HYPERSENSITIVE RESPONSE IN LIVE OAK: CHARACTERIZATION AND EFFICACY AGAINST A HOST SPECIFIC GALL-FORMING WASP

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ABSTRACT

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The establishment and spread of host-plant-specific pathogens and insect herbivores are fundamentally affected by plant defenses. Hypersensitive response (HR) is a plant defense classically recognized as resulting from a gene-for-gene interaction between pathogens and plants. More recently HR arising from plant-insect herbivore ınteractions has been documented in select study systems. HR results in localized necrotic plant tissue in response to, and at the site of, attack from pathogens and insect herbivores. It is well established that HR is associated with increased mortality, reduced establishment, and decreased spread of pathogens. However for insect herbivores, most

studies of HR lesions in response to insect attack have investigated the mechanics of the process leaving assessment of the effect of HR on natural populations of insect herbivores relatively unexplored. Herein I examined necrotic lesions that arise in the leaves of Quercus fusiformis (plateau live oak) in response to oviposition by the host specific gall-forming wasp Belonocnema treatae to assess whether apparent HR functions in plateau live oak as a plant defense, and if so, to estimate its importance. To do this I examined the probability of gall establishment in the presence and absence of necrosis within and among individual live oak trees monitored in each of two years. The probability of B. treatae gall formation was significantly reduced by necrotic lesions thus illustrating that lesions act as a plant defense. HR was commonly observed and individual trees varied widely in HR effectiveness both within- and among- years. This study is the first to demonstrate the effectiveness of HR lesions against any of the 1300 species of cynipid wasps. Cynipid diversity is centered on oaks with individual gall- former species typically restricted to individual species of oak. My results make cogent the questions concerning the frequency of HR and the effectiveness of HR defense among oak species against their diverse gall-forming fauna.

INTRODUCTION

Plant defensive traits are powerful determinants of establishment (Thrall et al. 2001, Egan and Ott 2007), performance (Thrall et al. 2001, Johnson 2008), abundance and spatial distribution (Burdon and Thrall 1999, McIntyre and Whitman 2003, Forde et al. 2004) and population dynamics (Thrall et al. 2001, Johnson 2008) of both pathogen and insect populations on their host plants. A widely induced plant defense mechanism against pathogens is Hypersensitive Response (HR) (Morel and Dangl 1997, Gilchrist 1998, Heath 2000). Classically described as a defense against pathogens the genetic basis of HR resistance was explored by Flor in 1942 while studying flax-flax rust infection in which he described a simple gene-for-gene model leading to HR with a single dominant *R* gene in the host plant which recognizes the signal molecules (elicitors) of a complementary dominant *Avr* gene in the pathogen (Flor 1952). The "incompatibility" or specific recognition of *R* and *Avr* genes leads to a signal transduction cascade in the plant which often results in plant cell lysis due to pathogen invasion. This becomes evident as characteristic necrotic lesions in the plant tissue (Hammond-Kosack and Jones 1997, Morel and Dangl 1997, Heath 2000, Kombrink and Schmelzer 2001) and this rapid and localized death of plant cells is thought to restrict invasion of pathogens (Heath 2000).

More recently, plant response to insect oviposition and feeding of phytophagous insects have also been found that resemble pathogen-induced HR (Hilker and Meiners 2002, Hoglund et al. 2005, Kaloshian and Walling 2005, Little et al. 2007). HR has now

been proposed to act as a defense against various plant herbivores; including Lepidoptera (Shapiro and DeVay 1987, Hilker and Mieners 2006), Hessian flies (Grover 1995), Coleoptera (Garza et al. 2001), Cecidomyid flies (Fernandes 2001, Ollerstam et al. 2002), Homoptera (Espirito-Santo and Fernandes 2002), nematodes (Kaloshian 2004), as well as Hemipterans (Kaloshian and Walling 2005). Like pathogens, insects deliver elicitor molecules introduced to their host plant through oviposition (Hilker and Meiners 2006, Little et al. 2007) or from saliva delivered via mouthparts of piercing-sucking insects (Kaloshian and Walling 2005, Harris 2006) which triggers HR defense. While many induced plant defenses attack feeding larvae or adults, defense targeting herbivore eggs (Shapiro and DeVay 1987) is considered an "early attack" mechanism, preventing initiation of feeding (Hilker and Meiners 2006, Little et al. 2007).

Although a growing number of studies have recognized HR as a defense against various insect herbivores, relatively few studies have documented the efficacy of HR at reducing natural herbivore populations and any evolutionary implications. For instance, in a two year study involving a cecidomyiid gall midge population feeding on *Bauhinia brevipes*, HR induction caused >90% mortality of gall midge larvae (Fernandes 1998). In another study, HR contributed to a significant amount of mortality against larvae of eight different Cecidomyiidae species across four tree species, contributing to two-thirds of gall larvae mortality (Fernandes and Negreiros 2001). Additionally HR in a *Fagus* species attacked by two species of Cecidomyiidae larvae was responsible for 77% mortality of the gall-forming larvae across the two populations examined (Fernandes et al. 2003).

Gall formers, including Cynipid wasps, are a specialized group of sessile insect herbivores which produce nutritive tissue by modifying undifferentiated plant cells (Weis et al. 1988). Cynipid wasps are harmful to their plant hosts because they depend on mineral nutrients and photoassimulates provided by the plant, thus they act as nutrient sinks (Stone et al. 2002) and because of this they may face acute selection from their host plants. Sessile insect species, which have intimate relationships to their host plants, may be particularly susceptible to this plant defense (Weis et al. 1988, Espirito-Santo and Fernandes 2002, Harris 2006).

The majority of cynipid wasp species reside in the tribe Cynipini and are obligate herbivores on oak and other tree species in the Fagaceae family (Ronquist and Liljeblad 2001). One such cynipid-oak relationship includes the gall-forming wasp *Belonocnema treatae* and its host-plant *Quercus fusiformis*. HR in oak species against *B. treatae* or any of the approximately 1300 Cynipid gall-forming wasp species (Ronquist and Liljeblad 2001, Hayward 2005) have not been documented in the literature.

HR-like necrotic lesions (heretofore referred to as HR lesions) in association with oviposition of the gall wasp *B. treatae* are commonly observed on the leaves of plateau live oak, *Q. fusiformis* (personal observation). Here we characterize the association of HR-like lesions in response to oviposition into the leaves of *Q fusiformis* by the cynipid gall-former *B. treatae*. Specifically we 1. assess the incidence of HR lesions in response to oviposition (at both the individual tree and population level, 2. determine whether HR lesions function as a plant defense against this host-specific herbivore and lastly, 3. document factors that may influence HR incidence in a heterogeneously distributed population of the gall-forming wasp.

METHODS AND MATERIALS

Study System

Belonocenma traetae Mayr (Hyemenoptera: Cynipidae) is a host specific, gall-former of plateau live oak, *Q. fusiformis* Small (Mueller 1961) in the Edwards Plateau of Central Texas, USA (Lund et al. 1998). *B. treateae* displays a heterogonous life cycle in which an asexual generation that develops inside of uniocular galls on leaves of live oak alternates with a sexual generation, that develops in multiocular root galls (Lund et al. 1998). The sexual generation emerge from March through May concomitantly with new leaf flush, mate immediately upon emergence and oviposit into the lateral veins on the abaxial side of immature *Q. fusiformis* leaves (Cryer 2003). The asexual generation emerges from November through February and oviposits into the roots of its host tree to complete the life cycle (Lund et al. 1998).

Characterization of HR Lesions

Ovipositor insertion of the sexual generation into the leaves of plateau live oak results in a distinct and permanent leaf scar, hereafter referred to as an oviposition scar. Oviposition scars result from the host plants mechanical wound response and are typified by ocher scar tissue surrounding the site of oviposition insertion. Oviposition scars that lead to gall induction are typically seen only on secondary or tertiary veins of the abaxial side of the leaf. Distinguished from these prominent oviposition scars are smaller

superficial ovipositor scars frequently observed between veins which result from dragging the ovipositor over the leaf surface (Figure 1). These superficial scars may be related to female oviposition site preference but they do not lead to gall formation or HR and thus were not analyzed here. Oviposition scars thus provide a record of oviposition attempts and associated outcomes (i.e. whether or not a gall was established) and can be examined at the individual leaf, tree, population and year level. Hypersensitive Response lesions in this system were described on a phenotypic basis as necrotic lesions in association with oviposition scars which were of sufficient magnitude to perforate the leaf tissue. Because HR lesions are associated with *B. treatae* oviposition insertion they are almost always seen on secondary or tertiary veins of *Q. fusiformis* leaves. Oviposition is attended by four possible outcomes: 1. oviposition scar with no HR lesion and no gall establishment (Figure 1A), 2. oviposition scar with no HR lesion with gall establishment (Figure 1B and 1E), 3. oviposition scar with HR lesion and no gall establishment (Figure 1C) and 4. oviposition scar with HR lesion with gall establishment (Figure 1D). The above oviposition outcomes were scored on a per leaf basis for each of two years.

Field Site and Sampling Methods

The study site was located at Texas State University-San Marcos Freeman Ranch in Hays County, Texas, USA (29°55' N, 98°00' W) which sits on the eastern edge of the Edwards Plateau region of central Texas and is characterized by oak-juniper savanna. To assess HR incidence within and among trees (within years) and between years, 78 mature *Q. fusiformis* trees with known populations of *B. treatae* were initially surveyed from May - August 2008. In 2009, seventy *Q. fusiformis* were surveyed from July –

November. Trees in this study were selected based on *B. treatae* oviposition activity (any tree showing evidence of oviposition scars, gall formation and/or HR lesions was considered a candidate tree). Trees in each year were sampled along transects over a wide swath of the ranch (this ensured sampling of trees that were spatially similar (along transects) as well as sampling distinct spatial areas (between transects)). All tree positions were identified with GPS coordinates and labeled with metal tagging for follow up studies.

Leaves from each tree in 2008 and 2009 with one or more ovipositor insertion leading to ovipositor scars, gall establishment and/or HR lesions were selectively collected from each tree throughout the canopy in both years. In each year we sampled approximately 200 leaves from each tree. The four possible oviposition outcomes (Figure 1) were scored on a per leaf basis. This sampling procedure secured a pool of oviposition insertion scars per tree from which to assess HR incidence and the consequences of lesion formation. Collected leaves were stored in plastic bags and placed in a freezer until examined under a dissecting microscope at 50X.

A subset of 65 trees, from the total 78 trees surveyed in 2008 were resurveyed in 2009. Thirteen trees in 2008 were not surveyed in 2009 because of drought conditions leading to early leaf senescence, additionally 5 new trees were surveyed in 2009 that were not surveyed in 2008. Leaves from the first 20 trees examined in 2008 were collected before gall formation occurred, thus 20 trees in the 2008 leaf analysis provide leaf information on the first two oviposition outcomes only (i.e. 1. oviposition scar with no HR lesion and no gall establishment and 2. oviposition scar with no HR lesion with gall establishment). The leaf data from the remaining 58 trees in 2008 provide

information in all four possible oviposition outcomes. Due to the incongruence in sampling among years, 65 trees in 2008 and 2009 have corresponding leaf data on the first two oviposition outcomes, while 46 trees in 2008 and 2009 have corresponding leaf data on all four possible oviposition outcomes. The mean number of leaves analyzed per tree was 189 ± 3.56 in 2008 and 150 ± 5.34 in 2009 (mean ± SE).

Leaf Analysis

Leaves were examined under a Meiji brand dissecting scope at 50X. A total of four oviposition outcomes (i.e. oviposition scars leading to the presence of lesions with or without gall establishment or the absence of lesions with or without gall establishment) were analyzed on a per leaf, per tree and per year basis. To determine whether the general size of lesions influenced gall establishment, lesions were categorized as 'small' or 'large'. Lesions were categorized based on the relative size of lesions in comparison to the typical size of oviposition scars. Small lesions were considered to be smaller or equal the size of a typical oviposition scar (0.01 - 0.06 mm), large lesions were considered to range from 0.07 - 0.65 mm or larger. The inspection of lesion size was analyzed to determine the proportion of lesion incidence associated with each size category as well as to determine the effectiveness of lesions sizes at preventing gall establishment on a per tree, per year and population-wide scale.

Lesion Incidence Variation

To test the hypothesis that the variation in HR incidence within years (among trees) differed significantly between 2008 and 2009, a repeated measures ANOVA was

also conducted, with year assigned as a fixed effect and tree nested within year as a random effect. A single factor ANOVA was then conducted to determine whether a significant difference in HR incidence existed among years. A variance components analysis, with tree as the random factor, was then conducted to measure the proportion of HR incidence variation contained within years (among trees) and the proportion of HR incidence contained among years. To assess, on a per tree basis, whether HR incidence was correlated in 2008 and 2009 ($N = 65$), a nonparametric Spearman's rank correlation (r_s) of proportion of HR incidence was conducted.

Lesions as a Plant Defense

The hypothesis that gall establishment in the presence of HR lesions was significantly different than gall establishment in the absence of HR lesions was examined using a chi-square test, oviposition scars were tabulated for all leaves, across all trees in 2008 and 2009. If HR lesions functioned as a plant defense, then the probability of gall establishment given HR lesions would be significantly lower than the probability of gall establishment in the absence of HR lesions.

Variation in Lesion Effectiveness

Using a repeated measures ANOVA, with year assigned as a fixed effect and tree nested within year as a random effect, we tested the hypothesis that the variation of gall establishment probability given HR incidence differed significantly among trees between 2008 and 2009. A single factor ANOVA was used to assess whether a significant difference in gall establishment probability given HR incidence existed among years.

This ANOVA was followed by a variance components analysis, with tree as the random factor, to determine the proportion of variation in gall establishment probability given HR contained within years (among trees) and the proportion contained among years. To assess, on a per tree basis, whether gall establishment probability given HR incidence was correlated in 2008 and 2009 ($N = 46$), a nonparametric Spearman's rank correlation (r_s) of gall establishment probability given HR lesions was performed.

Oviposition Intensity and Lesions

To test the hypothesis that the intensity of *B. treatae* oviposition scars affected HR incidence variation, a regression of mean oviposition scars per leaf (x) and percent HR incidence per tree (y) was conducted. This relationship established whether oviposition scar intensity contributed to the incidence of lesions at the population scale within each year and determined whether HR lesions are a function of oviposition intensity.

Lesions and Gall Establishment

The correlation between mean HR incidence per tree and gall establishment per leaf was examined to assess whether lesion incidence was associated with gall establishment in this *B. treatae* population. Because gall establishment is linked to overall population establishment and thus population dynamics of the gall-former, the correlation addresses population consequences at a geographic scale that are associated with host plant factors.

Lesion Size and Effects

To determine whether lesion size functioned as a determinant of gall establishment (i.e. using lesion size as a proxy for HR lesion rigor) a chi-square test was used to assess whether gall establishment in the presence of small lesions significantly differed from those in the presence of large lesions. If gall establishment was lower in the presence of large lesions, the size of lesions was directly related to gall establishment prevention.

Statistical analysis

All data were analyzed using The R project (R code) http://www.r-project.org/. Homoscedasticity and normality violations for ANOVA and regression data were repaired using log transformations.

RESULTS

Characterization of HR Lesions

HR lesions were described as necrotic perforated *Q. fusiformis* leaf tissue that developed following *B. treatae* ovipositior insertion. We described *B. treatae* ovipositor insertion scars and HR lesions as categorically different due to the perforation of the leaf tissue associated with lesion formation. Oviposition insertion always led to tissue scarring while HR manifest as lesions, irregular to circular in shape, which emanate from the site of ovipositor insertion. Typically HR lesions were associated with a ring of dead cell tissue (scarring) surrounding the lesion site (Figure 1C), however, occasionally clean cut edges around lesions were observed with no apparent scarring (Figure 1E). Lesions were categorized in sizes, small and large, to determine whether size of lesions was a determinant of gall establishment. Small lesions ranged from 0.01 - 0.06 mm while large lesions ranged from 0.06 - 0.65 mm. The total proportion of small lesions in 2008 and 2009 equaled 39% and 19%, large lesions had higher proportions equaling 61% and 81% in each respective year.

Lesion Incidence

HR lesions associated with oviposition into leaves of plateau live oak by the sexual generation of *B. treatae* is a common phenomenon. In 2008, across all trees,

lesions were associated with 13.8% of the 174,823 oviposition scars examined while in 2009 lesions were associated with 30% of the 78,968 oviposition scars examined (Figure 2). In 2008, six percent of the 14,737 leaves examined and in 2009, forty-three percent of the 10,500 leaves examined with one or more oviposition scar showed evidence of lesion incidence. On average 1.63 ± 0.18 (mean ± SE) (range 0.23 - 3.9) lesions were observed per leaf in 2008 while in 2009 lesions equaled to 2.23 ± 0.15 (mean ± SE) (range 0.44 – 8) per leaf. Overall population-wide HR incidence increased significantly by 54% in 2009 relative to 2008.

Variation in HR Incidence

HR incidence differed widely among and within years. The proportion of oviposition scars associated with lesions differed significantly among individual trees within years ($F = 51.94$, $df = 64$, $P < 0.0001$) and among years ($F = 42.47$, $df = 1$, $P < 0.0001$). At the level of the individual tree, variance components analysis revealed that within year variation of HR incidence exceeded variation among trees, with the majority of HR incidence variability contained among trees (within years) measuring 82% and between years measuring 18%. The larger proportion of HR incidence variation within year is noticeably evident when comparing the range of HR incidence distribution per tree in 2008 (3.16 - 46.5%; $N = 78$) to the range of HR incidence distribution per tree in 2009 (6.19 - 60.21%; $N = 70$). Comparison of the distribution of HR incidence at the individual tree level shows larger numbers of trees with lower HR incidence in 2008 as compared to 2009 (Figure 3). There was a weak but significant positive correlation ($r_s = 0.26$, $P = 0.03$; Figure 4) between incidence of HR observed for the 65 trees that were

surveyed in both 2008 and 2009, suggesting HR incidence among trees varies with respect to heritability.

Lesions and Plant Defense

It was expected HR lesions functioned as a plant defense against the gall former, as a result the presence of lesions would be negatively related to the incidence of gall establishment. A chi-square test, with data pooled across all leaves and all trees in each year, revealed that the incidence of gall establishment given lesions was significantly reduced compared to the incidence of gall establishment in the absence of lesions (X^2 = 1078.4, df = 3, $P < 0.001$ (2008); X^2 = 3344.02, df = 3, $P < 0.001$ (2009)). In the absence of lesions, mean gall establishment equaled 17% and 13% in 2008 and 2009 respectively, while mean gall establishment in the presence of lesions was 8% and 1% respectively, thus gall establishment was reduced via HR lesions by 53% in 2008 and 92% in 2009 (Figure 5). These results demonstrate that lesions act as a plant defense in this plant-insect system.

Variation in Effectiveness

The effectiveness of HR lesions at preventing gall establishment was examined to determine whether a similar trend in variation occurred among and within years, to that observed in HR incidence existed. The incidence of gall establishment in the presence of HR lesions differed significantly among individual trees within years (among trees) (F = 34.00, df = 45, $P < 0.0001$) and among years (F = 45.41, df = 1, $P < 0.0001$). At the level of individual trees, the effectiveness of HR at preventing gall establishment partitioned

by a variance components analysis found the majority of variation existed within years measuring 74% as compared to variation found among years measuring 26%. Thus more variation in the probability of HR incidence effectiveness is seen among individual trees within a year rather than trees among years.

The probability of gall establishment when in the presence of HR lesions observed per tree (from the subset of trees surveyed in both years) in 2008 equalled 1.1% (range 0 – 3.6%; $N = 58$) compared to 0.37% (range 0 – 3.4%; $N = 58$) (make this 70) in 2009 (Figure 6A and 6B). Thus the effectiveness of lesions at preventing gall establishment varied greatly among years. Moreover, the proportion of trees that established zero galls in the presence of HR lesions (i.e. trees which prevented 100% of gall formation when accompanied by lesions) equaled 1.5% in 2008 (1 of 65 trees) and 43% in 2009 (30 of 70 trees). Thus the proportion of trees ($N = 65$, 2008; $N = 70$, 2009) displaying "killer" phenotypes (those preventing 100% of gall establishment accompanied by HR lesions) differed dramatically from one year to the next. Overall, these results illustrate an impressive 97% variation in population-wide HR lesion effectiveness at preventing gall establishment among years. There was not a significant correlation in the probability of gall establishment in the presence of HR for the subset of trees surveyed between 2008 by 2009 ($r_s = 0.21$, $N = 46$, $P = 0.158$; Figure 7). The lack of a significant correlation demonstrates that variation in the effectiveness of individual trees at preventing gall establishment in the presence of lesions is not associated between years. The graph reveals fewer gall establishment associated with lesions in 2009.

Oviposition Frequency and HR Incidence

An inverse regression of mean HR incidence per tree and mean number oviposition scars per leaf per tree (oviposition intensity) explained 19% of the variation observed in HR incidence on a per tree basis in 2008 ($r^2 = 0.19$, $N = 78$, $P < 0.001$; Figure 8A). This relationship in 2009 however was not significant ($r^2 = 0.02$, $N = 70$, $P = 0.21$; Figure 8B). Comparing the 2008 and 2009 graphs reveals similar response functions in HR incidence outcomes in individual trees with low mean oviposition, however these similarities in HR incidence are not seen in individual trees expressing high mean oviposition. The lack of individual trees expressing high mean oviposition in 2009 (most likely due to environmental stochasitcity) possibly explains the regression outcomes in 2009 are different than that in 2008.

HR Incidence and Gall establishment

Lesion incidence among trees in both 2008 and in 2009 was significantly correlated to variation in mean gall density per leaf per tree ($r = 0.45$, $N = 58$, $P < 0.002$; Figure 9A.) and ($r = 0.37$, $N = 70$, $P < 0.008$; Figure 9B). These weak but significant correlations illustrate that HR incidence is associated with gall establishment per tree. Lesion incidence is thus connected to the spatial structure of *B. treatae* in this population.

Lesion Size

A chi-square test showed a significant difference between galls associated with small lesions versus large lesions in both 2008 and 2009 ($X^2 = 7{,}826$, $df = 3$, $P < 0.0001$; $X^2 = 660$, $df = 3$, $P = 0.001$). Gall establishment associated with small HR lesions averaged 6.1 ± 0.77 per leaf (mean $\pm$ SE) and gall establishment for large HR lesions

averaged 3.1 ± 0.39 per leaf (mean ± SE) in 2008 (N =58). Gall establishment associated with small HR lesions averaged 3.9 ± 1.0 per leaf (mean ± SE), while gall establishment associated with large HR lesions averaged 1.0 ± 0.24 per leaf (mean ± SE) in 2009 (N =70). These results reveal that gall establishment in the presence of large lesions were reduced compared with small lesions. Thus the size of the phenotypic expression of necrotic lesions is important in determining gall establishment.

DISCUSSION

This study characterizes and examines HR lesions and effectiveness in a naturally occurring Cynipid-Oak system. Demonstrations of HR have been extensively studied in pathogen-plant systems (Harris et al. 2006), yet fewer investigations of HR have been documented in insect herbivore-plant systems (Fernandes 1998, Kaloshian 2004, Hilker and Mieners 2006). This study, to our knowledge, is the first to recognize HR defense in a *Quercus* sp. acting against any of the approximately 1300 Cynipid gall-forming wasp species (Ronquist and Liljeblad 2001). Our study reveals population-wide HR incidence is significant within and among years. The hypothesis that HR in *Q. fusiformis* acts as a plant defense in response to oviposition from the cynipid wasp *B. treatae* is supported. Differentiation in HR effectiveness against gall establishment on individual host trees suggests that HR is a template of gall establishment differentiation. And the correlation between population-wide gall establishment and HR incidence suggests that *B. treatae* heterogeneity is associated with host plant defense factors.

HR Description

HR lesions described in this system are similar to insect-mediated HR lesions in other plant systems, i.e. localized necrotic lesions surrounding herbivorous insect invasion sites (Fernades 1998, Fernandes and Negreiros 2001, Fernandes et al. 2003, Hoglund et al. 2005). Hypersensitive Response lesions in live oak associated with *B.*

treatae oviposition attempts are a common feature in this system with 23% (13.8% and 30% averaged over 2008 and 2009) of total oviposition scars resulting in HR lesions. Since it is unlikely that all oviposition attempts are associated with egg insertion our estimate of 13.8% and 30% of total oviposition attempts (in each respective year) leading to HR lesion formation is a conservative estimate of HR incidence in response to oviposition. This interpretation however depends on whether HR lesions are in response to ovipositor insertion or egg deposition. Superficial oviposition scars (found in interveinal space) are less common than prominent oviposition scars (found on secondary and tertiary veins) while HR lesions are more commonly associated with prominent scars (personal observation). These observations suggest that HR lesions are more commonly associated with egg deposition.

Our ANOVA results indicate a significant difference in HR incidence within and among trees and years at the landscape level. Findings of among-plant HR incidence variation corresponds to documented HR variation among other plant-herbivore systems (Hoglund et al. 2005), variation in HR effectiveness between years are also consistent with other demonstrations of oscillations in resistance against pathogen and insect invasion (Burdon and Thrall 1999, Thrall et al. 2001, McIntyre and Whitham 2003, Forde et al. 2004). There was a small but significant correlation between HR incidence between 2008 and 2009. The most obvious explanation for the relationship may be heritable variation in HR among trees, but because the correlation is weak, factors such as genotype-by-environment interactions, plant physiology and difference in resource quality between years and/or environmental stochasticity may also play a role in determining HR incidence outcomes.

Lesions act as a Plant Defense

We predicted that HR incidence would act as a plant defense due to the fact that Cynipid gall-formers can be harmful to their hosts (Stone 2002). The reduction of gall establishment when in the presence of HR lesions by 53% and 92% in each respective year establishes that HR lesions act as a plant defense against *B. treatae*. The discovery of HR as a plant defense in this system adds to the growing number of demonstrations in plant communities that show HR as a resistance mechanism to pathogens and as well as insect pests (Ollerstam et al. 2002, Harris 2006, Chen et al. 2009).

Mortality against the *B. treatae* asexual generation in 2008 and 2009 was estimated at 21% (HR lesions without gall establishment / total ovipositor insertion scars), range from12 – 30% in each respective year. Because of uncertainty in whether every ovipositor insertion results in a deposited egg, this estimate of mortality is conservative. The average 21% estimated contribution of HR lesion mortality occurs soon after egg insertion (mainly in April). Under these assumptions the major contribution to mortality is exposure to natural enemies, which leads to approximately 97% mortality (range 95 - 99%), of the remaining gall-former population (occurring mainly in July) (Hall 2001). Studies done by Fernandes (1998) and Fernandes et al. (2003) found that HR was responsible for the majority of galling insect mortality. While natural enemy exposure contributes to greater absolute individual gall-former mortality in this system, detected HR mortality nonetheless contributes to an important amount of gall-former deaths at the egg stage.

A potentially concealed factor in gall establishment mortality could be HR which shows no phenotypic expression of lesion formation. The willow species *S. viminalis*

shows two types of resistance against the gall midge *D. marginemtorquens* one which displayed lesions and one which did not express necrotic lesions to larvae infestation (Hoglund et al. 2005). If resistant genotypes, which show no phenotypic expression of HR in this system *B. treatae* mortality due to HR may be of greater importance than the noted average 21% seen in 2008 and 2009. To more accurately determine the mortality associated with HR lesions, the proportion of oviposition insertion scars associated with egg deposition needs to be addressed.

Effectiveness of HR Lesions

Variation in HR effectiveness at preventing gall establishment was demonstrated by a conspicuous 82% difference in 2009 compared to 2008 (Figure 6A and 6B). On a per leaf basis gall establishment probability in the presence of HR incidence was 13.8% in 2008 and 3.1% in 2009. This disparity in HR effectiveness is especially clear when examining the frequency distributions of the effectiveness of gall establishment in the presence of HR lesions between years. The percent of trees showing zero gall establishment in the presence of HR lesions (in effect trees displaying 'killer' phenotypes) varies widely among years from 1.5% (1 of 65 trees) in 2008 to 43% (30 of 70 trees) in 2009. The large variation among years in HR effectiveness is generally reflected in the non-significant correlation coefficient characterizing gall establishment probability given HR incidence in 2008 by 2009. The non-significant correlation may be explained by environmental stochasticity and may demonstrate differentiation in plant resource allocation. In wetter years trees may allow gall establishment as opposed to

inducing HR lesions, which may be more beneficial to the plant while the opposite outcome may be more beneficial in drought years.

Fernandes (1998) showed variation in HR effectiveness at a population level changed minimally over a two year study. There are no studies to corroborate the extreme yearly fluctuations of HR incidence or HR effectiveness described here, this may be because most studies examining HR were completed in one year (Fernandes and Negreiros 2001, Fernandes et al. 2003). To rule out the possibility that HR incidence and its effectiveness in the *B. treatae* - live oak system is especially vulnerable to abiotic events, long term studies involving HR incidence in other systems are needed. The fundamental questions of the direct effects of HR on rates of gall development, attained gall size and parasitism rates should also be addressed.

Oviposition Rate and HR Incidence

In 2008 mean number of oviposition scars per leaf per tree (oviposition intensity) explained 19% of the variation in HR incidence among trees, however, in 2009, mean number of oviposition scars per leaf did not account for among tree HR variation. This discovery is informative because it illustrates that while oviposition intensity is responsible for some variation in HR incidence in particular years, it is not a consistent predictor of HR incidence patterns between years. Our results demonstrate that HR incidence is not consistently dependent on insect attack intensity. This result is exemplified by trees with high oviposition intensity but relatively low amounts of HR incidence. Because trees with low mean oviposition in 2008 and 2009 show similar HR incidence response outcomes, while the 2009 graph lacks trees with high mean

oviposition (possibly due drought conditions in that year), this may explain the non-significant2009 regression. Removing oviposition rate as a cause of HR incidence suggests that HR variation may be sustained through other means, such as a gene-for-gene relationship and/or genotype-by-environment interaction or both and is not induced by oviposition rate exclusively.

Lesion size

Our prediction of larger lesions associated with reduced gall establishment was supported. The reduction of gall establishment when associated with larger lesions, given that resources diverted to the gall are most likely restricted when lesions are present, larger lesions would mean lower resources and a higher chance of mortality. To our knowledge this is the first study to examine lesion size and gall establishment.

Spatial Structure

Realized gall establishment, measured as the observed number of galls per leaf, is associated with HR incidence per leaf per tree. This is illustrated by the weak correlation of gall establishment and HR incidence observed in both 2008 and 2009. This result reveals that population-level consequences of among tree variation in HR effectiveness may in turn effect variation in *B. treatae* establishment among trees. Resistance and virulence are fundamental outcomes of processes that shape pathogen population dynamics and recruitment leading to spatial structure within host populations (Burdon and Thrall 1999, Thrall et al. 2001, Forde et al. 2004) and host plant susceptibility due to genotype is known to produce population variation in gall densities (Stone 2002). Yet

few studies have shown that spatial variation in phytophagous insect population distributions are effected by resistance and susceptibility to a bottom-up defense (Mopper and Simberloff 1995, Mopper 1996). These results suggest that population dynamics of *B. treatae* may be intimately related to HR incidence and should be studied further.

Patchy distribution of *B treatae* on oaks leading to deme formation has been linked to plant quality (Egan and Ott 2007). Determining whether resistant-virulent defense interactions can cause and maintain deme formation in coevolved plant-insect populations is of general importance to understanding the processes that lead to adaptive deme formation. If natural selection on insect herbivores results from induced plant defenses (Agrawal 1999) and internal feeders experience stronger bottom-up selection pressures than their external feeding counterparts (Mopper 1996), then Cynipid gall forming wasps, which are sessile internal feeders may be subject to important selection pressure and their populations predisposed to exhibiting genetic substructure.

Environmental Influence on lesion Incidence

The dramatic increase in HR incidence and effectiveness in the second year of this study, corresponding to the 2009 drought in the study area, suggests that environmental fluctuations may have affected the outcomes of HR incidence in addition to the effectiveness of HR lesions against gall establishment. Environmental parameters are known to affect plant allocation and resistance to insects, for instance, nutrient and light availability effected gypsy moth populations on eight different genotypes of aspen (Osier and Lindroth 2005) and Maddox and Cappuccino (1986) demonstrated that water scarcity reduced aphid populations on different genotypes of *Solidago* sp. Increased

population-wide HR incidence probability (47%) as well as increased probability of HR effectiveness (82%) in 2009 indicates these aspects of the interaction are influenced by environmental stochasticity. The dynamics of HR variability during fluctuating abiotic events suggests that to HR defense is a phenotypically plastic trait. To better understand the dynamics influencing lesions as a defense the impacts of environmental conditions should be explored further in this system. The effects of sequential genotype-by-environment interactions could obscure the impact of gene-for-gene interactions and/or plant quality effects alone that might have been revealed under more environmentally stable conditions. The level at which HR incidence is impacted by environmental conditions should be explored in further studies.

Summary

This study provides the first description of HR against a Cynipid gall forming wasp. HR is demonstrated as an important plant defense while variation in HR incidence leads to differential insect establishment among individual trees. The variability of HR incidence under-environmental stresses may reveal important aspects of how this defense functions over time. We suggest long term studies be undertaken to understand the processes that govern HR and its effects on *B. treatae* populations in this system. Hypersensitive Response is an under-explored defense in herbivorous galling insects and our results support the possibility that HR affects a broader array of insect-host plant interactions (especially those involving internal feeding herbivores).

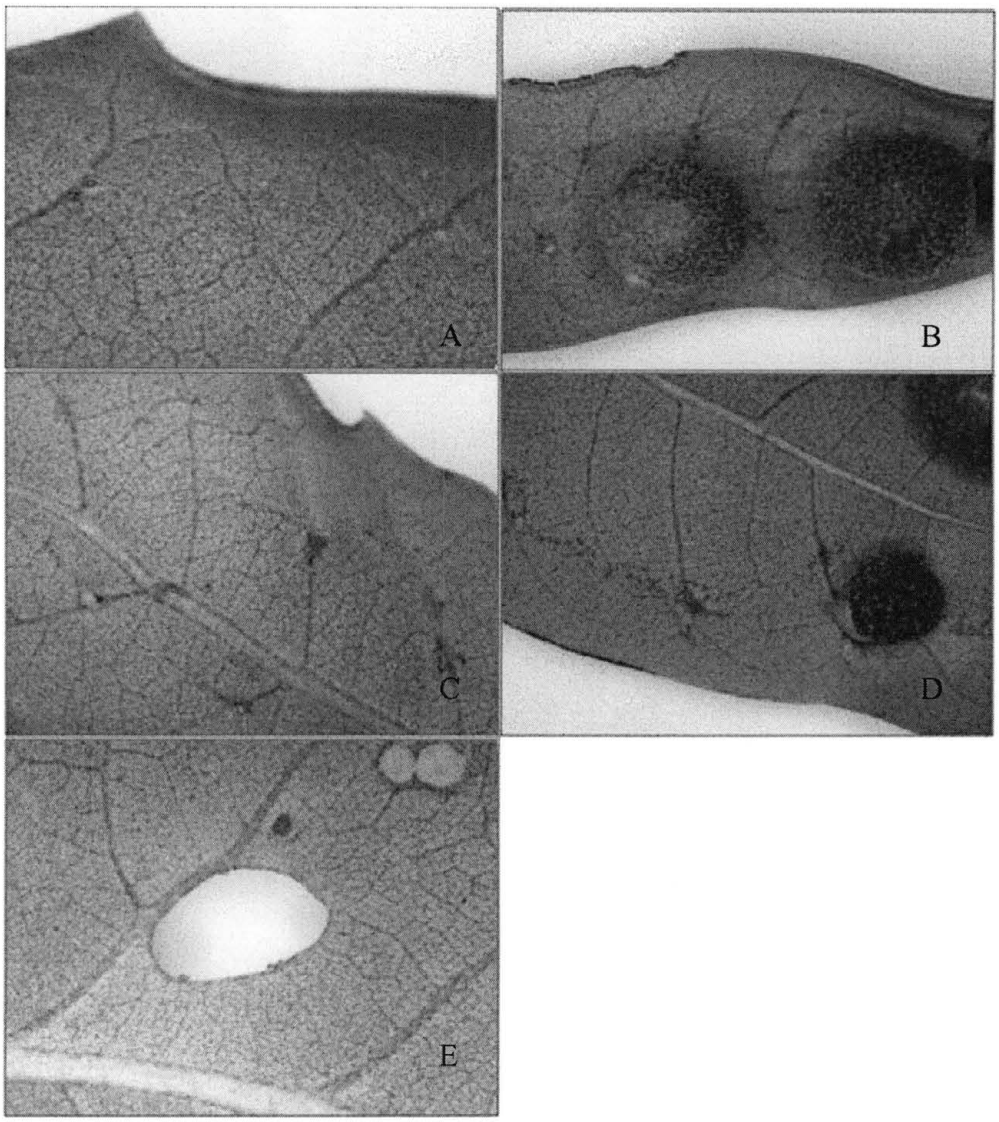

Figure 1. Oviposition scars and their associated outcomes. The four possible outcomes of oviposition are: (A) *B. treatae* oviposition insertion scars observed on secondary veins of leaves (evident as scarring), superficial oviposition scars are seen between primary veins. (B) gall establishment seen on lower half of leaf, oviposition insertion scars line the secondary veins of the leaf. (C) HR lesions and oviposition insertion scars on the secondary veins. (D) HR lesion adjacent to gall establishment (lower right corner) along with HR lesion and superficial oviposition attempts (lower left). (E) HR lesion with little scarring around circular necrotic edge.

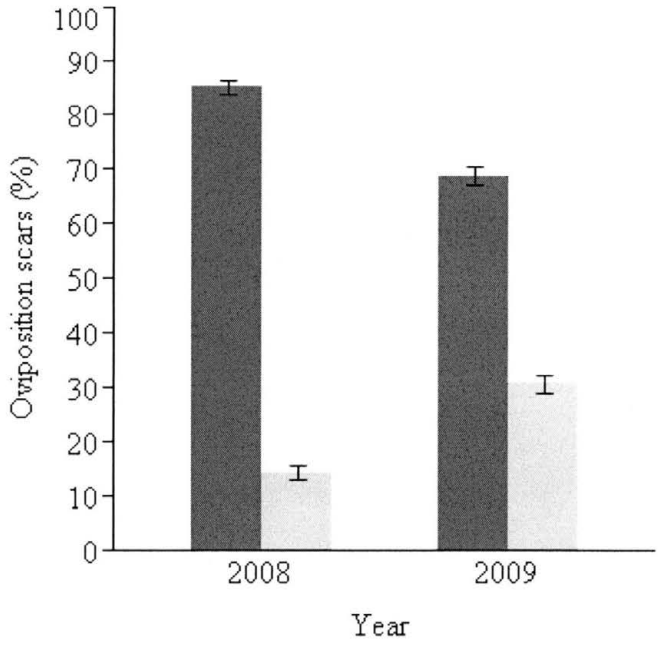

Figure 2. Oviposition in the absence and presence of HR formation. Population wide proportion of oviposition in the: absence of HR lesions (± SE) (black bar = 86.2%) and in the presence of HR lesions (grey bar = 13.8%) for each of 78 trees in 2008; as well as absence of HR lesions (± SE) (black bar = 70%) and presence of HR lesions (grey bar = 30%) in each of 70 trees in 2009.

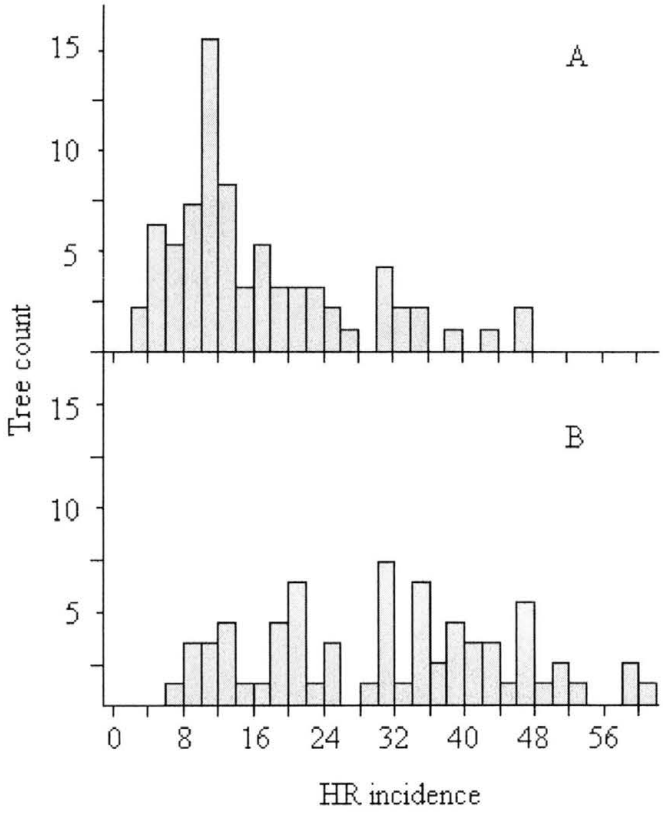

Figure 3. Frequency distributions of HR incidence. The frequency distribution of HR incidence (proportion of HR lesions per tree) for 78 trees in 2008 (A) and 70 trees 2009 (B). The degree to which the proportion of HR incidence varies within- and among- years is depicted by the divergent distributions.

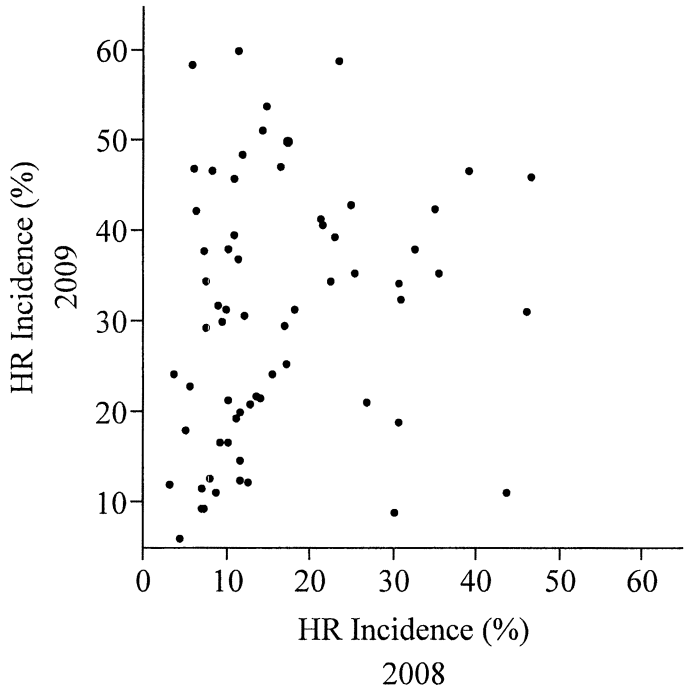

Figure 4. Correlation of HR incidence. Correlation between of HR incidence in 2008 and 2009 between paired trees ($N = 65$) ($r_s = 0.26$, $P = 0.03$).

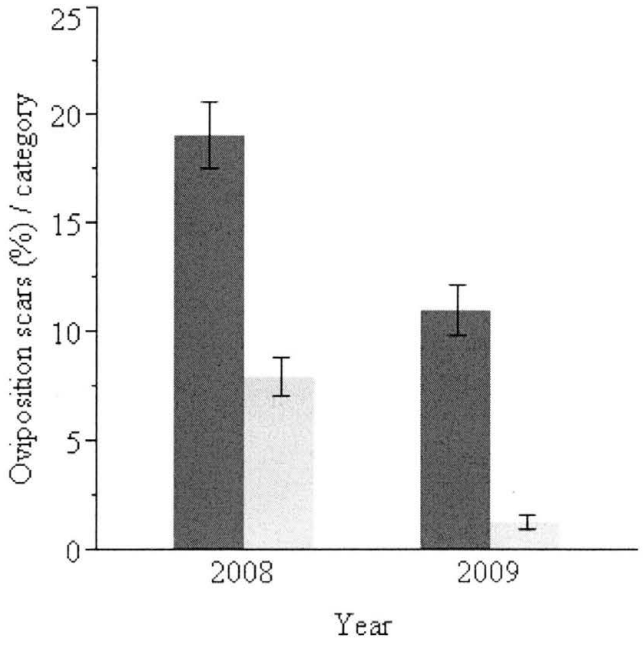

Figure 5. Gall establishment in the absence and presence of HR formation. Proportion of gall establishment in the absence of HR lesions in 2008 (N = 58) equaled 17% (± SE) and 13% (± SE) in 2009 (N = 70) (black bars); while the proportion of gall establishment in the presence of HR lesions equaled 8% (± SE) in 2008 and 1% (± SE) in 2009 (grey bars).

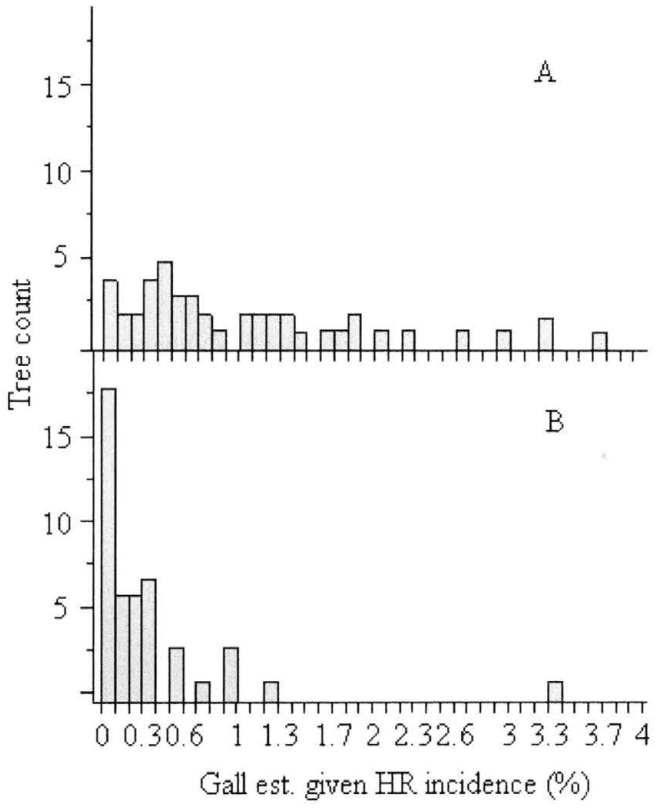

Figure 6. Frequency distributions of gall establishment given lesions. Frequency distribution of the proportion of gall establishment in the presence of HR lesions for 46 trees in 2008 (A) and 46 trees in 2009 (B). The degree to which the proportion of gall establishment in the presence of HR lesions varies within- and among- years is illustrated by the widely divergent distributions. Note the proportion of trees represented in the bar to the far left (trees expressing killer phenotypes) is dramatically different among years.

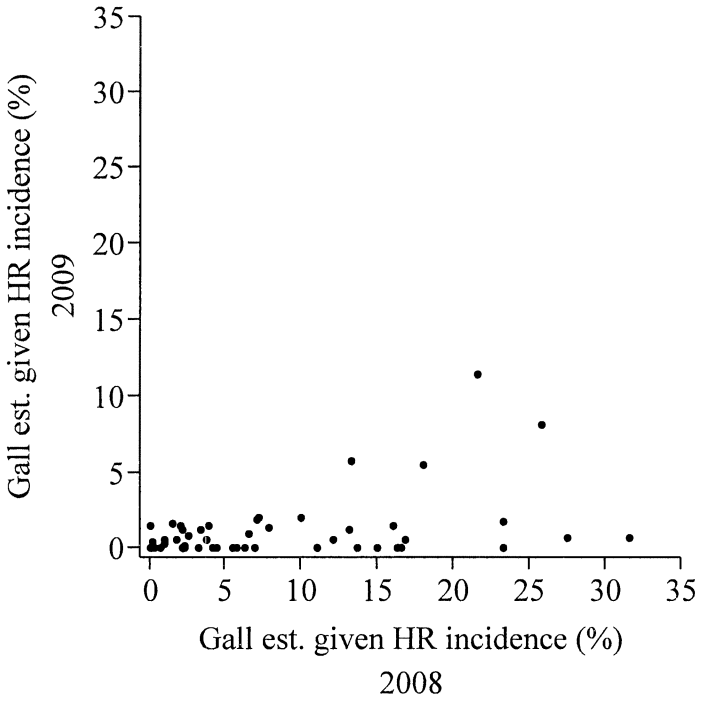

Figure 7. Correlation of gall establishment given HR incidence. Correlation between of the probability of gall establishment given HR incidence for paired trees ($N = 46$) in 2008 and 2009 ($r_s = 0.21$, $P = 0.158$).

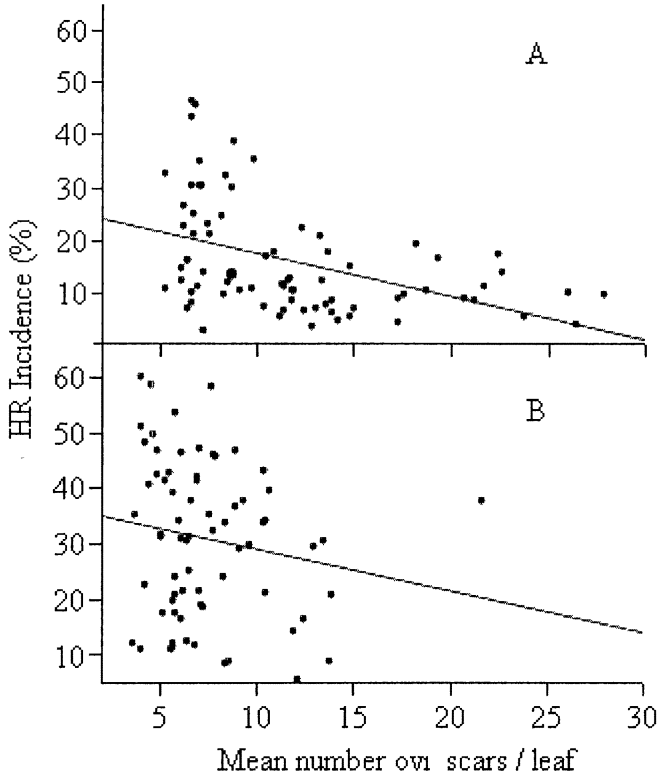

Figure 8. Relationship between oviposition and HR incidence. The relationship between mean number oviposition scars per leaf in 2008 (A) ($N = 78$) and 2009 ($N = 70$) (B) and HR incidence per tree. Mean number oviposition scars per leaf (oviposition intensity) explains a small portion of HR incidence ($r^2 = 0.19$, $P = 0.001$) in 2008, however in 2009 oviposition intensity did not explain HR incidence variation ($r^2 = 0.023$, $P = 0.21$).

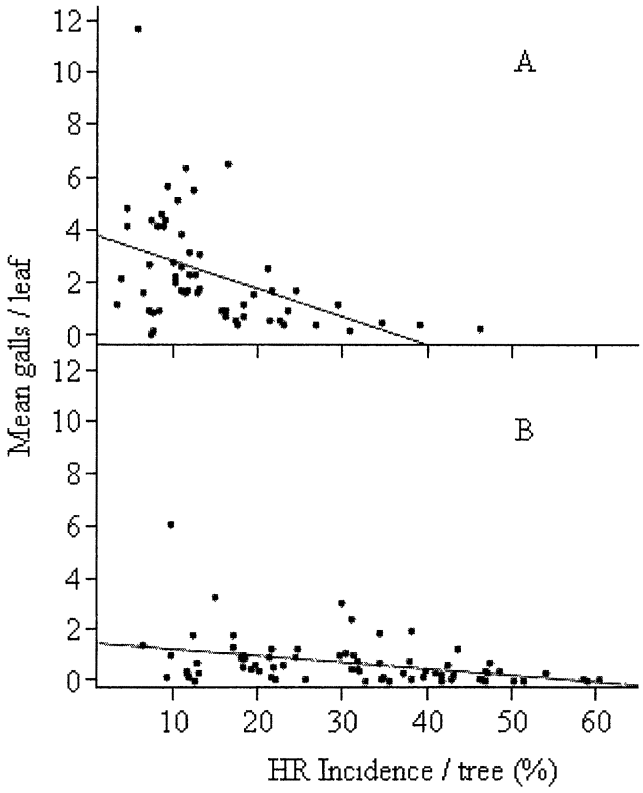

Figure 9. Correlation between HR incidence and gall establishment. The correlation between the proportion of HR incidence per tree and mean galls per leaf in 2008 (A) (N = 58) and 2009 (B) (N = 70). The significant relationship in each respective year (r_s = 0.45, $P < 0.002$; r_s = 0.37, $P < 0.008$) illustrate that mean gall establishment per leaf is associated with the proportion of HR incidence per tree.

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