Experimental

4.1 Pathogenicity test of *F. oxysporum* on different cultivars of soybean

Pathogenicity of *F. oxysporum* was tested on ten cultivars of soybean (Soymax, Bragg, Punjab1, 19-PK 466, PK-327, JS-2, UPSM-19, PK-564, 17-PK 472 and 18-PK-564). Inoculation technique and disease assessment have been described in detail under materials and methods. Results are presented in Table 1, Fig. 1 and Plate II (Figs. A&B). Disease assessment was carried out after 14 and 28 days of inoculation with *F. oxysporum.* Assessment was on the basis of percentage loss in dry weight of inoculated roots in relation to respective control as well as on the colour intensity of infected roots. Results revealed that among all the cultivars tested UPSM-19 with a percentage loss of 75.2% was the most susceptible, followed closely by JS-2 with 73.4% loss in dry weight. Soymax was the most resistant followed by Bragg with percentage losses in dry weight of roots being 27.2 and 31.4 respectively. On the basis of significant test of the different cultivars in relation to the most susceptible and the most resistant, the cultivars could be broadly divided into resistant and susceptible types. Soymax, Bragg, 19-PK-466, PK-327 and Punjab-1 were the resistant group where the percentage loss in dry weight varied from 27.2% to 38.9% 28 days after inoculation. Colour intensity of infected roots of these groups did not show much variation, the maximum intensity being deep brown.The other cultivars i.e. JS-2, UPSM-19, PK-564, 17-PK 472 and 18-PK-564 were the susceptible ones and percentage loss in dry weight of roots varied from 62.2% (PK-564) to 75.2% (UPSM-19) ofter 28 days of inoculation. Colour intensity of the infected roots also varied from deep brown to black.

4.2. Cultural characteristics of microorganisms

Three microorganisms have been mainly used in this study. These are *Fusarium oxysporum, Bradyrhizobium japonicum* and *Trichoderma harzianum.* The cultural characteristics of these have been studied and are described below.

Table 1 : Pathogenicity test of *Fusarium oxysporum* on different cultivars of Soybean.

Cultivars	% loss in dry mass of roots (mg)*		Colour intensity of infected roots**		Root rot index plant ***	
	14 days	28 days	14 days	28 days	14 days	28 days
Soymax	14.1 ± 1.1	27.2[a] ± 2.1	-	+	0.25	0.50
Bragg	22.5 ± 1.4	31.4[a] ± 3.1	+	++	0.25	0.50
Punjab 1	25.2 ± 2.1	38.1[a] ± 1.2	-	+	0.25	0.50
19-PK-466	27.3 ± 1.7	38.9[a] ± 3.8	+	++	0.50	0.50
PK-327	28.5 ± 2.3	37.2[a] ± 2.5	-	+	0.50	0.50
JS-2	43.5 ± 3.4	73.4[b] ± 3.8	++	++++	0.50	0.75
UPSM-19	39.2 ± 2.2	75.2[b] ± 2.9	++	+++	0.50	0.75
PK-564	33.4 ± 2.8	62.2[b] ± 2.5	+	+++	0.50	0.75
17-PK-472	37.0 ± 0.98	63.5[b] ± 1.9	+	++	0.50	0.75
18-PK-564	34.8 ± 3.0	65.6[b] ± 3.5	+	++	0.50	0.75

* In relation to control on the basis of 3 separate trials of 10 plants each. Values with same superscript not significant at P = 0.01.

** + = light brown; ++ = deep brown, +++ = blackish brown ; ++++ = black.

*** Root rot index computed on the basis of % loss in dry weight of roots : 1-25% loss = 0.25 ; 26-50 % = 0.50 ; 5 -75% = 0.75 and 76 -100% = 1.

Plate II (figs. A & B) : A - Healthy and *F.oxysporum* infected plants of soybean (cv.JS-2); **B** - Portion of infected roots

Pathogenicity test of *F.oxysporum* on soybean cultivars

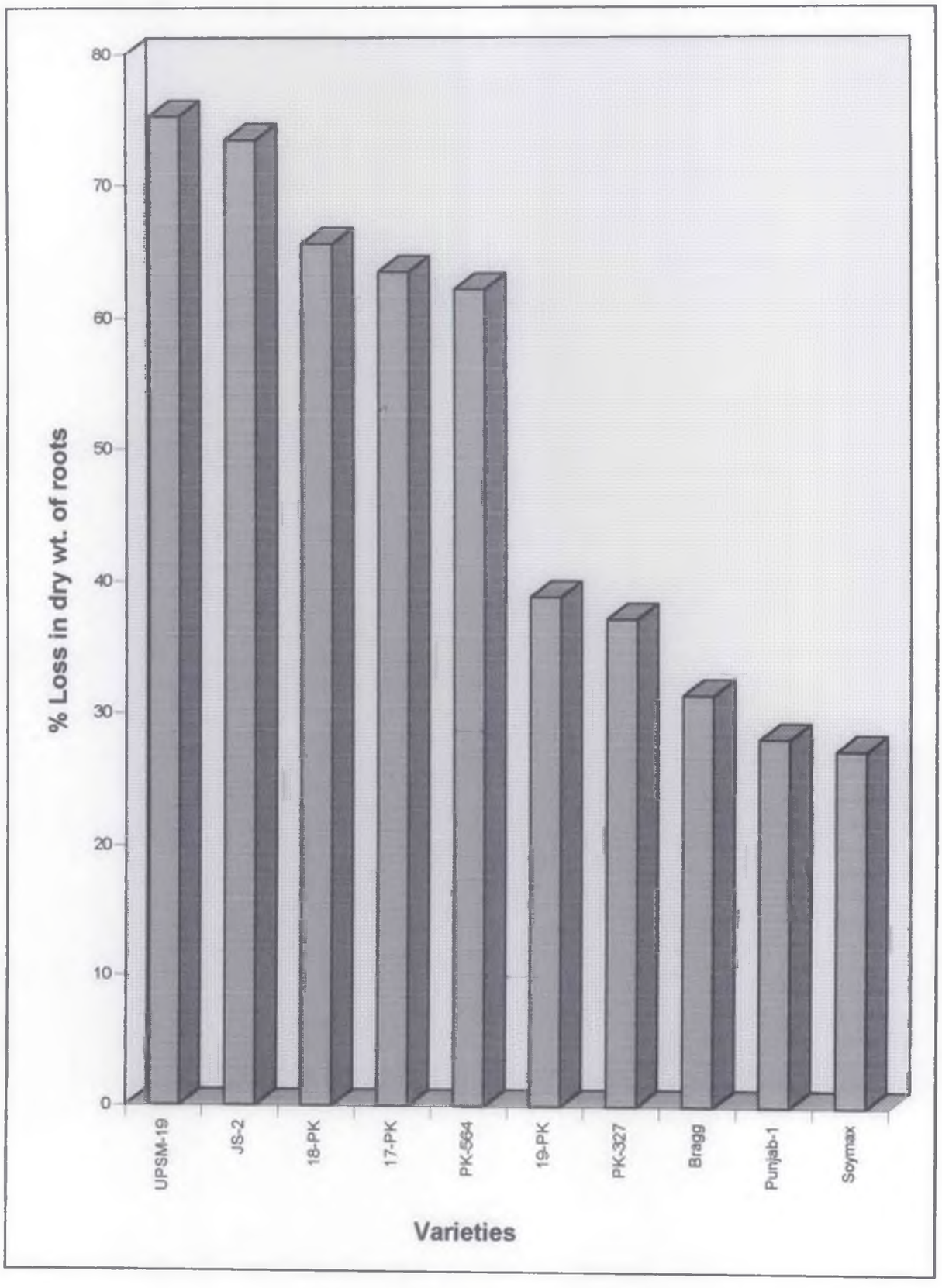


Fig.1

4.2.1. *F. oxysporum*

This is a fast growing fungus reaching 4-6 cm diameter within 4 days. Aerial mycelium sparse to abundant and floccose, becoming felted usually with a purple or violet tinge, more intense at the stromatic agar surface. Microconidia generally abundant, borne on sparsely branched conidiophores, never forming chains; not septate, ellipsoidal to cylindrical, 5-12x2-4 µm. Macroconidia fusiform, moderately curved, pointed at both ends, basal cells pedicillate, 3-4 septate, 20-60 x 3-5µm (Plate III, fig. A). Chlamydospores terminal or intercalary in hyphae.

Besides the general microscopic observations regarding morphology of the conidia, attempts were made to charecterize conidial wall by studying the agglutination effect of four lectins (Con A, HPA,UEA-1, WGA) on conidia. Agglutination procedure has been described in materials and methods. Agglutinations were examined under the light microscope and arbitrarily scaled from 0-4 (0= no agglutination; I=1-25% ; 2=26-50% ; 3-51-75% : and 4=76-100% agglutination.Microscopical examination revealed that conidia were strongly agglutinated by con A and HPA and to lesser degrees by WGA and UEA-1(Table 2&Plate III, figs. B-D). Strong agglutination with Con A suggest that surface components are glycoconjugates containing L -D glucopyranoside and / or L -D mannopyranoside residues, while agglutination with HPA suggest the presence of also glycoconjugates containing N-acetyl L -D galactosaminyl residues. Agglutination with WGA and UEA - 1 lectins which have affinity for N-acetyl L -D glucosamine and L- fucose suggested the presence of these sugar residues, though to a lesser extent on the conidial wall surface.

4.2.2. *B. japonicum*

This is a slow growing bacterium with slow and scanty growth, streak slightly raised, glistening, opaque white and grows well in medium containing mannitol. Gram negative rods varying in size from 3-5 µm in length and 1.5-3µm in breadth. Bacteroids present, sometimes branched forming T or V shaped structure.

Table 2 : Agglutination response of conidia of *F. oxysporum* to different lectins.

Name of lectins	Agglutination of conidia of *F. oxysporum* *
Concanavalin A (Con A)	4
Helix pomatia (HPA)	4
Ulex europaeus (UEA -1)	3
Wheat germ agglutinin (WGA)	2

* 0 = No agglutination; 0-25 = 1; 26-50 = 2 ; 51-75 = 3 ; 76.100 = 4.

4.2.3. *T. harzianum*

Fast growing hyaline colonies bearing repeatedly branched conidiophores in tufts with divergent, often irregularly bent, flask shaped phialides. These grow best in day light and in the dark they quickly loose the capacity to sporulate. Colonies reaching over 9 cm diameter in 5 days at 20°C . Conidia subglobose to short oval, measuring approximately 2.8-3.2 x 2.5x2.8 µm (Plate VI, fig. A)

4.3. Factors affecting growth

The effect of various cultural conditions on the growth of *F. oxysporum* and *B. japonicum* were tested, since the growth of microorganisms both *invitro* and in natural conditions is greatly influenced by different factors like temparature, pH of the sorrounding medium and available nutrients.

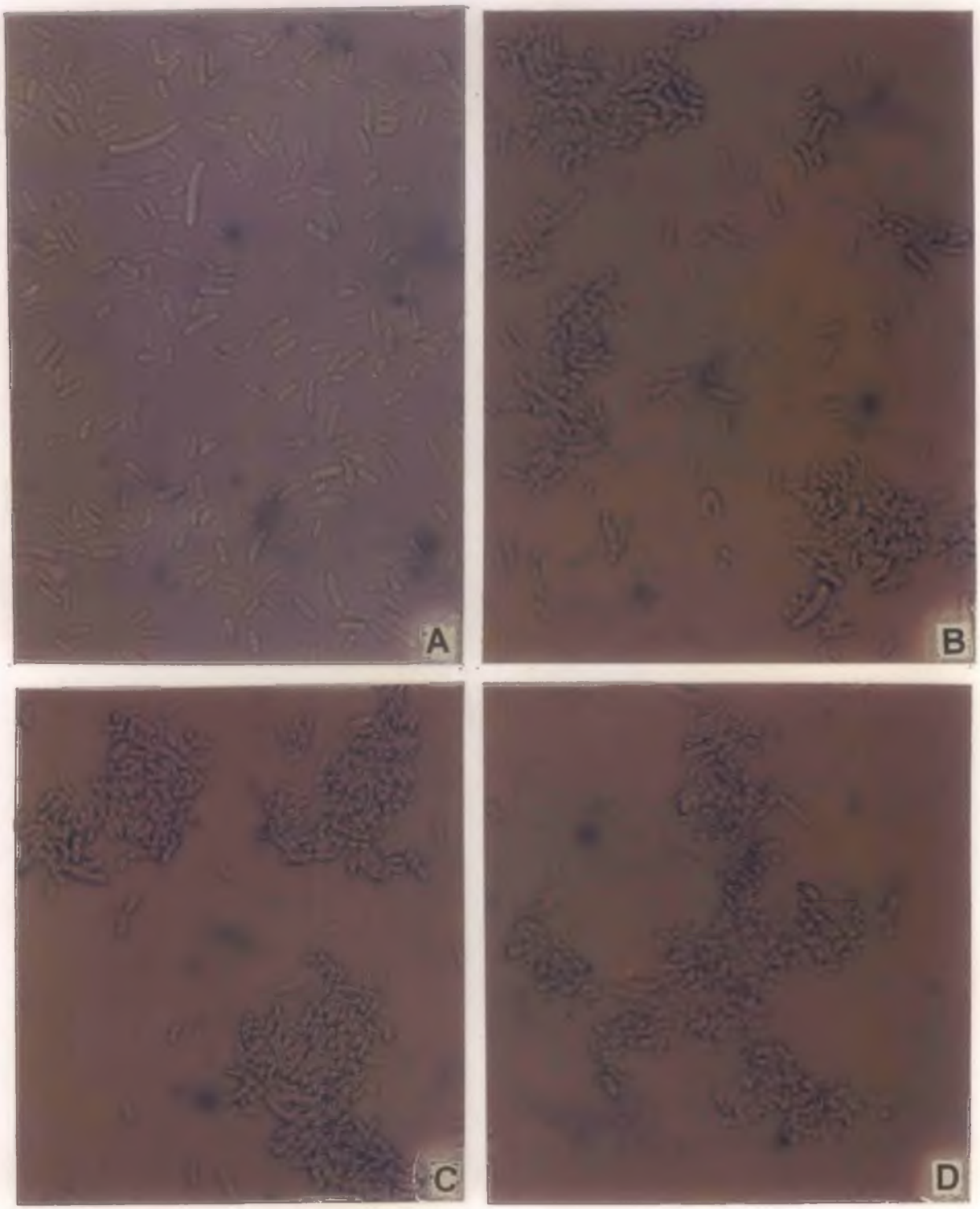


Plate III (figs. A-D) : Agglutination response of conidia of *F.oxysporum.* Control **(A)** ; After treatment with UEA-1 **(B)**, ConA **(C)** & HPA **(D)**

4.3.1. *F. oxysporum*

The growth and sporulation of *F. oxysporum* varies depending upon the available cultural conditions. The cultural conditions affecting the growth of *F. oxysporum* which were tested in this study are different media, incubation period, pH, different nitrogen and carbon sources.

4.3.1.1. Media

The growth of *F. oxysporum* was tested on a number of media, both solid and liquid and results were recorded.

4.3.1.1.1. Solid

F. oxysporum grew well in all the solid media tested (Potato dextrose agar, Richards agar, Yeast extract mannitol agar, Joffe's agar, Czapek dox agar and Carrot juice agar). Results (Table-3) revealed that two days after inoculation maximum growth was observed in PDA (25.3mm) and the least in Richards agar (17.3mm). This increased to 69.6 and 61.6 mm respectively after 6 days of growth while, after 8 days the mycelial growth in most of the media covered the petridish area (90mm). In Joffes agar medium the growth was submerged while in all other media, growth on the upper sufface of the petridish was white and fluffy. On the reverse side of the petridish a purplish colouration was observed after 8 days of growth in YEMA while black colour was observed in Czapek-dox agar.

4.3.1.1.2. Liquid

Mycelial growth was assessed in five different liquid media (Potato dextrose broth, Richards, Yeast extract mannitol, Joffes, and Czapek dox). Mycelial dry weights were determined after 6,9 and 12 days of growth. Maximum growth was obtained in Richards medium after 9 days of growth (581.3 mg dry weight) and minimum in Joffe's medium (Table-4). Czapek dox and potato dextrose broth also supported good growth while in yeast extract mannitol medium growth was rather suppressed. (Fig. 2).

Table 3 : Effect of different media on mycelial growth of *F. oxysporum*

Medium	Diameter of mycelia (mm)[a]			
	2 *	4 *	6 *	8 *
Potato dextrose agar	25.3 ± 0.72	51.6 ± 0.72	69.6 ± 0.98	90.0 ± 0.57
Richards agar	17.3 ± 0.72	43.6 ± 0.91	61.6 ± 1.26	90.0 ± 1.15
Yeast extract mannitol agar	23.6 ± 0.72	50.6 ± 1.18	68.0 ± 1.15	89.3 ± 0.27
Joff's agar	22.6 ± 0.54	46.3 ± 0.50	65.3 ± 0.98	90.0 ± 1.73
Czapek dox agar	21.3 ± 0.88	38.0 ± 2.31	58.3 ± 1.18	81.6 ± 0.72
Carrot juice agar	23.3 ± 0.54	49.6 ± 1.18	66.6 ± 0.54	86.3 ± 1.09

a Average of 3 replicates

* Days after inoculation

± Standard error.

4.3.1.2. Incubation period

F. oxysporum was grown in Richards medium for a period of 18 days. Mycelial growth was recorded after 3,6,9,12,15 and 18 days. Maximum Mycelial growth (553.0 mg dry weight) was recorded after 9 days of growth (Table 5, Fig. 3). After 3 days of growth mycelial dry weight was only approximately 52.0 mg which sharply increased to 413.5 mg after a further 3 days of growth. The reduction in growth after 9 days was gradual and after 18 days the growth was 276.5 mg dry weight. No sporulation was observed after 3 days growth while abundant sporulation appeared after 6 days.

Effect of different media on mycelial growth of *F.oxysporum*

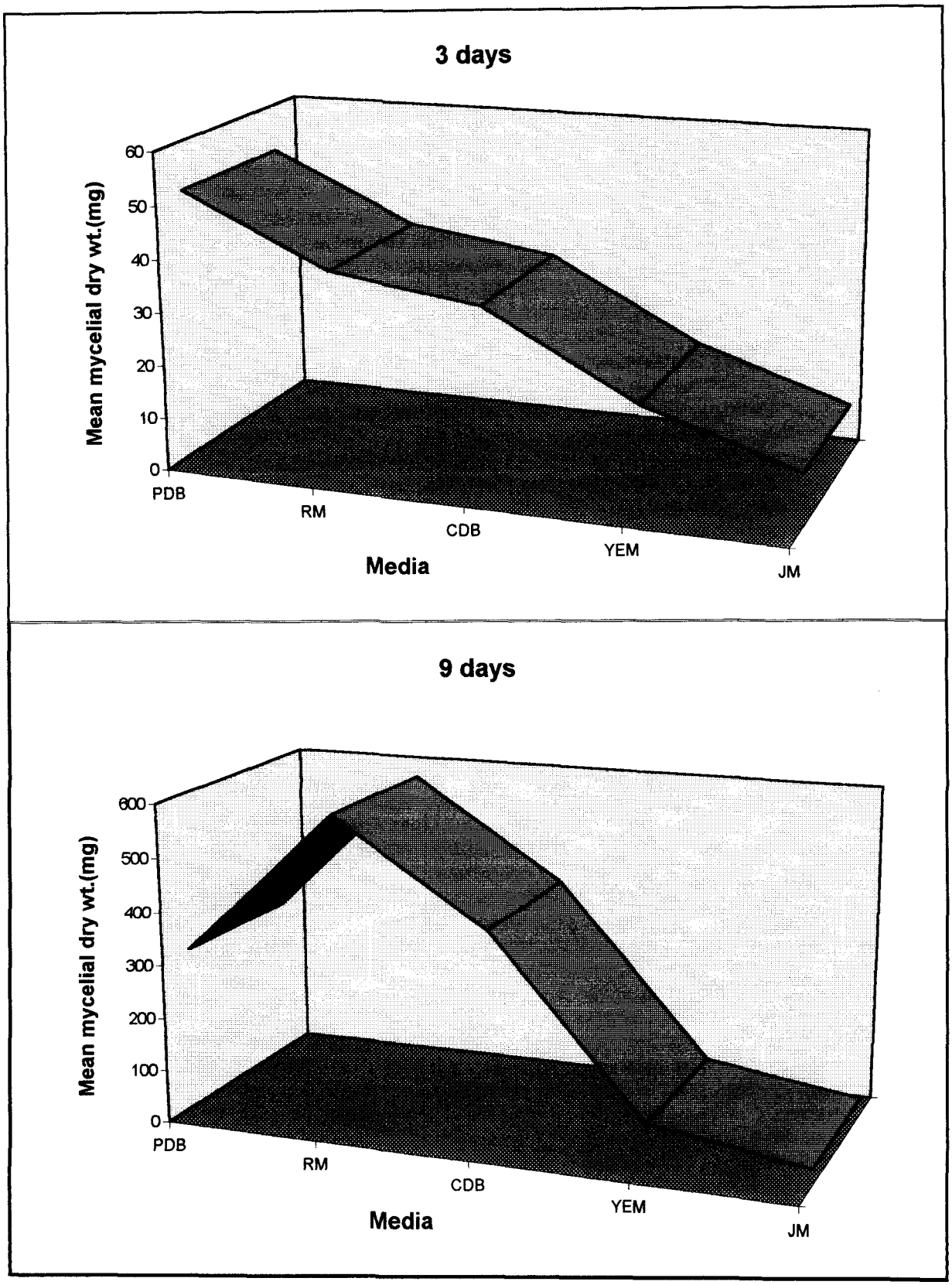


Fig.2

Table 4 : Effect of different liquid media on mycelial growth of *F. oxysporum*

Medium	Mean mycelial dry wt. (mg)[a]		
	6 *	9 *	12 *
Potato dextrose	253.6 ± 1.65	306.0 ± 2.16	260.0 ± 1.24
Richards	417.6 ± 1.18	581.3 ± 1.65	376.5 ± 2.60
Yeast extract Mannitol	60.3 ± 1.36	73.6 ± 2.22	52.5 ± 1.44
Joffe's	27.3 ± 0.78	30.3 ± 1.44	17.0 ± 1.82
Czapek dox	299.5 ± 2.60	396.3 ± 1.96	370.3 ± 0.78

a = Average of 3 replicates.

* = Days after inoculation.

± = Standard error

4.3.1.3. pH

The growth of all microorganisms are affected to a great degree by the pH of the medium in which they grow. To determine the effect of pH, buffer systems have to be used to stabilize the pH. In the present investigation buffer solutions with pH values ranging from 3 to 10 (3,4,5,6,7,8,9,10) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of M/30. The pH was finally adjusted using N/10 HCl or N/10 NaOH in each case. Double strength medium and the buffer was sterilized separately by autoclaving for 15 minutes at 15 lbs p.s.i. Equal parts of the buffer solution and the medium (RM) were mixed before use. Each flask containing 50 ml of the medium was then inoculated with mycelial block of *F. oxysporum* and incubated for 9 days at 28 ± 1°C. Results (Table-6) revealed that *F. oxysporum* grew to a lesser or greater extent

Effect of incubation period on growth of microorganisms

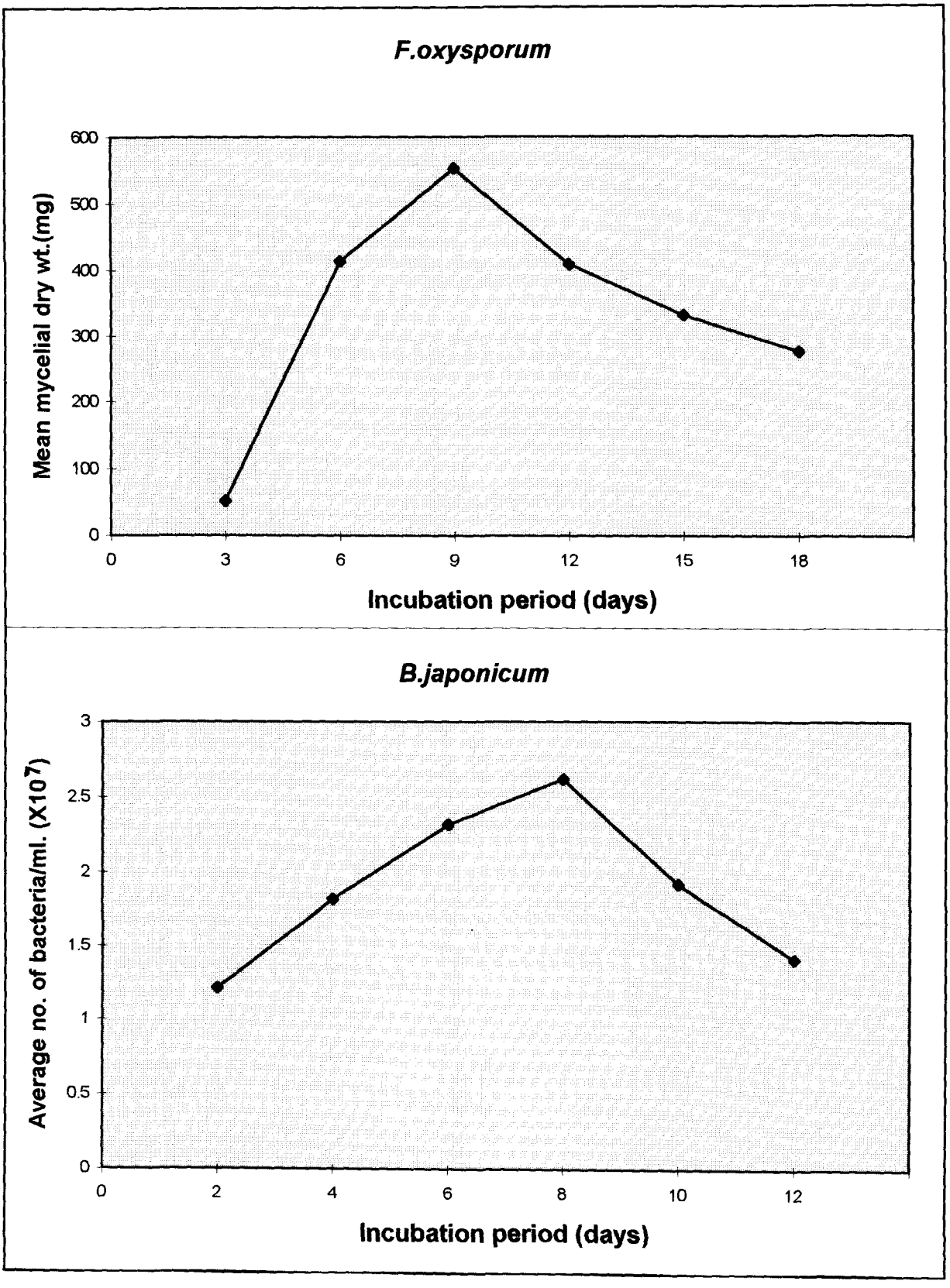


Fig.3

Table 5 : Effect of incubation period on Ithe growth of *F. oxysporum.*

Incubation time (Days)	Average dry wt. of mycelia (mg)*
3	51.6 ± 1.73
6	413.5 ± 1.15
9	553.0 ± 2.02
12	409.3 ± 1.96
15	331.6 ± 2.62
18	276.5 ± 1.51

* = Average of 3 replicates.

± = Standard error.

in all the pH tested, though maximum growth was recorded at pH 5 (642.5 mg dry weight).Minimum growth was recorded in pH3 followed by pH10.

4.3.1.4 Nitrogen Sources

The growth of any organism is dependent on the available nutrition and nitrogen is undoubtedly the single most important nutrient. The availability of nitrogen depends to a great degree on the form in which it is supplied. Thus the most suitable nitrogen source for any particular microorganism can only be determined by testing a number of sources including both inorganic and organic. In this study the effect of different nitrogen sources (both inorganic and organic) on the mycelial growth of *F. oxysporum* was tested. Basal Richards medium without any nitrogen source was considered as control. Mycelial dry wt was determined after 6,9 and 12 days of inoculation. Results (Table -7) revealed that among all the sources tested, both inorganic and organic, yeast extract was the best source followed by peptone. Organic sources were in general better source than inorganic ones. Minimum growth was evident when ammonium sulphate was used as nitrogen source. In the basal medium without nitrogen insignificant growth was recorded.

Table 6 : Effect of different pH on mycelial growth of *F. oxysporum.*

pH of medium[a]	Mean mycelial dry wt (mg)*
3	287.5 ± 2.60
4	530.0 ± 4.62
5	642.5 ± 3.75
6	606.0 ± 1.15
7	557.3 ± 4.06
8	525.3 ± 3.31
9	428.5 ± 2.08
10	391.0 ± 2.31

[a] Richards medium

* Average of 3 replicates

± Standard error.

4.3.1.5. Carbon sources

The ability of fungi to grow in different media depends on their capacity to utilise the available nutients of which carbohydrates are the major ones. All carbohydrates are not utilized by the fungus in the same rate and so the growth rate varies with different carbon sources. Eight different carbon sources were used in the present investigation, keeping Richard's medium as the basal. Data were recorded after 6, 9 and 12 days of inoculation. Among the different sources, dextrose was the optimum source while lactose supported minimum growth (Table - 8). All other sources supported moderately good growth. Growth without any carbohydrate was minimum.

Table 7 : Effect of different nitrogen sources on mycelial growth of *F. oxysporum*

Nitrogen sources	Mycelial dry wt. (mg)*		
	6 days	9 days	12 days
Inorganic			
Potassium nitrate	439.5 ± 2.02	604.5 ± 1.44	417.0 ± 1.71
Calcium nitrate	250.0 ± 4.92	536.6 ± 4.40	481.3 ± 1.97
Sodium nitrate	276.0 ± 6.34	349.0 ± 6,02	307.0 ± 5.20
Ammonium sulphate	71.0 ± 1.70	108.0 ± 4.04	82.3 ± 1.91
Organic			
Yeast extract	658.0 ± 2.62	1.009 ± 2.56	681.0 ± 2.87
Peptone	279.0 ± 5.40	951.6 ± 4.84	771.3 ± 6.44
Beef extract	260.3 ± 3.60	862.3 ± 1.79	623.0 ± 1.97
Casein acid hydrolysate	433.3 ± 1.52	836.6 ± 3.23	749.0 ± 3.46
Control (without nitrogen)	10.8 ± 0.98	50.6 ± 0.72	41.2 ± 1.02

[a] Basal medium - Richards.

* Average of 3 replicates.

± Standard error.

4.3.2. *B. japonicum*

Effect of various factors on the growth of *B japonicum invitro* were also determined in order to ascertain the optimum growth conditions. The various factors tested in this investigation include incubation period, pH as well as nitrogen and carbon sources in the medium. Growth of bacteria was assessed on the basis of OD values as described in materials and methods.

Table 8 : Effect of different carbon sources on mycelial growth of *F. oxysporum.*

Carbon sources[a]	Mycelial dry wt (mg)*		
	6 days	9 days	12 days
Sucrose	426.5 ± 0.86	547.0 ± 3.46	413.5 ± 3.17
Dextrose	688.0 ± 1.73	904.0 ± 3.46	560.5 ± 2.02
Fructose	556.5 ± 3.75	723.0 ± 2.89	650.0 ± 0.57
Lactose	55.3 ± 0.98	155.0 ± 3.46	88.0 ± 2.62
Sorbose	208.0 ± 1.73	596.5 ± 1.44	464.5 ± 2.60
Starch	297.0 ± 0.57	468.0 ± 2.94	409.6 ± 1.51
Mannitol	184.5 ± 0.86	702.3 ± 1.96	547.6 ± 3.31
Sorbitol	179.5 ± 3.17	617.0 ± 1.15	505.0 ± 2.89
Control (without sugar)	-	-	-

[a] Basal medium - Richards

* Average of 3 replicates

± Standard error.

4.3.2.1. Incubation Period

The bacterium was allowed to grow in yeast extract mannitol broth and growth measurements were done at every two day intervals, since this was a slow growing bacterium. Results presented in Table 9 and Fig. 3 revealed that growth increased for a period of upto 8 days after which it declined. The average number of bacteria /ml after 8 days of growth was more than double that of two days growth.

4.3.2.2. pH

It is well known that the pH of the medium usually plays on important role in the growth of microorganisms. The utilization of nutrients depends partially upon the pH of the culture medium. Hence in the present investigation the

Table 9 : Effect of different incubation period on the growth of *Bradyrhizobium japonicum.*

Incubation time (Days)	Average number of bacteria / ml.
2	$1.21x10^7$
4	$1.82x10^7$
6	$2.32x10^7$
8	$2.62x10^7$
10	$1.92x10^7$
12	$1.41x10^7$

effect of different pH was also studied on the growth of *B. japonicum.* The pH range varied from 3 to 10. It was observed that *B. Japonicum* grew best at pH 7 and also in pH 6 while the growth in pH either above or below this range was lesser. There was a sharp decline in growth at pH 10 while at pH 3 also the bacterium grew to a moderate extent. (Table 10)

4.3.2.3. Nitrogen Sources

The growth of *B. japonicum* in media with different nitrogen sources was tested, using yeast extract mannitol broth as the basal medium. Maximum growth was obtained with either yeast extract or casein acid hydrolysate as the nitrogen source. Among the inorganic sources calcium nitrate supported maximum growth followed by ammonium sulphate (Table 11)

Table 10 : Effect of different pH on the growth of *B. japonicum.*

pH of medium[a]	Average number of bacteria / ml
3	$2.32x10^7$
4	$2.48x10^7$
5	$2.62x10^7$
6	$2.69x10^7$
7	$2.79x10^7$
8	$1.73x10^7$
9	$1.37x10^7$
10	$8.09x10^7$

a Yeast Extract Mannitol

Incubation period - 8 days.

4.3.2.4. Carbon Sources

Eight different carbohydrates were tested to study their effect on the growth of *B. japonicum.* Among the tested carbohydrates mannitol supported maximum growth followed by sorbitol while minimum growth took place in fructose and sorbose. (Table -12). The results seen to indicate that the most easily utilizable sugars for this bacterium are the sugar alcohols (mannitol and sorbitol).

4.4. Effect of pre-inoculation on disease development.

The rhizosphere of a plant does not contain any single organism but rather, a large number of micro-organisms interact in the rhizosphere which finally determines how the particular host responds to a pathogen. In the present investigation it was considered worthwhite to study how two microorganisms i.e. *B. japonicum* and *T. harzianum,* both beneficial to the plant affect the development of root root disease caused by *F. oxysporum.* The effects were studied both singly as well as jointly.

Table 11 : Effect of different nitrogen sources on the growth of *B. japonicum*

Nitrogen sources[a]	Average number of bacteria / ml.		
	6 days	9days	12 days
Inorganic			
Potassium nitrate	3.03×10^6	8.09×10^6	4.04×10^6
Calcium nitrate	9.10×10^6	2.02×10^7	8.09×10^6
Sodium nitrate	3.03×10^6	5.05×10^6	4.04×10^6
Ammonium sulphate	7.07×10^6	1.11×10^7	8.09×10^6
Organic			
Yeast extract	2.42×10^7	2.73×10^7	1.82×10^7
Peptone	1.11×10^7	2.32×10^7	1.31×10^7
Beef extract	1.31×10^7	2.12×10^7	1.51×10^7
Casein acid hydrolysate	2.32×10^7	2.73×10^7	1.71×10^7
Control (without nitrogen)	1.01×10^6	1.51×10^6	5.05×10^5

[a] Basal medium - Yeast extract mannitol

4.4.1. *B. japonicum*

In order to study the effect of preinoculation with *B. japonicum* on root rot disease, four sets of plants were grown in earthenware pots containing soil : sand mixture as described in materials and methods. These four sets included - (i) uninoculated seed sown in noninfested soil; (ii) uninoculated seeds grown in soil infested with *F. oxysporum* : (iii) seeds inoculated with *B. japonicum* and sown in noninfested soil and (iv) seeds inoculated with *B. japonicum* and sown in soil infested with *F. oxysporum*. All the sets were watered regularly and uprooted after 14 and 28 days following which disease assessment was made as described in materials and methods. Results are presented in Table 13 and 14, Fig. 4 and Plate IV (figs. A & D). Bacterization of seeds reduced disease intensity in all tested cultivars (Soymax, Bragg, Punjab 1, JS-2, UPSM-19 and

Table 12 : Effect of different carbon sources on the growth of *B. japonicum.*

Carbon sources[a]	Average number of bacteria / ml.		
	6 days	9 days	12 days
Sucrose	1.61×10^7	1.92×10^7	1.31×10^7
Dextrose	2.12×10^7	2.22×10^7	1.71×10^7
Fructose	4.04×10^6	5.05×10^6	1.01×10^6
Lactose	1.71×10^6	1.92×10^7	1.71×10^7
Sorbose	3.03×10^6	4.04×10^6	2.02×10^6
Starch	5.05×10^6	9.10×10^6	7.07×10^6
Mannitol	2.52×10^7	2.83×10^7	1.92×10^7
Sorbitol	1.82×10^7	2.32×10^7	1.71×10^7
Control (without carbon)	-	-	-

[a] Basal medium - Yeast extract mannitol

PK-564) both after 14 and 28 days of inoculation. The difference in all cultivars was significant at both 5% and 1% level after 28 days of inoculation.

Production of nodules in the bacterized seeds was affected to a certain degree when grown in presence of *F. oxysporum* as the number of nodules per plant registered a reduction in this case.

4.4.2. *T. harzianum*

T. harzianum is a commonly used biocontrol agent and in this study experiments were conducted to determine the effect of *T. harzianum* on root rot disease development. Both *T. harzianum* and *F. oxysporum* were inoculated in the soil as described in detail in materials and methods. Plants were uprooted and disease assessment was done as before. Results (Table 15 and Fig.5)

Effect of pre-inoculation with *B.japonicum* on soybean root rot

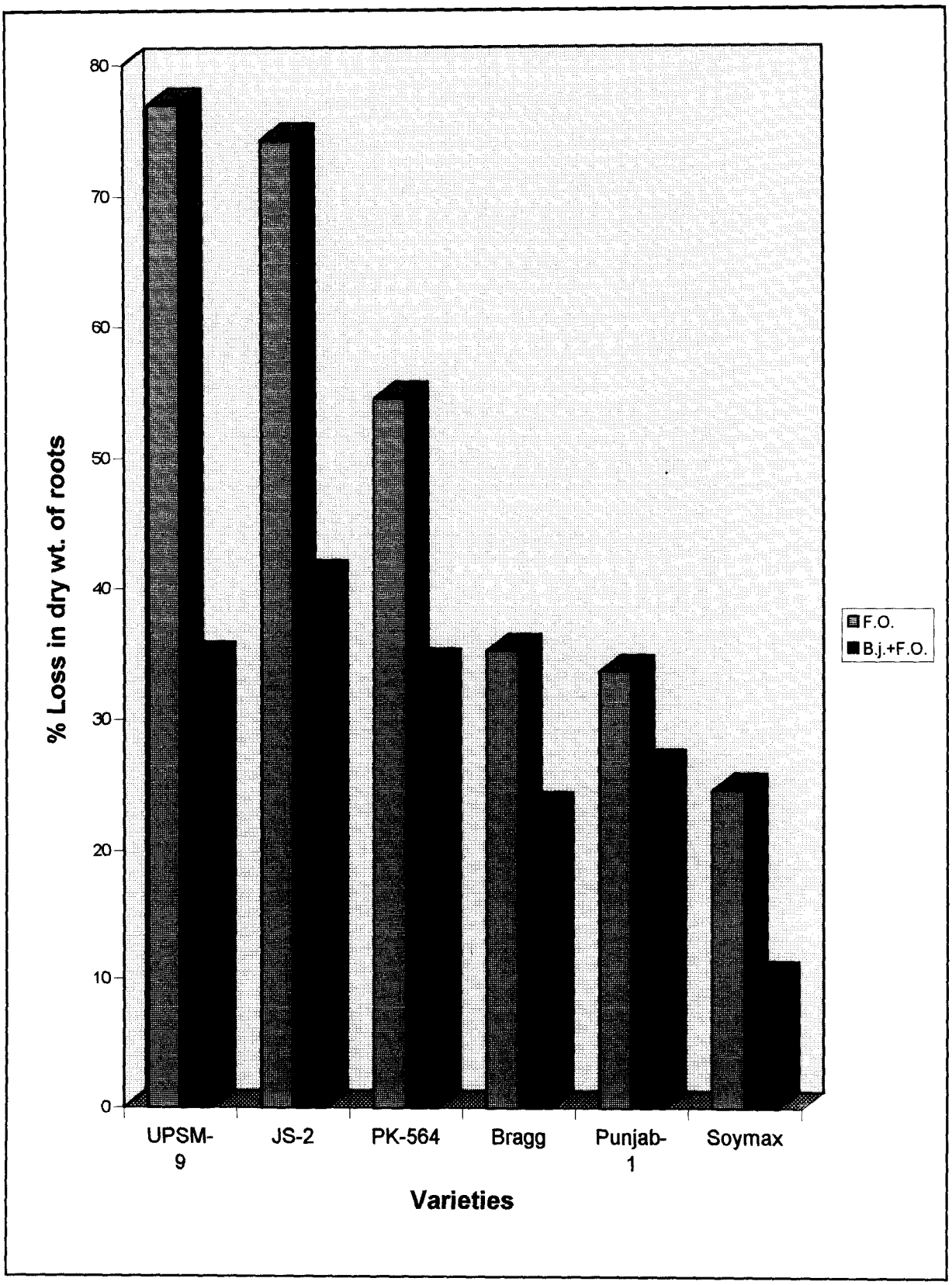


Fig.4

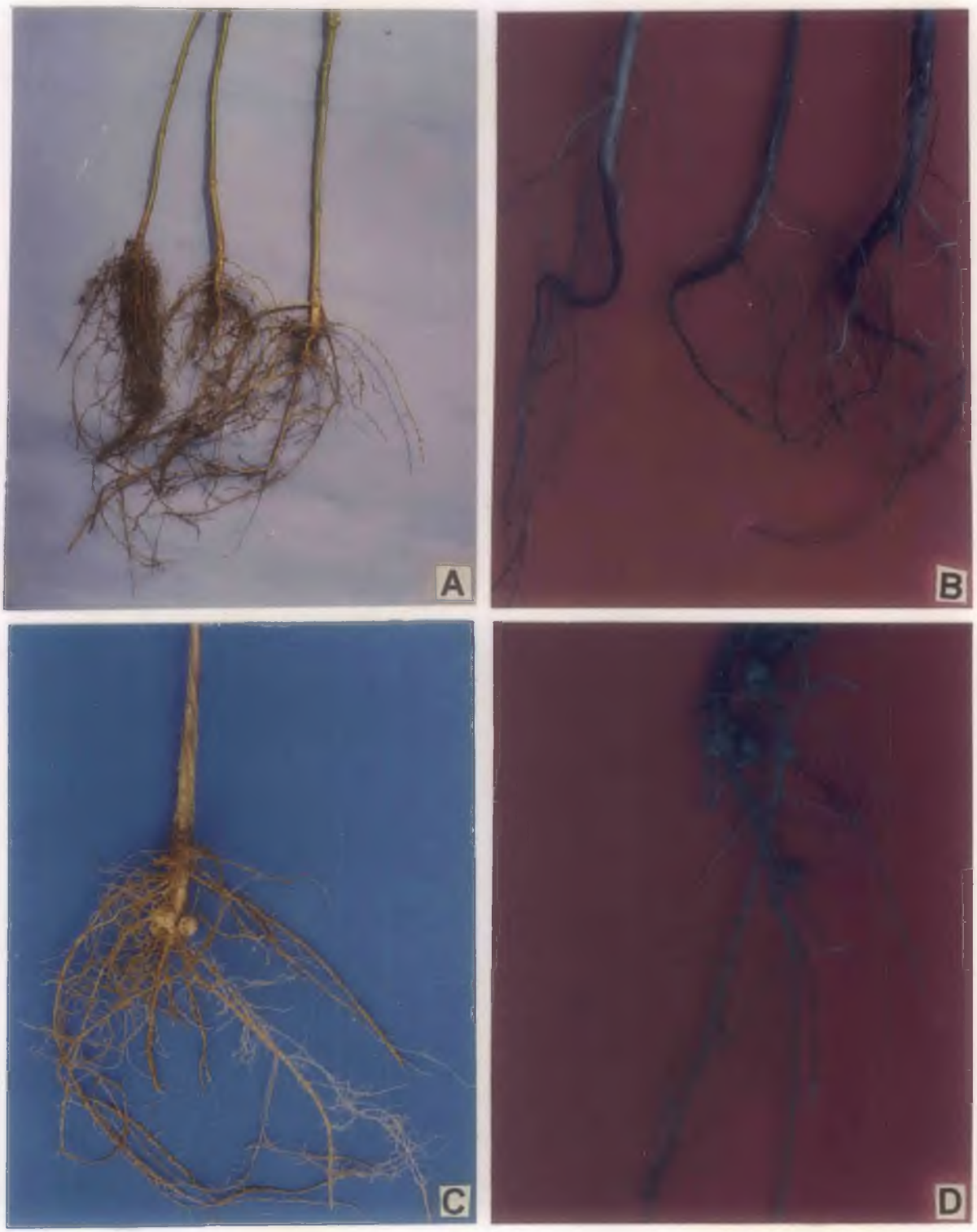


Plate IV (figs. A - D) : Portion of soybean roots . Healthy **(A)** ; Inoculated with *F. oxysporum* **(B)** , *B.japonicum* (**C**) & *B.japonicum* + *F.oxysporum* **(D)**

Table 13 : Effect of pre inoculation of soybean seeds with *Bradyrhizobium japonicum* on root rot disease development (14 days after inoculation)

Cultivar	Dry wt. of roots/plant*		% loss in dry wt.	Colour intensity*	Dry wt. of roots/plant*		%loss in drywt.	Colour intensity
	Healthy	Inoculated			*B.j*	*B.j +F.o*		
Soymax	130.3 ± 1.22	110.6 ± 0.72	15.12[a] ± 1.02	-	158.0 ± 1.24	140.3 ± 0.98	11.39[a] ± 0.82	-
Bragg	121.6 ± 1.65	89.3 ± 1.18	26.56[a] ± 1.18	+	137.0 ± 0.98	107.3 ± 1.90	21.68[a] ± 1.04	-
Punjab 1	114.0 ± 1.18	81.3 ± 1.44	28.69 ± 1.14	+	156.6 ± 1.15	119.3 ± 1.63	23.82 ± 1.81	-
JS-2	112.6 ± 1.65	66.6 ± 0.98	40.32[a,b] ± 1.89	++	128.0 ± 1.44	93.3 ± 1.96	27.11[a,b] ± 1.21	+
UPSM-19	129.3 ± 0.98	71.6 ± 0.98	44.49[a,b] ± 1.36	++	126.3 ± 1.65	96.3 ± 2.37	23.75[a,b] ± 1.45	+
PK-564	119.6 ± 1.44	78.3 ± 2.12	34.53 ± 1.89	+	146.3 ± 2.05	100.6 ± 2.12	31.24 ± 1.14	-

* In relation to control on the basis of 3 separate trials of 10 plants each.

** + - Light brown ; + + - Deep brown ; +++ - Blackish brown ++++- Black

a Difference between % loss in dry wt. due to *F. oxysporum* inoculation and due to *B. japonicum* inoculation significant at 5% level, and a, b = difference significant at 5% and 1% level in 't' test.

Table 14 : Effect of pre-inoculation of soybean seeds with *Bradyrhizobium japonicum* on root disease development (28 days after inoculation)

Cultivar	Dry wt. of roots/plant*		% loss in dry wt.	Colour* intensity	Dry wt. of roots/plant*		%loss in dry wt.	Colour inensity
	Healthy	Inoculated			*B.j*	*B.j +F.o*		
Soymax	213.0 ± 1.96	160.6 ± 2.59	24.60 ± 1.02	+	234.0 ± 2.16	210.3 ± 2.37	10.13 ± 1.04	-
Bragg	171.3 ± 1.18	111.6 ± 3.03	35.20 ± 1.25	++	204.3 ± 2.23	157.0 ± 2.83	23.15 ± 1.62	+
Punjab 1	169.0 ± 2.02	112.3 ± 1,65	33.55 ± 1.25	+	199.3 ± 1.96	146.6 ± 2.16	26.44 ± 1.14	-
JS-2	160.6 ± 3.42	41.3 ± 3.44	74.20 ± 2.51	++++	173.6 ± 2.37	102.6 ± 2.65	40.90 ± 2.05	++
UPSM-19	184.3 ± 2.59	42.6 ± 3.03	76.89 ± 2.14	+++	189.6 ± 2.67	124.0 ± 2.83	34.60 ± 2.18	+
PK-564	165.3 ± 2.32	75.3 ± 2.94	54.45 ± 1.89	+++	184.0 ± 1.90	121.3 ± 1.18	34.08 ± 2.24	+

* In relation to control on the basis of 3 separate trials of 10 plants each

** + - light brown; ++ Deep brown ; +++ Blackish brown ; ++++ - Black.

Difference in % loss in dry wt. due to inoculation with *F. oxysporum* and with *B. japonicum* + *F. oxysporum* significant at 1% level in 't' test in all cases.

revealed that disease intensity in both the cultivars tested (Soymax and JS-2) was reduced markedly by *T harzianum. T harzianum* also reduced nodulation since the number of nodules was lesser when plants were grown in presence of *T harzianum.*

Table 15 : Effect of *T. harzianum* on disease development by *F. oxysporum* in soybean (28 days after inoculation)

Cultivar	Treatment	Dry wt. of roots/plant (mg)*	Colour intensity **
	Healthy	265.0 ± 1.81	+
	T. harzianum	262.8 ± 2.04	+
Soymax	*F. oxysporum*	190.2 ± 1.30	++
	F. oxysporum + *T. harzianum*	216.3 ± 1.40	
	Healthy	271.3 ± 2.06	+
	T. harzianum	269.4 ± 2.10	+
JS-2	*F. oxysporum*	94.6 ± 1.08	+++ +++
	F. oxysporum + *T. harzianum*	203.8 ± 2.06	+

* Based on 3 separate trials of 10 plants each.

**+ - light brown; ++ - Dark brown : +++ - Blackish brwon ;

++++ - Black.

Effect of pre-inoculation with *T.harzianum* on soybean root rot

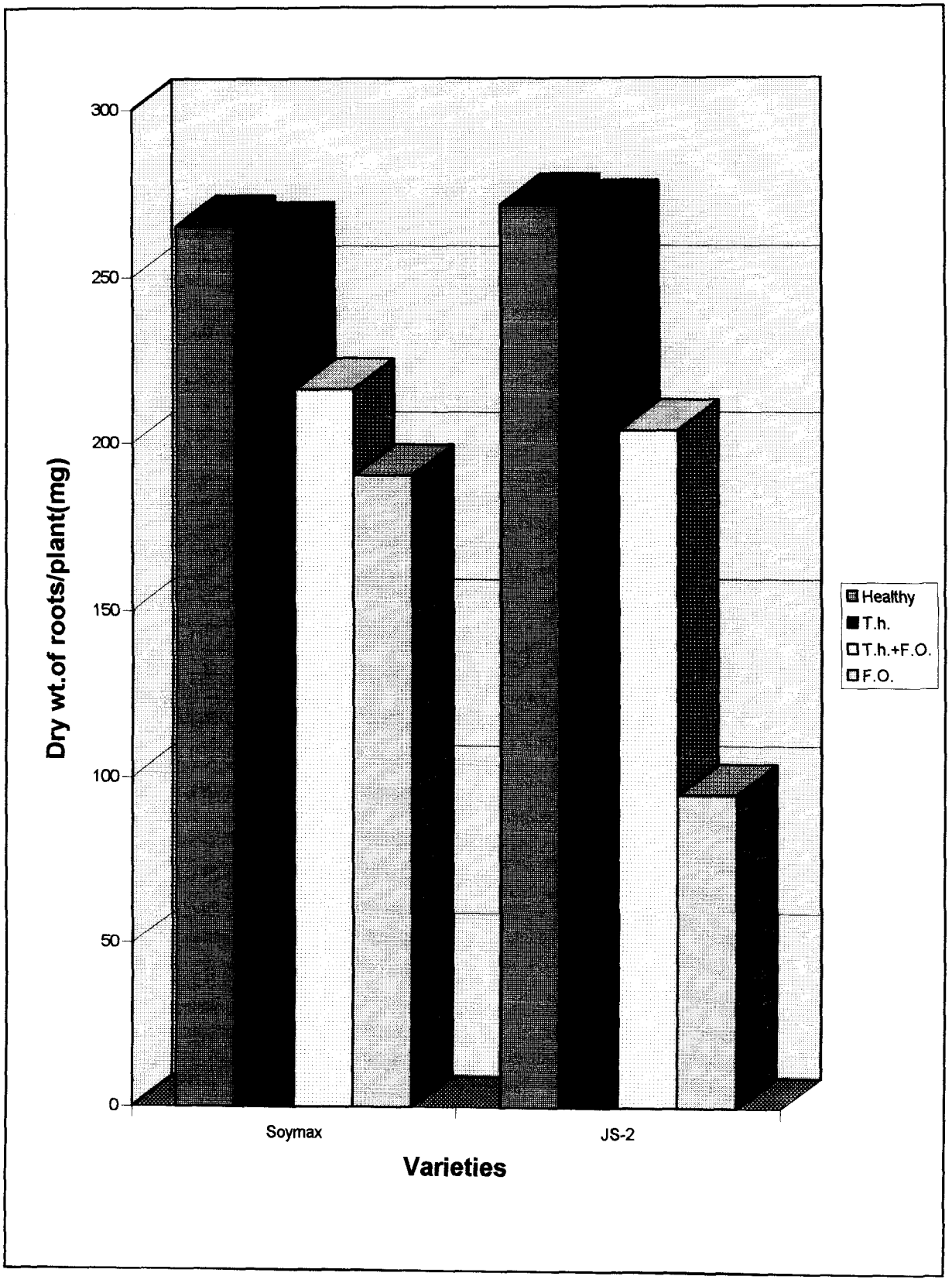


Fig.5

4.4.3. *B. japonicum* + *T. harzianum*

Since both *B. japonicum* and *T. harzianum* reduced disease intensity when treated separately, it was next decided to determine how joint inoculation with both of these microorganisms affect the development of root rot disease. For this, eight sets of plants were grown as follows, (i) uninoculated control, inoculated with (ii) *B. japonicum, (iii) T. harzianum ; (iv) B. japonicum + T. harzianum ; (v) F. oxysporum ; (vi) B. japonicum + F. oxysporum ; (vii) T. harzianum+ F. oxysporum (viii) B. japonicum + F. oxysporum + T. harzianum.* Assessment of disease revealed that though both *B. japonicum* and *T. harzianum* reduced disease intensity when treated separately, the effect of joint inoculation was hightly significant. Only about 4% loss in dry weight of roots was observed in joint inoculation as against 70% loss in dry weight when. *Fusarium* alone was inoculated (Table 16, Fig. 6). Inoculation with *B. japonicum* alone reduced percentage loss to a approximately 35% while *T. harzianum* reduced it to about 20%.

4.5. *Invitro* interaction studies

Results of the previous experiments indicated that both *B. japonicum* and *T. harzianum* reduced root rot disease intensity caused by *F. oxysporum.* This reduction in disease could be caused either due to inhibition of growth of the pathogen by the biocontrol agent or due to modification of biochemical changes in the host due to preinoculation with the respective organisms. Therefore in order to determine the mechamism by which disease reduction is brought about by *B. japonicum* and *T. harizianum,* a series of experiments were conducted. Initially *invitro* studies were carried out to determine whether any of the two organisms had antagonistic property.

4.5.1. *B. japonicum* and *F. oxysporum*

To determine the effect of *B. japonicum* on the growth of *F. oxysporum* and vice-versa, experiments were conducted in both solid and liquid medium.

Table 16 : Effect of *B. japonicum* and *T. harzianum* on disease development by *F. oxysporum* (cv. JS-2)

Treatment		Dry wt. of roots / plant (mg)*	Colour intensity**
Uninoculated	Control	252.6	-
Treated	*B. japonicum*	278.4	-
	T. harzianum	256.4	-
	B. japonicum + *T. harzianum*	259.1	-
Inoculated	*F. oxysporum*	80.0 (68.33)	++++
Treated	*B. japonicum* + *F. oxysporum*	182.9 (34.30)	++
	T. harzianum + *F. oxysporum*	204.2 (20.36)	++
	B. japonicum + *F. oxysporum* + *T. harzianum*	249.2 (3.82)	+

* Based on 3 separate trials of 10 plants each after 28 days of inoculation with *F. oxysporum*

** + - Light brown ; ++ - Dark brown ; +++ Blackish brown : ++++ Black

Figures in parenthesis indicate percentage loss in dry wt. of roots in relation to respective control.

Effect of *B.japonicum* and *T.harzianum* on soybean root rot

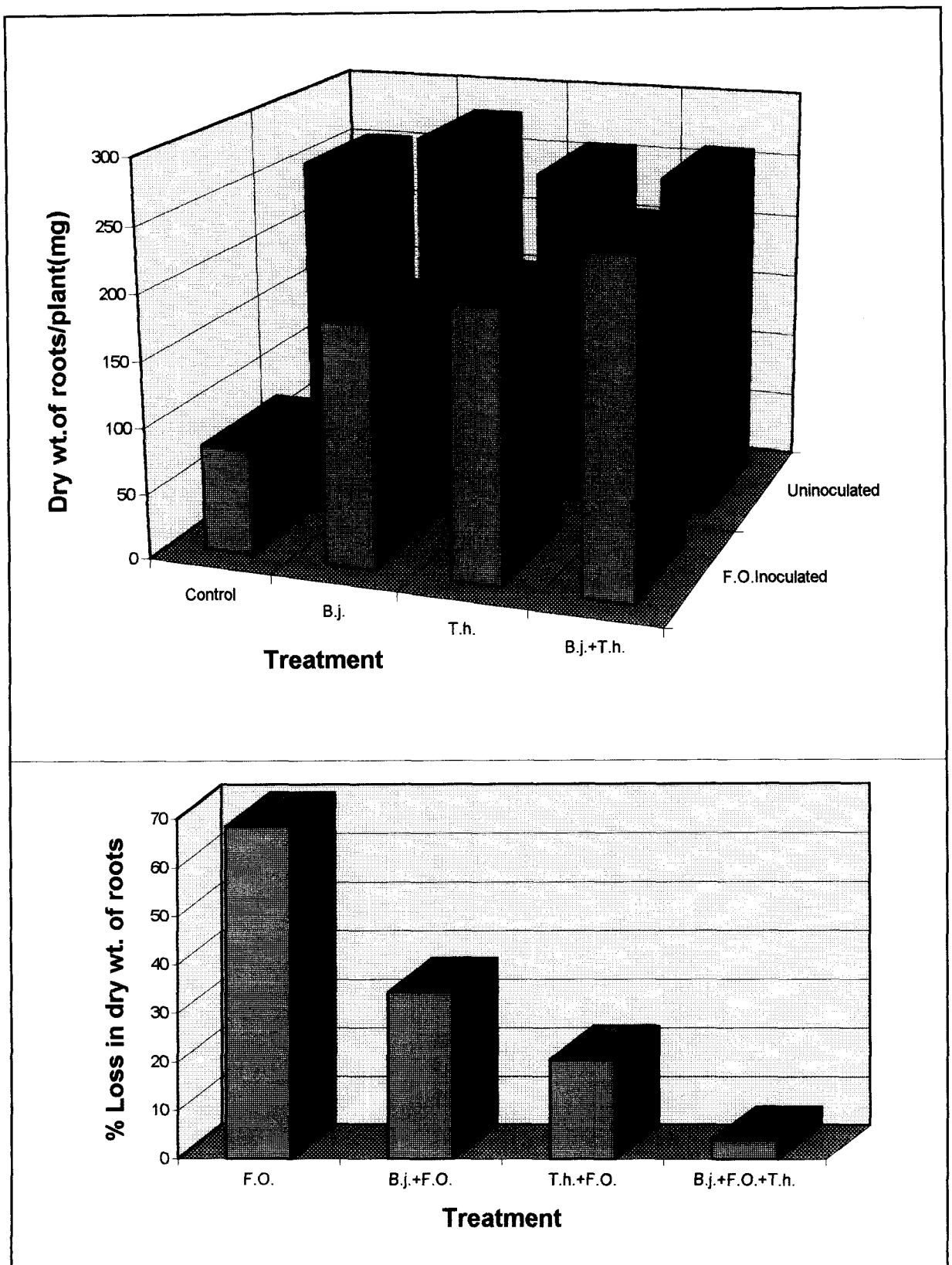


Fig.6

4.5.1.1. Solid medium

Pairing experiments were carried out with *B. japonicum* and *F. oxysporum* on YEMA. In the petridish (9 cm dia) a block of *F. oxysporum* was placed near one edge, while a streak of *B. japonicum* was made on the opposite edge. A block of *F. oxysporum* alone served as control. The petridishes were incubated for a period of 7 days after which observations were made. It was observed that *F. oxysporum* overgrew the bacterium and covered the petridish (Plate V, figs. A&B). This indicated that *B. japonicum* was not antagonistic to *F. oxysporum.*

4.5.1.2. Liquid medium

Interaction between *B. japonicum* and *F. oxysporum* was also studied in liquid culture. For this 50 ml YEM were taken in 250 ml Ehrlenmeyer flasks and inoculated either with a 4mm block of *F. oxysporum,* 0.5 ml of bacterial suspension ($1X10^7$ bacteria / ml) or both together. At 3 day intervals mycelial dry weight and bacterial growth were assessed as described in materials and methods. Results (Table 17) revealed that the growth of both organisms was inhibited to a slight degree in mixed culture. Growth of *F. oxysporum* was only reduced insignificantly whereas there was a greater reduction in growth of *B. japonicum.*

Invitro studies between *B. japonicum* and *F. oxysporum* therefore revealed that *B. japonicum* was not antagonistic to *F. oxysporum* and possibly reduced disease by changes in the host metabolism.

4.5.2. *T. harzianum* and *F. oxysporum*

Since T. hazianum significantly reduced root rot disease caused by *F. oxysporum invivo,* the effect of *T. harzianum* on the growth of the pathogen was determined *invitro.* Invitro interactions were studied on solid medium following which the effect of the culture filtrate of *T. harzianum* on the mycelial growth of *F. oxysporum* was also determined.

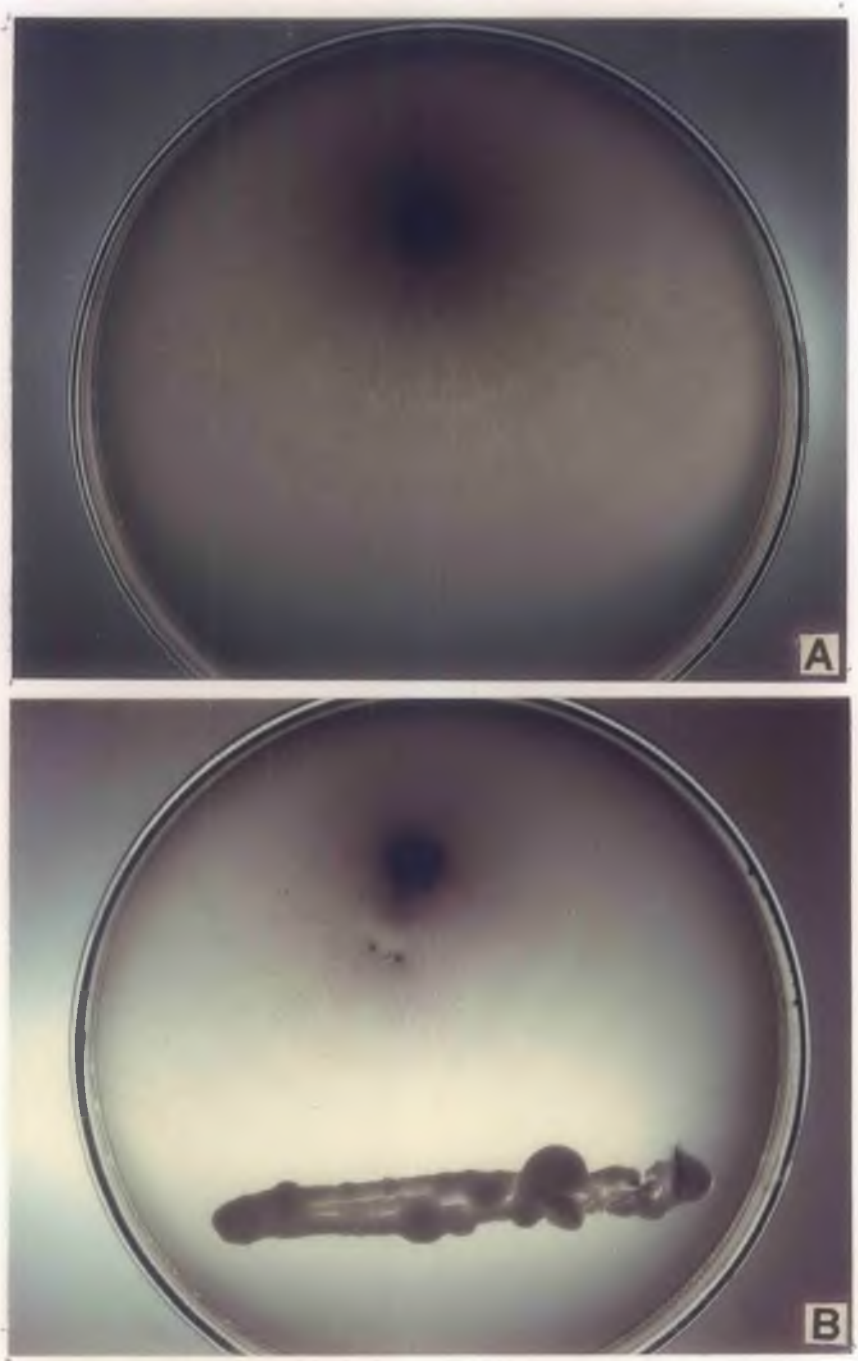


Plate V (figs. A & B) : Growth of *F.oxysporum* on YEMA.
A - Control ; **B** - Paired with *B. japonicum*

Table 17 : Interaction between *F. oxysporum* and *B. japonicum* in liquid culture.

Organism	Average dry wt. of mycelia (mg)**			Average no. of bacteria /ml		
	6*	9*	12*	6*	9*	12*
F. oxysporum	56.6 ± 0.98	71.6 ± 1.18	47.0 ± 1.24	-	-	-
B. japonicum	-	-	-	1.61×10^7	2.93×10^7	1.41×10^7
F. oxysporum + *B. japonicum*	49.6 ± 0.54	68.3 ± 1.18	43.3 ± 0.72	8.09×10^6	1.21×10^7	1.01×10^7

* Days after inoculation

** Mean mycelial dry wt. of 3 replicates

Growth medium - Yeast extract mannitol

± Standard error.

4.5.2.1. Solid medium

For this experiment, mycelial blocks of both *T. harzianum* and *F. oxysporum* were placed on PDA medium in petriplates. The two fungi were allowed to grow separately (control) as well as jointly. Observations were made after a period of 8 days and it was seen that *T. harzianum* inhibited the growth of *F. oxysporum* significantly (Plate VI, figs. B&C). *T. harzianum* overgrew *F. oxysporum*, thus inhibiting its growth. The growth and sporulation of *T. harzianum* was not affected by *F. oxysporum*.

4.5.2.2. Culture filtrate

As the growth of *F. oxysporum* was inhibited by *T. harzianum* it was decided to test whether *T. harzianum* secretes any antifungal compounds in culture. For this initially *T. harzianum* was grown in PDB for 6 days after which the culture filtrates were collected by harvesting and centrifugation. Half of the

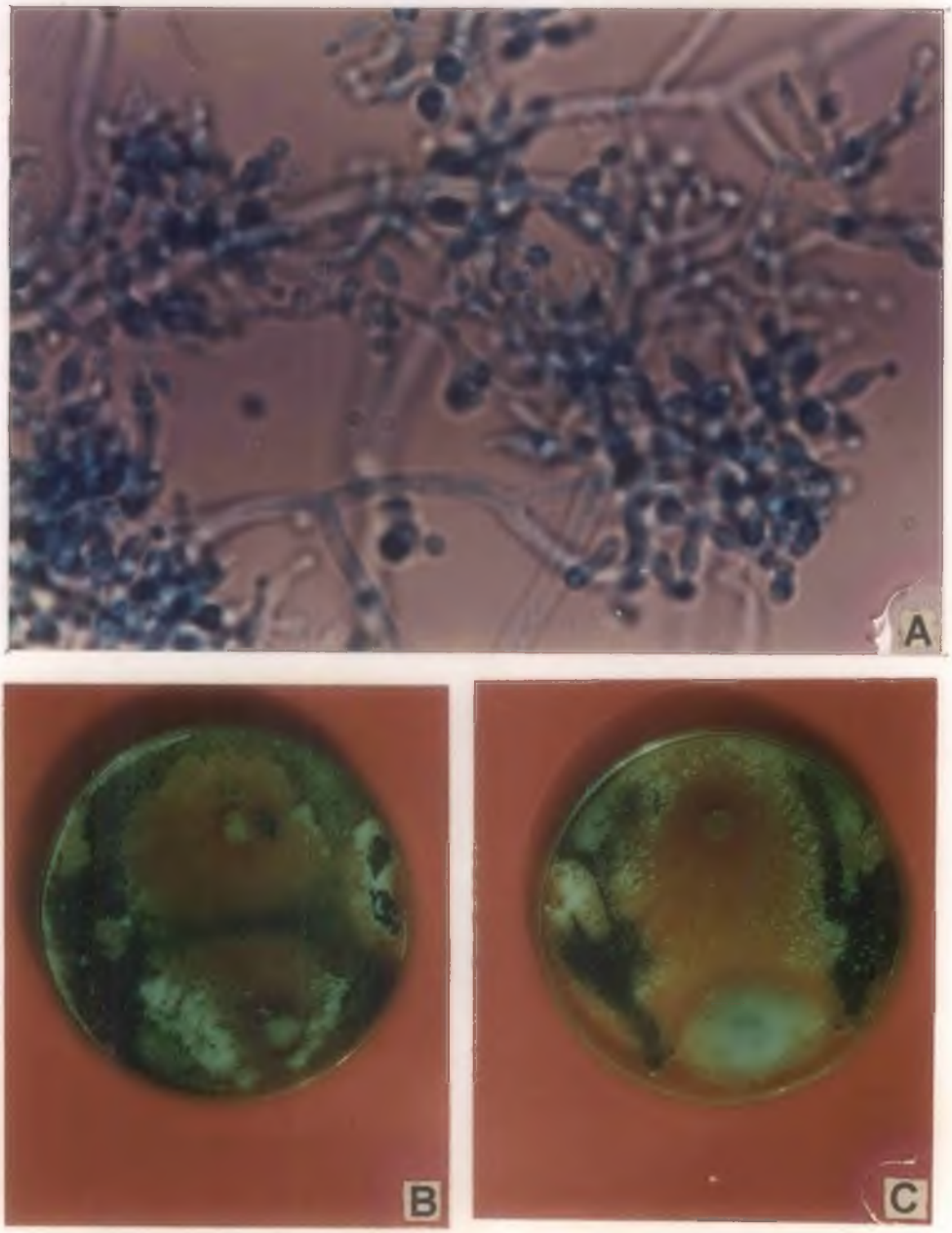


Plate VI (figs. A - C) : A - Mycelia and conidia of *T.harzianum*;
B & C - Pairing of *T.harzianum* and *F.oxysporum* on PDA;
B - *T.harzianum* alone; **C** - *T.harzianum* and *F.oxysporum*

culture filtrate was sterilized by autoclaving at 15 lbs p.s.i pressure for 15 minutes and the other half was sterilized by passing through a G5 bactofilter. Mycelial blocks of *F. oxysporum* was put into both outoclaved and non autoclaved culture filtrates and allowed to grow for a period of 9 days. *F. oxysporum* was also allowed to grow in fresh PDB which served as control. Results (Table 18) revealed that the culture filtrate of *T. harzianum* inhibited and growth of *F. oxysporum* to about 50%. The inhibition was evident in both autoclaved and cold sterilized culture filtrate. The antifungal principle secreted into culture by *T. harzianum,* therefore seems to be heat stable.

Results of *invitro* interaction studies between *F. oxysporum* and *T. harzianum* therefore revealed that *T. harzianum* was antagonistic to *F. oxysporum.* It probably reduced disease intensity by inhibiting the growth of the pathogen and not by bringing about any change in the biochemical mechanisms of the host.

Table 18 : Effect of culture filtrate of *T. harzianum* on the growth of *F. oxysporum.*

Treatment *	Average dry wt. of mycelia (mg)[a,b]
Control	539.6 ± 2.37
Cell free culture filtrate	
Autoclaved	377.6 ± 3.73
Unheated	369.0 ± 3.21

* Basal medium - Potato dextrose broth.

a Incubation period - 9 days

b Average of 3 replicates

± Standard error.

4.5.3. *B. japonicum* and *T. harzianum*

In the next phase of *invitro* interaction studies the interaction between *B.japonicum* and *T. harzianum* were also studied, since it was previously observed that joint inoculation with both *B. japonicum* and *T. harzianum* reduced disease significantly. Hence *invitro* studies were carried out both on solid as well as in liquid medium.

4.5.3.1. Solid medium

In paired experiments involving *T.harzianum* and *B. japonicum* both of these were allowed to grow on YEMA in petridishes as described previously. It was observed that within a period of 8 days *T. harzianum* overgrow the bacterial streak and covered the petriplates. (Plate VII, figs. A&B).

4.5.3.2. Liquid medium

T. harzianum and *B. japonicum* were inoculated into YEM jointly as well as separately as already described. Mycelial dry weights and bacterial growth were determined after 6,9 and 12 days of growth. Results (Table 19) showed that the growth of *T. harzianum* was only affected slightly by *B. japonicum* but the growth of *B. japonicum* was reduced to a greater extent by *T. harzianum.*

Thus, results of all *invitro* interaction studies between the three microorganisms clearly brings out the fact that while *T. harzianum* was antagonistic to *F. oxysporum, B. japonicum* was not antagonistic to either *F. oxysporum* or *T. harzianum.* The observed disease reduction by *T. harzianum* seems to be by its antagonistic effect on the pathogen while that of *B. japonicum* seems to be by other means, probably changes in the host metabolism. Hence further biochemical studies to determine the mechanism by which disease reduction occurs was carried out only with *B. japonicum* and no further tests were done with *T. harzianum.*

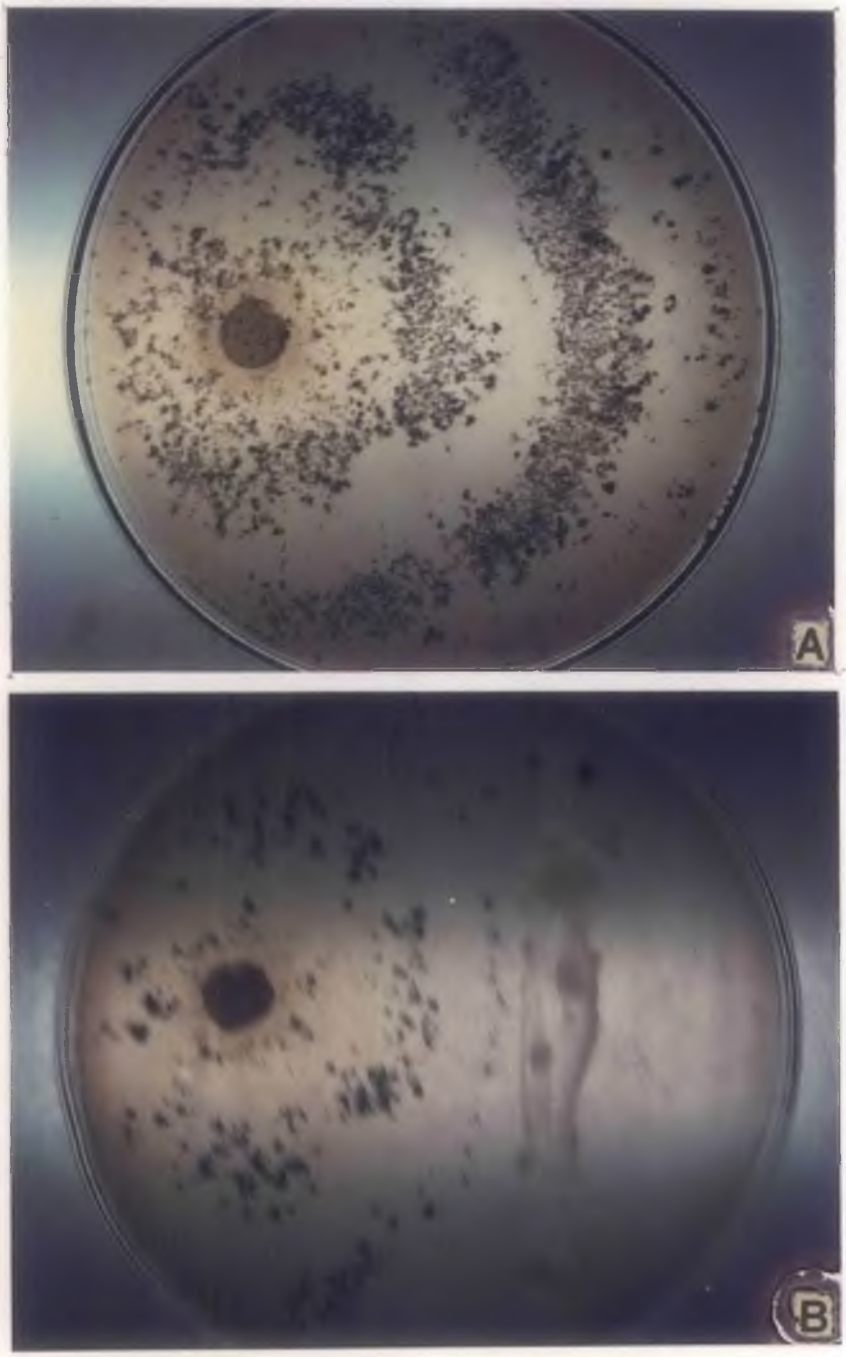


Plate VII (figs. A & B) : Growth of *T.harzianum* on YEMA;
A - Control ; **B** - Paired with *B.japonicum*

Table 19 : Interaction between *T. harzianum* and *B. japonicum* In liquid culture.

Organism	Average dry wt. of mycelia (mg)**			Average no. of bacteria /ml		
	6*	9*	12*	6*	9*	12*
T. harzianum	32.6 ± 1.18	47.3 ± 0.72	42.6 ± 0.27	-	-	-
B. japonicum	-	-	-	1.71×10^7	2.83×10^7	1.51×10^7
T. harzianum + *B. japonicum*	18.6 ± 0.54	38.3 ± 0.27	32.3 ± 0.98	6.06×10^6	1.11×10^7	9.10×10^6

* Days after inoculation

** Mean mycelial dry wt. of 3 replicates

Growth medium - Yeast extract mannitol

± Standard error.

4.6. Assay of phenylalanine ammonia lyase activity in soybean roots

Phenylalanine ammonia lyase was extracted from soybean roots at 4h intervals after inoculation with *F. oxysporum* and activity was assayed as described under materials and methods. For PAL assay two cultivars were selected, one resistant (Soymax) and other susceptible (JS-2) to *F. oxysporum*. Four sets of treatments were prepared i.e. healhy, inoculated with *F. oxysporum*, *B. japonium* and *B. japonicum* +*F. oxysporum*. Results are expressed as µg cinnamic acids produced by one gm root tissue /minute. Results are presented in Tables 20 and 21 and Figs. 7 & 8. It was observed that in general Soymax had higher PAL activity than JS-2 and following inoculation with *F. oxysporum* PAL activity increased from 4 h onwards till 16 hours of inoculation after which

it started declining. Inoculation with *B. japonicum* also increased PAL activity to some extent in relation to control but not as much as inoculation with *F. oxysporum.* In both cultivars, maximum increase in PAL activity was obtained following inoculation with *B. japonicum* + *F. oxysporum.*

Table 20 : Phenylalanine ammonia lyase (PAL) activity in soybean roots (cv. Soymax)

Time after inoculation with *F.o. (h)*	µg cinnamic acid produced / g / min*.			
	Healthy	Inoculated with *F.o.*	Inoculated with *B.j.*	Inoculated with *B.j* + *F.o*
4	2.97± 0.02	5.98 ± 0.01	3.90 ± 0.05	8.05 ± 0.05
8	3.08 ± 0.02	6.95 ± 0.18	3.43 ± 0.07	8.73 ± 0.07
12	3.20 ± 0.01	7.19 ± 0.52	4.21 ± 0.03	9.23 ± 0.02
16	2.98 ± 0.02	7.93 ± 0.09	4.18 ± 0.01	9.93 ± 0.23
20	3.01 ± 0.06	6.93 ± 0.02	4.21 ± 0.03	8.66 ± 0.23
24	2.98 ± 0.03	6.86 ± 0.07	4.16 ± 0.01	8.57 ± 0.05

* On the basis of 3 experimental sets.

± Standard error.

4.7. Assay of peroxidase activity in soybean roots.

Peroxidase is also one the enzymes generally involved in defence mechanism of a host. In this study therefore other than PAL, peroxidase activity was also assayed in soybean roots following inoculation with different microorganisms as already mentioned. Detailed procedure of extraction and

PAL activity in healthy and inoculated soybean roots (cv.Soymax)

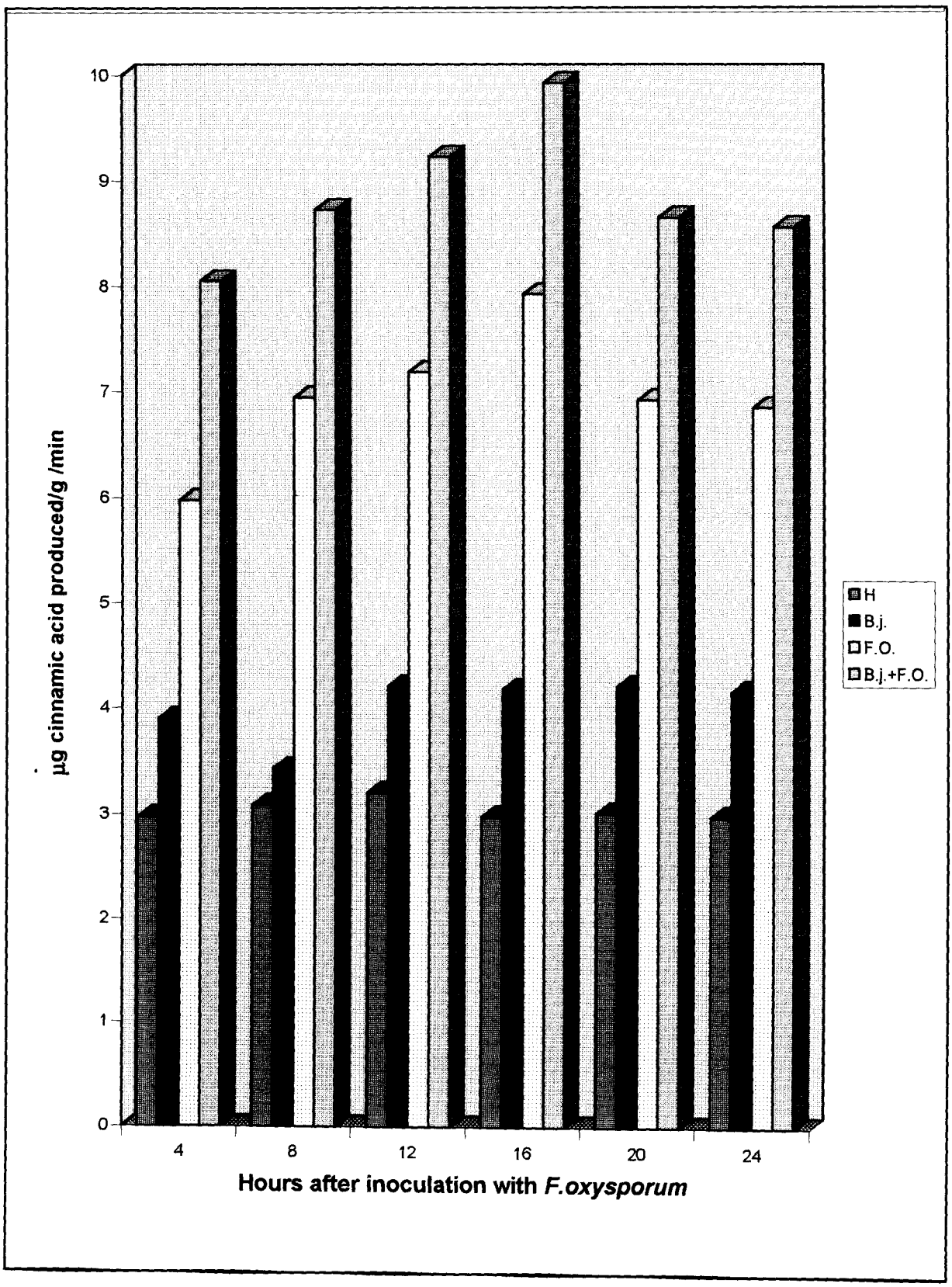


Fig.7

PAL activity in healthy and inoculated soybean roots (cv.JS-2)

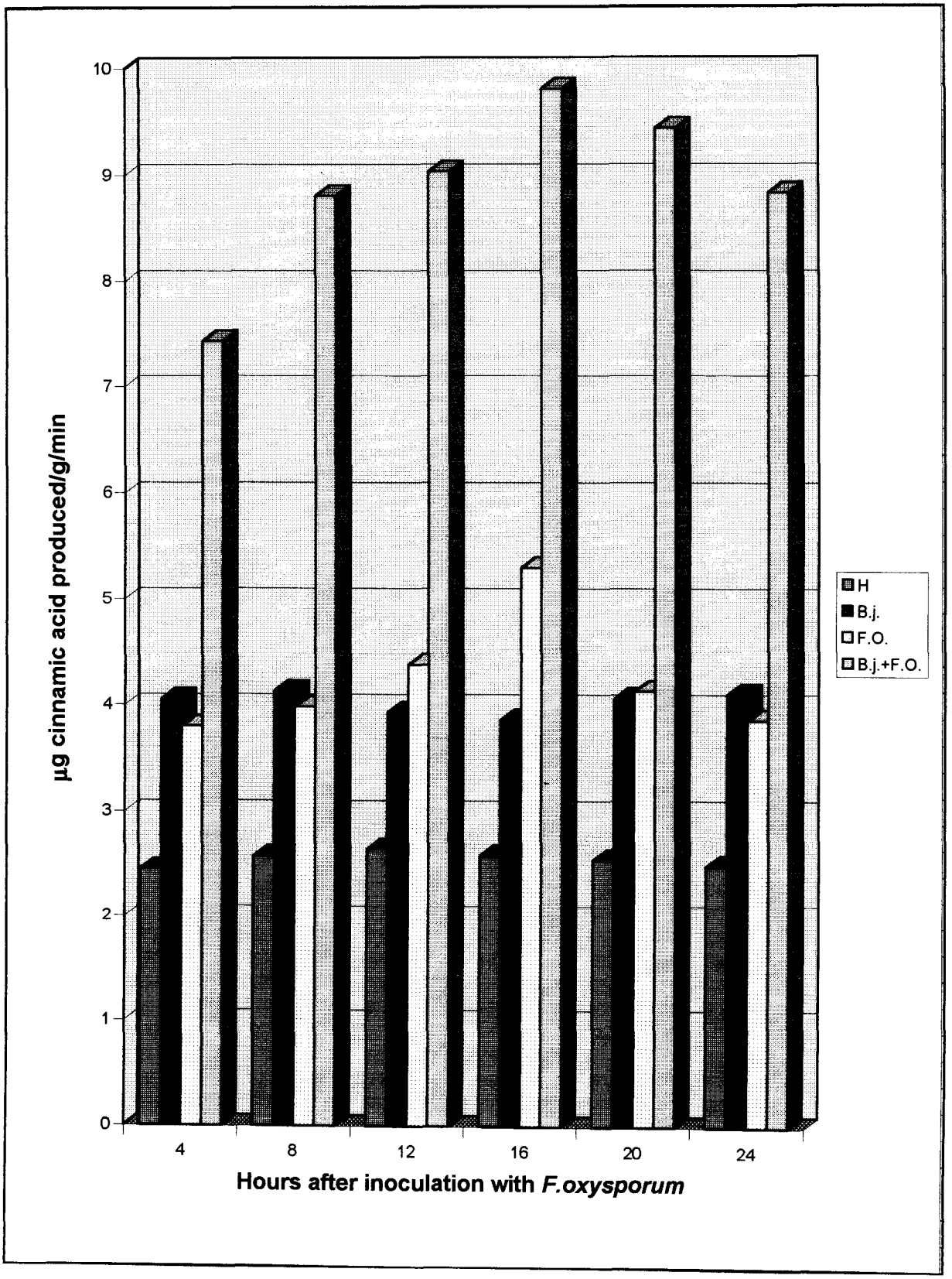


Fig.8

Table 21 : Phenylalanine ammonia lyase (PAL) activity in soybean roots (cv. JS-2)

Time after inoculation with *F.o. (h)*	µg cinnamic acid produced / g / min*			
	Healthy	Inoculated with *F.o.*	Inoculated with *B.j.*	Inoculated with *B.j + F.o*
4	2.45 ± 0.02	3.80 ± 0.05	4.05 ± 0.04	7.43 ± 0.07
8	2.57 ± 0.02	3.98 ± 0.03	4.13 ± 0.05	8.80 ± 0.05
12	3.64 ± 0.04	4.38 ± 0.03	3.93 ± 0.02	9.03 ± 0.10
16	2.58 ± 0.02	5.30 ± 0.05	3.86 ± 0.05	9.80 ± 0.16
20	3.54 ± 0.01	4.13 ± 0.02	4.06 ± 0.02	9.43 ± 0.07
24	2.48 ± 0.04	3.86 ± 0.02	4.10 ± 0.05	8.83 ± 0.09

* On the basis of 3 experimental sets.

± Standard error.

assay have been given in materials and methods. Peroxidase activity has been expressed as change in absorbance at 460 nm brought about by enzyme extract from one gram root tissue / minute. Results as presented in Tables 22 and 23 and Figs. 9 and 10 showed that maximum activity was obtained after 12h of inoculation with *F. oxysporum* in both the cultivars. Activities in both cultivars were more or less similar and inoculation with *B. japonicum* did not greatly affect the activity. Neither was the activity increased following inoculation with both *F. oxysporum* + *B. japonicum*.

Table 22 : Peroxidase activity In healthy and inoculated roots of soybean (cv. Soymax)

Time after noculation with *F.o. (h)*	Δ A 460 nm / g tissue / min			
	Healthy	Inoculated with *F. o*	Inoculated with *B. j*	Inoculated with *B.j + F.o*
4	6.92	6.03	6.84	5.75
8	6.21	6.09	6.17	6.10
12	6.72	11.63	6.92	7.96
16	7.15	8.15	7.00	8.92
20	6.35	6.70	6.40	7.14
24	5.66	5.62	5.42	4.92

Table 23 : Peroxidase activity in healthy and inoculated roots of soybean (cv.JS-2)

Time after inoculation with *F.o. (h)*	Δ O.D at 460 nm / g tissue / min			
	Healthy	Inoculated with *F. o*	Inoculated with *B. j*	Inoculated with *B.j + F.o*
4	7.30	7.20	7.50	7.45
8	6.93	6.85	7.08	6.84
12	6.75	12.80	7.02	9.64
16	7.42	9.25	7.36	8.54
20	7.22	7.68	7.46	7.02
24	6.86	6.58	6.90	6.74

Peroxidase activity in healthy and inoculated soybean roots (cv.Soymax)

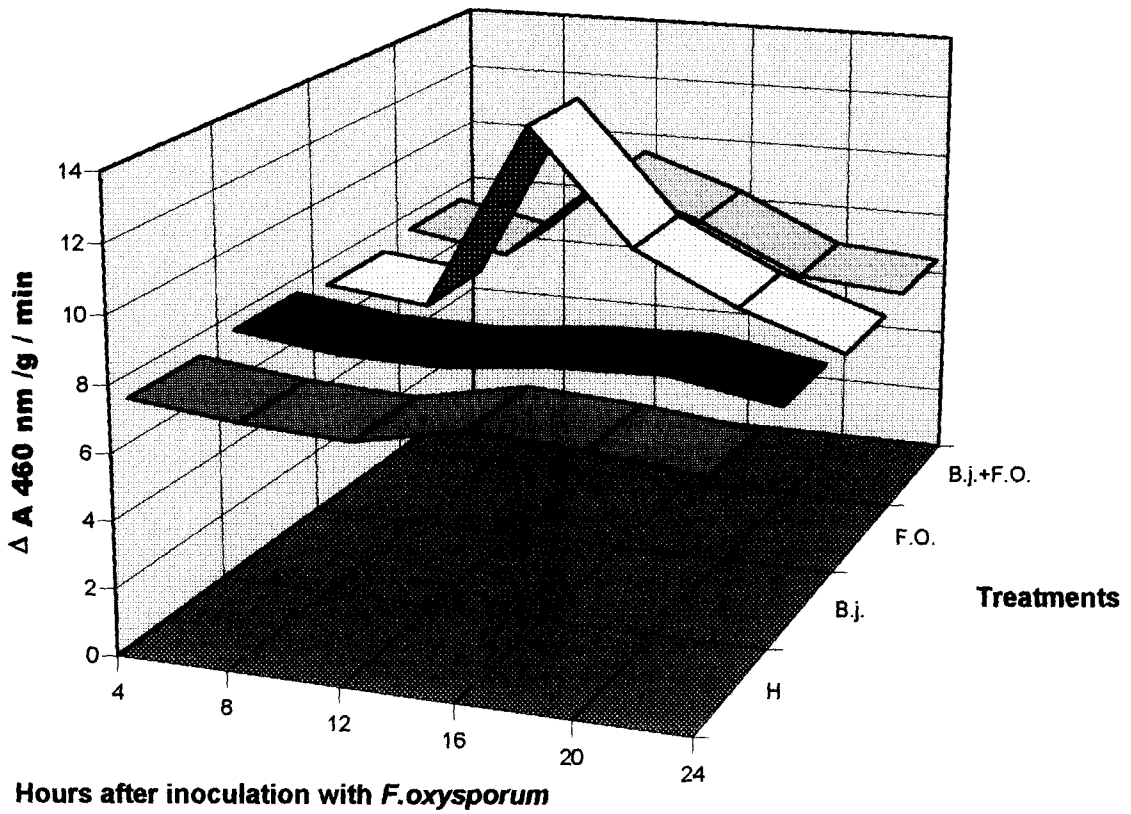


Fig.9

Peroxidase activity In healthy and inoculated soybean roots (cv.JS-2)

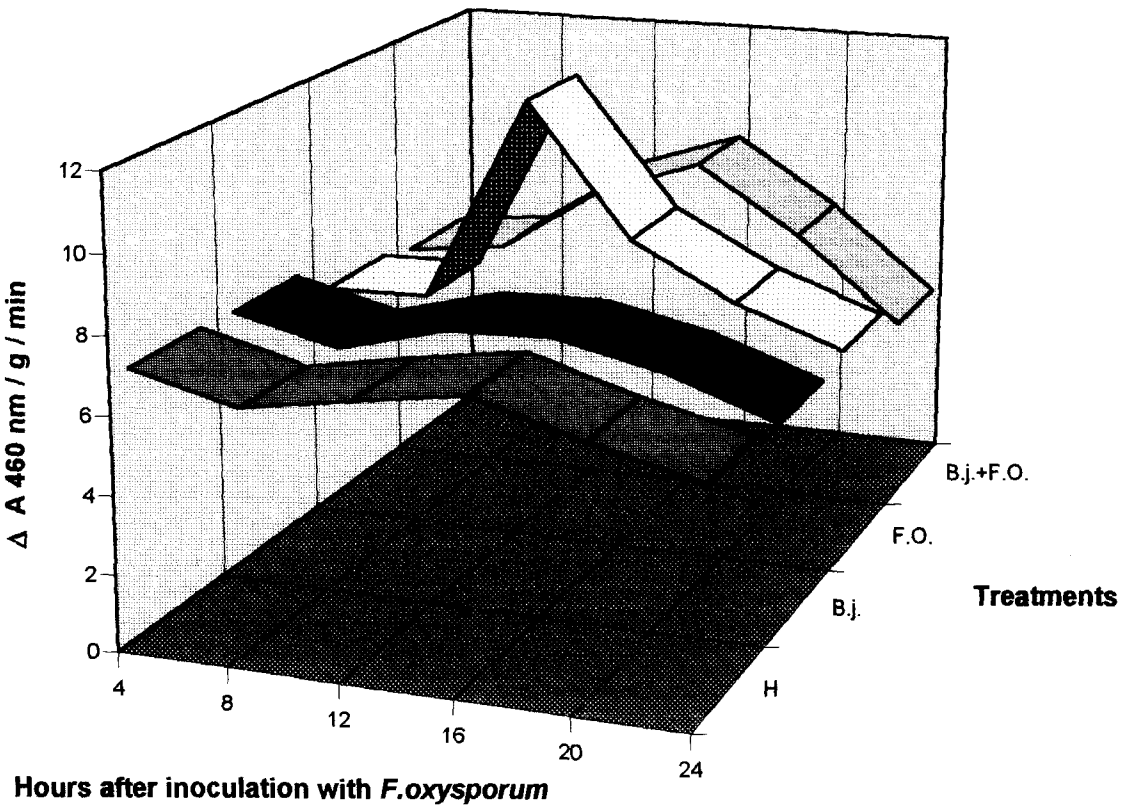


Fig.10

4.8. Glyceollin content

Among the biochemical changes in plants following inoculations with pathogen, accumulatin of phytoalexin is perhaps the most well documented (Bhattacharyya and Ward, 1986; Paxton, 1995; Avazkhodjaev et. at, 1995). Increased production of glyceollin (phytoalexin) has been reported in soybean following inoculation with a number of agents where resistant responses have been elicited. In this study therefore it was considered worthwhile to determine changes in glyceollin accumulation in a resistant and susceptible cultivar following inoculations with different microorganisms. Glyceollin extraction has been done from roots as well as from callus induced from cotyledons.

4.8.1. Roots

Glyceollin was extracted from roots of the two cultivars Soymax (resistant) and JS-2 (susceptible) after 24h of inoculation with *F. oxysporum.* Extraction procedure and quantification have already been described under materials and methods. Four treatments were considered in this case also. Glyceollin was initially detected on thin layer chromatograms on the basis of the appearance of yellow spots after spraying with p-nitroaniline. Glyceollin could only be detected in treatments with *F. oxysporum* and *B. japonicum* + *F. oxysporum*. Presence of glyceollin in the crude extracts of *F. oxysporum* as well as *B. japonicum* + *F. oxysporum* inoculated roots was further confirmed by petridish bioassay of th extracts. In petridishes where the medium was mixed with the above extracts inhibition in mycelial growth of *F. oxysporum* was obtained to about 75 %..

These extracts were further partially purified by thin layer chromatography and quantified from the absorbance values obtained at 286 nm. Results revealed that glyceollin content was much higher in the resistant roots following inoculation with *F. oxysporum* than in the susceptible ones (Table 24, Fig. 11). Further a marked increae in glyceoliin content was obtained following inoculation with both *B.japonicum* and *F. oxysporum.*

Table 24 : Glyceollin content of soybean roots after different treatments.

Treatment	Glyceollin content (µg / g fresh wt. of roots)	
	cv. Soymax	cv. JS-2
F. oxysporum	462 ± 5.8	250 ± 2.0
B. japonicum + *F. oxysporum*	541 ± 7.3	428 ± 5.5

Mean of 3 separate experiments
No glyceollin was detected in roots of healthy or *B. japonicum* inoculated plants.

4.8.2. Callus

Since the pathogen of root rot disease of soybean, *F. oxysporum* has also been reported as a latent infection in seeds (Sinclair, 1991) this created an interest to determine whether glyceollin accumulated in seed tissues following inoculation with *F. oxysporum* at rates comparable to those in roots. For this purpose initially calli were induced from cotyledons of cv. Soymax (Plate - VIII, figs. A&B) as described in materials and methods. About 2 mounth - old calli were inoculated with spore suspension of *F. oxysporum* and glyceollin was extracted from this tissue as already mentioned. The extracts were tested for the presence of glyceollin on both TLC and by petridish bioassay. Both of these confirmed the presence of glyceollin in the extracts after inoculation. Quantification of glyceollin content indicated that the cells of the callus accumulated glyceollin following inoculation at rates comparable to those in infected roots (450 µg / g tissue), while no glyceollin could be detected in healthy cells.

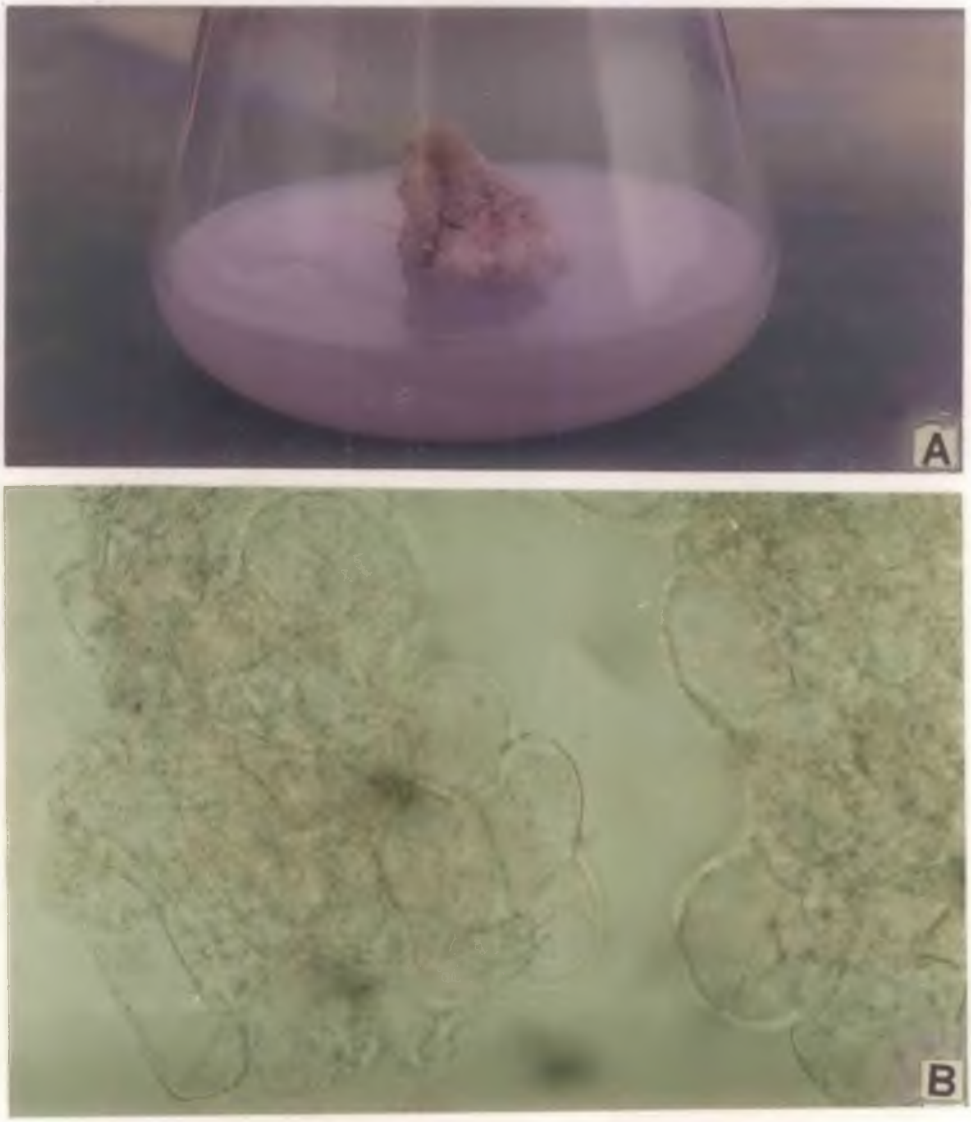


Plate VIII (figs. A & B) : A - Cotyledonary callus of soybean;
B - Cells from the callus

Glyceollin content of healthy and inoculated soybean roots

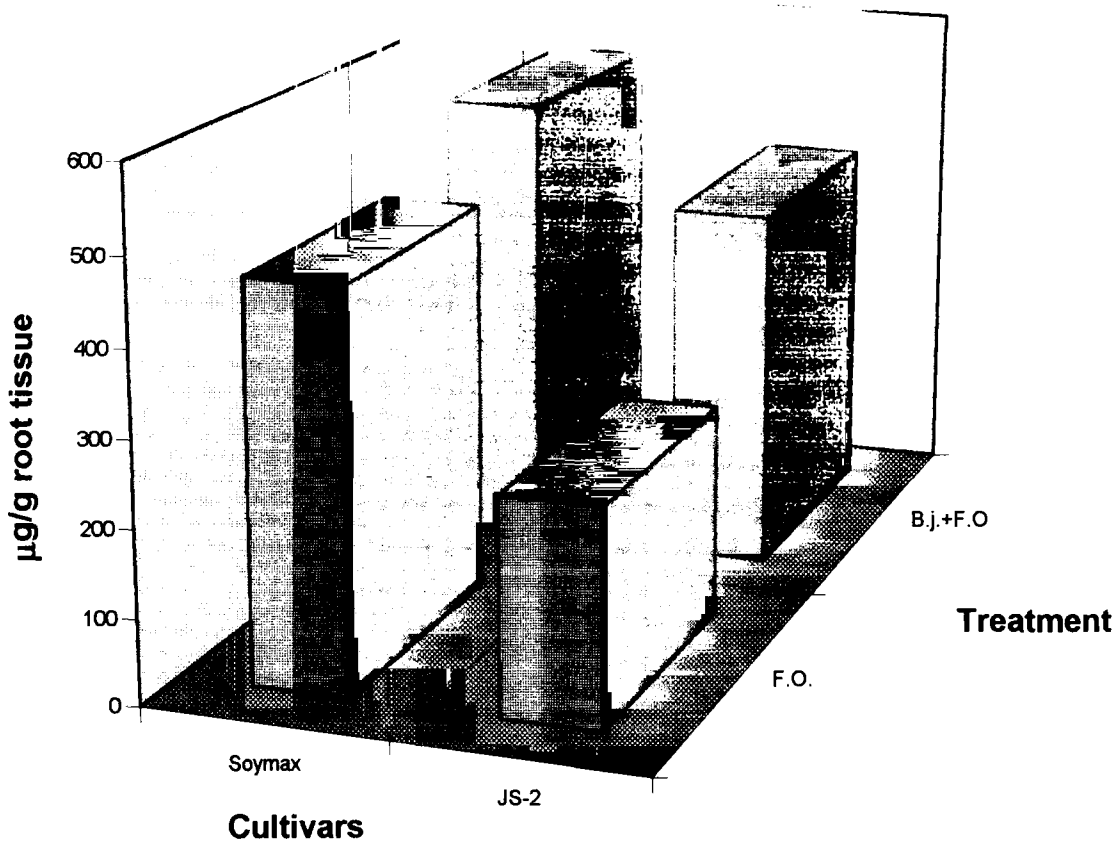


Fig.11

4.9. Analysis of proteins

Proteins are major biochemical components in all plants, Proteins are generally known to either increase or decrease due to infection by pathogen and more importantly their patterns may also change. Hence for a thorough investigation into the biochemical changes following inoculation, it was considered imperative to determine changes in the protein contents as well as protein patterns in soybean tissues. For this purpose a number of experiments were designed.

4.9.1. Protein content

Protein was extracted from the mycelia of *F. oxysporum,* soybean roots and soybean seeds and contents were estimated. Detailed procedures for extraction and estimation have been presented under materials and methods.

4.9.1.1. *F. oxysporum*

Estimation of mycelial protein content revealed that *F. oxysporum* had protein content of 2.37 mg / g/ fresh weight tissue.

4.9.1.2. Soybean roots

Proteins were extracted from roots of both Soymax (resistant) and UPSM-19 (susceptible) and estimated. Susceptible cultivar had higher protein content then the resistant cultivar while in both cultivars inoculation with either *B. japonicum*, *F. oxysporum* or *B. japonicum* + *F. oxysporum* increased protein content (Table 25, Fig. 12). This increase is greatest with *F. oxysporum* inoculation.

Table 25 : Protein contents of healthy and infected soybean roots

Cultivars	Protein content (mg / g root tissue)*			
	Healthy	Inoculated with *B. j*	Inoculated with *F. o*	Inoculated with *B.J + F.o*
Soymax	1.16	2.75	2.26	2.50
UPSM-19	1.42	3.65	1.80	3.25

* 28 days after inoculation with *F. oxysporum*.

4.9.1.3. Soybean seeds

Seeds were considered for studies in proteins since in nature latent infection of *F. oxysporum* is also to be found in the seeds. Surface sterilized seeds of two cultivars were placed on PDA medium in petridishes containing actively growing mycelia of *F. oxysporum*. For control, seeds were placed on media without any fungal growth, After a period of 6 days the seeds were taken out, external mycelia were washed off after which proteins were extracted and contents estimated. Results (Table 26) revealed that soybean seeds of both cultivars had much higher protein content than the roots. In this case also, following inoculation protein content increased in relation to control (Fig. 13).

Table 26 : Protein contents of healthy and *F. oxysporum* inoculated soybean seeds

Cultivars	Protein content (mg/ g tissue) *	
	Healthy	Inoculated with *F.o.*
Soymax	57.12	67.5
UPSM - 19	55.24	68.0

* 6 days after inoculation.

Protein contents of healthy and inoculated soybean roots

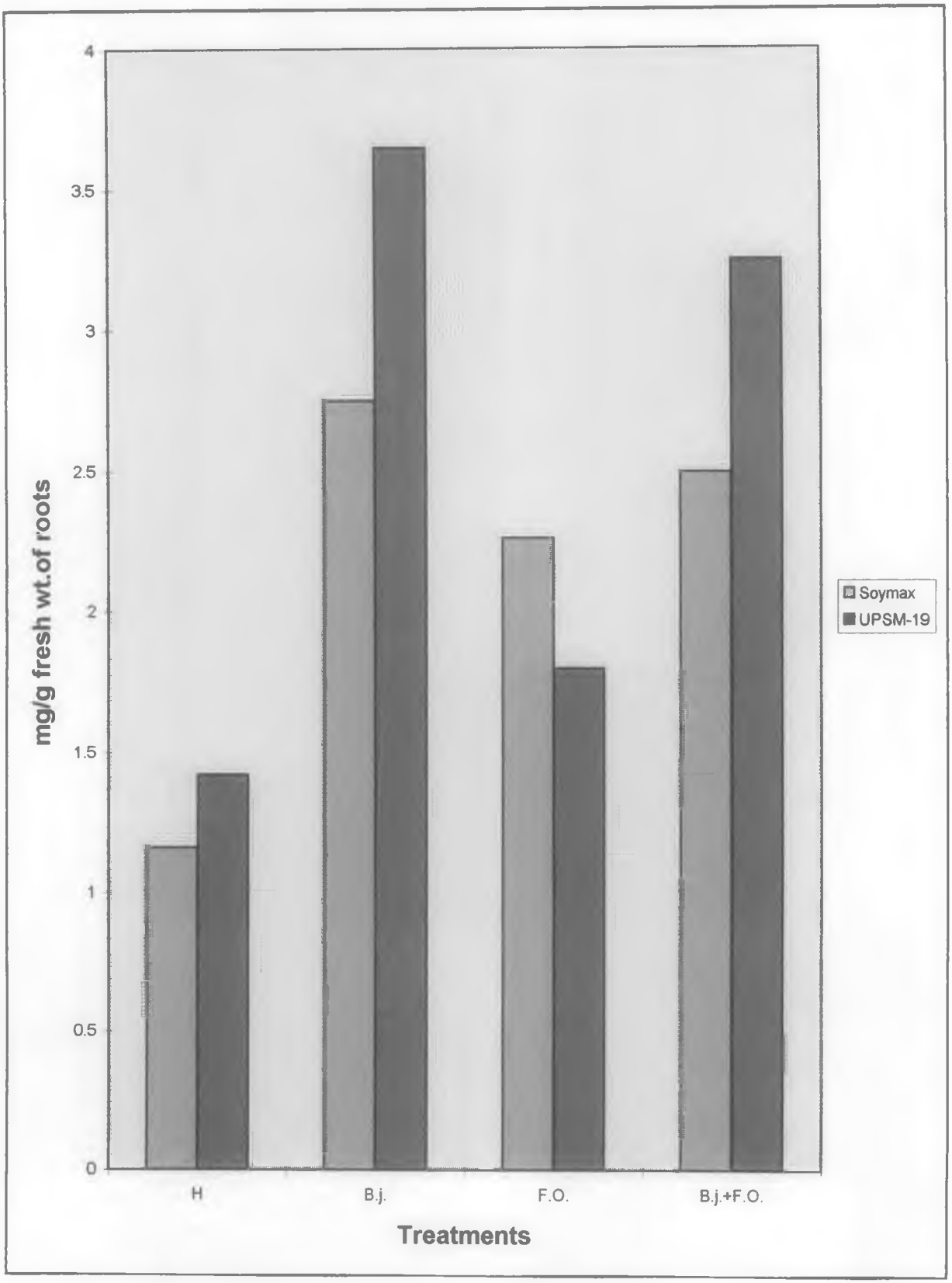


Fig.12

Protein content of healthy and inoculated soybean seeds

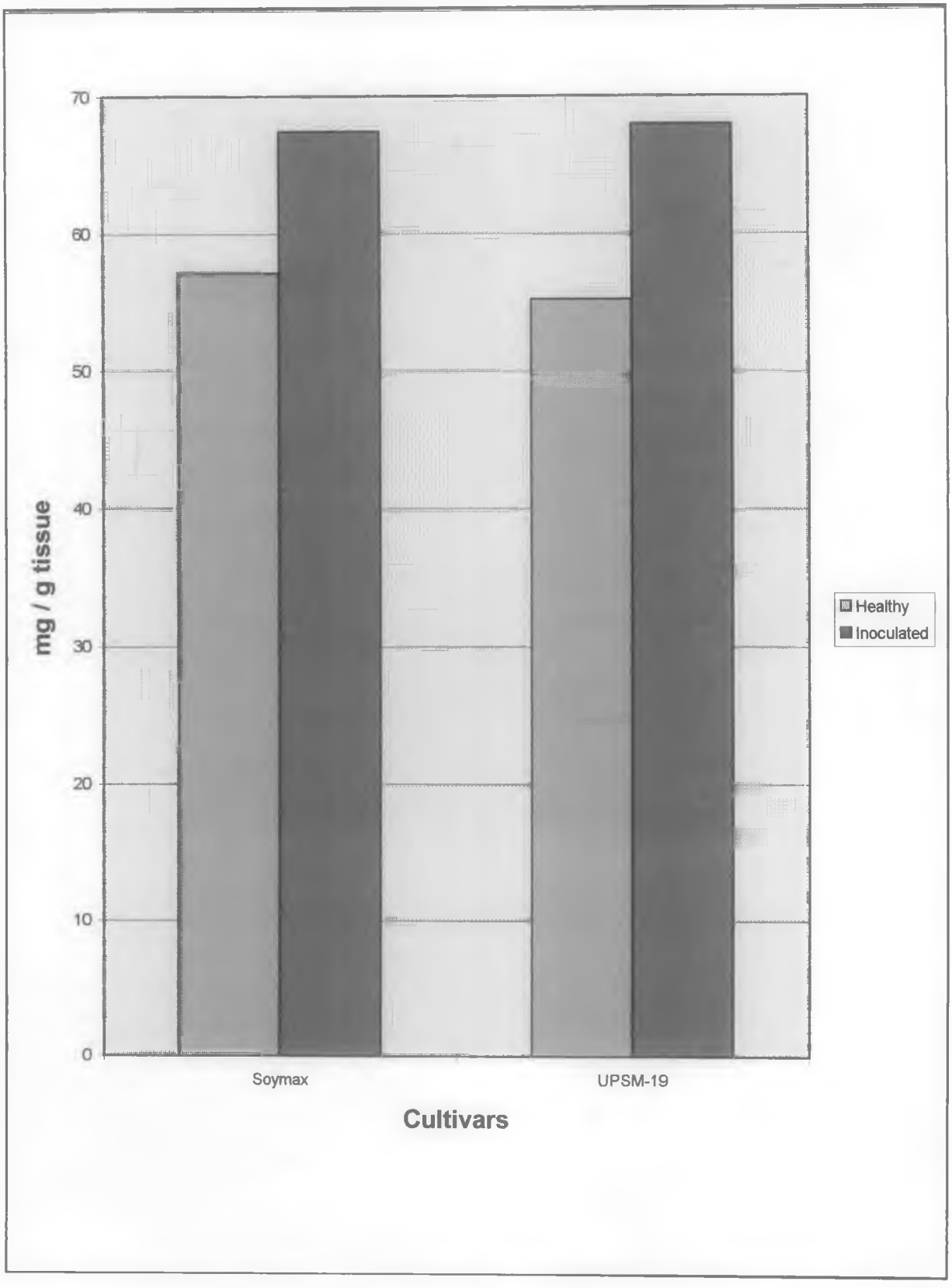


Fig.13

4.9.2. SDS - PAGE

Proteins extracted from the different sources were further analysed by SDS - PAGE following the method described under materials and methods. A number of bands were visualized in all cases following staining with coomassie blue. Molecular weights were determined from the known molecular weights of a mixture of 6 proteins as mentioned in materials and methods.

4.9.2.1. *F. oxysporum*

Mycelial protein exhibited about 35 bands ranging in molecular weight from 200 KDa to 14 KDa. The bands were of varying intensity and the lower molecular weight proteins were more prominent (Plate IX, fig. A).

4.9.2.2. Soybean roots

SDS - PAGE analysis of the different proteins revealed that the protein patterns in all four treatments were more or less similar. Roots after inoculation with *B. japonicum* had some extra bands specially in the lower molecular weight region. Two extra bands of high molecular weight were noticed following inoculation with any of the organisms (Plate IX, fig. B).

4.9.2.3. Soybean seeds

Extracts of healthy seeds of soybean exhibited some very prominent bands of approximate molecular weights 95, 70, 65, 50 KDa and very prominent one at a very low molecular weight. Following infection in the susceptible cultivar all the prominent bands disappeared excepting the one at 50 KDa. In the resistant cultivar most of the bands were still present but the intensity were much reduced. In this case two new bands at low molecular weight were evident. (Plate IX, fig. B).

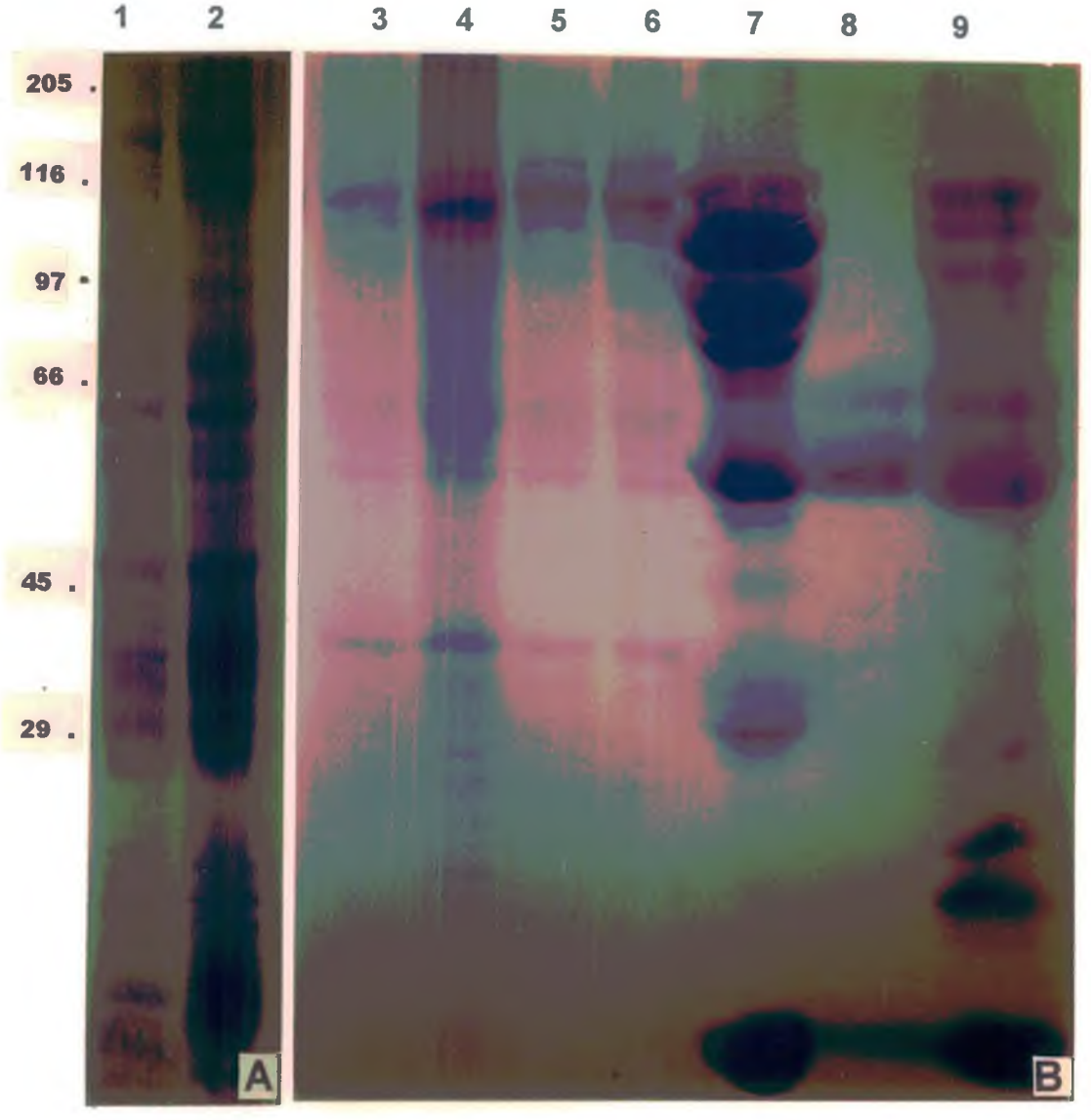


Plate IX (figs. A & B) : SDS-PAGE analysis of proteins . **A** - Mycelial proteins of *F. oxysporum* (**lanes 1 & 2**) ; **B** - Soybean root and seed proteins (**lanes 3 - 9**) ; **Lanes 3 - 6** : Root proteins of cv. Soymax (**3** - Healthy ; **4** - *B. japonicum* inoculated ; **5** - *F.oxysporum* inoculated & **6** - *B.japonicum* + *F.oxysporum* inoculated) ; **Lanes 7 - 9** : Seed proteins ; **7** - Healthy ; **8 & 9**- *F.oxysporum* inoculated ; **8** - cv. UPSM-19 & **9** - cv .Soymax

4.10. Detection of cross reactive antigens (CRA) between *F. oxysporum* and soybean cultivars

Existing studies on plant or animal host and their pathogens suggests that whenever an intimate and continuing association of cells of host and parasites occurs partners of this association have a unique resemblance to one another involving one or more antigenic determinants. Resistance or susceptibility of a plant to infection and disease development may be dependent also on the antigenic relationship of host and pathogen. The greater the antigenic similarity the greater the possibility of immunological tolerance and likelihood that the pathogen will become established in the host and cause disease. Conversely greater antigenic disparity may trigger off certain responses which lead to biochemical changes resulting in resistant responses.

Considering the above, in the present investigation following studies on several biochemical changes in soybean after inoculation with *F. oxysporum* and / or *B. japonicum,* attempts have been made to determine the serological relationship between soybean and *F. oxysporum* as well as to determine changes due to inoculation with *B. japonicum.* For this purpose immunodiffusion tests, immunoelectrophoresis, ELISA and immunofluorescence have been used.

4.10.1. Immunodiffusion

For immunodiffusion tests, initially antisera were raised against *F. oxysporum* as well as a susceptible soybean cultivar (UPSM-19) as described in materials and methods. The effectiveness of antigen preparation from soybean cultivar (UPSM-19) and *F. oxysporum* in raising antibodies was checked initially by homologous cross reaction following agar gel double diffusion tests. Control sets involving normal sera and antigens of both the host and pathogen were all negative. Antiserum of *F. oxysporum* was tested with homologous antigens as well as antigens of 10 soybean cultivars and also a non-host (*Camellia sinesis*) and a non-pathogen (*Glomerella cingulata*). Strong precipitin reactions occurred when antiserum of *F. oxysporum* was exposed to its own antigen or antigens of susceptible cultivars (JS-2, UPSM-19, PK-564, 17-PK-472,18-PK564). With resistant cultivars (Bragg, Punjab 1, Pk-327, 19.PK-466), weak precipitin reaction was observed while in case of the most resistant cultivar (Soymax) no precipitin band could be discerned (Table 27, Plate X). No reaction was observed

with antigen of non-host or non-pathogen.

In reciprocal test using antiserum raised against cv. UPSM-19 precipitin reactions were observed when this antiserum was exposed either to antigen of *F. oxysporum* or antigens of the different soybean cultivars. In this case also no precipitin reaction was observed when reacted with antigen of *C. sinensis* or *G. cingulata.*

Table 27 : Detection of cross reactive antigens among soybean cultivars and *F. oxysporum* using agar gel double diffusion[a]

Antigens	Antisera	
	F. oxysporum	**Soybean (cv. UPSM-19)**
Host		
Soybean cultivars		
Soymax	-	+
Bragg	±	+
Punjab - 1	±	+
19-PK-466	±	+
PK-327	±	+
JS-2	+	+
UPSM-19	+	+
PK-564	+	+
17-PK-472	+	+
18-PK-564	+	+
Nonhost		
Camellia sinensis	-	-
Pathogen		
Fusarium oxysporum	+	+
Non - pathogen		
Glomerella cingulata	-	-

[a] Common precipitin band :

+ Strong precipitin band

± Weak precipitin band.

- Precipitin band absent.

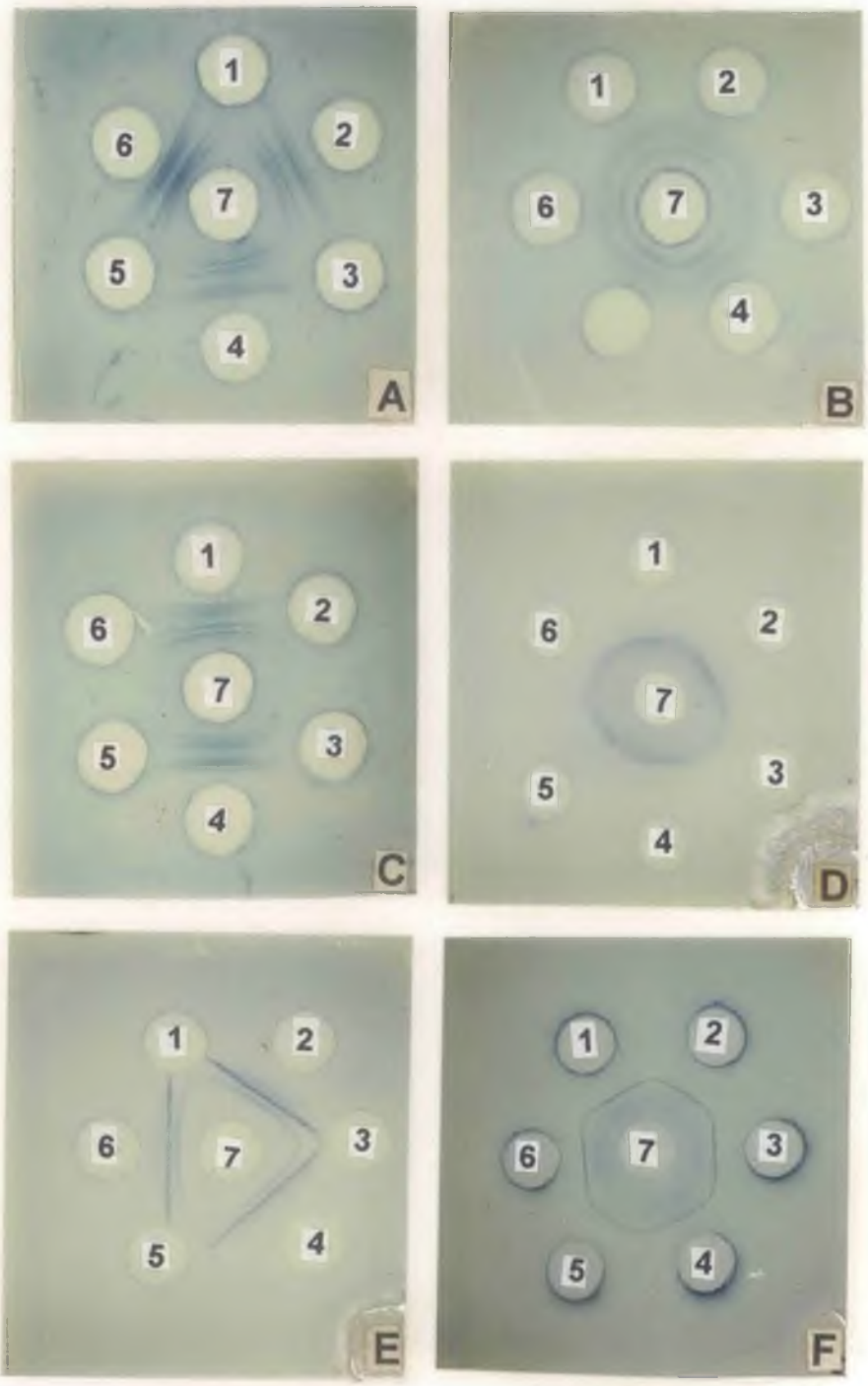


Plate X (figs. A- F) : Agar gel double diffusion tests. Central wells (7) contain antisera of *F.oxysporum* **(A,C,&E)** and soybean (cv.UPSM-19) root **(B,D & F).** Peripheral wells (1-6) contain antigens. **A** - *F.oxysporum* (2,4,6), *G.cingulata* (1,3,5) ; **B** - cv.UPSM-19 (1 ,2, 3 , 4, 5, 6) ; **C** - *F.oxysporum* - (1,4), cv.Soymax (2,3) *C.sinensis* (5,6); **D** - *F.oxysporum* - (1,2,3,4,5,6) ; **E** - cv.UPSM-19 (2,4,6) ; cv.Soymax (1,3,5) ; **F** - cv.Soymax (1,2,3,4,5,6)

4.10.2. Immunoelectrophoresis

Results of immunodiffusion tests were confirmed by immunoelectrophoresis using similar antiserum antigen combinations. Results of immunodiffusion tests revealed the presence or absence of cross reactive antigens (CRA) between *F. oxysporum* and soybean cultivars. Many of the cross reactions in the immunodiffusion gave diffused precipitin bands which could not be clearly distinguised. It was also not clear whether precipitin reactions in all cases were due to single or several antigenic substances. Therefore, further resolution was attempted by subjecting the antigens to electrophoresis before exposing them to antisera. Details of immunoelectrophoretic technique have been described under materials and methods.

Following the conventional immunoelectrophoretic set up, antigenic comparison of the ten cultivars of soybean, *F. oxysporum,* one non-host species (*Camellia sinensis*) and a non-pathogen of soybean (*Glomerella cingulata*) using antisera of pathogen (*F.oxysporum*) and host (UPSM-19) was done and results are presented in Table 28.

In homologous cross reactions, *F. oxysporum* and UPSM-19 antigens exhibited 6 and 5 precipitin arcs, each. In reactions of *F. oxysporum* antiserum with antigens of soybean cultivars; 4 precipitin lines were observed with susceptible cultivars and the number with other cultivars varied from 3-1. With Soymax (resistant cultlvar), no precipitin line was discerned. UPSM-19 also exhibited antigenic similarity with other cultivars. No lines were observed in reactions with either non-host or non-pathogens.

4.10.3. Enzyme linked immunosorbent assay

ELISA is an important technique in recent serological investigations which can specifically detect the presence of cross reactive antigens between host and pathogen or also detect the presence of pathogen infection in the host at very early stages. Several formats of ELISA have been developed in recent years of which direct antigen coated (DAC) ELISA is the most commonly used one in case of fungal pathogens. Besides DAC ELISA, Dot Blot ELISA

Table 28 : Antigenic comparison of soybean cultivars, pathogen, non-pathogen and non-host by immunoelectrophoresis.

Antigens	Total no of precipitin lines	
	Antisera	
	F. oxysporum	UPSM-19
Host		
Soybean cultivars		
Soymax	0	3
Bragg	1	3
Punjab-1	3	3
19-PK-466	3	4
PK-327	2	3
JS-2	4	4
UPSM-19	4	5
PK-564	3	3
17-PK-472	3	3
18-PK-564	3	3
Pathogen		
Fusarium oxysporum	6	4
Non-host		
Camellia sinensis	0	0
Non-pathogen		
Glomerella cingulata	0	0

can also be used for rapid detections. In the present investigation most of the tests have been performed using DAC ELISA while one emperiment was carried out using dot blot ELISA.

For ELISA, initially a series of experiments were carried out to determine the optimum conditions of the different variables i,e. enzyme, antiserum and antigen.

4.10.3.1. Optimization of ELISA

For optimization, 3 major variables were considered i,e. enzyme, antiserum and antigen dilutions. Optimization was carried out in homologous reactions.

4.10.3.1.1. Enzyme dilution

Various dilutions of the enzyme (alkaline phosphatase) was tested in ELISA using *F. oxysporum* antiserum (1 : 125 dilution) and homologous antitgen (5 µg/ml). Dilutions ranged from 1 : 10,000 to 1 : 50,000, Results (Table - 29, Fig - 14) revealed that absorbance values at 405nm decreased with increasing enzyme dilution. Absorbance values at 1 : 10,000 dilution was 1.7 which decreased to 0.4 to for 1 : 50,000 dilution. Enzyme dilution of 1 : 10,000 was used for all further experiments.

Table 29 : ELISA reaction of anti *F. oxysporum* antiserum and homologous antigen with various dilution of alkaline phosphatase.

Enzyme dilution[a]	Absorbance at 405 nm			
	Exp-1	Expt-2	Expt-3	Mean
1:10,000	1.687	1.724	1.692	1.701 ± 0.001
1:20,000	1.438	1.329	1.355	1.374 ± 0.028
1:30,000	1.201	1.226	1.192	1.206 ± 0.001
1:40,000	0.842	0.838	0.856	0.845 ± 0.005
1:50,000	0.421	0.422	0.428	0.424 ± 0.002

Mycelial antigen concentration 5 µg / ml

Anti *F. oxysporum* antiserum (1:125 dilution)

[a] Alkaline phosphatase.

± Standard error.

Effect of enzyme dilution on ELISA reaction

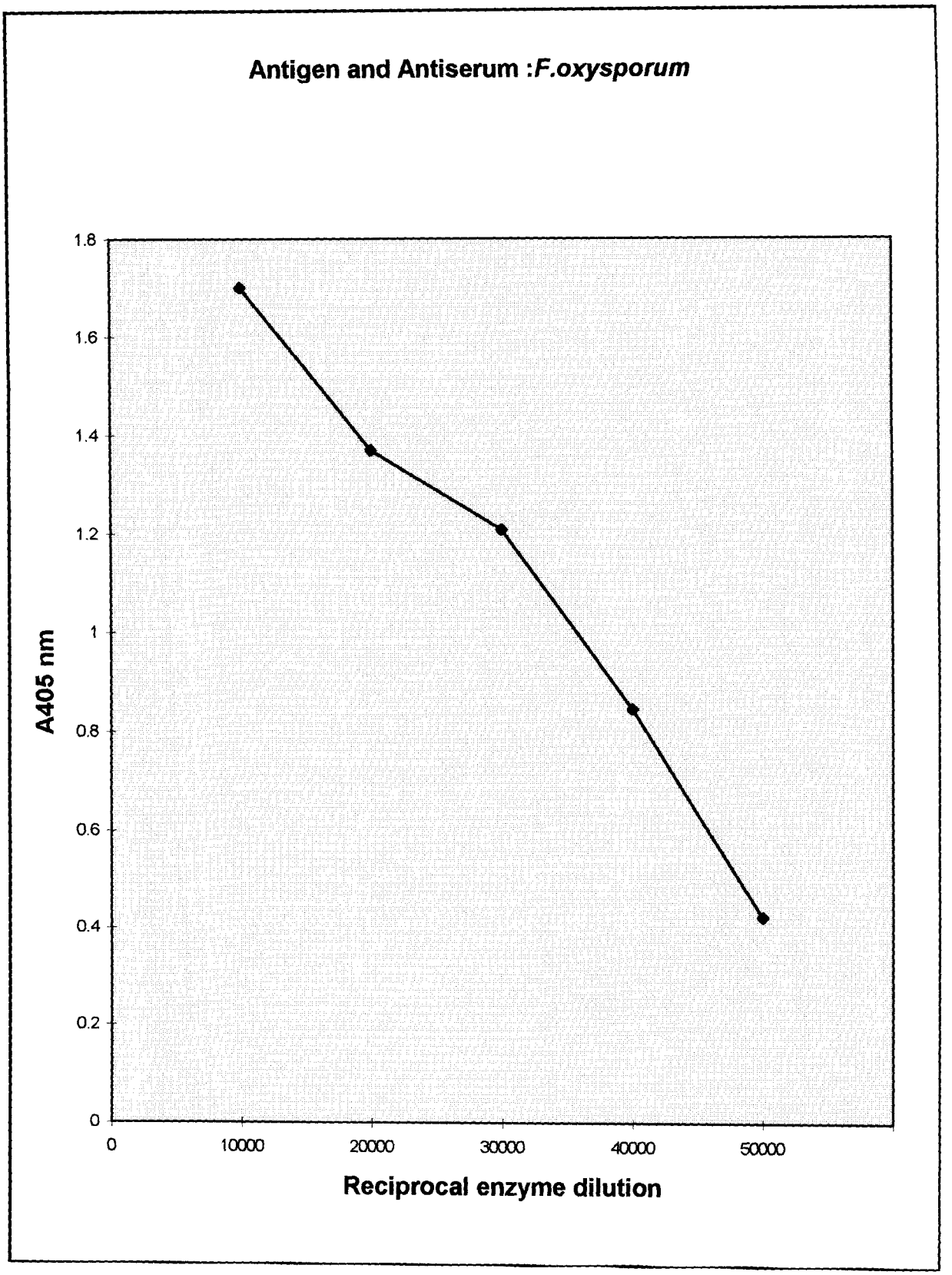


Fig.14

4.10.3.1.2. Antiserum dilution

Antiserum raised against *F. oxysporum* was used at doubling dilutions from 1 : 125 to 1 : 4000 against homologous antigen at a concentration of 5 µg/ ml in DAC ELISA. ELISA responses as indicated by A 405 nm values revealed that ELISA reactivity decreased with increasing dilutions. At 1 : 125 dilution absorbance value was 1.851 which decreased to 0.46 incase of 1.4000 dilution (Table 30, Fig. 15). Hence 1 : 125 dilution was used in further experiments.

Table 30 : ELISA reaction with various dilution of anti *F.oxysporum* antiserum and homologous antigen.

Antiserum dilution	Absorbance at 405 nm			
	Exp-1	Expt-2	Expt-3	Mean
1:125	1.863	1.844	1.836	1.847 ± 0.006
1:250	1.525	1.531	1.525	1.527 ± 0.001
1:500	1.015	0.997	1.008	1.006 ± 0.004
1:1000	0.863	0.903	0.876	0.880 ± 0.009
1:2000	0.578	0.582	0.575	0.578 ± 0.002
1.4000	0.467	0.462	0.465	0.464 ± 0.001

Mycelial antigen concentration - 5 µg / ml
Enzyme dilution -1 : 10,000
± Standard error.

4.10.3.1.3. Antigen dilution

Antigen dilution used for this experiment ranged from 3200 ng (3.2 µg) / ml to 25 ng / ml, ELISA reactivities were tested using two antiserum dilutions i,e. 1 : 125 and 1 : 250. In both cases antigen concentrations as low as 25 ng/ ml was still detectable by ELISA. ELISA reactivities decreased with increasing dilutions (Table 31, Fig. 16).

Effect of antiserum dilution on ELISA reaction

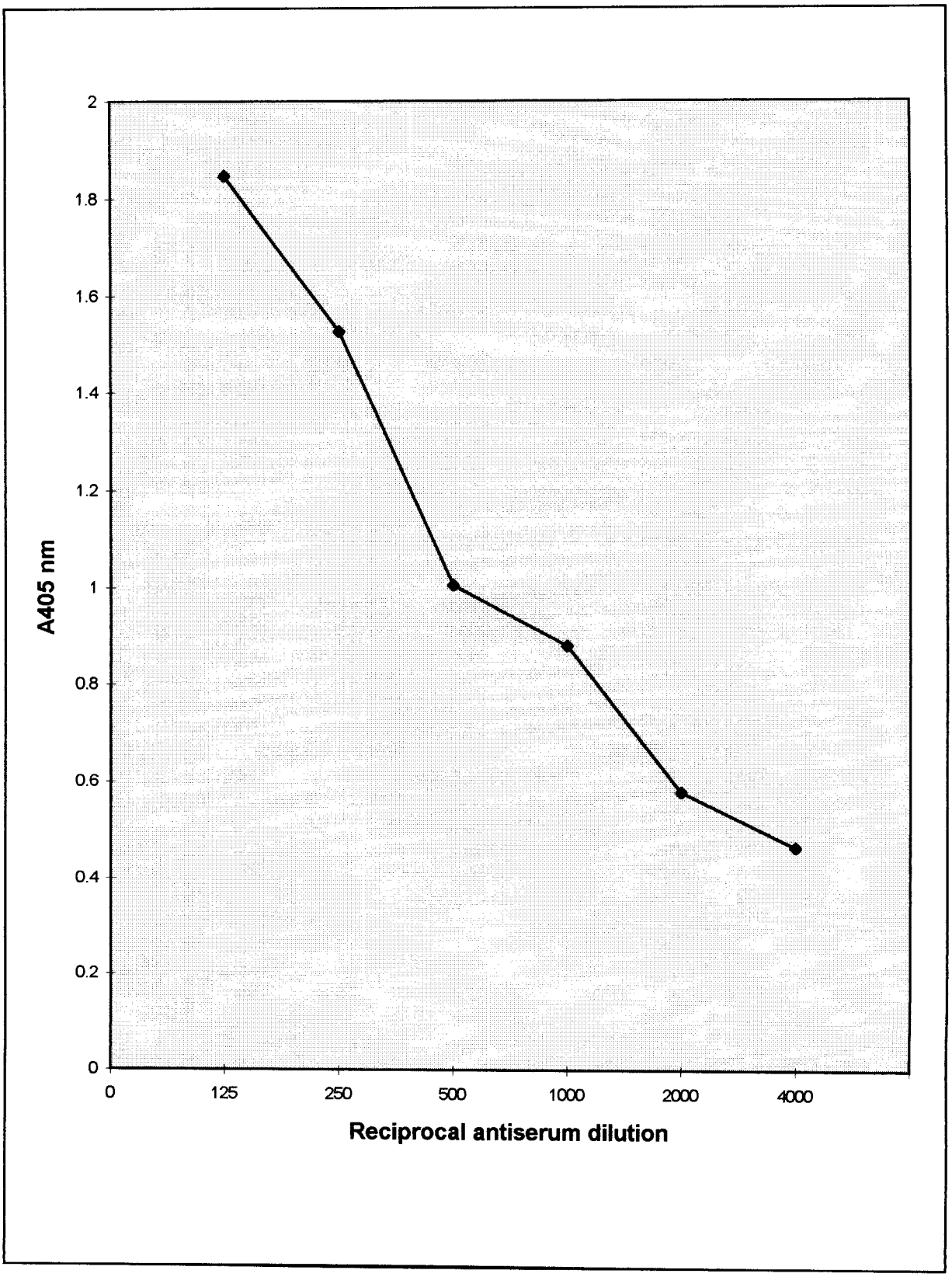


Fig.15

Table 31 : ELISA reaction with various concentration of mycelial antigen of *F. oxysporum* and homologous antiserum.

Antigen conc. (ng / ml)	Absorbance at 405 nm			
	Expt.-1	Expt.-2	Expt.-3	Mean
25	0.591	0.573	0.594	0.586 ± 0.005
50	0.676	0.674	0.693	0.681 ± 0.004
100	0.746	0.741	0.701	0.729 ± 0.011
200	0.803	0.804	0.816	0.807 ± 0.003
400	0.979	0.936	0.932	0.949 ± 0.012
800	1.019	0.997	1.003	1.006 ± 0.005
1600	1.213	1.294	1.210	1.239 ± 0.022
3200	1.531	1.551	1.528	1.530 ± 0.007

* Anti *F. oxysporum* antiserum dilution - 1:125

Enzyme dilution 1 : 10,000

± Standard error.

4.10.3.2. Comparison of ELISA reactivity among antigens of different soybean varieties against antiserum of *F. oxysporum*.

For detection of CRAs between soybean and *F. oxysporum* ELISA reactions were carried out with *F. oxysporum* antiserum and antigens of different soybean cultivars. Absorbance values in ELISA reavealed that higher absorbance values were obtained for the susceptible cultivars (JS - 2, UPSM - 19, PK - 564) than the resistant ones (Soymax, 19 - PK 466, PK - 327). In ELISA involving antigens of non-host very low values were recorded (Table 32, Fig 17). In reciprocal tests when antiserum raised against the susceptible soybean cultivar (UPSM - 19) was reacted with antigens of other varieties A 405 values did not differ significantly except in homologous combinations (Table 33, Fig. - 18) : The values varied among the different cultivars but no correlation could be drawn in this case.

Effect of antigen dilution on ELISA reaction

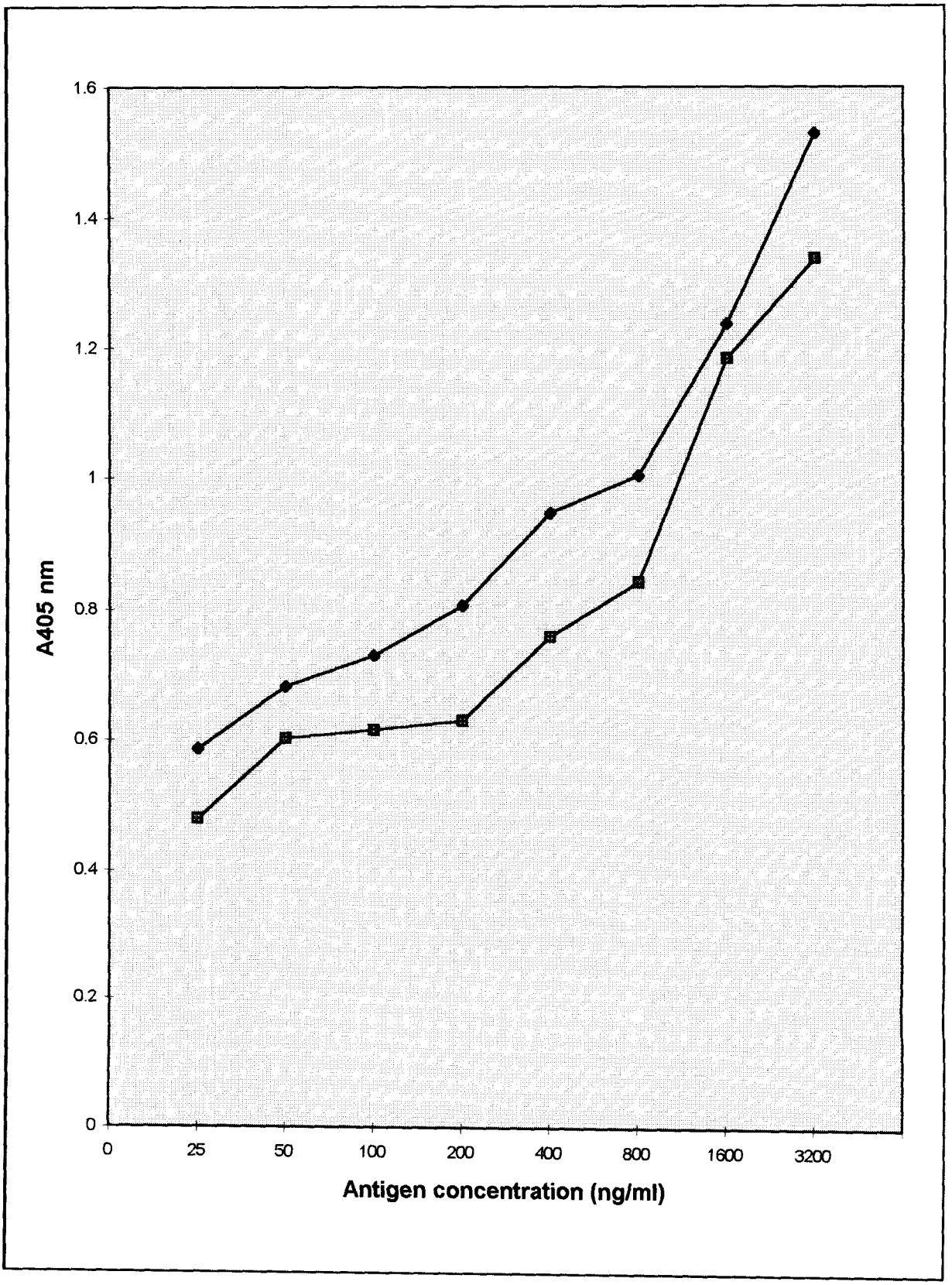


Fig.16

Table 32 : Indirect ELISA values (A 405) of combinations of antigens of host varities, non-host, pathogen and anti-serum of *F. oxysporum*.

Antigens	Absorbance at A 405nm			
	Expt - 1	Expt - 2	Expt. - 3	Mean.
Host				
Soymax	0.882	0.862	0.851	0.865 ± 0.010
JS - 2	1.538	1.506	1.529	1.524 ± 0.007
UPSM - 19	1.542	1.563	1.522	1.542 ± 0.014
Bragg	1.021	0.936	0.981	0.979 ± 0.020
Punjab - 1	1.038	1.121	0.989	1.049 ± 0.037
PK - 564	1.428	1.391	1.402	1.407 ± 0.038
17 - PK-412	1.218	1.114	1.238	1.190 ± 0.014
19 - PK-466	0.918	0.904	0.893	0.905 ± 0.014
PK - 327	0.929	0.939	0.962	0.943 ± ,0.016
18 - PK-564	1.318	1.298	1.307	1.306 ± 0.001
Non-host				
Camellia sinensis	0.320	0.285	0.369	0.325 ± 0.001
Pathogen				
Fusarum oxysporum	1.826	1.842	1.856	1.841 ± 0.001

Anti *F. osysporum* antiserum -1 : 125 dilution
Antigen concentration 40 μg/ml.
± Standard error.

DAC-ELISA response of *F.oxysporum* antiserum

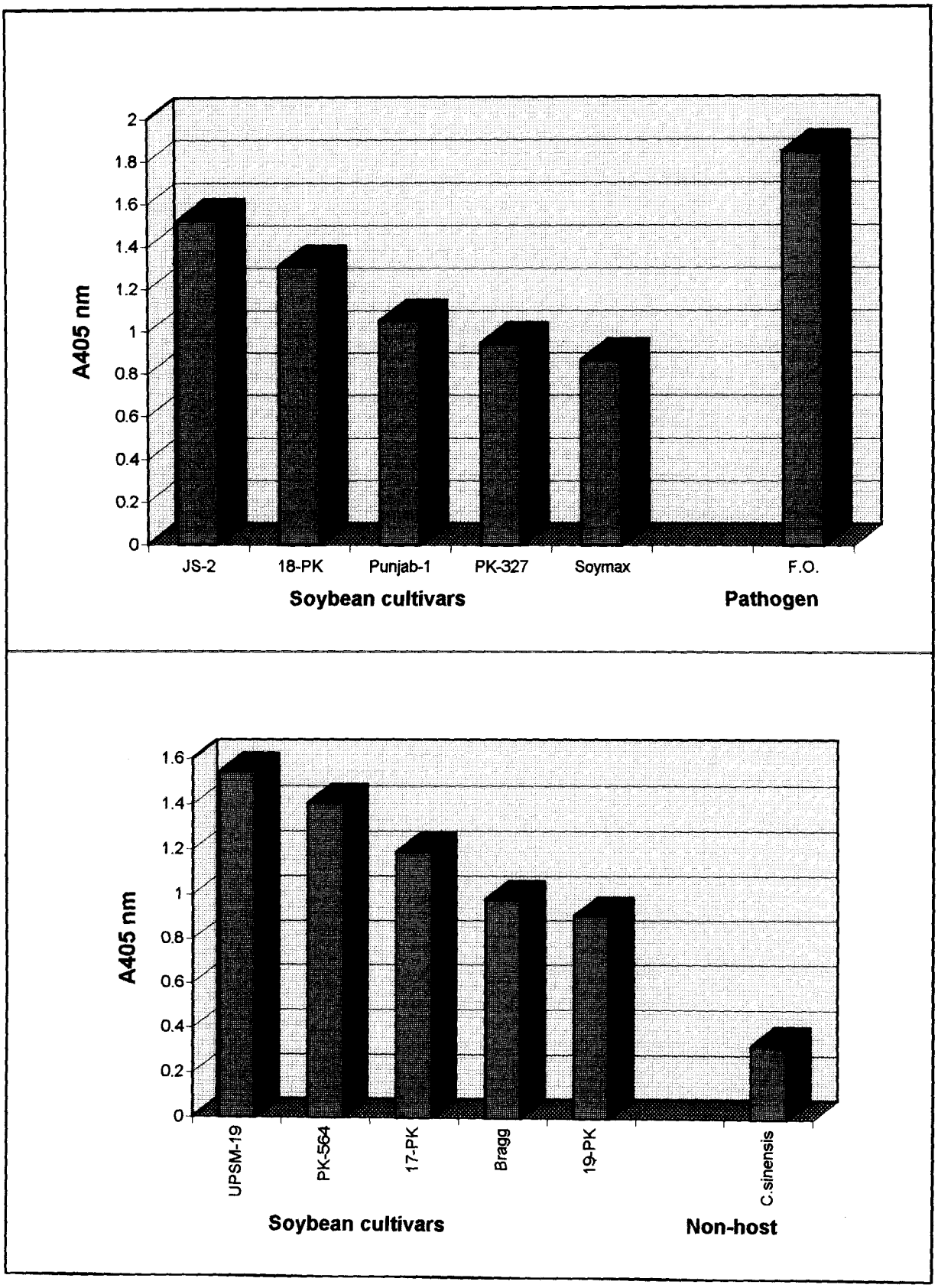


Fig.17

Table 33 : Indirect ELISA values of combinations of antigens of host varities, non-host, pathogen and antiserum of soybean (UPSM-19)

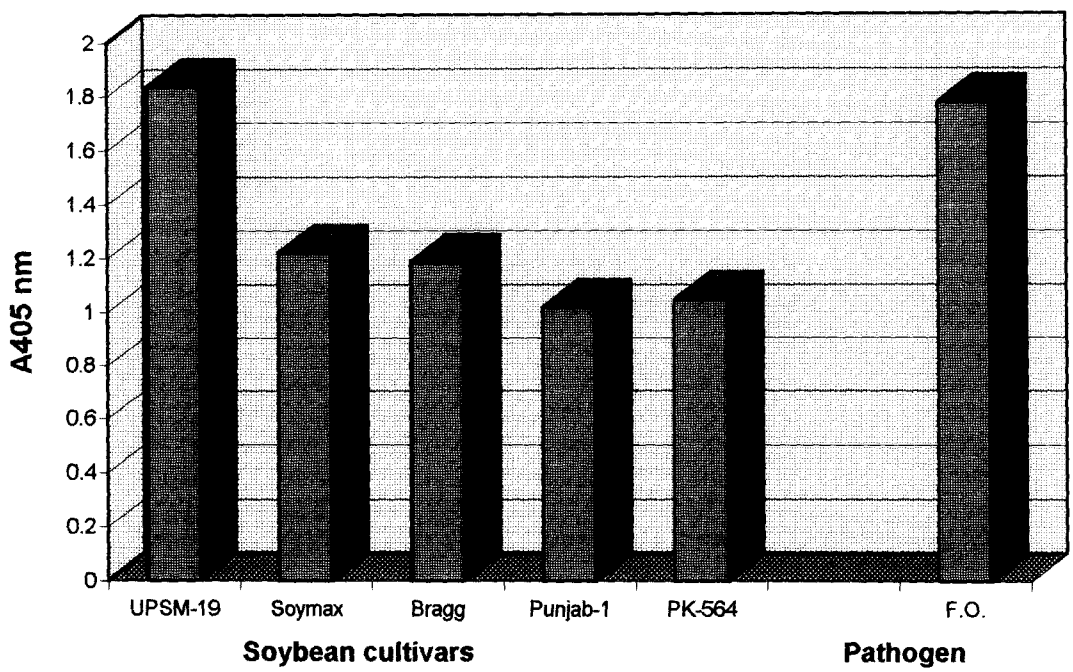
Antigens	Absorbance at 405 nm			
	Expt - 1	Expt.- 2	Expt. - 3	Mean
Host				
Soymax	1.218	1.208	1.232	1.219 ± 0.003
JS - 2	1.508	1.512	1.518	1.513 ± 0.002
UPSM - 19	1.828	1.834	1.842	1.835 ± 0.003
Bragg	1.226	1.228	1.102	1.185 ± 0.034
Punjab 1	0.982	1.062	1.004	1.016 ± 0.040
PK - 564	1.013	1.148	0.965	1.042 ± 0.045
17 - PK-472	1.124	0.998	1.062	1.061 ± 0.030
19 - PK-466	1.058	0.988	1.092	1.046 ± 0.025
PK - 327	0.842	0.839	0.892	0.858 ± 0.014
18 -PK. 564	1.046	1.128	1.039	1.071 ± 0.023
Pathogen				
F.oxysporum	1.742	1.763	1.819	1.775 ± 0.020
Non pathogen				
G. cingulata	0.245	0.249	0.250	0.248 ± 0.001

Antiserum dilution 1 : 125

Antigen concentration 40 μg/ml.

± Standard error.

DAC-ELISA response of soybean (cv.UPSM-19) antiserum



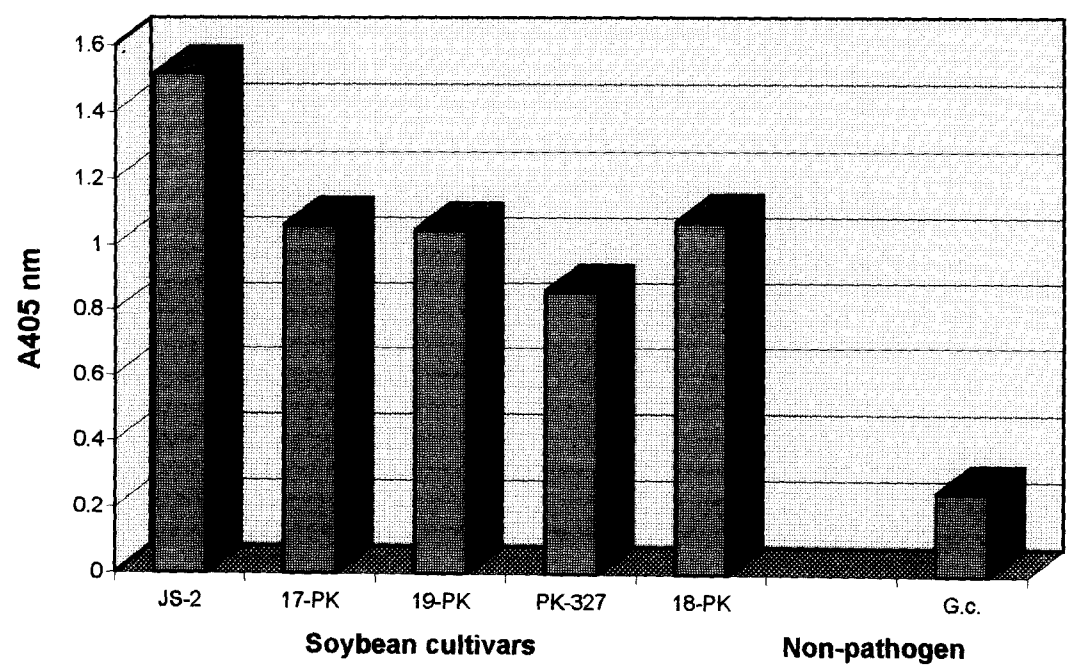


Fig.18

4.11. Detection of pathogen in infected tissues

4.11.1. *F. oxysporum* inoculated

Since a serological relationship exists between *F. oxysporum* and soybean as evidenced by previous experiments, the possibility of detection of *F. oxysporum* in soybean tissues by serological means was considered. Pathogen detection in host tissues by the use of ELISA with the antiserum raised against the pathogen is an effective method. Difference in ELISA readings between healthy and infectecd antigens indicates the measure and extent of infection. In the following experiments attempts have been made to detect *F. oxysporum* in root and seed tissues.

4.11.1.1. Root

In this experiment antigens were prepared from healthy and *F. oxysporum* infected roots of soybean of two cultivars - UPSM-19 and Soymax. These antigens at a concentration of 40 µg / ml were tested against anti *F. oxysporum* antiserum at 1:125 dilution using two ELISA formats.

4.11.1.1.1. DAC ELISA

This is the commonly used ELISA format for quantitative detection of fungal pathogen in host tissues. DAC ELIS A tests were performed as mentioned above and results are presented in Table 34 and Fig.19. Absorbance values for infected root antigen preparations of both the cultivars were significantly higher than their respective healthy extracts at P=0.01

4.11.1.1.2. Dot Blot ELISA

The efficacy of antiserum raised against *F. oxysporum* in detection of infection was further confirmed by dot blot ELISA. Dot blot ELISHA has been judged in many cases as the most sensitive and simple method for pathogen charecterization and detection (Lazerovits, 1990) In the presnt investigation observation of the membrane after the assay revealed deep coloured spots in homologous reactions. In healthy extracts faint coloured spots appeard whereas in case of infected extracts the colour intensity of the spots was much higher (Table 35)

Table 34 : ELISA values showing reaction of antiserum of *F. oxysporm* with antigens of healthy and and inoculated roots of soybean.

Cultivars	Treatment	Absorbance at 405 nm			
		Expt 1	Expt 2	Expt 3	Mean
UPSM-19	Healthy	1.428	1.432	1.446	1.435 ± 0.004
	Inoculated[a]	1.828	1.841	1.838	1.836 ± 0.003
Soymax	Healthy	0.850	0.864	0.861	0.858 ± 0.005
	Inoculated[a]	1.262	1.258	1.236	1.252 ± 0.001

Antigen concentration - 40 µg / ml

Anti *F. oxysporum* antiserum used at 1: 125 dilution

[a] 28 days after inoculation with *F. oxysprum*

± Standard error

Difference between healthy and inoculated values significant at P = 0.01.

Table 35 : Dot blot ELISA values of healthy and *F. oxysporum* inoculated soybean tissues.

Antigen preparation	Antiserum of *F. oxysporum*	
	Intensity of Spots	
	Healthy	Inoculated
cv. UPSM-19		
Root	+	++
Seed	+	++
cv. Soymax		
Root	+	++
Seed	+	++
F. oxysporum		+++

Colour intensity : Low(+); Medium(++); High (+++).

ELISA values of soybean root antigens with pathogen antiserum

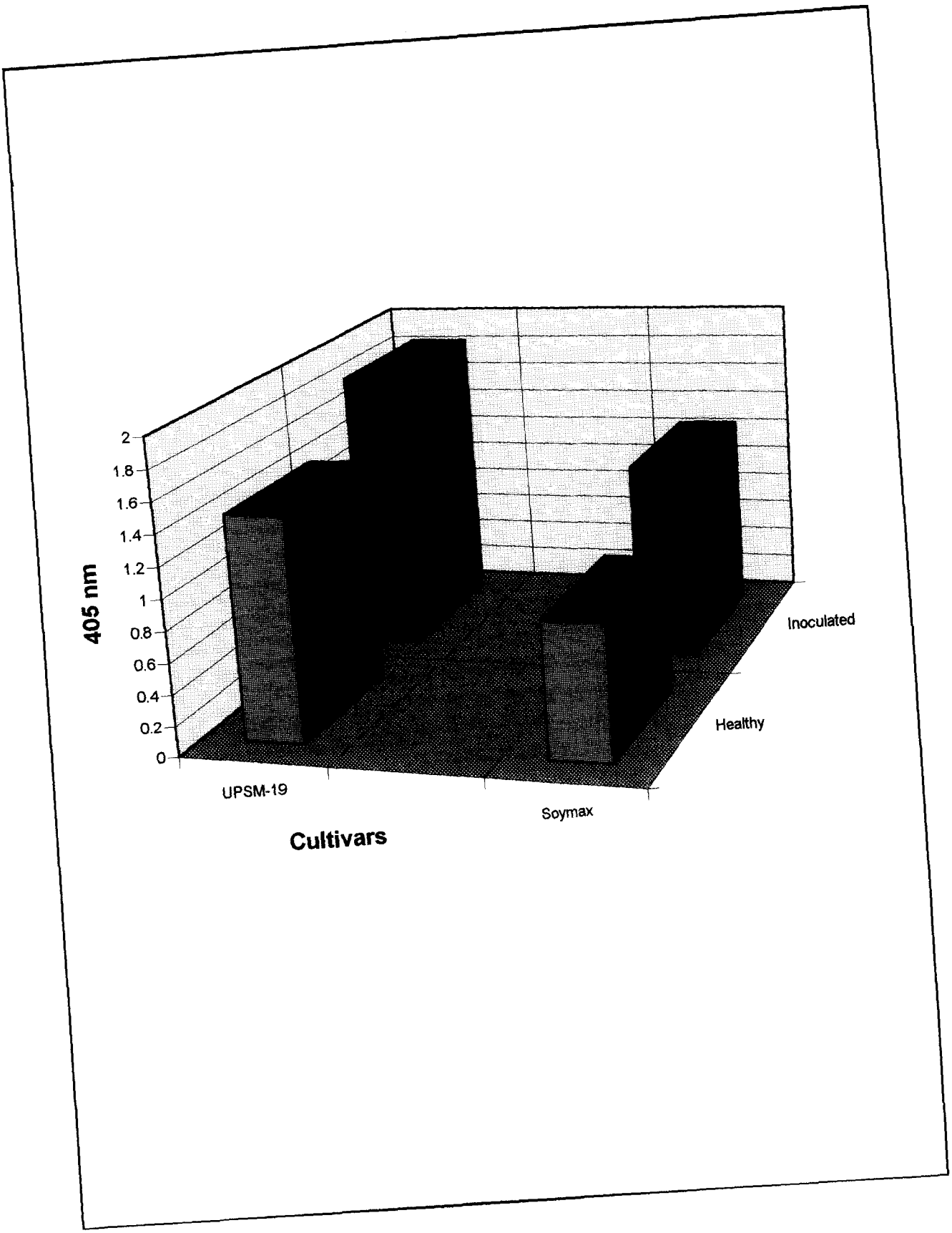


Fig.19

4.11.1.2. Seed

Latent infection of seeds by various pathogens is of quite common occurrence and *F. oxysporum* has also been previously reported to cause latent infection. In such cases serological techniques become all the more important because these can detect even very small amounts which by other techniques may be undetected. Hence ELISA was carried out with antigens of healthy and inoculated seed sample and antiserum raised against *F. oxysporum.*

4.11.1.2.1. DAC ELISA

Results of DAC ELISA performed as mentioned above revealed that absorbance values of infected seed extracts were significantly higher than those of the healthy extracts at P=0.01 (Table 36, Fig-20) Infection could be detected in both the cultivars tested.

4.11.1.2.2. Dot blot ELISA

Infected seed extracts exhibited deep coloured spots in dot blot ELISA as compared to less intense spots in healthy extracts (Table - 35). Thus infection in seeds could also be detected by visual estimation of the intensity of spots. It was graded for partial quantification.

4.11.2. Inoculated with *B. japonicum* and *F. oxysporum*

Results of previous experiments had established that preinoculation with *B. japonicum* reduced root rot discase caused by *F. oxysporum* and these were attributed to biochemical changes occurring in the soybean tissues. It has also been established that ELISA values of infected extracts was greater than healthy ones. Hence in the next experiment it was decided to investigate the changes in ELISA responses due to preinoculation with *B. japonicum.*

The antigens were prepared from healthy, *B. japojnicum* inoculated, *F-oxysporum* inoculated and *B. japonicum* + *F. oxysporum* inoculated roots of the two cultivars (UPSM-19 and Soymax) as described previously. These antigens were then tested against anti *F. oxysporum* antiserum in DAC ELISA. Results (Table 37, Fig. 21) revealed no significant differences between healthy

Table 36 : ELISA values showing reaction of antiserum of *F. oxysporum* with antigens of healthy and inoculated seeds of soybean.

Cultivars	Treatment	Absorbance at 405 nm			
		Expt 1	Expt 2	Expt 3	Mean
UPSM-19	Healthy	1.350	1.344	1.361	1.351 ± 0.004
	Inoculated[a]	1.840	1.828	1.836	1.834 ± 0.002
Soymax	Healthy	0.876	0.875	0.880	0.877 ± 0.001
	Inoculated[a]	1.256	1.390	1.306	1.317 ± 0.031

Antigen concentration - 40 µg / ml
Anti *F. oxysporum* antiserum used at 1: 125 dilution
[a] 6 days after inoculation with *F. oxysporum*
± Standard error.
Difference between healthy and inoculated values significant at P = 0.01.

and bacterized root antigens. Inoculation with *F. oxysporum significanlly* increased the ELISA values in both cultivars. Values for *B. japonicum* + *F. oxysporum* inoculated extracts were higher than those of either healthy or *B. japonicum* inoculated roots but lesser than those of *F. oxysporum* inoculated ones.

4.12. Cellular location of major cross reactive antigen by immunofluorescence.

In the present study the presence of CRA shared by soybean and *F. oxysporum* has been detected by immunodiffusion, immunoelectrophoresis and ELISA. Changes in ELISA responses due to infection with *F. oxysporum* as well as preinoculation with *B. japonicum* has also been recorded. It was next

ELISA values of soybean seed antigens with pathogen antiserum

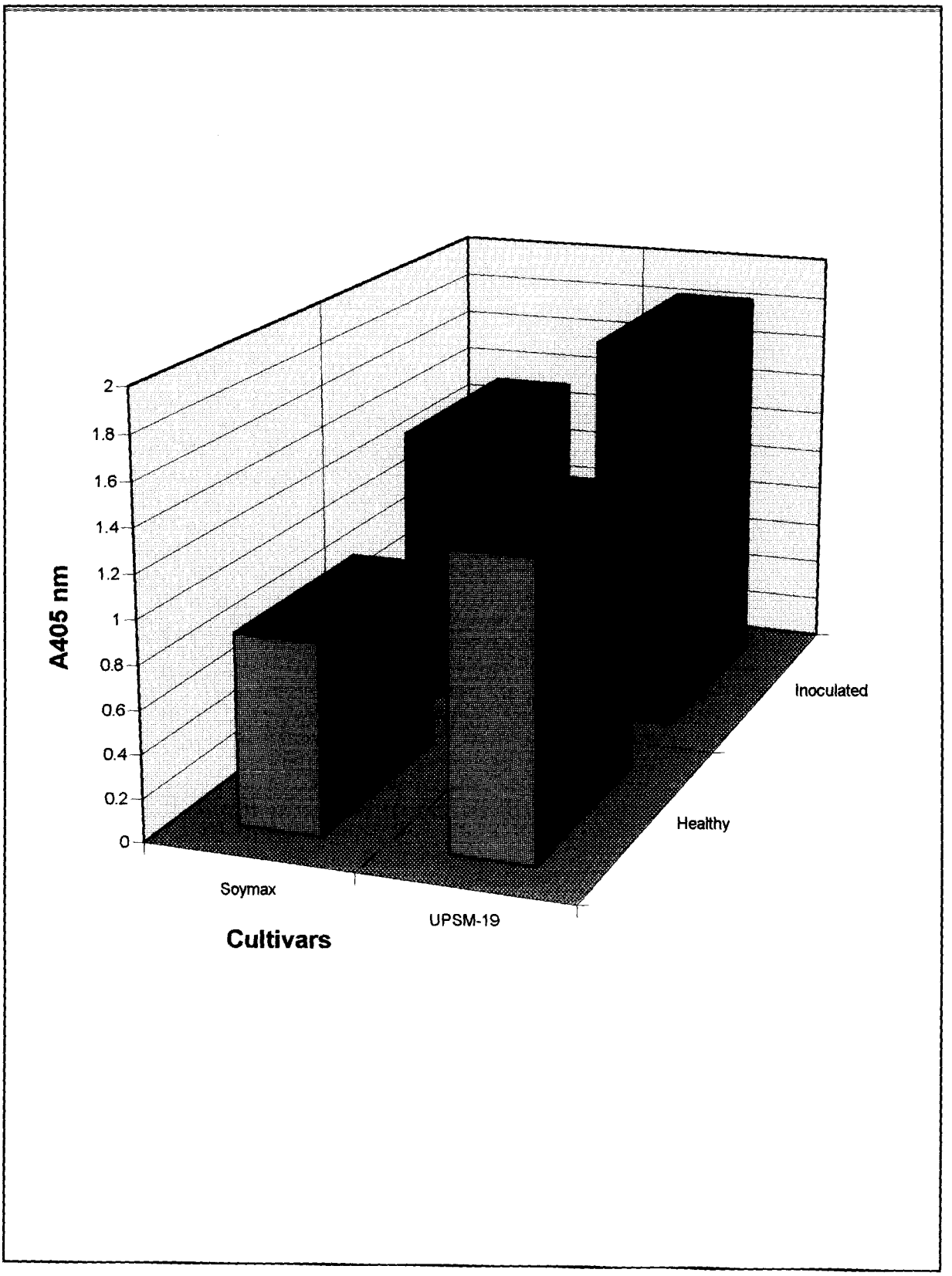


Fig.20

Table 37 : ELISA values of healthy and treated root antigens of two soybean cultivars reacted with pathogen antiserum.

Cultivars	Treatment*	Absorbance at 405 nm			
		Expt 1	Expt 2	Expt 3	Mean
UPSM-19	Healthy	1.439	1.448	1.526	1.471 ± 0.023
	Inoculated *(F.o)*	1.836	1.872	1.849	1.852[a] ± 0.009
	Inoculated *(B.j)*	1.462	1.386	1.475	1.441 ± 0.022
	Inoculated *B.j ± F.o.*	1.642	1.569	1.602	1.604[a] ± 0.017
Soymax	Healthy	0.850	0.863	0.861	0.858 ± 0.017
	Inoculated *(F.o)*	1.238	1.214	1.302	1.251[a] ± 0.021
	Inoculated *(B.j)*	0.836	0.840	0.844	0.841 ± 0.003
	Inoculated *B. j + F.o*	1.062	1.092	0.992	1.049[a] ± 0.024

Antigen concentration 40 µg / ml

Anti *F. oxysporum* antiserum used at 1:125 dilution.

* 28 days after inoculation with *F. oxysporum*

± Standard error.

[a] Difference with respective healthy extracts significant at P = 0.01.

ELISA response of healthy and inoculated soybean root antigens

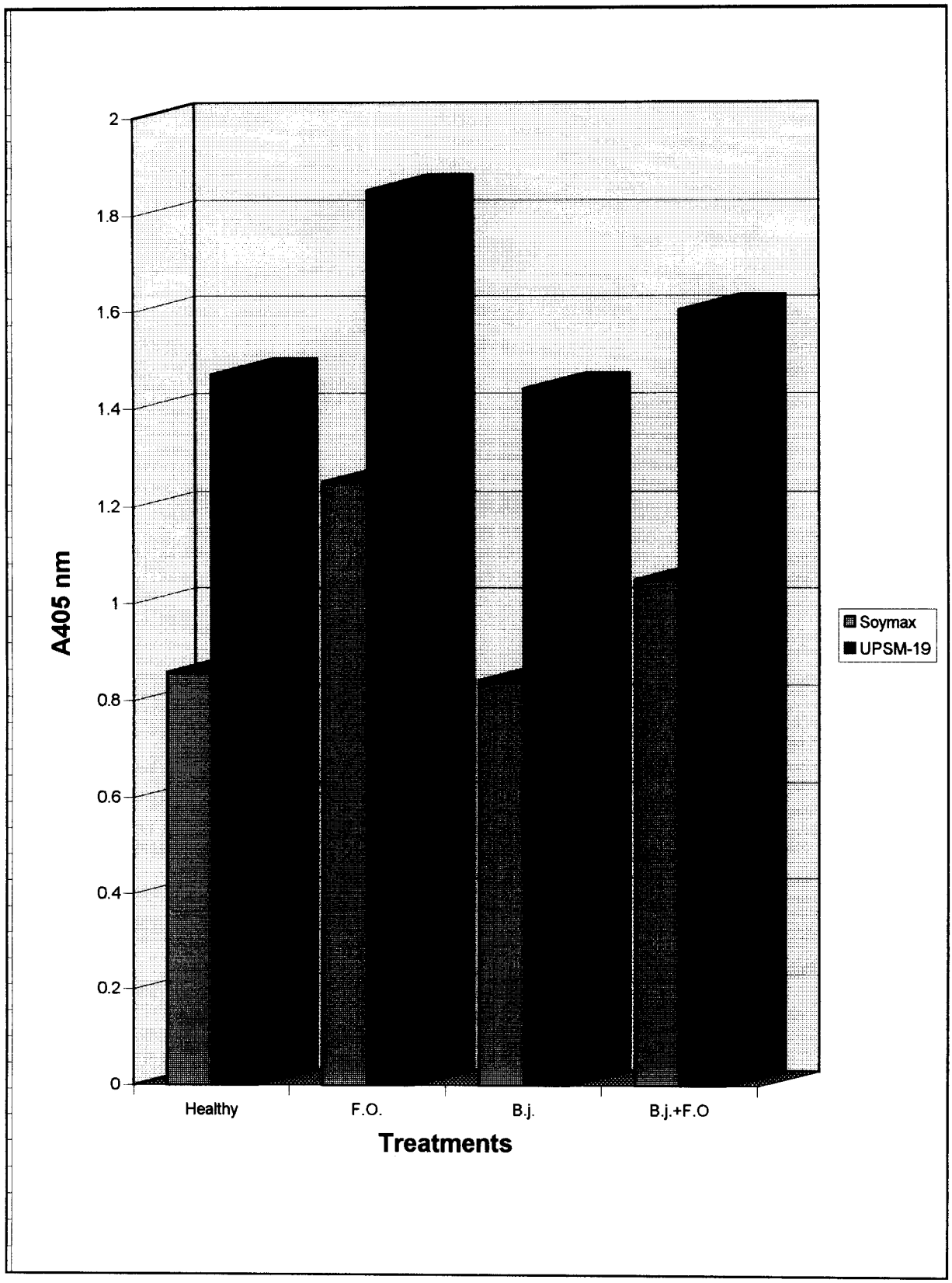


Fig.21

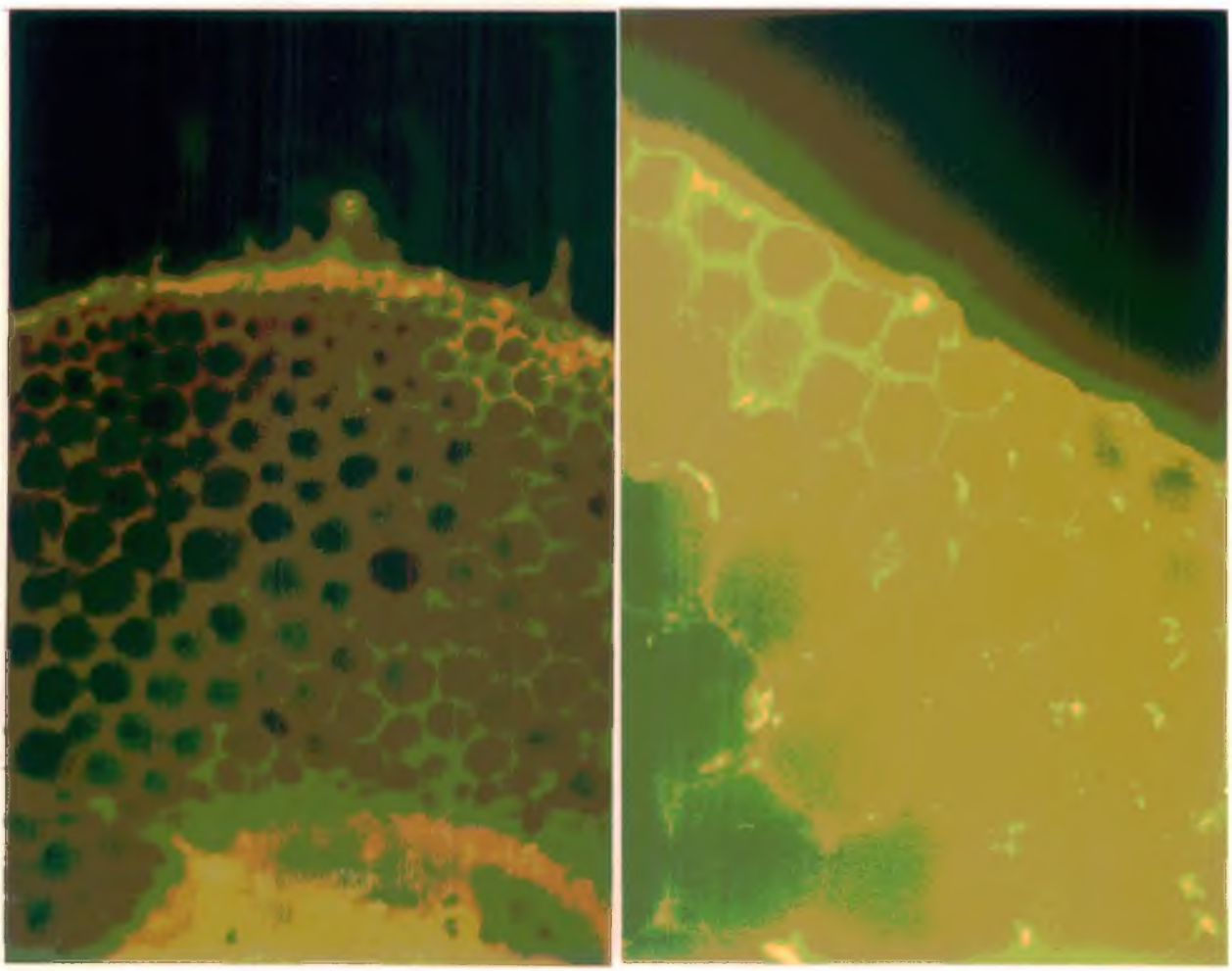


Plate XI (figs. A & B) : Fluorescent antibody staining of soybean root tissues (cv.UPSM -19) for cross reactive antigens shared with *F.oxysporum* . Root sections treated with antiserum to *F.oxysporum* and FITC - antibodies of goat specific for rabbit globulins ; **A** - Portion of root section ; **B** - magnified view of epidermal and cortical region

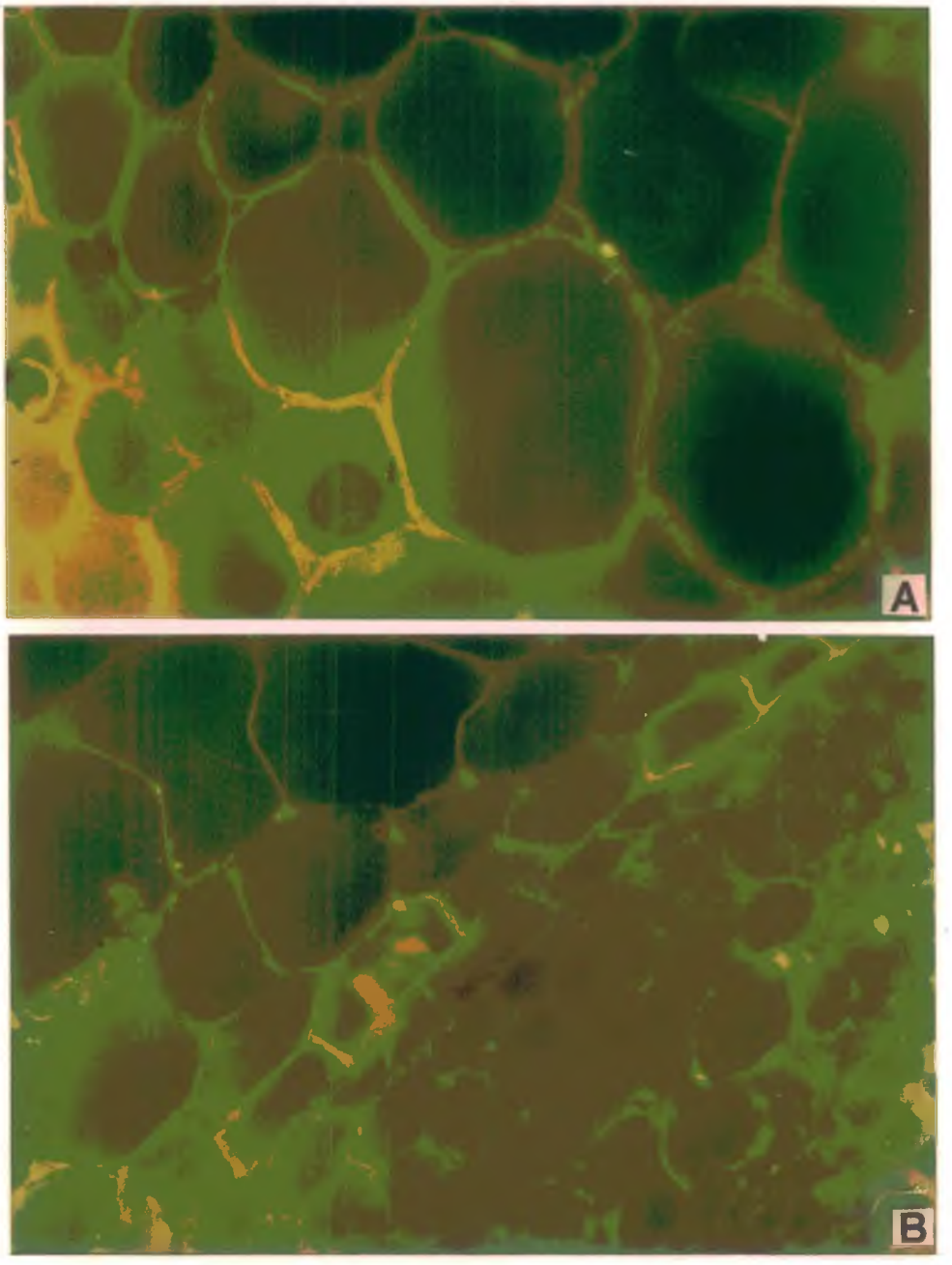


Plate XII (figs. A & B) : Fluorescent antibody staining of soybean root tissues (cv. UPSM -19) for cross reactive antigens shared with *F.oxysporum* . Root sections treated with antiserum to *F.oxysporum* and FITC - antibodies of goat specific for rabbit globulins. Enlarged view of : **A** - cortical region ; **B** - cortical,endodermal and stelar region

decided to determine the tissue and cellular location of CRA in root sections as well as in mycelia, conidia and chlamydospores of *F. oxysporum* by flourescent antibody labelling with fluorescein isothiocynate (FITC). Detailed methods of antibody staining have already been described in materials and methods.

4.12.1. Root Sections

Fresh cross sections of roots of two cultivars (Soymax and UPSM-19) were made and tested either with antiserum of UPSM-19 or *F. oxysporum* after indirectly labelling with FITC. When the sections were treated with antiserum of F. oxysporum indirectly labelled with FITC it was observed that in the sections of susceptible cultivar (UPSM-19), CRA was concentrated around xylem elements, endodermis, cortical tissues and epidermal cells (Plate XI, figs. A&B & XII, figs. A&B) while in the resistant cultivar (Soymax) fluorescence was concentrated mainly in the epidermal cells and partly in the cortical cells (Plate XIII, figs. A&B).

4.12.2. Mycelia, Conidia and Chiamydospore

Treatment of *F. oxysporum,* mycelia, microconidia, macroconidia as well as chlamydospores with its own antiserum as well as antiserum of UPSM-19 followed by FITC labelling resulted in general fluorescence in all cases. (Plate XIV, figs. A-C). None of these were autofluorescent nor did they fluoresce when treated with normal serum followed by FITC.

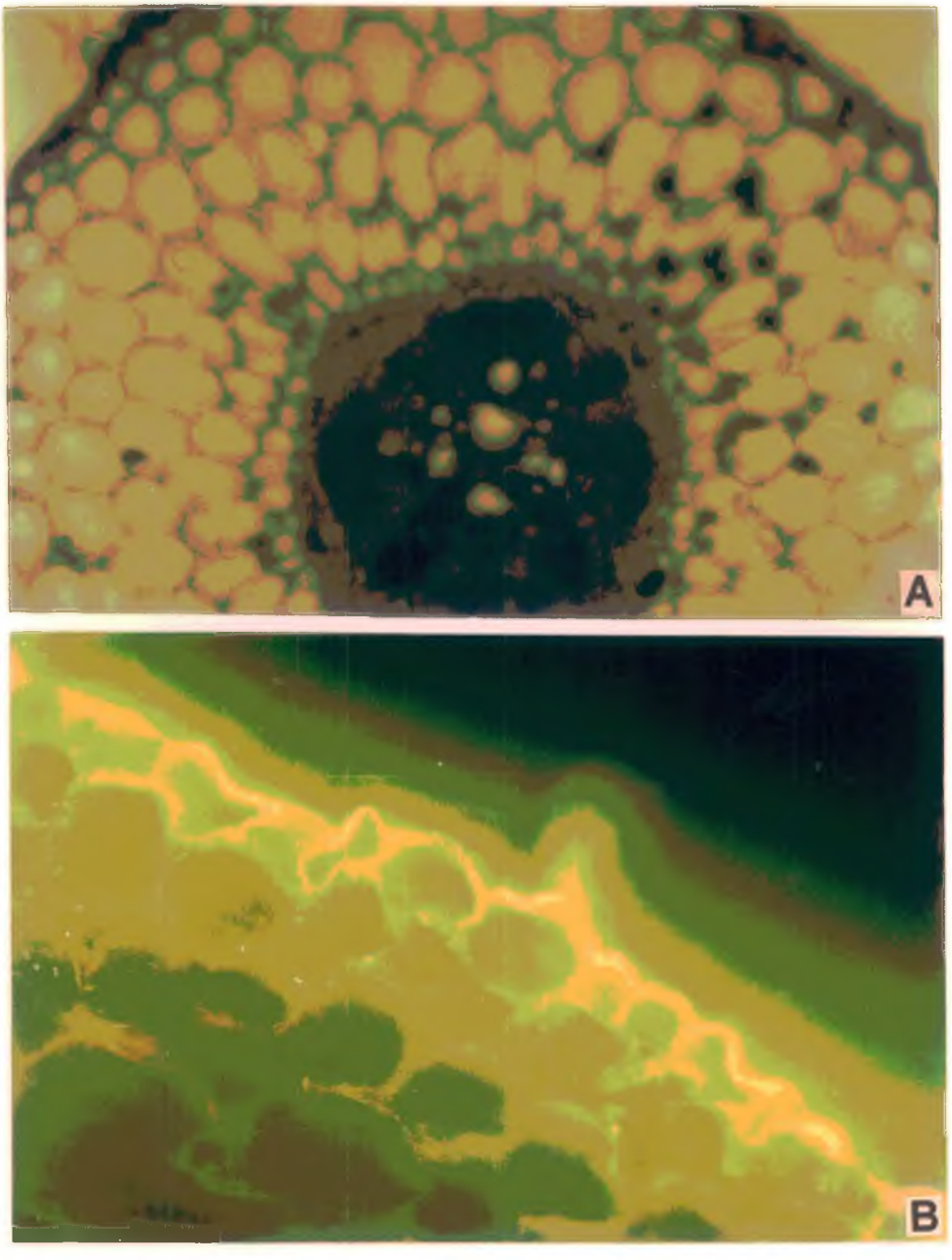


Plate XIII (figs. A & B) : A - Unstained root section of soybean (cv.UPSM -19) ; **B** - Portion of root section of cv. Soymax treated with antiserum to *F.oxysporum* and FITC-antibodies of goat specific for rabbit globulin

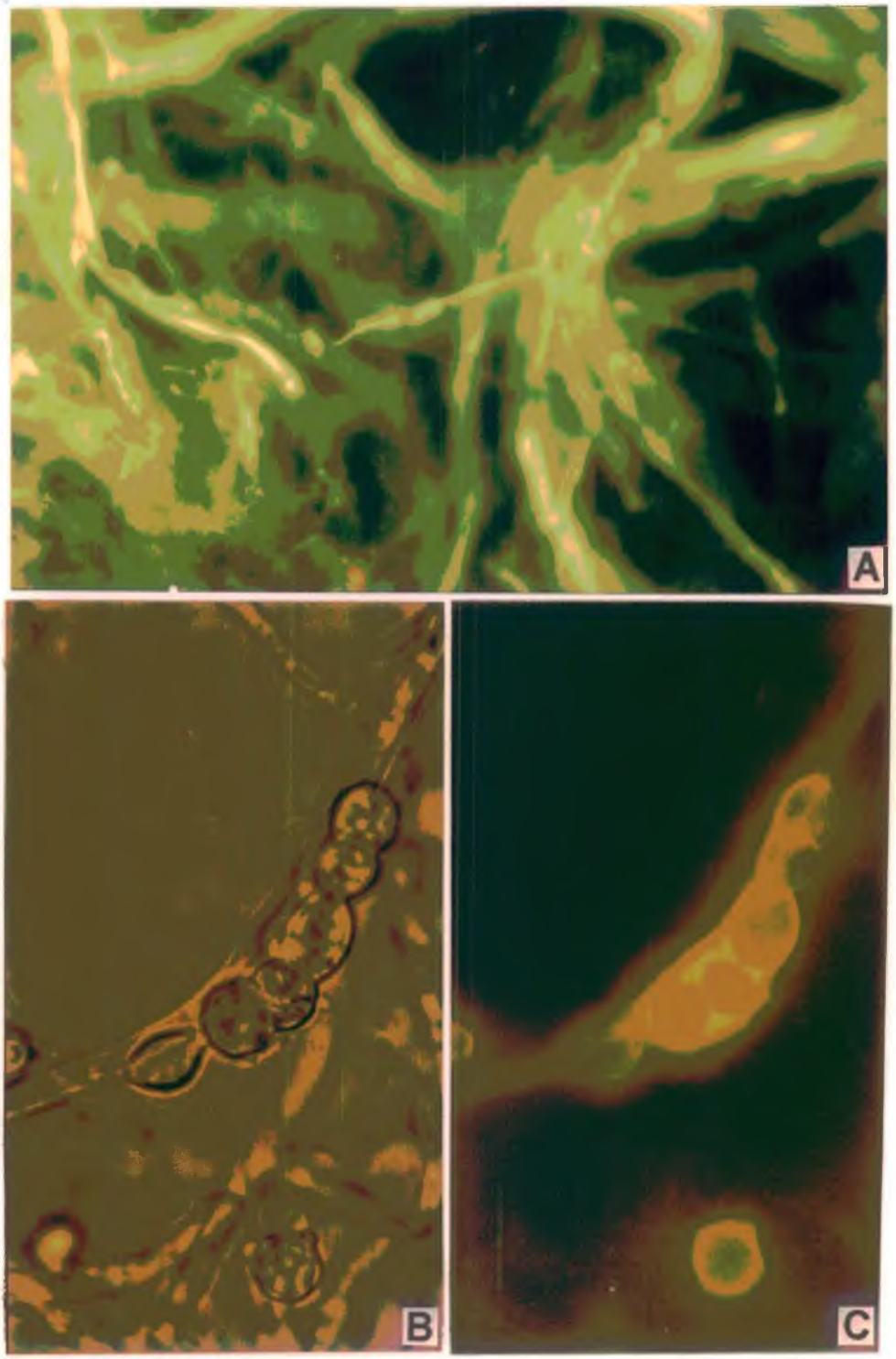


Plate XIV (figs. A - C) :Mycelia (**A**) and chlamydospores (**B&C**) of *F.oxysporum*. **A & C** - Treated with antiserum to *F.oxysporum* and FITC - antibodies of goat specific for rabbit globulin; **B** - Under light microscope