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Cytochemical investigation of resistance to root-knot nematode *Meloidogyne naasi* in cereals and grasses using cryosections of roots

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Summary – Some enzymes and plant constituents mobilized in the development of hypersensitive reactions were localized in healthy and infected roots of two plants fully resistant to *Meloidogyne naasi* (the grass *Aegilops variabilis,* and the wild barley *Hordeum chilense*), as well as from susceptible wheat and barley (*Triticum aestivum* cv. Chinese Spring and *Hordeum vulgare* cv. Doublet). Soon after infection, resistant roots showed induced reactions due to increased peroxidase activity and production of free radicals. Acid phosphatase activity in necrotic sites was detected after disruption of lytic vacuoles. Callose was mobilized early in *A. variabilis,* whereas lignin accumulated in *H. chilense.* In both cases, phenol oxidation was an early response to nematodes, whereas suberization was found later in cells contacting the endodermis. The role of these substances in the resistance of graminaceous hosts is discussed.

Résumé – *Recherche sur la cytochimie de la résistance au nématode à galle* Meloidogyne naasi *chez les céréales et les graminées sauvages* – La localisation histologique de certaines enzymes et constituants végétaux généralement mobilisés dans les réactions d'hypersensibilité a été étudiée sur du matériel sain et infesté provenant de deux plantes totalement résistantes à *Meloidogyne naasi* – les graminées sauvages *Aegilops variabilis* et *Hordeum chilense* – ainsi que sur les plantes sensibles – *Triticum aestivum* cv. Chinese Spring et *Hordeum vulgare* cv. Doublet. Chez les plantes résistantes, et contrairement aux plantes sensibles, certains changements sont observés peu de temps après l'infestation : activité de la péroxidase et production de radicaux libres accrues aux sites de réaction induite. Dans les jours suivants, après rupture des vacuoles lytiques, une activité de la phosphatase acide a été décelée dans les cellules nécrotiques. Chez *A. variabilis,* un dépôt rapide de callose dans les parois des cellules au contact des nématodes intervient alors que chez *H. chilense* on assiste à une accumulation de lignine. Dans les deux cas, une oxydation précoce des phénols se produit dans les cellules hypersensibles ainsi qu'une subérisation tardive au contact de l'endoderme. Le rôle de ces substances dans la résistance des graminées est discuté.

Key-words : *Meloidogyne naasi,* hypersensitivity, enzyme histo-localization, *Aegilops variabilis, Hordeum chilense.*

Plants show a variety of responses when attempting to resist attacks by pathogens, as well as mechanical or chemical stresses. When these responses are successful and prevent or inhibit pathogen growth, the plant is considered to have a complete or fully functional resistance. Most of these responses are found to be hypersensitive-type reactions that involve : *i)* changes in enzyme activities, e.g. peroxidases (Mohan & Kollatukudy, 1990), acid-phosphatase (a marker of lysosomes and senescence programme; Jordan & De Vay, 1990); *ii)* changes in phenol metabolism such as the accumulation of oxidized phenolic substances which may behave like a phytoalexin (Rhodes & Wooltorton, 1978); *iii)* the deposition of newly synthesized material in cell walls, i.e. callose, suberin and lignin (Kollatukudy, 1978; Rhodes & Wooltorton, 1978; Vidhyasekaran, 1988 *a, b*); and *iv)* changes in plasma membrane production and regulation of free radicals of oxygen (Doke, 1983; Sutherland, 1991).

Although no causal relationship has yet been clearly established between the resistance to pathogens and hypersensitive reactions (Lindgren *et al.*, 1992), these reactions are thought to be responsible for resistance and can be used to study and compare the response of hosts with different compatibilities.

Early hypersensitive-type reactions characterized the response of two plants, *Aegilops variabilis* and *Hordeum chilense,* which were found to be fully resistant to the cereal root-knot nematode *Meloidogyne naasi* (Balhadère & Evans, 1995.). The reactions were characterized by the autolysis of a few cells in the endodermis or the cortex adjacent to the nematode, accompanied by some early wall changes (i.e. the deposition of material stained in green by fast green, possibly callose in *A. variabilis;* and some material stained in red by safranin, possibly lignin, in *H. chilense*).

Cytological and histochemical changes were studied, using freezing microtomy followed by enzyme histoche-

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mistry, to further investigate the important first few days which determine whether a susceptible, resistant or intermediate response is produced.

Peroxidase and acid-phosphatase activities were studied as well as the localization of callose, suberin, lignin, oxidized phenols, acidic vacuoles and Nitroblue tetrazolium (NBT) reduction (used as a marker of metabolism), in uninfected and infected susceptible and resistant plants.

Materials and methods

Host plants studied

Two susceptible hosts, the wheat cv. Chinese Spring and the barley cv. Doublet, a resistant accession of wheat " x^8 " (with resistance from the wild grass *A. variabilis*) and the resistant accessions of *H. chilense* " PI283374 " and " PI283375 " were studied.

Source of nematodes and nematode inoculation

Second-stage juveniles of *M. naasi* were extracted from infested soil, obtained from the Institute of Grassland and Animal Production, Aberystwyth.

Three-day old seedlings were inoculated in agar plates and incubated at 20 °C (14 h light), (Balhadère & Evans, 1996). Observations on infected roots were made 1, 5 and 10 days later.

Tissue preparation for sectioning

A method modified from Sanderson (1972) was used for the rapid freezing of healthy and infected 1 cm long portions of roots. Root segments cut from the plant were immediately plunged into cold " OCT Compound " at 2 °C for embedding (Tissue Tek, Miles Company) and then stuck onto a holder made of filter paper. While dipping this holder with the root segments attached into liquid nitrogen, cold " OCT Compound " was also spread onto the section-holder of the microtome whose base was immersed in liquid nitrogen; the top was therefore surrounded by an atmosphere of cold nitrogen vapor. Using the paper holder, the section was quickly placed into the freezing layer of medium and orientated vertically. The paper holder was then removed and more cold " OCT Compound " was added before melting could occur. The metal section holder of the microtome, now with embedded plant segments, was quickly immersed in liquid nitrogen. More embedding medium was added if necessary, to entirely surround the specimen, before immersion again in liquid nitrogen.

The holder was put into a cryostat (Bright Ltd, model 5030 OTF/AS/M). The temperature of the specimen, measured with a thermocouple, was brought to – 24 °C, a favourable temperature for sectioning freeze-embedded plant material (Gahan *et al.*, 1967). Transverse or longitudinal sections, approximatively 12 μm thick were cut. Sections were picked up with a fine brush, placed on a cold slide, and brushed with a minimal quantity of cryostat-cold butanol in order to limit the loss of water during thawing. Slides were warmed slowly to room temperature in a tightly closed container and checked for their structural integrity under a low power light microscope. After evaporation of most of the butanol, slides could be safety washed using several changes of distilled water (DW).

Cytochemical tests

Enzyme activities were visualised in sections cut from within 0-3 mm of the root tip. All tests were done at room temperature (close to 20 °C), appropriate for plant tissues, with times of incubation often increased compared to procedures developed for mammalian tissues (Jensen, 1962).

For peroxidase activity, each section was covered for about 5 min by a droplet of localization medium (Gahan, 1984) consisting of 20 ml of 0.1 M Tris pH 7.6 with 10 mg p-phenylediamine, 20 mg pyrocatechol with 0.2 ml of freshly diluted 1 % H_2O_2 solution (Gahan, 1984). Control sections were made by incubation in localization solution lacking H_2O_2. After rinsing in DW, sections were mounted in glycerin jelly and viewed under a Dialux 20ER light microscope. A dark-brown deposit indicated sites of activity.

An azo-dye method proved unsuccessful for acid-phosphatase localization even when using sections previously fixed for 1/2 hr in a buffered cold solution of 10 % formalin at 4 °C, instead of being treated with butanol. Acid-phosphatases often lose significant activity in water and ethanol but formalin post-fixation often increases enzyme retention (Nachlas *et al.*, 1956). Adding 20 % polyvinyl alcohol into the test-medium, to increase its viscosity and attempt to reduce the loss of enzyme (as used with dehydrogenase enzymes; Gahan, 1984), was unsuccessful.

In an alternative approach, unsectioned root segments were incubated for 10 min in the test-solutions immediately after excision from the main root. They were then treated in a sonicator, until a colour change was visible. Root segments were then processed by freeze-sectioning, washed in DW and mounted in glycerin jelly as before.

The incubation/sonication medium used for acid-phosphatase consisted of 25 ml of 0.2 M acetate buffer Ph 5, containing 3 mg naphthol ASBI phosphate and 20 mg fast blue BB. Controls were made by incubating root sections for 10 min in the solution lacking substrate or in the solution to which 10 mM sodium fluoride had been added (Gahan, 1984). A blue colour was expected as a positive response, red-purple in the case of dissolution into lipidic substances (Pearse, 1968).

In the test for metabolic activity using the reduction of yellow NBT, roots were also incubated first in a solution

of 10 ml 50 mM Tris pH 7.4 containing 40 mg NBT for times 5-30 min, before freeze-sectioning. Positive activity precipitated the deep-blue insoluble formazan salt.

Callose was sought in infected root segments of accession " x^8 ", 3 days after inoculation. After freeze-sectioning and washing into DW, sections were stained overnight in 0.01 % aniline blue in 2.5 % KH_2PO_4 buffer pH 9.3 (Beckman *et al.*, 1982), rinsed in DW, mounted in glycerin jelly and examined under a Leitz Ortholux Microscope, using either bright field, phase contrast or fluorescence (filter block A RKP 400 : filter 1 BP 360 together with filter 2 LP 430 or filter block D RKP 455 : filter 1 LP 460 together with filter 2 BP 380).

For suberin, Gurr's (1973) procedure with oil red was used on fixed (10 % formalin) and frozen roots of accessions " x^8 " and *H. chilense* " PI283375 ", 10 days after infection. Lipid substances appeared brillant crimson, and the nuclei blue to blue-black.

The presence of lignin in sections of *H. chilense* " PI283375 " was sought using the phloroglucinol-HC1 test (Gahan, 1984). A red staining was the expected positive result.

Phenolic compounds were tested for with incubation in fast blue BB (Gahan, 1984) with a yellow-brown colouration indicating presence.

Neutral red staining (Gahan, 1984) was used to localize acidic vacuoles on frozen sections of healthy root tips of accession " x^8 " and infested tips of accessions " x^8 " and *H. chilense* " PI283374 ", taken 10 days after inoculation.

Results

Enzyme activities

The peroxidase reaction in uninfected roots of all cultivars was more pronounced in the tip region than in the differentiated, older parts (Fig. 1). A dark-brown staining accumulated in all parts of the cell (the wall, cytoplasm and nucleus). Most staining was seen in the epidermis (Fig. 1 A), endodermis and xylem vessels. In meristematic regions, the cytoplasm was as heavily stained as the walls (Fig. 1 A, B), whereas in differentiated regions, the walls were more intensely stained (Fig. 1 C). Controls without H_2O_2 showed a very faint reaction, but its intensity increased slowly with time.

In infected roots, two different responses were seen :

i) in the susceptible cv. Doublet, the activity around second-stages juveniles in the cortex was unchanged 3 days after infestation (Fig. 2 A). Even after 10 days, the galls of cvs Doublet and Chinese Spring revealed no higher peroxidase activity in nematode-induced giant-cells (Fig. 2 D).

ii) on the contrary, in the roots of the resistant accessions " x^8 " and *H. chilense* " PI283375 ", 3 days after inoculation, a higher peroxidase activity was observed around nematodes in meristematic or cortical cells which corresponded to the reacting cells observed in a

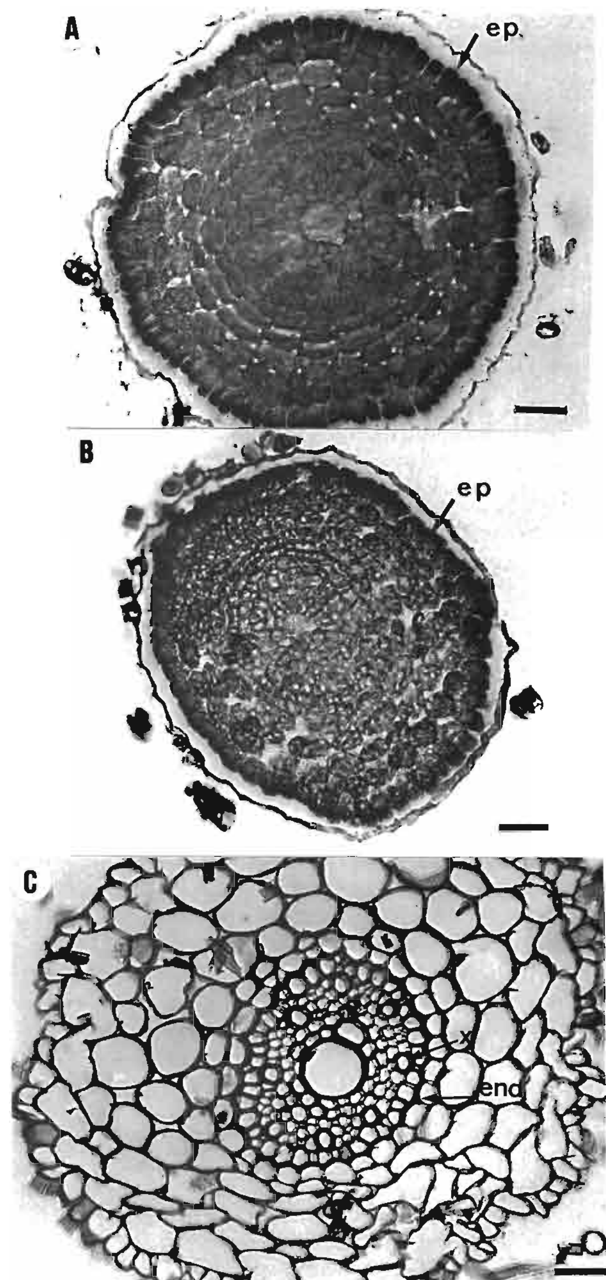

Fig. 1. *Histo-localisation of peroxidase in healthy root tips of the barley cv. Doublet and the wheat accession " x^8 ". A : Transverse section cv. Doublet (meristem), showing the localization of peroxidase in all meristematic tissues (in cell walls, cytoplasm and nuclei; note the more intense staining in epidermis); B : Transverse section accession " x^8 " (meristem), showing the same localization of peroxidase in meristematic tissues as in A; C : Transverse section accession " x^8 " (differentiated zone), showing a localization of peroxidase in all cell walls, with a greater intensity in xylem vessels and endodermal cells* (end = endodermis; ep = epidermis; x = xylem. Bar equivalents : A, B, C = 25 μm).

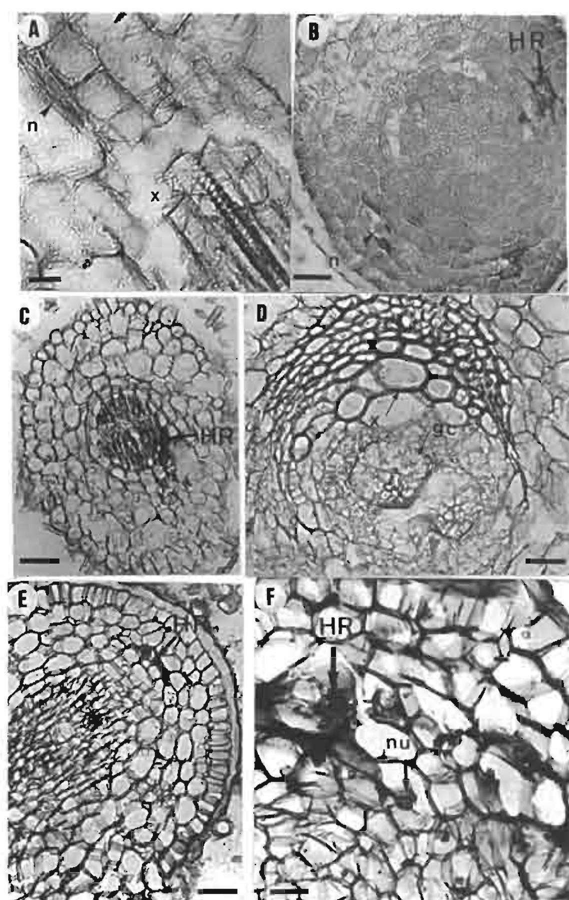

Fig. 2. Histo-localisation of peroxidase in root tips of the barley cv. Doublet and the wheat accession "$\times^8$" and the Hordeum chilense *accession "PI283375" infested by the Welsh population of* Meloidogyne naasi. *A : Longitudinal section cv. Doublet (differentiated zone), 3 days after inoculation; B : Transverse section accession "$\times^8$" (meristematic zone), 3 days after inoculation. (Note the localization of peroxidase in epidermis and in cell walls of hypersensitive cells around nematodes); C : Transverse section* H. chilense *accession "PI283375" (differentiated zone), 3 days after inoculation; D : Transverse section cv. Doublet (differentiated zone), 10 days after inoculation. Note the absence of any stronger activity for peroxidase in giant cells; E : Transverse section accession "$\times^8$" (differentiated zone), 10 days after inoculation; F : Transverse section accession "$\times^8$" (differentiated zone), 10 days after inoculation.* (gc = giant-cells; HR = hypersensitive reaction; n = nematode; nu = nucleus; x = xylem. Bar equivalents : A = 5 μm; B, C, D, E = 25 μm; F = 10 μm).

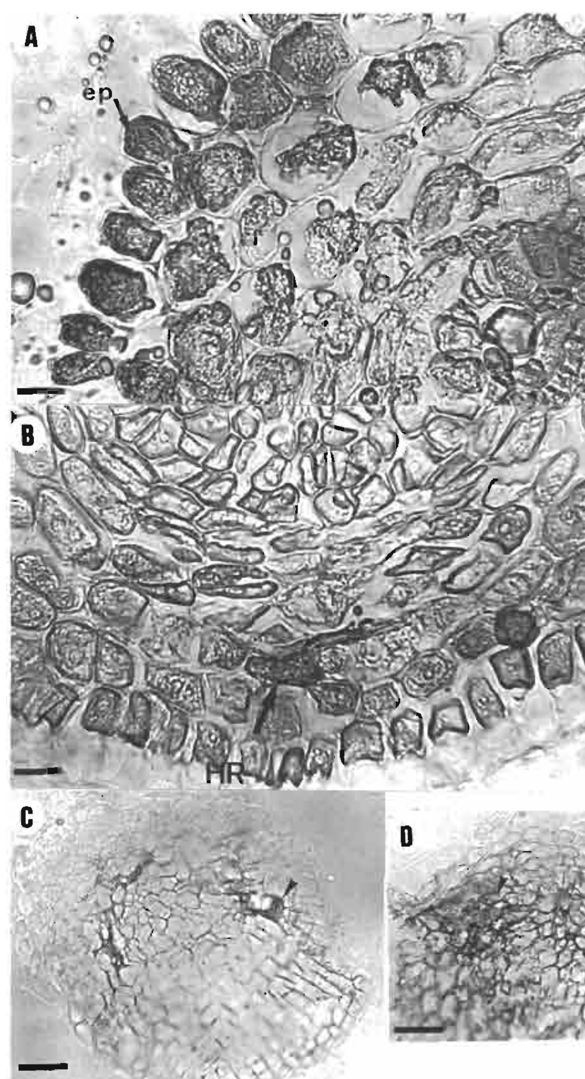

Fig. 3. Histo-localisation of acid-phosphatase in the root tips of the wheat accession "$\times^8$", either healthy or infested by the Welsh population of M. naasi. *A : Transverse section healthy accession "$\times^8$", showing the localization of acid-phosphatase in epidermis (elongating zone); B : Transverse section infested accession "$\times^8$", 3 days after inoculation, showing a faint reaction for acid-phosphatase in the hypersensitive cells (elongating zone); C, D : Transverse sections infested accession "$\times^8$", 10 days after inoculation.*
The arrows indicate the lysis of the hypersensitive cells (differentiated zone) – ep = epidermis; Hr = hypersensitive reaction. Bar equivalents : A, B = 10 μm; C = 25 μm).

preliminary study (Balhadère & Evans, 1995) (Fig. 2 B, C). The intensity of staining (localized in walls, cytoplasm and nucleus) was similar to the epidermis. Controls lacking H_2O_2 showed a significant endogenous reaction. After 10 days, root tips of accession "$\times^8$" still showed high activity of peroxidase at the site of hypersensitive reaction, mainly localized in walls (Fig. 2 E, F). Controls showed only the faint reaction obtained in healthy tips.

Acid-phosphatase activity was successfully demonstrated by incubating and sonicating roots in substrates before freeze-sectioning.

Cultivars differed in the degree and extent of acid-phosphatase endogenous activity in uninfected roots. Both roots of cv. Doublet and accession " ×8 " showed acid-phosphatase activity (reddish color) present in cap and epidermal cells of the meristematic region, with a granular sub-localization (Fig. 3 A). In contrast to accession " ×8 ", cv. Doublet had a less intense staining which appeared only after longer incubation periods.

In infected roots, no activity was detected in 5-day old giant-cells of cv. Doublet, but accession " ×8 " (3 days after inoculation), revealed a faint activity for acid-phosphatase in the same cells showing a hypersensitive reaction around the nematode (Fig. 3 B) as shown in Fig. 2 B. At 10 days such hypersensitively reacting cells showed a great accumulation of red azo-dye conjugate (Fig. 3 C, D). Controls without substrate showed no activity.

Other cytochemical tests

In uninfected roots of the susceptible barley cv. Doublet and the resistant wheat accession " ×8 ", NBT reduction was easily detected in tips, but with prolonged times of incubation, activity further from the root tip was seen. Cap and meristematic cells were all heavily stained, but in the meristem highest activity was observed in epidermal cells. In infected roots of cv. Doublet, no staining was visible around the nematodes (3 days after inoculation; Fig. 4 A, B) or in giant-cells (5 days after inoculation). In contrast, infested tips of accession " ×8 " 3 days after inoculation, showed heavy staining around nematodes in the cortex (Fig. 4 C).

Using either of the two filter blocks, a yellow-green fluorescence was observed 3 days after inoculation, in cell walls of hypersensitively reacting roots of the accession " ×8 " treated with aniline blue, revealing the presence of callose (Fig. 5 A, B); after 10 days, suberin was detected in the walls of reacting cells in the endodermis (Fig. 5 C).

In infected roots of accession *H. chilense* " PI283375 ", 1 day after inoculation, the test for lignin revealed a pink-red staining in thickened walls of cells on the track of nematodes (Fig. 5 D); after 10 days, suberin was found in walls of hypersensitively reacting cells, as was the case also with accession " ×8 ".

When infested tips of accession " ×8 "(3-10 days after inoculation) were incubated with fast blue BB, a brown endogenous staining was localized in hypersensitively reacting cells (Fig. 6 A). However phenols could be seen under bright field without any staining, as soon as 1 day after inoculation.

Use of neutral red allowed red staining of granules in cap cells of healthy roots from accession " ×8 ". Infested tips of accessions *H. chilense* " PI283374 " and " ×8 " after 10 days showed red staining of all the cellular content of the hypersensitively reacting cells (Fig. 6 B).

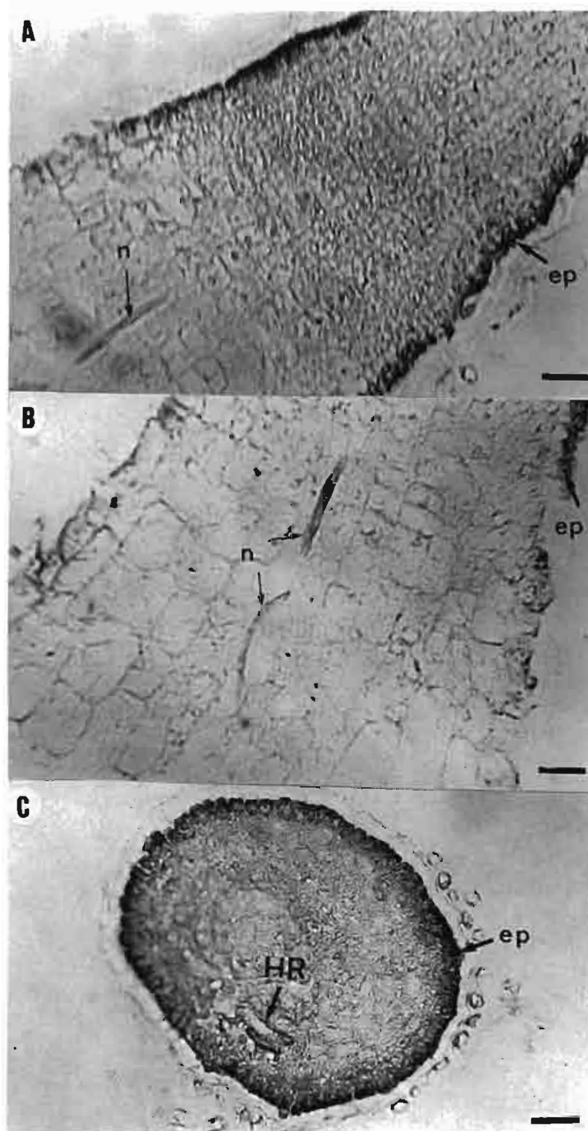

Fig. 4. *Histo-localisation of NBT in the root tips of the barley cv. Doublet and the wheat accession " ×8 " infested by the Welsh population of* M. naasi. *A, B : Longitudinal sections cv. Doublet (A in meristem, B in elongating zone), 3 days after inoculation, showing a blue reaction for NBT reduction in epidermis. Note the absence of any reaction in cells close to nematodes; C : Transverse section accession " ×8 ", 3 days after inoculation, showing the localization of NBT reduction in epidermis and in walls of hypersensitive cells (meristem).* (ep = epidermis; HR = hypersensitive reaction; n = nematode. Bar equivalents : A, B, C = 25 μm).

Discussion

This histochemical study of frozen plant tissue showed the extent to which susceptible and resistant plants differed in the immobilization of plant constituent involved in the hypersensitive response even though the

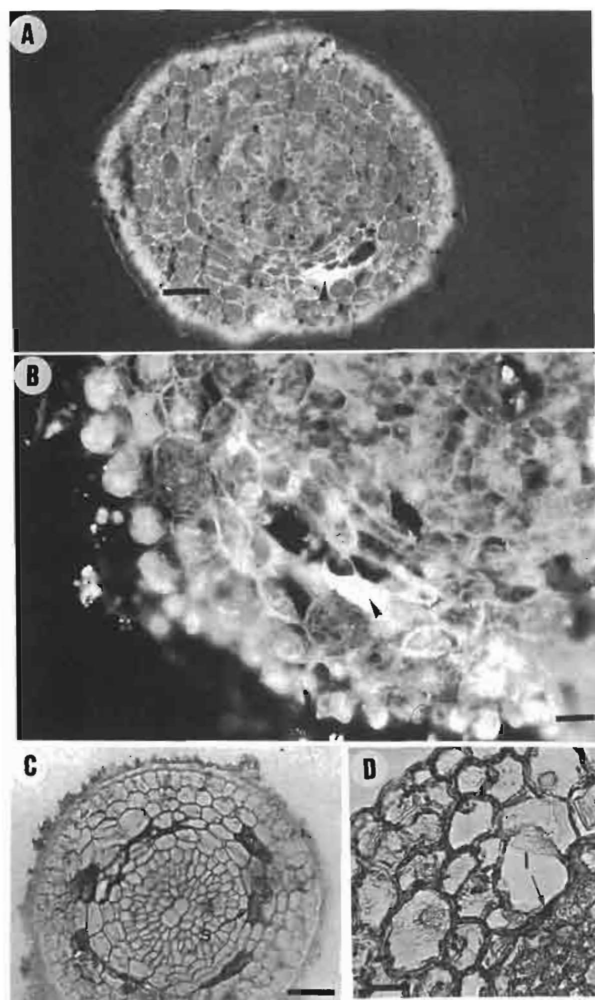

Fig. 5. Histo-localisation of some structural compounds in root tips of the wheat accession "×^{8}" and the accession "PI823375" of H. chilense *infested by the Welsh population of* M. naasi. *A : Transverse section fluorescence microscopy, test for callose in the accession "×^{8}", 3 days after inoculation (meristem). The arrow indicates the deposition of callose; B : Transverse section fluorescence microscopy, test for callose in the accession "×^{8}", 3 days after inoculation (meristem); C : Transverse section, test for suberin in the accession "×^{8}", 10 days after inoculation (differentiated zone), showing a localization in hypersensitive cells on a circle corresponding to endodermis; D : Transverse section, test for lignin in the accession "PI283375" of H. chilense, 1 day after inoculation (differentiated zone)* (l = lignin; s = suberin. Bar equivalents : A, C = 25 μm; B, D = 5 μm).

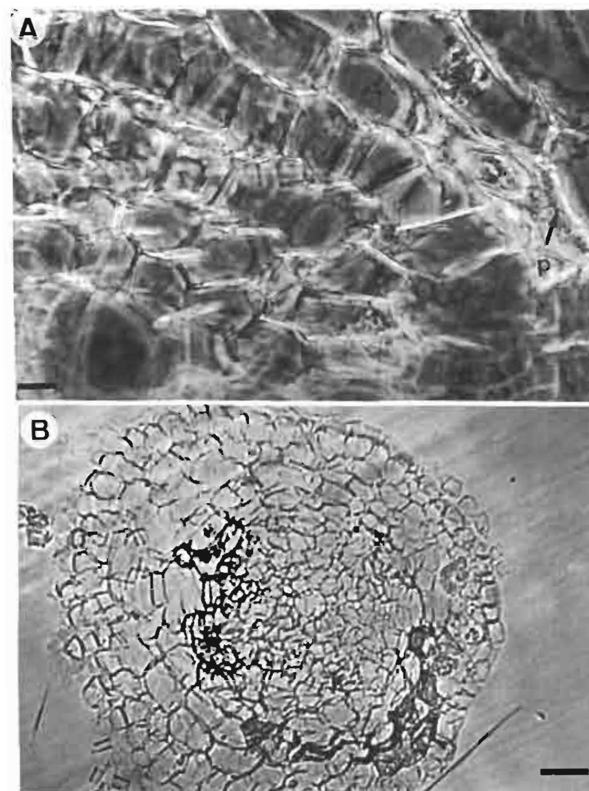

Fig. 6. Cytochemical tests in root tips of the wheat accession "×^{8}" infested by the Welsh population of M. naasi. *A : Transverse section phase contrast, a test for phenolics, 10 days after inoculation (differentiated zone), showing the accumulation of oxidized phenols (yellow colour by fast blue) in the cytoplasm of an hypersensitive cell; B : Transverse section, a test for acidic vacuoles, 10 days after inoculation (differentiated zone).* The arrow indicates the lysis of hypersensitive cells – p = phenols. Bar equivalents : A = 5 μm; B = 25 μm).

mechanisms of the elictation of the hypersensitive response, their specifity and how the cascade of events is organised, remain to be elucidated.

Freezing microtomy on unfixed, fresh sections of plant material was chosen as it is advised to preserve enzyme activity and determine enzyme histo-localization (Gahan, 1984). However, the method itself needed some practice and improvement, as successful sectioning of frozen tissue required that i) the tissue was frozen quickly and homogeneously (Meryman, 1956); *ii)* the sections were not disrupted by handling (Sanderson, 1972) and that *iii)* the sections were securely attached to glass slides without loss of activity.

Acid-phosphatase in the root sections was labile and present in small quantities. The alternative method of first incubating in substrate before freeze-sectioning, did allow the histo-localization of this compound. It can thus be infered that the acid-phosphatase is lost during freeze-sectioning or staining procedures. We have assumed that the pre-sectioning incubation followed by sonication produced no artefacts. Naphthol-diazonium salt complexes (poorly water-soluble and very easily seen; Gahan, 1984), allowed good localization of the

colour precipitate, and no further redistribution occurred inside the root.

Cellular localization of acid-phosphatase activity and acidic vacuoles in healthy roots resembled the description given by Sutcliffe and Sexton (1968), with a higher reaction in epidermal cells of accession « x^8 » compared to cv. Doublet.

Cellular and subcellular localization of peroxidase activity in healthy roots of all cultivars conformed to the general pattern proposed by Van Fleet (1963, 1972) and Kolattukudy (1981), wherein a shift of distribution of peroxidase with age was noted from cytoplasm of young cells to a lipid-bound polymer form in cell walls of older cells.

Differing from the two fully resistant plants *A. variabilis* and *H. chilense*, infected roots of susceptible cvs Doublet and Chinese Spring did not show any changes for the enzymes and compounds tested at the times of observation. Both resistant hosts showed the same changes (early increase as soon as one day in peroxidase activity, early wall changes, early oxidation, of cytoplasmic phenols, early membrane degradation, late suberization of cell walls from reacting cells after 5 days and release of acid-phosphatase), the only difference being an early accumulation of callose in *A. variabilis,* compared with early lignin accumulation in *H. chilense.*

These changes are similar to those reported in dicotyledons in response to pathogen attack or wounding, where for example peroxidase activity was noted (Giebel *et al.,* 1971; Melillo *et al.,* 1990; Guida *et al.,* 1992). However, lignin accumulation is probably a more specialized response of Graminae in response to pathogen attack (Moerschbacher, 1989).

In response to nematode invasion, both early phenol oxidation and polymerisation into lignin as well as late suberization of hypersensitive cell walls after 5 days, can be seen as wound responses, resulting from an overall activation of oxidative metabolism (Kolattukudy, 1978; Rhodes & Wooltorton, 1978). This wound response activation was clearly expressed through increase of peroxidase activity, associated with a possible involvement in lignification, suberization, protection from and production of free radicals (Rhodes & Wooltorton, 1978; Arrigoni, 1979; Espelie *et al.,* 1986).

The early deposition of callose in *A. variabilis* resistance, seen as soon as one day after inoculation, is also a wound response, due to activation of (1-3) β glucan synthase (Kauss, 1985). Through its low permeability to small molecules, callose could impede the cell-to-cell movement between the pathogen and the host (Braecker & Littlefield, 1973) and act as a physical and physiological barrier to the nematode.

The strong acid-phosphatase detected in accession " x^8 " at 10 days after inoculation supports its involvement in autolysis of hypersensitive cells of resistant varieties. The diffuse cytoplasmic staining for acid-phosphatase activity and acidic vacuoles could originate from the release of hydrolytic enzymes after disruption of autophagic vacuoles (Pitt & Galpin, 1973).

Evidence for involvement of free radicals has been offered by many studies (Thompson, 1988; Zacheo & Bleve-Zacheo, 1988; Sutherland, 1991). One of the primary events in hypersensitive reactions, possibly after membrane related signals (Mayer & Ziegler, 1988), could be the production of free radicals (e.g. HO_2/O_2^-) by a membrane-associated NADPH oxidase. These superoxide radicals would lead, together with activity of lipoxygenase (Croft *et al.,* 1990; Rickauer *et al.,* 1990), to enzyme dependent and enzyme independent membrane lipid peroxidation, physiological disruption (electrolyte leakage through alteration of protein channels of passive diffusion; Pavlokin & Novacky, 1986) and further production of active oxygen species. Among them, H_2O_2 was produced in large concentrations in the controls for infected roots of resistant hosts. NBT reduction into formazan precipitate can be used as a method for measuring O^{2-} generation (Doke, 1983). Therefore, the presence of this reaction in hypersensitive cells of accession " x^8 " could indicate a greater release of superoxide radicals. However, to verify this interpretation, a control using the enzyme superoxide dismutase to destroy the superoxide anion produced, would have been necessary. It was not done as the first purpose was to find differences in reducing activities between the resistant and susceptible cultivars. Also it would have been interesting to include mechanically wounded roots as controls to determine those reactions only provoked by the nematode attack.

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