Tolerance of Japanese larch to drought is modified by nitrogen and water regimes during cultivation of container seedlings

Mitsutoshi Kitao[1,*], Evgenios Agathokleous[2], Hisanori Harayama[3], Satoshi Kitaoka[4], Akira Uemura[3], Kenichi Yazaki[1], Hiroyuki Tobita[3]

[1]Hokkaido Research Center, Forestry and Forest Products Research Institute, Hitsujigaoka 7, Sapporo 062-8516, Japan; [2]Department of Ecology, School of Applied Meteorology, Nanjing University of Information Science & Technology (NUIST), Nanjing 210044, P.R. China; [3]Department of Plant Ecology, Forestry and Forest Products Research Institute, Matsunosato 1, Tsukuba 305-8687, Japan; [4]Research Faculty of Agriculture, Hokkaido University, Sapporo 062-0809, Japan; [*]Correspondence: Mitsutoshi Kitao, E-mail: kitao@ffpri.affrc.go.jp

Abstract

Improving drought tolerance of container seedlings of Japanese larch is of high importance to afforestation. We hypothesized that adequate nitrogen (N) and limited water supply would increase the tolerance of container seedlings to water deficit stress, circumventing photoinhibition, by means of (i) enhanced photosynthetic capacity with higher leaf N and (ii) decreased water loss from leaves with lower biomass allocation into aboveground parts. Container seedlings of Japanese larch were grown under the treatment combinations of adequate (+N: 300 mg N container^{-1}) or limited (−N: 150 mg N container^{-1}) N and adequate (+W: daily irrigation) or limited (−W: twice-a-week irrigation) water. Then, seedlings were subjected to a progressive drought treatment. Higher leaf N was observed in container seedlings grown under +N and −W. During progressive drought, lower stomatal conductance and net photosynthetic rate were observed in leaves with higher leaf N at a given predawn leaf water potential. Furthermore, the maximum efficiency of PSII photochemistry (F_v/F_m) was lower in leaves with higher leaf N, suggesting that higher leaf N might impair intrinsic tolerance to drought at the leaf level contrary to expectations. Conversely, −N and −W seedlings with lower shoot biomass delayed soil drying as a whole-plant response via a reduction in leaf transpiration, leading to delayed photoinhibition as indicated by a decline in F_v/F_m. To circumvent stress at the initial stage of water deficit, lower leaf N via limited N regime, and smaller shoot biomass driven by limited N and water regimes would be important.

38   **Declarations**

44   **Conflicts of interest:** The authors declare that they have no conflict of interest.

45   **Availability of data and material:** Data are available within the article and its
46   supplementary materials

47   **Code availability:** Not applicable

48   **Authors' contributions:** M.K., E.A. and H.T. designed the study. M.K. and H.H. collected
49   the photosynthetic data; A.U. prepared the seeds of Japanese larch; S.K. and K.Y.
50   collected the leaf N data, performed the analysis, and hence equally contributed to this
51   study. All authors also discussed the results and commented on the manuscript. M.K. led
52   the writing with input from E.A. and H.T.

53

54   **Key message:** Container-seedlings of Japanese larch grown with N and water limitation,
55   and having lower shoot biomass, had a higher ability to cope with subsequent prolonged
56   drought, by circumventing photoinhibition for a longer period.

Introduction

Container-grown seedlings, with soil-covered root system, are advantageous in the early establishment after planting compared with bare-rooted seedlings (Grossnickle and El-Kassaby 2015; Yamashita et al. 2016). Containers with relatively small volume (generally up to 300 ml) are used, where roots bind the growing medium together into a cohesive plug (Landis 1990). Japanese larch (*Larix kaempferi*) is a deciduous coniferous species, widely used for artificial afforestation in northern Japan. Recently, containerized seedlings of Japanese larch have been used increasingly, while production of container seedlings with a high ability of survival and growth is needed (Agathokleous et al. 2020). In northern Japan, soils are generally moist during the springtime irrespective of precipitation because of melting snow. Conversely, a low survival rate of containerized seedlings has been observed during a period with little precipitation in the summertime, although container-grown seedlings of Japanese larch have generally higher tolerance against water deficit stress than bare-root seedlings (Harayama et al. 2016, 2021). Hence, a further improvement of the tolerance of container seedlings of Japanese larch to water deficit stress is expected for afforestation throughout the seasons.

Regarding tolerance to drought, a preferable balance of biomass allocation into root, i.e. reduced biomass allocation into shoot, leading to lower ratio of shoot to root (S:R ratio), is an important ecological strategy to increase water acquisition by root and decrease water loss from leaves (Lloret et al. 1999; Villar-Salvador et al. 2004; Agathokleous et al. 2019). Stomatal closure is also an effective way to circumvent water deficit stress; however, it concomitantly suppresses photosynthetic rate via lower intercellular CO_2 concentration (C_i), leading to carbon starvation (Cornic and Fresneau 2002; Medrano et al. 2002; McDowell et al. 2008; McDowell 2011). To cope with the lower C_i under drought, an increase in leaf nitrogen (N), accompanied with an increase in the RuBP carboxylation rate (Evans 1989), can maintain photosynthetic rate under stomatal closure (Kitao et al. 2003, 2006, 2007; Flexas et al. 2006; Kitao and Lei 2007). Furthermore, electron flow through photosystem II (PSII) consumed via photosynthesis and photorespiration increases with increasing leaf N (Niinemets et al. 1999), which contributes to dissipating absorbed light energy efficiently to prevent photoinhibition (Flexas et al. 2006; Kitao et al. 2019).

Low to moderate N addition increases photosynthesis (Sun et al. 2018). Conversely, moderate to high drought decreases photosynthesis by lowering CO_2 intake through stomatal closure (Liang et al. 2019), whereas the decrease in electron transport rate is relatively small because of an increase in the ratio of photorespiration to photosynthesis (Cornic and Fresneau 2002; Flexas et al. 2009). Photorespiration is

93   considered an alternative pathway of electron flow, which contributes to circumventing

94   photoinhibition in cooperation with regulated thermal energy dissipation (Flexas and

95   Medrano 2002). However, severe drought beyond the photoprotective mechanisms,

96   such as photorespiration and thermal energy dissipation, induces production of active

97   oxygen, leading to photooxidative damage in plant tissues (Kasajima et al. 2011;

98   Sperdouli and Moustakas 2014a; Moustaka et al. 2015; Liang et al. 2019). Circumvention

99   from photooxidative damage is essential for container seedlings survival under drought

100   after transplanting.

101   Restricted water supply decreases S:R ratio, but N fertilization increases it

102   (Lloret et al. 1999; Kitao et al. 2003, 2005, 2007; Villar-Salvador et al. 2004).

103   Manipulation of S:R ratio and leaf N is expected by applying combinations of different

104   levels of water supply and N fertilization. We hypothesized that adequate N and limited

105   water supply increase drought tolerance, circumventing photoinhibition in container

106   seedlings of Japanese larch, by means of enhanced photosynthetic capacity with

107   increased leaf N, and greater water acquisition capacity and smaller water loss from

108   leaves with lower S:R ratio. To test this hypothesis, we investigated growth, carbon

109   partitioning, and photosynthetic traits in relation to photoinhibition, assessed by gas

110   exchange and chlorophyll fluorescence measurements, in container seedlings of

111   Japanese larch grown under combinations of different levels of water supply and N

112   fertilization.

113

Materials and methods

114

Plant materials

115

116   Container seedlings of Japanese larch (*Larix kaempferi* (Lamb.) Carr.) were grown from

117   seeds in a natural-light glasshouse of Hokkaido Research Center, Forestry and Forest

118   Products Research Institute (43°N, 141°E; 180 m a.s.l.). The size of container was 5.5 cm

119   in diameter, 13 cm in height, and 200 ml in volume, which is commonly used for

120   container seedlings. Coco peat (topcocopeat, Top, Osaka, Japan) was used as cultivation

121   medium. The fertilization treatments were conducted only once during the container-

122   seedlings cultivation. We added 2 and 1 g pot^{-1} of commonly-used fertilizer (Osmocote

123   Exact Standard 15-9-11 +TE, HYPONeX Japan, Osaka, Japan), corresponding to 300 mg N

124   pot^{-1} (+N) and 150 mg N pot^{-1} (−N). We considered N the major factor limiting

125   photosynthetic capacity (Evans 1989), although phosphate and potassium were reduced

126   concomitantly in the low fertilization (−N) treatment. Seeds were directly sown in the

127   pots at the beginning of June, 2016 (Fig. 1). Seeds germinated ≈10 days after sowing.

128   Different irrigation regimes were applied from the end of July 2016 onward: i) daily water

129   supply of 70 ml (+W) or ii) twice a week water supply of 140 ml (−W). Both regimes could
130   just saturate the water-holding capacity of the cultivation medium, but minimize the
131   runoff of supplied water. Plants were exposed to the treatments in 2 consecutive
132   growing seasons. All seedlings were moved into a dark room with a continuous
133   temperature of 4 °C at the beginning of Dec 2016, when all leaves had shed. This is a
134   common practice to nurse seedlings against snow and freezing damage. At the end of
135   May 2017, after snowmelt, the seedlings were moved back to the glasshouse. At the
136   experimental area, naturally grown Japanese larch generally flushes new leaves in the
137   middle of May. The experimental seedlings flushed new leaves at the beginning of June,
138   only a few days after the transfer.

139

140   *Drought or adequate-irrigation treatments after transplanting*
141   In the second growing season, 16 container seedlings (2 N treatments x 2 water regimes
142   x 4 replicates) in total were transplanted from 200-ml containers into larger pots (4 L),
143   simulating planting of container seedlings in the field with no limitation of root growth.
144   According to the Hokkaido Tree Seeds and Seedlings Association, the threshold of
145   Japanese larch container-seedlings is (i) height > 30 cm and diameter > 6 mm for the first
146   grade and (ii) height > 25 cm and diameter > 4 mm for the second grade. In the present
147   study, all the seedlings satisfied the threshold of second grade for shipping (cf. Table 3).
148   Transplantation was conducted on 29th August, 2017, and slow-released fertilizer was
149   added (40 g, Osmocote Exact Standard 15-9-11 +TE, HYPONeX Japan), which
150   corresponded to the adequate N treatment (+N) described above. Soils, consisting of
151   clay loam soil and Kanuma pumice soil (1:1 in volume), in the pots had been completely
152   dried in the glasshouse without irrigation. Initial irrigation (500 ml water) was conducted
153   at the transplanting. Sixteen additional container seedlings were also transplanted into
154   the larger pots on 29th August with adequate N fertilization (40 g, fertilizer) and
155   periodical irrigation (500 ml water supplied once a week), and then grown until 2nd
156   November 2017 for two months. Regarding summer precipitation in Sapporo, Hokkaido,
157   monthly precipitation smaller than 50 mm was recorded three times in July and
158   September and one time in August during the period 2001–2020 (Japan Meteorological
159   Agency, https://www.jma.go.jp/jma/index.html). Thus, transplanting at the end of
160   August was appropriate for the objective of the present study.

161

162   *Measurements of gas exchange and chlorophyll fluorescence*
163   Measurements of gas exchange and chlorophyll fluorescence were conducted in late
164   summer of the second growth season. Measurements were conducted for 1) container-

seedlings grown under the N and water treatment combinations under full irrigation (on 11th, 15th, 16th, 17th, 23rd, and 24th August, 2017), 2) container-seedlings under the most dried condition for each treatment combination, i.e. right before regular irrigation (18th August), and 3) seedlings transplanted into the progressive drought treatment periodically from 30th August (day 1 after transplanting) to 22nd September (day 24), 2017. Regarding the first set of the measurements, to assess photosynthetic potential, container seedlings of Japanese larch were fully irrigated on the evening of the day before the measurements. Measurements of gas exchange and chlorophyll fluorescence were conducted in fully-expanded mature leaves randomly selected from Japanese larch seedlings. Net photosynthetic rate (A), stomatal conductance (g_s), and chlorophyll fluorescence parameters were measured with a portable photosynthesis system (LI-6400, Li-Cor, Lincoln, NE, USA) with a chamber fluorometer (LI-6400-40, Li-Cor) with a PFD of 1500 μmol m^{-2} s^{-1} and a leaf temperature of ≈25 °C, under an ambient CO_2 concentration of 380 μmol mol^{-1}. The measurements of chlorophyll fluorescence were simultaneously conducted with measurements of gas exchange. The pulse flush was applied to determine F_m and F_m': maximum fluorescence under dark and illumination, respectively. The fluorescence flash was set to rectangular flash method; duration = 1 s, pulse intensity ≈ 7000 μmol m^{-2} s^{-1}, measuring frequency = 20 kHz and filter = 50 kHz. F_m and the minimum fluorescence (F_o) were measured after overnight dark adaptation. The maximum efficiency of PSII photochemistry (F_v/F_m) was determined as $F_v/F_m = (F_m - F_o)/F_m$. Quantum yield of PSII electron transport (YPSII), quantum yield of non-regulate non-photochemical quenching in PSII (YNO), and quantum yield of regulated non-photochemical quenching in PSII (YNPQ) were calculated based on the simple approach: YPSII = (F_m' − F)/F_m', YNO = F/F_m, and YNPQ = F/F_m' − F/F_m,

where F is relative fluorescence yield at steady state illumination (Hendrickson et al. 2004; Klughammer and Schreiber 2008). To assess the photosynthetic capacity independently of g_s, we calculated the maximum rate of Rubisco carboxylation ($V_{c,max}$). $V_{c,max}$ was estimated based on the 'one-point method' (de Kauwe et al. 2016), assuming that light-saturated net photosynthetic rate (A) was limited by Rubisco carboxylation, and respiration rate under light (R_d) was 1.5% of $V_{c,max}$ (Collatz et al. 1991) as follow:

$V_{c,max} = A_{sat} / \{(C_i+K_m)/(C_i-\Gamma) − 0.015\}$,

where C_i is intercellular CO_2 concentration, K_m is Michaelis-Menten constant, and Γ is CO_2 compensation point. K_m is defined as $K_m = K_c (1+O/K_o)$, where K_c is Michaelis constant for CO_2, and K_o is that for O_2, and O is intercellular concentration of O_2. K_m and Γ at 25 °C was estimated from the temperature dependencies of K_c, K_o and Γ after (Bernacchi et al. 2001).

201

Predawn leaf water potential

Predawn leaf water potential (WP) is assumed to be equivalent to soil water potential. To assess how the soils were dried by the N and W treatment combinations during the cultivation of container-seedlings, WP was measured by a pressure chamber (Model 1505D, PMS Instrument Company, Albany, OR, USA) on the morning right before regular irrigation on 18th August 2017, i.e. at the driest conditions during the periodic water supply. WP was also measured on days 1, 3, 6, 9, 13, 17, and 24 (the same days of photosynthetic measurements) during the progressive drought treatment, where day 0 indicates the day of transplanting (29th August). Measurements of WP were conducted in the morning for leaves not used for the measurements of gas exchange and chlorophyll fluorescence.

Growth and biomass allocation

Harvest was conducted to determine growth and biomass allocation into shoot (needles + stems) and root for 1) container-seedlings grown under different N and water supplies before transplanting (harvested on 25th August 2017), 2) seedlings transplanted and subjected into the progressive drought treatment for 27 days (harvested on 25th September 2017), and 3) seedlings transplanted and subjected to the adequate irrigation treatment for two months (harvested on 2nd November 2017). As for progressive drought treatment, the 27-day treatment was enough for plants to start wilting. We harvested them at that time to prevent implications of leaf shedding. For the same reason, we harvested plants transplanted into adequate irrigation at the beginning of November, when short-shoot needles turned yellow but long-shoot needles were still green. Plants were separated into shoots and roots, and then dried (70 °C) to constant weight. We also measured shoot height and basal diameter for the seedlings harvested on 25th August, transplanted on 29th August, and harvested on 2nd November after adequate irrigation.

Needle N content

Needle N contents on the basis of dry mass (N_{mass}) were determined in needles used for the gas exchange measurements by the combustion method using an analysis system composed of an N/C determination unit (SUMIGRAPH, NC 800, Sumika Chem. Anal. Service, Osaka, Japan), a gas chromatograph (GC 8A, Shimadzu, Kyoto, Japan), and a data processor (Chromatopac, C R6A, Shimadzu). Leaf mass per area (LMA) was determined using a scanner (LiDE210, Canon, Tokyo, Japan) and an image analysis software (LIA32

237   ver 0.3781, http://www.agr.nagoya-u.ac.jp/~shinkan/LIA32/). Based on N_{mass} and LMA,

238   area-based needle N content (N_{area}) was estimated, since photosynthetic properties

239   determined by the gas exchange measurements were expressed on the area basis. We

240   also calculated the total N amount in the needles ($N_{needles}$): $N_{needles}$ (g N plant^{-1}) = N_{mass} x

241   [total needle dry weight], for container-seedlings grown under different N and water

242   supplies before transplanting (harvested on 25th August, 2017).

243

244   *Statistical analyses*

245   Two factorial ANOVA was employed to test the effects of N and water supply on leaf

246   morphological and photosynthetic traits, growth and biomass allocation, and needle N

247   content in the container seedlings before and after transplanting (R Core Team 2020). A

248   linear mixed model was applied to analyze predawn leaf water potential (WP), N_{area}, and

249   photosynthetic traits (A, g_s, F_v/F_m, YPSII, YNPQ, and YNO) during progressive drought,

250   with nitrogen and water treatments, and days after withholding water (set as categorical

251   variables) as fixed factors and individual seedlings (n=4 for each treatment combination)

252   as random effect. Interactions among the fixed factors were also investigated.

253   Furthermore, we also analyzed the photosynthetic traits during the progressive drought,

254   using a linear mixed model with WP, 1/WP, −ln(−WP), or 1/(−ln(−WP)) and N_{area} as

255   explanatory variables and individual seedlings as random effect. We determined the best

256   model among those using the four variables (WP, 1/WP, −ln(−WP), or 1/(−ln(−WP)),

257   based on the lowest value of AIC (Akaike's information criterion). We used the lmer

258   function of the R package lme4 for the model fitting (Bates et al. 2015), and the ANOVA

259   function of the R package car for the analysis of the deviance table (Fox and Weisberg

260   2019). We applied linear regression analyses 1) to investigate the effects of N_{area} on

261   photosynthetic traits, setting N_{area} as the independent variable, and photosynthetic

262   traits (A, g_s, $V_{c,max}$, YPSII, YNPQ, and YNO) as the dependent variables; 2) to estimate

263   initial shoot dry mass from D^2H, setting D^2H as the independent variable, and shoot dry

264   mass as the dependent variable, assuming the regression line passes through the origin;

265   3) to investigate the effect of initial shoot dry mass on the harvested shoot dry mass,

266   also assuming the regression line passes through the origin; and 4) to investigate the

267   effect of shoot dry mass on soil drying rate, setting shoot dry mass as the independent

268   variable and WP as the dependent variable. The level of significance was 0.05.

269

270

271   **Results**

272   *Leaf morphological and photosynthetic traits in container seedlings grown under*

273 *different N and water supplies*

274 Seedlings of Japanese larch were grown under adequate (+N) or limited N (−N),
275 combined with adequate (+W) or limited water (−W) supplies for two growing seasons.
276 As Japanese larch is a deciduous conifer species, needles used for the measurements
277 had developed during the second growing season. Leaf mass per area (LMA) was
278 increased by +W, whereas area-based leaf N (N_{area}) was increased by both +N and −W
279 treatments (Table 1). To assess the potential of photosynthetic traits, measurements of
280 gas exchange and chlorophyll fluorescence were conducted in the presence of full
281 irrigation. g_s showed no significant difference among different treatment combinations.
282 A significantly higher $V_{c,max}$ was observed in +N-grown seedlings, whereas no significant
283 effect of water treatment was observed. Similar to A, YPSII increased in plants grown in
284 both +N, and −W. Conversely, quantum yield of YNPQ decreased in plants grown in +N
285 and −W. YNO increased only in plants grown in +N treatment. F_v/F_m showed no significant
286 difference among treatment combinations. Higher A, g_s, $V_{c,max}$, YPSII, and YNO were
287 observed with higher N_{area} across the treatment combinations, while YNPQ decreased
288 with higher N_{area} (Fig.2).

289   Predawn leaf water potential is a measure of soil water status because the
290 water potential of leaves is considered to be in equilibrium with that of soils before dawn.
291 Predawn water potential just before regular irrigation was significantly lower in −W
292 treatment, reaching −1.4 and −1.1 MPa in +N −W plants and −N −W plants, respectively
293 (Table 2). Accordingly, A and g_s declined to almost 0 in the −W plants before irrigation.
294 Although YPSII in −W-grown plants was significantly lower than those grown in +W
295 treatment, YPSII in −W plants was relatively high, maintained at over 60 % of +W plants.
296 Therefore, the response of YPSII was not in good agreement with the response of A and
297 g_s.

298

299 *Photosynthetic performance in seedlings transplanted into 4L-pots subjected to*
300 *progressive drought*

301 Predawn water potential decreased with the day progressing after the withholding water
302 supply, where the drought progress was significantly faster in +N treatment (Fig. 3a,
303 Supplemental Table S1). N_{area} was higher in +N plants, but gradually decreased during
304 the progressive drought (Fig. 3e). A and g_s steeply declined within 10 days after the onset
305 of water withholding, where lower A and g_s were observed in +N treatment (Fig. 3b & c).
306 YPSII declined steeply until 6 days after the water withholding, and thereafter decreased
307 gradually with no significant effect of the treatment combinations (Fig. 3d). F_v/F_m
308 declined earlier in +N plants (Fig. 3f). YNPQ declined earlier in +N plants, while YNO

309 increased earlier in +N plants (Fig. 3g and h). Regarding YNPQ and YNO, the interactions
310 between N treatment and days after withholding water were significant, which might
311 reflect a relatively large decline in YNPQ, and a relatively large increase in YNO in +N
312 plants on 24 days after withholding (Fig. 3g and h).

313   Photosynthetic performance was also investigated as a function of soil water
314 status, indicated by the predawn leaf water potential, taking effects of N_{area} into
315 consideration (Fig. 4, Supplemental Fig. S1). A and g_s declined to reach the minimum
316 values by a water potential of −1 MPa (Fig. 4a, b). YPSII further decreased below −1 MPa
317 (Fig. 4c). The effects of progressive drought were observed in F_v/F_m, YNPQ, and YNO after
318 water potential reached around −2 MPa, where F_v/F_m and YNPQ started to decrease but
319 YNO increased (Fig. 4d, e, f). Regarding the effects of N_{area}, A and g_s were significantly
320 lower in leaves with higher N_{area} at a given WP. No significant effect of N_{area} was observed
321 in the relationship between YPSII and soil water status. Conversely, significantly lower
322 F_v/F_m was observed in leaves with higher N_{area} at a given WP. Although the coefficient of
323 N_{area} was negative for YNPQ, but positive for YNO, significant effects of N_{area} were not
324 observed in the relationship between YNPQ or YNO and soil water status.

325

326 *Growth and biomass allocation into plant organs*
327 Sixteen container-grown seedlings were harvested on 25th August, 2017, before the
328 onset of drought treatment. Total dry mass and shoot dry mass were higher in seedlings
329 grown in adequate N or adequate water supply compared to limited N or limited water
330 supply, respectively (Table 3). However, root dry mass increased only in seedlings grown
331 in the presence of adequate N supply. S:R ratio was not significantly different among the
332 treatment combinations. The total amount of needle N ($N_{needles}$) was greater in +N
333 seedlings than in −N seedlings, whereas no significant difference was observed between
334 +W and −W seedlings (Fig. 5).

335   Container-grown seedlings transplanted on 29th August, 2017, and subjected to
336 progressive drought treatment until 25th September, 2017, showed little increment in
337 total biomass during the 27 days of treatment compared to container seedlings before
338 transplanting (harvested on 25th August 2017) (Table 3). Based on the relationship
339 between shoot dry mass and D^2H (= [basal diameter]^2 x [shoot height]) for seedlings
340 harvested on 25th August, and on 2nd November, initial shoot dry mass was estimated for
341 the seedlings transplanted into progressive drought (Fig. 6a). Little increment in shoot
342 growth was observed during the progressive drought (Fig. 6b). Significantly higher total
343 dry mass, shoot dry mass, and S:R ratio were observed in +N-grown plants compared
344 with −N-grown plants after a 27-day drought, whereas no difference was observed in

345   root dry mass among the treatment combinations.

346   Based on the relationship between shoot dry mass measured after the

347   progressive drought treatment (which might be almost constant during the treatment;

348   as described above) and predawn water potential, higher shoot dry mass was related to

349   lower leaf water potential during the progressive drought treatment (Fig. 7).

350   As an additional experiment, container-grown seedlings were also transplanted

351   into 4L pots with adequate N and water supplies. After two months of adequate N and

352   water supply, total biomass nearly doubled (Table 3). The increment of shoot dry mass

353   was estimated at ca. 60% after transplanting (Fig. 6b), whereas little increase in shoot

354   height was observed (Table 3). The effects of N and water treatments on total biomass,

355   shoot dry mass, and root dry mass before transplanting persisted even after 2 months

356   of adequate N and water. Conversely, biomass increment was apparently higher in root

357   than shoot, resulting in lower S:R ratio compared to before transplanting.

358

359

360   **Discussion**

361   Leaves developed under long-term moderate drought have higher area-based leaf N

362   (N_{area}) than well-irrigated ones, which can contribute to maintaining photosynthetic

363   carbon assimilation and preventing photoinhibition (Kitao et al. 2003; Flexas et al. 2006;

364   Kitao and Lei 2007) and enhancing photosynthetic activity following re-watering (Xu et

365   al. 2009). In the present study, both adequate N and limited water supplies increased

366   N_{area} (Table 1). Differences in the photosynthetic traits such as A, $V_{c,max}$, YPSII, YNPQ, and

367   YNO, measured under adequate water supply, were well explained by the differences in

368   N_{area} as a consequence of N and water treatments. A, $V_{c,max}$, and YPSII increased with

369   increasing N_{area} as reported previously (Niinemets et al. 1999; Kitao et al. 2018). YNPQ,

370   defined as regulatory light-induced non-photochemical quenching, decreased with

371   increasing YPSII as an increase in light energy consumption through electron transport

372   reduced the need of thermal energy dissipation (Hendrickson et al. 2004; Kramer et al.

373   2004; Lazár 2015). YNO, non-regulated non-photochemical quenching can be a measure

374   of oxidative stress (Sperdouli and Moustakas 2014a). YNO increased with increasing N_{area},

375   partly due to an increase in absorbed light energy by chlorophyll pigments, which

376   generally increases in N-fertilized plants (Koike et al. 2001; Kitao et al. 2019). Lower N_{area}

377   in +W-grown seedlings (Table 1) might not be attributed to runoff of fertilizer from the

378   pots, but rather to dilution of N inside the plants due to the greater amount of needle

379   biomass (cf. Table 3, Fig. 5,).

380   As a function of days after withholding water supply, predawn leaf water

381   potential decreased earlier in the +N treatment, which is considered to be the cause of
382   earlier declines in A and g_s in +N seedlings (Fig. 3). Greater amount of shoot biomass in
383   +N-grown seedlings suggests greater amount of transpiration through leaves (Fig. 7)
384   (Lloret et al. 1999; Villar-Salvador et al. 2004). This suggests that seedlings grown under
385   -N might exhibit delayed drought stress as a whole plant response. Corresponding to the
386   earlier decline in A, F_v/F_m also declined earlier in plants grown under +N than those
387   under −N, suggesting drought-induced photoinhibition (Krause 1994). At the end of
388   progressive drought, F_v/F_m substantially decreased below ≈0.6 as reported in *Quercus*
389   *petraea* under severe dehydration (Epron and Dreyer 1992). As an increase in YNO
390   indicates an increased risk of photooxidation, a drought-induced dysfunction in YNPQ
391   might result in an increase in YNO, leading to photoinhibition (Flexas and Medrano 2002;
392   Moustaka and Moustakas 2014; Sperdouli and Moustakas 2014a; Moustaka et al. 2015,
393   2018).

394   The decline in g_s, indicating stomatal closure, might result in suppressed A under
395   low leaf water potential (Cornic and Fresneau 2002). Although A steeply declined, with
396   g_s decreasing to the minimum by the predawn leaf water potential of −1 MPa, YPSII still
397   decreased beyond −1 MPa (Fig. 4). This suggests that there was some amount of electron
398   consumption by photorespiration when stomata had completely closed around −1 MPa;
399   then, biochemical activity of Rubisco per se might be inhibited by further drought
400   progress (Flexas and Medrano 2002; Cornic and Fresneau 2002; Liang et al. 2019).
401   Similarly, YNPQ, as a photoprotective mechanism (Lazár 2015), appeared to work until
402   the predawn leaf water potential reached around −2 MPa, which might be around the
403   leaf wilting point of container seedlings of Japanese larch (Harayama et al. 2016).
404   Damage to thylakoid membranes might be a cause of the reduced YNPQ under severe
405   water deficit (below −2 MPa) via a reduced proton gradient (Epron and Dreyer 1992;
406   Sperdouli and Moustakas 2012). Limitation in photoprotection under severe drought
407   consequently leads to photooxidative damage indicated by an increase in YNO as well as
408   a decrease in F_v/F_m (Sperdouli and Moustakas 2012, 2014b). Thus, photosynthetic
409   carbon gain was substantially inhibited under drought stress around −1 MPa of the
410   predawn leaf water potential, whereas photooxidative damage was not apparent up to
411   −2 MPa of predawn leaf water potential (Sperdouli and Moustakas 2014a). Regarding
412   regular water regimes in the present study, as limited water supply (−W) decreased WP
413   to −1 MPa or below at the most dried conditions (Table 2), photosynthetic carbon gain
414   was completely inhibited periodically, but photochemical process (cf. YPSII in Table 2)
415   and photoprotection might still be functional to prevent photooxidative damage in −W-
416   grown seedlings.

Although N_{area} changed as expected by the treatment combinations, the leaf-level drought tolerance was impaired in leaves with higher N_{area}, based on the relationships between photosynthetic parameters and soil water potential (indicated by the predawn leaf water potential) when the effect of N_{area} was taken into account (Fig. 4, Supplemental Fig. S1). Here, stomatal closure, accompanied by a decline in A, was observed in leaves with higher N_{area} at a given WP, which might result from impaired osmotic adjustment induced by N fertilization (Graciano et al. 2005). Conversely, no effect of N_{area} on YPSII was observed, suggesting that the decreased electron consumption by photosynthesis appeared to be compensated by an increase in electron flow into photorespiration partly due to higher N_{area} (Niinemets et al. 1999; Flexas et al. 2006; Kitao et al. 2006, 2019). YNPQ, a measure of photoprotective thermal dissipation (Lazár 2015), showed a decreasing trend, whereas YNO, a measure of photooxidative stress (Sperdouli and Moustakas 2012, 2014b), oppositely showed an increasing trend with higher N_{area} during the progressive drought. As a consequence, leaves with higher N_{area} might be more vulnerable to photoinhibition under water deficit stress, indicated by a decrease in F_v/F_m (Krause 1994). Thus, leaves with higher N_{area} might decrease drought tolerance regarding CO_2 uptake and photoinhibition in the container seedlings of Japanese larch contrary to expectations.

Additionally, container-grown seedlings were also transplanted into soils with adequate N and water supply. Total biomass after two months of treatment was greatest in the +N and +W seedlings, which might reflect the greatest shoot biomass before transplanting (cf. Table 3), i.e. largest amount of photosynthetic apparatus (Poorter and Remkes 1990). It is also noteworthy that S:R ratio largely decreased after transplanting, suggesting faster root growth relative to shoot growth under adequate space. Root growth was saturated before transplanting by the limited volume of containers (200 ml), as no significant difference was observed in root biomass before transplanting among the treatment combinations. Therefore, shoot biomass would be a major determinant of the rate of soil drying at the initial stages following transplantation.

Conclusion

In the present study, higher leaf N content might impair the leaf-level tolerance to drought, where lower photosynthetic rate and exacerbated photoinhibition were observed at a given soil water potential during progressive drought. Furthermore, enhanced shoot biomass in the presence of adequate N and water with limited growth of root systems within the containers might accelerate water deficit in the soils through higher amount of transpiration under little precipitation, leading to earlier

photoinhibition. Smaller shoot biomass, driven by cultivation regimes of low N and water supply, as well as lower leaf N content, lead to preferable status of seedlings to survive under prolonged water deficit stress, whereas it might simultaneously downgrade the shipping grade of container seedlings based on shoot biomass. Therefore, in the case of prolonged drought after transplanting, container-seedlings grown in the presence of limited N and water would be suitable to circumvent water deficit stress for a relatively longer period at the expense of initial growth. Conversely, if adequate precipitation is anticipated, container-seedlings grown in the presence of adequate N and water supply are expected to grow well after transplanting.

Author Contributions

M.K., E.A. and H.T. designed the study. M.K. and H.H. collected the photosynthetic data; A.U. prepared the seeds of Japanese larch; S.K. and K.Y. collected the leaf N data, performed the analysis, and hence equally contributed to this study. All authors also discussed the results and commented on the manuscript. M.K. led the writing with input from E.A. and H.T.

References

Agathokleous E, Belz RG, Kitao M, et al (2019) Does the root to shoot ratio show a hormetic response to stress? An ecological and environmental perspective. Journal of Forestry Research 30:1569–1580. https://doi.org/10.1007/s11676-018-0863-7

Agathokleous E, Kitao M, Komatsu M, et al (2020) Effects of soil nutrient availability and ozone on container-grown Japanese larch seedlings and role of soil microbes. Journal of Forestry Research. https://doi.org/10.1007/s11676-019-01056-y

Bates D, Mächler M, Bolker B, Walker S (2015) Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67:1–48. https://doi.org/10.18637/jss.v067.i01

Bernacchi CJ, Singsaas EL, Pimentel C, et al (2001) Improved temperature response functions for models of Rubisco-limited photosynthesis. Plant, Cell and Environment 24:253–259. https://doi.org/10.1111/j.1365-3040.2001.00668.x

Collatz GJ, Ball JT, Grivet C, Berry JA (1991) Physiological and environmental regulation of stomatal conductance, photosynthesis and transpiration: a model that includes a laminar boundary layer. Agricultural and Forest Meteorology 54:107–136. https://doi.org/10.1016/0168-1923(91)90002-8

Cornic G, Fresneau C (2002) Photosynthetic carbon reduction and carbon oxidation cycles are the main electron sinks for photosystem II activity during a mild drought. Annals of Botany 89:887–894. https://doi.org/10.1093/aob/mcf064

de Kauwe MG, Lin Y, Wright IJ, et al (2016) A test of the 'one-point method' for estimating maximum carboxylation capacity from field-measured, light-saturated photosynthesis. New Phytologist 210:1130–1144. https://doi.org/10.1111/nph.13815

Epron D, Dreyer E (1992) Effects of severe dehydration on leaf photosynthesis in *Quercus petruea* (Matt.) Liebl.: photosystem II efficiency, photochemical and nonphotochemical fluorescence quenching and electrolyte leakage. Tree Physiology 10:273–284. https://doi.org/doi: 10.1093/treephys/10.3.273

Evans JR (1989) Photosynthesis and nitrogen relationship in leaves of C3 plants. Oecologia 78:9–19. https://doi.org/10.1007/BF00377192

Flexas J, Barón M, Bota J, et al (2009) Photosynthesis limitations during water stress acclimation and recovery in the drought-adapted *Vitis* hybrid Richter-110 (*V. berlandieri×V. rupestris*). Journal of Experimental Botany 60:2361–2377. https://doi.org/10.1093/jxb/erp069

Flexas J, Bota J, Galmés J, et al (2006) Keeping a positive carbon balance under adverse conditions: Responses of photosynthesis and respiration to water stress.

Physiologia Plantarum 127:343–352. https://doi.org/10.1111/j.1399-3054.2006.00621.x

Flexas J, Medrano H (2002) Energy dissipation in C3 plants under drought. Functional Plant Biology 29:1209–1215. https://doi.org/10.1071/FP02015

Fox J, Weisberg S (2019) An R companion to applied regression, Third. Sage Publications, Thousand Oaks, CA, USA

Graciano C, Guiamét JJ, Goya JF (2005) Impact of nitrogen and phosphorus fertilization on drought responses in *Eucalyptus grandis* seedlings. Forest Ecology and Management 212:40–49. https://doi.org/10.1016/J.FORECO.2005.02.057

Grossnickle SC, El-Kassaby YA (2015) Bareroot versus container stocktypes: a performance comparison. New Forests 2015 47:1 47:1–51. https://doi.org/10.1007/S11056-015-9476-6

Harayama H, Kita K, Kon H, et al (2016) Effect of planting season on survival rate, growth and ecophysiological properties of container seedlings of Japanese larch (*Larix kaempferi*). Journal of the Japanese Forest Society 98:158–166. https://doi.org/10.4005/jjfs.98.158

Harayama H, Tobita H, Kitao M, et al (2021) Enhanced summer planting survival of Japanese larch container-grown seedlings. Forests 12:1115. https://doi.org/10.3390/f12081115

Hendrickson L, Furbank RT, Chow WS (2004) A simple alternative approach to assessing the fate of absorbed light energy using chlorophyll fluorescence. Photosynthesis Research 82:73–81. https://doi.org/10.1023/B:PRES.0000040446.87305.f4

Kasajima I, Ebana K, Yamamoto T, et al (2011) Molecular distinction in genetic regulation of nonphotochemical quenching in rice. Proc Natl Acad Sci U S A 108:13835–40. https://doi.org/10.1073/pnas.1104809108

Kitao M, Kitaoka S, Harayama H, et al (2018) Canopy nitrogen distribution is optimized to prevent photoinhibition throughout the canopy during sun flecks. Scientific Reports 8:503. https://doi.org/10.1038/s41598-017-18766-0

Kitao M, Koike T, Tobita H, Maruyama Y (2005) Elevated CO_2 and limited nitrogen nutrition can restrict excitation energy dissipation in photosystem II of Japanese white birch (*Betula platyphylla* var. *japonica*) leaves. Physiologia Plantarum 125:64–73. https://doi.org/10.1111/j.1399-3054.2005.00540.x

Kitao M, Lei TT, Koike T, et al (2007) Interaction of drought and elevated CO_2 concentration on photosynthetic down-regulation and susceptibility to photoinhibition in Japanese white birch seedlings grown with limited N availability. Tree Physiol 27:727–735. https://doi.org/10.1093/treephys/27.5.727

555 Kitao M, Lei TT, Koike T, et al (2003) Higher electron transport rate observed at low
556   intercellular CO_2 concentration in long-term drought-acclimated leaves of Japanese
557   mountain birch (*Betula ermanii*). Physiologia Plantarum 118:406–413.
558   https://doi.org/10.1034/j.1399-3054.2003.00120.x

559 Kitao M, Lei TTT (2007) Circumvention of over-excitation of PSII by maintaining electron
560   transport rate in leaves of four cotton genotypes developed under long-term
561   drought. Plant Biology 9:69–76. https://doi.org/10.1055/s-2006-924280

562 Kitao M, Tobita H, Kitaoka S, et al (2019) Light energy partitioning under various
563   environmental stresses combined with elevated CO_2 in three deciduous broadleaf
564   tree species in Japan. Climate 7:79. https://doi.org/10.3390/cli7060079

565 Kitao M, Yoneda R, Tobita H, et al (2006) Susceptibility to photoinhibition in seedlings of
566   six tropical fruit tree species native to Malaysia following transplantation to a
567   degraded land. Trees 20:601–610. https://doi.org/10.1007/s00468-006-0075-1

568 Klughammer C, Schreiber U (2008) Complementary PS II quantum yields calculated from
569   simple fluorescence parameters measured by PAM fluorometry and the Saturation
570   Pulse method. PAM Application Notes 1:27–35. https://doi.org/citeulike-article-
571   id:6352156

572 Koike T, Kitao M, Maruyama Y, et al (2001) Leaf morphology and photosynthetic
573   adjustments among deciduous broad-leaved trees within the vertical canopy profile.
574   Tree Physiology 21:951–958. https://doi.org/10.1093/treephys/21.12-13.951

575 Kramer DM, Johnson G, Kiirats O, Edwards GE (2004) New fluorescence parameters for
576   the determination of Q_A redox state and excitation energy fluxes. Photosynthesis
577   Research 79:209–218. https://doi.org/10.1023/B:PRES.0000015391.99477.0d

578 Krause GH (1994) Photoinhibition induced by low temperature. In: Baker NR, Bowyer JR
579   (eds) Photoinhibition of Photosynthesis: from Molecular Mechanisms to the Field.
580   BIOS Scientific Publishers, Oxford, pp 331–348

581 Landis TD (1990) Containers: Types and Functions. In: Landis TD, Tinus RW, McDonald SE,
582   Barnett JP (eds) The Container Tree Nursery Manual. U. S. Department of
583   Agriculture, Forest Service, Washington, DC, pp 1–39

584 Lazár D (2015) Parameters of photosynthetic energy partitioning. Journal of Plant
585   Physiology 175:131–147

586 Liang G, Bu J, Zhang S, et al (2019) Effects of drought stress on the photosynthetic
587   physiological parameters of *Populus × euramericana* "Neva." Journal of Forestry
588   Research 30:409–416. https://doi.org/10.1007/s11676-018-0667-9

589 Lloret F, Casanovas C, Peñuelas J (1999) Seedling survival of Mediterranean shrubland
590   species in relation to root:shoot ratio, seed size and water and nitrogen use.

591   Functional Ecology 13:210–216. https://doi.org/10.1046/j.1365-
592   2435.1999.00309.x

593 McDowell N, Pockman WT, Allen CD, et al (2008) Mechanisms of plant survival and
594   mortality during drought: Why do some plants survive while others succumb to
595   drought? New Phytologist 178:719–739

596 McDowell NG (2011) Mechanisms Linking Drought, Hydraulics, Carbon Metabolism, and
597   Vegetation Mortality. Plant Physiology 155:1051–1059.
598   https://doi.org/10.1104/pp.110.170704

599 Medrano H, Escalona JM, Bota J, et al (2002) Regulation of photosynthesis of C_3 plants
600   in response to progressive drought: Stomatal conductance as a reference parameter.
601   Annals of Botany 89:895–905. https://doi.org/10.1093/aob/mcf079

602 Moustaka J, Moustakas M (2014) Photoprotective mechanism of the non-target
603   organism *Arabidopsis thaliana* to paraquat exposure. Pesticide Biochemistry and
604   Physiology 111:1–6. https://doi.org/10.1016/J.PESTBP.2014.04.006

605 Moustaka J, Ouzounidou G, Sperdouli I, Moustakas M (2018) Photosystem II is more
606   sensitive than photosystem I to Al^{3+} induced phytotoxicity. Materials 11:1772.
607   https://doi.org/10.3390/ma11091772

608 Moustaka J, Tanou G, Adamakis ID, et al (2015) Leaf age-dependent photoprotective and
609   antioxidative response mechanisms to paraquat-induced oxidative stress in
610   *Arabidopsis thaliana*. International Journal of Molecular Sciences 16:13989–14006.
611   https://doi.org/10.3390/ijms160613989

612 Niinemets Ü, Bilger W, Kull O, Tenhunen J (1999) Responses of foliar photosynthetic
613   electron transport, pigment stoichiometry, and stomatal conductance to interacting
614   environmental factors in a mixed species forest canopy. Tree Physiol 19:839–852.
615   https://doi.org/10.1093/treephys/19.13.839

616 Poorter H, Remkes C (1990) Leaf area ratio and net assimilation rate of 24 wild species
617   differing in relative growth rate. Oecologia 83:553–559.
618   https://doi.org/10.1007/BF00317209

619 R Core Team (2020) R: A Language and Environment for Statistical Computing

620 Sperdouli I, Moustakas M (2014a) A better energy allocation of absorbed light in
621   photosystem II and less photooxidative damage contribute to acclimation of
622   *Arabidopsis thaliana* young leaves to water deficit. Journal of Plant Physiology
623   171:587–593. https://doi.org/10.1016/J.JPLPH.2013.11.014

624 Sperdouli I, Moustakas M (2012) Differential response of photosystem II photochemistry
625   in young and mature leaves of *Arabidopsis thaliana* to the onset of drought stress.
626   Acta Physiologiae Plantarum 34:1267–1276. https://doi.org/10.1007/s11738-011-

627   0920-8

628 Sperdouli I, Moustakas M (2014b) Leaf developmental stage modulates metabolite
629   accumulation and photosynthesis contributing to acclimation of *Arabidopsis*
630   *thaliana* to water deficit. Journal of Plant Research 127:481–489.
631   https://doi.org/10.1007/s10265-014-0635-1

632 Sun J, Yao F, Wu J, et al (2018) Effect of nitrogen levels on photosynthetic parameters,
633   morphological and chemical characters of saplings and trees in a temperate forest.
634   Journal of Forestry Research 29:1481–1488. https://doi.org/10.1007/s11676-017-
635   0547-8

636 Villar-Salvador P, Planelles R, Oliet J, et al (2004) Drought tolerance and transplanting
637   performance of holm oak (*Quercus ilex*) seedlings after drought hardening in the
638   nursery.   Tree   Physiology   24:1147–1155.
639   https://doi.org/10.1093/treephys/24.10.1147

640 Xu Z, Zhou G, Shimizu H (2009) Are plant growth and photosynthesis limited by pre-
641   drought following rewatering in grass? Journal of Experimental Botany 60:3737–
642   3749. https://doi.org/10.1093/jxb/erp216

643 Yamashita N, Okuda S, Suwa R, et al (2016) Impact of leaf removal on initial survival and
644   growth of container-grown and bare-root seedlings of Hinoki cypress
645   (*Chamaecyparis obtusa*). Forest Ecology and Management 370:76–82.
646   https://doi.org/10.1016/j.foreco.2016.03.054

647

648

	+N		-N		F-statistics		
	+W	-W	+W	-W	N ($F_{1,16}$)	W ($F_{1,16}$)	N x W ($F_{1,16}$)
LMA (g m^{-2})	66.2±3.4	60.5±2.4	72.8±1.9	63.5±3.5	1.05[ns]	5.26[*]	0.33[ns]
N_{area} (g m^{-2})	0.80±0.04	1.11±0.11	0.60±0.03	0.69±0.04	23.1[***]	11.8[**]	3.56[ns]
A ($\mu mol\ m^{-2}\ s^{-1}$)	9.17±0.44[b]	11.5±0.53[a]	8.57±0.34[b]	9.12±0.22[b]	9.82[**]	13.5[**]	5.26[*]
g_s (mol $m^{-2}\ s^{-1}$)	0.15±0.02	0.16±0.02	0.13±0.02	0.14±0.01	1.99[ns]	0.22[ns]	0.02[ns]
$V_{c,max}$ ($\mu mol\ m^{-2}\ s^{-1}$)	64.0±6.0	75.8±0.6	55.8±2.6	59.1±3.5	7.68[*]	2.84[ns]	0.86[ns]
F_v/F_m	0.79±0.005	0.80±0.001	0.79±0.001	0.79±0.002	0.47[ns]	1.86[ns]	0.94[ns]
YPSII	0.076±0.005	0.095±0.004	0.070±0.003	0.075±0.002	8.22[*]	8.91[**]	2.72[ns]
YNPQ	0.76±0.005	0.74±0.001	0.78±0.005	0.77±0.003	28.4[***]	15.2[**]	3.23[ns]
YNO	0.16±0.003	0.17±0.003	0.15±0.002	0.15±0.002	16.9[***]	3.13[ns]	0.67[ns]

664

665

666 **Table 2.** Leaf physiological traits, measured just before the watering, of container
667 seedlings of Japanese larch grown in the presence of adequate (+N) or limited N (-N)
668 supply combined with daily (+W) or twice-a-week irrigation (-W). Measurements were
669 conducted on 18th August; +W-seedlings were irrigated on 17th August, but -W-seedlings
670 received the last irrigation on 14th August. Photosynthetic gas exchange and chlorophyll
671 fluorescence were measured at ambient CO_2 concentration (380 µmol mol^{-1}) and
672 saturating light intensity (1500 µmol m^{-2} s^{-1}). WP denotes predawn water potential, A
673 net photosynthetic rate, g_s stomatal conductance, YPSII quantum yield of electron
674 transport through PSII. Values are means ± se (n=4). Significant effects are indicated by
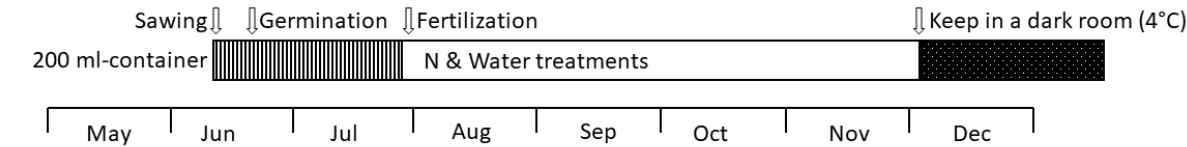675 ***: $P \leq 0.001$, **: $P \leq 0.01$; ns: non-significant.

676

	+N		-N		F-statistics		
	+W	-W	+W	-W	N ($F_{1,12}$)	W ($F_{1,12}$)	N x W ($F_{1,12}$)
WP (MPa)	-0.33±0.02	-1.36±0.30	-0.32±0.01	-1.08±0.20	0.69^{ns}	24.4***	0.57^{ns}
A (µmol m^{-2} s^{-1})	6.82±1.32	-0.57±0.92	6.43±1.18	0.37±0.88	0.06^{ns}	38.0***	0.37^{ns}
g_s (mol m^{-2} s^{-1})	0.088±0.015	0.012±0.010	0.101±0.016	0.014±0.005	0.39^{ns}	43.5***	0.19^{ns}
YPSII	0.074±0.006	0.057±0.010	0.078±0.005	0.050±0.003	0.07^{ns}	12.2**	0.74^{ns}

677

678

679 **Table 3.** Growth and biomass allocation in container seedlings of Japanese larch grown
680 in the presence of adequate (+N) or limited (-N) nitrogen supply combined with daily
681 (+W) or twice-a-week (-W) irrigation, 1) before transplantation, 2) transplanted into
682 progressive drought, or 3) transplanted into adequate water supply. Values are means ±
683 SE (n= 4 for each treatment combination). Note: Height and basal diameter of seedlings
684 harvested after the progressive drought were not recorded. Significant effects are
685 indicated by ***: $P \leq 0.001$, **: $P \leq 0.01$, *: $P \leq 0.05$; ns: non-significant.

686

	+N		-N		F-statistics		
	+W	-W	+W	-W	N ($F_{1,12}$)	W ($F_{1,12}$)	NxW ($F_{1,12}$)
1) Container seedlings before transplanting (harvested on 25th August 2017)							
Shoot height (cm)	41.1±1.2	35.3±1.5	37.0±1.2	32.8±1.1	6.61*	15.4**	0.40[ns]
Basal diameter (mm)	7.51±0.27	6.50±0.17	6.05±0.11	5.71±0.22	31.6***	11.5**	2.80[ns]
Total dry mass (g)	10.8±0.6	8.1±1.0	7.9±0.5	7.0±0.4	9.84**	8.10*	1.83[ns]
Shoot dry mass (g)	8.5±0.4	6.2±0.8	6.3±0.5	5.2±0.4	8.64*	10.1**	1.24[ns]
Root dry mass (g)	2.4±0.2	1.9±0.4	1.6±0.1	1.8±0.1	4.72[ns]	0.50[ns]	1.74[ns]
Shoot/Root ratio	3.6±0.2	3.4±0.6	3.9±0.4	3.0±0.3	0.02[ns]	2.28[ns]	1.02[ns]
2) Seedlings transplanted into progressive drought (harvested on 25th September 2017)							
Total dry mass (g)	10.6±1.2	9.0±1.0	6.9±1.1	6.0±1.1	9.24*	1.27[ns]	0.10[ns]
Shoot dry mass (g)	8.4±0.9	7.1±0.8	5.2±0.8	4.5±0.8	11.5**	1.55[ns]	0.12[ns]
Root dry mass (g)	2.2±0.4	1.9±0.1	1.7±0.3	1.5±0.3	2.60[ns]	0.40[ns]	0.04[ns]
Shoot/Root ratio	4.1±0.6	3.6±0.3	3.2±0.1	3.0±0.1	5.00*	1.00[ns]	0.08[ns]
3) Seedlings transplanted into adequate irrigation (harvested on 2nd November 2017)							
Shoot height (cm)	50.6 ±1.6	45.8±0.9	40.8±1.5	37.6±1.0	49.6***	9.80**	0.47[ns]
Basal diameter (mm)	8.85±0.31	8.19±0.31	7.86±0.29	7.62±0.43	5.26*	1.71[ns]	0.39[ns]
Total dry mass (g)	23.5±1.5	21.8±0.4	17.0±0.9	15.1±1.3	35.1***	2.51[ns]	0.01[ns]
Shoot dry mass (g)	14.2±0.8	12.4±0.3	9.9±0.5	8.2±0.6	52.5***	8.89*	0.01[ns]
Root dry mass (g)	9.3±0.8	9.4±0.4	7.0±0.6	6.8±0.7	13.7*	0.00[ns]	0.07[ns]
Shoot/Root ratio	1.55±0.08	1.33±0.06	1.43±0.10	1.23±0.09	1.65[ns]	6.09*	0.01[ns]

687

688 **Figure legends**

689

First growth season (2016)

Sawing⤒ ⤒Germination ⤒Fertilization   ⤒ Keep in a dark room (4°C)

200 ml-container ||||||||||||| N & Water treatments ░░░░░░░░░░░░░░░░░░░

| May | Jun | Jul | Aug | Sep | Oct | Nov | Dec |

Second growth season (2017)

Photosynthesis
measurements

Move back   ⤒Harvest (Sep 25)
to glasshouse⤒⤒Leaf flush   Harvest (Aug 25)⤒ ▨▨▨▨ Progressive drought

░░░░░░░░ N & Water treatments   Transplant into 4L-pot with fertilization

▨▨▨▨▨▨▨▨ Adequate water supply

Photosynthesis
measurements ⤒Initial height
Initial diameter   ⤒Harvest (Nov 2)

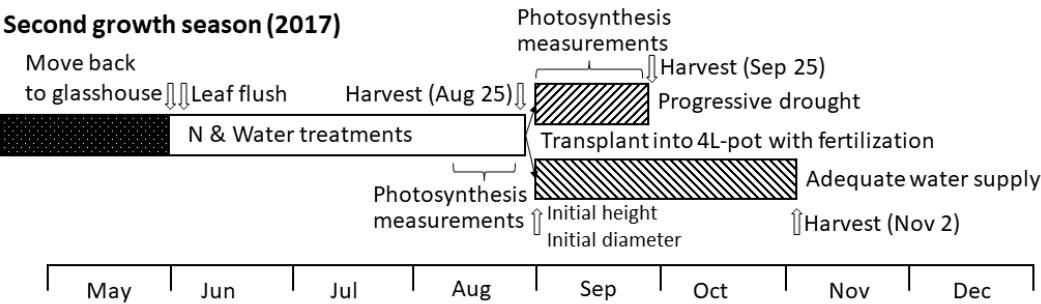| May | Jun | Jul | Aug | Sep | Oct | Nov | Dec |

690

691 **Figure 1.** Time course of the container larch experiments. Photosynthesis
692 measurements: WP, gas exchange, chlorophyll fluorescence, LMA, and N_{area}. Harvest:
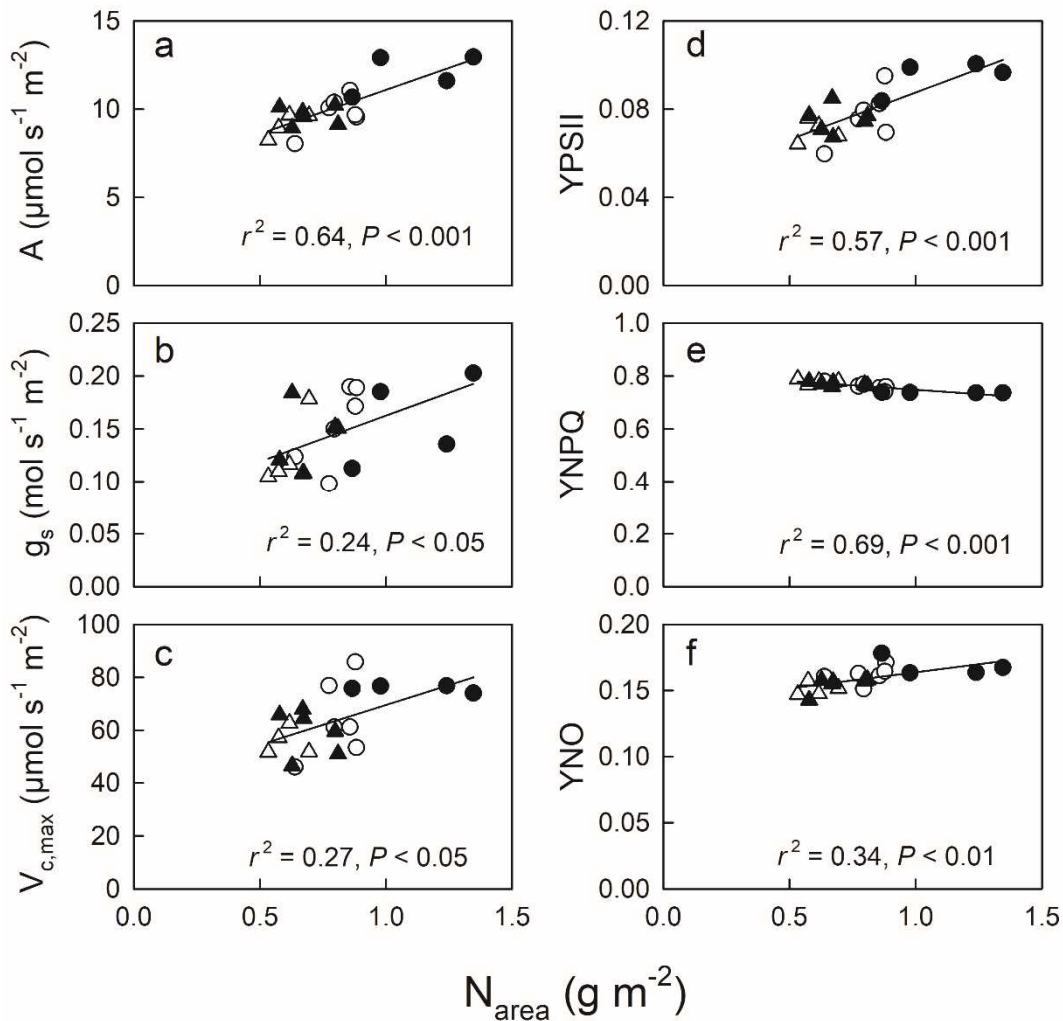693 measurements of dry mass of each organ, shoot height, and basal diameter.

694

Figure 2. Relationship between area-based leaf N (N_{area}) and A, g_s, $V_{c,max}$, YPSII, YNPQ, and YNO in individual seedlings of Japanese larch grown in adequate (circle) and limited (triangle) N combined with adequate (open) and limited (closed symbols) irrigation. Gas exchange measurements were conducted at a photon flux density of 1500 μmol m^{-2} s^{-1}, a leaf temperature of ≈25 °C, and an ambient CO_2 concentration of 380 μmol mol^{-1}. 'One-point method' (de Kauwe et al. 2016) was used to calculate $V_{c,max}$. Linear regression analysis was conducted for the pooled data across the treatment combinations.
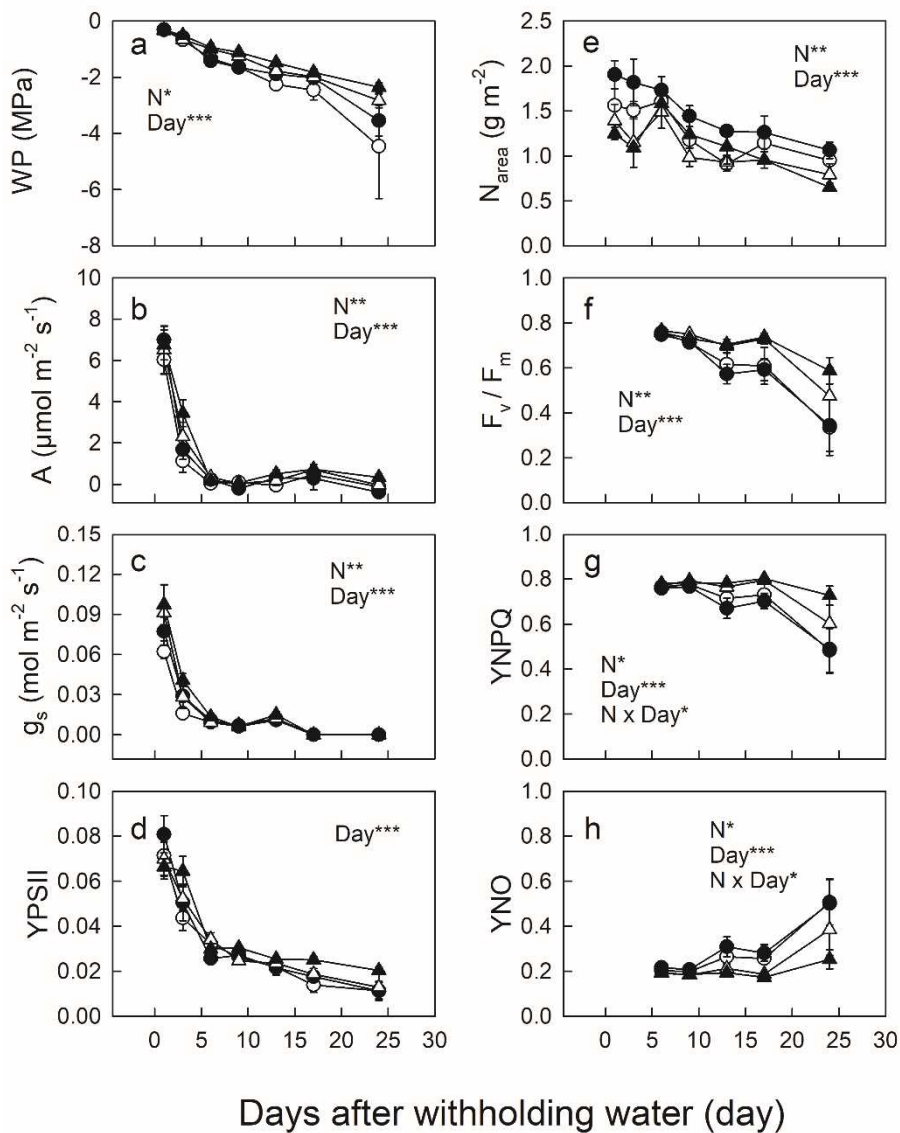
704
705

Figure 3. Predawn leaf water potential (WP), net photosynthetic rate (A), stomatal conductance (g_s), quantum yield of electron flow through PSII (YPSII), area-based leaf N content (N_{area}), the maximum efficiency of PSII photochemistry (F_v/F_m), quantum yield of regulated non-photochemical quenching (YNPQ) and quantum yield of non-regulated non-photochemical quenching (YNO) in seedlings of Japanese larch grown in adequate (circle) and limited (triangle) N combined with adequate (open) and limited (closed symbols) irrigation, as a function of days after withholding water supply. Significant effects of N, water, day and their interactions are indicated by *** $P \leq 0.001$, **$P \leq 0.01$,* $P \leq 0.05$ (details are shown in Supplemental Table S1). Data of F_v/F_m, YNPQ and YNO were not available on days 1 and 3.
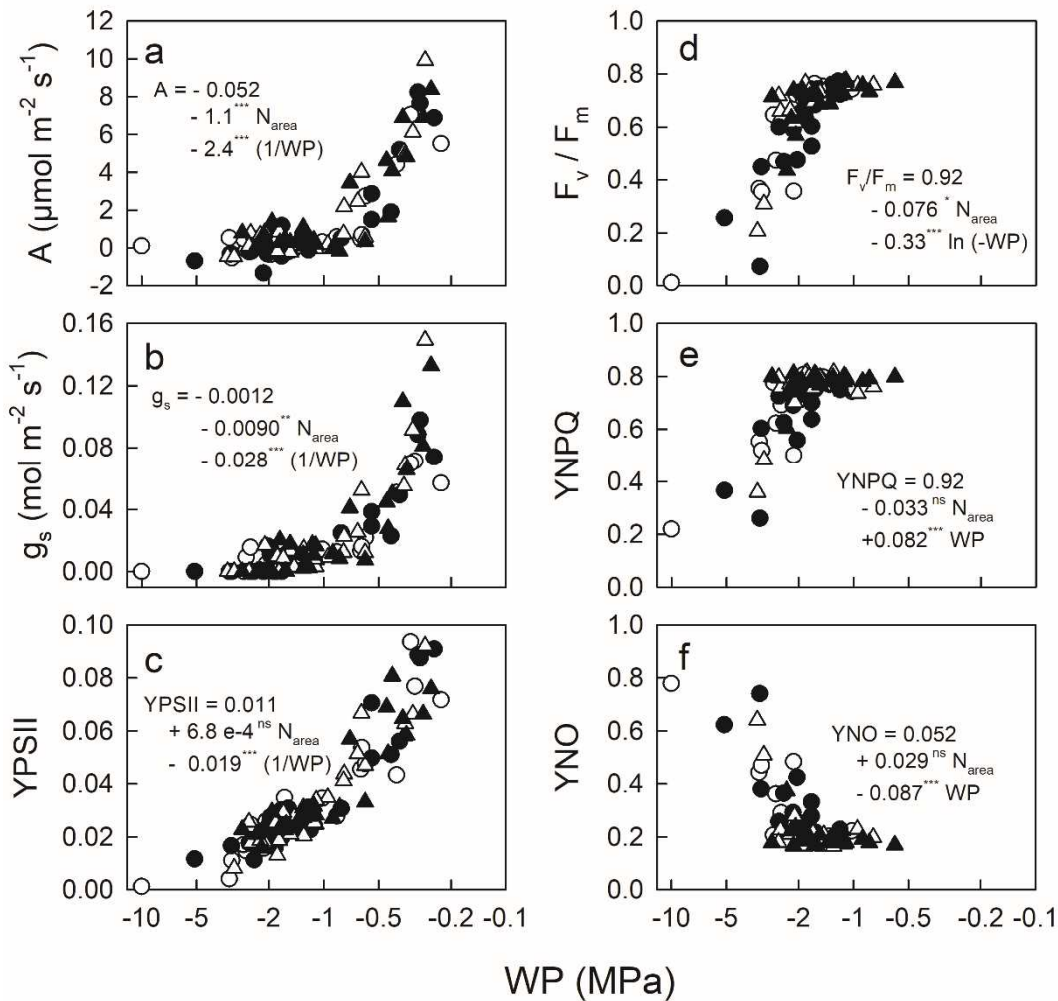
Figure 4. Photosynthetic traits of container seedlings of Japanese larch grown in adequate (circle) and limited (triangle) N combined with adequate (open) and limited (closed symbols) irrigation as a function of pre-dawn leaf water potential during the progressive drought. Data of F_v/F_m, YNPQ and YNO were not available on days 1 and 3. Significant effects of N_{area} and WP are indicated in the panel by [***] $P \leq 0.001$, [**]$P \leq 0.01$, [*]$P \leq 0.05$; ns indicates non-significant. To present responses to moderate water deficit stress more clearly, x-axis is log-scaled.

N: $F=33.8$, $P<0.001$
W: $F=0.07$, $P=0.79$
NxW: $F=1.10$, $P=0.31$

726

727

728 **Figure 5.** Total amount of needle N per plant ($N_{needles}$) in container seedlings of Japanese
729 larch, which were grown in the presence of adequate (+N) or limited (-N) nitrogen supply
730 combined with daily (open) or twice-a-week (closed bars) irrigation. Plants were
731 harvested before transplanting on 25th August 2017. Values are means + se (n = 4 for
732 each treatment combination). The data were analyzed with a two-factorial ANOVA. The
733 level of statistical significance was α = 0.05.

734

735

736

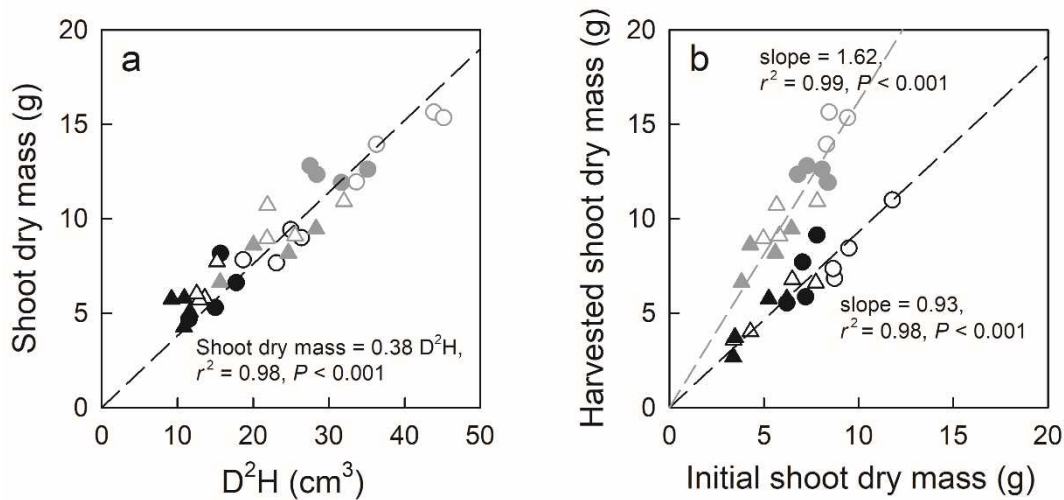737 **Figure 6.** Relationship between shoot dry mass and D^2H (= (basal diameter)^2 x (shoot

738 height) in container seedlings of Japanese larch grown in adequate (circle) and limited

739 (triangle) N combined with adequate (open) and limited (closed symbols) irrigation,

740 harvested before transplanting on 25th August (black) and after transplanting into

741 adequate irrigation, then harvested on 2nd November (grey) (a), and the relationship

742 between harvested shoot dry mass and initial shoot dry mass estimated from D^2H in

743 seedlings transplanted into progressive drought (black) and those transplanted into

744 adequate irrigation (grey) (b). Linear regression analysis through the origin was

745 conducted for the pooled data in the left panel (a). Linear regression analysis through

746 the origin was conducted for progressive drought and adequate irrigation, respectively

747 in the right panel (b).

748

749

Figure 7. Relationship between predawn leaf water potential (WP) and shoot dry mass during the progressive drought treatment in individual seedlings of Japanese larch grown in adequate (circle) and limited (triangle) N combined with adequate (open) and limited (closed symbols) irrigation. Shoot dry mass corresponds to that in the seedlings harvested 27 days after transplanting into the progress drought treatment, which showed little change from the initial shood dry mass estimated from D^2H (cf. Fig. 5). The wilting individual on Day 24 is included in the regression analysis.

Table S1. Analysis of deviance table summary using type II Wald chi-square (χ^2) tests for linear mixed-effects model fit for predawn leaf water potential (WP), light-saturated net photosynthetic rate (A), stomatal conductance (g_s), the maximum photochemical efficiency of PSII (F_v/F_m), area-based leaf N content (N_{area}), quantum yield of electron flow through PSII (YII), quantum yield of regulated non-photochemical quenching (YNPQ), and quantum yield of non-regulated non-photochemical quenching (YNO) in seedlings of Japanese larch grown under the N and water treatment combinations during progressive drought (Day: days after withholding water supply). Bold font indicates significant effects of fixed factors (N, Water, Day, and their interaction) at the level of $P \leq 0.05$.

Factor	WP χ^2	df	P	A χ^2	df	P	g_s χ^2	df	P	F_v/F_m χ^2	df	P	N_{area} χ^2	df	P	YPSII χ^2	df	P	YNPQ χ^2	df	P	YNO χ^2	df	P
N	5.03	1	**0.025**	6.19	1	**0.012**	7.94	1	**0.005**	6.91	1	**0.009**	8.50	1	**0.035**	2.26	1	0.132	6.04	1	**0.014**	6.25	1	**0.012**
Water	1.16	1	0.28	2.18	1	0.140	1.46	1	0.227	0.01	1	0.906	1.96	1	0.161	1.94	1	0.164	0.04	1	0.834	0.06	1	0.802
Day	180	6	**<0.001**	777	6	**<0.001**	671	6	**<0.001**	105	4	**<0.001**	102	6	**<0.001**	570	6	**<0.001**	72.0	4	**<0.001**	75.0	4	**<0.001**
N x Water	0.03	1	0.873	0.08	1	0.781	0.03	1	0.861	0.19	1	0.660	1.27	1	0.261	0.184	1	0.668	0.578	1	0.447	0.64	1	0.423
N x Day	9.94	6	0.127	7.92	6	0.244	12.4	6	0,055	9.47	4	0.0504	9.05	6	0.171	11.4	6	0.077	11.4	4	**0.023**	10.9	4	**0.0272**
Water x Day	2.83	6	0.830	4.73	6	0.579	8.07	6	0.232	1.73	4	0.786	3.75	6	0.711	6.67	6	0.353	2.77	4	0.596	2.71	4	0.608
N x Water x Day	0.56	6	0.997	2.03	6	0.917	0.66	6	0.995	1.01	4	0.908	2.69	6	0.847	3.72	6	0.714	1.36	4	0.852	1.33	4	0.857

a

$A = -0.052$
$- 1.1^{***} N_{area}$
$- 2.4^{***} (1/WP)$

y-axis: A ($\mu mol\ m^{-2}\ s^{-1}$)

b

$g_s = -0.0012$
$- 0.0090^{**} N_{area}$
$- 0.028^{***} (1/WP)$

y-axis: g_s ($mol\ m^{-2}\ s^{-1}$)

c

$YPSII = 0.011$
$+ 6.8\ e\text{-}4^{ns} N_{area}$
$- 0.019^{***} (1/WP)$

y-axis: YPSII

d

$F_v/F_m = 0.92$
$- 0.076^{*} N_{area}$
$- 0.33^{***} \ln(-WP)$

y-axis: F_v / F_m

e

$YNPQ = 0.92$
$- 0.033^{ns} N_{area}$
$+0.082^{***} WP$

y-axis: YNPQ

f

$YNO = 0.052$
$+ 0.029^{ns} N_{area}$
$- 0.087^{***} WP$

y-axis: YNO

x-axis: WP (MPa)

Supplemental Figure S1. Photosynthetic traits in container seedlings of Japanese larch grown in adequate (circle) and limited (triangle) N combined with adequate (open) and limited (closed symbols) irrigation as a function of pre-dawn leaf water potential during the progressive drought. Data points are categorized into two groups with higher or lower N_{area}; leaves with $N_{area} > 1.19$ mg g^{-1} (the median of N_{area}) (n=56) (red), or those with $N_{area} < 1.19$ mg g^{-1} (n=56) (black) for A, g_s, and YPSII, and leaves with $N_{area} > 1.10$ mg g^{-1} (n=40) (red) or those with $N_{area} < 1.10$ mg g^{-1} (n=40) (black) for F_v/F_m, YNPQ, and YNO. Data of F_v/F_m, YNPQ and YNO were not available on days 1 and 3. Significant effects of N_{area} and WP are indicated by *** $P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$. ns indicates non-significant effects.