

**Interaction of Rhizosphere, nodule microflora and seed protectants with *Rhizobium leguminosarum* incitant of Pea (*Pisum sativum* L.) root nodules**

मटर की जड़गंधियों के प्रेरक राइजोबियम लेग्यूमिनोसेरम की मूल परिवेष्टी, सूक्ष्म वनस्पतिजात एवं बीज संरक्षी के साथ अन्योन्य क्रियाएँ

**Surendra Kumar Tak**

Thesis  
**Doctor of Philosophy in Agriculture**  
(PLANT PATHOLOGY)



**2002**

**DEPARTMENT OF PLANT PATHOLOGY**  
**RAJASTHAN COLLEGE OF AGRICULTURE**  
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Thesis

Submitted to the

Maharana Pratap University of Agriculture and Technology

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for the Degree of

***Doctor of Philosophy in Agriculture***

**(Plant Pathology)**

By

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**2002**

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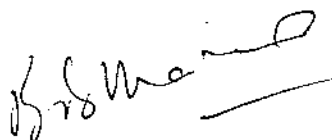
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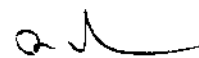
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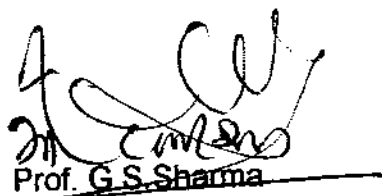
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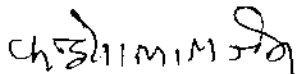
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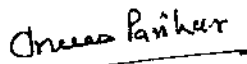
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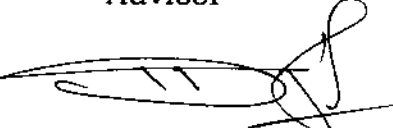
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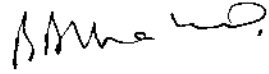
  
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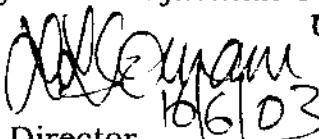
  
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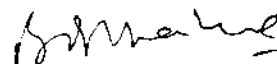
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# Contents

Chapter No.	Title	Page No.
1	Introduction	1-2
2	Review of Literature	3-14
3	Materials and Methods	15-34
4	Experimental Results	35-89
5	Discussion	91-95
6	Summary	97-98
	Bibliography	100-107
	Abstract (English)	108
	Abstract (Hindi)	109
	Appendices	110-115

## LIST OF TABLES

Table No.	Title	Page No.
1.	Nodulation status of Pea crop in different localities of Jaipur district in Rajasthan during rabi 1999. - 01.	35
2.	Quantitative test of different isolates of <u>Rhizobium leguminosarum</u> under sterilized soil conditions in pots.	41
3.	Comparative population counts of broth using Erlenmeyer flask and glass fermentor plate count method.	43
4.	Screening of different Pea genotypes to different isolates of <u>Rhizobium leguminosarum</u> under pot house conditions during rabi 2000. - 01.	49-50
5.	Screening of different Pea genotypes to different isolates of <u>Rhizobium leguminosarum</u> under field conditions (Pooled data for the year 2000 and 2001-02).	52-54
6.	CFU of different fungi associated with Pea root nodules	58
7.	CFU of <u>Mycoffora</u> associated with root nodules of Pea plants raised from fungicide-treated seeds.	61
8.	Actinomycetes (A) and bacteria (B) associated with pea root nodules	63
9.	Antagonistic activity of culture filtrate of fungi isolated from pea root nodules towards <u>Rhizobium leguminosarum</u> .	64
10.	Growth inhibition of <u>Rhizobium</u> due to antagonistic activity of actinomycetes	66
11.	Growth inhibition of <u>Rhizobium</u> due to antagonistic activity of bacteria.	67
12.	Variation in microbial population (colonies of fungi, bacteria and actinomycetes) of Pea rhizosphere at different stage of crop growth as influenced by chemical seed protector and <u>Rhizobium</u> inoculation.	68
13.	Effect of <u>Rhizobium</u> inoculated Pea seeds on growth and nodulation of the plants and nitrogen status of the soil under myco-colonized soil conditions.	69
14.	<u>In vitro</u> effect of fungicides on the growth of Pea <u>Rhizobium leguminosarum</u> .	72
15.	Inhibition of <u>Rhizobium leguminosarum</u> when pea seeds were treated with fungicides.	74
16.	Inhibition of <u>Rhizobium leguminosarum</u> growth on fungicidal treated pea seeds.	74-75
17.	Effect of chemical seed protectants on Pea (Azad-P-1) inoculated with <u>Rhizobium leguminosarum</u> in pot conditions during rabi 2000. - 01.	76
18.	Effect of chemical seed protectants on Pea (Azad-P-1) inoculated with <u>Rhizobium leguminosarum</u> in field conditions during rabi, 2000. - 01.	78
19.	Effect of chemical seed protectants on Pea (Azad-P-1) inoculated with <u>Rhizobium leguminosarum</u> in field condition during rabi, 2001. - 02.	80
20.	Available nitrogen in the soil after the harvesting of Pea crop during rabi season of 2000 and 2001. - 02.	82
21.	Leg-haemoglobin contents in Pea nodules at the initiation of flowering.	84
22.	Effect of carbofuron on root-knot nematode ( <u>Meloidogyne. incognita</u> and <u>Rhizobium</u> )	85
23.	Combined effect of seed protectants and bioagents on Pea (Azad-P-1) inoculated with pea <u>Rhizobium leguminosarum</u> under pot conditions during rabi 2001. - 02.	89

## List of Figures

Figure No.	Title	Page No.
Fig 1.	Quantitative test of different isolates of <u>Rhizobium leguminosarum</u> under sterilized soil conditions.	42
Fig 2.	Comparative population counts of broth using Erlenmeyer flask and glass fermentor plate count method.	44
Fig 3.	Nodule characteristics in Pea genotypes (pot experiment).	45
Fig 4.	Average fresh and dry weights of plant, nitrogen content and grain yield in Pea genotypes (pot experiment).	51
Fig 5.	Nodule characteristics in Pea genotypes (field experiment).	54
Fig 6.	Average fresh and dry weights of plant, nitrogen content and grain yield in Pea genotypes (field experiment).	56
Fig 7.	CFU of different fungi associated with pea root nodules	60
Fig 8.	Effect of different fungicides on the CFU of fungi associated with pea root nodules.	62
Fig 9.	Antagonistic activity of culture filtrate of fungi isolated from Pea root nodules towards <u>Rhizobium leguminosarum</u> .	65
Fig 10.	Effect of <u>Rhizobium</u> inoculated Pea seeds on growth and nodulation of the plants and nitrogen status of the soil under myco-colonized soil conditions.	70
Fig 11.	<i>In vitro</i> effect of fungicides on the growth of Pea Rhizobium.	73
Fig 12.	Inhibition of Rhizobium when Pea seeds were treated with fungicides.	73
Fig 13.	Effect of chemical seed protectants on Pea (Azad-P-1) inoculated with Pea <u>Rhizobium leguminosarum</u> in pot conditions during rabi 2000-01.	77
Fig 14.	Effect of chemical seed protectants on Pea (Azad-P-1) inoculated with <u>Rhizobium leguminosarum</u> in field conditions during rabi 2000-01.	78
Fig 15.	Effect of chemical seed protectants on Pea (Azad-P-1) inoculated with <u>Rhizobium leguminosarum</u> in field conditions during rabi 2001-02.	81
Fig 16.	Available nitrogen in the soil after the harvesting of pea crop during rabi-season of 2000-2001.	83
Fig 17.	Effect of carbofuran on root knot nematode ( <i>Meloidogyne incognita</i> ) and Rhizobium.	86
Fig 18.	Combined effect of seed protectants and bioagents on Pea (Azad-P-1) inoculated with <u>Rhizobium leguminosarum</u> under pot conditions during rabi 2001-02.	88

## LIST OF PLATES

Plate No.	Title	Page No.
1. a.	<i>Rhizobium leguminosarum</i> bacterioids from pink colour nodule, after Gram's staining	36
b.	Rod shape <i>Rhizobium leguminosarum</i> from CRYEMA culture, after Gram's staining.	36
2. a.	Pea plants inoculated with P <sub>1</sub> and P <sub>2</sub> isolates of <i>Rhizobium leguminosarum</i> .	38
b.	Uninoculated Pea plant showing lesser number of nodules.	38
3.	Assembled fermentor containing <i>Rhizobium leguminosarum</i> inoculum [glass fermentor connected to a glass tube (single inflow and three outlet) and aquarium pump].	40
4. a.	<i>Rhizobium</i> treated Pea (Azad-P-1) plants showing effect of indofil-M 45.	46
b.	Pea (Azad-P-1) plants treated with <i>Rhizobium</i> alone.	46
c.	Pea (Azad-P-1) plant untreated control.	46
5. a.	Pea genotype Azad-P-1 showing vigorous plants growth treated with <i>Rhizobium leguminosarum</i> in pots.	48
b.	Pea genotype PMR-19 showing vigorous plant growth treated with <i>Rhizobium leguminosarum</i> in pots.	48
6. a.	Photomicrograph of <i>Myrothecium verrucaria</i> isolated from Pea root nodules and rhizosphere.	57
b.	Photomicrograph of <i>Fusarium oxysporum</i> isolated from Pea root nodules and rhizosphere.	57
c.	Photomicrograph of <i>Aspergillus niger</i> isolated from Pea root nodules and rhizosphere.	58
d.	Photomicrograph of <i>Pencillium oxalicum</i> isolated from Pea root nodules and rhizosphere.	58
e.	Photomicrograph of <i>Drechslera</i> state of <i>Cochliobolus spicifer</i> isolated from Pea root nodules and rhizosphere.	58
7.	Root-knot showing Pea (Azad-P-1) plant infected with <i>Meloidogyne incognita</i> .	40

## List of Appendices

Appendix No.	Title	Page No.
I	ANOVA for different isolates of <i>Rhizobium leguminosarum</i> under sterilized soil conditions in pots.	110
II	ANOVA for comparative population counts of broth using Erlenmeyer flask and glass fermentor plate count method.	110
III	ANOVA For Screening of different Pea genotypes to different isolates of <i>Rhizobium leguminosarum</i> under pot house conditions during rabi 2000.-01.	111
IV	Screening of different Pea genotypes to different isolates of <i>Rhizobium leguminosarum</i> under pot house conditions during rabi 2000.-01.	111
V	ANOVA for microbial population (colonies of fungi, bacteria and actinomycetes) of Pea rhizosphere at different stage of crop growth as influenced by chemical seed protector and <i>Rhizobium</i> inoculation.	112
VI	ANOVA for effect of <i>Rhizobium</i> inoculated Pea seeds on growth and nodulation of the plants and nitrogen status of the soil under myco-colonized soil conditions.	112
VII	ANOVA for <i>In vitro</i> effect of fungicides on the growth of Pea <i>Rhizobium leguminosarum</i> .	113
VIII	Effect of chemical seed protectants on Pea (Azad-P-1) inoculated with <i>Rhizobium leguminosarum</i> in pot conditions during rabi 2000.-01.	113
IX	ANOVA for effect of chemical seed protectants on Pea (Azad-P-1) inoculated with <i>Rhizobium leguminosarum</i> in field conditions during rabi, 2000.-01.	114
X	ANOVA for the effect of chemical seed protectants on Pea (Azad-P-1) inoculated with <i>Rhizobium leguminosarum</i> in field condition during rabi, 2001.-01.	114
XI	ANOVA for the effect of carbofuron on root-knot nematode ( <i>Meloidogyne incognita</i> and <i>Rhizobium</i> )	115
XII	Combined effect of seed protectants and bioagents on Pea (Azad-P-1) inoculated with pea <i>Rhizobium leguminosarum</i> under pot conditions during rabi 2001.-02.	115

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(S.K. Tak)

# **Introduction**

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## INTRODUCTION

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Pea (*Pisum sativum* L.) is an important rabi legume. It is known to be one of the oldest pulse crop cultivated some ancient times both in Asia and Europe. Being a legume, it helps in maintaining soil fertility, and having a capacity of fixing atmospheric nitrogen through root nodule bacteria incited by *Rhizobium*.

On global basis, pea is the second most important pulse crop after beans it is being cultivated on 6515 million hectares with a annual production of 10945 million tonnes (vital Agriculture Statistics, 2000-2001). The major pea producing countries are France, Australia, Canada, Myanmar and Tanzania.

In India, the area under pea cultivation was 620 million hectares with a production of 6 lakh metric tonnes annually and productivity of 2589 k/ha (vital Agriculture statistics, 2000-1).

In Rajasthan state the crop has an acreage of 12553.76 ha with annual production of 2436 lakh tonnes. The productivity in the state is 1947 kg/ha. Jaipur and Kota districts are the main producer of pea in Rajasthan. The cultivation of pea has gained the importance since last decade with increased area due to expanding canning industry in the country.

The green peas are an excellent source of protein, low fat, calcium, iron, thiamine, riboflavin, niacin, vitamin C and vitamin A. The dried peas contain still higher protein, minerals and fibre contents (Gopalan, 1971).

Being a legume crop pea has an excellent advantage of fixing atmospheric nitrogen symbiotically with *Rhizobium* bacterium, which converts atmospheric nitrogen into the available form. The *Rhizobium* (family Rhizobiaceae) is an aerobic rod shaped (0.5-0.9 x 1.2-3 µm), gram negative, motile with one polar or sub-polar flagella or 2-6 peritrichous flagella. The cells of *Rhizobium* are host specific and invade the root nodules symbiotically. The bacteriod zone becomes pink due to accumulation of leg-haemoglobin in the nodular tissues. These bacteriods are remain in *in situ* condition. According to Hardy and Holsten (1972) nodule bacteria in association with leguminous plant can fix  $90 \times 10^6$  metric tonnes of nitrogen annually.

Interaction among soil micro organisms, competition for their survival, nutrition and soil environment are some of the important factors which affect survival of *Rhizobium* in the soil and rhizosphere of leguminous plants. These factors are known to play an important role in the formation of nitrogen fixing nodules depending on compatibility of host and rhizobial strain used.

Number of diseases are reported to be major constraints in pea production throughout the world. Majority of them are seed borne in nature and caused by fungal and bacterial pathogens viz., pea wilt (*Fusarium oxysporum* f. sp. *pisii*), Fusarium root rot (*F. solani* f. sp. *pisii*) (*F. poae*), leaf and pod spots (*Ascochyta pisii*), blight (*Ascochyta pinodes*, *Mycosphaerella pinodes*); root rot of pea (*Phoma medicaginis* var. *pinodella*). Leaf spot of pea (*Alternaria alternata*) (Wang Kuanchag et al., 1997) and bacterial blight of pea (*Pseudomonas pisi*). To overcome these constraints seed treatment with chemicals along with *Rhizobium* inoculants is a common practice for better management of seed born pathogens and nitrogen fixing ability. These chemicals may have stimulatory or inhibitory interaction with rhizosphere microflora nodule microflora. To study these interactions, the present investigation was undertaken on the following aspects and results <sup>obtained and summarized</sup> are presented in this thesis.

- Estimation of nodule status in different pea fields located around Jaipur.
- Isolation of *Rhizobium* from the pea root nodules from different localities and evaluation of the isolated culture by qualitative and quantitative methods.
- Quick multiplication of *Rhizobium leguminosarum* in broth by using simple glass fermenter.
- Isolation of rhizosphere microflora and nodule microflora and to study their antagonistic effects on *Rhizobium leguminosarum*.
- Study of compatibility of seed dressing fungicides with *Rhizobium* in *in vitro*, pot house and field condition.
- Interaction between selected Pea genotypes and *Rhizobium* isolate for better biological nitrogen fixing ability.

# **Review of Literature**

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## REVIEW OF LITERATURE

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Biological nitrogen fixation (BNF) has been estimated to contribute more than 175 million tonnes of N out of which legume N<sub>2</sub> fixation accounts for almost 40% (Burns and Hardy, 1975). Although, the role of legumes in improving soil fertility was known since long, it was only much later that the organism responsible was *Rhizobium*. Presently only 8.9% of the 14000 or so known species of leguminous plants have been examined for nodulation and less than 0.5% have been studied relative to their symbiotic relationship with nodule bacteria.

During last five decades, a large number of workers namely Brockwell *et al.* (1982); Howieson and Kwing (1986) and Keyser *et al.* (1992) have listed desirable characteristics in rhizobial strains for inoculant production and utilization. These include not only the ability to fix N but also the genetic stability, compatibility with agro-chemicals tolerance to stress etc. Another aspect of importance is the inoculum production technology. Perusal of literature reveals that more emphasis is being given to biological nitrogen fixation technology for improving leguminous crop.

### **INFECTION PROCESS AND NODULE DEVELOPMENT IN PEA**

The nodule bacteria (*Rhizobia* spp., rhizobia) are soil saprophytes occurring irregularly and often in relatively low numbers. A well established population in the rhizosphere of a potential host is a pre-requisite for prompt nodulation of seedlings. Host cells which will form the central tissue of nodules, usually become infected, at least initially, by penetration of ramifying infection threads. In peas, this process continues as long as the nodule grows. Infection thread penetration occurs in the narrow zone of cells just proximal to the meristem. Along most of their length in each cell, the infection threads are lined on their inner surface with cellulose and on the host cytoplasm side, by a membrane which is continuous with (Goodchild and Bergersen, 1966) and structurally closely related to (Tu 1974, Verma *et al.* 1978), the host plasma membrane. The infection thread is thus a tube which is really invaginated cell surface. The bacteria lie end to end within this tube; subsequently nodule central tissue cells arise by division of these infected cells. Determinate nodule generally have a transient phase of infection thread penetration, followed by division of infected cells to produce the young nodule, which than grows by enlargement of those cells. These various processes have been described in more detail by Libbenga and Bogors (1974).

Release of bacteria from infection threads into the host cells seems to be similar in all nodules. At or near the tip of a penetrating infection thread, a swelling develops, whose inner surface lacks or is deficient in cellulose. Bacteria become adherent to the inner surface of the

membrane which lines the swelling and then pass into the cytoplasm endocytotically (a sort of inverted phagocytosis; Goodchild and Bergersen 1966) part of the membrane enveloping each bacteriod in a vesicle. Kijne (1975) described changes in the vacuoles of pea nodule cells following release of bacteria from infection threads.

### ***Isolation of Rhizobium from nodule***

An important objective in legume inoculant research is to select highly effective strains of rhizobial for a particular host plants. Several laboratory methods have been developed for screening the isolates of *Rhizobium* by Vincent (1970) in the book entitled "A manual for practical study of root nodule bacteria". Thompson (1984) mentioned different methods for production and quality control carrier base inoculants. Somasegaran and Hoben (1985) in their manual "methods in legume-*Rhizobium* technology" described the use of symbiotic nitrogen fixation through *Rhizobium* technology. Somasegaran and Hoben (1994) in "Hand book of rhizobia" revised for identification of effective strains of *Rhizobium*. All these techniques described in the respective manuals were used to find out effective isolates of the pea *Rhizobium*. Werner (1993) in his new publication entitled "Symbiosis of plants and Microbes" described the symbiotic relationship, agricultural and environmental application, including the techniques of *Rhizobium* biology.

For preparation and multiplication of broth culture of *Rhizobium*. Gulati (1978) found that using a fermentor over shake culture gave quick multiplication of *Rhizobium* culture in broth.

### ***Factors affecting nodulation of Rhizobium***

Number of times the use of *Rhizobium* inoculant as seed inoculation is not effective. There may be several factors for the ineffectiveness of culture. Nambiar and Dart (1983) observed that physical factors which influence nitrogen fixation in legumes are soil p<sup>H</sup>, soil moisture, soil temperature and light intensity. Biological factors such as microbial antagonisms strain competition, role of nodule microflora in the development of nodule and chemical factors like root exudates, seed exudates, availability of micro and macro nutrients in the soil for establishment of nodular infection and nodule development and compatibility of chemical seed portectants with *Rhizobium* Spp. affect the process of symbiotic biological nitrogen fixation.

## **Physical Factors**

### **i. Soil Type**

Soil and the organisms on and within them beautifully reflect the outcome of biological nitrogen fixation. Bowen and Rovira (1976) showed that the growth rate of *Rhizobium* in soil without plants is low as compared to that in rhizosphere of legumes. In arid lands, nodulation takes

place in majority of the leguminous plants by the slow growing root nodule bacteria as these bacteria and their associated legumes are adapted the dry hot environments. Legumes associated with fast growing rhizobia are well established in semi-arid areas but are restricted to soils of heavy texture which contain protective colloids material thus affecting protection against dry heat. Size of clay along with porosity are also important in determining the survival of rhizobial in soil (Nutman, 1974). The clay minerals have direct and indirect effect on soil micro-organisms. Indirect effect is through the modification of the physico-chemical characterisation of the microbial habitats which direct effects are through the surface interactions (adhesion) between days and microbes (Storzky, 1986).

## ii. Soil pH

Soil pH influences the survival and nitrogen fixation of rhizobial in soil. Although in saline or salt affected soils host has more problem than micro-symbion. This may because of the high concentration of salts, the plant is not able to absorb nutrients as there is a coupled problem of water availability under osmotic potential of soil and reduced availability of P, Fe, Zn, Mn and B due to high pH. Some strains of *Rhizobium* can grow in solution with salinity as high as 92% of the salinity of sea water (Singhleton *et al.*, 1982). *Rhizobium* strains varied with respect to nodulation ability in saline condition leading to effective strain competition even in saline soils where host did not have good growth (Bhardwaj, 1975). The problem of survival of rhizobial in acidic soils is two fold. First, the survival may be affected due to acidity. Second, the availability of some of the nutrients is increased to toxic levels. However, bacteria can manage to grow by regulating their internal pH and by synthesizing specific and more of polysaccharides (O' Hara *et al.*, 1989).

Dughani and Battomley (1984) clearly brought out that soil pH has role in determining strain competition. Chickpea *Rhizobium* strain showed significantly poor competitive ability at 5.4 pH as compared with soil with 7.1 pH (Chandra and Pareek, 1991).

## iii. Soil temperature

Soil temperature influences the ability of *Rhizobium* to persist in soil. Marshall (1964) observed that the symbiotic bacteria did not survive at high temperature and causing mortality of cells due to the excessive heat. Chetal and Parker (1973) observed that certain group of root nodule bacteria, did not survive in hot summer when soil was dry and rhizobial cells lost their variability and resulted in poor nodulation. In green house study, Munever and Wollum (1981) showed that through increased temperature reduces nodulation, the extent of reduction varied with tolerance level *B. japonicum* to temperature rise. Hungaria and Franco (1993) observed the adverse effect of high temperature on nodulation and nitrogen fixation by *Phaseolus vulgaris* L.

#### **iv. Soil Moisture**

Soil moisture is an important factor affecting growth and activity of soil micro-organisms because availability of nutrients and enzymatic activity are influenced by moisture levels of soil. In un-sterilized Australian peat 40-50 per cent moisture was found suitable for development of nodules whereas moisture below 30 per cent or above 60 per cent was found to be unfavourable for survival of the bacteria (Roughley and Vincent, 1967).

Reduced O<sub>2</sub> and accumulation of organic acid due to the fermentative character of the microflora under high soil moisture condition may account for reduced population of rhizobia in such environment. Flouds (1971) studied the moisture level tolerance of various species viz., *R. trifoli*, *R. meliloti* and *Rhizobium* of lotus group and observed that *R. trifoli* was more tolerant to drought than cells of *R. meliloti* and *Rhizobium* of lotus group.

#### **v. Light intensity**

Light intensity plays an important role to decide rate of nitrogen fixation. Forty per cent reduction in light intensity caused considerable reduction in the rate of nitrogen fixation by *Rhizobium* inoculated peanut crop (Nambiar and Dart, 1983).

### **Biological Factors**

Interaction among soil micro-organisms is very common in the soil. Various types of micro-organisms such as bacteria, fungi, algae, protozoa and bacterial viruses are present in the soil. In soil these live in close proximity particularly in the rhizosphere and they interact in unique way and cause beneficial and harmful effects. Because of these interrelationships introduction of an organism in soil really leads to its establishment in the soil.

#### ***Microbial antagonism among soil micro-organisms***

The antagonistic effects of soil micro-organisms such as, bacteria, fungi and actinomycetes may reduce rhizobial population in the soil. The failure of nodulation in some parts of western Australia has been found to be due to the process of soil micro-organisms antagonistic to *Rhizobium* spp. (Hely *et al.* 1957), Charya *et al.* (1985) found that *Rhizobium* inoculation with *Fusarium oxysporum*, *Phoma exigua* and *Rhizobium solani* in sterilized soil inhibited nodule formation but *Phoma exigua* when inoculated with *Rhizobium* improved nodulation in *Vigna radiata*. Rhizobial have also been found to be inhibited by toxic metabolites produced by fungi *in vitro*. Sethi and Rao (1972) found that fusaric acid at 8 µg/ml inhibited the growth of *Rhizobium trifoli*. Production of fusaric acid by *Fusarium oxysporum* affected adversely formation of nodules but no such effect was found in case of *Rhizobium meliloti* even at 270 µg/ml.

## i. Soil actinomycetes

In Belgium, Patel (1974) observed antagonism of actinomycetes against rhizobial. The analysis of soil samples from pastures revealed 23.70 per cent actinomycetes to be inhibitory to clover *Rhizobium* and cause poor nodulation. On the contrary Kosslak and Bohlool (1985) reported that the outcome of competition between *B. japonicum* strains USDA 110 and USDA 123 was not affected by the presence of antagonistic actinomycetes in the soybean rhizosphere.

## ii. Soil bacteria

*Erwinia herbicola*, an epiphytic bacterium, commonly associated with seeds of *Medicago sativa*. Handelsman and Brill (1985) suggested that *E. herbicola* may suppress initial nodulation of *R. meliloti* mutant by producing a toxin thereby blocking rhizobial attachment sites on *M. sativa* root hairs. Parker and Grover (1970), and Stolp (1973) reported that *Bdellovibrio* an intercellular bacterial parasite of *Rhizobium* and is capable of infecting and lysing large populations of rhizobia.

## iii. Nematodes

*Meloidogyne* spp. inciting root knots are known to inhibit the symbiotic nodule formation. Singh and Gupta (1983) and Barker *et al.* (1972) studied the root knot of cowpea and *Rhizobium* interaction. These authors further observed a gradual reduction in root and shoot length of plant when *Rhizobium* treated cowpea seed were sown in *Meloidogyne* infested soil, other nematode like *Heterodera glycines* also inhibits the soybean root development. Kavathiya and Pandey (2000) studied the interaction of *Meloidogyne javanica*, *Rhizobium* and *Macrophomina phaseolina* in mungbean plants under artificial conditions. It was observed that when *Rhizobium* treated seeds were sown in mycocolonized soil and nematode infested mungbean plant gave less nodulation. Perusal of literature reveals that interaction of nematode and *Rhizobium* spp. has not been properly investigated.

## iv. Rhizobiophages

The lytic and lysogenic viruses that infect rhizobia are known as rhizobiophages. Different strains within the same species respond differentially to the presence of rhizobiophages. Some are sensitive and others resistant to lysis by phages. This characteristic has been used in ecological studied type strains by Lesley (1982). Barnett (1980) and Evans *et al.* (1979) reported that addition of virulent bacteriophages to paired strains of *R. trifoli* decreased the number of sensitive strains on the roots of clover plants. While resistant strains were favoured for nodulation. Schwinghamer and Brockwell (1978) reported that lysogenic strains of *R. trifoli* grown in sterile broth and peat strongly suppressed the growth of sensitive strain.

## v. Soil fungi

Number of fungi present in the soil or rhizosphere may or may not act as antagonistic to rhizobia thus may or may not affect the nodulation. Sethi and Subba Rao (1968) observed that culture filtrate of *Fusarium*, *Aspergillus*, *Thielaria* and *Trichoderma* inhibited the growth of rhizobial cells *in vitro* conditions. Orellana and Workey (1976) reported non-functional root nodules of soybean in the presence of *Rhizoctonia solani* infested soil. Similarly Charya *et al.* (1985) inoculated *Rhizobium* dressed mung (*Vigna radiata*) seeds to myco-colonized soil of *Fusarium oxysporum*, *Phoma exigua* and *Rhizoctonia solani* inhibited nodule formation. On the contrary authors observed that myco-colonized soil of *Phoma exigua* exhibited improved nodulation in mungbean. It seems that rhizobitoxin produced by *Rhizobium* inhibit the growth of *Phoma exigua*. Ehteshamulhaque and Ghaffar (1993) used rhizobial in the control of root rot diseases of sunflower, okra, soybean and mungbean. Ghaffar (1993) observed that rhizobia can be used as bio-control organism as it produces a toxic metabolite for the control of *Macrophomina phaseolina*, *Rhizoctonia solani* and *Fusarium* spp. of pathogenic soil organisms. Rhizobia may protect the root of legumes and non-legumes by producing a toxic metabolite, rhizobitoxin, (Chakraborty and Purkayashtra, 1984), by producing antibodies (Malajckuk, 1983), by parasiting the hyphae of pathogenic fungi directly (Tu, 1978) and by lysing them (Malajckuk *et al.*, 1984).

### **Fungi associated with root nodules**

Fungi associated with the surface of root nodules play an important role inhibiting the growth of developing nodules. Chonkar and Subba Rao (1966) isolated and identified eleven fungi viz., *Cephalosporium*, *Alternaria*, *Aspergillus*, *Pencillium*, *Rhizopus*, *Acrothecium*, *Pythium* and *Trichoderma* from nodules of nine common legumes namely, *Trifolium alexandricum*, *Trifolium* sp., *Melilotus parviflora*, *Trigonella foenum-graecum*, *Cicer arietinum*, *Pisum sativum*, *Glycine max*, *Phaseolus mungo* and *Arachis hypogaea*. These authors also observed definite relationship to the age of plant and volume of nodules. The isolated fungi were further screened for antibiotic activity towards 6 species of *Rhizobium*, *R. trifolium*, *R. phaseoli*, *R. leguminosarum* and *R. japonicum* were found more susceptible to fungal antibiotics than other two species *R. meliloti* and *Rhizobium* species belonging to cowpea group. Chonkar and Subba Rao (1966) further studied the effect of myco-colonized soil on the development of nodule and observed that an isolate of *Cephalosporium* sp. effect adversely affect the plant growth and nodule development on *Trifolium alexandricum*.

Orellana *et al.* (1976) reported that *Rhizoctonia solani* significantly reduced the nodule weight and shoot growth when soybean seeds were inoculated with *R. japonicum*. Bhattacharya

and Mukherjee (1990) reported that the presence of *Sclerotium rolfsi* reduced the population of *Rhizobium* sp. in the rhizosphere of peanut.

### **Strain competition**

Competition exhibits among strains of *Rhizobium* (*Bradyrhizobium*) sp. for occupying the root. Strain competition is measured by comparing the nodule occupying capacity of inoculated strain with that of the native strain of *Rhizobium* sp. strain competition between inoculated and native strain of *Rhizobium* for nodulation, however does not allow harnessing the full potential inoculum strain. Franco and Vincent (1976) reported strain competition for nodulation under field condition to range between 30 to 40 per cent. Since strain competition is a major problem of success of inoculation in pulses, a great deal of work has been put in improving the competitive ability of inoculated strain in field situation. *Rhizobium* strains for better nodulation at low soil p<sup>H</sup> (Chandra and Pareek, 1991) temperature not moisture (Chandra and Pareek, 1990) were isolated or trained.

The native strains of rhizobial have enormous diversity (Noel and Brill, 1980) and this provides them the capacity to cope up with the fluctuating environment in rhizosphere. Strains of rhizobia differ in number of ways, for example, in their ability to colonize root surface, reaction to chemicals, root surface binding mobility nutritional requirements, extra cellular polysaccharides, as well as their ability to adjust with the change in physical environment.

### **Saprophytic competence**

In the absence of host, rhizobial have to persist in the soil and compete for nodule sites on the roots of the host. The ability to colonize roots and soil by rhizobia has been described as saprophytic competence.

Higher the saprophytic competence the chance of survival by rhizobial are better. Marked differences in saprophytic competence among different strains of rhizobial has been reported (Chatel and Parker, 1973; Chatel and Greenwood, 1973).

### **Chemical factors**

#### **Availability of macro and micro nutrients**

Apart from the minerals needed for plant growth as whole, the legume *Rhizobium* symbiosis imposes additional nutritional requirements. Nitrogen fertilization adversely affects the nodulation of many legumes (Franco, 1977). Available nitrogen, particularly nitrates, is known to be inhibitory for nodulation Mc Dormett and Graham (1990) found strain differences in nodulation

of soybean crop in presence of nitrate while Kosslak *et al.* (1990) did not observed the difference while using two sero groups of *R. japonicum*.

Groundnut cultivars may vary in their response to nitrogen fertilizer (Cox, 1982). In general with increase in nitrogen fertilizer application the rate of nitrogen fixation decreases (Nambiar, 1984). Hence, attempts to meet the crops extra demand for nitrogen by the application of nitrogen fertilizer have not usually been successful (Cox, 1982).

Franco (1977) noted that soil acidity together with aluminum and/or manganese toxicity, deficiency of phosphorus, sulphur, Calcium and molybdenum are some of the factors that may limit grain yield of tropical legumes dependent on symbiotic nitrogen fixation for growth. Cartwright and Hallsworth (1970) reported that boron and zinc also increase nodulation in legumes.

Almendras and Bottomloy (1985) observed that when the soil having low  $p^H$  was limited to increase P availability the dominant sero group was out competed by a minor one however, addition of P and lime restored the dominance of original sero-group suggesting that P limitation is exacerbated by  $p^H$  and the combination of  $p^H$  and P levels can have strong influence on competition for nodulation.

Munns *et al.* (1977) observed that liming of an oxisol, increased  $p^H$  from 4.7 to 7.1, but did not affect any significant change in groundnut nodulation. Application of cobalt increased nodulation in high  $p^H$  soils. Evans and Rusell (1971). All *Rhizobium* strains grow rapidly on a mineral salt medium containing yeast extract and one of a wide variety of carbohydrates, particularly mannitol, glucose arabinose, fructose, glucose and sucrose (Vincent, 1970).

Perusal of literature reveals that availability of macro and micro nutrients which are present in soil play an important role in the establishment of nodular infection and development of nodule.

## Relationship of leg haemoglobin with nodule development

Review of literature reveals that leg-haemoglobin helps in quick multiplication of bacterioids in inner nodular cortex and thus there is a definite relationship between leg-haemoglobin and nodule development. Evans *et al.* (1971) studied biochemical and physiological role of leg-haemoglobin in relation to biological nitrogen fixation. Similarly, Bergersen (1980) find out that primary function of leg-haemoglobin was to augment the diffusive supply of dissolved oxygen to nitrogen fixing rhizobia of legume root nodules. Bisseling *et al.* (1980) described that decrease of nitrogen fixing ability is caused by a decrease in leg-haemoglobin content of the pea root nodules and not by suppression of the nitrogen synthesis.

## Root exudates

Legumes secrete a large number of substance into the rhizosphere. These root exudates provide nutrition to the growth of antagonists which could suppress the growth of rhizobia in soil.

Araujo and Medeiros (1992) observed the effect of root extract on nodulation of cowpea.. Kandaswamy and Prasad (1979) indicated that as the plants become older, the quantity of roots phenolics exuded was reduced and there was increase in the *Rhizobium* population in the rhizosphere of the three plants species. Truchet (1986) observed that clover root exudates contains a particular form of the lectin-trifolin A, which binds *Rhizobium trifoli*.

## Compatibility of chemical seed protectants with pea *Rhizobium*

In order to manage seed borne plant pathogens several chemical seed protectants have been recommended for dressing the legume seeds. It is more or less a common practice to dress the legume seeds with chemical protectants and also at the same time these legume seeds are inoculated with *Rhizobium* in order to ensure biological nitrogen fixation. But many times fungicides dressed and *Rhizobium* inoculated seeds are not compatible and toxic to rhizobial which reduced the number of cells of rhizobia coated over the seed surface. Thus toxicity caused reduced number of nitrogen fixing symbiotic nodules.

Allington (1945) and Wrobel (1963) observed that organo mercurial seed dressing treatment of soybean seeds have been shown to inhibit the rhizobial population coated over the seed surface. These authors also observed that that captan seed dressing treatment did not affect adversely to the symbiotic nodulation process. Milthrope (1945) dressed the pea seed with cerasan and noted that rhizobial population coated over the seed surface was markedly reduced. Hofer (1958) observed that mercury alone was found toxic to symbiotic process, while studying selective action of fungicides on *Rhyzobium* from lucerne. Wrobel (1963), also tested the compatibility of some organic mercury seed dressing fungicides with *Rhizobium* on pea and lupin and observed that these fungicides delayed nodule formation and plant growth.

Dalela and Lodha (1976) observed the effect of seed dressing fungicides on nodulation and yield of groundnut crop when inoculated with *Rhizobium* strain and revealed that maximum inhibition of nodules and minimum yield was recorded in phenyl mercury acetate. However, a combination of phenyl mercury acetate and captan was not found to exhibit any toxic effect on nodulation. These authors further observed that mancozeb alone did not show any inhibition of nodules and on the contrary it enhanced nodule formation and yield.

Sidhu and Kahlon (1970) studied the compatibility of *R. leguminosarum* isolated from pea with 6 chemical seed protectants viz., cersin, captaf, brassicol, thiram, benlate and aureofungin. They observed that brassicol, benlate and aureofungin did not show any adverse effect on the

growth of *Rhizobium leguminosarum* under *in vitro* condition. Kis *et al.* (1977) tested compatibility of 7 seed dressing fungicides with *Rhizobium* and it was observed that rhizobial activity was affected only slightly by benlate, thiram and captan seed treatment. However, higher yield were found with thiram seed dressing.

Abdel-Monem and El-Sawha (1984) tested compatibility of systemic fungicides viz., vitavax (carboxin), benlate (benomyl), bavistin (carbendazim), topsin M-70 (thio-phanate-methyl) and PN 50.50 (maneb + quintozone) with *R. japonicum* and observed that seed dressing with benlate, bavistin and topsin M-70 increased the nodulation in soybean crop. Patel *et al.* (1985) studied the compatibility of seed dressing fungicides with *Rhizobium leguminosarum* and their effect on nodulation, nodule weight and yield of green gram under field conditions and observed that seed dressed with captan and dithane M-45 treatments revealed maximum weight and dry weight of nodules. These authors observed that use of organo-mercurials like agrosan GN was toxic to *Rhizobium leguminosarum*. Thomas and Vyas (1985) observed that carbendazim and metalaxyl seed treatment with pigeonpea *Rhizobium* gave a significant increase in nodule number and nodule dry weight/plant.

Patel *et al.* (1989) tested the compatibility of fungicides with the growth of *Rhizobium leguminosarum* isolated from mungbean out of the six fungicides viz., brassicol, dithane M-45, bavistin, agrosan GN, captan and thiram *in vitro*. Among these chemicals brassicol, dithane M-45 and bavistin did not show any inhibition of *Rhizobium* growth even at concentration of 2000 ppm.

Reddy *et al.* (1990) tested compatibility of groundnut seed with dithane M-45 and observed that seed treatment with dithane M-45 increased the seed germination and number of nitrogen fixing nodules. Ram (1998) observed that seed treatment with ridomil MZ was found most compatible among six fungicides viz., thiram, bavistin, topsin-M, indofil M-45 and captan. Longiswaran *et al.* (1991) reported that the effect of seed treatment with fungicide carbendazim and the insecticides carbo-sulfan and acephate along with the *Rhizobium* seeds inoculation to black gram. The results revealed that treatment of seed with carbendazim followed by carbo-sulfan or Acephate did not affect nodulations incited by *Rhizobium* sp. Nagar *et al.* (1992) dressed the groundnut seeds with vitavax and these seeds were also inoculated with *R. lupini*. Study reveals that *Rhizobium lupini* was found compatible with vitavax and it also controlled the incidence of crown rot disease, incited by *Aspergillus niger*. Kler and Gupta (1993) reported that in irrigated groundnut (CV-M-13) yields were decreased by seed inoculation with *Rhizobium*.

However, seed treatment with thiram (3 g/kg) reduced the depressing effect of *Rhizobium*. Singh and Agrawal (1993) studied the compatibility of *Rhizobium japonicum* with bavistin, bayleton, captan, dithane M-45, copper oxychloride, ridomil, thiram and vitavax in soybean crop.

They observed that bavistin and bayleton seed protectants were found compatible with *Rhizobium japonicum*.

Sharma (1994) tested eight fungicides viz., agrosan GN, bavistin, benlate, captan, cersan, dithane M-45, ridomil MZ and thiram in *in vitro* conditions and among these chemicals bavistin, benlate captan and ridomil-MZ did not show any inhibition of growth of cowpea *Rhizobium*. while thiram showed maximum inhibition. Krishna-Chandra *et al.* (1996) observed that cersan was found toxic to *Bradyrhizobium japonicum* and *R. leguminosarum* from soybean and pea. Castro *et al.* (1997) observed that mancozeb did not show adverse effect on nodulation when it was used with *Rhizobium* seed inoculation in groundnut. Singh and Lodha (1997) studied the compatibility of five seed protectants viz., thiram, dithane M-45, captan, ridonil-MZ and bavistin with *Bradyrhizobium japonicum* in soybean to determine symbiotic nodulation and management of seed borne pathogen ridonil-MZ and bavistin were found compatible. Thus it appears from the literature that behaviour of *Rhizobium* strain with chemical seed protectants is variable depending upon type of legume crop and *Rhizobium* strain used.

### ***Interaction of Rhizobium and root knot nematode on the development of nodules and plant growth***

Root knot nematode (*Meloidogyne* spp.) are known to inhibit the symbiotic nodule formation. Subramanyam *et al.* (1992) reported that peanut root-knot caused by *Meloidogyne arenaria* and *M. hapla* which produces galls on roots, pegs and pods on infected plants. The roots infected with *M. hapla* tend to form branches near the point of nematode invasion. This frequently produces a dense, bushy root system. Singh and Gupta (1983) observed a gradual reduction in root and shoot length of plant when *Rhizobium* treated cowpea seed were sown in *Meloidogyne* infested soil.

Krishinovski *et al.* (1992) applied methyl bromide as soil fumigant along with peanut seed inoculation with *Rhizobium*. Ram (1998) observed that *M. hapla* (incitant of root knots in peanut) infested soil exhibited fewer number of nodules. These authors observed that methyl bromide substantially decreased the number of nodules containing native rhizobial strains. However, interaction of nematode and *Rhizobium* sp. has not been properly investigated in case of pea.

### ***REACTION OF CULTIVARS TO Rhizobium strains:***

Number of nodules formed by legumes and their effectiveness is governed by host cultivars and *Rhizobium* strain with large differences in nodulation and nitrogen fixing ability. Such variation in nitrogen fixing ability among pea cultivars was observed by Holl and La Rue

(1976). Perusal of literature reveals that host genotypes and *Rhizobium* strains have been shown to influence distribution of nodules in legumes.

# **Materials and Methods**

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## **MATERIALS AND METHODS**

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### ***Survey and collection of nodules***

Survey was undertaken of major pea growing localities in Jaipur district viz., Boraj, Bobas, Jobner, Sambhar, Phulera, Lalpura, Hirmoda, Naga ki Dhani, Bagru and Durgapura at the time when the plants were nearing flowering. Ten randomly selected pea plants were uprooted from each locality for counting of nodules. The effective nodules having pink colour were counted after thoroughly washing in water, these were categorized into following three categories:

Poor-	Below 10 nodules/plant
Moderate-	11-20 nodules/plant
Good-	21-40 nodules/plant
Very good-	41 and above.

### ***Glass ware***

All glass ware used for the experiments were of corning make. These were first thoroughly washed with cleaning powder and then in tap water. There after these were soaked overnight in chromic acid solution ( $K_2Cr_2O_7$  80g. distilled water 300 ml, to which concentrated  $H_2SO_4$  400 ml was added drop by drop with constant stirring). The soaked glass ware were washed in tap water, finally rinsed with distilled water and dried before use.

### ***Chemicals***

All chemicals used for laboratory work were either of Himedia Laboratories Pvt. Ltd., Mumbai or Glaxo Laboratories (India) Ltd., Mumbai or Sarabhai Chemicals, Baroda.

### ***Seeds***

Pea seeds were obtained from MULLaRP Scheme (M-Mung, U-urd, L-lentil, La-Lathyrus, R-Rajmah, P-field peas) Agricultural Research Station, Durgapura, Jaipur. For all pot house and field experiments pea genotype Azad-P-1 was used. For interaction of genotypes with *Rhizobium* eleven pea genotypes viz., Arkil, Azad-P-1, OSP, DPR-68, PMR-19, JP-83, VL-3, VL-7, Linolin, JP-585, IP-3 were used.

## **Rhizobium culture used**

Effective pea root nodules were collected from the surveyed localities of Jaipur district and *Rhizobium* was isolated and one *Rhizobium* culture was procured from All India Pulse Improvement Project, Agricultural Research Station, Durgapura, Jaipur. Isolated cultures were tested qualitatively and quantitatively, during 1999. The same was used during further experimentation.

## **COMPOSITION OF MEDIA**

- Micro-biological grade yeast extract, bacteriological agar and peptone were used.
- Double distilled water was used to prepare 1000 ml medium.
- Agar was used at the rate of 20 g per litre of medium for solidification, wherever, solid media were used.
- pH was tested initially by pH meter and maintained pH 7.0 by 0.1 N NaOH or 0.1 N HCl solution.

## **Yeast Extract Mannitol Agar**

Used for the maintenance of *Rhizobium leguminosarum* culture:

Yeast extract	1.0g
Mannitol	10.0g
Potassium hydrogen phosphate	0.5g
Magnesium sulphate	0.2g
Sodium chloride	0.1g
Agar-agar	20.0g
Dist. water	1000 ml

For isolation and purification of *Rhizobium* sterilized 10 ml (1/400 aqueous solution) of congo red is to be added to YEMA just before pouring of plates and the medium is referred as CRYEMA.

## **Yeast Extract Mannitol Broth**

Used for preparation of inoculum cultures.

Yeast extract	1.0 g
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Mannitol	10 g
Potassium hydrogen phosphate	0.5 g
Magnesium sulphate	0.2 g
Sodium chloride	0.1 g
Calcium carbonate	1.0 g

### Glucose Peptone Agar Medium

Used for the qualitative testing of mass cultures.

Glucose	10.0 g
Peptone	20.0 g
Sodium chloride	5.0 g
Bromocresol purple solution of ethyl alcohol solution	10.0 ml

### Martin's Rose Bengal Agar Medium

Used for nodule mycoflora isolation.

Dextrose	10.0 g
Peptone	5.0 g
Potassium dihydrogen phosphate	1.0 g
Magnesium sulphate	0.5 g
Rose bengal	0.033 g
Streptomycin	30.0 g
Agar-agar	20.0 g

One gram of streptomycin sulphate was dissolved in 100 ml of sterile distilled water and 3 ml of streptomycin solution was added to the above medium after it was cooled at 45°C.

### Potato Dextrose Agar Medium (PDA)

Used for maintenance of fungal cultures.

Peeled potato	250.0 g
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Dextrose	20.0 g
Agar-agar	20.0 g

### Richard's Synthetic Medium

Used for fungal culture filtrate studies.

Potassium nitrate	10.0 g
Potassium hydrogen phosphate	5.0 g
Magnesium sulphate	2.5 g
Ferric chloride	0.02 g
Sucrose	50.0 g

### Starch Ammonium Agar

Used for isolation of soil actinomycetes.

Starch	10.0 g
Ammonium sulphate	1.0 g
Potassium hydrogen phosphate	1.0 g
Magnesium sulphate	1.0 g
Sodium chloride	1.0 g
Calcium carbonate	3.0 g
Agar-agar	20.0 g

### Thornton's Standardised Medium

Used for isolation of soil bacteria

Potassium hydrogen phosphate	1.0 g
Magnesium sulphate	0.2 g
Calcium chloride	0.1 g
Ferric chloride	Trace
Potassium nitrate	0.5 g
Asparagines	0.5 g

Mannitol	1.0 g
Yeast extract	0.25 g
Agar-agar	20.0 g

### **Czapek's Medium with Glucose**

Used for maintenance of actinomycetes cultures.

Sodium nitrate	3.0 g
Potassium hydrogen phosphate	1.0 g
Magnesium sulphate	0.5 g
Potassium chloride	0.5 g
Ferrous sulphate	Trace
Glucose	30.0 g
Agar-agar	20.0 g

### **Nutrient agar medium**

Used for maintenance of bacterial cultures.

Beef extract	3.0 g
Peptone	5.0 g
Agar-agar	20.5 g

### ***Gram's Strain Reagents***

Hucker's ammonium oxalate crystal violet stain

#### **Solution A**

Crystal violet	2.0 g
Ethyl alcohol (95%)	20.0 ml

#### **Solution B**

Ammonium oxalate	0.8 g
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Distilled water	80.0 ml
Mix solution A and B	
Gram's iodine solution	
Iodine	1 g
Potassium iodide	2 g
Distilled water	300 ml

Dissolve the first two ingredients in water with a mortar and pestle.

Safranine solution	
Safranine	0.5 g
Distilled water	100 ml

Dissolve the safranine in water.

### **Litmus Milk**

Used for qualitative testing of *Rhizobium* sp.

Skimmed milk	750.0 ml
Bromocresol purple	0.02 g
Water	250.0 ml

Five ml of the above mentioned composition was dispensed in test tube and sterilized by steaming for 30 minutes on 3 successive days. Milk medium was inoculated with a loopful of 48 hours old growth of *Rhizobium* sp. and incubated. Observations upto 30 days were recorded at intervals for curd peptonization and acid or alkaline reaction. The milk changes from blue to yellow, if reaction is acidic and to violet, if it is alkaline. Peptomization is indicated by clearing of milk and usually proceeds slowly. An uninoculated control was also kept for comparison.

### **Seed protectants**

The following seed protectants were used to test the compatibility with *Rhizobium*.

Trade name	Common Name	Chemical Name	Dose kg <sup>-1</sup> seed(ing)
Bavistin	Carbendazim	50% methyl-2-benzimidazole-2-carbonate	1.0

Captan	Captan	N-(Tetrachloromethylthio)-4-cyclohexene-1,2,dicarboxamide	2.0
Indofil M-45	Mancozeb	75% Zinc ion and manganese ethylene bisdithiocarbamate	2.0
Ridomil-MZ	Metalaxyl	N-(2,6-Dimethylphenyl)-N-methyl ester(Ridomil : Mancozeb, 1 : 8	2.0
Thiram	Thiram	75% tetra methylthiuram disulphide	2.0
Topsin-M	Thiophanate methyl	75% 1,2, bis (3ethoxycarbonyl-2-thioureido)	1.0

### ***Isolation***

**Requirements** : Petridishes, sterile tap water, mercuric chloride (0.1%), ethyl alcohol (70%), test tubes containing 5 ml sterile water, scalpel yeast extract mannitol agar (YEMA) medium. With congo red, glass rod, forceps

### **Pouring of medium**

CRYEMA (congo-red) growth medium was prepared according to the above mentioned composition and sterilized in an autoclave at  $1.045 \text{ kg/cm}^2$  for 20 minutes. Twenty ml of lukewarm medium ( $45^\circ\text{C}$ ) was poured aseptically in each of the Petri plates (90 mm diameter). The Petri plates were rotated and tilted for uniform spreading of medium and allowed to solidify.

### **Procedure**

- \* Healthy pea plants were uprooted.
- \* Roots were washed with tap water.
- \* From the roots healthy pink colored nodules were selected, from the tap root.
- \* Nodule were separated by giving diagonal incisions from two sides to cut nodule, along with root portion.
- \* Nodules were washed in running water.
- \* Nodules were transfer to sterile beaker and these were surface sterilized with 0.1% mercuric chloride 60 seconds these were further placed in 70% ethyl alcohol for 30 seconds.
- \* Finally these nodules were washed with sterile water by giving six washings.
- \* These nodules were placed in a small vial containing sterile water.
- \* These were crushed with sterile glass rod.

- \* Clear suspension was poured in Petri plates (3 to 4 drops in each Petri plate) and then 20 ml. luke warm CRYEMA was added to each of the Petri plates.
- \* These plates were incubated at  $28 + 2^{\circ}\text{C}$  for 72 hours till white glistening colonies appear.
- \* These single white colonies were transferred to CRYEMA test tubes containing media by using single streaking method.
- \* Pure culture was maintained on YEMA slants in refrigerator.

## Preparation of broth cultures for seed inoculation

Yeast extract mannitol broth was prepared by putting 100 ml aliquots of the broth into 250 ml conical flask and each flask was then plugged with non absorbent cotton, these were autoclaved at  $1.045 \text{ kg/cm}^2$  for 20 minutes and cooled. Broth was inoculated with 7 days old *Rhizobium* culture. These flasks were incubated at  $28 + 2^{\circ}\text{C}$  and shaken periodically during incubation period for quick multiplication of *Rhizobium* in broth.

## Qualitative test

### i. Visual observation and pH

The medium was checked for visible contaminants. The  $\text{p}^{\text{H}}$  of *Rhizobium* inoculated broth was determined by  $\text{p}^{\text{H}}$  meter.

To determine pH, a loopful of *Rhizobium* culture was inoculated aseptically to five (250 ml) conical flasks each containing 150 ml glucose peptone broth. The pH was adjusted to 7.0. These flasks were incubated at  $28 + 2^{\circ}\text{C}$  for 48 hours. The pH was again tested by pH meter. If the pH is below 6.5 or above 8.0 it indicates the presence of contaminants.

### ii. Gram's staining

24 h growth of *Rhizobium* culture was diluted in sterile water. Thin smear was prepared on a glass slide. Smear was dried on slide by using flame. Smear was flooded with Hucker's ammonium oxalate crystal violet stain for 1 min and then washed in tap water. Slide was flooded with Gram's iodine solution and it was allowed to remain for 1 min. The slide was decolorized with 95% alcohol until only faint violet colour remains. Cover the slide with counter stain safranin for 30 seconds. Again washed the slide in tap water, drain, and air dried. After drying the slide was observed under the oil-immersion objective. Observe the rods for their colour.

### iii. Growth on glucose peptone agar medium

A loopful of the broth was streaked into the glucose peptone agar medium contained in a Petriplate and incubated at  $28 + 2^{\circ}\text{C}$ . The purple colour of the medium due to Bromocresol purple should not change in case there is no contamination. If colour changes to yellow (acidic reaction) or blue (alkaline reaction) the culture is contaminated and it should be rejected.

### iv. Reaction with litmus milk

A loopful broth was introduced into litmus milk and observed for the change in growth and acidity. The blue colour of the milk shall not change if there is no contamination. A change in colour indicates, presence of contaminant. Hence the culture should be rejected.

### v. Testing on CRYEMA Medium

A loopful of broth culture of the *Rhizobium* was streaked on the Petri plates of CRYEMA medium and incubated at  $28 + 2^{\circ}\text{C}$  for 48 hrs. White translucent, glistening and elevated streak was observed after the incubation period. Contaminated colonies observed red colour of congo red.

### Quantitative test

#### i. Testing of *Rhizobium* culture

Pea *Rhizobium leguminosum* cultures were tested for their efficacy in sterilized and unsterilized soil in pot house. Pea genotype Azad-P-1 was used for pot and field experiments. For each isolate, 10 surface sterilized earthen-pots (30 cm size) were filled with sterilized soil. Surface sterilization was done by 5% copper sulphate solution. Ten pots filled with unsterilized soil served as control. Five seeds of each genotype were inoculated with *Rhizobium leguminosum* using the sticker solution of powdered gum Arabic and sown in pots. Seeds without treatment with *Rhizobium* culture served as control. Before sowing treated seeds were air dried for 2 hours under shade. Uniform plant population of 3 plants/pot were maintained.

At the initiation of flowering following observations were taken:

1. Number of active nodules (pink coloured) per plant.
2. Fresh weight of pea plant and nodules.
3. Dry weight of pea plants and nodules.
4. Soil samples were taken before sowing and after harvesting of the crop the estimation of available nitrogen. Grain yield was recorded after harvesting of crop.

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## ii. Preparation of *Rhizobium* broth culture

### a. Multiplication of broth in simple Erlenmeyer flask

Yeast extract mannitol broth was prepared by dispensing 100 ml aliquots of the broth into 250 ml conical flask. These were plugged with non absorbent sterilized cotton. The flasks were autoclaved at 121.6°C or 1.045 kg/cm<sup>2</sup> for 20 minutes and then cooled. Sterilized yeast extract mannitol broth was inoculated aseptically with 7 day old *Rhizobium* culture.

The culture was examined by Gram's staining test method to eliminate contamination. Inoculated flasks were incubated at 28 + 2°C and shaken periodically for 7 days for uniform distribution of *Rhizobium* in broth.

### b. Multiplication of broth culture by using glass fermentor

For quick multiplication of *Rhizobium* culture glass fermentor was used multiplied broth culture was inoculated to seed with Arabic gum powder which was used as sticker.

#### a. Material used for installing simple glass fermentor

All materials required are readily available. The items needed are ~~for one~~ one litre Erlenmeyer flask to which a 35 mm long glass tube (7 mm outer diameter) has been fitted near its base and parallel to the bottom; glass tubing, 800 mm x 7 mm OD (cut into 6 pieces of 1 @ 400 mm, 5 @ 80 mm), two pieces of large diameter glass tubing, 120 mm x 30 mm OD (the barrels of two disposals 50 ml plastic polypropylene syringes may be used instead); a piece of solid glass rod 3 cm x 8 mm OD; surgical rubber tubing 800 mm x 12 mm OD (6 mm ID); four 30 (25) OD (ID) rubber stoppers; one 55 (40) OD (ID) rubber stopper; small roll of aluminum foil; small roll of non-absorbent cotton wool; tubing clamp; 300 mm flexible steel wire; autoclavable masking + tape; small aquarium pump; yeast mannitol broth. Adjust p<sup>H</sup> to 7 and then autoclave it.

#### b. Assembly

The assembly is shown in plate 3. It is important to pack the cotton in the filter barrels uniformly but loosely enough to facilitate air passage. All glass tubes and stoppers have to be tightly fitted to avoid leaks, supports for the filters is achieved by a wire ring surrounding the lower neck of the vessel. Two small pieces of wire are bent into little hooks which are taped to end of the filters. The filters may then be hooked into the wire ring photograph is showing one fermentor attached with aquarium pump photograph is showing three small glass fermentor attached with a devised glass tube (prepared by glass blower) with three outlets attached to fermentor(s) and one in flow tube, where aquarium pump was joint.

### c. Operation

A maximum of two litres of yeast extract mannitol broth may be filled into the flasks. The mouth of the flask is then tightly stoppered and secured with a wrapper of cotton wool followed by aluminium foil. The air inflow is closed with a clamp near that point where the inflow filter connects to the 400 mm inlet tubing. The unit is then autoclaved at 1.045 kg/cm<sup>2</sup> and 121.6°C for 20 min. Inoculation is done on a clean bench of laminar flow air at room temperature. Twenty ml of inoculant broth ( $5 \times 10^8$  cells/ml) from a shaker culture is aseptically injected through the latex inlet tubing with a sterilized syringe filled with a 18 number needle. An aquarium pump is then attached to the inflow tubing, the clamp is removed and the air flow activated. *Rhizobium* population was counted periodically at 24, 48, 72 and 96 hours after inoculation. A control was also kept where one Erlenmeyer flask was inoculated without using aquarium pump. The flask was shaken after 24 hrs. at regular interval to homogenize inoculum for ten days.

### iii. Quantification of growth of *Rhizobium*

Haemocytometer was used to determine the total and viable number of cells in pure broth culture of *Rhizobium*. The grid system consist of 25 larger squares, each of which is divided into 16 small squares. Knowing the volume of each square. Concentration of rhizobia (total cell/ml) was calculated.

A well grown broth culture from fermentor was diluted to 1 : 10 dilution with sterile water, to bring the suspension within a countable range. Another dilution series was prepared in sterile water from this dilution. A small drop of diluted culture suspension was poured to haemocytometer grid by pipette, the plates were inverted and incubated at  $28 \pm 2^\circ\text{C}$  for 4 days, colonies are then counted and counts per ml of the broth were recorded.

## **Reaction of different pea genotypes to two selected isolates of *Pea Rhizobium leguminosarum***

### Pot experiments

A pot house experiment was conducted to observe the interaction of eleven genotypes of with two selected *Pea Rhizobium leguminosarum* isolates. Seeds of each genotype were inoculated ( $10^8$  cells/gm of lignite as carrier) with two isolates of *Pea Rhizobium leguminosarum* (P<sub>1</sub> and P<sub>2</sub>). For each genotype and each isolate three replications (pots) were taken *Rhizobium* inoculated seeds were sown in each pot. A population of three plants/pot was maintained. At the initiation of flowering nodule number, fresh and dry weight of plants and nodules were observed and estimation of nitrogen was done by Microkjeldhal method.

## Field experiments

Field trails were conducted in Randomized Block Design in factorial at Agricultural Research Station, Durgapura, Jaipur during Rabi 2000<sup>-01</sup> and 2001<sup>-02</sup>. Eleven genotypes were taken with three replication each. All the 11 Pea genotypes were inoculated with two pea *Rhizobium* isolated before sowing. Row to row distance was 30 cm and plant to plant distance was maintained 10 cm. Irrigation and other intercultural operations were done as per requirement of the experiment.

At the initiation of flowering five plants were selected randomly for counting the nodule number, wet and dry weight of plant and nodules. Dry weight of nodules and plants were taken by keeping samples in hot air oven at 60°C for twelve hours. Grain yield was also recorded after harvesting of the crop.

## ***Isolation and identification of fungi associated with Pea root nodules***

Effective Pea root nodules of different shapes and size were collected from Agricultural Research Station, Durgapura, Jaipur and washed thoroughly to remove all soil from the nodules, finally the nodules were washed by giving several changes in sterile distilled water. The washed nodules were then transferred to Petri plates (4 nodules/plate) containing Martin's Rose bengal agar medium with streptomycin and incubated at 28 + 2°C for a week. The fungi isolated were transferred to PDA slants and tentatively identified and frequency of each fungus per 100 nodules was calculated as below

$$\text{Frequency of fungus/100 nodules} = \frac{\text{No. of isolates of each fungus}}{\text{No. of nodules plated}} \times 100$$

Fungi were also isolated from the surface of root nodules from the fungicide treated and *Rhizobium* inoculated plants infield. bavistin, captan, indofil M-45, ridomil MZ, thiram and topsin-M fungicides were used for the study. The frequency of fungi/100 nodules was also calculated as above.

## Isolation of actinomycetes

Pea root nodules were plated on starch ammonium agar medium. Each Petri plate contained four nodules, ten replications were taken. These were then incubated in an incubator at 28 + 2°C for 72 hrs. The colonies of actinomycetes were transferred to test tubes containing starch ammonium agar slant.

## Isolation of bacteria

Thornton's standardized medium was used for isolation of bacteria. Four nodules were plated in each Petri plate containing 20 ml of solidified medium. Ten replications were taken. Petri plates were incubated for 48 hrs. at 28 + 2°C. The bacterial colonies were transferred to nutrient agar slants.

## Screening of fungi for antagonistic activity of *Rhizobium*

Number of fungi associated with Pea nodules were isolated identified and maintained on 100 ml aliquot of Richard's synthetic medium for 21 days at 28 + 2°C. The fungal mats were discarded and the culture filtrates were adjusted to pH 7.0. These filtrates were evaporated to dryness in large flat dishes at 40°C in oven and the residue taken up in 100 ml of sterile distilled water.

The filter paper disc method was adopted for testing antagonistic activity Whatman No. 1 filter paper disc (10 mm in diameter) were soaked in the concentrated filtrates and placed 4 cm apart on *Rhizobium* inoculated CRYEMA medium plates. Four discs were plated/Petri plate. The plates were incubated for 48 hrs. and inhibition zones were measured.

## Screening of actinomycetes and bacteria for antagonistic activity against *Rhizobium*

The cross streak method was adopted for testing antagonistic activity on CRYEMA medium. The plates were incubated at 28 + 2°C for 48 hrs. observations were taken for antagonistic activity of bacteria and actinomycetes by measuring inhibition zones.

For screening of Actinomycetes and bacteria five replications were taken for their antagonistic activity.

## Rhizosphere microflora studies

Isolation of fungal, bacterial and actinomycetes species from Pea rhizosphere.

Plants at the stage of initiation of flowering were carefully uprooted and gently shaken to remove excess soil adhering to the roots. The roots later cut into bits with the help of a sterilized blade and these bits were put in sterile 150 ml distilled water in 250 ml flask and were shaken thoroughly. This suspension served as the stock solution and from this serial dilutions were made in 9 ml sterile water blanks with the help of one ml pipettes sterilized previously. Martin's rose bengal streptomycin agar medium was used for isolation of soil fungi from dilutions 10<sup>-2</sup> and 10<sup>-3</sup>. Sterile ammonium agar medium was used for the isolation of rhizosphere actinomycetes from

dilutions  $10^{-4}$  and  $10^{-5}$ . Rhizosphere bacteria were isolated from dilutions  $10^{-6}$  and  $10^{-7}$  on Thornton's standardized medium.

Fungi after isolation were maintained on PDA, actinomycetes on czapek's medium with glucose and bacteria on nutrient agar.

## Microbial population changes in Pea rhizosphere as influenced by different fungicides

Seventy two pots (30 cm) were filled with unsterilized soil. Seeds of Pea genotype Azad-P-1 were treated with seed dressing fungicides viz., bavistin, captan, indofil M-45, ridomil-MZ, thiram and topsin-M were inoculated with *Rhizobium* culture and there after sown in pots. Two controls i.e. seeds treated with *Rhizobium* and another of seeds without *Rhizobium* treatment were maintained. Each treatment had 3 pots with 3 seeds/pot. Three plants from each of the treatments were carefully uprooted after 30, 45 and 60 days of sowing and fungal, bacterial and actinomycetes counts in the rhizosphere of each treatment at particular interval were taken by serial dilution method from rhizosphere soil suspension. Thornton's standardized medium was used for counting bacteria, Martin's rose-bengal streptomycin agar medium for fungi and starch ammonium agar medium for taking actinomycetes counts. Bacterial count were taken after 24 hrs of incubation, fungal actinomycetes counts after 48 hrs. of incubation.

## Influence of MYCO-colonized root infecting fungi on symbiotic nodulation of Pea

### I. Pot conditions

Sterilized soil was filled in 30 cm diameter earthen pots. In each pot, 7 kg soil was filled and 5 g single super phosphate was added in each pot.

### II. Preparation and mixing of fungal culture

Fungal culture of *Myrothecium verrucaria*, *Fusarium oxysporum*, *Drechslera* state of *Cochliobolus Spicifer*, *Aspergillus niger*, *Pencillium oxalicum* found on nodule surface were grown in 500 ml flasks, containing 180 ml synthetic Richard's broth medium. The flasks were incubated at  $28 \pm 2^{\circ}\text{C}$  for 7 days. When fungal mat were fully developed, then these culture were mixed in sterilized soil pots for mycocolonization.

### **iii Incubation of seeds with Pea *Rhizobium leguminosarum* culture and their sowing in pots**

Pea *Rhizobium leguminosarum* inoculated pea seeds were sown in pots and population of 3 plants/pots were maintained. Sampling and observations were taken on growth nodulation and nitrogen uptake of each plant was estimated with micro Kejl Dahl digestion flask method.

### **Compatibility of seed dressing chemicals**

#### **In-vitro studies**

#### **Fungicidal assay**

For fungicidal assay the method described by the Waksman and Reilly (1945) was adopted. Seed dressing chemicals used were bavistin, captan, indofil M-45, ridomil-MZ, thiram and topsin-M.

The assay medium used was CRYEMA. Twenty four hours old culture of *Rhizobium* was added to the lukewarm medium and poured in thin layer into sterilized Petri plates and allowed to solidify. Filter paper discs (10 mm diameter) were soaked in different concentration of each chemical plated (4 discs/plates) to CRYEMA.

Inhibition zone was measure after incubation period of 72 hrs by method given below:

Zone of inhibition ( $\text{mm}^2$ ) = Area of zone of inhibition - Area of original disc.

#### **Plating of fungicides dressed seed on *Rhizobium* inoculated medium**

Different seed dressing fungicides viz., bavistin, captan, indofil M-45, ridomil-MZ, thiram and topsin-M were used to treat the Pea seeds. Treated seeds were plated aseptically on Petri plates containing *Rhizobium* inoculated CRYEMA medium. Four replications were taken for each treatment and each Petri plate contained 4 seeds. These were incubated at  $28 \pm 2^\circ\text{C}$  for 72 hrs. and inhibition zones were measured.

#### **Plating of fungicides dressed and *Rhizobium* inoculated seeds**

Pea seeds were surface sterilized by soaking in mercuric chloride (1: 1000) solution for 1½ min and washed with sterile distilled water 4 times to remove traces of mercuric chloride. Fungicides dressed and *Rhizobium* inoculated seeds were plated on *Rhizobium* inoculated CRYEMA medium and observations were taken on inhibition of *Rhizobium* growth after incubating the plates for 72 hrs.

## **Studies under pot and field conditions**

Seeds of pea genotype Azad-P-1 were treated with each of the six fungicides including bavistin, captan, indofil M-45, ridomil-MZ, thiram and topsin-M then inoculated with *Rhizobium* ( $10^8$  cells/g lignite as carrier 25g/kg seed) using a sticker solution of powdered gum Arabic. Each treatment was having 3 replications with 3 plants/pot only *Rhizobium* treated seeds and uninoculated seeds served as control. The pots were watered at regular intervals throughout the experimentation.

## **Field experiments**

Similar set of treatments was also sown in field with plot size 2m x 3m in Randomized Block Design conducted during 2000<sup>-01</sup> and 2001<sup>-02</sup> rabi season. Each treatment was replicated four times row to row and plant to plant spacing was 30 and 10 cm, respectively. Single super phosphate was applied at the time of sowing @ 70 kg/ha. Irrigations were applied at regular intervals throughout the experimentation.

At the initiation of flowering 3 plants/pot were selected randomly for counting number of effective nodules and fresh and dry weight of plant and nodules.

## **Sampling and observations**

Observations were recorded at the onset of flowering. The number of nodules/ plant/ treatment were counted. Fresh weight/ plant/ treatment and fresh weight of nodules/plant/treatment was taken and then the plants and nodules were air dried. Dry weight/plant/treatment and dry weight of nodules/plant/treatment were taken after drying the samples in oven at 60°C for 48 hrs. Available nitrogen of the soil was also estimated with micro kjeldhal method.

## **Estimation of Nitrogen Contents in Nodules and in Shoot:**

(Official method of Analysis of Association of Official Analytical Chemists AOAC, 1965).

100 mg dried powdered sample of nodule or shoot transferred 30 ml micro Kjeldahl digestion flask. To which 200 mg of catalysy mix of  $K_2SO_4 + CuSO_4$  in the ratio of 98:2) and 2 ml of 98%  $H_2SO_4$  was added. Mixture was digested for about 6-8 hours till the contents of the flask were colourless sample was cooled and then minimum quantity of water was added to it. Digested sample was transferred to distillation apparatus, followed by addition of 10 ml of 40% NaOH.

In 100 ml conical flask 5 ml of 4% Boric acid solution was taken. To this four drops of indicator (2 parts of methyl red 0.2% + one part of methylene blue 0.2%) was added. The flask

was kept at the receiver tip of distillation apparatus so that ammonia gas liberated during steam distillation passes into 4% Boric acid solution. After 15 minutes, the flask was removed and the contents were titrated against 0.02 NHCL. A blank was also run simultaneously. Percentage N was calculated by following formula

$$N \text{ in } \% = \frac{(S - B) \times \text{Normality of HCl} \times \text{Equivalent Wt. of N}}{\text{Weight of sample in mg}} \times 100$$

S = ml HCl required for sample

B = ml HCl required for blank

### **Estimation of total Available Nitrogen in Soil (AOAC 1965)**

One gram of soil was digested in 30 ml microkjeldahl flask with 5 ml of H<sub>2</sub>SO<sub>4</sub> 2% CuSO<sub>4</sub> and 1 g anhydrous Na<sub>2</sub>SO<sub>4</sub> for 1.5 hours. It was cooled and diluted with distilled water and about 20 ml of 40% NaOH was added to the flask just before connecting the flask to Kjeldahl distillation apparatus.

10 ml of 4% boric acid solution was placed in 100 ml conical flask to which 2 drops of mixed indicator (2 parts of methyl red 0.2% + one part methylene blue 0.2%) was added. The NH<sub>3</sub> liberated during the steam distillation of the sample was allowed to pass in the above boric acid solution (for about 15-20 minutes). This was then titrated against 0.02% N HCl. The blank containing without sample was titrated in similar manner. Percentage nitrogen was calculated according to the following formula:

$$\%N = \frac{T \times N \times 0.014 \times 100}{W}$$

where,

T = Volume in ml of standard acid required for soil sample.

N = Normality of standard acid

0.014 = Meq weight of nitrogen

W = Weight of sample in gram.

### **Estimation of Leg-haemoglobin in Nodules**

From each treatment 100 mg nodules were taken. These were washed and dried 5 ml pyridine and 500 mg sodium hydrosulphite were added to nodules. The mixtures was crushed in mortar and pestle and centrifuged at 3000 rpm for 10 minutes. Supernatant was taken and total volume was made upto 5 ml by adding pyridine. Determination of haematin was made on the

basis of absorption at 530 nm (standard solution was prepared on hematin HCl compound isolated from normal human blood). Leg-haemoglobin content of nodule was calculated by following formula:

$$\text{Concentration of leg-haemoglobin (mg)} = \frac{\text{Concentration of standard}}{\text{Density of standard}} \times \frac{\text{Density of unknown}}{5\text{ml}} \times \frac{x}{100}$$

X = mg nodules from single plant.

***Estimation of Haemoglobin from normal human blood:***

(Sahil's method : In clinical Hematology, ed. by M. Maxwell and M. D. Wintrobe 5<sup>th</sup> edition, 1961 pub. Lea and Febiger Philadelphia pp. 392-400).

i. In 50 ml volumetric flask 0.5 ml of well mixed oxalated human blood was taken by using a micro pipette. To this solution 2 ml of iron free concentrated sulfuric acid and 2 ml of saturated potassium sulphate solution was added and the solution was diluted in 25 ml with distilled water. To this, 2 ml of 10% sodium tungstate solution was added (for precipitation of protein). The solution was cooled under the tap water and distilled water was added to make the volume upto 50 ml. The same was filtered in dried flask.

For the preparation of standard solution, instead of blood, 0.5 ml of standard iron solution (0.1 ml each of unknown standard blank solution was taken in separate test tubes. Blank was prepared by taking to 10 ml of distilled water. To this, 0.5 ml of saturated potassium per sulphate solution and 2 ml of 3 N potassium thiocyanate solution was added to each of them. The solution in each test tube was mixed by inversion method. Optical density was measured at 480  $\mu\text{m}$  within 30 minutes after the colour development. Blood haemoglobin content was calculated by using the following formula :

$$\text{Concentration of haemoglobin/100 ml blood (g)} = \frac{\text{Density of unknown}}{\text{Density of standard}} \times 0.25 \times \frac{100}{0.5} \times \frac{1}{3.4}$$

***Preparation of Standard Acid Hematin Solution:***

Haemoglobin content of normal human blood was determined from the above method. On the basis of haemoglobin content the blood was diluted with 0.1 N HCl in volumetric flask so that, the resultant haemoglobin concentration became 3%. Standard solution was stored in a refrigerator for further use. From this stock solution, the dilute standard was prepared freshly, every week by diluting 5 ml of stock solution to 200 ml in a volumetric flask with 0.1 N HCl. Keep the solution in cold and dark. This dilute standard is an acid hematin solution isolated from normal human blood.

The estimation of leg-haemoglobin contents were done at Department of Biochemistry, SMS Medical College Jaipur.

## **Interaction of *Rhizobium* and root knot nematode (*Meloidogyne incognita*) on the development of root nodules and plant growth**

A pot experiment was conducted to find out the interaction of nematode and *Rhizobium* inoculation alone and in combination. The effect of carbofuran on the combination of root knot nematode was also studied. Thirty six pots of 15 cm size were taken filled with 1 kg sterilized soil in each pot. These were arranged for 5 treatments in 6 replications. Each pot contained one plant. Seeds of genotype Azad P-1 were used for the experiment.

The various treatments are

- (i) untreated check,
- (ii) root knot nematode alone (20 egg masses inoculated in 1 kg of soil),
- (iii) *Rhizobium* treatment alone ( $10^8$  cells /g of carrier based inoculant),
- (iv) *Rhizobium* + root knot nematode) and
- (v) root knot nematode + *Rhizobium* + carbofuran @ 2 kg/ha or 0.6 g/pot.

Observations were recorded after 60 days of germination by counting the number of galls, nodules, average length of root, shoot, fresh weight of root, shoots/plant.

## **Compatibility of chemical protectants and antagonistic bioagents with Pea *Rhizobium leguminosarum***

Seeds of pea genotype Azad-P-1 were moistened with 5 per cent gum Arabic solution applied at 100 ml kg<sup>-1</sup> of seed. These moistened seeds were treated in the sequence as bioagent ( $10^6$  spores/ml/10g seed), test fungicides (indofil M-45 and bavistin @ 1 g/kg) and then *Rhizobium* ( $10^8$  cells/g of lignite as carrier) @ 25 g/kg seed. A check was also maintained where Pea seeds were treated with *Rhizobium* and un-treated check. Size of the pots, methods of sowing and watering were the same as mentioned under pot experiments.

The details of the fungicides, bioagents and *Rhizobium* combination are mentioned as under:

Fungicides	Bioagents	Combination with pea
Bavistin	<i>Trichoderma viride</i>	Bavistin + <i>T. v.</i> + <i>Rhizobium</i>
	<i>Trichoderma harzianum</i>	Bavistin + <i>T. h.</i> + <i>Rhizobium</i>
	<i>Gliocladium virens</i>	Bavistin + <i>G. v.</i> + <i>Rhizobium</i>
	<i>Pseudomonas fluorescens</i>	Bavistin + <i>P. f.</i> + <i>Rhizobium</i>

<b>Fungicides</b>	<b>Bioagents</b>	<b>Combination with pea</b>
Indofil M-45	<i>Trichoderma viride</i>	Indofil M-45 + <i>T. v.</i> + <i>Rhizobium</i>
	<i>Trichoderma harzianum</i>	Indofil M-45 + <i>T. h.</i> + <i>Rhizobium</i>
	<i>Gliocladium virens</i>	Indofil M-45 + <i>G. V.</i> + <i>Rhizobium</i>
	<i>Pseudomonas fluorescens</i>	Indofil M-45 + <i>P. f.</i> + <i>Rhizobium</i>

# **Experimental Results**

# EXPERIMENTAL RESULTS

## 1. Survey and collection of pea root nodules

Root nodules of pea were collected from ten localities of Jaipur district in Rajasthan during rabi 1999 - 2000.

Average number of active nodules/plant and their categorization in different localities is shown in Table 1.

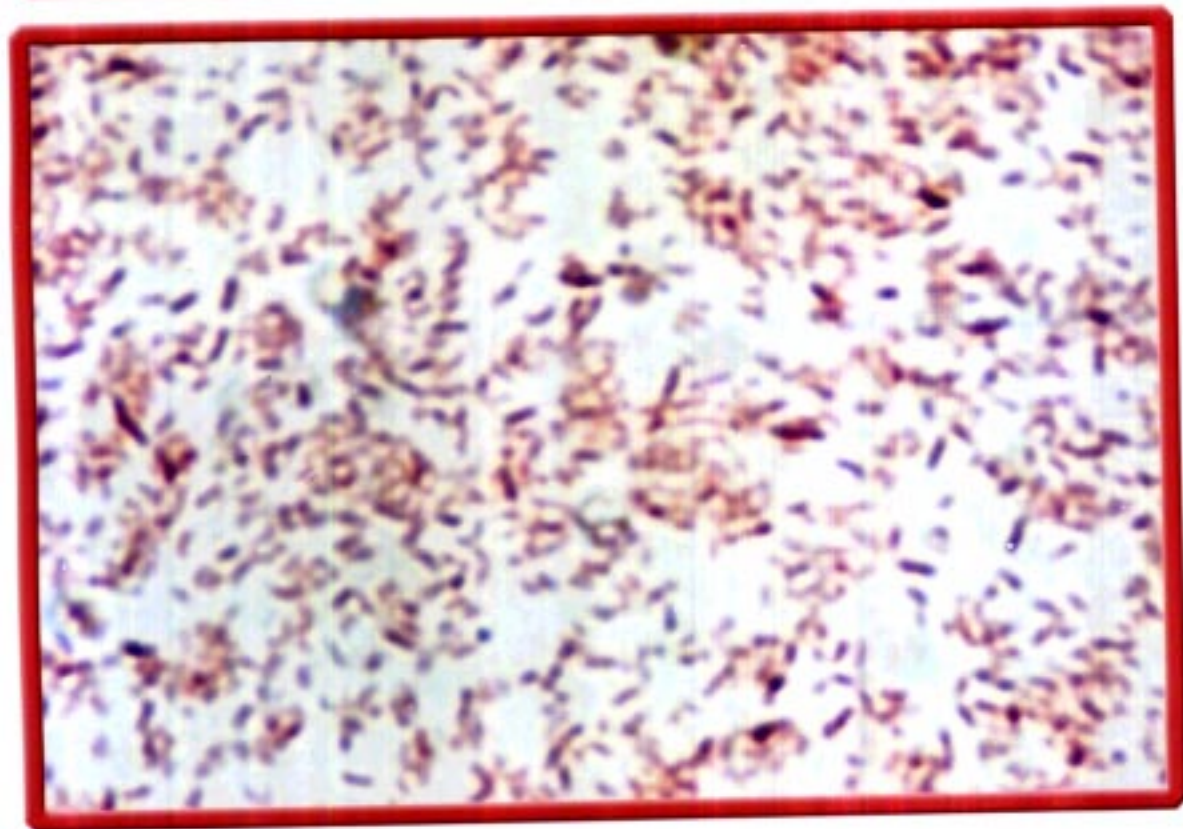
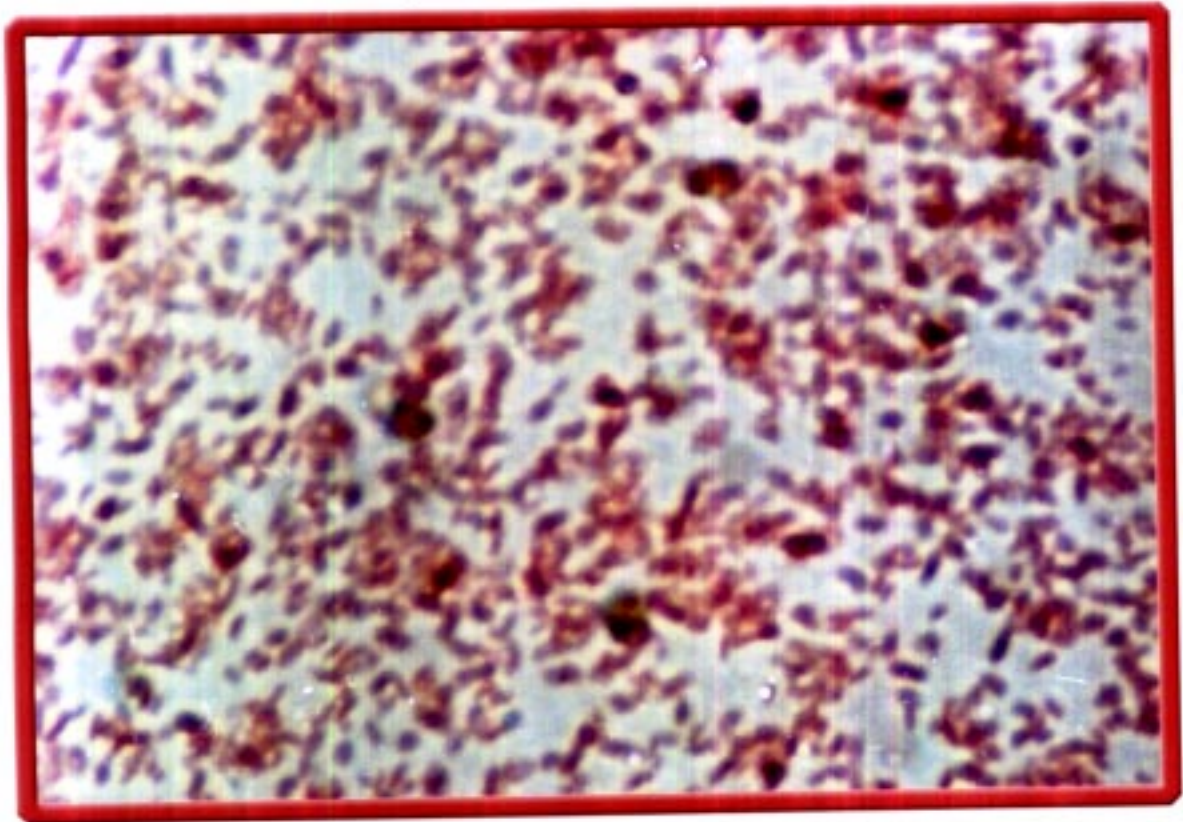
*Table 1. Nodulation status of Pea crop in different localities of Jaipur district in Rajasthan during rabi 1999 - 2000.*

Name of locality	Name of isolate	Average number of active nodules/plant	Category *
Boraj	P <sub>1</sub>	32	+++
Bobas	P <sub>2</sub>	30	+++
Sambhar	P <sub>3</sub>	28	+++
Phulera	P <sub>4</sub>	25	+++
Lalpura	P <sub>5</sub>	23	+++
Hirnoda	P <sub>6</sub>	20	++
Durgapura	P <sub>7</sub>	18	++
Jobner	P <sub>8</sub>	06	+
Nagonki Dhani	P <sub>9</sub>	08	+
Bagru	P <sub>10</sub>	16	++

\* +++ = Good nodulation, ++ = Moderate nodulation, + = Poor nodulation

Scale for categorization of nodules:

*1(a) Rhizobium leguminosarum bacterioids from pink colour nodule, after Gram's staining.*



*1(b) Rod shape Rhizobium leguminosarum from CRYEMA culture, after Gram's staining.*

Poor = Less than 10 nodules/plant  
Moderate = 11-20 nodules/plant  
Good = 21-40 nodules/plant  
Very good = 41 and above nodules/plant

Nodulation status of each locality is shown in Table 1. Perusal of data reveals that good nodulation status was observed in Boraj, Bobas, Sambhar, Phulera and Lalpura. Isolated *Rhizobium* were named as P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>, P<sub>4</sub> and P<sub>5</sub> isolates respectively. However, nodule status was categorized moderate in Jaipur, Bagru and Hirmoda, whereas poor nodulation were observed at NagokiDhani and Jobner. Isolations were made under aseptic conditions from active pea nodules collected during the survey in sterilize silica gel tubes and *Rhizobium* culture so obtained was tested by qualitative and quantitative methods. MULLaRP isolate (AICPIS, Durgapura) DP<sub>3</sub> was also tested by qualitatively and quantative tests.

## 2. Qualitative tests

Identification of *Rhizobium* culture by

### i. pH Test

For determination of p<sup>H</sup> *Rhizobium* culture was inoculated to glucose peptone broth medium (adjusted to p<sup>H</sup> 7.0). These flasks were incubated at 28 ± 2°C for 48 hrs. The p<sup>H</sup> was tested by p<sup>H</sup> meter. After incubation period of 48 hrs, p<sup>H</sup> was found to be in the range of 6.8 to 7.0 (remain unchanged). The culture was uncontaminated.

### ii. Gram's staining

A smear was prepared from 24 hrs old diluted suspension of *Rhizobium leguminosarum* culture and stained by Gram's staining method. Under high magnification, it was found that bacterial cells were rod shape and pink coloured. The pink colouration confirming their identity as gram negative. (Plate 1 a & b).

### iii. Growth on glucose peptone agar medium

A loopful of 48 hrs old *Rhizobium* culture was streaked to glucose peptone agar plates. The purple colour of the medium (due to bromocresol purple) remained uncharged. The test showed that culture was pure.



2(a) Pea plants inoculated with  $P_1$  and  $P_2$  isolates of *Rhizobium leguminosarum*.

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2(b) Uninoculated pea plant showing lesser number of nodules

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#### iv. Reaction with litmus milk

A loopful of 48 hrs old inoculated broth was poured in test tubes containing litmus milk and incubated for 48 hrs. Observations were recorded upto 30 days at regular intervals. Blue colour of the litmus milk remained uncharged, indicating that culture was uncontaminated. For comparison uninoculated control was also maintained.

#### v. Testing on CRYEMA medium

A loopful of broth culture was <sup>o</sup>pure on YEMA congo-red medium in Petri plates. These were incubated at  $28 \pm 2^{\circ}\text{C}$  for 48 hrs. white, translucent, glistening and elevated colonies were observed in all cases. None of the colonies absorbed red colour of congo red. Thus, the culture was pure.

### 3. Quantitative tests

#### i. Test for nodulation

Eight different *Rhizobium* isolates were further tested for nodulation. Pea seeds of genotype variety Azad-P-1 were inoculated with different isolates of pea *Rhizobium* viz., P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>, P<sub>4</sub>, P<sub>5</sub>, P<sub>6</sub>, P<sub>7</sub> and D P<sub>3</sub> at the concentration of  $10^8$  cells/g of carrier material (lignite) and using gum Arabic as sticker. Treated seeds were tested in 30 cm earthen pots containing sterilized soil. The average number of active nodules were counted, fresh and dry weight of the nodules per plant was recorded at the initiation of flowering and results are shown in Table 2. The analysis of Table 2 reveals that the *Rhizobium* isolate P<sub>1</sub> (Boraj) was found most effective with maximum number of average nodules (36.66/plant), fresh and dry weight of nodules/plant (547.66 mg/plant and 216 mg/plant) as compared to other isolates i.e. P<sub>2</sub> (Bobas), P<sub>3</sub> (Sambhar), P<sub>4</sub> (Phulera), P<sub>5</sub> (Lalpura), P<sub>6</sub> (Himoda), P<sub>7</sub> (Durgapura) and DP<sub>3</sub> (MULLaRP) as shown in the Table 2. Isolates P<sub>1</sub> and P<sub>2</sub> gave higher nodule counts and these were used for further experimental work (Plate 2).

#### ii. Quantification growth of Rhizobium

Comparative population of *Rhizobium* was analysed after 24, 48, 72 and 96 hours by using simple Erlenmeyer flask (shake culture method) and glass fermentor and results are shown in Table 3.

From the results it is evident that after 96 hrs higher viable counts were observed by using glass fermentor method  $\{54 \times 10^8$  (P<sub>1</sub> isolate) $\}$ , as compared to shake culture method  $\{50 \times 10^8$  (P<sub>1</sub> isolate) $\}$ . Isolate P<sub>2</sub> also gave higher counts by using fermentor method  $(30 \times 10^8)$  after 96 hours of incubations (Plate 3).

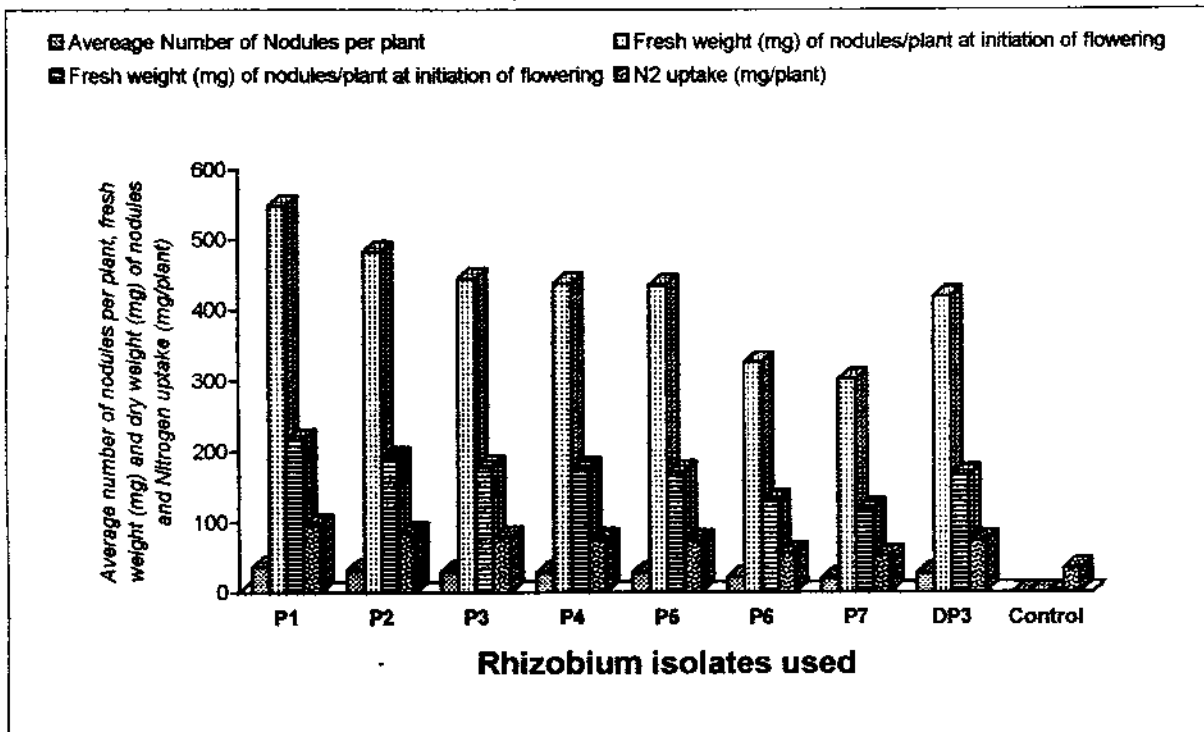
3. Assembled fermentor containing *Rhizobium leguminosarum* inoculum [glass fermentor connected to a glass tube (single inflow and three outlet) and aquarium pump].



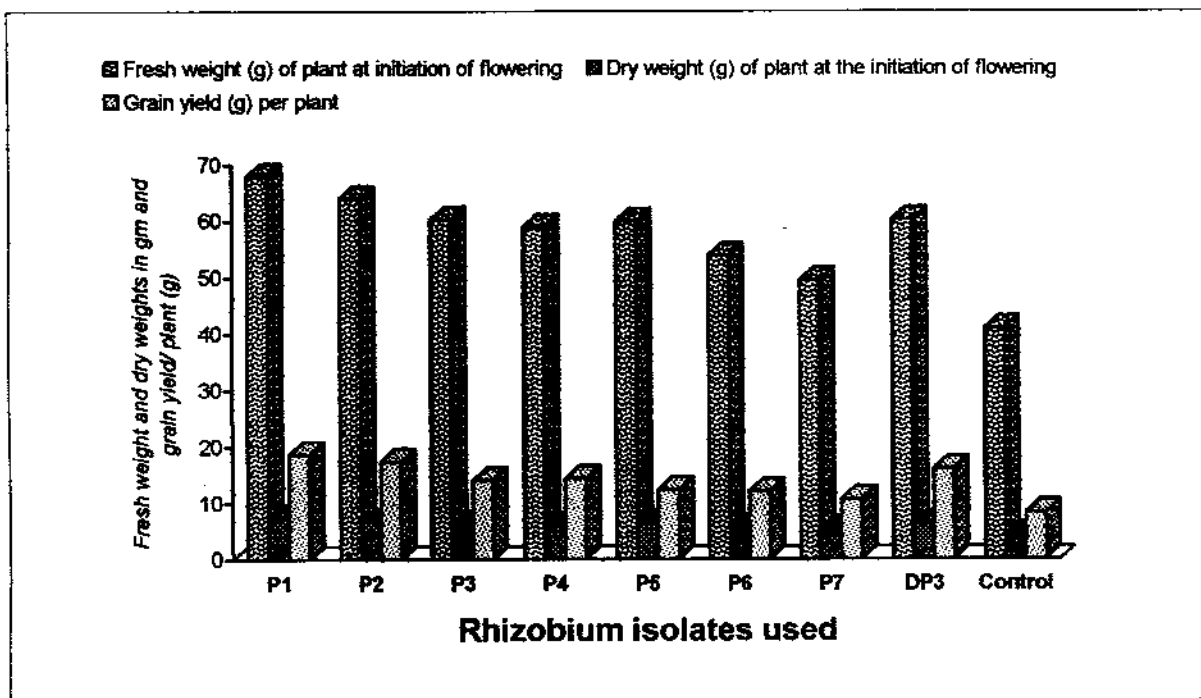
7. Root - Knot showing Pea (Azad-P-1) plant infected with *Meloidogyne incognita*.

Table 2. Quantitative test of different isolates of *Rhizobium leguminosarum* under sterilized soil conditions in pots.

Treatments	Av. No. of nodules/plant	Av. Weight of nodules/plant at the initiation of flowering		Av. Weight of /plant at the initiation of flowering		Grain yield/plant (g)	N <sub>2</sub> uptake mg/plant
		Fresh wet weight (mg)	Dry weight (mg)	Fresh wet weight (mg)	Dry weight (mg)		
P <sub>1</sub> (Boraj)	36.66 [6.2033]	547.66	216.00	68.00	7.33	18.66	96.33
P <sub>2</sub> (Bobas)	32.00 [5.8167]	482.33	190.33	64.00	6.66	17.33	84.33
P <sub>3</sub> (Sambhar)	30.00 [5.6567]	444.33	178.33	60.33	6.33	14.00	78.00
P <sub>4</sub> (Phulera)	29.00 [5.4067]	437.66	175.00	59.00	6.00	14.33	75.66
P <sub>5</sub> (Lalपुरा)	28.66 [5.4167]	435.00	170.33	60.00	6.33	12.33	73.66
P <sub>6</sub> (Hirmoda)	22.00 [94.7267]	325.00	130.00	54.00	5.66	12.00	55.33
P <sub>7</sub> (Jaipur)	20.00 [4.5400]	301.33	118.00	49.66	5.33	10.66	52.00
① P <sub>3</sub> (MuLLarp)	28.00 [5.2367]	419.33	166.33	60.33	6.33	16.00	73.66
Untreated control	00.00 [0.7600]	000.00	000.00	41.00	4.33	8.33	33.66
Mean	25.14	376.96	149.37	57.37	6.03	13.74	69.18
SEm±	0.18	5.03	4.25	2.86	0.34	1.52	1.99
C.D. at 5%	0.54	14.96	12.64	8.50	1.01	4.53	5.92
C.D at 1%	0.74	20.50	17.32	11.64	1.39	6.21	8.11
C.V.%	6.51	2.31	4.93	8.63	9.84	19.25	4.99



**A. Nodulation characteristics**



**B. Plant characteristics**

**Fig 1. Quantitative test of different isolates of *Rhizobium leguminosarum* under sterilized soil conditions.**

**Table 3 Comparative population counts of broth using Erlenmeyer flask and glass fermentor plate count method.**

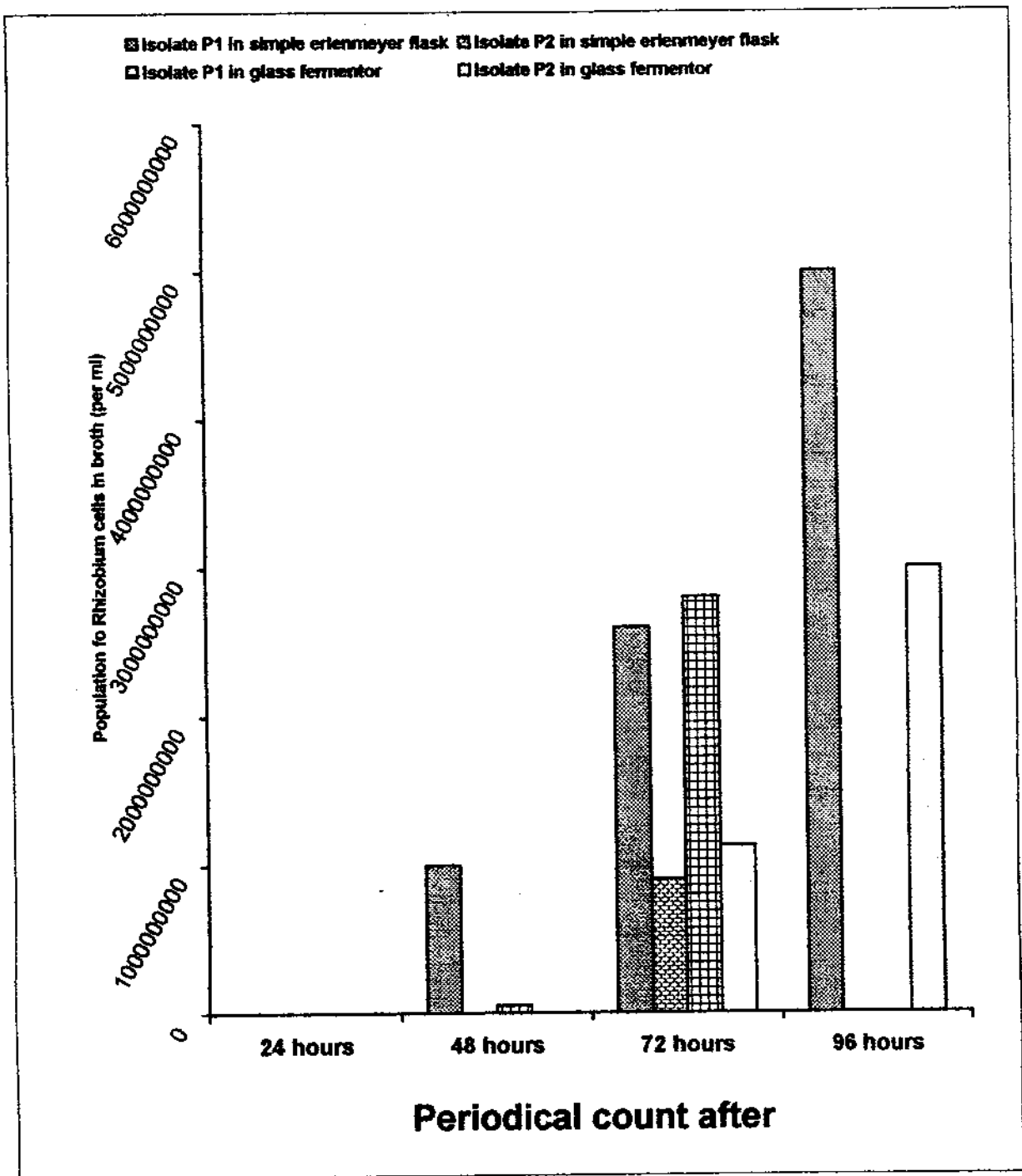
Periodical count after	Population of <i>Rhizobium</i> cells in per ml broth **				Mean
	Simple Erlenmeyer flask		Glass fermentor		
	Isolate (P <sub>1</sub> )	Isolate (P <sub>2</sub> )	Isolate (P <sub>1</sub> )	Isolate (P <sub>2</sub> )	
24 hours	0.00	0.00	0.00	0.00	0.00
48 hours	10.00x 10 <sup>8</sup>	0.00	12 x 10 <sup>8</sup>	0.00	5.500
72 hours	26.00x 10 <sup>8</sup>	9 x 10 <sup>8</sup>	30 x 10 <sup>8</sup>	11.25x 10 <sup>8</sup>	19.062
96 hours	30.00x 10 <sup>8</sup>	28 x 10 <sup>8</sup>	54 x 10 <sup>8</sup>	50.00x 10 <sup>8</sup>	40.500
Mean	15.3750		17.1563		

\*\* Average number of 4 replication

0.00 Indicates no *Rhizobium* population

Mean P<sub>1</sub> = 22.7500, P<sub>2</sub> = 9.7813

	SEm ±	CD at 5%
Method (M)	0.1342	0.3823
Isolate (P)	0.1342	0.3823
Hours (H)	0.1899	0.5407
M x P	0.1899	0.5407
M x H	0.2685	0.7646
P x H	0.2685	0.7446
M x P x H	0.3297	NS



**Fig 2. Comparative population counts of broth using Erlenmeyer flask and glass fermentor plate count method.**

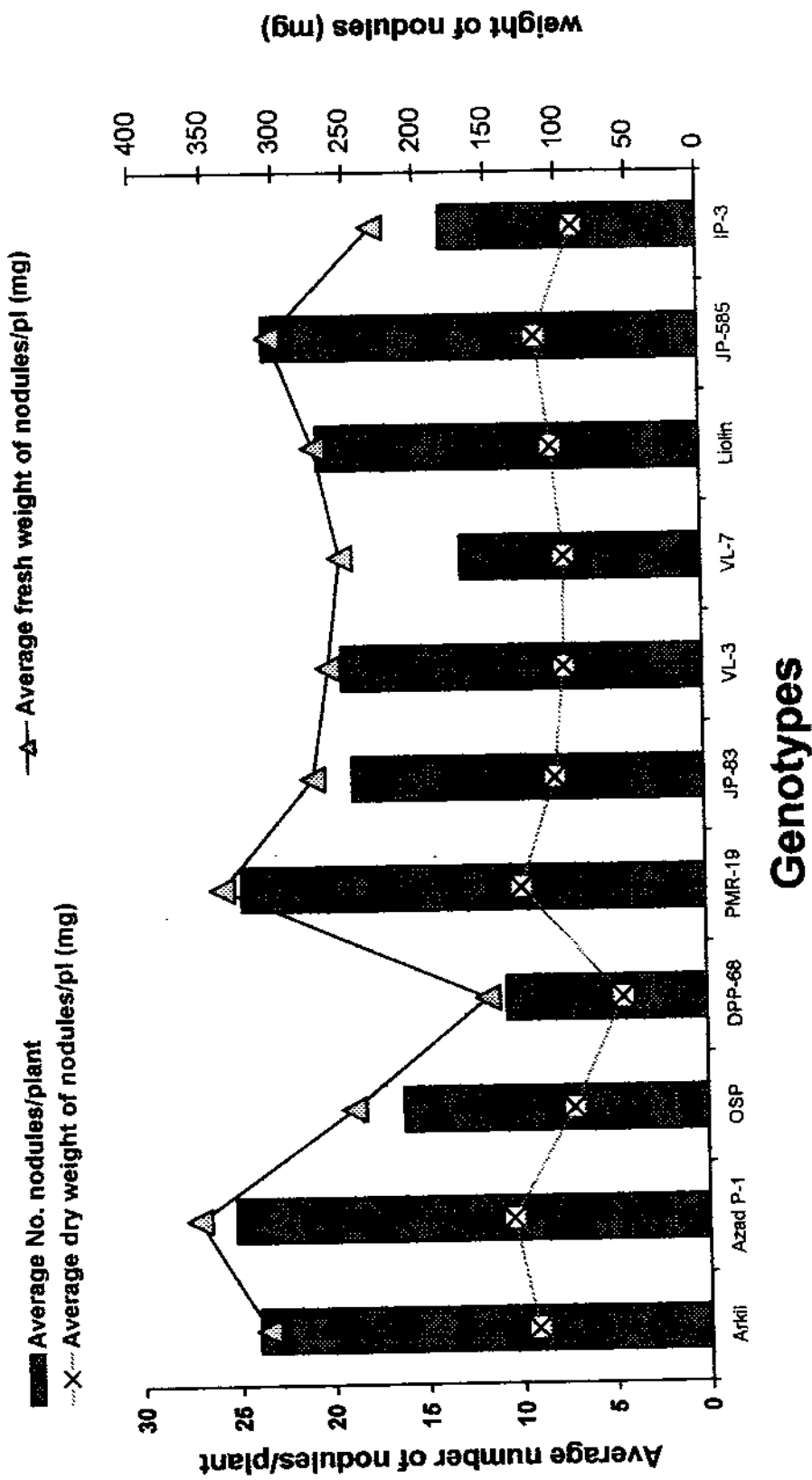


Fig 3. Nodule characteristics in Pea genotypes (pot experiment).



4(a) Rhizobium treated pea (Azad-P-1) plants showing effect of indofil- M 45.



4(b) Pea (Azad-P-1) plants treated with *Rhizobium* alone.



Attaining from the results is evident that after 96 hours higher viable counts were observed by using glass fermentor method [ $54 \times 10^8$  ( $P_1$  isolate),  $50 \times 10^8$  ( $P_2$  isolate)] for quick multiplication of *Rhizobium* population, glass fermentor in ideal method.

The statistical results are significantly at 5 per cent level.

#### **4. Screening of different Pea genotypes with different Rhizobium isolates of pea**

##### **i. Pot experiment**

Eleven pea ~~genotypes~~ <sup>genotypes</sup> were screened against 2 effective pea *Rhizobium* isolates ( $P_1$  and  $P_2$ ) under pot house conditions. At the initiation of flowering, average number of nodules/plant and fresh and dry weight of nodules per plant were recorded and results are presented in Table 4.

Screening of eleven genotypes of pea against two isolates of *Rhizobium* showed significant differences with respect to number of nodules and fresh and dry weight of nodules. However, maximum number of nodules were observed in Azad-P-1 (30 nodules/plant) which was significantly more over any other genotype against both the isolate. The two isolate also show significant differences among themselves. Whereas, isolate x genotype interaction were non significant.

The genotype (Azad-P-1) exhibited significant superiority. Fresh and dry weight, grain yield and  $N_2$  uptake was also significantly higher in genotype Azad-P-1 (58.66 g/plant) fresh weight, (13.83 g/plant) grain yield and (72.00 mg/plant)  $N_2$  uptake. Whereas, interactions among isolates genotype for these parameter were also non significant and it was used for further experimentation. Isolate x genotype interactions were non significant for these two parameter also (Plate 5).

##### **ii. Field experiment**

The analysis of 2 year (2000<sup>01</sup>-2001<sup>02</sup>) pooled data (Table 5) reveals in the screening of eleven genotypes of pea against two isolates of *Rhizobium* showed significant differences with respect to number of nodules and fresh and dry weight of nodules. Maximum number of nodules were produced by Azad-P-1 (32.50 nodules/plant) which was significantly more over any other genotype against both the isolate. The two isolate also show significant differences among themselves. Where year x genotype, year x isolate, genotype x isolate interaction were found non significant.

Fresh and dry weight, grain yield and  $N_2$  uptake was also significantly higher in genotype Azad-P-1 (60.07 g/plant) fresh weight, (6.24 g/plant) dry weight, (15.92 g/plant) grain yield and

5(a) - Pea genotype Azad- P- 1 showing vigorous plants growth treated with *Rhizobium leguminosarum* in pots.



5(b) - Pea genotype PMR -19 showing vigorous plants growth treated with *Rhizobium leguminosarum* in pots.

Table 4. Screening of different Pea genotypes to different isolates of *Rhizobium leguminosarum* under pot house conditions during rabi 2000-01.

Genotypes	Av. No. of nodules/plant				Average weight of nodules at the initiation of flowering				
	P <sub>1</sub>		P <sub>2</sub>		Fresh weight (mg)		Dry weight (mg)		Mean
	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	
Arkil	25.00 [4.99]	23.00 [4.79]	24.00 [4.892]	375.00	252.66	313.833	148	98	123.000
Azad-P-1	30.00 [5.46]	25.33 [5.02]	25.16 [5.243]	445.00	278.33	361.667	171.66	106.66	139.167
OSP	18.33 [4.27]	14.00 [3.72]	16.16 [4.000]	278.33	223.33	250.833	106.33	85.33	95.833
DPP-68	11.66 [3.38]	9.66 [3.09]	10.66 [3.240]	170.66	141.00	155.833	65.00	53.66	59.333
PMR-19	28.33 [5.31]	21.00 [4.40]	24.66 [4.942]	415.00	269.66	342.333	159.00	103.00	131.000
JP-83	21.66 [4.64]	15.66 [3.95]	18.66 [4.298]	325.00	228.33	276.667	124.33	87.33	105.833
VL-3	21.66 [4.64]	16.66 [4.07]	19.16 [4.360]	315.66	214.00	264.833	114.66	81.66	98.167
VL-7	20.66 [4.53]	18.33 [4.26]	12.80 [4.400]	309.33	201.33	255.333	118.33	77.00	97.667
Linolin	22.33 [4.71]	18.33 [4.26]	20.33 [4.488]	331.33	223.33	273.333	126.33	85.33	105.833
JP-585	25.00 [4.98]	21.33 [4.61]	23.16 [4.798]	372.00	236.66	304.333	142.33	90.66	116.500
JIP-3	16.33 [4.07]	11.00 [3.10]	13.66 [3.687]	247.00	213.66	230.333	94.66	82.00	88.333
Mean	21.905 [4.639]	15.976 [4.152]		325.848	255.667		124.606	86.424	
SEm ±	[0.133]	[0.057]	[0.187]	[4.191]	[1.787]	[5.927]	[2.052]	[0.875]	[2.902]
CD at 5%	[0.379]	[0.161]	[INS]	[11.977]	[5.107]	[NS]	[5.865]	[2.501]	[NS]
	G	P	G x P	G	P	G x P	G	P	G x P

Genotypes	Average weight of plant at the Initiation of flowering												Grain yield/plant (g)						N <sub>2</sub> uptake/plant (mg)																	
	Wet weight (g)						Dry weight (g)						P <sub>1</sub>			P <sub>2</sub>			Mean			P <sub>1</sub>			P <sub>2</sub>			Mean								
	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean									
Arkil	57.00	51.00	54.000	5.86	5.25	5.558	12.00	8.66	10.333	64.33	52.66	58.500	57.00	51.00	54.000	5.86	5.25	5.558	12.00	8.66	10.333	64.33	52.66	58.500	57.00	51.00	54.000	5.86	5.25	5.558	12.00	8.66	10.333	64.33	52.66	58.500
Azad-P-1	61.66	55.66	58.667	6.34	5.68	6.013	16.33	11.33	13.833	80.00	64.00	72.000	61.66	55.66	58.667	6.34	5.68	6.013	16.33	11.33	13.833	80.00	64.00	72.000	61.66	55.66	58.667	6.34	5.68	6.013	16.33	11.33	13.833	80.00	64.00	72.000
OSP	46.00	40.33	43.167	4.72	4.10	4.413	11.66	6.66	9.167	48.00	38.00	43.000	46.00	40.33	43.167	4.72	4.10	4.413	11.66	6.66	9.167	48.00	38.00	43.000	46.00	40.33	43.167	4.72	4.10	4.413	11.66	6.66	9.167	48.00	38.00	43.000
DPP-68	45.00	39.00	42.000	4.62	3.98	4.305	11.66	7.00	9.333	29.00	20.00	24.500	45.00	39.00	42.000	4.62	3.98	4.305	11.66	7.00	9.333	29.00	20.00	24.500	45.00	39.00	42.000	4.62	3.98	4.305	11.66	7.00	9.333	29.00	20.00	24.500
PMR-19	59.66	52.33	56.000	6.14	5.38	5.765	13.66	8.00	10.833	73.00	60.33	66.667	59.66	52.33	56.000	6.14	5.38	5.765	13.66	8.00	10.833	73.00	60.33	66.667	59.66	52.33	56.000	6.14	5.38	5.765	13.66	8.00	10.833	73.00	60.33	66.667
JP-83	50.66	45.66	48.167	5.21	4.73	4.973	12.00	8.33	10.167	55.33	41.33	48.333	50.66	45.66	48.167	5.21	4.73	4.973	12.00	8.33	10.167	55.33	41.33	48.333	50.66	45.66	48.167	5.21	4.73	4.973	12.00	8.33	10.167	55.33	41.33	48.333
VL-3	51.00	43.00	47.000	5.23	4.41	4.827	12.33	7.33	9.833	55.33	41.00	48.167	51.00	43.00	47.000	5.23	4.41	4.827	12.33	7.33	9.833	55.33	41.00	48.167	51.00	43.00	47.000	5.23	4.41	4.827	12.33	7.33	9.833	55.33	41.00	48.167
VL-7	52.00	46.33	49.167	5.35	4.76	5.060	13.33	8.00	10.667	55.00	40.33	47.667	52.00	46.33	49.167	5.35	4.76	5.060	13.33	8.00	10.667	55.00	40.33	47.667	52.00	46.33	49.167	5.35	4.76	5.060	13.33	8.00	10.667	55.00	40.33	47.667
Linolin	54.00	48.33	51.167	5.62	4.70	5.163	15.66	8.66	12.167	58.33	44.00	51.167	54.00	48.33	51.167	5.62	4.70	5.163	15.66	8.66	12.167	58.33	44.00	51.167	54.00	48.33	51.167	5.62	4.70	5.163	15.66	8.66	12.167	58.33	44.00	51.167
JP-585	56.66	51.00	53.833	5.71	5.19	5.455	14.33	8.33	11.333	66.66	48.00	57.333	56.66	51.00	53.833	5.71	5.19	5.455	14.33	8.33	11.333	66.66	48.00	57.333	56.66	51.00	53.833	5.71	5.19	5.455	14.33	8.33	11.333	66.66	48.00	57.333
JP-3	47.00	40.00	43.500	4.83	4.22	4.530	12.33	6.33	9.333	44.00	34.66	39.333	47.00	40.00	43.500	4.83	4.22	4.530	12.33	6.33	9.333	44.00	34.66	39.333	47.00	40.00	43.500	4.83	4.22	4.530	12.33	6.33	9.333	44.00	34.66	39.333
Mean	52.788	46.606	49.700	5.426	4.768	5.097	13.212	8.061	10.640	57.182	44.030	50.606	52.788	46.606	49.700	5.426	4.768	5.097	13.212	8.061	10.640	57.182	44.030	50.606	52.788	46.606	49.700	5.426	4.768	5.097	13.212	8.061	10.640	57.182	44.030	50.606
SEm ±	1.521	0.649	2.151	0.058	8.025	0.082	1.015	0.433	1.435	2.372	1.011	3.354	1.521	0.649	2.151	0.058	8.025	0.082	1.015	0.433	1.435	2.372	1.011	3.354	1.521	0.649	2.151	0.058	8.025	0.082	1.015	0.433	1.435	2.372	1.011	3.354
GD at 5%	4.347	1.854	NS	0.165	0.070	NS	2.900	1.237	NS	6.777	2.890	NS	4.347	1.854	NS	0.165	0.070	NS	2.900	1.237	NS	6.777	2.890	NS	4.347	1.854	NS	0.165	0.070	NS	2.900	1.237	NS	6.777	2.890	NS

Figures in parenthesis are angular transformed values.

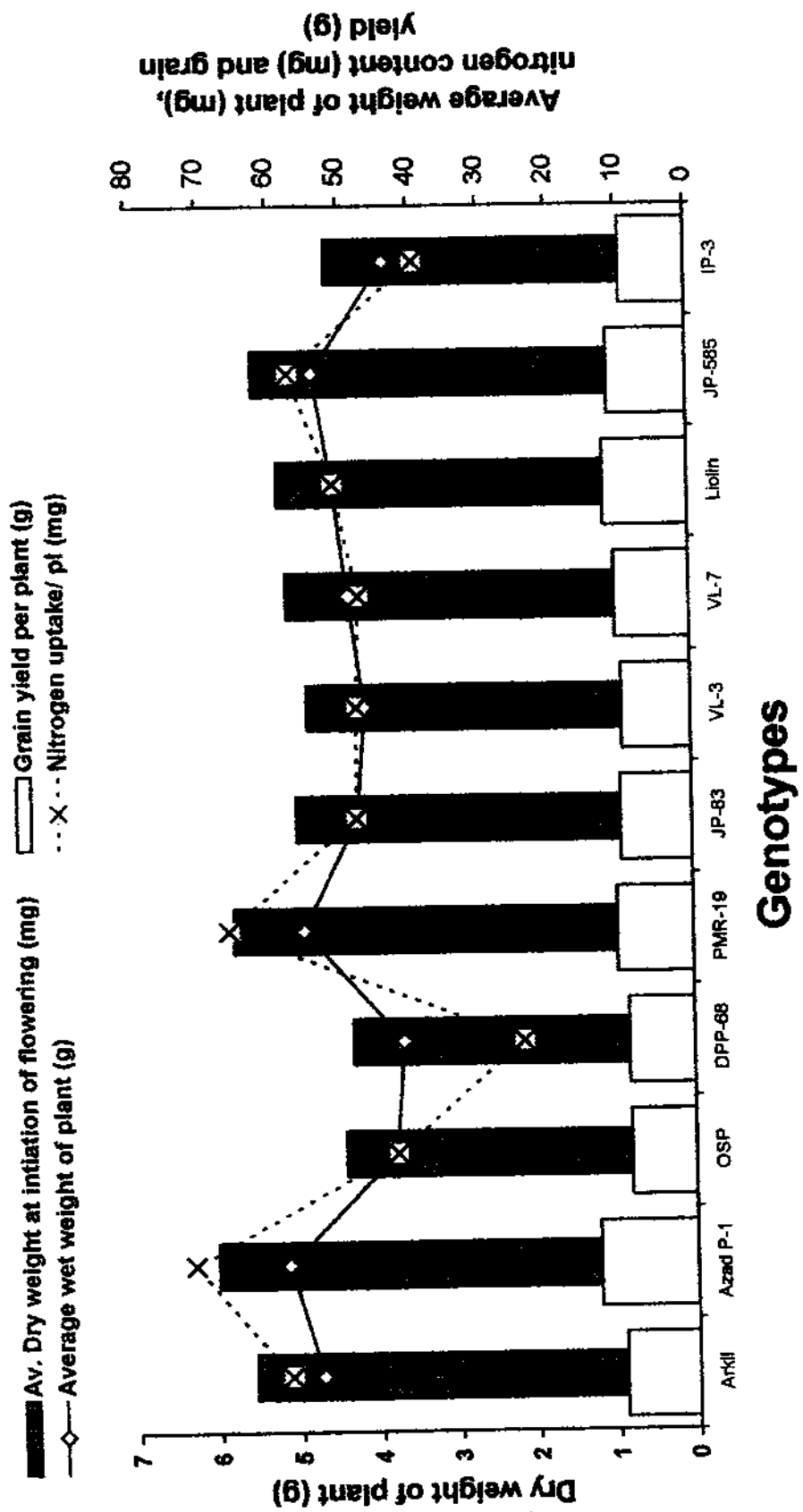


Fig 4. Average fresh and dry weights of plant, nitrogen content and grain yield in Pea genotypes (pot experiment).

Table 5. Screening of different Pea genotypes to different isolates of *Rhizobium leguminosarum* under field conditions (Pooled data for the year 2000-01 and 2001-02).

Genotypes	Av. No. of nodules/plant				Average weight of nodules at the time of initiation of flowering				Dry weight (mg)																											
	P <sub>1</sub>		P <sub>2</sub>		Mean		P <sub>1</sub>		P <sub>2</sub>		Mean		P <sub>1</sub>		P <sub>2</sub>		Mean																			
Arkil	35.50 [6.31]	26.33 [5.12]	28.41 [5.71]	452.17	403.67	427.92	182.67	162.67	172.63	35.17 [5.90]	30.00 [5.46]	32.85 [5.68]	503.67	453.17	478.42	200.67	183.33	192.00	25.00 [4.99]	20.00 [4.47]	22.5 [4.73]	351.00	302.83	326.92	141.83	122.33	132.08	183.33	105.25	154.83	144.83	150.00	147.58	157.50	163.00	125.58
Azad-P-1	18.83 [4.33]	14.66 [3.82]	16.74 [4.07]	410.83	353.67	382.25	166.50	134.17	144.83	18.83 [4.33]	14.66 [3.82]	16.74 [4.07]	410.83	353.67	382.25	166.50	134.17	144.83	150.00	18.83 [4.33]	14.66 [3.82]	16.74 [4.07]	410.83	353.67	382.25	166.50	134.17	144.83	150.00	147.58	157.50	163.00	125.58			
DPP-68	29.00 [5.20]	24.33 [4.92]	26.66 [5.06]	385.17	331.67	358.42	155.50	139.83	150.00	29.00 [5.20]	24.33 [4.92]	26.66 [5.06]	385.17	331.67	358.42	155.50	139.83	150.00	147.58	29.00 [5.20]	24.33 [4.92]	26.66 [5.06]	385.17	331.67	358.42	155.50	139.83	150.00	147.58	157.50	163.00	125.58				
PMR-19	26.83 [5.16]	20.66 [4.55]	23.74 [4.85]	395.17	341.67	368.42	160.17	137.17	147.58	26.83 [5.16]	20.66 [4.55]	23.74 [4.85]	395.17	341.67	368.42	160.17	137.17	147.58	150.00	26.83 [5.16]	20.66 [4.55]	23.74 [4.85]	395.17	341.67	368.42	160.17	137.17	147.58	157.50	163.00	125.58					
JP-83	27.16 [5.19]	22.00 [4.98]	24.58 [4.89]	391.00	348.83	369.92	158.00	146.67	157.50	27.16 [5.19]	22.00 [4.98]	24.58 [4.89]	391.00	348.83	369.92	158.00	146.67	157.50	163.00	27.16 [5.19]	22.00 [4.98]	24.58 [4.89]	391.00	348.83	369.92	158.00	146.67	157.50	163.00	125.58						
VL-3	27.50 [5.23]	21.50 [4.62]	24.5 [4.92]	414.50	363.83	389.17	173.83	152.17	163.00	27.50 [5.23]	21.50 [4.62]	24.5 [4.92]	414.50	363.83	389.17	173.83	152.17	163.00	125.58	27.50 [5.23]	21.50 [4.62]	24.5 [4.92]	414.50	363.83	389.17	173.83	152.17	163.00	125.58							
VL-7	28.50 [5.31]	22.33 [4.71]	25.41 [5.01]	428.50	374.83	401.67	127.00	124.17	125.58	28.50 [5.31]	22.33 [4.71]	25.41 [5.01]	428.50	374.83	401.67	127.00	124.17	125.58	125.58	28.50 [5.31]	22.33 [4.71]	25.41 [5.01]	428.50	374.83	401.67	127.00	124.17	125.58								
Linolin	28.50 [5.42]	24.83 [4.97]	27.16 [5.91]	313.67	306.33	310.00	159.05	140.11	125.58	28.50 [5.42]	24.83 [4.97]	27.16 [5.91]	313.67	306.33	310.00	159.05	140.11	125.58	125.58	28.50 [5.42]	24.83 [4.97]	27.16 [5.91]	313.67	306.33	310.00	159.05	140.11	125.58								
JP-585	21.83 [4.66]	16.33 [4.03]	19.08 [4.34]	393.58	346.95	346.95	SEm ±	0.73	NS	21.83 [4.66]	16.33 [4.03]	19.08 [4.34]	393.58	346.95	346.95	SEm ±	0.73	NS	125.58	21.83 [4.66]	16.33 [4.03]	19.08 [4.34]	393.58	346.95	346.95	SEm ±	0.73	NS	125.58							
IP-3	27.26 [5.24]	22.09 [4.66]	24.66 [5.15]	393.58	346.95	346.95	SEm ±	0.73	NS	27.26 [5.24]	22.09 [4.66]	24.66 [5.15]	393.58	346.95	346.95	SEm ±	0.73	NS	125.58	27.26 [5.24]	22.09 [4.66]	24.66 [5.15]	393.58	346.95	346.95	SEm ±	0.73	NS	125.58							
Mean	27.26 [5.24]	22.09 [4.66]	24.66 [5.15]	393.58	346.95	346.95	SEm ±	0.73	NS	27.26 [5.24]	22.09 [4.66]	24.66 [5.15]	393.58	346.95	346.95	SEm ±	0.73	NS	125.58	27.26 [5.24]	22.09 [4.66]	24.66 [5.15]	393.58	346.95	346.95	SEm ±	0.73	NS	125.58							
year (Y)	0.0596		NS	0.3930		2.39		5.62		0.85		1.99		2.39		5.62		0.85		1.99		2.39		5.62		0.85		1.99		2.39		5.62				
Genotypes (G)	0.596		0.1676	NS		NS		NS		0.85		2.83		7.95		NS		NS		NS		7.95		NS		2.41		3.41		9.59						
Y x G	0.1976		NS	NS		NS		NS		0.85		2.83		7.95		NS		NS		NS		7.95		NS		2.41		3.41		9.59						
Y x P	0.0843		NS	NS		NS		NS		0.85		2.83		7.95		NS		NS		NS		7.95		NS		2.41		3.41		9.59						
G x P	0.1976		NS	NS		NS		NS		0.85		2.83		7.95		NS		NS		NS		7.95		NS		2.41		3.41		9.59						
Y x G x P	0.2795		NS	NS		NS		NS		0.85		2.83		7.95		NS		NS		NS		7.95		NS		2.41		3.41		9.59						
CD at 5%	0.0596		NS	0.3930		2.39		5.62		0.85		1.99		2.39		5.62		0.85		1.99		2.39		5.62		0.85		1.99		2.39		5.62				

Figures in parenthesis are angular transformed values

Genotype	Average weight of plant at the Initiation of flowering										Grain yield/plant (g)			N <sub>2</sub> uptake/plant (mg)		
	Fresh weight (g)					Dry weight (g)					P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean
	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean							
Arkil	61.50	56.00	58.75	6.32	5.76	6.05	16.67	13.00	14.83	83.67	73.10	78.42				
Azad-P-1	63.50	57.83	60.67	6.54	5.93	6.24	18.33	13.50	15.92	91.17	79.50	85.33				
OSP	54.00	49.17	51.58	5.45	5.07	5.27	14.50	10.00	12.25	64.50	52.83	58.67				
DPP-68	50.33	44.67	47.55	5.18	4.61	4.90	13.00	8.50	10.75	64.50	52.83	58.67				
PMR-19	58.83	52.67	55.75	6.06	5.43	5.75	16.00	11.33	13.67	72.83	59.83	66.33				
JP-83	56.83	50.50	53.67	5.84	5.20	5.52	14.83	10.50	12.67	71.17	58.83	65.00				
VL-3	57.17	50.50	53.83	5.89	5.23	5.58	16.33	10.67	13.50	73.83	60.67	67.25				
VL-7	57.83	50.83	54.37	5.96	5.24	5.60	16.00	10.17	13.08	72.17	60.50	66.33				
Linolin	60.00	51.50	55.75	6.18	5.35	5.76	15.83	9.17	12.50	74.33	61.83	68.08				
JP-585	60.33	52.83	56.58	6.23	5.37	5.80	16.50	10.17	13.33	75.33	63.83	69.58				
IP-3	51.00	45.33	48.17	5.19	4.69	4.94	12.17	9.17	10.67	56.00	45.00	50.50				
Mean	57.39	51.08	54.23	5.90	5.26	5.62	15.47	10.56	13.01	72.68	60.80	66.74				
	SEm ±	SEm ±	CD at 5%	SEm ±	SEm ±	CD at 5%	SEm ±	SEm ±	CD at 5%	SEm ±	SEm ±	CD at 5%				
year (Y)	0.44	0.44	NS	0.01	0.01	0.03	0.316	0.316	NS	0.464	0.464	NS				
Genotype (G)	1.04	1.04	2.92	0.027	0.027	0.074	0.741	0.741	2.084	1.089	1.089	3.062				
Y × G (P)	0.44	0.44	1.24	0.011	0.011	0.0319	0.316	0.316	0.889	0.464	0.464	1.306				

Genotype	Average weight of plant at the Initiation of flowering										Grain yield/plant (g)			N <sub>2</sub> uptake/plant (mg)		
	Fresh weight (g)					Dry weight (g)					P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean
	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean	P <sub>1</sub>	P <sub>2</sub>	Mean							
Y x G	1.47	1.47	NS	0.037	0.037	0.105	1.048	1.048	NS	1.530	1.530	4.33				
Y x P	0.62	0.62	NS	0.016	0.016	0.045	0.447	0.447	1.257	0.656	0.656	NS				
G x P	1.47	1.47	NS	0.037	0.037	0.105	1.048	1.048	NS	1.539	1.539	NS				
Y x G x P	2.08	2.08	NS	0.053	0.053	NS	1.482	1.482	NS	2.177	2.177	NS				

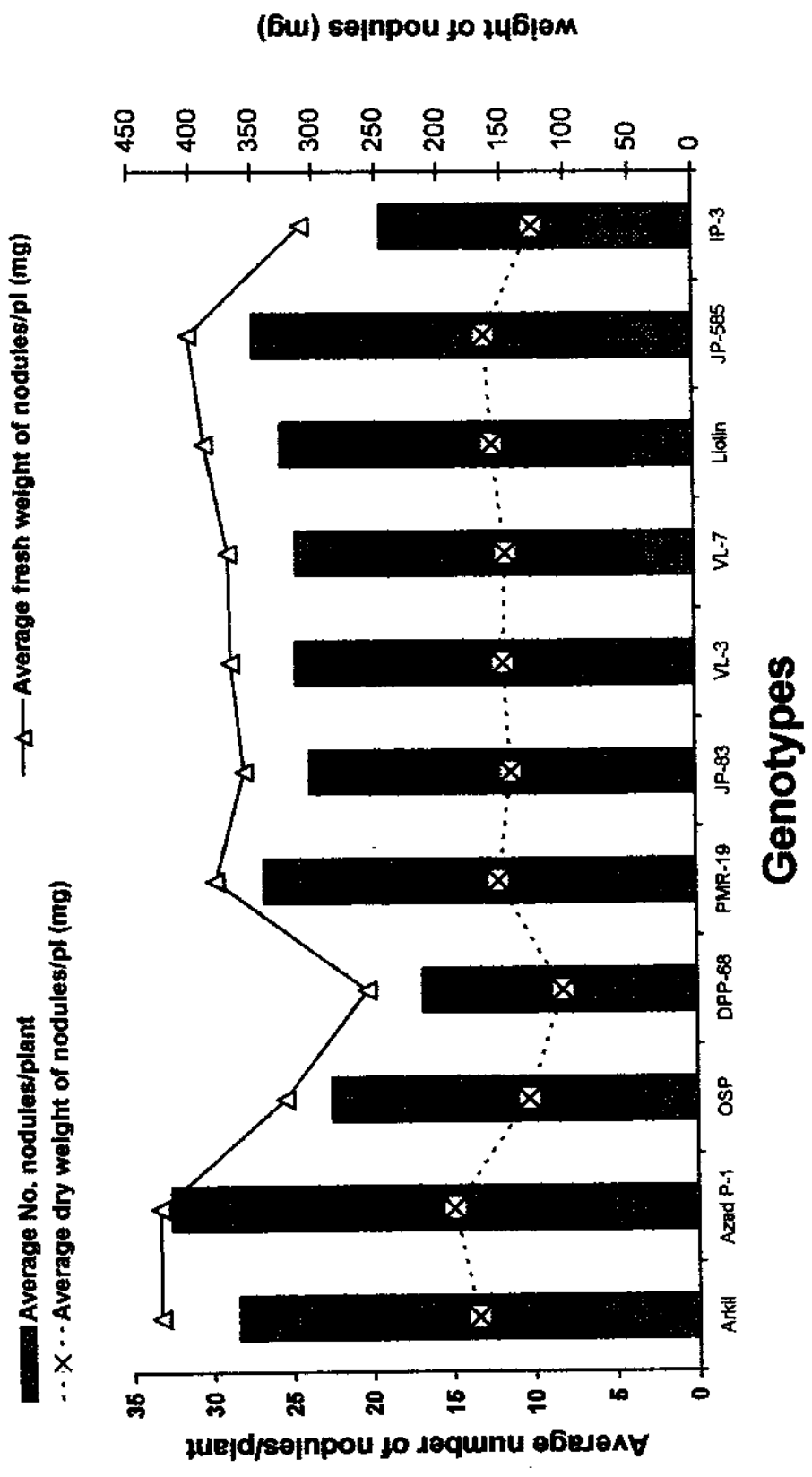


Fig. 5. Nodule characteristics in Pea genotypes (field experiment).

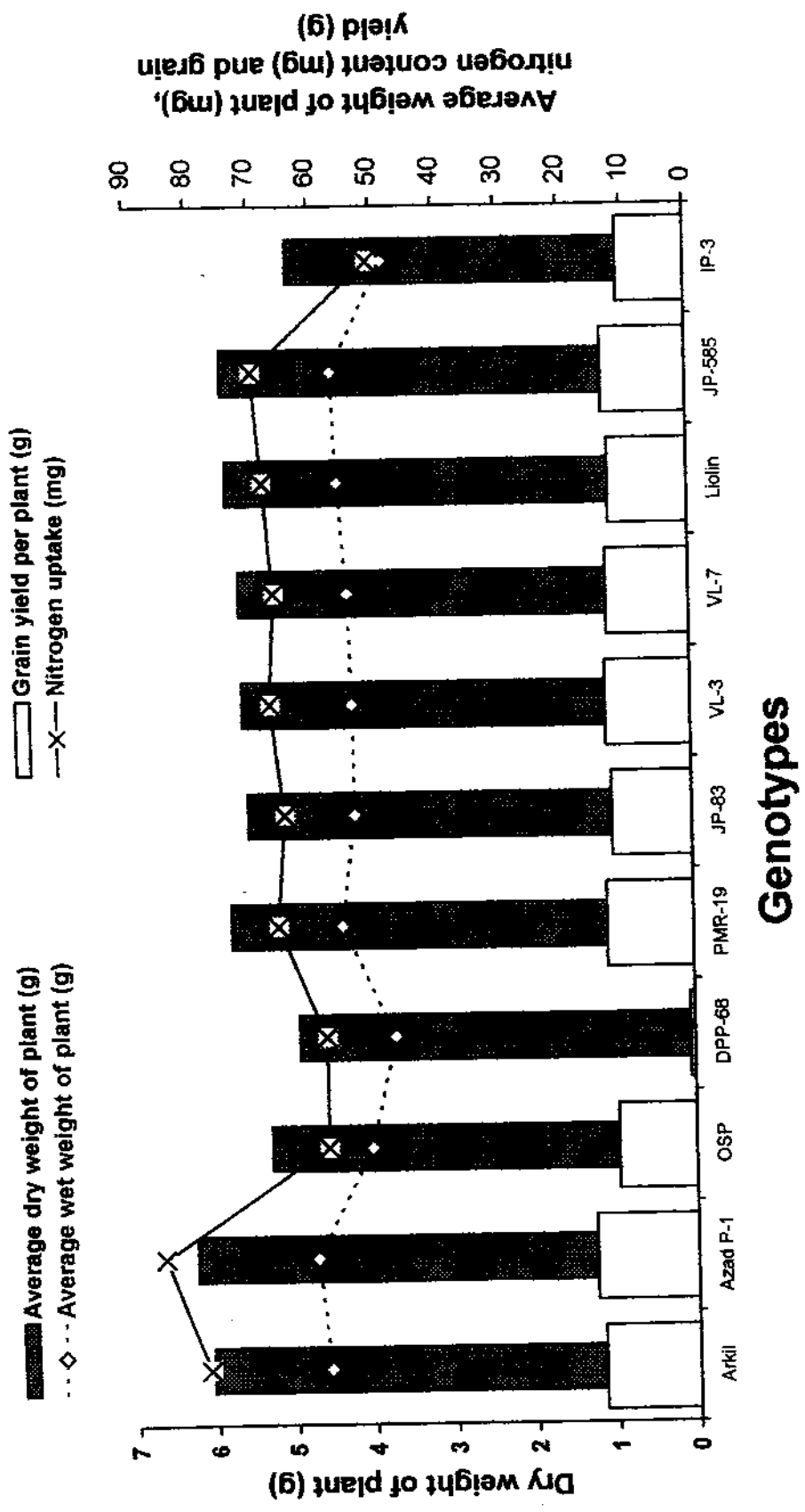
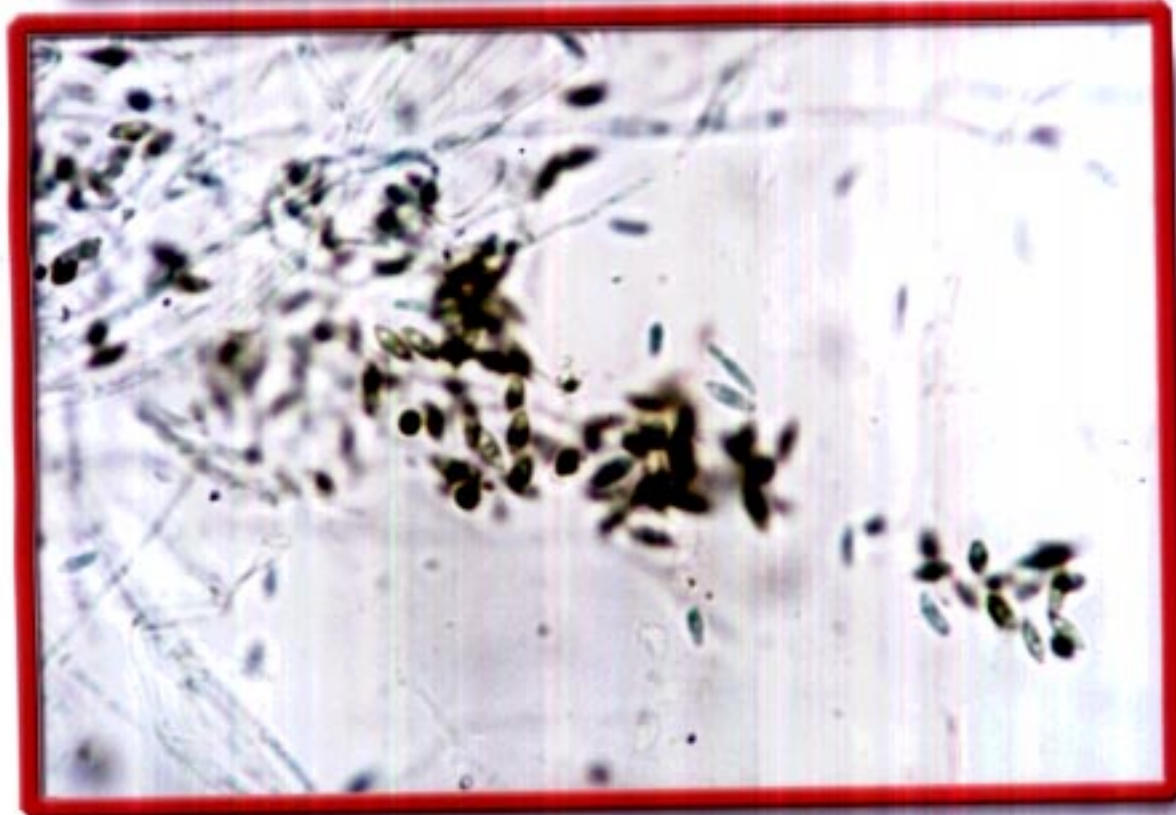


Fig 6. Average fresh and dry weights of plant, nitrogen content and grain yield in Pea genotypes (field experiment).

**Genotypes**

6(a) Photomicrograph of *Myrothecium verrucaria* isolated from Pea root nodules and rhizosphere



6(b) Photomicrograph of *Fusarium oxysporum* isolated from Pea root nodules and rhizosphere

(85.33 mg/plant) N<sub>2</sub> uptake. Whereas interactions among genotype x isolate and year x genotype x isolate for these parameter were also non significant.

## 5. Nodules microflora studies in vitro

Fungi, bacteria and actinomycetes were isolated from the surface of root nodules of pea plant by using specific media.

### i. Isolation of fungi from nodular surface

Pea nodules were plated (4 nodule/plate) on Martin's rose bengal agar medium. After incubation for 72 hrs. different fungi viz., *Aspergillus niger*, *Penicillium oxalicum*, *Drechslera state of Cochliobolus p<sub>h</sub>icifer*, *Fusarium oxysporum* and *Myrothecium verrucaria* were isolated, colony forming units (CFU) of fungi/100 nodules were counted (Table 6)

Table 6. CFU of different fungi associated with Pea root nodules

S. No.	Fungi	CFU/100 nodules
1	<i>Aspergillus niger</i>	25
2	<i>Penicillium oxalicum</i>	15
3	<i>Drechslera state of Cochliobolus p<sub>h</sub>icifer</i>	20
4	<i>Fusarium oxysporum</i>	30
5	<i>Myrothecium verrucaria</i>	32

Out of the five fungi isolated after 72 hours of incubation on Martin's rose Bengal medium, CFU of *Myrothecium verrucaria* (32/100 nodules) was found to be maximum followed by *Fusarium oxysporum* (30/100 nodules) and *Aspergillus niger* (25/100 nodules), *Penicillium oxalicum* (15/100 nodules) showed the least CFU.

### ii. Mycoflora associated with root nodules of plant raised from treated seeds

Out of the six seed dressing fungicides tested, root nodules formed in plants treated with inoloofil M-45, and bavistin supported maximum control of nodule mycoflora with respect to all five dominant fungal species as compared to untreated check. However, captan and thiram proved least effective in checking growth of all the five fungi (Table 7 and Plate 6).



6(c) Photomicrograph of *Aspergillus niger* isolated from Pea root nodules and rhizosphere.



6(d) Photomicrograph of *Penicillium oxalicum* isolated from Pea root nodules and rhizosphere.



6(e) photomicrograph of *Drechslera* state of *Cochliobolus spicifer* isolated from Pea root nodules and rhizosphere

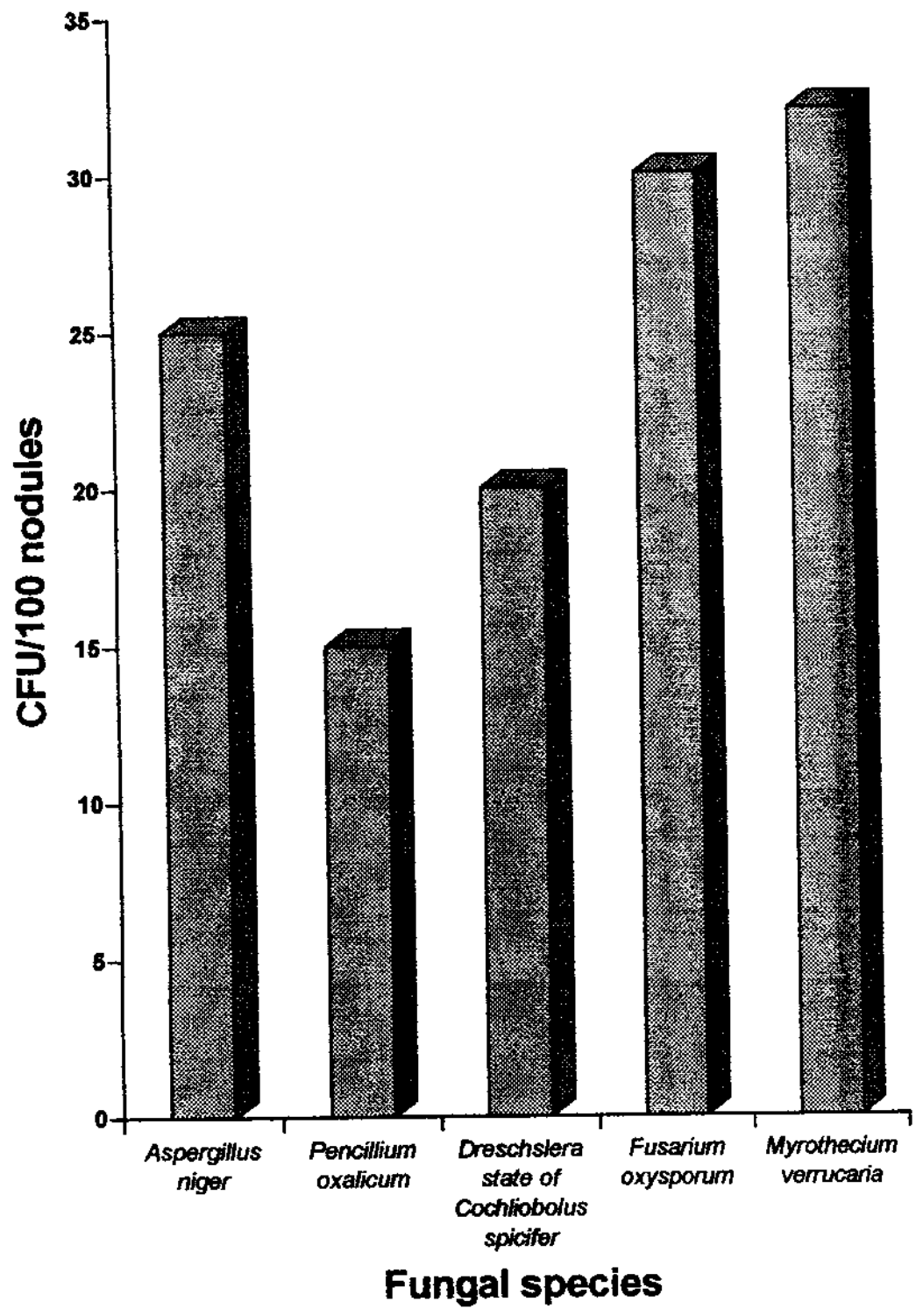


Fig 7. CFU of different fungi associated with pea root nodules

**Table 7. CFU of *Mycospora* associated with root nodules of Pea plants raised from fungicide-treated seeds.**

Treatment	Dose (g/kg)	<u>Aspergillus niger</u>	<u>Penicillium oxalicum</u>	<u>Drechlera state of cochliobolous spicifer</u>	<u>Fusarium oxysporum</u>	<u>Myrothecium verrucaria</u>
Indofil M-45	2.0	2	1	2	3	-
Bavistin	1.0	3	1	3	-	2
Ridomil-MZ	2.0	3	3	4	4	4
Captan	2.0	7	5	4	8	10
Thiram	2.0	6	3	4	8	10
Topsin-M	1.0	4	2	3	7	6
Check	-	8	6	6	10	12

- indicate Nil

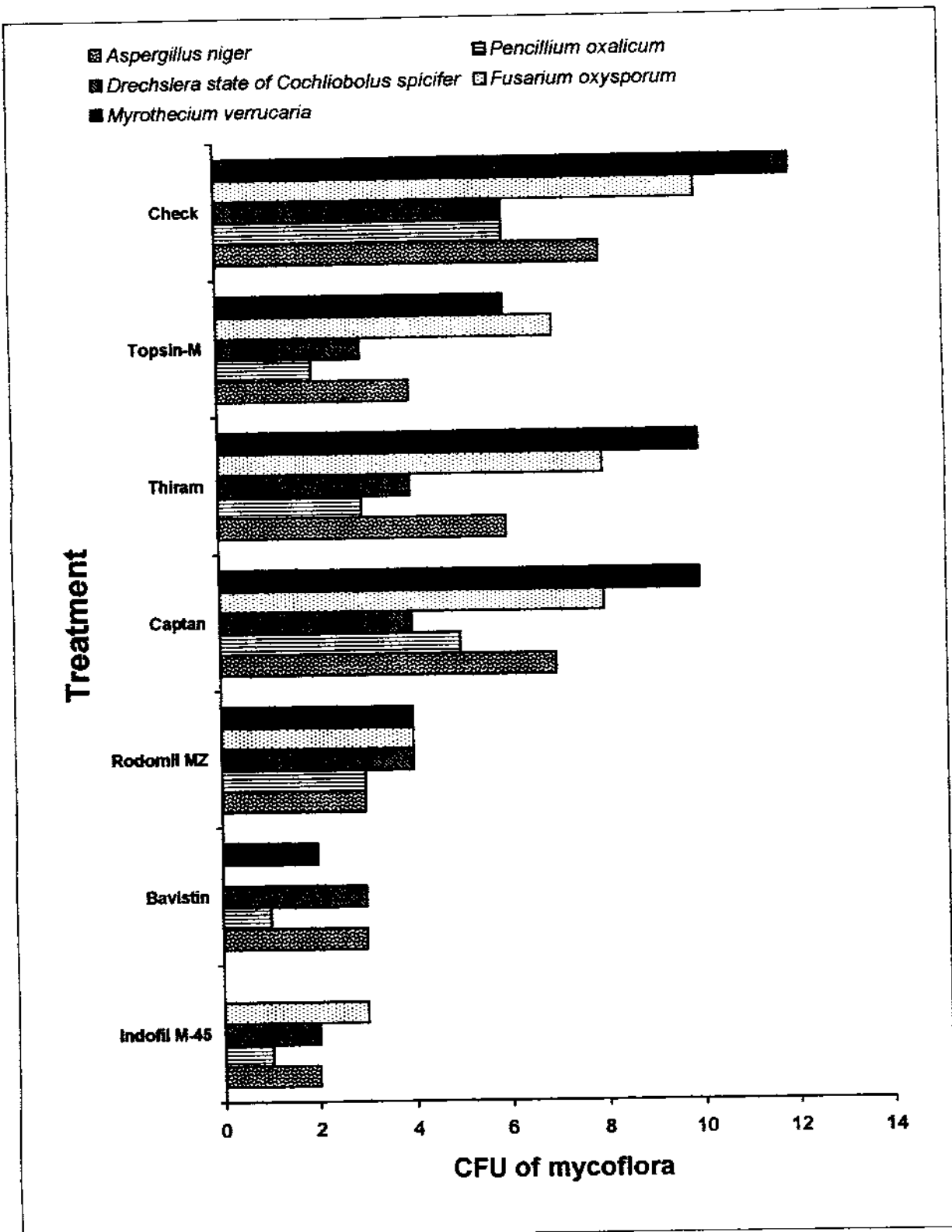


Fig 8. Effect of different fungicides on the CFU of fungi associated with pea root nodules.

*Table 8. Actinomycetes (A) and bacteria (B) associated with pea root nodules*

Replication/plate	Plated nodules							
	1		2		3		4	
	A	B	A	B	A	B	A	B
1	+	-	-	+	+	+	+	-
2	-	+	-	-	+	-	-	-
3	-	+	+	-	-	+	+	-
4	+	+	+	-	-	+	+	+
5	-	+	+	+	+	-	-	+
6	-	-	-	+	-	-	+	+
7	+	+	+	-	-	+	-	+
8	+	-	+	-	+	-	-	+
9	+	-	-	+	-	-	+	-
10	-	+	-	-	-	-	-	+
Total	5	6	5	4	4	4	5	6
Average	1.90 (A)		2.00 (B)					

A = Actinomycetes

B = Bacteria

+ = Actinomycetes and bacteria present

- = Actinomycetes and bacteria are not present.

### iii. Actinomycetes and bacteria associated with pea root nodules

Pea nodules were plated (4 nodules/ plate) on starch ammonium agar and thorton's standardized medium in Petri plates, for the isolation of actinomycetes and bacteria, respectively. These plates were incubated at  $28 \pm 2^{\circ}\text{C}$  for 48 hours. The observations were recorded as shown in Table 8.

Results reveals that average number of Actinomycetes/plate were (1.90) and average number of bacterial colonies were (2.00).

## 6. Antagonistic activity of fungi, actinomycetes and bacteria isolated from pea root nodules towards *Rhizobium leguminosarum* in in vitro conditions

### i. Fungi

Isolated fungi were tested for their antagonistic activity against pea *Rhizobium*. The culture filtrate of these fungi were dried and the residue was dissolved in the sterile distilled water (Table 9).

**Table 9. Antagonistic activity of culture filtrate of fungi isolated from pea root nodules towards *Rhizobium leguminosarum*.**

S. No.	Fungi (cultural filtrate)	Average inhibition zone in mm <sup>2</sup>
1	<i>Aspergillus niger</i>	5.25
2	<i>Penicillium oxalicum</i>	3.50
3	<i>Drechslera state of Cochliobolus sicifer</i>	1.50
4	<i>Fusarium oxysporum</i>	5.00
5	<i>Myrothecium verrucaria</i>	2.50
	SEm $\pm$	0.64
	CD at 5%	1.93
	CD at 1%	2.67

Whatman filter paper disc (dia 10mm) were soaked in aqueous solution of various fungal filtrates and placed on CRYEMA Petri plates, pre inoculated with pea *Rhizobium* suspension. These were incubated at  $28 \pm 2^{\circ}\text{C}$  for 72 hours. Observation were recorded on the zone of inhibition developed as a result of antifungal activity of fungal filterates. It was observed that the culture

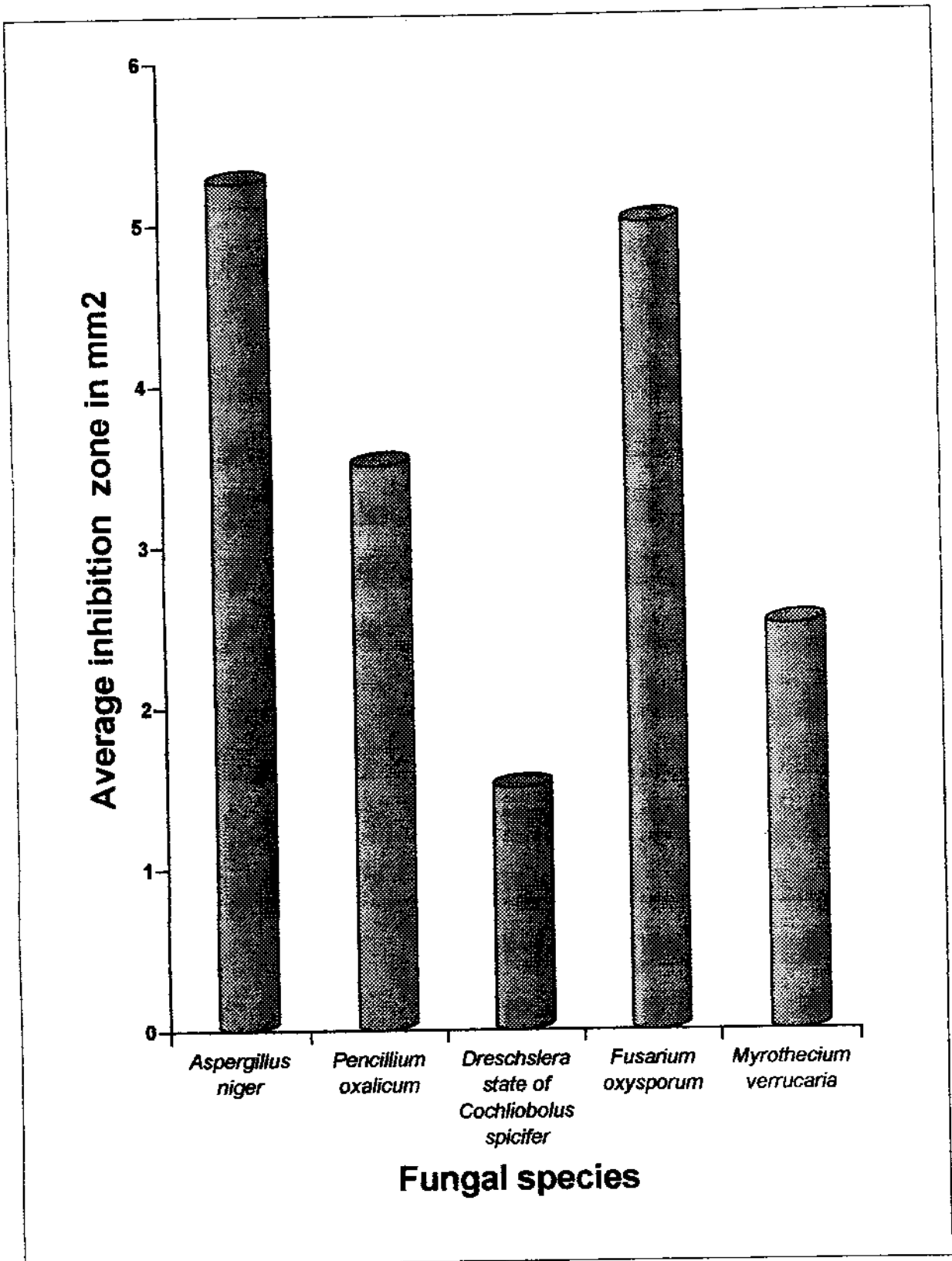


Fig 9. Antagonistic activity of culture filtrate of fungi isolated from Pea root nodules towards *Rhizobium leguminosarum*.

filtrate of *Aspergillus niger* developed maximum inhibition zone (5.25 mm<sup>2</sup>) followed by *Fusarium oxysporum* (5.00 mm<sup>2</sup>) though at par with each other. While it was minimum in *Drechslera* sp. (1.50 mm<sup>2</sup>).

## ii. Actinomycetes

Antagonistic activity of the isolated actinomycetes towards *Rhizobium* was tested *in vitro* by using cross streak method on CRYEMA medium. The observation was recorded on growth inhibition of *Rhizobium* as shown in Table 10.

**Table 10. Growth inhibition of *Rhizobium* due to antagonistic activity of actinomycetes**

Actinomycetes	Growth inhibition
R <sub>1</sub>	+
R <sub>2</sub>	+
R <sub>3</sub>	+
R <sub>4</sub>	+
R <sub>5</sub>	+

+ = Inhibits the growth of *Rhizobium*.

- = No inhibition

Table 10 shows that the actinomycetes inhibited the growth of *Rhizobium leguminosarum* *in vitro* conditions.

Inhibition of growth of *Rhizobium leguminosarum* was observed in all the replications.

## iii. Bacteria

Antagonistic activity of the isolated bacteria towards *Rhizobium* was tested *in vitro* by using streak method on CRYEMA medium. The observation was recorded on growth inhibition of *Rhizobium* (Table 11).

**Table 11. Growth inhibition of *Rhizobium* due to antagonistic activity of bacteria.**

Bacteria	Growth inhibition
R <sub>1</sub>	+
R <sub>2</sub>	+
R <sub>3</sub>	+
R <sub>4</sub>	+
R <sub>5</sub>	+

+ = Inhibits the growth of *Rhizobium*

- = no inhibition

Table 11 shows that the inhibition of growth of *Rhizobium leguminosarum* was observed in all the replications.

#### iv. Variation in microbial population in pea rhizosphere at different stages of crop growth as influenced by different chemical seed protectants

Rhizosphere soil from treated plant (fungicides and *Rhizobium* inoculated seeds of pea) was collected at different stages of plant growth and plated on Martin's rose bengal streptomycin agar medium for fungi and starch ammonium agar medium for taking actinomycetes and Thornton's standardized medium for counting bacteria and results are presented in Table 12.

Perusal of results revealed that maximum number of fungal colonies were found in untreated check plots ( $24.67 \times 10^3$ ) and it was significantly higher than all the fungicidal treatment. Among fungicidal treatment maximum number of fungal colonies were observed in thiram ( $20.67 \times 10^3$ ) followed by captan ( $19.33 \times 10^3$ ) as compared to *Rhizobium* treated control ( $20.33 \times 10^3$ ). However, minimum fungal colonies were recorded in the treatment of indofil M-45 ( $11 \times 10^3$ ) followed by ridomil MZ ( $13.33 \times 10^3$ ). It was also observed that number of fungal colonies increased in increasing order at increased interval.

In case of actinomycetes, the results revealed that maximum number of actinomycetes colonies were found untreated check plots ( $71.33 \times 10^5$ ) and it was significantly higher than all the fungicidal treatments. Among fungicidal treatments maximum number of actinomycetes colonies were observed in captan ( $60.33 \times 10^5$ ) followed by thiram ( $59.67 \times 10^5$ ) and bavistin ( $59.67 \times 10^5$ )

Table 12. Variation in microbial population (colonies of fungi, bacteria and actinomycetes) of Pea rhizosphere at different stage of crop growth as influenced by chemical seed protector and *Rhizobium* inoculation.

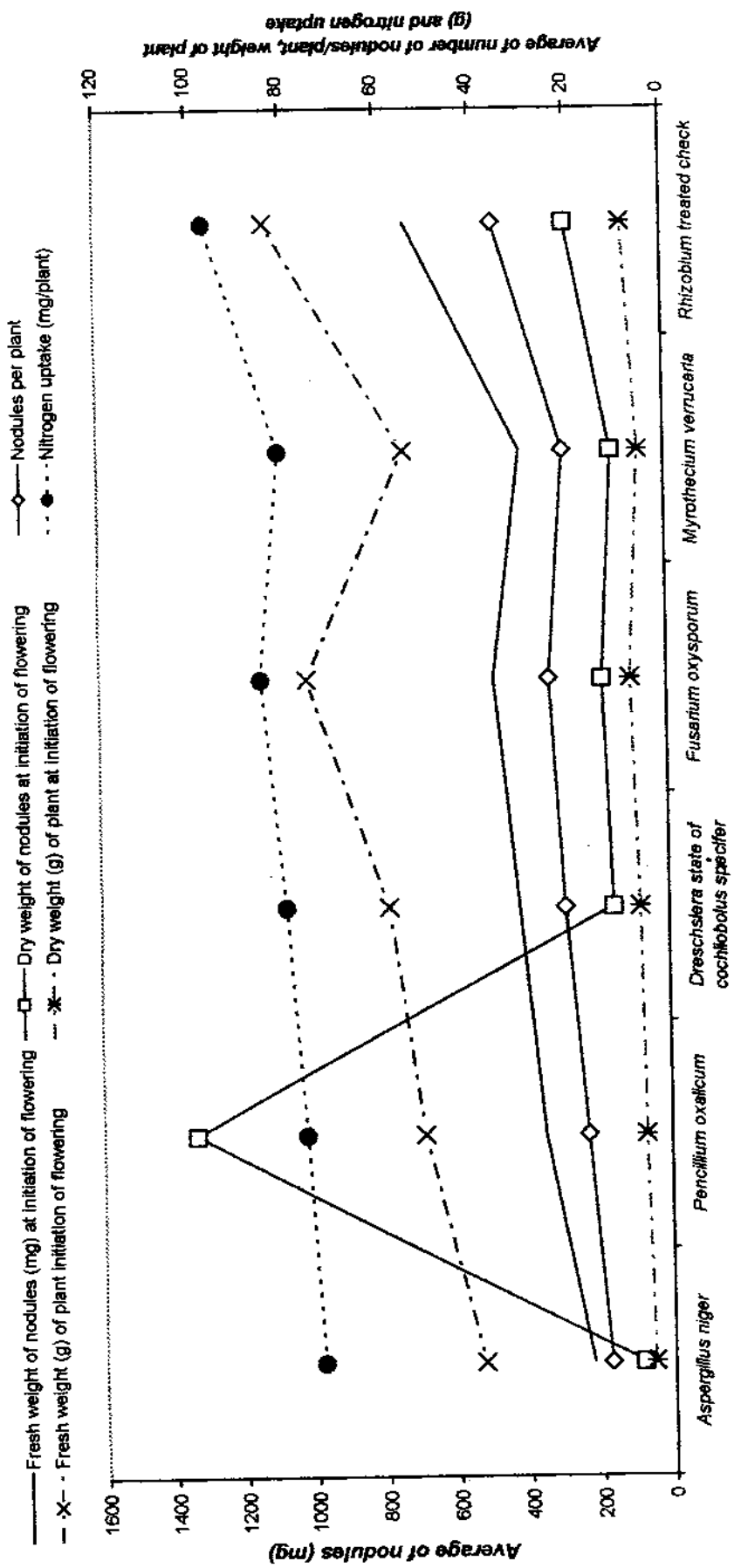
Fungicides	Microbial population																	
	Fungi						Actinomycetes						Bacteria					
	30 days	45 days	60 days	Mean	30 days	45 days	60 days	Mean	30 days	45 days	60 days	Mean	30 days	45 days	60 days	Mean		
Indofil M-45	8 x 10 <sup>3</sup> [3.901]	10 x 10 <sup>3</sup> [3.993]	15 x 10 <sup>3</sup> [4.170]	11 x 10 <sup>3</sup> [4.021]	50 x 10 <sup>5</sup> [6.698]	52 x 10 <sup>5</sup> [6.716]	61 x 10 <sup>5</sup> [6.785]	54.33 x 10 <sup>5</sup> [6.733]	42 x 10 <sup>5</sup> [6.623]	45 x 10 <sup>5</sup> [6.653]	55 x 10 <sup>5</sup> [6.740]	46.33 x 10 <sup>5</sup> [6.672]	43 x 10 <sup>5</sup> [6.633]	47 x 10 <sup>5</sup> [6.672]	60 x 10 <sup>5</sup> [6.778]	50 x 10 <sup>5</sup> [6.694]		
Bavistin	10 x 10 <sup>3</sup> [4.601]	14 x 10 <sup>3</sup> [4.143]	18 x 10 <sup>3</sup> [4.253]	14 x 10 <sup>3</sup> [4.332]	51 x 10 <sup>5</sup> [6.707]	60 x 10 <sup>5</sup> [6.778]	68 x 10 <sup>5</sup> [6.832]	59.67 x 10 <sup>5</sup> [6.773]	43 x 10 <sup>5</sup> [6.633]	43 x 10 <sup>5</sup> [6.672]	60 x 10 <sup>5</sup> [6.778]	59.67 x 10 <sup>5</sup> [6.773]	47 x 10 <sup>5</sup> [6.671]	62 x 10 <sup>5</sup> [6.792]	70 x 10 <sup>5</sup> [6.839]	59.67 x 10 <sup>5</sup> [6.767]		
Captan	15 x 10 <sup>3</sup> [4.174]	20 x 10 <sup>3</sup> [4.299]	23 x 10 <sup>3</sup> [4.360]	19.33 x 10 <sup>3</sup> [4.278]	55 x 10 <sup>5</sup> [6.740]	58 x 10 <sup>5</sup> [6.763]	69 x 10 <sup>5</sup> [6.839]	60.33 x 10 <sup>5</sup> [6.781]	40 x 10 <sup>5</sup> [6.602]	43 x 10 <sup>5</sup> [6.633]	58 x 10 <sup>5</sup> [6.763]	47 x 10 <sup>5</sup> [6.666]	40 x 10 <sup>5</sup> [6.602]	44 x 10 <sup>5</sup> [6.643]	56 x 10 <sup>5</sup> [6.748]	46.67 x 10 <sup>5</sup> [6.664]		
Thiram	16 x 10 <sup>3</sup> [4.202]	21 x 10 <sup>3</sup> [4.319]	25 x 10 <sup>3</sup> [4.398]	20.67 x 10 <sup>3</sup> [4.306]	52 x 10 <sup>5</sup> [6.715]	55 x 10 <sup>5</sup> [6.740]	72 x 10 <sup>5</sup> [6.857]	59.67 x 10 <sup>5</sup> [6.771]	40 x 10 <sup>5</sup> [6.600]	44 x 10 <sup>5</sup> [6.643]	56 x 10 <sup>5</sup> [6.748]	46.67 x 10 <sup>5</sup> [6.664]	40 x 10 <sup>5</sup> [6.600]	44 x 10 <sup>5</sup> [6.643]	60 x 10 <sup>5</sup> [6.778]	51 x 10 <sup>5</sup> [6.704]		
Ridomil-MZ	16 x 10 <sup>3</sup> [3.994]	13 x 10 <sup>3</sup> [4.110]	17 x 10 <sup>3</sup> [4.208]	13.33 x 10 <sup>3</sup> [4.104]	51 x 10 <sup>5</sup> [6.707]	54 x 10 <sup>5</sup> [6.732]	65 x 10 <sup>5</sup> [6.813]	56.67 x 10 <sup>5</sup> [6.751]	44 x 10 <sup>5</sup> [6.643]	49 x 10 <sup>5</sup> [6.690]	60 x 10 <sup>5</sup> [6.778]	51 x 10 <sup>5</sup> [6.704]	44 x 10 <sup>5</sup> [6.643]	49 x 10 <sup>5</sup> [6.690]	63 x 10 <sup>5</sup> [6.799]	55.67 x 10 <sup>5</sup> [6.743]		
Topsain-M	13 x 10 <sup>3</sup> [4.111]	14 x 10 <sup>3</sup> [4.143]	18 x 10 <sup>3</sup> [4.255]	15 x 10 <sup>3</sup> [4.170]	53 x 10 <sup>5</sup> [6.724]	55 x 10 <sup>5</sup> [6.740]	67 x 10 <sup>5</sup> [6.826]	58.33 x 10 <sup>5</sup> [6.763]	44 x 10 <sup>5</sup> [6.643]	51 x 10 <sup>5</sup> [6.724]	63 x 10 <sup>5</sup> [6.799]	55.67 x 10 <sup>5</sup> [6.743]	51 x 10 <sup>5</sup> [6.704]	53 x 10 <sup>5</sup> [6.724]	80 x 10 <sup>5</sup> [6.903]	71.67 x 10 <sup>5</sup> [6.841]		
Treated control(Rhizobium)	16 x 10 <sup>3</sup> [4.202]	21 x 10 <sup>3</sup> [4.322]	24 x 10 <sup>3</sup> [4.379]	20.33 x 10 <sup>3</sup> [4.301]	58 x 10 <sup>5</sup> [6.762]	60 x 10 <sup>5</sup> [6.778]	79 x 10 <sup>5</sup> [6.897]	61.67 x 10 <sup>5</sup> [6.789]	60 x 10 <sup>5</sup> [6.724]	65 x 10 <sup>5</sup> [6.724]	80 x 10 <sup>5</sup> [6.903]	71.67 x 10 <sup>5</sup> [6.841]	60 x 10 <sup>5</sup> [6.724]	65 x 10 <sup>5</sup> [6.724]	80 x 10 <sup>5</sup> [6.903]	71.67 x 10 <sup>5</sup> [6.841]		
Check (untreated)	20 x 10 <sup>3</sup> [4.298]	25 x 10 <sup>3</sup> [4.397]	29 x 10 <sup>3</sup> [4.462]	24.67 x 10 <sup>3</sup> [4.386]	65 x 10 <sup>5</sup> [6.813]	70 x 10 <sup>5</sup> [6.845]	85 x 10 <sup>5</sup> [6.952]	71.33 x 10 <sup>5</sup> [6.852]	45.88 x 10 <sup>5</sup> [6.778]	45.88 x 10 <sup>5</sup> [6.778]	62.75 x 10 <sup>5</sup> [6.903]	55.67 x 10 <sup>5</sup> [6.743]	45.88 x 10 <sup>5</sup> [6.778]	51 x 10 <sup>5</sup> [6.843]	62.75 x 10 <sup>5</sup> [6.903]	55.67 x 10 <sup>5</sup> [6.743]		
Mean	13.5 x 10 <sup>3</sup> [4.185]	17.25 x 10 <sup>3</sup> [4.216]	21.13 x 10 <sup>3</sup> [4.310]	18.33 x 10 <sup>3</sup> [4.306]	54.38 x 10 <sup>5</sup> [6.733]	58 x 10 <sup>5</sup> [6.761]	68.5 x 10 <sup>5</sup> [6.834]	61.67 x 10 <sup>5</sup> [6.852]	45.88 x 10 <sup>5</sup> [6.657]	45.88 x 10 <sup>5</sup> [6.657]	62.75 x 10 <sup>5</sup> [6.903]	55.67 x 10 <sup>5</sup> [6.743]	45.88 x 10 <sup>5</sup> [6.657]	45.88 x 10 <sup>5</sup> [6.657]	62.75 x 10 <sup>5</sup> [6.903]	55.67 x 10 <sup>5</sup> [6.743]		
	SEM ±	CD at 5%			SEM ±	CD at 5%			SEM ±	CD at 5%			SEM ±	CD at 5%				
Days (D)	0.009	0.024			0.003	0.008			0.003	0.008			0.003	0.010				
Fungicide (F)	0.014	0.040			0.005	0.013			0.006	0.016			0.006	0.016				
D x F	0.025	0.069			0.008	0.022			0.010	0.027			0.010	0.027				

Figures in parenthesis are angular transformed values

Table 13. Effect of *Rhizobium* inoculated Pea seeds on growth and nodulation of the plants and nitrogen status of the soil under myco-colonized soil conditions.

Fungi used for myco-colonization	Average of four replications (pots)						N <sub>2</sub> uptake mg/plant
	Average number of nodules/plant	Average weight of nodules at the initiation of flowering		Average weight of plant at the initiation of flowering		dry weight (g)	
		Fresh weight (mg)	dry weight (mg)	Fresh weight (g)	dry weight (g)		
<i>Aspergillus niger</i>	13.00(14.38)	223.50	83.00	39.50	4.10	73.25	
<i>Penicillium oxalicum</i>	17.25(16.60)	350.00	1333.75	51.50	5.31	76.50	
<i>Drechslera state of Cochliobolus spicifer</i>	21.50(18.54)	414.50	153.25	58.50	5.95	80.25	
<i>Fusarium oxysporum</i>	24.50(19.69)	481.25	179.25	75.50	7.49	85.25	
<i>Myrothecium verrucaria</i>	21.00(19.32)	402.00	147.25	54.00	5.34	81.00	
<i>Rhizobium</i> treated check	35.25(23.74)	717.00	268.25	83.50	8.21	96.50	
SEm ±	0.09	3.12	2.12	1.18	0.82	0.84	
CD at 5%	0.28	9.28	6.31	3.51	0.21	2.49	

Figures in parenthesis are angular transformed values.



### Fungi used for myco-colonized conditions

Fig 10. Effect of *Rhizobium* inoculated Pea seeds on growth and nodulation of the plants and nitrogen status of the soil under myco-colonized soil conditions.

as compared to *Rhizobium* treated control ( $61.67 \times 10^5$ ). Whereas minimum actinomycetes colonies were recorded in indofil M-45 treatment ( $54.33 \times 10^5$ ). It was also observed that number of actinomycetes colonies increased in increasing order at increased interval.

Bacterial colonies were also found maximum in untreated control ( $71.67 \times 10^5$ ) and it was significantly higher than all the fungicidal treatment. Among fungicidal treatment maximum number of bacterial colonies were observed in captan <sup>( $59.67 \times 10^5$ )</sup> followed by topsin-M ( $51 \times 10^5$ ) treatment as compared to *Rhizobium* treated control ( $55.67 \times 10^5$ ).

Whereas minimum bacterial colonies were recorded in the treatment indofil M-45 ( $46.33 \times 10^5$ ) and ridomil MZ ( $46.67 \times 10^5$ ) treatments. However, thiram ( $47 \times 10^5$ ) also showed the bacterial colonies at par with indofil M-45 and ridomil MZ.

## **7. Myco-colonization in sterilized soil**

The effect of 5 dominating fungi viz., *Aspergillus niger*, *Penicillium oxalicum*, *Drechslera* state of *Cochliobolus spicifer*, *Fusarium oxysporum* and *Myrothecium verrucaria* were observed on growth, nodulation and nitrogen uptake of the plant *in vitro* by using myco-colonized soil of individual fungi in sterilized soil. The control was also maintained by using inoculated seeds in sterilized soil. The results reveals that (Table 13) *Aspergillus niger* myco-colonized soil exhibited significant decrease in nodule number (13.00/plant), least fresh weight (39.50 g/plant) and dry weight (4.10 g/plant) of the pea plant. The nitrogen uptake was also comparatively less (73.25 mg/plant).

The myco-colonized soil of *Fusarium oxysporum* gave moderate nodulation (24.50/plant), 75.50 g fresh weight/plant and 85.25 mg/plant nitrogen uptake as compared to other myco-colonized soil whereas, in check (*Rhizobium* treated seed) maximum nodulation (35.25/plant), fresh weight/plant (83.50 g/plant) and dry weight (8.21 g/plant) was observed.

## **8. Compatibility of chemical seed protectants in vitro studies**

### **i. Fungicidal assay**

Different concentrations of fungicides viz., 100, 250, 500, 1000 and 2000 ppm were prepared and 10 mm filter paper discs were soaked in each concentration. The soaked discs were placed in Petri plates containing *Rhizobium* inoculated CRYEMA medium. After 72 hours of incubation inhibition zones ( $\text{mm}^2$ ) were measured and results are shown in Table 15. This table reveals that out of six fungicides tested indofil M-45 and bavisitin did not showed any inhibition zone at any of the concentration used. Filter paper discs soaked in thiram gave maximum inhibition zone i.e. 2.50, 5.00, 6.75, 8.25, and 13.75  $\text{mm}^2$  at 100, 250, 500, 1000 and 2000 ppm

concentration, respectively. Captan was not found effective at 100 ppm concentration. However, it gave 3.50, 5.25, 8.25, 14.50 inhibition zone at 250, 500, 1000 and 2000 ppm concentration, respectively. topsin-M showed inhibition zone at 500 ppm (4.25), 1000 ppm (6.25) and 2000 ppm (7.25) concentration, respectively. Ridomil MZ was not found effective at lower concentrations. However, at higher concentrations i.e. 1000 and 2000 ppm, it showed 3.5 and 5.75 zone of inhibition, respectively. Topsin-M showed inhibition zone at 500 ppm (4.25), 1000 ppm (6.25) and 2000 ppm (7.25) concentration, respectively. Ridomil MZ was not effective at lower concentrations.

## ii. Plating of fungicides dressed seeds on *Rhizobium* inoculated CRYEMA medium

Pea seeds were dressed with six different fungicides viz., indofil M-45, bavistin, captan, ridomil-MZ, thiram and topsin-M at their respective concentration. Dressed seeds were plated (4 seeds/plate) on *Rhizobium* inoculated CRYEMA medium. After 72 hours of incubation, inhibition zones were recorded (Table 15) in thiram (18.50) followed by captan (12.50) while minimum inhibition zone was recorded in topsin-M (3.30) and ridomil-MZ (5.50 mm<sup>2</sup>). However, indofil M-45 and bavistin did not show any inhibition zone and were at par with untreated control.

Table 14. *In vitro* effect of fungicides on the growth of Pea *Rhizobium leguminosarum*.

Fungicides	Inhibition zones (mm <sup>2</sup> )					Mean
	100*	250	500	1000	2000	
Indofil M-45	**	-	-	-	-	0.00
Bavistin	-	-	-	-	-	0.00
Captan	-	3.50	5.25	8.25	14.50	6.300
Ridomil-MZ	-	-	-	3.50	5.25	1.750
Thiram	2.50	5.00	6.75	8.25	13.75	7.250
Topsin-M	-	-	4.25	6.25	7.25	3.550
Mean	0.417	1.417	2.708	4.375	6.792	
		SEm ±		CD at 5%		
	Fungicide (F)	0.083		0.239		
	Concentration (c)	0.077		0.218		
	F x C	0.190		0.534		

\* values in ppm and \*\* no inhibition

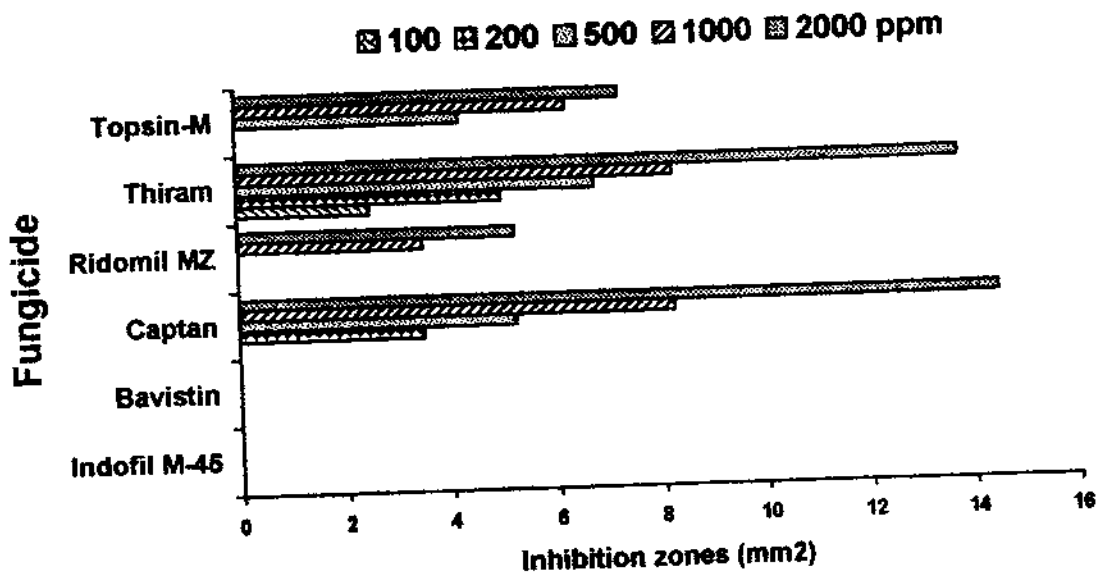


Fig 11. *In vitro* effect of fungicides on the growth of Pea *Rhizobium*.

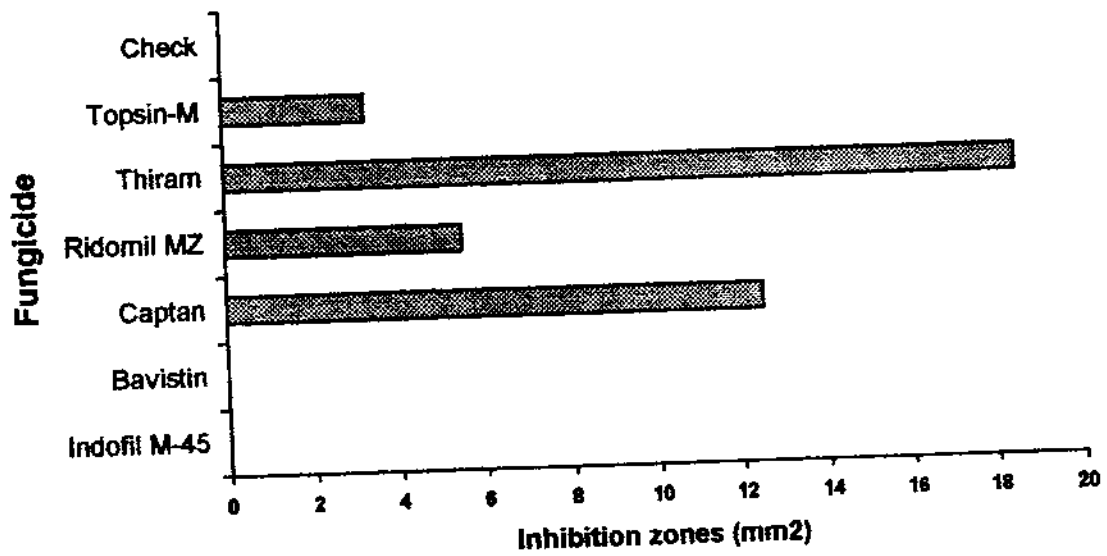


Fig 12. Inhibition of *Rhizobium* when Pea seeds were treated with fungicides.

**Table 15. Inhibition of *Rhizobium leguminosarum* when pea seeds were treated with fungicides.**

Seed dressing fungicides	Inhibition zone in mm <sup>2</sup>
Indofil M-45	Nil
Bavistin	Nil
Captan	12.50
Ridomil-MZ	5.50
Thiram	18.50
Topsin-M	3.30
Control (without fungicide)	Nil

### iii. Plating of fungicides dressed and *Rhizobium leguminosarum* inoculated seeds on CRYEMA medium

Pea seeds were dressed with chemical seed dressing fungicides viz., indofil M-45, bavistin, captan, ridomil-MZ, thiram, topsin-M at their respective concentrations and then inoculated with *Rhizobium* inoculant. Four seeds/plate were placed at equal distance on CRYEMA medium in Petri plates. After 72 hours of incubation, inhibition of *Rhizobium leguminosarum* growth was observed.

Maximum per cent inhibition of *Rhizobium leguminosarum* growth was observed in thiram (58.33%) and captan (54.16%) followed by topsin-M (33.33%). However, minimum inhibition was recorded in bavistin (8.33%) and ridomil-MZ (25.00%). No inhibition was recorded in Indofil M-45 seed treatment (Table 16).

**Table 16. Inhibition of *Rhizobium leguminosarum* growth on fungicidal treated pea seeds.**

Fungicides	Dose (g/kg)	Total number of seed plated	Growth observed in number of seeds	Growth inhibited in number of seeds	Per cent inhibition
Indofil M-45	2.0	24**	24	0*	-
Bavistin	1.0	24	22	2	8.33
Captan	2.0	24	11	13	54.16
Ridomil-MZ	2.0	24	18	6	25.00

Fungicides	Dose (g/kg)	Total number of seed plated	Growth observed in number of seeds	Growth inhibited in number of seeds	Per cent inhibition
Thiram	2.0	24	10	14	58.33
Topsin-M	1.0	24	16	8	33.33
Rhizobium treated (control)	-	24	24	-	-

\*No growth inhibition of seeds, \*\* Four seeds were plated in each plate and replicated 6 times.

## 9. Compatibility of chemical seed protectants with *Rhizobium leguminosarum* in pots and field conditions.

### i. Studies under pot conditions

Fungicides dressed and *Rhizobium leguminosarum* inoculated seeds were sown in 30 cm size pots. At the initiation of flowering number of nodules, average fresh and dry weight of nodules, fresh and dry weight of plant and nitrogen uptake by pea plants were recorded and results are presented in Table 17. The analysis of the data show that indofil M-45 was found most compatible with *Rhizobium leguminosarum* and exhibited maximum number of nodules (40/plant), fresh weight (80.66 g/plant). While grain yield/plant in indofil M-45 and bavis'tin were at par at 5% level of significance. Bavistin, thiram, topsin and ridomil MZ did not show any adverse effect to symbiotic nodulation. However, toxic effect of Captan seed dressing to *Rhizobium leguminosarum* was observed as compared to control.

### ii. Compatibility of seed dressing fungicides with *Rhizobium leguminosarum* under field conditions

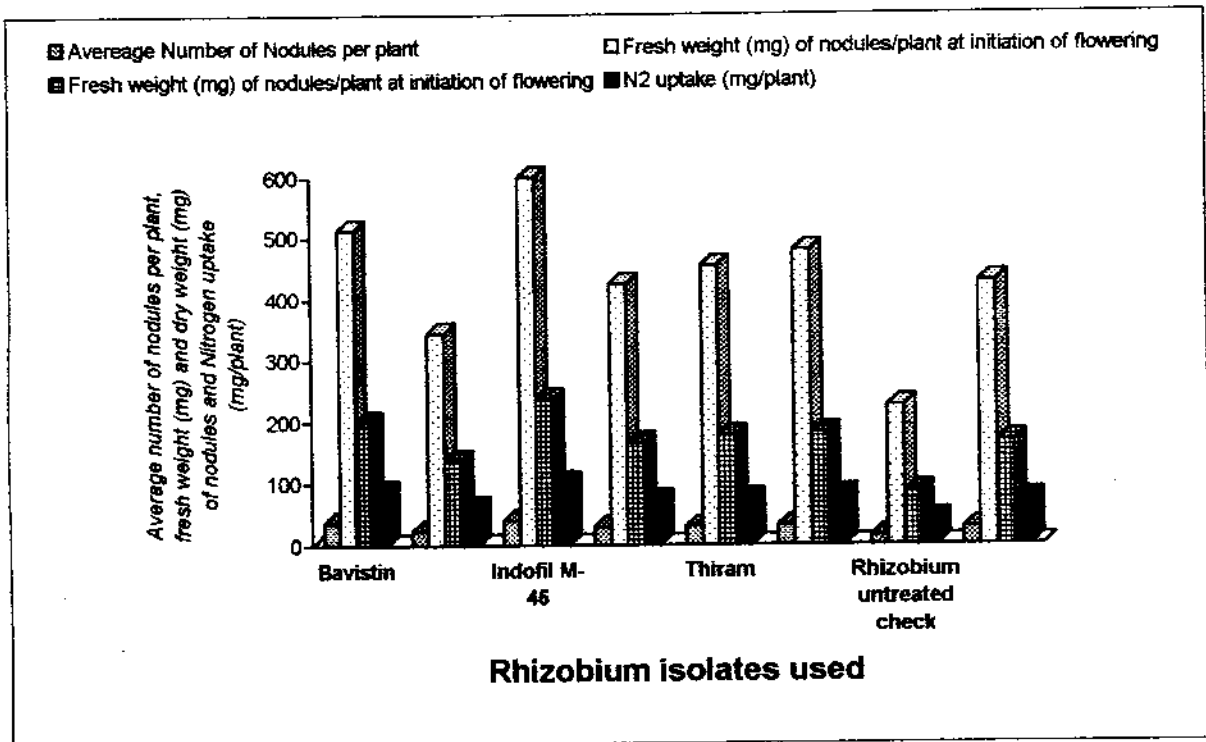
In order to confirm pot house experimental results a Randomized Block Design field experiment was conducted at Agricultural Research Station, Durgapura, Jaipur during 2000<sup>-01</sup> and 2001-02.

In year 2000<sup>-01</sup> Randomized Block Design experiment was conducted by using eight treatments and three replications. At the initiation of flowering average number of nodules per plant, average fresh and dry weight of nodules per plant, grain yield per plant and nitrogen uptake per plant were recorded and results are presented in Table 18. The data revealed that indofil M-45 and bavis'tin was found most compatible with pea *Rhizobium leguminosarum* and did not show any adverse effect on nodulation and recorded 41.66 and 37.00 nodule/plant respectively. These treatments also exhibited maximum fresh and dry weight of plant, grain yield and nitrogen uptake by the plant seed treatments with topsin-M, thiram and ridomil-MZ did not inhibit nodules as

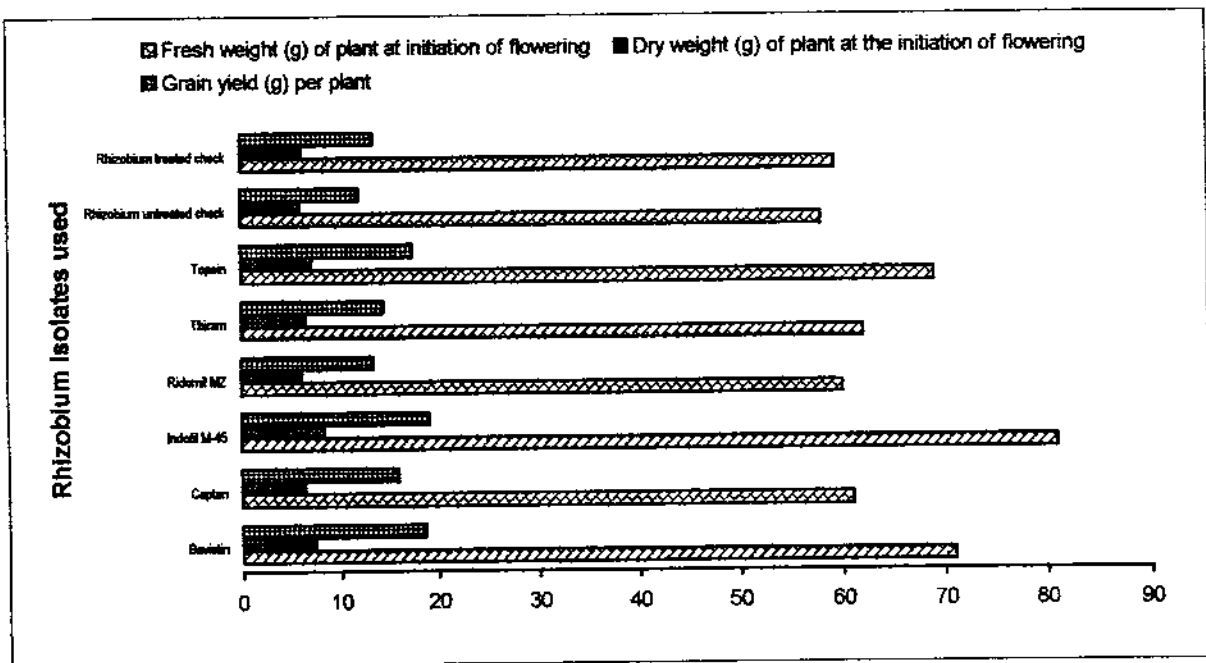
Table 17. Effect of chemical seed protectants on Pea (*Azad-P-1*) inoculated with *Rhizobium leguminosarum* in pot conditions during rabi 2000-01.

Treatment	Conc. g/kg of seed	Av. No. of nodules/plant	Av. Weight of nodules/plant at the initiation of flowering		Av. Weight of /plant at the initiation of flowering		Grain yield/plant (g)	N <sub>2</sub> uptake mg/plant
			Fresh weight (mg)	Dry weight (mg)	Fresh weight (mg)	Dry weight (mg)		
Bavistin	1.0	34.33 (5.86)	513.00	200.66	71.00	7.31	18.66	87.33
Captan	2.0	23.33 (2.83)	345.66	136.00	61.00	6.28	16.00	61.20
Indofil M-45	2.0	40.00 (6.32)	600.00	235.33	80.66	8.24	19.00	102.00
Ridomil-MZ	2.0	29.66 (5.45)	425.00	165.33	60.00	6.00	13.46	74.00
Thiram	2.0	30.00 (5.48)	454.00	177.00	62.00	6.41	14.50	75.33
Topsin	1.0	32.00 (5.65)	480.00	181.66	69.00	7.06	17.50	81.33
Check (Rhizobium untreated)	-	15.33 (3.91)	225.00	87.00	58.00	5.90	12.00	41.00
Control (Rhizobium)	-	28.00 (5.29)	428.33	167.66	59.33	6.06	13.50	74.00
SEm ±		0.109	5.02	4.88	1.50923	0.0680	0.1344	1.8596
CD at 5%		0.328	15.07	14.65	4.5248	0.2040	0.4029	5.5755

Figures in parenthesis are angular transformed values



### A. Nodulation characteristics



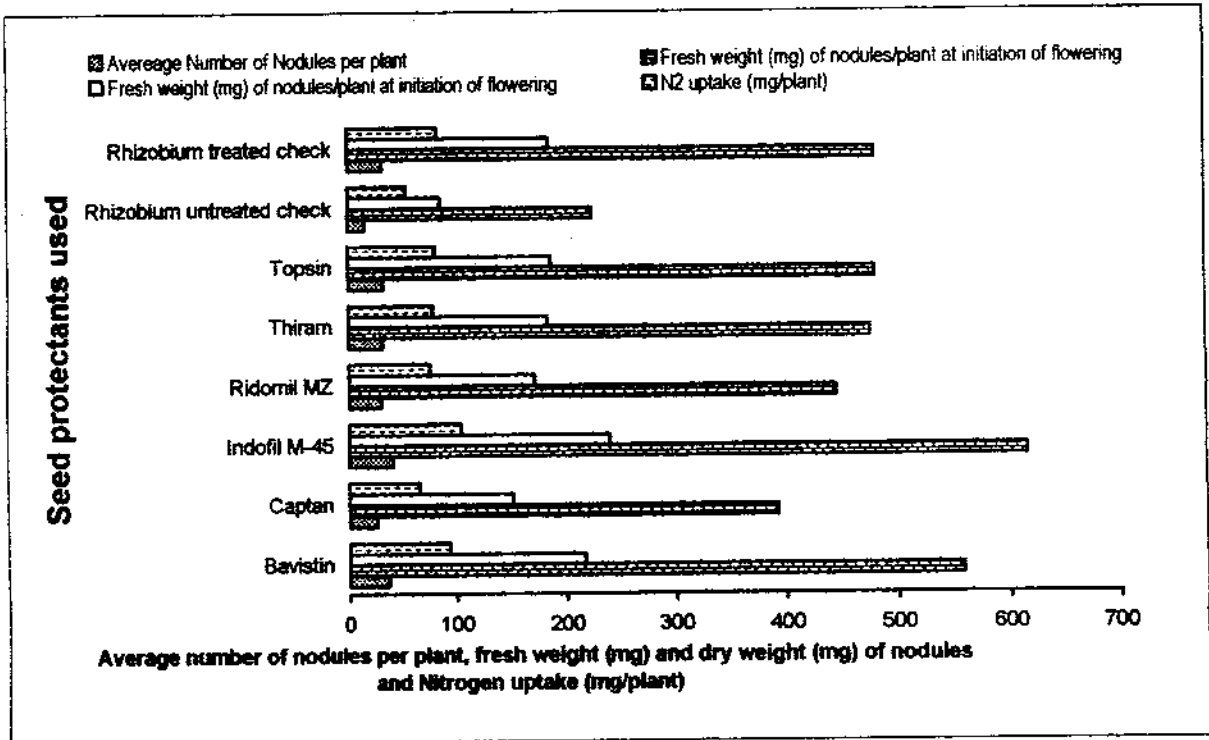
### B. Plant characteristics

Fig 13. Effect of chemical seed protectants on Pea (Azad-P-1) inoculated with Pea *Rhizobium leguminosarum* in pot conditions during rabi 2000, -01.

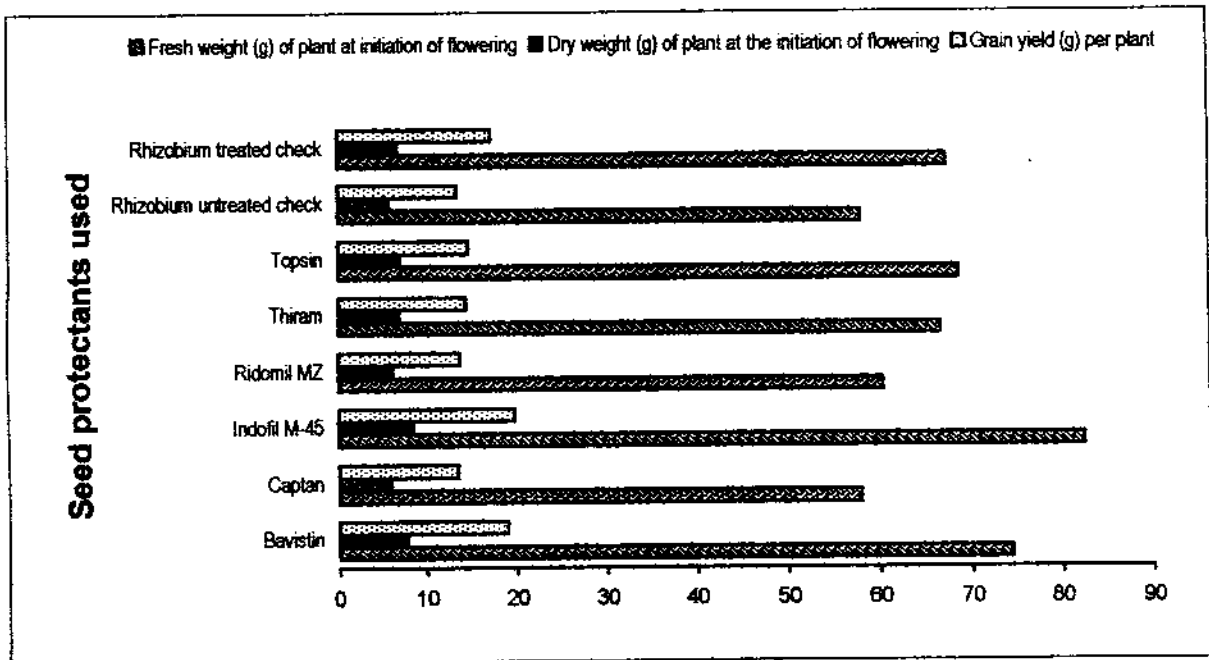
Table 18. Effect of chemical seed protectants on Pea (*Azad-P-1*) inoculated with *Rhizobium leguminosarum* in field conditions during rabi, 2006-07.

Treatment	Conc. g/kg of seed	Av. No. of nodules/plant	Av. Weight of nodules at the initiation of flowering		Av. Weight of plant at the initiation of flowering		Grain yield/plant (g)	N <sub>2</sub> uptake mg/plant
			Fresh weight (mg)	Dry weight (mg)	Fresh weight (mg)	Dry weight (mg)		
Bavistin	1.0	37.00 [6.08]	558.00	217.00	74.33	7.60	19.00	94.00
Captan	2.0	26.00 [5.10]	392.00	152.33	58.00	5.95	13.50	66.66
Indofil M-45	2.0	41.66 [6.45]	615.00	240.00	82.33	8.47	19.75	104.66
Ridomil-MZ	1.0	30.00 [5.47]	445.00	172.66	60.33	6.20	13.75	77.00
Thiram	2.0	31.00 [5.63]	475.33	183.66	66.66	6.96	14.50	79.66
Topsin	1.0	32.00 [5.66]	480.66	187.33	68.66	7.10	14.75	81.66
Check (Rhizobium untreated)	-	15.00 [3.86]	225.00	87.66	58.00	5.96	13.50	55.00
Control (Rhizobium untreated)	-	32.00 [5.66]	480.00	186.33	67.33	6.86	17.33	85.00
SEM ±		0.15	3.02	2.79	2.22	0.07	1.07	1.63
CD at 5%		0.46	9.11	8.46	6.74	0.22	3.24	4.96
CD at 1%		0.63	11.75	2.70	5.75	0.18	11.73	3.52
CV		4.78%	1.14%	2.70%	5.75%	0.18%	11.73%	3.52%

Figures in parenthesis are angular transformed values



### A. Nodulation characteristics



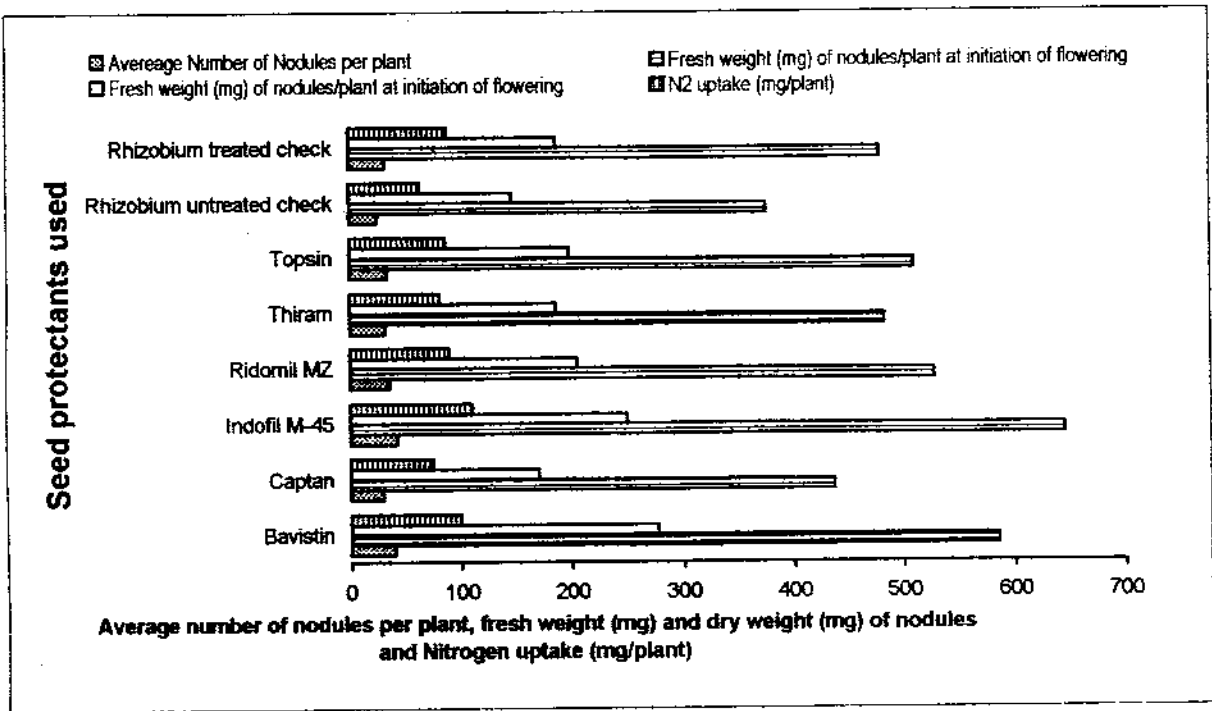
### B. Plant characteristics

Fig 14. Effect of chemical seed protectants on Pea (Azad-P-1) inoculated with *Rhizobium leguminosarum* in field conditions during rabi 2000 - 01.

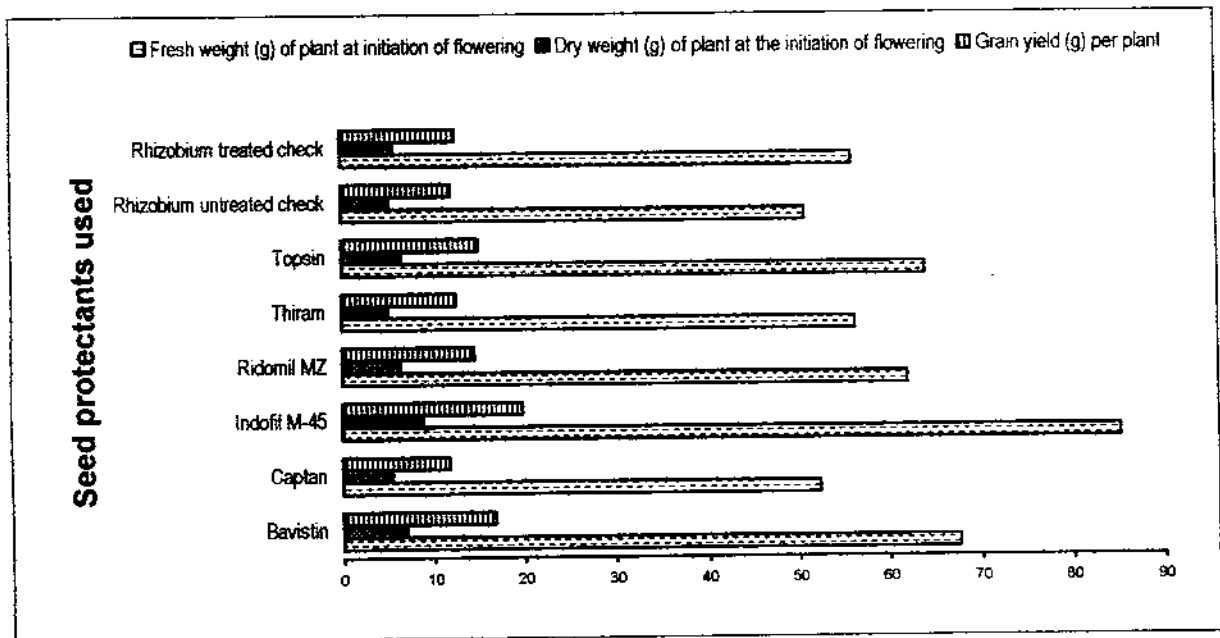
Table 19. Effect of chemical seed protectants on Pea (*Azad-P-1*) inoculated with *Rhizobium leguminosarum* in field condition during rabi, 2001-02.

Treatment	Conc. g/kg of seed	Av. No. of nodules/plant	Av. Weight of nodules/plant at the initiation of flowering		Av. Weight of /plant at the initiation of flowering		Grain yield/plant (g)	N <sub>2</sub> uptake mg/plant
			Fresh weight (mg)	Dry weight (mg)	Fresh weight (mg)	Dry weight (mg)		
Bavistin	1.0	89.00(6.24)	585.00	277.33	67.00	6.96	16.75	99.66
Captan	2.0	29.00(5.38)	437.00	170.00	52.00	5.40	11.75	74.00
Indofil M-45	2.0	43.00(6.55)	645.00	250.00	85.00	8.80	19.50	110.00
Ridomil-MZ	2.0	35.00(5.91)	528.00	205.00	62.00	6.35	14.50	89.00
Thiram	2.0	32.00(5.65)	483.33	186.66	56.00	5.80	12.51	81.33
Topsin	1.0	34.00	510.00	198.66	64.00	6.56	15.00	86.33
Check ( <i>Rhizobium</i> untreated)	-	25.00(5.00)	375.00	146.66	50.00	5.20	12.00	63.00
Control ( <i>Rhizobium</i> untreated)	-	32.00(5.32)	480.00	187.00	56.00	5.68	12.50	88.33
SEm ±		0.15	2.61	1.92	3.05	0.09	0.16	1.48
CD at 5%		0.47	7.94	5.85	9.26	0.29	0.49	4.5
CD at 1%		0.66	11.01	8.12	12.85	0.40	0.68	6.25
CV		4.73%	0.89%	1.69%	8.54%	2.61	1.96%	2.97%

Figures in parenthesis are angular transformed values



### A. Nodulation characteristics



### B. Plant characteristics

**Fig 15. Effect of chemical seed protectants on Pea (Azad-P-1) inoculated with *Rhizobium leguminosarum* in field conditions during rabi 2001-02 .**

compared to the *Rhizobium leguminosarum* control treatment. However, seed treatments with captan gave adverse effect and recorded less nodules (26 nodules/plant), fresh (58.00 g) and dry weight (5.95 g/plant) of plants as compared to *Rhizobium leguminosarum* inoculated control. The data were analysed statistically and found significant at 5 per cent level.

In order to confirm the field data of experiment trial, the experiment was repeated in the year 2001<sup>-02</sup> and results are shown in the Table 19. It is evident from the data that maximum average number of nodules/plant was 43.00, fresh and dry weight/plant (85.00 and 8.80 respectively). However maximum grain yield (19.50 g/plant) was observed in indofil M-45 seed dressing treatment, followed by bavistin treatment which showed 39.00 nodules/plant, 67.66 g, 6.96 g and 16.75 g fresh and dry weight of plant and grain yield respectively. However, indofil M-45 was found most compatible and gave maximum fresh and dry weight of nodules (645.00 mg/plant, 250.66 mg/plant respectively) and nitrogen uptake 110.00 mg/plant (Plate 4).

Experiment was repeated and analysed in 2001<sup>-02</sup> statistically. It did not show significant variation at 5% level.

### **10. Estimation of available nitrogen in the soil as influenced by the *Rhizobium leguminosarum* inoculated seed and dressed with chemical protectants.**

Soil samples were taken from the pea field before sowing the crop and after harvesting the crop to find out the per cent increase in available nitrogen of the soil, fungicides dressed seeds of pea were sown in the field to find out the per cent increase nitrogen as influenced by seed dressing treatments and results are presented in Table 20

**Table 20. Available nitrogen in the soil after the harvesting of Pea crop during rabi season of 2000<sup>-01</sup> and 2001<sup>-02</sup>.**

Treatment	Nitrogen (%)		Increase nitrogen % in soil	
	2000 <sub>1</sub>	2002 <sub>2</sub>	2000 <sub>1</sub>	2002 <sub>2</sub>
<i>Rhizobium</i> + Bavistin	0.0420	0.0440	0.0194	0.0200
<i>Rhizobium</i> + Captan	0.0416	0.0430	0.0190	0.0190
<i>Rhizobium</i> + Indofil M-45	0.0428	0.0448	0.0202	0.0208
<i>Rhizobium</i> + Ridomil-MZ	0.0416	0.0436	0.0190	0.0196
<i>Rhizobium</i> + Thiram	0.0401	0.0422	0.0175	0.0182
<i>Rhizobium</i> + Topsin-M	0.0408	0.0429	0.0182	0.0189
Control <i>Rhizobium</i>	0.0393	0.0420	0.0167	0.0180



Fig 16. Available nitrogen in the soil after the harvesting of pea crop during rabi-season of 2000-01 and 2001-02.

The data reveals that plots shown with fungicides dressed seeds, exhibited the per cent increase in nitrogen in the soil. Maximum per cent increase in nitrogen was observed in indofil M-45 followed by bavistin and ridomil-MZ treatment. It was observed that seed dressing treatments did not show any significant effect in nitrogen per cent increase.

### **11. Leg-haemoglobin content in pea nodules at the initiation of flowering as influenced by the chemical seed treatments**

Leg-haemoglobin contents were estimated in fungicide dressed and *Rhizobium leguminosarum* inoculated seed treatments. Nodules were harvested at the <sup>time</sup> of flowering <sup>initiation</sup> for leg-haemoglobin estimation. The Table 21 reveals that maximum leg-haemoglobin contents (mg/100 mg of nodules) was observed in plants dressed with indofil M-45. It was observed that untreated check exhibited minimum leg-haemoglobin contents (0.40 mg).

**Table 21. Leg-haemoglobin contents in Pea nodules at the initiation of flowering.**

Treatment	Leg-haemoglobin (mg/100 mg of nodules)
Bavistin	0.58
Captan	0.50
Indofil M-45	0.64
Ridomil-MZ	0.55
Thiram	0.46
Topsin-M	0.56
Untreated check	0.40
Rhizobium treated control	0.57

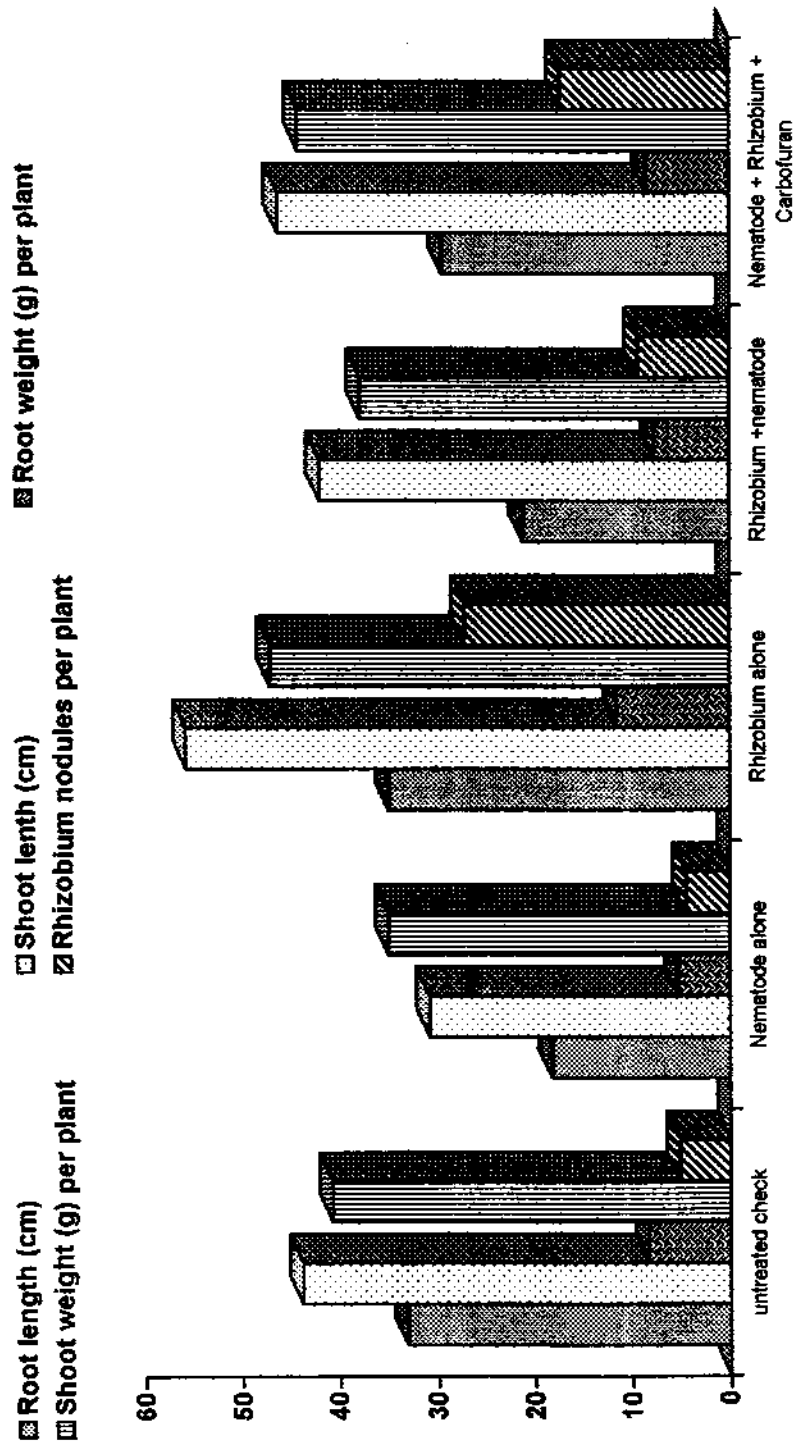
### **12. Interaction of *Rhizobium* and root knot nematode (*Meloidogyne incognita*) on the development of root nodules and plant growth**

*Rhizobium* inoculated seeds of pea were sown in *M. incognita* nematode infested soil to observe effect of root knot nematode on the nodule development (plate <sup>71</sup>Table 22). It was observed that plants in nematode infested soil exhibited small and less number of nodules (4.50) as compared to *Rhizobium* treated seeds (27.16 nodules/plant) sown in uninfested soils. Pea seeds sown in nematode infested soil exhibited reduced growth of root length (18.28 cm), plant shoot length (30.81 cm) and fresh weight (35.00 g/plant).

Table 22. Effect of carbofuran on root-knot nematode (*Meloidogyne. incognita* and *Rhizobium*)

Treatments	Av. root length/ plant (cm)	Av. shoot length/ plant (cm)	Av. fresh root weight/ plant (cm)	Av. fresh shoot weight/ plant (cm)	Average Number of galls/ plant	Av. No. of <i>Rhizobium</i> nodules/ plant.
Untreated check	33.15	43.80	8.32	40.80	0.00 (0.71)	5.16 (2.28)
Nematode alone	18.28	30.81	5.25	35.00	64.66(5.07)	4.50(2.12)
<i>Rhizobium</i> alone	34.98	55.75	11.56	47.18	0.00(0.71)	27.16(5.21)
<i>Rhizobium</i> + nematode	21.23	42.00	7.69	37.85	30.83(5.60)	9.33(3.05)
Nematode + <i>Rhizobium</i> + carbofuran	29.50	46.28	8.52	44.16	15.00(3.94)	17.33(4.16)
SEM ±	0.06	0.43	0.32	0.91	0.04	0.04
CD at 5%	0.19	1.27	0.94	2.65	0.11	0.13

Average of six replications. Figures in parenthesis are angular transformed values



## Treatment

Fig 17. Effect of carbofuran on root knot nematode (*Meloidogyne incognita*) and *Rhizobium*.

All the growth parameters including root and shoot length as well as fresh root and shoot weight were found increased in check (untreated) and *Rhizobium* alone. These parameters were found to be declined when plants were inoculated with nematode only. However, the plant growth as a whole was found to be increased when seeds were treated with *Rhizobium* along with simultaneous soil treatment of carbofuran @ 2.0 kg/ha.

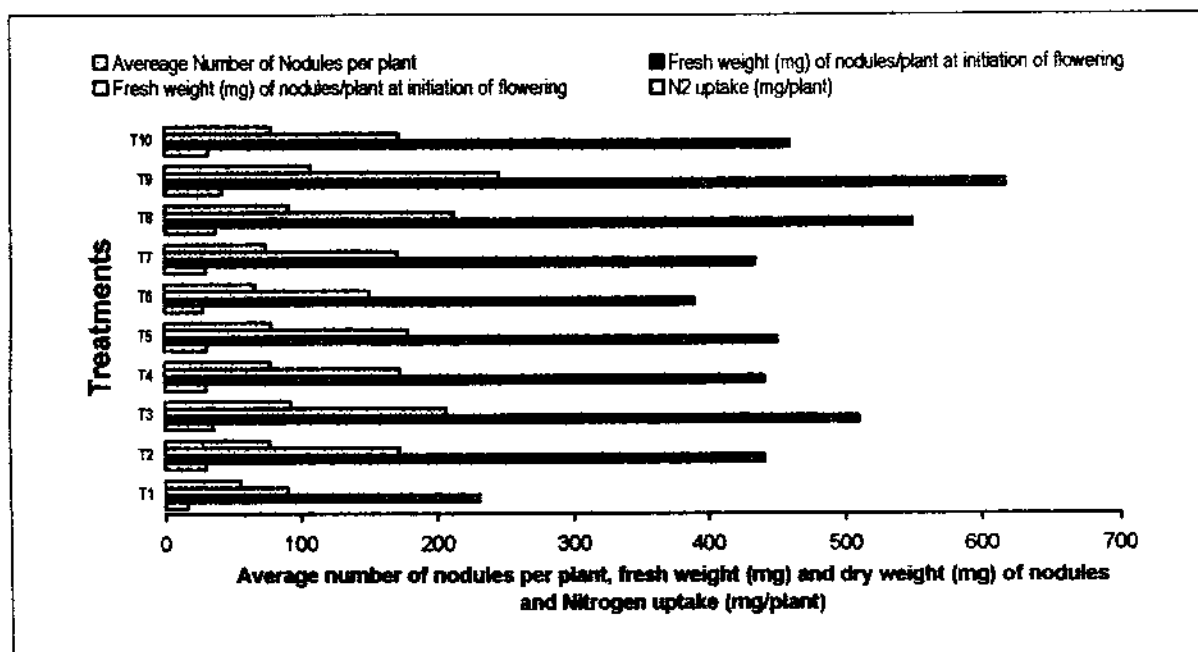
#### i. Phytonematode galls/plant

The number of galls and egg masses/pot were found to be maximum in the treatment of nematode alone (64.66 galls/plant) which got reduced (30.83 galls/plant) when inoculation was made with *Rhizobium* +nematode. The minimum number of galls (15.00 galls/plant) were recorded in the treatment *Rhizobium* + carbofuran @ 2.0 kg/ha over the untreated check (Plate 7).

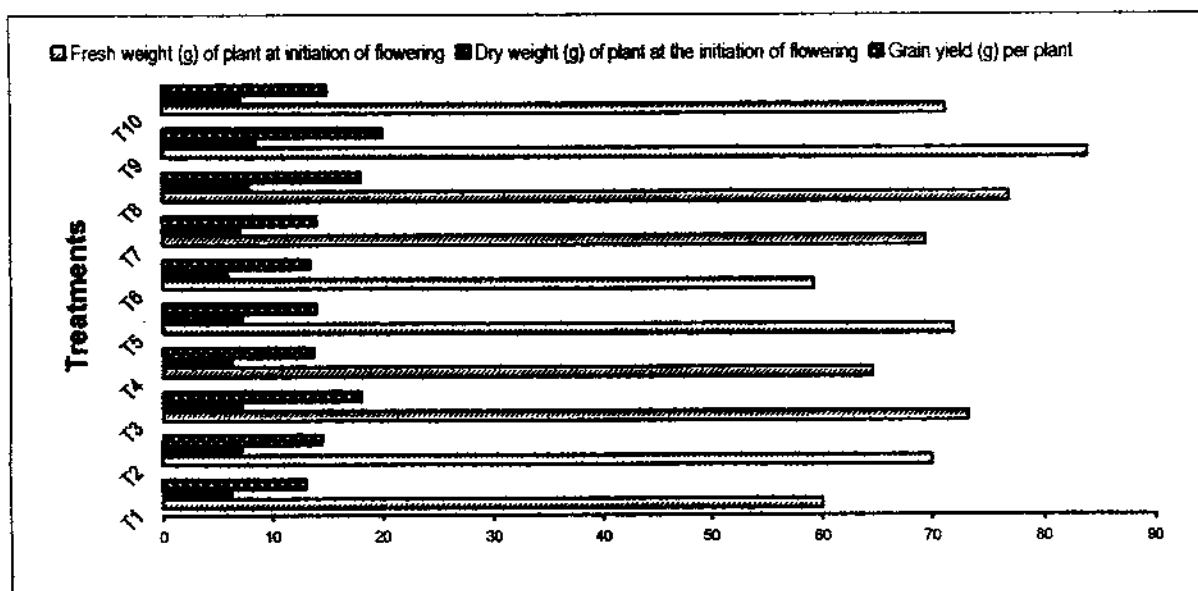
### **13. Compatibility of seed dressing fungicides and bioagents with *Rhizobium leguminosarum* under pot house conditions**

Pea seeds were treated in the sequence as bioagents ( $10^6$  spores/ml/10g) test fungicides (1 g/kg) and then *Rhizobium* ( $10^8$  cells/g of lignite base carrier material). These seeds were sown in 30 cm size earthen pots. A check was also maintained where pea seeds were treated with *Rhizobium* only.

Perusal of Table 23 showed the maximum nodulation (42 nodules/plant) when seeds were treated with indofil M-45 + *Pseudomonas fluorescens* + *Rhizobium* as against *Rhizobium* alone (32.0 nodules/plant). It was significantly superior over all other treatments with respect to number of nodules, fresh and dry weight of nodules and plant fresh weight as well as  $N_2$  uptake/plant. It also gave the highest yield (20.0 g/plant) which was significantly superior than *Rhizobium* alone (15.0 g/plant). Next treatment superior in its efficacy was bavisitin + *Pseudomonas fluorescens* + *Rhizobium*, bavisitin + *Gliocladium virens* + *Rhizobium* and indofil M-45 + *Gliocladium virens* + *Rhizobium* proved least compatible with respect to all the parameters.



### A. Nodulation characteristics



### B. Plant characteristics

Fig 18. Combined effect of seed protectants and bioagents on Pea (Azad-P-1) inoculated with *Rhizobium leguminosarum* under pot conditions during rabi 2001-02.

T1= untreated check  
 T2= Bavistin + T.v. + Rhizobium  
 T3= Indofil M-45 + T.v. + Rhizobium  
 T4= Bavistin + T.h. Rhizobium  
 T5= Indofil M-45 + T.h. + Rhizobium

T6= Bavistin + G.v. Rhizobium  
 T7= Indofil M-45 + G.v. Rhizobium  
 T8= Indofil M-45 + G.v. + Rhizobium  
 T9= Indofil M-45 + P.f. + Rhizobium  
 T10= Treated control with Rhizobium

Table 23. Combined effect of seed protectants and bioagents on Pea (*Azad-P-1*) inoculated with pea *Rhizobium leguminosarum* under pot conditions during rabi 2001.

Treatments	Av. No. of nodules/ plant	Av. weight of nodules at the initiation of flowering		Av. weight of plant at the initiation of flowering		Grain yield/ plant (g)	N <sub>2</sub> uptake mg/plant
		Fresh wet weight (mg)	Dry weight (mg)	Fresh wet weight (mg)	Dry weight (mg)		
Untreated check	16.00 [4.003]	230.00	90.00	60.00	6.20	13.00	55.00
Bavistin + <i>T. v.</i> + <i>Rhi.</i>	30.00 [5.433]	440.00	171.66	70.00	7.15	14.50	76.50
Indofil M-45 + <i>T. v.</i> + <i>Rhi.</i>	35.00 [5.903]	510.00	206.00	73.33	7.18	18.00	92.00
Bavistin + <i>T. h.</i> + <i>Rhi.</i>	30.00 [5.450]	440.66	172.00	64.66	6.25	13.70	77.00
Indofil M-45 + <i>T. h.</i> + <i>Rhi.</i>	31.00 [5.553]	450.00	178.33	72.00	7.23	14.00	78.00
Bavistin + <i>G. v.</i> + <i>Rhi.</i>	28.00 [5.270]	390.00	150.00	59.33	5.86	13.45	66.00
Indofil M-45 + <i>G. v.</i> + <i>Rhi.</i>	30.00 [5.450]	434.00	171.00	69.50	7.05	14.00	74.00
Bavistin + <i>P. s.</i> + <i>Rhi.</i>	37.00 [5.990]	550.00	212.00	77.00	7.80	18.00	91.00
Indofil M-45 + <i>P. f.</i> + <i>Rhi.</i>	42.00 [6.466]	618.00	245.00	84.00	8.50	20.00	107.00
Treated control with <i>Rhi.</i>	32.00 [5.646]	460.00	172.33	71.33	7.18	15.00	78.00
SEm ±	0.1672	4.88	4.14	2.25	0.06	1.44	2.23
CD at 5%	0.4933	14.40	12.22	6.64	0.18	4.25	6.59
CV	5.6279	1.87	4.05	5.56	0.15	16.25	4.87

Figures in parenthesis are angular transformed values

# Discussion

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## DISCUSSION

Biological nitrogen fixation (BNF) has been estimated to contribute more than 175 million tonnes of N out of which legume N<sub>2</sub> fixation accounts for almost 40% (Burns and Hardy, 1975). Although the role of legumes in improving soil fertility was known since long, it was only much later that the organism responsible-*Rhizobium* was isolated, identified and presently more than 7 cross-inoculation groups; based on host specification are known. The taxonomy of root nodulating bacteria is now in a state of transition and the latest system of classification (Jordan, 1984) divides, root nodule bacteria into 2 genera- *Rhizobium* and *Bradyrhizobium* based on DNA mole% G + C values, numerical taxonomy, serological relationship and other characteristics. Presently only 8-9% of the 14000 or so known species of leguminous plants have been examined for nodulation and less than 0.5% have been studied relative to their symbiotic relationship with nodule bacteria. Although rhizobia are common soil bacteria, they do not occur every where. There are many different types of rhizobia and not all types occur in all soils. This is important because certain plant species will only form a nitrogen fixing symbiosis with certain rhizobia. Which may not present in soil in which the legume is to be planted. This situation occurs most often when a new crop requiring a specific type of rhizobia or in other way they are host specific. Thus it is very essential to exploit the native strain which can be adaptable under local soil conditions for better biological nitrogen fixation process.

During present investigation, a brief survey was conducted in different localities of Jaipur district in Rajasthan in order to know the nodulation status of pea crop. Survey was conducted at ten localities of Jaipur district, out of which nodulation status was found to be good at five locations viz., Boraj, Bobas, Sambhar, Phulera and Lalpura; moderate at three locations, Hirnoda Durgapura, Bagru and poor at two locations, Jobner and Nagonki Dhani.

Variation in nitrogen fixing ability by native rhizobia may be due to several factors. These factors may be the organisms present in the rhizo sphere (Bhattacharya and Mukherjee, 1990 on peanut) and on the surface of root nodules (Chonkar and Subba Rao, 1966). These micro organism may act antagonistically to *Rhizobium* in soil and may also retard the growth of developing nodules. Besides these other physical factors like temperature (Hungria and Franco, 1993 on bean) soil moisture (Flounds, 1971) nature of clay (Nutman, 1976) and chemical factors like soil p<sup>H</sup> (Lowendarf and Alexander, 1983 on soybean) root exudates (Araiya and Medrios, 1992 on cowpea), availability of macro and micro nutrients (Cartwright and Hallsworth, 1970 on legumes), inoculation of pea seeds with chemical seed protectants along with incompatible pea *Rhizobium* strains play an important role in the formation of pea nodules.

To ensure biological nitrogen fixation pea seeds were inoculated with *Rhizobium* culture prior to sowing. But many a times *Rhizobium* inoculants do not form ample nitrogen fixing nodules. One of the reason may be the non-adoptability of treated *Rhizobium* inoculants to local soil. In order to exploit the native pea *Rhizobium* isolates, effective pink coloured nodules were harvested from 10 localities of Jaipur district and brought to the laboratory for isolation of pea *Rhizobium leguminosarum*. After isolations, the *Rhizobium* culture was analysed by standard qualitative and quantitative methods as described by Somasegaran and Hoben (1985, 1994). After through screening of different isolates, the *Rhizobium* isolate from the nodules collected from Boraj and Bobas locality were found promising and used for further experimentation.

Pea seeds were coated with *Rhizobium* by using carrier based inoculant prior to planting. This carrier based legume *Rhizobium* was prepared by using fully multiplied broth *Rhizobium* culture. During present investigation a simple glass fermenter was used for quick multiplication of *Rhizobium* culture in less time. It was observed that desired bacterial population  $54 \times 10^8$  cells/ml was obtained in 96 hours, as compared to the broth inoculated in simple Erlenmeyer flask (shake culture) which gave  $50 \times 10^8$  cells/ml in the same period by simple shaking method. This may be used to meet the requirements of large field experiments of pea *Rhizobium* inoculation. In addition, this glass fermenter may also be suitable for the production of starter cultures for medium sized commercial fermenters. Gulati (1978) also observed that using a fermentor over shake culture gave quick multiplication of rhizobial culture in broth.

It is an established fact that sowing of legumes increases the fertility of soil. Burns and Hardy (1975) used FAO statistics on land use to calculate estimated annual global  $N_2$  fixation ( $139 \times 10^6$  metric tonnes/annum) could be accounted for legume crops. This also help in nourishing the subsequent crop due to beneficial exudates released by the legume roots in the soil, which may be helpful in maintaining the equilibrium of beneficial microflora. During present investigation attempt has been made to analyse the soil for nitrogen level estimation before sowing and after harvesting of the crop. The analysis revealed positive trend of increase in per cent total and available nitrogen. Alexander (1984) has also reported the increase in the amount of available N for uptake by plants due to *Rhizobium*. Thus the nitrogen in pea sown soil increased due to pea *Rhizobium* inoculation through the seed.

The number of nodules formed by legume and their effectiveness is governed by both the host plants and *Rhizobium* species. Host cultivars and *Rhizobium* strain studies can be utilized for improving biological nitrogen fixation in pea crops. Attempts has been made to evaluate 11 selected genotypes for two rabi seasons for their nitrogen fixing ability, under pot house and field conditions. The results revealed that genotype Azad- P-1 and PMR-19 exhibited maximum nodulation under pot house conditions while under field conditions genotype Azad-P-1 and Arkil showed maximum nodulation. Since variations in nitrogen fixing ability is closely related to

infection of plant cells and development of bacterioids, it is attributed to the action of cellulolytic enzymes (Verma *et al.* 1978), Kijne (1975) described changes in the vacuoles of pea nodule cells following release of bacteria from infection threads. Such conditions could influence genotype differences in nitrogen fixation.

Mycoflora associated with surface of root nodules play an important role in inhibiting the growth of developing nodules. Nodulation may also be inhibited due to rhizosphere mycoflora. During the present investigation five fungi namely *Aspergillus niger*, *Penicillium oxalicum*, *Drechslera* state of *Cochliobolus spicifer*, *Fusarium oxysporum* and *Myrothecium verrucaria* were isolated from the surface of nodule *in vitro*, among these, *Myrothecium verrucaria*, *Fusarium oxysporum* and *Aspergillus niger* were found dominating. Such observations also supported by Chonkar and Subba Rao (1966) in nine common legumes including *Pisum sativum*.

During the course of present investigation the isolated fungi from the surface of nodules were further screened for their antagonistic activities toward *Rhizobium leguminosarum*. *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium oxalicum* showed antagonistic activity.

It may be attributed to the damaging and disorganizing nature of these fungi to the nodular tissues of the cortex. Thus myco-colonization of nodules by pathogenic fungi may be one of the factors, which may act in the rhizosphere and affect adversely to the biological nitrogen fixation process. Drapeau, Fortin and Gagnon (1973) reported that *Rhizobium* isolates show antifungal activity *in vitro* against eight fungi tested including *Fusarium* sp. *Pyrenochaeta terreotris*, *Colletotricum destructor*, *Phytophthora caetorum* and *Coniothyrium* sp.

Variation in the microflora in pea rhizosphere was studied at different stages of plant growth after seed dressing of pea seeds with fungicides. It was observed that at 30, 45 and 60 days of plant growth, actinomycetes were found most dominating followed by bacterial and fungal counts. These microbial counts were higher in undressed seeds, change in microflora was observed in pea plants during different plant growth stages. Out of the 6 seed dressers used in the present study, treated with indofil M-45 followed by bavisitin showed minimum counts of actinomycetes, bacterial and fungal colonies from the rhizosphere at all the three different stages i.e. 30, 45 and 60 days after sowing.

Seed borne pathogens are playing major role in inciting diseases in pea crops. These includes *Fusarium oxysporum* f. sp. *pisi*, *F. solani*, *F. sp. pisi* *Ascochyta pisi*, *A. pinodes* (*Mycosphaella pinodes*) *Phoma medicagnis* var. *pinodella*, *Alternaria alternata*, *F. poae*, *F. sporotrichoides*, *F. sambucinum* (Wang Kuan Chang *et al.* 1997). It is more or less common practice to dress pea seeds with chemical protectants and also at the same time pea seeds are inoculated with *Rhizobium* in order to ensure biological nitrogen fixation.

In the present study, six seed dressers were taken to study their compatibility with pea *Rhizobium*. Indofil M-45 was found to be most compatible with *Rhizobium leguminosarum* inciting maximum number of nodules (40 nodules/plant) and N<sub>2</sub> uptake (102 mg/plant). It was followed by bavisitin, thiram, topsin M. and ridomil-MZ, Krishan Chandra *et al.* (1996) also reported Indofil M-45 to be most compatible with *Rhizobium leguminosarum* and recorded maximum number of effective nodules. However, maximum nodulation was recorded when soybean seeds were treated with topsin-M. Singh and Lodha (1997) also reported the compatibility of 5 seed dressing fungicides viz., thiram, dithane M-45, captan, ridomil MZ, among these ridomil MZ and bavisitin were found best compatible.

In recent years there has been renewed interest in the use of bioagents and PGPR, which when applied to seeds, tubers or roots are able to colonize plant roots and stimulate plant growth and crop yield. This is due to production of plant hormones, enhancement of plant nutrient uptake or suppression of pathogenic or deleterious organisms via antibiotic or siderophore production may be involved (Azcon 1989). In the present studies pea seeds treated with fungicides, bioagents and *Rhizobium* showed best compatibility with indofil M-45 + *Pseudomonas fluorescens* followed by bavisitin + *P. fluorescens* with respect to increase in number of nodules, fresh dry weight of nodules biomass grain yield and N<sub>2</sub> uptake. Number of workers including Singh *et al.* (1995) also reported the compatibility of fungicide + bio-agent *Rhizobium* in leguminous crop. Dubey and Patel (2001) has also determined the tolerance in *Tanacetophorus cucumeris*, *T. v. G. v* and *Rhizobium* sp. to fungicides, carbendazim and Carboxin were more compatible with *Rhizobium* than thiram, thiophanate-methyl and captan.

Root knot nematodes alter the physiology of roots and induce galling, resulting in reduced crop yield. Root knot nematodes are known to inhibit the symbiotic nodules formation. During the course of nematode infection process of cell diffusion and proliferation of root cell take place resulting in hypertrophy of roots. In these hypertrophied cells frequency of establishment of infection of *Rhizobium* cells through root hairs is neutralized. This causes less nodular gland formation. Studies on interaction of *Rhizobium* - carbofuran and root knot nematode (*Meloidogyne incognita*) on the development of root nodules and plant growth, exhibited small and less number of nodules as compared to *Rhizobium* treated seeds sown in un-infested soils. Such finding were also observed by Singh and Gupta (1983) in cowpea and Barker *et al.* (1972) in soybean crop. Kishnovski *et al.* (1992) applied methyl bromide as soil fumigant along with peanut and inoculation with *Rhizobium*. These authors observed that methyl bromide substantially decrease the number of nodules. So soil fumigants was not compatible with peanut native *Rhizobium* and affect adversely to biological nitrogen fixation process. Thus compatible nematicides should be thoroughly analysed before their use under field conditions. It appears that interaction of nematode and *Rhizobium* sp. has not been properly investigated and detailed study on this aspect is needed.

33

Compatibility of seed protectants or nematicides and bio-agents with *Rhizobium* isolates in a relative term i.e. it may be compatible or less compatible or not compatible at all. While selecting the effective *Rhizobium* strain for inoculant, a number of criteria should be considered before it is used in field. These are:

1. Competitive ability with other strains for infection sites on the roots of the host legume.
2. Nitrogen fixing ability over a range of environmental conditions.
3. Nodule forming and nitrogen fixing abilities in the presence of soil nitrogen.
4. Ability to multiply in both and survive in peat.
5. Ability to survive when incorporated in seed pellets.
6. Persistence in the soil.
7. Ability to migrate from the initial site of inoculation.
8. Ability to colonize soil away from the influence of host roots.
9. Ability to survive adverse physical conditions such as desiccation, heat or freezing.
10. Strain stability during storage and growth.
11. Compatibility of seed protect ant with *Rhizobium*.
12. Compatibility of bio-agents with *Rhizobium*.
13. Compatibility of nematicides in nematode infested soil.

Although testing of *Rhizobium* for inoculants is a difficult task. During present studies attempt has been made to evaluate pea isolates under the local soil condition but more detail investigation above aspects are needed to be alone under field conditions, before any recommendations are made and for this sustained research both applied and basis on these aspects is needed to be done.

# Summary

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## SUMMARY

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Present study was undertaken to investigate the interaction of rhizosphere nodule microflora and seed protectants with *Rhizobium leguminosarum* incitant of pea root nodules and genotype differences in nitrogen fixation. Experiments were undertaken *in vitro* under laboratory and pot conditions, field trials were undertaken during the year 2000 and 2001 at Agricultural Research Station, Durgapura, Jaipur and the brief findings of the investigation are given below:

Survey was undertaken of major pea growing localities in Jaipur district during *Rabi* season 1999, at the time, when the plants were nearing flowering. Ten randomly selected pea plants were uprooted from each locality for counting the nodules. These were brought to the laboratory for the isolation of *Rhizobium* in silica gel tubes. The identity of the culture was confirmed by qualitative and quantitative tests. *Rhizobium* culture isolated from Boraj locality ( $P_1$ ) was found promising and used for further experiments in addition to Bobas locality isolate ( $P_2$ ).

Nitrogen of soil was also estimated before sowing and after harvesting of the crop during 2000 and 2001 *Rabi* seasons. It was found that there was a marginal increase in per cent total and available nitrogen of the soil in both the years.

For quick multiplication of *Rhizobium leguminosarum* culture in broth, a small glass fermentor was used to give desired population of  $54 \times 10^8$  and  $50 \times 10^8$  cells/ml of  $P_1$  and  $P_2$  *Rhizobium* isolate respectively in 96 hrs as compared to simple shake culture which took 10 days in obtaining the desired population of *Rhizobium* in broth.

Eleven pea genotypes were screened against  $P_1$  and  $P_2$  isolate of *Rhizobium leguminosarum* for their nitrogen fixing ability. Genotype Azad-P-1 exhibited maximum number of symbiotic nodules. Nodule microflora studies revealed that actinomycetes, bacteria and five fungi viz., *Aspergillus niger*, *Penicillium oxalicum*, *Fusarium oxysporum*, *Drechslera* state of *Coeliobolus spicifer* and *Myrothecium verrucaria* were found associated with pea nodules. *Myrothecium verrucaria* was most frequently observed during microflora studies.

Antagonistic activity of these micro-organisms towards *Rhizobium leguminosarum* revealed that actinomycetes and bacteria inhibited the growth of pea *Rhizobium leguminosarum*. Fungi *Aspergillus niger* and *Fusarium oxysporum* exhibited maximum antagonistic activity and inhibited the growth of *Rhizobium leguminosarum in vitro*. Compatibility of chemical seed dresser with pea *Rhizobium* was tested *in vitro*, in pot house and under field conditions. Indofil M-45 and

bavistin were found compatible with *Rhizobium leguminosarum*. Maximum number of nodules were observed in indofil M45 *Rhizobium* inoculated pea plants.

Compatibility of seed dressing fungicides and biocontrol agents with *Rhizobium leguminosarum* revealed that sequential treatment with indofil M-45 + *Pseudomonas fluorescens* + *Rhizobium leguminosarum* exhibited maximum number of nodules and plant growth.

When pea seeds were inoculated with *Rhizobium leguminosarum* and planted in soil infested with *Meloidogyne incognita* in pots, nodulation was suppressed. Application of carbofuron @ 2.00 kg/ha in nematode infested soil gave increased number of nodules and reduced the nematode galls in pea roots in pot house condition.

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## **Abstracts**

## ABSTRACT

### Interaction of Rhizosphere, nodule microflora and seed protectants with *Rhizobium leguminosarum* Incitant of pea [*Pisum sativum* L.] root nodules

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Interactions of Rhizosphere, nodule microflora, seed protectants with *Rhizobium leguminosarum* were studied *in vitro* and under field soil conditions. Survey was conducted at ten pea growing localities for symbiotic nodule status. Good nodulation were observed in Boraj, Bobas, Sambhar, Phulera and Lalpura localities of Jaipur district.

*Rhizobium leguminosarum* isolate P<sub>1</sub> (Boraj) and P<sub>2</sub> (Bobas) were found superior in quantitative and qualitative tests.

Eleven pea genotypes were screened against P<sub>1</sub> and P<sub>2</sub> *Rhizobium* isolate. Genotype Azad P-1 gave maximum nodulation.

Simple glass fermentor method was used for quick multiplication of pea *Rhizobium*. Actinomycetes, bacteria and five fungi were found antagonistic to *Rhizobium leguminosarum*. Fungi *Myrothecium*, *verrucaria* and *Fusarium oxysporum* sp. were found more frequently associated with pea root nodules.

At different stages of plant growth, change in rhizosphere microflora was observed and it was found that actinomycetes were most dominant among microflora followed by bacteria and fungi.

Indofil M-45 and bavistin used as seed dressers were found compatible with *Rhizobium leguminosarum*.

Indofil M-45 + *Pseudomonas fluorescens* + *Rhizobium* treatment with pea seeds exhibited maximum number of nodules and plant weight showed compatibility of all three treatments.

*Meloidogyne incoginta* (incitant of root knot in pea) infested soil exhibited fewer number of nodules. Application of carbofuran in soil near the vicinity of seed reduced the infection and enhanced the nodule formation.

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## अनुक्षेपण

**मटर की जड़ग्रन्थियों के प्रेरक राइजोबियम लेग्यूमिनोसेरम की मूल परिवेधी  
—सूक्ष्म वनस्पतिजात एवं बीज संरक्षी के साथ अन्योन्य क्रियाएँ.**

सुरेन्द्र कुमार टाक  
अनुसंधानकर्ता

डा. पी. सी. लोढा  
मुख्य परामर्शदाता

मटर की जड़ग्रन्थियों के प्रेरक राइजोबियम लेग्यूमिनोसेरम की मूल परिवेधी सूक्ष्म वनस्पतिजात एवं बीजसंरक्षी के साथ अन्योन्य क्रियाएँ पायी गयी। दस स्थानों पर सहजीवी ग्रन्थि के स्तर के लिये सर्वेक्षण किया गया। बोरज, बोबास, साँभर, फुलेरा और लालपुरा में उत्तम ग्रन्थन पाया गया।

राइजोबियम लेग्यूमिनोसेरम वियुक्तों का गुणात्मक एवं मात्रात्मक परीक्षण करने पर वियुक्त पी-1 ( बोरज) एवं पी-2 (बोबास) सर्वोत्तम पाये गये।

राइजोबियम वियुक्त पी-1 एवं पी-2 के विरन्ध मटर की 11 जीनरूप की जाँच की गई, जिसमें जीनरूप आजाद पी-1 में अधिकतम ग्रन्थन पाया ।

राइजोबियम लेग्यूमिनोसेरम के त्वरित गुणन के लिये सरल काँच किण्वन विधि उपयोग में ली गयी । एक्वीनोमाईसीटस, जीवाणु एवं 5 कवक मटर के जड़ग्रन्थि प्रेरक राइजोबियम पर विरोधी सूक्ष्मजीव देखे गये। कवक माइरोथेसियम वेरूकेरिया एवं प्यूजेरियम ओक्सीस्पोरम बारम्बार एवं प्रभावी रूप से पाये गये।

मटर के पौध वृद्धि की विभिन्न अवस्थाओं में मूल परिवेधी सूक्ष्म वनस्पति जातियों में परिवर्तन पाया गया ,साथ ही इनमें कवक और जीवाणु की अपेक्षा एक्विनोमाईसीटस अधिक प्रभावी रहे।

इन्डोफिल एम-45 और बावस्टिन बीज संरक्षी रसायन की मटर ग्रन्थि प्रेरक राइजोबियम लेग्यूमिनोसेरम के साथ संगतता पायी गयी।

इन्डोफिल एम-45 + स्ट्यूडोमोनास प्यूओरेसेन्स, राइजोबियम की मटर की ग्रन्थि प्रेरक राइजोबियम के साथ संगतता से मटर में ग्रन्थियाँ एवं पौध द्रव्यमान अधिक पाया गया ।

मिलोडोगाइनी इन्कोगनिटा ( जो मटर में मूल गाँठ पैदा करती है ) से दुर्पित भूमि में कम सख्या में ग्रन्थिका प्रकट हुई। भूमि में बीज के समीप कार्बोफ्यूरान के अनुप्रयोग से संक्रमण में कमी एवं ग्रन्थि निर्माण वृद्धि हुई।

# **Appendices**

Appendix I. ANOVA (CRD) for different isolates of Rhizobium leguminosarum under sterilized soil conditions in pots.

Source of variation	Df	M.S.						N <sub>2</sub> uptake mg/plant	
		Av. No. of nodules/ plant		Av. Weight of nodules/plant at the initiation of flowering		Av. Weight of /plant at the initiation of flowering			Grain yield/ plant (g)
		Fresh wet weight (mg)	Dry weight (mg)	Fresh wet weight (mg)	Dry weight (mg)	Fresh wet weight (mg)	Dry weight (mg)		
Between isolates	8	7.888**	12004.206**	76556.625**	196.32**	2.204**	32.398**	1078.175**	
Within treatment	18	0.100	54.370	76.111	24.556	0.353	7.000	11.926	

\* Significant at p=0.05 and \*\* Significant at p=0.01

Appendix II. ANOVA for comparative population counts of broth using Erlenmeyer flask and glass fermentor plate count method.

Source	d.f.	MS
Replication	3	0.2656 NS
Treatments	15	1305.1823**
Method (M)	1	50.7656**
Isolate (P)	1	2691.0156**
Hours (H)	3	5203.1821**
M x P	1	8.2656**
M x H	3	9.4323**
P x H	3	395.6823**
M x P x H	3	0.9323 NS
Error	45	0.5767

\* Significant at p=0.05 and \*\* Significant at p=0.01

APPENDIX III. ANOVA For Screening of different Pea genotypes to different isolates of *Rhizobium leguminosarum* under pot house conditions during rabi 2000.

Source of variation	Df	Average number of nodules per plant	M.S.E.					
			Average weight of nodules at the initiation of flowering		Average weight of plant at the initiation of flowering		Grain yield per plant	N <sub>2</sub> uptake per plant
			Fresh weight	Dry weight	Fresh weight	Dry weight		
Replications	2	0.25	27.27	28.34	19.74	0.04	6.86	4.65
Treatments	21	1.19**	18353.27**	2749.36**	118.63**	1.25**	27.24**	630.72**
Genotypes (G)	10	2.86**	19011.13**	2907.48**	184.89**	1.88**	11.76	1025.84**
Isolate (P)	1	3.91**	165600.35**	24054.56**	630.54**	7.14**	437.87**	2853.87**
G x P	10	0.06	2970.69**	460.71**	1.18	0.02	1.65	13.28
Error	42	0.11	105.40	25.26	13.889	0.02	6.18	33.75

\* Significant at p=0.05 and \*\* Significant at p=0.01

APPENDIX IV. Screening of different Pea genotypes to different isolates of *Rhizobium leguminosarum* under pot house conditions during rabi 2000.

Source of variation	d.f.	Av. No. of nodules/plant	MSI					
			Av. Weight of nodules/fresh weight (mg)	Plant at the initiation of flowering	Av. Plant weight/plant	At the initiation of flowering	Grain yield/plant (g)	N <sub>2</sub> uptake/plant (mg)
Replication	2	0.86	322.81	72.67	8.22	0.03	21.18	26.19
Treatment	43	1.06	11705.81	1878.82	81.20	0.85	29.03	369.88
Year (Y)	1	0.13 NS	556.42**	48.49 NS	16.71 NS	0.19**	13.36 NS	3.06 NS
Genotype (G)	10	2.87**	41228.86**	6572.02**	193.88**	2.08**	28.93*	1067.59**
Isolate (P)	1	11.31**	71726.36**	11837.09**	1317.35**	13.26**	795.27**	4656.46**
Y x G	10	0.26 NS	621.82**	107.81	16.60 NS	0.17**	4.59 NS	51.75*
Y x P	1	0.03 NS	27.32 NS	3.87 NS	3.34 NS	0.13**	27.27*	16.02 NS
G x P	10	0.15 NS	556.83**	92.75 NS	3.27 NS	0.05**	3.73 NS	1.95 NS
Y x G x P	10	0.14 NS	696.46**	117.81*	117.81*	0.02 NS	4.00 NS	1.66 NS
Error	86	0.23	47.88	34.89	6.59	0.01	6.59	14.22

\* Significant at p=0.05 and \*\* Significant at p=0.01

**APPENDIX V. ANOVA for microbial population (colonies of fungi, bacteria and actinomycetes) of Pea rhizosphere at different stage of crop growth as influenced by chemical seed protector and *Rhizobium* inoculation.**

Source of variation	d.f.	MS		
		Fungi	Actinomycetes	Bacteria
Replication	3	0.015035	0.00071	0.0005
Treatment	23	0.10161	0.01265	0.02824
Days (D)	2	0.13586**	0.08789**	0.15283**
Fungicide (F)	7	0.18926**	0.01481**	0.04606**
B x F	14	0.05289**	0.00083 NS	0.00152*
Error	69	0.00240	0.00025	0.00037

\* Significant at p=0.05 and \*\* Significant at p=0.01

**Appendix VI: ANOVA for effect of *Rhizobium* inoculated Pea seeds on growth and nodulation of the plants and nitrogen status of the soil under myco-colonized soil conditions.**

Source of variation	df	M.S...					
		Average weight of nodules/plant at the initiation of flowering		Average weight/plant at the initiation of flowering		N <sub>2</sub> uptake mg/plant	
		Fresh weight (mg)	dry weight (mg)	Fresh weight (g)	dry weight (g)	Fresh weight (g)	dry weight (g)
Between fungi	5	2.47**	97032.24**	15136.26**	1057.76**	9.28**	261.76**
Within fungi	18	0.03	39.04	18.00	5.61	0.02	2.80

\* Significant at p=0.05 and \*\* Significant at p=0.01

**APPENDIX VII : ANOVA for *In vitro* effect of fungicides on the growth of Pea *Rhizobium leguminosarum*.**

Source of variation	d.f	MS
Replication	3	0.075
Treatments	29	69.719
Fungicide (F)	5	152.596**
Concentration	4	194.788**
F x C	20	21.876**
Error	87	0.144

\* Significant at p=0.05 and \*\* Significant at p=0.01

**APPENDIX VIII: Effect of chemical seed protectants on Pea (*Azad-P-1*) inoculated with *Rhizobium leguminosarum* in pot conditions during rabi 2000.**

Source of variation	d.f.	M.S.E.						
		Av. No. of nodules/plant	Av. Weight of nodules at the initiation of flowering		Av. Weight of plant at the initiation of flowering		Grain yield/plant (g)	N <sub>2</sub> uptake/plant (mg)
			Fresh weight	Dry weight	Fresh weight	Dry weight		
Within fungicide	7	1.57**	37675.63**	5766.57**	183.61**	1.98**	20.51**	947.28**
Between fungicide	16	0.03	75.83	71.70	6.83	0.01	0.05	10.37

\* Significant at p=0.05 and \*\* Significant at p=0.01

**APPENDIX IX: ANOVA for effect of chemical seed protectants on Pea (Azad-P-1) inoculated with *Rhizobium leguminosarum* in field conditions during rabi, 2000.**

Source of variation	d.f.	MS						N <sub>2</sub> uptake/plant (mg)
		Av. No. of nodules/plant	Av. Weight of nodules at the initiation of flowering		Av. Weight of Plant at the initiation of flowering		Grain yield/plant (g)	
			Fresh weight	Dry weight	Fresh weight	Dry weight		
Replication	2	0.01	2.62	18.37	2.16	0.016	3.26	6.16
Fungicide	7	1.77**	40609.32**	6176.28**	213.56**	2.26**	20.57**	703.89**
Error	14	0.06	27.43	23.37	14.83	0.01	3.43	8.02

\* Significant at p=0.05 and \*\* Significant at p=0.01

**APPENDIX X: ANOVA for the effect of chemical seed protectants on Pea (Azad-P-1) inoculated with *Rhizobium leguminosarum* in field condition during rabi, 2001.**

Source of variation	d.f.	MS						N <sub>2</sub> uptake/plant (mg)
		Av. No. of nodules/plant	Av. Weight of nodules at the initiation of flowering		Av. Weight of plant at the initiation of flowering		Grain yield/plant (g)	
			Fresh weight	Dry weight	Fresh weight	Dry weight		
Replication	2	0.00	55.12	28.50	18.50	0.01	0.29	9.19
Fungicide	7	0.71**	21016.57**	3135.47**	386.56**	4.025**	22.19**	624.27**
Error	14	0.03	20.55	11.16	27.97	0.02	0.07	6.63

\* Significant at p=0.05 and \*\* Significant at p=0.01

APPENDIX XI: ANOVA for the effect of carbofuron on root-knot nematode (*Meloidogyne incognita* and *Rhizobium*)

Source of variation	d.f.	MS					
		Av. root length/ plant (cm)	Av. shoot length/ plant (cm)	Av. fresh root weight/ plant (cm)	Av. fresh shoot weight/ plant (cm)	Average Number of galls/ plant	Av. No. of <i>Rhizobium</i> nodules/ plant.
Between treatments	4	324.07**	512.88**	30.47**	141.26**	60.87**	10.32**
Within treatments	25	0.02	1.15	0.63	4.97	0.01	0.01

\* Significant at p=0.05 and \*\* Significant at p=0.01

APPENDIX XII: Combined effect of seed protectants and bioagents on Pea (Azad-P-1) inoculated with pea *Rhizobium leguminosarum* under pot conditions during rabi 2001-02

Source of variation	df	MS							
		Av. No. of nodules/ plant		Av. weight of nodules at the initiation of flowering		Av. weight of plant at the initiation of flowering		Grain yield/ plant (g)	N <sub>2</sub> uptake mg/plant
		Fresh wet weight (mg)	Dry weight (mg)	Fresh wet weight (mg)	Dry weight (mg)	Fresh wet weight (mg)	Dry weight (mg)		
Within treatments	9	1.219**	5033.57**	31461.96**	168.07**	1.83**	17.33	625.74**	
Between treatments	29	0.084	51.50	71.56	15.20	0.01	6.23	14.97	

\* Significant at p=0.05 and \*\* Significant at p=0.01

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