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SHORT COMMUNICATION



Assessing root nodule microsymbionts in healthy and declined rooibos (*Aspalathus linearis* burm f.) at a plantation in South Africa

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ABSTRACT

Aspalathus linearis (burm f.), commonly known as rooibos, grows in nutrient and organic matter poor sandy soils that limit its growth. In this study, samples of nodules from both declined and healthy rooibos plants were collected to determine the frequency of nodule nitrogen-fixing and endophytic bacteria. Standard microbiological procedures as well as sequence analysis of the 16S rRNA revealed that more than 75% of the bacterial isolates from the healthy plants contained microsymbionts belonging to the *Rhizobium* group and the remaining 25% were characterized as *Pseudomonas* and *Burkholderia* spp. The nodule from the declined plants lacks a sufficient number of rhizobia and was mostly white in color, small and contains the free-living endospore-forming *Bacillus* and other endophytic *Burkholderia* and *Pseudomonas* spp. The results provide a baseline data on the microsymbionts of rooibos nodules in Citrusdal and highlighted the need for further investigation using additional techniques.

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Rhizobium; free-living
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Introduction

Aspalathus linearis (burm f.), commonly known by the name rooibos, is an economically important beverage legume endemic to the Cape floristic region in South Africa (Hassen et al. 2012). Like in many other legumes, the presence of both the symbiotic root nodule rhizobia and the free-living indigenous soil microbes in the soil plays crucial roles in growth and normal functioning of this legume crop. While the symbiotic rhizobia mainly control the key functioning of nitrogen nutrition, the free-living microorganisms influence all other functions in the ecosystem including carbon cycling, soil structure maintenance and biological control (Kennedy 1999).

In the 2015/2016 growing seasons of rooibos, some crop decline were observed in rooibos plantations in Citrusdal, Western Cape in South Africa. The major reasons for such rooibos decline in this particular plantation have not yet been established. Among the various factors that contributed to crop decline in many agricultural fields is a change or decrease in beneficial soil microbial communities. A good example of the role of soil microbiota in controlling crop decline is the shift in soil microbial communities that favors the growth of antagonists of the pathogen *Gaeumannomyces graminis* (Weller et al. 2002). In addition, agricultural practices that result in change or reduction in the beneficial soil microbial communities may favor the proliferation of pathogens resulting in crop decline. Naturally, soil microorganisms such as *Bacillus*, *Trichoderma* and *Pseudomonas* spp. increase the level of soil suppressiveness in many agro ecosystems and contribute to prevent crop declines caused by *Fusarium* wilt in many crops, potato scab and take-all of wheat. With regard to biological control, many free-living rhizobacteria and some *Trichoderma* are associated with increasing the suppressiveness of the soil to different phytopathogens dwelling in the soil. The major modes of action in the suppressive effects of these rhizobacteria include antibiosis

and siderophore-mediated iron competition with the pathogens (Kloepper et al. 1980).

In the legumes, including rooibos, that mainly derive their nitrogen (N) from the process of biological nitrogen fixation in a symbiotic interaction with rhizobia, the absence of effectively nodulating and nitrogen-fixing strains of rhizobia in the soil and the root nodules contributes to a huge crop decline and yield loss unless inoculated. In the current observation of the rooibos plants at the Citrusdal plantation, the majority of the plants had stunted development with very ineffective nodules. The plants were observed to have poor vigor with chlorotic types of leaves. The aim of this study is therefore to determine the major bacterial symbionts associated with the root nodules of the stunted and healthy rooibos plants and to generate a baseline information on the status of the indigenous rhizobium involved in the nodulation and nitrogen fixation of rooibos in this particular area.

Materials and methods

Collection of plant materials

A simple random sampling technique (Thompson 2012) was used in the collection of root nodule samples to increase the likelihood of obtaining representative nodule samples within both the healthy and declined plants population. Twenty plants with intact roots were carefully dug up from the soils of both healthy and declined rooibos and were placed in a sterile plastic bag and put in cooler boxes, which was then transferred to the laboratory for bacterial isolation.

Isolation of bacteria

After proper surface sterilization, each single nodule was transferred into a 1.5 ml Eppendorf tube containing 100 µl

sterile H₂O. The nodules were crushed using a sterile glass rod and a loopful of the suspension was streak plated on Yeast Mannitol (YM) agar that supports the growth of the root nodule rhizobia as well as on Luria Bertani (LB) agar for the cultivation of a wide range of non-fastidious bacteria (Bertani, 1951). The plates were incubated at 28°C for 24–48 h for the fast growers and for 3–5 days for the slow-growing *Bradyrhizobium* group.

DNA extraction and 16S rRNA sequence analysis

DNA was extracted using Promega Genomic DNA purification Kit according to the manufacturer's instruction (Promega, Madison, USA) and the concentration of DNA was determined using Nano drop spectrometer. About 5 µl of this DNA was used as a template for PCR reaction. Polymerase chain reaction of the 16S rRNA was conducted using the forward and reverse primers fd1 (AGA AGT TTG ATC CTG GCT CAG) and rD1 AAG GAG GTG ATC CAG CC, respectively (Weisburg et al. 1991). The purified PCR products were sent to Inqaba Biotech (South Africa) for sequencing. The sequences were edited using Bioedit and Chromas Lite programs after which they were aligned with an online sequence alignment program (MAFFT) (Katoh et al. 2002). In order to elucidate the phylogenetic relationships of the isolates with known sequences on the NCBI database library, an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree was constructed using MEGA 7 program (Saito & Nei 1987; Kumar et al. 2015).

Results

Fifty bacterial isolates were initially isolated from both healthy and declined nodules. Preliminary cultural morphology and microscopic analysis of these isolates indicated that most of the isolates are duplicates or multiple copies of the same isolates. That resulted in the selection of 12 morphologically distinct colonies from each of the healthy and declined plants for the molecular identification. The nodules collected from the soils with the healthy plants were more effective in terms of their size, color, number and positions and the plants look greener and taller (Figure 1(a,b)). On the other hand, the declined rooibos plants were characterized by stunted and pale green to yellowish tiny leaves with very few small nodules (Figure 1(c,d)).

Sequence analysis of the 16S ribosomal RNA of the isolates revealed that more than 75% of the isolates from the nodules of healthy soils belonged into the *Rhizobium*–*Bradyrhizobium* group and the remaining 25% were identified as *Bacillus*, *Burkholderia* and *Pseudomonas* spp. (Figure 2). Phylogenetic tree constructed from the analysis of the 16S ribosomal RNA sequence of bacterial isolates from healthy and declined rooibos nodules grouped the bacteria into five major groups in which 75% of the isolates that fall under the *Rhizobium*–*Bradyrhizobium*–*Mesorhizobium* clades (group I) are those isolated from the nodules of the healthy plants (Figure 2). In the nodules from the declined plants, the proportion of the root-nodulating rhizobia is very low and most of the isolates were grouped under groups II–IV in the neighbor-joining tree that contains strains of *Burkholderia*, *Pseudomonas* and *Bacillus* spp. (Figure 2).



Figure 1. Photo showing rooibos plants in the healthy and declined plants. Note that plants in the healthy soil look greener and taller (a) with many big and pink nodules (b) whereas plants in the declined soil have less growth with stunted and yellowish appearance (c) and very few tiny nodules (d).

Discussions

The occurrence of a large percentage of rhizobia group in the nodules of the healthy rooibos plants, as compared to the declined rooibos, means that the environmental conditions are conducive to the survival of the *Rhizobium* strains and hence suitable for the nodulation process. Under this condition, the active rhizobial strains can fix sufficient amount of atmospheric nitrogen and the plants look healthier. In the rhizobium legume symbiosis, the efficiency of the nitrogen-fixing rhizobium strain could be affected by several limiting factors, which result in reduced growth of the legume. By the time this study was conducted, it was not possible to establish the real cause of the rooibos decline. The most problematic conditions associated with nodulation and nitrogen fixation efficiency by rhizobium leading to crop decline

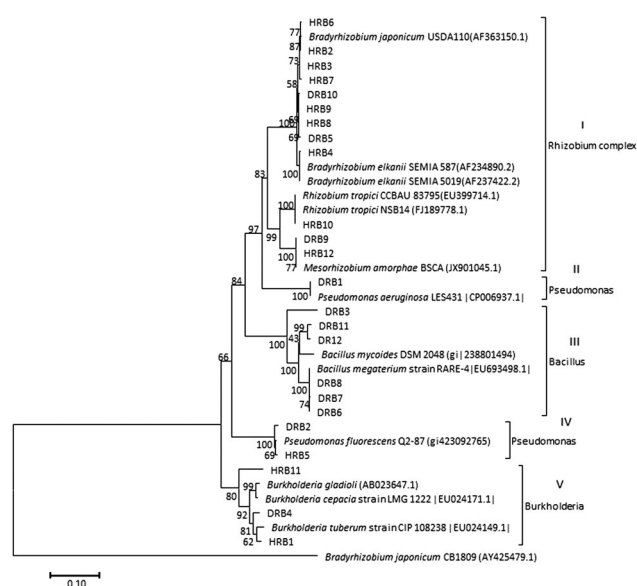


Figure 2. Neighbor Joining (NJ) phylogenetic trees constructed from the 16S rRNA sequences of 24 bacterial isolates from the nodules of healthy and declined rooibos. Note: the majority of the isolates from healthy rooibos (HRB) belong to the rhizobium complex (*Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*) whereas nodules from declined plants contain very little *Rhizobium* but contained isolates belonging to endophytic/rhizosphere bacteria (DRB).

include unfavorable soil pH, nutrient deficiency, extremes of temperature and poor water holding capacity or excessive application of synthetic chemicals (Zahran 1999). Screening for tolerant strains of rhizobia is an important strategy to tackle this problem as the population of *Rhizobium* and *Bradyrhizobium* species varies in their tolerance to major environmental factors (Keyser 1993).

Although a few *Bradyrhizobium* strains were isolated from the declining rooibos roots, these could only be competitive for root nodulation, but not effective for nitrogen fixation. The absence of viable effective *Rhizobia* and the isolation of tolerant *Bacillus* and other endophytic *Burkholderia* and *Pseudomonas* from the nodules in the decline plants probably suggests an early death of the rhizobia. Intensive migration of free-living bacteria from soil niche towards the root leading to premature consumption of the niche's resources could cause a cell death of rhizobia before they form a symbiosis with legume plants (Vorobyov & Provorov 2015) which probably led to no fixation of atmospheric nitrogen. This, in turn, could contribute to the plants to be susceptible to external damages including pathogens. The absence of rhizobia in these nodules could also be attributed to several external limiting factors such as considerable acidification and nutrient depletion, as is the case in many South African soils (Mills & Fey 2003) and which could lead to crop declines as many such bacteria have been found to be associated with legume nodules. For instance, the impact of such limiting factors on the failure of nodulation of soybean (*Glycine max* L.) by a locally developed commercial strain of *Bradyrhizobium japonicum* strain WB74 has been reported in our previous work (Hassen et al. 2014).

Although the highest proportion of the bacteria isolated from the healthy soils is mainly that of the symbiotic rhizobia group, it was possible to detect other endophytic bacteria from the healthy soil nodules that include *Bacillus*, *Burkholderia* and *Pseudomonas* spp. in this study. This concurs with previous studies that endophytes such as *Pseudomonas*, *Bacillus* and *Burkholderia* spp. have been isolated from the root nodules of various legumes (Xu et al. 2014). The existence of such rhizobacteria as *Pseudomonas* spp. in the soil with the ability to produce certain metabolites effective against soilborne pathogens could trigger soils to be suppressive to certain infections that affect plant growth and crop yield. In the USA for instance, soils with a high buildup of fluorescent *Pseudomonas* spp. that produce the metabolite 2,4-diacetyl phloroglucinol are able to suppress take-all infection in wheat (Zhao et al. 2013).

Conclusion

From the results of the study at this particular rooibos plantation, it is possible to conclude that the presence of symbiotic rhizobia and their persistence in the soil as well as in the nodules is very crucial for a healthy nodulating and nitrogen-fixing rooibos plant. It is observed in this study that the proportion of rhizobia in the declined plants was very low suggesting that for a plant that solely depends on symbiotic nitrogen fixation for its nitrogen (N) requirement, absence of sufficient rhizobia and hence fixed atmospheric nitrogen, along with other limiting factors, would contribute to the observed rooibos decline. While such decline is still observed at this plantation, further investigation on the microsymbionts of the nodules and the rhizosphere soil microbial diversity of the healthy and declined plants using additional molecular techniques is warranted. In addition, a study of

the physicochemical properties of the soils would elucidate more information on the potential limiting factors that hindered the persistence and functioning of the symbiotic nitrogen-fixing rhizobia from developing in the nodules of the decline rooibos plants.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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