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# Storage and breakdown of starch aid *P. parviflorus* in leaf re-greening after nitrogen deficiency

Kevin A. Korus
*University of Nebraska-Lincoln*, kevinak333@hotmail.com

M. Elizabeth Conley
*University of Nebraska-Lincoln*, mconley2@unl.edu

Erin E. Blankenship
*University of Nebraska-Lincoln*, eblankenship2@unl.edu

Ellen T. Paparozzi
*University of Nebraska-Lincoln*, etp1@unl.edu

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**Cover Page Footnote**
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## 1. Introduction

*Plectranthus parviflorus*, also known as *P. australis* and common Swedish ivy, displays a remarkable resilience towards death in the form of delayed leaf senescence. The presence of carbohydrates either in soluble or insoluble form (starch) is essential for biosynthetic reactions in plants (Buchanan et. al, 2000). The carbon nitrogen balance is also key in leaf senescence with the presence of sugars involved in signaling to the plant that senescence may proceed and low levels of nitrogen inducing premature leaf senescence (Gan, 2007). This research focuses on the concentration of soluble (reducing) sugars and presence of starch in leaves before and after repeated nitrogen (N) deficiency. Understanding the storage and solubility of sugars in these leaves is one step towards understanding how *Plectranthus* delays leaf senescence and allows leaves to regain a green color (re-green) without leaf loss.

## 2. Materials and Methods

Cuttings of *Plectranthus parviflorus* were rooted in vermiculite under intermittent mist (water only – no added nutrient elements) for 5 weeks. Once well rooted and yellow-green in color, cuttings were transferred to a hydroponics system. Each plant was started in a 3.8 L plastic container lined with a plastic bag and covered by a 24 $cm^2$ extruded polystyrene lid with a center hole to support the cutting and another hole for the insertion of an air-supply line. Black tape was used to close any gaps in order to prohibit light in the root zone. Later during the experiment, larger plants were transferred to 7.6 L plastic containers (with 29-34 $cm^2$ lids) to accommodate expanded root size.

Plants were grown in Lincoln, Nebraska (latitude 41° N) in an acrylic-sided glass-roofed greenhouse. Air temperature settings ranged from 20 to 27 °C. Four-hour night interruption (22:00 to 2:00) using high intensity discharge (HID) lights insured that plants stayed vegetative.

Two hydroponic experiments were run: The Fall Experiment (10/3/07 to 12/11/07) and the Spring experiment in the winter-spring (1/23/08 to 5/7/08). The experimental design was an incomplete, unbalanced block (with a 2X7 factorial treatment design). The 2X7 factorial treatment design is based on the 14 combinations that are possible when switching 0 and 150 mg $L^{-1}$ N repeatedly over time. Due to space and time limitations only 12 treatments and 24 plants were used. Plants initially received either 0 or 150 mg $L^{-1}$ N as solution treatments (Table 1). After 3 weeks, all plants in the 150 treatment had re-greened. At that time plants were harvested for dry weight analysis and carbohydrate analysis of total reducing sugars (Tables 1, 2). To evaluate changes in leaf color, top, middle, and lower leaves were

examined before harvest using the Royal Horticultural Society RHS Colour Chart (1995). Colors were matched to Yellow-Green Group 144 for yellow leaves and Green Group 137 for control and re-greened leaves. This was the criteria for determining when the leaves were completely green and for determining when to switch nutrient treatments from 0 to 150 mg $L^{-1}$ N or from 150 to 0 mg $L^{-1}$ N.

**Table 1.** Timetable of treatment switches and plant harvests for Fall Experiment.

| Plant | 10/3/07 | 10/24/07 | 11/14/07 | 12/11/07 |
|---|---|---|---|---|
| 1 | 0 | H | - | - |
| 2 | 0 | H | - | - |
| 3 | 150 | H | - | - |
| 4 | 150 | H | - | - |
| 5 | 0 | 150 | 150 | H |
| 6 | 150 | 0 | 0 | H |
| 7 | 150 | H | - | - |
| 8 | 0 | 150 | 150 | H |
| 9 | 150 | 150 | 150 | H |
| 10 | 0 | 150 | 0 | H |
| 11 | 0 | 0 | 0 | H |
| 12 | 150 | 0 | 150 | H |
| 13 | 150 | 0 | 150 | H |
| 14 | 0 | 150 | 150 | H |
| 15 | 150 | 150 | 150 | H |
| 16 | 0 | 0 | 0 | H |
| 17 | 0 | 150 | 0 | H |
| 18 | 150 | 0 | 0 | H |
| 19 | 150 | 0 | H | - |
| 20 | 150 | 150 | H | - |
| 21 | 0 | 0 | H | - |
| 22 | 150 | 0 | H | - |
| 23 | 0 | 150 | H | - |
| 24 | 0 | 150 | H | - |

0 = 0 mg $L^{-1}$ N, 150 = 150 mg $L^{-1}$ N, H = Harvest/Sampling date

**Table 2.** Timetable of treatment switches and plant harvests for Spring Experiment.

| Plant | 1/23/08 | 2/13/08 | 3/5/08 | 4/2/08 | 5/7/08 |
|---|---|---|---|---|---|
| 1 | 0 | H | - | - | - |
| 2 | 0 | 0 | 0 | 150 | H |
| 3 | 150 | 0 | H | - | - |
| 4 | 150 | 0 | 0 | 150 | H |
| 5 | 0 | H | - | - | - |
| 6 | 150 | 0 | 0 | H | - |
| 7 | 150 | 0 | 0 | H | - |
| 8 | 0 | 150 | 150 | H | - |
| 9 | 150 | 150 | 150 | H | - |
| 10 | 0 | 150 | 0 | 150 | H |
| 11 | 0 | 0 | 0 | H | - |
| 12 | 150 | 0 | 150 | H | - |
| 13 | 150 | 0 | 150 | H | - |
| 14 | 0 | 150 | 150 | H | - |
| 15 | 150 | 150 | 150 | H | - |
| 16 | 0 | 0 | 0 | H | - |
| 17 | 0 | 150 | 0 | H | - |
| 18 | 150 | H | - | - | - |
| 19 | 150 | H | - | - | - |
| 20 | 150 | 150 | H | - | - |
| 21 | 0 | 0 | H | - | - |
| 22 | 150 | 0 | H | - | - |
| 23 | 0 | 150 | H | - | - |
| 24 | 0 | 150 | H | - | - |

0 = 0 mg $L^{-1}$ N, 150 = 150 mg $L^{-1}$ N, H = Harvest/Sampling date

Four sets of leaf samples were tested for starch content (Johansen, 1940) by dripping iodine on a small leaf cross-section. The remaining part of the leaf was used for confocal (fresh) and transmission electron microscopy (TEM). The remainder of the plants were either held at the same N treatment or switched to the other treatment. After leaves had re-greened once again (approx. 3 weeks), similar data were taken and remaining plants were either switched to the other N treatment or maintained in the same N treatment. After leaves had re-greened once again, four weeks this time, similar data were taken and the experiment was terminated (15 weeks total).

Nutrient solutions were discarded and replaced with new solution as needed. This was determined by solution levels (roots covered by solution) and usually occurred 1-2

times per week. Solutions for macronutrients were prepared from potassium phosphate ($KH_2PO_4$), magnesium sulfate ($MgSO_4 \bullet 7H_2O$), potassium nitrate ($KNO_3$), ammonium nitrate ($NH_4NO_3$), calcium chloride ($CaCl_2 \bullet 2H_2O$), and/or calcium phosphate [$Ca(H_2PO_4)_2 \bullet H_2O$]. The pH of nutrient solutions was adjusted to 6.0 using 1 M potassium hydroxide (KOH). The 150 mg $L^{-1}$ N solution consisted of 68% $NO_3$-N and 32% $NH_4$-N. A stock solution of micronutrients was prepared from boric acid ($H_3BO_3$), sodium molybdate ($Na_2MoO_4 \bullet 2H_2O$), copper EDTA, iron EDTA, manganese EDTA, and zinc EDTA. Nutrients supplied (in mg $L^{-1}$) were 48.5 Ca, 24 Mg, 75 P, 95-150 K, 32 S, 0.5 B, 0.02 Cu, 1 Fe, 0.5 Mn, 0.01 Mo, 0.05 Zn.

Microscopy samples were collected from fully expanded leaves (3$^{rd}$ full pair from the top). Half of a selected leaf was fixed for TEM samples (Paparozzi, 1981) and also used as fresh tissue for confocal microscopy. The other half was fixed in formalin-acetic acid-alcohol (FAA) (Berlyn and Miksche, 1976) to be later embedded in paraffin and stained for future anatomical study.

At harvest for drying, each plant was separated into top, middle, bottom, peduncles/stems, and roots. Samples were dried at 60 °C and dry weights measured. A Wiley mill with a 20-mesh screen was then used to grind samples. For carbohydrate analysis, ground samples from leaves at the tip (and middle if necessary to get 100 mg) were combined and the soluble carbohydrates (specifically reducing sugars) were extracted using a hot water extraction and the Nelson-Somogyi assay (Nelson, 1944; Somogyi, 1952).

Data were analyzed using PROC MIXED such that each treatment switch could be compared within and between in a pair-wise fashion (T-test) (Littell et al., 2006). This results in 78 possible pair-wise comparisons. An example of how these are set up and interpreted is in Table 3.

## 3. Results and Discussion

### *Plant growth*

Visually, foliage, stems and roots displayed the typical N deficiency symptoms of yellowing leaves, reddened petioles and stems and reduced root growth. Photographs of roots, stems and leaves (pictures not shown) and dry weights indicated that if plants received N at any time during the 15 weeks of the experiments, root growth was promoted and sustained. For the variable total dry weight of the plant (roots and shoots) all but 10 (Fall) and 15 (Spring) of the pair-wise comparisons were significantly different from one another (Table 4). As expected, whenever a plant received nitrogen, at any time, and then was compared to one that never received nitrogen, there was a significant increase in growth. The treatments that were not different included all comparisons among the 0 N treatments. As expected, if a plant was with-

out N for one or more of the 3-week intervals then they weighed less and thus, grew less. Of particular interest were plants that received no N over 6 or 10 weeks. These plants, which received no nitrogen, were comparable in dry weight to plants receiving 150 mg $L^{-1}$ N for 3 weeks only or 0 N for 3 weeks and then 150 mg $L^{-1}$ N for 3 weeks. This implies that there is a lag in growth response once N is re-introduced to the plant after the plant has received no N for 3 weeks in hydroponics and/or during the rooting of the cuttings (5 weeks).

Plants were generally heavier and larger in the Spring (Table 4), however, generally the same statistical significance between treatments occurred.

*Carbohydrate content*

Regardless of treatment, all leaves sampled tested positive for the presence of starch (Korus et al., 2008). This was a bit puzzling as one would predict that the amount of stored carbohydrate should decrease as leaves lose their green color (chlorophyll) because less chlorophyll implies less photosynthesis and thus, less sugar production. However, when leaves were viewed under the confocal and transmission electron microscope (Figures 1-3), starch grains were present in the chloroplasts of all plants regardless of treatment with obvious differences in number and starch grain size.

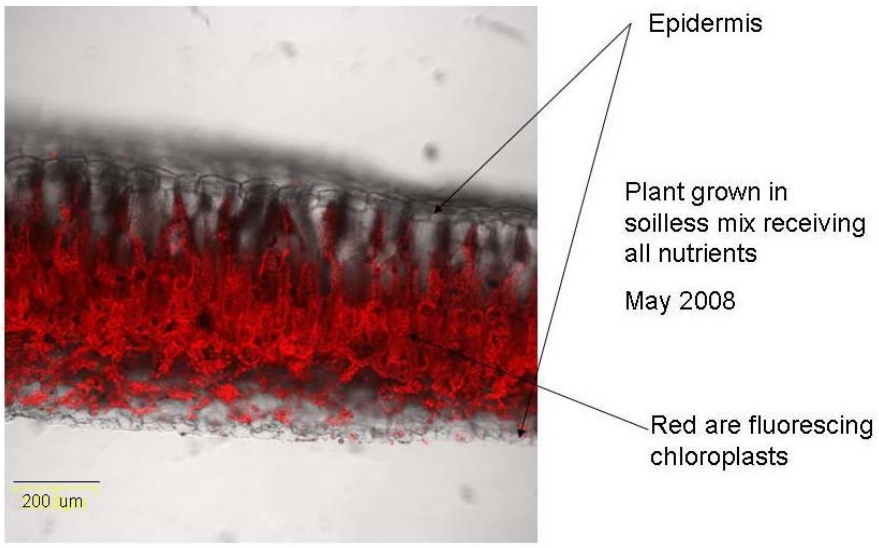
**Table 3.** Five examples of pair-wise comparisons testing the effect of either 0 or 150 mg $L^{-1}$ N on the subsequent combinations of N for the variable dry weight – Fall Experiment. For example – in line 1 - plants that received 0 N for 10 weeks (0 first, 00 next) are compared to plants that received 0 N for 6 weeks (0 first, 0 next). Line 2- plants that received 0N for 10 weeks (0 first, 00 next) are compared to plants that received 0 N for 3 weeks, 150 mg $L^{-1}$ N for 3 weeks and then 0 N for the last 4 weeks (0 first, 1500 next). n = no further treatment/harvested.

| Effect | first | next | _first | _next | Estimate | Error | DF | *t* Value | Pr > \|*t*\| |
|---|---|---|---|---|---|---|---|---|---|
| first*next | 0 | 00 | 0 | 0n | 0.1650 | 1.7646 | 12 | 0.09 | 0.9270 |
| first*next | 0 | 00 | 0 | 1500 | –8.5800 | 1.4408 | 12 | –5.95 | <.0001 |
| first*next | 0 | 00 | 0 | 150150 | –9.8783 | 1.3153 | 12 | –7.51 | <.0001 |
| first*next | 0 | 00 | 0 | 150n | –1.6600 | 1.4408 | 12 | –1.15 | 0.2717 |
| first*next | 0 | 00 | 0 | nn | 0.2050 | 1.4408 | 12 | 0.14 | 0.8892 |

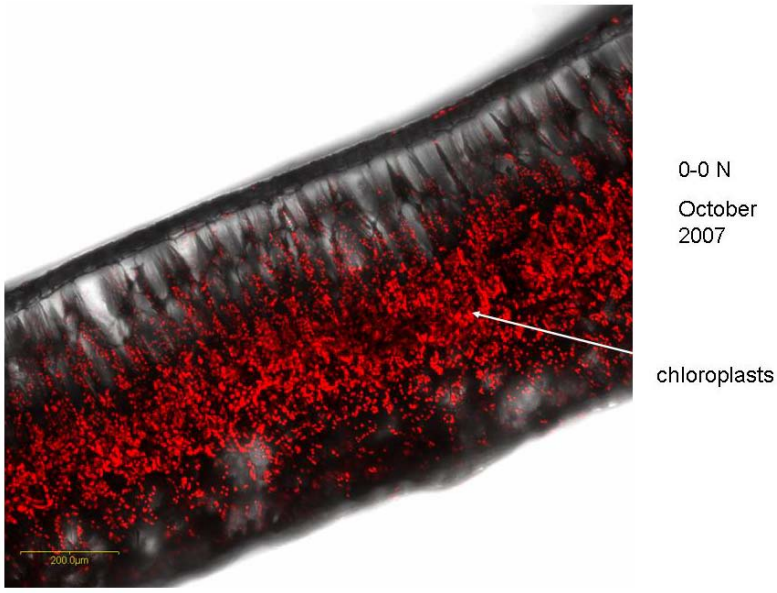
**Table 4.** Total dry weight of *Plectranthus parviflorus* leaves upon harvest. Fall – Pair-wise comparisons that were NOT statistically different were treatment 2 vs. 1, 3, 4, 7; trt 4 vs. 3, 7, 12; trt 6 vs. 10 and trt 9 vs. 6, 8. For Spring, pair-wise comparison that were NOT statistically different were trt 2 vs.1,3,4,7; trt 4 vs. 3, 7, trt 1 vs. 3, 7; trt 3 vs. 7; trt 5 vs. 6, 8; trt 8 vs. 6, 10 and trt 9 vs. 6, 10.

| **First sampling - Fall** | | | **Second Sampling** | | | **Third sampling** | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | Nitrogen $mg \cdot L^{-1}$ | Dry weight g | Treatment | Nitrogen $mg \cdot L^{-1}$ | Dry weight g | Treatment | Nitrogen $mg \cdot L^{-1}$ | Dry weight g |
| 1 | 0 | 0.66 ± 1.01 | 3 | 0-0 | 0.70 ± 1.44 | 7 | 0-0-0 | 0.87 ± 1.02 |
| 2 | 150 | 1.98 ± 0.83 | 4 | 0-150 | 2.53 ± 1.02 | 8 | 0-150-0 | 9.45 ± 1.02 |
| | | | 5 | 150-0 | 8.42 ± 1.02 | 9 | 0-150-150 | 10.74 ± 0.83 |
| | | | 6 | 150-150 | 13.63 ± 1.44 | 10 | 150-0-0 | 15.37 ± 1.02 |
| | | | | | | 11 | 150-0-150 | 27.60 ± 1.02 |
| | | | | | | 12 | 150-150-150 | 41.72 ± 1.02 |
| **First sampling - Spring** | | | **Second Sampling** | | | **Third sampling** | | |
| Treatment | Nitrogen $mg \cdot L^{-1}$ | Dry weight g | Treatment | Nitrogen $mg \cdot L^{-1}$ | Dry weight g | Treatment | Nitrogen $mg \cdot L^{-1}$ | Dry weight g |
| 1 | 0 | 1.42 ± 1.38 | 3 | 0-0 | 1.64 ± 1.95 | 7 | 0-0-0 | 1.57 ± 1.38 |
| 2 | 150 | 2.93 ± 1.38 | 4 | 0-150 | 4.20 ± 1.38 | 8 | 0-150-0 | 16.98 ± 1.95 |
| | | | 5 | 150-0 | 12.95 ± 1.38 | 9 | 0-150-150 | 20.91 ± 1.38 |
| | | | 6 | 150-150 | 15.95 ± 1.95 | 10 | 150-0-0 | 21.26 ± 1.02 |
| | | | | | | 11 | 150-0-150 | 37.33 ± 1.38 |
| | | | | | | 12 | 150-150-150 | 74.57 ± 1.38 |

**Figure 1.** Confocal micrographs of a trans-section through fresh leaves taken from a plant that was grown with the experiment but not subjected to any treatments (A) and a plant that had not received any nutrients during rooting and 6 weeks of the experiment (B). bar = 200 um



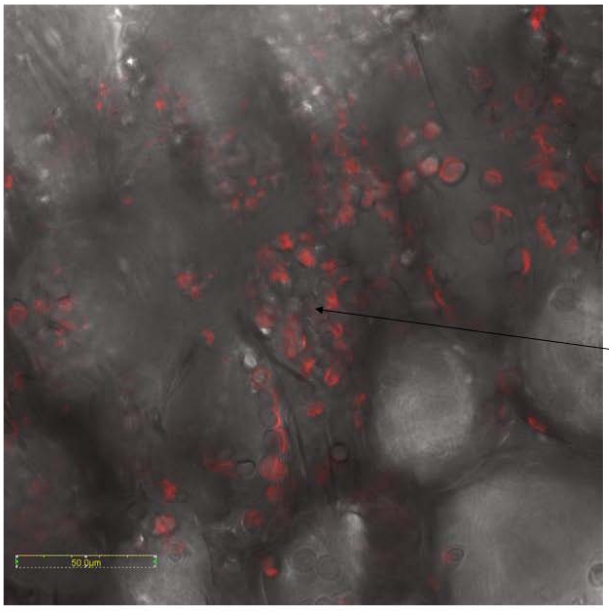

A.




B.

**Figure 2.** Confocal micrographs of a trans-section through fresh leaves taken from a plant that without nitrogen during the whole experiment (A) and a plant that had not received any nutrients during rooting and then was given nitrogen for 3 weeks (leaves re-greened) and then deprived of nitrogen for the last 3 weeks of the experiment (B). bar = 50um

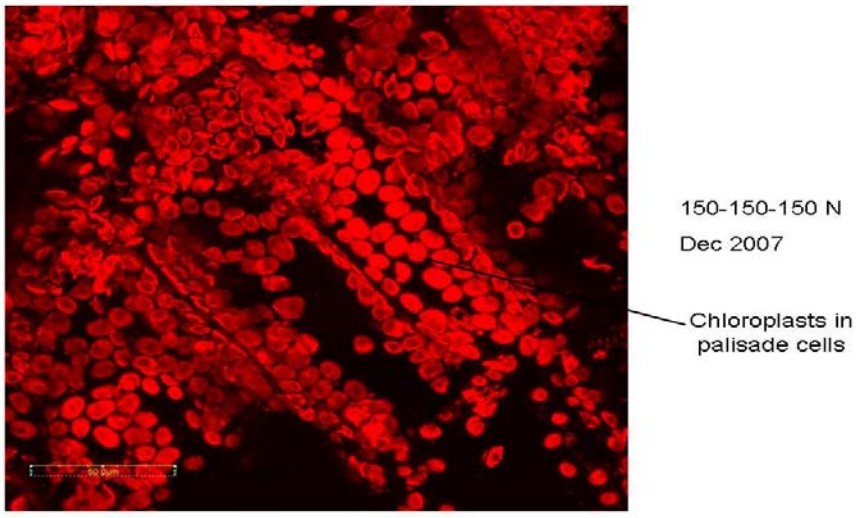
A.



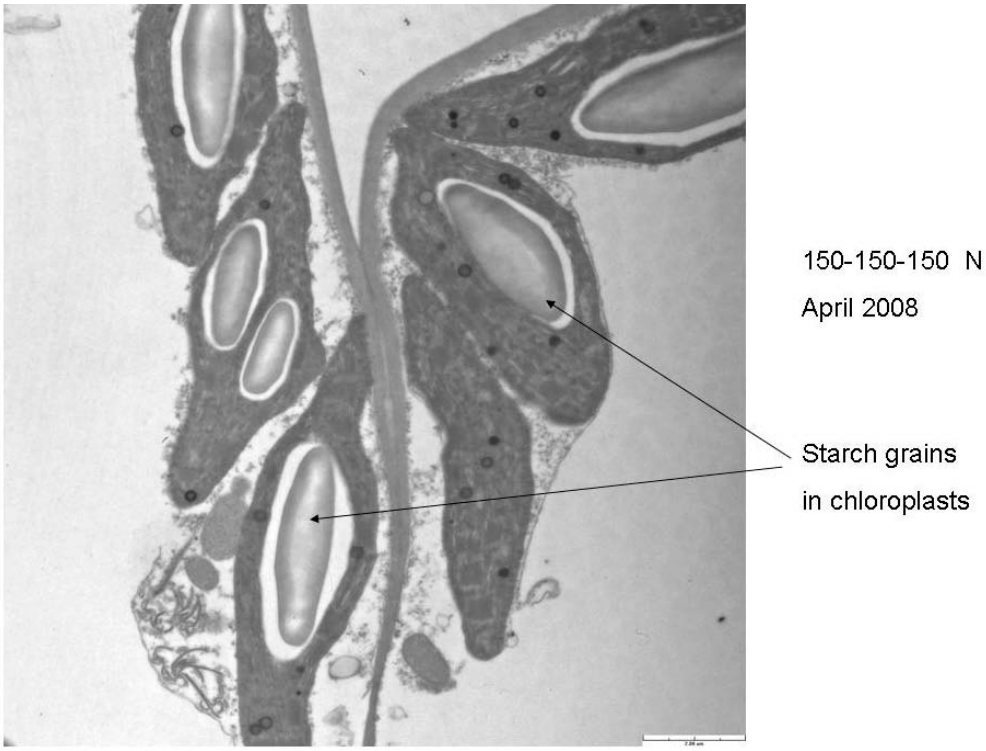

B.

**Figure 3.** Confocal micrograph (A; bar = 50 um) and a transmission electron micrograph (B; bar =2 um) of a trans-section through a leaf taken from a plant that received nitrogen during the whole experiment.

A.




B.



For the soluble carbohydrate (reducing sugars) data, it is helpful to think of the data presented as associated with three different physiological periods in the plant's growth. The first physiological phase is comprised of the first three weeks when plants were either growing well (150 mg $L^{-1}$ N treatment 2) or were ceasing to grow due to nitrogen stress (0 mg $L^{-1}$ N; treatment 1). The second physiological phase is comprised of the next 3 weeks when plants either cease growing due to nitrogen stress (150 mg $L^{-1}$ N to 0; treatment 5), start to recover (0 to 150 mg $L^{-1}$ N; treatment 4), are fully nitrogen stressed (0 to 0 mg $L^{-1}$ N; treatment 3) or a growing well (150 to 150 mg $L^{-1}$ N; treatment 6). The third physiological phase, comprised of the last four weeks, is designed to impose repeated nitrogen stress (0-150-0 mg $L^{-1}$ N; treatment 8 and 150-0-0 mg $L^{-1}$ N; treatment 10), recovery of plant growth due to added nitrogen (0-150-150 mg $L^{-1}$ N; treatment 9; and 150-0-150 mg $L^{-1}$ N; treatment 11) as compared to extreme nitrogen stress (0-0-0 mg $L^{-1}$ N; treatment 7) and ideal growing conditions (150-150-150 mg $L^{-1}$ N; treatment 12).

During the Fall when plants are growing well (receiving 150 mg $L^{-1}$ N during any or all 3 physiological stages) reducing sugar concentration ranged from 1.23 to 3.16 mg·100 $mg^{-1}$leaf tissue (Table 5). However, when leaves were nitrogen stressed after receiving nitrogen (150-0 and 150-0-0; treatments 5 and 10, respectively), there was a significant increase in the sugar concentration. This increased concentration should signal promotion of the senescence process and allow for abscission of these leaves (Gan, 2008). However, plants receiving nitrogen after nitrogen stress (150-0-150; treatment 11) re-green and contain levels of sugars comparable to plants that have received 2 or 3 periods of nitrogen (0-150-150 and 150-150-150; treatments 9, 12). The three treatments that were a series of 0 mg $L^{-1}$ N showed a similar concentration of reducing sugars when compared to treatments receiving nitrogen. Since net carboxylation rates (photosynthesis) are extremely low in plants under these three treatments (Paparozzi data unpublished), it is probable that the starch present in these leaves is being slowly broken down to supply the plant with soluble carbohydrates. This would keep respiration and other cellular processes going and allow for the subsequent re-greening and growth (Table 4) of these leaves. This hypo-thesis is supported by the appearance of cracks or fissures (presumptive) in the starch grains (Figure 4).

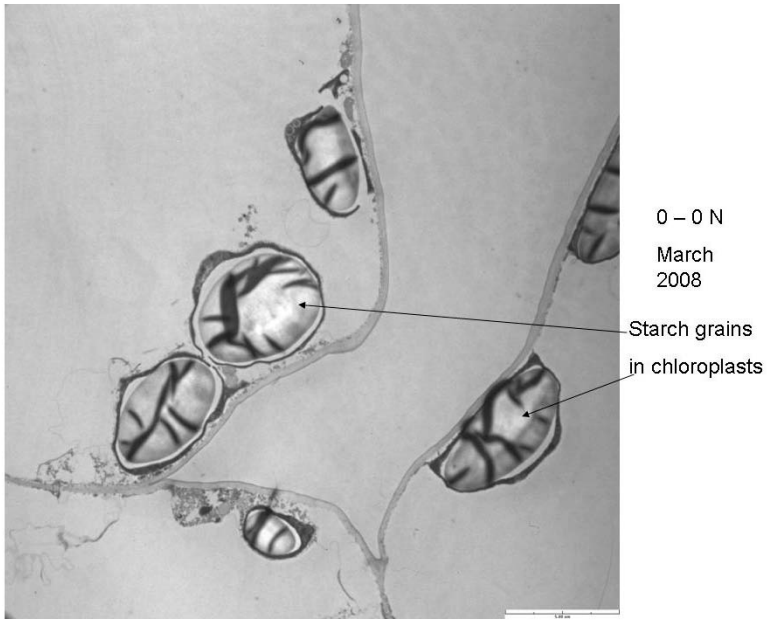
In the Spring experiment, rooted cuttings started with a higher concentration ofreducing sugars than the rooted cuttings used in the Fall experiment. This is reflected in the sugar concentration of all the treatments, but particularly those receiving no nitro-gen for the entire experiment or those grown for only one 3-week physiological period. As a result, plants that never received any nitrogen during the three physiological periods (0; 0-0; 0-0-0 mg $L^{-1}$ N) contained significantly more reducing sugars than all other treatment combinations except the 0-150-0 mg $L^{-1}$ N (treatment 8).

**Table 5.** Soluble carbohydrate (reducing sugars) concentration of *Plectranthus* leaves upon harvest. For Fall – Significantly different pairwise comparisons (p=0.05) were treatment 10 vs. all others; and treatment 5 vs. 1, 2, 9, 11, 12. For Spring, Treatment 1 vs. all but 8; Trt 2 vs. 3, 4, 11, 12; and Trt 7 versus 6, 9, 11.

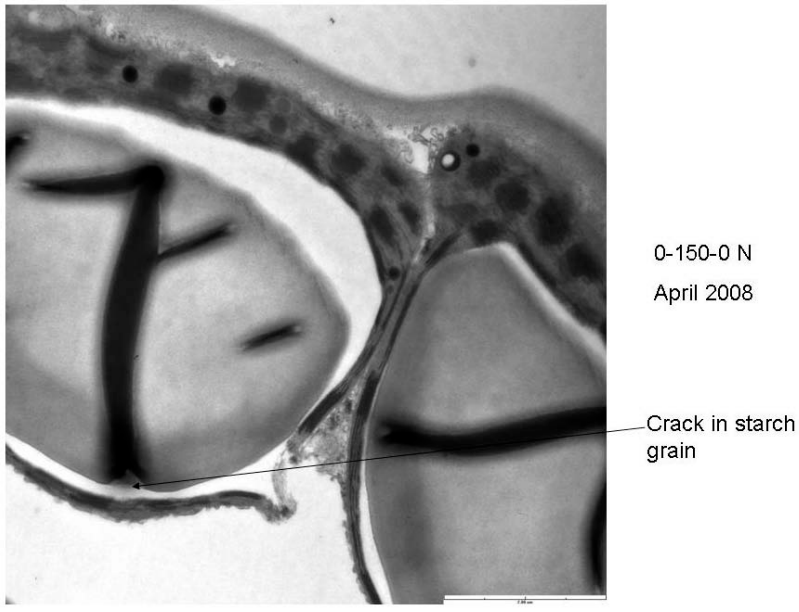
| **First sampling - Fall** | | | **Second Sampling** | | | **Third sampling** | | |
|---|---|---|---|---|---|---|---|---|
| Treatment | Nitrogen $mg \cdot L^{-1}$ | Reducing Sugars $mg \cdot 100\ mg^{-1}$ leaf tissue | Treat-ment | Nitrogen $mg \cdot L^{-1}$ | Reducing Sugars $mg \cdot 100\ mg^{-1}$ leaf tissue | Treatment | Nitrogen $mg \cdot L^{-1}$ | Reducing Sugars $mg \cdot 100\ mg^{-1}$ leaf tissue |
| 1 | 0 | 1.95 ± 0.56 | 3 | 0-0 | 2.35 ± 0.79 | 7 | 0-0-0 | 2.79 ± 0.56 |
| 2 | 150 | 2.08 ± 0.45 | 4 | 0-150 | 2.75 ± 0.56 | 8 | 0-150-0 | 2.84 ± 0.56 |
| | | | 5 | 150-0 | 3.72 ± 0.56 | 9 | 0-150-150 | 1.47 ± 0.45 |
| | | | 6 | 150-150 | 3.16 ± 0.79 | 10 | 150-0-0 | 6.94 ± 0.56 |
| Initial cuttings | | 2.23 ± 0.92 | | | | 11 | 150-0-150 | 1.23 ± 0.56 |
| Rooted cuttings | | 0.87 ± 0.16 | | | | 12 | 150-150-150 | 1.27 ± 0.56 |
| **First sampling - Spring** | | | **Second Sampling** | | | **Third sampling** | | |
| Treatment | Nitrogen $mg \cdot L^{-1}$ | Reducing Sugars $mg \cdot 100\ mg^{-1}$ leaf tissue | Treat-ment | Nitrogen $mg \cdot L^{-1}$ | Reducing Sugars $mg \cdot 100\ mg^{-1}$ leaf tissue | Treatment | Nitrogen $mg \cdot L^{-1}$ | Reducing Sugars $mg \cdot 100\ mg^{-1}$ leaf tissue |
| 1 | 0 | 5.32 ± 0.72 | 3 | 0-0 | 4.03 ± 1.02 | 7 | 0-0-0 | 4.25 ± 0.72 |
| 2 | 150 | 3.69 ± 0.72 | 4 | 0-150 | 3.00 ± 0.72 | 8 | 0-150-0 | 3.77 ± 1.02 |
| | | | 5 | 150-0 | 3.13 ± 0.72 | 9 | 0-150-150 | 1.42 ± 072 |
| | | | 6 | 150-150 | 1.40 ± 1.02 | 10 | 150-0-0 | 3.18 ± 0.72 |
| Initial cuttings | | 2.40 ± 0.31 | | | | 11 | 150-0-150 | 1.24 ± 0.72 |
| Rooted cuttings | | 2.37 ± 0.62 | | | | 12 | 150-150-150 | 2.90 ± 0.72 |

**Figure 4.** Transmission electron micrographs of a trans-section through a leaves taken from a plant that received no nitrogen during the 6 weeks of the experiment (A) and a plant that had not received any nutrients during rooting and for 3 weeks, then was given nitrogen for 3 weeks (leaves re-greened) and then deprived of nitrogen for the last 3 weeks of the experiment (B). bar = 2um

A.




B.




The overall pattern however is the same as in the Fall experiment. When a plant is receiving adequate N and all other environmental factors are stable, it is pho-

tosynthesizing, thus, adequate amounts of reducing sugars are available. So, even though storage of large amounts of carbohydrates as starch may not be necessary, *Plectranthus* does indeed store starch at all three physiological periods. This is important as once these plants are nitrogen-stressed starch breakdown is necessary to make reducing sugars available.

From both experiments, it appears that as long as nitrogen is present, the plant continues to grow, have reducing sugars available for growth and store starch. When this plant is nitrogen-stressed, it must receive a signal(s) to slow down metabolism, possibly allow more starch to accumulate and then slowly break down the starch so that respiration and other metabolic processes can maintain the plant but not promote growth. Plants that store starch are often termed 'starch accumulators' (Frantz and Bugbee, 2005). We propose that *P. parviflorus* is one of these types of plants and that this feature aids this plant in surviving nitrogen stress.

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