Isolation and identification of plant growth-promoting bacteria associated with tall fescue

J. MONK, E. GERARD, S. YOUNG, K. WIDDUP and M. O'CALLAGHAN
AgResearch Limited, Private Bag 4749, Christchurch 8140, New Zealand
jana.monk@agresearch.co.nz

Abstract

Tall fescue (*Festuca arundinacea*) is a useful alternative to ryegrass in New Zealand pasture but it is slow to establish. Naturally occurring beneficial bacteria in the rhizosphere can improve plant growth and health through a variety of direct and indirect mechanisms. In this study, we isolated bacteria colonising the roots of established tall fescue plants and screened isolates for their plant growth promotion characteristics *in vitro*. To date, 10% of rhizosphere bacteria and 25% of endorhiza bacteria produced the plant growth hormone auxin. A small proportion of auxin-producing bacteria could also produce iron-chelating agents (siderophores) and solubilise phosphorus, which promote plant growth and health. These bacteria will be tested to determine their potential to enhance tall fescue establishment in pot and field trials.

Keywords: rhizosphere, endorhiza, auxin, siderophore, P-solubilisation

Introduction

Regions in New Zealand that are seasonably dry, and which may be exacerbated by climate change, require the use of pasture species other than ryegrass. Tall fescue (*Festuca arundinacea*) could be a useful alternative to ryegrass but achieving good seedling establishment and a dense sward is currently problematic. Tall fescue has other advantages in addition to drought tolerance; it is tolerant of several important pasture pests e.g. grass grub and Argentine stem weevil (Milne & Fraser 1990; Prestidge *et al.* 1986). Some improvements have been made through selection for enhanced seedling vigour in the field (Easton *et al.* 1994) but a large improvement in tall fescue seedling vigour is required to lift farmers' confidence in using this species. The application of beneficial bacteria to seeds may improve pasture establishment, particularly if seed-applied bacteria subsequently become established in the root zone of the plant and contribute to longer-term plant growth promotion and health.

There is growing international interest in the beneficial role of endophytic microorganisms in plant health and development (Backman 2008). Bacterial endophytes are defined as bacteria that colonise the internal tissue of a plant, showing no external sign of infection or negative effect on their host (Schulz & Boyle 2006). Bacteria-induced plant growth promotion is achieved either through fixation of atmospheric nitrogen, solubilisation of minerals, production of iron-chelating siderophores and plant growth regulators or by a combination of these mechanisms (Kloepper 1997). Most bacterial endophytes appear to originate from the rhizosphere, the zone that surrounds the roots of plants, but some can be transmitted through the seed. The ability of microorganisms to produce and release various metabolites affecting plant growth and health is considered one of the most important factors in soil fertility (Asghar *et al.* 2004).

Auxins are key hormones that control plant growth and development (Davies 1995). Several studies have demonstrated the ability of rhizosphere bacteria to synthesise auxin (indole-3-acetic acid) *in vitro* (Loper & Schroth 1986; Sawar & Kremer 1995) and auxin production by rhizobacteria has been shown to improve plant productivity in brassicas (Asghar *et al.* 2004).

Siderophores are iron-chelating agents secreted by some bacteria and fungi under iron-limiting conditions (Neilands 1981). Many soil and rhizobacteria have the ability to release phosphorus (P) from sparingly soluble mineral forms found in soils, improving P availability to plants (Yadav & Dadarwal 1997). Rengel (1999) found a positive correlation between the number of P-solubilising bacteria in the rhizosphere and plant P-uptake and grain yield. There are numerous reports indicating that soil inoculation with P-solubilising bacteria can enhance solubilisation of fixed soil P and applied fertiliser P, resulting in higher crop yields (Rodriguez *et al.* 1999; Kumar *et al.* 2001).

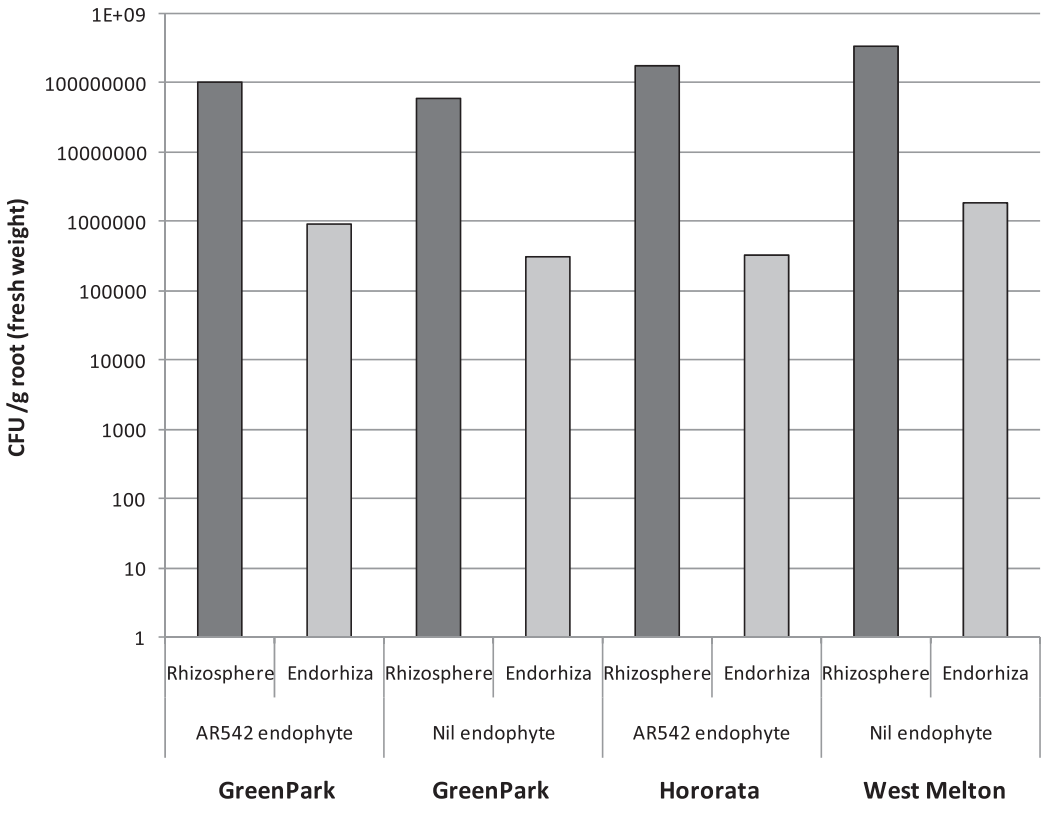
The aim of this preliminary study was to isolate and identify bacteria from tall fescue roots and select isolates that have the ability to produce auxin, release siderophores and solubilise P. Selected bacterial isolates harbouring all three traits will be considered for seed treatment to improve establishment of tall fescue in pasture systems.

Materials

Sampling of tall fescue

Samples were collected from three locations in Canterbury. GreenPark, near Lincoln, was the only site at which two tall fescue types were established: *Festuca arundinacea* cv. 'Grasslands Advance'

Figure 1 Numbers of bacteria around and inside of tall fescue roots. AR542 = commercial endophyte, Nil = no endophyte.



(AR542 endophyte) and cv. 'Grasslands Advance' without endophyte (Nil). Samples were also collected from a pasture in Hororata ('Grasslands Advance' AR542) and from West Melton from a ca. 15 year old stand of cv. 'Grasslands Roa' (Nil). At each site, plant roots with adhering soil (approximately 50 g) were collected from each corner of a randomly selected square meter and were placed into a sterile plastic bag to give a composite sample. Four composite samples were taken at each location. The microbial fraction was dislodged from the roots according to the protocol of Costa *et al.* (2005). For the isolation of bacterial endophytes, roots were surface sterilised in 2% NaOCl for 3 minutes, followed by rinsing with sterile water. Before homogenisation, a root fragment was imprinted on nutrient agar to serve as a sterility check. Roots were homogenised with a sterile mortar and pestle.

Counts of root-associated bacteria

The root washing solutions (rhizosphere) and homogenised roots (endorhiza) were serially diluted and plated on R2A medium (Difco Laboratories, Detroit, MI, USA). Plates were incubated for 5 days at 20°C and bacterial colonies were counted to calculate the mean number of colonies based on root fresh weight. For each site and tall fescue type, 80 colonies (20 per replication) from rhizosphere plates and 80 colonies from endorhiza plates were randomly selected and sub-cultured on nutrient agar (Merck, Germany). Bacterial isolates were screened for auxin-production, siderophore-production and P-solubilisation. Numbers of colony forming units per gram root fresh weight were log-transformed before analysis of variance (ANOVA) was conducted.

Screening for auxin-producing bacteria

Almost 600 bacteria were screened for their ability to produce auxin. The microplate method developed by Sawar & Kremer (1995) was used with the following modifications. All isolates were grown on half-strength tryptic soy agar (TSA, Gibco, Paisley, UK). For the colorimetric IAA assay, each isolate was suspended in 10 ml of growth medium. Controls were prepared by substituting bacterial suspension with sterile water. Tubes were incubated in the dark for 72 h at 20°C. Before analysis for auxin (IAA equivalents), 1 ml of growth medium of each isolate was centrifuged for 10

Figure 2 Percentage of auxin-producing bacteria around and inside of tall fescue roots. AR542 = commercial endophyte, Nil = no endophyte.

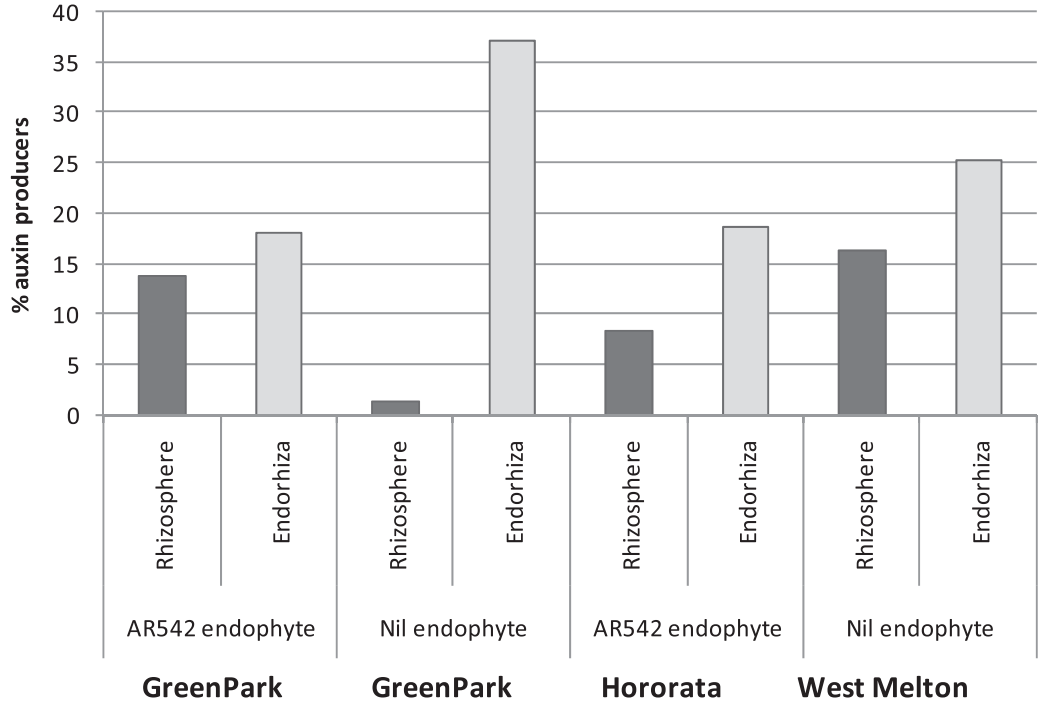


Table 1 Plant growth promotion characteristics of bacteria from three different locations.

Locations / Tall fescue	Total number of isolates tested	Auxin+ isolates	Siderophore + isolates[a]	P-solubilising isolates[a]
GreenPark				
Rhizosphere AR542	78	11	9%	36%
Endorhiza AR542	76	14	20%	10%
Rhizosphere Nil	74	1	0	0
Endorhiza Nil	69	25	50%	37%
Hororata				
Rhizosphere AR542	73	6	0	0
Endorhiza AR542	73	13	38%	23%
West Melton				
Rhizosphere Nil	75	12	50%	42%
Endorhiza Nil	76	19	42%	21%

[a] only auxin-positive bacteria were tested for siderophore-production and P-solubilisation

min at 13000 rpm at room temperature. The supernatant of each sample (90 µl) was dispensed into wells of 96-well microplates followed by addition of 60 µl of freshly prepared Salkowski reagent (Gordon & Weber 1951). After incubation for 30 min in the dark, the colour intensity was measured at 530 nm using a microplate reader (FLUOstar OPTIMA, BMG Labtechnologies, Germany). The statistical analysis of the percentages of auxin-producing bacteria was performed using a logistic regression method.

Table 2 Identification of bacteria with plant growth-promoting potential.

Location	Isolate	Auxin production	Siderophore production	P-solub.	Species identification
GreenPark	1R2	+	-	+	*Pseudomonas putida*
GreenPark	1R10	++	+	+	*Pseudomonas syringae*
GreenPark	1E56	+	+	-	*Pseudomonas putida*
GreenPark	1E63	+	+	+	*Pseudomonas sp.*
GreenPark	1nE2	++	+	+	*Rahnella aquatilis*
GreenPark	1nE16	++	+	+	*Rahnella sp.*
GreenPark	1nE66	+	+	(+)	*Pseudomonas putida*
Hororata	2R31	+++	-	-	*Aminobacter niigataensis*
Hororata	2E22	++	+	+	*Pseudomonas sp.*
Hororata	2E26	+	+	+	*Pseudomonas sp.*
Hororata	2E35	+	+	+	*Pseudomonas sp.*
West Melton	3R11	+	+	+	*Pseudomonas putida*
West Melton	3R25	+	+	+	*Pseudomonas sp.*
West Melton	3R32	+	(+)	+	*Enterobacter sp.*
West Melton	3R43	+	+	+	*Rahnella aquatilis*
West Melton	3E35	+	+	+	*Enterobacter cloacae*
West Melton	3E36	++	+	(+)	*Pseudomonas brassicacearum*
Lincoln	E3a	+	+	-	*Serratia proteamaculans*

-, no activity; (+), weak or delayed activity; +, moderate activity; ++, high activity; +++, very high activity

Screening for siderophore production and P-solubilisation

All auxin-positive isolates were screened for their ability to produce siderophores using a plate assay (Schwyn & Neilands 1987). The solubilisation of inorganic phosphate was tested on a double layer medium according to the method of Illmer & Schinner (1992) and Harris *et al.* (2006). The basal layer contained no P source whereas the top layer contained two different P sources.

Identification of beneficial isolates

The 16S rDNA gene fragments were PCR amplified with primer U16a (Wang & Wang 1996). Column purified PCR products were then submitted for sequencing. The sequences were aligned with data from the NCBI GenBank (www.ncbi.nlm.nih.gov/BLAST) using the megablast algorithm.

Results and Discussion

Counts of root-associated bacteria

The number of bacteria recovered from the rhizosphere was on average 10^8 colony forming units (CFU) /g root (fresh weight) and did not differ significantly between the locations (Fig. 1). A large number of bacteria with different colony morphologies and colours were found. Endophytic bacteria were less diverse and with a mean of 10^6 CFU /g root (fresh weight), populations were significantly lower ($P<0.001$) than in the rhizosphere (Fig. 1).

Screening for auxin production

A total of 300 rhizobacteria (root surface) and 294 bacteria from the endorhiza (inside of root) were tested for their ability to produce auxin *in vitro*. The proportion of auxin-positive isolates in the rhizosphere ranged from 1 to 16% and differed between locations (Fig. 2). Significantly higher numbers of auxin-producers were isolated from the endorhiza (18-37%) in comparison to the rhizosphere ($P<0.001$). During a screening of rhizosphere bacteria from potato Lottmann *et al.* (1999) found up to 67% auxin-positive bacteria.

Screening for siderophore production and P-solubilisation

Only bacteria capable of *in vitro* auxin-production were screened for siderophore production and P-solubilisation capability. The number of bacteria

with the ability to produce siderophores was higher for endorhiza bacteria, especially for bacteria isolated from tall fescue containing no fungal endophyte (Nil) or the introduced endophyte AR542 (Table 1). As endophytic bacteria have to compete with plant cells for their iron (Fe) supply, siderophore production may be important for endophytic growth (Sessitsch *et al*. 2004). The highest number of siderophore-producing bacteria was isolated from West Melton (from plants containing no endophyte).

Some bacteria from the rhizosphere and endorhiza were also able to solubilise P with the highest numbers from West Melton (Nil endophyte). Plants have the highest P demand during early development thus the existence of P-solubilising bacteria in association with roots could be of great benefit for the plant (Das *et al*. 2003).

Identification of beneficial isolates

Bacteria with two to three beneficial traits were identified to ensure no plant pathogens are applied in later seed-treatments (Table 2). A total of 18 isolates were identified by 16S rDNA sequencing and comparison of sequences with entries in the NCBI data base. The majority of isolates belonged to the genus *Pseudomonas*. Fluorescent pseudomonads are ubiquitous bacteria that are common inhabitants of the rhizosphere, and are the most studied group within the genus (Thomashow & Weller 1995). *Rahnella aquatilis*, a species which is usually found in fresh water, was isolated from the rhizosphere and endorhiza of tall fescue. Except for its nitrogen fixing ability, little is known about the ecological role of *R. aquatilis*. The strain identified as *Aminobacter niigataensis* produced a significantly higher amount of auxin than all other strains. *Aminobacter* spp. is mainly found in marine and freshwater habitats and little is known about it (Anzai *et al.* 2000). Some *Serratia* species, such as *S. proteamaculans* and *S. liquefaciens*, have been reported to have beneficial effects on plant growth of legumes (Zhang & Smith 1996). One plant pathogenic species, *Pseudomonas syringae* was identified and this strain will be excluded from further investigation. The fluorescent pseudomonad *P. brassicacearum* can have both pathogenic and plant growth-promoting effects on tomato plants (Belimov *et al.* 2007).

Conclusions

This study revealed that many bacteria isolated from tall fescue roots have characteristics that suggest the potential to promote plant growth, in particular bacteria isolated from the inside of tall fescue roots (endorhiza). Work is in progress to test these bacteria for other beneficial traits such as the ability to antagonise fungal pathogens due to antibiotic production or the release of fungal cell wall-degrading enzymes. The most promising isolates are also being tested in pot trials and in the field for enhancing the establishment of tall fescue. Plant growth-promoting bacteria will be delivered to the plant roots via seed priming. Tall fescue seeds will be imbibed in the bacterial solution for a critical period before drying back to the optimal water activity for storage. Seed priming offers an ideal delivery method for beneficial bacteria to seedlings (Niranjan *et al.* 2004; Müller & Berg 2008). During germination the bacteria colonise the root tissue and provide their plant growth promoting traits to the plants.

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REFERENCES

Anzai, Y.; Kim, H.; Park, J.; Wakabayashi, H.; Oyaizu, H. 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *International Journal of Systematic and Evolutionary Microbiology 50*: 1563–1589.

Asghar, H.N.; Zahir, Z.A.; Arshad, M. 2004. Screening rhizobacteria for improving the growth, yield, and oil content of canola (*Brassica napus* L.) *Australian Journal of Agricultural Research 55*: 187-194.

Backman, P.A.; Sikora, R.A. 2008. Endophytes: An emerging tool for biological control. *Biological Control 46*: 1-3.

Belimov, A.A.; Dodd, I.C.; Safronova, V.I.; Hontzeas, N.; Davies, W.J. 2007. *Pseudomonas brassicacearum* strain Am3 containing 1-aminocyclopropane-1-carboxylate deaminase can show both pathogenic and growth-promoting properties in its interaction with tomato. *Journal of Experimental Botany 58*: 1485-1495.

Costa, R.; Götz, M.; Mrotzek, N.; Lottmann, J.; Berg, G.; Smalla, K. 2005. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS Microbiology and Ecology 56*: 236–249.

Das, K.; Katiyar, V.; Goel, R. 2003. 'P' solubilization potential of plant growth promoting *Pseudomonas* mutants at low temperature. *Microbiol. Res. 158*: 359–362.

Davies, P. 1995. The plant hormone concept: concentration, sensitivity, and transport. pp.13-18. *In*: Plant hormones: physiology, biochemistry, and molecular biology. Ed. Davies, P. Kluwer Academic Publishers, Dordrecht.

Easton, H.S.; Lee, C.K.; Fitzgerald, R.D. 1994. Tall fescue in Australia and New Zealand. *New Zealand Journal of Agricultural Research 37*: 405-417.

Gordon, S.A.; Weber, R.P. 1951. Colorimetric estimation of indole acetic acid. *Plant Physiology 26*: 192-195.

Harris, J.N.; New, P.B.; Martin, P.M. 2006. Laboratory tests can predict beneficial effects of phosphate-solubilising bacteria on plants. *Soil Biology and Biochemistry 38*: 1521-1526.

Illmer, P.; Schinner, F. 1992. Solubilization of inorganic phosphates by microorganisms isolated from forest soils. *Soil Biology and Biochemistry 24*: 389-395.

Kloepper, J.W. 1997. Plant growth-promoting rhizobacteria (other systems). pp.137-166. *In*: *Azospirillum*/plant associations. Ed. Okon, Y. CRC Press, Boca Raton.

Kumar, V.; Behl, R.K.; Nurula, N. 2001. Establishment of phosphate solubilizing strains of *Azotobacter chroococcum* in rhizosphere and their effect on wheat cultivars under green house conditions. *Microbiological Research 156*: 87–93.

Loper, J.E.; Schroth, M.N. 1986. Influence of bacterial sources of indole-3-acetic acid on root elongation of sugar beet. *Phytopathology 76*: 386-389.

Lottmann, J.; Heuer, H.; Smalla, K.; Berg, G. 1999. Influence of transgenic T4 lysozyme-producing potato plants on potentially beneficial plant-associated bacteria. *FEMS Microbiology Ecology 29*: 365–377.

Milne, G.; Fraser, T. 1990. Establishment of 1600 hectares in dryland species around Omarau/Timaru. *Proceedings of the New Zealand Grassland Association 52*: 133-137.

Müller, H.; Berg, G. 2008. Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on *Verticillium* wilt in oilseed rape. *Biological Control 53*: 905-916.

Neilands, J.B. 1981. Microbial iron compounds. *Annual Review of Biochemistry 50*: 715-731.

Niranjan, R.S.; Shetty N.P.; Shetty, H.S. 2004. Seed bio priming with *Pseudomonas fluorescens* isolates enhances growth of pearl millet plants and induces resistance against downy mildew. *International Journal of Pest Management 50*: 41–48.

Prestidge, R.A.; Badam, D.; van der Zijpp, S. 1986. The effect of Argentine stem weevil on cocksfoot and tall fescue when sown with ryegrass. Proceedings of the 39th New Zealand Weed and Pest Control Conference: 33-37.

Rengel, Z. 1999. Mineral nutrition of crops: fundamental mechanisms and implications. Food Products Press, New York, 399 pp.

Rodriguez, H.; Fraga, R. 1999. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances 17*: 319–339.

Sawar, M.; Kremer, R.J. 1995. Determination of bacterially derived auxins using a microplate method. *Letters in Applied Microbiology 20*: 282–285.

Schulz, B.J.E.; Boyle, C.J.C.; Sieber, T.N. 2006. Microbial Root Endophytes. ISBN: 978-3-540-33525-2.

Schwyn, B.; Neilands, J.B. 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry 160*: 47–56.

Sessitsch, A.; Reiter, B.; Berg, G. 2004. Endophytic bacterial communities of field-grown potato plants and their plant-growth-promoting and antagonistic abilities. *Canadian Journal of Microbiology* 50: 239–249.

Thomashow, L.S.; Weller, D.M. 1995. Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. pp. 187-235. *In*: Plant–microbe interactions. Eds. Stacey, G.; Keen, N. T. Chapman and Hall, New York.

Wang, G.C.; Wang Y. 1996. The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. *Microbiology 142*: 1107–1114.

Yadav, K.S.; Dadarwal, K.R. 1997. Biotechnological approaches in soil microorganisms for sustainable crop production. pp. 293-308. *In*: Scientific publishers. Jodhpur, India.

Zhang, F.; Smith, D.L. 1996. Inoculation of soybean [*Glycine max* (L.) Merrill] with genistein-preincubated *Bradyrhizobium japonicum* or genistein directly applied into soil increases soybean protein and dry matter yield under short season conditions. *Plant and Soil 179*: 233–241.