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Evidence that Cell Death is Associated with Zebra Chip Disease in Potato Tubers

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Abstract Zebra chip (ZC) is an established and highly destructive disease of potato (*Solanum tuberosum* L.) that occurs in several southwestern states of the United States, Mexico, Central America, and New Zealand. The causal agent for this disease has not been identified. However, the bacterium "*Candidatus* Liberibacter solanacearum" and the potato psyllid, *Bactericera cockerelli* (Šulc), its insect vector, are associated with the disease. Tubers from ZC-affected potato plants exhibit dramatic browning of vascular tissue concomitant with "necrotic flecking" both of which can affect the entire tuber. Upon frying, these tubers develop a characteristic striped pattern of discoloration rendering them unmarketable. These characteristic ZC symptoms in the tubers have been suggested to be associated with general cell death, though no evidence to confirm this hypothesis has been shown. In order to determine if cell death is associated with ZC disease, a series of experiments were undertaken. Cell death was initially quantified by comparing cellular ion leakage from ZC-affected and ZC-free tubers. Levels of ion leakage were found to be significantly higher in ZC-affected tubers compared to ZC-free tubers. To examine further the association of cell death with ZC disease, ZC-affected and ZC-free tubers were compared using classical histochemical staining methods in conjunction with optical microscopy, which revealed layers of dead cells surrounding numerous, small, irregularly-shaped lesions throughout the parenchymatic medullary region, vascular ring and cortex of ZC-affected tubers. This cell death was confirmed using high-resolution, field-emission scanning electron microscopy (FE-SEM) of fresh-cut tuber tissue.

Resumen Zebra chip (ZC) es una enfermedad establecida y altamente destructiva de papa (*Solanum tuberosum* L.) que se presenta en varios estados del suroeste de los Estados Unidos, México, América Central y Nueva Zelandia. El agente causal de esta enfermedad no ha sido identificado. No obstante, la bacteria "*Candidatus* Liberibacter solanacearum" y el psílido de la papa *Bactericera cockerelli* (Šulc), su insecto vector, están asociados con la enfermedad. Los tubérculos de plantas afectadas por ZC presentan oscurecimiento dramático del tejido vascular concomitante con "pecas necróticas" que en ambos casos pueden afectar al tubérculo completo. Al freírse, estos tubérculos desarrollan un patrón característico de rayado haciéndolos no comerciales. Se ha sugerido que estos síntomas característicos en los tubérculos estén asociados con muerte general de las células, aún cuando no se ha mostrado evidencia para confirmar esta hipótesis. Se llevaron a cabo varios experimentos a fin de determinar si la muerte de las células está asociada con la enfermedad de ZC. La muerte celular se cuantificó inicialmente comparando el lixiviado iónico celular de tubérculos con y sin ZC. Se encontró que los niveles de iones lixiviados fueron significativamente más altos en tubérculos afectados con ZC comparados con los libres de ZC. Para examinar mas la asociación de la muerte de la célula con la enfermedad ZC,

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a tubérculos infectados y a libres de ZC se les comparó usando los métodos de la clásica tinción histoquímica, junto con microscopía óptica, lo cual reveló capas de células muertas rodeando a numerosas lesiones pequeñas, de forma irregular, a través de la región medular parenquimatosa, el anillo vascular y el cortex de tubérculos afectados por ZC. Esta muerte celular se confirmó usando microscopía electrónica de barrido de alta resolución (FE-SEM) de tejido de cortes de tubérculo fresco.

Keywords *Bactericera cockerelli* · Potato psyllid · *Candidatus* Liberibacter solanacearum · *Solanum tuberosum* · Cultivar Atlantic · FE-SEM

Introduction

Zebra chip (ZC) is a very destructive disease of potato (*Solanum tuberosum* L.) that has been documented in many parts of the world, including the southwestern United States, Mexico, Central America, and most recently New Zealand (Munyaneza et al. 2007a, b, 2008, 2009; Liefting et al. 2008; Secor et al. 2009). This disease causes severe economic losses to the potato industry by affecting both potato yield and marketability of processed tubers (Goolsby et al. 2007a, b; Munyaneza et al. 2007a, b, 2008, 2009). In Texas alone, the economic losses due to ZC disease could reach $100 million (CNAS 2006). Tubers from ZC-affected plants present a wide range of symptoms including collapsed stolons, enlarged lenticels, vascular tissue browning of the parenchymatic medullary region and cortex, and "necrotic flecking" throughout the tuber (Munyaneza et al. 2007a, b). Upon frying, ZC-affected tubers develop a characteristic striped "Zebra" pattern of discoloration rendering them unmarketable. The causal agent for this disease has been not been identified. However, the bacterium "*Candidatus* Liberibacter solanacearum", also known as *Ca.* Liberibacter psyllaurous, and the potato psyllid, *Bactericera cockerelli* (Šulc) the insect vector, have recently been associated with ZC (Hansen et al. 2008; Liefting et al. 2008, 2009; Lin et al. 2008; Abad et al. 2009; Crosslin and Munyaneza 2009; Secor et al. 2009).

A multitude of disorders of potato tubers result in discoloration 'browning' of the vascular tissue, which has been shown to be associated with elevated levels of reducing sugars and/or polyphenolics, as well as general cell death "necrosis". While some of these disorders are clearly non-pathologic or 'physiological', many have been positively linked to pathogens, including late blight (*Phytophthora infestans*) (Levin et al. 2001), potato soft rot (*Erwinia carotovora* pv. *atroseptica*) (Ghanekar et al. 1984; Nourian et al. 2002), potato leaf roll virus 'net necrosis' (Murphy 1968), potato virus Y (PVY) (Hinrichs-Berger et al. 1999), potato virus X (PVX) (Mondy and Kock 1978; Cruz and Baulcombe 1993), and common scab (*Streptomyces scabies*) (Blaszczak et al. 2005). The end result of the above-mentioned diseases is the rendering of the tuber unusable as food or as seed for future plantings.

When plants are attacked by pathogens, they defend themselves by deploying a variety of defense mechanisms designed to evade the colonization of pathogens. These include but are not limited to: hypersensitive response (HR), which is defined as incompatible plant-pathogen reactions or non-host-pathogen interaction leading to cell death; increased production of antimicrobial compounds (phenolics and phytoalexins), pathogenesis-related proteins, reactive oxygen species, and salicylic acid (SA); induced activity and/or biosynthesis of polyphenol oxidases (PPOs) and peroxidase enzymes; increased ion fluxes across the plasma membrane; the deposition of lignin and callose at the infection sites; and rapid dehydration of the infected cells (Lyon and McGill 1988; Nicholson and Hammerschmidt 1992; Bennett and Wallsgrove 1994; Richael and Gilchrist 1999; van Doorn and Woltering 2005; Reape et al. 2008; Almagro et al. 2009). Many of these morphological, physiological, and molecular changes occur coincident with the final phenotype of the HR, programmed cell death (PCD).

Cell death is generally subdivided into three main categories: apoptosis (Type I), autophagic cell death (Type II); which are classified as physiological cell death, and necrosis (Type III) (Kerr et al. 1972; Wyllie et al. 1984; Reape et al. 2008). Apoptosis and autophagic are types of programmed cell death (PCD), which is defined as a sequence of events that lead to the controlled and organized destruction of the cell (Lockshin and Zakeri 2004). In contrast, necrosis is a passive, uncontrolled type of cell death without active regulatory mechanisms (Lockshin and Zakeri 2004; Reape and McCabe 2008). Programmed cell death is a vitally important component of development as well as defense mechanisms in plants.

The main internal symptoms observed with tubers from ZC diseased plants (i.e., vascular tissue browning and "necrotic" flecking), which are linked with many of the above-mentioned diseases, have recently been associated with elevated levels of either reducing sugars (glucose) (Gao et al. 2009) or phenolics (namely tyrosine, a phenolic 'aromatic' amino acid) (Navarre et al. 2009) or to general tissue necrosis. Reducing sugars, which are elevated as a result of potato-pathogen interactions, mechanical damage and cold stress, have the proclivity to react with free amino acids, peptides and proteins during high-temperature processing (Maillard 'non-enzymatic' browning) giving the potato product an off taste and overall burnt appearance (reviewed in Friedman 1996). The other form of potato tissue browning ('enzymatic') involves polyphenol oxidase

enzymes (EC 1.14.18.1 and EC 1.10.3.2) (PPOs), which act in disease resistance, mechanical damage and wounding by catalyzing *o*-hydroxylation of monophenols to *o*-diphenols. They can further catalyse the oxidation of *o*-diphenols to *o*-quinones, which can rapidly undergo polymerisation to form polyphenols (pigmented compounds), which are deposited at the site of infection and are often more toxic to the pathogen than the original parent phenolic (Pendharkar and Nair 1995; Friedman 1997; Lulai and Corsini 1998; Gandia-Herrero et al. 2005; Poiatti et al. 2009). Whether the altered reducing sugar and/or phenolic content found in tubers from ZC-affected plants have anything to do with the characteristic striping and/or "necrotic flecking" found in tubers from ZC-affected plants has yet to be determined. To the best of our knowledge, the involvement of cell death in ZC disease in potato tubers has not been documented.

The objective of this work was to establish whether ZC disease causes cell death in potato tubers. Using ion leakage assays and conventional histochemical methods in conjunction with optical, fluorescence and electron (FE-SEM) microscopy, we have established that ZC disease involves cell death in potato tubers.

Materials and Methods

Plant Material

The potatoes (cv. Atlantic) used in this study were grown at the USDA-ARS Research Farm in Weslaco, TX. Briefly, the potatoes for this study were grown from certified clean potato seed obtained from Black Gold Farms, Pearsall, TX. Potatoes were hand-planted in plots on 6 January 2009, and the plots were arranged in a randomized complete block design with six potato plants per plot and each treatment replicated four times. The potatoes used in this study were grown either under field conditions or controlled cage conditions. The design for cage-grown treatments consisted of caged plants without psyllids (controls), and caged plants with psyllids (ZC-affected tubers). At no time during this experiment were insecticides applied to the plants. All potatoes were harvested by hand on 20 May 2009 and immediately shipped to the USDA-ARS laboratory in Wapato, WA, for processing. For a complete and detailed description of the field experiment conducted in Weslaco, TX, refer to Munyaneza et al. (2008).

Ion Leakage Assay of Tuber Slices

Measuring ion leakage from plant tissue is a long-standing and well established method for determining cell death in plants (Lewosz and Lojkowska 1985; Mittler et al. 1999; Weber et al. 1996; Aviv et al. 2002; Epple et al. 2003). Cell death was assayed by measuring ion leakage from tuber tissue using protocols adapted from (De Weerd et al. 1995; Mittler et al. 1999). Twenty healthy (clean) tubers from ZC-free plants (nine from a potato field; 11 grown under cage conditions) and 34 tubers from ZC-affected plants (17 from a potato field; 17 under cage grown conditions) were hand-collected from individual plants (one tuber per plant) and stored at 10°C for two weeks before use. It is interesting to note that over the many years of working with ZC disease, we have not found a single tuber, which having been harvested from a ZC-affected plant, not to exhibit the characteristic flecking and stripped pattern indicative of ZC disease. We have, however, found a small number of tubers from ZC-affected plants that do not show any internal symptoms after the initial slicing, i.e., fresh cut. These seemingly "clean" tubers all show the characteristic flecking and striped pattern upon frying. All clean tubers were fry-tested according to Munyaneza et al. (2007a) to confirm that they were free of ZC symptoms. Tuber sizes ranged from 6.0–6.5 cm in diameter and were devoid of any obvious damage and morphological abnormalities. Tubers were hand-washed using deionized water immediately prior to tissue sampling to remove any remaining soil. Tissue was collected from each tuber by making a transverse section, using a No. 9 cork-borer, at the upper quarter (stem-end) of the tuber. Each core was removed, and the skin was carefully cut-away using a new razor blade. The remaining tissue was sectioned into a sample consisting of 60 (9×1-mm) slices. The slices were successively washed three times in deionized water, randomly divided into three sub-samples consisting of 20 slices each and finally placed separately into tubes each containing 25 ml of deionized water. These sub-samples were incubated at 25°C on a gyratory shaker at 100 rpm for 5 h. After incubation, the slices were removed from the solution and the conductivity (in micro-Siemens, μS) was measured using a Pinnacle series Check-Mite™ (model Duo-60; USA) conductivity meter. This conductance (μS) value is hereafter designated as value A. The potato slices were then returned to the bath solution, frozen overnight, and then thawed and boiled in a water bath for 25 min. After cooling to room temperature, the conductivity of each sub-sample was again measured; this is designated as value B. For each measurement, ion leakage was expressed as percent leakage, i.e., (value A/value B) ×100.

Percent ion leakage was compared between ZC-affected and ZC-free plants in cage-grown vs field-grown tubers using a 2×2 (affected status × tuber source) analysis of variance. In the event of a significant interaction between affected status and source, simple effects contrasts were extracted to compare ion leakage in ZC-free and ZC-affected tubers separately for field-grown and cage-grown

tubers. Analyses were done using PROC MIXED and the SLICE command (for simple effects) in SAS (SAS Institute 2003).

Vital Staining Using Trypan Blue

To establish whether the tuber symptoms (i.e., flecking and striping) found in fresh-cut tuber tissue from ZC-affected plants consists of dead and dying cells, staining of whole tuber slices with trypan blue was performed (Coder 1997; Aviv et al. 2002; Epple et al. 2003). Potato slices from tubers of ZC-affected and ZC-free plants were washed with deionized water for 2 min prior to staining with trypan blue. The slices were then incubated for 2 min in boiling lactophenol trypan blue (20 ml of ethanol, 10 ml of liquid phenol, 10 ml of lactic acid, 10 ml of water, and 10 mg of trypan blue), following the protocols of Koch and Slusarenko (1990). The stained slices were left in 70% ethanol for 1 h and then rinsed in deionized water. The images of the slices were captured through a digital scanner (EPSON Perfection 4490 digital scanner). All staining was performed in triplicate with similar results.

Fluorescence Microscopy

Potatoes were cut in half to determine disease status. Symptomatic tissue was carefully sectioned by hand and stained with a 1% aqueous solution of toluidine blue O for 5 min (to quench background autofluorescence of plant tissue). Excess stain was removed with deionized water. Images were taken with a Leica DM6000 B fluorescent microscope, using a L5 fluorescent filter cube (excitation wave length of 480 nm and emission wave length of 527 nm). Images were captured with a Leica DFC480 camera (Leica Microsystems Inc. IL USA).

Phloroglucinol Staining (Wiesner Reaction) for Lignin

In acidic conditions, phloroglucinol gives a red-pink product when it reacts with coniferyl and sinapyl aldehyde units in lignins (Sarkanen and Ludwig 1971; Nakono and Meshituka 1992). Cross sections (thickness 10–15 mm) were made from the upper quarter (stem end) of potatoes from both ZC-free and -affected plants. A modified version of the Wiesner reaction (Vermerris et al. 2002; Ruelle et al. 2007) was used. Briefly, the Wiesner reaction was performed on freshly cut potato tuber slices by first aliquoting three milliliters of a 2% phloroglucinol ethanol (70%) solution and allowing to incubate for 10 min, followed by a 10 min incubation in 25% HCl. Treated tuber slices were rinsed three times in distilled water and immediately photographed using a Nikon Coolpix S220 digital camera under natural light. A Nikon Coolpix 4500 digital camera mounted atop a Leica dissecting scope (model MZ6) using a Volpi light source (model NCL 150) was used for high magnification pictures. Three replicates were completed with similar results.

LR White Sample Preparation and Histochemical Staining with Toluidine Blue O and Periodic Acid-Schiff

Samples were taken from the upper quarter (stem end) of ZC-free and ZC-affected tubers and cut into 1×1, 1×2, or 1×3 mm pieces, prior to fixation with 3% glutaraldehyde, 0.06 M phosphate buffer for 1.5 h at room temperature. Samples were washed thrice (15 min each) with phosphate buffer, then dehydrated through an ethanol series (30, 50, 60, 70, 80, 95, 100%—3 times). Potato tissues were infiltrated with increasing concentrations of resin over a period of 5 days (ethanol (LR White, acrylic embedding resin) 3:1, 2:1, 1:1, 100%—3 times) prior to embedding and overnight curing at 72°C.

Toluidine blue O is a metachromatic dye used to identify cellular structures and polyphenolic substances such as lignins and tannins (O'Brien et al. 1964; Young and Ashford 1995; Ray and Hammerschmidt 1998). Histochemical staining using toluidine blue O was done in the following manner. Slides with 1–2 mm resin (LR White) sections (500–800 nm) were incubated in a solution of 1% toluidine blue and 1% borax for 1.5 min, heated, rinsed with water, allowed to air dry, and then covered with a coverslip. The periodic acid-Schiff (PAS) reaction is a method for histochemical detection of insoluble polysaccharides (starch) and glycoconjugates (Geier 1980, and references therein). Histochemical staining using periodic acid-Schiff (PAS) staining was adapted from the method of Ruzin (1999). Slides with resin sections (500–800 nm) were incubated in periodic acid for 30 min at room temperature followed by washing under running water for 2 min. Slides were then incubated in Schiff's reagent for 1 h, rinsed in distilled water at room temperature for 10 s and allowed to air dry, followed by addition of a coverslip and immersion oil.

Field Emission Scanning Electron Microscopy (FE-SEM)

Field emission scanning electron microscopy was selected for this study based on its ability to image biological samples in their natural, hydrated state without the problems of conventional SEM techniques, which include sample shrinkage and distortion, the loss of soluble material, and mechanical damage. Using a clean razor blade, multiple triangular-shaped, transverse sections were taken from the upper quarter (stem end) of tubers from both ZC-free and ZC-affected plants. The microstructure of the tissue samples was examined using field-emission scanning

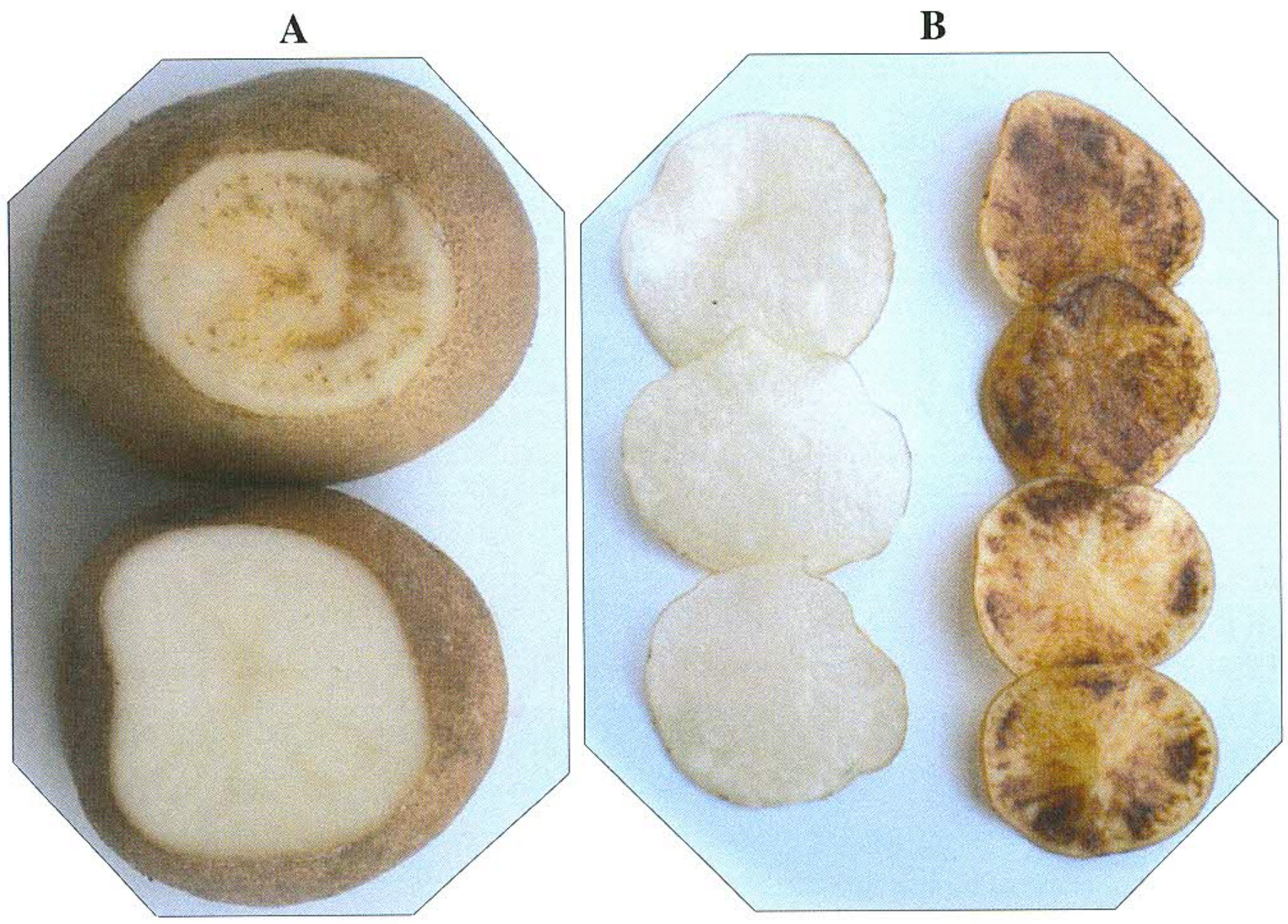

Fig. 1 Potato tubers with characteristic symptoms of ZC disease exhibiting "necrotic-like flecking" concomitant with browning and streaking of medullary ray tissue throughout the tuber (**a**), and (**b**), potato chips processed from ZC-affected tubers. The top two and bottom two chips in (**b**, right) are from two different tubers, respectively. Discoloration of both inner- and outer-medullary tissues, the former associated with potato eye, are clearly evident in the ZC tuber (**a**). ZC-free tubers are shown alongside ZC-affected tubers

electron microscope (FE-SEM) (FEI, Quanta 200 F, Netherlands). The following standard instrument settings were used for all samples: HV, 20.00 kV; spot, 3.0; det, LFD; pressure, ~120 Pa, and a scan time of 30 μs.

Results and Discussion

As mentioned above, ZC is a destructive disease that is causing losses in the millions of dollars to the potato industry by impacting both potato yield and marketability of processed chips (Goolsby et al. 2007a, b; Munyaneza et al. 2007a, b, 2008). The internal symptoms found in raw tubers from ZC-affected plants include vascular tissue browning, medullary ray discoloration, and "necrotic-like flecking" (Munyaneza et al. 2007a, b) (Fig. 1). Upon frying, ZC-affected tubers develop a characteristic striped "Zebra" pattern of discoloration rendering them unmarketable (Fig. 1).

Ion Leakage

In the present study, cell death was initially quantified by monitoring cellular ion leakage (conductance) in tuber tissue from both ZC-free and -affected plants (Fig. 2). Levels of ion leakage in ZC-affected vs ZC-free tubers depended in part on source of the plants, as shown by a significant infection status × source interaction (Fig. 2; $F_{1,50}$=8.6, P=0.005). Because of the significant interaction, we examined tests on simple effects. Levels of ion leakage were significantly higher in tubers from ZC-affected plants than tubers from ZC-free plants in both cage-grown ($F_{1,50}$= 52.6, P<0.0001) and field-grown ($F_{1,50}$=7.8, P<0.007) plants. Ion leakage was similar in clean tubers, irrespective of source ($F_{1,50}$=0.2, P=0.68). Conversely, source did significantly affect ion leakage in ZC-affected plants, with those grown in cages exhibiting a significantly higher mean percent ion leakage than shown by tubers from the field source ($F_{1,50}$=18.3, P<0.0001). This latter difference in percent ion leakage may have been due to a greater psyllid pressure existing in the caged experiment compared with that in the field experiment, as the level of ZC incidence in

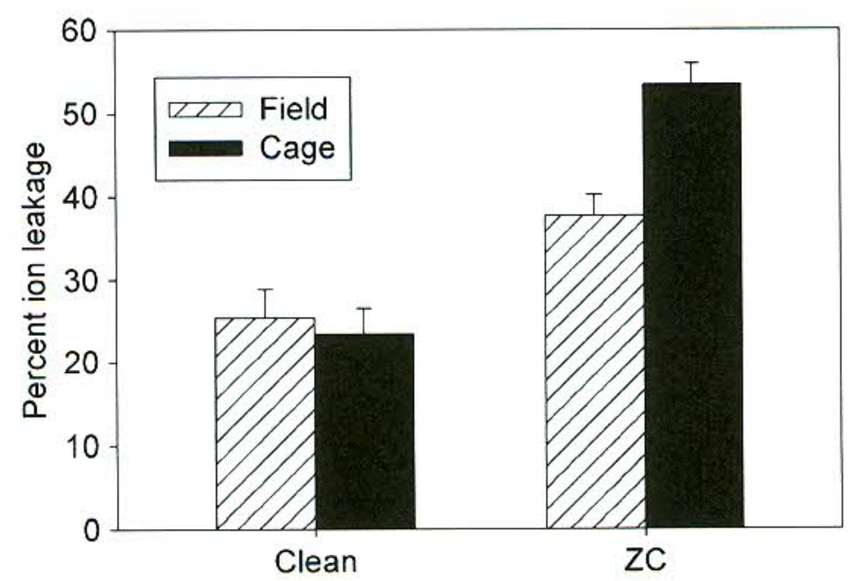

Fig. 2 Cell death measured as mean percent (± SEM) ion leakage in tubers from both ZC-affected and free plants. The graph shows enhanced cell death between tubers from ZC-affected plants compared to tubers from ZC-free plants from field and cage sources. For each measurement, ion leakage was expressed as percent leakage, i.e., [(value A/value B) ×100], where value A is the conductance (μS) of the bathing solution at 5 h and value B is the conductance (final) after overnight freezing followed by boiling for 25 min. Each sample value is the mean of the three sub-samples

field-grown tubers was relatively light compared to levels seen in previous years (Munyaneza, unpublished data).

Although no statistical tests were performed to correlate mean ion leakage (cell death) to tuber tissue discoloration, the latter has been used as a qualitative measure of disease severity. In the present study, there was no correlation between the degree of discoloration and ion leakage. That is, a small number of tubers displaying heavily pigmented tuber tissue had lower levels of ion leakage compared to tubers with lighter pigmentation.

Trypan Blue Staining

Trypan blue is a vital stain which is frequently used to selectively identify dead and dying tissues and cells, which stain blue due to loss of membrane integrity. Figure 3 (b–c) clearly show an increased uptake of trypan blue throughout the tuber from the ZC-affected plant compared to the tuber from the ZC-free plant (Fig. 3a), indicating the presence of cell death. The potato slice on the left in (Fig. 3b) was cut from the tuber slice on the right showing/exhibiting the dark blue "halo" surrounding intense brown, fibrous tissue regions (Fig. 3c). In ZC-free tuber slices light blue staining was evenly distributed across the tuber face as a result of damage incurred during tuber cutting (Fig. 3a).

Autofluorescence and Histochemical Staining of Lignified Tissues in the Vasculature of ZC-affected Tubers

As stated above, there are a number of molecular, physiological and morphological changes that have been shown to occur coincident with the rapid cell death

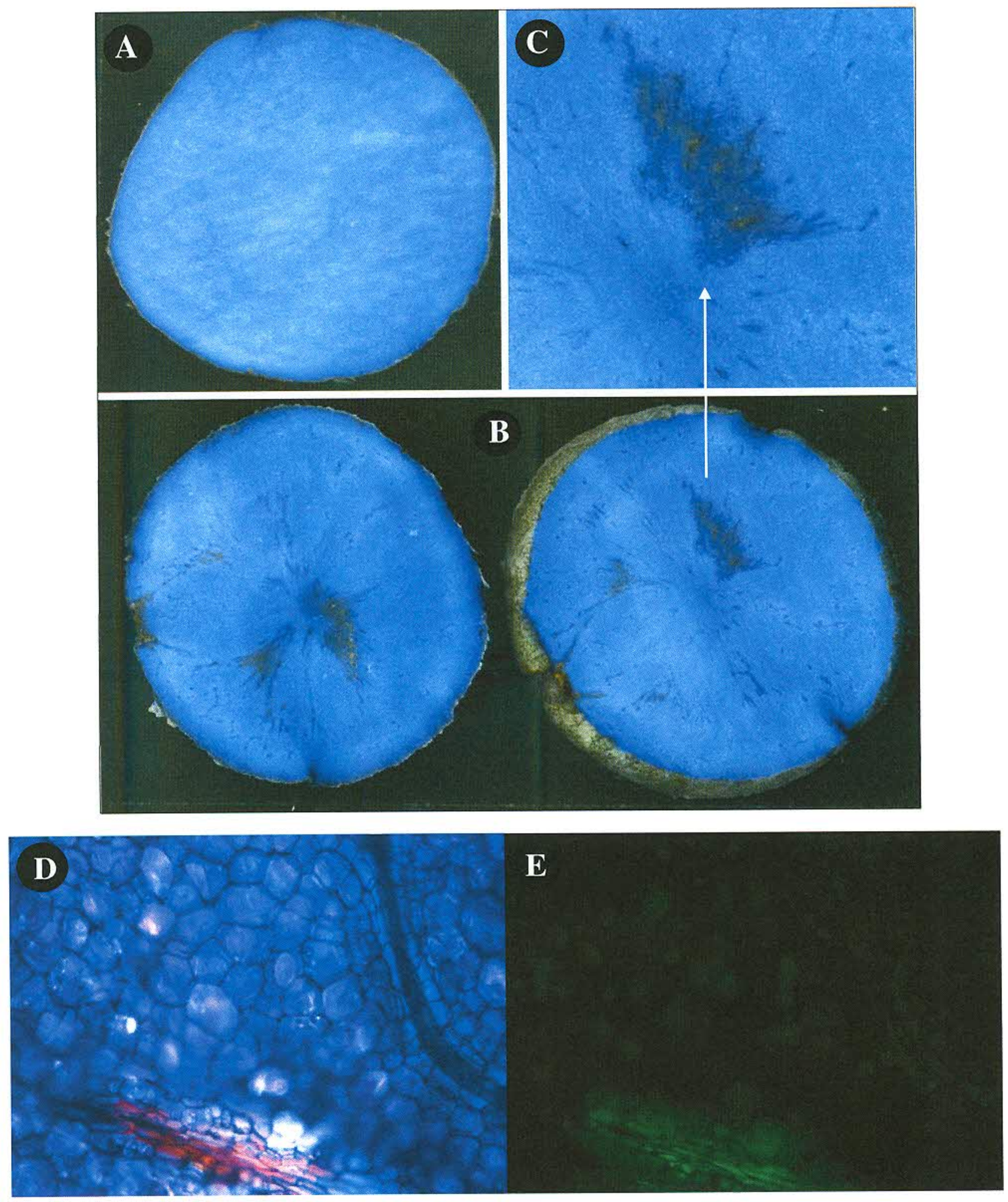

Fig. 3 Vital staining using trypan blue of tubers from both ZC-free and -affected plants. Trypan blue staining of potato tuber tissue from a ZC-affected plant (**b, c**) shows an increased uptake of trypan blue throughout the tuber, compared to tuber tissue from a ZC-free plant (**a**), indicating ZC-associated cell death in the tuber tissue from ZC-affected plants. In (**b**), the potato slice on the left was cut from the tuber slice on the right. Notice the dark blue "halo" surrounding a heavily "necrotic" region in (**c**). All experiments were completed in triplicate with similar results. Toluidine blue-stained (**d**) tuber tissue from a ZC-affected plant shows both healthy (*blue*) and discolored (*reddish-brown*) vascular bundles. The same tissue sample (**e**) shows natural autofluorescence (*green*) in and around the discolored tissue of the vascular bundle

component of the hypersensitive response in plants. One such response is the deposition of autofluorogenic phenolic compounds, including lignin and lignin-like species, at and around the site of pathogen interaction. These autofluorogenic phenolic compounds can be identified by various techniques including autofluorescence microscopy and histochemical staining, making them a useful biomarker of hypersensitive response to pathogen infection.

Tuber tissue (Fig. 3d) from a ZC-affected plant shows both healthy and discolored vascular bundles. Toluidine blue O was used to both show general cellular architecture and to quench background autofluorescence of phenolic materials in the tuber tissue. Figure 3(e) is the same tissue sample (Fig. 3d) showing autofluorescence at and around the vascular lesion, whereas, the vascular bundle in healthy tuber tissue (Fig. 3d, upper right) has no detectable autofluorescence. Autofluorescence found in the cell walls, the vascular lesion in (Fig. 3d), has been associated with resistance (HR) to pathogens, whereas, accumulation of phenolics is suppressed in non-resistance plant-pathogen interactions (reviewed in, Vidhyasekaran 2008).

To determine the extent of lignification in tubers from ZC-affected plants, the phloroglucinol-HCL (Wiesner reaction) method (Vermerris et al. 2002; Ruelle et al. 2007) was

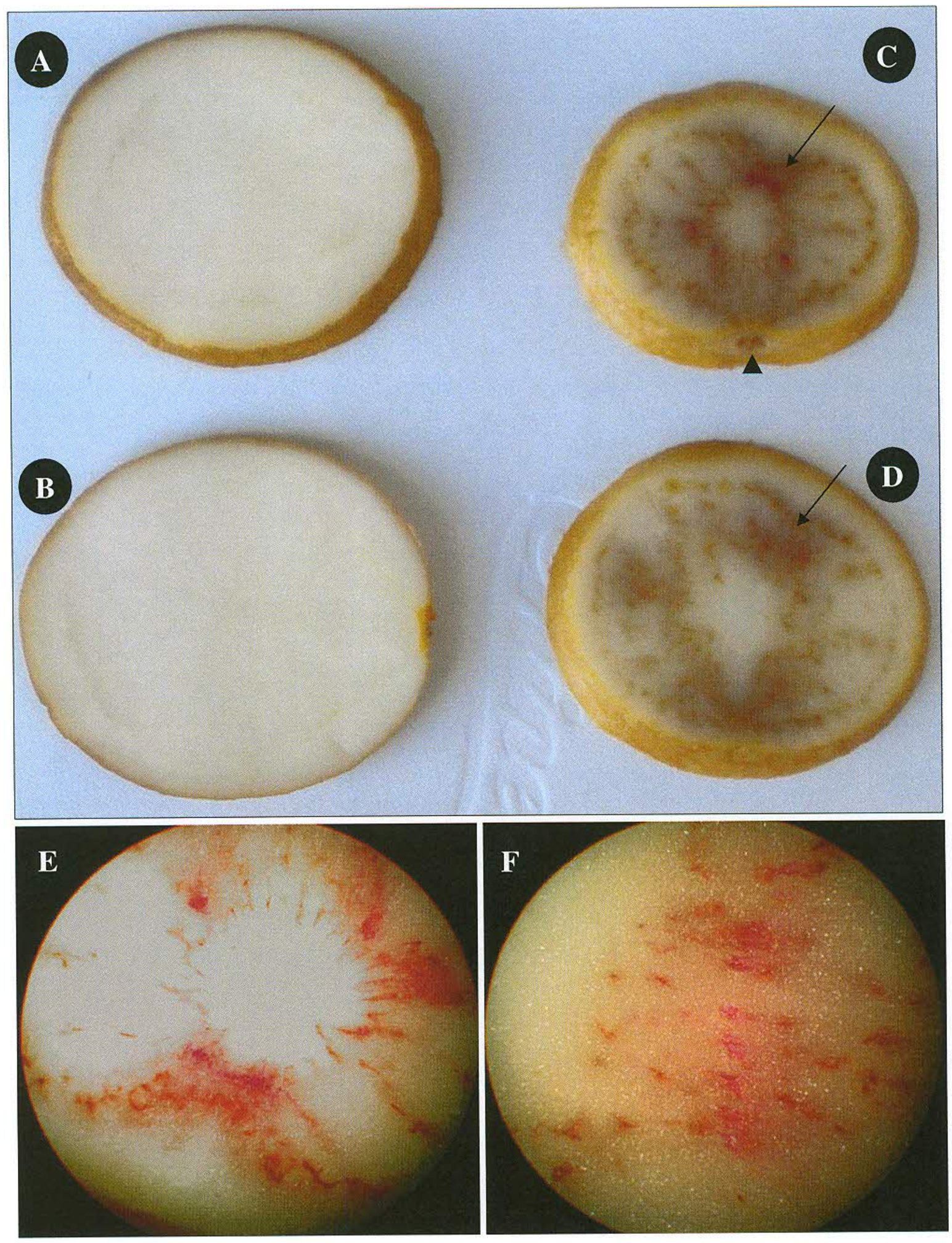

Fig. 4 Phloroglucinol staining of tuber tissue from both ZC-affected and -free plants for lignin. Transverse sections from the upper quarter (stem-end) of tubers from both ZC-free and -affected plants after Wiesner reaction. In tuber tissue from a ZC-free plant, there is no detectable staining (**a** and **b**), whereas, reddish-pink staining of lignified tissue is clearly visible at and around the vascular ring and throughout the medullar region (**c** and **d**) of tuber tissue from ZC-affected plant. Close-up images of areas of the ZC-affected tuber slices indicated by *arrows* in (**c** and **d**) are shown in (**e** and **f**), respectively. The arrow head (**c**) identifies phloroglucinol staining of a potato eye in the tuber from the ZC-affected plant. All experiments were completed in triplicate with similar results

employed. In tuber tissue from ZC-free plants (Fig. 4a–b), there was no detectable staining, whereas reddish-pink staining is readily apparent in the vascular ring and medullar region of tuber tissue from the ZC-affected plant (Fig. 4c–d). Magnified images of the tuber tissue from ZC-affected plants (Fig. 4c–d, see arrows) are shown in Figs. 4e and f, respectively. These high magnification pictures clearly show deposition of lignified materials in and around the vascular regions of the ZC-affected tuber. There is also detectable phloroglucinol staining of the vascular tissue associated with the potato eye in the tuber from the ZC-affected plant (Fig. 4c, arrow head). All experiments were completed in triplicate with similar results.

Histochemical Staining of Tuber Tissue from ZC-affected and ZC-free Plants Using Toluidine Blue O and Periodic Acid-Schiff (PAS)

Toluidine blue O staining of tuber tissue from ZC-free plants (Fig. 5a) shows healthy vasculature, whereas tuber tissue from ZC-affected plants (Fig. 5b–c) is decorated with numerous lesions. The tuber from the ZC-affected plant cell walls and extracellular matrix primarily associated with the vasculature stained dark blue to pale blue, indicating the presence of polyphenolics. While parenchymatous tissues in both ZC-free (Fig. 5a) and ZC-affected tubers (Fig. 5b) show a purplish color, which is indicative of pectin-

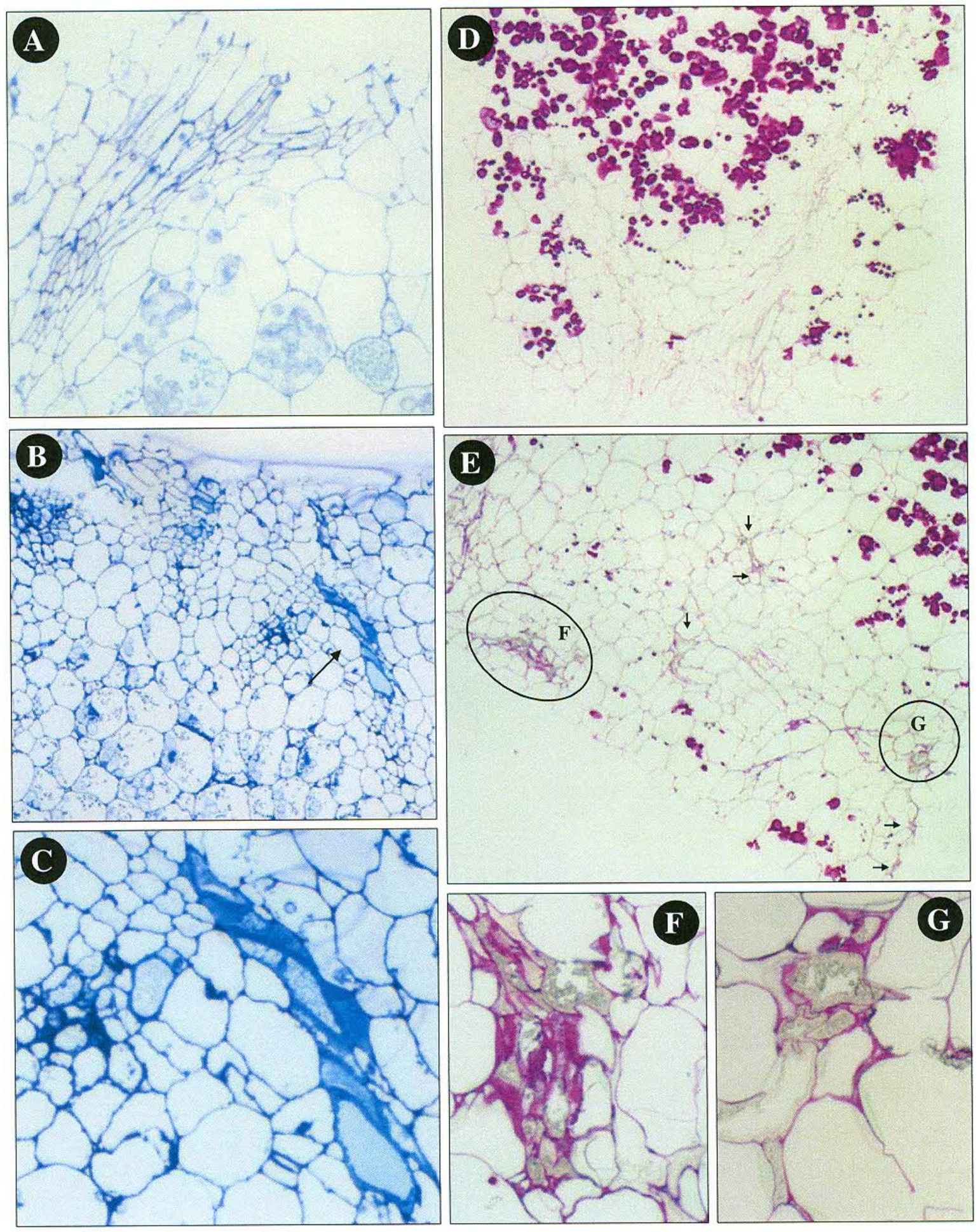

Fig. 5 Histochemical staining of tuber tissue collected from both ZC-affected and ZC-free plants. Toluidine blue staining (**a–c**) of tuber tissue collected from a ZC-free plant (**a**), with healthy vasculature, and tuber tissue from a ZC-affected plant (**b** and **c**) showing numerous lesions. Image (**c**) is a higher magnification of the large lesion (*arrow*) in image (**b**). Periodic acid-Schiff (PAS) staining (**d–g**) of tuber tissue from ZC-free (**d**) and ZC-affected (**e**) plants shows a large number of starch granules in the tuber from the ZC-free plant, whereas, the number of starch granules in the tuber from the ZC-affected plant is greatly reduced. Healthy, lesion-free vasculature can be seen in the ZC-free tuber tissue (**d**), while numerous lesions (indicated by *arrows*) are easily visible throughout the tuber from the ZC-affected plant (**e**). Two large lesions identified by the letters (**f** and **g**, *circles*), are shown at higher magnification and show intense PAS staining in and around these lesions

containing primary cell walls. Figure 5c is a high magnification version of the large lesion (arrow) found in Fig. 5b, and shows heavy deposition of polyphenolic materials.

Periodic acid-Schiff staining of tuber tissue from ZC-free (Fig. 5d) and ZC-affected (Fig. 5e) plants shows a large number of starch granules in the tuber tissue from ZC-free plants, whereas the number of starch granules in the tuber tissue from ZC-affected plants is greatly reduced. Both the degradation and loss of starch, most notably at and around the site of infection, has been observed in potato tubers infected with any of a number of pathogens (Blaszczak et al. 2005). Healthy, lesion-free vasculature can be seen in the tuber tissue from ZC-free plants (Fig. 5d), while numerous lesions are easily visible throughout the tuber from ZC-affected plants (Fig. 5e). Both photographed areas (Fig. 5d and e) are 2×2 mm in area. There are at least 10 lesions visible in (Fig. 5e), see arrows. Two large lesions, identified (by the letters F and G in Fig. 5e) are also shown at high magnification (Fig. 5f–g). Note also the heavy PAS staining in and around these lesions, which appears to have an ordered deposition throughout the lesion. The lesions contain what appear to be cellular materials, which are unstained. All experiments were completed in triplicate with similar results.

Field Emission Scanning Electron Microscopy (FE-SEM) of Potato Tubers from ZC-Free and ZC-affected Plants

Field emission scanning electron microscopy of transverse sections from the upper quarter (stem-end) of tubers from both ZC-free and affected plants are shown in Fig. 6a–b), respectively. Vasculature in the potato tuber from the ZC-free plant (Fig. 6a) is free of lesions (oval) and storage parenchyma cells are full of starch granules, whereas numerous lesions (circles) are visible throughout the tuber tissue from the ZC-affected plant (Fig. 6b). These lesions are associated with vascular tissues of the potato tuber. Interestingly, the storage parenchyma cells are nearly devoid of starch in the tuber from the ZC-affected plant, which is consistent with the PAS staining results (Fig. 5e) and the results of Gao et al. (2009). Other than the dead cells associated with the lesions in the tubers from ZC-affected plants, and the reduction in the amount and average size of the starch granules, there were no additional differences noted. High magnification images of lesions in ZC-affected tubers identified in Fig. 6 are shown in Fig. 7 (a, c, e). Micrographs (Fig. 7b and d) show higher magnifications of Fig. 7a and c), respectively. Micrograph (Fig. 7f) is a representative vascular bundle, designated by (*) from the ZC-free tuber shown in Fig. 6a. The arrows in Fig. 6 indicate direction of skin.

As mentioned above, the internal discoloration observed in tubers from ZC-affected plants has been largely attributed to elevated levels of reducing sugars, phenolics or general tissue necrosis. The discoloration of the fried potato products is determined to a large extent by the reducing sugar content in the potatoes. This non-enzymatic dark color of fried potato products results from the Maillard reaction between amine groups of free amino acids and reducing sugars (Schallenberger et al. 1959; Feather 1984) leading to the production of pigmented melanoidins (Hodge et al. 1972). Elevated levels of reducing sugars concomitant with tissue browning have been associated with storage at low temperatures as well as numerous diseases of potato. The elevated level of reducing sugars (glucose) in tubers from ZC-affected plants (Gao et al. 2009) likely contributes to the overall brown discoloration via the Maillard reaction. Although deposits of starch were observed within a number of lesions in ZC-affected tubers (Fig. 5f and g), additional

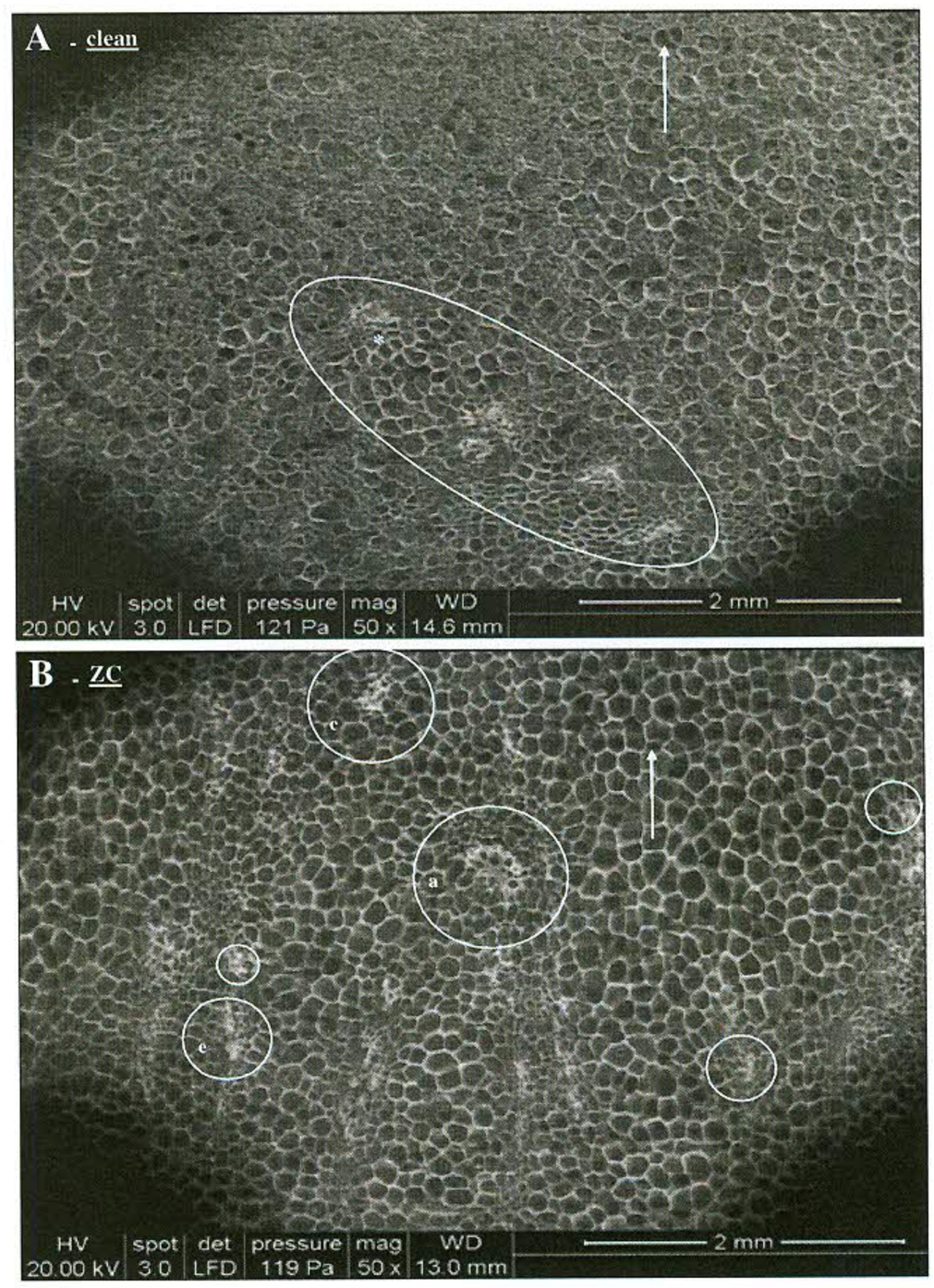

Fig. 6 High-resolution field emission scanning electron microscopy (FE-SEM) of transverse sections from the upper quarter (stem-end) of tubers from both ZC-free (**a**) and -affected (**b**) plants. The vasculature in the potato tuber from the ZC-free plant (**a**) is healthy and otherwise free of lesions (*oval*) and storage parenchyma cells are full of starch granules, whereas, numerous lesions (*circles*) are visible throughout the tuber from the ZC-affected plant (**b**) and are associated with vascular tissues. Interestingly, the storage parenchyma cells are nearly devoid of starch in the tuber from the ZC-affected plant. *Arrows* indicate direction of skin

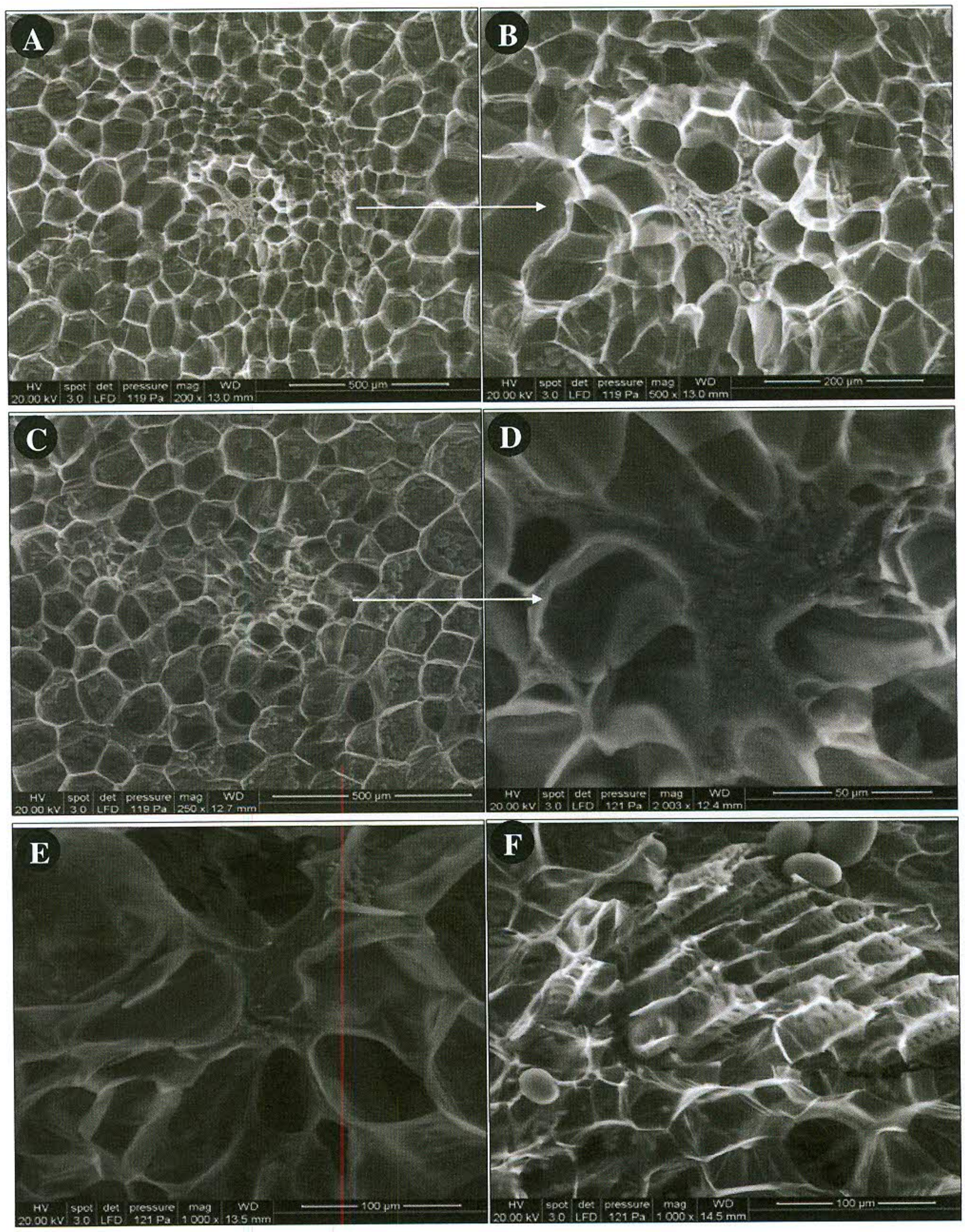

Fig. 7 High-resolution field emission scanning electron microscopy (FE-SEM) showing lesions associated with the vasculature in potato tuber tissue from ZC-affected plants. Micrographs (**a**, **c**, and **e**) are high magnification images of lesions identified in Fig. 6. Micrographs (**b** and **d**) are higher magnifications of (**a** and **c**), respectively. Micrograph **f** is a representative vascular bundle, designated by (*), from the ZC-free tuber shown in Fig. 6

work is needed to determine whether this altered sugar content contributes in any way to the intensity or spatiality of characteristic striping and/or "necrotic flecking" found in tubers from ZC-affected plants.

It is likely that increased accumulation of polymeric polyphenolics (pigmented compounds), in association with lesions, are responsible for the characteristic flecking and dark striping in medullary ray tissue in tubers from ZC-affected plants. Navarre et al. (2009) reported that tubers from ZC-affected plants show a marked increase in aromatic amino acids, namely tyrosine, and other phenolic compounds, which are substrates for polyphenol oxidase enzymes (PPOs). Through a series of oxidation and condensation reactions, polymeric polyphenolic compounds (pigmented species such as melanins), are produced and deposited at and around the site of infection (reviewed in Friedman 1997). The observation that heavy deposits of polyphenolic compounds were localized at and around the lesions in tubers from ZC-affected plants in this study suggest a direct role for these pigmented compounds in the flecking and striping patterns found in tubers from ZC-affected plants. It is possible that when chips from ZC-affected tubers are fried, deposits of pigmented compounds, which are associated with the vascular lesions, "bleed" together resulting in both enlarged flecking and medullary striping patterns characteristic of ZC disease. In addition to being a substrate for PPOs, tyrosine may also contribute to the overall browning by reacting with free

glucose (and other reducing sugars) during the Maillard reaction. Additional work is needed to determine the overall contribution that reducing sugars, polymeric polyphenolic compounds, and cell death play in the tuber discoloration found in ZC-affected tubers.

The data presented in this study clearly establish that ZC disease involves cell death in potato tubers and that this cell death is manifested as numerous, small, irregularly-shaped lesions which are associated with the tuber vasculature. The data also show heavy deposition of polyphenolic (e.g., lignin) compounds at and around the lesions, as well as an overall reduction in the level of starch, in tubers from ZC-affected plants compared to tubers from ZC-free plants. These findings, as well as the work by Navarre et al. (2009) showing elevated levels of aromatic amino acids, tyrosine and other phenolics as well as salicylic acid, a key regulator of plant defenses, strongly suggest that ZC disease induces a hypersensitive response leading to PCD in potato tubers.

It is evident that PCD is an integral part of many aspects of the plant's life including, defense, development, and response stress but it is not clear how many types of PCD occur and what their relationship may be. There is an increasing amount of plant PCD research in the scientific literature most of which simply refers to cell death without any attempt to define the type of cell death under investigation. This is most notably the case with the use of "necrosis" which is a catch-all term used to describe plant cell death. This is partly due to the fact that rigorous definitions of plant cell death mechanisms are limited in number, and the use of mammalian-based definitions to explain plant cell death. This practice has led to some confusion in terms and, in many cases, overly precise dichotomies such as between apoptosis and necrosis, as well as other types of cell death (reviewed in Lockshin and Zakeri 2004). Although PCD induced by an HR response is likely the main type of cell death occurring in ZC-affected tubers, this does not preclude the possibility of necrosis also occurring. An important aspect to consider is that cell death can be influenced by the level, timing and duration of the stress (reviewed in Reape et al. 2008), and PCD is highly susceptible to environmental conditions, such as thermal stress.

Elucidation of the precise underlying molecular and biochemical mechanisms which are involved in ZC-associated cell death in potato tubers, as well as in the aerial tissues (which is outside the scope of this paper) is fundamentally essential to the overall understanding of this disease, as well as other potato-related diseases where cell death occurs, and may lead to a number of insights with respect to general plant-pathogen interactions.

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