Plant carbohydrate depletion impairs water relations and spreads via ectomycorrhizal networks.

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Main Text

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Abstract

Carbon and water relations are fundamental to plant life and strongly interact. Under drought, the ability of plants to assimilate carbon is reduced, which increases their consumption of stored labile carbon in the form of non-structural carbohydrates (NSC). Stored NSC depletion may impair plant water relations, but mechanisms are not clear, and we do not know if its effects are independent of water deficit. If so, carbon costs of fungal symbionts could also indirectly influence plant drought tolerance through stored NSC depletion. We connected well-watered *Pinus ponderosa* seedling pairs via ectomycorrhizal (EM) networks where one seedling was shaded and the other experienced full light and compared responses to seedling pairs in the light. We measured plant water relations and traced carbon movements using $^{13}CO_2$ to explore the mechanisms linking stored NSC to water relations, and to identify potential tradeoffs between the ability to endure low water potentials and maintaining EM fungi under carbon-limiting conditions. Even in the absence of drought, mild NSC depletion impaired osmoregulation capacity and turgor maintenance, a critical strategy to tolerate drought. This demonstrates that NSC storage influences plant water relations independently of plant water status. We also found that EM networks propagated NSC depletion and its negative effects on water relations from carbon stressed hosts to non-stressed hosts. These results highlight carbon allocation tradeoffs between supporting fungal symbionts and retaining water via stored NSC and have implications for biotic interactions and forest drought responses.

Significance Statement

The potential effects of future drought on global carbon cycles, vegetation-climate feedbacks, species distributions and their ecological impacts, urgently call for a clear understanding of factors influencing vegetation tolerance to drought. Key to this is the understanding of mechanisms and processes by which plants tolerate drought and how prevalent plant-fungal interactions may influence these processes.

We demonstrate that even mild depletion of plant non-structural carbohydrate (NSC) storage readily decreases plant water retention capacity, therefore decreasing tolerance to drought. Because plant-fungal interactions depend on NSC exchange, plants face carbon-allocation tradeoffs between maintaining drought tolerance and feeding fungal symbionts. The impacts of these tradeoffs extend across plants connected via ectomycorrhizal networks as fungi propagate NSC depletion from NSC-limited plants to non-stressed individuals.

Main Text

Introduction

Under future climate, more intense and frequent droughts are expected to increase forest mortality and to impact forest ecosystems and global carbon cycles [1]. Predicting the extent of these impacts requires a full understanding of the mechanisms and processes associated with drought-induced mortality (DIM) [2,3]. Under drought, plants ultimately die from desiccation because water supply does not keep pace with water loss. Desiccation and death are generally associated with failure of the vascular system to transport water due to embolism formation (hydraulic failure, Adams *et al.* 2017). Reduced photosynthesis under prolonged drought eventually leads to the depletion of stored non-structural carbohydrates (NSC) and carbon limitation, a process thought to contribute to desiccation by decreasing the plant's ability to retain

66 and move water [5–8]. Hydraulic failure is thought to be the main driver of DIM with NSC depletion
67 playing a secondary, interacting role. However, it is not known whether NSC depletion *per se*
68 impairs plant water relations because its effects have not been experimentally isolated from those
69 caused by drought (e.g., reduced water transport) (Hartmann and Trumbore 2016, Adams et al.
70 2017). Yet, NSC depletion may be more relevant than currently thought as the wide array of
71 physiological processes that demand NSC may limit carbon allocation to storage and its positive
72 effects on water relations. For instance, in addition to metabolic costs, many trees incur significant
73 carbon costs to maintain symbiotic partnerships with ectomycorrhizal (EM) fungi [11]. Consequently,
74 plants may face carbon allocation tradeoffs between sustaining symbionts and maintaining water
75 relations via NSC storage under carbon-limiting conditions. Whether these tradeoffs exist has not
76 been tested despite their potential implications for forest mortality under global change. In this
77 study, we examine the independent effects of stored NSC for plant water relations and assess
78 potential tradeoffs between maintaining water relations and sustaining EM symbionts when
79 carbon becomes limiting.

80 Stored NSC have been classically understood as a carbon safeguard for growth and energy.
81 However, NSC may play an important role in the movement and retention of water [5] including
82 lowering tissue osmotic potential to increase tolerance to low water potentials under drought [12].
83 While the osmotically active soluble fraction of NSC plays a direct osmotic role, starch could also
84 play an indirect role as a source of osmotically active organic compounds and energy [13]. Under
85 carbon-limiting conditions of any kind, NSC-consuming processes such as respiration, defense
86 production, and maintenance of ectomycorrhizae rely on the same pool of stored NSC potentially
87 involved in osmoregulation [14]. Consistently, complete consumption of stored NSC is rare under
88 natural and experimental conditions, and plants rarely deplete their stored NSC below ca. 40% of
89 their seasonal maximums [4,15]. While NSC have been associated with maintenance of hydraulic

90 conductivity, capacitance, and turgor [9,16,17], it is unclear how much of the total stored NSC pool
91 plants must conserve to maintain these functions, even under non-drought conditions [18]. If a
92 significant fraction of stored NSC must remain unconsumed to maintain the capacity to move and
93 retain water, we may have underestimated the role of NSC depletion in DIM, which could have
94 important implications for modelling and prediction of tree mortality.

95 In temperate coniferous and boreal ecosystems, virtually all trees and seedlings form
96 associations with EM fungi and establish underground EM networks through which resources can
97 be exchanged [19–21]. These networks have been shown to enhance seedling establishment and
98 increase ecosystem-level nutrient cycling [22,23]. Plant hosts allocate up to 30% of their assimilated
99 carbon to EM fungi [11,24] in exchange for nutrients. As such, EM fungi could indirectly influence
100 tolerance to drought via network-mediated carbon redistribution and host NSC depletion under
101 carbon-limiting conditions. For instance, if stochastic or microsite effects (e.g., herbivory, shading,
102 microsite low moisture) cause carbon deficit and NSC depletion on some plant hosts but not
103 others within the network, NSC-depleted hosts could decrease carbon supply to their associated
104 EM fungi and trigger fungal carbon deficit. Non-NSC-depleted hosts may then offset the deficit of
105 the EM network with their own stored NSC, a response that could indirectly compromise their own
106 water relations especially if they cannot regulate carbon flow to EM fungi. There is debate on
107 whether carbon can be transferred among connected hosts via EM networks [20,25,26] or whether
108 NSC simply stays within the EM fungi [27]. In either case, network-mediated carbon redistribution
109 may influence how individual plants and entire forest stands respond to drought. Whether and
110 under what circumstances fungal carbon demand and redistribution compromise allocation to
111 plant NSC storage and water relations is unknown but deserves attention.

112 Here, we used an experimental design that depletes plant NSC pools by combining shading
113 treatments and EM networks among well-watered ponderosa pine seedlings (*Pinus ponderosa*

114 Douglas ex C. Lawson). This design allowed us to unveil the mechanisms (and their importance)
115 linking stored NSC and plant water relations without the confounding effects of water deficit, as
116 well as the carbon dynamics between ectomycorrhizal fungi and their plant hosts under carbon-
117 limiting conditions. Our experiment involved seedlings as they often determine forest distributions
118 through establishment [28]. Also, because of their small size and little storage capacity, seedlings
119 may be especially susceptible to carbon allocation tradeoffs between maintaining water relations
120 and EM fungi. Specifically, we asked 1) does stored NSC depletion affect host water relations even
121 without water deficit?; and 2) do ectomycorrhizal networks extend the effects of NSC depletion on
122 plant water relations to non-stressed neighbors? We found that, even in the absence of drought,
123 mild NSC depletion indirectly decreased plant tolerance to drought by impairing osmoregulation
124 capacity and turgor maintenance. These results unambiguously link NSC storage to plant water
125 relations. We also found that EM networks propagated NSC depletion and impaired critical
126 drought tolerance strategies in non-stressed hosts. This demonstrates the existence of resource-
127 allocation tradeoffs between supporting fungal symbionts and retaining water via stored NSC and
128 suggests that EM networks can affect tree and possibly entire forest tolerance to drought.

129 **Results**

130 NSC decreased in LD and D seedlings

131 Shading caused a 72% reduction in whole-plant NSC concentrations in dark (D) relative to
132 light (LL) seedlings ($p < 0.001$). Light seedlings paired with dark (LD) plants also experienced NSC
133 depletion (45% reduction) and showed intermediate whole-plant NSC pools relative to LL and D
134 seedlings ($p < 0.001$, and $p = 0.009$, respectively) (Fig. 2). When broken down to each NSC
135 compound, we found that starch was higher in LL seedlings ($p < 0.001$) relative to LD and D
136 seedlings. LD seedlings had intermediate starch levels although only marginally higher than D

137 seedlings ($p = 0.079$). Both sucrose and glucose + fructose were lower in D seedlings than in LL and
138 LD seedlings (sucrose: LL: $p < 0.001$, LD: $p = 0.040$; glucose and fructose: LL: $p < 0.001$, LD: $p =$
139 0.018). However, no differences were detected between LL and LD seedlings. Patterns within each
140 organ were consistent with those observed at the whole-plant level (Figure S6).

141 Labeled carbon stayed within ectomycorrhizal fungi

142 Labeled LL and LD seedlings showed significant increases in $^{13}C/^{12}C$ ratios ($\delta^{13}C$) across all
143 organs relative to pre-labeling base-line values (Fig. 3, Figure S7) while non-labeled seedlings
144 stayed at base-line levels. This indicates that only labeled seedlings incorporated the C-isotope and
145 that there was no contamination during the labeling process. In labeled seedlings, ^{13}C progressively
146 declined from needles to roots ($p < 0.001$ in all pairwise contrasts across seedling types and
147 organs, Fig. 3, Figure S7: Panels a-c). High levels of ^{13}C were also present in fungal tissue associated
148 with labeled LL and LD seedlings (Fig. 3, Figure S8: Panel a). To a lesser extent, ^{13}C was also present
149 in fungal tissue associated with non-labeled LL plants paired with labeled LL plants (Fig. 3, Figure
150 S8: Panel b). ^{13}C in fungi associated with non-labeled LL plants confirmed the existence of a
151 mycorrhizal network between seedlings at harvesting (Figure S1 and S2). However, ^{13}C was not
152 detected in colonized root tips of non-labeled D plants paired with labeled LD seedlings (Fig. 3,
153 Figure S8: Panel b). In all cases, ^{13}C levels within non-labeled seedlings were similar to base-line
154 values regardless of organ or seedling type (Fig. 3, Figure S7: Panels d-f).

155 LD and D seedlings exhibited lower turgor

156 Hydraulic conductivity, which describes the capacity to transport water, did not
157 significantly differ among seedling types in any organ (Figure S9). However, osmotic potentials,
158 which describe the ability to retain cell water at low water potentials, as is typical under drought,
159 were up to ca. 1.5 MPa lower (i.e., higher water retention capacity) in LL seedlings than in LD and

160 D seedlings (Fig. 4a-c). These differences were observed in needles ($p < 0.001$ in all pairwise
161 contrasts), stems (D: $p = 0.002$, LD: $p < 0.001$), and roots (D: $p < 0.001$, LD: $p = 0.001$). High osmotic
162 potentials were associated with low pressure potentials (i.e., turgor) in D and LD needles (D: $p <$
163 0.001, LD: $p < 0.001$, Fig. 4d) and stems (D: $p = 0.012$, LD: $p < 0.001$, Fig. 4e). Pressure potentials in
164 LD and D stems were low enough to bring stems to turgor loss (i.e., pressure potential lower than
165 0). As a result, we observed lower stem relative water content in NSC-depleted seedlings than in
166 non-depleted plants (D: $p < 0.001$, LD: $p = 0.047$, Figure S10).

167 NSC depletion impaired water relations

168 NSC depletion in needles was associated with less negative (higher) needle osmotic
169 potentials ($p < 0.001$, $R = -0.83$, Table S2, Fig. 5a). This relationship was linear when NSC depletion
170 was large and plateaued when NSC were ca. 20% depleted relative to non-depleted seedlings. The
171 pressure potential (necessary for positive turgor) mirrored this pattern ($p < 0.001$, $R = 0.64$, Table
172 S2, Fig. 5b) and plateaued at 1.5 MPa when NSC was ca. 20% NSC depleted.

173 Stored NSC depletion also influenced the relationship between needle pressure potential
174 and needle water potential (WP) ($p < 0.001$, $R = 0.78$, Fig. 6; Table S2). Plants with greater NSC
175 depletion showed lower pressure at any given WP and higher WP at turgor loss (NSC deviation
176 from controls: $p < 0.001$, Fig. 6). Additionally, plants with greater NSC depletion lost more turgor
177 per unit decline in WP than less NSC-depleted plants with (Leaf WP x NSC deviation from controls:
178 $p = 0.006$, Fig. 6).

179 **Discussion**

180 It has long been known that plants exchange water (lost through stomata) for carbon
181 (assimilated from atmospheric CO_2). Our results demonstrate that plants also need stored labile
182 carbon in the form of non-structural carbohydrates (NSC) to retain water. We asked 1) whether

183 stored NSC depletion affect host water relations even without water deficit and 2) whether
184 ectomycorrhizal networks extend the effects of NSC depletion on plant water relations to non-
185 stressed neighbors. We found that NSC depletion impaired turgor maintenance -a critical drought
186 tolerance strategy- even in well-watered plants. We also found that carbon-limiting conditions can
187 result in a tradeoff between supporting symbionts and retaining water via stored NSC, and that
188 this effect can be propagated by the EM network to otherwise non-stressed plants. During periods
189 such as drought, when carbon supply via photosynthesis is limited, carbon allocation tradeoffs
190 may be exacerbated by EM fungi and influence plant survival.

191 Depletion of stored NSC impairs a critical drought tolerance strategy

192 Despite being well-watered, seedlings that experienced stored NSC depletion below 20%
193 of non-NSC-depleted plants experienced declines in turgor and osmoregulation capacity (Figs. 4-6).
194 Leaf osmotic potentials shifted from values typical of drought-tolerant temperate conifers (-2.3
195 MPa) to values greater than those found in drought-vulnerable croplands (-1.3 MPa) (Bartlett *et al.*
196 2012) (Fig. 4), which led to turgor loss (Fig. 6). These shifts were consistent with declines in total
197 NSC concentrations but were not always reflected by declines in the osmotically active fraction of
198 NSC (glucose, fructose, and sucrose). In fact, shifts in osmotic potential were mainly associated
199 with declines in osmotically inactive starch. These results suggest that the effects of NSC depletion
200 on osmoregulation are largely indirect, perhaps via the synthesis of osmotically active molecules
201 other than soluble sugars (e.g., sugar alcohols, proteins, amino acids) [29–31]. Regardless of the
202 specific mechanisms, however, our results show that stored NSC depletion impairs a critical
203 drought tolerance strategy in plants. The link between stored NSC and maintenance of osmotic
204 and pressure potentials could explain previous findings that NSC-depleted plants experience lower
205 phloem turgor under well-watered conditions [9], lower water content at any degree of water deficit
206 [6], and reach lower stem WP and die at faster rates than NSC-enriched plants when exposed to

207 similar water deficit [16]. Based on our results, and given that osmotic potentials decrease from
208 moist to dry biomes to compensate for low water potentials typical of dry regions [12], drought-
209 tolerant plants from dry biomes might require greater NSC pools to maintain lower osmotic
210 potentials than those from moist biomes and be more prone to experience carbon-allocation
211 tradeoffs.

212 Drought impairs plant water transport and depletes NSC, and both have negative effects
213 on plant water relations. Therefore, it is not surprising that both hydraulic failure and NSC
214 depletion are often associated with drought-induced mortality (DIM) [4,16]. However, despite an
215 increasing interest in the potential role of stored NSC on plant water relations [5,9,10], evidence that
216 NSC affects plant water relations independent of drought has been lacking. The negative impact of
217 NSC depletion on osmoregulation capacity and turgor maintenance we show here provides such
218 evidence. Our findings are consistent with the observation that plants prevent NSC depletion
219 below certain thresholds at all costs [15,32], as well as observed increases in stomatal conductance in
220 response to increases in osmotic potential [33]. Together, these findings suggest that NSC depletion
221 may further decrease drought tolerance by preventing total stomatal closure under drought.
222 While still hypothetical, this could explain sudden increases in stomatal conductance at low water
223 potentials [32], accelerated water loss [6] and accelerated declines in WP observed in drought-
224 stressed, NSC-depleted plants [16] leading to early hydraulic failure and death. Our results provide
225 support for a greater focus on plant water balance (turgor maintenance) in our efforts to
226 understand interactions between water and carbon to better monitor and predict DIM [6,7,34].
227 Mechanistic models that consider turgor loss may achieve higher species-specific predictive
228 capacity because turgor loss marks the onset of many processes that lead to plant death.
229 Additionally, turgor loss integrates many processes, including regulation of water transport, water
230 loss and water retention. Additional research is needed to understand the specific mechanisms by

231 which NSC depletion impairs osmoregulation across plant types, drought strategies, and
232 environments and how species-specific carbon allocation strategies, including to storage and
233 osmoregulation, influence tolerance to drought.

234 Ectomycorrhizal networks can exacerbate NSC depletion and carbon allocation tradeoffs

235 We hypothesized that shaded seedlings (D) would indirectly increase carbon demand on
236 connected, non-shaded seedlings (LD) due to a disruption of carbon allocation to EM fungi from D
237 seedlings. Our results supported this hypothesis: LD seedlings became NSC-depleted relative to LL
238 seedlings (Fig. 2), indicating that carbon demand in LD seedlings exceeded supply. Additional
239 measurements confirmed that assimilation did not decrease in LD relative to LL seedlings (i.e.
240 supply did not decrease; Figure S5). Likewise, there were no differences in growth and respiration
241 between LD and LL seedlings (Figure S5). Clearly, stored carbon was lost in LD seedlings during the
242 three weeks of shading, but it did not go to any other sink we could measure, leaving the EM
243 network as the most likely sink. However, we did not find more ^{13}C in the EM network in the LD
244 pair than the LL pair, and the fungal tissue closest to the D seedling was not enriched, which is
245 opposite to what we expected if the LD seedling bore a greater cost for supporting the network.
246 This is not because the EM network connecting the seedlings was lacking. We confirmed the
247 presence of the network both visually (Figures S1 and S2) and isotopically as ^{13}C travelled from
248 labeled seedlings to fungal tissue connected to neighboring, unlabeled seedlings (Fig 3). We
249 believe that the most likely explanation is that, by the time labeling occurred, the LD seedling had
250 either reduced carbon allocation to the EM network as costs exceeded benefits [24,35,36], or the EM
251 fungi closest to the starved D seedling had died or become dormant, reducing the overall carbon
252 demand on the LD seedling. While we cannot unambiguously attribute the missing carbon in LD
253 seedlings to increased allocation to EM fungi (and possibly D seedlings [20]), it does not change the
254 fact that the EM network propagated NSC depletion to a non-stressed plant and that this had

255 consequences for drought tolerance strategies. Thus, although EM fungi may increase survival by
256 hydraulic lift [19], they may also reduce plant tolerance to drought [37] by contributing to the carbon
257 allocation tradeoff between supporting symbionts and retaining water via stored NSC.

258 Carbon costs of sustaining EM may increase when plant or fungal carbon demand
259 increases [38] and during periods of carbon limitation such as prolonged drought [39]. Costs of
260 sustaining EM networks might also increase substantially when some individuals connected to the
261 network die (e.g. fire, drought, disease, and insect induced mortality) and the surviving trees must
262 subsidize carbon supply to EM until fungal biomass re-adjusts to new levels of carbon supply. In
263 these situations, EM networks might increase NSC depletion rates in hosts, hinder their
264 osmoregulation capacity, and hasten desiccation rates. It is possible that our pairwise approach
265 overestimates costs to individual seedlings and that EM network-driven NSC depletion is less
266 substantial in complex networks of highly interconnected plants where increased costs are
267 subsidized by several hosts [40,41]. As such, EM networks may even enhance community resilience to
268 drought, although this will likely depend on the proportion of stressed hosts within the network.
269 The carbon costs of EM-networks under carbon-limiting conditions could also be offset over time
270 by positive effects on overall performance or fitness that occur at times when carbon is not
271 limiting (e.g., enhanced growth, photosynthetic rate, nutrient acquisition) [42,43]. Studying all the
272 factors that influence costs and benefits of EM networks at different spatiotemporal scales is
273 critical to predict when and where they might have positive or negative effects under future
274 climates [44]. While studies often assess costs and benefits of mycorrhizal associations based on
275 whether they increase or decrease plant growth [45], growth is only one of several functions
276 requiring NSC and may not reflect costs and benefits for other vital plant functions, especially
277 under stressful conditions that inhibit growth. Using a carbon balance approach, we can integrate

278 all the physiological processes that require carbon (e.g., growth, defense, osmoregulation,
279 reproduction) and provide a more comprehensive cost-benefit assessment.

280 While our novel results highlight a critical role of stored NSC on plant water relations and a
281 potentially delicate balance between costs and benefits of EM networks under carbon stress,
282 much research is still needed. For instance, we need to elucidate the specific mechanisms
283 (including the involved organic solutes) by which stored NSC depletion decreases osmoregulation,
284 and how these mechanisms vary across species and climate gradients. We also need a better
285 understanding of the potential effect of plant-fungal interactions on NSC depletion and plant
286 water relations in complex EM networks under climate change. For instance, does individual-level
287 (as opposed to stand level) mortality caused by low intensity fire, drought or insects cause rippling
288 effects of similar magnitude to the ones we document when multiple trees are interconnected?
289 We hope that our research motivates such work.

290 **Methods**

291 Experimental Design

292 Ponderosa pine is one of the most widely distributed species in North America and has
293 been extensively used as a representative gymnosperm in ecological, physiological, and forestry
294 studies. In the spring of 2016, we planted eighty, one-year-old ponderosa pine seedlings (source:
295 Zone IV-V block of the Missoula Ponderosa Pine Seed Orchard, Department of Natural Resources)
296 in forty 19 L pots at the University of Montana greenhouse. Each pot contained two seedlings. We
297 used a soil mixture consisting of 40% sand, 30% topsoil, and 30% peat moss, which was kept at
298 field capacity throughout the duration of the experiment to avoid water deficit. Seedlings came
299 already inoculated with ectomycorrhizal Pezizales fungi from source. However, we also inoculated
300 the rhizosphere of each seedling with a mixture of *Rhizopogon* spores (Mycorrhizal Applications.

301 Grants Pass, OR) to ensure colonization. Both *Rhizopogon* and Pezizales form ectomycorrhizal
302 structures that potentially redistribute resources among plants [20,46]. Half of the pots contained a
303 stainless-steel mesh barrier that separated seedlings (139.7 µm wire diameter and 177.8 µm pore
304 diameter) which was penetrable by hyphae but not roots. The remaining pots contained a
305 Plexiglass barrier to prevent both root and ectomycorrhizal connections between seedlings to
306 create a control treatment and observe patterns in the absence of ectomycorrhizal networks. Both
307 barrier types were secured using silicone sealant. After inoculation, seedlings grew unperturbed
308 for 47 weeks and established mature ectomycorrhizal associations (Figure S1 and S2). Despite our
309 efforts, roots in pots with plexiglass barriers pierced the silicon sealant to form ectomycorrhizal
310 networks. While this precluded a treatment without EM networks, our main goal was to assess
311 how NSC depletion of neighbor seedlings influenced NSC pools and water relations in non-
312 stressed, connected hosts. Because all key variables measured did not differ between pots with
313 and without root connections (Table S1), the two barrier treatments were pooled for statistical
314 analyses. All subsequent treatments described below were applied uniformly in each of the two
315 underground barrier treatments.

316 In the fall of 2017, we split pots from each treatment into two groups (NSC-depleted and
317 non-depleted) and applied a 3-week NSC depletion treatment to one group using light-blocking
318 covers that reduced photosynthetically photon flux density below compensation point (Fig. 1,
319 Figure S3). In each pot within the NSC-depleted treatment, we placed a cover over one of the two
320 seedlings (dark, D) to inhibit photosynthesis while the other seedling (light paired with dark, LD)
321 was left undisturbed. Each cover consisted of a wire scaffolding (20 x 40 cm) overlaid with
322 aluminum foil that blocked incoming light while keeping air temperatures similar to those in non-
323 covered neighbor seedlings (ca. 24 ºC). We pierced 5 mm diameter holes evenly across the cover
324 walls which facilitated air flow, kept the atmosphere around the plant unsaturated at less than

325 50%, and allowed canopy transpiration. Relative humidity was measured using a Vaisala HMP35C
326 sensor (Vantaa, Finland). We maximized ventilation inside the covers while maintaining PAR below
327 0.40 µmol quanta m^{-2} s^{-1} which is far below compensation point (Figure S4). We measured PAR
328 with a Licor LI-190SA Quantum sensor (Lincoln, NE, USA). Combining shading with ectomycorrhizal
329 networks allowed us to alter the overall carbon budget of a networked system and indirectly
330 modify NSC pools in non-shaded plants. This procedure generated non-depleted networks with
331 both connected seedlings in the light (light, LL) and NSC-depleted networks with one seedling in
332 the light (LD) connected to one seedling in the dark (D). After three weeks of light deprivation, we
333 randomly selected plants from ten pots (five from each underground barrier treatment) in both
334 NSC-depleted and non-depleted treatments to assess physiological status (see below) of LL, LD,
335 and D seedlings and harvested them to establish base-line $^{13}C/^{12}C$ ratios ($\delta^{13}C$) in all organs across
336 seedling types. We then labeled with ^{13}C (see below) the remaining LD seedlings from NSC-
337 depleted networks and one of the two LL seedlings (chosen at random) from the non-depleted
338 NSC networks to assess potential carbon transfer between individuals in the presence and
339 absence, respectively, of carbon-limiting conditions seven days later. Comparing carbon transfer
340 between non-depleted and NSC-depleted networks let us assess how carbon transfer responds to
341 carbon limitation. One week after the labeling, we re-assessed physiological status and harvested
342 the remaining seedlings in both treatments to measure changes in $\delta^{13}C$ across all organs (Fig. 1).

343 Isotopic Labeling and Carbon Transfer Variables

344 Labeling was performed following similar methods to Song *et al.* (2015). We enclosed the
345 entire canopy of each labeled plant in a clear 2 L plastic bag. Each bag was injected with 20 mL of
346 Carbon-^{13}C dioxide (99 atom % 13C, <3 atom % 18O, Sigma Aldrich, (St. Louis, MO, USA)) for a ratio
347 of 10 mL of $^{13}C \cdot L^{-1}$ of air and was left undisturbed for 2 hours. ^{13}C injections were performed at
348 midday over two consecutive days. After each labeling event, enriched air was removed from bags

349 and directed outside the greenhouse facilities using an industrial vacuum, thus preventing
350 contamination of neighboring seedlings. Additionally, extra seedlings were randomly interspersed
351 throughout the treatments and their needles were used to assess possible contamination during
352 labeling. No signs of increased $\delta^{13}C$ were observed in these seedlings. Ground samples of dry
353 needles, stems, and roots of all remaining seedlings (labeled or not) were sent to the Stable
354 Isotope Facility at University of California, Davis and analyzed to obtain $\delta^{13}C$ values. We also
355 collected a representative amount of fungal material from ectomycorrhizal root tips of LL, LD, and
356 D seedlings (5 samples) before the labeling event to obtain base-line $\delta^{13}C$ fungal values as well as
357 several labelled LL (4) and LD (3) seedlings, and non-labelled LL (9), and D (10) seedlings after
358 labeling and analyzed their $\delta^{13}C$. Fungal samples were collected to ensure that the applied ^{13}C label
359 traveled from needles to fungal tissue on the labeled side as well as to fungal tissue of the paired,
360 non-labeled plants connected through the ectomycorrhizal network. These descriptive
361 measurements served as an extra validation of the existence of an ectomycorrhizal network
362 between seedling pairs already observed at the time of harvest (Figure S1).

363 Measurements of water relations

364 After re-acclimating D seedlings to light for 2 hours (see Figure S5, panel F), we measured
365 assimilation, stomatal conductance, and respiration in each seedling with a Licor 6400XT (Lincoln,
366 NE, USA). We then measured midday leaf and stem water potential (WP) (i.e., xylem tension due
367 to water deficit) immediately before harvesting (Fig. 1) using a pressure chamber (PMS Instrument
368 Company, (Corvallis, OR, USA)) following methods in Kaufmann (1968). Stem WP was estimated by
369 equilibrating the WP of a needle bundle with that of the stem following methods from Begg &
370 Turner (1970) and measuring the equilibrated bundle as above. Subsequently, we harvested
371 seedlings and collected tissue samples (needles, stem, and roots) for osmotic potential
372 measurements (i.e., water retention capacity via solutes potentially derived from NSC). Tissue

373 samples were wrapped in aluminum foil to prevent artificial changes in osmotic potential due to
374 water loss or photosynthetic activity, placed in small Ziploc bags, frozen in dry ice and transported
375 to the lab within 2 hours. The rest of the seedling was placed in a Ziploc bag containing a wet
376 paper towel partially covered in tin foil to prevent desiccation without introducing external
377 moisture into the tissues in contact with the towel [32], placed in a cooler, and transported to the
378 lab. There, we measured relative water content (RWC) and hydraulic conductivity (i.e., capacity to
379 transport water [49]). We describe the methods used to measure water content and hydraulic
380 conductivity in Methods S1. We also used the rest of the seedling to measure plant NSC pools (see
381 below). Upon arrival to the lab, samples collected for osmotic potential were pressed to extract
382 cellular liquid contents and the expressed solution was used to saturate 28 mm^2 filter paper disks
383 following methods from Grange (1983) [50]. This method avoids potential osmotic artifacts due to
384 starch mobilization or low sample water content. Disks were then placed in a C-52 sample
385 chamber attached to a Psypro data logger (Wescor, Inc. Logan, UT, USA) to measure osmotic
386 potential. Finally, pressure potential (i.e., positive pressure exerted by cells against cell walls when
387 turgid [12]) was calculated in stems and needles as the difference between their respective water
388 and osmotic potential. Pressure potential was not calculated in roots because we lacked root WP
389 measurements.

390 Non-structural carbohydrates

391 Non-structural carbohydrates were analyzed in all tissue types (needles, stems, and roots)
392 collected at harvest. A sample of each tissue was microwaved for 180 seconds at 900 Watts in
393 three cycles of 60 seconds to stop metabolic consumption of NSC pools, and oven-dried at 70 ^{o}C.
394 Samples were dried to a constant mass and finely ground into a homogenous powder.
395 Approximately 11 mg of needle tissue and 13 mg of stem or root tissue were used to analyze NSC
396 dry mass content following the procedures and enzymatic digestion method from Hoch, Popp &

397 Körner (2002) and Galiano, Martínez-Vilalta, Sabaté & Lloret (2012). NSC, starch, sucrose, and
398 glucose + fructose tissue concentrations were then multiplied by their respective tissue fraction
399 (the dry mass of a tissue relative to whole-plant dry mass) to obtain whole-plant concentrations.
400 Whole-plant dry mass for each seedling was calculated by combining the dry mass of all samples
401 and the remaining biomass. These methods are described in Methods S1.

402 Statistical Analyses

403 We observed no differences between pots with (Plexiglas barrier) and without (mesh
404 barrier) root connections for all key variables measured (Table S1), and data from the two barrier
405 treatments were pooled. We tested differences among seedling types (LL, LD, D) in all variables
406 using ANOVA and Tukey's Honestly Significant Difference tests. Note that sample size of LL
407 seedlings is twice that of LD and D seedlings because both plants in non-depleted networks lack of
408 covers and are LL seedlings (Fig. 1). However, because treatment comparisons excluding a random
409 half of the LL seedlings yielded the same results, we decided not to exclude any data. To test
410 differences in NSC depletion, we compared total NSC concentration and concentrations of each
411 NSC component (i.e., starch, sucrose, and glucose and fructose together) among seedling types at
412 both organ and whole-plant levels. To test the effectiveness of the labeling process and assess
413 carbon translocation among organs, for each organ and treatment, we compared δ^{13}C values after
414 labelling to those prior to labeling. Note that D seedlings were excluded because they were never
415 labeled. We only analyzed a few fungal samples for δ^{13}C (see above) as an additional check that
416 the ^{13}C label could travel from the labelled seedling to the fungal network of the non-labelled
417 seedling, thus ensuring that plants were indeed connected via fungal networks. Likewise, to assess
418 potential carbon transfer to non-labeled seedlings, for each organ and treatment, we compared
419 δ^{13}C values after labeling the paired seedling to those before labelling. LD seedlings were excluded
420 because they were all labeled. We also tested differences in hydraulic conductivity, WP, osmotic

421 potential, pressure potential and relative water content in each organ for which we collected data
422 among seedling types.

423 To assess both direct and indirect effects of NSC depletion on water relations, we assessed
424 whether osmotic potential and pressure potential changed in response to NSC depletion. We used
425 two linear regressions with needle % NSC deviation from control as predictor and either leaf
426 osmotic or pressure potential as response variables. We focused on needles because they are the
427 organ with most living cells exposed to dry conditions and, therefore, most susceptible to turgor
428 loss. Finally, we assessed whether NSC depletion influences turgor loss as WP decreases using
429 linear regressions. Leaf pressure potential was the response variable and the interaction between
430 leaf WP and whole-plant % NSC deviation from control as predictor. In this analysis, we focus only
431 on differences in intercepts among NSC levels (i.e. NSC effect on leaf WP at turgor loss point, a
432 broadly used indicator of drought tolerance) and among their slopes (i.e. rate of turgor loss per
433 unit change in water potential) because a significant relationship between leaf pressure potential
434 and leaf WP is expected given that the former is calculated from the later.

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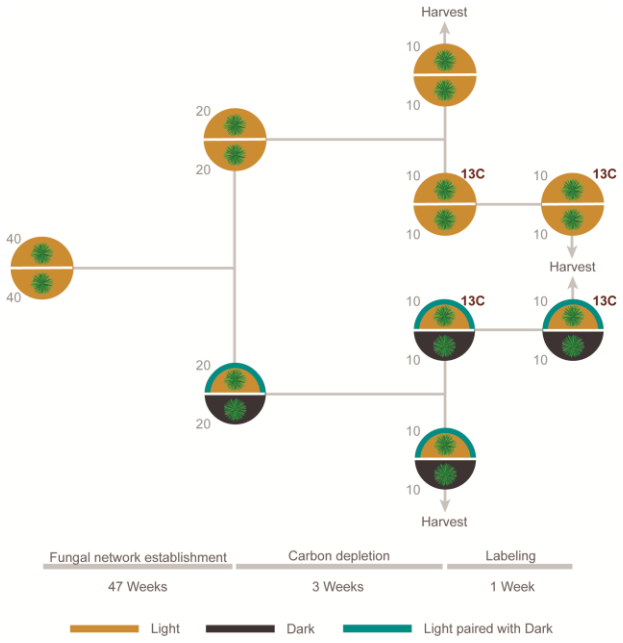
571 **Figure legends**



572

573 **Fig. 1. Experimental design.** Circles represent pots with barriers and a seedling on each side.

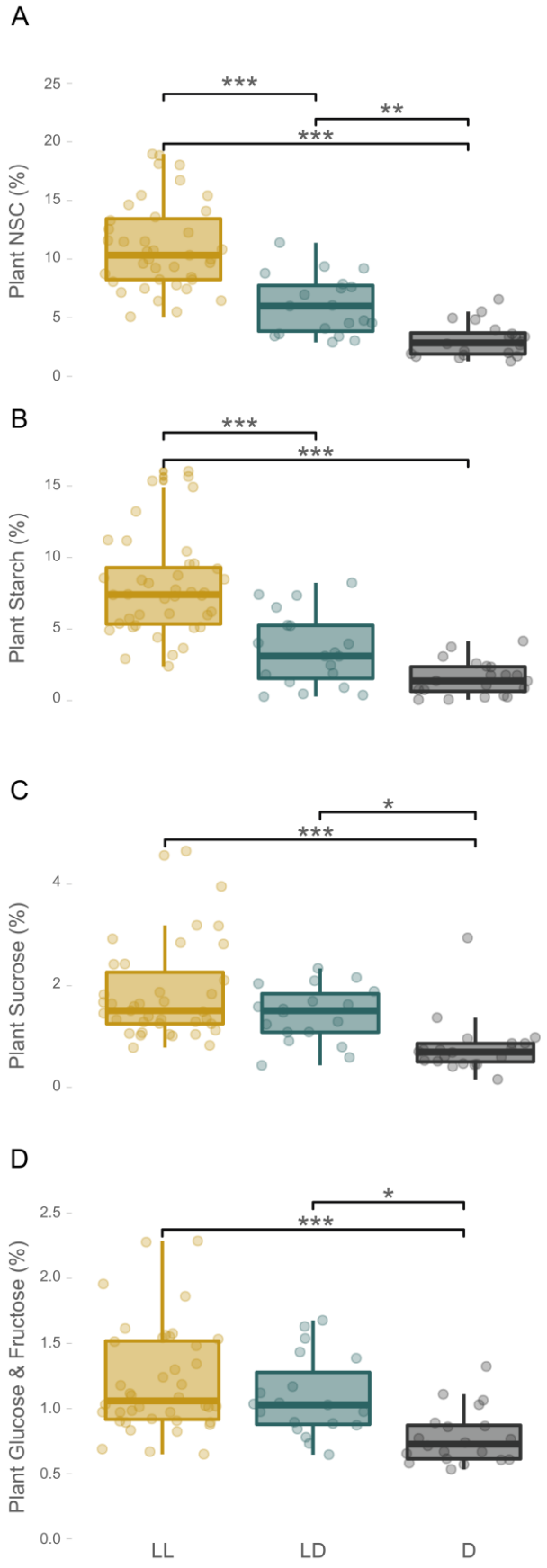
574 Colors represent LL seedlings exposed to natural light (golden), D seedlings with light-blocking

575 covers (black), and LD seedlings exposed to natural light paired with plants with covers (teal

576 border). Numbers on the side of each circle indicate the sample size of the seedling type

577 represented in that side of the pot at a given point on time. Red 13C annotations indicate pots

578 labeled with ^{13}C. Arrows indicate harvesting events.


A
Plant NSC (%)
25
20
15
10
5
0
***
**
***
B
Plant Starch (%)
15
10
5
0
***
***
C
Plant Sucrose (%)
4
2
0
*
***
D
Plant Glucose & Fructose (%)
2.5
2.0
1.5
1.0
0.5
0.0
*
***
LL
LD
D

579

580 **Fig. 2. Stored NSC decreased in seedlings of the NSC-depletion treatment.** Panels correspond to
581 whole-plant concentrations in percent dry weight. Colors represent LL seedlings exposed to
582 natural light (golden), D seedlings with light-blocking covers (black), and LD seedlings exposed to
583 natural light paired with plants with covers (teal). Lines within boxes represent the median and top
584 and bottom hinges represent 25th and 75th percentiles. Whiskers indicate highest and lowest
585 value no further than 1.5 times the inter-quartile range represented by the hinges. Dots represent
586 the distribution of the data. Asterisks indicate the degree of significance between groups (* = 0.05,
587 ** = 0.01, *** = < 0.001).

588

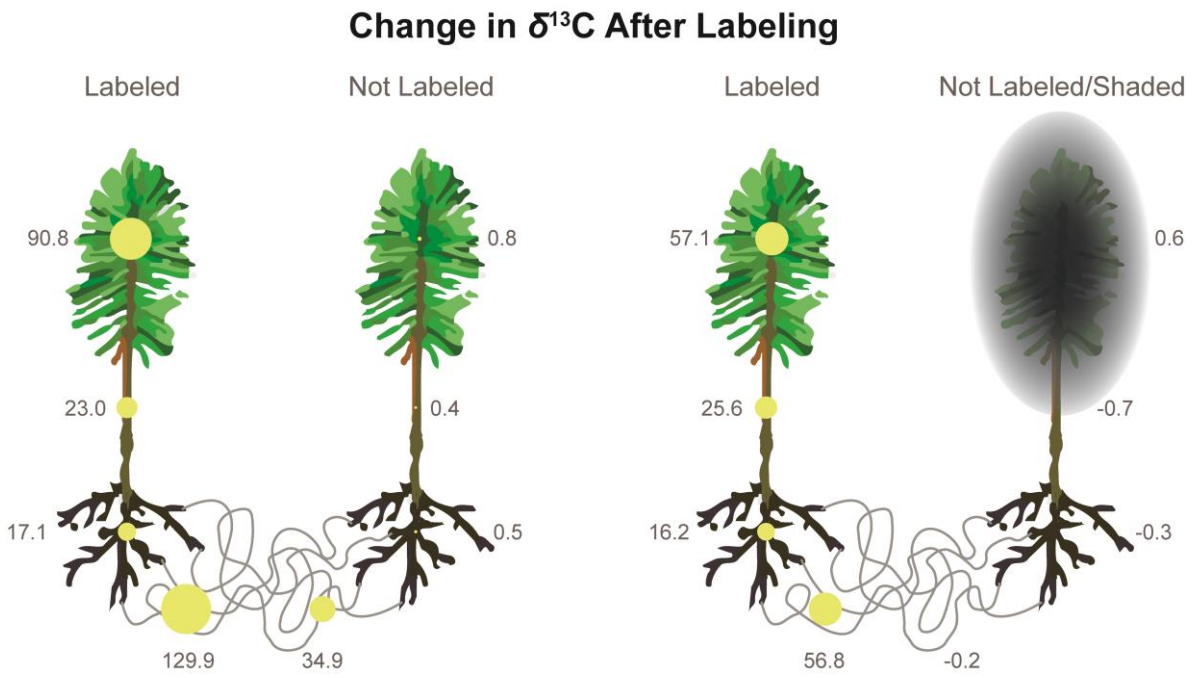


Fig. 3. ^{13}C isotope reached all organs in labeled plants and the ectomycorrhizal network but was not transferred to NSC-depleted hosts. The area of the yellow circles represents the average change in $^{13}C/^{12}C$ ratios in each tissue as a result from labeling (mean tissue $\delta^{13}C$ after labeling – mean tissue $\delta^{13}C$ before labeling) in parts per thousand (‰; also, annotated besides each circle). Positive values indicate an increase in $\delta^{13}C$. See Figure S7 and S8 for raw $\delta^{13}C$ values for each tissue and labeling time, confidence intervals, and statistical significances.

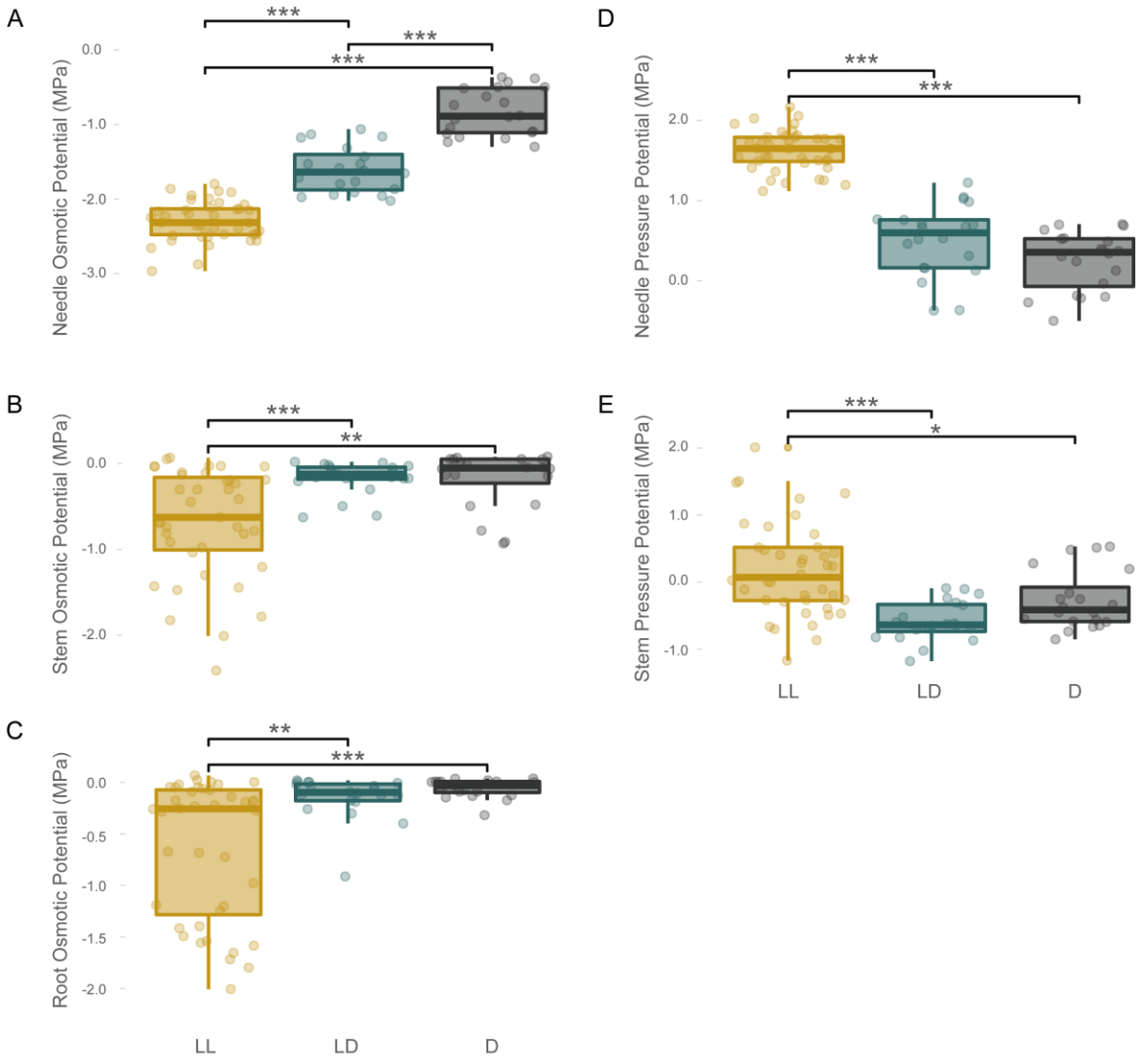


Fig. 4. All organs in NSC-depleted seedlings exhibited an increase of osmotic potential and a decrease in turgor. Panels on the left correspond to osmotic potentials of A) needles, B) stems, and C) roots. Panels on the right correspond to pressure potentials of D) needles and E) stems. Colors represent LL seedlings exposed to natural light (golden), D seedlings with light-blocking covers (black), and LD seedlings exposed to natural light paired with plants with covers (teal). Lines within boxes represent the median and top and bottom hinges represent 25th and 75th percentiles. Whiskers indicate highest and lowest value no further than 1.5 times the inter-quartile

605 range represented by the hinges. Dots represent the distribution of the data. Asterisks indicate the

606 degree of significance between groups (* = 0.05, ** = 0.01, *** = < 0.001).

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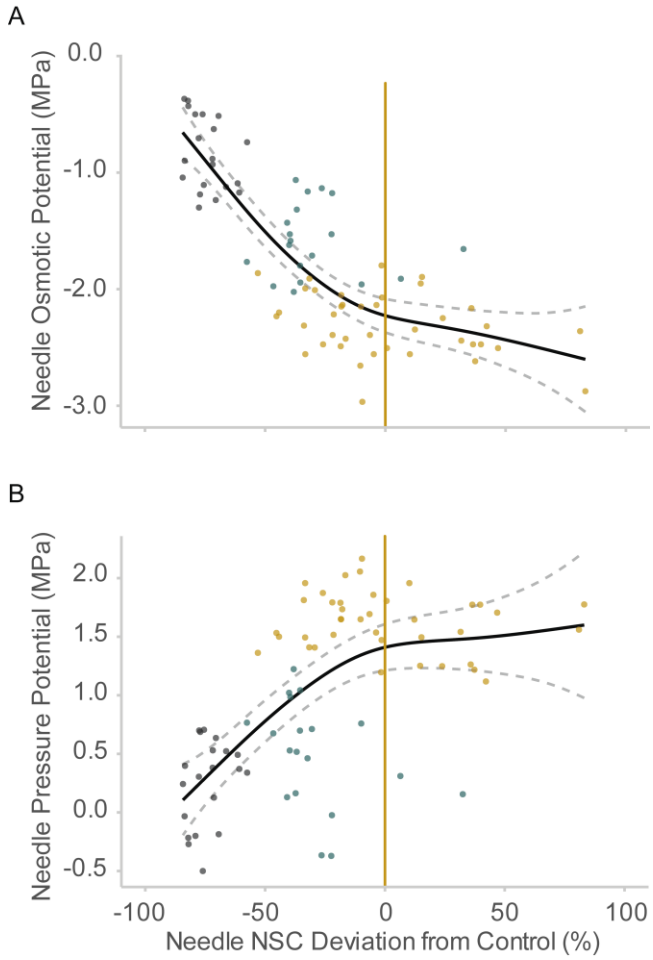

Fig. 5. Water relations are impaired when NSC drop below values corresponding to non-stressed plants. Panels correspond to A) osmotic potential and B) turgor pressure. All seedling types are merged for this analysis. Vertical golden lines indicate average leaf NSC content in non-depleted pots (i.e., light plants). A loess function was fit to the data to best represent the relationship between variables. Dashed lines indicate 95% confidence interval of the regression lines.

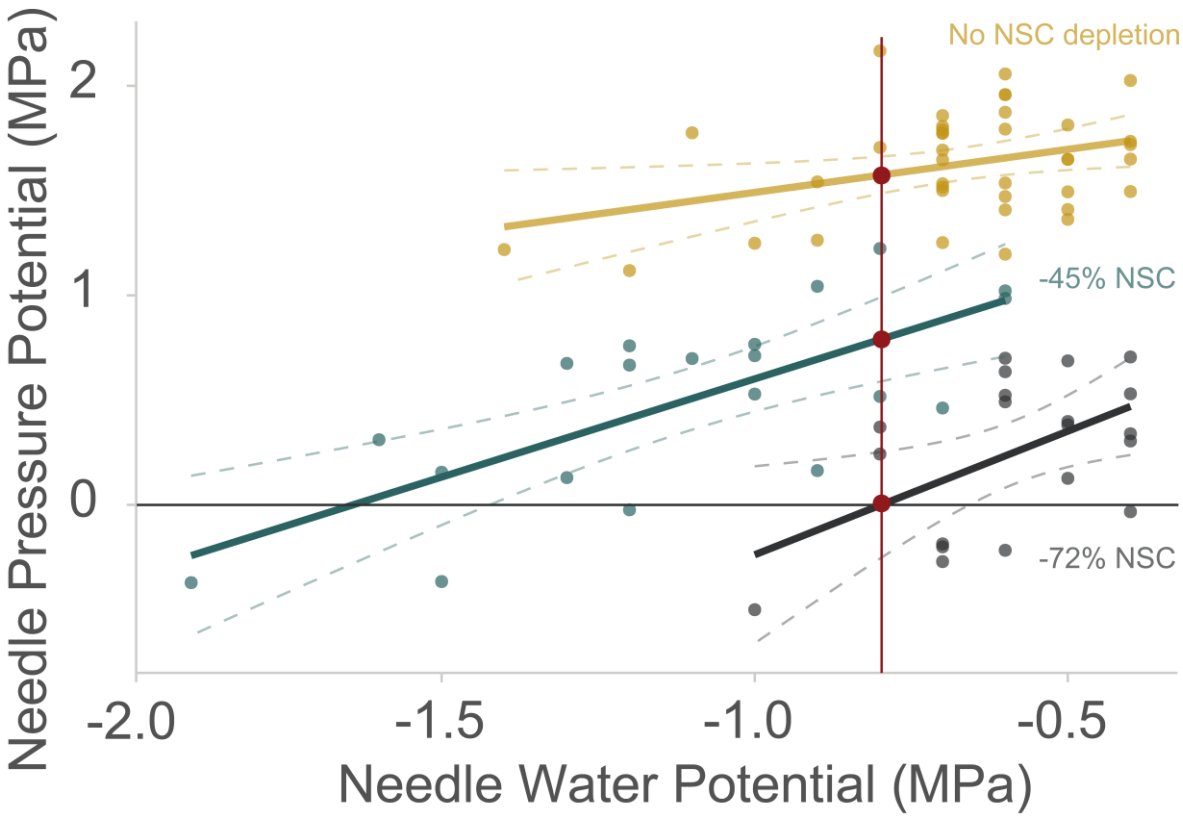

Fig. 6. NSC depletion is associated with turgor loss at high water potentials. Colors represent LL seedlings exposed to natural light (golden), D seedlings with light-blocking covers (black), and LD seedlings exposed to natural light paired with plants with covers (teal). Vertical red line and points indicate differences in turgor at a given water potential. Horizontal line indicates turgor loss point. Dashed lines indicate 95% confidence interval of the regression lines.