RESEARCH ARTICLE

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Visualization of extracellular DNA released during border cell separation from the root cap[1]

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PREMISE OF THE STUDY: Root border cells are programmed to separate from the root cap as it penetrates the soil environment, where the cells actively secrete >100 extracellular proteins into the surrounding mucilage. The detached cells function in defense of the root tip by an extracellular trapping process that also requires DNA, as in mammalian white blood cells. Trapping in animals and plants is reversed by treatment with DNase, which results in increased infection. The goal of this study was to evaluate the role of DNA in the structural integrity of extracellular structures released as border cells disperse from the root tip upon contact with water.

METHODS: DNA stains including crystal violet, toluidine blue, Hoechst 33342, DAPI, and SYTOX green were added to root tips to visualize the extracellular mucilage as it absorbed water and border cell populations dispersed. DNase I was used to assess structural changes occurring when extracellular DNA was degraded.

KEY RESULTS: Complex masses associated with living border cells were immediately evident in response to each stain, including those that are specific for DNA. Treating with DNase I dramatically altered the appearance of the extracellular structures and their association with border cells. No extracellular DNA was found in association with border cells killed by freezing or high-speed centrifugation. This observation is consistent with the hypothesis that, as with border cell extracellular proteins, DNA is secreted by living cells.

CONCLUSION: DNA is an integral component of border cell extracellular traps.

KEY WORDS corn; extracellular traps; exDNA; Fabaceae; pea; *Pisum sativum*; Poaceae; rhizosphere; root border cells; root caps; *Zea mays*

> "*One can find in various texts the statement that the root-cap cells of plants die and are sloughed off, and it is probably the general opinion among botanists that the root-cap cells are either dead when they are sloughed off or that they die soon thereafter…. That the root-cap cells, when sloughed off, are not necessarily dead or short-lived but may persist for many days, seems to be substantiated by various observations made by the writer with a number of different plants.*" —L. Knudson, 1919, *American Journal of Botany* 6: 309–310.

Most plant species are programmed to synthesize and deliver populations of metabolically active cells from the root tip into the soil environment (Knudson, 1919; Hawes and Pueppke, 1986; Hawes et al., 1998; Hawes et al., 2016a, b). Gene expression patterns in these root "border" cells are distinct from progenitor cells in the root cap, and the cells actively export a complex matrix that includes >100 extracellular proteins (Brigham et al., 1995, 1998; Knox et al., 2007). The fact that the extracellular protein delivery is an active process was confirmed by the observation that no measurable release of proteins occurs when border cells are treated with a secretion inhibitor or are killed by freezing or high speed centrifugation (Wen et al., 2007). The surprising discovery that histone is among the secreted proteins led to recognition of a parallel phenomenon in animal systems: In 2004, the Zychlinsky laboratory reported that neutrophils export histone-linked extracellular DNA (exDNA) as part of complex structures that immobilize pathogens and inhibit infection (Brinkmann et al., 2004). Treating these "neutrophil extracellular traps" (NETs) with DNase reverses trapping, and virulence is reduced in pathogens with reduced extracellular DNase (exDNase) activity (Buchanan et al., 2006; Brinkmann and Zychlinsky, 2012; Nasser et al., 2014). Subsequent studies revealed

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for the first time that exDNA also is a component of the border cell extracellular matrix (Wen et al., 2009a). Degrading the exDNA by adding DNase at the time of inoculation with a fungal pathogen results in 100% loss of root tip resistance to infection (Hawes et al., 2011, 2012, 2016b). DNase also reverses trapping of bacteria by border cells (Curlango-Rivera et al., 2013; Tran et al., 2016). As in animal pathogens, including Group A *Streptococcus* (Buchanan et al., 2006), knockout mutations of secreted DNases in the plant pathogen *Ralstonia solanacearum* results in reduced virulence and systemic spread through the host (Tran et al., 2016).

Knudson (1919) illustrated a key point about the "sloughed root-cap cells" from pea and corn grown in hydroponic culture: Their production and dispersal are so dynamic that, unless care is taken to detect them, it is easy to overlook their presence, let alone their distinctive characteristics. Only when he examined material that had collected at the base of his flasks did he understand the magnitude of the cell delivery, and the longevity of their survival: Based both on cell structure and on the ability to undergo reversible plasmolysis, the cells remained 100% viable for weeks. One complicating factor makes it especially easy to miss seeing the cells in vitro or in situ: The mucilage surrounding border cells can hold 1000× its weight in water, but even at 98% humidity or more no water absorption occurs (Guinel and McCully, 1986, 1987; Odell et al., 2008). Therefore, the cell populations remain tightly appressed to the root cap surface with no obvious evidence of their presence (Fig. 1A). Upon immersion of the root tip into water, water uptake into the mucilage occurs immediately (Fig. 1B), and border cell separation begins in seconds (Fig. 1B). Within minutes, the entire border cell population is dispersed into the liquid, again leaving the root tip surface smooth (Fig. 1C). The detached cells have a prominent nucleus and >1 μm-wide lignified cell walls (Fig. 1C, inset), and retain functional plasmodesmata (Zhang et al., 1995). The use of cytoplasmic streaming (Hawes and Pueppke, 1986) or uptake of the vital stain fluorescein diacetate, which only accumulates inside living cells with an intact plasma membrane, confirmed Knudson's (1919) report that the cell populations are viable (Fig. 1D).

Even easier to miss than the border cells per se is the presence of exDNA among the polysaccharides, proteins, and other components of the root cap "slime" that has been characterized in many elegant studies describing in detail this natural product of plant roots (reviewed by Chaboud and Rougier, 1990; Hawes et al., 1998, 2003; Hamamoto et al., 2006; Jones et al., 2009; Kabouw et al., 2012; Lynch and Whipps, 1990). Even when DNA was detected, the long-standing dogma that sloughed root cap cells were dead on arrival led to the reasonable presumption that the exDNA was leaking from broken cells (e.g., Voeller et al., 1964; Esau, 1967; Levy-Booth et al., 2007). As in mammalian systems, the presence of DNA outside the cell was presumed to be a byproduct of necrotic cells before it was recognized as a central player in the immune system (Wen et al., 2009a; Hawes et al., 2012, 2015). Recognition of the importance of understanding the structural dynamics of NETs is reflected in numerous emerging studies focused on imaging exDNA in mammalian systems (e.g., Brinkmann et al., 2010; De Buhr and von Köckritz-Blickwede, 2016; Kraaij et al., 2016; Masuda et al., 2016; Naccache and Fernandes, 2016; Sil et al., 2016). The goal of the current study is to visualize for the first time the dynamics of plant exDNA delivery as living border cell populations separate from the root caps of pea and corn, the same model species that Knudson (1917, 1919) employed a century ago.

MATERIALS AND METHODS

Plant materials—*Pisum sativum* L. seeds (cv. Little Marvel; Meyer Seed Co., Baltimore, Maryland, USA) were immersed for 10 min in 95% (v/v) ethanol followed by 60 min in 6.15% sodium hypochlorite (Wen et al., 1999). Seeds that floated to the surface during handling were discarded. The remaining seeds were rinsed 5 times with sterile double-distilled water (ddH$_2$O), followed by a 6-h imbibition in sterile ddH$_2$O. *Zea mays* L. seeds (Golden Bantam; Burpee Seed Co., Warminster, Pennsylvania, USA) were immersed in 95% ethanol for 10 min, followed by 10 min in 6.15% sodium hypochlorite,

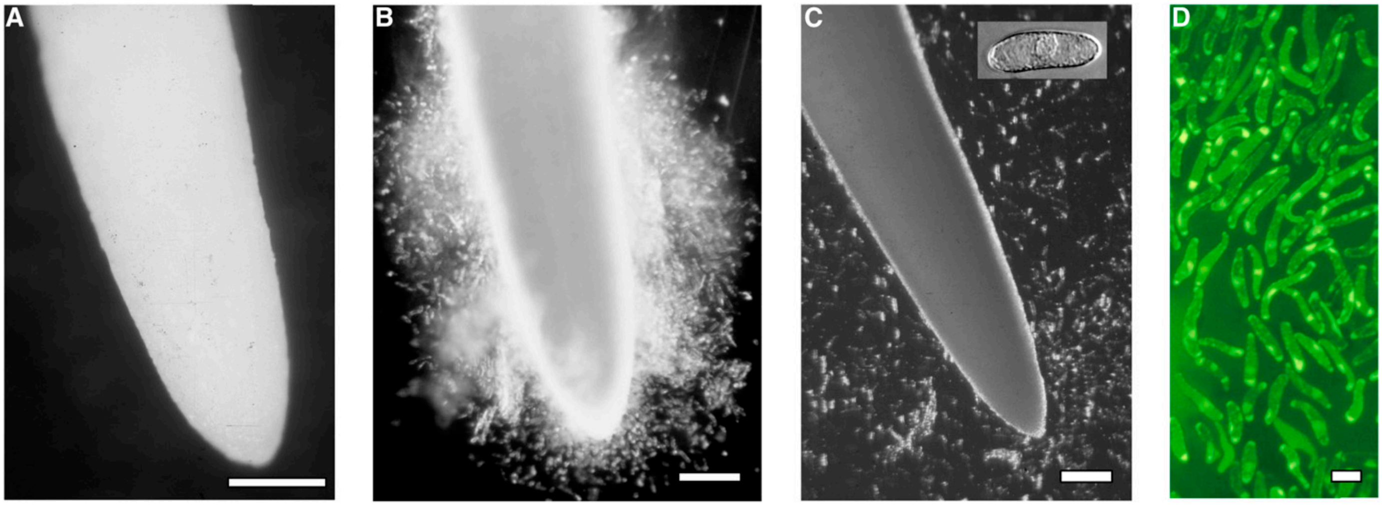

FIGURE 1 Border cell appearance on *Pisum sativum* root tips (A) maintained at 99% humidity in the absence of free water; (B) after immersion in water for 30–60 s; and (C) after dispersal of the cell population by gentle agitation of the water. Individual cells (inset) have a prominent nucleus and lignified cell walls >1 μm in diameter. Size markers = 1 mm. (D) Viability of the cells revealed by staining with fluorescein diacetate, which accumulates only within living cells with an intact plasma membrane. Scale bar = 25 μm.

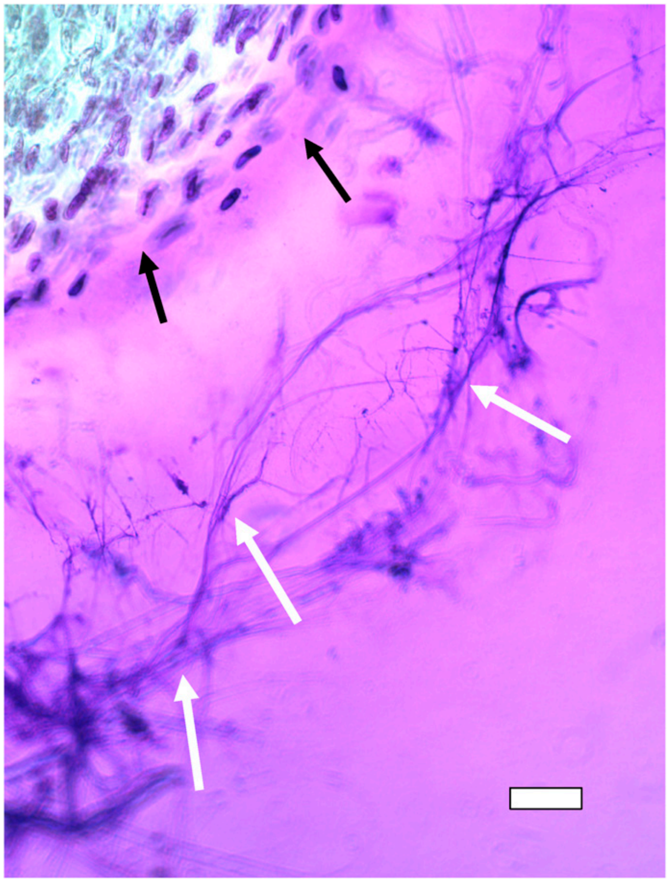

FIGURE 2 Intertwined strands (white arrows) at the edge of the extracellular matrix surrounding border cell populations at the root cap periphery (black arrows) are revealed by staining with crystal violet. Scale bar = 50 μM.

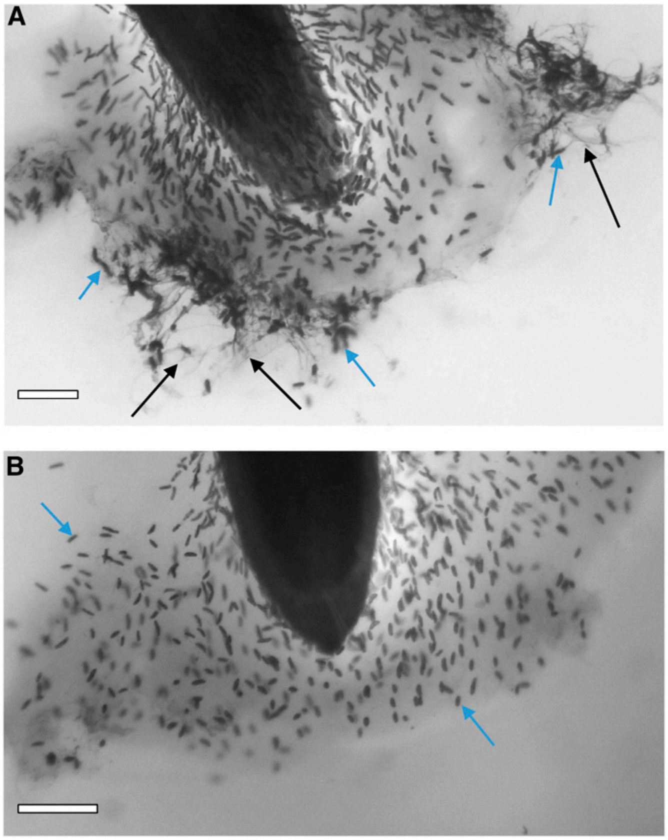

FIGURE 3 (A) Root tips of *Zea mays* immersed in water and stained with crystal violet reveals strands (black arrows) entangled with border cells (blue arrows); and (B) dissolution of strands and dispersal of border cells (blue arrows) after treatment with DNase I. Scale bars = 1 mm.

then rinsed 5× in ddH$_2$O, and imbibed for 1 h in sterile ddH$_2$0. Imbibed seeds were maintained at 99% humidity during the process of germination, by placing onto 1% agar (Bacto TM Agar, Becton Dickinson and Co., Baltimore, Maryland, USA) overlaid with sterile germination paper. Contaminated seeds were discarded. Border cell viability was measured based on cytoplasmic streaming (Hawes and Pueppke, 1987) and/or uptake of fluorescein diacetate (Sigma-Aldrich, St. Louis, Missouri, USA).

Histochemical staining—Crystal violet solution was made by dissolving 0.50 mg of crystal violet powder (Sigma-Aldrich) in 8 mL of ddH$_2$O, then adding 2 mL of methanol and mixing well. Root tips

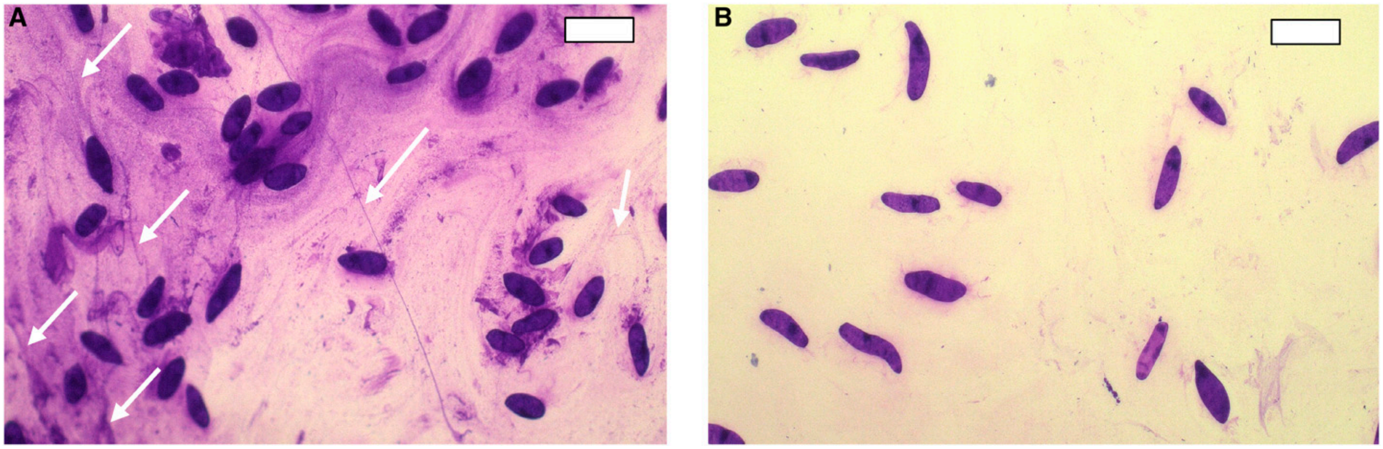

FIGURE 4 (A) Use of crystal violet to visualize strands (arrows) and other matrix components of border cells of *Zea mays* after root tip immersion into water followed by gentle agitation to disperse border cells; and (B) after adding DNase I. Scale bars = 40 μm.

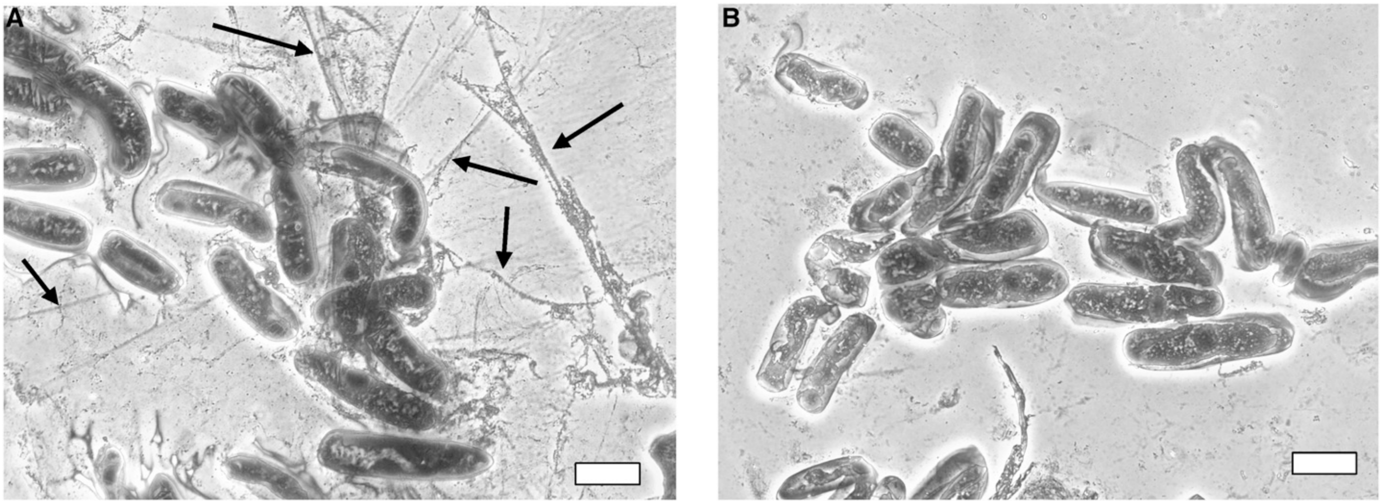

FIGURE 5 (A) Use of crystal violet to detect extracellular strands (arrows) after incubation of border cells of *Pisum sativum* in water for 2 h; and (B) disappearance of most strands after treatment with DNase I. Scale bars = 10 μm.

from roots 15–20 mm long were used; at least five replicates in three independent experiments were used for each assay. A single root tip was immersed in 50 μL of water or water containing 1 U of DNase 1 (Worthington Biochemical, Lakewood, New Jersey, USA) for 1.5 h. The root tip was then removed and placed into a 30-μL drop of water on a microscope slide. Within 10–20 s, 2 μL of 0.5% crystal violet was added and mixed gently. Within 1–3 min, the stained root tip was examined using an Olympus BX60 light microscope (Olympus, Tokyo, Japan). Border cell samples were dispersed from root tips in water or DNase I solution and placed on a microscope slide before adding 1 μL crystal violet. Images were captured using a Leica DFC290 HD digital camera with Leica LAS EZ software V4.0.0 (Leica Microsystems, Wetzlar, Germany).

A solution of 0.05% toluidine blue O (5 mg/100 mL ddH$_2$O; Sigma-Aldrich) was used as a stock solution. Root tips were removed from agar plates and immersed in 50 μL ddH$_2$O samples with or without DNase I and mixed with 2 μL of toluidine blue stock solution. Control samples included comparable mixtures of toluidine blue mixed with bacto-peptone (Sigma-Aldrich), chitosan (Sigma-Aldrich), nutrient agar (Thermo Fisher Scientific, Waltham, Massachusetts, USA), pectin (Sigma-Aldrich), yeast broth (Thermo Fisher Scientific), pea lectin (Sigma-Aldrich), and salmon sperm DNA (Thermo Fisher Scientific). Samples were placed onto a microscope slide and viewed using an Olympus microscope. Images were captured using a Leica DFC290 HD digital camera with Leica LAS EZ software V4.0.0.

Staining border cell exDNA with fluorescent dyes—DNA-specific dyes were DAPI (4′,6-diamidino-2-phenylindole, Sigma-Aldrich), Hoechst 33342 (Thermo Fisher Scientific), and SYTOX green (Invitrogen, Carlsbad, California, USA; Thermo Fisher Scientific). Stock solutions were made according to the manufacturer's instructions. Border cells were collected from root tips 15–20 mm long. Three root tips were immersed in 100 μL of ddH$_2$O in a microfuge tube. After 5 min, border cells were dispersed from the root tip by gentle agitation, the root tips were removed, and a 20-μL sample of border

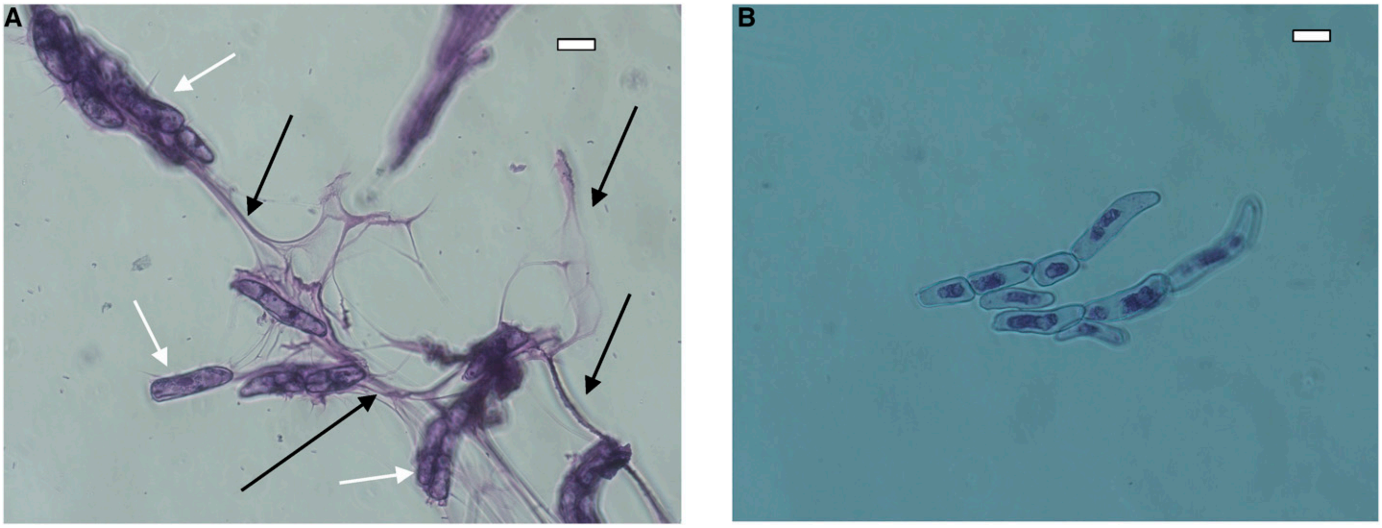

FIGURE 6 (A) Extracellular strands (black arrows) interconnecting *Pisum sativum* border cells (white arrows) revealed by staining with toluidine blue; and (B) absence of strands on border cells stained with toluidine blue after treating with DNase I. Scale bars = 25 μm.

cells was placed onto a microscope slide. A 5-µL sample of DAPI (0.5 µg/mL), Hoechst 33342 (0.5 µg/mL), or SYTOX green (5 µM) was added. Cells were covered with an ultraviolet permeable cover slip (VWR International, Radnor, Pennsylvania, USA) and incubated for 5 min before observing with an Olympus fluorescence microscope equipped with a UV lamp and corresponding wavelength of filter cubes. Images were captured using a Leica digital camera with Leica LAS EZ software, as described above (Leica Microsystems, Wetzlar, Germany).

Staining root cap surface mucilage exDNA with SYTOX Green—To detect exDNA in surface mucilage, a root tip was touched to the surface of a glass slide, and 10 µL of SYTOX green (50 µM) was added to the sample, which was then covered with an ultraviolet permeable cover slip (VWR). Samples were viewed using an Olympus fluorescence microscope. Images were captured with a Leica digital camera.

RESULTS

Appearance of root cap mucilage stained with crystal violet and toluidine blue—Within 1–2 min of immersing corn root tips in water and adding crystal violet, intensely stained entangled strands with the appearance of barbed wire (Fig. 2, white arrows) were revealed within the mucilage surrounding the root cap periphery (Fig. 2, black arrows). The structures remained obvious when viewed in grayscale (Fig. 3A, arrows). After treating with DNase I, the "barbed wire" structures disappeared and aggregated border cells dispersed, but residual staining of the matrix remained (Fig. 3B). Crystal violet is a synthetic dye that stains DNA and other acidic polymers including pectin (Revuelta et al., 2016), which is a component of root cap mucilage (Wen et al., 1999); thus, the residual staining of the root cap-border cell mucilage mass after DNase I treatment was not unexpected. Similar results occurred when border cell populations were dispersed from the root by gentle agitation with a pipette tip after several minutes in water: Staining with crystal violet revealed strands (Fig. 4A, arrows) throughout a strong background reaction. After treating with DNase I, border cells dispersed, and strands disappeared, but some residual staining remained at the cell wall surface and periphery of border cells (Fig. 4B).

Among pea border cells stained with crystal violet 2 h after dispersal of cells into water (Fig. 5), strands were readily apparent when crystal violet was added (Fig. 5A), but were digested almost entirely after treatment with DNase I (Fig. 5B).

Staining border cell populations with toluidine blue immediately revealed complex strands linking border cells together in clusters (Fig. 6A). The strands disappeared after treatment with DNase I (Fig. 6B). A control survey of materials including bacto-peptone, chitosan, nutrient agar, pectin, yeast broth, pea lectin, and salmon sperm DNA was carried out to see if similar structures might occur in response to proteins, polysaccharides, or complex biological mixtures. Identical complex strands (as in Fig. 6A) occurred only when toluidine blue was mixed with salmon sperm DNA (not shown).

Use of DNA-specific fluorescent stains to reveal dynamics of border cell exDNA delivery—Hoechst 33342 (Fig. 7A) and DAPI (Fig. 7B) can penetrate the cell membrane and, therefore, can stain nuclear DNA inside living or dead cells as well as exDNA structures surrounding border cells (arrows). In contrast, SYTOX green does not penetrate living cells and, therefore, revealed exDNA strands (Fig. 7C, block arrows) outside cell walls (arrows) of living border cells, which remained virtually invisible in the absence of SYTOX green uptake.

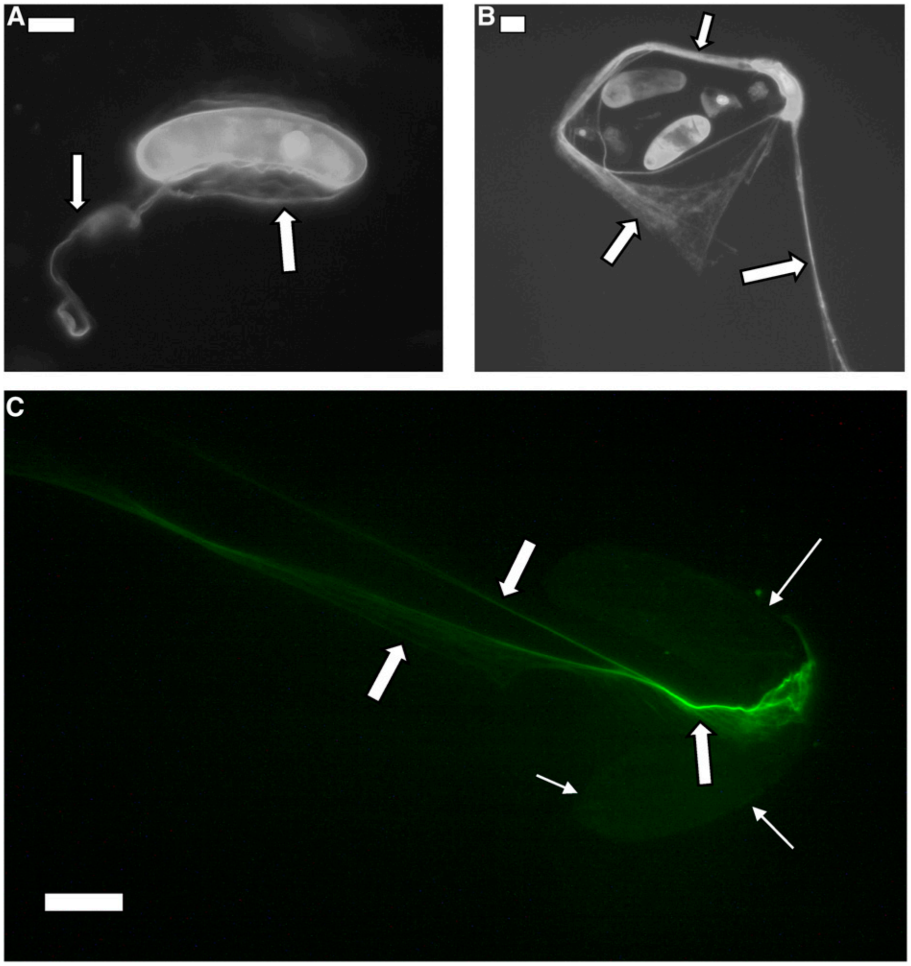

FIGURE 7 exDNA strands (block arrows) on border cells of (A) *Zea mays* and (B, C) *Pisum sativum* revealed by staining with Hoechst 33342 (A); DAPI (B); and SYTOX green (C). No SYTOX green (C) is taken into the nucleus inside the walls of a living border cell (arrows) attached to exDNA strands (block arrows). Scale bars = 10 µm.

When samples were stained with SYTOX green upon immersion of corn root tips into water, exDNA structures were immediately evident (Fig. 8A–C, arrows) surrounding border cells after dispersal (Fig. 8A, B, block arrows) and at the surface of the peripheral root cap (Fig. 8C, yellow arrows). No staining was evident within the border cells. After treatment with DNase I, exDNA structures were no longer detectable (Fig. 8D).

Up to 5% of pea border cell populations are nonviable under the test conditions (Hawes and Pueppke, 1986; Wen et al., 2009a). In one sample, in addition to complex exDNA structures (Fig. 9A, blue arrows) intermingled with masses of living border cells with no SYTOX green uptake (Fig. 9A, white arrows), two cells revealed staining of the nucleus as an indicator of cell death (Fig. 9B, orange arrow). If cell death is a significant contributor to exDNA delivery, then killing all the cells would be predicted to result in increased exDNA. To evaluate the potential role of cell death in exDNA delivery, we killed 100% of the border cell population by high speed centrifugation (Fig. 9C) or by freezing at −80° (Fig. 9D). Penetration of SYTOX green into all the dead cells was evident, but no exDNA was detected, and border cell populations were dispersed instead of aggregated together in groups.

When, where, and how exDNA is synthesized and exported is not known. Experiments were carried out to determine whether the process occurs before, after, or in parallel with induction of border cell separation. When root tips were immersed into water followed by gentle agitation to remove the border cells (as in Fig. 1), no exDNA was revealed at the root surface by staining with SYTOX green at time 0 after dispersal of border cells (Fig. 10A). Upon removal of border cells, mitosis in the root cap meristem is induced within 5 min, and new viable border cells begin to detach from the root cap periphery (Hawes and Lin, 1990; Brigham et al., 1998; Wen et al., 2009b). Within 30 min, staining the root tip with SYTOX green water revealed renewed exDNA structures (Fig. 10B) associated with emerging border cells (Fig. 10B, arrow). Uptake of SYTOX green inside the border cells did not occur. Uptake of the vital stain fluorescein diacetate confirmed that the cells were 100% viable (Fig. 10B, inset). When the washed root tips were immersed into water with DNase I, no exDNA strands were detected even after a 1-h incubation (Fig. 10C), despite dispersal of several hundred border cells. The viability of the border cell populations was evident from negative SYTOX green staining in all but one cell (Fig. 10C, arrow) and positive fluorescein diacetate staining (Fig. 10C, inset).

DISCUSSION

Understanding how disease develops is critical to understanding how to prevent it, but tracking the process using destructive approaches to dissect complex tissues in plants and animals is a challenge. Recognition of the robust nature of the detached cell populations from the root cap and the ability to collect them without cell

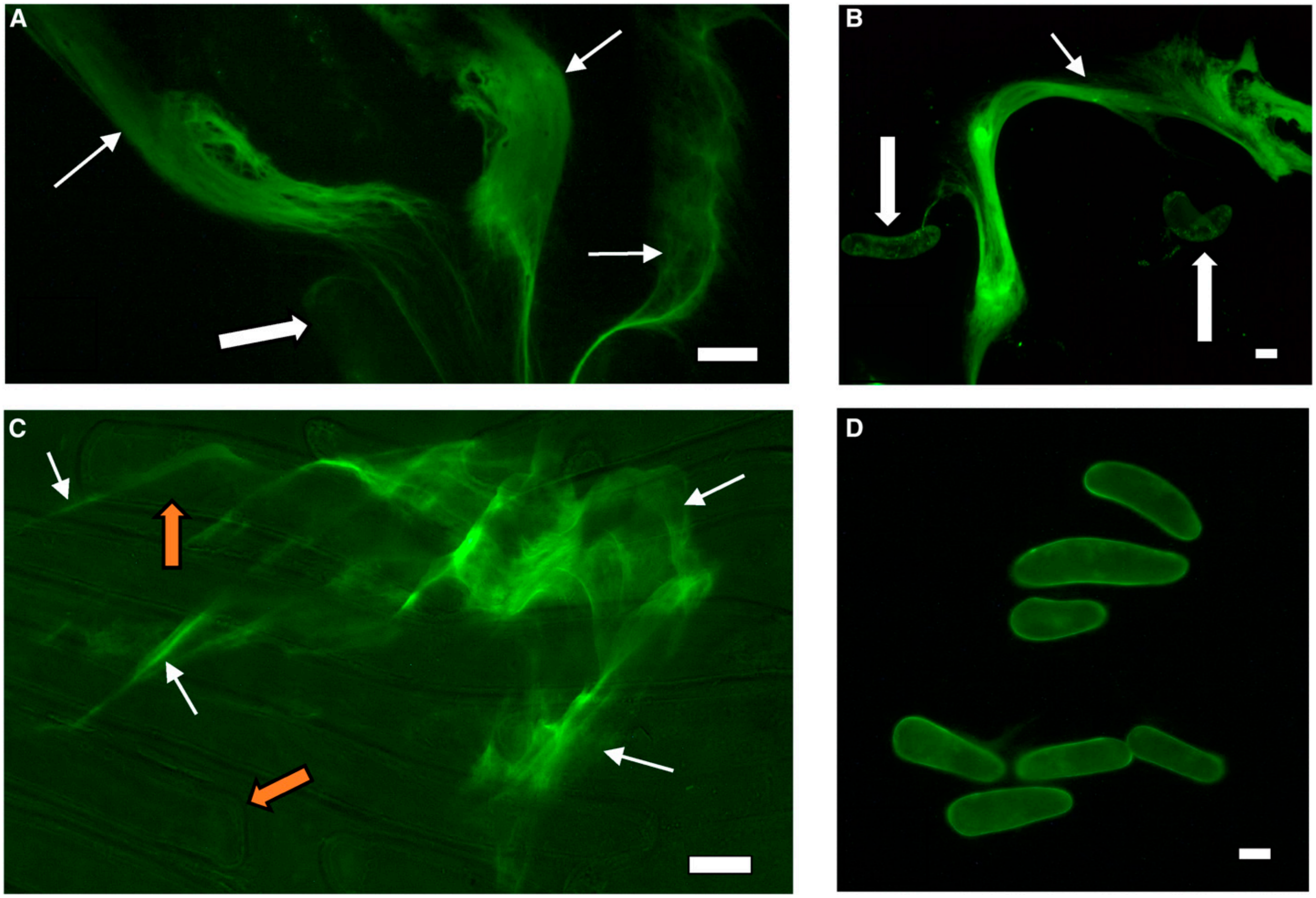

FIGURE 8 (A, B) Use of SYTOX green to detect exDNA strands (arrows) surrounding individual viable detached border cells (block arrows) of *Zea mays* as they dispersed from the root tip surface (C, orange arrows), upon addition of water to the root. (D) Absence of border cell exDNA strands after treatment with DNase I. Scale bars = 10 μm.

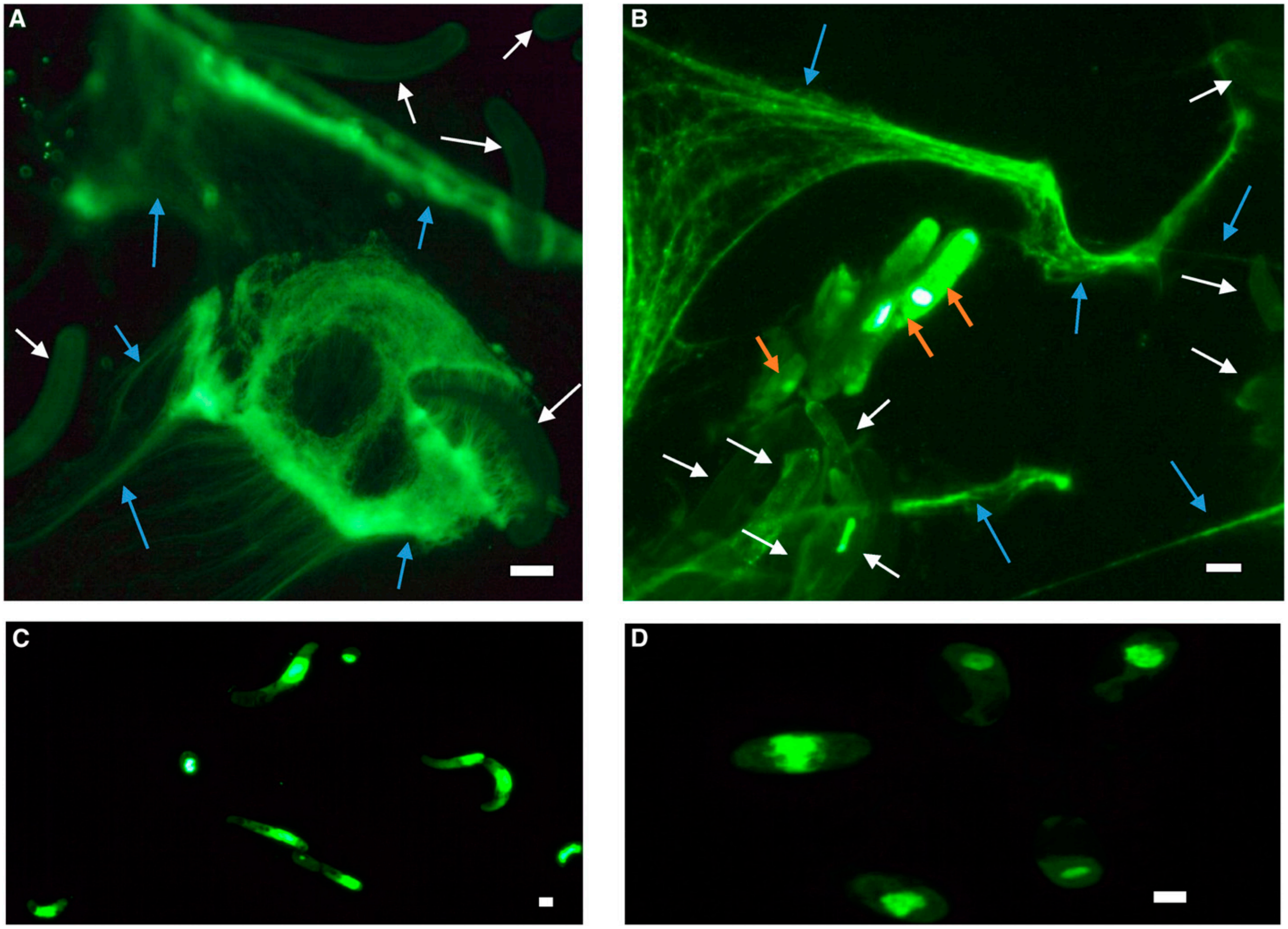

FIGURE 9 (A, B) SYTOX green staining reveals complex masses and strands of exDNA (blue arrows) intercalated with aggregated border cells (white arrows) of *Pisum sativum*. In three of the cells (orange arrows) (B), loss of viability is revealed by intracellular uptake of SYTOX green into the nucleus. (C) Intracellular uptake of SYTOX green into the nucleus occurs in all border cells killed by high-speed centrifugation for 5 min or (D) freezing at −80° for 15 min. No exDNA is evident. Scale bars = 10 µm.

or tissue damage made them of interest for studying plant–microbe infection at the cellular level using *Agrobacterium tumefaciens* as a model (Hawes and Pueppke, 1986, 1987). Initial results were very encouraging: Host–microbe-specific chemotaxis and binding of pathogenic strains resulted in the accumulation of thousands of bacteria on the cell wall and the surface of ropelike structures that appeared during incubation and linked border cells and bacteria into aggregated masses (Hawes et al., 1998, 2003, 2012). This appeared to involve a pathogen-induced virulence process that would facilitate invasion of the host cells and reveal critical aspects of the infection process. However, despite many replicated experiments, in no case did infection of the cells occur.

Host-specific chemotaxis and accumulation on border cells also occurred with other pathogens including nematodes, zoospores, and fungi, which in some cases did penetrate and kill individual border cells (Sherwood, 1987; Goldberg et al., 1989; Hawes et al., 1998; Zhao et al., 2000; Gunawardena and Hawes, 2002). However, in each case, the immediate attraction and aggregation was rapidly followed by induced quiescence in the pathogen population. When the inoculation was carried out with whole roots, the root tip passed by the border cell–pathogen masses without becoming infected (Gunawardena et al., 2005). This discovery provided insight into the long-standing recognition that root tips of most species are largely resistant to infection by most pathogens, which instead infect primarily in the region of elongation despite the vulnerability of the nonlignified root tips moving through soil (Curl and Truelove, 1986). The hypothesis that border cells function as a decoy to neutralize threats intrinsic to the newly synthesized tissues of the root tip was proposed (Brigham et al., 1995). However, efforts to define the mechanism by which the decoy lured its prey using standard approaches yielded few insights, and there were no hints that exDNA might be involved (e.g., Hawes et al., 1989, 1998; Hawes and Lin, 1990; Wen et al., 1999, 2009b; Woo et al., 2004). The fact that DNA is synthesized in cells at the root cap periphery at the same rate as cells within the meristem was clearly documented in independent studies with corn and *Convolvulus* (Clowes, 1968; Phillips and Torrey, 1971), but there was no basis for interpretation of this surprising observation until NETs were discovered (Brinkmann et al., 2004). Subsequent studies have established the role of exDNA in plant

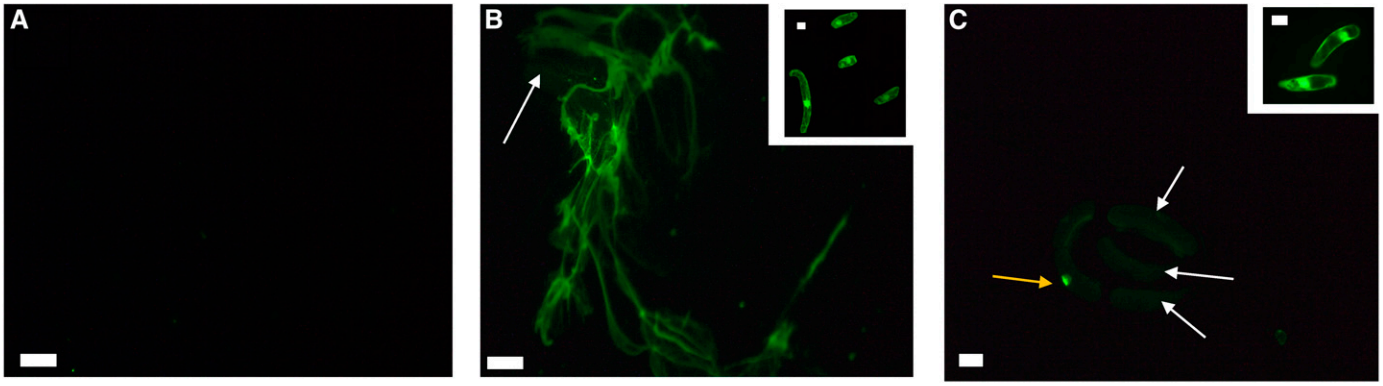

FIGURE 10 (A) Absence of exDNA detected by SYTOX green in the absence of border cells. Pea root tips were immersed into water, and the water was agitated to disperse border cells; when the root tip was removed and immersed into water containing SYTOX green at time 0, no border cells were present, and no exDNA was detected. (B) Within 30 min of incubation in water, new border cells began to separate (arrow), and new exDNA strands were detected using SYTOX green. Staining with the vital stain fluorescein diacetate (inset) revealed 100% cell viability. When the root was placed into water with DNase for 1 h and then stained with SYTOX green, no exDNA strands were detected (C). Viability of all but one (orange arrow) of the emerging border cells (white arrows) was evident from negative SYTOX green staining and from positive fluorescein diacetate staining (inset). Scale bars = 10 µm.

extracellular trapping (Curlango-Rivera et al., 2013; Hawes et al., 2016a; Tran et al., 2016; Wang et al., 2015; Wen et al., 2009a).

In the current study, constitutive traps that are delivered in the absence of pathogens were visualized. Data presented in this paper demonstrated that exDNA is secreted as new border cells disperse from the root cap periphery. Furthermore, exDNA plays a critical role in the structural integrity of the complex extracellular trap structures surrounding border cells. Future studies to define the process and how it varies in response to pathogens, toxins, metals (Hawes et al., 2016b), and other dangers are needed. Rather than providing a convenient system to observe how infection occurs at the cellular level (e.g., Hawes and Pueppke, 1986, 1987), border cells instead may provide a tool to define how infection and injury are prevented.

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