Studies on the interaction between *Heterodera daverti*, *Fusarium avenaceum* and *F. oxysporum* on *Trifolium subterraneum*

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SUMMARY

The interaction between *Heterodera daverti*, *Fusarium avenaceum* and *F. oxysporum* on two clones of subterranean clover was studied. Simultaneous inoculation of *H. daverti* with *F. avenaceum* or *F. oxysporum* did not reduce the yield of the two clones of subterranean clover more than the additive losses caused by inoculation of each pathogen separately. When the nematode was inoculated one or two weeks after either fungus, plant dry weight was reduced synergistically. Nematode penetration and development was generally increased when the nematode was inoculated after the fungus. Increasing the interval between *F. avenaceum* and *H. daverti* inoculations decreased the number of cysts produced. Leaf reddening, a symptom often observed under field conditions, was developed only on plants inoculated with both *H. daverti* and *F. avenaceum*. The root-rot tolerant ecotype 48G formed lesions after penetration of *H. daverti*.

RÉSUMÉ

Études sur l'interaction entre Heterodera daverti, Fusarium avenaceum *et* F. oxysporum *sur* Trifolium subterraneum

L'interaction entre *Heterodera daverti*, *Fusarium avenaceum* et *F. oxysporum* sur deux clones de trèfle souterrain a été étudiée. L'inoculation simultanée n'a pas réduit le rendement des deux clones de trèfle souterrain plus que les pertes additives causées par des inoculations de chaque pathogène faites séparément. Quand les nématodes ont été inoculés une ou deux semaines après l'un ou l'autre champignon, le poids sec de la plante a été réduit synergétiquement. La pénétration et le développement du nématode ont été généralement élevés quand le nématode a été inoculé après le champignon. L'augmentation de l'intervalle entre les inoculations de *F. avenaceum* et *H. daverti* a diminué le nombre de kystes formés. Le rougissement de la feuille, un symptôme fréquent dans les pâturages n'a été observé que sur les plantes inoculées avec *H. daverti* and *F. avenaceum*. L'écotype "48G" tolérant à la pourriture des racines a formé des lésions après la pénétration de *H. daverti*.

Australian clones of subterranean clover (*Trifolium subterraneum* L.) were introduced into Northwest Tunisia in 1967-69 as an alternative fodder legume ; large field trials were started by Jaritz (1972). Two years later the first signs of clover decline were observed. The symptoms included root-rot, which often occurred in connection with an abnormal alteration of the root as well as stunting and discoloration of the plants.

Sikora (1972) isolated the cyst nematode, *Heterodera daverti* Wouts & Sturhan, and the fungi *Fusarium oxysporum* Schlecht *ex* Fr. and *F. avenaceum* (Corda *ex* Fr.) Sacc. from diseased plants. Nordmeyer, Sikora and Jaritz (1978) found that *Pythium irregulare* Biusm. was also associated with the root-rot complex. The symptoms observed in the field could not be reproduced by inoculations of the individual pathogens under controlled conditions (Nordmeyer, 1979). These results indicated that combinations of the organisms might be the cause of the root-rot disease. The purpose of this study was to determine whether an interaction occurred between *H. daverti* and *(i) F. avenaceum* and *(ii) F. oxysporum*.

Materials and methods

H. daverti isolated from *T. subterraneum* from Northwest Tunisia was maintained in the greenhouse on *T. subterraneum* cv. Clare. *F. avenaceum* and *F. oxysporum* were isolated from diseased plants in Tunisia and kept as permanent cultures in soil tubes (Miller, 1945).

Seeds of *T. subterraneum* were submerged in 95 % alcohol for 10 seconds, rinsed in water, immersed in 0.5 % NaOCl for 15 minutes and rinsed in sterile water. The seeds were germinated on moist filter paper in Petri dishes. The seedlings were planted in 250 cm³ plastic pots, ten per pot, containing steam pasteurized sand, which was screened to a particle size of 0.5 mm and smaller. The pots were fertilized weekly with a 0.5 N Hoagland's solution. Each experiment was run for four weeks at 20° and 80 % relative humidity. The root-rot susceptible cultivar Clare and the root-rot tolerant ecotype "48G" were used. To collect second stage juveniles for inoculum, nematode cysts were extracted from infested *T. subterraneum* plants, placed on a milk filter over a plastic screen and exposed to water. Freshly hatched juveniles were transfered daily to fresh water. The juveniles were then pipetted on the sand surface of the pot and washed into the sand with a small amount of water. Macroconidia of the fungi were either mixed into the sand before planting, or pipetted on the sand surface after planting and washed into the sand.

A disease index of root lesions formed either by the fungus or the nematode was determined by the length and severity of lesions (after McGee & Kellock, 1974) : 0 = no visible lesions ; 1 = lesions 1 mm long, light brown ; 2 = lesions 1 to 5 mm long, brown ; 3 = lesions longer than 5 mm, dark brown ; 4 = root totally infected and brown ; 5 = root destroyed and plant dying or dead.

Influence of different inoculum densities of *H. daverti* and *F. oxysporum* on cyst production of the root-rot susceptible cultivar Clare

H. daverti was inoculated at rates of 500, 1 000 and 2 000 juveniles/100 cm³ sand and *F. oxysporum* was inoculated at rates of 10^2, 10^4 and 10^6 macroconidia/25 cm³ sand and each nematode treatment was inoculated in combination with each fungus treatment. The number of cysts was recorded. Treatments were replicated six times.

Influence of different inoculation sequences of *H. daverti* and *F. oxysporum* on cultivar Clare

Nematodes (2 000 juveniles/100 cm³ sand) and fungus (10^6 macroconidia/25 cm³ sand) were inoculated alone and in the following combinations : nematodes and fungus inoculated simultaneously (NFs) ; fungus inoculated one or two weeks before nematodes (FN1, FN2) ; nematodes inoculated one or two weeks before fungus (NF1, NF2). The number of cysts, plant dry weight and disease index were recorded. Treatments were replicated five times.

Predisposition of *T. subterraneum* to nematode penetration and development after *F. avenaceum* infection

Seedlings (cv. Clare) were inoculated in a seed tray with *F. avenaceum* (10^6 macroconidia/25 cm³ sand). Five days later the seedlings were removed from the sand, the roots washed and ten plants were transplanted per pot in steam pasteurized sand. Control plants for each treatment were not infected with the fungus. The pots were inoculated with 2 000 *H. daverti* 5, 7, 9, 13 and 21 days after transplanting. Treatments were replicated six times. For comparisons with other treatments, the cyst production of the control plants was set to zero. The number of cysts was recorded.

Influence of different inoculation sequences of *H. daverti* and *F. avenaceum* on the root-rot tolerant ecotype "48G"

Nematodes (2 000 juveniles/100 cm³ sand) and fungus (10^3 macroconidia/25 cm³ sand) were inoculated alone and the nematodes were inoculated one and two weeks after the fungus. The number of cysts, plant dry weight and disease index were recorded. Treatments were replicated five times.

For all experiments, treatments were arranged in a completely randomized design. Data wsre analyzed by analysis of variance followed by least significance difference comparisons.

Results

Influence of different inoculum densities of *H. daverti* and *F. oxysporum* on cyst production of the root-rot susceptible cultivar Clare

A significant increase in the number of cysts was detected with inoculations of 10^6 macroconidia plus 500 and 2 000 *H. daverti* juveniles and with 10^4 macroconidia plus 1 000 juveniles (Fig. 1). Conversely, cyst production decreased significantly when 500 juve-

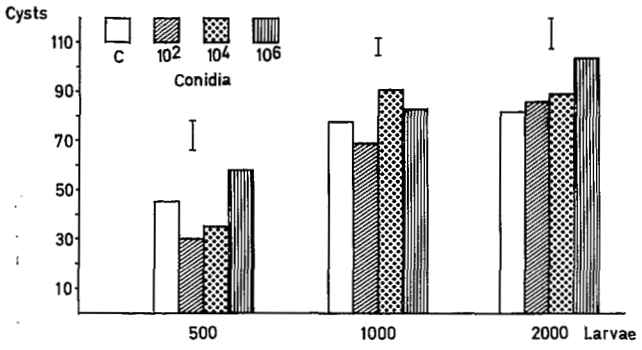

Fig. 1. Influence of different inoculum densities of *F. oxysporum* on the cyst production of *H. daverti* on *T. subterraneum* cv. Clare. The bars represent the $LSD_{0.05}$.

niles were combined with 10^2 and 10^4 macroconidia. No effects were detected in the other nematode-fungal combinations.

Influence of different inoculation sequences of *H. daverti* and *F. oxysporum* on cultivar Clare

Separate inoculations of the fungus and the nematode reduced yields (dry weight) by 15 % and 17 %, respectively (Fig. 2). No reduction in yield over the additive effect of the individual pathogens occured when the nematode was inoculated one or two weeks prior to the fungus. The respective disease indices were about the same as for the nematode alone, but less than with the fungus alone (Fig. 2).

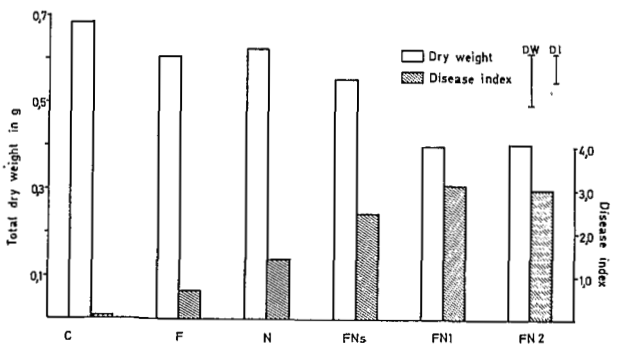

Fig. 2. Influence of different inoculation sequences of *H. daverti* and *F. oxysporum* on dry weight (DW) and disease index (DI) of *T. subterraneum* cv. Clare. The bars represent the $LSD_{0.05}$.

A 40 % reduction in yield was obtained when both organisms were inoculated simultaneously, but this was not significantly different from the 32 % cumulative loss caused by the individual organisms. Even though the disease index of the simultaneous inoculation was significantly greater than the one of the fungus alone, it did not reach the cumulative value of the individual inoculations.

Inoculations of the nematode one and two weeks after the fungus resulted in a 50 % yield loss, which was greater than the additive losses from treatments of the nematode and fungus alone. The respective disease indices were the same as for the fungus alone.

There were significant increases in cyst number when the nematode and the fungus were inoculated simultaneously (NFs) and when the nematode was inoculated one and two weeks after the fungus, FN1 and FN2 (Tab. 1). When the nematode was inoculated one week after the fungus (FN1) the number of cysts was significantly greater than in any of the other treatments.

Table 1

Influence of different inoculation sequences of *F. oxysporum* on the cyst production of *H. daverti* on *T. subterraneum* cv. Clare (Abbrev. see text. $LSD_{0.05}$ = 21.5.

Treatment	*Cysts/pot*
N	108
NF1	102
NF2	110
NFs	130
FN1	200
FN2	130

Predisposition of *T. subterraneum* to nematode penetration and development after *F. avenaceum* infection

Plants inoculated with *H. daverti* five and seven days after exposure to the fungus showed 69 % and 68 % more cysts than the control plants (Fig. 3). Inoculations 9, 13 and 21 days after exposure to the fungus resulted in significantly smaller numbers of cysts. The number of cysts on plants inoculated with the fungus was always greater than on those free of fungus.

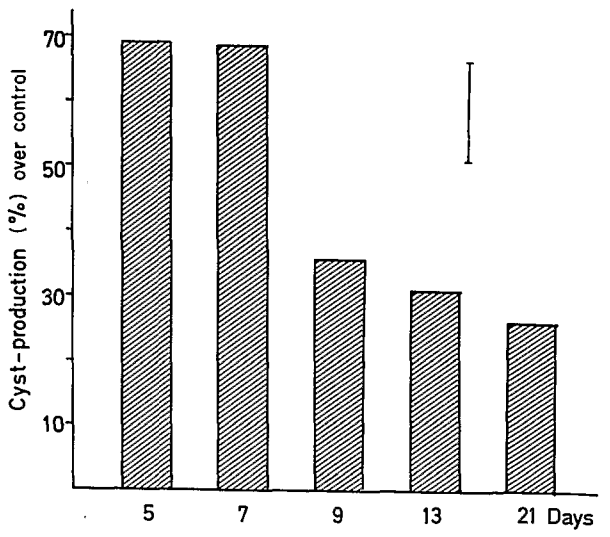

Fig. 3. Influence of the interval between exposure of *T. subterraneum* cv. Clare to *F. avenaceum* and inoculation of *H. daverti* on cyst production. The bar represents $LSD_{0.05}$.

INFLUENCE OF DIFFERENT INOCULATION SEQUENCES OF *H. daverti* AND *F. avenaceum* ON THE ROOT-ROT TOLERANT ECOTYPE „48G"

Inoculations of the nematode or the fungus alone did not cause significant reductions in dry weight (Fig. 4). Simultaneous inoculations of nematode and fungus reduced the yield significantly when compared with the control. Inoculations of nematodes one and two weeks after the fungus resulted in a 40 % yield reduction. The disease indices of these combined inoculations (FN1 and FN2) were significantly greater than the added indices of the individual pathogens.

The number of cysts per gram of root was greatest when the nematode was added two weeks after the fungus (Tab. 2). The percentage of dead plants increased from 6 % for the fungus alone to 12 % when both organisms were inoculated simultaneously. The percentage of dead plants increased further to 24 % and 28 % when the nematode was inoculated one or two weeks after the fungus, respectively.

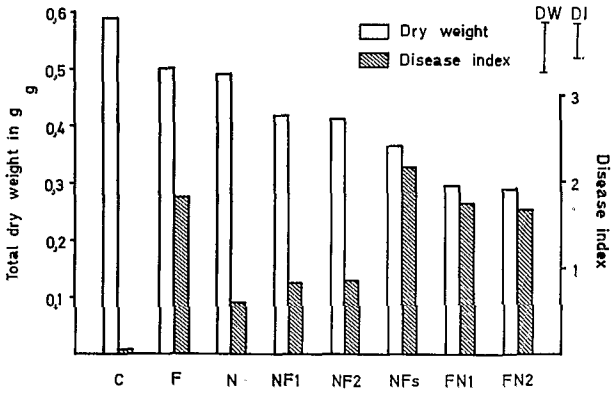

Fig. 4. Influence of different inoculation sequences of *H. daverti* and *F. avenaceum* on dry weight (DW) and disease index (DI) of *T. subterraneum* cv. 48G. The bars represent $LSD_{0.05}$.

Table 2

Influence of different inoculation sequences of *H. daverti* and *F. avenaceum* on number of dead plants, change in leaf pigmentation and cyst production on *T. subterraneum* cv. 48G (Abbrev. see text $LSD_{0.05}$ for cysts = 5.5.

Treatment	*Dead plants* %	*Number of yellow leaves*	*Number of red leaves*	*Cysts/ g roots*
C	0	0	0	
F	6	0	0	
N	0	0	0	2
FNs	12	6	0	3
FN1	24	34	41	5
FN2	28	14	7	18

Chlorosis of the leaves was observed for all combined treatments and leaf reddening was observed when the nematode was inoculated after the fungus. These alterations in leaf pigment were most distinct when the nematode was inoculated one week after the fungus. Chlorosis occured mainly in the intercostal leaf area, but was occasionally observed to cover the entire leaf. Generally, these leaves were smaller than those from healthy plants. Leaf reddening started from the tip of the first true leaf and covered the leaf margin. Occasionally, the entire leaf would dry up.

Discussion

Yield losses may be increased by fungal infection after or at the same time as nematode penetration (Dittmann, 1963 ; Powell, 1963). This effect has been demonstrated specifically for interactions between *Heterodera* spp. and *Fusarium* spp. by Capus (1917) and Powell (1971), as well as for *Heterodera* spp. and other soil-borne fungi (Adeniji *et al.*, 1975 ; Corbett & Hide, 1971 ; Harrison, 1971 ; Meagher & Chambers, 1971).

In our study the nematode did not predispose the host to the fungus as in the „classic" interactions mentioned above. Yield losses increased when nematode inoculation followed fungal infection of the plant. In the case of *H. daverti* and *F. avenaceum*, the disease index was increased significantly when the nematode was inoculated one or two weeks after the fungus. Also there were more dead plants in the same treatments.

Sometimes a synergistic effect becomes apparent as a more rapid occurence of plant symptoms (Jenkins & Coursen, 1957 ; Sasser, Powers & Lucas, 1955 ; Schlang & Sikora, 1978). Leaf chlorosis associated with simultaneous inoculations of *H. daverti* and *F. avenaceum* on „48G" appeared earlier than in the single inoculations. Furthermore, leaf reddening as an additional symptom was produced by this interaction. This symptom did not appear after inoculation with either pathogen alone. The leaf reddening was observed in fields in Tunisia when *Fusarium* spp. and *H. daverti* were present. This discoloration of clover is often observed when plants are under severe stress (Hiltner, 1933).

Nordmeyer (1979) showed that disease indices increased significantly when *T. subterraneum* was inoculated with increasing inocula of *F. oxysporum*. In the present study, *H. daverti* seemed to suppress establishment of *F. oxysporum*, as the disease indices decreased when the nematode was inoculated prior to the fungus. However, since in these treatments the fungus was inoculated one and two weeks after the control plants, development of an age resistance cannot be excluded. Miller (1975) demonstrated that tomato infected with *H. tabacum* was less susceptible to *Fusarium* wilt.

The high disease index caused by *H. daverti* on "48G" was considered to be due to a hypersensitive reaction of the host, as root lesions generally are not produced by cyst nematodes. In a study of 36 cultivars and ecotypes of subterranean clover, „48G" was the only one to show lesions after invasion of the nematode (Nordmeyer, 1979).

It is uncertain whether synergistic yield reductions were caused by an increase in the nematode or by increased damage due to the fungus. The yield reductions might be related to increased penetration and development of the nematode in the presence of the fungus. This is shown specifically in the third experiment where plants inoculated with *F. avenaceum* allowed more nematode penetration and development than the respective control plants. This effect has also been demonstrated when plants were pretreated with *F. avenaceum* culture filtrates that increased the rate of nematode penetration (Nordmeyer & Sikora, 1982). Inoculating susceptible seedlings with increasing densities of macroconidia of *F. oxysporum* may have had some positive effect on the number of cysts formed (first experiment) but results were not consistent.

Increased nematode reproduction due to nematode-fungus interaction, has been shown in several reports (Goswami *et al.*, 1970 ; McKeen & Moutain, 1960 ; Ross, 1965 ; Tchatchoua & Sikora, 1978). In the present study, cyst production was increased when the nematode was inoculated after *F. oxysporum* with „Clare" or after *F. avenaceum* with "48G". However, more extensive root systems at later inoculation dates might have influenced the number of cysts produced.

Our studies show that inoculations of *F. oxysporum* before *H. daverti* and *F. avenaceum* before *H. daverti* decrease plant weights of two clones of *T. subterraneum* synergistically. The yield decrease might have been the result of increased penetration and development by nematodes. Increasing the interval between *F. avenaceum* and *H. daverti* inoculations decreased the number of cysts produced. This might have been due to decreasing root mass, competition for nutrients and/or the release of fungal metabolic by-products. The latter possibility has been demonstrated with culture filtrates of *F. avenaceum* (Nordmeyer & Sikora, 1982).

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