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Transient de-coupling of photosynthesis and stomatal conductance in response to leaf primary vein cut in Helianthus annuus

Laura Elizabeth Green

Laura Elizabeth Green
Candidate

Biology
Department

This thesis is approved, and it is acceptable in quality and form for publication:

Approved by the Thesis Committee:

_____, Chairperson

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B.F.A., Visual Arts, University of New Mexico, 1992

THESIS

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ABSTRACT

Control of stomatal aperture is the primary way plants regulate gas exchange in the short-term, but what triggers stomatal responses to water stress is still debated. Chlorophyll-a fluorescence imaging, local leaf temperature, and gas exchange were measured simultaneously following a cut to primary leaf vein of *Helianthus annuus* to access the effect of local leaf xylem cavitation on leaf function. The treatment was repeated under 3 different vapor pressure deficit (VPD) conditions. Surprisingly, photosynthesis (A) and stomatal conductance (g_s) responded inversely immediately following the treatment, indicating that A was not CO_2 limited by stomatal closure. Comparisons of fluorescence images and temperature data showed that while both A and g_s responded heterogeneously across the measured leaf area, local responses did not correspond spatially or temporally, suggesting that each was the result of a different mechanism and/or was initiated by a separate signal. Since the stomatal response varied with VPD but A did not, it is likely that only g_s was ultimately responding to a hydraulic signal. Both A and g_s recovered to near steady state levels by 900s after the cut. These results indicate that stomata respond immediately to a sudden hydraulic perturbation and that hydraulic redundancy in sunflower is sufficient to allow quick recovery to local interruption of vascular system. This experiment also provides evidence of transient de-coupling of A and g_s following wounding.

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INTRODUCTION

In most environments, plant growth and survival depends upon balancing water loss with CO_2 fixation. Although changes in leaf area and leaf energy balance affect canopy transpiration in the longer term, short term regulation of water loss is controlled primarily by changes in stomatal aperture. Stomata respond to a complex signal transduction process that is influenced by CO_2 concentration, red and blue light signals, leaf water potential and transpiration rate (Buckley, 2005; Messinger, et al., 2006; Shimazakie et al., 2007; Sperry and Pockman, 1993; Mott and Parkhurst, 1991). Although single factor responses have been widely studied, many of the intricacies of stomatal response to fluctuating conditions remain poorly understood. In this study, I address the role of stomata in short-term dynamic responses of leaf gas exchange to changes in hydraulic conductance caused by manipulations of a downstream leaf vein.

Hydraulic conductance (k) is the ratio of the rate at which water moves to the magnitude of its driving force and is a measure of efficiency of water movement through a system (Sack and Holbrook, 2006). As a result, transpiration (E) can be expressed in terms of hydraulic conductance from soil to leaf ($k_{whole\ plant}$) and the difference between soil (Ψ_{soil}) and leaf (Ψ_L) water potential using Ohm's law:

$$E * A_{leaf} = k_{whole\ plant} * (\Psi_{soil} - \Psi_L) \qquad (1)$$

where A_{leaf} is the total leaf area of the canopy.

Stomata, because of their position, are sensitive to water potential caused by changes in the evaporative gradient driving transpiration and changes in the flow path that transports water from the soil to the leaf. The transpiration rate of a patch of leaf tissue is determined by stomatal conductance to water vapor (g_s) and the vapor pressure deficit (VPD). By rearranging equation 1, the water potential in the same location is determined by E, Ψ_{soil}, and $k_{whole\text{-}plant}$. In the absence of stomatal regulation, an increase in the evaporative gradient will cause a proportional increase in E and decrease in Ψ_L.

Likewise, a decrease in $k_{whole\text{-}plant}$ will decrease Ψ_L without a change in VPD unless g_s also decreases proportionately.

Hydraulic conductance determines stomatal sensitivity to higher E, and is a crucial component in maintaining stomatal aperture and therefore photosynthesis. Since hydraulic pathways through the leaf and root contribute most to whole plant hydraulic resistance (Yang and Tyree, 1994), k_{leaf} is particularly important in maintaining g_s and photosynthetic rate (A).

The mechanism by which stomata respond to changes in plant water status is still debated. As predicted by modeling studies (Tyree and Sperry 1988), stomata respond to changes in xylem hydraulic conductance (k) (Sperry and Pockman 1993) and Ψ_L (Saliendra et al 1995), reflecting their integration of conditions up- and down-stream in the soil-plant-atmosphere continuum, suggesting a purely hydraulic mechanism is sufficient to explain short term stomatal regulation. A likely hydraulic control mechanism is one in which a change in Ψ_L, as a result of change in the evaporative gradient or hydraulic tension, triggers a stomatal response since stomatal aperture has a predictable relationship to guard cell turgor (Franks, et al., 1995). However, while correlations between g_s and k or Ψ_L associated with xylem cavitation thresholds have been demonstrated (Mencuccini and Comstock, 1999; Saliendra, et al., 1995; Salleo, et al., 2000; Sperry and Pockman, 1993), observations of seemingly contradictory stomatal responses have led to confusion regarding whether the hydraulic signal is feed-forward (preventing Ψ_L change) or feed-back (responding to Ψ_L change). Additional studies have argued that an additional feed-forward mechanism must exist in which stomata are able to respond quickly to some hydraulic cue and vary conductance to water vapor before leaf water status is negatively affected (e.g. Meinzer, 2002) Isohydric behavior, in which daytime Ψ_L is maintained regardless of Ψ_S, has been thought to require a feed-forward signal transmitted from roots (Tardieu, 1992). Some data have suggested root signals may primarily control stomata in anisohydric species like sunflower, since while g_s varies with Ψ_L under stress, the relationship is inconsistent over all conditions (Tardieu, et al., 1996).

The time frame at which these relationships are observed is important in understanding a potential signal, since $\Delta\Psi$ oscillations that trigger stomata may be small, local, or transient, further confounding the relationship between g_s and Ψ_L (Sperry, et al., 1993; Saliendra, et al., 1995).

Stomata may behave heterogeneously across leaves, but little is known about what initiates this phenomenon (Lange, et al., 1971;Eckstein, et al., 1998; Mott and Buckley, 1998). Oscillating heterogeneity in g_s has been observed following changes in ambient humidity, suggesting that guard cells are sensitive to small variations of water potential across leaves and that some level of interaction occurs among groups of leaf cells. This "patchy" behavior is thought to occur as neighboring cells interact with guard cells and transiently affect turgor pressure (Mott and Franks, 2001). Heterogeneous, small-scale stomatal responses might allow finer tuning of water balance even if perturbation is large. Small, transient adjustments in stomatal aperture (and local cell Ψ) would be undetectable in net measurements of Ψ_L, E, and g_s and so give the impression that Ψ_L was controlled by some other mechanism. (Nardini and Salleo, 2003; Saliendra, et al, 1995; Lawson, et al., 1998)

Since leaf water balance is a function of both supply and demand (Lange, et al, 1971), patchiness observed following changes in VPD (West, et al., 2005; Mott and Franks, 2001) might also occur following a sudden heterogeneous change in k caused by local leaf vein cavitation, if stomata respond to changes in Ψ_L (Terashima, 1992). And a more rapid step change in Ψ of cells caused by sudden loss of conductance in a vein directly supplying them with water might possibly induce more dramatic patchy behavior. Some loss of xylem function may be tolerated to optimize gas exchange, (Jones and Sutherland, 1991, Sperry, et al, 1998, Mencuccini and Comstock, 1999), and may cause a drop in leaf cell water potentials resulting in initiation of stomatal closure. However, Nardini, et al. (2001) found Laurel leaf hydraulic architecture to be redundant and water to move through the leaf in parallel pathways, rather than a series. In this case, water could easily

bypass a cavitated vein making the effect on stomata and gas exchange minimal or temporary.

The objective of this study was to determine whether a local change in leaf hydraulic conductance leads to changes in leaf gas exchange associated with a heterogeneous stomatal response. By varying humidity, West et al. (2005) induced a patchy photosynthetic response across leaves of *Xanthium strumarium*, detected as changes in spatial patterns of chlorophyll a fluorescence. Comparison with simultaneous thermal imaging showed that CO_2 limitation caused by heterogeneous stomatal behavior was the cause of the patchy photosynthetic response they observed, since leaf temperature is a function of E (and therefore a function of gs). Sunflower, like *X. strumarium,* exhibits heterobaric anatomy and compartmentation of mesophyll, which can limit lateral gas diffusion (McClendon, 1992; Pieruschka, et al., 2005). In this experiment, a major vein in sunflower leaves was cut at steady-state in one of three different VPD conditions and the response followed using simultaneous measurements of gas exchange, chlorophyll fluorescence imaging and leaf temperature using a thermocouple array on abaxial side of the leaf. In the absence of metabolic limitation, chlorophyll fluorescence was expected to increase where stomatal closure caused CO_2 limitation of photosynthesis following vein-cutting.

METHODS

Plant material

Helianthus annuus seeds were germinated and grown in a Conviron growth chamber (Winnipeg, Manitoba, CA) for 25-30 days where they received 12 hours of 500µmol m^{-2} s^{-1} light per day. Relative humidity was approx. 50% and temperature was controlled at 23^{o} C during the dark period and 27^{o} C during the light period. Plants were fertilized 3x/week with Jacks water soluble 20-20-20 (N- P-K) and were well watered.

Treatment

With the entire plant inside the growth chamber, a 2 x 2 cm area of one fully expanded leaf per plant, including the primary vein 1cm from petiole, was enclosed in a gas-exchange cuvette (LiCor 6400, Lincoln, Nebraska, USA) where air temperature was maintained at 25^{o}C and reference CO_2 was constant at 400 ppm. A fluorescence camera (fluorcam prototype, Photon Systems Instruments, Ltd., Czech Republic) was attached to the cuvette to provide spatially explicit measurements of chlorophyll-a fluorescence. For comparison of spatial stomatal behavior with fluorescence imaging, a thermocouple array made up of 13 evenly spaced copper-constantan thermocouples (36 gauge, Omega), in contact with the abaxial side of the leaf and measured by a datalogger (Campbell Scientific model CR7, Logan, Utah, USA), was used to measure spatial changes in leaf temperature (Fig. 1.).

Each leaf was dark adapted for 20 minutes, after which the quantum efficiency of open photosystem II centers (F_v/F_m) was measured using a saturating flash for 5s and measuring light (PAR=0.03 µmol quanta m^{-2} s^{-1}). Saturating flash intensity was varied in trial experiments (during dark and light) to ensure the intensity used was sufficient to saturate PSII (data not shown) The blue (peak=450 nm) and red (peak=628 nm) actinic lights of the fluorescence camera were then turned on to a level which matched light intensity in the growth chamber (approx. 500 µmol quanta $m^{-2}s^{-1}$) outside of the cuvette (50% each red and blue). The leaf was allowed to reach steady state photosynthetic and

transpiration rates before a saturating pulse was applied to determine the quantum yield of Photosystem II photochemistry (Φ_{PSII}). The leaf was again allowed to reach steady state following the saturating pulse, at which time the fluorescence camera measuring light was turned on so that fluorescence in the light (F') was measured every 5s for 15 minutes. Simultaneously, measurements of net CO_2 and H_2O exchange were stored every 5s and thermocouple temperatures were recorded every 1s. A cut was made through the primary leaf vein just outside the cuvette, 1 cm from the petiole and the junction between the main vein and the 2 secondary veins, without damaging surrounding leaf tissue. Fluorescence, and thermocouple temperature data were logged from steady state to 900 seconds following the cut while gas exchange data continued to be logged until 30 minutes following the cut. After 30 minutes, a second Φ_{PSII} measurement was taken. This protocol was repeated 6 times for each of 3 reference VPD treatments: 2 kPa, 1.25 kPa, and 0.5 kPa corresponding to approximately 15, 40 and 70% relative humidity. Reference humidity was controlled manually using the LI-6400 desiccant.

Data analysis

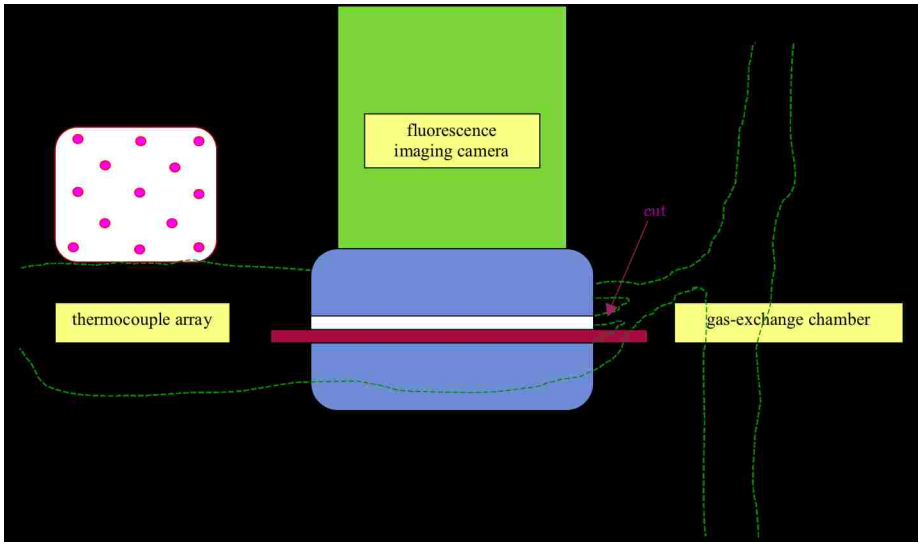
Pixels within a 0.2 cm radius of the estimated position of each thermocouple were averaged and used to spatially compare the F' response with temperature response corresponding to individual thermocouple position. These 13 circular areas are referred to as "sub-areas". The average F' of these sub-areas is used as average leaf F' when compared to average leaf temperature (the average of the13 discrete areas). When average F' response was compared to net gas exchange, whole leaf F' averages (all pixels included) were used.

Net g_s was calculated using the average temperature for all thermocouples.

Spatial heterogeneity of fluorescence and temperature magnitude within each leaf was estimated by calculating standard deviation of fluorescence/temperature in each of the 13 leaf sub-areas at four time intervals. The time intervals were defined as: I)before the cut,

Figure 1

a.



b.

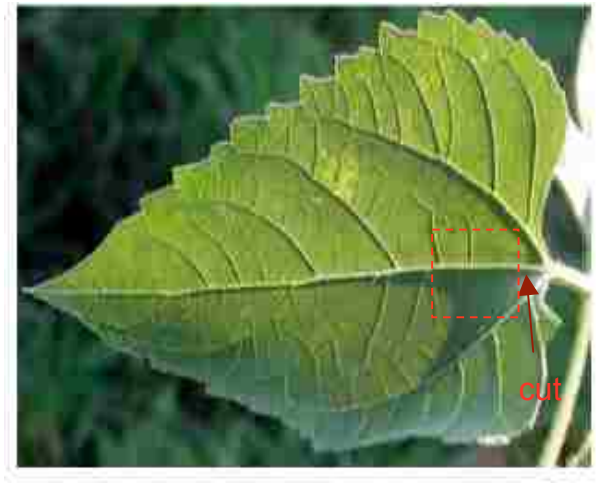


Fig. 1.
Leaf was clamped in cuvette and allowed to reach steady state (a). Following treatment (cut just outside the 2 x 2 cm measured area (b)), gas-exchange measurements (of both CO_2 and H_2O) were made providing an average measure of photosynthetic and transpiration rates. At the same time fluorescence was imaged for spatial measurements of photosynthesis. Temperature measurements were made with a 13- thermocouple array that contacted the bottom of the leaf. (Inset is a plan diagram of the thermocouple array.) Temperture decreases as transpiration rate increases, so the thermocouples provided a spatial meausurement of transpiration rate. Stomatal conductance was later calculated. Measurements were made every 5s for 900s (15m). The plant was inside a growth chamber for the whole experiment where conditions were similar to those in the cuvette.

II)at the peak of the response (time of highest F' or lowest temperature), III)at 300s, and IV)at 900s. Heterogeneity of recovery time was estimated by calculating standard deviation of peak response times (time at which parameter changed direction) in each of the 13 leaf sub-areas.

Parameter and VPD treatment means were compared using 2-way t-tests with un-equal variance.

RESULTS

Whole leaf response

Across all VPD treatments, cutting the main vein initiated opposite responses in net carbon assimilation (A) and transpiration rate. Typical leaves responded to treatment with an immediate and rapid decrease in A and a simultaneous *increase* in E and calculated stomatal conductance (g_s) (Fig. 2a). The average decrease in A was 8.5 ±4.1 μmol CO_2 $m^{-2}s^{-1}$ (all errors are standard deviations), while the average increase in E was 1.3 ±1 mmol H_2O $m^{-2}s^{-1}$, and g_s increased 0.22 ±0.23 mol H_2O $m^{-2}s^{-1}$ across VPD treatments (see below for treatment averages). The increase in E is only consistent with an increase in g_s, suggesting that the observed decline in photosynthesis was not the result of a CO_2 limitation that might occur with decreased g_s.

The response of A and E were not synchronous, with minimum A preceding maximum E in all leaves across all VPD treatments (e.g. Fig 2a). Photosynthesis reached its lowest rate an average of 64 ±11 seconds after the cut and E reached its highest rate significantly later ($p=.0006$), at an average of 143 ±80 s after the cut (estimated g_s reached its highest rate 156 ± 78 s after the cut) and varied with VPD treatment. Initial responses to the cut were followed by rapid recovery of both A and E to near pre-cut rates by 900 s (Fig. 3).

Spatial variation across leaf

The decrease in A and increase in E appeared simultaneously and immediately upon cutting the vein. Both photosynthetic and stomatal responses displayed spatial heterogeneity as measured by fluorescence imaging and variation of temperature across the thermocouple array. However following initiation, A and E changed at different rates and the spatial pattern of the response differed between parameters.

Figure 2

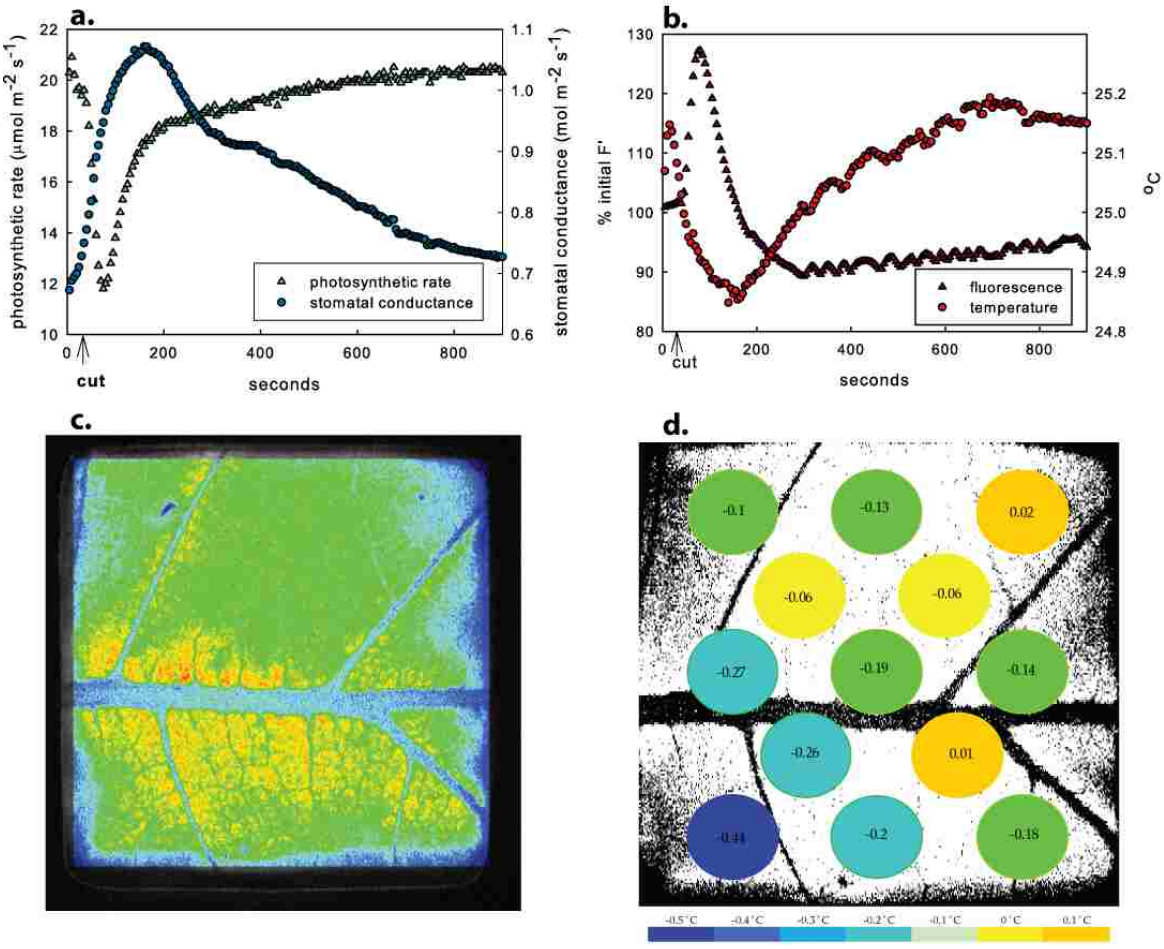


Fig. 2. Response of example leaf in 1.25 kPa VPD treatment: Mean response to leaf vein cut in entire measured leaf area of photosynthetic rate (green) and stomatal conductance (blue) (a) and fluorescence (purple) and temperature (red) (b) showing typical inverse relationship of photosynthetic rate and stomatal conductance. Image of fluorescence (c) and temperature of 13 leaf sub areas (d) temp change (ºC) at t=peak of average fluorescence response following cut. Both fluorescence and temperature responded heterogeneously to the cut, but areas of greatest fluorescence did not have greatest temperature increases.

Figure 3

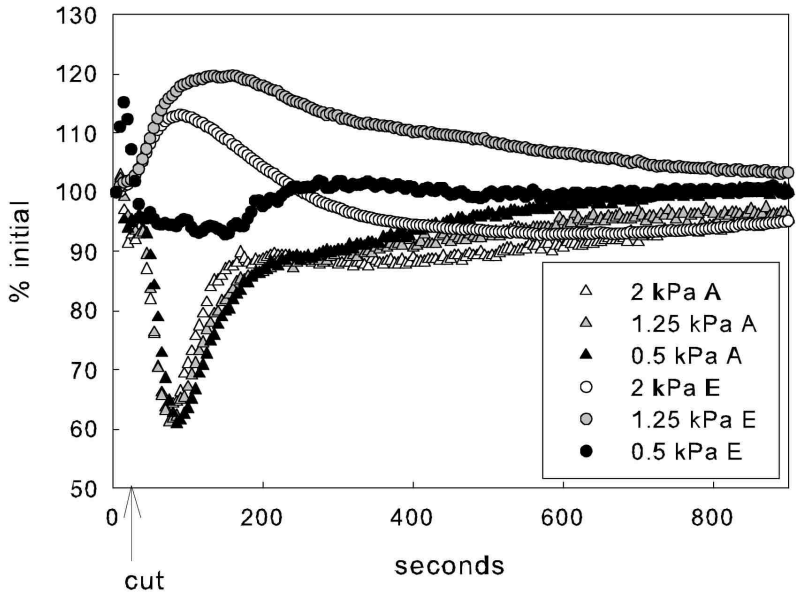


Fig. 3. Mean response of gas exchange by VPD treatment as % of initial (steady state) values.

The peak fluorescence response preceded the peak temperature response in all areas of the leaf by an average of 82 s, although the time by which the extremes were separated varied across the leaf (mean std dev across leaf sub-areas was 55s). Photosynthetic heterogeneity of leaf sub-areas increased significantly following the cut ($p<0.01$) and returned to pre-cut variability by 300s, but heterogeneity of T_{leaf} did not significantly increase in response to the cut (Fig. 4 a,b), although temperature did respond differentially across the leaf (Fig 4 c). While often the same general area of a leaf saw the greatest overall changes in both F' and temperature, at the time of peak fluorescence (65 ± 10s following cut), many sub-areas where a decrease in electron transport was observed did not show evidence of stomatal closure (ie temperature increase) (Fig 2 c, d). Many areas in which F' increased saw temperature decreases, reinforcing the transient inverse relationship between measured net rates of photosynthesis and stomatal conductance. However, some sections of the leaf saw changes in F' with no corresponding change in temperature or vice versa.

No significant differences were found between Φ_{PSII} or F_v/F_m values across treatments either before or after leaf vein cuts, indicating that biochemical adjustments (non-photochemical quenching) were probably not a factor. F_v/F_m averaged 0.80±0.02; Φ_{PSII} before cut averaged 0.51±0.02: Φ_{PSII} 30 minutes after cut averaged 0.50±0.02.

Figure 4

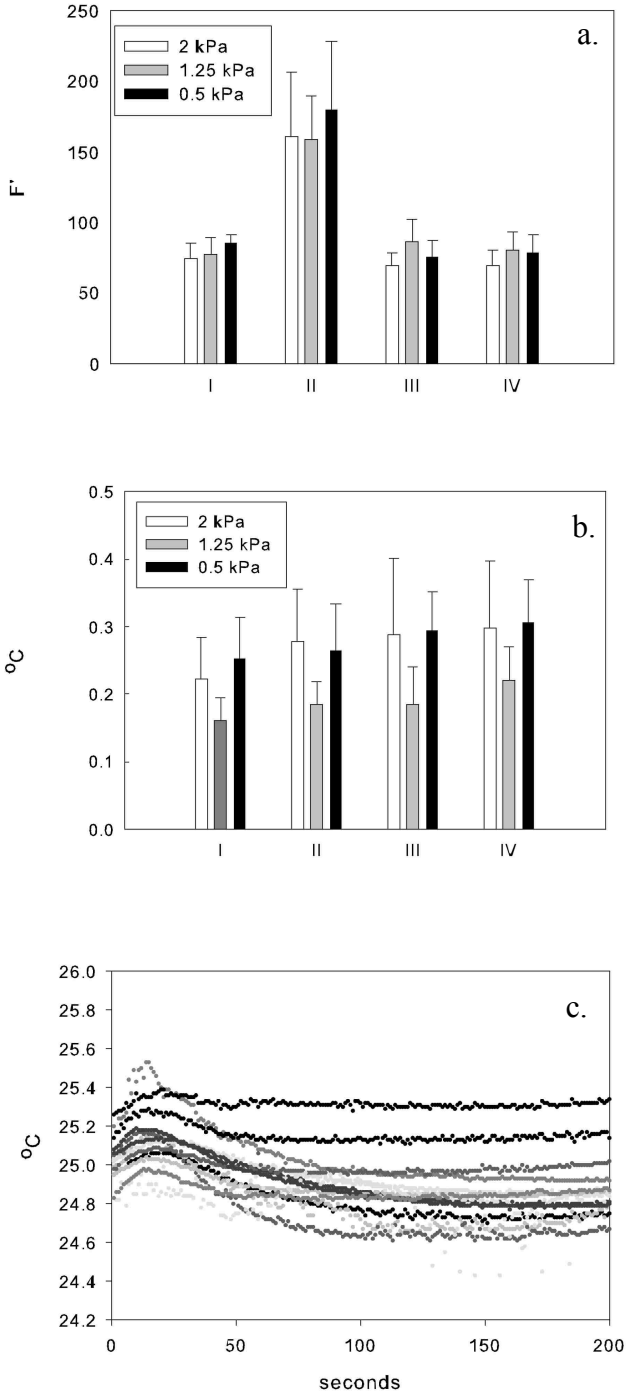


Fig. 4. Mean variation (standard deviation) of 13 leaf sub-areas of fluorescence signal (a) and temperature (b) at times I, steady state (pre-cut); II, peak of response; III, 300s; IV, 900s by VPD treatment (all error bars are 2SE), and response of temperature for 13 leaf sub-areas for example leaf (c) demonstrating that leaf temperature response was heterogeneous although mean variation did not significantly increase following the cut.

Most leaves responded with what could be described as a 3-phase response: 1.) 0s- peak of photosynthetic response, in which A and E are inversely related, in most cases, 2.) start of A recovery- peak of transpiration response (A and E are directly related) and 3.) start of E recovery- 900s (A and g_s are again inversely related, but the nature of the relationship varies widely between leaves; fig 5a).

Although individual leaf sub-areas also exhibited the 3-phase response characteristic to the net gas exchange response, no consistent relationship between temperature and fluorescence was found across the leaf, suggesting that net relationships observed in entire measured leaf areas are not representative of smaller scale responses (fig 5b).

Since the photosynthetic and stomatal responses were separated in time, regressions between extremes of fluorescence and temperature at any time were analyzed to assess the possibility that a time lag clouded the relationship between parameters. But of 18 leaves, only 4 were found to have significant negative correlation between temperature and fluorescence at a 5% confidence level and 1 leaf was found to have a significant positive correlation between temperature and fluorescence (also at the 5% level). Therefore, A and E were likely responding independently and at different rates to the treatment, creating the appearance of a relationship that varied through time.

Figure 5

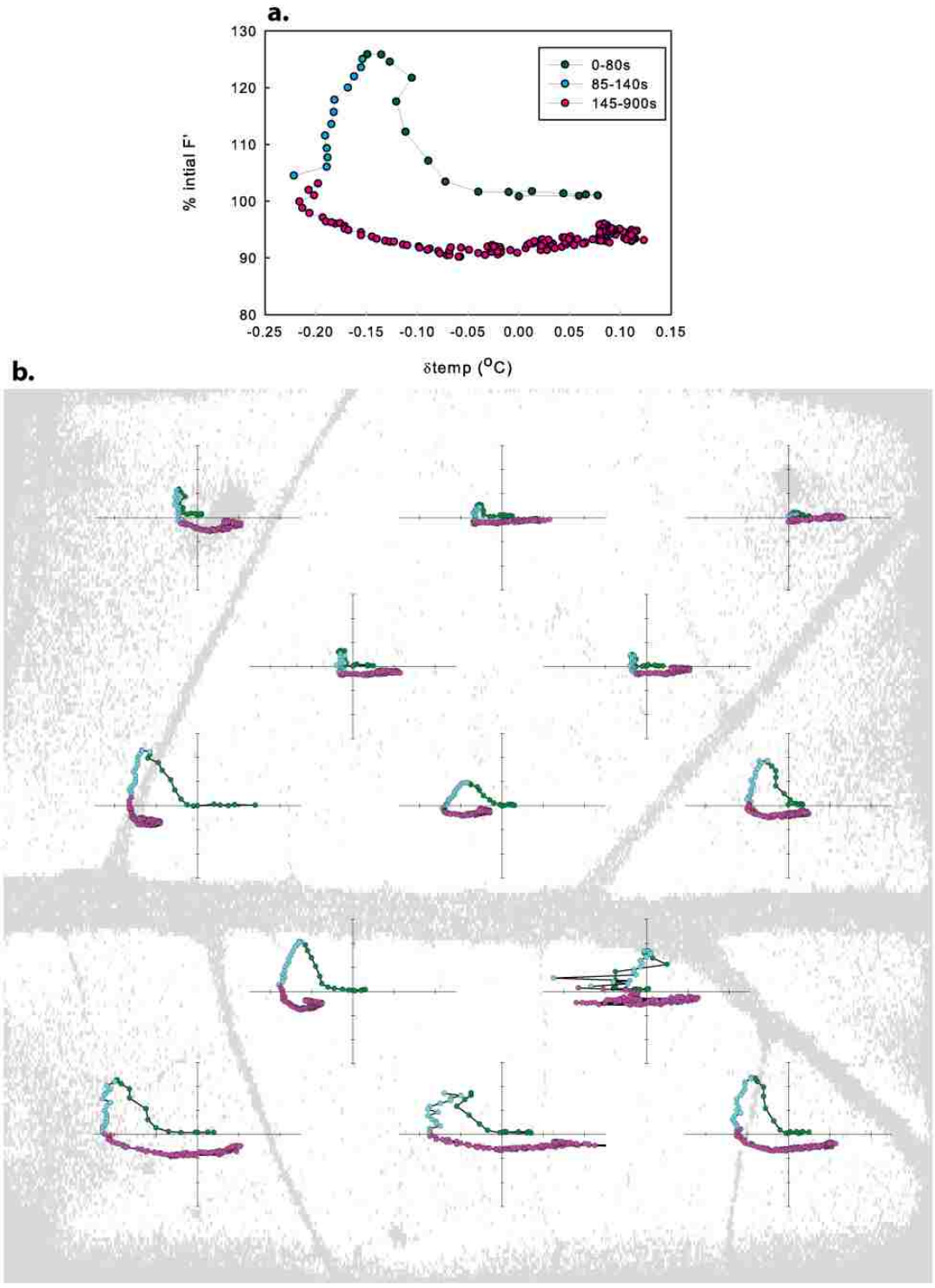


Fig. 5. Relationship between δF' and δtemp when averaged across leaf (a) and when averaged in leaf sub-areas (b) for example leaf in 1.25 kPa VPD treatment.

VPD Treatment Comparisons

Steady state

At steady state (pre-cut), the three VPD treatments exhibited significantly higher E (p=.046 between 2 and 1.25 kPa, p=.008 between 1.25 and 0.5 kPa, and p=.002 between 2 and 0.5 kPa VPD treatments) and lower g_s, (p=.07 between 2 and 1.25 kPa (at 10% confidence level), p=.004 between 1.25 and 0.5 kPa, and p= .001 between 2 and 0.5 kPa VPD treatments) with increased VPD (Fig 6). Therefore leaves in higher VPD treatments most likely experienced lower water potentials. At steady state, leaves in the 2 kPa, 1.25 kPa, and 0.5 kPa VPD treatments averaged E of 10.8 (±3.2), 8.8 (±0.8), and 7.3 (±0.9) mmol H_2O $m^{-2}s^{-1}$ and g_s of 0.4 (±0.11), 0.61(±0.12), and 1.18 (±0.29) mol H_2O $m^{-2}s^{-1}$, respectively.

Average initial photosynthetic rates were nearly identical for leaves in both the 1.25 kPa and 0.5 kPa VPD treatments in spite of differing stomatal conductance (21.6μmol CO_2 $m^{-2}s^{-1}$ ±2.7 and 3.1, respectively), indicating that photosynthesis was likely limited by RuBP regeneration, not CO_2 diffusion rates through stomata. Leaves in the 2 kPa VPD treatment averaged lower initial A (18.4 μmol CO_2 $m^{-2}s^{-1}$ (±3.2) (significant at 10% confidence level: p= .08 between 2 kPa and 1.25 kPa and p= 0.096 between 2 kPa and 0.5 kPa VPD treatments), probably due to carbon limitation caused by lower stomatal conductance.

Magnitude of response

No significant differences in the average decrease in A or increase in average F' were detected among VPD treatments (Fig 7). Following the cut, A dropped to an average of 9.5 (±6.4), 12.7 (± 3.2), and 12.6 (±4.5) μmol CO_2 m^{-2} s^{-1} which was an average change from steady state of -7.75 (± 5.2), -8.87 (± 1.3), and -8.9 (± 5.3) μmol CO_2 m^{-2} s^{-1} for the 2, 1.25, and 0.5 kPa treatments, respectively. % initial F' averaged 122.69 (± 14.8) %,

Figure 6

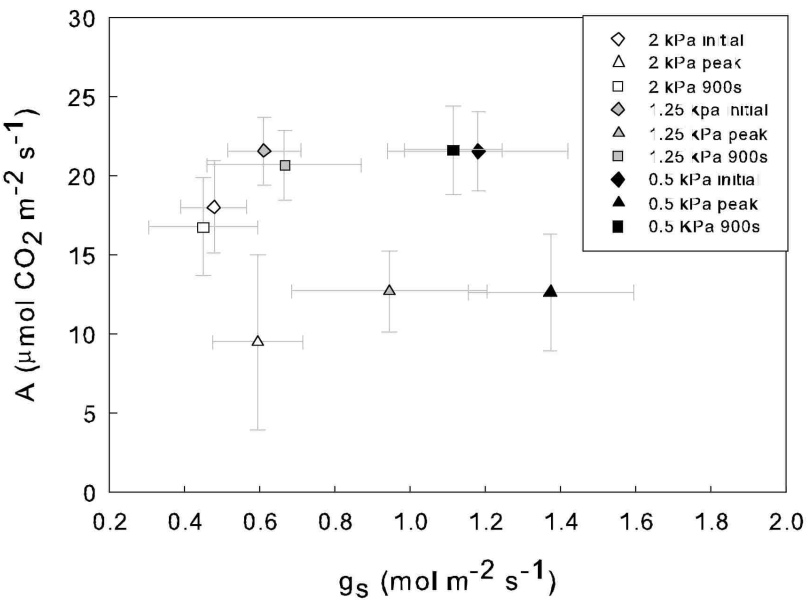


Fig. 6. Relationship between mean photosynthetic rate and mean stomatal conductance by treatment. "Peak" is the greatest change for each parameter.

Figure 7

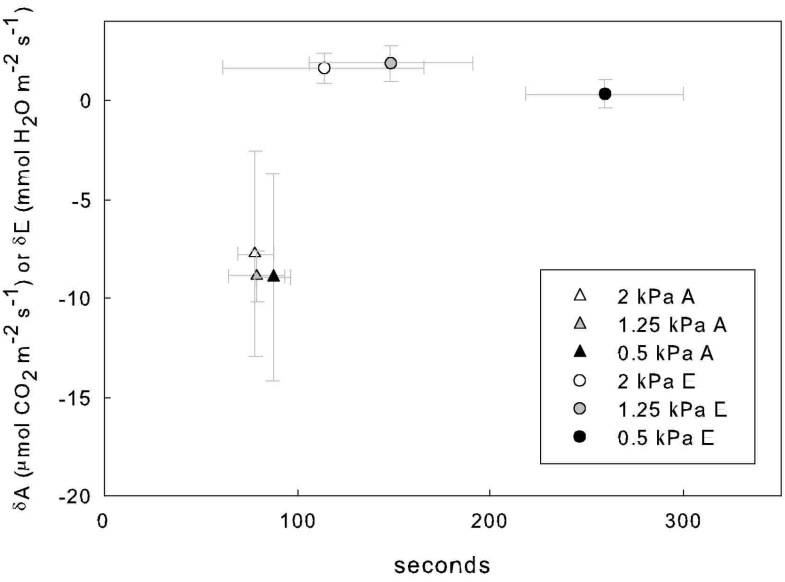


Fig 7. Relationship between magnitude of gas exchange responses and time of response peak.

124.94 (± 5.8) %, and 126.15 (±17.3)% for the 2, 1.25, and 0.5 kPa VPD treatments, respectively.

Increases in mean E were significantly higher for leaves in both the 2 and 1.25 kPa VPD treatments than leaves in the 0.5 kPa VPD treatment (p-value=0.04 and 0.008, respectively). The E response varied widely for leaves in the 0.5 kPa treatment where the average E increase was not statistically different from 0. The 1.25 kPa VPD treatment averaged the greatest transpiration rate increase among humidity treatments, so average E increase in response to the cut did not vary linearly with VPD treatment, but the difference between the E increase in the 1.25 kPa and 2 kPa treatments was not significant. (However the average estimated g_s increase for leaves in the 1.25 kPa treatment (0.33 ±0.2 mol H_2O $m^{-2}s^{-1}$) was found to be significantly greater (p=.05) than the average 2 kPa g_s increase (0.13 ±0.2 mol H_2O $m^{-2}s^{-1}$.) The average leaf temperature also followed this pattern, although no differences in temperature between VPD treatments were found to be statistically significant. E increased an average of 1.65 ±0.8, 1.90 ±0.9, and 0.34 ±0.7 mmol H_2O $m^{-2}s^{-1}$ and leaf temperature decreased an average of -0.21 ± 0.19 ^oC, -0.26 ±0.12 ^oC, and -0.14 ± 0.09 ^oC for the 2 kPa, 1.25 kPa, and 0.5 kPa VPD treatments, respectively.

Relative to steady state, leaves increased transpiration rates by 116.2 ±6.9 %, 120.9 ±9, and 105.3±10.2 % a significantly greater proportional increase in the 1.25 kPa treatment than the 0.5 kPa treatment (p=.02).

Initiation of response recovery

Across VPD treatments, there were no differences in the average timing of the minimum photosynthetic rate, as measured by either gas exchange or fluorescence (A rates were lowest at 61 ± 12 s, 63 ±13 s, and 68 ±9 s after the cut for the 2kPa, 1.25kPa, and 0.5kPa treatments, respectively). In contrast, the time it took the transpiration rate to reach its peak following the cut increased with decreasing VPD although with more relative variability than the photosynthetic response (18% in the A response vs 56% in the E

response between all leaves). E peaks were reached at 76 ± 41 s, 113 ±31 s and 239 ±41 s after the cut for the 2 kPa, 1.25 kPa, and 0.5 kPa VPD treatments, respectively. Average E peaks of leaves in the 0.5 kPa VPD treatment were significantly later than both the 2 kPa and 1.25 kPa VPD treatments (p= .00004 and p=.0003, respectively) Leaves in the 2 kPa VPD treatment averaged E peaks over 35s before those in the 1.25 kPa VPD treatment, a difference that was almost significant at a 10% confidence interval (p=.11). No significant differences between average time of lowest average leaf temperature were found between treatments, although they followed a similar pattern.

Contrasts between spatial heterogeneity of photosynthetic and stomatal conductance rates following a leaf vein cut further suggest that A and E responses are not the result of the same mechanism. In all VPD treatments F' heterogeneity increased transiently and was significantly greater at the peak of each leaf's average F' response than at any other time (p= 0.01, 0.002, 0.01 for 2 kPa, 1.25 kPa, and 0.5 kPa VPD treatments, respectively; Fig. 4a). Measurements of local temperature changes suggest stomatal response to the treatment was non-uniform across the leaf area (Fig. 4c), but average steady state heterogeneity of temperature was not significantly different from heterogeneity at time of peak F' (Fig. 4b; or time of average temperature minimum; data not shown).

By 300s, photosynthesis had returned to pre-cut uniformity across the leaf, while the degree of stomatal heterogeneity still showed no significant change. In fact, no significant change occurred in temperature heterogeneity at any time in any VPD treatment with the exception steady-state (pre-cut) and 900s within the 1.25 kPa VPD treatment (p= 0.4).

No significant differences in average fluorescence heterogeneity were found between VPD treatments at any time before or after the cut, but treatments did differ in average temperature heterogeneity. Temperature heterogeneity of leaves in the 1.25 kPa VPD treatment was often significantly lower than that of leaves in other VPD treatments at the same times. Significant differences in average temperature heterogeneity were found between VPD treatments 1.25 kPa and 0.5 kPa VPD treatments before leaf vein cut (p value=0.012), between 1.25kPa and both 2 kPa (p value=0.034) and 0.5 kPa (p

Figure 8

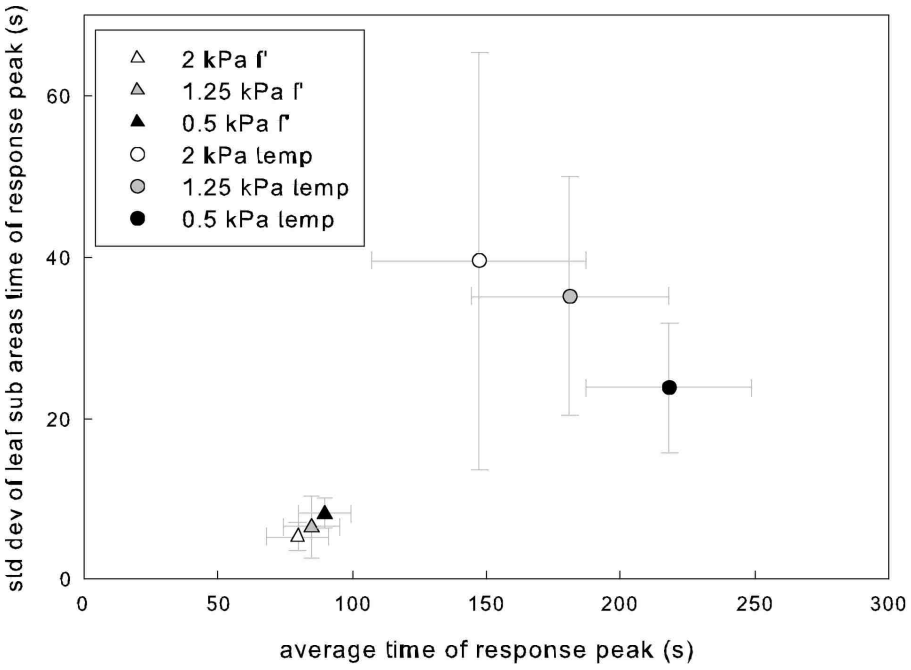


Fig 8. Relationship between mean variation (standard deviation) of time of response peak across leaf sub-areas and mean time at which response peak occurred by VPD treatment for fluorescence (Δ) and temperature (o).

value=0.036) VPD treatments at the peak of the response, and between 1.25 kPa and 0.5 kPa VPD treatments at both 300s(p value= 0.007) and 900s (p value=0.026).

Heterogeneity of response recovery initiation

Much more variability in the times of peak responses was found in temperature than in fluorescence across each leaf (Fig. 8), demonstrating that the initiation of photosynthetic recovery was more temporally coordinated than that of the stomatal response. The average standard deviation of fluorescence in 13 sub-areas averaged across all leaves was 8 ± 3 s, while the standard deviation of temperature for these same sub-areas averaged 32 ±11 s, a significantly different spread (p=.00001).

Furthermore the temperature response recovery times showed a trend towards greater variability between sub-areas as VPD increased, although these differences were not significant due to the small sample size and variation among individual leaves. Standard deviations of times of temperature lows (interpreted as just before stomatal aperture began to decrease) across the leaf averaged 40 ± 26 s, 35 ± 15 s, and 24 ± 8 s for 2 kPa, 1.25 kPa, and 0.5 kPa VPD treatments, respectively. The fluorescence response recovery time varied very little with VPD treatment. Standard deviations of fluorescence response peak times were 5 ± 2 s, 7 ± 4 s, and 8 ± 2 s for 2 kPa, 1.25 kPa, and 0.5 kPa VPD treatments, respectively. The increase in the variability of response recovery times with increasing VPD that was observed in the temperature response would be expected if the mechanism at work were hydraulic. The spread between hydraulic flow rates across a leaf should increase with evaporative demand since differential conductance in different hydraulic pathways to leaf sub-areas would be magnified as the driving force increased.

Recovery

By 900 seconds, all leaves had recovered and gas exchange rates were at or near pre-cut values regardless of VPD treatment. No significant differences in gas exchange recovery rates were detected between VPD treatments at 300 s. Although, by 900s, the average A in leaves in the 0.5 kPa VPD treatment of 100.2 ± 2.9 % pre-cut rate was significantly higher (p-values =.01 and .02 for 2 kPa and 1.25 kPa VPD treatments, respectively)

than that of other treatments (92.2 ± 5.04% and 95.8 ± 2.8% pre-cut A in 2 kPa and 1.25 kPa VPD treatments, respectively.

DISCUSSION

Surprisingly, cutting a primary leaf vein caused opposite responses in transpiration and photosynthesis in the region of the leaf closest to the affected vein. This pattern, transient increases in transpiration and concomitant decreases in photosynthesis, was consistent across measurements at three levels of VPD. Concurrent spatially-explicit measurements of leaf temperature and chlorophyll a fluorescence showed that the responses measured by gas exchange were the net effect of underlying variation across the leaf, but that the inverse response of photosynthesis and stomatal conductance was common in many leaf sub-areas. Leaves in all treatments exhibited rapid recovery, with E and A returning to within 10% of steady state (pre-cut) values within 15 minutes of the treatment that initiated the response. These data suggest that the increase in transpiration was due to changes in water potential following the cut but that the photosynthetic response reflects a non-stomatal limitation triggered by the treatment. Evaluating the basis of the non-stomatal limitation of assimilation will require more detailed analysis of magnitude and spatial extent of water potential changes and action/variation potentials triggered by the treatment.

Inverse response of photosynthesis and stomatal conductance and heterogeneity

Depression of leaf photosynthesis observed after our vein cutting treatment was not the direct result of stomatal limitation of diffusion of CO_2 from the atmosphere. At the same time that photosynthesis was decreasing, stomatal conductance increased significantly. Independent spatially explicit measurements of leaf temperature indicated that temperature decreased in many leaf sub-areas where fluorescence increased, although no consistent relationship (negative or positive) between the magnitude of the responses was found (Fig. 2b, c). Additionally, VPD had no effect on the photosynthetic response to the cut but did affect the magnitude and timing of the E response and recovery, indicating a hydraulic component in the response and recovery of stomata that was not evident in the photosynthetic response (Fig. 3).

Chlorophyll-a fluorescence imaging has been used as a non-destructive way to detect and record dynamics of heterogeneous behavior across the leaf that cannot be measured by gas exchange methods. Previous studies have assumed that uneven increases in fluorescence images were caused by CO_2 limitation resulting from stomatal closure and have equated this patchiness with an image of stomatal patchiness. Using thermal imaging, West et al. (2005) demonstrated that fluorescence patchiness measured following changes in humidity was in fact caused by stomata, since areas of increased F' values correlated with increased temperature. But in the present study, although temperature data did suggest non-uniform stomatal conductance in response to vein-cutting, fluorescence heterogeneity also observed following the treatment was not indicative of a resulting differential stomatal limitation.

Wrong way stomatal response: sudden decrease in water potential

Stomatal conductance (g_s) increased immediately following the treatment, indicating that stomatal aperture increased. This change occurred faster than osmotic potentials could change actively, and because the manipulation of the hydraulic architecture supplying the measured leaf area occurred at the leaf, it is unlikely that signals from the subtending stem or roots played a role. Therefore the most likely explanation for the stomatal response is a passive effect of sudden Ψ changes produced by the cut.

A similar transient increase in E and g_s has also been observed in experiments in which a whole leaf was excised at the petiole. Described as the "wrong way" stomatal response, it is thought to be an effect of sudden loss of turgor pressure in subsidiary epidermal cells, releasing pressure on guard cells and increasing aperture (Darwin and Pertz, 1911; Willis, et al., 1963; Raschke, 1970). Raschke (1970) described a stomatal response on a time scale similar to that observed in the present study in which a decrease in xylem Ψ of *Zea Mays* was transmitted to stomata in 0.1s, causing stomatal conductance to increase. Willis, et al. (1963) reported that in *Vicia faba* leaves, both the magnitude of initial stomatal opening and the time required to reverse the effect increased with leaf water potential. The positive relationship between pre-cut water status (assuming higher water potentials in leaves in higher humidity) and the time it took stomata to begin closing was

also observed in the current study, but the relationship between magnitude of initial stomatal opening and VPD treatment was more complicated. Increases in g_s were significantly larger in the 1.25 kPa than 2 kPa VPD treatment ($p=.049$; Fig. 6). Because of the large variability of the g_s response in the 0.5 kPa VPD treatment, the average stomatal response in this treatment did not differ significantly from either the 2 kPa or the 1.25 kPa VPD treatments. In fact, average g_s change in leaves of the 0.5 kPa VPD treatment was not statistically different from 0. This could be explained if stomata in the high humidity treatment were fully open at steady state. Guard cells may not have been able to open further, even with the pull of subsidiary epidermal cells as Ψ_L decreased.

Since stomata respond to the changes in turgor that were almost certainly caused by the disruption to the leaf water supply caused by the leaf vein cut, it is likely that the "wrong-way" stomatal response observed in the present study was triggered by a similar sudden decrease in epidermal Ψ. While a transient increase in water potential would have occurred initially in some parts of the leaf, this effect must have been negligible to the overall stomatal response. Furthermore, the estimated volume of water that would have been temporarily released from tension (contained in the cut vessels) would not have accounted for the transpiration rate increase observed following the cut.

The rapid recovery of E in the first 900 seconds after the vein was cut suggests that the hydraulic conductance of alternate flow paths in sunflower was sufficient to restore transpiration. Nardini, et al. (2001) found high redundancy in leaves of *Prunus laurocerasus* such that the leaf midrib contributed relatively little to overall leaf conductance, suggesting that hydraulic pathways within some leaves may be in parallel with each other, rather than strictly in series. Although sunflower leaves have two large veins in addition to the mid-rib, which may provide some redundancy in major vein distribution, it is assumed that an interruption of the mid-rib would increase the distance water must travel via non-vascular pathways of greater hydraulic resistance to the sites of evaporation.

The time at which transpiration rate reached its maximum could be interpreted as the time at which stomata reverse direction following their initial "wrong way" response to the vein excision- -the point at which re-hydration from alternative pathways begins. Re-hydration was found to occur sooner but with greater rate variability with higher VPD (Fig 8). This was probably because greater evaporative driving force would increase the rate of water movement through alternative pathways, but as water found alternative pathways of differential hydraulic conductance to move through, conductance would have a greater effect on the rate of water movement, and so the rate of recovery, as driving force (VPD) increased.

Possible scenarios for photosynthetic decline

While the observed changes in g_s and E are consistent with stomatal responses to a perturbation of hydraulic architecture and water potential, the mechanism responsible for the transient decrease in assimilation is more difficult to explain. What triggered the temporary photosynthetic decline when the observed change in g_s should have increased conductance of CO_2 to photosynthetic tissues?

Photosynthesis can be metabolically limited at low Ψ_L as a result of depressed ATP synthesis, RuBP regeneration or Rubisco activity (Tang, et al., 2002; Parry, et al., 2002). . However, impaired photosynthetic metabolism has been measured only when cell turgor loss is severe (Bota, et al., 2004), which is unlikely to have been the case in the extremely transitory response to this treatment. Increased non-photochemical quenching has been observed in sunflower when water stress is less severe, but it has been associated with stomatal closure (Tezara, et al., 2008), which did not occur in this study.

Photosynthesis has been found to decline following tissue injury. Electrical potentials transmitted from the site of injury are thought to suppress photosynthesis by increasing the pH gradient, depressing enzyme activity in cell walls (Davies, 1987; Bulychev and Kamzolkina, 2006). A sudden and transient decline in photosynthesis was observed in leaflets of mimosa and poplar trees in response to flame induced wounding (Koziolek et al., 2003; Lautner et al., 2005). In both cases, the decline in photosynthesis was

associated with a measured change in electrical potential, although it was inconclusive whether the signal was a direct result of wounding or initiated by a hydraulic signal (Malone, 1994). A chemical signal released by the cut could also explain A decline, although Koziolek, et al. (2004) and Lautner et al. (2005) eliminated the possibility of a hydraulically independent chemical signal in mimosa and poplar, respectively, based on the comparatively slow rate of translocation through phloem. The photosynthetic depression observed in sunflower occurred on a similar time frame as that measured in mimosa and poplar. A chemical signal transported through xylem also seems unlikely since the photosynthetic response and its propagation varied little with initial transpiration rate.

Initiation of decoupled response

The observed decoupling of the rates of photosynthesis and transpiration could indicate that either 1. the two processes responded differently to the same (unified) signal or 2. each process ultimately responded to a separate signal that differed in transmission path and rate (two signals).

unified signal

The decline in A could have been triggered by the sudden decrease in xylem water potential transmitted to cells that must have caused the increase in stomatal conductance following the cut, but the mechanisms that caused each response might have occurred at different rates. For example, metabolic inhibitions might depress photosynthesis before a decrease in epidermal turgor could close stomata accounting for A declining faster than E increased. However, data from the current study do not support this hypothesis. No relationship was found between the magnitudes of the photosynthetic and transpiration rate changes in leaf sub-areas; And in some sub-areas, only one parameter was found to respond to the cut. Leaf hydraulic architecture could cause differential hydraulic resistance between leaf xylem and non-vascular pathways (Tyree, et al., 1981; Salleo, et al. 2000; Trifilo, et al., 2003) and could be responsible for the non-congruent responses of A and g_s observed. But heterobaric species (like sunflower) have bundle sheath extensions which can function as hydraulic conduits, directly connecting vascular tissue

to the epidermis (McClendon, 1992; Pieruschka, et al., 2005) and separating leaf regions between the extensions. This anatomy suggests that in sunflower the transpiration stream would be linked more directly to the epidermis than the photosynthetic mesophyll, a type of hydraulic partitioning that would buffer mesophyll cells from sudden Ψ change (Zwieniecki, et al., 2007) and would not have resulted in the immediate response of photosynthesis observed here if the trigger were water potential alone. Furthermore, g_s was affected by VPD treatment, A was not, suggesting that A did not respond to the same hydraulic signal.

Koziolek et al. observed a concurrent "opposite" stomatal response in mimosa leaves, similar to what was observed in sunflower in this study. Kaiser and Grams (2006) re-visited the work of Koziolek, et al. and attributed the phenomenon to the "wrong way" response described above, suggesting that epidermal cells lost turgor in response to wound initiated e-potential signals by a mechanism thought to occur in the specialized pulvinar extensor cells responsible for leaf movement in mimosa and concluded that stomata were responding indirectly to the electrical signals. Although a similar net effect in gas exchange was observed in sunflower following mid-rib cutting as was observed in *Mimosa pudica* after flaming a neighboring leaflet, local measure of g_s in the present study (the thermocouple array) showed the spatial pattern of stomatal response to be different from that of photosynthesis, suggesting that change in stomatal behavior is not the indirect result of the same signal affecting photosynthesis.

Two signals

The observed spatial and temporal differences between the responses of A and E could occur if the cut produced two separate signals, both initiated by vein-cutting, which propagated independently across the leaf. Based on the similarity of the A response we observed and other A responses attributed to e-potential, the most likely second signal is an electrical signal, either initiated by the wound itself or hydraulically triggered

Electrical signals, propagated as variation potentials (VP), have previously been detected in sunflower in response to flaming and light induction (Stankovic, 1998) and were found to directly follow sudden pressure increases in the xylem. In the present study, xylem Ψ

would have risen to 0 (atmospheric) at the site of the cut, regardless of transpiration rate and downstream resistance of cells and stomata. The magnitude and propagation of a resulting VP would have been similar for all VPD treatments. Once initiated, a VP can be transmitted to cells lateral to affected xylem through plasmodesmata and into the phloem pathway (Lautner et al., 2005), until the signal fades with time and distance from the point of stimulation. The short time frame, transience, and pattern (which radiated from main leaf veins) of photosynthetic response observed in sunflower, are consistent with the manner in which variation potentials travel through tissue (Fromm, 2007). On the other hand, the water potential of epidermal and guard cells would be most affected by hydraulic resistances and evaporative demand. The pressure increase at the excision site would be somewhat buffered by progressively lower k of the hydraulic pathway, as transpiration pathway size decreases and/or water moves into living cells near the site of evaporation (Sack and Holbrook, 2006). The water potential of stomata and epidermis might have primarily decreased as the upstream hydraulic supply was interrupted and xylem resistance increased. The trigger for both the photosynthetic and stomatal responses could have been sudden local changes in leaf water potential, but A might have responded to VP caused by a transient *increase* in Ψ, while g_s responded to a relative *decrease* in Ψ of subsidiary epidermal cells causing the two separate but simultaneous responses observed in sunflower following a leaf vein cut. However data from the present experiment are not sufficient to determine if or how an electrical signal was initiated.

Conclusion

The unexpected results of this experiment demonstrate short-term uncoupling of photosynthesis and stomatal conductance. More data, including cell water potential and e-potential measurements, are needed to elucidate the mechanisms responsible, although it is likely that two separate mechanisms were initiated by the primary vein cut, causing spatially and temporally different responses of A and g_s. Transient physiological adjustments to perturbations of hydraulic flow such as these must be considered to develop a more comprehensive picture of stomatal regulation of leaf gas exchange and how it is related to plant water status.

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