

Research article

Facilitating AMF activities in Moringa species for reforestation purposes in Lake Victoria basin, Kenya.

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ABSTRACT

Current knowledge in improved AMF symbiosis integrates plant growth promoting rhizobacteria (PGPR) inoculum to increase mycorrhizal populations in tripartite synergism *in planta*. A biotest was conducted on the three rhizospheric soil types representative of Lake Victoria basin (LVB) using AMF host *Plantago major* L. as inoculant for Moringa plants. PGPR inoculum in chickpea rhizobia was added into the experiment. Within six months, full arbuscule occupancy (>90%) in the root cortex was realized in samples established in paddy vertisols/histosols. Dual inoculation (AMF+PGPR) gave higher yields than single AMF inputs or control samples. The findings support integration of dual (AMF+PGPR) inoculum as a viable method in improving growth conditions of Moringa species in its habitat in LVB, Kenya. **Copyright © ASETR, all rights reserved.**

Key words: Freshwater ecosystems, mycorrhiza, plant growth-promoting rhizobacteria, Moringa

Introduction

Lake Victoria is eutrophic. Fresh water biomes like Lake Victoria basin (LVB) face extirpation with exponential growth rate of 3%, (>30 million residents) exerting high pressure on soils, with persistent mineral restocking. However N and P are highly soluble leading to mass water pollution. Njiru et al. (2002) reported on water quality decline caused by increased inflow of applied fertilizers from agriculture. For LVB ecosystems improved biological methods such arbuscular mycorrhizal fungi (AMF) and plant growth promoting rhizobacteria (PGPR) in legume rhizobia or in combination with organic ligands in Moringa plant growth enhancer (MPGE, Knopf, 2012) could supplement or even replace conventional fertilizers.

Mycorrhizas improve plant nutrient uptake, through filamental networks in hyphae within microsites, increase plant survival and growth rates, tolerance to edaphic stresses and pathogenic protection among several other functions in the soils (Barea et al., 2005; Augé et al., 2001; Medina and Azcón, 2010; Gianinazzi-Pearson and Schüepp, 2002). Glomeraceae taxa categories representing natural classification based on molecular phylogenetic analyses at present are recognized as *Glomus macrocarpum* and Claroideoglomeraceae of Glomeraceae family (Schüßler and Walker, 2010) with updated taxon nomenclature within *Glomeromycota* (Schüßler et al., 2001).

It is also known that most rhizospheric colonizing bacteria typically produce metabolites like siderophores, biosurfactants or organic acids that stimulate plant growth (Glick, 1995). Moreover, mycorrhizas enhance growth, composition and activities of microbial communities by altering root exudation (Wamberg et al., 2003), stirring biochemical changes (acid phosphatase and nitrate reductase) in P and N metabolism in plants thereby promoting plant nutrition. In the complex interaction with bacterium encodes at symbiosis, the basic enzymatic machinery for converting molecular N into NH_4^+ plus a number of genes required, a yield of up to 300 kg N $\text{ha}^{-1} \text{yr}^{-1}$ (Heckman, 2006) could be realized. However in the absence of AMF formation, P scarcity in soil limits legume rhizobia establishment and N^2 fixation (Barea et al., 2005).

Developments in several investigations have confirmed improvement of plant performance through dual AMF+ PGPR compared to single AMF inoculation. The studies of Negi et al. (1989) demonstrated that interactions between mycorrhiza and PGPR increased growth and nutrition of the barley plants, where mycorrhizal plants alone had no effects. Wander et al. (1994) using three farming systems: (1) animal-based (cover crops and animal manure only), (2) legume based (cover crop only), and (3) conventional (N-fertilizer) observed that combined AMF+ PGPR model had higher levels of microbial activities and diverse species. Requena et al. (2001) showed inoculation with AMF and rhizobial N-fixers not only enhanced the establishment of key plant species, but similarly increased soil fertility and quality. The dual symbiosis increased the soil N content, organic matter, hydrostable soil aggregates and enhanced N transfer from N-fixing to non-fixing species associated within the natural succession. Rabie et al. (2005) reported that mycorrhized plants exhibited improvements in all measurements compared to non-mycorrhized ones at all salinity levels, especially in the presence of PGPR. However soil heterogeneity and mycorrhizal *autochthony* (Knopf et al., 2013) may influence performance of certain plants such as Moringa species dependant on growth stages.

Moringa (class: Moringaceae; genera: Rosidae) is a fast-growing tropical multipurpose tree. The importance of Moringa species are multiple, from domestic to industrial applications. *M. stenopetala* is indigenous to LVB, Kenya and other parts of East African regions. De Saint-Sauveur, (1991), Makkar and Bekker, 1997, Sajidu et al. (2006) Ayssiwede et al. (2011), have reported on the uses of Moringa in human food, multivitamins,

animal/poultry feeds, medicinal/antioxidants, fungicidal/pesticidal, fertilizer, water coagulants/water purifier and even soil-water resource remediation. In particular, Moringa species, in symbiosis with AMF can aid reforestation in degraded regions of LVB in simplicity.

MATERIALS AND METHODS

Soil sampling, treatment and storage

Soils were sampled from three regions representative of Lake Victoria basin (LVB), Kenya with altitudinal heights of 800, 1300 and 1800 meters above sea level. Paddy vertisol/histosols were cored at 00° 42' 00'' N and 37° 22' 00'' E, in a rice growing district. At 00° 05' 04.77'' S and 35° 07' 57.51'' E location. Clay alfisols were sampled from a commercial sugar plantation area at 1300m above sea level and loamy oxisol came from humid highlands with high rainfall at 1800m above sea-level. The fields are frequently-tilled using multiple techniques. Soil coring points were in the arable lands mostly used for annual crops. The fourth type was a standard growth substrate "Biotopferde®" used for optimal germination in growth chambers or greenhouses. The rhizospheric soils had been air-dried in the shade for two weeks, organic matter removed, pulverised at 2 mm size, stored in a laboratory at room temperature, analysed using VDLUFA MB I, D2.1 (Fingerprobe) A 5.1.1 (pH-Wert) and A 6.2.1.1 CAL-Methods, version 2002 with the following chemical contents (TABLE 1). The standard soil with pH (CaCl₂):5, 8, KCl in g/l 1, 1 Solutes 150mg/l N, (CaCl₂) 150mg/l P₂O₅, phosphate (CAL) 210mg/lK², potassium oxide (CAL) organic substance, 85% was used concurrently.

TABLE 1: Chemical characteristic of rhizospheric soil types

Soil	Paddy	Clay	Loam
Organic Carbon (%)	2,07	2,25	2,39
Total Nitrogen (%)	0,14	0,13	0,24
Nitrat-N (CaCl ₂) mg/100g	3,04	1,48	1,07
Ammonium-N (CaCl ₂) mg/100g	0,28	0,06	0,96
CaCO ₃ (%)	< 0,2	4,5	28
P (P ₂ O ₅ -CAL m) mg/100g	6	6	2
K (K ₂ O ₅ -CAL m) mg/100g	42	11	33
Cr mg/kg	67	44	28
Cu mg/kg	27	10	1
Ni mg/kg	39,7	23,6	16,9
Co mg/kg	26,9	18,7	11,9
pH Value CaCl ₂	5,7	7,7	4,3
Calcium	<0,2	4,5	<0,2

* **Standard (Boitopferde®) chemical contents;** pH 5, 5 (CaCl₂); Salts, (KCl) in g/l 1, 1; Solutes 150mg/l N, 150mg/l P₂O₅; Phosphate (CAL) 210mg/lK₂; potassium oxide (CAL) and 85% organic substance.

Seed collection, preparation and planting

Seeds from Kenya were acquired from Orongo village and at the Kenya Forestry Research Institute (KEFRI), Maseno sites within LVB within co-ordinates (UTM) 699, 285.37 S and 9,986,154.13 E; 34° 45' / 0° 00': 0° 00' / 35° 00'; 34° 45' / 0° 15': 0° 15' / 35° 00' respectively. The first set of germplasm was developed from Moringa seeds grown in paddy “sticky black cotton soils” of vertisol/ histosol origin. The second set had acidic loamy oxisol background. The seeds were packed in plastic bags for use in this study.

The seeds were dehulled, the nuts soaked in sterile water for three days under room temperature to accelerate germination with frequent water changes to avoid fungal contamination. The seeds were planted in vermiculite initially and transplanted into different soil types according to treatments after fourteen days of germination. These were replicated three times into blocks according to soil types.

Inoculum development

Cultured AMF propagules were acquired from the following species: A *Glomus* species which was earlier affiliated to *Glomus hoi* (origin: culture UY110 from University of York) but identified to most likely represent a different species currently (Redecker et al., 2013), registered as BEG 104, *Funelliformis mosseae* (or *G. mosseae*), BEG 68 (Schüßler and Walker, 2010); Nicolson and Gerderman, (1963); Berch and Trappe, (1985), *Rhizophagus irregularis* (Schüßler and Walker, 2010) and *Funelliformis mosseae* used as (*Glomus mosseae* and *G. intraradices*) culture in cocktail form was used to produce AMF inoculum via *Plantago major* L. raised from seeds collected from open sources.

After incubating the seeds for 2-3 days, the germinated seedlings were transplanted into 5x5x5 cm of 650 ml plastic containers filled with autoclaved sand of 0.5-1mm size, collected from “Kronthaler Kieswerk,” Freising, Germany. The sand was washed several times with running tap-water and finally in sterile water. A cocktail of 2g inoculum was applied to each pot for 15 pots in total. These were irrigated daily using Hoagland and Arnon (1950) nutrient solution in 1/10 strength.

After 14 days of germination Moringa seedlings were inoculated with AMF and legume rhizobia in chick pea, set in blocks as follows; block 1→AMF inoculum, block 2→ AMF+ PGPR, block soil types and transferred into greenhouse with ambient light condition; block 3→harnessed autochthonous AMF and block 4→ AMF (*G. mosseae*, *G. intraradices* and *G. hoi*), as allochthonous mycorrhiza in this research. The blocks were set on a greenhouse bench with mean temperature of 20°C under ambient light supply.

Irrigation was done *via* water pipe sprinkler once every day at the beginning of the experiment according to plant need. Soil moisture contents in the rootainers were assessed using time domain reflectometry (TDR; TEKTRONIX sensor, 1502 C/Tektronix Oregon, USA). A 12 cm head with two pins was installed in each root compartment for soil moisture contents reading.

In all experiments data was taken on basal stem diameter (BSD) below cotyledon defoliation point, plant height and biomass. BSD and plant height were taken at two weeks intervals for at least a period of 2-6 months in each case.

Mycorrhiza Identification, quantification and evaluation

Fine roots were harvested from different soil types. The roots were washed carefully in running water. Root segments were soaked in KOH 10% at room temperatures 18-22°C. KOH solution was removed and HCl applied for 1-2 hours. In case of intensive coloration depigmentation using alkaline solution in 3 ml of NH₄OH to 30 ml of 10% H₂O₂ and 567 ml of tap water (Brundrett and Abbott, 1994) was applied. The cleared roots were incubated in ink and vinegar according to Vierheilig (1998) for 12–24 hours at room temperature. The stained segments were mounted on slides for light microscopy, using Leitz Aristoplan® microscope (Wetzlar, Germany) connected to KAPPA® digital camera for root analysis. Morphological criteria (Brundrett and Abbott, 1994) were used to define anatomical key mycorrhizal features. AMF scoring procedure was done according to Trouvelot et al. (1986) where abundance was based on mycorrhizal structures such as intraradical vesicles, hyphae, and arbuscles and coils presence.

Statistics

A two-way analysis of variance (ANOVA) based on soil and inoculum effects on BSD, height and biomass increments values were tested using GenStat 9th Edition for windows (VSN) International Ltd., U.K.), to ascertain if there were significant differences between soils and treatments. Data were checked for homogeneity of variance and normality by analysis of the residual. Predictions from regression model based on soil and inoculum effects on BSD, height increments were applied for data robustness. Differences were considered significant if $P < 0.05$.

RESULTS

Plant response

AMF inoculated Moringa seedlings grew faster than control plants; however dual inoculation (AMF+ PGPR) constantly gave higher values in basal stem diameter (BSD) and heights compared to single AMF inoculation or controls, with larger variation (Fig. 1). Inoculated samples did not shed their fine roots much as was observed among control samples in native /rhizospheric soils. With increasing time, leaf defoliation was higher in non-inoculated samples compared to either AMF or dual AMF+PGPR inocula. Special was the performance in standard and paddy vertisol/histosol types. Although inoculum promoted growth of *M. oleifera* and *M. stenopetala*, soil factor equally gained importance. Plants grown in paddy soils and standard soils had larger BSD values compared to clay alfisols and loam oxisols. Faster growth rates and plant vigour were recorded in inoculated Moringa seedlings, although it was not clear whether growth rates were influenced by plant competition in the rhizospheric soils or mycorrhizal effects, before weed control. Single AMF inoculated Moringa were slightly shorter than dual (AMF+ PGPR) inoculated seedlings and lost leafy biomass more easily in the three native soils.

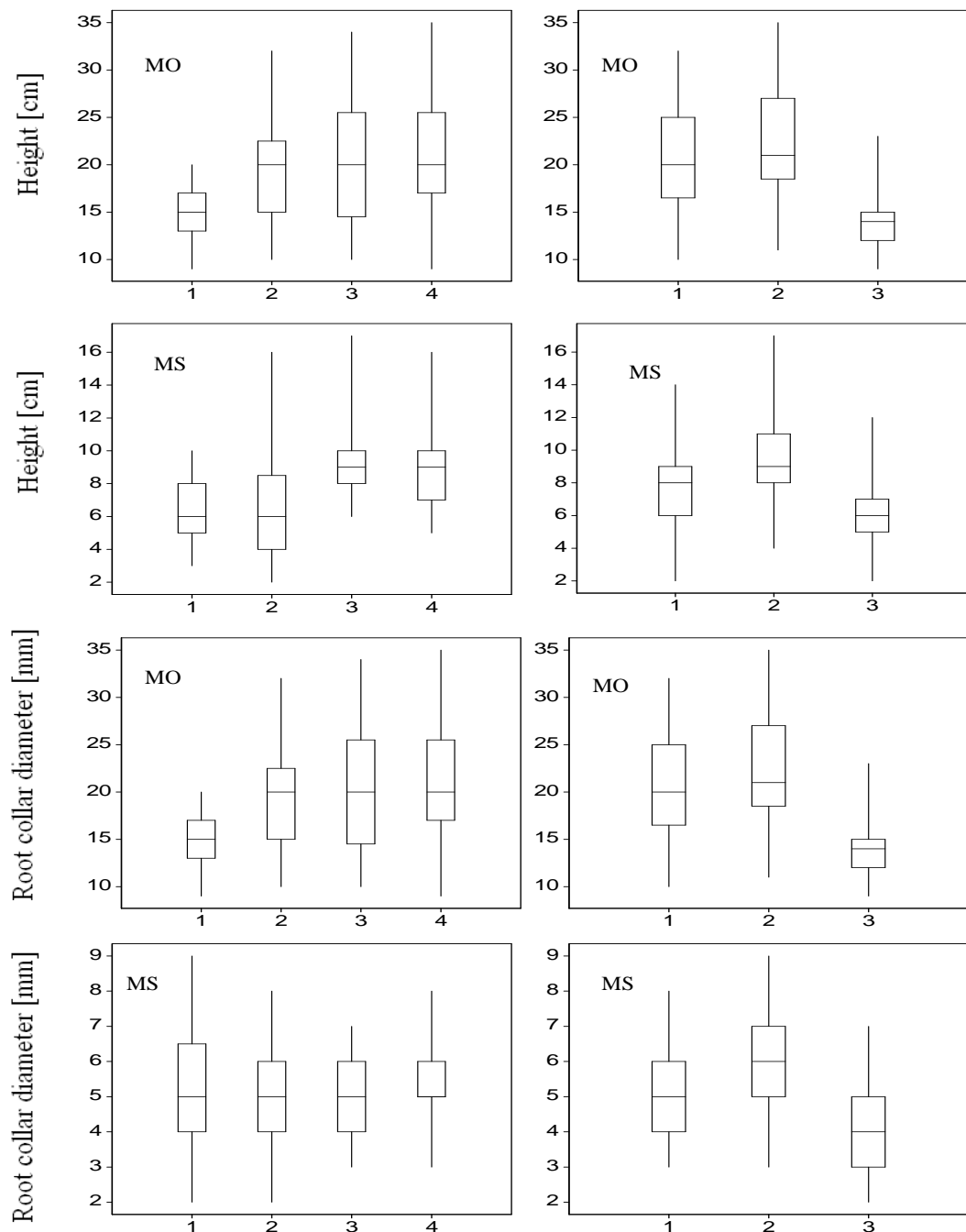


Figure 1: Interactions between (A) soil types, 1-4 (loam oxisols, clay alfisols, paddy vertisols/histosols and standard soils respectively); (B) treatments 1-3; arbuscular mycorrhizal fungi (AMF), AMF+ Plant growth promoting bacteria (PGPR) and control respectively on root collar diameter/BSD and height increments of twenty months old *Moringa stenopetala* (MS) and *Moringa oleifera* (MO). Box range 25-75th and 50th percentile median. Whisker represents highest and lowest values. (n=6, Significant $\alpha=0.05$).

Between paddy vertisols/histosols and standard types, there was only minor or no differences in *Moringa* growth increment values. AMF inoculated plants were more vigorous and taller than controls without inoculum. BSD of seedlings grown in standard “Biotopferde®” soils were larger than those in paddy soil types. Greater differences were evident in *M. oleifera* height values compared to *M. stenopetala*.

In a two way ANOVA, Soil and inoculum influenced plant heights of both *M. stenopetala* and *M. oleifera* with significant differences ($P < 0,001$). Soil versus inoculum interaction was equally significant to height increments of *M. oleifera* species unlike *M. stenopetala*.

Inoculation induced biomass increments (Fig 2). While larger variations could be observed on *M. stenopetala*, *M. oleifera* showed mixed response to treatments. AMF+ PGPR showed larger variations compared to single or non-inoculated plants. Although treated samples exhibited higher root:shoot ratios, the differences were obviously not so large especially on *M. oleifera*. AMF cocktail inoculants had no influence on biomass increments.

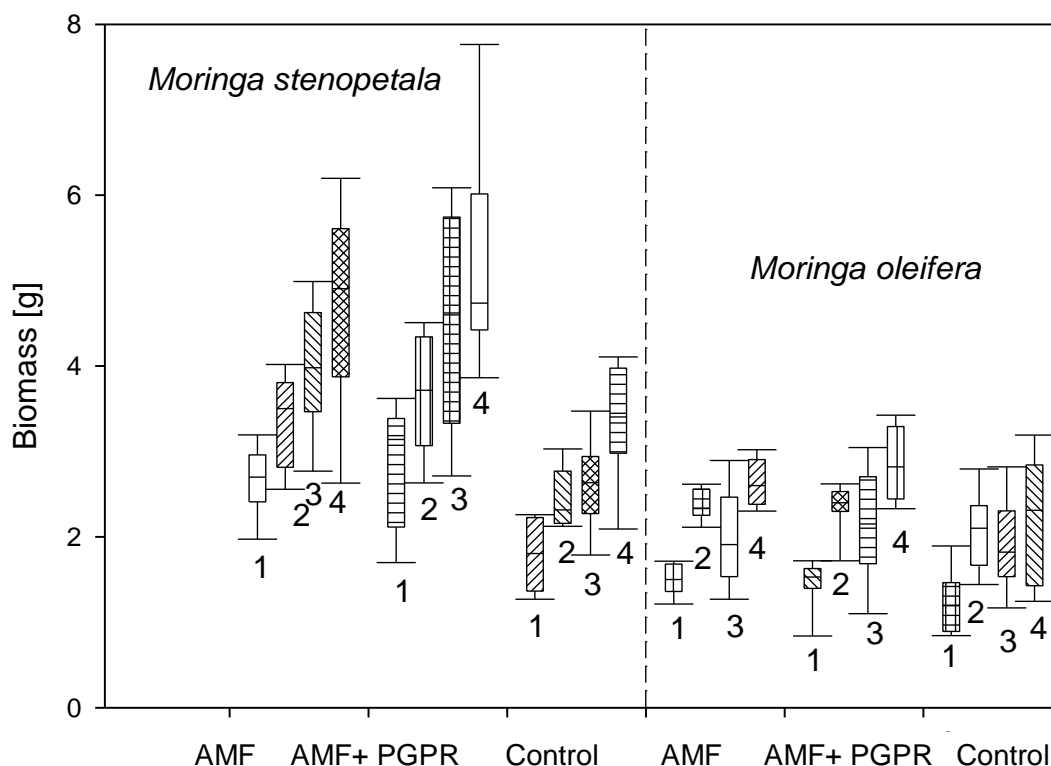


Figure 2: Changes in biomass at inoculation of *Moringa stenopetala* and *M. oleifera* seedlings 4 different soil types (F) e.g. F1: loamy oxisols, F2: clay alfisols, F3: paddy vertisol/histosols) and F4: as a standard substrate “Biotopferde®”) grown under ambient light in greenhouse. Treatments: arbuscular mycorrhizal fungi (AMF) and AMF + PGPR in chickpea rhizobia. Control samples are non-inoculated seedlings with simple watering. Box range 25-75th and 50th percentile median. Whiskers indicate highest and lowest values. (Significant $\alpha = 0.05$).

AMF bioassays

Microscopy into the hyphopodium revealed diverse mycorrhizal structures, mostly of *Glomus tenuis* taxa, occupying the corticle cells of fine roots. ‘Arum-type’ intracellular arbuscles in infection units and hyphal coils in ‘Paris-type’ mycorrhiza were quantified from the paddy histosols. Rootlets sampled from the paddy soils registered upto 90% and above degree of colonization. Apart from arbuscle colonization, the dark septate endophytes (DSE) that mostly identified among weedy endemics occupied fine roots of plant diversity

(TABLE 2). Biomass records from plant samples with this characteristic (dual AMF+DSE root colonization) had higher values.

TABLE 2: Estimates of arbuscular mycorrhizal fungi (AMF) and dark septate fungi (DSE) quantified in 6-18 months old plant rootlets from Moringa and plant diversity identified from the rhizospheric native and standard soils. *) Colonization intensity %.

		Loamy	Clay-loamy	Paddy
2009	Arbuscles	○	○	●
	Dark septate endophytes	●	●	●
2010	Arbuscles	○	●	●●
	Dark septate endophytes	●●●●●	●●●	●●●●●
2011	Arbuscles	○	○●●	●●●●●
	Dark septate endophytes	●●●●●	●●●●	●●●●●
*) ○ is 0%		●	●●<10%	●●●<50%
				●●●●>50%
				●●●●●>90%)

DISCUSSION

Smith and Read (2008), Lodha and Burman (2000), Rosemeyer et al. (2000), Requena et al. (2001), Mantelin and Touraine (2004) and Barea (2005) already reported on effects of PGPR on plant performance and the need to increase rhizobia in the host plants. Moringa plant growth rates, biomass and vigour were observed to be dependant on inoculum application, the two factors concurrently depicting significant differences at ANOVA tests. Dual (AMF+ PGPR) inoculum showed better plant performance in BSD, height and biomass increments than single AMF inoculation or non-inoculated seedlings, consistent with these reports. Degree of variation in turn was observed to be dependant on levels of inoculum application (dual or single) although to an extent growth behaviour was more time-dependent. Moreover it is clear that at initial stages of plant growth, mineral depletion activities depends on plant development rather than uptake functions (De Freitas et al., 1997; Mantelin and Touraine, 2004). Although dry weight biomass of *M. stenopetala* depicted higher values, it was not always consistent in *M. oleifera* when subjected to tests. In all cases AMF+PGPR inoculation proved to be an effective tool in bioavailable plant nutrients, increasing growth rates and vigour, indicated by increased assimilation processes (Flores et al., 2002) enabling effective nutrient and water uptake, resulting in plant growth. The combination (AMF+ PGPR) was however more efficient in improving yields compared to AMF singly in this model. The reason for the constant growth and biomass differences may well be explained by plant competition for scarce nutrients in native soils exerted by weedy competitors and to some extent, inoculum biomass. Further research is needed in establishing inoculum banks that take care of resultant donor-sponsor resource competition in such soil conditions.

The intense colonization by the dark septate endophytes (DSE) in weedy endemics in rhizospheric soils gave new insight into increased plant competition where survival of plant diversity depended on degree of coalition (Knopf et al., 2013) within rhizospheric soils. Moringa lost vigour at this face, being non-obligate amidst soil poverty in the experiments. The frequently dark septate endophytes, DSE, explained the complex nutrient

acquisition (Mandyam et al., 2010) developments in plants at stress, increasing nutrient sequestration and scavenging potentials within mycorrhizosphere.

The R:S (Root-shoot) ratio depended much on treatment variability. Plants without treatments defoliated faster than inoculated seedlings resulting in lesser biomass values frequently. In both species (*M. stenopetala* and *M. oleifera*) situations, AMF+PGPR had greater R:S ratios while control samples without inoculum showed lesser R:S ratios. Inoculum did not always increase R:S ratios in Moringa although differences in leaf, stem and roots at treatments or soil factor were evident. The seedlings established in paddy soils showed rather larger variation compared to those in standard. The higher root biomass indicated heavy root investment in Moringa tubers, a feature of most desert plants with food reserve functions in developments. There was a slight difference between root and shoot biomass values in *M. oleifera* at two months. Roots are strong sinks of assimilates under P stress, increasing the R:S ratio (Mollier and Pellerin, 1999). The R:S ratio explains degree of acquisition of edaphic and aboveground resources (Brouwer, 1983) that affected Moringa in most experiments. Mineral acquisition increased with plant mineral availability, explaining allometric growth differences, depending on treatments and conditions of growth. The root tuber growth dimensions were positively responsive to inoculum indicating efficient food reserve investments in roots at inoculum. Samples treated with AMF+PGPR had larger tuberous diameter compared to AMF singly showing significant differences in tuber development. This is an indication of improved nutrient uptake due to larger food reserve formation. Interesting were the tuber parameter values in AMF+PGPR treatments which induced larger tuber diameter. Improved nutrition due to symbiosis enables improved stress tolerance (Augé et al., 2001; Allen, 2007) a factor that could improve Moringa growth conditions ecologically.

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