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The Possible Induction of Resistance in *Lupinus termis* L. Against *Fusarium oxysporum* by *Streptomyces chibaensis* and its Mode of Action: I. Changes in Certain Morphological Criteria and Biochemical Composition Related to Induced Resistance

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ABSTRACT

Much attention has been focused on examining the sequence of different changes that are triggered upon invasion of a pathogenic organism to a higher plant. Of the most rapid changes are those related to the morphological appearance of the infected plant. In the present work, growth of *L. termis* in *Fusarium*–pathogenized soil points to marked increases in root and shoot length, decrease in fresh and dry weights of roots and obvious increase and decrease in fresh and dry weights of shoots, respectively. Such changes were associated with marked decreases in soluble sugars, starch, Ca and Mn contents of the roots. The present results revealed also a prominent increase in soluble proteins and P content of roots at the 1st and 2nd stages of growth. With the exception of an elevated level of soluble sugars and Mn, the shoot system of *L. termis* suffered from marked decreases in starch, soluble proteins, P, Ca contents as a result of *Fusarium* pathogenesis. The chlorophyll content of leaves subjected to obvious decrease (chl. *a)* and marked increase (chl. *b*) in response to *Fusarium* investment. Growth promotion induced by *Streptomyces chibaensis* was underlain with an obvious increases in shoot length, fresh and dry weights of roots and shoots of *L. termis*. Enhanced growth of shoots was synchronized with marked increases in soluble sugars, starch, soluble proteins and the chlorophyll a and b contents. Moreover, there was an obvious increase in the shoot content of Ca, P and Mn. On the other hand, a marked decrease in root length and its content of soluble sugars and Ca was observed and was associated with increase in the content of starch, soluble proteins, P and Mn. In response to *F. oxysporum* the yielded seeds were characterized by a relatively low content of starch, soluble proteins, total nitrogen, P, Ca, Fe, Zn and Mn, associated with high level of soluble sugars and obvious decrease in the activity level of peroxidase and catalase. *S. chibaensis* when applied alone or in combination with *F. oxysporum*, on the other hand, induced marked increases in the seed content of all the above mentioned parameters. Moreover, *S. chibaensis* had provided the seeds with unique proteins which playing crucial role against pathogenic invasion, providing the seeds with rapid reactivity against pathogens. Moreover, some of these proteins have antimicrobial activity and others initiate the lyses of fungal cell walls.

Key Words: *Lupinus termis;* Streptomycetes; *Fusarium;* Resistance; Biological control

INTRODUCTION

Use of intensify farming and mass monoculture resulted in the extensive use of chemical pesticides to control plant pests and diseases which account for considerable economic loss annually. It is estimated that $26 billion is invested in pesticides to control plant pests; otherwise, an additional $330 billion in losses would be incurred (Agriose, 1997). However, the extensive use of chemicals is restricted due to concerns for the environment and health. Thus, the biologists are facing the challenge of finding more effective, safer and economical ways to protect plants against pests. Utilization of plants' own defense mechanisms to induce resistance can minimize this problem (Tuzun & Kloepper, 1995). In this regards, Osbourn (2001) stated also that the application of plant's own defense response, utilization of antimicrobial compounds produced by the plant, might be option to traditional crop protection.

Because of the enhanced protection afford by induction of the resistance through exposure to a pathogen, the term "induced resistance" has been used synonymously with "acquired resistance", "acquired immunity" and "immunization" (Kuc, 1983). The term immunization is misleading (Urban *et al.,* 1990), because plants, unlike animals, neither possess circulatory system, nor immune surveillance. Induced disease resistance has been adopted as a general term and defined as "the process of active resistance dependent on the host plant's physical or chemical barriers, activated by biotic or abiotic agents (Van loon, 1997).

Systemic acquired resistance (SAR), has been well characterized in relation to primary inoculation with foliar pathogens while inoculated plants with non-pathogenic strains exhibit a form of systemic resistance known as induced systemic resistance (ISR) (Van loon *et al.,* 1998; Pieterse *et al.,* 1998). Foliar diseases were intensively studied (Ellis *et al.,* 2000; Feys & Parker, 2000). In contrast, reports are scanty about factors that determine the outcome

of interactions between soil borne pathogens and their hosts, even though root diseases have considerable economic impact (Deacon, 1996).

A subgroup of rhizosphere bacteria and fungi can effectively induce resistance, while providing other beneficial effects to plants (Tuzun & Kloepper, 1994). Free living root and soil bacteria as inoculants for enhancing plant defensive responses may increase the chance of their applicability and offer a practical way to deliver plant immunization (Tuzun & Kloepper, 1995).

Plant growth promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that are able to aggressively colonize plant roots and stimulate plant growth when applied to roots, tubers or seeds (Weller, 1988). Among other effects, PGPR induce systemic resistance (Kloepper *et al.,* 1993) and act as effective agents against several pathogenic fungi (Leeman *et al.,* 1995).

Various plant products encoded by host defense genes which induce cell wall modifications such as lignifications, an accumulation of secondary metabolites, such as phytoalexins, phenolics, and pathogen related (PR) proteins and other defense related proteins were induced by PGPR **(**Collinge *et al.,* 1993). These proteins have an advantage over other phytochemicals from a biotechnological viewpoint , in that each protein is encoded by a single gene which can be isolated and used to genetically engineer crops for enhanced pest resistance (Constabel, 1999).

Protease inhibitors (PIs) are proteins that tightly bind proteolytic enzymes and thereby inhibit their activity (Richardson, 1991). Plant PIs are also known to inhibit microbial proteases, and since PIs may also be pathogen-induced, they may play a role in defense against pathogens (Duffey & Stout, 1996).

Several studies provide evidence that immunization of plant with biotic or abiotic inducers effectively controls disease in the field. Utilization of pathogenic organisms in the field, however, may create problems of handled carelessly. Culture filtrates of pathogenic organisms may provide a safer alternative, and several studies have been indicated induction of resistance in many crop species by microbial metabolites (Kopp *et al.,* 1989). Barker (2000) stated that the latest discoveries in genetics can engineer disease resistance in plants which was impossible by traditional breeding. On the other hand, Kuc (2001) indicated that utilization of SAR to control disease could be used in combination with the genetic application. Sensitization of plants by SAR and traditional breeding for broad genetic resistance could be an alternative strategy for genetic modification.

Streptomycetes spp. is gram positive filamentous bacteria. They produce and secrete a wide array of biologically active compounds, including antibiotics, ionophores and a wide range of hydrolytic enzymes (Madigan *et al.,* 2000). These bacteria share characteristics with PGPR, make them attractive candidates as biological control agents against soil borne plant pathogens (Samac & Kinkel, 2001). White lupin (*Lupinus termis* L.) seems to be grown as a minor winter crop all over Egypt as economically useful leguminous crop. *F. oxysporum*. is a vascular pathogen, responsible for the bayoud diseases of lupin.

The aim of the present work is to search for the definition of possible changes induced in certain growth parameters and in biochemical composition related to induced resistance in lupin, type of defense induced via exploring *Streptomyces* mode of action.

MATERIALS AND METHODS

Biological materials. White Lupin (*Lupinus termis* L. cv. Giza 1) seeds were supplied by the Ministry of Agriculture, Giza, Egypt. Surface sterilization was done with 70% (v/v) ethanol for 60 min and 30% (v/v) hydrogen peroxide for 20 min and then soaked for 4h in sterile distilled water (Kudryavtseva *et al.,* 1998).

The present work also employed the use of water culture suspension of both *Streptomyces chibaensis* and *Fusarium oxysporum* which were kindly provided by Microbiology Section, Department of Botany, Faculty of Girls , Ain Shams University, Cairo.

Experimental design. Earthen pots (35 cm diameter) were equally filled with garden soil, and divided into three main groups, each of 32 pots. Pots were arranged inside the botanical garden of Botany Department, Faculty of Girls, Ain Shams University. Previously sterilized seeds were sown at the rate 10 per pot at the first of December. After emergence, seedlings were thinned per pot as possible for comparable ones. Plants were irrigated regularly with tap water. The experiment was conducted under natural conditions of temperature and irradiance.

First treatment. Twenty days after sowing (DAS), the pots were divided into three groups: the 2nd group of pots was inoculated with *Streptomyces* culture suspension at the rate of 30 mL per pot (T_1), while the 3rd group of pots was invested with 30 mL of *Fusarium* culture suspension per pot (T_2). Same supporting dose was given again to 2nd & 3rd groups of pots after 10 days later. The first group of pots was left untreated serving as control.

Second treatment. The 2nd group of pots previously inoculated with *Streptomyces chibaensis* (T_1), was subdivided into two batches. One batch of them was further inoculated with 30 ml of *Fusarium* culture suspension after ten days of *Streptomyces* treatment (T_3). The 3rd group of pots (*Fusarium* treated ones) was also subdivided into two batches, one of them was further inoculated with 30 mL of *Streptomyces* culture suspension after ten days of *Fusarium* treatment (T_4).

Harvesting times. The plants were harvested in their 35, 55 and 75 days old. To get yield, 8 pots of each treatment was left aside. For the determination of carbohydrate fractions and certain elements content of shoots and roots of untreated and treated plants the tissues were oven dried at 80°C for 48

h. The chlorophyll, soluble protein contents were determined in fresh samples. The oven-dried seeds of untreated and variously treated plants subjected to analysis of their contents of total-N, certain elements, and certain carbohydrate fractions. To detect the activity level of some enzymes (peroxidase & catalase), soluble protein and proteins pattern, the fresh seeds were cooled in liquid nitrogen and stored at -4°C in the refrigerator.

Growth Parameters. Certain growth criteria of untreated and variously treated plants were recorded and were statistically analyzed using estimated marginal means at 0.05% of probability.

Molecular Analysis. Continuous denaturating polyacrylamide gel electrophoresis (SDS-PAGE) in 1mm thick 15% acrylamide gel was performed by means of vertical slabe gel (Bio Rad, Protean IIXI cell) for protein pattern. For extracting soluble proteins, one grame of frozen tissue (seeds) was grounded with 0. 1 ml of buffer containing 0. 125 M Tris-HCl; pH 6. 8; 4% SDS; 20% glycerol; 10% 2-mercaptoethanol and 1% bromophenol blue as a tracking dye.

The homogenates were boiled in a water bath (100°C) for 1min using a micro centrifuge tubes. After which samples were left at room temperature then centrifuged for few seconds. The previous samples (140 µL) were loaded onto gel wells for electrophoresis as described by Laemmli (1970). Separation was performed at constant current of 20 mA per gel, left over night at room temperature. Molecular mass markers ranged from 8-200 KD were used. Gel was stained with Coommassie brilliant blue (R-250) and destained with methanol acetic acid mixture. Gel was photographed, and scanned for data analysis.

Biochemical methods. The chlorophyll content of leaves was determined according to the method of Hiscox and Israelstam (1979).

For soluble protein extraction two grames of frozen tissues were homogenized with 10 mL of 50 mM phosphate buffer solution (pH 7. 0) containing 0. 07% of NaH_2PO_4. $2H_2O$ and 1. 6% Na_2HPO_4. $12H_2O$ grounded with a mortar and pestle, and centrifuged at 15, 000g for 25 min in a refrigerated centrifuge (Fu *et al*., 2000). Estimation was carried out according to Lowry *et al.* (1951) with little modification (Copeland, 1994), using soluble bovine serum albumin as a standard.

Soluble sugars and starch were extracted following the method of McCready *et al*. (1958). The anthrone-sulphuric acid reagent was used for the determination of soluble sugars and starch (Malik & Singh, 1980).

For extraction of certain elements, oven dried samples were digested with acid mixture (conc. nitric, sulphuric, perchloric acids) as described by of Chapman and Pratt (1978). Calcium and phosphorus content were determined spectrophotometrically (Adrian & Stevens, 1977; Holman & Elliott, 1983). Potassium content was determined following Mengel and Kirkby (1980) method. Iron, zinc and manganese were determined spectrophotometrically according to Allan (1961). The total nitrogen content was determined according to Jacobs (1978). Frozen seed samples were used for peroxidase and catalase extraction (Fu *et al.,* 2000). Peroxidase, catalase enzyme activity were estimated according to Aebi (1974) and Kar and Mishra (1976).

RESULTS AND DISCUSSION

The results (Table I) indicated that growth of *Lupinus termis* in pathogenized soil (soil invested with *Fusarium oxysporum*) was greatly affected. Generally significant increments were observed in length of roots and shoots throughout the three developmental stages of growth (Table I).

The increase in root and shoot length may be related to the action of cellulases and pectinases of pathogen on host cell walls which would decrease the level of lignin cell wall – bound phenolic compounds (Rioux & Biggs, 1994; Ikegaw *et al.,* 1996), affect mechanical properties of cell

Table I. Effect of soil inoculation with *Streptomyces chibaensis, Fusarium oxysporum* or both of them on certain growth parameters of *Lupinus termis* L. shoots, roots at different stages of growth and development. Values represent the means of four separate measurements

	Parameters	Shoot Length (cm)			Fresh Weigh (g)			Dry Weight (g)		
		DAS			DAS			DAS		
	Treatments	35	55	75	35	55	75	35	55	75
Shoots	Control	6.75	12.00	20.00	2.80	4.60	8.20	0.42	0.77	0.98
	T1	8.00*	15.50*	29.00*	5.40*	8.70*	9.80	1.42*	1.50*	1.97*
	T2	8.00*	17.75*	35.00*	4.00	5.00	9.17	0.40	0.65	0.83
	T3		16.00*	32.00*		9.50*	12.50*		1.37*	1.80*
	T4		17.00*	31.50*		6.05	10.40*		0.87	1.24
Roots	Control	20.30	35.00	42.00	1.04	6.50	8.40	0.19	0.69	0.94
	T1	17.10*	29.50	40.00	1.20	10.80*	12.50*	0.41*	1.01	1.31
	T2	22.40	50.00*	86.00*	0.90	5.96	8.00	0.14*	0.62	0.74
	T3		44.00*	59.80*		8.70*	13.50*		1.10*	1.50*
	T4		55.00*	69.00*		7.70	9.00		0.90	1.20

DAS : days after sowing; T1: *S.chibaensis* treatment, T2: *F.oxysporum* treatment, T3: *S.chibaensis* followed by *F.oxysporum* treatment, T4: *F.oxysporum* followed by *S.chibaensis* treatment; Asteriks indicate a significant difference from the control at P=.05

Table II. Effect of soil inoculation with *Streptomyces chibaensis, Fusarium oxysporum* or both of them on the content of certain charbohydrates of *Lupinus termis* L. shoots, roots at different stages of growth. Values listed are expressed as mg g^{-100} Dry weight

Parameters		35 DAS		55 DAS		75 DAS	
Treatments		Soluble Sugars	Starch	Soluble Sugars	Starch	Soluble Sugars	Starch
Shoots	Control	10000	15700	11000	28260	11800	12900
	T1	18000	18360	19060	31930	20500	24620
	T2	12260	9090	12000	18260	15000	12000
	T3			13150	19720	14570	24590
	T4			11500	11290	14300	16500
Roots	Control	5000	5900	6190	6940	6860	8420
	T1	4820	7420	5750	11040	6590	11280
	T2	3400	5090	3690	5390	3780	8010
	T3			5760	7760	6020	8890
	T4			4520	7200	5850	8550

DAS : days after sowing; T1: *S.chibaensis* treatment, T2: *F.oxysporum* treatment, T3: *S.chibaensis* followed by *F.oxysporum* treatment, T4: *F.oxysporum* followed by *S.chibaensis* treatment

walls, result in cell wall length (Boudet, 2000).

The fresh weight of roots decreased infected with *Fusarium oxysporum* which may be due to the presence of low concentration of manganese ions (Tables I and V) relative to the control. It may decrease the degree of water retention of root cells (Yagodin, 1984). Decrease in fresh weight also be related to the action of phenolics produced from cell wall degradation (lignin) mainly via depolymerization resulted from fungal infection (Steijl *et al.,* 1999). The phenols induce impairment of cell membranes leading to efflux of water (in addition to metabolites), and maceration of roots. Moreover, the decrease in fresh weight of roots may be due to toxins produced by the fungi which affected K^+ uptake and stomata function leading to uncontrolled transpiration and excessive loss of water, leading to wilted plants (Aducci *et al.,* 1997).

The general significant decrease in dry weight of roots infected with *F. oxysporum*-pathogenized soil may be due also to accumulation of phenols in roots particularly at the 1st and 2nd stages of growth (Nafie, in preparation). Accumulated phenols in roots will affect metabolic activities via certain metabolic keys as: increasing permeability of membranes, thus increasing the accessibility of respiratory enzymes to their substrates, hence an increase in respiratory rate is expected which account for the decrease encountered in dry weight of roots. The observed decrease in dry weight of roots may also be due to high rate of efflux of metabolites into the growth medium, under the influence of toxins produced by *F. oxysporum* (Nemec, 1995). Increased rate of leakage of metabolites could partly be responsible for the general marked decrease detected in contents of roots soluble sugars, starch and soluble proteins (3rd stage) (Tables II, III). On the other hand, part of the decrease recorded in roots contents of soluble sugars, starch and soluble proteins may be related to the obvious decrease in K^+ content (Nafie, in preparation). Low level of K^+ results in a decrease of osmotic potential of host tissues (Orcutt & Nilsen, 2000) which facilitate the flow of nutrients from host to pathogen. This may be important in controlling infection and the spread of the pathogen.

The marked increases observed in fresh weight of shoots (associated with obvious decrease in dry weight) in *F. oxysporum* pathogenized soil, may be due to increase in water content of tissues (Table V).

Table III. Effect of soil inoculation with *Streptomyces chibaensis, Fusarium oxysporum* or both of them on soluble protein content of *Lupinus termis* L. shoots, roots at different stages of growth. Values listed are expressed as mg g^{-100} fresh weight

Stages		Days after sowing		
Treatments		35	55	75
Shoots	Control	5710	14260	7600
	T1	5830	14720	9480
	T2	5220	7660	3500
	T3		6990	8600
	T4		9690	5770
Roots	Control	1970	4460	3960
	T1	2140	5210	6880
	T2	2000	4800	2600
	T3		3190	5100
	T4		2660	6660

T1: *S.chibaensis* treatment, T2: *F.oxysporum* treatment, T3: *S.chibaensis* followed by *F.oxysporum* treatment, T4: *F.oxysporum* followed by *S.chibaensis* treatment

The decrease in dry weight of the shoots may be due to increased rate of respiration , decompartmentalization due to membrane degradation, (Orcutt & Nilsen, 2000), and low rate of photosynthesis. Decrease in chlorophyll *a* (Table IV) was observed, may be a consequence of the fungal effect on the release of transported toxins which leads to the liberation of reactive oxygen species (ROS) (Elstner *et al.,* 1985). Toxins produced by the *Fusarium* were also reported to induce inhibition of chlorophyll biosynthesis (Achor *et al.,* 1993). The changes induced by pathogens can influence partition of photoassimilates among the important organic compounds used in metabolism (Orcut & Nilsen, 2000). The *F. oxysporum* induced a considerable shift in roots

toward the formation of nitrogenous constituents particularly during the acute period of infection (35 & 55 DAS), while this blocked at latter stage (75 DAS) a sharp decrease in soluble proteins was also observed (Table III). The marked decrease in content of carbohydrate and soluble proteins at late stage of growth (Tables II, III) can be related to leakage of soluble metabolites.

The shoot system, on the other hand, respond to *F. oxysporum* by accumulating of soluble sugars which were associated with significant decrease in nitrogenous compounds (soluble proteins). Accumulation of soluble sugars in the shoot may be due to retarded rate of translocation under the influence of cell wall degrading enzymes or toxins produced by *F. oxysporum* which may induce blockage of transfer via phloem elements (Heiser *et al.,* 1998) or inhibit the synthesis of sucrose translocator (Emes & Neuhaus, 1997). Moreover, retarded rate of synthesis of the main nitrogenous compounds in shoots (Table III) may be related to a depletion of energy demand. It is hard to predict how the presence of *F. oxysporum* and/or its metabolites can influence the capacity of tissues in energy conservation. However, it may be speculated that the toxins produced by *F. oxysporum* may act as uncouplers and inhibit ATP synthesis (OBwald & Die Wirt, 1995).

Several inorganic nutrients have been associated with changes in disease development in plants. Calcium is one of the essential elements which plays crucial role in disease development. The marked decrease obtained in Ca^{2+} content of both roots and shoots of plants grown in *F. oxysporum* invested soil (Table V) may be responsible of many biochemical changes, as well as, symptoms of diseases induced by *F. oxysporum*. Thus, the decrease in Ca^{2+} content of treated plants may be responsible of decreasing the integrity of cell membranes and hence increase their permeability and increased leakage from tissues invaded by *F. oxysporum* or their metabolites. Moreover, decreased Ca^{2+} under *Fusarium* pathogenesis may interfere with uptake of toxic ions from the soil (Orcutt & Nilsen, 2000).

Phosphorus also was reported among the macronutrients which affect resistance or susceptibility of plants to pathogenic organisms. In the present work, marked increase in phosphorus content of root system of treated plants was observed at the 1st and 2nd stages of growth followed by slight decrease at the third (Table V). Increased level of phosphorus of treated plants may be responsible for increasing susceptibility of *Lupinus termis* to fungal diseases induced by *F. oxysporum*. In this connection, phosphorus was reported to reflect the strategy and growth requirement of fungi or viruses (Van Veen *et al.,* 1997). Phosphorus was also reported to be important in maintaining a proper balance of nutrients within the plant. The shoot system of *L. termis* suffered from obvious decrease in phosphorus content under the influence of *F. osysporum* (Table V). This decrease may be due to the accumulation of phosphorus in roots of the same plants as a result of *Fusarium* invasion which may convert the root

Table IV. Effect of soil inoculation with *Streptomyces chibaensis, Fusarium oxysporum* or both of them on cholorophylls level of *Lupinus termis* L. leaves at different stages of growth. Values listed are expressed as µg g^{-1} Fresh weight

Parameters / Treatments	35 DAS			55 DAS			75 DAS		
	Chl *a*	Chl *b*	Total Chl (*a*+*b*)	Chl *a*	Chl *b*	Total Chl (*a*+*b*)	Chl *a*	Chl *b*	Total Chl (*a*+*b*)
Control	800	337	1137	1000	430	1430	1100	500	1600
T1	1067	750	1817	1600	934	2534	1800	1000	2800
T2	700	360	1060	840	650	1490	839	601	1439
T3				1440	1050	2490	1455	1083	2537
T4				900	690	1590	1200	1100	2300

Table V. Effect of soil inoculation with *Streptomyces chibaensis, Fusarium oxysporum* or both of them on the content of certain elements of *Lupinus termis* L. shoots, roots. Values listed are expressed as mg g^{-100} Dry weight

	Parameters / Treatments	Manganese			Calcium			Phosphorus		
		DAS			DAS			DAS		
		35	55	75	35	55	75	35	55	75
Shoots	Control	3.1	5.8	8.3	1407	1243	1165	390	350	400
	T1	3.5	6.1	13.2	1424	1247	1249	400	410	410
	T2	5.3	6.9	10.0	1205	1137	582	370	330	360
	T3		8.6	10.8		1254	1119		350	380
	T4		6.0	8.6		1250	648		380	140
Roots	Control	2.3	2.1	2.1	1297	1123	1003	150	280	400
	T1	2.7	4.0	2.8	1129	678	1004	380	330	430
	T2	1.6	2.0	2.0	1263	1053.	571	410	350	360
	T3		3.1	3.1		509	710		362	380
	T4		2.5	2.0		681	1108		300	250

DAS : days after sowing; T1: *S.chibaensis* treatment, T2: *F.oxysporum* treatment, T3: *S.chibaensis* followed by *F.oxysporum* treatment, T4: *F.oxysporum* followed by *S.chibaensis* treatment

Table VI A. Effect of soil inoculation with *Streptomyces chibaensis, Fusarium oxysporum* or both of them on the activity of peroxidase, catalase enzymes, certain carbohydrates, nitrogen constituents of the yielded seeds of *Lupinus termis* L. plants

Parameters Treatments	Peroxidase Activity $g^{-1}h^{-1}$	Catalase Activity $g^{-1}h^{-1}$	Soluble Protein mg g^{-100}F.wt.	Soluble Sugar mg g^{-100}Dry wt.	Starch mg g^{-100}Dry wt.	Total Nitrogen mg g^{-100}Dry wt.
Control	170.0	200.0	5000	20000	16900	22000
T1	218.7	289.4	9400	29870	24100	38000
T2	162.8	195.1	4070	24720	15930	20000
T3	197.1	238.3	5280	43240	17260	30200
T4	226.8	252.6	4460	21000	17180	20500

Table VI B. Effect of soil inoculation with *Streptomyces chibaensis, Fusarium oxysporum* or both of them on the contents of certain elements of the yielded seeds of *Lupinus termis* L. plants, values listed are expressed as mg g^{-100} Dry weight of seeds

Parameters Treatments	Phosphorus	Calcium	Potassium	Iron	Zinc	Manganese
Control	450.0	1170.0	900.0	2.50	0.39	2.10
T1	520.0	1208.0	1650.0	3.00	0.48	2.68
T2	440.0	1166.0	700.0	2.32	0.32	2.08
T3	530.0	1200.0	1200.0	9.44	0.66	3.20
T4	430.0	650.0	550.0	3.05	0.20	2.62

T1: *S.chibaensis* treatment, T2: *F.oxysporum* treatment, T3: *S.chibaensis* followed by *F.oxysporum* treatment, T4: *F.oxysporum* followed by *S.chibaensis* treatment

system to an active sink for the most important elements pre-requisite for successful invasion and pathogenesis.

Streptomyces chibaensis, is the element used in the present work as a tool to counteract or decrease the above mentioned unfavorable biochemical changes induced by the *F. oxysporum*. Its mode of action in this respect involves improving the metabolic state of the host (inducing resistance) prior to pathogen invasion as a primitive strategy and/or curing if it is already affected by the pathogen. Streptomycetes are gram positive filamentous bacteria that produce and secrete a wide array of biologically active compounds including antibiotics, hydrolytic enzymes and enzyme inhibitors. Many species of Streptomycetes are rhizobacteria that effectively colonize plant roots, influence plant growth and protect plant roots from pathogens. They are resistant to desiccation and nutrient stress. Streptomycetes recommended belonging to the plant growth promoting rhizobacteria (PGPR). PGPR not only can stimulate plant growth when applied (Weller, 1988), but were also reported to interact with plant roots and have the capacity to activate plant defense (Kloepper *et al.,* 1992). They were also observed to induce resistance systemically in plants (Kloepper *et al.,* 1999).

These characteristics make Streptomycetes attractive candidates as resistance inducer to protect plant against soil borne plant pathogens (Samac & Kinkel, 2001).

In the present work, soil drench with *Streptomyces* either alone or in combination with *Fusarium* (before or after *Fusarium* investment) induced prominent changes in different growth parameters and certain metabolic activities of *L. termis* plants (Table I-V). Moreover, most of the disease symptoms induced by *F. oxysporum* as wilt, chlorosis, curling, were mostly alleviated (Tables IV, V).

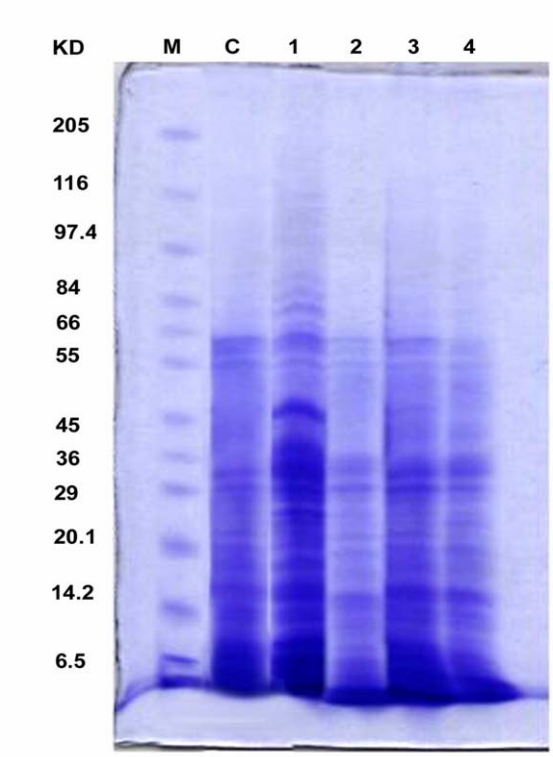
So, it was concluded that the addition of *Streptomyces* or its metabolites to the growth medium of *L. termis* may induce resistance against pathogens, whether they were present or absent in rhizosphere. Hence, the mechanisms induced by *Streptomyces* (applied alone or in combination with *Fusarium)* and employed in changing growth parameters, metabolic activities and in induction of resistance to *F. oxysporum* will be discussed together.

With the exception of the marked decrease observed in root length, *Streptomyces chibaensis* significantly enhanced growth of root and shoot systems represented as marked increases in shoot length, fresh and dry weights of roots and shoots (Table I).

The obvious decrease obtained in root length may refer to that one of the principle defense mechanisms induced by *Streptomyces* which may involve mechanical mechanisms from early stages of pathogenesis. This will limit the action of cellulases and pectinases of *F. oxysporum* on cell walls of roots of *L. termis*. At latter stages, chemical mechanisms will predominates which might inhibit the biosynthesis of these pathogenic extra cellular cell wall-degrading enzymes.

In accordance to this explanation, Steijl *et al.* (1999), El Modafer and Boustani (2000), De Ascensao and Dubery (2000) suggest that resistance inducer to *F. oxysporum* affected carnation, radish; date palm and banana roots respectively may be due to enhanced polymerization of monoligonal phenol compounds resulting in increased lignin levels via activating enzymes involved in cell wall strengthening (peroxidase, polyphenol oxidase).

Fig. 1. SDS-PAGE electrophoresis analysis for yielded seeds. Lanes: C- Seeds of untreated plants. 1- Seeds of plants grown in *S. chibaensis* inoculated soil. 2- Seeds of plants grown in soil inoculated with *S. chibaensis* followed by *F. oxysporum*. 3- Seeds of plants grown in soil infested with *F. oxysporum*. 4- Seeds of plants grown in soil infested with *F. oxysporum* followed by *S.chibaensis*. M- indicates molecular mass marker.



The marked increase in length of the shoot system of *L. termis* induced by *Streptomyces chibaensis* could be due to their efficiency in the production of growth hormones (Lippman *et al.,* 1995). Moreover, this increase could be due to increases in calcium content of the shoot system (Table V). Such increase will enhance cell division and expansion (Jauneau *et al.,* 1994).

The marked increase in fresh weight of both roots and shoots of *L. termis* in response to *Streptomyces* could be due to increased level of the water retained in tissue as a result of the marked increase recorded in their content of manganus ions (Table V). Increased lignin level induced by PGPR (Steijl *et al.,* 1999) may act as an efficient water proofing polymer at the surface of plant tissues which control the water loss via these surfaces and hence an increase in fresh weight could be explained on this base.

In response to soil drench with *Streptomyces* an obvious increase in dry weight of both shoots and roots was observed. Such change refer to the interference of *Streptomyces chibaensis* with different biochemical activities as it is evident from the marked changes induced in the main metabolic constituents of roots and shoots. The marked increase obtained the main nitrogenous fractions of roots and shoots (Table III) particularly the soluble protein (an absolute necessity for increasing dry weight), may be due to the efficiency of rhizobacteria in enhancing nitrogen fixation process, increasing the nitrate availability (Chanway, 1997). In addition, the efficiency of rhizobacteria in increasing the available phosphorus (Table V) is harnessed to generate the prerequisite of high rate of biosynthesis of carbohydrate and nitrogenous constituents.

It is worthy mentioning here that the prevalence of carbohydrate fractions in the roots (Table II) in the form of starch rather than as soluble sugars, represents one of the strategy imposed by *Streptomyces chibaensis* in retarding or controlling infection process via root system (Bahme & Schroth, 1987; Madigan *et al.,* 2000). These circumstances (reduced level of soluble sugars) assist in osmotic regulation of roots, a candidate which retard the flow of nutrients from *L. termis* roots to the site of invading pathogen. On the other hand, an obvious increase in both soluble sugars and starch of shoot system was observed in response to *S. chibaensis* (Table II).

The data obtained indicate a relatively high level of photosynthetic pigments in response to *Streptomyces chibaensis* throughout the three developmental stages of growth (Table IV). The increased level of photosynthetic pigments induced by *Streptomyces chibaensis* may be related to their enhancement effect on the rate of its biosynthesis. Moreover, increased level of photosynthetic pigments could be attributed to the phytohormones produced by rhizobacteria (Samac & Kinkel, 2001) affecting plant hormonal balance or increasing the host cytokinin level, resulting in preservation of chlorophyll and retardation of its loss. Here it is worthy to mention that chlorosis score negatively correlated with chlorophyll concentration, and plants grown in *Streptomyces chibaensis* inoculated soil remained symptomless. Increase in chlorophylls concentration may directly affect photosynthetic rate leading to the recorded increase in both soluble sugars and starch in shoot system (Table II).

The yielded seeds of *L. termis* plants which were grown in soil infested with *F. oxysporum* were characterized by a relatively low content of starch, soluble protein and total nitrogen. This change was associated with high level of soluble sugars (Table VI). The decline in the content of macromolecules may not be attributed to a restriction in the supply of assimilates to the ripening seeds, as there was an elevated level of soluble sugars, but to fall in the capacity of seeds to utilize these building blocks (Agrawal, 1998). It seems that there exist factors, other than assimilates, operating within the seed itself which may be induced under the influence of pathogen which was important determinant of capacity of seeds to accumulate starch and proteins. Moreover, the obvious decline in the element content of seeds (P, Ca, K, Fe, Zn and Mn) (Table VI B) may be responsible for the impairment of the synthetic machinery as

those responsible for the synthesis of major storing stuffs of the seeds as starch and proteins. Reduced activity level of catalase and peroxidase in seeds in response to *F. oxysporum* may retard the polymerization of lignin monomers, reduce lignifications of cell walls, hence the seeds could be easily attacked by pathogens (Duffey & Stout, 1996) Moreover, reduced level of these enzymes may lead to accumulation of peroxides which are predicted to induce lipid per oxidation and the derangement of cell membranes.

The appreciable increase of the seed content of major metabolites (soluble sugars, starch, soluble proteins and total nitrogen) in addition to the existence of elevated level of the elements P, Ca, K, Fe, Zn and Mn (Tables VI A, B) in response to *S. chibaensis* are likely to indicate an enhanced metabolic activity. These results may refer to the interference of *S. chibaensis*, through different mechanisms, with the pivotal synthetic processes of carbohydrate and nitrogenous compounds. Expected increased metabolic activities of seeds in response to *S. chibaensis* may be related to its behavior as PGPR (Samak & Kinkel, 2001). PGPR as plant growth hormones producer will enhance the metabolic activity via increasing the sink activity by elevating the net amount of photoassimilates transferred from the maternal tissues to the developing seeds.

The obvious increase observed in the activity levels of peroxidase and catalase of seeds in response to *S. chibaensis* is predicted to play a crucial role in minimizing the hazards of translocated toxins produced by *F. oxysporum*. Moreover, increased activity of these enzymes may enhance lignifications of cell walls of testa and hence contribute to increased rigidity of cell walls and resistance against pathogen attacks.

Data analysis of SDS-PAGE (Fig. 1) of seeds revealed that there were three novel proteins appeared in the seeds in response to *S. chibaensis* with the molecular mass of 27, 32. 9 and 24 KD. In addition of having antifungal activity, the proteins with molecular mass 27 and 33 KD were identified to act as chitinase and β-1, 3- glucanase which catalyze the hydrolysis of chitin and β-1, 3- glucanase polymer respectively of fungal cell walls (Mauch *et al.,* 1988). The protein having molecular mass of 24 KD is related to osmotin, a member of the pathogen-related protein (PR-5) and proved to have antimicrobial activity (Woloshuk *et al.,* 1991). In response to *F. oxysporum* a protein having molecular mass 50 KD accumulated in the yielded seeds it may had critical role in *F. oxysporum* pathogenesis, as it had been induced systemically in plant leaves (Nafie, in preparation).

As a result of treatment with *S. chibaensis* followed by *F. oxysporum* and *F. oxysporum* followed by *S. chibaensis* yielded seeds SDS-PAGE analysis revealed that novel bands were recorded denoting to proteins having molecular mass of 70, 90, and 12. 5, 3. 9 KD, respectively. Among them, the protein having molecular mass of 70 KD was identified as calcium-dependent kinase protein (CDKP) which acts as a signal assistant in increasing the reactivity of tissues with pathogens and activate the synthesis of proteinase inhibitors (Farmer & Ryan, 1992).

It may be concluded that *S. chibaensis* could act professionally in the field of biological control via:

- Changing the morphological criteria of plants implicated in increasing resistance against pathogens.
- Predominance of carbohydrate fraction of roots in the form of starch rather than sugars which retard the flow of nutrients to the pathogen via osmotic regulation.
- Altering the gene expression or the over expression of genes in the yielded seeds which sustain the prevalence of conditions unfavorable for microbial attack and for vigor growth of seedlings.

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