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PISUM SATIVUM-RHIZOBIUM INTERACTIONS UNDER DIFFERENT ENVIRONMENTAL STRESSES

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Abstract

Effect of low temperature, salinity, nutrient level and photoperiod has been studied on 3 varieties of *Pisum sativum* var., P-48, meteor and AM-1inoculated with *Rhizobium* strain PS-1. The plants were grown at 5, 10, 15, 20 and 25°C. The number of viable rhizobial cells was counted at each temperature by most probable number technique. The number of viable cells were constant at 5 and 10°C but increased between 15-30°C. Nodule formation was not observed at 5, 10 and 25°C at 15°C the nodules were less in number, whereas at 20°C best nodulation was observed. The pea plants were also subjected to different salinity levels i.e., 0, 1, 2, 3, 4, 5, 10 and 15dsm^{-1}. High number of nodules was formed at 0 and 1dsm^{-1} whereas at 2 and 3dsm^{-1} the nodule number was reduced and yet at higher salinity levels no nodules were formed. Pea plants nodulated best at 12 hours photoperiod and 1/6th concentration of Hoagland's nutrient solutions. The mature nodules developed at different temperatures and salinity levels were assayed for nitrogenase activity and processed for light and electron microscopy which supported our study.

Introduction

Environmental stresses like temperature extremes, salinity, drought and nutrient supply have detrimental effects on nitrogen fixation (Biosca *et al*., 1996; Manhan & Steck, 1997; Denison & Kiers, 2004). In biological nitrogen fixation not only the micro but also the macrosymbiont plays an important role and their co-selection under given set of environmental conditions may enhance the amount of fixed nitrogen (Hafeez *et al.*, 1991; 2000, Gage, 2004). Low and high temperature affects the symbiotic nitrogen fixation severely (Hafeez *et al*., 1995; Michiels *et al*., 1994). Temperature regulates the metabolism of the bacteria (Vincent, 1977) and the plant (Delvin, 1975) as well as bacteria-plant association (Young, 2006). *Rhizobium* strains differ in their ability to grow, nodulate their host plants and expression of nitrogenase activity at extreme temperatures. The low temperature limits for plants native to temperate zone is 2°C and for tropical species about 10°C (Werner, 1992). Sometimes the sensitivity of host towards low temperature affects the nitrogen fixation severely leading to abrupt cut off at temperatures where the bacterial cells can still grow and metabolize. Temperature affects N_2-fixation at any stage of nodulation from the attachment of bacteria to the host root hair upto the number of bacteroids within the host cell. Root hair infection is much more temperature sensitive than nodule development (Hafeez *et al*., 1995; Werner 1992). Lipsanen & Lindstrom (1986) and Roughly *et al.*, (1970) have reported delayed infection process at low temperature in various legume species.

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Detrimental effects of salt on growth and survival of *Rhizobium* have been reported (Saxena & Rewari 1992; Zahran, 1992). This may be due to the sensitivity of the host (Newcomb *et al*., 1979; Sprent 1984; Hafeez *et al*., 1988), the microsymbiont (Tu, 1981) or their symbiosis (Subba Rao *et al*., 1972). Tolerance towards high salt concentrations has been reported in several *Rhizobium* strains isolated from different saline soils, that have shown to be efficient inoculants (Rasool *et al*., 1987; Elsheikh & Wood, 1995; Zou *et al*., 1995).

The legume crops are highly sensitive towards salinity hence successful growth of crops in saline soils may possibly be achieved by the use of salt tolerant host genotype and *Rhizobium* strain. Studies have revealed that salt stress causes an alteration of root hair curling, a reduction in the number of rhizobia attached to root hair and the amount of nitrogen fixed per unit weight of nodules also declines with salt stress (Miller, 1996).

Effect of photoperiod and nutrient supply on nodulation has been studied (Frings, 1976). It is shown that pea plants need a minimum amount of organic substrates in order to enable nodulation. When the plants are grown at high temperature, less substrate is available, because under these conditions a greater part is respired. Day and night temperature and photoperiod should be adjusted to counterbalance the respiration and photosynthesis.

Pisum sativum commonly known as peas is a cold season legume. Low temperature and salinity effects nodulation to greater extent in peas (Frings, 1976). Different studies have shown that pea shows problem in nodulation even in the soils with rich *Rhizobium* population. Different cultivars of peas behave differently in their compatibility with different strains of *Rhizobium*. Although the pea crop is cultivated in large areas in the world, the parameters related to symbiotic performance had not been evaluated up till now specially the effect of low temperature, photoperiod and nutrient concentrations on the symbiotic performance of different cultivars of peas (Frings, 1976).

The objectives of the present study were to identify the most compatible *Rhizobium* strain with different cultivars of pea and also to study the effect of different parameters on their symbiotic performance.

Materials and Methods

Experiment-1. *In vitro* growth of *Rhizobium leguminosarum* bv. *viciae* strains at different temperatures

***Rhizobium* strains:** Four *Rhizobium leguminosarum* bv. *viciae* strains viz., PS-1, PS-2, LC-31 and LC-12 isolated from the root nodules of pea and lentil respectively were used (Shah *et al*. 1995). The strains were taken from BIRCEN (culture collection section), NIBGE.

***In vitro* study of bacteria at different temperatures:** The cultures were grown in yeast mannitol broth media YEM (Vincent, 1970). The number of viable cells was counted at 5, 10, 15, 20, 25 and 30^{o}C. The initial reading was taken after one hour of inoculation whereas the final reading was taken after 3 days of inoculation. The cells were counted by viable cell count technique (Shah *et al.,* 1996, Hafeez *et al.,* 1991). Data are collected as mean of three readings and recorded as log of the reading.

Experiment-2. *Rhizobium* legume compatibility

Plant materials and plant growth conditions: Three varieties of *Pisum sativum* viz., AM-I, P-48 and Meteor were used in this experiment. The seeds were obtained from Ayub

Agricultural Research Institute, Faisalabad. The seeds of pea plants were surface sterilized by 0.1% $HgCl_2$ and then washed with sterilized distilled water. The seeds were germinated on water agar plates and then transferred to the growth pouches after 2 days of germination of seeds. The plants were watered with sterilized nitrogen free 1/6 strength Hoagland nutrient solution. The plants were kept in growth chambers under controlled conditions. The inoculum was given in the form of liquid culture (2 mL per plant. 2.1 X 10^9 cells per mL) after four days of germination of seeds.

Optimization of nutrient concentration: Three varieties of pea plants were supplied with different concentrations of Hoagland nutrient solution i.e., $1/2^{nd}$, $1/4^{th}$, $1/6^{th}$ and $1/8^{th}$. The dilutions were made by autoclaved distilled water. All the four strains of *R. leguminosarum* described in Exp-1 were used as inoculum individually. Data are means of four replicates. Readings were recorded as mean of all the three varieties of pea plants.

Determination of photoperiod: Three varieties of pea plants used in above experiments were grown at different photoperiods i.e., 10, 12, 14 and 16 hrs. The plants were grown in growth chambers under controlled conditions of day and night light and watered with $1/6^{th}$ and $1/8^{th}$ strength of Hoagland nutrient solution. All the four strains of *R. leguminosarum* were used as inoculum individually. Data are mean of four replicates. Nodulations time was determined at each photoperiod, the readings are the mean of three varieties.

Effect of low temperature: Three varieties of *Pisum sativum* described in Exp-2 a, were subjected to different temperature i.e., 5, 10, 15, 20 and 25^oC (Day temperatures). The growth conditions were same as mentioned in Exp-2. Four strains of *Rhizobium leguminosarum*, PS-1, PS-2, LC-31 and LC-12 were used to inoculate all the varieties of pea plants individually. Data was collected after 5 weeks of the germination of seeds. Data are the means of four replicate. The control were the plants grown without any inoculum. The night time temperature was adjusted 2 degrees below than the day time temperature.

Effect of salt stress: Same three varieties of *Pisum sativum* and four strains of *Rhizobium leguminosarum* were used in this experiment. The culture maintenance and plant growth procedure were exactly the same as for Exp-1 and 2. The plants were grown at different salinity levels i.e., 0, 1, 2, 3, 4, 5, 10 and 15 dsm^{-1}. The salinity levels were prepared according to the procedure of Hafeez *et al*., 1988. The plants grown at 0 dsm^{-1} were taken as control. For the purpose double distilled water was used. Data was collected after 5 weeks of the germination of the seeds. Data are the mean of four replicate of all the three varieties.

Acetylene reduction activity: *Pisum sativum* root nodules developed at different temperature and salinity levels were assayed for the nitrogenase activity by the method of Hafeez *et al*., (1995) by using FID (flame ionizer detector) gas chromatograph. For this assay 2 weeks old nodules were used. For this purpose the nodules developed at different nodulation time were allowed to be mature for 2 weeks individually from the onset of their specific nodulation time and then picked for the assay. The nitrogenase activity and rate was expressed in n moles h^{-1} g^{-1} nodule dry weight.

Study of infection process and cell occupancy: Three varieties of pea plants grown at different temperatures and salinity levels were studied for root hair curling. After 3 days of inoculation, 4-5 mm long sections were examined for root hair curling. The root sections were double stained with safranine and crystal violet and examined under Ziess Phase contrast light microscope fitted with 35 mm camera at X40 magnification. The plants with inoculation and without any stress were taken as control.

Thick hand sections (80-100 um) of two weeks old nodules, of all the three varieties, developed at different temperatures and salinity levels were cut and fixed in a mixture of paraformaldehyde and gluteraldehyde in 0.1 M phosphate buffer (pH 7.2). The sections were then post fixed in 1% Osmium tetroxide. The sections were stained with 5% Uranyl acetate and then dehydrated by passing through a series of ethanol i.e., 30, 50, 70, 80, 90% and absolute. After dehydration the sections were embedded in Spurr resin (Spur, 1969). Ultrathin plastic sections were cut with the help of ultra microtome and observed under Jeol 1010 transmission electron microscope at 80 K (Hameed, 2003). Photomicrographs were processed.

Results

Experiment 1

Effect of different temperature on the *In vitro* growth of *R. leguminosarum* bv. viciae strain: *In vitro* studies have shown pronounced effect of temperature on the bacterial growth (Table 1). At 5 and 10^{o}C there was no significant increase in the number of cells of the bacteria. At 15^{o}C there was a little increase in the number of cells suggesting that the cells were still growing under stress conditions. At 20, 25 and 30^{o}C there was a significant increase in the number of cells of bacteria of all the strains. Also the growth rate was quite high at these temperatures.

Experiment 2

***Rhizobium* legume compatibility:** Among four strains of *R. leguminosarum* viz., PS-1, PS-2, LC-31 and LC-12 used in this study. PS-1and PS-2 were found to be compatible with all the three varieties of *Pisum sativum* but PS-2 formed very less number of nodules whereas PS-1 strain formed high number of nodules on all the three varieties of peas while LC-12 and LC-31 strains did not form any nodule on any plant. Therefore, only PS-1 strain was selected to carry out all the further studies.

Effect of nutrient concentration on the nodulation: Nodulation was completely inhibited at Hoagland's nutrient concentration of half and one fourth strength whereas better nodulation was observed at one sixth and one-eighth nutrient concentrations after 14 days of inoculation.

Effect of photoperiod on the nodulation: All the three varieties of pea plants at one sixth and one eighth nutrient levels were subjected to different photoperiods to optimize the best photoperiod for the *Rhizobium* pea symbiosis. Results showed that the plants subjected to 12h and 14h photoperiod showed good growth and smaller nodulation time i.e., 14 days, whereas in the plants grown at 10h & 16h photoperiod nodulation was completely absent.

Table 1. Effect of different temperatures on the growth of *Rhizobium leguminosarum* bv *viciae* strains (*In vitro* study).

Strains	5°C		10°C		15°C		20°C		25°C		30°C	
	I	II	I	II	I	II	I	II	I	II	I	II
PS-1	7.52	7.77	8.01	7.90	8.46	9.82	7.23	9.49	7.38	9.38	7.12	9.02
PS-2	7.73	8.01	8.90	7.71	8.26	9.42	6.93	9.04	7.15	8.16	4.99	6.79
LC-12	8.34	8.21	8.32	8.38	7.70	8.96	7.93	9.28	7.32	9.32	7.20	9.40
LC-31	8.02	8.12	6.38	8.54	7.79	7.66	8.07	9.12	7.31	9.31	7.58	9.06

I: Initial reading, II: Finalreading Bact. Cells/mL. Data are mean of three replicates. Cells were harvested 3 days after initial reading. Readings are taken in log.

Table 2. Effect of different temperatures on the nodule numbers per plant, nitrogenase activity and nodulation time in *Pisum sativum* varieties.

Temperature °C	No. of nodules plant^{-1}	Nodule dry wt. grams	n.mole of C_2H_4 $h^{-1}g^{-1}$ nodule dry wt.	Nodulation time days
5	0	0	0	0
10	11	0.99	4.9	30
15	23	1.2	269.5	14
20	23	1.1	250.5	14
25	6	0.1	8.82	22

Values are the means of three varieties of with four replicates each

iv. Effect of temperature: *R. leguminosarum* strain PS-1 was selected as it showed high nodulation for further study. At 5°C nodulation was absent and the overall growth of the plant was completely suppressed. At 10°C the phenotypic growth of plant was better than at 5°C but the mature nodules were observed after 30 days of inoculation and the number of nodules per plant was 11. At 15 and 20°C plants showed excellent growth, the mature nodules appeared after 14 days. The number of nodule per plant was also quite high (23 nodules) at these temperature. At 25°C although the growth of plant was good but the nodules appeared 22 days and the nodule number per plant was reduced (Table 2).

Two weeks old nodules developed at each temperature and salinity levels were assayed for nitrogenase activity. Results did not show a direct correlation between the nodule number and the specific activity at 10 and 25°C. At 10°C nodules showed very low activity i.e., 4.9 nmoles of C_2H_2 h^{-1} g^{-1} of nodule dry weight, whereas at 25°C the activity was 8.2 nmole of C_2H_2 h^{-1} g^{-1}, Although the number of nodules at 10°C was almost double to that at 25°C but the activity at 10°C was almost half to that at 25°C. At 15 and 20°C the nodule number was same but the value of specific activity was slightly higher at 15°C than that at 20°C (Table 2).

Light microscopy at X 40 magnification have shown that at 5°C the root hair development was quite poor. The number of root hair per unit area was quite low. There was no curling at any part of root at this temperature (Fig. 1). At 10°C the number of root hair per unit area was little better as compared to that at 5°C but again no curling was observed at this temperature (Fig. 2). At 15 and 20°C the number of root hair was very high and also a high number of root hair showed tight curling. In some of the root hair the development of infection thread could also be observed (Fig. 4). At 25°C the number of root hair per unit area was high but very little root hair showed curling. Again the curling was rather loose showing delayed in infection process. Swollen root hair tips could easily be seen (Fig. 2).

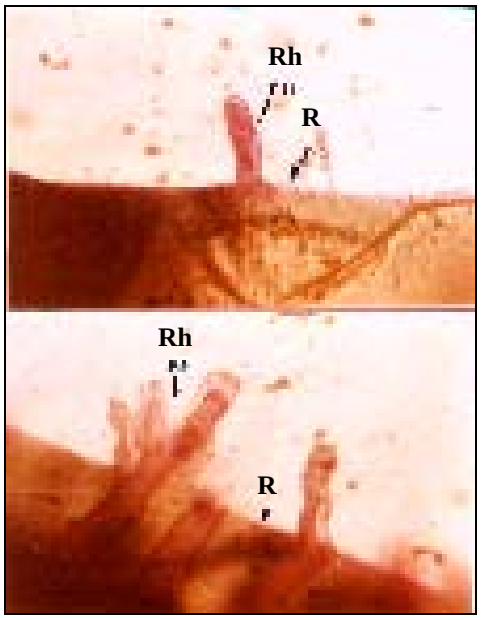

Fig. 1a. *P. sativum* plant roots showing very low number of root hairs (Rh) without curling, developed at 5°C. X 40
Fig. 1b. *P. sativum* plant roots showing very high number of root hairs (Rh) and root (R) with curling, developed at 20°C. X 40

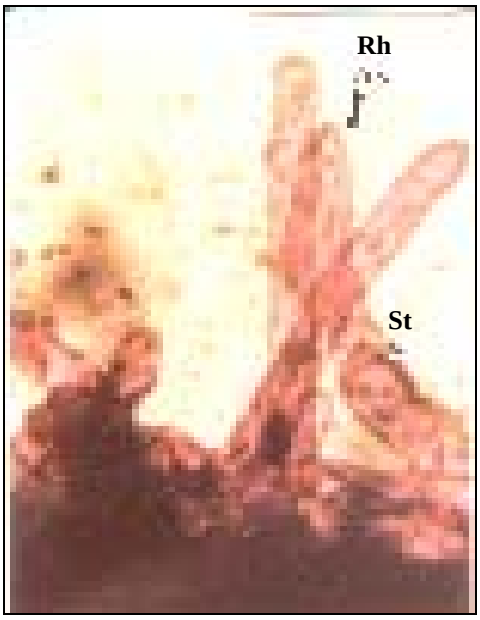

Fig. 2. *P. sativum* plant roots showing root hairs (Rh) and roots ® without curling, developed at 25°C; swollen tips(St) can be seen. X40

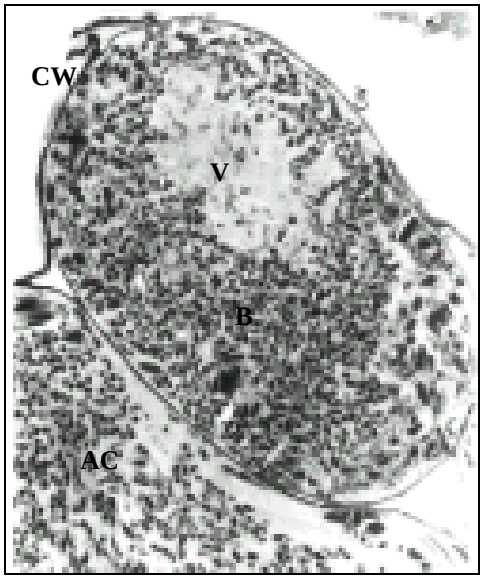

Fig. 3. Photomicrograph of *P. sativum* root nodule cells developed at normal temperature showing bacteroids (B), cell wall (CW) vacuole (V) and adjacent cells (AC). X 80K.

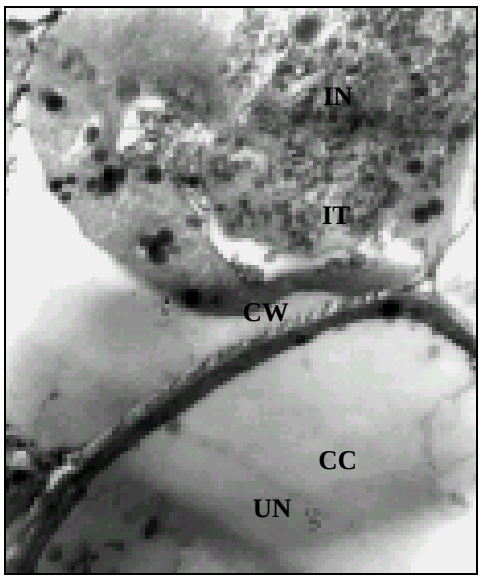

Fig. 4. Photomicrograph of *P. sativum* root nodule cells developed at low temperature showing one infected cell (IN) and infected cell (UC) with under developed infection thread (IT), large number of cell contents (CC) and cell wall (CW) uninfected cell X 80K (UN).

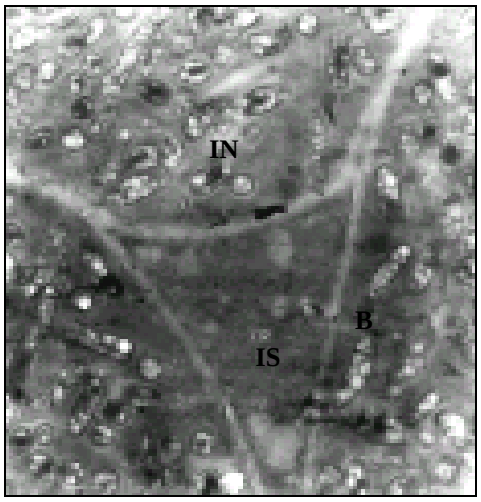

Fig. 5. Photomicrographs of *P. sativum* root nodule cells developed at 0dsm^{-1} salinity level showing many infected cells (IN) with bacteroids (B) and intercellular spaces (IS). X 80K

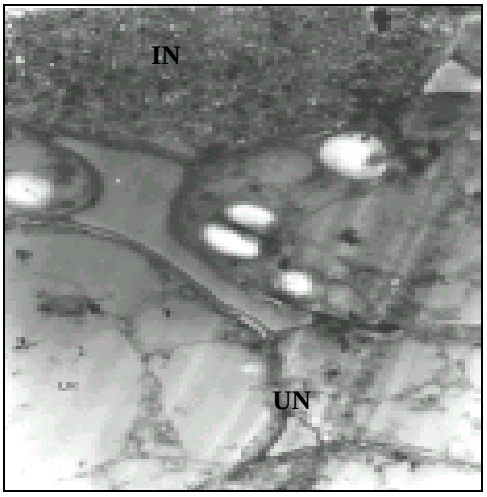

Fig. 6. Photomicrographs of *P. sativum* root nodule cells developed at 1dsm^{-1} salinity level showing many infected cells (IN) and uninfected cells (UN). X 80K

Discussion

Poor nodulation in peas in the field have often been attributed to its symbiosis with non-compatible rhizobial strains established in soils and the optimization of temperature range, photoperiod, salinity levels and nutrient levels of soil. Many studies have already pointed out the importance of plant genotypes to the success of symbiosis (Esseling *et al*., 2004). Therefore, the present study has been carried out to understand the competitiveness problems and other factors affecting the poor nodulation in peas which are often affected by high salt and nutrient concentrations and also the temperature during the pea cropping season is very low in this area.

Biological nitrogen fixation is a complex process and a successful symbiosis depends on the genetic background of both symbiotic partners (Hungria & Bohrer 2000). In the present study three varieties of *Pisum sativum* i.e., P-48, Meteor and AM-I were inoculated with 4 rhizobial strains individually to identify the most compatible strain of *Rhizobium leguminosarum* with all the three varieties of pea plants. Results have shown that only one strain PS-1 showed best nodulation in all the three varieties whereas PS-2 showed poor nodulation and LC-12 and LC-31 that was isolated from the lentil did not form any nodules in any variety. This showed the compatibility difference and highly host specificity of these strains. This was according to the earlier studies, which pointed out the importance of plant genotype to the success of symbiosis (Lewis *et al*., 2005). Several plants factors were considered responsible for the control of symbiotic relationship such as photosynthetic ability, hormonal balance and activity of enzymes related to symbiosis (Nutman, 1981).

Present investigations also confirmed the results of Frings (1976), who reported that 12 hr photoperiod was optimum for best nodulation in pea. Plants grown at 12 and 14 hr photoperiod showed nodulation whereas at 10 and 16 hr photoperiod there was no nodulation at all. At the same time the supply of different nutrient concentrations also showed quite interesting results, where the plants supplied with 1/6th and 1/8th concentration of N-free

Hoagland nutrient solution showed nodulation whereas plants supplied with 1/2 and 1/4th concentration of N-free Hoagland nutrient solution did not show nodulation in peas.

These facts explain the reasons that why pea plants fail to nodulate which are usually rich in nutrients. It is suggested that to get the best nodulation in pea plants the photoperiod and nutrient supply should be adjusted to counter balance the photosynthesis and respiration in pea plants (Frings, 1976).

Low temperature can affect the nodulation at any stage of nodulation process from the increase in the rhizobia population in the rhizosphere of the pea plants upto the nodule maturation and activation of nitrogenase activity. It was reported that attachment of the rhizobia to the roots is adversely effected by temperatures (Frings, 1976). It is generally assumed that rhizobia have to multiply in the rhizosphere to considerable numbers before being able to invade the root hair and reduced multiplication might be involved in early type of inhibition (Manns, 1968). It is assumed that at low temperature the rhizobial population might fail to reach the required density essential for nodulation process to be initiated. The above mentioned assumption is contrary to our findings, which indicates that failure in the attachment of rhizobia to the root hair is not because of the low population in the rhizosphere but it is the poor development of root hair at different temperatures which inhibit the rhizobial attachments. In pea, specific rhizobia are able to attach to more than 95% of the growing root hair, whereas only 25% of these show tight curling (Ek Jander, 1971). Rhizobial cells present on the root surface bring about root hair deformation, before being entering the roots, usually *via* the deformed root hairs, which then pass in an infection thread. In addition to the attachment of the bacteria to the root, the development of root hair is also affected by low temperatures (Hafeez *et al.*, 1995; Serraj, 2003). In the present investigation, it was found that at 5^{o}C not only the root hair development was significantly reduced but also the number of root hair per cm of the root length was quite low. In this case rhizobia had hardly any effect because of their low number in the rhizosphere. At 10^{o}C the root hair development was such that provided a chance to very low number of rhizobial cells to get attached to the roots and cause the root hair curling. At 15 and 20^{o}C the conditions were favorable for the development of root hair and large number of infections was observed. Frings (1976) found that above 22^{o}C the root hair of pea plants acquired a spherical shape or become swollen at their tips. This type of structure inhibits the attachment of rhizobial cells and may be unsuited for the process involved in nodulation. This was also confirmed by the present studies where at 25^{o}C, the rhizobial population was quite high in the rhizosphere, but the nodulating ability of the plant was reduced. The swollen tips of the root hair could easily be observed (Fig. 2), which may be responsible for the failure in the attachment of rhizobia to the root hair. Initial attachment of rhizobia in a root hair is followed by the formation of infection thread containing the invading rhizobia. The present studies have shown the formation of infection thread within nodule cells subjected to low temperature stress, but the rhizobial cells fail to release into the host cells (Fig. 4). On the other hand the nodules where the stress was not applied the cells were full of bacteroids (Fig. 3). These results showed that low temperature slows down the development of infection thread and release of bacteroids within the cells which consequently results into prolonged nodulation time at low temperatures (Table 2). It has been reported that the release of bacteroids from the infection thread is influenced by bacterial genes (Werner, 1992), so it can be assumed that low temperatures suppress some physiological processes in bacteria which inhibit the expression of these genes. Ultrastructural studies have revealed that between the large infected cells filled with bacteroids there are often many smaller non-infected cells, Studies have shown that at temperatures below or suboptimum, the ratio of empty cells to fully occupied cells is variable (Fig. 4). This

suggests that the sub-optimum temperatures affect the growth of bacteroids even within the nodule and also affects the cell to cell movement of the bacteroids. Reduced nitrogenase activity in such nodules can also be explained on the basis of above mentioned fact. At optimum temperatures not only the nodule number was high but also the cell occupancy by the bacteroids was quite high, whereas in nodules developed under stress the number of bacteroids was reduced and these exhibited the reduced nitrogenase activity (Table 2).

Sexana & Rewari (1992), suggested that symbiotic performance of legumes in saline soils depends on salt tolerance of both symbionts. Detrimental effects of salt on growth and survival of rhizobia have been reported by many authors (Zahran 1992; El-Sheikh & Wood 1995; Zou *et al*., 1995; Hafeez *et al*., 1988; Räsänen & Lindström, 2003). Rigand (1987) found that different *Rhizobium* strains can tolerate the salt concentration upto varying degrees. The reason which is explained by different authors, (Botsford & Lewis 1990; Ghittani & Bueno 1995; Breedveld *et al*., 1993) is that rhizobia generally response to increased environmental osmotic pressures by an import of K+ ions and concomitant synthesis and accumulation of glutamic acid in cytoplasm. Den Herder *et al*., (2007) found that changes in osmotic pressure, concentrations and pH etc. changes the structure of lipopolysaccharides of bacteria in response to salt stress and rhizobia accumulate several compatible solutes to overcome the stress induced by salt. Now the question is "Are the rhizobial osmoadaptive responses integrated with these of plants? What is the effect of changing osmotic pressure on the nodule forming ability of pea plants and also on their general growth conditions"?

Answer to these questions may be given by the results shown in the Table 3, where plants not subjected to any salt stress showed good growth and the nodule number per plant was also significantly high. On the other hand plants subjected to even very low salt concentration i.e., 1 and 2 dms^{-1} exhibited shrinkage of leaves just after three weeks of plantations. Whereas plants grown at 4 dsm^{-1} and above salinity levels did not stand even after 1 week of plantation. This suggests the high sensitivity of plants toward salinity. These results have shown that with an increase in salinity, there was a significant decrease in number and diameter of nodules. These results are in accordance to the findings of Qureshi *et al*., (1977) who found a gradual reduction in seed germination and plant height from 0.1 to 1% NaCl salt concentration as compared to controls. These present observations are also according to the recommendations of Soil Society of America that defined the saline soils as having Ec_e value upto $2dsm^{-1}$ or above (Qureshi *et al*., 1977*)*. Reduced nodulation in pea plants under salt stress can be explained on the basis of results obtained during the study of root hair curling at different salinity levels. Results have shown that plant root hair formation is more salinity sensitive than the rhizobial cells. There are reports that *Rhizobium* can survive salinity upto 4.5 to 5.2 dsm^{-1} (Sexana & Rewari, 1992). Whereas even $1dsm^{-1}$ salinity levels affects the root hair formation in pea drastically and upto $4dsm^{-1}$ there were no root hair development at all, which reduced the bacterial attachment. At 2 and 3 dsm^{-1} although the root hair formation was significantly reduced but a few bacterial cells may get the chance to start the initiation which resulted into reduced nodulation. It can be concluded that in moderate saline environment the high sensitivity of the host towards salinity is the major factor in poor nodulation. The ultrastructure of nodules examined under electron microscope showed that salinity also affects the cell occupancy of bacteroids (Figs. 5, 6). This suggests that not only the growth of the bacteroids but also their infection within the cell is greatly effected by salinity. The results of acetylene reduction assay also explains the above mentioned facts. At high salinity levels the number of bacteria was very low, hence their nitrogenase activity was very low and *vice versa* (Table 3).

Table 3. Effect of different salinity levels on the nodule numbers per plant nitrogenase activity and nodulation time in *Pisum sativum* varieties.

Salinity dsm^{-1}	Nodule number $plant^{-1}$	Nodule dry weight. (grams)	n moles of C_2H_2 $h^{-1}g^{-1}$ nodule dry wt.	Nodulation time days
0	20	1.0	215.5	14
1	15	0.7	200	14
2	6	0.1	44.6	14
3	3	0.05	8.9	21
4	-		-	-
5	-		-	-
10	-		-	-
15	-		-	-

Values are the means of three varieties of with four replicates each.

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References

Biosca, E.G., C. Amaro, E. Marto–Noales and J.D. Oliver. 1996. Effect of low temperature on starvation-survival of cell pathogen *Vibrio vulnificus* biotype 2. *Appl. Environ Microbiol*., 62: 450-455.

Botsford, J.L. and T.A. Lewis. 1990. Osmoregulation in *Rhizobium meliloti*. Production of glutanic acid in response to osmotic stress. *Appl. Environ. Microbiol*., 56: 533-540.

Breedveld, M.W., C. Dijkema, L.P.T.M. Zevenhuizen and A.J.B. Zehnde. 1993. Response of intercellular carbohydrates to a NaCl shock in *R. leguminosarum* biovar *trifolii* TA-I and *R. meliloti* Sci-47. *J. Microbiol*., 139: 3157-3163.

Delvin, R. 1975. *Plant physiology.* 3rd edition. D Van Nostrand Company, New York 600p.

Denison, R.F. and E.T. Kiers. 2004 Lifestyle alternatives for rhizobia: mutualism, parasitism, and forgoing symbiosis. *FEMS Microbiol Lett*., 237(2): 187-93.

Den Herder, J., C. Vanhee, R. De Rycke, V. Corich, M. Holsters and S. Goormachtig. 2007. Nod factor perception during infection thread growth fin-tunes nodulation. *Mol Plant Microbe Interact*, 20: 129-137.

EK-Jander, J. and G. Fakraeus. 1971. Adaptation of *Rhizobium* to sub-arctic environment in Scandinavia. *Plant and Soil Special*, 129-137.

El-Sheikh, E.A. and M. Wood. 1995. Nodulation and N_2-fixation by Soybean inoculated with salt tolerant rhizobia or salt sensitive bradyrhizobia in saline soil. *Soil Biol. Biochem.*, 27: 657-661.

Esseling, J.J., F.G.P. Lhuissier and A.M.C. Emons. 2004. A nonsymbiotic root hair tip growth phenotype in NORK mutated legumes: implications for nodulation factor–induced signaling and formation of a multifaceted root hair pocket for bacteria. *Plant Cell*., 16: 933-944.

Frings, J.F.J. 1976. *The Rhizobium pea symbiosis as affected by high temperatures*. Thesis: Medelingen Landbouwhoges School, Wageningen, Netherland.

Gage, D.J. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiol Mol Biol Rev*., 68: 280-300.

Ghittani, N. and M.A. Bueno. 1995. Peanut rhizobia under salt stress: role of trehalose accumulation in strain ATCC 51466. *Can. J. Microbiol*., 22: 41-85.

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.) Wilckzek. *Plant and Soil*, 106: 3-8.

Hafeez, F.Y., M.A. Khan, S. Hameed, E. Rasul and K.A. Malik. 1995. Use of Gus marked *Rhizobium* and *Bradyrhizobium* strains for studying the effect of temperature on infection process. *Pak. J. Bot*., 27(1): 55-62.

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(2): 285-294.

Hafeez, F.Y., N.H. Shah and K.A. Malik. 2000. Field evaluation of lentil cultivars inoculated with *R. leguminosarum* bv. *viciae* strains for nitrogen fixation using nitrogen-15 isotope dilution. *Biol Fertil Soils*, 31: 65-69.

Hameed, S. 2003. *Molecular characterization and significance of rhizobial exopolysacchrides*. Ph.D. Thesis. NIBGE/ Quaid-i-Azam University. Islamabad, Pakistan.

Hungaria, M. and T.R.J. Bohrer. 2000 Variability of nodulation and dinitrogen fixation capacity among soybean cultivars. *Biol Fertil Soils*, 31: 45-52.

Lipsanen, P. and Lindstrom. 1986. Adaptation of red clover rhizobia to low temperatures. *Plant and Soil*, 92: 55-62.

Lewis, G., B. Schrire, B. MacKinder and M. Lock. 2005. *Legumes of the world, Kew: Royal Botanic Gardens*. ISBN 1 900 34780 6.

Räsänen, L.A. and K. Lindström. 2003. Effects of biotic and abiotic constraints on the symbiosis between rhizobia and the tropical leguminous trees: *Acacia* and *Prosopis*. *Ind. J. Exp Bot*., 41: 1142-1159.

Manhan, S.H. and T.R. Steck. 1997. The viable but non culturable state in *Agrobacterium tumefaciens* and *Rhizobium meliloti FEMS. Microbiol. Ecol*., 22: 29-37.

Manns, D.N. 1968. Nodulation of *Medicago sativa* in solution culture. 1. Acid sensitive steps. *Plant and Soil*, 28: 129-146.

Miller, K.J. and J.M. Wood. 1996. Osmoadaptation by rhizosphere bacteria. *Annual Rew of Microbiol*., 50: 101-136.

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

Newcomb, W., D. Sipell and R.L. Peterson. 1979. The early morphogenesis of *Glycine max* and *Pisum sativum* root nodules. *Can. J. Bot*., 57: 2603-2616.

Nutman, P.S. 1981. Hereditary host factors affecting nodulation and nitrogen fixation .In: *Current perspectives in nitrogen fixation*. (Eds.): A.H. Gibson & W.E. Newton. Academy of Science, Canberra, pp. 194-204.

Qureeshi, R.H., M. Saleem, Z. Aslam and G.R. Sandhu. 1977. An improved gravel culture technique for salt tolerance studies in plant. *Pak. J. Agri. Sci.*, 14(2-3): 11-18.

Rasool, E., I. Ali, F.Y. Hafeez, K.A. Malik and A.N. Ahmed. 1987. Effect of salinity and *Rhizobium* inoculation on nodulation, nitrogen fixation and yield of *Vigna radiata* (L.) Wilczek, In: *Mod.Trends Pl. Sci. Pak.* (Ed.): I. IIahi. Dep. Bot. Univ. Peshawer, pp 121-125.

Rigand, J. 1987. Salt tolerance of *Medicago* nodules and bacteroids, In: *Plant Genes involved in Nitrogen Fixation and productivity of Alfalfa*, USDA-INRA Workshop, Auzeville.

Roughly, R.J., P.J. Dart, P.S. Nutman and C. Rodriguez-Burrueco. 1970. The influence of root temperature on root hair infection of *Trifolium subterraneum* L., by *Rhizobium trifolli* Dang. *Proc. 11th Int. Grasslands Congr*., pp. 451-455.

Saxena, A.K. and R.B. Rewari. 1992. Differential responses of chickpea (*Cicer arientinum* L.) *Rhizobium* combination to saline soil conditions. *Biol Fertil Soils*, 13: 31-34.

Serraj, R. 2003. Effects of drought stress on legume symbiotic nitrogen fixation: Physiological mechanisms. *Ind. J. Exp Bot*., 41: 1136-1141.

Shah, N.H., F.Y. Hafeez, S. Asad, A. Hussain and K.A. Malik. 1995. Isolation and characterization of indigenous *Rhizobium leguminesarum* bv *vicia* nodulating *Lens culinaris* Medik. from four Pakistani soils. In: *Biotechnology for Sustainable Development*. (Eds.): K.A. Malik, A. Nasim & A.M. Khalid. NIBGE, Faisalabad, Pakistan, pp. 211-219.

Shah, N.H., F.Y. Hafeez, A. Hussain and K.A. Malik. 1996. Influence of seasonal variation on the indigenous ability of introduced rhizobia in lentil. *Lens Newsletter*, 23(1/2): 32-37.

Sprent, J.I. 1984. Effects of drought and salinity on hetrotrophic nitrogen fixing bacteria and on infection of legumes by rhizobia. In: *Advances in nitrogen fixation research*. (Eds.): C. Veeger & W.E. Newton. Martinus Nijhoff/Drw Junk, The Hague, pp. 295-302.

Subba Rao, N.S., M. Lakshmi-Kumari, C.S. Singh and S.P. Magu. 1972. Nodulation of lucern (*Medicago sativa* L.) under the influence sodium chloride. *Indian J. Agric Sci*., 42: 384-386.

Tu, J.C. 1981. Effect of salinity on *Rhizobium* root hair interaction, nodulation and seed growth of soybean. *Can. Ja. Plant Sci*., 61: 231-239.

Vincent, J.M. 1977. *Rhizobium* General biology. In: *A treatise on Dinitrogen Fixation Section-III*. (Eds.): R.W.F. Hardy and W.S. Silver. John Willey and Sons, New York. pp. 237- 266.

Werner, D. 1992. Physiology of nitrogen fixing legume nodules-compartments and functions. In: *Biological Nitrogen Fixation*. (Eds.): G. Stacey, R. Birris and H.J. Evans, Chapman Hal, New York pp. 399-341.

Young, J.P.W., L.C. Crossman, A.W.B. Johnston, N.R. Thomson, Z.F. Ghazoui, K.H. Hull, M. Wexler, A.R.J. Curson, J.D. Todd, P.S. Poole, T.H. Mauchline, A.K. East, M.A. Quail, C.I. Churcher, C. Arrowsmith, I. Cherevach, T. Chillingworth, K. Clarke, A. Cronin, P. Davis, A. Fraser, Z. Hance, H. Hauser, K. Jagels, S. Moule, K. Mungall, H. Norbertczak, E. Rabbinowitsch, M. Sanders, M. Simmonds, S. Whitehead and J. Parkhill. 2006. The genome of *Rhizobium leguminosarum* has recognizable core and accessory components. *Genome Biol*., 7: 1-20.

Zahran, N.H. 1992. Conditions for successful *Rhizobium* legume symbiosis in saline environments. *Biol Fertil Soils*, 12: 73-80.

Zou, N., P.J. Dart and N.E. Marcar. 1995. Interaction of salinity and Rhizobial strain on growth and nitrogen fixation by *Acacia ampliceps. Soil Biol. Biochem*., 27: 409-419.

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