Universidade Estadual de Londrina

AMARAL MACHACULEHA CHIBEBA

CARACTERIZAÇÃO DE RIZÓBIOS ISOLADOS DE SOJA EM MOÇAMBIQUE E ESTRATÉGIAS VISANDO INCREMENTAR A CONTRIBUIÇÃO DA FIXAÇÃO BIOLÓGICA DE NITROGÊNIO

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Tese apresentada ao Departamento de Agronomia da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Doutor em Agronomia.

Orientadora: Prof. Dr[a]. Maria de Fátima Guimarães
Co-orientadora: Dr[a]. Mariangela Hungria

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BANCA EXAMINADORA

_____
Orientadora Prof. Dr[a]. Maria de Fátima Guimarães
Universidade Estadual de Londrina - UEL

_____
Dr[a]. Mariangela Hungria
Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA

_____
Dr. Stephen Kyei-Boahen
International Institute of Tropical Agriculture - IITA

_____
Dr. Felix Dapare Dakora
Tshwane University of Technology - TUT

_____
Dr. Marco Antonio Nogueira
Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA

_____
Dr[a]. Adriana Pereira da Silva
Universidade Estadual de Londrina - UEL

Londrina, 01 de junho de 2016.

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RESUMO

A inoculação da soja com estirpes de rizóbios eficientes no processo de fixação biológica de nitrogênio (FBN) torna dispensável o uso de fertilizantes nitrogenados nos trópicos. Um problema frequentemente relatado é a falta de capacidade de estirpes identificadas como elite e usadas em inoculantes comerciais em superar a competição imposta pelas populações indígenas ou naturalizadas de rizóbios. A seleção de rizóbios indígenas, adaptados às condições locais, com alta eficiência para o uso em inoculantes representa uma estratégia promissora para evitar fracassos na inoculação. Por outro lado, as fortes evidências de sucesso de inoculação em áreas com populações altas de rizóbios publicadas no Brasil abrem uma oportunidade para a pesquisa sobre inoculação em outras regiões geográficas no mundo. Assim, os objetivos desta tese foram: 1) Caracterizar estirpes indígenas de rizóbio e identificar estirpes com potencial para serem incluídas em inoculantes para variedades de soja tanto de nodulação promíscua como não promíscua nas condições agroclimáticas de Moçambique; 2) Avaliar o desempenho de quatro estirpes elite brasileiras (SEMIAs 587, 5019, 5079 e 5080) e uma norte-americana (USDA 110) no Brasil e em Moçambique. Para o primeiro objetivo, 105 isolados foram obtidos de nódulos de soja promíscua cultivada em 15 locais, em Moçambique, e avaliados pela eficiência simbiótica na casa de vegetação, juntamente com quatro estirpes usadas em inoculantes no Brasil, *Bradyrhizobium japonicum* SEMIA 5079, *B. diazoefficiens* SEMIA 5080, *B. elkanii* estirpes SEMIA 587 e SEMIA 5019, e uma estirpe usada na África, *B. diazoefficiens* USDA 110. Oitenta e sete isolados confirmaram a habilidade de formar nódulos efetivos em fixar N_2 com a soja e foram usados na caracterização genética, por rep-PCR (BOX) e sequenciamento do gene 16S rRNA, e eficiência simbiótica. Os perfis obtidos por BOX - PCR revelaram uma notável diversidade genética com 41 agrupamentos formados, considerando o nível arbitrário de similaridade de 65%. A análise do gene 16S rRNA posicionou os isolados nos clados *Bradyrhizobium* (75%) e *Agrobacterium – Rhizobium* (25%). Variabilidade elevada no desempenho simbiótico foi observada entre os isolados indígenas de Moçambique com dez isolados tendo melhor desempenho que USDA 110, a melhor estirpe de referência, e 50 isolados tendo menor desempenho que todas as estirpes de referência. Treze dos isolados mais eficientes foram avaliados, juntamente com as cinco estirpes de referência, em duas variedades promíscuas (TGx 1963-3F e TGx 1835-10E) e uma não promíscua (BRS 284) de soja, em um segundo ensaio de casa de vegetação. Os 13 isolados também foram caracterizados quanto à tolerância à acidez e alcalinidade (pH 3,5 e 9,0, respectivamente), salinidade (0,1, 0,3 e 0,5 mol L^{-1} de NaCl) e temperaturas altas (35, 40 e 45 ºC) *in vitro*. Cinco isolados, três (4, 19 e 22) pertencentes ao sub-grupo *B. elkanii* e dois (27 e 61) ao sub-grupo *B. japonicum*, mostraram, consistentemente, alta eficiência de fixação de N_2, sugerindo que a inoculação com estirpes indígenas de rizóbios adaptadas às condições locais representa uma estratégia possível para a produção de soja em Moçambique. Para o segundo objetivo, as cinco estirpes de referência testadas no primeiro estudo foram avaliadas nas safras 2013/2014 e 2014/2015 no Brasil (quatro locais) e em Moçambique (cinco locais), em solos com populações de rizóbios compatíveis com soja, variando de < 10 a 2×10^5 células g^{-1} solo. Os ensaios foram conduzidos com variedades não promíscuas de soja e os seguintes tratamentos: (1) NI, controle não inoculado e sem N-fertilizante; (2) NI+N,

controle não inoculado e com 200 kg de N ha^{-1}; e, inoculado com (3) *B. japonicum* SEMIA 5079; (4) *B. diazoefficiens* SEMIA 5080; (5) *B. elkanii* SEMIA 587; (6) *B. elkanii* SEMIA 5019; (7) *B. diazoefficiens* USDA 110; (8) SEMIAs 5079 + 5080 (tratamento avaliado somente no Brasil). A nodulação, crescimento de plantas e rendimento foram avaliados em ambos países. Os melhores tratamentos com inoculação no Brasil, considerando a média dos locais e safras, foram com as estirpes SEMIAs 5079 + 5080, SEMIA 5079 e USDA 110, com ganhos médios de rendimento de grãos de 4–5% em relação ao tratamento NI. As estirpes SEMIA 5079, SEMIA 5080, SEMIA 5019 e USDA 110 foram as melhores em Moçambique, com ganhos médios de rendimento de 20–29% em relação ao tratamento NI. Adicionalmente, as quatro estirpes com melhor desempenho em Moçambique resultaram em rendimentos semelhantes ou melhores do que o tratamento NI+N, confirmando a FBN como uma alternativa sustentável aos N-fertilizantes, poluidores ambientais. Os resultados também confirmam a viabilidade da transferência de tecnologias relacionadas à FBN com a soja entre países com condições agroclimáticas similares.

Palavras-chave: Fixação biológica de nitrogênio. Simbiose. Variedades promíscuas de soja. *Bradyrhizobium.* Inoculação.

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ABSTRACT

Soybean inoculation with effective rhizobial strains makes unnecessary the use of nitrogen fertilizers in the tropics. A frequently reported problem is the failure of the inoculant strain to overcome the competition imposed by indigenous or naturalized rhizobial populations. The screening of indigenous rhizobia, adapted to local conditions, for highly effective strains for use as inoculants represents a promising strategy in overcoming inoculation failure. On the other hand, the strong evidences of inoculation success in areas with high rhizobial populations in Brazil open a window for inoculation research in other geographic regions in the world. Therefore, the objectives of this thesis were: 1) To characterize indigenous rhizobia and to identify strains that hold potential to be included in inoculant formulations for soybean production, with both promiscuous and non-promiscuous varieties, in Mozambican agro-climatic conditions; 2) To compare the performance of four elite strains from Brazil (SEMIAs 587, 5019, 5079 and 5080) and another strain from the US (USDA 110) in trials carried out with non-promiscuous soybean varieties in Brazil and Mozambique. For the first objective, 105 isolates were obtained from nodules of promiscuous soybean grown at 15 sites in Mozambique and screened for N-fixation effectiveness in the greenhouse along with four strains used in inoculants in Brazil, *Bradyrhizobium japonicum* SEMIA 5079, *B. diazoefficiens* SEMIA 5080, *B. elkanii* strains SEMIA 587 and SEMIA 5019, and one strain used in Africa, *B. diazoefficiens* USDA 110. Eighty-seven isolates confirmed the ability to form effective nodules on soybean and were used for genetic characterization, by rep-PCR (BOX) and DNA sequencing of the 16S rRNA gene, and symbiotic effectiveness. The BOX-PCR fingerprinting revealed remarkable genetic diversity, with 41 clusters formed, considering an arbitrary similarity level of 65%. The 16S rRNA analysis assigned the isolates to the *Bradyrhizobium* (75%) and *Agrobacterium – Rhizobium* (25%) clades. Great variability in symbiotic effectiveness was detected among the indigenous rhizobia from Mozambique with ten isolates performing better than USDA 110, the best reference strain, and 50 isolates with lower performance than all the reference strains. Thirteen of the isolates with the highest symbiotic effectiveness were evaluated, along with the five reference strains, in two promiscuous (TGx 1963-3F and TGx 1835-10E) and one non-promiscuous (BRS 284) soybean varieties in a second greenhouse trial. The 13 isolates were also characterized for tolerance to acidity and alkalinity (pH 3.5 and 9.0, respectively), salinity (0.1, 0.3 and 0.5 mol L^{-1} of NaCl) and high temperatures (35, 40 and 45 ºC) *in vitro*. Five isolates, three (4, 19 and 22) belonging to the sub-group *B. elkanii* and two (27 and 61) assigned to the sub-group *B. japonicum,* consistently showed high symbiotic effectiveness, suggesting that the inoculation with indigenous rhizobia adapted to local conditions represents a possible strategy for soybean production in Mozambique. For the second objective, the five reference strains tested in the first study were evaluated in the 2013/2014 and 2014/2015 crop seasons in Brazil (four sites) and Mozambique (five sites). In both countries, the trial sites were in tropical and temperate climate zones and the areas had soybean rhizobial population ranging from < 10 to over 1×10^3 cells g^{-1} of soil. The treatments were: (1) NI, non-inoculated control with no N-fertilizer; (2) NI+N, non-inoculated control with 200 kg of N ha^{-1}; and inoculated with (3) *Bradyrhizobium japonicum* SEMIA 5079; (4) *B. diazoefficiens* SEMIA 5080; (5) *B. elkanii* SEMIA 587; (6) *B. elkanii* SEMIA 5019; (7) *B. diazoefficiens* USDA 110; (8) SEMIAs 5079 + 5080 (treatment only tested in Brazil). The best inoculation treatments across sites and crop

seasons in Brazil were SEMIAs 5079 + 5080, SEMIA 5079 and USDA 110, with average grain yield gains of 4–5% in relation to the NI treatment. Strains SEMIA 5079, SEMIA 5080, SEMIA 5019 and USDA 110 were the best in Mozambique, with average 20–29% grain yield gains over the NI treatment. Moreover, the four best performing strains in Mozambique resulted in similar or better yields than the NI+N treatment, confirming the BNF as a sustainable option to the use of environmentally polluting N-fertilizers. The results also confirm the feasibility of transference of technologies related to BNF with soybean between countries with similar agro-climates.

Keywords: Biological nitrogen fixation. Symbiosis. Promiscuous soybean varieties. *Bradyrhizobium*. Inoculation.

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LISTA DE ABREVIATURAS E SIGLAS

Al	*Aluminum*
BNF	*Biological nitrogen fixation*
bp	*Base pairs*
°C	*Degree Celsius*
Ca	*Calcium*
cm	*Centimeter*
$cmol_c$	*Centimol of charge*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CPAC	Centro de Pesquisa Agropecuária dos Cerrados
C.V.	*Coefficient of Variation*
DNA	*Deoxyribonucleic Acid*
dNTP	*Deoxynucleotide triphosphates*
EDTA	*Ethylenediaminetetraacetic acid*
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
FAO	*Food and Agriculture Organization*
FBN	Fixação Biológica de Nitrogênio
IITA	*International Institute of Tropical Agriculture*
K	*Potassium*
L	*Liter*
mg	*Miligram*
Mg	*Magnesium*
mL	*Mililiter*
MLSA	*Multilocus sequence analysis*
mm	*Milimeter*
mmol	*Milimol*
N	*Nitrogen*
N2Africa	*Nitrogen to Africa*
ng	*Nanogram*
nm	*Nanometre*
OD	*Optical Density*
PCR	*Polymerase Chain Reaction*
pmol	*Picomol*
RNA	*Ribonucleic acid*
rRNA	*Ribossomal Ribonucleic Acid*
SEMIA	Seção de Microbiologia Agrícola
TGx	*Tropical Glycine cross*
TUT	*Tshwane University of Technology*
UEL	Universidade Estadual de Londrina
USDA	*United States Department of Agriculture*
UV	*Ultraviolet*
V	*Volt*
v/v	*Volume/volume*
w/v	*Weight/volume*
YM	*Yeast Mannitol*
YMA	*Yeast mannitol agar*
µL	*Microlitre*
µm	*Micrometre*

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1 INTRODUÇÃO

A soja [*Glycine max* (Linnaeus) Merrill] tem potencial para desempenhar um papel importante na resposta à insegurança alimentar resultante da crescente pressão demográfica. A população mundial está projetada para superar 10 bilhões de pessoas em 2100 (GERLAND et al., 2014; SAKSCHEWSKI et al., 2014; UN, 2015), e grande parte desse crescimento deve ocorrer na África (CLELAND, 2013; GUPTA, 2013; UN, 2015), onde a fome já é uma ameaça. Com um elevado teor de proteínas nas sementes (~40%), que proporcionam todos os aminoácidos essenciais para a saúde humana (YOUNG, 1991), e alto teor de óleo nas sementes (~20%), a soja é uma excelente fonte de proteína para o consumo humano, assim como para ração animal. Os grãos de soja são também utilizados na indústria farmacêutica (NR et al., 2013; KUSUNOKI et al., 2015) e, mais recentemente, têm sido reconhecidos como uma fonte alternativa aos combustíveis fósseis (VIEIRA et al., 2010; REQUENA et al., 2011). Esta cultura proporciona muitas vantagens aos sistemas de cultivo sustentáveis, incluindo a capacidade de fixar nitrogênio atmosférico (N_2), diminuindo a necessidade de aplicação de fertilizantes nitrogenados. Esta vantagem é particularmente importante na África, onde existem grandes limitações econômicas ao uso de fertilizantes (CHIANU et al., 2011; PARR, 2014).

O cultivo da soja fora do Sudeste Asiático, centro de origem e domesticação da soja (XU et al., 2002; LI et al., 2010), requer inoculação com estirpes exóticas eficazes (PULVER et al., 1985; ABAIDOO et al., 2007; GILLER et al., 2011). Na África, onde a distribuição de inoculantes representa outra limitação, uma estratégia que consiste no uso de variedades promíscuas de soja capazes de formar nódulos com rizóbios indígenas (PULVER et al., 1985; ABAIDOO et al., 2007; TEFERA, 2011) foi sugerida e é usada há décadas. Todavia, com a intensificação da cultura, a procura por variedades mais produtivas mas que requerem inoculação está a aumentar.

Numerosas pesquisas reportam insucessos da inoculação da soja, principalmente atribuídos à competição imposta pelos rizóbios indígenas ou naturalizados (THIES et al., 1992; STREETER, 1994; VLASSAK et al., 1997; VIEIRA et al., 2010). Entretanto, fortes evidências de inoculação bem sucedida em áreas com populações elevadas de rizóbios, de 10^3 a 10^6 células g^{-1} de solo, têm sido relatadas no Brasil (HUNGRIA et al., 1998; HUNGRIA et al., 2005; HUNGRIA et al., 2006a; CAMPO et al., 2009; HUNGRIA et

al., 2013; HUNGRIA; MENDES, 2015), o que representa uma oportunidade para a investigação da inoculação em outras regiões geográficas do mundo.

A ocorrência de rizóbios indígenas compatíveis com variedades promíscuas e com alta capacidade de fixação biológica do nitrogênio (FBN) na África (ABAIDOO et al., 2000; SANGINGA et al., 2000; MUSIYIWA et al., 2005; ABAIDOO et al., 2007; KLOGO et al., 2015; GYOGLUU et al., 2016) sugere que a seleção de estirpes efetivas, competitivas e adaptadas às condições locais para uso em inoculantes representa uma estratégia promissora para a produção de soja. Evidências recentes indicam a ocorrência de rizóbios indígenas capazes de estabelecer simbiose efetiva com variedades de soja de nodulação promíscua e não promíscua em Moçambique (GYOGLUU et al., 2016).

O sucesso da inoculação e FBN com a cultura da soja no Brasil é primordialmente atribuído aos programas de seleção de estirpes levados a cabo por mais de meio século e ao desenvolvimento de métodos apropriados de aplicação dessas bactérias no solo (HUNGRIA et al., 2006a; HUNGRIA; MENDES, 2015). Ao contrário, em Moçambique a soja é uma cultura relativamente nova, sendo cultivada principalmente com variedades promíscuas e sem uso de inoculantes (GYOGLUU et al., 2016). Em anos recentes, no entanto, o aumento da demanda por grãos para abastecer a indústria de frango e para exportação (DIAS; AMANE, 2011; MUANANAMUALE et al., 2012), resultou na demanda por variedades não promíscuas mais produtivas mas que requerem inoculação. As condições agroclimáticas das áreas de produção de soja em Moçambique são consideravelmente similares às principais zonas de produção de soja no Brasil, o que levanta a questão sobre se as várias tecnologias de inoculação que levaram mais de 50 anos para serem desenvolvidas poderiam ser transferidas para Moçambique, poupando significativamente tempo e dinheiro.

Assim, os objetivos desta tese foram: 1) Caracterizar rizóbios indígenas e identificar estirpes com potencial para serem usadas como inoculantes para variedades promíscuas e não promíscuas de soja, nas condições agroclimáticas de Moçambique; 2) Avaliar o desempenho de quatro estirpes do Brasil (SEMIAs 587, 5019, 5079 e 5080) e uma estirpe norte-americana (USDA 110) adotada como inoculante padrão em muitos países africanos, em ensaios conduzidos com variedades de soja não promíscuas no Brasil e em Moçambique.

2 REVISÃO BIBLIOGRÁFICA

2.1 OS RIZÓBIOS

O termo rizóbio é referente a todas as bactérias do solo capazes de formar nódulos nas raízes e caules de plantas leguminosas (WILLEMS, 2006; ZHANG et al., 2012; BERRADA; FIKRI-BENBRAHIM, 2014). No nódulo, o rizóbio reduz o nitrogênio molecular (N_2) em amônia (NH_3), que em seguida passa a íon amônio (NH_4^+), portanto, transformando-o de uma forma não utilizável para outra diretamente assimilada pelas plantas (BERRADA; FIKRI-BENBRAHIM, 2014).

2.1.1 Caracterização

Uma célula típica de rizóbio é de tamanho pequeno a médio (0,5 a 0,9 × 1,2 a 3,0 μm), Gram-negativa, móvel quando jovem, com grandes quantidades de grânulos de poli β-hidroxibutirato (PHB), formando 40-50% do peso seco da célula, que podem ser facilmente observados usando corantes de grânulos metacromáticos (GUPTA et al., 2007). Os genes simbióticos, que codificam a nodulação e a FBN estão localizados em plasmídeos ou ilhas simbióticas e determinam a gama de hospedeiros e a capacidade de fixar N_2 nos nódulos (VELÁZQUEZ et al., 2010). Em geral, os microssimbiontes são muito diferentes em entre si em relação à morfologia da colônia (AL-FALIH, 2002; SESSITSCH et al., 2002), tolerância à acidez (LI et al., 2011; ZHANG et al., 2011; TIAN et al., 2012), à salinidade (HAN et al., 2009; ZHANG et al., 2011), a altas temperaturas (ZAHRAN, 2010), sorologia e outras características bioquímicas (AL-FALIH, 2002; SESSITSCH et al., 2002).

2.1.2 Taxonomia

A taxonomia pode ser definida como a ciência da descrição, identificação, nomenclatura e classificação ou estabelecimento da relação de parentesco evolutivo entre os organismos (WHITEHEAD, 1990; ZAKHIA; DE LAJUDIE, 2001; CHAMBEL; TENREIRO, 2004). A necessidade de se identificar os organismos e estabelecer as relações

entre eles tem resultado na crescente aplicação da taxonomia a outras disciplinas (CHAMBEL; TENREIRO, 2004), contribuindo para a redução da ambiguidade e incerteza que obstruem a reprodutibilidade dos resultados de pesquisas (BENNETT; BALICK, 2014).

Até aos anos 1980 os rizóbios eram classificados em um único gênero, *Rhizobium,* que incluía seis espécies, *R. leguminosarum, R. meliloti, R. trifolii, R. phaseoli, R. lupini e R. japonicum* (YOUNG; HAUKKA, 1996; ZAKHIA; DE LAJUDIE, 2001). A ideia prevalecente era de que os rizóbios podiam ser separados em espécies com base na gama de hospedeiros que nodulavam e, reciprocamente, as leguminosas podiam ser agrupadas com base em simbiontes mútuos (WILLEMS, 2006; VELÁZQUEZ et al., 2010). Entretanto, esta taxonomia foi abandonada em virtude de observações frequentes de sobreposições entre grupos de hospedeiros (YOUNG; HAUKKA, 1996; WILLEMS, 2006), e ao reconhecimento de que a classificação dos rizóbios devia seguir a taxonomia geral das bactérias e incluir um painel de características fenotípicas e genotípicas (ZAKHIA; DE LAJUDIE, 2001; BARCELLOS et al., 2007; ZHANG et al., 2012; BERRADA; FIKRI-BENBRAHIM, 2014).

Assim, foram introduzidas características genéticas como as hibridizações DNA-DNA e DNA-rRNA, catálogos rRNA e sequenciamento rDNA o que revelou maior diversidade entre os rizóbios e evidenciou as relações entre grupos de bactérias (YOUNG; HAUKKA, 1996; WILLEMS, 2006; MENNA et al., 2009a; BERRADA; FIKRI-BENBRAHIM, 2014). Até Dezembro de 2014 tinham sido descritas 98 espécies pertencentes a 14 gêneros de rizóbios, que incluem 11 da classe Alfaproteobacteria: *Rhizobium*, *Ensifer* (anteriormente *Sinorhizobium*), *Shinella*, *Mesorhizobium*, *Phylobacterium*, *Methylobacterium*, *Microvirga*, *Ochrobactrum*, *Azorhizobium*, *Devosia* e *Bradyrhizobium*; dois da classe Betaproteobacteria: *Burkholderia* e *Cupriavidus* (anteriormente *Ralstonia*); e um da classe Gamaproteobacteria: *Pseudomonas* (BERRADA; FIKRI-BENBRAHIM, 2014). As espécies *Bradyrhizobium japonicum* (JORDAN, 1982; MAN et al., 2008; HUNGRIA et al., 2013; ZERPA et al., 2013), *B. diazoefficiens* (DELAMUTA et al., 2013; SIQUEIRA et al., 2014), *B. elkanii* (KUYKENDALL et al., 1992; HUNGRIA et al., 1998; SUZUKI et al., 2008; SHIRO et al., 2013; HERRMANN et al., 2014; PARR, 2014), *B. liaoningense* (XU et al., 1995; HAN et al., 2009), *B. yuanmingense* (HAN et al., 2009; JAISWAL et al., 2012), *B. canariense* (VINUESA et al., 2005; WU et al., 2011), *Rhizobium* spp. (HUNGRIA et al., 2006b; VIEIRA et al., 2010), *Rhizobium etli* (JAISWAL et al., 2012), *Mesorhizobium tianshanense* (WU et al., 2011), *Ensifer fredii* (SUZUKI et al., 2008; HAN et al., 2009) e *E. xinjiangense* (PENG et

al., 2002; VIEIRA et al., 2010) têm sido reportadas como microssimbiontes da soja em diferentes regiões geográficas do mundo.

A caracterização da estirpe é o elemento chave da taxonomia das bactérias (FOURNIER et al., 2006; TINDALL et al., 2010). Os procariotos são identificados usando uma "abordagem polifásica" (DE LAJUDIE et al., 1994; ROSSELLO-MORA; AMANN, 2001), que consiste em comparar as características fenotípicas e genotípicas de uma estirpe com as das estirpes tipo, representantes de espécies descritas anteriormente (FOURNIER et al., 2006; CHUN et al., 2007; TINDALL et al., 2010; RAMASAMY et al., 2014). As características fenotípicas incluem propriedades morfológicas (cor, dimensões e forma da colônia), fisiológicas (crescimento em diferentes temperaturas, pH, concentração de sais) e bioquímicas (composição química da parede celular, composição de proteínas) (ROSSELLO-MORA; AMANN, 2001; OREN, 2004; GEVERS et al., 2006; TINDALL et al., 2010).

As técnicas mais usadas para descrever as características genotípicas dos procariotos são: hibridização DNA–DNA (DDH*, DNA-DNA hybridization*), sequenciamento do gene ribossomal 16S rRNA, teor G+C (guanina + citosina) e análise de sequências de multilocus (MLSA, *multilocus sequence analysis*). A técnica DDH consiste na mistura, desnaturação e reassociação de moléculas de DNA de dois organismos diferentes (ROSSELLO-MORA; AMANN, 2001). O princípio desta técnica é que organismos geneticamente similares terão um maior número de sequências de bases de nucleotídeos semelhantes e formarão mais moléculas de DNA híbridas. O resultado é medido pela taxa relativa de anelamento (RBR*, Relative binding rate*) e a diferença termal de desnaturação (ΔT_m) (OREN, 2004; TINDALL et al., 2010). Atualmente, considera-se que valores de RBR $\geq$ 70% e $\Delta T_m \leq 5°C$ indicam que os organismos comparados pertencem à mesma espécie (ROSSELLO-MORA; AMANN, 2001; OREN, 2004; TINDALL et al., 2010). As principais desvantagens desta técnica incluem ser muito laboriosa, não permitir comparação entre laboratórios e a inexistência de um banco de dados (KIM et al., 2014).

O gene 16S rRNA foi proposto para estudo da taxanomia dos procariotos por ser uma molécula grande, altamente conservada e com uma considerável informação genética (WOESE et al., 1990; ROSSELLO-MORA; AMANN, 2001). A maior vantagem desta técnica é a existência de uma ampla base de dados com sequências de várias espécies (KONSTANTINIDIS; TIEDJE, 2005; GEVERS et al., 2006; TINDALL et al., 2010), que pode ser usada para identificação de novos isolados. A uniformidade do gene 16S rRNA, entretanto, restringe esta técnica à comparações entre gêneros, tendo baixo poder de resolução

para delinear espécies (FOX et al., 1992; STACKEBRANDT; GOEBEL, 1994; ROSSELLO-MORA; AMANN, 2001; COOPER; FEIL, 2004).

A técnica G+C baseia-se na constância da relação entre as quatro bases de nucleotídeos adenina, (A), timina (T), guanina (G) e citosina (C), na molécula de DNA, e é calculada como percentagem molar de G+C =(G+C)/(A+T+C+G) × 100 (ROSSELLO-MORA; AMANN, 2001). Organismos que diferem em mais de 10 mol% em teores de G+C do DNA não pertencem ao mesmo gênero e 5 mol% é o limite máximo para organismos da mesma espécie (ROSSELLO-MORA; AMANN, 2001; RAMASAMY et al., 2014). Entretanto, estes limites não são aplicáveis a todos os gêneros (LUDWIG; KLENK, 2000; ROSSELLO-MORA; AMANN, 2001; TINDALL et al., 2010; RAMASAMY et al., 2014).

A análise de sequências multilocus é baseada na premissa de que os genes de manutenção celular (*housekeeping genes*) são de evolução lenta (mais rápida que o gene rRNA), codificam produtos essenciais às bactérias e, consequentemente, estão presentes em todas as estirpes de um gênero (GEVERS et al., 2005; HANAGE et al., 2006). Sequências de fragmentos internos dos genes *housekeeping* são determinadas e a identificação de estirpes é feita por comparação com sequências existentes na base de dados disponível na internet (URWIN; MAIDEN, 2003; COOPER; FEIL, 2004; HANAGE et al., 2006). Esta técnica tem alto poder de resolução para inferência de gêneros e espécies, para além de permitir a identificação de novas espécies (URWIN; MAIDEN, 2003; COOPER; FEIL, 2004).

2.1.3 Ecologia

Os rizóbios e as plantas hospedeiras estão expostos a condições ambientais adversas que afetam a sua sobrevivência, desenvolvimento e produtividade. Em termos globais, os fatores abióticos que mais limitam a simbiose rizóbio-leguminosa são a deficiência hídrica, acidez do solo, altas temperaturas e salinidade (BOTTOMLEY, 1992; ZAHRAN, 2001; AL-FALIH, 2002).

Os rizóbios divergem na susceptibilidade à seca. Em geral, os de crescimento lento sobrevivem melhor à dessecação do solo do que os de crescimento rápido (ZAHRAN, 2001; AL-FALIH, 2002) e os rizóbios que produzem grandes quantidades de polissacarídeos extracelulares são mais sensíveis à dessecação do que os que produzem pouca ou nenhuma (AL-FALIH, 2002). A seca pode afetar a FBN de diversas formas, incluindo a sobrevivência e crescimento dos rizóbios, a sua estrutura populacional no solo, a formação e

longevidade dos nódulos, a síntese de leghemoglobina e o funcionamento do nódulo (HUNGRIA; VARGAS, 2000).

A acidez é um problema típico dos solos tropicais que está associada a concentrações tóxicas de alumínio e manganês (HUNGRIA; VARGAS, 2000) e deficiências de fosfato e molibdênio (AL-FALIH, 2002), o que pode prejudicar o rizóbio, o hospedeiro e a simbiose. Tanto as leguminosas como os rizóbios exibem diferenças quanto à tolerância à acidez do solo. Em geral, o gênero *Bradyrhizobium* tolera valores de pH mais baixos do que *Rhizobium* (VIEIRA et al., 2010; ZAHRAN, 2010). A acidez afeta as primeiras etapas do processo de infecção, incluindo a troca de sinais moleculares entre parceiros simbióticos e a aderência do rizóbio às raízes, além de várias etapas da simbiose (HUNGRIA; VARGAS, 2000).

As temperaturas máximas do solo nos trópicos frequentemente superam 40 °C aos 5 cm e 50 °C a 1 cm de profundidade (HUNGRIA; VARGAS, 2000), o que pode afetar negativamente a fixação biológica, uma vez que a temperatura crítica da simbiose está entre 35 e 40 °C (ZAHRAN, 1999; AL-FALIH, 2002; ZAHRAN, 2010). Temperaturas elevadas prejudicam a sobrevivência do rizóbio, a infecção dos pelos radiculares, a diferenciação do bacteróide, a formação e funcionamento dos nódulos (HUNGRIA; VARGAS, 2000; ZAHRAN, 2010).

A salinidade é um fator que afeta a produção agrícola em regiões áridas e semiáridas do mundo (ZAHRAN, 2010). Os níveis de salinidade que inibem a simbiose entre a leguminosa e o rizóbio são diferentes dos que limitam o crescimento de cada simbionte. Em geral, as concentrações de cloreto de sódio que afetam a simbiose são mais baixas do que as que afetam a leguminosa ou o rizóbio e as leguminosas são mais sensíveis à salinidade do que os rizóbios (AL-FALIH, 2002). As espécies *Sinorhizobium/Ensifer* spp. têm sido reportadas como dominantes nas regiões biogeográficas com altos teores de sais (HAN et al., 2009; ZHANG et al., 2011). A salinidade alta diminui a sobrevivência da população de rizóbios no solo e na rizosfera (BOTTOMLEY, 1992; ZAHRAN, 2010).

2.2 FIXAÇÃO BIOLÓGICA DE NITROGÊNIO

A fixação biológica de nitrogênio (FBN) é o processo no qual os rizóbios capturam o nitrogênio atmosférico e convertem as moléculas não reativas de N_2 em NH_3, que depois incorporam íons hidrogênio e se transformam em amônio (NH_4^+), uma forma que é

disponibilizada para as plantas. Este mecanismo é catalisado pela enzima nitrogenase, presente na bactéria, pela reação (SKORUPSKA et al., 2010):

$$N_2 + 8H + 8e^- + 16\ ATP \xrightarrow{Nitrogenase} 2NH_3 + H_2 + 16\ ADP + 16\ Pi$$

O impacto da FBN na agricultura é grande. Estima-se que dos 100 – 290 milhões de toneladas de N fixados pelos microrganismos por ano, 50 – 70 milhões de toneladas de N ocorrem nos sistemas agrícolas, muito próximo dos 83 milhões fixados pela indústria (SKORUPSKA et al., 2010). Além da redução da necessidade do uso de adubos nitrogenados, a FBN diminui o risco de contaminação ambiental, uma vez que o N fixado simbioticamente é menos suscetível a perdas por lixiviação, escoamento superficial ou desnitrificação do que o fixado industrialmente.

O objetivo da inoculação é fornecer rizóbios simbioticamente efetivos em quantidade suficiente para induzir uma rápida colonização da rizosfera e desenvolver nódulos na planta (ARCHANA, 2010). Muito frequentemente os rizóbios inoculados devem competir com populações de rizóbios indígenas ou naturalizados, muitas vezes não efetivos, mas altamente competitivos e já adaptados ao ambiente (AL-FALIH, 2002). As etapas pelas quais passa uma nova estirpe de rizóbio inoculada incluem a adaptação ao ambiente, a multiplicação no solo e na rizosfera do hospedeiro e, por fim, a competição com populações de rizóbios indígenas pelos locais de infecção e nodulação (AL-FALIH, 2002; VIEIRA et al., 2010).

Uma vez que os rizóbios podem viver saprofitamente por longos períodos no solo, o cultivo repetido da soja na mesma área promove o estabelecimento de população naturalizada de estirpes de rizóbio (MCLOUGHLIN et al., 1990; MENDES et al., 2004), que resulta da saída de rizóbios dos nódulos quando estes se desintegram (VIEIRA et al., 2010). Esta população naturalizada pode constituir um obstáculo competitivo para uma posterior inoculação de estirpes novas e mais eficientes (STREETER, 1994; VLASSAK et al., 1997; AL-FALIH, 2002; VIEIRA et al., 2010). A magnitude deste problema depende do ambiente (solo e clima), do hospedeiro (variedade), das estirpes do inoculante (capacidade competitiva), do tamanho e composição da população naturalizada de rizóbios e do tipo de inoculante e forma de aplicação (KVIEN et al., 1981; LÓPEZ-GARCÍA et al., 2009).

Considerando que na África existem rizóbios indígenas efetivos para a soja promíscua (ABAIDOO et al., 2000; MPEPEREKI et al., 2000; ABAIDOO et al., 2007;

TEFERA, 2011; ATIENO et al., 2012; WASIKE et al., 2012; HERRMANN et al., 2014) pode-se deduzir que a prospecção entre os rizóbios indígenas de estirpes altamente eficientes para uso em inoculantes é uma estratégia promissora. Esta estratégia é particularmente importante porque populações indígenas superiores a 2×10^1 células g^{-1} de solo geralmente têm vantagem competitiva sobre as estirpes exógenas do inoculante, anulando o efeito da inoculação (SINGLETON; TAVARES, 1986; THIES et al., 1991a; STREETER, 1994; ARCHANA, 2010; BALA et al., 2011; CHIANU et al., 2011). Além disso, há evidências de que, em muitos locais, as populações indígenas não são muito eficientes ou não ocorrem em densidades suficientes para atender a demanda em N das variedades promíscuas de soja (SANGINGA et al., 2000; OKOGUN; SANGINGA, 2003; ABAIDOO et al., 2007; KLOGO et al., 2015).

Tendo em conta o potencial da soja para a nutrição humana e animal, bem como de enriquecimento do solo com restos culturais, especialmente no contexto da África, onde há carência nutricional e dificuldade de acesso a insumos agrícolas, a prospecção e uso de microrganismos capazes de fixação de N_2, promovendo o aumento da produção agrícola com menores impactos ambientais, é uma estratégia promissora.

2.3 FERTILIZAÇÃO NITROGENADA

O nitrogênio (N) é o elemento que determina a produção de grãos, mormente em espécies com elevado teor de N nas sementes (HIROSE et al., 2005), como a soja. Se para cada tonelada de grãos de soja produzida são necessários 80 kg de N (65 kg de N alocados às sementes, e 15 kg alocados às raízes, caule e folhas) (HUNGRIA et al., 2005; HUNGRIA; MENDES, 2015), para produzir 2000 kg ha^{-1} de grãos seriam necessários 160 kg de N. Em solos com baixo teor de N, e considerando que os fertilizantes nitrogenados raramente ultrapassam uma eficiência de 60% (HUNGRIA et al., 2006c), seriam necessários aproximadamente 250 kg de N. Isto significaria um custo adicional direto de cerca de 100 kg ha^{-1}, pela ineficiência dos fertilizantes nitrogenados, e custos adicionais indiretos relacionados ao consumo de energia não renovável para a síntese de amônia, contaminação de águas superficiais e subterrâneas (por escoamento superficial e lixiviação de nitrato), e emissão de óxidos de nitrogênio de efeito estufa (CASSMAN, 1999; GILLER, 2001).

No Brasil, pequisas realizadas com diferentes fontes, doses e frequências de aplicação de nitrogênio mostraram, consistentemente, que a fertilização nitrogenada da soja

não é lucrativa (NISHI; HUNGRIA, 1996; HUNGRIA et al., 2006c; HUNGRIA; MENDES, 2015) e, por isso, não se recomenda o uso de fertilizantes nitrogenados para a cultura (HUNGRIA; MENDES, 2015). Estes resultados contrastam com os obtidos na China, centro de origem da soja, que indicam que, sozinha, a FBN é incapaz de suprir as necessidades da cultura em nitrogênio (GAN et al., 2003). Todavia, os rendimentos obtidos são inferiores aos reportados na América do Sul (HUNGRIA et al., 2006c), o que sugere a necessidade de uma pesquisa contínua que favoreça a FBN.

A fertilização nitrogenada aumenta os rendimentos da soja nos Estados Unidos da América (PUEPPKE, 2005; TAYLOR et al., 2005), o que pode ser explicado pelo melhoramento de variedades efetuado na presença N mineral no solo, contrariamente à estratégia usada na América do Sul (HUNGRIA; MENDES, 2015).

A experiência da América do Sul tem mostrado que a soja pode ser produzida exclusivamente com FBN, desde que as pesquisas de melhoramento de variedades sejam realizadas em campos sem fertilizantes nitrogenados (HUNGRIA; MENDES, 2015).

ARTIGO A: NOVAS ESTRATÉGIAS PARA AGRICULTURA SUSTENTÁVEL EM ÁFRICA: CARACTERIZANDO E SELECIONANDO BRADYRHIZOBIA PARA AUMENTO DA PRODUÇÃO DE SOJA [*GLYCINE MAX* (L.) MERRILL] EM MOÇAMBIQUE

RESUMO

A inoculação da soja com estirpes de rizóbios efetivas torna dispensável o uso de fertilizantes nitrogenados nos trópicos. Um problema frequentemente relatado é a falta de capacidade da estirpe inoculante em superar a competição imposta pelas populações indígenas de rizóbios. A seleção entre os rizóbios indígenas, adaptados às condições locais, de estirpes com alta eficiência para o uso em inoculantes representa uma estratégia promissora para evitar fracassos na inoculação. Caracterizar estirpes indígenas de rizóbio e identificar estirpes com potencial para serem incluídas em inoculantes para variedades de soja tanto de nodulação promíscua como não promíscua nas condições agro-climáticas de Moçambique. Um total de 105 isolados obtidos de nódulos de soja promíscua cultivada em 15 locais, em Moçambique, foram avaliados pela eficiência simbiótica na casa de vegetação juntamente com quatro estirpes usadas em inoculantes no Brasil, *Bradyrhizobium japonicum* SEMIA 5079, *B. diazoefficiens* SEMIA 5080, *B. elkanii* strains SEMIA 587 e SEMIA 5019, e uma estirpe usada na África, *B. diazoefficiens* USDA 110. Oitenta e sete isolados confirmaram a habilidade de formar nódulos efetivos em fixar N_2 com a soja e foram usados na caracterização genética, por rep-PCR (BOX) e sequenciamento do gene 16S rRNA, e eficiência simbiótica. Os perfis obtidos por BOX-PCR revelaram uma notável diversidade genética, com 41 agrupamentos formados, considerando o nível arbitrário de similaridade de 65%. A análise do gene 16S rRNA posicionou os isolados nos clados *Bradyrhizobium* (75%) e *Agrobacterium – Rhizobium* (25%). Variabilidade elevada no desempenho simbiótico foi observada entre os isolados indígenas de Moçambique com dez isolados tendo melhor desempenho que USDA 110, a melhor estirpe de referência, e 50 isolados tendo menor desempenho que todas as estirpes de referência. Treze dos isolados mais eficientes foram avaliados, juntamente com as cinco estirpes de referência, em duas variedades promíscuas (TGx 1963-3F e TGx 1835-10E) e uma não promíscua (BRS 284) de soja, em um segundo ensaio de casa de vegetação. Os 13 isolados também foram caracterizados quanto à tolerância à acidez e alcalinidade (pH 3,5 e 9,0, respectivamente), salinidade (0,1, 0,3 e 0,5 mol L^{-1} de NaCl) e temperaturas altas (35, 40 e 45 ºC) *in vitro*. Cinco isolados, três (4, 19 e 22) pertencentes ao sub-grupo *B. elkanii* e dois (27 e 61) ao sub-grupo *B. japonicum*, mostraram, consistentemente, alta eficiência de fixação de N_2, sugerindo que a inoculação com estirpes indígenas de rizóbios adaptados às condições locais representa uma estratégia possível para a produção de soja em Moçambique.

Palavras-chave: Fixação biológica de nitrogênio – simbiose – variedades promíscuas de soja – *Rhizobium* – 16S rRNA – BOX-PCR

3 ARTIGO A: NEW STRATEGIES FOR A SUSTAINABLE AGRICULTURE IN AFRICA: CHARACTERIZING AND SELECTING BRADYRHIZOBIA TO INCREASE SOYBEAN [*GLYCINE MAX* (L.) MERRILL] YIELD IN MOZAMBIQUE

ABSTRACT

Soybean inoculation with effective rhizobial strains makes unnecessary the use of nitrogen fertilizers in the tropics. A frequently reported problem is the failure of the inoculant strain to overcome the competition imposed by indigenous rhizobial populations. The screening of indigenous rhizobia, already adapted to local conditions, for highly effective strains for use as inoculants represents a promising strategy in overcoming inoculation failure. The objective of this study was to characterize indigenous rhizobia and to identify strains that hold potential to be included in inoculant formulations for soybean production, with both promiscuous and non-promiscuous varieties, in Mozambican agro-climatic conditions. A total of 105 isolates obtained from nodules of promiscuous soybean grown at 15 sites, in Mozambique, were screened for N-fixation effectiveness in the greenhouse along with four strains used in inoculants in Brazil, *Bradyrhizobium japonicum* SEMIA 5079, *B. diazoefficiens* SEMIA 5080, *B. elkanii* strains SEMIA 587 and SEMIA 5019, and one strain used in Africa, *B. diazoefficiens* USDA 110. Eighty-seven isolates confirmed the ability to form effective nodules on soybean and were used for genetic characterization, by rep-PCR (BOX) and DNA sequencing of the 16S rRNA gene, and symbiotic effectiveness. The BOX-PCR fingerprinting revealed remarkable genetic diversity, with 41 clusters formed, considering an arbitrary similarity level of 65%. The 16S rRNA analysis assigned the isolates to the *Bradyrhizobium* (75%) and *Agrobacterium – Rhizobium* (25%) clades. Great variability in symbiotic effectiveness was detected among the indigenous rhizobia from Mozambique with ten isolates performing better than USDA 110, the best reference strain, and 50 isolates with lower performance than all the reference strains. Thirteen of the isolates with the highest symbiotic effectiveness were evaluated, along with the five reference strains, in two promiscuous (TGx 1963-3F and TGx 1835-10E) and one non-promiscuous (BRS 284) soybean varieties in a second greenhouse trial. The 13 isolates were also characterized for tolerance to acidity and alkalinity (pH 3.5 and 9.0, respectively), salinity (0.1, 0.3 and 0.5 mol L^{-1} of NaCl) and high temperatures (35, 40 and 45 ºC) *in vitro*. Five isolates, three (4, 19 and 22) belonging to the sub-group *B. elkanii* and two (27 and 61) assigned to the sub-group *B. japonicum,* consistently showed high symbiotic effectiveness, suggesting that the inoculation with indigenous rhizobia adapted to local conditions represents a possible strategy for soybean production in Mozambique.

Keywords: Biological nitrogen fixation – symbiosis – promiscuous soybean varieties – *Rhizobium* – 16S rRNA – BOX-PCR

3.1 INTRODUCTION

Soybean [*Glycine max* (Linnaeus) Merrill] stands out as the best-bet legume to feed the growing world population, projected to be between 9.6 and 12.3 billion in 2100 (GERLAND et al., 2014; TAAGEPERA, 2014; UN, 2015), with much of the increase expected to happen in Africa (GUPTA, 2013; GERLAND et al., 2014; UN, 2015). With approximately 40% seed protein and 20% seed oil content (ARSLANOGLU et al., 2011; PINHEIRO et al., 2013; SHARMA et al., 2014), soybean is an excellent source of food, fodder and biofuels. Like most legumes, soybean has the ability to reduce atmospheric nitrogen (N_2) to a biologically usable ammonia (NH_3), in association with bacteria, collectively known as rhizobia (SINGLETON et al., 1992; GILLER, 2001), obviating the need for N fertilizers. This is particularly important in Africa, where the predominantly subsistence farmers can hardly afford the limited available agricultural inputs (SINGLETON et al., 1992; MAINGI et al., 2006; CHIANU et al., 2011). In Mozambique, the demand for soybean has increased notably in recent years (LAVA KUMAR et al., 2011; CUNGUARA et al., 2012), to supply the growing poultry industry and for exportation (DIAS; AMANE, 2011; MUANANAMUALE et al., 2012).

Many reports have established that when soybean is grown for the first time outside Southeast Asia, its centre of origin and domestication (GILLER, 2001; XU et al., 2002; LI et al., 2010), it yields poorly (PULVER et al., 1985; MAINGI et al., 2006; ABAIDOO et al., 2007; CHIANU et al., 2011; GILLER et al., 2011; GRÖNEMEYER et al., 2014), presumably due to the lack of coevolved rhizobial strains in soils abroad (MPEPEREKI et al., 2000; GILLER, 2001; PARR, 2014). Successful introduction of soybean into new regions is, therefore, dependent on inoculation with exotic rhizobia.

In Africa, where economic and farmer scale problems have limited the possibility of distribution of commercial inoculants for decades, a practical alternative to the dependence on inoculation for developing countries was proposed. Researchers at the International Institute of Tropical Agriculture (IITA) developed soybean TGx genotypes (Tropical Glycine cross), known as promiscuous varieties, due to their capacity of forming effective symbiotic relationships with a broad range of rhizobia indigenous to African soils (PULVER et al., 1985; SANGINGA et al., 1996; ABAIDOO et al., 2007; TEFERA, 2011). Considerable evidence, nevertheless, indicates that, in many locations, indigenous rhizobial populations are either not effective, or do not occur in sufficient number to meet N demand of

promiscuous varieties (SANGINGA et al., 2000; OKOGUN; SANGINGA, 2003; ABAIDOO et al., 2007; KLOGO et al., 2015). This suggests that it is safer to inoculate soybean with effective rhizobial strains than relying on resident strains of unknown potential (GILLER, 2001; OSUNDE et al., 2003).

Soybean response to inoculation is influenced by a number of factors including soil temperature (HUNGRIA; VARGAS, 2000; AL-FALIH, 2002; NISTE et al., 2013), N availability (THIES et al., 1991b; SINGLETON et al., 1992), salinity (TU, 1981; ZAHRAN, 1999; ZAHRAN, 2010; NISTE et al., 2013), pH (GILLER, 2001; AL-FALIH, 2002), and indigenous rhizobial population (THIES et al., 1992; OSUNDE et al., 2003). Very often, inoculant strains must compete with populations of indigenous or naturalized rhizobia, frequently not effective, but highly competitive and already adapted to the environment (STREETER, 1994; AL-FALIH, 2002; GRÖNEMEYER et al., 2014). It is widely believed that inoculation responses are more likely to occur when there are less than 10 cells of indigenous or naturalized rhizobia per gram of soil (THIES et al., 1991b; THIES et al., 1992; SANGINGA et al., 1996; SESSITSCH et al., 2002; OKOGUN; SANGINGA, 2003), or when a substantial component of the population is not effective (BROCKWELL et al., 1995; OSUNDE et al., 2003). However, in Brazil, soybean responses to reinoculation in soils with over 10^3 cells g^{-1} of soil (HUNGRIA et al., 1998; HUNGRIA et al., 2005; HUNGRIA et al., 2006a; CAMPO et al., 2009; HUNGRIA et al., 2013) and even 10^6 cells g^{-1} of soil (HUNGRIA; MENDES, 2015) are frequently reported.

The occurrence of indigenous strains compatible with promiscuous soybean varieties and with high symbiotic effectiveness in Africa (ABAIDOO et al., 2000; SANGINGA et al., 2000; MUSIYIWA et al., 2005; ABAIDOO et al., 2007; KLOGO et al., 2015; GYOGLUU et al., 2016) suggests that effective, competitive and locally adapted strains can be selected for use in inoculants for soybean. Recently published evidence from Mozambique indicates that indigenous rhizobia capable of establishing effective symbiosis with both promiscuous and non-promiscuous soybean varieties do occur in the country (GYOGLUU et al., 2016). The objective of this study was to characterize indigenous rhizobia and to identify strains that hold potential to be included in inoculant formulations for soybean production under Mozambican agro-climatic conditions, for both promiscuous and non-promiscuous soybean varieties.

3.2 MATERIAL AND METHODS

3.2.1 Site and Soil Description, and Nodule Sampling

To trap indigenous rhizobia, seven promiscuous soybean varieties were sown at 15 sites within research stations owned by IITA in Manica (4), Nampula (2), Tete (6) and Zambézia (3) provinces (Table 3.1 and Supplementary Fig. S4), which represent the main soybean production region in Mozambique. Selected fields had no known history of soybean cultivation or rhizobia inoculation. The climate types, based on the Köppen-Geiger climate classification system (PIDWIRNY, 2011), were Cwa (dry winter, wet summer) in Manica, Tete and Zambézia, and Aw (savanna) in Nampula. Soil types, according to the FAO/UNESCO soil classification (FAO, 2016), were Rhodic Ferralsols in Manica and Zambézia, Ferric Luvisols in Nampula, and Orthic Ferralsols in Tete.

Sixty days before sowing, 20 soil subsamples were collected at each site from the 0–20 cm layer for chemical and particle size analyses (Table 3.1). For chemical analysis, samples were oven dried at 60°C for 48 h and sieved (2 mm). Soil pH was determined in H_2O (1/2; soil/water) 1 h after shaking (PEECH, 1965). Calcium, Mg, K, Al and P were determined after extraction with Mehlich-3 (0.2 mol L^{-1} $C_2H_4O_2$, 0.25 mol L^{-1} $N_2H_4O_3$, 0.015 mol L^{-1} NH_4F, 0.013 mol L^{-1} NHO_3, and 0.001 mol L^{-1} $C_{10}H_{16}N_2O_8$) (1/10; soil/solution) (SIMS, 1989) using inductively coupled plasma optical emission spectroscopy (ICP-OES). Soil organic carbon (SOC) was determined based on the Walkley-Black chromic acid wet oxidation method (WALKLEY; BLACK, 1934) and soil organic matter (SOM) was determined considering SOM = 1.724 × SOC. Soil particle sizes were determined by the hydrometer method (KILMER; ALEXANDER, 1949).

Nodules were sampled in March – April 2013. At each site, five to ten nodules per plant were harvested from five randomly selected healthy plants, about 50 days after sowing. A minimum of fifteen nodules were randomly selected from each sampling site.

3.2.2 Bacteria Isolation from Root Nodules

At the laboratory, undamaged nodules were immersed in 70% (v/v) C_2H_2O for 10 s, and then in 10% (v/v) NaClO for 4 min. They were subsequently rinsed six times with sterile water to remove traces of NaClO. The isolation and purification of bacteria were performed as previously reported (VINCENT, 1970).

Table 3.1: Location, soil characteristics and soybean varieties from where indigenous rhizobia were obtained in Mozambique.

Sampling sites	Coordinates		Varieties	Isolates (Number sampled)[1]	Soil characteristics								
					Silt	Sand	Clay	SOM[2]	pH[3]	P[4]	K	Ca	Mg
	South	East			--------- g kg^{-1} ---------			g dm^{-3}	$CaCl_2$	---------- mg dm^{-3} ----------			
Tete province													
$Ntengo_1$	14°32.8'	34°11.2'	TGx 1485-ID	1-7 (7)	133	537	330	21.9	5.3	2.2	219	1,240	234
$Ntengo_2$	14°35.8'	34°11.2'	TGx 1835-10E	8-11, 14 (5)	173	420	407	22.8	6.3	8.0	786	1,740	428
$Ntengo_3$	14°32.8'	34°11.2'	TGx 1937-1F	15, 17-20 (5)	114	459	427	24.1	6.1	15.6	259	2,250	255
$Nkhame_1$	14°37.5'	33°58.9'	TGx 1904-6F	22-28 (7)	134	719	147	25.2	5.5	19.1	122	734	132
$Nkhame_2$	14°37.6'	33°58.9'	TGx 1740-2F	29-35 (7)	133	640	227	34.7	7.2	29.9	269	4,080	223
$Nkhame_3$	14°37.6'	33°58.9'	TGx 1908-8F	36-42 (7)	114	699	187	16.6	5.4	16.0	84	604	159
Zambézia province													
$Ruace_1$	15°14.1'	36°41.3'	TGx 1740-2F	43-46, 48 (5)	113	817	70	18.1	5.3	28.5	148	722	116
$Ruace_2$	15°14.0'	36°41.4'	TGx 1987-38F	50, 52, 53, 55, 56 (5)	114	797	89	14.7	5.2	31.0	126	584	97
Mutequelesse	15°19.2'	36°42.7'	TGx 1908-8F	57-63 (7)	114	799	87	17.9	5.5	25.5	150	735	116
Nampula province													
$Muriaze_1$	15°16.4'	39°18.8'	TGx 1937-1F	64-67, 69, 70 (6)	56	664	280	27.4	5.9	3.9	219	1,450	173
$Muriaze_2$	15°16.4'	39°19.0'	TGx 1908-8F	71-77 (7)	74	699	227	38.6	7.3	5.4	205	3,800	148
Manica province													
$Sussundenga_1$	19°18.9'	33°14.5'	TGx 1485-ID	78-82 (5)	36	897	67	16.2	5.5	16.5	63	490	74
$Sussundenga_2$	19°18.9'	33°14.5'	TGx 1740-2F	85-88, 90, 91 (6)	73	797	130	22.9	5.5	18.5	285	836	167
$Zembe_1$	19°15.8'	33°23.1'	TGx 1904-6F	92-97 (6)	136	577	287	37.8	5.3	11.6	305	728	164
$Zembe_2$	19°15.9'	33°23.0'	TGx 1485-ID	99, 100 (2)	114	799	87	27.2	5.5	7.9	176	779	158

[1] Number of sampled isolates at each site consider only those (in total of 87) used for genetic and symbiotic characterization.
[2]SOM, Soil Organic Matter = 1.724 × soil organic carbon.
[3] pH $CaCl_2$ was estimated based on the equation pH ($CaCl_2$) = pH (H_2O) × 0.923 - 0.373 (AHERN et al., 1995).
[4] Organic P.
Source: This author.

The surface-sterilized nodules were crushed individually and the nodule suspension was streaked onto plates containing yeast-mannitol agar (YMA) medium (VINCENT, 1970) modified to contain 5 g L^{-1} of mannitol and 0.00125% Congo red (w/v). After confirming the purity of each single type of colony, the isolates were maintained on YMA slants at 4 °C for short-term storage. For long-term storage isolates were maintained on YM with 30% (w/v) glycerol at both -80 °C and -150 °C, and lyophilized. A total of 256 isolates were obtained and of these, seven were randomly selected from each of the 15 sampling sites, resulting in 105 isolates used in this study.

3.2.3 Morphophysiological Characterization

The ability of the isolates to grow under stressed conditions *in vitro*, including salinity, acidity, alkalinity and high temperature was assessed as described elsewhere (CHEN et al., 2002). The isolates and reference strains were grown in the dark in tubes with 100 µL of YM medium with pH adjusted to 6.8–7.0, at 28 °C on a rotary shaker operating at 90 cycles per minute and optical density (OD) was measured on the seventh day in a spectrophotometer (Spectronic Genesys®2, Spectronic Instruments®, New York, USA) at λ = 600 nm as control readings. To assess tolerance to salinity, the samples were grown in YM medium supplemented with 0.1, 0.3 and 0.5 mol L^{-1} of NaCl. Sensitivity to acidity or alkalinity was evaluated in YM medium adjusted to pH 3.5 or 9.0. The ability to grow under high temperatures was assessed at 35, 40 and 45 °C. All evaluations were made with three replicates and tolerance was expressed as the percentage of OD in relation to the control treatment.

3.2.4 Genetic Characterization

3.2.4.1 DNA extraction

Isolates and reference strains were grown at 28 °C on a rotary shaker operating in the dark at 90 cycles per minute for three to seven days and DNA was extracted with DNeasy Blood & Tissue kit (QIAGEN®, Germany). Mini-gels (8 cm × 10 cm) of 1.0% (w/v) agarose and 0.5× Tris-acetate/EDTA (TAE) were employed in electrophoresis at 60 V for 35 min, using DNA Mass® Ladder, to confirm DNA purity (KASCHUK et al., 2006). Gels were then stained with $C_{21}H_{20}BrN_3$, visualized and photographed under UV light.

3.2.4.2 PCR amplification with primer BOX A1R

The DNA of each isolate or reference strain was amplified with BOX A1R (5'-CTACGGCAAGGCGACGCTGACG-3', Invitrogen® Life Technologies®, São Paulo, Brazil) (VERSALOVIC et al., 1994). The final volume of the PCR reaction was a 25 μL mixture containing sterile milli-Q water, 13.8 μL; dNTPs (1.5 mmol L^{-1} of each), 5.0 μL; $MgCl_2$ 50 mmol L^{-1}, 1.5 μL; buffer 10x (500 mmol L^{-1} KCl; 100 mmol L^{-1} Tris–HCl, pH 8.3), 2.5 μL; BOX A1R primer (50 pmol μL^{-1}), 1.0 μL; Taq DNA polymerase (5 U μL^{-1}), 0.2 μL; sample DNA 50 ng μL^{-1}, 1.0 μL.

The reaction was carried out in a thermocycler (Eppendorf® Mastercycler Gradient®, Hamburg, Germany), as follows: one cycle of denaturation at 95 °C for 7 min; 30 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min, and extension at 65 °C for 8 min; one cycle of final extension at 65 °C for 16 min; and a final soak at 4 °C. PCR fragments were separated by horizontal electrophoresis on a 1.5% agarose gel (20 cm × 25 cm), at 120 V, for 6 h. A 1 kb DNA marker (Invitrogen®) was placed at both ends and in the middle of each gel.

After electrophoresis, gels were stained with $C_{21}H_{20}BrN_3$, visualized and photographed under UV light, with a digital camera (Kodak®, China). The obtained BOX A1R-PCR products were grouped considering a level of similarity of 70% (KASCHUK et al., 2006) in the cluster analysis with UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm and Pearson´s correlation. All analyses were performed with the software Bionumerics® 7.5 (Applied Mathematics, Sint-Martens-Latem, Belgium).

3.2.4.3 Amplification of the DNA region coding for the 16S rRNA gene

The amplification of the DNA was performed as reported earlier (MENNA et al., 2006) with universal primers fD1 and rD1 (WEISBURG et al., 1991). Each reaction was carried out with a total volume of 50 μL containing: ultrapure water, 37.30 μL; dNTPs (300 μmol L^{-1} of each), 3.0 μL; 10 × buffer (Tris-base 20 mmol L^{-1}, KCl 50 mmol L^{-1}, pH 8.4), 5.0 μL; $MgCl_2$ (1.5 mmol L^{-1}), 1.5 μL; fD1 and rD1 primers (10 pmol μL^{-1} of each), 1.0 μL each primer; Taq DNA polymerase (5 U), 0.2 μL; sample DNA (20 ng), 1.0 μL^{-1}. PCR reaction conditions included: an initial denaturation at 95 °C for 2 min; 30 cycles of denaturation at 94 °C for 15 s, 93 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 2 min; a final extension cycle of 72 °C for 5 min. The obtained amplified products

were purified using a PureLink® Quick PCR Purification Kit (Invitrogen® by Life Technologies®, Löhne, Germany). The concentration of the samples was verified in 1% (w/v) agarose gels, adjusted to 40 ng DNA μL^{-1} and stored at -20 °C until further processing.

3.2.4.4 Sequencing of the 16S rRNA gene

The PCR reactions were performed in 96-well-full-skirt microplates. Purified PCR products of each isolate or reference strain (15µg mL^{-1}) were mixed with 1.3 µL of BigDye (3500XL Genetic Analyzer, Hitachi® Applied Biosystems®, California®, USA) and 3.2 pmol of each primer. Five reactions were performed to obtain the complete sequence of the 16S rRNA gene, with five primers: fD1, 362f, Y2, 786f and 1203f (MENNA et al., 2006). PCR procedure was as follows: denaturation at 96 °C for 1 min; 35 cycles of denaturation at 96 °C for 15 s, 50 °C for 15 s, and extension at 60 °C for 4 min; and a final soak at 4 °C. The obtained sequences for each isolate or strain were assembled into contigs using program software Bionumerics® 7.5. Confirmed sequences in both the 5' and 3' directions were submitted to the public database of nucleotide sequences GenBank (http://www.ncbi.nlm.nih.gov/blast) to seek for significant alignments. To confirm phylogenetic positions multiple alignments of sequences were performed with the program *ClustalW* of the software MEGA (Molecular Evolutionary Genetics Analysis), version 6.0 (TAMURA et al., 2013). Phylogenetic tree reconstruction was performed by maximum likelihood analysis, bootstrap method (FELSENSTEIN, 1985), with 1000 replications (HEDGES, 1992), based on the best DNA model in MEGA 6 (TAMURA et al., 2013).

3.2.5 Characterization of Symbiotic Properties

3.2.5.1 Isolates, reference strains and soybean varieties

The 105 indigenous rhizobial isolates from Mozambique were screened for symbiotic N_2-fixation effectiveness in a greenhouse along with four reference strains used in commercial inoculants in Brazil, *B. japonicum* SEMIA 5079 (=CPAC 15), *B. diazoefficiens* SEMIA 5080 (=CPAC 7), *B. elkanii* strains SEMIA 587 and SEMIA 5019 (=29w), and a strain used in inoculants in Africa, *B. diazoefficiens* USDA 110. The trial was performed with soybean variety BRS 133 (non-transgenic, genealogy FT-Abyara X BR 83–147), a typical modern genotype of non-promiscuous nodulation.

Subsequently, a second greenhouse trial was performed, with 13 of the most effective isolates identified in the first trial, in addition to the five reference strains. In this trial, two promiscuous (TGx 1963-3F and TGx 1835-10E) and one non-promiscuous (BRS 284, non-transgenic, genealogy Mycosoy-45 × Suprema) soybean varieties were employed. Two non-inoculated treatments, control with (control + N) and without (control – N) nitrogen, were included in both trials. Plants treated with control + N received 80 mg of KNO_3 per plant per week (HUNGRIA, M. et al., 2001), equivalent to 11 mg of N $plant^{-1}$ per week.

3.2.5.2 Inocula preparation, trial management and experimental design

Each bacterium was grown in YM medium for five days and then adjusted to a concentration of 10^9 cells mL^{-1}. Soybean seeds were surface-sterilized as described in item 3.2.2. Sowing was carried out in December 2013 and June 2015, for the first and second trials, respectively.

Four seeds were sown in each of the pre-sterilized modified Leonard jars (VINCENT, 1970) containing a mixture of sand and coal (1/3, v/v) and N-free autoclaved nutrient solution with pH adjusted to 6.6 – 6.8 (ANDRADE; HAMAKAWA, 1994). Each seed was individually treated with 1 mL inoculum, equivalent to 1.2 10^6 cells $seed^{-1}$. Jars were thinned to contain two seedlings at five and ten days after emergence (DAE), for the first and second trials, respectively. All through the trials, plants were kept with an adequate volume of N-free solution. Air temperature and relative humidity inside the greenhouse were daily recorded at 09h00 and 15h00 throughout the trials. In the first trial, the daily mean air temperatures at 09h00 and 15h00 were 26.0 ± 1.9 and 30.3 ± 2.9 ºC (mean ± SD),

respectively, whereas the daily mean air relative humidity records were 67.0 ± 9.6 and 54.6 ± 7.1%, respectively. In the second trial, the daily mean air temperatures at 09h00 and 15h00 were 22.1 ± 1.6 and 25.0 ± 2.8 ºC, respectively, whereas the daily mean air relative humidity records were 69.1 ± 6.3 and 66.1 ± 8.1%, respectively.

The first trial was laid out in a randomized complete block design (RCBD) with four replicates. For the second trial, a factorial 20 × 3 (18 inoculants + non-inoculated control without N + non-inoculated control with N × three soybean varieties) fitted in RCBD with four replications was used.

3.2.5.3 Evaluation of nodulation, plant growth and nitrogen accumulation in shoots

The plants were harvested at 35 and 41 DAE, respectively, for the first and second trials, both at R2 [reproductive stage, one open flower at one of the two uppermost nodes on the main stem with a completely developed leaf (FEHR; CAVINESS, 1977)]. Stems were cut at the cotyledonary node separating plant shoots from roots. Shoots were placed in labeled paper bags, with each bag containing shoots from only one jar, and dried at 50 ºC for 72 h. Samples were then weighed to determine shoot dry weight (SDW) and ground (18 mesh) to quantify total N accumulation in shoots (TNS) by the indophenol-blue method (FEIJE; ANGER, 1972). Roots and adhering rooting medium were dislodged and washed over 1 mm mesh screen. Soil particles adhering to the roots were carefully rinsed off with a gentle stream of water. Roots and nodules were placed in paper bags and dried for 72 h at 50 ºC and weighed to determine root dry weight (RDW) (only in the second trial). Nodules were then detached from the roots, counted, to determine nodule number (NN), and allowed to dry further before weighing to determine nodule dry weight (NDW). At a later stage, relative effectiveness (RE) was determined as the percentage of SDW of plants supplied with N (Control + N) (RUFINI et al., 2014).

3.2.5.4 Statistical analyses

As most data failed to meet ANOVA assumptions, nonparametric statistics were performed to analyze data from the first trial. Spearmen's rank correlation was used to assess relationships among soybean nodulation, plant growth and production variables with software Statistica® 10.0 (STATSOFT, 2011). Relationships among isolates and sampling sites were explored with principal component analysis using software Analyse-it® (Analyse-it Software Ltd, Leeds, UK). In the second trial, original TNS data were transformed with $x^{½}$ prior to ANOVA testing to attain Gaussian data distribution and homoscedasticity. When differences among treatments were detected (ANOVA, $p < 0.05$), Tukey's test ($p < 0.05$) was performed to compare treatment means. The software Sisvar® (FERREIRA, 2011) was used for data analyses.

3.3 RESULTS

3.3.1 Isolates used in the study

Of the 105 isolates obtained from soybean nodules collected in Mozambique, 18 did not nodulate the non-promiscuous soybean variety BRS 133 and, as the objective of the study was to select isolates able to nodulate both promiscuous and non-promiscuous varieties, they were not considered in the analyses. Hence, the screening for N_2-fixation and genetic characterization were performed with 87 isolates.

3.3.2 Genetic Characterization

3.3.2.1 BOX A1R-PCR genomic fingerprinting

DNA profiles with an average of 200 bands and sizes between 200 and 5000 bp were obtained for the 87 isolates and five reference strains, after performing PCR with the primer BOX A1R. The isolates were grouped in 41 phylogenetic clusters (Fig. 1). Thirty-two of the 41 clusters (78%) were composed of less than three isolates, three clusters (14, 16 and 19) were composed of four isolates and there was a cluster (15) with 15 isolates.

In general, isolates were unevenly distributed across sites. $Ntengo_2$, $Ruace_2$, $Zembe_1$, $Zembe_2$ were the most heterogeneous sites with 80% or more isolates clustered in different phylogenetic groups (Fig. 1 and Table 3.2). On the other hand, $Ntengo_1$ (71%), $Nkhame_1$ (71%) and $Sussundenga_2$ (67%) were the most homogenous sampling sites with more than 65% of the isolates from the same cluster. The dendrogram also showed that the two *B. elkanii* reference strains clustered with 19 isolates from Mozambique. Strains *B. japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 were each joined to one isolate, while *B. diazoefficiens* strain USDA 110 was not clustered to any isolate (Fig. 1).

3.3.2.2 Phylogeny based on the 16S rRNA gene

Considering the genetic relatedness with known species and the clustering with reference and type strains, the majority of the isolates were assigned to the *Bradyrhizobium* (75% of the isolates) and *Agrobacterium–Rhizobium* clades (25%) (Fig. 2 and Table 3.2).

In general, the BOX A1R-PCR (Fig. 1) analysis was consistent with the 16S rRNA gene sequencing (Fig. 2). A total of 30 isolates, represented by isolates 1, 4, 17, 20 and 39 were clustered with or closely to the reference strains *B. elkanii* SEMIA 587 and SEMIA 5019 in the BOX-PCR analysis (Fig. 1) and they were all positioned in the sub-group *B. elkanii* in the 16S rRNA phylogram (Fig. 2). Moreover, the five isolates clustered tightly with type strain *B. elkanii* USDA 76^T (Supplementary Fig. S1). In both BOX-PCR and 16S rRNA analyses, isolates 95 and 97 were clustered with reference strains *B. diazoefficiens* SEMIA 5080 and USDA 110^T (Fig. 1 and Fig. 2), suggesting genetic relatedness.

Overall, 41 of the 65 *Bradyrhizobium* isolates (63%) were clustered with sub-group *B. elkanii* and 24 isolates (37%) fit into the sub-group *B. japonicum* (Fig. 2 and Table 3.2). Some *Bradyrhizobium* isolates might represent novel species. For example, isolate 27 had identity ≤ 97% with described *Bradyrhizobium,* and other putative new species might be represented by isolates 9, 14, 31, 45, 64, 70, 72 and 76 (Fig. 2 and Supplementary Fig. S1). There was a clear biogeographic pattern with the superabundant *Bradyrhizobium* present at all sites, except $Sussundenga_2$ and $Zembe_2$, and this was the only genus recorded at six ($Nteng_1$, $Ntengo_3$, $Nkhame_1$, $Nkhame_2$, $Ruace_1$, $Sussundenga_1$) out of the 15 sampled sites (Table 3.2).

In the *Agrobacterium-Rhizobium* clade, isolates 55, 73 and 90 were clustered closely in both BOX-PCR and 16S rRNA analyses (Fig. 1 and Fig. 2), and were grouped with strain *Rhizobium pusense* NRCPB10^T with a bootstrap value of 99%, confirming the robustness of the clustering (Fig. 2 and Supplementary Fig. S2). Isolates 92 and 93 formed a cluster distant from other isolates, reference and type strains (Fig. 2 and Supplementary Fig. S2), and exhibited low genetic relatedness to known *Rhizobium,* as indicated by identity ≤ 98% with the nearest species *R. etli* CFN 42^T, suggesting that they might represent a novel species. Overall, in the *Agrobacterium-Rhizobium* clade, tightly clustered groups of one (8) and four (55, 73, 88 and 90) isolates were closely related to *Agrobacterium tumefaciens* and *Rhizobium pusense,* respectively. The *Agrobacterium-Rhizobium* clade was represented in 50% of the sampled isolates at $Zembe_1$ and was exclusively recorded at $Sussundenga_2$ and $Zembe_2$ (Table 3.2).

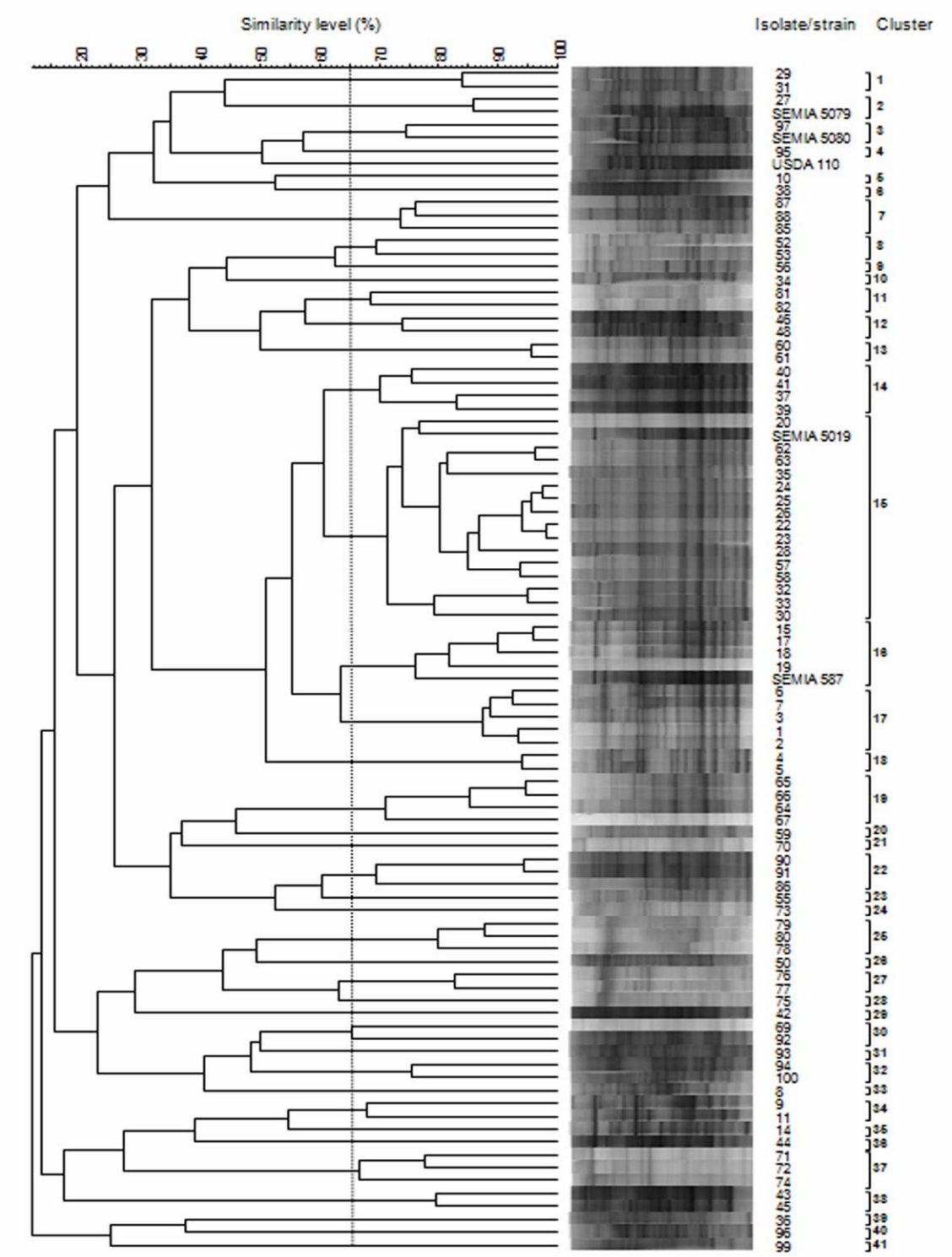

Figure 1: Dendrogram of 87 isolates from Mozambique and five reference strains used in commercial inoculants, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110 based on cluster analysis of BOX-PCR products using the UPGMA algorithm and Pearson correlation.
Source: This author.

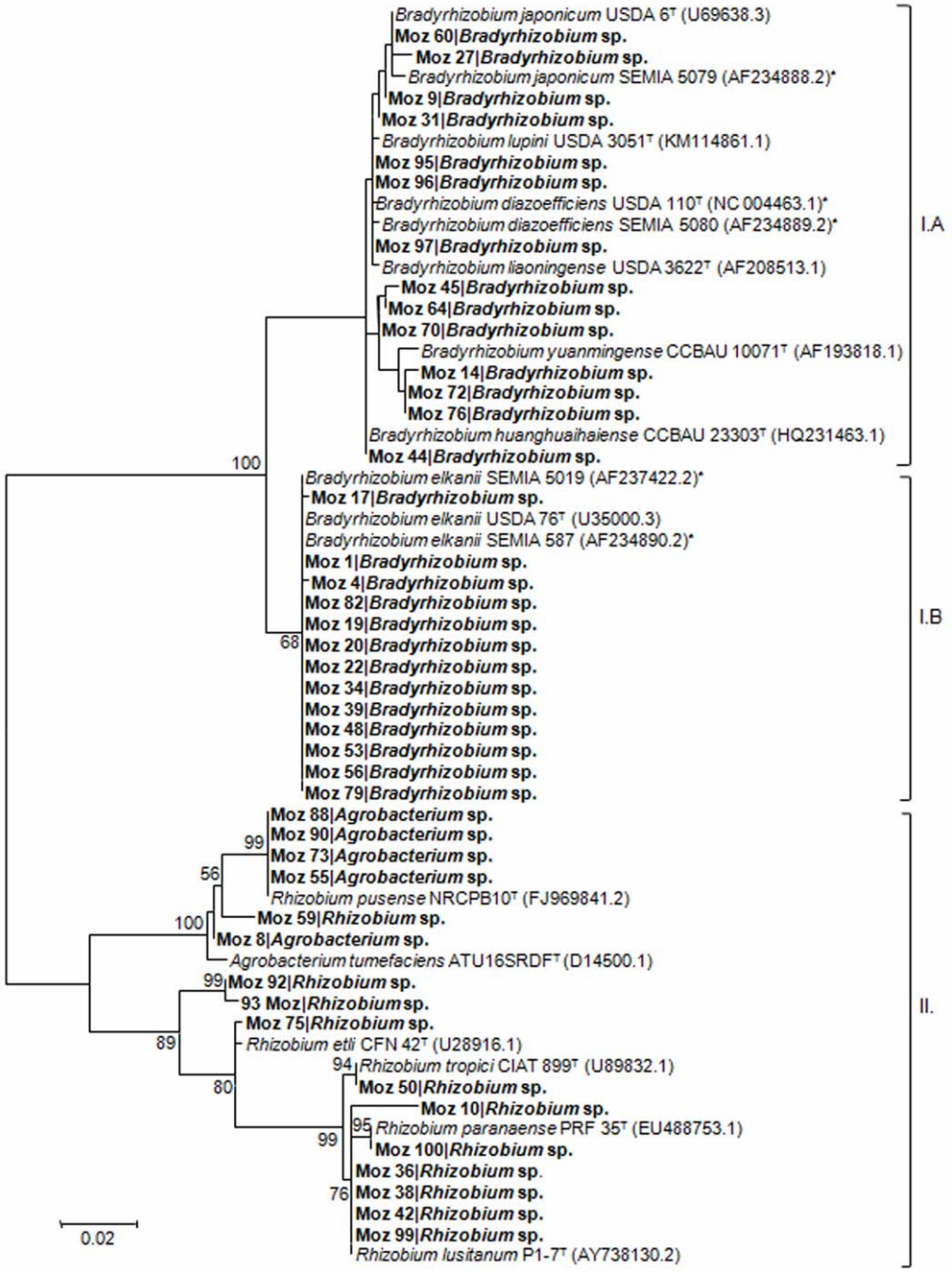

Figure 2: Phylogenetic tree of 16S rRNA gene sequences showing the relationships among representative rhizobial isolates from Mozambique (in bold) with type (^{T}) and reference strains used in commercial inoculants, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110 (with an asterix). The evolutionary history was inferred using the nearest-neighbor-interchange method. Only bootstrap confidence levels > 55% are shown at the internodes. The scale bar indicates 2% nucleotide substitutions. I.A and I.B represent the sub-groups *B. japonicum* and *B. elkanii*, respectively; and II represents the *Rhizobium-Agrobacterium* clade.
Source: This author.

3.3.3 Symbiotic Performance

3.3.3.1 First trial

Nonparametric Spearman Rank analyses revealed positive and highly significant correlations between NN and NDW (r = 0.91, $p < 0.001$), NDW and SDW (r = 0.88, $p < 0.001$), NDW and TNS (r = 0.89, $p < 0.001$), SDW and TNS (r = 0.94, $p < 0.001$), and between SDW and RE (r = 0.93). Considering that SDW has been suggested as the best variable for assessing symbiosis (HAYDOCK; NORRIS, 1980; HUNGRIA; BOHRER, 2000; DE SOUZA et al., 2008a; DE SOUZA et al., 2008b), the high correlation between SDW and RE, and the practicality of using RE, this variable was used to make the general symbiotic assessment of the 87 isolates from Mozambique.

Great variability in symbiotic effectiveness was detected among the indigenous rhizobia from Mozambique with ten isolates performing better than USDA 110, the best reference strain, and 50 isolates with lower performance than all the reference strains.

Thirty-seven isolates had outstanding symbiotic performance, as indicated by RE > 80%, a similar performance to that of the reference strains (Table 3.2 and Supplementary Table S1). There was an uneven geographical distribution of very effective isolates across sampling sites, with high proportions found in only six out of the 15 sampling sites, $Ntengo_1$ (100% = all isolates had RE > 80%), $Ntengo_3$ (100%), $Nkhame_1$ (100%), $Nkhame_2$ (57%), $Nkhame_3$ (86%) and Mutequelesse (86%) (Supplementary Table S1).

A total of 40 isolates had low symbiotic performance, as shown by RE < 40%. High proportions of these isolates were recorded in nine sampling sites, $Ntengo_2$ (60%), $Ruace_1$ (100%), $Ruace_2$ (100%), $Muriaze_1$ (83%), $Muriaze_2$ (86%), $Sussundenga_1$ (100%), $Sussundenga_2$ (83%) and $Zembe_2$ (100%) (Supplementary Table S1).

The symbiotic effectiveness of the 41 phylogenetic clusters, based on the BOX-PCR results, is presented in Table 3.2. Eleven clusters (2, 3, 4, 6, 13, 14, 15, 16, 17, 18 and 29) had RE > 80% and 24 clusters had RE < 40%. Principal component analyses were performed to explore the symbiotic performance of the representative isolates (Fig. 3) and their biogeographic distribution across the sampling sites (Supplementary Fig. S3). The analyses considered two components that together explained 99% of the variation in NN, NDW, SDW, TNS and RE. The 11 phylogenetic clusters with RE > 80% are located on the left side along with the reference strains, whereas the 24 poorly effective clusters are positioned on the right side of the graph (Fig. 3). Similarly, the sampling sites from where large proportions of isolates with RE > 80% were recorded are shown on the left side and

sites with high proportion of isolates with RE < 40% are in the inferior and superior quadrants on the right side (Supplementary Fig. S3).

Interestingly, isolates of the same phylogenetic cluster tended to show similar symbiotic performance. For example, the RE variation among the highly effective clusters was as follows, cluster 13: mean = 112.0%, SD = 6.7% (n = 2, range = 107.3 – 116.8); cluster 14: mean = 109.4%, SD = 12.4% (n = 4, range = 92.3 – 119.8); cluster 15: mean = 120.5%, SD = 14.9% (n = 15, range = 84.6 – 138.4) (Supplementary Table S2). The variations in RE among the worst three poorly effective phylogenetic clusters were as follows, cluster 12: mean = 21.0%, SD = 8.0 (n = 2, range = 15.3 – 26.7); cluster 25: mean = 18.6%, SD = 2.9 (n = 3, range 15.6 – 21.5); and cluster 38: mean = 20.8%, SD = 3.2 (n = 2, range = 18.5 – 23.0).

Table 3.2: Nodule number (NN, n° plant^{-1}) and dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), total N accumulation in shoots (TNS, mg plant^{-1}) and relative effectiveness (RE, %) of soybean, variety BRS 133, inoculated 87 isolates from Mozambique, for each BOX-PCR cluster and five reference strains, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110. Trial performed under greenhouse conditions in Londrina, Brazil, and plants harvested at 35 days after emergence.

Cluster [1]	Isolates	Source [2]	Species name [3]	NN [4]	NDW	SDW	TNS	RE [5]
1	29, 31	5	*Bradyrhizobium* sp.	79.9	456.66	3.1	59.47	73.0
2	27*	4	*Bradyrhizobium* sp.	65.5	493.08	5.2	146.15	129.5
3	97	14	*Bradyrhizobium* sp.	68.8	352.67	3.6	92.65	88.0
4	95*	14	*Bradyrhizobium* sp.	68.4	397.79	4.2	102.18	98.9
5	10	2	*Rhizobium* sp.	7.0	29.14	0.8	6.51	18.2
6	38*	6	*Rhizobium* sp.	73.0	541.83	4.0	100.71	96.4
7	85, 87, 88	13	*Rhizobium* sp.	29.1	121.02	1.7	38.00	40.0
8	52, 53	8	*Bradyrhizobium* sp.	22.2	79.05	1.0	12.60	22.9
9	56	8	*Bradyrhizobium* sp.	11.8	54.59	0.8	6.97	18.3
10	34	5	*Bradyrhizobium* sp.	29.1	236.00	2.1	30.74	48.8
11	81,82	12	*Bradyrhizobium* sp.	10.9	37.42	0.8	8.14	19.4
12	46, 48	7	*Bradyrhizobium* sp.	14.3	56.58	0.9	9.80	21.0
13	60, 61*	9	*Bradyrhizobium* sp.	77.3	419.79	4.7	130.59	112.0
14	37, 39*, 40*, 41 20, 22*, 23, 24*, 25	6 3,4	*Bradyrhizobium* sp.	75.5	555.01	4.7	124.22	109.4
15	26, 28, 30, 32, 33, 35, 57, 58, 62*, 63	4,5 5, 9	*Bradyrhizobium* sp.	75.7	519.38	5.2	132.27	120.5
16	15, 17*, 18, 19*	3	*Bradyrhizobium* sp.	96.0	546.24	5.2	121.29	122.9
17	1, 2, 3, 6*, 7	1	*Bradyrhizobium* sp.	76.4	438.13	4.3	91.59	100.9
18	4*, 5	1	*Bradyrhizobium* sp.	68.3	532.74	5.6	136.66	130.9
19	64, 65, 66, 67	10	*Bradyrhizobium* sp.	37.6	138.28	1.6	29.69	35.0
20	59	9	*Rhizobium* sp.	37.4	310.11	2.9	53.69	70.4
21	70	10	*Bradyrhizobium* sp.	38.0	161.78	1.5	32.01	34.9
22	86, 90, 91	13	*Rhizobium* sp.	17.1	86.63	1.1	16.56	25.9
23	55	8	*Rhizobium* sp.	11.4	55.44	0.9	10.98	21.2
24	73	11	*Rhizobium* sp.	23.6	63.90	0.9	12.94	21.6
25	78, 79, 80	12	*Bradyrhizobium* sp.	6.5	42.02	0.8	8.32	18.6
26	50	8	*Rhizobium* sp.	5.5	18.93	0.8	5.76	20.5
27	76, 77	11	*Bradyrhizobium* sp.	25.0	120.43	1.8	27.65	42.3
28	75	11	*Rhizobium* sp.	16.1	53.29	0.8	13.35	19.3
29	42	6	*Rhizobium* sp.	64.3	488.68	3.8	107.68	88.9
30	69, 92	10, 14	*Rhizobium* sp.	26.7	116.13	1.5	30.61	33.8
31	93	14	*Rhizobium* sp.	12.5	39.39	0.8	8.12	18.3
32	94, 100	14, 15	*Rhizobium* sp.	10.4	33.99	1.0	12.40	23.8
33	8	2	*Agrobacterium* sp.	38.3	241.22	2.7	40.80	64.0
34	9, 11	2	*Bradyrhizobium* sp.	21.2	105.63	1.5	20.96	34.9
35	14	2	*Bradyrhizobium* sp.	10.0	27.53	1.1	9.20	24.3
36	44	7	*Bradyrhizobium* sp.	9.8	19.71	0.7	4.74	17.2
37	71, 72, 74	11	*Bradyrhizobium* sp.	21.2	83.10	1.4	17.88	32.7
38	43, 45	7	*Bradyrhizobium* sp.	19.4	48.37	0.9	9.18	20.8
39	36	6	*Rhizobium* sp.	9.4	29.68	0.8	7.19	19.1
40	96	14	*Bradyrhizobium* sp.	10.3	41.55	0.9	13.87	22.2
41	99	15	*Rhizobium* sp.	31.4	70.24	1.0	13.14	23.3
Reference strains								
USDA 110		USA	*B. diazoefficiens*	61.5	408.81	5.4	140.20	127.9
SEMIA 587		Brazil	*B. elkanii*	59.8	265.06	3.9	84.60	93.9
SEMIA 5019		Brazil	*B. elkanii*	56.4	513.58	5.0	119.28	118.8
SEMIA 5079		Brazil	*B. japonicum*	73.4	350.91	3.4	89.16	81.5
SEMIA 5080		Brazil	*B. diazoefficiens*	81.6	391.29	3.7	77.31	86.4

[1]Phylogenetic cluster as defined by BOX-PCR analysis (Fig. 1).
[2] Sampling sites: 1 – Tengo$_1$; 2 – Tengo$_2$; 3 – Tengo$_3$; 4 – Khame$_1$; 5 – Khame$_2$; 6 – Khame$_3$; 7 – Ruace$_1$; 8 – Ruace$_2$, 9 – Mutequelesse.

10 – Muriaze$_1$; 11 – Muriaze$_2$; 12 – Sussundenga$_1$; 13 – Sussundenga$_2$; 14 – Zembe$_1$; 15 – Zembe$_2$.
[3] Based on www.ncbi.nlm.nih.gov/blast genbank database (section 3.2.4.4) and 16S rRNA gene analysis (Fig. 2).
[4] Values of each isolate represent average of four replications.
[5] Expressed as the percentage of shoot dry weight of plants supplied with N (Control + N) (RUFINI et al., 2014).
[*] Isolates selected for the second greenhouse trial.
Source: This author.

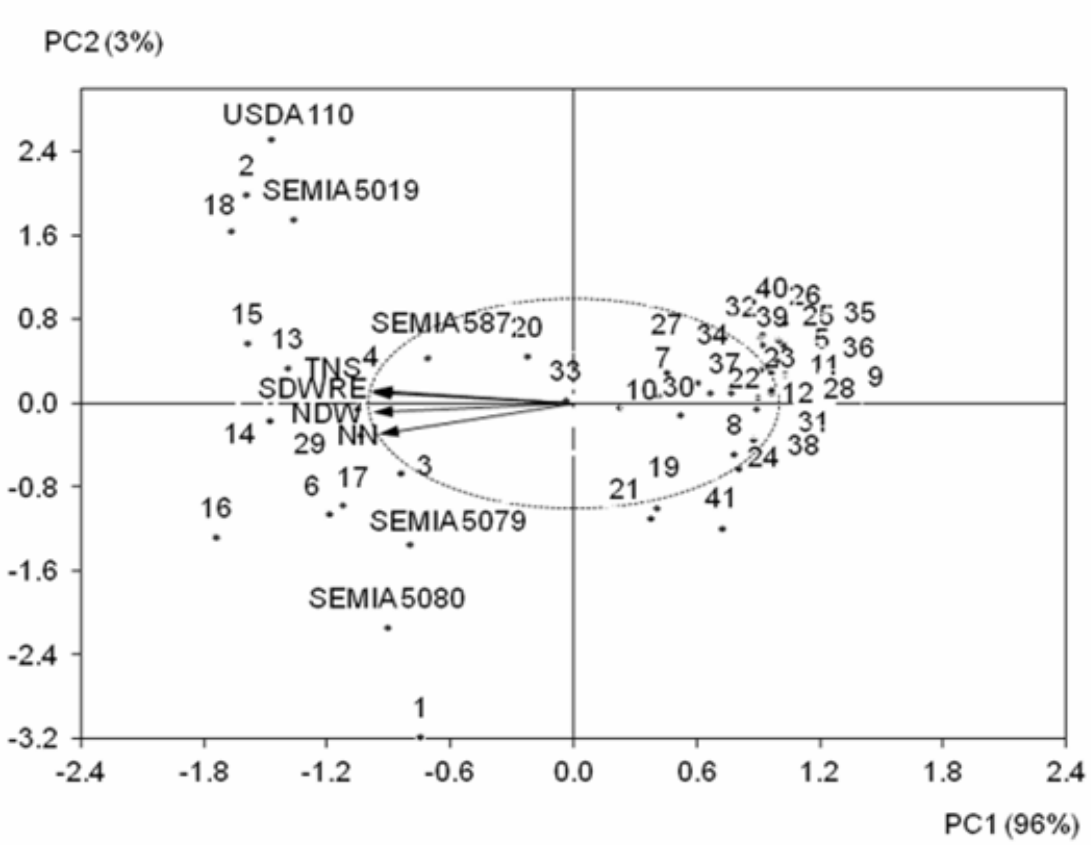

Figure 3: Principal component analysis exploring the symbiotic performance of the representative isolates from Mozambique and five reference strains, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110 based on nodule number (NN) and dry weight (NDW), shoot dry weight (SDW), total N accumulated in shoots (TNS) and relative effectiveness (RE). Numbers represent phylogenetic clusters as defined by BOX-PCR analyses (Figure 1).
Source: This author.

3.3.3.2 Second trial

The thirteen best performing isolates in the first trial were selected for a second greenhouse trial with two promiscuous (TGx 1963-3F and TGx 1835-10E) and one non-promiscuous (BRS 284) soybean varieties. Significant differences were observed among the soybean varieties in terms of root dry weight (RDW), with TGx 1963-3F recording significantly ($p < 0.05$) higher mean than TGx 1835-10E and BRS 284 (Table 3.3). Among the reference strains, USDA 110 was the most effective, resulting in the highest RDW. Considering the effect across varieties, isolates 38, 40 and 95 had the lowest means. Similarly to the observed in the first trial, SDW was positively and significantly correlated with NDW ($r = 0.38$, $p < 0.001$), TNS ($r = 0.73$, $p < 0.001$) and RE (0.84, $p < 0.001$), but in general

correlation coefficients were lower in the second trial. TGx 1963-3F was revealed to be more promiscuous than TGx 1835-10E as it had significantly higher SDW ($p < 0.05$) with more isolates (17, 22 and 95 and SEMIA 587) (Table 3.3). TGx 1835-10E responded to inoculation with isolates 38, 61 and 95 with significantly ($p < 0.05$) lower SDW than BRS 284. The symbiotic performance of isolates 38 and 61 on the promiscuous variety TGx 1963-3F resulted in significantly ($p < 0.05$) lower SDW than the non-promiscuous BRS 284. Promiscuous varieties responded to inoculation with isolates 19 and 40 with improved ($p < 0.05$) growth, compared to the non-promiscuous variety. In general, variety TGx 1963-3F had better ($p < 0.05$) growth than TGx 1835-10E and BRS 284.

Table 3.3: Root dry weight (RDW, g plant^{-1}) and shoot dry weight (SDW, g plant^{-1}) of two promiscuous (TGx 1963-3F and TGx 1835-10E) and one non-promiscuous (BRS 284) soybean varieties inoculated with 13 indigenous rhizobial isolates from Mozambique and five reference strains, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110. Trial conducted under greenhouse conditions in Londrina, Brazil, and plants harvested at 41 days after emergence.

Isolate/	RDW				SDW			
Strain	TGx 1963	TGx 1835	BRS 284	Mean	TGx 1963	TGx 1835	BRS 284	Mean
4	0.91 $^{Aab\ 1}$	0.74 $^{Ba-d}$	0.73 ^{Bab}	0.79 ^{abc}	2.19 $^{Aa\ 1}$	1.91 $^{Aa-d}$	1.96 $^{Aa-d}$	2.02 ^{abc}
6	0.86 ^{Aab}	0.68 $^{Ba-e}$	0.65 ^{Bab}	0.73 $^{b-f}$	1.95 ^{Aa}	1.65 $^{ABb-e}$	1.44 ^{Bde}	1.68 ^{de}
17	0.76 ^{Abcd}	0.60 ^{Bdef}	0.72 ^{ABab}	0.69 $^{c-f}$	2.07 ^{Aa}	1.49 ^{Bde}	1.80 ^{ABbcd}	1.79 $^{b-e}$
19	0.81 $^{Aa-d}$	0.78 $^{Aa-d}$	0.72 ^{Aab}	0.77 $^{a-e}$	2.14 ^{Aa}	2.09 ^{Aabc}	1.67 ^{Bcde}	1.97 $^{a-d}$
22	0.99 ^{Aa}	0.79 $^{Ba-d}$	0.76 ^{Bab}	0.85 ^{a}	2.26 ^{Aa}	1.80 $^{Ba-d}$	1.83 $^{Ba-d}$	1.96 $^{a-d}$
24	0.81 $^{Aa-d}$	0.70 $^{Aa-d}$	0.81 ^{Aa}	0.77 $^{a-e}$	1.93 ^{Aa}	1.60 $^{Ab-e}$	1.77 ^{Abcd}	1.77 $^{b-e}$
27	0.85 ^{Aab}	0.81 ^{Aabc}	0.78 ^{Aab}	0.81 ^{ab}	2.08 ^{Aa}	2.13 ^{Aab}	1.97 $^{Aa-d}$	2.06 ^{ab}
38	0.47 ^{Be}	0.46 ^{Bf}	0.66 ^{Aab}	0.53 ^{h}	1.21 ^{Bb}	1.11 ^{Be}	1.62 ^{Ade}	1.31 ^{f}
39	0.84 ^{Aabc}	0.79 $^{Aa-d}$	0.72 ^{Aab}	0.79 ^{abc}	2.03 ^{Aa}	1.88 $^{Aa-d}$	1.75 ^{Abcd}	1.88 $^{b-e}$
40	0.74 ^{Abcd}	0.66 $^{ABb-e}$	0.60 ^{Bb}	0.67 ^{efg}	1.92 ^{Aa}	1.72 ^{Abcd}	1.17 ^{Be}	1.60 ^{ef}
61	0.79 ^{Abcd}	0.66 $^{Bb-e}$	0.79 ^{Aab}	0.75 $^{a-e}$	1.98 ^{Ba}	1.85 $^{Ba-d}$	2.40 ^{Aa}	2.08 ^{ab}
62	0.78 ^{Abcd}	0.84 ^{Aab}	0.75 ^{Aab}	0.79 ^{abc}	1.80 ^{Aa}	1.82 $^{Aa-d}$	1.78 ^{Abcd}	1.80 $^{b-e}$
95	0.72 ^{Abcd}	0.49 ^{Bef}	0.69 ^{Aab}	0.63 $^{f-h}$	1.91 ^{Aa}	1.45 ^{Bde}	1.92 $^{Aa-d}$	1.76 $^{b-e}$
USDA 110	0.74 ^{Abcd}	0.87 ^{Aa}	0.75 ^{Aab}	0.79 ^{abc}	2.21 ^{Aa}	2.35 ^{Aa}	2.25 ^{Aab}	2.27 ^{a}
SEMIA 587	0.63 ^{Ade}	0.49 ^{Bef}	0.61 ^{ABb}	0.57 ^{gh}	1.83 ^{Aa}	1.43 ^{Bde}	1.71 $^{ABb-e}$	1.66 ^{de}
SEMIA 5019	0.71 ^{Abcd}	0.64 $^{Ac-f}$	0.69 ^{Aab}	0.68 $^{d-g}$	1.73 ^{Aab}	1.55 ^{Acde}	1.74 ^{Abcd}	1.67 ^{de}
SEMIA 5079	0.72 ^{Abcd}	0.67 $^{Ab-e}$	0.71 ^{Aab}	0.70 $^{c-f}$	1.84 ^{Aa}	1.63 $^{Ab-e}$	1.64 ^{Ade}	1.70 ^{cde}
SEMIA 5080	0.66 ^{Acde}	0.71 $^{Aa-d}$	0.79 ^{Aab}	0.72 $^{b-f}$	1.75 ^{Bab}	2.09 ^{ABabc}	2.23 ^{Aabc}	2.02 ^{abc}
Mean	0.76 ^{A}	0.69 ^{B}	0.72 ^{B}	0.72	1.93 ^{A}	1.75 ^{B}	1.81 ^{B}	1.83
Control + N^{2}	0.82	0.83	1.03		3.14	3.16	3.74	
Control - N^{2}	0.35	0.30	0.37		0.60	0.58	0.66	
C.V. (%)				10.77				12.25

[1] Means of four replications and when followed by same letter, uppercase on the same line or lowercase on the same column, are not statistically different by Tukey's test at $p < 0.05$.
[2] Not included in statistical analysis.
Source: This author.

Inoculation resulted in significantly ($p < 0.05$) higher nodule number (NN) in the non-promiscuous variety BRS 284 than in the promiscuous TGx 1963-3F and TGx 1835-10E varieties (Table 3.4). BRS 284 had greater ($p < 0.05$) NN when inoculated with isolates 17, 19, 24, 38 and 62 than TGx 1963-3F and TGx 1835-10E. Inoculation with isolates 38 and 40 resulted in significantly higher ($p < 0.05$) NN than that of all the other isolates, except 22 and 39. On average, TGx 1963-3F had significantly ($p < 0.05$) lower NDW than TGx 1835-10E and BRS 284 (Table 3.4). Both promiscuous varieties had significantly ($p < 0.05$) higher NDW than BRS 284 when inoculated with isolate 6, but had significantly ($p < 0.05$) lower NDW when inoculated with isolates 24 and 38. Isolates 19, 22, 27, 39 and 62 had the highest NDW and outperformed ($p < 0.05$) all the reference strains.

Table 3.4. Nodule number (NN, n° plant^{-1}) and nodule dry weight (NDW, mg plant^{-1}) of two promiscuous (TGx 1963-3F and TGx 1835-10E) and one non-promiscuous (BRS 284) soybean varieties inoculated with 13 indigenous rhizobial isolates from Mozambique and five reference strains, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110 grown under greenhouse conditions in Londrina, Brazil, and harvested at 41 days after emergence.

Isolate/ Strain	NN				NDW			
	TGx1963	TGx 1835	BRS 284	Mean	TGx 1963	TGx 1835	BRS 284	Mean
4	28.00 $^{Abc\ 1}$	29.17 $^{Aa-e}$	35.75 ^{Ac}	30.97 ^{cd}	267.75 $^{Aabc\ 1}$	259.27 ^{Abc}	254.13 $^{Aa-e}$	260.38 ^{bcd}
6	27.75 ^{ABbc}	21.63 ^{Bcde}	35.38 ^{Ac}	28.25 ^{cd}	275.42 ^{Aab}	266.52 ^{Abc}	176.05 ^{Bg}	239.33 ^{def}
17	28.38 ^{Babc}	26.50 $^{Bb-e}$	40.50 ^{Abc}	31.79 ^{bcd}	254.62 ^{Abcd}	234.68 ^{Acde}	261.38 $^{Aa-d}$	250.23 ^{cde}
19	23.75 ^{Bc}	21.00 ^{Bcde}	40.50 ^{Abc}	28.42 ^{cd}	253.33 ^{Bbcd}	292.76 ^{Aab}	254.83 $^{Ba-e}$	266.97 ^{bc}
22	43.00 ^{Aab}	44.75 ^{Aa}	36.67 ^{Ac}	41.47 ^{ab}	307.52 ^{Aa}	313.48 ^{Aa}	286.46 ^{Aab}	302.49 ^{a}
24	31.13 ^{Babc}	23.25 ^{Bcde}	42.83 ^{Abc}	32.40 ^{bcd}	227.87 ^{Bdef}	234.22 ^{Bcde}	295.13 ^{Aa}	252.41 ^{cde}
27	29.38 ^{Aabc}	32.63 $^{Aa-d}$	36.00 ^{Ac}	32.67 ^{bcd}	248.04 ^{Bbcd}	299.65 ^{Aab}	289.11 ^{Aa}	278.93 ^{ab}
38	35.75 ^{Babc}	32.33 $^{Ba-d}$	62.83 ^{Aa}	43.64 ^{a}	140.04 ^{Bh}	161.70 ^{Bgh}	226.07 $^{Ac-f}$	175.94 ^{hi}
39	28.13 ^{Aabc}	38.00 ^{Aabc}	39.00 ^{Abc}	35.04 ^{abc}	271.40 ^{Bab}	299.22 ^{Aab}	264.08 ^{Babc}	278.23 ^{b}
40	45.50 ^{ABa}	34.22 $^{Ba-d}$	54.50 ^{Aab}	44.74 ^{a}	222.35 ^{ABdef}	247.04 ^{Acd}	202.10 ^{Bfg}	223.83 ^{f}
61	38.88 ^{Aabc}	21.25 ^{Bcde}	35.75 ^{Ac}	31.96 ^{bcd}	218.43 ^{ABdef}	210.07 ^{Bdef}	246.00 $^{Ab-e}$	224.83 ^{f}
62	29.88 ^{Babc}	27.17 $^{Bb-e}$	42.33 ^{Abc}	33.13 ^{bcd}	248.23 ^{Bbcd}	308.83 ^{Aa}	286.52 ^{Aab}	281.19 ^{ab}
95	31.25 ^{ABabc}	21.25 ^{Bcde}	37.00 ^{Ac}	29.83 ^{cd}	193.11 ^{ABf}	175.12 $^{Bf-h}$	213.75 ^{Aefg}	194.00 ^{gh}
USDA 110	29.13 ^{Aabc}	33.13 $^{Aa-d}$	31.75 ^{Ac}	31.33 ^{cd}	238.44 $^{Ab-e}$	247.07 ^{Acd}	227.60 $^{Ac-f}$	237.70 ^{def}
SEMIA 587	22.75 ^{ABc}	14.00 ^{Be}	34.25 ^{Ac}	23.67 ^{d}	151.51 ^{ABgh}	138.38 ^{Bh}	175.63 ^{Ag}	155.17 ^{i}
SEMIA 5019	30.38 ^{ABabc}	24.25 ^{Bcde}	37.63 ^{Abc}	30.75 ^{cd}	205.58 ^{Bef}	201.78 ^{Befg}	254.75 $^{Aa-e}$	220.70 ^{f}
SEMIA 5079	24.50 ^{Bc}	19.33 ^{Bde}	38.13 ^{Abc}	27.32 ^{cd}	238.91 $^{Ab-e}$	232.74 ^{Acde}	231.10 $^{Ac-f}$	234.25 ^{ef}
SEMIA 5080	33.88 ^{Aabc}	42.13 ^{Aab}	35.75 ^{Ac}	37.25^{abc}	191.75 ^{Bfg}	232.89 ^{Acde}	222.30 ^{Adef}	215.65 ^{fg}
Mean	31.19 ^{B}	28.11 ^{C}	39.81 ^{A}	33.03	230.79 ^{B}	241.97 ^{A}	242.61 ^{A}	238.46
Control + N^2	0.00	0.00	0.00		0.00	0.00	0.00	
Control - N^2	0.00	0.00	0.00		0.00	0.00	0.00	
C.V. (%)				20.97				6.91

[1] Means of four replications and when followed by same letter, uppercase on the same line or lowercase on the same column, are not statistically different by Tukey's test at $p < 0.05$.
[2] Not included in statistical analysis.
Source: This author.

Soybean varieties differed in N accumulated in shoots (TNS), with TGx 1963-3F fixing significantly ($p < 0.05$) more N than TGx 1835-10E and BRS 284 (Table 3.5). Inoculation with isolates 19, 40 and 95 favored higher ($p < 0.05$) N accumulation in the promiscuous varieties than in the non-promiscuous one but inoculation with isolate 38 and reference strains USDA 110 and SEMIA 5080 resulted in significantly lower TNS in the promiscuous varieties. Isolates 38, 61 and were the best among the 13 tested. Overall, the promiscuous varieties had significantly greater ($p < 0.05$) relative effectiveness (RE) than the non-promiscuous BRS 284. The inoculation with isolates 19, 27, 39 and 40 was more favorable ($p < 0.05$) to the promiscuous varieties than to BRS 284. Considering all varieties, isolates 19, 22, 27 and 61 had the best symbiotic performance among the indigenous rhizobia and were significantly superior ($p < 0.05$) than three of the four reference strains from Brazil (Table 3.5). USDA 110 outperformed ($p < 0.05$) all the other reference strains, except SEMIA

5080 (Table 3.5), but was not statistically different to isolates 19, 22, 27 and 61. USDA 110 and isolates 4, 19, 22, 27 and 61 had the highest SDW and RE in both the first (Supplementary Table S1) and second (Tables 3.3 and 3.5) trials. In contrast, isolate 38 had the lowest RE among the 13 isolates tested in the two trials (Table 3.5 and Supplementary Table S1).

Table 3.5: Total nitrogen accumulation in shoots (TNS, mg plant^{-1}) and relative effectiveness (RE, %) of two promiscuous (TGx 1963-3F and TGx 1835-10E) and one non-promiscuous (BRS 284) soybean varieties inoculated with 13 indigenous rhizobial isolates from Mozambique and five reference strains, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110 grown under greenhouse conditions in Londrina, Brazil, in 2015, and harvested at 41 days after emergence.

Isolate/	TNS				RE[3]			
Strain	TGx 1963	TGx 1835	BRS 284	Mean[2]	TGx 1963	TGx 1835	BRS 284	Mean
4	54.69 Aab 1	44.52 ABb-f	40.32 Bde	46.51 d-g	63.32 Aab 1	50.55 Bb-e	46.23 Bbcd	53.36 b-e
6	52.16 Aabc	33.55 Bef	26.52 Bef	37.41 h	61.40 Aab	45.38 Bc-g	36.88 Bde	47.89 def
17	54.21 Aab	40.74 Bc-f	31.50 Bdef	42.15 f-h	65.50 Aab	42.34 Bd-g	38.23 Bde	48.69 de
19	53.47 Aabc	54.35 Aa-d	32.89 Bdef	46.91 d-g	68.10 Aab	70.77 Aa	41.62 Bcde	60.16 ab
22	56.21 Aab	43.56 Bc-f	36.21 Bde	45.33 d-h	73.26 Aa	56.45 Ba-e	46.61 Bbcd	58.77 abc
24	42.94 Abc	38.66 Adef	38.19 Ade	39.93 f-h	61.65 Aab	45.73 Bc-g	45.24 Bcde	50.87 cde
27	45.19 Aabc	49.28 Aa-d	43.46 Acd	45.98 d-g	66.11 Aab	64.18 Aab	49.76 Ba-d	60.02 ab
38	43.86 Babc	33.44 Cef	73.70 Aab	50.33 c-f	38.03 ABc	32.52 Bg	47.09 Abcd	39.21 f
39	53.91 Aab	40.93 Bc-f	33.81 Bdef	42.88 e-h	60.57 Aab	57.80 Aa-d	44.12 Bcde	54.16 b-e
40	53.40 Aabc	39.85 Bdef	23.18 Cf	38.81 gh	59.02 Aab	47.37 Bc-g	30.03 Ce	45.47 ef
61	60.14 Aa	48.34 Ba-e	67.28 Ab	58.59 bc	63.16 Aab	52.41 Bb-e	60.93 ABab	58.83 abc
62	45.50 Aabc	38.38 Adef	39.85 Ade	41.24 f-h	57.64 Ab	52.38 ABb-e	45.25 Bcde	51.76 b-e
95	57.23 Aab	56.89 Aabc	40.29 Bde	51.47 cde	63.89 Aab	32.98 Cfg	49.00 Ba-d	48.62 de
USDA 110	57.68 Bab	61.94 Bab	92.16 Aa	70.59 a	62.90 Aab	70.32 Aa	64.14 Aa	65.79 a
SEMIA 587	58.75 Aab	40.20 Bdef	59.44 Abc	52.80 cd	53.37 Abc	41.13 Befg	43.80 ABcde	46.10 ef
SEMIA 5019	37.42 ABc	32.63 Bf	45.12 Acd	38.39 gh	55.21 Ab	48.57 ABb-f	43.99 Bcde	49.26 de
SEMIA 5079	52.49 Aabc	42.48 Ac-f	44.02 Acd	46.33 d-g	59.18 Aab	44.98 Bc-g	41.23 Bcde	48.46 de
SEMIA 5080	45.90 Cabc	66.38 Ba	93.32 Aa	68.53 ab	55.76 Ab	58.21 Aabc	56.63 Aabc	56.86 a-d
Mean	51.40 A	44.78 B	47.85 B	48.01	60.45 A	50.78 B	46.15 C	52.46
Control + N[4]	93.83	91.51	122.75		100.00	100.00	100.00	
Control - N[4]	4.55	7.91	8.30		18.97	18.28	17.73	
C.V. (%)				6.31				11.86

[1] Means of four replications and when followed by same letter, uppercase on the same line or lowercase on the same column, are not statistically different by Tukey's test at $p < 0.05$.
[2] Original data transformed with x½, to meet ANOVA assumptions.
[3] Expressed as the percentage of shoot dry weight of plants supplied with N (Control + N) (RUFINI et al., 2014).
[4] Not included in statistical analysis.
Source: This author.

3.3.4 Tolerance to Acidity/Alkalinity, High Temperature and Salinity

The 13 best performing isolates in the first trial were also evaluated in relation to the ability to grow under stressed conditions and the results are summarized in Table 3.6. Most isolates grew well in YM supplemented with 0.1 mol L^{-1} of NaCl, with two isolates (38 and 40) growing better than the control. However, only three (17, 38 and 40) and two (17 and 40) isolates showed tolerance to 0.3 and 0.5 mol L^{-1} of NaCl, respectively. In relation to the tolerance to acidity/alkalinity, all isolates grew remarkably well in YM at pH 9.0, as shown by OD higher than 65% in relation to control, but ten isolates (77%) had growth inhibited at pH 3.5, as indicated by OD lower than 7%. While all isolates tolerated 35 °C, as shown by OD values greater than 40% in relation to control, ten isolates (77%) had inhibited growth at 40 °C, as indicated by OD below 9%, and only two isolates (17 and 19) had OD higher than 10% at 45 °C. Isolates 17 and 38 were the most endurable as shown by OD values greater than 50% when grown in YM supplemented with 0.3 mol L^{-1} of NaCl and 40 °C. Among the reference strains, USDA 110 was the most sensitive and SEMIA 5019 was the most endurable.

Table 3.6: Tolerance (in % of control OD readings) to salinity, acidity/alkalinity and high temperature of 13 indigenous rhizobial isolates from Mozambique and five reference strains, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110.

Isolate/	Salinity (mol L^{-1} of NaCl)			Acidity/Alkalinity (pH)		High temperatures (°C)		
Strain	0.1	0.3	0.5	3.5	9.0	35	40	45
4	56.9 [1]	2.2	0.0	0.5	79.2	61.3	5.1	2.5
6	97.7	7.5	9.6	31.0	108.3	82.1	8.8	5.7
17	81.5	51.2	16.5	10.5	70.6	68.5	65.7	10.4
19	41.7	5.7	0.0	6.5	65.1	60.3	20.0	10.3
22	27.4	2.9	0.0	1.2	101.6	75.0	7.3	2.2
24	27.5	2.6	0.0	0.6	104.2	81.3	4.2	2.2
27	40.6	4.3	0.0	1.6	87.8	51.6	6.3	2.5
38	124.3	97.5	0.0	14.8	118.5	68.5	55.4	0.6
39	76.0	5.4	0.0	1.9	96.4	76.0	6.0	2.5
40	172.9	71.1	47.9	0.0	73.7	72.9	4.8	1.0
61	12.8	3.3	0.0	0.0	103.4	41.5	5.5	0.2
62	52.5	2.1	0.0	0.1	75.8	60.8	3.4	1.8
95	4.5	3.2	0.0	0.2	95.0	49.5	6.6	0.0
USDA 110	2.4	2.8	1.3	1.6	86.3	37.5	4.8	0.0
SEMIA 587	6.0	3.3	3.7	0.8	90.4	71.6	6.3	0.1
SEMIA 5019	49.3	7.7	8.8	6.8	105.3	99.6	15.5	3.1
SEMIA 5079	70.7	5.1	5.2	4.0	95.4	75.4	5.2	3.3
SEMIA 5080	18.6	5.6	5.3	1.4	98.4	39.0	9.1	2.2

[1] Mean of three replications and values represent rhizobia sample growth measured as percentage of control optical density at 600 nm.
Source: This author.

3.4 DISCUSSION

A total of 87 indigenous isolates trapped by promiscuous soybean varieties (TGx) from soils of Mozambique were studied. The isolates were assigned to the *Bradyrhizobium* (75%) and *Agrobacterium-Rhizobium* (25%) clades (Table 3.2). Most (63%) of the *Bradyrhizobium* isolates clustered with the sub-group *B. elkanii* and the remaining 24 isolates (37%) showed genetic relatedness to the sub-group *B. japonicum* (Fig. 2 and Table 3.2).

Bradyrhizobium has been repeatedly reported among indigenous rhizobia in Africa. In a study conducted in Malawi, *B. elkanii* was the dominant species that formed nodules with soybean (PARR, 2014). A survey conducted in Kenya identified all indigenous rhizobia nodulating soybean as *B. elkanii* (HERRMANN et al., 2014). In addition, a study conducted with indigenous rhizobia isolated from soybean in Benin, Cameroon, Ghana, Nigeria, Togo and Uganda revealed the genera *Bradyrhizobium* and *Rhizobium* as the most abundant, and *B. elkanii* and *B. japonicum* were the most common species identified (ABAIDOO et al., 2000).

BOX-PCR and 16S rRNA analysis were not always fully congruent. For example, isolates 72 and 76 clustered tightly together in the 16S rRNA (Fig. 2) but were far apart in the BOX-PCR analysis (Fig. 1). Similarly, isolate 96 clustered with reference strain *B. diazoefficiens* USDA 110 in the 16S rRNA phylogram (Fig. 2) but exhibited weak genetic relatedness in the BOX-PCR reconstruction tree (Fig. 1). Because in BOX-PCR strains belonging to same species are often positioned in more than one cluster this method is not reliable enough to be used as primary evidence for inferring species or even genera (HUNGRIA et al., 2006b; BINDE et al., 2009; MENNA et al., 2009b). However, BOX-PCR is a robust means for detecting diversity among strains (BINDE et al., 2009; MENNA et al., 2009b). It is noteworthy the high genetic diversity detected in this study, as indicated by the 41 clusters formed, considering a 65% level of similarity. Furthermore, all isolates joined at final similarity level of less than 15%, confirming the great genetic diversity among the indigenous rhizobia from Mozambique.

While 16S rRNA is a precise tool for defining kingdom and genera, alone, this method is inappropriate for inferring species (STACKEBRANDT; GOEBEL, 1994; VAN BERKUM et al., 2002). Indeed, considerable evidence indicates that strains with 16S rRNA identity > 99% may represent different species (FOX et al., 1992; DELAMUTA et al., 2013; DURÁN et al., 2014; GRÖNEMEYER et al., 2014). Therefore, further analyses, with robust

tools, such as the MLSA (LU et al., 2011), will be required to determine lower taxonomic levels of the isolates from Mozambique.

The high variability in N_2-fixation effectiveness and uneven distribution of symbiotically effective isolates among the sampling sites observed in this study corroborate evidence from elsewhere in Africa. Studies conducted in six (ABAIDOO et al., 2000) and nine (ABAIDOO et al., 2007) African countries have consistently reported both great variation in symbiotic effectiveness among indigenous rhizobial isolates sampled at the same sites and broad geographic distribution of effective isolates. This study contributes to evidence that indigenous rhizobia capable of establishing highly effective symbiosis with soybean do occur in Africa (ABAIDOO et al., 2000; MUSIYIWA et al., 2005; ABAIDOO et al., 2007; TEFERA, 2011; YOUSEIF et al., 2014a; KLOGO et al., 2015; GYOGLUU et al., 2016) and confirms that the indigenous strains are also capable of nodulating non-promiscuous soybean varieties (KLOGO et al., 2015).

The development of TGx varieties that nodulate with indigenous rhizobia aimed at obviating the need for inoculation in Africa (PULVER et al., 1985; TEFERA, 2011). The high proportion of very effective isolates recorded at Ntengo, Nkhame and Mutequelesse (Table 3.2 and Supplementary Table S1) suggests that, at these sites, TGx varieties may be successfully grown without inoculation, providing that the population sizes are large enough for an effective nodulation that supports the plant N demand.

USDA 110 was the best and most consistent reference strain recording the highest SDW, TNS and RE in the first trial (Supplementary Table S1), and outcompeting the other strains in all variables in the second trial (Tables 3.3, 3.4 and 3.5). This corroborates the evidence that strain USDA 110 has superior N_2-fixation abilities (DOWDLE; BOHLOOL, 1985; SINGLETON et al., 1985; ISRAEL et al., 1986; SOMASEGARAN; BOHLOOL, 1990; PAZDERNIK et al., 1997; YOUSEIF et al., 2014b; AGOYI et al., 2016). Moreover, in the second trial, USDA 110 recorded the highest performance in all varieties, supporting previous evidence that this strain is effective with a large number of soybean varieties (PAZDERNIK et al., 1997; AGOYI et al., 2016).

Fast-growing rhizobia, assigned to the *Agrobacterium-Rhizobium* clade, represented a large (25%) proportion of the studied isolates. *Agrobacterium* (CHEN et al., 2000; YOUSEIF et al., 2014b) and *Rhizobium* (ABAIDOO et al., 2000; HONG et al., 2010; ALAM et al., 2015) strains have previously been isolated from soybean nodules. Fast-growing rhizobia are believed to have a number of advantages including high competitiveness, facility for commercial production, easier establishment in the soil

(CHATTERJEE et al., 1990; BUENDÍA-CLAVERÍA et al., 1994) and high N_2-fixation capacity (YOUSEIF et al., 2014b; ALAM et al., 2015). In this study, however, fast-growing rhizobia were medium to poor symbionts (Supplementary Table S1). Isolate 38, the best fast-growing rhizobia in the first trial (Supplementary Table S1), was the worst symbiont in the second trial (Tables 3.3, 3.4 and 3.5).

In general the promiscuous varieties (TGx 1963-3F and TGx 1835-10E) responded markedly better to inoculation than the non-promiscuous one (BRS 284) (Tables 3.3, 3.4 and 3.5) validating previous reports that TGx varieties establish effective symbioses with a wide range of rhizobia (MUSIYIWA et al., 2005; PULE-MEULENBERG et al., 2011; TEFERA, 2011; GYOGLUU et al., 2016).

The different response to inoculation between the TGx varieties observed in this study substantiates previous findings (MUSIYIWA et al., 2005; KLOGO et al., 2015; AGOYI et al., 2016; GYOGLUU et al., 2016) and highlights the need for screening varieties to determine the best inoculant – TGx combination. These trials may serve to decide whether inoculation is required. For example, from the four TGx varieties tested in Mozambique, one of the three varieties that did not respond to inoculation had 2.0 t ha^{-1} of grain exclusively attributed to symbiosis with indigenous rhizobia and was recommended for use by resource-poor farmers without inoculation (GYOGLUU et al., 2016).

The results of rhizobia tolerance to stressed conditions reported here are in line with previous observations. In an evaluation of rhizobia isolated from soybean grown in Paraguay, SEMIAs 587, 5019 and 5080 were used as reference strains and also had OD values lower than 10% in relation to the control treatment when grown under high salinity (0.3 and 0.5 mol L^{-1} of NaCl), acid conditions (pH 3.5) or high temperatures (40 and 45 °C) (CHEN et al., 2002). Twelve of the 13 examined isolates were *Bradyrhizobium* (Table 3.2) and of these ten (83%) had inhibited growth, as indicated by OD below 10%, at 40 °C (Table 3.6), corroborating with similar studies on slow-growing rhizobia (CHEN et al., 2002; YOUSEIF et al., 2014a). The observation that most (77%) of the 13 isolates had inhibited growth at pH 3.5, as indicated by OD values below 7% (Table 3.6), is consistent with the much higher pH values (5.2 – 7.3) of the sampling sites (Table 3.1) and supports previous observations that rhizobial optimum pH is neutral to moderately alkaline (YADAV; VYAS, 1971).

In conclusion, indigenous rhizobia isolated from nodules of soybean grown in Mozambique have been characterized. Large differences in the capacity to grow under stressed conditions including acidity/alkalinity, salinity and high temperature were observed.

Isolates also exhibited high phylogenetic and symbiotic variability. Five representative isolates (4, 19, 22, 27 and 61) consistently showed high N_2-fixation effectiveness, suggesting that the inoculation with indigenous rhizobia already adapted to local conditions is a possible strategy for soybean production in Mozambique. Multi-site field trials with those promising isolates should be conducted to ascertain their superiority in fixing N in the presence of other indigenous and/or commercial strains.

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ARTIGO B: É POSSÍVEL TRANSFERIR TECNOLOGIAS RELACIONADAS À INOCULAÇÃO ENTRE PAÍSES? UM ESTUDO DE CASO COM A CULTURA DA SOJA NO BRASIL E EM MOÇAMBIQUE

RESUMO

A simbiose soja-*Bradyrhizobium* pode ser muito eficiente em fixar nitrogênio e suprir toda a demanda desse nutriente pela planta, eliminando a necessidade do uso de N-fertilizantes. O Brasil tem investido em pesquisa e uso de inoculantes para a soja por décadas e com a expansão da cultura em países africanos as possibilidades de transferência de tecnologias de fixação biológica do nitrogênio (FBN) entre países devem ser investigadas. Aqui nós relatamos experimentos conduzidos para avaliar o desempenho de quatro estirpes elite brasileiras e uma norte-americana nas safras 2013/2014 e 2014/2015, no Brasil (quatro locais) e em Moçambique (cinco locais). Em ambos países os experimentos foram montados em zonas de clima tropical e temperado e em solos com populações de rizóbios compatíveis com soja variando de < 10 a 2×10^5 células g^{-1} solo. Os tratamentos foram: (1) NI, controle não inoculado e sem N-fertilizante; (2) NI+N, controle não inoculado e com 200 kg de N ha^{-1}; e inoculado com (3) *B. japonicum* SEMIA 5079; (4) *B. diazoefficiens* SEMIA 5080; (5) *B. elkanii* SEMIA 587; (6) *B. elkanii* SEMIA 5019; (7) *B. diazoefficiens* USDA 110; (8) SEMIAs 5079 + 5080 (somente avaliado no Brasil). A nodulação, crescimento de plantas e rendimento foram avaliados em ambos países. Os melhores tratamentos com inoculação, considerando a média dos locais e safras, no Brasil foram com as estirpes SEMIAs 5079 + 5080, SEMIA 5079 e USDA 110, com ganhos médios de rendimento de grãos de 4–5% em relação ao tratamento NI. As estirpes SEMIA 5079, SEMIA 5080, SEMIA 5019 e USDA 110 foram as melhores em Moçambique, com ganhos médios de rendimento de grão de 20–29% em relação ao tratamento NI. Adicionalmente, as quatro estirpes com melhor desempenho em Moçambique resultaram em rendimentos semelhantes ou melhores do que o tratamento NI+N, confirmando a FBN como uma alternativa sustentável aos N-fertilizantes, poluidores ambientais. Os resultados também confirmam a viabilidade da transferência de tecnologias relacionadas à FBN com a soja entre países com condições agroclimáticas similares.

Palavras-chave: Fixação biológica do nitrogênio, *Glycine max*, *Bradyrhizobium*, inoculação, N-fertilizante

4 ARTIGO B: IS IT FEASIBLE TO TRANSFER INOCULATION-RELATED TECHNOLOGIES BETWEEN COUNTRIES? A CASE STUDY OF STRAINS FOR THE SOYBEAN CROP IN BRAZIL AND MOZAMBIQUE

ABSTRACT

The soybean-*Bradyrhizobium* symbiosis can be very effective in fixing nitrogen and supply all plant's demand on this nutrient, obviating the need for N-fertilizers. Brazil has been investing in research and use of inoculants in soybean for decades and with the expansion of the crop in African countries, the feasibility of transference of biological nitrogen fixation (BNF) technologies between the continents should be investigated. We evaluated the performance of four rhizobial strains from Brazil and one from the USA in the 2013/2014 and 2014/2015 crop seasons in Brazil (four sites) and Mozambique (five sites). In both countries, the trial sites were in tropical and temperate climate zones and the areas had soybean rhizobial population ranging from < 10 to over 1×10^3 cells g^{-1} of soil. The treatments were: (1) NI, non-inoculated control with no N-fertilizer; (2) NI+N, non-inoculated control with 200 kg of N ha^{-1}; and inoculated with (3) *Bradyrhizobium japonicum* SEMIA 5079; (4) *B. diazoefficiens* SEMIA 5080; (5) *B. elkanii* SEMIA 587; (6) *B. elkanii* SEMIA 5019; (7) *B. diazoefficiens* USDA 110; (8) SEMIAs 5079 + 5080 (only tested in Brazil). The best inoculation treatments across sites and crop seasons in Brazil were SEMIAs 5079 + 5080, SEMIA 5079 and USDA 110, with average grain yield gains of 4–5% in relation to the NI treatment. Strains SEMIA 5079, SEMIA 5080, SEMIA 5019 and USDA 110 were the best in Mozambique, with average 20–29% grain yield gains over the NI treatment. Moreover, the four best performing strains in Mozambique resulted in similar or better yields than the NI+N treatment, confirming the BNF as a sustainable option to the use of environmentally polluting N-fertilizers. The results also confirm the feasibility of transference of technologies related to BNF with soybean between countries with similar agro-climates.

Keywords: Biological nitrogen fixation, *Glycine max*, *Bradyrhizobium*, inoculation, N-fertilizer

4.1 INTRODUCTION

Soybean [*Glycine max* (Linnaeus) Merrill] has potential to play a major role in responding to global food insecurity that results from mounting demographic pressures. The world population is projected to grow beyond 10 billion by 2100 (GERLAND et al., 2014; SAKSCHEWSKI et al., 2014; UN, 2015), and much of the increase will occur in Africa (CLELAND, 2013; GUPTA, 2013; UN, 2015), where hunger is already a threat. With high concentration of seed protein (~40%), that provides all the essential amino acids for human health (YOUNG, 1991), and high seed oil content (~20%), soybean has many uses, encompassing human food, animal feed and biofuels. Furthermore, soybean offers a number of advantages in sustainable cropping systems, including the ability to symbiotically fix atmospheric nitrogen (N_2), which obviates the reliance on expensive and environmentally polluting N-fertilizers.

When soybean is grown for the first time outside Southeast Asia, its centre of origin and domestication (GILLER, 2001; XU et al., 2002; LI et al., 2010), it generally requires inoculation with exotic rhizobial strains (PULVER et al., 1985; HUNGRIA; BOHRER, 2000; HUNGRIA, M et al., 2001; HUNGRIA et al., 2006c; ABAIDOO et al., 2007; GILLER et al., 2011; HUNGRIA; MENDES, 2015). In Africa, where the distribution of inoculants is limited, a strategy consisting in the use of promiscuous soybean varieties capable of forming nodules with indigenous rhizobia (PULVER et al., 1985; ABAIDOO et al., 2007; TEFERA, 2011) has been used for decades. Nevertheless, with cropping intensification, the search for soybean varieties with higher yield potential, but requiring inoculation, is scaling up.

Soybean response to inoculation is dependent on environmental factors including soil N availability (THIES et al., 1991b; SINGLETON et al., 1992), soil temperature (HUNGRIA; VARGAS, 2000; NISTE et al., 2013), soil pH (GILLER, 2001; AL-FALIH, 2002), salinity (ZAHRAN, 1999; ZAHRAN, 2010; NISTE et al., 2013), and more importantly indigenous rhizobial populations (THIES et al., 1992; OSUNDE et al., 2003). Very often, elite inoculant strains fail to overcome the competition for nodule occupancy imposed by indigenous or naturalized rhizobia (THIES et al., 1992; STREETER, 1994; VLASSAK et al., 1997; AL-FALIH, 2002; FURSETH et al., 2012), most times ineffective but very competitive and adapted to the environment (STREETER, 1994; AL-FALIH, 2002; GRÖNEMEYER et al., 2014). However, inoculation success in areas with high rhizobial population, $10^3 – 10^6$ cells g^{-1} of soil, has been achieved in South America (HUNGRIA et al.,

1998; HUNGRIA et al., 2005; HUNGRIA et al., 2006a; CAMPO et al., 2009; HUNGRIA et al., 2013; HUNGRIA; MENDES, 2015), opening a window for inoculation research in other geographic regions.

Several ecological studies on rhizobia have established that exogenous inoculant strains undergo genetic changes (PROVOROV; VOROBYOV, 2000; SCHLOTER et al., 2000; SILVA et al., 2003; BARCELLOS et al., 2007) and may acquire superior competitive abilities as they become naturalized (DUNIGAN et al., 1984; DOWDLE; BOHLOOL, 1987; HUNGRIA et al., 1998). The success of BNF in soybean in Brazil is chiefly ascribed to *Bradyrhizobium* strain selection programs that took place for over half a century, in addition to the development of proper inoculation methods (HUNGRIA et al., 2006a; HUNGRIA; MENDES, 2015). On the contrary, soybean is a relatively new crop in Mozambique and the production is primarily based on promiscuous varieties without inoculation (GYOGLUU et al., 2016). In recent years, nevertheless, the increased demand for soybean grain, to supply the chicken industry and for exportation (DIAS; AMANE, 2011; MUANANAMUALE et al., 2012), has led to search for more productive non-promiscuous varieties, which are generally responsive to inoculation. The agro-climatic conditions of the soybean production areas in Mozambique are considerably similar to the major soybean growing areas in Brazil, raising the question on whether the inoculant strains that perform well in a variety of agro-climatic zones in Brazil could be successfully transferred to Mozambique, with an expressive saving of time, labor and money.

The objective of this study was to compare the performance of four elite rhizobial strains from Brazil (SEMIAs 587, 5019, 5079, and 5080) and one strain adopted as standard inoculant in many African countries (USDA 110), in trials carried out with non-promiscuous soybean varieties in Brazil (four sites) and Mozambique (five sites).

4.2 MATERIAL AND METHODS

4.2.1 Sites description: location, climate and soil characterization

Climate and soil classification (Table 4.1 and Supplementary Fig. S5), soil chemical properties and rhizobial counts (Table 4.2), and rainfall data (Table 4.3) of the trial sites are summarized below. Sixty days prior to commencing the experiments, 20 soil sub-samples (0–20 cm) were collected at each site to evaluate biological, physical and chemical properties. Rhizobial population sizes were estimated by the most probable number (MPN) method (VINCENT, 1970) with soybean variety BMX Potência RR (in Brazil) or Storm (in Mozambique). Silt, sand and clay fractions were determined by the hydrometer method (KILMER; ALEXANDER, 1949). In Mozambique, soil pH was determined in H_2O (1/2; soil/water) 60 min after shaking (PEECH, 1965), Ca, Mg, Al, K and P were determined by inductively coupled plasma optical emission spectroscopy (ICP-OES) after extraction with Mehlich-3 (0.2 mol L^{-1} $C_2H_4O_2$, 0.25 mol L^{-1} $N_2H_4O_3$, 0.015 mol L^{-1} NH_4F, 0.013 mol L^{-1} NHO_3, and 0.001 mol L^{-1} $C_{10}H_{16}N_2O_8$) (1/10; soil/solution) (SIMS, 1989). In Brazil, chemical analyses were performed as previously described (SPARKS et al., 1996). Soil pH was determined in 0.01 mol L^{-1} $CaCl_2$ (1/2.5; soil/solution). Exchangeable Al, Mg and Ca were extracted with 1 mol L^{-1} KCl (1:10; soil/solution) after agitation for 10 min, P and K were extracted with Mehlich-1 (0.05 mol L^{-1} HCl + 0.0125 mol L^{-1} H_2SO_4) (1/10; soil/solution) after 10 min agitation. Aluminum was determined by titration with 0.015 mol L^{-1} standardized NaOH with indicator bromothymol blue, K was determined in a flame photometer, Ca and Mg were determined in an atomic absorption spectrophotometer, and P by the molybdenum-blue method with $C_6H_8O_6$ as reducing agent. In both countries soil organic carbon (SOC) was determined by the Walkley-Black chromic acid wet oxidation method (WALKLEY; BLACK, 1934) and soil organic matter (SOM) was obtained considering SOM = 1.724 × SOC.

In Mozambique, all trials were established in areas with no previous soybean cropping history or rhizobial inoculation, whereas in Brazil, the experiments were conducted in areas with and without soybean cultivation history. Based on the results of the soil analyses, where applicable, lime was applied to rise bases saturation to 70% (LOPES et al., 1991).

Table 4.1: Location, climate, soil type and textural class of the sites where the field trials were conducted in Brazil and Mozambique during the 2013/2014 and 2014/2015 crop seasons.

Trial site	Georeference			Climate[1]	Soil type[2]	Textural class[3]
	Latitude	Longitude	Altitude (m)			
Brazil						
Londrina	23°11′S	51°11′W	620	Cfa	Rhodic Ferralsols	Clay
Maracaí	22°36′S	50°40′W	475	Cfa	Ferric Luvisols	Sand
Ponta Grossa	25°13′S	50°01′W	880	Cfb	Orthic Ferralsols	Sandy clay loam
Rio Verde	17°47′S	50°54′W	730	Aw	Acric Ferralsols	Sand clay
Mozambique						
Muriaze	15°16′S	39°19′E	363	Aw	Ferric Luvisols	Sandy clay loam
Nkhame	14°38′S	33°59′E	1115	Cwa	Orthic Ferralsols	Sandy loam
Ntengo	14°33′S	34°11′E	1225	Cwa	Orthic Ferralsols	Clay
Ruace	15°08′S	36°25′E	673	Cwa	Rhodic Ferralsols	Sand
Sussundenga	19°19′S	33°15′E	611	Cwa	Rhodic Ferralsols	Sand

[1] Based on Köppen-Geiger climate classification (PIDWIRNY, 2011). [2] Based on FAO soil classification (FAO, 2016). [3]Based on USDA textural soil classification (USDA, 1987).

Table 4.2: Rhizobial count (MPN g^{-1} of soil), soil chemical properties (pH, $CaCl_2$; soil organic matter, g dm^{-3}; Organic P, mg dm^{-3}; Exchangeable K, Ca and Mg, $cmol_c$ dm^{-3}; exchangeable acidity, $cmol_c$ dm^{-3}) and soil granulometry (silt, sand and clay, g kg^{-1}) of the locations where the field trials were conducted in the 2013/2014 and 2014/2015 crop seasons in Brazil and Mozambique.

Soil characteristic	Trial sites in Brazil[1]					Trial sites in Mozambique[2]									
	2013/14 season			2014/15 season		---------- 2013/14 crop season ----------					---------- 2014/15 crop season ----------				
	Lon	Mar	Rio	Lon	Pon	Mur	Nkh	Nte	Rua	Sus	Mur	Nkh	Nte	Rua	Sus
Rhizobia (MPN g^{-1} soil)	2×10^5	< 10	< 10	5×10^5	3×10^4	< 10	1×10^3	75	1×10^3	< 10	na[9]	na	na	na	na
pH ($CaCl_2$)[3]	5.6	5.4	5.0	5.7	5.5	5.9	5.5	6.3	4.9	5.4	5.9	5.5	5.3	5.3	5.5
SOM (g dm^{-3})[4]	23.56	8.41	50.74	23.79	30.86	41.38	12.41	22.80	13.53	11.38	27.41	25.17	21.90	18.10	16.21
Organic P (mg dm^{-3})	22.01	6.57	2.45	41.00	2.55	13.20	27.60	7.96	22.40	4.12	3.94	19.10	2.17	28.50	16.50
K ($cmol_c$ dm^{-3})	0.61	0.05	0.17	1.13	1.11	0.65	0.22	2.02	0.27	0.22	0.56	0.31	0.56	0.38	0.16
Ca ($cmol_c$ dm^{-3})	4.47	1.20	3.46	5.05	3.02	9.45	3.11	8.70	2.12	3.38	7.25	3.67	6.20	3.61	2.45
Mg ($cmol_c$ dm^{-3})	2.48	0.34	0.94	2.46	1.53	1.38	1.13	3.57	0.49	0.83	1.44	1.10	1.95	0.97	0.62
EA[5] ($cmol_c$ dm^{-3})	4.62	1.12	3.03	3.28	3.63	1.02	0.78	0.82	1.30	0.83	0.85	0.99	2.19	1.30	0.66
SB[6] ($cmol_c$ dm^{-3})	7.56	1.59	4.57	8.64	5.66	11.48	4.45	14.29	2.88	4.44	9.25	5.08	8.71	4.96	3.23
CEC[7] ($cmol_c$ dm^{-3})	12.18	2.71	7.60	11.92	9.29	12.50	5.23	15.11	4.18	5.27	10.10	6.07	10.90	6.26	3.89
BS[8] (%)	62.07	58.67	60.13	72.48	60.93	91.88	85.07	94.57	68.88	84.17	91.62	83.74	79.92	79.17	83.01
Silt (g kg^{-1})	166	8	96	208	30	128	128	173	84	43	56	134	133	113	36
Sand (g kg^{-1})	80	904	540	82	732	542	682	420	842	861	664	719	537	817	897
Clay (g kg^{-1})	754	88	364	710	238	330	190	407	74	96	280	147	330	70	67

[1] Trial sites in Brazil: Lon – Londrina; Mar – Maracaí; Rio – Rio verde; Pon – Ponta grossa.
[2] Trial in Mozambique: Mur – Muriaze; Nkh – Nkhame; Nte – Ntengo; Rua – Ruace; Sus – Sussundenga.
[3] In Mozambique pH was estimated based on the equation pH ($CaCl_2$) = pH (H_2O) × 0.923 - 0.373 (AHERN et al., 1995).
[4] SOM, Soil Organic Matter = 1.724 × soil organic carbon.
[5] EA, Exchangeable Acidity = (Al + H).
[6] SB, Sum of Bases = (K + Ca + Mg).
[7] CEC, Cation Exchangeable Capacity = (EA + SB).
[8] BS, Bases Saturation = SB/CEC × 100.
[9] na, not available: due to logistic difficulties, rhizobial populations were not estimated in the 2014/2015 crop season in Mozambique.

Table 4.3: Sowing dates and rainfall recorded during soybean growth at the trial sites in the 2013/2014 and 2014/2015 crop seasons in Brazil and Mozambique.

Trial site	Sowing date	Rainfall (mm) recorded in different soybean growth stages[1]														
		VE	VC	V1	V2	V3	V4	V5	V6	R1	R3	R4	R5	R6	R7	R8
Brazil, crop season 2013/2014																
Londrina	24-Oct-13	17.7	10.3	19.1	23.5	37.0	14.9	14.5	0.0	0.0	60.2	104.3	13.0	2.5	131.0	65.3
Maracaí	23-Oct-13	23.0	11.5	47.6	22.7	34.9	31.0	24.2	3.1	0.0	1.4	209.0	61.4	116.9	32.5	33.7
Rio Verde	06-Nov-13	79.2	24.0	145.2	17.6	75.6	27.4	70.0	3.8	56.4	28.8	24.8	11.6	42.6	30.6	168.0
Mozambique, crop season 2013/2014																
Muriaze	25-Dec-13	160.6	54.7	58.9	117.9	17.7	0.0	4.4	38.5	8.5	194.4	39.5	11.6	16.1	72.7	25.8
Nkhame	20-Dec-13	64.0	104.0	28.0	55.0	5.0	53.0	30.0	56.0	3.0	89.0	47.0	7.0	9.0	81.0	7.0
Ntengo	17-Dec-13	104.2	8.9	63.1	22.5	65.2	3.9	22.3	19.4	55.5	29.5	92.9	67.0	26.2	97.3	9.4
Ruace	20-Dec-13	61.8	20.1	56.6	111.7	25.7	115.7	66.8	28.1	49.0	60.7	129.4	101.0	40.6	9.1	107.1
Sussundenga	21-Dec-13	259.0	67.2	68.0	24.9	10.2	109.4	344.5	20.5	210.8	208.3	127.0	208.6	32.3	59.9	31.5
Brazil, crop season 2014/2015																
Londrina	04-Nov-14	11.2	8.2	95.4	4.6	35.5	19.6	21.9	0.1	117.8	9.6	2.9	61.6	57.5	90.7	50.4
Ponta Grossa	18-Nov-14	88.1	0.0	27.1	66.8	2.3	112.9	27.8	39.9	8.3	66.3	14.6	103.3	118.0	90.9	29.3
Mozambique, crop season 2014/2015																
Muriaze	05-Jan-15	245.5	41.6	2.3	53.9	35.9	75.1	21.2	39.5	32.9	48.2	226.1	60.9	23.1	3.5	7.4
Nkhame	23-Dec-14	118.0	16.0	35.0	18.0	25.0	48.0	98.0	62.0	6.0	28.0	84.0	122.0	30.0	47.0	15.0
Ntengo	22-Dec-14	89.7	40.9	32.7	49.6	70.6	5.9	48.6	3.9	14.0	21.1	100.6	65.3	32.4	29.6	6.8
Ruace	21-Jan-15	48.9	43.4	33.1	12.6	44.9	43.2	43.4	6.9	77.9	6.2	20.0	0.6	17.2	0.0	3.0
Sussundenga	29-Dec-14	530.6	0.2	37.5	9.1	1.2	32.2	164.0	0.0	0.0	87.6	62.7	10.9	76.9	127.7	81.5

[1]As defined by FEHR; CAVINESS (1977).

4.2.2 Treatments and trials management

Thirty days before sowing, the areas were weeded with 2.5 L ha^{-1} of glyphosate ($C_3H_8NO_5P$) (in Brazil only). The experiments consisted of the following treatments: (1) NI, non-inoculated and non-N-fertilized control (symbiosis relied on indigenous or naturalized rhizobial populations); (2) NI+N, non-inoculated control with 200 kg of N ha^{-1} as urea (CH_4N_2O, 46.6%N), applied 50% at sowing and 50% at R2 [reproductive stage, open flower at one of the two uppermost nodes on the main stem with completely developed leaf (FEHR; CAVINESS, 1977)]; (3) SEMIA 5079*,* inoculated with *Bradyrhizobium japonicum* strain SEMIA 5079; (4) SEMIA 5080*,* inoculated with *B. diazoefficiens* strain SEMIA 5080; (5) SEMIA 587*,* inoculated with *B. elkanii* strain SEMIA 587; (6) SEMIA 5019*,* inoculated with *B. elkanii* strain SEMIA 5019; (7) USDA 110*,* inoculated with *B. diazoefficiens* strain USDA 110; (8) SEMIAs 5079 + 5080, inoculated simultaneously with *B. japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080 (only in Brazil, as this is the most common combination used in the country). All inoculants were prepared on a peat basis.

Colony Forming Units (CFU) of each inoculant were verified before sowing to estimate the amount of inoculant that should be applied to release the same number of cells per treatment, of 1.2×10^6 cells $seed^{-1}$. The inoculation was performed by adding a sucrose solution (10%), to adhere the peat, and mixing seeds and inoculant vigorously and allowing the mixture to dry under the shade for 2 h before sowing. Seeds received no pesticide treatment.

Plot sizes were 6 m × 4 m (in Brazil) or 9 m × 3 m (in Mozambique) and seeds were sown in rows 0.50 m apart to achieve a final population of approximately 300,000 plants ha^{-1} in both countries. The experiments were laid out in randomized complete block design (RCBD) with six (Brazil) or five (Mozambique) replicates. At all trial sites the plots were separated by 0.50 m-wide lines and 1.5 m-wide terraces to avoid cross contamination with bacteria and/or fertilizer contained in superficial run-off. All trials relied on natural rainfall (Table 4.3).

Immediately before sowing, 300 kg ha^{-1} of triple superphosphate and potassium chloride [0–20–20(N-P_2O_5-K_2O)] were applied in furrow. In Brazil, at V4 [vegetative stage, four nodes on the main stem with completely unrolled leaves beginning with the unifoliolate nodes (FEHR; CAVINESS, 1977)], plants were sprayed with herbicide, 2.5 L ha^{-1} of $C_3H_8NO_5P$, and micronutrients, 20 g ha^{-1} of Mo (as $Na_2MoO_4 \cdot 2H_2O$) and 2.5 g

ha^{-1} of Co (as $CoCl_2 \cdot 6H_2O$). In Mozambique, weeding was performed in weekly intervals using manual hoe and, apart from the NI+N treatment, no fertilizer was applied.

4.2.3 Evaluation of nodulation, plant growth, N accumulation, yield and relative effectiveness

Five randomly selected plants were dug out from each plot at V4 (in Brazil) or R3 [reproductive stage, pod is 5 mm in length at one of the four uppermost nodes on the main stem with a completely developed leaf (FEHR; CAVINESS, 1977)] (in Mozambique) and taken for assessment of nodulation, plant growth and N accumulation. At the laboratory, plants were cut at the cotyledonary node to separate roots from shoots. Shoots were washed and placed in an air-forced drier at 50 ºC for 72 h and weighed to determine shoot dry weight (SDW). Entire shoots were ground (18 mesh) and employed to determine total N accumulation in shoots (TNS) by the indophenol-blue method (FEIJE; ANGER, 1972) , with readings taken at the wavelength of 697 nm. Roots and nodules were dried at 50 ºC for 72 h. Nodules were then detached from roots, counted, to determine nodule number (NN), before determination of nodule dry weight (NDW).

At physiological maturity, all plants within the central area of 8 m^2 (in Brazil) or 20 m^2 (in Mozambique) of each plot were harvested and used to determine the above ground biomass (AGB) (only in Mozambique), grain yield (GY), and grain dry weight (GDW). To determine AGB, plants were cut at the cotyledonary node, dried at 50 ºC for 72 h and weighed. For determination of GY, grains were weighed and values adjusted to 13% of moisture content, considering the humidity in a grain moisture tester. One hundred seeds were weighed to determine GDW. Relative effectiveness (RE) was determined as a percentage of SDW of any treatment over that of the NI+N treatment, in the same block (RUFINI et al., 2014).

4.2.4. Statistical analysis

Data were checked for normality of errors and homogeneity of variances prior to the statistical analyses. One-way general linear model ANOVA was employed to determine differences among treatments. When significant differences among treatments were detected, Duncan's test was employed to classify the means of the treatments. Differences were considered significant at $p \leq 0.10$, a level acceptable for strain or inoculant technology

recommendation in Brazil (MAPA, 2011). All statistical analyses were performed with software SAS® 9.3 (SAS Institute, North Carolina, USA).

4.3 RESULTS

4.3.1 Soil physical and chemical properties

The trial sites in Brazil were in four textural classes, Clay, Sand, Sandy clay loam and Sand clay (USDA, 1987), all of which were represented in Mozambique, apart from Sand clay (Table 4.1). In relation to chemical characteristics, the sites in Mozambique were in relatively more fertile soils, as shown by lower exchangeable acidity and higher bases saturation (Table 4.2).

4.3.2 Indigenous/naturalized rhizobia populations

In Brazil, the population density of naturalized rhizobia varied from < 10 (Maracaí and Rio Verde) to over 10^5 (Londrina) cells g^{-1} of soil (Table 4.2). In Mozambique, the population sizes of indigenous rhizobia were estimated only in the 2013/2014 crop season, due to logistic difficulties, and were much lower than those of the Brazilian sites, ranging from < 10 (Muriaze and Sussundenga) to over 10^3 cells g^{-1} (Nkhame and Ruace) (Table 4.2).

4.3.3 Climate and rainfall

Climate (Table 4.1) and rainfall data (Table 4.3) recorded all through soybean growth stages at the trial sites are summarized below. In both countries the experiments were carried out in two climate regions, tropical and temperate, according to the Köppen climate system (PIDWIRNY, 2011). In Brazil, the rainfall was particularly low during the transition of soybean from the vegetative to the reproductive growth stages in the 2013/2014 crop season at Londrina and Maracaí. In Mozambique, the rainfall recorded during the transition of soybean from the vegetative to the reproductive growth stages was lower in the 2014/2015 compared to the 2013/2014 crop season (Table 4.3).

4.3.4 Nodulation (nodule number and nodule dry weight)

In Brazil, the effect of inoculation on nodulation was observed at Londrina, where all inoculation treatments, except for SEMIA 5080, resulted in increased nodule number (NN) when compared to the non-inoculated control (NI) in the 2013/2014 crop season (Table 4.4). In the 2014/2015 crop season, plants inoculated with strains SEMIA 5079 and USDA 110 at Londrina had significantly greater NN and nodule dry weight (NDW) when compared to the NI control (Table 4.4). Inoculation with SEMIA 5019 and SEMIAs 5079 + 5080 at Londrina also increased significantly NDW in relation to the NI control in the 2014/2015 crop season, although this was not accompanied by a statistically higher NN. No effects of inoculation on NN or NDW were observed at Maracaí, Rio Verde and Ponta Grossa (Tables 4.5 and 4.6).

Strong responses to inoculation were observed at all sites in Mozambique. Plots treated with strains SEMIAs 5079, 5080, and 5019 at Muriaze (Table 4.7) had significantly higher NN and NDW in relation to the NI control in the 2013/2014 and 2014/2015 crop seasons. Inoculation with strain SEMIA 5019 at Nkhame (Table 4.8) in the 2014/2015 crop season, and at Ntengo (Table 4.9) in both crop seasons also resulted in increased NN and NDW in relation to the NI treatment. Strain SEMIA 587 improved nodulation in both crop seasons only at Ruace (Table 4.10). At Sussundenga (Table 4.11), the nodulation responses were similar to those observed at Muriaze.

Although the use of N-fertilizer decreased nodulation in Brazil, as indicated by a significant reduction of NN and/or NDW at Londrina (Table 4.4) and Rio Verde (Table 4.5), the detrimental effects of N-fertilizer application on nodulation were more evident in Mozambique, where significant reduction on NN and/or NDW was observed at Muriaze (Table 4.7), Nkhame (Table 4.8), Ntengo (Table 4.9) and Ruace (Table 4.10).

4.3.5 Plant growth and nitrogen accumulation

In Brazil, strains SEMIAs 587, 5079, and 5080, and the combination of 5079 + 5080 significantly improved shoot dry weight (SDW) and total N accumulated in shoots (TNS) when compared to the NI control at Maracaí (Table 4.5). The combination of 5079 + 5080 also resulted in statistically higher TNS at Londrina (2014/2015) than the NI treatment (Table 4.4).

In Mozambique, inoculants carrying strains SEMIAs 5079 and 587 at Nkhame (2014/2015) (Table 4.8), SEMIA 587 and USDA 110 at Ntengo (2013/2014) (Table 4.9), SEMIAs 5079, 5080 and 587 at Ruace (2014/2015) (Table 4.10) and SEMIAs 5079, 5080 and 587 at Sussundenga (both seasons) (Table 4.11) had higher SDW than the NI treatment.

4.3.6 Above ground biomass at harvest, grain yield and grain dry weight

The effect of inoculation on grain yield (GY) was observed at two sites in Brazil. Compared to the NI control, treatments SEMIAs 5079 + 5080 and SEMIA 5079 significantly increased GY by 9 and 8%, respectively, at Londrina (2013/2014) (Table 4.4), while USDA 110 improved GY by 7% at Rio Verde (Table 4.5). USDA 110 was the best performing strain across sites and crop seasons with grain yield gains of 5% in relation to the NI control (Supplementary Table S6). Plots treated with strains SEMIAs 5079, 5080, 587, USDA 110 and SEMIAs 5079 + 5080 had significantly higher grain dry weight (GDW) compared to the NI control at Londrina (2013/2014) (Table 4.4) and Ponta Grossa (Table 4.6). GY gains attributable to N-fertilizer varied from 11% at Ponta Grossa (Table 4.6) to 25% at Londrina (2013/2014 crop season) (Table 4.4). The average N-fertilizer gain on GY across sites and crop seasons was 11% in relation to the NI treatment, compared to 5% of USDA 110 (Supplementary Table S6).

Remarkable inoculation effects on above ground biomass (AGB), GY and GDY were observed in Mozambique. Plots treated with strains SEMIA 5079 at Muriaze (2014/2015) (Table 4.7), SEMIA 5080 and SEMIA 5019 at Sussundenga (2013/2014) (Table 4.11) had higher AGB than the NI control. Analysis across sites revealed that in the 2013/2014 crop season plants inoculated with strain SEMIA 5080 had the best and significantly higher (8%) AGB than the NI control (Supplementary Table S5). In the 2014/2015 crop season all inoculation strains resulted in higher (16–23%) and significant AGB gains relatively to the NI control, and strains SEMIAs 5080, 5019 and USDA 110 presented significantly higher AGB gains of 12, 10 and 9%, respectively, in relation to N-fertilized control (Supplementary Table S5).

In the 2013/2014 crop season, inoculants with strains SEMIAs 5080, 5019 and USDA 110 at Muriaze (Table 4.7), all strains at Ruace (Table 4.10), and strains SEMIAs 5079, 5080 and 5019 at Sussundenga (Table 4.11) significantly increased GY in relation to

the NI control. In the following crop season, all inoculated plants at Muriaze (Table 4.7) and Ruace (Table 4.10) improved significantly GY compared to the non-inoculated ones. Inoculation with strains SEMIAs 5079 and USDA 110 at Nkhame (Table 4.8) also resulted in increased GY in relation to the NI treatment in the 2014/2015 crop season. All inoculated plants had significantly higher GY than the non-inoculated ones across trial sites in both 2013/2014 (GY gains range 5–21%) and 2014/2015 (24–57%) crop seasons (Supplementary Table S5). In the 2014/2015 crop season, inoculation with SEMIA 5079 and USDA 110 resulted in significant GY gains of 25 and 18%, respectively, in relation to the NI-fertilized treatment (Supplementary Table S5). SEMIAs 5079, 5080, 5019 and USDA 110 were the best strains across trial sites and crop seasons with grain yield gains of 20–29% over the NI control, a statistically similar or better performance than the 22% yield gains obtained with the NI+N control (Supplementary Table S7).

Inoculants with strains SEMIA 5079, SEMIA 587 and USDA 110 at Nkhame (2014/2015) (Table 4.8), and all strains in the 2013/2014 crop season at Ruace (Table 4.10) resulted in significant increased grain dry weight (GDW) compared to the NI control. N-fertilizer application significantly improved GDW compared to the NI control at Nkhame (Table 4.8) and Ruace (2013/2014 crop season) (Table 4.10). Interestingly, in the 2014/2015 crop season, N-fertilizer treatment was outperformed by treatments with strains SEMIAs 5080, 587 and 5019 and USDA 110 at Ruace (Table 4.10).

4.3.7 Relative effectiveness

Plants inoculated with SEMIAs 5079 + 5080 had significantly higher relative effectiveness (RE) compared to those that relied on naturalized rhizobia at Londrina (2014/2015) (Table 4.4), Maracaí (Table 4.5) and Ponta Grossa (Table 4.4). Inoculation with strains SEMIAs 5079, 5080 and 587 at Maracaí (Table 4.5), SEMIA 5019 and USDA 110 at Ponta Grossa (Table 4.6), also resulted in increased RE in relation to the NI treatment.

In Mozambique, plants treated with strains SEMIA 587 at Ntengo (Table 4.9) and SEMIAs 5079, 5080 and 5019 at Sussundenga (Table 4.11) had significantly greater RE than the NI control in the 2013/2014 crop season. In the 2014/2015 crop season, inoculation with strains SEMIAs 5079 and 587 and USDA 110 at Nkhame (Table 4.8), SEMIAs 5079 and 5080 at Ruace (Table 4.10) and SEMIA 5080 at Sussundenga (Table 4.11) significantly increased RE in relation to the NI treatment.

Table 4.4: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), total N accumulation in shoots (TNS, mg plant^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, varieties BMX Potência–RR and BRS 360 –RR, grown with or without inoculation treatment, in the 2013/2014 and 2014/2015 crop seasons, respectively, at Londrina, Brazil

Treatment[1]	Londrina, 2013/2014 crop season							Londrina, 2014/2015 crop season						
	NN	NDW	SDW	TNS	GY	GDW	RE[2]	NN	NDW	SDW	TNS	GY	GDW	RE[2]
NI	11.8^{b} [3]	25.17^{a}	0.8^{ns}	29.61^{ab}	2220^{cd}	9.7^{d}	114.4^{ns}	15.8^{c} [3]	26.78^{bc}	3.2^{b}	138.90^{bc}	3052^{ns}	15.6^{bc}	95.4^{bc}
NI+N	5.6^{c}	6.03^{b}	0.7	32.12^{a}	2773^{a}	10.7^{a}	100.0[4]	12.4^{d}	17.15^{c}	3.3^{b}	157.35^{ab}	3081	16.1^{a}	100.0[4]
SEMIA 5079	17.5^{a}	31.30^{a}	0.6	22.83^{bc}	2401^{b}	10.1^{b}	82.5	22.1^{a}	40.76^{a}	2.9^{b}	126.85^{cd}	3316	15.3^{c}	89.9^{bcd}
SEMIA 5080	15.0^{ab}	25.32^{a}	0.6	22.49^{c}	2129^{d}	10.0^{bc}	82.9	18.8^{b}	34.27^{ab}	2.9^{b}	126.37^{cd}	3306	15.5^{c}	88.8^{bcd}
SEMIA 587	17.0^{a}	29.88^{a}	0.6	24.42^{bc}	2182^{cd}	10.0^{bc}	87.1	17.0^{bc}	28.12^{b}	3.0^{b}	119.75^{cd}	3222	15.7^{bc}	88.2^{cd}
SEMIA 5019	16.4^{a}	28.48^{a}	0.6	25.34^{bc}	2294^{bc}	9.8^{cd}	89.4	17.2^{bc}	44.55^{a}	3.4^{b}	153.18^{ab}	3388	15.3^{c}	102.6^{b}
USDA 110	17.8^{a}	31.91^{a}	0.5	19.97^{c}	2285^{bc}	10.1^{b}	75.0	18.3^{b}	40.17^{a}	2.5^{c}	108.45^{d}	3366	15.4^{c}	75.5^{d}
5079 + 5080	17.4^{a}	33.95^{a}	0.7	23.34^{bc}	2429^{b}	10.0^{bc}	95.5	17.5^{bc}	40.22^{a}	3.9^{a}	175.01^{a}	3191	15.9^{ab}	118.9^{a}
p - value	0.00	0.00	0.17	0.04	0.00	0.00	0.15	0.00	0.00	0.01	0.00	0.80	0.00	0.00
C.V. (%)	24.65	31.52	24.45	25.33	5.87	2.15	26.45	11.74	29.93	17.66	15.86	13.08	2.06	13.95

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; 5079 + 5080, inoculated with *B. japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080; All rhizobia were applied at the rate of 1.2×10^{6} cells seed^{-1}.

[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).

[3] Means of six replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan test).

[4] Not included in the statistical analysis.

Table 4.5: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), total N accumulation in shoots (TNS, mg plant^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, variety BMX Potência – RR, grown with or without inoculation treatment in the 2013/2014 crop season, at Maracaí and Rio Verde, Brazil.

Treatment[1]	Maracaí, 2013/2014 crop season							Rio Verde, 2013/2014 crop season						
	NN	NDW	SDW	TNS	GY	GDW	RE[2]	NN	NDW	SDW	TNS	GY	GDW	RE[2]
NI	15.3$^{ns\ 3}$	75.62^{ns}	1.3^{d}	33.27^{b}	1588^{ns}	13.3^{a}	86.7^{d}	27.4$^{ns\ 3}$	107.65^{a}	2.5^{ns}	69.36^{b}	2569^{cd}	13.0^{a}	133.5^{a}
NI+N	13.3	69.38	1.6^{bc}	51.27^{a}	1892	13.0^{b}	100.0[4]	20.8	40.09^{b}	2.4	92.81^{a}	2949^{a}	12.9^{ab}	100.0[4]
SEMIA 5079	13.5	80.52	1.6^{abc}	44.57^{a}	1553	13.3^{a}	112.5^{bc}	26.0	103.53^{a}	2.2	70.10^{b}	2581^{bcd}	13.1^{a}	114.9^{abc}
SEMIA 5080	17.5	78.77	1.7^{ab}	48.53^{a}	1485	12.9^{c}	117.1^{ab}	26.6	97.06^{a}	2.5	69.47^{b}	2596^{bcd}	12.7^{bc}	127.8^{ab}
SEMIA 587	12.2	86.56	1.7^{ab}	47.67^{a}	1473	13.0^{b}	123.4^{ab}	23.1	91.05^{a}	2.0	61.80^{bc}	2692^{bc}	12.9^{ab}	106.9^{c}
SEMIA 5019	15.2	72.05	1.4^{cd}	38.15^{b}	1576	12.9^{c}	96.2^{cd}	24.6	90.71^{a}	2.0	59.03^{c}	2482^{d}	12.7^{c}	112.1^{bc}
USDA 110	9.1	68.73	1.3^{d}	36.50^{b}	1805	12.8^{c}	86.7^{d}	23.6	91.04^{a}	2.0	58.22^{c}	2755^{b}	13.1^{a}	107.5^{c}
5079 + 5080	13.9	64.83	1.9^{a}	50.42^{a}	1481	13.4^{a}	132.5^{a}	27.6	110.54^{a}	2.3	68.39^{b}	2697^{bc}	13.1^{a}	129.2^{ab}
p - value	0.11	0.66	0.00	0.00	0.15	0.00	0.00	0.28	0.00	0.23	0.00	0.00	0.02	0.08
C.V. (%)	32.54	27.93	15.15	14.69	18.69	1.12	17.30	20.56	23.33	19.72	13.06	6.27	1.92	15.64

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; 5079 + 5080, inoculated with *B. japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080; All rhizobia were applied at the rate of 1.2×10^{6} cells seed^{-1}.

[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).

[3] Means of six replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).

[4] Not included in the statistical analysis.

Table 4.6: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), total N accumulation in shoots (TNS, mg plant^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, variety BRS-359–RR, grown with or without inoculation treatment in the 2014/2015 crop season at Ponta Grossa, Brazil.

Treatment[1]	NN	NDW	SDW	TNS	GY	GDW	RE[2]
NI	115.1$^{ns\ 3}$	402.89^{ns}	5.3^{ns}	196.85^{bc}	2825^{b}	12.7^{c}	67.3^{c}
NI+N	93.2	341.69	7.4	312.67^{a}	3126^{a}	13.5^{a}	100.0[4]
SEMIA 5079	111.0	447.73	6.1	215.83^{bc}	2903^{b}	13.1^{ab}	72.9^{bc}
SEMIA 5080	103.4	365.31	5.4	180.88^{c}	2928^{b}	13.1^{ab}	69.1^{bc}
SEMIA 587	111.1	469.49	6.0	248.73^{b}	2880^{b}	13.1^{ab}	71.3^{bc}
SEMIA 5019	130.4	421.40	6.5	237.31^{bc}	2870^{b}	12.9^{bc}	81.2^{ab}
USDA 110	96.7	356.11	6.4	245.77^{b}	2719^{c}	12.9^{bc}	87.9^{a}
5079 + 5080	107.8	392.09	7.4	250.62^{b}	2927^{b}	12.9^{bc}	90.0^{a}
p - value	0.20	0.11	0.22	0.02	0.00	0.04	0.01
C.V. (%)	21.29	20.23	25.58	24.81	3.62	1.79	15.93

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; 5079 + 5080, inoculated with *B. japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080; All rhizobia were applied at the rate of 1.2×10^{6} cells seed^{-1}.
[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).
[3] Means of six replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).
[4] Not included in the statistical analysis.

Table 4.7: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), ground biomass (AGB, kg ha^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, variety Storm, grown with or without inoculation treatment in the 2013/2014 and 2014/2015 crop seasons at Muriaze, Mozambique.

Treatment[1]	Muriaze, 2013/2014 crop season							Muriaze, 2014/2015 crop season						
	NN	NDW	SDW	AGB	GY	GDW	RE[2]	NN	NDW	SDW	AGB	GY	GDW	RE[2]
NI	6.2^{d} [3]	27.40^{c}	12.9^{c}	5506^{ns}	1284de	15.6 ^{ns}	68.5^{ns}	18.5^{f} [3]	150.15^{e}	22.5^{ns}	3199^{b}	824^{f}	15.8^{ab}	100.8^{ns}
NI+N	3.4^{e}	27.60^{c}	19.8^{a}	5610	1243^{e}	16.4	100.0[4]	13.5^{g}	73.70^{fg}	23.5	1955^{c}	902^{f}	15.9^{ab}	100.0[4]
SEMIA 5079	11.3^{c}	123.80^{b}	11.9^{c}	5402	1365de	15.9	66.3	27.1^{e}	272.11^{d}	18.1	5346^{a}	2204^{a}	14.1^{c}	81.3
SEMIA 5080	14.9^{b}	108.72^{b}	11.8^{c}	5651	1800^{a}	16.8	62.1	30.2^{d}	419.40^{b}	20.7	3986^{b}	1357^{d}	15.2^{bc}	94.2
SEMIA 587	7.2^{d}	38.52^{c}	12.6^{c}	5181	1324de	16.6	69.8	33.8^{c}	343.96^{c}	18.6	3539^{b}	1203^{e}	14.5^{bc}	86.1
SEMIA 5019	23.7^{a}	181.73^{a}	18.8^{ab}	5373	1664^{ab}	17.5	87.6	46.1^{a}	508.70^{a}	18.4	4203^{b}	1509^{c}	14.9^{bc}	81.8
USDA 110	7.4^{d}	37.64^{c}	15.0^{bc}	5575	1580^{bc}	15.8	76.9	35.4^{b}	89.20^{f}	21.4	3742^{b}	1813^{b}	14.8^{bc}	94.4
p - value	0.00	0.00	0.00	0.35	0.00	0.21	0.22	0.00	0.00	0.25	0.00	0.00	0.06	0.38
C.V. (%)	13.97	29.74	25.50	8.54	9.88	6.97	23.00	2.89	11.35	21.29	22.72	6.45	8.72	18.80

[1] NI, no inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; All rhizobia were applied at the rate of 1.2×10^{6} cells seed^{-1}.

[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).

[3] Means of five replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).

[4] Not included in the statistical analysis.

Table 4.8: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), above ground biomass (AGB, kg ha^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, variety Storm, grown with or without inoculation treatment in the 2013/2014 and 2013/2014 crop seasons at Nkhame, Mozambique.

Treatment[1]	Nkhame, 2013/2014 crop season							Nkhame, 2014/2015 crop season						
	NN	NDW	SDW	AGB	GY	GDW	RE[2]	NN	NDW	SDW	AGB	GY	GDW	RE[2]
NI	24.8$^{a\,3}$	29.50^{cd}	36.5^{ns}	6995^{ns}	2947^{bc}	15.6^{bcd}	66.0^{ns}	8.9$^{d\,3}$	91.63^{c}	17.2^{e}	3810^{ns}	1384^{e}	14.8^{d}	59.6^{d}
NI+N	16.7^{cd}	16.95^{d}	58.6	8032	3964^{a}	17.3^{a}	100.0[4]	9.6^{d}	68.93^{d}	31.3^{b}	4797	1772^{cd}	15.7^{ab}	100.0[4]
SEMIA 5079	22.6^{ab}	18.65^{d}	50.2	7111	2998^{bc}	17.0^{a}	92.1	22.1^{b}	93.58^{c}	37.8^{a}	4223	2287^{a}	16.0^{a}	130.8^{a}
SEMIA 5080	17.7^{bcd}	15.96^{d}	43.4	7772	3299^{b}	15.7^{bcd}	79.5	16.4^{c}	99.74^{c}	21.3^{de}	4343	1526de	15.0^{cd}	70.7^{cd}
SEMIA 587	17.3^{cd}	42.20^{c}	54.0	7131	2936^{bc}	15.0^{d}	94.9	10.0^{d}	122.63^{b}	36.8^{a}	4961	1487^{e}	15.4^{abc}	124.3^{a}
SEMIA 5019	22.5^{ab}	90.01^{a}	62.7	7209	3161^{bc}	16.0^{bc}	100.5	33.4^{a}	156.57^{a}	16.8^{e}	4621	1649^{cde}	15.1^{cd}	57.7^{d}
USDA 110	20.4^{abc}	38.40^{c}	52.4	7463	3278^{b}	16.1^{b}	93.2	16.4^{c}	52.00^{de}	24.3^{cd}	4913	2052^{ab}	15.5^{abc}	81.7^{c}
p – value	0.00	0.00	0.35	0.61	0.02	0.00	0.24	0.00	0.00	0.00	0.42	0.00	0.02	0.00
C.V. (%)	21.92	31.21	28.83	13.18	17.56	4.94	24.96	31.23	20.19	18.41	19.31	13.25	3.29	17.38

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; All rhizobia were applied at the rate of 1.2×10^6 cells seed^{-1}.

[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).

[3] Means of five replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).

[ns] not statistically different ($p \leq 1.0$, Duncan's test).

[4] Not included in the statistical analysis.

Table 4.9: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), above ground biomass (AGB, kg ha^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, variety Storm, grown with or without inoculation treatment in the 2013/2014 and 2014/2015 crop seasons at Ntengo, Mozambique.

Treatment[1]	Ntengo, 2013/2014 crop season							Ntengo, 2014/2015 crop Season						
	NN	NDW	SDW	AGB	GY	GDW	RE[2]	NN	NDW	SDW	AGB	GY	GDW	RE[2]
NI	6.0^{f} [3]	96.40^{b}	19.1^{d}	6081^{ns}	2397^{ns}	14.9^{ns}	80.3^{b}	29.0^{b} [3]	77.90^{d}	18.8^{ns}	2740^{ns}	1105^{ns}	16.2^{ns}	102.1^{ns}
NI+N	6.8^{ef}	90.85^{b}	24.0^{ab}	5265	2312	15.8	100.0[4]	18.0^{c}	44.70^{e}	18.6	3543	1444	16.4	100.0[4]
SEMIA 5079	9.0^{cd}	128.15^{b}	19.5^{cd}	6423	2686	15.5	82.8^{b}	39.9^{a}	282.21^{a}	21.8	3167	1459	16.0	118.2
SEMIA 5080	22.2^{a}	181.00^{a}	20.0^{cd}	5581	2167	15.2	84.2^{b}	28.6^{bc}	92.38^{d}	16.2	3202	1337	16.3	88.0
SEMIA 587	10.6^{c}	127.35^{b}	26.5^{a}	5790	2404	16.1	111.7^{a}	29.5^{b}	125.45^{c}	19.8	3475	1609	15.9	108.1
SEMIA 5019	13.4^{b}	201.56^{a}	19.7^{cd}	5505	2398	15.9	81.9^{b}	42.7^{a}	195.38^{b}	18.3	3219	1374	15.7	99.3
USDA 110	8.2^{de}	118.84^{b}	22.4^{bc}	5395	2222	15.8	93.5^{b}	22.0^{bc}	80.49^{d}	16.8	3403	1455	16.0	90.7
p – value	0.00	0.00	0.00	0.19	0.21	0.25	0.00	0.00	0.00	0.77	0.46	0.14	0.26	0.74
C.V. (%)	14.90	23.42	12.41	12.19	11.96	5.57	13.94	31.18	20.22	28.14	17.30	18.76	3.94	29.82

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; All rhizobia were applied at the rate of 1.2×10^{6} cells seed^{-1}.

[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).

[3] Means of five replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).

[4] Not included in the statistical analysis.

Table 4.10: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), above ground biomass (AGB, kg ha^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, variety Storm, grown with or without inoculation treatment in the 2013/2014 and 2014/2015 crop seasons at Ruace, Mozambique.

Treatment[1]	Ruace, 2013/2014 crop season							Ruace, 2014/2015 crop season						
	NN	NDW	SDW	AGB	GY	GDW	RE[2]	NN	NDW	SDW	AGB	GY	GDW	RE[2]
NI	9.9^{e} [3]	100.00^{e}	26.8^{b}	8806^{ns}	2813^{d}	17.5^{e}	81.7^{a}	7.4^{d} [3]	48.68^{de}	7.8^{cd}	2267^{ns}	729^{c}	14.2^{ns}	54.9^{bcd}
NI+N	2.4^{f}	17.64^{f}	32.8^{a}	9167	3694^{a}	18.3^{d}	100.0[4]	6.2^{d}	29.24^{e}	15.0^{a}	2526	938^{bc}	15.6	100.0[4]
SEMIA 5079	21.6^{c}	296.85^{b}	28.0^{b}	8791	3774^{a}	18.8^{cd}	86.1^{a}	22.3^{b}	151.67^{b}	15.0^{a}	2766	1116^{ab}	14.8	106.3^{a}
SEMIA 5080	41.1^{a}	426.48^{a}	26.2^{b}	9178	3421^{bc}	19.4^{bc}	80.1^{a}	34.5^{a}	198.28^{a}	17.4^{a}	3156	1245^{a}	16.1	119.8^{a}
SEMIA 587	23.5^{c}	217.60^{c}	25.2^{b}	8920	3375^{bc}	19.6^{b}	77.6^{a}	25.1^{b}	124.12^{c}	10.4^{b}	2497	1031^{ab}	15.0	72.5^{b}
SEMIA 5019	37.9^{b}	409.28^{a}	26.2^{b}	8531	3490^{b}	20.2^{a}	80.4^{a}	12.3^{c}	67.12^{d}	7.2^{cd}	2799	996^{b}	14.7	48.5^{cd}
USDA 110	9.9^{e}	139.92^{d}	22.0^{c}	8304	3289^{c}	19.2^{bc}	67.5^{b}	11.7^{c}	37.80^{e}	5.6^{d}	2644	1012^{ab}	14.8	38.2^{d}
p - value	0.00	0.00	0.00	0.16	0.00	0.00	0.02	0.02	0.00	0.00	0.35	0.03	0.34	0.00
C.V. (%)	12.31	15.88	9.53	7.48	4.54	2.82	9.47	9.47	23.72	20.80	22.61	19.85	8.35	23.91

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; All rhizobia were applied at the rate of 1.2×10^6 cells seed^{-1}.

[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).

[3] Means of five replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).

[ns] not statistically different ($p \leq 0.10$, Duncan's test).

[4] Not included in the statistical analysis.

Table 4.11: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), above ground biomass (AGB, kg ha^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, variety Storm, grown in the 2013/2014 and 2013/2014 crop seasons at Sussundenga, Mozambique.

Treatment[1]	Sussundenga, 2013/2014 crop season							Sussundenga, 2014/2015 crop season						
	NN	NDW	SDW	AGB	GY	GDW	RE[2]	NN	NDW	SDW	AGB	GY	GDW	RE[2]
NI	8.9^{e} [3]	118.98^{ef}	8.2^{c}	6056^{cde}	1322^{d}	9.1^{ns}	35.8^{b}	5.5^{d} [3]	65.80^{fg}	11.4^{c}	5519^{ns}	1506^{ns}	13.5^{ns}	138.5^{bc}
NI+N	4.1^{f}	64.30^{f}	23.3^{a}	6728^{bcd}	1693^{c}	9.5	100.0[4]	5.8^{d}	53.04^{g}	8.0^{d}	6512	1880	15.3	100.0[4]
SEMIA 5079	20.8^{c}	334.72^{c}	17.0^{b}	7250^{a-d}	1581^{c}	9.3	73.3^{a}	15.5^{c}	129.80^{d}	10.8^{c}	5836	1622	14.0	128.8^{c}
SEMIA 5080	30.0^{b}	439.03^{b}	16.9^{b}	7867^{ab}	2341^{a}	9.4	71.5^{a}	34.0^{ab}	188.10^{c}	15.4^{a}	6912	1809	13.9	163.6^{a}
SEMIA 587	15.4^{d}	176.35^{de}	8.9^{c}	5861de	1288^{d}	9.2	38.6^{b}	32.2^{b}	276.30^{b}	12.8^{abc}	6022	1577	14.2	138.2^{bc}
SEMIA 5019	39.5^{a}	594.65^{a}	17.0^{b}	8528^{a}	1922^{b}	9.5	72.3^{a}	35.4^{a}	351.48^{a}	14.8^{ab}	6482	1343	15.9	160.9^{ab}
USDA 110	18.4^{cd}	246.38^{cd}	10.8^{c}	7437^{abc}	1340^{d}	9.1	47.3^{b}	5.7^{d}	85.41^{ef}	12.5^{bc}	6298	1831	15.3	141.6^{abc}
p - value	0.00	0.00	0.00	0.00	0.00	0.84	0.00	0.00	0.00	0.00	0.39	0.19	0.13	0.02
C.V. (%)	20.65	28.76	25.13	17.50	11.04	6.23	28.78	15.66	12.04	19.36	17.20	19.83	9.70	14.11

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; All *Bradyrhizobium* spp. were applied at the rate of 1.2×10^6 cells seed^{-1}.

[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).

[3] Means of five replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).

[ns] not statistically different ($p \leq 0.10$, Duncan's test).

[4] Not included in the statistical analysis.

4.4 DISCUSSION

Brazilian soils are originally devoid of rhizobia capable of nodulating soybean, but strain selection programs started early with soybean expansion, in the 1960s (HUNGRIA et al., 2006a; HUNGRIA; MENDES, 2015). Elite inoculant strains from Australia and the USA were field tested in Brazil to verify their adaptability to the local agro-climatic conditions, N_2-fixation effectiveness and ability to compete for nodule occupancy (HUNGRIA; MENDES, 2015). Following years of extensive trials and research improvements, four strains, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 are currently employed in commercial inoculants for the crop in Brazil, in single or double combinations (HUNGRIA et al., 2000; CAMPOS et al., 2001; HUNGRIA et al., 2005; LOUREIRO et al., 2007; CAMPO et al., 2009). The combination SEMIA 5079 + SEMIA 5080 represents over 80% of the commercial inoculants sold in the country (HUNGRIA et al., 2013) and is the farmers' choice in the Cerrado region (HUNGRIA; MENDES, 2015), whose climate is savanna (Aw) (PIDWIRNY, 2011).

The superiority of the combination SEMIA 5079 + SEMIA 5080 was confirmed in this study, where it consistently resulted in the highest nodulation, plant growth, N accumulation in shoots, grain dry weight and symbiotic effectiveness (Tables 4.4, 4.5, 4.6 and Table S4). This combination of strains resulted in grain yield gains over the NI control of 9 and 5%, respectively, in the 2013/2014 and 2014/2015 crop seasons at Londrina (Table 4.4), the site with the highest naturalized rhizobial population, estimated at 10^5 cells g^{-1} of soil (Table 4.2). These yield gains are within the 3.2–14.5% interval of re-inoculation yield benefit reported in Brazil (NISHI; HUNGRIA, 1996; ALVES et al., 2003; MENDES et al., 2004; HUNGRIA et al., 2013).

Interestingly, USDA 110, a strain that has never been used in commercial inoculants in Brazil, was among the best performing strains even at Londrina trial site (Table 4.4). This is in agreement with reports of outstanding competitiveness (GEORGE et al., 1987; ABAIDOO; VAN KESSEL, 1989; ABAIDOO et al., 1990; MCDERMOTT; GRAHAM, 1990; THIES et al., 1992) and N_2-fixation effectiveness (ABAIDOO et al., 2007; MUTUMA et al., 2014; YOUSEIF et al., 2014b; AGOYI et al., 2016) of this strain.

In Mozambique, where three out of the five sampled fields had < 100 cells g^{-1} of soil (Table 4.2), inoculation responses were much stronger, as indicated by average yield gains over the NI control of 5–57% (Supplementary Table S5). Despite a general positive response to inoculation, particularities were observed at each site. Grain yield gains

were far greater at Ruace (17–34%) (Table 4.10) than Nkhame (2–12%) (Table 4.8), in the 2013/2014 crop season, although both sites had a similar rhizobial population size of 1×10^3 cells g^{-1} of soil (Table 4.2). Considering the lower shoot dry weight and grain yields obtained without inoculation at Ruace, we suppose that this location had a larger proportion of ineffective indigenous bacteria.

In general, in the 2013/2014 crop season the yields recorded at the trial sites in Brazil were lower than in previous crop seasons [Tables 4.4 and 4.5; (CONAB, 2016)]. This can be ascribed to inadequate rainfall during R3 [reproductive stage, pod is 5 mm in length at one of the four uppermost nodes on the main stem with a completely developed leaf (FEHR; CAVINESS, 1977); Table 4.3], when low rainfall substantially reduces grain yields (SIONIT; KRAMER, 1977; ECK et al., 1987; SOUZA et al., 2013).

In Mozambique, relatively better grain yields were recorded in the first compared with the second crop season at Muriaze, Nkhame, Ntengo and Ruace (Tables 4.7, 4.8, 4.9 and 4.10). The lower rainfall recorded during R3 stage at the four trial sites in the 2014/2015 compared with the 2013/2014 crop season (Table 4.3) may have contributed to the decrease in grain yield from the first to the second crop season.

Soybean inoculation success in Brazil can be explained by the positional difference between inoculant and naturalized strains in the root profile. Inoculant strains typically dominate occupancy of crown root nodules (MCDERMOTT; GRAHAM, 1989; GRAHAM, 2008), but are unable of sustaining high population levels beyond the crown root area, 0–5 cm from the stem base (MADSEN; ALEXANDER, 1982; MCDERMOTT; GRAHAM, 1989; WADISIRISUK et al., 1989). Because of this, the strains already in the soil have positional advantage on the competition for lateral root infections sites (VLASSAK et al., 1997; LÓPEZ-GARCÍA et al., 2002; ALTHABEGOITI et al., 2008). Furthermore, crown root nodules usually undergo a senescence process around R4 [reproductive stage, pod 2 cm in length and one of the four uppermost nodes on the main stem with completely developed leaf (FEHR; CAVINESS, 1977)] (BERGERSEN, 1958; ESPINOSA-VICTORIA et al., 2000; ALESANDRINI et al., 2003) just before N_2-fixation reaches maximum levels (THIBODEAU; JAWORSKI, 1975). This means that symbiosis will markedly be influenced by the symbiotic effectiveness of naturalized rhizobia. It is, therefore, possible that the observed re-inoculation responses represent a combined effect of the N fixed on the crown and lateral nodules, predominately occupied by inoculant and naturalized strains, respectively (MCDERMOTT; GRAHAM, 1989; LÓPEZ-GARCÍA et al., 2002; GRAHAM, 2008). In annually cropped soybean areas in Brazil, inoculated soybean plants frequently exhibit

profuse nodulation on the crown root, contrasting with delayed infections occurring at 1–2 cm below the crown on control plots (HUNGRIA; MENDES, 2015), which elucidates the positional difference of inoculant and naturalized strains in the root profile.

N-fertilizer use reduced nodule number and dry weight in both countries, supporting previous observations that increased levels of mineral N in the rhizosphere inhibit soybean nodule formation and functioning (KEYSER; LI, 1992; ARRESE-IGOR et al., 1997; HUNGRIA et al., 2006c; HUNGRIA; MENDES, 2015). Moreover, in Mozambique, inoculation with strains SEMIA 5079 and USDA 110, the best performing strains across sites in the 2014/2015 crop season, resulted in significant GY gains, of 31 and 23%, respectively, in relation to the N-fertilized control (Supplementary Table S5). This corroborates previous evidence of the profitably of inoculation compared to N-fertilizer application (HUNGRIA et al., 2006a; 2006c; HUNGRIA; MENDES, 2015). In Brazil, however, N-fertilizers increased grain yield in three out of five experiments compared to the NI treatment (Tables 4.4, 4.5 and 4.6). The low rainfall recorded at the trial sites, particularly during R3 (Table 4.3), may explain the low yields. In addition, it is broadly reported that under water stressing conditions BNF is more affected than the assimilation of mineral N (SERRAJ et al., 2001; GIL-QUINTANA et al., 2012; DWIVEDI et al., 2015). Despite the observed yield gains, N-fertilizer is not profitable, considering the price in the Brazilian market. However, concerns are raised in Brazil that the increasing periods of water stress, due to the global climatic changes, might lead to the need of application of N fertilizers, with serious economic and environmental impacts. On the contrary, in Mozambique, analysis across trial sites and crop seasons showed that the use of N-fertilizer did not provide better results than those obtained with the best performing strains, SEMIA 5079, SEMIA 5080, SEMIA 5019 and USDA 110 (Supplementary Table S7).

In conclusion, elite strains either selected in Brazil or in USA improved soybean growth, yield and grain dry weight in Brazil and Mozambique. The best treatments across trial sites in Brazil were SEMIA 5079 + SEMIA 5080, SEMIA 5079 and USDA 110, with average grain yield gains of 4–5%. In Mozambique, the best treatments were SEMIA 5079, SEMIA 5080, SEMIA 5019 and USDA 110, with overall grain yield gains of 20–29%. These results suggest that the strains SEMIA 5079, SEMIA 5080 and USDA 110 hold the best potential as commercial inoculants in both countries. Strains SEMIA 5079 and SEMIA 5080 have shown to be very effective in fixing nitrogen and tolerant to the harsh conditions of the Brazilian Cerrados (HUNGRIA et al., 2006c; HUNGRIA; MENDES, 2015).USDA 110 is also very effective (ABAIDOO et al., 2007; AGOYI et al., 2016) and competitive (GEORGE

et al., 1987; MCDERMOTT; GRAHAM, 1990). Therefore, these strains are likely to adapt well not only in Brazil and Mozambique, but also in other countries with similar agro-climatic conditions. The feasibility of transference of BNF-related technologies between continents and countries with relatively similar agro-climatic conditions can save time, labor and money, and speed up the introduction of productive and sustainable cropping systems, as is the case of the soybean in Africa.

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5 CONSIDERAÇÕES FINAIS

No primeiro estudo, rizóbios indígenas isolados da soja em Moçambique foram avaliados pela capacidade de FBN na casa de vegetação e foram caracterizados geneticamente no Brasil. Quatro estripes usadas em inoculantes no Brasil, *B. japonicum* SEMIA 5079, *B. diazoefficiens* SEMIA 5080, *B. elkanii* strains SEMIA 587 e SEMIA 5019, e uma norte americana *B. diazoefficiens* USDA 110 foram usadas como referências. Cinco isolados (4, 19, 22, 27 e 61) apresentaram, consistentemente, alta eficiência de FBN, sugerindo que a inoculação com estirpes indígenas de rizóbios adaptadas às condições locais representa uma estratégia possível para a produção de soja em Moçambique. Ensaios mutilocais com estes isolados promissores devem ser conduzidos em Moçambique para verificar a sua superioridade de FBN na presença de outros rizóbios indígenas e/ou estirpes comerciais.

No segundo estudo, as cinco estirpes de referência usadas no primeiro trabalho foram testadas em ensaios a campo com variedades de soja de nodulação não promíscua no Brasil e em Moçambique. Resultados bem sucedidos foram obtidos no Brasil e em Moçambique com as estirpes SEMIA 5079, SEMIA 5080 e USDA 110 o que sugere que estas estirpes têm potencial para serem usadas em inoculantes em ambos países. Os resultados também indicam a viabilidade da transferência de tecnologias relacionadas à FBN com a soja entre países com condições agroclimáticas similares.

A estirpe USDA 110 apresentou o melhor desempenho nos experimentos em casa de vegetação, bem como uma ótima performance nos ensaios conduzidos a campo em Moçambique e no Brasil. Isso ocorreu inclusive em Londrina, onde essa estirpe foi inoculada pela primeira vez em um solo com população de rizóbios naturalizados superior a 10^5 células g^{-1} de solo. Curiosamente, alguns isolados indígenas de Moçambique apresentaram performance similar ou superior à USDA 110, o que abre uma janela de oportunidades para a pesquisa com os rizóbios indígenas de Moçambique.

6 CONCLUDING REMARKS

In the first study, indigenous rhizobia isolated from nodules of soybean grown in Mozambique were screened for N_2-fixation in the greenhouse and genetically characterized in Brazil. Four strains used in commercial inoculants in Brazil, *B. japonicum* SEMIA 5079, *B. diazoefficiens* SEMIA 5080, *B. elkanii* strains SEMIA 587 and SEMIA 5019, and a strain used in inoculants in Africa, *B. diazoefficiens* USDA 110 were used as references. Five representative isolates (4, 19, 22, 27 and 61) consistently showed high N_2-fixation efficiency, suggesting that the inoculation with indigenous rhizobia already adapted to local conditions is a possible strategy for soybean production in Mozambique. Multi-site field trials with those promising isolates should be conducted to ascertain their superiority in fixing N in the presence of other indigenous and/or commercial strains.

In the second study, the five reference strains used in the first work were evaluated on non-promiscuous soybean varieties in field trials conducted in Brazil and Mozambique. Successful results were obtained in Brazil and Mozambique with strains SEMIA 5079, SEMIA 5080 and USDA 110 indicating that these strains have potential to be included in commercial inoculants in both countries. The results also confirm the feasibility of transference of BNF-related technologies in soybean between countries with similar agro-climatic conditions.

USDA 110 was the best reference strain in both greenhouse trials and had an excellent performance in the field trials conducted in Mozambique and Brazil, including at Londrina where this strain was inoculated for the first time and there was a rhizobial population size of over 10^5 cells g^{-1} of soil. Interestingly, some indigenous isolates from Mozambique had similar or better performance than USDA 110, which opens a window for research with indigenous rhizobia from Mozambique.

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ANEXOS

MATERIAL SUPLEMENTAR

APPENDICES

SUPPLEMENTARY MATERIAL

Table S1: Nodule number (NN, n° plant^{-1}) and dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), total N accumulation in shoots (TNS, mg plant^{-1}) and relative efficiency (RE, %) of soybean, variety BRS 133, inoculated with 87 isolates from Mozambique and five reference strains, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110 screened in a greenhouse trial in Londrina, Brazil, in 2014.

Isolate	Source	Species name [1]	NN[2]	NDW	SDW	TNS	RSE[3]
1	Ntengo	*Bradyrhizobium* sp.	79.9	453.80	4.0	98.93	96.3
2	Ntengo	*Bradyrhizobium* sp.	56.3	380.43	3.4	73.17	80.4
3	Ntengo	*Bradyrhizobium* sp.	81.4	465.54	4.9	105.32	115.2
4*	Ntengo	*Bradyrhizobium* sp.	63.4	508.00	5.7	142.03	135.5
5	Ntengo	*Bradyrhizobium* sp.	73.3	557.48	5.4	131.29	126.3
6*	Ntengo	*Bradyrhizobium* sp.	103.6	498.81	4.9	104.15	116.9
7	Ntengo	*Bradyrhizobium* sp.	60.6	392.05	3.9	76.36	95.7
8	Ntengo	*Agrobacterium* sp.	38.3	241.22	2.7	40.80	64.0
9	Ntengo	*Bradyrhizobium* sp.	36.4	191.06	2.2	36.93	51.1
10	Ntengo	*Rhizobium* sp.	7.0	29.14	0.8	6.51	18.2
11	Ntengo	*Bradyrhizobium* sp.	6.0	20.19	0.8	4.98	18.6
14	Ntengo	*Bradyrhizobium* sp.	10.0	27.53	1.0	9.20	24.3
15	Ntengo	*Bradyrhizobium* sp.	86.5	531.65	5.0	108.19	107.7
17*	Ntengo	*Bradyrhizobium* sp.	88.0	586.10	5.8	123.32	139.4
18	Ntengo	*Bradyrhizobium* sp.	102.6	527.30	5.0	114.44	120.6
19*	Ntengo	*Bradyrhizobium* sp.	106.8	539.89	5.1	139.19	124.0
20	Ntengo	*Bradyrhizobium* sp.	78.1	594.66	5.3	131.68	128.1
22*	Nkhame	*Bradyrhizobium* sp.	76.0	474.31	6.2	152.70	135.3
23	Nkhame	*Bradyrhizobium* sp.	79.9	595.50	5.8	146.57	137.4
24*	Nkhame	*Bradyrhizobium* sp.	102.0	588.26	5.8	146.78	138.4
25	Nkhame	*Bradyrhizobium* sp.	65.8	478.60	5.3	141.01	114.1
26	Nkhame	*Bradyrhizobium* sp.	70.8	520.29	5.3	143.88	126.7
27*	Nkhame	*Bradyrhizobium* sp.	65.5	493.08	5.2	146.15	129.5
28	Nkhame	*Bradyrhizobium* sp.	77.9	601.06	5.5	136.19	129.1
29	Nkhame	*Bradyrhizobium* sp.	77.5	465.88	3.1	61.75	73.2
30	Nkhame	*Bradyrhizobium* sp.	46.1	340.00	3.5	58.64	84.6
31	Nkhame	*Bradyrhizobium* sp.	82.3	447.43	3.1	57.18	72.9
32	Nkhame	*Bradyrhizobium* sp.	84.8	511.89	4.6	121.66	109.8
33	Nkhame	*Bradyrhizobium* sp.	82.6	527.56	4.7	113.38	112.3
34	Nkhame	*Bradyrhizobium* sp.	29.1	236.00	2.1	30.74	48.8
35	Nkhame	*Bradyrhizobium* sp.	68.1	538.13	5.4	144.08	129.7
36	Nkhame	*Rhizobium* sp.	9.4	29.68	0.8	7.19	19.1
37	Nkhame	*Bradyrhizobium* sp.	87.3	592.80	4.5	90.67	108.4
38*	Nkhame	*Rhizobium* sp.	73.0	541.83	4.0	100.71	96.4
39*	Nkhame	*Bradyrhizobium* sp.	83.5	638.99	5.0	152.92	119.8

Isolate	Source	Species name [1]	NN[2]	NDW	SDW	TNS	RSE[3]
40*	Nkhame	*Bradyrhizobium* sp.	60.6	512.78	5.0	142.85	117.2
41	Nkhame	*Bradyrhizobium* sp.	70.8	475.45	4.3	110.42	92.3
42	Nkhame	*Rhizobium* sp.	64.3	488.68	3.8	107.68	88.9
43	Ruace	*Bradyrhizobium* sp.	30.0	64.43	1.0	10.08	23.0
44	Ruace	*Bradyrhizobium* sp.	9.8	19.71	0.7	4.74	17.2
45	Ruace	*Bradyrhizobium* sp.	8.9	32.30	0.8	8.27	18.5
46	Ruace	*Bradyrhizobium* sp.	9.4	30.85	0.6	3.93	15.3
48	Ruace	*Bradyrhizobium* sp.	19.3	82.31	1.1	15.67	26.7
50	Ruace	*Rhizobium* sp.	5.5	18.93	0.8	5.76	20.5
52	Ruace	*Bradyrhizobium* sp.	18.8	76.99	1.1	14.04	24.7
53	Ruace	*Bradyrhizobium* sp.	25.6	81.10	0.9	11.16	21.1
55	Ruace	*Rhizobium* sp.	11.4	55.44	0.9	10.98	21.2
56	Ruace	*Bradyrhizobium* sp.	11.8	54.59	0.8	6.97	18.3
57	Mutequelesse	*Bradyrhizobium* sp.	58.4	435.56	4.4	127.64	105.3
58	Mutequelesse	*Bradyrhizobium* sp.	59.6	452.94	5.0	137.59	106.6
59	Mutequelesse	*Rhizobium* sp.	37.4	310.11	2.9	53.69	70.4
60	Mutequelesse	*Bradyrhizobium* sp.	90.9	399.29	4.5	114.61	107.3
61*	Mutequelesse	*Bradyrhizobium* sp.	63.8	440.28	4.9	146.57	116.8
62*	Mutequelesse	*Bradyrhizobium* sp.	98.5	593.78	5.4	147.83	129.6
63	Mutequelesse	*Bradyrhizobium* sp.	86.3	538.11	5.0	134.41	120.1
64	Muriaze	*Bradyrhizobium* sp.	81.9	276.54	2.6	68.17	60.0
65	Muriaze	*Bradyrhizobium* sp.	29.0	181.61	1.8	22.25	36.8
66	Muriaze	*Bradyrhizobium* sp.	21.5	51.89	0.9	18.30	19.9
67	Muriaze	*Bradyrhizobium* sp.	17.8	43.08	1.0	10.04	23.1
69	Muriaze	*Rhizobium* sp.	10.8	40.45	1.1	18.24	24.1
70	Muriaze	*Bradyrhizobium* sp.	38.0	161.78	1.5	32.01	34.9
71	Muriaze	*Bradyrhizobium* sp.	21.6	82.16	1.5	16.05	35.1
72	Muriaze	*Bradyrhizobium* sp.	26.5	89.63	1.4	19.03	33.5
73	Muriaze	*Rhizobium* sp.	23.6	63.90	0.9	12.94	21.6
74	Muriaze	*Bradyrhizobium* sp.	15.5	77.51	1.2	18.56	29.4
75	Muriaze	*Rhizobium* sp.	16.1	53.29	0.8	13.35	19.3
76	Muriaze	*Bradyrhizobium* sp.	23.0	75.06	1.3	17.78	31.3
77	Muriaze	*Bradyrhizobium* sp.	27.0	165.80	2.3	37.51	53.3
78	Sussundenga	*Bradyrhizobium* sp.	5.0	36.74	0.7	7.08	15.6
79	Sussundenga	*Bradyrhizobium* sp.	6.8	47.30	0.8	7.53	18.6
80	Sussundenga	*Bradyrhizobium* sp.	7.6	42.03	0.9	10.36	21.5
81	Sussundenga	*Bradyrhizobium* sp.	10.9	35.96	0.8	8.24	18.5
82	Sussundenga	*Bradyrhizobium* sp.	11.0	38.88	0.8	8.03	20.3
85	Sussundenga	*Rhizobium* sp.	6.6	22.24	0.9	7.22	21.6
86	Sussundenga	*Rhizobium* sp.	15.0	79.25	1.0	19.25	23.2
87	Sussundenga	*Rhizobium* sp.	63.6	309.55	3.2	97.65	76.3
88	Sussundenga	*Rhizobium* sp.	17.1	31.28	0.9	9.13	22.2
90	Sussundenga	*Rhizobium* sp.	15.4	88.21	1.1	12.29	25.9
91	Sussundenga	*Rhizobium* sp.	20.9	92.43	1.3	18.14	28.5
92	Zembe	*Rhizobium* sp.	42.5	191.81	1.8	42.97	43.6

Isolate	Source	Species name [1]	NN[2]	NDW	SDW	TNS	RSE[3]
93	Zembe	*Rhizobium* sp.	12.5	39.39	0.8	8.12	18.3
94	Zembe	*Rhizobium* sp.	10.4	26.95	0.8	8.27	18.3
95*	Zembe	*Bradyrhizobium* sp.	68.3	397.79	4.2	102.18	98.9
96	Zembe	*Bradyrhizobium* sp.	10.3	41.55	0.9	13.87	22.2
97	Zembe	*Bradyrhizobium* sp.	68.8	352.67	3.6	92.65	88.0
99	Zembe	*Rhizobium* sp.	31.4	70.24	1.0	13.14	23.3
100	Zembe	*Rhizobium* sp.	10.3	41.03	1.2	16.53	29.3
Reference strains							
USDA 110	EUA	*B. diazoefficiens*	61.5	408.81	5.4	140.20	127.9
SEMIA 587	Brasil	*Bradyrhizobium elkanii*	59.8	265.06	3.9	84.60	93.9
SEMIA 5019	Brasil	*Bradyrhizobium elkanii*	56.4	513.58	5.0	119.28	118.7
SEMIA 5079	Brasil	*B. japonicum*	73.4	350.91	3.4	89.16	81.5
SEMIA 5080	Brasil	*B. diazoefficiens*	81.6	391.29	3.7	77.31	86.4
Control + N			0.0	0.00	4.3	98.34	100.0
Control - N			0.0	0.00	0.7	4.75	16.7
C.V. (%)			31.1	22.40	20.0	13.14	10.4

[1] Based on www.ncbi.nlm.nih.gov/blast genbank database (section 3.2.4.4).
[2] Means of four replicates.
[3]Expressed as the percentage of shoot dry weight of plants supplied N (Control + N) compared to treatment with inoculant (RUFINI et al., 2014);
Highlighted isolates had better symbiotic efficiency than three (SEMIAs 587, 5079 and 5080) of the four Brazilian reference strains.
Source: This author.

Table S2: Relative efficiency (range, mean and standard deviation) of soybean, variety BRS 133, inoculated with 87 rhizobial isolates from Mozambique, for each BOX – PCR cluster and five reference strains, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110 inoculated on soybean, variety BRS 133, and screened for N_2-fixation in a greenhouse trial in Londrina, Brazil, in 2014.

Clusters[1]	Number of isolates	RE[2] Range (%)	RE Mean (%)	Std deviation (%)
1	2	72.9 – 73.2	73.0	0.2
2	1		129.5	
3	1		88.0	
4	1		98.9	
5	1		18.2	
6	1		96.4	
7	3	21.6 – 76.3	40.0	31.4
8	2	21.1 – 24.7	22.9	2.6
9	1		18.3	
10	1		48.8	
11	2	18.5 – 20.3	19.4	1.3
12	2	15.3 – 26.7	21.0	8.0
13	2	107.3 – 116.8	112.0	6.7
14	4	92.3 –119.8	109.4	12.4
15	15	84.6 – 138.4	120.5	14.9
16	4	107.7 – 139.4	122.9	13.1
17	5	80.4 – 116.9	100.9	15.2
18	2	126.3 – 135.5	130.9	6.5
19	4	19.9 – 60.0	35.0	18.2
20	1		70.4	
21	1		34.9	
22	3	23.2 – 28.5	25.9	2.6
23	1		21.2	
24	1		21.6	
25	3	15.6 – 21.5	18.6	2.9
26	1		20.5	
27	2	31.3 – 53.3	42.3	15.6
28	1		19.3	
29	1		88.9	
30	2	24.1 – 43.6	33.8	13.8
31	1		18.3	
32	2	18.3 – 29.3	23.8	7.8
33	1		64.0	
34	2	18.6 – 51.1	34.9	23.0
35	1		24.3	
36	1		17.2	
37	3	29.4 – 35.1	32.7	3.0
38	2	18.5 – 23.0	20.8	3.2
39	1		19.1	
40	1		22.2	
41	1		23.3	
Reference strains				
B. diazoefficiens USDA 110			127.9	
B. elkanii SEMIA 587			93.9	
B. elkanii SEMIA 5019			118.8	
B. japonicum SEMIA 5079			81.5	
B. diazoefficiens SEMIA 5080			86.4	

[1] Phylogenetic cluster as defined by BOX-PCR analysis (Fig. 1).
[2] Each isolate represented by four replications and RE expressed as the percentage of shoot dry weight of plants supplied with N (Control + N) (RUFINI et al., 2014).
Source: This author.

Table S3: Sowing dates and temperature recorded during soybean growth stages at the trial sites in the 2013/2014 and 2014/2015 crop seasons in Brazil and Mozambique.

Trial site	Sowing date	Temperature (°C) in different soybean growth stages[1]														
		VE	VC	V1	V2	V3	V4	V5	V6	R1	R3	R4	R5	R6	R7	R8
Brazil, crop season 2013/2014																
Londrina	24-Oct-13	22.7	21.5	24.6	21.6	23.3	24.9	23.0	23.3	23.2	24.3	23.7	24.0	27.3	24.1	23.3
Maracaí[2]	23-Oct-13															
Rio Verde	06-Nov-13	23.6	24.1	22.6	23.9	24.4	23.8	23.2	24.3	22.6	23.6	24.0	23.2	22.9	24.3	22.4
Mozambique, crop season 2013/2014																
Muriaze	25-Dec-13	26.8	26.1	26.7	25.6	26.5	27.4	27.1	27.5	27.1	24.9	25.5	26.5	27.1	25.4	23.6
Nkhame[2]	20-Dec-13															
Ntengo	17-Dec-13	22.9	23.9	23.5	23.7	23.5	21.9	24.0	23.0	21.7	23.2	22.5	21.7	23.5	23.2	21.6
Ruace	20-Dec-13	25.2	24.7	24.5	23.9	24.3	24.8	24.8	23.1	25.2	24.4	23.2	24.1	24.9	24.5	23.3
Sussundenga	21-Dec-13	25.8	24.9	24.2	25.5	24.2	25.0	24.2	26.5	25.7	24.7	24.3	25.0	25.0	24.1	21.2
Brazil, crop season 2014/2015																
Londrina	04-Nov-14	23.5	22.0	22.1	23.9	23.2	22.7	23.6	24.6	19.9	24.9	24.5	24.7	23.3	22.7	22.8
Ponta Grossa	18-Nov-14	21.5	21.6	21.4	21.4	21.8	21.8	22.8	22.3	22.6	23.2	22.4	22.2	21.8	21.8	20.7
Mozambique, crop season 2014/2015																
Muriaze	05-Jan-15	25.8	25.8	25.5	26.2	25.8	25.1	26.1	26.0	25.6	26.0	22.9	25.2	26.7	25.7	25.5
Nkhame[2]	23-Dec-14															
Ntengo	22-Dec-14	24.0	22.3	21.9	20.9	21.7	22.0	23.0	22.0	19.3	21.2	23.1	21.8	21.4	23.5	20.9
Ruace	21-Jan-15	24.2	23.8	23.8	24.8	23.5	24.5	24.1	23.9	20.9	24.1	24.7	24.5	23.5	22.4	22.5
Sussundenga	29-Dec-14	23.8	24.7	25.3	24.2	24.7	25.7	26.3	24.7	25.0	24.2	24.1	23.2	25.5	22.0	22.6

[1]As defined by FEHR; CAVINESS (1977). [2]Due to logistic difficulties, temperature data were not recorded at Maracaí and Nkhame.

Table S4: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), total N accumulation in shoots (TNS, mg plant^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, varieties BMX Potência–RR and BRS-359–RR, grown with or without inoculation treatment in the 2013/2014 and 2014/2015 crop seasons, across four trial sites (Londrina, Maracaí, Ponta Grossa and Rio Verde) in Brazil.

Treatment[1]	2013/2014 crop season, across all sites							2014/2015 crop season, across all sites						
	NN	NDW	SDW	TNS	GY	GDW	RE[2]	NN	NDW	SDW	TNS	GY	GDW	RE[2]
NI	18.2^{a} [3]	69.48^{a}	1.5^{c}	44.08^{bc}	2126^{c}	12.0^{b}	84.7^{b}	65.4^{ns} [3]	214.83^{abc}	4.2^{b}	167.88^{cd}	2938^{ns}	14.2^{c}	71.3^{c}
NI+N	13.2^{b}	38.50^{b}	1.8^{a}	58.74^{a}	2538^{a}	12.2^{a}	100.0[4]	52.8	179.42^{c}	6.0^{a}	235.01^{a}	3103	14.6^{a}	100.0[4]
SEMIA 5079	19.0^{a}	71.79^{a}	1.5^{cd}	45.84^{b}	2178^{bc}	12.2^{a}	84.0^{b}	66.5	244.24^{ab}	4.5^{b}	171.34^{cd}	3110	14.2^{bc}	77.0^{bc}
SEMIA 5080	19.7^{a}	67.05^{a}	1.6^{b}	46.83^{b}	2070^{c}	11.8^{cd}	91.6^{a}	61.1	199.79^{bc}	4.2^{b}	153.62^{d}	3117	14.3^{bc}	71.0^{c}
SEMIA 587	17.4^{a}	69.17^{a}	1.4^{cd}	44.63^{bc}	2116^{c}	12.0^{bc}	82.0^{b}	64.1	248.80^{a}	4.5^{b}	184.23^{bcd}	3051	14.3^{b}	75.3^{bc}
SEMIA 5019	18.7^{a}	63.75^{a}	1.4^{cd}	40.84^{cd}	2117^{c}	11.8^{d}	77.9^{bc}	73.8	232.98^{ab}	4.9^{b}	195.24^{bc}	2991	14.1^{c}	84.4^{b}
USDA 110	16.8^{a}	63.90^{a}	1.3^{de}	38.23^{d}	2282^{b}	11.9^{bc}	73.1^{c}	57.5	198.14^{bc}	4.4^{b}	177.11^{cd}	3043	14.1^{c}	76.8^{bc}
5079 + 5080	19.6^{a}	69.77^{a}	1.6^{b}	47.39^{b}	2203^{bc}	12.1^{a}	92.4^{a}	62.7	216.16^{abc}	5.7^{a}	212.81^{ab}	3059	14.4^{b}	95.5^{a}
p - value	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.02	0.00	0.00	0.77	0.00	0.01
C.V. (%)	16.93	17.28	7.70	8.09	5.83	0.93	7.81	18.98	17.30	14.75	16.09	6.58	1.36	13.96

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; 5079 + 5080, inoculated with *B. japonicum* strain SEMIA 5079 and *B. diazoefficiens* strain SEMIA 5080; All rhizobia were applied at the rate of 1.2×10^{6} cells seed^{-1}.
[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).
[3] Means of six replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).
[4] Not included in the statistical analysis.

Table S5: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), above ground biomass (AGB, kg ha^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, variety Storm, grown with or without inoculation treatment in the 2013/2014 and 2014/2015 crop seasons across five trial sites (Muriaze, Nkhame, Ntengo, Ruace and Sussundenga) in Mozambique.

Treatment[1]	2013/2014 crop season, across all sites							2014/2015 Crop season, across all sites						
	NN	NDW	SDW	AGB	GY	GDW	RE[2]	NN	NDW	SDW	AGB	GY	GDW	RE[2]
NI	11.2^{f} [3]	76.24^{f}	20.7^{c}	6689^{bc}	2152^{c}	14.8^{e}	66.4^{c}	13.8^{e}	86.83^{d}	15.5^{e}	3506^{d}	1110^{c}	14.9^{ns}	96.0^{c}
NI+N	6.7^{g}	43.47^{g}	31.7^{a}	6960^{ab}	2581^{a}	15.5^{bc}	100.0[4]	10.6^{f}	53.92^{f}	19.3^{abc}	3866^{c}	1325^{b}	15.8	100.0[4]
SEMIA 5079	17.1^{c}	180.43^{c}	25.3^{b}	6995^{ab}	2481^{a}	15.3^{c}	80.1^{ab}	25.4^{c}	180.34^{c}	20.7^{a}	4073^{bc}	1738^{a}	15.0	118.3^{ab}
SEMIA 5080	25.2^{b}	234.24^{b}	23.7^{bc}	7210^{a}	2606^{a}	15.6^{b}	75.5^{b}	28.7^{b}	199.58^{b}	18.2^{bcd}	4320^{ab}	1455^{b}	15.3	122.6^{a}
SEMIA 587	14.8^{d}	120.40^{e}	25.5^{b}	6576^{c}	2265^{bc}	15.3^{c}	78.5^{b}	26.1^{c}	198.49^{b}	19.7^{ab}	4099^{bc}	1382^{b}	15.0	118.4^{ab}
SEMIA 5019	27.4^{a}	295.45^{a}	27.0^{b}	7029^{ab}	2527^{a}	15.8^{a}	87.8^{a}	34.0^{a}	255.85^{a}	15.1^{e}	4265^{ab}	1374^{b}	15.3	103.9^{bc}
USDA 110	12.8^{e}	114.46^{e}	24.5^{b}	6835^{abc}	2342^{b}	15.0^{d}	75.7^{b}	18.2^{d}	68.98^{e}	16.1^{de}	4200^{ab}	1633^{a}	15.3	99.8^{c}
p - value	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.14	0.04
C.V. (%)	7.98	10.31	12.4	4.78	5.35	1.22	9.81	9.33	6.91	11.50	6.19	8.22	3.14	12.99

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; All rhizobia were applied at the rate of 1.2×10^6 cells seed^{-1}.

[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).

[3] Means of five replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).

[4] Not included in the statistical analysis.

Table S6: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), total N accumulation in shoots (TNS, mg plant^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, varieties BMX Potência–RR and BRS-359–RR, grown with or without inoculation treatment across two crop seasons (2013/2014 and 2014/2015) and four trial sites (Londrina, Maracaí, Ponta Grossa and Rio Verde) in Brazil.

Treatment[1]	NN	NDW	SDW	TNS	GY	GDW	RE[2]
NI	41.8^{ab} [3]	142.16^{ab}	2.9^{b}	105.98^{cd}	2532^{b}	13.1^{cd}	78.0^{b}
NI+N	33.0^{c}	108.96^{c}	3.9^{a}	146.87^{a}	2821^{a}	13.4^{a}	100.0[4]
SEMIA 5079	42.8^{ab}	158.02^{a}	3.0^{b}	108.59^{cd}	2644^{b}	13.2^{bc}	80.5^{b}
SEMIA 5080	40.4^{ab}	133.42^{b}	2.9^{b}	100.22^{d}	2594^{b}	13.1^{cd}	81.3^{b}
SEMIA 587	40.7^{ab}	158.98^{a}	3.0^{b}	114.43^{cd}	2583^{b}	13.2^{bc}	78.6^{b}
SEMIA 5019	46.3^{a}	148.36^{ab}	3.1^{b}	118.04^{bc}	2554^{b}	13.0^{d}	81.1^{b}
USDA 110	37.2^{bc}	131.02^{b}	2.9^{b}	107.67^{cd}	2662^{b}	13.1^{cd}	74.9^{b}
5079 + 5080	41.2^{ab}	142.96^{ab}	3.6^{a}	130.10^{b}	2631^{b}	13.3^{b}	94.0^{a}
p - value	0.03	0.00	0.00	0.00	0.01	0.00	0.00
C.V. (%)	15.28	14.29	11.34	12.86	4.78	0.91	7.77

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; All rhizobia were applied at the rate of 1.2×10^6 cells seed^{-1}.

[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).

[3] Means of five replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).

[4] Not included in the statistical analysis.

Table S7: Nodule number (NN, n° plant^{-1}), nodule dry weight (NDW, mg plant^{-1}), shoot dry weight (SDW, g plant^{-1}), above ground biomass (AGB, kg ha^{-1}), grain yield (GY, kg ha^{-1}), grain dry weight (GDW, g 100 seeds^{-1}) and relative effectiveness (RE, %) of soybean, variety Storm, grown with or without inoculation treatment across two crop seasons (2013/2014 and 2014/2015) and five trial sites (Muriaze, Nkhame, Ntengo, Ruace and Sussundenga) in Mozambique.

Treatment[1]	NN	NDW	SDW	AGB	GY	GDW	RE[2]
NI	12.5^{e} [3]	81.53^{f}	18.1^{d}	5098^{e}	1631^{d}	14.7^{c}	81.2^{c}
NI+N	8.7^{f}	48.69^{g}	25.5^{a}	5413^{bcd}	1984^{b}	15.6^{a}	100.0[4]
SEMIA 5079	21.2^{c}	183.16^{c}	23.0^{b}	5632^{ab}	2109^{a}	15.1^{b}	99.2^{a}
SEMIA 5080	27.0^{b}	216.91^{b}	20.9^{bc}	5765^{a}	2030^{ab}	15.3^{ab}	99.1^{a}
SEMIA 587	20.5^{c}	159.45^{d}	22.6^{bc}	5338^{cde}	1823^{c}	15.1^{b}	98.5^{a}
SEMIA 5019	30.7^{a}	275.65^{a}	21.0^{bc}	5647^{ab}	1951^{b}	15.5^{ab}	95.9^{ab}
USDA 110	15.5^{d}	91.72^{f}	20.3^{cd}	5518^{abc}	1987^{b}	15.2^{ab}	87.7^{bc}
p - value	0.00	0.00	0.00	0.00	0.00	0.01	0.01
C.V. (%)	5.88	6.93	9.19	4.57	4.56	2.27	9.18

[1] NI, No inoculation and no N-fertilizer applied; NI+N, no inoculation with 200 kg of N ha^{-1}, split twice, applied at sowing and R2; SEMIA 5079, inoculated with *B. japonicum* strain SEMIA 5079; SEMIA 5080, inoculated with *B. diazoefficiens* strain SEMIA 5080; SEMIA 587, inoculated with *B. elkanii* strain SEMIA 587; SEMIA 5019, inoculated with *B. elkanii* strain SEMIA 5019; USDA 110, inoculated with *B. diazoefficiens* strain USDA 110; All rhizobia were applied at the rate of 1.2×10^{6} cells seed^{-1}.

[2] Determined as a ratio between the SDW of the inoculant and that of the treatment NI+N (RUFINI et al., 2014).

[3] Means of five replicates and when followed by same letter in the same column are not statistically different ($p \leq 0.10$, Duncan's test).

[4] Not included in the statistical analysis.

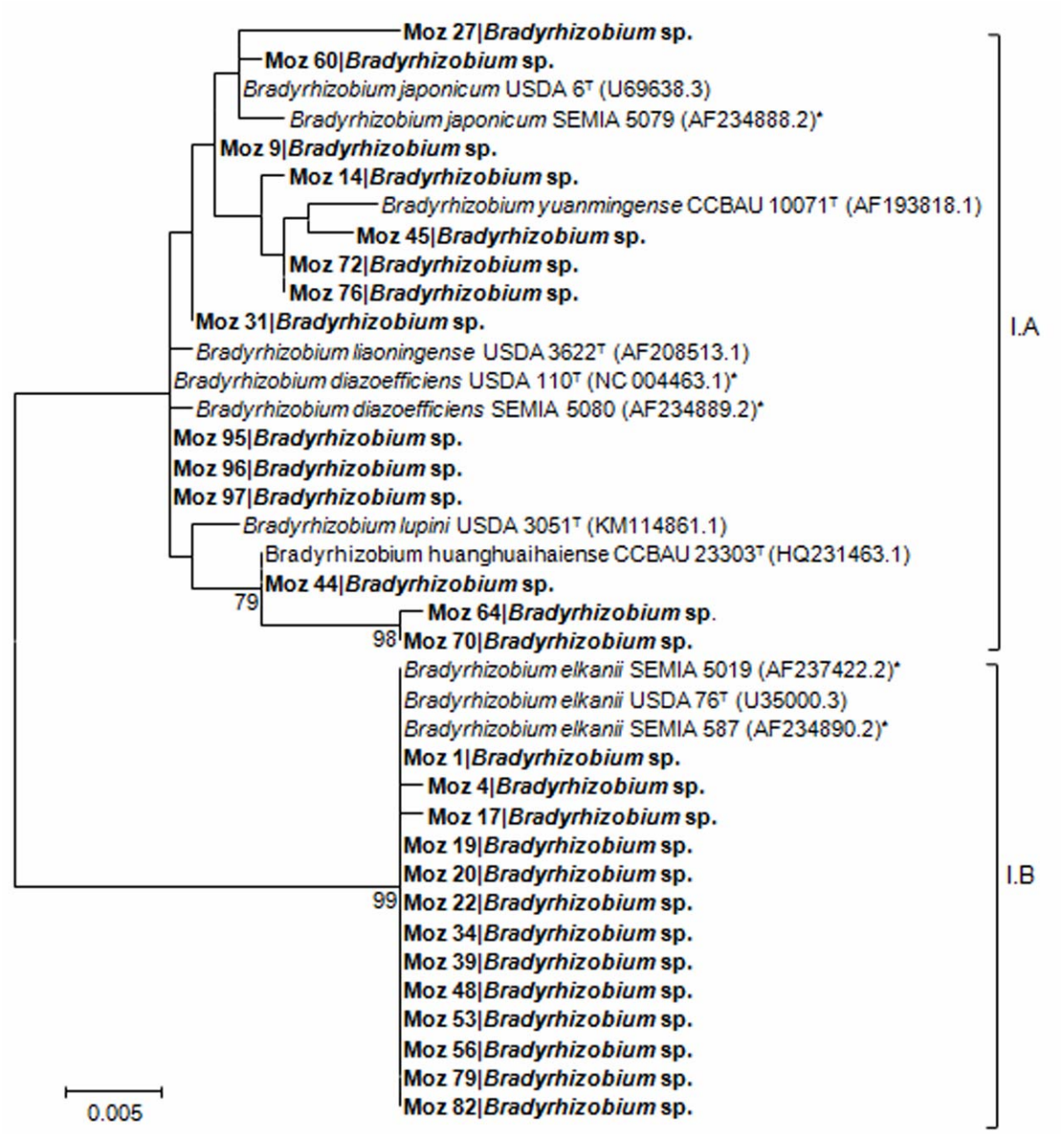

Figure S1: Phylogenetic tree of 16S rRNA gene sequences showing the relationships among representative *Bradyrhizobium* isolates from Mozambique (in bold) with *Bradyrhizobium* type (^{T}) and reference strains used in commercial inoculants, *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110 (with an asterix). The evolutionary history was inferred using the nearest-neighbor-interchange method. Only bootstrap confidence levels > 55% are indicated at the internodes. The scale bar indicates 0.5% nucleotide substitutions. I.A and I.B represent sub-groups *B. japonicum* and *B. elkanii,* respectively.
Source: This author.

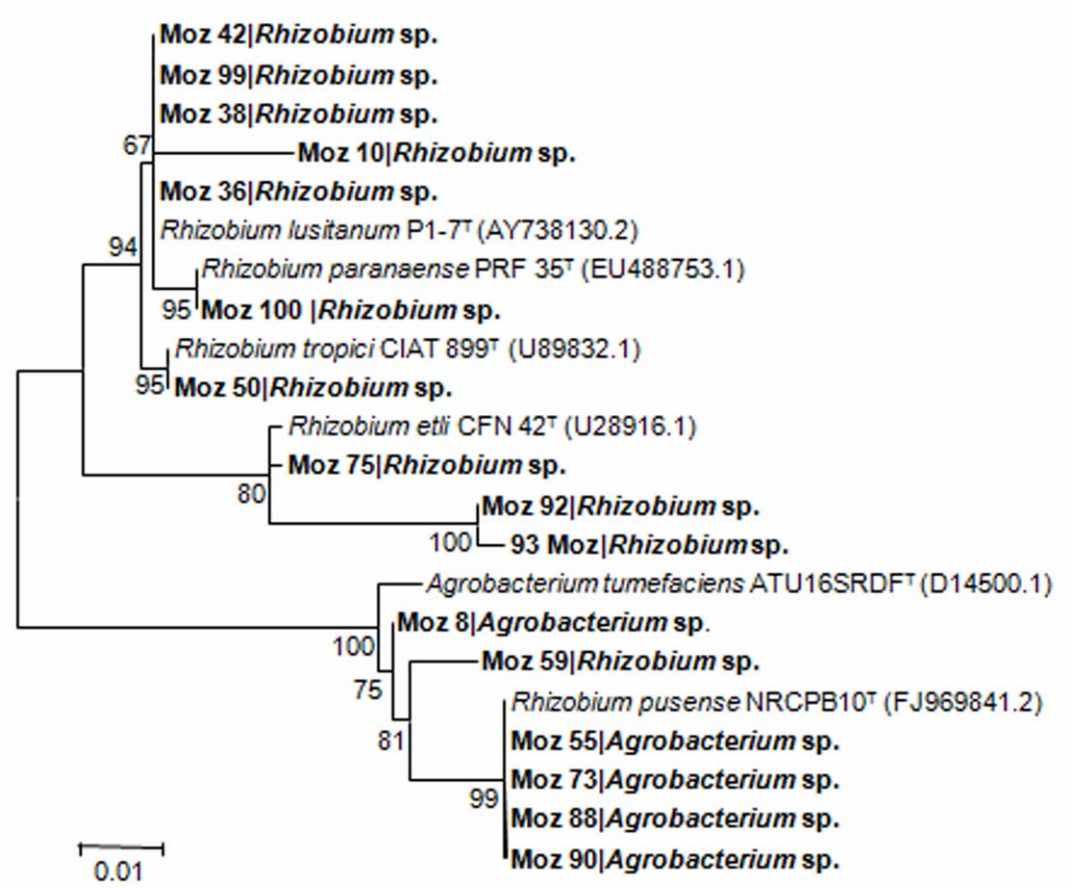

Figure S2: Phylogenetic tree of 16S rRNA gene sequences showing the relationships among representative *Agrobacterium* - *Rhizobium* isolates from Mozambique (in bold and with suggested species names) with *Agrobacterium* - *Rhizobium* type strains (^{T}). The evolutionary history was inferred using the nearest-neighbor-interchange method. Only bootstrap confidence levels > 55% are indicated at the internodes. The scale bar indicates 1% nucleotide substitutions.
Source: This author.

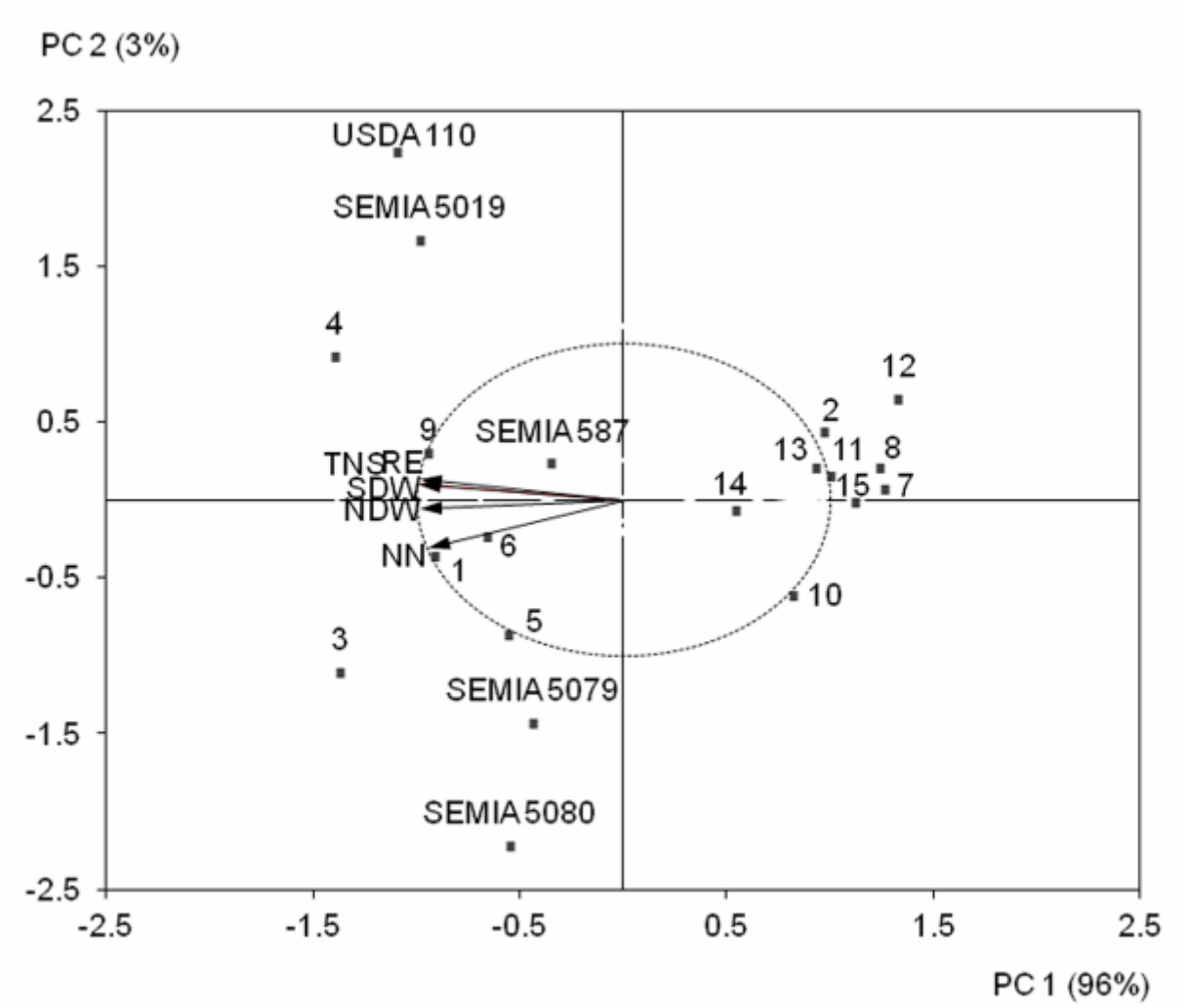

Figure S3: Principal component analysis exploring the relationships among sampling sites as sources of high or poor performing representative isolates. Reference strains *B. elkanii* SEMIA 587 and SEMIA 5019, *B. japonicum* SEMIA 5079, and *B. diazoefficiens* SEMIA 5080 and USDA 110 represent the ideal sources of good performing strains. Sources of isolates and strains are compared considering variables NN and NDW, SDW, TNS and RE. Number represent sampling sites: 1 – $Tengo_1$; 2 – $Tengo_2$; 3 – $Tengo_3$; 4 – $Khame_1$; 5 – $Khame_2$; 6 – $Khame_3$; 7 – $Ruace_1$; 8 – $Ruace_2$; 9 – Mutequelesse; 10 – $Muriaze_1$; 11 – $Muriaze_2$; 12 – $Sussundenga_1$; 13 – $Sussundenga_2$; 14 – $Zembe_1$; 15 – $Zembe_2$.
Source: This author.

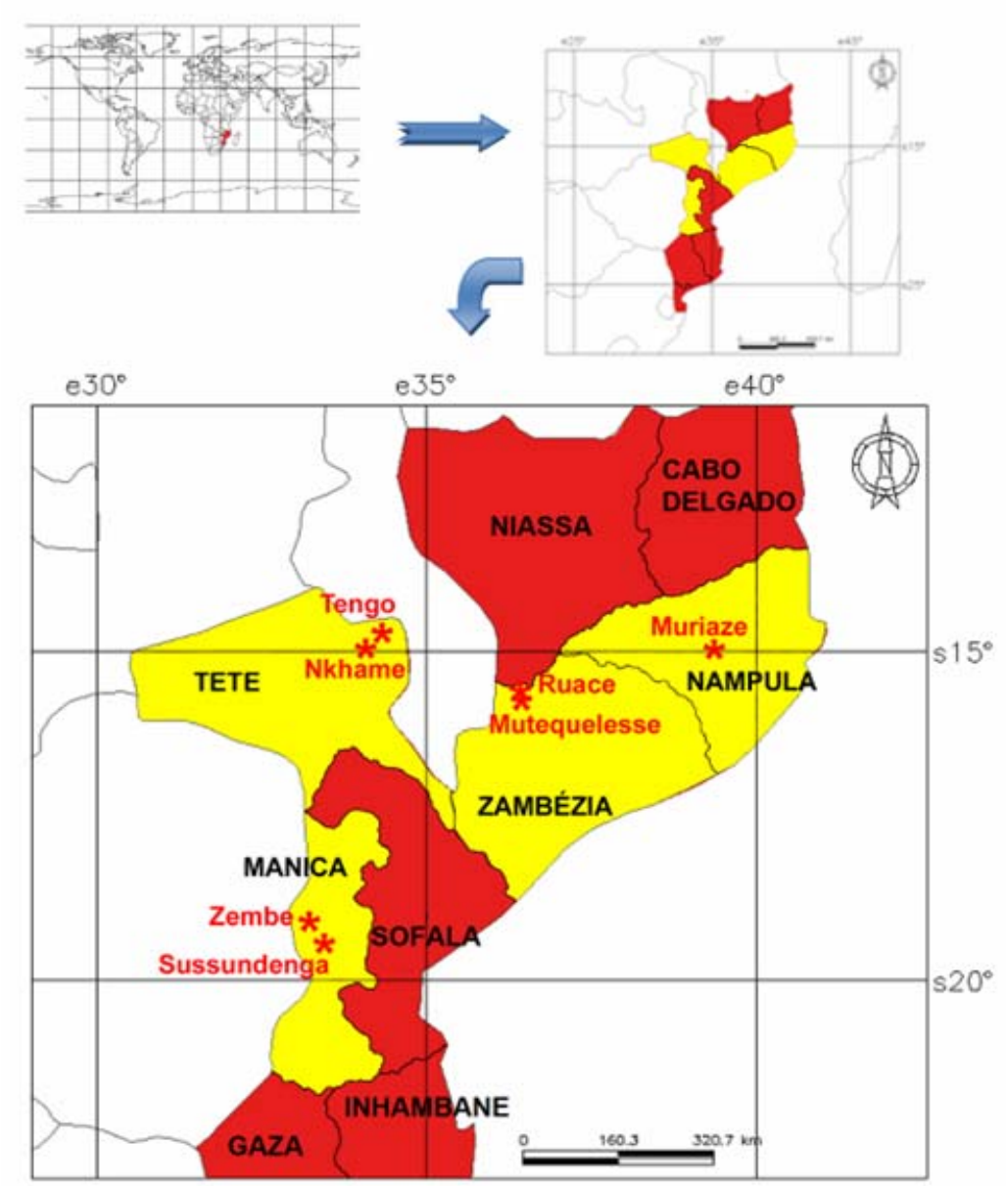

Figure S4: Locations and climates (in brackets) of the sites from where promiscuous soybean nodules were sampled in Manica, Nampula, Tete and Zambézia provinces, which represent the major soybean production area in Mozambique. Map with courtesy from Dr. Osvaldo Coelho Pereira Neto (Universidade Estadual de Londrina).

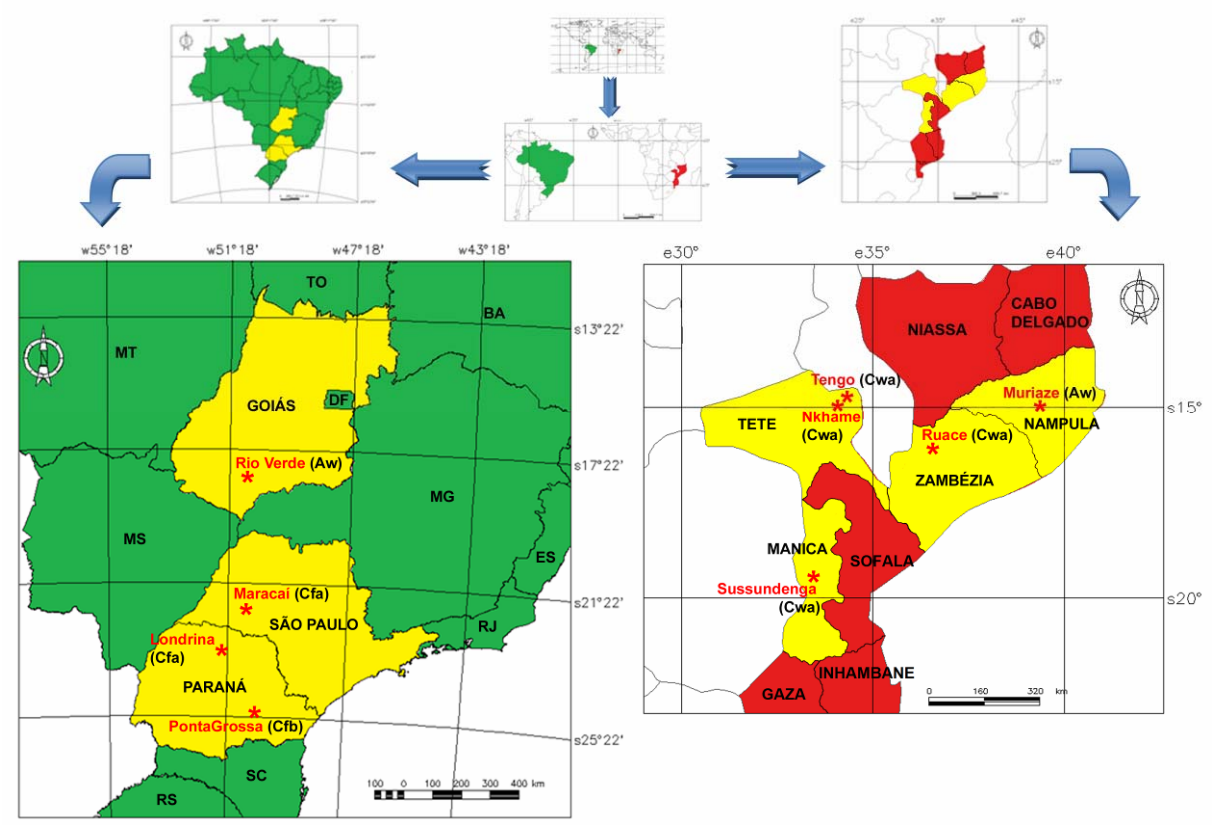

Figure S5: Locations and climates (in brackets) of the trial sites in Brazil (left) and Mozambique (right). Map with courtesy from Dr. Osvaldo Coelho Pereira Neto (Universidade Estadual de Londrina).