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Evaluation of MALDI-TOF mass spectrometry for the competitiveness analysis of selected indigenous cowpea (*Vigna unguiculata* L. Walp.) *Bradyrhizobium* strains from Kenya

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Abstract

Cowpea N_2 fixation and yield can be enhanced by selecting competitive and efficient indigenous rhizobia. Strains from contrasting agro-ecologies of Kilifi and Mbeere (Kenya) were screened. Two pot experiments were established consisting of 13 *Bradyrhizobium* strains; experiment 1 (11 Mbeere + CBA + BK1 from Burkina Faso), experiment 2 (12 Kilifi + CBA). Symbiotic effectiveness was assessed (shoot biomass, SPAD index and N uptake). Nodule occupancy of 13 simultaneously co-inoculated strains in each experiment was analyzed by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) to assess competitiveness. Strains varied in effectiveness and competitiveness. The four most efficient strains were further evaluated in a field trial in Mbeere during the 2014 short rains. Strains from bacteroids of cowpea nodules from pot and field experiments were accurately identified as *Bradyrhizobium* by MALDI-TOF based on the SARAMIS™ database. In the field, abundant indigenous populations 7.10×10^3 rhizobia g^{-1} soil, outcompeted introduced strains. As revealed by MALDI-TOF, indigenous strains clustered into six distinct groups (I, II, III, IV, V and VI), group III were most abundant occupying 80% of nodules analyzed. MALDI-TOF was rapid, affordable and reliable to identify *Bradyrhizobium* strains directly from nodule suspensions in competition pot assays and in the field with abundant indigenous strains thus, its suitability for future competition assays. Evaluating strain competitiveness and then symbiotic efficacy is proposed in bioprospecting for potential cowpea inoculant strains.

Keywords *Bradyrhizobium* · Cowpea · Symbiotic effectiveness · Nodule occupancy · Protein profile · Bacteroid

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Introduction

Optimal biological nitrogen fixation (BNF) by legumes can be achieved in the presence of efficient soil bacteria which are generally referred to as rhizobia, whose strains can either be indigenous to the soil or introduced in form of inoculants (Giller 2001; Rengel 2002). In this regard, BNF is considered as a sustainable alternative to the use of nitrogen fertilizers which are mostly inaccessible to smallholder farmers due to their high cost (Gopalakrishnan et al. 2015).

Cowpea (*Vigna unguiculata* L. Walp.) production by smallholder farmers could be improved by exploiting its symbiotic association with rhizobia by enhancing yields and soil N through BNF (Singh et al. 2003; Ulzen et al. 2016). It is one of the most important crops in mixed farming systems of the arid and semi-arid regions in Africa and other countries across the globe due to its drought tolerance (Ehlers and Hall 1997;

Langyintuo et al. 2003). In Kenya, the harvested area under cowpea production is estimated at 300,000 ha with an average grain yield of 492 kg ha^{-1} (FAOSTATS 2014). This yield is below the estimated potential of selected improved cowpea varieties ranging from 800 to 1800 kg ha^{-1} (Karanja et al. 2006). In coastal and eastern Kenya, smallholder farmers cultivate cowpea due to its drought tolerance and high nutrition value, it is mainly intercropped with maize or sorghum (Kimiti and Odee 2010; Ndiso et al. 2015). Cowpea production offers versatility by utilization of both foliage and grain while used for food by humans or animal feed, and as green manure (Singh et al. 2003).

Cowpea is considered promiscuous since it forms nodules with a wide range of rhizobia strains (Giller 2001; Guimarães et al. 2012). Indigenous rhizobia forming symbiosis with cowpea are abundant in many tropical soils (Ampomah et al. 2008; Giller 2001; Kimiti and Odee 2010). This abundance coupled with the promiscuity of cowpea is a possible limitation to strains introduced in form of inoculants that could be used for growth promotion and yield enhancement. Previous studies on cowpea inoculation have recorded contrasting results with some showing that cowpea responded to inoculation and that crop growth and yield could be enhanced (Martins et al. 2003; Rufini et al. 2014; Ulzen et al. 2016) while others did not (Awonaike et al. 1990; de Freitas et al. 2012; Mathu et al. 2012). Abundant indigenous populations are unfortunately and generally poor in their ability to fix nitrogen and can compete at the same time strongly with introduced *Rhizobium* inoculants (Pérez-Giménez et al. 2011; Slattery et al. 2001) since they are more adapted to local soil conditions and/or present in very high numbers.

A strategy to improve the effectiveness of inoculation and N_2 fixation could be to select the most competitive and efficient strains from locally adapted rhizobia populations that are associated with cowpea (Ampomah et al. 2008; Mathu et al. 2012). While the successful nodulation is a precondition for N_2 fixation, an efficient symbiosis depends also on the potential of the bacteroids to fix nitrogen, the availability of soil N for the plant and the cost of N_2 fixation to the plant (Zahran 1999). Also important to symbiotic functioning is the adequate availability of nutrients (P, K, Ca, Mg, S, Zn and Fe) that promote plant growth and rhizobia survival in the soil (O'Hara 2001; Zahran 1999). The effectiveness of rhizobia strains can be assessed in controlled pot trials by measuring increase in biomass and plant nitrogen compared to a non-inoculated and non-fertilized plant (Howieson and Dilworth 2016). The competitiveness of rhizobia strains for nodulation can be assessed within so-called "competition studies" and for this the use of suitable tools enables the accurate identification of the strain(s) present in the nodules (Novák 2011; Pistorio et al. 2002).

These tools include the use of antibiotic resistance markers, enzyme linked immunosorbent assay (ELISA) (Spriggs and Dakora 2009), DNA-based methods [polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Krasova-Wade et al. 2006), enterobacterial repetitive intergenic consensus (ERIC) PCR (Ampomah et al. 2008), quantitative polymerase chain reaction (qPCR) (Checcucci et al. 2016) and fluorophore-enhanced, repetitive, extragenic, and palindromic-PCR (HFERP) DNA fingerprinting (Wongphatcharachai et al. 2015)] and proteomic profiling by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry (MS) (Ziegler et al. 2012, 2015).

MALDI-TOF MS is a technique based on the analysis of intracellular proteins from cell lysates, which in turn are ionized and separated according to their mass to charge ratio, recorded as distinct peaks that together form a complex mass spectrum, also named fingerprint (Mehta and Silva 2015; Uhlik et al. 2011; Ziegler et al. 2012). Protein mass fingerprints can be used for the identification of bacteria at the genus, the species and, in some cases, the subspecies level when matched to reference spectra in databases based on well characterized strains (Biswas and Rolain 2013; Ziegler et al. 2012). The advantage of MALDI-TOF compared to other identification techniques is the great discrimination power, its reproducibility and low cost as well as the rapid sample preparation directly from isolates on agar plates or from root nodules. Recently MALDI-TOF MS has proven to be a powerful tool to assess nodule occupancy in competition assays of two closely related *Ensifer* (formerly *Sinorhizobium*) strains that were co-inoculated to cowpea (Ziegler et al. 2012).

The use of strains carrying antibiotic resistance suffer from inaccuracy due to cross-reaction with indigenous rhizobia and reduced strain competitiveness, while the ELISA technique lacks proper resolution among closely related strains. Although DNA-based approaches (PCR-RFLP, ERIC-PCR, qPCR, HFERP DNA fingerprinting) may have better resolution they are laborious, time consuming and expensive and more specifically the application of genetic markers is only suitable under controlled conditions or areas where use of genetically modified organisms is permitted (Pérez-Giménez et al. 2011; Spriggs and Dakora 2009).

In the above-mentioned competition studies (Checcucci et al. 2016; Krasova-Wade et al. 2006; Spriggs and Dakora 2009; Wongphatcharachai et al. 2015), assays of two co-inoculated strains were assessed. Using ERIC-PCR, Ampomah et al. (2008) carried out a competition assay in which up to 5 strains were assessed simultaneously. However, to our best knowledge there was no attempt to trace the co-inoculation of more than five different rhizobia strains simultaneously in competition assays. In this study, we evaluated whether MALDI-TOF MS could be used to

study competitiveness in a more complex assay with 13 *Bradyrhizobium* strains.

Several studies have reported that indigenous rhizobia are abundant in different soils and proposed their screening for effectiveness and competitiveness to develop inoculants for different legumes (Batista et al. 2015; Mathu et al. 2012; McInnes and Haq 2007). Following this, we collected a total of 202 indigenous strains from two contrasting agro-ecologies in Kenya [Kilifi County (Coastal lowland) and Mbeere district (lower midland)] by sampling 15 cowpea fields and 5 from uncultivated fields per region. One hundred and seventy two strains were characterized as *Bradyrhizobium* by MALDI-TOF MS (Ndungu et al. 2018) and from each region the most diverse strains were selected.

The aims of this study were to (i) evaluate 12 selected strains (8 derived from cowpea fields and 4 derived from uncultivated fields) of each agro-ecology for their symbiotic effectiveness in pot experiments in order to choose the most promising candidate strains for field inoculation experiments, (ii) test, and if proven feasible, implement a MALDI-TOF MS technology to study competitiveness of co-inoculated multiple strains in controlled inoculation assays, (iii) introduce selected candidate strains into fields of Mbeere to validate effectiveness and test via MALDI-TOF MS the competitiveness under natural conditions and against indigenous populations.

Materials and methods

Bradyrhizobium strains

Bradyrhizobium strains were isolated directly from cowpea nodules (red coloured) from the field or trap cultures as described by Ndungu et al. (2018). In brief, dried root nodules from the field were rehydrated in sterile distilled water prior to surface-sterilization, while the nodules of the trap cultures were immediately surface-sterilized. After immersion in 70% ethanol for 30 s, nodules were immediately transferred to 3.85% NaOCl solution for 2 min before three thorough rinses in sterile distilled water. Each nodule was crushed in 50 μl of sterile 40% glycerol in a sterile 1.5 ml Eppendorf tube, using a sterile plastic pestle. A wire loop full of the nodule homogenate was dilution-streaked on yeast extract mannitol agar (YMA) plates (Somasegaran and Hoben 1994). Plates were incubated in the dark at 28 °C for 3–7 days to allow for growth of *Bradyrhizobium* isolates. Single-strain isolates were obtained by repeated further dilution-streaking of subsamples of single colonies. Glycerol stocks for long-term storage at − 80 °C were prepared in yeast extract mannitol (YM) broth supplemented with 20% (*v*/*v*) glycerol.

The twenty-five most diverse *Bradyrhizobium* strains based on MALDI-TOF MS protein mass fingerprints were selected; 12 and 11 from Kilifi and Mbeere, respectively, plus 2 references (from Biofix, a commercial inoculant produced in Kenya by MEA Ltd., Nakuru and the other from Burkina Faso). Selection of these strains (Table 1) was based on their different proteomic profiles obtained by MALDI-TOF MS in order to cover a great diversity as described by Ndungu et al. (2018). The strains grouping and affiliation were verified by 16S rRNA sequencing. The sequences were deposited in the European Nucleotide Archive of the European Molecular Biology Laboratory under the accession numbers LT618843-LT618867 (Ndungu et al. 2018).

Pot trials under controlled conditions

Two growth chamber experiments were set up in Zurich (Group of Plant Nutrition, ETH) to identify *Bradyrhizobium* strains forming effective symbiotic association with cowpea. Experiment 1 and experiment 2 consisted of 12 and 11 strains from Kilifi and Mbeere, respectively (Table 1). Strain CBA (isolated from Biofix CB 1015, a commercial inoculant sold in Kenya by MEA ltd., was used as a reference strain in both experiments. Strain (BK1) from Burkina Faso, isolated from nodules of cowpea plants that exhibited good growth as a result of N_2 fixation under greenhouse conditions (provided by Dr. Abidine Traore) was evaluated against native strains in experiment 2 (Table 1). In each experiment, a mixture treatment consisting of 13 strains co-inoculated (12 plus the reference CBA) was included to assess strain competitiveness (Table 1).

In total, 16 treatments were assessed per experiment; 12 *Bradyrhizobium* strains from Kilifi and 11 strains from Mbeere plus BK1, a negative control without inoculation and no N source, a positive control without inoculation with N applied as KNO_3 (240 mg N pot^{-1}), a reference strain CBA and in each experiment, the mixture of all 13 strains (12 individual strains plus CBA) (Table 1).

An axenic sand culture system (Howieson et al. 1995) with modifications was used. In brief, plants were grown in free-draining plastic pots (1 kg; 12 cm diameter and 11 cm length) sterilized with 75% ethanol and lined with a sterilized mesh at the base. Seven hundred grams of quartz sand and sterilized vermiculite (2:1 *v*/*v*) was filled per pot and 100 ml of Millipore water applied to each pot in preparation for sowing.

Rhizobia inoculant cultures were prepared by picking single purified colonies of the individual strains from plate cultures on Modified Arabinose Gluconate (MAG) media (Sadowsky et al. 1987; Van Berkum 1990). Each colony was transferred into 20 ml of fresh MAG broth (same media without agar) in 100 ml Erlenmeyer flasks and incubated at 28 °C at 200 rpm for 3–5 days until growth in the log phase of the individual strains.

Cowpea (*Vigna unguiculata* L. Walp.) cultivar, K80 [improved cultivar, from Kenya Agricultural and Livestock Research Organization (KALRO)] commonly cultivated in

Table 1 Description of treatments, strains as identified by MALDI-TOF MS and strain origin (where they were obtained) used in experiment 1 and 2 of the pot trials

	Treatment	Strains as identified by MALDITOF-MS	Region	Strain origin
	Control	No inoculation, no N	–	–
	N (+)[c]	Not inoculated, +N	–	–
Experiment 1	K1h	*Bradyrhizobium elkanii*	Kilifi	Cultivated site
	K3c	*Bradyrhizobium elkanii*	Kilifi	Cultivated site
	K6e	*Bradyrhizobium* cf. *diazoefficiens*	Kilifi	Cultivated site
	K7a	*Bradyrhizobium* sp. I	Kilifi	Cultivated site
	K9f	*Bradyrhizobium* cf. *diazoefficiens*	Kilifi	Cultivated site
	K10e	*Bradyrhizobium* cf. *japonicum*	Kilifi	Cultivated site
	K14h	*Bradyrhizobium* cf. *diazoefficiens*	Kilifi	Cultivated site
	K15c	*Bradyrhizobium elkanii*	Kilifi	Cultivated site
	K16c	*Bradyrhizobium* cf. *diazoefficiens*	Kilifi	Uncultivated site
	K17f	*Bradyrhizobium elkanii*	Kilifi	Uncultivated site
	K19a	*Bradyrhizobium* cf. *japonicum*	Kilifi	Uncultivated site
	K20f	*Bradyrhizobium* cf. *diazoefficiens*	Kilifi	Uncultivated site
	CBA	*Bradyrhizobium* cf. *diazoefficiens*	Kenya	Biofix
	Mixture[a]		–	–
Experiment 2	Control	No inoculation, no N	–	–
	N (+)[c]	Not inoculated, + N	–	–
	M1h	*Bradyrhizobium* cf. *japonicum*	Mbeere	Cultivated site
	M2h	*Bradyrhizobium elkanii*	Mbeere	Cultivated site
	M3h	*Bradyrhizobium* sp. II	Mbeere	Cultivated site
	M6h	*Bradyrhizobium* cf. *japonicum*	Mbeere	Cultivated site
	M9i	*Bradyrhizobium* cf. *diazoefficiens*	Mbeere	Cultivated site
	M11c	*Bradyrhizobium* cf. *diazoefficiens*	Mbeere	Cultivated site
	M12f	*Bradyrhizobium elkanii*	Mbeere	Cultivated site
	M13j1	*Bradyrhizobium* sp. IV	Mbeere	Cultivated site
	M18f	*Bradyrhizobium elkanii*	Mbeere	Uncultivated site
	M19c	*Bradyrhizobium* sp. V	Mbeere	Uncultivated site
	M20a	*Bradyrhizobium* cf. *diazoefficiens*	Mbeere	Uncultivated site
	BK1	*Bradyrhizobium sp.* III	Burkina	Burkina Faso
	CBA	*Bradyrhizobium* cf. *diazoefficiens*	Kenya	Biofix
	Mixture[b]		–	–

[a] Mixture of strains (K1h + K3c + K6e + K7a + K9f + K10e + K14h + K15c + K16c + K17f + K19a + K20f + CBA) in experiment 1

[b] Mixture of strains (M1h + M2h + M3h + M6h + M9j + M11c + M12f + M13j1 + M18f + M19c + M20a + BK1 + CBA) in experiment 2

[c] N+ (nitrogen applied as KNO_3 at 240 mg N pot^{-1}), Biofix (commercial inoculant sold in Kenya by MEA ltd. contains strain CBA), BK1 (Strain isolated from nodules of cowpea plants grown in soil from Burkina Faso). Cultivated sites (farmers' fields) and uncultivated sites (no prior history of crop production). Regions (coastal Kilifi and eastern Mbeere) representing contrasting agro-ecologies from where strains were obtained

the Mbeere and Kilifi regions of Kenya, was used in both experiments. Seeds were surface-sterilized by washing in ethanol (70%; 30 s), hydrogen peroxide (2%; 2 min) and rinsed thoroughly several times with sterile distilled water. Surface-sterilized seeds were immersed in water for 1 h to initiate germination, and afterwards placed in Petri dishes with moistened sterile cotton wool for germination in a growth chamber at 28 °C in the dark for 24 h for the radicle to emerge and then three pre-germinated seeds were sown per pot.

Inoculum of each strain was adjusted to an $OD_{540\ nm}$ of 1.3 in sterile physiological water (9 g l^{-1} NaCl) to target 10^6 colony-forming units (CFU) ml^{-1}. Inoculant for the mixture treatments was prepared by using 1 ml of liquid culture of each strain and adding to a separate sterile tube and vortexed for 1 min. For each pot, 3 ml of the inoculant was added at the base of each seedling (1 ml per seedling), at 4 days after sowing. Plants were thinned to two healthy plants per pot at 3 days after inoculation.

In both experiments, the pots were laid out following a completely randomized design (CRD) with four replicates per treatment. The plants were grown for 5 weeks under growth chamber conditions set to: 12 h of light [Growth chamber 1; Grolux (1000 Lumen) and Sylvania white cool (5000 lumen) lamps for experiment 1 and Sylvania bulbs (780 lumen) for experiment 2] and to 27/20 °C (day/night) temperature. Air humidity fluctuated between 60 and 70% throughout the growth period.

Essential nutrients with the exception of N (only added to the positive control: 240 mg N) were added per pot [16 mg phosphorus (P), 160 mg potassium (K), 16 mg calcium (Ca), 16 mg magnesium (Mg), 0.16 mg manganese (Mn), 0.16 mg zinc (Zn), 0.05 mg copper (Cu), 0.03 mg boron (B), 0.05 mg molybdenum (Mo) and 0.05 mg cobalt (Co)]. Nutrients were applied as solutions of potassium chloride (KCl), potassium phosphate (KH_2PO_4), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), magnesium sulphate ($MgSO_4$), manganese chloride ($MnCl_2$), zinc chloride ($ZnCl_2$), copper chloride ($CuCl_2$), cobalt chloride ($CoCl_2$), sodium borate ($Na_2B_4O_7$) and sodium molybdate (Na_2MoO_4) at sowing and at 3 and 6 weeks after sowing.

Sampling and data assessment of pot trials

Plants were harvested during the vegetative growth phase 5 weeks after sowing. Prior to harvest, the soil plant analysis development (SPAD) index of the youngest fully developed cowpea leaves was determined using a Minolta SPAD-502 chlorophyll meter (Minolta corporation, Ltd., Osaka, Japan). Shoots of two plants per pot were separated from roots by cutting using a clean, sharp knife at 1 cm above the sand surface. Parameters measured included shoot dry weight (SDW), nodule dry weight (NDW) and the shoot nitrogen uptake (SNU) (see below). In both experiments, 20 nodules were selected per individual treatment (5 nodules per pot/rep) and forty nodules per treatment (10 per pot/rep) from the two treatments consisting of mixtures for strain identification by MALDI-TOF MS. Nodules for occupancy assessment were surface-sterilized in 70% ethanol and immediately stored at − 20 °C until analysis. Shoots and remaining nodules were oven-dried at 60 °C for 48 h to constant dry weight.

The mean dry weight of shoots (X) was used to calculate an index of symbiotic effectiveness (SE), where SE = 100 ($X_j - X_{TO}$ $X_{TN} - X_{TO}$) where j is the inoculated isolate, TO is the uninoculated control and TN is the nitrogen-fertilized treatment, modified from Ferreira and Marques (1992).

Field trial design, data assessment and sampling

The best strains [K1h and K17f from Kilifi (experiment 1) and M19c and M20a from Mbeere (experiment 2)] in terms of improved cowpea shoot dry weight and SPAD measurements were selected for validation of the symbiotic effectiveness in a field trial in Mbeere during the short rainy season 2014.

The site was located in Mbeere on a farmers' field (S 00° 46′ 59.1″, E 037° 40′ 46.4″) at an altitude of 1048 m above sea level. The rainfall is bimodal, falling in two seasons: the long rains (LR) from March to June and short rains from October to December. An annual rainfall ranging from 640 to 1100 mm and annual temperature ranging from 15 to 30 °C is experienced in this area. The soils are classified as Rhodic and Orthic Ferralsols (Jaetzold and Schimdt 1983; Jaetzold et al. 2006). According to the climate data obtained for the growing season from October 2014 to January 2015, the total precipitation was 183 mm. The mean maximum temperature was 29 °C and the minimum 18 °C. Temperature and rainfall pattern data for the 2014 and 2015 growing seasons at the study sites were obtained from the aWhere weather module http://www.awhere.com.

Soil sampling and analysis

At sowing, soil was sampled to a depth of 0–20 cm by pooling 30 cores into a composite sample, placed in polythene bags and transported in a cooler box and stored in the refrigerator at 4 °C prior to analysis. The soil sample was divided into two parts; one part was for the enumeration of indigenous rhizobia population in the soil, while the other part was for the chemical and physical analyses. This second part was air-dried and sieved to ≤ 2 mm prior to determining their chemical and texture properties at the MEA Ltd. soil and tissue testing laboratories (Nakuru, Kenya). The following parameters were determined: total nitrogen based on the Kjeldahl procedure (Bremner 1960), organic carbon (Walkley and Black 1934), Olsen (1954), Mehlich K, Ca, Mg and Na (Mehlich et al. 1962), pH in H_2O and soil texture using the hydrometer method (Bouyoucos 1962).

Treatments and experimental design of the field trial

The field had no history of rhizobia inoculation and was previously cropped with an intercrop of cowpea, pigeonpea (*Cajanus cajan* (L.) Millsp.) and pearl millet (*Pennisetum glaucum* (L.) R. Br). Each treatment received a basal application of Sympal fertilizer (101 g kg^{-1} P, 125 g kg^{-1} K, 71 g kg^{-1} Ca, 40 g kg^{-1} S, 6 g kg^{-1} Mg and 1 g kg^{-1} Zn). This was applied at a rate of 150 kg ha^{-1}; this implies 15 kg P ha^{-1}, 19 kg K ha^{-1}, 11 kg Ca ha^{-1}, 6 kg S ha^{-1}, 0.9 kg Mg ha^{-1} and 0.12 kg Zn ha^{-1}. The trial was set up in a randomized complete block design replicated four times. Treatments included inoculation with either CBA, K17f, M19c or mixture (M19c + M20a + K1h + K17f + CBA). Filter-mud-based inoculants for each strain (10^7 rhizobia g^{-1} filter mud) were applied at a rate of 10 g of inoculant kg^{-1} of seed resulting in 10^4 to 10^5 CFU rhizobia per seed, on surface-sterilized cowpea seeds using

gum arabic (3 g l^{-1}) as an adhesive. The following data were assessed: shoot dry weight (SDW), shoot nitrogen concentration (SNC), shoot nitrogen uptake (SNU) and nodule dry weight (NDW) (at 50% flowering; 53 days after sowing (DAS) and grain yield (at maturity; 80 DAS). To evaluate competitiveness of selected strains under field conditions, nodule occupancy was assessed by collecting fresh nodules from cowpea plants at mid flowering. Nodules were transported from the field in cooler boxes to the lab and immediately surface-sterilized by immersion in ethanol (70%; 30 s), NaOCl (0.5 M; 2 min) and rinsed thoroughly several times with sterile distilled water and stored in 40% glycerol in 2 ml cryo-vials at − 20 °C until analysis.

Shoot nitrogen analysis

Dried cowpea shoots from pot and field experiments were milled (particle size ~ 1 mm) using a cutting mill (Retsch GmbH, Germany) and a subsample milled into fine powder using a ball mill (Retsch GmbH, Germany). Shoot total N concentration (SNC) was measured using a CN analyzer coupled with mass spectrometer (IsoPrime 100 IRMS system, Isoprime Ltd). The shoot nitrogen uptake (SNU) was calculated as follows; SNU = SNC × SDW.

Enumeration of indigenous *Bradyrhizobium* populations from field sites

A most probable number (MPN) experiment in sterile sand using dilutions of soil from the field experiment in Mbeere was carried out according to Brockwell (1963) to estimate the size of the indigenous rhizobia population nodulating with cowpea. Briefly, 10 g of soil was diluted in 90 ml sterilized distilled water and serial dilutions done to obtain a fivefold serial dilution of 1:50, 1:250, 1:1250, 1:6250 and 1:31250 with four replicates per dilution. Sterilized sand was used as the growth medium and 600 g weighed into sterile plastic pots (diameter 9 cm, length 11 cm). The same cowpea cultivar was used and procedures for seed sterilization and pre-germination were done as described above for the pot experiments. Plants were thinned to one healthy seedling per pot at 4 days after sowing and 1 ml of soil diluent was then used to inoculate each seedling in the pots. Twenty milliliters of Broughton and Dilworth nutrient solution (Broughton and Dilworth 1970) was applied every second day in alternation with distilled water according to plant need. Conditions in the greenhouse were set to 12 h of light, 27/20 °C (day/night) temperature and air humidity fluctuated between 60 and 70%. Presence or absence of root nodules was assessed after 30 days and rhizobia population estimates calculated using MPN tables (Vincent 1970).

Nodule lysates and MALDI-TOF MS analysis to assess strain competitiveness in pot and field conditions

Forty (40) nodules were analyzed from each competition assay of pot experiment 1 and 2, while a total of 96 (24 per treatment) nodules from the field trial were assessed. Sample preparation and MALDI-TOF MS analysis were carried out as described in Ziegler et al. (2012). Prior to isolation of endosymbiotic bacteria, nodules were surface-sterilized in 70% ethanol and crushed into 400 μl of sterile ddH_2O. The crude supernatants were transferred free of nodule debris into new microfuge tubes and centrifuged at 20,000×*g* to collect bacteroids. The bacterial pellet was rinsed up to three times with 200 μl of sterile ddH_2O to wash off abundant plant leghaemoglobin proteins and transferred into 20 μl of 25% formic acid.

Aliquots of 1 μl of the suspension were spotted in quadruplicate (or in duplicate in the case of field nodule isolates) on a MALDI steel target plate and air-dried at room temperature. Then each spot was covered with 1 μl of a mixture of sinapinic acid (SA; Sigma Aldrich, Buchs, Switzerland) diluted in 60% acetonitrile and 0.3% trifluoroacetic acid (TFA) as ionising matrix. After complete co-crystallisation at room temperature, target plates were introduced into the MALDI-TOF Mass Spectrometer Axima™ Confidence machine (Shimadzu-Biotech Corp., Kyoto, Japan) using the linear positive detection mode, a laser frequency of 50 Hz and a mass range of 4 to 20 kDa. For each sample, spectra consisting of 50 to 100 profiles were averaged and processed using the LaunchPad™ 2.8 software (Shimadzu-Biotech). For identification of bacteroids, mass spectra were matched against the rhizobia-specific module of the SARAMIS™ databases previously described by Ziegler et al. (2015). For unsupervised hierarchical clustering of protein masses, a binary matrix indexing the presence/absence of each mass signal was generated using the SARAMIS™ superspectra tool.

Database update and mass spectral analysis for tracing *Bradyrhizobium* strains in pot and field trials

Strain-specific sets of biomarkers were calculated using 8 to 12 reference mass spectra [in total 134 spectra for experiment 1 (Kilifi strains), and 119 spectra for experiment 2 (Mbeere strains)] obtained from two to four nodules per strain collected from pot experiments 1 and 2 under controlled conditions (corresponding to the single inoculation treatments). By using the SARAMIS™ Superspectra tool and according to the SARAMIS™ user guideline, a consensus spectrum was calculated first, considering all reference mass spectra of each individual strain used for each experiment. The consensus spectrum which includes masses that are present in more than 60% of all reference spectra for all strains was stored as an

exclusion list. Subsequently, to calculate strain-specific Super Spectra (SSp)'s enabling the update of current rhizobial database, masses present in the exclusion list were reduced in order to select 25 strain-specific biomarkers.

Statistical analysis

Data on SDW, NDW and SNU from the pot and field experiments, SPAD (pot experiments only) and grain yield were analyzed using the mixed procedures of SAS software (ver. 9.4, SAS Institute, Cary, NC). Square or log transformations were applied where necessary to protect against violation of homoscedasticity and normality of the ANOVA. The treatment effects when significant were compared using the Tukey's HSD test, significance of difference was evaluated at $P \leq 0.05$.

Results

Assessment of N fixation and symbiotic effectiveness in pot experiments

Parameters used to assess symbiotic effectiveness included SDW, SPAD value and SNU. There were significant ($P < 0.05$) differences between the different strains in the mentioned parameters for both experiments (Tables 2 and 3).

In experiment 1, inoculation with strains K19a, K1h, K17f and the mixture resulted in SE of above 100% while the lowest SE of 10% was observed in plants inoculated with strain K6e (Table 2). SPAD values ranged from 14.5 to 50.3 in the uninoculated control and inoculation with strain K3c, respectively. Inoculation with all strains except K6e resulted in a significant increase in SPAD and SNU relative to the uninoculated control. Shoot nitrogen uptake ranged from 3.2 to 31.0 mg N pot^{-1} in the uninoculated control and N (+) treatment. Shoot dry weight ranged from 0.22 to 0.84 g pot^{-1} in the uninoculated control and inoculation with strain K17f, respectively. Inoculation with all strains except K6e, K10e, and K16c resulted in a significant increase in SDW relative to the uninoculated control.

In experiment 2, inoculation with strains M11c, mixture, M2h, CBA, M20a, M19c and M3h resulted in symbiotic effectiveness (SE) of above 100% while inoculation with strains M6h and M13j1 had the lowest SE values of 29 and 55%, respectively (Table 3). SPAD values ranged from 8.2 to 48.8 in the uninoculated control and inoculation with strain M19c, respectively. Inoculation with all strains resulted in a significant increase in SPAD and SNU relative to the uninoculated control. Shoot nitrogen uptake ranged from 2.2 to 39.4 mg N pot^{-1} in the uninoculated control and N (+) treatment, respectively. Shoot dry weight ranged from 0.22 to 1.09 g pot^{-1} in the uninoculated control and inoculation with strain M3h, respectively.

Nodulation and nodule occupancy of pot experiments

Nodulation in both experiments varied in response to inoculation by individual *Bradyrhizobium* strains and nodules were formed on roots of all inoculated cowpea plants with none on the control and N+ treatment without inoculation (Tables 2 and 3). Inoculation with the reference strain from Biofix inoculant (CBA) resulted in the highest NDW of 96.9 and 112.7 mg pot^{-1}in experiment 1 and 2, respectively.

In experiment 1, NDW ranged from 24.7 to 96.9 mg pot^{-1} in plants inoculated with strains K6e and CBA, respectively. There was no significant difference between inoculation with strain CBA and other strains except strains K17f, K16c, K15c and K6e that had low NDW of 62.3, 60.4, 58.1 and 24.7 mg pot^{-1}, respectively.

In experiment 2, NDW ranged from 57.6 to 112.7 mg pot^{-1} in plants inoculated with strains M6h and CBA, respectively. The highest nodulation by strain CBA was not significantly different from all other strains except M6h.

Competitiveness of strains in pot experiments

To establish the use of MALDI-TOF MS for nodule occupancy, the primary step involved acquisition of mass spectra of the 25 free-living strains (pure strains cultivated on nutrient agar) to confirm that fingerprint profiles of each strain were distinct and that resolution down to strain (sub-species) level is given (data not shown). Secondly, mass spectra of nodule bacteria were acquired to confirm that fingerprint profiles of bacteroids were distinct and allowed for resolution down to strain (sub-species) level. This was performed by analysing the bacteroids of nodules collected from the single inoculation treatments. Unfortunately, some strains did not show sufficient differences and could not be distinguished by the analysis (see "Materials and methods"). These strains had to be combined into a shared SSp., and are defined as "strain-group". These strains included the combinations K14h/K16c/K9f and K3c/K6e from experiment 1 and M3h/M6h from experiment 2. Subsequently, sets of biomarkers were calculated for each strain or "strain-group". A blinded set of reference nodules was then tested to evaluate the sensitivity and specificity of the MALDI-TOF approach.

In experiment 1, by using the calculated sets of strain-specific SSp's, a set of "blinded" nodules were validated first. Considering an SSp confidence value threshold of 90% (equals the presence of 18 of 25 biomarker masses), 46 of 52 mass spectra were positively identified. Furthermore, when applying an additional minimal threshold of two (out of four) positive identifications per nodule, each blinded sample was

Table 2 Mean SPAD value, shoot dry weight (SDW), shoot nitrogen uptake (SNU), nodule dry weight (NDW) and symbiotic effectiveness index (SE) from experiment 1 (strains from Kilifi) harvested 5 weeks after sowing

Treatment	SPAD value		SDW (g pot^{-1})		SNU (mg N pot^{-1})		NDW (mg pot^{-1})		SE (%)
Control	14.5	d	0.22	c	3.2	c	–	–	–
N (+)[b]	31.9	cd	0.75	a	31.0	a	–	–	–
K1h	48.1	ab	0.83	a	28.8	a	86.4	ab	116
K3c	50.3	a	0.74	a	26.9	a	75.2	abc	99
K6e	19.9	d	0.27	bc	6.3	bc	24.7	c	10
K7a	39.9	bc	0.73	a	24.1	a	74.8	abc	96
K9f	45.0	ab	0.63	ab	22.7	a	62.8	abc	77
K10e	41.1	abc	0.59	abc	20.0	ab	71.9	abc	70
K14h	42.6	abc	0.64	ab	22.4	a	65.0	abc	80
K15c	46.8	ab	0.71	a	23.6	a	58.1	bc	93
K16c	43.8	abc	0.63	abc	22.3	a	60.4	bc	87
K17f	45.4	ab	0.84	a	30.1	a	62.3	bc	116
K19a	43.1	ab	0.79	a	23.9	a	67.8	abc	108
K20f	45.2	ab	0.63	ab	18.9	ab	67.2	abc	77
CBA	42.6	abc	0.67	ab	20.6	ab	96.9	a	85
Mixture[a]	49.6	ab	0.80	a	27.6	a	85.8	ab	109
CV%	18.7		24.42		27.2		41.6		

Means followed by different letters are significantly different at $P < 0.05$. Means separated according to Tukey's test

CV coefficient of variation

[a] Mixure of strains (K1h + K3c + K6e + K7a + K9f + K10e + K14h + K15c + K16c + K17f + K19a + K20f + CBA)

[b] N+ (nitrogen applied as KNO_3 at 240 mg N pot^{-1}), Biofix (commercial inoculant sold in Kenya by MEA ltd. Contains strain CBA)

correctly identified either at "strain" or at "strain-group" level (K14h/K16c/K9f or K3c/K6e).

Bacteria derived from 40 nodules of the mixture treatment in experiment 1 were classified as following: 29 nodules occupied by strain K20f (72.5%), 5 nodules by K17f (12.5%), 4 nodules by K1 h (10%), one nodule by K10e (2.5%) and one nodule by K7a (2.5%) as shown in supplemental Fig. S2a.

In experiment 2, identical to the concept described for experiment 1 strains, 48 of 52 mass spectra acquired from blinded nodule samples were positively identified. All blinded nodule samples were correctly identified either at "strain" or at "strain-group" level (M3h/M6h).

The following strains were identified in the mixture treatment: 28 nodules as strain group M3h/M6h (70.0%), 6 nodules as M1h (15.0%), 3 nodules as M18f (7.5%), one nodule as M2h (2.5%), one nodule as M12f (2.5%) and one nodule remained unassigned due to insufficient spectrum quality (supplemental Fig. S2b).

Additional nodules were obtained to enable discrimination between strains M3h and M6h by MALDI-TOF MS. These two strains were inoculated separately and in a mixture (supplemental Fig. S3). When comparing the phenotype of plants inoculated separately with either strain M3h or M6h it was evident that M3h was efficient (dark green leaves) and M6h inefficient (pale green to yellow leaves) in forming symbiosis with cowpea (supplemental Fig. S3). This was also the case in the earlier established experiment 2.

Data on SDW, SPAD and SNU confirmed that M3h was efficient in N_2 fixation as compared to M6h (Table 2; supplemental Fig. S3).

Results of the field trial

The field soil in which the trial was conducted was a sandy loam with a slightly acidic pH. Soil concentrations of N and C were below 1 g kg^{-1}. Soil P and Ca were 3.2 mg kg^{-1} and 0.4 cmol$_c$ kg^{-1}, respectively (Table 4).

The soil Mehlich K, Mg and Na values were lower than the detection limits. Results from the MPN assessment revealed a high abundance of indigenous *Bradyrhizobium* strains estimated at 7.1×10^3 CFU rhizobia g^{-1} of dry soil. No significant ($P > 0.05$) difference in shoot biomass, nodulation, shoot N content and grain yield between the uninoculated control and inoculated treatments (CBA, K17f, M19c and the mixture) was observed (data not shown).

The effectiveness of our inoculated strains could not be validated based on the mentioned agronomic parameters. We used MALDI-TOF MS to trace the presence of our introduced strains in the nodules of inoculated plants and in this way explain the lack of response to inoculation.

Table 3 Mean SPAD value, shoot dry weight (SDW), shoot nitrogen uptake (SNU), nodule dry weight (NDW) and symbiotic effectiveness index (SE) from experiment 2 (strains from Mbeere) harvested 5 weeks after sowing

Treatment	SPAD value		SDW (g pot^{-1})		SNU (mg N pot^{-1})		NDW (mg pot^{-1})		SE (%)
Control	8.2	e	0.22	c	2.2	d	–	–	–
N (+)[b]	29.4	d	0.96	ab	39.4	a	–	–	–
M1h	45.6	ab	0.92	ab	26.5	ab	72.3	ab	96
M2h	44.2	ab	1.02	ab	33.1	a	75.9	ab	109
M3h	43.0	ab	1.09	a	33.2	a	79.8	ab	118
M6h	31.5	d	0.49	bc	13.9	bc	57.6	b	29
M9i	41.4	bc	0.86	ab	26.9	ab	107.3	a	87
M11c	44.3	ab	0.98	ab	31.3	a	86.3	ab	103
M12f	45.6	ab	0.88	ab	31.6	a	78.5	ab	90
M13j1	34.7	cd	0.63	abc	12.6	c	86.4	ab	55
M18f	44.8	ab	0.94	ab	34.1	a	89.4	ab	98
M19c	48.8	a	1.08	a	36.6	a	93.1	ab	116
M20a	48.6	a	1.04	a	32.1	a	83.8	ab	112
BK1	45.4	ab	0.87	ab	26.2	ab	90.3	ab	88
CBA	43.2	ab	1.04	a	23.3	abc	112.7	a	112
Mixture[a]	45.0	ab	1.01	ab	33.6	a	98.2	ab	108
CV%	13.9		21.07		8.6		20.7		

Means followed by different letters are significantly different at $P < 0.05$. Means separated according to Tukey's test

CV coefficient of variation

[a] Mixure of strains (M1h + M2h + M3h + M6h + M9j + M11c + M12f + M13j1 + M18f + M19c + M20a + BK1 + CBA), [b] N+ (nitrogen applied as KNO_3 at 240 mg N pot^{-1}), Biofix (commercial inoculant sold in Kenya by MEA ltd. Contains strain CBA), BK1 (Strain isolated from nodules of cowpea plants grown in soil from Burkina Faso). The two best treatments consisting of strains K1h and K17f; and M19c and M20a from experiment 1 and 2, respectively, were selected for field testing based on their SDW and SPAD data

Table 4 Soil physiochemical properties from site C, composite samples were taken at a depth of 0–20 cm

Property	Site C
pH (H_2O)	5.5
Total N[a] (g kg^{-1})	0.2
Organic C[b] (g kg^{-1})	0.6
P[c] (Olsen) (mg kg^{-1})	3.18
Mehlich K[d] (cmolc kg^{-1})	BDL
Mehlich Ca[e] (cmolc kg^{-1})	0.38
Mehlich Mg[f] (cmolc kg^{-1})	BDL
Mehlich Na[g] (cmolc kg^{-1})	BDL
Sand[h] (g kg^{-1})	720
Clay[i] (g kg^{-1})	150
Silt[j] (g kg^{-1})	130
MPN[k] (CFU rhizobia g^{-1} soil)	7.10×10^3
Textural class	Sandy loam

BDL below detection limit

[a] Total N: Kjeldahl method (Bremner 1960), [b] Organic C according to Walkley and Black (1934), [c] P Olsen according to Olsen (1954), available [d] K, [e] Ca, [f] Mg and [g] Na using AAS Mehlich double acid method according to Mehlich et al. (1962), [h] sand, [i] clay and [j] silt using the hydrometer method according to Bouyoucos (1962), [k] MPN Most probable number (Brockwell 1963)

Nodule occupancy analysis of field nodules

From a total of 96 nodules analyzed from CBA, K17f, M19c and the mixture treatments, none of the strains used as inoculants were present in nodules as revealed by MALDI-TOF MS analysis. Protein mass fingerprints from these nodules did not correspond to reference mass spectra of individual bacteroids of either strains CBA, K17f, M19c, M20a or K1h that were included in the mixture treatment.

All nodules analyzed were occupied by other indigenous strains distributed in six distinct groups (I, II, III, IV, V and VI) as revealed by MALDI-TOF mass fingerprints (Table 5; supplemental Fig. S1). The groups were defined based on unsupervised hierarchical clustering of protein masses and the distinctness of these groups is illustrated in supplemental Fig. S1. Strains in groups I, II and III showed high similarity to *Bradyrhizobium* sp., *B. japonicum* and *B. diazoefficiens*, while those in groups IV, V and VI remained unassigned in comparison to the reference library SARAMIS™ of Mabritec AG (Ziegler et al. 2015) (data not shown).

Mass profiles that grouped together as group III were dominantly present representing 80% of analyzed nodules. This was followed by strains from groups I and II while mass profiles IV, V and VI occurred only once (Table 5).

Table 5 Summary of nodule occupancy by inoculated strains and indigenous strains classified into six different MALDI-TOF MS groups (I to VI) of cowpea nodules expressed in percentages

Treatment	Nodule occupancy by inoculated strains (%)					Nodule occupancy of indigenous strains (%)						No. of analyzed nodules
	CBA	K1h	K17f	M19c	M20a	I	II	III	IV	V	VI	
CBA	0	0	0	0	0	4	8	79	4	4	0	24
K17f	0	0	0	0	0	4	8	88	0	0	0	24
M19c	0	0	0	0	0	8	0	92	0	0	0	24
Mixture	0	0	0	0	0	21	13	63	0	0	4	24

To evaluate whether this group represented only one type of mass spectrum (one clonal strain) or several ones (different closely related strains), we calculated a group-III-SSp which showed that all mass profiles within this cluster were identified accordingly thus, we concluded that group III is represented by a single clonal strain rather than by several (supplemental Fig. S1). Since strains from this group were present in the majority of nodules analyzed, independent of the sampled treatment in the field trial, we concluded that these indigenous strains outcompeted the introduced candidate strains. According to MALDI-TOF cluster analysis and the SARAMIS database, group III showed high similarity to strain M11c collected 1 year before from the same field (Ndungu et al. 2018) and also included in experiment 1of this study (Tables 1 and 3).

Discussion

In the present study, the effectiveness of individual strains was observed under axenic conditions in pot experiments and the best performing strains (M19c, M20a, K1h and K17f) based on SDW and SPAD values were selected for inoculation and validation in field trials. In both pot experiments, the mixture treatments showed high symbiotic effectiveness with an efficiency above 100%. Our results agree with the findings of Fening and Danso (2002), who observed that indigenous isolates from Ghanaian soil varied in their symbiotic effectiveness on cowpea.

Similar findings were also reported by Ampomah et al. (2008) for indigenous isolates not only on cowpea but also on groundnut (*Arachis hypogeae* L.), mung bean (*Vigna radiata* L. Wilczek) and soybean (*Glycine max* L. Merr.) where strains varied in symbiotic effectiveness and only few were superior in N_2 fixation. At a global scale, studies on different legumes with indigenous strains have also shown varying effectiveness in symbiosis (da Costa et al. 2017; de Almeida Ribeiro et al. 2015; Thrall et al. 2011; Xu et al. 2013).

The ability of MALDI-TOF MS to discriminate closely related species of rhizobia is an advantage when it comes to strain tracing in mixed populations of closely related strains in competition assays and in field trials as shown in this study. This was previously demonstrated by Ziegler et al. (2012) where MALDI-TOF MS could clearly resolve bacteroids of two closely related *Ensifer* (formerly *Sinorhizobium*) strains in competition assays.

At Mabritec AG, the enlarged SARAMIS™ database was updated with reference spectra obtained from free-living and also endosymbiotic rhizobia enabling an accurate identification of the bacteroids present in cowpea nodules obtained from our pot and field experiments. The majority of these strains were identified as belonging to the genus *Bradyrhizobium* (Ndungu et al. 2018; and this study).

In a few instances, the use of MALDI-TOF MS analysis could not immediately discriminate bacteroids of different strains, i.e. K6e/K3c, K9f/K14h/K16c and M3h/M6h. This was rapidly solved by the use of a supervised approach, linear discriminant analysis (LDA) earlier applied by Wittwer and Müller (Müller et al. 2013; Wittwer et al. 2011). As described by Müller et al. (2013), we used shrinkage discriminant analysis (SDA) for constructing the LDA model which enhanced discrimination between highly similar strains (data not shown). However, when the free-living form (colonies on nutrient media) of these bacteria were analysed, discrimination between them was instant. This could be attributed to the difference in form and lifestyle of strains in their free-living state (colonies on nutrient medium) or as bacteroids (inside root nodules) since they display different metabolism in each form. Inside the nodule, the environment is more controlled or limited to the function of N_2 fixation thus fewer proteins may be expressed as compared to when the bacteria exist in their free-living state.

Therefore, fewer distinct peaks of protein masses are available to discriminate closely related strains in this form. Contrary to the pot experiments, all strains could clearly be resolved from field nodules by use of MALDI-TOF MS using the unsupervised biomarkers analysis. However, from a computational point of view, it needs to be highlighted that the unsupervised approach is fully dependent on the manual selection of strain-specific markers by the user, in order to obtain a solid SuperSpectrum, while the supervised analysis is a fully automated and iterative approach. Due to this, highly similar fingerprints of two different strains can be discriminated because of few

distinct mass peaks in a supervised approach, while a solid identification via a SuperSpectrum expects a set of around 20 to 40 mass peaks. Effectiveness for the majority of the strains evaluated in either experiment was not correlated with competitiveness. For instance, competition assays in pot experiments revealed M3h and K10e as superior competitors in their respective experiments, M3h was also effective in N_2 fixation with cowpea, while K10e was not as effective. Also, in experiment 2, strains M20a and M19c were effective in N_2 fixation but not competitive as revealed by nodule occupancy analysis.

In the field, nodule occupancy by MALDI-TOF MS revealed that indigenous strains were prevalent. Strains from group III had very high similarity to the originally sampled strain M11c collected from the same farm a year before the experiment setup and screened in experiment 2 (see Ndungu et al. 2018). This result reiterates the high-resolving power of MALDI-TOF MS since the SARAMIS™ database was updated with reference spectra from the pot trials. In pot experiments, strain M11c was effective in N_2 fixation but was not among the competitive strains in the competition assay. However, under field conditions strains from group III were dominant. This could in part be explained by the difference in environment and possibly strain M11c could be more adapted to harsh field conditions rather than the favourable environment in the pot trials. Previous studies demonstrated that environment is a key proponent dictating the competitiveness of a strain and can differ under varying growth conditions (Ji et al. 2017; Zhang et al. 2014). The dominance of strains from group III in the field is in agreement with findings of several studies that demonstrated dominance of particular strains within indigenous populations of nodule occupants of cowpea under field conditions (Mathu et al. 2012; Pule-Meulenberg et al. 2010; Wade et al. 2014).

In our study, the abundance of indigenous strains recovered from field nodules can be attributed to the adaptation of these strains to the adverse dryland field conditions of Mbeere in soils of low nutrient availability (Table 4). The cropping of cowpea in this particular field for over 40 years either as a sole crop or in rotation with other crops (Ndungu et al. 2018) is also another factor. Continuous cropping of the host plant has an influence on indigenous rhizobia (Bottomley 1992).

This could explain the high abundance of rhizobia (7.1×10^3 CFU g^{-1} soil) obtained from this field soil and could have presented a major limitation to colonization of the rhizosphere and roots of cowpea by our introduced strains.

Despite having used inoculum containing a high amount of *Bradyrhizobium* strains (approximately 10^4 to 10^5 CFU rhizobia per seed), occupancy of nodules by our introduced strains was not attained. Several studies have reported low or no recovery of the inoculant strain where resident rhizobial populations exceeded 10^3 rhizobia g^{-1} soil (Danso and Owiredu 1988; Mathu et al. 2012; Thies et al. 1991). In contrast, other studies have shown responses to inoculation even against a high background of indigenous strains (Asad et al. 1991; Zhang et al. 2014). In these mentioned studies, success of inoculation was attributed to the high competitiveness of introduced strains as compared to indigenous strains. Also, an alternative strategy applied to enhance strain competitiveness in soils with high populations of *Bradyrhizobium* entailed re-inoculation in several seasons that resulted in increased soybean yield (Hungria et al. 2006).

In our study, besides abundant indigenous rhizobia, the lack of response to inoculation can also be attributed to the inoculation technique and formulation. This may have reduced the survival of our strains under field conditions and inability to successfully compete with indigenous rhizobia to colonize the rhizosphere of cowpea plants and initiate nodules. Seed coating by use of filter mud-based inoculant might not have been adequate to ensure the survival of the introduced strains on the seed surface and in the soil until germination. Poor survival of rhizobia on the seed surface and in the soil due to harsh environmental conditions has been documented as a cause of inoculation failure (Deaker et al. 2004; Law et al. 2007). Mbeere has a hot and dry climate usually characterized by erratic rainfall and frequent droughts. Therefore, new inoculant formulations that will offer adequate nutrients, humidity, protect the strains against desiccation and enhance cell multiplication in the rhizosphere will be required to enhance the competitiveness of introduced strains against indigenous populations.

In conclusion, rapid screening due to simple sample preparation and short processing time of multiple rhizobia strains makes MALDI-TOF MS attractive for studying nodule occupancy as demonstrated in the pot and field experiments. This approach of studying nodule occupancy is suitable for assessing the competitiveness of a large number of strains simultaneously. Although the maximum number of strains that can be simultaneously traced in competition assays is yet to be investigated, this tool provides an opportunity that would allow for selection of superior competitors if assays including 20 or more strains can be developed.

The accuracy of strain identification by MALDI-TOF MS when using complex mixtures of strains will allow future studies simulating more the natural conditions of native soils. Considering the high concentration of indigenous rhizobia populations in soils used in this study, it might be beneficial to first screen for competitiveness and then test the symbiotic efficacy in order to develop new inoculants for cowpea production. In addition, future genome sequencing of the two strains M3h and M6h will allow investigation into the genetic basis and possibly identify candidate genes responsible for the poor efficacy of M6h and high efficacy of M3h.

Future research could investigate the possible applications of MALDI-TOF MS to compare and relate available protein mass spectra to the proteins or metabolites that might be important for competitiveness and/or symbiotic efficacy, and development of biomarkers for such a large set of data if available.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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