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Activation of defense responses to *Fusarium* infection in *Asparagus densiflorus*

Chenyang He[1], Tom Hsiang[2] and David J. Wolyn[1,*]
[1]*Department of Plant Agriculture,* [2]*Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1;* [*]*Author for correspondence (Phone: +1 519 824 4120; Fax: +1 519 767 0755; E-mail: dwolyn@uoguelph.ca)*

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Abstract

Defense responses to *Fusarium oxysporum* f. sp. *asparagi* and *F. proliferatum* were compared after root inoculation of the asparagus fern, *Asparagus densiflorus* vars. Myersii and Sprengeri, and cultivated asparagus, *A. officinalis* cv. Guelph Millennium. Both varieties of *A. densiflorus* exhibited a hypersensitive response with rapid death of epidermal cells within 8–24 h and restricted the fungal growth. In *A. officinalis* roots, rapid cell death was not found, and necrotic lesions were observed 8–14 d after fungal inoculation. Peroxidase and phenylalanine ammonia-lyase activities increased significantly in inoculated *A. densiflorus* but not *A. officinalis* plants. Local and systemic induction of peroxidase activity was detected after pathogen inoculation in root and spear tissues, respectively, of *A. densiflorus*. POX activity decreased in roots of inoculated *A. officinalis* by 8 d post-inoculation. Germination and germ tube growth were inhibited when spores of *F. oxysporum* f. sp. *asparagi* were incubated in root exudates and on root segment surfaces of inoculated *A. densiflorus* plants exhibiting hypersensitive cell death. Spore germination of *F. proliferatum* and three fungi non-pathogenic to cultivated asparagus was inhibited as well. Rapid induction of hypersensitive cell death in *A. densiflorus* was associated with restriction of fungal growth, and activation of peroxidase and phenylalanine ammonia-lyase, two defense enzymes thought to be important for plant disease resistance.

Abbreviations: Foa – *Fusarium oxysporum* f. sp. *asparagi*; Fp – *F. proliferatum*; HR – hypersensitive response; PAL – phenylalanine ammonia-lyase; POX – peroxidase; SAR – systemic acquired resistance.

Introduction

Plants have coevolved with pathogens to develop complex, constitutive and inducible mechanisms to defend against infection (Kombrink and Somssich, 1995; Agrios, 1997). When a plant recognizes an invading pathogen, active defense mechanisms are induced which can include a hypersensitive response (HR), accumulation of antimicrobial phytoalexins, synthesis of hydrolases and pathogenesis-related proteins, reinforcement of cell walls through callose deposition and lignification, and activation of defense-related genes (Hammond-Kosack and Jones, 1996). Defense mechanisms in a susceptible plant are induced more slowly than those of a resistant plant, and the time required to induce a variety of defense responses appears to be a key factor resulting in a resistant phenotype (Yang et al., 1997).

Asparagus crown and root rot, caused by *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *asparagi* Cohen & Heald (Foa) and *F. proliferatum* (Matsu.) Nirenberg (Fp), is considered the primary agent of decline in major asparagus-producing areas of the world (Elmer et al., 1996). The cultivated asparagus, *A. officinalis*,

is susceptible to *Fusarium* crown and root rot, while the asparagus fern, *A. densiflorus*, is resistant to the disease, and is assumed to be a nonhost (Lewis and Shoemaker, 1964; Stephens et al., 1989). The mechanisms of disease resistance, however, are currently unknown. Sexual incompatibility between *A. densiflorus* and *A. officinalis* prevents development of resistant *A. officinalis* by traditional breeding approaches (Stephens et al., 1989). An improved understanding of resistance mechanisms in *A. densiflorus* to *Fusarium* species which are pathogenic or non-pathogenic to cultivated asparagus could provide insights into developing strategies to engineer durable and broad-spectrum resistance. The purpose of this study was to examine whether active defense mechanisms, such as hypersensitive cell death, activation of peroxidase (POX) and phenylalanine ammonia-lyase (PAL), and production of antifungal compounds are induced in *A. densiflorus* plants in response to inoculation with *Fusarium* species pathogenic and nonpathogenic to *A. officinalis*.

Materials and methods

Fungal isolates and cultures

Isolates of *Fusarium oxysporum* f. sp. *asparagi* (isolate FO50) and *F. proliferatum* (isolate FPM6374) pathogenic to asparagus were provided by Dr. W.H. Elmer (Connecticut Agricultural Experimental Station, Connecticut, USA). Other fungal isolates, pathogenic on tomato (*F. oxysporum* f. sp. *lycopersici* (isolate FOL67)), cyclamen (*F. oxysporum* f. sp. *cyclaminis* (isolate HYC-7)) and tobacco (*Colletotrichum destructivum* (isolate CD001)), were obtained from the Department of Environmental Biology, University of Guelph. All isolates used in this study were grown on sterile millet kernels and maintained for long-term storage at 4 °C (Escandi and Echandi, 1991). Cultures were initiated by inoculating potato dextrose agar (PDA, Difco) plates with fungus-colonized millet kernels and incubating plates at 24 °C in darkness. Fungal conidia were harvested from 2-week-old cultures by dislodging the spores with a glass rod into distilled water. Spore suspensions were filtered through cheesecloth to remove mycelium and agar chunks, the filtrate was centrifuged at 6000 × *g* for 5 min, and the spore pellet was re-suspended in sterile 0.5% malt extract. Spores were pre-germinated overnight at 24 °C in darkness. After centrifugation of the pre-germinated spore suspension described above, the resulting supernatant (spore germination fluid) was collected. Germinating spores were washed, serially diluted with distilled water and adjusted to 1×10^2, 1×10^4 and 1×10^6 spores ml^{-1} for plant inoculations. Mycelial homogenates and culture filtrates for plant inoculation were produced by inoculating a 50 ml culture of 0.5% malt extract with a mycelial plug (10-mm-diameter) of Foa (FO50) and allowing the culture to grow on a shaker (100 rpm) for 4 weeks at 24 °C in darkness. Culture filtrates were obtained by filtering liquid cultures through Whatman #4 filter paper. The filtered mycelium was aseptically homogenized with 20 ml distilled water in a pre-chilled mortar and ground with a pestle for 2 min. The resulting suspension, containing 4.5×10^9 hyphal fragments ml^{-1} with lengths of 150–250 μm and widths of 3–4 μm, was directly used for inoculation in experiments.

Asparagus plants

Seeds of *Asparagus densiflorus* varieties Myersii and Sprengeri (Ball Seed Co., West Chicago, IL, USA) and *A. officinalis* cultivar Guelph Millennium (University of Guelph) were disinfected first in sodium hypochlorite and subsequently in a solution of benomyl in acetone according to Stephens and Elmer (1988). The seeds were aseptically germinated in 9 cm Petri plates on moistened sterile filter paper at 24 °C under a 12 h photoperiod (∼40 μmol m^{-2} s^{-1}). Ten days after sprouting, individual seedlings were transferred to Hoagland's solid medium (Hoagland and Arnon, 1950) for further growth under the same conditions described above. For soil inoculation experiments, 4-week-old seedlings of asparagus were transplanted into individual 10 cm diameter pots containing a commercial greenhouse soil (Promix Bx, Rivière-du-Loup, PQ, Canada) and grown in a greenhouse at 25 °C (day)/20 °C (night) for an additional 12 weeks before pathogen inoculation. For hyphal-sandwich root inoculation experiments described later, asparagus seedlings were grown in Hoagland's solid medium for 8 weeks before inoculation.

Assessment of Fusarium *resistance in greenhouse trials*

An inoculation technique with *Fusarium*-colonized millet (Stephens et al., 1989) was used to evaluate disease resistance of asparagus in greenhouse trials. To

prepare fungal inoculum, 200 g of millet seeds were combined with 100 ml of distilled water in 1-l flasks and autoclaved twice for 1 h on two consecutive days. A 10-mm-diameter plug from the margin of a 7-d-old fungal culture grown on PDA was placed aseptically into individual flasks of sterile millet. After 14 d incubation at 24 °C in darkness, the colonized millet was incorporated into greenhouse soil at the rate of 7 g millet kg^{-1} soil. Subsequently, plants of *A. densiflorus* vars. Myersii and Sprengeri and *A. officinalis* cv. Guelph Millennium grown in soil as described above, were individually transplanted into 10 cm diameter pots containing greenhouse soil which was either inoculated or not inoculated with Foa, Fp, or both pathogens. Plants were grown in the greenhouse at 25 °C (day)/20 °C (night). Each treatment consisted of nine replicate plants. The experiment was repeated once. After 8 weeks, plants were harvested and evaluated for disease severity. *Fusarium* crown and root rot was visually rated on a 1–5 scale: 1 = no symptoms or lesions; 2 = lesions present on 1–25% of root system; 3 = lesions on 26–50% of root system; 4 = 51–75% of root tissues covered with lesions; 5 = >76% of root tissues covered with lesions (Stephens et al., 1989; Blok et al., 1997).

Assessment of HR in asparagus genotypes

Hyphal-sandwich root inoculation. For hyphal-sandwich inoculation (Xue et al., 1998), 8-week-old seedlings of *A. densiflorus* vars. Myersii and Sprengeri and *A. officinalis* cv. Guelph Millennium were removed from Hoagland's solid medium, washed with distilled water, blotted dry and transferred to sterile Pyrex glass trays (38 × 27 × 6 cm) that were lined with sterile paper towels. The root systems of these plants were placed between two PDA strips (6 × 3 × 0.5 cm) containing hyphae from 2-week-old cultures of Foa, Fp or both, with the hyphal side of each strip facing the roots. Uninoculated PDA strips were used as controls. Two replicate experiments were performed each consisting of 2 sets of 3 replicate glass trays each with 12 treatment combinations (4 inoculation treatments (Foa, Fp or both, plus uninoculated control) × 3 plant genotypes). Each treatment combination consisted of 2 plants in each replicate tray which were inoculated together between PDA strips. From the first set of glass trays, individual plants were sampled from each treatment at 8 h and 24 h post-inoculation, and plants from the second set of glass trays were sampled at 8 d and 14 d. Each root was sampled twice where 100 contiguous epidermal cells were observed for each subsample.

Root inoculations with mycelial homogenate, culture filtrate and spores. Petri dishes containing 8-week-old seedlings of *A. densiflorus* var. Myersii and *A. officinalis* cv. Guelph Millennium were each inoculated with 1 ml of mycelial homogenate, culture filtrate, pre-germinated spores (1×10^2, 1×10^4, 1×10^6 spores ml^{-1}), 3% malt extract, or distilled water according to Asiegbu et al. (1994). Each treatment combination (7 inoculation treatments × 2 plant genotypes) consisted of 3 replicate plates each with 3 plants, and the entire experiment was repeated once. The inoculated plant roots were repeatedly sampled at 8 h, 24 h, 8 d and 14 d post-inoculation for examination of HR.

Examination of hypersensitive cell death. Death of asparagus root cells after *Fusarium* inoculation was investigated using trypan blue as both a vital dye and a fungal stain. Plant cell death was monitored as described by Tang et al. (1999). Root samples were placed in 0.05% trypan blue in lactophenol, boiled for 5 min, rinsed first in 50% ethanol, then in water and stored in 50% glycerol until use. Fungal hyphae and plant root cells stained by trypan blue were examined microscopically for quantification of cell death. At least 100 epidermal cells of sampled root were assessed under 400 to 1000× magnification and the numbers of dead cells were counted to estimate the percentage of cell death.

Analysis of POX and PAL activities in asparagus challenged by Foa

Fungal inoculations. Eight-week-old seedlings of *A. densiflorus* vars. Myersii and *A. officinalis* cv. Guelph Millennium were inoculated with Foa (FO50) using the hyphal-sandwich root method described above. Each experiment consisted of 2 sets of 3 replicate glass trays each with 4 treatment combinations (Foa inoculation treatment plus uninoculated control × 2 plant genotypes). Each treatment combination consisted of 3 plants in each replicate tray which were inoculated together between PDA strips. From the first set of glass trays, individual plants were sampled for each treatment at 0, 8 h and 2 d post-inoculation, and those from the second set of glass

trays were sampled at 4 d, 8 d and 14 d. The entire experiment was repeated once.

Enzymatic assays. Root and spear tissues were each homogenized with 10 mM sodium phosphate buffer (pH 6.0) in a chilled mortar on ice by grinding with a pestle for 50 s. The homogenate was filtered through Whatman #4 filter paper and filtrates were centrifuged at 12,000 × *g* for 20 min at 4 °C. Supernatant protein concentrations were determined by the method of Bradford (1976).

POX activity was measured according to Hammerschmidt et al. (1982) with minor modifications. The assay reaction contained 7.5 µl of 10 mM guaiacol in 50 mM sodium phosphate buffer, pH 6.0, 100 µl diluted extract supernatant (diluted 10-fold with 5 mM sodium phosphate buffer, pH 6.0), 792.5 µl of 5 mM sodium phosphate buffer, pH 6.0, and 100 µl of 600 mM H_2O_2. Assays were initiated by addition of H_2O_2 and the change in optical density at 470 nm was measured for 1 min (DU-64, Beckman Instruments Inc., CA, USA). POX activity was expressed as the change in optical density mg^{-1} protein min^{-1}.

PAL activity was assayed following Beaudoin-Eagan and Thorpe (1985). The assay reaction consisted of 100 µl of plant extract supernatant and 900 µl of 6 µM L-phenylalanine in 500 mM Tris–HCl buffer, pH 8.5. The mixture was incubated at 37 °C for 1 h and measured spectrophotometrically at 290 nm. PAL activity was expressed as units g^{-1} protein, where one unit was determined as 1 µM of L-phenylalanine converted to trans-cinnamate and NH_3 min^{-1} for a standard sample of PAL from *Rhodotorula glutinis* (Sigma, St. Louis, MO, USA).

Survey of antifungal activity from asparagus roots inoculated with Foa

Root inoculations. Hyphal-sandwich root inoculation with Foa (FO50) was performed for *A. densiflorus* var. Myersii and *A. officinalis* cv. Guelph Millennium. Two replicate experiments each consisted of 3 replicate glass trays, each with 4 treatment combinations (Foa inoculation treatment plus uninoculated control × 2 plant genotypes). Each treatment combination consisted of 3 plants in each replicate tray which were inoculated together between PDA strips. After 24 h inoculation, two plants were sampled for the preparation of root exudates and the third plant was sampled for root segments.

Fungal inhibitory trial in root exudates. Fungal germination and growth in plant root exudates was performed according to Fernandez and Heath (1989a) with modifications. Foa-inoculated plant roots of *A. densiflorus* var. Myersii and *A. officinalis* cv. Guelph Millennium and those from uninoculated controls, were each submerged in 20 ml of sterile distilled water in darkness for 24 h. The water that presumably contained plant root exudates was collected and stored at 4 °C. Spore suspensions (1×10^6 ml^{-1}) of Foa (FO50), Fp (FPM6374), *F. oxysporum* f. sp. *lycopersici* (FOL67) and *F. oxysporum* f. sp. *cyclaminis* (HYC-7) and *C. destructivum* (CD001) were diluted with an equal volume of root exudate solution, or spore germination fluid, or sterile distilled water. The resulting spore suspensions (5×10^5 ml^{-1}) were incubated at 24 °C and percent spore germination and/or germ tube length were measured.

Foa inhibitory trial on root segment surfaces. Fungal spore germination and growth on root segment surfaces was assayed following Fernandez and Heath (1989a,b). Two Foa-challenged root segments (2-mm-diameter and 25-mm-length) from individual *A. densiflorus* var. Myersii or *A. officinalis* cv. Guelph Millennium plants were placed together on a glass slide and covered with 200 µl of 5×10^5 ml^{-1} spore suspension of Foa. Three replicate glass slides were produced from each inoculated plant. Glass slides were incubated at 24 °C in parafilm-wrapped Petri dishes. The root segments of unchallenged plants were used as controls. At least 100 spores per slide were examined microscopically for effects of root segments on spore germination and germ tube growth.

Results

Macroscopic reaction of asparagus genotypes to inoculations with Foa and Fp

A. densiflorus vars. Myersii and Sprengeri displayed a resistant response 8 weeks after inoculation with Foa or Fp, while *A. officinalis* cv. Guelph Millennium was susceptible to these pathogens (Figure 1). Synergistic effects were not found when plants were inoculated simultaneously with both *Fusarium* species. No macroscopic disease symptoms or lesions were observed in *A. densiflorus*, while vascular discoloration in rotted crowns, collapse of feeder roots, and plant death were observed for *A. officinalis* within 8 weeks.

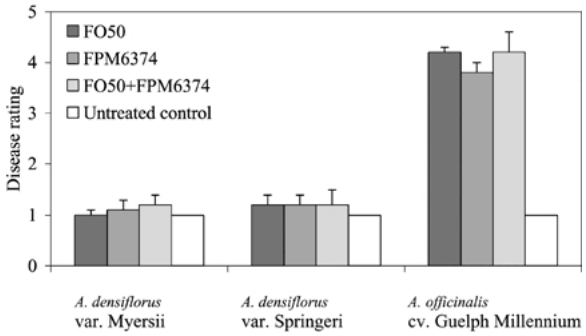

Figure 1. Disease resistance reactions of *Asparagus densiflorus* vars. Myersii and Sprengeri and *A. officinalis* cv. Guelph Millennium 8 weeks after inoculation with *Fusarium oxysporum* f. sp. *asparagi* (FO50) or *F. proliferatum* (FPM6374) in a soil inoculation greenhouse trial. Disease rating 1 = no symptoms or lesions on roots; 2 = lesions present on 1–25% of root system; 3 = lesions on 26–50% of root system; 4 = 51–75% of root tissues covered with lesions; and 5 = >76% of root tissues covered with lesions. Mean±SE.

Hypersensitive cell death in response to Fusarium *inoculation*

Root cells of *A. densiflorus* displayed hypersensitive cell death when inoculated with Foa and Fp (Figure 2). The HR was first observed 8 h post-inoculation in root epidermal cells of *A. densiflorus* var. Myersii (Figure 2A), and no infected fungal hyphae were found in the cells adjacent to those that were dead (Figure 2B), indicating the HR prevented growth and spread of the pathogen into healthy tissue. In contrast, susceptible plants of *A. officinalis* var. Guelph Millennium did not show HR after fungal challenge; the invading hyphae rapidly penetrated and colonized the root epidermal tissues 24 h post-inoculation (Figure 2C). Necrotic root lesions were first observed 8 d post-inoculation, and were well established by 14 d post-inoculation (Figure 2D).

Hyphal-sandwich root inoculations with Foa and Fp, individually, and in combination induced similar percentages of cell death in the root epidermis of *A. densiflorus* vars. Myersii and Sprengeri (Table 1). At 8 h post-inoculation 2–6% of root epidermal cells exhibited HR; this value increased to 9–15% at 24 h. No further increases in percent cell death were observed at 2, 4, 8 and 14 d post-inoculation (data not shown).

Mycelial homogenates, culture filtrates and pre-germinated conidia of Foa each induced rapid cell death in roots of *A. densiflorus* var. Myersii (Table 2). The highest percent cell death was found with mycelial homogenates and high concentrations of pre-germinated conidia (10^4 to 10^6 ml^{-1}), while the lowest levels occurred with fungal culture filtrates and a low concentration of pre-germinated conidia (10^2 ml^{-1}). The percentage of dead epidermal cells did not increase significantly after 24 h post-inoculation. No rapid cell death occurred in roots of *A. officinalis* var. Guelph Millennium when inoculated either with mycelial homogenates, culture filtrates or pre-germinated conidia of Foa within 8 to 24 h (C.Y. He and D.J. Wolyn, unpublished).

Changes in POX and PAL activities accompanying HR

In *A. densiflorus* var. Myersii, POX activity increased significantly in roots and spears after hyphal-sandwich root inoculations with Foa compared to the untreated control (Figure 3). In response to hyphal-sandwich inoculation, POX activity increased eight-fold in plant roots after 8 h (Figure 3A), which coincided with HR induction in infected root epidermal cells (Table 1, Figure 2A). Elevated levels of the enzymatic activity were maintained 2, 4, 8 and 14 d post-inoculation (Figure 3). POX activity increased 350% by 2 d post-inoculation in spears of *A. densiflorus* plants, then decreased 30% from 2 d to 14 d post-inoculation (Figure 3B). In roots of *A. officinalis* cv. Guelph Millennium, POX activity decreased 30–50% following root inoculation with Foa (Figure 3A). The decline was first observed 8 h after inoculation and levels remained constant to 8 d post-inoculation. By 14 d post-inoculation, POX activity increased to levels greater than that of the uninoculated control, when tissue collapse and necrosis became apparent (Figure 2D). POX activity increased slightly in spears of *A. officinalis* plants with Foa-inoculated roots, while no significant changes in POX activity were observed in spears of the untreated control (Figure 3B).

PAL activity increased significantly in roots of *A. densiflorus* var. Myersii challenged with Foa (Figure 4). Two days post-inoculation, PAL activity increased approximately 300% compared to the control and subsequently decreased 50% from day 4 to day 14. PAL activity decreased in inoculated roots of *A. officinalis* from 8 h to 8 d post-inoculation compared to the untreated control plants (Figure 4A). PAL activities in spears did not significantly differ between treated and untreated *A. densiflorus* or *A. officinalis* (Figure 4B).

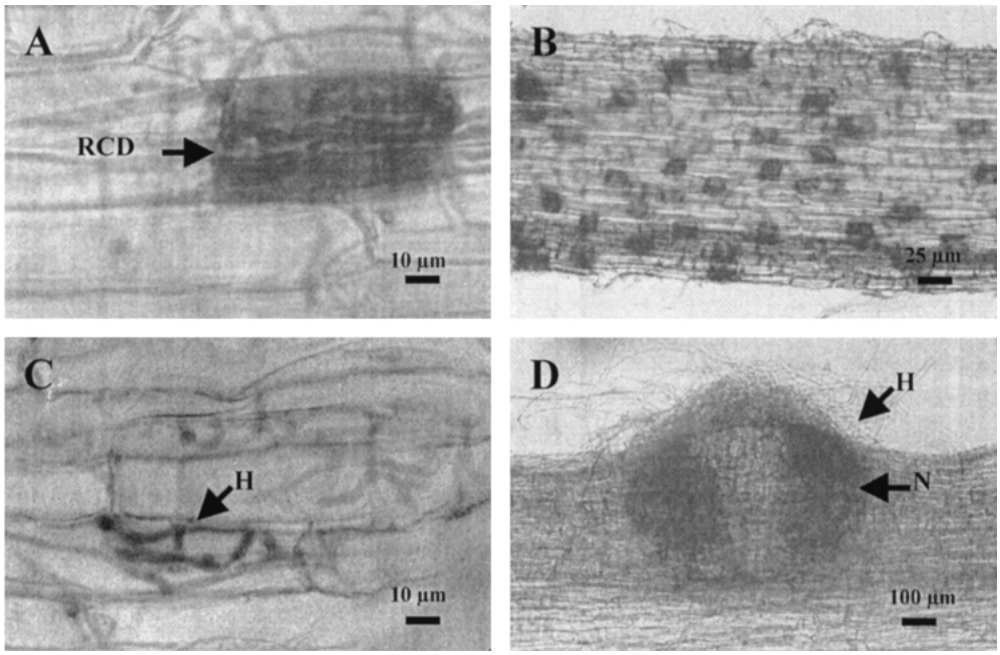

Figure 2. Rapid cell death and necrosis in root tissues of *Asparagus* species inoculated with *Fusarium oxysporum* f. sp. *asparagi* (FO50). Fungal hyphae and dead plant cells were visualized by trypan blue staining. In *A. densiflorus* var. Myersii, rapid cell death (RCD, arrow) occurred at 8 h post-inoculation (A), and remained limited to epidermal cells at 24 h post-inoculation (B). In *A. officinalis* cv. Guelph Millennium, infected plant roots showed no visible cell death; fungal penetration was followed by branching, elongation and colonization by hyphae (H, arrow) within infected tissues at 24 h post-inoculation (C), and typical disease necrosis (N, arrow) developed at 14 d post-inoculation with abundant hyphae (H, arrow) surrounding diseased tissues (D).

Table 1. Percent dead cells[1] of epidermal cells in *Asparagus densiflorus* roots inoculated for 8 h and 24 h, singly and in combination with *F. oxysporum* f. sp. *asparagi* (FO50) and *F. proliferatum* (FPM6374) using a hyphal-sandwich root inoculation method

Inoculation treatments	*A. densiflorus* var. Myersii		*A. densiflorus* var. Sprengeri	
	8 h	24 h	8 h	24 h
FO50	2	14 ± 6[2]	2 ± 1	10 ± 8
FPM6374	5 ± 2	11 ± 2	6 ± 4	9 ± 5
FO50 + FPM6374	3	15 ± 6	5 ± 4	14 ± 2
Untreated control	0	0	0	0

[1]Percent dead cells represent the proportion of dead cells in all root epidermal cells examined.
[2]Means ± SE, each mean is based on 6 replicates of 100 root cells each.

Table 2. Percent dead cells[1] of epidermal cells in roots of *Asparagus densiflorus* var. Myersii inoculated with mycelial homogenate, culture filtrate and pre-germinated spores of *Fusarium oxysporum* f. sp. *asparagi* (FO50)

Inoculation treatments	Time post-inoculation			
	8 h	24 h	8 d	14 d
Mycelial homogenates	2	21 ± 2[2]	20 ± 2	16 ± 2
Culture filtrate	3	8 ± 2	8 ± 2	6
Spore suspension				
10^2 ml^{-1}	6	12 ± 9	10	16 ± 2
10^4 ml^{-1}	6 ± 4	18 ± 14	24 ± 9	26 ± 2
10^6 ml^{-1}	8 ± 4	15 ± 4	24 ± 8	25 ± 4
3% Malt extract	0	0	0	0
Distilled water	0	0	0	0

[1]Percent dead cells represent the proportion of dead cells in all root epidermal cells examined.
[2]Means ± SE, each mean is based on 6 replicates of 100 root cells each.

Antifungal properties of A. densiflorus *roots exhibiting HR*

Incubation of Foa spores with root exudates and root segments from plants of *A. densiflorus* exhibiting HR from Foa-challenged tissues inhibited spore germination and germ tube elongation (Figure 5). Values were 50–70% less than those for uninoculated plants, fungal spore germination fluid or distilled water. The greatest

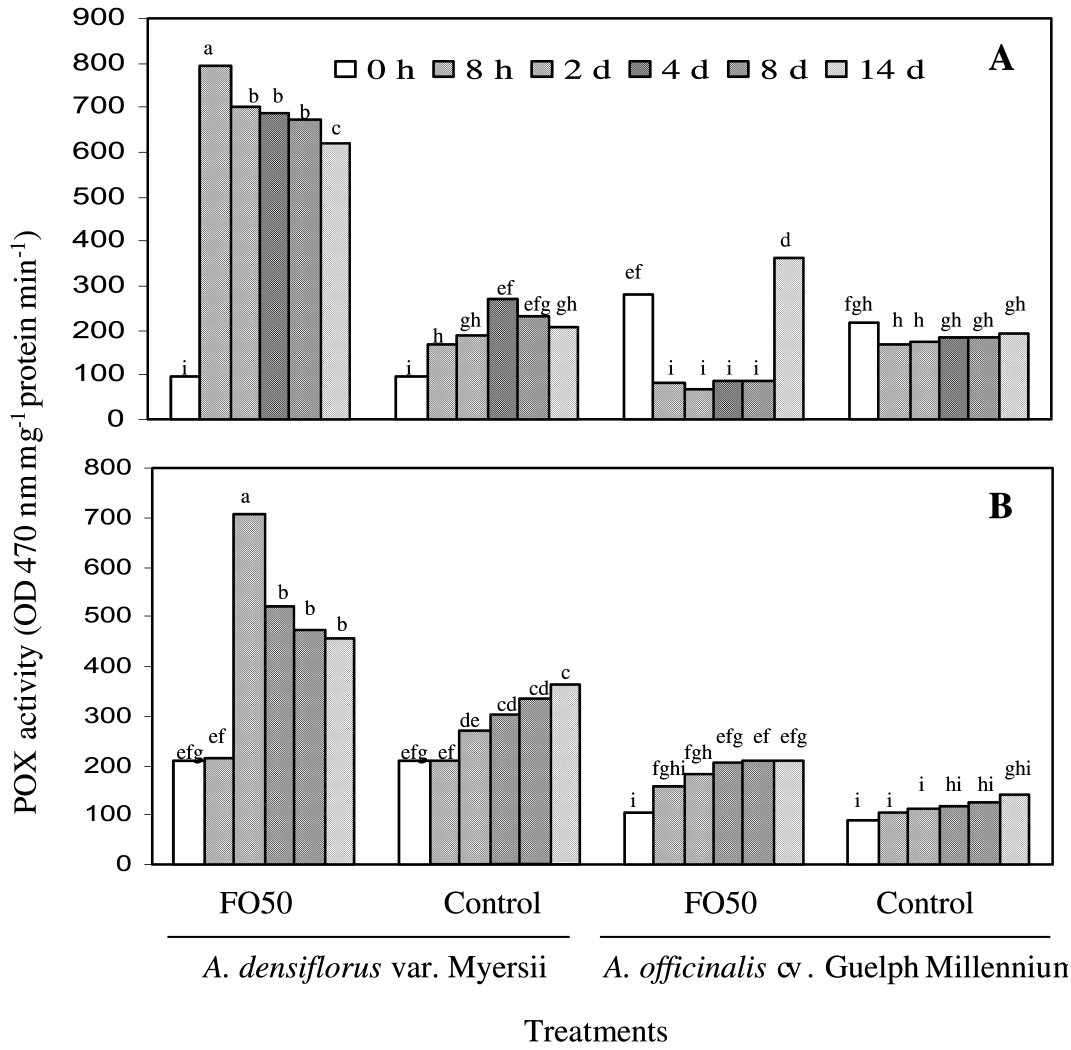

Figure 3. Peroxidase (POX) induction in roots (A) and spears (B) of 8-week-old asparagus plants either inoculated with *Fusarium oxysporum* f. sp. *asparagi* (FO50) or uninoculated (control) using hyphal-sandwich root inoculation. Within each tissue type, means with the same letter are not significantly different at $P = 0.05$ (test of least significant difference) and each mean is based on 6 replicates.

response was 71% inhibition of spore germination with root exudates. No antifungal activity was observed for exudates from either Foa-challenged or unchallenged control plants of *A. officinalis* (C.Y. He and D.J. Wolyn, unpublished). The pH of root exudates did not change between the challenged and unchallenged plants, and treatments of root exudates with proteinase K and boiling did not affect the inhibitory activities (data not shown).

Root exudates of *A. densiflorus* significantly inhibited spore germination *in vitro* for several plant pathogenic fungi, including *F. proliferatum*, *F. oxysporum* f. sp. *lycopersici* and f. sp. *cyclaminis* and *Colletotrichum destructivum* (Figure 6). The greatest inhibition was observed for spores of *F. proliferatum*. No inhibitory activity to these pathogens was observed for exudates from either Foa-challenged or unchallenged plants of *A. officinalis* (data not shown).

Discussion

Both varieties of *A. densiflorus*, Myersii and Sprengeri, displayed an incompatible reaction with the *Fusarium*

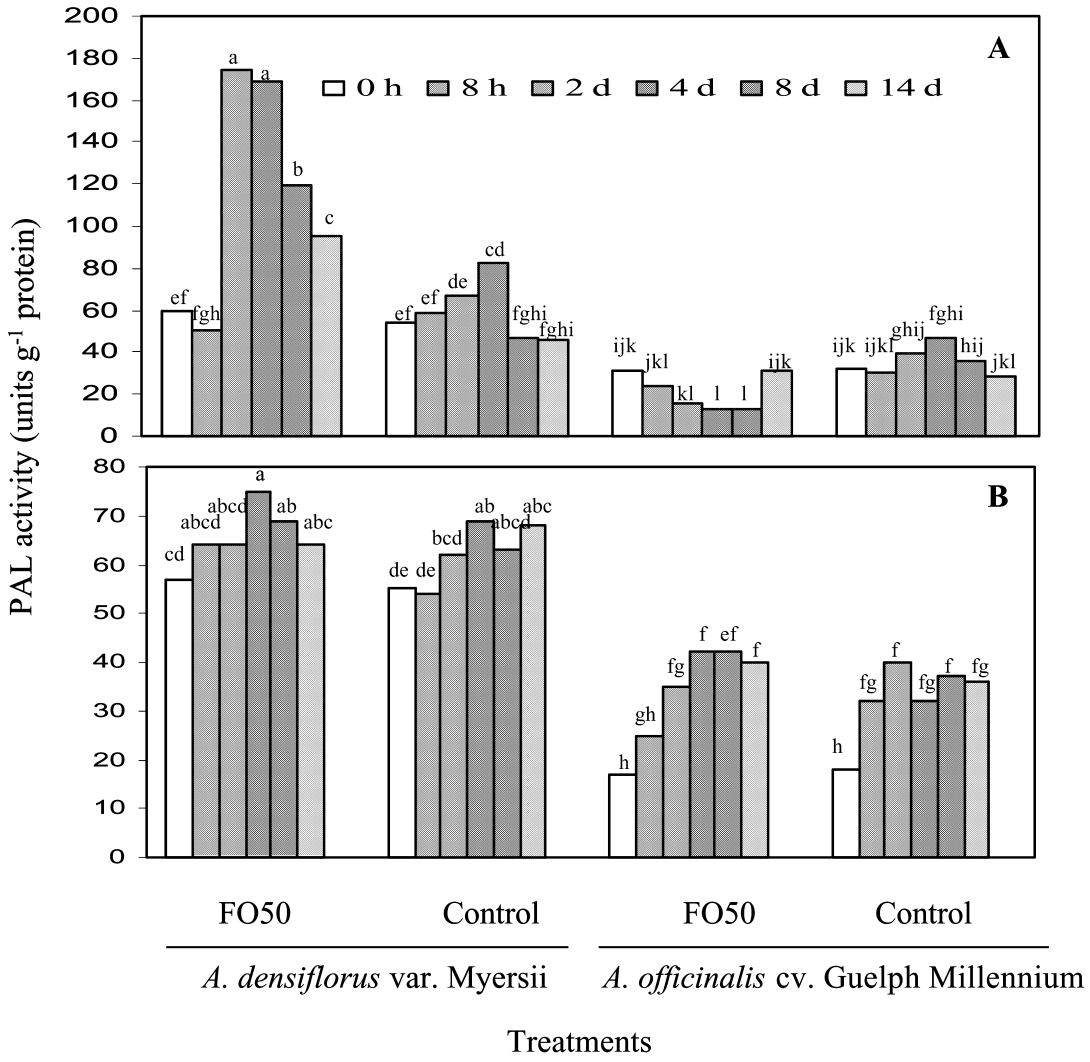

Figure 4. Phenylalanine ammonia-lyase (PAL) induction in roots (A) and spears (B) of 8-week-old asparagus plants either inoculated with *Fusarium oxysporum* f. sp. *asparagi* (FO50) or uninoculated (control) using hyphal-sandwich root inoculation. Within each tissue type, means with the same letter are not significantly different at $P = 0.05$ (test of least significant difference) and each mean is based on 6 replicates.

species tested in a greenhouse trial, which was consistent with previous research showing that *A. densiflorus* was highly resistant or immune to infection by Foa and Fp (Stephens et al., 1989). *A. densiflorus* responded rapidly after inoculation by the *Fusarium* species with the HR, which is typically associated with disease resistance (Dangl et al., 1996; Pontier et al., 1998). Susceptible *A. officinalis* did not show HR, and progressive fungal growth led to disease and necrosis of infected tissues. HR has been shown to be a powerful defense mechanism for most examples of race/cultivar-specific resistance and many cases of nonhost resistance (Jahnen and Hahlbrock, 1988; Somssich and Hahlbrock, 1998; Kamoun et al., 1999). The HR may act to confine the pathogen, locally activate defense genes, and induce systemic acquired resistance (SAR) (Pontier et al., 1998). In the present study, rapid and localized cell death in response to pathogen challenge directly prevented the spread of the pathogen into healthy tissues of *A. densiflorus*. Death of root epidermal cells was concurrent with the activation of POX and PAL defense enzymes and the release of compounds from

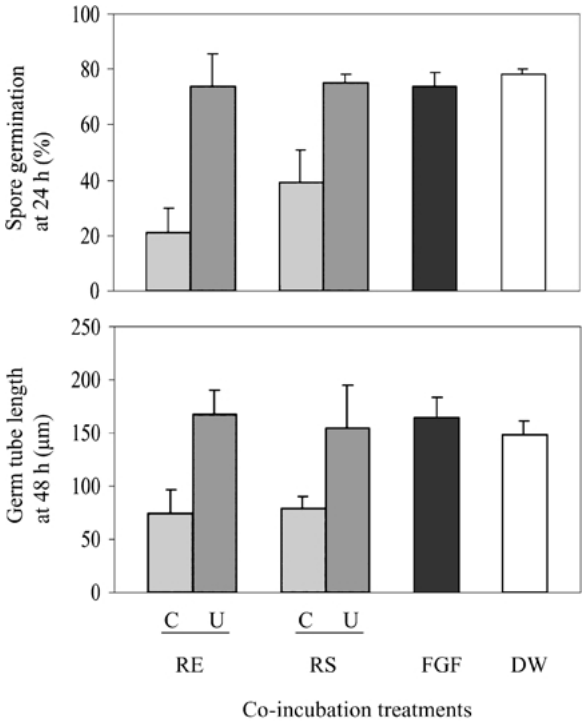

Figure 5. Spore germination and germ tube length of *Fusarium oxysporum* f. sp. *asparagi* (FO50) when co-incubated with root exudates (RE) and root segments (RS) from Foa-challenged (C) and unchallenged (U) *Asparagus densiflorus* var. Myersii plants, fungal germination fluid (FGF) and distilled water (DW). Each mean (±SE) is based on 6 replicate observations of 100 spores.

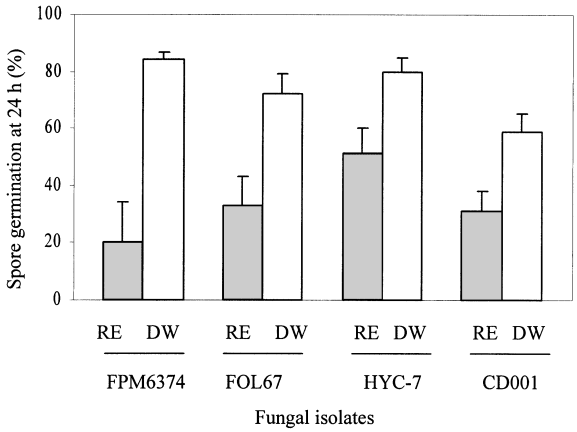

Figure 6. In vitro inhibitory effects of root exudates (RE) from *Asparagus densiflorus* var. Myersii challenged for 24 h with *Fusarium oxysporum* f. sp. *asparagi* (FO50) on spore germination of *F. proliferatum* (FPM6374), *F. oxysporum* f. sp. *lycopersici* (FOL67), *F. oxysporum* f. sp. *cyclaminis* (HYC-7) and *Colletotrichum destructivum* (CD001). Fungal spore germination in distilled water (DW) was used as controls. Each mean (±SE) is based on 6 replicate observations of 100 spores.

roots which could inhibit fungal spore germination and germ tube elongation.

Local or systemic changes in POX and PAL activities upon *Fusarium* inoculation were associated with HR induction in *A. densiflorus*. POX has been extensively implicated in multiple functions of plant disease resistance, including reinforcement of cell wall, lignin biosynthesis and hydrogen peroxide generation (Brisson et al., 1994; Milosevic and Slusarenko, 1996). At 8 h post-inoculation, HR was observed in roots of *A. densiflorus*. POX activity increased significantly at 8 h post-inoculation in roots and 2 d post-inoculation in spears, demonstrating systemic induction of POX since only roots were inoculated. Activation of POX might result in production of hydrogen peroxide which was involved in hypersensitive cell death, as observed in barley (Hückelhoven et al., 1999) and lettuce (Bestwick et al., 1998).

PAL, the first committed enzyme in the phenylpropanoid and flavonoid pathways, is involved in biosynthesis of phytoalexins, lignins and salicylic acid associated with disease resistance expression (Mauch-Mani and Slusarenko, 1996). Activation of PAL in *A. densiflorus* could directly affect accumulation of secondary toxic compounds, such as phytoalexins, which might be released in root exudates and on root segment surfaces from the inoculated plants to inhibit fungal spore germination and growth. The antifungal activity of root exudates was not affected by treatments with proteinase K and boiling, suggesting the production of a secondary metabolite with antifungal properties. Most importantly, the broad-spectrum inhibition of spore germination for several plant pathogenic fungi by root exudates may be one of the crucial defense mechanisms underlying *Fusarium* resistance of *A. densiflorus*. Identification and characterization of the inducible antifungal compounds could further facilitate the understanding of the resistance in *A. densiflorus*.

The occurrence of HR, systemic induction of POX and broad-spectrum antifungal properties in inoculated *A. densiflorus* plants may indicate the induction of SAR, as observed in other plants (Sticher et al., 1997; Van Loon et al., 1998). SAR was induced in numerous crops after inoculation with nonpathogenic *Fusarium* strains; subsequent infection of pathogenic *Fusarium* strains was suppressed (Alabouvette and Couteaudier, 1992; Larkin and Fravel, 1999).

In summary, we described activation of defense responses in *A. densiflorus* upon *Fusarium* inoculation. Rapid formation of the hypersensitive response as a result of attempted fungal invasion was accompanied by induction of POX and PAL activities and was correlated with production of antifungal compounds which inhibited spore germination and growth.

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References

Agrios GN (1997) How plants defend themselves against pathogens. In: Plant Pathology (pp 93–114) Academic Press, San Diego

Alabouvette C and Couteaudier Y (1992) Biological control of *Fusarium* wilt with nonpathogenic Fusaria. In: Tjamos EC, Papavizas GC and Cook RJ (ed) Biological Control of Plant Diseases (pp 415–426) Plenum Press, New York

Asiegbu FO, Daniel G and Johnsson M (1994) Defence related reactions of seedling roots of Norway spruce to infection by *Heterobasidion annosum* (Fr.) Bref. Physiological and Molecular Plant Pathology 45: 1–19

Beaudoin-Eagan LD and Thorpe TA (1985) Tyrosine and phenylalanine ammonia-lyase activities during shoot initiation in tobacco callus cultures. Plant Physiology 78: 438–441

Bestwick C, Brown I and Mansfield JW (1998) Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce. Plant Physiology 118: 1067–1078

Blok WJ, Zwankhuizen MJ and Bollen GJ (1997) Biological control of *Fusarium oxysporum* f. sp. *asparagus* by applying nonpathogenic isolates of *F. oxysporum*. Biocontrol Science and Technology 7: 527–541

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248–254

Brisson LF, Tenhaken R and Lamb CJ (1994) Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. Plant Cell 6: 1703–1712

Dangl JL, Dietrich RA and Richberg MH (1996) Death don't have no mercy: cell death program in plant–microbe interactions. Plant Cell 8: 1793–1807

Elmer WH, Johnson DA and Mink GI (1996) Epidemiology and management of the diseases causal to asparagus decline. Plant Disease 80: 117–123

Escandi AR and Echandi E (1991) Protection of potato from *Rhizoctonia* canker with binucleate *Rhizoctonia* fungi. Plant Pathology 40: 197–202

Fernandez MR and Heath MC (1989a) Interactions of the nonhost French bean (*Phaseolus vulgaris*) with parasitic and saprophytic fungi. I. Fungal development on and in killed, untreated, heat-treated, or blasticidin S treated leaves. Canadian Journal of Botany 67: 661–669

Fernandez MR and Heath MC (1989b) Interactions of the nonhost French bean (*Phaseolus vulgaris*) with parasitic and saprophytic fungi. II. Fungal development after inoculation by injection or wounding. Canadian Journal of Botany 67: 670–675

Hammerschmidt R, Nuckles EM and Kuc J (1982) Association of enhanced perioxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiological and Molecular Plant Pathology 20: 43–47

Hammond-Kosack KE and Jones JDG (1996) Resistance gene-dependent plant defense response. Plant Cell 8: 1773–1791.

Hoagland DR and Arnon DI (1950) Circular 347, California Agriculture Experimental Station, Berkeley

Hückelhoven R, Fodor J, Preis C and Kogel KH (1999) Hypersensitive cell death and papilla formation in barley attacked by the powdery mildew fungus are associated with hydrogen peroxide but not with salicylic acid accumulation. Plant Physiology 119: 1251–1260

Jahnen W and Hahlbrock K (1988) Cellular localization of nonhost resistance reactions of parsley (*Petroselinum crispum*) to fungal infection. Planta 173: 197–204

Kamoun S, Huitema E and Vleeshouwers VGAA (1999) Resistance to oomycetes: a general role for the hypersensitive response? Trends in Plant Science 4: 196–200

Kombrink E and Somssich IE (1995) Defense responses of plants to pathogens. In: Callow JA (ed) Advances in Botanical Research, Vol 21 (pp 1–34) Academic Press

Larkin RP and Fravel DR (1999) Mechanisms of action and dose-response relationships governing biological control of *Fusarium* wilt of tomato by nonpathogenic *Fusarium* spp. Phytopathology 89: 1152–1161

Lewis GD and Shoemaker PB (1964) Resistance of asparagus species to *Fusarium oxysporum* f. sp. *asparagi*. Plant Disease Reporter 48: 364–365

Mauch-Mani B and Slusarenko AJ (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Perenospora parasitica*. Plant Cell 8: 203–212

Milosevic N and Slusarenko AJ (1996) Active oxygen metabolism and lignification in the hypersensitive response in bean. Physiological and Molecular Plant Pathology 49: 143–157

Pontier D, Balague C and Roby D (1998) The hypersensitive response – a programmed cell death associated with plant resistance. Proceedings of the Academy of Sciences (Series III Science of Life) 321: 721–734

Somssich IE and Hahlbrock K (1998) Pathogen defence in plants – a paradigm of biological complexity. Trends in Plant Science 3: 86–89

Stephens CT and Elmer WH (1988) Comparison of technique for eliminating contaminants from asparagus seeds. HortScience 23: 1031–1032

Stephens CT, Vries RMD and Sink KC (1989) Evaluation of *Asparagus* species for resistance to *Fusarium oxysporum* f. sp. *asparagi* and *F. moniliforme*. HortScience 24: 365–368

Sticher L, Mauch-Mani B and Metraux JP (1997) Systemic acquired resistance. Annual Review of Phytopathology 35: 235–270

Tang XY, Xie MT, Kim YJ, Zhou JM, Klessig DF and Martin GB (1999) Overexpression of *Pto* activates defense responses and confers broad resistance. Plant Cell 11: 15–29

Van Loon LC, Bakker PAHM and Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology 36: 435–483

Xue L, Charest PM and Jabaji-Hare SH (1998) Systemic induction of perioxidase, 1.3-glucanase, chitinase and resistance in bean plants by binucleate *Rhizoctinia* species. Phytopathology 88: 359–365

Yang Y, Shah J and Klessig DF (1997) Signal perception and transduction in plant defense responses. Genes & Development 11: 1621–1639