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SUSCEPTIBILITY OF CLOVER SPECIES TO FUNGAL INFECTION: THE INTERACTION OF LEAF SURFACE TRAITS AND ENVIRONMENT[1]

DEVON J. BRADLEY,[2] GREGORY S. GILBERT,[3,4] AND INGRID M. PARKER[2]

[2]Department of Ecology and Evolutionary Biology, University of California, 1156 High St., Santa Cruz, California 95064 USA; and
[3]Department of Environmental Studies, University of California, 1156 High St., Santa Cruz, California 95064 USA

Many foliar pathogens require free water to germinate; therefore, disease pressure should favor plants that are able to repel water. For a suite of 18 sympatric clover species (*Trifolium* and *Medicago*, Fabaceae), we evaluated leaf traits affecting leaf wetness and susceptibility to infection by the fungal pathogen *Stemphylium* sp., causal agent of Stemphylium leaf spot. Spore germination increased with time in free water, and the relative susceptibility of host plants to infection was proportional to the duration of water retention on leaves. Larger leaves captured more water and retained it longer. Unexpectedly, trichomes and leaf wettability did not affect water capture. For clovers planted within natural clover populations at two sites, infection was threefold greater at the wetter site. At the drier site, water retention on the leaf surface was an important predictor of infection rates across host species, but persistent fog and dew at the wetter site reduced the importance of rapid leaf drying. Our results suggest that plant adaptations that reduce water retention on leaves may also reduce disease incidence, but the selective advantage of these traits will vary among habitats.

Key words: Fabaceaee; foliar diseases; fungal infection; leaf wettability; *Medicago*; spore germination; *Stemphylium*; *Trifolium*.

Adaptations that repel water from leaf surfaces are common features among plant species in habitats exposed to daily precipitation during the summer growth period, but are less common in drier habitats (Smith and McClean, 1989). Leaf morphologies that reduce water capture on leaf surfaces are much more common among plants in open-meadow habitats, where dew formation occurs at high frequency, compared with plants in the drier forest understory (Brewer and Smith, 1997). The selective pressure for reducing the wettability of leaf surfaces implied by these studies is usually thought to be physiologically driven. Because CO_2 diffuses about 10 000 times more slowly through water than air, excess leaf water may impede photosynthesis if a significant portion of the leaf surface is covered by a film of water (Smith and McClean, 1989; Brewer et al., 1991; Brewer and Smith, 1994). Indeed, leaf surface properties that promote the beading of water allow maintenance of a high photosynthetic rate and increased water use efficiency (Smith and McClean, 1989). However, although physiological factors are undoubtedly important in mediating natural selection on leaf surface properties, foliar pathogens may be equally important selective agents driving adaptations to reduce water capture on leaves. Few studies have addressed how repelling water from leaf surfaces may confer differential susceptibility to fungal infection (Cook, 1980; Statler and Nordgaard, 1980).

The persistence of leaf surface moisture, a condition critical for the development of most foliar fungal pathogens (Jones, 1986), plays a key role in the epidemiology of fungal diseases. Numerous studies have shown an increase in the incidence and severity of disease with increasing duration of leaf wetness (Cowling and Gilchrist, 1982; Evans et al., 1992; Filajdic and Sutton, 1992; Montesinos et al., 1995; Basallote-Ureba et al., 1999; Suheri and Price, 2000). Many foliar pathogens require extended periods in free water for spore germination, germ tube growth, and host penetration (Everts and Lacy, 1990; Wadia and Butler, 1994; Vloutoglou et al., 1996; Gilles et al., 2000). Therefore, plant traits that reduce the time that leaves remain wet after rain or dew could reduce susceptibility to foliar pathogens and potentially slow disease spread. In natural ecosystems, foliar pathogens can exert important selection pressures on plants (Alexander and Burdon, 1984; Esquivel and Carranza, 1996; see review in Gilbert, 2002), suggesting that the ability to repel water from a leaf surface may be an adaptation for the prevention of fungal infection. The mechanistic links between leaf water retention and fungal infection can provide a unifying framework for understanding variation in infection among plant species and across different habitats.

The amount of water captured and retained by a plant is influenced by leaf characteristics, including size, shape, and surface wettability. Wettability is a function of surface properties including smoothness and chemical composition of the cuticle (Holloway, 1970; Smith and McClean, 1989; Brewer et al., 1991). An irregular and/or waxy surface tends to repel water, leading to the formation of droplets, which evaporate from a leaf surface more rapidly than a film of water (Grammatikopoulos and Manetas, 1994). Increasing the surface area of a water drop increases the rate of evaporation, so that the smallest drops evaporate the most quickly (Brain and Butler, 1985). Therefore, variations in leaf surface characteristics that promote droplet formation reduce leaf wettability. The contact angle formed between a drop of water and the leaf surface can be used to compare the wettability of leaves of different plant species, with larger contact angles indicating less wettable leaf surfaces (Adam, 1963).

Eighteen species of native and exotic clovers (*Trifolium* and *Medicago* spp., Fabaceae) live sympatrically in the coastal

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[4] Author for reprint requests (phone: (831) 459-5002; Fax: (831) 459-4015; e-mail: ggilbert@ucsc.edu).

TABLE 1. Summary of results for leaf-wetting properties of 18 species of clovers (*Medicago* and *Trifolium* spp.). For each species, we measured the amount of water captured by individual leaves, time to dryness throughout the day and night, leaf area, leaf wettability, and density of trichomes. Values are means with standard deviations. See text for sample sizes.

Species	Leaf area (mm^2)	Total water capture (mg)	Time to dryness		Adaxial trichomes ($no./mm^2$)	Abaxial trichomes ($no./mm^2$)	Wettability (contact angle, °)
			Day (min)	Night (min)			
M. arabica (L.) Hudson	919 ± 59	102.4 ± 6.4	56 ± 8	125 ± 12	0	1.5 ± 0.2	109 ± 2
M. lupulina L.	377 ± 34	52.4 ± 10.7	51 ± 5	60 ± 6	1.5 ± 0.2	3.0 ± 0.3	136 ± 3
M. polymorpha L.	346 ± 25	42.3 ± 8.2	47 ± 6	71 ± 8	0	0	129 ± 3
T. barbigerum Torrey	94 ± 13	14.8 ± 6.9	43 ± 8	62 ± 5	0	0	128 ± 3
T. bifidum A. Gray	76 ± 6	11.0 ± 2.5	12 ± 5	26 ± 5	0	1.6 ± 0.2	134 ± 2
T. campestre Schreber	118 ± 15	15.5 ± 7.4	38 ± 6	59 ± 7	0	0.2 ± 0.04	125 ± 5
T. dubium Sibth.	180 ± 6	23.7 ± 10.6	52 ± 5	76 ± 10	0	0	131 ± 3
T. fucatum Lindley	275 ± 32	48.9 ± 18.6	42 ± 3	82 ± 6	0	0	128 ± 2
T. glomeratum L.	99 ± 15	35.7 ± 15.9	51 ± 5	63 ± 5	0	0	129 ± 3
T. gracilentum Torrey & A. Gray	134 ± 15	31.3 ± 11.7	21 ± 8	53 ± 5	0	0	133 ± 4
T. macrei Hook. & Arn.	111 ± 8	27.2 ± 6.0	25 ± 11	81 ± 11	10.1 ± 0.8	7.9 ± 0.8	121 ± 3
T. microcephalum Pursh	158 ± 15	39.9 ± 10.6	45 ± 2	81 ± 8	4.4 ± 0.4	5.2 ± 0.4	133 ± 2
T. microdon Hook. & Arn.	92 ± 15	28.6 ± 16.3	30 ± 8	73 ± 9	0	0	127 ± 4
T. repens L.	287 ± 43	55.6 ± 30.4	38 ± 2	69 ± 5	0	0	125 ± 3
T. subterraneum L.	430 ± 79	70.6 ± 59.4	67 ± 5	103 ± 10	0.4 ± 0.1	2.8 ± 0.2	123 ± 4
T. variegatum Nutt.	110 ± 15	25.2 ± 14.5	42 ± 7	67 ± 8	0	0	130 ± 3
T. willdenovii Sprengel	67 ± 5	18.8 ± 9.6	46 ± 7	83 ± 11	0	0	129 ± 4
T. wormskioldii Lehm.	213 ± 30	50.1 ± 11.5	54 ± 4	80 ± 5	0	0	117 ± 4

prairie at the Bodega Marine Reserve, California, USA. A common disease of clovers at the site is leaf spot caused by multiple species of the ascomycete anamorph *Stemphylium*, which often have sexual states in *Pleospora* [including *S. botryosum* Wallr. (teleomorph *P. tarda* E. Simmons), *S. alfalfae* E. Simmons (teleomorph *P. alfalfae* E. Simmons), *S. globuliferum* (Vestergr.) E. Simmons, *S. trifolii* (Graham), *S. vesicarium* species complex, *S. herbarum* E. Simmons (teleomorph *P. herbarum* (Pers.:Fr.) Rabenh.), and *S. sarciniforme* (Cav.) Wiltsh.]. *Stemphylium* spp. are important fungal pathogens of cultivated alfalfa (*Medicago sativa* L.) and clover (*Trifolium* spp.), responsible for foliar necrosis, leaf spots, and early leaf senescence (Smith, 1937; Nelson, 1955; Graham, 1957; Pierre and Millar, 1965; Cowling et al., 1981; Irwin and Bray, 1991; Berg, 1996). *Stemphylium* spp. cause the majority of foliar disease on clovers at the Bodega Reserve (G. S. Gilbert and I. M. Parker, unpublished data).

Infection occurs through air-dispersed asexual conidia that germinate on the leaf surface and then penetrate the host. Previous work on *Stemphylium* in agricultural systems has associated longer wetness periods with increased sporulation (Bashi and Rotem, 1975), infection (Johnson and Lunden, 1986; Suheri and Price, 2000), and disease severity (Cowling and Gilchrist, 1982; Lacy, 1982); the moist coastal areas of California provide conditions suitable for *Stemphylium* to produce viable conidia throughout the year (Gilchrist, 1990). However, sparse attention has been paid to the specific moisture conditions necessary for spore germination. Because spore germination is a gatekeeping stage in the life cycle of a pathogen, understanding abiotic effects on this critical process is essential to ecological studies of plant–pathogen interactions.

Here we used laboratory and greenhouse experiments to trace the mechanistic links among spore germination, water retention, leaf characteristic, and susceptibility to infection. First, we investigated whether time in free water drives *Stemphylium* spore germination. Then we tested whether variation in leaf characteristics among sympatric clover species causes differences in leaf water retention among species and whether susceptibility to infection by *Stemphylium* is correlated with continuous water retention time. Finally, in two field experiments, we tested whether predictions that arose from the laboratory and greenhouse studies explain relative infection rates among sympatric clover species in the field, and how environmental conditions in natural clover habitats influence *Stemphylium* infection.

MATERIALS AND METHODS

Site description and source of plants and fungi—Eighteen species of clovers (Fabaceae) (Table 1) were grown from seeds collected from wild plants at the University of California Natural Reserve at Bodega Bay in Sonoma County, California, USA (38°19′ N, 123°04′ W). The Reserve experiences cool, wet winters and foggy summers, with average annual rainfall of 784.9 mm and monthly averages ranging from 174 mm in January to 3 mm in July. Mean daily temperature ranges from 9°C in January to 13°C in August (mean = 11.7°C) (Chow, 2002). Field studies were performed at two sites, ''Bluff'' and ''Dorms.'' The Bluff site is a coastal prairie habitat, 40 m above sea level (a.s.l.) on a cliff overlooking the Pacific Ocean. The Dorms site is located 1.0 km inland (3 m a.s.l.) in a disturbed roadside habitat along the sheltered Bodega Bay. The Bluff site is much more exposed to wind, rain, fog, and salt spray from the ocean than is the Dorms site.

The strain of *Stemphylium* used in all experiments was isolated from a diseased leaf of *Trifolium fucatum* at the Bluff site. The leaf was surface sterilized (1 min 70% ethanol, 1 min 0.525% sodium hypochlorite) and then placed on 2% malt extract agar (MEA). The resulting isolate was maintained frozen at −80°C and subsequently used for experiments described later. Because of great overlap in morphological characteristics among described *Stemphylium* spp. as well as variation within species, identification based on morphology is difficult. A recent molecular phylogeny of *Stemphylium* spp. (Câmara et al., 2002) indicates there are five major clusters among 16 species, with pathogens of *Trifolium* and *Medicago* included in three clusters. Comparison of ITS sequence data showed that our strain (Bfuca39C, GenBank accession AY211983) fits their Group E, which includes *S. triglochinicola*, *S. loti*, and two clover pathogens, *S. trifolii* and *S. sarciniforme*. As do the strains in Group E, our isolate had smooth spores and did not produce sexual reproductive structures in culture.

Conidia were obtained by growing the fungus on V8 media (163 mL V8 juice (Campbell Soup Co., Camden, New Jersey, USA), 742 mL distilled water, 1.8 g calcium carbonate, 15 g agar) at room temperature for approximately 4 wk. Conidia were harvested by adding 60 mL of sterile distilled

water to the culture plate and swiping the colony surface with a rubber policeperson to ensure detachment of conidia from the mycelium. The resulting suspension was filtered through four layers of cheesecloth to remove hyphal fragments.

Germination rates—To examine the effects of free water and desiccation on *Stemphylium* spore germination, we assessed germination rates of conidia that were air dried and kept desiccated for 3, 9, or 14 h, after which they were either resuspended in free water or kept at 100% relative humidity for 24 h. Air drying was performed in a laminar flow hood. Additionally, we measured germination of conidia kept continuously in free water (0 h desiccation). For each of the seven treatment combinations, five replicates of a 55-μL spore suspension were placed into individual wells on the lid of a 96-well microtitre plate (Becton-Dickinson, Franklin Lakes, New Jersey, USA). Conidia were dried in situ (or maintained continuously wet in the case of 0 h desiccation—free water) in a laminar flow hood. At the end of the desiccation period, 55 μL of sterile deionized water were added to rewet the free-water treatments. The plates were then suspended over 100 mL of water in a sealed 355 mL plastic container at 25°C to maintain relative humidity at 100%. Using an inverted microscope, conidia were checked for germination at 0, 2, 4, 6, 9, 12, 20, and 24 h after placement in the container; to minimize disturbance to the conidia, a separate microtitre plate was prepared for each of the eight time points, with five replicates per treatment in each plate. Conidia with germ tubes equal to or longer than the length of the spore were considered germinated. All conidia in each well were counted (range 55–120 conidia per well). The proportion of germinated conidia after 24 h was compared using ANOVA (JMP 3.1.2, SAS Institute, Cary, North Carolina, USA); data were arcsine square-root transformed prior to analysis to reduce heteroscedasticity.

Water capture and retention—We assessed patterns of leaf wettability among 18 clover species (Table 1) by misting leaves and measuring the amount of water captured and the duration of time water remained on leaf surfaces. We germinated seeds on moistened filter paper and then planted seedlings into Ray Leach Conetainers (2.5 cm × 16.5 cm; Stuewe and Sons, Corvallis, Oregon, USA) filled with Premier brand Pro-Mix PGX growth medium (Premier Tech, Rivière-du-Loup, Quebec, Canada). Plants were grown and maintained in the greenhouse. After 4 wk, the plants were transferred to an incubator 24 h prior to experimental treatments and maintained at 17.7°C with 16 h of light per day.

We sampled three leaves on each of five plants per species to determine the amount of water captured. Leaves were sprayed until runoff, and the water was left to settle for approximately 30 s. Moisture was collected from the adaxial and abaxial surfaces of individual leaves with filter paper fragments (mean mass 1162 mg) previously placed in 1.5-mL Eppendorf microfuge tubes (Eppendorf, Hamburg, Germany), oven dried and pre-weighed. After absorption of water from the leaf surface was complete, the filter paper was placed back into the Eppendorf tube, resealed and reweighed to determine the amount of water absorbed off the leaf.

To measure how long leaves retained surface moisture both in light and dark conditions, the same plants were misted with distilled water until runoff and then monitored for presence or absence of water every 5 min for the first 30 min, and every 15 min thereafter, until complete dryness. Three replicate leaves from each of five individuals per species were monitored in random order in experiments in both light (from 0900 until 1800) and dark (from 2100 until 0400) periods.

To quantify leaf surface area, each individual leaf was removed, scanned into a PICT-format data file using Adobe PhotoShop 6.0 software (Adobe, San Jose, California, USA), and measured using NIH Image software (U.S. National Institutes of Health, available at http://rsb.info.nih.gov).

We compared water capture, leaf area, and length of time each species retained water on the leaf surface using nested ANOVA (leaves nested within plants, plants nested within species) in the SAS GLM procedure (SAS v6.12, SAS Institute, Cary, North Carolina, USA), using Type III expected mean square error term to test for significant effect of species. The rate of water loss from leaf surfaces for all species over time throughout the light and dark periods was compared using the log-rank χ^2 test of homogeneity between groups of the Kaplan-Meier product-limit survival analysis (Everitt, 1994).

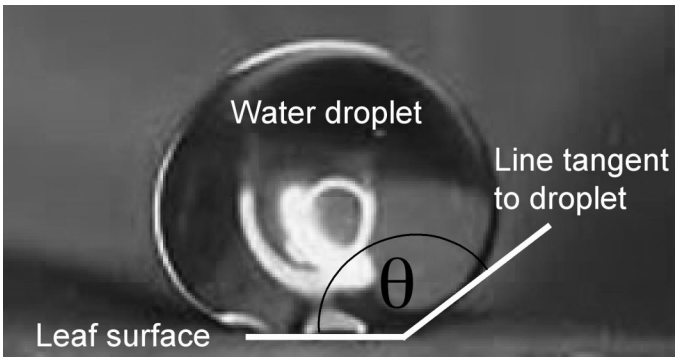

Fig. 1. The contact angle (θ) of a line tangent to a water droplet on the leaf surface of *Trifolium glomeratum* provides an estimate of leaf wettability, following Crisp (1963).

Water repellency—We quantified the degree of leaf wettability of different clover species by measuring the contact angle of a water droplet on the leaf surface (Adam, 1963). Individual leaves were placed on a piece of cardboard mounted on a horizontally oriented stereoscope equipped with a camera. For all 18 species (two leaves per plant, five plants per species), a 5-μL water droplet placed on the horizontal adaxial leaf surface was photographed. From the scanned image (Adobe PhotoShop 6.0), the contact angle (θ) of a line tangent to the droplet through the point of contact was measured using NIH Image software (Fig. 1). A less wettable (more repellent) leaf surface leads to the formation of a more spherical water droplet and a higher value of θ (Smith and McClean, 1989). Leaves with $\theta < 110°$ are considered wettable, and leaves with $\theta > 130°$ are nonwettable, or repellent (Crisp, 1963). Differences in wettability among species were compared using nested ANOVA (leaves nested within plants, plants nested within species) in the SAS GLM procedure (SAS v6.12, SAS Institute), using the Type III expected mean square error term to test for significant effect of species.

Trichomes—For each of the seven species that possessed trichomes, we counted the number of trichomes present on each of two leaflets on five plants. Trichomes were counted on both the abaxial and adaxial surfaces of the leaflet, but not on the petiole.

Determinants of water capture—The relative influence of leaf area, trichome density, and water repellency on water capture was tested using a mixed stepwise multiple regression on the mean values for each species, with a threshold $\alpha = 0.25$ to enter and leave the function (JMP 3.1.2, SAS Institute).

Infection rates: microclimate effects and variation among species—*Greenhouse inoculations*—We selected six clover species to represent the range of leaf wettability: *M. arabica*, *T. gracilentum*, *T. wormskioldii*, *T. microdon*, *T. bifidum*, and *T. subterraneum*. Six-week-old plants ($N = 30$ per species) were inoculated with *Stemphylium* by dipping plants into a suspension of 1.9×10^3 conidia/mL. After inoculation, plants were held in plastic bags with wet paper towels to maintain moist conditions for 0, 12, or 48 h and then moved to the greenhouse. There were 10 replicates per species per treatment time. At 12 d after inoculation, five randomly selected leaves per plant were excised, surface sterilized, and plated on 2% malt extract agar to determine the proportion of leaves infected. Leaves were scored for infection based on the production of *Stemphylium* conidia after 7 d on agar.

Field experiments—We estimated the relative rates of infection under natural conditions for each of the species listed in Table 1, except for *T. willdenovii* and *T. variegatum*. At both the Bluff and Dorms sites, 40 6-wk-old plants of each species were planted in complete randomized block experiment

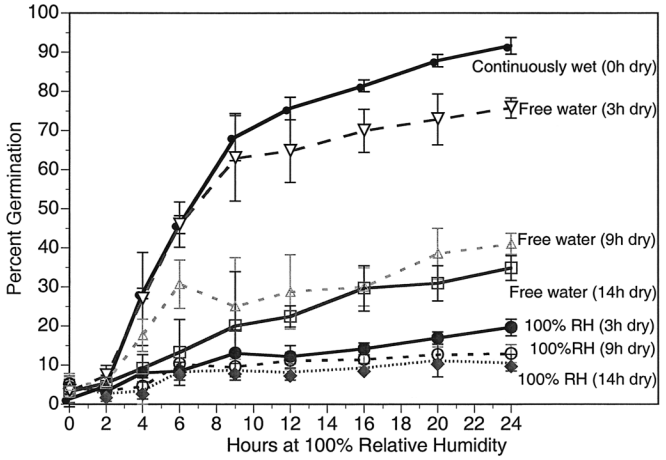

Fig. 2. Percentage germination of *Stemphylium* conidia under different moisture regimes. Conidia were dried for 3, 9, or 14 h, and then either rewetted to keep conidia in free water or placed at 100% relative humidity in the absence of free water. A seventh treatment kept conidia continuously in free water (0 h dry). Error bars are standard deviations ($n = 5$).

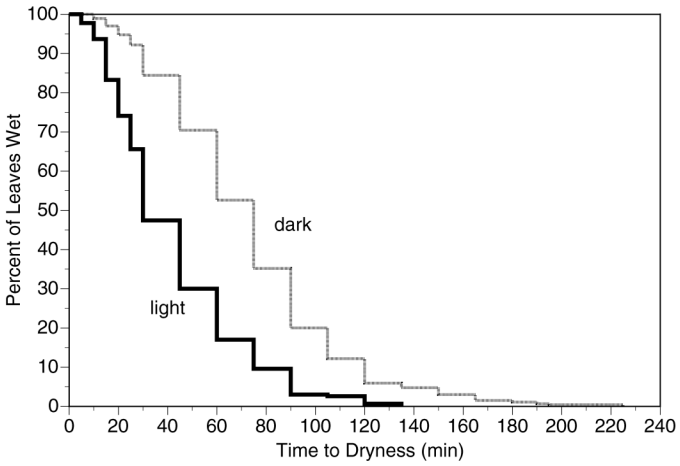

Fig. 3. Time that free water was retained on clover leaves after misting, in the light and in the dark, for 18 clover species pooled ($n = 270$ leaves per light treatment). Treatments were significantly different (log rank $\chi^2 = 107.9$, df = 1, $P \leq 0.0001$).

on 2 February 2000. On 21 May 2000, we isolated fungi from one terminal leaflet per plant. We placed surface-sterilized leaflets on 2% MEA and scored for typical production of *Stemphylium* conidia after 1 wk. For each site, we used linear regression (JMP 4.04, SAS Institute) to test whether the time to leaf dryness as determined in the growth chamber experiment was a good predictor of the proportion of leaves that became infected with *Stemphylium* in the field. Mean infection data for each species were arcsine square-root transformed before analysis to reduce heteroscedasticity.

In the same experiment, we estimated differences in rates of infection by *Stemphylium* at the moist Bluff and drier Dorms sites by comparing the mean proportion of leaves infected for each of the 16 species using a paired *t* test (JMP 4.04, SAS Institute). In a similar experiment in 2002 that involved only *M. polymorpha*, 36 plants were planted at each site (17 April 2002), and three leaves were harvested from each plant (1 June 2002) to determine infection rates.

To estimate the length of time that plant leaves would be wet under natural conditions in the Bluff and Dorms habitats, we used a Campbell Scientific (Logan, Utah, USA) wetness sensing grid (Model 237) connected to a Campbell Datalogger CR500. Sensors were oriented to the north and mounted on a post 50 cm above the ground. Sensor wetness is presented as the 1/resistance (per kilo-ohms); this value increases as more of the sensor plate is covered with water. Sensors were placed in the field from 12 to 27 May 2002.

RESULTS

Germination rates—The presence of free water was important for germination of *Stemphylium* conidia. Conidia kept at 100% relative humidity (dry) never exceeded 20% germination, whereas those continuously in free water reached 92% germination (Fig. 2). In optimal conditions (continuously wet), conidia reached 68% germination in as little as 9 h, and reached maximum germination of 92% after 24 h. Desiccation of conidia quickly reduced their ability to germinate. Drying the conidia for as little as 3 h reduced maximum germination to 20%, and 14 h of desiccation reduced maximum germination to only 11%, compared to 92% for continuously wet conidia. With the exception of conidia dried for 9 and 14 h and not rewetted, all treatments had significantly different percentage of germination at the end of the experiment (Fig. 2, $F_{6,28} = 988.6$, $P < 0.0001$, Tukey-Kramer HSD post-hoc test, $\alpha = 0.05$).

Water capture and retention—The 18 species of clovers had different patterns of leaf surface water retention after being misted. Clover species differed in the time required after wetting to reach complete dryness, both in the light ($F_{17,72} = 2.27$, $P = 0.0086$) and in the dark ($F_{17,72} = 2.83$, $P = 0.0011$) (Table 1). There was also significant variation among plants within species (light, $F_{72,180} = 3.43$, $P = 0.0001$; dark, $F_{72,180} = 5.10$, $P < 0.0001$). For all species combined, moisture was retained on leaf surfaces significantly longer in the dark (mean = 72.3 min) than in the light (mean = 42.5 min, log-rank $\chi^2 = 107.9$, df = 1, $P < 0.0001$) (Fig. 3). The time required for complete drying under light conditions was strongly and positively correlated with time required in the dark (Pearson's correlation coefficient, $r = 0.679$), suggesting that water loss was a simple function of evaporation.

The most important determinant of the length of time that water remained on leaf surfaces was the total amount of water captured by a leaf (time to dryness = 29.06 + 0.343(water captured), $F_{1,16} = 8.4$, $R^2 = 0.34$, $P = 0.01$). The amount of water captured on different species varied over an order of magnitude (Table 1, $F_{17,72} = 7.16$, $P < 0.0001$ among species, $F_{72,180} = 2.40$, $P < 0.0001$ among plants nested within species).

Clover species also varied in water captured per leaf area ($F_{17,72} = 2.14$, $P = 0.014$ among species, $F_{72,180} = 2.22$, $P < 0.0001$ among plants nested within species), although it was not a good predictor of time to dryness ($F_{1,16} = 0.08$, $R^2 = 0.01$, $P = 0.79$). Leaf area for 17 of the species was fairly evenly distributed between 67 and 430 mm^2, with *M. arabica* exceptionally large at 919 mm^2 (Table 1). Leaf area varied significantly among species ($F_{17,72} = 22.29$, $P = 0.0001$ among species, $F_{72,180} = 3.39$, $P = 0.0001$ among plants nested within species).

Leaf wettability, measured as the contact angle (θ) of water drops on the leaf surfaces, ranged from 109° to 136° (Table 1). There were highly significant differences among species ($F_{17,72} = 3.16$, $P = 0.0003$), but no significant effect of plant nested within species ($F_{72,89} = 1.32$, $P = 0.1068$). *Medicago arabica* was the only species with a wettable leaf surface ($\theta < 110°$, following Crisp, 1963); the remaining species were distributed evenly from 117 to 136°, including five species

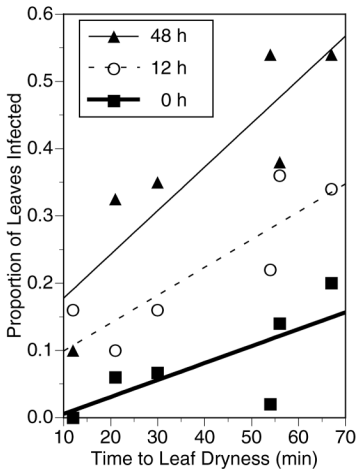

Fig. 4. The proportion of leaves of six clover species that became infected when dipped in a suspension of conidia of *Stemphylium*. Plants in a greenhouse were held in plastic bags at 100% relative humidity to prevent evaporation of free water from the leaves for 0, 24, and 48 h after inoculation. Linear regressions indicate that for 0 h (triangles), Infection = −0.0197 + 0.00252(time to dryness), $R^2 = 0.54$, $F_{1,4} = 4.68$, $P = 0.097$; for 12 h (circles), Infection = 0.0578 + 0.00414(time to dryness), $R^2 = 0.75$, $F_{1,4} = 11.89$, $P = 0.026$; and for 48 h (squares), Infection = 0.1129 + 0.00649(time to dryness), $R^2 = 0.77$, $F_{1,4} = 13.18$, $P = 0.022$.

above the traditional water-repellent threshold of 130° (following Crisp, 1963) (Table 1).

Seven species had abaxial trichomes, ranging from 0.2 to 7.9 trichomes/mm². Only four of these species also had trichomes on the adaxial surface (Table 1).

Using mean values of total water captured for each of the 18 clover species, separate regressions indicated that both leaf area (water captured = 15.346 + 0.102(area); $F_{1,16} = 94.8$; $P < 0.0001$; $R^2 = 0.86$) and wettability (θ) (water captured = 313.2 − 2.162θ; $F_{1,16} = 10.1$, $P < 0.006$, $R^2 = 0.39$) were significant predictors of water capture. The relationship between water capture and wettability, however, was driven entirely by *M. arabica* ($F_{1,15} = 1.14$, $P = 0.30$, $R^2 = 0.07$ without *M. arabica*). Neither adaxial nor abaxial trichome density were significantly correlated with water capture ($F_{1,16} = 0.24$, $R^2 = 0.02$, $P = 0.63$ for adaxial; $F_{1,16} = 0.20$, $R^2 = 0.01$, $P = 0.66$ for abaxial), nor were there significant differences in water capture between species with and without trichomes (unequal variance *t* test, $t_{7.3} = 0.88$, $P = 0.40$). In a stepwise regression including mean leaf area, abaxial and adaxial trichome density, and wettability for all 18 clover species, only leaf area contributed significantly ($P < 0.0001$) towards predicting the amount of water captured ($P > 0.48$ for all others). Wettability was not included in the model because of a significant negative correlation between leaf area and wettability (θ = 131.5 − 0.019(area); $F_{1,16} = 9.6$; $P < 0.007$; $R^2 = 0.38$), again driven entirely by *M. arabica* ($P = 0.75$ without *M. arabica*). Leaf area alone was a strong predictor of the amount of water a leaf would capture.

Infection rates: microclimate effects—For all six species inoculated in a greenhouse experiment, the percentage of leaves infected increased with time held in moisture post-inoculation. Both intercept and slope of the regressions for infection rates vs. time to leaf dryness increased with increasing time held in bags (Fig. 4).

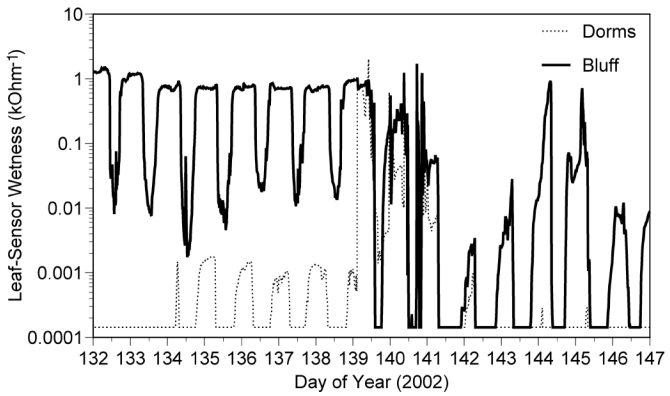

Fig. 5. Surface wetness from fog, dew, and rain on Campbell Wetness Sensing Grids from 12 May through 26 May 2002 at the drier inland site (Dorms) and wetter coastal prairie site (Bluff) at the UC Natural Reserve at Bodega Bay, California, USA. A greater number of water drops on the sensor surface reduces electrical resistance, measured in kilo-ohms. Using the inverse of the resistance as a measure of surface wetness, a dry sensor reads 0.00014 $kOhm^{-1}$ and a sensor in the rain approximately 2.0 $kOhm^{-1}$.

Field studies revealed large differences between clover habitats in terms of both available moisture and fungal infection (Fig. 5). Over a 2-wk period in 2002, clovers at the Bluff site experienced substantially wet leaves (less than 100 kOhm resistance) for 12 h or more on 64% of the 14 d (counting from noon to noon). At the Dorms site, clover experienced this degree of wetness on only 7% of the days. Additionally, at the Bluff site, only 21% of days had less than 3 h of wetness, whereas at the Dorms site, over 85% of days were that dry. For each of the 16 clover species tested, natural infection rates by *Stemphylium* were much higher in the moist Bluff site than at the drier Dorms site (Fig. 6), and overall infection rate was significantly greater at the Bluff site (80.1%) than at the Dorms site (24.4%) (paired *t* test, $t = 9.26$, df = 15, $P \leq 0.0001$). Similarly, in the 2002 experiment 100% of the leaves of *M. polymorpha* ($n = 69$) were infected at the Bluff site, but only

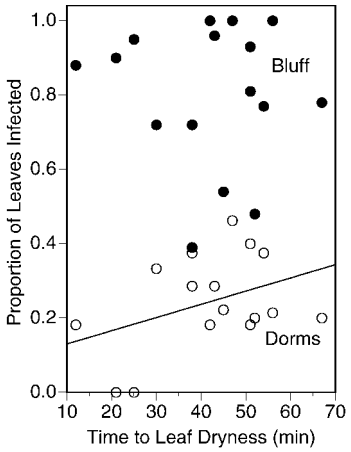

Fig. 6. The proportion of leaves naturally infected with *Stemphylium* in the wetter Bluff site (N = 19–32, one leaf per plant) and drier Dorms site (N = 4–15) in 2000. Each dot represents the mean for a *Trifolium* or *Medicago* species (as given in Table 1; *T. willdenovii* and *T. variegatum* were not included in the field experiment). Infection rates at the Dorms site increased with time leaves required to dry ($\text{asin}\sqrt{(\text{Infection})} = 0.191 + 0.0069$(time to dryness); $F_{1,14} = 3.91$, $P = 0.068$, $R^2 = 0.22$). The regression was not significant at the Bluff site ($F_{1,14} = 0.008$, $P = 0.93$, $R^2 = 0.001$).

60.3% of the leaves were infected at the Dorms site ($\chi^2 = 42.3$, $P \leq 0.0001$).

Infection rates: variation among species—For six clover species tested in greenhouse inoculations, the mean length of time that free water was retained on leaf surfaces was a good predictor of infection rates after inoculation with *Stemphylium* (Fig. 4). Those clover species that retained free water for longer times suffered a greater proportion of infected leaves. This relationship held whether plants were kept in moist conditions for 0, 12, or 48 h.

To test whether this relationship held for infection rates in the field, either at the moist Bluff site or the drier Dorms site, we used rates of infection by *Stemphylium* for the larger suite of 16 clover species. At the Dorms site, infection rates were greater ($P \leq 0.07$) in species that retained water on their leaves for longer times (Fig. 6), as expected from the greenhouse studies. In contrast, at the wet Bluff site, time to leaf dryness and natural infection rates in the field were not significantly related (Fig. 6).

DISCUSSION

These results suggest a mechanism for differential susceptibility to disease in clovers that is useful for making predictions about disease development across habitats and among species. The amount of water captured by a leaf upon contact with moisture is critical in determining how long water will remain on a leaf. As the time that water remains on a leaf surface increases, the probability that a spore will germinate and be able to infect the host also increases. Leaf properties such as surface area, but not wettability or trichomes in our study, determine how much water a leaf captures and partly how susceptible a host is to fungal infection. Realized infection rates in the field are driven in part by susceptibility, but are also constrained and modified by microclimatic variation and other factors.

Successful spore germination on a leaf surface is critical to the development of foliar plant diseases, and this ontogenetic step is often regulated by moisture availability. For *Stemphylium*, an important foliar pathogen of many plants including 18 species of clovers in the coastal prairie at Bodega Bay, California, the presence of free water was critical for spore germination, and short periods of desiccation greatly reduced germination rates. Through a series of laboratory, greenhouse, and field experiments, we found that morphological attributes of leaves that decreased the length of time that moisture was retained also decreased the susceptibility of hosts to infection. These results were further substantiated by field experiments that showed that habitats with longer periods wetted by dew or fog supported greater rates of infection by *Stemphylium* on *Trifolium* and *Medicago*. These findings suggest that understanding how different plant species capture and retain water on the leaf surface is critical for making predictions about foliar disease development in natural communities.

The 18 clover species differed in water capture and retention after misting. The total amount of water captured by leaves was the principal determinant of the length of time a leaf remained wet, and the amount of water captured was in turn primarily a function of leaf size. While trichome density and leaf wettability (as measured by contact angle) varied greatly among host species, neither trait had a significant effect on the amount of water captured by a leaf. This result was unexpected, given that a strong linear relationship between leaf surface wettability and water capture has previously been documented in other systems (Cook, 1980) and that repellent leaf morphologies have reduced leaf water films and their associated physiological effects on plants (Smith and McClean, 1989; Brewer and Smith, 1997).

Equally unexpected was the lack of a positive correlation between leaf size and water repellency. Because larger leaves capture more water, which should lead to greater disease development, plants with larger leaves should be under greater selection than small-leaved plants for characteristics that shed water from leaves. We thus expected that species with larger leaves should have less wettable leaf surfaces. For example, Cook (1980) found that the larger apical leaves on peanut (*Arachis hypogaea*, Fabaceae) were less repellant than the smaller basal leaves, although this pattern was confounded by basal leaves being older. The lack of a correlation between leaf size and wettability in clovers and the overall lack of relationship between leaf surface properties and water capture may reflect constraints on the correlations among traits or the influence of herbivory or other selective agents on leaf properties. In addition, plants with large leaves may moderate water capture not through wettability, but through attributes we did not measure, such as leaf shape and orientation, petiole flexibility, nocturnal leaf folding, and biochemical defenses.

Although many *Stemphylium* species are known from both *Trifolium* and *Medicago*, host species and cultivars vary in their responses to individual isolates of *Stemphylium* (Smith, 1937; Borges et al., 1976; Irwin and Bray, 1991; Berg, 1996), and different species and isolates of *Stemphylium* vary in their virulence on particular hosts (Borges et al., 1976; Heiny and Gilchrist, 1991; Irwin and Bray, 1991). Several biochemical factors may be involved in these differential interactions, including the ability of the fungus to detoxify phytoalexins (Soby et al., 1996), cyanogenic potential of the host (Wilkinson, 1978), and host sensitivity to stemtoxin produced by the pathogen (Heiny and Gilchrist, 1991). However, only one of the species included in this study (*T. repens*) is cyanogenic (K. J. Hayden and I. M. Parker, unpublished data), and the importance of stemtoxin in virulence is uncertain (Heiny and Gilchrist, 1991). In contrast, there is strong evidence that moisture conditions during the infection process are key to development of disease caused by *Stemphylium*.

The amount of water captured on a leaf surface and the length of time that water was held on the leaf surface after wetting were good predictors of infection by *Stemphylium* after inoculation in the greenhouse. These results are consistent with those of Cook (1980) who found infection of peanut by the rust *Puccinia arachidis* was positively correlated with the amount of water retained on the leaves. Similarly, Statler and Nordgaard (1980) increased the wettability of leaves of wheat varieties by adding the surfactant Tween-20 or by rubbing the leaf surface with a wet finger prior to spraying with a suspension of spores of *Puccinia recondita*, and in doing so increased infection rates. Like Statler and Nordgaard's wheat varieties, all the clover species tested were intrinsically susceptible to infection by *Stemphylium* (note high levels of infection when kept wet for 48 h in the greenhouse [Fig. 4] and natural infection at the wet Bluff site [Fig. 6]), and differences in infection were due to the effects of surface water retention on spore germination.

Infection was so tightly linked to leaf wetness because *Stemphylium* conidia required extended, continuous time in free

water to germinate successfully. This requirement is similar to that of other foliar pathogens such as *Alternaria* and *Phaeoisariopsis* that require extended leaf wetness to germinate (Wadia and Butler, 1994; Vloutoglou et al., 1996) and explains earlier observations that increased wetness periods increase infection rates by *Stemphylium* (Cowling and Gilchrist, 1982; Johnson and Lunden, 1986; Montesinos et al., 1995; Basallote-Ureba et al., 1999; Suheri and Price, 2000) and other pathogens (Everts and Lacy, 1990; Evans et al., 1992; review in Huber and Gillespie, 1992). This critical need for continuous, extended periods of leaf wetness for conidia to germinate and infect host plants indicates that understanding leaf wetness patterns may be key to understanding variation among habitats in severity of infection from *Stemphylium*.

We found striking differences in infection rates by *Stemphylium* in two nearby habitats that differed strongly in how long leaves remained wet. At the Bluff site, leaves remained heavily wet long enough from dew and fog to ensure high rates of spore germination on most days, whereas at the Dorms site leaves did not experience more than a small amount of moisture for a short time on most days. Our lab experiments indicate that the continuity of moisture on the leaf surface is particularly important, since even brief periods of desiccation strongly reduced spore germinability. As such, spore germination and subsequent plant infection may be limited only to rainy days at the Dorms site, whereas at the Bluff site conditions are conducive to infection on most days. This postulation is consistent with the much greater rates of infection by *Stemphylium* at the Bluff site (80.2%) than at the Dorms (24.4%) when the same host species was planted at each site in both 2000 and 2002. Such striking environmentally driven differences in disease pressure may influence the natural distribution of host plants, as well as translate into substantially different local selection pressures for development of resistance mechanisms, including rapid drying of leaves.

Contrary to our expectations, selection pressures to reduce water retention may actually be greater at drier sites than wet sites. At the wet Bluff site, extended periods of fog and dew provided a continuous input of moisture to the leaves for more than 12 h per day, long enough for most leaves to become infected, provided *Stemphylium* conidia were present. Even fast-drying leaves would have experienced a large enough window of opportunity for infection prior to the mid-morning drying of leaves. At the Dorms site, fog and dew formation lasted only a few hours, so differences in the length of time leaves took to dry may be critical in determining whether spore germination and subsequent infection could take place. Interestingly, over a much drier range of environmental conditions, Talley et al. (2002a) found that antifungal leaf chemistry in sagebrush populations was correlated with pathogen pressure, and that both fungal abundance and defensive chemistry were more prevalent in moister habitats (Talley et al., 2002b). In addition to selection pressures on the host plant, local microenvironmental conditions may select for the development of more rapid germination rates in the pathogens at drier sites.

Understanding how leaf morphology affects surface water retention and in turn fungal infection led to testable predictions about the distribution of *Stemphylium* in natural communities both across potential host species and among habitats with different environmental conditions. As we learn more about the mechanisms governing plant–pathogen interactions in natural systems, we also acquire new insights into how environmental conditions can drive local adaptation in plants and their pathogens.

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