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BARD

FINAL REPORT
PROJECT NO. I-878-85

**Sequestering of Iron as a Significant Virulence
Factor of *Verticillium Dahliae* and it's
Manipulation as a Potential Method for the
Control of *Verticillium* Diseases**

I. Barash, R. A. Olsen, J. Krikun, Y. Kashman,
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Appendix VI

Standard BARD Cover Page for Scientific Reports

Date: December 1989.

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BARD project No. 1-0878-85

Title

SEQUESTERING OF IRON AS A SIGNIFICANT VIRULENCE FACTOR OF VERTICILLIUM
DAHLIAE AND ITS MANIPULATION AS A POTENTIAL METHOD FOR THE CONTROL
OF VERTICILLIUM DISEASES.

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Project's starting date: September 1986

Type of Report: 1st Annual 2nd Annual Final X

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A. ABSTRACT

The objectives of the present project were: a) to evaluate the effect of iron applications on host tolerance towards Verticillium dahliae. b) to gain an insight into the role of iron in virulence and c) to characterize iron sequestration systems by V. dahliae and other fungal pathogens. Results presented indicate that when either FeEDDHA or foliar microelement applications containing iron were made to peanuts grown under field conditions in soil infested with the pathogen, a highly beneficial influence on plant vigor, green color of foliage and yield was obtained. No such effects were noted using the same treatments on plants grown in non-infested soil. Iron application may therefore be recommended for controlling verticillium wilt in peanuts. Iron limiting conditions increased the sensitivity of peanut, tomato, eggplant, potato and cannola to V. dahliae. Supplement of iron significantly alleviated disease symptoms in all the above mentioned crops.

The advancement and distribution of V. dahliae in iron-deficient and iron-sufficient peanut or tomato plants was similar. Iron deficiency as well as infection significantly induced phytoalexins production in peanuts. Medicago, the major phytoalexin detected, was 18.6, 23.8 and 43.4 times higher in iron-deficient healthy, iron-sufficient infected and iron-deficient infected peanuts than the healthy control. Nevertheless, phytoalexins production was not correlated with retardation of the pathogen. On the basis of the foregoing results, it was hypothesized that iron may exert its influence by rendering plants more tolerant towards virulence factors of the pathogen such as pectic enzymes, toxins or others.

Extensive studies on siderophores production and characterization of iron uptake systems was carried out with several phytopathogenic fungi. V. dahliae, Stemphylium botryosum (tomato pathogen) and Gaeumannomyces tritici produce dimerum acid and coprogen B as major hydroxamate siderophores whereas Geotrichum candidum was identified as a non-siderophore producer. All the 4 fungi appeared to possess receptors for hydroxamate siderophores of foreign origin. The kinetics specificity and mechanisms of the siderophore-mediated systems as well as iron uptake systems which do not involve siderophores (in S. botryosum and G. candidum) were characterized. Peanut and wheat plants could utilize iron from dimerum acid and coprogen B.

B. OBJECTIVES OF ORIGINAL RESEARCH PROPOSAL

Iron is an essential nutrient for most living cells and Fe^{3+} is the predominant oxidation state prevailing in aerobic environment. In spite of its abundance in nature, the availability of iron is extremely low owing to the profound insolubility of the ferric ion. Therefore, microbes, plants and animals have developed sequestering systems for the ferric ion to ensure its utilization. In bacterial and mycotic diseases of animals, iron acquisition has been established as a critical element of virulence.

The ultimate goal of the present proposal was to gain a deeper insight into the role of iron in virulence of plant pathogens and to demonstrate that iron can be manipulated for inducing tolerance towards verticillium wilt. The following points were investigated: a) The effect of iron applications on tolerance towards verticillium wilt in the field, b) mechanisms involved in the reponse of the verticillium-infected plants to iron and c) characterization of different systems present in V. dahliae and other fungal pathogens for competition of iron. Although we originally proposed to use potato as the major host for investigation in Israel, peanut was selected instead. The latter exhibits higher sensitivity to iron deficiency and was easier to grow and analyze in various field and greenhouse experiments.

C. BODY OF THE REPORT

The body of the report is presented as individual and independent manuscripts, some of which have already been published.

EFFECT OF IRON STATUS ON VERTICILLIUM WILT
DISEASE AND ON IN VITRO PRODUCTION OF
SIDEROPHORES BY VERTICILLIUM DAHLIAE

KEY WORDS: Verticillium dahliae, iron deficiency,
peanut, eggplant, siderophores.

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ABSTRACT

In peanuts cultivated in plots that were either artificially infested with Verticillium dahliae or non-infested, soil application of FeEDDHA or foliar application of Ryplex (Fe, Zn, Cu and Mn) significantly increased yield only in plots where the fungus was present. Addition of FeEDDHA to eggplants infected with V. dahliae significantly reduced disease severity in calcareous soil.

Coprogen B was the predominant siderophore produced by seven different strains of V. dahliae during a growth period of up to 18 days. Low amounts of dimerum acid and an additional unidentified siderophore were also detected. However, a decrease in coprogen B with a concomitant increase in dimerum acid and pH were measured in culture filtrates during a longer incubation period. A correlation was established between the pH value of the medium and the relative concentrations of the two siderophores. The magnitude of siderophore repression by iron varied among different isolates.

INTRODUCTION

Verticillium dahliae Kleb. is a destructive and widespread soilborne pathogen which infects numerous crop plants (Pegg, 1974). The disease is expressed by stunting, wilting and premature senescence. The fungus survives in the soil as microsclerotia which are associated with infected plant debris. As the crop residues decompose, the fungal propagules are released into the soil (Pegg, 1974).

Verticillium dahliae retards the development of peanut plants and causes irregular chlorosis and wilt. Krikun and Frank (1975) have reported that some cultivars and experimental breeder lines of peanut which has previously been found to be highly tolerant to the disease in sandy soils, succumbed to V. dahliae in calcareous loess, except when Sequestrene Fe 138 (FeEDDHA) was added into the soil close to the plants at the age of approximately 7 weeks. These results indicated that iron deficiency may increase the sensitivity of peanuts to Verticillium wilt.

Sesquestration of iron via siderophores has been established as an important determinant of virulence in bacterial and mycotic diseases of animals (Weinberg, 1984). A similar function could be assumed for siderophores in plant diseases (Neilands and Leong, 1986). A recent study with Erwinia chrysanthemi supports the latter hypothesis (Expert and Toussaint, 1985). Dimerum acid has been reported to be the sole siderophore produced by V. dahliae (Harrington and Neilands, 1982). The present study was undertaken to examine the capability of V. dahliae to sequester iron under natural conditions and further characterize the production of siderophores by this pathogen.

MATERIALS AND METHODS

Plant Materials

Peanut plants (Arachis hypogaea L. cv. Shulamit) were cultivated in infested and non-infested plots in which potatoes had been grown previously. In the infested plots, the potatoes were infected by V. dahliae. Test plots were 5 m long and consisted of three beds (3 rows/bed). The experiment was arranged in a randomized complete block design with five replications per treatment. The soil was sandy loam. The treatment with Sequestrene Fe 138 (FeEDDHA, Ciba Geigy) was applied one month after the sowing date (May 15). Foliar application of Ryplex were sprayed 2, 2.5 and 3 months after sowing. Ryplex concentrations were

0.5% for the Fe and 0.2% for Mn, Zn and Cu. Ryplex is a lignosulfonate natural chelate containing approximately 6%* w/w of either Fe, Zn, Cu or Mn. It is sprayed with urea (1%w/v) to facilitate penetration. Three meters of the center bed were hand dug at maturity for yield determination.

Eggplant seeds (*Solanum melongena* L. cv. Black Beauty) were planted in pots containing washed sand and irrigated with half Hoagland solution without iron. Roots of seedlings approximately 3 cm high were immersed in a mycelial and spore suspension for 1 h and transferred to pots (15 x 12 cm) containing mountain rendzina light clay soils with a 63% CaCO₃ content. FeEDDHA was added at the rate of 50 mg Fe/pot with the irrigation water on the 7th and 14th days after seeding. The experiment was terminated after 35 days. Shoots were oven dried at 70°C for 48 h and weighed. Chlorophyll content of leaf samples was analyzed and calculated by the method of Mackinney (1941) as described by Barak and Chen (1982). There were three replications of 7 plants for each treatment (Table 2).

Isolates and culture media

The following isolates of *V. dahliae* were obtained from infected potatoes of different countries: dv-1 (Israel), pl, pb, sb (Canada), pk (England) and C-3, C-6 (USA). The fungi were maintained on potato dextrose agar or grown in liquid medium as described previously (Manulis et al., 1984). The iron limited medium for siderophores production contained the following compounds: glucose, 20g; asparagine, 2 g; KH₂PO₄, 1.5 g; MgSO₄·7H₂O, 100 mg; ZnSO₄·7H₂O, 20 mg; MnSO₄, 20 mg; pyridoxin, 5 mg; thiamine Hcl, 10 mg and double glass distilled water (pH 4.8).

Isolation and identification of siderophores

At the end of the growth period the siderophores were extracted from the culture filtrates by benzyl alcohol and purified to homogeneity as described previously (Manulis et al., 1987). The amount of total siderophores was measured according to Subramanian et al. (1965). To facilitate structure determination, the siderophores were deferrated with 8-hydroxyquinoline (Weibe and Winkelmann, 1975). ¹HNMR spectra were recorded in D₂O on a Bruker AM360WB spectrometer and IR spectra were obtained as described previously (Manulis et al., 1984). Authentic sample of dimerum acid was obtained from D. J. B. Neillands and coprogen B was isolated from *Stemphylium botryosum* (Manulis et al., 1987). Results presented are averages of at least three different experiments.

TABLE 1
Effect of Soil Application of FeEDDHA and Foliar Applications of Minor Elements on Yield of Peanuts Growing in Verticillium dahliae-Infested and Non-infested Soil.

Treatment	Yield per plant (g)	
	Infested Soil	Non-infested soil
Ryplex* (1 spray)	76.0 ^{b**}	106.8 ^a
Ryplex (2 sprays)	88.2 ^{ab}	107.2 ^a
Ryplex (3 sprays)	79.6 ^b	91.8 ^a
FeEDDHA (10 kg ha ⁻¹)	104.8 ^a	109.2 ^a
Control	50.6 ^c	96.0 ^a

*Ryplex contained Fe, Zn, Mn and Cu

**Means within columns followed by the same letter are not significantly different (LSD=0.05)

RESULTS

Effect of iron on tolerance to V. dahliae

The presence of V. dahliae in the soil caused a reduction of approximately 50% in the yield of peanut plants (Table 1). Soil application of FeEDDHA in infested soil one month after sowing significantly increased the yield, which reached a level close to that in the non-infested soil. A significant but smaller increase in yield was obtained by one to three foliar applications of Ryplex which were applied 2 months after seeding. No significant effect of any of these treatments on yield was recorded in non-infested soil. No visible disease symptoms could be detected in the plants during the experimental period.

The effect of iron on the severity of Verticillium wilt in eggplants grown under iron limited conditions is shown in Table 2. Addition of FeEDDHA significantly increased the height and dry weight of shoots in infected plants, as well as the chlorophyll content. Addition of iron to healthy eggplants did not induce a significant effect on the height or dry weight of shoots, but increased significantly the chlorophyll content. The dry weight of shoots from infected plants to which iron was added was significantly higher than that of healthy plants which did not receive additional iron.

TABLE 2
Effect of FeEDDHA on Disease Severity Caused by Verticillium dahliae in Eggplant Growing in Calcareous Soil.

Treatment	Shoot height (cm)	Shoot weight (mg dry wt)	Chlorophyll content (mg/l)
Healthy plants	6.7 ^{a*}	150 ^{bc}	76 ^b
Healthy plants +FeEDDHA	6.6 ^a	180 ^{ab}	102 ^a
Infected plants	5.3 ^b	120 ^c	54 ^c
Infected plants +FeEDDHA	7.2 ^a	220 ^a	81 ^b

*Within columns, means followed by a common letter are not significantly different (LSD=0.05).

Identification and production pattern of siderophores

Siderophores were extracted from filtrates of 18-day-old cultures and initially purified by gel filtration through a column of Bio-Gel P-2 (2.5 x 115 cm). Three major components which eluted with double distilled water from the column were designated as A, B and C according to their migration rate towards the cathode in paper electrophoresis. Siderophore C was a highly cationic compound, whereas B and A were only slightly cationic in pyridine-acetate buffer, pH 5.2. Further purification was achieved by chromatography on Cellex CM column which exhibited differential adsorption towards the three siderophores. Each siderophore was brought to homogeneity by employing preparative paper electrophoresis and Sephadex LH-20 column, as described by Manulis et al. (1987). Initial identification of siderophore B as dimerum acid and C as coprogen B was demonstrated by obtaining identical R_fs following co-chromatography with authentic samples in different solvents (Manulis et al., 1987). Identical migration rates of the two siderophores with authentic samples were also obtained with high voltage electrophoresis (2000 V) in pyridine-acetate buffer pH 5.2. Comparative ¹H-NMR and IR spectra of the deferrated siderophores with deferrated authentic samples supported the identity of siderophore B and siderophore C as dimerum acid and coprogen B, respectively. ¹NMR

Growth, pH changes and production of siderophores as a function of incubation period are shown in Fig. 2. Maximum total siderophore production was observed on the 24th day after the organism reached

Fig. 1. Dimerum acid (A) Coprogen B

spectra of these siderophores were identical with previously reported spectra (Zahner et al., 1963; Diekmann, 1970; Harrington and Nielsands, 1982; and Manulis et al., 1987).

the stationary phase. The concentration of total siderophores in the medium decreased sharply as the culture aged. Initial growth was followed by a decrease in pH from 4.8 to approximately 3.8 after 11 days, with a subsequent increase to 6.9 at the end of the growth period. Coprogen B was the predominant siderophore (>60%) produced by different strains of *V. dahliae* during a growth period of 18 days, whereas the amount of dimerum acid or siderophore A was minor (<20%) (Fig. 3).

FIG. 2. Production of siderophores as function of growth and pH siderophores (□); pH (○) and growth (●).

FIG. 3. Production of siderophores by different isolates after 18-day-growth period. Dimerum acid (□); siderophore A (■) and coprogen B (▨).

FIG. 4. Production of individual siderophores as a function of time. Siderophore A (A); dimerum acid (B) and coprogen B (C).

Results in Fig. 4 illustrate that the amount of coprogen B is significantly reduced after 14 days with a corresponding increase in dimerum acid. After 30 days, dimerum acid became the major siderophore produced by *V. dahliae*. Because a significant elevation in pH was recorded after 14 days (Fig. 2), it was assumed that the changes in the relative amounts of the two siderophores after this period might be influenced by pH. A sequential decrease in coprogen B and an increase in dimerum acid were correlated with a rise of the pH (Fig. 5a). A correlation was also established between an increase in total siderophores production and elevation in pH to 6.5 (Fig. 5b). The response of different isolates to repression of siderophore production by iron varied among different isolates (Fig. 6). The concentration of iron required for total repression of siderophores was in the range of 0.2 to 3 mg/l.

DISCUSSION

The development of non-infected peanut plants in plots heavily infested with *V. dahliae* (Table 1)

FIG. 5. Production of total and individual siderophores as a function of pH. Siderophores were determined after 20 days growth. Top: Production of individual siderophores: Coprogen B (C); dimerum acid (B) and siderophore A (A). Bottom: Production of total siderophores in various buffers at (0.1 M); citrate (X); phosphate (O); citrate-phosphate (Δ).

permitted study of the capability of the pathogen to deprive the plant of iron even when the former was present ex planta. Peanuts are highly sensitive to iron deficiency and have been used for evaluation of iron status in the soil (Barak and Chen, 1982). The dramatic increase in yield following FeEDDHA application to infested, but not to non-infested soil (Table 1) indicates that this fungus can efficiently sequester iron from the environment and compete for iron with plants and presumably with common microorganisms as well. Although peanut may serve as a host to *V. dahliae* (Krikun and Frank, 1975), the absence of *Verticillium* wilt in spite of the presence of the pathogen in the soil could result from differential host specificity. As indicated earlier,

FIG. 6 Effect of iron concentration on siderophore production by various isolates. dv-1 (O); pl (Δ); sb (●).

the pathogenic isolate originated from infected potatoes and was presumably avirulent to peanut. Nevertheless, the possibility that the root system of the peanut plants was infected cannot be entirely excluded.

Iron deficiency caused by calcareous soil significantly increased disease severity in eggplants as expressed by growth retardation and chlorophyll content (Table 2). Addition of FeEDDHA completely restored normal growth and partially restored chlorophyll content. Aggravation of disease symptoms due to iron limitation was also demonstrated during the infection of bean by *Fusarium solani* (Guerra and Anderson, 1985). *Botrytis cinerea*, for example, produced lesions on leaves of broad bean more rapidly and many more spreading lesions developed under conditions of iron deficiency (Brown and Swinburne, 1982). In the latter case the withdrawal of iron by chelates was responsible for a decrease in phytoalexins production and consequently impaired the defense response. Reduction in lignin formation in beans

infected with F. solani under iron limiting conditions (Guerra and Anderson, 1985) could facilitate penetration and colonization by the pathogen. The mechanism for increases susceptibility of eggplants to V. dahliae due to iron deficiency is not yet clear.

Dimerum acid has been previously reported as the sole siderophore produced by V. dahliae (Harrington and Neilands, 1982). In the present work, coprogen B seemed the major siderophore produced by seven different isolates of this pathogen, whereas dimerum acid and an additional unidentified hydroxamate siderophore were present in minor quantities (Fig. 3). The discrepancy between the two studies is most likely due to variations in cultural conditions. The initial pH of the culture medium used by Harrington and Neilands (1982) for example, was 6.3 as opposed to 4.8 in the present work. As illustrated in Fig. 5a, the increase in production of dimerum acid with a corresponding decrease in coprogen B was correlated with elevation of the pH to 6.3. The pattern of siderophore production as a function of growth (Fig. 2) strongly resembles the kinetics of siderophore secretion in Fusarium dimerum which also forms dimerum acid and coprogen B (Diekmann, 1977). The inverse correlation in the production of the two siderophores (Figs. 4, 5) could imply that dimerum acid is formed as a breakdown product of coprogen B. The latter phenomenon could occur by enzymatic cleavage of the ester bond which links a fusarinine unit and dimerum acid to form coprogen B (Fig. 1).

The ability of microbial pathogens to compete successfully for a vital nutrient such as iron is considered a prerequisite for infection (Neilands, 1981). In animal systems, there is a substantial body of evidence correlating siderophore production with virulence (Weinberg, 1984). The role of siderophores in the pathogenicity of V. dahliae is still unclear. The capability of the pathogen to withdraw iron from the rhizosphere and induce iron deficiency (Table 1) may occur via siderophores.

Hydroxamate siderophores have been isolated from soil (Powell et al., 1980) and it is quite reasonable to assume that they are being secreted by V. dahliae for its survival ex planta. Whether conditions exist for the production of siderophores in planta is still an unresolved question. Harrington and Neilands (1982) failed to detect siderophores in cotton plants infected with V. dahliae. Moreover, iron depletion of the host plant during infection (Table 2) may be caused by

mechanisms other than siderophores. Thus, citrate, endogenous phenolic compounds, heme or iron proteins, rather than siderophores, may be the major iron source for V. dahliae within the host plant.

ACKNOWLEDGMENTS

Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, No. 2043-E, 1987 series. The work was supported by a grant from Binational Agricultural Research and Development Funds IS-0878-85. The authors wish to thank Dr. J. B. Neillands for providing a sample of authentic dimerum acid.

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Effect of FeEDDHA soil additions on yield of peanuts
grown under field conditions

in plots free and plots infested with V. dahliae

J. Krikun, R. Barak, L. Livescu, A. Nachmias

Previous studies conducted at the Gilat Experiment Station, Negev, Israel showed that when either foliar microelement applications or FeEDDHA soil applications were made to peanuts grown in soil which was infested with V. dahliae, such treatments had a highly beneficial influence on plant vigor, green color of foliage and yield. No such effects were noted using the same treatments on plants grown in non-infested soils. In order to confirm the results of the Fe EDDHA treatments, a further experiment was conducted where four rates of Fe EDDHA applications were tested.

Materials and methods

The experiment was conducted in a loessial soil, pH. 7.9; CaCO_3 , 18%. Two plots, adjacent to each other, were used. Four years previously one of the plots was artificially infested with V. dahliae by incorporating chopped potato stems containing microsclerotia of the fungus into the soil. Both plots were identical with regard to previous cultural practices. After inoculum incorporation the plots were used for various Verticillium studies, comparing the effects of the fungus in infested and, as a check, in non-infested soil.

Prior to sowing peanuts, potato was grown on both plots. In the infested plot, Verticillium infection of potato was 100%; in the non-infested plot, no infection was observed.

The plots were prepared for peanut culture as for commercial growing. Sowing was on raised beds, two rows per bed. The cv. was Shulamit. Sowing date was 24 April. Six weeks after sowing differential treatments using Sequestrene 138 (FeEDDHA) were conducted by manually opening small furrows near the plants and applying 0, 50, 100 or 200 kg ha⁻¹ of the chelated iron. After application the furrows were covered with soil and 300 m³ ha⁻¹ of irrigation water applied. There were six replications. Plots were 5 meters long and consisted of three beds. At time of planting Bradyrhizobium was injected into the soil near the seeds.

Observations were made during the growing season for disease symptom development. To test the possibility that plants may be infected but not showing typical disease symptoms, isolations were made from the vascular bundles of symptomless plants.

In order to determine if infected plants supplied with Fe EDDHA had different V. dahliae colonization levels as compared with plants growing with no Fe EDDHA added, a study was made which compared numbers of propagule forming units in stems of infected plants growing in 0 and 100 kg ha⁻¹ Fe EDDHA amended plots. This was done by blenderizing in water 1 cm stem sections and after proper dilution, distributing 0.1 ml aliquots on sorbose agar. After two weeks incubation at 25°C V. dahliae colonies could easily be counted by noting those colonies which produced microsclerotia on this medium.

At the end of the growing season, three meters from the center bed of each plot were dug up and the yield weighed. Yield data is provided on a plant basis.

Results and discussion

Germination and growth during the first six weeks were similar in both plots. At this time, chlorosis symptoms and a slowdown of growth of the plants in the infested plots began to appear. With the application of the chelate treatments these symptoms disappeared in the treated plots in the infested plot.

Typical V. dahliae symptoms did not begin to appear until 2.5 months after sowing. They were never greater than 10% in any one treatment in the infested plot. When typical leaf symptoms appeared, consisting of intervenal chlorosis and usually marginal necrosis, vascular discoloration of the stem was always present, and V. dahliae could always be isolated. The pathogen was never isolated from the above ground portion of plants which showed no symptoms.

Counts of Verticillium colony forming units from stems from infected plants growing in 0 and 100 kg ha⁻¹ Fe EDDHA treated plots suggested no difference in colonization due to treatment; counts from 0 Fe EDDHA plots were 126 per plate as compared to 171 per plate from chelate treated plots (differences not significant).

In the infested plots, plants in the 0 Fe EDDHA plot remained chlorotic, whereas those receiving the chelate recovered from the chlorosis and developed normally. No chlorosis or stunting was observed in the 0 Fe EDDHA plots in the non-infested plots.

The yield data is presented in Table 1. As can be seen from the results, Fe EDDHA treatments greatly affected yield in the infested plots, but not in the non-infested one.

These results, which have also been obtained previously, are difficult to explain. We can at present not put forth a hypothesis

which can explain the above results. From our data it would seem that either the previous presence of a heavily infected crop (potato) influenced the soil Fe status in the following crop (peanut) or that the very presence of the pathogen in the soil and/or the rhizosphere limits Fe uptake by peanut.

In discussions with Dr. R. Chenney from the A.R.S., Beltsville, Md. the possibility was raised that saprophytic colonization of the potato stems by V. dahliae after incorporation into soil may have an affect of immobilizing soil Fe. Very preliminary laboratory results indicate that when V. dahliae produced microsclerotia there is much greater siderophore production than when the fungus is not producing microsclerotia. It may be that, if in fact this occurs under field conditions, a partial explanation of the above results may be hypothesized.

Table 1. Effect of FeEDDHA soil applications on yield of peanut growing in Verticillium dahliae infested and non-infested soil (gr. plant⁻¹)

Treatment	Infested	Non-infested
FeEDDHA kg ha ⁻¹		
0	31 a ¹ A ²	70 b B
50	61 a B	85 a B
100	72 a B	81 a B
200	82 a B	93 a B

1 - numbers followed by lower case letters different between columns at 0.05% level.

2 - numbers followed by upper case letters different within columns at 0.05% level.

Effect of nutrient supplements in Verticillium wilt
of potato

J. Krikun, S. Shabtai, R. Barak, L. Livescu, A. Nachmias

As shown in the previous report, a combination of Fe, Zn, Cu and Mn as foliar applications to potato foliage of Verticillium infected plants reduced yield loss in such plants, while no effect was noted in control (non-infected) plants. This research was continued in the spring season of 1989 with the following modifications:

1. Two cvs. were tested: Blanca and Nicola
2. Rather than using a combination of minor element sulfates, a commercial foliar nutrient preparation which contains the minor elements in chelated form was used for foliar applications.
3. Iron, supplied as Fe EDDHA was used as a soil treatment.

As in previous years, the experiments were conducted in infested and non-infested plots at Gilat Experiment Station, in the Negev Region.

Materials and Methods

Cvs. used: Blanca and Nicola

Dates of sowing: Control plot 22 February 1989

Verticillium plot 24 February 1989

Seedbed Preparation: Pre-irrigation with $600 \text{ m}^3 \text{ ha}^{-1}$, plowing to 35 cm, disced and beds prepared.

Fertilization: 1,200 kg double superphosphate ha^{-1}

300 kg KCl ha^{-1}

400 kg NH_4SO_4 ha^{-1}

incorporated into soil prior to sowing.

During the growing season 270 kg ha^{-1} N, as NH_4NO_3 supplied via the irrigation system.

Plots were 10 meters long; 4 rows. There were four replications.
Supplemental nutrition:

1) Sequestrene 138 - Fe EDDHA applied at the rate of 10 kg ha^{-1} on April 23, 1989, by diluting in sand, and hand spread between the rows. After spreading, an irrigation of 300 m^3 ha^{-1} was used to infiltrate the chelated Fe into the root zone.

2. Microelement sprays: Wuxal preparation 7: obtained from Aglukon, Dusseldorf, West Germany, at rates of 1.0 and 1.5% of the formulated material. The sprays on both cvs. were conducted on 25 April and 8 and 28 of May. A further spray was conducted on the cv. Nicola on 8 June.

Disease ratings

Vine vigor ratings as related to Verticillium symptoms were conducted on 28 April and on 16 and 28 May; ratings were from 0-5; 0=completely healthy; 5 - all plants dead.

Yield analysis

The plots were dug up after complete drying in the non-infested plots, in this case for Blanca on 25 June and for Nicola, on 3 July.

Five meters of row from the two center rows of the plots were dug up; total 10 meters row. Rows were 0.9 meters apart; total 9 m^2 .

The yield was separated into three size classes: less than 100 gr.;

100-200 gr. and over 200 gr. per tuber.

At harvest haulms were examined for vascular discoloration. Twenty tubers of over 200 grams in weight from each replication were examined for stolon end vascular discoloration.

Results and discussion

The results of Verticillium ratings are presented in Table 1. The effects of the treatments on the yield of the cvs. Blanca and Nicola are presented in Tables 2 and 3.

As can be seen from the results presented in Tables 1, 2, and 3, all three treatments reduced Verticillium symptom expression in both cvs. Yield improvement due to the treatments was noted only in the cv. Blanca. It is interesting to note that the yield improvement was apparent in the over 200 gr. category of tuber size. It is accepted that Verticillium usually causes a reduction in tuber size, therefore the results obtained with the cv. Blanca are in keeping with the proposed hypothesis that the supplemental fertilization treatments maintained the plants in a better physiological state and permitted greater translocation of photosynthate to the tubers than in untreated plants. That the cv. was not lacking any of the usual soil supplied nutrients when non-infected can be seen from the results in the non-infested plots; and from the fertilizers added before sowing.

The added yield effect due to the treatments is in the order of 10 metric tons ha ; and particularly as it is with large tubers, quite meaningful. As can be seen from Table 2, the yield in the non-infested plots was higher than in the infested ones; although strict comparisons cannot be made as the two plots were ca 1.0 km apart.

It should be pointed out that in the infested plots there was 100% plant infection; there was 0% infection in the non-infested plots.

As can be seen from Table 3, the treatments had no effect on yield of Nicola under both disease levels. In contrast to Blanca most of the yield was in the 100-200 gr. range. Nicola has a longer growing period than Blanca, and as is apparent from the results Blanca is a heavier yielder. It may be that the rate of bulking is important in determining the effect of the nutrient treatments. That the treatments were partially effective can be seen from the results in Table 1, with regard to symptom expression. It can also be observed that symptom expression in Nicola at the earlier stages of growth was less severe.

Our results, and those of previous years, suggest that certain nutritional treatments can greatly alleviate yield loss in at least certain cvs. of potato. These treatments are very inexpensive, and ecologically sound. More needs to be understood about the possible mechanisms. In last year's report we showed lowered K levels in infected plants, and that foliar microelement treatments increased the K level.

Bolle-Jones showed that an interaction existed between K and Fe in potato. Increasing Fe greatly increased yield at three out of four levels of K tested, the differences being 11 fold at the lowest K level. Thus it is possible that in *Verticillium* infected plants, besides the positive effects of Fe in itself, side effects pertaining to K uptake and translocation may be operative.

Jolley et al have also shown an interaction of K and Fe in tomato and soybean, which may have a bearing on the work reported here.

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Table 1. Effect of foliar or soil treatments on Verticillium symptoms of potato; cvs. Blanca and Nicola

	Blanca		Nicola	
	days after sowing		days after sowing	
	82	94	82	94
Treatment				
Control	2.13* a	2.88 a	1.00 a	2.13 a
Wuxal				
Type 7				
1.0%	1.38 b	1.75 b	1.00 a	1.50 b
1.5%	1.25 b	1.63 b	0.25 ab	1.63 b
FeEDDHA				
10 kg ha ⁻¹	1.13 b	1.50 b	0.0 b	1.38 b

* - Verticillium ratings: 0-5, 0 = no symptoms; 5 = all plants dead

Mean separation in columns by Duncan's multiple range test, 5% level

Table 2. Effect of foliar or soil treatments on yield ($\text{kg } 9\text{m}^{-2}$) of potato, cv. Blanca grown in infested and non-infested plots

	Infested Plot			Non-infested Plot		
	up to 100 g	100-200 g	>200 g	up to 100 g	100-200 g	>200 g
Treatment						
Control	6.24 a	16.47 a	11.38 a	11.25 a	19.78 a	23.63 a
Wuxal						
Type 7						
1.0%	5.06 a	19.47 a	20.03 b	9.38 a	21.50 a	29.38 a
1.5%	5.63 a	19.01 a	22.00 b	9.50 a	19.88 a	26.88 a
FeEDDHA	5.30 a	20.06 a	21.43 b	9.18 a	20.95 a	30.25 a
10 kg ha^{-1}						

Mean separation in columns by Duncan's multiple range test, 5% level

Table 3. Effect of foliar or soil treatments on yield ($\text{kg } 9\text{m}^{-2}$) of potato, cv. Nicola grown in infested and non-infested plots

	Infested Plot			Non-infested Plot		
	up to 100 g	100-200 g	>200 g	up to 100 g	100-200 g	>200 g
Treatment						
Control	14.78 a	21.83 a	4.48 a	20.36 a	28.03 a	5.48 a
Wuxal						
Type 7						
1.0%	13.28 a	21.59 a	5.06 b	19.28 a	26.88 a	6.85 a
1.5%	14.80 a	20.98 a	3.30 b	20.28 a	25.73 a	7.43 a
FeEDDHA	17.70 a	20.75 a	3.81 b	19.83 a	28.10 a	7.10 a
10 kg ha^{-1}						

Mean separation in columns by Duncan's multiple range test, 5% level

The Relationship Between Iron Nutrition and Verticillium Wilt Resistance
in Tomato

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ABSTRACT

The effect of iron nutritional status on Verticillium Wilt disease in tomato possessing single gene resistance to Race 1 of Verticillium dahliae was investigated using hydroponic culture media. Iron limiting conditions increased the sensitivity of resistant tomatoes to the pathogen as expressed by wilting. Distance of longitudinal invasion was approximately the same in both iron replete and iron limited treatments. However, the magnitude of wilting in resistant near-isoline Pixie II was considerably less than that expressed by the susceptible Pixie variety. Infection of tomato did not enhance iron stress severity as quantified by root peroxidase activity and chlorophyll content of young leaves.

INTRODUCTION

Nearly all living cells require iron for their survival. Though relatively abundant in nature, the low solubility of iron under aerobic conditions limits its availability to organisms. Thus, plants and microbes utilize iron sequestering systems to maintain a well regulated supply of this essential element.

Acquisition of iron has been reported to be a critical virulence factor in a number of plant-pathogen interactions (Kloepper et al., 1980; Expert and Toussaint, 1985). The influence of iron nutritional status on *Verticillium* wilt severity has been observed in peanut and eggplant (Krikun and Frank, 1975; Barash et al., 1988). Krikun and Frank (1975) reported that peanut varieties which were highly tolerant to *Verticillium* Wilt in sandy soils, succumbed to the disease when grown in iron limiting, calcareous loess soil. The addition of an iron amendment to the calcareous soil allowed the plants to retain tolerance to the disease. A similar study by Barash et al. (1988) utilizing eggplant revealed comparable results. Enhancement of disease expression through iron limitation has also been observed during infection of bean by *Fusarium solani* (Guerra and Anderson, 1985) and *Botrytis cinerea* (Brown and Swinburne, 1982). Reduction in lignin formation and decreased phytoalexin production were cited as reasons for the aggravation of disease symptoms under iron deficient conditions. The mechanisms involved in iron's effect on sensitivity of peanut and eggplant to *Verticillium dahliae* are not yet clear.

Plants exhibit complex physiological and anatomical responses to various forms of stress induced by different biotic and abiotic factors. A considerable amount of information is known about iron stress response

mechanisms in tomatoes (Bienfait, 1985) and tomato response to infection by Verticillium spp (Pegg, 1981). The present study was undertaken to examine the interaction and combined effect of these two stress inducing conditions in tomato.

MATERIALS AND METHODS

Plants, Pathogen, and Inoculation

Pixie II-resistant and Pixie-susceptible tomato varieties were used as near-isolines differing in the presence of the Ve gene for resistance to race 1 of V. dahliae. Ace VF (resistant) and Marglobe (susceptible) were also utilized in experiments. Seeds purchased from W. Atlee Burpee Co. (Warminster, PA) were surface sterilized for three minutes in 0.5% sodium hypochlorite and germinated on stainless steel screens covered with moist cheesecloth. The tomato seedlings were transferred to opaque 10L polyethylene tubs (24 seedlings per tub) containing 8L of standard nutrient solution plus 36 μ M FeEDTA. The standard nutrient solution was composed of 1.90 mM $\text{Ca}(\text{NO}_3)_2$, .47 mM $\text{Mg}(\text{NO}_3)_2$, .24 mM KCl, .61 mM KNO_3 , .60 mM K_2HPO_4 , .32 mM $(\text{NH}_4)_2\text{SO}_4$, 7.4 μ M MnCl_2 , 41.3 μ M H_3BO_4 , 1.9 μ M ZnSO_4 , 0.5 μ M CuSO_4 , and 0.4 μ M Na_2MoO_4 with a pH of approximately 7.0. All nutrient solutions in the experiments were continuously aerated and were changed weekly. At the late 3-leaf stage, plants were inoculated by submergence of the roots into an aerated conidial suspension (1.0×10^6 conidia/ml) of V. dahliae for 24 hours. After inoculation, individual seedlings were placed into opaque polyethylene bottles containing 1L of standard nutrient solution. To allow induction of iron deficiency, 5.0 mM NaHCO_3 , 0.25 g CaCO_3 , and 54 μ M EDTA were added to all treatments. Iron replete treatments also contained 54 μ M FeCl_3 . The growth environment consisted of

16 hours light with an approximate energy level of $210 \mu\text{E m}^{-2} \text{ s}^{-1}$ followed by 8 hours of darkness. The day and night temperatures were $22^\circ\text{C} \pm 0.7$ and $21^\circ\text{C} \pm 0.7$, respectively, while the average relative humidity was about 70%. Analysis of specific physical and biochemical parameters was initiated after 20 additional days of growth.

Evaluation of Disease

Wilting was used as the criterion for disease expression in the tomato plants (Visser and Hattingh, 1980). Wilt symptoms were quantified by measurement of leaf stomatal resistance with a MK3 automatic porometer (Delta - T devices LTD.). Measurements were taken on terminal leaflets of lower leaves.

Fungal Infection

Longitudinal fungal progression through the vascular system was measured 23 days after inoculation. Leaves and lateral roots were removed and the main axis was dipped into 95% ethanol for 5 seconds followed by submergence in 0.5% sodium hypochlorite for 3 minutes. Segments 0.5 cm in length were cut and placed on czapeks agar media containing 100 ppm kanamycin monosulfate. After 7 days of incubation, the segments were visually inspected for outgrowth of V. dahliae.

Evaluation of Iron Deficiency

Iron deficiency was quantified by a visual chlorosis rating (1 = no chlorosis, 5 = severe chlorosis) and determination of chlorophyll content.

Chlorophyll content was measured in a manner described by Inskeep and Bloom (1985).

Peroxidase Activity

Root samples of Pixie and Pixie II were assayed for peroxidase activity using the technique modified from Reuveni and Ferreira (1985). Immediately following excision, 0.3 g root samples were placed in dry ice. The frozen tissues were homogenized with a chilled mortar and pestle. The pulverized material was added to 3.0 ml of cold 15 mM sodium phosphate buffer, pH 6.0, and the homogenate was centrifuged at 10,000 g for 10 min at 4°C.

Aliquots of the enzyme extract (50 μ L) were added to 3.0 ml of the assay solution consisting of 15 mM sodium phosphate buffer, pH 6.0, 1.0 mM H_2O_2 , and 0.1 mM O-methoxyphenol (guaiacol). Enzyme activity was expressed as change in absorbance (470 nm) $\text{min}^{-1} \text{g}^{-1}$ fresh weight.

Total Iron

Total iron analysis of leaf and petiolar tissue was conducted on the susceptible Marglobe variety. Perchloric and nitric acid tissue digests were analyzed for total iron using atomic absorption spectrophotometry (Perkin-Elmer Model 560 atomic absorption spectrophotometer).

O-dihydroxyphenols

The method utilized for analysis of total O-dihydroxyphenols was modified from Collier (1979). Root material (0.25 g) was added to 3.0 ml 95% ETOH and homogenized for 10 minutes (Virtis 23 homogenizer). The homogenate was filtered (0.45 μ m) and loaded on 4.0 x 0.8 cm acid alumina

columns (Activity Grade 1). The columns were rinsed with two bed volumes of 95% ethanol followed by diazotization of the O-dihydroxyphenols with two bed volumes of a 5% NaNO_2 plus 5% acetic acid solution. The diazotized compounds were eluted with two bed volumes of 5.0 N NaOH and assayed spectrophotometrically at 520 nm. Caffeic acid was used as the standard.

Siderophore Assay

Siderophore assay agar media was prepared according to the method outlined by Schwyn and Neilands (1987). This assay is non-specific and is very sensitive to molecules possessing iron chelating properties. The assay was conducted to test for the presence of iron chelates in the vascular system. Xylem fluid was squeezed out of stem cuttings and placed on the assay media. After 24 hours the media was visually inspected for color change.

RESULTS

Measurements of stomatal resistance provide evidence indicating that iron deficiency of tomato plants bearing major gene resistance to V. dahliae increases their sensitivity to the pathogen (Fig. 1). Iron deficient resistant plants exhibited significant wilting while iron replete plants showed little wilting. However, visual observation revealed slight stunting of iron replete Pixie II lower leaves. Though sensitivity of resistant varieties was significantly increased by iron deficiency, the magnitude of wilting was considerably less than that expressed by the susceptible Pixie variety. The iron status of infected Pixie had no significant effect on stomatal resistance.

The assay used to determine extent of longitudinal invasion indicated the pathogen was able to advance a significantly greater distance in Pixie versus Pixie II (Table 1). The distance invaded in the resistant varieties was approximately the same for both iron replete and iron limited treatments. Nearly all petiole segments excised from lower leaves of inoculated treatments tested positive for presence of the pathogen.

Infection of either resistant or susceptible tomato did not aggravate iron stress in the host as quantified by root peroxidase activity, visual chlorosis rating and determination of chlorophyll content (Table 1). However, iron stress of diseased Pixie was considerably less severe than any other iron limited treatment. In addition, peroxidase activity in the infected, iron deficient Pixie treatment was significantly higher than other treatments (Fig. 2). All other iron limited treatments demonstrated reduced activity. Root peroxidase is a sensitive indicator of iron stress and an increase in iron stress is correlated with a decrease in enzyme activity (Sijmons, 1985).

In contrast, infection by vascular wilt pathogens is correlated to elevation in peroxidase activity (Pegg, 1981). Under the present conditions, elevation of peroxidase activity due to infection was only observed in conjunction with iron stress. Reduced activity, which would be expected under iron limitation, was apparently delayed or masked by the additional effect of infection. In a similar experiment conducted with the susceptible Marglobe variety, total iron analysis of leaf and petiole tissue indicated that infected plants subjected to iron limitation contained 31% more iron than non-infected plants. No difference was observed in the iron replete treatment.

Analysis of 0-dihydroxyphenols yielded results too variable for discernment of significant differences between treatments. All iron deficient treatments tested positive for the presence of siderophores and/or chelates in the xylem fluid. The iron replete treatments tested negative. These results may be attributed to high concentration of iron free EDTA in the nutrient solution of iron deficient treatments.

DISCUSSION

The use of hydroponic culture media permitted study of the interaction between iron deficiency and pathogenicity of V. dahliae in tomato. Incorporation of near-isolines Pixie (susceptible) and Pixie II (resistant) into the experiments allowed for a viable comparison between resistant and susceptible cultivars. The results showed that infected tomatoes possessing major gene resistance expressed an increase in wilting upon iron limitation. However, the level of wilting expressed by iron stressed Pixie II was considerably less than that expressed by the susceptible Pixie variety.

In addition, longitudinal progression of the pathogen in stems of Pixie II and Ace VF was not significantly different between iron replete and iron deficient treatments. These results suggest that enhanced expression of disease upon iron limitation is due to an increase in sensitivity of resistant plants to the pathogen rather than restriction of fungal invasion.

Iron is directly involved in many enzymatic and electron transport reactions in cells. Thus, its limitation can severely impact plant metabolism and subsequently increase a plant's sensitivity to virulence factors such as toxins, hydrolytic enzymes, and/or hormones. Iron

limitation may also cause impairment of one or more of the resistance factors regulated by the Ve gene. Although many of these factors are associated with restriction of fungal invasion, i.e., tylose formation (Sinha and Wood, 1958), hypersensitivity, phytoalexin accumulation (Tjamos and Smith, 1975), and secretion of vascular coatings (Robb et al., 1987), physiological mechanisms have also been identified as critical to resistance (Dixon and Pegg, 1969). The specific Ve gene regulated physiological mechanisms are currently unknown.

Stunting induced by iron limitation may be directly responsible for enhancement of wilt symptoms in resistant varieties. A reduction in total plant volume (quantified by height, Table 1) may have allowed for a more concentrated build-up of symptom inducing compounds as compared with non-stunted plants.

A positive correlation between elevated peroxidase activity and presence of the Ve gene has been observed in tomato (Reuveni and Ferreira, 1985). Guerra and Anderson (1985) have suggested that peroxidases involved in resistance to disease are directly impacted by iron deficiency. Peroxidases are iron containing enzymes especially sensitive to iron deficiency (Sijmons et al., 1985). They catalyze redox reactions with H_2O_2 and phenols. One of the mechanisms associated with iron stress response in tomato is accumulation and subsequent root exudation of phenolics resulting from reduced peroxidase activity. Phenolics, in turn, are known to play a complex role in pathogenesis of wilt diseases, i.e., metabolism of IAA and other growth substances and regulation of enzyme activity including polygalacturonase (Pegg, 1981). The complex interaction between phenols and resistance has yet to be fully understood.

Research conducted by Barash et al. (1988) suggested that V. dahliae was able to induce iron deficiency in peanut plants. They proposed that sequestration of iron may be a significant virulence factor in the disease. In the current study, the pathogen demonstrated no significant ability to withhold iron from plants although iron did play a role in expression of disease. However, the use of peroxidase activity and chlorophyll content as parameters for quantification of iron stress may not be valid in this plant-pathogen interaction. Nevertheless, the results imply that iron sequestration may not be a significant virulence factor in this system.

We acknowledge and appreciate financial support from BARD Grant # 1878-85.

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Fig. 1. Disease symptoms were measured by stomatal resistance (1/transpiration) 20 and 21 days after inoculation. A: Ace VF (resistant). B: Near-isolines Pixie (susceptible) and Pixie II (resistant) ($P = 0.05$).

Fig. 2. All 0.0 μM iron treatments exhibited depressed peroxidase activity with the exception of infected Pixie. Infection of iron replete treatments showed no influence on enzyme activity ($P = 0.05$).

fig. 1

Table 1. Comparison of stunting, distance of longitudinal fungal invasion, and quantified iron stress (1 = no stress, 5 = severe stress) in near-isolines ($P = 0.05$).

variety	treatment	plant height (cm)	fungal invasion (cm)	chlorosis rating	chlorophyll content (mg/cm ²)
Pixie II	0.0 uM iron inoculated	13.7 a	8.2 a	3.3 b	1.0 a
	control	13.7 a	--	3.3 b	0.6 a
	54.0 uM iron inoculated	20.5 b	9.0 ab	1.0 a	14.5 d
	control	21.9 b	--	1.0 a	14.6 d
Pixie	0.0 uM iron inoculated	10.9 c	10.9 b	2.0 c	4.3 b
	control	16.8 d	--	3.5 b	0.8 a
	54.0 uM iron inoculated	18.3 d	14.5 c	1.0 a	12.4 c
	control	24.4 e	--	1.0 a	16.2 e

EFFECT OF IRON ON THE RESPONSE OF PEANUTS TO VERTICILLIUM DAHLIAE

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Abstract

Soil application of Fe-EDDHA suppressed virulence of Verticillium dahliae in peanuts cultivated in calcareous soil. Reduction in virulence was expressed by inhibition of characteristic disease symptoms such as plant stunting, reduced leaf size and chlorosis. The phytoalexins medicarpin, demethylmedicarpin, vestiton and formononetin were detected in stems of both infected as well as noninfected peanuts. However, the total amount of these phytoalexins in infected plants was about 4.8 times higher than in noninfected plants which were supplied with iron. The amount of medicarpin, the major phytoalexin that was detected, was higher x18.6 in uninfected plants grown under iron stress and x23.8 and x43.4 in infected plants grown with and without iron. Since iron application did not influence the advancement and distribution of the pathogen within the plant it was concluded that phytoalexins may not be involved in the suppression of virulence in this system.

Introduction

Previous studies (2) have shown that in peanuts cultivated in plots that were either artificially infested with Verticillium dahliae or non-infested, soil application of Fe-EDDHA significantly increased yield only in plots where the fungus was present. The present study was undertaken to further characterize the effect of iron on the development of the pathogen within the host plant and on the defense mechanisms of peanuts to verticillium wilt disease.

Materials and Methods

Peanut plants (Arachis hypogaea L. cv. Shulamit) were germinated in vermiculite for 8 days before being transferred to pots containing rendzina calcareous soil (pH 7.6). The plants were placed in a growth chamber at 25°C under fluorescence and incandescence illumination (100 w m^{-2}) and a photoperiod of 12h. The plants were regularly irrigated with double distilled water and once per week with 0.5 x Hoagland solution. The growth period was 22-24 days.

The plants were separated into the following 4 different treatments (10 plants per each treatment): a) plants infected with V. dahliae and grown without iron b) infected plants grown with iron. In the latter case 15 mg Fe-EDDHA dissolved in 50 ml 0.5 x Hoagland was added after 2 days c) healthy plants grown without iron and d) healthy plants grown with iron.

The pathogen (isolate Dvir) was kept on PDA plate for inoculation, the fungus was grown in liquid medium as previously described (1). Prior to inoculation the mycelium was filtered through 4 layers of cheesecloth and macerated with Waring Blendor for 30 seconds. Six grams of wet weight mycelia was used per 1 liter of distilled water. Eight-day-old peanut seedlings were removed from the vermiculite and rinsed with water. The roots were immersed in the mycelial suspension for 1 h and then were planted in the pots. Control plants were immersed in sterile water instead of mycelial suspension.

The amount of the pathogen within the plant was estimated by colony forming units (cfu). Stem or roots of each plant were separated and surface sterilized by 1% sodium hypochloride for 2 min following by excessive washings with sterile water. They were then extracted with 10 ml of sterile water with Polytron in maximal speed for 1 min. The extract (0.2 ml) was uniformly distributed on PDA plates containing chloramphenicol (250 ug/ml) and streptomycin (200 ug/ml). Colonies were counted after 5 days incubation at 25°C.

Phytoalexins were extracted from stems of 6 plants of each treatment as described by Keen (4). The obtained dry material was dissolved in acetonitril (0.5 ml per g. fresh wt.). The phytoalexins in 0.2 ml were then determined by calibrated and computerized HPLC (Perp LC/system 500 Waters Associates). Peroxidase and chlorophyll was determined as described by Guidotti et al. (3) and Barak and Chen (1) respectively.

Results and Discussion

The effect of Fe-EDDHA on Verticillium wheat development in peanuts grown under calcareous soil is described in Table 1. It can be observed that addition of iron to the healthy plants did not show any significant effect on light, shoot weight or leaf area but significantly increased the chlorophyll content of the chlorotic leaves. On the other hand, addition of iron to infected plants significantly increased the height and shoot weight of the plants as well as leaf area and chlorophyll to the level of the healthy control. Thus iron virtually neutralized most of the major symptoms of verticillium wilt during early development of peanut plants.

The next question which was addressed is whether the defense mechanisms of the plants, as far as it expressed by phytoalexins production, was affected. Major phytoalexins which were previously identified in peanuts include the isoflavanones vestitone and formononetin and the pterocarpan, demethyl medicarpin and medicarpin. The content of these phytoalexins in infected and healthy peanuts with and without iron is given in Table 2. The level of total phytoalexins was induced by either iron deficiency and/or by infection. Thus healthy plants which were grown under iron deficiency contained 2.8 times more phytoalexins than healthy plants with iron. Infected peanut plants with iron contained 3.6 times higher phytoalexins and infected plants without iron 4.8

fold than the healthy control. Medicarpin was the major phytoalexin detected and its concentration as compared to healthy plants with iron was 18.6, 23.8 and 43.4 higher in healthy plants without iron, infected with iron and infected without iron stress respectively.

The foregoing results indicate that iron stress is capable of inducing phytoalexins production but at the same time aggravates the symptoms of Verticillium wilt in peanuts. Thus the phytoalexins detected may not be involved in the defense mechanism. Since peroxidase activity is known to be associated with plant response to infection, the activity of the enzyme in roots was monitored (Table 3). Results indicate that the level of peroxidase was significantly increased by infection but reduced under iron deficiency.

Further experiments were aimed at elucidating the effect of iron on the advancement of the pathogen within the host. The pathogen could be isolated from the roots and stem up to the 5th leaf in both iron-treated and non-treated infected plants. Results in Table 4 indicate that the CFU in plants with iron was even slightly higher than plants grown under iron deficiency. These and previous results clearly indicate that the advancement of the pathogen within the host and the defense mechanisms that might inhibit its progress are not significantly affected by the iron status of the plant. We therefore, hypothesize that iron may exert an effect on expression of virulence mechanisms such as toxins, pectic enzymes or hormones released by the pathogen or alternatively, render the plant more tolerant towards these virulence determinants.

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Table 1

Effect of Fe-EDDHA on disease severity caused by Verticillium dahliae in peanuts growing in calcareous soil.

Treatment	Shoot height (cm)	Shoot weight (d.f.wt.)	Leaf area (cm)	Chlorophyll content (mg.l ⁻¹ /mg f.wt.)
Healthy plants	19 ^a	11.6 ^a	23 ^a	18 ^b
Healthy plants + Fe-EDDHA	21 ^a	12 ^a	23 ^a	48 ^a
Infected plants	13 ^b	8 ^b	19 ^b	12 ^b
Infected plants + Fe-EDDHA	18 ^a	11 ^a	25 ^a	44 ^a

Means within columns followed by the same letter are not significantly different (LSD=0.05).

Table 2

Effect of Fe-EDDHA on phytoalexins production in stems of peanut plants infected and non-infected with Verticillium dahliae

Phytoalexins	Non-infected		Infected	
	+Fe	-Fe	+Fe	-Fe
	mg/gr.F.wt.			
(-) Demethylmedicarpin	56.8	81.8	75.2	28.9
Vestitone	5.29	2.17	37.8	11.07
(-) Formonoretin	4.86	28.19	31.6	61.49
Medicarpin	<u>4.94</u>	<u>92.09</u>	<u>117.8</u>	<u>214.62</u>
Total	71.89	204.28	262.4	315.44

Table 3

Effect of Fe-EDDHA on peroxidase activity in peanut roots of healthy and infected plants with Verticillium dahliae¹

Treatment	Peroxidase activity (units/ml)
Healthy plants	34 ^a
Healthy plants	
+ Fe-EDDHA	48 ^b
Infected plants	69 ^c
Infected plants	
+ Fe-EDDHA	87 ^d

¹Plants were grown in calcareous soil as described in Table 1. Each treatment contained 10 plants. Means within columns followed by different letters are significantly different.

Table 4

Effect of Fe-EDDHA on colonization of Verticillium dahliae in peanut plants as expressed by colony forming units.

Plant Part	Colony forming units	
	with iron	without iron
Lower root	650	350
Upper root	2540	600
Lower stem	1150	1800
Upper stem	<u>3250</u>	<u>2250</u>
Total	6590	5000

Results are an average of 5 different experiments.

The effect of Verticillium dahliae infection on the
nutrient status of two crop species

J. Krikun

As part of the ongoing study of mineral nutrition effects on Verticillium wilt, an opportunity was afforded to conduct tissue analysis in two crops which were grown under controlled conditions and inoculated or non-inoculated with V. dahliae. The two crop species were eggplant (Solanum melongonea) and four cvs. of canola (1 mustard, 3 rapeseed).

The analyses were conducted in an attempt to relate infection effects on plant nutrient composition under conditions of full nutrient availability, in plants grown under controlled (greenhouse) conditions and under controlled pathogen levels.

Materials and methods

The plants were grown in peat-vermicullite mix containing added nutrients. During the growing period additional nutrients were supplied with the irrigation water, the fertilizer being 20-20-20.

The experiments were conducted in a greenhouse, with added illumination as needed. Temperature range was 20-27°C.

Substrate infestation was carried out by adding ca. 50 microsclerotia per cc. substrate mix in the case of canola. In eggplant, young seedlings growing in jiffy pots were inoculated and transferred to large pots.

At the end of each experiment, the portions to be analyzed were

prepared for tissue analysis by washing, oven drying and weighing. Analysis was conducted by the techniques employed in the Plant Testing Laboratory at Volcani Center.

For eggplant, the plant was divided into upper and lower sections and analyzed separately. For canola, because of the normal leaf shed, only the stem was analyzed.

In eggplant the plants were harvested 24 days after inoculation; in canola 50 or 63 days after seeding.

Symptom onset and expression were monitored every two days.

At the conclusion of the experiment portions of the stem were used to confirm infection by isolation techniques.

In eggplant two isolates were used; one from potato and one from pea. In canola only the potato isolate was used.

Results and discussion

The results of the effects of infection on dry weight and nutrient composition of eggplant are presented in Table 1.

The results of the effect of infection on the dry weight of the stem, including pods, and on selected nutrients in canola are presented in Table 2.

Infection in eggplant was 100% with both isolates; in canola it varied from 67-100% depending on the cultivar.

In eggplant symptom onset was eight days after inoculation; in canola symptom onset was 29 days after sowing. In canola the symptoms appeared earlier in the cvs. Tobin and Domo, which flowered earlier than Karat and Westar.

As can be seen from Table 1, the most obvious symptoms of inoculation with both isolates of Verticillium dahliae in eggplant is a

decrease in dry weight of the upper half of the plant. Examination of the nutrient analysis results shows that when the upper half of the plant is considered there is a marked decrease in the K content; there are no differences in the N and P contents. There is a higher Ca level in the lower half of infected plants, there are no differences in Mg, Mn, Cu and Zn. There is a lower Fe content in both lower and upper portions of infected as compared to non-infected plants. The differences with regard to K and Fe would become even more striking when we consider that the upper portion of healthy plants was 130% and 90% greater in dry weight than in the pea and potato isolate infected plants. Thus total uptake of K and Fe is greatly reduced in eggplant infected with V. dahliae. In contrast N and P per-cent values of the tissues are not affected.

Table 2 presents the results of infection on stem and pod weight; and on K, Ca, Fe and Mn content of these tissues. Examination of the results shows that infection significantly affected plant dry weight, and lowered the K, Ca and Fe content in the cv. Domo. In the cv. Karat there was also less Ca and Fe. The most striking feature, however, with regard to nutrient composition in infected as contrasted to non-infected plants was their lowered Mn content; there being only half the concentration in infected plants, in all four cvs. tested. The results per se of the Mn contents may also be of interest as there seem to be large differences in uptake between cvs., without any regard to infection; the same applies to Ca.

The above results and those presented in Table 3 from a previous study for tomato grown under hydroponic conditions demonstrate that rather profound changes in the mineral nutrition of V. dahliae infected

plants occurs. The major differences occur with regard to K and Fe. Increasing Fe availability by the addition of chelated Fe in potato and eggplant (Barash et al.) decreases symptom expression and increases plant size or yield. It remains to be seen if infection effects are due to uptake, translocation or both. In cotton, the suggestion has been made that decreased K content found in plants infected with *Verticillium* is due to decreased uptake (Hafez et al.)

In this regard it may be useful to point out that Mikkelsen has shown that highly *Verticillium* resistant cultivars of cotton take up significantly more K from nutrient solutions low in K, and produce higher dry weights, than more susceptible cvs. (Kerby & Adams).

The results of the K analysis, conducted in plants grown under controlled nutrient conditions clearly demonstrate a *Verticillium* induced effect, and not a K effect on infection as suggested for pistachio (Ashworth et al.).

Recognizing that there are interactions between nutrient should perhaps be of priority in attempting to understand the effects between nutrition, nutrient composition and *Verticillium* infection. It is obvious that further research is needed to better understand the above. Such studies may suggest avenues of alleviating disease losses caused by *Verticillium dahliae*, and perhaps other pathogens as well.

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Table 1. Effect of V. dahliae infection of eggplant on plant dry weight (gr) and mineral components of the tissues

Treatment	Dry wt.	Upper half of plant								
		% dry matter						PPM		
		N	P	K	Ca	Mg	Mn	Cu	Zn	Fe
Control	2:98	5.5	1.0	6.5	2.18	0.64	242	14.3	29	158
<u>V. dahliae</u> potato isolate	1.27	5.3	0.8	5.1	2.04	0.61	228	12.4	34	133
<u>V. dahliae</u> pea isolate	1.52	5.0	0.8	5.1	1.93	0.63	203	17.5	32	126
				*1						*
	Dry wt.	Lower half of plant								
		% dry matter						PPM		
Control	1.45	6.8	1.0	5.4	0.46	0.36	132	9.8	42	159
<u>V. dahliae</u> potato isolate	1.15	6.8	1.0	4.8	0.87	0.34	129	18.6	47	127
<u>V. dahliae</u> pea isolate	1.48	6.8	1.0	4.3	0.93	0.39	129	16.4	43	124
					*					*

1Denotes significant differences between control and V. dahliae infected plants at 0.05% level.

Table 2. Effect of V. dahliae infection on dry weight (gr.) of stem and pods and on selected mineral components of these tissues of four cvs. of canola.

Cultivar	Dry	%		PPM	
	weight	K	Ca	Fe	Mn
<u>Domo</u>					
control	10.6	3.13	1.26	38.7	74.3
infected	7.5	2.17	0.92	26.8	35.0
	* ¹	** ²	**	**	**
<u>Tobin</u>					
control	11.4	2.68	0.53	21.8	34.0
infected	8.5	3.10	0.72	30.5	14.5
	**			**	**
<u>Karat</u>					
control	16.4	2.00	1.63	32.3	61.0
infected	11.6	1.87	1.16	25.0	31.2
	**		**	**	**
<u>Wester</u>					
control	17.0	2.50	0.48	20.0	34.0
infected	15.4	2.97	0.71	21.7	15.8
			*		*

1. Denotes sig. diff. between control and inoculated at 0.05% level.

2. Denotes sig. diff. between control and inoculated at 0.01% level.

Table 3. The effect of V. dahliae infection of tomato grown in half-Hoagland's nutrient solution on N, P and K content in the lower, middle and upper portions of the foliage (percent of dry matter).

Nutrient	Plant portion	Control plants	Infected plants
N	U	6.00	6.25
	M	5.25 a ¹	6.50 b
	L	5.75	5.25
P	U	0.88	1.05
	M	0.70	0.80
	L	0.63	0.60
K	U	4.75	4.54
	M	4.17 a	3.81 b
	L	4.09 a	2.38 b

U = Upper, M = middle, L = lower, portions.

1. Numbers followed by different letters between columns are sig. different at the 0.05% level.

D. DESCRIPTION OF COOPERATION

Cooperation between the labs of Dr. Barash at TAU and Drs. Olsen and Stobel at MSU was achieved by visits of Dr. Barash (1987, 1989) to MSU and frequent bitnet communication. Dr. Stobel visited Israel in 1988 and Mr. Macur, a graduate student of Dr. Olsen, participated in an international meeting on Iron and plants which was held last June in Jerusalem. Parts of the work supported by the project were presented during this meeting as well as other scientific meetings in previous years.

E. EVALUATION OF THE RESEARCH ACHIEVEMENTS WITH RESPECT TO THE ORIGINAL AIMS

As pointed out previously, the main goal of the present project was to define the role of iron in virulence of plant pathogens, particularly in Verticillium dahliae. In order to gain an insight into the interaction of iron with virulence, we sought answers to the following questions

- a) What is the relationship between the iron status of the host plant and verticillium wilt severity?
- b) How does iron limitation affect development of Verticillium dahliae within the plant and plant defense mechanisms?
- c) What are the mechanisms for iron sequestration by plant pathogens during saprophytic life and pathogenesis? Are siderophores involved or other mechanisms? Results obtained provided unequivocal answers to some of these questions whereas only partial answers were obtained for others. Each of this questions will be addressed in the following discussion.

1. Relationship between iron status of host plant and disease severity

In case of verticillium wilt caused by Verticillium dahliae, plants grown under iron deficient conditions were significantly more sensitive to symptoms development and yield reduction than plants grown under iron sufficiency. In the present project, this phenomenon has been demonstrated in peanut, tomato, eggplant, potato and cannola under field as well as greenhouse conditions.

Supplemental of iron either applied via the soil (as EDDHA) or the foliage generally alleviated disease symptoms and increased yields compared to control in infested plots. These supplements had no effect on yield of plants growing in non-infested plots. In peanuts, soil application of iron was significantly more effective than foliar application. Moreover, the highly significant effect of FeEDDHA soil

additions on yield increase of peanuts grown in Verticillium infested soil justifies it as a practical control measure against the disease. As a matter of fact, FeEDDHA application has been recently successfully used in Israel to recover stunting and chlorotic peanuts in fields infested with Verticillium. Such results encouraged the recommendation of iron application as a practical mean of control. It should however be stressed that peanuts are known to be particularly sensitive to iron deficiency. Although, as demonstrated, supplemental of iron reduced Verticillium wilt in other crops as well, it may not be significant enough to recommend such a treatment as a control mean against the disease. Iron application as a control measure against verticillium wilt should be considered for each crop separately. It may be justified in other plants which exhibit high sensitivity to iron deficiency (e.g. avocado, mango, olives, etc.).

During the course of this project, the effect of Verticillium dahliae infection on the nutrient balance of several host species was investigated. The results of our studies (Krikun et al) indicate that the primary effect of infection with regard to macroelement composition is a decrease in K. Depending on the crop and CV. there is also a decrease in Fe. In canola, in all 4 CVs examined, infected plants had one-half the Mn content of control plants. That there seem to be intereactions between Fe, Mn and K is known from the literature and from some of the results presented here.

2. Effect of iron on advancement of V. Dahliae within the host and host defense mechanisms.

Further experiments were aimed at elucidating the effect of iron on the development of the fungus in the plant. No significant difference could be detected in peanut (Bitan & Barash, Krikun et al.) or tomato (Macur & Olsen) between iron-deficient and iron-sufficient plants. In peanuts colony forming units were examined under greenhouse and field conditions with similar results in treated and control plants. In tomatoes, the distance of longitudinal invasion was approximately the same in both, iron replete and iron limited conditions.

The fact that the increased sensitivity of iron-deficient plants to V. dahliae is not due to enhanced colonization by the pathogen was also

supported by following phytoalexins production in peanuts (Bitan & Barash). It is noteworthy that the content of phytoalexins was significantly induced not only by infection but also due to iron deficiency. Thus, if the phytoalexins examined in peanut exert any effect on the progress of the mycelium within the host, than iron-stressed plants should become more resistant which was not the case in our studies. We therefore hypothesize that iron may exert an effect on expression of virulence mechanisms such as toxins, pectic enzymes or hormones production by the pathogen or alternatively, render the plant more tolerant towards these virulence determinants

3. Mechanisms for iron acquisition by plant pathogens

Many efforts have been invested in the present project in characterizing mechanisms for iron uptake by phytopathogenic fungi. These studies were dual-purposed: a) to elucidate the survival capacity of these pathogens as far as competition for iron is concerned and b) to find out whether V. dahliae can deprive the host of iron. In recent years, the role of iron competition in biological control exerted by pseudomonads has been elucidated. Although the latter is certainly not the only mechanisms involved in biocontrol, in several cases it has been proven to be a significant factor. Obviously fungal pathogens, especially soil-born fungi, should possess mechanisms for iron sequestration in order to survive. In view of the very scanty information on such mechanisms in phytopathogenic fungi we have extended this portion of the project beyond what was originally planned. Thus, in addition to V. dahliae, other pathogens were extensively investigated.

Production of siderophores and siderophore-mediated iron uptake were investigated in V. dahliae, Stemphylium botryosum f. sp. lycopersici (causal agent of leaf spot and foliage blight in tomato), Gaeumannomyces graminus var tritici (causal agent of take-all disease in wheat) and the non-siderophore producer Geotrichum candidum (causal agent of sour rot in citrus). Quite interestingly, all the first three pathogens, which belong to the same or close groups of the Ascomycetes (all produce perithecia during sexual stage) secrete coprogen B and dimerum acid as their major siderophores. The

investigated transport systems generally exhibit characteristic properties for siderophore-mediated iron transport detected in other fungi. Properties unique to each system has been discussed in the papers presented in the "Body of the report". However, some general comments might be of interest. All of the investigated fungi were capable of utilizing iron from foreign hydroxamate siderophores produced by other fungi of special interest is Geotrichum candidum, which cannot produce siderophores but nevertheless exhibit high capacity to utilize iron from an array of various hydroxamate siderophores. These results suggest that fungi are generally competitive for hydroxamate siderophores regardless of their origin. Hydroxamate siderophores have been detected in the rhizosphere (Reid et al. 1986 in Iron, siderophores and plant diseases). Another point which should be emphasized is that the siderophore-mediated transport systems are designed for iron-stress conditions (i.e. high iron deficiency). Such conditions may not be available during pathogenesis and sequestration of iron may be achieved by mechanisms other than siderophores. Examples for such mechanisms have been demonstrated in Stemphylium botryosum (Menulis et al. 1987) and Geotrichum candidum (Mor et al. 1988). In the former case, the pathogen released ferric chelates (i.e. stemphytoxins I & II) to facilitate iron uptake. These chelates have been also shown to be phytotoxic.

The question of whether V. dahliae can deprive the host of iron has been examined. The work of Macur & Olsen with tomato indicate that Verticillium dahliae did not enhance iron stress severity as quantified by root peroxidase and chlorophyll content of young leaves. This conclusion may also be supported by the facts that peanuts could utilize iron from hydroxamate siderophores of V. dahliae and siderophores could not be detected in plants. Thus, the siderophore-mediated iron uptake systems may not be involved in iron sequestration during pathogenesis. However, although V. dahliae may not cause iron deficiency to the host plant during infection, it may do so indirectly. Thus a dramatic increase in peanut yield following FeEDDHA application to infested but not to non-infested soil was observed (Barash et al. 1988). This may indicate that the fungus can

efficiently sequester iron from the rhizosphere and compete indirectly for iron with the host plant.

F. LIST OF PUBLICATION RESULTING FROM THE RESEARCH PROJECT

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