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Strain origin drives virulence and persistence of *Xylella fastidiosa* in alfalfa

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Generalist pathogens frequently exist as a complex of genetically differentiated strains, which can differ in virulence and transmissibility. A description of the extent to which strain variability mediates host species competence is needed to understand disease dynamics for systems with both host and pathogen strain diversity. This study tested the hypothesis that strain-specific variation of a generalist vector-borne plant pathogen, *Xylella fastidiosa*, affects disease severity in alfalfa (*Medicago sativa*) and competence of this crop as a reservoir host. Alfalfa seedlings were inoculated with one of 23 *X. fastidiosa* isolates collected from different hosts, eight identified as belonging to an almond strain, and the remainder from a grape strain. Pathogen population, symptom severity and infection incidence were compared over five successive harvests. Infected plant size, measured mainly by plant height, internode length and above ground biomass, was reduced up to 50% compared to buffer-inoculated controls, and more severe symptoms were observed at later harvests and for higher pathogen populations. Grape isolates had higher bacterial populations within alfalfa than almond isolates. In addition, infection with grape isolates resulted in more severe alfalfa stunting than that caused by almond isolates. Moreover, there was a strong positive relationship between isolate multiplication rate and both symptom severity and infection persistence (i.e. maintenance of chronic infection within host). Isolates with low initial populations had low incidence at the final harvest, with one isolate dying out altogether. The results showed that *X. fastidiosa*-genetic diversity contributed to variation in alfalfa disease severity. The results also suggest that pathogen strain may mediate host competence via differences in bacterial population density and persistence.

Keywords: disease reservoir, disease spread, infection dynamics, pathogen strain

Introduction

For generalist pathogens, for which host species may vary in their susceptibility, disease prevalence depends on host community composition (Power & Mitchell, 2004) and proximity to alternative hosts (Groves *et al.*, 2002). Such variation may result in pathogen spillover from competent alternative hosts (i.e. reservoirs) to focal hosts. In addition to host-specific variation in infectivity or susceptibility to infection, there exists variation associated with genetic differentiation within the pathogen itself. Widespread and generalist pathogens commonly exist as a complex of different strains (Brisson & Dykhuizen, 2004; Schuenzel *et al.*, 2005). These strains can differ biologically with respect to their vector transmissibility (Power, 1996; Lucio-Zavaleta *et al.*, 2001) or impact on hosts (Almeida *et al.*, 2008). Plant pathogens, in particular, can show large differences in virulence (herein defined as pathogen multiplication) and reductions in host growth, development, reproduction or survival (i.e. aggressiveness; Hopkins, 1985; Kema *et al.*, 1996), to the extent that relatively avirulent strains have been investigated for their therapeutic potential against more virulent strains (Jaynes & Elliston, 1980). The relative prevalence of different strains may vary temporally, with vector (Power, 1996) or host species (Brisson & Dykhuizen, 2004) playing a role in strain turnover or maintenance. Thus host and strain diversity may interact to determine virulence (Nesme *et al.*, 1994) and host competence for generalist pathogens (Lopes *et al.*, 2009). An understanding of these interactions is important for predicting and managing pathogen outbreaks.

One disease system for which both host species and pathogen strain variation influence disease dynamics is for the plant pathogen *Xylella fastidiosa*. This xylem-limited bacterium is widespread throughout the Americas, spread primarily by a group of xylem sap-feeding leafhoppers (Severin, 1949). *Xylella fastidiosa* is a generalist pathogen, able to infect a wide range of native, weedy and agricultural plants. However, both pathogen

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population density (Hopkins & Thompson, 1984; Hill & Purcell, 1995; Almeida & Purcell, 2003) and disease severity differ greatly among hosts (Almeida *et al.*, 2008). As with other generalist pathogens, *X. fastidiosa* prevalence is affected by surrounding plant communities. In northern coastal California, disease prevalence in vineyards follows a pattern of primary spread from alternative hosts in riparian habitats (Purcell, 1975). Meanwhile, in the central valley of California linkages exist among a complex of *X. fastidiosa* diseases, with spread occurring among three economically important crops: grape, almond, and alfalfa fields, causing Pierce's disease of grape, almond leaf scorch, and alfalfa dwarf disease, respectively (Thomson *et al.*, 1978; Purcell & Frazier, 1985).

Historically, interest in alfalfa dwarf (AD) disease focused on alfalfa as a pathogen source to vineyards rather than on understanding the alfalfa-*X. fastidiosa* association (Weimer, 1931, 1936). Widespread stunting of alfalfa plants was observed throughout California in the 1930–40s (Weimer, 1937), which coincided with Pierce's disease epidemics in the region (Hewitt *et al.*, 1949). Studies at that time also documented a disease gradient in vineyards, with higher incidence near alfalfa (Hewitt & Houston, 1941). However, neither the causal agent of AD nor its vectors were identified at the time. It was not shown until decades later that *X. fastidiosa* could be recovered from alfalfa plants (Thomson *et al.*, 1978), with the same group of leafhopper vectors responsible for transmission (Hewitt *et al.*, 1946; Purcell & Frazier, 1985). Collectively this work supports the hypothesis that alfalfa can function as a pathogen reservoir. However, detailed measures of *X. fastidiosa* virulence in alfalfa are lacking, making it difficult to describe accurately the importance of either disease in alfalfa or the role of alfalfa as a pathogen source for other hosts.

The epidemiology of *X. fastidiosa* diseases is complicated further by underlying genetic variation in the pathogen. *Xylella fastidiosa* shows significant genetic structure that is attributable largely to host plant type (Hendson *et al.*, 2001; Schuenzel *et al.*, 2005; Almeida *et al.*, 2008). This is important epidemiologically because *X. fastidiosa* strains also differ biologically with respect to pathogen infectivity, population growth rate, within-host movement rate and host symptom severity (Hopkins, 1985; Hill & Purcell, 1995; Almeida & Purcell, 2003; Almeida *et al.*, 2008). Given that *X. fastidiosa* was not isolated from alfalfa during disease epidemics of the 1930s and 40s and that AD has received little attention since then, it is not know what role alfalfa has played in strain dynamics. The significance of pathogen strain variation is unknown for AD disease severity and the potential of alfalfa as a reservoir host. Because *X. fastidiosa*'s within-host population mediates transmission by vectors (Hill & Purcell, 1997), strains reaching higher populations in alfalfa may be disseminated more frequently to susceptible plants, potentially replacing previously dominant genotypes (Power, 1996). Such turnover could affect the epidemiology of secondary hosts if the focal host also functions as a pathogen reservoir. In this study, the hypothesis that pathogen strain affects susceptibility to infection and competence of alfalfa as both a focal crop plant and an alternative host of *X. fastidiosa* was tested.

Materials and methods

Bacterial isolates

A total of 23 *X. fastidiosa* isolates were used in this study, representing both grape (n = 15) and almond (n = 8) genetic groupings. Isolates were collected from diseased grape (n = 10) and almond (n = 11) or symptomless alfalfa (n = 2) plants in different regions of California (North Coast, Central Valley and South) and Mexico (Baja California) (Table 1). All isolates were triply-cloned and stored at −80°C. 'Strain origin' is used throughout to refer to grape vs. almond genetic groupings, whereas 'isolate' refers to one of the 23 collected *X. fastidiosa* lines. Frozen cells of all 23 isolates were plated on Periwinkle wilt gelrite solid medium (Hill & Purcell, 1995) and were grown for two passages at 28°C. Because some of the isolates (SN1, M35, ALS1, ALS2, ALS9, M12, M23 and SJV1) had not been typed previously, their identity as grape or almond strain origins was confirmed using diagnostic primers RST31 and RST33 (Minsavage *et al.*, 1994). Total DNA was extracted from colonies of all plated isolates using a commercial kit (Qiagen DNeasy), and amplified using polymerase chain reaction (PCR). The RST31-33 PCR amplified-product was digested with the restriction enzyme *Rsa*I. Strain origin was determined based on the number and size of resulting fragments (Hendson *et al.*, 2001).

Test plants and bacterial inoculation

Seedlings of the commercial alfalfa cultivar WL625HQ were grown individually in 10 cm 0·5 L pots with Supersoil potting soil (Rod Mclellan Company). Four weeks after germination, one of each of the 23 *X. fastidiosa* isolates was mechanically inoculated into these seedlings. *Xylella fastidiosa* suspensions with densities of 10^8 to 10^9 colony forming units (CFU) mL^{-1} estimated by dilution-plating were prepared for inoculation by suspending cells in 0·5 mL of succinate-citrate buffer (Hopkins & Thompson, 1984) after 7–10 days of growth of the second passage; isolates in the grape strain grew faster than those in the almond strain. Inoculations with these suspensions were made on the primary stem of 12 to 15 cm tall plants at 5 cm above soil level, following the pin-prick method described by Hill & Purcell (1995). The same grape isolate suspensions were inoculated into the stem of 3–5 healthy plants of grapevines (*Vitis vinifera* cv. Cabernet Sauvignon) and almond isolates were inoculated into sweet almond (*Prunus dulcis* cv. Sonora-Hansen) to serve as positive controls for inoculum infectivity. Groups of alfalfa plants inoculated with buffer alone were used as healthy negative controls. Because of the large number of isolates tested, it was not possible to make all

Table 1 Pathogen incidence and population density of *Xylella fastidiosa* isolates in plants of *Medicago sativa* at successive harvests after mechanical inoculation

Inoculation date/Isolate (California county or country)	Host of origin (strain group)	Proportion of infected plants				Mean concentration [log(CFU g^{-1} tissue)] ±SE			
		1st cut (8[a])	2nd cut (14)	3rd cut (21)	5th cut (35)	1st cut (8)	2nd cut (14)	3rd cut (21)	5th cut (35)
15/03/2007									
Hopland (Mendocino)	Grape (G)	6/12[b]	1/7	9/9	10/14	6·6 ± 0·5	8·0	8·6 ± 0·2	7·9 ± 0·5
Napa Silverado (Napa)	Grape (G)	5/11	4/9	4/9	4/8	6·7 ± 0·6	7·8 ± 0·4	8·2 ± 0·4	7·4 ± 1·0
STL (Napa)	Grape (G)	7/9	4/8	4/7	5/12	7·2 ± 0·4	8·7 ± 0·2	7·6 ± 0·5	7·0 ± 0·5
SN1 (Fresno)	Alfalfa (G)	7/9	6/8	7/8	10/13	8·1 ± 0·5	8·6 ± 0·2	7·9 ± 0·3	8·2 ± 0·3
M12 (Kern)	Almond (A)	7/9	5/8	4/8	3/12	6·3 ± 0·4	7·6 ± 0·2	7·3 ± 0·5	6·3 ± 0·4
Mock	–	0/14	0/14	0/14	0/14	–	–	–	–
30/03/2007									
Medeiros (Fresno)	Grape (G)	6/6	6/9	6/6	9/9	8·1 ± 0·1	8·6 ± 0·2	8·4 ± 0·2	9·5 ± 0·1
Conn (Napa)	Grape (G)	9/9	4/8	4/8	9/11	7·3 ± 0·4	6·5 ± 0·5	7·4 ± 0·5	8·8 ± 0·4
M23 (Kern)	Almond (G)	7/7	7/7	6/7	6/6	8·0 ± 0·4	8·8 ± 0·2	8·6 ± 0·1	9·3 ± 0·2
ALS1 (San Joaquin)	Almond (G)	8/8	8/8	7/7	7/8	7·9 ± 0·2	8·2 ± 0·2	8·7 ± 0·2	9·0 ± 0·2
ALS6 (San Joaquin)	Almond (A)	7/7	6/8	5/7	7/7	7·1 ± 0·3	7·9 ± 0·4	7·4 ± 0·2	9·1 ± 0·4
Butte (Butte)	Almond (A)	9/9	9/9	Ct[c]	12/13	8·3 ± 0·2	7·6 ± 0·3	–	9·0 ± 0·2
ALS4 (San Joaquin)	Almond (A)	7/9	4/7	1/8	4/12	7·7 ± 0·2	6·8 ± 0·2	7·7	7·4 ± 0·5
ALS9 (San Joaquin)	Almond (A)	6/9	5/8	Ct[c]	8/12	8·0 ± 0·2	7·6 ± 0·4	–	7·0 ± 0·5
Mock	–	0/7	0/6	0/6	0/11	–	–	–	–
10/04/2007									
Buena Vista (Kern)	Grape (G)	9/9	9/9	9/9	10/10	8·4 ± 0·2	8·4 ± 0·1	8·7 ± 0·1	8·2 ± 0·4
Traver (Tulare)	Grape (G)	8/9	9/9	7/7	10/10	8·7 ± 0·0	8·6 ± 0·1	8·8 ± 0·2	8·8 ± 0·3
Pavich (Kern)	Grape (G)	8/9	9/9	9/9	10/10	8·3 ± 0·1	8·6 ± 0·2	8·2 ± 0·1	9·2 ± 0·2
Baja#5 (Mexico)	Grape (G)	6/9	7/8	7/9	6/10	7·3 ± 0·2	8·1 ± 0·3	8·7 ± 0·1	8·1 ± 0·4
UCLA (Los Angeles)	Grape (G)	5/9	0/9	1/9	0/13	6·3 ± 0·3	–	5·7	–
M35 (Fresno)	Alfalfa (G)	9/9	6/8	6/7	8/10	8·3 ± 0·1	8·6 ± 0·4	7·6 ± 0·4	8·2 ± 0·5
SJV1 (San Joaquin)	Almond (G)	4/9	2/9	5/9	3/8	6·0 ± 0·2	7·3 ± 0·5	7·2 ± 0·6	7·9 ± 1·1
ALS2 (San Joaquin)	Almond (A)	6/9	3/9	4/9	5/12	7·4 ± 0·2	7·8 ± 0·4	7·1 ± 0·3	6·3 ± 0·4
Glenn (Glenn)	Almond (A)	6/9	5/8	1/9	11/13	7·9 ± 0·2	8·4 ± 0·2	5·3	7·0 ± 0·4
Dixon (Solano)	Almond (A)	3/9	3/9	1/9	12/16	7·5 ± 0·2	8·3 ± 0·5	8·0	7·7 ± 0·5
Mock	–	0/7	0/7	0/7	0/10	–	–	–	–

[a]Number within parenthesis represents time (weeks) after inoculation.
[b]Number of infected plants over the total number inoculated.
[c]Data not obtained due to plate contamination during bacterial isolation.

inoculations on the same day. Instead, groups of isolates plus separate healthy control groups were inoculated on one of three days, with 50 plants per isolate and a buffer-inoculated healthy control group (Table 1).

Once alfalfa plants reached the flowering stage (after 6–8 weeks of growth) all plants inoculated with *X. fastidiosa*, along with their corresponding control plants, were cut to 5 cm above soil surface to mimic natural harvesting that occurs in alfalfa fields. The experiment was conducted for five successive harvests. Throughout the experiment, plants were kept in a heated (25 ± 5°C) vector-proof greenhouse and irrigated three to four times per day. At the second harvest, a slow-release fertilizer (Osmocote 14-14-14; 3-month formulation) was applied on the soil surface of each potted alfalfa plant.

Xylella fastidiosa infection and plant response among successive harvests

Xylella fastidiosa infection in plants and plant response to infection were estimated at 8, 14, 21 and 35 weeks post inoculation, corresponding with the first, second, third and fifth harvests, respectively. At each of these times 7–16 replicate alfalfa plants per *X. fastidiosa* isolate, and healthy controls, were randomly selected from the initial group of 50 plants per isolate or control group. Tissue samples were collected from this set of plants to estimate infection via plate culturing and measurements were made of plant morphological characters. A different set of plants was selected at each harvest number to ensure that replicates were independent across harvests.

Samples for bacterial culturing consisted of 0·15 g of stem section removed from each plant at 5 cm above soil level, which was surface sterilized and ground in a tissue homogenizer prior to being dilution plated (10^0, 10^{-2} and 10^{-4}) to determine the incidence of infection and pathogen population within plants, as described by Hill & Purcell (1995). At the first harvest, samples were taken from the inoculated stem, whereas axillary stems growing off the inoculated stem were sampled in following harvests. An individual plant was considered infected if the

X. fastidiosa population density (no. of *X. fastidiosa* CFU g^{-1} plant material) recovered from sample culturing was greater than zero.

Several plant morphological characters were measured among harvests to determine the effect of *X. fastidiosa* infection on alfalfa growth, including: i) number of stems, ii) plant height (from soil surface to the tip of tallest stem), iii) number of nodes on the tallest stem, iv) internode length, v) leaflet length, vi) leaflet width, and vii) above ground biomass. For leaflet size the apical leaflet of the seventh node on the tallest stem was selected. For biomass, all stems and leaves harvested above 5 cm from each plant were weighed after drying at ~60°C for 72 h.

Statistical analysis

All measures among alfalfa harvests were made on independent plants. Infections by *X. fastidiosa* were compared among treatments in two ways. First, the proportion of plants infected as a function of harvest number and *X. fastidiosa* strain (almond vs. grape origins) was compared in a generalized linear mixed model with binomial errors and pathogen isolate as a random variable (function 'lmer' in R; (Crawley, 2007)). This framework was required to appropriately describe the nature of different *X. fastidiosa* isolates as random, nested samples within either almond or grape strains. Next, *X. fastidiosa* population densities in infected plants (log[no. of *X. fastidiosa* CFU g^{-1} of plant tissue]) were compared among treatments using a linear mixed effects ANOVA (function 'lme' in R; (Crawley, 2007)) with *X. fastidiosa* strain and alfalfa harvest as fixed effects, and pathogen isolate as a random variable. Significant interactions were followed up with pairwise *t*-tests among harvests within a strain and between strains within a given harvest number, with adjustment of α for multiple comparisons.

Plant infection incidence (proportion of plants infected) and infection levels (bacterial population density) were also used to investigate pathogen persistence (i.e. maintenance of chronic infection within host) in this system, in two different ways. First, mean bacterial population densities in infected plants and the proportion of plants infected were correlated for a given *X. fastidiosa* isolate across all harvests and strains. In addition, a generalized linear model with binomial errors (Crawley, 2007) was used to test for effects of the initial (first alfalfa harvest) mean bacterial populations in infected plants and strain origin on the final (fifth alfalfa harvest) proportion of plants infected for a given isolate.

The final set of analyses related to alfalfa response to infection with *X. fastidiosa*. To estimate stunting due to infection, seven plant characteristics were compared among alfalfa harvests and between almond and grape origins: plant height, above ground biomass, number of stems, number of nodes, leaflet width, leaflet length, and internode length. Because of high variation among the healthy controls in different inoculation groups, these seven measures for each plant were divided by the mean character value for the appropriate buffer-inoculated control group to yield relative plant character values. Thus individual plant metrics were expressed as a percentage of the control group ($[x_{infected}/\bar{x}_{control}]*100$), which standardizes differences among inoculation groups.

For comparisons among treatments, principal components (PC) analysis was used on these seven relative plant responses to simplify description of the plant response to infection (Crawley, 2007). The effects of alfalfa harvest, bacterial population density and strain origin on only the most important principle components were tested using separate linear mixed effects ANOVAS with harvest and origin as fixed effects, *X. fastidiosa* population density as a covariate, and isolate as a random factor. α values were adjusted to reflect the multiple tests. Stepwise deletion of non-significant interactions from the full model, with *F* tests to determine whether deletion significantly increased model deviance, was used to determine the minimum adequate description of the data (Crawley, 2007). Significant effects on PCs were followed up with linear mixed effects ANOVAS on the individual plant traits that had high loading scores in a given PC.

Results

All isolates of *X. fastidiosa* were recovered from inoculated grape or almond positive control plants up to 3 months after inoculation, and all samples recovered were confirmed to be *X. fastidiosa* via PCR with diagnostic primers. Moreover, all isolates caused disease symptoms in their respective grape or almond control host plants, indicating their viability and pathogenicity in homologous inoculations. Conversely, none of the buffer-inoculated control plants tested positive for *X. fastidiosa*, indicating that results were not influenced by contamination.

Plant infection dynamics

The proportion of plants that tested positive for *X. fastidiosa* was affected significantly by a harvest number by pathogen strain interaction ($\chi^2 = 11{\cdot}380$; d.f. = 3; $P = 0{\cdot}0098$). Grape isolates tended to have higher infection incidence than almond, with that difference being significant at the third harvest (Fig. 1). *Xylella fastidiosa* population densities in infected plants showed significant effects of not only *X. fastidiosa* strain ($F_{1,21} = 4{\cdot}664$; $P = 0{\cdot}0425$), but also harvest number ($F_{3,520} = 8{\cdot}301$; $P < 0{\cdot}0001$) and a harvest by strain interaction ($F_{3,520} = 3{\cdot}521$; $P = 0{\cdot}0150$). Bacterial populations of almond isolates were relatively low and consistent across harvests, whereas grape isolates increased after the first harvest (Fig. 2).

Overall, pathogen persistence was high. Across all isolates and harvests, only two inoculation groups had no infected plants, both from the same isolate (UCLA) that was identified as belonging to the grape strain. However, there was a range of low to intermediate infection incidence, with a strong positive correlation between mean

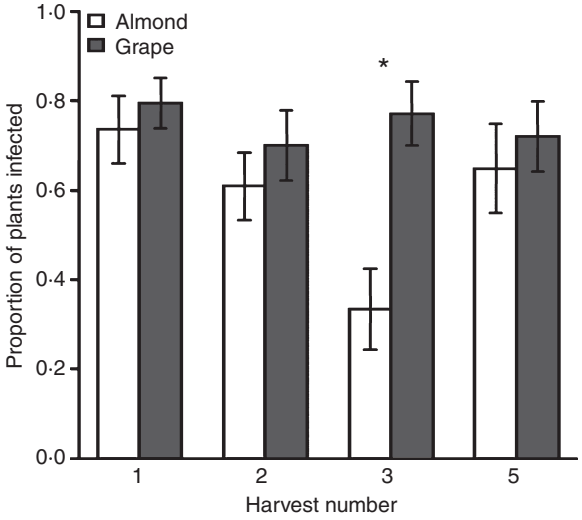

Figure 1 Proportion of plants testing positive for *Xylella fastidiosa* among successive alfalfa (*Medicago sativa*) harvests, for grape vs. almond isolates. Error bars denote the standard error of proportions infected for individual isolates within grape or almond strains for a given harvest number. *denotes a significant difference in the proportion infected between grape and almond isolates for a given harvest.

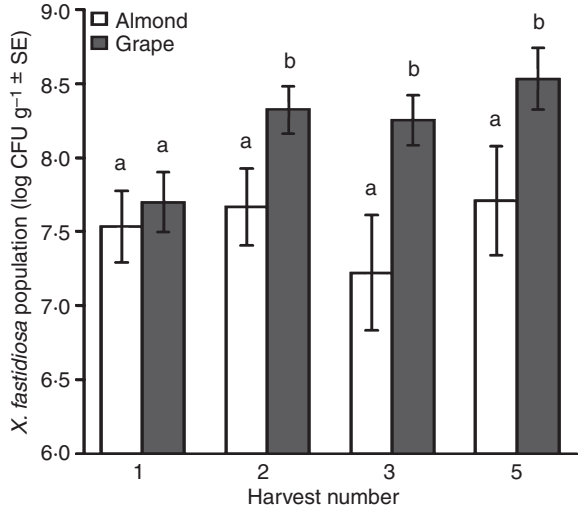

Figure 2 Mean bacterial population density in infected *Medicago sativa* plants (log [no. *Xylella fastidiosa* colony forming units] g^{-1} plant tissue) among successive alfalfa harvests, for grape vs. almond isolates. Different letters denote significant differences in *X. fastidiosa* populations among harvests or between pathogen origins.

X. fastidiosa population density and proportion of plants infected (r = 0·5703; t_{86} = 6·439; P < 0·0001). This correlation was similar for almond and grape isolates, separately. The final proportion of plants infected did not differ significantly between grape versus almond isolates (χ^2 = 3·278; d.f. = 1; P = 0·0700), but it was strongly dependent on the mean *X. fastidiosa* population of a given isolate at the first harvest (χ^2 = 51·580; d.f. = 1; P < 0·0001). Isolates with low initial population densities led to low final infection incidence, whereas isolates with high initial populations led to high proportions of infected plants at the end of the experiment (Fig. 3).

Plant response to infection

The principle components analysis of plant morphological characters identified four PCs that were important for describing alfalfa response to *X. fastidiosa* infection. The first component explained nearly 44% of the variation in the seven plant characters, while the remaining three PCs explained approximately 21, 15, and 12%, respectively. For the first component, relative plant height, above ground dry mass and internode length all had loadings that were strongly negative. Thus, PC 1 was essentially a measure of stunted plant size. PC 2 encapsulated stunting of leaf size, with strong negative loadings of leaflet width and length. PC 3 represented a measure of alfalfa 'bushiness', with positive loading of stem number. Finally, PC 4 was composed of a negative loading of number of nodes and positive loading of internode length.

Tests on the individual PCs showed significant effects of harvest number on all PCs (Table 2). Follow-up tests on the individual relative plant responses showed effects of harvest number on all plant characters (Table 3). The plant characters were indistinguishable from buffer-inoculated plants (i.e. approximately 100% relative response) at the first harvest, but relative plant height, biomass, number of stems, number of nodes, internode length and leaflet length and width were all reduced (i.e. stunted) at later harvests (Figs 4, 5). The only significant effect of *X. fastidiosa* population density was on PC 2, whilst PC 1

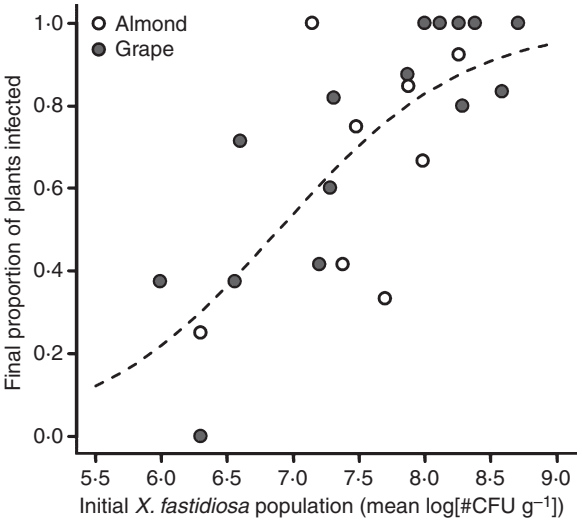

Figure 3 Relationship between mean *Xylella fastidiosa* population density in infected *Medicago sativa* plants at the first harvest and the proportion of plants still infected at the fifth harvest. The dotted line represents the fit of a generalized linear model with binomial errors, irrespective of pathogen strain (grape vs. almond).

Table 2 Statistical results for response of *Medicago sativa* to infection by *Xylella fastidiosa* as a function of harvest number, *X. fastidiosa* population density (CFU), and strain origin (grape vs. almond). Results of separate minimum adequate linear mixed effects ANOVAS on individual principle components. Tests evaluated at $\alpha \leq 0{\cdot}0125$

	PC 1		PC 2		PC 3		PC 4	
Source	$F_{df,dfe}$	*P*	$F_{df,dfe}$	*P*	$F_{df,dfe}$	*P*	$F_{df,dfe}$	*P*
Harvest	$87{\cdot}032_{3,\ 519}$	<0·0001	$15{\cdot}618_{3,\ 522}$	<0·0001	$15{\cdot}618_{3,\ 522}$	<0·0001	$4{\cdot}978_{3,\ 522}$	0·0021
CFU	$3{\cdot}046_{1,\ 519}$	0·0815	$5{\cdot}490_{1,\ 522}$	0·0195	$2{\cdot}500_{1,\ 522}$	0·1182	$2{\cdot}261_{1,\ 522}$	0·1333
Origin	$1{\cdot}477_{1,\ 21}$	0·2377	$1{\cdot}576_{1,\ 21}$	0·2231	$0{\cdot}665_{1,\ 21}$	0·4240	$0{\cdot}800_{1,\ 21}$	0·3813
Harvest × Origin	$4{\cdot}002_{3,\ 519}$	0·0078	–	–	–	–	–	–

Table 3 Statistical results for the response of alfalfa (*Medicago sativa*) plants to *Xylella fastidiosa* infection: plant height, above ground dry mass, internode length, leaf width, leaf length, number of stems and number of nodes. Results of separate best fit linear mixed effects ANOVAS on individual plant characters. Only characters with high loadings from principle components analysis were tested. *P* values in bold are significant after controlling for multiple comparisons

	Harvest		CFU		Origin		Harvest × Origin	
Response	$F_{df,dfe}$	*P*	$F_{df,dfe}$	*P*	$F_{df,dfe}$	*P*	$F_{df,dfe}$	*P*
Height[a]	$125{\cdot}3723_{3,519}$	**<0·0001**	$2{\cdot}712_{1,519}$	0·1002	$3{\cdot}905_{1,21}$	0·0614	$3{\cdot}970_{3,519}$	**0·0082**
Biomass[a]	$31{\cdot}959_{3,519}$	**<0·0001**	$6{\cdot}475_{1,519}$	**0·0112**	$0{\cdot}191_{1,21}$	0·6664	$1{\cdot}292_{3,519}$	0·2764
Internode[a]	$76{\cdot}472_{3,519}$	**<0·0001**	$2{\cdot}543_{1,519}$	0·1179	$0{\cdot}447_{1,21}$	0·5109	$2{\cdot}919_{3,519}$	**0·0337**
Width[b]	$10{\cdot}171_{3,522}$	**<0·0001**	$0{\cdot}884_{1,522}$	0·3475	$0{\cdot}114_{1,21}$	0·7390	–	–
Length[b]	$27{\cdot}817_{3,522}$	**<0·0001**	$0{\cdot}424_{1,522}$	0·5150	$0{\cdot}246_{1,21}$	0·6251	–	–
Stems[c]	$7{\cdot}859_{3,522}$	**<0·0001**	$7{\cdot}626_{1,522}$	**0·0060**	$0{\cdot}306_{1,21}$	0·5863	–	–
Nodes[c]	$34{\cdot}508_{3,522}$	**<0·0001**	$0{\cdot}350_{1,522}$	0·5543	$4{\cdot}955_{1,21}$	**0·0371**	–	–

[a] $\alpha_c = 0{\cdot}01667$.
[b] $\alpha_c = 0{\cdot}025$.
[c] $\alpha_c = 0{\cdot}05$.

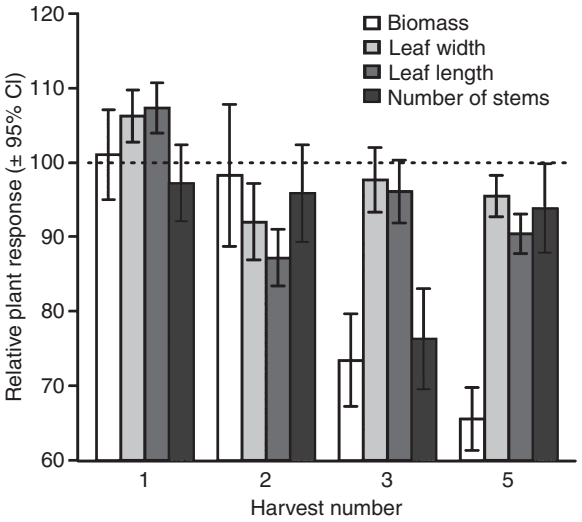

Figure 4 Mean relative biomass, number of stems and leaflet length and width across successive harvests in alfalfa (*Medicago sativa*) plants. Plant characters are expressed as a relative percentage of infected plants to buffer-inoculated plants ($[x_{infected}/\bar{x}_{control}]^*100$). The dotted line represents equivalence between *Xylella fastidiosa*-infected and buffer-inoculated plants, with means below this line denoting stunting of infected plants.

was marginally significant (Table 2). More specifically, higher population densities were associated with lower alfalfa biomass and fewer stems (Fig. 6). Finally, there were no significant main effects of pathogen strain origin on any of the PCs, but there was a significant effect of strain by harvest interaction for PC 1 (Table 2). Follow-up tests on individual plant characters showed this interaction to be significant for plant height and marginally significant for internode length (Table 3). Plant height and internode length decreased across harvests, but the severity of stunting was higher in grape isolates than in almond (Fig. 5).

Discussion

Alfalfa is a perennial crop plant that has been bred for a high tolerance to harvesting (i.e. rapid regrowth) to maximize forage yield. *Xylella fastidiosa*-infected alfalfa plants were reduced not only in overall height, but also all other measured phenotypic characters. Reduction of some of these characters in infected plants was greatest at higher pathogen populations and at later harvests, suggesting that there was a cumulative effect of infection to reduce alfalfa tolerance, which is consistent with the vessel occlusion hypothesis (Goodwin *et al.*, 1988). It is also worth noting that the severity of plant stunting varied among pathogen isolates, with grape isolates tending to cause more severe stunting than almond isolates. Similar strain-specific virulence has been noted for other animal (Berger *et al.*, 2005) and plant pathogens (Nesme *et al.*, 1994; Kema *et al.*, 1996).

Given that *X. fastidiosa* can be pathogenic to alfalfa, it is curious that outbreaks of AD that occurred historically

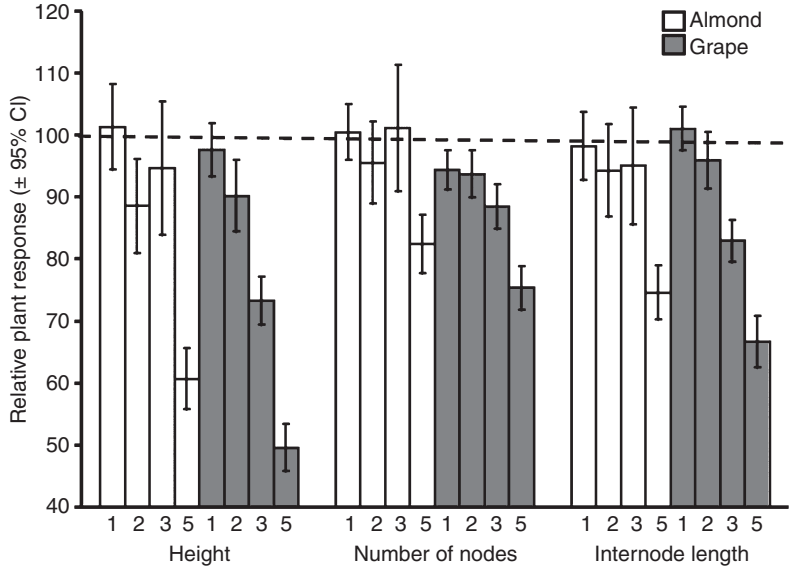

Figure 5 Mean relative height, number of nodes and internode length across successive harvests in alfalfa (*Medicago sativa*) plants, for grape vs. almond *Xylella fastidiosa* isolates. The dotted line represents equivalence between infected and buffer-inoculated plants, with means below this line denoting stunting of infected plants.

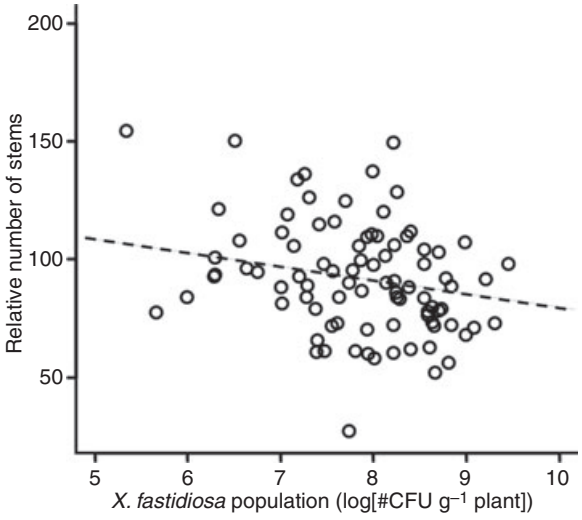

Figure 6 Relationship between *Xylella fastidiosa* population density in infected plants and relative number of stems. The dotted line represents linear regression model fit [slope (±SE) = −4·151 (±1·575), intercept (±SE) = 128·442 (±12·489)]. A qualitatively similar reduction at higher pathogen population densities was seen for relative plant biomass [slope (±SE) = −3·850 (±1·860), intercept (±SE) = 138·469 (±13·970)].

in California (Harris & Schlocker, 1943) have not been reported since then. This may be because drought causes similar symptoms in alfalfa (Johnson & Tieszen, 1994), promoting misinterpretation and underreporting of the disease. However, outbreaks may not have occurred because of strain turnover (Power, 1996), with replacement of a virulent *X. fastidiosa* strain by a less virulent strain. It is also possible that changes in cultivation practices, such as quicker turnover of alfalfa fields, affected disease prevalence. Unfortunately, because the pathogen was not isolated from plants during those early alfalfa dwarf outbreaks, it was not possible to explicitly evaluate these hypotheses. Regardless, the results suggest that pathogen strain variability plays a role in disease severity, which may also be important for alfalfa as a pathogen source.

For generalist pathogens the likelihood that a given host species promotes pathogen spillover or dilution depends on its infectivity and pathogen multiplication relative to other hosts in the community (Power & Mitchell, 2004). This study identified strain-specific levels of pathogen multiplication, with almond isolates averaging up to 15-fold lower bacterial population densities in alfalfa than grape isolates. This difference is important because the transmission efficiency of vectors in this system depends on bacterial population density. Below a certain *X. fastidiosa* population density ($\sim 10^4$ CFU g^{-1} of plant material in the case of grapevines) sharpshooter transmission is inefficient (Hill & Purcell, 1997). Therefore transmission efficiency varies as a function of time since inoculation (Hill & Purcell, 1997) and host species (Lopes *et al.*, 2009) due to differences in pathogen populations. In the case of alfalfa, overall pathogen populations were above the putative threshold for sharpshooter transmission suggesting that it is a competent host. However, isolates varied greatly in their bacterial population density, with some isolates producing mean population densities as low as 10^5 CFU g^{-1}. A companion study showed that such differences in pathogen populations mediated sharpshooter transmission efficiency (Lopes *et al.*, 2009). Similar strain-specific transmission

efficiency has also been noted in other systems (Power, 1996; Lucio-Zavaleta *et al.*, 2001). Collectively, these results provide support for pathogen strain affecting host competence.

Host competence may also be a function of its ability to maintain pathogen infection over time. Disease ecologists have long been concerned with factors contributing to disease persistence. Theory suggests that there is a population level threshold for persistence that is based on host density or abundance (Lloyd-Smith *et al.*, 2005). This study did not address persistence at this scale, however it did provide evidence that host-pathogen interactions affect infection persistence at the individual scale, because of differences in isolate virulence. The observed temporal variability in incidence over time among a few specific isolates may be explained by heterogeneous distributions of *X. fastidiosa* infection within individual hosts (Daugherty *et al.*, 2010) and that quantification method used (i.e. dilution plating) may not detect very low bacterial populations. Regardless of strain, isolates with low initial population densities also had lower incidence at the end of the experiment. For example, the isolate with the lowest initial population (UCLA) died out completely, and a few other weakly virulent isolates had relatively low incidence. This relationship is interesting because it suggests that some pathogen isolates may result in 'dead end' infections that do not readily propagate. Host recovery has been documented for this pathogen in other species (Feil *et al.*, 2003), and also occurs for other plant pathogens, such as chrysanthemum yellows phytoplasma (Palermo *et al.*, 2001). The mechanism of host recovery is not known, but in the case of *X. fastidiosa*-infected grapevines it has been suggested to result partly from seasonal effects and pruning of infected host material (Feil *et al.*, 2003). The latter explanation is particularly likely for alfalfa, which is harvested several times each year. Regardless of the mechanism, recovery is an important element of epidemiological theory that factors into a pathogen's basic reproductive ratio (R_o; Anderson & May, 1991) and whose effect reduces directly host incidence (i.e. loss of infected individuals), thereby constraining secondary spread.

Increasingly, infectious diseases are recognized as threats to plant health, and to the sustainability of natural and managed ecosystems. Disease outbreaks may occur because of changes in the host community (LoGiudice *et al.*, 2008), establishment of a novel vector species, or introduction of a novel pathogen or pathogen strain (Lanciotti *et al.*, 1999; LoGiudice *et al.*, 2008). Here, *X. fastidiosa* strain diversity is shown to underlie variation in disease severity in a focal host. These differences in strain virulence may have important implications for spread of this pathogen to other hosts, through effects on source host recovery rate and vector acquisition efficiency. Testing this hypothesis will require explicit measures of strain variability in the field coupled with measures of vector movement and infectivity rates among hosts.

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