Use of ELISA to Rapidly Screen Hazelnut for Resistance to Eastern Filbert Blight

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ABSTRACT

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A rapid and accurate screening system was developed to more rapidly identify resistance to eastern filbert blight based on an indirect enzyme-linked immunosorbent assay (ELISA) of greenhouse-inoculated hazelnut. Polyclonal antibodies were obtained from rabbits following immunization with antigens from pure cultures of Anisogramma anomala, the pathogen. One-thousand-fold dilution of the antiserum produced positive reactions to 1.7×10^5 dilutions of A. anomala–infected hazelnut tissue extracts, but did not react to 1.7×10^2 dilutions of healthy hazelnut tissue extracts. Symptomless plants infected by A. anomala were detected by the indirect ELISA 3 to 5 months after inoculation, an improvement over the 13- to 27-month incubation period required for susceptible genotypes to develop external symptoms of infection (cankers). ELISA was more sensitive and efficient than conventional microscopic assays (i.e., visualizing A. anomala mycelium in hand-sectioned plant tissue) in both healthy and infected extracts. The screening system was tested on selected progenies from populations segregating for a single, dominant resistance gene. ELISA detected 100% of the infections while microscopic examination detected only 36% of the infected samples. ELISA rapidly and reliably identifies hazelnut progeny with a gene conferring a high level of resistance derived from the cv. Gasaway.

Oregon's Willamette Valley hazelnut (*Corylus avellana* L.) production, which constitutes 98% of the U.S. crop and 5% of the world market, is threatened by eastern filbert blight (EFB). This disease is caused by *Anisogramma anomala* (Peck) E. Müller, an endemic parasite of *C. americana* Marsh. that is native to northeastern North America (18). All current hazelnut cultivars are susceptible (19) and infected trees are killed in 5 to 15 years (10). The disease was first identified in southwest Washington State in 1970 (6) and has spread to approximately a third of the hazelnut production area in the Pacific Northwest (20). The life cycle of the pathogen requires a minimum of 2 years, and the period from infection to symptom expression ranges from 13 to 27 months (21). The fungus *A. anomala* infects actively growing shoots and buds from bud break (mid to late March) through early shoot elongation (late April to early May) (14). Three to five fungicide applications are required during this time each spring to control the disease (14).

Genetic resistance offers the most promising solution to this important disease. One obsolete pollenizer, Gasaway, appears to possess such resistance (17). Neither cankers nor evidence of hyphal growth have been found in field-grown trees or in controlled inoculations of Gasaway scions (19). Screening progeny of this cultivar in the field, however, is slow and expensive, and depends on canker development up to 2 years after exposure (19). Field screening also requires a location isolated from commercial orchards and from breeding activity. In seedlings of highly susceptible cultivars (e.g., Royal or Ennis), microscopic examination of asymptomatic shoots 2 to 4 months after inoculation was relatively efficient to determine incidence of infection (14,21). However, progeny segregating for the Gasaway-derived resistance may have an additional moderate to high level of quantitative resistance (19), which renders microscopic detection of infections laborious (5). Progress in breeding for genetic resistance has been hindered by the lack of a rapid and reliable method to annually screen hundreds of selected genotypes that are segregating for Gasaway-derived resistance.

Enzyme-linked immunosorbent assay (ELISA) has been widely applied in plant pathology, including detection of fungal pathogens in host plant tissues (2). ELISAs based on polyclonal antibodies have been reported for the detection of many fungal plant pathogens in plant tissue. In some reports, cross-reaction with nontarget fungi was observed (7,8,15); however, other polyclonal antisera were highly specific (9, 12,13). Our objective was to develop an ELISA system for sensitive detection of A. anomala in inoculated C. avellana plants. The goal was to distinguish infected and noninfected genotypes segregating for Gasaway-derived resistance to EFB. A preliminary report has been published (4).

MATERIALS AND METHODS

Polyclonal antibody production. Ascospores of A. anomala were germinated aseptically on modified Murashige and Skoog agar medium (22) without the addition of bovine serum albumin, subcultured to the same medium without agar, and grown in the dark at 20°C. Mycelium for antibody production was filtered and washed with phosphate-buffered saline (PBS, pH 7.4), ground in liquid nitrogen, and lyophilized. Antiserum was obtained from New Zealand white, female rabbits injected subcutaneously with A. anomala mycelium (11). ELISA results reported here were obtained with antiserum taken after the fifth booster injection.

Antiserum reactivity. Reactivity and specificity of the antiserum prepared against A. anomala was first tested by immunoblotting (11). Fresh A. anomala mycelium, and healthy and infected hazelnut shoots, were prepared for immunoblotting by first grinding in liquid nitrogen and lyophilizing the samples. These samples, along with molecular weight standards (161-0304, Bio-Rad, Hercules, CA) were separated in a 12% gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (16), and the Western blot method of Towbin et al. applied (24).

ELISA standardization. For indirect ELISA standardization, the procedure of Harlow and Lane (11) was utilized. Homogenized, lyophilized C. avellana shoot-tissue samples were suspended in 1 ml of PBS (pH 7.4) and a dilution series was prepared in combination with serial dilutions of antiserum and alkaline phospha-

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tase-conjugated goat anti-rabbit immuno gamma globulin (IgG) (A8025, Sigma, St. Louis, MO). Reagent dilutions were selected that provided maximal differentiation between infected and healthy hazelnut samples.

For evaluation of double antibody sandwich–ELISA (DAS-ELISA), anti-*A. anomala* IgG was partially purified with sodium sulfate precipitation (11). The DAS-ELISA procedure of Clark and Adams (3) was used. The same dilutions of antigen, antiserum, conjugate, and substrate were used as in the assay standardization for indirect ELISA.

Inoculation of resistant and susceptible hazelnut. Dormant scion wood of *C. avellana* current commercial cultivars, progeny selected from populations segregating for resistance, and the resistant and susceptible parents used to create the populations, were collected from the Oregon State University research farm in December, stored at 0°C, and whip grafted onto rootstock layers in January to February 1992, 1993, and 1994, and then grown in the greenhouse (24°C day/18°C night). Four trees of each genotype were grafted. In 1992, 1993, and 1994, the total number of trees grafted was 772, 712, and 286 trees, respectively. All trees were grown in 3.7-liter pots containing a 1:1:1 growth medium of peat, pumice, and fine fir bark

supplemented with ground limestone and complete fertilizer (Peters 20-20-20, Osmocote 19-6-12, and Micromax Plus). The trees were ready for inoculation in 6 to 8 weeks when the shoots were expanded to four to five nodes.

Cankered shoots with stromata collected from diseased orchards in November to December of 1991, 1992, and 1993 were stored at –20°C. Inoculum was prepared as described by Stone et al. (21). Three to five expanding shoots per tree were labeled to identify the shoot apex at the time of inoculation. The labeled shoots were inoculated with the suspension three times each, at weekly intervals, with a hand-held pump sprayer. The trees were placed on benches in a randomized incomplete block design and inoculated in sets of approximately 60 trees per block over a 2-month period. The trees were covered with polyethylene bags for 5 days after each inoculation to maintain high humidity (14). Two layers of shade cloth were placed over benches in the greenhouse to minimize heat buildup under the bags. Noninoculated control trees were sprayed with deionized water and incubated similarly. Known susceptible genotypes included in each set served as controls on inoculum viability.

Detection of *A. anomala* in samples. The standardized indirect ELISA was first applied to the resistant and susceptible par-

ents used in the crosses, and several cultivars. Shoots for ELISA were collected from inoculated trees and noninoculated trees starting at 2 months up to 6 months postinoculation. Each shoot was sampled from the marked sites by hand-sectioning cambial and subcambial tissue with a razor blade, after which they were ground in liquid nitrogen and lyophilized. Microtiter plates were coated with a serial dilution of 6 mg of lyophilized plant tissue in PBS ($1:1.7 \times 10^2$ through $1:1.7 \times 10^6$). Antiserum and conjugate dilutions were 1:1,000 and 1:2,000 respectively. There were three replications of each sample per assay and the assays were done three times. The threshold optical density value for positive ELISA reactions was set at the average negative control plus 3.5 times the standard deviation (23). Diagnoses obtained by ELISA were confirmed by extensive microscopic examination of the remaining inoculated shoots (21). Cross-reaction with healthy plant tissue and two other fungi isolated from greenhouse-grown trees was evaluated with the indirect ELISA procedure. The fungi, *Cladosporium cladosporioides* (Fresen.) G. A. De Vries and a *Fusarium* sp., were isolated by culturing shoots on water agar and on potato dextrose agar. Tissue samples of these fungi were prepared for ELISA by scraping the mycelia from agar plate cultures and grinding in liquid nitrogen, followed by lyophilization.

The ELISA was further compared with microscopic examination on selected progeny from segregating seedling populations inoculated in the greenhouse. One comparison of ELISA and microscopic examination was performed with one shoot from 60 seedlings, and three susceptible and two resistant genotypes (parents and cultivars). Shoots were harvested by pruning two nodes above and two nodes below the inoculated shoot segment (approximately 10 to 15 cm of shoot) 20 weeks after inoculation. One shoot from each tree was divided into two samples by first cutting transversely and then cutting lengthwise, resulting in four pieces. Opposing quarters were evaluated for infection by indirect ELISA (10 mg of PBS per ml) or by microscopic examination (21). Shoots were prepared for microscopic examination by hand-sectioning xylem, and the sections were stained with trypan blue in lactoglycerol or lacto-phenol (21). The sections were examined for the presence of *A. anomala* hyphae by bright field microscopy at 100× to 400× magnification. For ELISA, shoots were prepared as described above and each shoot extract was replicated three times per assay. Diagnoses were confirmed by microscopic examination of all the remaining inoculated shoots from the 60 seedlings. The comparison was repeated with three inoculated shoots from each of a second set of 60 trees from the 1994 inoculated selections, with ELISA
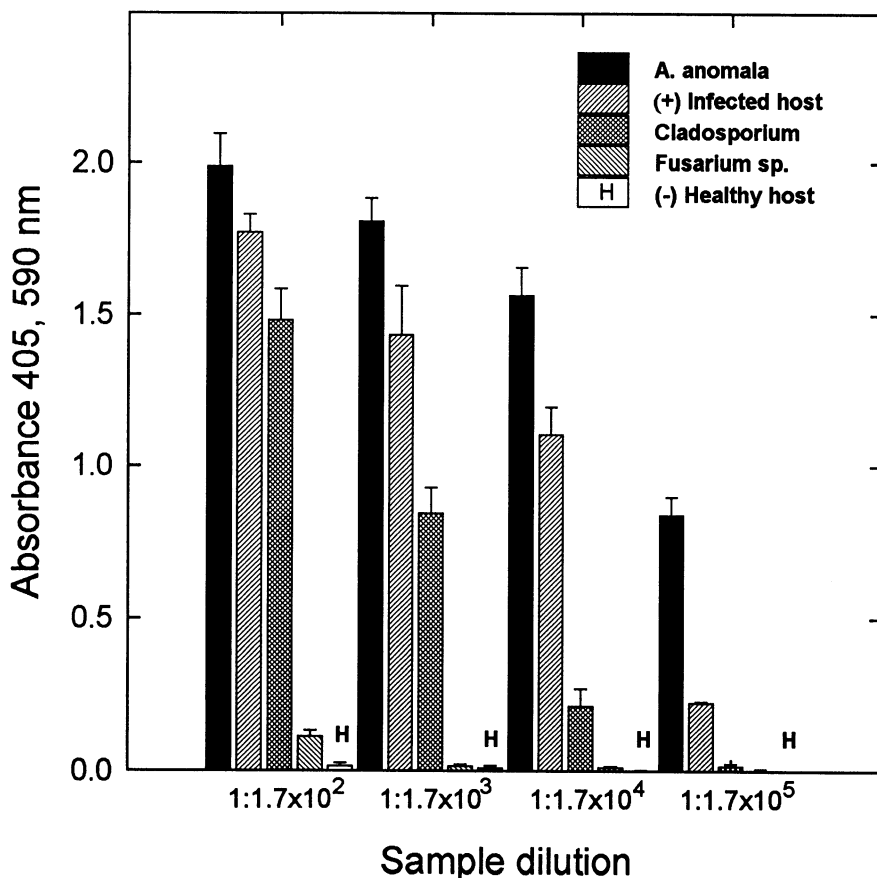

Fig. 1. Sensitivity and reactivity of an antiserum prepared against *Anisogramma anomala* with extracts from *A. anomala*, *Cladosporium cladosporioides*, and a *Fusarium* sp., and infected and healthy hazelnut tissue evaluated by indirect enzyme-linked immunosorbent assay.

and microscopic examination evaluated on all three shoots from each tree. Five known susceptible and four known resistant genotypes (parents and cultivars) were included in the second comparison. Discrepancies between the two methods were reevaluated either with ELISA or microscopically to confirm the diagnoses and to determine which method produced the false negative or false positive.

RESULTS

ELISA standardization. The maximum differentiation of infected and healthy plant tissues was obtained at an antiserum dilution of 1:1,000 for each conjugate concentration tested (data not shown). Cross-reaction with host tissue occurred only at the lowest antiserum dilution evaluated, 1:500. An antiserum dilution of 1:1,000 and conjugate dilution of 1:2,000 were selected for the standardized, indirect ELISA. Partial purification of the antiserum to IgG resulted in a reduced ability to detect *A. anomala* mycelium and to detect this fungus from infected host tissues relative to indirect ELISA (data not shown). No reaction to healthy hazelnut tissue occurred with DAS-ELISA; however, indirect ELISA was more sensitive than DAS-ELISA for the detection of *A. anomala* in hazelnut with this polyclonal antibody.

Immunoblots of fungal and plant protein extracts, with antiserum prepared against *A. anomala,* resulted in the reaction with many proteins from *A. anomala* and many proteins from infected hazelnut shoots, but none from healthy hazelnut shoots (data not shown). With ELISA, the antiserum did not react to healthy hazelnut extracts at dilutions of $1:1.7 \times 10^2$ (data not shown). Lyophilized mycelium of *A. anomala* and infected hazelnut tissue reacted positively at dilutions of $1:1.7 \times 10^6$ (data not shown) and $1:1.7 \times 10^5$ (Fig. 1), respectively. The antiserum cross-reacted with *C. cladosporioides* at $1:1.7 \times 10^3$ dilution, but cross-reactivity with this fungus was relatively insignificant at a dilution of $1:1.7 \times 10^4$ (Fig. 1). The antiserum also failed to react significantly with healthy hazelnut tissue or with lyophilized mycelium of a *Fusarium* sp. (Fig. 1).

Detection of *A. anomala* in hazelnut. Indirect ELISA was first tested on greenhouse-inoculated trees (cultivars with known reaction) with postinoculation intervals ranging from 2.7 to 6 months (Table 1). The standardized, indirect ELISA detected 98% of infected shoots and produced no false positives from shoot tissue extracts diluted up to $1:1.7 \times 10^5$. In several noninfected samples, the absorbance readings were close to the positive-test threshold at the highest dilutions of $1:1.7 \times 10^4$ and $1:1.7 \times 10^5$. The average reading for one infected tissue diluted $1:1.7 \times 10^5$ fell below the positive threshold resulting in a false negative in one assay. Based on ELISA, greenhouse inoculations

resulted in infection of 92% of inoculated shoots of all trees of the susceptible cultivars, whereas genotypes with Gasaway-derived resistance remained healthy (data not shown). Infected trees were thereby correctly detected by ELISA, as indicated by careful reexamination of all the shoots microscopically.

The first comparison of indirect ELISA with microscopic examination resulted in the ELISA procedure detecting infection in 13 trees, including the three known susceptible cultivars (Table 2). Examination of hand sections of shoot tissue by microscopy, however, failed to detect any infections. Subsequent reexamination of the

hand sections resulted in detection of only 36% of the infected shoots. Indirect ELISA failed to detect one microscopically detected infected shoot. This failure could be attributable to a sampling error since only one quarter of one shoot was tested by ELISA.

In the second comparison, indirect ELISA detected 23 infected trees, including the five known susceptible cultivars (Table 2). After the ELISA was conducted, the presence/absence of *A. anomala* was investigated by microscopic examination of all three shoots from each tree and 24 trees were found to be to be infected. Retesting by ELISA of the one tree nega-

Table 1. Detection of *Anisogramma anomala* with indirect enzyme-linked immunosorbent assay (ELISA) on greenhouse- inoculated hazelnut cultivars

Genotype	Expected disease reaction[a]	Months from inoculation	Absorbance[b]	Diagnosis[c]
Healthy control	S	6	0.02 ± 0.00	Healthy
Barcelona	S	3.3	0.39 ± 0.06	Infected
		4.3	1.68 ± 0.06	Infected
		5.6	0.01 ± 0.00	Healthy
Casina	S	2.7	1.95 ± 0.05	Infected
		2.7	0.04 ± 0.01	Healthy
		3.3	0.44 ± 0.04	Infected
Cosford	S	3.3	1.50 ± 0.06	Infected
		4.2	1.75 ± 0.11	Infected
		6	1.49 ± 0.01	Infected
Creswell	S	2.7	0.82 ± 0.04	Infected
		3.3	0.14 ± 0.02	Infected
		3.3	0.18 ± 0.05	Infected
Ennis	S	3.3	1.64 ± 0.05	Infected
		4.3	0.55 ± 0.10	Infected
		5.8	0.81 ± 0.03	Infected
Gasaway	R	2.7	0.04 ± 0.01	Healthy
		3.3	0.02 ± 0.01	Healthy
		4.3	0.04 ± 0.01	Healthy
		5.3	0.03 ± 0.00	Healthy

[a] R = resistant and S = susceptible; the disease reaction expectation was based on previous field studies (19). The healthy hazelnut control genotype was the cv. Ennis.

[b] ELISA was based on 6 mg of lyophilized hazelnut tissue in 1 ml of phosphate-buffered saline and reagent dilutions of 1:1,000 for antiserum and 1:2,000 for conjugate. Absorbances (A_{405}) are means of three wells followed by the standard deviation.

[c] ELISA-based positive threshold set at the average of healthy host plus 3.5 times the standard deviation. Each diagnosis based on sampling inoculated shoots from one tree. All the shoots were reexamined microscopically to reconfirm the diagnoses.

Table 2. Results of two tests comparing indirect enzyme-linked immunosorbent assay (ELISA) with microscopic examination of stained, hand-sectioned, inoculated hazelnut shoot tissue for determining the presence of *Anisogramma anomala* 20 weeks after inoculation

Test no./type	Positive diagnosis	Negative diagnosis	False positive	False negative	Total
Test 1[a]					
Indirect ELISA	13	47	0	1[b]	60
Microscopic examination	0	60	0	14[c]	60
Test 2[d]					
Indirect ELISA	23	37	0	1[b]	60
Microscopic examination	24	36	0	0	60

[a] Test 1 utilized one shoot divided into two samples: one for ELISA, one for microscopic examination.

[b] Retesting of this sample by ELISA resulted in a positive diagnosis for infection.

[c] Retesting of all 60 shoots by microscopic examination found five of 14 infected trees (36%). Contradictions were clarified by examining the remaining inoculated shoots to confirm the diagnoses.

[d] Test 2 utilized three inoculated shoots per test tree by first sampling each shoot for ELISA and repeated microscopic examination of all three hand-sectioned shoots from each tree.

tive in the first ELISA resulted in a positive diagnosis after hyphae were found microscopically.

DISCUSSION

An effective, indirect ELISA system was developed for detection of *A. anomala* infections in hazelnut in a relatively short time after inoculation (3 to 5 months). The time after inoculation when ELISA can detect infections represents a substantial improvement over the 13- to 27-month period that is required for external symptoms to develop in the field. Unlike many other polyclonal antisera produced against plant pathogenic fungi, the antiserum we obtained proved specific to *A. anomala* without cross-reactivity to healthy tissue. The indirect ELISA did cross-react to relatively high concentrations of antigens prepared from a pure culture of *C. cladosporioides*. This cross-reactivity was significantly reduced, however, by diluting antigen extracts to greater than $1:1.7 \times 10^4$. The risk of cross-reaction is likely even lower in tree tissue samples because young, newly emergent hazelnut tissue has no other important pathogens and epiphytic fungi do not grow in high concentration on expanding shoots.

Indirect ELISA allowed for more efficient and rapid sampling of hazelnut tissue than did microscopic examination. In addition, a low number of infections was detected (36%) with microscopic examination when compared directly with ELISA. Previous reports have stated that microscopic examination was efficient for the detection of *A. anomala* in hazelnut (14,21). We attribute the relatively high numbers of false negatives obtained from microscopic examinations in this study to the use of progenies of crosses with moderate to high levels of quantitative resistance. Our DAS-ELISA was less sensitive than indirect ELISA, possibly due to the loss of IgG in the purification process or the loss of other immunoglobin classes important to *A. anomala* detection sensitivity.

Greenhouse inoculations provided reliable screening of hazelnut genotypes for resistance to EFB (14,21). Replication (three to four trees) of each genotype was important as escapes were noted in the 3 years of inoculation studies (5). Similarly, Johnson et al. (14) achieved 79% infection in greenhouse inoculations of the susceptible cvs. Ennis and Royal. Indirect ELISA was used to identify genotypes with

Gasaway-derived resistance to *A. anomala* in the segregating populations of hazelnut. Progress in breeding for genetic resistance to EFB will be hastened by this technique, as hundreds of selected genotypes can be efficiently screened in the greenhouse. The earliest consistent detection following inoculation in the greenhouse with ELISA was about 3 months. The indirect ELISA data reported in this paper did not evaluate quantitative resistance, since genotypes were classified either as infected or healthy. Identification of quantitative resistance to this destructive pathogen on *C. avellana* will continue to depend on field evaluations at a remote location (19). Antiserum used in these studies is available to researchers interested in using ELISA to detect *A. anomala* in hazelnut, upon request to S. A. Mehlenbacher.

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