Soil & Environ. 26(1): 81-91, 2007

Isolation and screening of rhizobia for improving growth and nodulation of lentil (*Lens culinaris* Medic) seedlings under axenic conditions

M. Zafar-ul-Hye, Z.A. Zahir*, S.M. Shahzad, U. Irshad and M. Arshad
Institute of Soil & Environmental Sciences, University of Agriculture Faisalabad-38040, Pakistan

Abstract

A screening experiment to evaluate the effectiveness of rhizobia for lentil was carried out under controlled environment. Using dilution plate technique, seventy five cultures of rhizobia were isolated. A pouch experiment was conducted for screening of efficient cultures. Results of pouch study showed that inoculation with selected cultures increased the root length, shoot length, root weight, shoot weight, root dry weight and shoot dry weight of lentil up to 135, 82, 175, 161, 150 and 140%, respectively over uninoculated control, while up to 11 nodules plant^{-1} were recorded in case of inoculation with rhizobial cultures. Results indicated that screening of the effective cultures of rhizobia for growth promotion under gnotobiotic conditions could be a useful approach for selecting the efficient cultures before testing their potential under field conditions.

Key words: Screening, rhizobium, axenic conditions, lentil

Introduction

In the recent years, use of biological techniques for sustainable crop production is attaining popularity in various parts of the world. The rhizobium-legume symbiosis has been examined extensively. Biological N_2-fixation represents the major source of N input in agricultural soils including those in arid regions. The major N_2-fixing systems are the symbiotic systems, which can play a significant role in improving the fertility and productivity of low-N soils (Almendras and Bottomley, 1987; Abaidoo *et al*., 1990)

Nodules-the sites for symbiotic nitrogen fixation are formed as a result of series of interactions between rhizobia and leguminous plants. However, there are number of factors which affect the nodulation on legume roots including host micro-symbiont compatibility, physicochemical conditions of the soil and presence of both known and unknown bio-molecules such as flavonoides, polysaccharides and hormones (Antoun *et al*., 1978; Tisdale *et al*., 1990). It is a molecular dialogue between the host plant and a compatible strain of rhizobium, which serves as an initiate or of the development of nodules (Murray *et al*., 2007). The rhizobial infection begins, when the rhizobium enters the root in a host-controlled manner (Limpens *et al*., 2003).

A critical aspect of rhizobium-legume association is the fact that it is often manipulated under nitrogen limiting field conditions in such a way that crop production could be enhanced easily and inexpensively (Hubbell *et al*., 1979; Freiberg *et al*., 1997). The amount of nitrogen fixed, depends upon the presence of effective nodules on the host roots which are a prerequisite for the potential gain of nitrogen from the system. Although, the rhizobia commonly occur in soils but often fail to cause nodulation, either because of some unspecified type of antagonism that prevents root colonization by the rhizobial strain (Jadhav et *al*., 1994). Inoculation is a management practice by which the rhizobium-legume symbiosis is exploited through overcoming nodulation failure or ineffective nodulation. Bermer *et al*. (1990) carried out a screening program to evaluate the effectiveness of *R. leguminosarum* for lentil. They initially carried out experiments under controlled environments followed by evaluation under field conditions. In two separate growth room experiments, the effectiveness of 185 and 24 different strains of *R. leguminosarum* were tested for Laird and Eston varieties of lentil. After 5 to 7 weeks, the study revealed that the strains were significantly different in number of nodules, shoot weight, nitrogenase activity and plant height. In field, both varieties were tested at two different locations. Inoculation increased yield up to 135% and total N_2 fixed ranged from 0 to 76 and 0 to 105 kgha^{-1}, respectively. Nitrogen fixing activity was site specific and higher soil NO_3-levels resulted in lower N_2-fixing activity. However, total plant weight and total N of lentil grown under growth conditions were highly correlated with field parameters and were most reliable screening parameters for the selection of superior rhizobial stains (Moawad and Beck, 1991; Shah *et al*., 1994). The isolation and screening of highly efficient and competitive strains from native rhizobial population to be used as inoculum proves much beneficial as the strains most competitive and persistent in particular field environment are often those isolated from similar environments (Chatel and Greenwood, 1973).

Keeping in view the above discussion, a pouch study was conducted to isolate and screen the indigenous rhizobial cultures from lentil crop growing at different locations of the Punjab.

*E-mial: bio@fsd.paknet.com.pk.

Materials and Methods

Collection of nodule samples from lentil, growing at different locations

Lentil root samples were collected from Faisalabad, Layyah, Sialkot and Gujranwala districts of the Punjab. The root samples were collected, saved in polythene bags and transferred to the laboratory.

Isolation of rhizobial cultures

In the laboratory, the roots were washed gently with tap water to remove the soil. Then nodules were separated from the roots and placed in petri-plates. The collected nodules were surface sterilized by momentary dipping in 95% ethanol solution followed by dipping in 0.2% $HgCl_2$ solution for 3-5 minutes and 6-7 times washings with sterilized water. The surface sterilized nodule were crushed in a minimal volume of sterilized water with the help of a sterilized glass rod to obtain a milky suspension. A loopful of the suspension was streaked out on yeast extract mannitol (YEM) agar medium plates and incubated at 28 ± 1 ^{0}C. Well isolated single colonies were picked and restreaked on clean plates to obtain the pure cultures. In this way, seventy five rhizobial cultures were isolated from the lentil nodules. . The collected and purified rhizobial cultures were coded as Z1, Z2 ……. Z75. These cultures were stored at 4 ± 1 ^{0}C on slants and maintained for further experimentation. Out of these seventy five cultures, Z1 to Z19 were from Faisalabad, Z20 to Z39 from Layyah, Z40 to Z56 from Sialkot, and Z57 to Z75 from Gujranwala districts of the Punjab.

Screening of rhizobial cultures under gnotobiotic conditions

A laboratory experiment was conducted in the growth room for screening of the rhizobial cultures under gnotobiotic conditions. Liquid broth was prepared by using YEM medium. The test tubes containing 60 mL of medium were inoculated with rhizobial cultures and incubated at 28±1^{0}C for three days. An optical density of 0.5 recorded at a wavelength of 535 nm, was achieved by dilution to maintain uniform cell density (10^8-10^9 CFU mL^{-1}).

Lentil (*Lens culinaris* Medic) cv. Masoor-93 seeds were surface-sterilized by momentary dipping in 95% ethanol solution followed by dipping in 0.2% $HgCl_2$ solution for 3-5 minutes and 6-7 thorough washings with sterilized water (Russel *et al*., 1982). Surface-sterilized lentil seeds were inoculated by dipping for five minutes in the broth of respective rhizobial culture prepared as explained above. Three inoculated seeds were placed in each sterilized (autoclaved) growth pouch. In case of control, seeds were dipped in sterilized YEM broth. Modified nitrogen free sterilized Hoagland solution was used for nutrients supply (Fahraeus, 1957). Each treatment was replicated thrice. Growth pouches were placed in growth room at 20±1 ^{0}C adjusted to 10.0 hours light and 14.0 hours dark period. After sixty days of sowing, data regarding root length, shoot length, total biomass, fresh root weight, fresh shoot weight, oven dry root and shoot weight and number of nodules $plant^{-1}$ were recorded. Standard error of means were calculated (Steel and Torrie, 1980).

Ten promising rhizobial cultures with maximum effect on seedling growth were selected for further experimentation.

Results

The rhizobial cultures were tested in growth pouches to assess their effects on seedling growth and nodulation of lentil under gnotobiotic condition. The results showed that inoculation with rhizobia increased shoot length ranging from 9.1 to 81.8%, as compared to uninoculated control (Figure 1). The rhizobial culture Z22 gave highest increase in shoot length i.e. 81.8% higher than the control. Ten most promising cultures (Z1, Z3, Z4, Z7, Z9, Z14, Z22, Z23, Z38 and Z45) increased the shoot length from 64 to 81.8%, as compared to the control. Forty three cultures were less effective but still upto 47.5% increase in shoot length, over control was recorded.

Data revealed that the same ten rhizobial cultures out of seventy five, gave higher increase in root length, which ranged from 96.5 to 135.9%, over uninoculated control (Figure 2). Twenty three cultures showed moderate increase in root length which was upto 92.5%, as compared to the control. Remaining forty two rhizobial cultures produced comparatively less increase in root length that was upto 59.5% higher than the control.

The results showed that inoculation with rhizobial cultures increased fresh shoot weight ranging from 11 to 161%, as compared to the control (Figure 3). The highest increase (161%) was recorded in case of inoculation with the rhizobial culture Z22. Thirty six cultures gave moderate increase in the shoot weight that was upto 116.7%, over control. Twenty nine cultures were less effective but still 55.6% increase in shoot weight, over control was observed.

An increase in fresh root weight over control was recorded in case of inoculation with ten rhizobial cultures, ranging from 137 to 175% (Figure 4). Seventeen cultures enhanced fresh root weight that ranged from 62 to 106% higher than the control, while remaining forty eight rhizobial cultures gave relatively less increase in root weight, which was upto 59.9% higher as compared to the control.

The results revealed that ten rhizobial cultures were the most effective in increasing shoot dry weight from 120 to 140%, over control (Figure 5). Thirty two microbial cultures increased the shoot dry weight up to 100%. Remaining thirty

three rhizobial cultures gave lesser increase in shoot dry weight, which was 20 to 40% higher as compared to the control.

The results showed that the rhizobial inoculation was effective in increasing root dry weight as compared to the control (Figure 6). Ten rhizobial strains were categorized as the most promising cultures. They increased the root dry weight from 125 to 150%, over control. The culture Z22 exhibited highest increase (150%) in root dry weight. Thirty cultures increased dry root weight up to 100%. While remaining rhizobial cultures gave lesser increase in root dry weight, which was 25 to 47.5% higher than the control.

The data regarding number of nodules showed that inoculation with rhizobia was effective for producing nodules in lentil seedlings (Figure 7). In case of inoculation, higher number of nodules was observed in ten rhizobial cultures which ranged from 6.0±0.5 to 11.0±1.2 as compared to the control. Inoculation with rhizobial culture Z22 produced maximum number of nodules. After that, twenty four rhizobial cultures produced 4 to 5 nodules per plant. Twenty eight rhizobial cultures, on an average produced 3 nodules per plant. No nodulation was recorded in thirteen microbial cultures as well as in case of uninoculated control.

Discussion

Agriculture depends heavily on biologically fixed nitrogen from the symbiotic association between rhizobia and plants. Therefore, a laboratory study was conducted to isolate rhizobial cultures and subsequently screening of selected cultures for growth promoting activity in lentil in the growth room under axenic conditions.

The results of the study revealed that almost all the rhizobial cultures showed the growth promoting activity in lentil but with variable efficacy. In pouch trial, in which seventy five rhizobial cultures were tested, the inoculation gave upto 82, 135, 160 and 175% increase in shoot length, root length, shoot weight and root weight respectively, over control. Rhizobial cultures produced upto 11 nodules per plant while no nodulation was recorded in the control. Some of the rhizobial cultures did not produce any nodules.

In addition to (better) nodulation, certain rhizobial cultures also produced considerable increase in shoot length and shoot weight and root length and root weight in lentil seedlings as compared to the control. The reason behind might be the production of some of the phytohormones, additional to the positive responses on the seedlings growth exhibited by the nitrogen- fixing interactions (Sevilla *et al*., 2001).

As far as increase in shoot fresh weight and root fresh weight is concerned, similar work was conducted by Chanway *et al*. (1989). They isolated certain strains of rhizobia from lentil seedlings and obtained the similar results. The similar data were reported by Yanni (1992). He observed the response of lentil, chickpea and pea. Pal *et al*. (2000) also reported the same trend in the results in case of root dry weight and shoot dry weight. They stated that inoculation with rhizobium increased the growth, yield and nutrient uptake significantly in pot and field experiments. Comparable results were reported by Sindhu *et al*. (1999). It is concluded from the data and the above discussion that growth of lentil seedlings can be enhanced by inoculating the seeds with the effective rhizobial strains.

It is evident from the data that certain rhizobial strains produced upto 11 nodules per plant. Yahalom *et al*. (1987) also conducted an experiment and observed similar results. The rhizobial infection starts, when the bacterium enters the root in a host-controlled manner. In the legumes, the rhizobium becomes trapped in a cavity formed by the curling of root hair. The root hair plasma membrane invaginates the cavity and a tube like structure is formed by which the rhizobium enters the plant and reaches the base of the root hair. Consequently, the infection thread reaches a nodule primordium in the cortex of the root that develops into a nodule, upon release of the rhizobium (Limpens *et al*., 2003).

On the other hand, no nodulation was recorded in thirteen microbial isolates. Paul and Verma (1999) also reported no nodulation in case of inoculation with some of the rhizobial cultures. The reason behind failure of nodulation might be some unspecified type of antagonism that prevented the colonization of root surfaces by rhizobium (Jadhav *et al*., 1994). Sometimes no nodulation occurs in spite of inoculation with certain rhizobial cultures, because the strains used in such cases, become exopolysaccharide deficient due to mutation or any unspecified reason (van Rhijn *et al*., 2001).

It is concluded from the data and the above discussion that growth of lentil seedlings can be enhanced by inoculating the seeds with the effective and compatible rhizobial strains. Moreover, by screening under axenic conditions, comparatively more efficient, effective and infective rhizobial strains could be obtained. On the other end of the spectrum, using rhizobia to improve nodulation and biological nitrogen fixation, might be an economical and environmental friendly technique to obtain increased and better yield of lentil and the other leguminous crops.

Acknowledgement

We are thankful to the Higher Education Commission (HEC), Pakistan for funding this research project.

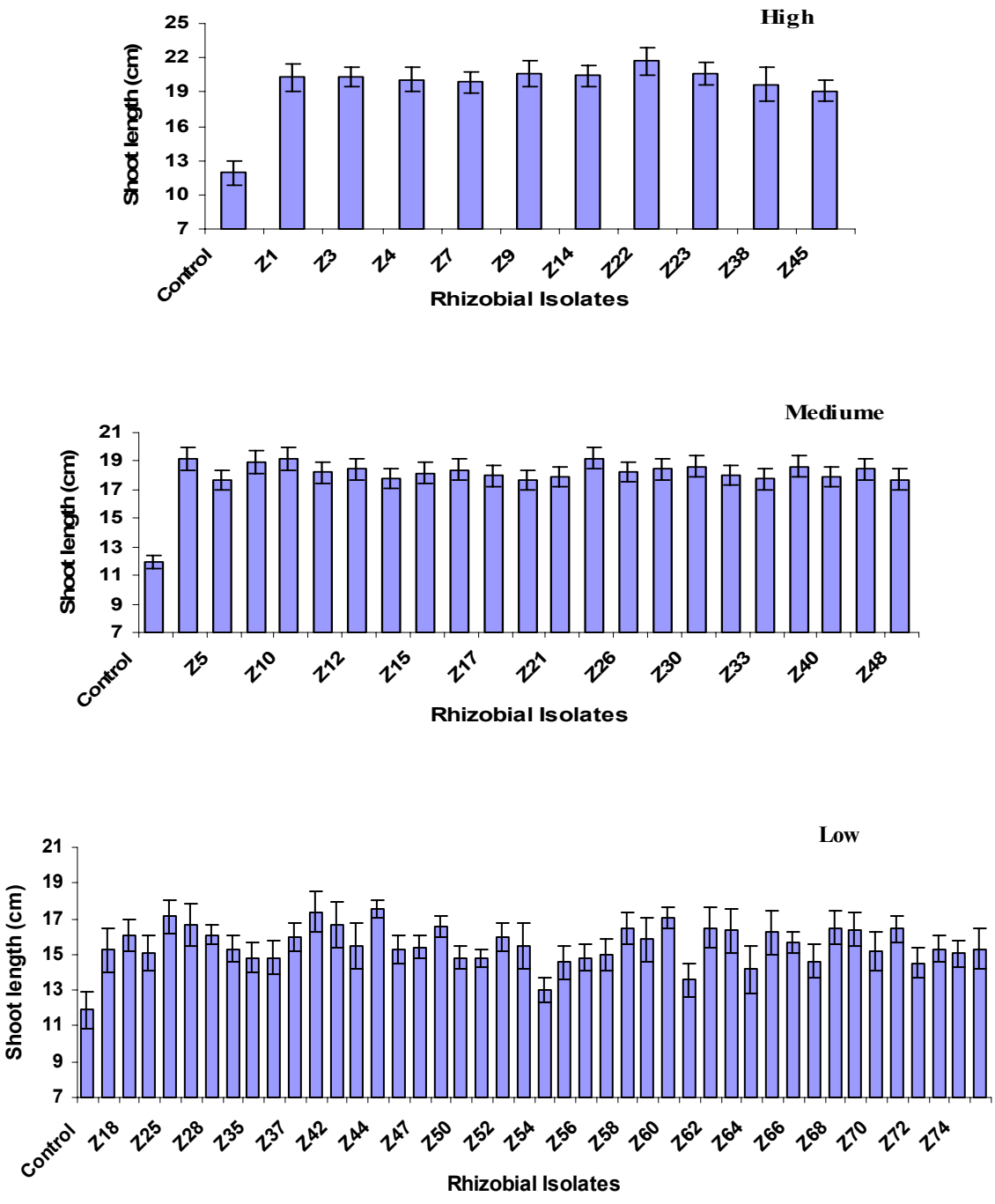

Figure 1. Potential of different rhizobial isolates for improving growth of lentil seedlings under axenic conditions.
High: 61.0 - 82.0% increase over control
Medium: 49.0 - 60.0% increase over control
Low: 9.1- 47.5% increase over control

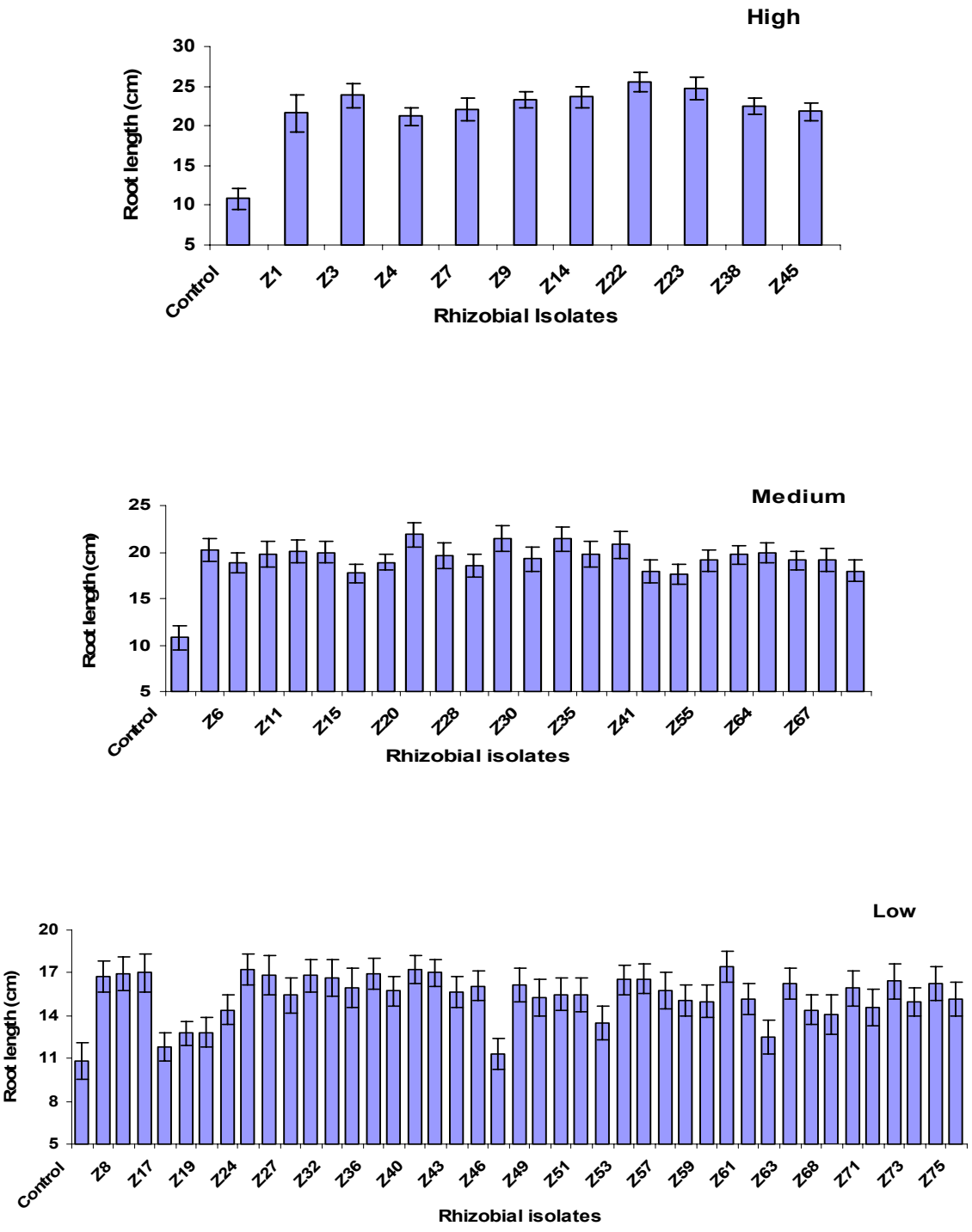

Figure 2. Potential of different rhizobial isolates for improving growth of lentil seedlings under axenic conditions.
High: 96.5 - 135.9% increase over control
Medium: 60.0 - 92.5% increase over control
Low: 11.5 - 59.5% increase over control

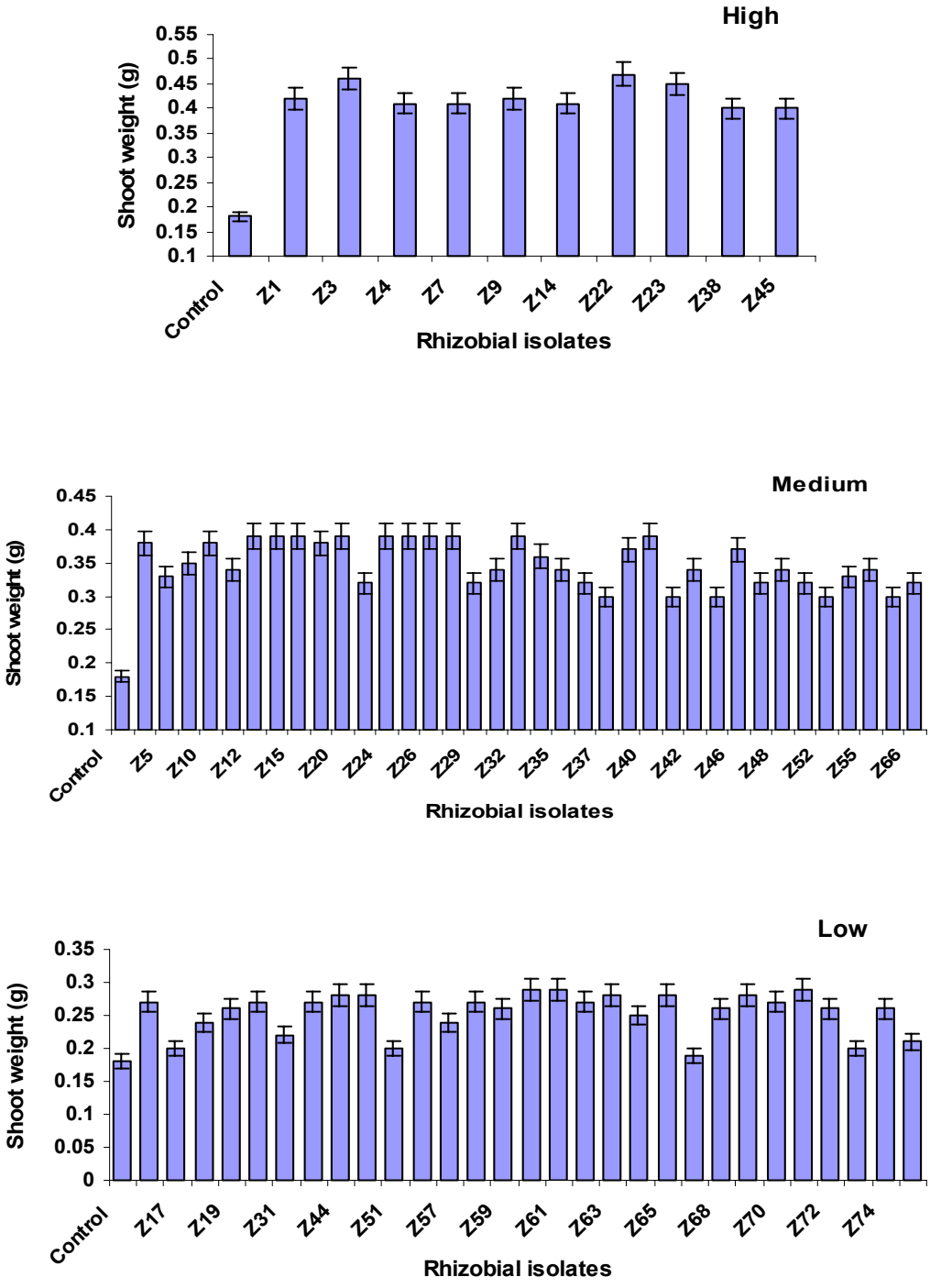

Figure 3. Potential of different rhizobial isolates for improving growth of lentil seedlings under axenic conditions.
High: 129.0 - 161.0% increase over control
Medium: 60.0 - 116.7.0% increase over control
Low: 11.0 - 55.6% increase over control

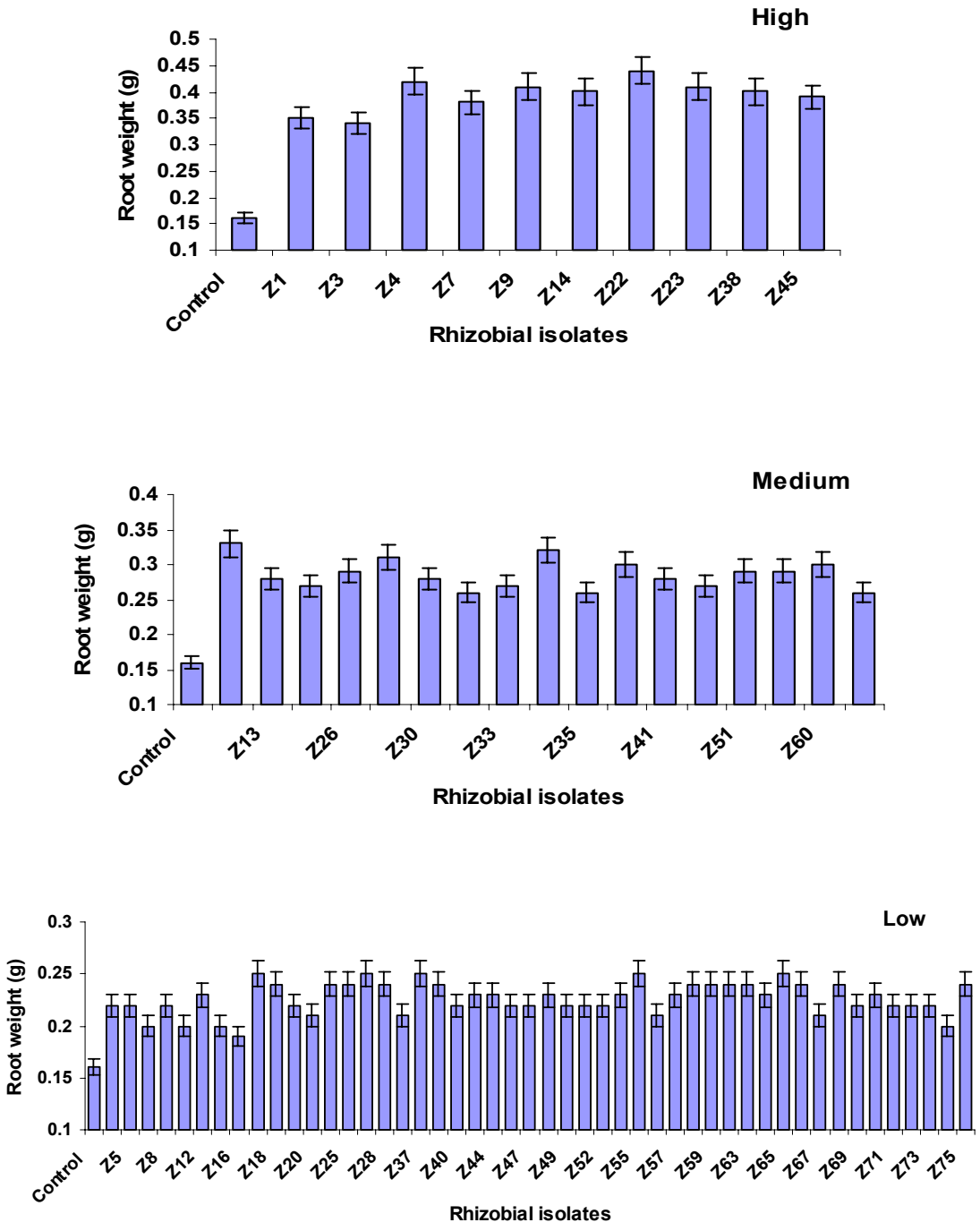

Figure 4. Potential of different rhizobial isolates for improving growth of lentil seedlings under axenic conditions.
High: 137.0 - 175.0% increase over control
Medium: 62.0 - 106.5.0% increase over control
Low: 19.5 - 59.9% increase over control

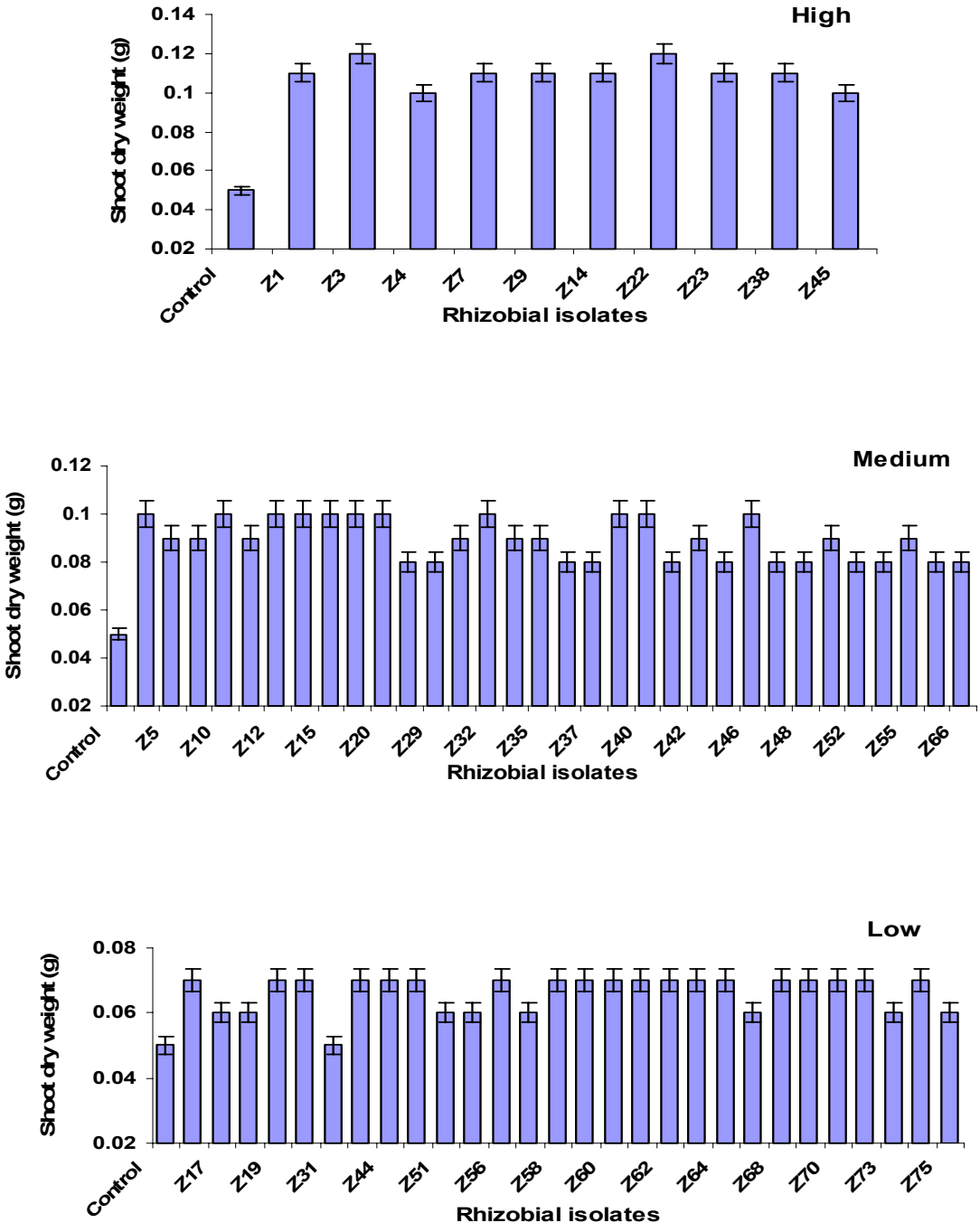

Figure 5. Potential of different rhizobial isolates for improving growth of lentil seedlings under axenic conditions.
High: 120.0 - 140.0% increase over control
Medium: 60.0 - 100.0% increase over control
Low: 20.0 - 40.0% increase over control

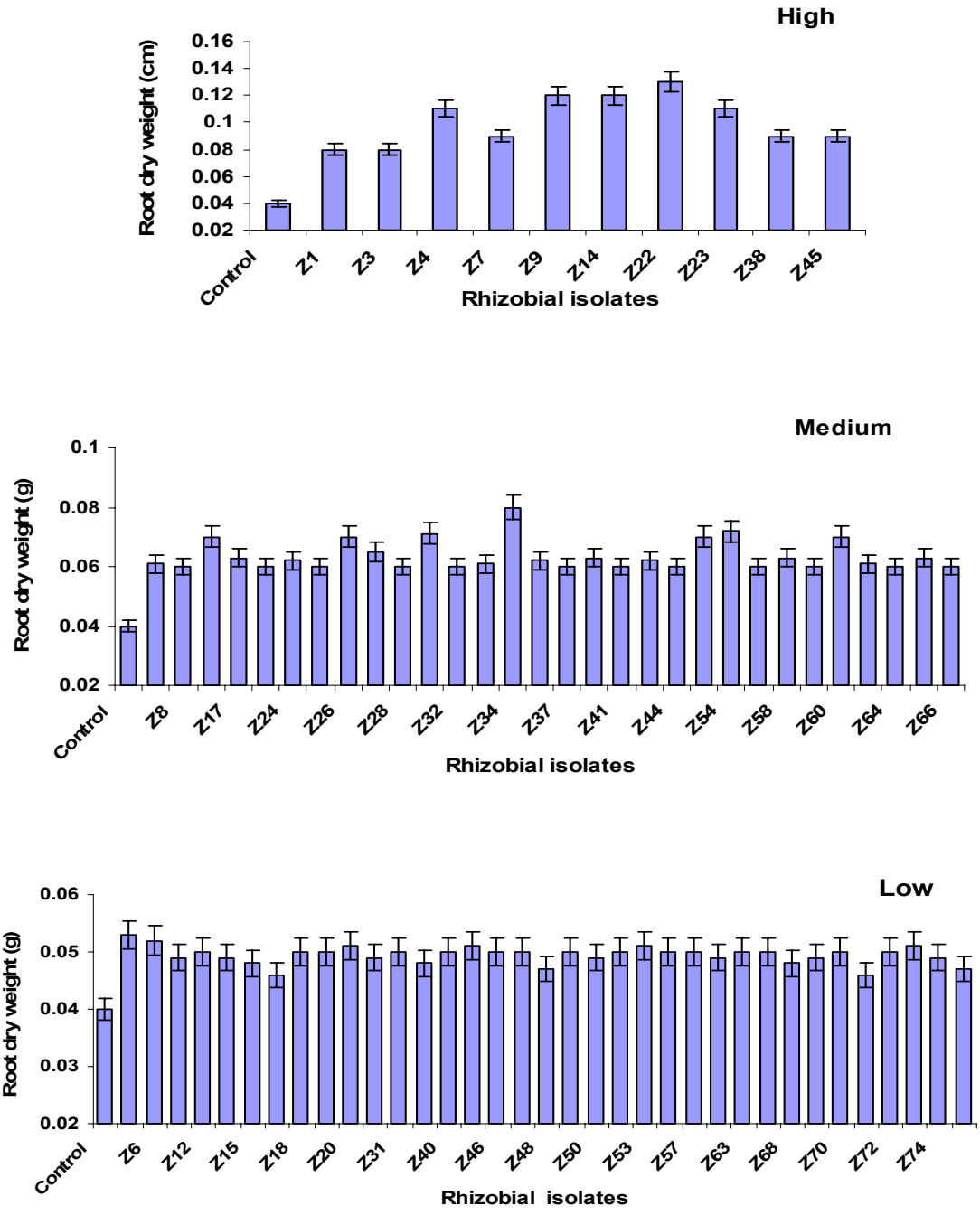

Figure 6. Potential of different rhizobial isolates for improving growth of lentil seedlings under axenic conditions.
High: 125.0 - 150.0% increase over control
Medium: 61.0 - 100.0% increase over control
Low: 25.0 - 47.5% increase over control

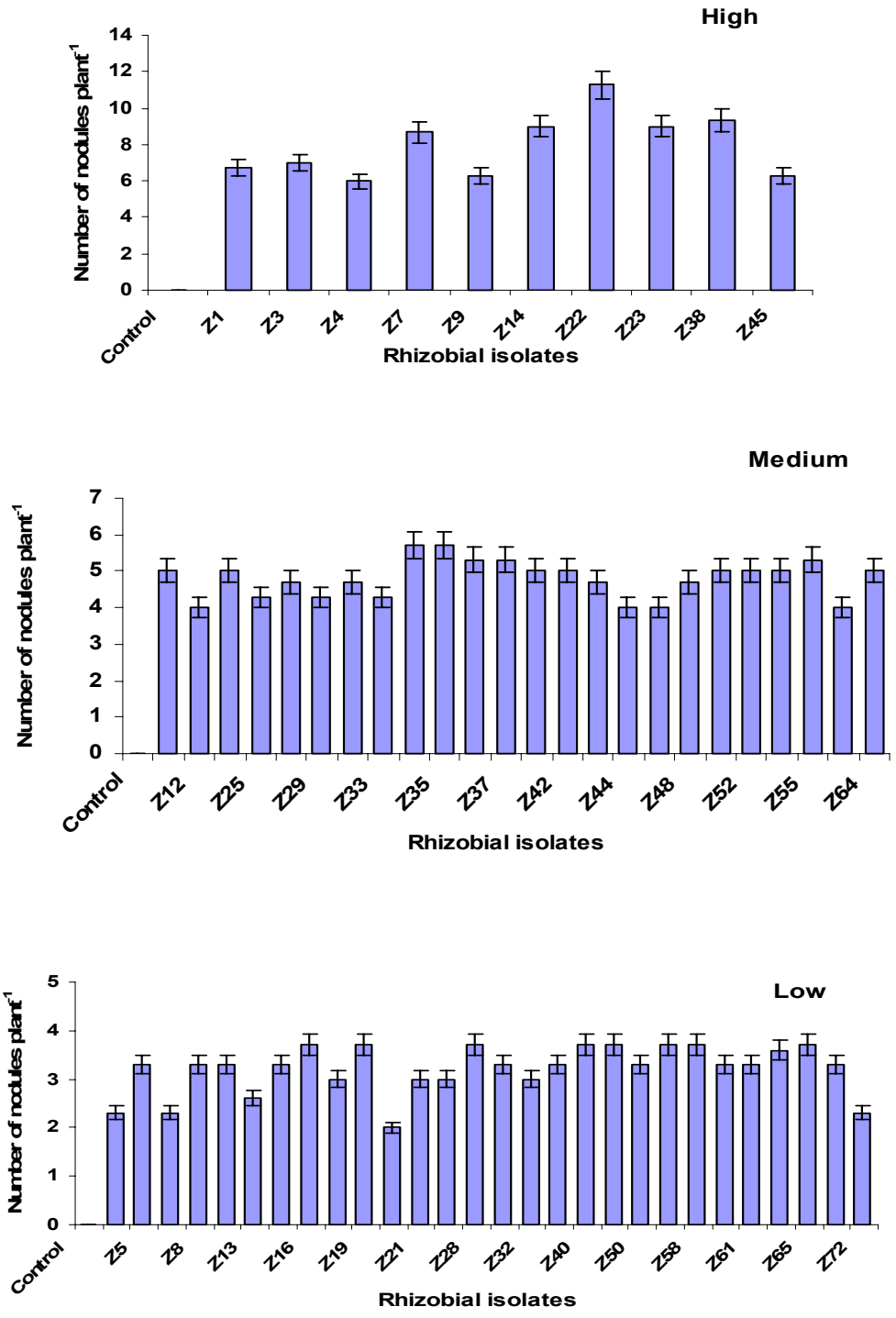

Figure 7. Potential of different rhizobial isolates for improving growth of lentil seedlings under axenic conditions.
High: 6.0 - 11.0% increase over control
Medium: 4.0 - 5.0% increase over control
Low: 2.0 - 3.0% increase over control

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