



**Full Length Article**

# Variation in Phytohormone Production in *Rhizobium* Strains at Different Altitudes of Northern Areas of Pakistan

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## ABSTRACT

The present investigation was aimed to isolate *Rhizobium* strains from high altitudes of northern Pakistan and their biochemical characterization regarding phytohormone production and ability to form nodules on re-inoculation. *Rhizobium* species from the soil of northern areas were isolated in yeast mannitol agar medium. They were identified and characterized on the basis of morphological characters and biochemical tests. The maximum level of phytohormone production was observed in the isolates from Naran, showing the pattern of quick miniaturized testing system QTS-24 test different from other isolates. A significant positive correlation was found between altitude and gibberellic acid versus indole acetic acid ratio (GA/IAA). Beneficial effects of *Rhizobium* inoculation were obtained on soybean plants grown in sterilized soil. The maximum increase in fresh and dry weight and the greater number of nodules were obtained in the *Rhizobium* isolates from Shinkyari as compared to that of higher altitudes. It is inferred that strain isolated from Naran was most effective inoculant.

**Key Words:** Phytohormone; Altitudinal variation; GA/IAA; *Rhizobium*

## INTRODUCTION

With increasing altitude, legumes are more patchily distributed where nitrogen deposition and symbiotic nitrogen fixation together represent a significant source of nitrogen for the grassland ecosystem while at low altitudes these nitrogen inputs appear to be much less important (Jacot *et al.*, 2000). Inoculation of chickpea with rhizobial strains isolated from wild chickpeas (*C. anatolicum*) of high altitudes was demonstrated to substitute costly nitrogen fertilizers in chickpea production even in cold highland areas such as Erzurum (Kantar *et al.*, 2003). Rhizobial inoculants have contributed to increased nitrogen fixation and yield in legume crops that represents 70-80% of total nitrogen accumulated in plants (Catroux *et al.*, 2001). The microbial symbiosis is suggested to be the ideal solution for the improvement of soil fertility and the rehabilitation of arid lands (Zahran, 1999).

Phytohormone production by *Rhizobium* in both symbiotic as well as free living state has been reported. Inoculation of chickpea (*Cicer arietinum* L. cv. Aziziye-94) with eight *Rhizobium leguminosarum* sp. biover ciceri isolated from wild chickpeas (*C. anatolicum*) of high altitudes (2000-2500 m) had significantly increased host plant shoot dry weight, nodule dry weight, nitrogen%, chlorophyll, seed yield, total biomass and nitrogen fixation efficiency of symbiosis compared with the un-inoculated controls (Kantar *et al.*, 2003). Boiere *et al.* (2007)

demonstrated IAA, GA and zeatin production in *Bradyrhizobium japonicum*.

We report here the altitudinal variation in physiology of *Rhizobium* strains found in northern areas of Pakistan. These isolates were identified and characterized by the morphological and biochemical characters along with infectivity tests. The isolated strains were further tested for production of plant growth hormones (IAA & GA) in pure culture. In addition, their effect on growth and nodulation of Soybean cv. *Ajmeri* were also studied.

## MATERIALS AND METHODS

Soil samples (3-4) were collected from each site according to the quadrat method at the depth of 5-10 cm from rhizosphere at altitudes ranging 940-3090 m.a.s.l. in the northern areas of Pakistan i.e., Shinkyari, Naran, Shughran, Siri and Pay. The soil samples were analyzed for availability of K, Mg, Ca, Fe and Zn by ammonium acetate EDTA method (Cottenie *et al.*, 1982). The pH and electric conductivity of samples was also measured according to Radojevic and Bashkin (1999).

In order to isolate *Rhizobium* from soil, the soil paste was used for the inoculation studies on soybean. The nodules thus formed were used to isolate and characterize rhizobia by plant infection methodology test of reinoculation (Rao, 1999). The appearance of nodules on the host plant grown under sterile conditions confirmed the

presence of rhizobia. Soybean seeds cv. *Ajmeri* (obtained from National Agriculture Research Council, Islamabad) were surface sterilized with 0.1% HgCl<sub>2</sub> and washed several times with sterile water and sown in soil from Islamabad (570 m.a.s.l.). Soil was air dried and passed through a 2 mm sieve, autoclaved and filled in the pre-sterilized plastic pots (14 x 14 cm<sup>2</sup>). Initially 5 seeds were sown in each pot; 7 days after germination, the roots of the seedlings were inoculated with a soil paste made up of 10 g soil in 100 mL distilled water. The recommended irrigation practices were followed through out the course of study. The harvesting was done 45 days after sowing.

Large sized healthy nodules (4-5) detached from the roots were surface sterilized and crushed in 100 µL of sterile water on a glass slide; the suspension thus formed was streaked on YMA plates containing Congo red, incubated at 28°C and examined for appearance of colony 48 h after incubation. Morphological identification of *Rhizobium* was made by observing colony size, shape, color and growth on selective medium. To study the cell motility and shape, single colony from agar plates was transferred to a drop of sterile water on a glass slide and observed under light microscope (Nikon, Japan) at 100x using oil immersion. Oxidase test was performed according to Steel (1961) and the catalase test was made according to Faddin (1980). Physiological and biochemical tests were performed using QTS-24 miniaturized identification system (DESTO Laboratories Karachi, Pakistan). The bacterial cultures (24 h old) grown on YMA plates were suspended in saline solution (0.85% NaCl) and used to inoculate QTS kits.

Phytohormones were extracted from overnight broth cultures following the method of Tien *et al.* (1979). For analyses of IAA and GA pure cultures of five *Rhizobium* strains of different altitudes were grown in yeast Mannitol broth (YMB). Then 100 mL of YMB inoculated with 24 h cultures were placed on a shaker (80 rpm) for one week and harvesting was done by centrifugation at 10,000 rpm for 15 min; supernatant was used for the extraction of growth hormones. The extracted samples were analyzed on HPLC (Shimadzu, Japan) equipped with C-18 cartridge column (3.9-150 mm) at 30°C with methanol, acetic acid and water (30:1:70) at flow rate of 1 mL min<sup>-1</sup> and average run time of 15 min per sample. The wavelength used for detection of IAA was 278 nm and for GA analysis 254 nm (Li *et al.*, 1994). For identification of hormone, purified samples were filtered (0.45 µm) and injected (10 µL) into column. Pure IAA and GA (Sigma, USA) were used as standards. These growth hormones were identified on the basis of retention time and peak area of the standards.

Rhizobial strains isolated from the soil of northern areas were tested for their ability to induce nodulation on the host seedling cv. *Ajmeri* under natural climatic conditions. The *Rhizobium* strains isolated from soybean roots were grown in YMB (Vincent, 1970). The cell pellets were obtained by centrifugation at 10,000 rpm and re-suspended in sterile water equal in amount to original culture medium

to get approximately 10<sup>8</sup>-10<sup>9</sup> cells mL<sup>-1</sup>. Cell suspension (5 mL per plant) was applied to one week old soybean seedlings. For control treatment heat killed bacterial cell suspension in sterile water was used. The plants were harvested and numbered along with fresh and dry weight of nodules, diameter of pink bacteroid tissue was measured, root length, shoot length, fresh and dry weight of plant were also recorded. Fresh nodules were collected and thin sections (5 µm) of the nodules were cut and diameter of the pink bacteroid tissues containing leghaemoglobin in the nodule cortex was measured under light microscope at 4 x (Nikon Research Microscope optiphat with HFX-II camera). The data were analyzed by analysis of variances (ANOVA) and the means were compared following t-test.

## RESULTS

At higher altitude (3000 m.a.s.l. & above) the electric conductivity (EC) value was low in all cases whereas the EC value of relatively lower altitude (2270 m.a.s.l. & below) was about two fold higher. The highest EC was observed in soil samples collected from Naran (2270 m.a.s.l.), while those from Siri (3070 m.a.s.l.) showed the lowest value. The soil samples were more acidic at lower altitude i.e., 2270 m.a.s.l. and below compared to that at higher altitude i.e., 3000 m.a.s.l. and above. Moreover, the concentration of Mg, Fe, Zn, K and Ca were relatively higher at high altitude i.e., 3000 m.a.s.l. than that at lower altitude i.e., 2270 m.a.s.l. and below.

Nodules of *Rhizobial* colonies isolated from soils of different sites grown on soybean failed to take up red color of the Congo red medium. The colony size was 1-2 mm after 48 h on YMA. Under the microscope all these isolates were motile and rod shaped cells. Among the isolates, the strain of Naran (2270 m.a.s.l.) showed the pattern of QTS-24 test different from other four strains. The strains of Naran could be differentiated from the remaining isolates on the basis of indole and inositol tests, acid from sorbitol test was positive in the strains of lower altitude i.e., 2270 m.a.s.l. and below and highest altitude i.e., 3090 m.a.s.l. (Table I).

All the *Rhizobium* strains isolated from the soil of northern areas, produced GA in Yeast Mannitol growth medium. At higher altitude i.e., 3000 m.a.s.l. and above the GA as well as IAA was low and it was comparatively higher at lower altitude. The highest amount of GA produced by *Rhizobium* strain was at 2270 m.a.s.l. whereas maximum IAA was produced by strain isolated from Shinkyari (940 m.a.s.l.). The GA/IAA ratio increased with the altitude and was highest at 3090 m.a.s.l. A significant ( $p < 0.01$ ) positive correlation existed between altitude and GA/IAA ratio (Fig. 1).

Rhizobial inoculation to soybean plants cv. *Ajmeri* resulted in a significant ( $p < 0.001$ ) increase in nodule number as compared to control (un-inoculated). Maximum number of nodules per plant was observed in plants inoculated with *Rhizobium* from Naran (2270 m.a.s.l.) as compared to other inoculation treatments. The fresh and dry weight of nodules

**Table I. Morphological and biochemical characteristics of the bacterial isolates obtained from soil of northern areas of Pakistan**

Test	Shinkyari (940 m.a.s.l.)	Naran (2270 m.a.s.l.)	Shughran (3030 m.a.s.l.)	Siri (3070 m.a.s.l.)	Payi (3090 m.a.s.l.)
Cells	Motile rods Gram-ive	Motile rods Gram-ive	Motile rods Gram-ive	Motile rods Gram-ive	Motile rods Gram-ive
Colonies	Round off-white	Round off-white	Round off-white	Round off-white	Round off-white
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
ONPG	+	+	+	+	+
ADH	-	-	-	-	-
H <sub>2</sub> S	-	-	-	-	-
URE	-	-	+	-	-
TDA	-	-	-	-	-
IND	-	+	-	-	-
GLU	+	+	+	+	+
MAL	+	+	+	+	+
SUC	+	+	+	+	+
MAN	+	+	+	+	+
ARA	+	+	+	+	+
RHA	+	+	+	+	+
SOR	+	+	-	-	+
INO	-	+	-	-	-
MEL	+	+	+	+	+
MOT	+	+	+	+	+

(Values are expressed as mean  $\pm$  SE. All means that share a common letter are non-significantly different otherwise they differ significantly at  $P < 0.01$ )

**Table II. Root length, shoot length, fresh weight and dry weight of Soybean plant and nodules, number of nodules, diameter of pink bacteroid tissue of nodule following *Rhizobium* inoculation**

Sites (altitude m.a.s.l.)	Control (570)	Shinkyari (940)	Naran (2270)	Shughran (3030)	Siri (3070)	Payi (3070)
Root length (cm)	9.00a	18.67b	21.67bc	14.33bd	18.00bcde	18.67bcde
Shoot length (cm)	17.67a	28.33b	30.00bc	20.67abcd	22.67abde	28.00bcde
Fresh weight of Soybean Plant (g)	1.93a	5.67b	6.00bc	4.27d	4.50de	4.90bde
Dry weight of Soybean Plant (g)	0.54a	1.78b	2.77c	1.25abd	1.57bde	1.91bd
Number of nodules	NA	16.67a	26.00b	12.33ac	14.33acd	15.33acd
Fresh weight of nodules (g)	NA	0.26a	0.32ab	0.12c	0.17cd	0.13c
Dry weight of nodules (g)	NA	0.09a	0.24b	0.0c	0.04d	0.04d
Diameter of pink bacteroid tissue of nodule (mm)	NA	4.01a	4.12b	3.23c	3.80abcd	3.95d

Microbial identification kits (QTS-24h; DESTO Labs., Karachi) were used for these biochemical tests. For these tests 24-hour old bacterial cultures were used and results were noted after 18 hours of incubation at 30°C. ONPG=Ortho nitro phenyl  $\beta$ -D-galactopyranoside; ADH: Arginine dihydrolase; H<sub>2</sub>S: Hydrogen sulphide production; URE: Urea Hydrolysis; TDA: Tryptophane deaminase; IND: Indole; GLU: Acid from glucose; MAL: Acid from maltose; SUC: Acid from sucrose; MAN: Acid from mannitol; ARA: Acid from arabinose; RHA: Acid from rhamnose; SOR: Acid from sorbitol; INO: Acid from inositol; MEL: Acid from melibiose; MOT: Motility

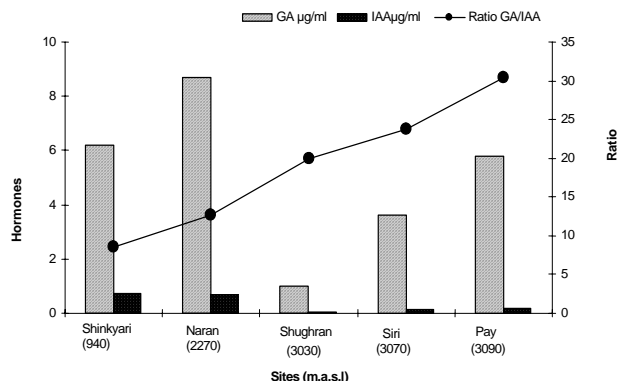
was significantly ( $p < 0.001$ ) greater when *Rhizobium* strain from Naran was used as inoculant. Maximum diameter of pink bacteroid tissue was observed in nodules produced from inoculants of Shinkyari and Naran strains. A significant ( $p < 0.001$ ) increase in root and shoot length was observed due to the inoculation with *Rhizobium* strains as compared to control (un-inoculated). The inoculation of all the isolated strains from the soil of northern areas proved more efficient. The most significant increase in root and shoot length was observed following inoculation with rhizobial strains of Naran (2270 m.a.s.l.) as compared to other altitudes. The root length and shoot length were lower in plants treated with rhizobial strain of altitude 3030 m.a.s.l (Table II).

Fresh and dry weight of soybean plants increased following inoculation with *Rhizobium* as compared to control. A significant ( $p < 0.001$ ) increase of fresh and dry weight of soybean plants was observed following inoculation of rhizobial strain from Naran soil as compared to other isolated strains. However, the isolated strain from Shinkyari, Shughran, Siri and Payi also showed significant

( $p < 0.001$ ) increase as compared to that of control.

## DISCUSSION

The gradient in IAA and GA concentration probably represents a decline in natural resources like radiation intensity, soil moisture and soil nutrients. It has been reported that over 80% of the bacteria isolated from rhizosphere can produce IAA (Arshad & Frankenberger, 1998) by obtaining tryptophan either through root exudates or from the proteins released by the dead bacterial cells (Patten & Glick, 1996). IAA is produced by *Rhizobium* strains in association with plants (Costacura & Vanderleyden, 1995) as well as in free-living state (Sekine *et al.*, 1988). We report that all the isolates from northern areas produced GA in pure culture irrespective of the altitude. It has also been previously detected by Bastian *et al.* (1998). Moreover, growth rate and IAA were positively correlated probably because environmental factors that increase endogenous auxins can increase growth (Salisbury & Ross, 1985).

**Fig. 1. Phytohormone levels in *Rhizobium* strains at different altitudes**

Maximum number of nodules was seen in inoculated treatments with isolates from Naran (2270 m.a.s.l.) probably due to the maximum production of IAA and GA by that strain. Similar results have been reported previously as the number of nodules in *Lotus corniculatus* is increased by Indole 2-Phenyl-n-butyric acid (Rao, 1999). Beneficial effects of inoculation with other isolated *Rhizobium* strains of northern areas at altitude of 940, 3030, 3070 and 3090 m.a.s.l. were observed on the soybean as compared to control. Similar results have also been reported after inoculation of chickpea (*C. arietinum* L cv. Aziziye-94) with eight *Rhizobium leguminosarum* sp. Ciceri strains isolated from wild chickpeas (*C. anatolicum*) of high altitude (2000-2500 m.a.s.l.) in comparison with un-inoculated control at an altitude of 1850 m.a.s.l. (Kantar *et al.*, 2003). These beneficial effects have also been observed previously on *Rhizobium* legume symbiosis (Bianca *et al.*, 2001).

Nodulation by *Rhizobium* strains is a complex process that can be modified by several factors like strains used, plant genotype, nutrient status and environmental factors (Wollaston, 1997). Diameter of pink bacteroid tissue, the active site of nitrogen fixation was found maximum in plants inoculated with Naran strain. This increase in diameter is reflected by the greater size of nodules in inoculated plants. Pawlowski (1997) demonstrated that the nitrogen fixation per plant increases as nodule grows possibly indicating the greater volume of pink bacteroid tissue.

Inoculated seedlings had greater plant height and stem width as compared to control (Muthukumar *et al.*, 2001). The increase in plant height of inoculated treatments is due to the stimulatory effects of microbe induced growth regulators i.e., IAA and GA (Rabie, 1996). Wall (2000) has suggested that plant hormones stimulate root development and consequently enhance absorption capacity of water and nutrients leading to plant growth (Camacho, 2001).

IAA and GA produced at low altitude were greater than that of high altitude, which is reflected from the greater biomass of the inoculated treatments. At altitude of 570 m.a.s.l. greater dry weights due to the improved root and

shoot development in inoculated plants has previously been reported (Bianca, 2001). The positive effect on growth can be explained by an enhancement of root growth due to the production of growth promoting hormones resulting in improved efficiency of mineral uptake in inoculated treatment (Okon & Vanderleyden, 1997).

Inoculation of plant with microbes increases dry matter content (Alagawadi & Gaur, 1988). Gaur (1990) has also shown increased fresh and dry matter yield. Inoculation of *Rhizobium* strain isolated from Naran showed increased root and shoot dry weight. That has also been reported by Kumar *et al.* (2001). This increase in dry matter production of inoculated plants may be attributed to enhanced nodulation, higher nitrogen fixation rate and a general improvement of root development.

## CONCLUSION

Altitudinal variation exists in the ability of phytohormone production of *Rhizobium* isolates in culture. There appears a critical level of altitude beyond which the efficiency decreases both for nodule formation and phytohormone production. The most efficient rhizobial isolates from Naran at altitude of 2270 m.a.s.l. may be included in bio fertilizer production and possibly applied for inoculation under the abiotic stresses.

## REFERENCES

- Alagawadi, A.R. and A.C. Gaur, 1998. Associative effect of *Rhizobium* and phosphate solubilizing bacteria on the yield and nutrient uptake of chickpea. *Plant Soil*, 15: 241-6
- Arshad, M. and W.T. Frankenberger, 1998. Plant growth regulating substances in the rhizosphere: Microbial production and function. *Advan. Agron.*, 62: 45-151
- Bastian, F., A. Cohen, P. Piccoli, V. Luna, R. Baraldi and R. Bottini, 1998. Production of indole-3 acetic acid gibberellins A<sub>1</sub> and A<sub>3</sub> by *Acetobacter diazotrophicus* and *Herbaspirillum seroopedicae* in chemically defined culture media. *Plant Growth Regul.*, 24: 7-11
- Bianca, H., J. Abbadi, S. Burdman, Rasid, S. Sarig and Y. Okon, 2001. Effects of inoculation with *Azospirillum brasilense* on chickpea (*Cicer arietinum*) and faba beans (*Vicia faba*) under different growth conditions. *J. Agron.*, 21: 553-60
- Boiere, L., D. Perrig, O. Masciarelli, C. Penna, F. Cassan and V. Luna, 2007. Phytohormone production by three strains of Bradyrhizobium japonicum and possible physiological and technological implications. *J. Appl. Microbiol. Biotechnol.*, 74: 874-80
- Camacho, M., C. Santamaria, F. Temprano Rodriguez-Navarro and A. DN Daza, 2001. Co-inoculation with *Bacillus* species. Cect 450 improves nodulation in *Phaseolus vulgaris* L. *Canadian J. Microbiol.*, 47: 1058-62
- Catroux, G., A. Hartmann and C. Revellin, 2001. Trends in *Rhizobium* inoculant production and use. *Plant Soil*, 230: 21-30
- Costacura, A. and J. Vanderleyden, 1995. Synthesis of phytohormones by plant associated bacteria. *Crc. Rev. Microbiol.*, 21: 1-18
- Cottenie, A., M. Verloo, L. Kiekens, G. Velghe and R. Camerlynck, 1982. *Chemical Analysis of Plant and Soils*, p. 41. Laboratory of Analytical and Agrochemistry, State University, Ghent-Belgium
- Faddin, M., 1980. 'Biochemical Tests for Identification of Medical Bacteria', p. 51. Williams and Wilkins, Baltimore, USA
- Gaur, A.C., 1990. 'Phosphate Solubilizing Microorganism as Biofertilizer', p. 57. Omega scientific Publishers, New Delhi, India

- Jacot, K.A., A. Luescher, J. Noesberger and U.A. Hartwig, 2000. Symbiotic N<sub>2</sub> fixation of various legume species along an altitudinal gradient in the Swiss Alps. *Soil Biochem.*, 32: 1043–52
- Kantar, F., E. Elkoca, H. Ogutcu and O.F. Algur, 2003. Chickpea yields in relation to *Rhizobium* inoculation from wild chickpea at high altitudes. *J. Agron. Crop Sci.*, 189: 291–7
- Kumar, D., B.S.I. Berggen and A.M. Maartensson, 2001. Potential for improving pea production by coinoculation with fluorescent *Pseudomonas* and *Rhizobium*. *Plant Soil*, 229: 25–34
- Li, J.C., J. Shi, X.L. Zhao, G.Y.U. Wang and Y.J. Ren, 1994. Separation and determination of three kinds of plant hormones by high performance liquid chromatography. *Fenxi-Huaxue*, 22: 801–4
- Muthukumar, T., K. Udaiyan and V. Rajeshkannan, 2001. Response of neem (*Azadirachta indica* A. juss) to indigenous arbuscular mycorrhizal fungi, phosphate solubilizing and symbiotic nitrogen fixing bacteria under tropical nursery conditions. *Biol. Fertil. Soils*, 34: 417–20
- Okon, Y. and J. Vanderleyden, 1997. Root associated *Azospirillum* species can stimulate plants. *ASM News*, 93: 370–399
- Patten, C.L. and B.R. Glick, 1996. Bacterial biosynthesis of indole-3-acetic acid. *Canadian J. Microbiol.*, 42: 207–20
- Pawlowski, K., 1997. Nodule-specific gene expression. *Plant Physiol.*, 99: 617–37
- Rabie, K.A.E., 1996. Studies on the interaction between gibberellin and benzyladenine in regulating growth, yield and phytohormone content in wheat plants. *Annal. Agric. Sci.*, 41: 99–110
- Radojevic, M. and V.N. Bashkin, 1999. 'Practical Environmental Analysis. In: *Soil Sediment, Sludge and Dust Analysis*', p: 300. The Royal Society of Chemistry, UK
- Rao, N.S., 1999. 'Soil Microbiology. In: *Rhizobium and Legume Root Nodulation*', p: 116. Oxford and IBH Publication, Co. Pvt Ltd., New Delhi
- Salisbury, F.B. and C.W. Ross, 1985. 'Plant Physiology. In: *Hormone and Growth Regulators: Auxin and Gibberellins*', p: 309. Wadsworth publication Company Inc., USA
- Sekine, M., T. Techikawa, N. Kuga, M. Kobayashi, A. Sakurai and K. Syono, 1988. Detection of the IAA biosynthesis pathway from tryptophan via indole-3- acetamide in *Bradyrhizobium* specie. *Plant Cell Physiol.*, 202: 159–66
- Steel, K.J., 1961. The oxidase reaction as a toxic tool. *J.G. Microbiol.*, 25: 297–306
- Tien, T.M., M.H. Gaskind and D.H. Hubbel, 1979. Plant growth substances produced by *Azospirillum brasilense* and their effect on growth of pearl millet (*Pennisetum americanum* L.). *Appl. Environ. Microbiol.*, 37: 1016–24
- Vincent, J.M., 1970. 'A Manual for the Practical Study of the Root Nodule Bacteria', p: 45. Burgess and Son Ltd., Great Britain
- Wall, L.G., A. Hellsten and K. Huss-Danell, 2000. Nitrogen, phosphorus and the ratio between them affect nodulation in *Alnus incana* and *Trifolium pratense*. *Symbiosis.*, 29: 91–105
- Wollaston, B.V., 1997. The molecular biology of leaf senescence. *J. Exp. Bot.*, 48: 181–99
- Zahrn, H.H., 1999. *Rhizobium* legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol. Molecular Biol.* 63: 968–89

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