Changes in nucleic acids and ribonuclease in tomato plants during infection by the root-knot nematode, *Meloidogyne incognita* [1]

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SUMMARY

Studies on the sequential changes in nucleic acids and ribonuclease in healthy and *Meloidogyne incognita* infected tomato plants on Pusa ruby (susceptible) and SL-120 (resistant) varieties were conducted. Nematode infection resulted in an alteration of nucleic acid metabolism in both varieties but to different degree. Ribonuclease activity showed a bimodal response in Pusa ruby, while only a unimodal response in SL-120. Polyacrylamide gel electrophoresis revealed the presence of a ribonuclease isoenzyme synthesized *de novo* in the inoculated resistant plants.

RÉSUMÉ

Variation de l'activité ribonucléasique et des acides nucléiques de plants de tomates après inoculation par Meloidogyne incognita

Il a été effectué une étude des variations chronologiques en acides nucléiques et en ribonucléase chez des variétés de tomates sensibles (Pusa ruby) et résistantes (SL-120), saines ou infestées. Après infestation, on observe une variation plus ou moins importante du métabolisme des acides nucléiques suivant la variété considérée. La réponse de l'activité ribonucléasique au parasitisme est bimodale dans la variété sensible et unimodale dans la variété résistante ; cette dernière présente une isoribonucléase supplémentaire synthétisée *de novo*.

The root-knot nematode, *Meloidogyne incognita* has been suggested to evoke the latent synthetic potential of the plant especially during the resistant response (Mote & Dasgupta, 1979). Increased levels of nucleic acids during post-infection period are commonly observed in many pathogen-induced plant diseases (Heitefuss, 1966). Increasing reports indicate rapid turnover in nucleic acid metabolism in response to nematode invasion (Bird, 1961 ; Rubinstein & Owens, 1964 ; Owens & Specht, 1966 ; Epstein, 1974). Due to their role in post-transcriptional processing of different ribonucleic acids (RNAs) in the protein-synthesizing system of the cell, ribonuclease has also received much attention in molecular physio-pathology. Enhanced levels of ribonuclease activity are known in plants after infection by viruses (Reddi, 1959), fungi (Rohringer, Samborski & Person, 1961), bacteria (Reddi, 1966) and as a result of senescence and stress in the form of osmotic shock, water shortage or heat (Wyen, Erdei & Farkas, 1971) or after mechanical injury (Bagi & Farkas, 1967). While ribonuclease activity decreased in carrot plants tolerant to *Meloidogyne hapla*, it was observed to increase in the secondary phloem and xylem tissues of susceptible plants (Knypl & Janas, 1975).

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(1) Forms part of the thesis submitted by the senior author for the degree of Doctor of Philosophy in Nematology.

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The present studies seek information concerning the sequential development of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and ribonuclease in tomato plants after inoculation with *M. incognita*, and its relation to resistance.

Materials and methods

Plant materials and nematode species

Two cultivars of tomato (*Lycopersicon esculentum* Mill.) and one population of the root-knot nematode, *Meloidogyne incognita* were used. Varieties Pusa ruby and SL-120, respectively susceptible and resistant to the nematode (Prasad & Dasgupta, 1964) were obtained from the Division of Horticulture, I.A.R.I., New Delhi. The procedure for raising the tomato seedlings, culturing and axenizing the nematodes were essentially the same as described by Dasgupta and Ganguly (1975).

Inoculation

39 day-old tomato seedlings of each variety were inoculated with surface-sterilized, active, second-stage larvae ($\approx$ 5 000 larvae/seedling). Uninoculated plants of the same age of both the varieties served as controls. At pre-determined intervals (one, three, fourteen and 28 days) the seedlings from each treatment were harvested, surface-sterilized and used for biochemical studies.

Estimation of nucleic acids

The nucleic acids from the root samples were extracted as per the method given by Smillie and Krotkov (1960). DNA was estimated by the diphenylamine reaction (Burton, 1968) and RNA by the orcinol reaction (Markham, 1955).

The root samples were homogenized in two volumes of K^+ phosphate buffer (pH 5,7 - 0,2 M). The resulting homogenate was centrifuged at 10 000 g for 15 minutes at 1° and the supernatant was used for ribonuclease assay according to the method of Beopoulos, Esnault and Buri. (1978) with slight modifications. The reaction mixture consisted of 100 µl of 1% yeast RNA in 0,2 M phosphate buffer, pH 5,7, 300 µl of the crude enzyme extract, and 100 µl of 0,2 M phosphate buffer pH 5,7. The reaction mixture was incubated at 37° for 30 minutes and then the reaction was terminated by chilling the test-tube in ice. The acid-insoluble nucleotides were precipitated by the addition of 200 µl of cold 12,5% perchloric acid containing 0,75% uranyl acetate. After ten minutes of precipitation, the precipitates were removed by centrifugation at 10 000 g for ten minutes at 4°. The supernatant (200 µl) was diluted to 2,5 ml and read spectrophotometrically at 260 nm. One unit of ribonuclease is defined as that amount which produced an increase in absorbance of 0,1 unit at 260 nm in 30 minutes at 37°. The specific activity of ribonuclease was defined as units/mg protein. The estimation of soluble proteins was done according to Lowry *et al.* (1951).

Assay of the nematode body homogenate for ribonuclease activity

The collection and concentration of *M. incognita* larvae, were done by the same method used by Dasgupta and Ganguly (1975). The larvae (5×10^5) were surface-sterilized in 0,5% hibitane diacetate for ten minutes. The homogenization was done in phosphate buffer, (pH 5,7 - 0,2 M) by sonication using a vibrionics 250 W ultrasonic tissue homogenizer model VPL P-2 at a frequency of 25 000 c.p.s. for five minutes at 4°. The homogenates were centrifuged at 16 000 g for 30 minutes at 1° and the supernatants used for ribonuclease assay as described earlier.

Disc electrophoresis of ribonuclease

Electrophoresis on 7,5% polyacrylamide gels was carried out as per the method of Wilson (1971) ; 28 day-old samples were ground in 15 mM citric acid and 42 mM HEPES buffer (pH 5,5). Electrophoresis was carried out using the alkaline system of Davis (1964) at 2 mA/gel for ten minutes and later at 4 mA/gel for 30 minutes. The gels were transferred to tubes containing a pre-incubation buffer (0,1 M sodium cacodylate, pH 5,8) and incubated for fifteen minutes at 37°. The solution was changed to yeast RNA (4 mg/ml) in 50 mM cacodylic acid-imidazole, pH 6,2 and incubated for another fifteen minutes at 37°. Finally the solution was changed to a post-incubation buffer which was the same as the pre-incubation buffer. The gels were stained in 0,2% toluidine blue in 0,5% acetic acid (pH 3,0) for 30 minutes and later destained in the same solvent. The dye free bands of enzyme activity were visible soon.

Results

Changes in DNA content after inoculation with *M. incognita*

Our observations on the development of DNA during the progression of disease showed a certain amount of variation (Tab. 1). Firstly the increase in DNA content was maintained in Pusa ruby, but not in SL-120. Secondly, the increase in DNA levels in the resistant variety was detectable only three days after inoculation, while in the case of the susceptible Pusa ruby, the difference was apparent within 24 hours after inoculation.

Changes in total RNA content after inoculation with *M. incognita*

The effect of nematode infection on the sequential development of RNA in root extracts of susceptible and resistant varieties is shown in Table 2. We stress the following features. Firstly, RNA in extracts

Table 1

Sequential changes in DNA[a] content in the roots of Pusa ruby and SL-120 after inoculation with *M. incognita*

Period after inoculation (day)	*Pusa ruby*				*SL-120*				*Corresponding value of t*	*P*[c]	*significance*
	Uninoculated	*Inoculated*	*Ratio*[b] *(I/U)*	*S.O. of ratio*	*Uninoculated*	*Inoculated*	*Ratio*[b] *(I/U)*	*S.O. of ratio*			
1	112	150	1.33	0.014	152	152	1.00	0.02	43.87	< 0.001	**
3	150	158	1.29	0.020	108	147	1.85	0.008	6.87	< 0.001	**
14	236	282	1.20	0.024	280	285	1.12	0.015	10.46	< 0.001	**
28	359	438	1.22	0.022	535	517	8.87	0.019	28.73	< 0.001	**

[a] DNA content is expressed as µg/g of wet tissue ; [b] I : Inoculated ; U : Uninoculated (control) ; [c] P at 22 degrees of freedom ; ** : Indicates highly significant; The data presented is the mean of three separate experiments, carried out during the year, each having four replicates.

Table 2

Sequential changes in RNA[a] content in the roots of Pusa ruby and SL-120 after inoculation with *M. incognita*

Period after inoculation (day)	*Pusa ruby*				*SL-120*				*Corresponding value of t*	*P*[c]	*significance*
	Uninoculated	*Inoculated*	*Ratio*[b] *(I/U)*	*S.D. of ratio*	*Uninoculated*	*Inoculated*	*Ratio*[b] *(I/U)*	*S.D. of ratio*			
1	870	1 220	1.17	0.014	750	960	1.28	0.026	12.29	< 0.001	**
3	757	1 127	1.48	0.026	640	1 340	2.48	0.028	89.96	< 0.001	**
14	1 455	1 287	0.87	0.021	1 425	1 480	1.04	0.021	83.19	< 0.001	**
28	2 290	3 281	1.42	0.095	3 330	2 835	0.85	0.019	19.08	< 0.001	**

[a] RNA content is expressed as µg/g of wet tissue ; [b] I : Inoculated ; U : Unicoculated ; [c] P at 22 degrees of freedom; ** : Indicates highly significant ; The data presented is the mean of three separate experiments, carried out during the year, each having four replicates.

of uninoculated roots of the resistant variety was, in general, less than that in the susceptible variety. Secondly, inoculation of the susceptible plants led to elevated levels of RNA subsequently maintained. Thirdly, inoculation of the resistant plants led to at least a two fold increased level of RNA within three days of inoculation. Fourthly, the values we obtained for inoculated resistant variety were lower only during the initial period of observation, but generally were more than those for the comparable inoculated susceptible plants.

Changes in ribonuclease activity after inoculation with *M. incognita*

Sequential changes in ribonuclease activities in the root extracts of uninoculated and inoculated plants of both the varieties are given in Figure 1. It is evident from the data that ribonuclease activities underwent remarkable changes during nematode infection in resistant as well as susceptible plant varieties. A bimodal increase in ribonuclease activity, one in the early stage and another in the late post-infection stage was observed in the susceptible variety. In contrast, we noticed only a unimodal increase in the resistant variety.

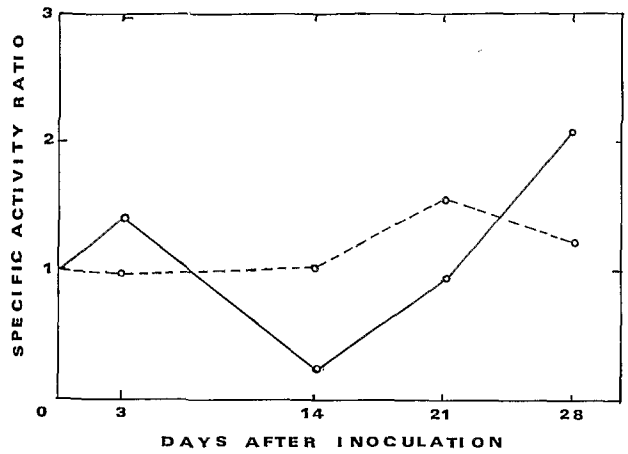

Fig. 1. Sequential changes in the ratio (inoculated : uninoculated) of the specific activity of ribonuclease in roots of tomato varieties Pusa ruby (continuous line) and SL-120 (broken line) after inoculation with *Meloidogyne incognita*. The data presented is the mean of three separate experiments carried out during the year, each having four replicates.

Disc electrophoresis

The zymogram (Fig. 2) clearly indicated a distinctly different isozyme pattern of ribonuclease in uninoculated and inoculated SL-120 resistant cultivar.

The possible role of the nematode as contaminants and thereby enhancing the level of enzyme activity in the infected plant tissue was also examined. The results (Tab. 3) indicate that the inoculum used and the degree of penetration achieved is unlikely to aid in the increased enzyme levels observed.

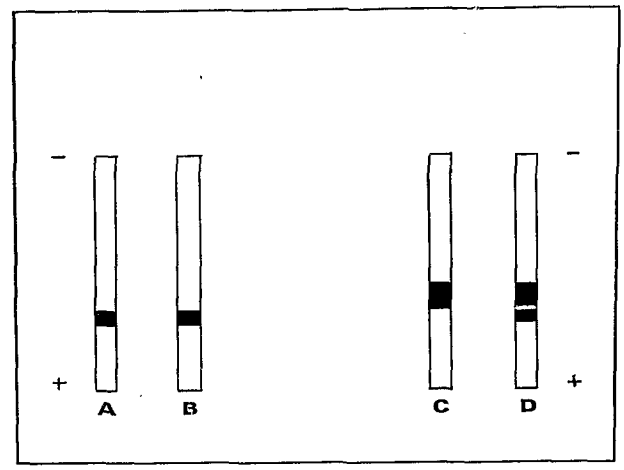

Fig. 2. Electrophoretograms (from cathode to anode) of ribonuclease from roots of tomato varieties 28 days after inoculation with *Meloidogyne incognita*. A = Uninoculated Pusa ruby ; B = Inoculated Pusa ruby ; C = Uninoculated SL-120 ; D = Inoculated SL-120.

Discussion

The results presented herein lead us to conclude that plant-nematode interaction results in altered nucleic acid metabolism, both in the case of DNA and RNA, and is in consistency with the observations made by Heitefuss and Wolf (1976), with various other pathogens. Our studies provide convincing evidence for the occurence of a preferential synthesis of total RNA quite early during the infection in the susceptible as well as the resistant variety. The newly formed RNA molecules possibly utilize the increased nucleotide pool in the plant cell (Premachandran, unpubl.) thus stepping up the protein-synthesizing machinery of the cell and account for the increased protein in the inoculated plant.

Table 3

Comparative data on the ribonuclease activity from tomato plants and from *Meloidogyne incognita*

Ribonuclease source	*Ribonuclease activity (units/mg) range*	
	Uninoculated[a]	*Inoculated*[a]
Varieties of tomato		
Pusa ruby	0.149-0.240	0.109-0.178
SL-120	0.123-0.288	0.155-0.304
Nematodes		
A	0.628	
B	0.006	
C	0.002-0.003	

[a] = Values obtained for different intervals of observation ; A = Enzyme activity obtained from homogenate of 5 × 10^5 larvae ; B = Calculated enzyme activity for 5 × 10^3 larvae used as inoculum/seedling ; C = Calculated enzyme activity for the number of larvae that probably penetrated the roots (30-50% of the initial inoculum).

In our studies there exists a striking parallelism between the accumulation of RNA and enhanced ribonuclease activity particularly in the resistant variety. In this context, it may be relevant to emphasize *(i)* the appearance of ribonuclease in two well-defined phases in Pusa ruby : an early phase and a late phase in contrast to only one phase of ribonuclease increase in the resistant variety and *(ii)* the appearance of a new isozyme in the inoculated resistant variety. We feel that the initial increase in ribonuclease activity in both the varieties during the early phase plays a decisive role in deciding the course of disease development. Apparently, the initial increase of ribonuclease activity in the inoculated plants leads to the synthesis of macromolecules which initiate restricting the parasite activity in the resistant variety or favouring the parasite activity in the susceptible variety. The significance of the appearance of the new ribonuclease isozyme may be more meaningful when we consider the synthesis of new isozymes like that of phenylalanine ammonia lyase in the inoculated resistant plants (Mote & Dasgupta, 1979). The possibility that the "new band" appearing in the inoculated resistant variety (Fig. 2) may be of nematode origin is eliminated because the number of the nematodes that have entered the roots in this case were too few to account for this. The comparative quantitative studies on the RNases of plant varieties and that of the nematodes adds support to this fact. Furthermore, although the susceptible variety harboured many more nematodes than the resistant variety, there was absolutely no evidence of the band in the former case.

The role of ribonuclease with altered kinetic properties in pathogen-induced disease development has been well documented (Chakravorty & Shaw, 1977). It is plausible that since the resistant varieties involve an incompatible relationship there is no *per se* demand on the protein biosynthetic system of the cell. Since the ribonuclease from the inoculated host is distinct from that of its healthy counterpart, it is logical to assume the role of modified or late phase ribonuclease in the post-transcriptional processing of RNA molecules occuring only in the susceptible variety towards the growth and multiplication of the nematode. The present studies thus provide information on the early events at molecular level, in the plant-nematode interaction which may be vital to the understanding of the actual mechanism of resistance.

ACKNOWLEDGEMENTS

The authors wish to express their gratitude to the Head of the Divion of Nematology, Indian Agricultural Research Institute, New Dehli, for providing the facilities for this work and to Drs. V. Shankar and R.M. Kothari, Biochemists, Sarabhai Research Center, Baroda for critically reviewing the manuscript.

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Accepté pour publication le 20 avril 1982.