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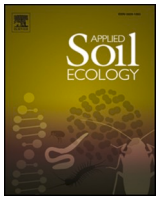

# Rate of hyphal spread of arbuscular mycorrhizal fungi from pigeon pea to finger millet and their contribution to plant growth and nutrient uptake in experimental microcosms

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ABSTRACT

Intercropping is a sustainable agroecological tool known to provide multiple benefits to farmers. Several studies have shown that arbuscular mycorrhizal fungi (AMF) play a key role for the improved grain yields in intercropping systems through facilitative nutrient and water uptake via the common mycorrhizal network (CMN), yet little is known on the rate of hyphal spread. Here we hypothesized that AMF species differ in the rate of extraradical hyphae to spread from one plant to another, thereby affecting the growth of the intercropped plants. To test our hypothesis, we established experimental microcosms in the greenhouse, in which one pigeon pea (*Cajanus cajan*) and two finger millet (*Eleusine coracana*) plantlets were kept in separate pots, connected by soil bridges of 5 or 12 cm length, inaccessible to roots but accessible to fungal hyphae. The pigeon pea plants were pre-inoculated with *Claroideoglomus etunicatum*, *Rhizophagus fasciculatus* or *Rhizophagus irregularis*. All three AMF species led to a strong growth promotion compared to uninoculated control of the short microcosms and more than doubled the biomass of pigeon pea. The biomass as well the phosphorus content of finger millets connected by AMF to the pigeon pea differed with the length of the soil bridge and the species of AMF. By applying $^{15}N$ isotopes to the soil of pigeon pea pots we revealed that in both lengths of the microcosms *R. fasciculatus* and *C. etunicatum* transported nitrogen from pigeon pea to finger millet across distances of up to 12 cm but *R. irregularis* did not. Furthermore, by destructive sampling, we estimated a hyphal spread of 4.1 mm $d^{-1}$ by *C. etunicatum* across a 12 cm soil bridge. We conclude that the row distance between the crops and the choice of AMF species play a crucial role for the application of AMF as biofertilizer.

## 1. Introduction

Intercropping is growing two or more different crop species and genotypes in the same field coexisting for a time (Brooker et al., 2015). Besides improving soil quality, intercropping systems can also act as a buffer against extreme events when one crop is more resilient than the other, which stabilizes yields over time. Improving productivity under intercropping systems requires better understanding of the above and belowground interactions, particularly the interplay between the crop species and beneficial soil microbes. Inoculation of crop species using mutualistic root soil microorganisms like arbuscular mycorrhizal fungi (AMF) can substantially improve yield (Lekberg and Koide, 2005). According to our meta-analysis, AMF facilitated yield responses in crops are particularly high in dry climate and at low available Phosphorus (P) content (Schütz et al., 2018).

Pigeon pea is a deep rooting nitrogen fixing legume while finger millet is a shallow rooted cereal. Pigeon pea is generally a popular crop used for mixed cropping with 65 different companion crops, including finger millet, particularly in India (Ahlawat et al., 2005). There is also a temporal complementarity between the two crops as finger millet is

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harvested earlier. In certain regions, including parts of Southern India, pigeon pea is pre-grown in a nursery before the onset of the monsoon, and transplanted. The transplanting method has the advantage of a longer vegetative phase compared to direct sown system, leading to higher yields (Praharaj et al., 2015). Nevertheless, the success largely depends on the onset of the monsoon and planting time (Pavan et al., 2011). Pre-growing pigeon pea in a nursery is also ideal for the inoculation with AMF, ensuring colonization with a preselected AMF strain. In a recent field study, we have shown improved grain yield in transplanted pigeon pea pre-inoculated with AMFs (Mathimaran et al., 2020). Yet in our field study we did not address whether the pigeon pea inoculated with AMF could also colonize neighbouring finger millet plants, and if so, how fast the AMF hyphae could spread from pigeon pea to finger millet. Since finger millet is grown in rows, it is interesting to know how many rows of finger millet can be colonized from the inoculated pigeon pea. Although it is well known that AMF can connect different plant species to form a common mycorrhizal network (CMN) (van der Heijden and Horton, 2009), there are few studies with regard to the rate of

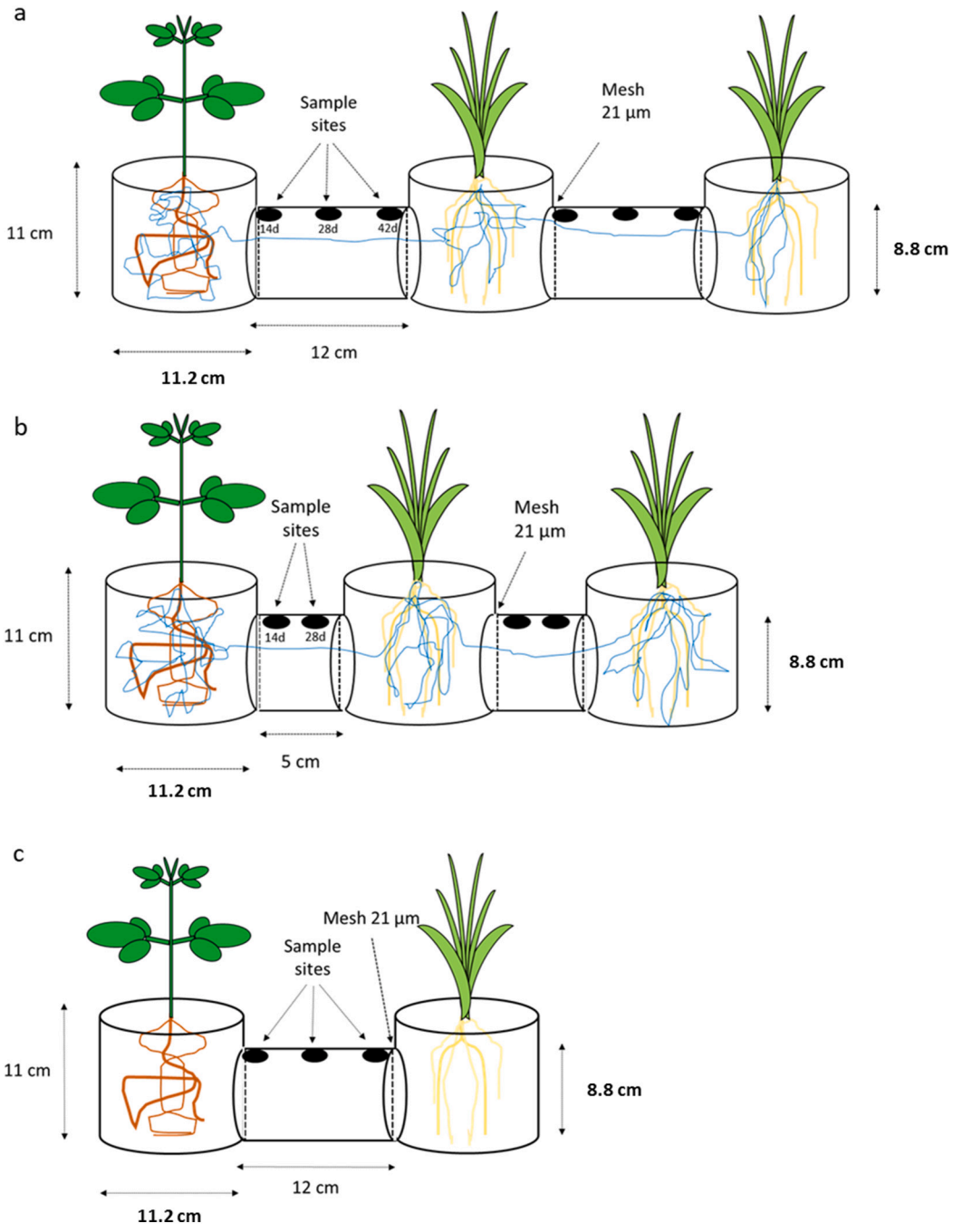


**Fig. 1.** Design of the experimental microcosms. (a, b) Microcosms used in experiment 1, with the inoculated pigeon pea plant (left) and two finger millet plants (right) in individual pots, connected by root-free hyphal compartments, separated by 21 µm mesh size nylon nets (dashed lines). The hyphal compartments (with sampling sites indicated including the day of sampling at a distance of 1.1 cm, 5.9 cm and 10.2 cm) are 12 cm long for the long microcosms (a) and 5 cm long for the short microcosms (with sampling sites indicated including the day of sampling at a distance of 1.1 cm and 5.9 cm) (b). Blue lines represent fungal hyphae at an advanced stage of the experiment where they have already colonized both finger millet plants; (c) Microcosms used in experiment 2, with one pigeon pea plant (left) and one finger millet plant (right), connected by a root-free hyphal compartment (12 cm long), separated by 21 µm mesh size nylon nets on both sides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spread of hyphae eventually forming CMN.

Here we studied, using microcosm experiments, the rate of spread of different AMFs species to connect pigeon pea and two plants of finger millet, representing two rows of finger millet. This knowledge may be important to choose ideal AMF species as "biofertilizer" in pigeon pea-finger millet intercropping systems, particularly for the transplanting system practiced in parts of the Southern India. Optimization of the AMF species could also help in better nutrient and water transfer from pigeon pea-finger millet "bioirrigation" system (Saharan et al., 2018; Singh et al., 2020).

Thus the following hypotheses are addressed with this study:

(i) We hypothesize that AMF species, inoculated on pigeon pea, differ in their ability to connect with finger millet plants, to influence the growth of finger millet and pigeon pea, and to transport nutrients via the CMN. We thus characterised three AMF species under our experimental intercropping setting in experiment 1 and identified the AMF species with the strongest hyphal growth and growth promotion of finger millet for a detailed measurement of the hyphal growth rate in experiment 2; (ii) We hypothesize that inoculation of pigeon pea by AMF during pre-growth, which then colonizes finger millet in the intercropping field, is sufficient for the "biofertilization" of finger millet and will improve its growth and yield.

## 2. Material and methods

### 2.1. Experimental setup

#### 2.1.1. Experiment 1

The experimental setup of the "microcosms" with one pigeon pea plant inoculated with AMF, and two non-inoculated finger millet plants, is shown in Fig. 1. The finger millet plant closest to pigeon pea is hereafter referred to as finger millet 1 (FM1) and the more distant one as finger millet 2 (FM2). The three plant pots were connected by hyphal compartments (HC) with a diameter of 8.8 cm, filled with the same substrate but separated by a 21 μm nylon mesh, which allows passage of fungal hyphae but not roots. A fiber glass mesh with a larger pore size was attached adjacent to the nylon mesh to provide mechanical support; it was fixed by a ring-adapter fitting tightly into the tube. Whenever long HC (12 cm) were used (Fig. 1a) these microcosms are termed long microcosms and whenever short HC (5 cm) they are termed short microcosms (Fig. 1b). Containers were washed with detergent and rinsed with tap water. Then they were sterilized by spraying with 70% ethanol before they were filled with substrate. The growth substrate consisted of Sorbix US Premium oil binder (Chem-Sorb) (Maagtechnic AG, 8600 Dübendorf, Switzerland), quartz sand (0.6–1.2 mm, Alsace, Kaltenhouse, Trafor AG Basel) and sieved (< 3 mm) Loess (Biel-Benken, Switzerland) in a mixture of 1:4:1 (*w/w/w*). Loess was autoclaved with 50 ml water per 5 kg. Quartz Sand and Chem-Sorb were heated for 9 h at 80 °C for sterilization. Nutrient content of the whole mixture was 101 mg P/kg, 200 mg K/kg, 331 mg Mg/kg (all three analyzed via ICP-OES after extraction with nitric acid), 9.58 mg nitrate/kg (water extract and analyzed with UV/Vis spectroscopy), pH 6.3 (Umweltanalysezentrum, Salucor GmbH, Filderstadt, Germany). All treatments and the controls consisted of four replicates. A non-mycorrhizal control was set up for both lengths of microcosms. Microcosms were randomized weekly. Hyphae were sampled by taking soil cores (core diameter 13 mm) at the following distances and times after the microcosms were joined: after 14 d at 1.1 cm, after 28 d at 5.9 cm and in the long microcosm after 42 d at 10.7 cm. The sample sites were refilled with the growth substrate after each sampling to prevent dehydration of the soil and allow regrowth of hyphae.

### 2.2. Microbial inoculants

Three species of AMF were tested and inoculated to pigeon pea: *Rhizophagus fasciculatus* (Thaxt.) C. Walker & Schuessler (formerly *Glomus fasciculatum* Gerdemann & Trappe), strain originating from Bangalore, India (Govinda Rao et al., 1983); *Claroideoglomus etunicatum* C. Walker & Schuessler (formerly *Glomus etunicatum* W.N. Becker & Gerd), strain ISCB 31, originating from DOK field site, Therwil, Switzerland; and *Rhizophagus irregularis* (N.C. Schenck & G.S. Sm.) C. Walker & Schuessler (formerly *Glomus intraradices* N.C. Schenck & G.S. Sm.), strain BEG75. AMF species were maintained in pot cultures with leek as a host plant. Spore numbers were counted after they were isolated with a sugar gradient (Talukdar and Germida, 1993); *C. etunicatum* had 132 spores/g, *R. fasciculatus* 53 spores/g, and *R. irregularis* 15 spores/g. All inocula were adjusted to 15 spores/g. Microbial wash was obtained by wet sieving 100 g of inoculum with 1 l of water through a 32 μm sieve and through a folded filter (Schleicher and Schuell, LS 14 ½). Ten ml of the microbial wash were added to each control microcosm (Koide and Elliott, 1989). All pigeon pea seeds were additionally inoculated with *Bradyrhizobium* sp. strain IHP 195, DSM No. 5969, which was grown on yeast mannitol (YM) Agar plates for 5 days and then transferred into liquid YM Medium. One ml of this culture (adjusted to $10^6$ CFU in 1 ml) was pipetted to the pigeon pea plants.

### 2.3. Plant materials and growth conditions

Seeds of pigeon pea and finger millet originating from India belong to the variety TTB7 and GPU28 respectively (Ankur Seeds Pvt. Ltd., Bangalore, India). Seeds were surface-sterilized by soaking them 30 s in ethanol 96% and 2 min in 5% NaOCl (commercial bleach), then washed first once with 0.01 N HCl and then 8 times with sterile water (Somasegaran and Hoben, 1985). Three pigeon pea seeds were placed into one pot together with 5 g of the AMF inoculant, containing 15 spores/g. After 10 days they were thinned to one seedling per pot and grown for 30 days in their compartment to establish symbiosis. The connection sites were covered by aluminum foil. Finger millet seeds were pre-germinated in vermiculite and covered by sand. Seedlings of a similar size were selected for transplanting into the experimental pots. The hyphal compartment was then filled with substrate as well as the compartments of finger millet, and seedlings (7 d old) of finger millet were transplanted into the finger millet compartments. Plants were watered every two days to field capacity (39%) again without any signs of water stress. Watering was regularly adjusted by measuring the evapotranspiration. The plants were grown in a greenhouse under controlled conditions with 16 h light at 25 °C–35 °C and 220 μE $m^{-2}$ $s^{-1}$, 8 h dark at 20 °C, and constant relative humidity of 65%. Six weeks after transplanting finger millets, 5 ml of a full strength Hoagland's solution without $NH_4H_2PO_4$ was applied to pigeon pea (Gamborg and Wetter, 1975).

### 2.4. Application of $^{15}N$

Six weeks after the finger millet seedlings were transplanted, $^{15}N$ was applied to the soil around the pigeon pea plant. 4 g of $^{15}NH_4^{15}NO_3$ (Cambridge Isotope Laboratories) were dissolved in 4 l water, 4 g of $FePO_4$ was added and the mixture constantly stirred. A portion of 100 ml (containing 100 mg $^{15}NH_4^{15}NO_3$ and 100 mg of $FePO_4$) was then applied to each pigeon pea. $FePO_4$ was applied as the only P source as it is known that pigeon pea can mobilize phosphate from hardly soluble forms by way of root exudates (Ae et al., 1990; Shibata and Yano, 2003). Thereafter the plants were watered to ensure infiltration of $^{15}N$, taking care that no liquid leaked out of the pot.

### 2.5. Harvest

In experiment 1, plants were harvested 23 weeks after pigeon pea was sown. Finger millet plants were brought to maturity to assess earhead weight and 1000 seed weight. Nodulation was assessed visually. Shoots and roots were separated, dried for 22 h at 105 °C, and ground to a fine powder at 30 Hz using a mixer mill (MM2224, Retsch, Haan,

Germany) for subsequent P and $^{15}N$ analysis.

### 2.6. AMF analysis

Harvested roots were washed and cut into pieces of 1 cm. They were then bleached and stored in 10% (*w/v*) KOH at 4 °C and in the case of pigeon pea afterwards heated for 8 min at 90 °C in a water bath. They were then stained with trypan blue (0.05% lactic acid, glycerol, water 1:1:1) for 15 min at room temperature. After de-staining in water they were examined for possible colonization of AMF. Proportion of roots colonized by AM hyphae, arbuscules and vesicles was calculated after Brundrett and McGonigle, 1994, examining 100 intersections on 25 randomly chosen root pieces for each root sample. Soil cores were sieved through a 500 µm and a 32 µm sieve. The resulting material in the 32 µm was homogenized with 100 ml $H_2O$ in a blender. The suspension was then diluted to a total of 500 ml and stirred. After stop of stirring subsamples of 1 ml were taken after 10, 20, 30, 40 and 50 s 2 cm below the surface. Those five subsamples were combined into one. This sample was then placed on a membrane filter with a millimeter grid and water was removed by suction. The wall of the filtration unit was washed with water and the filter was stained with 1 ml trypan blue for 2 min. Under the microscope (200×) all hyphae were counted, which intersected with crosses on the grid (Newman, 1965; Sylvia, 1992; Thingstrup et al., 2000) and hyphal length density (HLD) was calculated per g of dry soil.

### 2.7. Plant analysis

P-content of shoots and roots was measured using the molybdate blue method on a Shimadzu UV-160 spectrophotometer (Shimadzu Biotech, Duisburg, Germany) after acid digestion (Murphy and Riley, 1962). The $^{15}N$ content and total N content of plants was analyzed with isotope ratio mass spectrometry (Delta V Plus, Thermo Fisher Scientific, Germany). Relative $^{15}N$ uptake was calculated as the percentage of total N content of the plants.

### 2.8. Experimental setup

#### 2.8.1. Experiment 2

A second experiment was conducted to analyse, in more detail, the hyphal growth of *Claroideoglomus etunicatum*, which had fast growing hyphae with the best growth promotion of finger millet. Three seeds of pigeon pea were sown. In five microcosms no germination occurred after one week and separately grown seedlings were carefully transplanted to these pots. In the other microcosm pigeon pea plants were then thinned to one plant per system after two weeks, and inoculated for one month to establish symbiosis. Then one HC (12 cm length) was attached and one FM compartment was attached (Fig. 1c). The pieces were taped with a transparent tape to control visually that no roots entered the HC across the ring connecting the compartments. FM were sown as five seeds per pot and thinned to one seedling 15 days after sowing. In the HC hyphae were sampled by taking soil cores at pre-defined sample sites at a distance from the pigeon pea compartment of 1.1 cm, 5.9 cm and 10.7 cm; at each distance two weeks, three weeks, four weeks, five weeks and eight weeks after the microcosms were joined (Hart and Reader, 2005). After each sampling the sampled microcosms were discarded. Each sampling and thus treatment was replicated 5 times. Sampling and analytical methods were the same as described above in experiment 1.

### 2.9. Statistics

SPSS (v20) software was used for statistical analysis. All treatments and parameters were tested for normal distribution with Shapiro-Wilk test and homoscedasticity with Levene's test before using other statistical methods. One-way ANOVA and Tukey test was used for post-hoc analysis to identify significant differences between the plant's response to the inoculants at a significance level of $p < 0.05$. If data were not normally distributed, Welch test and Games Howell test were used as post-hoc test. If data showed heterogeneous distribution of variance, Kruskal-Wallis and Dunn-Bonferroni were used as a post-hoc test. For normally distributed data with homogeneous distribution of variance, interactions between factors were tested for the two sizes of the microcosm and the different inoculants with a two-way ANOVA if data quality was sufficient. In experiment 1 two replicates of the control in the long microcosm were colonized by mycorrhizal fungi (PP: 85 and 76%, FM1: 79 and 68%, FM2: 49 and 5%), and therefore the control in the long microcosm was removed from statistical analysis. In experiment 2 hyphal growth per day was estimated with the package "Scatterplot3D" (Ligges and Mächler, 2003) in the R Software, Version 3.2.3 and the interface R-Studio Version 0.99.491. Two pigeon pea plants that had a very low colonization were considered outliers and were excluded from the analysis. Similarly, treatments with limited hyphal growth into the HC (6 replicates in total) were treated as outliers and excluded from analysis. Their values were maximally a sixth of the next largest replicate and did not exceed 3.9 m hyphae/g. A linear regression plane was calculated for hyphal length density as dependent on sampling time and distance from pigeon pea and the speed of growth calculated from the resulting formula. Results are presented as mean value and standard error (SEM) for each individual treatment. Further results of experiment 2 were tested for significance with a t-test in the R Software Version 3.2.3.

## 3. Results

### 3.1. Effect of microbial inoculation on colonization

In experiment 1, all three AMF species colonized pigeon pea well (> 85%) (Fig. 2a). For the finger millet, the colonization depended on the distance from the pigeon pea plant, with the more distant being less colonized. The colonization values had a large variance, but finger millet 1 was well colonized by *C. etunicatum* and *R. irregularis* compared to *R. fasciculatus* (Fig. 2b). In finger millet 2, in short microcosms, *C. etunicatum* trended to have better colonization. However, in long microcosm *R. irregularis* trended to be a better colonizer (Fig. 2c).

### 3.2. Effect of microbial inoculation on plant biomass

The biomass of inoculated pigeon pea was significantly increased compared to un-inoculated controls in the short microcosm (Fig. 3a). In pigeon pea, *R. fasciculatus* promoted the highest increase compared to the control in the short microcosm (Fig. 3a). AMF inoculation also clearly improved nodulation of pigeon pea roots with rhizobia after visual assessment in both the short and long microcosms (Table S1). For finger millet 1 in the long microcosm, *C. etunicatum* promoted growth significantly more than the other inoculants, however not in the short microcosms (Fig. 3b). With *R. irregularis*, biomass of the finger millet plants was significantly decreased compared to the control in the short microcosm. For finger millet 2 a similar trend for *C. etunicatum* was observed (Fig. 3c), differences in treatments were less than for finger millet 1. Results could be confirmed by weight of the earhead (Fig. S1), however here inoculation with *R. fasciculatus* improved earhead weight significantly more than the other inoculants in the short microcosm. No significant differences were found for 1000 seed weight in finger millet 1 and 2 (Table S2).

### 3.3. Effect of microbial inoculation on plant nutrient uptake

In general, P content in pigeon pea was considerably enhanced by mycorrhization (Fig. 4a). In the long microcosm, compared to other AMF species, *C. etunicatum* significantly improved the P content of finger millet 1 (Fig. 4b). When compared to the control of the short microcosm, there was two-fold increase of finger millet P contents in long microcosm. P concentration in shoot and root was significantly increased in

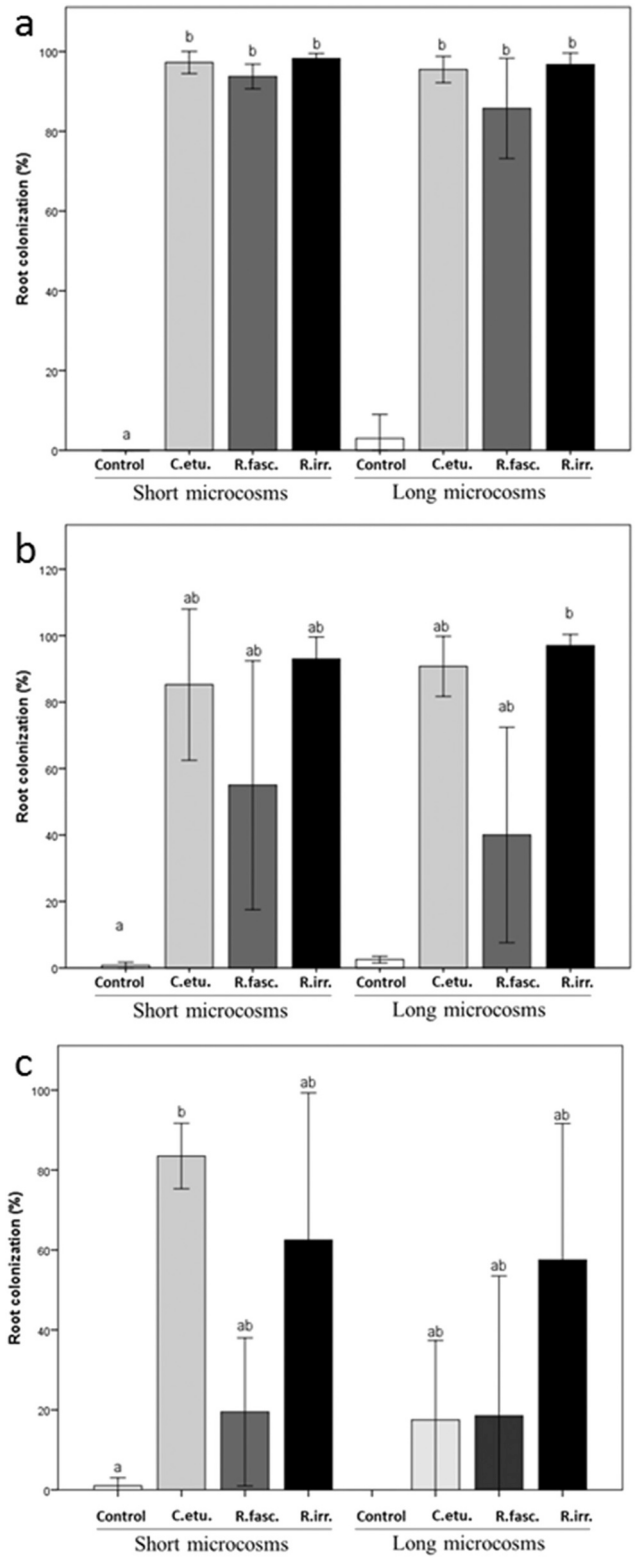


**Fig. 2.** Root colonization of the plants inoculated with three AMFs in the microcosms of experiment 1. Results of the root colonization of (a) pigeon pea, (b) finger millet 1, (c) finger millet 2. The values represent the mean ± SE of four replicates. Treatment means with a letter in common cannot be considered different at a $p > 0.05$ according to Dunn-Bonferroni test for root colonization. $n = 2$ for control long microcosms (excluded from statistical analysis). C.etu. = *Claroideoglomus etunicatum*, R.fasc. = *Rhizophagus fasciculatus*. R.irr. = *Rhizophagus irregularis*.

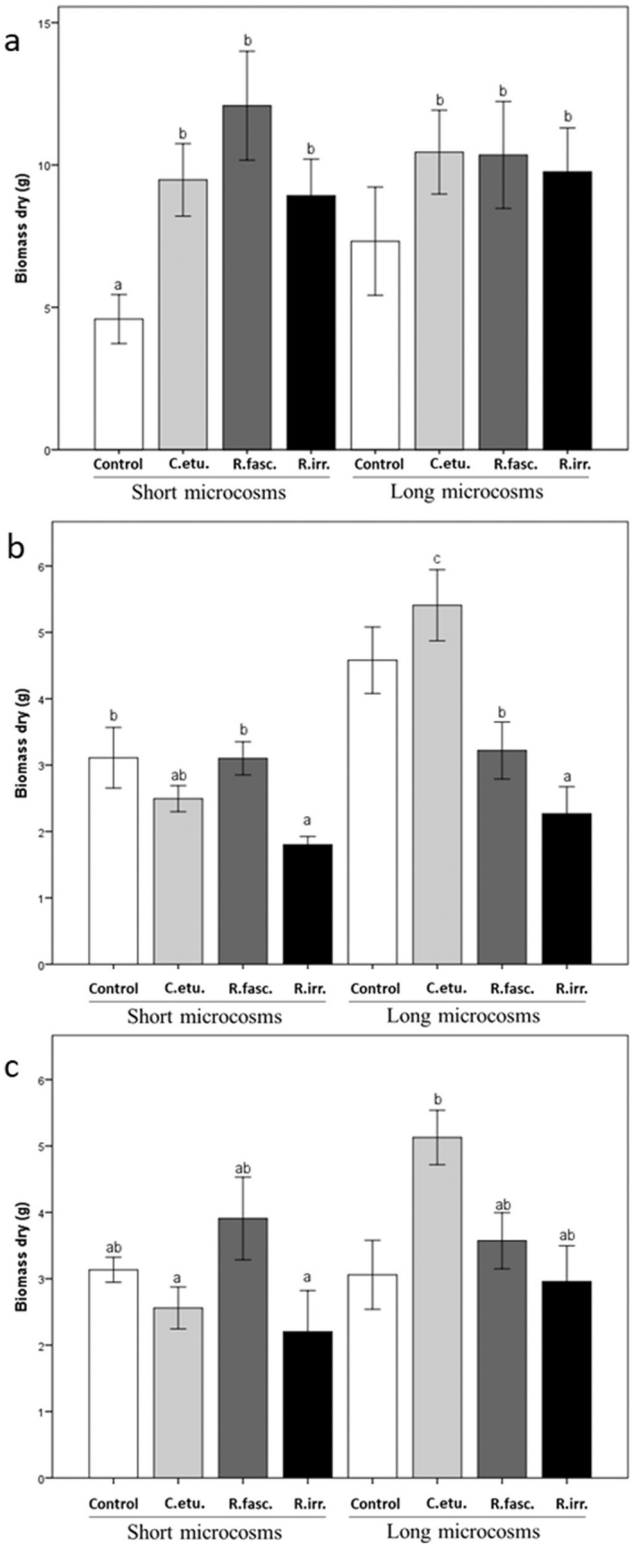


**Fig. 3.** Biomass of the plants in the microcosms of experiment 1 after inoculation of pigeon pea with three AMFs. Biomass of (a) pigeon pea, (b) finger millet 1, (c) finger millet 2. The values represent the mean ± SE of four replicates. Treatment means with a letter in common cannot be considered different at a $p > 0.05$ according to Tukey-HSD for pigeon pea and finger millet 1, according to Games-Howell Test for finger millet 2. $n = 2$ for control long microcosm (excluded from statistical analysis). C.etu. = *Claroideoglomus etunicatum*, R.fasc. = *Rhizophagus fasciculatus*. R.irr. = *Rhizophagus irregularis*.

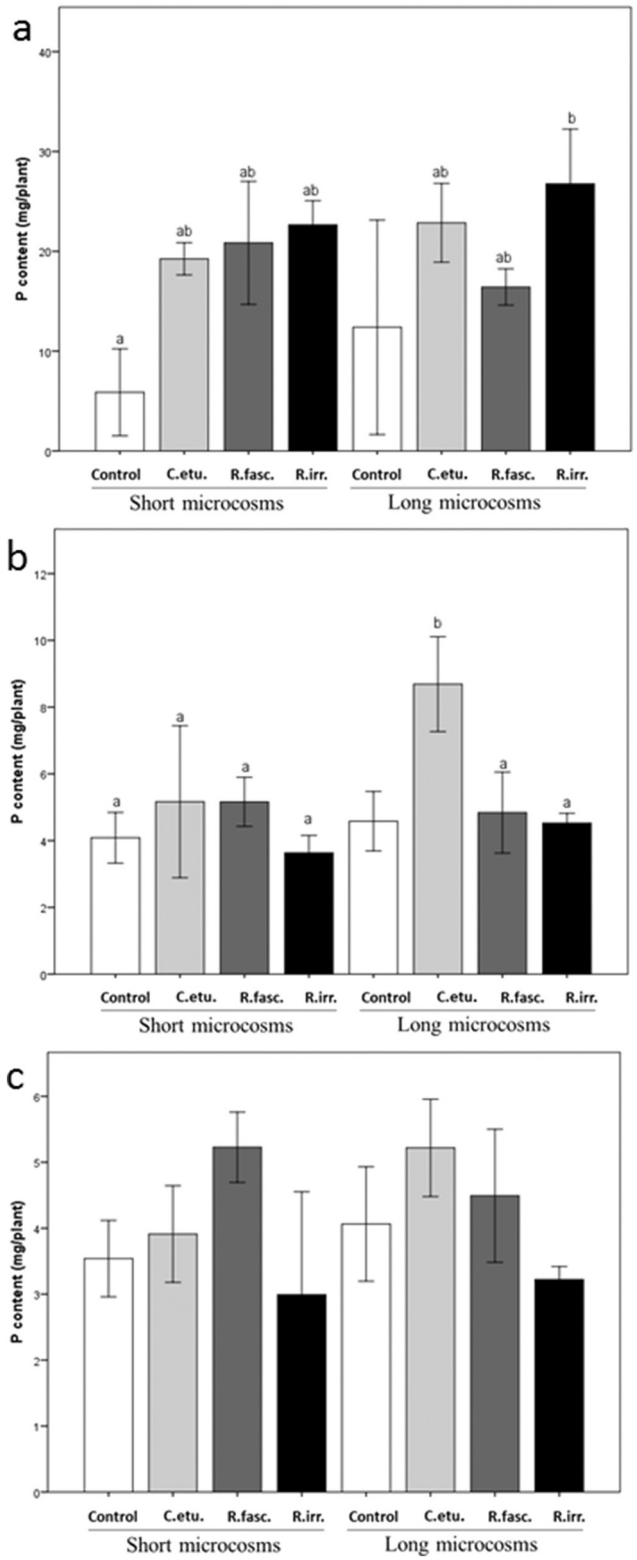


**Fig. 4.** Phosphorus content of the plants inoculated with three AMFs in the microcosms of experiment 1. Phosphorus content of (a) pigeon pea, (b) finger millet 1; (c) finger millet 2. The values represent the mean ± SE of four replicates. Treatment means with a letter in common cannot be considered different at a $p > 0.05$ according to Dunn-Bonferroni test for pigeon pea, according to Tukey-HSD for finger millet 1, no significant differences were found in finger millet 2. n = 2 for control long system (excluded from statistical analysis). C. etu. = *Claroideoglomus etunicatum*, R.fasc. = *Rhizophagus fasciculatus*. R.irr. = *Rhizophagus irregularis*.

pigeon pea after inoculation with *R. irregularis* (Fig. S2). In finger millet 1 this effect was also significant in the long microcosms compared to the control of the short microcosms (Fig. S2c). In finger millet 2 grown in short microcosms, inoculation with *C. etunicatum* significantly increased the shoot P content than in the long microcosms (Fig. S2e). Analysis of the nitrogen isotopes revealed that in the microcosms with *C. etunicatum*, the first finger millet plants in the short microcosm were enriched up to 3.71% $^{15}$N of total nitrogen content by *C. etunicatum* (Fig. 5a) while the second finger millet was enriched to 1.87% (Fig. 5b). In both lengths of the microcosms, *R. fasciculatus* did not appear to transport any $^{15}$N via their hyphae; the level of $^{15}$N in the finger millet plants was 0.4%, close to the level of natural abundance of 0.3663% (Fig. 5). Total N content in finger millet 1 and 2 in the short microcosm with *R. irregularis* was significantly decreased compared to inoculation with *R. fasciculatus* (Table S3). Inoculation with *C. etunicatum* in the long microcosm in both finger millet 1 and 2 resulted in significantly higher values than when inoculated with *R. irregularis* or *R. fasciculatus* (Table S3).

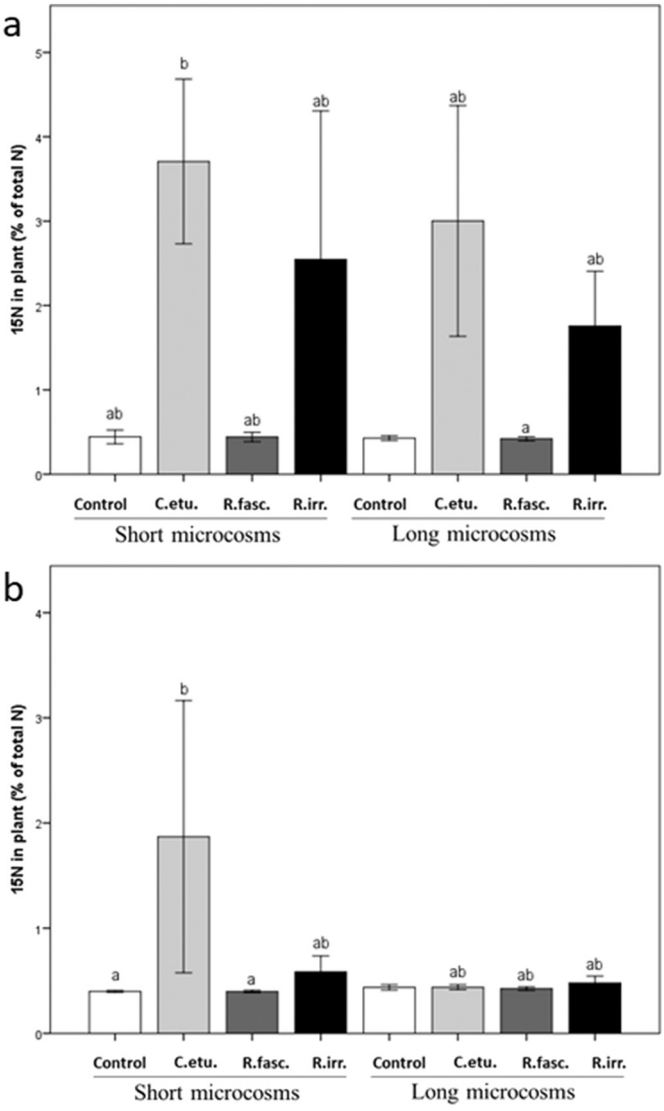


**Fig. 5.** $^{15}$N relative to the total N content in finger millets inoculated with three AMFs in the microcosms of experiment 1. $^{15}$N in (a) finger millet 1 and (b) finger millet 2. The values represent the mean ± SE of four replicates. Treatment means with a letter in common cannot be considered different at a $p > 0.05$ according to Games-Howell test for finger millet 1, according to Dunn-Bonferroni test for finger millet 2. $N = 2$ for control in long system (excluded from statistical analysis). C.etu. = *Claroideoglomus etunicatum*, R.fasc. = *Rhizophagus fasciculatus*. R.irr. = *Rhizophagus irregularis*.

### 3.4. Hyphal length density and rate of hyphal spread

Hyphal length densities (HLD) of all species were similar with lower HLD in the long microcosms (Fig. 6). HLDs of *R. irregularis* showed a higher trend than *C. etunicatum* and *R. fasciculatus*. However, after 28 days and 5.9 cm, the trend changed and *C. etunicatum* had the highest HLD in both lengths of the microcosm (Fig. 6b). Subsequently, 42 days later at 10.2 cm in the long microcosm, the HLD trend between *C. etunicatum* and *R. irregularis* became larger (Fig. 6c).

In the second experiment, with *C. etunicatum* in a "long microcosm" with one donor pigeon pea and only one receiver finger millet, hyphae covered the distance of 12 cm and colonized the neighbouring finger millet. Over the five time points hyphal length densities increased continuously until a plateau of about 40 m hyphae per g dry soil was reached (Fig. S3). The full length of the HC was colonized between the 4th and 5th week after the HC was attached. By using data from all weeks on HLD, without outliers, a regression plane was fitted in R (for visualization see Fig. 7), with an $R^2$ of 0.54. The time of sampling was highly significant ($p \leq 0.0001$) and the distance to pigeon pea was moderately significant ($p \leq 0.05$).

The following formula was extracted for the regression plane:

$$\text{HLD (m/g)} = 3.75 + 4.73\text{*Week} - 1076.53\text{*Distance (m)}$$

Resolving the formula for HLD = 0 (m/g) and by setting Week to 1/7 (1 d) results in a distance of 4.1 mm which addresses the question at which distance does the HLD reach 0 per day. This is the average rate of hyphal spread per day of *C. etunicatum*. After 8 weeks pigeon pea was well colonized by AMF hyphae and also many arbuscules could be found (Fig. S4). This is reflected also in the growth promotion and P content (Figs. S5 and S6). The roots were also well nodulated, and first nodules were already observed at the first harvest. For finger millet, however, the colonization level by AMF and rate of arbuscule formation (Fig. S4) was low. Nevertheless, the growth and P content of finger millet was significantly increased in the AMF treatments (Fig. S5 and S6).

## 4. Discussion

We found that AMF hyphae can spread readily through root free soil bridges from the roots of pigeon pea to the roots of finger millet, covering distances of up to 35 cm in 20 weeks with one finger millet halfway, and have growth promoting effects there. Hyphal spread was measured by several studies (Harinikumar and Bagyaraj, 1995; Jakobsen et al., 1992; Jansa et al., 2003), but never between two different crop plant species.

### 4.1. Characterization of the hyphal spread of the different AMF species

Pigeon pea benefitted significantly from mycorrhization by all three tested AMF species, both with regard to biomass and P content, in the experiment with short microcosms. In the short microcosm the biomass of pigeon pea almost tripled when inoculated with *R. fasciculatus*. Such a growth promotion is not unusual in laboratory experiments and had been found also in other studies (Saharan et al., 2018). Although all AMF species reached and colonized the most distant finger millet after 5 months, their HLD at three sample distances and sample times differed. Our sampling strategy assumed that the hyphae would regrow into the freshly supplied soil and/or hyphae would continue growing from outside the removed soil core. In experiment 1 we cannot exclude that differences in the HLD between the three AMF from the second sampling onward measured also their ability to recover from the previous sampling. Different hyphal growth strategies were found for the different species and include the unknown component of the recovery from sampling. Although there are differences in the growth promotion by AMF inoculants, other factors may interfere with the growth promotion of the finger millet plants e.g. AMF could confer competition with the pigeon pea plant (Hodge and Storer, 2015; Watkinson and Freckleton,

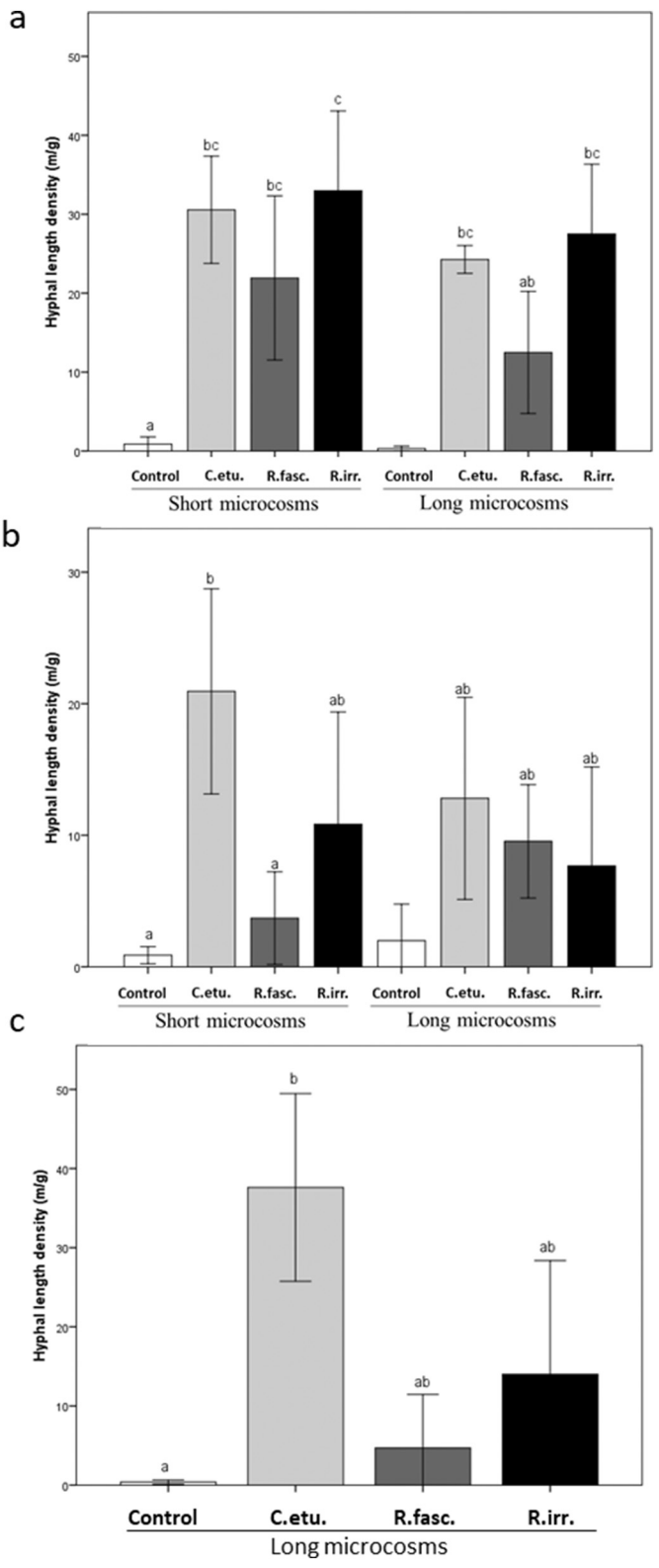


**Fig. 6.** Hyphal length density of three AMFs in the hyphal compartment between pigeon pea and finger millet 1 in the microcosms of experiment 1. Hyphal length density at (a) 1.1 cm and after 14 days, (b) 5.9 cm after 28 days and (c) 10.7 cm after 42 days (only from long microcosms). The values represent the mean ± SE of four replicates. Treatment means with a letter in common cannot be considered different at a $p > 0.05$ according to Tukey-HSD for (a) and (b), according to Dunn-Bonferroni test for (c). $n = 2$ for control long system (excluded from statistical analysis). C.etu. = *Claroideoglomus etunicatum*, R.fasc. = *Rhizophagus fasciculatus*. R.irr. = *Rhizophagus irregularis*.

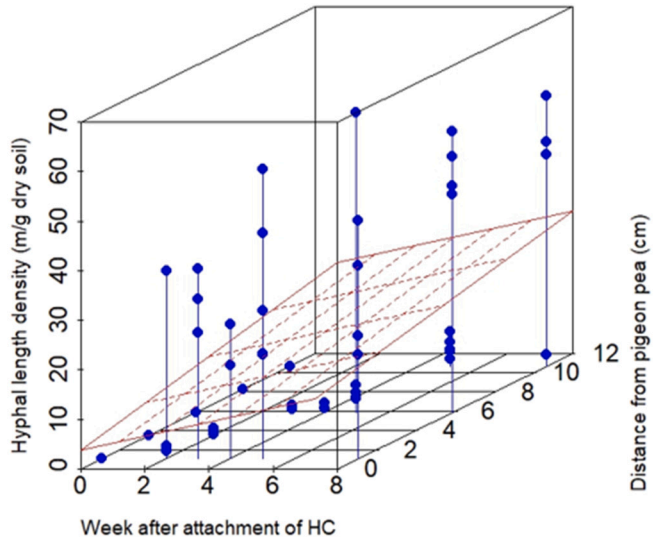


**Fig. 7.** Hyphal length density in the hyphal compartment of the microcosms for experiment 2 at three distances (1.1 cm, 5.9 cm, 10.7 cm) from the pigeon pea compartment and five time points after the hyphal compartment was attached. Blue lines show samples of one replicate. The red layer is the regression plane which was fitted through all data with an $R^2$ of 0.54. Outliers were excluded; Week 2: $n = 5$; Week 3: $n = 3$; Week 4: n = 2; Week 5: n = 5; Week 8: $n = 4$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1997). Furthermore, AMF may transport allelopathic substances such as juglone (Achatz and Rillig, 2014). Pigeon pea is known to contain substances with an allelopathic potential (Hepperly et al., 1992).

*R. fasciculatus* has previously been shown to be the best isolate out of three local Indian AMF strains and one American strain for growth promotion in finger millet (Govinda Rao et al., 1983). But here, the effects of *R. fasciculatus* on growth of finger millet were unclear. *R. fasciculatus* only showed a lower root colonization and lower HLD than the other inoculants. Interestingly *C. etunicatum* only promoted the growth of finger millet in the long microcosms. In contrast, *R. irregularis* had no effect on growth of finger millet despite its rapid spread and the relatively high HLD at different sampling times. Thus *R. irregularis* seems to invest little into promotion of the growth of finger millet. Data on P content per plant showed larger differences than with biomass data alone. Different growth strategies in *R. irregularis*, *C. etunicatum* and *Gigaspora gigantea* were also identified by Hart and Reader in a root observation chamber without a HC (Hart and Reader, 2005), e.g. *R. irregularis* and *C. etunicatum* produced extensive root colonization and many external structures like runner hyphae, absorptive hyphae and hyphal bridges. In their experiment *R. irregularis* showed an earlier (3 weeks) colonization than *C. etunicatum* and reached a lower plateau of external structures earlier. We observed a similar trend for these two species.

Thus, we could observe differences between our inoculants in their hyphal spread and growth promotion of pigeon pea and finger millet. However, as the length of the microcosm had a significant effect on the biomass and P content of finger millet 1, we cannot exclude effects of competition between the plants or better nutrient supply in the longer HC on our results.

In experiment 1, *C. etunicatum* showed the fastest hyphal spread. Its hyphal growth rate can be translated to a speed of 2.43 mm $d^{-1}$. Nevertheless HLD indicate that on that day hyphae had already grown further, which we could not assess in experiment 1. In our second experiment with destructive sampling hyphal growth rate was in fact higher with 4.1 mm per day, although our regression plane showed only a moderate level of fit to our data. To our knowledge this result is the fastest hyphal spread rate of AMF recorded. With *Trifolium subterraneum* Jakobsen et al. (1992) found the maximum hyphal growth of 3.1 mm $d^{-1}$ with *Acaulospora laevis* at a similar distance inside a hyphal compartment at 7 and 11 cm, however without a plant compartment behind. Also much slower rates have been detected e.g. 0.6 mm $d^{-1}$ in *Glomus fasciculatum* and *Festuca rubra*, however estimated via the root colonization (Warner and Mosse, 1982). Hyphal growth of course depends on soil parameters. Temperature and moisture have been found important factors for the growth and dieback of hyphae (Hernandez and Allen, 2013).

### 4.2. Nitrogen transport

In the first experiment we could show that nitrogen can be transported across distances up to 12 cm by AMF. The diffusion of $^{15}N$ through the hyphal compartment to finger millet was negligible as the control had a very small $\delta^{15}N$ value, close to the one in the atmosphere. The idea that nitrogen can benefit an intercropped cereal is not new. In our case we cannot distinguish whether $^{15}N$ was taken up by the legume and then transferred to AMF or whether $^{15}N$ was taken up directly by AMF. In our experiment, *R. fasciculatus* appeared to deliver almost no $^{15}N$ to finger millet, in contrast to *R. irregularis* and to *C. etunicatum*, which supplied $^{15}N$ even to the more distant second finger millet plant. It is not surprising that AMF species differ in their capacity to deliver nitrogen (or phosphorus) to their host plant. Similar differences were found by Walder et al. (2012), who studied mycorrhizal $^{15}N$ supply to flax and sorghum by *R. irregularis* (formerly *Glomus intraradices*) and *Glomus mosseae*. Large differences depending on the fungal isolate were also found in the study by Mårtensson et al. (1998), who found that N obtained by chicory from pea ranged from three to 50% of total N when the plants were grown in root-separated microcosms.

Some N assimilated by the finger millet may have been derived from the intercropped pigeon pea; such transfer from legumes to cereals upon AMF inoculation has been reported earlier (Johansen and Jensen, 1996; Meng et al., 2015), and has been suggested to follow a source sink relationship between N donor and recipient plants (Bethlenfalvay et al., 1991; Jalonen et al., 2009; Smith and Smith, 1990). In our experiment 1, unlabelled ammonium nitrate was supplied to pigeon pea only; thus, the increased total N values of finger millet with *C. etunicatum* in the long microcosms could be an indicator for the transport of elemental N taken up from the soil. Ammonium seems to be the form of nitrogen that is most transported by AMF hyphae to the root versus nitrate which is taken up but less readily transferred; Tanaka and Yano (2005) found that compared to nitrate, a 10 times higher amount of ammonium was transported to the root by *Glomus aggregatum*. Three ammonium transporters are known in *R. irregularis* including one that has been recently discovered (Calabrese et al., 2016).

### 4.3. Suitability of different AMF species as inoculants for different planting densities

We characterised three AMF species in a pigeon pea-finger millet intercropping system, yet the growth promotion of finger millet by AMF was variable with no growth promotion in the short microcosms and significant increases in the long microcosms. Finger millet 1, in both lengths of the microcosms, was connected with AMF hyphae of *C. etunicatum* after about four to five weeks of age and probably also colonized soon after. In the short microcosm the hyphae may have transported nutrients mostly to pigeon pea, leaving little nutrients to the finger millet, but in the longer microcosm AMF could contribute more to the nutrition of the finger millet due to the larger distance. The pigeon pea plants were one month older and had also in the end of the experiment much more biomass with a larger transpiration and water suction in the soil including nutrients in solution, which can be seen in the larger plants in the long microcosms that had more substrate and more nutrients in the HC. Another influencing factor is that AMF have been shown to favour plants which are older and have a higher rate of photosynthesis

(van der Heijden and Horton, 2009). In the field, the density and distance between plants has been shown to be crucial for the success of an intercropping system (Dhima et al., 2013) and also for pigeon pea-finger millet intercropping systems (Padhi et al., 2010). Hence, also with AMF inoculants the density and distance between plants is crucial. In a simulated pigeon pea-finger millet intercropping system we found CMN facilitates "bioirrigation" under drought condition (Singh et al., 2020). Under such a scenario, AMF species with relatively faster spread from pigeon pea to finger millet could have a positive impact on the survival of the finger millet from the neighbouring "bioirrigating" pigeon pea.

## 5. Conclusions

The design of our microcosms for the study of intercropping in the greenhouse allowed us to study the complex tripartite interaction among pigeon pea, finger millet and AMF in great detail. Our work provides an insight of the rate of forming a CMN between two different plant species, a preferable trait for selecting an ideal AMF inoculant in inter/mixed cropping system, and in particular in a pigeon pea and finger millet system. We characterised three AMF species, addressing our first hypothesis, and found significant differences in their growth promotion of pigeon pea and finger millet, their rate of hyphal spread and their transport of labelled nitrogen, although we cannot exclude effects of competition between the plants or better nutrient supply in the longer microcosms. Such effects may explain why *C. etunicatum* only promoted the growth of finger millet in the long microcosms.

Addressing our second hypothesis, we compared three AMF species in experiment 1 and made detailed measurements of the rate of hyphal spread for one AMF species, *C. etunictaum*, in experiment 2. The three AMF species that we tested had some influence on the growth and yield of finger millet, but at this stage, we cannot recommend any of them for field application, because the growth promotion would not be relevant for farmers. Our study suggests that it is important to select AMF species not only for their compatibility with both the plant species but also to consider the inter and intra plant-plant spacing and other interactions with the environment.

## CRediT authorship contribution statement

L.S., P.M., T.B. and N.M. designed research; L.S. performed research, analyzed data, wrote the paper. K.S., P.M., T.B. and N.M contributed to writing. The authors declare no conflict of interest.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2021.104156.

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