Development of Tree Carbohydrate Budget Based Methods for Sustainable Management of Almonds under Changing Central Valley Climatic Conditions

Project No.: **15-PREC8-Zwieniecki**

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Objectives:

- Establish experimental tools for studying shoot physiology under variable environmental conditions in the lab and in the field including detailed analysis of starch and soluble sugar concentration dynamics, carbohydrate metabolism enzyme activity, and expression pattern of genes encoding enzymes from carbohydrate metabolism pathways.
- Using existing and novel tools to determine the seasonal pattern of carbohydrate management by the almond tree with a special focus on winter to determine the dynamics between trees' activity in spring and shoot carbohydrate reserves.
- Determine cumulative impact of environmental stresses on carbohydrate management including the combined effects of thermal and water stress (and possibly include salinity stress in the future).

Interpretive Summary:

Vegetative life of any plant can be described as a continuous struggle to acquire, transfer, and store energy that is necessary to grow, reproduce, and protect from environmental abiotic and biotic stress. Carbohydrates (sugars) are responsible for the majority of long distance energy transfers and long term storage of energy in plants [1-3]. They are the ultimate currency that the plant has to interact with environment. The understanding of carbohydrate management in plants responding to environmental stress while accomplishing their reproductive functions (yield) is of key importance to yield predictions, determination of plant stress level, and their ability to mediate salinity, drought, or winter survival [4]. Understanding of carbohydrate management is especially important in long lived perennial crops like *almond* that must balance between short-term (seasonal) vs multi-year benefits with effects being carried out over many years. This management of tree resources is an evolved trait that is under significant strain resulting from slow changes in climatic conditions of Central Valley. California's Central Valley experienced the most severe drought in more than 1,200 years. Simultaneously the drought's effects are magnified by the slow climatic shift that is reducing the valley's fog cover [5]. The net result is an increased incidence of extreme thermal condition during winter including: higher average temperatures, more severe frost nights, and hot sunny

days. These factors combined with the increasing use of saline ground water supplies in some locations necessitated by decreasing surface water supplies have produced an unprecedented set of new abiotic stresses that affect horticultural production. Crop growth and yield is dependent on a complex set of interactions involving the plant genotype, the physiological and developmental processes that occur within the crop plant, and the interaction of these processes with the environment that the plant grows in. Successful mediation of abiotic stresses by horticultural manipulation of the plant by the crop manager depends on precise knowledge of plant physiological responses to applied treatments.

This effort focuses on the development of new approaches to measure trees physiological status that complement the currently used methods and will provide new options for orchard management.

We have already determined yearly carbohydrate reserve status showing that short term accumulation of carbohydrates in stems and branches in the fall is followed by winter redistribution across the tree. It is an open question if the fall level of carbohydrates in branches is related to success of bloom and yield in the following year.

We have developed and tested simple lab protocols to determine carbohydrate levels in almond wood and bark. Protocols will be used in all subsequent research and shared among collaborating labs. We have also developed simple protocols for collecting samples in the field that we will distribute to farm advisors, growers and farm managers in case they are interested in providing samples for seasonal analysis of carbohydrate levels. Participation in this 'citizen research project' would speed up discovery process in determination of carbohydrate status usefulness in assessing orchard performance.

Methods, Results and Discussion:

The perennial habit of Almond trees implies that during the growing season they have to store energy in the form of nonstructural carbohydrates (NSCs) before entering dormancy. These NSCs will be mobilized during winter dormancy and support bloom. One of our main objectives in the first year of study was to determine temporal (seasonal) and spatial (allocation within the tree) PATTERNS of NSC content in almond trees. It is generally assumed that wood and roots are the main storage compartments for carbohydrate reserves (starch) during winter. Yet, it is not known how these storage compartments interact, when they are mobilized, and how local and distal to buds' storage compartments influence biology of bud-break. Almond trees are highly dependent on stored carbohydrates during spring, and they need an integrated remobilization of carbohydrates (starch degradation) for a successful bloom (vital flowers) that has a direct effect on yield. That is winter carbohydrate management might have a direct influence on the tree reproductive functions, such as blooming time, synchronization of bloom time within a tree and between trees, may influence the number of vital flowers and their fate post pollination [6-10]. As our previous work showed that carbohydrates management is highly responsive to temperature. Thus we think that gaining a better understanding of the underlying mechanism of dormancy through carbohydrate management will improve our prediction of dormancy breaking time and provide a mechanistic understanding to improve chilling hours models.

In regards to stated objectives, we started and continue to collect data for analysis of seasonal variations of nonstructural carbohydrates in vegetative branches (current year extension), spur bearing branches, trunks of almond trees (**Figure 1**).

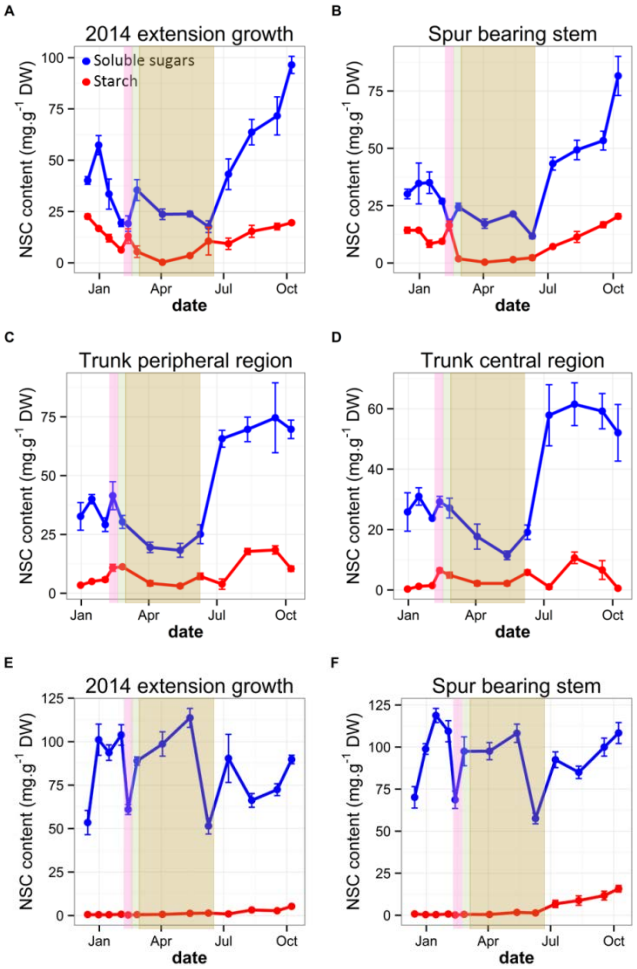

Figure 1. Seasonal variations of nonstructural carbohydrates (NSC) in the wood (A-D) and bark (E-F) of different organs of almond trees (n=5). Blue and red data points represent mean values of soluble sugars content (SC) and starch content respectively for 5 trees. Pink line represents blooming time. Green line represents leafing time. Brown area represents nut fill.

The study of seasonal patterns of carbohydrate mobilization by almond trees (**Figure 1**) provided insight of how trees manage NSCs in an integrative manner over the course of a year. Our main focus during this past year, was analysis of the NSCs utilization pattern during winter. We observed mobilization of wood carbohydrates during winter in the branches, which was followed by an accumulation of starch in wood just prior to bud-break. That starch shift coincided with mobilization of soluble sugars. Later in the spring and early summer no accumulation of NSCs in wood was observed (time of nut fill) despite that all the leaves were mature and photosynthetically active. It seemed that vegetative growth and nut growth was exceeding photosynthetic capacity of the tree and production was dependent on available storage raising the question on how important is pre-bloom storage size is for successful yield and how long it will last. We observed that stem reserves were drained to near zero in the middle of the summer. Once that occurs, one can hypothesize that any interruption in production of new carbohydrates (photosynthesis) will strongly influence performance of the tree, most likely shutting down nut fill first followed by growth, potentially making this time of the year most fragile to any abiotic/biotic stress. We can expect that a trade-off between developing or dropping nuts and survival could be experienced by these trees in this moment

of time. Once the nuts are dropped or are harvested, restoration of the starch reserves becomes the main goal of tree in preparation for dormancy. We observed an intense accumulation of NSC starting August and continued into the fall (**Figure 1**).

Mobilization of NSC during winter. Influence of temperatures on respiration and NSC depletion: We observed the mobilization of wood carbohydrates during winter of 2015 in the branches (**Figure 1, A and B**). In these compartments, almost 80 % of the stored carbohydrates were depleted before bloom. This intense mobilization can be explained by maintenance respiration, reallocation, and finally providing required energy for blooming. The last aspect has a direct link with yield and represents a good starting point for explanation of the discrepancy between bud set and number of flowers in spring. That is if not enough sugar is provided to the buds at the time of blooming, they can't develop fertile flowers.

Respiration has a direct relationship with temperature. Higher temperatures during milder winters are responsible for excessive NSC depletion, especially in twigs that are characterized by a high volume of living cells. Expected climate change may bring highly variable diurnal temperatures with large number of hot days in the winter. To calculate and model future NSCs losses due to climate change, we studied the temperature variations experienced by the trees during the time course of the year in different organs (trunk, limb stems; **Figure 2**) and we measured the thermal response of respiration of dormant almond twigs. This relationship is also known as Q10 (**Figure 2**). This new research will provide an exciting tool for predicting the depletion of carbohydrates during winter, and help orchard management to predict the impact of winter weather on timing and success of flowering. In addition, we plan to learn about the physiology of chill substitute treatments, and we hope to provide specific knowledge on their application in relation to weather conditions and tree physiological status. We plan to study the effect of oil/dormax/paint applications on respiration, and model the temperature response of stem respiration over the winter in order to determine treatment role on the NSC depletion.

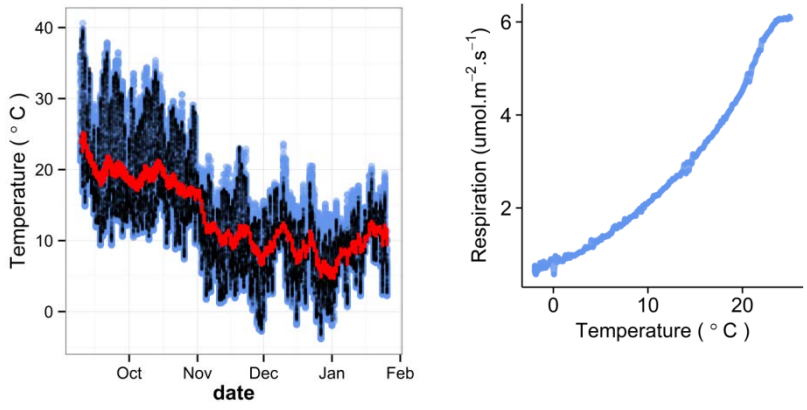

Figure 2. Seasonal temperature variation of an extension growth of almond tree (blue) in comparison to air (black) and soil (red) temperature. Respiration response to temperature of an extension stem of almond.

Mobilization of NSC during bud-break.
A key but understudied time of tree phenology is the blooming time. The timing, synchronism of different trees and quality of blooming is essential for the reproductive success of the tree and for orchard yield. Error in synchronization between branches and trees can lead to no pollination. Also a decrease in flower number and fertility because of limited accessibility/mobility to NSC will affect the yield of an orchard. In addition, thermal condition of the tree at the time of blooming might influence carbohydrate redistribution.

Our anatomical results performed on cuttings (**Figure 3**) showed the proof of concept that blooming and bud-break relies on stored carbohydrates. Another interesting aspect of the observation was that this storage seems to be very local. To assess the carbohydrate cost of a flower from local wood storage, we compared the percentage of flowers blooming on stems of various length from 1 to 5 cm (**Figure 4**). Our results showed that a minimum of 5 cm of wood was necessary for a successful bloom. Besides in order to estimate the carbohydrate cost of a flower we measured the respiration of a flower during blooming over 10 days (**Figure 4**). Knowing the carbohydrate content of the stems over the time course of the winter and the carbohydrate cost of a flower, we want to predict whether the stored NSCs become limiting because of a too mild winter, as well how distal stored starch is mobilized. This knowledge could potentially help in timing of chill substitute treatments.

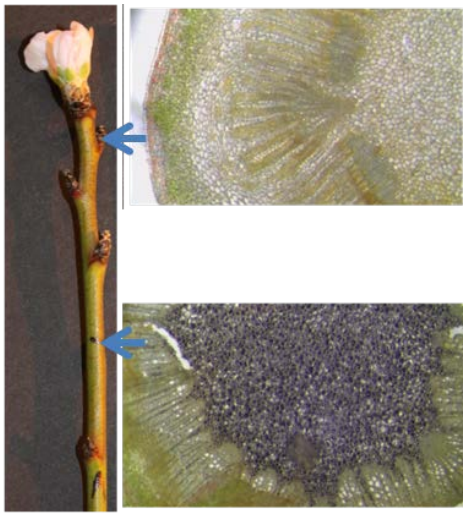

Figure 3. Local depletion of starch in a blooming cutting kept at 21°C. Cross sections were performed on the cutting at the blue arrows position. Then they were stained with an iodine solution that stains starch black.

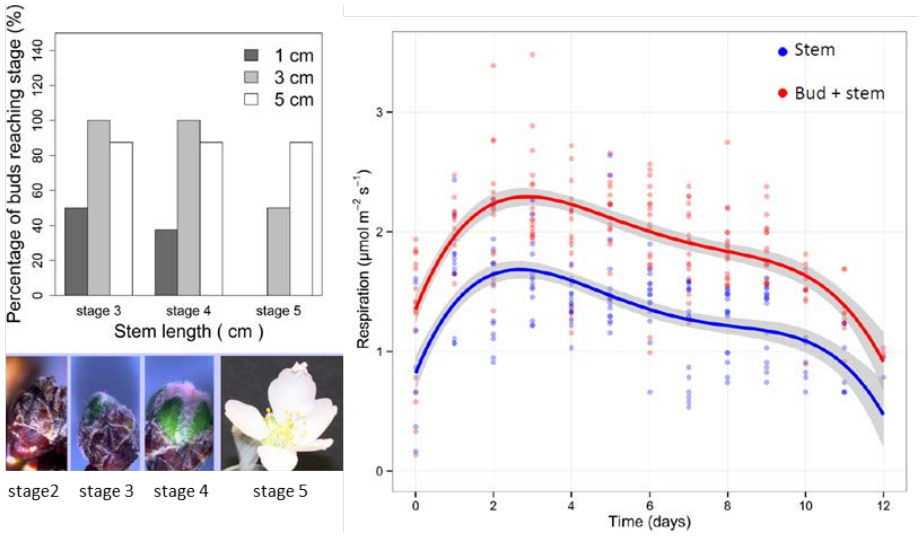

Figure 4. Carbohydrates cost of flowers. Different stem lengths with a floral bud were studied for bud phenological stages. 1 cm and 3 cm showed significantly lower percentage of successful bloom (reaching full bloom, stage 5). Comparison of the time course of respiration of stem with a bud or de-budded until full bloom. Budbreak increases respiration of a stem linearly.

Projects under development

Influence of temperature variations on NSCs mobility during winter

The winter pattern of NSC distribution in almond trees showed increase in NSCs in the aboveground organs. Since no photosynthesis occurred at that time, it means these carbohydrates where mobilized from another location, probably stems or roots. We think these observations are of special importance for the mobilization of carbohydrates for blooming. During the 2015/2016 winter we measured the underground organs NSCs seasonal variations in addition to the variations in the tree crown. Interestingly when observed in the field, the blooming time coincide with mobilization of carbohydrates in the whole tree, and when trees are grown in variable temperatures, no local depletion is observed close to the bud (as was observed in cut samples). Thus, carbohydrates seem to be also mobilized from distal storage sources. It is intriguing because no transpirational stream can be considered for transpors and the phloem is still dormant. Another important aspect we would like to address is the physiology of dormancy breaking in stems. Traditionally the focus has been on the buds, and very little is known about the way the whole tree breaks dormancy. Mobility of carbohydrates in trunk and branches before bud-break suggests that there is internal activity preceding the bud swelling, and that trees break dormancy in an integrated manner (all tree or nothing). To get a better look at these processes we started to quantify enzymatic activity (starch degrading enzymes) in the wood of branches in order to perform a quantitative characterization of dormancy (**Figure 5**), and plan to also study assay for ATP (product of respiration in active tissue used to drive all cellular reactions) levels.

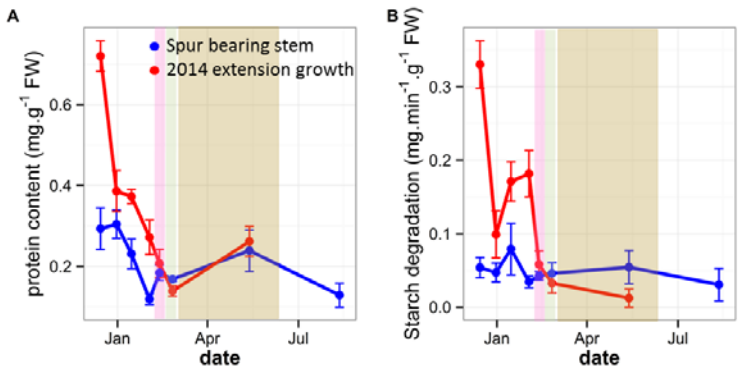

Figure 5. Seasonal variations of protein content (A) and starch degradation rate (B) in the wood of spur bearing stems and 2014 extension growth.

Quantification of ATP (cellular energy source) will be done for wood, phloem, and buds during winter and spring. We expect to see an increase in ATP in buds and wood during dormancy breaking, and obtain a finer resolution of how the tree breaks dormancy along the stem.

Interesting from this perspective is to determine who wakes up first in the tree. Is it possible that buds sense temperature and trigger branches to leave dormancy, or does the wood deliver signals to the buds via carbohydrates depletion in response to temperature? We have started preliminary experiments with heating devices that are controlled with thermocouple sensors. These newly developed tools allowed us to warm only buds or branch sections in order to understand the integrative temperature sensing in the tree, and analyze forced dormancy break on carbohydrate mobilization in not heat-treated parts of the tree. The methods have proven promising and may provide exciting perspectives for next winter (**Figure 6**) as we can deprive some buds of chilling or mimic spring thermal conditions on a bud, a branch, or a trunk.

Figure 6. Bud heating devices allowing to break dormancy in buds. 29 January 2016

Materials & Methods:

Protocol for non-structural carbohydrate analysis in almond tissue

Sample preparation

- Sample stem material from tree always at the same time of the day (diurnal variation).
- Remove bark from wood in the field and put sample in paper envelope
- Size of sample: 10 cm long stem sample
- Dry samples in an oven at 70-80^{o}C quickly after sampling (within an hour) or put them on dry ice
- Let the sample dry for 48 hours
- Grind the samples into a fine homogeneous powder using ball mill and weigh ~25 mg (± 2 mg) of tissue in centrifuge tube.

Soluble sugars extraction

- Add 1 mL of ultra-pure (UP) water to the sample and vortex.
- Incubate at 70 C for 15 minutes, vortex, and centrifuge at 15000 RPM for 10 minutes.
- Extract 50 µL of supernatant to new centrifuge tube. Add 1000 µL of UP water. Vortex. Use supernatant tube for soluble sugars analysis.
- Keep pellet for starch quantification

Measure soluble sugars

- Make **Standard solution**: 1mg/ml glucose. Store at 4 ^{o}C
  SD1: 300 µL glucose + 700 µL water
  SD2: 100 µL glucose + 900 µL water
  SD3: 30 µL glucose + 970 µL water
  SD4: 0 µL glucose + 1000 µL water
- Make **Anthrone reagent** (0.1% in sulfuric acid)
- Pipette 50 µL of supernatant and each standard solution into plate.
- Add 150 µL of anthrone reagent to plate (work under a fume hood). Pipette up and down vigorously 10 times to mix the solution.
- Incubate the plate at 100 ^{o}C for 20 minutes. Incubate the plate at room temperature for 10 minutes.
- Measure absorbance in spectrophotometer at 620 nm. (**Figure 7**)

Removing residual soluble sugars in starch sample

- Discard the supernatant. Add 1 mL ethanol to pellet, vortex, Centrifuge at **15000 RPM for 10 min**.
- Discard the supernatant. Add 1 mL UP water to pellet, vortex. Centrifuge at **15000 RPM for 10 min**.
- Discard the supernatant. Possibility to store samples at -20°C at this stage

Enzymatic degradation of starch

- Boil samples at 100 °C for 10 minutes. Let at room temperature for 20 minutes.
- Add 500 µl of 0.2 M Na acetate (pH5.5), 100 µl of 70 units/ml amyloglucosidase and 100 µl 7 units/ml alpha amylase.
- Vortex. Incubate at 37 °C for 4 hours. Gently shake during incubation.

Measure starch

- Centrifuge at **15000 RPM for 5 min**
- Extract 50 µL of supernatant to new centrifuge tube. Add 1000 µL of UP water. Vortex.
- Pipette 50 µL of supernatant and each standard solution into plate.
- Add 150 µL of anthrone reagent to plate. Pipette up and down vigorously 10 times to mix the solution.
- Incubate the plate at 100 C for 20 minutes.
- Incubate the plate at room temperature for 10 minutes.
- Measure absorbance in spectrophotometer at 620 nm.

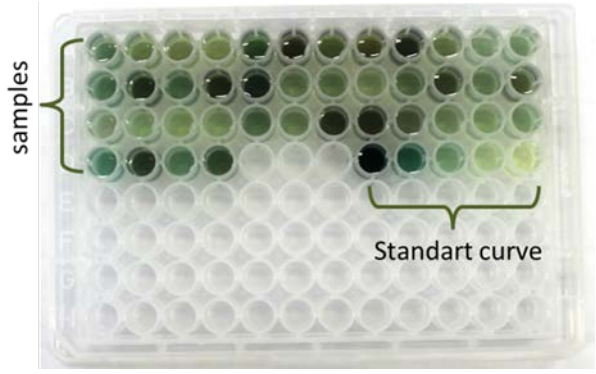

Figure 7. Representative plate with anthrone based sugar concentration determination

Research Effort Recent Publications:

Two publications related to annual carbohydrate management and temperature role in flowering are being prepared and will be submitted to appropriate journals.

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