ALTERATIONS IN PEROXIDASE ACTIVITIES INDUCED BY ROOT KNOT NEMATODE ON TOMATO[1]

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Abstract

Root knot nematode infections on tomato stems and roots altered the peroxidase contents in these organs considerably. Peroxidase levels in tomato stems began to rise 24 hrs after inoculation with *Meloidogyne incognita*. The rise in enzyme level chronologically coincided with the initiations of giant cells and hyperplasia. The enzyme level rapidly ascended to a peak on around 10 th day after inoculation when a drastic drop ensued. It remained low until the 17 th day. The period of low enzyme levels corresponded to the III and IV stages of the pathogen in galls. Young adults began to appear 17 days after inoculation. Rapid ascent in peroxidase level also resumed around this time and reached a second peak on 27 th day. Abnormal tracheary element differentiations commenced on about 9 th day after inoculation and by 23 rd day prominent tracheary network was histologically detected. Mature females were observed 23 days after inoculation. From there on ovipositions and secondary infections were detectable within stem galls. The latters were thought to have additive effects on the rise in peroxidase activities. Histochemical tests demonstrated that the enhanced peroxidase activities were localized in hyperplastic tissues and occassionally giant cells of stem galls.

Introduction

Infections of many fungi are known to enhance peroxidase activities in their host plants (Fehrman and Dimond 1966, Jennings *et al.* 1969, Maxwell and Bateman 1967, Mace 1964, Wood 1967 and Weber *et al.* 1967). Tobacco leaves inoculated with tobacco mosaic virus (Lovrekovich *et al.* 1968b) or injected with dead cells of *Pseudomonas tabaci* (Loverekovich *et al.* 1968a) are also reported to result in increased peroxidase activities. The only reports of heighten peroxidase activities due to nematode infections known to the

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authors are those by Myuge about *Ditylenchus destructor* on potato tubers and *M. incognita* induced root galls of cucumber and radish (Krusberg 1963). Details of the reports, however, are not available to us.

The authors have recently demonstrated that infections of tomato stems by root knot nematodes resulted in rapid increase in the number of peroxiease isoenzymes which was detectible 12 hrs. after inoculation (Huang *et al.* 1971). As part of our attempts to elucidate the physiological roles of the individual peroxidase isoenzymes in gall developments, it was thought of importance to follow the changes of peroxidase level in the process of galling manifestation and histological significance of enzyme activities in the galls.

Materials and methods

Tomato (*Lycopersicon esculentum* var. Break' 0 Day) stems were inoculated with *Meloidogyne incognita* according to the method previously described (Huang and Lin 1970). Inoculation media and unpenetrated nematodes were removed five days after inoculation. Root galls are obtained by pouring nematode inoculum into the root systems. All of the experimental plants, inoculated or otherwise, were maintained in a growth chamber where diurnal temperature fluctuated between 27–30°C. Twelve hours of fluorescent lighting, 7000 lux on the level of seedling top, was given daily.

At the presence of H_2O_2, peroxidase carries out the oxidation of guaiacol to form tetraguaiacol whose concentration can be determined colorimetrically. The guaiacol test (Maehly and Chance 1959) employed to assay peroxidase in the tissues is based on this principle. Preparation of the reaction mixtures and assay procedures were conducted as follows: 2 ml. of 0.02 M aqueous guaiacol solution (0.22 ml. of guaiacol stock solution, Sigma Lot No. 38B-1310, in 100 ml. of water), 2 ml. of 0.01 M phosphate buffer at pH 7.0 and 10 μ of tissue sap freshly expressed from a sample were placed in a test tube of Bausch & Lomb Spectronic—20 colorimeter. Immediatly after 10 μl of 0.01 M H_2O_2 was added, the contents in the test tube were momentarily mixed with an electric vibrator and the tube placed in the colorimeter. ODs at 470 mμ were then recorded for the reaction mixture continuously at 15 seconds intervals for two minutes.

For histochemical localizations of peroxidase in the tissues, benzidine-blue method (Van Duijn 1955) was employed. The reaction mixture was prepared as follows: to 200 ml of 0.1 M phosphate buffer at pH 7.0, 6 g of benzidine acetate, 10 g of NH_4Cl and 2 ml. of 35% H_2O_2 were added and thoroughly mixed. The final test solution thus contained benzidine acetate (saturated), NH_4Cl 5% (w/v) and H_2O_2 0.1 M and had a pH of around 6.5. The mixture was used immediately after prepared.

To localize the enzyme activities, fresh sections of 200 μ thick were made with Oxford Vibratome Sectioning System* in a 0.1 M phosphate buffer bath at pH 7.0. The sections were washed briefly in the same buffer and floated in the test solution for 10 minutes. The "stained" sections were then passed through two water baths briefly to remove crystals formed superficially and observed under a microscope within 10 minutes. Paraffin sections of 15 μ thick were prepared according to standard TBA procedures (Jensen 1962) and stained with safranin-fast green.

Results

Total peroxidase activities in various tomato organs.

To compare relative peroxidase activities in various organs, guaiacol tests were conducted with fresh tissue saps expressed respectively from organs of two-and-half-month old tomato. As shown in Fig. 1, total peroxidase activities in root system is substantially higher than that in any other norml organs of the same plant. It is to be noted further that both stem and root galls contain significantly higher peroxidase activities than their respective counterparts.

Changes in peroxidase activities in developing galls.

Fig. 1 demonstrated that gall tissues do have higher peroxidase activities. It is therefore of interest to see how the enzyme levels change with the process of gall development. Inoculated tomato stems were periodically harvested and assayed for total peroxidase activities. For each sample (10 μl fresh tissue sap) change in optical density per 15 second at 470 mμ ($\triangle$OD/15 sec) obtained during the initial linear reaction rate of the guaiacol test was taken to represent the magnitude of enzyme activity. Uninoculated portion of the same stem (right next to a gall) was assayed concurrently with the gall to serve as a control.

With respect to the ages of galls, following pattern of peroxidase changes can be deduced (Fig. 2). (1), Two days after inoculation, the enzyme level began to ascend rapidly until 9th day when it reached the first peak. (2), The enzyme level dropped rapidly 10 days after inoculation and remained low until the 17 th day when a drastic rise commenced again. (3), A second peak was reached on 27 th day which is considerably higher than the first one and a rapid descent ensued from there again.

Histological significance of peroxidase activities in gall development.

Portions of the same galls used in the previously described assays were fixed and serial paraffin sections prepared so that histological changes can be studied in terms of peroxidase levels. In the first week of inoculation when

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a gall is not large enough to be separated for the two tests, however, separate galls of equivalent ages were employed for the histological purposes.

Table 1 summarizes the histological events which took place chronologically in the process of stem gall development. Data for each vertical columns

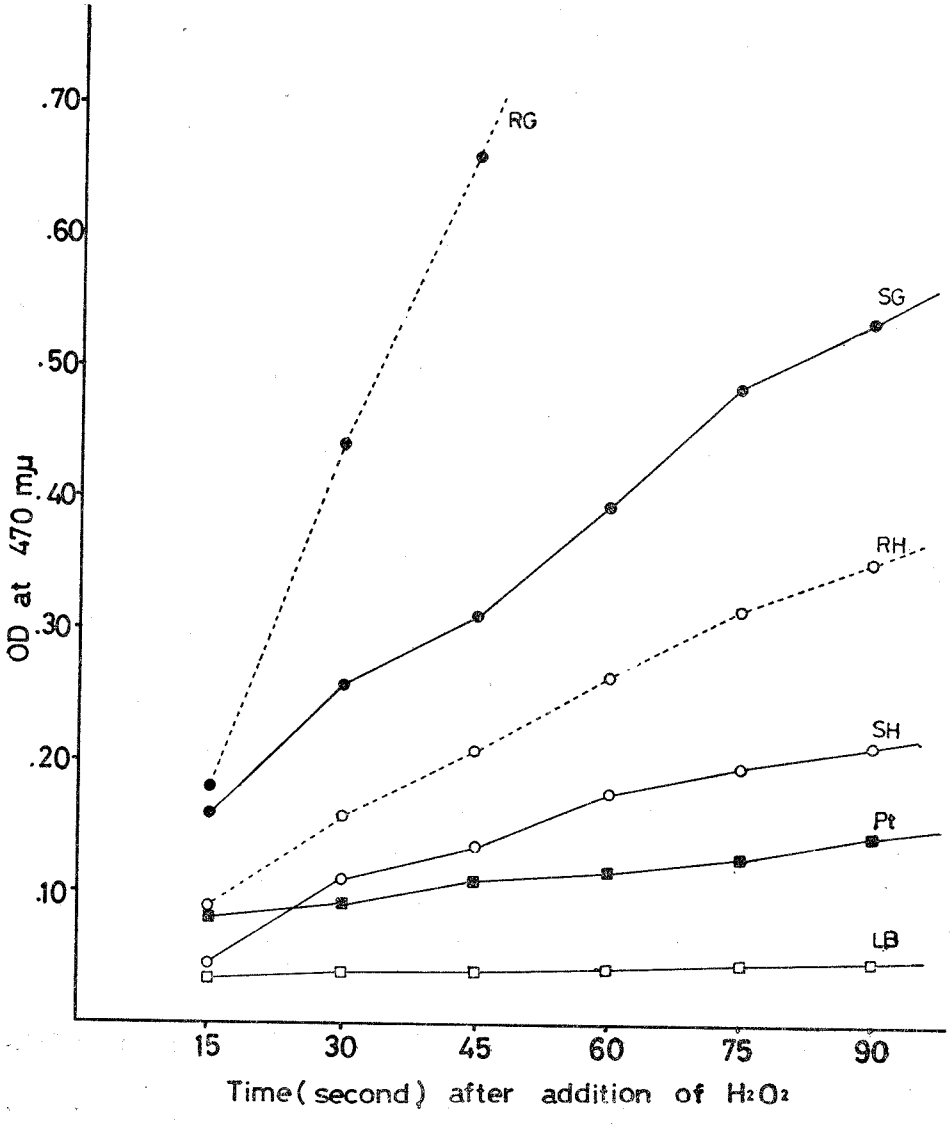


Fig. 1. Relative peroxidase activities in various organs of tomato. RG=root galls induced by root knot nematode 78 days after inoculation. SG=stem gall induced by root knot nematode 61 days after inoculation. RH=normal root system. SH=normal stem. Pt=petioles. LB=leaf blade. SG, RH, SH, Pt and LB are from the same tomato plant whereas RG belongs to a separate inividual. Each reaction mixture contained 2 ml of 0.02 M aqueous guaiacol, 2 ml of 0.01 M phosphate buffer at pH 7.0, 10 μl of fresh tissue sap and 10 μl of 0.01 M H_2O_2.

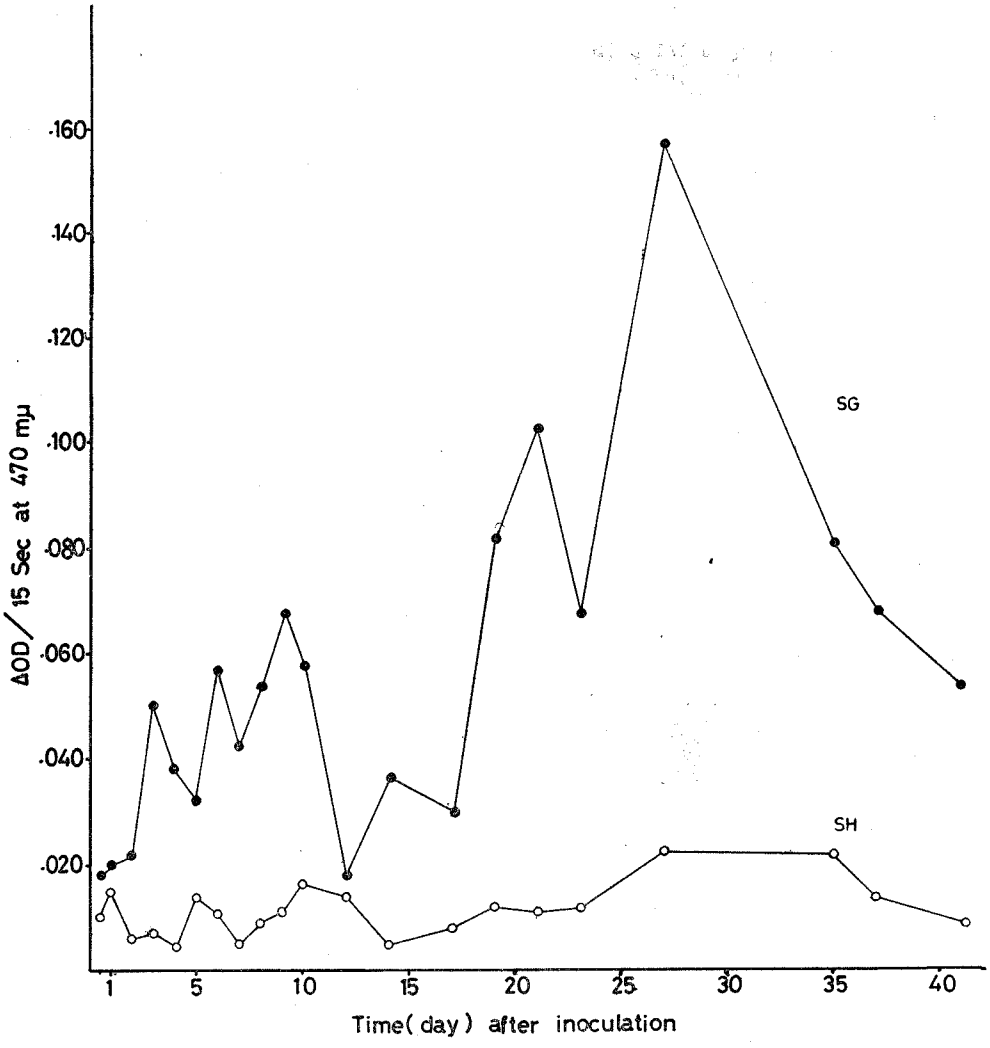


Fig. 2. Peroxidase activities in stem galls of various ages as measured by the changes in OD at 470 mμ per 15 sec for 10 μl tissue saps in guaiacol $-H_2O_2$ assay system (Maehly and Chance 1959). SG=stem galls. SH=healthy stems.

(ages of gall) in Table 1 are from the serial sections of three galls or half-galls.

The process of penetration commenced 24 hrs after inoculation and lapsed for a few days under the system (Fig. 3A, B). Giant cells, hyperplasia and abnormal tracheary element differentiation (Fig. 3C, D, E, F, G, H, I) which followed the nematode penetration are therefore not chronologically uniform between galls of the supposedly similar ages. So are the stages of nematodes in the galls.

Table 1. *Histological events associated with tomato stem gall development induced by* ***Meloidogyne incognita***

Gall features in section	Days after inoculation												
	½	1	1½	2	3½	9	10	12	14	23	35	37	41
Migrating II larvae	−	+[1]	+[2]	+[2]	+[2]	−	−	−	−	+[2]	−	−	−
Giant cells	−	−	−	±	+	+	+	+	+	+	+	+	+
Hyperplasia	−	−	−	±	+	+	+	+	+	+	+	+	+
Abnormal tracheary elements[3]	−	−	−	−	−	+	+	+	++	+++	+++	+++	+++
Oviposition	−	−	−	−	−	−	−	−	−	+	+	+	+
Stages of nematode[4]	−	II	II	II	II	II	II III	III	III	II IV V	IV V	V III	IV V

+ =Positive occurrance.
− =Negative occurrance.
± =Presence uncertain.
1=Under epidermis.
2=In cortex.
3=In this column + indicates trace amount, ++ in small amount but easily detected under 10×4 optical system and +++ prominently present.
4=In this column, II indicates second stage larvae, III, third stae, IV, fourth stage and V, adult females (Triantaphyllou and Hirschmann 1960).

With some fluctuations, the general pattern of rise in the total peroxidase activities (Fig. 2) chronologically matches well with the appearances of giant cells, hyperplasia and abnormal tracheary elements in stem galls. Histochemical tests revealed that the enzyme was most active in the hyperplastic tissues. Giant cells generally yielded negative localization but occassionally intensive peroxidase reaction can also be seen in these multinucleated cells.

Discussion

Preliminary tests indicated that even under the growth chamber condition, peroxidase level in a normal tomato stem was slightly higher during "light-period" as compared to "dark-period" (unpublished data). All samplings throughout the studies therefore were conducted between 8:00–9:00 AM. Even so, slight variations in the enzyme contents were detected between the uninoculated stems of different individuals (Fig. 2). Similar variables undoubtedly were also incorporated into the stem gall curve in Fig. 2. Additional variables could of course come from the time individual larvae took to penetrate and establish their feeding sites which accoring to histological studies (Table 1) should be in the neighborhood of three days. Irrespective of those uncontrolled variables, however, the changes in peroxidase levels with respect to the ages of galls are evident.

Histological studies (Table 1 and Fig. 3) indicated that the first ascent of

peroxidase level (Fig. 2) corresponded chronologically with the giant cell formation, hyperplasia and abnormal tracheary differentiation. From around 23rd day, the galling manifestations were compounded by oviposition in the tissues (Fig. 3J), secondary infections (Fig. 3K) and enhanced tracheary differentiations (Fig. 3F, G, H, I). These probably would account for the reason why the second rise in peroxidase was much more than the first one (Fig. 2).

Bird (1959) demonstrated that the growth curve of *M. javanica* is characterized by a plateau starting at the onset of second molting and extending to the completion of last molt when a rapid increase in body size follows. During this period, i.e., the Ⅲ and Ⅳ stages, the nematode ceases feeding completely. The young adults resume active feeding. Since *M. hapla* also seemed to behave in the same manner (Bird 1959), it is likely that the nematode used in this investigation is of no exception. If such an assumption is correct, there must be a lag of "no-feeding" between 10th to 23rd day after inoculation when only Ⅲ and Ⅳ stages were found in the gall sections (Table 1). Interestingly enough, this "no-feeding" period corresponded well with the period of low peroxidase level in the galls (Fig. 2). How the nematode feeding is related to the enzyme level, we believe, is a fertile area for further research.

Enhanced peroxidase localization is known to take place prior to or accompanying cell divisions in wound meristems and other meristematic tissues (Van Fleet 1959). Localization of the enzyme found in hyperplastic tissues of the galls seems to agree with such general rule; for these tissues are actively dividing. Giant cells occupy large area and their multinuclei are known to divide synchronously (Huang & Maggenti 1969). Since cessation of cell divisions are also known to associate with loss or inhibition of the enzyme activity (Van Fleet 1959), it is not surprising that one does not always find positive or negative peroxidase localization in these multincleated cells.

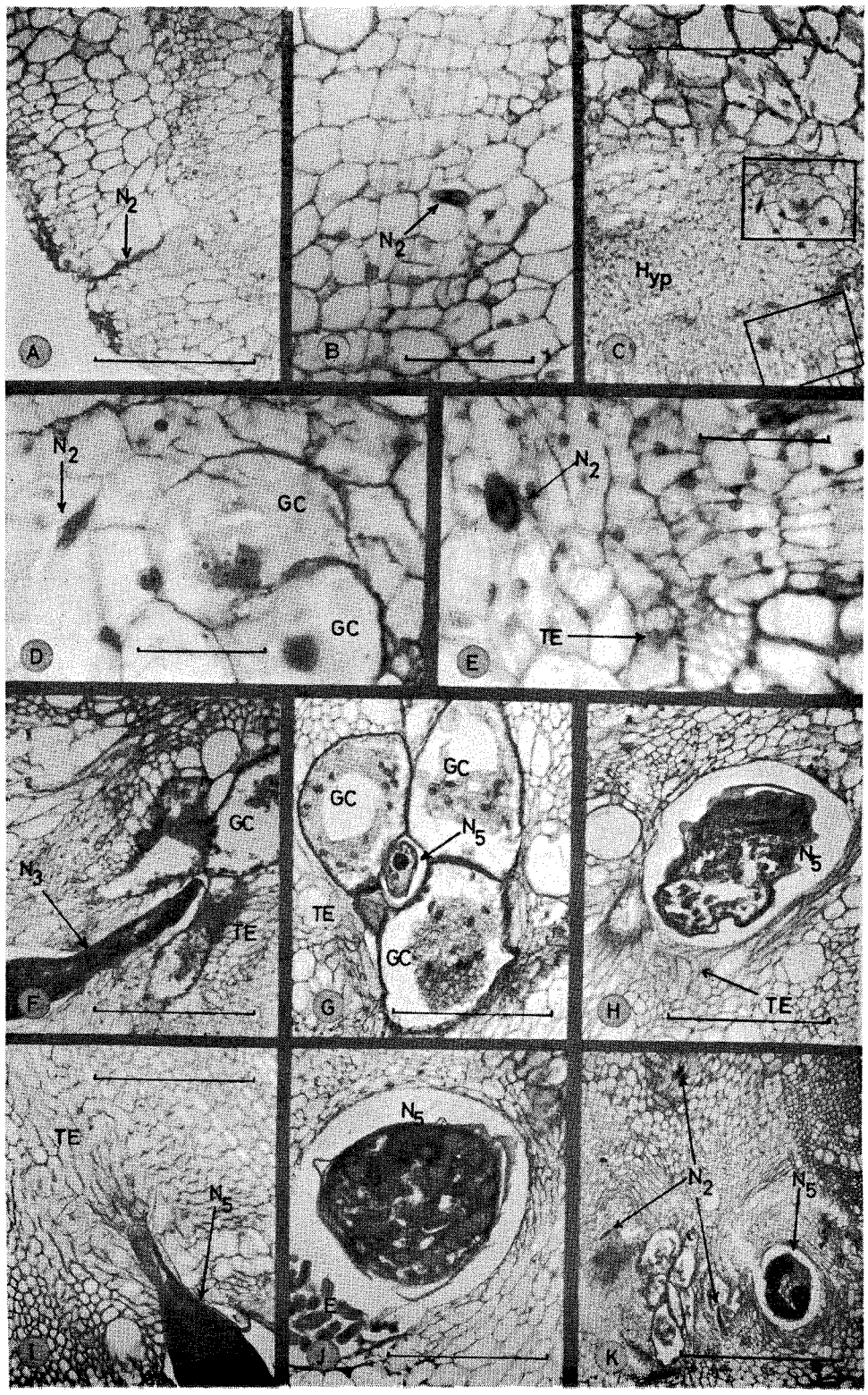
Infection by root-knot nematode and the subsequent gall development are known to induce numerous peroxidase isoenzymes which otherwise are not present or present in trace amount in a normal stem. (Huang *et al.* 1971). It is possible that the enhanced enzyme levels in the galls (Fig. 2) are related to the gall specific peroxidase isoenzymes.

根瘤線蟲誘致蕃茄瘤腫所引起之過氧化酵素變化

黃炤雄 林麗華 黃修斌

蕃茄器官中過氧化酵素之活性依根、莖、葉柄及葉片之次序遞減。根瘤線蟲可引起根莖中過氧化酵素活性之激烈變化。莖部接種根瘤線蟲24小時後過氧化酵素之活性開始上昇。酵素活性之激變與巨形細胞及增生組織（Hyperplasia）之形成在時間上相吻合。酵素活性在接種後第10天左右達到第一個高峯，而後急遽下降。這個酵素活性的低潮正值瘤中線蟲第三、四齡期。感染後第17天出現初期成蟲。此時過氧化酵素之活性又開始急遽上昇。直到第27天達到第二個頂峯。病理解剖顯示感染後第9天開始有異常之輸導系統分化。至第23天可觀察到甚多此種異常組織及成蟲。從此產卵及第二次感染也都陸續出現。第二次感染似可導致過氧化酵素活性之倍增。組織化學之研究顯示過氧化酵素的活性集中於分裂旺盛的增生組織，偶而也發現在巨形細胞內。

Fig. 3. Histological changes of tomato stems due to *Meloidogyne incognita* infections. (A), A second stage larvae (N_2) penetrating through cortex 24 hrs. after inoculation. (B), 3½ days after inoculation, a second stage larvae (N_2) reached cambium region. (C), Nine days after inoculation, multinucleated giant cells (upper square) and hyperplasia (Hyp) are evident. Abnormal tracheary elements (lower square) are also initiated. (D), Enlarged figure of the upper square in (C). N_2=a section of second stage larvae, GC=giant cell. (E), Enlarged figure of the lower square in (C). Note the abnomal tracheary element initial (TE). N_2=a section of second stage larvae. (F), 14 days after inoculation, nematode developed to third stage (N_3) and the abnormal tracheary elements (TE) are more developed (++ in Table 1). (G) and (H), 23 days after inoculation, the nematode developed to maturity (N_5). Giant cells (GC) and abnormal tracheary elements (TE) are prominent. N_5 in (G) is a cross section of nematode esophageal region and that in (H) is a cross section of the hind portion of the same nematode. (I), 27 days after inoculation, abnormal tracheary elements (TE) are very pronounced. (J), 27 days after inoculation. N_5=a cross section of the hind portion of a mature female. E=eggs. (K), 27 days after inoculation. The three second stage larvae (N_2) are progenies of the primary inoculum and at least one of them have established secondary infection (with giant cells). Note the extensive hyperplastic development. N_5=a section of mature female which is the primary inoculum. The scales in (A), (C), (F), (G), (H), (I) and (J) are 300 μ, those in (B), (D) an (E) are 50 μ whereas the one is (K) is 750 μ.


A
N2
B
N2
C
Hyp
D
N2
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E
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N5
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GC
H
N5
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I
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N5
J
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K
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