Research

Systemic Hypersensitive Resistance to *Turnip mosaic virus* in *Brassica juncea* is Associated With Multiple Defense Responses, Especially Phloem Necrosis and Xylem Occlusion

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Abstract

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Systemic hypersensitive resistance (SHR) caused by *Turnip mosaic virus* (TuMV) was studied by light microscopy and histochemical analysis in stem cross sections of *Brassica juncea* (Indian mustard) plants. Ten TuMV isolates were inoculated to leaves of susceptible line JM 06006, cv. Oasis CI, which carries TuMV systemic hypersensitivity gene *TuRBJU 01*, and F3 progeny plants obtained from a cross between them. Systemic mosaic (SM) symptoms were induced by all 10 isolates in plants of JM 06006, and by resistance-breaking isolate NSW-3 in all cv. Oasis CI and F3 plants. With the other nine isolates, cv. Oasis CI plants developed SHR while F3 progeny plants segregated for both phenotypes; mock-inoculated control plants never became infected. Presence of SHR did not delay systemic invasion as this commenced within 2 hours after inoculation (hai) and was almost complete by 72 hai regardless of whether plants subsequently developed SHR or SM. When stem cross sections sampled 9 to 12 days after inoculation were examined for the plant defense responses, phloem necrosis, hydrogen peroxide accumulation, and additional lignin deposition, sections from plants with SHR demonstrated all of these characteristics, but sections from plants with SM or mock-inoculation did not. Based on consolidated data from all isolates except NSW-3, stems developing SHR had significantly more occluded xylem vessels ($P < 0.001$) compared with stems from plants developing SM or mock-inoculated plants. Both light microscopy and histochemical tests with phloroglucinol-HCl and toluidine blue O indicated that the xylem occlusions could be gels. Thus, phloem necrosis, xylem occlusion, lignification, and hydrogen peroxide accumulation were all associated with the SHR in *B. juncea* plants carrying TuMV hypersensitivity gene *TuRBJU 01*. In addition, virus inclusion bodies were fewer in sections from plants with SHR. Phloem necrosis was apparently acting as the primary cause of SHR and xylem occlusion as an important secondary cause.

Systemic hypersensitive resistance to virus infection (SHR) occurs when plants inoculated with infective sap develop localized necrotic lesions in inoculated leaves followed by systemic necrosis that sometimes leads to plant death (e.g., del Pozo and Lam 2003; Hajimorad et al. 2005; Jones and Smith 2005; Wahlroos and Susi 2015). Localized hypersensitive resistance (HR) differs from SHR in that no systemic invasion occurs (e.g., Aguilar et al. 2015; Kim et al. 2008). Both involve *R* gene recognition and a gene-for-gene interaction, and are sometimes controlled by the same *R* gene (Kim et al. 2008; Ravelo et al. 2007). HR and SHR share similarities at the biochemical and molecular level, such as being associated with programmed cell death and triggering rapid accumulation of reactive oxygen species (ROS) (Mandadi and Scholthof 2013). Moreover, viruses inducing HR on sap inoculation often cause SHR following graft-inoculation (e.g., Jones 1990). Despite lack of virus localization, SHR still fits the definition of an incompatible reaction as it involves rapid recognition of an invading virus followed by effective activation of defense responses (e.g., Lamb and Dixon 1997; Yang et al. 1997). HR limits virus spread within individual infected plants while SHR limits its plant-to-plant spread in the field, so SHR operates at the plant population level whereas HR operates at the individual plant level (e.g., Jones et al. 2003; Kim et al. 2008). SHR is most effective when infected plants are killed rapidly thus removing them as internal virus infection foci for spread by contact or vector acquisition to other plants, as with *Bean yellow mosaic virus* (genus *Potyvirus*) spread in crops planted with lupin cultivars carrying hypersensitivity gene *Nbm-1* (Cheng et al. 2002; Jones et al. 2003; Jones and Smith 2005; Thackray et al. 2002).

At the cellular level, phloem necrosis occurs in association with virus-induced SHR, the necrosis then spreading from the phloem to other plant tissues (e.g., Edwards and Agrios 1981; Xu and Roossinck 2000). Accumulation of hydrogen peroxide and callose deposition also occur in association with virus-induced SHR (Xu et al. 2003). Although lignin deposition remains unstudied for virus-induced SHR, it occurs in association with HR in virus-inoculated leaves (e.g., Appiano et al. 1977; Faulkner and Kimmins 1975; Kimmins and Wuddah 1977; Sundaresan and Kimmins 1981; Vance et al. 1980). Wan et al. (2015) found that *Turnip mosaic virus* (TuMV; genus *Potyvirus*) moves systemically via xylem and phloem, but virus movement through the xylem involved transport of viral vesicles in hollow vessels of mature xylem and occurred at a slower rate. Edwards and Agrios (1981) found that the xylem vessels of virus-infected plants with SHR were clogged with brown colored material of undetermined composition. Infection with other types of plant pathogens sometimes induces vascular occlusions, such as tyloses or gel (= gum) formation, which seal off the xylem and can cause plant death (e.g., Yadeta and Thomma 2013). Their involvement in virus-induced SHR remains to be investigated.

TuMV causes a serious disease in *Brassica juncea* (Indian mustard), which is an important oilseed brassica crop in many countries (e.g., Odeh et al. 2011; Oram et al. 2005). SHR is a common TuMV resistance phenotype in this species (Kehoe et al. 2010; Nyalugwe et al. 2015a). The different TuMV-induced phenotypes that develop in *B. juncea* plants include $+_N$ (systemic infection with some necrosis), $+_{ND}$ (systemic hypersensitivity and plant death) and + (susceptibility). When F2 generation progeny plants from the cross between parent JM 06006

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(susceptible) and parent Oasis Cl (carries gene *TuRBJU 01*) were used for segregation ratio studies with TuMV infection, they gave a 3:1 (systemic necrosis:susceptible) ratio at an early stage, but a 1:2:1 ratio (phenotypes $+_{ND}$:$+_{N}$:+) at a late stage of infection. This showed that a single incompletely dominant resistance gene, designated *TuRBJU 01* (TuMV resistance in *B. juncea* 01), was responsible for systemic necrosis phenotypes $+_{ND}$ (homozygous) and $+_{N}$ (heterozygous) in *B. juncea* (Nyalugwe et al. 2015a). In subsequent segregation ratio studies using F3 generation plants from the same cross at an early stage of TuMV infection, a 3:1 (systemic necrosis:susceptible) ratio was obtained. This ratio developed with nine of 10 TuMV isolates used. However, it did not occur with the remaining isolate (NSW-3), which was resistance-breaking for gene *TuRBJU 01* as all F3 generation plants inoculated with it developed susceptible phenotype + (Nyalugwe et al. 2016).

This study used the TuMV-*B. juncea* pathosystem and gene *TuRBJU 01* to establish what alterations are visible when stem cross sections in plants developing SHR are subjected to histochemical tests and light microscopy. Ten isolates belonging to at least four different TuMV pathotypes were used, one of which overcame gene *TuRBJU 01*. The aim was to understand the defense responses involved in plants expressing SHR, and to study this by comparing *B. juncea* plants expressing systemic necrotic symptoms (phenotypes $+_{N}$ or $+_{ND}$) with plants expressing mosaic symptoms (phenotype +). The main focus was to establish whether the plant defense responses phloem necrosis or xylem dysfunction, or both acting together, were associated with and acting as primary or secondary causes of SHR. Possible occurrence of the plant defense responses lignin deposition and hydrogen peroxide accumulation in association with SHR were also investigated, along with occurrence of virus inclusions. All four of these plant defense responses were found associated with SHR in *B. juncea* plants carrying TuMV hypersensitivity gene *TuRBJU 01*. In addition, there were fewer virus inclusions in plants with SHR.

Materials and Methods

Virus isolates, plants, and inoculations. All of the 10 Australian TuMV isolates used (WA-Ap, WA-Ap1, NSW-1 to NSW-6, 12.1, and 12.5) were from previous work (Nyalugwe et al. 2014, 2015a,b, 2016; van Leur et al. 2014). These isolates belong to *B. napus* pathotypes 1 (NSW-2, NSW-6), 7 (NSW-1, NSW-5), 8 (WA-Ap, WA-Ap1), and unnamed new pathotype (NSW-3, NSW-4) (Nyalugwe et al. 2015b, references therein), or are as yet unpathotyped (12.1 and 12.5) (Nyalugwe et al. 2016). As mentioned above, NSW-3 overcomes gene *TuRBJU 01* causing mosaic instead of SHR (Nyalugwe et al. 2016). Virus cultures were kept in plants of TuMV-susceptible parental *B. juncea* line JM 06006. The plants used in the experiments were JM 06006, gene *TuRBJU 01* carrying TuMV-resistant parent cv. Oasis Cl (Nyalugwe et al. 2015a) and F3 progeny plants arising from self-pollinated F2 plants themselves derived from F1 generation plants produced by crosses

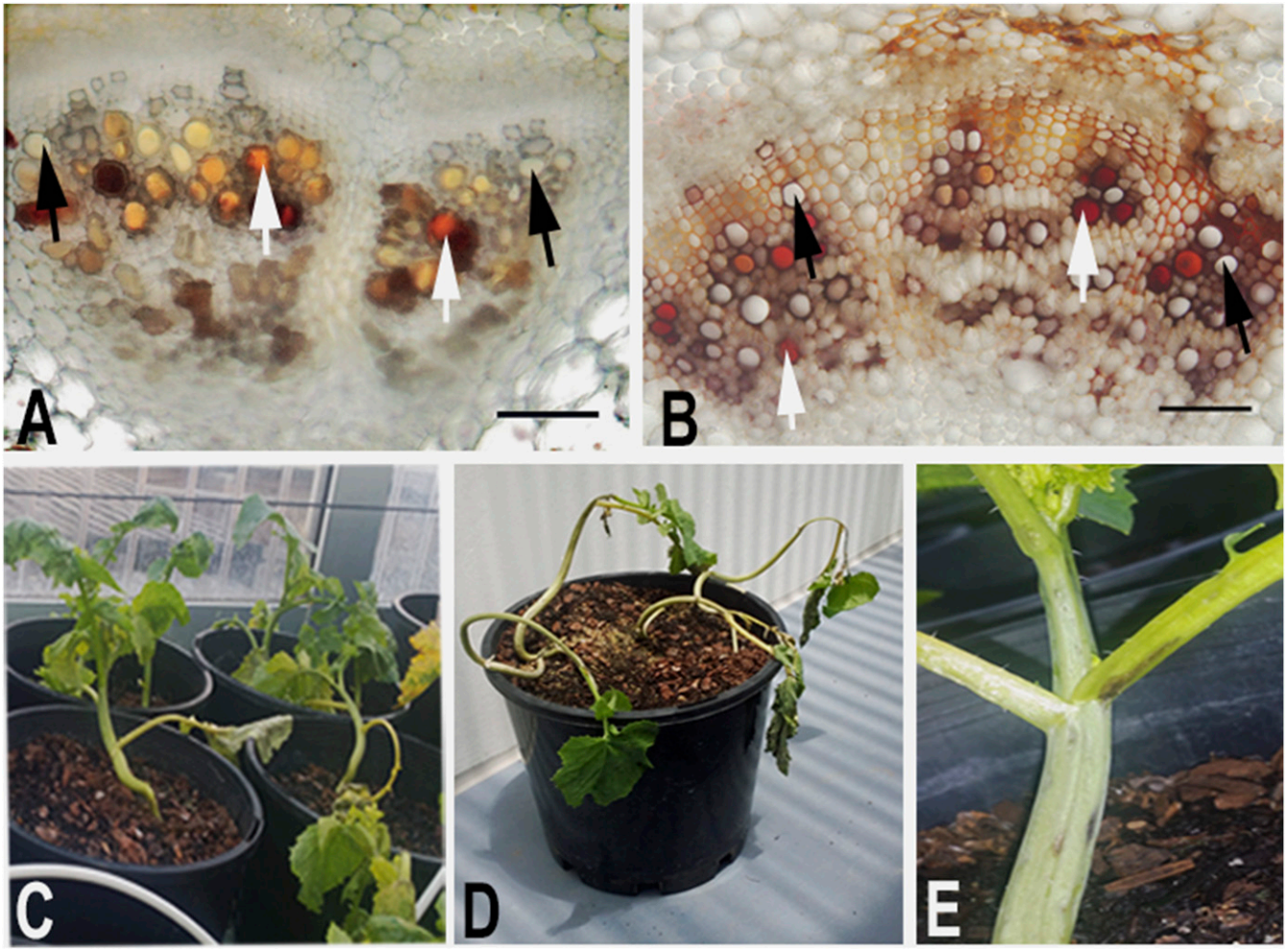

Fig. 1. At 12 days after inoculation (dai) with *Turnip mosaic virus* (TuMV), stem cross sections were cut by hand **(A)** or vibratome **(B)** from plants of *Brassica juncea* Oasis Cl which carries hypersensitivity gene *TuRBJU 01*. Light microscopy images shown are from plants inoculated with isolate NSW-2 without (A) or with (B) phloroglucinol-HCl staining. Occluded xylem vessels appeared brown (A) or red (B) (white arrows) in comparison with non-occluded xylem vessels, which appeared grayish white (A) or pure white (B) regardless of staining (black arrows). **C through E,** F3 progeny plants that carried hypersensitivity gene *TuRBJU 01* developing a systemic hypersensitive phenotype caused by infection with TuMV isolate NSW-2 (C and E) or TuMV 12.1 (D). C, Plants on the left show initial wilting symptoms while plants on the right show wilting and stem bending. D, Plant shows typical advanced wilting symptoms in remaining leaves and systemic necrosis. E, Plant stem and petiole show necrotic stem streaking (black arrows). Plants photographed at 6 dia (C) or 12 dai (D and E). Scale bars: A and B = 200 μm.

between them. To produce culture and experimental plants, each pot was filled with potting mix (pine bark:peat:sand at 2.5:1.0:1.5 w/w). Sap inoculation was by extracting the sap from leaves of TuMV-infected culture plants in buffer containing 11.5 g/liter Na_2HPO_4 and 3 g/liter NaH_2PO_4 (pH 7.2), and mixing this sap with "celite" before rubbing it onto healthy plant leaves. To provide control plants for the experiments, extraction buffer was used in mock-inoculations. All plants were grown in an air-conditioned, aphid-proofed glasshouse at 26/22°C (day/night) under natural light conditions.

Section preparation. To observe anatomical changes in stems, plants of JM 06006, Oasis Cl, and their F3 progeny plants were inoculated at the 2 to 3 true leaf stage with infective sap containing the 10 TuMV isolates (10 to 15 plants of each type/isolate). Mock-inoculated plants were used as controls (5 to 7 plants of each type). For anatomical observations on JM 06006, Oasis Cl, and F3 progeny plants, samples were taken at 9 to 12 days after inoculation (dai) by cutting stem sections from 3 to 6 stems/isolate (1 stem segment/plant) just below the first inoculated true leaf. For each isolate, stem sections were taken from one or more plants with necrotic leaf symptoms and one or more plants with mosaic leaf symptoms. The type of plant selected was recorded but choice of plants was based entirely on leaf symptoms and independent of whether they were parental or F3 progeny plants. From each stem, segments 5 to 10 cm long were prepared and cleared in 95% ethanol overnight, dehydrated in 70% ethanol, and rinsed in deionized water. They were then cleared again before being stored in chloral hydrate (2.5 g/ml) until use. Hand cut (>150 μm thickness) or vibratome cut (80 to 150 μm thickness) cross sections were prepared from stem segments and used to visualize necrotic tissue/areas, lignification, and virus inclusion bodies (5 to 10 sections/type of plant/isolate). The data referred to in the Results section are for both parental and F3 plants. No anatomical differences between stem sections were evident between cv. Oasis Cl and F3 plants with necrotic foliage symptoms or between line JM 06006 and F3 plants with mosaic.

Necrotic tissues in stem cross sections. To visualize necrotic tissues or areas, stem cross sections obtained as described above were stained using phloroglucinol-HCl and toluidine blue O (0.02%) as described by Mitra and Loqué (2014) and unstained stem cross sections were also examined. A Zeiss Axioplan 2 microscope with an AxioCam Digital photograph system was used. With stained sections, necrotic areas were stained red (with phloroglucinol-HCl) or blue (with toluidine blue). With unstained sections, necrotic areas appeared brownish. In addition, autofluorescence of unstained sections was examined by ultraviolet (UV) light microscopy using an exciter filter (G365) and a barrier filter (LP397) inserted into a beam of incident light from a mercury vapor lamp. Necrotic tissues then appeared darkish or brownish.

Virus inclusion bodies in stem cross sections. The method of Christie and Edwardson (1986) was used to detect virus inclusion bodies. Briefly, stem cross sections obtained as described above were floated on 2-methoxyethanol for 30 min to remove extra chlorophyll. Sections were stained by immersing them in 0.1% azure A in 2-methoxyethanol and buffered with 0.2M Na_2HP0_4 for 10 min. Stained sections were washed with several changes of 95% ethanol until the excess dye was removed (for about 5 to 10 s with each change) and then immersed in 2-methoxyethyl acetate for 5 min. Finally, they were mounted in a drop of Euparal on a glass slide and examined by light microscopy as described above.

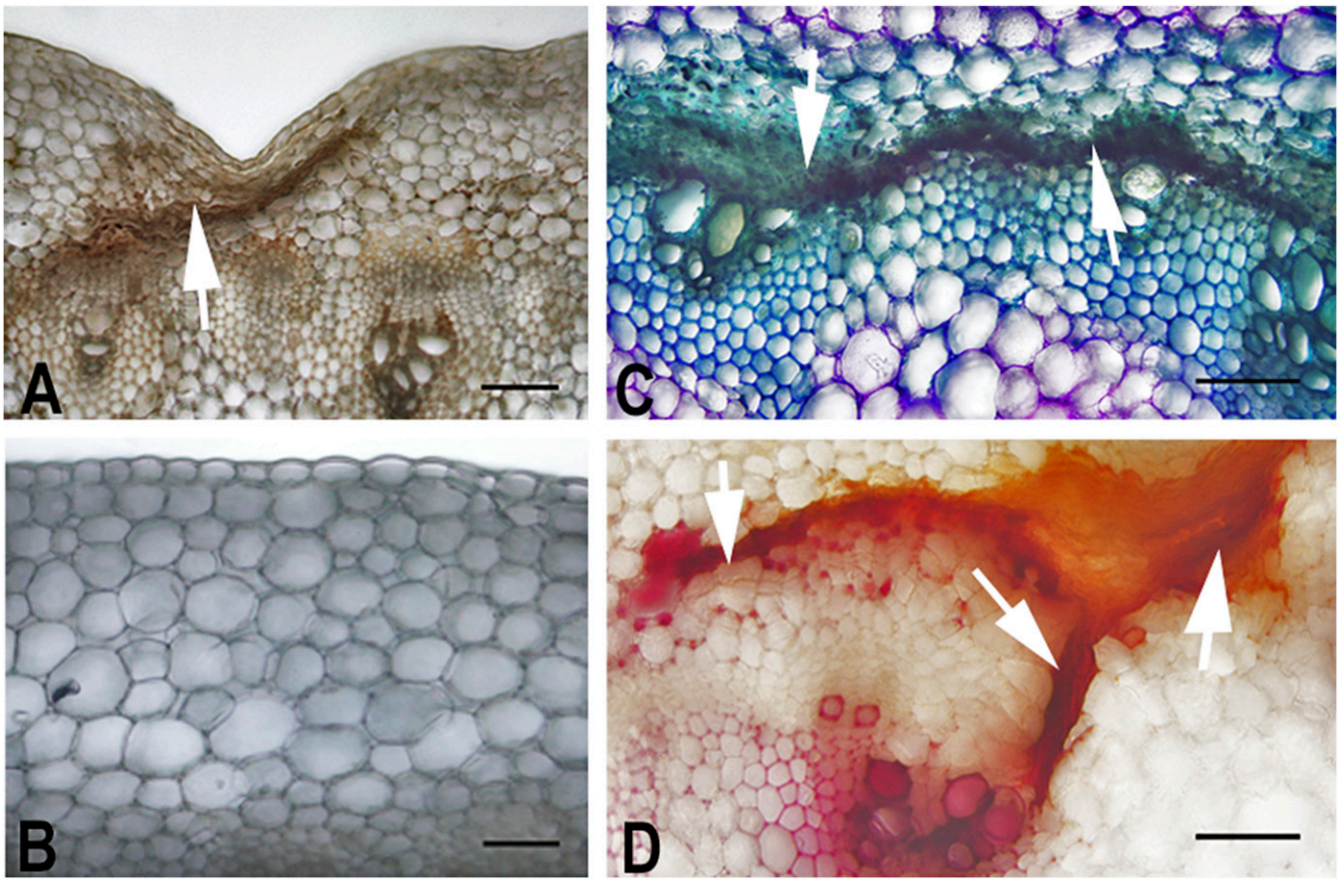

Fig. 2. Light microscopy images. At 12 days after inoculation (dai) with *Turnip mosaic virus* (TuMV), stem cross sections were cut by hand **(A and B)** or vibratome **(C and D)** from plants of *Brassica juncea* cv. Oasis Cl, which carries hypersensitivity gene *TuRBJU 01* (A, C, D) or line JM 06006 (B), which does not. The plants sectioned were infected with TuMV isolates NSW-4 (A and B) or NSW-6 (C and D). A and B were unstained, whereas C was stained with toluidine blue O, and D with phloroglucinol-HCl. Sections from cv. Oasis Cl plants developing systemic hypersensitive reactions showed necrosis in cortical cells, phloem, and phloem fibers (A, C, D) (white arrows) in comparison with sections from JM 06006 plants which lacked any necrosis (B). In some cases, necrosis spread laterally (C and D) or inwards and occasionally penetrated the xylem (D). Scale bars = 200 μm.

In addition, stem sections obtained as described above were stained with toluidine blue O (Littlefield 2003) to observe virus inclusion bodies following the procedure of Mitra and Loqué (2014).

Lignification in stem cross sections. To visualize additional lignification, stem cross sections obtained as described above were stained using phloroglucinol-HCl as described and photographed above under "Necrotic tissues in stem cross sections." A positive lignin reaction with phloroglucinol-HCl staining was indicated by a pink to brilliant or cherry red/mauve color, and a positive reaction with toluidine blue O by green, green blue, blue, or turquoise color. In addition, autofluorescence of unstained sections was studied as described above.

Although the range of colors produced by the stains phloroglucinol-HCl and toluidine blue O in stem sections were the same for tissues with lignification or necrosis, the two types of defense response were differentiated by presence of tissue damage. Necrotic tissue, which occurred mainly in phloem and cortical cells but rarely in the xylem, was identified by visible damage (cell distortion). In contrast, lignification caused no damage and was mainly associated with the xylem.

Hydrogen peroxide localization in stem cross sections. Using stem segments prepared as described above, hydrogen peroxide (H_2O_2) was localized in stems using 3,3′-diaminobenzidine (DAB) following the procedure of Thordal-Christensen et al. (1997). Briefly, stem segments were immersed in 1 mg/ml DAB (pH 3.8), incubated overnight to allow DAB uptake and the reaction between H_2O_2 and peroxidase to occur. After DAB incubation, the stem segments were boiled in 95% ethanol and then de-stained in chloral hydrate (2.5 g/ml). Thick cross sections were cut using a vibratome (80 μm) or hand sections (>80 μm); 5 to 10 sections were used for each isolate and type of plant. Sections were mounted on a glass slide using chloral hydrate, and then examined and photographed as described above. H_2O_2 was visualized as a reddish-brown polymerization product.

Occlusion of xylem vessels. Stem segments prepared as described above were used to determine the number of occluded xylem vessels. Since, regardless of the inoculum applied all treatments developed

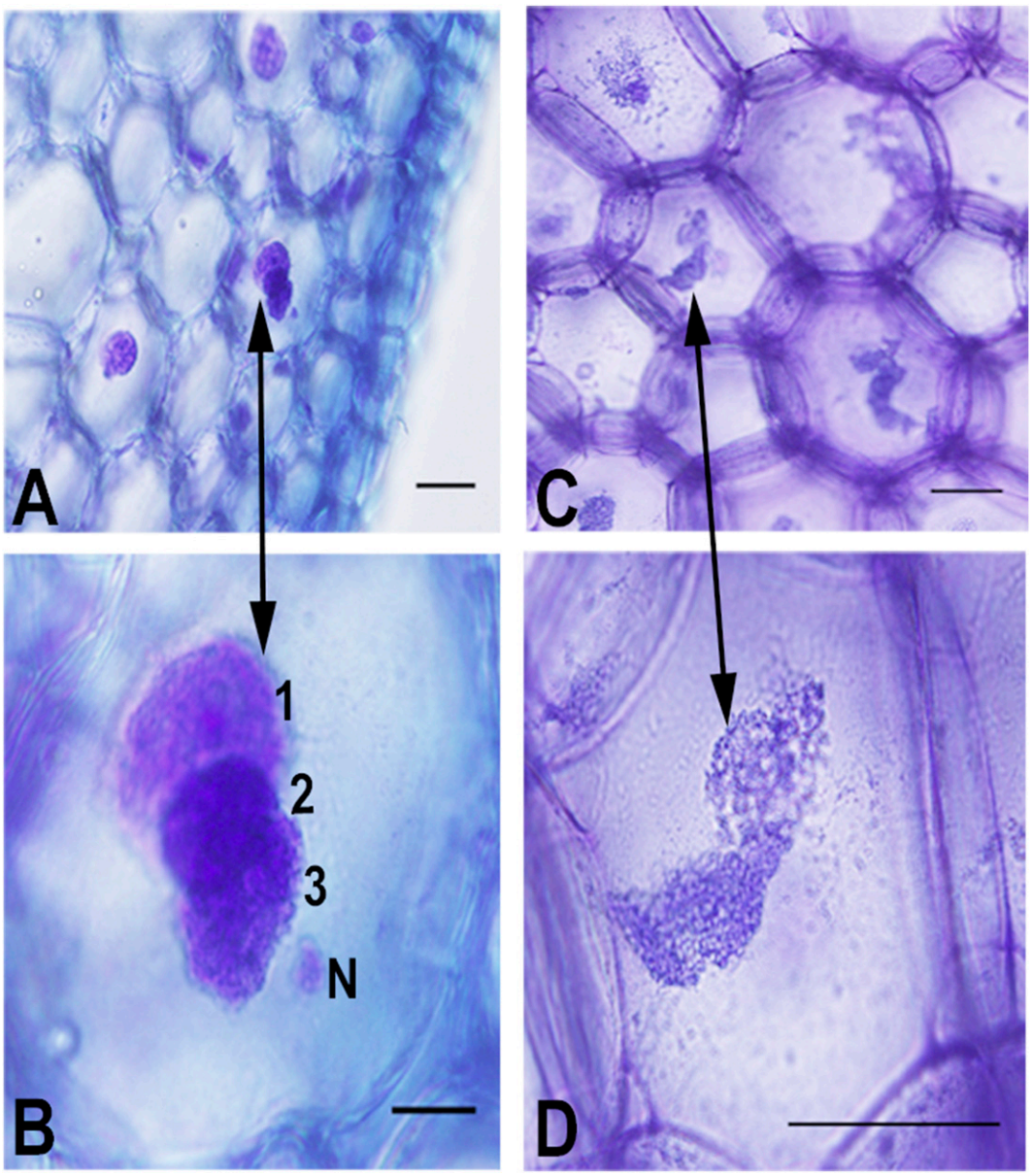

Fig. 3. Light microscopy images. At 12 days after inoculation with *Turnip mosaic virus* (TuMV) isolate NSW-4 **(A and B)** and NSW-2 **(C and D)**, stem cross sections were cut by hand from plants of *Brassica juncea* line JM 06006 (which lacks hypersensitivity gene *TuRBJU 01*). The sections were stained with azure A (A and B) or toluidine blue O (C and D). Black arrows indicate amorphous inclusion bodies. Sections A (cortical cells) and C (pith) are lower magnifications of sections B and D, respectively. B shows the nucleolus (N) to the right and three inclusion bodies in a row (1, 2, and 3) down the middle. D shows a mass of inclusion bodies. Scale bars: A and C = 200 μm, B and D = 50 μm.

occlusion of xylem vessels, data from all isolates except NSW-3 was used for analysis. Randomly chosen stem cross sections were used (1 section/isolate) from plants developing SHR (Oasis CI and F3), plants developing SM (JM 06006 and F3), and mock-inoculated plants. The main vascular areas (4 to 5 main xylem bundles/section) containing 40 to 80 vessels were studied on each section (Fig. 1A and B) and then the percentage of occluded xylem vessels calculated using the method of Inch et al. (2012) as follows:

$$\frac{\text{Number of occluded xylem vessels}}{\text{Total number of xylem vessels}} \times 100$$

Means from 4 to 5 main xylem vascular bundles from one stem section for each treatment were used in an analysis of variance appropriate for a randomized complete design. To compare means, GenStat 15th Edition (Lawes Agricultural Trust, Hemel, Hempstead,

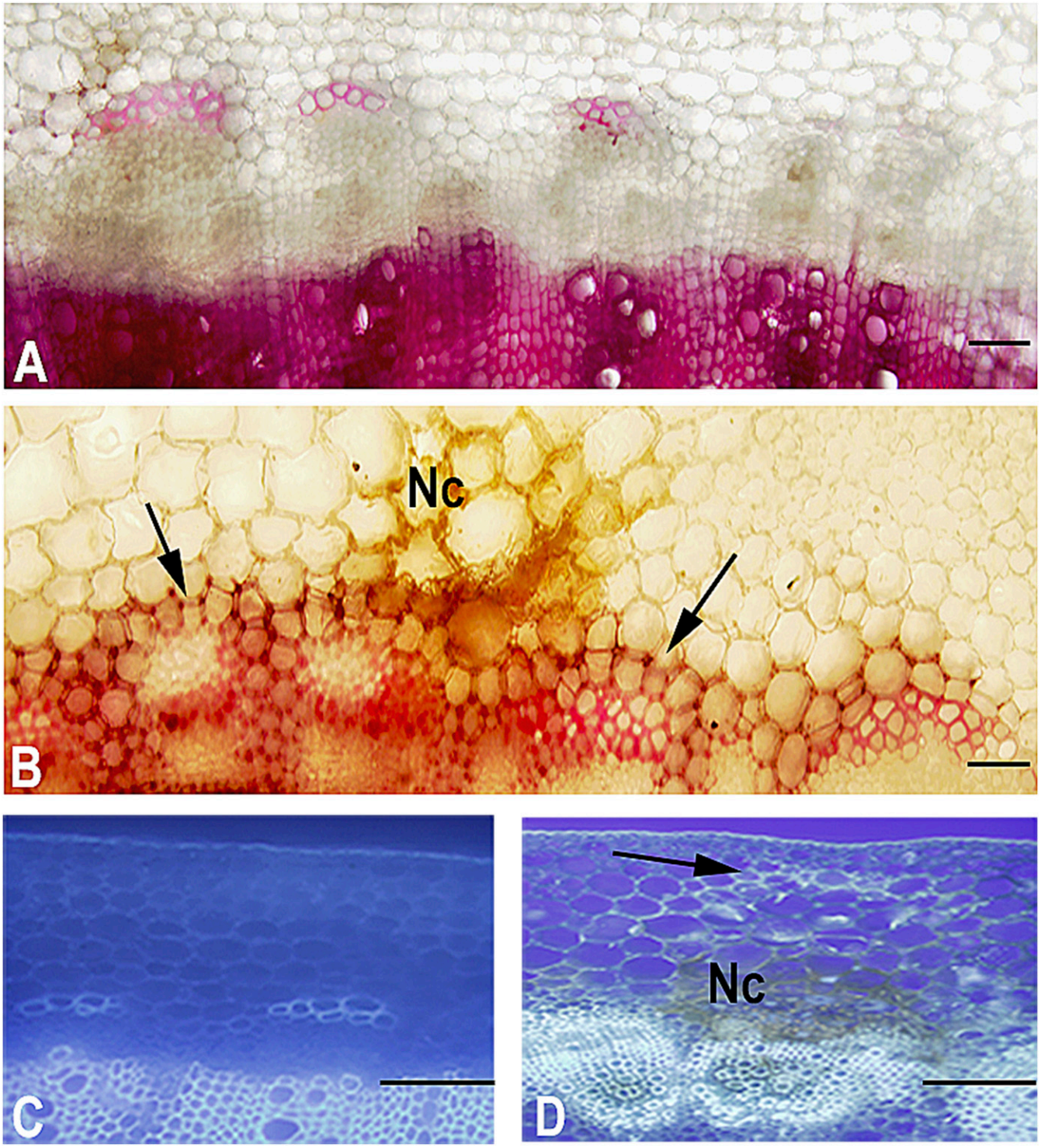

Fig. 4. Light microscopy images. At 9 to 12 days after inoculation (dai) with *Turnip mosaic virus* (TuMV) isolate NSW-5, stem cross sections were cut by hand **(A and B)** or vibratome **(C and D)** from plants of *Brassica juncea* line JM 06006, which lacks hypersensitivity gene *TuRBJU 01* (A and C) and cv. Oasis CI, which carries *TuRBJU 01* (B and D). The sections were stained with phloroglucinol-HCl (A and B) or examined unstained using ultraviolet (UV) light (C and D). A and B show different colored reactions to phloroglucinol-HCl because they were stained on different dai. A only shows normal lignification in the phloem fibers and xylem. B shows a continuous intense lignified band in close association with the endodermis cells and positioned between the cortex and phloem fibers (black arrow) but above necrotic tissue (nc). With UV light, no additional lignification was observed in line JM 06006 (C), but additional lignification near necrotic tissue was evident in the cortex (black arrow) in cv. Oasis C1 (D). Scale bars = 500 μm.

United Kingdom) was employed, along with Fisher's least significant differences ($P < 0.05$).

Lignin deposition in inoculated leaves. To establish whether formation of necrotic spot local lesions is associated with lignification in inoculated leaves of plants that subsequently developed SHR, TuMV isolates NSW-1, NSW-2, NSW-4 to NSW-6, WA-Ap, and WA-Ap1 were sap inoculated to F3 progeny plants at the 2 to 3 true leaf stage (20 plants/isolate). Five mock-inoculated plants were always included as controls. The virus-inoculated leaves developed necrotic spot local lesions at 3 dai. At 4 dai, leaf samples consisting of leaf pieces (2.5 cm^2) were cut from them and each leaf piece from a plant which later developed SHR contained at least 10 necrotic spots. Five to seven leaf pieces each from a different plant were sampled per isolate and from control plants, cleared in 95% ethanol overnight, dehydrated in 70% ethanol, and rinsed in deionized water. The leaf pieces were then cleared in chloral hydrate (2.5 g/ml) in which they were stored prior to examination. For each inoculum, 2 to 3 cleared leaf pieces selected at random were then stained using phloroglucinol-HCl following the procedure of Mitra and Loqué (2014). Once stained, they were air dried for 10 min at room temperature before being photographed and the images acquired digitally. A positive reaction with phloroglucinol-HCl staining was indicated by pink to brilliant or cherry red/mauve color.

Commencement of systemic movement. To establish when virus begins to move systemically, plants of JM 06006 and cv. Oasis Cl were inoculated with isolates WA-Ap1 and 12.5. Inoculated leaves were detached at 2, 48, and 72 h after inoculation (hai). Plants were inoculated at the 2 to 3 true leaf stage. There were nine plants/inoculum/leaf detachment time, nine plants without their inoculated leaves detached, and 30 mock-inoculated control plants. The plants were monitored for systemic infection daily until 14 dai. At 14 dai, a tip leaf sample was taken from every plant without systemic necrosis and tested for TuMV infection by ELISA (Nyalugwe et al. 2014).

Results

Symptoms in inoculated plants. SHR (i.e., foliage necrosis in presence of gene *TuRBJU 01*) started to appear at 4 to 7 dai in all cv. Oasis Cl and three quarters of F3 plants inoculated with nine TuMV isolates (NSW-3 excluded), and was accompanied by local necrotic spots which developed in inoculated leaves at 4 to 5 dai. The initial systemic symptom was plant wilting, and this was followed by stem bending, systemic leaf necrosis and some mosaic, necrotic streaking of stems and petioles (Fig. 1C, D, and E), leaf distortion, and plant stunting. At the stage when stem sections were taken (i.e., at 9 to 12 dai), the different types of necrotic symptoms (phenotypes $+_N$ $+_{ND}$) could not be distinguished in the F3 plants that developed necrosis, and resembled the necrosis in cv. Oasis Cl plants. Subsequently, however, the necrosis became more intense in all the Oasis Cl plants and about a quarter of the F3 plants resulting in plant death (phenotype $+_{ND}$). In the remaining F3 plants with necrosis, this symptom spread less and the plants were not killed (phenotype $+_N$). With all 10 TuMV isolates, at 4 to 7 dai, a systemic susceptible response (i.e., SM in absence of gene *TuRBJU 01*) started to appear in plants of line JM 06006 and the remaining quarter of the F3 plants, gradually affecting all of them. In inoculated leaves, these systemic symptoms were associated with local chlorotic spots or faint mosaic which developed at 4 to 5 dai. When inoculated with isolate NSW-3, all Oasis Cl and F3 plants developed SM and the same symptoms in inoculated leaves. No virus-infection or symptoms developed in mock-inoculated parental or F3 plants.

Necrotic tissues in stem cross sections. Except with isolate NSW-3, bright field and UV microscopy of stem sections showed that by 9 to 12 dai, necrosis was present in stems from >90% of plants with gene *TuRBJU 01* following inoculation with the different TuMV isolates. This necrosis developed in cortical cells (collenchyma or parenchyma), phloem fibers, and phloem (Fig. 2A, C, and D). By contrast, xylem vessels were only penetrated occasionally (Fig. 2D). The phloem showed most necrosis (Fig. 2C and D), and sometimes a band of necrosis spread laterally along the phloem (Fig. 2C). In >60% of stem sections, necrosis was observed passing around or through the phloem (Fig. 2A, C, and D). In contrast, no necrosis occurred in stem cross section samples from any TuMV-inoculated plants without gene *TuRBJU 01* (Fig. 2B), plants with *TuRBJU 01* inoculated with isolate NSW-3, or the mock-inoculated control plants.

Virus inclusion bodies in stem cross sections. In plants without gene *TuRBJU 01* inoculated with all 10 TuMV isolates, toluidine blue O and azure A staining both detected presence of virus inclusion

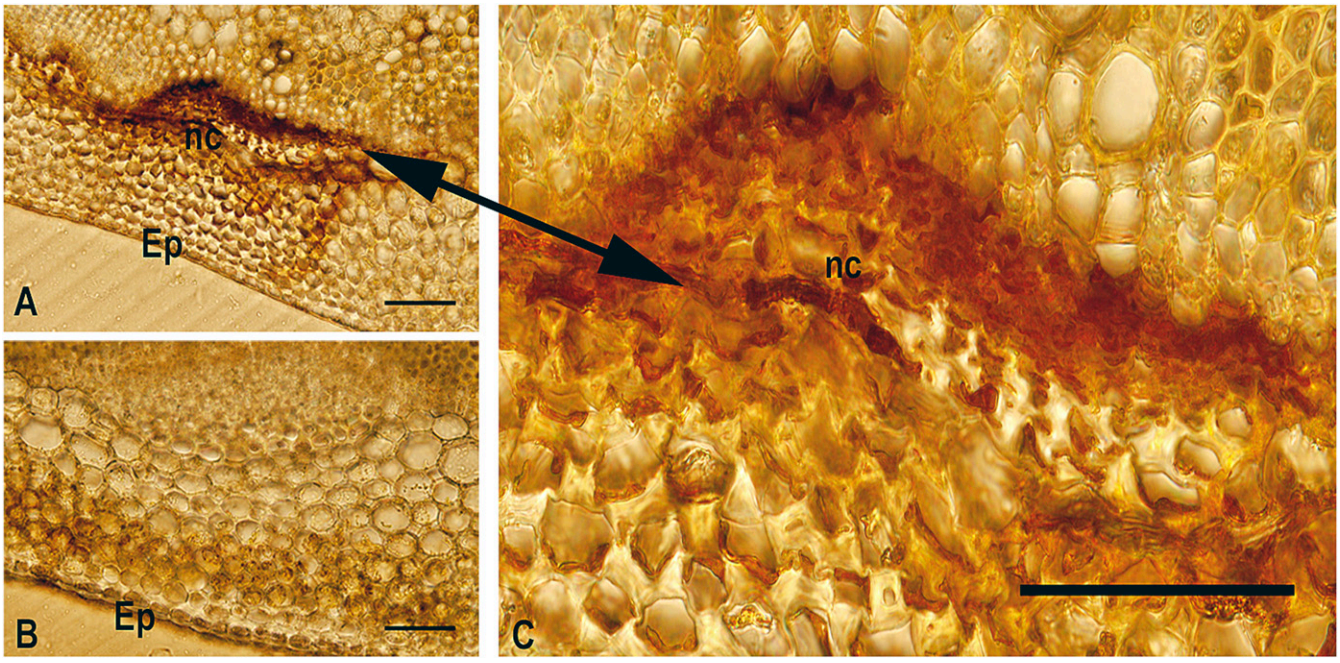

Fig. 5. Light microscopy images. At 9 days after inoculation (dai) with *Turnip mosaic virus* (TuMV) isolate WA-Ap1, stem cross sections were cut using a vibratome from plants of *Brassica juncea* cv. Oasis Cl, which carries hypersensitivity gene *TuRBJU 01* **(A and C)** or line JM 06006, which lacks *TuRBJU 01* **(B)**. The sections were stained with 3,3′-diaminobenzidine to detect localization of hydrogen peroxide (H_2O_2). A shows browning of the necrotic area (nc) near or along the phloem due to H_2O_2 deposition. B lacks this browning and the necrotic area. C is a magnified view of A highlighting numerous deposits of H_2O_2. Ep = epidermis. Scale bars = 200 μm.

bodies in stem sections from all plants (Fig. 3). The inclusion bodies mostly had an amorphous appearance and consisted of aggregates of HC-Pro. Inclusion bodies were rare in stem sections from plants with gene *TuRBJU 01*, and when present in them were mainly confined to the cortex. No inclusion bodies were observed in stem sections from mock-inoculated plants.

Lignification in stem cross sections. Upon staining with phloroglucinol-HCl following inoculation with all 10 isolates, stem sections from plants without gene *TuRBJU 01*, plants with gene *TuRBJU 01* inoculated with isolate NSW-3, and mock-inoculated plants all showed normal lignification of the phloem fibers and xylem (Fig. 4A). In stem sections from the immediate vicinity of necrotic tissue from plants with gene *TuRBJU 01* inoculated with all nine isolates except NSW-3, extra lignification was prominent in the endodermis (Fig. 4B), cortex, and occasionally in the pith. These included bands like striated springs in the endodermis (Fig. 4B). When stem sections from plants i) without gene *TuRBJU 01*, ii) with gene *TuRBJU 01* inoculated with isolate NSW-3, or iii) that were mock-inoculated were viewed under UV light, they all showed normal fluorescence of phloem fibers and xylem (Fig. 4C). In contrast, when stem sections from plants with gene *TuRBJU 01* inoculated with the other nine isolates were viewed under UV light, strong autofluorescence was observed in areas in or near necrotic endodermal or cortical tissues (Fig. 4D).

Hydrogen peroxide localization in stem cross sections. When all isolates except NSW-3 were inoculated to plants of cv. Oasis Cl,

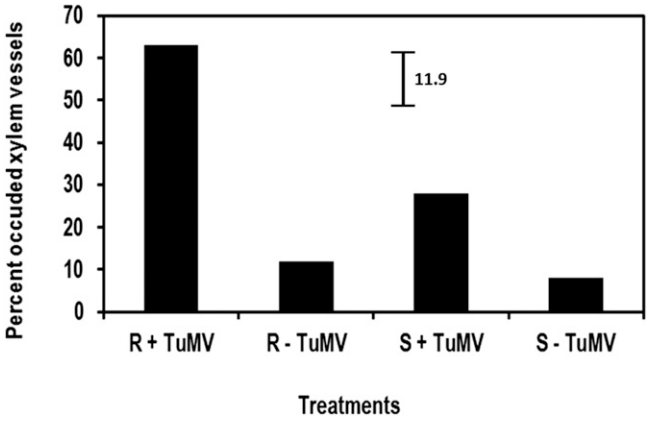

Fig. 7. Quantitative assessment of xylem vessel occlusions. Plants of *Brassica juncea* cv. Oasis Cl (carries gene *TuRBJU 01*), line JM 06006 (lacks gene *TuRBJU 01*), and their F3 progeny plants (segregating for gene *TuRBJU 01*) were inoculated with nine *Turnip mosaic virus* (TuMV) isolates (NSW-3 excluded). Horizontal axis: R = resistant (+ gene *TuRBJU 01*), S = susceptible (- gene *TuRBJU 01*), + TuMV = infected, - TuMV = mock-inoculated. Stem cross sections were sampled 9 to 12 days after inoculation (dai). For each isolate, stained (phloroglucinol-HCl and toluidine blue O) or unstained stem sections were examined by light microscopy for xylem vessel occlusions. Data represent means of the percentages of occluded xylem vessels within the main vascular bundle(s) of 40 to 80 xylem vessels/section for each of the nine isolates. Scale bar = lsd.

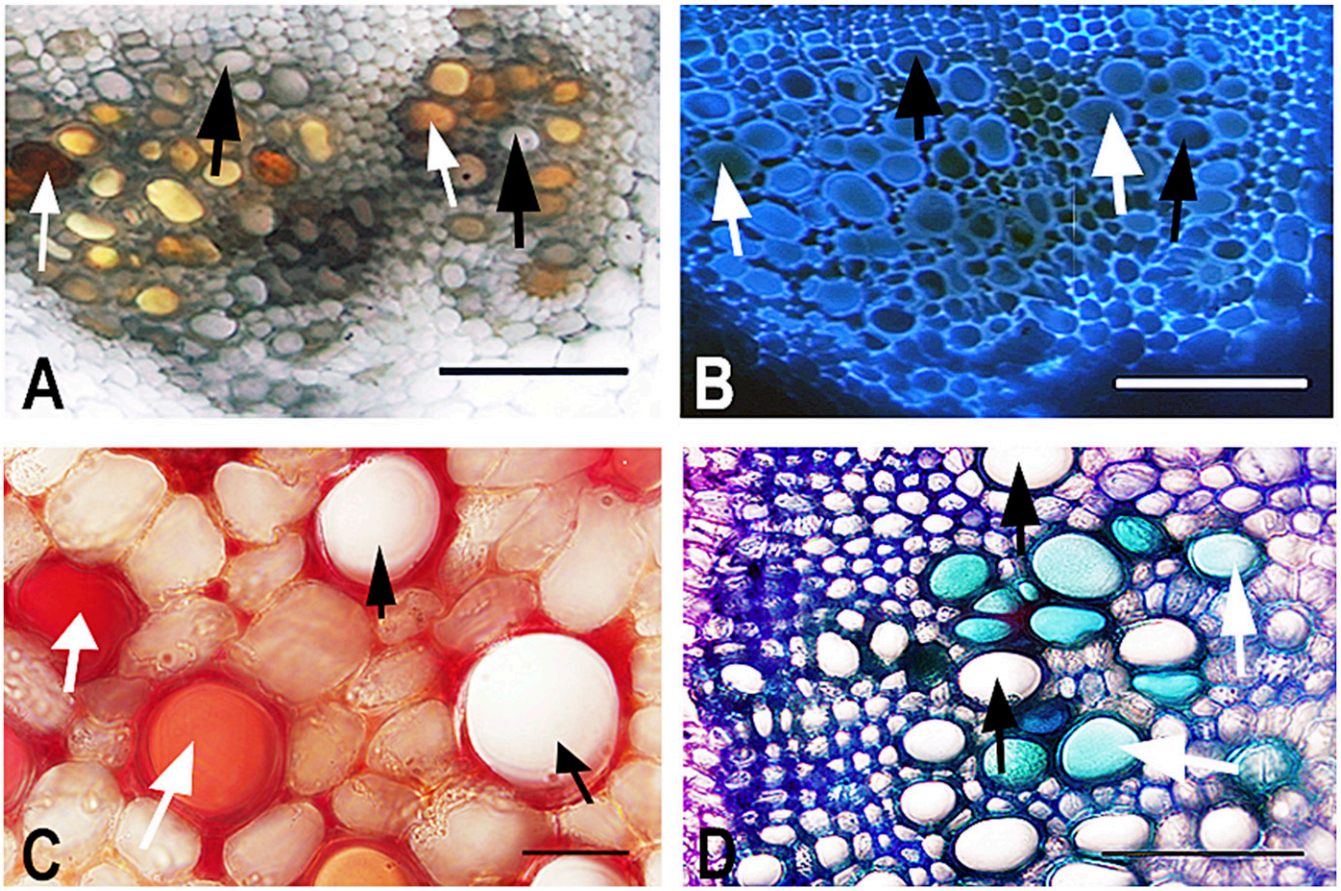

Fig. 6. Light microscopy images. At 12 days after inoculation (dai) with *Turnip mosaic virus* (TuMV), stem cross sections were cut by hand **(A and B)** or a vibratome **(C and D)** from plants of *Brassica juncea* cv. Oasis Cl, which carries hypersensitivity gene *TuRBJU 01* (A-C), or line JM 06006, which lacks *TuRBJU 01* (D). A and B were inoculated with isolate NSW-4, C with isolate NSW-6, and D with isolate WA-Ap1. A and B were from the same plant and stem sample position, whereas C and D were from different plants. Unstained stem sections were viewed using ultraviolet light (A) or under bright field microscopy (B) to observe occluded xylem vessels, and the stem sections in C and D show the responses of occluded xylem vessels to staining with phloroglucinol-HCl (C) or toluidine blue O (D). A, occluded xylem vessels appear sealed (white arrows) compared with non-occluded xylem vessels, which were not sealed (black arrows). B, occluded xylem vessels appear yellowish or brownish (white arrows), whereas non-occluded xylem vessels appear white (black arrows). Occluded xylem vessels stained deep orange or reddish in C, or turquoise in D (white arrows). Non-occluded xylem vessels stained white (black arrows) in C and D. Scale bars: A, B, and D = 200 μm, C = 50 μm.

which carries gene *TuRBJU 01*, H_2O_2 was mainly associated with areas of necrotic tissue in the stem sections (Fig. 5A). These areas with H_2O_2 included the phloem, phloem fibers, and cortex. In contrast, when inoculated with all 10 isolates, plants of line JM 06006, which lacks gene *TuRBJU 01* (Fig. 5B), and mock-inoculated plants were devoid of H_2O_2 in all tissues, as were plants of cv. Oasis Cl inoculated with isolate NSW-3 (Fig. 5B). Examination of the areas with H_2O_2 localization in cv. Oasis Cl showed numerous H_2O_2 deposits (Fig. 5C).

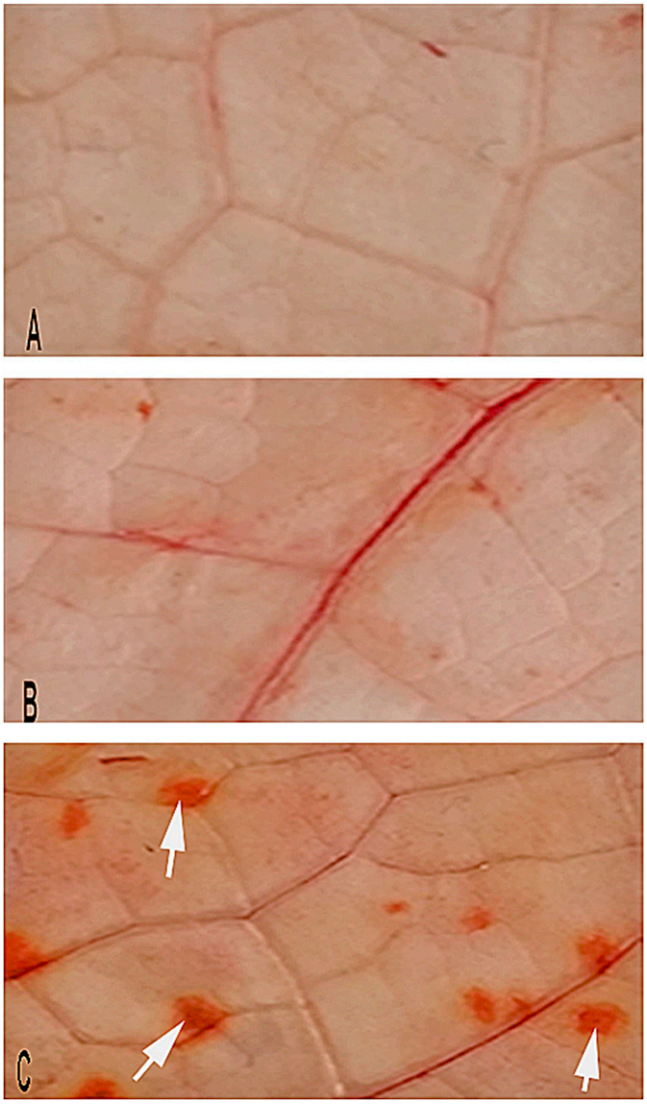

Fig. 8. Veinal necrosis in the leaf epidermis of F3 *Brassica juncea* plants inoculated with *Turnip mosaic virus* (TuMV) isolate NSW-6 at 4 days after inoculation. Staining was with phloroglucinol-HCl and photographs were taken 10 min after staining. **A,** leaf piece from a mock-inoculated control plant showing no effect of stain. **B,** leaf piece from a plant with a susceptible response (non-necrotic) showing staining of leaf veins with occasional red coloration of the cells. **C,** leaf piece from a plant developing a hypersensitive (necrotic) response showing intense brownish red coloration of necrotic lesions (white arrows) and leaf veins. Scale: 54 × 150 mm (150 × 150 DPI).

Occlusion of xylem vessels. Regardless of whether the plants from which stem sections came were inoculated with TuMV or mock-inoculated with buffer, some xylem vessels were always occluded. The natural color of the occluding material varied from yellow to dark brown when viewed using bright field light microscopy (Fig. 6A). When viewed with UV light, the occluded vessels appeared sealed with gel-like substances whereas the non-occluded vessels did not (Fig. 6B). When treated with phluoroglucinol-HCl or toluidine blue O, occluded xylem vessels showed different tones of yellow, orange, and red (Fig. 6C), or stained light blue or turquoise with toluidine blue O (Fig. 6D). In general, colored xylem vessels stained by phloroglucinol-HCl were also stained by toluidine blue O. In contrast, normal/non-occluded xylem vessels stained with these two stains appeared white in color (Fig. 6C and D). This suggests that the xylem occlusions found could be gels or tyloses. There were significant differences between categories in the percentages of occluded xylem vessels ($P < 0.001$) (Fig. 7). Stem sections from plants developing SHR had a significantly higher percentage (63%) of occluded xylem vessels than those from plants developing SM (27%), or any of the mock-inoculated plants (9 to 11%). Virus inoculation induced a significantly higher percentage of occluded xylem vessels than mock-inoculation.

Lignin deposition in inoculated leaves. With phloroglucinol-HCl staining, leaf piece samples from mock-inoculated leaves occasionally showed very faint red coloration in their veins (Fig. 8A). In leaf piece samples from inoculated leaves of plants developing SM, red coloration was evident in their veins, and red stained spotted areas occasionally appeared in the epidermis (Fig. 8B). Leaf piece samples from inoculated leaves of plants developing the necrotic lesions and areas surrounding them exhibited strong red coloration showing the presence of additional lignification (Fig. 8C). As all seven TuMV isolates used in the inoculations (WA-Ap, WA-Ap1, NSW-1 NSW-2, and NSW-4 to NSW-6) behaved similarly, the images in Figure 8 are representative of how all isolates appeared.

Commencement of systemic movement. When inoculated leaves were detached at 72 hai, systemic virus symptoms developed in 33/36 TuMV-inoculated *B. juncea* plants (Table 1). After inoculated leaf detachment at 48 hai, 22/36 inoculated plants developed systemic symptoms, but with inoculated leaf detachment at 2 hai, only 5/36 did so. When inoculated leaves were not detached, all plants displayed systemic symptoms with every isolate/plant combination. None of the mock-inoculated plants developed virus symptoms. When tip leaf samples were taken from every TuMV-inoculated plant without systemic necrosis at 14 dai and then tested for infection by ELISA, TuMV was detected in all plants that developed SM, but in none that were symptomless. No symptoms developed and no TuMV-infection was detected in any of the 30 mock-inoculated control plants. At each sampling time, numbers of plants with systemic invasion differed little between the two isolates or between line JM 06006 or cv. Oasis Cl, except that only isolate 12.5 caused systemic infection at 2 hai in Oasis Cl

Table 1. Effect of detaching leaves at different times after inoculation with two *Turnip mosaic virus* (TuMV) isolates on systemic symptom development in *Brassica juncea* lines developing systemic hypersensitive (cv. Oasis C1) or susceptible (line JM 06006) phenotypes[a]

	JM 06006		Oasis Cl		
Inoculated leaf detachment	**WA-Ap1**	**12.5**	**WA-Ap1**	**12.5**	**Number positive/tested**
2[b]	2[c]	2	0	5	9/36
48	6	4	6	6	22/36
72	9	7	9	8	33/36
ND	9	9	9	9	36/36
Mean	7	6	6	7	-

[a] There were nine plants in each category. At 14 days after inoculation (dai), a tip leaf sample was taken from every plant without systemic necrosis and tested for TuMV infection by ELISA. No infection was detected in any of the 30 mock-inoculated control plants and none of them developed virus symptoms.
[b] Hours after inoculation before inoculated leaves were removed, ND = inoculated leaves not detached.
[c] Numbers of plants out of nine that developed systemic infection (= systemic necrosis or +ve ELISA test on tip leaves) by 14 dai.

plants and in JM 06006 isolate 12.5 was found in somewhat fewer plants than isolate WA-Ap1 at 48 and 72 hai.

Discussion

This research involving the TuMV-*B. juncea* pathosystem, hypersensitivity gene *TuRBJU 01*, and inoculation with 10 TuMV isolates (one of which overcame gene *TuRBJU 01*) provided new evidence of anatomical and physiological changes associated with virus-induced SHR in plants. Moreover, using histochemical analysis of stem cross sections with light microscopy proved a simple yet effective way to identify SHR-associated changes occurring at the cellular level following virus infection. This study revealed presence of the plant defense responses phloem necrosis, additional lignification, xylem occlusion, and hydrogen peroxide accumulation. Both light microscopy and histochemical tests with phloroglucinol-HCl and toluidine blue O indicated that the xylem occlusions could be gels. This appears to be the first time that xylem occlusion has been associated with virus-induced SHR development. Phloem necrosis was apparently acting as the primary cause of SHR and xylem occlusion as an important secondary cause. Virus inclusion bodies were fewer in sections from plants with SHR and there was no delay in systemic virus spread associated with SHR development or presence of gene *TuRBJU 01*.

With HR, infection is restricted to cells around the inoculation site (e.g., Kimmins and Wuddah 1977). In this study, neither presence of gene *TURBJU01* nor the development of necrotic local lesions, restricted TuMV systemic spread out of inoculated leaves to other parts of the *B. juncea* plant. Thus, regardless of whether i) *B. juncea* plants were with or without this gene, or ii) SHR did or did not develop subsequently, by 2 hai the virus had started to enter the stem vascular system and move systemically. Moreover, by 72 hai, it had entered the vascular systems of all (isolate WA-Ap1) or almost all (isolate 12.5) plants regardless of whether they carried gene *TURBJU 01* or later developed SHR or SM. This brief delay before systemic infection started resembles what occurs with some other viruses, e.g., with *Tomato aucuba mosaic virus* (genus *Tobamovius*), *Tobacco mosaic virus* (genus *Tobamovius*), and *Potato virus X* (genus *Potexvirus*), delays to move from inoculated leaves to petioles and stems were 3 to 4, 5, and 3 days, respectively (Capook 1949). In plants that developed SHR after TuMV inoculation, initial symptoms consisted of plant wilting and stem bending and were followed by leaf necrosis and mosaic, petiole and stem necrotic streaking, leaf distortion and plant stunting (phenotype $+_N$), or more generalized necrosis leading to plant death (phenotype $+_{ND}$). There was no necrosis in plants that developed SM.

In stem cross sections taken at 9 to 12 dai from *B. juncea* plants in which SHR was developing, there was necrosis of the cortex, endodermis, phloem and, occasionally, the xylem. However, no necrosis was present in sections from plants developing SM. Similar findings were reported in other pathosystems where SHR develops, e.g., *Tobacco ringspot virus* (TRSV, genus *Nepovirus*) infecting cowpea (Edwards and Agrios 1981) and the D satellite RNA of *Cucumber mosaic virus* (genus *Cucumovirus*) infecting tomato (Xu and Roossinck 2000). With the TRSV-cowpea pathosystem, after first appearing in the phloem, virus-induced necrosis then spread to the xylem, epidermis, collenchyma, and pith (Edwards and Agrios 1981). In this study, necrosis was observed passing around or through the phloem in more than 60% of stem cross sections. This suggests that necrosis may have been initiated in one of its components, viz. sieve tubes, companion cells, phloem parenchyma, or phloem fibers. This study did not determine where it started in the phloem, but an earlier investigation using a pathosystem in which virus-induced phloem necrosis occurred without SHR found that phloem necrosis started with sieve tube swelling (Dijkstra and de Jager 1998).

Lignin deposition constitutes a plant defense response associated with virus-induced HR but not reported yet with SHR (see Introduction). When stem cross sections from *B. juncea* plants in which SHR was developing were viewed i) without staining using UV light or ii) after staining with phloroglucinol-HCl, in both instances, additional lignification was prominent in the endodermis and cortex in areas near necrotic tissues. In contrast, the amount of lignification in sections from plants developing SM was no different from that in mock-inoculated plants. In leaf piece samples from inoculated leaves of plants in which SHR subsequently occurred, additional lignification was prominent in the necrotic lesions and areas surrounding them, as evidenced by intense red coloration produced by phloroglucinol-HCl staining. This contrasted with the less intense reddening evident in leaf veins of plants developing SM and very faint reddening of leaf veins in mock-inoculated plants. These results for lignification in TuMV-inoculated leaves resembled those found previously with HR in virus-inoculated leaves (Appiano et al. 1977; Faulkner and Kimmins 1975; Kimmins and Wuddah 1977; Sundaresan and Kimmins 1981).

Triggering rapid accumulation of ROS, such as hydrogen peroxide, constitutes a plant defense response associated with virus-induced SHR (see Introduction). Hydrogen peroxide accumulation was detected in necrotic tissues in stem cross sections of *B. juncea* plants developing SHR, but not in sections from plants developing SM. These results resembled those found previously with virus-induced SHR in tomato plants (Xu et al. 2003).

Presence of vascular occlusions in xylem vessels constitutes a plant defense response associated with plant death caused by some other types of plant pathogen but remains unreported for viruses (see Introduction). Vascular occlusions were present in stem cross sections from all plants regardless of whether they were developing SHR or SM, or were mock-inoculated. Based on consolidated data from stem cross sections across nine isolates (NSW-3 excluded), the percentages of occluded vessels were significantly larger for SHR (63%) than for SM (27%) or mock-inoculation (9 to 11%), and the values for mock-inoculation were significantly smaller than those for the other two categories. Histochemical studies using different stains with bacterial and fungal pathogens have shown that gels and tyloses contain polysaccharides, lignin, lipids, phenolics, and pectin (e.g., Kpémoua et al. 1996; Sun et al. 2008). With gels, xylem lumens mostly appear completely sealed but tyloses rarely seal vessels completely (Sun et al. 2008; Wang et al. 2014). In this study, when viewed under UV light, occluded vessels appeared sealed by gel-like substances as their lumens were completely sealed. Nyalugwe et al. (2016) found that the same 10 TuMV isolates used here differed in virulence and associated rate of plant death: NSW-2 induced the most severe and WA-Ap the least severe SHR, while resistance-breaking isolate NSW-3 only caused SM. In the present study, no attempt was made to quantify differences in xylem occlusions based on isolate virulence and associated rate of plant death because numbers of stem cross sections examined/isolate were too small. Such a study could be a focus of future research.

Virus inclusion bodies with an amorphous appearance were observed readily in stem cross sections of *B. juncea* plants developing SM, but rarely in sections from plants developing SHR. In the latter case, their rarity may reflect the effects of plant defense responses associated with SHR in diminishing TuMV multiplication in cells.

In conclusion, by employing histochemical analysis of stem cross sections with light microscopy to investigate the TuMV-*B. juncea* pathosystem and the behavior of hypersensitivity gene *TuRBJU 01* at the cellular level, this paper demonstrated that the plant defense responses phloem necrosis, xylem occlusion, additional lignification, and hydrogen peroxidase accumulation were all associated with SHR development. The effect of phloem necrosis in preventing translocation of nutrients through the stem seems to be the primary factor leading to the spread of necrosis and eventual plant death that occurs with SHR. However, this effect seems likely to be exacerbated by occlusions, which seal off xylem vessels and suppress water conduction, thereby contributing to the spread of necrosis. Further research with this pathosystem would be worthwhile to explore the roles of phloem necrosis and xylem occlusion, and to establish the importance of lignin deposition and hydrogen peroxide accumulation on SHR development. Furthermore, future investigations should also focus on the roles of callose deposition and the activities of general defense and stress related genes in contributing to SHR.

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