Effects of CO₂ enrichment and drought pretreatment on metabolite responses to water stress and subsequent rehydration using potato tubers from plants grown in sunlit chambers.

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Main Conclusion

Several osmolytes that accumulated in water stressed potato tubers were unaffected by rehydration. This suggested that tubers, unlike other plant tissues, do not remobilize specific soluble carbohydrates and amines that ultimately diminish tuber quality. Total metabolite change were very distinct between source and sink tissues. Single or double drought treatment itself did not affect metabolite change in tuber, but the difference was evident when CO₂ effect was added.

Abstract

Experiments were performed using naturally sunlit Soil–Plant–Atmosphere Research chambers that provided ambient or twice ambient CO₂. Potato plants were grown in pots that were water sufficient (W), water insufficient for 12 to 18 days during both vegetative and tuber development stages (VR), or water insufficient solely during tuber development (R). In the ambient CO₂ treatment, a total of 17 and 20 out of 31 tuber metabolites differed when comparing the W to the R and VR treatments, respectively. Hexoses, raffinose, mannitol, branched chain amino acids, phenylalanine and proline increased, although most organic acids remained unchanged or decreased in response to drought. Osmolytes, including glucose, branched chain amino acids and proline, remained elevated following 2 weeks of rehydration in both the ambient and elevated CO₂ treatments, whereas fructose, raffinose, mannitol and some organic acids reverted to control levels. Failure of desiccated plant tissues to mobilize specific osmolytes after rehydration was unexpected and was likely because tubers function as terminal sinks. Tuber metabolite responses to single or double drought treatments were similar under the same CO₂ levels but important differences were noted when CO₂ level was varied. We also found that metabolite changes to water insufficiency and/or CO₂ enrichment were very distinct between sink and source tissues, and total metabolite changes to stress were generally greater in leaflets than tubers.

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Key words

abiotic stress, osmolytes, soluble carbohydrates, plant metabolism, water potential

Abbreviations

Amb, ambient; Elv, elevated; R, reproductive drought treatment; VR, vegetative and reproductive drought treatment; W, well-watered treatment; Deh, dehydrated treatment; Reh, rehydrated treatment; SWC, soil water content

Introduction

Potato is the foremost root crop cultivated globally and its importance derives from the production of abundant starch filled tubers. The potato plant is cultivated as an herbaceous annual that transfers assimilates from leaves to underground stolons, where tuber formation occurs (King and Stark 1997). Potato performs well in varying climates but optimal tuber yields occur when plants are grown in cooler regions with abundant soil moisture. With advanced farming techniques, potato yields can exceed 40 Mt ha⁻¹ (Ojala et al. 1990). The maximal soil temperature for tuber formation is approximately 25 °C (Menzel 1985) but yield potential may diminish as future climates become warmer and drier (Evers et al. 2010). Potato is shallow rooted and tuber yield is particularly susceptible to soil water deficits (King and Stark 1997; Ojala et al. 1990). Potato tubers that are grown with water deficits are often unsuitable for processing and sale, partly because of decreased starch and elevated sugar levels (Ojala et al. 1990; Kumar et al. 2004). This condition contributes to browning and/or acrylamide formation at high temperatures used for cooking (Ohara-Takada et al. 2005). Therefore, adequate hydration, especially during key developmental stages, is essential for producing and marketing potato tubers.

Because potato is a C₃ crop, significant yield increases occur in response to CO₂ enrichment (Kimball 1983). A 40% increase in tuber yields was observed when CO₂ concentrations were increased from 460 to 660 µmol mol⁻¹ CO₂ using a Free Air CO₂ Enrichment technique (Migletta et al. 1998). A similar increase in tuber yields was observed when experiments were performed in open-topped chambers in the field (Sicher and Bunce 1999). Both of these prior studies observed increased tuber number in response to CO₂ enrichment. Therefore, it is possible that large below ground tubers function as sinks for excess assimilates formed by exposure to Elv CO₂. Although potato leaflets accumulate high starch levels in response to Elv CO₂ treatments (Sicher and Bunce 2001), tuber development can partially relieve the feedback inhibition of net photosynthesis that is attributed to leaf starch (Ludewig et al. 1998; Sicher and Bunce 1999). Stomatal aperture also is decreased by Elv CO₂ concentrations in the atmosphere and this can result in enhanced water use efficiencies for many C₃ crops (Bunce 2004; Barnaby et al. 2012). However, the effects of CO₂ enrichment on plant water relations generally are greater for mild rather than severe drought conditions (Sicher and Barnaby 2012).

Fleisher et al. (2013; 2014) recently reported results of experiments on potato plants grown in Soil-Plant-Atmosphere-Research chambers that were exposed to cyclic drought treatments under ambient or elevated CO₂. One important observation was that drought treatments increased the harvest index of potato. This was consistent with data from other species showing that shoot growth was retarded and root growth was promoted by water deficits (Sicher et al. 2012). Several prior authors (Bussis and Heineke 1998; Vasquez-Robinet et al. 2008; Evers et al. 2010; Kondrak et al. 2012; Barnaby et al. 2015) reported

metabolite changes in drought treated potato leaflets or potato tubers (Muttucumaru et al. 2015; Juhasz et al. 2014; Maggio et al. 2008). In general, reducing sugars and specific amino acids, including Pro, accumulated, whereas starch and certain organic acids decreased in response to drought stress. These prior studies confirmed that low molecular weight metabolites were integrally involved in abiotic stress mitigation in plants (Kaplan et al. 2004; Pinheiro et al. 2004; Sperdouli et al. 2012; Pedrol et al. 2000). The above mentioned metabolite studies on potato were all single treatment experiments. However, it is unclear how drought pre-treatments, CO₂ enrichment or the reversal of water stress by rehydration affected these results. This detailed information is important if we are to understand the effects of complex future environments on potato production. In addition, no one has compared stress dependent changes of tuber and leaf metabolites from the same experiment. It is highly likely that the extent of drought stress differs for below and above ground tissues and this should affect metabolite responses to water stress.

Greenhouse gas concentrations in the atmosphere are increasing and current predictions are that this will result in rising temperatures and altered precipitation patterns (Cao et al. 2011). The objective of this paper was to determine metabolite responses to CO₂ enrichment and water insufficiency in potato tubers and to compare this to foliar data from the same experiment. Because climate change should also increase the frequency and duration of drought, we questioned whether previous episodes of water stress affected metabolite responses to subsequent water stress treatments. To accomplish this, drought treatments were imposed solely during reproductive growth (R) or during both vegetative and reproductive growth (VR) combined. Tuber samples were also collected on the final day of the treatment (Deh, dehydration samples) and 2 weeks of after watering was restored (Reh, rehydrated samples). We further postulated that elevated CO₂ treatments would preserve soil moisture and mitigate the effects of water depletion on potato tubers.

Materials and methods.

Plant materials

Two consecutive plantings were performed during the 2011 growing season using six naturally sunlit Soil-Plant-Atmosphere-Research chambers at the Beltsville Agricultural Research Center, in Beltsville, Maryland, U.S.A. Air temperature, chamber air CO₂ concentrations, irrigation, nutrient supply and humidity were controlled as described previously (Fleisher et al. 2013; Barnaby et al. 2015). Twelve, 16 l pots per chamber were filled with a 3:1 ratio of washed sand and vermiculite (Grace Construction Products, Cambridge, MA., USA). Sections of seed tubers (*Solanaum tuberosum* L. var. Kennebec), that

averaged 50 g FW, were sown in each pot. Sprouts were thinned to two main stems per pot between 5 and 6 d after emergence. Chamber air was maintained at either 400 (Amb) or 800 (Elv) $\mu\text{mol mol}^{-1} \text{CO}_2$ during daylight and was uncontrolled at night. Air temperatures were $22 \pm 0.3^\circ\text{C}$ during the day and $17 \pm 0.3^\circ\text{C}$ during the night. The relative humidity varied between 62 and 78%. The average daily integral of photosynthetically active radiation was 43.9 ± 10.1 and $24.7 \pm 12.4 \text{ mol m}^{-2}$ in the first and second plantings, respectively. First, reproductive drought treatments (R) commenced between 42 and 45 days after shoot emergence using one SPAR chamber from each CO_2 treatment. Water was withheld for 12 to 18 d and then all pots were rehydrated when leaf water potential (LWP) was -1.5 MPa or less (Barnaby et al. 2015). A second pair of SPAR chambers was pre-treated with drought during the vegetative growth phase prior to receiving the above reproductive drought treatments (VR). For the vegetative drought pre-treatment, water was withheld beginning 10 d post emergence. Watering was resumed approximately 14 d later based on changes of leaf water potential as described above. The final two SPAR chambers served as well-watered controls (W). Leaf water potential was measured with a model HR-33T dewpoint microvoltmeter after a 1 h incubation period (Wescor, Logan UT, USA). The volumetric soil water content of the potting medium was determined by time domain reflectometry and data were collected hourly using seven pots in each SPAR chamber (Fleisher 2013).

Metabolite measurements

A random tuber was harvested from four separate, individual pots in each of the six SPAR chambers on the last day of drought treatment of both plantings. A second random tuber from four additional pots in each chamber was harvested 14 d later, when all pots were fully rehydrated. The selected tuber from each pot was removed from the soil medium, quickly rinsed and a 1 cm diameter core of tissue was removed from the center of the tuber with a cork borer. The periderm of each core sample was removed with a sharp razor and the remaining medulla tissue was transferred to labeled envelopes and immediately frozen in liquid N_2 . Samples were then freeze-dried and stored at -20°C for up to two months. Lyophilized tuber tissue was ground to a fine powder in a mortar and pestle and 30 mg dry weight was pulverized in a TissueLyser II bead mill (Qiagen, Valencia, CA, USA) using 2 ml SealRite microcentrifuge tubes (USA Scientific, Ocala, FL USA) containing a 6.4 mm diameter ceramic bead (MP Biomedicals, Santa Ana, CA USA). Soluble metabolites were extracted in aqueous methanol and were quantified by gas chromatography coupled to mass spectrometry as described previously (Sicher and Barnaby 2012; Barnaby et al. 2015). Standard curves were prepared with four point curves using known concentrations of soluble sugars, sugar alcohols, organic acids and amines.

Statistical and Hierarchical Clustering analysis

Mean results from both plantings were combined for data presentation and analysis. Significant differences were determined by an Analysis of Variance (ANOVA) procedure (StatView 5.0, Mountain View, CA, USA). Clustering analysis was performed with data for each of the six treatments on both harvest dates. First, leaf metabolite ratios were obtained by dividing metabolite concentrations from drought treated samples by corresponding values from the well watered control treatment. The resultant ratios were averaged among replicate samples and used to generate slopes, which were then employed for calculating P-values for Hierarchical Clustering analysis. Two-way Hierarchical Clustering identified relationships among various water stress responsive metabolites and was used to quantify the interacting effects of CO₂ enrichment and rehydration. Metabolite data were clustered using Euclidean distance to generate distance matrices or Pearson correlation to produce correlation matrices, and these were compared to various treatment clusters. Hierarchical clusters were constructed as described by Barnaby et al. (Barnaby et al 2013). A scatterplot matrix was constructed to determine if a linear relationship existed between multiple treatments (InfoStat, Córdoba, Argentina). Combinations of treatments at the top of each heat map are described using a series of abbreviations. A Reh or Deh in the first position indicates a sample that was from the rehydration or drought treatment, respectively, and numbers 400 and 800 represent the Amb and Elv CO₂ treatments, respectively. Letters, VR (drought treatment during vegetative and reproductive stages) and R (drought treatment only during reproductive stage), in the last position represent the two watering treatments in this study. Additional technical details were discussed elsewhere (Sicher and Barnaby 2012).

Results

Changes of SWC in response to water stress with Amb or Elv CO₂

Changes of volumetric SWC are shown in Figure 1. Pots in the well-watered treatment contained 32 and 33% water in the Amb and Elv CO₂ treatments, respectively, when measured on the first harvest date. The volumetric SWC in the W watering treatment decreased to 24-25% water in both CO₂ treatments on the second harvest. After 12 to 18 d of drought treatment, pots in the R and VR treatments contained 7 and 8% water, respectively, when plants were grown using Amb CO₂. In comparison, SWC values contained approximately 10 and 11% water in the R and VR treatments in chambers provided with Elv CO₂. When pots in the R and VR treatments were rehydrated for an additional 2 week period, SWC values in the R and VR treatments increased to 24-25% water in both CO₂ treatments. This SWC value was similar to pots in the W watering treatment on this date.

Potato tuber metabolite responses to water stress in Amb CO₂

A mixture of 31 metabolites (7 soluble carbohydrates, 2 polyols, 12 organic acids and 10 amino acids) were quantified using potato tuber tissue in this study. Out of this total, 20 and 17 metabolites differed significantly ($P \leq 0.05$) when comparing the W to the VR or R treatments, respectively, and when plants were grown with Amb CO₂ (Fig. 1, Suppl. Tab. S1A). Note that treatment comparisons for metabolite data in this Figure differ by 2-fold or more. Of the seven soluble carbohydrates measured in this study, all but trehalose and *myo*-inositol increased 1.5-fold or more in response to water stress imposed by the R and VR watering treatments. However, ribose increased in the R but not the VR watering treatment. Combined concentrations of fructose and glucose increased 2.1- and 2.8-fold, respectively, in the VR and R compared to the W watering treatment. In addition, sucrose increased approximately 2-fold in response to drought in both the VR and R watering treatments. These three carbohydrates accounted for over 50% of the total accumulation of tuber metabolites that were measured in this study. One of two polyols measured here also accumulated in response to water deficits. Mannitol increased 2.5- and 5.6-fold, respectively, in the VR and R compared to the W watering treatments when plants were grown with Amb CO₂. Conversely, *myo*-inositol was unaffected by either drought treatment in tubers from Amb CO₂ grown plants.

Gln/Glu was the most prevalent amino acid measured in potato tubers in this study and levels of this amino acid increased 1.3- and 1.7-fold, respectively, in the VR and R compared to the W watering treatment. Asp and Gly did not respond to drought in either the VR or R drought treatments in this study. There was a 2.8- and 5.6-fold increase of Ser, respectively, in the VR and R compared to the W watering treatment. Pro also accumulated in response to drought. There was a 7.2- and 29.6-fold increase in tuber Pro levels from the VR and R drought treatments, respectively, versus that of the W watering treatment. Even after accumulating almost 30-fold, Pro was still a minor amino acid in drought treated potato tubers. The branched chain amino acids, Ile, Leu and Val, and the related amino acid, Thr, increased a minimum of 4-fold in response to both the R and VR drought treatments in potato tubers. There was over a 60-fold accumulation of Thr in potato tubers in the R drought treatment.

Although there were notable exceptions, the majority of organic acids in this study were either unchanged or decreased in the water stressed compared to the control treatments. When plants were exposed to Amb CO₂, three and six out of twelve organic acids were affected by water deficits in the R and VR treatments, respectively. Citrate increased 1.5 and 1.8-fold in the VR and R treatments respectively. However, malate, fumarate, aconitate and adipate all decreased in response to drought. Aconitate decreased over 80% in the R compared to the W watering treatment. Conversely, maleate, malonate, quinate, 2-

oxoglutarate and shikimate concentrations in potato tubers were unaffected by water stress and glycerate increased in the VR but not the R drought treatment.

Potato tuber metabolite responses to rehydration in the Amb CO₂ treatment

A total of 14 and 16 metabolites differed in comparisons between the W and the VR or R watering treatments, respectively, when tubers were harvested 2 weeks after drought treatments were terminated in the Amb CO₂ treatment (Fig. 2). Interestingly, some tuber metabolites reverted to control values in response to 2-weeks of rehydration, whereas other compounds that accumulated in potato tubers in response to drought remained elevated through the end of the experiment. For example, fructose, raffinose and sucrose accumulated 2-fold or more in response to drought but these compounds returned to well-watered control levels after rehydration (Suppl. Tab. S1B). Glucose and mannitol, which are two important osmolytes in potato tubers, did not revert to control levels fully 2 weeks after plants in the VR and R watering treatments were rehydrated.

The eight amino acids that accumulated under drought in the ambient CO₂ treatment generally remained elevated in water stressed tubers following rehydration. For example, Gln/Glu levels in tubers of plants rehydrated for 2-weeks were 1.6 and 2.2-fold, greater, respectively, in the VR and R compared to the control watering treatment. In addition, Ser, Thr, Val, Ile and Leu all were substantially greater in tubers from the VR and R watering treatments that were rehydrated in comparison to the well watered controls. Concentrations of these amino acids in rehydrated potato tubers were up to 10-fold greater than in the well-watered control tubers.

Similar to carbohydrates, the effects of rehydration on changes of organic acids due to drought were variable. Compounds, such as aconitate, fumarate and malate, that decreased in response to drought were largely unaffected by subsequent rehydration. Also, shikimate, adipate, quinate, malonate and maleate, which were unresponsive to drought, also were unaffected by rehydration. Citrate and glycerate were affected by rehydration when examining samples in the R watering treatment but did not differ from control values in the VR watering treatment.

Effects of CO₂ enrichment on metabolite responses to water stress in potato tubers.

A total of 14 and 18 individual compounds differed in the VR and R compared to the W watering treatment, respectively, when plants were grown using Elv CO₂ (Fig. 2, Suppl. Tab. S1C). Similar to Amb CO₂, fructose, glucose, sucrose and raffinose differed in both the VR and R compared to the W

watering treatment when plants were grown with Elv CO₂. Almost all of the amino acids measured here, including Pro and the branched chain amino acids, also were elevated in one or both drought treatments under Elv CO₂. The only organic acid that was consistently altered by drought was succinate, which decreased in water stressed tubers of plants grown using Elv CO₂. The latter finding suggested that CO₂ enrichment mitigated changes of organic acids in potato tubers exposed to drought. However, this was less true for many soluble carbohydrates and amino acids.

A total of 11 and 16 metabolites differed in comparisons between the W and the VR or R watering treatments, respectively, when tubers from Elv CO₂ grown plants were harvested after 2 weeks of Reh (Suppl. Tab. S1D). This represented the least number of metabolites that differed among the various watering treatments employed in this study. Nevertheless, total metabolite levels in tubers from both the VR and R watering treatments remained about 60% greater than was observed in well-watered tubers. This difference was mainly attributed to enhanced levels of glucose, branched chain amino acids and Pro. Regardless of CO₂ treatment, rehydration only partially reversed metabolite changes in potato tubers that were attributed to water stress.

We also performed ANOVA comparisons on metabolite measurements for all tuber samples in the Amb versus the Elv CO₂ treatments (Suppl. Tab. S2). For this analysis, CO₂ effects were determined using combined data for the W, VR and R watering treatments on the final date water was withheld and after 2-weeks of rehydration. Using this approach, only Pro, malate and fumarate differed among CO₂ treatments on the final date of drought treatment. A total of 13 compounds differed among the Amb and Elv CO₂ treatments when data for all watering treatments were combined using tuber samples collected after 2 weeks of rehydration.

Effects of a pre-drought treatment during vegetative growth on metabolite levels in well-watered and drought treated potato tubers

Metabolite responses to water stress generally were diminished by a prior drought treatment that was given during vegetative growth. When individual values for all 31 tuber metabolites were summed, the metabolite totals increased about 85 and 125% in the VR and R watering treatments, respectively, relative to well-watered tubers (see Suppl. Tab. S1A). These numbers were obtained on the final date water was withheld in the Amb CO₂ treatment. Observed differences among the R and VR treatments involved nine compounds including, fructose, mannitol, Pro, and branch chain amino acids. However, there were no changes to organic acids. Without exception, metabolite levels on this harvest date were lower in the VR compared to the R watering treatment. Note that 11 compounds differed among the R and VR treatments

when drought treated plants were grown in Elv CO₂ (Suppl. Tab. S1C). Similar to Amb CO₂, metabolite accumulation in response to drought was greater in the R than in the VR treatment when examining samples from the Elv CO₂ treatment.

Summed metabolites increased 78 and 58%, respectively, in the R and VR compared to the W watering treatment when plants grown in Amb CO₂ were rehydrated for 2 weeks (Suppl. Tab. S1B). Only four individual compounds differed among the two drought treatments in the Amb CO₂ grown tubers and this increased to 7 compounds when plants were grown with Elv CO₂ (Suppl. Tab. 1D). In agreement with the above results, metabolite accumulation in response to drought was generally lessened by drought pre-treatment.

Clustering relationships for metabolite responses to drought in potato tubers

Metabolite responses to eight combinations of watering and CO₂ treatments in this study were clustered by Euclidean distance (Fig. 4A). Compounds that accumulated, decreased or were unchanged in response to treatment are shown in yellow, blue and black colors, respectively. The color bar at the top of the Figure depicts up to a 5-fold positive or negative concentration change for a given metabolite. Clustering relationships and relative responses to treatment are shown in descending order on the left side of the heatmap. Numbers on the upper left hand side of the heatmap give absolute fold-change responses to treatment for individual metabolites relative to corresponding well watered control samples. Metabolites were clustered into three groups indicating those that declined (group 1), those that were little changed (group 2), and those that accumulated (group 3) in response to treatment. Eight treatment combinations are clustered at the top of the heatmap. There was a clear separation between drought treated samples, which clustered on the left half of the heatmap and rehydrated samples which clustered on the right. Relative metabolite accumulation in response to drought treatment was greater in desiccated compared to rehydrated tubers. Also, the R and VR treatments clustered together within both the desiccated and rehydrated treatments but there was no clear clustering pattern for the Amb and Elv CO₂ treatments.

Metabolite changes in drought treated potato leaflets and tubers

Table 1 identified 17 metabolites that responded to drought in both potato leaflets and tubers. Some of the leaflet data are from a prior publication by Barnaby et al. (2015) that was performed on the same plants as in the current study. Data are presented as ratios of metabolite levels from leaflet and tuber samples harvested from drought treated versus well-watered plants on the final day of reproductive

drought treatment for the R and VR watering treatments and from the Amb and Elv CO₂ treatments. The relative responses of fructose, *myo*-inositol, citrate and shikimate to drought were greater in potato leaflets than in tubers following a full course of drought treatment. The opposite was true for Val and succinate, which had greater responses to drought in tubers than in leaflets. Also, Ile, Leu and Pro had larger responses to drought in tubers compared to leaflets but this was more evident for the R compared to the VR watering treatments. The highest concentrations of fructose, glucose and mannitol on the last day of drought treatment were observed in leaflets from the elevated CO₂ treatment. It is likely that CO₂ enrichment augmented the osmotic adjustment of potato leaf tissue in response to water insufficiency but there was little evidence that CO₂ enrichment affected the osmotic adjustment of water stressed tubers at this sampling.

Comparisons of metabolite responses to treatment combinations in tubers or leaflets

A scatter plot matrix was constructed to determine linear regressions of tuber metabolites using combinations of all eight treatment variables (Fig. 5A). The purpose of this approach was to quantify the impact of combined treatment effects on metabolite changes in potato tubers. Correlation coefficients among the R and VR treatments at two CO₂ partial pressures (i.e., Deh400, Reh400, Deh800 or Reh800) were all greater than 0.85 indicating that metabolite responses to the VR and to the R drought treatments were broadly similar. Note that a high correlation coefficient indicates that the two treatment combinations had similar effects on metabolite levels in potato tubers. In comparison, correlations between the 800R and 800VR treatments were 0.63 and 0.64, respectively, whereas those for the 400R and 400VR treatments were 0.56 and 0.27, respectively. This finding suggested that metabolite differences between the Deh and Reh samples were greater in the Amb than in the Elv CO₂ treatment. When different CO₂ treatments were compared using Deh samples, correlation coefficients for a single drought treatment were greater than that for two drought treatments, i.e., compare Deh400R vs Deh800R (0.75) and Deh400VR vs Deh800VR (0.37). The opposite was true in the Reh treatment, i.e., compare Reh400R vs Reh800R (0.52) and Reh400VR vs Reh800VR, (0.75). This result suggested that the effects of CO₂ enrichment were greater when Deh plants were subjected to two drought treatments (VR) compared to single drought treatment (R). This finding was reversed for tuber samples from the Reh treatment, such that the effects of CO₂ enrichment on metabolite levels were greater in the VR compared to the R treatment.

A scatter plot matrix also was constructed from data in Table 1 that compared treatment effects on metabolites from potato tubers with that of leaflets (Fig. 5B). Correlation values between the R and VR

watering treatments under both Amb and Elv CO₂ treatments (Dehtuber400, Dehleaf400, Dehtuber800, Dehleaf800) were between 0.62 to 0.87. Therefore, leaf and tuber metabolite responses to both the R and VR drought treatments and to Amb and Elv CO₂ treatments were similar. However, correlation coefficients were greater for leaf samples (Dehleaf400 and Dehleaf800; 0.76 and 0.62, respectively) than for tuber samples (Dehtuber400 and Dehtuber800; 0.86 and 0.87, respectively). This result suggested that single or double drought treatment effect varies more in the leaf samples. When various treatment effects on metabolites from leaf and tuber samples were compared, correlations of all four combination of treatments were low (0.001 to 0.21) indicating that the metabolic response to Deh treatment differed for tubers and leaflets. Also, differences among CO₂ treatments were greater ($P \leq 0.001$) for the VR treatment (Tuber400VR vs Tuber800VR, 0.51; Leaf400VR vs Leaf800VR, 0.42) compared to that in the R treatment (Tuber400R vs Tuber800R, 0.69; Leaf400R vs Leaf800R, 0.60). Note that comparisons between leaf and tuber samples were low (i.e., ≤ 0.29), suggesting that Deh effects were dissimilar among the two tissue types.

Discussion

Metabolite responses to water stress in potato tubers

One of the most important responses of plants to water stress is an increase in the osmotic potential of affected tissues (Morgan 1984). This adjustment normally involves the accumulation of low molecular weight sugars, amino acids and ions that counteract the effects of drought (Jones et al. 1980). With one or two exceptions, all of the sugars and sugar alcohols measured in this study accumulated in water deficient samples. This clearly indicated that soluble carbohydrate accumulation is an important mechanism of drought tolerance in potato tubers. In agreement with prior studies using potato leaflets (Kondrak et al. 2012; Barnaby et al. 2015) the two reducing sugars, glucose and fructose, were the principal osmolytes that accumulated in potato tubers in response to water stress. These two hexoses are derived from the enzymatic hydrolysis of sucrose and their accumulation in stressed plant tissues is normally attributed to the induction of a specific vacuolar invertase (Kim et al. 2000). Sucrose also doubled in response to water stress in this study, which suggested that this disaccharide may function as an osmolyte in potato tubers. It is likely that water stress inhibited starch accumulation in potato tubers and this resulted in a buildup of sucrose and other sugars. Raffinose increased 7.5-fold or more in tuber tissue exposed to water stress, regardless of CO₂ treatment and a drought dependent accumulation also was observed in water stressed potato leaflets (Kondrak et al. 2012; Barnaby et al. 2015). Raffinose is a galactose containing trisaccharide that is involved in scavenging reactive oxygen species in plant tissues

experiencing abiotic stress (Nishizawa et al. 2008). It is interesting that *myo*-inositol was unaffected by drought in the water deficient samples. This sugar alcohol is a precursor of galactinol and raffinose, and because of this association, the accumulation of these three compounds is sometimes coordinated in plant tissues undergoing abiotic stress (Nishizawa et al. 2008).

In the current study, Gln/Glu, Ser, Thr, Pro, Phe, Ile, Leu and Val, accumulated in potato tubers experiencing water stress. The accumulation of multiple amino acids during drought has been observed in various other plant tissues (Witt et al. 2012; Bowne et al. 2012; Barnaby et al. 2015). Amino acid accumulation in water deficient potato tubers may be responsible for acrylamide formation during processing (Muttucumaru et al. 2015). Pro accumulation is a widely recognized response of plant tissues exposed to water deficits (Delauney and Verma 1993). In maize roots tips, for example, Pro functions as an important osmolyte during drought (Sharp et al. 2004). Transcriptional events associated with abiotic stress initiate Pro synthesis in plants (Verbruggen and Hermans 2008). Water deficits also normally induce the accumulation of all three branched chain amino acids, and sometimes Thr (Binder 2010). These amino acids share a common biosynthetic pathway and concentration changes in plant tissues frequently occur in a coordinated manner (Bowne et al. 2012). The accumulation of branched chain amino acids in water stressed potato tubers also is likely under transcriptional control (Binder 2010). It is possible that branched chain amino acids are required for the synthesis of secondary compounds that function in mitigating abiotic stress. Nitrogen sequestration and ammonia detoxification are additional, possible reasons that amino acid levels increased in water stressed tubers (Jones et al. 1980). It also is possible that the nitrogen stored in soluble amino acids is available for remobilization during rehydration when growth resumes (Sicher and Barnaby 2012).

The majority of organic acids in potato tubers were either unchanged or decreased in response to water stress. The exception to this rule was citrate, which increased under drought in the Amb CO₂ treatment both here and in previous studies employing potato leaflets (Vasquez-Robinet et al. 2008; Barnaby et al. 2015). Other than citrate, specific organic acids associated with the TCA cycle decreased up to 80% in response to drought. Tuber concentrations of malate, aconitate, fumarate and succinate were diminished by either the R or VR drought treatments when plants were grown in Amb CO₂. This result was in agreement with prior drought studies using wheat (Bowne et al. 2012) and maize (Foyer et al. 1998; Sicher and Barnaby 2012), although (Witt et al. 2012) reported that pyruvate and succinate increased in water stressed maize leaves in a greenhouse study. A large reduction of TCA cycle activity also was observed in soybean leaflets grown at above ambient temperatures (Sicher 2013). Interestingly, decreased organic acid levels in soybean leaflets due to high growth temperatures were partly reversed by CO₂ enrichment. The above findings supported the suggestion that the TCA cycle is down regulated in

potato tubers and in other plant tissues during abiotic stress (Atkin and Macherel 2009). Organic acids also are used as carbon skeletons in various biosynthetic reactions and this process likely was diminished in tubers experiencing water stress.

Tuber metabolite responses to rehydration

It is widely accepted that the effects of sub-lethal desiccation on plant tissues are reversed by rehydration (Morgan 1984; Jones et al. 1980). The resurrection plant is an extreme example of this concept. Osmotic adjustments in plants due to water insufficiency also are reversed by rehydration and small molecule concentrations normally are restored to control levels within a few to several days after drought treated tissues were rehydrated (Prasad et al. 1982; Sicher et al. 2012). Therefore, it was interesting that specific osmolytes in potato tubers failed to revert to control values when water depleted tubers were rehydrated for 2 weeks. In the Amb CO₂ treatment, fructose, raffinose and sucrose reverted to control values 2 weeks after rehydration. The mobilization of raffinose in Reh tuber samples may indicate that stress due to reactive oxygen species abated. However, important osmolytes like glucose, Gln/Glu, the branched chain amino acids and Pro unexpectedly remained elevated in potato tubers fully 2 weeks after drought treatments were terminated. This unique observation may indicate that metabolite changes in response to Reh are less likely to occur in terminal sinks in comparison to vegetative tissues on the same plant. Current findings help explain why growers, processors and extension specialists are concerned that drought events will diminish the marketability of potato tubers (King and Stark 1997). Nevertheless, Reh partially mitigated the effects of drought on potato tubers and this mitigation was particularly apparent in the Elv CO₂ treatment. Enhanced glucose and amino acids levels in water deficient potato tubers were little changed following 2 weeks of rehydration in both CO₂ treatments. However, CO₂ enrichment further diminished the total number of metabolites in rehydrated potato tubers that differed among watering treatments in this study, and also the difference in relationship between D and R on metabolic responses was greater under Amb CO₂ compared to Elv CO₂ condition.

Metabolite responses to drought pre-treatments and CO₂ enrichment

It is well known that plants acclimate to adverse environmental conditions and through exposure become increasingly tolerant of subsequent episodes of biotic or abiotic stress (Bray 2001; Chaves et al. 2002). Acclimation to drought stress involves numerous processes including, starch accumulation (Ackerson 1985; Chaves et al. 2002), increased calcium signaling (Knight 1999), the induction of ABA related genes

(Shinozaki and Yamaguchi-Shinozaki 2007) and the accumulation of late-embryogenesis abundant proteins (Hundertmark and Hinch 2008). In the current study, total osmolyte accumulation was less evident in the VR than in the R watering treatment in both dehydration and rehydration treatments. However, overall relationship between R and VR on metabolic responses was similar whether plants are subjected to Amb CO₂ or Elv CO₂, or subjected to dehydration or rehydration treatment. When different CO₂ conditions were compared on each treatment, CO₂ effect was more in the VR treatment when plants are subjected to dehydration; however, this was opposite in the rehydrated samples in that CO₂ effect was more evident in the R treatment. Mitigation of metabolite accumulation in response to drought also was more evident in the samples from the VR compared to R watering treatment when plants were grown with Elv compared to Amb CO₂. In contrast to current findings, canopy level measurements from this same study did not show enhanced acclimation to drought as a result of prior drought cycles (Fleisher et al. 2014).

Comparative responses to drought in leaflets and tubers

It was previously reported (Fleisher et al. 2013) that water stress during the R growth stage decreased vegetative dry weight by 25 and 10% under Amb and Elv atmospheric CO₂, respectively, relative to control plants. However, total biomass decreased about 70% relative to well-watered controls in response to the VR watering treatment independent of the CO₂ concentration used for plant growth. Interestingly, tuber biomass in the R watering treatment increased 22 and 17% in the Amb and Elv CO₂ treatments, respectively, relative to control samples. However, there was about a 30% decrease in tuber biomass in the VR-treated tuber samples from either CO₂ treatment (Fleisher et al. 2013).

A total of 17 metabolites were identified that varied between water sufficient and water depleted samples in both leaves and tubers of potato plants that were grown with ambient or elevated CO₂. This analysis only used drought-treated samples that were exposed to a single (R) or double drought (VR) treatments. Overall, metabolite responses to water insufficiency were broadly similar between single (R) and double drought (VR) treatments. However, the correlation between leaf and tuber samples was very low indicating that the degree that individual metabolites responded to drought varied among leaf and tuber samples. For example, maximal Pro accumulation was about 8-fold in leaflets but was almost 40-fold in tubers. A greater accumulation of branched chain amino acids also was observed in tubers compared to leaflet samples in the Amb CO₂ treatment. Conversely, accumulations of *myo*-inositol, citrate and shikimate in response to water stress were greater in leaflets than in tubers. There also was an enhanced accumulation of hexoses in water stressed leaves in comparison to tubers and this effect was greater under

elevated than ambient CO₂. It is possible that enhanced hexose accumulation in leaflets was attributable to increased photosynthetic activity in the Elv compared to Amb CO₂ treatment (Barnaby et al. 2015).

Conclusions

Water stress induced the accumulation of multiple soluble carbohydrates and amines in potato tubers but most organic acids were either unchanged or decreased by this treatment. Drought pretreatments, CO₂ enrichment and Reh incrementally mitigated the effects of drought on metabolite levels in both control and water stressed tuber tissues. Unlike most vegetative plant tissues, the accumulation of osmolytes in potato tubers was only partially reversed 2 weeks after drought treated plants were rehydrated. This study identified 14 metabolites that are potentially responsible for the degradation of marketing quality of tubers by water stress. Metabolite responses to drought in leaflets and tubers were broadly similar but concentrations of individual metabolites were affected by photosynthetic activity, CO₂ enrichment or differing water potentials between the two tissues. The accumulation of fructose, glucose, and mannitol in response to drought was greater in the R- compared to the VR-treated samples, and this was true for both potato leaflets and tubers.

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Author Contribution Statement

JY and DF conceived, designed and conducted the experiment. JY, RS, JK, VB, and VR analyzed the data. JY and RS wrote the manuscript. All of the authors approved the manuscript.

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Figure legends

Figure 1. Effects of watering treatments and CO₂ enrichment on the volumetric water content of potting medium used for growing potato. Measurements were from time domain reflectometry that took place during tuber development after 12 to 18 d of drought treatment and followed by 14 d of rehydration. (W), well-watered; (R), reproductive drought; (VR) vegetative and reproductive drought. Values are means $\pm$ SE.

Figure 2. Effects of drought treatments and CO₂ enrichment on potato tuber metabolites measured after 12 to 18 d of water stress. Primary metabolites experiencing a 2-fold or greater concentration change versus well-watered controls are shown in yellow (increase) or blue (decrease). Watering treatments (VR and R) were as in Figure 1. Probabilities (*, **) are for $P \leq 0.05$ and ≤ 0.01 , respectively. Amb/Elv are 400/800 $\mu\text{mol mol}^{-1}$ CO₂.

Figure 3. Effects of rehydration and CO₂ enrichment on potato tuber metabolites measured 14 d after 12 to 18 d of water stress ended. Primary metabolites experiencing a 2-fold or greater concentration change versus well-watered controls are shown in yellow (increase) or blue (decrease). Watering treatments (VR and R) were as in Figure 1. Probabilities (*, **) are for $P \leq 0.05$ and ≤ 0.01 , respectively. Amb/Elv treatments are 400/800 $\mu\text{mol mol}^{-1}$ CO₂.

Figure 4. Metabolite responses to CO₂ enrichment and water stress in potato tubers. Heatmap with Hierarchical Clustering analysis showing either the increase (shown in yellow) or decrease (shown in blue) of tuber metabolites in response to drought treatment (Dehydration or Rehydration), CO₂ enrichment (400 vs 800), or frequency of drought (dehydration during Reproductive stage only (R) or dehydration during both Vegetative and Reproductive stages (VR)) (A). Values are given as ratios obtained from drought treated and control samples from various treatments in this study and are shown in gradations of from 5 to 5 $\times$ (see scale bar bottom left). Ratios that were unchanged are shown in black. Clustering relationships were based on Euclidean distance. Scatter plot matrix of metabolite responses to CO₂ enrichment and water stress in potato tubers (B). Variables are crossed against each other (upper right). In each scatterplot a red line illustrates linear regression. Values of pairwise linear regression (R^2) and p-values were presented on the left side in the matrix table. * $p < 0.05$, ** $p < 0.01$. Clustering relationships were based on pearson correlation, and was shown on the left side of the matrix table. au,

Approximately unbiased P-values (0–100%, the higher the number the more significant). D and R before a number denote dehydration (drought) and rehydration; 400/800 are CO₂ concentrations in μmol mol⁻¹ CO₂; R and VR behind a number are watering treatments during vegetative stage (V), and both vegetative and reproductive stage (VR) (see Figure 1).

Figure 5. Comparison of metabolite responses to CO₂ enrichment and water stress in potato leaf and tubers. Scatter plot matrix of metabolite responses to CO₂ enrichment and water stress in potato tubers (B). Variables are crossed against each other (upper right). In each scatterplot a red line illustrates linear regression. Values of pairwise linear regression (R²) and p-values were presented on the left side in the matrix table. **p*<0.05, ***p*<0.01. Clustering relationships were based on pearson correlation, and was shown on the left side of the matrix table. au, Approximately unbiased P-values (0–100%, the higher the number the more significant). D before a number denote dehydrated samples; 400/800 are CO₂ concentrations in μmol mol⁻¹ CO₂; R and VR behind a number are watering treatments during vegetative stage (V), and both vegetative and reproductive stage (VR) (see Figure 1).

Table 1. Relative responses of leaf and tuber metabolites to water stress treatments. Values are ratios of water insufficient and water sufficient tuber samples. The R drought treatment was imposed during reproductive growth and the VR watering treatment was applied during both vegetative and reproductive growth. Plants were grown in sunlit chambers provided with 400 or 800 mmol mol⁻¹ CO₂ (Amb and Elv CO₂, respectively).

	Amb CO ₂				Elv CO ₂			
	Tuber		Leaf		Tuber		Leaf	
	R	VR	R	VR	R	VR	R	VR
Fructose	2.73	1.76	8.32	6.29	1.93	2.07	16.26	6.44
Glucose	3.2	2.62	2.43	1.58	4.04	2.63	5.97	4.42
Mannitol	5.56	2.54	2.89	3.4	6.21	1.81	7.29	3.24
<i>myo</i> -inositol	1.31	1.49	4.96	5.88	1.21	1.53	2.42	2.86
Sucrose	1.93	1.95	2.28	3.86	1.13	0.85	1.4	1.29
Asp	1.28	1.39	0.98	1.03	0.81	1.15	0.78	1.07
Gly	0.62	0.85	0.51	0.82	1.66	1.16	0.57	1.27
Ile	9.74	4.29	3.83	6.09	13.9	5.22	1.23	2.36
Leu	6.98	4.42	3.14	2.86	20.17	4.6	3.64	4.35
Pro	29.87	7.29	5.2	8.15	39.81	27.11	2.96	5.16
Val	18.9	7.04	2	2.74	3.44	3.36	2.46	2.53
Citrate	1.77	1.47	2.1	2.07	1.36	1.25	2.56	3.43
Fumarate	0.52	0.45	0.52	0.77	1.49	1.03	0.71	0.91
Glycerate	0.58	2.33	0.73	0.54	0.77	1.55	0.52	0.6
Malate	0.56	0.31	1.13	1.9	1.36	0.9	1.28	1.42
Shikimate	1.08	1.09	1.69	2.51	0.83	0.74	1.35	2.7
Succinate	0.44	0.35	0.71	0.82	0.25	0.51	1.33	1.34

Figure 1. Effects of watering treatments and CO₂ enrichment on the volumetric water content of potting medium used for growing potato. Measurements were from time domain reflectometry that took place during tuber development after 12 to 18 d of drought treatment and a subsequent 14 d of rehydration. (W), well-watered; (R), reproductive drought; (VR) vegetative and reproductive drought. Values are means $\pm$ SE.

Figure 2. Effects of drought treatments and CO₂ enrichment on potato tuber metabolites measured after 12 to 18 d of water stress. Primary metabolites experiencing a 2-fold or greater concentration change versus well-watered controls are shown in yellow (increase) or blue (decrease). Watering treatments (VR and R) were as in Figure 1. Probabilities (*, **) are for $P \leq 0.05$ and ≤ 0.01 , respectively. Amb and Elv indicate 400 or 800 $\mu\text{mol mol}^{-1}$ CO₂ treatments.

Figure 3. Combined effects of rehydration and CO₂ enrichment on potato tuber metabolites measured 14 d after 12 to 18 d of water stress ended. Primary metabolites experiencing a 2-fold or greater concentration change versus well-watered controls are shown in yellow (increase) or blue (decrease). Other treatment details were as in Figure 2.

Figure 4. Metabolite responses to CO₂ enrichment and water stress in potato tubers. Heatmap with Hierarchical Clustering analysis showing either an increase (yellow) or decrease (blue) of tuber metabolites in response to drought treatment [Dehydration (Deh) or Rehydration (Reh)], CO₂ treatment (400 or 800 $\mu\text{mol mol}^{-1}$), or growth stage of drought treatment (dehydration during Reproductive stage only (R) or dehydration during both Vegetative and Reproductive stages (VR). Values are given as ratios obtained from drought treated and well watered control samples from various treatments in this study and are shown in gradations of from -5 fold to +5 fold (see scale bar at bottom). Ratios that were unchanged are shown in black. Clustering relationships were based on Euclidean distance.

Figure 5. Comparisons of metabolite responses to treatment combinations in tubers or leaflets. Scatter plot matrix of metabolite responses to CO₂ enrichment (Amb CO₂ vs Elv CO₂) and water stress (Deh vs Reh; R vs VR) in potato tubers (A) and CO₂ enrichment and water stress (R vs VR) in dehydrated potato leaflets and tubers (B). Treatment variables are arrayed from upper left to lower right. Regressions of metabolite data were constructed using X/Y treatment combinations from columns and rows on the top half of the matrix. Values of pairwise linear regression (R²) and P-values [* ($P \leq 0.05$) and ** ($P \leq 0.01$)] correspond to cells on the right side of the matrix table. Clustering relationships were based on Pearson correlation, and was shown on the left axis. au, Approximately unbiased P-values (0–100%, the higher the number the more significant). D and R before a number denote dehydration (drought) and rehydration; 400/800 are CO₂ concentrations in $\mu\text{mol mol}^{-1}$ CO₂; R and VR behind a number are watering treatments during vegetative stage (V), and both vegetative and reproductive stage (VR) (see Figure 1)