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Cowpea (*Vigna unguiculata* L. Walp) hosts several widespread bradyrhizobial root nodule symbionts across contrasting agro-ecological production areas in Kenya

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A B S T R A C T

Cowpea (*Vigna unguiculata* L. Walp.) is an important African food legume suitable for dry regions. It is the main legume in two contrasting agro-ecological regions of Kenya as an important component of crop rotations because of its relative tolerance to unpredictable drought events. This study was carried out in an effort to establish a collection of bacterial root nodule symbionts and determine their relationship to physicochemical soil parameters as well as any geographical distributional patterns. *Bradyrhizobium* spp. were found to be widespread in this study and several different types could be identified at each site. Unique but rare symbionts were recovered from the nodules of plants sampled in a drier in-land region, where there were also overall more different bradyrhizobia found. Plants raised in soil from uncultivated sites with a natural vegetation cover tended to also associate with more different bradyrizobia. The occurrence and abundance of different bradyrhizobia correlated with differences in soil texture and pH, but did neither with the agro-ecological origin, nor the origin from cultivated (n = 15) or uncultivated (n = 5) sites. The analytical method, protein profiling of isolated strains by Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS), provided higher resolution than 16S rRNA gene sequencing and was applied in this study for the first time to isolates recovered directly from field-collected cowpea root nodules. The method thus seems suitable for screening isolate collections on the presence of different groups, which, provided an appropriate reference database, can also be assigned to known species.

1. Introduction

Cowpea (*Vigna unguiculata* L. Walp) is an important food legume and an essential component of sustainable cropping systems in the sub humid tropics and, generaly, dry regions across the globe (Singh et al., 2002). In Kenya, it is grown in the drier eastern area around Mbeere, as well as, in the humid coastal area around Kilifi, where it makes up an important part of the diet of small-scale farmers (Kimiti et al., 2009).

Cowpea is considered promiscuous in its association with root nodule-colonising bacteria, so-called rhizobia. It was shown to establish symbioses with several species and genera of the phyla Alpha- and Betaproteobacteria (de Souza Moreira et al., 2006; Pule-Meulenberg et al., 2010). Symbiotic association with effective rhizobia is a prerequisite to attain maximal benefits from symbiotic N_2 fixation. Symbiotic N_2 fixation can compensate for missing soil nitrogen (N) and thus potentially save costly mineral N fertilizer (Guimarães et al., 2012; Rashid et al., 2012). Rhizobial inocula for inoculating legumes increasingly account for differences in symbiotic specificity and effectivity, two parameters that are often correlated (Batista et al., 2015). Yet, the agro-ecological origin of rhizobial inoculants and thus most probably edaphic and climatic adaptation are often not considered sufficiently to make inoculation successful. A variety of biotic and abiotic factors, such as host plant, cultivation history, drought, soil pH, salinity, mineral nutrient availability, soil organic carbon content and

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texture, are known to affect rhizobial diversity and distribution (Giller, 2001; Law et al., 2007; Grönemeyer et al., 2014; Wade et al., 2014). However, collections of strains for inoculum development linked to such information are still rare.

In order to find adapted strains with a chance to establish and persist after inoculation, it is thus important to know their geographical and ecological distribution and physicochemical soil requirements. Differences in strain occurrence and abundance depending on these environmental parameters can provide this information, if based on observations across many sites.

To discriminate and identify rhizobial strains, there is a wide array of mostly DNA-based analytical tools. Strains can be discriminated based on Polymerase Chain Reaction-Restriction Fragment Length Polymorphisms (PCR-RFLP) and assigned to taxa by phylogenetic analysis of nucleotide sequences, such as those of the 16S rRNA and 23S rRNA genes, or the 16S-23S rRNA intergenic transcribed spacer (IGS) (Krasova-Wade et al., 2003; Qian et al., 2003; Pule-Meulenberg et al., 2010). Recently, however, a rapid high-throughput assignment technique emerged that relies on the cellular protein profiles, as characterized by Matrix Assisted Laser Desorption/ionization Time of Flight (MALDI-TOF) Mass Spectrometry (MS) (Ferreira et al., 2011; Ziegler et al., 2012). This protein profile-based approach to strain identification is yet mostly applied in the clinical diagnostics, where it has partially replaced biochemical assays and DNA-based discrimination and identification methods (Singhal et al., 2015). However, MALDI-TOF MS protein profiling has also already been used to assign *Bradyrhizobium* symbionts isolated from root nodules of cowpea, siratro (*Macroptilium atropurpureum* (DC.) Urb.) and soybean (*Glycine max* (L.) Merr) to species (Ziegler et al., 2012).

The objectives of the present study were to (i) determine the root nodule-colonising rhizobia of cowpea in the field (i.e. at cultivated sites) and of cowpea raised in pots with soil from uncultivated sites in two contrasting agro-ecological regions of Kenya and (ii) assess how their occurrence and abundance relates to geography, cultivation of cowpea and other grain legumes as well as physicochemical soil parameters at the collection sites. This was done with the intention to compile a collection of isolates with known edaphic requirements to develop site condition-adapted inoculants for cowpea.

2. Materials and methods

2.1. Study regions and sites, soil and root nodule sampling and soil analyses

Root nodule and soil samples were collected in the two most important cowpea-growing areas of Kenya, belonging to two different agro-ecological regions, the area around Mbeere (lower midland) and Kilifi (coastal lowland), about 600 km distance apart (Table 1). The Mbeere area is a dry and high elevation area and the Kilifi area is next to the coast with a more humid and buffered climate (i.e. less extreme temperatures and drought spells). The annual rainfall follows a bimodal pattern, allowing for two cropping seasons in both agro-ecological regions (Table 1). The Mbeere area is considered as part of the Arid and Semi-Arid Lands (ASALS) of Eastern Kenya and is characterized by frequent droughts due to erratic and unreliable rainfall, while the Kilifi area is hot and humid throughout the year (Jaetzold et al., 2006). The soils around Mbeere are predominantly rhodic and orthic ferralsols, thus well drained, moderately deep to deep dark red to yellowish red, friable sandy-loams, while the soils around Kilifi are mostly cambisols, phaeozems, and rendzinas, less weathered clayey soils with high amounts of organic matter in the topsoil (Jaetzold et al., 2006). Information on soil texture for all study sites is listed in Table S1.

This field survey relied on a sampling scheme with 20 sites per agro-ecological region; 15 farmers' fields (i.e. 'cultivated sites') with a history of cowpea cultivation and five sites with no prior history of crop production (i.e. 'uncultivated sites'). The geographical co-ordinates of each sampled site are also listed in Table S1. The sites differed in topography, microclimate and soil physico-chemical properties (Table 1). The sites were selected in consultation with regional agricultural extension officers, knowing smallholder farmers who grew cowpea and were willing to allow root nodule sampling. The sampling areas around Kilifi and Mbeere covered about 33 km^2 and 17 km^2, respectively. None of the selected sites had a previous known history of inoculation with rhizobia (pers. com. with farmers by Samuel Mathu Ndungu). Nodule and soil samples of cultivated fields were collected at the flowering stage that was for Mbeere in May 2013, and for Kilifi in August 2013 due to later planting time. At uncultivated sites only soil samples could be collected, which were used for physico-chemical characterisation and trapping indigenous rhizobia with cowpea in pot cultures (Zilli et al., 2004; Silva et al., 2012).

The soils were sampled to a depth of 15 cm by pooling five cores into a composite sample per site. After air-drying, the soil samples were passed through a 2 mm sieve before the chemical properties were determined by the MEA Ltd. soil and tissue testing laboratories (Nakuru, Kenya) and the texture was determined by the International Centre for Tropical Agriculture (CIAT) soil laboratory (Nairobi, Kenya). The measured parameters were total nitrogen, based on the Kjeldahl procedure (Bremner, 1960), organic carbon, using the method of Walkley and Black (1934), pH (H_2O), and soil texture, using the hydrometer method (Bouyoucos, 1962). The bio-available inorganic phosphorus (P) was measured in Zurich (Plant Nutrition Group, ETH) as resin-extractable P (Pres) and was determined in triplicate by extraction with anion exchange resin membranes. In brief, 2–3 g moist soil was shaken with 30 ml of double-distilled water and two resin strips of 3 cm × 2 cm (BDH Laboratory Supplies product 55164 2S, Poole, England) for 16 h at 160 rpm on a horizontal shaker. The membranes were rinsed with water, and P was eluted with 0.1 M NaCl/HCl, followed by colorimetric concentration measurements, using malachite green (Ohno and Zibilske, 1991).

2.2. Cowpea cropping system of the sampled fields

Cowpea is the main crop during the short and long rainy seasons in the Kilifi and Mbeere areas, which leads to a nearly continuous presence of cowpea as a host of rhizobia. In both regions farmers grow additionally common bean (*Phaseolus vulgaris* L.), green gram (*Vigna radiata* (L.) Wilczek.), and pigeonpea (*Cajanus cajan* (L.) Millsp.) (Table S1). These also form root nodules with *Rhizobium* spp. and *Bradyrhizobium* spp. as symbionts and may thereby increase the diversity of cowpea-nodulating rhizobia. Cowpea can be grown as a sole crop, but is mostly intercropped with maize (*Zea mays* L.), sorghum (*Sorghum bicolor* (L.) Conrad Moench) and pearl millet (*Pennisetum glaucum* (L.) R.Br) and in the coastal region of Kenya also with cassava (*Manihot esculenta* Crantz) (Table S1).

In both agro-ecological regions soils are infertile because of nutrient depletion as a consequence of little mineral and organic fertilizer use by the resource-poor farmers. Typical cropping involves alternating rows of cereals, such as maize, sorghum and millet, and legumes, such as common bean, green gram, pigeonpea, and cowpea, with the latter being the most dominant in both regions (Table S1).

2.3. Nodule collection in farmers' fields and from trap culture plants

Root nodules were collected from cowpea plants in farmers' fields, giving samples for the 'cultivated sites'. At each site, five healthy cowpea plants were selected for uprooting and collection of nodules. Nodules were stored in McCartney glass vials with dehydrated silica gel for transport to the laboratory and storage at 4 °C until bacterial isolation.

To trap rhizobia from the soil samples of the 'uncultivated sites', two approaches were used: (1) trapping of rhizobia in 300 g plastic pots filled with a 2:1 (v:v) mixture of native soil and autoclaved quartz sand (grain size: 0.7-1.2 mm) planted with one cowpea plant (Zilli et al.,