Published March, 2012

RESEARCH

Characterization and Effectiveness of Co-inoculation of *Sinorhizobium* Strains on Annual Medics

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ABSTRACT

Selection and identification of better-adapted annual medics with an appropriate rhizobia inoculum is crucial for improved forage quality and yield. The objectives of this study were to characterize 11 rhizobial strains used for an effectiveness study on annual medics and generate an inoculum blend suitable for annual medic species adapted to the southern Great Plains. Each strain was compared to reference rhizobia strains by polymerase chain reaction (PCR) based on repetitive sequences (rep-PCR) and ribosomal gene sequences and analyzed for plant growth promoting (PGP) activities. Genetic characterization separated the strains into two groups belonging to *Sinorhizobium medicae* (seven isolates) and *Sinorhizobium meliloti* (four isolates). Three of the 11 strains tested positive for the PGP activities of indole acetic acid production and phosphate solubilization. A blend of three strains (NFmix3, consisting of WSM1115, 102F85, and M49), of which two were positive for PGP activities, was used in an effectiveness study for the annual medics *Medicago minima* (L.) Bartal., *Medicago orbicularis* (L.) Bartal., *Medicago rigidula* (L.) All., and *Medicago rigiduloides* E. Small. The NFmix3 association with three of the four annual medic species resulted in equivalent biomass production as that of the single specific strain. The NFmix3 blend would be suitable and more economic for use with a mixture of medic species that may provide expansion of the grazing season.

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Abbreviations: DAE, days after emergence; IAA, indole acetic acid; IGS, intergenic spacer; NFmix3, M49, 102F85, and WSM1115; NJ, neighbor joining; PCR, polymerase chain reaction; PGP, plant growth promoting; rDNA, ribosomal DNA; rep-PCR, polymerase chain reaction based on repetitive sequences; SM, simple matching; TrN, Tamura and Nei; UPGMA, unweighted pair-group method using arithmetic average; UTC, untreated control; YEM, yeast extract mannitol.

The genus *Medicago* (Leguminosae) comprises a large number of species of annual herbs and herbaceous perennials (Lesins and Lesins, 1982). Many species of this genus have significant and wide-ranging agricultural and environmental applications (Rochon et al., 2004). Moreover, the annual species collectively known as medics have potential to increase forage production and improve seasonal distribution for pastures of the southern Great Plains (Tivoli et al., 2006). The ability of these species to establish a N-fixing symbiotic relationship with rhizobia makes them excellent candidates for use in sustainable agricultural systems (Howieson et al., 2000).

Establishment of medics in U.S. pasture systems is truly a challenge as different biotic and abiotic stresses can affect both medics and their N-fixing symbiotic partner (Zahran, 1999). Recently, there has been greater emphasis on reducing the application of commercial inorganic fertilizers due to economic and environmental issues. Hence, bioinocula have gained great agricultural value by potentially improving soil fertility and helping to establish medics in diverse environments (Bowman et al., 1998; Graham,

Published in Crop Sci. 52:932–942 (2012).
doi: 10.2135/cropsci2011.08.0459
Freely available online through the author-supported open access option.
Published online 8 Dec. 2011.

1992). Therefore, inoculation of medics with efficient rhizobia has significant economic and ecological impacts. To establish forage legumes in different pasture systems it is important to characterize the rhizobia with optimal N fixation efficiency and the factors influencing their composition and population (Gandee et al., 1999).

The annual medics *Medicago minima* (L.) Bartal, *Medicago orbicularis* (L.) Bartal, *Medicago rigidula* (L.) All., and *Medicago rigiduloides* (E.) Small have gained limited attention and very little information is available regarding their symbiotic association with rhizobia when compared to *Medicago sativa* L. and *Medicago truncatula* Gaertn. However, all four annual medics can sustain low water stress and high grazing pressure (Busso et al., 1998; Francis and Poole, 1973) and have potential to extend the grazing period (Hoveland et al., 1997), therefore making them potential candidates for pasture systems.

Three bacterial species, *Sinorhizobium meliloti*, *Sinorhizobium medicae*, and *Sinorhizobium mongolense*, have been found to form symbiotic associations with the genus *Medicago* (Rome et al., 1996; van Berkum et al., 1998). Preferential relationships occur between *S. meliloti* and tetraploid *Medicago* spp. while *S. medicae* has specificity for diploid species such as many annual medics (Biondi et al., 2003). *Medicago* species can be clustered into three groups depending on their symbiotic partners: (i) species known to establish effective symbioses with both *S. meliloti* and *S. medicae*, such as *M. truncatula*, (ii) species that interact only with *S. medicae*, such as *Medicago polymorpha* L., and (iii) species that interact only with *S. meliloti*, such as *M. rigiduloides* (Brockwell et al., 1988).

A polyphasic approach based on a combination of all the information derived from phenotypic and genotypic data sets is most commonly used to characterize microorganisms (Diouf et al., 2000; Janecka et al., 2002). Phenotypic information is based on the morphology, growth rate (Mateos et al., 2002), utilization patterns of different carbohydrates and amino acids (Wolde-meskel et al., 2004), protein profiling (Liu et al., 2011; Zahran et al., 2003), and fatty acid analysis (Tighe et al., 2000). Genetic information is derived from the complete genome such as polymerase chain reaction (PCR) based on repetitive sequences (rep-PCR) (Bernal and Graham, 2001; Han et al., 2008; Montecchia et al., 2002; Muresu et al., 2005; Yan et al., 2007; Zhang et al., 2010) or analysis of targeted genes such as the RNA operon, *rec*A, *atp*D, and other housekeeping genes (Johnston et al., 2008; Katoh and Toh, 2010; Martir et al., 2007; Ramirez-Bahena et al., 2009; Tian et al., 2011; Wu et al., 2011; Yokoyama et al., 2006).

There is also an interest to assess rhizobia for their plant growth promoting (PGP) potential because this can be important for increasing productivity in cultivated legumes and for large-scale biomass production of legumes and nonlegume mixtures. The PGP properties can include production of plant growth hormones such as auxins (indole acetic acid [IAA]) (Egamberdieva et al., 2010) as well as the ability to solubilize inorganic phosphate present in the soil (Qureshi et al., 2009). An increase in the availability of phosphate can result in more cell replication, effective nodulation, and better N fixation leading to greater biomass production. These types of additional PGP activities might help in the selection of rhizobia as a bioinoculum and in the establishment of more productive pastures (Biswas et al., 2000; Hafeez et al., 2004).

Recently seven annual medics (*Medicago arabica* (L.) Huds., *Medicago lupulina* L., *M. minima*, *M. orbicularis*, *M. polymorpha*, *M. rigidula*, and *M. rigiduloides*) were evaluated for their ability to form effective symbiotic associations with 11 rhizobial stains, WSM1115, M2, 102A13, 102B11, 102H2, WSM540, W118, 102F85, M18, M49, and RR1128, and significant difference in rhizobia specificity to these annual medics was found (Interrante et al., 2011). To identify reasons for the difference in sinorhizobial specificity noted by Interrante et al. (2011), characterization of these 11 *Sinorhizobium* strains was performed using a polyphasic approach. Strains were genotypically characterized by rep-PCR using BOX elements and sequence analysis of 16S and intergenic spacer (IGS) region and assessed for the PGP activities of phosphate solubilization and IAA production. These data were then correlated with nodulation specificity and performance.

MATERIALS AND METHODS

Sinorhizobium Strains and Morphological Characterization

Strains used in this study are listed in Table 1. All strains were maintained on yeast extract mannitol (YEM) agar (10 g mannitol, 0.5 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g NaCl, 0.4 g yeast extract, 3.0 g $CaCO_3$, and 10 g agar L^{-1} [pH 6.8]) (Vincent, 1970) amended with 25 mg L^{-1} Congo red and 10 mg mL^{-1} chloramphenicol (Graham, 1969). Cultures were incubated at 28°C for 2 to 3 d. Selective media (YEM agar amended with 25 mg L^{-1} bromothymol blue) was used for the initial characterization of rhizobia in which fast growers result in yellow colonies and slow growers are blue (Rathore et al., 2009). Fast-growing yellow colonies were selected and restreaked 4 to 5 times on YEM agar amended with 25 mg L^{-1} Congo red dye to differentiate between rhizobia (white colonies) and *Agrobacterium* spp. (red colonies). Rhizobial strains were characterized as fast-growing gram-negative rods. All pure strains were stored at –80°C in YEM broth containing 50% (v/v) glycerol.

Plant Growth Promoting Activity of Strains

Phosphate Solubilization

Log phase bacterial cultures grown in YEM were spot inoculated on to Pikovaskaya's agar (0.5 g yeast extract, 10 g dextrose, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 100 μg $MnSO_4 \cdot H_2O$, 100 μg $FeSO_4 \cdot 7H_2O$, and agar 10 g L^{-1}

Table 1. Rhizobia strains, original host, site of origin, proposed taxonomic position, and plant growth promoting activity.

Strain identification†	Original host	Source of strains	Proposed taxonomic position	Phosphate solubilization‡	Indole acetic acid production†
102A13	*Medicago arabica*	Costa Rica	*Sinorhizobium medicae*	+	–
102B11	*Medicago polymorpha*	Florida, U.S.	*S. medicae*	+	+
102H2	*Medicago rugosa* Desr.	Tunisia	*S. medicae*	–	+
WSM540	*M. polymorpha*	Italy	*S. medicae*	+	–
W118	*M. rugosa*	Australia	*S. medicae*	–	+
WSM1115§	*M. polymorpha*	Greece	*S. medicae*	+	+
M2	*Medicago lupulina*	United States	*S. medicae*	–	+
M18	*Medicago rigidula*	Syria	*Sinorhizobium meliloti*	–	–
102F85§	*Medicago sativa*	Canada	*S. meliloti*	+	+
M49§	*M. rigidula*	Syria	*S. meliloti*	–	–
RR1128	*Medicago truncatula*	Australia	*S. meliloti*	–	–

†The strains 102A13, 102B11, 102H2, WSM540, and W118 are blended together in the Nitragin N inocula (EMD Crop Bioscience, Novozymes, Brookfield, WI). Strain 102F85 is commercially available as Nitragin A. Strains WSM1115, M2, and M49 are available by Plant Probiotics (Indianapolis, IN). M18 is an experimental strain and RR1128 is a commercial strain for alfalfa received from Australia by R. Ballard in 2010. Basic information about the strains was obtained by J. Kosamke, EMD Crop Bioscience, R. Ballard, South Australian Research and Development Institute (Adelaide, SA), and T. Wacek, Plant Probiotics (Indianapolis, IN) (personal communication, 2010).

‡+ indicates positive for phosphate solubilization or indole acetic acid production; – indicates no phosphate solubilization or indole acetic acid production.

§Strains in bold were used for the NFmix3 blend (WSM1115, 102F85, and M49) to test the interaction of blending strains.

[pH 6.8]) (Nautiyal, 1999) in triplicate and incubated at 28°C for 72 h. Strains positive for phosphate solubilization were identified by formation of a clear zone around the colonies and scored as positive (Nautiyal, 1999).

Indole Acetic Acid Production

To test for IAA production, each strain was spot inoculated onto YEM agar plates supplemented with 0.1% tryptophan and overlaid with 82 mm diameter nitrocellulose membrane and then incubated at 28°C for 2 d. Following incubation the nitrocellulose membrane, including rhizobial colonies, was soaked in approximately 15 mL of Salkowski reagent (2% 0.5 M $FeCl_3$ in 35% perchloric acid) (Srinivasan et al., 1996). The reaction was allowed to proceed at room temperature in the dark until adequate color developed (typically within 1 h of incubation). Strains positive for IAA production were identified by the formation of a characteristic red halo within the membrane and recorded as positive (+).

Genotypic Characterization of *Sinorhizobium* Strains

Rhizobial DNA was extracted by colony PCR (Fukui and Sawabe, 2007). Cultures were grown on YEM agar plates for 24 h after which a single colony was picked and resuspended in 50 μL of sterilized water and then incubated at 95°C for 5 min. Samples were spun in a centrifuge for 2 min at high speed and 2 μL of supernatant was used for each PCR reaction. Polymerase chain reaction was performed in 5x PCR buffer (GoTaq, Promega, Madison, WI) containing $MgCl_2$, 0.2 mM of each deoxyribonucleotide triphosphate (dNTP), 1 μM of each primer, and 1 U of GoTaq polymerase (Promega).

All strains were subjected to rep-PCR genomic fingerprinting using the BOXA1R (GATCGGCAAGGCGACGCTGACG) primer corresponding to BOX elements (Versalovic et al., 1994). The reaction conditions were as follows: 95°C denaturation for 7 min followed by 39 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 6 min.

Primers fD1 (CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG) and rD1 (CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC) were used to amplify 16S ribosomal DNA (rDNA) region (Weisburg et al., 1991), and IGS11F (TGCGGCTGGATCACCTCCTT) and IGS12R (CCGGGTTTCCCCATTCGG) were used to amplify the IGS region (Weisburg et al., 1991). The reaction conditions for 16S rDNA amplification were as follows: initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 54°C for 40 s, 72°C for 90 s, and a final extension of 72°C for 7 min. The IGS amplification conditions were initial denaturation at 95°C for 7 min followed by 35 cycles of 94°C for 1 min, 47°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min.

The PCR products representing the BOX elements were separated on 1.5% agarose gel and 16S and IGS PCR products were separated on a 0.7% agarose gel for 45 min at 90 V followed by staining with ethidium bromide and viewed by a geldoc bioimaging system (Gel Doc-it 300, UVP Bioimaging Systems, Upland, CA). Amplified fragments for both regions (16S and IGS) were purified by using QIAquick PCR purification kit (Qiagen, Valencia, CA) and then sequenced using BigDye terminator 3.1 standard procedure. Sequencing reactions were purified with Agencourt CleanSEQ dye-terminator removal system (Beckman Coulter Inc., Brea, California) following the manufacturer's protocol with Biomek FXP (Beckman Coulter Inc., Brea, CA). Purified sequencing samples were analyzed with the 3730 Xl DNA analyzer (Applied Biosystems Inc., Foster City, CA) following the manufacturer's protocol. The GenBank accession numbers (Benson et al., 2011) for 16S and IGS are from HQ143387 to HQ143389, HQ143391, HQ143399 to HQ143404, and HQ143407 to HQ143411.

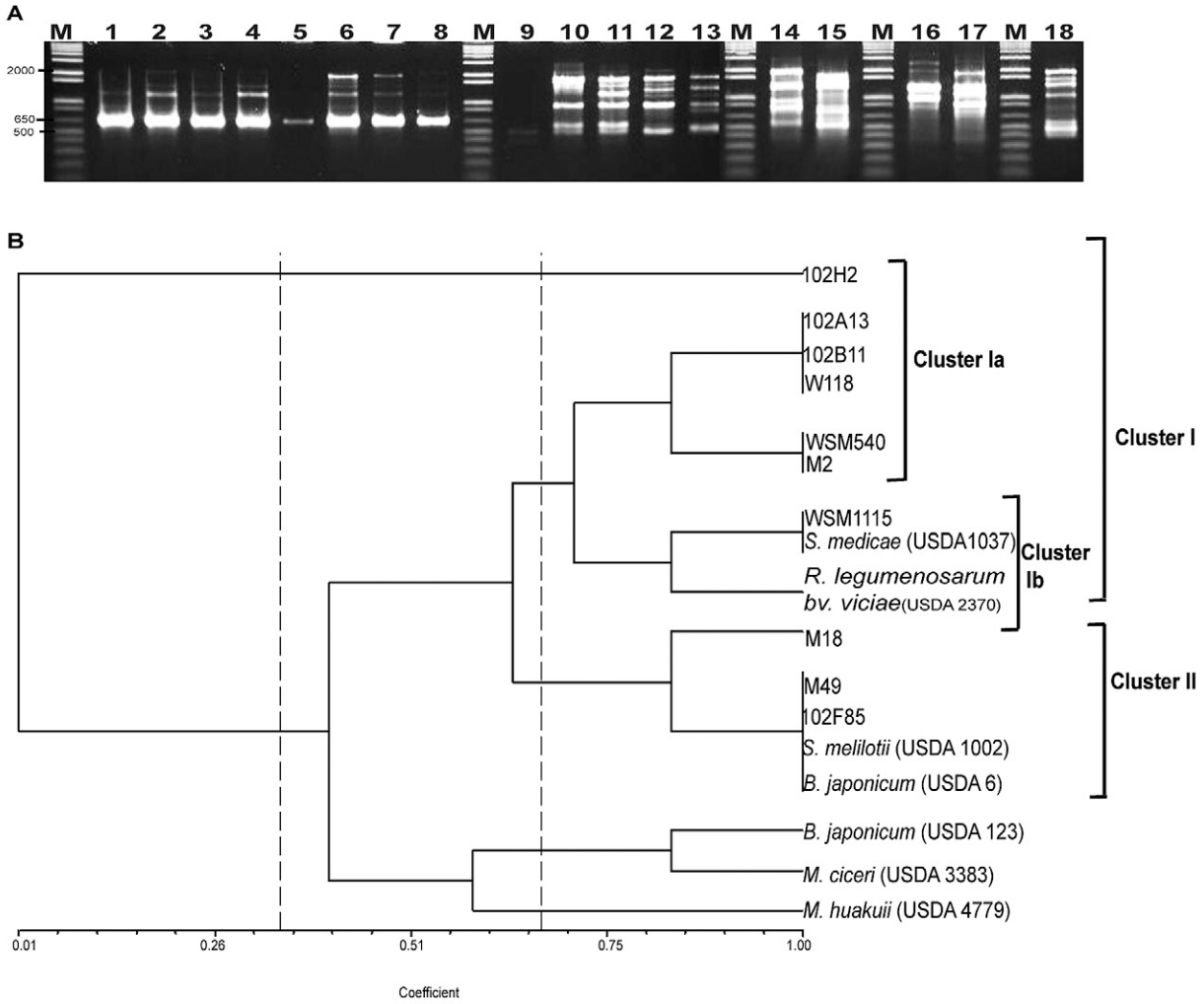

Figure 1. Polymerase chain reaction (PCR) based on repetitive sequences (rep-PCR) analysis of rhizobial strains. (A) BOXA1R-PCR fingerprinting of rhizobial strains used as bioinocula along with related species from the genus *Rhizobium*. M) Marker, 1Kb Plus DNA Ladder (Invitrogen, Life Technologies, Grand Island, NY). 1) 102H2, 2) 102A13, 3) 102B11, 4) WSM540, 5) W118, 6) M2, 7) WSM1115, 8) *Sinorhizobium medicae* USDA 1037, 9) RR1128, 10) M18, 11) M49, 12) 102F85, 13) *Sinorhizobium meliloti* USDA 1002, 14*)* *Bradyrhizobium japonicum* USDA 6, 15) *Bradyrhizobium japonicum* USDA 123, 16) *Mesorhizobium ciceri* USDA 3383, 17) *Mesorhizobium huakuii* USDA4779, and 18) *Rhizobium legumenosarum* bv. *viciae* USDA 2370. (B) Cluster analysis using simple matching (SM) coefficient and unweighted pair-group method using arithmetic average (UPGMA) clustering method of BOXA1R-PCR fingerprinting showing diversity of rhizobial strains. The scale bar represents similarity coefficient.

Phylogenetic Analysis for Identification

The banding pattern of rep-PCR was analyzed by NTsys software (Rohlf, 1993) and the phenetic tree (Fig. 1B) constructed using simple matching (SM) similarity coefficient and unweighted pair-group method using arithmetic average (UPGMA) clustering parameters.

Sequences of 16S and IGS regions were manually edited in Sequencher 4.10.1 (Gene Codes, 2011). An alignment was made using MAFFT, a multiple sequence alignment program (Katoh and Toh, 2010). Aligned sequences were verified manually and were edited with Geneious Pro. 5.1.0 Software (Drummond et al., 2011). The relationship between strains and other members of Rhizobiacae family was based on partial 16S and IGS sequence data sets. These two gene data sets were analyzed independently using MODELTEST (Posada, 2008) to determine the best-fit substitution model. The optimal model defined by MODELTEST was used to determine the genetic distances for neighbor joining (NJ) analysis using the Phylogenetic Analysis Using Parsimony program (PAUP version 3.1) (Swofford, 1993) for both 16S and IGS data sets. The analysis was performed using the heuristic search algorithm holding 10 trees at each step and branch swapping on all trees using the steepest descent option.

Effect of Co-inoculation of *Sinorhizobium* Strains on Annual Medics

A greenhouse trial was conducted to compare the effectiveness of single and co-inoculation of *S. medicae* and *S. meliloti* with the annual medics *M. minima* 'Devine', *M. orbicularis* 'Estes', *M. rigidula* PI 495552, and *M. rigiduloides* PI 227850 in 2009 and 2010 at the Samuel Roberts Noble Foundation in Ardmore, OK (34°19′ N, 97°08′ W). Selection of the rhizobia strains and annual medic varieties was made on the basis of strain identification and the results obtained by Interrante et al. (2011). The greenhouse conditions were maintained at approximately

20°C, 45% relative humidity, and 700 W m^{-2} irradiance with a photoperiod of 16 h light and 8 h dark. The experimental design was a factorial combination of five rhizobium inoculum treatments, M49, 102F85, WSM1115, co-inoculum NFmix3 (a 1:1:1 ratio by volume of M49, 102F85, and WSM1115), and untreated control (UTC) with four annual medic species (n = 20) arranged in three replications of a randomized complete block design. The inoculants M49, 102F85, WSM1115, and NFmix3 were evaluated for their effectiveness against the four annual medic species. Information about the strains used for inoculation is listed in Table 1.

Seeds were surface sterilized in 95% (v/v) ethanol (15 min) and 0.2% $HgCl_2$ (v/v) for 60 min and then rinsed thoroughly with sterile water. Twenty seeds of a single species were planted per pot and grown in 1.9-L bottom-watered pots (Apollo Plastics Ltd., Mississauga, ON) containing 1:1 ratio by volume of washed and autoclaved masonry sand and vermiculite as described by Interrante et al. (2011). Inoculant strains were cultured in YEM broth for 4 d at 28°C. The number of cells per milliliter was counted by the most probable number for viable cell count and maintained at approximately 10^9 cells mL^{-1}. Each strain of the NFmix3 was grown separately and maintained at 10^9 cells mL^{-1} and then an equal volume of each strain was mixed at the time of inoculation. The rhizobium treatment was applied by adding 50 mL suspension of the appropriate treatment of rhizobial culture to each pot. Untreated control pots were inoculated with 50 mL of sterilized water, and all pots were watered with 350 mL of sterilized water after planting. Growth of the medics was limited by N deficiency except when they were effectively nodulated. All plants were given regular N-free nutrient and sterile water as required (McKnight, 1949). Pots were thinned to 10 plants per pot approximately 7 d after emergence (DAE). The pot surfaces were covered with a thin layer of autoclaved heat-treated montmorillonite clay (Profile Products LLC, Buffalo Grove, IL) when seedlings were approximately 3 cm tall to minimize surface rhizobial contamination. Plant shoots and roots were excised 45 DAE, carefully washed, assessed for nodule number and color, dried for 2 d at 60°C, and then weighed.

Rhizobial Effectiveness Index

The rhizobium effectiveness index was calculated as described by Interrante et al. (2011). Plants were visually scored on a 1 to 5 color scale: 1 represents purple (ineffective nodulation) and 5 represents dark green (very effective nodulation). The number of nodules per plant was counted and nodules were scored as 1 represents pink (effective nodules), 0 represents no nodules, and –1 represents white (ineffective nodules). Effectiveness index was calculated as plant color × number of nodule × nodule color.

Statistical Analysis

Experiments were analyzed using PROC MIXED of SAS (SAS Institute, 2002). Medic species, rhizobium inoculum, and their interaction were considered as fixed effects and year, replicate, and their interaction were treated as random effects. Medic species was included in the model as a subplot treatment in a split-plot arrangement, with the rhizobium inoculum treatment being the main plot. Significance was determined at $p \leq 0.05$, and the PDIFF function of the LSMEANS procedure was used to compare treatment means.

RESULTS

Eleven rhizobial strains were characterized by rep-PCR using BOX profiling. The rep-PCR yielded multiple fragments (1–7 per stain) ranging in size from 500 to 1650 bp. (Fig. 1A). According to the BOX profiles, two different banding profiles emerged grouping seven strains, 102A13, 102H2, M2, 102B11, WSM1115, WSM540, and W118, and three strains, M49, 102F85, and M18. The RR1128 strain did not effectively amplify with the BOX1A primer and was therefore excluded from further rep-PCR analysis. The dendrogram obtained by numerical analysis of the distance matrix was based on presence (1) or absence (0) of bands and UPGMA clustering was performed using the SM coefficient. All strains, including the standard *Sinorhizobium* strains, divided into two major clusters (Fig. 1B). Strains 102A13, 102B11, and W118 grouped together with 100% similarity and WSM540 and M2 grouped together in cluster Ia while WSM1115 shared 100% similarity with the standard strain of *S. medicae* (USDA1037) and formed cluster Ib. Cluster Ia and Ib showed more that 70% similarity among them on the coefficient scale. Different strains of the same species usually show similarity greater than 70% in BOX PCR analysis (Binde et al., 2009). Strains 102F85 and M49 grouped with the USDA1002 reference strain of *S. meliloti* with 100% similarity. Strain M18 was intermediate between the *S. medicae* and *S. meliloti* cluster and shared 60% similarity to both clades.

A NJ phylogenetic tree (Fig. 2A) was constructed with 16S rDNA sequences using distance setting from best-fit model of DNA substitution Tamura and Nei (TrN) (Tamura and Nei, 1993) as determined by MODELTEST (Posada, 2008). Bootstrap confidence intervals of the various clusters NJ tree were estimates from 1000 replications. Figure 2A shows the phylogenetic tree obtained with the 16S rDNA aligned sequences of the 11 rhizobial strains as well as type and reference rhizobial strains using *Bradyrhizobium japonicum* strain (USDA6) as an outgroup. Results based on 16S rDNA sequences indicate that seven strains (WSM540, 102A13, 102B11, 102H2, M2, WSM1115, and W118) grouped with the taxonomic position of *S. medicae* (bootstrap value of 68.5%) while three strains (RR1128, M49, and 102F85) grouped with *S. meliloti* (bootstrap value of 61.6%). M18 was separated in the lineage with *S. meliloti* and supported by a bootstrap value of 84.6%. The relationship of each strain was also determined using IGS. All strains produced a single band of approximately 850 bp. In the IGS phylogenetic tree, the strains were compared to IGS sequences of five different *Sinorhizobium* spp. along with other members of *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium* group using *B. japonicum* (USDA6) as an outgroup. The NJ phylogenetic tree was constructed with settings from the best-fit model TrN selected by Akaike information criterion (AIC) in MODELTEST (Posada, 2008) (Fig. 2B). Bootstrap confidence intervals

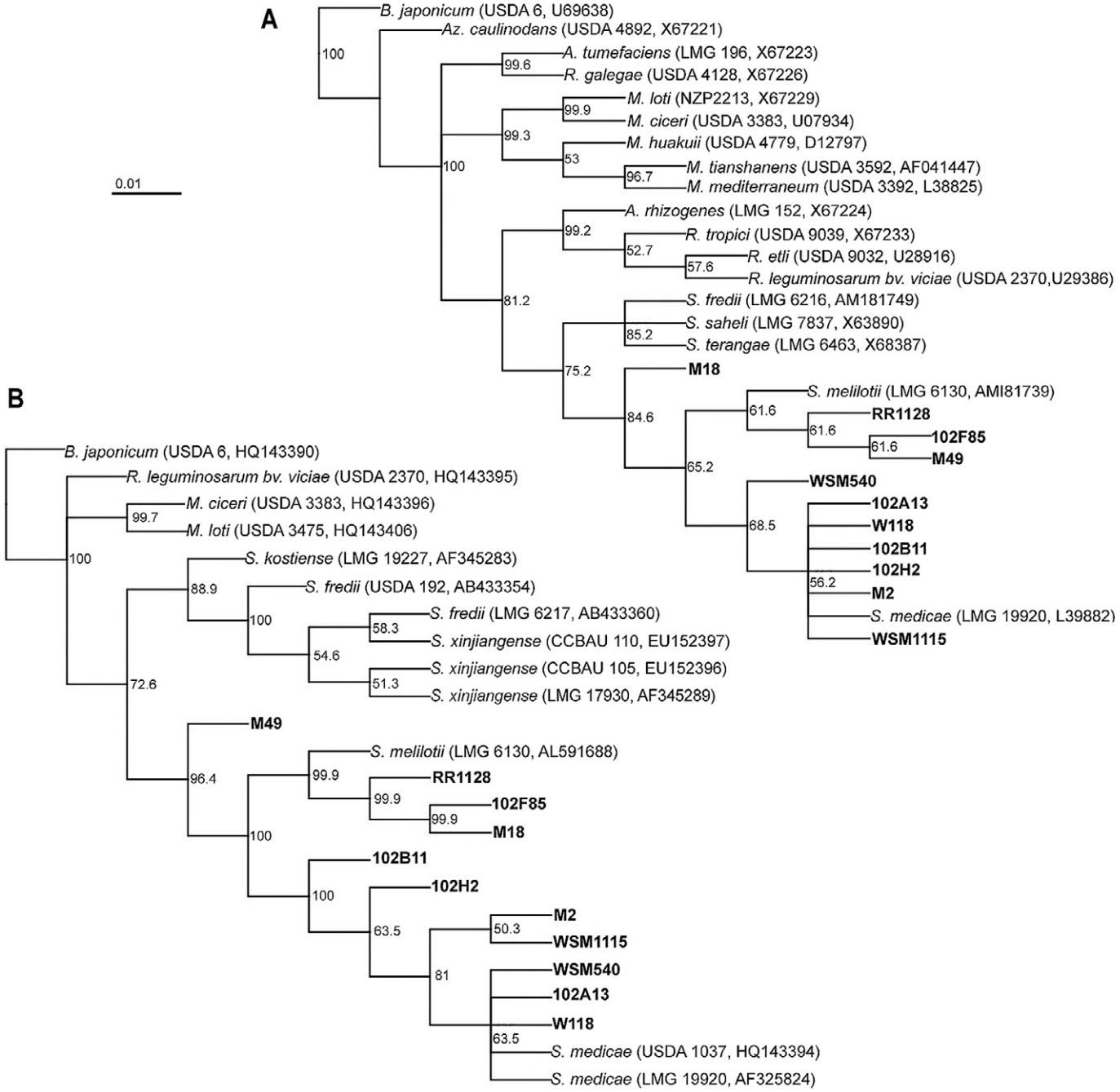

Figure 2. Neighbor joining phylogenetic tree based on 16S and the intergenic spacer (IGS) region between the 16S and 23s ribosomal DNA (rDNA) genes. (A) Phylogenetic tree based on 16S gene sequences. (B) Phylogenetic tree based on IGS sequences showing the position of different strains compared with those of related species of genus *Rhizobium*. Bioinoculum isolates are in bold. Bootstrap values calculated for 1000 replications are indicated on branch nodes. The bar represents one nucleotide substitution per 100 nucleotides. *Bradyrhizobium japonicum*, *Azorhizobium caulinodans*, *Agrobacterium tumefaciens*, *Rhizobium galegae*, *Mesorhizobium loti*, *Mesorhizobium ciceri*, *Mesorhizobium haukuii*, *Mesorhizobium tianshanens*, *Mesorhizobium mediterraneum*, *Agrobacterium rhizogenes*, *Rhizobium tropici*, *Rhizobium etli*, *Rhizobium leguminosarum* bv. *viciae*, *Sinorhizobium fredii*, *Sinorhizobium saheli*, *Sinorhizobium terangae*, *Sinorhizobium melilotii*, *Sinorhizobium medicae*, *Sinorhizobium kostiense*, and *Sinorhizobium xinjiangense*.

of the various clusters NJ tree were estimated from 1000 replications with values greater than 70% shown in Fig. 2B. The IGS tree was supported by stronger bootstrap values than the 16S rRNA tree. The *S. meliloti* strain is strongly grouped with 102F85, RR1128, and M18 supported by 99.9% bootstrap value while M49 is separated with 96.4% bootstrap support. W118, 102A13, 102B11, 102H2, WSM540, M2, and WSM1115 grouped broadly in a cluster with the standard *S. medicae* strain. The three strains 102A13, 102B11, and 102H2 were found to be genetically diverse with a relationship showing more than 80% bootstrap support.

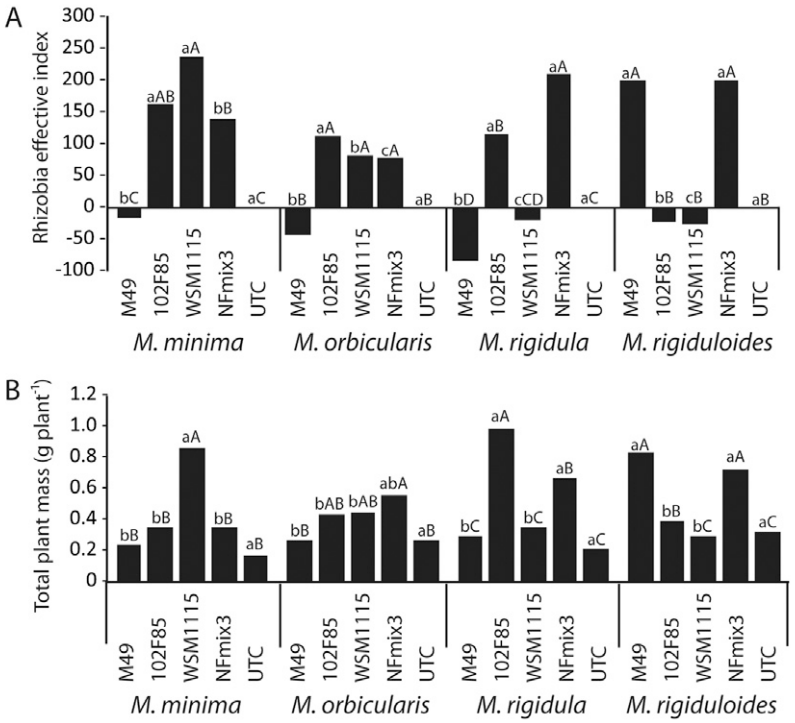

Figure 3. Effectiveness study comparing single specific Rhizobial strains with the NFmix3 blend (WSM1115, 102F85, and M49) on the annual medics *Medicago minima*, *Medicago orbicularis*, *Medicago rigidula*, and *Medicago rigiduloides*. (A) Rhizobia effective index as affected by medic species × rhizobium interaction ($p < 0.0001$) at 45 d after emergence. SE = 31.6 of the interaction mean. (B) Comparison of total plant dry biomass (g per plant) as affected by medic species × rhizobium interaction ($p < 0.0001$) at 45 d after emergence. SE = 0.13 of the interaction mean. Means followed by the same letter above the bar do not differ by the least squares means test ($p > 0.05$) whereby lowercase letters are within each rhizobial treatment and uppercase letters are within each plant species. UTC, untreated control.

The 11 strains were also examined for their PGP activity of phosphate solubilization and IAA production (Table 1). Five strains, 102A13, 102B11, WSM540, WSM1115, and 102F85, were able to solubilize phosphate and six strains, 102H2, WSM540, W118, WSM1115, M2, and 102F85, were able to produce IAA. Only strains 102B11, WSM1115, and 102F85 were positive for both phosphate solubilization and IAA production while M49, RR1128, and M18 were not able to solubilize phosphate or produce IAA (Table 1).

The previous study by Interrante et al. (2011) found positive interactions between the entries *M. minima* and *M. orbicularis* with both WSM1115 and 102F85 while *M. rigidula* and *M. rigiduloides* were effectively nodulated by 102F85 and M49, respectively. Therefore, a greenhouse experiment was conducted to check the effect of co-inoculation of the effective strains (NFmix3) on biomass production of annual medics with the aim to produce one effective bioinoculum for these four annual medics.

To test for biomass production and rhizobium effectiveness, data was combined across both years and the medics × rhizobia interactions are presented in Fig. 3A and 3B. Comparative data of dry weight and effectiveness index provided strong evidence for selective nodulation by annual medics. These results are in accordance with the finding of Interrante et al. (2011) whereby total plant dry biomass followed the same trends as was observed by rhizobia effectiveness index (Fig. 3A and 3B).

Medicago minima was effectively nodulated by WSM1115, 102F85, and NFmix3 as indicated by rhizobia effective index (Fig. 3A). The interaction between *S. medicae* strain WSM1115 and *M. minima* resulted in the formation of effective nodules and produced 70% more total biomass than the UTC (Fig. 3B). There was no significant difference between the biomass production when inoculated with NFmix3 and 102F85 whereby both produced 19% more total biomass than the UTC (Fig. 3B) but were significantly less than WSM1115. There was no significant difference in total plant dry biomass production of M49 and the UTC (Fig. 3B).

102F85, WSM1115, and NFmix3 were equally effective at nodulating *M. orbicularis* as indicated by rhizobium effectiveness index (Fig. 3A). However, the NFmix3 interaction with *M. orbicularis* produced 25% more total biomass than UTC and 10% more than single inocula WSM1115 and 102F85 (Fig. 3B). Strain M49 had a negative rhizobium effectiveness index and therefore the biomass production performed similarly to the UTC.

Medicago rigidula was effectively nodulated by NFmix3 followed by 102F85 while WSM1115 and M49 did not form effective nodules as indicated by rhizobium effectiveness index (Fig. 3A). Although the rhizobia effective

index showed that NFmix3 formed significantly more effective nodules than 102F85, the total biomass production was less than 102F85 but was significantly greater than M49, WSM1115, and UTC. There was no significant difference found for biomass production between M49 and the UTC (Fig. 3B).

Medicago rigiduloides was effectively nodulated by both M49 and NFmix3 (Fig. 3A), in which both produced 45% more total biomass when compared to the UTC and WSM1115 (Fig. 3B). No significant difference was observed in biomass production with WSM1115 inoculated *M. rigiduloides* as compared to the UTC.

The NFmix3 performed as effectively for *M. orbicularis* and *M. rigiduloides* when compared to the most effective isolate as reported by Interrante et al. (2011). However, NFmix3 was not as effective on *M. minima* as the single specific strain WSM1115 but performed equally as well as 102F85, which was reported as the second most effective strain by Interrante et al. (2011). While NFmix3 was more effective on *M. rigidula* based on rhizobia effective index, 102F85 produced more total biomass.

DISCUSSION

The polyphasic approach used to characterize the rhizobium strains showed there was considerable diversity among the 11 rhizobia strains used for bioinoculation of the annual medic species *M. minima*, *M. orbicularis*, *M. rigidula*, and *M. rigiduloides*. The rhizobia inoculants that differed in their specificity on medic species were genetically different and appeared to belong to different *Sinorhizobium* species.

Interspersed repetitive DNA sequences, such as the BOX elements, serve as primer binding sites separated by varying distances in the bacterial chromosome (Koeuth et al., 1995) and therefore, BOX profiling can be a useful tool to estimate the intraspecific genetic diversity of strains. Analysis of rep-PCR using BOXA1R indicated genetic diversity among the tested strains. These findings are in agreement with the results obtained by other groups (Binde et al., 2009; Gao et al., 2001; Lu et al., 2009; Zhang et al., 2010) in that rep-PCR is a reliable tool that can distinguish between closely related strains, even those belonging to the same species.

Although rep-PCR is a powerful means of fingerprinting and can detect high genetic diversity, it is not always sufficient for species identification (Laguerre et al., 1997). The RNA operon-based phylogeny is important for identification of closely related strains (Hoque et al., 2011; Wolde-meskel et al., 2005). Therefore, 16S rDNA and IGS were used to identify strains at the species level by comparison with reference strains. Ribonucleic acid operon-based sequencing separated M49 into a lineage with the *S. meliloti* group. Soil origin and plant genotype are known to influence diversity of *Sinorhizobium* species (Gao et al., 2001; Wu et al., 2011; Zribi et al., 2005). The strains WSM1115, M2, 102A13, 102B11, and 102H2 were originally isolated from different *Medicago* species, and while they all grouped with *S. medicae*, there was diversity among them. Similar results have been reported by (Paffetti et al., 1998) whereby plant genotype is a major factor influencing the genetic structure of rhizobial populations.

Compatible plant and rhizobia associations result in better N fixation and ultimately more biomass production (Robin et al., 2005; Zhu et al., 1998; Zribi et al., 2005). The medic × rhizobia interaction study confirmed the same symbiotic pattern as shown by Interrante et al. (2011) and Zhu et al. (1998). *Medicago minima* and *M. orbicularis* formed effective associations with both *S. medicae* (WSM1115) and *S. meliloti* (102F85) strains, which is consistent with reports that both *S. medicae* and *S. meliloti* have been isolated from *M. minima* and *M. orbicularis* growing in different geographical locations (Rome et al., 1996; Sebbane et al., 2006). *Medicago rigidula* and *M. rigiduloides* were found to only form effective associations with *S. meliloti* strains 102F85 and M49, respectively. While *M. rigidula* and *M. rigiduloides* are known to have specific symbiotic associations with *S. meliloti*, these associations are always found to be independent of each other (Ballard and Charman, 2000; Bena et al., 2005).

Plant growth promoting bacteria are beneficial for biomass production due to mechanisms such as the production of growth hormones (e.g., IAA) that can increase plant cell metabolic activity and through phyto-active substances that can cause morphological and physiological changes in the root system resulting in increased biomass production (Etesami et al., 2009; Pena and Reyes, 2007; Vargas et al., 2009; Yanni et al., 2001). Most PGP studies have focused on mixing non-rhizobial strains such as those found from the genus *Pseudomonas*, *Azotobacter*, and *Bacillus* and many other microorganisms to benefit plant growth. While rhizobia are best known for N fixation in legumes, they have now gained attention for their potential PGP activities that might positively influence biomass of non-legume plants in mixed pasture systems (Shaharoona et al., 2006). WSM1115 and 102F85 were found to produce IAA and were able to solubilize phosphate, which may enhance early and rapid root development due to increased phosphate uptake to leguminous and non-leguminous plants.

The NFmix3 blend of strains WSM1115, 10F85, and M49 was developed as a single inoculum that would effectively nodulate a wide variety of annual medics and provide PGP activities to the system. *Medicago orbicularis*, *M. rigidula*, and *M. rigiduloides* were all effectively nodulated by NFmix3 and performed as well as or better than the single specific inocula for *M. orbicularis* and *M. rigiduloides*. Unfortunately, while the NFmix3 contained WSM1115 it was not as effective on *M. minima* as WSM1115 alone.

This could indicate that *S. meliloti* 102F85 is more competitive than *S. medicae* WSM1115 on *M. minima*. There was no evidence of increased plant biomass through PGP activity on the annual medics in this study. However, it is possible that the presence of the bioinoculum in the soil would help to increase soil fertility and may positively influence the growth of nonlegume forages when grown in a mixed pasture system.

This study was conducted to understand the genetic differences between the annual medic specific isolates as determined by Interrante et al. (2011). A blend of rhizobia consisting of *S. medicae* and two *S. meliloti* strains was developed as a broad-spectrum inoculum for the use with multiple annual medics as monocultures and as mixtures. Benefits of using annual medics in mixtures include extension of the grazing season through the inclusion of early- and late-maturing varieties as well as increased pasture vigor against different soil properties, pests, and diseases (Busso et al., 1998; Hoveland et al., 1997). Future research would be required to determine the effectiveness of NFmix3 in the field and potential PGP benefits to nonlegume plants.

Acknowledgments

The authors thank Tom Wacek (Plant Probiotics), John Kosamke (EMD Crop Bioscience), and Ross Ballard (South Australian Research and Development Institute) for providing rhizobia inoculants and thank the USDA-ARS National *Rhizobium* Germplasm Collection for providing reference rhizobial strains. The authors also thank Jimmy Stein for providing support with greenhouse study and the Microarray Core facility at The Samuel Noble Research Foundation, Ardmore, OK.

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