Necrosis in *Solanum tuberosum* Stems Infected with Potato Virus *Y* by Grafting

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This work examined the distribution of necrosis on stems of two cultivars of potato (*Ania*, *Glada*) with different levels of resistance to PVY infection. Potato virus Y particles and/or cytoplasmic (CI) and amorphous inclusions (AI) were identified in insert and offshoot potato cells of susceptible cv. *Glada*. Cytoplasmic inclusions were not observed in insert and offshoot stems of resistant cv. *Ania*, although there were numerous deformations, degeneration and tissue necrosis. It was found that (1) necrotic reactions were the form of plant cell response for both the PVY-resistant and susceptible cultivars, (2) development of necrosis in vascular tissue did not prevent the pathogen from spreading outside the necrotic region in the less resistant cultivar (*Glada*), and (3) extreme resistance to PVY in potato plants, determined by the Ry_{sto} gene, was manifested in the absence of virus particles and cytoplasmic inclusions in infected plant cells.

Key Words: Necrotic reaction, PVY inclusions, Ry_{sto} gene, cell ultrastructure, PVY spread.

INTRODUCTION

Potato virus Y (PVY) is one of the major viral pathogens of potato cultures. It can decrease yields by as much as 80%. Protection against this pathogen is based on breeding and cultivation of resistant cultivars (Valkonen, 2007).

Two main types of potato resistance to PVY infection are known. One is field resistance based on polygenes. It is found in several cultivars, which can be classified according to their degree of resistance. The most resistant plants are infected only accidentally. This relative classification remains practically unchanged in various epidemiological conditions; what changes is the number of infected plants (Rohloff, 1979; Weidemann, 1988).

The other type of resistance to PVY, recognized in wild and cultivated varieties of potato (*Solanum tuberosum* spp.) is based on a single gene. This resistance, controlled by a single dominant gene, comprises two types of reactions: hypersensitive response (HR) and extreme resistance (ER) (Ross, 1986). The hypersensitive response, determined by *Ny* genes, is often specific for the PVY strain (Celebi-Toprak et al., 2002), while extreme resistance controlled by *Ry* genes is

effective against all strains of this virus (Cookerham, 1970; Jones, 1990). Plants with *Ny* gene expression are characterized by the development of necrotic damage after mechanical inoculation of the leaves and by necrosis in systemically infected parts of the plants. Extremely resistant plants whose genome contains the *Ry* gene do not show symptoms after inoculation and are rarely infected (Ross, 1986; Valkonen et al., 1994; Marczewski, 2001). Occasionally, limited necrosis can develop in systemically infected leaves of several genotypes after inoculation through grafting. In extremely resistant plants the virus is undetectable by the ELISA test even after infection (Ross, 1986). Potato cultivation programs have employed resistance determined mainly by two major genes: Ry_{adg}, derived from *Solanum tuberosum* subspecies *andigena* (Brigneti et al., 1997), or Ry_{sto}, orginating from *Solanum stoloniferum* (Ross, 1986; Hamalainen et al., 1997; Brigneti et al., 1997).

Our study compares the cytological reactions of two varieties of potato: the extremely resistant potato cv. *Ania*, with resistance determined by the Ry_{sto} gene, and susceptible potato cv. *Glada* after infection by PVY[N] Wi, introduced by grafting.

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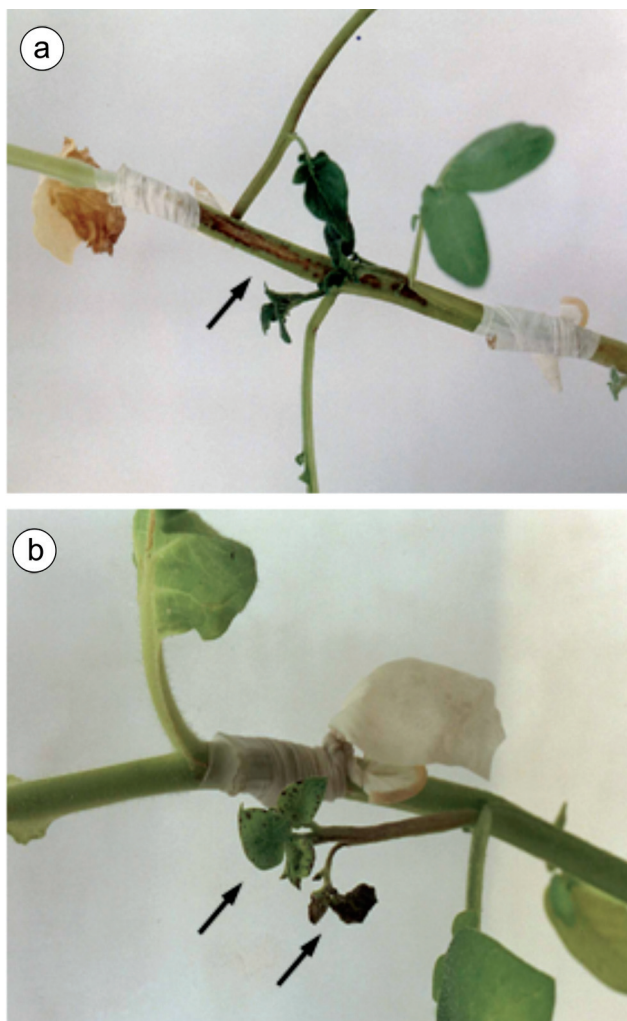[**]e-mail: katarzyna_otulak@sggw.pl

Fig. 1. Morphology of potato cv. *Ania* stem infected with PVY[N] Wi. **(a)** Longitudinal necrosis (arrow) on potato stem insert between tobacco scion infected with PVY[N] Wi and noninfected tobacco stock, **(b)** Necroses and tissue browning on leaves and stem of offshoot (arrows).

MATERIALS AND METHODS

PLANT MATERIAL

We used two genera of *Solanaceae* family plants: tobacco (*Nicotiana tabacum* L.) cv. *Samsun* and potato (*Solanum tuberosum* L.) cvs *Ania* and *Glada*. The choice of cultivars was based on their differing levels of resistance to PVY infection. *Ania* is an extremely resistant variety, 9 on the 1-to-9 scale of field resistance. *Glada* is a relatively resistant variety in field conditions, 7.5 on the scale, but after grafting or mechanical inoculation with PVY it can be systemically infected; in our study it was treated as the susceptible variety.

Disease symptoms were observed on potato stems infected with necrotic strain PVY[N] Wi (*Wilga*) in the experiment with bridge grafting inoculation. A healthy tobacco cv. *Samsun* plant was used as rootstock. The potato stem (resistant cv. *Ania* or susceptible cv. *Glada*) was used as insert, and tobacco cv. *Samsun* infected with PVY[N] Wi served as infection graft (Fig. 1). After 2-3 weeks, new lateral shoots (offshoots) were growing from the insert. The plant material was obtained from M. Chrzanowska (IHAR, Młochów, Poland).

SAMPLE PREPARATION FOR LIGHT AND TRANSMISSION ELECTRON MICROSCOPY

Plant tissue samples (~ 1 mm^3) were fixed by a classical method. The tissue samples were placed for 2 h in Karnowsky's fixing agent (1965), a mixture of 2% glutharaldehyde and 2% paraformaldehyde in sodium cacodylate buffer (pH 7.2), and then rinsed with 0.1 M sodium cacodylate buffer 4 times and additionally fixed with 2% osmium tetraoxide for 2 h at 4°C (Watson, 1958). The tissue sections were dehydrated in an ethanol series, then in acetone or propylene oxide, after which they were embedded in Epon 812 (Fluka) and polymerized for 24 h at 60°C.

The Epon blocks with plant material were cut in the following ways: (a) for LM, with a Supercut 2065 microtome (Leica/Reichert-Jung) into 0.3 mm sections and stained with methylene blue in 2% borax and 1% Azure B solution; (b) for TEM, with an Ultracut E microtome (Reichert) into ultrathin sections ~ 90 nm thick, which were collected on Formvar-coated slot-grids and contrasted with 1% lead citrate and 2% uranyl acetate (Venable and Coggeshall, 1965).

Semithin sections were examined and documented with the use of an Axioscope (Zeiss) light microscope with a Contax 167 MT camera, and ultrathin sections with the use of JEM 100C or JOEL 1220 transmission electron microscopes in the Electron Microscopy Laboratory of the Warsaw University of Life Sciences – SGGW.

RESULTS AND DISCUSSION

Potato virus Y particles and viral protein inclusions (CI, AI) were identified only in the cells of susceptible cv. *Glada*; in the resistant cv. *Ania* no pathogenic agent structures were found. Since both the PVY-resistant and the susceptible cultivars were inoculated, it can be assumed that the presence/absence of the virus in the observed cells differentiates the cultivars according to their resistance to that pathogen. However, regardless of the degree of resistance of the investigated plants, necrosis symptoms can develop in tissues in response to the infecting agent. In the insert stem of susceptible potato cv. *Glada* infected with strain PVY[N] Wi, virions and cytoplasmic inclu-
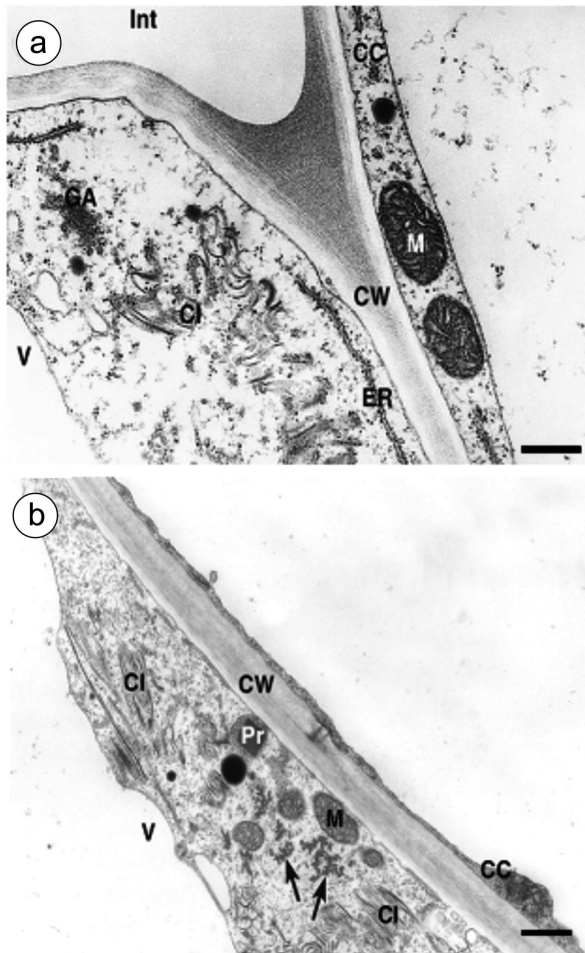
Fig. 2. Ultrastructure of vascular tissue and phloem parenchyma cells of potato cv. *Glada* stem insert infected with PVY^N Wi. **(a)** Internal phloem of potato stem in transverse section. Cytoplasmic viral inclusion (CI) located in neighboring Golgi apparatus (GA) and rough ER in phloem parenchyma cytoplasm, **(b)** PVY^N Wi particles (arrows) and cytoplasmic inclusions in phloem parenchyma cells of potato stem insert cv. *Glada*. Bar = 0.2 μm.
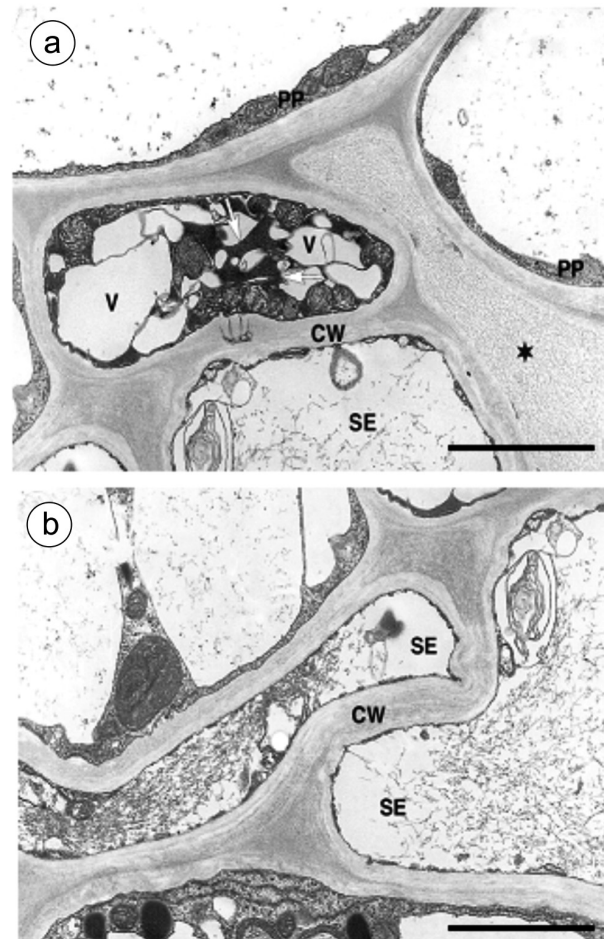

Fig. 3. Ultrastructure of phloem potato cv. *Glada* stem insert infected with PVY^N Wi. **(a)** Internal phloem in transverse section: sieve element (SE) cell wall extension (star), coagulated protoplast of companion cell (white arrows) with numerous vacuoles (V). PP – phloem parenchyma, **(b)** Cell wall (CW) deformations in sieve elements (SE). Bar = 1 μm

sions (CI) were observed in parenchymatous cells of the cortex (Fig. 2). Cytoplasmic inclusions were also found in vascular tissue. The phloem cells underwent irreversible structural changes (Fig. 3). Similarly, in the stem of the offshoots in potato cv. *Glada* there were PVY particles (Fig. 2b) and viral protein inclusions, both cytoplasmic (CI) (Fig. 4a) and amorphous (AI) (Fig. 4b). In the stem of the insert in potato cv. *Ania* infected with PVY^N Wi, cortex collenchyma cells with thin cell walls became split (Fig. 5a). Widespread necrosis was observed in vascular tissue (Fig. 5b). Coagulated protoplasts and split cell walls were observed in offshoot potato cv. *Ania* (Fig. 6a, b). Anatomical observations (Fig. 7) showed large internal necroses in the cortex and axial cylinder in the stem of offshoot potato cv. *Ania*.

Hinrichs-Berger et al. (1999) noted two phenotypes of necrotic reactions on the cellular level. Early in the necrotization process, protoplast plasmolysis, cell wall thickening and deformation are seen. Later the protoplasts remain in the form of a highly osmophilic mass of membranous structures in which the cell organelles are completely degraded.

For an understanding of the scope of resistance it is important to characterize the distribution of necrotic reactions in plants.

In mechanically inoculated highly resistant potato cultivars, PVY did not spread systemically and the necrotic reaction was restricted to leaf epidermis, mesophyll and collenchyma cells. To explain the lack of necrosis in the vascular system we note that the resistance reaction is effective at the stage of infection
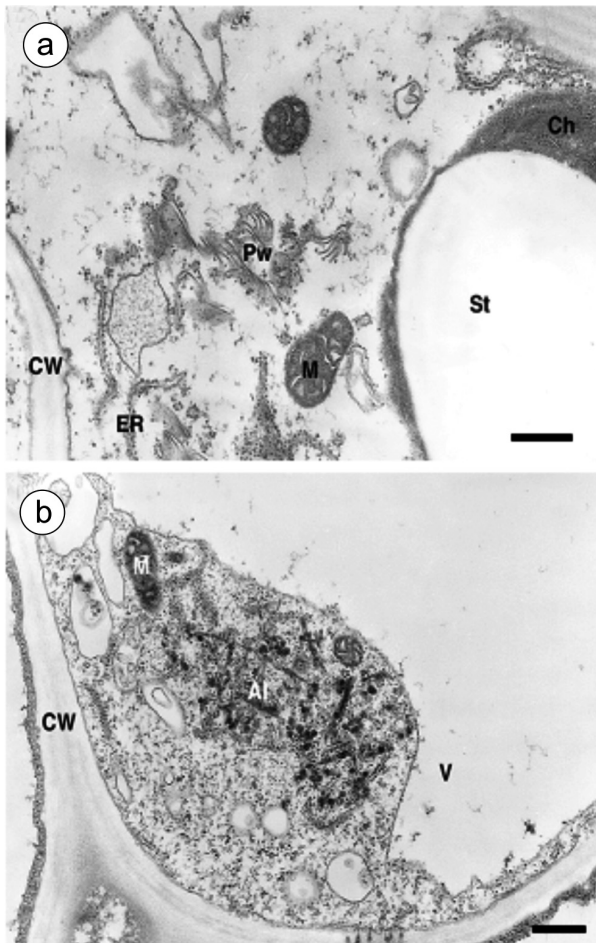
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Fig. 4. Ultrastructure of phloem parenchyma of new stem (scion) of potato cv. *Glada* infected with PVY^N Wi. **(a)** Pinwheels (Pw) in cytoplasm of internal phloem parenchyma, **(b)** Amorphous viral inclusions (AI) in cytoplasm of internal phloem parenchyma. Bar = 0.2 μm

Fig. 5. Ultrastructure of cortex and vascular tissue of potato cv. *Ania* stem insert cells infected with PVY^N Wi. **(a)** Angular collenchyma cells in transverse section. Deformed thin cell wall (thin arrows) with thickenings at angles (thick arrow) and degenerated chloroplasts (arrowheads), **(b)** External phloem in necrotic region in transverse section. In disrupted companion cells and sieve elements, electron-dense and coagulated protoplasts (thin arrows) were observed. Flexous cell wall deformations of these cells shown by thick arrows. Bar = 1 μm

when the virus is still outside the vascular tissue (Hinrichs et al., 1998). In mechanically inoculated potato, Hinrichs-Berger et al. (1999) found necrotic damage in leaves of both the resistant potato cv. *Pirola* and susceptible cv. *Quarta*, but the virus induced systemic infection only in the susceptible variety.

Ultrastructural studies showed necrosis on the inserts and the offshoots of the two Polish potato varieties, resistant cv. *Ania* and susceptible cv. *Glada*. Necroses were within the bicollateral vascular bundle. In the susceptible variety the pathogen also occurred outside necroses (Fig. 3a,b). In the resistant variety there was only necrosis (Fig. 5b) involving individual cells as well as large ensembles of tissue.

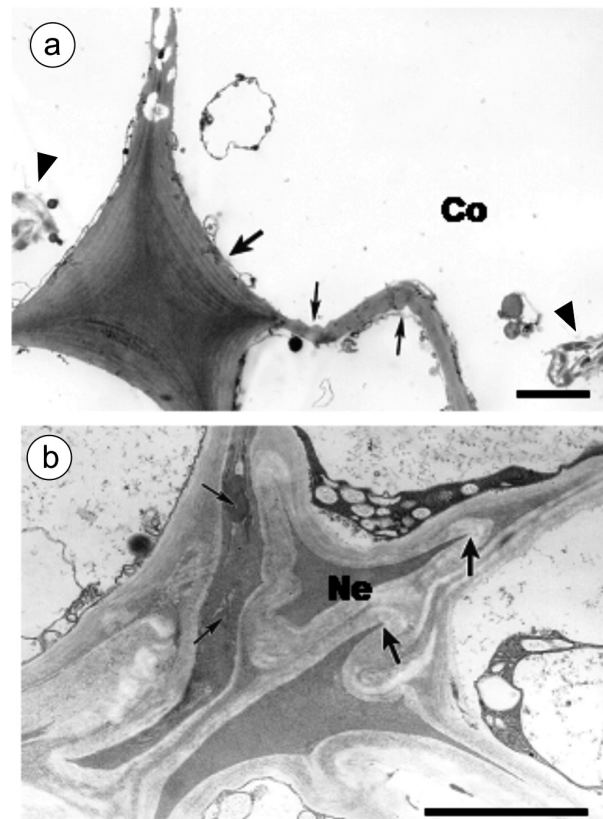The presence of internal necrosis in the vascular tissue of resistant potato cv. *Ania* (Fig. 5b) confirms the effectiveness of the resistance mechanism at the stage of systemic infection. Possibly such localization of the necroses is due to the grafting method of inoculation, in which transport of the virus is largely conditioned by the vascular tissue.

In every multicellular organism, death signals, when released, trigger the active process of programmed cell death (PCD) (Gilchrist, 1998; Lam et al., 1999; Baehrecke, 2002). In animal cells the characteristic form of this process is apoptosis, involving fragmentation and absorption of decaying cells by neighboring ones. In plant cells the trigger of programmed cell death is the hypersensitive response of cells affected by metabolically active necrotization (Heath, 2000; Shirasu and Schulze-Lefert, 2000; Goldbach et al., 2003). The mechanism of cell death is regulated via the expression of
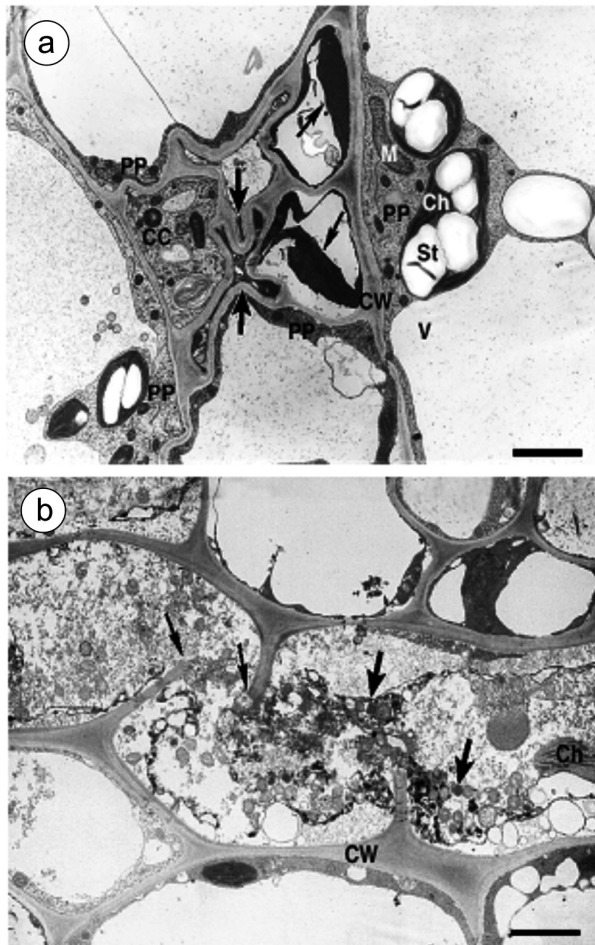
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Fig. 6. (a) Ultrastructure of internal phloem in potato stem insert cv. *Ania* infected with PVY^N Wi. Group of phloem cells in transverse section, (b) Ultrastructure of external phloem of new stem of potato cv. *Ania* infected with PVY^N Wi. Disrupted phloem parenchyma cell walls (thin arrows) and multivesicular remnants of protoplasts (thick arrows). Bar = 1 μm.

Fig. 7. Anatomical structure of potato stem cv. *Ania* (resistant) infected with PVY^N Wi in transverse section. (a) Large internal necroses (arrows) in cortex (PCP) and external phloem (ExPh) of new potato stem (scion), (b) Large internal necrosis (arrow) in phloem parenchyma cell. The stem was used as an insert in grafting inoculation. Bar = 50 μm ED – epidermis; Co – collenchyma; InPh – internal phloem; X – xylem.

genes encoding proteins that activate (Bax) or silence (BCL-2, BCL-XL) (Doukhanina et al., 2006; Hofius, 2007) this process with agents released from mitochondria, such as cytochrome c and reactive oxygen species (ROS) (Wojtaszek, 1997; Lam, 1999). ROS production during pathogen recognition can also be conditioned by membrane-associated NADP(H) oxidase or by peroxidase in the apoplast. Severe oxidative stress associated with a high local concentration of ROS can participate directly in killing the pathogen affecting the plant cell, due to cell death in the hypersensitive response (Hofius et al., 2007).

In potato plants extremely resistant to PVY, after mechanical inoculation the rapid and localized hypersensitive response is activated. Cell death occurre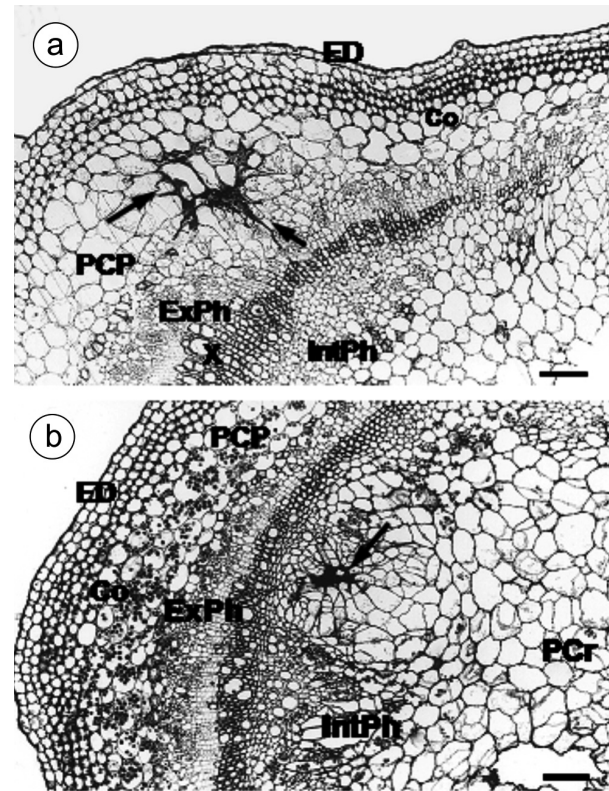d at sites of pathogen attack and the virus was blocked and restricted to necrotic damaged areas. (Hinrichs-Berger et al., 1999). In PVY-susceptible potato plants, virus replication could proceed unrestricted for 4–5 days after inoculation. After that period, necroses began to develop. Late activation of this reaction enables the virus to spread outside the cells reacting with necrosis and caused by PVY systemic transport (Hinrichs-Berger et al., 1999).

CONCLUSIONS

1. Both the PVY-resistant and susceptible potato cultivars responded to infection with necrotic reactions. Necroses could develop in all plant tissue types.

2. The development of necroses in vascular tissues in upper shoots of the observed potato plants differing in their degree resistance to PVY may have been due to the method of inoculation by grafting. In highly resistant potato plants (*Ania*)

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systemically infected by grafting, the development of internal necroses in the vascular tissue of upper shoots blocked the spread of potato virus Y outside the necrotic regions. These necroses developed in the highly resistant cultivar as a result of the resistance reaction. In the plants with lower field resistance (*Glada*), development of necroses in the vascular tissue did not prevent the virus from spreading outside the necrotic regions.

3. High resistance to PVY in potato plants determined by the Ry_{sto} gene was manifest in the lack of viral particles or viral protein inclusions in infected plant cells.

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