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Strain-specific alfalfa water stress induced by *Xylella fastidiosa*

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Abstract Differences in the virulence of a pathogen among host species can occur because hosts differ in their resistance or tolerance to infection or because of underlying genetic variation in the pathogen. The xylem-limited bacterium *Xylella fastidiosa* is pathogenic to dozens of plant species throughout the Americas, and is structured into genetically and biologically distinct strains. In some plants *X. fastidiosa* causes striking leaf scorch symptoms and in others, such as alfalfa, stunting is the primary symptom. The mechanism by which these symptoms occur has been debated. We tested the hypothesis that symptoms result from *X. fastidiosa*-induced water stress, and that the magnitude of water stress is strain-dependent. We mechanically inoculated alfalfa plants with one of 14 isolates (5 identified genetically as "almond" and 9 as "grape" isolates), and compared stable carbon isotope ratios among isolates. Infected plants showed significant isotopic shifts (up to 2% on average) relative to healthy plants that were consistent with water stress. Moreover, there were significant differences in water stress among isolates, with a tendency for grape isolates to cause more severe water stress than almond isolates. There was also a positive relationship between plant infection level and isotopic shift (slope ± SE=0.273±0.068), which supports the hypothesis that *X. fastidiosa* symptoms result from bacterial multiplication and vessel occlusion. Unexpectedly, however, water stress was not correlated with measures of alfalfa stunting. These results suggest *X. fastidiosa* induces strain-specific levels of water stress, but factors other than water stress alone are responsible for stunting.

Keywords Drought stress · Stable isotopes · Alfalfa dwarf · Pierce's disease · Sharpshooter

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Introduction

Disease ecologists have long recognized that vector transmission is the end product of a series of pairwise interactions among pathogen, vector, and host. For pathogens with wide host ranges, disease severity can differ among host species. Some of this variation may be due to differences in host resistance or tolerance to the pathogen (Tooley and Kyde 2007). In addition, disease severity may vary within a host species because of underlying genetic variation in the

pathogen (Stanghellini et al. 1977). One pathogen for which these two sources of variation may be important epidemiologically is the plant pathogen *Xylella fastidiosa*.

Xylella fastidiosa is a xylem-limited bacterium that is endemic to the Americas and is widespread throughout the southeastern and western United States (Purcell 1997). This pathogen is transmitted by several species of xylem sap-feeding insects. There is no evidence of specificity in vector-*X. fastidiosa* interactions (Frazier 1965), though there is variability in transmission efficiency among vector species (Purcell 1980). The most important vectors in California are the sharpshooter leafhoppers (Hemiptera, Cicadellidae; Severin 1949).

In addition to having a diverse vector complex, *X. fastidiosa* has a wide host range. This pathogen is able to infect dozens of agricultural, native, and weedy plant species in the western United States (Hopkins and Purcell 2002). Severity of disease, however, differs greatly among hosts. The most pronounced diseases occur as leaf scorch symptoms in certain agricultural crops, such as Pierce's disease of grapevines (Krivanek et al. 2005), oleander leaf scorch (Purcell et al. 1999), and almond leaf scorch (Davis et al. 1980). In grapevines, for example, infection causes progressive reddening along leaf margins, leaf loss, "matchstick" petioles, and cane or whole plant death within a few years (Krivanek et al. 2005). In other crops, symptoms manifest primarily as a delayed onset stunting of plants, such as alfalfa dwarf disease (Harris and Schlocker 1943) and phony peach disease (Turner and Pollard 1959). In still other plant species, most notably weeds, *X. fastidiosa* causes no apparent disease (Wistrom and Purcell 2005). Among host species *X. fastidiosa* reaches different cell populations within plants, which may explain differences in disease severity (Hill and Purcell 1995).

X. fastidiosa exhibits substantial genetic variation that appears to be associated with adaptations to specific host plants. Recent work has identified four main pathogen genetic groups associated primarily with i) grapes, ii) citrus and coffee, iii) oleander, and iv) almond and other hosts (Scally et al. 2005). This is important because the different strains behave differently biologically. For example, grape and almond strains differ substantially in population growth rates; and isolates belonging to the grape strain cause disease in both grape and almonds, whereas almond isolates do not cause disease in grape (Almeida and Purcell 2003). Thus, different isolates of these *X. fastidiosa* genetic groups may contribute to variability in disease severity within and among host plant species.

The precise cause of *X. fastidiosa*-induced leaf scorch and stunting symptoms has been debated in the literature. The most frequently cited explanation is that, as a xylem-limited bacterium, *X. fastidiosa* replication causes occlusion of vessel elements and restricted water transport (Goodwin et al. 1988; McElrone et al. 2008). Symptom onset is favoured when infected hosts are also drought stressed (Choat et al. 2009; McElrone et al. 2001). Support for this vessel occlusion hypothesis comes from direct measures of leaf water potential, hydraulic conductance and stomatal resistance in *X. fastidiosa* infected plants (Goodwin et al. 1988; McElrone et al. 2001, 2008), and from vascular wilt diseases that result from pathogen-induced water stress in hosts (Tyree and Sperry 1989). If this is true, a higher pathogen population in plants should be associated with more severe stress in the plant, which is not always the case (Gambetta et al. 2007). Moreover, more virulent *X. fastidiosa* strains should cause more severe water stress. In this study we used stable carbon isotopes to quantify water stress of alfalfa plants that were inoculated with different *X. fastidiosa* isolates. We then investigated how the magnitude of alfalfa water stress was related to the severity of plant stunting.

Materials and methods

Stable carbon isotopes and plant water stress

Carbon exists in two common stable forms, ^{12}C and ^{13}C, with the heavier isotope being far more rare in atmospheric CO_2 (approx. 1/91). Increasingly, the ratio of these two stable carbon isotopes is being used as an indicator of plant water status (Dawson et al. 2002; Ferrio et al. 2003). For plants, water shortage leads to stomatal closure. This closure increases water use efficiency, but also reduces gas exchange. Plant biochemical pathways discriminate against ^{13}C during fixation of atmospheric carbon. Therefore, less gas exchange means less potential for discrimination against ^{13}C (i.e. less isotopic "fractionation"). In other

words, all else being equal, a water stressed plant has a higher relative ratio of ^{13}C to ^{12}C than a well watered plant (Dawson et al. 2002; Ferrio et al. 2003).

Isotopic ratios are typically expressed in "delta" notation, relative to a standard of known isotopic ratio. For comparison of carbon isotopes the standard used is the PeeDee belemnite. The typical formulation of delta for an unknown sample, expressed per thousand (‰) rather than percent, is:

$$\delta^{13}C = [(R_{unknown}/R_{standard}) - 1] \times 1000$$

where R is the ratio of ^{13}C to ^{12}C. A typical value of $\delta^{13}C$ for C3 plants is approximately −29‰. The negative value reflects the lower relative concentration of ^{13}C in plant tissues relative to the standard. More importantly, water stressed plants are enriched in ^{13}C (i.e. less negative $\delta^{13}C$) compared to well watered plants, with severe water stress resulting in positive changes in $\delta^{13}C$ of 5‰ or more (Dawson et al. 2002; Ferrio et al. 2003).

Experiment set-up and sample processing

In the spring of 2007 we inoculated alfalfa plants with one of 14 *X. fastidiosa* isolates. Five of these isolates (ALS6, ALS9, Butte, Dixon, Glenn) were identified as being grouped genetically as almond strains, and the remaining 9 isolates (ALS1, Buena Vista, Conn Creek, M35, Medeiros, Pavich, SN1, Traver) are related to grape strains (Table 1). A recent proposal would place the grape and almond strains used here into the *X. fastidiosa* subspecies *fastidiosa* and *multiplex*, respectively (Scally et al. 2005; Schaad et al. 2004).

Inoculations were made on alfalfa seedlings (cv. WL625 HQ), ten days after transplanting to 10 cm pots filled with Supersoil potting soil (Rod Mclellan Company, San Mateo, CA). Plants were pin-inoculated on the primary stem 5 cm above soil surface with a 10^8–10^9 colony forming units (CFU) ml^{-1} suspension of one of the *X. fastidiosa* isolates. Inoculations were made in three groups, approximately one week apart, with mock inoculations with buffer only to serve as controls for each inoculation group. We successfully inoculated a total of more than 160 alfalfa plants, with 8–10 replicate plants for each *X. fastidiosa* isolate and at least 10 control plants for each inoculation group.

Following inoculation, plants were housed in the Oxford tract greenhouses at UC Berkeley for the next 35 weeks. To mimic harvesting of alfalfa as it occurs in the field we periodically cut plants as they began to flower at 5 cm above the soil surface. Five such harvests occurred over the duration of the experiment. We measured several morphological characteristics of both control and *X. fastidiosa*-inoculated plants, including: plant height, dry above-ground weight, and internode length (# nodes/height). We also estimated *X. fastidiosa* infection level in plants by plate culturing ground stem tissue on PWG medium (Hill and Purcell 1995). Finally, we measured alfalfa stable carbon isotope ratios, total carbon, and total nitrogen of both *X. fastidiosa*-inoculated and control plants at 35 weeks post inoculation.

After harvesting we oven dried all plant material, then collected alfalfa leaflets from near the top of the plant. We used a Wigglebug mill for one minute to grind these leaflets to a fine homogeneous powder. For each sample 3–4 mg of ground leaf material was loaded into a 4×6 mm tin capsule (Costech Analytical, Valencia, CA), and was processed via elemental analyzer/continuous flow isotope ratio mass spectrometry (ANCA/SL elemental analyzer coupled with a PDZ Europa Scientific 20/20 Mass Spectrometer) at the UC Berkeley Center for Stable Isotope Biogeochemistry. We included a range of standard weights to correct for any nonlinearities in measurement due to variation in sample mass. This analysis provided carbon isotope ratios, total carbon, and total nitrogen for all control and *X. fastidiosa*-inoculated plants. Total carbon and nitrogen were used to calculate a C to N ratio for each sample.

Statistical analyses

In this study we consider the affect of *X. fastidiosa* on alfalfa plants after 35 weeks of infection. Mean $\delta^{13}C$ in the three control groups ranged from −31.75‰ to −32.98‰. Therefore, to control for variation among the three inoculation groups, isotopic and C:N values for infected plants were standardized relative to control plants. This was done by calculating mean $\delta^{13}C$ and C:N values for each control group, separately, then subtracting these means from measurements for individual *X. fastidiosa*-inoculated plants [e.g., $\delta^{13}C_{sample} - mean(\delta^{13}C_{control\ group})$; $C{:}N_{sample} - mean(C{:}N_{control\ group})$]. Thus, positive relative ^{13}C

Table 1 Alfalfa plant mean (±SE) height, mass, internode length, and principal component score among *X. fastidiosa* isolates evaluated 35 weeks after inoculation

Isolate	Strain[1]	n	Height[2]	Mass[2]	Internode[2]	PC1[3]
ALS6	Almond	7	52.79±5.72	57.56±11.11	67.14±2.97	1.37±0.39
ALS9	Almond	8	63.87±5.57	58.46±9.87	74.60±5.97	0.77±0.36
Butte	Almond	12	56.84±4.25	81.02±7.10	62.26±4.31	0.98±0.24
Dixon	Almond	12	56.24±4.30	63.88±7.75	82.59±4.59	1.18±0.36
Glenn	Almond	11	58.66±5.37	65.23±5.25	74.79±5.23	0.83±0.37
ALS1	Grape	7	52.09±11.62	60.10±10.94	63.42±7.55	1.51±0.66
Buena Vista	Grape	10	38.28±4.14	52.15±8.12	63.20±4.34	2.21±0.38
Conn	Grape	9	57.74±6.69	79.70±10.19	68.21±4.69	1.02±0.36
Hopland	Grape	10	60.77±5.52	70.28±4.29	87.79±7.88	1.08±0.37
M35	Grape	8	67.66±9.28	74.71±15.21	86.25±8.97	0.63±0.65
Medeiros	Grape	9	48.23±4.33	75.87±6.09	59.09±4.55	1.17±0.30
Pavich	Grape	10	34.16±3.25	33.61±5.25	53.94±4.15	2.84±0.28
SN1	Grape	10	51.19±4.64	89.28±9.04	57.56±3.85	0.93±0.33
Traver	Grape	10	34.98±0.00	48.24±8.76	45.69±5.96	2.40±0.31

[1] strain type denoting "almond" versus "grape" *X. fastidiosa* genetic groupings as determined using diagnostic primers RST31 and RST33 (Almeida and Purcell 2003)

[2] plant morphological characters are expressed as a percentage relative to the mean for a group of uninoculated control plants $\left(\left[x_{infected}/\bar{x}_{control}\right]*100\right)$

[3] most important score from principal component analysis, which incorporated 44% of all variation in infected alfalfa plants across all isolates and all alfalfa harvests. Inversely related to plant height, mass, and internode length

fractionation values equate to enrichment in ^{13}C, which is consistent with increased water use efficiency and increased water stress relative to control plants (Dawson et al. 2002). Conversely, negative relative ^{13}C fractionation values equate to depletion of ^{13}C, which is consistent with decreased water use efficiency and decreased water stress.

We used analysis of covariance (Crawley 2007) to test for effects of *X. fastidiosa* infection on the relative ^{13}C fractionation, as a fixed effect, and as a function of *X. fastidiosa* population in the plant [log(# CFU g^{-1} plant tissue)]. A significant effect of Isolate was followed up with pair-wise comparisons among isolates within groups identified as originating from almond or as originating from grape, with adjustment of α to control for multiple comparisons. We also used ANCOVA to test for effects of infection on the relative carbon to nitrogen ratio among isolates and as a function of *X. fastidiosa* population in the plant. Finally, to understand the relationship between water stress and alfalfa stunting, we correlated relative plant ^{13}C fractionation with a composite measure of plant stunting, the most important component (PC #1) identified in an earlier principal components analysis that incorporates 44% of all variation in infected alfalfa morphological characters across all harvests (J. Lopes, *unpublished data*). This metric is associated with reductions in height, mass, and internode length relative to control plants (Table 1).

Results

Raw $\delta^{13}C$ values of infected plants ranged over 5‰, from −33.75‰ to −28.37‰, and relative fractionation values ranged from −1.58‰ to 2.54‰. There were significant effects of both Isolate ($F_{13,118}$=3.285, P=0.0003) and *X. fastidiosa* population ($F_{1,118}$= 47.285, P<0.0001) on the relative ^{13}C fractionation of infected plants. Conversely, relative C:N did not differ significantly among isolates ($F_{1,118}$=2.610, P=0.1089) or as a function of *X. fastidiosa* population ($F_{13,118}$=1.592, P=0.0969). Certain isolates caused mean isotopic shifts as high as 1.5–2‰ relative to

controls, whereas others did not differ from control plants (Fig. 1). ALS6, ALS1, Conn and Traver showed the strongest isotopic shifts, whereas ALS9, Dixon, Glenn, Hopland, and SN1 showed no fractionation relative to controls (i.e. Fig. 1—error bars overlap zero). Some of this variation appears to break down according to isolate genetic group (i.e. strain). According to 95% confidence intervals, proportionately fewer isolates from the almond grouping (2/5) showed significant ^{13}C fractionation than did grape isolates (7/9). Across all isolates, higher *X. fastidiosa* population in plants caused more positive ^{13}C fractionation (Fig. 2, slope ± SE=0.273±0.068). The largest shifts were associated with grape isolates that had high *X. fastidiosa* populations. Finally, relative ^{13}C fractionation was not clearly related to plant stunting (Fig. 3). These two plant characteristics were weakly correlated (r=0.098), but not significantly so (t=1.1301, df=131, P=0.2605).

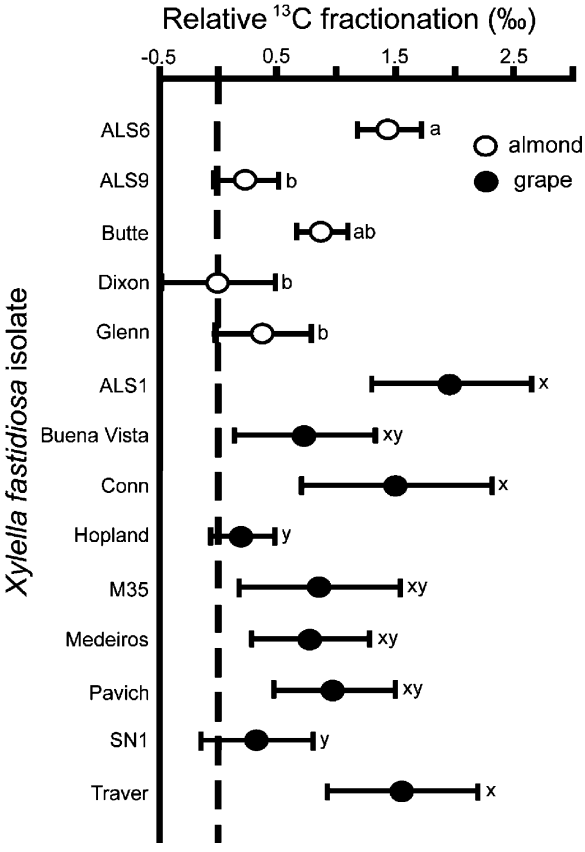

Fig. 1 Alfalfa ^{13}C fractionation among *X. fastidiosa* isolates (±95% confidence intervals) relative to non-inoculated control plants. Points followed by different consecutive letters differ significantly from each other

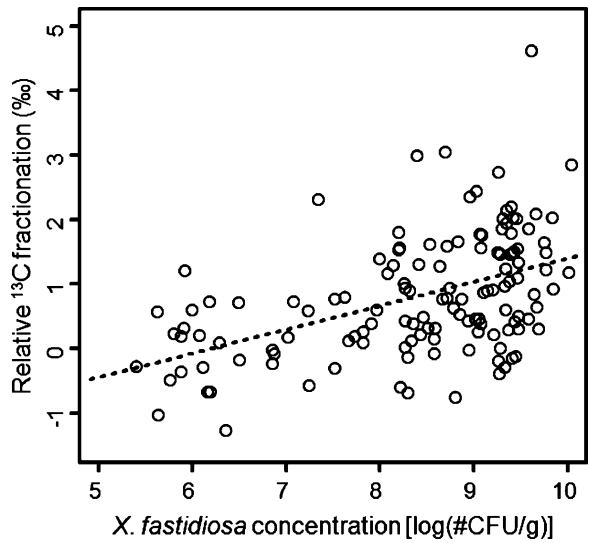

Fig. 2 Relative ^{13}C fractionation as a function of alfalfa plant infection level. Slope ± SE=0.273±0.068

Discussion

In many host plant species *X. fastidiosa* infection produces pronounced leaf scorch symptoms, such as Pierce's disease (Krivanek et al. 2005), oleander leaf scorch (Purcell et al. 1999), or almond leaf scorch (Davis et al. 1980). For other susceptible plant hosts,

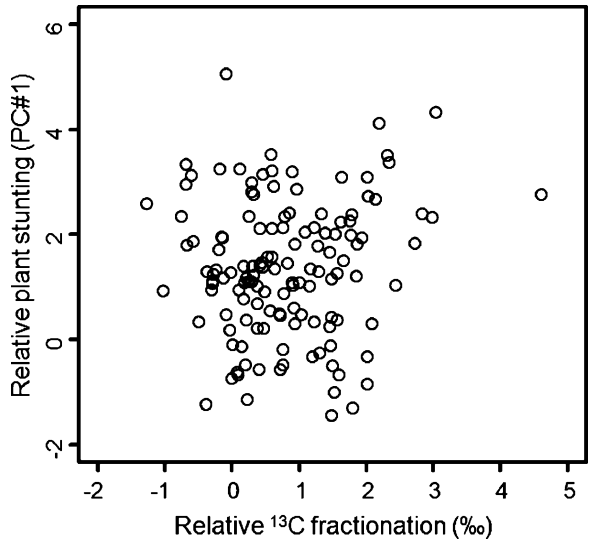

Fig. 3 Correlation between ^{13}C fractionation and plant stunting. Stunting is expressed as a composite reduction (most important principal component) of plant height, dry mass, and internode length relative to non-inoculated control plants

such as alfalfa and peach, symptoms are more cryptic, manifesting as stunting long after initial infection (Turner and Pollard 1959). In this study we used stable carbon isotopes to understand how different *X. fastidiosa* isolates affect plant water relations.

We found significant shifts in carbon isotopes in infected alfalfa plants that were consistent with increased water stress (Dawson et al. 2002). The magnitude of these isotopic shifts also differed among *X. fastidiosa* strains, with almond isolates tending to produce less severe water stress than did grape isolates (Fig. 1). These results complement other findings (J. Lopes, *unpublished data*) of large differences in infection level and alfalfa plant stunting among *X. fastidiosa* isolates, with the highest pathogen populations and most severe stunting occurring from infections with grape isolates (Table 1). Compared to healthy plants, infected plants had reduced aboveground biomass, shorter internode length, and reduced leaflet size; symptoms were not apparent early on, they manifested only after the second or third cut. These characteristics match field observations made during the 1940s of alfalfa dwarf disease (Harris and Schlocker 1943). It is important to note, however, that ratios of stable carbon isotopes are not a direct measure of water stress. Instead, they are a measure of plant tissue discrimination against ^{13}C during fixation of atmospheric carbon, which tends to be correlated with water use efficiency (Dawson et al. 2002). Many factors can potentially cause shifts in carbon isotopic ratio, including irradiance level, atmospheric CO_2 concentration, temperature, and soil nutrients (Ferrio et al. 2003). Nonetheless, carbon stable isotopes are considered a reliable method of assessing plant water relations in a variety of systems, in part, because they provide a time integrated measure of plant status rather than an instantaneous measure (Dawson et al. 2002; Ferrio et al. 2003). At least two lines of evidence support the conclusion that the noted isotopic shifts in alfalfa represent accurately *X. fastidiosa*-induced water stress. First, all control and inoculated plants were housed in the same 10 m×10 m greenhouse under the same temperature, watering, and soil conditions. These controlled conditions should limit the influence of other extraneous factors on stable carbon isotope ratios. Second, the water stress symptoms noted in these plants match observations of drought stressed alfalfa from greenhouse and field studies (Hall 1993; Johnson and Tieszen 1994).

Alfalfa plants grown under drought conditions had higher water use efficiency and enrichment in ^{13}C (Johnson and Tieszen 1994; Nicolodi et al. 1988). Although we did not include a water stress treatment in our experiment, the carbon shifts of infected plants of 2% or more are on par with or even exceed levels of fractionation observed for healthy water stressed versus irrigated alfalfa plants in the field (Nicolodi et al. 1988)—indicating that *X. fastidiosa* induces biologically meaningful levels of water stress. It is worth noting that the samples we analyzed included both plant and any bacterial carbon. However, given what we know about *X. fastidiosa* infection level in these samples and cell size, bacterial biomass accounted for an extremely small proportion of total sample biomass (approximately 10^{-7}). Thus it is unlikely that this source of contamination influenced overall estimates of water stress-induced carbon fractionation. The relationship between stable carbon isotope fractionation and alfalfa water use efficiency is strong enough that stable carbon isotopes have been used in alfalfa breeding programs to identify germplasm with higher drought resistance (Johnson and Tieszen 1994).

There are two main hypotheses for the mechanism by which *X. fastidiosa* causes disease symptoms in infected hosts. The vessel occlusion hypothesis posits that *X. fastidiosa* replication leads to clogging of vessel elements, embolism, and restricted water transport in the plant (Goodwin et al. 1988; McElrone et al. 2001; Choat et al. 2009). An alternative explanation is that the pathogen releases phytotoxins or other compounds that illicit a systemic response from the plant without obstruction of water transport (Gambetta et al. 2007; Goodwin et al. 1988; Pérez-Donoso et al. 2007). Although our experiment was not designed to test explicitly the relative importance of these two mechanisms, it does provide some support for the former. One prediction of the vessel occlusion hypothesis is that symptom severity should be positively related to pathogen population size within the plant (Gambetta et al. 2007). Not only did we find different *X. fastidiosa* isolates caused differential water stress in alfalfa, but there was also a significant positive relationship (across all isolates) between pathogen population size and the magnitude of water stress as measured by stable carbon isotopes (Fig. 2). This result contradicts Gambetta et al. (2007), who found no such relationship for Pierce's

disease leaf scorch symptoms in grapevines. This difference in results is likely explained by the different characters being considered as symptoms in the two studies. Leaf scorch symptoms are typically evaluated using rankings of different disease categories (Krivanek et al. 2005). Such methods are clearly an indication of the chronic disease state of plants, however there is potential to underestimate other physiological changes occurring in plants induced by *X. fastidiosa* infection. Conversely, stable isotopes and instantaneous metrics of plant water relations offer a continuous metric of plant condition, though the relationship to disease induced mortality is not as well established.

As a forage crop, alfalfa has been bred to have a high tolerance to biomass removal, to facilitate rapid re-growth between harvests. *Xylella fastidiosa* infection appears to have a cumulative effect on alfalfa, by reducing this high tolerance. We did not find, however, conclusive evidence that plant water stress (as measured by C isotopes) explains alfalfa stunting (Fig. 3). This result is counterintuitive in that studies manipulating alfalfa water availability show that drought stressed plants have reduced re-growth following harvest (Hall 1993). Our result may be due to plant tissue sampling, which only occurred above ground. Many plants, including alfalfa (Johnson and Tieszen 1994) are plastic in their allocation to roots versus shoots depending on water availability. Alfalfa roots can host *X. fastidiosa* at concentrations even greater than in portions of the aboveground biomass (M. Daugherty, *unpublished data*). Moreover, roots frequently show characteristic tissue discolouring associated with *X. fastidiosa* infection. However, because we did not inspect roots in this study we cannot determine definitely the response of these plants' roots to infection. If alfalfa is shifting its allocation to roots following infection, looking at above-ground biomass alone may provide an incomplete metric of the effects of infection on plant growth.

We have presented evidence that different strains of a widespread xylem-limited pathogen cause differential water stress in an economically important crop. These results may also be important in an epidemiological sense because of vector behavior. The most important vectors of *X. fastidiosa* in California are the sharpshooter leafhoppers (Severin 1949). As xylem feeders, it is plausible that infected plants with restricted water transport may serve as relatively poor hosts for these insects. This prediction is supported by a study showing that sharpshooters stop feeding when xylem tension in water stressed plants increases beyond approximately 2.1 MPa (Andersen et al. 1992). Moreover, sharpshooters appear to prefer healthy over symptomatic *X. fastidiosa* infected citrus (Marucci et al. 2005) and grapevines (Perring et al. 2009). In addition, other non-vector leafhoppers favour well-watered alfalfa plants over drought stressed plants (Hoffman and Hogg 1992). Given that the preference of vectors for healthy versus infected plants can impact rates of disease spread (McElhany et al. 1995), studies are needed that quantify sharpshooter preference and transmission efficiency in experiments offering the choice among healthy, asymptomatic, and symptomatic host plants. Such experiments would provide insights into the significance of infection-induced water stress for pathogen spread.

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