Apple Δ¹³C Discrimination Is Related to Shoot Ash Content

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Abstract. Plant ash content has been highly correlated with plant water use efficiency (WUE) and Δ¹³C (Δ) in field crops and grassland species and proposed as a selection criteria for WUE. Δ¹⁸O (δ) has also been correlated with transpiration in herbaceous plants. The objectives of the study were to 1) evaluate the relationship of shoot ash (ASH) with Δ and δ in ‘Empire’ apple over a 3-year period; 2) determine if yearly variation significantly affected the relationship of ASH with Δ and δ; and 3) evaluate the value of the relationship between ASH content with Δ and δ for a population of Malus sieversii. ‘Empire’ leaf area index (LAI) was negatively correlated with ASH content and positively correlated with Δ. Δ was negatively correlated with ‘Empire’ ASH. There were no yearly effects at a site. Within the Malus sieversii accessions, there was a grouping based on ASH that could be identified and this outlier group also had the lowest δ of the accessions. There were no correlations of leaf area, number, length, width, or stomata number with δ, Δ, or ASH for the Malus sieversii accessions. Rather than a substitute for Δ measurement in assessing WUE, ash content analysis adds an additional dimension to understanding the dynamics of WUE in apple. This work has identified a unique population of Malus meriting further study.

Masle et al. (1992) demonstrated that plant ash content was related to WUE (dry matter accumulation per unit of transpiration) and Δ in a range of C₃ species. These findings have been confirmed in many studies of wheat (Araus et al., 1998; Misra et al., 2006; Monneveux et al., 2005; Zhu et al., 2008). Studies of grassland species have demonstrated an inconsistent correlation of WUE with Δ that varied between years and species (Tsialtas et al., 2002). The biological principle relating ash content with Δ and WUE is based on the passive transport of minerals in the xylem and their accumulation in growing and transpiring tissues. The greater the amount of transpiration, the greater the amount of mineral transport to the transpiring tissues leading to increased ash content (Araus et al., 1998; Misra et al., 2006; Monneveux et al., 2005; Zhu et al., 2008). The relationship between plant ash and Δ has been exploited in wheat breeding to select for increased WUE (Araus et al., 1998; Misra et al., 2006; Monneveux et al., 2005; Zhu et al., 2008) as a result of the low analytical cost and minimal technical requirements of measuring plant ash compared with Δ.

Barbour et al. (2004) have demonstrated a strong negative relationship between oxygen isotope ratios of wheat leaves and stomatal conductance (gₛ) that is independent of photosynthesis (A). They also demonstrated that enrichment of δ can be the result of either increased transpirational demand, primarily through an increased vapor pressure deficit (VPD), or an increase in gₛ. When the relationship of oxygen and carbon isotopic ratios has a shallow slope, it indicates variation in carbon isotopic ratios, and so WUE may be attributable primarily to gₛ, an insensitivity of δ to gₛ, or changes in photosynthetic capacity (Barbour and Farquhar, 2000). Cabrera-Bosquet et al. (2009) established a negative relationship between δ and cumulative transpiration. Conversely, Sheshshayee et al. (2005) have demonstrated a positive correlation between δ enrichment of leaf tissues and mean transpiration rate in which the degree of δ discrimination can serve as a measure of gₛ at a specific VPD.

Identifying and improving WUE in tree fruit production has been accomplished on a limited scale (Glenn, 2010; Glenn et al., 2000, 2006), and international breeding programs are recognizing the need for improved WUE and/or drought tolerance in high-value tree fruit crops (Yan et al., 2008; Yang and Yang, 1996). Plant exploration of the apple center of diversity identified progenitor apple germplasm from xeric sites in Kazakhstan and resulted in the propagation and maintenance of several genetically distinct collections in Geneva, NY (Forsline et al., 2003) for use as a genetic resource for apple improvement. Among the populations collected were trees flourishing in a xeric site (site 6.01) where little else survived. These trees have been established at Geneva, NY, and a subpopulation of plantings from Kazakhstan site 6.01 was characterized phenotypically and genotypically; this core subpopulation of 33 individuals represents over 90% of the genetic diversity of the original population (Volk et al., 2005).

The objectives of the study were to 1) evaluate the relationship of ASH with Δ and δ in ‘Empire’ apple over a 3-year period; 2) determine if yearly variation significantly affected the relationship of ASH with Δ and δ; and 3) evaluate the value of the relationship between ASH with Δ and δ for a population of Malus sieversii.

Materials and Methods

Irrigated ‘Empire’/‘M.7A’ were planted at a density of 500 trees/ha in 1992 at the USDA/ARS Appalachian Fruit Research Station, Kearneysville, WV (lat. 39.3 N, long. 77.9 W, elevation 175 m). In all years, the trees were hand-thinned post-bloom. Conventional orchard practices were used in tree training and weed control. Description of the study area and environmental conditions is published (Glenn, 2009, 2010; Glenn and Puterka, 2007). Tree water requirements were based on 70% of pan evaporation (Glenn, 1995, 1999) and evapotranspiration (ET) was assumed to be equivalent to the tree water requirement. Data from only the control and irrigated trees in 2005 to 2007 were used in the present study. After harvest, the trees were covered with a netting to capture all the leaves when they abscised. The leaves were collected and air-dried at 70 °C for ≈1 week. At leaf harvest, a sub-sample of ≈3 kg fresh weight was separated, leaf area was measured, and the ratio of air-dried weight:leaf area calculated. This ratio was used to convert the total air-dried weight of each tree to total leaf area. LAI was calculated as the quotient of the total leaf area divided by the area of the canopy shadow measured within 1 h of solar noon.

Four 30-cm terminal shoot samples per tree were collected in the dormant season and the buds removed. Four trees were sampled in 2005 and five trees in 2006 and 2007. The tissue was dried at 70 °C for 72 h, ground, and analyzed for ¹³C and ¹⁸O content (University of California, Davis Stable Isotope Facility, Department of Plant Sciences, Davis, CA). Carbon isotope discrimination (Δ) was calculated according to Farquhar et al. (1989). The carbon dioxide isotope composition in air (δ¹³Cair) was assumed to be −7.8 parts per thousand (Francey et al., 1995). Oxygen isotope composition (δ) was calibrated against a cellulose standard that was calibrated against Standard Mean Ocean Water. The same shoot tissue used for isotopic analysis was ground as described previously and then re-dried after grinding at 75 °C, weighed, and ignited at 550 °C to determine shoot ASH.

The USDA National Malus Germplasm Collection at Geneva, NY (lat. 42°52' N, long. 76°59' W, elevation 151 m) provided plant material from the core Kazakhstan collection (Forsline et al., 2003) planted in 1993. Thirty-three accessions from a xeric site in Kazakhstan (site 6.01) were selected for study (Table 1). All plant material from Geneva, NY, was planted in three double rows with 1.8-m spacing between rows and 1.5 m in-row spacing on their own roots. All trees received conventional pest control and light pruning. Plant
material from the core collection was sampled in the winter of 2006 and treated as previously described for stable isotope analysis. No biomass or yield data were collected from these trees in Geneva, NY. Leaf area, leaf length and width, and stomatal density were measured on five leaves at about the same position from five shoots of equal length per tree. Stomatal density was measured according to Glenn et al. (2000).

Data were analyzed using SAS (Version 8; Cary, NC) with PROC REG for simple linear regression at \( P \leq 0.05 \). Yearly effects on ‘Empire’ ASH were analyzed using analysis of covariance with independent covariates of \( \Delta \) and \( \delta \) using SAS (Version 8).

**Results**

‘Empire’ LAI was negatively correlated with ASH but positively correlated with \( \Delta \) (Fig. 1). \( \Delta \) was negatively correlated with ‘Empire’ ASH (Fig. 2). There was no significant effect of year on the relationship between ‘Empire’ ASH and \( \Delta \) based on analysis of covariance (data not shown). The Kazakhstan collection of accessions had lower ASH (2.27%) than ‘Empire’ apple (3.97%) for similar levels of \( \Delta \) (Fig. 3B). Within the Kazakhstan accessions, there was a grouping of outliers (3684.a, 3684.b, 3684.l, 3687.d, 3989.k, 3683.i, 3683.n, 3689.n, 3989.f, 3682.f, 3688.n, 3975.l) that could be identified (Fig. 3A) as having higher ASH than the remainder of the population for equivalent levels of \( \Delta \). This outlier group also had the lowest \( \delta \) of the Kazakhstan accessions (Fig. 3B). There was not a significant correlation for ‘Empire’ apple between ASH and \( \delta \) (Fig. 3B). Although there was a significant correlation between ASH and \( \delta \) for all the Kazakhstan accessions (Fig. 3B), this is likely artificial because the two groups (outliers and pooled population) had no significant within-group correlation (data not presented). The leaf area, number, length, width, or stomatal number did not significantly correlate with \( \delta \), \( \Delta \), or ASH of the Kazakhstan accessions (data not presented). \( \Delta \) was not significantly correlated with \( \delta \) for ‘Empire’ apple or the Kazakhstan accessions. There was no significant correlation between ASH and \( \Delta \) for either ‘Empire’ or the Kazakhstan accessions.

**Discussion**

Glenn et al. (2006) previously documented a negative correlation between LAI and WUE and seasonal WUE and \( \Delta \) (Glenn, 2010). The current study further demonstrated that ASH is negatively correlated with LAI while \( \Delta \) is positively correlated with LAI (Fig. 1). Others have shown that specific leaf weight also declines with increasing shade resulting from increased LAI (Barden, 1974, 1977), so it is expected that ASH would also decline with increasing LAI. The fundamental assumption that ASH is negatively related to \( \Delta \) (Farquhar and Richards, 1984) in apple is supported in this work (Fig. 2). Findings of this study suggest that ASH can be used as a measure of seasonal WUE in apple similar to work with agronomic crops (Araus et al., 1998; Masle et al., 1992; Misra et al., 2006; Monneveux et al., 2005; Zhu et al., 2008) because apple seasonal WUE is negatively correlated with \( \Delta \) (Glenn, 2010). As transpiration increases, mineral transport to the transpiring tissues and ash content are expected to increase, whereas increasing WUE will decrease the ash content (Araus et al., 1998; Misra et al., 2006; Monneveux et al., 2005; Zhu et al., 2008). Kearneysville, WV, has a higher ET than Geneva, NY (data not presented) as a result of the more southern latitude. The higher transpiration demand at Kearneysville, WV, is likely the reason for the higher ASH at equivalent \( \Delta \) compared with Geneva, NY (Fig. 3A). Differences in mineral content of the soil may also influence the mineral content of the xylem stream. Although there were no yearly differences measured in Kearneysville, WV, there are likely site differences that will influence the relationship of ash content with iso-discrimination. This also indicates that ash levels should only be compared within a site.

The value of the correlation of ASH with \( \Delta^{13}C \) discrimination lies in its simple, low-cost measurement for large numbers of samples as is typically found in breeding programs. A range of ASH and \( \Delta \) was identified in the Kazakhstan accessions (Fig. 3A). Outliers in the Kazakhstan accessions had ASH greater than the remainder of the sample population for equivalent levels of \( \Delta \), which illustrates the independent response of ASH to \( \Delta \) (Fig. 3A). Likely, these outliers had reduced WUE resulting in increased ash accumulation compared with the remaining Kazakhstan accessions in Geneva, NY. This interpretation of an outlier group is supported by the relationship between ASH and \( \delta \) (Fig. 3B) in which the outlier group had the lowest \( \delta \) of the Kazakhstan accessions. Glenn (2010) demonstrated a positive relationship between \( \delta \) and seasonal ET similar to Sheshshayee et al. (2005) suggesting this outlier group had reduced transpiration together with higher ASH. Morphological parameters of the leaf and stomata density in the Kazakhstan accessions did not explain the unique nature of these outliers (data not presented). Further research will be needed to confirm the physiological basis of these differences in the Kazakhstan accessions and determine if they are the result of stomatal behavior or metabolic differences.

The lack of association between \( \Delta \) and \( \delta \) was in contrast to others (Barbour and Farquhar, 2000; Saurer et al., 1997). Barbour and Farquhar (2000) suggest that low correlation between \( \Delta \) and \( \delta \) could indicate that either the sensitivity of \( \delta \) to changes in \( g_s \) is low and/or that part of the variation in \( \Delta \) is caused by changes in the photosynthetic capacity. At this point, there is no physiological data to understand this lack of correlation.

If ASH data alone were used to measure WUE in the Kazakhstan accessions, there would be errors in assigning relative WUE because the highest ASH would be assigned the lowest WUE (Fig. 2) when \( \Delta \) does not uniformly support this interpretation. In the Kazakhstan accessions, there is an interaction between ASH and \( \Delta \) (Fig. 3A) that can only be detected by \( \Delta \) and \( \delta \) in conjunction with ASH analysis. Rather than a substitute for \( \Delta \) in assessing WUE, ASH analysis adds an additional dimension to understanding the dynamics of WUE in apple. This work has
identified a unique population of Malus for further study of WUE.

Literature Cited

Araus, J.L., T. Amaro, J. Casadesus, A. Asbati, and M.M. Machit. 1998. Relationships between ash content, carbon isotope discrimination and yield in durum wheat. Aust. J. Plant Physiol. 25:835–842.

Barbour, M.M. and G.D. Farquhar. 2000. Relative humidity- and ABA-induced variation in carbon and oxygen isotope ratios of cotton leaves. Plant Cell Environ. 23:473–485.

Barbour, M.M., J.S. Roden, G.D. Farquhar, and J.R. Ehleringer. 2004. Expressing leaf water and cellulose oxygen isotope ratios as enrichment above source water reveals evidence of a Péclet effect. Oecologia 138:426–435.

Barden, J.A. 1974. Net photosynthesis, dark respiration, specific leaf weight and growth of young apple trees as influenced by light regime. J. Amer. Soc. Hort. Sci. 99:547–551.

Barden, J.A. 1977. Apple tree growth, net photosynthesis, dark respiration and specific leaf weight as affected by continuous and intermittent shade. J. Amer. Soc. Hort. Sci. 102:391–394.

Cabrera-Bosquet, L., G. Molero, S. Nogues, and J.L. Araus. 2009. Water and nitrogen conditions affect the relationships of $\Delta^{13}$C and $\Delta^{18}$O as exchange and growth in durum wheat. J. Expt. Bot. 60:1633–1644.

Farquhar, G.D., J.R. Ehleringer, and K.T. Hubick. 1989. Carbon isotope discrimination and photosynthesis. Annu. Rev. Plant Physiol. Mol. Biol. 40:503–537.

Farquhar, G.D. and R.A. Richards. 1984. Isotopic composition of plant carbon correlates with water-use-efficiency of wheat genotypes. Aust. J. Plant Physiol. 11:539–552.

Forsline, U.J., P.P. Tans, C.E. Allison, I.G. Enting, J.W.C. White, and M. Troller. 1995. Changes in oceanic and terrestrial carbon uptake since 1982. Nature 373:326–330.

Glenn, D.M. 1995. Irrigation scheduling, p. 161–168. In: Hogmire, H. (ed.). Mid-Atlantic orchard monitoring guide. Northeast Regional Agricultural Engineering Service, Ithaca, NY.

Glenn, D.M. 1999. Analysis of trickle and pulse microsprinkler irrigation of processing apples. J. Tree Fruit Prod. 2:11–17.

Glenn, D.M. 2009. Particle film mechanisms of action that reduce the effect of environmental stress in ‘Empire’ apple. J. Amer. Soc. Hort. Sci. 134:314–321.

Glenn, D.M. 2010. Environmental and cultural factors affecting gas exchange and water use efficiency in ‘Empire’ apple under field conditions. J. Amer. Soc. Hort. Sci. 134:314–321.

Glenn, D.M. and G.J. Puterka. 2007. The use of plastic films and sprayable reflective particle films to increase light penetration in apple canopies and improve apple color and weight. HortScience 42:91–96.

Glenn, D.M., R. Scorza, and C. Bassett. 2000. Physiological and morphological traits associated with increased water use efficiency in the narrow-leaf peach. HortScience 35:1241–1243.

Glenn, D.M., R. Scorza, and W.R. Okie. 2006. Genetic and environmental effects on water use efficiency in peach. J. Amer. Soc. Hort. Sci. 131:290–294.

Masle, J., G.D. Farquhar, and S.C. Wong. 1992. Transpiration ratio and plant mineral content are related among genotypes of a range of species. Aust. J. Plant Physiol. 19:709–721.

Misra, S.C., R. Randive, V.S. Rao, M.S. Sheshshayee, R. Serraj, and P. Monneveux. 2006. Relationship between carbon isotope discrimination, ash content and grain yield in wheat in the Peninsular zone of India. J. Agron. Crop Sci. 192:352–362.

Monneveux, P., M.P. Reynolds, R. Trehowian, H. Gonzalez-Santoyo, R.J. Pena, and F. Zapata.
2005. Relationship between grain yield and carbon isotope discrimination in bread wheat under four water regimes. Eur. J. Agron. 22: 231–242.

Saurer, M., K. Aellen, and R. Siegwolf. 1997. Correlating $\delta^{13}$C and $\delta^{18}$O in cellulose of trees. Plant Cell Environ. 20:1543–1550.

Sheshshayee, M.S., H. Bindumadhava, R. Ramesh, T.G. Prasad, M.R. Lakshminarayana, and M. Udayakumar. 2005. Oxygen isotope enrichment ($\Delta^{18}$O) as a measure of time-averaged transpiration rate. J. Expt. Bot. 56:3033–3039.

Tsialtas, J.T., M. Kassioumi, and D.S. Veresoglou. 2002. Evaluating leaf ash content and potassium concentration as surrogates of carbon isotope discrimination in grassland species. J. Agron. Crop Sci. 188:168–175.

Volk, G.M., C.M. Richards, A.A. Reilley, A.D. Henk, P.L. Forsline, and H.S. Aldwinckle. 2005. Ex situ conservation of vegetatively propagated species: Development of a seed-based core collection for *Malus sieversii*. J. Amer. Soc. Hort. Sci. 130:203–210.

Yan, G., H. Long, W. Song, and R. Chen. 2008. Genetic polymorphism of *Malus sieversii* populations in Xinjiang, China. Genet. Resources Crop Evol. 55:171–181.

Yang, J. and E. Yang. 1996. A study on drought resistance of seedlings. Acta Agriculturae Boreali-Sinica (China) 2:81–86 [in Chinese].

Zhu, L., Z.S. Liang, X. Xu, and S.H. Li. 2008. Relationship between carbon isotope discrimination and mineral content in wheat grown under three different water regimes. J. Agron. Crop Sci. 194:421–428.