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CHANGES OF ASCORBATE FREE RADICAL REDUCTASE IN PEA ROOTS INFESTED BY *HETERODERA GOETTINGIANA*

by
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There is much evidence which correlates ascorbic acid with plant resistance to various pathogens (Tonzig and Bracci, 1951; Gerola and Laudi, 1956; Farkas *et al.*, 1960; Maine and Kelman, 1961; Van Lelyveld, 1975). Van Lelyveld (1975 a) suggested that ascorbic acid may be involved in the hypersensitive reaction in plant and the formation of lesions; mango fruits showing lesions as a result of bacterial infection had a lower level of ascorbic acid than in healthy plants.

Decrease in ascorbic acid obtained by the application of lycorine, an inhibitor of ascorbic acid biosynthesis, was associated with a reduction of plant resistance to nematodes. On the other hand, the application of ascorbic acid to susceptible tomato plants rendered them partially resistant to *Meloidogyne incognita* (Arrigoni *et al.*, 1979). Moreover, it has been demonstrated that the synthesis of ascorbic acid increased in resistant plants attacked by nematodes (Arrigoni *et al.*, 1979). It seems, therefore, that ascorbic acid is utilized by the plant to activate the biological mechanisms of defence; during the process large quantities of ascorbic acid are oxidized as it functions as an electrons donor in the cell. Oxidation of the ascorbic acid (AA) results in the formation of two compounds through the successive loss of two electrons: ascorbic free radical (AFR), an intermediate compound after the loss of the first electron, and dehydro ascorbic acid (DHA), the final product of the reaction. Of the two, only AFR can efficiently be reconverted into AA because of the action of the AFR reductase (EC 1.6.5.4) present in the cells, which

catalizes transfer of electrons from NADH (α-Nicotinamide Adenine Dinucleotide) to AFR (Arrigoni *et al.*, 1981).

The aim of the work described here was to determine differences in the AFR reductase activity between pea (*Pisum sativum* L.) lines susceptible or partially resistant to the pea cyst nematode, *Heterodera goettingiana* Wollenweber, and to ascertain if enzymatic activity varies during nematode attacks.

Materials and Methods

Seeds of ten pea lines (Table I) from the germplasm collection of the Istituto del Germoplasma del CNR, Bari, were soaked overnight and planted into 30 cm plastic pots containing sterilized sandy soil. Nematode inoculation was performed by thoroughly mixing the soil, before sowing, with eggs and juveniles of *H. goettingiana* to obtain an inoculum level of 30 nematodes/g of soil. The pots were then placed in a growth chamber (16 °C, 65% RH, 3,000 lux) and 25 days later the plants were removed. A few grams of roots were stained with lactophenol-acid fuchsin to assess the extent of penetration of the nematodes. Roots for the determination of ascorbic acid and enzyme activities were washed thoroughly in distilled water and dried with filter paper. Five grams of roots were homogenized in a solution of 5% metaphosphoric acid and centrifuged at 25,000xg for 15 min; 0.1 ml of this extract was added to 2.9 ml of 0.1 M citrate 0.2 M phosphate buffer at pH 6.2, in a quartz cuvette and the OD at 265 nm was determined with a UV-visible spectrophotometer ACTA C III (Beckman Instruments Inc., Fullerton, CA). The ascorbic acid was oxidized by adding a small quantity of ascorbic acid oxidase (Boehringer) and the relative decrease in OD was determined again (Dawson and Magee, 1955). Concentrations of ascorbic acid were expressed as μg/g of dry weight and compared with a standard curve (10-100 μg) of pure ascorbic acid. Twenty grams of roots of each line were homogenized in a Waring blendor for 8 sec. in a medium containing 50 mM Tris-HCl, 0.3 M mannitol, 1 mM EDTA, 10 mM $MgCl_2$, 0.1% bovine serum albumin, 0.05% cystein, 0.1% polyvinylpyrrolidon. The homogenate was centrifuged at 600xg to remove cell debris and nuclei. Mitochondria were precipitated at 7000xg for 20 min, washed twice and resuspended in the same medium, without cystein and pyrrolidon. The microsomal fraction was precipitated from the supernatant at 100,000

xg for 2h and resuspended in the same medium as the mitochondria. The mitochondrial fraction was sonicated with a Branson sonifier. AFR-reductase activity was assayed by measuring the rate of NADH oxidation at 340 nm in the presence of AFR generating systems (Arrigoni *et al.*, 1981). The reaction mixture contained 0.2 mM NADH enzyme (~ 400 µg protein), 1 mM AA and 1 U AA-oxidase. Proteins were determined by the method of protein-dye binding (Bradford, 1976).

Results

Among the ten lines tested three were defined, arbitrarily, as resistant on the basis of the nematode densities detected in their roots. Nematode numbers were much lower in them than in three other lines considered to be susceptible (Table I).

The concentrations of AA determined in the roots of the plants indicated that, following nematode invasion, there are two different trends: the AA content tends to increase, with respect to the uninfested plants, in the so called resistant lines and abruptly decreases in the susceptible ones (Table I). The increase was of about 180% in the lines MG 101877 c and MG 101956 a which supported the least

Table I - *Density of population of* Heterodera goettingiana *and ascorbic acid content in resistant (R) and susceptible (S) pea lines.*

Lines	No. nematodes/g roots	Ascorbic acid µg/gdw
MG 101877 c (R)		1.47
» + Nematodes	1,407	4.10
MG 101956 a (R)		1.55
» + Nematodes	1,619	4.26
MG 101877 b (R)		1.59
» + Nematodes	2,407	2.86
MG 101956 b (S)		2.85
» + Nematodes	4,506	1.75
MG 101877 a (S)		4.49
» + Nematodes	4,820	1.93
MG 101793 (S)		7.16
» + Nematodes	5,893	3.05

number of nematodes in the roots. There was an increase of about 80% for the other resistant lines which had a slightly higher infestation. Conversely, there was a decrease of 40-57% of AA in the susceptible lines, with the larger decreases occurring in the more highly infested lines. The correlation between severity of infestation and content of AA is clearly shown in Fig. 1.A.

The activity of the AFR-reductase, tested on the soluble fraction, mitochondria and microsomes of the same roots, also was considerably affected by the attack of the nematodes. Again the resistant lines always showed an increase and the susceptible ones a decrease in the activity of the enzyme (Table II). Changes of the AFR-reductase activity occurred in all three cellular fractions examined, although they were largest in the microsomes and least in the mitochondria.

Among the resistant lines the increase of AFR-reductase activity in the microsomes, following nematode attack, was 51% in MG 101877 b, 158% in MG 101877 c and 230% for MG 101956 a, while decreases ranged from 60 to 72% for the susceptible lines. A direct correlation between AFR-reductase activity and the AA content in the microsomes of nematode infested plants is shown in Fig. 1.B.

Table II - *AFR-reductase content in resistant (R) and susceptible (S) pea lines infested and not by* Heterodera goettingiana.

Lines	AFR reductase as µg NADH / min / mg proteins		
	Mitochondria	Microsomes	Soluble fraction
MG 101877 c (R)	10.47	1.37	4.03
» + Nematodes	12.12	3.53	4.84
MG 101956 a (R)	11.00	1.69	1.78
» + Nematodes	12.69	5.60	2.80
MG 101877 b (R)	6.33	1.61	1.33
» + Nematodes	8.63	2.43	1.87
MG 101956 b (S)	10.40	2.87	3.09
» + Nematodes	8.23	1.14	1.59
MG 101877 a (S)	6.09	3.46	1.92
» + Nematodes	3.31	1.14	0.97
MG 101793 (S)	12.72	4.57	5.76
» + Nematodes	7.69	1.25	1.50

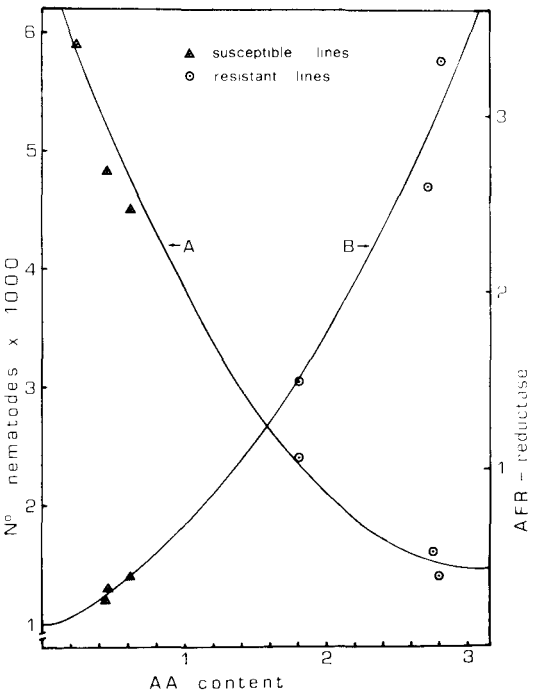

Fig. 1 - Relationship between changes in ascorbic acid content and nematode populations in roots (A) and microsomal activity of AFR-reductage (B); ascorbic acid and AFR-reductase activity expressed as ratio infested/healthy.
A: $y = 4.81 - 1.59x + 0.14x^2$
B: $y = 0.10 + 0.32x + 0.26x^2$.

Discussion

The results of these studies further confirm the close relationship in plants between their capability of synthesizing AA following nematode attack and their capacity to overcome the injuries caused by the parasite.

The pea lines tested as resistant or susceptible to *H. goettingiana* showed similar biochemical behaviour to tomato plants resistant and susceptible to *Meloidogyne incognita* (Arrigoni *et al.*, 1979). In the pea lines, as in the tomato plants, nematode attack caused an increase of AA in resistant plants and a decrease in susceptible ones.

It is also shown that in resistant plants subject to nematode attack, there is an increase in the activity of AFR-reductase, the key enzyme which maintains AA in its reduced form.

Therefore the resistant lines, beside having an increased capability of biosynthesizing and utilizing AA, also have an increased capability of reconverting the main product of its oxidation, AFR, to AA by means of electrons obtained from the system of the piridinnucleotides.

It is interesting that AFR-reductase activity increases particularly in the microsome fraction; that is in the endoplasmic reticulum, a cellular constituent actively engaged in hydroxylation processes. The AFR-AFR-reductase systems is clearly involved in the hydroxylation processes, but only as regenerator of AA and not directly as assumed by Schneider and Staudinger (1965).

The increase of AFR-reductase that occurred in the microsomes of resistant plants following nematode attack indicates that AFR is produced in large quantities and consequently the utilization of AA is very high in this cell component.

This agrees with two demonstrated facts:

i) AA is required for the *in vivo* synthesis of proteins containing hydroxyproline; in this process AA acts as a donor of electrons during the hydroxylation of the proline present in the polypeptidic chain (Arrigoni *et al.*, 1977);
ii) the synthesis of hydroxyproline containing proteins increases in resistant plants following nematode attack (Zacheo *et al.*, 1977).

According to the recently proposed hypothesis of a biological mechanism of defence (Arrigoni, 1979), it would be really the ascorbic dependent synthesis of hyproproteins to permit development of the cyanide resistant respiration (Arrigoni *et al.*, 1976), which is the metabolic event initiated by the cell to counteract the effects of the pathogen.

SUMMARY

The relationship between ascorbic free radical (AFR)-reductase and ascorbic acid (AA) content was studied in pea roots of plants resistant and susceptible to the pea cyst nematode *Heterodera goettingiana*. The nematode induced in resistant plants a large increase and in susceptible ones a decrease of both AFR-reductase and AA. The resistant plants showed an increased capability of biosynthesis and a better utilization of AA through the reconversion of AFR to AA, by means of the AFR-reductase.

LITERATURE CITED

ARRIGONI O., 1979. A Biological Defence Mechanism in Plant. *In*: Root-knot Nematodes (*Meloidogyne* spp.) Systematics, Biology and Control (Eds. F. Lamberti and C. E. Taylor) Acad. Press, London and New York, pp. 457-467.

ARRIGONI O., ARRIGONI-LISO R. and CALABRESE G., 1976. Ascorbic acid as a factor controlling the development of cyanide-insensitive respiration. *Science, 194*: 332-333.

ARRIGONI O., DE SANTIS A., ARRIGONI-LISO R. and CALABRESE G., 1977. The increase of hydroxyproline-containing proteins in Jerusalem artichoke mitochondria during the development of cyanide-insensitive respiration. *Biochem. Biophys. Res. Commun., 74*: 1637-1641.

ARRIGONI O., ZACHEO G., ARRIGONI-LISO R., BLEVE-ZACHEO T. and LAMBERTI F., 1979. Relationship between ascorbic acid and resistance in tomato plants to *Meloidogyne incognita*. *Phytopathology, 69*: 579-581.

ARRIGONI O., DIPIERRO S. and BORRACCINO G., 1981. Ascorbate free radical reductase, a key enzyme of the ascorbic acid system. *FEBS Letters, 125*: 242-244.

BRADFORD M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem., 72*: 248-254.

DAWSON C. R. and MAGEE R. J., 1955. Ascorbic Acid Oxidase. *In*: Methods of Enzymology. Vol. II, Acad. Press, London, New York, S. Francisco, pp. 831-835.

FARKAS G. L., KIRALY Z. and SOLYMOSY F., 1960. Role of oxidative metabolism in the localization of plant viruses. *Virology, 12*: 408-421.

GEROLA F. M. and LAUDI G., 1956. Ricerche sulla fisiologia delle piante virosate. I. Contenuto in acido ascorbico nelle foglie di *Spinacia oleracea* affette da mosaico del cetriolo. Rendic. Acc. Naz. Lincei, Cl. Sc. Fis. Ser. VIII, 20: 89-94.

MAINE E. C. and KELMAN A., 1961. The influence of reducing substances on resistance to bacterial wilt in tobacco. *Phytopathology, 51*: 491-492.

SCHNEIDER W. and STAUDINGER Hj., 1965. Reduced nicotinamide-adenine nucleotide-dependent reduction of semidehydroascorbic acid. *Biochim. Biophys. Acta, 96*: 157-159.

TONZIG S. and BRACCI L., 1951. Ricerche sulla fisiologia dell'acido ascorbico. VI. Attività tubercoligena del *Rhizobium leguminosarum* e acido ascorbico. *N. Giorn. Bot. Ital., 58*: 258-270.

VAN LELYVELD L. J., 1975. Bacterial black spot in mango (*Mangifera indica* L.) fruits. Ascorbic acid and the hypersensitive reaction as a means of resistance. *Agroplantae, 7*: 45-50.

VAN LELYVELD L. J., 1975 a. Ascorbic acid content and enzymes activities during maturation of the mango fruit and their association with bacterial black spot. *Agroplantae, 7*: 51-54.

ZACHEO G., LAMBERTI F., ARRIGONI-LISO R. and ARRIGONI O., 1977. Mitochondrial protein-hydroxyproline content of susceptible and resistant tomatoes infected by *Meloidogyne incognita*. *Nematologica, 23*: 471-476.

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