higher ($p < 0.05$) in the roots of CMP, SL and SL+SA treated plants than CS treatment. In leaves, there were no significant effect of any of the treatments on the gene abundances of bacterial 16S rRNA and fungal ITS (Table 3.13).

3.4.8 Diversity and composition of rhizosphere and endophytic microbial community

Diversity indices for bacterial and fungal community in the rhizosphere and plant tissues were evaluated and compared between the treatments. Shannon and Simpson diversity indices account for the measurement of both richness (measurement of OTU abundances) and evenness (measure of relative abundance of different species consisting of a community) of species present in a sample with more weightage of species richness in Shannon index and that of species evenness on Simpson index (Kim et al., 2017a). Chao1 is used to only estimate the richness (measurement of OTUs expected in a given sample) and is sensitive to changes in the rare species (Wang et al., 2018b).

Treatments CMP, GYP and SL+SA increased ($p < 0.1$) the bacterial diversity compared to CS (Figure 3.6). There were no significant differences between other treatments. Shannon indices for fungal OTUs showed opposite trends in response to treatments, as they were at a lower range compared to CS treatment (Figure 3.7). Shannon indices were significantly lower in GYP and SL+SA treatments. No significant

differences were observed in root and leaf endophytes between the treatments and control (CS) (Table 3.14 and 3.15). OTU numbers, Simpson index and Chao1 values are shown in Table 3.14 and Table 3.15.

Permanova test and PCOA was performed using Bray-Curtis distances for pairwise comparison between experimental treatments for bacterial (Table 3.16) and fungal (Table 3.17) OTUs. A PCoA plot of Bray-Curtis distances for bacterial and fungal OTUs in rhizosphere and plant tissues are shown in Figure 3.8 and 3.9 respectively. For bacterial community, a clear separation was exhibited by CMP, SA and COU in the rhizosphere; CMP, SA and SL+SA in roots; and SA, SL+SA and COU in leaves compared to CS treatment (Figure 3.8). For fungal community in the rhizosphere, all treatments clustered together and showed separation from the CS treatment (Figure 3.9A). PCoA for root and leaf fungal community showed no separation between any treatment and CS indicating no significant impact of treatments on leaf fungal endophytes (Figure 3.9B, 3.9C).

Permanova showed slight significant differences ($p < 0.1$) in the composition of treatments that were clearly separated from controls in PCoA plots above mentioned.

Table 3.11. Gene abundances of 16S rRNA, ITS, AMF and *nifH* in rhizosphere of experimental treatments measured at 6 WAG.

Treatment	Log (16S rRNA gene copies g^{-1} soil)	Log (ITS gene copies g^{-1} soil)	Log (AMF gene copies g^{-1} soil)	Log (*nifH* gene copies g^{-1} soil)
CMP	8.10 ± 0.10ab	7.94 ± 0.09a	7.15 ± 0.14a	7.35 ± 0.31abc
SL	8.18 ± 0.36ab	7.89 ± 0.17a	7.05 ± 0.24a	6.95 ± 0.38bc
SA	8.32 ± 0.09ab	7.26 ± 1.73a	6.84 ± 0.17a	7.37 ± 0.14abc
SL+SA	8.52 ± 0.24a	8.14 ± 0.03a	7.23 ± 0.05a	7.56 ± 0.34a
COU	8.32 ± 0.45ab	7.99 ± 0.26a	6.89 ± 0.34a	6.81 ± 0.29c
COU+SL	8.10 ± 0.64ab	7.94 ± 0.24a	6.88 ± 0.26a	6.85 ± 0.52c
GYP	8.29 ± 0.46ab	8.29 ± 0.07a	7.36 ± 0.31a	7.54 ± 0.09ab
GYP+MYCO	8.13 ± 0.15ab	7.76 ± 0.36a	6.79 ± 0.45a	7.16 ± 0.38abc
CS	7.74 ± 0.39b	7.63 ± 0.31a	6.86 ± 0.45a	7.04 ± 0.17abc

Note: data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

Table 3.12. Gene abundances of 16S rRNA, ITS, AMF and *nifH* in roots of experimental treatments measured at 6 WAG.

Treatment	Log (16S rRNA gene copies g^{-1} root)	Log (ITS gene copies g^{-1} root)	Log (AMF gene copies g^{-1} root)	log (*nifH* gene copies g^{-1} root)
CMP	7.95 ± 0.45abcd	7.87 ± 0.34a	6.86 ± 0.36ab	7.84 ± 0.45ab
SL	8.41 ± 0.48a	8.27 ± 0.36a	7.06 ± 0.02a	8.04 ± 0.36a
SA	8.20 ± 0.47abc	7.95 ± 0.24a	6.46 ± 0.19abc	7.36 ± 0.38abc
SL+SA	8.32 ± 0.24ab	8.29 ± 0.09a	7.08 ± 0.26a	8.05 ± 0.38a
COU	7.52 ± 0.40bcd	7.68 ± 0.26a	6.27 ± 0.34bc	7.30 ± 0.36abc
COU+SL	8.12 ± 0.16abcd	8.12 ± 0.38a	6.87 ± 0.38ab	7.73 ± 0.09abc
GYP	7.85 ± 0.26abcd	7.83 ± 0.17a	6.30 ± 0.39bc	7.20 ± 0.52bc
GYP+MYCO	7.46 ± 0.31cd	7.64 ± 0.78a	5.87 ± 0.39c	7.02 ± 0.31c
CS	7.34 ± 0.79d	7.78 ± 0.64a	5.93 ± 0.38c	7.06 ± 0.45c

Note: data presented are the means of 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

Table 3.13. Gene abundances of 16S rRNA and ITS in leaves of experimental treatments measured at 6 WAG.

Treatment	Log (16S rRNA gene copies g^{-1} leaf)	Log (ITS gene copies g^{-1} leaf)
CMP	7.18 ± 0.19a	7.22 ± 0.52a
SL	6.68 ± 0.62a	7.01 ± 0.31a
SA	6.15 ± 1.04a	6.36 ± 0.84a
SL+SA	6.85 ± 0.23a	6.72 ± 0.09a
COU	7.15 ± 0.17a	7.06 ± 0.50a
COU+SL	6.71 ± 0.58a	6.12 ± 1.14a
GYP	6.58 ± 0.83a	6.57 ± 0.73a
GYP+MYCO	7.07 ± 0.48a	7.35 ± 1.00a
CS	6.58 ± 0.51a	6.17 ± 0.21a

Note: data presented are the means of 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

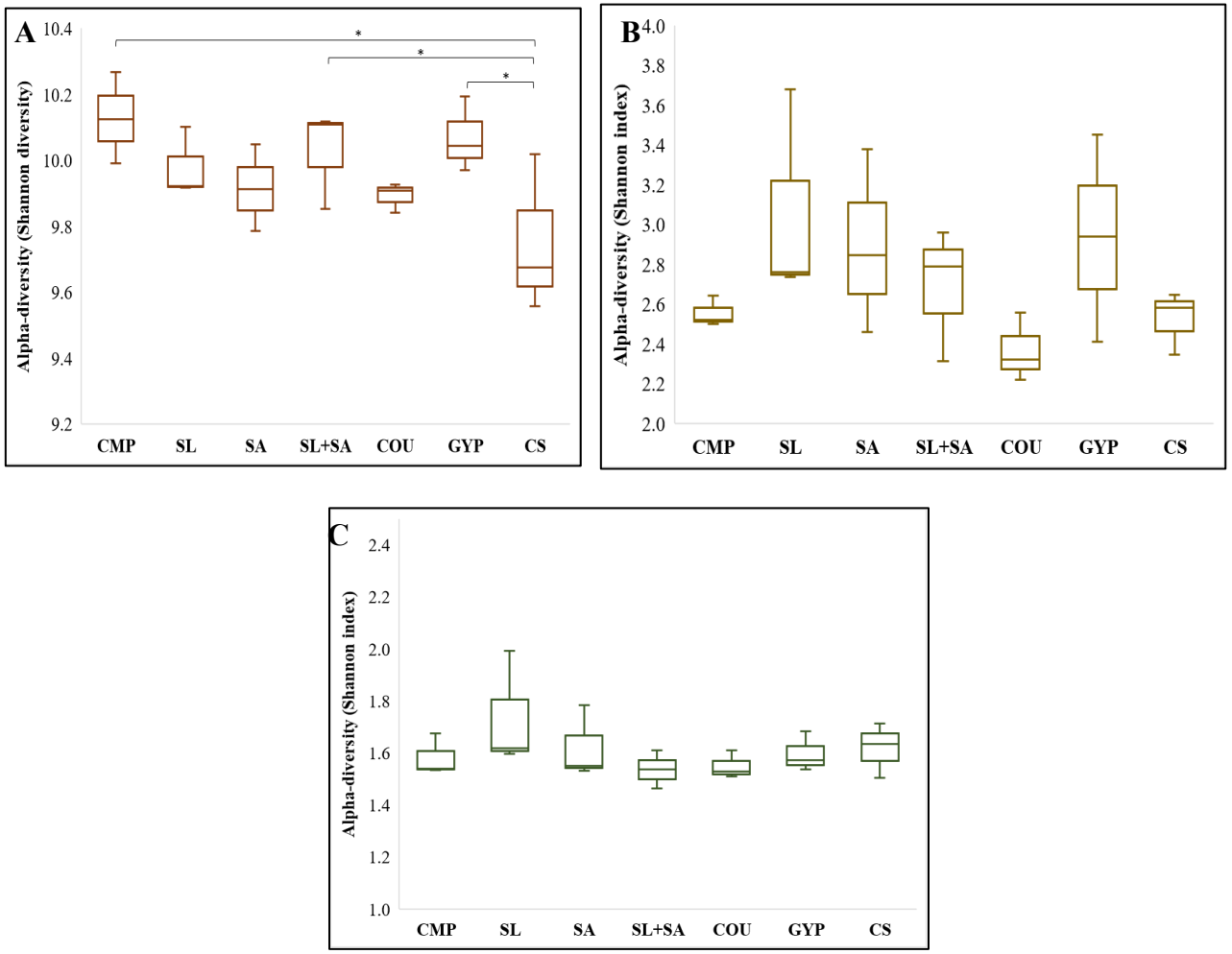

Figure 3.6. Shannon indices (Alpha-diversity) of bacterial community in the rhizosphere (A), roots (B) and leaves (C) of cowpea bean. Statistical analyses were performed by ANOVA and significance is denoted by asterisks where *P < 0.1.

Table 3.14. OTU numbers, Simpson and Chao1 for bacterial community in rhizosphere, roots and leaves of experimental treatments.

Treatment	Rhizosphere			Root			Shoot		
	OTUs	**Simpson**	**Chao1**	**OTUs**	**Simpson**	**Chao1**	**OTUs**	**Simpson**	**Chao1**
CMP	3404a	0.9971a	5578ab	307a	0.6699ab	720ab	58a	0.5067a	320ab
SL	3238a	0.9969a	5392ab	462a	0.7025a	944a	52a	0.5519a	158b
SA	3146a	0.9966a	5037b	403a	0.6867ab	787ab	59a	0.5249a	210b
SL+SA	3370a	0.9969a	5650ab	363a	0.6733ab	808ab	64a	0.5017a	490a
COU	3249a	0.9967a	5576ab	219a	0.6434b	495b	67a	0.5054a	353ab
GYP	3405a	0.9970a	5757a	410a	0.6935ab	796ab	53a	0.5198a	165b
CS	3318a	0.9933a	5751a	285a	0.6598ab	528ab	50a	0.5217a	148b

Note: data presented are the means for 3 replicates followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

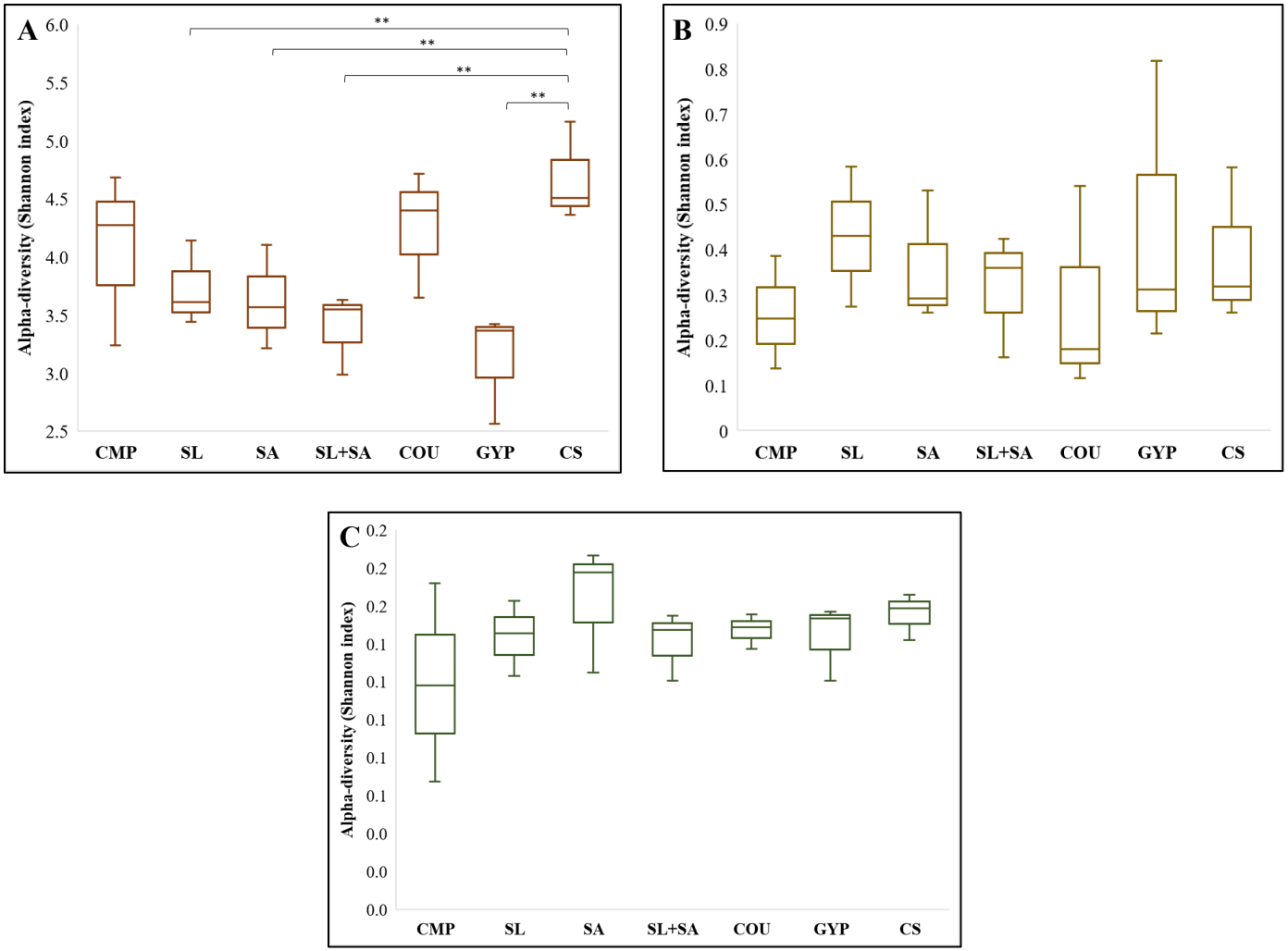

Figure 3.7. Shannon indices (Alpha-diversity) of fungal community in the rhizosphere (A), roots (B) and leaves (C) of cowpea bean. Statistical analyses were performed by ANOVA and significance is denoted by asterisks where **P < 0.05.

Table 3.15. OTU numbers, Simpson and Chao1 for fungal community in rhizosphere, roots and leaves of experimental treatments.

Treatment	Rhizosphere			Root			Leaf		
	OTUs	**Simpson**	**Chao1**	**OTUs**	**Simpson**	**Chao1**	**OTUs**	**Simpson**	**Chao1**
CMP	432a	0.7790abc	515a	90a	0.0509a	150a	25b	0.0282a	45a
SL	407a	0.7603abc	494a	101a	0.0861a	138a	35a	0.0339a	55a
SA	402a	0.7546abc	488a	97a	0.0705a	141a	27ab	0.0415a	41a
SL+SA	404a	0.6778bc	501a	104a	0.0565a	156a	29ab	0.0338a	41a
COU	414a	0.8341ab	499a	72a	0.0554a	96a	27ab	0.0362a	42a
GYP	401a	0.6540c	503a	99a	0.1059a	134a	28ab	0.0346a	57a
CS	441a	0.8846a	537a	75a	0.0789a	120a	310ab	0.0388a	48a

Note: data presented are the means for 3 replicates followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

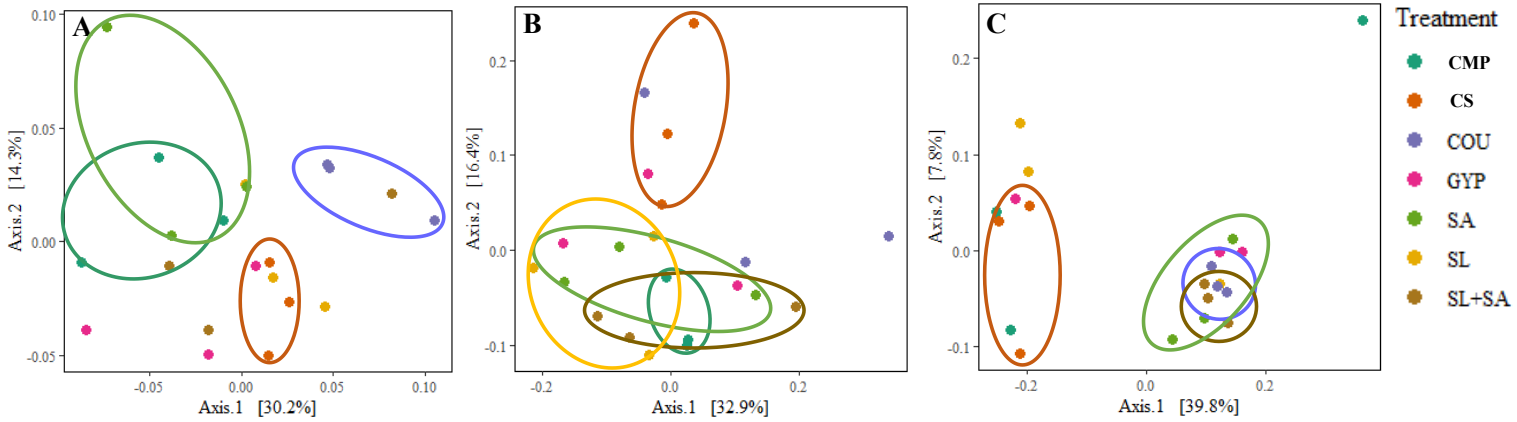

Figure 3.8. Principal Coordinate Analysis (PCOA) of bacterial community in rhizosphere (A), roots (B) and leaves (C) for individual samples from all the treatments using Bray-Curtis dissimilarity distance matrix. The 3 replicates of each treatment are surrounded by an oval of its corresponding color. Ovals are only shown for treatments which are distinctly separated from CS (red) treatment.

Table 3.16. PERMANOVA p-values from pairwise comparisons of the experimental treatments for bacterial OTUs based on Bray-Curtis dissimilarity index.

Compartments		CMP	SL	SA	SL+SA	COU	GYP	CS
Rhizosphere	CMP		0.0997	0.6994	0.5076	0.0983	0.3049	0.0996
	SL	0.0997		0.0997	0.6011	0.1045	0.1022	0.0987
	SA	0.6994	0.0997		0.6100	0.0985	0.1993	0.0966
	SL+SA	0.5076	0.6011	0.61		0.2977	0.4981	0.3952
	COU	0.0983	0.1045	0.0985	0.2977		0.0981	0.1003
	GYP	0.3049	0.1022	0.1993	0.4981	0.0981		0.0983
	CS	0.0996	0.0987	0.0966	0.3952	0.1003	0.0983	
Root	CMP		0.1026	0.3035	0.5973	0.1963	0.2005	0.0964
	SL	0.1026		1	0.8976	0.1022	0.4952	0.0983
	SA	0.3035	1		0.8025	0.1955	0.4007	0.0995
	SL+SA	0.5973	0.8976	0.8025		0.3034	0.596	0.0968
	COU	0.1963	0.1022	0.1955	0.3034		0.1988	0.4037
	GYP	0.2005	0.4952	0.4007	0.596	0.1988		0.1029
	CS	0.0964	0.0983	0.0995	0.0968	0.4037	0.1029	
Leaf	CMP		0.7029	0.5009	0.3999	0.3981	0.7938	1
	SL	0.7029		0.4066	0.4007	0.4023	0.3976	0.4974
	SA	0.5009	0.4066		0.8954	0.4976	0.7031	0.0995
	SL+SA	0.3999	0.4007	0.8954		1	0.2	0.0968
	COU	0.3981	0.4023	0.4976	1		0.7053	0.1006
	GYP	0.7938	0.3976	0.7031	0.2	0.7053		0.1029
	CS	1	0.4974	0.0995	0.0968	0.1006	0.1029	

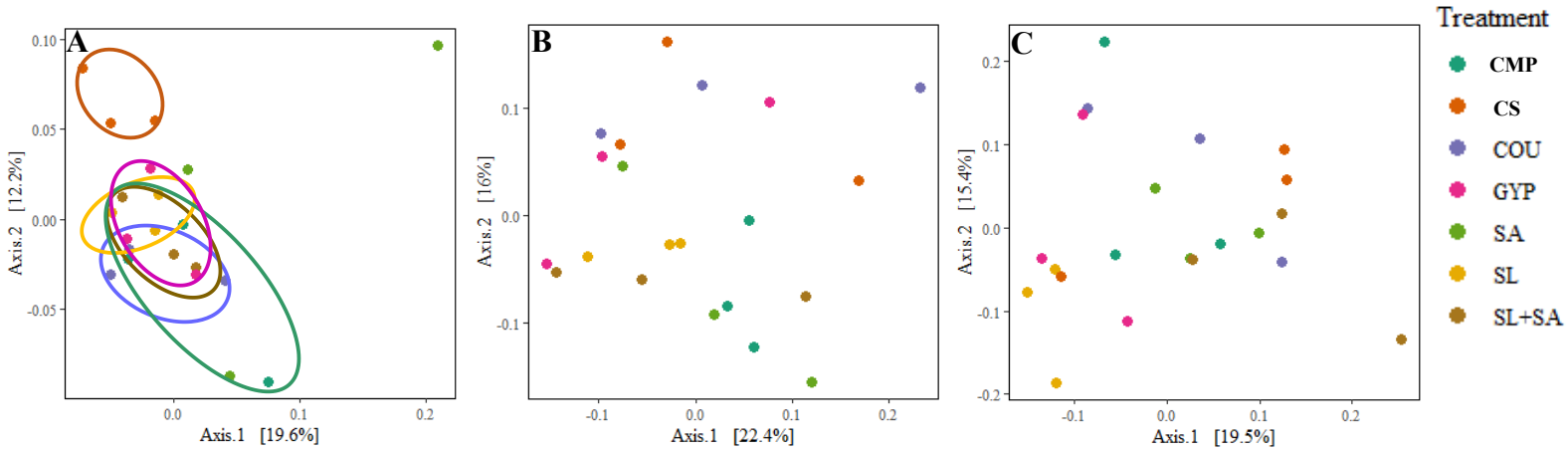

Figure 3.9. Principal Coordinate Analysis (PCOA) of fungal community in rhizosphere (A), roots (B) and leaves (C) for individual samples from all the treatments based on Bray-Curtis dissimilarity distance matrix. The 3 replicates of each treatment are surrounded by an oval of its corresponding color. Ovals are only shown for treatments which are distinctly separated from CS (red) treatment.

Table 3.17. PERMANOVA p-values from pairwise comparisons of all the treatments with Control for fungal OTUs based on Bray-Curtis dissimilarity index.

Compartments		CMP	SL	SA	SL+SA	COU	GYP	CS
Rhizosphere	CMP		0.1004	0.3982	0.103	0.1023	0.0973	0.1037
	SL	0.1004		0.5952	0.2052	0.4927	0.294	0.0995
	SA	0.3982	0.5952		0.707	0.7002	0.7967	0.1031
	SL+SA	0.103	0.2052	0.707		0.6044	0.3027	0.0974
	COU	0.1023	0.4927	0.7002	0.6044		0.8045	0.099
	GYP	0.0973	0.294	0.7967	0.3027	0.8045		0.0962
	CS	0.1037	0.0995	0.1031	0.0974	0.099	0.0962	
Root	CMP		0.1924	0.4969	0.2089	0.1026	0.0994	0.0963
	SL	0.1924		0.4995	0.4007	0.0986	0.2042	0.4002
	SA	0.4969	0.4995		0.2959	0.1009	0.1007	0.0967
	SL+SA	0.2089	0.4007	0.2959		0.0972	0.0977	0.102
	COU	0.1026	0.0986	0.1009	0.0972		0.197	0.5943
	GYP	0.0994	0.2042	0.1007	0.0977	0.197		0.2993
	CS	0.0963	0.4002	0.0967	0.102	0.5943	0.2993	
Leaf	CMP		0.9016	0.3044	0.0958	0.7025	0.901	0.1982
	SL	0.9016		0.5981	0.1013	0.6116	0.6942	0.8002
	SA	0.3044	0.5981		0.4998	0.292	0.2004	0.8992
	SL+SA	0.0958	0.1013	0.4998		0.2981	0.104	0.9077
	COU	0.7025	0.6116	0.292	0.2981		0.3985	0.5037
	GYP	0.901	0.6942	0.2004	0.104	0.3985		0.2013
	CS	0.1982	0.8002	0.8992	0.9077	0.5037	0.2013	

Relative abundancies of bacterial and fungal phyla are presented in Figure 3.10 and 3.11 respectively. The predominant bacterial phyla in all the compartments included *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes, Proteobacteria*, *Planctomycetes* and *Verrucomicrobia* (Figure 3.10). In rhizosphere, relative abundance of *Acidobacteria* and *Firmicutes* were significantly higher ($p < 0.05$) while *Planctomycetes* and *Cyanobacteria* were lower in CS treatment compared to other treatments (Figure 3.10,3.12). Additionally, LEfSe was performed on OTU abundance data to identify significantly different microbial taxa between individual treatment and CS treatment (Figure 3.12-3.15). Several taxa were significantly more abundant in some treatments than CS. For example, order *Rhizobiales*, family *Hyphomicrobiaceae* and class *Alpha proteobacteria* were found to be significantly more abundant in CMP treatment than CS treatment (Figure 3.12A). Also, genus *Bacillus* was significantly more abundant in CS treatment than SL, SA, and SL+SA treatments (Figure 3.12B-D). In roots, phyla *Actinobacteria* and *Chloroflexi* were significantly more abundant in CMP, SL, SA and SL+SA treatments than CS treatment (Figure 3.10,3.13). Additionally, LEfSe revealed significantly higher relative abundance of order *Xanthomonadales* in SL treatment and genus *Streptomyces* in CMP, SA and SL+SA treatments than CS roots (Figure 3.13). No significantly abundant bacterial taxa were observed in the leaf tissues between the treatments and control.

The predominant fungal phyla in the rhizosphere, root and leaves were *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, *Glomeromycota* and *Zygomycota* (Figure 3.11). In the rhizosphere, relative abundance of *Ascomycota* was significantly higher ($p < 0.05$) in CS

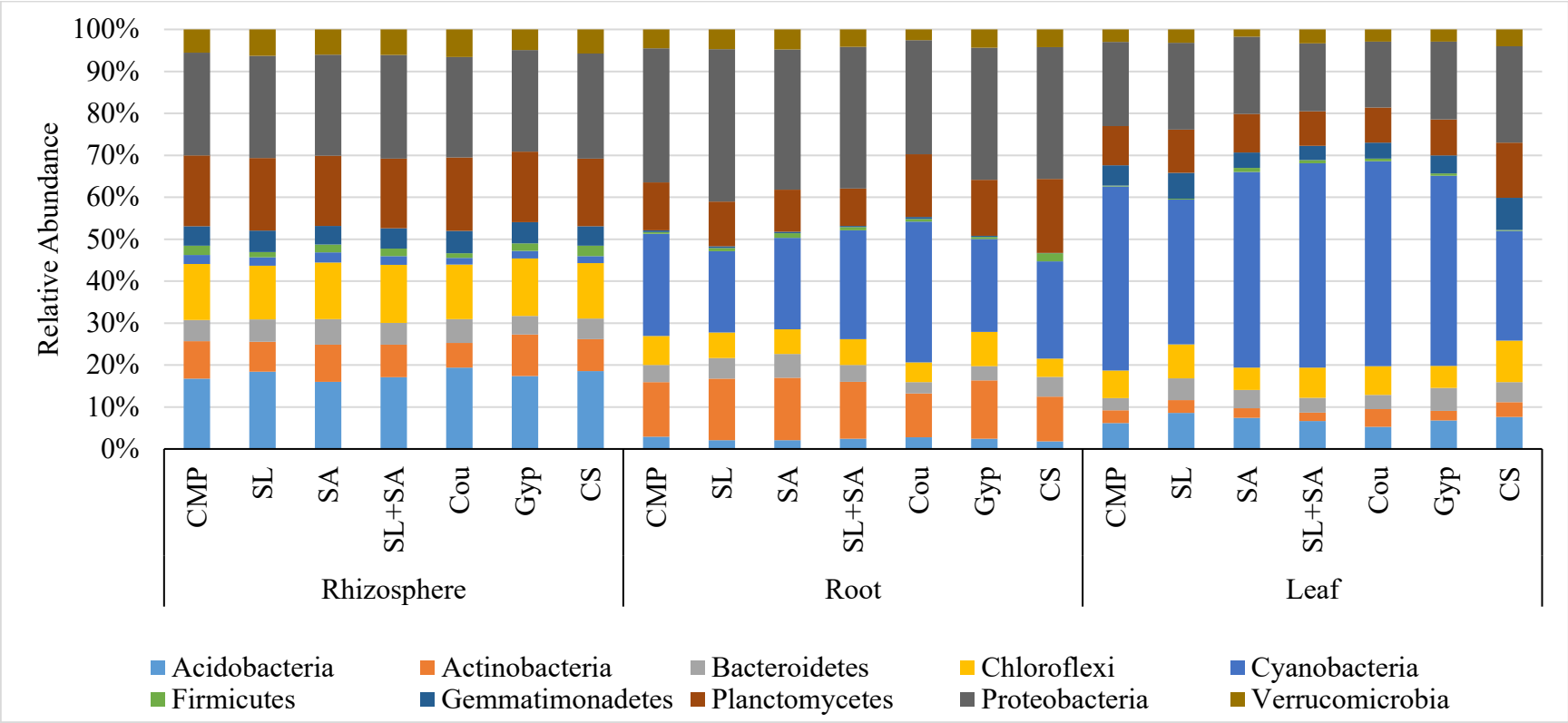

Figure 3.10. The relative abundance of bacterial phyla in the experimental treatments in rhizosphere and endosphere.

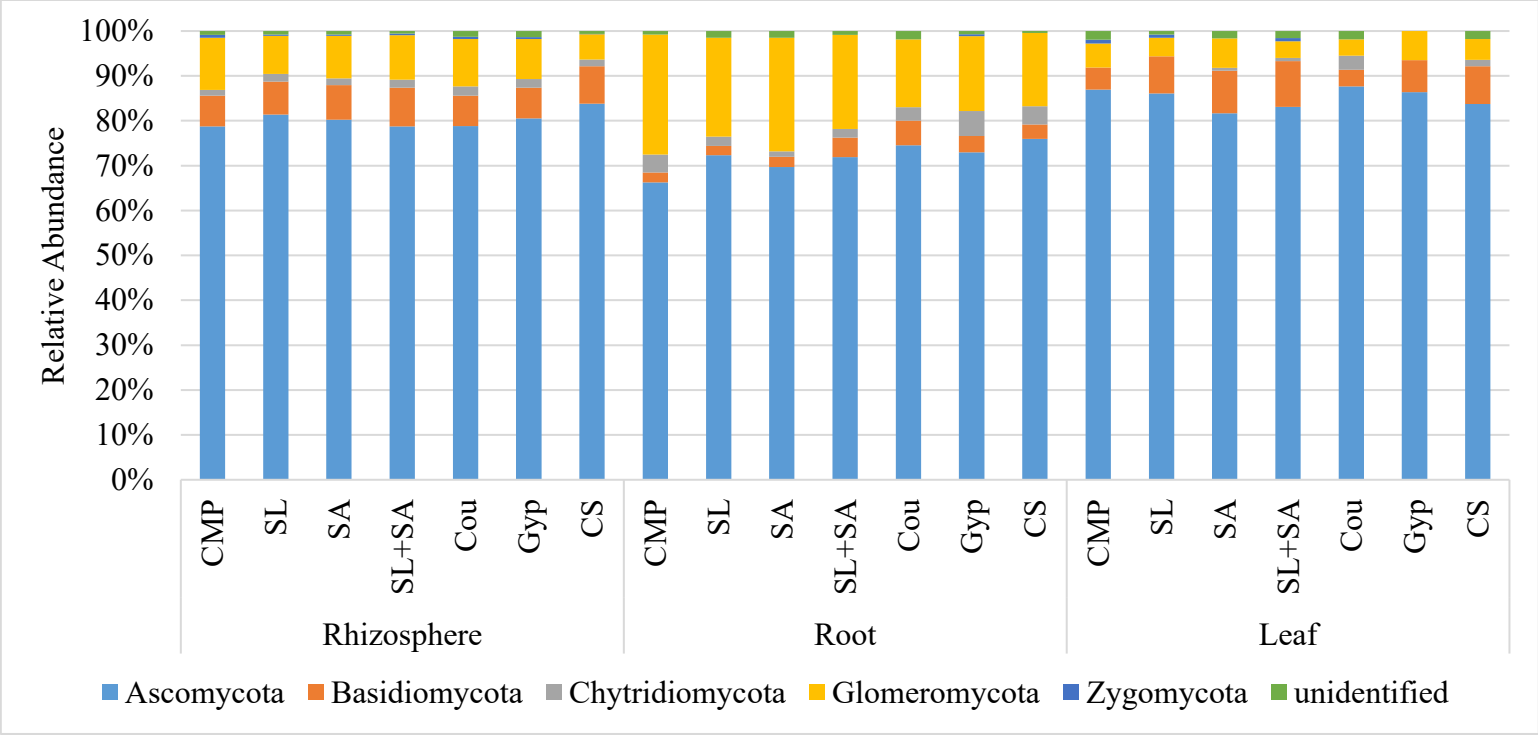

Figure 3.11. Relative abundance of fungal phyla in the experimental treatments in the rhizopshere and endopshere.

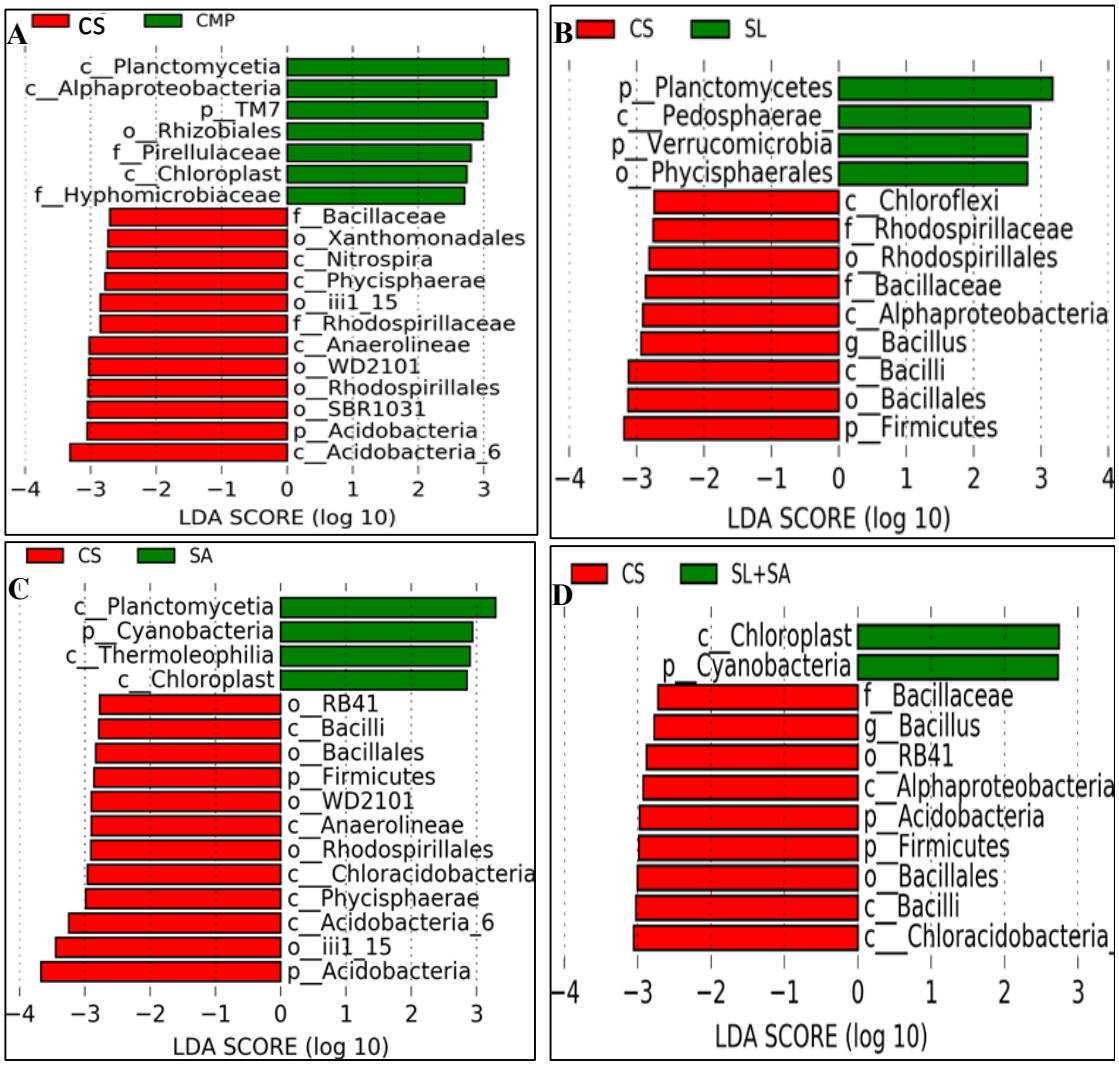

Figure 3.12. Significantly different bacterial taxa in the rhizosphere between pair-wsie treatment comparisons based on linear discriminant analysis effect size (LEfSe) method. Only taxa meeting a linear discriminant analysis (LDA) significance threshold of > 2.5 are presented.

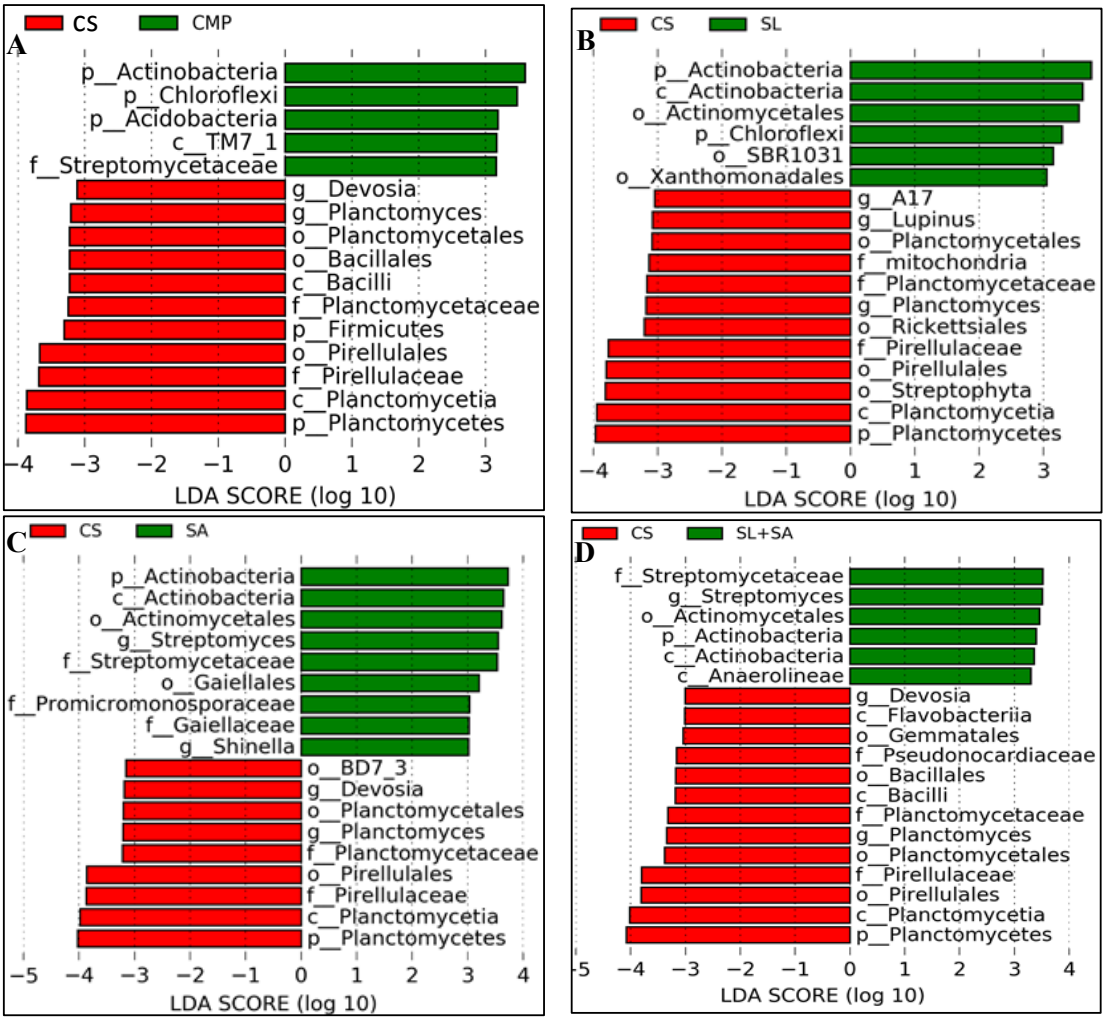

Figure 3.13. Significantly different bacterial taxa in the roots between pair-wise treatment comparisons based on linear discriminant analysis effect size (LEfSe) method. Only taxa meeting a linear discriminant analysis (LDA) significance threshold of > 2.5 are presented.

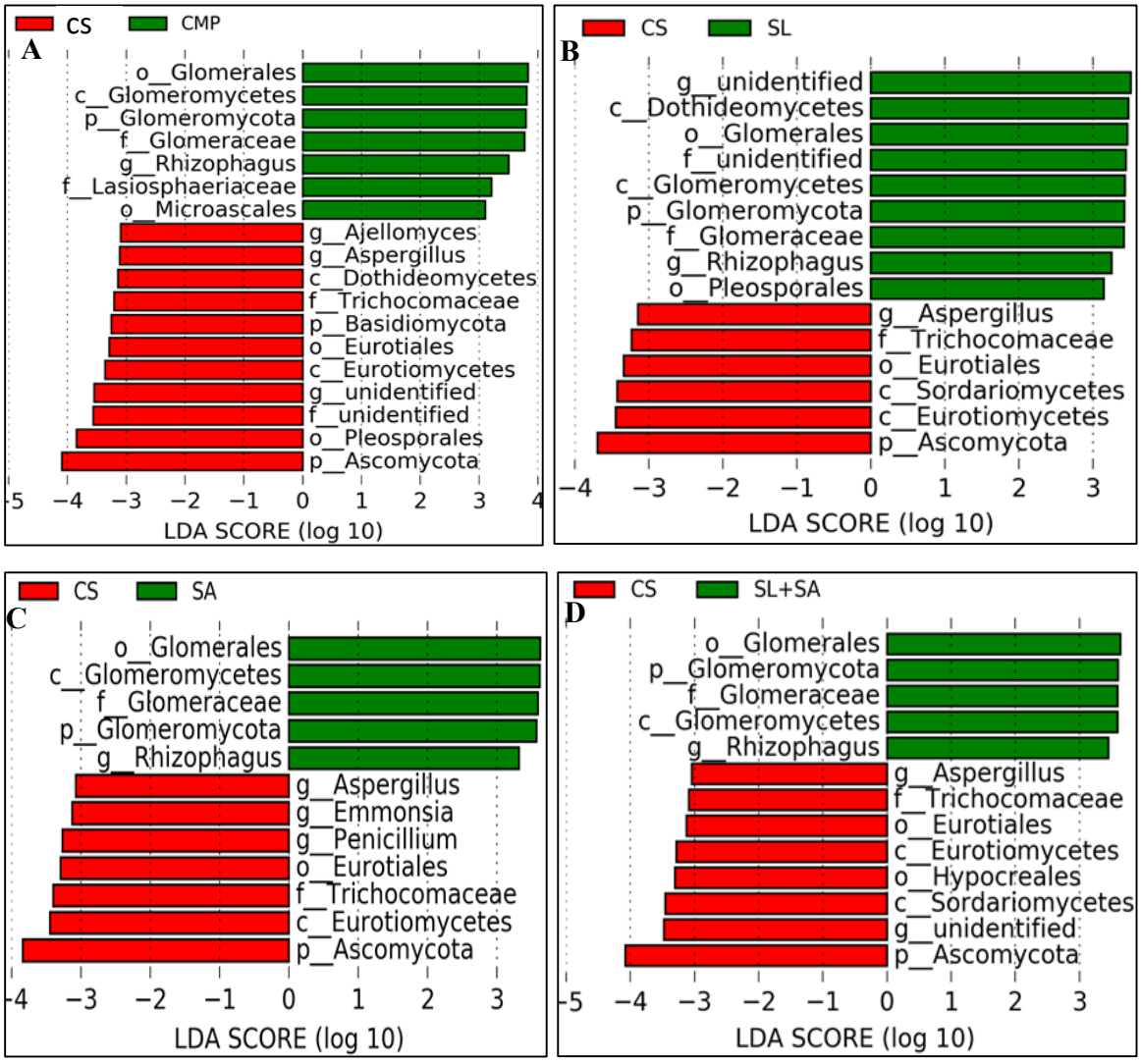

Figure 3.14. Significantly different fungal taxa in the rhizosphere between pair-wise treatment comparisons based on linear discriminant analysis effect size (LEfSe) method. Only taxa meeting a linear discriminant analysis (LDA) significance threshold of > 2.5 are presented.

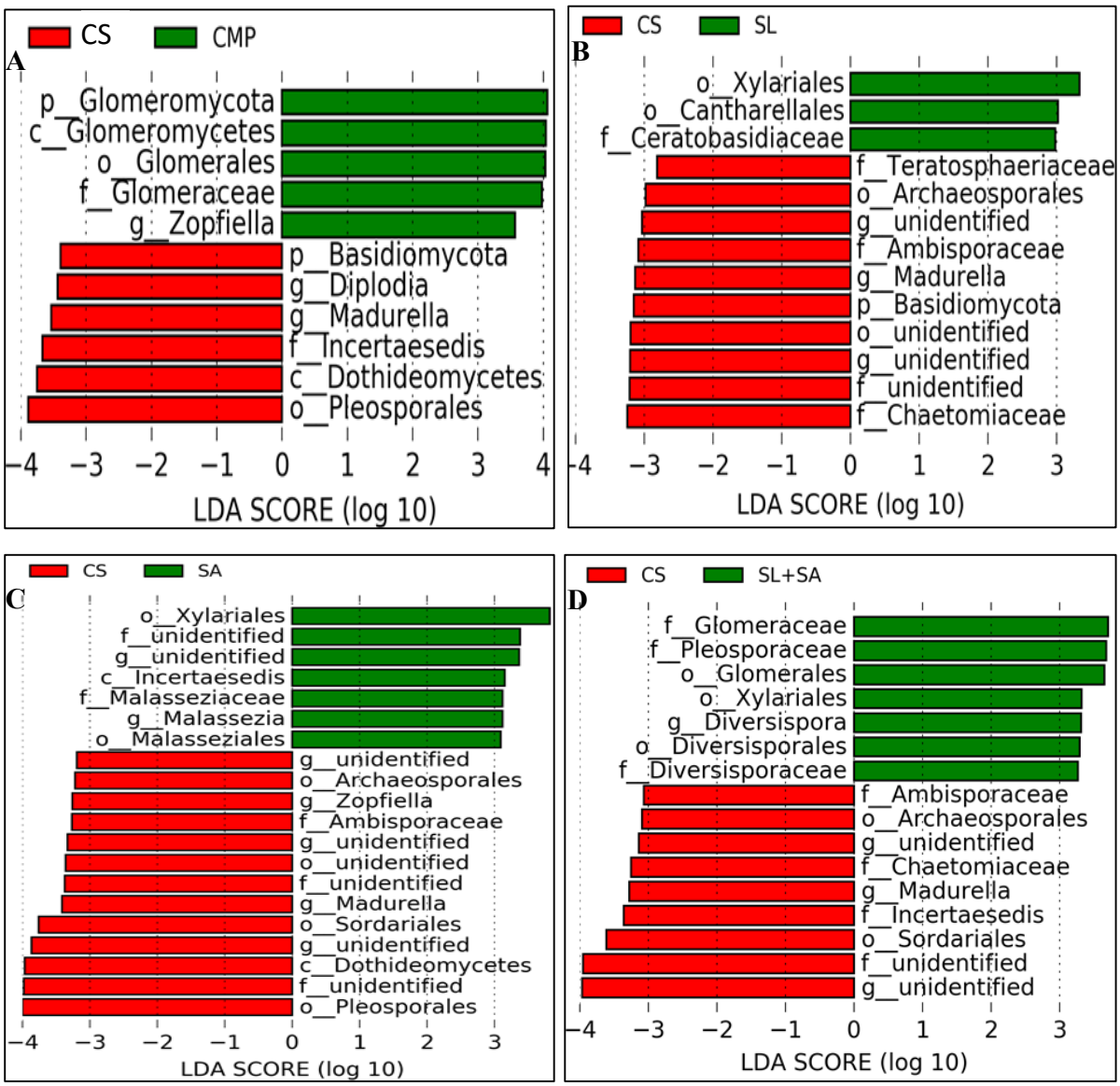

Figure 3.15. Significantly different fungal taxa in the roots between pair-wise treatment comparisons based on linear discriminant analysis effect size (LEfSe) method. Only taxa meeting a linear discriminant analysis (LDA) significance threshold of > 2.5 are presented.

treatment than all other treatments. Whereas, relative abundance of AMF phylum *Glomeromycota* was significantly lower in the CS than all other treatments (Figure 3.11,3.14). LEfSe analysis indicated that treatments CMP, SL, SA and SL+SA significantly increased several AMF taxa such as genus *Rhizophagus*, order *Glomerales* and family *Glomeraceae* (Figure 3.14). Also, genus *Aspergillus* and family *Trichocomaceae* were significantly more abundant in rhizosphere of CS treatment than other treatments (Figure 3.14). In roots, relative abundance of *Glomeromycota* was significantly higher ($p < 0.05$) in CMP, SA, SL and SL+SA treatments than CS (Figure 3.11, 3.15). In addition, AMF genus *Diversispora was* more abundant in the roots of SL+SA treated plants (Figure 3.15D).

3.4.9 Influence of soil and plant growth parameters on microbial abundance

Mantel test was performed to estimate Pearson's correlations between soil and plant parameters (soil pH, RLD, nutrient uptake and K^+:Na^+ ratio) and rhizosphere and endophytic community composition (based on Bray-Curtis distances) (Table 3.18, 3.19). Results of Mantel test showed that the abundance of bacterial community in the rhizosphere was most significantly correlated (positively) with K^+:Na^+ ratio ($p < 0.05$) (Table 3.18). Additionally, bacterial community in the rhizosphere were also found to be positively correlated with root RLD ($p < 0.1$). Most significant correlation observed between abundance of bacterial community in roots was observed with RLD ($p < 0.05$) followed by potassium (K) uptake in plants ($p <0.1$). No significant correlations were observed between abundance of fungal population in rhizosphere and any of the soil and

Table 3.18. Mantel tests between soil and plant growth parameters and composition of bacterial community in the experimental treatments using Pearson's correlation method.

Parameters	Rhizosphere		Root		Leaf	
	R	p-value	r	p-value	r	p-value
Soil pH	-0.098	0.8238	-0.023	0.5505	-0.0538	0.6759
RLD	0.152	0.0657	0.227	**0.0162**	0.0445	0.2730
Leaf N	0.053	0.2926	0.006	0.4416	-0.1003	0.8455
Leaf P	-0.130	0.9347	0.051	0.2687	0.0156	0.3874
Leaf K	-0.189	0.9615	0.201	0.0618	-0.0525	0.6330
Leaf Ca	-0.022	0.5820	0.109	0.1013	-0.0714	0.8002
Leaf Fe	-0.105	0.8864	-0.018	0.5448	0.1438	**0.0557**
K: Na	0.330	**0.0049**	0.047	0.3101	0.0598	0.2599

Note: Pearson correlation coefficients were used to test for the correlations between dissimilarity matrices using 9999 permutations. Bray Curtis dissimilarities were used for bacterial community while Euclidean distance dissimilarities were used for soil and plant growth parameters; r: Pearson's correlation coefficient; pH: rhizosphere soil pH; RLD: root length density; N, P, K, Ca, Fe denotes to total concentration of these nutrients in leaf tissues; K: Na is K^+: Na^+ ratio in leaves.

Table 3.19. Mantel tests between soil and plant growth parameters and composition of fungal community in the experimental treatments using Pearson's correlation method.

Parameters	Rhizosphere		Root		Leaf	
	r	p-value	r	p-value	r	p-value
pH	-0.157	0.9270	0.012	0.4162	-0.1526	0.9442
RLD	0.131	0.1033	0.289	**0.0018**	0.0826	0.1787
Leaf N	-0.036	0.5947	-0.156	0.9628	0.0825	0.2112
Leaf P	0.044	0.3189	0.032	0.3331	0.0762	0.1902
Leaf K	0.004	0.4698	-0.034	0.6109	-0.0052	0.4984
Leaf Ca	-0.047	0.6784	-0.028	0.6221	-0.0086	0.5218
Leaf Fe	0.020	0.3986	-0.081	0.8412	-0.0407	0.6659
K:Na	0.106	0.1963	-0.036	0.6342	-0.1337	0.9028

Note: Pearson correlation coefficients were used to test for the correlations between dissimilarity matrices using 9999 permutations. Bray Curtis dissimilarities were used for fungal community while Euclidean distance dissimilarities were used for soil and plant growth parameters; r: Pearson's correlation coefficient; pH: rhizosphere soil pH; RLD: root length density; N, P, K, Ca, Fe denotes to total concentration of these nutrients in leaf tissues; K: Na is K^+: Na^+ ratio in leaves.

plant variables (Table 3.19). However, fungal community in roots were found to be significantly correlated to RLD ($p < 0.05$).

3.5 Discussion

3.5.1 Impact of experimental treatments on rhizosphere pH and root traits in saline soil

Addition of 5% CMP (pH ~7.0) in saline soil did not significantly alter the soil pH. However, other studies noted varied impacts of composting as soil pH either increased (Wong et al., 1998), or decreased (Walker et al., 2004) after compost application. It was dependent on the type, maturity and amount of CMP used (Duong, 2013; Sarwar et al., 2020). No significant change in soil pH was observed in COU and SA treatments compared to the CS treatment. Treatments of SL, COU+SL, SL+SA significantly decreased the pH to around 8.0 with highest decrease noted in SL+SA treatment to around 7.6. It is possible that SL application increased the organic acids production by roots as noted under Pi deficient conditions (Gamir et al., 2020). Organic acid production by plants can significantly reduce soil pH in the root zone of saline soils as noted in other studies (Oburger et al., 2011; Ström et al., 2005).

Root biomass and RLD were lowest in the CS treatment indicating the adverse impacts of salt stress on Cowpea root growth. Decreased root biomass and RLD under saline conditions has been observed in different crops including legumes (Cordeiro et al., 2014; Puvanitha and Mahendran, 2017; Shrivastava and Kumar, 2015; Yang et al., 2016). Compost (CMP) treatment significantly increased both root biomass and RLD, potentially due to improving the physical properties of soil such as porosity and hydraulic

conductivity (Leogrande and Vitti, 2019). Treatments of SL, SA and SL+SA also significantly increased the root biomass and RLD compared to CS treatment at both plant growth stages. SL has been shown to influence root system architecture such as root hair elongation and lateral root development in plants under N and P deficient conditions in other studies (Kapulnik et al., 2011; Sun et al., 2014). Saline soil used in the study was deficient in both N and P content. It has been suggested that under conditions of nutrient deficiency, root architecture was modified by SL through its crosstalk with phytohormones auxin and ethylene (Andreo-Jimenez et al., 2015; Koltai et al., 2010), the two hormones which regulate the root growth and development in plants (Růžička et al., 2007). In a recent study, use of SA under saline conditions promoted salt tolerance by plants by regulating the expression of genes involved in development of root system architecture (Miao et al., 2020). Exogenous application of SA upregulated the expression of genes responsible for growth and development of lateral roots, differentiation of root hairs and cell expansion of secondary lateral roots under salt stressed conditions in cucumber seedlings. Therefore, increase in RLD in the SL+SA treatment could be to the combined effect of SL and SA on root system architecture of cowpea beans under salt stressed and nutrient deficient conditions. Coumarin treatments (COU and COU+SL) had no significant impact on root biomass and RLD on cowpea beans.

Collectively, SL had a significant impact on pH of saline soil possibly due to induced production of organic acids by plant roots under P deficiency. Root biomass and RLD were severely impacted by higher Na^+ concentrations in the root zone of a saline soil. Treatments of SL, SA and SL+SA significantly improved root biomass and RLD as

compared to CS treatment whereas COU treatment had no impact on soil pH and root growth traits in saline soil.

3.5.2 Impact of treatments on nodulation and AMF colonization in saline soil

Nodule numbers were significantly lower in CS treatment compared to other experimental treatments (CMP, SL, SA and SL+SA). Inhibition of nodulation due to salt stress has been evident in many studies, as nitrogenase activity and oxygen permeability in nodules of many legumes were affected (Faghire et al., 2011; Farhangi-Abriz and Torabian, 2018). Moreover, higher salt concentration inhibits growth of NFB in soil and disrupts nodulation by impairing bacterial ability to infect root hairs (Tu, 1981). In this study, nodulation was significantly improved by CMP, SL, SA and SL+SA treatments. Several studies noted similar effects of SL, which serves as a signaling molecule under N-starvation to increase symbiosis with rhizobia and promote nodulation (Foo and Davies, 2011; Foo et al., 2013; Marzec et al., 2013). Similarly, McAdam et al. (2017) reported that the SLs may induce infection thread formation by rhizobia which can promote nodulation. They showed that infection thread formation in SL deficient *ccd8* mutants of pea (*Pisum sativum*) was greatly reduced as compared to wild type plants. Increased number of nodules in SA treated plants could be due to protection of root nodules by antioxidant enzymes against the adverse effects high salt concentrations (Palma et al. (2013). It was noted that foliar application of SA regulated the redox balance in root nodules by inducing the production of various antioxidant enzymes such as peroxidases, SOD and APX and thus reducing the oxidative stress caused by salinity. In a recent study by Sedaghat et al.

(2017), increased activity of antioxidants SOD, POD, APX and CAT was observed in winter wheat cultivars under drought stressed conditions by foliar treatment of SL and SA and the maximum increase was noted under the combined treatment of SL and SA. Therefore, highest number of nodules in SL+SA treatment in this study could be due to increased salt tolerance of plants by increased activity of antioxidants. Increased nodulation by CMP treatment under saline conditions could be a result of decreased impact of Na^+ ions on plant-rhizobia symbiosis by the increase in exchangeable Ca^{2+} ions in the soil due to increased CEC by CMP which prevents uptake of toxic Na^+ ions in the nodules preventing its negative effect on nodulation (Lawson et al., 2004; Lawson et al., 1995). Treatments based on COU (COU, COU+SL) did not have any significant effect on nodulation. It was shown previously that coumarins inhibit *nodABC* genes and thus may prevent the nodule initiation (Bhattacharya et al., 2010; Djordjevic et al., 1987).

Salt stress significantly decreased percentage of root colonized by AMF possibly due to inhibition of hyphal growth in roots by high salt content as reported in multiple studies (Hajiboland et al., 2010; Ruiz-Lozano and Azcón, 2000). AMF colonization was significantly increased in CMP, SL, SA, SL+SA and COU+SL treatments whereas it was significantly decreased in COU treatment. It is well established that SLs can initiate the symbiosis between plant roots and AMF under P deficient conditions, and increase colonization (Carvalhais et al., 2019; Foo et al., 2013). Moreover, SLs induce hyphal growth and branching in AMF fungi during pre-symbiotic stage and thus increases the chances of colonization in the roots (Akiyama et al., 2010; Besserer et al., 2006). Foliar application of SA significantly increased root AMF colonization as compared to CS

treatment. SA treatment was noted to increase AMF colonization in roots of salt stressed plants in few studies (Ansari et al., 2016; Garg and Bharti, 2018). The increased AMF colonization could be due to the increased allocation of sugars from leaves to roots by SA treatment providing AMF with more carbon (Ansari et al., 2016) and thus increasing colonization and symbiosis with plant roots (Qiang-Sheng et al., 2011). However, some reports have also mentioned decreased and no effect of SA on root AMF colonization and suggested that this might be due to the upregulation of defense-related genes (systemic acquired resistance) by SA during the early stages of AM symbiosis which is inhibited during the later stages (García-Garrido and Ocampo, 2002). Highest percentage of AMF colonization in SL+SA treated plants among the treatments used in the study was due to the combined positive effects of both the stimulants leading to more signaling in the plants increasing the root colonization.

The inhibitory effect of coumarins on AMF colonization is not known clearly and there are no reports available on direct influence of COU application on AMF colonization. It was recently mentioned in a review by Stringlis et al. (2019) that COU are excreted by roots under P deficient conditions and thus could potentially impact AMF colonization in P deficient soils. Furthermore, it was proposed by Chutia et al. (2019) that under conditions of both Fe and P deficiency, a common scenario in saline soils, plant-microbe responses could lead to antagonistic effects. If only P availability is impacted, but not Fe, then COU exudation is decreased, and thus its adverse effect on AMF colonization could be decreased. Addition of CMP in soil increased AMF colonization in the present study. The positive effect of compost on root AMF colonization has been noted in few

studies (Cavagnaro, 2015; Yang et al., 2018). Compost (CMP) is rich in humic acid which stimulates AMF hyphal growth and sporulation (Gryndler et al., 2009). Moreover, CMP provide a sustained release of P to the plant maintaining a moderate level of available P in the soil which enhances AMF root colonization (Yang et al., 2018).

In conclusion, soil salinity adversely impacted symbiotic interactions of plant roots with AMF and NFB. CMP, SL, SA and SL+SA improved AMF colonization and nodulation significantly as compared to CS treatment. Among these, SL+SA treatment produced the highest number of nodules and percentage of AMF colonization.

3.5.3 Nutrient concentration in soil and leaves and ratio of K^+: Na^+ in leaves

The ratio of K^+: Na^+ in leaves were significantly decreased in the CS treatment as compared to GYP confirming the ionic imbalance in these plants due to soil salinity. This was in agreement with several previous reports which indicated similar K^+: Na^+ ratio in leaves under salinity stress (Ashraf et al., 2010; Pakar et al., 2016). Lower K^+: Na^+ ratios in plant cells disrupts many enzyme activity, protein synthesis, turgor maintenance and stomatal movement reducing plant photosynthetic efficiency and growth (Evelin et al., 2019). Reduced P uptake in leaf tissues in CS treatment as compared to CMP, SL and SL+SA treatments was due to reduced P availability, probably because of the fixation of P with other cationic salts in the saline soil mainly Ca^{2+}, Mg^{2+} and Zn^{2+} (de Aguilar et al., 1979). Salt stress also caused reduced the number of nodules and total N in the leaf tissues, suggesting that salinity stress was detrimental to NFB symbiosis and efficiency of N_2 fixation (Allito et al., 2020; Aydi et al., 2008). Compost (CMP) treatment increased soil P content, leaf uptake of all the nutrients measured (N, P, K, Ca, Mg) and K^+: Na^+ in leaves.

Increased nutrient uptake and K^+: Na^+ ratio in compost amended plants was shown in other studies, which was alluded to increased CEC and exchangeable K^+ in soil (Palanivell et al., 2013; Rosenani et al., 2016; Walker and Bernal, 2008). Plants treated with SL+SA accumulated higher concentrations of N, P and K in soil, and higher K^+: Na^+ ratio in leaves compared to CS treatment. However, individual treatment of SL and SA accumulated lower range of nutrient concentrations than SL+SA treatment. This result indicates a synergistic effect of SL and SA interactions leading to greater salt tolerance. In a study by Sedaghat et al. (2017), increased drought tolerance was noted in winter wheat cultivars treated with both SL and SA together and they suggested that this could be due to significantly higher antioxidant activity than in the individual SL and SA applications. Highest percentage of AMF root colonization was observed under SL+SA treated plants which may have contributed to higher K^+: Na^+. Previous studies noted increased K^+: Na^+ ratio in leaves of AMF colonized plants than non-colonized plants under saline soil conditions (Chang et al., 2018; Chen et al., 2017b; Sannazzaro et al., 2006). Chen et al. (2017b) showed that mycorrhizal colonization increased the expression genes encoding for membrane transport proteins involved in maintaining K^+: Na^+ in leaves of black locust plants under salt stressed conditions. No significant impact of COU treatment on concentration of nutrients and K^+: Na^+ ratio in leaves was observed as compared to CS treatment. It was reported that beneficial effects of exogenously applied COU is dose dependent and higher salinity tolerance was noted at 100 ppm COU than at 50 ppm (Sultana et al. (2020). For this study, 50 ppm COU was used which could be the reason for no significant difference in K^+: Na^+ ratio in the leaves. This could also be the major

reason for insignificant impact of COU on leaf Fe concentration of plants as compared to CS treatment. This was in contrast to a few studies that showed COU increased the Fe availability in saline soil by chelation and/or reduction of Fe^{3+} to Fe^{2+} increasing its uptake by the root cells (Rajniak et al., 2018; Schmidt et al., 2014). While, SL treated plants (SL and SL+SA) significantly increased Fe leaf concentration compared to COU and CS treatments. One reason could be exudation of organic acids in the rhizosphere induced by SLs under nutrient deficient conditions (Gamir et al., 2020). Several organic acids can solubilize complexed-Fe and increase availability in saline soil (Tsai and Schmidt, 2017). In addition, SL can also improve the interaction of plants with PGPR that produce siderophores, which can chelate Fe^{3+} under low iron concentrations and transform the insoluble iron (Fe^{3+}) into plant available iron (Fe^{2+}) (Schlemper et al., 2018; Zhou et al., 2018).

In summary, soil salinity significantly impacted ionic homeostasis in plant tissues resulting in reduced K^+: Na^+ ratios in leaves. Treatments CMP, SL and SL+SA significantly improved salinity tolerance as indicated by significantly higher K^+: Na^+ ratios in leaves. Moreover, these treatments also improved plant N, P and Fe concentration in leaves protecting plants from nutrient deficiencies that are prevalent under saline soil conditions. There was no significant impact of COU treatment on Fe uptake whereas SL significantly improved Fe concentration in leaves.

3.5.4 Impact of salt stress on diversity, abundance and composition of rhizosphere and endophytic microbes

Shannon diversity index and beta-diversity (based on PERMANOVA test) for bacterial community in rhizosphere significantly decreased in salt stressed conditions than GYP reclaimed soil indicating the adverse effects of salinity on bacterial diversity like observed in other studies (Ibekwe et al., 2010). However, Shannon diversity index for fungal community was significantly higher in the rhizosphere of saline soil than GYP treated soil. No significant change in bacterial and fungal diversity (both alpha and beta diversity) was observed in the root and shoot tissues of salt stressed plants indicating that microbial community inside the plant tissues are more stable in response to fluctuating environment than in rhizosphere (Han et al., 2020; Xiao et al., 2017). In addition, the diversity and composition of endophytic community are more dependent on the host type and growth stage in contrast to rhizosphere microbiome which is more influenced by physicochemical conditions (such as salinity) of soil (Xiao et al., 2017).

Most dominant bacterial phyla found in saline conditions in the present study were *Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Gemmatimonadetes, Proteobacteria, Planctomycetes* and *Verrucomicrobia.* This was similar to a recent study by Shi et al. (2019) and Szoboszlay et al. (2019) who noted these phyla to be dominant under saline soil conditions. Salt stressed conditions led to increased abundance of phyla *Acidobacteria* and *Firmicutes* while abundance of *Planctomycetes* and *Cyanobacteria* were decreased in the rhizosphere of saline soil. Some studies noted that phylum *Acidobacteria* was highly abundant in salt affected soils (Xu et al., 2020b;

Zhao et al., 2018), however, some other studies reported a decrease in abundance with increase in salt content of soil (Han et al., 2020; Xu et al., 2020a). Firmicutes increased in saline soil and was found significantly higher in abundance than most of the treatments. *Bacillus* was at higher relative abundance in the rhizosphere of saline soil. *Bacillus* was shown to increase plant salt tolerance by various mechanisms such as iron acquisition, phytohormone synthesis, regulating the expression of sodium transporter *HKT1* in roots and shoots and thereby decreasing Na^+ accumulation in plants (Kim et al., 2017b; Xie et al., 2009).

Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota and Zygomycota were dominant fungal phyla in the present study. These phyla were also found dominant in saline agricultural soils in a recent study by Zhao et al. (2019). Among these, *Ascomycota* was found to be significantly higher in relative abundance in rhizosphere of unamended natural saline soil than amended and treated conditions similar to few other studies (Kim et al., 2019; Yang et al., 2020a). Phylum *Glomeromycota* (a phylum entirely composed of AMF species) was significantly depleted in rhizosphere and roots of plants under natural saline soil. This suggests that AMF were largely sensitive to salinity stress. It is well known that salinity impacts AMF spore germination and hyphal growth inhibition (Hajiboland et al., 2010).

3.5.5 Impact of experimental treatments on diversity, abundance and composition of rhizosphere and endophytic community

Effect of CMP on alpha and beta diversity of bacterial community in rhizosphere was similar to a few recent studies where addition of CMP led to increased microbial

diversity under salt stressed conditions (Manasa et al., 2020; Shi et al., 2019) and indicated that this may be due to the improved physicochemical characteristics of soil such as lower pH and higher nutrients indirectly affecting soil microbial community composition and structure (Shi et al., 2019). Compost (CMP) treatment however decreased the fungal diversity in the rhizosphere. Treatment of SL did not have any significant impact on alpha diversity of bacterial and fungal community; however, the beta-diversity was significantly different for fungal OTUs in the rhizosphere. This was in agreement with a recent study by Carvalhais et al. (2019) who showed that alpha diversity did not change significantly between the bacterial and fungal community of SL deficient mutant of *Arabidopsis max4* and wild type while the composition of fungal community was significantly different in *max4* rhizosphere. No significant difference in microbial diversity was found in the rhizosphere of COU treatment while SA treatment only impacted the diversity of fungal community in rhizosphere. No impact of SA on rhizosphere bacterial diversity was also observed in a study by (Liu et al., 2018). However, the combined effect of SL and SA was found to significantly impact the alpha and beta diversity of bacterial and fungal community in rhizosphere. No reports are available on effect of combined application of SL and SA on microbial diversity and composition in plant rhizosphere under saline conditions. In a study by Sedaghat et al. (2017) on the impact of combined foliar spray of SL and SA on wheat plants under drought stressed conditions, showed that the SL and SA together enhanced antioxidant enzymes significantly and suggested there might be a cross talk among these two compounds which is responsible for their positive effect on plants drought tolerance. This might also be the reason for the impact of SL and SA together on

microbial community diversity and composition in the present study, however this needs further investigation to prove.

Furthermore, alpha-diversity of bacterial and fungal endophytic community did not change for root and leaf tissues. However, community structure (beta diversity) of endophytes was significantly impacted by treatments CMP, SL, SA and SL+SA as evident from PCoA plot and PERMANOVA test results. Microbial community shifts are typically in response to environmental conditions (Wang et al., 2020b), which in this study was primarily due to change in soil pH and soil salinity stress.

Compost (CMP) application in soil significantly increased the abundance of order *Rhizobiales* (class *Alphaproteobacteria*) in the rhizosphere similar to other studies (Daquiado et al., 2016; Zhou et al., 2019). Members of *Rhizobiales* play a dominant role in N_2-fixation and organic phosphate solubilization (Long et al., 2018), and may have also mineralized organic matter in CMP treatment (Zhou et al., 2019). Phyla *Actinobacteria* and *Chloroflexi* were significantly more abundant in roots of CMP, SL, SA and SL+SA treatments. Within phylum *Actinobacteria*, genus *Streptomyces* was significantly more abundant in roots of CMP, SL, SA and SL+SA treatments. *Streptomyces* are major PGPB and promote plant growth under saline soil conditions (Olanrewaju and Babalola, 2019). Many species were noted promote salinity tolerance by various mechanisms such as production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase (Palaniyandi et al., 2014), plant growth regulators like IAA (Sadeghi et al., 2012) and iron chelators (Tokala et al., 2002). Under salt stressed conditions, ethylene regulates plant homeostasis resulting in reduced root and shoot growth (Shrivastava and Kumar, 2015). ACC

deaminase hydrolyzes ACC, the precursor of ethylene in plants to ammonia and α-ketobutyrate, thus prevents accumulation of ethylene in plants (Glick, 2005). Additionally, the siderophore producing ability of many *Streptomyces* under saline conditions may have increased Fe availability and uptake noted in SL and SL+SA treated plants compared to CS treatment (Sadeghi et al., 2012). It is well known that SLs improve signaling between plants and siderophore producing PGPR in the soil (Schlemper et al., 2018). Many *Streptomyces* induce plant gene expression of various antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (PO), ascorbate peroxidase (APX) and guaiacol peroxidase (GPX), which are known to minimize ROS impacts (Singh and Gaur, 2017). Thus it is proposed that salinity tolerance was increased by higher relative abundance of *Streptomyces* and treatments (CMP, SL, SA and SL+SA) that increased their relative abundance produced higher yield. Among major fungal phyla, the relative abundance of *Glomeromycota* was significantly increased in most treatments compared to CS. AMF genera *Rhizophagus* was significantly higher in the rhizosphere of CMP, SL, SA and SL+SA treatments. These results confirm previous assumption that improving physicochemical properties of soil by compost (Maji et al., 2017) and increased signaling between plant and AMF through SA and/or SL application increase AMF interactions (Besserer et al., 2006; Medina et al., 2003) in conditions of P deficiency. *Rhizophagus*, in addition to providing plants with the well-known benefits of AMF symbiosis, has also shown to protect its host under abiotic stressed conditions (Li et al., 2014). Moreover, a new genus of AMF, *Diversispora* was observed in roots of SL+SA treatment. This AMF species was isolated from an arid region (Symanczik et al., 2014), suggesting that it may

be one of the saline tolerant native AMF species. It was detected only in the treatment of SL+SA, suggesting that native AMF were responsive to this combination of signaling compound application, and were primarily responsible for higher root colonization in this treatment, more than the GYP+MYC treatment, which received commercial AMF species. These results highlight the importance of using signaling compounds for modulating native microbial community interactions, which appears to be a more suitable management practice for improving salinity tolerance, rather than exogeneous supply of microbial inoculum.

3.5.6 Influence of soil and plant growth parameters on microbial abundance

Results obtained from Mantel tests showed that K^+: Na^+ ratio and RLD were the most influential factors for altering the rhizosphere and endosphere microbial community composition divergence between the treatments. It was noted in a previous study that concentration of K^+ or Na^+ was a major corelating factor with microbial community composition in a saline soil (Kim et al., 2019; Zhao et al., 2019). It is not clear how RLD changes influence microbiome composition in the rhizosphere under saline conditions. One suggestion was that root architecture influences microbiome composition by modifying the surface area of soil-root interactions, and as RLD increases more microbes are able to interact with root exudates and their abundance in the rhizosphere and endosphere of plants (Stewart et al., 2017). A significant correlation between leaf Fe content and bacterial community composition of leaf endosphere was noted. This result underscores the importance of Fe nutrition in saline soils, which is mostly dependent on

recruitment of siderophores producing bacteria in the rhizosphere and endosphere (Rout et al., 2013).

3.5.7 Experimental treatment implications on soil fertility management and improving plant production in saline soils

Use of CMP in soil and combined application of SL and SA on cowpea plant leaves produced more comprehensive salinity tolerance and higher yields. These treatments outperformed gypsum treatments (GYP and GYP+MYC). Many studies have previously shown the importance of compost treatment in increasing plant yield under saline conditions mainly due to its impact on improving soil fertility through enhanced nutrient availability in soils (Palanivell et al., 2013; Rosenani et al., 2016; Walker and Bernal, 2008). In the present study, SL+SA treatment increased cowpea pod yields more than compost (CMP) treatment. Higher nodules, P concentration in shoots and AMF colonization was observed in this treatment, which appears to be the major reason for higher cowpea bean yields. Plant growth promoting bacteria *Streptomyces* and AMF genera *Rhizophagus* and *Diversispora* were increased under SL+SA treatment which may have further contributed to salt stress tolerance, as noted in other studies (Li et al., 2014; Olanrewaju and Babalola, 2019; Symanczik et al., 2014). Higher nutrient uptake (N, P and Fe) and K^+: Na^+ ratio in this treatment was also attributed to beneficial effects of symbiotic interactions in the rhizosphere and endosphere. Beneficial impact of SL and SA have been previously observed under stressed conditions (Tari, 2002; Van Ha et al., 2014). Thus, it can be concluded from these results that SL+SA produced most comprehensive beneficial impacts on cowpea plants grown in saline soil conditions.

3.6 Conclusions

It can be concluded from this study that soil salinity adversely impacted plant growth, nutrient uptake, nodulation, AMF colonization, yield and overall diversity and composition of beneficial bacterial and fungal community in rhizosphere and endosphere. Treatments of CMP, SL, SA and SL+SA showed a positive impact on overall plant growth, nutrient uptake, nodulation and BPMI, particularly AMF colonization. Abundance and composition of AMF was also impacted by these treatments, with greatest impacts on SL+SA followed by CMP treatment. Bacterial genus *Streptomyces*, a salt tolerant PGPB and AMF genus *Rhizophagus* were significantly more abundant in CMP, SL, SA and SL+SA treated plants and probably contributed to increased N, P and Fe, salt tolerance and higher yields. Microbial community divergence among the treatments correlated significantly with changes in K^+: Na^+ ratio, RLD and leaf Fe concentration, suggesting that these factors were principally influenced by rhizosphere and endophytic microbial community interactions Both CMP and SL+SA treatments produced the highest AMF colonization, nodulation and pod yields. Therefore, use of either compost as a soil amendment and or foliar application of SL and SA are recommended for improving BPMI and salinity tolerance, and increasing crop growth and yield in saline soils.

SUMMARY

The first experiment detailed in chapter II was conducted to investigate the impacts of soil amendment of biochar (BC) and foliar application of salicylic acid (SA) on nodulation, root colonization by arbuscular mycorrhizal fungi (AMF) and on diversity and composition of rhizosphere and endophytic microbiome of cowpea plants grown in an acidic soil. Treatments were also evaluated for their impacts on soil pH, nutrient concentrations in the rhizosphere and plants, root biomass and plant yield. Results indicated that plants grown under acidic soil accumulated higher Al concentrations in the leaves and showed adverse impacts on nutrient availability, root and plant growth and pod yield. Soil acidity significantly decreased nodulation and leaf nitrogen (N) concentrations. However, no significant impact of soil acidity was observed on AMF colonization of roots. Biochar (BC) amendment increased soil pH, nutrient availability in the rhizosphere, nutrient concentration in leaf tissues and pod yield, significantly more than unamended acidic control (AC) treatment. In addition, BC treatment improved nodulation, percent AMF colonization and the abundance of many plant beneficial taxa such as *Bacillus*, *Pseudomonas, Penicillium* and N_2-fixing bacteria (NFB) such as *Rhizobium* and *Bradyrhizobium* in the rhizosphere and endosphere. Foliar application of SA decreased Al concentrations and increased nutrient concentrations in leaf tissue compared to AC treatment but did not significantly change the soil pH. Foliar spray of SA also increased the percent AMF colonization and abundance of several key microbes such as *Burkholderia* spp., *Trichoderma* spp. and AMF *Glomus* spp. in the rhizosphere and root

endosphere, significantly more than AC treatment. However, nodulation, leaf N concentrations and pod yields were lower than the BC treatment. Based on the results of this study it was clear that cowpea nodulation was more sensitive to soil acidity than root AMF colonization. Thus, improving nodulation and N-uptake in plants under acidic conditions through pH correction is critical. Addition of BC to acidic soil produced more comprehensive benefits on microbial interactions and plant growth and development and must be considered for improving soil health and productivity in acid soils.

The second experiment detailed in chapter III was conducted to evaluate compost (CMP) and gypsum (GYP) as soil amendments and foliar application of several signaling compounds such as strigolactones (SL), salicylic acid (SA) and coumarins (COU) for their impacts on diversity and composition of rhizosphere and endophytic microbiome, AMF colonization, nodulation, plant nutrient concentrations and pod yield. Results showed that soil salinity adversely impacted plant growth, nutrient uptake, nodulation, AMF colonization, yield and overall diversity and composition of beneficial bacterial and fungal community in the rhizosphere and endosphere. Treatments of CMP, SL, SA and SL+SA showed a positive impact on overall plant growth, nutrient uptake, K^+: Na^+ ratio, nodulation and AMF colonization. Abundance and composition of AMF was also impacted by SL+SA and CMP treatments. Bacterial genus *Streptomyces*, a salt tolerant plant-growth-promoting bacterium and AMF genus *Rhizophagus* were significantly more abundant in CMP, SL, SA and SL+SA treated plants. Treatments CMP, SL and SL+SA also accumulated higher P and Fe in the leaves. Microbial community divergence among the treatments correlated significantly with changes in K^+: Na^+ ratio, RLD and leaf Fe

concentration, suggesting that these factors were principally influenced by the rhizosphere and endophytic microbial community composition. Both CMP and SL+SA treatments produced the highest AMF colonization, nodulation and pod yields. Use of either compost as a soil amendment or foliar application of SL and SA were most effective in improving beneficial plant-microbe interactions and cowpea plant yield grown in a saline soil.

It was demonstrated by these two studies that several beneficial microbes in the rhizosphere and endosphere of a legume crop were sensitive to acidity and salinity stress. It was also clear that various soil amendments and exogenous application of signaling compounds significantly altered rhizosphere and endosphere microbiome structure of a legume crop, and improved cowpea interactions with AMF and NFB. Using effective soil amendments such as biochar in acidic soil and foliar application of SL and SA for plants grown in saline soils are potential agriculture management avenues for improving soil health and productivity in acidic and saline soils.

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APPENDIX A

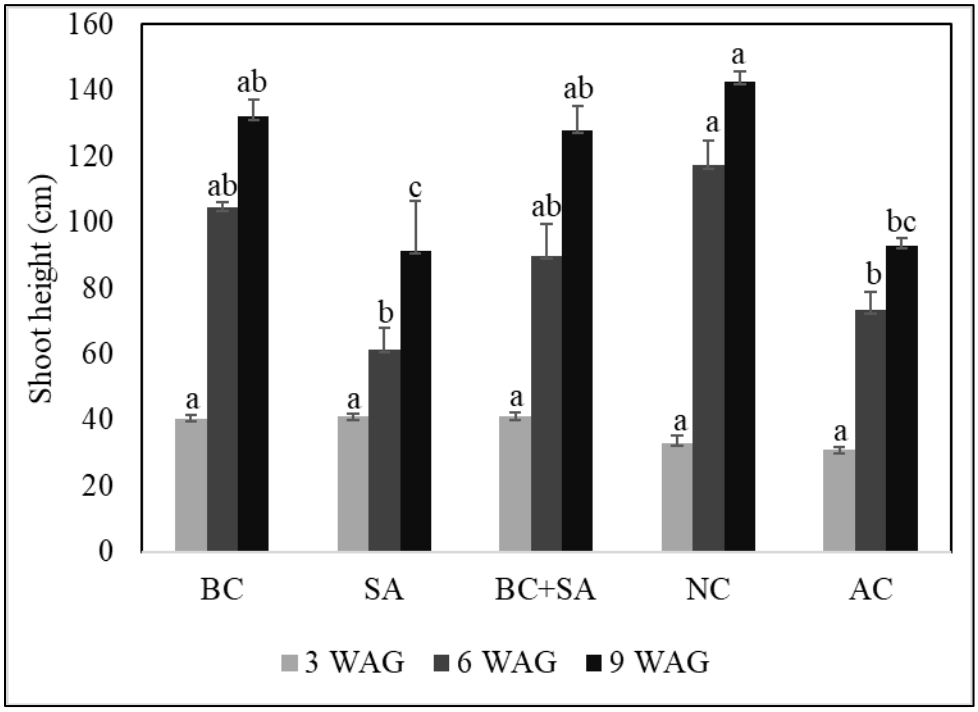

Figure 1. Impact of experimental treatments on plant shoot height measured at 3, 6 and 9 WAG.

Note: data presented are the means for 3 replicates with standard deviation. Within each time point, means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3 for all time points.

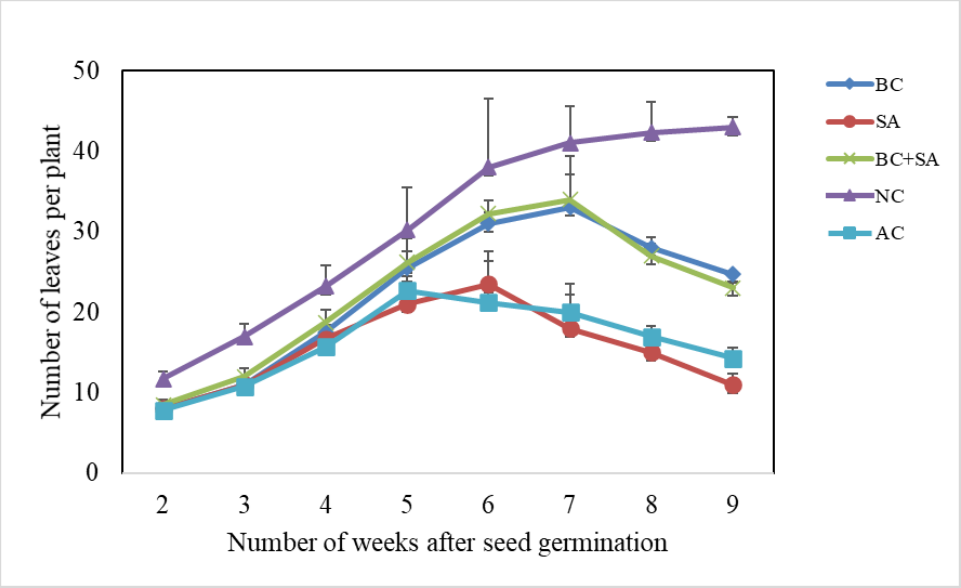

Figure 2. Impact of experimental treatments on number of leaves per plant measured at 3, 6 and 9 WAG.
Note: Data presented are the means for 3 replicates with standard deviation.

Figure 3. Cowpea plants of experimental treatments at 6 WAG (Flowering stage).

Figure 4. Cowpea plant showing symptoms of leaf yellowing under SA treatment after 6 WAG.

Figure 5. Cowpea plants of experimental treatments at 9 WAG (Pod maturity stage).

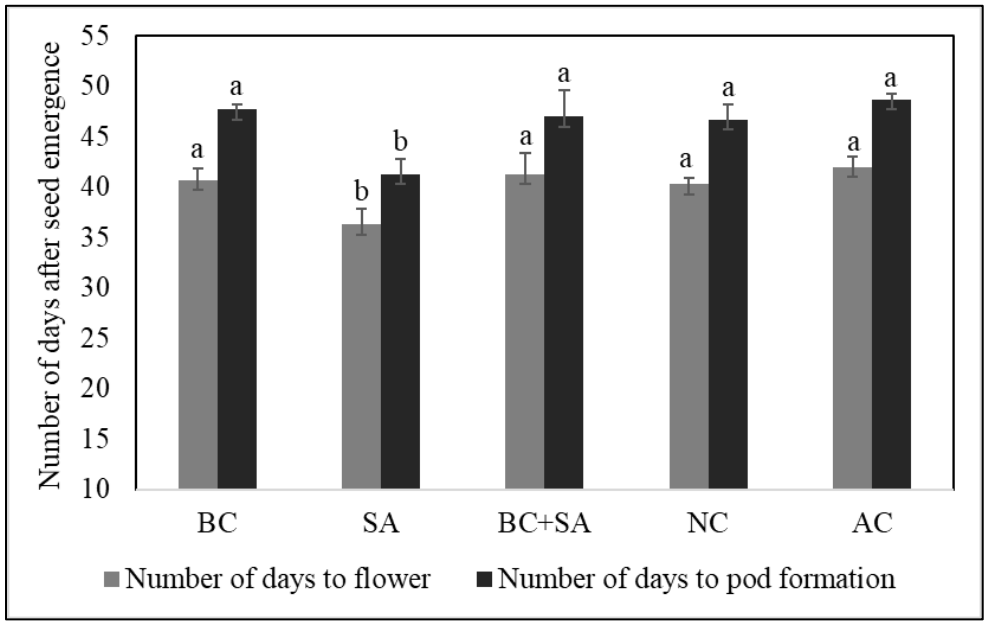

Figure 6. Impact of experimental treatments on number of days to flowering (gray) and pod formation (black).
Note: Data presented are the means for 3 replicates with standard deviation. Means followed by different letters indicate significant difference among treatments ($p < 0.05$) by Duncan's multiple range test; n=3.

APPENDIX B

Figure 1. Cowpea plants grown in greenhouse in second experiment at 2 WAG.

Figure 2. Cowpea plants under CMP treatment at 6 WAG.

Figure 3. Cowpea plants under SL treatment at 6 WAG.

Figure 4. Cowpea plants under SA treatment at 6 WAG.

Figure 5. Cowpea plants under SL+SA treatment at 6 WAG.

Figure 6. Cowpea plants under COU treatment at 6 WAG.

Figure 7. Cowpea plants under COU+SL treatment at 6 WAG.

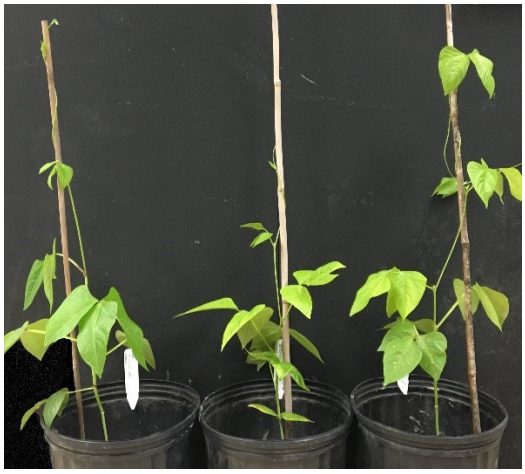

Figure 8. Cowpea plants under GYP treatment at 6 WAG.

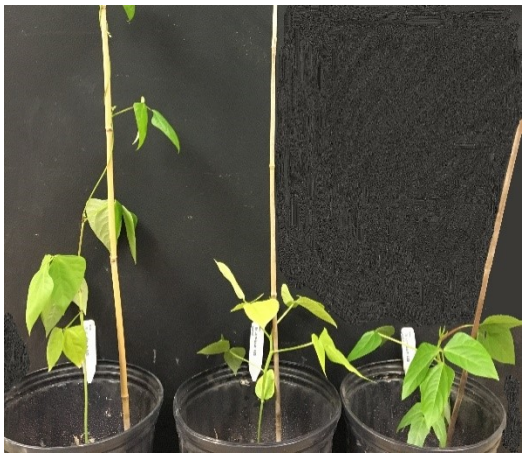

Figure 9. Cowpea plants under GYP+MYCO treatment at 6 WAG.

Figure 10. Cowpea plants under CS treatment at 6 WAG.