Pak.J.Bot., 27(1): 55-62, 1995.

USE OF GUS-MARKED *RHIZOBIUM* AND *BRADYRHIZOBIUM* STRAINS FOR STUDYING THE EFFECT OF TEMPERATURE ON THE INFECTION PROCESS

FAUZIA Y. HAFEEZ, M. AURANGZAIB KHAN*, SOHAIL HAMEED, EJAZ RASUL* AND KAUSER A. MALIK

Institute for Biotechnology and Genetic Engineering, P.O. Box 577, Faisalabad, Pakistan.

Abstract

Effect of diurnally administered temperature regimes on the infection process of 3 *Vigna* spp., and 1 *Medicago* sp., inoculated with GUS-marked (brady) rhizobial strains was examined. Conditions optimized for blue color development to indicate the presence of GUS-marked *Rhizobium* and *Bradyrhizobium* strains in the roots and nodules of inoculated plants showed significant results with 50 μg ml^{-1} X-gluc buffer and 3 and 5 minutes of vacuum infiltration for young and mature nodules, respectively. Root hair colonization, curling, infection thread and nodule formation were observed in all the legumes at 30°C without any temperature shock. In *V. radiata*, no infection thread was found even at optimum temperature of 30°C. Root hair curling and infection thread formation were not observed in the genotypes exposed to temperature stresses, except in *V. unguiculata*, where the infection thread aborted in the root hair. Root hair colonization was observed a day after inoculation in plants subjected to both high or low temperature stresses, indicating the survival of *(Brady)rhizobium* strains under temperature stress conditions. The strains were unable to nodulate the host plants due to poor root hair development.

Introduction

Biological nitrogen fixation, symbiotic or asymbiotic, which is carried out by certain prokaryotic microorganisms is the major source of renewable combined nitrogen available to the biosphere (Postgate, 1982). The most important system for biological nitrogen fixation involves the symbiotic relationship between bacteria of the genus *Rhizobium* and plants of the family Leguminosae. The development of a legume nodule is a finely tuned process. Generally rhizobia invade its specific host through the root hair, forming an infection thread. This infection process involves a continued interplay between the two symbiotic partners, where gene expression from both the symbionts must be temporally or spatially coordinated. This multistep infection process fails more frequently than it succeeds (Hirsch, 1991) and that can be due to a number of inhibitory factors. Attempts are being made to identify the factors limiting the establishment and maintenance of the legume-*Rhizobium* symbiosis in adverse environments. Environmental factors such as salinity, high temperature and drought may affect the nodulation and nitrogen fixation of leguminous plants (Hafeez *et al.*, 1991; Hafeez *et al.*, 1988; Hartel & Alexander 1984; Kishinevsky *et al.*, 1992; Michiels *et al.*, 1994). The effect of high temperature on nodulation and growth of *V. radiata* with different bradyrhizobial

*Department of Botany, University of Agriculture, Faisalabad, Pakistan.

strains has been reported (Hafeez *et al.*, 1991). In the present study, GUS-marked *Rhizobium/Bradyrhizobium* strains were used to examine the effect of temperature on the compatibility of (brady)rhizobial strains and host genotypes through infection process. The *gusA* gene encodes an enzyme ß-glucuronidase which converts X-gluc to an indigo (colored) product. The colored product indicates the presence of *Rhizobium* and *Bradyrhizobium* strains in the plant roots (Jefferson *et al.*, 1986; Jefferson & Wilson 1991; Wilson *et al.*, 1991; Wilson, 1994).

Materials and Methods

The GUS-marked strains Rm 1021 and NC 92 were obtained from Dr. Kate Wilson, CAMBIA, Canberra, Australia. Seeds of tropical legume plants viz., *Vigna unguiculata, V. mungo* and *V. radiata* were obtained from the Mutation Division of NIAB, Faisalabad, Pakistan and that of temperate legumes *Medicago sativa* from NifTAL, Hawaii, USA. The plants were grown in self designed hydroponic assemblies (Fig.1).

Surface sterilized seeds were germinated on water agar plates and 72 h old seedlings transferred aseptically into hydroponic assemblies (Fig.1). They were grown in nitrogen free nutrient solution (Arnon & Hoagland, 1940), at 30°C and inoculated with rhizobial strain @ 10^9 cfu ml^{-1} . *M. sativa* seedlings were inoculated with *Rhizobium meliloti* strain Rm 1021 (Gus-1) and *V. mungo, V. radiata* and *V. unguiculata* seedlings with *Bradyrhizobium* strain NC 92 (Blue-1). Uninoculated seedlings were kept as controls. The seedlings were harvested daily till nodule formation for upto 14 days. The excised roots were dipped in different dilutions (1:1 to 1:5) of X-gluc buffer in distilled water: 50mM Na_2HPO_4, 1mM Na_2EDTA, 0.1% Sarkosyl, 0.05% SDS, 50 μg ml^{-1} X-gluc (Wilson *et al.*, 1991). The plant roots were vacuum infiltrated in each dilution for 1-7 min.

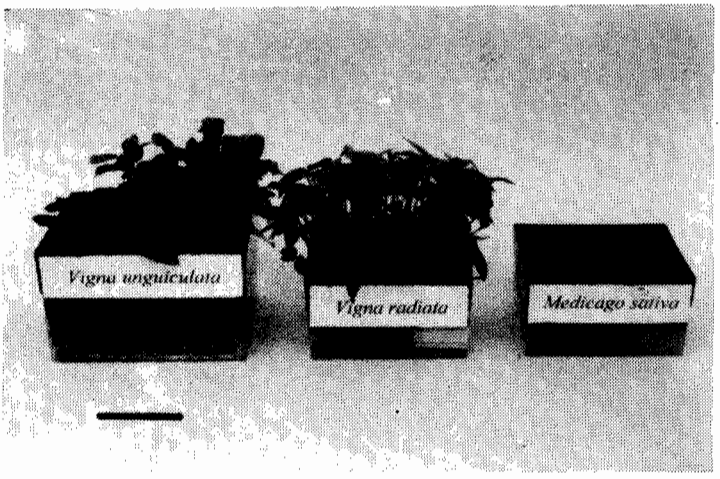

Fig. 1. Specially designed hydroponic growth assemblies to study temperature effect on the root infection by GUS-marked strains.

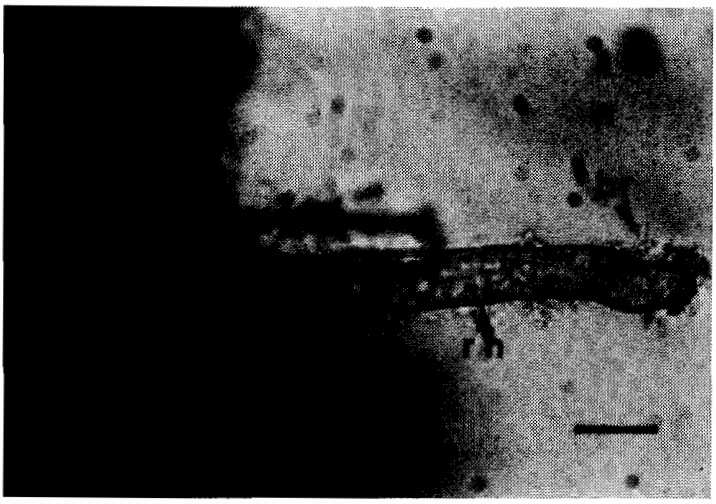

Fig. 2. Root hair (RH) colonization by GUS-marked *Bradyrhizobium* (BR) strain after 24 hrs of inoculation in *Vigna unguiculata* seedling at 30°C. X 40.

In the second experiment, *Vigna* spp., were given high temperature shocks of 36, 42 and 48°C for 2 h daily and the day and night temperature maintained at 30°C (Hafeez *et al.*, 1991). *M. sativa* plants were given temperature shocks of 15, 18 and 22°C and the day and night temperature maintained as low as 10°C. The desired root temperature was achieved within 1 h. The temperature of solution inside the assemblies was continuously monitored using a thermometer with a precision of ± 1.0°C. The seedlings were exposed to a 14 h photoperiod and a photon flux density of 392 μmole m^{-2} sec^{-1}. Three seedlings of each genotype were harvested daily in the first week and the final harvest was made 2 weeks after inoculation. GUS-marked rhizobia and bradyrhizobia were detected in plant root tissues by immersing the excised root system in X-gluc buffer.

Results and Discussion

Blue colour development, characteristic of *gusA* gene present in (brady)rhizobial strains, in the root and nodules of inoculated plants were obtained with 50 μg ml^{-1} X-gluc buffer and 3 minutes of vacuum infiltration for young seedlings and 5 minutes of vacuum infiltration for both mature nodules and 2-4 week old roots. GUS-marked *Bradyrhizobium* strain Nc 92 produced nodules on *V. unguiculata* and *V. mungo* but was unable to form nodules on *V. radiata*. *Rhizobium* strain Rm 1021 formed nodules on *M. sativa* within 10 days of inoculation. The root systems of all the legume hosts at 30°C were well developed. Bacterial cells colonized mostly at the tip of root hair in *V. unguiculata, V. mungo, V. radiata* and *M. sativa* (Fig.2). The infection time of *Rhizobium* and *Bradyrhizobium* strains was between 50-60 h after inoculation. Root hair deformation and curling was observed on the second day of inoculation. Blue

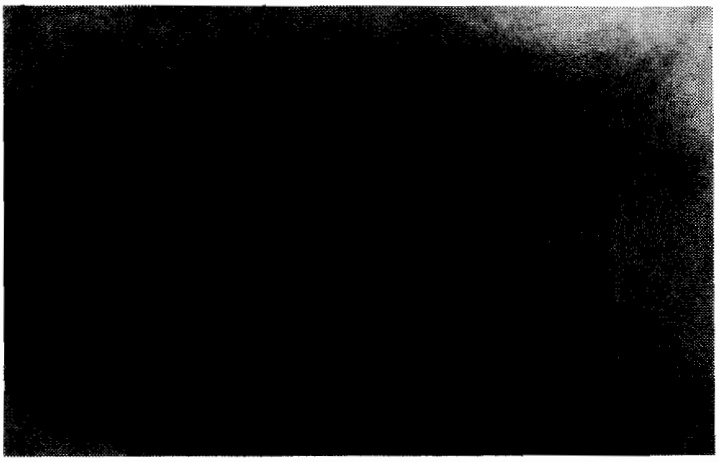

Fig.3a. Blue stained infection thread (IT) in root hair of *Vigna unguiculata* after 72 hrs of inoculation at 30°C . X 40.

stained infection threads indicating the presence of rhizobia were observed in the curled root hair on the third day (Fig.3a) which entered cortical cells on the fifth day (Fig.3b). Blue staining was not observed in mature root hair zone. This supports the findings of Wilson *et al.*, (1991) that colonization by compatible bacteria was restricted to certain zones of the growing root. The nodule initiation was also observed on the fifth day that

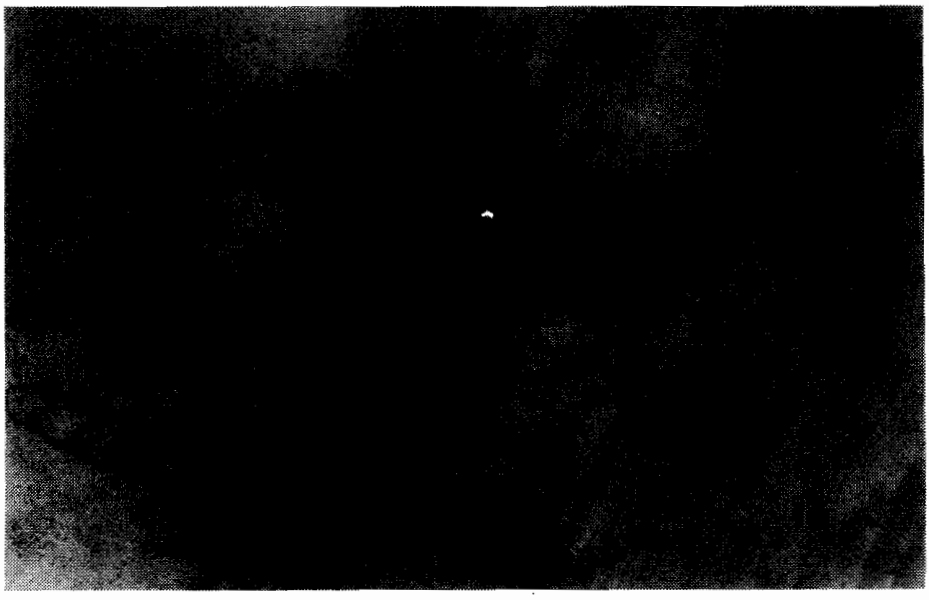

Fig.3b. Photomicrography showing infection thread (IT) in the root hair (RH) and extending to the inner cortical zone (IC) before nodule initiation at 30°C. X 100.

reached maturity after 12 days. Nodules formed by GUS-marked strains on their respective hosts stained blue. The young *Medicago* nodules stained bright blue (Fig.4), where good color development was obtained after 24 h of incubation in the buffer.

For *Vigna* spp., the optimum incubation time in buffer was 72 h. In *V. radiata*, bacterial colonization was observed at the tips and within the root hair one day after inoculation at 30°C. Root hair deformation and curling were observed on the second day of inoculation, but no infection thread or nodule formation was observed, showing the incompatibility of bradyrhizobial strain NC 92 with *V. radiata*. In a previous study rhizobial strains isolated from Siratro showed less compatibility with *V. radiata* for invasiveness and effectiveness than other *Vigna* spp., (Hafeez *et al.*, 1994). Nitrogen deficiency in non-nodulated test plants and controls was marked visually by the change of dark green color of leaves in the early 5-6 days to light green and then yellowing and early shedding.

Plants subjected to high and low temperature stress showed suppressed growth. Root hair development was very poor, that remained small (Fig.5a) and were present on few sites of secondary roots. Root hair colonization was observed a day after inoculation. Root hair curling and deformation was not found in the temperature stressed seedlings except in *V. unguiculata* in which some root hair were infected but infection threads aborted within the root hair. Blue staining was only observed in the outer zone of root hair but not close to the root (Fig.5b). The temperature stress had lesser inhibitory effect on the root hair development of *V. unguiculata* as compared to other tested hosts (Fig.5a,b).

Root hair colonization was observed in all the plants studied under stress conditions but failed to nodulate the hosts suggesting that the bacterial strains may be more tolerant to temperature stress than the host. High and low temperature stress has previously

Fig.4. Young nodule of *Medicago sativa* at 30°C showing blue stained central infected zone (IZ) and outer uninfected cortical zone (UZ) X 40.

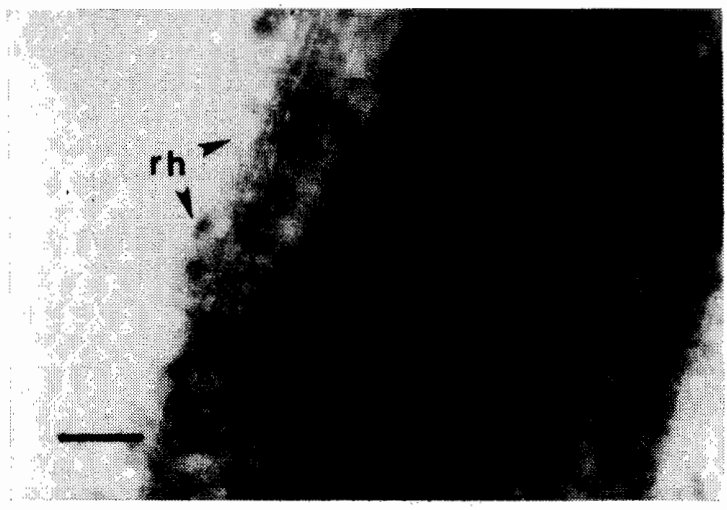

Fig.5a. Effect of high temperature stress showing stunted root hair (RH) development and inhibition of bacterial infection in *Vigna mungo* after 72 hrs of inoculation. X 10.

been shown to suppress nodulation by affecting the host genotype (Hafeez *et al.*, 1991; Munevar & Wollum 1982; Roughley *et al.*, 1976). In the present study temperature stresses inhibited the development of root hair and resulted in distortion of root hair at the site where rhizobia enter the host, prior to the establishment of symbiosis. It would suggest that while selecting legume genotypes which could tolerate stress factors, the ability to form normal root hair should be a major consideration. *Rhizobium* and

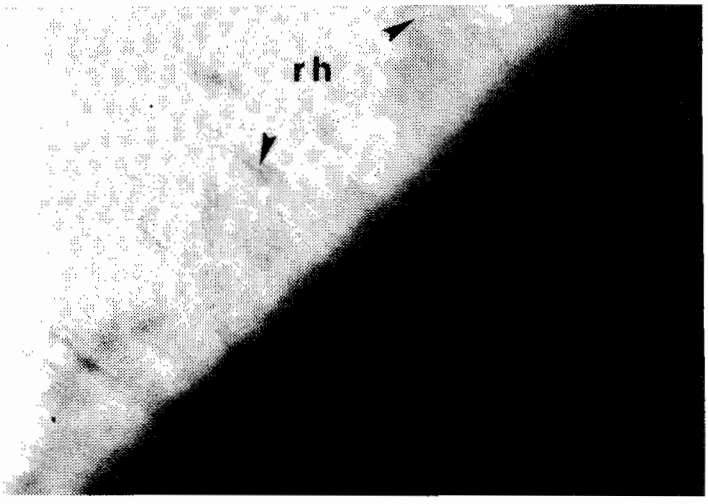

Fig.5b. Photomicrograph showing blue staining in the outer zone of root hair (RH) of *Vigna unguiculata* after 48 hrs of inoculation under high temperature stress. X 10.

Bradyrhizobium strains tolerant to temperature stress have been isolated and can be identified more easily than tolerant host plants (Hafeez *et al.*, 1991; Kishinevsky *et al.*, 1992).

The use of a marker gene enables a quick selection of compatible strain and any given crop/cultivar for a specific environment. This marker gene greatly facilitates the study of early stages of infection before nodules are visible. Bottomely (1992) reported that marker genes would offer great advantages, they would both enable analysis of population changes and visualization of the marker strain in its interaction with the plant host. A careful selection of compatible thermotolerant microsymbiont and host genotype is required for maximum production of legumes under temperature stress condition. The use of GUS-marked strains may be a very useful tool to study the compatibility or specificity between rhizobial strains and host cultivars under different climatic conditions.

Acknowledgements

We are grateful to Dr. Kate J. Wilson, CAMBIA, Canberra, Australia for improving and critically reviewing the manuscript. Thanks are also due to Mr. Tanvir Ahmad and Mr. Tariq Mahmood for technical assistance.

References

Arnon, D. I. and D. R. Hoagland. 1940. Crop production in artificial culture solution and in soil with special reference to factors influencing yield and absorption of inorganic nutrients. *Soil Sci.*, 50:463-483.

Bottomely, P. J. 1992. Ecology of *Bradyrhizobium* and *Rhizobium*. pp. 239-348. In: *Biological nitrogen fixation*. (Eds.): G. Stacey, H.J. Evans and R. H. Burris. Routledge, Chapman and Hall, New York, USA.

Hafeez, F. Y., S. Asad, T. Ahmad and K.A. Malik. 1994. Host specificity and characterization of fast growing rhizobia from *Macroptilium atropurpureum* cv siratro in Pakistan. *Soil Biol. Biochem.*, (in press).

Hafeez, F. Y., S. Asad and K.A. Malik. 1991. The effect of high temperature on *Vigna radiata* nodulation and growth with different bradyrhizobial strains. *Environ. Exp. Bot.*, 31:285-294.

Hafeez, F. Y., Z. Aslam and K. A. Malik. 1988. Effect of salinity and inoculation on growth, nitrogen fixation and nutrient uptake of *Vigna radiata* (L.) Wilczek. *Plant Soil*, 106: 3-8.

Hartel, P. G. and M. Alexander. 1984. Temperature and desiccation tolerance of cowpea rhizobia. *Can. J. Microbiol.*, 30: 820-823.

Hirsch, A. M. 1992. Developmental biology of legume nodulation. *New Phytol.*, 122: 211-237.

Jefferson, R. A., S.M. Burgess, and D. Hirsch. 1986. ß-Glucuronidase from *Escherichia coli* as a gene fusion marker. *Proc. Acad. Sci.* USA., 86: 8447-8451.

Jefferson, R. A. and K.J. Wilson. 1991. The GUS gene fusion system. pp. B 14/1-B 14/33. In: *Plant molecular biology manual*. (Eds.): S. Gelvin, R. Schilperoort and D. P. Verma. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Kishinevsky, B. D., D Sen and R.W. Weaver. 1992. Effect of high root temperature on *Bradyrhizobium*-peanut symbiosis. *Plant & Soil*, 143: 275-282.

Michiels, J., C. Verreth and J. Vanderleyden. 1994. Effects of temperature stress on bean-nodulating *Rhizobium* strains. *Appl. Environ. Microbiol.*, 60: 1206-1212.

Munevar, F. and A.G. Wollum. 1982. Response of soybean plants to high temperature as affected by plant cultivar and *Rhizobium* strain. *Agron. J.*, 74: 138-142.

Postgate, J. R. 1982. *The fundamentials of nitrogen fixation.* Camb. Univ. Press, Cambridge. UK.

Roughley, R. J., P.J. Dart and J.M. Day. 1976. The structure and development of *Trifolium subterraneum* L., root nodules. II. In plants grown at sub-optimal root temperatures. *J. Exp. Bot.*, 27: 431-440.

Wilson, K. J., K.E. Giller and R.A. Jefferson. 1991. ß-Glucuronidase (GUS) operon fusions as a tool for studying plant-microbe interactions. pp. 226-229. In: *Advances in molecular genetics of plant-microbe interactions*, vol.1. (Eds.): H. Hennecke and D.P.S. Verma. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Wilson, K. J. 1994. The role of molecular genetics in understanding microbial ecology. *Soil Biol. Biochem.*, (in press).

(Received for Publication 5 October 1994)