Improving Moringa Growth by Using Autochthonous and Allochthonous Arbuscular Mycorrhizal Fungi in Lake Victoria Basin

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Abstract

Biological methods such as mycorrhiza biotechnology used for raising and sustaining soil fertility in agricultural ecosystems close to freshwater biomes are gaining attention. However, ubiquitous ecological conditions may subject mycorrhizal management to choices that depend on inoculum sources (autochthonous or cultured). Effects of AMF on growth of *M. stenopetala* and *M. oleifera* were evaluated using three native soil types, representative of Lake Victoria basin and a standard substrate. Autochthonous AMF was harnessed from the native soils while allochthonous AMF cocktail was acquired from culture banks of *Glomus hoi*, *G. mosseae* and *G. intraradices*, using *Plantago major* as plant indicator and trap-culture. The soils were blocked according to tillage intensities. *P. major* supplied Moringa with AMF inoculum. To facilitate mycorrhization, nitrogen fixing bacteria (NFB) in chickpea rhizobia inoculum was integrated. The hyphopodium assays revealed > 90% arbuscle occupancy in root cortex of plants established in paddy LT soils. AMF inoculation improved growth and biomass of both Moringa species. Autochthonous AMF inoculum had relatively higher biomass turnover compared to cultured AMF. The presence of dark septate endophytes (DSE) in plant biodiversity gave a new insight into target plant performance at plant competition for nutrients. Results reveal that inoculum AMF and NFB are potential candidates in optimizing plant production technology applicable in eco-sensitive-oriented low in-put agriculture.

Introduction

Methods of soil fertility improvements and sustenance in Lake Victoria ecosystem are still rudimentary. Effective replacement to fertilizer has not been discovered suitable for such eco-sensitive large water systems. Although benefits of arbuscular mycorrhiza fungi (AMF) have been richly explored globally, implementation is still at infancy or non-existent in Lake Victoria region. Madadi *et al.* (2007) reported on P flux in the basin where atmospheric deposition and land runoff accounted for 90% of P and 94 % of N inputs from agricultural fields, reflecting growing dependency on fertilizer, which has caused the eutrophic state of the lake.

AMF phylum *Glomeromycota* (Schüßler *et al.*, 2001) transfer phosphorus (P) to the root system in exchange of photosynthates ranging between 5 to 85% of carbon element, depending on plant species (Treseder &Allen, 2002) mostly in *P* deficient soils. These are organisms packaged in microscopic units (Allen, 2007) known to grow in soil pores down to 2ƒn˜m and even penetrate the rock matrix, while individual hyphae may only be 2-10ƒn˜m in diameter (Staddon *et al.*, 2003).

Glomeromycotan intracellular fungi form trunk hyphae (3-6 mm), fine arbuscules (0.8-1.5 mm) and vesicles (20-30 mm). The monomeric glycoprotein structures in hyphae

are coated with glomalin, bound together by hydrophobic interactions (Nichols, 2003) Knopf *et al.*: Improving Moringa growth by using arbuscular mycorrhizal fungi 53 forstering stable soil macro-/microaggregates > 0. 25 mm (Tisdale & Oades, 1975; Tisdall & Nelson, 1979). Research trends in the Glomeraceae taxa categories representing natural classification based on molecular phylogenetic analyses at present are recognized as *Glomus macrocarpum* and *Claroidoglomeraceae* of Glomeraceae family (Schüßler & Walker, 2010).

Allochthonous mycorrhiza are commonly cultured off-site while autochthonous cultures are communities in rhizospheric soils that can be harnessed to develop inoculum banks for possible application within the same environment. Limited availability of propagules could invite allochthonous mycorrhizal cultures in biological soil fertility improvement domains. Autochthonous preferences in inoculation strategy guarantees successful mycorrhizal re-establishment in degraded soil and are physiologically and genetically adapted to environment (Caravaca *et al*., 2003). Effectiveness of either autochthony or allochthony of fungal strains could be much dependent on species, edaphic factors, etc. Marulanda *et al*. (2007) documented that autochthonous AMF strains formed highest intraradical arbuscular development and produced higher lavender root biomass with efficient N and K absorption than with the inoculation of similar allochthonous strains under drought conditions. Requena *et al.* (2001) confirmed that dual inoculation with autochthonous AMF and rhizobial NFB not only enhanced the establishment of key plant species, but increased soil fertility, quality, soil N content, organic matter, hydrostable soil aggregates and enhanced N transfer from N-fixing to non-fixing species associated within the natural succession. Schreiner (2007) observed that plant growth was not affected by either native or non-native *G. mosseae* isolates in one case, although in another set-up non-native isolates were registered to be more effective in promoting growth and nutrient uptake compared to native ones.

Nevertheless, long term ecological implications of cultured specimens are unspecified. Situations such as water stress, invasive species or ruderal weeds may alter benign association in mycorrhization with gross impacts. Conditions fluctuate enough that any given association moves back and forth along mutualism-parasitism continuum (Bellgard & Steven, 2011), interfering with organism fitness, resiliency and functions. Like introduced species that often become invasive, exotic propagules may require caution before general application. Currently dark septate fungal endophytes (DSE) are gaining attention in mutualistic symbiosis, parasitism or even antagonistic impacts in the mycorrhizosphere, identified frequently in grasses and forbs (Jumpponen & Trappe, 1998), as well as tall grass prairie in complex nutritional acquisition (Mandyam *et al*., 2010). Jaison *et al.* (2012) recently reported that 33% of fruit crops in their study that was associated with DSE fungi, similarly, contained AMF, requiring further investigations based on endophytic roles in mycorrhization. Developments in AMF biotechonlogy also consider rhizobial-mycorrhizal-legume symbiosis where P is bioavailed by legume rhizobia establishment and N2 fixation (Barea et al. 2005) facilitating AMF formation. Studies have confirmed that mycorrhiza is mutualistically associated with N-fixing prokaryotes, encouraging dual

inoculum in effective mycorrhizal management.

Being native to Lake Victoria ecology, Moringa, in beneficial symbiosis with AMF is practicable Knopf *et al.*: Improving Moringa growth by using arbuscular mycorrhizal fungi 53 Knopf *et al.*: Improving Moringa growth by using arbuscular mycorrhizal fungi 49 when applied in Agroforestry, Moringa (class

Moringacea; genera *Rosidae*) is a monotypic drought tolerant tropical species, falling under "underutilized" plants of the tropics (Price, 2000) that adjust to almost all soil types within altitudes from 600 m to1200 m within 25-40 °C temperature ranges but can survive at 2000 m above sea level and outlive light frosts (Reyes, 2006). *M. stenopetala* is endemic to Kenya, Ethiopia and Somalia, while *M. oleifera* is indigenous to the sub-Himalayan tracts of north-west India, Asia (Makkar *et al*., 2001). The multiple uses of Moringa range from ecosystem services, human/animal/poultry food or multivitamins, soil/ water bioremediation/phytoremediation activities, medicinal usage, fertilizer, fungicide/pesticide and other diverse industrial usages such as oil production. The basis of this study was to improve tropical Moringa plant performance using eco-sensitive methods of enhancing soil fertility relevant to Lake Victoria basin.

Materials and methods

Soil sampling, treatment and storage

Native soil sample selection (Table 1) was based on altitude range of 800-1800 m above sea level, representative of Lake Victoria basin, Kenya. The soils were categorized according to tillage frequency, high tillage (HT), medium tillage (MT), low tillage (LT) and standard substrate (ST) as effective soil control. The paddy LT frequency types were cored from a rice growing district at 00° 42' 00'' N and 37° 22' 00'' E, and an altitude of about 1300 m above sea level. The land tillage is up to 10-16 cm deep.

Clay MT Alfisol came from sugarcane growing area 00° 05' 04.77'' S and 35° 07' 57.51'' E location, an important water catchment area for Lake Victoria. Tillage systems range from heavy machinery tillage, mould ploughs down to small tills privately applied by farmers. The well-drained acidic-red oxisol came from the humid highlands with high rainfall situated at approximately 1800 m above sea-level. The fields are frequently tilled using multiple techniques. The arable lands are mostly used for annual crops but accommodate perennial cropping like tea bushes. The soils were air-dried in the shade for 2 weeks, pulverised and passed through 2-mm size sieve to remove organic matter, stored in a laboratory at room temperature, analysed using VDLUFA MB I, D2.1 (Fingerprobe) A 5.1.1 (*p*H-Wert) and A 6.2.1.1 CAL-Methods, version 2002 in soil analyses methods in an earlier study based in the research centre Helmholtzzentrum, München.

Seed collection, preparation and planting

Seeds from Kenya were acquired from Orongo village in Lake Victoria basin at (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' and Kenya Forestry Research Institute (KEFRI), Maseno within Lake Victoria basin. The first set of germplasm was developed from Moringa seeds grown in paddy "sticky black cotton soils" of Vertisol and Histosol origin while the second set had acidic loam oxisol

background. The seeds were packed in plastic bags and transported to Germany for the study.

The seeds were dehulled and the nuts soaked in sterile water, for approximately about 72 hr under room temperature in order to speed up germination. The water was changed frequently to avoid fungal contamination. The seeds were planted in vermiculite initially and left to germinate before transplant into different soil types according to treatments and space. The blocks were set on a greenhouse bench with mean temperature of 20 °C under ambient light supply.

G. mocrocarpum clade". *Glomus mosseae*, BEG 68 (Berch & Trappe, 1985; Nicolson & Gerderman, 1963), *Glomus intraradices* BEG 31 (Schenck & Smith, 1982) in cocktail form was used to develop an AMF inoculum bank using *Plantago major* from open sources and were left to incubate for around 36 h. Approximately 2 g of AMF inoculum were applied on germinated seedlings and transplanted into $5 \times 5 \times 5$ cm of 650 ml plastic containers, filled with autoclaved sand of 0.5–1mm size, originating from "Kronthaler Kieswerk," Freising, Germany. These were irrigated daily using Hoagland

TABLE 1
Chemical characteristics of native soil types representative of Lake Victoria basin, Kenya

Soil	*Paddy LT*	*Clay MT*	*Loam HT*
Organic carbon (% TM)	2.07	2.25	2.39
Total nitrogen (% TM)	0.14	0.13	0,24
Nitrat-N ($CaCl_2$) mg/100 g	3.04	1.48	1.07
Ammonium-N ($CaCl_2$) mg/100 g	0.28	0.06	0.96
$CaCO_3$ (% TM)	< 0.2	4.5	28
P (P_2O_5-CAL m) mg/100 g	6	6	2
K (K_2O_5-CAL m) mg/100 g	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
*p*H Value $CaCl_2$	5.7	7.7	4.3

*Stnadard soil properties: *p*H 5, 5 (CaC12); Salts, (KC1) in g/l 1, 1; Solutes 150 mg.l N, 150 mg/l P_2O_5; Phosphate (210 mg/1K_2) and 85% organic substance.

Inoculum development

Cultured AMF propagules, *Glomus hoi,* of University of York origin, registered at BEG, (BEG 104), updated as "revised sequences from UY110 (accession numbers KC182044 and KC182045) and BEG104 (KC182048-KC182048) cluster within the

& Arnon (1950) nutrient solution with 10% of its original ionic force.

Moringa seedling establishment in native soils and inoculation

Using flexible Rootrainers® with open book design and deep rooting system,

germinated Moringa was transplanted into 55 cm^3 size trainers of 195 total seedlings replicated into 3 × 65 blocks of plantlets, each in vermiculite for germination under a temperature of 23 °C and a humidity of 70%. After 14 days of germination, the seedlings were transplanted into the native and standard soils. The seedlings were inoculated with AMF cocktail and NFB. Block 1→AMF inoculum, block 2 → AMF + NFB, block 3 ® harnessed autochthonous AMF and block 4 → AMF (*G. mosseae, G. intraradices* and *G. hoi*) as allochthonous inoculum cocktail.

In all experimental blocks, Moringa seedlings were inoculated after 2 weeks with harnessed mycorrhiza, cultured AMF cocktail and rhizobacteria in legumes after transplant. Autochthonous *versus* allochthonous mycorrhizal experiment was set to identify differences in plant performances based on the higher performing native soil with sand at ratio 2:1 (v/v) of paddy LT soils and the standard type without any modifications or fertilizer. Non-inoculated seedlings were used as controls in the experiments.

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 HC1 applied for 1–2 hr. In case of intensive coloration depigmentation using alkaline solution in 3 ml of NH4OH to 30 ml of 10% H_2O_2 and 567 ml of water (Brundrett & Abbott, 1884) was applied. The cleared roots were incubated in ink and vinegar according to Vierheilig (1998) for 12–24 hr at room temperature. The stained segments were mounted on slides for light microscopy, Leitz Aristoplan® microscope (Wetzlar, Germany) connected to KAPPA® digital cameras for root analysis. Morphological criteria (Brundrett & Abbott, 1994) were used to define anatomical key mycorrhizal features. AMF scoring procedure was done according to Trouelot *et al.* (1986) where abundance was based on mycorrhizal structures such as intraradical vesicles, hyphae, and arbuscles and coils presence.

Statistical analysis

Data analysis was done using GenStat 9th Edition for windows (VSN) International Ltd., UK). Data were assessed for homogeneity of variance and normality through analysis of the residuals and *posthoc* comparisons of means of Moringa plants. Linear regression model was applied in a two-way analysis of variance (ANOVA), based on soil and inoculum effects on growth parameters, to ascertain if there were significant differences between soils and treatments. Differences were considered significant if $P < 0.05$.

Results

Plant response and performance

In the biotest, a higher germination rate at 62.5% in paddy LT compared to other native soils (33% and 58%) in total squares per tray was realized. Growth and biomass records were higher compared to clay MT and loam HT. However, a combination of loam HT type and vermiculite improved plant germination (58%) compared to HT soil type without vermiculite, registering 33%. Clay MT with 58% had delayed germination with slower growth rate compared to paddy LT. Paddy LT samples exhibited high mycorrhizal presence in the soils observed in rootlet bioassays. Intensive AMF colonization (Fig.

4) characterized by the presence of arbuscular mycorrhizal structures in the cortical cells was evident.

From microscopic assays in the hyphopodium, a range of mycorrhizal structures, mostly of *G. tenuis* clade was observed, occupying the corticle cells of plant rootlets. Tree-like intracellular arbuscles in 'Arum-type' infection units, AM hyphae, appressoria, arbuscles and intracellular hyphal coils in 'Paris-type' mycorrhiza were quantified from the paddy LT soils. Rootlets sampled from the paddy LT soils registered more than 90% degree of colonization. Abundant dark septate endophytes (DSE) colonization was realized among weedy competitors. At phase II, colonization in paddy LT was still higher compared to other soils, apart from DSE hyphal strands and resting spores.

Changes in Moringa growth characteristics confirmed that soil and inoculum (AMF, NFB) had influence on plant height and basal stem diameter (BSD) of Moringa (Fig. 1). Inoculated Moringa grew faster than controls. Greater heights were achieved with either single AMF or dual (AMF + NFB) inoculation Although AMF inoculated Moringa seedlings revealed higher BSD increments, compared to non-inoculates, AMF+NFB input constantly gave higher values in BSD and heights. Special was the performance in standard and paddy LT soils. Although inoculum promoted growth of *M. oleifera*

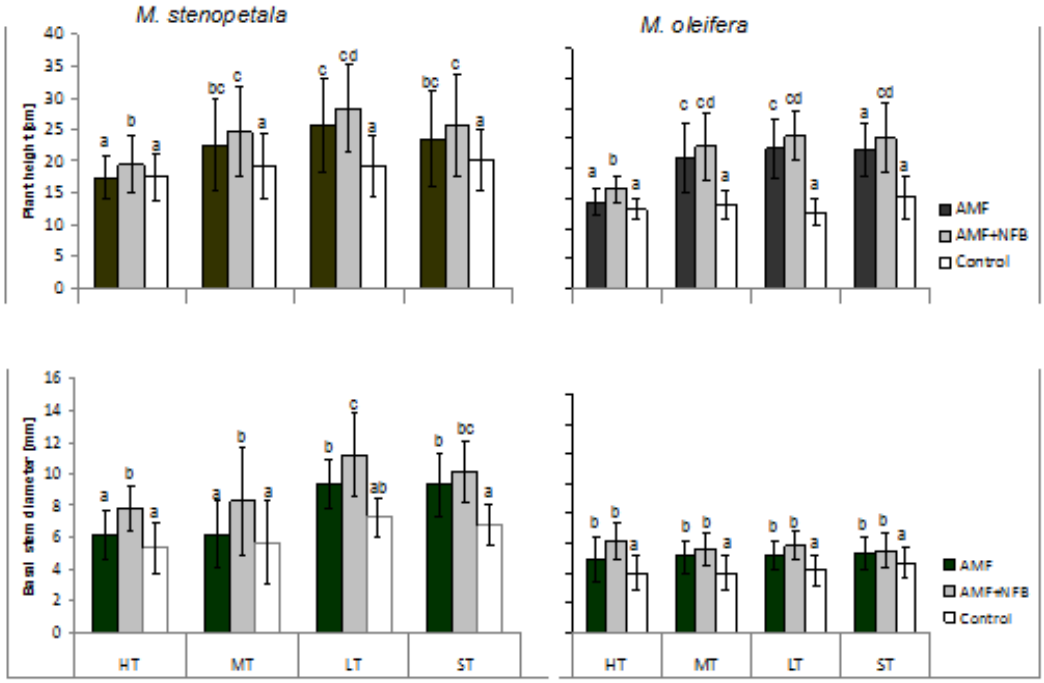

Fig. 1. Development of basal stem diameter and height of *M. stenopetala* and *M. oleifera* relative to treatments (AMF and NFB). The seedlings were established in native and standard soils under ambient light and mean temperature of 20 °C in greenhouse. Data collected at different time frames from 2009–2011. Different letters indicate significant differences by Duncan's multiple-range test. (Mean ± SD; n = 6).

and *M. stenopetala*, soil factor was equally important. Plants grown in paddy LT and standard soils had larger BSD values compared to clay MT and loam HT soils.

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 or mycorrhizal effects initially before weed control.

Comparing the two high performing substrates, paddy LT and standard types exhibited minor or no differences in BSD and height parameters of Moringa growth (Fig. 2). Larger BSD values were realized on plants established in standard soils compared to the paddy LT types.

Autochthonous mycorrhiza seemed to have induced growth but the effect was not so evident in *M. oleifera*. In most cases, seedlings established in cultured AMF cocktail showed larger BSD and greater heights compared to harnessed autochthonous mycorrhizal effects from paddy LT soils.

Even though plant growth improvements were recorded in combined AMF + NFB treatments, autochthony showed mixed responses on height and diameter increments.

Soil factor and treatments showed significant differences on height growth of both Moringa species at P <0,001. Soil factor alone neither influenced BSD growth of *M. oleifera* nor *M.stenopetala* in the variance test. Cultured AMF cocktail had no

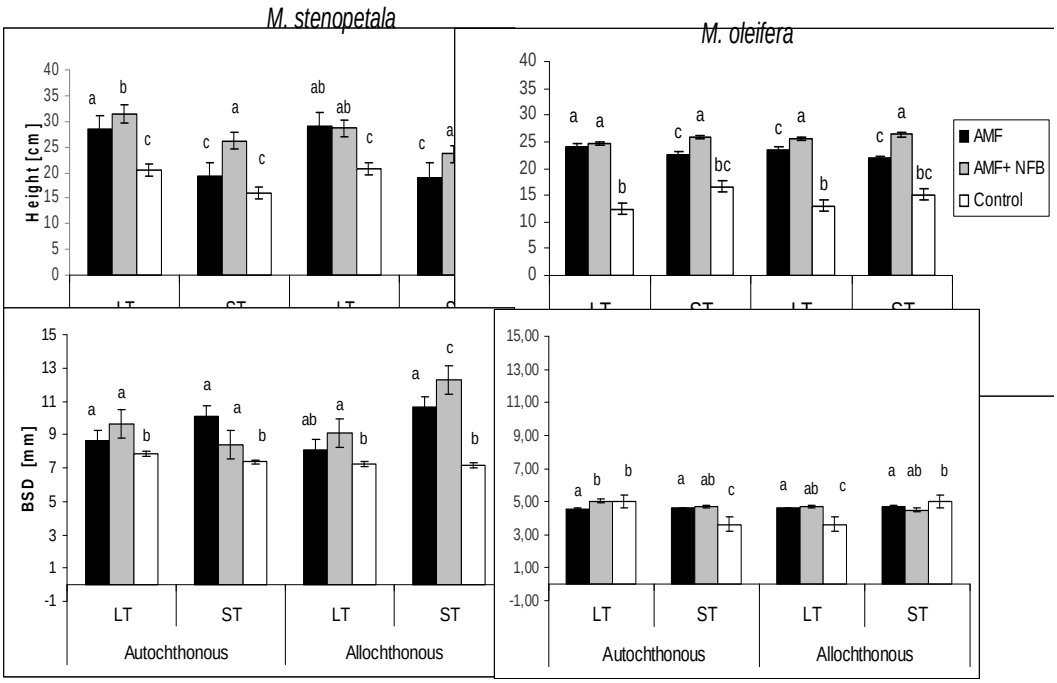

Fig. 2. Soil and inocula influence on basal stem diameter (BSD) and height of *M. stenopetala* and *M. oleifera*. Treatments: NFB, AMF cocktail (*G. intraradicies*, *G. hoi* and *G. mosseae*) and harnessed mycorrhiza from native soils. Different letters indicate significant differences by Duncan's multiple-range test, $P < 0,05$; n = 4.

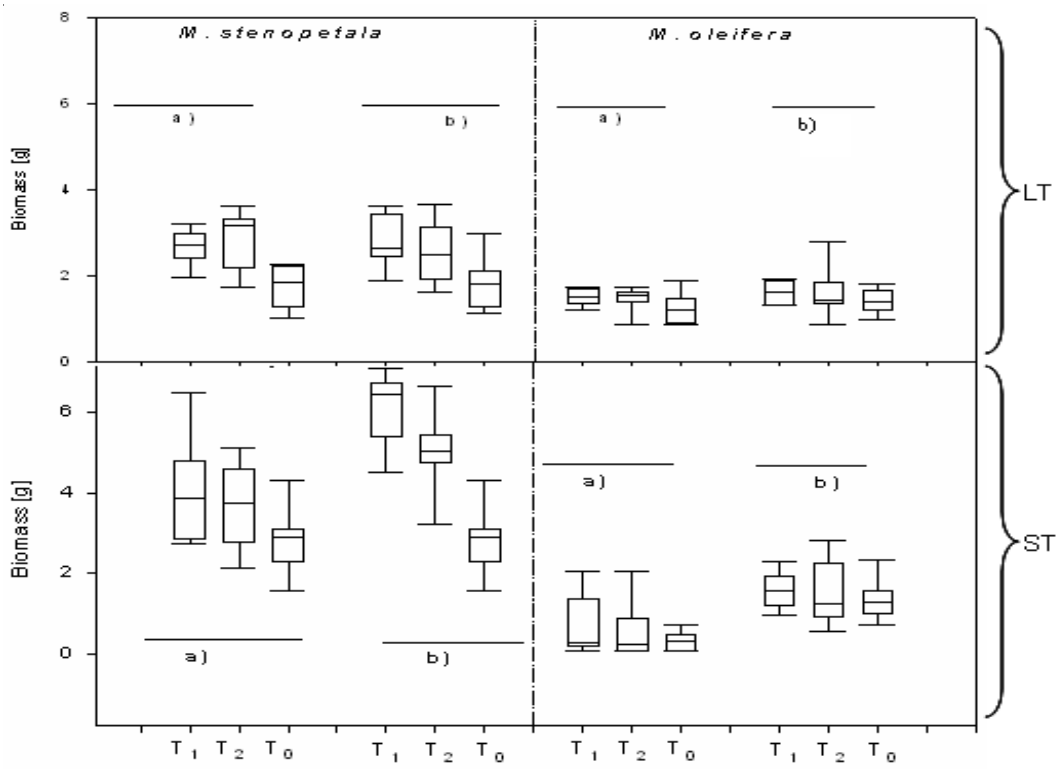

Fig. 3. Changes in biomass at T_1 arbuscular mycorrhizal fungi (AMF), T_2 AMF + nitrogen fixing bacteria (NFB) inoculation on total biomass of 2 year old *M. stenopetala* and *M. oleifera* established in paddy (LT) and standard soil (ST). T_0 are non-inoculated with simple watering, a) allochthonous AMF cocktail, and b) harnessed autochthonous AMF treatments. Box range 25-75th and 50th percentile median. Whiskers indicate highest and lowest values. (Significant á = 0.05).

significant differences in biomass variations (Fig. 3); but indeginous mycorrhizal inoculum showed a slightly higher value on Moringa biomass returns. However the picture was different on *M. stenopetala* especially the plants established on paddy LT native soil which slowed in time.

Larger variations were, however, visible on autochthonous AMF inoculated species compared to cultured cocktail types on biomass records. While changes could be observed on *M. stenopetala*, *M. oleifera* showed mixed responses to inoculation withal. Unlike *M. stenopetala* with evenly distributed median in the analysis, *M. oleifera* biomass distribution was more skewed.

Discussion

Ouahmane *et al*. (2007) observed that cultured *G. intraradices* seemed to promote growth of *Cupressus atlantica* compared to autochthonous mycorrhiza, inoculated plants with the latter mobilized P from rock phosphates more efficiently than allochthonous *G. intraradices.* In this study, autochthonous mycorrhiza seemed to

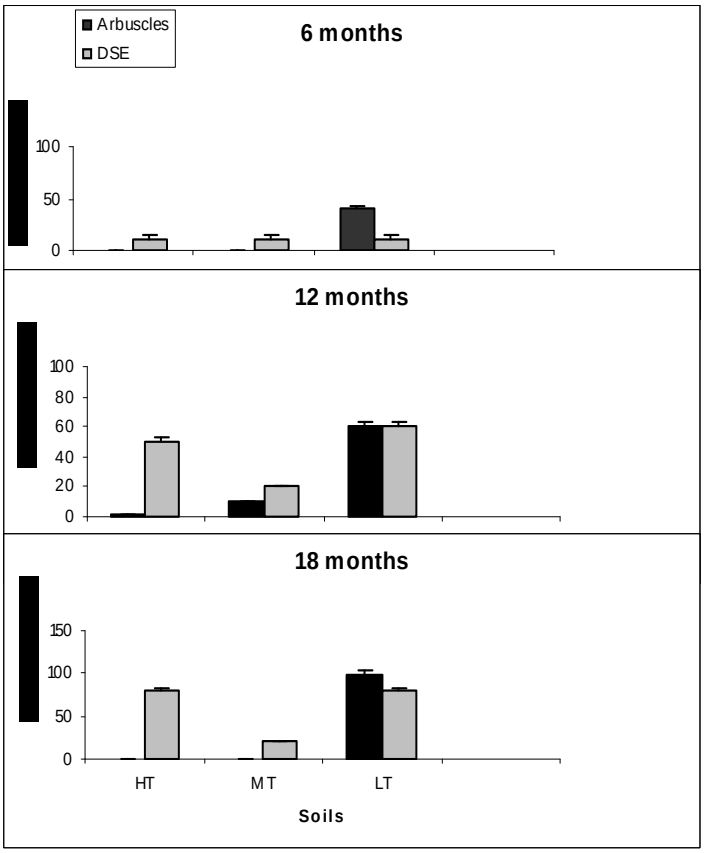

Fig. 4. Estimates of arbuscular mycorrhizal fungi (AMF) and dark septate fungi (DSE) quantified in 6-18 months old plant rootlets from Moringa and plant diversity identified from the rhizospheric native and standard soils.

promote growth but in a much slower rate compared to cultured cocktail, consistent with this literature. Both harnessed indigenous mycorrhiza and cultured AMF cocktail applied on Moringa species established in paddy LT and standard soil improved plant growth in *M. stenopetala* and *M. oleifera*. Basal stem diameter and height values reflected improved plant nutrition at inoculum. It was not clear about the differences in basal stem diameter and height increments in plants inoculated with autochthonous mycorrhiza and the cultured AMF cocktail since performance indicators continued to alternate with time. Although Schreiner (2007) noted conflicting plant growth characterics at AMF inoculation, where no significances were observed by either autochthony or allochthonous *G. mosseae* isolates in a situation, and by contrast, another experimental set-up revealed that non-native isolates were more effective in promoting growth and nutrient uptake compared to native ones. From the experiments, autochtony showed mixed responses in height, diameter and biomass turnover.

Whereas Marulanda *et al*. (2007) reported that autochthonous strains of *G. intraradices* and *G. mosseae* increased root growth by 35% and 100%, respectively, when compared to similar allochthonous strains, the studies on Moringa demonstrated only mixed or slight differences in response to inoculum autochthony. Emergence of weedy competitors from the native soils highly colonized by DSE, affected growth of Moringa probably through increased sequestration and mineral scavenging potentials within mycorrhizosphere (Mandyam *et al.*, 2010). Clear differences were, however, observed on total biomass returns of Moringa species consistent with this literature, where root biomass outweighed shoot biomass in plant developments. Observations on growth parameters showed that treatments rather than indigeneity induced growth and biomass output of *M. stenopetala* and *M. oleifera* however, significant differences existed between dual inoculations and non-inoculates.

Mycorrhizal inoculum was found to be significant in biomass yield increments, supporting (Puente *et al.,* 2004; Barea *et al.,* 2005; Schnepf *et al.,* 2007; Atul-Nayyar *et al.*, 2009) that inoculum significantly promotes plant nutrition and growth. Although mycorrhizal presence affect growth, composition and activity of microbial communities by altering root exudation (Wamberg *et al.*, 2003), it is not clear whether different strains of AMF can produce different effects on soil biochemical properties via enzymes, or conditions which enable native or allochthonous to be more effective (Aguacil *et al.,* 2008). For better conclusions, field tests are important to gain insight into plant behaviour concerning effective mycorrhization on Moringa species.

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