Drought stress and fluctuating asymmetry in *Quercus undulata* leaves: confounding effects of absolute and relative amounts of stress?

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Abstract

One of the most general types of stress experienced by plants is water-limitation, which becomes particularly pronounced during periods of drought. We evaluated fluctuating asymmetry (FA) in *Quercus undulata* leaves for two subsequent dry years: 2001, when precipitation was 25% below average, and 2002, when precipitation was 65% below average, from a plot receiving ambient water and one in which water was excluded. In the first and less severe drought year, ambient-water trees had a slightly higher index of FA than the water-exclusion trees, contrary to expectations. However, in the second and much more extreme drought year, water-exclusion trees exhibited greater FA as expected, but in additional observations water-supplement trees exhibited by far the greatest amount of FA, contrary to expected. Further data on plant water potential confirmed that degree of plant stress corresponded to plot treatments: water exclusion > ambient water > water supplement. Stable carbon isotope ratios indicated that trees on the water-supplement plots were less stressed than ambient-water and water-exclusion trees, and leaf size was much greater for water-supplement trees than ambient-water or water-exclusion trees. We

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hypothesize that the complexity of the results could be due to the confounding effects of relative vs. absolute stress.

1. Introduction

Evaluation of fluctuating asymmetry (FA) of an organism's morphology is rapidly emerging as an important tool for evaluating biological responses to stress. Leaves can be highly sensitive organs of trees as they are subject to the environment and phonological growth cycles. Deciduous plants with new leaves developing every year offer the repeated chance to investigate the developmental stability of that plant for that current growing season. The highest level of stability has been observed under conditions that are optimal for the existence of that species (Zakharov, 1982) where developmental stability is reduced under conditions of environmental and genetic stress (Zakharov, 1987). FA or the deviation in growth patterns in bisymmetrical organisms is one of the most common indicators of developmental stability. Indeed, developmental stability has proved to be a useful tool for assessing the impacts of environmental stress on organisms, particularly for stress-adapted plants (Alados et al., 2001). Specifically, fluctuating in plants has been found to be correlated with such potential stressors as urbanized areas (Sherzhykova et al., 2002), contaminated sites (Møller, 1998; Chistyakova and Kryazheva, 2001), serpentine soils (Alados et al., 1999), herbivory (Lempa et al., 2000; Díaz et al., 2004), and climate (Hódar, 2002; see also Møller and Shykoff, 1999). Developmental stability, in turn, has been shown to be directly related to fitness in plants by increasing pollinator preference (Møller and Eriksson, 1995; Møller and Sorci, 1998). However, Hochwender and Fritz (1999), Martel et al. (1999), and Lempa et al. (2000) found in *Salix* and *Betula*, respectively, that FA increased under optimal growth conditions due to the rapid growth rate that triggers developmental instability. This could undoubtedly lead to misleading conclusion about stress if an increase in FA was found in a population of trees in the field. Therefore, we might expect an increase in FA in trees that either experiences a short and rapid growing season in optimal conditions or that experiences stress with a shorter growth spurt. However, Kozlov (2003) found no correlation between FA and growth rate in *Betula pubescens*.

One of the most general types of stress experienced by plants is water-limitation, which becomes particularly pronounced during periods of drought. The very definition of drought accepted by many includes a reduction in plant growth due to a historical decrease in average precipitation amounts (Tucker and Goward, 1987). Drought can be a very important environmental factor, especially in the arid south-western North America where it occurred in 43% of a 40-year period following 1944 (Holechek et al., 1989). Extreme and short-term drought has also been found to cause entire and long-term ecotone shifts of forests (Allen and

Breshears, 1998). Water shortage can cause plants to reduce their metabolic activity, causing a decrease in photosynthesis, carbon fixation, and ultimately, growth (Depuit and Caldwell, 1973; Younis et al., 1993; see also Møller and Shykoff, 1999 for review of developmental stability in plants).

During periods of drought, most plants are likely to be under stress but differences in the degree of stress can have important implications for plant survival and function. However, few studies to date have evaluated responses in FA to various degrees of water stress associated with drought. There are several metrics related to plant water stress that may be reflected by patterns of FA. Temporal dynamics of soil water and plant water potential are two such metrics that can indicate potential plant water stress and availability (Waring and Running, 1998). Additionally, stable isotope analyses of nitrogen and carbon in leave can provide valuable information about plant physiological status (Farquhar et al., 1989). Prolonged droughts restrict the mobility of N through dehydrated soils. Measurements of stable isotopes have been used in several studies of the response of plants to water stress (Robinson et al., 2000; Warren et al., 2001; DaMatta et al., 2002; see also Ehleringer et al., 1993 for a review). Warren et al. (2001) concluded that $\delta^{13}C$ might be useful as an indicator of water availability or drought stress, but only in seasonally dry climates. Another study found that shoot–root $\delta^{15}N$ may be a more sensitive indicator of stress than using $\delta^{15}N$ from the entire plant (Robinson et al., 2000).

Our objective was to test for morphological responses as measured by FA in leaves of a deciduous woody plant, *Quercus undulata,* in response to drought conditions. *Q. undulata* is widely distributed in south-western North America and northern Mexico. Following a dry year, we evaluated FA in leaves for two subsequent dry years: 2001, when precipitation was 25% below average, and 2002, when precipitation was 65% below average. For both years, we measured FA in leaves from a plot receiving ambient water and one in which water was excluded; in the second year we also measured FA on a plot receiving supplemental water. In support of our overall objective, we also obtained supplemental data on soil water content, plant water potential, leaf size, and C and $\delta^{15}N$ content and C^{13}ratios in leaves.

2. Materials and methods

2.1. Study site and plot design

Our study plot and transect were located within semi-arid piñon-juniper woodland stands dominated by *Pinus edulis* Englem. and *Juniperus monosperma* (Englm) Sarg at the Mesita del Buey site on Los Alamos National Laboratory, NM, USA (latitude 35.50°N, longitude 106.16°W). Our study species was *Q. undulata* Torr. Although *Q. undulata* is considered as a separate species by some, it is probably a hybrid of *Q. gambelii* and *Q. grisea* (Williams et al., 2001). All trees used in the experiment fell under the description of *Q. undulata* and were all similar in morphology. The dominant herbaceous species in these stands is *Bouteloua gracilis*, located primarily in intercanopy patches (Breshears et al., 1998). The elevation of the plots is 2140 m.

In general, Los Alamos has a temperate montane climate with four distinct seasons. Annual precipitation is ca. 40 cm of precipitation, mainly in the form of winter snowfall and late-summer precipitation. The mean ambient air temperature is ca. 9 °C, ranging from −2 °C in January to 21 °C in June (Breshears et al., 1998). The soils are Hackroy sandy loam, derived from volcanic tuff (Nyhan et al., 1978); soil depth varies from 33 to 125 cm (Davenport et al., 1996). This site has been the subject of many previous studies (e.g. Lajtha and Barnes, 1991; Breshears et al., 1997; Wilcox et al., 2003 and references therein).

In the first year of the study, we evaluated trees on two plots, referred to as ambient-water trees and water-exclusion trees. For water-exclusion trees, we installed a water barrier on the ground surface of a roughly 50 m × 50 m plot. Study trees were limited to the interior 30 m × 30 m of the plot, such that all trees had at least a 10 m buffer of water exclusion around them. The water barrier consisted of a ventilation system and a composite plastic sheet on the ground. The ventilation system was comprised of ~10 cm diameter perforated pipes that are ventilated along the sides. Every 5 m along the plot and parallel to the slope, running from the top of the plot downslope to the bottom (to encourage air flow), perforated pipe sections (3.05 m length) were fit together and placed on the ground surface such that the ventilation holes were not covered by ground. Rebar stakes were inserted into the ground and wired to the pipes to hold the pipes in place. We fabricated a composite ground cover of plastic sheeting that was white vinyl-coated polyester truck tarp (primarily 14 oz with a few sections of 18 oz; Shelton, Reynolds Inc., Milwaukee). The plastic sections were glued together with vinyl tarp glue in the field and over the ventilation tubes, with the ventilation tubes remaining exposed at the upslope and downslope ends of the plot. Special sections were fabricated for installation around the base of each woody plant. These pieces were generally sealed to the stems of the woody plants with a section of bungee cord. Prior to installation of the water barrier, time-domain reflectometry (TDR) probes for measuring soil water were installed in the intercanopy sections of each of eight of the nine 10 × 10 quadrats of the 30 m × 30 m central plot area. The soil water measurements were used to verify the effectiveness of the water-exclusion plot, which did not completely exclude all water inputs; no direct measurement of the amount of excluded water was obtained.

2.2. Measurements and analysis

We studied the *Q. undulata* trees that were located on and around the drought plot. The leaves of *Q. undulata* are alternate, oblong or elliptical, and the margins are serrated to undulated tapering to the base. At the end of the 2001-growing season (November), we located and marked twelve *Q. undulata* trees in both the ambient-water plot and the water-exclusion plot (Table 1). At the end of the 2002-growing season, we added trees on a water-supplement plot, which received direct runoff from a parking area and buildings. We randomly collected approximately 50 total leaves from all sides and heights of a tree during a given sampling period. Due to the unique shape of the oak leaves, a simple measurement between the leaf edge and vein could be misleading, we therefore focused on total leaf area. We selected and sliced

Table 1
Study design and treatment sample sizes

	Water supplement (*N*)	Ambient water (*N*)	Water supplement (*N*)
Pre-drought	None	12	12
Post-drought	4	12	12

30 of the 50 leaves with a razor blade in half along the leaf vein. Measurement error along the leaf vein was extremely small (0.01% of leaf area) and all leaves from all treatment locations were treated the same. Using a leaf area meter (LI-3100, LI-COR Inc., Lincoln, NE, USA) we calculated the leaf area of each half and repeated each measurement twice to estimate measurement error from the machine. We estimated the appropriate sample size of the number of trees for leaf water potentials for the detection of differences between the plots to be eight trees per plot for a greater than 90% detection probability.

The statistical effects of interest are the right vs. left side of the leaf, individuals, and repeated measurements. The mean square for the side × individual interaction includes measurement error and nondirectional asymmetry, which consists of antisymmetry and FA. Antisymmetry is distinguished from FA by producing a platykurtic or bimodal distribution, rather than a normal distribution, of the right–left difference around a mean of zero of FA (Palmer and Strobeck, 1986). Thus, antisymmetry, which represents the exaggerated development of one side or the other, can be detected by testing the right minus the left difference for normality. Measurement error is estimated directly.

Analysis of FA included the determination of measurement error by comparing the duplicate measurements of each leaf. The pattern of variation in the area of leaf sides between individuals and between right and left trees was quantified for each leaf by a two-way, mixed-model analysis of variance (ANOVA) according to Palmer and Strobeck (1986). Repeat measurements were used from each leaf on each side to estimate measurement error. Three statistical tests were used in this analysis: (1) F([tree∗leaf]/error) estimates the magnitude of nondirectional asymmetry (antisymmetry and FA), (2) F(tree/[tree∗leaf]) estimates the magnitude of size variation among individual trees, and (3) F(leaf/[tree∗leaf]) estimates the magnitude of directional asymmetry. Antisymmetry is expressed as platykurtosis in the distribution of right (*R*)–left (*L*) differences (Palmer and Strobeck, 1986) and is estimated. Accordingly, the *R*–*L* distributions were tested for each leaf 1–5 for normality. However, to avoid the mixing of multiplicative and additive measurement error, we computed the natural $\log E(l) - \log E(r)$ as recommend by Graham et al. (2003). This transformation did not change the results of the analysis when compared to untransformed data. The variance component *V* was also calculated for the leaves 1–30 of each individual as the square of the normalized difference of the left and right sides (Fair et al., 1999):

$$V = \left[\frac{2(R - L)}{R + L}\right]^2.$$

An FA index was then calculated for each individual as the square root of the variance components of each leaf one and five, which is a measure of the individual contribution to FA expressed as a decimal fraction of the average leaf area. Thus,

$$FA_j = \sqrt{\frac{\sum_{i=1}^{N} V_{ij}}{N}}.$$

FA for each tree was then analysed in a multi-way ANOVA in which the effects were treatment and year. We also determined whether FA was correlated with soil moisture and tree water potentials in analyses of covariance with treatment entered as an effect.

Soil moisture was measured by TDR (Topp et al., 1980) using a system similar to that described by Baker and Allmaras (1990). The TDR probes, each 30 cm in length, were installed vertically in the soil at each of the ambient-water sampling stations ($n = 30$) and on the water-exclusion plot ($n = 8$).

For the midday plant water potentials we collected oak twig samples from trees once per month for July, August, September, and October in 2002 from 12:00 to 12:30 p.m. Three twigs were collected per tree from each subject tree from different branches. These were sealed in locking plastic bags and kept cool and in the dark until analysed. The time between samples collection and analysis was kept to a minimum of under an hour. To confirm that plant water potentials did not change during the time from collection and analysis, we collected a subset of twigs ($n = 6$) for comparison with samples collected an hour previously and there was no difference ($F_{1,10} = 0.25$, $p = 0.62$) and there was no increase in variance from time from collection ($F_{1,10} = 0.48$, $p = 0.5$, Levene's test). Midday plant potentials were measured using a PMS model 1000 pressure chamber (Corvalis, OR, USA) to the nearest 0.05 MPa. Two twigs were used with the third twig being used in the event of damage to one of the original two during insertion into the pressure chamber. Using the same procedure, we also had one predawn collection for all trees on August 21, 2002.

For analysis of C and N stable isotope ratios, oak leaves were washed in detergent and thoroughly rinsed to remove all oil, dirt, and residual detergent. Oak leaves were then oven dried and a small amount (2.0–6.0 mg) was analysed for $\delta^{13}C$ and $\delta^{15}N$. The leaf was then analysed by elemental analyser—continuous flow—isotope ratio mass spectrometry utilizing a Micromass Isoprime located at Los Alamos National Laboratory.

3. Results

Precipitation for the year prior to the study, 2000, was 25% below normal. The drought continued over the 2 years of the study, with precipitation being 25% below normal in 2001 and 65% below normal in 2002. The drought of 2000–2002 in the south-western USA, although not unprecedented (Allen and Breshears, 1998), has

been one of the most severe in 50 years (Fig. 1). In 2001, all oak trees had leaves during the growing season. In 2002, there was a clear effect of the drought with 50% of the trees not budding and growing any leaves during the summer season. Sixty-six percent of the trees on the ambient-water plot and 33% of trees on the water-exclusion plot did not grow leaves in 2002. All of the trees in the added area with supplement water had leaves. This decrease in the number of trees that leafed out between the 2001 and 2002 was significant ($\chi^2 = 16$, $p < 0.001$).

The repeatability of leaf width measurements was 95% ($F_{15,435} = 6.76$, $p < 0.001$) for both years and the measurement error was less than 4.6%. We did not detect that skewness or kurtosis values differed from zero, indicating no directional asymmetry. For the 2001-growing season there was no difference in the FA (index of 30 leaves per tree) between the water-exclusion plot and the ambient-water plot (Fig. 2), the leaves of trees on the transect were larger than leaves on the water-exclusion plot ($F_{1,2878} = 341.27$, $p < 0.0001$). There was little variance in the FA of leaves within trees for 2001 ($F_{29,667} = 1.04$, $p = 0.41$). However, there was significant variation in FA between trees ($F_{23,667} = 3.18$, $p < 0.0001$). In 2002 when the drought was more severe, the total area of leaves varied for all three locations ($F_{2,1917} = 796.5$, $p < 0.0001$). In 2002, the water-supplement trees had significantly larger leaves, followed by the water-exclusion plot tree leaves, and then by ambient-water plot tree which had the smallest leaves. Similar to 2001, there was little variance in the FA of leaves within trees ($F_{29,435} = 0.84$, $p = 0.71$) and there was again significant variation in FA between trees ($F_{29,435} = 6.76$, $p < 0.0001$). For the FA indexes calculated for each tree there was heteroscedasticity between locations ($F_{2,37} = 4.05$, $p = 0.03$, Levene's test for homogeneity of variance). Numerous transformations did not eliminate the unequal variances, so locations and years were compared nonparametrically. In 2001, FA was slightly greater in the trees on the water-ambient plot ($\chi^2 = 8.17$, $p = 0.004$, Kruskal–Wallis) (Fig. 3). In 2002, the water-exclusion plot trees had a higher degree of FA than the water-ambient and exclusion plots ($\chi^2 = 9.08$, $p = 0.01$, Kruskal–Wallis) (Fig. 3). Within trees, there was no correlation with the size or area of a leaf and FA. Leaf size was the largest for the water-supplemented trees (Fig. 3B) and all three locations varied in leaf size
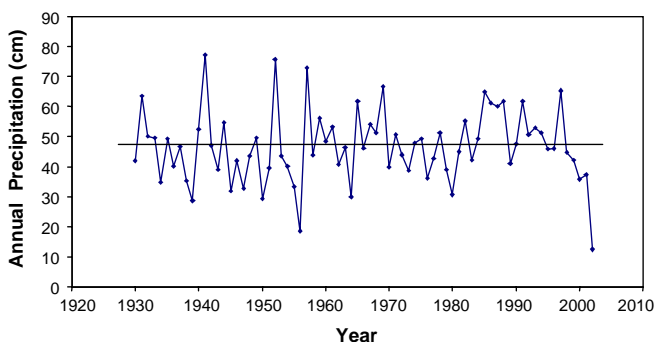

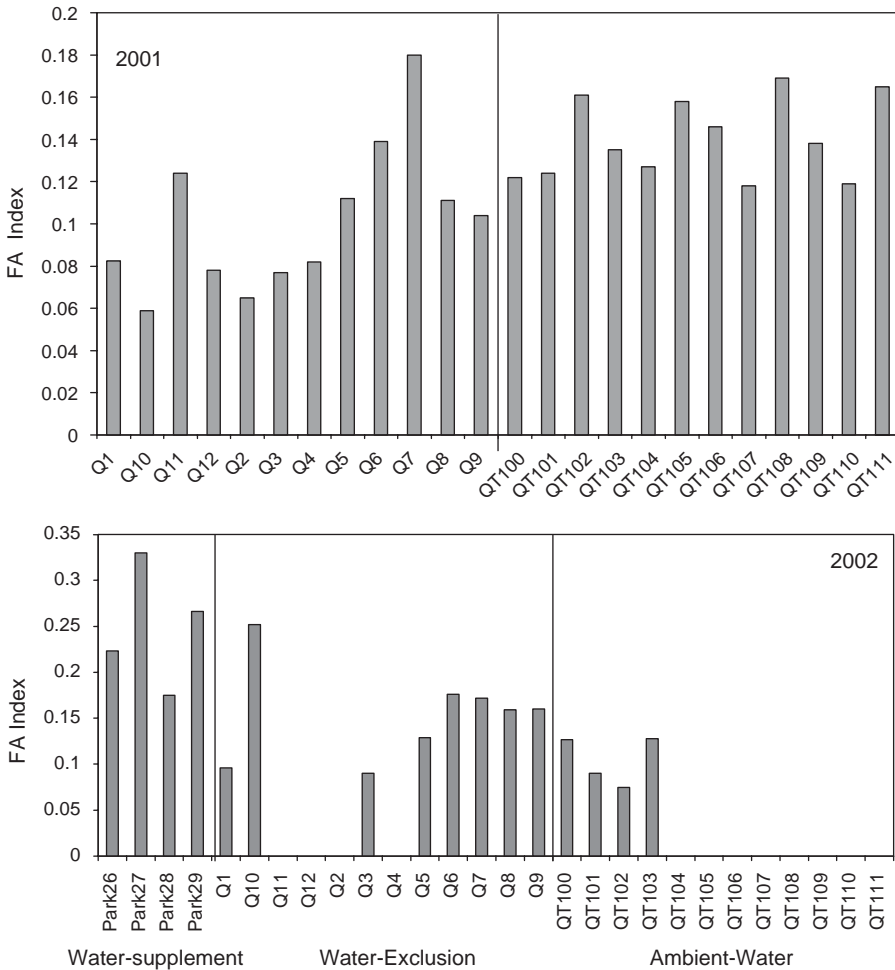Fig. 1. Annual precipitation for Los Alamos, New Mexico from 1930 to August 2002.

Fig. 2. FA (FA index) of 30 leaves per tree for the water-exclusion plot and ambient-water oak trees for the 2001- and the 2002-growing seasons.

($F_{2,477} = 186.9$, $p < 0.0001$). The water-exclusion tree leaves were the next largest and the ambient tree leaves were the smallest.

For the midday plant water potentials measured in 2002 there were differences between trees in the three plot areas ($F_{3,19} = 13.61$, $p < 0.0001$, repeated measures ANOVA) (Fig. 4). The water-exclusion plot exhibited the less negative midday water potentials, corresponding to less water stress, than the ambient-water plot in July (measurements on water-supplemented plots were not initiated until July). Overall, all three locations followed the same trend in August having the highest water potentials and then decreasing in September after some rainfall. Throughout the
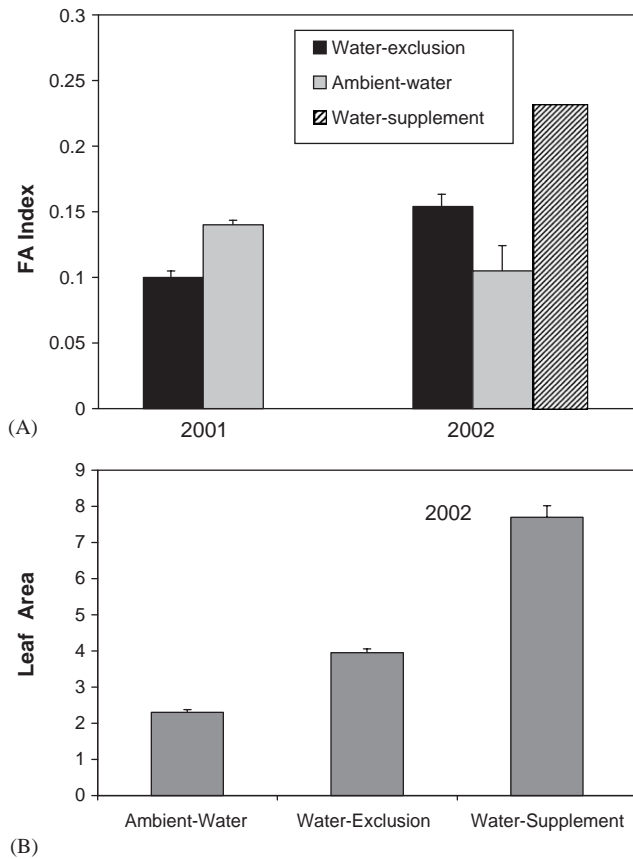
Fig. 3. (A) Mean FA (FA index) (+S.E.) of 30 leaves per tree for the water-exclusion plot and ambient-water oak trees for both the 2001- and 2002-growing seasons. No data for 2001 for the water-supplement area. (B) Mean total leaf area (cm) (+S.E.) for the plots for 2002 only.

summer, the water-supplemented trees that received direct runoff had significantly lower water potentials. There was no difference in heteroscedasticity for water potentials within collection times except for in the October of 2002 collection period ($F_{2,24} = 5.76$, $p = 0.009$, Levene's test for homogeneity of variance). Soil water content for both locations increased in September and followed the pattern of plant water potentials. However, as expected from the treatment the water-exclusion plot increased only slightly in September.

For percent C and N in the leaves no trees were different between the three treatments ($F_{2,8} = 0.47$, $p = 0.64$, $F_{2,8} = 1.2$, $p = 0.36$, respectively). There was also no difference in total amount of N in leaves between the treatments ($F_{2,8} = 1.8$, $p = 0.23$). However, $\delta^{13}C$ in the water-supplement tree leaves were less than both of the other treatments ($F_{2,8} = 9.5$, $p = 0.02$).
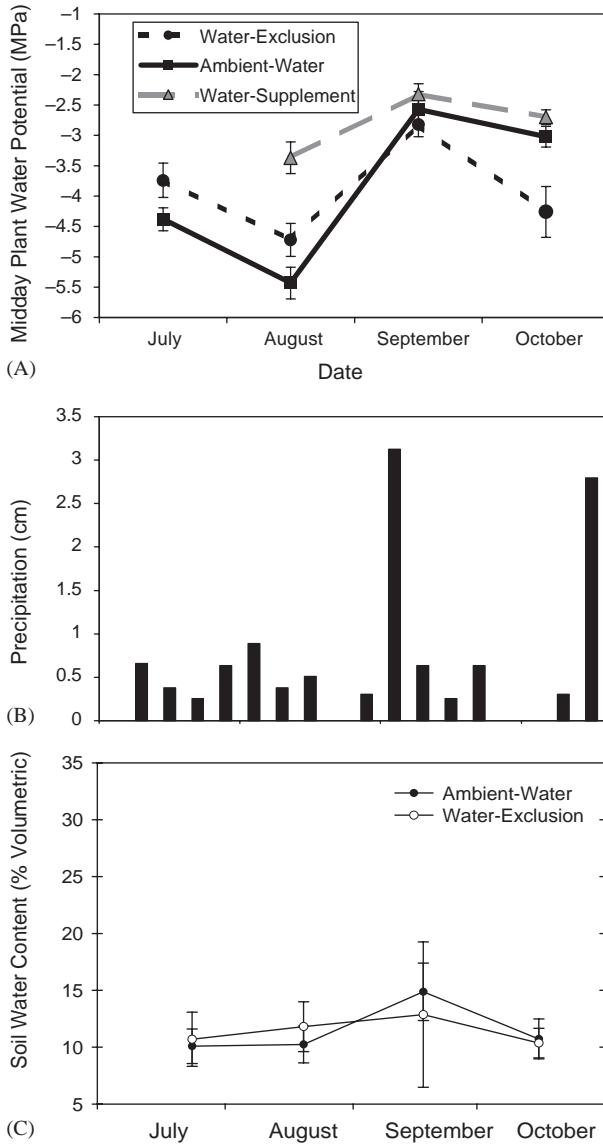
Fig. 4. (A) Midday plant water potentials for the summer of 2002 for the three tree locations. (B) Precipitation from July through October 2002. (C) Soil water content measured by TDR for water-exclusion and ambient-water locations for summer 2002.

4. Discussion

Our study contributes to quantifying the severity of the regional drought of 2002. A significant number of trees on both the treatment and the ambient-water plot did

not even grow leaves during 2002. No acorns were recorded for any of the trees, showing a direct effect of the drought on fitness. Plant water potentials also exceeded $-5.0\,MPa$, indicating a high degree of plant water stress. Thus, drought impacts were apparent both in terms of plant physiology, productivity, and fitness. We found no significant variation in FA in leaves within trees thereby suggesting that FA is an indicator of whole-plant response for *Q. undulata*. This finding in consistent with that of Hódar (2002) for the Holm oak (*Quercus ilex*), and contrasts with that of Cowart and Graham (1999), who found that FA in the fig (*Ficus carica*) depended on the height and position of the leaves in the tree. Hence, genera and species variation need to be considered in the use of FA for whole-plant response to stress. More generally, Bussotti et al. (2000) found that leaf mass per area explained the most variation in field performance in broadleaved trees under conditions of stress. However, our results are similar to the findings of Auslander et al. (2003), who reported no effect of slope orientation on leaf asymmetry, even though south-facing slopes receive six times higher solar radiation, and from Duda et al. (2003), who found no correlation with FA a disturbance gradient in plants. Lappalainen et al. (2000) also found that nitrogen enrichment increased levels of FA in birches that confirms that not only stress but also any deviation from normal resource availability may increase leaf developmental instability in plants.

Our results do not show clear consistent responses in FA to drought stress. For both years, there was little variation in FA within trees and substantial variation among trees, which yielded significant differences among plot types that varied by year. In the first and less severe drought year, ambient-water trees had a slightly higher index of FA than the water-exclusion trees, contrary to expectations (water-supplement trees were not evaluated this first year). However, in the second and much more extreme drought year, water-exclusion trees exhibited greater FA as expected, but water-supplement trees exhibited by far the greatest amount of FA, contrary to expected. Additional data on plant water potential confirmed that degree of plant stress corresponded to the expected plot treatments: water exclusion > ambient water > water supplement, adding further support to our conclusions even though we cannot fully discount other secondary factors or differences associated with the drought or supplement plots.

While it is true that water was for the most part kept from trees to soil water uptake, other species of trees were found to exhibit foliar absorption (Breshears unpublished data). Foliar absorption could be particularly important for plant in drylands where plants are commonly stressed and where precipitation frequently occurs as small events that wet foliage without substantially increasing available soil water. This ability to absorb water through foliage could have allowed trees continual growth on the restricted water plot.

In addition, stable carbon isotope ratios indicated that trees on the water-supplement plots were less stressed than ambient-water and water-exclusion trees, and leaf size was much greater for water-supplement trees than ambient-water or water-exclusion trees. $\delta^{13}C$ has been used to screen C_3 genotypes for potential water use efficiency (WUE) (Ehleringer et al., 1993) due to the fact that stable isotopes may

integrate many other physiological variables that would be impractical to measure. In our study, we found that $\delta^{13}C$ was decreased in the water-supplement trees. Lower $\delta^{13}C$ in leaves results from higher intercellular CO_2 concentrations relative to ambient CO_2 concentrations, which will tend to occur when stomata are more open. When intercellular carbon dioxide concentrations are closer to ambient concentrations more of the fractionation associated with carbon fixation is expressed. WUE is inversely correlated with c_i and $\delta^{13}C$ leaf, so $\delta^{13}C$ of leaves is positively correlated with WUE if leaf to air vapor deficit (LAVD) is assumed constant (c_a can be assumed constant): WUE $= (c_a - c_i)/(1.6(LAVD))$ (Farquhar and Richards, 1984). Due to the fact that the water-supplement trees have had long-term water availability over years that the other two treatments did not have, we might predict that in a drought year, these trees might show a more pronounced decreased in WUE than trees that developed under more variable conditions. Long-term temporal variation could be an important variable in field studies of stress on organisms.

The lack of consistent support for drought-induced effects on FA that we found could be due to a number of factors, two of which are of particular note. First, Llorens et al. (2002) completed a similar study with FA with bilberry (*Vaccinium myrtillus* L.) and found that experimental drought increased leaf area FA, and also that warming of the trees decreased physiological stress during leaf development because of the alleviation of temperature constraints. This factor is not likely applicable to the hot and arid conditions of our study. Second, and more importantly, another possibility for greater FA in less stressed plants has been shown by Hochwender and Fritz (1999) and Martel et al. (1999), who found in *Salix* and *Betula*, respectively, increased FA under optimal growth conditions due to the speed of growth that is very fast and triggers developmental instability. Their studies lead us to consider the potential importance of absolute levels of stress vs. relative levels of stress. In this study, we focused on absolute levels of stress and found contrary to expectations FA was greatest in the water-supplement plots. However, while all of the plants likely experienced an increase in stress in conjunction with the drought, the water-supplement plots may have had the greatest relative stress, in terms of the ratio of water available pre-drought to drought. Our study is insufficient to rigorously evaluate this possibility, but leads us to hypothesize that both relative and absolute amounts of stress may produce effects evident in FA. In summary, our results, do not show clear consistent responses in FA to drought stress, highlight potential complexities in evaluating responses of FA to stress and rapid growth and point to the need for future studies of the effects of relative vs. absolute stress on FA.

Acknowledgements

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