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Research

Physiological performance of an Alaskan shrub (*Alnus fruticosa*) in response to disease (*Valsa melanodiscus*) and water stress

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Summary

- Following the decades-long warming and drying trend in Alaska, there is mounting evidence that temperature-induced drought stress is associated with disease outbreaks in the boreal forest. Recent evidence of this trend is an outbreak of Cytospora canker disease (fungal pathogen *Valsa melanodiscus* (anamorph = *Cytospora umbrina*)) on *Alnus* species.
- For *Alnus fruticosa*, we examined the effects of water stress on disease predisposition, and the effects of disease and water stress on host physiology. In two trials, we conducted a full-factorial experiment that crossed two levels of water stress with three types of inoculum (two isolates of *V. melanodiscus*, one control isolate).
- Water stress was not required for disease predisposition. However, the effects of water stress and disease on host physiology were greatest near the peak phenological stage of the host and during hot, dry conditions. During this time, water stress and disease reduced light-saturated photosynthesis (−30%), light saturation point (−60%) and stomatal conductance (−40%).
- Our results depended on the timing of water stress and disease in relation to host phenology and the environment. These factors should not be overlooked in attempts to generalize predictions about the role of temperature-induced drought stress in this pathosystem.

Introduction

In the circumpolar north, there is considerable and compelling evidence that the climate to which plants are currently adapted is shifting (Jump & Penuelas, 2005; Sturm *et al.*, 2005; Tape *et al.*, 2006). High-latitude climate changes often operate at a faster pace than the scale at which plants are able to migrate or adapt to the altered climate (Jump & Penuelas, 2005; Garrett *et al.*, 2006). This may push plants beyond the physiological limits of their current ranges (Garrett *et al.*, 2006), resulting in long-term exposure to stresses such as high temperature or low precipitation. For example, long periods of warmth and dryness in the boreal forest over the last several decades have caused accelerated evapotranspiration and soil water deficits (Barber *et al.*, 2000; Oechel *et al.*, 2000), which in turn resulted in temperature-induced drought stress and reduced growth of many forest species (Brandt *et al.*, 2003; Juday *et al.*, 2005; Hogg *et al.*, 2008; Nossov, 2008). As a consequence of climate-related stressors, plants may not have the capacity to provide sufficient structural or biochemical defenses against diseases (Ayres, 1984; McPartland & Schoeneweiss, 1984; Boyer, 1995) or recover from disease damage (Ayres, 1984, 1991; Paul & Ayres, 1987). For these reasons, it is generally predicted that plants will be more vulnerable to disease (Coakley *et al.*, 1999; Juday *et al.*, 2005) and experience higher disease incidence and severity with a shifting climate (Larsson, 1989; Mitchell *et al.*, 2003; Rodriguez *et al.*, 2004).

These predictions appear to be unfolding for Alaskan keystone shrubs, *Alnus* species, which are the dominant, symbiotic nitrogen-fixing shrubs in the boreal forest (Uliassi & Ruess, 2002; Mitchell & Ruess, 2009). An outbreak of canker disease has caused significant dieback in *Alnus incana* ssp. *tenuifolia* (thin leaf alder) and *Alnus fruticosa*

(green alder), resulting in mortality and reduced nitrogen fixation throughout central and south-central Alaska (Ruess *et al.*, 2009). The disease is associated with the fungus *Valsa melanodiscus* (anamorph = *Cytospora umbrina*) and is characterized by long, girdling cankers (Adams, 2007; Stanosz *et al.*, 2008). The rapid development of this disease coincided with suppressed radial growth in *Alnus tenuifolia* (Nossov, 2008) during one of the hottest, driest summers on record in 2004 (Ruess *et al.*, 2009). Drought stress has been classically cited as a predisposing factor to Cytospora canker disease (Bier, 1953; Bloomberg, 1962; Bloomberg & Farris, 1963), and the drought event of 2004 prompted the working hypothesis that temperature-induced drought stress was a principal factor in the development of the disease epidemic (Ruess *et al.*, 2009).

The working hypothesis for the causal conditions of canker disease in Alaska remains untested. Establishing causality between the summer conditions of 2004 and the canker disease epidemic requires long-term disease records in addition to crucial information about the three parts of the disease triangle: the host, the pathogen and the environment (Harvell *et al.*, 2002; Woods *et al.*, 2005). The current canker epidemic on alder is the first on record for Alaska, so it is difficult to historically determine whether this disease is related to the warming trend or is part of natural population cycles. Instead, we will have to rely heavily on information from the disease triangle to ascertain if the disease epidemic could be related to temperature-induced drought. Drought-related decline in host condition has been correlated to disease outbreaks in the boreal forest (Brandt *et al.*, 2003; Juday *et al.*, 2005; Hogg *et al.*, 2008), but there are no studies on the effects of canker disease and drought on the condition of *Alnus* species. For other hosts of Cytospora canker fungi, only static indicators of water stress, such as water potential (Guyon *et al.*, 1996; Kepley & Jacobi, 2000) or relative water status (Bloomberg, 1962; Tao *et al.*, 1984), have been used to gauge host condition. Our study measures host physiological response to canker disease and water stress using photosynthetic performance, stomatal conductance, sapflow and water-use efficiency.

Our study is an experimental investigation of two types of disease–water stress relationships for *Alnus fruticosa*: an effect of water-limitation on the susceptibility of hosts to disease (the predisposition concept), and the combined effects of disease and water-limitation on host physiology (the multiple stress concept) (Desprez-Loustau *et al.*, 2006). The goal of the predisposition approach was to test the idea that the Cytospora canker pathogen will characteristically attack *A. fruticosa* hosts that have been weakened or compromised by water stress (Christensen, 1940; Manion, 1991; Worrall, 2009), as observed on other hardwoods in natural systems and tested in experimental settings (Bier, 1953; Bloomberg, 1962; Bloomberg & Farris, 1962; Kamiri & Laemmlen, 1981; Guyon *et al.*, 1996; Kepley & Jacobi, 2000). The goal of the multiple stress approach was to evaluate the effects of simultaneous disease and water limitation on the physiological performance of *A. fruticosa*, as one stressor is likely to exacerbate the effects of the other and reduce the capacity of the host to compensate or recover from disease (Ayres, 1984, 1991; Paul & Ayres, 1987).

Materials and Methods

Plant material

In March 2005, *A. fruticosa* (*A. viridis* subsp. *fruticosa* (Rupr.) Nym., (synonym = *Alnus crispa*) seeds were collected at nine sites within 50 km of the University of Alaska, Fairbanks, Alaska (64°51′28″ N 147°51′23″ W). Seeds from the cones of 36 plants were germinated in a soil media with a ratio of two parts peat, one part vermiculite and one part coconut coir, and established seedlings were transplanted to 328 cm^3 'cone-tainers' (Stuewe and Sons, Tangent, OR, USA). After 2 yr of growth, plants were transplanted into larger 983 cm^3 pots using the same peat–vermiculite–coconut soil media. Individual plants (genets) developed between one to four stems (ramets) and were pruned several times during the course of their growth. Five weeks before experimental treatments began (July, 2007), all ramets were pruned to a height of 200 mm. Five containers were placed with equal spacing in a rack, and the 34 racks were rotated weekly around the glasshouse benches.

Fungal isolates

Two *Valsa melanodiscus* isolates were used to produce inoculum: 'Jim's Landing 2' (06-08) and 'Helmaur 1' (06-12), hereafter referred to as Isolate 1 and Isolate 2. Both of these isolates were obtained from cankers on *Alnus tenuifolia* in Alaska and were collected and identified by Adams (2008). Cultures were maintained on potato dextrose agar (Fisher Scientific, Houston, TX, USA) at 17°C.

Experimental design

The experiment was conducted in two trials. Trial I began on 13 July 2007 and Trial II began *c.* 1 month later, on 23 August. Each trial was conducted as a completely randomized full-factorial design with two water treatment levels (well-watered or water-limited) crossed with three levels of inoculum type (Isolate 1, Isolate 2, or plain potato dextrose agar as a control inoculum), which resulted in six treatment combinations. There were 15 replicates (alders) per treatment combination and 90 plants per trial. Plants were randomly assigned to a water treatment level and then one ramet per plant was randomly assigned to an isolate type.

Glasshouse conditions

The glasshouse temperature was set in the range 18–26°C with a photoperiod of 21 h (maximum photoperiod for interior Alaska). Supplemental lighting from high-pressure sodium and mercury lamps provided 300 μmol m^{-2} s^{-1} at bench height. Environmental conditions in the glasshouse zone (80 m^2) were recorded with the climate monitoring system (Hortimax, Pijnacker, the Netherlands), including relative humidity, temperature, and light.

Inoculation

Plugs of inoculum (10 mm × 5 mm) bearing mycelium were cut from the active margins of 12-d old cultures of *V. melanodisus*. The inoculation site (on the stem 10 mm above the soil surface) was wiped with 95% ethanol, and a scalpel was used to make a single wound (10 mm × 5 mm) exposing the sapwood. Inoculum plugs were positioned on the wound with mycelium facing the sapwood and secured with Parafilm (American National Can, Greenwich, CT, USA), which was kept in place for 2 wk. Stem wounds of this size and larger can be naturally associated with snowshoe hare browsing, heavy snow-loading, or frost damage.

One ramet per plant was inoculated; if there were multiple ramets on the plant, the ramet to be inoculated was randomly selected. Necrotic lesions began developing beneath the Parafilm 1 wk after inoculation, and the dimensions of developing cankers were measured at 2 wk intervals for 3 months following inoculation. Disease incidence was recorded as positive if necrosis advanced > 2 mm around the initial wound (typical necrotic response to control inoculum). The extent of the canker was estimated as an elliptical area based on length and width measurements. We measured internal colonization of the pathogen on a subset of 35 plants not used for physiological measurements. On this subset, the bark was peeled away to expose vascular tissue and the vertical extent of pathologically darkened tissue was measured. *Valsa melanodiscus* was recultured from all experimental cankers to confirm that the fungal pathogen was consistently associated with the disease symptoms. The cultures stained the agar reddish and often produced conidiomata (asexual fruiting structures).

Water treatments

Plants were watered by hand with reverse osmosis (RO) water. Water-soluble fertilizer (Sunshine technigro 10–30–20 (Sun gro Horticulture, Vancouver, Canada) combined with 20–10–20 and Sprint 330 iron chelate micronutrient) was applied once a week in equal volumes (150 ml^3) to all plants. In both trials, the water limitation treatment began 2 wk before inoculation and involved the application of low volumes of water for an average of 4 d followed by a short period (1–2 d) of no water. By contrast, well-watered alders were watered daily and generally received three to four times the water volume of water-limited alders. We adjusted the watering regime in accordance with plant growth and glasshouse conditions. For example, during warmer glasshouse conditions in July and August, the well-watered group received 450–600 ml^3 water while the water-limited group received 150 ml^3. The level of water stress was carefully determined using physiological measurements and observing physical signs of water stress. Our objective was to maintain moderate water stress that would still enable leaves to respond to light curve and gas exchange measurements. We avoided high levels of water stress that resulted in full stomatal closure, wilting, excessive leaf shedding, or mortality.

Growth measures

Ramet height, leaf number, and ramet diameter were measured in each trial just before inoculation and 8 wk after inoculation. At the end of the experiment, in September, aboveground biomass was measured using dry weights of ramets and leaves from a subset of 80 randomly harvested plants. Specific leaf area (cm^2 g^{-1}) for alders in each of the water treatments was also measured for at the end of the experiment using 15–20 leaf punches from leaves on a subset of 50 randomly selected plants.

Plant water status

Our priority was to maintain intact experimental plants and avoid the risk of additional infections from further wounding within a close vicinity of high inoculum loads. Therefore, we did not perform any destructive water status measurements on the experimental plants.

We used several types of physiological measurements as an index of plant water status. First, we took weekly measurements of stomatal conductance and transpiration rates (Li-Cor 6400; Li-Cor Biosciences, Lincoln, NE, USA) on plants in Trial I and II. Second, we measured monthly water potential (PMS pressure chamber; PMS Instrument Company, Albany, OR, USA) on ramets from a set of 20 plants that had been randomly selected for destructive measurements. We also continually monitored sapflow (Flow 32 Sapflow monitoring system; Dynamax, Inc., Houston, TX, USA) on ramets from a set of eight well-watered plants as a long-term indication of plant water loss over the entire experiment. Physiological measurements were possible as the majority of ramets only developed sublethal cankers (necrotic lesions that did not girdle the entire stem).

Sapflow

Intact ramets on eight randomly selected plants from the well-watered treatment were fitted with small, external

sapflow gauges (micro flow gauges SGA3, SGA5; Dynamax, Inc.) and transpirational water loss was estimated by a heat balance method described by Baker & van Bavel (1987). Sapflow was only measured on ramets from well-watered plants because it was difficult to detect the heat signal from low water flow in the water-limited treatment. Sapflow was monitored throughout the experiment on four ramets from the Isolate 1 treatment and three ramets that were not treated. The gauges and adjacent portions of the stem were wrapped with foam insulation and then reflective foil to minimize radiating heating of the stem. A gauge on one ramet was operated without power to the heater to be certain that the foil and foam insulation shielded the stem from external temperature fluctuations (Gutierrez *et al.*, 1994). For a 2 wk period at the end of Trial II, we rearranged the gauges so that sapflow could be measured below and above the stem canker. Four ramets were fitted with two sensors, and each sensor was attached to the stem adjacently to the upper or lower region of a canker. A data logger (Model CR10x; Campbell Scientific Corporation, Logan, UT, USA) continuously recorded mass flow of sap and averages were logged every 15 min.

Light response curves

We measured light response curves (LRC) using a LI-6400 (Li-Cor Biosciences). A split-plot design was used, where water treatment was applied at the whole-plot level (individual plant) and the inoculum treatment was applied at the subplot level (ramet). This split-plot design was used for two groups of plants: a disease group and a no-disease group. For plants in the disease group, we tested the effects of disease on light response. Light response was measured on leaves from different ramets on the same plant: an untreated ramet (control ramet) and a ramet wounded and treated with inoculum from Isolate 1 or 2 (diseased ramet). For each trial, LRC measurements were made on three to four plants from each treatment combination that were selected based on the similarity of diameter, height and leaf number of the paired ramets. The same split-plot design was used for plants in the no-disease group, which tested the effect of the inoculation procedure (wounding and agar application) on light response. Light response was measured on leaves from paired ramets on the same plant: an untreated ramet (control ramet) and a ramet that was wounded and received an agar-only plug (control inoculum). For each trial, we measured four to six alders in the no-disease group, which were also selected based on similar morphology of the paired ramets. Multivariate ANOVA confirmed that the small wound and agar plug did not affect light response, as all LRC parameters were similar between the paired ramets from the no-disease group. Wounding only explained 6% of the variation in light response in August ($F_{3,14}$ = 0.096, P = 0.438) and < 1% of the variation in September ($F_{3,14}$ = 0.07, P = 0.977). Therefore, we only report results that describe the differences between the paired ramets (control vs diseased) in the disease group.

We measured LRCs on these plants in the beginning of August and September. The most recently-expanded leaf was used for the LRC measurements. A portion of the leaf was enclosed in a cuvette with an area of 100 mm^2, which was regulated for temperature, air flow, humidity and irradiance. Leaves were measured between 11:30 h and 15:30 h each day. Automatically programmed LRCs were used starting with a high light level (2500 $\mu mol\ m^{-2}\ s^{-1}$), constant reference CO_2 (400 $\mu mol\ CO_2\ mol^{-1}$) a constant air flow (500 $\mu mol\ s^{-1}$), and set points for chamber humidity, and leaf temperature were established based on ambient conditions. Leaves were illuminated by the LED light source mounted on the sensor head. The infrared gas analysers (IRGAs) were matched before launching each light response autoprogram.

Data analysis

Two-way ANOVA was used to analyse growth measurements, with ramet height, diameter, and leaf number as response variables and treatment and isolate as explanatory variables. Repeated measures MANOVA was used to analyse canker area expansion over time, where the within-subject factor (response variable) was canker area over time and the between-subjects factors were isolate type and water treatment. *G*-tests were conducted to test the independence of water treatment from disease incidence and disease-related mortality. Rates of water loss at the beginning and end of the experiment were analysed with a one-way ANOVA, using sapflow as the response variable and disease severity as the explanatory variable.

Each LRC was fit separately with the Mitscherlich function (Potvin *et al.*, 1990) using the NLIN procedure in SAS (SAS Inst. version 9):

$$A_{max}[1 - e^{-Aqe(PPFD-LCP)}]$$

(A, net photosynthesis; A_{max}, the asymptote of photosynthesis; A_{qe}, the initial slope of the curve or apparent quantum yield; PPFD, incident photosynthetic flux density; LCP, the light compensation point that corresponds to the *x*-intercept (where photosynthetic carbon uptake and respiratory carbon release are in equilibrium)). For each LRC, the adequacy of the Mitscherlich function was evaluated and consistently showed a good fit to the data ($r^2 \geq 0.90$). This Mitscherlich function was used to estimate the following parameters: light-saturated rate of photosynthesis (A_{max}), apparent quantum yield (A_{qe}), and the light compensation point (LCP). The slope (A_{qe}) needed to be rescaled by a factor of 0.0001 because of convergence

problems (Peek *et al.*, 2002). Using the Mitscherlich function, the light saturation point (LSP) was calculated as the PPFD where A_{max} was reached. For each LRC, we also calculated instantaneous water use efficiency (WUE_i = photosynthesis/transpiration) at light saturated values. These LRC parameters, in addition to WUE_i, were analysed as the response variables in a mixed-model, split-plot ANOVA using the Mixed procedure in SAS. In these analyses, treatment, isolate and treatment × isolate interactions were included as fixed effects and alder individuals were included as random effects. The Satterthwaite approximation was used for determining the denominator degrees of freedom for hypothesis testing. Although a nonlinear mixed model (NLMixed) approach has been used to analyse photosynthetic response curves (Peek *et al.*, 2002), we were not able to use this approach as NLMixed does not allow for two random statements, which are necessary to estimate the two error terms of a split-plot design.

Results

Water treatment effects

Stomatal conductance measurements indicated that water-limited plants were more water-stressed in Trial I (beginning of July) than Trial II (late August). Trial I physiological measurements were taken during conditions of high evaporative demand (Fig. 1a), when air temperature and light ranged between 30°C to 33°C and 622 μmol m^{-2} s^{-1} to 1116 μmol m^{-2} s^{-1}, respectively. Water-limited alders

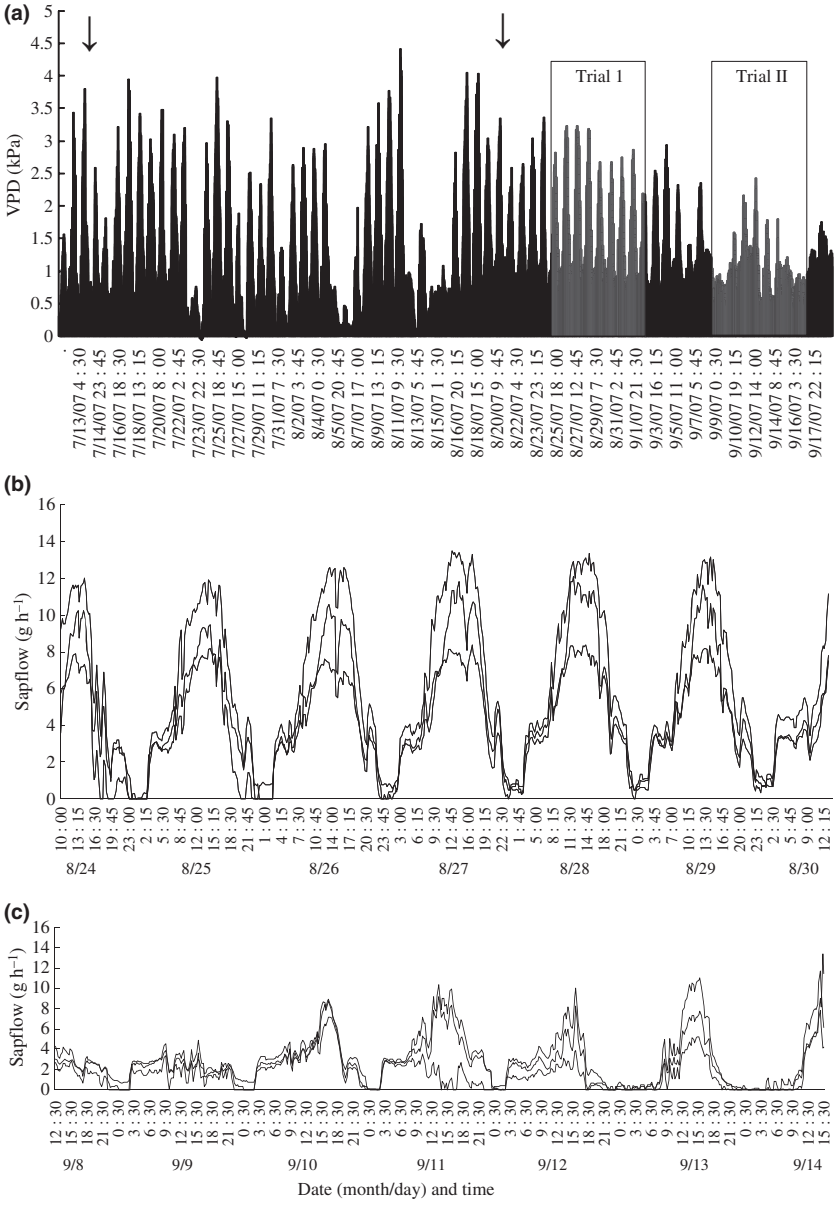

Fig. 1 Glasshouse vapor pressure deficit (VPD) throughout the experiment and sapflow during Trial I and II. Arrows on the VPD graph (a) indicate the dates on which inoculation began in Trial I and Trial II. The squares on (a) enclose the period during which light response curves were measured for Trial I and Trial II. For these periods, corresponding sapflow in healthy alders is shown in the lower graphs (b,c), depicting differences in plant water loss (n = 3 for each period of sapflow).

in Trial I functioned over a lower range of stomatal conductance (60–80 mmol m^{-2} s^{-1}) than well-watered plants (80–200 mmol m^{-2} s^{-1}) ($F_{1,106}$ = 15.97, P = 0.0001). By September, vapor pressure deficit (VPD) had dropped by 50%, temperatures declined by an average of 8°C, and maximum light intensity was 50% less (483–600 μmol m^{-2} s^{-1}) (Fig. 1a). The lower driving conditions for evaporative water loss were reflected in decreased rates of transpiration in the well-watered alders of Trial II (1.74 ± 0.13 mmol m^{-2} s^{-1}) vs Trial I (2.28 ± 0.15 mmol m^{-2} s^{-1}) ($F_{1,108}$ = 7.46, P = 0.0074). In Trial II, stomatal conductance was similar in well-watered (92.0 ± 8 mmol m^{-2} s^{-1}) and the water-limited alders (86.6 ± 7 mmol m^{-2} s^{-1}) (P > 0.1).

Monthly measurements of midday and predawn water potential (ψ) also indicated lower water status in water-limited plants. Predawn measurements averaged between −1.43 and −0.79 MPa in water-limited group vs −0.49 and −0.39 MPa in the well-watered group ($F_{1,15}$ = 15.76, P = 0.0014). Water potential in water-limited plants was typically restricted to lower values (−1.75 to −1.0 MPa) during the day (8:00–17:00 h), while well-watered alders had higher morning values of ψ (−0.5 MPa) that gradually declined over the course of the day (Table 1).

Sapflow

Sapflow decreased over the course of the experiment, in accordance with the decline in VPD. Measurements over the course of 9 wk indicated greater water loss during Trial I (Fig. 1b) compared to Trial II (Fig. 1c). We also measured sapflow using two sensors per ramet, with each sensor placed adjacent to either the upper or lower region of a canker. During the midday highs in VPD, between 12:00 h and 16:00 h, gauges on nondiseased alders measured a sapflow difference between 0.09–0.37 g H_2O h^{-1} compared with a range of 1.11–1.49 g H_2O h^{-1} for diseased alders ($F_{1,31}$ = 245.67, P < 0.0001) (Fig. 2). This indicates that water flowed at a slower rate past the diseased part of the stem.

Plant size

Water-limited plants exhibited reduced plant size in several ways. First, water-limited plants were shorter than well-watered plants by an average of 9 cm in Trial I ($F_{2,82}$ = 6.19, P = 0.015) and by an average of 14 cm in Trial II ($F_{1,59}$ = 5.45, P = 0.232). Second, water-limited plants in Trial II had an average of 31 fewer leaves than well-watered plants ($F_{1,60}$ = 7.69, P = 0.0075), while leaf weight ratios (leaf mass : plant mass) were similar between treatments (P > 0.01). Third, stem diameters of water-limited alders were narrower than well-watered alders by an average of 1.25 mm in Trial 1 ($F_{1,61}$ = 18.87, P < 0.0001) and 2.24 mm in Trial II ($F_{1,57}$ = 9.06, P = 0.0039). These differences resulted in lower mean aboveground biomass (26.63 ± 1.94 g) in the water-limited group than in the well-watered group (32.05 ± 1.92 g) ($F_{1,80}$ = 4.09, P = 0.0468). As plants in both water and isolate treatments had similar leaf specific area (236.00 ± 7.14 cm^2 g^{-1}), we used area-based measurements of photosynthesis for treatment comparisons.

Test of predisposition concept

Trial I Disease incidence was high in both water treatments and was independent of water treatment (G = 0.582, 1 df, P = 0.445). Eighty-seven per cent of Trial I plants developed disease, which was similar to the frequency with which *V. melanodiscus* was recultured for both trials (85%). There was only one case of disease-related mortality in Trial I. Canker area steadily increased until 60–90 d after inoculation, when the majority of alders developed callusing (70%) (Fig. 3). Horizontal callus dimensions were greater in well-watered alders (7.86 ± 0.45 mm) compared to water-limited plants (5.74 ± 0.41 mm) ($F_{1,55}$ = 11.71, P = 0.0012). Well-watered plants produced less vertical callus (9.55 ± 0.82 mm) than the water-limited group (13.78 ± 1.02 mm) ($F_{1,55}$ = 10.21, P = 0.0024).

Water-limitation affected disease severity for Isolate 2 alders. During the first trial, disease severity was greatest in

Table 1 Leaf-level measurements indicating water treatment effects in the water-limited (−H_2O) and well-watered (+H_2O) groups

	Leaf water potential (MPa)					g_s (mmol m^{-2} s^{-1})		E (mmol m^{-2} s^{-1})	
Treatment	8:00	12:00	16:00	18:00	19:00	July	Aug.	July	Aug.
+H_2O	−0.47 (0.06)	−0.72 (0.07)	−0.90 (0.05)	−0.92 (0.08)	−0.47 (0.06)	199 (24)	81.2 (11)	3.99 (0.36)	1.79 (0.22)
−H_2O	−1.27 (0.23)	−1.43 (0.12)	−1.53 (0.20)	−1.40 (0.10)	−0.65 (0.06)	118 (28)	67.3 (10)	2.88 (0.56)	1.89 (0.18)

An example of the daily fluctuation in water potential is shown from 8:00 h to 19:00 h on 18 July when water potential measures were taken after a short period (2 d) of no water in the water-limited (−H_2O) treatment (n = 3 for each time period). The standard protocol for the −H_2O treatment was the application of low volumes of water for an average of 4 d followed by a short period (1–2 d) of no water. After the measurement at 18:00 h, plants were watered and water potential was fully restored to early morning values in the +H_2O group. Stomatal conductance (g_s) and transpiration (E) measurements (on healthy controls) are also shown for July and August after a similar period of withheld water followed by restored water (n = 8–10). Mean ± SE.

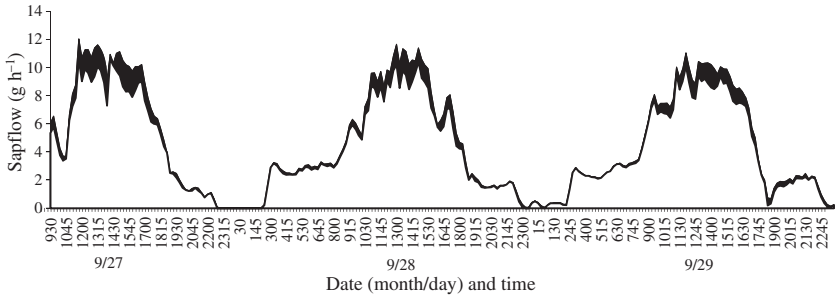

Fig. 2 Paired sapflow measurements from adjacent regions to a stem canker. The curves represent sapflow over 3 d. The area of each curve is divided into two parts. The open area represents the total amount of water transported above the canker. The dark portion of the curve represents the amount of water transported just below the canker, indicating the difference in sapflow between the upper and lower regions adjacent to the canker. The greatest differences in water transport between the gauges occured during peak sapflow, when driving variables (light, temperature, vapor pressure deficit) are high.

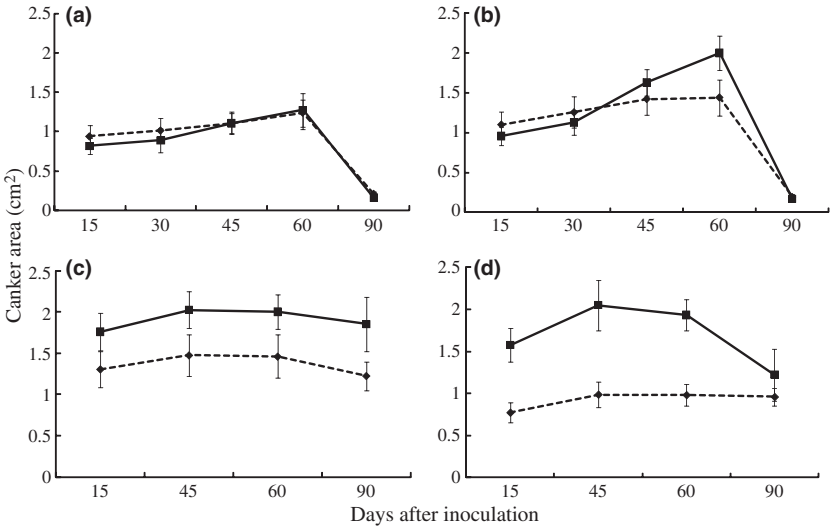

Fig. 3 Changes in canker area for Trial I (a,b) and Trial II (c,d). Each period after inoculation shows mean canker area ± 1 SE for Isolate 1 (dotted line) and Isolate 2 (solid line) (n = 15 for each isolate).

the water-limited, Isolate 2 alders and peaked 60 d after inoculation (Trial I) (Fig. 3). Time–isolate and time–water treatment interactions affected disease severity, but only during in the first trial (Table 2). Conidiomata (asexual reproductive structures) developed during the first 5 wk after inoculation, with 13 of the 30 inoculated plants bearing a total of 37 conidiomata. Nine of the 13 ramets with conidiomata were in the water-limited treatment.

Trial II In Trial II, water treatment did not affect disease incidence, severity or disease-related mortality. Ninety-two per cent of inoculated plants developed disease symptoms, with eight inoculations resulting in mortality. Disease-related mortality (G = 0.582, 1 df, P = 0.445) and disease incidence (G = 2.09, 1 df, P = 0.148) were independent of water treatment. The majority of alders (63%) developed callusing, which caused sunken necrotic tissue and decreased canker area from 60 to 90 d after inoculation (Fig. 3, Table 2). Well-watered plants developed more vertical callusing (12.42 ± 6.18 mm) than water-limited plants (9.47 ± 2.42 mm) ($F_{1,55}$ = 21.22, P < 0.0001).

Table 2 Repeated measures MANOVA and time contrasts for the effects of water treatment, isolate type (1,2) and time on canker area

	Trial I	Trial II
Source	F-value	F-value
Time	59.12***	8.22***
Time × isolate	3.11*	1.60 NS
Time × treatment	3.08*	1.66 NS
Between subject		
Isolate	0.24 NS	6.69**
Treatment	6.02*	2.56 NS
Within subject		
Time	125.79***	11.33***
Time × isolate	4.22**	2.84 NS
Time × treatment	6.67***	0.95 NS

MANOVA tests use Roy's Greatest Root with 4 numerator degrees of freedom and 47 denominator degrees of freedom. Significance level: ***, P < 0.001; **, P < 0.01; *, P < 0.05; NS, not significant. The Glasshouse–Geisser Epsilon Adjustment was used to adjust degrees of freedom for within subject tests.

During the second trial, alders inoculated with Isolate 2 generally had greater disease severity than Trial I (Fig. 3). High conidiomata production reflected greater disease severity. Conidiomata development peaked *c.* 5 wk after inoculation when 16 out of 30 ramets bore a total 194 conidiomata. A similar number of ramets (6–7) developed conidiomata in each water treatment.

Internal vs external canker dimensions

The length of external cankers was small (13.1 ± 1.5 mm in Trial I and 12.5 ± 1.1 mm in Trial II) when compared with the overall length of the stem (935.3 ± 19.2 mm). However, the length of discolored sapwood was much greater. Each millimeter of vertical necrosis on the bark surface corresponded to an average of 15.7 mm of pathologically darkened tissue. The length of external cankers was positively correlated with the length of discolored sapwood (r^2 = 0.32, P = 0.0012).

Multiple stressors concept

Light response curve parameters *Trial I.* As expected, the highest A_{max} in Trial I was maintained by leaves from control, well-watered ramets (9.13 ± 0.69 μmol CO_2 $m^{-2}s^{-1}$) (Fig. 4, Table 3). However, leaves from the water-limited, control ramets (untreated) maintained a similar A_{max} as the water-limited, diseased ramets, indicating that one stress did not exacerbate the other (Fig. 5, Table 3). Therefore, similar downregulatory effects on A_{max} were found in leaves from the ramets that were either water-limited or diseased. These groups all maintained an A_{max} between 6.33 and 6.93 μmol CO_2 $m^{-2}s^{-1}$ (Table 4).

We also tested the effects of multiple stressors on other parameters of the LRC (A_{qe}, LCP and LSP). In Trial I, the slope of the LRC (A_{qe}) and the light saturation point (LSP) were affected by disease. For both water treatments, A_{qe} was higher in leaves from diseased ramets. Leaves from the water-stressed, diseased ramets had the highest A_{qe} (109.43 ± 13.76 mol CO_2 mol $quanta^{-1}$) (Tables 3,4), in contrast to the lowest A_{qe} measured for the well-watered, control ramets (55.67 ± 12.74 mol CO_2 mol $quanta^{-1}$). The steep slope and quick curvature of the LRC led to a low LSP for leaves from the water-stressed, diseased treatment (667 ± 190 μmol m^{-2} s^{-1}) (Fig. 5, Table 4). However, in leaves from the well-watered control ramets, the lower slope of the LRC led to a curvature point and light saturation at higher light levels (1643 ± 178 μmol m^{-2} s^{-1}) (Fig. 4, Table 4).

Trial II. Trial II did not confirm Trial I results. In contrast, several Trial II alders showed increased photosynthetic performance after inoculation. Trial II LRCs indicated that A_{max} was upregulated in well-watered ramets treated with Isolate 1 inoculum (10.56 ± 1.29 μmol CO_2 $m^{-2}s^{-1}$) compared with the Isolate 2 ramets (5.75 ± 1.19 μmol CO_2 $m^{-2}s^{-1}$) (Tables 3,4). Isolate 1 was also associated with the smallest cankers in both trials (Fig. 3). To confirm the upregulatory response, we remeasured photosynthetic rates on Trial II plants in October, but did not find the same trend in the upregulation of well-watered, Isolate 1 plants. We did not detect a water or disease treatment effect for any of the other LRC parameters in the second trial (Tables 3,4).

Photosynthesis as a function of conductance *Trial I.* The water-limited plants photosynthesized over a lower range of conductance values (60–80 mmol m^{-2} s^{-1}). However, leaves from all ramets receiving either the water-limitation or disease treatment in Trial I were restricted to photosynthesis over the lowest values of conductance (Fig. 6). Well-watered ramets operated over a higher and broader range of stomatal conductance values (67–137 mmol m^{-2} s^{-1}) at light saturation (Fig. 6). Diseased ramets from the well-watered treatment maintained a higher instantaneous WUE_i (4.90 ± 0.36) than the control ramets (4.09 ± 0.37) (Tables 3,4).

Trial II. Consistent with the first trial, leaves from the well-watered ramets operated at the highest and widest ranges of conductance values in Trial II. The upper and lower limits of light-saturated stomatal conductance were similar between trials (Fig. 6), as well as the range in which the water-limited, diseased ramets operated (60–80 mmol m^{-2} s^{-1}). Also similar between trials was the higher WUE_i in diseased ramets (6.25 ± 0.79) compared with the control ramets (5.07 ± 0.06). Contrary to the results from Trial I, the two pathogenic isolate treatments had opposite effects in the well-watered plants from Trial II. Isolate 2 ramets photosynthesized at conductance values of 60 ± 10 mmol H_2O m^{-2} s^{-1}, while leaves from Isolate 1 ramets operated at higher values of conductance (150 ± 40 mmol H_2O m^{-2} s^{-1}).

Discussion

Predisposition concept

Drought stress has been a working hypothesis for the increasing incidence of Cytospora canker disease on *Alnus* spp. in Alaska (Ruess *et al.*, 2009). At the landscape scale, temperature-induced drought stress and suppressed radial growth in *A. tenuifolia* suggest that summer drought may be associated with increased host susceptibility in *A. tenuifolia* (Nossov, 2008; Ruess *et al.*, 2009). However, drought stress was not related to disease incidence in our study, as the majority of inoculated *A. fruticosa* became infected and developed disease regardless of water treatment. Disease incidence also did not differ between the trials. This was surprising as we expected higher incidence of disease during Trial I, when alders were more water stressed and the environment was hotter and drier. Threshold levels of water stress are often

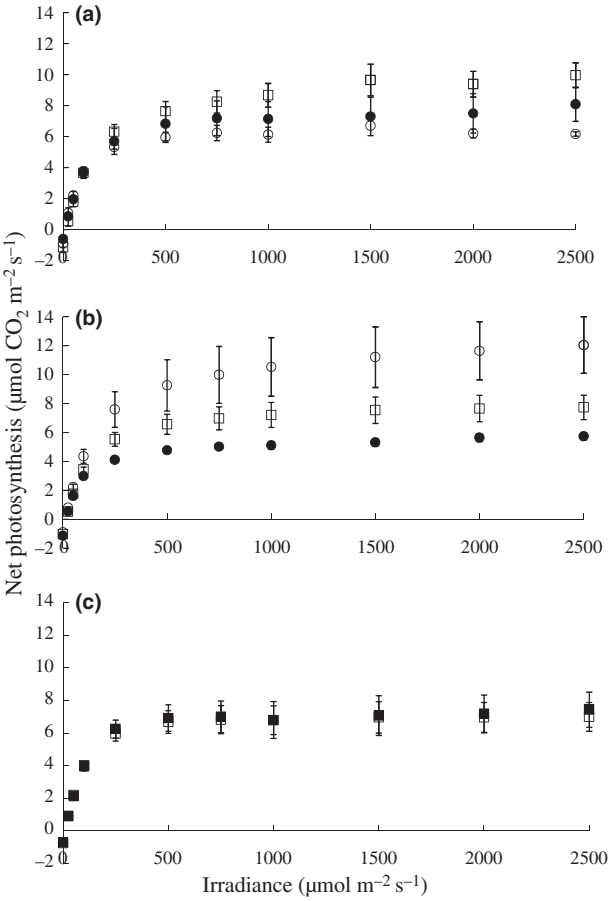

Fig. 4 Light response curves for paired ramets in the well-watered treatment for Trial I (a), Trial II (b) and the no-disease control group (c). Measurements are based on a split-plot design, where water treatment is applied at the whole-plot level (alder) and isolate type is applied at the subplot level (ramet). Each point is the mean ± 1 SE from ramets treated with either Isolate 1 (open circles), Isolate 2 (closed circles), or untreated, control ramets (open squares). In the no-disease control groups for August and September, light response curves were not different between the untreated ramet (open squares) and the ramet treated with wound + agar only (closed squares). Therefore, only the September group (c) is shown (n = 3 ramets for each isolate and n = 6 ramets for controls).

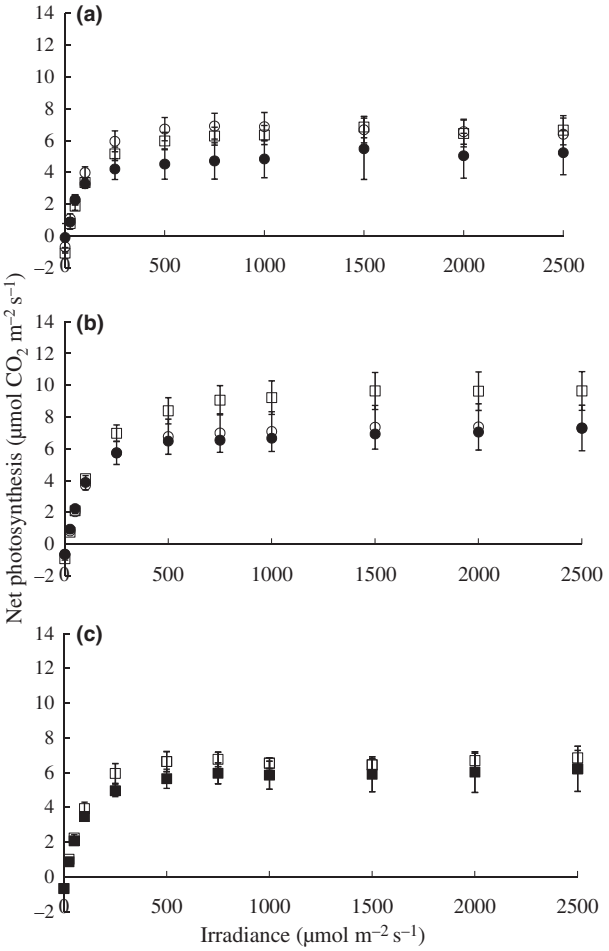

Fig. 5 Light response curves for paired ramets in the water-limited treatment for Trial I (a), Trial II (b) and the September no-disease control group (c). Each point is the mean ± 1 SE from ramets treated with either Isolate 1 (open circles), Isolate 2 (closed circles) or untreated, control ramets (open squares). In the no-disease control groups for September, light response is shown for the untreated ramet (open squares) and the ramet treated with wound + agar only (closed squares).

required for predisposition to nonaggressive pathogens (Schoeneweiss, 1975), but our study indicates that the Cytospora pathogen isolates were aggressive enough to infect *A. fruticosa* regardless of water status. Drought stress was also not required for disease predisposition in field studies that inoculated Alaskan hosts, *A. tenuifolia* and *A. fruticosa*, with the same *V. melanodiscus* isolates used in this study (Stanosz *et al.*, 2008; J. K. Rohrs-Richey unpublished data).

We also expected disease severity to be greater during the more stressful environment in Trial I; however, severity was greatest during the cooler conditions of Trial II. One explanation for higher severity is that environmental conditions may have been more suitable for pathogen growth. Various epidemiological stages typically require specific ranges of temperature and humidity (Berger *et al.*, 1997) and optimal conditions for canker expansion have been determined for some species within the *Cytospora* genera (Kamiri & Laemmlen, 1981). Optimal conditions for canker expansion are unknown for *Cytospora umbrina* on *Alnus*, but it is possible that the hot, dry conditions of Trial I discouraged canker growth.

Alternatively, there are several lines of evidence indicating that greater disease severity during Trial II was based on the timing of host water-stress relative to host phenological stage. First, alders were entering the height of their phenological stage at the beginning of Trial I (16 July). It is likely that costly defense responses were fully maintained during Trial I, which began just as alders typically enter the peak stage of their phenology (third week of July) when rates of nitrogen fixation and plant growth are at their highest (Mitchell &

Table 3 Results from the mixed-model, split-plot ANOVA on the effects of treatment and isolate type (1, 2) on the light response curve parameters

		Trial I			Trial II		
Variable	Effects	Num. df	Den. df	*F*-value	Num. df	Den. df	*F*-value
A_{max}	Treatment	1	11	2.77 NS	1	8.52	0.00 NS
	Isolate(s)	1	11	6.41*	2	11.3	2.36 NS
	Treatment × Isolate	1	11	2.36 NS	2	11.3	5.43*
A_{qe}	Treatment	1	11	3.62 NS	1	9.37	0.02 NS
	Isolate(s)	1	11	5.21*	2	12	1.29 NS
	Treatment × Isolate	1	11	0.15 NS	2	12	1.49 NS
LCP	Treatment	1	11	0.89 NS	1	7.2	2.42 NS
	Isolate(s)	1	11	2.53 NS	2	10.8	1.81 NS
	Treatment × Isolate	1	11	0.23 NS	2	10.8	0.84 NS
LSP	Treatment	1	11	8.07*	1	8.72	0.46 NS
	Isolate(s)	1	11	1.73 NS	2	10.7	0.70 NS
	Treatment × Isolate	1	11	0.33 NS	2	10.7	0.70 NS
WUE_i	Treatment	1	11.1	0.99 NS	1	8.9	0.04 NS
	Isolate(s)	1	11.6	9.77**	2	10.9	6.81*
	Treatment × Isolate	1	11.6	6.09*	2	10.9	0.03 NS

A_{max}, the light saturation point (μmol CO_2 m^{-2} s^{-1}); A_{qe}, the quantum efficiency (mol CO_2 mol^{-1} quanta); LCP, the light compensation point (μmol m^{-2} s^{-1}); LSP, light saturation point (μmol m^{-2} s^{-1}); WUE_i, instantaneous water use efficiency (μmol CO_2 m^{-2} s^{-1} (μmol H_2O m^{-2} s^{-1})). In Trial I, statistical differences could not be detected between isolates, so they were pooled for the analysis. Significance level; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; NS, not significant. Denominator (Dem.) degrees of freedom were approximated using the Satterthwaite method. Numerator (Num.) degrees of freedom depended on whether isolates were pooled in the analysis.

Table 4 Estimates of light response curve parameters and instantaneous water use efficiency (WUE_i)

Trial	Treatment	Isolate	A_{max}	A_{qe}	LCP	LSP	WUE_i
1	$-H_2O$	0	6.87 (0.74)	89.11 (13.76)	18.74 (4.53)	833 (220)	4.97 (0.39)
	$-H_2O$	1	6.33 (0.74)	109.43 (13.76)a	10.31 (4.53)	667 (190)a	5.06 (0.39)
	$+H_2O$	0	9.13 (0.69)b	55.67 (12.74)b	12.41 (4.19)	1643 (178)b	4.09 (0.37)a
	$+H_2O$	1	6.93 (0.69)a	84.30 (12.74)	7.87 (4.19)	1214 (301)	4.90 (0.36)b
2	$-H_2O$	0	9.49 (1.06)	65.02 (13.11)	9.93 (2.56)	1000 (209)	5.07 (0.55)a
	$-H_2O$	1	7.09 (1.32)	83.08 (16.28)	7.98 (3.27)	1200 (200)	5.72 (0.67)b
	$-H_2O$	2	7.02 (1.58)	89.15 (19.53)	9.31 (3.98)	–	6.25 (0.79)b
	$+H_2O$	0	7.41 (0.93)	79.87 (11.57)	14.94 (2.22)	1250 (122)	5.28 (0.49)a
	$+H_2O$	1	10.56 (1.29)a	57.05 (16.06)	7.79 (3.26)	1250 (120)	5.84 (0.65)b
	$+H_2O$	2	5.75 (1.19)b	94.09 (14.82)	17.53 (2.94)	–	6.17 (0.61)b

Values for A_{max} (the light saturation point, μmol CO_2 m^{-2} s^{-1}), A_{qe} (the quantum efficiency, mol CO_2 mol^{-1} quanta), LCP (the light compensation point, μmol m^{-2} s^{-1}), LSP (light saturation point, μmol m^{-2} s^{-1}), and WUE_i (μmol CO_2 m^{-2} s^{-1} (μmol H_2O m^{-2} s^{-1})) are least square means estimates with standard errors in parentheses. For Trial I, both isolate types (1,2) were pooled (Isolate 1) for statistical tests. Otherwise, control = 0, Isolate 1 = 1, Isolate 2 = 2. Tests for differences between means based on the Tukey–Kramer adjustment in the ANOVA mixed procedure. Significant differences at the $\alpha = 0.05$ level for water–isolate combinations are indicated by letters.

Ruess, 2009). At this stage, higher water status may have supported additional defensive strategies that can be effective against Cytospora canker, such as increased water supply to the bark and maintenance of cell turgor (Bier, 1953; Bloomberg, 1962).

Trial II alders inoculated with Isolate 2 immediately produced larger cankers, developed more conidiomata and had higher mortality in response than Trial I alders. Furthermore, the Trial II alders did not produce the healing response of Trial I alders, which had adequate stem growth and callus production to close off the canker almost entirely. This high disease severity during Trial II could be explained by lower active and passive defense responses at later phenological stages. Alders in Trial II were inoculated when alders in the field are typically resorbing nutrients and beginning senescence (Mitchell & Ruess, 2009). During that time, it is likely that resources were not heavily invested in costly processes to prevent canker advance, including suberin and lignin production for mechanical barriers (Bloomberg, 1962; Bloomberg & Farris, 1962), nonspecific wound healing (necrophylactic periderms and nonsuberized impervious tissue) (Maxwell *et al.*, 1997), or synthesis of secondary metabolites (McPartland & Schoeneweiss, 1984; Boyer, 1995).

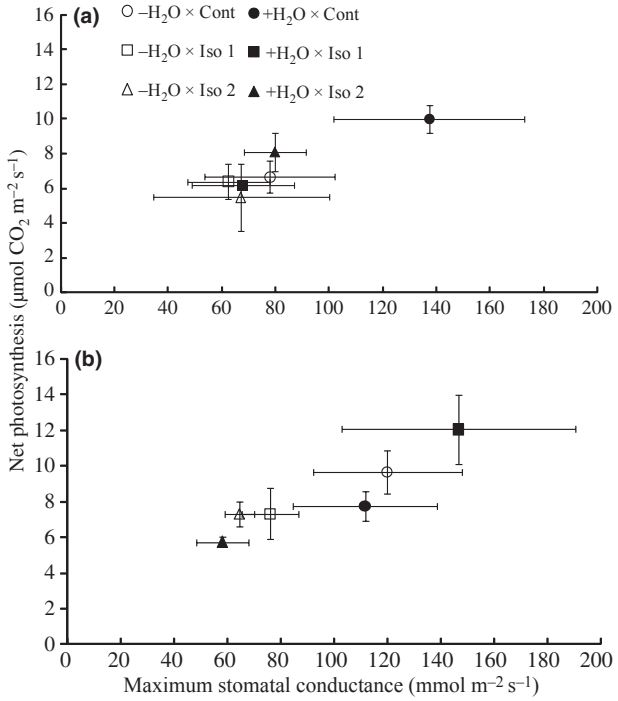

Fig. 6 Net photosynthesis as a function of maximum stomatal conductance for Trial I (a) and II (b). At maximum stomatal conductance under the highest irradiance level, the mean photosynthetic rate ± 1 SE is plotted as a function of mean stomatal conductance ± 1 SE for each water treatment (WW = well-watered, WL = water-limited) and isolate combination (n = 3 for each isolate and n = 6 for controls).

We can only speculate on the reason for greater disease severity during Trial II. This could be experimentally resolved with an inoculation experiment using a factorial design that crosses levels of phenological stage with different environmental conditions. The environmental parameters of such an experiment would be best informed by more specific studies on the optimal temperature and humidity ranges required for the epidemiological stages of *V. melanodiscus* in Alaska.

Multiple stress concept

We evaluated the multiple stress concept by examining how drought stress and disease influenced host photosynthetic performance. We predicted that well-watered plants challenged by only one stress would maintain a higher light-saturated photosynthetic rate (A_{max}) than plants challenged by the simultaneous stresses of water-limitation and disease. As expected, the well-watered, healthy ramets reached the highest A_{max} in Trial I. However, Trial I plants maintained similar values of A_{max} regardless of whether treated with the individual or combined stresses of water-limitation or disease. These Trial I results indicate that one type of stress did not exacerbate the other; rather, the stresses resulted in a generalized depression in A_{max}. These results do not support the multiple stress concept but instead suggest that reduced A_{max} reflected systemic downregulation and generalized stress response from both water stress and the localized stem canker (Chapin, 1991; Isaac, 1992; Flexas *et al.*, 2004).

Although A_{max} did not reflect a multiple stress response, the light response parameters A_{qe} and LSP did support the multiple stress concept for Trial I. Leaves of well-watered, healthy ramets reached light-saturated photosynthesis at light intensities that were more than double the light intensity at which the leaves from water-limited, diseased ramets reached light saturation. The low LSP measured for the water-limited, diseased ramets was achieved by high A_{qe} (steep slope) and quick curvature of the LRC. Low LSP for the water-limited, diseased ramets likely reflect stomatal limitation as well as metabolic limitation to carbon fixation, as these leaves operated over a range of conductance values below the threshold level (100 mmol m^{-2} s^{-1}) at which ribulose-1,5-bisphosphate (RuBP) regeneration is considered to be resistant to water stress (Flexas *et al.*, 2004). Low LSP is also indicative of the inability to use high light intensities, which can increase the risk of photoinhibition in water-limited, diseased ramets during daily maxima of light and temperature (Ayres, 1984). Low-intensity saturation has been found previously for diseased plants (Niederleitner & Knoppik, 1997) and suggests that water stress and disease can mechanistically limit the ability to fix carbon in addition to risking photosystem damage under high-light, high-temperature conditions.

We only detected downregulation of light response in water-stressed, diseased plants during Trial I. Treatment effects may have been easier to detect during Trial I, as it overlapped with peak phenology when plants operate close to physiological potential. During this stage, we captured the reduction in LSP and A_{max} under drought stress and disease, a mechanistic explanation of how carbon resources are limited for water stressed alders with Cytospora canker.

Despite later phenology during Trial II, light parameters during this trial suggest an important mechanism by which alders may compensate for disease. We measured upregulation of A_{max} in well-watered ramets inoculated with Isolate 1, which maintained an A_{max} twice that of those treated with Isolate 2. Alders may have upregulated A_{max} for two reasons. First, the low disease damage associated with Isolate 1 could have allowed compensatory photosynthesis in the host. Alternatively, the Isolate 1 pathogen may have placed a higher metabolic demand on its host and plants responded by upregulating photosynthesis. Photosynthetic upregulation can be a compensatory response to the earlier stages of fungal infection and colonization, when the host may be able to support the increased carbon costs associated with pathogen biomass (Isaac, 1992; Lucas, 1998). Upregulation of A_{max} may also be a mechanism by which plants tolerate this disease. As upregulation was only found in well-watered plants, this suggests that water availability may affect the

capacity for compensatory photosynthesis and potential tolerance in response to disease. However, even in the well-watered alders, upregulation was a temporary response (it was not found in measurements 2 wk later), which was not sustained during later phenological stages.

Stomatal regulation of water loss

In addition to the effects of water stress and disease on light-response parameters, the canker disease also decreased the amount of functional sapwood tissue and reduced water transport during daily periods of high VPD. Pathogen colonization of the vascular system can decrease functional sapwood by causing resistance to water flow, interfering with osmotic gradients, or blocking and embolizing conduits (Ayres, 1981; Sutic & Sinclair, 1991), all of which may be exacerbated by water stress. We found that alders consistently used stomatal regulation to ameliorate the interference of cankers with water transport, as diseased ramets in both trials consistently had higher peak WUE_i than healthy ramets. In Trials I and II, we found that leaves from the water-limited, diseased ramets operated within a narrow range of stomatal conductance values (63–76 mmol m^{-2} s^{-1}). This range is much lower than the maximum conductance values in our experiment (137–146 mmol m^{-2} s^{-1}), the range of stomatal conductance values previously reported for water stressed alders (181–268 mmol m^{-2} s^{-1}) (Hibbs *et al.*, 1995; Schrader *et al.*, 2005) and the typical range for woody plants (Eschenbach & Kappen, 1999). Stomatal regulation is not necessarily a given in alders (e.g. *A. glutinosa*, Eschenbach & Kappen, 1999) or in diseased plants (Ayres, 1981). Our study indicates that stomatal regulation is generally used as a disease-coping strategy for *A. fruticosa*, whereas photosynthetic upregulation appears to be a strategy conditional on water status. As plant pathogens influence all physiological processes throughout the plant (Sutic & Sinclair, 1991; Isaac, 1992; Lucas, 1998), the capacity for these types of adjustments in physiological performance may buffer individuals against the effects of multiple stresses (Helmuth *et al.*, 2005).

Conclusions

Our results are not entirely aligned with the general assumption that climate-related stressors will physiologically compromise plants and reduce their capacity to defend against or recover from disease damage (Larsson, 1989; Mitchell *et al.*, 2003; Rodriguez *et al.*, 2004). In our study, the greatest disease damage did not correspond to the most stressful environmental conditions. Instead, disease severity was greatest in alders inoculated during later phenological stages (Trial II) and under a less stressful environment. The most suppressed disease levels were in Trial I, well-watered alders, which were inoculated during peak phenological stage. These alders experienced the most demanding environmental conditions and had lower physiological performance under the simultaneous stresses of water-limitation and disease. Directional changes in temperature may be the primary driver behind changes to plant–pathogen dynamics; however, the dependence of our results on host phenological stage and environment makes it difficult to accept that increased temperatures will consistently lead to higher levels of disease for this pathosystem.

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